Compatibility of the parasitoid *Aphidius matricariae* with BotaniGard for the control of greenhouse aphids

by

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Abstract

The simultaneous use of multiple biological control agents can be effective in suppressing pest populations, but the intraguild interactions that occur between biological control agents can disrupt biological control. I hypothesized that the combined use of the parasitoid, *Aphidius matricariae,* and the entomopathogenic fungi *Beauveria bassiana* (in the form of commercialized product, BotaniGard) for controlling the greenhouse aphid pest, *Myzus persicae*, would have a positive effect on pest control. Experiments were conducted in the laboratory over short periods and in greenhouses over multiple generations. Although BotaniGard had negative interactions with the parasitoids in short time scale experiments, over an extended time the combined use of BotaniGard and parasitoids had synergistic effects on aphid suppression. The number of parasitoids present at the end of the experiments in longer-scale experiments was higher in treatments with BotaniGard application than in treatments with parasitoids alone.

Keywords: *Beauveria bassiana;* BotaniGard®*; Aphidius matricariae; Myzus persicae; interaguild interactions;* biological control

Dedication

To my parents, Akram Arefi and Hassan Norouzi, who give me endless love and support and who encourage me to see the world and never stop to learn. Baba, you have been my biggest inspiration. And, Maman, you are the most incredible woman I know in the world. You both have been exemplary in my life. I love you so much.

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1. Introduction

1.1. History of Biological control

Biological control, the use of one organism to reduce the population density of another, has been practiced since the ancient times (Howarth, 1991; van Lenteren, 2012). The use of biological control may be considered to go back to the ancient Egyptians who used cats to control rodents. In terms of insect biological control there is evidence that Chinese citrus growers were using predaceous ants, *Oecophylla smaragdina* (Fabricius, 1775) (Hymenoptera: Formicidae), to control foliage-feeding insects in 400AD (Howarth, 1991; Symondson et al., 2002).

Biological control of insect pests primarily uses predatory insects, parasitoids or pathogens. There are different types of biological control: natural, conservation, classical, and augmentative biological control. Natural biological control is the reduction of pests by naturally occurring enemies without direct human intervention (van Lenteren, 2012). This type of biological control is the greatest economic contribution of biological control in agriculture. Losey and Vaughan (2006) estimated that the regulation of herbivore pests by native, non-domesticated and naturally occurring insects is valued at about 4.49 billion dollars in the United States. Conservation biological control involves human actions that manipulate habitats (including agricultural habitats) in a way that conserves, protects, enhances performance and increases the number of natural enemies of pests to reduce pest problems (Losey and Vaughan, 2006; Snyder et al., 2006; Straub et al., 2008; Symondson et al., 2002; van Lenteren, 2012). Classical

biological control often involves the suppression of invasive pests by the release of natural enemies from the pest's native region (Howarth, 1991; Shaw et al., 2011; Simberloff, 2012). Classical biological control is done with the aim of the establishment of the control agent in the environment for long-term control of the pest (van Lenteren, 2012). For most annual or seasonal crops or in protected horticulture, the long-term establishment of the natural enemies is neither possible nor required. In these cases the augmentation of natural enemies is often used. The aim of augmentative biological control is short-term pest control (Collier et al., 2004) and is the release of mass-reared biological control agents with the goal of "augmenting" the population of the natural enemies. If the release of the natural enemies takes place only at the beginning of the crop and the natural enemies naturally reproduce the method is called "seasonal inoculative release" but if the release takes place repeatedly, the method is called "inundative" release of biological control agents (Messelink et al., 2013; Polack et al., 2011; van Lenteren, 2000a; van Lentern et al., 1996). Inundative release is the most common strategy for applying insect pathogens.

Species richness enhances the functioning and the processes of many ecosystems that have ecological and/or socio-economical importance (Casula et al., 2006; Flombaum and Sala, 2008; Loreau et al., 2001; Tilman, 1996; Yachi and Loreau, 1999). Therefore, it is possible that the simultaneous use of multiple species of biological agents can be beneficial for pest suppression (Jabbour et al., 2011; Stiling and Cornelissen, 2005). However, the performance of multiple natural enemies against prey populations can be difficult to predict because of the potential interactions that occur between them (Bigler et al., 2006; Boivin et al., 2012; Myers et al., 1989; Rosenheim, 1998). Therefore, before releasing more than one species of biological control agent on

a particular target pest it is important to understand the interactions that take place between biological control agents.

Root (1967) used the word “guild” to define “a group of species that exploit the same class of environmental resources in a similar way”. This environmental resource can be a prey item that is the resource that members of a guild exploit and for which they compete and interact with one another. Intraguild interactions can be direct through intraguild predation (IGP) or indirect through competition for the same prey (Brodeur and Rosenheim, 2000; Polis and Holt, 1992; G. Polis et al., 1989; Rosenheim et al., 1993; Rosenheim et al., 1995; Schmitz, 2007; van Veen et al., 2006). These interactions occur widely in most ecosystems and are recognized as functionally important since they are the primary elements that shape or reshape ecosystems by influencing the distribution, evolution and abundance of organisms in communities (Brodeur and Rosenheim, 2000; Polis and Holt, 1992; Polis et al., 1989; Schmitz, 2007; Sih et al., 1998). The original definition of a guild did not cover possible taxonomic differences among the members of a guild and I use the term “guild” broadly in this review to include members of different kingdoms. My focus is on the interactions that take place within a pest management system. Different biological control agents that share the same pest as a food resource can be considered as members of the same guild. Through intraguild interactions, the biological control agents can improve pest suppression (Baverstock et al., 2008; Jabbour et al., 2011; Vergel et al., 2011; Ramirez and Snyder, 2009; Snyder et al., 2008; Snyder et al., 2006) or interfere with each other’s performance and reduce the strength of the potential exploitation which results in a negative impact on pest management (Denoth, Frid, & Myers, 2002; Finke & Denno, 2003; Rosenheim, 2005; Spiller & Schoener, 1990).

1.2. Direct interactions between biological control agents

Intraguild predation differs from the classical concept of predation. First of all, because intraguild predation occurs between the members of the same trophic level, it is a combination of competition and predation and this sets it apart from the classical concept of predation. Second, when an organism preys on another organism from the same guild, aside from the energy that it obtains, it also reduces competition for the food and the risk of being predated upon in case of a mutual IGP (Polis et al., 1989).

Such interactions can lead to the spatial and/or temporal segregation of predators and the prey (Walzer et al. , 2001). The following example demonstrates how IGP among biological control agents changes the distribution and abundance of organisms in a system. The two predatory mites, *Phytoseiulus persimilis* (Athias-Henriot*,* 1957) and *Amblyseius andersoni* (Chant, 1957) (Acari: Phytoseiidae)*,* share the spider mite *Tetranychus urticae* (Koch, 1836) (Acari: Tetranychidae) as their prey (Walzer and Schausberger, 2012). Although the guild members can prey on each other, the IGP is asymmetric with *A. andersoni* being the intraguild predator (Walzer and Schausberger, 2011). However, the intraguild prey, *P. persimilis,* has the capability to recognize the chemosensory cues of *A. andersoni.* Thus it not only avoids ovipositing in areas where *A. andersoni* eggs or female traces exist, but also *P. persimilis* responsiveness to chemosensory cues of *A. andersoni* increases if the female *P. persimilis* has previous experience of encountering the intraguild predator (Walzer and Schausberger, 2012).

Other than the mechanisms that the members of the same guild have acquired to separate spatially, in nature there may be temporal separation between natural enemies. For example, fungal entomopathogens are usually most effective in cool humid

conditions (which generally occur at the start and the end of growing seasons), which are the opposite of the preferred environment for most arthropod predators (Pell, 2007). Also, in terrestrial communities when natural enemies consume each other instead of the herbivores, the IGP can reduce the intensity of predation on herbivores and because of cascading effects, reduce plants' productivity (Brodeur and Rosenheim, 2000; Finke and Snyder, 2010; Snyder and Wise, 2001; Spiller and Schoener, 1990). It has also been suggested that the efficacy of a generalist predator can be reduced by feeding on alternative hosts (Debach & Rosen, 1991 reviewed by Stiling & Cornelissen, 2005). However, the use of generalist natural enemies as successful biocontrol agents is often advocated because they are more efficient control agents than specialist natural enemies and in many studies they reduce the pest numbers significantly (Cabanillas and Jones, 2013; Stiling and Cornelissen, 2005; Symondson et al., 2002).

For growers, the neutral effect of biodiversity is another type of negative effect. Negative effects happen when the biological control agents become functionally redundant (Casula et al., 2006; Straub et al., 2008; Wilby and Thomas, 2002). Functional redundancy occurs when natural enemies fill the same feeding niche and can form a single functional group (Casula, Wilby, and Thomas 2006; Schmitz 2007; Sih, Englund, and Wooster 1998). Therefore, by increasing the biodiversity of the natural enemies, they get more involved in intraguild competition without any gain or disruption in pest suppression (Wilby and Thomas, 2002). In this case, investment in different natural enemies is not worthwhile because the same results can be obtained by adding the most effective biological control agent. For example, in a study done by Straub and Snyder (2006) the lady beetles *Coccinella septempunctata* (Linnaeus, 1758) (Coleoptera: Coccinellidae) and *Coccinella transversoguttata* (Mulsant, 1850)

(Coleoptera: Coccinellidae) were the key biological control agent species for controlling the green peach aphids, *Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphididae). They tested the effect of increasing biodiversity of a system in treatments that contained one or multiple natural enemies: *Nabis americoferus* (Carayon, 1961) (Hemiptera: Nabidae) and *Nabis alternatus* (Parshley, 1922) (Hemiptera: Nabidae), *Geocoris bullatus* (Say, 1832) (Hemiptera: Lygaeidae), *Geocoris pallens* (Stal, 1854)(Hemiptera: Lygaeidae), *Misumenops Lepidus* (Thorell, 1877) (Araneae: Thomisidae), *Harpalus pensylvanicus* (Degeer, 1774) (Coleoptera: Carabidae), *C. septempunctata* (Coleoptera: Coccinellidae), *C. transversoguttata* (Coleoptera: Coccinellidae), and *Aphidius matricariae* (Haliday, 1834) (Hymenoptera: Braconidae). In this study increasing biodiversity had no effect on controlling green peach aphids (GPA) on potatoes but the treatments that contained the *Coccinella* beetles provided better control of GPA than communities without *Coccinella.*

1.3. Indirect interactions between biological control agents

Adding multiple natural enemies to control pests can lead to indirect positive effects. Natural enemies might consume different sub-populations of a single pest species due to traits that affect how, where and when they consume the prey. If natural enemies completely partition resources in such a way that there is no overlap between them then the effect of having those species together will be additive to pest management (Casula et al., 2006; Straub et al., 2008). An example of predators attacking different sub-populations occurs when they attack different developmental stages of the pest and enhance pest suppression (Finke & Snyder, 2010; Jabbour et al., 2011) or when they occupy different habitat domains (Schmitz, 2007; Straub and

Snyder, 2008). Schmitz (2007) showed that the combined presence of different predatory spiders *Pisaurina mira* (Walckenaer, 1837) (Araneae: Pisauridae), *Hogna rabida* (Walckenaer, 1837) (Araneae: Lycosidae) and *Phidippus rimator* (Walckenaer, 1837) (Araneae: Salticidae), that respectively occupy the top, bottom and middle parts of an open canopy results in an additive effect on suppression of *Melanoplus femurrubrum* (De Geer, 1773) (Orthoptera: Acrididae) grasshoppers that exist and move in all parts of an open field canopy.

Another way that the combined use of natural enemies can lead to a positive effect on biological control is through functional facilitation (Finke & Snyder, 2010; Jabbour et al., 2011; Straub et al., 2008). This type of interaction between biological control species, also known as synergistic interaction, usually results in a greater effect than that predicted by individual impacts (Sih, 1997). Functional facilitation occurs when one natural enemy increases prey (i.e. the pest) availability to one or multiple species. For example, the presence of foraging coccinellid beetles on foliage causes their prey, pea aphids, to drop from the foliage to the ground and makes the aphids more susceptible to predation by ground foraging carabid beetles (Losey and Denno, 1998). Also, several studies have suggested that the presence of predators could enhance pathogen transmission to the pest by increasing movement and thus pathogen contact (Baverstock et al., 2009, 2008; Goertz and Hoch, 2013; Pell et al., 1997; Roy et al., 1998; Roy et al., 2001; Roy and Pell, 2000). Another situation in which one predator can make the prey more available to another happens when the presence of one predator can enhance the dispersal of a pathogen by transporting it to other areas normally inaccessible to the pathogen. Studies which have demonstrated enhanced fungal infection of pests in the presence of predatory insects or parasitoids suggest that this

increase was a result of the prey's escape response (Baverstock et al., 2009; Baverstock et al., 2010; Roy et al., 1998). However, I would argue that the increase in fungus transmission may not only be a result of the behavioral response of the prey; the parasitoid may also carry fungal conidia and vector these to new areas. Such passive dispersal is also observed for other organisms. For example, Collembolans can pick up soil borne entomopathogenic fungi and disperse them by transporting conidia that become attached to their cuticle, or pass through their guts (Broza et al., 2001; Dromph, 2001, 2003). Moreover, recently it has been shown that in greenhouses bumblebees can be used to vector entomopathogens to control greenhouse pests (Kapongo et al., 2008; Kevan et al., 2008; Shipp et al., 2012).

Most research on functional facilitation and synergism focuses on interactions between predators and how the presence of one predator physically exposes the prey to the other natural enemy. However, increasing prey susceptibility to another predator can work in ways other than making the prey physically more available. For example, the presence of one natural enemy can make the prey more susceptible to another natural enemy: the larvae of the herbivorous Colorado potato beetle *Leptinotarsa decemlineata* (Say, 1824) (Coleoptera: Chrysomelidae) feed above the ground and are exposed to predators such as active searching lady beetles, *Hippodamia convergens* (Guerin-Meneville, 1824) (Coleoptera: Coccinellidae), ground beetles *Pterostichus melanarius* (Illiger, 1798) (Coleoptera: Carabidae), and also sit and wait predatory bugs, *N. alternatus,* on foliage (Ramirez & Snyder, 2009). When *L. decemlineata* pupates inside the ground it gets exposed to entomopathogenic nematodes such as *Steinernema carpocapsae* (Weiser, 1955) (Nematoda: Rhabditida) and *Heterorhabditis marelatus* (Liu and Berry, 1996) (Rhabditida: Heterorhabditidae) and entomopathogenic fungi such as

Beauveria bassiana (Deuteromycotina: Hyphomycetes). Ramirez and Snyder (2009) showed that a synergistic interaction takes place between the predators and the pathogen. In their study, if the herbivore was exposed to a predator, its immune system weakened and it was more likely to be killed by the entomopathogens later in its development. Therefore, predators may enhance a pathogen's performance not only by eliciting a behavioral escape response in the pest that increases their movements and increases their chance of encountering conidia, but also by vectoring the pathogen or by making the immune system of the pest weaker and thus making it more susceptible to infection. Considering these examples, one can understand the potential benefit of the combined use of predators, parasitoids and pathogens for controlling insect pests, especially in greenhouses where natural enemy diversity can be more readily manipulated.

Biological control is particularly common in greenhouses because the enclosed environment makes it easy and efficient to apply treatments: there is less environmental stochasticity than in the field, growers can control the negative effect of the climatic changes such as light intensity and temperature, the number of pest species developing are limited compared to field crops, and only a few species of natural enemies are required. Even though greenhouses make up only 0.02% of global agricultural areas, the use of biological control is more prevalent in greenhouses than in field crops and over half of the commercially produced biological control agents have applications in nurseries or greenhouses (Lopes et al., 2009; Paulitz and Bélanger, 2001; Pilkington et al., 2010; van Lenteren, 2000). Moreover, not only is the use of biological control in greenhouses rapidly growing, in some places it has even replaced chemical pesticides for controlling pests (Boissard et al., 2008; Driesche et al., 2008; Lopes et al., 2009;

Merino-Pachero, 2007; van Lenteren, 2000a). This makes the study of intraguild interactions between biological control agents of greenhouse pests a necessity.

1.4. Entomopathogenic Fungi

Microbial biological control agents (MBCAs) that target insect pests are becoming recognized as important organisms for insect pest control. MBCAs are naturally occurring, disease-causing microorganisms, known as entomopathogens, and usually include viruses, fungi, protozoa and bacteria, plus entomopathogenic nematodes. An advantage of these MBCAs is that they have a more complex mode of action (infection) than chemical insecticides, and thus it is much less likely that the pest will develop resistance to MBCAs (Demirci et al., 2013; Khan et al., 2012; Polar et al., 2005). However, while there are reports of insects developing resistance to some types of MBCA (Boyer et al., 2012; Cory and Franklin, 2012; Lacey et al., 2001) there are none yet for fungal biological control agents. Fungi are unusual among all the entomopathogens because of their mode of infection (Khan et al., 2012; Meyling and Hajek, 2010; St Leger and Wang, 2010). Unlike other insect pathogens, entomopathogenic fungi do not need to be ingested to initiate infection and they can be directly transmitted by contact between susceptible hosts (Cory and Ericsson, 2010), making them important biological control agents for controlling insects which do not feed directly on foliage such as sap suckers (Butt et al., 2001; Khan et al., 2012; St Leger and Wang, 2010). In addition, their ease of delivery in a variety of formulations and the large number of different strains, make fungal entomopathogens ideal candidates for pest management in numerous situations (Ibarra-Cortés et al., 2013; Khan et al., 2012; St Leger and Wang, 2010). Entomopathogenic fungi are normally applied as inundative

sprays but the pathogen has the capacity to grow, disperse and persist in the environment. Therefore, they have the potential to be used even in long-term pest control programs (Cory and Ericsson, 2010; Inglis et al., 2001). However, their use for such programs is understudied, and except for a few examples of classical biological control, the effectiveness of entomopathogenic fungi over time is rarely exploited (Shanley et al., 2009; Ugine et al., 2013).

Entomopathogenic fungi are diverse in terms of host specificity. Species of fungi differ in terms of their number of potential hosts, but also in terms of whether they are obligate pathogens or are also capable of growing outside of their host (facultative entomopathogens) (Vega et al., 2009). Obligate fungal pathogens usually have a narrower host range than those that have alternative lifestyles as their saprophytic or endophytic characteristics allow them to stay alive in absence of a viable host in their local environment (Cory and Ericsson, 2010). Although a broad host range makes a fungus a good candidate for controlling a variety of pests, this also means that the risk to non-targets is increased and several studies have shown that arthropod natural enemies are susceptible to infection by generalist entomopathogenic fungi (Askary and Brodeur, 1999; Vergel et al., 2011; Rashki et al., 2009; Seiedy et al., 2012). Thus, it is important to carry out appropriate risk assessments before their application with other natural enemies.

1.5. Parasitoids

Parasitoids are frequently used in classical and augmentative biological control. Parasitoids are either koinobiont or idiobiont depending on their effect on the host's feeding behavior and growth. The larva of a kionobiont parasitoid consumes the host

while the host continues feeding and growing whereas an idiobiont parasitoid larva stagnates the host's feeding or growth (Askew and Shaw, 1986; Harvey et al, 2013). Most parasitoids that are produced and used for biological control purposes are in the Hymenopteran families: Ichneumonidae and Braconidae, and the chalcidoid families, Eulophidae, Encyrtidae, Pteromalidae and the Eurytomidae, and some are from the Cecidomiidae (Diptera) family (Gurr et al., 2007). Braconidae (the Aphidiinae subfamily) and Aphelinidae are among the most commonly used parasitoids in greenhouses (Boivin et al., 2012; Hopper, 2003; Völkl, Mackauer, Pell, & Brodeur, 2007; Yano, 2003) and are the key parasitoids for controlling aphids (Stary, 1988 in Brodeur and Rosenheim, 2000, Vollhardt et al., 2010). The Aphidiinae subfamily in particular are solitary koinobiont endoparasitoids that specialize on aphids (Barrette et al., 2009; Boivin et al., 2012; Brodeur and Rosenheim, 2000) and are widely distributed around the world mainly in the northern hemisphere (Brodeur and Rosenheim, 2000; Ratnasingham and Heber, 2012).

The high price of mass rearing parasitoids is the main constraint to their use in biological control because, in addition to rearing the parasitoid, it is also necessary to rear its host as well as host's food plant. However, the costs vary from country to country and most mass production programs take place in countries with low manpower costs. Other alternatives that have been examined to reduce the cost of rearing parasitoids include producing parasitoids on cheaper alternate hosts, on hosts that can be reared on low cost artificial media or rearing parasitoids on artificial media that mimic the nutritional value of the host (Boivin et al., 2012; Larocca et al., 2005; Vafaie et al., 2013). However, it is necessary to ensure that these alternative solutions would not affect the quality of the parasitoid.

Parasitoids have high potential for reducing pest populations in a short time. For

example, aphidiid females have a potentially high fecundity (300-1800 eggs) and because of their short generation time, many offspring can be produced in a short time (Mackauer and Völkl, 1993). However, fecundity is usually measured under optimal laboratory conditions and parasitoids are likely to have shorter lives in the field (Manfred Mackauer, 1983). Also they parasitize only a small portion of the hosts available to them (Barrette et al., 2009; Mackauer and Völkl, 1993; Völkl et al., 2007); therefore, parasitoids are commonly used with predators or pathogens (Völkl et al., 2007).

Parasitoid performance can be influenced by interaction with other parasitoids, predators and entomopathogens (Aqueel and Leather, 2013; Lacey et al., 1997; Lazreg et al., 2009; Nielsen et al., 2005; Rashki et al., 2009; Rosenheim, 1998; Snyder and Ives, 2001; Traugott et al., 2012). Pathogens, in comparison with predators, could potentially interact with parasitoids in complex ways making it hard to predict the outcome of their interaction (Brodeur and Rosenheim, 2000; Brooks, 1993). This could involve direct or indirect interactions, and at different developmental stages of the parasitoids, that could lead to lethal or sub-lethal effects on the fungal pathogen or parasitoid fitness. Parasitoid larvae can ingest fungal blastospores and hyphae inside an infected host (Askary and Brodeur, 1999) or produce fungistatic factors that decrease the viability and pathogenicity of an entomopathogen (Askary and Brodeur, 1999; Dillon and Charnley, 1991; Powell et al., 1986). However, as an adult the effect of a parasitoid on a fungal pathogen is more likely to be assisting pathogen dispersal (as discussed earlier). Other than variation in environmental factors, the relative dose of the pathogen and the timing of pathogen application influence the end-result of the competition between pathogen and parasitoids. For example, adult *Aphidius colemani* (Viereck, 1912) (Hymenoptera: Aphididae) and *Aphidius nigripes* (Ashmead, 1901) (Hymenoptera:

Aphididae) can be infected by direct exposure to *Lecanicillium* spp. (Deut.: Moniliales) and the rate of death of the parasitoid depends on the concentration of the suspension of fungal conidia (Aiuchi et al., 2012; Askary & Ajam Hassany, 2009).

These interactions are likely to be asymmetric and to the pathogen's advantage most of the time (Brodeur and Rosenheim, 2000).. The pathogen usually competes with the parasitoid at the parasitoid's larval stage and by reducing the host quality impedes parasitoid development (Askary and Ajam Hassany, 2009; Askary and Brodeur, 1999; Brooks, 1993; Hamdi et al., 2011; Hochberg et al., 1990; Hochberg and Lawton, 1990; Milner et al., 1984; Milner, 1989; Rosenheim et al., 1995). Because of this, the timing of host infection relative to parasitism plays an important role in the parasitoid's successful of completion development; this can only happen when it has had enough time to reach a stage in its development where it can tolerate the effect of the pathogen (Mesquita and Lacey, 2001; Milner et al., 1984; Milner, 1989; Rashki et al., 2009). It is also important to point out that while the parasitoid needs a suitable host to complete development, some entomopathogens can survive as a facultative saprophyte (Aqueel & Leather, 2013). Because entomopathogens are increasingly being used as microbial insecticides in biological control programs, methods for studying the interaction and impacts on biological control outcome are required (Aiuchi et al., 2012; Alma et al., 2010; Baverstock et al., 2012; de Faria and Wraight, 2007; Frewin et al., 2012; Goettel et al., 1995; Lacey et al., 2001; Ludwig and Oetting, 2001; Mesquita et al., 1997).

1.6. Study system

Aphids are attacked by a complex of pathogens, parasitoids and predators and the nature of this complex varies with time and space, and can significantly affect aphid

population growth (Brodeur and Rosenheim, 2000; Frazer, 1988; Van Veen et al., 2008). Species in all of these functional groups are used as biological control agents against aphids in greenhouses, so their interactions are relevant to IPM. *Beauveria bassiana,* a generalist entomopathogen, is the only registered fungal entomopathogen in greenhouses in Canada and is available in commercialized form BotaniGard® ("The Pest Management Newsletter," 2013). It is registered for use against aphids, thrips, and whiteflies but can also infect beneficial insects such as parasitoids. The compatibility of *B. bassiana* or BotaniGard with arthropod biological control agents is understudied. Moreover, like other fungal entomopathogens, the interactions between *B. bassiana* and beneficial insects over time have not been studied.

I investigate the intraguild interactions between *B. bassiana* and *A. matricariae* and the compatibility of using BotaniGard with *A. matricariae* for controlling *M. persicae* in greenhouses. *Aphidius matricariae* is an important parasitoid of *M. persicae* (Giri, Pass, Yeargan, & Parr, 1982) and is an excellent organism for studying intraguild interactions because parasitoid mummies remain attached to the plant substrate and as thus remain vulnerable to pathogen exposure. I studied whether the presence of parasitoids enhances *B. bassiana* infection in the aphid population; whether BotaniGard impedes *A. matricariae* development, reproduction and population growth; and the effect of BotaniGard-parasitoid interaction on aphid mortality, reproduction and population control. My first experimental chapter reports results of studies on the short-term effects of BotaniGard on *M. persicae* and *A. matricariae.* The experiments in this chapter were designed to determine the susceptibility of *A. matricariae* to BotaniGard and the effect of BotaniGard on GPA. Host plant identity and concentration of BotaniGard-mix were studied as factors that could affect the efficacy of BotaniGard. I also studied the effect of

BotaniGard on parasitoid development and fitness (measured by dry weight, longevity and the ratio of female to male offspring, which are common metrics of fitness) as well as studying the foraging behaviour of adult female parasitoids in response to healthy vs infected aphid hosts. My second results chapter reports results of studies on the longer-term effects of the combined use of BotaniGard and parasitoids on aphid populations as well as the effects of BotaniGard on parasitoids in greenhouses. The experiments investigated how BotaniGard and *A. matricariae* interact with each other and with GPA, and examined the consequences of pathogen-parasitoid-aphid interactions on pest control and crop productivity.

1.7. Reference:

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2. The short-term effects of BotaniGard (*Beauveria bassiana*) on the green peach aphid, *Myzus persicae*, and the parasitoid *Aphidius matricariae*

2.1. Introduction

Biological control in greenhouses is rapidly growing worldwide because of the advantage that a closed environment has for applying biocontrol agents (Boissard et al. 2008; Driesche et al., 2008; Lopes et al., 2009; van Lenteren, 2000a). In some systems, biological control has good potential for replacing chemical methods of arthropod pest control (Gillespie et al., 2002; van Lenteren, 2000b). For example, biological control of thrips has almost completely replaced the use of chemical insecticides in greenhouses in Spain (Merino-Pachero, 2007). One of the reasons why greenhouse growers might prefer using biological control over chemical insecticides is its practicality. For example, the release of natural enemies can take less time, has less risk to employees than chemicals, is also compatible with the use of bumble bees and honeybees for pollination and there is no required safety period between application and harvesting fruit (van Lenteren, 2000b, 2012).

Biological control of insects has been practiced since the ancient times (900 AD in China) (Howarth, 1991). In the 1930s, parasitoid *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) was shipped to countries outside of Europe, such as Canada, for the first time to be used in greenhouses (van Lenteren et al., 1996), but after World War II the distribution of *E. formosa* was terminated when insect pest control in European countries became strongly chemical-based (van Lenteren, 2000b; van Lenteren et al., 1996). However, the interest in the use of natural enemies increased in 1970-1999, because many pests (for example, whiteflies in greenhouses) had developed chemical insecticide resistance, which increased the need for using natural

enemies; as a result, many new natural enemy species became available (van Lenteren et al., 1997, 2000b, 2012, 1996). Cock et al. (2010) list more than 170 species of invertebrates that are used in augmentative biological control in Europe against more than 100 pests. Most of the natural enemies used worldwide in pest management belong to the Arthropoda, and within the arthropods, Hymenopteran parasitoids comprise most of the natural enemy species that are used in augmentative biological control (van Lenteren, 2012). Parasitoids are commonly used in greenhouse agriculture because of their specificity in targeting pests compared to predators (Messelink et al., 2013; van Lenteren & Woets, 1988; van Lenteren, 2000b, 2012).

Natural enemies are not limited to invertebrate natural predators. Microbial agents are also increasingly used and are very often merely substituted for chemical pesticides (Jaronski, 2010). Bacteria, fungi, oomycetes, viruses and protozoa are used as biopesticides against insects, pathogens and weeds in agricultural pest management (Chandler et al., 2011) and are usually applied in a similar fashion to chemical pesticides, that is, onto high density pest populations (Alma et al. 2010; Khan et al. 2012). Among microbial biological control agents, entomopathogenic fungi are becoming widely available and are being used worldwide, and are applied on high-value crops (Alma et al., 2010; Butt et al., 2001; Shipp et al., 2012). De Faria and Wraight (2007) listed 171 mycoinsecticide products (129 of which are registered and commercially available) that were mostly based on *Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria fumosotrosea*, and *Beauveria brongniartii* for controlling insects and mites. Most target pests of these fungal entomopathogens are in the orders Hemiptera, Coleoptera, Lepidoptera, Thysanoptera and Orthoptera (De Faria & Wraight, 2007).

The productivity and functioning of many ecosystems has improved because of biodiversity (Casula et al., 2006; Flombaum and Sala, 2008; Loreau et al., 2001; Tilman, 1996; Yachi and Loreau, 1999). Included within ecosystem functioning are the effects that biodiversity has on natural pest control (Casula et al., 2006; Wilby and Thomas, 2002). This positive effect is because having more natural enemies in the system can provide “more solutions” to pest management (Casula et al., 2006; Straub et al., 2008; Straub and Snyder, 2008; Wilby and Thomas, 2002). However, the simultaneous introduction of different biological control agents can have a variety of outcomes on pest control since the biocontrol agents may engage in intraguild interactions directly or

indirectly (Askary and Ajam Hassany, 2009; Brodeur and Rosenheim, 2000; Brooks, 1993). These interactions could impair, enhance, or not affect the outcome of biological control. Intraguild interactions can be in the form of predation (including infection) of one biological control agent on the other (also known as IGP) or competition for the same resources (Brodeur and Rosenheim, 2000; Denoth et al., 2002; Van Veen et al., 2008), and they can exist between individuals of different taxa (Hochberg and Lawton, 1990; Rashki et al., 2009). When natural enemies compete for the same resources (i.e. prey or host) or kill competing biological control agents, they may reduce pest suppression compared to when a single species is applied (Denoth et al., 2002; Finke and Snyder, 2010; Messelink et al., 2011; Snyder and Ives, 2001; Traugott et al., 2012). For example, some entomopathogenic fungi have a wide host range, compared to other insect pathogens, and could potentially harm non-target species and beneficial insects, such as other natural enemies (Roy and Pell, 2000). Parasitoids, in particular, can be influenced in several ways as they depend on host quality. As Brooks (1993) explains, entomopathogens like fungi can reduce the suitability of the host for parasitoid larval development, alter the parasitoids' oviposition and mating behaviour, infect the parasitoid adult or larvae by invading their tissues, or change parasitoid fitness in terms of longevity and fecundity.

Studies have demonstrated direct and indirect ways that a fungal entomopathogen can influence parasitoids. Fungal entomopathogens have been shown to reduce parasitoid longevity, fecundity and emergence success, and alter sex ratio, foraging behaviour and development (Aiuchi et al., 2012; Aqueel and Leather, 2013; Askary and Ajam Hassany, 2009; Askary and Brodeur, 1999; Brobyn et al., 1988; Mesquita and Lacey, 2001). There is some evidence that indicates that parasitoids, too, can have a negative effect on fungi if the parasitoid larva digests or releases chemicals that inhibit fungal growth (Brodeur and Rosenheim, 2000; El-Sufty and Furher., 1981; Führer & Willers, 1986; Powell et al., 1986; Willers et al., 1982). Thus, the simultaneous use of entomopathogenic fungi and parasitoids might disrupt a parasitoid-driven biological control system.

On the other hand, it is possible that the foraging of the natural enemies does not overlap. For example, if natural enemies attack different developmental stages, the biological control agents can have an additive effect on pest suppression (Castrillo et al.,

2008; Casula et al. 2006; Finke and Snyder, 2010; Griffiths et al. 2008; Jabbour et al. 2011; Ramirez and Snyder, 2009; Snyder et al. 2008; Straub et al. 2008). Natural enemies can also have non-lethal effects on their prey, known as trait-mediated effects, that change the victim's behaviour, life history, physiology, and morphology that can modify the predator-prey interaction and expose the pest to other natural enemies (Abrams, 1995; Kunert & Weisser, 2003; A Sih, 1997). An example of this effect is when one predator changes the behaviour of the prey in a way that makes the prey more vulnerable to other natural enemies (Casula et al., 2006; Griffiths et al., 2008). In such cases the predators will facilitate one another and have synergistic effects, which is a greater effect than the sum of the independent impact of each predator alone (Casula et al., 2006; Griffiths et al., 2008; Losey & Denno, 1998; Schmitz, 2007; Straub, Finke, & Snyder, 2008). Therefore, the compatibility of parasitoids and fungi must be tested before using them together for pest management.

In greenhouses, aphids attack a wide variety of crops including a range of vegetables and ornamental flowers (Driesche et al., 2008; Gillespie et al., 2002; Yashima and Murai, 2013). They can deplete plants of nutrients by sucking plant sap, transmit important viruses, and increase the likelihood of mould infestations by depositing honeydew on the crops (Boivin et al., 2012; Emden and Harrington, 2007a; Hogenhout et al., 2008; Ng and Perry, 2004; Sylvester, 1989; Ye et al., 2005). The green peach aphid (GPA), *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), is one of the principal pests of greenhouse crops worldwide (Capinera, 2005; Zamani et al., 2007) and is notorious for developing resistance to chemical pesticides (Foster, Denholm, & Thompson, 2003). As the name suggests, peach trees are its primary host plant (Emden and Harrington, 2007a), but GPA has more than 875 secondary host plants and is a pest of a wide variety of vegetables, ornamentals and field crops (Gillespie et al., 2002; Zamani et al., 2007). In 2002 and 2003, several greenhouses in British Columbia (Canada), suffered uncontrollable outbreaks of GPA on bell peppers (Bennett et al., 2009; Janmaat et al., 2011).

In greenhouses biocontrol agents are usually augmented in a seasonal, inundative fashion, which provides relatively long-term pest control and the control agents are removed at the end of the growing season along with the pest (van Lenteren, 1988). Biological control has become a common approach for controlling GPA in

greenhouses in Canada and a range of predators, parasitoids and pathogens have been shown to be effective (Gillespie et al., 2002). Four parasitoids, *Aphidius matricariae, Aphidius colemani* Viereck (Hymenoptera: Braconidae), *Aphidius ervi* Haliday and *Aphelinus abdominalis* (Dalman) are commonly used for aphid pest control in greenhouses (Gillespie et al., 2002). Among the pathogens, *Beauveria bassiana* has been shown to have promising effects on controlling aphids (Gillespie et al., 2002). Several products which contain *B. bassiana* as their active ingredient have been produced and registered in different countries, (see Khan et al., 2012 for a list). One such product is BotaniGard® 22WP (Laverlam International Corporation, Butte, MT) which uses *B. bassiana* strain GHA conidia as its active ingredient available in the form of a wettable powder. *Beauveria bassiana* is the only registered entomopathogen against aphids in greenhouses in Canada ("The Pest Management Newsletter," 2013). This entomopathogen was registered in Canada in 2009 as a foliar spray against a wide range of insect pests, including aphids and on a variety of crops such as ornamentals, vegetable and greenhouse products. Since then, it is increasingly being adopted as a control strategy in greenhouses (Shipp et al., 2012).

Beauveria bassiana has a host range of > 700 species (Inglis et al., 2001; Shipp et al., 2012). This pathogen is widely used to control many different orders of arthropod pests on greenhouses produce, outdoor crops, pastures and grassland: Homoptera, Heteroptera, Thysanoptera, Coleptera, Lepidoptera, Orthoptera, and Diptera (Goettel et al., 1995; Islam and Omar, 2012; Johnson and Goettel, 1993; Shah and Goettel, 1999; Shipp et al., 2012). However, it can also potentially be harmful to beneficial insects such as the natural predators and parasitic wasps that control pest populations (Goettel et al., 1990; Ludwig and Oetting, 2001; Shipp et al., 2003; Shipp et al., 2012). For example, Shipp et al. (2003) reported 31-83% infection rates for the adult parasitoids *E. formosa*, *Eretmocerus eremicus* Rose & Zolnerowich (Hymenoptera: Aphelinidae), *A. colemani*, *Dacnusa sibirica* Telenga (Hymenoptera: Braconidae) *Aphidoletes aphidimyza* (Rondani) (Diptera: Cecciidomyiidae), and the adults and juveniles of the predatory bug *Orius insidiosus* (Say) (Heteroptera: Anthocoridae) in conditions of 75% or 95% humidity in Petri dishes. Such small-scale laboratory studies are valuable for exploring the potential host range of entomopathogens. However, environmental conditions in greenhouses or the field may be very different and when parasitoid and fungal

entomopathogen releases are going to be combined, factors such as the timing of releases, host life stage, application methodology and fungal concentration also need to be considered (Brodeur and Rosenheim, 2000). Few studies have investigated the use of entomopathogenic fungi with parasitoids under more realistic conditions.

In this study I investigate the interaction between *B. bassiana*, in the form of Botanigard, and the commonly used parasitoid *Aphidius matricariae* (Hymenoptera) for the control of GPA. *A. matricariae* is a solitary koinobiont endoparasitoid belonging to the subfamily Aphidiinae [Braconidae] (Boivin et al., 2012). It was introduced to Canada in the 1950s and is now available widely commercially (Clausen, 1978 reviewed by Gillespie et al., 2002). The larva develops and pupates inside the aphid at which time the cuticle of the aphid changes to a shiny gold color, a structure referred to as the mummy. When the development is complete, the adult parasitoids carve an emergence hole to exit. Mating usually occurs within 24 hours of emergence and mated females are immediately ready to parasitize aphids if the latter are present in their vicinity.

The aims of the experiments in this chapter were to examine how effective BotaniGard was for suppressing GPA, and to determine whether BotaniGard had negative effects on *A. matricariae*. My null hypotheses were that BotaniGard does not affect the parasitoids' oviposition behaviour and does not impact the development and fitness of parasitoid larvae that develop inside infected hosts. I first investigated the susceptibility of GPA to BotaniGard in terms of longevity and reproduction. I then asked whether the method of applying BotaniGard and the host plant identity would influence the effectiveness of BotaniGard. I predicted that BotaniGard would reduce aphids' longevity and reproduction and that the rate of reduction will be faster with the higher concentrations of the fungus than lower concentrations. Then, I tested the effect of the application technology and host plant identity on the infection of aphids and I predicted that leaves with a hairier surface and wet application of BotaniGard would increase the level of aphid mortality as the conidia would be more likely to stick. In regard to the interaction between the parasitoid and the pathogen, I predicted that BotaniGard would infect parasitoids when applied to mummies directly or to the parasitized aphid hosts; however, the negative effect would be limited as I predicted that parasitoids would be able to identify an infected host as a bad host and would parasitize them less than a healthy host. Finally, I predicted that the timing of the BotaniGard application would

influence the degree to which BotaniGard affects the parasitoids negatively (i.e. the less time the parasitoid larva has for development before competition with the fungus, the less likely that it will complete its development).

2.2. Materials and Methods

2.2.1. Plant and Insect production

Pepper plants (*Capsicum annuum*), cv. Bell Boy were grown from seed on greenhouse benches at Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre in Agassiz, British Columbia (PARC). These plants were used for experiments, and the maintenance of aphid and parasitoid colonies. The temperature in the greenhouses was set at 20°C and the light regime was set for 16hr light and 8h dark.

The GPA colony was initiated from a colony at the Agriculture and Agri-Food Canada Research Centre. This colony originated from a few light-green individuals on a single leaf that were collected from a greenhouse at the research centre and maintained in isolation since 2005 in small cages (60 x 60 x 60cm BugDorm- 2120 Insect rearing tent (BD2120), MegaView Science Co. Ltd. Taiwan). The GPA colony was maintained in an isolated insectary room with controlled environment (16L:8D photoperiod, 23°C ± 2 °C and 60±10% RH) in two ways. The stock colony was maintained on pepper plant leaves that were cut to fit in cups. The cups used for this purpose were 237ml Styrofoam cups (Solo Cup Company, Lake Forest, CA, US). Aphids were also produced on whole plants in a cage (60 x 60 x 60cm BugDorm- 2120 Insect rearing tent (BD2120), MegaView Science Co. Ltd. Taiwan). To start this colony, leaf pieces from the stock colony were placed on aphid-free 6-week-old plants with about eight fully expanded leaves. These plants were used for producing the *A. matricariae* colony.

The parasitoid colony was maintained in cages in an isolated insectary room with controlled environment (16L:8D photoperiod, 23°C ±2 °C and 60±10% RH). The *A. matricariae* colony was initiated from stock material provided by Applied Bionomics (Sidney, BC). Pepper plants that were colonized by aphids were transferred to the parasitoid cage on a weekly basis. The old plants were cut after four weeks and left on the bottom of the cage to allow some time for the mummies to emerge.

To obtain cohorts of aphids of the same age for all the experiments, eight adult aphids were placed on a pepper leaf inside a 237ml Styrofoam cups (Solo cup company, Lake Forest, CA, US) and left to reproduce for a day. After the first day of reproduction, adults were removed and offspring were maintained in the same cup until they reached the age to be used in the experiments. The age at which these aphids were used in experiments depended on the requirement of the experiment.

,To produce *A. matricariae* of the same age for each experiment, fifteen female *A. matricariae* were introduced to approximately 300 3-day-old aphids feeding on pepper leaves inside insect rearing cages (60x 60 x 60cm BugDorm- 2120 Insect rearing tent (BD2120), MegaView Science Co. Ltd. Taiwan). *A. matricariae* development from eggs to adults takes approximately 12 days at room temperature and mummies form within about 8 days after parasitization. If parasitoid mummies were used in the experiment, they were gently removed from the leaves using fine forceps (BioQuip, USA), placed in 60ml plastic cups (Solo Cup Company, Lake Forest, CA, US) treated within the first 24h after formation. For other experiments, the female adults that emerged out of the mummies were collected from the emergence cage and transferred to the experimental system using an aspirator. These parasitoids were collected within 24h after emergence and were one day old, mated and naïve to aphids.

2.2.2. BotaniGard application rate

The product label of BotaniGard 22WP recommends the preparation of a BotaniGard-water mix at a rate of 500g/400L against aphids. The label concentration for BotaniGard is, $4.4x10^{13}$conidia/kg of the material, which is equal to a concentration of $5.5x10^{7}$ conidia/mL of the material in water. This concentration will herein be described as the recommended concentration.

2.2.3. Leaf disk preparation

For the laboratory experiments aphids were maintained on leaf disks inside 60ml plastic cups (Solo Cup Company, Lake Forest, CA, US) with holes for ventilation. Leaf disks (3cm diameter) were cut from the waxy leaves from the middle part of the pepper plants. Each leaf disk contained a leaf vein, which was covered with a piece of moist

cotton cosmetic pad (Rexall Quilted Round Cosmetic Pads, Canada) to keep it turgid. The disks and the cotton pads were changed every three days to provide fresh leaves for the experimental aphids, and the excess moisture in the cups was wiped with a paper towel.

2.2.4. Experiment 1: The effect of BotaniGard on the longevity and reproduction of Myzus persicae

This study was designed to test the effect of BotaniGard on the longevity and reproduction of eight-day-old (new adult) aphids. Eight-day-old aphids are at the age when they will start reproducing within a short time, which allows for the measurement of their reproduction, as well as longevity, after fungal exposure. The aphids were individually dipped in one of four concentrations of BotaniGard for one second: 10x recommended concentration (extremely high concentration), recommended concentration (high concentration), 1/100 recommended concentration (low concentration), 1/10,000 recommended concentration (extremely low concentration) and water (control). These concentrations were designed to differ from the next concentration by 100x so as to study a wide a range of concentrations as possible. The exception was the 10x concentration because the 100x recommended concentration was a paste instead of a suspension. There were 15 replicates per treatment.

After treatment, each aphid was placed on a pepper leaf disk inside a 60ml plastic cup (Solo Cup Company, Lake Forest, CA, US). The cups were kept in an insectary room (16L:8D photoperiod, 23°C ±2 °C and 60±10% RH). Aphids were monitored every one to two days and their date of death was recorded to estimate longevity. Aphids are reproductive until death. When the aphids were checked every 2 days, the date of death of the aphid was determined based on the presence or absence of offspring: if the dead aphid had no offspring it meant that it died on the first day, but if there were any offspring on the leaf disk, it meant that the aphid died on the second day. The number of offspring produced by each aphid was recorded every 3 days when the leaf disks were changed.

Aphid longevity in response to exposure to different concentrations of BotaniGard was analyzed using a Kaplan-Meier survival analysis with a critical α of 0.05. When significant differences in the survival plots occurred, pairwise comparisons were carried out using the same method. The mean daily offspring production was first subjected to logarithmic transformation and checked for normality before being analyzed with a one-way analysis of variance (ANOVA).

2.2.5. Experiment 2: How do application methodology and host plant identity influence the efficacy of BotaniGard on aphids?

BotaniGard can be applied as the dry product rather than in a wet formulation. This study was designed to compare the two approaches to examine whether moisture makes the *B. bassiana* conidia stick better to the leaf surface or the insect cuticle. In addition, I compared whether the effect of BotaniGard was likely to vary with the host plant by contrasting the effect of BotaniGard on radish, *Raphanus stivus* L. and bell pepper. Radish and bell pepper leaves differ from each other in many ways; however, as entomopathogenic fungi are acquired by the insect through contact, rather than ingestion, leaf surface properties are particularly important. Thus how well the fungal conidia stick to the surface, and how evenly distributed they are, are likely to be very important in terms of the probability that aphids encounter an infective dose. The most obvious difference between the two leaf types is that radish leaves are hairy and pepper leaves are smooth and waxy; however, there will be other more subtle differences that might also affect the transmission process. The radishes were planted and maintained in the same manner as the pepper plants. I hypothesized that if moisture makes conidia stick better to both plants and insects then the infection rate of aphids in wet treatments should be higher than dry treatments, resulting in greater mortality, a faster speed of kill and reduced reproduction. My second hypothesis was that if the hair on the leaves holds the conidia and provides more contact surface for the aphids, infection (and consequently aphid mortality) should be higher or more rapid on radish compared to pepper plants. Furthermore, the effect of BotaniGard that was sterilized by chemo-sterilization was compared with the original form of BotaniGard on aphids to determine if the other components in the mix were toxic to the aphids.

This method of sterilizing BotaniGard was adapted from Paulitz & Linderman (1991). Sterilized BotaniGard was prepared by placing 40g of powder inside a 400ml glass beaker (KIMAX®, Rockwood, TN, US) and covering the powder with cotton balls that were saturated with 40ml Propylene Oxide in a sealed 5L glass container, to maintain the propylene oxide evaporations inside the system. The container was sealed for 3 days and opened under a fume hood and left open for 2 days. To verify sterility, the chemo-sterilized BotaniGard was plated on 2% Agar-water (Anachemia, Montreal, CA) and the test was repeated 10 times. The treated agar plates were maintained on a lab bench for a week and the sterilization of BotaniGard was confirmed when no germination and growth was observed on any of the plates.

Three-centimeter diameter leaf disks were prepared for all treatments and the two controls, which were left untreated. These treatments were BotaniGard application (live powder and sterile powder), applied to two different plants (pepper and radish), in two different ways (wet and dry). There were 24 replicates per treatment.

To apply BotaniGard, the abaxial sides of the leaf disks that were cut from the middle of leaves and contained a leaf vein, were dusted with either the sterile or the original dry powder of BotaniGard. A fine paintbrush was dipped in the powder and was gently shaken twice to remove the excess powder before being rubbed lightly on the leaf. Although the number of conidia that was applied is not known, the same method of application was used for all leaves and the amount of powder applied is therefore likely to be similar among treatments. Therefore, while this is an imprecise method there should be no systematic bias among treatments. Dusting leaves with the powder was the final step for the dry treatments before the introduction of the aphids to the system. For wet treatments an additional step was taken: after applying BotaniGard the treated leaves were sprayed with water using an airbrush (Vaper Dual-action Gravity-feed Airbrush, Renton, WA, USA). The amount of water that was sprayed was only enough to wetten the surface of the leaves. The wet leaves were then air-dried for an hour before the aphids were placed on the leaves (both dry and wet treatments). For each plant type, a control group was assigned that was not dusted with BotaniGard (live form or sterile form). After the leaves were prepared, a six-day-old aphid (3rd instar) was placed on the treated abaxial side of the leaf in a 60 ml plastic cup (Solo Cup Company, Lake Forest,

CA, US). Organisms were transferred and maintained in an insectary room with controlled environment (16L: 8D photoperiod, 23°C ±2 °C and 60±10% RH).

After 24hr, the aphids were checked for survival and the leaf disks were replaced with a fresh, untreated leaf disk of the original host plant. In preliminarily tests, aphids treated with BotaniGard started dying a week after being treated with that material. As a result of these tests, the experiment was left unchecked for the first 6 days. After that period, the aphids were monitored every 1 to 2 days and leaf disks were changed twice per week. Each time the leaf disks were replaced the adult aphid was transferred to the new leaf disk. The experiment was done only once.

The aphids that died within 24 hr of treatment were excluded from the analysis, as they were likely to be due to handling error. Aphid longevity was compared using a Kaplan-Meier survival analysis with a critical α of 0.05. When significant differences in the survival plots occurred, pairwise comparisons were carried out using the same method. However, because almost all aphids in BotaniGard treated groups unexpectedly died within the 6 day period that the experiment was left unchecked, no confidence intervals were obtained for these treatments. All remaining aphids were monitored until they all died.

2.2.6. Experiment 3: The effect of BotaniGard on the longevity and development rate of Aphidius matricariae.

Parasitoid cages were prepared as described earlier. On the same day that the mummies were formed (i.e. 8 days after the aphids were parasitized) they were randomly collected from pepper leaves and treated with different concentrations of BotaniGard. I hypothesized that if BotaniGard can infect the immature parasitoid inside the mummy, then parasitoids would have a shorter life span and/or have slower development after exposure to high concentrations of BotaniGard. Thus, four different concentrations of BotaniGard were prepared relative to the recommended concentration: 10x recommended concentration, recommended concentration, 1/100 recommended concentration, 1/10000 recommended concentration and water as a control. Parasitoid mummies were gently detached from the pepper leaves and they were immersed in one

of the BotaniGard suspensions for one second using fine forceps (BioQuip, USA). The mummies were then placed in 60 ml plastic cups (Solo cup company, Lake Forest, CA, USA) size and were reared in an insectary room with controlled environment (16L:8D photoperiod, 23°C ±2 °C and 60±10% RH).

The insects were monitored on a daily basis for parasitoid emergence and death and there were 48 replicates per treatment. The number of parasitoids that completed development (i.e. emerged), the speed of development (the number of days from aphid parasitization to parasitoid emergence), and the sex ratio of the emerged parasitoids were recorded.

Parasitoid emergence and sex ratio were compared across treatment groups using a Pearson's chi-squared test. Parasitoids' development time (the period between the application of the treatment and emergence) in response to exposure to different concentrations of BotaniGard was analyzed using a Kaplan-Meier survival analysis with a critical α of 0.05.

2.2.7. Experiment 4: Oviposition of A. matricariae in healthy vs Beauveria-infected M. persicae

The Optimal diet theory (ODT) predicts that parasitoids are expected to select resources that would maximize their lifetime fitness (Stephens and Krebs, 1986). I assumed that the infection status of an aphid host would affect the host's suitability for oviposition. The purpose of the experiment was to investigate the behavior of parasitoids in response to aphid infection status. This experiment included two different age cohorts of aphids, and to ensure that parasitoids' preference for a specific instar of aphids would not affect the results, the preferred aphid instar (second instar) was not included in the experiment (Barrette et al., 2009).

Each parasitoid was exposed to an aphid before the start of the experiment to gain previous experience of encountering aphids. These aphids were always untreated and were the same age as the aphids that the parasitoids were exposed to during the experiment. During the trials, the parasitoid was presented with a new aphid at the onset

of the experiment that was either infected or healthy, depending on the treatment that they were assigned to.

One-day-old, recently mated, naïve parasitoids were transferred from the insect-rearing cage to 3mL shell vials with an aspirator. To prepare the parasitoid for the experiment, a healthy, untreated aphid on a $2cm^2$ square leaf patch was placed inside the shell vial, close to the parasitoid for 10mins (Figure 1) to ensure that the parasitoid gained experience with the hosts. The parasitoid was then removed and deprived of aphids for 1hr and the aphid and the leaf patch were discarded. The parasitoid was then ready to use in the experiment.

During the experiment the parasitoid's behavior was observed in response to the healthy or infected aphid that it encountered. This aphid was prepared as follows: a 1µL drop of either water (control groups) or the recommended concentration of BotaniGard mix (treatment groups) was placed for one minute on one-day old aphids. After a minute of exposure to the liquid, the liquid was removed by gently holding a tissue against it to avoid the aphid being drowned. The aphids were then maintained on a 3cm diameter pepper leaf disk in a plastic cup (Solocup, Lake Forest, CA, US) for five or seven days depending on the treatment group that they were assigned to. Once the 1hr deprivation period was over for the parasitoid, it was exposed to an aphid from either the treated group (BotaniGard-treated) or the control group (water-treated). These aphids were on a leaf piece that was carefully placed in the parasitoids' shell vials to avoid stressing the parasitoid. The design of the experiment was a 2 x 2 factorial design (aphid age x fungus treatment): 5-day old BotaniGard treated (N=19), 5-day old water-treated (N=30), 7-day old BotaniGard-treated (N=23), and 7-day old water-treated (N=11). In this experiment the age of the aphid is confounded with pathogen development i.e. pathogen infection should have progressed further in the older aphids; however, it still provides an initial assessment of whether pathogen infection influences oviposition behavior.

The experiment lasted for a maximum of 22mins. This was the maximum time that I could allocate to each trial due to time constraints. A trial was stopped if the parasitoid ignored the aphid, i.e. it did not search for the aphid or was not in the vicinity of the aphid for more than two minutes. The number of stinging attempts by the parasitoid (zero, once, more than once) was recorded for each trial. Parasitoids' average

number of stinging attempts was compared for all treatments to understand if there was a difference between parasitoids' stinging attempts for different treatments. This metric was compared using a nominal logistic regression fit.

2.2.8. Experiment 5: Is BotaniGard compatible with Aphidius matricariae for the control of green peach aphids in greenhouses?

Two separate experiments (A and B) were completed to determine the effect of BotaniGard on populations of *A. matricariae* in terms of development, sex ratio, longevity and weight. In "experiment A" potentially parasitized aphids were treated with BotaniGard, whereas in "experiment B" parasitoids encountered aphids that had been previously treated with BotaniGard. Both experiments explored the effect of timing on the end result of the competition between the parasitoid and the fungus.

For both experiments, 4-week-old pepper plants were maintained in individual pots in a greenhouse at PARC, Agassiz. Leaf disks harboring one-day old aphids were prepared and transferred onto the pepper plants. The number of aphids on each leaf disk was recorded for each pot. The aphids were then left for a day so that they moved to the plants from the leaf pieces. BotaniGard mix, 1.5 ml or water (control) was applied to plants harboring aphids using an airbrush (Vaper Dual-action Gravity-feed Airbrush, Renton, WA, USA) at 100x recommended concentration to ensure infection. The amount of liquid that was sprayed on plants was enough to wet the surface completely. Each of the four-week-old pepper plants was considered a replicate. Introduced parasitoids were one day old, mated and naïve to aphids. All organisms (aphids, parasitoids and plants) in both experiments were contained inside perforated bags placed over each plant to prevent the insects from escaping while still allowing ventilation.

Experiment A: Parasitization Before Inoculation

On average, 92 aphids (μ= 92.46 ± 1.6 SE, min 70, max 117) were transferred to each pepper plant. In this experiment aphids were introduced to parasitoids before being exposed to *B. bassiana* conidia. One day after the aphids were transferred, 3

female *A. matricariae* parasitoids were introduced to each plant. The parasitoids were left to oviposit in healthy aphids for 24 hours, after which they were removed. These potentially parasitized aphids were subsequently sprayed with 1.5 ml of 10x recommended solution BotaniGard or water (control groups) at one of two times: immediately after the removal of the parasitoids or 2 days later. There were 15 replications per treatment. The purpose of having two different times of spraying was to measure the effect of parasitoids' developmental stage on competition against *B. bassiana*.

After checking for normality, the number of mummies produced and the dry weight of the female parasitoids were compared across treatments with a full factorial ANOVA. The percentage of parasitoids' emergence and the ratio of the female parasitoids to the total parasitoids emerged were compared across treatments using a GLM model with a binomial distribution and logit link with over-dispersion. The longevity of parasitoids was compared with a Kaplan-Meier survival plot.

Experiment B: Parasitization After Inoculation

Pepper plants were colonized by an average of 52 aphids per plant (μ=52.21 ± 1.15 SE, minimum 28 and maximum 79). In this experiment, aphids were sprayed with 1.5 ml of 10x recommended solution BotaniGard before being introduced to the parasitoids. As in experiment A, a single pepper plant was a replicate. To test the effect of the progress of the pathogen infection on parasitoid survival, aphids were sprayed at different ages (2 day old, 3 day old and 4 day old). However, all aphids were introduced to parasitoids on the same day when they were 5 days old. Therefore, the parasitoids encountered aphids of the same age but the infection had presumably progressed more in the aphids that were sprayed at 2 days of age than the ones that were sprayed one day before the introduction of parasitoids (i.e. 4-day old aphids). There were 15 replicates per treatment.

In both experiments all mummies formed seven days after the introduction of parasitoids to the system. All mummies were collected within 24hrs of formation and kept in 0 size gelatin capsules. In addition, thirty mummies from each treatment (2 per replicate) were kept separately inside 60mL plastic cups (Solo cup company, Lake Forest, CA, US). Both the gel capsules and the 60mL plastic cups containing mummies

were kept inside a controlled environment room (16L:8D photoperiod, 23°C ±2 °C and 60±10% RH). The parasitoids inside the gel capsules were used to calculate the rate of emergence, sex ratio of the emerging parasitoids and the dry weight of the females. The parasitoids inside the cups were used to measure the longevity of the emerging parasitoids.

The cups were monitored on a daily basis to record the emergence date of the parasitoids. As soon as the adult parasitoids emerged, they were provisioned with cotton wicks (1 x 10 cm; Richmond Dental, Charlotte, NC, USA) soaked with a 5% sugar-water solution. The emerged parasitoids were sexed and longevity of female parasitoids was monitored as a surrogate of parasitoid fitness. It was expected that all parasitoids that could complete their development would emerge within the first week after mummy formation. Therefore, seven days after the collection of the mummies all of the gel capsules were placed in a -80°C freezer. All the capsules were inspected for parasitoids' emergence. The emerged adult parasitoids were sexed and air-dried for a week. In addition, to ensure all female parasitoids were completely dry, the gel capsules containing females were heated at 50°C for 90 mins inside heat blocks (VWR Digital Dry Block Heaters, Radnor, PA, USA).

In part B of the experiment, unlike part A, not all the aphids were parasitized and there were live aphids on the leaves on the day that the mummies were collected. Due to time constraints, it was not possible to count all the aphids remaining on all of the plants; however, the total number of aphids residing on 6 out of 15 plants in each treatment was recorded.

The number of mummies produced was subjected to a root function ($^2\sqrt{x}$) transformation and compared across treatments using a full factorial ANOVA. The ratio of emerged parasitoids to the total number of mummies and the ratio of females to the total emerged parasitoids was compared using a GLM with a binomial distribution and a logit link with overdispersion. The average dry weight of female parasitoids emerged from the mummies was compared using ANOVA and the longevity of female parasitoids was compared using the Kaplan-Meier survival plot. The number of aphids at the end of the experiment in each trial was compared using a 2 x 2 ANOVA, considering the number of initial aphids that were introduced to colonize the pots as a covariate.

2.3. Results

2.3.1. Experiment 1: The effect of BotaniGard on the longevity and reproduction of Myzus persicae

As expected, BotaniGard affected the longevity of aphids (χ^2 = 24.11, *df* = 4, *p* = 0.0001). The untreated aphids in the control group lived longer than those treated with BotaniGard, but the concentration of the BotaniGard did not affect the longevity of the aphids (χ^2 = 5.53, *df* = 3, p = 0.1371) (Figure 2.2 and Table 2.1). Daily offspring production did not differ between any of the treatment groups and the controls ($F_{4, 65}$ = 0.69, p = 0.6008) (Figure 2.3).

2.3.2. Experiment 2: How does application methodology and host plant identity influence the efficacy of BotaniGard?

The longevity of aphids differed between treatments (χ^2 = 132.59, *df* = 9, p = 0.0001) and BotaniGard application affected aphids' longevity (χ^2 = 124.93, *df* = 1, p = 0.0001); however, because of lack of confidence intervals for BotaniGard-treated groups, it was not possible to detect statistically where among the BotaniGard treated groups the difference resided (Table 2.2) (Figure 2.4). There was no statistical difference between the longevity of aphids residing on different host plants (χ^2 = 1.64, *df* = 1, p = 0.1998) and the method of application (wet or dry) did not affect aphids' longevity (wet or dry) (χ^2 = 1.2, *df* = 1, p = 0.2738) (Figure 2.4). The longevity of aphids treated with sterile BotaniGard was not different from that of the aphids in the control groups (χ^2 = 5, *df* = 5, p = 0.4153).

2.3.3. Experiment 3: The effect of BotaniGard on the longevity and development rate of A. matricariae

Successful emergence was not affected by treatment with BotaniGard (χ^2 = 2.69, *df* = 3, p=0.4407) and neither was sex ratio (χ^2 = 1.37, *df* = 3, p=0.7136) (Figure 2.5).

Parasitoids emerged over a span of four days but their speed of development was not affected by treatment (χ^2 = 3.42, *df* = 3, p=0.3305) (Figure 2.6).

2.3.4. Experiment 4: Can A. matricariae identify a healthy and infected aphid hosts during oviposition?

The parasitoids' pattern of stinging aphids did not differ regardless of the age and the infection status of the aphids (effect of age, χ^2 = 2.01, *df* = 2,6, p= 0.3652; effect of treatment, χ^2 = 1.16, *df* = 2,6, p=0.56; effect of age and treatment, χ^2 = 0.51, *df* = 2,6, p=0.7734) (Figure 2.7).

2.3.5. Experiment 5: How would BotaniGard application affect parasitoid reproduction, fitness and development on green peach aphids?

Experiment A: Parasitization Before Inoculation

BotaniGard treatment reduced the number of *A. matricariae* mummies that were produced (BotaniGard, $F_{1,56}$ = 26.09, p=0.0001) but this was not influenced by the timing of the spray ($F_{1,56}$ = 0.9607, p=0.3312, BotaniGard and timing of spray interaction, $F_{1,56}$ = 1.69, p=0.1987) (Figure 2.8a). Similarly, a lower percentage of parasitoids completed their development as adults when the host aphids were sprayed with BotaniGard (BotaniGard, $F_{1,56}$= 71.32, p = 0.0001), but again there was no impact of timing of spray, $F_{1,56}$ = 0.6, p=0.4389, BotaniGard and timing of spray interaction, $F_{1,56}$ = 0.06, p=0.8077) (Figure 2.8b). However, the proportion of the female parasitoids that emerged out of the mummies was similar across treatments (BotaniGard, $F_{1,56}$ = 0.09, p = 0.7557; timing of spray, $F_{1,56}$ = 0.05, p = 0.8229, BotaniGard and timing of spray interaction, $F_{1,56}$ = 2.11, p = 0.1468) (Figure 2.9a). There was no difference between the weights of these females across treatments (BotaniGard, $F_{1,53}$ = 0.0001, p = 0.9926; timing of spray, $F_{1,53}$ = 0.83, p=0.3657; BotaniGard and timing of spray interaction, $F_{1,53}$ = 0.0045, p = 0.9468) (Figure 2.9b). Also, there was no difference in the longevity of females (χ^2 = 5.44, *df* = 3, p = 0.1422) (Figure 2.10 and Table 2.3).

Experiment B: Parasitization After Inoculation

Treatment with BotaniGard reduced the number of mummies formed (BotaniGard, $F_{1,83}$ = 239.05, p = 0.0001) as well as the ratio of adult parasitoids that emerged out of mummies ($F_{1,83}$ = 101.87, p = 0.0001). That is, fewer mummies formed and fewer adults emerged out of the formed mummies if aphid hosts were treated with BotaniGard (Figure 2.11). The age of the aphids at the time of spraying did not influence mummy formation ($F_{2,83}$ = 1.03, p = 0.3627) or the ratio of emerged parasitoids ($F_{2,83}$ = 0.94, p = 0.6235) (Figure 2.11). Spraying and the age of the host aphids at the time of spraying affected the sex ratio of the parasitoid offspring; aphids that were treated with BotaniGard when they were 4 days old had a lower proportion of females than in other treatments ($F_{2,83}$ = 6.86, p = 0.0323), and resulting in a near significant main effect for aphid age ($F_{2,71}$ = 5.79, p = 0.0552) but not BotaniGard treatment ($F_{1,71}$ = 1.75, p = 0.1856) (Figure 2.12a). Also, female longevity was marginally different between treatments (χ^2 = 10.55, *df* = 5, p = 0.0611) in that female parasitoids that emerged out of aphids that were treated with BotaniGard when they were 4 days died sooner than the other female parasitoids in the other treatment groups (Figure 2.13 and Table 2.4). The weight of the female parasitoid offspring was higher in hosts treated with BotaniGard ($F_{1,63}$ = 5.37, p = 0.0238) but the age of host aphids at the time of treatment had no effect on this ($F_{2, 63}$ = 0.74, p = 0.4811) (Figure 2.12b).

The spraying treatment affected the number of the aphids present at the end of the experiment ($F_{1, 30}$ = 7.96, p = 0.0087) since there were fewer aphids in BotaniGard treatments, but the age of the aphid at the time of spraying did not influence this number ($F_{1, 30}$ = 2.02, p = 0.1507) (Figure 2.14).

2.4. Discussion

Many studies have suggested that the effect of a fungal entomopathogen on the developing parasitoid inside the host depends on the timing of exposure or infection (Mesquita and Lacey, 2001; Powell et al., 1986; Rashki et al., 2009). The effect of fungus on parasitoid larvae can be directly related to the time interval between exposure to the parasitoid and the entomopathogen (Mesquita and Lacey, 2001; Powell et al., 1986; Rashki et al., 2009). A study by Rashki et al (2009) suggested that the timing of

application of *B. bassiana* on *M. persicae* affects the number of mummies and the ratio of *A. matricariae* that complete development but not the sex ratio of the emerged *A. matricariae*. In their study the number of *A. matricariae* mummies produced and the percentage of emergence of F1 generation from GPA hosts was higher if *B. bassiana* application took place 72 or 96 hours after parasitization rather than 24 or 48 hours after parasitization. It is important to remember that time is a surrogate of the development of the parasitoid larva and indicates the developmental stage. In other words the susceptibility of a developing larva inside a host varies at different developmental stages. However, unlike the Rashki et al (2009) study there was no effect of timing of BotaniGard application on *A. matricariae* survival in the current study, but spraying BotaniGard on 4-day-old aphids that were parasitized a day later reduced the female ratio and longevity in F1. This contradicts my assumption that if *B. bassiana* colonizes an aphid host for a longer time then their influence would be stronger on the developing parasitoid larvae. The method used in the Rashki et al. (2009) study was different from that used in my study. The biology of entomopathogenic fungi is highly dependent on humidity (Baverstock, Clark, Alderson, and Pell, 2009; Ibarra-Cortés, Guzmán-Franco, González-Hernández, Suarez-Espinosa, and Baverstock, 2013; Polar et al., 2005) and Rashki et al. did their experiment inside Petri dishes that were sealed with parafilm to maintain saturated humidity. However, my experiments were done at plant scale inside greenhouses, where humidity is lower than the relative humidity that is actually recommended for periods of BotaniGard applications (Shipp et al., 2003). Therefore, it is possible that Rashki et al. (2009) observed different results on the effect of timing of application of *B. bassiana* in relation to parasitization on parasitoid development because their environment was optimal for fungal growth and the parasitoids were extremely vulnerable to the fungus.

The exposure of *A. matricariae* mummies to high and low concentrations of BotaniGard showed that the emergence, sex ratio and the speed of development did not differ between different concentrations of BotaniGard and the control group. Eighty nine percent of the parasitoids in the control group, 94% in the low concentration, 94% in the recommended concentration and 85% in the high concentration were able to complete development successfully and emerged over a span of 4 days. This suggests that *B. bassiana* does not affect *A. matricariae* at the pupa stage inside the aphid host. This is a

result consistent with other studies that investigated parasitoids' susceptibility to fungal entomopathogens at the mummy stage. Adult *Aphidius nigripes* Ashmead (Hym.: Aphididae) emerged successfully out of mummies that were inoculated by high and low concentrations of the fungus *Lecanicillium muscarium* (Deuteromycota: Moniliaceae) despite mycelial growth on the surface of mummies (Askary et al., 2006; Askary and Ajam Hassany, 2008). There was no difference in the development time of parasitoids or the sex ratio of *Aphelinus asychis* larvae when their host aphids were treated with the entomopathogenic fungus *Paecilomyces fumosoroseus* after parasitization (Mesquita and Lacey, 2001). Aiuchi et al. (2012) observed that when the mummies of *Aphis gossypii* were dipped in a range of high to low concentrations of the entomopathogen Lecanicillium spp. products (Vertalec, Mycotal and 2aF43) the emergence success and longevity of the *Aphidius colemani* female adults that emerged out of those mummies was not affected. Askary and Ajam Hassany (2008) showed that, although the adult parasitoids that emerge out of mummies that were dipped in *L. muscarium* had a shorter lifespan, the parasitoids that emerged out of infected mummies and were surface sterilized before the parasitoid's emergence, were not affected by the fungus. Therefore, they concluded that the parasitoids become infected when they came in contact with the fungus that grew on the mummies during emergence, but the *L. muscarium* did not penetrate through the cuticle of the mummies. Aiuchi et al. (2012) suggested that although *Lecanicillium* species penetrated the aphid's cuticle after parasitization, fungal penetration into the aphid host declined significantly at mummification and by a day after mummification, they observed no fungal penetration. The physical and chemical changes in the hosts' cuticle at the time of mummification appeared to prevent the fungi penetrating the insects' cuticle (El-Sufty and Furher, 1981).

Some studies have suggested that *B. bassiana* will not harm adult parasitoids. For example, parasitoid *Trichogramma pretisoum* Riley development and emergence was not affected when the parasitoid oviposited inside *Anagasta kuechniella* Zeller eggs that were treated with *B. bassiana* (Potrich et al., 2009). The mortality of adult parasitoids *Spathius agrili* Yang, *Tetrastichus planipennisi* Yang was not affected after exposure to *B. bassiana* (Dean et al., 2012). In my experiments, although *B. bassiana* did not impose a significant risk to parasitoids at adult or the mummy stage, parasitoids were still at risk before mummification as they develop within the host. The aphids that

were sprayed with BotaniGard in part A and part B of Experiment 5 were potentially parasitized aphids that had not turned into mummies at the time of spraying. In both experiments the number of mummies and the percentage of emergence of adult *A. matricariae* were lower if aphids were treated with BotaniGard compared to the control groups, regardless of the age of the aphid (part B) or the timing of spraying with regards to parasitization (part A). Previous studies suggested that the failure of parasitoids to complete development is because the pathogen reduces the quality of the host and sometimes even causes early host mortality (Brooks, 1993; Hamdi et al., 2011; Hochberg et al., 1990; Rosenheim et al., 1995). Fewer mummies of *Aphidius rhopalosiphi* De Stefani-Perez were formed when the host rose grain aphids, *Metopolophium dirhodum* (Walker), were infected with the fungus *Erynia neoaphidis* Remaudiere & Hennebert less than four days after parasitization (Powell et al.1986); however, histological samples showed no evidence of the fungus invading the parasitoid developing inside the aphid (Powell et al., 1986). Similarly, larvae of the parasitoid *Trioxy complanatus* developing inside spotted alfalfa aphids, *Therioaphis maculata,* exposed to *Zoophthora radicans* and the body of parasitoid larvae *Aphidius sonchi* inside snow thistle aphids, *Hyperomyzus lactuace* exposed to *Erynia neoaphidis* were not invaded by the fungal pathogens (Milner et al., 1984). The only recorded incident of a fungal pathogen penetrating a parasitoid larva directly is from a study done by Askary and Brodeur (1999). In their study, light micrographs showed that the fungal entomopathogen *Vertacillium lecanii* (Strain DAOM 1987499) formed a dense aggregation of hyphal bodies after colonizing the tissues of the aphids *Macrosiphum euphorbiae* that induced a localized penetration of the fungus into the *Aphidius nigripes* larva. However, evident from the noticeable inward dent on the cuticle of the parasitoid larva in the images, the physiochemical properties of *A. nigripes* cuticle appear to have prevented the direct penetration of *V. lecanii* and the fungus was only able to penetrate inside the larval cuticle through imposing a mechanical pressure (Askary and Brodeur, 1999). This evidence also suggests that in my study, the fungal infection did not influence the parasitoid directly but altered the quality of the aphid host in such a way that some individuals could not reach the pupal stage or that some of those who did pupate were not able to complete their development to adulthood. Surprisingly, the female parasitoids that emerged out of BotaniGard-treated aphids weighed more than the females from the control group (part B of Experiment 5). This effect was not

observed in Part A of Experiment 5. In Part A although there were fewer adults that emerged in treatments with BotaniGard application, several adults per each treatment were weighed: on average 10 females in control groups and 7-8 females for BotaniGard treated groups. It is important to note that in part B, while many females emerged in the control groups and were available for weighing, very few females were available for weighing in the BotaniGard-treated groups. This happened because of the low mummification and low emergence rate in these groups. The effect was so strong that no females were obtained in half of the replicates in the BotaniGard-treated aphids and in these only 1-2 females were available for weighing in the BotaniGard-treated. Since very few females were able to complete their development in these treatments and they weighed the most, one can conclude that the females that were weighed in the BotaniGard treatment groups of Part B were the "largest and fittest" females that emerged.

If the infection of aphid hosts hinders the development of parasitoid offspring inside the aphid then the competition with the pathogen could be relaxed and the parasitoid could gain a selective advantage if the ovipositing female parasitoid could avoid ovipositing in infected aphids (Baverstock et al., 2005; Brobyn et al.,1988). Some parasitoids are capable of receiving and responding to volatiles, such as those that plants release in response to herbivores, to find hosts (Fatouroset al., 2008; Salernoet al., 2013; Tamiru et al., 2011). Also, females of many parasitoid species are capable of distinguishing between already parasitized and unparasitized hosts using chemical cues while assessing their quality for oviposition and avoiding superparasitism (Chow and Mackauer, 1986; Fatouros et al., 2008; Goubault et al., 2011; Hoffmeister and Roitberg, 1997). Although there is no record of a chemical released by a pathogen that would assist a parasitoid in host selection, some studies have shown that parasitoids can discriminate between healthy and *Beauveria*-infected aphids (Brobyn et al., 1988; Fransen and van Lentern, 1993; Landa, 1984; Mesquita and Lacey, 2001). However, in this study there was no difference in the number of stinging attempts of parasitoids towards healthy or potentially *Beauveria*-infect aphids. Baverstock et al. (2005) carried out an experiment that investigated the foraging behaviour of parasitoids of the same genus as in my study, *Aphidius ervi.* Parasitoids in their study were given, at their host finding stage, a choice of entering host-plant complexes of different qualities. Their

results showed that there was no difference in entry rate of *A. ervi* to host-plant complex of healthy *Acyrthosiphon pisum* (Harris) and the entry rate of the parasitoid to a host-plant complex with *Pandora neoaphidis*-sporulating cadavers. In the same study, Baverstock and co-authors showed that the time that *A. ervi* spent searching for hosts in host-plant complexes with healthy hosts was not different from the time they spent searching for hosts in complexes with *P. neoaphidis*-sporulating cadavers. Finally, similar to my study, they showed that in Petri dish bioassays, *A. ervi* attack rate is not different for uninfected aphids or living aphids that were infected with *P. neoaphidis* for 1, 24, 48, 72 or 96 hours and that the parasitoid only avoids attacking sporulating cadavers. They concluded that *A. ervi* does not discriminate between healthy aphids and aphids infected with *Pandora neoaphidis* for oviposition. Baverstock et al. (2005) suggested that the lack of effect of the fungus on the parasitoids' ovipositing behaviour could be the result of fungus not releasing the relevant chemical cues that would signal the infection status of the aphid, or if it did release them, the parasitoid either didn't receive them or it did not respond to them. However, it is also possible, in the current experiment, that the reduced airflow and odours from the infected host might have saturated the environment and habituated the parasitoids to those odours. Further experiments would be needed to understand this interaction further, but the preliminary results suggest that the reduction in female offspring ratio and longevity of the female offspring in Experiment 5 (part B) was not due to the alteration of the oviposition behaviour of *A. matricariae* in response to the infection status of GPA.

When aphids were exposed to parasitoids before BotaniGard application, 100% mortality of aphids was observed and no living aphids were the plants at the end of the experiment. When aphids were first treated with BotaniGard before exposure to parasitoids many plants harboured aphids. However, the number of aphids had increased fivefold from the beginning until the end of the experiment when only parasitoids were used as biocontrol agents, whereas that increase was just above 2.5 times higher when aphids were treated with BotaniGard and exposed to parasitoids after. As expected, BotaniGard reduced the longevity of the aphid, *M. persicae*, but the effect of BotaniGard on aphids' longevity was not different on radish and pepper, which was contrary to my original hypothesis. The experiment was compromised by the rapid mortality, but the result is supported by the first experiment that showed that the

concentration of BotaniGard that aphids were exposed to did not influence longevity. Therefore, even if the trichomes on the leaf surface attract and hold the conidia for a longer period than plants without trichomes, as long as there are sufficient conidia to initiate infection, the actual concentration may not be that important. The method of application of BotaniGard or Chemo-sterilized BotaniGard did not affect the aphids' longevity, and neither did Chemo-sterilized BotaniGard (both wet and dry) alone. This experiment needs to be repeated for more clarification.

BotaniGard reduced the aphids' longevity in Experiments 1 and 2, and there were fewer aphids on BotaniGard-treated plants at the end of the fifth experiment than in the control group. While BotaniGard did not influence parasitoids at the pupal stage, parasitoid larva development was highly impacted by the presence of BotaniGard. Fewer mummies formed and fewer parasitoids emerged from mummies in the presence of BotaniGard. This effect was observed both when BotaniGard was applied after parasitization and when it was applied before parasitization. However, the period between BotaniGard application and parasitization in greenhouses did not affect the number of mummies and percentage emergence. Additional research is required to understand why the sex ratio and longevity of parasitoid offspring changed when aphid hosts were pre-treated with BotaniGard at 4 days of age and exposed to parasitoids at 5 days of age.

Table 2.1 The median survival times (days plus 95% upper and lower confidence intervals (CI) of Myzus persicae in relation to different concentrations of BotaniGard in relation to the recommended concentration (RC), $5.5x10^7$ *B. bassiana* conidia/mL of water.

Treatment	Median number of days to death	Lower 95% CI	Upper 95% CI
Control (water)	31A	14	31
1/10000 x RC	11B	7	23
1/100 x RC	7B	4	11
RC	7B	4	9
10 x RC	9B	4	11

Table 2.2. Median survival time (days) of, *Myzus persicae* reared on pepper or radish leaf disks that were treated with wet or dry application of BotaniGard (in its original form) or the chemo-sterilized BotaniGard (SB). No 95% confidence intervals (CI) were obtained for the BotaniGard treated groups because almost all aphids in these groups died within the first 6 days that the experiment was left unmonitored.

Treatment	Median number of days to death	Lower 95% CI	Upper 95% CI
Pepper-Control	17	10	19
Radish-Control	19	15	19
Pepper- Dry-SB	19	10	19
Radish- Dry-SB	11	7	19
Pepper- Wet-SB	15	15	19
Radish- Wet-SB	15	11	19
Pepper-Dry-BotaniGard	7		
Radish- Dry-BotaniGard	7		
Pepper- Wet-BotaniGard	7		
Radish- Wet-BotaniGard	7		

Table 2.3. Comparison of the survival of female *A. matricariae* parasitoids. Host aphids *Myzus persicae* were exposed to female parasitoids for oviposition for 24 hours. Aphids were treated with BotaniGard or with water after the removal of the parasitoids (0 days) or 2 days later. There was no difference in the survival of the emerging female parasitoids.

Treatment and timing of spray since parasitization	Median number of days to death	Lower 95% CI	Upper 95% CI
Water – 0 days	9	6	12
Water – 2 days	6	5	8
BotaniGard – 0 days	9	4	12
BotaniGard – 2 days	6	5	8

Table 2.4. Comparison of the survival of female *A. matricariae* parasitoids. The parasitoids emerged out of 5-day old aphids, *Myzus persicae*, that were treated with BotaniGard or water at different ages (2, 3 or 4 days old) and were exposed to female *A. matricariae*. There was no difference across treatments.

Treatment application and age of aphid	Median longevity	Lower 95% CI	Upper 95% CI
Water – 2 days old	8	4	10
Water – 3 days old	7	3	8
Water – 4 days old	4	4	7
BotaniGard – 2 days old	8	7	10
BotaniGard – 3 days old	6	4	12
BotaniGard – 4 days old	4	2	5

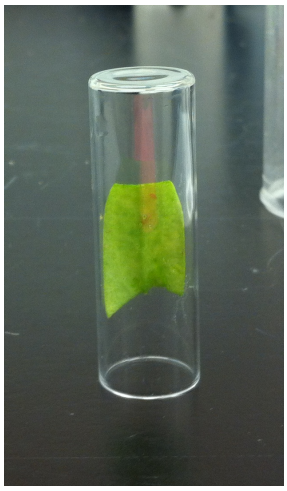

Figure 2.1. *A. matricariae* females were maintained in a 3mL shell vial and the aphids were introduced to parasitoid on 2cm^2 leaf patches.

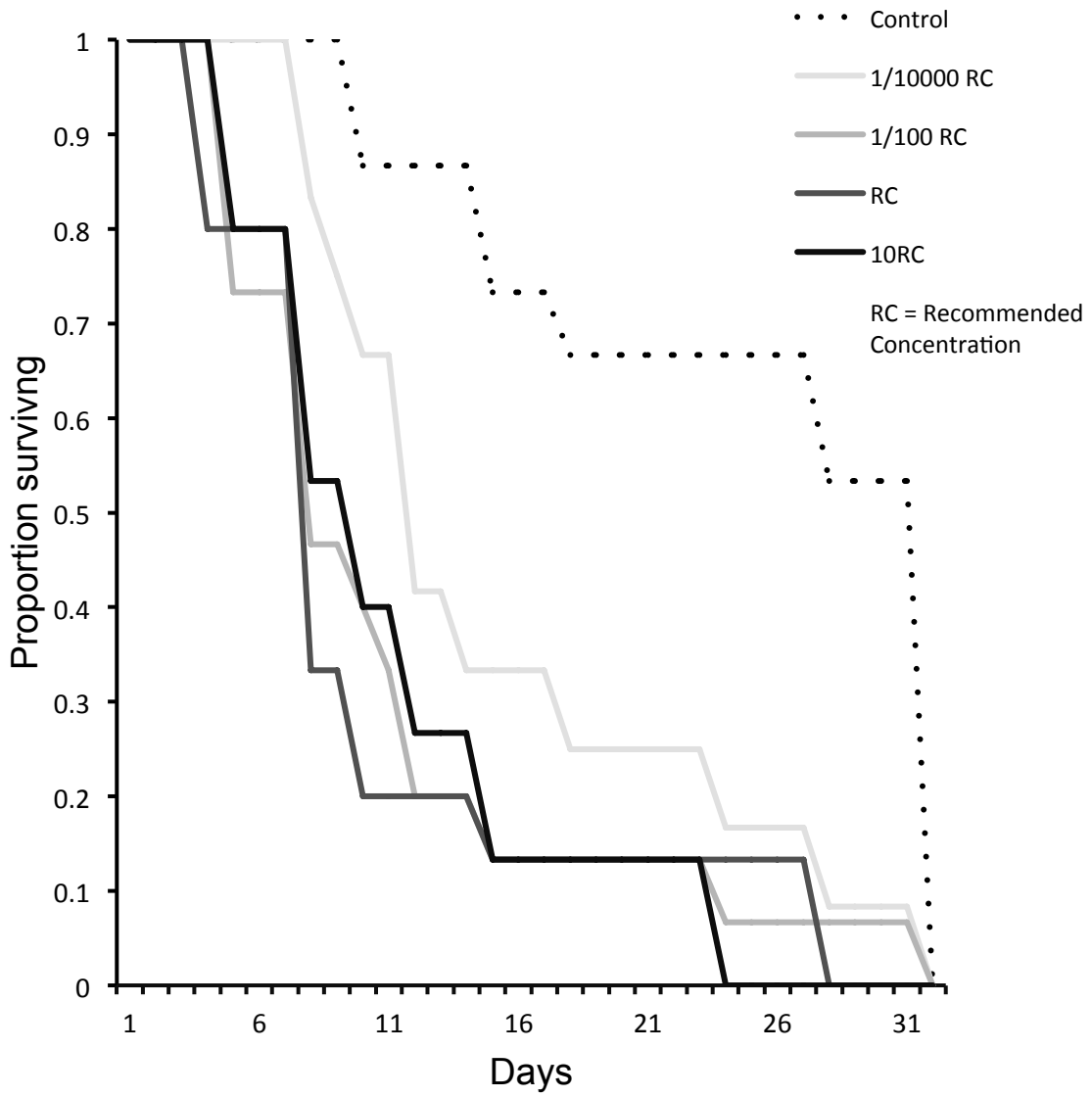

Figure 2.2. Survival curves for 8-day old *Myzus persicae* exposed to different concentrations of BotaniGard with relation to the recommended concentration (N = 15 per treatment).

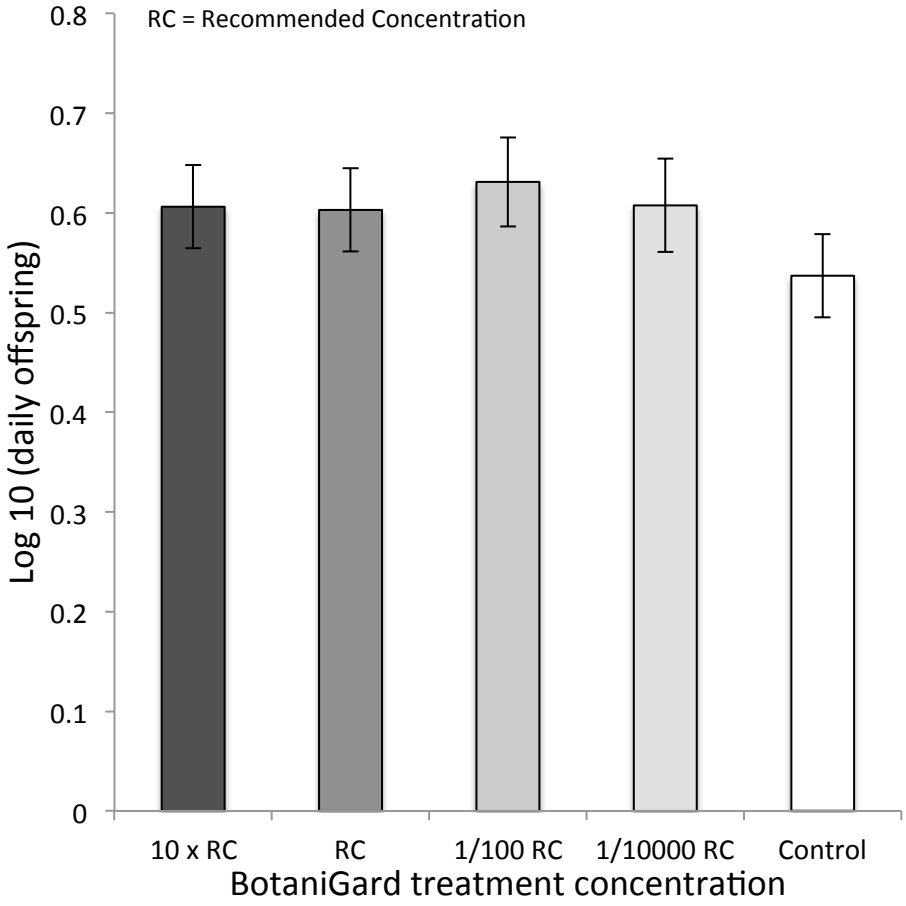

Figure 2.3. Daily reproduction (+/- Standard Error) of *Myzus persicae* after treatment with different concentrations of BotaniGard (N = 15 per treatment).

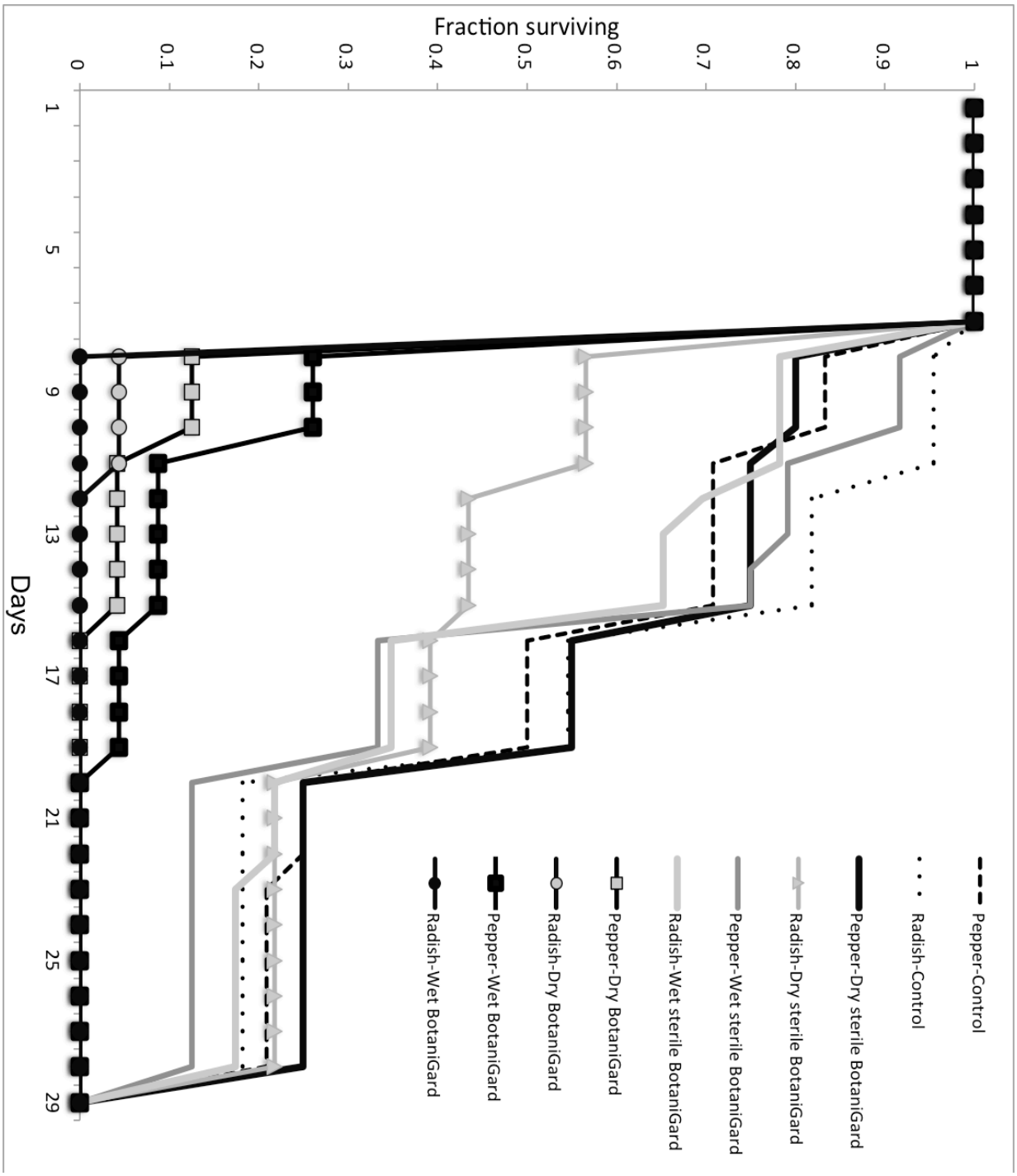

Figure 2.4. Survival of *Myzus persicae* maintained on pepper or radish leaf disks that were treated with BotaniGard in its original form, chemo-sterilized BotaniGard (Sterilized BotaniGard), or untreated controls (N = 24 per treatment).

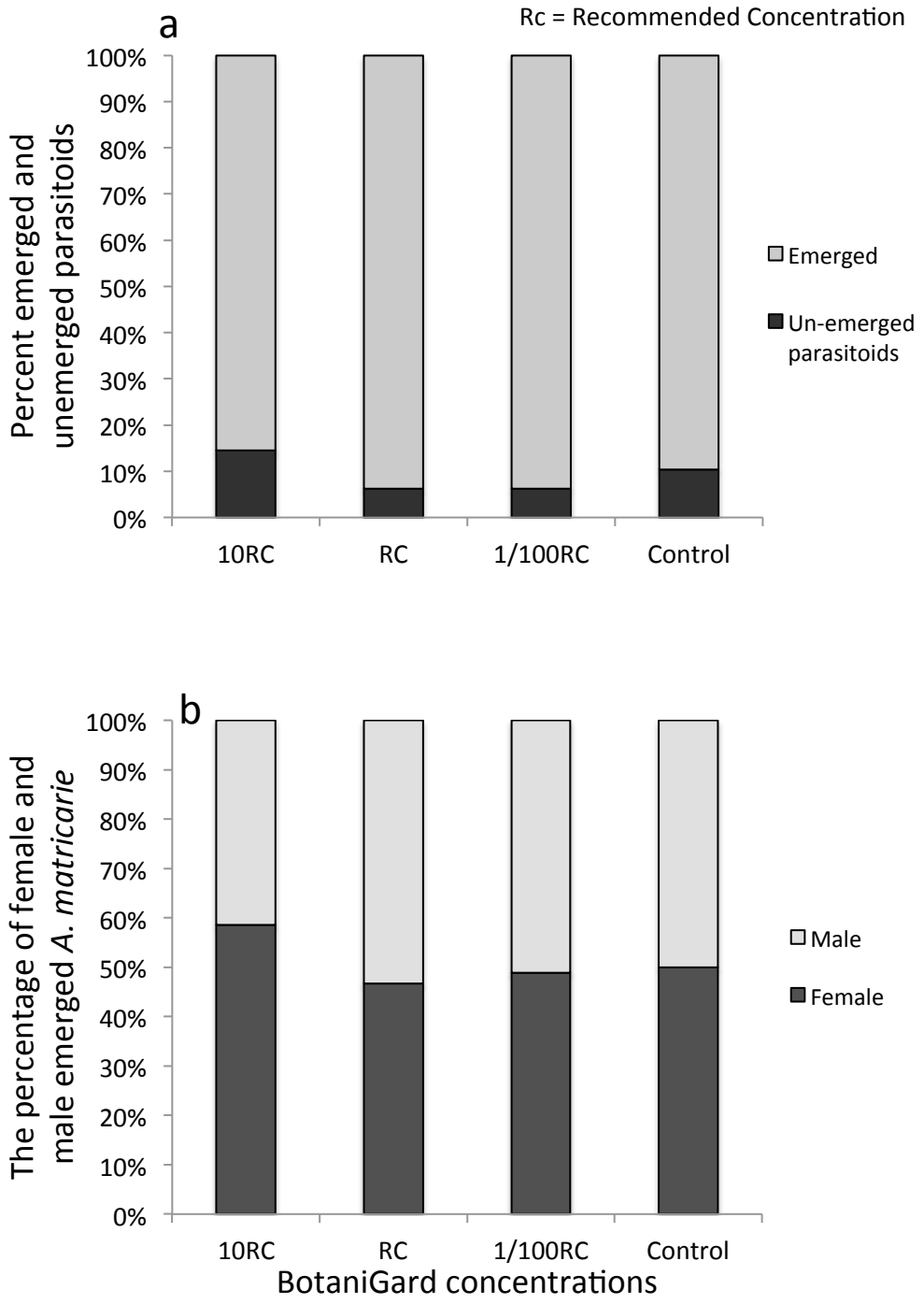

Figure 2.5. (a) Percentage emergence of *Aphidius matricariae* and (b) the sex ratio of the emerged parasitoids (b) after mummies were dipped in different concentrations of BotaniGard in relation to the recommended concentration (RC). For both measurements no significant difference was observed between the treatments (N = 48 per treatment).

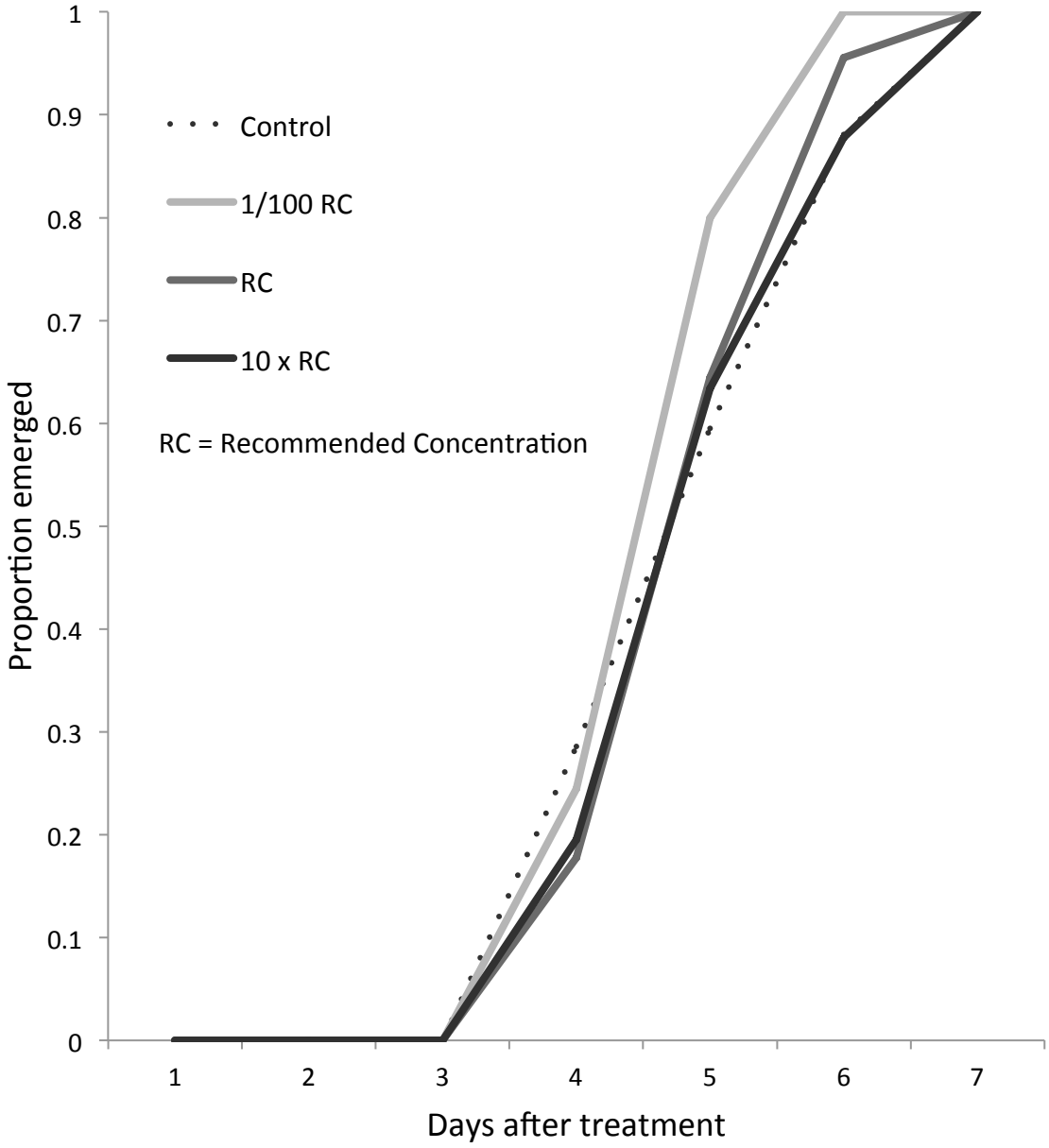

Figure 2.6. Emergence period of the parasitoid *Aphidius matricariae* after treatment with different concentrations of BotaniGard in relation to the manufacture's recommended concentration (N = 48 per treatment)

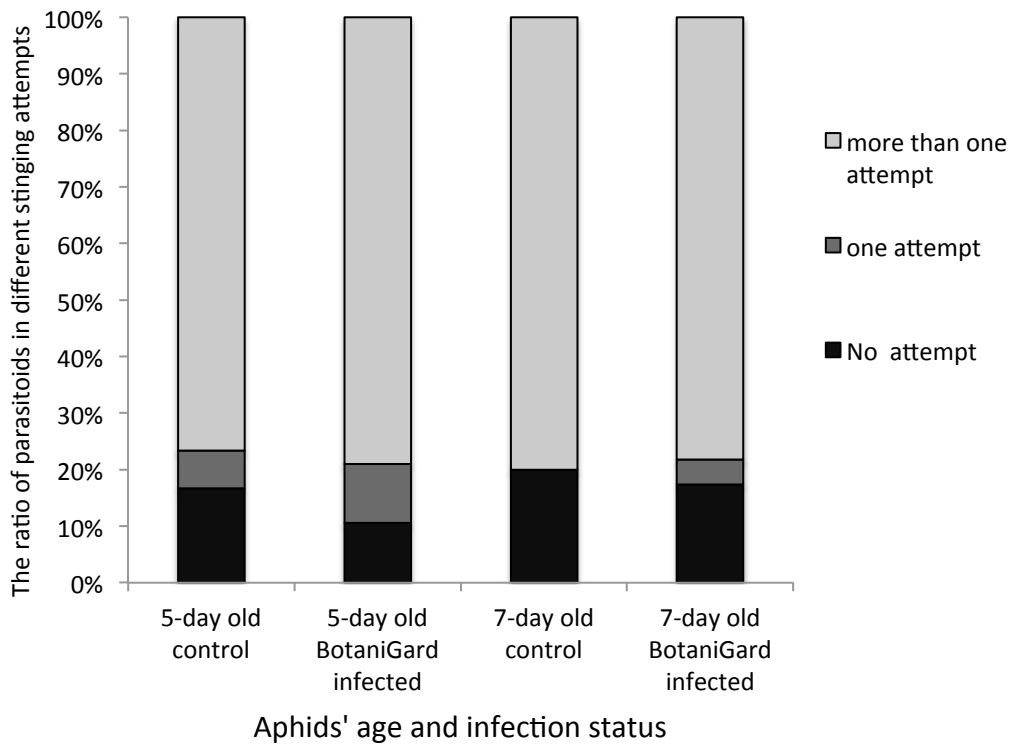

Figure 2.7 Distribution of the number of attacks on *M. persicae* attempted by *A. matricariae* after encountering 5-day old and uninfected aphids (N = 30), 5 days old and infected with BotaniGard (N= 19), 7-day old and not infected (N = 11), 7 days old and infected with BotaniGard (N = 23).

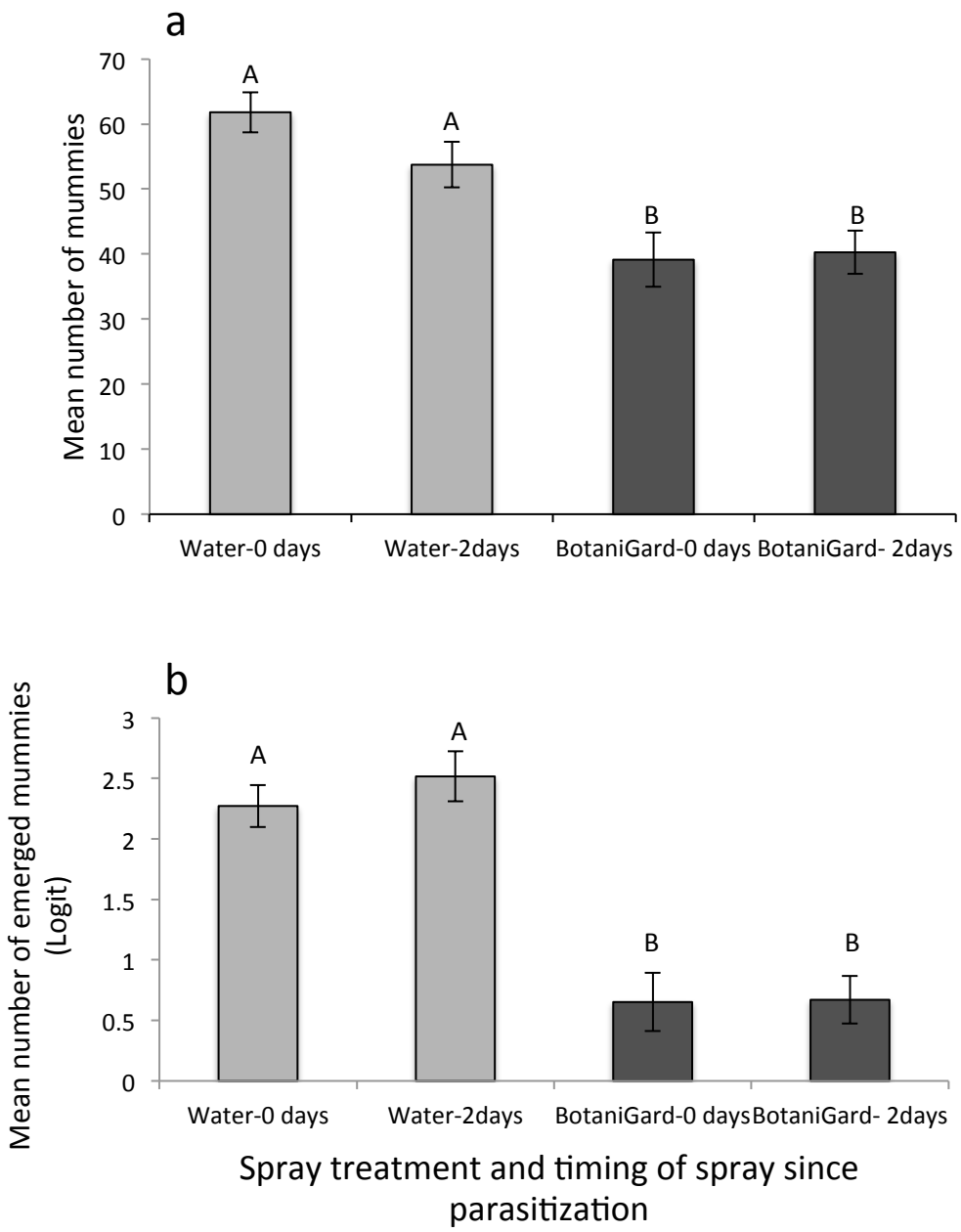

Figure 2.8 (a) The number of *Aphidius matricariae* mummies that formed, and (b) the proportion of the parasitoids completed when parasitized host aphids were subjected to different spray treatments (Water or BotaniGard) either immediately after parasitization (0 days) or 2 days after parasitization (2 days) (N = 15 per treatment). Different letters represent significant difference. Error bars present ±SE.

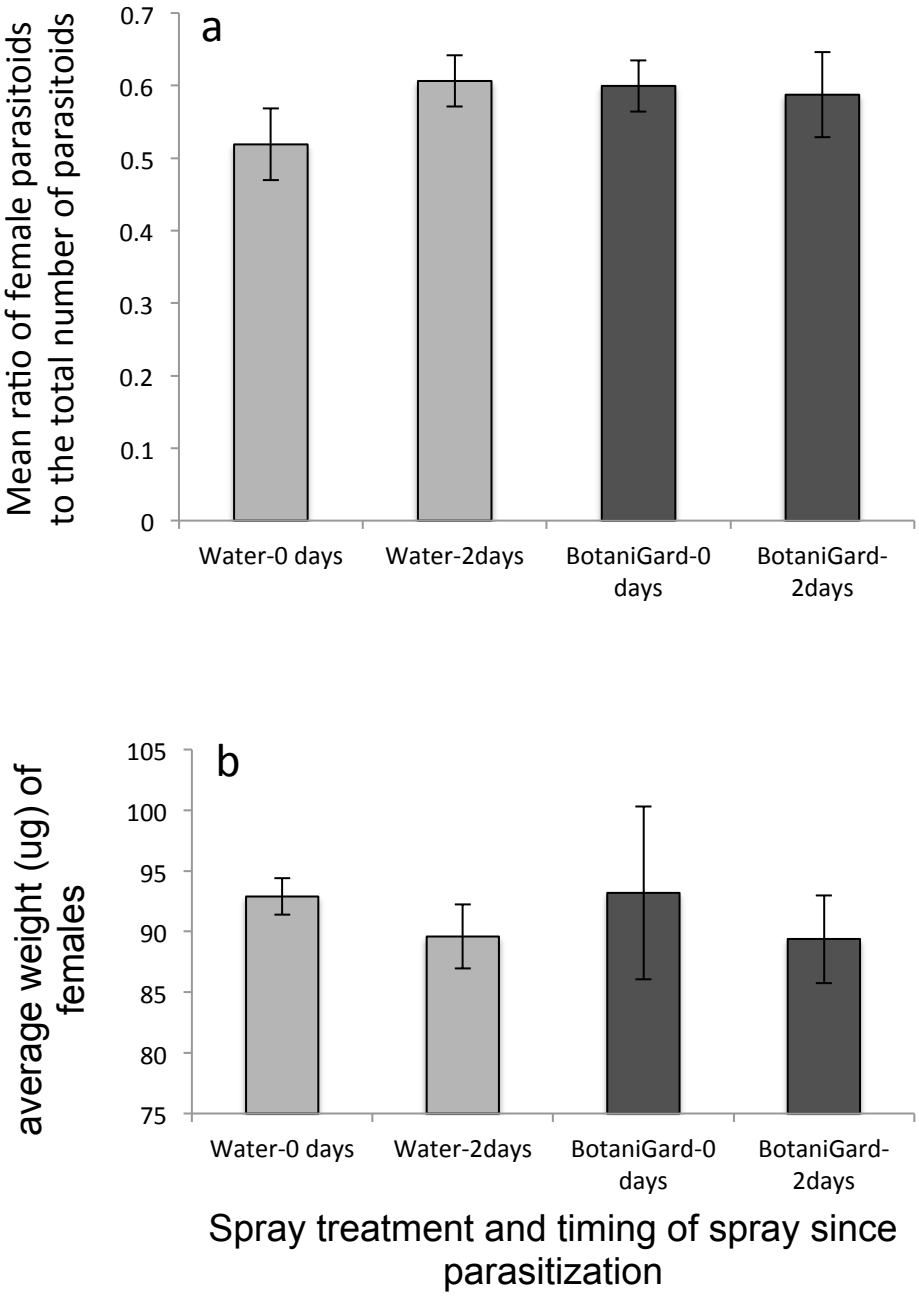

Figure 2.9 (a) The ratio of female offspring and (b) average weight of the female offspring when the parasitized host aphids were subjected to different spraying treatments (BotaniGard or Water) immediately after parasitization (0 days) or 2 days after parasitization (2 days) (N = 15 per treatment).

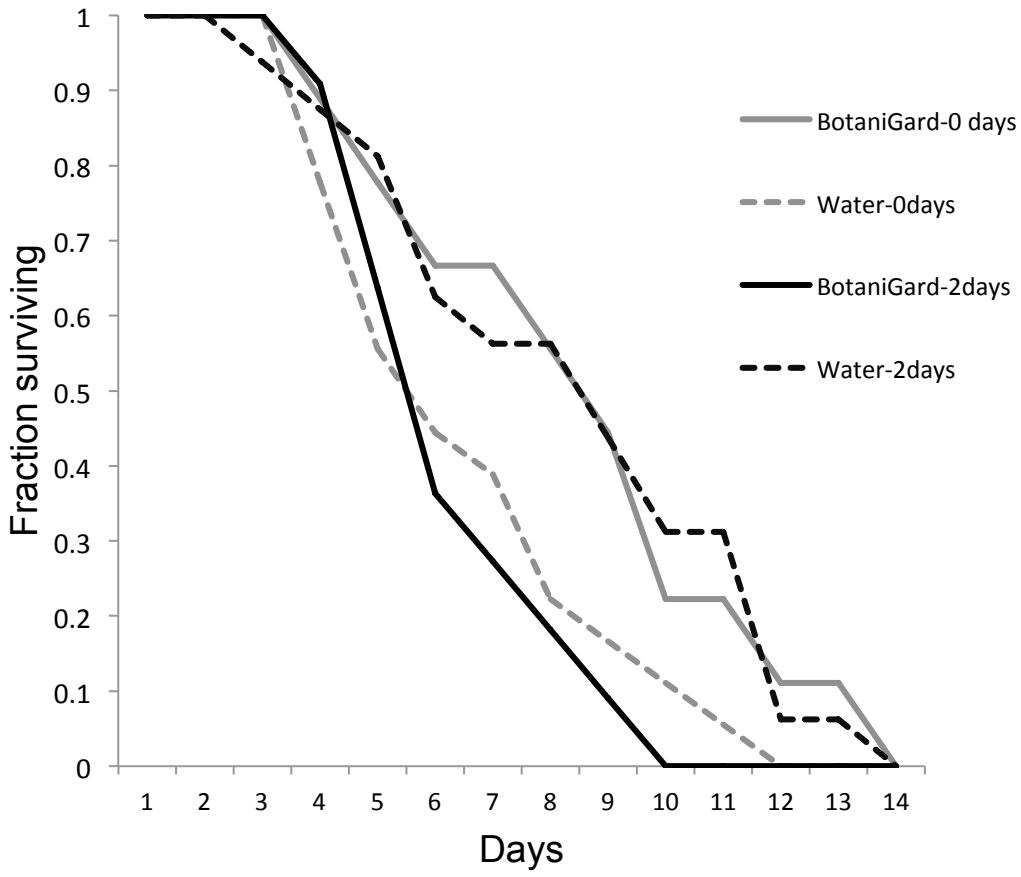

Figure 2.10 Longevity of female parasitoid offspring after emergence from host aphids that were treated with different spraying treatments (Water or BotaniGard) immediately after parasitization (0 days) or 2 days after parasitization (2 days).

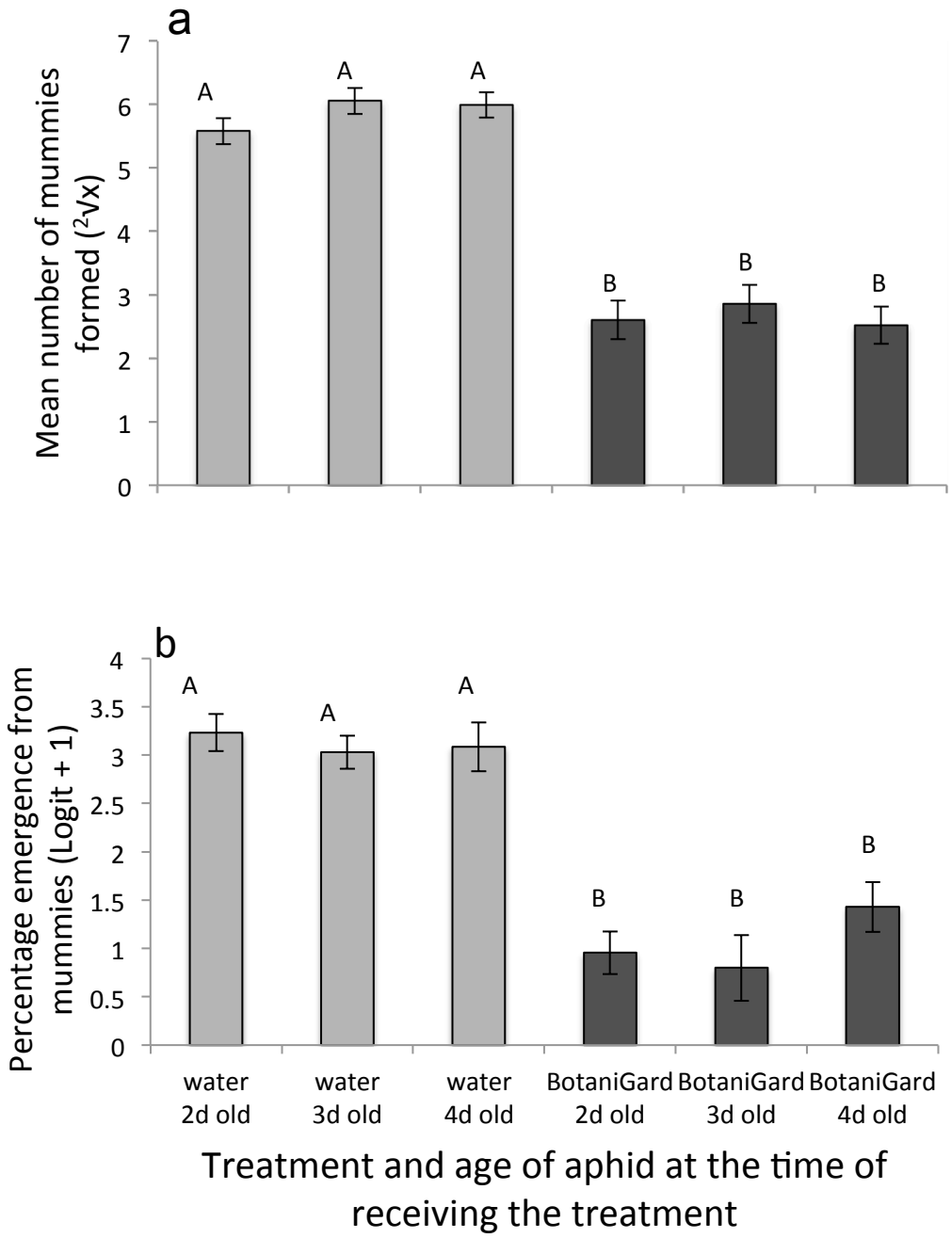

Figure 2.11 (a) The number of *Aphidius matricariae* mummies that formed, and (b) the percentage of the parasitoids that completed development. Host aphids were subjected to different spraying treatments (water or BotaniGard) when they were 2 days old (2d old), 3 days old (3d old) or 4 days old (4d old) and exposed to parasitoid when they were 5 days old (N = 15 per treatment). Different letters represent significant difference. Error bars present ±SE.

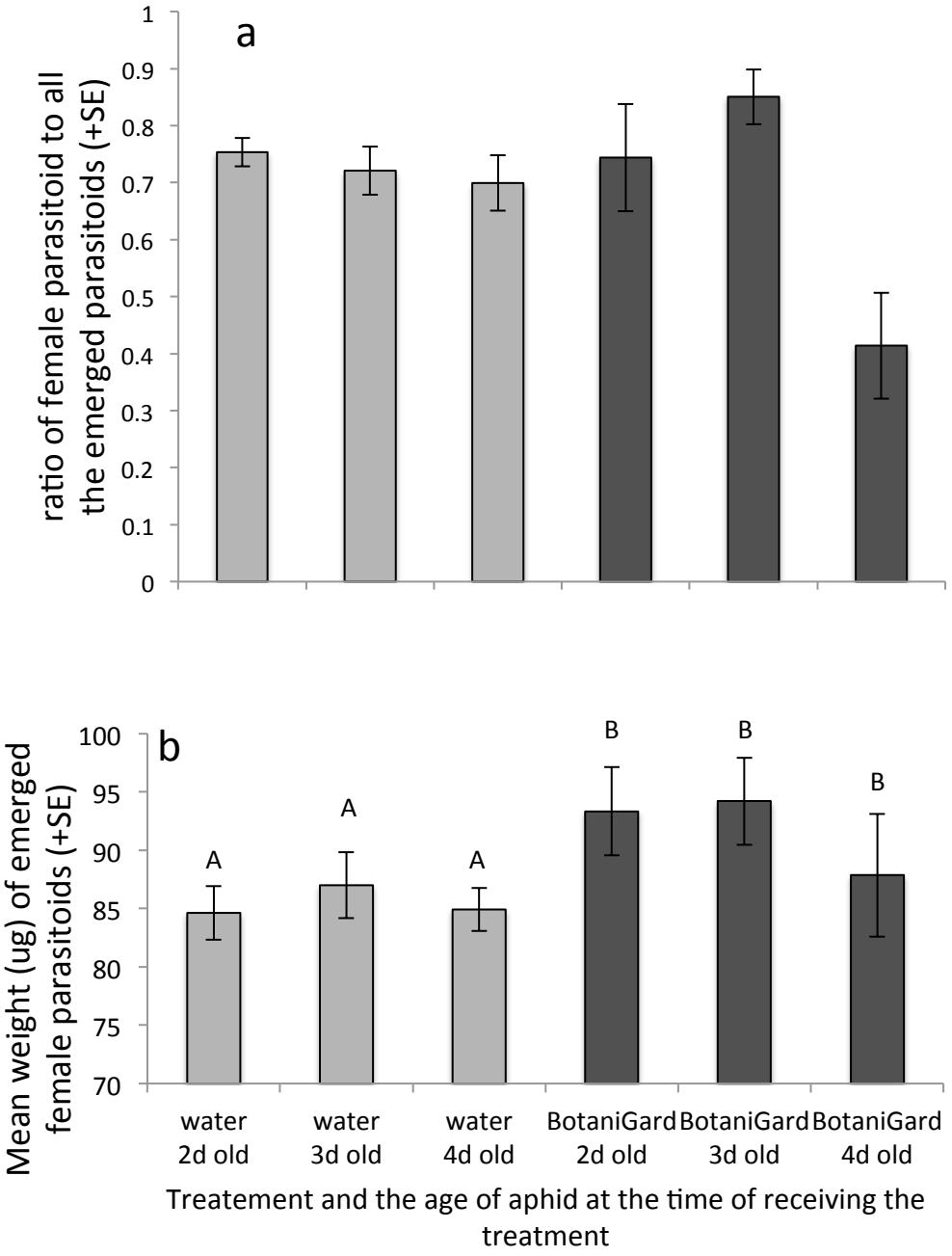

Figure 2.12 The ratio (a) and weight (b) of female offspring that emerged out of aphid hosts that were subjected to a spraying treatment (water or BotaniGard) when they were 2 days old (2d old), 3 days old (3d old) or 4 days old (4d old) and then exposed to parasitoids when they were 5 days old for 24h (N = 15 per treatment). Different letters represent significant differences between the mean weight of female parasitoid offspring.

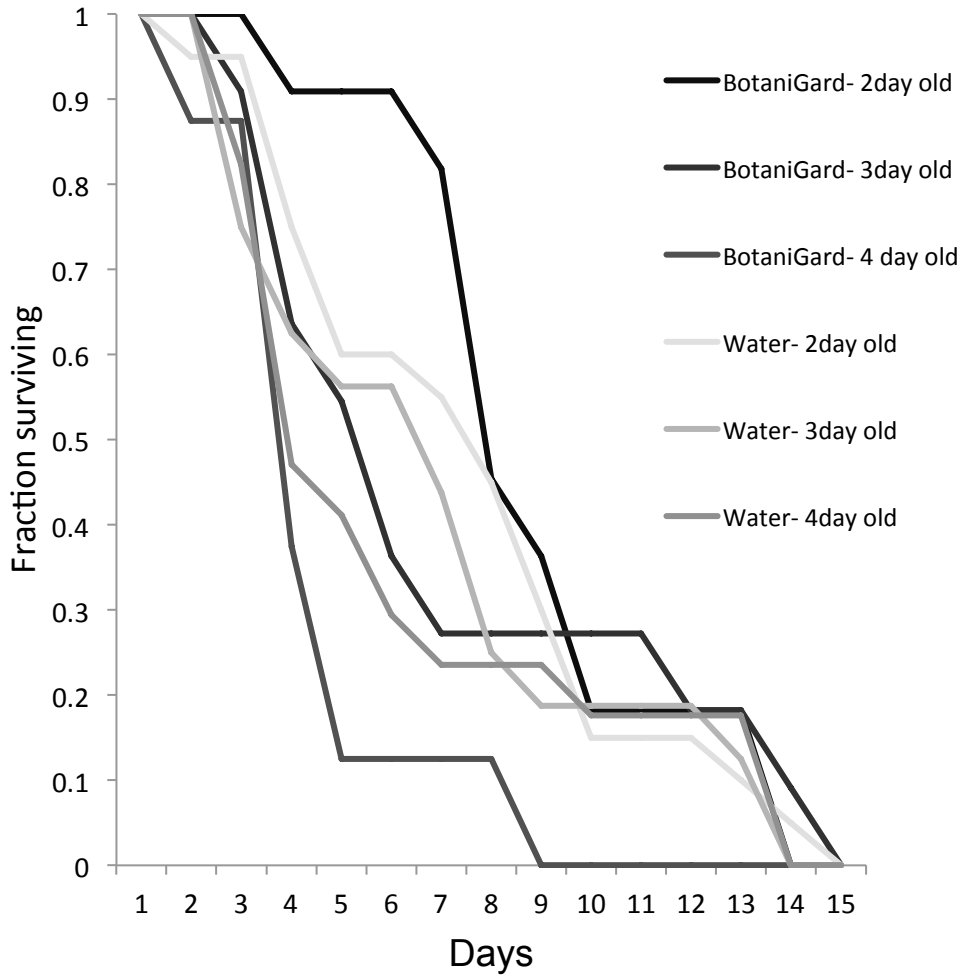

Figure 2.13 Longevity of female offspring of parasitoids that oviposited inside of 5 days old aphid hosts that were subjected to either Water (control) or BotaniGard spraying treatments at different ages (2 days, 3 days, or 4 days old) (N = 15 per treatment).

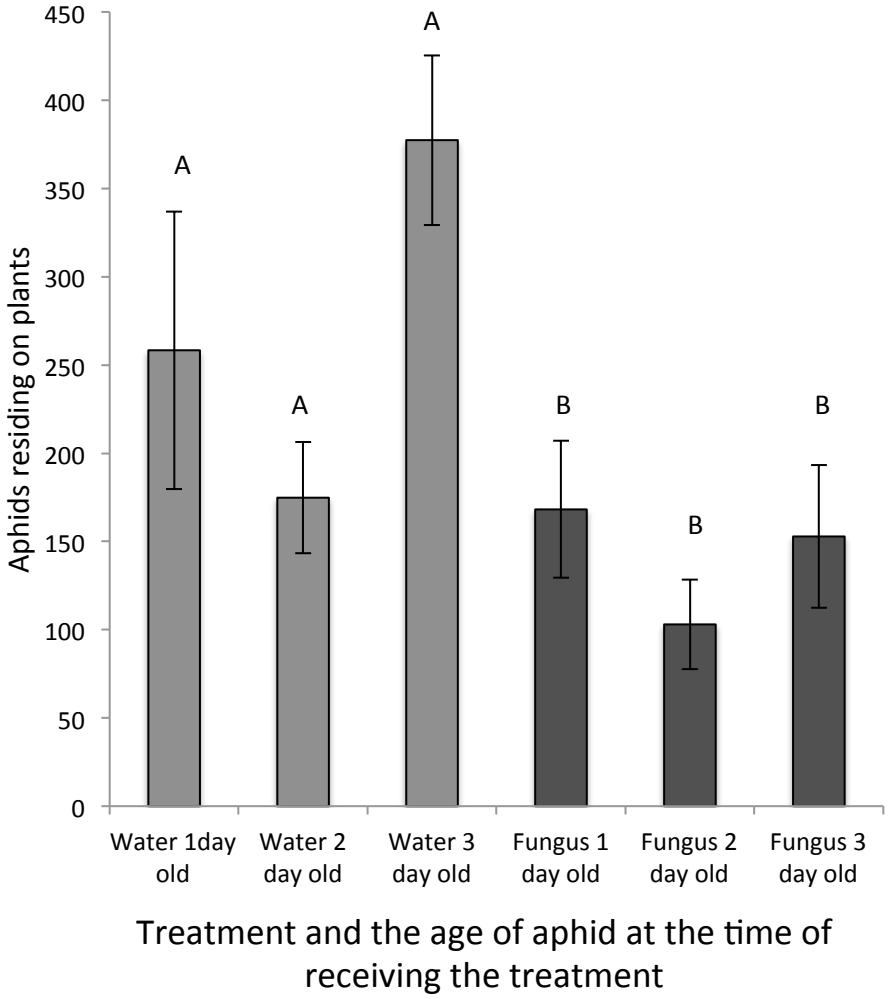

Figure 2.14. Number of aphids remaining at the end of the experiment on the plants that were colonized by aphids that were subjected to a spraying treatment (water or BotaniGard) when they were 2 days old (2d old), 3 days old (3d old) or 4 days old (4d old) and then exposed to parasitoids when they were 5 days old for 24h (N = 6). Different letters represent significant difference.

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3. How does BotaniGard interact with *Aphidius matricariae* and *Myzus persicae* across multiple generations?

3.1. Introduction

Biological control can effectively replace chemical control of aphids and its use is growing in greenhouses worldwide (Gillespie et al., 2002; Lopes et al., 2009; van Lenteren, 2000a). The closed environment in greenhouses brings several benefits for biological control application: 1- it is easy to organize pest management programs separately for each greenhouse unit, 2- the interference from pest management of neighbouring greenhouses is limited and the drift of pesticides on natural enemies, which is a problem in open field crops, does not happen, 3- greenhouses can potentially be cleaned of pests before the cropping season and during the winter, 4- massive immigration of pest organisms is prevented; thus, usually the number of pests species is limited and natural enemies of only a few species will be introduced (Boissard et al., 2008; Driesche et al., 2008; Lopes et al., 2009; Paulitz and Bélanger, 2001; Pilkington et al., 2010; van Lenteren, 2000). Since the functioning and the processes of many ecosystems that have ecological and/or socio-economical importance benefit from species richness (Flombaum and Sala, 2008; Loreau et al., 2001; Tilman, 1996; Yachi and Loreau, 1999), it has been suggested that the simultaneous use of multiple species of biological agents against pests can be beneficial (Jabbour et al., 2011; Stiling and Cornelissen, 2005). In greenhouses this diversity is achieved by the supplemental release (augmentation) of naturally occurring natural enemies (Messelink et al., 2013; Polack et al., 2011; van Lenteren, 2000a; van Lentern et al., 1996). However, the effects of natural enemies on prey populations can be reduced because of the competition and/or predation that take place between the natural enemies (top-down effect) and resources (bottom-up effects) (Rosenheim, 1998) that can lead to undesirable effects on the pest control in a greenhouse (Bigler et al., 2006; Boivin et al., 2012).

A guild is defined as “a group of species that exploit the same class of environmental resources in a similar way” (Root, 1967). For natural enemies, such resources can be a prey item, i.e. the pest. The natural enemies will have interactions with one another, known as intraguild interactions. Intraguild interactions can be in the form of competition for the pest, or direct predation on each other, also known as IntraGuild Predation (IGP). The definition of a guild does not include taxonomic differences and I use the term “guild” broadly in this review to include members of different kingdoms.

Intraguild interactions between natural enemies (referred to as intraguild interactions) can result in a spectrum of positive, neutral, or negative effects on pest suppression (Rosenheim et al., 1995). On the one hand, the top-down control of pests may be enhanced by using multiple biological control agents in augmentative biological control in two ways: (i) the complementarity effect, which happens when biocontrol agents prey on different subsets (different species or different developmental stages) of pests, and (ii) facilitation, which takes place when the presence of one predator enhances the predation by another predator; (Casula et al., 2006; Ponzio et al., 2013; Sih, 1997; Sih et al., 1998; Straub et al., 2008). The complementarity effects leads to additive effects on pest control but facilitation has an effect which is stronger than the additive effect and is known as synergistic effects on pest control (Finke and Snyder, 2010; Jabbour et al., 2011; Losey and Denno, 1998; Meyling and Pell, 2006). In the case of using pathogens with natural predators as the biological control agents, predators can increase the chance of infection by acting as a stress factor and reducing the prey’s immunity (McCauley et al., 2011; Ramirez and Snyder, 2009). Moreover, several studies have suggested that the presence of predators can possibly enhance pathogen transmission to the pest by increasing movement and thus pathogen contact (Baverstock et al., 2009, 2008; Goertz and Hoch, 2013; Pell et al., 1997; Roy et al., 1998; Roy et al., 2001; Roy and Pell, 2000). With significant differences in ecology, physiology, resource and habitat use strategies, prey preference, mode of hunting, and reproduction, pathogens and predators may play a significant role in pest management together (Casula et al., 2006; Ramirez and Snyder, 2009; Straub and Snyder, 2008).

Alternatively, increasing the diversity of natural enemies can also be disruptive to pest management. Intraguild predation occurs when one predator species (the intraguild

predator) kills and consumes another predator (the intraguild prey) with whom it competes for a shared prey (Messelink et al., 2013; Polis and Strong, 1996; Polis et al., 1989). Generalist predators have a broad diet that is composed of almost anything that the generalist predator is physically capable of consuming, which sometimes also includes the intraguild predator as well as the shared prey. The concept of a generalist predator can also be applied to generalist entomopathogens that can infect the predatory biological control agents as well as the pest. Therefore, intraguild interactions, both in the form of IGP or competition over the same resources, can disrupt biological control performances and affect pest management negatively (Martin et al., 2013; Messelink et al., 2011; Rosenheim et al., 1993; Traugott et al., 2012).

Among pathogens, fungal biological control agents have a considerable potential for use in pest management. Unlike most other entomopathogens, they do not need to be ingested to cause infection. Instead they can be directly transmitted by contact with the susceptible host (Cory and Ericsson, 2010). This characteristic makes them important biological control agents for controlling sap-sucking insects (Butt et al., 2001; Khan et al., 2012; St Leger and Wang, 2010). Entomopathogenic fungi are usually applied via inundative sprays for short-term pest control but with their potential for rapid growth, persistence, and passive dispersal they have the capacity to be used in long-term pest control programs (Cory and Ericsson, 2010; Inglis et al., 2001). Entomopathogenic fungi have a diverse host specificity, not only in terms of the number of species that they can infect, but also in terms of whether they grow outside of their host (facultative) or not (obligate) (Vega et al., 2009). Compared to obligate fungal pathogens, fungi which have alternative nutritional sources have a broader host range and their saprophytic and endophytic characteristics allow them to establish outside of their insect host in their local environment (Cory and Ericsson, 2010). Although a broad host range makes a fungus a good candidate for controlling a variety of pests, many laboratory studies have shown that arthropod natural enemies are susceptible to generalist entomopathogenic fungi as well (Askary and Brodeur, 1999; Vergel et al., 2011; Rashki et al., 2009; Seiedy et al., 2012).

Studies that have addressed the effect of fungal entomopathogens on predatory insects tend to use Petri dishes or single-plant scenarios and there is the possibility that these results do not reflect the complex interactions in natural environments. Laboratory

studies are valuable for investigating the details of host-pathogen interactions under a controlled environment. However, they are unlikely to reflect natural situations where numerous factors interact and influence the outcome of intraguild interactions at the same time (Jaronski, 2010; Straub et al., 2008). These factors include competitors, response to prey density, environmental variation, sunlight, microhabitat use and phenology. In other words, laboratory studies might demonstrate the "physiological susceptibility" of arthropod natural enemies but not their "ecological susceptibility". Hence, it is unclear whether the fungi have similar effects under realistic field conditions where the environment is usually suboptimal for fungus growth and reproduction (Roy and Pell, 2000). For example, Jaronski et al. (1998) showed that although *Beauveria bassiana* (strain GHA) infected *Eretmocerus* sp. parasitoids at significantly high rates in laboratory bioassays, the impact was minimal in the field on *Eretmocerus* sp. as well as on predators such as reduviids, *Nabis* spp. *Orius* spp., *Geocoris* spp., *Collops* spp., Coccinellids or chrysopids. Similarly, Ludwig and Oetting (2001) tested the infection rate of predatory mites and parasitoid *Aphidius colemani* Viereck and concluded that the infection rate of *B. bassiana* (Stain GHA), especially for the parasitoids, was higher under laboratory conditions than in greenhouses. Therefore, laboratory experiments may not reflect the interactions, and the outcome of those interactions at larger spatial scales. This indicates the necessity of studying intraguild interactions in natural settings, alongside the laboratory experiments, before conclusions can be made about the compatibility of organisms for pest management.

There are studies that have monitored changes in the pest population over time after application of different control agents (Gillespie and Acheampong, 2012; Henry et al., 2010; Labbé et al., 2009; Numa Vergel et al., 2011; Snyder et al., 2006; Walzer et al., 2001), but only a few studies have monitored intraguild interactions between biological control agents and changes in the pest population over time scales of >1 predator/prey generation (Finke and Snyder, 2010). However, the study of population dynamics over several generations is necessary for understanding the long-term effects of intraguild interactions.

In this study, I examined the compatibility of BotaniGard®22WP (Laverlam International Corporation, Butte, MT), which is a commercially available product in the form of a wettable powder that uses *B. bassiana* strain GHA conidia as its active

ingredient, with the parasitoid *Aphidius matricariae* (Hymenoptera) for controlling Green Peach Aphids (GPA), *Myzus persicae* in greenhouses. Green peach aphid is one of the principal pests of greenhouse crops worldwide (Capinera, 2005; Zamani et al., 2007). *B. bassiana* (strain GHA), one of the most widely used fungal entomopathogens, has shown promise in controlling aphids (Gillespie et al., 2002). About 15 mycopesticides have been produced and registered in different countries across the world that have *B. bassiana* as their active ingredient (Cuddeford and Kabaluk, 2010; Khan et al., 2012). In Canada BotaniGard® 22WP (Laverlam International Corporation, Butte, MT) is a registered product for foliar spray against a wide range of insect pests, including aphid pests of greenhouses. The specialist insect biological control agent in this study, *A. matricariae,* is a parasitoid of aphids. It is a commonly used and commercially available biocontrol agent for controlling aphids, particularly GPAs in greenhouses (Bannerman et al., 2011; Gillespie et al., 2002). In this manuscript I describe two experiments that were designed to examine the effect of the simultaneous use of BotaniGard and *A. matricariae* in greenhouses. In both experiments I studied the effect of BotaniGard on parasitoid population density, the influence of the presence of parasitoids on the infection rate of aphids, and the effect of both biocontrol agents on pest population suppression. In the first study, I focused on the compatibility of using the two biological control agents simultaneously versus using each biocontrol agent individually. The second study focused on the parasitoids and examined the effect of adding parasitoids twice in a six-week period to the system and whether this reduced the negative effects of BotaniGard on the parasitoids' performance, in addition to estimating crop yield.

The null hypothesis of this study was that there would be no relationship between the two control agents and they would act independently (an additive effect). The alternative hypothesis was that the combined use of *A. matricariae* and BotaniGard would provide a better control of GPA than would be predicted from each of the biological control agents alone (a non-additive effect). From my results in chapter 2, I predicted that BotaniGard would reduce the parasitoid population but parasitoids would enhance the rate of aphid encounter with *B. bassiana* conidia; and this would result in a synergistic interaction between BotaniGard and parasitoids that would reduce aphids' population to lower levels and at a faster rate than each biological control agent applied separately. In the second experiment, the null hypothesis was that repeated release of

parasitoids would not affect the suppression of aphids whether BotaniGard was present or not. I predicted that the repeated release of parasitoids would enhance aphid control, which in turn would increase crop yield, and the effect would be stronger in the presence of BotaniGard.

3.2. Methods:

The target crop was bell pepper, *Capsicum annuum*, cv. Bell Boy, placed in large netting cages (1.75m^3) (BioQuip Products, Rancho Dominguez, Canada) in greenhouses to mimic a realistic pest control scenario. In these greenhouses temperature was set at 22 °C during day and 18 °C during night with 70% RH at all times. These greenhouses were smaller than an average vegetable greenhouse in British Columbia, which are 2.3 hectares on average (*BC Jobs Plan Special Report: The BC Greenhouse Sector*, 2011), but the environment inside these greenhouses is equivalent to the environment found in a typical vegetable grower's greenhouse. Twenty cages were set up and split evenly between two greenhouses. Inside the cages one 4-week-old plant (approximately 15cm height and each with four leaves) was placed at each of the corners of the cages, equidistant from one another.

3.2.1. Insect preparation

An even-aged cohort of Green Peach Aphids, *M. persicae,* was obtained by placing adult aphids on pepper leaves inside 237ml plastic cups (Solo cup company, Lake Forest) to reproduce for 24 hours. The adults were removed and their offspring were maintained in the same environment until they were used in the experiments, when they were 7-8 days old (fourth instars).

Using a similar method to that was explained in the previous paragraph a cohort of 3-day old aphids was produced. *A. matricariae* were prepared by allowing 15 female parasitoids to parasitize approximately 300 3-day-old *M. persicae* residing on five pepper leaves inside an insect rearing cage (BugDorm- 2120 Insect rearing tent, 60 x 60 x 60 cm). Since these parasitoids were introduced to aphids of uniform age, all the offspring parasitoids pupated and emerged on the same date. The parasitoids were used in the

experiments immediately after emergence. All organisms were maintained in a controlled environment room at 16L: 8D photoperiod, 21°C ± 2 °C and 60 ± 10% RH.

3.2.2. Fungal application

The recommended rate for the application of BotaniGard 22WP for aphid control is a BotaniGard-water mix at a rate of 500g/400L. According to the product label, there are 4.4 x 10^{13} conidia/kg of the material. This translates to a concentration of 5.5 x 10^{7} conidia/mL against aphids. This concentration is herein referred to as the "recommended concentration". The application of the fungus was carried out using a 50µL droplet size nozzle with a CO_2 sprayer. In order to limit the evaporation rate and to avoid killing the fungus, plants were sprayed until runoff in the evening when the temperature and the UV irradiation were low.

3.2.3. Experiment 1- Is the dual application of BotaniGard and Aphidius matricariae more effective than either biological control agent alone? (First big-cage experiment)

Five adult aphids were placed on each plant in the cages and left to settle for one week before the start of the experiment. This produced aphids with a range of ages at the start of the experiment. Experimental treatments were randomly assigned to the cages containing plants that were colonized by aphids: water treatment (to control for spraying effect) (N=5 cages), release of parasitoids (N= 5 cages), application of BotaniGard (N=4 cages), application of both BotaniGard and parasitoids (N=6 cages). Thus, seven days after the introduction of the aphids to the cages, four 1-day-old, recently mated parasitoids were released into the cages assigned to a parasitoid treatment. Two days later, the plants were sprayed until runoff with water or BotaniGard at a rate of 10x recommended solution. This spraying took place on a weekly basis for 6 weeks. However, by the end of week 4 some plants were suffering from an aphid population explosion and plants in treatments with no parasitoids were dead (Figure 3.1.); therefore, sampling in these cages was terminated.

Measurements:

Every week before spraying, three leaves were removed haphazardly from different plant levels (top, middle, and bottom leaves) of two plants diagonally opposite each other in each cage. Sampling of the plants was rotated every week to relax the stress that was caused by cutting the leaves off the plant. The total numbers of aphids, parasitoid mummies and infected (brown) aphids on the detached leaves were counted. However, because of the time that the parasitoids and the fungus took to develop inside the aphids, no mummies and no infected aphids were found on the first week of sampling.

The number of aphids residing on the sampled leaves was recorded for all plants. Data were gathered for healthy aphids starting from the first week, and infected aphids from the second week. It was possible to distinguish healthy aphids from the infected ones because the former were light green in color whereas the infected aphids were brown and dull. However, from the fourth week of sampling, because of the overcrowding effect, the aphids were generally much smaller and were covered with honeydew making their colors duller and harder to distinguish. Therefore, I did not collect data on the number of infected aphids for the last two sampling periods. Parasitoid mummies were counted from the second week of sampling because they take about a week to pupate inside aphids. Sampling of the mummies continued until the end of the sixth week.

Statistical Analysis

Analysis was carried out on the average values of the organisms (number of aphids, parasitoid mummies and infected aphids) per leaf per cage for both experiments. By analyzing the values per cage I avoided pseudo-replication within cages. The terms “density” and “number” of organisms are used interchangeably because they were averaged for each cage.

Statistical analyses were carried out using JMP version 9 (SAS Institute, Cary, NC, USA). The confidence interval for all of the tests was set at 95% and the significant cut-off for the p-value was 0.05.

The effect of the treatments was compared for each sampling date separately. All data were tested for the assumptions of normality with a Shapiro-Wilk test before the analysis and subjected to transformation if needed. In case of a significant result, treatment means were compared with a Tukey's test, for 2x2 factorial analyses of variance (ANOVA). A positive interaction in the factorial design is indicative of a synergistic response. For the last two sampling points, where only two of the treatments were sampled, if a significant difference was observed between the means of the two treatments the treatment means were compared with a t-test for one-way ANOVA statistical analyses.

Effect of parasitoid release and BotaniGard application on aphid population control

Aphid densities were first subjected to a $\log_{10}$ transformation. The first four weeks of aphid density data were analyzed with a 2x2 factorial design. Since data were collected only from the cages with parasitoids in the last two weeks, data regarding these two weeks were analyzed with a one-way ANOVA to examine the effect of BotaniGard application on aphid density in the presence of parasitoids.

The aphid population growth rates (PGR) between each two consecutive sampling periods (between first and second, second and third, third and fourth, fourth and fifth, fifth and sixth sampling periods) were estimated (Enns, 2011):

$$PGR= (\ln(P(t_2))- \ln(P(t_1)))/(t_2-t_1)$$

where $P(t_1)$ and $P(t_2)$ are the population numbers at time t_1 and t_2, and ln is the natural logarithm function. The PGRs were compared using a 2x2 factorial design. A significant interaction response was indicative of non-additive effects. The positive interaction represented synergism and a negative interaction represented disruption.

Cross contamination of BotaniGard was low as the number of infected aphids in treatments without BotaniGard application was negligible (mean value= 4.5 ± 2.6SE over the 4 weeks of sampling). To assess the influence of parasitoids on the infection of the aphids with *B. bassiana*, the numbers of infected aphids were compared between the cages of BotaniGard treatment and cages of BotaniGard and parasitoids. These

numbers, for all the sampling periods, were subjected to a $\log_{10}^{(x+1)}$ transformation before analysis using a one-way ANOVA.

Effect of BotaniGard application on parasitoid populations

The rate of parasitization was almost zero for the cages without parasitoid release (0.018 ± 0.0185SE over the 6 weeks of sampling). The densities of mummies were first transformed with a $\log_{10}$ function and then compared using a one-way ANOVA only across the two treatments where parasitoids had been added.

Population Growth Rate (PGR) was used to calculate the rate of parasitoid population growth between two sampling periods. PGRs were tested for normality and the data were compared using a one-way ANOVA to study the influence of BotaniGard on the parasitoids' population growth rate.

3.2.4. Experiment 2- Does BotaniGard affect the development, sex ratio and recycling of parasitoids in a greenhouse pest management system? (Second big-cage experiment)

The purpose of this study was to examine whether BotaniGard interfered with the development and recycling of *A. matricariae* and whether parasitoid augmentation would decrease the negative effects of BotaniGard on parasitoids. Because the aphids became overcrowded in the previous experiment, fewer were released onto the plants at the beginning of this experiment. In this experiment, unlike the previous one, all cages contained parasitoids and the effect of BotaniGard application and parasitoid augmentation during the experiment was measured for these cages The experimental treatments were assigned as follows: single release of parasitoids and water treatment (N=4 cages), double release of parasitoids and water treatment (N=6 cages), single release of parasitoids and BotaniGard application (N=6 cages), and double release of parasitoids and BotaniGard application (N=4). Cages were sprayed with either BotaniGard or water to compare the effect of BotaniGard on parasitoids. In some cages one release of parasitoids took place whereas in other cages parasitoids were released again to understand whether the rerelease of parasitoids would decrease the effect of BotaniGard on parasitoid populations and whether this would enhance pest control.

Four pepper plants were placed at the four corners of each cage as described in the first experiment. Two adult aphids were placed on the plants and left for five days, before the first release of parasitoids, to build a population with aphids of different ages. Four mated, one-day-old female parasitoids were released in each cage at the beginning of the experiment for all treatments. The second release of four female parasitoids took place 7 days after the first release in the cages with the double release treatment. The cages were sprayed with water or the recommended concentration of BotaniGard to runoff two days after the release of the first parasitoids, and every 10 days for a total of three times (see figure 3.2. for a timeline of these applications) Thirty days after the introduction of the aphids, the experiment was terminated. I sampled three leaves from each plant for all plants in the cages and recorded the number of organisms on the leaves.

Effect of BotaniGard application and parasitoid release on aphid population control

To assess the effect of the biological control agents on aphids, the number of aphids on each plant was counted. In addition, the number of adult aphids and juvenile aphids in each cage population was counted separately to assess the age structure of the population. It was possible to distinguish adults from the juveniles based on their sizes. Adult aphids are usually about 2mm long, and the young aphids are between 1-2 mm.

The method of counting brown aphids on the leaves to estimate the infection rate in the previous experiment was extremely laborious and did not show significant results. I assumed that the difference in infection rates would be reflected on the number of *Beauveria*-sporulating cadavers on the soil at the end of the experiment. Hence, in this experiment the surface of the soil was examined carefully for aphids that had been colonized by the fungus and were sporulating and appeared as white and fuzzy cadavers. The number of the fuzzy aphids was recorded for each pot and compared between cages to gather evidence on the effect of BotaniGard in the biological control system. *Beauveria bassiana*-sporulating aphids have a characteristic appearance and a careful eye-inspection was done to determine that the sporulated organisms were GPA that were infected with *B. bassiana.*

Effect of BotaniGard application on parasitoid populations

Data were gathered on the parasitoid populations in two ways. First, for on-site data collection: the total number of parasitoid mummies on the leaves was recorded, as described above, inside greenhouses at the end of the experiment. Preliminary experiments indicated that parasitoids kept at room temperature would develop into mummies within 8 days and emerge as adults in 12 days. As a result, it was assumed that all the emerged parasitoids belonged to the first generation of parasitoids along with some early emergence of the second generation (Figure 3.2.). Considering this assumption, mummies were examined for emergence holes and the number of emerged parasitoids was recorded.

Secondly, 30 un-emerged mummies were collected from each plant and were kept separately in 60ml plastic cups (Solo cup company, Lake Forest) in a controlled room environment (16L:8D photoperiod, 23°C ±2 °C and 60±10% RH). These mummies were assumed to be the pupa stage of the second generation of the first and second release of parasitoids, but the F2 of the first release of parasitoids inside these mummies were at a more advanced stage in development than the F2 of the second release parasitoids (Figure 3.2.). After the parasitoids emerged, data were collected on the percentage parasitoid emergence and their sex ratio.

Effect of BotaniGard application and parasitoid release on crop yield

The fresh weight of the plants, as an estimate of the effect of the biological control agents on the crop yield, was assessed. All plants were cut at the bottom of the stem, at the base of the cotyledon leaves attachment, and were immediately weighed to avoid water loss.

Statistical Analysis

One cage of the single parasitoid release with BotaniGard spray and one cage of the double parasitoid release with BotaniGard spray were excluded from the analysis because the parasitoids did not establish in these cages and the number of aphids was extremely high.

Effects of BotaniGard application and parasitoid release on aphid numbers

Data for the total number of aphids were subjected to a $\log_{10}$ transformation before statistical analysis and then analyzed using a two-way ANOVA. The numbers of fuzzy aphid cadavers were transformed with a root function $\sqrt[2]{x}$ and analyzed using a two-way ANOVA (Frequency x BotaniGard treatments).

Effect of BotaniGard application on parasitoid population and aphid age structure

Analysis of the data collected on-site: to compare the rate of parasitoid emergence between treatments, the number of emerged parasitoids in relation to the number of mummies was compared using a GLM with a binomial distribution and a logit link. Although aphid numbers could affect the number of parasitoid mummies, my preliminarily results showed that the ratio of parasitoids to aphids in the cages was not different between treatments. Therefore, I assumed that the number of aphids would not affect the analysis of parasitoid numbers or percentage emergence. The proportion of adult aphids to all aphids in the aphid population was compared among treatments using a GLM with a binomial distribution and a logit link. Overdispersion was checked for both analyses.

Analysis of the data gathered after the experiment: data for emergence of parasitoids and also the ratio of females to males were fitted using a GLM with a binomial distribution and a logit link to compare the proportion of emergence and sex ratio across treatments. Overdispersion was checked for all analyses.

Effect of BotaniGard application and parasitoid release on crop yield

Plant wet weight data were tested for normality with the Shapiro-Wilk test. Since all data were normal, they were compared among treatments using a two-way ANOVA without any transformation.

3.3. Results

Experiment 1

Effect of parasitoid release and BotaniGard application on aphid population control

Aphid densities did not differ between treatments at the first sampling point (parasitoid release, $F_{1,16}$= 0.17, p = 0.6876; BotaniGard, $F_{1,16}$= 0.22, p = 0.6439; parasitoid and BotaniGard interaction, $F_{1,16}$= 2.07, p=0.1690) (Figure 3.3). This is consistent with my expectation, because at this point parasitoids had been recently introduced to the system and BotaniGard had not been applied yet (Figure 3.1).

At the second sampling point, cages containing parasitoids had fewer aphids in them (parasitoid release: $F_{1,16}$= 12.19, p= 0.0030) than treatments without parasitoids (Figure 3.3). Aphid densities in cages that were treated with BotaniGard were not different from those in the cages that had not received fungus application (BotaniGard, $F_{1,\ 16}$ = 2.97, p = 0.1041) and there was no interaction between parasitoids and BotaniGard (parasitoid and BotaniGard interaction, $F_{1,16}$ = 1.28, p=0.2742). Despite the differences in aphid numbers between the treatments, the population growth rates did not differ across treatments from week one to week two (Parasitoid release, $F_{1,16}$ = 1.68, p = 0.2136; BotaniGard, $F_{1,\ 16}$ = 0.08, p = 0.7756; parasitoid and BotaniGard interaction, $F_{1,\ 16}$ = 2.68, p = 0.12) (Figure 3.4). Also, there was no difference in the number of infected aphids in the presence and absence of parasitoids ($F_{1,8}$ = 2.61, p = 0.1448) (Figure 3.5).

In the third week of sampling, the parasitoids plus BotaniGard had a marginally synergistic (i.e. non-additive) effect (shown by a positive interaction term) with fewer aphids than the other treatments (parasitoid and BotaniGard interaction, $F_{1,\ 16}$ = 4.2, p=0.0572; parasitoid release, $F_{1,\ 16}$= 2.36, p=0.1438; BotaniGard, $F_{1,\ 16}$= 0.99, p=0.3349) (Figure 3.3). However, like the second sampling period, the rates of population growth between the treatments were not different (Parasitoid release $F_{1,16}$ = 2.3, p = 0.1482; BotaniGard, $F_{1,16}$ = 0.27, p = 0.6119; parasitoid and BotaniGard interaction, $F_{1,16}$ = 2.74, p = 0.1171) (Figure 3.4) and the presence of parasitoids did not affect the number of infected aphids ($F_{1,8}$ = 0.4907, p = 0.5035) (Figure 3.5).

By week four, cages with parasitoids had the lowest density of aphids (parasitoid release, $F_{1,16}$ =9.59, p= 0.0069; BotaniGard, $F_{1,16}$ =2.05, p =0.1715; parasitoid and BotaniGard interaction, $F_{1,16}$ = 3.39, p =0.0842) (Figure 3.3) but the number of infected aphids, like previous sampling points, was not statistically different between cages ($F_{1,8}$ = 1.77, p = 0.2199) (Figure 3.5). The cages with both biological control agents had a high population growth rate of aphids between week 3 and 4, similar to that of the control groups, suggesting some kind of negative interactions between the two biological control agents (BotaniGard and parasitoid interaction: $F_{1,16}$ = 13.55, p = 0.002), both of which had much lower aphid population growth (Figure 3.4). Neither parasitoid or BotaniGard main effects were significant (parasitoid release, $F_{1,16}$ = 0.17, p = 0.6824; BotaniGard, $F_{1,16}$ = 0.0014, p =0.9709) (Figure 3.4).

As mentioned above, aphid numbers in cages without parasitoids were not recorded after week four as the aphid numbers rose too high and the plants were killed. In the two remaining treatments that contained parasitoids, fewer aphids were present when BotaniGard was applied with the parasitoid as compared to the parasitoid alone (week five, $F_{1,9}$ = 20.45, p = 0.0014; week six, $F_{1,9}$ = 14.82, p = 0.0039) (Figure 3.3). The number of aphids started to decrease in the mixed application, so the population growth rate became negative, whereas in cages without BotaniGard application population continued to grow ($F_{1,9}$ = 16.39, p = 0.0029) (Figure 3.4). By week six, aphid population growth rate in cages with parasitoids started to decrease at similar rate to that found in cages with both parasitoids and BotaniGard ($F_{1,9}$ = 2.46, p = 0.1511) (Figure 3.4).

Effect of BotaniGard on the parasitoid population

Parasitoid mummies started to form from week two, but remained low even in week three (mean number of parasitoid mummies = 17.82 ± 5.83SE parasitoid mummies/ plant/ cage) (Figure 3.6). At the second and third sampling points there was no difference in mummy density between cages with parasitoids that were treated with BotaniGard and those that were sprayed with water (second week sampling, $F_{1,9}$ = 0.816, p=0.7762; third week sampling, $F_{1,9}$ =0.0016, p=0.9692) (Figure 3.6). Following on from this, the population growth rate of the parasitoids was also very low for the period between weeks 2 to 3 and was not different in the presence or absence of BotaniGard ($F_{1,9}$ = 1.33, p = 0.2774) (Figure 3.7).

The number of mummies started growing exponentially after week 3 (Figure 3.6), but the growth rate between week three and four did not differ between the two treatments ($F_{1, 9}$ = 0.17, p = 0.6897) (Figure 3.7). Parasitoid populations were not different between treatments at week four ($F_{1, 9}$ = 0.06, p=0.8052) (Figure 3.6). From week four to five parasitoid mummies increased at a marginally faster rate in the cages with BotaniGard application than in the cages without BotaniGard ($F_{1, 9}$ = 4.96, p = 0.05) (Figure 3.7.). A non-significant trend in the number of mummies at the end of week five suggested that the number of mummies was higher in the cages that had received BotaniGard application ($F_{1, 9}$ =3.66, p=0.0879), which became more evident in week six ($F_{1, 9}$ = 7.1934, p=0.0251) (Figure 3.6). The rate of population increase between the fifth and sixth weeks did not differ between the treatments ($F_{1, 9}$ = 1.23, p = 0.2962) (Figure 3.7).

Experiment 2

Effects of BotaniGard application and parasitoid release on aphid population density

Aphid densities did not differ between treatments after 6 weeks (parasitoid release, $F_{1,14}$ = 3.37, p = 0.0877; BotaniGard application, $F_{1,14}$ = 0.28, p = 0.6025; interaction of parasitoids and BotaniGard, $F_{1,14}$ = 0.5278, p = 0.4795) (Figure 3.8.). The proportion of adults in the aphid populations did not differ between the treatments (parasitoid release, $\chi^2_{1,14}$ =0.53, p = 0.4668; BotaniGard, $\chi^2_{1,14}$ = 0.0005, p = 0.9821; parasitoids and BotaniGard interaction, $\chi^2_{1,14}$ = 0.45, p = 0.5014) (Figure 3.9.).

The extremely low number of aphid cadavers infected with fungus that were collected from the soil surface of the water-treated cages provides evidence that cross contamination was rare (mean number of infected aphids = 0.075 ± 0.05SE) between cages. At the same time, the higher number of white fuzzy cadavers in the BotaniGard-treated groups provides evidence of the effectiveness of the *B. bassiana* conidia ($F_{1,14}$ = 1.19, p = 0.0001). The release of parasitoids had no effect on the number of infected aphids ($F_{1, 14}$ = 1.19, p = 0.2943) (Figure 3.10.).

Effect of BotaniGard application on parasitoid populations

Results from the data collected on-site showed that there was no statistically significant difference between the numbers of mummies in each treatment (parasitoid release, $F_{1,14}$ = 3.47, p= 0.0836; BotaniGard application, $F_{1,14}$ = 0.07, p = 0.7934; interaction of parasitoids and BotaniGard, $F_{1,14}$= 0.002, p= 0.9628) (Figure 3.11.a). Similarly, there was no difference between the proportions of emerged mummies (parasitoid release, $\chi^2_{1,14}$= 1.37, p = 0.2417; BotaniGard application, $\chi^2_{1,14}$= 0.02, p = 0.8837; interaction of parasitoids and BotaniGard, $\chi^2_{1,14}$= 0.1, p= 0.9196) across all treatments (Figure 3.11.b).

Results from the data that were collected in the laboratory revealed that as expected, the number of parasitoid releases had no effect on the development of larval parasitoids and more parasitoids reached adulthood if mummies were not exposed to the BotaniGard (parasitoid release, $\chi^2_{1,14}$ = 1.84, p = 0.1751; BotaniGard application, $\chi^2_{1,14}$ = 5.81, p = 0.0159, interaction of parasitoid release and BotaniGard, $\chi^2_{1,14}$ = 0.55, p = 0.4593) (Figure 3.11.c). However, the percentage of females in the cups did not differ across treatments (parasitoid release, $\chi^2_{1,14}$= 0.47, p = 0.511; BotaniGard application, $\chi^2_{1,14}$ = 0.51, p = 0.7028; interaction of parasitoid release and BotaniGard, $\chi^2_{1,14}$ = 0.06, p = 0.7550) (Figure 3.12).

Effect of BotaniGard application and parasitoid release on crop weight

Comparison of the weight of the plants at the termination of the experiment revealed that neither the number of parasitoid releases nor BotaniGard application affected plant wet weight (parasitoid release, $F_{1,14}$= 0.01, p= 0.9373; BotaniGard, $F_{1,14}$= 0.04, p=0.8430; interaction of parasitoids and BotaniGard, $F_{1,14}$= 0.1, p=0.7560) (Figure 3.13).

3.4. Discussion

The first experiment was designed to test whether the combination of the parasitoid and BotaniGard could produce synergistic effects on aphid population suppression, or whether they acted independently or alternatively, interfered with each other, resulting in negative effects on aphid population control. Parasitoids and

BotaniGard showed synergistic interactions in controlling aphid populations in the first experiment in the third week. Although there was no interaction effect in week four, in week five and six there were fewer aphids in cages with BotaniGard plus the parasitoid than in those with the parasitoid alone. However, because cages with only BotaniGard application and the cages that had no control agents were not sampled it is not possible to distinguish whether this positive effect was additive or synergistic. In cages with both biological control agents, not only was the number of aphids generally lower, but the population growth rate of the aphids started to decrease. In the combination treatment, the PGR became negative between weeks four and five and the rate of decrease accelerated between weeks five and six. Data gathered in greenhouses in the second experiment suggest that the combination of the parasitoid and BotaniGard does not interrupt the parasitoid's performance and persistence in the system. In this experiment, there was no significant difference between aphid populations and BotaniGard did not affect the parasitoids' development or sex ratio. Some laboratory experiments have also shown that *B. bassiana* can be compatible when used with parasitoids. For example, Dean et al. (2012) showed that *B. bassiana* (strain GHA) did not affect parasitoids that were introduced to control the Emerald ash borer (EAB), *Agrilus planipennis* (Coleoptera: Buprestidae): the ectoparasitoids *Spathius agrili* Yang (Hymenoptera: Braconidae), the endoparasitoid *Terastichus planipennisi* Yang (Hymenoptera: Eulophidae) and the egg parasitoid *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae). In a bioassay Frewin et al. (2012) showed that BotaniGard did not influence the mortality of *Aphis glycines* Matsumura (Hemiptera: Aphididae) and was the least harmful product towards the parasitoid *Aphelinus certus* (Hymenoptera: Aphilinidae) among six registered or potential pesticides against soy bean aphids. More importantly, in greenhouses experiments, *B. bassiana* has shown promising effects when used with parasitoids. Hamdi et al. (2011) compared the interactions of the parasitoid *Encarsia formosa* with three mycoinsecticides tested individually. These mycoinsecticides were based on *B. bassiana*, *Isaria fumosorosea* or *Lecanicillium muscarium*. The results from Hamidi et al (2011) showed that *B. bassiana* induced the highest level of whitefly mortality and caused the least interruption of parasitoid activity compared to the other two entomopathogens. Based on this evidence, they concluded that *B. bassiana* has the best compatibility with the release of the parasitoid *Encarsia formosa* in the reduction of whitefly *Trialeurodes vaporariorum* populations. Also, in another study when *B. bassiana*

was used with *E. formosa* in greenhouses, the two biological control agents reduced the *T. vaporariorum* population effectively without reducing the parasitoid population (Labbé et al., 2009). Moreover, the rate of parasitization by *E. formosa* was higher in the presence of BotaniGard application than in compartments that contained only parasitoids (Labbé et al., 2009). Similarly, in my experiment, BotaniGard application was associated with a positive effect on parasitoids: at most sampling points, population growth rate of mummies was faster in cages treated with BotaniGard. In addition, there were more mummies in cages that were treated with BotaniGard than cages without BotaniGard at the last sampling point in the first experiment.

Compared to BotaniGard, parasitoids were a more effective control agent. Although the additional release of *A. matricariae* in the second experiment did not improve the aphid control significantly, a power analysis showed that four more reps would have demonstrated a significant result. Although, in general, augmentation of parasitoids would help to reduce the aphid population, most aphid parasitoids only parasitize a small percentage of the aphids in their natural systems (Cohen and Mackauer, 1987; Kfir and Kirsten, 1991; Mackauer and Völkl, 1993). In addition, most parasitoids show a preference for a particular developmental stage (Barrette et al., 2009; Mackauer and Völkl, 1993; Völkl et al., 2007). Therefore, in many cases, some aphids remain “untouchable” to parasitoids. The addition of an entomopathogen could potentially enhance pest control if it targets these “untouchable” aphids. Some preliminary experiments that I carried out in chapter 2 showed that BotaniGard infects all stages of aphids and with a similar level of virulence. More over, the results in chapter 2 showed that aphids are susceptible to *Beauveria*-infection regardless of the concentration of the pathogen that was applied to them. In the experiments described in this chapter, the results indicate that the parasitoid and *B. bassiana* acted in a more than additive manner, resulting in a synergistic effect. This suggests that BotaniGard or the parasitoid made the “untouchable” aphids more vulnerable to infection or parasitization.

The synergism that takes place between the entomopathogenic fungus and the parasitoid could be caused in one of the following ways: 1- parasitoids disperse the conidia and/or transfer it to uninfected aphids, 2- parasitoids increase the aphids’ movement and thus increase the chances of picking up conidia from the leaf surface, 3- parasitoids decrease the aphids’ immune system by causing stress and making them

more vulnerable to *Beauveria*-infection 4- parasitoids are able to distinguish between healthy and infected aphids and avoid the infected aphids, 5- the parasitoids emerging out of the infected aphids are potentially fitter than those that have not been exposed to fungus. I will discuss each of these possibilities in turn and evaluate the likelihood of each of these effects in explaining the synergism that was observed in the current study between *A. matricariae* and BotaniGard.

Some studies have shown that natural enemies of insects can passively vector pathogens to healthy individuals after feeding on or parasitizing infected organisms, or they can increase the movement of the pest and increase the chance of the pest coming into contact with the pathogen (Brooks, 1993; Goertz and Hoch, 2013; Ramirez and Snyder, 2009). Also some studies have shown that the stress responses in insects that are induced after encountering a predator will make them more vulnerable to other mortality factors (McCauley et al., 2011; Ramirez and Snyder, 2009). For example Ramirez and Snyder (2009) showed that the herbivorous beetles, *Leptinotarsa decemlineata,* that encountered their natural predators had higher pathogen-induced mortality. If the presence of parasitoids in my studies resulted in increased pathogen vectoring, aphid contact with *B. bassiana* conidia, or aphid vulnerability to *B. bassiana* then one would have expected to observe an increase in the number of *Beauveria*-infected aphids in the population. However, this was not seen in my experiments and in both of the large-scale experiments the presence of parasitoids had no effect on the number of infected aphids in cages. This suggests that *A. matricariae* is not likely to increase the likelihood of inducing stress-responses that make aphids more vulnerable to *B. bassiana* infection. Nor does *A. matricariae* increase the chances of aphid encounter with the pathogen by increasing aphid movement or transmitting the conidia into cryptic feeding sites. During data collection I noticed that the number of brown and infected aphids on the leaves was relatively high, but there were few sporulating bodies on the leaves and most sporulating individuals were only found on the soil. A probable explanation is that *B. bassiana,* like most fungi, is highly dependent on moisture for sporulation (Shipp et al., 2003; Wraight et al., 2000) and the soil provided the required moisture. This suggests that, although parasitoids and aphids can be exposed and susceptible to BotaniGard, it is unlikely that they will encounter a sporulating individual and pick up *B. bassiana* conidia on the plant. Therefore, the chances of secondary

transmission were low under the conditions of the greenhouse experiments. Also, if parasitoids trigger the aphids' anti-predatory response and caused aphid dispersal on the leaves, those aphids would probably not come into contact with the conidia. It is possible that parasitoids increased aphids' encounter rate with the fungus on the surface of the soil if they made the aphids jump off of the plants. However, that assumption is not supported because, although the number of sporulating aphid cadavers were statistically higher in treatments with single release than the double release of parasitoids, the difference is not likely to be biologically meaningful as there were on average 1-2 more individual aphid cadavers on the soil in those treatments. For parasitoids to increase the likelihood of the aphids contact with the pathogen, the prey must have a high degree of risk aversion (i.e. in the case of aphids, dropping off of the plant) (Henry et al., 2010). To my knowledge, there is no study that has directly studied risk aversion in GPA, but after working with GPAs I found that they rarely jump off the plants.

One of my previous studies (described in chapter 2) showed that females that emerge from BotaniGard-treated aphids tend to weigh more. I would argue that the first two BotaniGard applications selected for the fittest females early in the experiment. The selected females reproduced at a higher rate because they were larger and more fecund. As a result of these females reproducing, a faster population growth rate was observed by week five and more parasitoid mummies were found in week six. This argument can be supported by the fact that the densities of *A. matricariae* mummies in cages treated with BotaniGard were lower than the control groups early in the experiment (6 vs. 10 mummies per leaf on average), but started to increase towards the end of the experiment. At the last sampling point, there were more parasitoids in cages treated with BotaniGard than in cages with only parasitoids. These results, however, contrast with the laboratory study done by Rashki et al. (2009) which concluded that when *A. matricariae* develop inside *B. bassiana*-infected Green Peach Aphids, their net reproductive rate and the sex ratio of their progeny is not affected, but their intrinsic rate of increase decreases compared to parasitoids that develop in uninfected aphids. Moreover, in my experiment, mummies that I gathered in greenhouses but kept until emergence in a controlled environment showed that BotaniGard reduced the emergence of parasitoids. It is not clear why the results of parasitoid PGR from the greenhouses contradicts the result of emergence rate data that was gathered post experiment in the

insectary, and the Rashki et al. (2009) conclusion. A possible explanation is that the fluctuating abiotic conditions inside the greenhouses affected the competitive ability of *B. bassiana.* Baverstock et al. (2009) studied the compatibility of parasitoid *Aphidius ervi* with the entomopathogenic fungus *Pandora neoaphidis* against pea aphid, *Acyrthosiphon pisum* in microcosms and polytunnels. Their results showed that in microcosms the parasitoid's reproductive success was significantly reduced in the presence of the fungus but was not affected in polytunnel experiments. Since the biology of entomopathogenic fungi is highly dependent on temperature and humidity (Baverstock et al., 2009; Ibarra-Cortés et al., 2013; Polar et al., 2005) it is important to consider that while the environmental conditions fluctuate in the greenhouses, they were kept constant in a controlled environment room at 16L: 8D photoperiod, 21°C ± 2 °C and 60 ± 10% RH). .

In the current experiments I found limited evidence that BotaniGard reduced aphid density. However, BotaniGard showed high virulence against GPA in one of the laboratory experiments described in chapter 2. Other studies that were carried out in controlled environments also showed that entomopathogens are very effective in increasing pest mortality rates. Mortality of cereal aphids, *Rhopalosiphum padi* (L.) and *Sitobion avenae* (F.), on plants in growth chambers with controlled environment (at 22±2◦C, 60–65%RH and 16L: 8D photoperiod), increased linearly with increases in the dose of Mycotal® (a *Verticillium lecani* (Z.) based product) (Aqueel and Leather, 2013). *Beauveria bassiana* was shown to be highly virulent against shore flies, *Scatella stagnalis* in laboratory growth chambers (Jacobson et al., 1999). Rashki et al (2009) showed that in laboratory settings, 100% of the aphids that were sprayed with *B. bassiana* conidia solution died in 7 days, whereas only 10% of aphids in their control groups died during that period. BotaniGard has been shown to be highly effective against other pests such as the Emerald ash borer (EAB) in laboratory conditions (Dean et al., 2012). Unlike laboratory experiments, BotaniGard by itself did not effectively reduce whitefly populations (Labbé et al., 2009), Western flower thrips (Skinner et al., 2012) or the tunnelling response of mole crickets (Thompson and Brandenburg, 2005) in greenhouses.

As Labbé et al. (2009) suggest, it is possible that the poor performance of BotaniGard can be accounted for by the fact that the relative humidity inside the

greenhouses is lower than the relative humidity that is recommended for periods of BotaniGard applications (Shipp et al., 2003). Surprisingly, *B. bassiana* has also shown promising effects in the field. It has strong potential for controlling nymphal whiteflies *Bemisia argentifolii* on cucurbit crops in fields (Wraight et al., 2000). Liu and Bauer (2008b) observed that the effect of BotaniGard on EAB was stronger when it was sprayed on infested green ash trees in the field than when it was sprayed on infested logs in greenhouses. Also, in an experiment that is discussed in chapter 2, BotaniGard did reduce the number of aphids on individual plants that were covered with perforated bags to maintain the organisms in greenhouses. In that experiment, BotaniGard was applied to aphids infesting 4 week-old plants which then were exposed to parasitoids for 24 hours. After two weeks the number of aphids was lower in BotaniGard treated cages than cages with only parasitoids. It is unclear why the results from these two experiments contradict each other and why the effect of the fungus is lower in greenhouses compared to the field and laboratory experiments given that the temperature and moisture levels were similar in both experiments. However, as Liu and Bauer (2008) suggested, the observed difference could be the result of differences in log conditions, cage setups and other abiotic factors.

The weights of the plants were similar among all treatments regardless of the number of parasitoid releases and BotaniGard application. However, the weight of the plants is not the only parameter that affects crop production. Aphids can damage plants directly by depleting nutrients from the crops and depositing honeydew, but most importantly they vector plant viruses and cause damage indirectly (Boivin et al., 2012; Hogenhout et al., 2008; Ng and Perry, 2004). They are responsible of vectoring over 28% of identified plant viruses (Hogenhout et al., 2008) including non-persistent stylet-borne viruses, as well as persistent viruses that move through the haemocoel of the insect to the salivary glands where they are discharged (Emden and Harrington, 2007; Hogenhout et al., 2008; Katis et al., 2007; Ng and Perry, 2004; Sylvester, 1989). It is likely that although the weight of the plants did not reflect a significant difference among different treatments, plants with fewer aphids were healthier. However, measuring plant health in any other way except for comparing its weight was not possible in the experiment.

Overall this study showed that the parasitoids and BotaniGard are safe to be used together against GPA in greenhouses. Further research is required to understand the underlying mechanism of the effect of BotaniGard on aphids in different environmental conditions.

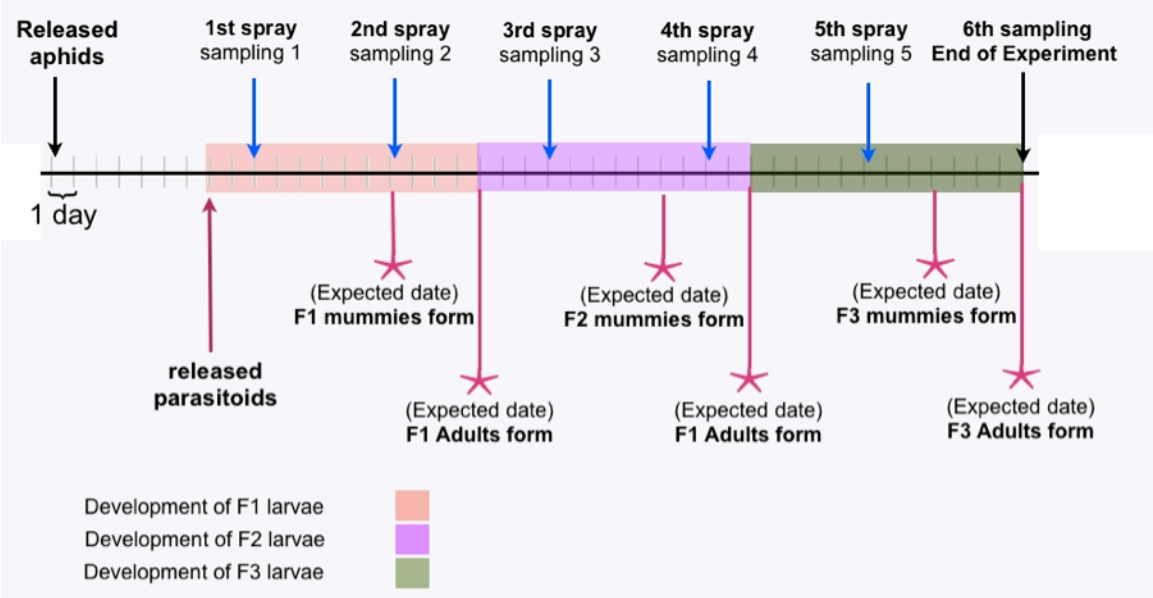

Figure 3.1. The timeline of the first experiment and the expected dates of parasitoids' developmetal stages

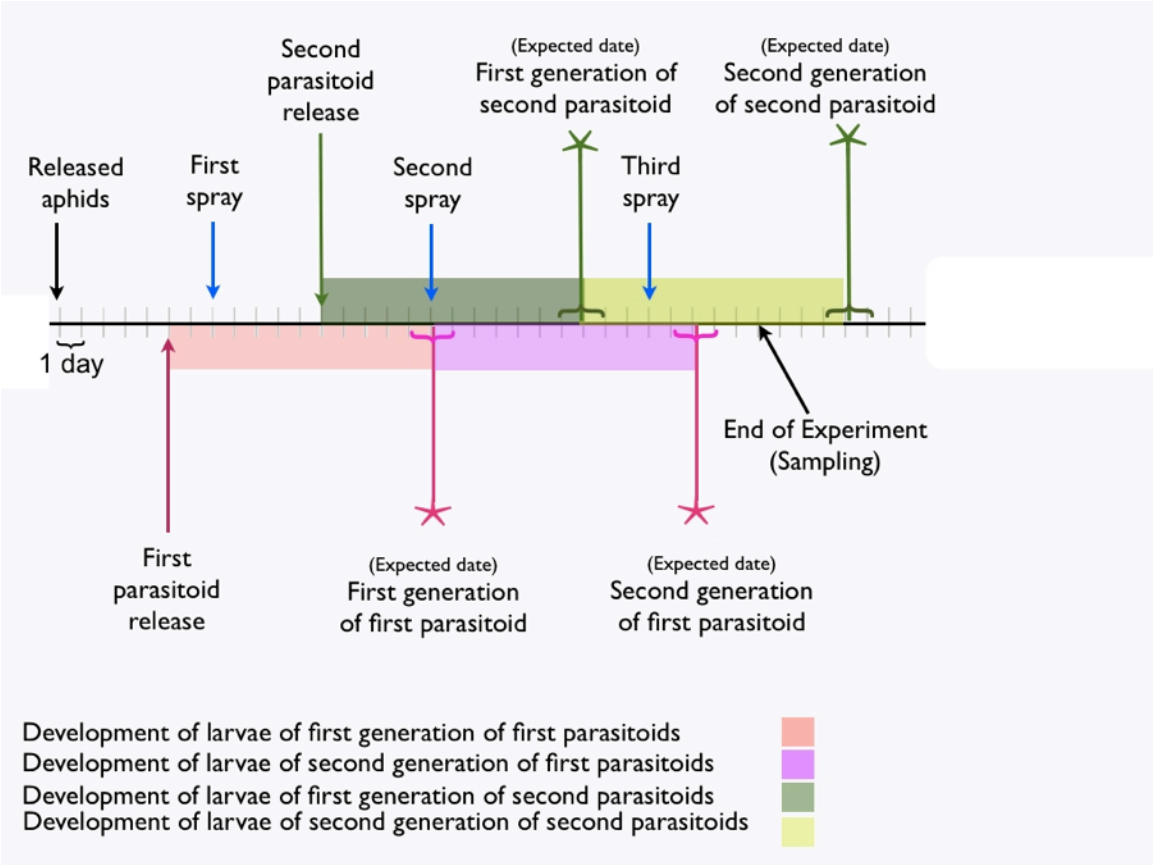

Figure 3.2. The timeline of the second experiment and the expected dates of parasitoids' developmental stages

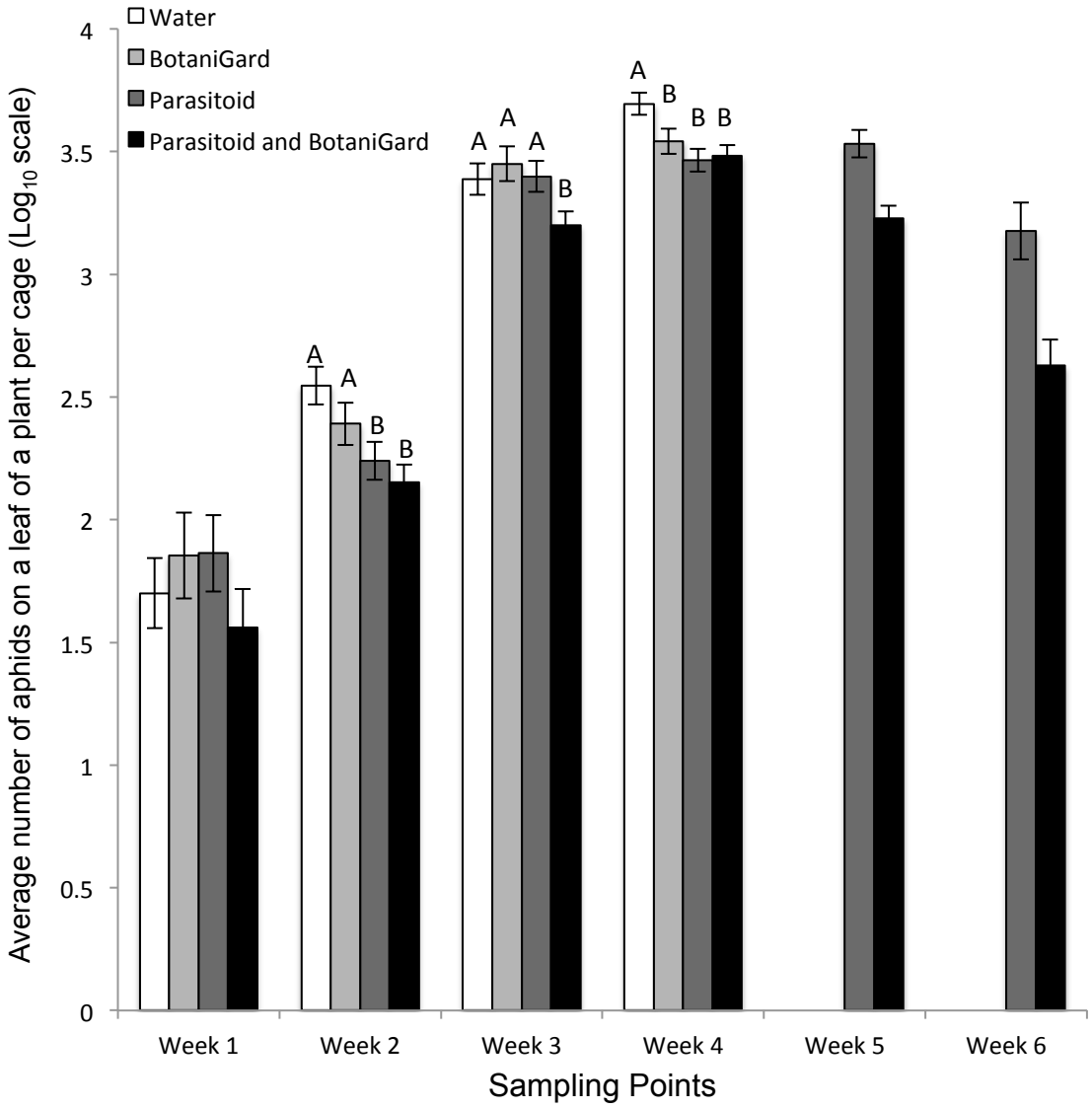

Figure 3.3. The density of M. persicae recovered from cages containing no natural enemies (Water) (N= 5 cages), Parasitoids (N= 5 cages), BotaniGard (N=4 cages), BotaniGard and Parasitoids (N=6 cages) at six successive sampling points that took place weekly. (each cage contained 4 plants). Treatments are compared for each sampling point separately. Tukey test results are denoted by different letters. Error bars represent ±SE.

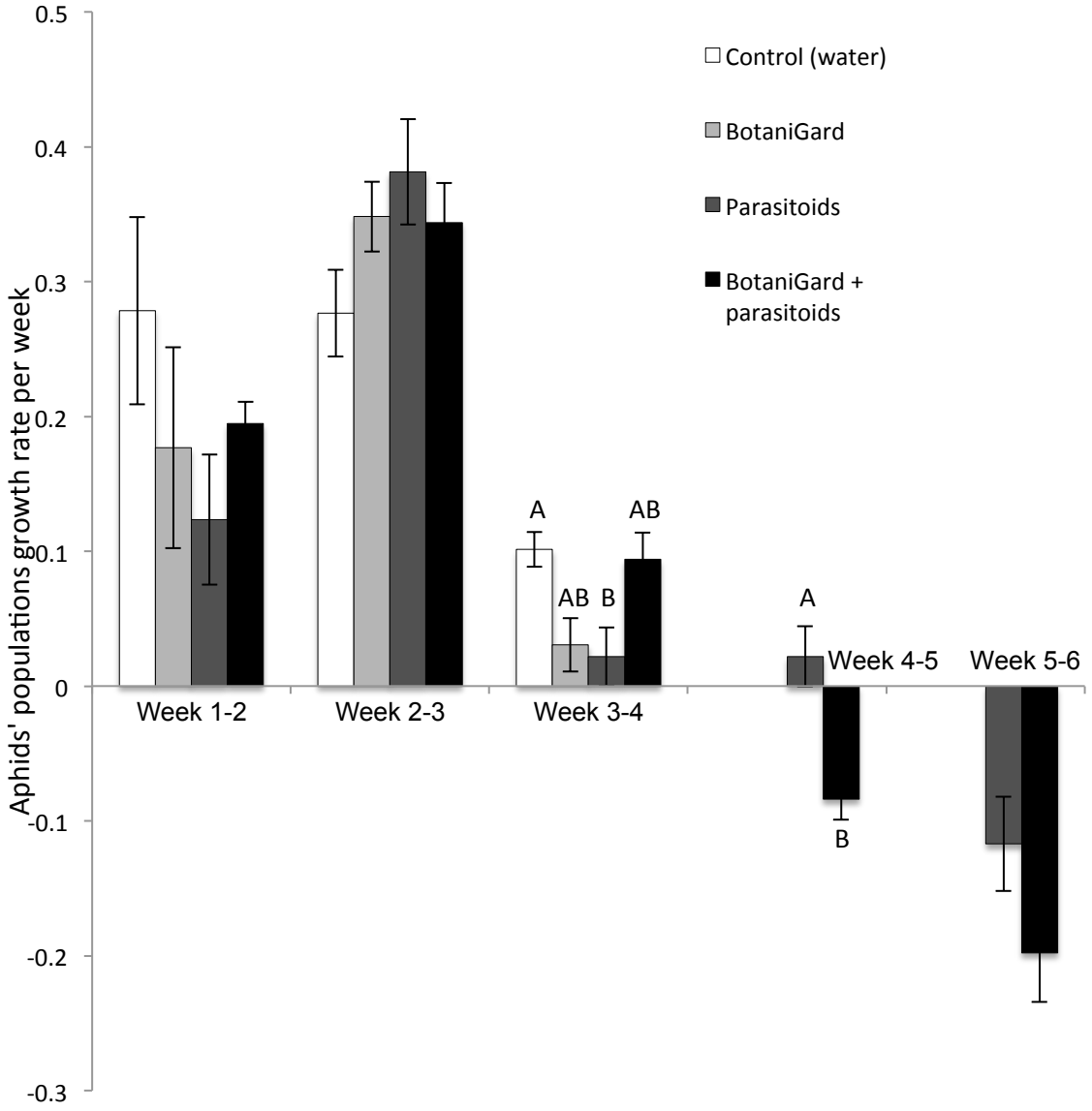

Figure 3.4. The population growth rate of aphids, *M. persicae,* in cages containing no natural enemies (Water) (N=5 cages), Parasitoids (N= 5 cages), BotaniGard (N=4 cages), BotaniGard and Parasitoids (N=6 cages) between the successive sampling points for six weeks. Sampling was stopped in cages without parasitoids after week four. The growth rates were compared for the sampling periods separately. Error bars present ±SE. Tukey test results are denoted by different letters.

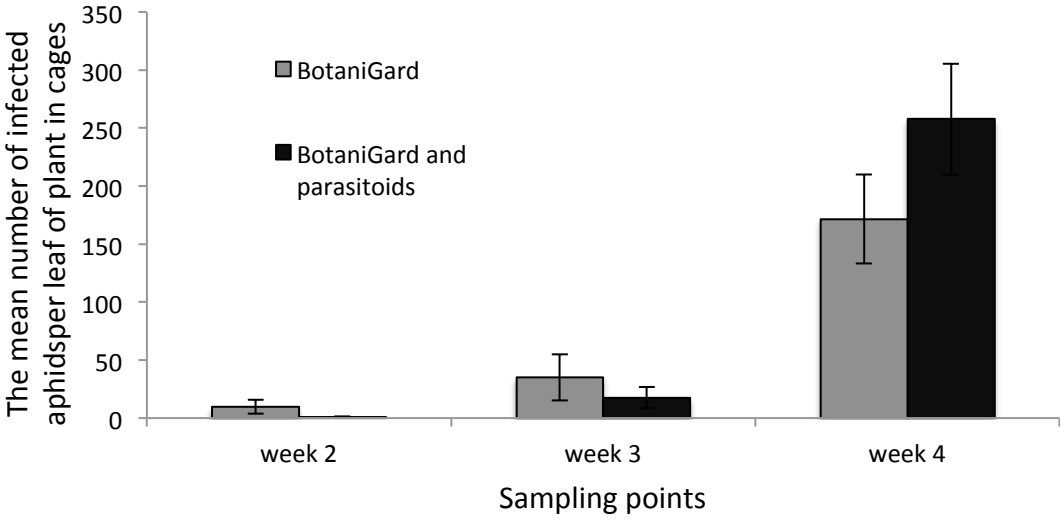

Figure 3.5. The average number of infected aphids, *M. persicae* counted on a leaf of a plant for the cages containing either BotaniGard (N=4 cages) or BotaniGard and *A. matricariae* (BotaniGard and parasitoids) (N=6 cages) at week 2,3, and 4 of sampling. Error bars present ±SE.

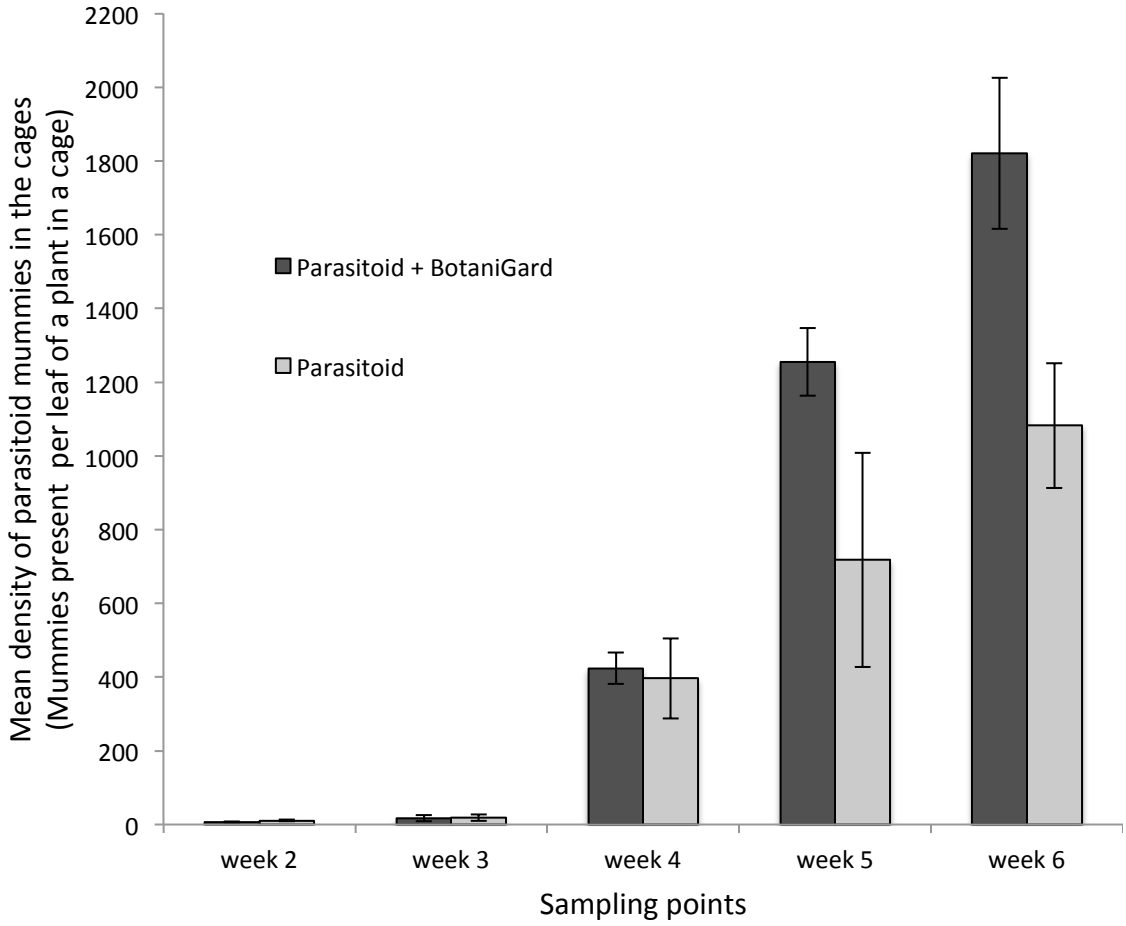

Figure 3.6. Comparison of the density of *A. matricariae* mummies present in *M. persicae* populations in cages containing either treated *A. matricariae* and BotaniGard (Parasitoid + BotaniGard) (N=6) or *A. matricariae* (Parasitoids) (N=5) at different sampling points. Error bars represent ±SE.

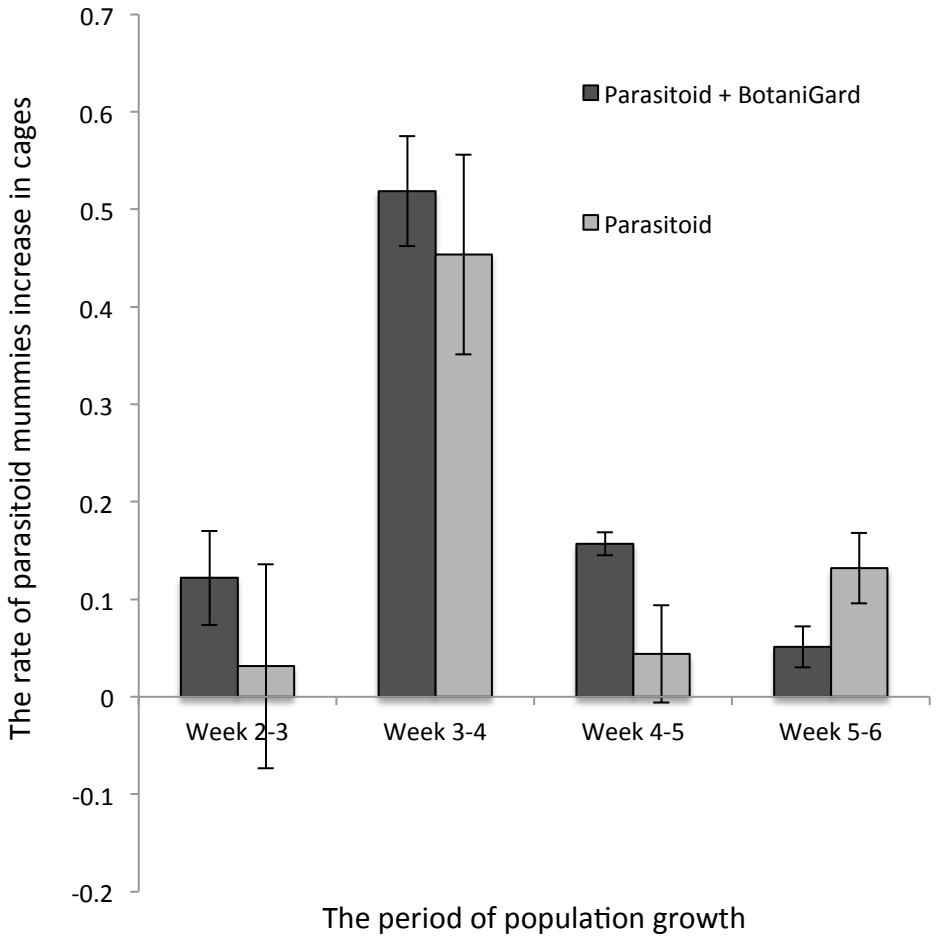

Figure 3.7. The growth rate in the number of *A. matricariae*'s mummies in a week in *M. persicae* populations treated *A. matricariae* and BotaniGard (Parasitoid + BotaniGard) (N=6) or *A. matricariae* (Parasitoid) (N=5) at different sampling points. Error bars represent ±SE.

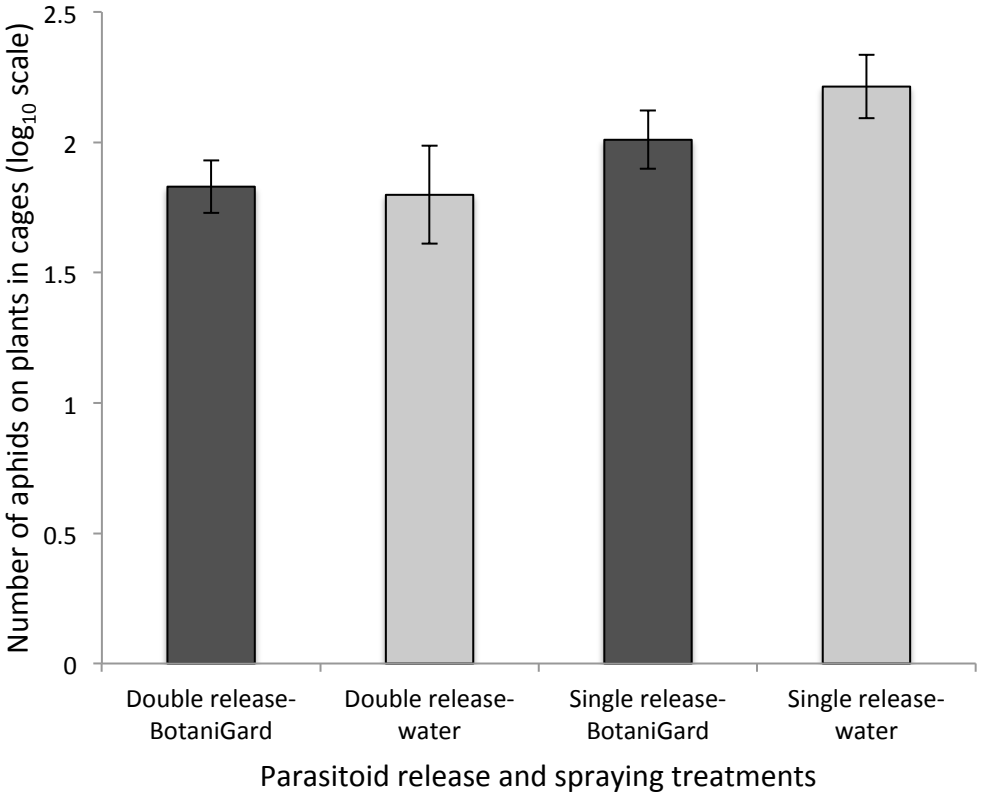

Figure 3.8. Average number of aphids, *M. persicae* per leaf of a pepper plant in cages subjected to different treatments of parasitoids *A. matricariae* and BotaniGard application: Double release of parasitoids plus BotaniGard (N=3), Double release of parasitoids plus water (control) (N=6), Single release of parasitoids and BotaniGard (N=5), Single release of parasitoids plus water (control) (N=4). Error bars represent ±SE.

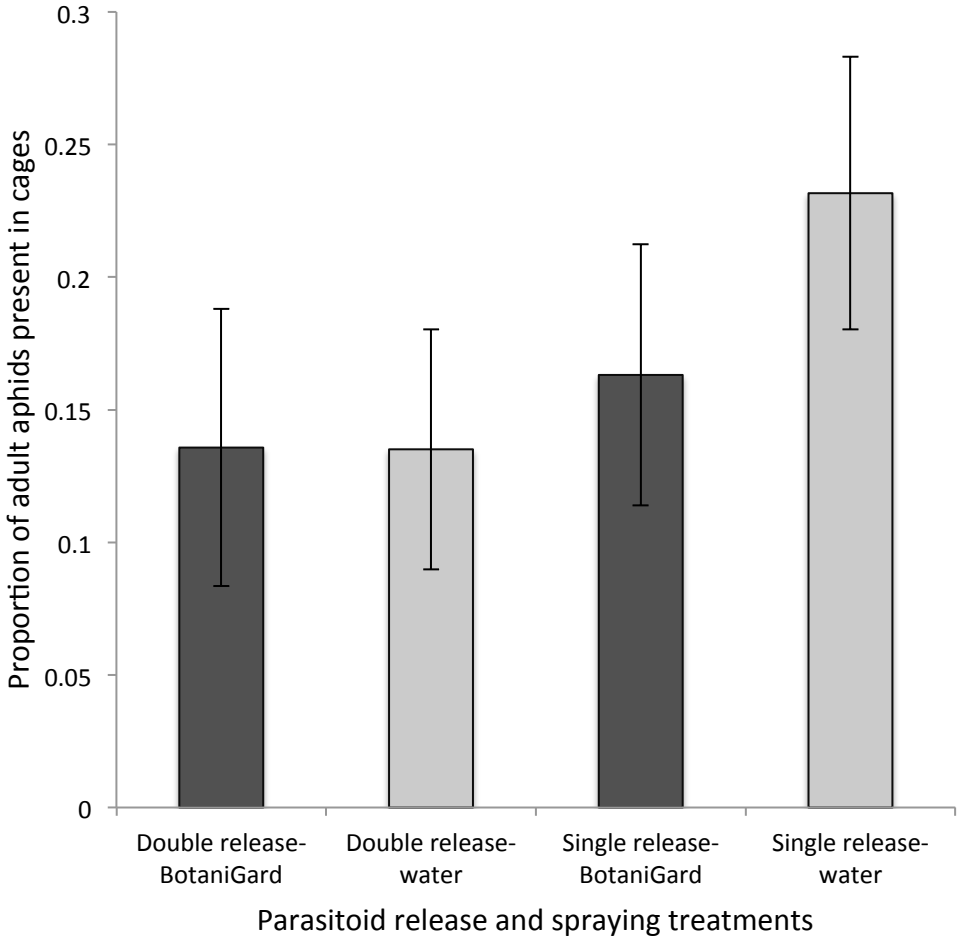

Figure 3.9. The proportion of adult *M. persicae,* in cages subjected to different treatments of parasitoids *A. matricariae* and BotaniGard application: Double release of parasitoids plus BotaniGard (N=3), Double release of parasitoids plus water (control) (N=6), Single release of parasitoids and BotaniGard (N=5), Single release of parasitoids plus water (control) (N=4). Error bars represent ±SE.

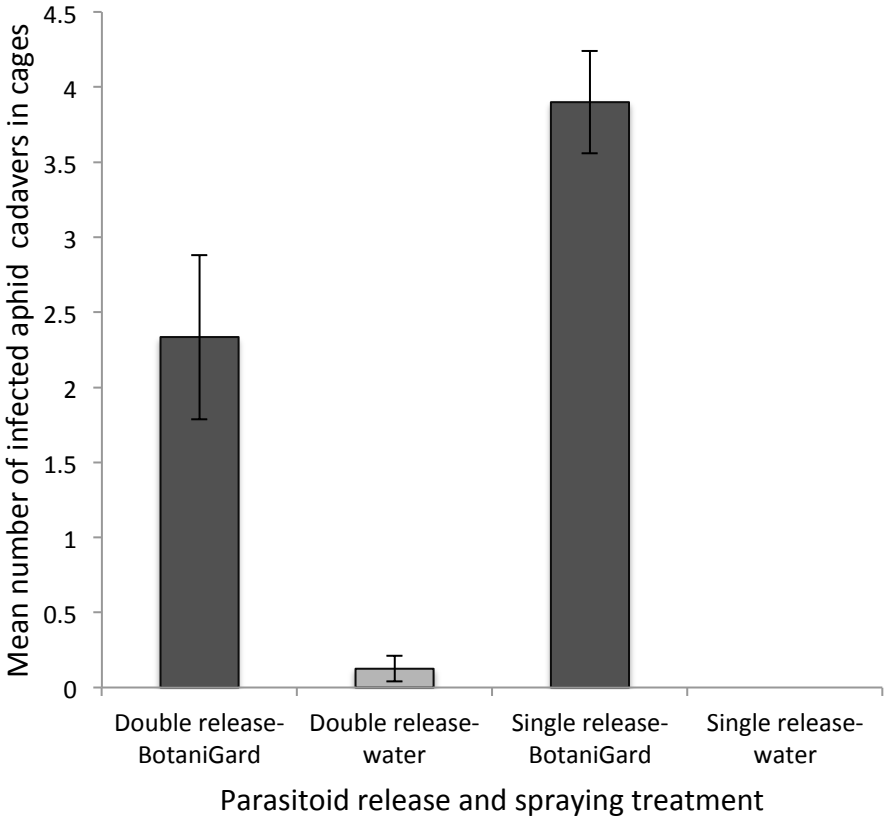

Figure 3.10. The number of infected aphid (*M. persicae*) cadavers covered in conidia of *B. bassiana* fungus, the main ingredient of BotaniGard. The cadavers were collected from the surface of the soil in the plant pots in cages subjected to different treatments of parasitoids *A. matricariae* and BotaniGard application: Double release of parasitoids plus BotaniGard (N=3), Double release of parasitoids plus water (control) (N=6), Single release of parasitoids and BotaniGard (N=5), Single release of parasitoids plus water (control) (N=4). Error bars represent ±SE.

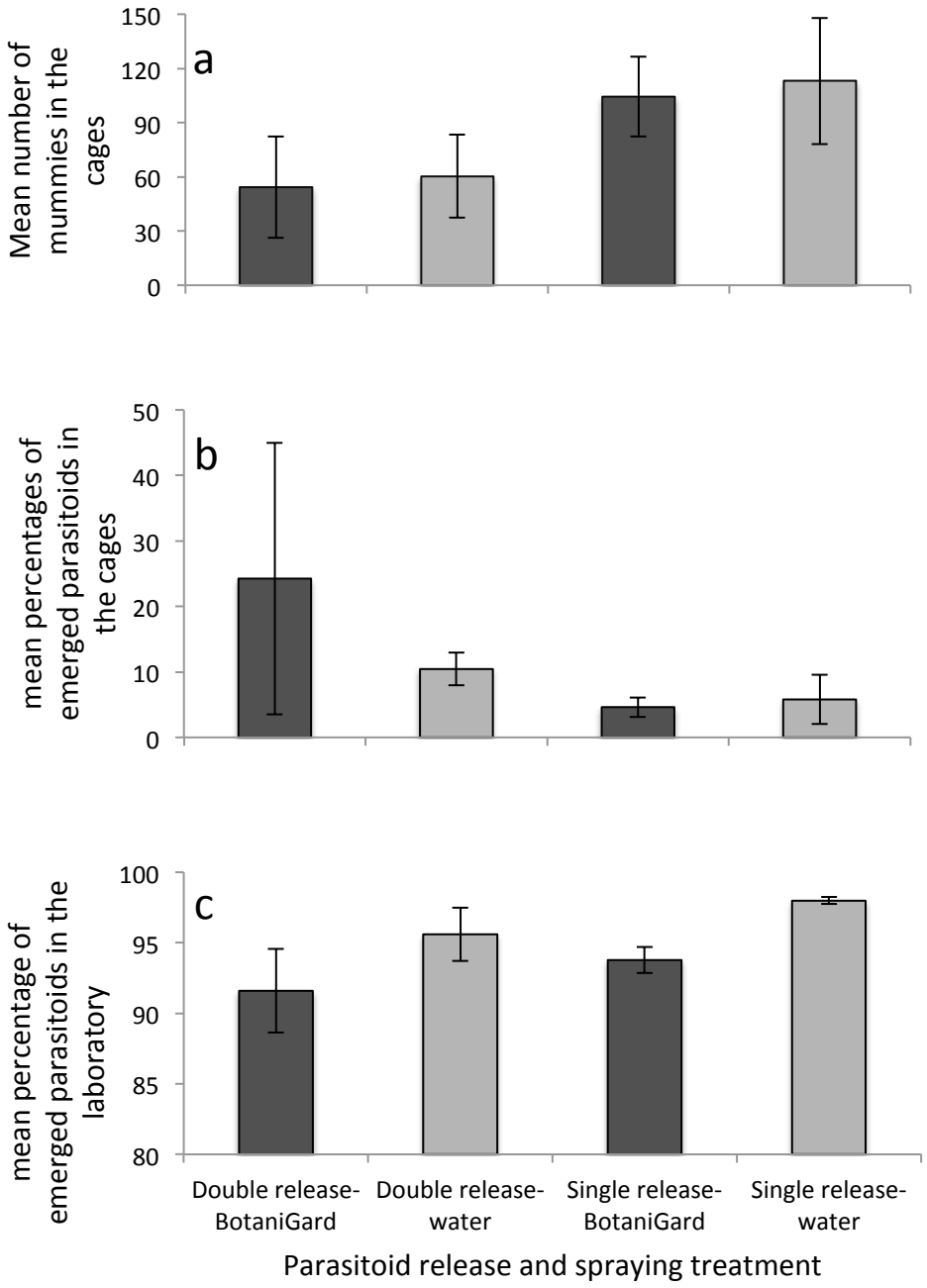

Figure 3.11. Mean number of *A. matricariae* mummies (a), mean percentage of emerged parasitoids in cages (b) and mean percentage of emerged parasitoids in laboratory (c). Mummies formed in *M. persicae* populations inside cages of Double release of parasitoids plus BotaniGard (N=3), Double release of parasitoids plus water (control) (N=6), Single release of parasitoids and BotaniGard (N=5), Single release of parasitoids plus water (control) (N=4). Error bars represent ±SE.

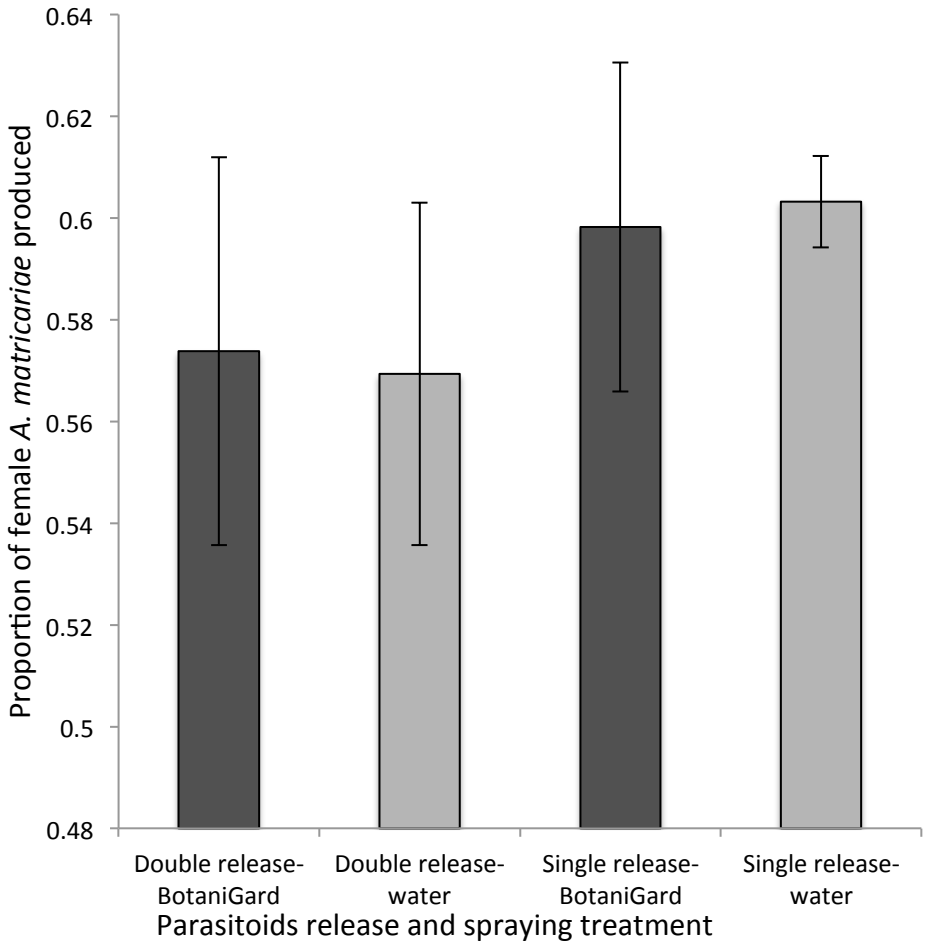

Figure 3.12. Proportion of the female *A. matricariae* to all the emerged adults from the mummies that were maintained in Laboratory until emergence. Mummies were formed in *M. persicae* populations inside cages of Double release of parasitoids plus BotaniGard (N=3), Double release of parasitoids plus water (control) (N=6), Single release of parasitoids and BotaniGard (N=5), Single release of parasitoids plus water (control) (N=4). Error bars represent ±SE.

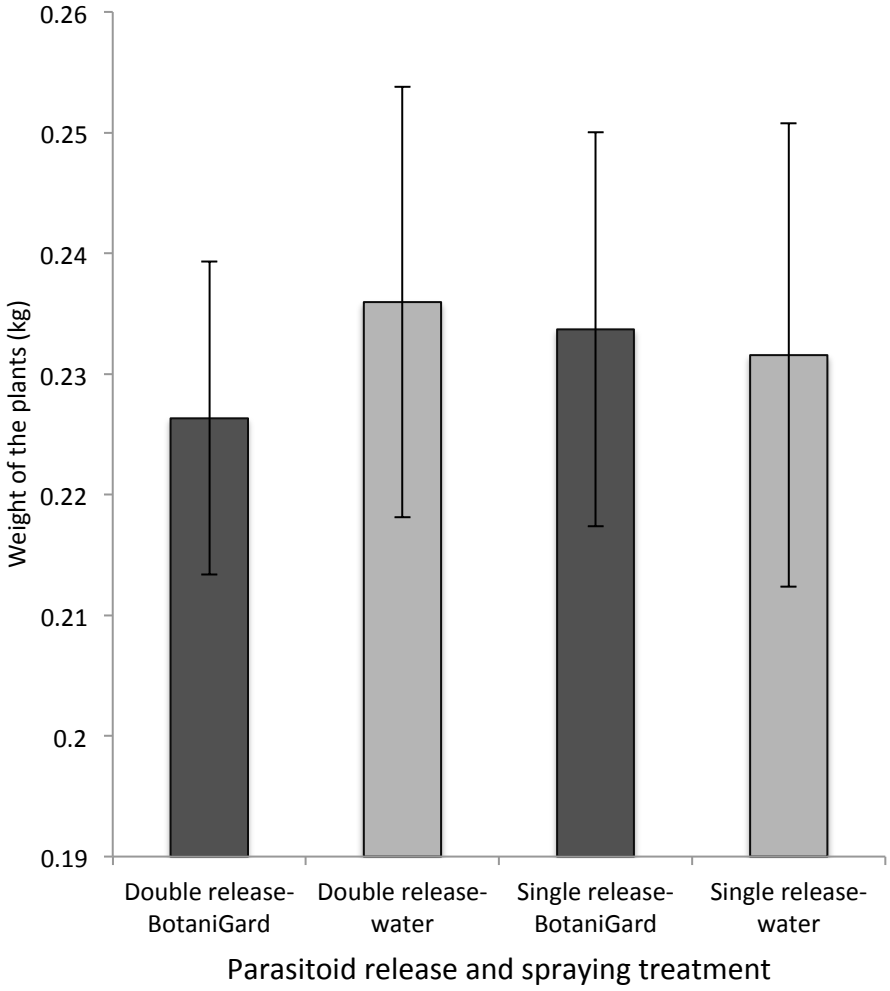

Figure 3.13. Average weight of plants inside cages cages of Double release of parasitoids plus BotaniGard (N=3), Double release of parasitoids plus water (control) (N=6), Single release of parasitoids and BotaniGard (N=5), Single release of parasitoids plus water (control) (N=4). Error bars represent ±SE.

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4. Conclusion and Discussion

Simultaneous use of multiple biological control agents for controlling pests is a common practice; however, intraguild interactions are likely to occur among these agents, making it necessary to determine whether biological control agents impact each other. These intraguild interactions can result in negative effects that disrupt the pest management system or in positive effects that enhance it. For simultaneous use of biological control agents to be economically practical the outcome of the intraguild interaction should be additive or synergistic.

My study showed that the simultaneous use of a parasitoid *Aphidius matricariae* and the fungus *Beauveria bassiana* (applied in the commercialized form BotaniGard22WP ®) has positive effects on control of green peach aphid, *Myzus persicae*, in greenhouses. In my long-term experiments, I studied the compatibility of BotaniGard and parasitoids in greenhouses over 3 generations of parasitoids (6 weeks) in a full factorial design. Treatments were assigned to large cages inside greenhouses where host plants, aphids and parasitoids were contained and BotaniGard was applied on a weekly basis. The number of healthy aphids, infected aphids and parasitoid mummies on plant leaves were counted each week. The results showed that by the second sampling point parasitoids kept aphid populations to half of the size of aphid population in experimental controls (no parasitoids). BotaniGard on the other hand, although causing reduction in aphid longevity and hence total reproduction in laboratory experiments, was not able to provide control of the aphid populations by itself in greenhouses, under the conditions of the experiment. However, the combined use of parasitoids and BotaniGard resulted in a stronger effect than would be expected from the effect of either biological control agent alone: a synergistic effect was observed between parasitoids and BotaniGard after 3 weeks. Positive effects of the combined use of parasitoids and BotaniGard were also evident at the 5^{th} and 6^{th} week of sampling, but, unfortunately, because sampling was terminated from the fourth week the experimental design was no longer a full factorial design, so it is not possible to determine whether

these positive effects happened due to synergism or additive effects. However, it was evident that aphid population growth rate (PGR) became negative for the first time in presence of both biological control agents in the period between the 4^{th}-5^{th} sampling point, and that fewer aphids were present when both biological control agents were present than parasitoids alone at the 5^{th} and 6^{th} sampling points. More interestingly, the parasitoid population was larger in cages with BotaniGard application, despite the fact that they contained fewer aphids than cages that only had parasitoids as the biological control agents in the system. Also, the growth of the parasitoid population was faster in cages with both biological control agents. These facts indicate that the higher number of parasitoids was not related to the number of aphids present in the cages. Looking at the results of small-scaled experiments can help to elucidate the mechanisms underlying these effects. The rest of this chapter is dedicated to understanding the mechanisms that resulted in the effects observed in this large-scale greenhouse experiment.

When parasitoid, *A. matricariae,* mummies were dipped in BotaniGard there was no detrimental effect in terms of emergence. Previous studies have suggested that fungal penetration into the aphid cuticle at mummy stage declines significantly, often to no penetration at all, and the fitness of female parasitoids in terms of development time, emergence success, sex ratio, longevity and fecundity would not be affected (Aiuchi et al., 2012; Askary et al., 2006; Askary and Ajam Hassany, 2008; El-Sufty and Furher., 1981; Mesquita and Lacey, 2001). This evidence suggests that in the large-scale experiments in the greenhouses BotaniGard affected parasitoids at a stage other than the mummy stage. However, the fitness of female parasitoids that emerged out of treated mummies and their offspring fitness were not measured in my experiments. As a result, although the possibility remains, there are limitations in concluding that BotaniGard would not affect *A. matricariae* fitness at mummy stage under more realistic conditions.

One of my short-time scale experiments was designed to study the effect of BotaniGard on parasitoids at the individual pot level in greenhouses. It consisted of 2 parts, Part A and Part B, and both parts investigated the effect of BotaniGard and timing of BotaniGard application on parasitoid development, emergence, sex ratio and fitness. In part A, plants harboring potentially parasitized Green Peach Aphids (GPA) were treated with BotaniGard (or were sprayed with water for control); in Part B plants that

harbored aphids were first treated with BotaniGard before parasitoids were introduced to the system. Results from both Part A and Part B showed that, the number of mummies and the percentage of adults emerging decreased in presence of BotaniGard. These results suggested that BotaniGard can have negative effects on *A. matricariae* at the larval stage. Previous studies have shown the susceptibility of parasitoid larva to fungus and a reduction in host quality (Hamdi et al., 2011; Hochberg et al., 1990; Milner et al., 1984; Powell et al., 1986). However future laboratory experiments that carefully test the effect of BotaniGard on the parasitoid larvae developing inside individually parasitized and infected aphids would be required to confirm this effect between BotaniGard and *A. matricariae*.

It could be possible that the positive effect between *A. matricariae* and BotaniGard in the long-term greenhouse experiment happened because the parasitoid could avoid oviposition in infected aphids and hence reduce competition. While some parasitoids are capable of receiving and responding to volatiles that signal the presence and the suitability of a potential host (Brobyn et al., 1988; Chow and Mackauer, 1986; Fatouros et al., 2008; Goubault et al., 2011; Hoffmeister and Roitberg, 1997; Landa, 1984; Mesquita and Lacey, 2001; Salerno et al., 2013; Tamiru et al., 2011), there is evidence that female *Aphidius* parasitoids cannot discriminate between fungus-infected aphid and healthy hosts (Baverstock et al, 2005). To understand if parasitoid host choice affected the positive effect on aphid control in the large-scale greenhouse experiment, as well as the reduction in mummy production in Part B of short-time scale pot-scale experiments, I tested parasitoid oviposition behavior in response to host infection status. I measured the number of times that an *A. matricariae* attacked an aphid host under laboratory conditions. The parasitoid in this experiment was not given a choice but individual parasitoids encountered a 5-day old healthy aphids, 7-day old healthy aphid, 5- day old infected aphid, or a 7-day old *Beauveria*-infected aphid. All the infected aphids were directly treated with BotaniGard at one-day of age. The age of the aphid at the time of the experiment was a surrogate of infection development inside the aphid. Results from this experiment revealed that there was no difference in the number of oviposition attempts of parasitoids from different treatments. Although the results of this experiment might suggest that parasitoid foraging behavior is not affected by aphid host infection status, there were limitations to this experiment: parasitoids were not given a choice, the

experiment needs to be repeated under different condition, and aphid infection development was cofounded by the age of aphid. Nonetheless, this experiment provided more evidence that it is more likely that, in the long-term scale greenhouse experiment, that BotaniGard affected parasitoids at the larval stage and that *B. bassiana* out competed the parasitoids by reducing the host's resources necessary for the parasitoid's development and not by affecting the oviposition behavior of adult parasitoids. To be able to test this suggestion, not only are experiments required that test parasitoid host selection behavior, but also it is necessary to understand the level to which *A. matricariae* larva depends on host resources and how *B. bassiana* can reduce host-suitability for the parasitoid larvae.

Synergism among biological control agents is usually attributed to either niche compartmentalization or functional facilitation. While, female parasitoids of the same species *Aphidius colemani*, have a preference for second instars aphids (Barrette et al., 2009), preliminary tests that I carried out showed that there is no difference in virulence of *B. bassiana* in response to aphid age. On the other hand results from my laboratory experiments (explained in detail in chapter 2) have shown that there was no difference in aphid mortality for different concentrations of BotaniGard applied directly on to aphids. This indicates that there is no indication of niche compartmentalization between *A. matricariae* and the fungal entomopathogen For facilitation to happen between *A. matricariae* and BotaniGard, at least one of the biological control agents should make *M. persicae* more susceptible to the other biological control agent (i.e. either the presence of parasitoid would increase infection probability of *M. persicae*, or BotaniGard application would expose or make *M. persicae* more vulnerable to oviposition by *A. matricariae*. For parasitoids to increase the chance of aphids coming in contact with the pathogen, the prey must have a strong degree of risk aversion (Henry et al., 2010). However, personal observations have shown that GPA does not have a high degree of risk aversion and as a result, the presence of parasitoids in the cages does not elicit aphid dispersal or to increase the chances of picking up conidia from leaf surfaces. The outcome might be different for aphids that have higher risk aversion but it was evident that, the number of infected aphids was not different in the presence and absence of parasitoids and augmentation of parasitoids to the system did not influence the number of infected aphids. This indicates that *A. matricariae* has no role in increasing the rate of

B. bassiana infection in the GPA. One might think that it is possible that BotaniGard influenced the immune system of aphids in a way that *M. persicae* either could not defend itself against *A. matricariae* during oviposition, or that the infected aphid became a better host for the parasitoid larvae. However, that is probably not the case, because in both Part A and Part B of the short-time scale greenhouse experiment the number of produced mummies were significantly lower in BotaniGard treated groups than the control groups. I concluded from the observations that are reported in this thesis that there is no functional facilitation either between *A. matricariae* and *B. bassiana* in *M. persicae*-pest control.

If there was neither functional facilitation nor niche compartmentalization then how did BotaniGard and *A. matricariae* have synergistic effects on aphid population control? After the female offspring of parasitoids used in Part B of the short-time scale experiment were weighed an interesting phenomenon appeared: female parasitoids that emerged out of BotaniGard treated aphids on average weighed more than parasitoids that emerged out of healthy aphids. I suggest that the synergism occurred because intraguild interactions between the fungus and the parasitoid led to intraguild selection over time. Intraguild selection happens when individuals with advantages in competition or with characteristics that give them the ability to survive intraguild predation contribute more offspring to the succeeding generation. In my experiment (Part B), very few female parasitoids completed their development to adulthood in the presence of BotaniGard. In this experiment, female parasitoids emerging out of BotaniGard-treated aphids on average weighed 8% more than female parasitoids that emerged out of un-treated aphids. In the case of parasitoids, female weight usually is a surrogate of fecundity and fitness (Roitberg et al., 2001). I propose that the susceptibility of *A. matricariae* larvae to *Beauveria*-infection of their host lead to selection of parasitoids that are highly fecund. Because weight is a surrogate of fitness for parasitoids, one can conclude that the "better" parasitoids were selected in the presence of BotaniGard. Many studies have demonstrated cases in which biological control agents have been selected for resistance to synthetic pesticides (Auger et al., 2004; Baker, Perez-Mendoza et al., 1998; Corino et al., 1986; Goulart et al., 2012; Havron et al., 1991; Mogeret Binti and Dzolkhifli, 2013). While it might be the case the *A. matricariae* might evolve to develop resistance to *B. bassiana* overtime, in this study selection happened in one or two generations. In my

study, parasitoid mortality is not relevant - , it is the greater reproduction of parasitoids in the presence of BotaniGard application compared to the control groups that is relevant. It seems unlikely that the effect is due to development of resistance to BotaniGard.

The other matter to note is that, the effect was only observed when parasitoids parasitized potentially *Beauveria*-infected aphids and similar results were not observed when aphids were parasitized before BotaniGard treatment. It is not clear why this result was observed. It might be tempting to suggest that in the presence of BotaniGard parasitoid weight increased because only large-size aphid hosts were present in the system. However, as explained earlier, *B. bassiana* has similar virulence for different aphids and no difference was observed in aphid population age structure. It is possible that the effect was an artifact of the experimental set-up since this study was not repeated. The validity of and the application of this effect for pest management issues require more extensive research. As a first step, laboratory experiments that study the emergence and fitness of female *A. matricariae* emerging out of BotaniGard treated *M. persicae* is necessary.

Although there are studies that have monitored the effect of different pest management practices on pest population over time (Gillespie and Acheampong, 2012; Henry et al., 2010; Labbé et al., 2009; Vergel et al., 2011; Snyder et al., 2006; Walzer et al., 2001), most studies took over less than one generation of predator/prey (Finke and Snyder, 2010). Finally, to my best of knowledge there is no study that has reported intraguild selection as a result of intraguild interaction. More research is required to study the long-term effects of pathogens on parasitoids and more research is required to better understand the underlying mechanisms of emerging effects of intraguild interactions. Understanding the mechanisms by which BotaniGard affects parasitoids has implications not just for inundative release but also for classical biological control programs.

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