COMPATIBILITY OF THE ENTOMOPATHOGENIC FUNGUS *LECANICILLIUM LONGISPORUM* (PETCH) ZARE & GAMS WITH THE PREDATORY MIDGE *APHIDOLETES APHIDIMYZA* RONDANI (DIPTERA: CECIDOMYIIDAE)

by

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Compatibility of the entomopathogenic fungus *Lecanicillium longisporum* (Petch) Zare & Gams with the predatory midge *Aphidoletes aphidimyza* Rondani (Diptera: Cecidomyiidae)

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ABSTRACT

The combined use of the predatory midge *Aphidoletes aphidimyza* Rondani (Diptera: Cecidomyiidae) and the entomopathogenic fungus *Lecanicillium longisporum* (Petch) Zare & Gams., biocontrol agents of the green peach aphid *Myzus persicae*, was evaluated in a semi-greenhouse setting. Results from this experiment showed a statistically significant additive effect of these organisms in controlling aphid populations: a higher reduction of aphid populations in cages with fungus plus predator was observed compared to predator-only or fungus-only treatments.

Lab experiments evaluating interactions between *A. aphidimyza* and *L. longisporum* showed no effect of the fungus on survival of the predator when sprayed directly on four-day old larvae. However, feeding on infected aphids decreased fitness proxies of the midge. Prey consumption was not affected by the presence of infected aphids and infected aphids did not affect the oviposition site selection by *Aphidoletes.* Based on these findings, the combined use of predator and fungus is recommended.

Keywords: *Aphidoletes aphidimyza*; *Lecanicillium longisporum*; interactions; biological control; entomopathogenic fungus; predator

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PREAMBLE

The green peach aphid *Myzus persicae* (Homoptera: Aphididae), is a world-wide insect pest capable of inflicting significant damage in agricultural systems both through direct feeding and by its ability to transmit plant viruses (Alavo & Accodji, 2004; Rabasse & Wyatt, 1985). This insect can resist a wide range of insecticides (Devonshire, et al., 1998) making the inclusion of biological control agents imperative in integrated pest management programs for *M. persicae*. The predatory midge *Aphidoletes aphidimyza* Rondani (Diptera: Cecidomyiidae) and the entomopathogenic fungus *Lecanicillium longisporum* (Petch) Zare & Gams., have been proven to be reliable biological control agents of the green peach aphid (Gardner et al, 1984; Gilkenson & Hill, 1987; Hall & Burges, 1979; Helyer et al., 1992; Meadow et al., 1985; Warner & Croft, 1982). However, the successful integration of both biocontrol agents depends on their mutual compatibility and this has yet to be documented.

Compatibility between entomopathogenic fungi and parasitoids as biocontrol agents has been suggested in different systems (Bethke & Parella, 1989; De La Rosa et al., 2000; Fransen & Van Lenteren, 1993). In addition, successful integration of entomopathogenic fungi and other biological control agents has been demonstrated with the omnivorous predator *Dicyphus hesperus* and the fungus *Paecilomyces fumosoroseus* in which an additive effect of both natural enemies was achieved on whitefly populations (Alma et al., 2007). Similarly, Labbé (2005) found that the entomopathogenic fungus *Beauveria bassiana* did not impair biological control on whitefly populations when it was

used in conjunction with the predator *D. hesperus* and the parasitoid *Encarsia formosa.* The final outcome of the combined use of natural enemies depends upon a complex set of interactions that determine their compatibility.

The aforementioned interactions between biological control agents have been subject of research particularly with respect to model systems (Alma et al, 2007; Bethke & Parella, 1989; Colfer & Rosenheim, 1995; De La Rosa et al., 2000; Labbé et al., 2006). Research on interactions between entomopathogenic fungi and other natural enemies has focused mainly on the susceptibility of the natural enemy to direct infection (Askary & Brodeur, 1999; De La Rosa et al., 2000; Sewify & El Arnaouty, 1998). However, since the susceptibility of natural enemies to direct infection by entomopathogenic fungus can be counteracted by their ability to discriminate between infected and uninfected insects for both feeding and oviposition, this aspect has received wide attention as well (Alma, 2005; Ashouri et al., 2003; Baverstock et al, 2005; Brobyn et al., 1988; Fransen & Van Lenteren, 1993; Labbé et al., 2006).

This thesis is comprised of three chapters. Chapter 1 is a review of the research work done during the last 25 years on compatibility and interactions between biological control agents. In chapter 2, I present results from a semi-greenhouse experiment which tested the compatibility of *A. aphidimyza* and *L. longisporum* on *M. persicae* populations growing on pepper plants. For this purpose, four treatments were set up: predator plus fungus, predator only, fungus only, and no natural enemies. Aphid populations, number of *Aphidoletes,* infected aphids, depredated aphids and dry weight of pepper plants at the end of the experiment were compared among treatments. Finally, laboratory experiments that tested direct interactions between these biological control agents are presented in

chapter 3. The susceptibility of *A. aphidimyza* to infection by direct application of *L. longisporum* and by feeding on infected aphids was tested. In addition, the ability of *A. aphidimyza* to discriminate between infected and uninfected aphids for both feeding and oviposition were studied.

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CHAPTER 1:
INTERACTIONS BETWEEN BIOLOGICAL CONTROL AGENTS: A HISTORICAL OVERVIEW

The question of whether the use of multiple biological control agents offers better results than the use of a single natural enemy for controlling pest populations has been debated for more than twenty five years. Different approaches have been used in an attempt to answer this question; however, consensus has not yet been achieved. This chapter will present a historical overview of different attempts to answer this question. It will outline the mechanisms underlying the outcomes of the use of multiple natural enemies such as competition and Intraguild Predation. Special attention will be given to Intraguild Predation (henceforth IGP), defined by Polis et al. (1989) as "the killing and eating of species that use similar, often limiting resources and are thus potential competitors". Finally, the effects of antagonistic interactions between natural enemies may be mitigated by insect behaviours. The role of such behaviours as mitigating factors will be explored.

1.1 Competition

The compatibility of biological control agents may be affected by the presence of interspecific competition. However, little attention has been given to indirect interactions in which a non-target species is affected by the biological control agent without suffering direct attack. The lack of experimental evidence on this topic may be the result of difficulties in documenting such interactions (Messing et al., 2006).

In some cases in which competition has been documented, the mechanisms underlying the competitive interactions have been elucidated. For example, Krause et al., (1990) showed that multiparasitism influences the parasitisation rates and parasitoid emergence of both *Cotesia melanoscelus* and *Glyptapanteles flavicoxis* (Hymenoptera: Braconidae), parasitoids of the Gypsy month *Lymatria dispar*. *Cotesia melanoscelus* emerged significantly more often than *G. flavicoxis* and out-competed *G. flavicoxis* following multiparasitism. Pijls et al., (1995) investigated the competitive ability of *Apoanagyrus lopezi* and *A. diversicornis* (Hymenoptera: Encyrtidae) in multiparasitized hosts and found a low survival probability of *A. diversicornis* following multiparasitism. The authors concluded that these results may explain the failure of this parasitoid to establish when introduced to Africa as part of a biological control program of the cassava mealybug *Phenacoccus manihoti*. Competition between immature endoparasitoids in multiparasitized hosts may involve physical combat, physiological suppression or resource competition (Pijls et al., 1995); however, this study did not identify which of these determined the outcome of competition through multiparasitism.

Since exploitation competition, a process that results in each consumer affecting others solely by reducing resource abundance (Amarasekare, 2002), has been demonstrated to have significant consequences for the structure and persistence of species assemblages (Human & Gordon, 1996), this may also be a relevant mechanism underlying the outcome of the use of multiple natural enemies in pest control. Exploitation competition may play a role in the competitive exclusion of the native parasitoid *Praon pequodorum* (Hymenoptera: Braconidae) by the exotic parasitoid *Aphidius ervi* (Hymenoptera: Braconidae) in alfalfa cropping systems. The superior

searching behaviour of *A. ervi;* searching longer on a plant after an aphid is encountered, moving more rapidly within plants, and attacking and parasitizing more aphids per unit time, may partially explain the decline of the *P. pequodorum* (Schellhorn et al., 2002).

One approach that can give insight into the issue of single natural enemy versus multiple natural enemies in the context of classical biological control has been the analysis of data already available in the literature. Based on a summary of biological control programs carried out between 1890 and 1968, Ehler & Hall (1982) found that the rate of establishment of exotic natural enemies released against exotic pests was inversely related to the number of species released at a given time and place. The same inverse relationship was found between number of exotic natural enemies released and number of previously introduced and established natural enemies. Based on these results, these authors argued in favour of the competitive-exclusion hypothesis. According to this hypothesis, an exotic species fails to establish because of competitive interference from other biological control agents.

Further evidence supporting the competitive-exclusion hypothesis has been found by Denoth et al., (2002). Data were taken from the BIOCAT database of biological control projects (Greathead & Greathead, 1992). The analysis showed that the mean establishment rate of control agents in multiple-agent projects was significantly lower than in single-agent projects against an insect pest. In addition, although no relationship was found between number of biological control species released and success rates, in over 50% of successful projects, one species was considered responsible for the outcome. This last result is consistent with findings by Myers et al., (1989). From this perspective, one agent can be enough to control a pest and the use of multiple agents can result in

injury to the project itself. Therefore, the release of a single species is recommended by Myers et al., (1989).

The hypothesis of competitive interference established by Ehler & Hall (1982), however, has been an object of debate. Keller (1984) argued that there was bias in the data to explain the results obtained by Ehler & Hall (1982): e.g., data from successful projects are published more often than data from unsuccessful projects; multiple release projects are published more often than a single release project since greater effort is usually expended on them; multiple-species release programs frequently are used as a filter to determine the most promising species, thus, greater rates of success can be expected among programs releasing fewer species. Finally, Keller (1984) considers the hypothesis of competitive exclusion inappropriate since, when beneficial insects are released the target species is usually at high density and therefore, it cannot be considered a limiting resource.

1.2 Intraguild predation

In addition to competition, IGP has been explored as one of the mechanisms impacting the success of biological control programs. While competition is more common than IGP among biological control agents of plant pathogens and weeds, IGP seems to be more frequent among biological control agents associated with nematode or arthropod pests (Rosenheim et al., 1995).

Polis et al. (1989) established a theoretical framework for the analysis of IGP. In that context, IGP may be asymmetric when one species is the intraguild predator and the other species is the intraguild prey, or may be symmetric with each species preying upon

the other. Different cases of IGP among biological control agents of arthropods pests are herewith presented and the impact of IGP on the success of biological control analyzed following the framework given by Polis et al (1989).

1.2.1 Parasitoid-parasitoid interactions

A parasitoid is an organism that spends a significant portion of its life span within a host which is finally killed. Three different scenarios can be considered as a source of IGP between parasitoids: facultative hyperparasitism, in which case a parasitoid can develop either as a primary or secondary parasitoid; facultative autoparasitism, a case in which hyperparasitic males develop either on conspecific parasitoid females or on other species of primary parasitoids (Rosenheim et al., 1995) and predatory feeding behaviour.

Hyperparasitism and predatory feeding have been documented within a guild of parasitoids of the coffee berry borer, *Hypothenemus hampei* (Coleoptera: Scolytidae). Perez-Lachaud et al., (2004) studied under laboratory conditions the interactions between the Bethylid wasps *Cephalonomia hyalinipennis*, *Prorops nasuta* and *Cephalonomia stephanoderis*. They reported hyperparasitism of *C. stephanoderis* and *P. nasuta* by *C. hyalinipennis.* In addition, *C. hyalinipennis* was observed feeding on eggs of *C. stephanoderis*. Thus, the authors did not encourage the introduction of *C. hyalinipennis* into coffee growing regions outside of its natural range. Additional evidence was given in an observational study on biological control of the Citrus Blackfly *Aleurocanthus woglumi* Ashby. Three parasitoids were introduced in Florida to control the Citrus Blackfly: *Encarsia opulenta* Silvestri (Hymenoptera: Aphelinidae), *Amitus hesperidum* Silvestri (Hymenoptera: Platygasteridae) and *Encarsia smithi* Silvestri, a facultative hyperparasite of *E. opulenta* (Thompson et al., 1987). Biological control was achieved

by *E. opulenta* and *A. hesperidum* but suppression of the pest was delayed in a location in which all these species were released. It was suggested that interactions between *E. opulenta* and *E. smithi* could explain the delay in suppression (Nguyen et al., cited by Thompson et al., 1987).

The scarcity of experimental trials involving facultative autoparasitoids in biological control makes not possible to draw general conclusions on this topic. However, data from one experiment suggests that autoparasitoids do not necessarily hinder biological control. The biological control of the Silverleaf Whitefly *Bemisia argentifolii* was enhanced when the facultative autoparasitoid *Encarsia pergandiella* Howard was added to cages in the greenhouse containing both the pest and the primary parasitoid *Encarsia formosa* Gahan. The number of *B. argentifolii* nymphs doubled when only one parasitoid was released (Heinz & Nelson, 1996).

An explanation of the phenomenon described above can be given by understanding the nature of autoparasitism. Male offspring of *E. pergandiella* are produced on immature stages of conspecific females or on *E. formosa* larvae. When *E. pergandiella* was released alone, all the costs of male production are on conspecific females. When *E. pergandiella* was released in conjunction with *E. formosa,* the costs of male production was split among both the conspecific females and the *E. formosa* larvae. If the mortality of *E. formosa* is compensated by the decrease in mortality of the autoparasitoid, we can thus expect that the release of both primary parasitoid and autoparasitoid leads to an increase in pest mortality when compared with the release of only one natural enemy. This argument hinges on the assumption that both parasitoids are equally efficient as biological controls.

The analysis is different for facultative hyperparasitoids. If the facultative parasitoid is released alone, it would behave as a primary parasitoid since the only host available is the pest insect. However, once it is released in conjunction with a primary parasitoid and potential host, the facultative hyperparasitoid would allocate part of the offspring to the primary parasitoid. Thus, mortality of the pest population by both the facultative hyperparasitoid and the primary parasitoid would decrease. This argument depends on the assumption that the only species interacting are the facultative hyperparasitoid and the primary parasitoid. In addition, the pest population should be a limited resource.

1.2.2 Predator-parasitoid interactions

Two types of asymmetric IGP can be recognized in predator-parasitoid systems. First, predators can prey directly upon parasitoids. Second, predators can prey upon parasitized hosts. On current experimental evidence, the former is associated with a disruption in biological control while the latter can enhance the suppression of a pest population. Rosenheim et al. (1995) gives insight into this issue by pointing out that in the first case mortality of the shared host is not required; thus IGP can lead to a high mortality for one or both of the natural enemies, while the mortality upon a target pest remains low. The same rule applies for IGP among predators.

The influence of adding a predator to a parasitoid-pest system when the predator feeds directly on adults parasitoids has been tested experimentally. Rees & Onsager, (1982), quoted by Rosenheim et al., (1995), reported the results of a large field cage experiment examining the interactions between parasitoids and predators of the migratory grasshopper *Melanoplus sanguinipes* (F). Survivorship of adult parasitoids, total

parasitism rates and the reduction in grasshopper population were lower in cages containing both dipteran parasitoids and predators compared with parasitoids alone. IGP was present, since predatory flies in the family Asilidae that feed on grasshopper were observed feeding upon adult parasitoids in the genus *Blaesoxipha* (Diptera: Sarcophagidae).

Rosenheim et al., (1995) quote two sources of experimental evidence for enhanced biological control in predator-parasitoid systems, despite IGP in which the predator feeds upon parasitized hosts. First, Colfer & Rosenheim (1995) demonstrated in a cotton agroecosystem that the addition of the predator *Hippodamia convergens* Guerin enhanced biological control of the cotton aphid *Aphis gossypii* Glover by the endoparasitoid *Lysiphlebus testaceipes* (Cresson), despite the fact that *H. convergens* feeds on both healthy and mummified aphids. Second, Heinz & Nelson (1996), demonstrated that biological control of the whitefly *B. argentifolii* by parasitoids is improved by the addition of the predator *D. pusillus*, despite the fact that *D. pusillus* consumes whitefly harbouring first and second instar parasitoid larvae. One possible explanation for this phenomenon may be found in a paper by Losey & Denno (1998). They found a synergistic interaction between the predators *Coccinella septempunctata* and *Harpalus pennsylvanicus* on pea aphid *Acyrthosiphon pisum* as a result of "predator facilitation". In this phenomenon, the foraging activity of one predator species influences the prey behaviour, making it more susceptible to another predator. In the case of predator-parasitoid system, the predator may drive the prey from one habitat, making more susceptible to attack by parasitoids. The increase in parasitoid attacks should produce mortality high enough to compensate for the decrease in mortality due to IGP.

1.2.3 Predator-predator interactions

Since many predators are generalists and may consume a broad array of prey, IGP among predators is widespread. Both symmetric and asymmetric IGP is common among guilds of predators (Rosenheim et al., 1995). In the scenario of predator-predator interactions, some factors influencing an organism's vulnerability to IGP and the symmetry of the interactions are the predator: predator size ratio, with smaller individuals being eaten by the larger ones; the mobility of each species, with sessile and slow moving stages being heavily attacked; the feeding specificity of the protagonists and the presence of extraguild prey (Lucas et al., 1998). Experimental evidence suggests that specialist predators are more likely to become IG prey when involved in IGP interactions. For example, *Aphidoletes aphidimyza*, a specialist predator of aphids, tends to be the IG prey in IGP interactions involving generalist predators such as *Chrysoperla rufilabris* (Neuroptera: Chrysopidae) and *Coleomegilla maculata* (Coleoptera: Coccinellidae). (Lucas et al., 1998). Finally, IGP decreases when extraguild prey is present. However, with some combination of predators, IGP can remain stable following the addition of extraguild prey (Lucas et al., 1998).

Models for predator-predator interactions consistently predict that IGP disrupts biological control. Rosenheim et al. (1995) point out that IGP among predators does not require mortality of the target pest; thus, IGP can be intense, resulting in high levels of mortality for one or both of the natural enemies, while the total mortality imposed on the target pest is minimal. There is experimental evidence supporting this argument. First, Rosenheim et al., (1993) evaluated the effect of IGP among generalist predators on aphid populations. By means of field experiments they found that the survivorship of lacewing

larvae, *Chrysoperla carnea,* was severely reduced in the presence of hemipteran predators. Biological control of the aphid *Aphis gossypii* by the lacewing *C. carnea* was disrupted when the hemipteran predators *Nabis* spp and *Zelus* spp. were added to the system. Second, Onzo et al., (2004) tested the combined effect of the phytoseiid predatory mites *Typhlodromalus aripo* De Leon and *Typhlodromalus manihoti* Moraes on populations of cassava green mite *Mononychellus tanajoa* (Bondar) in a screenhouse experiment. The suppression of the cassava green mite was disrupted by IGP of *T. manihoti* on *T. aripo* larvae. Despite evidence in favour of Rosenheim's argument, IGP between predators does not always hamper biological control. As an example, the polyphagous predator *Orius majusculus* Reuter (Hemiptera: Anthocoridae) preys upon the predator *Macrolophus caliginosus* Wagner (Heteroptera: Miridae). However, the suppression of *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) was not hampered by the asymmetric IGP of *O. majusculus* upon *M. caliginosus* (Jakobsen et al., 2004).

1.2.4 Fungus interactions with other biological control agents

IGP between pathogens, an infected agent that causes diseases, and other natural enemies occurs when a pathogen infects both an arthropod that is the target pest and its associated predators or parasitoids. This type of interaction has been demonstrated with all pathogen groups, but experimental evidence is mostly from laboratory trials. In laboratory studies, ecological and behavioural barriers are removed to assure contact between the pathogen and natural enemy; thus, results from bioassays cannot be easily extrapolated to field conditions (Rosenheim et al., 1995).

Coincidental IGP is another source of interaction between pathogens and natural enemies. This type of IGP occurs when a larger consumer eats a prey species and its inhabitants. For example, predators eat pathogens, parasites and parasitoids when they eat the host. Parasitoids can ingest pathogens when they feed upon infected hosts (Polis et al., 1989). One example of coincidental IGP is given by Askary & Brodeur (1999). They found that the parasitoid *A. nigripes* consumes blastospores and hyphae of *V. lecanii* while feeding on infected aphids. However, no evidence of internal invasion of parasitoid tissues by blastospores was observed. Only in those few cases where hosts were heavily infected did the parasitoid larvae become infected. The impact of coincidental IGP on the success of biological control has not been tested.

Most of the experimental work involving fungi and other natural enemies has been done to test for compatibility between them, but it is not always possible to infer from those studies the presence of IGP or its impact on biological control. In addition, most of that experimental work has been done on parasitoid-fungus systems while the study of predator-fungus systems has been largely neglected. One study on parasitoid-fungus systems tested the effect of *V. lecanii* on the leafminer parasite *Diglyphus beginii* (Hymenoptera: Eulophidae) (Bethke & Parella, 1989). The parasite longevity was only affected when exposed to fungal-infected aphids but not when exposed to direct fungal spray. In this study, the impact of IGP on biological control was not assessed.

One of the few studies on IGP in predator-fungus systems can be found in Alma et al., (2007). The intraguild interactions between *Dicyphus hesperus* and *Paecilomyces fumosoroseus*, natural enemies of the greenhouse whitefly *Trialeurodes vaporariorum*, were investigated using bioassays and greenhouse settings. Although *D. hesperus* suffers

38% mortality when exposed to leaf discs treated with *P. fumosoroseus,* the simultaneous use of predator and fungus in tomato greenhouse setting resulted in additive whitefly mortality. A predator-parasitoid-fungus system was evaluated by Labbé (2005). The author determined whether the fungus *Beauveria bassiana* could disrupted biological control of whitefly by interfering with the predator *D. hesperus* or the parasitoid *E. formosa*. Comparisons were made between predator, parasitoid and whitefly populations in greenhouse treated with *D. hesperus* plus *E. formosa* and those treated with *D. hesperus, E. formosa* and *B. bassiana.* Neither parasitoid nor predator populations were significantly reduced by the pathogen and compartments treated with *B. bassiana* had fewer immature whitefly (Labbé, 2005). .

When a fungus has a broad host range, it is most likely to be involved in IGP. That is the case with *B. bassiana* and *Metarhizium anisopliae*. De La Rosa et al., (2000) evaluated the effect of both *B. bassiana* and *M. anisopliae* on *Prorops nasuta* (Hymenoptera: Bethylidae), a parasitoid of the coffee berry borer. Despite the fact that *P. nasuta* can become infected by direct inoculation with both fungi, the parasitic and predatory capacity of *P. nasuta* were not affected. Thus, the presence of IGP involving an entomopathogenic fungus does not necessarily mean that biological control will be impaired. In another bioassay, IGP among the parasitic wasp *Cephalonomia tarsalis* (Hymenoptera: Bethylidae) and the fungus *B. bassiana* was tested. Both parasitoid and fungus are potential biological control agents of the sawtoothed grain beetle *Oryzaephilus surinamensis*. Exposure of adult wasps to 100 mg of *B. bassiana*/kg of wheat resulted in 52.7% mortality. The wasp did not avoid infected hosts for oviposition despite the fact that wasp larvae are susceptible to fungus infection (Lord, 2001).

1.3 Sublethal effects

In addition to direct infection, entomopathogenic fungi may cause sublethal effects in non-target arthropods such as reduced food consumption, reduced adult longevity, and decreased fecundity (Hajek & Goettel, 2000). The feeding capacity of the predator *Chrysoperla carnea* was impaired by *L. longisporum* infection with less aphids eaten by infected predators (Sewify & El Arnaouty, 1997). Moreover, the duration of both larval and pupal stage increased significantly when *C. carnea* larvae were fed infected aphids. The same effect on larval duration was observed in the predator *Serangium parcesetosum* after treatment with *B. bassiana* (Poprawski et al., 1998). Sublethal effects of entomopathogenic fungi on fecundity have been observed in *C. carnea* (Sewify & El Arnaouty, 1997) after treatment with *L. longisporum* and in *Harmonia axyridis*, a biological control agent of aphids and scales, when treated with *B. bassiana* (Roy et al., 2008). Finally, sublethal infection by entomopathogenic fungi may result in lower insect activity. *Paecilomyces fumosoroseus*-treated parasitoids were significantly less active than untreated parasitoids for percentage of time walked, walking speed and distance covered (Lacey et al., 1997).

1.4 Mitigating factors

The negative impact of IGP on biological control can be mitigated by the ability of the predators and parasitoids to avoid contact with the IG predator. In the context of pathogens and other natural enemy interactions, it usually means the ability of predators and parasitoids to avoid either the infected host or the infected prey. In addition, the selection of oviposition sites free of inoculum may increase the probability of survival for offspring. Thus, oviposition site selection and the avoidance of infected hosts and

infected prey have been objects of intense research activity, not only in the context of IGP but in the context of simultaneous use of multiple biological controls.

Most of the research on mitigating factors has focused on parasitoid-pathogen guilds. The parasitoid *E. formosa* and the fungus *Aschersonia aleyrodis* may be used as complementary biological control agents on the greenhouse whitefly *T. vaporariorum*. Fransen & Van Lenteren (1993) found that for oviposition, *E. formosa* prefers non-infected than infected whitefly. One factor modulating the ability to discriminate between non-infected and infected hosts is the degree of infection. For example, the aphid parasitoid *Aphidius rhopalosiphi* was able to discriminate between infected and non-infected aphids only when aphids where infected three days in advance of testing, but not when aphids were infected one and two days in advance (Brobyn et al., 1988). However, such discrimination is not universal. The parasitic wasp *C. tarsalis* did not show any sign of discrimination, despite the fact that the wasp larvae do not survive when oviposition occurs in heavily infected hosts (Lord, 2001).

Studies on discrimination in predator-pathogen guilds are less common. The generalist predator *Anthocoris nemorum* L (Heteroptera: Anthocoridae) and the entomopathogenic fungus *B. bassiana* are natural enemies of the aphid *Microlophium carnosum*. Observations of *A. nemorum* infected by *B. bassiana* have been made in the field. Under laboratory conditions, Meyling & Pell (2006) found that the predator *A. nemorum* prefers to prey on non-infected aphids rather than on aphids infected by *B. bassiana*. The predator oviposits preferentially on conidia-free leaves. In another study, Alma (2005) found that adult *D. hesperus* females were able to discriminate between infected and non-infected whitefly nymphs when the nymphs were offered to them five

days after spray application but not when nymphs were offered three days after treatment with the entomopathogenic fungus *P. fumosoroseus*.

1.5 *Aphidoletes aphidimyza*

The aphidophagous midge *A. aphidimyza* Rondani (Diptera: Cecidomyiidae) is a specialist predator of aphids commonly used in biological control programs. The egg stage lasts for 2-3 days at 23°C. The larvae which are effective predators of aphids develop in 4 days at room temperature and locate their prey mainly by olfactory means but vision may play an important role in prey location. The larvae usually attack aphids by biting their joins and injecting a toxin that paralyze the prey. Pupation takes place in the soil and lasts 9-10 days at 23°C. Adults live 1-2 week and feed on the honeydew secreted by aphids (Markkula & Tiittanen, 1985).

Oviposition by the predatory midge is influenced by olfactory, chemical and tactile stimulation via the aphids (Markkula & Tiittanen, 1985). The prey species has not been observed to influence oviposition activity, but the species of plant and the variety has a clear effect. The number of eggs oviposited by *Aphidoletes*' females is directly proportional to aphid density (Markkula & Tiittanen, 1985). Females deposit about 110 eggs with approximately 80% of the total number deposited between day 6 and 10 after mating (Havelka & Zemek, 1999).

Little is known about IGP involving *A. aphidimyza.* The predatory midge *A. aphidimyza* is susceptible to IGP by the aphid predators *Chrysoperla rufilabris* (Neuroptera: Chrysopidae) and *Coleomegilla maculata* (Coleoptera: Coccinellidae). IGP was asymmetric, in favour of the coccinelid and the lacewing (Lucas et al., 1998).

However, oviposition site selection of the predatory midge was not influenced by the presence of the coccinelid *C. maculata.* Thus, *A. aphidimyza* is not able to discriminate between plants colonized by the coccinelid and those that are not.

1.6 *Lecanicillium longisporum*

Lecanicillium longisporum (Petch) Zare & Gams. , is a well-known entomopathogenic fungus which was originally identified as *Verticillium lecanii* (Zimm) Viegas and re-identified according to Zare and Gams by DNA based methods (ITS sequences) (Kim et al, 2007). The commercial product Vertalec™ (Koppert Biological Systems, The Netherlands) which contains an aphid active isolate (a fungal strain with a particular host or geographical origin) of *Lecanicillium longisporum* was used in all the experiments described in this thesis. According to Roditakis et al (2008), the isolates KV42 and KV71 are the active constituent of the mycoinsecticide Vertalec. Although a literature review on *Verticillium lecanii* reveals a wide host range, *L .longisporum* itself may have a narrower host range since it is only one of several species previously known as *Verticillium lecanii*(Kim et al, 2007).

Lecanicillium longisporum readily produces conidia on the aerial mycelium while blastospores are formed by a yeast-like budding process in submerged cultures (Samson & Rombach, 1985). This fungus is not restricted to insect hosts. The species is commonly isolated from mouldy organic material and soil (Samson & Rombach, 1985). It is hyperparasitic on phytopathogenic fungi and its activity against cucumber powdery mildew has been confirmed by Kim et al. (2007).

Penetration of the host by *L. longisporum* results from both mechanical force and enzymatic hydrolysis by chitinases (Askary & Brodeur, 1999). The production of conidiophores and release of the fungus from aphid cadavers takes place after a massive invasion of internal tissues and assimilation of nutrients by fungal cells (Askary & Brodeur, 1999). Secondary metabolites with insecticidal properties are produced during colonization of the host tissue (Wang et al, 2005). On death of the insect host, the fungus emerges from the dead host and sporulation or conidiogenesis usually occurs on the outside of the cadaver (Shah & Pell, 2003). Spore dispersal and spread of infection occurs by a combination of contagion and dispersal of spores (Hall, 1981). An environment of high relative humidity is needed for conidial germination and for high levels of infection of aphids in greenhouse (Shah & Pell, 2003). Sporulating cadavers could be seen 7-10 days following application of Vertalec when temperatures are between 18-20°C and humidity is higher than 80% for several hours a day (Vertalec's label information)

With regard to IGP involving *Lecanicillium longisporum*, the fungus has shown to reduce the longevity of the parasitoid *D. begini* and species of the genus *Lecanicillium* have been found to cause mortality in the parasitoids *Aphidius matricariae* and *E. formosa* (Bethke & Parella, 1989). Finally, the predator *Nabis alternatus* can be infected by *L. longisporum* (Rosenheim et al., 1995).

1.7 *Myzus persicae*

The green peach aphid is a polyphagous species which ranks as one of the most serious pests of greenhouse and field crops. Aphids are small soft-bodied insects which live on plants in dense colonies. Two forms of adults occur: the apterae (wingless) and

the alatae (winged). The latter is readily produced under crowded conditions (Rabasse & Wyatt, 1985). *Myzus persicae* generally reproduces asexually. The young are born fully formed and able to feed immediately. The green peach aphid may develop fully from nymph to adult in 4 days at 23°C.

1.8 Characteristics of greenhouse production

Based upon data provided by the British Columbia Ministry of Agriculture and Fisheries (2003), Canada ranks worldwide third with regard to greenhouse vegetable production area. The greenhouse vegetable and ornamental industry has experienced an expansion in Canada with a surface area increasing from 6,648,347 square meters in 1981 to about 17,933,961 in 2001 (Statistics Canada, 2001). As the greenhouse industry expands, new challenges have arisen particularly in the area of pest management. Firstly, the international trade of flowers and ornamental plants has facilitated the spread of pests. Secondly, quality and cosmetic standards have led growers to apply intensive preventive chemical control. Consequently, pest resistance to the most-frequently-used pesticides has become a problem (Gullino et al., 1999).

The optimal climatic conditions within the greenhouse provide the perfect environment in which exotic pests can become established and polyphagous insects become prevalent. The succession of crops throughout the year in regions in which greenhouse production systems predominate, increase the likelihood of survival for polyphagous pests by providing a chance to migrate between greenhouses. Moreover, field crops may be a refuge for pests during periods in which no greenhouse crops are available and subsequent immigration of pests into the greenhouse may cause unpredictable pest density increase (Gullino et al., 1999).

As pest-resistance and consumer demand for pesticide-free products are increasing, the need for integrated systems for greenhouse pest control is growing. Finally, the use of natural enemies as part of an Integrated Pest Management system (IPM) in greenhouse may be facilitated by the possibility of modifying the greenhouse conditions to favour the effectiveness of biological control agents. The variables of light, temperature, air and soil humidity can be regulated to favour biological control agents (Gullino et al., 1999). Since more than one natural enemy may be necessary to control a pest, the study of compatibility between biological control agents is important to development of optimal IPM strategies.

In this study, interactions and compatibility between *L. longisporum* and *A. aphidimyza* were explored using both laboratory and semi-greenhouse experiments i.e., experimental units consisted of encaged individual pepper plants instead of greenhouse compartments. In Chapter Two, a semi-greenhouse experiment designed to assess the outcome of the combined use of *L. longisporum* and *A. aphidimyza* in controlling *Myzus persicae* is described. Aphid populations were sampled and the dry weight of pepper plants was measured at the end of the experiment to determine the compatibility of these biological control agents. The presence of asymmetric IGP between fungus and predator was tested by sampling predator populations. Finally, the number of depredated aphids and infected aphids were monitored in order to elucidate the nature of interactions between fungus and predator.

In Chapter Three, I describe laboratory experiments carried out to test whether asymmetric IGP between fungus and predator was present. In addition, the presence of mitigating factors such as avoidance of infected prey or oviposition site selection in

favour of non-infected colonies was tested. The relevance of this study is warranted by its implications to both biological control practices and applied ecology.

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CHAPTER 2:
THE COMBINED USE OF *LECANICILLIUM LONGISPORUM* AND *APHIDOLETES APHIDIMYZA* TO CONTROL *MYZUS PERSICAE*: A SEMI-GREENHOUSE STUDY

2.1 Abstract

The interactions of natural enemy complexes used in pest management may result in additive, synergistic or antagonistic effects. The overall effect will often depend upon the particulars of the interactions among those enemies. This chapter describes a semi-greenhouse experiment which aimed to determine the compatibility of the entomopathogenic fungus *Lecanicillium longisporum* (Petch) Zare & Gams and the predator *Aphidoletes aphidimyza* Rondani (Diptera: Cecidomyiidae) used for the suppression of the green peach aphid *Myzus persicae* Sulzer (Homoptera: Aphididae). Four treatments were compared: predator plus fungus, predator only, fungus only, and no natural enemies. Aphid populations, number of *Aphidoletes* larvae, infected aphids and depredated aphids were sampled during 3 weeks. Dry weight of host pepper plants was recorded at the end of the experiment. This experiment showed that *Aphidoletes aphidimyza* and *Lecanicillium longisporum* were compatible and that there was a statistically additive effect of these natural enemies on aphid populations and consequently on preserving the dry weight of pepper plants. The fungus did not have a significant effect on the number of predatory midge larvae. Conversely, the predator did

not influence the frequency of fungal infection on aphids. Further experiments are needed to elucidate the mechanisms that underlie the additive effect.

2.2 Introduction

The green peach aphid *Myzus persicae* Sulzer (Homoptera: Aphididae) is a cosmopolitan, polyphagous pest attacking plants in the field and infesting vegetables and ornamental plants grown in greenhouses. Direct damage is due to removal of sap and injection of active salivary substances into the plant tissue while indirect damage comes about through its ability to transmit plant viruses and excretion of honeydew. The honeydew encourages the growth of sooty moulds, which in turn decreases photosynthesis (Alavo & Accodji 2004; Rabasse & Wyatt 1985). Traditional aphid control methods have relied upon pesticides. However, emergence of pesticide resistant populations and concerns over the health effects of pesticides have led to an increasing interest in alternative control methods (Fournier & Brodeur, 2000).

Some resistance mechanisms have been identified in *M. persicae*. Firstly, the production of insecticide-detoxifying esterases which cause enhanced degradation and sequestration of insecticidal esters. These esterases give a broad spectrum of resistance to organophosphorus, carbamate and pyrethroid insecticides. Second, insecticide-insensitive acetylcholinesterase (AChE), the target for orgonophosphorus and carbamate insecticides confers strong resistance to pirimicarb and triazamate. Lastly, the modified sodium channels. This mechanism involves a mutation that confers a cross-resistance and was first identified in house fly (Devonshire et al, 1998).

The use of biocontrols was intended to eliminate the problem of resistance. However, there is now evidence that host species could be resistant to biological control methods. For example, Milner (1985) recognized a biotype of pea aphid, *A. pisum* resistant to certain isolates of the fungal pathogen *Erynia neoaphidis*. Adherence to host cuticle could be a factor determining the susceptibility of insets to fungal pathogens. Sitch & Jackson (1997) showed that species highly susceptible to *Verticillium lecanii* did not have spore loss following 24 hour incubation while resistance species showed up to 50% spore loss during the same period. Germination and germ-tube growth were possible on resistant non-target insects, indicating that resistance to infection does not occur at this stage (Sitch & Jackson, 1997). The overuse of biocontrol may exert a selective pressure upon the insect pest and resistant strain of insects may develop. Therefore, the excessive use of a single biological control should be avoided.

The aphid midge *Aphidoletes aphidimyza* Rondani (Diptera: Cecidomyiidae) and the entomopathogenic fungus *Lecanicillium longisporum* (Petch) Zare & Gams, formerly known as *Verticillium lecanii* (Zimm) Viegas, are both effective and alternative methods for aphid control. *Aphidoletes aphidimyza* is a specialist predator of aphids that has been used successfully for biological control in both greenhouse and field crops (Warner & Croft, 1982; Meadow et al., 1985; Gilkenson & Hill., 1987). *Lecanicillium longisporum* is available as Vertalec in a commercial formulation manufactured by Koppert Biological Systems in the Netherlands. Good efficacy against a number of aphid species including *M. persicae* has been demonstrated under greenhouse conditions (Hall & Burges 1979; Gardner et al 1984; Helyer et al., 1992).

Aphidoletes aphidimyza and *L. longisporum* may be used as complementary measures against *M. persicae*, particularly in greenhouse production systems where climate can be manipulated to keep conditions close to the optimum for biocontrol agents (Gullino et al, 1999). However, successful integration of multiple biocontrol agents depends upon the interactions among them. Three possible outcomes of such interactions are predicted. Firstly, a synergistic effect could occur i.e., observed pest mortality is higher than the individual mortalities combined. Secondly, if there were no interaction between natural enemies an additive effect would be observed, i.e., pest mortality is equal to the sum of individual mortalities. Finally, an antagonistic effect may result in which the combined mortality is lower than the sum of individual mortalities (Ferguson & Stiling, 1996).

During the last twenty years, there has been an increasing interest in the effect of multiple natural enemies in pest management. The issue of whether or not the use of multiple enemies is desirable has been the focus of a number of papers addressing both the interactions between biocontrol agents and the outcomes of such interactions (Myers et al., 1989; Rosenheim et al., 1995; Losey & Denno, 1998; Lucas et al., 1998; Perez-Lachaud et al., 2004). In regard to simultaneous use of entomopathogenic fungi and other biocontrol agents, most of the work has been laboratory studies of fungus-parasitoid systems (Brobyn et al., 1988; Bethke & Parella, 1989; Fransen & Van Lenteren, 1993; Fransen & Van Lenteren, 1994; De La Rosa et al., 2000; Lord, 2001; Baverstock et al., 2005; Kim et al., 2005) while fungus-predator systems and field experiments have received less attention. These experiments have mainly focused on survival of parasitoids after treatment with the fungus (Bethke & Parella, 1989; Fransen & Van

Lenteren, 1993; De La Rosa et al., 2000; Lord, 2001; Kim et al., 2005) and the ability of the parasitoid to discriminate between infected and uninfected hosts for parasitization (Brobyn et al., 1988; Fransen & Van Lenteren, 1994; Lord, 2001; Baverstock et al., 2005), two factors that are of paramount importance in predicting the outcome of complementary activity by parasitoids and fungi.

There are fewer studies on interactions between predators and entomopathogenic fungi relative to those on parasitoid-fungus interactions and once again, these have been mainly carried out in the laboratory. Although the conclusions from such experiments are limited by the context in which they were done and may not reflect the field situation, they give a preliminary prospective of possible outcomes in the more complex context of field environment (Roy & Pell, 2000). Laboratory studies have explored a wide range of possible interactions between predators and fungi. Antagonistic interactions include infection of the predator by the pathogenic fungus and reduction of pathogen density by foraging predators. Factors that may reduce such interactions and thus enhance the suppression of pest populations include detection and avoidance of infected prey and dispersal of pathogenic fungi by predators.

Avoidance of infected prey has been observed in the predators *Dicyphus hesperus*, *Coccinella septempunctata*, *Chrysoperla carnea and Episyrphus balteatus* (Roy & Pell, 2000; Labbé et al, 2006). Dispersal of the pathogenic fungus *Erynia neoaphidis* by the predator *C. septempunctata* was observed in laboratory and field experiments on colonies of the pea aphid *Acyrthosiphon pisum* (Roy et al, 2001). The presence of the predator resulted in a significant increase in transmission of the fungus to healthy aphids (Roy et al, 1998). Roy et al (1998) investigated antagonistic interactions

between fungi and predators and found that damage to infected and sporulating aphids by *C. septempunctata* reduced the number of conidia produced. Finally, infection of predators by entomopathogenic fungi may disrupt biological control. The coccinelid predator *Serangium parcesetosum* and the parasitoid species *Bracon hebetor* and *Aponagyrus lopezi* (Danfa & Van der Valk, 1999) were found to be highly susceptible to *Beauveria bassiana* (Poprawski et al., 1998; Danfa & Van der Valk, 1999) while L. *longisporum* is pathogenic to the aphid parasitoid *Aphidius nigripes* (Askary & Brodeur, 1999) but did not infect the red spider mite *Tetranychus urticae* or the spider predator *Phytoseiulus persimilis* (Hall, 1981).

Few experiments assessing interactions between fungi and predators have been carried out in greenhouse settings. Labbé et al (2006) examined the compatibility of B. *bassiana* with the parasitoid *Encarsia formosa* and the predator *D. hesperus* using a large-scale greenhouse experiment. It was found that neither the parasitoid nor the predator populations were significantly reduced by the pathogen. However, whitefly predation by *D. hesperus* was significantly reduced in *B. bassiana* treated compartments. Alma et al (2007) evaluated the interactions between *D. hesperus* and the entomopathogenic fungus *Paecilomyces fumosoroseus* on whitefly populations in a greenhouse setting. The findings suggest that interaction between those biological control organisms was not significant: predator populations were not affected by applications of the fungus and the suppression of whitefly population was enhanced when both biocontrol organisms were used together.

The current study assessed the compatibility of *L. longisporum* and *A. aphidimyza* to control the green peach aphid *M. persicae* on pepper plants. The evaluation was

carried out in a semi-greenhouse experiment with all possible factorial combinations of these natural enemies. Interactions between predator and fungus and their impact on host plant productivity were evaluated by sampling predator populations, depredated aphids, infected aphids, aphid populations and dry weight of pepper plants at the end of the experiment.

2.3 Materials and Methods

2.3.1 Insects: *Myzus persicae* and *Aphidoletes aphidimyza*

The green peach aphid *M. persicae* was obtained from the Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada(AAFC), Agassiz, British Columbia and reared on radish plants cv Sparkler at 23°C ± 1 (16L:8D). The strain was collected from pepper plants in a greenhouse at Agassiz, BC in 2002 and resistance patterns were determined by S. Foster (Division of Plant and Invertebrate Ecology, Rothamsted Research, Harpenden, Herts, AL5 2JQ, UK). This strain was identified with the following resistance characters: high Esterase activity, no modified acetylcholinesterase activity and high knockdown resistance.

Aphidoletes aphidimyza was provided by Applied Bio-Nomics as pupae and kept at 23±2°C (16L: 8D). Adults were fed on 5% sucrose solution and allowed to mate for 24 hours before release. Temperature and humidity in both the rearing room and greenhouse were recorded using a HOBO® data logger (Onset, MA, USA).

2.3.2 Fungus: *Lecanicillium longisporum*

Lecanicillium longisporum was supplied as Vertalec® by Koppert, B.V., The Netherlands and prepared using water as a carrier. Experiments were carried out under

research permit 05-RP-05 issued by the Pest Management Regulatory Agency, (Ottawa, Canada). Vertalec is produced as blastospores which are formulated with a nutrient source in a wettable power (Shah & Pell, 2003). Viability was determined using the methodology described by Goettel & Inglis (1997). A suspension of Vertalec in water was spread on to PDA medium amended with benomyl (Benlate®, 0.005%). Three Petri dishes were incubated in the dark at 23°C±2 for 20 hours. Propagules were stained with lactophenol cotton blue and the viability was evaluated for 300 of these. Spore deposition after spray application was determined from blocks of 5% water agar as described by Labbé (2005) and Alma (2005). Agar blocks were pinned on two leaves randomly chosen before Vertalec application and three microscope fields (400X) were scanned on each agar block. Propagules were quantified and dose expressed as number of propagules/mm^2 after adjustment for viability. Quantification of propagules per unit volume in the fungal suspension was assessed microscopically using a haemocytometer (Improved Neubauer). Number of propagules/ml was calculated and actual doses determined after correction for viability.

Vertalec was applied with a Melnor two-litre hand-pressure sprayer. Concentration of the suspension was determined to be 1.21 x 10^5 spores/ml and 1.07 x 10^5 spores/ml for the first and second applications, respectively. Spore deposition was found to be 9.1 spores/mm^2 and 10.9 spores/mm^2 for the first and second applications, respectively. Viability was established to be 87% and 84% for the first and second applications, respectively.

2.3.3 Experimental protocol

The experiment took place during December 2005 at the Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada (AAFC), Agassiz, British Columbia. Two blocks were set up in adjacent greenhouse compartments. The basic experimental unit was a single two-month-old pepper plant (cv. 4 Ever) in a 70x70x70 cm PVC frame cage, covered with a mesh fabric and transparent plastic.

The effect of the combined use of biocontrols was tested using four different combinations of natural enemies: predator-fungus (+,+), predator-no fungus (+,-), no predator-fungus (-,+) and no natural enemies (-,-). Eight replicates were conducted per treatment, four per block. Throughout the experiment, temperature ranged from 17 to 27°C in compartment 1 and from 17 to 26°C in compartment 2. Humidity (RH) varied between 44-97% for compartment 1 and 39-89% for compartment 2 during night time (18:00 – 6:00 hours).

Myzus persicae used for infestation was obtained by placing 10 one-day-old adults on pepper leaves kept in foam cups and allowing the aphids to reproduce for 5 days. After that, pepper leaves bearing aphid colonies were used for infestation. On day one, infested leaves that harboured between 180 and 220 aphids were placed adjacent to uninfested pepper plants. Aphids were allowed to transfer to the clean plants. On days 3 and 15, pepper plants bearing aphids were sprayed until run off with Vertalec. Control treatments were sprayed with water. *Aphidoletes aphidimyza* adults were released 4 days after infestation of plants with *M. persicae*. Six females and three males were released per cage. Adults used in this trial were one day post-eclosion to permit mating.

Considering the predator's life span, one release of *Aphidoletes* at the beginning of the experiment should be enough to observe predatory activity throughout the trial. At 23°C, it takes 1-2 days for the eggs to hatch, 4 days for larval development, 9-10 days for the pupal stage, and adults may live 1-2 weeks in cages (personal observations). Therefore, larvae from released adults should be present until about day 20 when a new generation should emerge. With regard to Vertalec, the first application was done early since it was determined in a preliminary experiment that at least 7 days were needed to observe the first sporulating aphids under similar humidity conditions. The second application was done since the number of sporulating aphids observed at day 10 was lower than expected.

Aphid populations were sampled 8 hours before the first Vertalec application. Sampling was conducted on days 10, 20 and 30 after starting the experiment. Numbers of aphids, *A. aphidimyza* larvae, sporulated aphids and depredated aphids were recorded. Sporulating *M. persicae* cadavers appear as white cottony particles and were easily distinguishable from healthy conspecifics. *Aphidoletes aphidimyza* sucked out the aphid's body fluids and the remaining aphid cuticle can be identified because they remain attached to the leaf. Depredated aphid remains can be distinguished from aphid exuviae by their darker colour and abdomen-concave shape. Three leaves were examined per plant, one located at the bottom, one at the middle and one at top level. Both upper side and underside of the leaves were examined for insects. Dry weight of aerial part of pepper plants was measured at the end of the experiment. Plants were cut off at soil level.

2.3.4 Statistical analysis

The effect of treatments on number of aphids, infected aphids, number of predator larvae and number of depredated aphids at day 10 and 20 with the initial number of aphids as a covariate were tested using the PROC GLM of SAS 9.1. An analysis with repeated measures using the PROC MIXED of SAS was used to test for non-additive effects on the same variables mentioned above. The covariance structures were compared using the Schwarz Bayesian Criterion (SBC). The number of aphids on day 30 was not considered when testing for additive effects since populations reached zero for two treatments.

The effect of treatments on the final dry weight of aerial part of pepper plants was tested with a two-way ANOVA after logarithmic transformation (PROC GLM, SAS 9.1). Normality was assessed using a Shapiro-Wilk test.

2.4 Results

The differences in initial number of aphids did not significantly affect the response variable number-of-aphids-on-day-10 and 20 (table 2.1, p=0.3493; table 2.2, p=0.9263 respectively). There was no significant predator by entomopathogenic fungus by time interaction (table 2.3, p=0.3909) nor predator by entomopathogenic fungus interaction (table 2.3, p=0.9132; figure 2.1), indicating that there was a statistically-additive effect of the natural enemies on aphid populations. This means that there was no interference between the predator and the entomopathogenic fungus which allowed them to reduce aphid populations to a greater extend than with each treatment alone.

The number of infected aphids, number of *Aphidoletes* larvae and number of depredated aphids on day 10 and 20 were not significantly affected by initial differences in the number of aphids (table 2.1, p=0.1882, p=0.1508, p=0.7492 respectively for day 10; table 2.2 p=0.4110, p=0.5394, p=0.4334 respectively for day 20). In addition, the presence of *Aphidoletes* did not significantly influence the number of fungus-infected aphids (table 2.3, p=0.5277). This tendency was consistent over the duration of the experiment (table 2.3, p=0.3431; figure 2.2) confirming the additive effect observed on aphid populations.

Additional evidence of an additive effect was found by analysing the effect of Vertalec on the number of *Aphidoletes* larvae (figure 2.3). The number of larvae was not significantly affected by the presence of the fungus (table 2.3, p=0.3028) throughout the experiment. However, interference of the fungus on the number of depredated aphids is suggested (table 2.3, p= 0.0321; figure 2.4).

There was no evidence for an interaction between predator and fungus in their effects on the mean dry weight of pepper plants recorded at the end of the experiment (table 2.4and table 2.5, p=0.8737).

Table 2.1: **Effect of treatments on response variables on day 10 with initial number of aphids as the covariate.**

Dependent variable	Source	DF	Type I SS	Mean Square	F-value	P
Aphid populations						
	Covariate	1	105366.6	105366.6	0.91	**0.3493**
	Block	1	741978.9	741978.9	6.43	0.0185
	Aphidoletes	1	5044205.4	5044205.4	43.7	<0.0001
	Vertalec	1	118872.9	118872.9	1.03	0.3208
	V*A	1	34047.6	34047.6	0.29	0.5923
Infected aphids						
	Covariate	1	1540.4	1540.4	1.97	**0.1882**
	Block	1	174.7	174.7	0.22	0.6457
	Treatment	1	34.2	34.2	0.04	0.8382
Aphidoletes larvae						
	Covariate	1	37.2	37.2	2.52	**0.1508**
	Block	1	13.2	13.2	0.90	0.3717
	Treatment	1	0.5	0.5	0.04	0.8562
Depredated aphids						
	Covariate	1	4463.7	4463.7	0.11	**0.7492**
	Block	1	139616.8	139616.8	3.40	0.0983
	Treatment	1	84538.6	84538.6	2.06	0.1852

Table 2.2: **Effect of treatments on response variables on day 20 with initial number of aphids as the covariate.**

Dependent variable	Source	DF	Type I SS	Mean Square	F-value	P
Aphid populations						
	Covariate	1	5508.3	5508.3	0.01	**0.9263**
	Block	1	57048.7	57048.7	0.09	0.7661
	Treatment	3	7796720.9	7796720.9	12.38	<0.0001
Infected aphids						
	Covariate	1	16959.3	16959.3	0.73	**0.4110**
	Block	1	282.2	282.2	0.01	0.9142
	Treatment	1	8068.6	8068.6	0.35	0.5674
Aphidoletes larvae						
	Covariate	1	55.5	55.5	0.41	**0.5394**
	Block	1	154.3	154.3	1.14	0.3165
	Treatment	1	78.8	78.8	0.58	0.4670
Depredated aphids						
	Covariate	1	21906.9	21906.9	0.67	**0.4334**
	Block	1	33817.1	33817.1	1.04	0.3349
	Treatment	1	213468.2	213468.2	6.55	0.0307

Table 2.3: **Repeated measures analysis of variance for effects of treatments on aphid density, infected aphids, *Aphidoletes* larvae and depredated aphids. Numerator (Num DF) and Denominator (Den DF) degrees of freedom are given.**

Dependent variable	Effects	Num DF	Den DF	F value	P value
Aphids					
	Aphidoletes	1	24	29.16	<0.0001
	Vertalec	1	24	13.33	0.0013
	Aphidoletes*Vertalec	1	24	0.01	**0.9132**
	Time	1	25	133.09	<0.0001
	Aphidoletes*Time	1	25	3.18	0.0866
	Vertalec*Time	1	25	27.08	<0.0001
	Aphidoletes*Vertalec*Time	1	25	0.76	**0.3909**
Infected aphids					
	Aphidoletes	1	12	0.42	**0.5277**
	Time	2	26	6.26	0.0060
	Aphidoletes * Time	2	26	1.11	**0.3431**
Aphidoletes larvae					
	Vertalec	1	9	1.19	**0.3028**
	Time	2	20	1.16	0.3347
	Vertalec*Time	2	20	0.49	**0.6209**
Depredated aphids					
	Vertalec	1	10	6.19	**0.0321**
	Time	2	22	7.12	0.0041
	Vertalec*Time	2	22	1.27	**0.3012**

Table 2.4: **Means ± SE for dry weight of the aerial part of pepper plants infected with aphids and treated with Vertalec, *Aphidoletes* or Vertalec + *Aphidoletes*. Untransformed data is presented and dry weight is expressed in grams.**

Treatment	Mean	Std Error
Aphidoletes	12.62	1.062
Control	7.76	0.920
Vertalec	7.76	0.920
Vertalec + Aphidoletes	13.50	0.986

Table 2.5: **Two-way ANOVA for effect of treatments on dry weight of host pepper plants.**

Source	DF	Type III SS	Mean Square	F-value	P
Block	1	0.90396603	0.90396603	27.89	<0.0001
Vertalec	1	0.00038886	0.00038886	0.01	0.9137
Aphidoletes	1	1.79212906	1.79212906	55.28	<0.0001
V*A	1	0.00083708	0.00083708	0.03	**0.8737**

Figure 2.1: **Mean number (±SE) of *Myzus persicae* on pepper plants treated with Vertalec, *Aphidoletes aphidimyza*, Vertalec plus *Aphidoletes*, no natural enemies. Vertalec was sprayed on day 3 and 15. *Aphidoletes* was released on day 4. For statistical test, see table 2.1 and 2.2.**

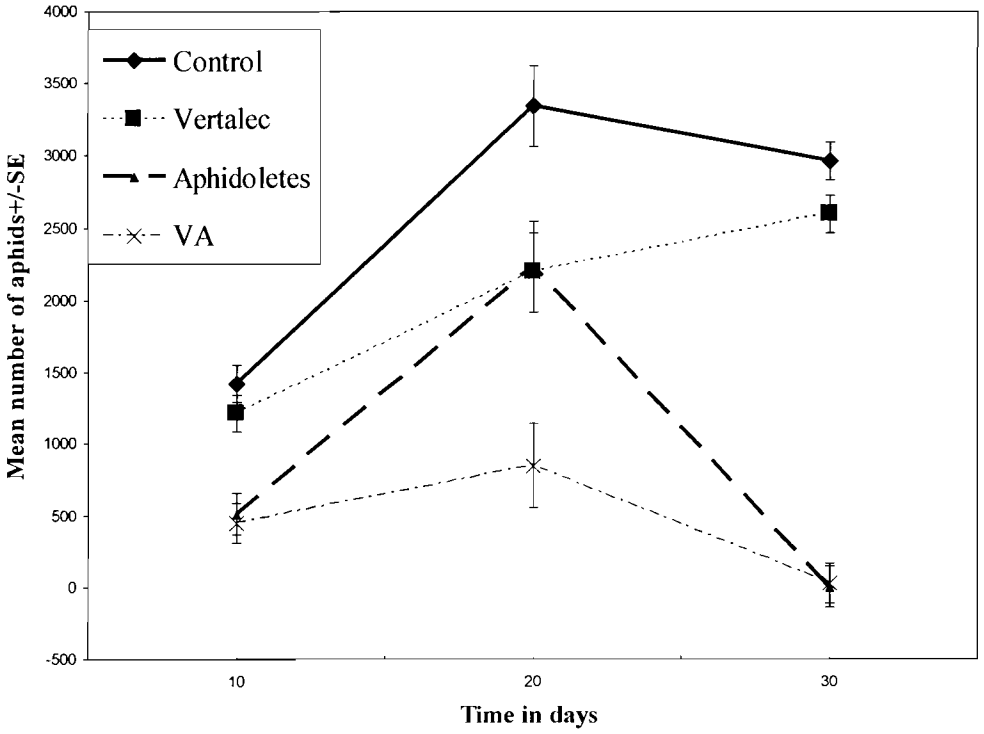


Figure 2.2: **Mean number (±SE) of *L. longisporum* infected aphids on pepper plants treated with either Vertalec or Vertalec plus *Aphidoletes*. No infected aphids were found in either the *Aphidoletes* treatment or in the control treatment. Vertalec was sprayed on day 3 and 15. *Aphidoletes* was released on day 4. For statistical test see table 2.1 and 2.2.**

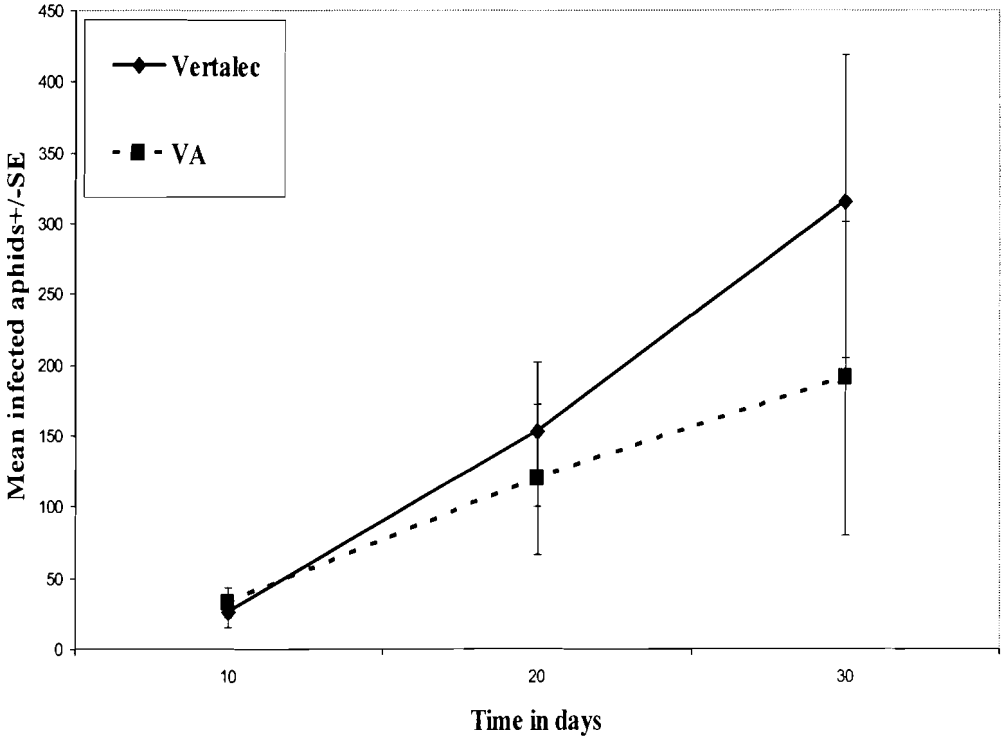


Figure 2.3: **Mean number (±SE) of *Aphidoletes* larvae on pepper plants treated with either *Aphidoletes* alone or Vertalec plus *Aphidoletes*. No larvae were observed on pepper plants treated with Vertalec alone or in control treatment cages. Vertalec was sprayed on day 3 and 15. Aphidoletes was released on day 4. For statistical test, see table 2.1 and 2.2.**

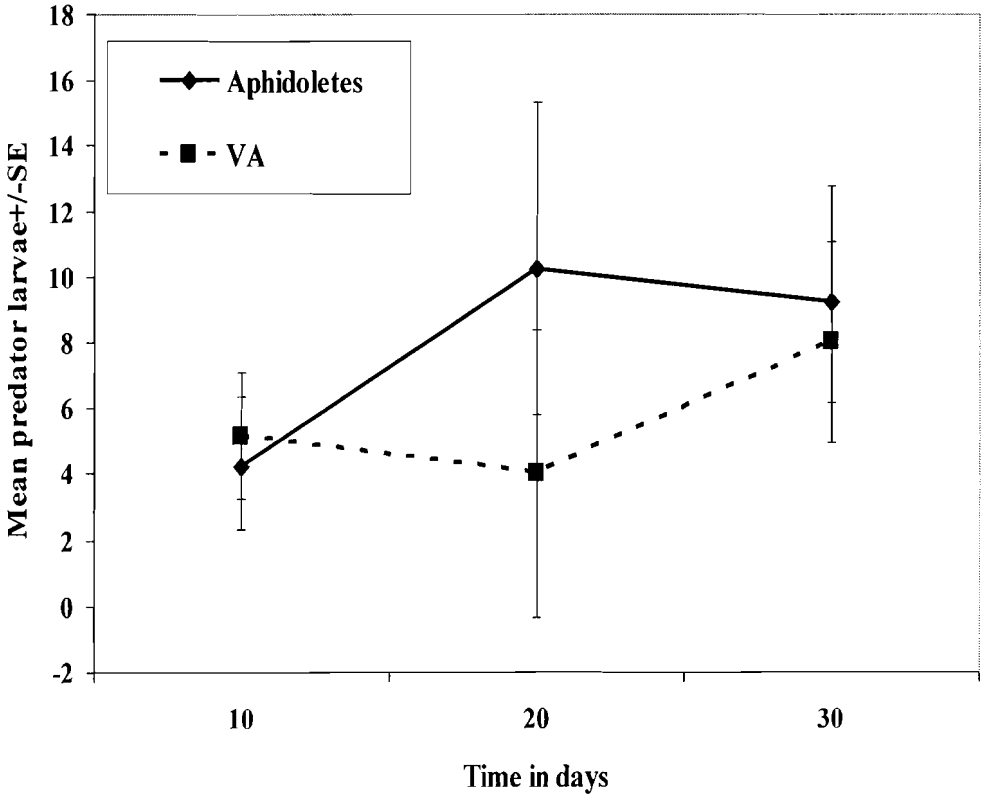
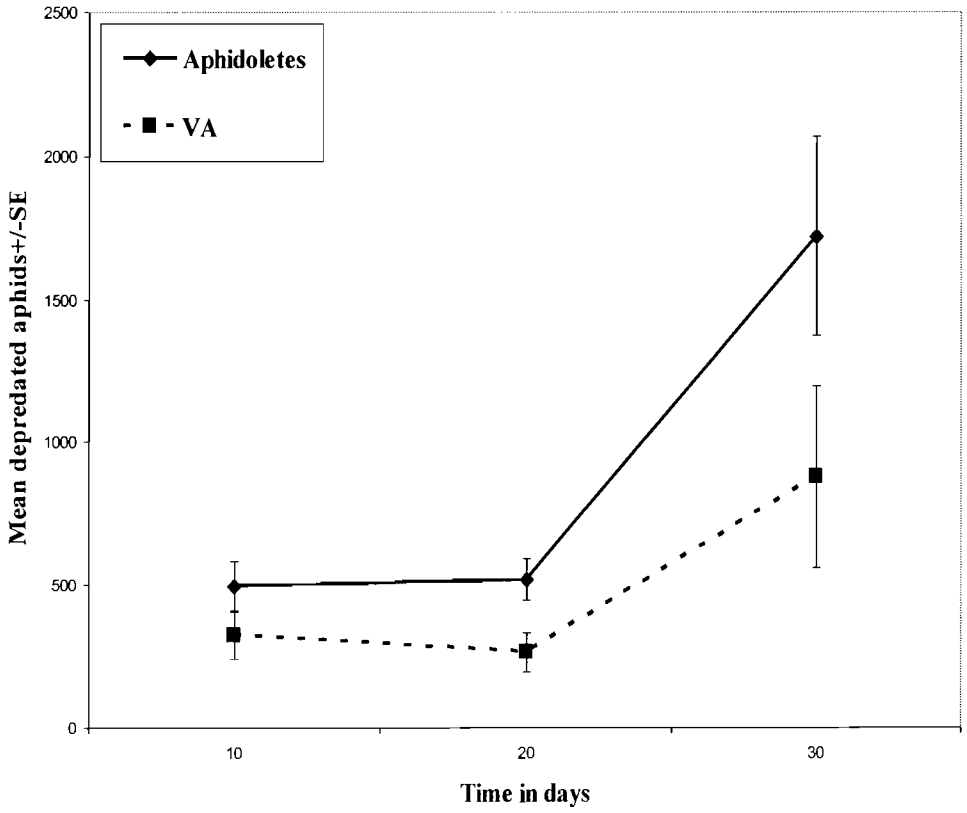


Figure 2.4: **Mean number (±SE) of depredated *Myzus persicae* per cage on pepper plants treated with either *Aphidoletes aphidimyza* or Vertalec plus *Aphidoletes*. No depredated aphids were observed on pepper plants treated with Vertalec or on the control treatment. Vertalec was sprayed on day 3 and 15. *Aphidoletes* was released on day 4. For statistical test, see table 2.1 and 2.2.**



2.5 Discussion

The use of multiple natural enemies in biological control may result in a synergistic, additive or antagonistic effect, depending on the interactions present among them (Ferguson & Stiling, 1996). Outcomes from the semi-greenhouse experiment strongly suggested an additive effect as a result of interactions between the predator *A. aphidimyza* and the entomopathogenic *L. longisporum*. No fungus by predator interaction was found for aphid populations, number of infected aphids, number of *Aphidoletes* larvae and dry weight of pepper plants at the end of the experiment. Positive interactions between entomopathogenic fungus and other natural enemies with regard to the control of pest insects have been demonstrated in other model systems.

Roy & Pell (2000) suggested that, in general, there is a positive interaction between arthropod natural enemies and fungal pathogens with respect to the control of insect populations. Alma et al (2007) found non-significant interaction effect between the predator *Dicyphus hesperus* and the fungus *Paecilomyces fumosoroseus* on whitefly populations under greenhouse conditions. Therefore, the combined use of *D. hesperus* and *P. fumosoroseus* was recommended. Labbé (2005) evaluated the combined use of the predator *D. hesperus*, the parasitoid *E. formosa* and the entomopathogenic *Beauveria bassiana* in a greenhouse trial on tomato plants. There was no significant difference in whitefly mortality between treated and control compartments suggesting compatibility between these natural enemies.

Changes in aphid densities over time could affect the *Aphidoletes-Lecanicillium* interactions. An increase in aphid density over time led to an exponential increase in the number of infected aphids. Consequently, it may be expected: 1) A greater exposure of

the predator to fungal inoculum 2) An increase number of encounters between predators and infected aphids as a result of predator foraging activity. Although *Aphidoletes-Lecanicillium* interactions could change over time as a result of changes in aphid density, a repeated measures analysis evaluating fungus-predator interactions on aphid populations failed to reveal such changes (table 2.3, Aphidoletes * Vertalec * Time P=0.3991).

Although the abundance of *A. aphidimyza* larvae was not significantly different in *Aphidoletes* plus Vertalec cages compared to Aphidoletes-only cages, predation by *Aphidoletes* was lower in the presence of the fungus. The observed reduction in predation may be explained by a non-lethal infection of the predator or by rejection of infected prey. Firstly, reduced food consumption is one of several possible sublethal effects caused by entomopathogenic fungi (Hajek &Goettel, 2000), e.g. the predator *Chrysoperla carnea* infected with *L. longisporum* consumed less aphids when compared to aphid consumption by uninfected predators (Sewify & El Arnaouty, 1998). Similarly, mycosis with *L. longisporum* significantly reduced food consumption by *M. persicae* (Roditakis et al., 2008) and predation rates were found to be lower in the predator *D. hesperus* after treatment with the fungus *P. fumosoroseus* (Alma, 2005). Secondly, rejection of infected prey has been observed in different species. The predator *D. hesperus* avoided feeding on infected whitefly (Labbé et al., 2006) and the generalist predator *Anthocoris nemorum* avoided sporulated aphids infected with *B. bassiana* (Meyling & Pell, 2006). However, one question remains to be answered. How can we have observed an additive effect when the reduction in predation is not compensated by a higher number of infected aphids in *Aphidoletes* plus Vertalec treatment?

Although it is not possible to give a conclusive answer to this question, these contradictory results may be explained by a predator-induced stress on the aphid population, which significantly and synergistically augments the sublethal effect of *L. longisporum* on aphid populations. Decrease in fecundity is one possible sublethal effect associated with entomopathogenic fungi (Hajek &Goettel, 2000). This sublethal effect has been documented on the aphid *Rhopalosiphum padi* after infection with *L. longisporum* (Hsiao et al, 1992). Predator-induced stress increased the mortality of the gray treefrog tadpoles *Hyla versicolor* exposed to the pesticide carbaryl by 2-4 fold (Relyea & Mills, 2001). Therefore, I suggest that a predator-induced stress may significantly increase the sublethal effect of *L. longisporum* on aphid fecundity. However, these hypotheses require further testing.

With regard to temporal patterns, no significant effect of Vertalec on aphid populations was observed 10 days after starting the experiment (Fig 2.1). However, aphid populations were significantly lower for treatments containing the predator when compared to no-predator treatments, indicating that the effect on aphid populations at that time was mainly due to predatory activity.

An additive effect of natural enemies on aphid populations was observed 20 days after setting up the experiment (Fig 2.1). Aphid densities increased dramatically in the control treatment. Vertalec alone and Aphidoletes alone treatments had comparable effects while aphid population were significantly lower in the Vertalec + Aphidoletes treatment.

On day 30, aphid populations reached zero for both Vertalec plus *Aphidoletes* treatment and *Aphidoletes* alone treatment impairing the evaluation of an additive effect

(Fig 2.1). Desiccation and weakness of pepper plants in the control treatment led to a decrease of aphid populations. The relative humidity in the experimental area could have affected Vertalec performance and it may explain the increase in aphid populations in the Vertalec alone treatment throughout the experiment. The manufacturer recommends, i.e. information on Vertalec package, a minimal relative humidity of 80% for 10-12 hours a day for several days after Vertalec application. The humidity requirements of Vertalec limit its use in open-field crops (Shah & Pell, 2003). However, in this experiment the relative humidity was higher than 70% only 4 hours per day during the 5 days following Vertalec application. The rest of the day the humidity fluctuated between 40% and 70%.

The efficacy of Vertalec in controlling *M. persicae* under greenhouse conditions has been demonstrated at higher doses and higher relative humidity. Fournier & Brodeur (2000), found that Vertalec (2×10^6 spores/ml) significantly reduced *M. persicae* populations under greenhouse commercial conditions. Likewise, Gardner et al., (1994) demonstrated that a single aqueous spray of Vertalec effectively controlled *M. persicae* on chrysanthemums in greenhouses at humidity levels ranging daily from 65 to 90%.

The findings reported here suggested that the combined use of *A. aphidimyza* and *L. longisporum* may provide better control of *M. persicae* than the use of each one of these natural enemies alone. Further studies are necessary to elucidate the mechanisms behind the observed additive effect and the reduction in prey consumption by *A. aphidimyza.* Complementary greenhouse studies would be needed to corroborate these findings in a broader spatial and time scale.

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CHAPTER 3:
INTERACTIONS BETWEEN THE PREDATORY MIDGE *APHIDOLETES APHIDIMYZA* AND THE ENTOMOPATHOGENIC FUNGUS *LECANICILLIUM LONGISPORUM*

3.1 Abstract

Aphidoletes aphidimyza Rondani (Diptera: Cecidomyiidae) and *Lecanicillium longisporum* (Petch) Zare & Gams are two biological control agents of the green peach aphid *Myzus persicae*, a pest found throughout the world attacking many field and greenhouse crops. It is important to determine the compatibility of both natural enemies before their combined use in pest management programs. Direct interactions between *A. aphidimyza* and *L. longisporum* were evaluated in a series of laboratory experiments. Direct application of *L. longisporum* on four-day old *A. aphidimyza* larvae did not affect the number of emerged adults. However, the number of emerged adults was significantly lower when *A. aphidimyza* was fed upon fungus-infected aphids throughout the larval stage. Fitness of the predatory midge was also affected with lower fresh weight, lower dry weight and a decrease in wing length for adults fed upon fungus-infected aphids in their larval stage. However, the presence of fungus-infected prey did not affect prey consumption and did not have a significant effect on the number of eggs laid by *A. aphidimyza* females. The predator-fungus interactions previously described are discussed in the light of their possible effects on the combined use of *L. longisporum* and *A. aphidimyza* in controlling aphids.

3.2 Introduction

The green peach aphid *Myzus persicae* is considered one of the most harmful pests to field and greenhouse crops throughout the world (Shean & Cranshaw, 1991). This aphid can cause direct harm to plants by extracting sap when feeding and indirect harm by transmission of viruses as well as by the excretion of honeydew which promote mildew growth in the plant (Alavo & Accodji, 2004; Rabasse & Wyatt, 1985). *Lecanicillium longisporum* and *Aphidoletes aphidimyza* are two natural enemies of aphids that have been used for controlling *M. persicae.*

Lecanicillium longisporum is an entomopathogenic fungus whose infection process involves the adhesion of spores to the insect's cuticle followed by the germination, penetration and internal colonization of the host, ending with the host's death (Jazzar & Hammad, 2004). The efficacy of this fungus for the control of *M. persicae* in greenhouse agriculture has been widely confirmed (Gardner et al, 1984; Hall & Burges, 1979; Fournier & Brodeur, 2000). *Aphidoletes aphidimyza* is a predator midge that feeds on close to 60 different aphid species, including *M. persicae* (Markkula & Tiittanen, 1985). The effectiveness of *A. aphidimyza* in controlling the green peach aphid has been proven both in greenhouses and in the field (Gilkenson & Hill, 1987; Meadow et al., 1985). The combined use of these beneficial organisms in biological control programs depends on their mutual compatibility. This compatibility is itself determined by the types of interactions between them.

Several factors determine the compatibility of entomopathogenic fungi and other biological controls. One of these factors is the predator's susceptibility to direct infection by the fungus (Lord, 2001). Hyphomycete entomopathogenic fungi are thought to have a

broad range of hosts and different studies have demonstrated both lethal and sub-lethal effects in non-target insects treated with *L. longisporum* (Roy & Pell, 2000).

In laboratory studies, *L. longisporum* was found to be highly pathogenic to larvae of the predatory lacewing *Chrysoperla carnea* (Neuroptera: Chrysopidae); the number of emerged adult lacewings was reduced as was their ability to feed (Sewify & El Arnaouty, 1998). Mortalities of 30 and 36% were observed in larvae and adults of the predator *Adonia variegata* (Coleoptera: Coccinellidae) treated in the laboratory with the fungus *L. longisporum* (Ashouri et al., 2003). Additionally, there are reports of *L. longisporum* infecting the aphid parasitoid *Aphidius nigripes* (Askary & Brodeur, 1999), reducing the longevity of the parasitoid *Diglyphus beginii* (Hymenoptera: Eulophidae) (Bethke & Parella, 1989) and reducing the emergence of another parasitoid, *Aphidius colemani* (Kim et al., 2005).

Other factors that determine the compatibility of entomopathogenic fungi and other biological controls are the mortality and sub-lethal effects from feeding on infected prey. The consumption of prey infected with the *Beauveria bassiana* caused a mortality of 86% in the predator *Serangium parcesetosum* (Coleoptera: Coccinellidae) (Poprawski et al, 1998). On the other hand, the predator's ability to discriminate between infected and non-infected insects (Ruberson et al., 1991) might mitigate such effects.

The ability of predators to discriminate between infected and non-infected prey has been documented in the omnivorous predator *Dicyphus hesperus*. This predator is has been shown to discriminate between uninfected and infected prey with either the fungus *Paecilomyces fumosoroseus* (Alma, 2005) or *B. bassiana* (Labbé et al., 2006). In other species tested, this ability to discriminate was not observed. For example, larvae of

the predator *Coccinella septempunctata* (Coleoptera: Coccinellidae) fed for similar periods on aphids whether or not they were infected with the fungus *Erynia neoaphidis* (Roy et al., 1998). Similarly, the parasitoid *Aphidius ervi* indiscriminately attacked aphids infected with the *Pandora neoaphidis* fungus as well as non-infected aphids (Baverstock et al., 2005).

One final aspect to consider when evaluating the compatibility of entomopathogenic fungi and other biological controls is the ability of females to discriminate between oviposition sites free of infected prey and those with presence of infected prey. When the properties of the oviposition habitats vary considerably, it is expected that females will choose those habitats that maximize offspring fitness (Kiflawi et al., 2003). For example, it is known that oviposition attempts by the parasitoid *Aphidius rhopalosiphi* (Hymenoptera: Aphidiidae) are greater on aphids in the early stages of infection than on those in the late stages of infection by the fungus *E. neoaphidis* fungus. *Erynia neoaphidis* infection in aphids prevents the development of parasitoid larvae (Brobyn et al., 1988).

I am not aware of any research on the effect of infected aphids on the selection of oviposition sites by *A. aphidimyza,* though the effect of other factors on this predator's egg-laying behaviour have been studied. It has been shown that *A. aphidimyza* females only lay their eggs on aphid-infected plants to which they are attracted by olfactory stimuli from the aphids or their secretions (Markkula & Tiittanen, 1985). Other factors affecting oviposition by *A. aphidimyza* are the plant species (Markkula & Tiittanen, 1985), the presence of honeydew excreted by aphids which acts as an olfactory stimulus, the aphid density (Choi et al., 2004), the aphid species (Havelka & Ruzicka, 1984), the

presence of conspecific oviposition-marking-pheromones and of oviposition-marking-allomones, the latter being produced by species of the Chrysopidae and Coccinellidae families (Ruzicka & Havelka, 1998). However, Lucas & Brodeur (1999) found that midge females do not discriminate between plants colonized by the predator *Coleomegilla maculata* (Coleoptera: Coccinellidae) and plants free of this predator, although this coccinelid not only competes for prey but also can prey upon *A. aphidimyza.*

This chapter presents the results of laboratory experiments that evaluated some direct interactions between the predator *A. aphidimyza* and the entomopathogenic fungus *L. longisporum*: 1) the direct effect of *L. longisporum* when it is applied to the predator's larvae, 2) the consequences of feeding upon *L. longisporum*-infected aphids on the survival of *A. aphidimyza*, and 3) the ability to discriminate between *L. longisporum* - infected and uninfected aphids while feeding or ovipositing.

3.3 Materials and Methods

3.3.1 Fungus: *Lecanicillium longisporum*

Lecanicillium longisporum was provided by Koppert Biological Systems (The Netherlands) as Vertalec, a commercial product based on a strain specifically selected for use against aphids (Alavo & Accodji, 2004). Vertalec is produced as blastospores which are formulated with a nutrient source in a wettable power (Shah & Pell, 2003). The product was prepared using sterilized water as a carrier and applied using a Paasche airbrush model VL-SET at a pressure of 15 psi.

Viability of the fungal propagules was determined using the methodology described by Goettel & Inglis (1997). A suspension of Vertalec in water was spread on to three replicas of PDA medium amended with benomyl (Benlate, 0.005%) and plates were incubated in the dark at 23°C±2 for 20 hours. After incubation, propagules were stained with lactophenol cotton blue and 300 of them were microscopically examined to determine the number of germinated propagules. Spore deposition was determined from blocks of 5% water agar as described by Labbé (2005) and Alma (2005). Agar blocks were placed on the surface to be sprayed and three microscope fields (400X) were scanned on each agar block. Propagules were quantified and dose expressed as number of propagules/mm^2 after adjustment for viability. Quantification of propagules per unit volume in the fungal suspension was assessed by twice loading an Improved Neubauer haemocytometer. Number of propagules/ml was calculated and actual doses determined after correction for viability.

A sub-sample of Vertalec was sterilized using Electron Beam Sterilization technology provided by Acsion Industries Inc (Manitoba, Canada). The sub-sample was exposed to a minimum irradiation dose of 42.4 kGy and a maximum of 48.2 kGy. Viability after irradiation was found to be 0%. The irradiated Vertalec sub-sample was used to test for possible effects of the inert material on *Aphidoletes* mortality.

3.3.2 Insects: *Myzus persicae* and *Aphidoletes aphidimyza*

The green peach aphid *M. persicae* was obtained from the Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada (AAFC), Agassiz, British Columbia and reared on radish plants (*Raphanus sativus* cv Sparkler) at 23°C ± 1 (16L:8D). The strain was originally collected from pepper plants in a greenhouse at Agassiz, BC in 2002

and resistance patterns were determined by S. Foster (Division of Plant and Invertebrate Ecology, Rothamsted Research, Harpenden, Herts, AL5 2JQ, UK). This strain was identified with the following resistance characters: high Esterase activity, no modified acetylcholinesterase activity and high knockdown resistance. Same-age (± 12 hr) green peach aphids were obtained by placing *M. persicae* on pepper leaves (cv.4 Ever) and allowing them to reproduce for a 24-hour period. Adults were then removed and offspring reared on pepper leaves until use in experiments. Pepper leaves bearing aphids were kept in 225 ml Styrofoam cups containing water and replaced every three days.

Aphidoletes aphidimyza were provided by Applied Bio-Nomics as pupae and kept at 23±2°C (16L: 8D) before and after emergence. Adults were fed on 5% sucrose solution and allowed to mate for 24 hours before use in experiments. To obtain synchronized cohorts of *A. aphidimyza* larvae, adults were allowed to oviposit for 24-h on pepper leaves bearing *M. persicae* colonies. If not otherwise specified, larvae were then fed on an excess of *M. persicae* before use in experiments.

Temperature and humidity in both rearing room and greenhouse were recorded using a HOBO data logger ™ (Onset Computer Corporation, Pocasset, MA, USA).

3.3.3 Description of Experiments

3.3.3.1 Effects of Vertalec on *Aphidoletes aphidimyza* larvae

Tests were performed to evaluate the effect of Vertalec and its inert material on four-day old *A. aphidimyza* larvae. Groups of five larvae received one of the following treatments: 1) Vertalec 2) Inert material 3) Sterilized water (control). Each group of five

larvae was considered as a replicate and twelve replicates were carried out per treatment, six per block.

The inert material and Vertalec treatments were prepared by suspending 0.2 grams of either sterilized Vertalec or non-sterilized Vertalec in 10 ml of sterilized water. Serial dilutions were made from these suspensions. Both the inert material and Vertalec were sprayed at a rate of 2 mg/ml. The actual dose of viable propagules for the Vertalec treatment was determined to be 3.9×10^6 spores/ml and spore deposition was determined to be 50.6 spores/mm^2. Spore viability was 87.6%.

Larvae were placed on filter paper inside a 100 mm diameter Petri dish and sprayed with 0.5 ml of sterile Vertalec, active Vertalec or sterilized water. Larvae were then transferred to a 60 ml plastic cup containing a mixture of sterilized Vermiculite and sterilized water provided as a substrate for pupation. Plastic cups were covered with a fabric mesh for ventilation and kept for 30 minutes under room conditions before placing them into a 4-liter plastic container with a saturated solution of potassium chloride to provide high humidity (79%-95%). Temperature throughout the experiment was 24°C+/-2 and photoperiod 16L: 8D. Each 4-liter plastic container was treated as a block. Each experimental unit was supplied with a piece of pepper leaf bearing *M. persicae* and *A. aphidimyza* was allowed to feed on them until pupation. The number of emerging adults was counted every 24 hours for 10 days starting on day 9 after spraying the larvae.

The experimental dose of active Vertalec was observed to cause 100% mortality in *M. persicae* in preliminary observations. Therefore, as a positive control, one day old *M. persicae* adults were sprayed at the same experimental dose and mortality recorded daily for the following eight days. Aphids were fed on pepper leaves during the

experiment and kept under the same environmental conditions described above for predators. One 225 ml Styrofoam cup containing between 9 and 13 *M. persicae* adults on a pepper leaf was considered as a replicate and two replicates were carried out per block. Pepper leaves were changed on Days 3 and 6. As expected, 100% mortality was observed in aphids on Day 8 after treatment.

3.3.3.2 Lethal and sub-lethal effects of fungus-infected prey on *A. aphidimyza*

An experiment was conducted to assess whether the survival of *A. aphidimyza* was affected by exclusively feeding on *L. longisporum*- infected aphids. *Aphidoletes aphidimyza* larvae received either uninfected *M. persicae* or *L. longisporum*- infected *M. persicae* throughout their larval stage. A 35 mm diameter ventilated Petri dish with 5 larvae was considered as a replicate. Petri dishes were covered at the bottom with a 35 mm diameter piece of pepper leaf (cv. 4 Ever) placed over a moistened filter paper as a food supply for aphids. Each treatment was replicated 20 times, 10 per block. Each block was set up in a 4-liter plastic container over a potassium chloride saturated solution to provide high humidity.

A cohort of *A. aphidimyza* eggs was obtained by introducing a mixed-sex population of adults into a 30 x 30 x 30 cm Plexiglas cage containing pepper leaves (cv. 4 Ever) bearing *M. persicae* colonies. *A. aphidimyza* adults were allowed to oviposit for an 8-hour period. Eggs were collected and transferred to experimental units using a fine paint brush. Ten eggs were initially transferred to a 35 mm diameter ventilated Petri dish and after eclosion the surplus larvae were removed with the help of a dissecting microscope.

Fungus-infected aphids were obtained by spraying Vertalec on pepper leaves bearing *M. persicae* adults four days before experimental use. After treatment, pepper leaves bearing aphids were placed on 225 ml Styrofoam cups and allowed to air-dry before incubation in a 4-liter plastic container containing a saturated solution of potassium chloride. Both infected aphids and *A. aphidimyza* larvae were kept at 24 ± 2°C, 85%-92% RH and a 16h light: 8h dark diel cycle photoperiod. A cohort of aphids was inoculated every day for five days. Aphids to be used in the control treatment were sprayed with sterilized water. A dose of 3.6×10^6 spores/ml-4.2×10^6 spores/ml; was used throughout the experiment and spore viability was 87.6%-89.1%. One ml of suspension was sprayed per pepper leaf. Experimental units were provided every day with either five uninfected or infected-aphids and checked twice a day to remove offspring and replace dead-infected aphids.

To verify aphid infection, mortality was recorded daily for a sub-sample of aphids from each cohort. Sub-sample size was 6 to 24 aphids per cohort. Mortality of 100% was observed between Day 7 and 9 after treatment except for the second aphid-cohort which reached 91.6% mortality on Day 10. In addition, to ensure that the observed mortality was due to infection by *L. longisporum,* ten non-sporulated aphid cadavers per block were surface sterilized following the guidelines given by Goettel & Inglis (1997) and placed on 100 mm diameter Petri dishes containing PDA. Once pure colonies of the fungus were observed, slide cultures were prepared (Goettel & Inglis, 1997) and fruiting bodies observed microscopically. In all cases, infection by *L. longisporum* was confirmed.

Four days after initiating the experiment, *A. aphidimyza* larvae were transferred to ventilated 60 ml transparent plastic cups containing a mixture of sterilized Vermiculite and sterilized water as a substrate for pupation. A piece of pepper leaf bearing either non-infected or infected aphids was added to each container. The number of emerging *A. aphidimyza* was recorded between day 13 and day 18 after egg eclosion. Measurements were made of the wing length, fresh weight and dry weight as proxies of fitness. Both wings were measured in each insect using a dissecting microscope with an ocular micrometer, and the average obtained. Wing length was measured from the junction of the Cubitus-two vein and the inner margin of the wing to the junction of the Radius-five and the outer margin of the wing. Fresh weight was recorded 24 hours after emergence and dried weight after keeping them for 48 hours in a container with Drierite. Chill-anaesthesia was used to immobilize emerged adults in order to determine fresh weight (-8°C for 45 seconds).

3.3.3.3 Effects of fungus-infected prey on prey consumption and discrimination by *A. aphidimyza*

To assess the effect of fungus-infected prey on prey consumption by *A. aphidimyza*, two levels of infection were evaluated: aphids that were infected 44 hours (experiment 1) or 4 days before experimental use (experiment 2) were offered to *A. aphidimyza* larvae. The experiment evaluating the effect of aphids infected 44 hours in advance was set up twice with 15 replicates/per treatment/per block. However, the experiment evaluating aphids infected 4 days in advance was done once, with 30 replicates per treatment.

Each experimental unit consisted of a 35 mm diameter Petri dish lid provided with ventilation and attached to a pepper leaf by two clothes pins. Pepper leaves were not detached from plants and Petri dishes were kept at 24±1°C. A single two-day old *A. aphidimyza* larva was placed inside each experimental unit and aphids were offered as follows: 1) Two uninfected aphids, or 2) Two *L. longisporum*-infected aphids, or 3) One uninfected plus one fungus-infected aphid. Infected aphids were marked with food colouring on their thorax as means of identification. Two-day old *A. aphidimyza* larvae were obtained following the methodology noted in section 3.3.2 and kept under starvation for a period of 20 hours before experimental use. *A. aphidimyza* larvae were then given a 24 hour period before the number and type of depredated aphids were recorded. Only aphids completely consumed were considered during the evaluation. It takes 6-8 hours for complete consumption of an adult aphid by *Aphidoletes* (personal observation). Therefore, lack of satiation is not likely going to lead to lack of discrimination.

Depredated aphids were distinguishable as dark empty exoskeletons with collapsed abdomen since *A. aphidimyza* sucks aphids dry after paralyzing them. Distinction between depredated-infected and depredated-uninfected aphids was made using the food-colouring marks on infected aphids. A preliminary experiment showed no effect of food colouring on prey selection by *A. aphidimyza.*

Fungus-infected aphids were obtained by spray application of Vertalec on pepper leaves bearing same-age adult aphids. A dose of 4.2×10^{6} spores/ml was used in experiment 1 and a dose of 3.7×10^{6} spores/ml was used in experiment 2. Doses are presented after correcting for viability. Blastospore viability was 85.8% % in experiment 1 and 86.8% in experiment 2. Aphids were kept at 24±1°C°C and 79% ±1 RH after

treatment and before experimental use. High humidity was provided by keeping the treated aphids within a 4-liter plastic container with a saturated solution of potassium chloride. The methodologies to determine dose, viability and to obtain an aphid cohort were the same as those described in sections 3.3.1 and 3.32. Mortality was recorded in a sub-sample of infected-aphids for both experiment 1 and 2, and slide cultures were prepared from dead infected aphids following the guidelines given by Goettel & Inglis (1997). Mortality of 100% was observed on Day 6 for the sub-sample from experiment 1 and on Day 7 for the sub-sample from experiment 2.

3.3.3.4 Effects of fungus-infected aphids on the oviposition behaviour of *A. aphidimyza*

A semi-greenhouse experiment was carried out to assess the effect of 1) *L. longisporum*-sporulating aphids on oviposition site choice by *A. aphidimyza* and 2) the effect of dead aphids (sporulating-dead aphids plus dead-uninfected aphids) on the number of eggs laid by *A. aphidimyza.*

The experiment was set up in two greenhouse compartments, each with 15 cages. Twelve cages per greenhouse were use to test for differences between mean number of eggs on pepper plants with dead-sporulating aphids and pepper plants with dead uninfected aphids. To this end, each one of the 12 cages contained one pepper plant with live aphids plus 10 dead-sporulating aphids and a second plant with the same number of live aphids plus 10 dead-uninfected aphids. The three remaining cages per greenhouse had two pepper plants with only live aphids and were set up to test for differences between the mean number of eggs laid on cages with only live aphids and cages with dead aphids. However, data from 3 cages with only live aphids in one of the greenhouses

was lost. Therefore, the test of the effect of dead aphids on oviposition behaviour of *A. aphidimyza* was done analyzing data from only one greenhouse.

The experimental unit was a cage made of 70 x 70 x 70 cm PVC frame covered with white mesh fabric. Each cage contained two, two-month old pepper plants cut back to one bottom leaf. Each plant was infested with aphids by transferring seven same-age adult aphids and allowing them to reproduce for three days. The number of aphids per plant was counted before starting the experiment. If needed, aphids were removed to ensure that both plants inside each cage had the same number of aphids at the beginning of the experiment. However, the number of aphids between cages differed. The mean number of live aphids per pepper plant at the beginning of the 48-hour experimental period was 46.8 ±13.9 (Mean ± SD).

Nine *A. aphidimyza* adults, three males and six females, were released per cage and they were given 48 hours for ovipositon before pepper plants were collected and the number of eggs per pepper plant was determined. *A. aphidimyza* was obtained as pupae from Bio-Nomics and adults were allowed to mate for 24 hours before release. Since a high number of eggs laid by *Aphidoletes* during the 48-h period could lead to lack of discrimination due to saturation, females were used in this experiment immediately after the mating period. Havelka & Zemek (1999) studied twelve geographic populations of *Aphidoletes* and showed that only 0.4-9.2% of the total number of eggs are oviposited by *Aphidoletes* during the first two days after mating. The oviposition period lasts 19 days.

Same-age adult aphids were obtained using the procedures previously described in this chapter. Fungus-infected aphids were obtained by spraying 2 ml of Vertalec solution (4×10^6 spores/ml and 86.5% viability) on pepper leaves bearing between 20

and 30 *M. persicae* adults. Pepper leaves were allowed to air-dry before incubation in a 4-liter plastic container containing a saturated solution of potassium chloride. Infected aphids were kept at 24°C ± 2, 85%-92% RH and 16L: 8D photoperiod. Sporulating aphids which died on days 5, 6 or 7 after Vertalec treatment were used in the experiment. Dead, uninfected aphids were obtained by freezing same age adults at -8 °C. Both sporulating aphids and dead, uninfected aphids were stuck to the underneath of pepper leaves using white liquid glue ® (Elmer's, Columbus, USA).

3.3.3.5 Statistical analysis

All analyses were performed using JMP version 7 (SAS Institute Inc., 2007) unless otherwise specified, and in all cases the accepted level of significance was $P < 0.05$. Normality of data was evaluated using the Shapiro-Wilk test and summary statistics are shown for untransformed data. A Randomized Block Analysis of Variance (ANOVA) was used to test for effects of Vertalec and its inert material on the number of *A. aphidimyza* emerged adults. Data from the experiment evaluating the effect of fungus-infected prey on prey consumption by *A. aphidimyza* was tested using a Chi-Square test (when aphids were infected 4 days prior to the experiment) and a Cochran-Mantel-Haenszel test (when aphids were infected 44 hours before the experiment). A Multivariate Analysis of Variance (MANOVA) was used to compare the number of eggs laid by *A. aphidimyza* on pepper plants with dead-infected aphids and those with dead-uninfected aphids. Since data from cages with only live aphids was lost for one greenhouse compartment, comparisons between the number of eggs laid on cages with dead aphids and cages with live aphids was done using only data from one greenhouse compartment. In this case, a One-Way ANOVA was used for between treatment

comparisons after performing an Analysis of Covariance (ANCOVA) to assess for any significant effect of initial number of aphids on number of eggs laid by the predatory midge.

Data from the experiment testing the effect of fungus-infected prey on *A. aphidimyza* survival and fitness were analyzed using SAS (version 9.1; SAS Institute Inc.). Randomized Block ANOVAs were performed to test for significant differences between treatment and control on fresh weight, dry weigh and wing length of males and females separately. The dry weight of females was log-transformed to correct problems of non-normality. The non-parametric Friedman test was used to determine whether there was significant difference between treatment and control on number of emerged adults.

3.4 Results

3.4.1 Effect of Vertalec on *A. aphidimyza* larvae

The number of *A. aphidimyza* emerged adults was not affected by Vertalec or by the Vertalec's inert material when sprayed on four-day old larvae (table 3.1 and table 3.2; $F_{2,32}=2.03$, $P=0.1473$).

Table 3.1: **Mean number of *A. aphidimyza* emerged adults per experimental unit ±SE after treatment of four-day old larvae with Vertalec, Vertalec's Inert Material or sterilized water.**

Treatment	Mean	Std Error
Control	1.91	0.342
Inert Material	2.66	0.342
Vertalec	2.83	0.342

Table 3.2: **Randomized Block ANOVA for effect of Vertalec and Vertalec's Inert Material on number of *A. aphidimyza* emerged adults.**

Source	DF	Sum of Squares	F-value	P
Block	1	42.2500	30.04	<0.0001
Treatments	2	5.7222	2.03	**0.1473**
Error	32	45.0000		
Total	35	92.9722		

3.4.2 Effects of fungus-infected prey on *A. aphidimyza* fitness proxies

Feeding exclusively on *L. longisporum*-infected aphids during the larval stage affected fitness of the predatory midge *A. aphidimyza*. The number of adults emerging from pupae was significantly lower for larvae fed on fungus-infected aphids compared to larvae fed on non-infected aphids, $X^2(1, N=40) = 31.14$, $P < 0.0001$. Fresh weight of both females and males was significantly lower when fed on a fungus-infected aphid diet ($F_{1,25}=11.91$, $P=0.002$ and $F_{1,11}=10.85$, $P=0.0072$ respectively; table 3.3 and 3.4). Although no significant effect of fungus-infected prey was found on dry weight of males ($F_{1,11}=1.12$, $P=0.3134$; table 3.3 and 3.4), consumption of infected aphids significantly reduced the dry weight of females ($F_{1,25}=28.51$, $P<0.0001$; table 3.3 and 3.4). Feeding on infected aphids also decreased the wing length of both females ($F_{1,25}=22.12$,

$P<0.0001$; table 3.3 and 3.4) and males ($F_{1,11}=9.11$, $P=0.0117$; table 3.3 and 3.4) of the predatory midge.

Table 3.3: **Effects of fungus-infected prey on fitness proxies of the predatory midge *A. aphidimyza*. Within dependent variables, values with the same letter are not significantly different (ANOVA, $P > 0.05$). N=28 and N=14 for females and males respectively.**

Dependent variable	Treatment	Means	Standard Error
Female fresh weight (g)			
	Fungus-infected prey	0.23 a	0.02
	Non-infected prey	0.31 b	0.01
Male fresh weight (g)			
	Fungus-infected prey	0.16 a	0.02
	Non-infected prey	0.23 b	0.01
Female dry weight (data before log-transformation) (g)			
	Fungus-infected prey	0.05 a	0.01
	Non-infected prey	0.09 b	0.00
Male dry weight (g)			
	Fungus-infected prey	0.06 a	0.01
	Non-infected prey	0.06 a	0.00
Female wing length (mm)			
	Fungus-infected prey	2.18 a	0.04
	Non-infected prey	2.41 b	0.03
Male wing length (mm)			
	Fungus-infected prey	2.22 a	0.06
	Non-infected prey	2.40 b	0.02

Table 3.4: **Analysis of variance for effects of fungus-infected prey on fitness proxies of the predatory midge *A. aphidimyza*. The accepted level of significance was P < 0.05. Female dry weight was log-transformed.**

Dependent variable	Source	DF	Mean Square	F value	P
Female fresh weight					
	Block	1	0.0092	3	0.0957
	Treatment	1	0.0367	11.91	**0.0020**
	Error	25	0.0030		
	Total	27			
Male fresh weight					
	Block	1	0.0013	1.72	0.2160
	Treatment	1	0.0086	10.85	**0.0072**
	Error	11	0.0007		
	Total	13			
Female dry weight					
	Block	1	0.4740	6.02	0.0215
	Treatment	1	2.2453	28.51	**<0.0001**
	Error	25	0.0787		
	Total	27			
Male dry weight					
	Block	1	0.00007	1.5	0.3059
	Treatment	1	0.00006	1.12	**0.3134**
	Error	11	0.00006		
	Total	13			
Female length of wing					
	Block	1	0.1022	7.85	0.0097
	Treatment	1	0.2881	22.12	**<0.0001**
	Error	25	0.0130		
	Total	27			
Male length of wing					
	Block	1	0.0046	0.73	0.4110
	Treatment	1	0.0574	9.11	**0.0117**
	Error	11	0.0063		
	Total	13			

3.4.3 Effects of fungus-infected prey on prey discrimination by *A. aphidimyza*

There was no significant difference in the proportion of *A. aphidimyza* that preyed upon zero, one and two aphids when offered two fungus-infected aphids, two uninfected aphids or one infected and one uninfected aphid. The same results were found regardless of whether aphids offered to the predatory midge were infected 48 hours before ahead, $X^2(4,N=90)=7.18$, $P=0.1265$, (table 3.5) or 4 days before ahead $X^2(4,N=90)=3.82$, $P=0.4301$, (table 3.6) of experimental use.

When *A. aphidimyza* larvae were offered just one aphid infected 44 hours ahead of experimental use and one uninfected aphid, 10% of the larvae fed upon only one aphid (table 3.5). The infected aphid was selected in all 3 cases. In contrast, when *A. aphidimyza* larvae were offered one aphid infected 4 days before and one uninfected aphid, 13% of them fed upon only one aphid and in all 4 cases the uninfected aphid was selected (table 3.6).

Table 3.5: **Percentage of *A. aphidimyza* which preyed upon zero, one or two aphids when offered two fungus-infected aphids, two uninfected aphids or one infected and one uninfected aphid. Aphids were infected 48 hours before. A Cochran-Mantel-Haenszel test stratified by blocks did not reveal any association between feeding choices and aphid consumption ($P=0.1265$). The number of predators feeding upon uninfected (U) and infected (I) prey are given within parenthesis. N=90**

Feeding choices	0 aphids preyed	1 aphid preyed	2 aphids preyed
Two Uninfected	3	27	70
Uninfected-Infected	23	10 (3I:0U)	67
Two Infected	20	13	67

Table 3.6: **Percentage of *A. aphidimyza* which preyed upon zero, one or two aphids when offered two fungus-infected aphids, two uninfected aphids or one infected and one uninfected aphid. Aphids were infected 4 days before. A Chi-square test did not reveal any association between feeding choices and aphid consumption (P=0.4301). The number of predators feeding upon uninfected (U) and infected (I) prey are given within parenthesis. N=90**

Feeding choices	0 aphids preyed	1 aphid preyed	2 aphids preyed
Two Uninfected	20	27	53
Uninfected-Infected	27	13 (0I:4U)	60
Two Infected	30	30	40

3.4.4 Effects of dead-infected aphids on oviposition by *A. aphidimyza*

A Multivariate Analysis of Variance (MANOVA) shows that *A. aphidimyza* females did not discriminate for oviposition between plants with dead-infected aphids and plants with dead-uninfected aphids, $F(1,22)=0.03$; $P=0.8584$ (table 3.7). A similar number of eggs was laid on pepper plants with dead-infected aphids (19.8 eggs/plant) and pepper plants with dead-uninfected aphids (19.1 eggs/plant).

Table 3.7: **Multivariate Analysis of Variance for effects of dead-infected aphids and dead-uninfected aphids on number of eggs laid by *A. aphidimyza*. The accepted level of significance was P < 0.05.**

Source	Exact F	Num DF	Den DF	P
Model	1.89	1	22	0.1825
Greenhouse	1.89	1	22	0.1825
Intercept	0.03	1	22	**0.8584**

An Analysis of Covariance (ANCOVA) revealed that initial number of aphids is not significant in explaining variability in the number of *A. aphidimyza*-laid eggs across

treatments (table 3.8). Although the average number of eggs per pepper plant was higher in the dead-aphids treatment than in the live aphids treatment (table 3.9), both ANCOVA and ANOVA (table 3.10) failed to reveal significant effect of treatments on the number of eggs laid by *A. aphidimyza*.

Table 3.8: **Analysis of Covariance assessing the significance of initial number of aphids in explaining the variability in the number of *A. aphidimyza*-laid eggs on pepper plants treated with dead-infected aphids and dead-uninfected aphids. The accepted level of significance was $P < 0.05$.**

Source	DF	Sum of Squares	F Ratio	P
Treatments	1	187.7526	0.17	0.6846
Initial Number of Aphids	1	315.0584	0.29	**0.5997**
Error	12	13009.858		
Total	14	13568.933		

Table 3.9: **Mean number of *A. aphidimyza*-laid eggs per cage. Each cage contained two pepper plants treated with either dead aphids (sporulating aphids + dead uninfected) or live aphids.**

Treatment	Mean	Std Error
Dead aphids	31.8	9.5
Live aphids	22.9	19.1

Table 3.10: **Analysis of Variance assessing the effect of dead aphids and live aphids on number of *A. aphidimyza*–laid eggs on pepper plants. The model does not include the initial number of aphids as covariate. The accepted level of significance was $P < 0.05$.**

Source	DF	Sum of Squares	F-value	P
Treatments	1	244.017	0.23	**0.6337**
Error	13	13324.91		
Total	14	13568.93		

3.5 Discussion

Non-target insects may be susceptible to fungal infection by entomopathogens with broad host range either by direct spray application (Roy & Pell, 2000) or by feeding on infected insects (Poprawski et al., 1998). In the present experiments, *A. aphidimyza* survival was not impaired by direct spray application of *L. longisporum*; however, consumption of *L. longisporum*-infected aphids by *A. aphidimyza* affected fitness proxies. The effect of fungus-infected prey on predator survival has been observed on *Serangium parcesetosum* (Coleoptera: Coccinellidae). Feeding on *B. bassiana*-infected whitefly caused 86% mortality in this predator compared to 13% in predators fed upon uninfected whitefly (Poprawski et al., 1998).

Several factors may explain why *A. aphidimyza* was not susceptible to direct spray application but was negatively affected when fed on infected aphids. First, the mode of fungal penetration may differ. The most common route of host invasion is through the external integument. However, infection through the digestive tract is also possible (Goettel & Inglis, 1997). Since moisture is high in the alimentary tract, spores may germinate readily in this environment (Tanada & Kaya, 1993). It is known that conidia of *B. bassiana* can germinate in the gut of certain insects regardless of the gut microflora (Poprawski et al., 1998). Similarly, large numbers of hyphae were found in the gut of the parasitoid *Aphidius nigripes* after feeding upon *L. longisporum*-infected aphids (Askary & Brodeur, 1999). This suggests that the parasitoid consumes blastospores and hyphae while feeding on infected hosts (Askary & Brodeur, 1999). Host integumental penetration is expected when *L. longisporum* is directly sprayed on four-day old *A. aphidimyza* larvae. However, penetration via the digestive tract may also

occur when larvae are fed upon infected aphids. Access from the digestive tract could enhance the chances of a fatal fungal infection.

Second, the predatory larvae were exposed to different type and quality of fungal propagules which may differ in pathogenicity. For example, four-day old *A. aphidimyza* treated directly with the fungus were exposed only to blastospores since Vertalec is produced as blastospores which are formulated with a nutrient source in a wettable power (Shah & Pell, 2003). In contrast, predatory larvae feeding upon infected aphids could ingest both blastospores and mycelium which are produced within the insect during the process of host colonization (Askary et al., 1999). Fungal pathogenicity could be higher for *L. longisporum* propagules coming from infected aphids since passing through an aphid host may result in enhancement of virulence (Taborsky, 1992). In addition, the mycelium but not the blastospores of *L. longisporum*, contains a cyclodepsipeptide toxin (Taborsky, 1992) and the digestion of fungal structures may cause death by toxicosis rather that by mycosis (Tanada & Kaya, 1993). Predators feeding upon infected aphids may then be exposed to fungal toxins.

Third, exposure time may play an important role in fungal infection. Four-day old *A. aphidimyza* larvae were exposed only once to a single Vertalec-spray application while predatory larvae feeding upon infected aphids were continuously exposed to the source of inoculum throughout the larval state increasing the chances for infection.

Fitness of the predatory midge was also affected by feeding on fungus-infected aphids. In addition to increased mortality rates, lower dry weight, lower fresh weight and lower wing lengths were observed for predatory midges fed upon fungus-infected aphids with regard to those fed upon uninfected aphids. Although the mode of penetration, the

type of infected propagules and exposure time may explain the effect of feeding on infected aphids on *A. aphidimyza* survival, the observed sub-lethal effects may result from unsuitability of the fungus-infected aphid as a food for predator development (Askary & Brodeur, 1999). *Lecanicillium longisporum*-infected prey may be nutritionally inferior in comparison to uninfected prey. In addition, metabolites with insecticidal properties produced by the fungus during colonization of the host (Wang et al., 2005) may render the aphid a unsuitable for the predatory larvae. This is especially true if we consider that aphids in the late state of infection (4 days) were used in this experiment.

It is important to distinguish between physiological and ecological host range when extrapolating lab results to greenhouse and field scenarios (Goettel et al., 2001). Many studies have shown that entomopathogenic fungi with broad host ranges can interact antagonistically with arthropod natural enemies under laboratory settings where environmental conditions usually are optimal for the fungi. However, the conditions in the field are likely to be suboptimal for fungal activity and therefore the final outcome of fungus-natural enemy interactions may be different (Roy & Pell, 2000). A demonstration of this comes from the fungus *Entomophaga maimaga*, a potential biological control for the gypsy moth *Lymantria dispar*. Laboratory experiments indicated that 35.6% of non-target species were susceptible; however, in field trials only 2% of non-target species were infected (Roy & Pell, 2000).

Under greenhouse conditions, several factors may attenuate the negative effect of *L. longisporum*-infected prey on survival and fitness of the predatory midge. Firstly, laboratory experiments were carried out under temperature and humidity that favoured

the entomopathogen. However, environmental conditions inside greenhouses are not always expected to be optimal for fungal infection. Secondly, *A. aphidimyza* larvae were subjected to maximum-challenge tests. Larvae were fed exclusively and throughout the larval stage upon infected aphids at a late state of infection. The findings described in this chapter show that some preference for uninfected aphids maybe expected when the predator is offered a choice between late-stage infected aphids and uninfected prey. Therefore, it is expected that the predatory midge feeds in greenhouses upon a random mix of early-stage infected aphids and uninfected aphids which would attenuate the impact of infected prey. These attenuated factors may partially explain the additive effect observed during the semi-greenhouse experiment described in chapter 2.

The presence of fungus-infected prey did not significantly affect the number of aphids eaten by *A. aphidimyza*. The percentage of predators feeding on zero, one or two aphids did not differ significantly when the predator was offered two uninfected aphids, two fungus infected aphids or one infected plus one uninfected aphids. The effect of fungus-infected prey on prey consumption has been previously investigated in different model systems. However, there is no consensus regarding this issue. In some cases, fungus-infected prey has been shown to cause a decrease in prey consumption. For example, prey consumption by *D. hesperus* was lower when it was offered *P. fumosoroseus*-infected whitefly (Alma, 2005) or *B. bassiana*-infected whitefly (Labbé et al., 2006) in late stage of infection. Similarly, the predator *C. septempunctata* fed less when presented with *E. neoaphidis*-infected aphids (Roy et al., 1998). However, in some cases, the infected prey is more readily consumed; e.g. *M. anisopliae*-infected locusts were more susceptible to predation than uninfected ones and some carabids (Coleoptera:

Carabidae) consumed infected aphids in preference to uninfected ones (Roy & Pell, 2000).

When *A. aphidimyza* was given a choice between a fungus-infected aphid and an uninfected one, the predatory midge showed preference for the infected aphid only if aphids were offered 44 hours after infection. After 4 days of infection the preference reversed to uninfected aphids. However, final conclusions regarding *A. aphidimyza* prey preferences cannot be drawn since prey preference could be evaluated only in few experimental units. This was due to the fact that prey selection can be only evaluated in the infected-uninfected treatment; particularly, in those experimental units in which the predator fed upon one aphid. However, the number of predators showing this behaviour could not be controlled.

The symptoms of fungal infection in insects include reduced mobility and loss of coordination (Tanada & Kaya, 1993). The preference for infected prey over uninfected controls may be the result of lack of mobility in infected prey which render them more susceptible to predators as they are less able to escape or to ward off attacks (Bell et al., 2004). Prey mobility and not nutritional quality is the criterion employed by the predator *Geocoris punctipes* (Heteroptera: Geocoridae) to select prey (Eubanks & Denno, 2000). Since *A. aphidimyza* larvae mainly locate their prey by olfactory means (Markkula & Tiittanen, 1985), the possible absence of chemical clues from late stages of prey infection may explain the lack of attractiveness of infected aphids. At late stages of infection, massive invasion of internal tissue occurs (Askary et al., 1999) and the metabolic activity of aphids has been reduced (Tanada & Kaya, 1993). Thus, chemical clues emanating from aphids may be no longer present but this has not been experimentally confirmed.

The occurrence of prey rejection at late stages of infection and prey preference at early stages of fungal infection in the same species has been reported in different model systems. Labbé et al. (2006) evaluated the effect of *B. bassiana*-infected whitefly on the predator *Dicyphus hesperus.* In those experiments, the predator rejected prey in a late stage of infection but not those in an early stage supporting my results that prey acceptance depends on the timing of infection. In other study, *D. hesperus* was able to discriminate between *P.fumosoroseus*-infected and uninfected prey when whitefly were treated with the fungus 5 days in advance but not when the infection occurred 3 days beforehand (Alma, 2005). In addition, infected-prey rejection has been documented for the generalist predator *Anthocoris nemorum* L (Heteroptera: Anthocoridae) which avoided *B. bassiana*-infected aphids and favoured the selection of control prey (Meyling & Pell, 2006).

Survival and other fitness components of the predatory midge *A. aphidimyza* may be reduced by feeding on *L. longisporum*-infected aphids. Therefore, a strong selection pressure to avoid situations that promote infection may be expected. *A. aphidimyza* may be able to avoid the risk of infected prey by discriminating between infected and uninfected prey or by avoiding oviposition in areas that increase the risk of feeding on infected prey. *Aphidoletes aphidimyza* larvae may discriminate between infected and uninfected prey at late stage of infection. However, results from a semi-greenhouse experiment do not support this hypothesis because *A. aphidimyza* showed no preference for oviposition site between aphid colonies with sporulating aphids and colonies free of sporulating aphids.

Blaustein et al., (2004) suggest that oviposition habitat selection is more likely to occur when: 1) the progeny are highly vulnerable to the natural enemy 2) the insect has few lifetime reproductive events, and 3) eggs for each reproductive cycle are laid together as a single clutch and are not spread across multiple sites. Therefore, there could be several reasons to explain that oviposition habitat selection with regard to infected aphids was not present in *A. aphidimyza*. First, *A. aphidimyza* larvae were not highly vulnerable to infection by *L. longisporum* as shown by the findings that *A. aphidimyza* survival was not affected by direct application of Vertalec. Moreover, these findings showed that discrimination between infected and uninfected prey by *A. aphidimyza* occurred but only when prey was at a late stage of infection. Such discrimination may have attenuated the effect of feeding on infected aphids in a greenhouse setting. Second, a single *A. aphidimyza* female can produce 148 eggs during her life time which are spread among several oviposition events (Havelka & Zemek, 1999). *Aphidoletes aphidimyza* females may be selected to allocate energy to egg production rather than to searching for good oviposition sites.

Finally, oviposition site selection by the aphidophagous gall midge *A. aphidimyza* is mediated mainly by olfactory stimuli and it is known that aphid honeydew volatiles are the main source of attraction (Choi et al., 2004). Other chemical stimuli include oviposition-marking pheromones and allomones which are known to impact the number of eggs laid by *A. aphidimyza* (Ruzicka & Havelka, 1998). The ability of *A. aphidimyza* to respond to chemical signs of danger has been demonstrated in a study by Ruzicka & Havelka (1998). The predatory midge laid fewer eggs in response to oviposition-marking allomones from the predators *Coccinella septempunctata*, *Chrysopa oculata* and

Chrysopa perla which are more aggressive predators that might endanger *Aphidoletes*' eggs and larvae if aphid prey become scare (Ruzicka & Havelka, 1998). These features may explain the lack of preference for oviposition sites free of sporulating aphids. First, there may be no repellent fungus-chemical cues to deter *A. aphidimyza* oviposition. Second, the honeydew and other aphid chemical stimuli may persist despite the fungal infection and favour the completion of the oviposition event (Lucas & Brodeur, 1999). However, there are no data from my experiment to conclude in favour of either of these hypotheses.

Although further studies are necessary to clarify the effect of infected prey on survival and fitness of the predatory midge, it is possible to suggest at this point that the integration of *A. aphidimyza* and *L. longisporum* to control the green peach aphid is feasible.

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GENERAL DISCUSSION

The successful use of multiple biological control agents in pest management depends on their mutual compatibility which in turn is moulded by their interactions. In order to predict the final outcome of the combined use of biological controls, their compatibility and interactions have been studied in many systems, including: parasitoid-parasitoid systems (Perez-Lachaud et al., 2004), predator-parasitoid (Rees & Onsager, 1982; Heinz & Nelson, 1996; Colfer & Rosenheim, 1995), predator-predator(Losey & Deno, 1998; Onzo et al., 2004), fungus-parasitoid (Fransen & Van Lenteren, 1993; Brobyn et al., 1988) and fungus-predator systems (Alma et al., 2007; Labbé, 2005).

In this study, a semi-greenhouse experiment and lab experiments were used to elucidate the compatibility and interactions between the entomopathogenic fungus *Lecanicillium longisporum* and the predatory midge *Aphidoletes aphidimyza*. The findings suggested a statistically significant additive effect (i.e. independent) of fungus and predator on *Myzus persicae* populations. Similarly, Alma et al., (2007) found an additive mortality when the fungus *P. fumosoroseus* and the predator *D. hesperus* were combined to control whitefly populations and Labbé (2005) found no negative effect of the fungus *B. bassiana* when combined with the parasitoid *E. formosa* and the predator *D. hesperus* on whitefly populations.

Although no effect on the number of emerged adults was observed after treatment of four day old *A. aphidimyza* larvae with *L. longisporum*, feeding on infected aphids reduced the number of emerged adults compared to predators fed upon uninfected prey.

In addition, feeding on infected aphids was observed to reduce the fresh weight, dry weight and wing length of the predatory midge. Similarly, Bethke & Parella (1989) found that longevity of the leafminer parasitoid *Diglyphus beginii* was not affected when treated directly with *L.longisporum* but adults kept in close confinement with fungal infected aphids had significantly reduced longevity when contrasted with controls. The reduced survival of predatory midges when fed upon infected aphids can be partially explained by a higher pathogenicity of fungal propagules coming from infected aphids compared to propagules coming from a commercial formulation (Taborsky, 1992). In addition, infected prey may be nutritionally inferior compared to uninfected aphids leading to sublethal effects on the predatory midge (Askary & Brodeur, 1999).

The ability to discriminate between infected and uninfected prey may play an important role as a mitigating factor on the negative effect resulting from feeding on infected aphids. Findings from lab experiments suggested that the presence of infected aphids need not affect the number of aphids consumed by the predatory midge, but it does affect prey selection with a preference for uninfected over infected prey when aphids were at late stage of infection. Same preference for uninfected prey has been found in the predator *A. nemorum* when offered with *B. bassiana*-infected aphids (Meyling & Pell, 2006), the predator *D. hesperus* when offered with both *P. fumosoroseus*-infected whitefly (Alma, 2005) and *B. bassiana*-infected whitefly (Labbé, 2006). The stage of infection seems to be an important factor determining the presence of prey preference (Labbé et al., 2006, Alma, 2005).

Aphidoletes aphidimyza did not show preference for oviposition sites free of infected aphids although survival of larvae could be compromised by feeding on infected

prey. Likewise, Lord (2001) found that the parasitic wasp *Cephalonomia tarsalis* did not avoid *B. bassiana*-infected host for oviposition although wasp larvae do not survive when oviposition occurred in heavily infected hosts. However, selection of oviposition site to avoid infected hosts has been observed in the parasitoids *E. formosa* (Fransen & Van Lenteren, 1993) and *Aphidius rhopalosiphi* (Brobyn et al., 1988). The lack of olfactory cues from infected aphids may be responsible for the absence of preference for oviposition sites free of fungus-infected aphids since oviposition by the predatory midge is mainly influenced by olfactory stimulation (Markkula & Tiittanen, 1985).

Further studies are needed to completely elucidate the compatibility and interactions between *Lecanicillium longisporum* and *Aphidoletes aphidimyza*, for several reasons, including: 1) The scope of the results from the semi-greenhouse experiment is limited since aphid populations crashed in two treatments 30 days after starting the experiment making it impossible to corroborate the presence of an additive effect at that point in time. Therefore, the setting up of a greenhouse experiment with a broader time scale would be desirable to give final insight into this issue. 2) Only the effect of feeding on aphids at late-stage infection was tested. It would be important to determine if infected aphids at early stage of infections can cause similar effects on *A. aphidimyza* survival since that can give insight into the mechanisms underlying an additive effect. 3) Additional experiments may be needed before final conclusions can be drawn with regard to the ability of the predatory midge to discriminate between uninfected and infected aphids at late stage of infect since only few replicates were available for observation.

In summary, a statistically additive effect of *A. aphidimyza* and *L. longisporum* on *M. persicae* was observed in semi-greenhouse conditions. Although feeding on infected

aphids may cause some lethal and sub-lethal effects on the predatory midge, its ability to discriminate between infected and uninfected aphids at late stage of infection can mitigate such negative effects. Although further studies are needed to completely elucidate the interactions between predator and fungus, minimal antagonistic interactions between *Aphidoletes aphidimyza* and *L. longisporum* may be expected. Therefore, the combined use of these biological control agents in pest management programs is recommended.

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