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Biological control of the cabbage root fly (*Delia radicum*) using an entomopathogenic fungus (*Metarhizium brunneum*)

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Abstract

Cabbage root flies are a damaging pest to cruciferous crops in the northern hemisphere. They are considered to be the main pest of brassica crops (EPPO, 2004). Their larvae feed on the roots and stems of brassica plants. Larval feeding causes quality reductions in crops like turnips, rutabaga and radishes, as well as stress to important crops like broccoli and cauliflower. Insecticide use is limited, and there are few alternative management strategies. Alternative options include using physical barriers like nets covering the crop or vertical net fences to block flies entering the enclosed area, using plant volatiles and trap cropping to lure cabbage root flies away from important crops, and using natural enemies to reduce cabbage root fly populations. Biological control agents including entomopathogenic fungi, like *Metarhizium brunneum,* can be considered a part of integrated pest management for cabbage root flies.

In this study two greenhouse experiments, and a field study were performed to assess the ability of *M. brunneum*, isolate Met. 250/O2, to reduce cabbage root fly populations by lethally infecting cabbage root fly larvae and pupae. The influence of *M. brunneum* inoculation of plant roots on how the female adult cabbage root fly lays her eggs was tested, as well as the egg-laying ability of surviving flies collected from Met. 250/O2 inoculated treatments. However, no significant effect of Met. 250/O2 was found; Met. 250/O2 treatments did not reduce pupae survival or reduce the number of cabbage root fly larvae and pupae samples collected or influence how many eggs adult female flies laid. However, *M. brunneum* should still be considered a part of integrated pest management for cabbage root fly populations, with further research.

Keywords: integrated pest management, *Delia radicum*, *Metarhizium brunneum*

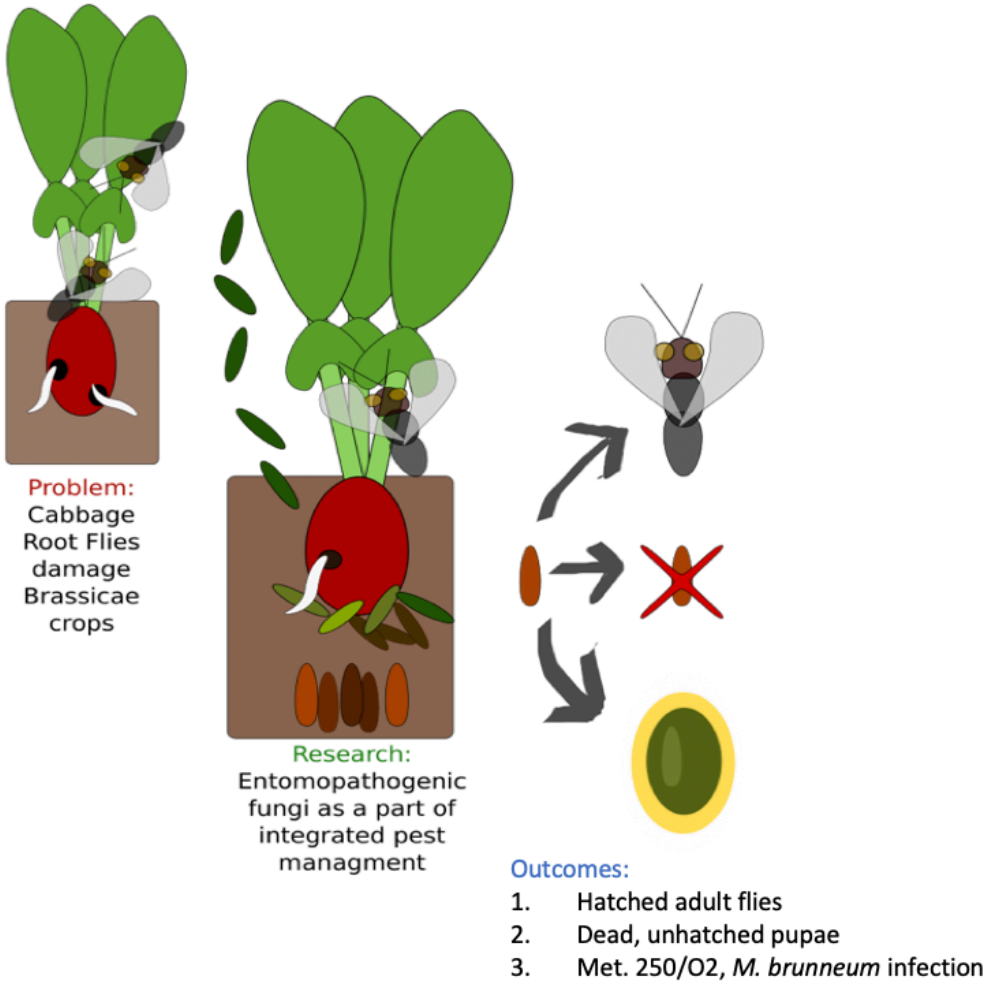

Figure 1: Graphical abstract of research problem, research focus and three resulting categories of the cabbage root fly pupa collected.

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1. Introduction

1.1. Integrated Pest Management and Biological Control Agents

Integrated pest management (IPM) is a foundation for sustainable crop production without relying heavily on chemicals. There will always be pests, weeds or pathogens that can cause damage to vegetable crops in the field. Shifting focus from elimination to reduction of target insect density with a variety of control strategies, a balance can be struck between insect pest damage, vegetable quality and yield (Finch and Collier, 2000b; Mesmin et al., 2019). IPM is defined as:

> "careful consideration of all available plant protection methods and subsequent integration of appropriate measures that discourage the development of populations of harmful organisms and keep the use of plant protection products and other forms of intervention to levels that are economically and ecologically justified and reduce or minimise risks to human health and the environment. 'Integrated pest management' emphasises the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanism."(European Parliament, 2009)

IPM has a long history, developing over a century ago before reliance on chemical pesticides became prevalent in agricultural systems (Kogan, 1998). Once the environmental, and human health consequences of chemical pesticides became better understood many chemical products were banned or are becoming more restricted (Chandler et al., 2008; Finch and Collier, 2000b; Kogan, 1998, Biever et al., 1994). Integrating biological, biological and cultural practices for pest management reclaimed attention of crop protection researchers in the 1960s (Kogan, 1998). Desire for alternative, environmentally sustainable products, such as biological control agents, is rising with the reduction of effective chemicals coupled with the need to keep crop yield and quality profitable (Chandler et al., 2008; Köhl et al., 2019). Research into alternative pest management strategies is important.

Biological control agents may be used as alternatives to chemical insecticides. Nematodes, parasitoids, predators, protozoa bacteria, entomopathogenic fungi, including endophytic and soil/rhizosphere-colonizing microbes can all be considered biocontrol agents (Chandler and Davidson, 2005; Finch and Collier, 2000b; Finch, 1993; Lacey et al., 2015). There are over 50 commercially produced microbial pesticides, representing approximately

1-2% of pesticides sold within the EU (Lacey et al., 2015). Sales and production are expected to rise with growing organic markets and sustainability-centered integrated pest management programs (Lacey et al., 2015). They are generally considered 'safer' as they have a narrow host range, thus minimizing risk to beneficial insects, less potential to cause resistance development within target insects, and pose no known risk to humans, unlike many of their chemical counterparts (Shapiro-Ilan et al., 2006)). In Norway, microbial biological control agents are ranked as a level one in terms of environmental risks, thus viewed with minor associated risks (OCED, 2000). Biological control agents ideally work to reduce pest populations; but it is challenging to make them work effectively in the field.

Biological control agents, including fungal entomopathogens, are the focus of laboratory, greenhouse and field studies for potential commercial application. Biological control with the aim of an epizootic event, a large outbreak of disease to control a target insect pest, requires a pathogen, a target host population, transmission within the target population, and is affected by environmental conditions (Hajek and Meyling, 2018; Shapiro-Ilan et al., 2006). These factors need to be considered when choosing and developing biocontrol strategies, including microbial control agents for target insect pest population reduction.

Once potential entomopathogenic agents are isolated from different agroecosystems, they need to be assessed for virulence and ability to kill or damage target pests. They must be able to produce enough infective material to contact, infect and kill insect pests (Lacey et al., 2015, Shapiro-Ilan et al., 2006). Isolates need to be robust enough to withstand a variety of environmental conditions that occur in field conditions including sunlight, humidity, and temperature (Lacey et al., 2015). Interactions with other applications to agricultural fields like fertilizer, chemical pesticides, fungicides, and soil nutrients need to be known to assess viability of using the biological control agent (Lacey et al., 2015). Sometimes interactions with the crop plant itself influences the control agent, volatiles and root exudates could affect the microbiome in which the control agent is present, as well as competition with other microbes and metabolites or toxins that they produce (Lacey et al., 2015). There are many factors to consider when developing a microbial or other biological control agent for agricultural use.

When using a biological control agent, how it is applied needs to accounted for. Choosing soil treatment or foliar applications, is dependent on where the target insect is usually found, where and when it is at its most vulnerable. The control agent usually needs to

be applied where the damage occurs to infect target insects. For example, to protect against root feeding pests, an entomopathogen should be applied in the soil root zone (Lacey et al., 2015). Within soil applications there are many ways of integrating the microbial pesticide to the field; drench application of a conidia suspension, as granular formula with just conidia or as nutrient based granules (Lacey et al., 2015). Finding the most effective way to deliver potential microbial control agents, that is also commercially viable for use by farmers is challenging.

Pests feed on, and cause damage to host plants (Meyling and Hajek, 2010). Pathogens of the target pest should work to reduce the pest population, indirectly benefitting the host plant (Meyling and Hajek, 2010). Biological control agents, such as entomopathogenic fungi, should infect insect pests leading to insect mortality, thus reducing pest population. The end result is that the crop plant does not have so many pests feeding on it (Meyling and Hajek, 2010). Synergism between potential biological control agents like entomopathogenic fungi, predators and nematodes could be a strategy (Lacey et al., 2015).

Often times a biological control agent is just one part of an integrated pest management strategy. Other parts could include using a push-pull system with attractive and/or deterrent volatiles, or companion trap crops, or physical exclusion fences to reduce the pest population below damaging thresholds. Cabbage root fly management is an example of how many different strategies can combine to be effective to reduce pest populations within a vegetable field.

1.2. Cabbage Root Flies:

1.2.1. Lifecycle

Cabbage root flies (*Delia radicum*), start their lifecycle as eggs. Eggs are laid at the base or stems of cruciferous plants. The eggs are white, oval-shaped, approximately 1.1mm long; they are visible to the naked eye (Capinera, 2008). Two distinct ridges among alternating stripes of opaque white and more translucent white run from end to end of the egg (Capinera, 2008). Eggs take around 3-5 days to hatch. Once hatched larvae go through three instar stages growing from 1mm to 8mm long between first instar and third instar stage. Larvae have black mouth hooks surrounded by 12 paired spiracles (Capinera, 2008; Smith, 1927). The larvae feed on the roots, stems and heads of brassica plants (Capinera, 2008). The larval stage is approximately 18-22 days depending on the weather before entering the pupal stage. Pupae range in colour from golden to red-bronze to dark brown and are approximately 6mm long

(Smith, 1927). In summer months, pupation takes 2-3 weeks before adult emergence occurs. Climate influences how many fly generations there will be, in Norway there can be 1-2 depending on the temperature (Johansen and Meadow, 2015), while other countries can have between 2-7 generations (EPPO, 2004). If overwintering, pupae can remain in the soil for 5-8 months until diapause is complete. Adult flies are bristly, around 5-7mm long and feed on nectar from plants (Capinera, 2008). The lifecycle of cabbage root flies is shown in Figure 1.

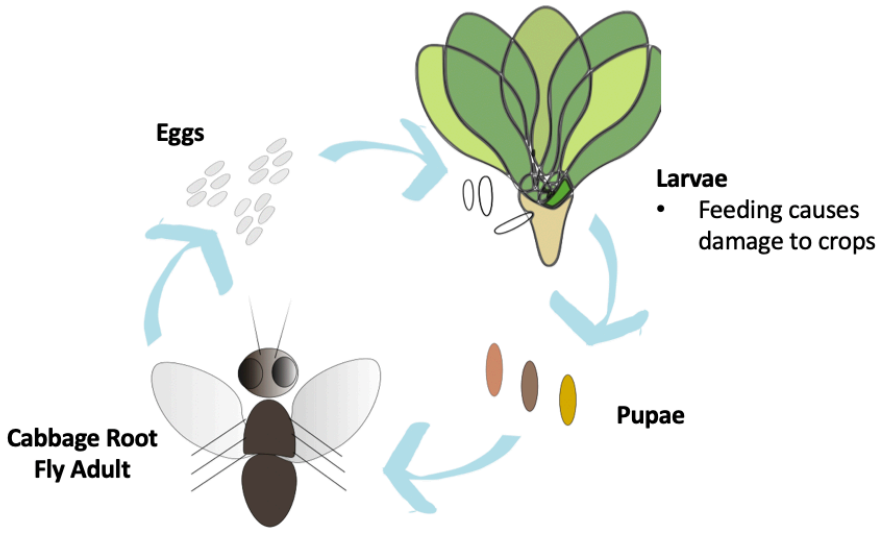


Figure 2. Lifecyle diagram of cabbage root flies. Eggs are laid by female flies at the base of acceptable host plants. Larva emerge and feed on plant host roots, stems and leaves. Larva go through three instar stages. Pupa develop in the soil, depending on time of year, can remain as pupa over winter. Adult flies emerge from pupae.

1.2.2. Emergence

Adult cabbage root fly emergence is influenced by temperature (Lepage et al., 2014); with early and late emerging cabbage root fly populations (Lepage et al., 2014). Pupal survival can vary in field studies, where some pupae are killed by physical damage, pathogens, or otherwise do not emerge (Finch and Skinner, 1980).

1.2.3. Dispersal

Cabbage root flies are capable of dispersing 2-3km from the point of infestation (Finch and Skinner, 1975). Abiotic factors like wind, precipitation and terrain affect dispersal ability (Finch and Skinner, 1975). Both male and female flies do not move much the first two days after emergence. After that, just before mating, and between mating and oviposition, male flies can travel around 100m a day, while female flies can travel around 1000m (Finch and

Skinner, 1975). Female flies do not always stop at the first host plant they find. Female flies continue searching until they find an appropriate host plant to lay eggs on (Finch and Skinner, 1975). Female flies can orient themselves upwind to host plants once within a certain range while males tend to be more random in their movements (Hawkes and Coaker, 1979; Hawkes 1974). Female flies are most active when gravid (Hawkes, 1974), and can move more slowly upwind towards their host plant (Hawkes et al., 1978). Once a desirable host plant is found, females oviposit.

1.2.4. Host choice and Oviposition

Female flies lay eggs based on visual and chemical cues. Studies have shown that female flies can detect desirable host plants based on leaf colour, and leaf area within patches of non-host plants (Propkopy et al., 1983; Kostal and Finch, 1994; Finch and Collier, 2000a). Volatiles released from host plants attract flying cabbage root flies (Finch and Collier, 2000a). These volatiles act as olfactory cues to that gravid females can sense via antennae receptors and use these volatiles to locate desirable host plants (Hawkes et al., 1978; Hawkes and Coaker, 1979; Nottingham, 1988). Once cabbage root flies have landed on a plant, they can use tarsal receptors to test if they are on a desirable host plant; this leads to acceptance of the host plant and subsequent oviposition or rejection and exit to find a more desirable plant (Finch and Collier, 2000a). To elicit oviposition both physical, visual cues and chemical stimuli can be important (Košťál et al., 2000; Košťál and Finch 1994). These cues can be used to manipulate cabbage root fly behaviour, as described in later sections.

1.2.5. Damage

The larval stage of cabbage root flies is the damaging stage. Larvae feed on roots and stems of cruciferous vegetables (EPPO, 2004). They cause drooping, wilting, delay maturation, and can cause stunting (Capinera, 2008). In dry weather the effect of larvae damaging roots and stems can cause plant stress or death (Capinera, 2008). Larvae feeding damages the quality of crops like radish and turnip, where the scars reduce marketability (Capinera, 2008; Eppo, 2004). Brussel sprouts, broccoli, cabbage, cauliflower, collards, kale, kohlrabi, radish, rutabaga, turnip and watercress are all susceptible brassica crops (Capinera, 2008). In addition to physical symptoms caused by feeding, larvae can introduce or create opportunities for bacteria to colonize plants and cause rot (Doane and Chapmen, 1964). Overall, cabbage

root fly larvae infestation is detrimental, leading to lower quality, yield and profitability of brassica vegetable crops.

1.3. Management Strategies

1.3.1. Chemical-Insecticides

Historically, insecticides have been effective at cabbage root fly management (Coaker and Finch, 1971). Many previously relied upon chemicals including organochlorines, chlorpyrifos, carbamate and organophosphorus are currently restricted in use or banned due to environmental damage, detrimental effects to human health and development of insect resistance (Ester et al., 2003). Spinosad is the only insecticide that can be used in Norway for cabbage root flies (Mattilsynet, 2012). Spinosad has been tested for effect against cabbage root flies and ability to indirectly reduce root damage; it has been found to work effectively (Herbst et al., 2017; Razinger et al., 2017). Film-coating of seeds with spinosad has been found to be moderately effective against cabbage root flies in field experiments (Ester et al., 2003). Approved insecticides, like spinosad can be applied as sprays or granules targeting the soil, close to the plant base or seed at sowing (Eppo, 2004). With only one available insecticide, other alternatives need to be investigated and incorporated into cabbage root fly management.

1.3.2. Non-chemical Controls

Over the past few decades many non-chemical controls have been tried and tested to manage cabbage root flies and mitigate the damage they do to important crops. Foam, cardboard, carpet or rubber collars around the base of the plant can work a little bit to reduce oviposition (Havukkala, 1988; Skinner and Finch, 1986). Less eggs are found around the plant, but they create a more humid environment underneath the discs which can be damaging to the plant, in addition to being tedious to apply in a commercial field setting (Skinner and Finch, 1986). Mulching with grasses or straw can reduce the symptoms from larval root feeding (Hellqvist, 1996), but has not been observed to reduce oviposition (Hellqvist, 1996, Herbst et al. 2017).

Other largely unsuccessful deterrents have been tested for their effect on cabbage root flies. Sphagnum moss, wood ash, rape seed oil residues, charcoal silica filter waste, and turpentine-soaked wood sticks have been found to have negligible to low effects (Havukkala, 1988). *Bacillus thuringiensis* strains have been tested as well (Havukkala, 1988). Treating adult flies with a myosupressin to inhibit nerve bundles has been shown to reduce muscular

activity but it is not lethal (Bell et al., 2019). Many non-chemical alternatives have been tried, however with minimal and variable effectiveness.

1.3.3. Barriers

Row covers can be used to reduce egg laying and damage on susceptible crops (IOBC-WPRS, 2016; Witkowska et al., 2018; Ester et al. 1994; Hough-Goldstein, 1987). They are extensively used, especially for crops like radishes or rutabaga where aesthetic quality needs to be high for marketability (Witkowska et al., 2018). Row covers are expensive. Row covers change the surrounding microclimate which can promote disease and weed development (Witkowska et al., 2018; Ester et al., 1994; Finch, 1989). However, row covers can be part of a cabbage root fly management strategy.

Vertical barrier fences can be used to interrupt adult fly flight; the fences surround the susceptible area and cabbage root flies are intercepted. Overhangs can be added to the top of the fence, to collect flies as they climb upwards as a result of meeting a barrier (Blackshaw et al., 2012), and further reduce cabbage root flies within the enclosed crop area (Bomford et al., 2000). These vertical barriers, also known as exclusion fences, have been shown to reduce the movement of cabbage root flies in small plot areas. Blocked access to rutabaga plots results in fewer cabbage root fly-damaged plants (Blackshaw et al., 2012; Vernon and Mackenzie, 1998). Best practices for using vertical fences requires meticulous attention; they need to be closed properly and carefully handled to prevent damage and flies from entering the plot. This method may be better for crops like rutabaga or radish, whose value is affected by aesthetic quality (Vernon and Mackenzie, 1998). Both types of barriers can be part of integrated pest management for cabbage root flies.

1.3.4. Intercropping and Mixed Cover Crops

Intercropping or mixing non-host crops with preferred cultivars can have an effect on cabbage root fly oviposition (Finch, 1989; Morley et al., 2005). There are many hypotheses as to why this is the case; non-host plants provide a physical mechanical barrier, and they can mask or alter host plant odors. Non-host plants increase the diversity of natural enemies. Non-host plants interfere with host selection by causing cabbage root flies to land on non-host plants with similar visual cues, leading to inappropriate landings and increased searching time (Finch and Collier, 2000a). Overall, companion, non-host plants diversify the agroecosystem and help to reduce insect pests, including cabbage root flies (Finch and

Collier, 2012; Morley et al., 2005). Examples of mixed cover and intercrops reducing cabbage root fly populations compared to monocultures are discussed below.

Female flies land on brassica host plants growing in bare soil more than those surrounded by non-host plants because they are using green as a visual cue to signal landing (Kostal and Finch, 1994; Finch and Collier, 2000a). It is hard for the cabbage root fly to tell the difference between host and non-host plants that also have leaves within the correct colour spectrum signalling a host plant (Finch and Collier, 2000a). Intercropping non-host species has been shown to reduce the number of cabbage root fly eggs than when compared to a monoculture of cabbage or Brussel sprouts (Coaker, N.D.). Similarly, fewer cabbage root fly eggs per host plant were observed in a cabbage and clover intercrop system than in a cabbage monoculture (Theunissen and Schelling, 1992). Companion plants that are planted close to host cauliflower crops influence oviposition of female cabbage root flies (Collier and Elliott 2014). Diversifying cruciferous fields through mixed cover crops, intercropping, and companion plants can be part management strategy to influence host finding and oviposition by cabbage root flies.

1.3.5. Cultivar Selection

Cabbage root flies prefer some varieties or cultivars of brassicas over others. Studies have looked at breeding resistant lines and finding cultivars that survive better, have less damage, or result in fewer, smaller pupae. One such study tested 56 accessions of different turnips, turnip tops and turnip greens in no-choice experiments (Santolamazza-Carbone et al., 2017). From those, the authors chose the top 10 most promising and 10 most susceptible to analyze in field test. They found that pupae number was different between more resistant and susceptible cultivars (Santolamazza-Carbone et al., 2017). Resistant cultivars still had pupae development with smaller, and whiter roots (Santolamazza-Carbone et al., 2017). Other studies have noted cabbage root fly resistance in canola and have hand crossed potential genes to rutabaga (Malchev et al., 2010). Isoline pairs were identified and through high performance liquid chromatography peaks for resistance markers were associated with glucosinolate profiles (Malchev et al., 2010). Glucosinolates are known to be involved in plant-insect interactions, but can also affect flavour profiles in plants (Malchev et al., 2010). From studies like these models can be made to predict potential resistance lines for various susceptible brassicas.

1.3.6. Plant Volatiles

Plants emit chemicals that can be sensed by cabbage root flies, and their predators. These volatiles can be artificially applied to manipulate insect behaviour (Ferry et al., 2009; Ferry et al., 2007; Lamy et al., 2018; Lamy et al., 2017). Laboratory studies indicate that odor can stimulate different levels of oviposition on different plants with in the brassica crops (Kergunteuil et al., 2015). Intercropping different cultivars of brassica that are more attractive with ones that are more susceptible can help to design systems to reduce insect pest density in susceptible plant crops (Kergunteuil et al., 2015). Chinese cabbage is known to be attractive to cabbage root flies, as are *Brassica. napus* "Yudal" variety and *Brassica. rapa* (Badenes-Pérez, 2019; Lamy et al., 2018; Rousse et al., 2003). Volatiles known to be released by attractive plants are terpenes like linalool, B-caryophyllene, humulene and a-farasene (Kergunteuil et al., 2015). Dimethyl disulfide is released from damaged plant roots and has been found to be an oviposition-deterrent, in addition to attracting natural predators to the damaged plant (Ferry et al., 2009). Eucalyptol is also an oviposition deterrent and has been shown to reduce oviposition on broccoli (Lamy et al., 2017). Groupings of synthetic deterrents and attractants in combination with more attractive plants can be used for push-pull trap cropping, which will be described in the next section.

1.3.7. Push-Pull and Trap Cropping

Push-pull strategies use combinations of stimuli to modify insect behaviour, changing abundance and distribution within an area (Cook et al., 2007; Eigenbrode et al., 2016). It is defined as:

> "The push pull strategy is a behavioural manipulation method that uses repellent/deterrent (push) and attractive/stimulant (pull) stimuli to direct movement of pest or beneficial insects for pest management." (Cook et al., 2007).

These attractants and deterrents can be olfactory such as different volatiles, visual or physical cues on the plants themselves such as leaf shape, trichomes or surface texture (Eigenbrode et al., 2016). These cues can interact synergistically, to get a net push-pull effect to change the distribution of pest insects in the cultivated area (Eigenbrode et al., 2016).

For cabbage root flies, push-pull systems can be made from known volatiles for deterring or attracting adult flies. The push consists of a deterrent, to push the cabbage root flies away from the main cash crop, such as dimethyl disulfide (Eigenbrode et al., 2016;

Lamy et al., 2018; Lamy et al., 2017). The pull element is an attractant, a reason that will draw the flies to another host plant (Eigenbrode et al., 2016). Chinese cabbage, particularly the *Brassica rapa* subsp. *pekinensis*, is known to be more attractive to cabbage root flies and can be used as a trap crop (Lamy et al., 2018; Lamy et al., 2020).

Trap crops can be a part of a push-pull system; they can be used as an attractant, or to intercept, acting as a mechanical barrier or to capture pest insects, like cabbage root flies (Badenes-Pérez, 2019). Trap crops are either a different species, cultivar or growth stage from main crop species that is favoured by the target insect (Cook et al., 2007). Groupings of attractive and repellent stimuli with trap crops can be used to manipulate cabbage root fly behaviour. Many studies have been performed to try and find the best push-pull-trap system for cabbage root flies, evaluating different parts and combinations; the trap crop, the push stimuli and the pull stimuli.

To evaluate different potential trap crops Rousse et al. (2003) tested six plants; radish, rutabaga, Chinese cabbage, turnip, white mustard and cauliflower. Plots with Chinese cabbage as a trap crop generally had more natural predators and less attack on the main broccoli crop on rows in close vicinity to the Chinese cabbage rows (Rousse et al., 2003). There is a preference of cabbage root flies for Chinese cabbage compared to broccoli, so it is used as a trap crop (Kergunteuil et al., 2015; Lamy et al., 2018). A recent study by Lamy et al. (2020) shows that more eggs are laid on the *pekinensis* subspecies of Chinese cabbage, than broccoli. These studies show that cabbage root flies have a preference for Chinese cabbage cultivars over broccoli crops, which can be applied to trap cropping (Lamy et al. 2020). In addition to the attractiveness of Chinese cabbage itself, pull attractants can be added. Z-3-hexenyl-acetate on Chinese cabbage strips increased oviposition beyond only having trap crops (Lamy et al., 2018)

To study the effect of deterrents, DMDS (dimethyl disulfide) was applied to a sub-set of broccoli plants within a field plot. It was found to attract predators with a lower number of eggs found in treated plots but overall damage and number of pupae remained similar to control plots (Ferry et al., 2009). DMDS been used as a push stimulus in several other studies, and consistently observed to be an oviposition deterrent, while also attracting predators in field studies (Lamy et al., 2017; Lamy et al., 2018). Eucalyptol has also been found to reduce oviposition of cabbage root flies and act as a push stimulus (Lamy et al., 2017). There are many combinations of stimuli and trap crops in different geometric

distributions within a plot that can be used to manipulate the distribution of cabbage root flies in a field.

1.3.8. Natural Enemies: Nematodes, Parasites and Predators

There are natural predators, and parasitoids that can affect cabbage root flies in their agroecosystems (Finch and Collier, 2000b). Entomopathogenic nematodes such as *Steinermema feltiae* can be used to reduce cabbage root fly populations by infecting larvae and pupae (Chen et al., 2003; Nielsen and Philipsen, 2004; Herbst and Hommes, 2015; Bracken, 1990; Finch, 1993). Temperature and soil moisture affect entomopathogenic nematode performance; they prefer drier soils (Herbst and Hommes, 2015, Chen et al., 2003). High densities of juvenile nematodes per plant is needed to have a negative effect on cabbage root fly populations, which can be challenging to achieve in field conditions. Field studies are less successful than greenhouse results, potentially due to poor host finding by nematodes, and environmental conditions (Chen et al., 2003; Nielsen and Philipsen, 2004; Herbst and Hommes, 2015).

Predators feed on the early stages of the cabbage root fly. *Aleochara* species have been found in Norwegian populations of cabbage root flies (Jonasson et al., 1995). Adult *Aleochara bilinetata and A. bipustulata* feed on cabbage root fly eggs and larvae (Fournet et al., 2000; Langlet and Brunel, 1996). Their larvae parasitize cabbage root fly pupae (Brunel and Fournet, 1996) and emerge as adults from the cabbage root fly puparium. In natural populations, the timing of adult beetle generations and parasitic larvae mismatch with vulnerable cabbage root fly stages (Finch, 1993; Finch, 1996). As a management strategy, they would have to be applied at appropriate times to best target vulnerable cabbage root fly stages (Finch, 1996).

Parasites can work to kill pupae during or shortly after pupation (Coaker and Finch, 1971). *Aleochara* larvae parasitize cabbage root fly pupae (Brunel and Fournet, 1996; Fournet et al., 2000; Brunel and Nénon, 1996) and emerge as adults from the cabbage root fly puparium. Another common parasitoid of cabbage root fly is *Trybilographa rapae*. *T. rapae* preferentially lays eggs in third instar cabbage root fly larvae, but will parasitize first and second instar larvae as well (Neveu et al., 2000; Kaecem et al., 1996). Adults will emerge from cabbage root fly puparium (Brunel and Fornet, 1996; Kaecem et al., 1996). Unlike *Aleochara* species, the lifecycle of *T. rapae* aligns better with cabbage root fly generations because it has a long adult life span so it can infect all larval stages (Kaecem et al., 1996).

Sometimes biological control agents' conflict with each other in practice; entomopathogenic nematodes have been observed to negatively affect both *Aleochara* and *T. rapae* (Nielsen and Philipsen, 2004). Adult *Aleochara* are attracted by volatiles emitted by cabbage root fly damaged plants (Ferry et al., 2007; Brunel and Nénon, 1996), but studies with volatiles should include responses of *Aleochara* species and *T. rapae* as they do not always respond the same (Lamy et al., 2017; Nielsen and Philipsen, 2004). Overall, natural enemies are an essential part of cabbage root fly management.

An example of how natural enemies can help to manage cabbage root fly pupae populations are in organic farms. Lower pupae to egg ratios are observed in organic farming systems than conventional (Meyling et al., 2013). This difference is partly due to natural enemy predation of eggs; the predation of pupae was found to be an insignificant difference between the two systems (Meyling et al., 2013). Oviposition is not reduced as a result of organic farming practices. Organic practices generally benefit natural predator populations which prey upon cabbage root fly eggs (Meyling et al., 2013). Natural predators have a role in cabbage root fly management, and should be encouraged when designing control strategies.

Overall, there many alternatives to chemical insecticides have been studied. Some are more promising than others, and many can be used as part of an integrated pest management strategy. Incorporating push-pull-trap crop systems with natural enemies and biocontrol agents is a possible management strategy. One biocontrol agent yet to be discussed, that has applications for cabbage root fly management, is entomopathogenic fungi.

1.4. Entomopathogenic Fungi

Many fungal entomopathogens belong to Ascomycota, the order Entomophthorales, Neozygitales, or Hypocreales (Boomsma et al., 2014; Hajek and Meyling, 2018). Hypocreales fungal entomopathogens commonly belong to the family Clavicipitaceae. This order generally has a broad insect host range (Vega et al., 2012). To reliably identify species of fungal entomopathogens, molecular based characteristics are used because many species are cryptic and hard to distinguish based on morphology (Hajek and Meyling, 2018; Bischoff et al., 2009). They are facultative, thus can survive outside of their insect host, as endophytes, or on non-living material (Vega et al., 2012). They can display both anamorph and

teleomorph stages (Vega et al., 2012), however anamorphic stages are more common among Hypocreales (Hajek and Meyling, 2018).

Entomopathogenic fungi are natural enemies of insects (Hajek and Delalibra, 2010; Hajek and Meyling, 2018; Meyling and Hajek, 2010). Entomopathogenic fungi produce spores, also known as conidia, which are the infective agent. Spores of fungal entomopathogens attach to the insect cuticle. Spores do not need to be consumed by an insect to be infectious (Hajeck and Meyling, 2018). Some fungi produce mucilage or adhesion proteins to assist in attachment or enzymes to help degrade insect cuticles like chitinases, proteases or lipases (Vega et al., 2012). After spores attach, they penetrate the insect cuticle. Spores penetrate the insect cuticle using mechanical pressure or by producing enzymes. The fungus uses up the insect's resources, and can produce toxic secondary metabolites (Hajek and Meyling, 2018; Roy et al., 2006; Vega et al., 2012). Fungal hypha grow into the insect hemocoel, using nutrients to grow and produce conidia, ultimately leading to insect death (Hajek and Meyling, 2018; Meyling and Hajek, 2010; Vega, 2018; Vega et al., 2012).

Conidia are produced on infected insects, and are passively dispersed via wind or rain, or associate with plant roots (Vega et al., 2012; Hajek and Meyling, 2018). Conidia are released upon host mortality; they can be released in a mass episode or in short cycles depending on environmental conditions (Hajek and Meyling, 2018). While no specialized duration structure is made by most Hyprocrealean entomopathogens, these fungi can be found in soil for years (Hajek and Meyling 2018). Once conidia come in contact with a susceptible target host, the cycle repeats itself (Hajek and Meyling, 2018). Infection cycles and lethal effects on target insects can be observed in lab and in the field.

1.4.1. Fungal Entomopathogens as Biological Control Agents

Insects can be crop pests, such as cabbage root flies. Using fungal entomopathogens to reduce harmful insect populations is a control strategy. Fungal entomopathogens have been used for decades for various insect pests (Helen E. Roy et al., 2010; Vega et al., 2012). There are over 170 products made from at least 12 fungal entomopathogen species (Roy et al., 2010; Vega et al., 2012). Despite the relatively long history of use, there is limited success with biological control agents, partly due to the unpredictable effects when compared to more consistent chemical pesticides (Roy et al., 2010). However, due to declining options for chemical control methods and increasing interest in sustainability, attention and research into fungal entomopathogens as biocontrol agents is rising (Chandler et al., 2008; Köhl et al., 2019).

There are advantages and disadvantages to using entomopathogenic fungi for insect control. Some advantages are that entomopathogenic fungi are largely ubiquitous; they can be found in many environments and geographic regions. Entomopathogenic fungi can associate with the rhizosphere of different plants so will be present to infect insect pests. They do not need to be consumed to be infective, they kill their hosts via infective spores once contact occurs (Meyling and Hajek, 2010). Their insect host range can be very specific or very broad, thus there is potential to find fungal entomopathogens for a variety of insect pests. They have few negative effects; they do not usually infect beneficial insects, or cause harmful reactions in plants (Vega, 2018; Hajek and Meyling, 2018). They generally act faster than some other natural enemies, like parasitoids, but are still slow when compared to chemical pesticides (Hajek and Meyling, 2018; Shapiro-Ilan et al., 2006). However, it is challenging to achieve consistent, reliable performance of fungal entomopathogens for insect control, especially in field environments (Chandler et al., 2008; Vega, 2018; Vega et al., 2012; Roy et al., 2009).

Fungal entomopathogens spend a lot of time outside of their insect host. They can be vulnerable to abiotic environmental factors. Temperature can cause heat damage. Moisture can be necessary for some lifecycle stages, but too much rain can wash away conidia from target field (Hajek and Meyling, 2018; Shapiro-Ilan et al. 2006). Oxygen levels, soil composition, and pH can have minor influences on effectiveness. A major disadvantage is sensitivity to UV light. Direct sunlight can damage spores or reduce the number of conidia produced due to damaging effects of UVB rays (Hajek and Meyling, 2018). Fungal entomopathogens need to be robust to environmental conditions in the field, including UVB or applied where it is not in direct sunlight, such as in the soil. Plants may also respond to and influence fungal entomopathogens via root exudates, surface chemistry and plant volatiles, which can affect success as well (Vega et al., 2018; Roy et al. 2009). Application to crops can be tricky; it requires knowing where the target insect is usually found, and when it is at its most vulnerable stage, and an entomopathogenic fungi isolate that can colonize the same environment and infects the targeted life stage.

Infections by entomopathogenic fungi are spread by horizontal transmission. Conidia, infect susceptible hosts through contact. These spores either exist in the soil, associate with plant rhizospheres, or spread by contact of infected living host with uninfected possible host at the time of conidia production (Hajek and Meyling, 2018). Thus, dispersal is dependent on

host density, conidia production and environmental conditions (Meyling and Hajek, 2010; Hajek and Meyling, 2018).

To cause a lethal infection there needs to be enough virulent conidia to overcome host defenses. Insect hosts can have defenses like avoidance behaviour and produce antimicrobial compounds that act as sanitizers. Melanised cuticle or manipulating temperature outside of the fungi's tolerance ranges also disrupts infection by fungal entomopathogens (Hajek and Meyling, 2018). Once these defenses are overwhelmed, and the insect cuticle penetrated by the fungal entomopathogen, infection can occur.

To cause an epizootic event, a large scale outbreak, to reduce target insect pest populations the three conditions need to be met: 1. Presence of susceptible hosts at a certain density, 2. High enough levels of fungal entomopathogen inoculum, such as concentration of conidia, for infection and insect death, 3. Environmental conditions have to favour fungal entomopathogen establishment, infection of target insect and dispersal of infective conidia (Hajek and Meyling, 2018; Shapiro-Ilan et al., 2006). When these conditions align, entomopathogenic fungi such as *Metarhizium* can infect and kill target insect pests, including cabbage root flies.

1.4.2. *Metarhizium brunneum* as a Fungal Entomopathogen

Metarhizium is a widespread, well-characterized genus of entomopathogenic fungi belonging to the Hypocreales family. Conidia infect many Diptera insect hosts and can be found in the soil, and associated with plant roots (Hajek and Delalibra, 2010; Hajek and Meyling, 2018; Meyling and Eilenberg, 2007; Vega et al., 2012). Generally, *Metarhizium* species are isolated from soils or insects in anamorphic phase, with green coloured conidia (Bischoff et al., 2009). Species within *Metarhizium* are often indistinguishable morphologically as colour, shape and size of conidia often overlap (Bischoff et al., 2009). Multi-locus molecular and DNA sequencing techniques have recently been used to parse out and identify different species within this cryptic genus (Bischoff et al., 2009; Rehner and Kepler, 2017). The main phylogenic clade researched for potential biocontrol agents is the PARB clade including *M. pinghaense, M, anisoplaie, M. robertsii, and M. brunneum*, with *M. brunneum* being the most basal lineage (Bischoff et al., 2009; Rehner and Kepler, 2017). Throughout some previous literature of *Metarhizium* species, different names have been given to different species, with some species excluded, and others added back in (Bischoff et al., 2009). This entomopathogenic fungus is cryptic and isolates can be challenging to identify.

Metarhizium species can be found in most countries, spread by wind, insect vectors or human activity, but they seem to have an inherent ability for long distance dispersal (Bischoff et al., 2009; Rhener and Kepler, 2017). Climate preferences and ecological adaptions have unique patterns linked to the origin of the *Metarhizium* species in question (Rhener and Kepler, 2017). *M. brunneum* is the dominant species in Europe, with a general Holarctic distribution (Rehner and Kepler, 2017). *Metarhizium brunneum* has been isolated from Norwegian soil (Klingen et al., 2015).

Within smaller geographic scales, such a field, or meadow, *Metarhizium* species can be found in association with plant roots and in insect hosts (Meyling and Eilenberg, 2007; Wyrebek et al., 2011). Some studies indicate that different species of *Metarhizium* associate with different plant types, but this hypothesis is not consistent in all agroecosystems. Wyrebek et al. (2011) found that *M. brunneum* most often associated with shrubs and trees, while *M. robertsii* was associated more with grass roots, potentially indicating some exclusion or competition for rhizosphere resources. Both species co-occurred with wildflowers (Wyrebek et al., 2011). On the other hand, Steinwinder et al. (2015), found that *M. brunneum* was most commonly isolated within fields of oats, rye and cabbages. Plants themselves recruit or influence different compositions of *Metarhizium* populations in and between different agroecosystems (Steinwender et al., 2015). Overall, *Metarhizium* species are potential biocontrol agents for the insect pests of several crop plants, with *M. brunneum* isolates being a focus of much interest.

1.5. Entomopathogenic Fungi and Cabbage Root Flies

Entomopathogenic fungi have been used for many years in various laboratory, greenhouse and field studies as a method to reduce pest populations. Ones most frequently studied include *Beauveria bassiana, Metarhizium anisopliae* and *Metarhizium brunneum* on cabbage root fly larvae and pupal stages (Bruck et al., 2005; Chandler and Davidson, 2005; Myrand et al., 2015; Razinger et al., 2018; Vänninen et al., 1999a; Vänninen et al., 1999b). Susceptibility of cabbage root flies and effectiveness of the fungal entomopathogen can depend on lifecycle stage of cabbage root flies, conidia concentration and application method of conidia, as well as the experimental system.

Studies have been performed on adult cabbage root flies and *Entomophthora muscae*, and found to cause epizootics (Klingen et al., 2000). Directly exposing second and third

cabbage root fly larvae to *M. anisopliae* at a concentration of $1.5x10^{10}$ conidia/dish has been shown to cause 40-50% mortality in laboratory studies (Vänninen et al. 1999a). However, applying $2x10^{8}$ conidia/plant in the greenhouse did not translate to a similar result in this particular study, as no reduction in pupae numbers was observed (Vänninen et al., 1999b). In greenhouse studies, *M. anisopliae* was observed to reduce the number of pupae found per plant, when (40ml of $1.x10^{8}$ conidia/ml) conidia applied as a soil drench (Chandler and Davidson, 2005). *M. anisopliae* has been observed to infect and sporulate on cabbage root fly eggs, larvae and pupae when conidia were directly applied, and in soil when conidia were applied at a $3.85x10^{6}$ conidia/g of soil (Razinger, et al., 2014a). In laboratory assays replicating in-furrow and broadcast application of *M. anisopliae* conidia to soil was observed to be lethal to second instar *D. radicum* larvae (Bruck et al., 2005). Drench application, applying a liquid suspension of conidia directly to the base of the plant, has been observed to reduce the numbers of larvae and pupae recovered from the plants in a greenhouse environment using *M. anisopliae* (Chandler and Davidson, 2005). Results in soil or in greenhouse environments can have conflicting outcomes between different experimental set ups, as shown by comparing the aforementioned studies. This research sets the foundation for further analysis of *M. brunneum* on cabbage root fly larvae and pupae.

1.5.1. *Metarhizium brunneum* and Cabbage Root Fly

Several studies have been performed in different laboratory, greenhouse and field conditions, at varying concentrations of conidia and by different application methods. Root associations have also been studied for *Metarhizium brunneum*. Roots of cauliflower have been tested for rhizosphere colonization, and show that *M. brunneum* can persist in soil environments and associate with plants (Razinger et al., 2014a). Application methods have been compared with regards to rhizosphere colonization by *M. brunneum*. Seed-coating cauliflower seeds with *M. brunneum* were compared to drench application of *M. brunneum* conidia in liquid suspension ($5.3x10^{5}$ conidia/mL). Drench application colonized the cauliflower rhizosphere better than seed-coating (Razinger et al., 2018). Being able to colonize the rhizosphere is important for an entomopathogenic fungi when looking to target pests that feed on roots and tubers of plants, because it will be in the same environment as the targeted insect.

Metarhizium brunneum has been shown to infect and kill cabbage root flies at the larvae and pupae stages. Two examples are studies by Myrand et al. (2015), and Razinger et

al. (2014b). Laboratory studies of *M. brunneum* (F52 isolate, $1x10^8$ conidia/ml) effectiveness on third instar larvae reduced hatching from pupae by up to 79% compared to control treatments when applied to sand that larvae were then added to (Myrand et al. 2015). Razinger et al (2014b.) also observed significant larval mortality as a result of directly inoculation larvae with conidia. Direct inoculation of $1.15\ x10^7$ conidia/mL of *M. brunneum* to cauliflower seedlings resulted in infection and mycosis of cabbage root fly pupae in a greenhouse environment (Razinger et al., 2014b). These in-vitro and greenhouse studies have some promising results for using *M. brunneum* isolates for potential cabbage root fly management in the field.

In field studies, *M. brunneum* was shown to non-significantly reduce the number of pupae found when liquid suspension of conidia ($1.15x10^7$ conidia/ml) was directly added to plant roots (Razinger et al., 2017). Another field study directly applied the same concentration of *M. brunneum* conidia but the number of pupae and larvae recovered did not differ from control treatments (Herbst et al., 2017). Overall, field studies using *M. brunneum* isolates is an area requiring further research for practical applications in integrated pest management for cabbage root flies.

1.6. Aims and Objectives

Metarhizium brunneum and its potential to manage cabbage root fly populations is the focus of this thesis. Two greenhouse studies and one field season will be discussed. The capability of *M. brunneum* to infect cabbage root fly larvae and pupae in greenhouse and field environments will be analyzed. The effects of this fungal entomopathogen on host choice and fecundity of surviving adult flies will also be analyzed. This thesis aims to answer three questions with one overarching theme:

1. Will exposure to *M. brunneum* in field or greenhouse conditions reduce cabbage root fly populations through pupal mycosis?
2. Does *M. brunneum* colonization of host plants affect oviposition by adult cabbage root flies?
3. Will flies that hatch from pupae collected in *M. brunneum* treated plots have reduced fecundity?

Overall, should *M. brunneum* be used as a biological control agent of cabbage root flies in integrated pest management?

2. Materials and Methods

2.1. *Metarhizium brunneum*, Met. 250/O2 procedures

2.1.1. Fungal Culture

Metarhizium brunneum 250/02 is the Norwegian isolate used in this study. To prepare a master plate to be a source of inoculum for future experiments, a sample (NCRI 250/02 4+GlyC 12/12/08) was taken from the -80°C freezer. Once thawed 50µL was added to two previously made SDA (Sabouraud Dextrose Agar) plates, and 10µL to a third. Plates were sealed with parafilm and placed in a plastic box, which was wrapped in aluminum foil and placed in a dark cupboard in the lab room (ca. 22°C). Plates were incubated for 21 days.

To ensure the correct fungus was grown without contamination, a master plate was checked. Some spores were scraped from one plate with a needle and added to 1-2 drops of lactic acid on a glass slide. Spores were observed under a light microscope and found to be the correct elongated oval shape, within the size range for *M. brunneum*.

2.1.2. Inoculum Plates

Inoculum plates were prepared from one master plate for the initial greenhouse study and field experiment. Spores were transferred from master plate to SDA plates with an inoculum loop in sterile conditions. Inoculum plates were incubated for 21 days before use.

For the initial greenhouse study 5 plates were prepared. For second greenhouse study 12 plates were inoculated from a second master plate made from the same isolate, as the original master plate became too wet to use. For the field season 25 plates were prepared.

2.1.3. Rice Preparation

200g parboiled rice was measured into clear plastic autoclave bags (approximately 2L). 90mL of deionized/distilled water was added to each. The bags were folded over three times and sealed with three stables across the top, carefully to prevent the staple from piercing the bag more than once. A strip, approximately 7cm of autoclave tape was added to one side of the bag to allow for sealing after inoculation with fungal suspension. Bags were labelled with the date, and prepared 1-2 months in advance of inoculation with Met. 250/O2. Bags were autoclaved for 5 minutes at 121°C. Sterile bags were laid flat at room temperature to dry,

mixing them every day for approximately 1 week. Once dry, they can be placed in the fridge (4°C) until use. Procedure follows what was described at the University of Copenhagen (S. Thapa and N.V. Meyling pers. comm. 2018).

2.1.4. Fungal Suspension Preparation – Rice Inoculation

After inoculum plates showed substantial growth and spore production, as indicated by green colour, suspensions were prepared following the procedure developed by S. Thapa and N.V. Meyling (pers. comm, 2018) at the University of Copenhagen. First 0.01%, Tween20 was added to sterile water with a pipette. This is with the exception of the first greenhouse study where 5% Tween20 was added due to a calculation error and using a different detergent from the Copenhagen procedure, who use 0.05% TritonX instead.

Pipette tips used had cut tips, where the ends were cut with scissors before autoclaving the tips to allows for better suction of spores, so they do not get caught in the smaller openings as easily. Tween20 was slowly added to each inoculum plate with a pipette and spores were scraped from each with a sterile spatula. Spores were transferred from plates to a 50ml centrifuge tube with 5mL pipettes. The suspension was centrifuged for 3 minutes and 3000RPM, supernatant discarded and more Tween20 added until the suspension had been washed twice. A dilution series was prepared from the stock tube to 10^{-3} concentration by adding 500µL of the previous dilution to 4.5mL Tween20 sequentially. The concentration of the stock solution was assessed by counting spores from the 10^{-3} dilution with an haemocytometer. Suspension was added between the cover slip and glass slide with a 20µL pipette after vortexing for at least 30 seconds. Spores in 5 squares (for example A, B, C, D, E in the haemocytometer) of 15 cells were counted for each side of the haemocytometer, and an average calculated from these 10 observations. This process was usually repeated 2-3 times, and average from all was used. Germination plates were prepared to assess viability by adding 100ul of 10^{-2} dilution to two SDA plates and incubating them in darkness for 24-26 hours. Spores are considered germinated if the tail is as long or longer than the spore itself. Conidia are evaluated by placing two coverslips, one on each side of the agar plate and the first 100 conidia observed are used to assess viability (Fig. 3c).

After germination viability was assessed and found to be within an acceptable range (greater than 95%), stock suspension is added to sterile rice. Volume of suspension added was specified by the number of spores / ml in the stock suspension. 25mL of Tween20 was measured and injected into a bag of rice with a 30ml syringe. The added piece of autoclave

tape is peeled back approximately 2cm, the needle tip is stabbed through the plastic bag and solution injected. The needle is removed and bag resealed with tape. For the fungal inoculation, $2.00x10^7$ conidia/mL is added to a sterile glass cylinder, remaining volume up to 25mL is made of Tween20 so a total of $5.00x10^8$ conidia are added to the sterile rice. Rice was incubated in darkness for approximately 17 days, and thoroughly mixed every other day, to encourage even growth on each grain of rice. Concentrations and volumes of stock solution used for inoculations is shown in Table 1.

Equation 1: [conidia / ml = average spores x 2000 x dilution factor]
where 2000 is the conversion for the volume in each cell of the haemocytometer, and dilution factor is 10 for 10^{-1}, 100 for 10^{-2} 1000 for 10^{-3} depending on which dilution was used.

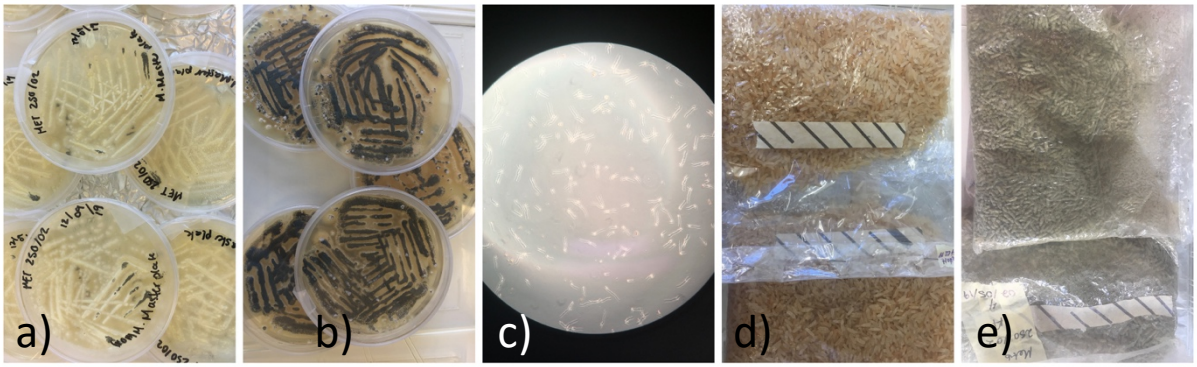


Figure 3: Inoculation of SDA media plates with Met 250/O2, (left to right) 1 week after inoculation and before harvesting 21 days after inoculation. Example of a germination viability test, where germinated conidia are shown at 200x magnification. Sterile rice and rice inoculated with Met 250/O2 suspension after 14 days.

2.1.5. Soil Inoculation – Preparation of Dosage

The concentration of spores per gram of rice was calculated by first mixing rice thoroughly within each bag. Bags were cut open and 1g of colonized rice was weighed.
This gram of rice was transferred to 10mL of 0.01%Tween20 in a 50ml centrifuge tube and vortexed for 1 min. This is the stock solution. Three 15mL tubes of 4.5mL Tween20 were prepared for a dilution series. 500µL of stock solution was transferred into the first dilution tube, tube was vortexed for 30 seconds. 500µL of 10^{-1} transferred to next tube, creates 10^{-2} dilution, after vortexing 500µL of from 10^{-2} was transferred to the remaining tube, creating 10^{-3} dilution. To assess germination ability 100µL of 10^{-2} dilution were added to 2 SDA

plates and incubated in darkness for 24-26 hours. Concentration of conidia in the stock solution created from 1g of rice was calculated using a haemocytometer.

Recommended soil application is $1.62x10^{12}$ conidia/m^3 of soil based off of the application rate of commercial product Met52 (Novozymes Biologicals Inc., USA; S. Thapa, pers. comm, 2018). The grams of rice needed to achieve this rate can be calculated based off of how many conidia are present in 1g of colonized rice and the desired soil volume to be inoculated. Volumes of rice applied to soil in each of the experiments is given in Table 1.

Equation 2: Z = conidia in 1 gram of rice; 1 conidium in 1 / (Z) g rice
$1.62x10^{12}$ conidia in (1 / Z)($1.62x10^{12}$) = Y g rice
Y g rice needs to be added to each m^3 of soil to achieve $1.62x10^{12}$ conidia /m^3
Y/(1000) to get g of rice per litre of soil.

2.1.6. Selective Media Preparation

Selective media for growth of Met. 250/O2 was made according to the recipe obtained from the University of Copenhagen (S. Thapa and N.V. Meyling, pers. comm. 2018). For 500mL of media: 5g of peptone, 10g glucose (dextrose) and 6g of agar were measured into a 500mL glass flask with 500ml of deionized water and a stirring magnet. 200µL of dodin (0.1g/mL) was added. PH was adjusted between 6.3-6.5. Media was autoclaved and cooled until approximately 60C. This was measured by touch; if it could be held at a temperature that was almost too hot it was considered to be at approximately 60C. Once cool enough, 500µL streptomycin (0.6g/ml), 500µL tetracycline (0.05g/ml) and 1000µL cycloheximide were added and media stirred for 1 minute with a magnetic stirrer. Once cool enough to hold, media was carefully poured from the glass flask into 5cm petri dishes until plate is just covered. Plates were stored in a cold room until use (4C).

Table 1. Summary table of concentration of *M. brunneum* conidia added and germination viability at each step of the performed studies.

	Greenhouse 1	**Field Study**	**Greenhouse 2**
Average conidia isolated from inoculum SDA plates	76 conidia/square (10^{-2} dilution)	Stock A: 41.88 conidia/square Stock D: 44.65 conidia/square (10^{-3} dilution)	15.2 conidia/square (10^{-3} dilution)
Initial germination viability	97%	Stock A: 97% Stock D: 97%	95.25%
Concentration of stock solution added to sterile rice bags	$1.52x10^{7}$ conidia/mL	Stock A: $8.38x10^{7}$ conidia/mL Stock D: $8.93x10^{7}$conidia/mL	$3.04x10^{7}$ conidia/mL
Total amount of conidia added to sterile rice bags	$1.99x10^{8}$ conidia	$5.0x10^{8}$ conidia in: Stock A: 5.969mL Stock D: 5.599mL	$5.0x10^{8}$ conidia in 16.447mL stock solution
Average conidia isolated from 1 g colonized rice	34 (10^{-2} dilution)	A1: 7 conidia/square A2: 8.1 conidia/square D1: 6.9 conidia/square (10^{-3} dilution)	10.1 conidia/square (10^{-3} dilution)
Germination viability of conidia from 1g colonized rice	98%	A1:97% A2: 96.25% D1:99%	97.5%
Conidia/gram of colonized rice	$6.9x10^{7}$ conidia/g	A1: $1.40 x10^{8}$ conidia/g A2: $1.62 x10^{8}$ conidia/g D1: $1.38 x10^{8}$ conidia/g	$2.02 x10^{8}$ conidia/g
Grams of rice used for soil inoculation (300mL of soil)	7g colonized rice	A1: 3.47g of rice A2: 3g of rice D1: 3.5g of rice	40.1g of rice (inoculation 5L of soil)

2.2. Greenhouse 1 Design

The greenhouse was set up with 11 replicates of 3 treatments. The three treatments were: Tween20 inoculated rice, Met. 250/O2 colonized rice and control (no rice). Within each treatment 3x300mL pots were filled with soil and 2 radish seeds (Cherry red variety) were planted. To minimize the risk of contamination, control treatments were planted first, followed by Tween20 and lastly Met. 250/O2 pots.

1. **Control**: soil was added to each pot, firmly pressed down and a fingertip indentation made in the center of the pot. Two seeds were added to the indent and covered with soil.
2. **Tween20**: soil was added to the pot, a medium sized hole (ca 2cm x 2cm) was made in the center and 7g of rice added to the pot. A small layer of soil was added to lightly cover the rice, and two seeds were added.
3. **Met. 250/O2**: treatments were potted with the same method as Tween20 but with Met. 250/O2 colonized rice.

The replicates were placed on the greenhouse table according to random design made in R, with six replicates on one table, and five on the other, with a set of spare plants for each treatment (Fig. 4). Radishes were watered once per day or every other day for the entirety of the study. After one week, pots that had multiple seedlings were thinned so that only one remained.

WALL	1.Tween	Met	Control	DOOR	S: Control	Met	Tween	WALL
	2.Tween	Control	Met		11.Tween	Control	Met	
	3.Met	Tween	Control	ASILE	10. Control	Tween	Met	
	4.Tween	Control	Met		9.Tween	Control	Met	
	5.Control	Met	Tween	WALL	8.Control	Met	Tween	
	6.Met	Tween	Control		7.Control	Tween	Met	

Figure 4. Randomized order for initial greenhouse experiment. Physical placement of trays on the greenhouse tables.

2.2.1. Egg Collection and Inoculation

Eggs were collected from the culture room at NIBIO. All eggs were laid within three days of collection, although flies were of different ages. Eggs were collected by scooping out sand from around turnips (the laying media) and floating in deionized water. They were then

filtered through a fine net and laid on dark fabric. Ten eggs were placed in individual 25mL cups with some deionized water. These were placed on control treatments, Tween20 treatments and then Met. 250/O2 treatments. Replicates 2, 3, 4, 5, 6, 7, 8 and 11 were inoculated with eggs on 26 April 2019. The other replicates were excluded from further study due to poor radish growth.

2.2.2. Radish Dissection and Pupae Collection

Radish dissection took place on 29 May 2019, 33 days after eggs were placed on radishes. Trays were harvested in order from 2, 3, 4, 5, 6, 7, 8 and 11. Control treatments were first, followed by Tween20 and Met. 250/O2 was harvested last for each replicate. Numbers of larvae and pupae were recorded. Radishes were cut in half and damaged assessed on a scale from 1-4 as described below:

1=no damage. **2**=evidence, ex. holes. **3**=tunneling present. **4**=more than 25% is damaged

Soil was washed and sorted through to collect pupae. Pupae from each treatment and replicate were placed into medicine cups, which were sealed with a lid and secured with tape. They were allowed to hatch at room temperature for three weeks.

2.2.3. Plating of collected pupae

Selective media was made according to the protocol outlined in section 2.1.6. Pupae were stored in a cold room (4C) until 10 September 2019. Unhatched pupae were surface sterilized by dipping them into 70% EtOH. Each was cut lengthwise with a razor blade on the lid of a petri dish, then was placed onto selective media with forceps. Each was assessed for mycosis under a dissection microscope. Plates were labelled and sealed with parafilm. They were stored in the dark at room temperature. Plates were incubated for 14 days, and observed for signs of mycosis every 3-4 days.

2.2.4. Data Analysis

Statistical analysis was performed in R (version 3.5.1, 2018-07-02). Numbers of pupae collected, survival, and mycosis of pupae were analysed with a Poisson Regression model, adjusted for over-dispersion and zero-inflation by the quasi-Poisson parameter.

2.3. Greenhouse 2 Design

This greenhouse study differs from the first in a few ways. Instead of inoculating small pots, trays with a volume of 7.3L (29cm x 50.5cm x 5cm depth) were used. Tween20 and Met. 250/O2 rice was portioned out the previous day into 5L plastic bags. One tray was lined with plastic to become water proof, and another was placed on top for planting. Treatments were placed in a randomly generated order and labelled with treatment and replicate number (Fig. 5). A total of 18 trays were prepared, 6 replicates of 3 treatments: Control, Tween20, and Met.250/O2. Soil was 60vol. % Sphagnumtorv H2-H4, 20vol. % Spahgnumtorv H6-H8, 10vol. % komposert bark and 10vol. % sand. Similarly, to previous studies control treatments were planted first, followed by Tween20, and concluding with Met. 250/O2.

1. **Control:** One litre of soil was added to the bottom of the top tray and spread evenly, followed by 5L of soil was added to the trays and firmly pressed down. Soil was measured in a 2L jug; large pieces of wood or rocks were removed as soil was added to it. A finger-tip depression was made approximately every 7cm, creating 3 rows of 5 holes. Two radish (Cherry Red variety) seeds were planted in each one, and lightly covered with soil. Tray was watered and a final 1L of soil added to the top, spread evenly and firmly pressed down. Trays were watered before placed in their designated order
2. **Tween20**: One litre of soil was added to the bottom of the tray, and 5L of soil was added to the bag containing rice. The bag was carefully, but thoroughly mixed by inverting and vigorously shaking overtop of the tray to be planted in case of breakage. The soil and rice mixture was spread evenly over the tray and pressed down. Small indentations were made 7cm apart, and two radish seeds placed in each hole, then lightly covered with soil. The tray was then watered, 1L of soil spread evenly across the top and pressed into place. This layer of soil covered the rice on the surface of the previous layer. The tray was watered before it was placed in its designated spot.
3. **Met. 250/O2**: The same process was repeated for Met. 250/O2 treatments as with Tween20 (Fig. 6). Additional care not to touch tween or control trays when placing Met. 250/O2 trays in their spots was taken. Gloves were worn throughout and regularly replaced.

The greenhouse room was set to 18-23C with 16hr photoperiods. Trays were watered every day for the first week, then every day to every other day. However, from October 23rd to November 18th 2019 radishes were watered with water containing fertilizer, instead of plain water. One week after planting seedlings were removed so there was only 15 plants/tray (Fig. 5). Control trays were thinned first, followed by Tween20, finishing with Met. 250/O2 Fungus gnats were observed as early as the 25th of October.

Interior Wall								
Replicate	1	2	3	4	5	6	Place	
Interior Wall	Tween	Control	Tween	Tween	Tween	Met. 250	Back	Exterior Wall
	Met. 250	Tween	Met. 250	Control	Met. 250	Control	Mid	
	Control	Met. 250	Control	Met. 250	Control	Tween	Front	
Center Aisle (middle of greenhouse room)								

Figure 5. Randomized order of treatments in the second greenhouse study. Physical placement in the greenhouse room

2.3.1. Fly Release

A tunnel was constructed over the greenhouse room table. A cage approximately 1m high, 1m wide and 4m long, and enclosed with insect netting. Velcro was attached along the length of the table, three equally spaced places in the net were cut with Velcro attached to create re-sealable openings for access to the radishes. The tunnel was constructed and radishes added on 31 October 2019 (Fig. 6). Yellow sticky traps were placed in the greenhouse room to catch any escapee flies.

Flies were caught with an aspirator from the culture kept at NIBIO into five vials. Both male and female flies were added in approximately equal numbers; 47 females and 57 males. They appeared to die and/or disappear within one day, so a second round of flies was added on November 8th. Approximately 55 females and 56 males were caught and released as previously performed. On 15 November 2019 yellow sticky traps were put up to catch surviving flies. The following day the netting was removed as no living flies were observed.

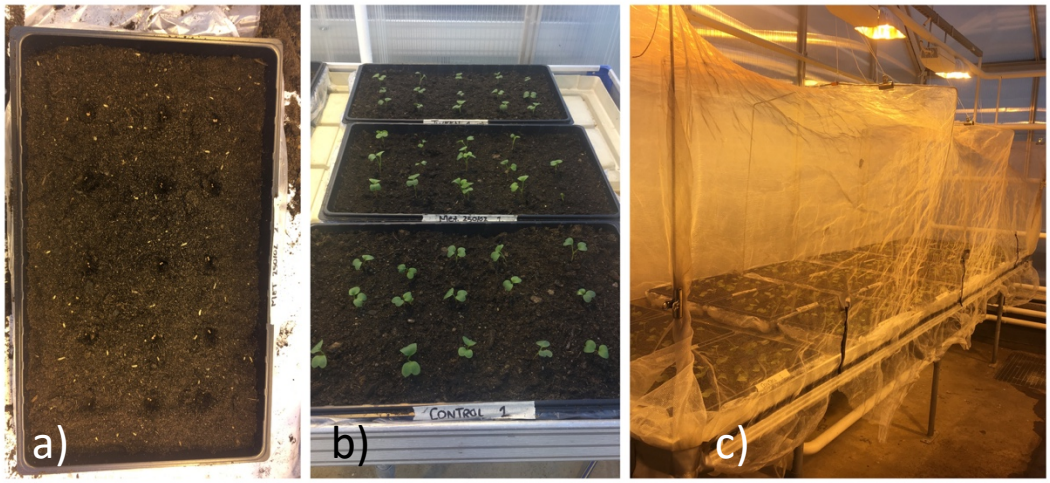


Figure 6. Experimental set up for the second greenhouse study: a) Radish seeds planted in three rows of five, Met. 250/O2 inoculated soil, b) Seedlings at time of thinning c) Constructed cage around greenhouse table, with 6 replicates of 3 treated radish trays inside

2.3.2. Radish Harvest

Pupae were collected from radishes on 11 and 12 December 2019. Each replicate, 1-6, was harvested in order of Control, Tween20, Met. 250/O2. Each radish was pulled out, loose soil rinsed off over a tray to collect any pupae that might fall out (Fig. 9). Radish was quartered and assessed for damage on a scale of 1-4. If larvae were found they were placed in a container of damp sand, one container per treatment per replicate. Radish number 3, 7 and 15 were collected for surface washing to check for colonization. Approximately 2 cm of the bottom part of the radish were collected, placed in individual plastic bags, sealed and stored in darkness at 4C until processing. Once all radishes were removed from the tray, soil was searched. Soil was sifted by hand into a tray, colleting pupae that were found (Fig. 7). Soil was washed with water to find any missed pupae that would float. Pupae were collected in individual 1.5ml centrifuge tubes. Work surface was sterilized with 70% ethanol after every replicate. Five days after harvesting, sand samples were washed out to find pupae from collected larvae. Each pupa was placed in an individual centrifuge tube, labelled with an L to represent larvae at date of collection. Samples were placed with the rest of the pupae to monitor hatching.

2.3.3. Surface Colonization

Loose soil was rinsed off. Approximately 1cm of root and adjoining 1cm of radish were measured, radish was trimmed to be less than 1cm tall and no more than 3cm wide (Fig. 7). This was to ensure radish would fit inside the petri plate. Root segments were placed in enclosed, perforated spoons. Each root was rinsed three times in sterile water, for 1 minute

each, and vigorously shaken to remove lingering soil. Spoons were placed on paper towel to dry before being firmly placed into selective media plates. Plates were sealed with parafilm. The work surface and tools were washed with ethanol after every treatment and paper towel replaced. Water was changed after every replicate. Plates were stored in darkness at room temperature for 14 days.

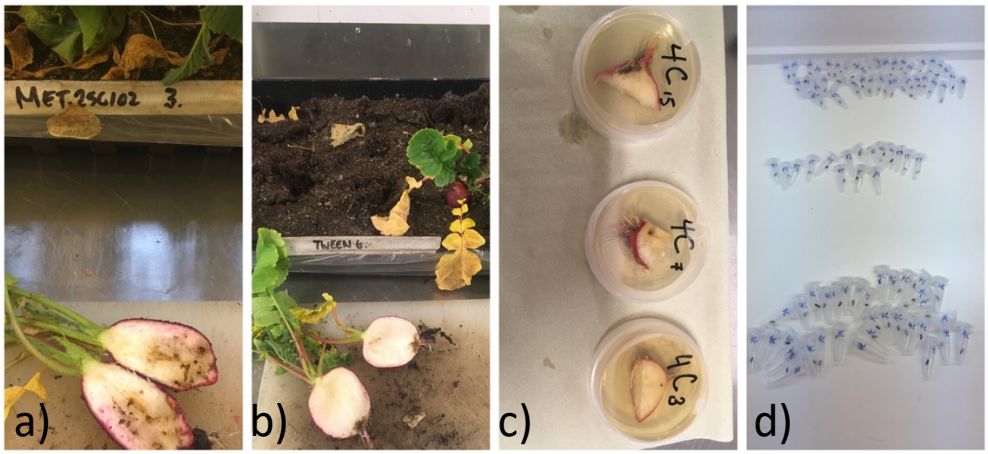


Figure 7. a) Radish harvesting, b) dissection and c) set up for surface colonization of radish roots by Met. 250/O2. d) Collected pupae stored in Eppendorf tubes and allowed to hatch

2.3.4. Hatching

Pupae in their individual Eppendorf tubes were laid out on in a lab room and left out in ambient daylight at approximately 20C (Fig. 7). They were checked 1-2 times a day. Once hatched, pupae were released into cages. Cages were organized based on treatment that each pupa was collected from: one for control flies, one for Tween20 flies and one for Met. 250/O2 flies. Each cage was placed under a desk lamp set for 16 hours of light (6am – 10pm) at room temperature. Cages contain a water source, and food source. This layout is similar to the culture room. Date, and sex or deformity, of the hatched fly was recorded.

2.3.5. Mycosis and Surface Sterilization

The method used consisted of a brief 70% Ethanol rinse in perforated spoons, followed by deionized water rinse, and a 1% sodium hypochlorite solution for 1 minute. The sodium hypochlorite wash was followed by two 30 second rinses in sterilized water (Lacey and Solter, 2012; Myrand et al., 2015). Perforated spoons were set on clean paper towel to air dry before each pupa was cut in half with a razor blade. Once cut in half, the pupa was firmly placed onto selective media plates prepared following the protocol in section 2.1.6. Plates were stored at room temperature (approximately 20C) in darkness. Pupae and plates were assessed at approximately 7 days, 14 days and 21 days. Several different isolates or species of

entomopathogenic and/or saprophytic fungi were observed. Small samples of each were taken and observed under a light microscope to observe structures and conidia to aid in identification. Slides were preserved in lactic acid and clear nail polish.

2.3.6. Data Analysis

Predictor variables, treatment and placement were chosen because treatment is of interest and where the trays were in the greenhouse room had an effect too. Some response variables, like the number of pupae and larvae collected, a normal distribution is assumed because these variables had a large data set with a normal distribution pattern. Others, such as the number of hatched flies, a Poisson distribution was assumed and a Poisson regression analysis used, because there was a large number of zeros, and a relatively small data set. If the residual deviance coefficient was larger than 1.5, a quasi-Poisson regression was used to account for over-dispersion. A binomial model was used to test M. brunneum infections due to the low frequency of infection, and observations in only the Met. 250/O2 treatment. Statistical analysis was performed in R (version 3.5.1, 2018-07-02). The following models were used:

Model: aov(Response Variable ~ Treatment + Placement)
Model: glm(Response Variable ~Treatment + Placement, family= poisson or quasipoisson)
Model: glm(Response Variable ~ Treatment + Placement, family="binomial")

2.4. Field Study Design

There were 12 replicates for the field study, each with 3 treatments of 20 plants. This requires 240 doses of Met. 250/O2 colonized rice and 240 doses of Tween20 inoculated rice. Three bags of rice were used to inoculate the plants; A1, A2 and D1 in Table 1. These bags had slightly different concentrations of conidia/g of rice, which led to 3 slightly different volumes of rice for field soil inculcation. Each bag was portioned out into 80 doses of colonized rice to give an inoculum of 1.62×10^{12} conidia/m^3 of soil, based on inoculating the surrounding 300ml of soil, similar to the first greenhouse study. The volume of Tween20 inoculated rice corresponded to the volume of Met. 250/O2 colonized rice used for the same replicate. These doses were measured out the day before planting.

For the field study, Chinese cabbage seedlings were obtained from a grower. Holes were made with planting pins approximately 50cm apart in 3 offset rows as demonstrated in Figure 8. Each treatment consisted of 20 plants, with a buffer zone of 7-10 untreated seedlings. In replicates 5-8 there were not enough cabbage seedlings to plant a buffer zone, bare soil was left open as a buffer zone instead. The order of treatments within a plot was determined by random sequence selection in R. To minimize potential for contamination all control treatment seedlings were planted first, followed by Tween20, and concluding with Met250/O2 for all replicates.

1. **Control**: Seedlings placed in an approximately 5-10cm deep, and covered with soil.
2. **Tween20**: Pre-measured Tween20 inoculated rice was poured into a hole, the cabbage seedling was placed overtop and covered with soil.
3. **Met 250/O2**: Pre-measured Met. 250/O2 colonized rice was poured into a hole the cabbage seedling was placed overtop and covered with soil.

Planting occurred in the rain, and all replicates were planted on 22 May 2019 and were regularly photographed (Fig. 9).

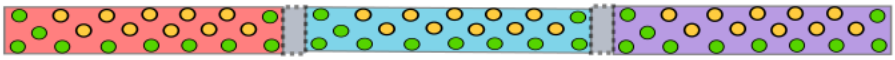


Figure 8. Schematic of a trial plot; three rows of staggered Chinese cabbage plants divided into three treatments represented by circles. Yellow circles at the interior of the treatment plot represent the plants inoculated with eggs, and later harvested. Grey box represents the buffer zone between treatments.

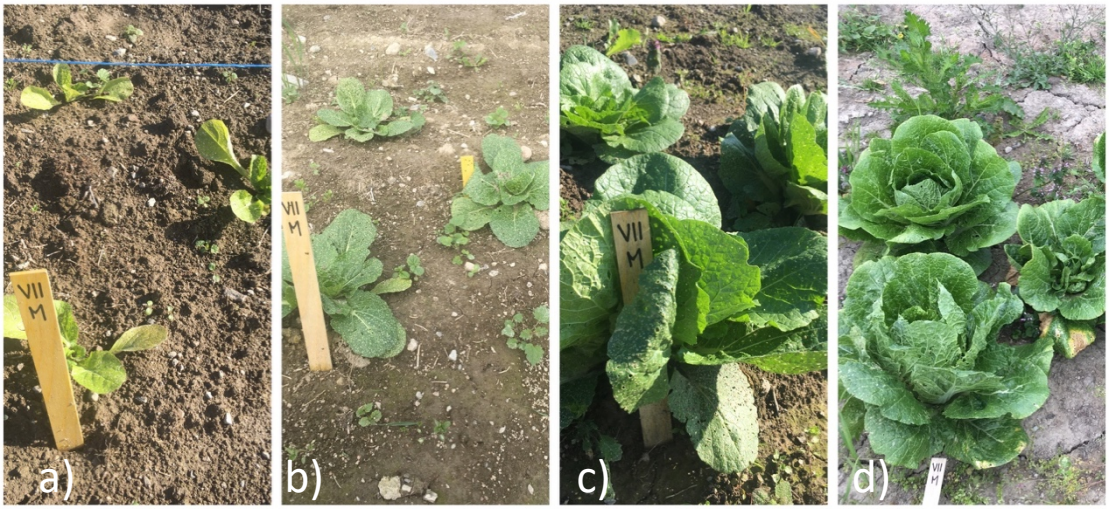


Figure 9. Growth over the field season of Chinese cabbage a) 1 week after planting, b) 3 weeks after planting, c) 5 weeks after planting and d) at harvest approximately, 8 weeks after planting.

2.4.1. Egg Inoculation

On 5 June 2019, 7 eggs were added to each of 10 plants per each treatment. The interior 10 plants were selected and a sub-plot containing them was marked with stakes (Fig. 8). In the morning, 7 eggs were portioned from a general container containing eggs that had been collected from the culture room on 3 June 2019 and 30 May 2019. Eggs were collected from fly cages of all ages, but were mixed together in a general collection container. From these 7 eggs were counted into individual 25ml medicine cups with distilled water. Egg suspensions were poured onto the base of the cabbage plants, then covered with 1-2 tablespoons of sand to prevent predation. This was done to supplement natural attack and ensure an even distribution of cabbage root fly pupae for further study.

2.4.2. Harvesting

Ten heads from each treatment were harvested, from the sub-plot that was inoculated with eggs on July 19 2019. Cabbage heads were cut at the base of the plant, close to the soil surface (Fig. 10). Heads were individually placed in labelled plastic bags, then placed in clear, larger plastic bags (one per treatment) for transport and storage. Heads were stored at approximately 4C until analysis. Roots and surrounding soil were collected with a soil corer. The corer was placed over top of the root and pushed into the soil; samples were taken between 8-12cm deep, and a soil volume of 600-800mL was collected. Two samples were taken with a shovel, due to rocks impeding the soil corer. If no roots were found to guide sampling, samples were taken from the approximate area that roots should have been; this occurred once. Some replicates had fewer samples due to plant death, some replicates had more, due to accidently harvesting the wrong heads; the correct ones were also harvested. Soil samples were individually placed in labelled plastic bags, and stored at 4C until analysis.

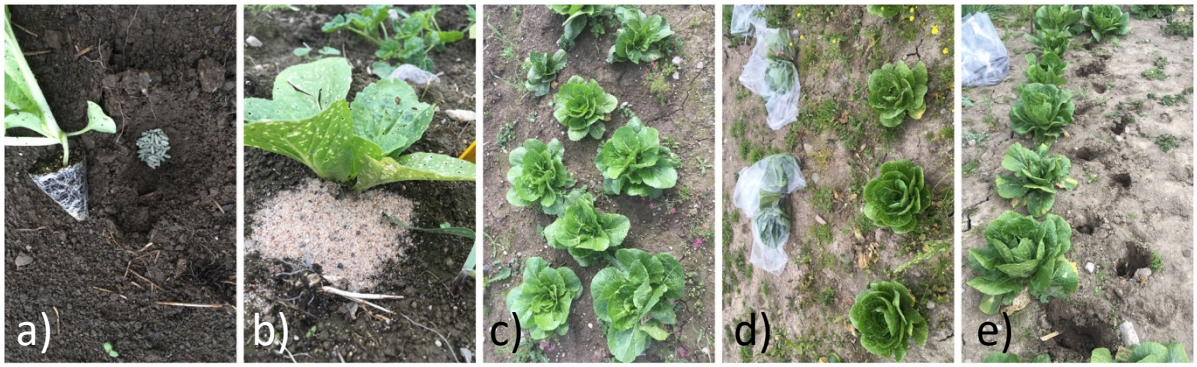


Figure 10. Photo timeline of Chinese cabbage field study from a) inoculation with Met. 250/O2 colonized rice and planting of seedling b). Plant after adding eggs and covering them with sand. c) Cabbage heads at harvest d) Harvesting cabbage heads, organized into individual bags e) Holes left by soil corer after the soil samples were collected

2.4.3. Dissection of Cabbage Heads and Roots, and Soil Samples

Cabbage heads were analyzed 3-5 days after harvest. The order of analysis was replicate 1 to 12, for each replicate the first treatment analyzed was control, followed by Tween20, and concluding with Met. 250/O2. The working area was cleaned after each treatment and replicate, 70% ethanol was used to sterilize the work area after each Met 250/O2 replicate.

For each head the number of larvae, number of pupae, and presence or absence of damage was recorded. Each head was cut in half, or in quarters, depending on size. Evidence of damage was visually assessed based on presence of holes, tunneling and/or rot. Photos of each head were taken (Fig. 11). Each leaf was peeled back, starting at the base, to check for larvae or pupae, larvae were gently pulled out of cabbage heads or stems with forceps. Larvae were placed in 250ml containers of sand, between ½ to ¾ full and dampened with tap water. All larvae from the same cabbage head were collected in the same container and the container was labelled with treatment and replicate (Fig.11). Containers were kept at room temperature for one week before storage at 4C. Pupae were individually stored in 2mL Eppendorf tubes, and stored at 4C in darkness. Replicates 1-4 were dissected on July 22, 5-8 on July 23 and 9-12 on July 24.

Soil samples were similarly assessed. Soil samples were placed in trays and visible pupae and larvae were collected. Roots were washed and cut in half to assess damage and collect the remaining larvae and pupae (Fig. 11). Soil samples were then washed with tap water and stirred, pupae that floated to the surface were collected. Larvae from each sample were placed in containers of damp sand and stored at room temperature for 1 week before storage at 4C. Pupae were placed in microcentrifuge containers. With the exception of replicate 9-12, 1 pupa was placed per tube (Fig. 11), but for these replicates up to four were stored in 1 tube until more tubes arrived the following week. Replicates 1-5, were assessed on July 29, replicates 6- 12 on July 31st.

Pupae that developed from collected larvae were assessed after two weeks. Pupa were stored in individual labelled Eppendorf tubes. All samples from control replicates were assessed, followed by Tween20, followed by Met. 250/O2. All samples were collected on August 12 for larvae collected from soil samples. Pupae from replicates 1-6 of the cabbage head larvae were collected 13 August, and 7-12 on the 15th. All pupae were stored in boxes to keep them in darkness, at 4^{o}C.

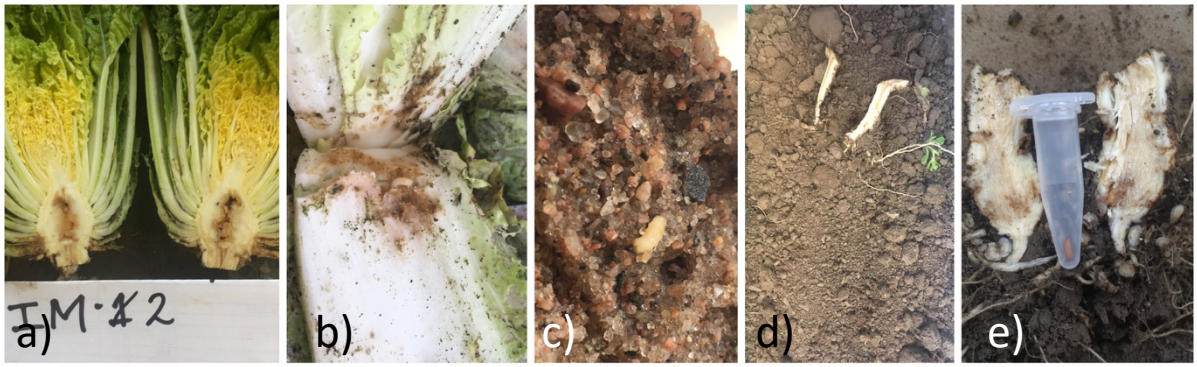


Figure 11. Dissection of Chinese cabbage heads and roots with surrounding soil sample. a) Cabbage head from Met. 250/O2 treatment showing damage, b) Cabbage head from control treatment with a larva in leaves c) Larva collected from heads into sand-filled container, until pupation d) Example of cabbage root in soil that was sifted through and filled with water to find floating pupa. e) Pupa collected from cabbage root stored in a Eppendorf tube until hatching

2.4.4. Hatching

Pupae that were originally collected from soil samples, or developed from larvae collected in soil samples were taken out of cold storage. They were set out to hatch at 19°C on January 22 2020 in their individual Eppendorf tubes. When hatched, the date, and sex or deformity of flies was recorded and the new fly was placed in a cage according to its treatment: Control, Tween20, and Met. 250/O2. Cages were stored at room temperature (approximately 20°C) with 16hr day lamps, food and water.

2.4.5. Egg Laying

One week after pupae stop hatching, a small slice, approximately 1cm by 4cm by 4cm, of rutabaga in sand was placed in each cage. Every few days eggs were collected and counted by rinsing sand with water and pouring floating eggs through black filter fabric. The number of eggs was divided by the number of female flies in the cage, and days between counting. Eggs were counted for two weeks. Egg laying was assessed by ANOVA statistical test in R.

2.4.6. Mycosis and Surface Sterilization

Pupae were placed in perforated, encapsulated teaspoons. They were dipped in 70% ethanol for 3-5 seconds, rinsed in distilled water, washed in 1% NaoCl for 1 minute, followed by 2x 30sec rinses in sterile water (Fig.12) (Lacey and Solter, 2012; Myrand et al., 2015). Spoons were air-dried on paper towel before the pupa was picked up with forceps and cut with a razor blade before being placed on prepared selective media.

Washes were changed after every treatment and equipment used disinfected with 70% ethanol. Plates were sealed with parafilm and stored in a dark cupboard at room temperature (approximately 21C) for 4 weeks, and checked for development approximately every 7 days. If the pupae displayed external mycosis before surface sterilizing the petri plates were marked.

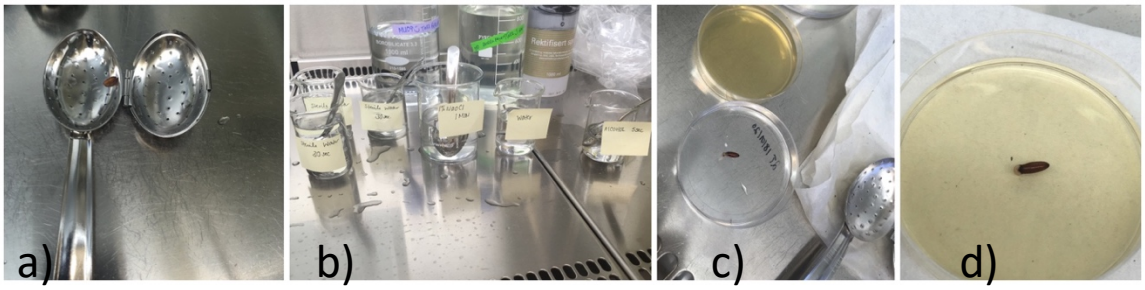


Figure 12. Surface sterilization process starting from a) placing a pupa into the enclosable perforated spoon b) into the surface sterilization washes, c) cut and d) placed on selective media.

2.4.7. Data Analysis

Similar statistical tests were performed for pupae results from field collections as in the second greenhouse study. Recovered pupae and larvae were analyzed as normally distributed while other variables were analyzed with Poisson regression models. *M. brunneum* infection was modelled with a binomial distribution similar to greenhouse 2. Egg laying was assessed by an ANOVA test, after log-transforming the data. The following models were used in R:

Model: aov(Response Variabel ~ Treatment)
Model: glm(Response Variable ~Treatment, family= poisson or quasipoisson)
Model: aov(logEggLaying ~ Treatment)

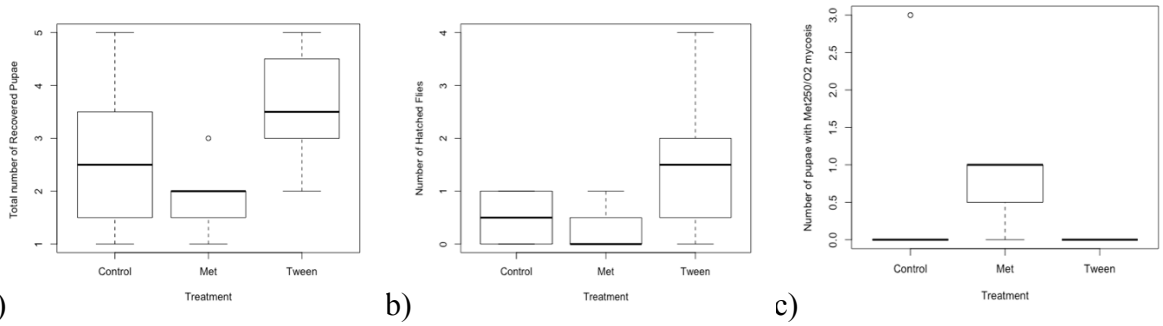
3. Results

3.1. Greenhouse 1 Study

3.1.1. Pupa Results

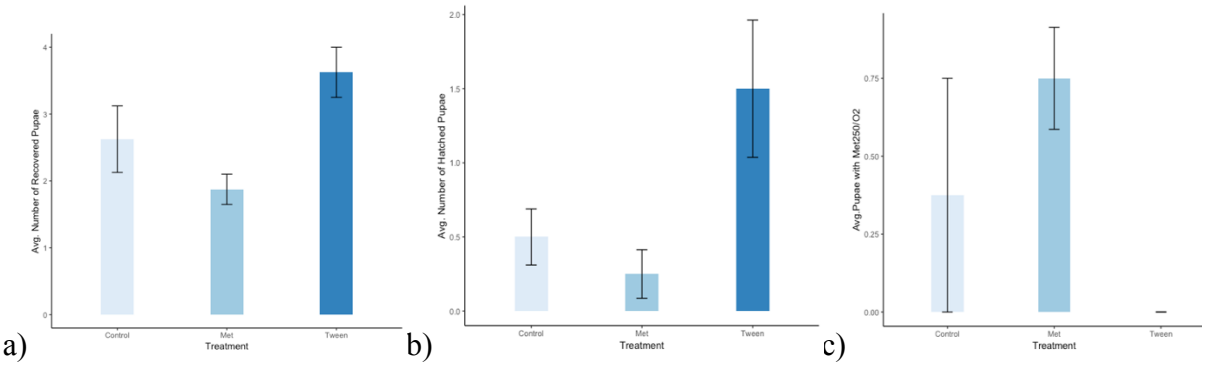
Few pupae were collected from the first greenhouse study, despite having 10 eggs added per radish. Distributions of recovered, hatched and Met. 250/O2 infected pupae are shown in Figure 13. Figure 14 shows the average numbers of recovered pupae, of which 18 hatched and 9 showed Met. 250/O2 infection. Met. 250/O2 infection was observed in control treatments. A summary of the main categories of pupa results is depicted in Figure 15.

Treatment did not have a significant effect on the number of pupae recovered (GLM, df=2, t=-1.38, p>0.05). Few pupae hatched, a Poisson regression analysis shows no significant effect of treatment on the number of hatched flies (Poisson Regression, df=2, z=-0.8, p>0.05). *M. brunneum* infections were observed in control and Met. 250/O2 trays, however treatment did not have a significant effect on mycosis (Poisson Regression, df=2, z=0.98, p>0.05). The majority of pupae died (Fig. 15). Treatment had no significant effect on pupa death (GLM, df=2, t=0.64, p>0.05). Damage to radishes was approximately the same from all treatments, with the majority of radishes rated between 3-4 on the damage scale.



a) b) c)

Figure 13. Boxplots showing distrubution of recovered (a), hatched (b) and *M. brunneum* (c) infected pupae from greenhouse study 1, eight replicates of three treatments; Control, Tween20 and Met. 250/O2.



a) b) c)

Figure 14. Average number of recovered, hatched and *M. brunneum* infected pupae collected from greenhouse 1 study, bars represent +/- SEM, n=8.

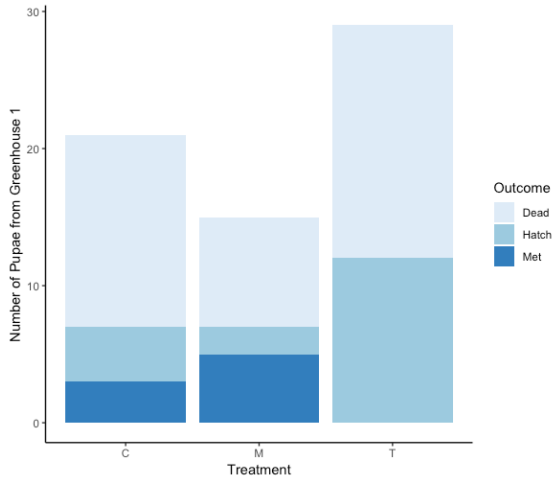
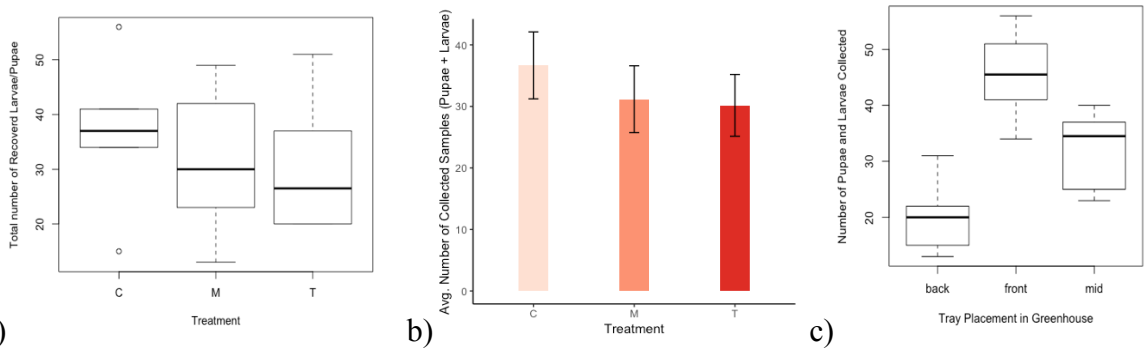


Figure 15. Results of pupae collected in greenhouse 1 study from 8 replicates of three treatments, C, T and M. Main categories of pupa results are either hatched into adult flies or Met. 250/O2 infection or unhatched and considered dead by other causes.

3.2. Greenhouse 2 Study

3.2.1. Pupa Results: Recovered Pupae and Larvae

The distribution and average number of pupae collected from each of the six replicates for the second greenhouse study are shown in Figure 16. Placement of trays in the greenhouse had a significant effect (GLM, df=2, F=23.63, $p<0.05$). Fewer pupae and larvae were collected from trays placed at the back of the table (Fig. 16c). More pupae and larvae were collected from trays in the middle and front of the table. Treatment is discussed in the oviposition section. Damage to radishes was uniform and severe, with the majority of radishes rated between 3-4 on the damage scale.

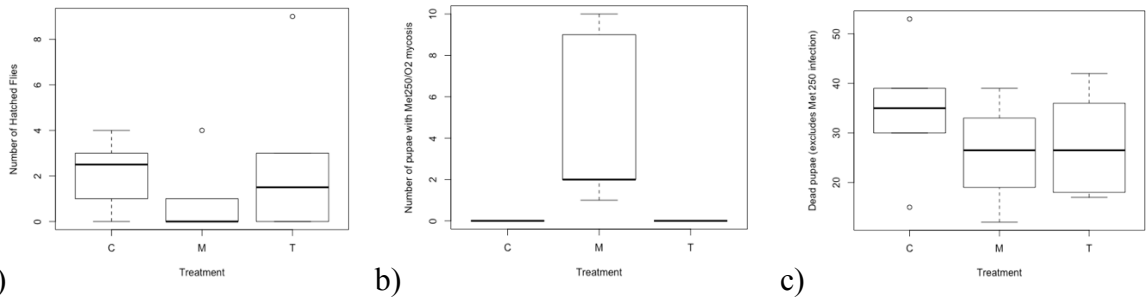


a) b) c)

Figure 16. Distribution of collected pupae and larvae by a) treatment (n=6), and, b) Average number of collected pupae and larvae in each treatment (n=6), bars represent +/- SEM. c) Distribution of collected larvae and pupae by tray placement (n=6)

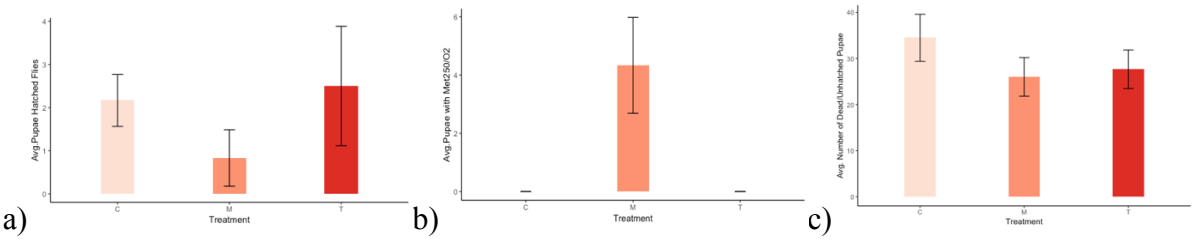
3.2.2. Hatched, Met. 250/O2 Infected and Dead, Unhatched Pupae and Larvae

Figure 17a and Figure 18a show the distribution and average number of hatched flies for each treatment. The number of hatched flies was analyzed with a Poisson regression because there was a high number of zeros and a relatively small data set. There are two apparent outliers: one in Met. 250/O2 treatments and one in Tween20, however, both are kept in the analysis due to small sample size. No significant difference of treatment was observed (Poisson Regression, df=2, t=-1.07, $p>0.05$) when model was adjusted to account for over-dispersion. Placement in the greenhouse had effect where trays at the front had a higher probability of having hatched flies after correcting for over-dispersion within the model (Poisson Regression, df=2, t=2.21, $p<0.05$).



a) b) c)

Figure 17. Boxplots showing the distribution (n=6) of hatched flies, Met. 250/O2 infected pupae and dead pupae over 6 replicates of C (Control), T (Tween20) and M (Met. 250/O2).

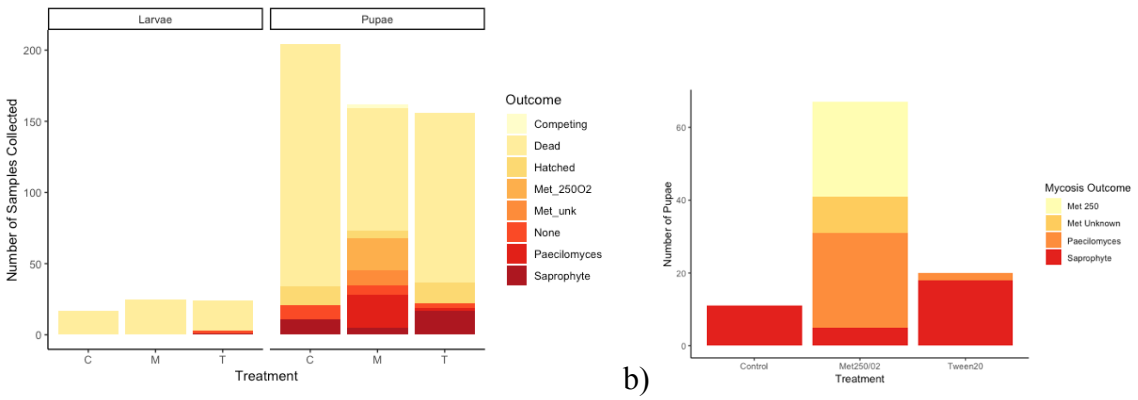


a) b) c)

Figure 18. Average number of a) hatched flies, b) Met. 250/O2 infected pupae c) otherwise dead pupae. Bars represent +/- SEM, n=6.

Figure 17b and 18b show the distribution and average number of pupae with Met. 250/O2 fungal infections. Only pupae from Met. 250/O2 treated trays were infected with M. *brunneum*. There was an uneven distribution of *M. brunneum* infections within Met. 250/O2 replicates, with most only having 1 or 2 infected pupae, and then two replicates with more than 9 infections. The effect of Met. 250/O2 treatment on *M. brunneum* infection is not significant (Binomial Regression, df=2, $z=1.26x10^5$, $p>0.05$).

The majority of collected pupae and larvae died from other causes than Met. 250/O2 infection. Figure 17c and Figure 18c show the distribution and averages of dead pupae and larvae from the three treatments. Treatment did not have a significant effect (GLM, df=2, F=1.38, p>0.05). Placement did (GLM, df=2, F=15.18, p<0.05). Figure 19a is a summary of the main results of the collected samples. Some pupae displayed mycosis from fungi other than the Met 250/O2 isolate: *Paecilomyces*, a different *Metarhizium* strain and general saprophytes were observed (Fig. 20). The largest pupae outcome was death/unhatched, usually accompanied with bacterial colonization (Fig. 21) or dead without signs of a cause (None – Fig. 20d).



a) b)

Figure 19. a) The results of larvae and pupae collected, different colours represent different results, either competing fungi with Met. 250/O2, dead pupae, hatched pupae, Met. 250/O2 infection, unknown *Metarhizium, Paecilomyces* or general saprophyte infection. None means that there was no sign of bacteria or fungi contamination; this category is counted as dead in other analyses. b) A close up of the numbers of fungal infection in greenhouse 2 pupae.

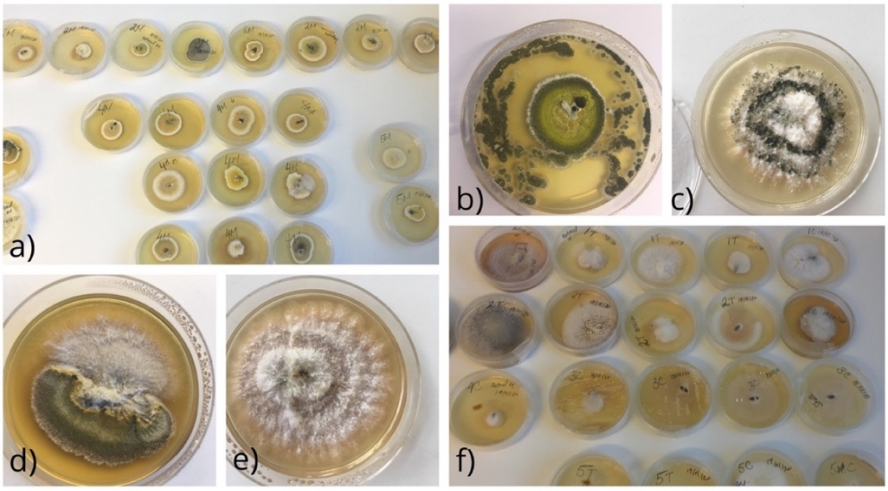


Figure 20. Fungi from greenhouse 2. a) examples of Met. 250/O2 infection b) close up of Met. 250/O2 c) Unknown *Metarhizium* species d) competing Met. 250/O2 with possible *Paecilomyces farinosus* e) *Paecilomyces* species contamination f) Saprophyte contamination from Tween and Control treatments.

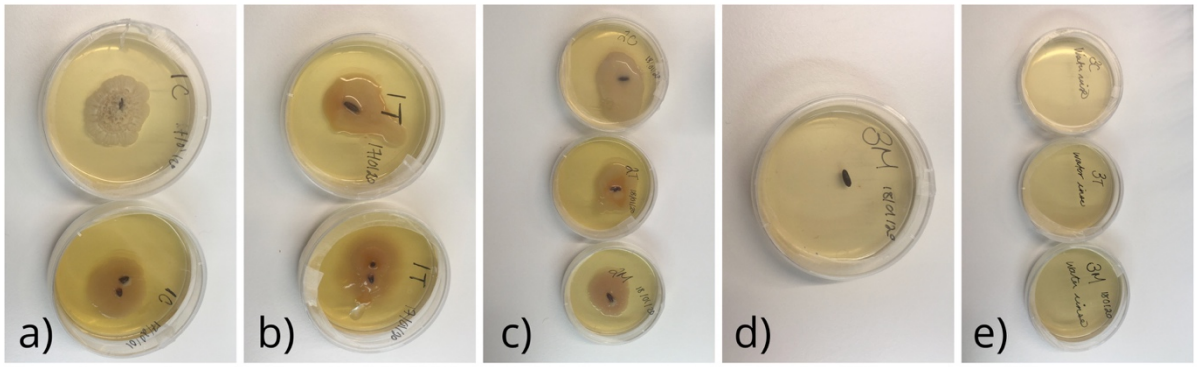


Figure 21. Examples of bacterial contamination from greenhouse 2 samples (a-c) Example of a 'None' outcome, where no growth observed (d) and e) example of water rinses

3.2.3. Oviposition

Cabbage root flies were released into a tunnel enclosing all radish trays to test the effect of the three treatments on oviposition. The number of pupae recovered from each tray was used as a proxy to indicate where adult female flies laid their eggs. The distribution and average number of pupae collected from each treatment is shown in Figure 16 a and b. Treatment did not have a significant effect on the number of pupae and larvae recovered (GLM, df=2, F=1.753, p>0.05).

3.2.4. Radish Colonization

Less than one-third of the radishes from Met250/O2 inoculated trays showed *Metarhizium* growth on selective media plates; 27.75% +/- 0.055% had *Metarhizium* growth. No radishes

sampled from control or Tween20 treatments showed signs of *Metarhizium* growth, however all plates grew unknown saprophytes and bacteria as demonstrated in Figure 22.

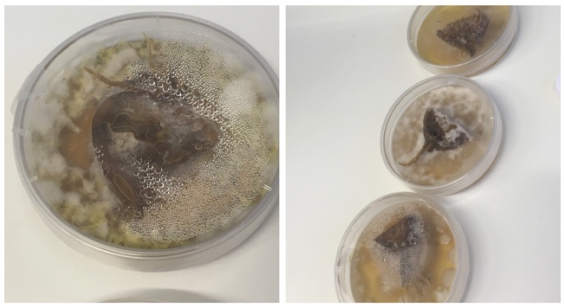
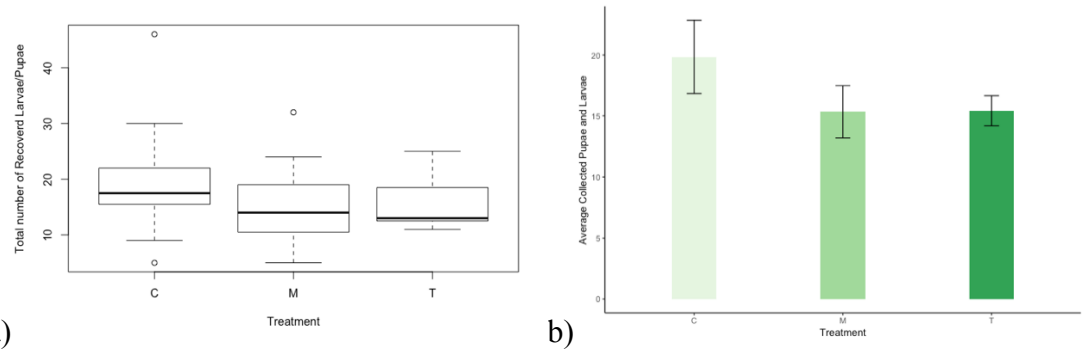


Figure 22. a) *Metarhizium* colonized radish root segment from replicate 2M b) Saprophyte and bacteria colonized radish root segment from replicate 5C.

3.3. Field Study

3.3.1. Pupa Results: Recovered Pupae and Larvae

Over 500 larvae and pupae were collected from the roots and surrounding soil of field grown Chinese cabbage. The distribution and average numbers of pupae and larvae for each treatment are shown in Figure 23. The average number of pupae and larvae recovered from each treatment is similar, with slightly more samples collected from Control treatments (Fig. 23b). However, treatment did not have a significant effect on the number of larvae and pupae recovered (GLM, df=2, F=1.31, p>0.05). One control replicate (4C) had more pupae collected than the other replicates, however removing it as an outlier did not change the statistical significance of treatment on the number of pupae and larvae recovered from the soil environment. The majority of cabbage roots were damaged by larval feeding.



a) b)

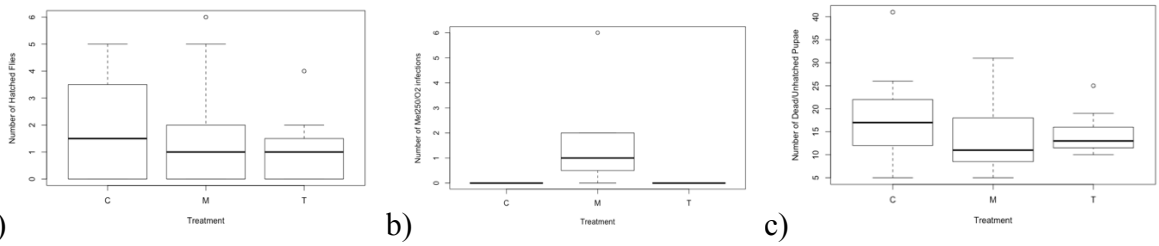
Figure 23. a) Boxplot showing the distribution of recovered pupae and larvae from the roots and soil of Chinese cabbage plants (n=12). b) The average number of recovered pupae and larvae per treatment, n=12 and bars represent +/- SEM.

3.3.2. Hatched, Met. 250/O2 Infected and Dead, Unhatched Pupae

Figure 24a and 25a show the distribution and average number of hatched flies. On average, control treatments had the most hatched flies, followed by Met. 250/O2, then Tween20. Treatment did not have a significant effect on the number of hatched flies, after adjusting for over-dispersion of zeros within the model (Poisson Regression, df=2, t=-0.33, p>0.05).

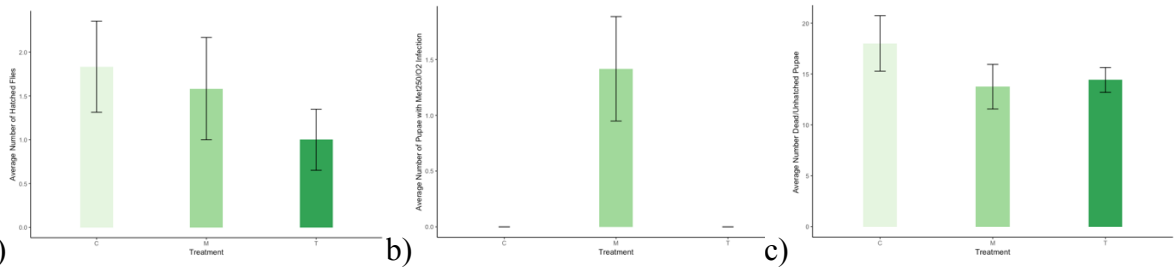
The distribution and average number of Met. 250/O2 infected pupae is shown in Figure 24b and 25b. Similarly, to greenhouse 2 results, *M. brunneum* infections were only observed in Met. 250/O2 treatments (Fig. 25b, 26b and 27). Again, a low frequency of mycosis by *M. brunneum* was observed, with no significant effect of treatment (Binomial Regression, df=2, $z=5.11x10^{3}$, p>0.05).

The majority of pupae and larvae died from other causes than *M. brunneum* infection. Figure 24c and 25c show the distribution and average number of dead/unhatched pupae in each treatment. Many pupae showed signs of bacterial colonization, or infections by other fungi (Fig. 26). A few pupae were parasitized by *T. rapae* (Fig. 26 and Fig. 27). Treatment did not have a significant effect on pupae death (GLM, df=2, F=1.41, p>0.05).



a) b) c)

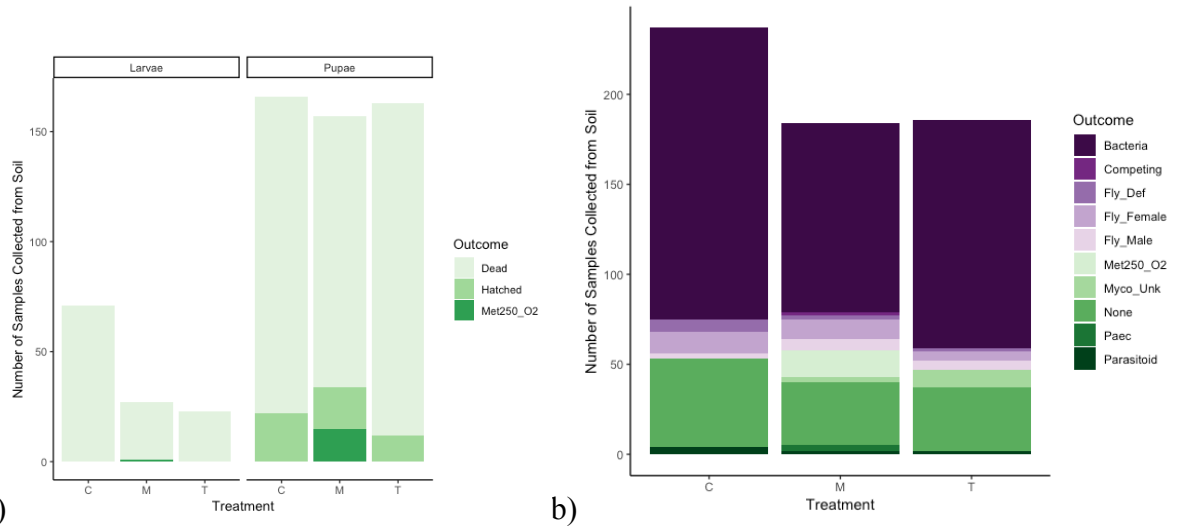
Figure 24. Boxplots showing the distribution of hatched flies, Met. 250/O2 infected pupae and dead pupae over 12 replicates of C (Control), T (Tween20) and M (Met. 250/O2).



a) b) c)

Figure 25. Plots showing the average number of hatched flies, Met. 250/O2 infected pupae and dead pupae over 12 replicates of C (Control), T (Tween20) and M (Met. 250/O2), bars represent +/- SEM.

Figure 26 is a summary of the general (Fig. 26a) and detailed (Fig. 26b) pupae results. Similar to the greenhouse 2 study, the majority of pupae and larvae died or remained unhatched. Some within Met. 250/O2 treatments showed signs of *M. brunneum* mycosis (Fig. 27a-c*)*. Some pupae were infected by *Paecilomyces* or other unknown fungi (Fig. 26b, Fig. 27b-c). A few pupae were parasitized by *T. rapae* (Fig. 27b, 28d-e).



a) b)

Figure 26. a) the main results of larvae and pupae collected, different colours represent three different categories: Dead, Hatched or Met. 250/O2 infection. b) a more detailed representation of pupae and larvae results: either bacteria, fungi competing with Met. 250/O2, dead pupae, hatched pupae divided by sex of the fly (Male, Female and Def-deformed), Met. 250/O2 infection, unknown fungi (likely saprophytes)*, Paecilomyces* infection or parasitoid infection (likely *T. rapae*). None means that there was no sign of bacteria, fungi or parasitoid activity; this category is also counted as dead in statistical analyses.

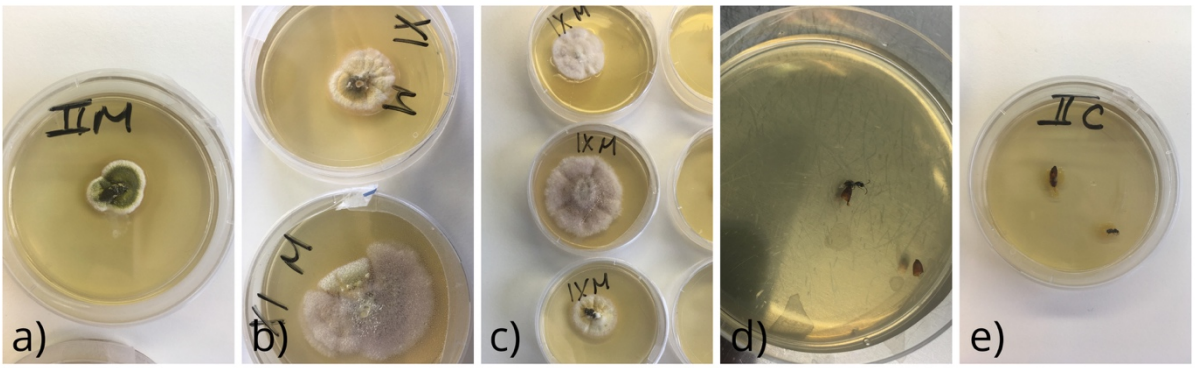


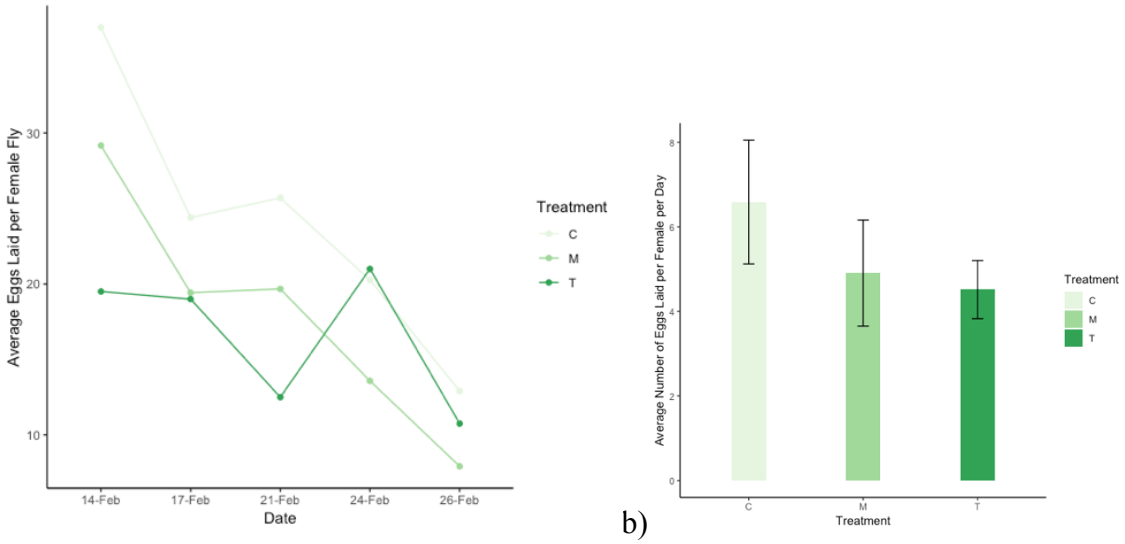
Figure 27. a) *M. brunneum* infection on pupa collected from replicate 2M b) *M. brunneum* infection (top) and *M. brunneum* competing with *Paecilomyces* (bottom), c) examples of unknown fungus, *Paecilomyces*, and *M. brunneum* on pupae collected from 9M. d) and e) show parasitized pupae with *T. rapae* parasitoid.

3.3.3. Oviposition

Flies that hatched from field collected pupae were placed in cages respective of treatment. Only male and female flies that displayed normal body morphism and flies that could completely hatch from their pupa shells were included in the cages (Table 2). The number of eggs laid per female fly in each treatment were counted on five dates over two weeks and is shown in Figure 28a. The average number of eggs laid per female fly per day was calculated and is demonstrated in Figure 28b.

Table 2. Number of adult flies in each treatment that hatched from field collected pupae.

	Control	**Tween**	**Met. 250/O2**
Female	10 (11 after Feb. 24)	4	12
Male	3	5	5
Deformed/Partially Hatched	7	4	2



a) b)

Figure 28. a) Total number of eggs collected from each treatment on the five treatment dates. b) average number of eggs laid per female per day, bars represent +/- SEM.

To test the potential effect of treatment on fecundity of adult flies an ANOVA was performed on the average of eggs laid per female per day. The egg counts were log transformed. No significant difference was found between treatments (ANOVA, df=2, F=1.042, p>0.05).

4. Discussion

The overall aim of these studies was to assess *M. brunneum* as a potential biological control agent to reduce cabbage root fly populations. This was done with greenhouse and field studies. The effect that *M. brunneum* colonization has on host selection by female flies was assessed in the second greenhouse study. The effect of Met. 250/O2 on egg-laying ability of hatched, surviving adult flies was assessed as a follow-up to the field study.

The greenhouse 1 study led to design changes for the field and second greenhouse experiment. Greenhouse 1 will be briefly mentioned before discussing trends in the second greenhouse study and field experiment. The total number of pupae and larvae collected from each treatment will be discussed. Each pupa collected was categorized based on three final results: hatched, Met. 250/O2 infected or unhatched, and thus considered dead by other factors. Trends in pupae from the three resulting categories will be discussed with respect to treatment: Control, Tween20 and Met. 250/O2. Trends in pupae collected from the greenhouse 2 experiment and the field study will be discussed together, because the trends from both studies are similar. To conclude the section, the resulting pupa categories will be compared to major trends in published literature.

After comparing the main results of the pupa collected, results relevant to greenhouse 2 will be discussed followed by results relative to the field study. Results relevant to the second greenhouse study are: oviposition, *M. brunneum* establishment around radish roots, and unexpectedly, contamination from other fungi. Finally, the field study will be examined relative to the egg-laying ability of surviving, hatched flies from each treatment. A summary and reflection for further research will be given at the end.

4.1. Greenhouse 1 Experiment

An aim of greenhouse 1 study was to figure out how to incubate and work with Met. 250/O2 in a different lab from where the technique was taught. It was performed to draft a timeline for cabbage root fly development and Met. 250/O2 incubation for the following field study. Another objective of greenhouse 1 was to test the effect of *M. brunneum* on cabbage root fly populations. The pupae collected from greenhouse 1 study had three final results (Fig. 13, Fig. 14, and Fig. 15):

1. Dead/unhatched
2. Hatched
3. Fungal infection

The majority of pupae did not hatch and were not infected with Met. 250/O2. There were signs of Met. 250/O2 infection on pupae from Control treatments in addition to Met. 250/O2 treatments (Fig. 15). Met. 250/O2 was not a significant cause of pupa death. These results led to some changes for the second greenhouse study.

The first change was to reduce the volume of rice necessary for Met. 250/O2 inoculum. This was done by increasing the number of initial fungal culture plates with the aim of increasing conidia concentration for inoculation. In the greenhouse, planting radishes changed from three 300ml pots of individual radishes per treatment to a 7L tray with 15 radishes per treatment. Instead of placing rice directly under radish seeds, rice was mixed throughout the soil layer where radish seeds were planted (Fig. 6). This was done to increase growing space for the radishes, while allowing for uniform colonization of radish seedlings with Met. 250/O2. This method would help to minimize the risk for contamination between treatments as additional soil was placed over top of the seeds and rice. Even though Met. 250/O2 did not have an effect on cabbage root fly pupae populations in the first greenhouse study, this study did induce important changes to make the following studies better.

4.2. Pupa Results: Greenhouse 2 Study and Field Experiment

4.2.1. Recovered Pupae and Larvae

The total number of recovered larvae and pupae from the greenhouse 2 and field studies were similar (Fig. 19, Fig. 28). On average, more larvae and pupae were recovered from the greenhouse 2 study, where flies were released to lay eggs naturally, than compared to the field study. In addition to natural cabbage root fly infestation, only seven eggs were

artificially added to cabbages in the field study, which may explain why fewer samples were collected in the field trial.

Between the three treatments, trends were similar in both studies. More samples were recovered from Control treatments on average, followed by approximately the same averages in Met. 250/O2 and Tween20 treatments (Fig.16 and Fig. 23). Treatment did not have a significant effect on the number of larvae and pupae recovered in both studies. The greenhouse 2 study had a broader distribution and variation in the number of pupae recovered from each replicate than the field study (Fig. 16 and Fig. 23) This may have been affected by placement within the greenhouse, which will be described in the oviposition section.

4.2.2. Hatched Pupae

An objective of the greenhouse and field studies was to investigate if *M. brunneum* reduces cabbage root fly populations. A way to assess the effect of *M. brunneum* on cabbage root flies is to test if there are fewer surviving, hatched flies from Met. 250/O2 inoculated replicates compared to the other treatments. If fewer adult flies hatch from Met. 250/O2 treatments then *M. brunneum* could be reducing the survival of pupae. However, both the greenhouse 2 study and field study showed no significant effect of Met. 250/O2 treatments.

No effect of treatment or placement was found on the number of flies hatched in the greenhouse 2 study. Met. 250/O2 treatment did not appear to reduce hatching in comparison to Control and Tween20 treatments. However, very few flies hatched (Fig. 19), which resulted in a zero-inflated statistical model, making analysis difficult. There are two outliers (Fig 17C), which may be driving the model. The data set is too small to remove the outliers, because they cannot be assumed to truly be outliers. Outliers may be exceptions or mistakes, but because there are only 6 replicates in the study there are too few data points to assume that two larger samples are exceptions. Similar outliers occurred in the field experiment.

In the field experiment, treatment did not have a significant effect on the number of flies hatched. Fig. 25 shows two outliers, however removing them did not change the statistical significance. Again, there was a zero-inflated model as a result of only having a few hatched flies. Tween20 had the fewest hatched flies, but is not statistically different from the other treatments. Figure 24a, 25a and 26 show that there was approximately the same number of hatched flies from each treatment, though with variations in the sex of hatched flies.

Both the greenhouse 2 study and field experiments also had deformed flies hatch. This could be due to inbreeding in the culture room. The flies released into the greenhouse 2

tunnel were from the culture at NIBIO, as well as the eggs artificially applied in the field. In the culture, flies have been breeding together for many generations. It is possible that some of the unhatched, dead pupae were the result of inbreeding as well.

4.2.3. *Metarhizium brunneum* Infected Pupae:

The main goal of these studies was to see if Met. 250/O2 reduces cabbage root fly populations by infecting and killing cabbage root fly larvae and pupae. Inoculating soil around radish or Chinese cabbage roots was done to expose larvae to infective conidia. The conidia could then attach to cabbage root fly larvae, and cause lethal infections. Therefore, pupae collected from Met. 250/O2 replicates were expected to have higher pupa mortality due to *M. brunneum* infection, but no significant effect of Met. 250/O2 causing lethal infections was observed. The low frequency of infection makes the effect of Met. 250/O2 hard to test; a binomial model was used for both the greenhouse 2 and the field study. *M. brunneum* infection was assessed by plating unhatched pupa on selective media, and identifying *M. brunneum* colonies (Fig. 20b, 27a).

In the second greenhouse study, only pupae from Met. 250/O2 treatments showed signs of *M. brunneum* infection (Fig. 17b, 18b, 19). On average, only four pupae were infected with Met. 250/O2 per replicate. This is less than 1/5 of recovered pupae from the Met. 250/O2 treatments (Figure 19). Treatment did not have a significant effect on *M. brunneum* infection. Results from the field study are consistent with the second greenhouse study.

Again, *M. brunneum* infected pupae were only isolated from Met. 250/O2 replicates. Similarly, few pupae from Met. 250/O2 were infected by *M. brunneum* (Fig. 27); on average less than 2 pupae collected from each Met. 250/O2 replicate showed signs of *M. brunneum* infection (Figure 24b, 25b, 26b). The infection frequency was so low, that it is hard to fit into a model for statistical testing. Met. 250/O2 is not considered to be a significant cause of death for cabbage root fly pupae in this study.

4.2.4. Unhatched, Dead Pupae

The majority of cabbage root fly larvae and pupae collected in greenhouse and field studies did not hatch. They were considered dead from other causes than *M. brunneum* infection. Other causes of death could be inbreeding, bacterial infection, other entomopathogenic fungi or opportunistic saprophytic fungi. The unhatched pupae could be affected by the

development conditions, such as storage in the cold room inducing diapause or the time of year. Most pupae died from factors that were not directly caused by *M. brunneum*.

In the greenhouse 2 study, most flies died (Figure 17c, 18c, 19). The majority of pupae did not hatch, and displayed either bacteria colonization or no symptoms of their cause of death (Fig.19). Based on a Poisson distribution adjusted for over-dispersion, treatment did not have an effect. Met. 250/O2 treatment did not result in more dead or unhatched pupae. Figure 17c, 18c and 19 show the majority of pupae from all treatments are unhatched or killed by other causes than *M. brunneum*.

Similarly, the majority of pupae and larvae from the field study did not hatch (Fig. 23c, 25c, 26), and were considered dead from causes other than *M. brunneum*. Treatment did not have an effect on the number of dead pupae. Many pupae showed signs of bacterial infection, or other fungal infections. Some pupae were parasitized by *T. rapae,* while others showed no signs of why they did not hatch (Fig. 26). The other fungal infections were identified based on approximately two weeks of incubation. Most of the other fungi and saprophytes were observed to be slow growing in the greenhouse 2 study, and should be further identified after at least 3 weeks, and will be observed when the lab is accessible again (At the time of writing I do not have access to the lab due to COVID-19 restrictions).

In comparison to previously published literature, the results of this greenhouse and field studies are alike. There is variation in application methods, several of the studies used drench applications, directly applying conidia to either eggs, larvae, or plants, sometimes with multiple applications (Chandler and Davidson, 2005; Herbst et al., 2017; Myrand et al., 2015; Razinger et al., 2018; Razinger et al., 2014a), or mixing conidia suspensions into the soil prior to planting (Chandler and Davidson 2005). In the field study, Met. 250/O2 did not reduce the number of cabbage root fly larvae and pupae recovered, which is similar to observations by Herbst et al. (2017), as well as Chandler and Davidson (2005).

Some of the other studies also observed mycotic pupae recovered from *M. brunneum* (Razinger et al., 2014a; Razinger et al., 2014b). Myrand et al. (2015) performed a laboratory assay with third instar larvae and directly applied a conidial suspension. They observed a much greater effect of *M. brunneum* on cabbage root larvae, as did Razinger et al. (2014b), than the effect of *M. brunneum* on cabbage root fly populations observed in our study.

The method and experimental design are very different between the two studies, it is hard to compare results. In our study conidia were grown in rice, and the colonized rice was either sown underneath the plantlet or the seed, or mixed into the soil. The success of *M.*

brunneum may have also been influenced by plant exudates (Klingen et al., 2002). Razinger et al. (2014a) found that the soil environment can influence the success of entomopathogenic fungi. Application may have had an influence on the exposure of larvae to conidia, as Myrand et al. (2015) hypothesize. Endogenous soil biota and environmental conditions may also have influenced *M. brunneum* success, as Razinger et al. (2014b) hypothesize.

It was a relatively wet field season, which may have influenced the time spent by larvae in the soil. Larvae may have preferred to feed in the heads of the Chinese cabbage instead of the roots, only going into the soil to pupate. Larvae in our studies were feeding on radishes and Chinese cabbages, they may have had reduced exposure to the infective conidia. In the greenhouse and field cabbage root fly larvae were exposed to many other fungi and conditions that may have reduced the success of *M. brunneum* infection.

4.3. Greenhouse 2 Study

4.3.1. Oviposition

The second greenhouse study was designed to allow adult female flies to choose their preferred radish for oviposition, to some extent. This was done to see if *M. brunneum* colonization of host plants affects oviposition choices by adult cabbage root flies. Flies could fly freely over the three treatments of radishes. The number of pupae collected from each treatment was used as a proxy for the number of eggs laid. More pupae and larvae should be collected from treatments female flies were attracted to, or less pupae should be collected from treatments that deterred female flies, if Met. 250/O2, Tween20 or Control treatments have an effect.

The distribution and average number of pupae and larvae collected from each treatment is shown in Figure 16. Control treatments had the highest average number of pupae and larvae (Fig. 16). Met 250/O2 and Tween20 treatments had broader distributions, and slightly fewer samples collected than Control treatments. A significant effect of placement in the greenhouse room was found. More eggs were collected from trays that were at the front and the middle of the table, than the trays closest to the back of the greenhouse room (Fig. 5). Additionally, more Tween20 trays were placed at the back position than Met. 250/O2 and Control treatments. There are a few improvements to consider for repeating this study.

A similar study should be performed to better test the effect of Met. 250/O2 on female fly host choice for oviposition. More replicates should be performed. Increasing replicates

may help to account for the effect of placement within the greenhouse room because there will be better distribution of back, middle and front placement for each treatment. Overall, there were more than 500 pupae and larvae collected in the greenhouse 2 study. There may have been too many female flies in the study to see a trend in oviposition. Two releases of approximately 55 female flies were performed because the first release was believed to have died rapidly. It would have been better to release fewer flies so there is more host choice due to treatments, and to limit the effects of potential over-crowding.

4.3.2. Radish Colonization

The ability of Met. 250/O2 to colonize the radish rhizosphere was tested in the greenhouse 2 study. Three randomly selected radishes were surface washed three times and plated on selective media for *M. brunneum* growth. Only Met. 250/O2 treated radishes showed *M. brunneum* colonization (Fig. 22). This indicates that there was minimal contamination between treatments because Met. 250/O2 growth was not observed on radishes from Control and Tween20 treatments. Only 27.75%, of radishes from Met. 250/O2 treatments were colonized by *M. brunneum*.

M. brunneum may have been introduced to the radishes from the soil inoculation of colonized rice, or by larvae feeding on radishes. All radishes tested for colonization were damaged by cabbage root fly larval feeding. To test the ability of Met. 250/O2 to associate with radishes and their rhizospheres, another experiment should be performed without introducing larvae. Testing radishes grown in Met. 250/O2 inoculated soil without larvae to cause feeding damage or introduce conidia to the radish, will give a better picture of how well Met 250/O2 can independently colonize radishes. Additionally, other isolates of *M. brunneum* should be tested for their ability to associate with radishes. Previously published literature has reported successful establishment of *M. brunneum* isolates in the rhizoplane and roots of broccoli and cauliflower, and other plants (Herbst et al., 2017; Razinger et al., 2018; Razinger et al., 2014b; Wyrebek et al., 2011). Perhaps different isolates will be better at establishing in the rhizosphere and lead to more cabbage root fly larvae infections, which could be tested in future studies.

4.3.3. Competition with Met. 250/O2

Multiple entomopathogenic fungi and saprophytic fungi were found in the second greenhouse experiment. Figure 19 and 20 show at least two other fungi, morphologically identified as *Paecilomyces (farinose)* and another strain of *Metarhizium*. In two instances, two species of

fungi were found to compete with each other on the same pupa (Fig. 20d, 20e). Bacteria colonisation was also prevalent (Figure 21). Competition with fungi and other microbes may have come from potting soil used, the radish seeds or the general greenhouse environment. The presence of other infective pathogens may have influenced the success of Met. 250/O2 to infect and kill cabbage root fly larvae and pupae.

Soil environments have vast, complex microbial communities (Fierer et al., 2007). Co-infection of insect hosts with more than one strain of fungi or other pathogens like nematodes, and bacteria can occur (Ghoul and Mitri, 2016; Staves and Knell, 2010). There are theories that hypothesize competition between pathogens infecting the same host can select for the species or strain with higher virulence (Staves and Knell, 2010). However, in practice there are many examples where less virulent species or strains are the more competitive species in co-infections (Hughes and Boomsma, 2004; Staves and Knell, 2010). This could be due to indirect competition, where pathogens compete for finite resources within the host insect or direct competition, where one pathogen produces toxins that can harm the other (Ghoul and Mitri, 2016). Two studies have been performed with *Metarhizium anisopliae* strains that show *Metarhizium* can influence the success of co-infecting pathogens (Staves and Knell, 2010; Hughes and Boomsma, 2004).

One study compared multiple strains of *M. anisopliae* and their virulence when co-infecting wax moth larvae. They found that more virulent strains of *M. anisopliae* out competed less virulent ones (Staves and Knell, 2010). In the greenhouse 2 study, there was at least one other strain of *Metarhizium* in addition to Met. 250/O2. Met. 250/O2 strain may not have been the most virulent one present. The Met. 250/O2 strain may have lost the competition, and did not successfully infect as many cabbage root larvae and pupae as it could have, if it were the only entomopathogenic fungus present.

A study between *M. anisopliae* and opportunistic fungi*, Aspergillus flavus* co-infecting leaf cutting ants indicates a similar phenomenon (Hughes and Boomsma, 2004). *A. flavus* on its own is less virulent than *M. anisopliae*. In co-infection with *M. anisopliae*, *A. flavus* performs better and has higher sporulation. These authors theorize that *M. anisopliae* supresses host immune systems, permitting other parasites pathogens to take advantage, and outcompete *M. anisopliae* for host resources.

In greenhouse 2, a similar situation may have happened. Co-infections of cabbage root fly larvae and pupae with opportunistic fungi, other *Metarhizium* species, and other entomopathogenic fungi such as *Paecilomyces* species occurred. Met. 250/O2 may have facilitated the success of the other fungi and Met. 250/O2 was the poorer, less virulent

competitor. Figure 19 shows that the Met. 250/O2 treatment has a higher variety of fungal infections, while Control and Tween20 treatments were limited to opportunistic saprophytes. Perhaps, Met. 250/O2 treatment facilitates mycosis, which could be further analyzed.

4.4. Field Study

4.4.1. Oviposition

A follow-up experiment to the field study was performed to assess if surviving adult flies from Met. 250/O2 treatments showed reduced fecundity, compared to flies surviving from Control and Tween20 treatments. Fecundity was measured by the averge number of eggs laid per female fly. Egg laying ability of adult flies hatching from field-collected pupae was tested. One week from the majority of adult hatching, a thin slice of rutabaga in a container of damp sand was introduced each treatment cage. There were at least 4 adult female flies in each cage (Table 2), with flies from pupae from the Tween20 treatments having the fewest and Met. 250/O2 the most. Eggs were counted periodically over two weeks, replacing sand and rutabaga each time. On each counting date, female flies from Control treatments laid the most eggs, while Tween20 laid the least, except for a spike in the second week (Fig. 28). However, Control female flies laid the most eggs on average per day, followed by Met. 250/O2 and Tween20. Statistical tests show no significant effect of treatment on the average of eggs laid per female per day. Fecundity of adult flies was not affected by Met. 250/O2 treatment in this study.

At least one adult fly from Met. 250/O2 treatments was infected by Met. 250/O2 and killed during the egg collection period. All adult flies were plated on selective media after the egg collection period. Unfortunately, whether or not they were colonized by Met. 250/O2 remains to be seen (due to COVID-19 restricted access). It would be interesting to see if Met. 250/O2 infected more than one adult fly, and if there was any infection from any other entomopathogenic fungi found in the field. While this experiment did not show an effect of *M. brunneum* on fecundity of adult cabbage root flies surviving Met. 250/O2 inoculation as larvae or pupae, it would be interesting to carry the experiment further. Rearing the eggs collected and assessing the larvae that hatched from each treatment may have shown some effects of Met. 250/O2 exposure from adult flies. Possibly larvae would have different feeding behaviour; eating less than larvae from Control or Tween20 treatments, even eventually succumbing to mycosis.

Additionally, while Tween20 did not have a significant effect on the average eggs laid per female per day, fewer flies hatched from this treatment. Tween20 should not have had an effect at all, as it is also used in the Met. 250/O2 suspension as a surfactant. Perhaps lowering the concentration to less than 0.01% or changing the surfactant to TritonX is worth considering.

4.5. *Metarhizium brunneum* and IPM Applications

From the research presented here, *M. brunneum*, Met. 250/O2 isolate, did not reduce the survival of cabbage root fly larvae, or pupae. It was hard to assess the effect of *M. brunneum* on cabbage root fly larvae and pupae because the majority of pupae did not hatch, even from Control and Tween20 treatments. *M. brunneum* did not significantly influence how female flies laid their eggs in the second greenhouse study. This fungus did not attract more female flies to the inoculated plants, but it also did not deter female flies from laying their eggs on inoculated plants. Unfortunately, the effect of *M. brunneum* on oviposition may have been clouded by the experimental design. *M. brunneum* did not reduce the number of eggs laid by hatched female flies collected from Met. 250/O2 field plots. From this follow up study, oviposition did not seem to be affected by *M. brunneum*. Despite the lack of statistical significance, *M. brunneum* did cause mycosis in some pupae in field and greenhouse conditions, may have facilitated infection by other fungi and did not seem to deter egg laying by female flies. *M. brunneum* can still be used as part of integrated pest management for cabbage root fly populations.

This entomopathogenic fungi, based on this study, should not be the only method of control applied to manage cabbage root flies. *M. brunneum* could be used in combination with trap crops and volatiles, as part of a push-pull and kill strategy. *M. brunneum* could be combined with other entomopathogenic fungi depending on competition; *E. muscae* targets the adult fly stage (Klingen et al., 2002), so perhaps combining multiple fungi that can target different life stages will be better at reducing cabbage root fly populations.

Studies could be performed to analyze how well multiple natural enemies work together to reduce cabbage root fly populations, in combination with *M. brunneum*. Further studies could be performed to assess the effect that *M. brunneum* may have on attracting predators and parasitoids to infested plants, or any negative effects that could impact how effective different parts of integrated pest management are in combination (de Azevedo et al., 2019). Integrated pest management is using combinations of different strategies to reduce the

use of chemicals and adverse effects on the environment and human health (Finch and Collier, 2000b; Mesmin et al., 2013; European Parliament, 2009). *M. brunneum*, and entomopathogenic fungi, can help to reduce cabbage root fly populations sustainably, but will need further research to refine the methods for best practices.

5. Conclusion

While *M. brunneum*, isolate Met. 250/O2 did not significantly reduce the survival of cabbage root fly pupae and larvae, it was able to infect some larvae and pupae in both field and greenhouse conditions. Observations from an oviposition choice experiment, *M. brunneum* soil inoculation neither attracts nor deters adult flies from laying eggs. Treatment with this entomopathogenic fungus did not affect the ability of hatched, surviving adult flies to lay eggs in comparison with Control groups. *M. brunneum*, isolate Met. 250/O2, has the potential to work with other integrated pest management strategies to reduce cabbage root fly populations. Further research into *M. brunneum* as a biological control agent, including improvements to this study, may help to better identify its effect on cabbage root fly pupae, larvae and even adult flies.

6. References

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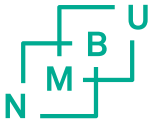

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