Asymptomatic pathogen infection alters interactions at higher trophic levels

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

Plants and insects have intimate and complex relationships. However, other species, such as plant pathogens, may change the way plants and their herbivores interact. Plant diseases can change the host plant quality, alter physical and physiological traits and defensive capability. These changes can have a consequential effect on insects associated with the infected plant, thus altering interactions between species. However, the effects of these interactions can quantitatively vary amongst individuals and may influenced by many factors including the severity of plant disease (e.g. if the infection is symptomatic or asymptomatic). To date, little is known on the effect of asymptomatic pathogen infection on insects at higher trophic levels. My research aims to examine how patterns of interactions between plants, microbes and insects change when plant pathogen infection is asymptomatic. I examined the effect of asymptomatic plant pathogen infection on the life history and behaviour of herbivorous insects, and its consequent effects on an insect community at higher trophic levels. In the laboratory, I found that asymptomatic pathogen infection altered plant traits and changed the life history and behaviour of an herbivorous insect and its parasitoid. In the field, the effect of asymptomatic pathogen infection differed between plant varieties and species of insect involved. Asymptomatic pathogen infection greatly affects the assemblage and population dynamics of aphids on one variety of lettuce (Tom Thumb), but no significant effects were found on Little Gem. However, both lettuce varieties were less attractive to natural enemies when asymptomatically infected. The asymptomatic pathogen infection also modulated the interaction of plants and insects on neighbouring plant. More aphids were found on the uninfected plant neighbouring an asymptomatic-infected plant, and thus influencing the number of natural enemies observed. These findings show the importance of asymptomatic pathogen infection to the ecological interactions at higher trophic levels in the laboratory and field. Pathogen infection, even if asymptomatic, can influence interactions between organisms. This is of considerable interest to both pure and applied ecologists, and can affect the success of biological control programme in agriculture systems.

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Chapter 1: Introduction

1.1. Plants as a basis of food webs

Recent estimates suggest that planet Earth is home to one to six billion species (Larsen et al., 2017). The greatest species diversity is dominated by bacteria (70 to 90% of all species), followed by the insects which account for forty million species known to science (Larsen et al., 2017). Half of all insects are herbivorous, with Coleoptera and Lepidoptera making up two-thirds of species (Wielkopolan and Obrępalska-Stęplowska, 2016). Despite plants being less species rich, with approximately 391,000 species, they form the basis of most food webs (Willis, 2017).

Plants are members of many biodiverse communities and interact simultaneously with their biotic environment, including other plants, animals and microorganisms; such interactions have been shown to shape the functioning of the Earth's ecosystem (Oldroyd and Robatzek, 2011; Austin and Ballaré, 2014). The interaction between plants and other organisms comes in several forms, such as antagonistic relationships where plants compete for resources or spread disease to their neighbours (Haag et al., 2004; Schädler et al., 2007) or mutualistic associations when plants provide shelter, oviposition sites and food to other organisms and as a return those organisms give protection to the plants from their enemies or help plant pollination (Bennett et al., 2006; Bronstein et al., 2006; Frago et al., 2012). These interactions are governed by many aspects such as physiology, ecology and behaviour of the organism involved. Interactions in the plant ecosystem, either direct or indirect, such as competition, are among the most important biotic factors which contribute to the structuring of the ecological communities.

As an example, plants globally are attacked by more than 10,000 species of insect herbivores and 100,000 plant diseases caused by fungi, bacteria, viruses and other microorganisms (Dhaliwal et al., 2010). Faced with this multitude of enemies, plants have an ability to implement an often-tailor-made defence response to resist those attacks (Gurevitch et al., 2000). Plants may either act directly upon their antagonist by producing substances such as toxins and digestibility reducers to reduce infestation by insect herbivores (Wouters et al., 2016) or by activating the different plant pathways such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) to suppress the pathogen infection (Pieterse and Dicke, 2007). They also may engage in 'biological warfare' by attracting and rewarding other animals such as predators and parasitoids to influence the performance of the insect herbivores (Bronstein et al., 2006). These defence mechanisms initiated by the plant then contributes to shaping the assemblage of, and interactions between, the other organisms such the herbivores, phytopathogens and the natural enemies of herbivores within a community (Dicke and Baldwin, 2010; Hare, 2011; Ponzio et al., 2013).

1.2. Plant attack by insect herbivores

Terrestrial plants are a food source for many insect species from diverse taxonomic groups. Insects use various feeding strategies such as chewing, sap sucking, seed predation, gall inducing and leaf mining to obtain nutrients from plants. Insect infestation usually causes mechanical damage to the plant. However, the quantity and severity of plant injury varies greatly depending on the insect feeding tactics (Howe and Jander, 2008). The piercing-sucking herbivores such as aphids form intimate and long-lasting association with their hosts, whereas chewing insect such as leaf-eating beetles or caterpillars are usually more mobile. Plants recognise and respond to insect herbivores based on the feeding style while insects interact with the plant depending on their dietary specialisation(Mello and Silva-Filho, 2002). In each of these plant-insect relationships, both partners send and receive chemical cues that determine the outcome of the interaction. Insects locate suitable plants as a food source through their mouthparts, antennae and tarsi, while plants recognise and mediate responses to insect movement and infestation by activating different metabolic pathways which considerably alter their chemical and physical aspects (Mello and Silva-Filho, 2002; Mithöfer and Boland, 2012).

As insect herbivores encounter variation in traits, plant quality and defensive compounds during their foraging activities, they have to make a decision either to accept or reject a plant as a food source and oviposition site. Since this insect foraging decision is primarily governed by secondary plant metabolites, such as deterrent and attractant allelochemicals (Schoonhoven et al., 2005; Becerra, 2007), they may depend on their sensory cues (olfactory, contact chemoreceptory and visual cues) to locate, identify and assess the suitable food and oviposition sites (Heisswolf et al., 2007; Stenberg and Ericson, 2007; Kuhnle and Muller, 2011; Anderson and Anton, 2014). Olfactory and visual cues are used by insects to locate and identify potential host plants (Stenberg and Ericson, 2007; Anderson and Anton, 2014), but the decision to either accept or reject the plant as a source of food or as a habitat for their offspring depends on the contact cues, which are often plant-surface compounds and texture (Schoonhoven et al., 1998). Unlike visual and contact chemoreception, olfactory cues are considered helpful to plant-feeding insects in either recognising or rejecting the host plants from a distance (Stout, 2007; Ballhorn et al., 2013). Based on the sensory cues of a plant-feeding insect, judgment on the appropriateness of a host plant is essential for the success of performance of the insect progeny.

Plant quality is a major guiding criterion when being chosen as food by an insect. Generally, plants rich in nitrogen are more attractive since these chemical compounds are of vital importance for insect growth (Coley et al., 2006). On the other hand, plants rely heavily on chemical defences and alter their phenotype through changes in the production of primary and secondary metabolites, morphological traits and architecture to protect themselves from attack by insect herbivores (Dicke and Baldwin, 2010; Hilker and Meiners, 2010). Plant compounds that exert repellent, anti-nutritive, or toxic effects directly on insect herbivores attack by suppressing the growth of the attackers and influencing their behaviour as well as changing the suitability of host plant to the other herbivores. Plant chemical composition (e.g. terpenoids, alkaloids, anthocyanins, phenols, quinones, flavones, and isoflavones) are compounds that are naturally produced in the plant and do not affect their normal growth and development, but may cause multiple challenges for the feeding insects (Lattanzio et al., 2008; Mierziak et al., 2014). These compounds are stored in the plant tissue and function as biochemical defences through their toxicity when digested by the insect, which allows plants to react immediately with the means to kill or to reduce damage caused by the intruder (Howe and Jander, 2008; Mithöfer and Boland, 2012). They act together as a toxin in a synergistic manner, affecting the physiological processes of their antagonist by inhibiting the intruder’s feeding and colonisation, reducing digestion or reproduction and disrupting metabolism, which will indirectly affect insect growth and performance (Després et al., 2007). As an example, it has been previously shown that leaves with condensed tannin concentration are negatively correlated with the abundance and species richness of herbivores in the community structure (Forkner et al., 2004). Tannin in plant tissues consumed by insects may decrease protein digestion in the insect gut (Barbehenn and Peter

Constabel, 2011). As a result, the fecundity, growth rate and longevity of aphids fed on plants containing tannin were reduced (Grayer et al., 1992), which then resulted in the disturbance in development of its population (Sylwia et al., 2006). Other alkaloids that may illicit harmful effects are glucosinates and hydroxamic acids. These compounds can decreased the fecundity of cabbage aphids *Brevicoryne brassicae* fed on the Brassica species (Hopkins et al., 1998) and reduce the population growth of *Rhopalosiphum padi* on wheat (Givovich et al., 1994).

Plants may repel insect herbivores by producing a chemical odour that make them less attractive (Mitchell et al., 2016). This odour acts as an insect repellent which affects the foraging behaviour of insect herbivores towards their potential host plants. As an example, tobacco plants release several volatile compounds to repel female moths *Heliothis virescens* (De Moraes et al., 2001), while Arabidopsis plants released the glucosinolates compound which make them less attractive to their herbivores (Kliebenstein et al., 2001). The emission of chemical volatile compounds by the plants can be a double-edged sword. Plants can repel a wide range of potential herbivores due to the nature of toxic compounds released in the air, but may also have an ability to produce cues to engage in 'biological warfare' by attracting the insects that prey on or parasitize the herbivores (Dicke and Baldwin, 2010; Clavijo McCormick et al., 2012; Reddy, 2012). Volatile cues emitted by plants may attract the enemies of herbivores such as parasitic wasps, predatory insects and possibly even insectivorous birds (Pichersky and Gershenzon, 2002; Unsicker et al., 2009). These cues are used by those enemies to locate the habitat of their prey, provide information about the activity and perhaps even the developmental stage of the attacking herbivore (Baldwin, 2010). The chemical properties in that chemical cue, such as green leaf volatiles (GLVs) and terpenoids were found to be important in the host location of parasitic wasps (Wei et al., 2007). As an example, tomato plants infected by *Myzus persicae* emitted volatiles that could attract the natural enemies of both aphids and whiteflies (Tan and Liu, 2014).

Plant physical barriers are considered as the first line of defence and important barriers that should be circumvented by insect herbivores before invading the plant (Mitchell et al., 2016). These defence mechanisms are mediated by the plants morphological traits such as the cell wall, leaf toughness and leaf structure (War et al., 2012). The morphological traits such as leaf surface wax, thorns or trichome may interfere with the feeding behaviour of the insects by slowing the insect movement (Steinite and Ievinsh, 2016) or preventing insect pests from attaching to the plant (White and Eigenbrode, 2000). As an example, leaf surface characteristics such as trichomes have deterrent effects on egg deposition by two-spot spider mites *Tetranychus uticae* on *Rubus idaeus* (Karley et al., 2016) and by *Plutella xylostella* on *Arabidopsis thaliana*. Physical plant surface properties may also affect herbivore performance, particularly in endophytic species that have to penetrate into plant tissue at the beginning and at the end of their development (Velten et al., 2008). The toughness of the leaf surface potentially contributes to protecting the leaves from damage by sap sucking feeders such as aphids, thus penetration by the aphids stylet to reach the phloem for sucking requires strong forces (Will and Vilcinskas, 2015).

As plants are ready for attack with a series of internal and external defences, it is crucial for insect herbivores to develop strategies to overcome these defences. The ability of insect herbivores to recognise, avoid or detoxify harmful substances will help them to successfully encounter the defences mechanisms of their host (Furstenberg-Hagg et al., 2013). If given a choice, insect herbivores will avoid plants that may contain toxins, which affect their fitness and performance (Ibanez et al., 2012). Insects may change their host plant to avoid lethal doses of plant defence compounds or simply feed on the non-toxic plant organs where toxins are absent (Pentzold et al., 2014). In some situations where the ability to choose a suitable host plant is not an option, insect herbivores need to build adaptations to tolerate the plant defence mechanisms (Gatehouse, 2002). Most insects have developed strategies, such as biochemical and molecular adaptations to overcome plant barriers and chemical defences. As an example, insects may detoxify compounds by producing powerful enzymes to neutralize the compound (Glauser et al., 2011; Maag et al., 2014), sequester the toxins and use the compound for their defence against natural enemies (Mello and Silva-Filho, 2002; Nishida, 2002), or alter the gene expression pattern to enhance their tolerance mechanisms (Tiffin, 2000; Stinchcombe, 2002). Insects have an ability to possess a powerful assemblage of enzymes to overcome plant chemical defences by oxidation, reduction, hydrolysis or conjugation of molecules (Scott and Wen, 2001). The production of detoxification enzymes produced by the insect such as esterases, glutathione-*S*-transferases, and cytochrome P450 monooxidases (Li et al., 2007; Tiwari et al., 2011) are used to neutralize plant toxins. As an example, *Papilio polyxenes* has an adaptation to feed on plants with toxic compounds through a diversification of cytochrome P450s (Scott and Wen, 2001). Another example is the enzyme in the saliva of *Helicoverpa zea* which contains glucose oxidase and has the ability to inhibit the signalling pathway in plants which then will prevent the induction of nicotine (Tian et al., 2012; Wang et al., 2017).

Another adaptation strategy adopted by insects includes the evolution of their physiological, morphological, and behavioural adaptations (Harris et al., 2003; Zhu-Salzman and Zeng, 2008). These strategies can be genetically determined, inherited, or learned by the insect and their progeny (Hawthorne, 2001; Després et al., 2007). Insects may increase their consumption rate, modify the nutritive quality of host plant tissues or establish associations with symbiont microorganisms (Mello and Silva-Filho, 2002). The way they adjust their food quality intake and assimilation rate assures the maintenance of their growth and development. As an example, *Helicoverpa armigera, Agrotis ipsilon* and *Helicoverpa zea* larvae are able to overcome the effect of various host plant PIs (proteinase inhibitor) by altering its midgut composition after PI ingestion (Mazumdar-Leighton and Broadway, 2001; Patankar et al., 2001). In addition, production of PI-digesting proteinases allows the insect to overcome the plant defences and also to use the digested inhibitor as a source of amino acids (Girard et al., 1998). Insects may also sequester the toxic phytocompounds that they get from the plant for their own benefit such as to use against their own predators (Müller et al., 2001; Opitz and Müller, 2009; Petschenka and Agrawal, 2016). As an example, the leaf beetles, such as the broad-shoulder leaf beetle *Chrysomela populi* and brassy willow beetle *Phratora vitellinae* sequester the salicinoid salicin from willow *Salix* spp. and channel it for their own defensive system (Kuhn et al., 2004; Opitz and Müller, 2009).

1.3. Plant attacks by pathogens

Apart from attacks by herbivorous insects, plants are often confronted with a variety of diseases caused by microbial pathogens, and their negative impacts on plants are equally well known (Agrios, 2005). With environmental changes such as global warming likely to make plants more vulnerable to disease infection, this will then reduce the crop quality and crop yield (Lychuk et al., 2015). The regional alterations in crop areas, type of crops, cultivation systems, distribution and abundance of vectors, and weed or cultivated reservoir hosts was also influenced the abundance and spread of plant diseases (Jones, 2016).

Physical barriers are the first defence mechanisms adopted by plants to inhibit pathogen infection (Gohre and Robatzek, 2008). In the same way as they respond to attacks by insect herbivores, plants also have an ability to recognise the pathogens that may cause them harm. Once infected, plants may activate their emergency strategies by triggering an increase or a decrease in synthesis or degradation of hormones in the plant systems (Overmyer et al., 2003; De Wit, 2007). This plant reaction may cause a variety of symptoms, such as the formation of adventitious roots, gall development, and epinasty (Ravichandra, 2013).

However, plant pathogens have evolved to enable them to invade plants, neutralise plant defence reactions, and colonise plant tissue to obtain plant nutrients for their growth, survival and reproduction (Hématy et al., 2009; Dodds and Rathjen, 2010; Wirthmueller et al., 2013). Generally, plant pathogens can penetrate into the plant interior, either by accessing the leaf or root surface directly or through wounds and placement by specific feeding insects or via a natural opening such as the stomata, hydathodes or lenticels (Shepherd and Wagner, 2012). In some cases, plant pathogens can also be transmitted by the host plant to their next generation through the seeds (Iwai et al., 2002; Sowley et al., 2010).

Plant pathogens can be divided into two categories based on their mode of attack: biotrophs and necrotrophs. Biotropic pathogens have an intricate biological interaction and need living tissue for their growth, killing the host tissue in the late stages of the infection (Mendgen and Hahn, 2002). Infection by biotropic pathogens affects the photosynthesis process, changes the translocation of nutrients and causes a net influx of nutrient into infected tissue. In contrast, the necrotrophic pathogens are considered to not have a real interaction with their host but often secrete enzymes and toxins to kill the host tissue at the beginning of the infection and feed on the plant's dead tissue (Laluk and Mengiste, 2010).

1.4. Consequences for plant fitness and conflict of plant defence

The interaction between plants and their antagonists may alter the quality and performance of plants (Inbar et al., 2001), which will usually end up with damage to the plant body and may kill it. Although the symptom of damage varies depending on the type of attacker; either necrotrophic or a biotrophic pathogen; or either chewing or sap sucking insects, the attack usually disturbs the plants physiological and biochemical processes resulting in abnormal structure, growth or function. Heavy infestation by insect pests or infection by plant pathogens will affect the plants respiration and transpiration process, disrupt translocation of nutrients, influencing the growth and development of the plants and may decrease the photosynthetic rate by defoliation and damaging chloroplasts in the plant cells (Clarkson, 1985; Lucas, 1998; Hématy et al., 2009).

The damage on the plant body caused by pathogen infection may also affect the water relations in plants, with the symptoms depending on the area infected. The symptoms of pathogen infection starts when the pathogen stunts the transpiration rate, at which point the plant will wilt rapidly (Ravichandra, 2013). Pathogens that infect the root system may influence the abilities of plants to absorb water, thus producing secondary symptoms such as wilting and defoliation of the plant's leaves (Okubara and Paulitz, 2005; Aguadé et al., 2015). Infection by pathogens in the plant vascular system will block the xylem vessels, thus, affecting the water movement in plants (Yadeta and Thomma, 2013). As a consequence, the nutrient source-sink pattern in plant systems will change and affect the plant growth and development (Mooney et al., 2012). On the other hand, the symptom of damage caused by the insect infestation varies depending on the insect feeding behaviour. Damage from insects with chewing mouthparts such as caterpillars and grasshoppers typically appear on leaves or stems as ragged edges, holes, or other missing tissue. Meanwhile the sap-sucking insects such as aphids, often cause yellowing or browning on plants, and possible wilting (Zvereva et al., 2010). Similar to the effect caused by the pathogen infection, heavy infestation by the insect pest will reduce quality, growth and reproduction of plants.

Plants are sessile, but rather than acting as passive victims towards attacks by their antagonists, plants employ induced defence mechanisms to coordinate the appropriate response in order to limit any possible damage (Rausher, 2001; Gatehouse, 2002). Although plants are already equipped with physical defences, it is more important for the plants to initiate defence strategies upon damage by their attackers (Karban and Baldwin, 1997). This secondary defence mechanism is considered as an alteration in a set of traits that play a major role in reducing the negative effect on plant fitness. Their effect includes conferring disease resistance against plant pathogens (Maleck and Dietrich, 1999), increased toxicity to disturb insect ingestion, delay of larval development, or increased attack by the insect herbivores natural enemies (Baldwin and Preston, 1999). These mechanisms allow plants to be phenotypically plastic depending on the type of attackers and the stage of damage (Dicke et al., 2003).

However, plant defence mechanisms are diverse in nature and often complicated by an extreme variability in biotic and abiotic factors such as plant ontogeny, genetic variation, plant age, health status of the plant and the environment surroundings (Dicke et al., 2003; Fujita et al., 2006; Kempel et al., 2011; Atkinson and Urwin, 2012; Rejeb et al., 2014; Dicke, 2016). The variability of the plants health may influence the type and strength of the defence mechanism in the plant. Thus, the quantity and quality of defence pathways induced by healthy and diseased plants towards the secondary attack by insect herbivores might be different. As an example, the composition of the released induced volatile is clearly dependent on the type of plant attacker (Leitner et al., 2005; Van Der Ent and Pieterse, 2012) such as between a leaf chewer and sap sucking insect (Leitner et al., 2005; Gosset et al., 2009; Duceppe et al., 2012) or between a biotrophic and necrotrophic pathogen (Glazebrook, 2005; Kliebenstein and Rowe, 2008; Al-Naemi and Hatcher, 2013). Chewers and necrotrophic pathogens often induce the jasmonic acid (JA) dependent defence pathway, while sap sucking insects and biotrophic pathogens induce the salicylic acid (SA) dependent pathways (Shoji et al., 2008; Spoel and Dong, 2008; Walling, 2008; Ballaré, 2011; Robert-Seilaniantz et al., 2011). As both of these defence pathways can crosstalk, the infection by pathogens can interfere in plant defence response against insect herbivores, and vice versa (Ponzio et al., 2013). As a result, this may influence the effectiveness of defence strategies. To make it more complicated, plants also induce different volatiles for different life stages and genders of the insect herbivores that attack the plants (Hern and Dorn, 2001; Gouinguené et al., 2003; Williams et al., 2005). The variability in mode of action applied by the plants to combat their intruders shows the complexity in plant defence systems that may affect the interactions between insects, plants and other organisms.

1.5 Effect of plant-pathogen-insect interactions on multitrophic levels

The interaction between plants, insect herbivores and the natural enemies of insect herbivores constitutes a tritrophic interaction, where the plant quality and derived compounds affect directly or indirectly an organism at the third trophic level. However, it is very important to consider the other factors that may influence the interaction between plants and those insects, such as the intervention by a third organism such as a microbial pathogen. As previously discussed, the infection by a pathogenic microorganism may alter the quality of the host plant (Hatcher, 1995; Hatcher et al., 1995), which may then indirectly affect the interaction between organisms in the food webs (Biere et al., 2002; Stout et al., 2006; Biere and Bennett, 2013). Plant phenotypic changes following pathogen infection may lead to an increase in non-digestible matter and the induction of the host plant’s resistance pathways. This leads to increased levels of secondary compounds and changes in water, carbohydrate and nitrogen content that can affect phytophagous insects sharing the same host plant (Biere et al., 2002; Cardoza and Tumlinson, 2006). Variation in resource quality may then affect species interactions by limiting energy transfer from the plants to their consumers, which may then affect the life history and behaviour of interacting species.

Host plant traits and quality are very important for many aspects of an herbivorous insect’s life history, such as growth, fitness and reproduction (Awmack and Leather, 2002). Changes in plant quality caused by pathogen infection indirectly change the herbivores performance. Pathogen infection can change the population growth rate of herbivore populations and can affect the initial population growth after colonization and the carrying capacity of the plant (Pruter and Zebitz, 1991). For example, the equilibrium density, reproduction and mortality of aphids *A. fabae* was reduced on rust-infected faba beans (Pruter and Zebitz, 1991). In contrast, the aphid *Euceraphis betulae* had a higher population growth rate on pathogen-infected branches of the *Betula pendula* (silver birch), possibly because of the higher concentrations of free amino acids (Johnson et al., 2003). The effect of pathogens on herbivore population dynamics may also vary within the infected plant, as shown by the mite *Tetranychus urticae* on tobacco plants infected by the tobacco necrosis virus. While population dynamics were not affected by systemic infection, the total number of mite offspring was reduced by more than 35% on the leaf part that received the inoculation (Apriyanto and Potter, 1990). Variation in plant traits and quality not only influenced the growth and performance of the current generation of insect herbivores, but also the next generation (Underwood, 2009). The maternal effect of a mother's phenotype or environment may affect their offspring's phenotype (Bonduriansky and Head, 2007), which then has the potential to influence the population dynamics of their future generations (Benton et al., 2005).

Infection by a plant pathogen may alter the emission of volatile molecules from the plant tissue (Holopainen and Gershenzon, 2010). As these olfactory cues have been recognised as an important component in the interaction between plants and insects, the changes in the composition of volatiles may influence the foraging behaviour of their insect herbivores (Dicke, 2000). As an example, beet armyworms *Spodoptera exigua* oviposited more on white mould-infected plants than on healthy plants. This insect preference is mediated by plant volatiles and other biochemical changes in plant chemistry caused by the fungal infection (Cardoza et al., 2003). In summary, these studies demonstrate that the effect of pathogen infection on both the preference and performance of the herbivore may strongly affect their population dynamics.

Host plant quality affects parasitism of insect herbivores indirectly through their effect on host accessibility or quality (Ode, 2006) and by introducing a modification in the traits, such as behaviour, morphology and/or life history (Harvey et al., 2003). The trait mediated indirect interactions will occur when a species affects a trait of another species, and the latter species affects a third species which then will cause the cascading effect on the interacted organism (Werner and Peacor, 2003). In this case, the variation in host quality altered by the pathogen infection indirectly affects the population dynamics of the insect herbivores' natural enemies (van Nouhuys and Laine, 2008). Any change in the interaction between the insect herbivore and its natural enemies is as a consequence of the alteration of the quality of the substrate that the insect herbivore is feeding on (Bottrell et al., 1998; Price et al., 2004; Ode, 2006). For instance, plant defensive chemistry and nutrient availability influences the rate of parasitism, as well as offspring size, rate of development and survival of the parasitoid (Barbosa et al., 1991; Turlings and Benrey, 1998; Rodriguez-Saona et al., 2005; Harvey et al., 2005, 2007). Furthermore, because resource needs and fitness values of male and female offspring differ (Charnov et al., 1981; Hardy et al., 1998) and adult female parasitoids control the sex of their progeny, host plant quality can affect parasitoid sex ratio (Fox et al., 1990). Parasitoids frequently alter sex allocation to reflect perceived differences in host quality by placing male eggs in relatively poorer quality hosts (Morris and Fellowes, 2002).

As host quality is the key determinant for the fitness of developing offspring of the parasitoid, natural selection is expected to lead the female parasitoid to choose the highest quality host to oviposit her eggs (Fellowes et al., 2005). Some parasitoids may depend on a variety of cues produced by both the plant and insects to locate and find their prey. A complex mixture of volatiles which act as an attractant and/or an arrestant influence the foraging behaviour of insect predators to fly toward a food source and to oviposit. These cues also help insect predators to choose a good quality food source before attacking their prey. Parasitoids are often specialised on one or a few host species and host stages. Successful host selection depends upon the execution of appropriate behavioural responses to an array of cues which are available during the parasitoid foraging process. Therefore, they have specialized sensory nervous systems that help them in using a variety of chemical and physical cues during the habitat location, host location, and host examination phases of host selection (Turlings and Wäckers, 2004; Schnee et al., 2006; D'Alessandro et al., 2014). Physical cues such as colour, sound, shape, movement and size of the prey influence parasitoid host choice (Demas et al., 2002; Kroder et al., 2007).

Several morphological and physiological factors can influence prey acceptance by predators. One underlying physical factor is prey size. As an example, different sizes of caterpillars were attacked with the same frequency by reduviid *Zelus longipes*, but successful attacks were more frequent in small larvae (Cogni et al., 2002). The colour polymorphism of prey may also influence the preference of visual foraging predators (Harmon et al., 1998). As an example, *the Coccinella septumpunctata* L. attacks red aphids more frequently than green ones. The behavioural, morphological, and weapon (chemical) of prey defence strategies may also determine whether a predator accepts or rejects the prey. Some prey such as aphids will kick, run, drop, fly away, or exude noxious chemicals when predators approach them. Sometimes, the prey uses a chemical as a weapon to induce the predators to vomit (Vencl, 2006).

One important element in the interaction between the pathogen infection and the insect infestation on plant is the possibility of hormone crosstalk. The production of hormones by the plant in response to pathogen infection may interfere with the production of hormones in response to herbivore infestation. For example, *Botrytis* infection induces the jasmonic acid pathway (JA) and ethylene (ET), while aphid infestation will induce salicylic acid defence pathways (SA). Both SA and JA-dependent pathways do not exist in isolation; rather there is cross-talk between them, with activation of the SA-dependent pathway often leading to a down-regulation of the JA-dependent pathway and vice versa (Thaler et al., 2002a, 2002b). There is evidence for antagonism between SA- dependent resistance to microbial pathogens and JA-dependent resistance to herbivorous insects; once a plant is conditioned to express resistance against microbial pathogens it may become more susceptible to attack by herbivores, and the reverse is also found (Pieterse et al., 2001). As a consequence, hormone crosstalk may disturb the olfactory cues used by the natural enemies to locate the insect herbivores.

1.6 Pathogen attack is not always obvious: How the pathogens lifestyle may affect organisms at a higher trophic level

There are cases where the infection by the pathogen is symptomless. In this case, the pathogen invades the plant, disturbs the plant’s physiology and chemistry, but the symptoms of pathogen infection are hidden. As an example, many leaf pathogens that infect the host just as the young leaves emerge from the bud do not form lesions until months later (Carrol, 1988). Recently, research carried out by Sowley et al. (2010) provided evidence that microbial pathogens may exist in the plant body as a persistent, symptomless and systemic infection, where the pathogen originates from the seed, infected during the flowering stage. Further research in this area has shown that asymptomatic infections are not rare. As an example, *Botrytis cinerea,* the necrotrophic pathogen that infects many plants, is capable of colonizing its host internally without causing any disease or stress symptoms (van Kan et al., 2014; Shaw et al., 2016). As a consequence, it has changed the traditional view of plant pathologists in studying, detecting and evaluating the damage caused by plant pathogen. As plant quality influences their insect herbivores performance and behaviour, this recent finding also highlights a gap in the entomological research.

Several studies have suggested that plant pathogen infection may alter the interaction between herbivorous insects and their natural enemies (Biere et al., 2002; Cardoza et al., 2003) by influencing or changing the production of volatile compounds within the host plants (Cardoza et al., 2003), which may in turn have a direct or indirect effect on the attraction of parasitoids (Turlings and Wäckers, 2004). The current view of plant defence mechanisms recognises that plants utilize multiple primary and secondary metabolites, as well as morphological and physiological traits to face the attack of their enemies and to recruit the natural enemies of the attackers (Stout et al., 2006).

However, to date, no research has been done to investigate the effect of hidden plant pathogen infections on insect herbivore performance, fitness and preference, and its consequent effects on higher trophic levels. Detailed research should be carried out to investigate the effect of the second attacker (insect herbivores) on the asymptomatic colonization of plant pathogens and whether they will build the antagonism or mutualism interaction. The antagonism interaction might appear if the plant releases volatile organic compounds which can interfere in a plant's defence response to herbivory and vice versa (Ponzio et al., 2013).

We carried out this study to investigate the effect of hidden pathogen infections on plants and their associated insect ecosystem. We predict that the asymptomatic pathogen infection may still interfere with the host plant physiology, which may have nutritional implications for the plant. This then may give a consequential effect on the organisms at a higher trophic level such as the insect herbivores and their natural enemies. We addressed this in both laboratory and field studies, using two varieties of the crop plant lettuce (*Lactuca sativa* L; family: Asteraceae) (Little Gem and Tom Thumb) as our host plants. The lettuces were infected with the plant pathogen *B. cinerea* in order to investigate the influence of plant traits and quality on the life history and behaviour of herbivorous insects. The lettuce plant was used in this study because *B. cinerea*, the cause of grey mould disease, is often present in lettuce plants as a symptomless, systemic, endophytic, infection which may arise from the seed. In this study, the effect of symptomatic and asymptomatic plant pathogen infection was considered. In Chapter 2, 3, 5 and 6, the green peach aphid *Myzius persicae* was the herbivorous insect used in this study and the parasitoid *Aphidius colemanii* (Chapter 2 and 3) was used to see the indirect effect of the plant pathogen at higher trophic levels.

1.7. Study system

1.7.1. Lettuce

Lettuce (*Lactuca sativa*) (Family: Asteraceae) is an annual herbaceous plant and is often grown as a vegetable crop. It is believed that lettuce is one of the oldest domesticated crops that was cultivated at the time of the Egyptian Pharaohs (Hancock, 2012). During that time, lettuce was used in cooking or as an ingredient in the preparation of traditional and herbal medicines (Watts, 2007; Duke, 2009). Lettuce was also planted for oil and to be used in religious ceremonies (Hart, 2005). This plant species was first described by Carl Linnaeus in the second volume of *Species Plantarum* published in 1753 (National History Museum, 2003). Since then, lettuce has been catalogued into many taxonomic groups, subspecies and varieties, based on their morphological characteristics and genetic variation (Křístková et al., 2008). Today, lettuce has become one of the most important leafy crops, ranking second after potatoes in terms of annual consumption per capita (AGMRC, 2017). In 2013, the worldwide production of lettuce exceeded 24.9 million tons and in 2015, the annual consumption of all types of lettuce in the USA alone was recorded as £24.50 per person (AGMRC, 2017).

Lettuce plants grow best in nitrogen-rich, loose soils that have a pH ranging between 6.0 and 7.0, with the optimum growth temperature ranging from 16 to 18 °C. Several types of lettuce are grown worldwide, but the four most common types are romaine, butterhead, iceberg and loose-leaf lettuce. These lettuces have a wide range of shapes and textures. As an example, romaine lettuce is a tall head type with oblong rigid, spoon shaped leaves and thick midribs running almost to the apex while butterhead have soft, tender and rounded-leaves. Generally, lettuce can grow up to 100 cm tall, forming a dense rosette or a head, before bolting and branching in the upper part when mature. Lettuce generally lives between 65–130 days from planting to harvesting, depending on the variety and time of planting. If the temperature is too high, lettuce will bolt and produce flowers.

As with many other plants, lettuce suffers from attacks by many species of insect pests and disease. Among the most serious lettuce diseases are lettuce mosaic virus (LMV)(Dinant and Lot, 1992), lettuce downy mildew caused by *Bremia lactucae* (Michelmore et al., 2009), lettuce drop caused by *Sclerotinia spp*. (Melzer and Boland, 1994) and grey mould caused by *B. cinerea* (Sowley, 2006). Infection by a fungal disease usually causes lettuce leaves to mould, rot and finally die. In some conditions, the infection by pathogenic fungi, such as *B. cinerea*, on lettuce is symptomless (Sowley et al., 2010). However, infection by this pathogen still affects the plant by reducing its growth and disturbing the physiology of the plant body (van Kan et al., 2014; Shaw et al., 2016). Lettuce are also attacked by insect pests such as cutworms, which cut seedlings off at the soil line (Theunissen and Fransen, 1984); nematodes which cause yellow, stunted plants (Zhang et al., 2010); and aphids such as the green peach aphid (*Myzus persicae*), lettuce aphid (*Nasonovia ribisnigri*) and lettuce root aphid (*Pemphigus bursarius*) which cause yellow and distorted leaves (Lebeda et al., 2014). The green peach aphid, possibly the most important leaf-feeding pest on lettuce because of their ability to transmit several important viruses, may stunt plant growth and deform leaves (e.g: Akhtar et al., 2010; Krenz et al., 2015).

1.7.2. *Botrytis cinerea*

Grey mould disease caused by *B. cinerea* has been considered as a minor disease in field-grown lettuce (Matheron and Porchas, 2008). However, reported incidences of this disease on lettuce have recently increased significantly (Matheron and Porchas, 2008). This pathogen causes damping-off in seedlings and rotting in mature plants (Pink and Keane, 1993). Recent studies found that this pathogen is often present in symptomless lettuce plants as a systemic, endophytic infection which may arise from seed (Sowley et al., 2010). As plants grow, the infection spreads from the roots to the stems and leaves (Sowley et al., 2010). Frequent systemic and asymptomatic infection by *B. cinerea* was also observed in cultivated primula and cyclamen (Barnes, 2002; Barnes and Shaw, 2002, 2003), and soft fruit such as strawberries (Helbig, 2001; Xiao et al., 2001; Donmez et al., 2011) and grapes (Gabler et al., 2003; Blanco-Ulate et al., 2013; Lorenzini et al., 2013).

Botrytis cinerea is ubiquitous in nature and can cause serious losses and disastrous economic impact on many other crop species worldwide. This airborne necrotropic fungi attacks over 230 pre- and post-harvest crop hosts worldwide (Zhao et al., 2010). Infection by this pathogen leads to considerable yield and quality loses which then will either reduce marketability of the harvested product or render them unmarketable. This pathogen is responsible for an annual loss of approximately 20% from vineyards in France, and costs to control this pathogen were approximately US$780 million in 2002 (Genescope, 2002), indirectly affecting the price and availability of food.

This polyphagous pathogen is most destructive on mature, senescent and/or dead tissues of dicotyledonous hosts (Elad et al., 2007). Seedlings attacked by *B. cinerea* may collapse from the water soaking of parenchyma tissue on the stem. *Botrytis cinerea* infects the tissues directly to enable rapid colonization and derive nutrients from host cells, resulting in serious damage which could lead to tissue death in plants (van Kan, 2006). Pathogenicity factors and phytotoxic excretions from the fungus act synergistically to kill, degrade and macerate host cells at very early stages of infection (Govrin and Levine, 2000; Bolton et al., 2006; Mengiste, 2012). The typical grey mould soon develops in the decayed tissue. High humidity in the environment will cause damping-off to the seedlings if the seeds are contaminated with sclerotia or mycelium of *Botrytis*, or if *Botrytis* is present in the soil, and living in marcescent vegetable tissues (Fiume and Fiume, 2005). Fungal hyphae actively penetrate the cell and enzymatically digest the leaf cells and alter the leaf integrity and physiology (Volpin, 1991; Jansen et al., 2009).

Botrytis cinerea conidia can be transported by wind or insects over long distances before infecting the next host (Jarvis, 1977). The *B. cinerea* infection begins with a conidium landing on the host plant surface. Germination of the *B. cinerea* spore occurs under moist conditions, resulting in the production of a germ tube that develops into an appressorium which penetrates the host tissue. *Botrytis cinerea* appressoria presumably secretes the enzymes cutinase and lipase to breach the plant surface. The underlying cells are killed and the fungus establishes a primary lesion, in which necrosis and host defence responses may occur (van Kan, 2006). *Botrytis cinerea* is difficult to control because it uses multiple strategies and modes of attack to subdue its host, has diverse hosts as inoculum sources, and can survive as mycelia and/or conidia, or for extended periods as sclerotia in crop debris (Ferreira et al., 2006). For these reasons, the use of any single control measure is unlikely to succeed and a more detailed understanding of the host–pathogen interaction, the micro environment in which the fungus operates and its microbial competitors on the host is essential.

In some cases, the infection by *B. cinerea* may remain hidden for an undefined length, in which fungal outgrowth is restricted with no symptoms appearing on the plant tissues (Prusky, 1996; van Kan et al., 2014) until the physical and chemical environment such as humidity, temperature, light and the availability of nutrients and water are at optimum levels for the pathogen to start to take action. The hidden infections are because the plant defence mechanisms are more active compared to the mechanisms of the pathogen's infection (Holz et al., 2007). When the environmental conditions are appropriate, the defence barriers are breached and the fungus starts a vigorous outgrowth to decompose plant biomass and convert it into a fungal mass. This results in a rapid maceration of plant tissue, on which the fungus eventually sporulates to produce inoculum for the next infection (Elad et al., 2007). Under optimal conditions, an infection cycle may be completed in as little as three to four days, depending on the type of host tissue attacked (Elad et al., 2007).

In fruit plants, *B. cinerea* predominantly infects the flowers and resides quiescent in the developing fruit tissue, often for several weeks. Fungal growth resumes at the onset of fruit ripening. It has been postulated that high levels of fungitoxic or fungistatic compounds in immature fruits contribute to grey mould quiescence. The level of these compounds decreases during the ripening process concomitant with fungal outgrowth. Since *B. cinerea* is capable of colonizing the plant internally without causing any disease or stress symptoms for an indefinite duration (Elad et al., 2007), the latent infection can occur at any stage of the plant life and at any stage of pathogen growth (Jarvis, 1977; Prusky, 1996) without giving any gross damage to the host. To an extent, the latent infection of *B. cinerea* will interfere with the plant's secondary metabolites, which may have nutritional implications for the plants (Shafia, 2009) as well as a consequential effect on the organisms at higher trophic levels. Even the inoculum of the *Botrytis* is thought to be spread by wind or splash dispersal of conidia into above-ground plant parts (Elad et al., 2007), the seedborne *Botrytis* also may be important. Seed-borne infection of *B. cinerea* has been isolated in many hosts, including flower, forage and vegetable species (Sowley et al., 2010).

1.7.3 Aphids

One of the notable insect pests of lettuce are aphids (Homoptera: Aphididae). Worldwide, approximately, 250 of 4000 aphid species described has been listed as a pest (Sadeghi et al., 2009), and 21 of those have infested lettuce (Blackman and Eastop, 2000). Among those, the green peach aphid, *Myzus persicae* Sulzer (1776) is the most economically important aphid on lettuce and other crops worldwide (van Emden and Harrington, 2007). There are a number of factors that have enhanced the status of this species as a pest, including its distribution, host range, mechanisms of plant damage, life cycle, capacity to disperse and ability to evolve resistance to insecticides. *Myzus persicae* is both an extremely cosmopolitan species with a worldwide distribution (see www.cabi.org for its current distribution) and highly polyphagous, with a host range of more than 400 species in 40 different plant families, including many economically important crop plants (Blackman and Eastop, 2000). This aphid species not only damages the plant through their sap sucking behaviour (Field et al., 2017), but it also is the most important aphid virus vector. It has been shown to transmit well over 100 plant virus diseases, in about 30 different families, transmitting to many major crops (Minorsky, 2005). On lettuce, the aphid *M. persicae* was identified as a vector for the lettuce mosaic virus (Nebreda et al., 2004). Serious attack by *M. persicae* may weaken the plant and cause a metabolic imbalance, twisting of the leaves and, in extreme cases, leaf loss. They also introduce toxins into the plant, systemically altering its development.

Aphids generally are considered to be the most important threat to crop production in temperate regions (Holman, 2009) and cause significant losses to the agricultural industry worldwide. As an example, losses to the soybean aphid *Aphis glycines* in the north-central region of the United States and southern Canada, if left untreated can reduce yield values by $2.4 billion annually (Tilmon et al., 2011). Aphids also have made themselves an enemy of farmers and gardeners by damaging crops through transmitting hundreds of pathogens, mostly viruses (e.g: Ng et al., 2000; Davis et al., 2005; Hodge and Powell, 2010; Finlay and Luck, 2011; Hodge et al., 2011), covering plants with honeydew (e.g: Fischer and Shingleton, 2001; Hogervorst et al., 2008; Leroy et al., 2011; Katayama et al., 2013) and altering normal plant physiology (e.g: Tjallingii, 2006; Giordanengo et al., 2010; Babikova et al., 2014; Züst and Agrawal, 2016) which then renders them unmarketable or unprofitable to grow. Aphids can transmit dozens of viruses from a diseased plant, with winged morphs dispersing viruses over a greater distance. Even if a virus fails to kill a plant, it may damage it enough to reduce its economic value (Hooks and Fereres, 2006).

Aphids also produce honeydew, a sugary excretion of carbohydrates, amino acids and water derived from plant phloem (Yao and Akimoto, 2002; Pringle et al., 2014). Ants are attracted to honeydew as a predictable, renewable food resource and consequently ‘tend’ honeydew-producing aphids, protecting the aphids from predators and parasitoids (Sharma and Sundararaj, 2011; Helms, 2013). The excretion of honeydew by aphids not only suppresses induced plant defences (Schwartzberg and Tumlinson, 2014), but also allows the growth of sooty moulds (Chomnunti et al., 2014; Schmidt et al., 2014). The deposition of this sooty mould on the foliage forms a barrier on the leaf, reducing photosynthesis, interfering with respiration, which then may lead to the death of the plant (de Lemos Filho and Paiva, 2006; Santos et al., 2013; Insausti et al., 2015).

Aphids have a soft cuticle; wings, if present, are membranous. Winged aphids are known as alatae and wingless aphids as apterae. They have both sexual and parthenogenetic reproduction, and can produce fertilized eggs that overwinter. Short developmental time and the ability of adult females to reproduce several nymphs per day enable aphids to achieve very high rates of population increase. If aphids develop on a plant that is crowded with many other aphids or when the quality of food is changing and not preferred, they may produce winged offspring and fly to a new host plant to start a new colony (Braendle et al., 2006). Aphids produce the phenotype which suits the environmental conditions they encounter. These kinds of environmentally induced discrete variants are called polyphenisms. These are a major reason for the success of the aphid, allowing them to partition life history stages, to adopt different phenotypes that best suit predictable environmental changes, to cope with temporally heterogeneous environments, and to partition labour within social groups. Aphid females respond to specific environmental cues by transmitting signals that have the effect of altering the development of their offspring. The mechanisms which allow aphids to switch between alternative morphs have remained obscure. It is believed that insect hormones (like juvenile hormone and ecdysone) regulate these switches (Nijhout, 1999). Some aphid species alter their feeding behaviour and the composition of their honeydew (e.g. by increasing the concentrations of amino acids) at the expense of their own growth and fecundity in the presence of tending ants (Yao and Akimoto, 2002).

Aphids feed by piercing the phloem of their food plant and are sensitive to changes in plant quality (Omacini et al., 2001). Host plant selection by aphids is generally governed by the olfactory and visual stimuli (Doring, 2014). This process is not random; these insects employ a variety of sensory and behavioural mechanisms to locate and recognize their host plants (Powell et al., 2006). Plant conditions may affect the probing behaviour of aphids. Aphids use their flexible and long stylets to obtain nutrients from the phloem sap (Jaouannet et al., 2014). Before that, aphids must conquer the host physical defences including trichomes and waxes before they can insert their stylets into the host. Surface resistance is the first barrier of plant defence against aphid infestation. The time between arriving on a leaf and making their first probe mainly reflects the physical barriers of the leaf surface including trichomes, repellent volatiles, and a thick or tough leaf surface.

Aphids may change their feeding location on the host plant or migrate to the new host to avoid plant defences or to feed on the better quality of plant; these behavioural responses can be extremely variable. As an example, infection of a microbial pathogen on a host plant was reported to increase aphid preference (Srininvasan et al., 2006; Ingwell et al., 2012) but some species of aphid, such as *Aphis fabae*, reacted negatively when the leaves were mechanically damaged (Nottingham et al., 1991). Aphids have an ability to secrete ‘effector’ proteins to suppress plant defences (Hogenhout and Bos, 2011) by inducing the changes in host plant metabolism and gene expression to enhance their feeding (Giordanengo et al., 2010).

Aphids display a number of antipredator behaviours to protect themselves from the foraging and attack of predators and parasitoids (Matheron and Porchas, 2008). These may be short term responses such as releasing an alarm pheromone that alerts conspecifics and alters predator foraging, shaking the body vigorously while kicking at the parasitoid with the hind legs, walking away from the threatened feeding site, dropping off the plant to avoid exposure, clustering together to reduce predation risk by the dilution effect, and selecting host plants and micro habitat free of predators (Villagra et al., 2002; Wu et al., 2010; Vandermoten et al., 2012). Antipredator responses may also be long term such as ingesting toxic allelochemicals which then have deterrent or toxic effects on predators, enhancing the production of winged morphs and enhancing the production of soldiers, which may eventually defend the colony (Kazana et al., 2007; Petschenka and Agrawal, 2016). The antipredator behaviour of the pea aphid, *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae) which can be found in red and green colour morphs, is one of the best understood. Pea aphids show genetically determined variation in escape behaviour, as shown in their response to the presence of foraging natural enemies (Stacey and Fellowes, 2002; Hazell et al., 2006). Laboratory experiments established that alarmed pea aphids are less likely to walk away from and drop off high-quality plants than poor-quality plants, and are also less likely to do so when climatic conditions are severe (i.e. hot and dry environments), suggesting that aphids display escape behaviours that minimize lost feeding opportunities and desiccation-induced mortality (Dill et al., 1990). Another study showed that recently moulted, adult stage pea aphids drop off and walk away from high-quality plants more frequently than older aphids, and they are also more likely to relocate to a new host plant after dropping, suggesting that the age-dependent physiological state affects antipredator decisions (Stadler et al., 1994).

1.7.4. Natural enemies

Aphid populations can be regulated by the action of natural enemies such as parasitoids and predatory insects. These natural enemies are commonly used in biological control programs and have been long known to contribute to a sustainable agricultural system (Bale et al., 2008). The use of natural enemies reduces the usage of pesticides, which is more environmental friendly as it reduces the effects on non-target species. There are cases where the application of a pesticide has failed to fully eliminate the insect pest because of the development of resistance mechanisms, either through biochemical, physiological or behavioural resistance (e.g. Foster et al., 2000; Kranthi et al., 2002; Nauen and Denholm, 2005). As such, the use of natural enemies in biological control if especially effective for insecticide-resistant pests.

The successful use of parasitoids as biocontrol agents is affected by the knowledge of their taxonomy, host selection behaviour, and ecology (Rehman and Powell, 2010). Among the common biological agents used to control the aphids are the parasitoid *Aphidius colemani* and predatory ladybirds such as *Harmonia axyridis* and *Adalia bipunctata*. The parasitoid *A. colemani* is a solitary endoparasitoid. This parasitoid is very effective in controlling aphids when aphid numbers are low. Ladybirds are voracious eaters and may eat up 50 aphids per day; 5,000 aphids during their lifetime (Bessin, 2007; Shelton, 2018). The application of both types of natural enemies especially at the early stage of aphid infestation may protect plants from devastated damage.

Most parasitoids are tiny wasps (0.08 - 0.12 inches) that belong to the Hymenoptera (Family: Braconidae and Aphelinidae)(Boivin et al., 2012). Parasitoids are commonly used as a biocontrol agent to control aphid populations belong to the order Hymenoptera (Boivin et al., 2012). They are slender, black or brown, and have a pinched or "wasp waist". The presence of a parasitoid can be determined by the presence of mummies (parasitized aphids) on the leaves (Raymond et al., 2016). Mummies appear as dark, “bloated” aphids and are a clear sign of parasitism. Parasitoids require one host to complete their lifecycles (Heimpel and Casas, 2008). Once a female finds an individual aphid or aphid colony, she will palpate the aphids with her antennae (Fellowes et al., 2005). If the aphid she is examining fulfils the criteria, such as having the correct body size, and has not already been parasitized by another parasitoid, she rapidly curls her abdomen under her body and stabs the aphid with her ovipositor (Sequeira and Mackauer, 1993). This process usually takes less than one second, but in this time the female checks the identity of the aphid with her ovipositor and lays an egg into it if she is satisfied. In about 48 hours after parasitism, the egg hatches and the small larvae starts consuming the aphid tissues, eventually killing its host. The larva is a tiny, white grub. When the larva completes its development, it cuts a slit in the hollowed-out shell of its host, and attaches the carcass to the leaf with silk. The larvae remain inside of the dead aphid and pupate turning the aphid body into a "mummy" (Boivin et al., 2012). The mummies are actually the aphid’s skin which are swollen, brown or blackish, and papery in appearance. After the completion of development, the tiny wasp cuts a circular trap door in the rear of the mummy to escape and become a free-living adult (Gutiérrez-Ibáñez et al., 2007). The development time from egg to adult can range from nine to 23 days (Heimpel and Casas, 2008). Factors such as rain, humidity and wind can affect the development and length of life of the parasitoid (Weisser et al., 1997). The speed of development depends on the temperature; the development is slower at low temperatures and faster at high temperatures (Heimpel and Casas, 2008). Adults may live from seven to 15 days and a female can parasitize over 300 aphids in her lifetime (Fellowes et al., 2005).

Host quality is the key determinant for the fitness of developing offspring of the parasitoid, thus natural selection is expected to lead the female parasitoid to choose the highest quality of host to oviposit her eggs (Fellowes et al., 2005). Parasitoids frequently alter sex allocation to reflect perceived differences in host quality by placing male eggs in relatively poor-quality hosts (Morris and Fellowes, 2002). The growth and development of parasitoids can also be influenced indirectly by the quality of plants, through the quality of their host or prey (Teder and Tammaru, 2002; Hunter, 2003). Allelochemicals in their host or prey diets can either inhibit or enhance the fitness of emerging parasitoids (Harvey et al., 2005; Ode, 2006).

Parasitoids are often specialised on one or a few host species and host stages. Successful host selection depends upon the execution of appropriate behavioural responses to an array of cues which are available during the parasitoid foraging process. Therefore, they have specialized sensory nervous systems that help them in using a variety of chemical and physical cues during the habitat location, host location, and host examination phases of host selection (Tumlinson et al., 1992). In the host selection behaviour of insect parasitoids, volatile cues derived from plants can have a similar influence to that exerted by signals emanating directly from the host (Tumlinson et al., 1992; Vet and Dicke, 1992), but those plant-derived cues are assumed to be more detectable from a distance because of the plant's relatively large biomass (Vet et al., 1990; Vet and Dicke, 1992). Parasitoids, while searching for the hosts' habitat, may exploit volatile cues derived from uninfested plants as well as from infested plants (Nordlund et al., 1988). The plant often provides the first cue in the chain of events that leads to host location, regardless of the nature of the orienting factor (Vinson, 1976). It is assumed that the release of plant volatiles due to herbivore-feeding damage serves as a mechanism that first evolved as a direct defence against herbivores and pathogens, and the function of attracting natural enemies evolved secondarily (Dicke and van Loon, 2000). This attraction of natural enemies by odours derived from plants has been widely studied in the laboratory using wind tunnel experiments showing that parasitoid females are able to locate their host habitat and discriminate between uninfested and infested plants (e.g: Lo Pinto et al., 2004). Besides volatile cues, physical cues such as colour, sound, shape, movement and size of the prey also influence parasitoid host choice (Battaglia et al., 1995). Intra specific variation in aphid communities also affects natural enemy assemblages in terms of attacking preference. For example, the aphidiine parasitoid *A. ervi* Haliday prefers to attack green pea aphid morphs compared to red morphs (Libbrecht et al., 2007). The other cues such as plant architecture, canopy stratification and height, and leaf surface area may also elicit a flight response in parasitoid females (Cloyd and Sadof, 2000; Jang et al., 2000). Several braconid aphid parasitoid species have been reported as responding to a variety of both contact and olfactory cues associated with the host or with the host’s habitat (Jang et al., 2000; Carver and Franzmann, 2001). The behavioural responses expressed by a foraging parasitoid at any one time are not necessarily influenced by chemical or physical cues, but largely determined by its genotype, physiological state and previous environmental learning experiences (Fellowes et al., 2005). Parasitoids show some associative learning which plays a role in host location and choice (Morris and Fellowes, 2002). This behavioural plasticity allows the parasitoid to make optimum use of the prevailing foraging opportunities. The capacity to learn through experience, and then modify their responses to environmental cues accordingly, plays an important role in endowing a foraging parasitoid with behavioural adaptability (Vet and Dicke, 1992).

Insect predators are characterized by a set of attributes that differentiate them from parasitoids. They are large relative to their prey, kill their prey immediately and consume many of them to complete their development; they have free-living predatory immature or adult stages, and many species of insect predators are predacious in both phases of their lives (Patterson and Ramirez, 2016). Many predators are generalist feeders, meaning that they eat many different species of prey (Bernays, 1989). Some predators are conspicuously selective in their diet (Snyder and Ives, 2003). Most of the predacious insects possess strong mandibles to capture or venom to kill their prey.

Some predators may depend on a variety of cues produced by both plants and insects to locate and find their prey (Clavijo McCormick et al., 2012). A complex mixture of volatiles which act as an attractant and/or an arrestant influence the foraging behaviour of insect predators to fly toward a food source and to oviposit (Leroy et al., 2012; Steidle and Loon, 2003). These cues also help insect predators to choose a good quality food source before attacking their prey. Knowing the influence of these compounds on predator behaviour forms a strong basis for manipulating the natural enemies in the field (Dicke et al., 1990). As an example, several species of predacious beetles and bolas spiders use chemical cues (plant terpenes, prey pheromones, or mixtures of terpenes and pheromones) to find their prey (Haynes et al., 2001; Nelson and Jackson, 2009).

Some immature insects start their life cycle as a predator. Not being equipped with wings to fly to a food source makes the immature predator’s life more challenging. To overcome this, many larval predators have evolved various means to locate and recognize their prey such as through vision, olfaction, sound or vibration detection and contact (Hodek, 1993). Predacious mites use chemical cues to find their prey (Sabelis and Bakker, 1992; Fadini et al., 2010). Their limited ability to search and encounter their prey have stimulated some larval predators to switch their behaviour from linear movements to area intensive searching (Kareiva and Odell, 1987). A predator's previous experience (learning) can influence its searching and foraging behaviour, as well as the characteristics of its prey and proportion of the prey to be attacked (Begon et al., 2005). Plant structure and architecture may also affect the pattern of predator movement (Eigenbrode et al., 1996; Heinz and Zalom, 1996). Species and biotypes of predators can differ radically in their habitat associations and in the micro habitats they choose. As an example, certain ladybirds have been found to forage in plant canopies, whereas others are more comfortable foraging in areas closer to the soil (Hodek, 1993) and *Chrysoperla rufilabris* (green lacewing) prefers moist habitats, whereas *Chrysoperla carnea* is well adapted to dry conditions (Tauber and Tauber, 1993). These differences in habitat association are reflected in the prey availability and suitability to predators. All of these criteria, play a very important role in the choice of species or biotypes to be used in specific biological control situations.

After the predator discovers its prey, it must pursue, subdue, and consume it. These three functions constitute the predator's handling time, which is a very important determinant of dietary breadth (Begon et al., 2005). Several morphological and physiological factors can influence prey acceptance by predators (Schmitz, 2017). One underlying physical factor is prey size (Milonas et al., 2011) and other physical characteristics of the prey, such as the presence of wax which may affect the predators' response (Agarwala and Yasuda, 2001). Losey et al. (1997) suggested that colour polymorphism of prey influences the preference of visual foraging predators. As an example, the *Coccinella septumpunctata* L. attacks red aphids more frequently than green ones (Losey et al., 1997). Predators affect prey directly, via consumption (i.e., consumptive effects), and indirectly, via the induction of energetically costly antipredator responses (i.e., non-consumptive effects). These responses can include changes in prey behavioural, morphological, or developmental traits (Podjasek et al., 2005; Preisser et al., 2005; Verheggen et al., 2009). The behavioural, morphological, and weapon (chemical) of prey defences strategies may also determine whether a predator accepts or rejects the prey. Some prey such as aphids will kick, run, drop, fly away, or exude noxious chemicals when predators approach them.

1.8 Research questions and objectives

In this thesis, I report my research which attempts to detangle the ecological consequences of asymptomatic pathogen infection on organisms at higher trophic levels, both in the laboratory and in the field. I use two varieties of lettuce plant (to investigate if plant genotype influences patterns) infected (and therefore asymptomatic or symptomatic) or uninfected by *B. cinerea*, to investigate how expression of pathogen symptoms affects interactions between plants, herbivores, predators and parasitoids, in both the laboratory and field. I report my findings in the following chapters, where I ask:

Chapter 2: Does asymptomatic *B. cinerea* infection alter plant traits and the life history of insects (aphid herbivore, parasitoid) in the laboratory?

Chapter 3: Does asymptomatic pathogen infection alter insect preference and escape behaviour (herbivore, parasitoid, ladybird) in the laboratory?

Chapter 4: Does asymptomatic pathogen infection influence the abundance and diversity of herbivores insects and their natural enemies recruited by plants in the field?

Chapter 5: Does asymptomatic pathogen infection shape the assemblage of an aphid’s natural enemies formed in the field?

Chapter 6: Does pathogen infection indirectly affect the outcome of interactions between plants and herbivores in the field, mediated by natural enemies?

Finally, in Chapter 7 I summarize the results of my work, place it in context, and suggest future opportunities for research.

Chapter 2: Symptomless host plant infection by the fungal pathogen *Botrytis cinerea* alters host plant, aphid and parasitoid traits

2.1. Abstract

Plant pathogens can profoundly affect host plant quality as perceived by their insect herbivores, with potentially far-reaching implications for the ecology and structure of insect communities. Changes in host plants may have direct effects on the life-histories of their insect herbivores, which can then influence their value as prey to their natural enemies. While there have been many studies that have explored the effects of infection when plants show symptoms of disease, very little is understood about how unexpressed infection may affect interactions at higher trophic levels. We examined how systemic, asymptomatic and seed-borne infection by the ubiquitous plant pathogen *Botrytis cinerea*, infecting two varieties of the lettuce *Lactuca sativa*, affected the life history of aphids (the green peach aphid, *Myzus persicae*) and their parasitoids (the widely used biocontrol agent *Aphidius colemani*) in a controlled environment. Lettuce varieties differed in host plant quality. Asymptomatic infection reduced chlorophyll content and dry weight of host plants, irrespective of plant variety. Aphids reared on asymptomatic plants were smaller, had reduced off-plant survival time and were less fecund. Parasitoids emerging from hosts reared on asymptomatic plants were smaller and showed reduced starvation resistance. These results suggest that the reduced nutritional quality of plants asymptomatically infected by *B. cinerea* negatively affects the life history of aphids and their parasitoids. Fungal pathogens are ubiquitous in nature, and this work shows that even when host plants are yet to show symptoms, pathogens can affect the life histories and interactions of insect herbivores and their natural enemies.

Key words: *Myzus persicae*, *Aphidius colemani*, lettuce, tritrophic interactions, biological control

2.2. Introduction

Plant pathogens are ubiquitous in nature, affecting the growth and development of many plant species, and reducing the quality of the plant as experienced by herbivores (Stout et al., 2006). They are of enormous economic importance. Besides direct crop loss, they cause indirect losses through the costs of prevention and treatment (Savary et al., 2012). Infection by plant pathogens frequently affects the respiration and transpiration capabilities of host plants, resulting in decreased rates of photosynthesis, which in turn alters rates of nutrient translocation, causing a net influx of nutrients into infected tissues (Berger et al., 2007). Many plant species also react to pathogen infection by triggering a change in the rate of hormone synthesis or degradation (Overmyer et al., 2003); these changes in turn alter the production of secondary defences and alter the host plant's normal resistance pathways (Hatcher et al., 2004; Bostock, 2005; Mauch-Mani and Mauch, 2005).

As plant pathogens and herbivorous insects may share the same host plant, changes in plant traits caused by infection can act as a feeding deterrent to herbivorous insects, and can also alter their physiology and development, resulting in reduced growth rates, reduced adult size, and increased mortality rates (Giamoustaris and Mithen, 1995; van Dam et al., 2000). However, plant pathogen infection can also have a positive effect on the fitness, performance and host plant preference of insect herbivores (Pruter and Zebitz, 1991; Kluth et al., 2002; Mondy and Corio-Costet, 2004; Cardoza and Tumlinson, 2006). Herbivorous insects may benefit from pathogen infection when the presence of the pathogen increases nutrient levels (e.g. by digesting the complex sugars in infected leaves) or when the pathogenic fungi changes the plant defence mechanisms in a way that makes it more susceptible to insect herbivores (Stout et al., 2006). These effects may also have consequences at higher trophic levels, with the predators and parasitoids of insect herbivores in turn affected by consequent changes in the quality of their hosts (Heil, 2011; Tack and Dicke, 2013). For example, pathogen infection can cause a change in the composition of plant volatiles (Cardoza et al., 2003) which in turn alters their attraction to parasitoids (Turlings and Wäckers, 2004). If parasitoids attack hosts on pathogen-infected plants, then they may alter their sex allocation behaviour to reflect perceived differences in host quality, as female parasitoids can choose to place male eggs in relatively poor quality hosts (Morris and Fellowes, 2002; Fellowes et al., 2005).

However, plant pathogen infections do not always result in visually obvious negative effects on the plant, such as wilting or defoliation of the leaves, which could affect the visual preference of the insects (Schowalter, 2013). Pathogen infection in which live pathogen is present in a host but does not cause gross damage to the host is referred to as a latent infection. Latency can occur at any stage of the crop life cycle and at any stage of pathogen growth (Jarvis, 1994; Prusky, 1996). Latent infection may take the form of quiescence, surviving but not growing until appropriate environmental triggers are perceived (Verhoeff, 1974). Alternatively, the pathogen may grow inside the host but still cause no or very slight symptoms. A number of studies have suggested that a range of plant species such as wheat (Zeng et al., 2010), grapes (Czemmel et al., 2015), basil (Farahani-Kofoet et al., 2012) and woody plants (Flowers et al., 2001, Palmer and Skinner, 2002) harbour such hidden infections by plant pathogens. We have little understanding of the consequences - whether positive or negative - of such hidden infections for insect herbivores and in turn their natural enemies. This is a fundamental question of considerable interest, given the ubiquity of plant pathogens.

The common and widespread generalist plant pathogen *Botrytis cinerea* Persoon: Fries s. lato has been the focus of many epidemiological and biocontrol studies (e.g. Elad and Shtienberg, 1995; Elad et al., 1996; Dal Bello et al., 2011), and causes extensive damage to a wide range of economically important crops worldwide (Elad et al., 2007). Known as ‘grey mould fungus’, this airborne fungus attacks over 200 plant species (Boddy, 2015). Infection by this pathogen will either reduce or eliminate the marketability of the harvested product (Elad et al., 2007). *Botrytis cinerea* multiplies through conidia which directly infect the host plant, typically resulting in spreading necrotrophic lesions (Elad et al., 2007). Once it has penetrated into the plant system, *B. cinerea* secretes a range of nonspecific chemical compounds, including oxalic acid (Rollins, 2003), the fungal toxin botrydial (Deighton et al., 2001), and hydrogen peroxide (Schouten et al., 2002). These compounds contribute to host-plant cell death and promote the growth of macerated lesions (Dulermo et al., 2009). In turn, the plants then activate resistance mechanisms to combat this pathogen attack (Méndez-Bravo et al., 2011). One of the plants first defences is to activate the hypersensitive response (HR), generating the oxidative burst that can trigger hypersensitive cell death (Govrin and Levine, 2000).

Botrytis cinerea also has been seen to remain quiescent in strawberry leaf epidermal cells (Braun and Sutton, 1988), grape flowers (Dugan et al., 2002), and quiescent infection, in which a few dead cells harbour localised but live *B. cinerea*, limited by host defences in the surrounding cells is probably a common cause of post-harvest infection in many fruit, including strawberries and raspberries (Rajaguru and Shaw, 2010). In some cases, *B. cinerea* can grow systemically, extending as the plant grows, without the plant showing symptoms of infection (Barnes and Shaw, 2003; Sowley et al., 2010). This plant pathogen is often present in what are otherwise visually healthy lettuce plants (Sowley et al., 2010; Jeyaseelan, 2015) as an asymptomatic, endophytic infection, which may also arise from seed (van Kan et al., 2014). As the host plant grows, infection spreads into roots, stem and leaves (Sowley et al., 2010). This form of infection is known in multiple *Botrytis* species and in hosts including hybrid primula plants (Barnes and Shaw, 2003), *Pelargonium* sp. leaves (Suarez et al., 2005), wild Primula and *Taraxacum vulgare* agg. (dandelion) (Rajaguru and Shaw, 2010; Shaw et al., 2016) and daylilies (Grant-Downton et al., 2014). The likelihood of quiescent infection by *B. cinerea* varies among plant species.

Asymptomatic infection by *B. cinerea* may still alter host plant physiology. This may then have a consequential effect on organisms at a higher trophic level. However, despite the potential ubiquity of hidden infection, little is known of the consequences for insect herbivores and their natural enemies (Yahaya, 2013). We addressed this in a laboratory study, using two varieties of lettuce, an economically valuable crop plant (production in the UK alone was valued at >£150 million in 2016; ONS, 2017) as our host plants. We asked if asymptomatic infection by *B. cinerea* (i) alters plant traits; (ii) influences the size and life history traits of an insect herbivore (the green peach aphid, *Myzus persicae*); and (iii) if asymptomatic pathogen infection indirectly affects parasitoid (*Aphidius colemani*) traits.

2.3. Materials and methods

2.3.1. Study system

Plant and insect rearing was carried out in a constant environment (CE) room at 18-20°C, 80 ± 5 % relative humidity and L16:D8 photoperiod. Both varieties of uninfected and infected lettuce were grown in 15 cm diameter pots with traditional potting compost (Vitax Grower, Leicester, England). Seeds of two commercial varieties of lettuce *Lactuca sativa* L. (Little Gem and Tom Thumb; Thompson and Morgan, Suffolk, UK; harvest year 2013) were used and grown in 15 cm diameter pots with traditional potting compost (Vitax Grower, Leicester, England). To ensure that plants used in experiments were otherwise identical, plants were grown from single source seed to produce plants for use in all later experiments. Plants grown for 'infected seeds' and 'uninfected seeds' were grown separately, to reduce infection rates of our control (uninfected) plants. Infected plants were grown from systemically infected seed collected from plants which had been previously inoculated with *B. cinerea* strain B05.10 at the flowering stage (following Sowley et al., 2010), while uninfected plants were grown from uninfected seeds collected from uninfected plants. Six-week-old plants which were free from any symptoms of disease were used in this study. Sixty replicates were set up per treatment, as it proved impossible to both guarantee infection in the treated plants and lack of infection in the control plants. A week before each experiment started, plant infection status of the plants was checked using *Botrytis* Selective Media Agar (BSM). Thirty infected/uninfected plants were then selected randomly from the tested plants for use in trials. It should be noted that some plants did show symptoms of infection, so final replicate numbers for all insect trials was lower than 30.

Both insects were reared in rectangular clear plastic cages (20cm X 20cm X 15cm) fitted with the cotton mesh windows. A monoclonal culture of the green peach aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae) and parasitoid *Aphidius colemani* Viereck (Hymenoptera: Branconidae) were reared on both varieties of uninfected and infected lettuce plants for five generations before the experiment, thus avoiding any confounding maternal effects (Dixon, 1985). Parasitoids were reared on aphids in a population cage on each of the four treatments to avoid learning effects. In order to obtain uniform aged *A. colemani*, mummies were collected from respective lettuce plants and placed individually in gelatine capsules. Upon emergence, female parasitoids were kept for 24 hours with male parasitoids to ensure mating, fed ad libitum with drops of pure honey, and then used for the experiments. Only female *A. colemani* were used in this experiment.

2.3.2. Effect of asymptomatic infection on plant traits

Plant height was measured on the first and last day (day 30) of the experiment (N = 30 for each treatment. At the end of the experiment, plant chlorophyll content was measured at three different positions on plant leaves using a handheld chlorophyll meter (Model atLeaf; FT Green LLC) (Zhu et al., 2012; Lundy et al., 2014). Plants were then harvested and dried in an oven at 75°C until reaching constant mass (approximately 48 h), and weighed using an electronic balance (Sartorius LC 6200S, Goettingen, Germany). The root: shoot ratio was calculated by dividing the dry mass of individual above ground plant material by the dry mass of their roots.

2.3.3. Effect of asymptomatic plant pathogen infection on insects

Aphid fecundity, longevity and size. Leaf clip-cages (30mm in diameter by 10mm in height; Noble, 1958) were used to prevent aphid escape or parasitism. Both clip cage rings were covered with fine muslin netting to allow air to flow to both leaves and aphids. Clip cage edges were lined with foam as a precaution against leaf damage. Adult apterous aphids were randomly chosen from the rearing colonies and one was placed into each individual clip cage, which was attached to a healthy, mature leaf. Aphids were permitted to produce nymphs for 24 hours, then the adult and surplus aphids were removed, leaving five aphid nymphs which were then allowed to grow until they reached maturity.

To evaluate aphid fecundity, the number of offspring produced by each individual aphid was recorded once every two days and these were removed; this was repeated five times (10 days fecundity recorded). To measure aphid longevity and size, the same methods as above were used with ten nymphs, which were allowed to grow in individual clip cages for approximately seven days. Aphids were then collected and transferred into a Petri dish without a food source or water to time to death. Observations were made every 12 hours until all aphids died. Aphid hind tibia length was measured using a high-performance stereomicroscope (Leica, MZ9.5, Texas, USA).

Parasitoid fecundity, longevity and size. Two mated female parasitoids were introduced to forty ten-day-old aphids growing on lettuce plants, and then covered with a mesh plastic bag. Thirty replicates were set up per treatment. Parasitoids were left to oviposit for 24 hours before removal. After 10 days, mummies were collected and counted on each of the plants. The mummies were placed individually in a gelatine capsule (16 x 5 mm) and kept in a CE room until they emerged. Observations were made at 12 hours intervals until all of the parasitoids had died. The time taken for parasitoids to emerge and die was recorded. The left hind tibia length of each parasitoid was then measured using a high-performance stereomicroscope (Leica, MZ9.5, Texas, USA).

2.3.4. Confirming asymptomatic infection

The bioassay to confirm plant health status was made before the plant was harvested. To test for the presence of systemic infection by *B. cinerea*, three mature leaf samples from each plant (1 cm in diameter), with no visible symptoms of infection were randomly harvested at the end of the experiment from each experimental plant. Leaf samples were first disinfected with 70% ethanol for one minute, and then in a 20% solution of bleach (Domestos, Unilever:5% NaOCl in alkaline solution with surfactants) for one minute. Samples were then rinsed three times in sterile distilled water and allowed to dry. This removes all surface inoculum, whether dusted or soaked in (Barnes, 2002). The leaf disk was then plated on a *Botrytis* Selective Media (BSM) agar and incubated at 18-20°C for at least 10 days in an incubator with alternating UV-A light (12h/day) and dark (12h/day) to determine the presence or absence of *B. cinerea*. Confirmation of presence was based on the sporulation of the pathogen and morphological observation of fungal colonies under a high-performance stereomicroscope (Leica MZ9.5, Texas, USA).

2.3.5. Statistical analyses

All statistical analyses were conducted using R-statistical software version 3.4.0 (R Core Team, 2017). The influence of plant variety and pathogen infection on plant traits, and aphid/parasitoid size and longevity were compared using Linear Models (LM), while aphid total fecundity was analysed using Generalized Linear Models (GLM) with Poisson errors. The proportion of parasitized aphids was analysed by using GLM with quasibinomial errors. The significance of differences between mean values were determined by using LSmeans and separation by post-hoc Tukey tests, with plant variety and infection status as explanatory variables.

2.4. Results

2.4.1. Plant traits

Plant varieties significantly differed in their physical traits (Table 2.1; N = 30 for each treatment), with Tom Thumb showing a lower chlorophyll index plant dry weight and plant height than Little Gem (Table 2.2). Asymptomatic infection by *B. cinerea* resulted in reduced chlorophyll and plant dry weight for both plant varieties, but there was no effect on plant height (Table 2.2). There was no effect of plant variety or of infection status on root:shoot ratios. All interaction terms were non-significant (Table 2.1).

Table 2.1: Summary of effects of plant variety and *B. cinerea* asymptomatic infection status on plant traits following analysis. Significant values are in bold.

Plant trait	Explanatory variable	Coefficient z value ± SE	P
atLEAF value	**Intercept**	**38.747 ± 0.648**	**<0.001**
	Variety	**-6.427 ± 0.935**	**<0.001**
	Infection status	**7.713 ±0.909**	**<0.001**
	Interaction	-0.215 ± 1.298	0.830
Shoot: root	**Intercept**	**20.786 ± 0.027**	**0.001**
	Variety	1.787 ± 0.032	0.088
	Infection status	0.984 ± 0.044	0.327
	Interaction	-0.175 ± 0.064	0.861
Dry weight (g)	**Intercept**	**21.022 ± 0.558**	**<0.001**
	Variety	**-4.442 ± 0.805**	**<0.001**
	Infection status	**5.744 ± 0.783**	**<0.001**
	Interaction	-1.280 ± 1.118	0.204
Plant height (mm)	**Intercept**	**98.515 ± 1.606**	**<0.001**
	Variety	**-34.003 ± 2.316**	**<0.001**
	Infection status	-0.875 ± 2.251	0.384
	Interaction	0.948 ± 3.216	0.345

Table 2.2: The effect of asymptomatic *B. cinerea* infection and plant variety on plant traits. atLEAF value represents the amount of chlorophyll present in the plant leaf. For each parameter, differences among treatment were examined by LSMEANS multicomparison tests ($P < 0.05$). Means within columns followed by the same letters are not significantly different.

Treatment	Plant traits (mean ± SE)			
	atLEAF value	Shoot: root	Dry weight (g)	Plant height (mm)
Uninfected Little Gem	32.15 ± 0.70^{a}	0.62 ± 0.02^{a}	16.25 ± 0.74^{a}	156.25 ± 2.00^{a}
Asymptomatic Little Gem	25.14 ± 0.62^{b}	0.57 ± 0.02^{a}	11.75 ± 0.50^{b}	158.22 ± 1.27^{a}
Uninfected Tom Thumb	25.86 ± 0.70^{b}	0.68 ± 0.03^{a}	11.23 ± 0.42^{b}	80.54 ± 1.50^{b}
Asymptomatic Tom Thumb	19.14 ± 0.52^{c}	0.65 ± 0.05^{a}	8.48 ± 0.50^{c}	79.46 ± 1.53^{b}

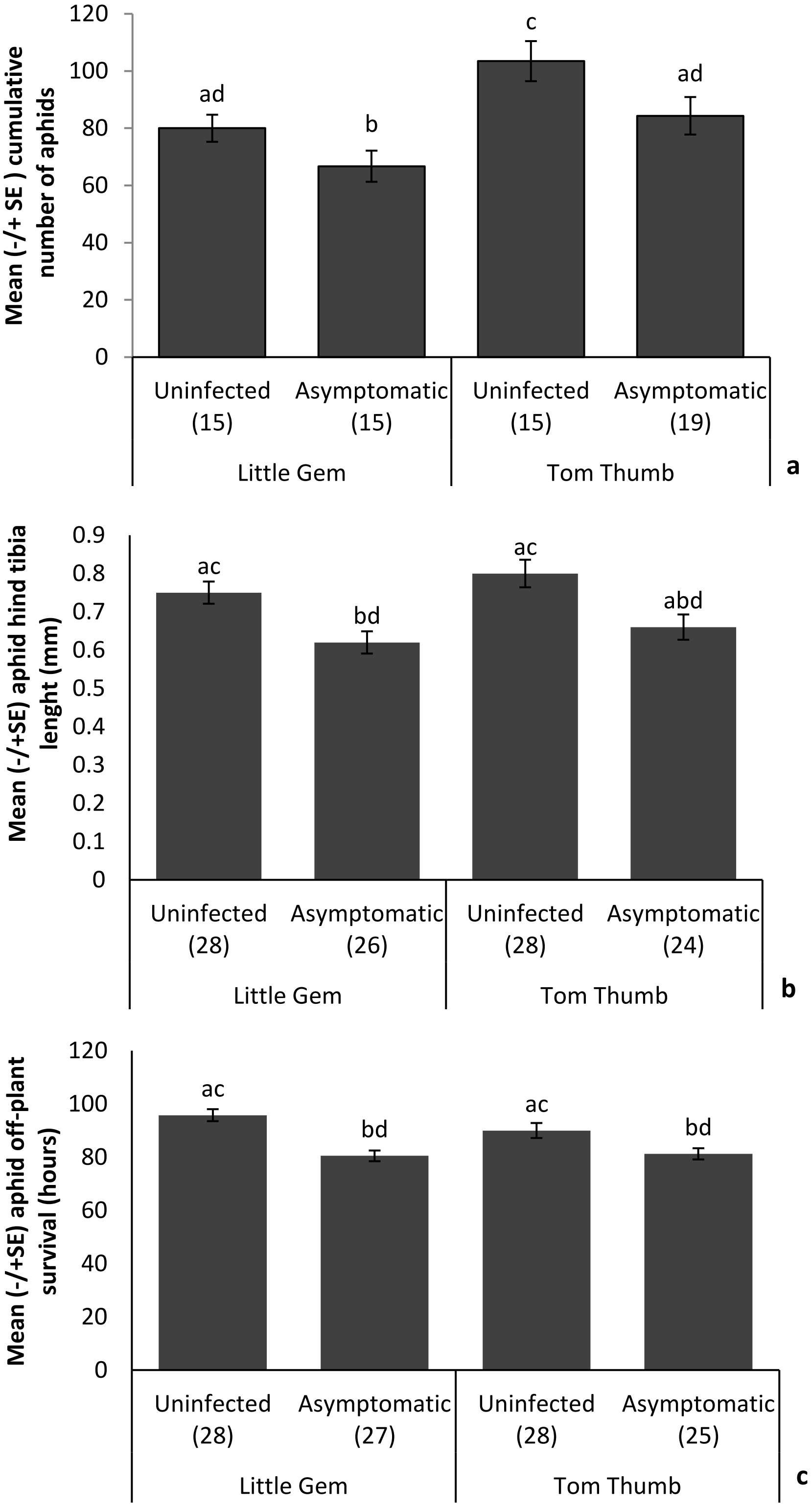
2.4.2. Aphid life history

Plant variety influenced the number of aphids produced, with aphid fecundity higher on Tom Thumb. Aphid size and off-plant survival time did not differ with plant variety (Table 2.3; Figure 2.1). Asymptomatic plant pathogen infection significantly reduced aphid fecundity, size and off-plant survival time. Overall, aphids had the best performance when reared on uninfected Tom Thumb plants, and the poorest when reared on infected Little Gem. Interaction terms were non-significant.

Table 2.3: Summary of effects of plant variety and *B. cinerea* asymptomatic infection status on aphid traits following analysis. Significant values are in bold.

Aphid trait	Explanatory variable	Coefficient value ± SE	P
Fecundity	**Intercept**	**132.904 ± 0.031**	**<0.001**
	Host plant variety	**5.821 ± 0.040**	**<0.001**
	Host plant infection status	**4.236 ± 0. 042**	**<0.001**
	Interaction	0.408 ± 0.055	0.683
Size	**Intercept**	**18.907 ± 0.032**	**<0.001**
	Host plant variety	0.894 ± 0.042	0.373
	Host plant infection status	**2.862 ± 0.130**	**0.005**
	Interaction	0.110 ± 0.007	0.912
Off-plant survival time	**Intercept**	**34.454 ± 2.336**	**<0.001**
	Host plant variety	0.221 ± 3.369	0.826
	Host plant Infection status	**4.668 ± 3.274**	**<0.001**
	Interaction	-1.393 ± 4.677	0.167

Figure 2.1: The effect of plant variety and asymptomatic pathogen infection status on Mean ± SE a) cumulative number of offspring; b) aphid hind tibia length; and c) aphid off-plant survival. Number of replicates per treatment is shown below each bar.



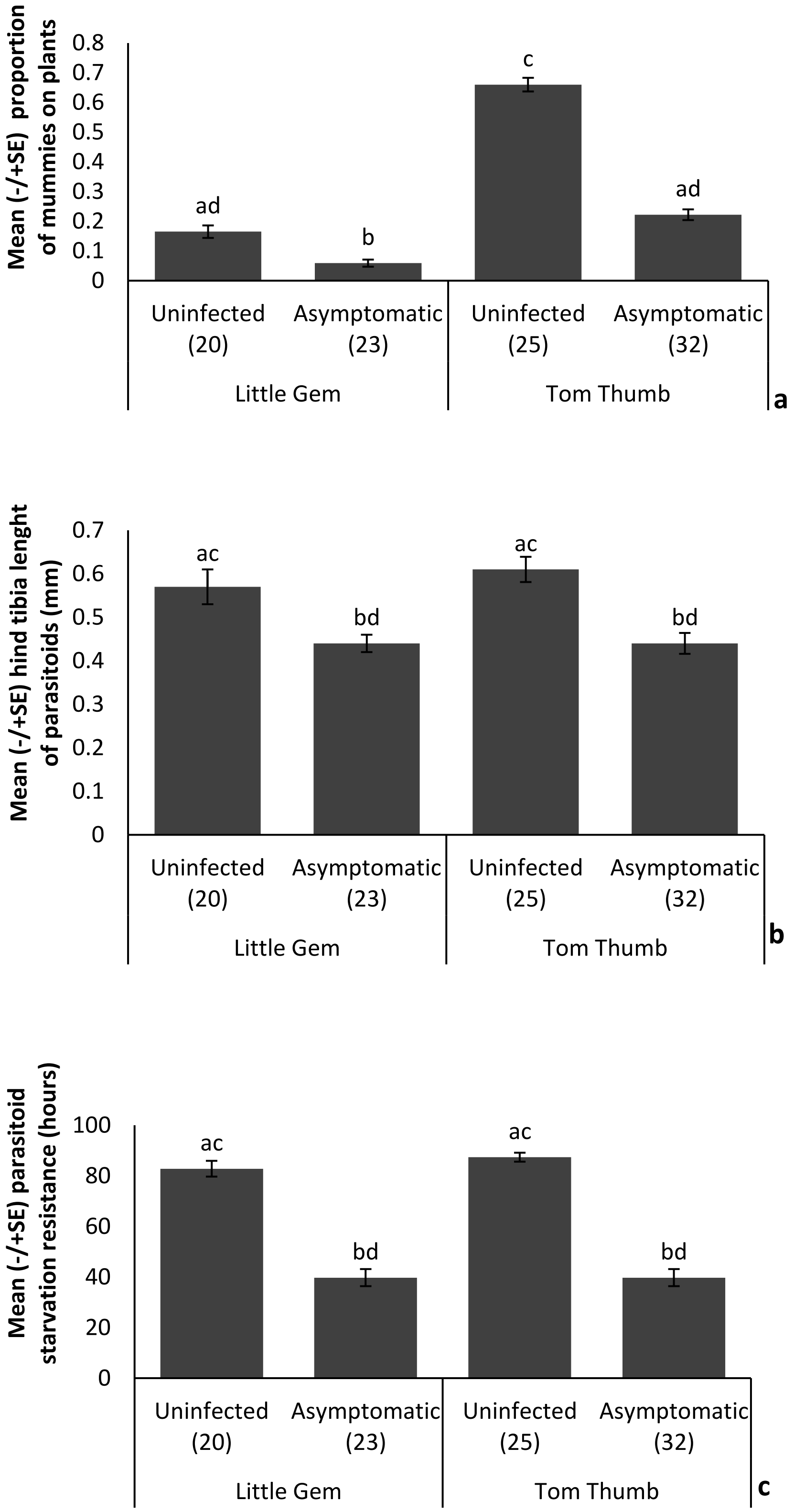
2.4.3. Parasitoid life history

Plant variety influenced parasitoid attack rates, with more mummies on Tom Thumb, but there was no effect of plant variety on parasitoid size or longevity (Table 2.4; Figure 2.2). The proportion of parasitoid mummies formed on infected plants was lower than that found on uninfected plants, and there was also a significant interaction between plant variety and infection status on parasitoid attack rates. Parasitoids emerging from aphids reared on infected plants were smaller and showed reduced starvation resistance.

Table 2.4: Summary of effects of plant variety and *B. cinerea* asymptomatic infection status on parasitoid traits following analysis. Significant values are in bold.

Parasitoid traits	Explanatory variable	Coefficient value ± SE	P
Parasitism rate	**Intercept**	**-12.415 ± 0.221**	**<0.001**
	Aphid host plant variety	**6.107 ± 0.246**	**<0.001**
	Aphid host plant infection status	**4.215 ± 0.269**	**<0.001**
	Interaction	**2.527 ± 0.308**	**0.013**
Size	**Intercept**	**14.447 ± 0.031**	**<0.001**
	Aphid host plant variety	-0.019 ± 0.040	0.985
	Aphid host plant infection status	**2.828 ± 0.045**	**0.005**
	Interaction	0.563 ± 0.060	0.574
Starvation resistance	**Intercept**	**12.845 ± 3.818**	**<0.001**
	Aphid host plant variety	-1.857 ± 5.006	0.066
	Aphid host plant infection status	**6.029 ± 5.599**	**<0.001**
	Interaction	1.864 ± 7.432	0.065

Figure 2.2: The effect of plant variety and asymptomatic pathogen infection status on Mean ± SE a) proportion of mummies formed; b) parasitoid hind tibia length; and c) starvation resistance. Number of plant replicates per treatment is shown below each bar.



2.5. Discussion

Asymptomatic infection by plant pathogens is likely to be widespread, yet we have almost no understanding of its effects on species interactions at higher trophic levels. Here, we show that asymptomatic host plant infection by *B. cinerea* of two lettuce varieties affects species at higher trophic levels. While our lettuce plants showed no overt, visible symptoms of infection, it is apparent that this hidden, asymptomatic infection did subtly affect host plants. Infected, asymptomatic plants had reduced chlorophyll content and showed reduced mass, although plant height and root:shoot ratio did not differ. Therefore, while effects were minimal and symptoms of disease absent, it is evident that asymptomatic infection did affect host plants, and this was consistent across host plant varieties. However, the consequences of asymptomatic infection on our model insect herbivore was more clear. Aphids reared on infected asymptomatic plants were smaller, produced fewer offspring and had reduced off-plant survival times. In turn, parasitoids reared on hosts feeding on infected asymptomatic plants, showed reduced rates of mummy formation, and their offspring were smaller and also showed reduced longevity. While there were differences in traits between lettuce varieties, this only appeared to affect aphid fecundity and parasitoid attack rate. The value of this work lies in the demonstration of the importance of hidden disease on insect life history; diseased plants, even when asymptomatic, alter the physiology of insect herbivores and their natural enemies and hence may affect how assemblages are formed and so affect the outcome of biological control efforts. Infection by this pathogen in apparently healthy wild growing host plants such as *T. vulgare* may reach 50% of plant samples (Shaw et al., 2016). These findings suggest plant pathogens have a strong influence on arthropod tritrophic systems, and are therefore of particular relevance to arthropod biocontrol.

The effect of *Botrytis* infection on postharvest products (Martinez-Romero et al., 2007; Xu et al., 2007; Celik et al., 2009; Sanzani et al., 2012) and plants under cultivation (Elad, 1988; Barnes and Shaw, 2002, 2003; Keller et al., 2003) is well studied. However, little has been reported on the effect of asymptomatic infection on plant traits. An investigation of the effect of *B. cinerea* infection on lettuce plants reported that latent *B. cinerea* infection on seed, root, stem and leaf was common (Sowley et al., 2010); asymptomatic lettuce with more *Botrytis* recovery were of greater mass than uninfected plants (Shaw et al., 2016), This is inconsistent with the findings here, but the methods differ in the two studies.

Aphid size, off-plant survival time and fecundity were reduced when reared on asymptomatic, infected plants (Table 3). If the presence of the pathogen either directly or indirectly results in a reduction in host plant quality, then such effects are not unexpected. Due to the reduction in plant quality caused by pathogen infection, it has been suggested that this could play a role in determining the structure of arthropod communities (Stout et al., 2006; Tack and Dicke, 2013). The low quality of diseased plants generally results in a decline in fecundity and an increase in developmental time of insect herbivores (Legrand and Barbosa, 2000). This change results from a reduction in available plant amino acids due to assimilation of resources by *B. cinerea* (Dulermo et al., 2009) and it would also influence the ability of aphids to effectively utilise the host plant (Douglas, 2006; Powell et al., 2006). We show that it is not only plants showing symptoms which affect their herbivores' life histories, but also that asymptomatic host plant infection also alters the growth, reproduction and starvation resistance of an insect herbivore.

Al-Naemi and Hatcher (2013) found that *B. cinerea* negatively affected the development, survival and fecundity of *Aphis fabae* on faba bean and these effects correlated with lesion density, and suggested that this was due to the decreased availability of nitrogen during pathogen infection. Mouttet et al. (2011) found that *Rhodobium porosum* (Sanderson) feeding on rose was negatively affected by *B. cinerea* infection, suggesting the existence of a negative bidirectional indirect interaction between the necrotrophic fungus and a piercing-sucking insect which is conveyed by decreased aphid growth rate and reduced fungal lesion area. It should be noted that the effect of symptomatic infection by *B. cinerea* is not always negative. For example, *Aphis fabae* performance improves on infected and symptomatic *Vicia faba* due to an increased availability of amino acids resulting from the degradation of mesophyll cells in the phloem, during pathogen-induced senescence (Zebitz and Kehlenbeck, 1991). The fungus may also have a direct benefit to non-aphid herbivores; caterpillars of the moth *Lobesia botrana* were better developed, had higher survival rates and improved fecundity when fed on an artificial diet containing *B. cinerea* fungus (Mondy and Corio-Costet, 2004).

Interactions between two trophic levels are predicted to have effects on the third trophic level (Price et al., 1980; Fritz, 1995; Müller et al., 2005). In this study system, the effect of pathogen infection on plants may provide a significant biotic factor which indirectly modulates the outcome of interspecific interactions at higher trophic levels. We observed that asymptomatic infection by *B. cinerea* has subsequent effects on our model herbivore, and so we may expect consequent changes at higher trophic levels. Indeed, we demonstrate that the parasitoid *A. colemani* was negatively affected by asymptomatic infection, exhibiting a reduction in parasitism rate, growth rate, and starvation resistance, suggesting that the consequences of such hidden infections may ramify through trophic interactions, although we do not know if this is a simple consequence of host size reduction, or some more subtle change in plant/pathogen chemistry. Nevertheless, asymptomatic plant pathogen infection may alter patterns of plant-herbivore and host-parasitoid interactions in natural and agro-ecosystems, with implications for biological control programmes.

Variation in plant quality resulting from differences in plant genotype plays an important role in shaping arthropod community structure (Johnson and Agrawal, 2005; Mooney and Agrawal, 2008). Such multitrophic effects may be mediated by either the nutritional, defensive, or physical qualities of the host plant. What is of interest here is whether there is an interaction between host plant variety and infection status. We found that the performance of both the aphids and their parasitoids differed between plant varieties, with the performance being better on lettuce variety Tom Thumb than on Little Gem. In part the latter may be the result of the differing growth forms (Little Gem is a relatively tight-headed Cos lettuce, while Tom Thumb is more open in structure) affecting parasitoid foraging behaviour. While this explains differences between the varieties, the effect of asymptomatic infection remains. In addition, we used one clone of *M. persicae* in this study. It is worth considering the interaction between variation among clones in traits such as resistance to parasitoid attack, escape behaviour, or competitive ability, and the presence of hidden pathogen infection would be instructive, as these factors affect aphid interactions in the field (Hazell et al., 2005; Hazell et al., 2006; Hazell and Fellowes, 2009). Unpicking the effects of such factors will prove worthwhile if we are to better understand the potential effects of asymptomatic infection on insect pest management.

Whether because they are assumed, ignored or dismissed, the ecological consequences of plant–pathogen-insect interactions and their importance is poorly understood. Here, we present experimental evidence that demonstrates that asymptomatic infection by a widespread, economically important plant pathogen can play an important role in determining the interaction between insect herbivores and their natural enemies. A very wide diversity of plant species host infections by *B. cinerea*, which may cause no visible symptoms on the plant at the initial time of infection (Shaw et al., 2016). This study suggests that hidden plant infections may have considerable direct and indirect effects on the structuring of species assemblages in both natural and agro-ecosystems. A challenge for the future is to consider how such effects may scale up to the larger processes that help determine insect population dynamics. Latent and asymptomatic infection by plant pathogens are likely to be widespread in nature; this is an early step in developing an understanding of the consequences of such hidden infections in the field.

Chapter 3: Symptomless host plant infection by the pathogen *Botrytis cinerea* alters the behaviour of aphids and their natural enemies

3.1. Abstract

Plant pathogen infection plays an important role in determining the dynamics and structure of insect communities. The infection either obvious or asymptomatic may influence the direct and indirect interactions between the plant and other organisms at higher trophic levels. As a consequence, these interactions could shape community structure and influence the assemblage of other species within the plant ecosystem. Little is understood as to how asymptomatic pathogen infection can induce changes in insect herbivores and parasitoid behaviour, and whether this varies among plant varieties. Here we report evidence that volatiles produced by asymptomatic *Botrytis cinerea* infected lettuce plants alter host selection by aphid *Myzus persicae* and its parasitoid *Aphidius colemani*, but does not affect behaviour of the ladybird *Adalia bipunctata*. When given a choice in an olfactometer, aphids preferentially chose uninfected lettuce plants of one variety (Tom Thumb) but showed no preference with the second (Little Gem) variety. Parasitoids preferentially attacked aphids on uninfected plants, irrespective of host plant variety, but ladybirds did not show any preference. We also show that the pathogen infected-plant even asymptomatic influenced the fitness of insect herbivores. Aphids feeding on asymptomatic *Botrytis* infected plants are more exposed to predation than aphids feeding on the healthy plants. Since the behaviour of the natural enemies of insect pests is an important factor in determining the efficacy of biological control, these findings have implications for disease and biological control management.

Key words: aphid, parasitoid, predator, ladybird, escape behaviour, host choice, lettuce

3.2. Introduction

Since insect herbivores depend on plant resources, optimal host plant selection is crucial in determining the success of their offspring (Heisswolf et al., 2005; Videla et al., 2012; Prager et al., 2014). Herbivorous insects can discriminate between potential host plants based on their nutritional quality (Janz and Nylin, 1997; Marchioro and Foerster, 2014), and plant quality greatly affects their performance and behaviour (Bernays, 2001; Wetzel et al., 2016). Variation in plant quality may affect herbivore fitness and reproductive strategies (Stadler et al., 1994; Awmack and Leather, 2002), influencing both their own growth and survival and also the fitness of their offspring (Mousseau and Fox, 1998). In turn, this will affect the quality of insect herbivores as prey or hosts to predators and parasitoids (Morris and Fellowes, 2002; Müller et al., 2005; Hazell et al., 2006). Variation in host plant quality results from differences in morphology, available nutrient content and the presence of defensive metabolites (Moore et al., 2014; Wiggins et al., 2016). Such variation may result from heritable differences between individuals, populations and species. However, variation in host plant quality may also be determined by factors such as water or nutrient availability (Müller et al., 2005) , or due to previous attack by other herbivore species (War et al., 2012). One of the most widespread causes of change in plant quality is as a result of infection by plant pathogens (Mittler, 2002; Korves and Bergelson, 2003; Berger et al., 2007; Dodds and Rathjen, 2010).

Infection by plant pathogens is common in both natural and agricultural ecosystems and can have a severe impact on plant health, where the infection may alter plant physiology, plant survival, and biomass production (Rottstock et al., 2014). It is estimated that annual agricultural losses due to plant pathogen infection in the United States alone amounts to c. US$33bn (Jose et al., 2013). For example, *Botrytis cinerea* Persoon: Fries s. lato, the causative agent of grey mould on over 200 plant species causing losses up to $100 billion annually worldwide (Boddy, 2015). Although there are fungicides to control this ubiquitous pathogen, many classes of fungicides have failed due to its genetic plasticity (Williamson et al., 2007) and their ability to infect the plant without showing the symptom of infection (van Kan et al., 2014; Shaw et al., 2016).

Infection by plant pathogens leads to changes in plant primary and secondary metabolism (Berger et al., 2007) which can have substantial ecological consequences (Termorshuizen, 2016). Pathogen infection may trigger defence mechanisms altering morphological, biochemical, and molecular structures to counter the effects of attack (Dangl and Jones, 2001; Hématy et al., 2009; Dodds and Rathjen, 2010), and thus can modify plant quality. The most rapid plant defence reaction after pathogen attack is the production of Reactive Oxygen Species (ROS), primarily in the form of superoxide, hydrogen peroxide and hydroxyl radical (H_2O_2) (Liu et al., 2010), which leads to the oxidative destruction of cells (Kaushik and Roychoudhury, 2014). This can lead to the hypersensitive response (HR), preventing further spread of the infection (Govrin and Levine, 2000). In addition to the production of ROS, the pathogen infection also produces compounds that are similar to plant hormones such as auxins, cytokinins (CKs), gibberellic acids (GAs), ethylene (ET), abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) (Chanclud and Morel, 2016). These hormones are crucial in controlling plant growth and development as well as in triggering plant defence mechanisms during pathogen invasion (De Vleesschauwer et al., 2013; Giron et al., 2013; De Vleesschauwer et al., 2014; Spence and Bais, 2015). Among these hormones, the SA and JA trigger defences against pathogen infection in an antagonistic manner (Thaler et al., 2012a; Gimenez-Ibanez and Solano, 2013). The JA pathway is primarily induced by and is effective in mediating resistance against chewing herbivores and necrotrophic pathogens, whereas the SA pathway is primarily induced by and effective in mediating resistance against sucking insects and biotrophic pathogens (Glazebrook, 2005; Walling, 2008; Thaler et al., 2010).

In certain conditions, the pathogen infection may remain undetected without causing any visible disease symptoms (Barnes and Shaw, 2002; Rajaguru and Shaw, 2010). For example, members of the fungal family Botryosphaeriaceae (Botryosphaeriales, Ascomycetes) reside in many plant hosts as a latent infection, and typically is only evident under conditions of host stress, when disease symptoms develop (Slippers and Wingfield, 2007; Marsberg et al., 2017). Members of this family such as *Sphaeropsis sapinea* has been isolated from the symptomless shoots of *Pinus* (Flowers et al., 2001), *Fusicoccum* sp from healthy shoots of pistachio (Mila et al., 2005), and latent *Neofusicoccum* infection has been found in many host plant species (Sakalidis et al., 2011; Czemmel et al., 2015; McGregor et al., 2016). Recently, *B. cinerea* has received increasing attention because of its ability to invade hosts without showing any symptoms of infection. This pathogen was found to asymptomatically infect hybrid primula (Barnes and Shaw, 2003), lettuce (Sowley et al., 2010; Shaw et al., 2016) and grapes (Pezet et al., 2003; Sanzani et al., 2012). The presence of asymptomatic infection, is not visible, and can be detected by isolating the pathogen on *Botrytis* Selective Media Agar (Edwards and Seddon, 2001). Since asymptomatic pathogen infections are likely to be common (Stergiopoulos and Gordon, 2014; Shaw et al., 2016), the importance of this type of infection should not be overlooked, although little work has been done to investigate the effects on plant physiology and chemistry (Barnes and Shaw, 2002, 2003; Sanzani et al., 2012).

Plant pathogen infections can have effects that extend well beyond the direct effects that they exert on the physiology and life-history of their host plants. As noted above, plant pathogens can alter host plant quality as perceived by their herbivores (Thaler et al., 2010; Lazebnik et al., 2014), and also with their natural enemies (Van Der Putten et al., 2001; Ponzio et al., 2014). The nature of the pathogen-altered interactions between plants, their insect herbivores and their natural enemies are complicated and depend on the species involved. Susceptibility to insects that feed on pathogen-infected plants are determined by a wide variety of mechanisms, including changes in apparency (Mauck et al., 2012), nutritional quality (Awmack and Leather, 2002) and defence chemistry (Stout et al., 1999). However, the influence of asymptomatic pathogen infection on interactions between plants, their herbivores and natural enemies is not known. In my previous work (Chapter 2), we showed that asymptomatic infection of two varieties of lettuce plants reduce plant quality, and results in reduced aphid size and population growth rate, and reduced the size and attack rate of aphid parasitoids in the laboratory. Such hidden pathogen infection may also influence insect behaviour. For example, pathogen infection interferes with plant volatile emission profiles (VOCs) (Mann et al., 2012; Groen et al., 2016) or visual cues of insects if infection alters plant morphology (Hodge and Powell, 2008b). Both of these cues play an important role in mediating ecological interactions among plants and insects (Campbell and Borden, 2006; Stenberg and Ericson, 2007; Heisswolf et al., 2007; Kuhnle and Muller, 2011), particularly in terms of host plant location and choice.

My previous work suggests that asymptomatic pathogen infection has direct consequences for insects at higher trophic levels, but we do not know if asymptomatic infections alter insect behaviour, particularly in terms of host/host plant choice, or escape behaviour. If this were the case, it would suggest that asymptomatic infection not only directly influences interactions in tritrophic systems, but that it could also indirectly affect interactions by modulating the behaviour of species at higher trophic levels. Using two varieties of lettuce as host plants, we asked if the presence of asymptomatic infection by *B. cinerea* affect (i) aphid host plant preference; (ii) parasitoid searching behaviour and host preference; (iii) predator prey preference, and (iv) the escape behaviour of aphids exposed to foraging predators.

3.3. Materials and methods

3.3.1. Plants

Two varieties of the lettuce *Lactuca sativa* (Tom Thumb, Little Gem; Thompson and Morgan, Suffolk, UK) were used to assay preference behaviour. Only Little Gem was used in the escape behaviour experiment. The methods for growing, selecting and checking the infection status of plants for the experiment is as described in Chapter 2.

3.3.2. Insects

The insects used in this experiment were the aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae), its parasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae) and ladybird predator *Adalia bipunctata* Linnaeus (Coleoptera: Coccinellidae). All insects for the preference behaviour experiment were reared on Brussels sprouts *Brassica oleracea,* and aphids for the escape behaviour experiment were reared on the lettuce variety Little Gem in rectangular clear plastic cages (20cm X 20cm X 15cm) fitted with the cotton mesh windows. The rearing process was conducted in CE rooms at 20 ± 1 °C, L16: D8 photoperiod and 60-70 % R.H. Insects were reared on Brussels sprouts to ensure that they were naive (no maternal influences or learning experience affecting preference behaviour). Parasitoids were reared on the aphid *M. persicae.* To obtain a standardised age of each parasitoid, mummies were collected and isolated before parasitoid emergence, in corked glass vials (8 x 60 mm). Emerging females used for the experiment were kept isolated after emergence, fed with a honey-water solution and then mated. Parasitoids were exposed to the experience of oviposition to enhance responsiveness to the host location cues. All parasitoids tested in the experiment were 48 hours old, and experienced solely with *M. persicae* reared on Brusselss sprouts. The *A. bipunctata* were purchased from Green Gardener (Yarmouth, UK) and reared in the laboratory for one week before they were used for the experiment. Ladybirds were fed with *M. persicae* and prior to the experiments, beetles were starved for 12 hours.

3.3.3. Preference behaviour experiment

The olfactometer: The trials were conducted in a four arm olfactometer (BLM4-300, Shanghai Billion Instrument Co. Ltd, Shanghai, China). The internal diameter of the olfactometer was 200 mm and 15 mm deep. The exposure arena was divided into five different zones; one central and four arm zones. Each arm has an inlet to which odours were applied. All four of the olfactometer arms were connected by the silicone tube to a plastic container which contained the different plants whose odours were tested. A vacuum pump was set to exhaust air from the centre of the arena at a flow of 250 ml/min per arm. Airtight seals at the inlet of each jar, were used to avoid external odours during the experiments. Before entering the tunnel, the air was filtered by a 5cm thick layer of activated charcoal. Odour-emitting samples were place in a 3L plastic container linked by plastic tube to the relevant olfactometer arm.

The bioassay: The preference behaviour experiment consisted of exposing aphid, parasitoid and predators to stimuli derived from simultaneous odour sources: (1) uninfected plant (2) asymptomatic-infected plant and (3) empty arms. The location of the tested plant in the olfactometer was randomly exchanged for each replicate to avoid physical bias. The olfactometer arena and its arms were cleaned with 70% alcohol and rinsed with distilled water between each replicate. Tests were replicated 30 times for each insect, using different insects and plants in every trial. Twenty aphids, or twenty parasitoids, or one predator were used for each replicate in this bioassay. Plants used for parasitoids and predator preference behaviour were infested with 200 adult aphids on each plant to encourage searching behaviour. The olfactometer was run for five minutes before each trial began to ensure a good circulation of odours. Insect preference was deemed to have been made when the insects fully left the arena and entered one of the collecting jars. This bioassay was carried out in a CE room at 20 ± 1 °C and 60-70% R.H in the dark to eliminate any possible visual cues.

3.3.4. Escape behaviour experiment

The escape behaviour of *M. persicae* fed on uninfected and asymptomatic-infected plants was assessed in a CE room at 20 ± 1 °C and 60 - 70% R.H. Forty adult aphids were placed on each experimental plant. The lettuce variety Little Gem was used as this plant has a more open growth form, and fewer refugia for test aphids. Aphids were exposed to one foraging *A. bipunctata* or an artificial stimulus as a control. A single ladybird was released at the base of the lettuce plant and allowed to search for aphids for 5 minutes. If experimental ladybird failed to forage, the trial was stopped and the replicate was discarded and replaced. For the control treatment, plants were slowly shaken by hand for five seconds to give an artificial stimulus, in an attempt to replicate normal plant movement. Aphids which escaped by dropping off the plant were recorded. Each treatment was replicated 30 times.

3.3.5. Statistical analyses

The preference behaviour of the aphids, predatory ladybirds and parasitoid wasps towards the experimental target were calculated. The preference of insects towards plant and blank odour were analysed using a generalised linear model (glm) with quasibinomial error, and either insect choosing infected, uninfected or blank odour were analysed using multinomial logistic regression analysis (UCLA, 2017). In the aphid and parasitoid preference behaviour bioassay, not all insects made a choice. To solve that problem, data of insect preference was counted as the number of insect heading to an odour source where the total number of insect that made a choice was treated as a weighting factor (Crawley, 2014). The escape behaviour of aphids was analysed using a generalized linear model (glm) with quasibinomial family. All analyses were carried out using R-statistical software version 3.4.0 (R Core Team, 2017).

3.4 Results

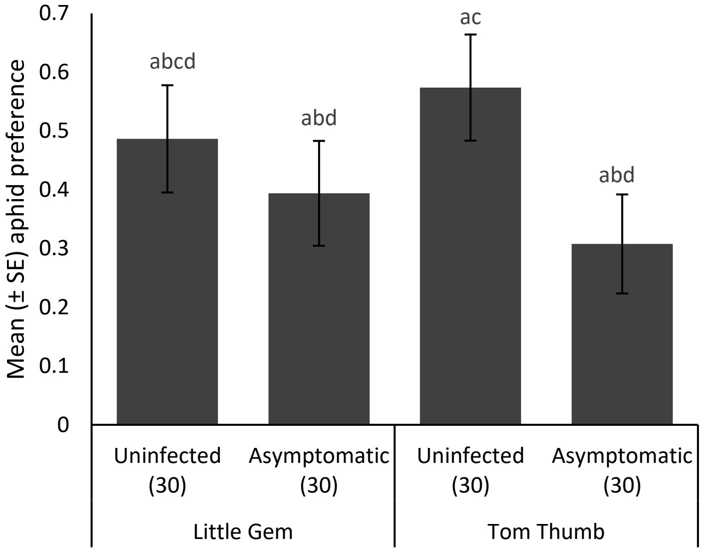
3.4.1. Preference behaviour

Aphid choice. Aphids preferred to move towards plant odours compared to the blank arm. When choosing between different host plant possibilities, aphids were significantly more likely to choose uninfected Tom Thumb than asymptomatically infected Tom Thumb. However, aphids showed no preference between uninfected and asymptomatic Little Gem (Table 3.1; Figure 3.1).

Table 3.1: Summary of effects of plant infection status on aphid host plant preference behaviour on two lettuce varieties. N = 30 for each treatment. Significant values are in bold.

Plant variety	Choice	Coefficient t value ± SE	P
Tom Thumb	**Plant vs Blank**	**8.394 ± 0.246**	**< 0.001**
	Infected plant vs Blank	**-0.926 ± 0.191**	**< 0.001**
	Uninfected plant vs Blank	**-1.549 ± 0.178**	**< 0.001**
	Infected plant vs Uninfected plant	**-0.623 ± 0.126**	**< 0.001**
Little Gem	**Plant vs Blank**	**8.588 ± 0.223**	**< 0.001**
	Infected plant vs Blank	**1.190 ± 0.205**	**< 0.001**
	Uninfected plant vs Blank	**1.402 ± 0.200**	**< 0.001**
	Infected plant vs Uninfected plant	0.211 ± 0.133	0.112

Figure 3.1: Mean ± SE proportion of aphids *Myzus persicae* orientating in an olfactometer trial towards two varieties (Little Gem, Tom Thumb) of uninfected or asymptomatically infected lettuce plants. Number of replicates per treatment is shown about each bar.

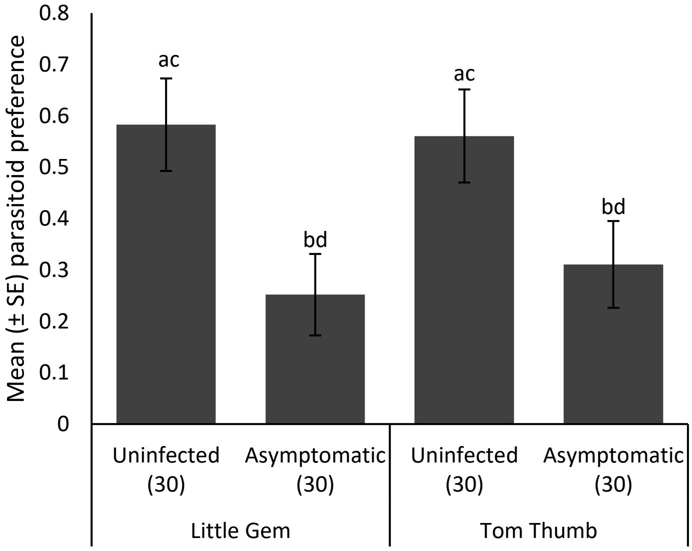


Parasitoid choice. *Aphidius colemani* showed a preference towards aphid/plant odour sources as opposed to a blank odour sources for both Tom Thumb and Little Gem. When given a preference between infected and uninfected plants, parasitoids significantly preferred aphids on uninfected Tom Thumb and uninfected Little Gem compared to their respective infected plants (Table 3.2; Figure 3.2).

Table 3.2: Summary of effects of plant infection status on parasitoid host preference behaviour with aphids reared on two lettuce varieties. N = 30 for each treatment. Significant values are in bold.

	Response variable	Coefficient t value ± SE	P
Tom Thumb	**Plant vs Blank**	**8.621 ± 0.222**	**< 0.001**
	Infected plant vs Blank	**0.882 ± 0.198**	**< 0.001**
	Uninfected plant vs Blank	**-1.472 ± 0.184**	**< 0.001**
	Infected plant vs Uninfected plant	**-0.590 ± 0.133**	**< 0.001**
Little Gem	**Plant vs Blank**	**8.74 ± 0.185**	**< 0.001**
	Infected plant vs Blank	**0.420 ± 0.194**	**0.030**
	Uninfected plant vs Blank	**1.259 ± 0.170**	**< 0.001**
	Infected plant vs Uninfected plant	**-0.838 ± 0.146**	**< 0.001**

Figure 3.2: Mean ± SE proportion of parasitoids *Aphidius colemani* orientating in an olfactometer trial towards aphids on two varieties (Little Gem, Tom Thumb) of uninfected or asymptomatically infected lettuce plants. Number of replicates per treatment is shown below each bar.



Predator choice. Significantly more ladybirds moved towards the Tom Thumb odour source (mean/SE: 0.733 ± 0.082) than a blank odour source (0.266 ± 0.082), and to Little Gem (mean/SE: 0.633 ± 0.089) than a blank odour (mean/SE: 0.366 ± 0.089). Asymptomatic pathogen infection did not influence the preference behaviour of *A. bipunctata* [Uninfected Tom Thumb (0.433 ± 0.092), Infected Tom Thumb (0.300 ± 0.085), Blank (0.266 ± 0.082); Uninfected Little Gem (0.366 ± 0.089), Infected Little Gem (0.266 ± 0.082), Blank (0.366 ± 0.089] (Table 3.3).

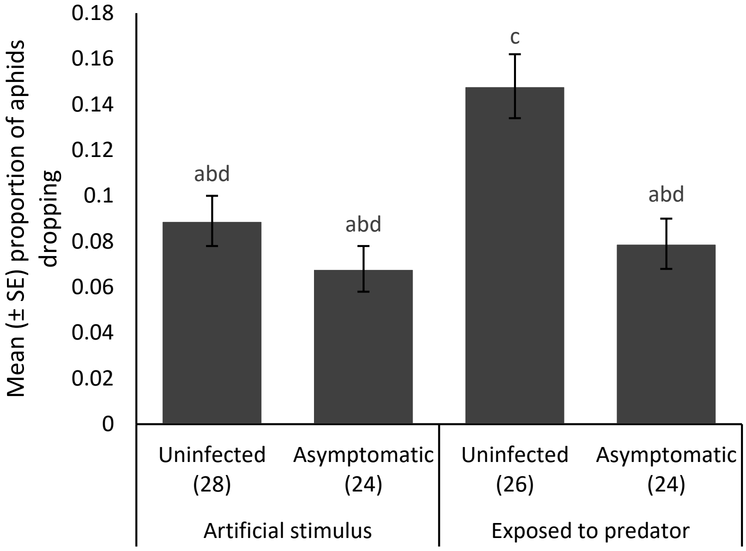
Table 3.3: Summary of effects of plant infection status on predator preference behaviour when offered prey reared on two lettuce varieties. N = 30 for each treatment. Significant values are in bold.

	Response variable	Coefficient z value ± SE	P
Tom Thumb	**Plant vs Blank**	**3.465 ± 0.583**	**< 0.001**
	Infected plant vs Blank	0.286 ± 0.573	0.774
	Uninfected plant vs Blank	1.343 ± 0.553	0.179
	Infected plant vs Uninfected plant	-1.067 ± 0.542	0.286
Little Gem	**Plant vs Blank**	**2.040 ± 0.535**	**0.041**
	Infected plant vs Blank	-0.830 ± 0.560	0.407
	Uninfected plant vs Blank	-0.001 ± 0.533	0.999
	Infected plant vs Uninfected plant	-0.830 ± 0.560	0.407

3.4.2. Aphid escape behaviour.

The proportion of aphids that dropped when *A. bipunctata* was present ($F_{1, 99} = 9.23$, $p < 0.003$) was significantly higher than the proportion dropped when the plant was shaken. There was a significant effect of plant pathogen infection on the proportion of aphids falling from the plant ($F_{1, 100} = 13.52$, $p < 0.001$) (Figure 3.3), with the aphids fed on the uninfected plants dropping more frequently than those fed on asymptomatic-infected plants.

Figure 3.3: Mean ± SE proportion of *Myzus persicae* showing escape behaviour (dropping) in response to an artificial stimulus (gentle shaking) and the presence of a foraging ladybird, *Adalia bipunctata* on uninfected or asymptomatically infected lettuce plants var. Little Gem).



3.5 Discussion

Asymptomatic infection by plant pathogens is likely to be widespread, yet we have no understanding of its effects on species interactions at higher trophic levels. Here, I show that hidden infection of lettuce by the economically damaging plant pathogen *B. cinerea* affects the behaviour of a widespread pest species, the aphid *M. persicae*, and two of its enemies, the ladybird *A. bipunctata* and the parasitoid *A. colemani*, a species widely used as a biocontrol agent. Furthermore, these effects varied with host plant variety. Aphids preferentially chose uninfected Tom Thumb variety plants over asymptomatically infected ones, but did not distinguish between infected and uninfected Little Gem plants. We found that ladybirds did choose aphids/plants over empty controls, but did not distinguish between infected and uninfected plants/aphids. In contrast, parasitoids did prefer plants with aphids over controls, and furthermore showed a strong preference for uninfected plants and their aphids over asymptomatically infected plants and aphids. This strongly suggests that a) plants vary in odours produced, and b) that ability to distinguish host plant infection status can be ranked parasitoids > aphids > ladybirds. Finally, we evaluated aphid predator escape behaviour, and found that aphids were less likely to attempt escape from foraging ladybirds when reared on asymptomatically infected plants. The novelty of this work lies in the demonstration of the importance of asymptomatic pathogen infection in altering the behaviour of insect herbivores and their natural enemies, hence may affect the distribution and assemblage of insects in the natural ecosystem. Since the *B. cinerea* is ubiquitous, these findings form the basis of a new perspective on how plant pathogens may influence tritrophic systems.

The effects of plant pathogen infection on insects are well documented (e.g. Lattanzio et al., 2006; Mauck et al., 2012; Bitas et al., 2013; Mauck et al., 2016) but most studies have overlooked the effect of plant disease severity on the performance and behaviour of the insect involved. In this study, we use *B. cinerea* as a model organism as this pathogen can spread throughout the plant without showing any evident symptoms (Sowley et al., 2010; van Kan et al., 2014). Generally, pathogen infection will induce changes in the volatile emission profiles of plants (Mann et al., 2012; Becker et al., 2014; Groen et al., 2016). Most insects depend on olfaction to use this information to locate their food source and to choose among potential plant resources (Dicke, 1999; Oppenheim and Gould, 2002; Arimura et al., 2005; Clavijo McCormick et al., 2012; Zakir et al., 2013; Clavijo Mccormick et al., 2014). The composition of volatiles released can vary widely between different plant varieties, their health status and type of stress they are exposed to (Kesselmeier and Staudt, 1999; Holopainen and Gershenzon, 2010). This variability may inform foraging insects about the identity and quality of the host plants (Van Der Putten et al., 2001; Pieterse and Dicke, 2007; Sisterson, 2008; Mauck et al., 2016).

Changes in plant quality affects the life history of insect herbivores which affects the behaviour of the aphids and their parasitoids (Biere and Bennett, 2013). For example, some aphids show preferences for pathogen infected plants (Jiménez-Martínez et al., 2004; Srininvasan et al., 2006; Alvarez et al., 2007) and others for healthy plants (Baker, 1960; Blua et al., 1992; Fereres et al., 1999). The black bean aphid (*Aphis fabae*) performance can be increased when feeding on *Botrytis*-infected plants (Zebitz and Kehlenbeck, 1991). However, for *Rhodobium porosum* (Mouttet et al., 2011) and *Aphis fabae* (Al-Naemi and Hatcher,

2013) species, feeding on infected plants reduces fecundity which would explain why here we found a preference of the aphids towards uninfected plants compared with the asymptomatically *Botrytis*-infected plants. Indeed, my previous work (Chapter 2) shows that *M. persicae* produce fewer offspring when reared on infected plants in the laboratory. However, while those aphids produced fewer offspring on both lettuce varieties, we find that aphids are able to distinguish the odour of both varieties from empty controls, but can only distinguish between uninfected and infected Tom Thumb plants. They did not discriminate between uninfected and infected Little Gem plants. This suggests that there are differences in response to infection by the lettuce varieties, leading to differences in volatile profiles, thus affecting aphid host plant choice. Studies show that plants infected with *B. cinerea* and showing symptoms emit a different cocktail of VOCs compared to healthy plants (Jansen et al., 2009), and it would be surprising if this did not differ between plant varieties.

The preference behaviour of the parasitoids was correlated with host suitability for offspring development, referred to as the 'mother knows best' principle (Mayhew, 2001; García-Robledo and Horvitz, 2012). According to this principle, parasitoid females locate the best insect host and/or their habitat to ensure the successful development of their progeny. Theoretically, the pathogen-infected plant will die earlier than an uninfected plant (and aphids reared on such plants are smaller; Chapter 2), so choosing hosts on uninfected plants increases the female's fitness. For example, volatiles of wheat infested by the fungi *Aspergillus sydowii* and *A. versicolor* repelled female parasitoids in an olfactometer experiment (Steiner et al., 2007). However, this is not universal, as peanut (*Arachis hypogaea*) stem infected by the white mould fungus *Sclerotium rolfsii* Saccodes increased wasp attraction to beet armyworms (Cardoza et al., 2003).

Contrary to the preference behaviour shown by the aphid *M. persicae* and its parasitoid *A. colemani*, my predatory insect *A. bipunctata* was not affected by the presence of asymptomatic *Botrytis* infection. Generally, this predatory insect depends on the chemical cues emitted by their potential prey and the plant associated with their prey, each alone or in association (Vet and Dicke, 1992; Francis et al., 2005; Dicke et al., 2009). As an example, the ladybird *Coleomegilla maculata* and lacewing *Chrysoperla carnea* are attracted by the semiochemicals released by prey species and their host plants (Zhu et al., 1999; Abassi et al., 2000). However, the effect of the plant cues on the predatory insect olfactory system may vary among insect species. The ladybird *Coccinella septempunctata* may use methyl salicylate as the olfactory cue for prey location, but *Harmonia axyridis* showed no preference when offered the same plant volatile (Zhu and Park, 2005). *Cyrtorhinus lividipennis* preferred rice plants infected by the pathogenic bacterium *Xanthomonas oryzae* pv. *Oryzae* after the brown planthopper *Nilaparvata lugens* fed, but preferred healthy rice plants without *N. lugens* feeding (Sun et al., 2016). My finding shows the absence of a clear preference of the ladybirds towards uninfected and asymptomatic infected plants, showing the cues emitted by the *Botrytis* infected-plants do not affect the foraging behaviour of this predator.

Asymptomatic infection by *Botrytis* may not affect *A. bipunctata* host choice, but it does affect *M. persicae* escape behaviour from the foraging ladybirds. Antipredator behaviour such as dropping, kicking or walking away from predators are fitness-related (Lind and Cresswell, 2005). Previous work (Chapter 2) showed that aphids reared on asymptomatically infected plants were smaller, had fewer offspring, and showed reduced off-plant survival times. Given the latter, it is not surprising that dropping behaviour (a trade-off between predation risk and of finding a suitable host plant before death through starvation or predation; Losey and Denno, 1998a, 1998b) was reduced. Aphid dropping is therefore a risky and energetically costly antipredator behaviour (Harrison and Preisser, 2016) and when the energetic internal stress of aphids is increased, aphid antipredator responses change from walking away and dropping to kicking behaviour (Villagra et al., 2002). Similar to my findings, the aphids *Acyrthosiphon pisum* and *Uroleucon jaceae* reduce their dropping rate when feeding on low-quality plants (Stadler et al., 1994).

My results show that asymptomatic infection by the economically important plant pathogen *B. cinerea* changes the behaviour of insects at higher trophic levels, and this influence depends on the species and the host plant genotype. Given the ubiquity of *B. cinerea*, this suggests that non-obvious infection can have consequences for interactions between crop pests and biocontrol agents and thus the disease status of plants should be considered as part pest management planning. Beyond this, my work suggests that these previously unconsidered effects could be widespread in plant-herbivore-enemy systems. However, I note that this is a laboratory study, and investigating if these changes to insect-enemy behaviour (this study) and performance (Chapter 2) matter in the field is of considerable value. There is little doubt that pathogen infection can be a highly important ecological driver of changes in patterns of interactions in tritrophic systems. I show that symptoms of disease are not necessary before this pathogen changes the behaviour of a crop pest and its enemies.

Chapter 4: Does asymptomatic infection by the plant pathogen *Botrytis cinerea* affect the structure of naturally formed insect assemblages?

4.1. Abstract

Plant quality varies as a result of many factors (e.g. genetic variation, nutrient availability, plant age, water stress, attack by herbivores and pathogens), and this variation can affect complex multitrophic interactions. However, most studies investigating how multitrophic interactions are affected by plant quality have neglected the influence of hidden (asymptomatic) disease caused by plant pathogens. Asymptomatic pathogen infection will not be readily detected as no overt symptoms appear, but may alter interactions between plants and their herbivores, and herbivores and their natural enemies in the laboratory (Chapters 2 and 3). However, it is not known if hidden pathogens affect interactions in the field. In a large replicated field experiment, I used two varieties of lettuce (Little Gem, Tom Thumb) infected by the ubiquitous plant pathogen *Botrytis cinerea* to examine how insect assemblages were affected by host plant infection status. Infection significantly affects plant quality by reducing chlorophyll content, height and dry mass of the plant, and that these effects differ between the lettuce varieties. The influence of pathogen infection depended on lettuce variety. The diversity and abundance of aphids did not differ between asymptomatically-infected and uninfected Little Gem lettuce, but for lettuce variety Tom Thumb, infection greatly affects the assemblage of aphids. However, both lettuce varieties were less attractive to natural enemies when asymptomatically infected. Considering the effects of asymptomatic pathogen infection on plant-insect systems will help improve our understanding of the consequences of pathogen infection for pest management, biological control and wider insect ecology.

Key words: Aphid, parasitoid, predatory insect, plant disease, multitrophic interaction

4.2. Introduction

Plant pathogens are ubiquitous in nature and their effects are well recognized in agriculture where they cause approximately $540 billion of damage every year (Burdon and Thrall, 2009; Termorshuizen, 2016; Reuters, 2017). The effects of plant pathogen infection can extend well beyond the direct effects that they exert on the physiology and chemistry of their host plant. The presence of disease elicits major phenotypic changes in host plant quality (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012; Barber et al., 2013), such as triggering a change in synthesis or degradation of hormones (Overmyer et al., 2003; Gimenez-Ibanez and Solano, 2013; Yang et al., 2015) and inducing secondary metabolites such as stilbenes and saponins (Elad et al., 2007; Ribera and Zuñiga, 2012; Pusztahelyi et al., 2015). These changes directly and indirectly affect the behaviour and abundance of insect herbivores and in turn their natural enemies (Utsumi et al., 2010; Biere and Bennett, 2013; Tack and Dicke, 2013).

The most obvious consequence of pathogen infection is the presence of disease symptoms on the plant. As an example, necrotrophic pathogens such as *Botrytis cinerea* (grey mould) and *Sclerotinia sclerotiorum* (stem rot) generally kill host cells when they are actively growing to provide themselves with food resources, which result in visible disease symptoms (van Kan, 2006). Plant appearance may influence the visual preferences of insect herbivores (Kuhnle and Muller, 2011; Reeves, 2011). As plant traits and quality are key determinants of the fecundity of insect herbivores (Price, 1999; Awmack and Leather, 2002), changes in host plant quality caused by the presence of a plant microbial pathogen may have consequences for the growth, fitness and behaviour of insect herbivores (Gange et al., 2012; Giron et al., 2013). Chemical defences produced by the plants during pathogen attack (such as antinutritives or toxins) may also suppress the growth and influence the behaviour of herbivores (Tack and Dicke, 2013). Plant volatiles produced by the infected plant may influence how insects locate resources required for nutrition and reproduction as many insects rely on olfactory and visual cues (Felton and Tumlinson, 2008; Beyaert and Hilker, 2014). These changes may have either a positive or negative effect, depending on the characteristics of the insect species concerned (Kaplan and Denno, 2007).

For example, plant conditions can affect the probing behaviour of aphids due to changes in plant properties, chemical contents of the sap, and/or physiological changes (Pegadaraju et al., 2005). Host plant selection by aphids is generally governed by olfactory and visual stimuli (Reeves, 2011; Knolhoff and Heckel, 2014). Aphids might change their plant-host selection behaviour when the plant is damaged or when the quality of the plant changes (Barber et al., 2013; Tack and Dicke, 2013). In turn, changes in aphid behaviour and fecundity are may lead to changes in the assemblage of natural enemies found attacking aphid colonies (Hazell et al., 2006; Hazell and Fellowes, 2009). The availability of prey can affect oviposition and attack rates (Seagraves and Lundgren, 2010; De Rijk et al., 2013), or alter the life-history traits resulting from attacking fewer or poorer quality hosts/prey (Utsumi et al., 2010; Mooney et al., 2012). Not surprisingly, studies have shown that plant pathogen infection can therefore alter the structure of insect communities at higher trophic levels (Marquis et al., 2001; Biere et al., 2002; Kruess, 2002; Bagchi et al., 2014).

Botrytis cinerea Persoon: Fries s. lato is the cause of grey mould disease, a necrotrophic pathogen that affects photosynthesis, reducing plant weight and inducing secondary metabolic defence compounds in plant (Govrin and Levine, 2000; Berger et al., 2004; Lecompte et al., 2010). It is regarded as a model necrotrophic organism, attacking over 200 crop hosts worldwide (Boddy, 2015). Infection can induce activation of jasmonic acid (JA) and ethylene (ET) dependent defence signalling (Glazebrook, 2005), with salicylic acid (SA) induced pathways only becoming important later if the necrotroph starts to behave as a hemi-biotroph (van Loon et al., 2006). Studies of the effects of *Botrytis* infection provide a good example of how interactions between plants and insect herbivores are modified by the presence of plant pathogens. *Botrytis cinerea* alters nutrient value and induces defence reactions of the plant, which then indirectly affect the performance and behaviour of insect herbivores (Al-Naemi and Hatcher, 2013). Plants induce different components of primary and secondary metabolites such as resveratrol and other stilbenes (Keller et al., 2003; Pezet et al., 2003) as well as proanthocyanidins (Jersch et al., 1989), α-Tomatine and saponines and cucurbitans (Van Baarlen et al., 2007) to inhibit *Botrytis* activity. Some metabolites such as saponines influence preference and performance in many different herbivores, including insects (De Geyter et al., 2007). Saponines negatively influenced aphid probing behaviour, reduced the aphids' reproduction and survival, and disturbed the development of the aphids' population (Sylwia et al., 2006). Other phenolic compounds may have negative, neutral, or positive effects on herbivores depending on the feeding guild (Torres et al., 2003; Lattanzio et al., 2006). It can be concluded that *Botrytis* infection may have an important role in plant-insect interactions since it involves antagonistic and/or synergistic cross-talk that may determine the outcome of such interactions (Mouttet et al., 2011; Desneux et al., 2012). As an example, symptomatic infection by this pathogen has an inhibitory effect on the development, survival, and fecundity of individual *Aphis fabae*; these effects increased with lesion or pustule density (Al-Naemi and Hatcher, 2013). In contrast, *B. cinerea* infection has been reported to increase aphid *Aphis fabae* performance (Pruter and Zebitz, 1991; Zebitz and Kehlenbeck, 1991) and the infected host plant provides an improved diet (Mondy and Corio-Costet, 2004).

However, the presence of infection is not always obvious (Newton et al., 2010; van Kan et al., 2014); some plants remain asymptomatic (i.e. there are no evident external signs of infection), and the consequences of asymptomatic infection for organisms at higher trophic levels has received remarkably little attention. Nevertheless, while overt symptoms of disease may be absent, plants do respond to infection. Plant morphology, as well as primary and secondary plant compounds, including emitted volatiles and plant nutrients, are some of the traits that can be altered by asymptomatic pathogen infection of plants (Verhoeff, 1974; Malcolm et al., 2013; Zhou et al., 2016).

Botrytis cinerea is capable of colonizing plants internally, presumably as an endophyte, without causing any evident disease or stress symptoms (van Kan et al., 2014) and has the ability to remain apparently asymptomatic in an infected plant for an indefinite duration (Sowley et al., 2010). Latency can occur at any stage of the plants life and at any stage of pathogen growth (Prusky, 1996). Since *B. cinerea* is capable of colonizing its host plant internally without showing any symptom of infection, this provides an excellent model system for exploring the consequences of such asymptomatic infection for species at higher trophic levels. The lettuce *Lactuca sativa* was used as a model host plant as it can harbour systemic and latent infection of *B. cinerea* (Sowley, 2006).

In this paper, we report the results of a study examining the consequences of asymptomatic infection on the structure of naturally formed insect communities at higher trophic levels. In the natural environment, insects can choose the best host plant for feeding and lay eggs, and so it is important to understand if 'hidden' pathogen infection will affect the insect community. We investigated the effects of asymptomatic, systemic and seed-borne pathogen infection on plant traits and quality, and if this affected recruitment of aphids and their natural enemies in the field, in which insects were left to colonize the plant naturally. We recorded (i) plant traits; (ii) aphid diversity and abundance; and (iii) the abundance and diversity of aphid natural enemies, found on a) control (uninfected); b) infected but symptom-free and c) infected and symptomatic plants.

4.3. Materials and methods

4.3.1. Study system

Lettuce, *L. sativa* (Asteraceae), of two different varieties (Tom Thumb and Little Gem) were used in this study. Each variety, either uninfected or infected with the fungus *B. cinerea*, were grown from a single source of seeds to ensure that all plants used in the experiment were otherwise near identical. *Botrytis*-infected lettuce were grown from seed systemically infected with *B. cinerea* strain B05.10 spores during their flowering stage (following Sowley et al., 2010), while uninfected lettuce was grown from seeds collected from the healthy plant. The mother plants for both uninfected and *Botrytis* infected seeds were grown in separate glasshouses in 2014 to avoid contamination by *Botrytis* spores during the fungal inoculation process. All uninfected varieties showed no infection after diagnostic staining of leaf samples, whereas at least two from three of the stained leaf samples of infected plants from each replicates had fungal hyphae when examined using *Botrytis* selective media agar (BSM) (Edwards and Seddon, 2001).

4.3.2. Establishment of plant materials

Experiments were carried out in May and July 2016. This experiment was repeated as a reflection of the temporal peaks in aphid/natural enemy abundance. Two varieties of uninfected and infected *L. sativa* (Little Gem and Tom Thumb) were grown in 15 cm diameter pots with traditional potting compost (Vitax Grower, Leicester, England) in a glasshouse. Sixty replicates were grown per treatment in each experiment as infection in the *Botrytis*-treated seeds and lack of infection in the control plants is not guaranteed. Two weeks before each experiment started, the infection status of the plants was checked using *Botrytis* Selective Media Agar (BSM). Thirty *Botrytis*-infected/uninfected plants were then selected randomly from the tested plants for use in each experiment. Six-week-old plants free from any symptoms of infection were used in this study. It should be noted that some plants were damage due to the environmental effect such as attacks by slug and deer during the experiment, so final replicate numbers were fewer than 30 per experiment.

The plants were randomly placed in a grid pattern in a field site within the experimental grounds, at the University of Reading, U.K (51.4414° N, 0.9418° W). The site was surrounded by crop and ornamental plants (e.g. broad bean and strawberry) with naturally occurring populations of aphids, as well as nearby gardens, glasshouses and the University buildings. The plants were approximately 1.7 meters apart to reduce direct competition between plants in the different treatments. Plants were watered as required and any weeds occurring were removed. Each plant pot was placed in a shallow plastic dish to minimize the effect of water stress or saturation.

The number of aphids, parasitoid mummies and predatory insects present on each plant was recorded every two days until all aphid colonies died, which took approximately 28 days. The mummies, predatory insects and a sample of aphids observed from each species were collected and identified in the laboratory. If more than one mummy was found on the plant, at least half of them (randomly chosen) were left for up to 72 hours on the plant to allow for hyperparasitoid attack. Prior to harvest, pathogen infection status of the plants (either symptomatic or asymptomatic) was recorded. We categorised “symptomatic plants” as plants that showed either restricted or dry lesions, or spreading soft rots with or without the appearance of conspicuous sporulating colonies (Elad et al., 2007), while “asymptomatic plants” were categorized as a plant grown from the infected seed but did not shows any of above symptoms.

Plant traits were measured at the end of experiment. Chlorophyll content was measured on three different randomly-chosen leaves of each plant replicate, using a handheld Chlorophyll Meter (Model atLeaf; FT Green LLC, Delaware, USA) before the plant harvested. The height of the plants was measured on the first day and on the final day of each experiment. The plants (including the roots) were harvested and dried in an oven at 75°C until reaching constant mass (which takes ~48 h), they were then weighed using an electronic balance (Sartorius, LC 6200S, Goettingen, Germany). The ratio of the root: shoot was measured by dividing the dry weight of shoot per root for each plant. The shoot section is above ground and includes the leaves and stems while the root section includes those parts of the plant below ground, such as the roots.

4.3.3. Infection status

The presence of *B. cinerea* in the plant body was determined twice using BSM agar; before the experiment started to choose suitable plants for each treatment and before the plant harvested to determine the infection status of the plant at the end of experiment (uninfected, infected but asymptomatic or infected and symptomatic). Three 1 cm diameter of mature leaf samples with no visual sign or symptoms of damage were randomly harvested at the end of the experiment from each experimental plant. Leaf samples were first disinfected with 70% ethanol for 1 min, followed by 1 min in 20% bleach solution (Domestos, Unilever; 5% NaOCl in alkaline solution with surfactants) and then rinsed three times in sterile distilled water and allowed to dry. This was shown by Barnes (2002) to remove all surface inoculum, whether dusted or soaked in. The leaf disk then was plated on BSM agar and incubated at 18-20°C for at least 10 days in an incubator with alternating UV-A light (12h/day) and dark (12h/day) to determine the presence or absence of *B. cinerea*. Confirmation of *B. cinerea* presence was based on the sporulation of the pathogen and morphological observation of colonies under a high-performance stereomicroscope (Leica, MZ9.5, Texas, USA) where the presence of *B. cinerea* was confirmed by the BSM staining brown.

4.3.4. Statistical analysis

All data were analysed using R-statistical software version 3.4.0 (R Core Team, 2017). Data of response variables were transformed when necessary to meet the assumptions of normality and homoscedasticity. Linear mixed effects models with a restricted maximum likelihood method were calculated to investigate the influence of plant variety and plant pathogen infection on the plant traits (chlorophyll content, dry weight, plant height and shoot: root ratio). The count data for aphids, parasitoid mummies, and predators were pooled and the cumulative number of aphids was analysed using generalized linear mixed models using the glmmADMB package and negative binomial family as the data were over-dispersed (Crawley, 2014). The effects of plant variety, infection status and the cumulative number of aphids on the numbers of predators and parasitoid mummies collected were analysed using glmer.nb with a Poisson distribution, where aphid number was treated as a covariate. The action of secondary parasitoid on mummies found on the plant was also investigated by using similar analysis with the number of mummies was treated as a covariate. In all the analyses, time of experiment was treated as a random effect. Significance of differences between means values were determined by using LSmeans and separation by post-hoc Tukey tests using plant variety and infection status as explanatory variables. Species diversity was estimated by the Shannon diversity index using the vegan package (Oksanen, 2017).

4.4. Results

4.4.1. Plant life-history traits

Lettuce variety Tom Thumb and Little Gem are differed in their physical traits (Table 4.1). Overall, infection by *B. cinerea* (either symptomatic or asymptomatic) substantially reduced chlorophyll content, plant height and shoot: root ratio of lettuce variety Little Gem (Table 4.2). There were no significant differences in the plant traits for uninfected and asymptomatic Tom Thumb. However, the symptomatic Tom Thumb were found to have a lower chlorophyll content and were shorter than uninfected Tom Thumb (Table 4.2).

4.4.2. Aphids

The cumulative number of aphids counted was 26,427 individuals, consisting of *Myzus persicae* (Sulzer)*, Myzus ornatus* (Laing)*, Macrosiphum euphorbiae* (Thomas)*, Acyrthosiphon lactucae* (Passerini) and *Aphis fabae* (Scopoli) (Table 4.3). The diversity of aphids was highest on the uninfected Tom Thumb (1.24), followed by infected Little Gem (1.09), infected Tom Thumb (1.00) and uninfected Little Gem (0.94) (Table 4.3). *Acyrthosiphon lactucae* (45.76%) and *M. euphorbiae* (34.77%) were the most abundant species of aphid observed on the plants. All five species of aphid infested the Tom Thumb variety and uninfected Little Gem. However, *A. fabae* and *M. ornatus* were not observed on infected Little Gem.

Table 4.1: Summary of effects of plant variety and *B. cinerea* infection status on plant traits following analysis. Significant values are in bold.

Plant traits	Explanatory variable	Coefficient t value ± SE	P
atLEAF value	**Intercept**	**8.026 ± 3.569**	**<0.001**
	Variety	**-3.040 ± 1.506**	**0.002**
	Uninfected	**4.946 ± 1.329**	**<0.001**
	Symptomatic	-1.579 ± 1.596	0.116
	Variety-Uninfected	**-2.785 ± 1.886**	**0.006**
	Variety-Symptomatic	-1.249 ± 2.157	0.213
Shoot: root	**Intercept**	**26.912 ± 0.065**	**<0.001**
	Variety	-0.244 ± 0.069	0.807
	Uninfected	**6.540 ± 0.061**	**<0.001**
	Symptomatic	-1.220 ± 0.073	0.224
	Variety-Uninfected	**-4.084 ± 0.087**	**<0.001**
	Variety-Symptomatic	1.840 ± 0.099	0.067
Dry weight (g)	**Intercept**	**19.884 ± 0.918**	**<0.001**
	Variety	**-5.174 ± 0.771**	**<0.001**
	Uninfected	-1.703 ± 0.680	0.090
	Symptomatic	-1.333 ± 0.816	0.184
	Variety-Uninfected	1.532 ± 0.966	0.127
	Variety-Symptomatic	0.717 ± 1.104	0.474
Plant height (mm)	**Intercept**	**12.322 ± 11.418**	**<0.001**
	Variety	**-11.414 ± 4.449**	**<0.001**
	Uninfected	**5.927 ± 3.928**	**<0.001**
	Symptomatic	1.032 ± 4.717	0.303
	Variety-Uninfected	**-3.337 ± 5.573**	**0.001**
	Variety-Symptomatic	**-2.164 ± 6.373**	**0.031**

There was no significant difference in aphid abundance between plant varieties (Table 4.4). The number of aphids on asymptomatic-infected and symptomatic-infected Tom Thumb was lower than that found on uninfected Tom Thumb (Figure 4.1). The asymptomatic infection of *B. cinerea* on Little Gem did not significantly affect the number of aphids when compared with the uninfected plant (Figure 4.1). The number of aphid on Little Gem was significantly reduced when the plant shows symptom of *Botrytis* infection.

Table 4.2: The plant traits of uninfected, asymptomatic and symptomatic *L. sativa* varieties Tom Thumb and Little Gem. The mean (± S. E) with same letters showed there are no significant differences between treatments.

Treatment	n	atLEAF value	Plant height (mm)	Dry weight (g)	Shoot: root
Uninfected Little Gem	38	$34.51\pm0.72_{a}$	$161.68\pm3.00_{a}$	$17.25\pm0.69_{abc}$	$2.14\pm0.07_{a}$
Asymptomatic Little Gem	25	$26.89\pm0.79_{bcd}$	$135.02\pm5.40_{bc}$	$18.63\pm0.53_{abc}$	$1.73\pm0.03_{bcdef}$
Symptomatic Little Gem	20	$27.82\pm2.07_{bcde}$	$151.03\pm3.95_{bc}$	$16.81\pm0.50_{abc}$	$1.68\pm0.03_{bcdef}$
Uninfected Tom Thumb	44	$24.93\pm0.82_{bcde}$	$93.10\pm2.42_{de}$	$14.69\pm0.25_{def}$	$1.77\pm0.01_{bcdef}$
Asymptomatic Tom Thumb	22	$23.15\pm1.39_{cde}$	$86.93\pm3.10_{def}$	$14.46\pm0.42_{def}$	$1.72\pm0.01_{bcdef}$
Symptomatic Tom Thumb	26	$19.37\pm1.45_{f}$	$82.66\pm2.98_{ef}$	$13.86\pm0.41_{def}$	$1.83\pm0.04_{bcdef}$

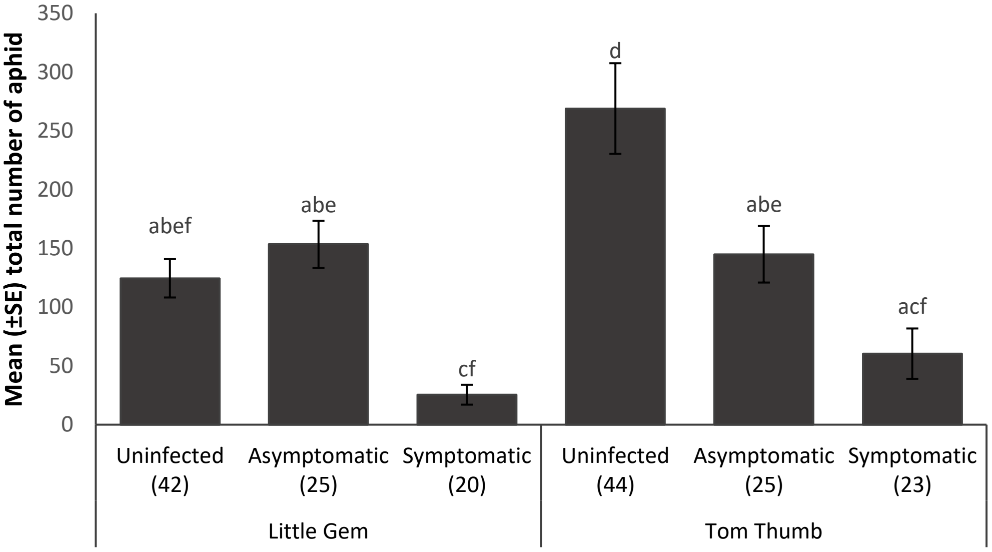
Table 4.3: Counts of insect species found on the plants. Plant treatment, ULG: Uninfected Little Gem, ALG: Asymptomatic Little Gem, SLG: Symptomatic Little Gem, UTT: Uninfected Tom Thumb, ATT: Asymptomatic Tom Thumb, STT: Symptomatic Tom Thumb. Number of plants as shown below each treatment heading.

Species	ULG (42)	ALG (25)	SLG (20)	UTT (44)	ATT (22)	STT (26)	Total
Herbivore			Number of insects				
Myzus persicae	319	1252	81	1847	403	249	4151
Myzus ornatus	28	0	0	371	12	3	414
Macrosiphum euphorbiae	2173	1331	195	4469	818	204	9190
Acyrthosiphon lactucae	2702	1255	232	4737	2295	873	12094
Aphis fabae	40	0	0	409	96	61	606
Parasitoid							
Asaphes vulgaris	6	2	0	28	3	0	39
Alloxysta victrix	19	2	0	41	3	1	66
Dendrocerus carpenteri	7	2	0	14	3	0	26
Aphidius ervi	37	12	1	75	8	3	136
Diaeretiella rapae	21	10	2	24	3	0	60
Praon gallicum	18	4	0	22	2	2	48
Aphidius matricariae	7	5	0	21	5	1	39
Predator							
Harmonia axyridis	4	1	0	2	2	1	10
Propylea quattuordecimpunctata	2	1	0	9	6	0	18
Coccinella septempunctata	8	0	0	13	1	1	23
Episyrphus balteatus	5	1	0	7	0	0	13
Syrphus ribesii	4	1	0	9	6	0	20
Anthocoris sp.	2	3	0	6	0	1	12
Tachyporus sp.	1	0	1	5	0	0	7
Lacewing (Family: Chrysopidae)	0	4	0	5	0	0	9

Table 4.4: Summary of effects of plant variety and *B. cinerea* infection status on the cumulative number of aphids, parasitoid mummies and predatory insect. Significant values are in bold.

Insects	Explanatory variable	Coefficient z value ± SE	P
Aphid	**Intercept**	**3.17 ± 1.161**	**0.001**
	Host plant variety	0.89 ± 0.133	0.371
	Uninfected	-0.16 ± 0.119	0.873
	Symptomatic	**-3.14 ± 0.166**	**0.001**
	Variety-Uninfected	**4.26 ± 0.168**	**<0.001**
	Variety-Symptomatic	-0.98 ± 0.222	0.325
Parasitoid mummies	Intercept	-1.487 ± 0.375	0.137
	Host plant variety	1.057 ± 0.295	0.290
	Uninfected	**4.079 ± 0.204**	**<0.001**
	Symptomatic	**-3.162 ± 0.375**	**0.001**
	Aphid	**2.869 ± 0.001**	**0.004**
	Variety-Aphid	-1.474 ± 0.001	0.140
Predatory Insect	**Intercept**	**-2.152 ± 0.905**	**0.031**
	Host plant variety	**2.712 ± 0.277**	**0.006**
	Uninfected	**2.904 ± 0.288**	**0.003**
	Symptomatic	-1.900 ± 0.568	0.057
	Aphid	-0.330 ± 0.001	0.741

Figure 4.1: Influence of infection status (uninfected/symptomatic/asymptomatic) and plant variety on mean (+/-SE) aphid numbers found on experimental lettuce plants.

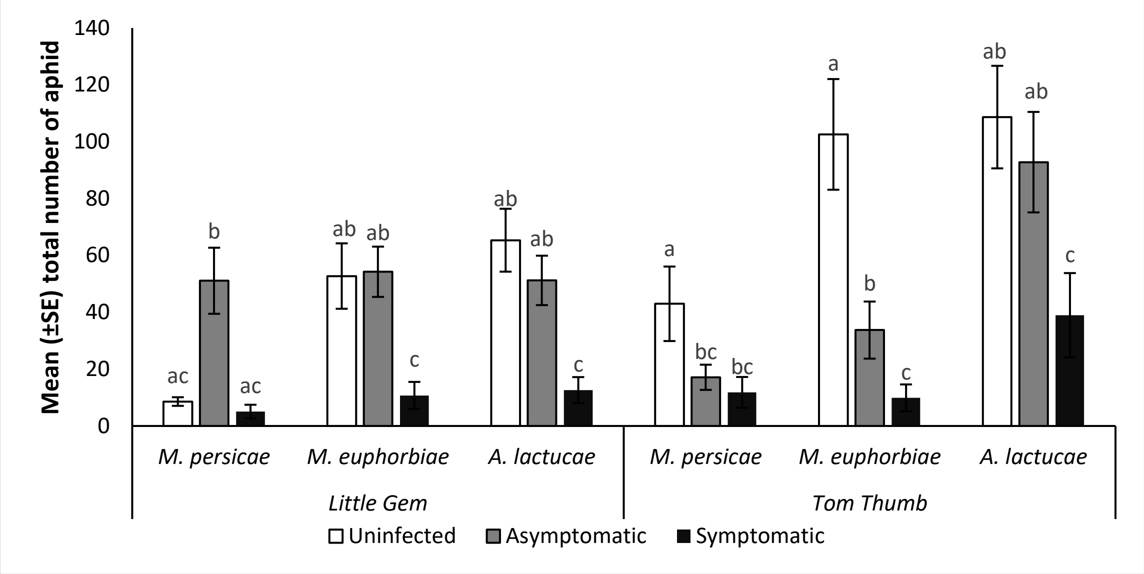


I then investigated the effect of plant variety and pathogen infection on the most abundance aphid species (*M. persicae*, *M. euphorbiae* and *A. lactucae*). Results depended on plant variety and type of pathogen infection (Table 4.5). There were no significant differences between the numbers of *M. persicae* and *M. euphorbiae* found on either plant variety, but more *A. lactucae* were found on Tom Thumb than Little Gem (Table 4.5). Asymptomatic pathogen infection did not influence the number of *M. euphorbiae* and *A. lactucae* on Little Gem; their numbers only reduced when the plant showed symptoms of infection. In contrast, the numbers of *M. persicae* was significantly higher on asymptomatic Little Gem compared to the uninfected or symptomatic Little Gem (Figure 4.2). On the other hand, asymptomatically or symptomatically-infected Tom Thumb had a significantly lower number of *M. persicae* and *M. euphorbiae* than found on uninfected plants. The number of *A. lactucae* showed same pattern as that seen as on Little Gem (Figure 4.2).

Table 4.5: Summary of effects of plant variety and *B. cinerea* infection status on the cumulative number of aphid *M. persicae, M. euphorbiae* and *A. lactucae.* Significant values are in bold.

Insects	Explanatory variable	Coefficient z value ± SE	P
M. persicae	**Intercept**	**3.60 ± 0.809**	**0.003**
	Host plant variety	-1.85 ± 0.338	0.064
	Uninfected	**-3.90± 0.309**	**<0.001**
	Symptomatic	**-3.03 ± 0.393**	**0.002**
	Variety-Uninfected	**4.57 ± 0.431**	**<0.001**
	Variety-Symptomatic	1.53 ± 0.526	0.125
M. euphorbiae	**Intercept**	**2.37 ± 1.127**	**0.018**
	Host plant variety	-0.67 ± 0.303	0.502
	Uninfected	0.42 ± 0.266	0.676
	Symptomatic	-0.17 ± 0.352	0.861
	Variety-Uninfected	**2.45 ± 0.377**	**0.014**
	Variety-Symptomatic	-1.59 ± 0.480	0.112
A. lactucae	**Intercept**	**2.73 ± 1.065**	**0.006**
	Host plant variety	**3.68 ± 0.124**	**0.002**
	Uninfected	1.89 ± 0.144	0.058
	Symptomatic	**-2.90 ± 0.181**	**0.003**

Figure 4.2: Influence of infection status (uninfected/symptomatic/asymptomatic) and plant variety on mean (+/-SE) number of aphid *M. persicae, M. euphorbiae* and *A. lactucae* found on experimental lettuce plants. The letters on the error bars only comparing the significant different between plant treatments for respective aphid species

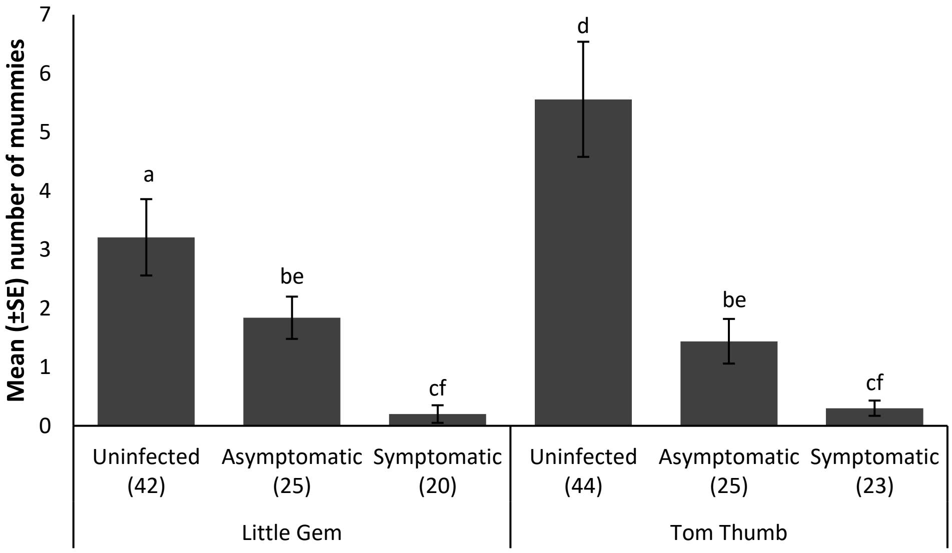


4.4.3. Natural enemies

Parasitoids: 473 mummies were collected in these experiments. 414 of those mummies emerged; four species were identified as primary parasitoids (53.72 %) and three as secondary parasitoids (46.28 %) (Table 4.3). The primary parasitoids were all members of the Family Braconidae [*Aphidius ervi* (Haliday), *Aphidius matricariae* (Haliday), *Praon gallicum* (Stary), *Diaeretiella rapae* (M'Intosh)], while the hyperparasitoids were *Dendrocerus carpenteri* (Curtis) (Hymenoptera: Ceraphronidae), *Asaphes vulgaris* (Walker) (Hymenoptera: Pteromalidae) and *Alloxysta victrix* (Westwood) (Hymenoptera: Cynipidae). The polyphagous parasitoid *A. ervi* was the most abundant species, with 136 individuals (32.85% of all individuals) followed with the secondary parasitoid *A. victrix* (15.94%) (Table 4.3). Parasitoid diversity was highest on infected Tom Thumb (1.81), followed by uninfected Tom Thumb (1.79), uninfected Little Gem (1.75) and the smallest number on the infected Little Gem (1.66).

While plant variety did not influence the number of mummies, the number of aphids and presence of plant pathogen infection played an important role in determining the number of mummies (Table 4.4). Infection by *B. cinerea* was associated with a reduction in the number of mummies on both plant varieties. The number of mummies was also reduced on the infected but asymptomatic plants of both varieties (Figure 4.3). Further investigation on the action by the secondary parasitoid found that plant variety and pathogen infection did not influence the parasitism behaviour of this insect. The number of secondary parasitoids emerging from each plant is correlated with the number of mummies present.

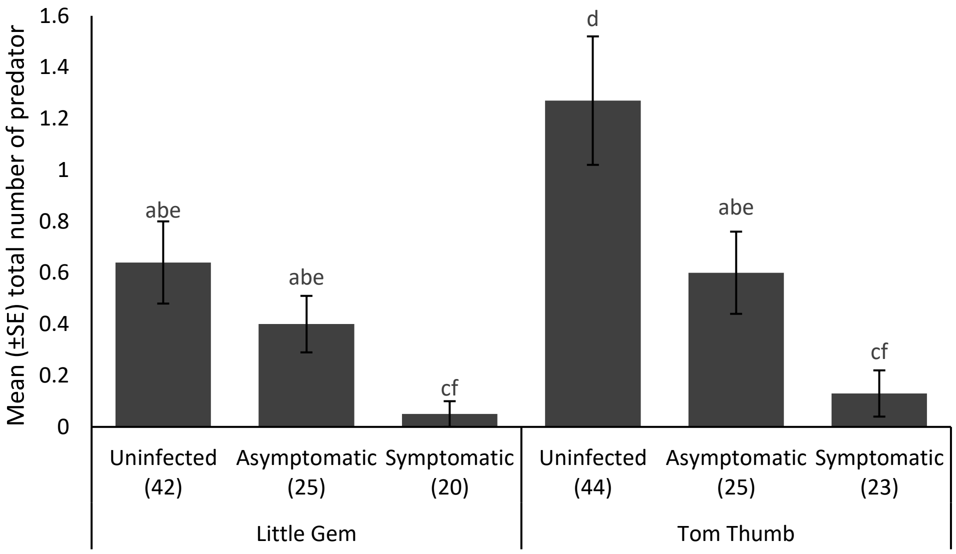
Figure 4.3: Effects of status of pathogen infection (uninfected/ symptomatic/asymptomatic) and plant variety on the number of mummies. Mean (±SE) number of mummies on all of the plant status were significantly differed for each variety.



Predators: 112 predatory insects (larvae and adults) were collected over both experimental periods (Table 4.3). The insects were the coccinellid ladybirds *Harmonia axyridis* (Pallas)*,* *Propylea quatuordecimpunctata* (Linnaeus) and *Coccinella septempunctata* (Linnaeus); hoverflies *Episyrphus balteatus* (De Geer) and *Syrphus ribesii* (Linnaeus); flower bug *Anthocoris nemorum* (Linnaeus), rove beetle *Tachyporus* sp, and lacewing (Family: Chrysopidae). Ladybirds were the most abundant predatory insect collected in the experiment (45.53% of all individuals). All species of ladybirds were found on most of the plant types except *C. septempunctata*, which were absent from infected Little Gem. The abundance of hoverfly larvae was also high (29.46%), followed by flower bugs (10.71%), lacewings (8.03%) and rove beetles (6.25%) (Table 4.3). There were no lacewings or rove beetles found on infected Tom Thumb plants. The diversity of predatory insects was highest on uninfected Tom Thumb (1.97), followed by uninfected Little Gem (1.77), infected Little Gem (1.74) and infected Tom Thumb (1.43).

The number of predatory insects was not influenced by the number of aphids, but was affected by plant variety (Table 4.4), with fewer predators collected on Little Gem (Figure 4.4). Overall, more predatory insects were recorded on uninfected plants than on infected plants, and there were significantly more on uninfected than asymptomatic plants (Figure 4.4).

Figure 4.4: Effect of pathogen infection status (uninfected/ symptomatic/asymptomatic) and plant variety on the number of predators. Plant infection status significantly affected the number of predators for each plant variety.



Taken together, plant variety (Tom Thumb, Little Gem) and plant infection status (uninfected, infected but asymptomatic, infected and symptomatic) influenced the assemblages of insect herbivores and their natural enemies found on the experimental plants (Figures 4.5 and 4.6).

Figure 4.5: Food web of uninfected, asymptomatic and symptomatic Little Gem. Trophic links from plant to aphids (lower panel) and natural enemies (upper panel) are represented as triangles shows the strength of interaction between insects and plant treatment, while triangle base represents the proportion of insects that counted on the plant.

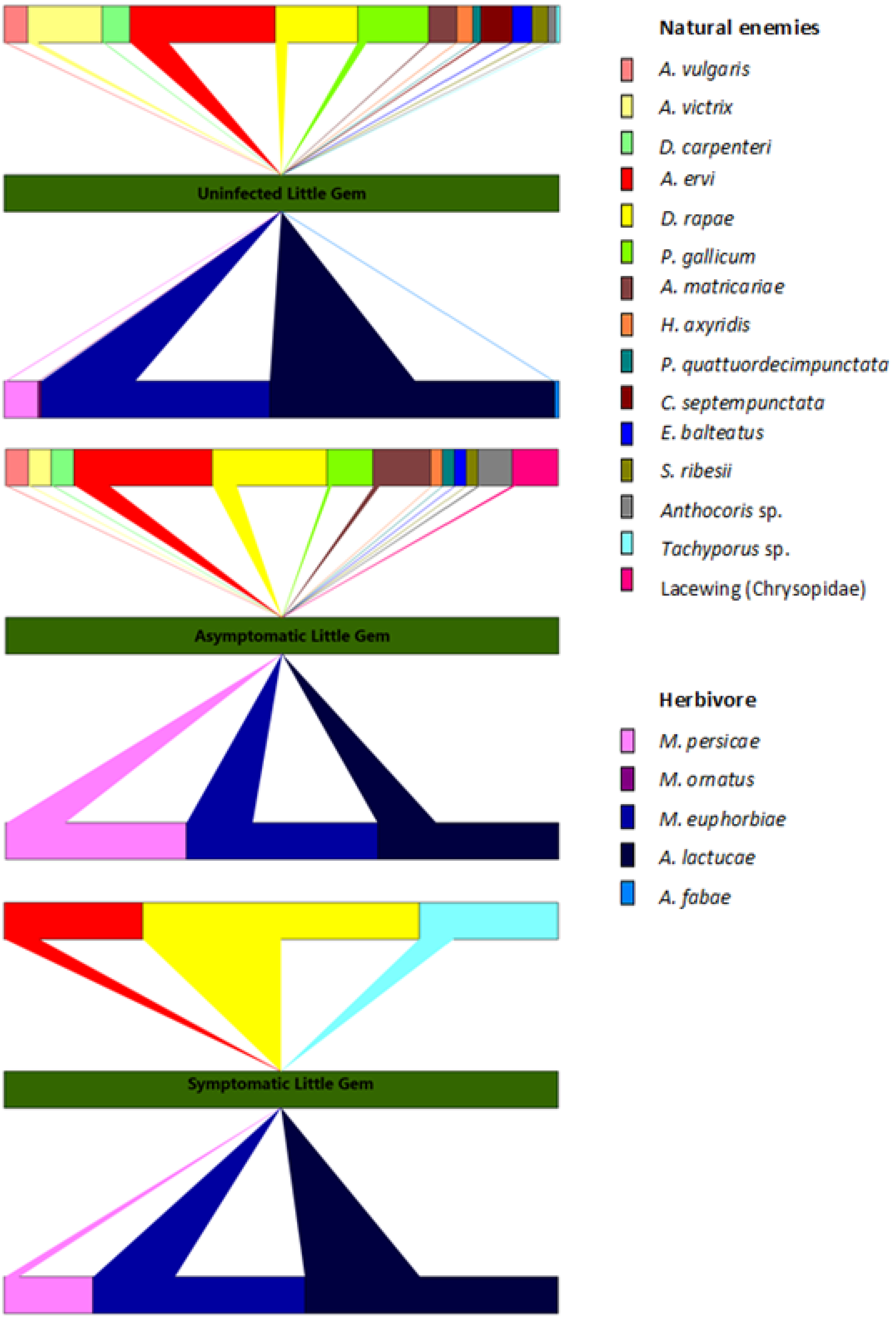
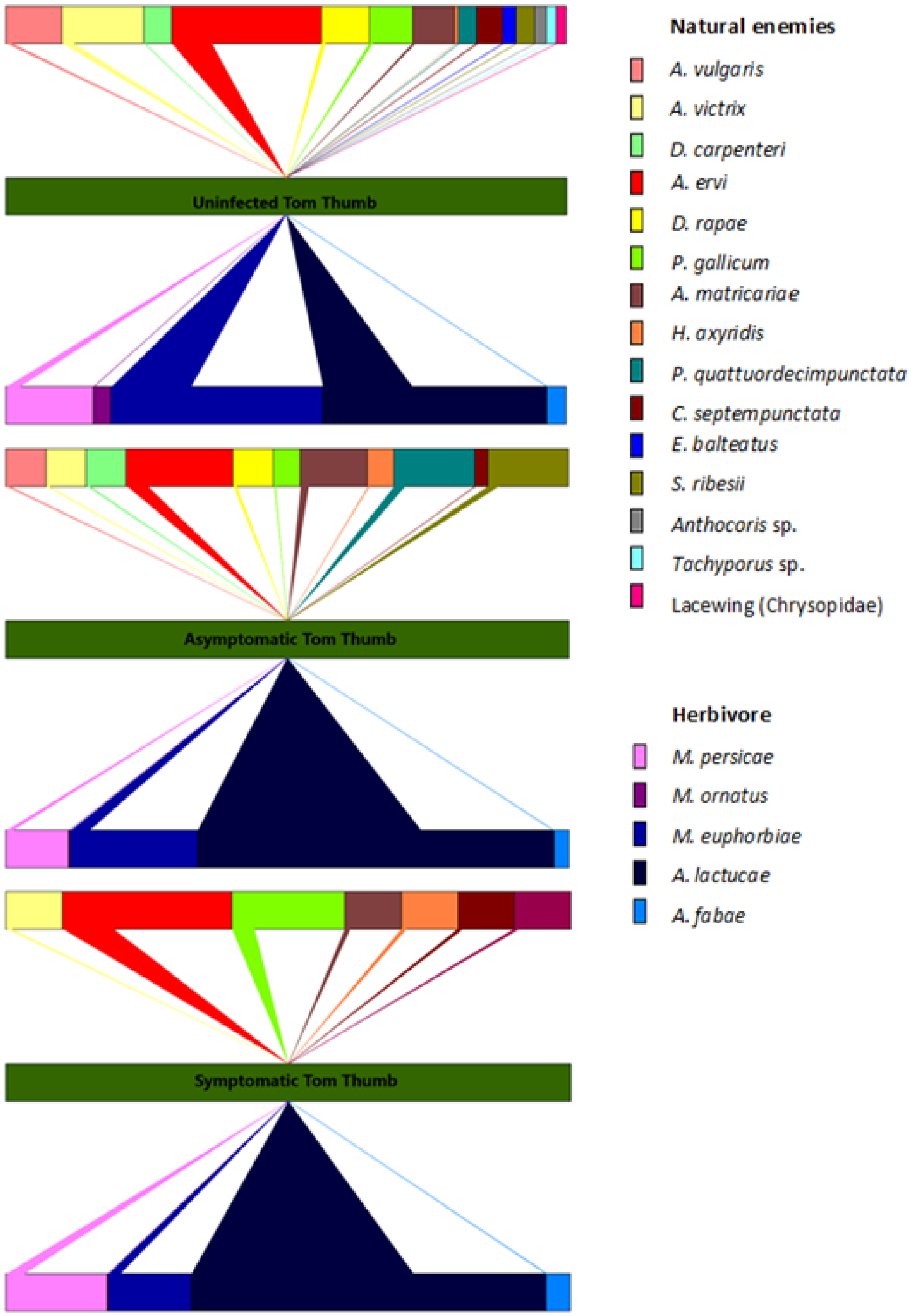


Figure 4.6: Food web of uninfected, asymptomatic and symptomatic Tom Thumb. Trophic links from plant to aphids (lower panel) and natural enemies (upper panel) are represented as triangles shows the strength of interaction between insects and plant treatment, while triangle base represents the proportion of insects that counted on the plant.



4.5. Discussion

I found that hidden infection by the ubiquitous plant pathogen *B. cinerea* can affect the abundance and diversity of naturally formed insect communities at higher trophic levels, and that this in turn was affected by host plant variety. The chlorophyll content, height and dry mass of Little Gem plants was higher than that of Tom Thumb. When infected with the plant pathogen, Little Gem experienced a reduction in height, chlorophyll content and shoot: root ratio, but had no effect on the dry weight. In contrast, the chlorophyll content and height of Tom Thumb was reduced when plants showed symptoms of infection. Pathogen infection on Tom Thumb, either symptomatic or asymptomatic, reduced the number of aphids colonizing the plants, and indirectly influenced the number of natural enemies. However, with Little Gem, the presence of the asymptomatic pathogen infection did not influence the number of aphids, but it did affect the number of natural enemies. Pathogen infection of Little Gem only mattered when plants showed symptoms, and symptomatic Little Gem harboured fewer aphids, which affected the numbers and diversity of predators and parasitoids. Overall, I found a significant difference in the number of aphids found on symptomatic and asymptomatic plants for both lettuce varieties, suggesting that the level of injuries found on the plants, such as the lesions found on symptomatic plants (Karban and Myers, 1989) and changes in plant traits (Price et al., 1980) play an important role in determining the performance of insects colonizing them.

Botrytis cinerea is responsible for a wide range of symptoms on diseased plants, from restricted lesions to dry or spreading soft rots with or without the appearance of conspicuous sporulating colonies (Elad et al., 2007). Sporulation and visible damage caused by this pathogen occurred only when infected tissues were stressed, or became mature or senescent (Shaw et al., 2016). In this experiment, we only quantified some of the morphological traits of the plant, as well as the chlorophyll content, which can be used to predict plant nitrogen content (Matsunaka et al., 1997; Hawkins et al., 2007). The mechanism of the asymptomatic infection by *Botrytis* was explored recently to understand how the fungus is able to grow within the plant host without causing visible disease symptoms (Rajaguru and Shaw, 2010; Sowley et al., 2010; van Kan et al., 2014; Shaw et al., 2016). Those studies suggest that *Botrytis* can exist in cryptic form and do not need to cause overt disease on host plants in order to complete their life-cycles.

In this experiment, both varieties of plants attracted five difference species of aphids. All aphid species were equally attracted to both uninfected and infected Tom Thumb. However, the aphids *A. fabae* and *M. ornatus* were not observed on infected Little Gem. The discrimination of these aphid species towards the *Botrytis*-infected plant may be related to plant nutrient quality, or to the repellent effect resulting from pathogen infection. While I cannot comment on the latter, data collected supports the hypothesis that plant quality was lower in infected plants. Aphids show a strong response to nitrogen levels in their host plants (White, 1984; Khan and Port, 2008). The nitrogen level in aphid diet is the most important factor affecting their performance (Awmack and Leather, 2002; Aqueel and Leather, 2011) and nitrogen deficiency lowers the intrinsic rate of increase of aphid populations (Douglas, 1993). In the laboratory, we found that asymptomatic *B. cinerea* infection altered the life history of the aphid *M. persicae* and their parasitoid *Aphidius colemani* (Chapter 2). The size, fecundity and longevity of aphids reared on asymptomatic Tom Thumb and Little Gem were greatly reduced. In turn, parasitoids reared on those aphid hosts showed reduced rates of mummy formation, and their offspring were smaller and had reduced starvation resistance.

Negative effects of symptomatic *B. cinerea* infection have been shown on the life history of aphids; *Aphis fabae* on *Vicia faba* (Al-Naemi and Hatcher, 2013) and *Rhodobium porosum* (Sanderson) on young rose plants, *Rosa hybrida* cv. Sonia (Mouttet et al., 2011). *Aphis fabae* had slower growth and lower fecundity when feeding on *Botrytis*-infected leaves, while there is an indirect two-way negative interaction between *B. cinerea* and *R. porosum* which is expressed by a reduction of aphid growth rate and a decrease in growth speed of fungal lesions on the plants. The *Botrytis*-infection also indirectly influences host choice behaviour, as aphids preferred uninfected plants when given a choice (Desneux et al., 2012). Contrary to our findings, *B. cinerea* infection has been reported to increase *A. fabae* performance on the broad bean *Vicia faba*, which may be a result of the senescence-like forced decomposition of the infected leaves increasing the amount of carbohydrates available for the aphids (Zebitz and Kehlenbeck, 1991).

Botrytis cinerea induces the jasmonic acid (JA) and ethylene (ET) pathway, thus down-regulating the salicylic acid (SA) dependent signalling pathway (Glazebrook, 2005) which may influence plant susceptibility to aphids on the shared host plant (Bostock, 2005; Goggin, 2007; Robert-Seilaniantz et al., 2011). However, the interaction between SA and JA varies depending upon hormone concentration and the relative timing of induction (Devadas et al., 2002; Thaler et al., 2002a). The role of SA and JA in determining plant–aphid interactions may vary among plant and insect species. For example, jasmonic acid has a direct negative impact on aphid *M. euphorbiae* survivorship on tomato plants (Cooper and Goggin, 2005) and limits the population growth of the aphid *M. persicae* on *Arabidopsis* (Ellis et al., 2002), while SA has a positive effect on aphid performance on *Arabidopsis* (Thompson and Goggin, 2006). In contrast, JA does not affect the survival and fecundity of *M. euphorbiae* but it enhances the aphid host preference on jai1-1 (jasmonic acid insensitive 1) tomato plants (Bhattarai et al., 2007), while the induction of SA in tomato plants mediated resistance to the aphid *M. euphorbiae* (Li et al., 2006). The level of injuries caused by this pathogen varies, and the degree of injury caused by the pathogen may influence the level of expression of plant defences, so it can be expected that the consequences for the herbivorous insects attacking the same host plants would also be variable.

Insect diversity and abundance is influenced by resource quality, competition and the action of natural enemies (Müller et al., 2005). The interaction between plants, aphids and natural enemies is complex because host plants may affect prey quality in unpredictable ways. As aphid life histories and population growth typically depend on the host plant, the life history and behaviour of natural enemies are affected (Bukovinszky et al., 2008) when a plant pathogen indirectly modified the traits of their herbivorous insect host. Here, the decrease in natural enemy abundance on the lettuce variety Tom Thumb is associated with the *Botrytis* infection and the availability of aphids on the plants. Pathogen infection did not affect the number of aphids on Little Gem, but reduced the number of natural enemies. This could be because the pathogen infection was lower, reducing the quality of the aphids and as a consequence they attracted fewer natural enemies (Stout et al., 2006). Some predators and parasitoids depend on a variety of cues produced by both plants and insects to locate and find their prey. For example, a complex mixture of volatiles could act as an attractant and/or an deterrent, influencing the foraging behaviour of these natural enemies to fly toward a food source and to lay their eggs (Hatano et al., 2008).

Plant traits play an important role in determining the dynamics and structure of insect communities. Changes in plant traits induced by the infection of a plant pathogen may have a cascading effect on both the direct and indirect interactions between the plant and other organisms at higher trophic levels. As a consequence, these interactions could shape community structure and influence the abundance of other species within this ecosystem. However, the effects of these interactions can be quantitatively variable amongst individuals. Much research has been done to examine the consequences of the effects of such interactions between and within plant-associated insect communities. However, research in the field of plant-mediated interactions between pathogens and insects are quite complex. As a consequence, this has received less interest from ecologists, although there is growing evidence that there are similar community-wide impacts when compared to plant-associated insect communities. Our study shows that under field conditions, plant pathogen infection may alter the assemblage of insects found on host plants by modulating plant quality. The strength and consequences of infection depends on the presence of symptoms and the genotype of the infected plant, but critically, the effects of infection can be seen even when there are no overt symptoms of disease. While it is still unclear how this system works, the results obtained suggest that systemic, asymptomatic and seed-borne infection by *B. cinerea* can influence the abundance and diversity of aphids and their predators and parasitoids in the field.

These findings reveal another dimension to the microbial pathogen-plant-insect interaction. The consequences of pathogen infection, even when symptomless, should not be underestimated. Since *Botrytis* is ubiquitous, infecting many plant species, and has the ability to infect plants without showing any symptoms, my findings may be of interest to agricultural production systems, where the presence of disease may change the patterns of interaction between insect pests and their natural enemies. Therefore, while we traditionally consider the direct implications of pathogen infection for productivity losses, it appears that hidden infection may also indirectly affect how insect pests affect crop productivity.

Chapter 5: Asymptomatic infection by the fungal plant pathogen *Botrytis cinerea* alters interactions between insect herbivores and their natural enemies in the field

5.1. Abstract

Bottom-up forces have often been proposed as presenting an important factor in mediating the influence of natural enemies on the structure of phytophagous insect communities. Pathogen infection changes plant quality and indirectly alters the life history and behaviour of herbivorous insects, thus affecting the interaction between natural enemies and their prey. These pathogen-plant-insect interactions are well studied in the laboratory, but little is known of the effect of this bottom-up factor on the structure of the assemblage of natural enemies in natural environments. In addition, some pathogen infection is symptomless and their existence therefore frequently overlooked, thus their importance in determining the success of biological control is unconsidered. We show experimentally that changes in natural enemy assemblages results from infection by a fungal pathogen of the host plant. This was tested in the field using the ubiquitous necrotrophic pathogen *Botrytis cinerea*, the aphid herbivore *Myzus persicae* and their host plant lettuce *Lactuca sativa*, of two varieties (Tom Thumb and Little Gem). We found that both symptomatic and asymptomatic plant pathogen infection altered plant quality, altering aphid colony population dynamics if natural enemies are excluded. However, these effects are varies among plant variety. Natural enemies reduced the number of aphids, and more predatory insects and parasitoid mummies were found on uninfected plants. This work suggests that hidden plant pathogens may have considerable influences on the structure of insect communities.

Key words: Aphid, natural enemies, plant pathogen, tritrophic level

5.2. Introduction

In the most simplistic of views, populations in typical tritrophic (plant-herbivore-natural enemy) systems can be regulated by direct and/or indirect top-down (predator or herbivore abundance) or bottom-up (plant quality, prey abundance) influences (Scherber et al., 2010; Meiners, 2015; Schuldt et al., 2017). For insect herbivores, particularly rapidly reproducing pest species such as aphids, variation in host plant quality is regarded as the most important factor in determining the abundance of insect herbivores (Tack et al., 2010; Joern et al., 2012). Variability in host plant quantity and nutritional quality can influence the fecundity, survival and development rates of herbivorous insects simply through changes in resource availability, and thus can indirectly affect the abundance and population dynamics of taxa at higher tropic levels (Stacey and Fellowes, 2002; Stang et al., 2006; Bukovinszky et al., 2008; Poelman et al., 2008; Schuman et al., 2016). Host plant quality can vary as a result of a variety of factors, such as differences in resource availability to the plant [e.g. fertiliser effects (Müller et al., 2005; Aqueel and Leather, 2011), or heritable variation in plant defences (Fritz et al., 1994; Roche and Fritz, 1997)]. More recently, the influence of the presence of fungi, in the guise of mutualists [e.g. fungal endophytes (Krauss et al., 2007; Faeth and Saari, 2012); arbuscular mycorrhizal species (Gange, 2001; Koricheva et al., 2009)) or pathogens (e.g. *Botrytis cinerea* (Mondy et al., 1998; Mondy and Corio-Costet, 2000; Al-Naemi and Hatcher, 2013)] on species at higher trophic levels has been studied.

Pathogen infection alters both plant morphology and chemistry (Heil, 2001; Heil and Bostock, 2002; Choudhary et al., 2007; Shah, 2009). Pathogen infection may affect the process of photosynthesis, thus altering the quality and quantity of plant nutritional content (Rahoutei et al., 2000; Berger et al., 2004; Bonfig et al., 2006). Upon sensing the invading microorganism, plants can activate defence mechanisms in an attempt to restrict pathogen growth (Dangl and Jones, 2001). General defence reactions employed by plants include cell wall reinforcement, phytoalexin production and the accumulation of antimicrobial proteins (González-Lamothe et al., 2009; Dodds and Rathjen, 2010; Miedes et al., 2014). These reactions alter plant biochemistry, which in turn then affects investment in plant growth and reproduction (Massad et al., 2012; Huot et al., 2014). Volatile and non-volatile secondary metabolites are activated in plants when pathogens invade. For example, plants biosynthesise phenolic defence hormones such as salicylic acid, jasmonic acid, ethylene, abscisic acid, gibberellins, auxins, and cytokinins in respond to pathogen infection (Vlot et al., 2009; Du Fall and Solomon, 2011; Bos et al., 2013; Dudareva et al., 2013; Dempsey and Klessig, 2017). These hormones have pivotal roles in the regulation of plant growth, development, and reproduction (Pieterse et al., 2012). The composition and timing of the hormonal blend produced by the plant can determine the plant susceptibility or resistance to the invading organism (Verhage et al., 2010).

As the performance of herbivorous insects is largely dependent on the quality of their host plant (Awmack and Leather, 2002; Wetzel et al., 2016), insects alter their performance and behaviour to maximise fitness given resource availability (De Roode and Lefèvre, 2012). Changes in herbivore behaviour includes altering feeding strategies such as total food consumption or time spent feeding, with consequent effects on reproduction or development rates. For example, herbivorous insects may increase their total consumption and spend more time feeding on low nutritional quality plants (Lavoie and Oberhauser, 2004).

Pathogen infection is usually detrimental to the plant, but this does not necessarily result in a reduction in host plant quality for insect herbivores. For example, herbivorous insects may show higher performance on pathogen-free (Al-Naemi and Hatcher, 2013) or diseased plants (Cardoza and Tumlinson, 2006; Mauck et al., 2010). Such variation in the effects of plant pathogen infection on herbivore traits is multifactorial. Plant pathogen infection may lead to an increase in non-digestible matter and the induction of the host plant's resistance pathways (Feys and Parker, 2000; Bezemer et al., 2003), increasing levels of secondary compounds and changes in water, carbohydrate and nitrogen content, that in turn can affect herbivorous insects (Rasmussen et al., 2008). The defensive compounds produced by pathogen-infected plants can have toxic or anti-feedant properties that directly affect insect's development (Pieterse and Dicke, 2007). In addition, such effects will be influenced by the severity of the disease symptoms (Dordas, 2008). Severity of plant disease is difficult to measure and will depend on the aggressiveness of the pathogen infection (Vale et al., 2001).

While pathogens can affect insect herbivores, they can also alter interactions between the herbivorous insects and their natural enemies (reviewed by Agrawal, 2000; Van Der Putten et al., 2001; Dicke and Baldwin, 2010). For example, pathogen infection alters plant nutritional quality and thus can slow herbivore development rates, widening the window of exposure to attack by natural enemies (Schmitz et al., 2004; Thaler et al., 2012b). Plant nutrient availability not only affects herbivorous insects, but also indirectly influences parasitoid attack rate, offspring size, development and survival (Teder and Tammaru, 2002). Furthermore, host plant quality can affect parasitoid sex ratio as the resource requirements and fitness values of developing male and female parasitoid are different (Morris and Fellowes, 2002). Plants also produce volatiles when infected with pathogenic microorganisms (Baldwin, 2010; Scala et al., 2013), which may affect olfactory responses of foraging herbivorous insects and their natural enemies (D'Alessandro et al., 2014).

However, pathogen infection does not always result in visible symptoms. The ubiquitous necrotrophic pathogen *Botrytis cinerea* Persoon: Fries s. lato causes considerable economic losses in a wide variety of crops mainly dicotyledonous plant species, including important protein, oil, fibre and horticultural crops. Symptoms of infection including soft rots on plant parts including fruits and flowers which this pathogen will produce prolific grey conidiophores and (macro) conidia typical of the disease (Williamson et al., 2007). Fascinatingly, this pathogen has an ability to exist as asymptomatic infection on their host plant (Shaw et al., 2016). The mechanism of asymptomatic infection by this pathogen on lettuce are well documented (Sowley et al., 2010). Previous work (see Chapters 2, 3, 4) showed that asymptomatic infection by *B. cinerea* in lettuce still causes measurable changes in host plants, and this varies between plant varieties. In the laboratory, I found that aphids (*Myzus persicae* Sulzer; Hemiptera: Aphididae) reared on asymptomatic infected plants were smaller, had fewer offspring and were less tolerant of starvation; parasitoids (*Aphidius eadyii* Viereck; Hymenoptera: Braconidae) emerging from such hosts were also smaller (Chapter 2). When given a choice, both aphids and parasitoids preferentially chose uninfected plants, showing that they could differentiate between plants varying in infection status, and furthermore, aphids were more likely to show escape (dropping) behaviours to foraging coccinellid predators when reared on infected plants (Chapter 3). In the field, we see that asymptomatic plants recruit a different assemblage and abundance of herbivores and their natural enemies (Chapter 4). However, these effects are varied between plant varieties. Aphids show discrimination between uninfected, symptomatic and asymptomatic infected Tom Thumb, but this is not so for Little Gem plants. Infection by pathogens on Little Gem only affected aphids when plants showed symptoms. Interestingly, both Tom Thumb and Little Gem when infected by either symptomatic or asymptomatic *B. cinerea*, were less attractive to aphid natural enemies.

However, this does not allow us to disentangle direct effects of infection (e.g. changes in aphid population growth rate, and hence density dependent effects on natural enemy foraging behaviour), from indirect effects of infection (changes in aphid host quality, rather than quantity affecting natural enemy behaviour). To address this, I performed a field experiment where infected lettuce plants, and uninfected plants of two varieties were placed in the field with a known number of aphids. Numbers of aphids, their predators and parasitoids were recorded to assess the consequences of asymptomatic infection on interactions at higher trophic levels.

5.3. Materials and methods

5.3.1. Study system

Plants. I used two lettuce *Lactuca sativa* L. (Asteraceae) varieties (Tom Thumb and Little Gem) in my trials. Both varieties differ in their morphology and leaf characteristics. Tom Thumb has smoother leaves with a more compact arrangement than Little Gem. The size of Little Gem is bigger and wider compared to Tom Thumb and grows to around 10 centimetres wide and 15 cm tall. Plants were grown from pathogen free and *B. cinerea* infected seed (following Sowley et al., 2010). Infected seeds were collected from plants which were systemically infected with the *B. cinerea* strain BO5.10 spores during their flowering stage, while uninfected seeds were collected from uninfected plants. Both uninfected and *B. cinerea* infected plants for seed collection were grown in 2014 in different glasshouses with the same conditions to avoid pathogen contamination.

Experimental plants were grown from seeds sown in individual cells of plug trays with professional seed and modular compost (Clover brand; Dungannon, UK) in a glasshouse (Temperature: 25-30°C, relative humidity 80 ± 5 % and L12:D12 photoperiod). Fourteen days after emergence, seedlings were transferred into 15 cm diameter pots with traditional potting compost (Vitax Grower; Leicester, UK). Plants were then allowed to grow for another four weeks in the glasshouse before use. Plants that showed the symptoms of pathogen infection were discarded immediately to avoid pathogen contamination in the glasshouse. Only plants with no injuries and free from symptoms of pathogen infection were used in the experiment.

Aphids. Our model herbivore was a single clone of the green peach aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae). This is a generalist phloem feeder, which had previously been reared for four generations on *Botrytis*-infected or uninfected plants prior to use to avoid confounding maternal effects. Aphids were reared in the laboratory at the ambient temperature, isolated using cylindrical clear plastic cages fitted with cotton mesh windows. The details of aphid rearing are as described in Chapter 2.

5.3.2. Field site

There were three treatments: 1) to examine the influence of infection status (using infected and uninfected plants), 2) to examine the effect of plant variety (Tom Thumb, Little Gem) and 3) to examine the influence of natural enemy attack on aphid colony population dynamics (using protected and exposed plants). Each treatment was replicated 30 times, with 240 plants and aphid colonies initially placed in the study site. However, some replicates are discarded due to the damage caused by environmental effects or because unexposed plants became infected by pathogens in the field.

No-predation plants were covered with breathable plastic bags to protect aphids from natural enemies, allowing the effects of plant infection status on aphid colony growth to be assessed. Plants and aphid colonies for the three treatments were randomly placed out in a grid pattern in the field site within the experimental grounds, University of Reading, U.K (51.4414° N, 0.9418° W). Plants were placed approximately 1.7 meters apart to avoid direct competition between each plant. Each plant pot was placed in a shallow plastic dish to make the watering process easier and to minimise disturbance to insects on plants when watered, and also to minimize the likelihood of water stress or saturation. Plants were watered as required and any weeds growing between plants were regularly removed. Each colony was established with five seven-day-old aphids which were placed the plants five days before being transferred to the field site to allow colonies to establish.

5.3.3. Data collection

The number of aphids and natural enemy assemblage. The numbers of aphids, mummies and predatory insects on each plant were counted and recorded every two days until all colonies of aphids died, which took 28 days. Predatory insects were collected for later identification. Parasitoid mummies were also collected. However, if more than one mummy was found on the plants, their presence was recorded and their location noted. Half were then randomly collected, and half were left on the plant for a further 72 hours giving the opportunity for hyperparasitoid attack.

The effect of plant pathogen infection on plant traits. Plant height was measured on the first day and once again on the final day of the experiment. At the end of the experiment, leaf chlorophyll content was recorded for three different mature leaves from each plant by using a Chlorophyll Content Meter (Model CL01; Hansatech Instruments Ltd, Norfolk, UK). Plants were then harvested (including the root) to measure their dry weight and shoot: root ratio. Plants were oven dried at 75°C until reaching constant mass (~48 hours), and weighed using an electronic balance (Sartorius, LC 6200S, Goettingen, Germany). The ratio of the root: shoot was measured by dividing the dry weight of shoot per root for each plant.

Assessing of B. cinerea infection. Following data collection, plants were visually inspected for symptoms of disease. The symptoms of *Botrytis* infection on leaves include spreading soft rots with or without the present of conspicuous sporulating colonies, or restricted or dry lesions (Elad et al., 2007). All plants (both experimentally infected and uninfected prior to placement) were then assessed for the presence of *Botyritis cinerea*. Three 1 cm diameter of mature leaf samples with no visible symptom of infection were randomly harvested at the end of the experiment from each plant. The leaf samples were sterilised before plating on *Botrytis* Selective Media (BSM) agar to confirm the *Botrytis* infection status of the plants. Leaf samples were disinfected with 70% ethanol for 1 min, followed by 1 min in 2% bleach solution (Domestos, Unilever; 5% NaOCl in alkaline solution with surfactants) and then rinsed three times in sterile distilled water to remove all surface inoculum, whether dusted or soaked in (following Barnes and Shaw, 2002). The sterile leaf disk then was plated on BSM agar and incubated at 18-20°C for at least 10 days in an incubator with alternating UV-A light (12h/day) and dark (12h/day). After fourteen days, the BSM plate was observed again to see whether there was evidence of *B. cinerea* growth, such as browning of the agar or sporulation of the fungus. Confirmation of presence was based on the sporulation of the pathogen and morphological observation of the colonies under a high-performance stereomicroscope (Leica, MZ9.5, Texas, USA). Plant health status was therefore categorized as (i) symptomatic if the symptom of *B. cinerea* infection appears on the leaf and was confirmed by the BSM agar test; (ii) asymptomatic infection if there is no symptom of *Botrytis* infection appearing on the leaf, but the plated BSM agar shows signs of *Botrytis* growth (such as browning or sporulation of fungi); and (iii) healthy if no symptom of *Botrytis* infection appears on the leaf and there is no sign of fungal growth on BSM agar.

5.3.4. Statistics

All analyses were performed using R statistical software version 3.4.0 (R Core Team, 2017). The influence of plant variety, plant exposure to aphid natural enemy and pathogen infection status on plant traits was compared using linear models. Data for shoot root ratio were log transformed, while chlorophyll content, dry weight and plant height were square root transformed to meet the assumptions of normality and homoscedasticity. The effect of plant variety, plant exposure and pathogen infection on the number of aphids was analysed using repeated measures analysis with generalized least squares function. The effects of plant variety and pathogen infection on the numbers of predators and parasitoid mummies collected were analysed using generalized linear models with a Poisson distribution, where aphid number was treated as a covariate. The significance differences between mean values of plant traits and number of aphid were determined by using LSmeans and separation by post hoc Tukey test, with plant variety, infection status and plant exposure to aphid natural enemies treated as explanatory variables.

5.4. Results

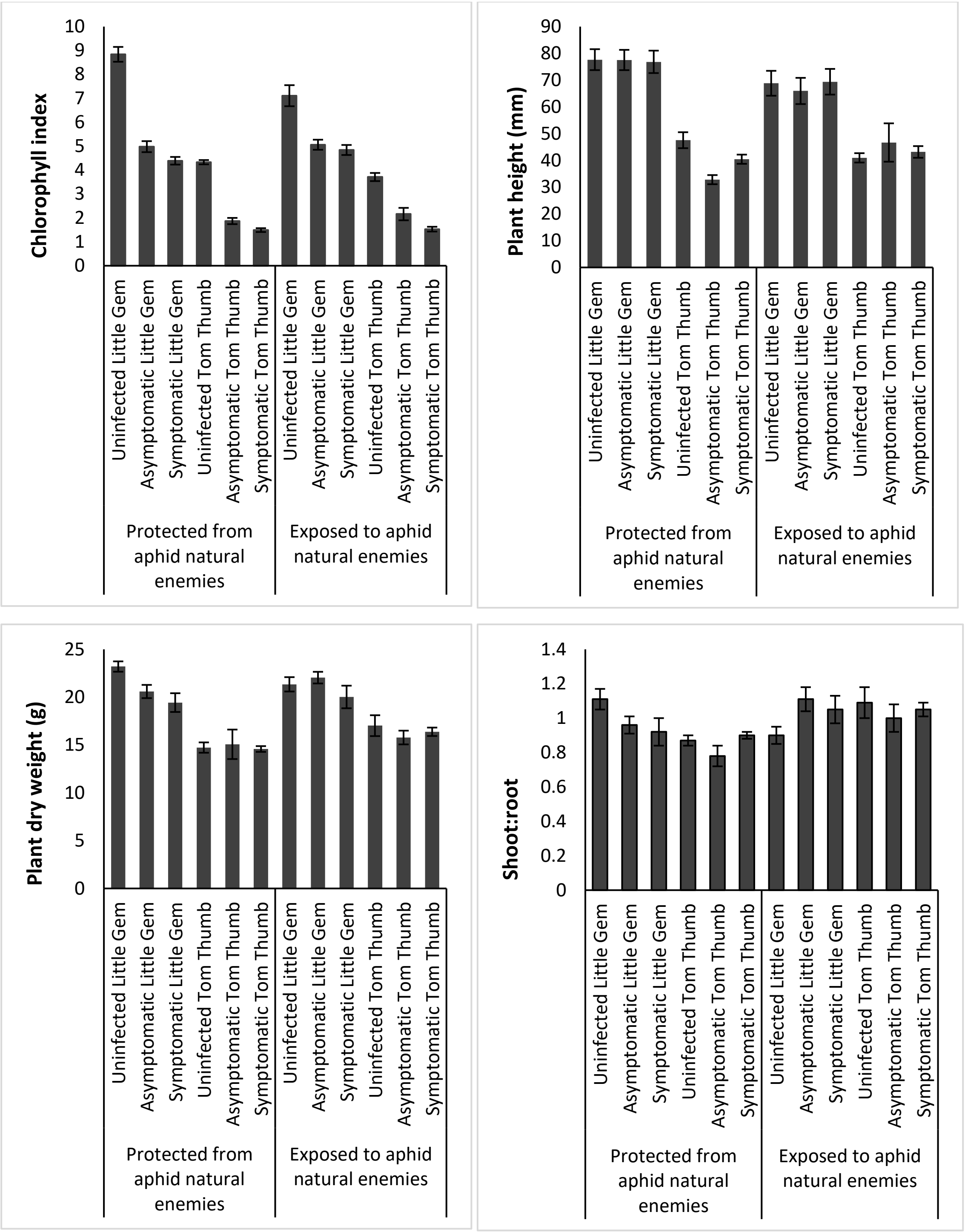
5.4.1. Plant life history traits

Lettuce varieties differed in chlorophyll content, dry weight, plant height and shoot: root (Table 5.1). *Botrytis cinerea* infection either symptomatic or asymptomatic reduced the chlorophyll content of Tom Thumb and Little Gem (Figure 5.1). Both symptomatic and asymptomatic pathogen infection only affected the dry weight of protected Little Gem and did not show any significant effect on Tom Thumb (Figure 5.1). On the other hand, symptomatic and asymptomatic pathogen infection affected the height of protected Tom Thumb and did not influence the height of Little Gem plants (Figure 5.1). The effect of pathogen infection on plant dry weight (Little Gem) and plant height (Tom Thumb) was eliminated when the plant was exposed to aphid natural enemies. If protected, the shoot:root ratio of uninfected Little Gem plants was significantly higher than asymptomatic or symptomatic Little Gem plants. These results were the converse when Little Gem plants were exposed to aphid natural enemies where the shoot: root ratio of uninfected Little Gem of exposed plant is lower than that of symptomatic and asymptomatically infected Little Gem.

Table 5.1: Summary of effects of plant variety and *B. cinerea* infection status on plant traits following analysis. Significant values are in bold.

Plant traits	Explanatory variable	Coefficient t value ± SE	P
Chlorophyll index	**Intercept**	**50.392 ± 0.045**	**<0.001**
	Variety	**-16.721 ± 0.053**	**<0.001**
	Plant exposure	**-2.641 ± 0.050**	**0.009**
	Uninfected	**12.063 ± 0.048**	**<0.001**
	Symptomatic	**-3.018 ± 0.052**	**0.003**
	Variety- Plant exposure	1.469 ± 0.074	0.144
Shoot: root	Intercept	0.374 ± 0.046	0.708
	Variety	**-2.500 ± 0.090**	**0.013**
	Plant exposure	-6.663 ± 0.048	0.508
	Uninfected	-0.539 ± 0.054	0.590
	Symptomatic	-0.496 ± 0.069	0.620
	Variety-Plant exposure	**2.951 ± 0.070**	**0.003**
	Variety-Uninfected	1.075 ± 0.099	0.284
	Variety-Symptomatic	1.144± 0.104	0.254
Dry weight (g)	**Intercept**	**74.816 ± 0.062**	**<0.001**
	Variety	**-7.176 ± 0.119**	**<0.001**
	Plant exposure	-0.802 ± 0.063	0.424
	Uninfected	1.195 ± 0.072	0.243
	Symptomatic	**-2.00 ± 0.092**	**0.046**
	Variety-Plant exposure	**2.994 ± 0.093**	**0.003**
	Variety-Uninfected	-0.147 ± 0.131	0.883
	Variety-Symptomatic	1.548 ± 0.139	0.123
Plant height (mm)	**Intercept**	**43.112 ± 0.202**	**<0.001**
	Variety	**-6.162 ± 0.389**	**<0.001**
	Plant exposure	**-2.807 ± 0.208**	**0.005**
	Uninfected	0.234 ± 0.236	0.815
	Symptomatic	0.313 ± 0.301	0.754
	Variety-Plant exposure	2.195 ± 0.304	0.029
	Variety-Uninfected	0.598 ± 0.429	0.551
	Variety-Symptomatic	-0.082 ± 0.453	0.935

Figure 5.1: The effect of plant variety, pathogen infection and exposure on Mean ± SE chlorophyll index, plant height, plant dry weight and shoot:root ratio of the plant.



5.4.2. Aphids

Overall, plant pathogen infection, plant variety and natural enemy attack influenced the number of aphids of plants (Table 5.2). More aphids were recorded on Tom Thumb than Little Gem for both exposed and protected plants (Figure 5.2). Pathogen infection, either symptomatic or asymptomatic, reduced aphid numbers and this effect has changed over time and it is differed between plant varieties (Figure 5.2). When protected from attack by natural enemies, the number of aphids on uninfected Tom Thumb plants was significantly higher than on asymptomatic and symptomatic Tom Thumb plants. However, the number of aphids on protected uninfected, asymptomatic and symptomatic Little Gem plants showed no significant difference. The number of aphids on all plant treatments were greatly reduced when exposed to the attack by their natural enemies (Figure 5.2). Here, there is no significant difference were detected on the number of aphids on uninfected, asymptomatic and asymptomatic plant for both lettuce varieties.

5.4.3. Parasitoids

In this experiment, we collected 525 parasitoid mummies of which 394 emerged and were identified to five species (the braconids *Aphidius ervi* Haliday, *Aphidius matricariae* Haliday, *Praon gallicum* Stary, *Diaeretiella rapae* M'Intosh, and the pteromalid *Asaphes vulgaris* Walker) (Table 5.3). *Aphidius ervi* was the most abundant (27.66%) species followed by *A. matricariae* (25.89%), *P. gallicum* (25.63%) and *D. rapae* (12.44%). The hyperparasitoid *As. vulgaris* formed 8.38% of records. The number of parasitoid mummies recorded on plants was significantly affected by *B. cinerea* infection, plant variety and the number of aphids on the plant (Table 5.4). More parasitoid mummies were recorded on uninfected plants compared to the asymptomatic plants for both lettuce varieties (Figure 5.3). Surprisingly, there was no significant difference between the number of aphids on uninfected and symptomatic plants for both Tom Thumb and Little Gem (Figure 5.3).

Table 5.2: Summary of effects of plant variety and *B. cinerea* infection status on the number of aphids per recording day

Explanatory variable	numDF	F- value	P
Intercept	1	410.723	<0.001
Plant exposure	1	7.335	0.006
Plant status	2	4.774	0.008
Plant variety	1	53.584	<0.001
Day	10	107.291	<0.001
Plant exposure: Plant status	2	2.749	0.064
Plant exposure: Plant variety	1	13.820	0.001
Plant status: Plant variety	2	21.739	<0.001
Plant exposure: Day	10	85.900	<0.001
Plant status: Day	20	1.384	0.119
Plant variety: Day	10	13.433	<0.001
Plant exposure: Plant status: Plant variety	2	1.236	0.290
Plant exposure: Plant status: Day	20	1.079	0.364
Plant exposure: Plant variety: Day	10	15.465	<0.001
Plant status: Plant variety: Day	20	1.830	0.013
Plant exposure: Plant status: Plant variety: Day	20	1.806	0.015

Figure 5.2: Influence of infection status (uninfected/symptomatic/asymptomatic) and plant variety on mean (+/- SE) number of aphid on experimental lettuce plants per recording day. Aphids recorded on plants where natural enemies were excluded (a and b) and plants exposed to aphid natural enemies (c and d). Plant treatment, UTT: Uninfected Tom Thumb, ATT: Asymptomatic Tom Thumb, STT: Symptomatic Tom Thumb, ULG: Uninfected Little Gem, ALG: Asymptomatic Little Gem, SLG: Symptomatic Little Gem.

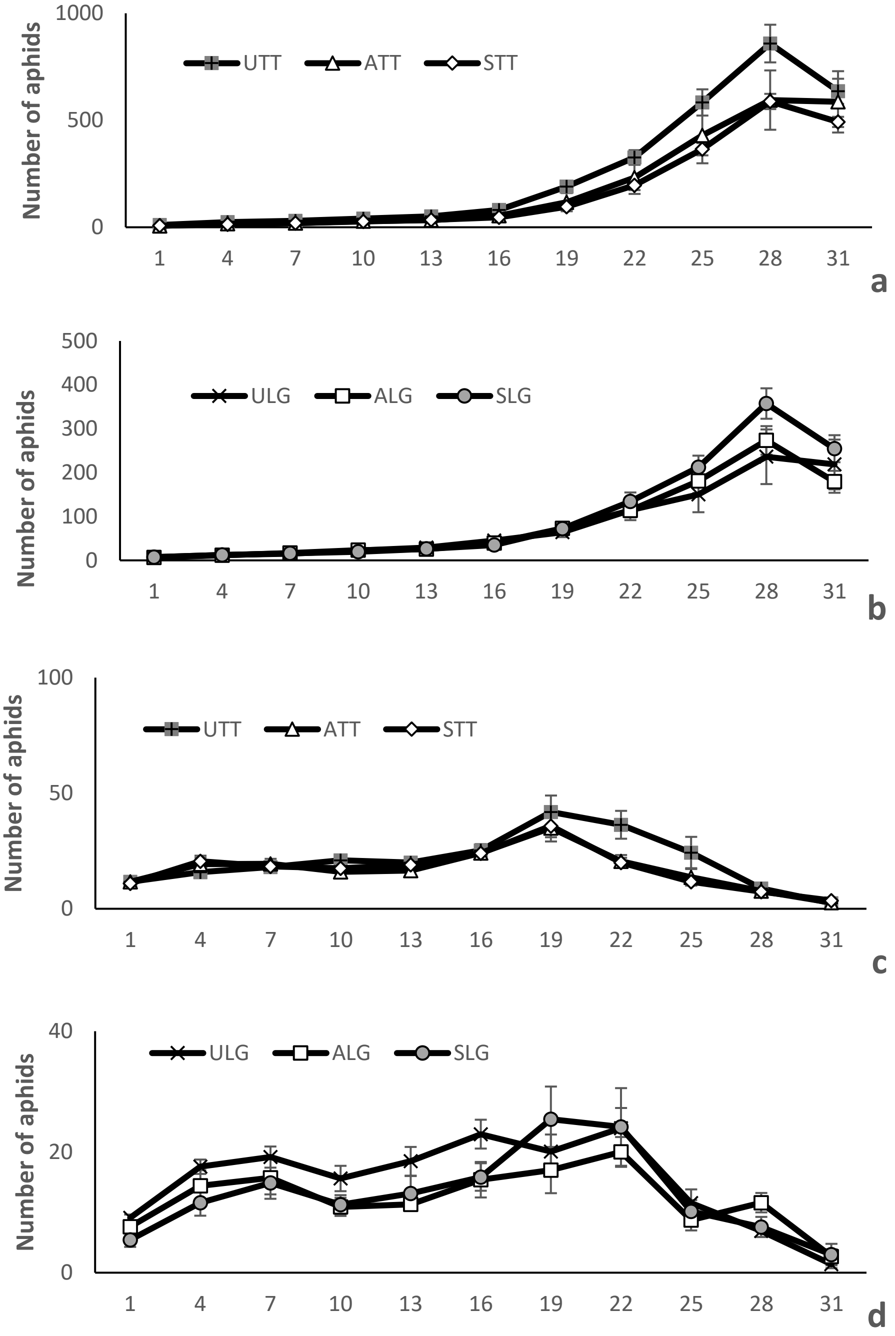


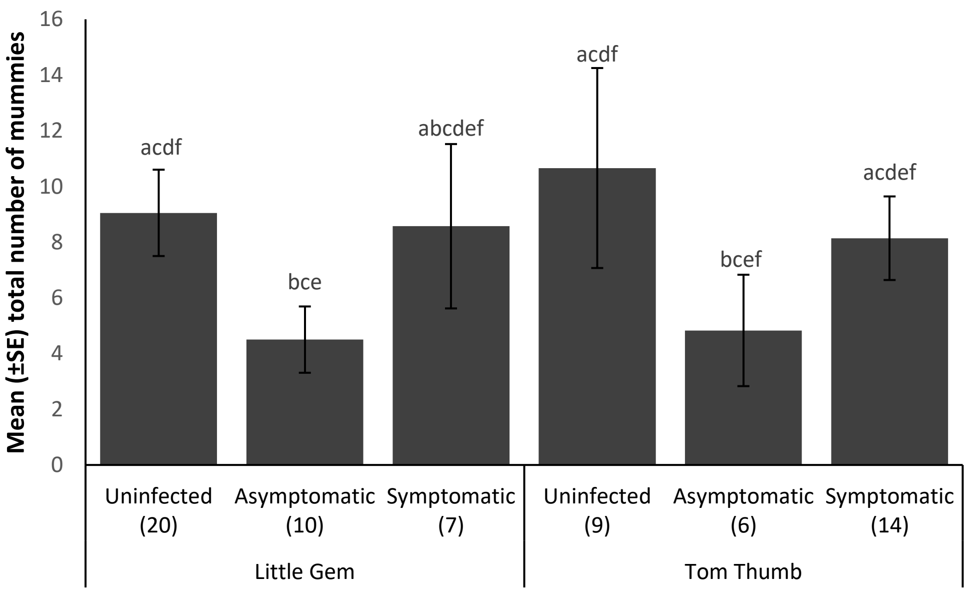
Table 5.3: Counts of aphid natural enemies found on experimental plants. UTT: Uninfected Tom Thumb, ATT: Asymptomatic Tom Thumb, STT: Symptomatic Tom Thumb, ULG: Uninfected Little Gem, ALG: Asymptomatic Little Gem, SLG: Symptomatic Little Gem. Numbers of plants are below.

Species	ULG (20)	ALG (10)	SLG (7)	UTT (9)	ATT (6)	STT (14)	Total
Parasitoids				Number of insect			
Asaphes vulgaris	17	0	0	12	0	4	33
Aphidius ervi	27	5	6	39	9	23	109
Diaeretiella rapae	27	1	3	6	0	12	49
Praon gallicum	32	21	17	3	7	21	101
Aphidius matricariae	45	7	14	16	3	17	102
Predators							
Harmonia axyridis	35	15	16	20	3	6	95
Coccinella septempunctata	42	5	4	4	8	16	79
Adalia bipunctata	34	1	2	19	4	15	75
Episyrphus balteatus	17	5	2	16	1	5	46
Syrphus ribesii	12	0	1	5	0	2	20

Table 5.4: Summary of effects of plant variety and *B. cinerea* infection status on the cumulative number of parasitoid mummies and predatory insects. Significant values are in bold.

	Explanatory variable	Coefficient z value ± SE	P
Parasitoid mummies	**Intercept**	**4.090 ± 0.165**	**<0.001**
	Variety	**-2.596 ± 0.104**	**0.009**
	Uninfected	**3.440 ± 0.137**	**<0.001**
	Symptomatic	**3.825 ± 0.141**	**<0.001**
	Aphid	**7.465 ± 0.001**	**<0.001**
Predatory Insect	**Intercept**	**3.911 ± 0.217**	**<0.001**
	Variety	-0.231 ± 0.134	0.817
	Uninfected	**5.594 ± 0.173**	**<0.001**
	Symptomatic	1.097 ± 0.198	0.273
	Aphid	0.748 ± 0.001	0.455

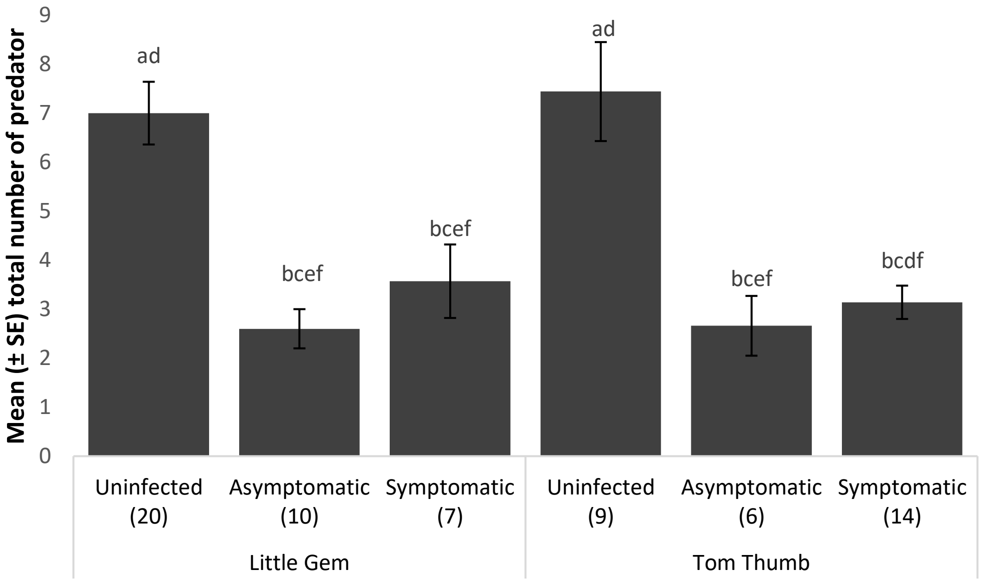
Figure 5.3: Effects of status of pathogen infection (uninfected/ symptomatic/asymptomatic) and plant variety on the Mean ± SE number of mummies.



5.4.4. Predators

A total of 315 predators were observed in this experiment, which only consisted of predatory ladybirds (79.05 %) and hoverfly larvae (20.95 %). The ladybirds were *Harmonia axyridis* Pallas, *Coccinella septempunctata* Linnaeus and *Adalia bipunctata* Linnaeus; while hoverfly larvae were *Episyrphus balteatus* De Geer and *Syrphus ribesii* Linnaeus (Table 5.3). *Harmonia axyridis* was the most abundant predator (30.16 %), followed by *C. septempunctata* (25.08 %), *A. bipunctata* (23.81 %), *E. balteatus* (14.60 %) and *S. ribesii* (6.35%). Overall there was no significant difference in the number of predators between plant varieties (Table 5.4). The number of aphids did not influence the number of predatory insects collected on the plants. However, symptomatic and asymptomatic infection by *B. cinerea* did affect the number of predators observed on both plant varieties, with more predators recorded on uninfected plants (Figure 5.4).

Figure 5.4: Effects of status of pathogen infection (uninfected/ symptomatic/asymptomatic) and plant variety on the Mean ± SE number of predatory insects.



5.5. Discussion

Fungi that inhabit living plants, wherever they lie on the spectrum from beneficial to pathogenic, have the potential to influence interactions between plants, their insect herbivores, and the insects' natural enemies (reviewed by Pieterse and Dicke, 2007; Hartley and Gange, 2009). Pathogenic microorganisms such as *B. cinerea*, cause symptomatic disease as they mature, and the presence of disease has a detrimental effect on plant quality (e.g. Elad et al., 2007; Choquer et al., 2007; Adrian and Jeandet, 2012; Blanco-Ulate et al., 2014), and so affects insect performance and behaviour (e.g: Zebitz and Kehlenbeck, 1991; Louis et al., 1996; Mondy et al., 1998; Mondy and Corio-Costet, 2000; Mondy and Corio-Costet, 2004; Desneux et al., 2012; Al-Naemi and Hatcher, 2013). However, recent work has shown that *B. cinerea* can be present in plants in an asymptomatic form (Sowley et al., 2010; van Kan et al., 2014; Shaw et al., 2016) and our previous work shows that asymptomatic *B. cinerea* infection influences interactions between herbivorous insects and their natural enemies in the laboratory (Chapter 2, 3). We have also demonstrated that asymptomatically infected plants recruit different numbers and species of aphids and their natural enemies in the field (Chapter 4). Here we placed known numbers of aphids on plants in the field, and we demonstrate that the symptomatic and asymptomatic pathogen infection affected the number of aphids, but this effect is varied with plant variety. However, this effect of pathogen presence was eliminated when aphids were exposed to their natural enemies. While symptomatic and asymptomatic infection by *B. cinerea* on exposed plants did not affect aphid numbers, it did influence the numbers of parasitoids and predators recorded. More parasitoid mummies and predators were recorded on uninfected plants. This finding suggests that uninfected plants received more natural enemies compared to symptomatic and asymptomatic plants, thus reducing the numbers of aphids. This points to a complex interplay between host plants with symptomatic or asymptomatic infection status and aphid physiology and behaviour, influencing the recruitment of predators and parasitoids in the field.

Botrytis cinerea is the causal agent of grey mould disease, is a ubiquitous necrotrophic pathogen, and is regarded as one of the most important crop diseases (Williamson et al., 2007). It infects many parts of the plant, and infection may be symptomless, aggressive, restricted or widely developing (Elad et al., 2007; Shaw et al., 2016). *Botrytis cinerea* kills host cells through the production of toxins, reactive oxygen species and the induction of a plant-produced oxidative burst (Choquer et al., 2007). *Botrytis cinerea* can be present in symptomless lettuce plants as a systemic, endophytic infection which may arise from seed (Sowley et al., 2010). Al-Naemi and Hatcher (2013) found that infection by *B. cinerea* reduces nitrogen content on *Vicia faba* leaves. In the laboratory, we found that asymptomatic infection resulted in reduced chlorophyll content and plant dry weight of lettuce. In our first field experiment (Chapter 4), we found that asymptomatic *B. cinerea* infection reduces plant chlorophyll content [likely to be a result of lost nitrogen levels (Biljana and Aca, 2009)], plant height and shoot: root ratio of Little Gem, but found no differences on plant dry weight. No effect of asymptomatic *Botrytis* infection was detected in Tom Thumb traits. In this field experiment, protection of aphids from attack by their natural enemies indirectly influenced the traits of both plant varieties. These changes might be influenced by the interaction between the numbers of aphids and the effects of pathogen infection on the protected plant. Asymptomatic infection by *B. cinerea* on protected plants changed the chlorophyll content, plant dry weight and shoot:root ratio of Little Gem, and only affected chlorophyll content and height of Tom Thumb. On the other hand, plants exposed to aphid natural enemies indirectly altered the chlorophyll content and shoot:root ratio of Little Gem, and reduced the chlorophyll content of Tom Thumb. This suggests that the effects of asymptomatic infection are subtle, and when environmental variation is present, may be masked.

Little work has been done to investigate the effects of *B. cinerea* infection on plant traits, with most research focusing on the effects of infection on the post-harvest product (e.g. Elmer and Reglinski, 2006; Seglie et al., 2009; Qin et al., 2010; Seglie et al., 2012; Jiang et al., 2015). While our measures of variation in plant quality were simple, investigation of *Botrytis fabae* infection found a significant effect of infection on chlorophyll concentration and photosystem II activity, which then affected plant quality by reducing the total soluble sugars, polysaccharides and total soluble proteins concentrations in faba bean leaves, as well as increasing the production of catalase and pectinase enzyme in the plant body (Mahmoud et al., 2004).

In this field experiment, when protected from aphid natural enemies, we found that *B. cinerea* either symptomatic or asymptomatic infection of Tom Thumb plants reduced the numbers of the aphid *M. persicae*. However, this effect was not detected when aphids on Tom Thumb were exposed to attack by natural enemies. Symptomatic and asymptomatic infection of *B. cinerea* did not affect the numbers of *M. persicae* recorded on Little Gem, whether exposed or protected from natural enemies. In our previous field experiment, we observed more aphids from the natural environment infested uninfected Tom Thumb than asymptomatically infected Tom Thumb, but no similar difference in aphid numbers on Little Gem (Chapter 4). Thus, we predict that the effect of *Botrytis* on the fecundity of aphids depends on the plant genotype and perhaps also the species of insect involved. However, our findings from our field experiment contradict those from our laboratory experiment, where we found that aphids produce more offspring on uninfected lettuce irrespective of variety compared to the asymptomatically-infected plants (Chapter 2). In contrast, Zebitz and Kehlenbeck (1991) found that increased numbers of black bean aphids (*Aphis fabae*) are found on symptomatic *Botrytis* infected *Vicia faba* cv. Bolero compared to the uninfected plants of the same variety, and suggested that this was due to increased availability of amino acids resulting from the pathogen infection. However, in the same experiment, they found that *Botrytis* infection did not influence the number of aphids on *Vicia faba* cv. Diana. In contrast, Desneux et al. (2012) found that population growth of the yellow rose aphid (*Rhodobium porosum*) was significantly reduced by pre-infection of rose plants by *B. cinerea*. Similarly, Al-Naemi and Hatcher (2013) showed that *Botrytis* infection had an inhibitory effect on the growth and fecundity of *A. fabae* reared on *V. faba*.

Changes in aphid performance may have a consequential effect on the life history and behaviour of predators and parasitoids (Blande et al., 2004; Silva et al., 2011). Parasitoid oviposition preference is affected by fitness costs in terms of opportunity time, energy, mortality risk, and potential fitness returns from oviposition in a particular host (Buitenhuis et al., 2004; Fellowes et al., 2005; Ode et al., 2005). In this experiment, we speculate that changes in aphid traits influenced the assemblage of natural enemies attacking them. *Myzus persicae* reared on asymptomatic *Botrytis*-infected lettuce plants are smaller than those from uninfected plants (Chapter 2), and thus may influence the preference behaviour of parasitoids (Chapter 3). Generally, parasitoids are expected to choose hosts that are optimal for offspring development and growth (Morris and Fellowes, 2002). As an example, the fitness of *A. colemani* is greater when reared on the larger *M. persicae* than on the smaller *Aphis gossypii* (Sampaio et al., 2008). Large hosts contain more resources for parasitoid development and hence presumably have higher quality; however, they require more time to subdue and prey are more likely to escape (Chau and Mackauer, 2000). Furthermore, there is a positive relationship between aphid size and resistance, suggesting that successful resistance to parasitoid attack may involve physical, as well as physiological, defences (Gwynn et al., 2005).

Similarly, female insect predators also optimise fitness by choosing oviposition sites and the availability of prey that contribute more to lifetime fitness for their offspring (Sadeghi and Gilbert, 2000; Venzon et al., 2002; Frechette et al., 2004). Predator preference behaviour is affected by aphid-associated chemical stimuli, aphid colony size, the spatial position of the aphid colony and host-plant characteristics (Francis et al., 2004; Schellhorn and Andow, 2005; Almohamad et al., 2006; Pettersson et al., 2008). In our experiment, we found that the numbers of both parasitoid mummies and predatory insects was higher on uninfected plants compared to asymptomatically *Botrytis*-infected plants, despite there being no significant difference in aphid numbers. Confusingly, even though parasitoid preferred to parasitize more aphids on uninfected plants, data obtained shows no significant difference on the number of mummies between uninfected and symptomatic plants. On the other hand, symptomatic and asymptomatic plants showed a similar effect in reducing the numbers of predatory insects compared to uninfected plants. These findings suggest that variation in plant quality resulting from symptomatic or asymptomatic infection by *B. cinerea* influences the foraging behaviour of insects at higher trophic levels (Lill et al., 2002; Hunter, 2003), even though some result obtained in this experiment might difficult to explain without further investigation.

Other studies have found effects of plant pathogen infection on foraging parasitoids. The parasitoid *Microplitis tristis* prefers to attack *Hadena bicruris* on uninfected host plants compared to hosts on *Microbotryum violaceum* -infected plants. *Microbotryum violaceum* infection of the host plant resulted lower host plant quality, causing reduced adult emergence of the parasitoids (Biere et al., 2002). Some studies also have reported the effects of plant pathogen-induced volatiles on the foraging behaviour of parasitoids. *Tamarixia radiate*, the parasitoid of the psyllid *Diaphorina citri,* was attracted more towards plants infected by the citrus greening bacterium '*Candidatus* Liberibacter asiaticus' than uninfected plants. This was due to the release of methyl salicylate; parasitism of *D. citri* nymphs on *Candidatus* Liberibacter asiaticus infected plants was found to be higher than that on uninfected controls (Martini et al., 2014). The parasitoid *Cotesia marginiventris* landed more frequently on white mould fungus *Sclerotium rolfsii* Saccodes infected plants than on healthy plants. This preference is mediated by plant volatiles and other biochemical changes in plant chemistry caused by fungal infection (Cardoza et al., 2003).

However, there are many exceptions to this basic framework, and recent work suggests that the co-occurrence of the infection by pathogen and insect infestation on the shared host plant may interrupt the foraging behaviour of natural enemies (Harvey et al., 2010; Ponzio et al., 2013; Tack and Dicke, 2013). Studies report that infection by *B. cinerea* triggers activation of jasmonic acid (JA) and ethylene (ET)-dependent defence signalling in the plant body (Zimmerli et al., 2001; Audenaert et al., 2002; Van Baarlen et al., 2007; Cabot et al., 2013), while aphid infestation triggers the production of salicylic acid (SA)-dependent pathways (Mohase and van der Westhuizen, 2002; Chaman et al., 2003; Walling, 2008; Donovan et al., 2012). Both pathways do not exist in isolation and cross talk between these pathways may occur. The activation of the SA-dependent pathway often leads to a down-regulation of the JA-dependent pathway and vice versa (Felton and Korth, 2000; Thaler et al., 2002a; Robert-Seilaniantz et al., 2011). If so, a change in the emission of many compounds should be the result, and members of the third trophic level may adapt their responses to optimize exploitation of the signals. Since the parasitoid olfactory system is dependent on the chemical pathways produced by the host or host habitat (Fellowes et al., 2005), down-regulation in defence pathways may affect parasitoid foraging behaviour. In our study, we did not measure the quality and quantity of defences metabolites produced by the plant, but based on the theory mentioned above, it is likely that symptomatic and asymptomatic infection of *B. cinerea* influences plant secondary defences, and this in turn influences the structure of the assemblage of the aphid natural enemies in this system.

In nature, plants are exposed almost constantly to attack by herbivorous insects and pathogenic microorganisms. While attack by external herbivores can readily be recorded, this is not necessarily so for asymptomatic infection by plant pathogens. As infection by hidden plant pathogens has the potential to shape the composition of insect communities across the landscape and can generate changes that ramify upward to higher trophic levels, this study provides novel insights to help improve our understanding of how these complex systems work. Findings from this experiment may also help to disentangle the relationships that occur between plants, pathogens, herbivorous insects and their enemies. In agricultural systems, such knowledge could enhance the effectiveness of biological control programmes and might help explain why some biological control programmes are successful whereas others fail.

Chapter 6: Both symptomatic and asymptomatic pathogen infection can cause herbivore-mediated indirect interactions between neighbouring plants

6.1. Abstract

Interactions between organisms at higher trophic levels can be modulated by host plant quality. For example, alterations in plant quality caused by the pathogen infection changes interactions between insect herbivores and species at higher trophic levels. Changes in interactions between plants and insects caused by pathogen infection is well understood. However, the effect of asymptomatic pathogen infection on interactions at higher trophic levels is not explored. We designed an experiment to investigate the indirect effects of hidden pathogen infection on the interactions between neighbouring plants measured in terms of plant quality and the assemblage of insect herbivores and their natural enemies found on the plants. We found that asymptomatic pathogen infection gives rise to a double jeopardy effect; infection not only altered plant traits and changed the structure of their associated insect community, but also reduced the quality and influenced the insect assemblage found on their uninfected neighbour. Uninfected plants neighbouring symptomatic *Botrytis*-infected or asymptomatic *Botrytis*-infected plants were infested with more aphids than uninfected plants neighbouring other uninfected plants. While no effect was observed on the diversity of natural enemies, the number of predators and parasitoids was higher on uninfected plants neighbouring infected plants. This finding has considerable implications for our understanding of the importance of plant pathogens in mediating both direct and indirect interactions at multiple trophic levels.

6.2. Introduction

Host plant traits are critical factors in determining the behaviour and success of insect herbivores (West and Cunningham, 2002; Peccoud et al., 2010; Forister and Wilson, 2013). Among these traits, factors which determine plant quality as perceived by the herbivore (e.g. plant secondary metabolites, nutrient content) affect the fecundity and longevity of insect herbivores (Prokopy and Owens, 1983; Patt and Sétamou, 2007; Mithöfer and Boland, 2012; Wetzel et al., 2016), and in consequence, can have alter species interactions and hence influence how communities are structured (Stout et al., 2006; Biere and Tack, 2013; Schuldt et al., 2017). Some of these plant-mediated effects are direct [e.g. plant quality directly affects the life history of insect herbivores (Awmack and Leather, 2002)], while others are indirect, where herbivore-natural enemy interactions are altered by changes to the plant's morphological and chemical phenotype (Werner and Peacor, 2003; Schmitz et al., 2004; Frago et al., 2012; Stam et al., 2014). Such direct and indirect consequences of variation in host plant quality affect the abundance and diversity of arthropods associated with plants (Lau et al., 2008; Everwand et al., 2014; Ohgushi and Hambäck, 2015).

Linking how the effects of such inter and intra-specific variation in host plant quality ramify through food webs is an active area of ecological research. In general, ecologists have studied the effects of biotic [e.g. previous damage (Hare, 2011; Lucas-Barbosa et al., 2011), or defence genotype (Agrawal, 2011; Ali and Agrawal, 2012)], and abiotic factors [e.g. nutrient availability (Franzke et al., 2010; Joern et al., 2012), or water availability (Gutbrodt et al., 2011; Jactel et al., 2012)] on the behaviour and fitness of insect herbivores. For example, plant morphological and physiological characteristics change over time as plants develop from seedlings to mature stages. These changes influence the nutrient quality of phloem sap (Boege and Marquis, 2005) which then alters the feeding behaviour of aphid herbivores (Karley et al., 2002). Plant quality can simply be altered by the addition of fertiliser, which can positively influence insect abundance (Müller et al., 2005), or increased nitrogen availability can lead to the increased expression of plant defences, reducing plant quality for herbivores (Mur et al., 2017).

Plant quality can also be altered by pathogen infection (Berger et al., 2007; Dodds and Rathjen, 2010). As an example, the necrotrophic pathogen *Botrytis cinerea* may infect plants with extensive fungal growth, giving the appearance of grey mould (Govrin and Levine, 2000). In some conditions, infection by this pathogen may cause only minimal damage to host tissues (Elad, 1997) or the infection might be hidden and not show any symptoms at all (Sowley et al., 2010). However, infection of *B. cinerea*, whether symptomatic or asymptomatic, may still affect plant traits, such as growth rates (Sowley et al., 2010; van Kan et al., 2014; Chapter 2). This is because the infected host plant may activate their defence mechanisms, and produce pathogenicity related proteins, phytoalexins or other phenolic compounds to combat the pathogen (Elad, 1997), thus reducing host plant quality. Most plants respond to the damage made by their enemies with the production of a variety of volatile organic compounds, either to combat attack or as host-location cues for insect natural enemies (Choudhary et al., 2008; Dicke et al., 2009; Ponzio et al., 2016), recruiting parasitoids and predators to reduce the consequences of insect herbivory (Kerchev et al., 2012).

It is not only plants with evident symptoms of disease that have altered interactions with their herbivores. In our previous experiment, we found the asymptomatic pathogen infection changed the life history (Chapter 2) and preference behaviour (Chapter 3) of insect herbivores in controlled settings. In the field, asymptomatic pathogen infection influenced the abundance and diversity of aphids (Chapters 4 and 5). Such hidden diseases also influenced insect natural enemies. As the asymptomatic pathogen infection affected their aphid hosts, the life history and preference behaviour of parasitoids was indirectly affected (Chapter 2 and Chapter 3). Therefore, the assemblage of natural enemies attacking aphids in the field was also altered (Chapter 4 and Chapter 5). The presence of a hidden plant pathogen altered ecological interactions between species at four trophic levels.

While there is therefore much evidence that aphids and their natural enemies are influenced by the infection status of the host plant, this begs the question of whether such influences are strong enough to cause indirect effects on ecological interactions. The most frequently considered form of indirect interaction is apparent competition, where an increase in the abundance of one (non-directly competing) species, indirectly leads to a reduction in the abundance of another, mediated by the presence of natural enemies. Given that asymptomatic infection alters host plant quality, the fitness of aphids, and the morphology and behaviour of natural enemies, it is reasonable to ask if the infection status of a plant can affect the dynamics of an insect herbivore on a neighbouring uninfected plant by influencing natural enemy behaviour, and if this in turn affects the fitness of the neighbouring plant.

Most studies have examined the indirect effects of plant pathogen infection on the insect abundance and diversity with focused on the insect community associated to the individual plant or entire plant community (e.g: Cory and Hoover, 2006; Hodge and Powell, 2008a, 2010; Johannesen et al., 2012; Mann et al., 2012; Martini et al., 2014; Pelz-Stelinski and Killiny, 2016). Fewer studies have examined the effect of plant pathogen infection on their neighbouring plant (e.g: Gilbert and Webb, 2007; Quintana-Rodriguez et al., 2015). The effects of apparent competition on species coexistence cause by the action of natural enemies are well studied (e.g: Holt and Lawton, 1993; Chaneton et al., 2000; van Veen et al., 2006). However, not much are known on the ability of hidden plant pathogen to mediate the apparent competition among herbivores insect that fed on neighbouring plant. Here, we attempt to bridge this gap by investigating the effect of plant pathogen in indirectly mediating interactions between species. In this experiment, we investigate the interactions between uninfected and *Botrytis* infected-plants in close proximity, in which the influence of plant health status either uninfected, asymptomatic-infected or symptomatic-infected on neighbouring plants in term of the assemblage of herbivorous insects and their natural enemies.

6.3. Materials and method

6.3.1. Study system

Lettuce, *Lactuca sativa* (Asteraceae) variety Tom Thumb either uninfected or infected with the fungus *B. cinerea* strain BO5.10 was used as a model system. The insect herbivore was the peach potato aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae). Both uninfected and *Botrytis*-infected plants were grown from seeds harvested in 2014. The *Botrytis*-infected seed were collected from plants inoculated at the flowering stage with dry spores of *B.*

cinerea (following Sowley et al., 2010), while uninfected seeds were collected from uninoculated plants*.* *Botrytis*-inoculated and uninoculated plants were grown in different glasshouses but under similar conditions to reduce pathogen contamination of uninoculated plants. Six-week-old plants were infested with five seven-day-old aphids, four days before the field experiment. Aphids were originally collected from cabbage plants at the experimental grounds of the University of Reading and have been reared on Brussel sprouts for several years in the laboratory. In 2014, aphids were transferred onto both uninfected and *Botrytis*-infected Tom Thumb plants and reared for several generations at ambient temperature and humidity in the laboratory prior to the experiment to avoid maternal effects. The experiment was conducted in July 2016.

6.3.2. Field study

Plants were grown in 15 cm diameter pots with traditional potting compost (Vitax Grower, Leicester, England) in a glasshouse. As there is no guarantee that plant grown from the *Botrytis*-infected seed will grow as infected plants and vice versa, the experiment was started with sixty plants for each treatment. Two weeks before the field experiment started, the plant infection status was checked. Forty replicates of plants with confirmed pathogen infection status were selected as hosts to rear aphids for the field experiment. From that, thirty replicates of plants with aphid colonies and visually free from the symptoms of pathogen infection or mechanical damage were selected for each treatment to use in this field experiment. Control plants were covered with breathable plastic bags to protect aphids from predation, parasitism or migration. Plant stations consisted of two pots of lettuce with the following combinations: A: uninfected-infected; B: infected-infected; C: uninfected-uninfected. Pairs of plants were randomly placed in a grid pattern in the field site within the experimental grounds, University of Reading, U.K (51.4414° N, 0.9418° W). The distance between each pot was 20 cm and each station was 3 m apart. Plants were watered as required and any weeds occurring at the field site were removed regularly. Each plant pot was placed in a shallow plastic dish to minimize the effect of water stress or saturation.

The numbers of aphids, parasitoid mummies and predatory insects were counted by looking on both the upper and lower surfaces of every leaf and recorded every two days until the colonies of aphids died, which took approximately 28 days. Mummies and predatory insects were collected from the plants and brought to the laboratory for identification. If more than one mummy was found on the plant, at least half were randomly left for up to an additional 72 h on the plant to allow for hyperparasitoid attack. The location of mummies left on the plant was marked to avoid recounting. Mummies were kept in 5 ml vials until the parasitoid emerged. The larvae of predatory insects were fed with aphids in 5 ml vials and kept until they were adult.

At the end of experiment, the infection status of plants was rechecked. Plants grown with uninfected seeds but showing disease symptoms (with symptoms such as dry lesions, or spreading soft rots with or without the appearance of conspicuous sporulating fungus colonies; Elad et al., 2007) were discarded from the experiment as these would have been infected at an undefined time later in the experiment. The remaining plants were then defined as being in the following pairs: 1: uninfected-uninfected; 2: uninfected-asymptomatic; 3: uninfected-symptomatic; 4: symptomatic-symptomatic; 5: symptomatic-asymptomatic and 6: asymptomatic-asymptomatic plants.

The chlorophyll content of the leaves was measured a day before the plant was harvested. Three different mature and apparently healthy leaves for each plant replicate were randomly chosen and the chlorophyll content of the leaves were measured by using a handheld Chlorophyll Meter (Model atLeaf; FT Green LLC, Delaware, USA). To investigate the plant growth, the height of the plant was measured on the first day and on the final day of the experiment. Whole plants (including the roots) were harvested and dried in an oven at 75°C until reaching constant mass (which takes ~48 h) and were weighed using an electronic balance (Sartorius, LC 6200S, Goettingen, Germany). The root: shoot ratio was measured by dividing the dry weight of shoot by root dry weight for each plant. The shoot section is above ground and includes the leaves and stems while the root section includes those parts of the plant below ground, such as the roots.

The status of *Botrytis* infection in the plant body was confirmed by examining leaf samples using *Botrytis* selective media agar (BSM) (Edwards and Seddon, 2001). Symptomatic plants were defined as those plants which showed symptoms of *Botrytis* infection and the presence of *Botrytis* in the plant body was confirmed by using BSM. If symptoms of infection were absent but the presence of *Botrytis* was confirmed by the BSM inspection, it was then categorized as an asymptomatic plant. The presence of *Botrytis* in the plant body was determined by plating three 1 cm diameter discs of leaf samples with no visual signs or symptoms of damage on BSM agar. The leaf disc was surface-sterilised by dipping the leaves in 70% ethanol for 1 min, followed by 1 min in 20% bleach solution (Domestos, Unilever; 5% NaOCl in alkaline solution with surfactants) and then rinsed three times in sterile distilled water and allowed to dry (Barnes, 2002). The leaf disk then was plated out on BSM agar and incubated at 18-20°C in an incubator with alternating UV-A light (12h/day) and dark (12h/day). The BSM agar was observed at intervals for up to 14 days to determine the presence of *B. cinerea*. Confirmation was based on the sporulation of the pathogen and morphological observation of the colonies under a high-performance stereomicroscope (Leica, MZ9.5, Texas, USA).

6.3.3. Statistical analysis

All statistical analyses were performed in R statistical software version 3.4.0 (R Core Team, 2017). The analyses were run to investigate two main questions: (i) the effect of pathogen infection on the plant traits and the number of insect; (ii) the influence of plant health status (either uninfected, asymptomatic-infected or symptomatic-infected) on neighbouring plants in term of plant quality and the assemblage of insects. For the first question, we used linear models and treated pathogen infection status and plant exposure as explanatory variables to measure the plant traits. Data for plant dry weight was log transformed and data for shoot:root ratio was square root transformed to meet the assumption of normality and homoscedasticity. The data of the cumulative number of aphids, parasitoid mummies and predatory insects were analysed using Generalized Linear Models (GLM) with quasipoisson errors.

To learn whether plant health status will indirectly affect their uninfected neighbour, only data of the focal plant (uninfected plant) of following pairs: 1: uninfected-uninfected; 2: uninfected-asymptomatic; 3: uninfected-symptomatic were subjected to statistical analysis. We analysed our focal plant traits data using linear models. Then we compared the cumulative number of aphids on focal plants (uninfected plants) on pairs 1, 2 and 3 using ANCOVA with the number of aphids on their neighbour as a covariate. The total number of predators and parasitoids on focal plants was also compared with the same manner which the cumulative number of aphids on the respective focal plant treated as a covariate.

6.4. Results

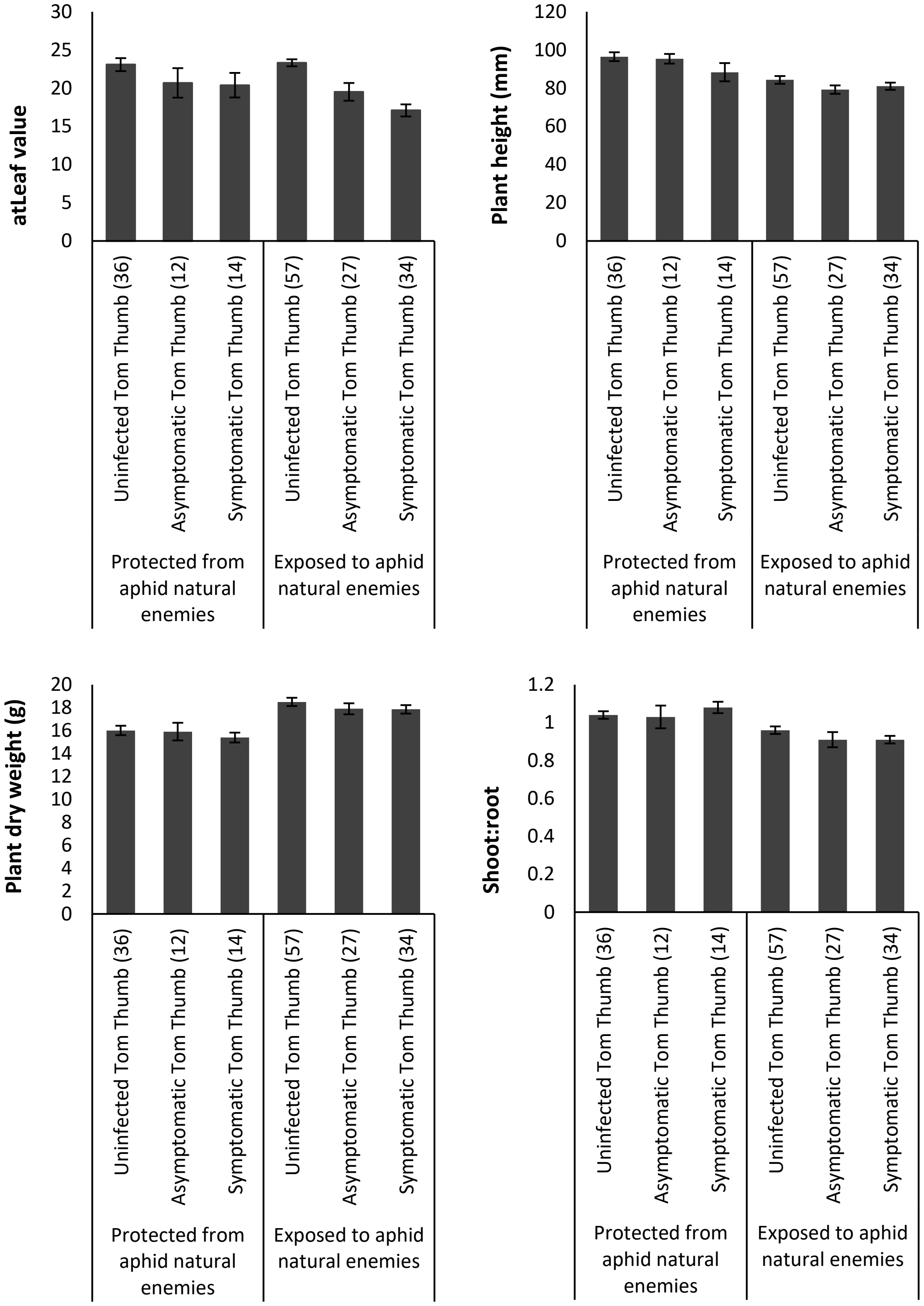
6.4.1. Plant life-history traits

The effect of pathogen infection and plant exposure varied depending on the traits measured (Table 6.1). Symptomatic pathogen infection altered the chlorophyll content of the plant on both exposed and covered plants, while the asymptomatic pathogen infection status only affected the chlorophyll content of exposed plants (Figure 6.1). No significant effect of either symptomatic or asymptomatic pathogen infection was observed on the other plant traits (Figure 6.1).

Table 6.1: Summary of effects of plant exposure and *B. cinerea* infection status on plant traits following analysis. Significant values are in bold.

Plant traits	Explanatory variable	Coefficient t value ± SE	P
atLeaf value	**Intercept**	**21.340 ± 0.961**	**<0.001**
	Plant exposure	-1.175 ± 0.782	0.241
	Uninfected	**3.451 ± 0.949**	**< 0.001**
	Symptomatic	-1.695 ± 1.070	0.091
Shoot:root	**Intercept**	**55.773 ± 0.018**	**<0.001**
	Plant exposure	**-3.945 ± 0.014**	**<0.001**
	Uninfected	1.262 ± 0.017	0.208
	Symptomatic	0.635 ± 0.020	0.526
Dry weight (g)	**Intercept**	**103.746 ± 0.026**	**<0.001**
	Plant exposure	**6.538 ± 0.021**	**<0.001**
	Uninfected	0.943 ± 0.026	0.347
	Symptomatic	-0.290 ± 0.029	0.772
Plant height (mm)	**Intercept**	**34.972 ± 2.641**	**<0.001**
	Plant exposure	**-5.491 ± 2.148**	**<0.001**
	Uninfected	1.487 ± 2.606	0.139
	Symptomatic	-0.296 ± 2.939	0.767

Figure 6.1: The effect of plant exposure and pathogen infection on Mean ± SE of atLeaf value, plant height, plant dry weight and shoot: root ratio of the plant. Number of plants as shown below each treatment heading.

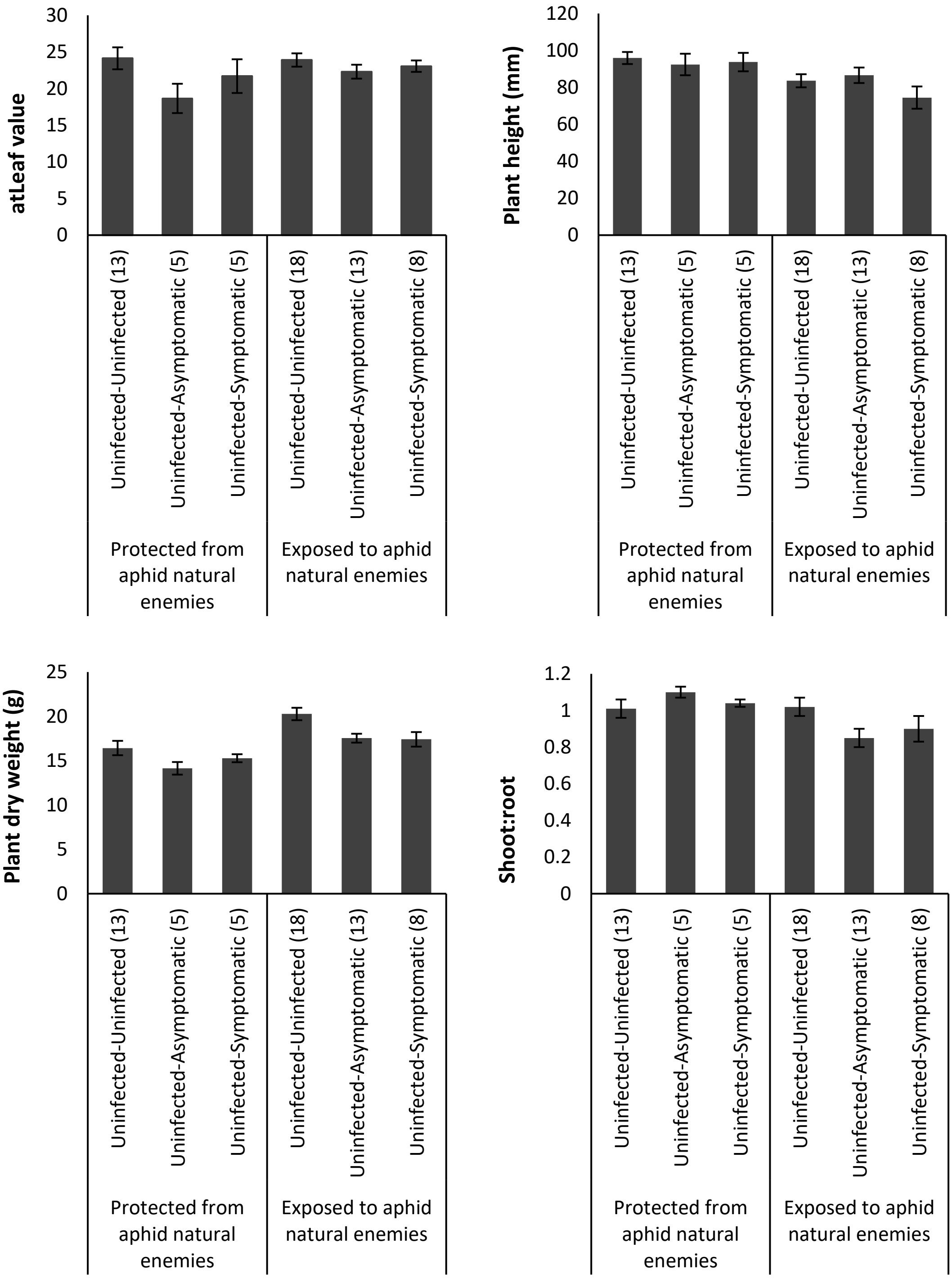


The traits of focal plants (uninfected plants) that neighboured uninfected, asymptomatic or symptomatic plants also varied (Table 6.2). The chlorophyll content of focal plants in pair 2 (uninfected-asymptomatic) was significantly lower than the focal plants in pair 1 (uninfected-uninfected). However, this pathogen effect was eliminated when plants were exposed to aphid natural enemies. Surprisingly, the chlorophyll content on focal plant in pair 1 and pair 3 (uninfected-symptomatic) showed no difference on both exposed and covered plants (Figure 6.2). Symptomatic and asymptomatic infected plants affected the dry weight of their uninfected neighbours. The focal plant in pair 1 was heavier than the focal plants in pair 2 and pair 3 (Figure 6.2). The same pattern was also observed for the shoot: root ratio of the plant. The focal plants on pair 1 had higher shoot: root ratios than the focal plants in pair 2 and pair 3, but this result only applied to exposed plants (Figure 6.2). Meanwhile, the height of the focal plants was not influenced by the health status of their neighbours (Figure 6.2).

Table 6.2: Summary of effects of the health plant status on the traits of their uninfected neighbour plant following analysis.

Plant traits	Explanatory variable	Coefficient t value ± SE	P
atLeaf value	**Intercept**	**16.184 ± 1.267**	**<0.001**
	Plant exposure	1.001 ± 1.106	0.321
	Uninfected-uninfected	**2.290 ± 1.246**	**0.025**
	Uninfected-symptomatic	0.891 ± 1.523	0.376
Shoot:root	**Intercept**	**16.502 ± 0.06**	**<0.001**
	Plant exposure	-1.718 ± 0.052	0.091
	Uninfected-uninfected	1.352 ± 0.059	0.181
	Uninfected-symptomatic	0.270 ± 0.072	0.788
Dry weight (g)	**Intercept**	**66.657 ± 0.039**	**<0.001**
	Plant exposure	**5.598 ± 0.034**	**<0.001**
	Uninfected-uninfected	**3.515 ± 0.039**	**<0.001**
	Uninfected-symptomatic	0.483 ± 0.047	0.630
Plant height (mm)	**Intercept**	**22.390 ± 4.330**	**<0.001**
	Plant exposure	**-3.209 ± 3.779**	**0.002**
	Uninfected-uninfected	-0.268 ± 4.259	0.789
	Uninfected-symptomatic	-1.438 ± 5.206	0.155

Figure 6.2: The effect of plant exposure and pathogen infection on Mean ± SE of atLeaf value, plant height, plant dry weight and shoot: root ratio of their uninfected neighbour



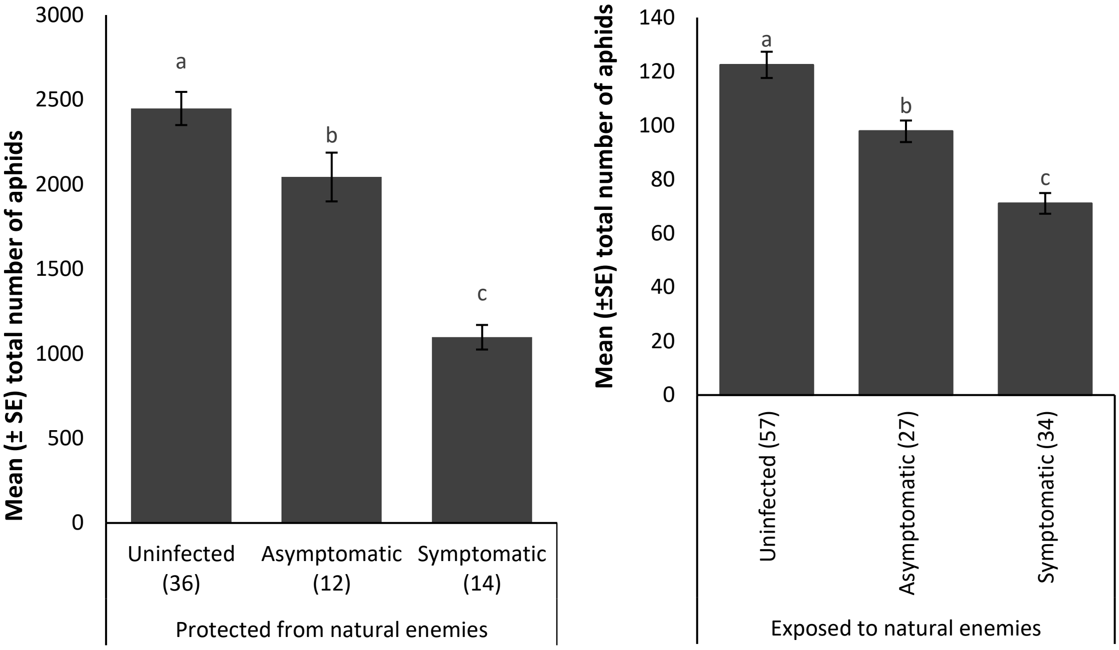
6.4.2. Aphids

Pathogen infection status and natural enemy exposure significantly altered the number of aphids on plants (Table 6.3). Plants that were protected from attack by aphid natural enemies have significantly more aphids than exposed plants (Table 6.3). Whether protected or exposed to natural enemies, the number of aphids on uninfected plants was significantly higher than on the symptomatic and asymptomatic plants (Figure 6.3).

Table 6.3: Summary of effects of *B. cinerea* infection status on the cumulative number of aphid, parasitoid mummies and predatory insect. Significant values are in bold.

	Explanatory variable	Coefficient t value ± SE	P
Aphid	**Intercept**	**174.642 ± 0.04**	**<0.001**
	Plant exposure	**-21.714 ± 0.139**	**<0.001**
	Uninfected	**3.663 ± 0.049**	**<0.001**
	Symptomatic	**-8.844 ± 0.070**	**<0.001**
Parasitoid mummies	Intercept	-0.607 ± 0.264	0.544
	Uninfected	**2.165 ± 0.295**	**0.032**
	Symptomatic	**-2.886 ± 0.685**	**0.004**
Predatory insect	**Intercept**	**-3.008 ± 0.404**	**0.003**
	Uninfected	**2.986 ± 0.430**	**0.003**
	Symptomatic	-0.014 ± 0.542	0.989

Figure 6.3: The effect of plant exposure and pathogen infection (uninfected/ asymptomatic/ symptomatic) on mean ± SE number of aphids.

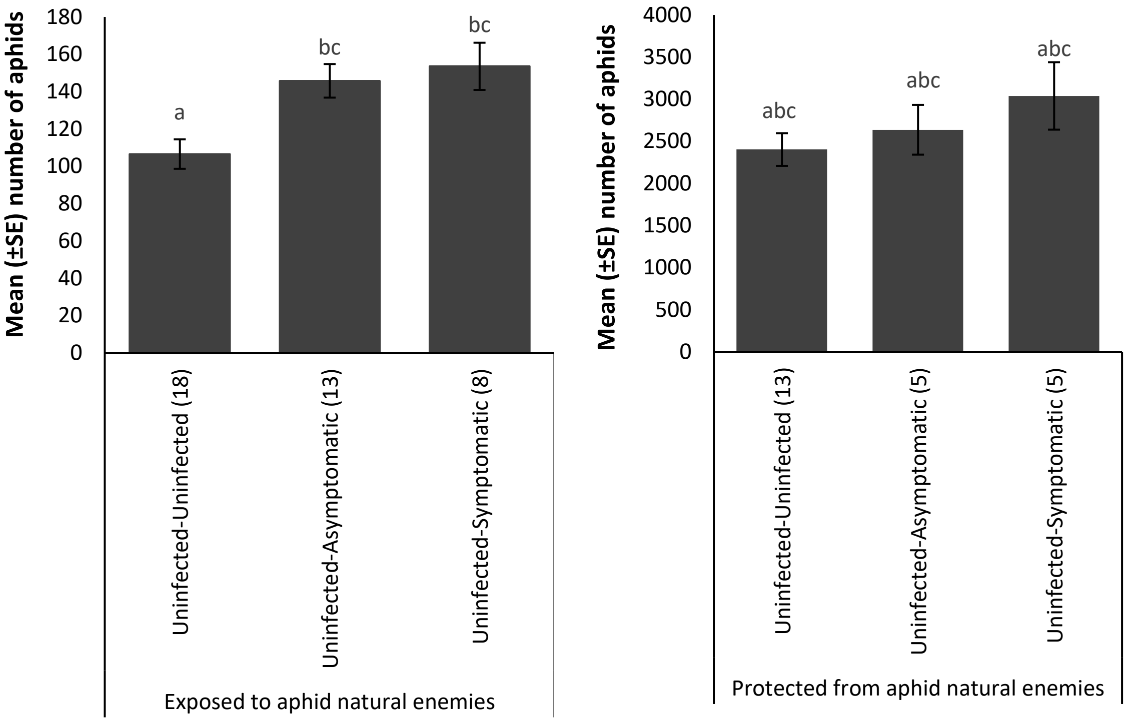


The neighbours' health status and the number of aphids on their neighbours influenced the number of aphids on the focal plants (uninfected plants) (Table 6.4). When exposed to aphid natural enemies, the focal plant in pair 1 (uninfected-uninfected) harboured significantly lower numbers of aphids than the focal plants in pair 2 (uninfected-asymptomatic) and focal plants in pair 3 (uninfected-symptomatic) (Figure 6.4). This effect was not observed when aphids on plants were protected from attack by their natural enemies (Figure 6.4).

Table 6.4: Summary of effects of *B. cinerea* infection status on the cumulative number of aphid, parasitoid mummies and predatory insect on neighbouring plant

	Explanatory variable	DF	SS	RSS	AIC	F value	P
				36763	275.10		
Aphid (Exposed)	**Plant set**	**2**	**15317.800**	**52081**	**284.680**	**7.291**	**0.002**
	Aphid on neighbouring plant	**1**	**8320.600**	**45083**	**281.060**	**7.921**	**0.007**
				7766374	300.79		
Aphid(Protected)	Plant set	2	765959	8532333	298.95	0.9369	0.4092
	Aphid on neighbouring plant	1	546243	8330617	300.40	1.3804	0.2545
				143.510	58.810		
Parasitoid	Plant set	2	7.755	151.260	56.862	0.945	0.398
Parasitoid	Aphid on focal plant	1	9.120	152.630	59.213	2.224	0.144
				68.014	29.690		
Predatory insect	Plant set	2	2.618	70.633	27.163	0.673	0.516
Predatory insect	Aphid on focal plant	1	0.686	68.701	28.082	0.353	0.556

Figure 6.4: The effect of pathogen infection (uninfected/ symptomatic/asymptomatic) on the mean ± SE number of aphids on neighbouring plants.



6.4.3. Natural enemies

We collected 119 parasitoid mummies and 79 predatory insects in this experiment. 101 of those mummies emerged; four species were identified as primary parasitoid (85.14%) and three as secondary parasitoids (Table 6.5). The primary parasitoids were all members of the family Braconidae [*Aphidius ervi* (Haliday), *Aphidius matricariae* (Haliday), *Praon gallicum* (Stary), *Diaeretiella rapae* (M'Intosh)], while the hyperparasitoids were *Dendrocerus carpenteri* (Curtis) (Hymenoptera: Ceraphronidae), *Asaphes vulgaris* (Walker) (Hymenoptera: Pteromalidae) and *Alloxysta victrix* (Westwood) (Hymenoptera: Cynipidae). *Aphidius matricariae* (34.65%) and *A. ervi* (24.75%) were the most abundant species of parasitoid collected in this experiment. The least common was *A. victrix*, with only one individual found on the uninfected plant.

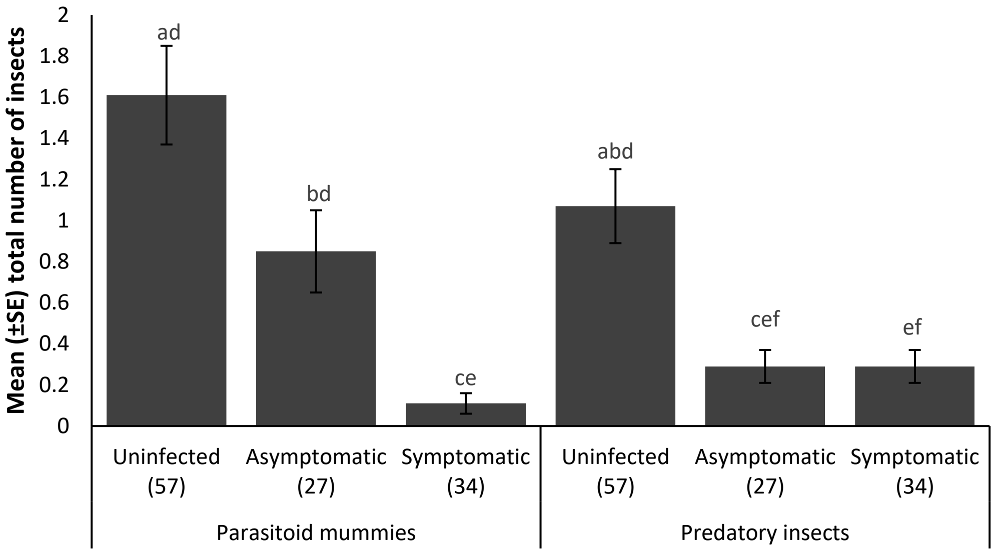
The predatory insects consisted of three species of ladybird (*Harmonia axyridis* Pallas, *Coccinella septempunctata* Linnaeus and *Propylea quatuordecimpunctata* Linnaeus) and one species of hoverfly (*Syrphus ribesii* Linnaeus) (Table 6.5). *Harmonia axyridis* was the most abundant predator (39.25 %), followed by *C. septempunctata* (31.64 %), *S. ribesii* (18.99%) and *P. quatuordecimpunctata* (10.12%).

Table 6.5: Counts of insect species found on the plants. Plant treatment; UTT: Uninfected Tom Thumb, ATT: Asymptomatic Tom Thumb, STT: Symptomatic Tom Thumb. Number of plants as shown below each treatment heading.

	UTT (57)	ATT (27)	STT (34)	Total
Species	Number of insects			
Parasitoid				
Asaphes vulgaris	8	0	1	9
Alloxysta victrix	1	0	0	1
Dendrocerus carpenteri	5	0	0	5
Aphidius ervi	18	5	2	25
Diaeretiella rapae	14	0	0	14
Praon gallicum	15	0	0	15
Aphidius matricariae	28	4	0	32
Predator				
Harmonia axyridis	22	3	6	31
Propylea quattuordecimpunctata	8	0	0	8
Coccinella septempunctata	17	5	3	25
Syrphus ribesii	14	0	1	15

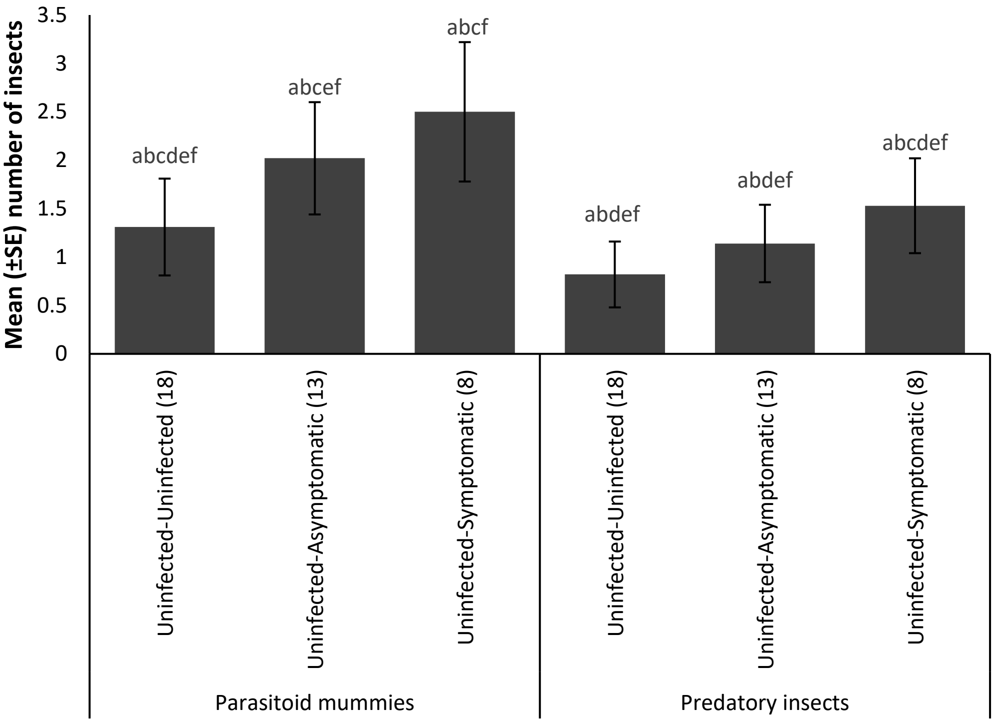
The results obtained confirmed that the pathogen infection status affects the number of parasitoid mummies and predatory insects observed on plants (Table 6.3). More aphid natural enemies were found on uninfected plants than on asymptomatic and symptomatic infected plants (Figure 6.5).

Figure 6.5: The effect of plant pathogen infection on Mean ± SE of mummies and predatory insects.



Our finding also confirmed that the pathogen infection status of neighbouring plant does not influence the assemblage of aphid natural enemies on uninfected plants (Table 6.4). However, pattern shows more mummies and predatory insects were collected on uninfected plants that neighboured symptomatic infected plants (pair 3) followed by uninfected plants in pair 2 and uninfected plants in pair 1 (Figure 6.6).

Figure 6.6: The effect of pathogen infection on the number of mummies and predatory insect on neighbouring plants.



6.5. Discussion

This study focussed on the direct and indirect effects of pathogen infection status on plant-insect interactions, and through that, the indirect effects of hidden infection on neighbouring plants. The direct effect of expressed plant pathogen infection on insect life history and performance are well studied (Van Der Putten et al., 2001; Pieterse and Dicke, 2007; Heil, 2011; Van Dam and Heil, 2011). Our previous work (Chapters 2 and 3) showed the direct effects of pathogen infection status, including when the plant was asymptomatic, on the preference and performance of insect herbivores and their natural enemies in the laboratory. In the field, pathogen infection status influences the structure of insect assemblages on the plants themselves. In this experiment, we asked if pathogen-infected plants indirectly influence the structure of the insect assemblage on their uninfected neighbours. We found that hidden pathogen infection not only altered plant traits and the structure of the associated insect community, but also significantly affected traits and the structure of insect assemblage of their healthy neighbour, most likely mediated by variation in herbivore behaviour. Uninfected plants neighbouring symptomatic *Botrytis*-infected or asymptomatic *Botrytis*-infected plants had more aphids than uninfected plants. While there was no significant effect on the assemblage of natural enemies, more predators and parasitoids were observed on uninfected plants neighbouring infected plants.

Aphid assemblages are structured by the quality of the host plant (Awmack and Leather, 2002; Bukovinszky et al., 2008) and the action of natural enemies (Walker and Jones, 2001; Kos et al., 2011). However, these bottom up and top-down forces can be altered by intraspecific plant variation (Crist et al., 2006; Karley et al., 2008; Barbour et al., 2016; Wetzel et al., 2016), for example by the differences in concentrations of metabolites and chemical cues that affect herbivore abundance and performance through the plant quality or interaction with natural enemies of this herbivores (Kos et al., 2011; Santolamazza-Carbone et al., 2014). In principle, the number of aphids on the focal (uninfected) plant in plants in this experiment should show no differences, and indeed this is the case for control plants covered to prevent access by colonizing insects. However, where the plants are exposed to attack, we find that the infection status of the neighbouring plant influences the herbivore load, and hence physical traits, of the focal, uninfected plant.

Previous research has shown that the impact of neighbouring plants on the population of arthropods on a focal plant may be mediated by chemical and visual cues (Barbosa et al., 2009). Plants have a variety of chemical defences that often increase in concentration following the attack by plant pathogen and herbivorous insect (Schardl, 2002; Chen, 2008; Choudhary et al., 2008; Smith et al., 2009; De Wit et al., 2013; Vos et al., 2013). These chemical cues may influence their neighbouring plants to alter their defences (Kost and Heil, 2006; Choh et al., 2013; Delaney et al., 2015) and have the ability to confuse or to interfere with the ability of natural enemies to hunt their prey (Dicke and Dijkman, 2001; Kost and Heil, 2006; Li et al., 2012). In this experiment, as the number of aphids on the focal plants (uninfected plants) in pair 2 (uninfected-asymptomatic) and pair 3 (uninfected-symptomatic) is significantly higher than the number of aphids on focal plants in pair 1 (uninfected-uninfected), it can be suggested that (i) the production of plant pathways by infected plant alters the defence mechanisms of their uninfected neighbour, thus influencing aphid performance and fecundity, and/or (ii) the chemical cues produced by infected plant disturb the foraging behaviour of natural enemies, thus they may be less able to locate aphids on their uninfected neighbour.

An accumulating body of literature shows plants infected with pathogens have developed a broad range of defence responses to cope with pathogenic infection (Wojtaszek, 1997; Berger et al., 2007; Bari and Jones, 2009; Naseem et al., 2015; Shigenaga and Argueso, 2016). Plants infected with *B. cinerea* vigorously attempt to prevent pathogen invasion and outgrowth by activating multiple defence pathways, including the production of antifungal metabolites and pathogenesis-related proteins (Van Baarlen et al., 2007; Williamson et al., 2007). Once infected, plants induce an oxidative burst and expression of genes associated with systemic acquired resistance (SAR) and encoded defence-related proteins that are considered to be markers for defence pathways governed by salicylic acid (SA), ethylene (ET) and jasmonate (JA) (Govrin and Levine, 2000; Thomma et al., 2001; Díaz et al., 2002; Govrin and Levine, 2002; Glazebrook, 2005; El Oirdi et al., 2011). As plants have a capability to recognize and respond to the information derived from their neighbours (Arimura et al., 2000; Karban and Maron, 2002; Kost and Heil, 2006), the chemical pathways produced by *Botrytis*-infected plants may activated the defence pathways in their neighbouring plants or there will be hormone crosstalk between neighbouring plants (Heil and Karban, 2010).

As an example, *Centaurea maculosa* can change its defensive strategy based on the identity of its plant neighbours. *Centaurea maculosa* exposed to the plant defence signalling molecule methyl jasmonate, accumulated higher levels of defence-related secondary metabolites and lower levels of primary metabolites when growing in conspecific (*C. maculosa*) versus heterospecific (*Festuca idahoensis*) field stands (Broz et al., 2010). Wild tobacco plants with clipped sagebrush neighbours had increased levels of the putative defensive oxidative enzyme, polyphenol oxidase, relative to control tobacco plants with unclipped sagebrush neighbours (Karban et al., 2000). These changes then indirectly influence the interaction between plant and their insect herbivores. For example, the *Agapeta zoegana* (knapweed moth) and *Urophora spp* (fruit flies) occurred more frequently in conspecific (*C. maculosa*) versus heterospecific (*Festuca idahoensis*) stands (Broz et al., 2010); and tobacco plants near clipped sagebrush experienced greatly reduced levels of leaf damage by grasshoppers and cutworms during three field seasons compared to unclipped controls (Karban et al., 2000). In my experiment, pathogen infection on plant may activate the production of volatiles which provide chemical information about the status of pathogen attack to their uninfected neighbour. Once received, the uninfected plant then alters their defence mechanisms to avoid the spread of pathogen infection. As the defence mechanism for pathogen infection in the uninfected plant is now activated, the performance of aphids fed on the uninfected plant might be affected.

Hormone crosstalk between defence pathways is thought to provide plants with useful information, which help it to prioritize and decide which defensive strategy to follow, depending on the type of attacker (Pieterse and Dicke, 2007). Induction of SA or JA/ABA-dependent defence responses by the biotrophic pathogen *Hyaloperonospora arabidopsidis* or the herbivorous insect *Pieris rapae*, respectively, was shown to reduce the level of induced JA/ET-dependent defence against subsequent infection with the necrotrophic pathogen *B. cinerea* (Vos et al., 2015). In this experiment, all plants were infested with aphids, where aphid infestation activated the production of SA dependent pathways (Walling, 2008). If the neighbouring plant with the *Botrytis* infection managed to trigger the activation of plant pathogen defence pathway (JA/ET) in their uninfected neighbours, the hormone crosstalk may also occur in uninfected plant. The SA dependent pathway often leads to a down-regulation of the JA dependent pathway and vice versa (Bostock, 2005; van Loon et al., 2006; Robert-Seilaniantz et al., 2011; Al-Naemi and Hatcher, 2013). Aphids may gain an advantage if the mechanism of hormone crosstalk managed to avoid, suppress, or eliminate host defence system. Aphids feed by piercing the phloem of their food plant and are sensitive to the changes in plant quality (Omacini et al., 2001). As host plant quality is a key determinant of the fecundity of herbivorous insects (Awmack and Leather, 2002), any changes in plant quality will affect the number of aphids on the focal plants.

Visual cues from neighbouring plants may also disturb the foraging behaviour of insect natural enemies (Barbosa et al., 2009). Although it is difficult to demonstrate the use of visual cues in host habitat location (Fellowes et al., 2005), the appearance of a plant may influence the visual cues natural enemies use to locate their prey. Some predatory insects such as ladybirds depend on visual cues to locate their prey. For example, the predation rate of *Coccinella septempunctata, Hippodamia convergens, Harmonia axyridis*, and *Coleomegilla maculata* varies depending on the colour of aphid's background (Harmon et al., 1998). Visual cues also play a role in host location and recognition by parasitoids. The parasitoid *Aphidius ervi* was significantly more likely to oviposit into colonies formed from green morphs when the neighbouring colony was formed from red morph aphids (Libbrecht et al., 2007) and attacked green aphids in preference to brown aphids (Powell et al., 1998). However, there is no significant difference of plant traits on both uninfected and infected plant in this experiment, except the amount of chlorophyll content, but the appearance of the symptoms of pathogen infection may influence the visual cues used by natural enemies. In this experiment, the plant set of uninfected-infected plants divided into symptomatic and asymptomatic plants. However, given that similar patterns were found with asymptomatic and symptomatic plants, and that there are no obvious visual differences between asymptomatic and uninfected plants, we speculate that visual cues are less likely to have played a significant role in affecting host plant and prey choice in our system. We cannot definitively state that other cues (e.g. odour) play a role. This will be a topic for future research.

The fascinating result from this work is that having an infected, but asymptomatic plant as a neighbour, alters the structure of the assemblage of insects found on a plant, and furthermore, results in a loss of plant fitness. To our knowledge, this is an important novel finding, with important implications for our understanding of the role fungal pathogens can play in both agricultural and more natural ecosystems. This study may help to explain on the failure of some pest management efforts. This plant–insect interaction mediated by the bottom up and top down effect may represent a common mechanism which adds a new facet to our understanding on the complex interactions among different trophic levels.

Chapter 7: General discussion

7.1 Summary of findings

Asymptomatic pathogen infection of plants affects interactions between organisms at higher trophic levels. Pathogen infection, even if asymptomatic, changes the life history of aphids that feed on a shared host plant and indirectly alters the life history of parasitoids that emerge from those aphids. The behaviour of those insects is also affected, but varies between host plant varieties. Therefore, hidden disease changes the density and distribution of insects associated with those plants. As there are differences in the life histories, densities and distributions of insect herbivores that feed on different quality plants, the assemblages of natural enemies associated with those insect herbivores is also altered. In natural environments, asymptomatic pathogen infection of a neighbouring plant also shapes the assemblage and distribution of insects on healthy plants. These findings are predictable since it is known that most pathogen infections will commonly have a detrimental effect on plant quality. The novelty here lies in the fact that asymptomatic pathogen infections on plants are overlooked since the infection is hidden and unnoticeable, thus the effect of this pathogen lifestyle in plant ecosystems has been neglected. The findings here have opened a new dimension on the research of interactions between plants, insects and plant pathogens. This may influence how insect pest managers look at their strategies and how ecologists should study the ecological interactions between plants, insects and plant pathogens.

7.2 Background of study

My research builds on findings by Shaw and his research group on the ability of plant pathogens to asymptomatically infect plants (e.g: Barnes and Shaw, 2002, 2003; Rajaguru and Shaw, 2010; Sowley et al., 2010; van Kan et al., 2014; Shaw et al., 2016). Most of this research focuses on the ability of *B. cinerea*, a ubiquitous broad host range necrotrophic fungal pathogen (Hahn et al., 2014), to asymptomatically infect many host plants in natural and controlled environments. As the spores of this pathogen can be found everywhere, such as in the soil, water and air, and can asymptomatically infect many host plants, it was hypothesised that there could be many interesting ecological events which could be explored. Although there was no evidence of this asymptomatic pathogen infection giving a deleterious effect on the growth of its host (Shaw et al., 2016), we could not simply assume that it may not affect another organism that shared the same host plant. It is understood that infection by *B. cinerea* activates the host plant's defence mechanisms, which involves changes at the biochemical, molecular and physiological levels of the infected plant (AbuQamar et al., 2017). The strength of the defence mechanism activated by the plant will determine whether the infection will end up with symptoms of disease or whether it will remain hidden (Aoun, 2017). Thus, all of the events taking place in the plant body in combating the infection may indirectly affect the insects that share the same host plant.

Here, I predicted that asymptomatic pathogen infection not only affects the life history of the plant, but also the assemblage of insect herbivores, and that it also interferes with the interactions between organisms at higher trophic levels. Some research had been carried out to evaluate the effect of *B. cinerea* on the life history of insects in a controlled environment but the findings have been variable (e.g: Mondy and Corio-Costet, 2004; Desneux et al., 2012; Al-Naemi and Hatcher, 2013). However, all previous studies focused on the life history and performance of insects reared on symptomatic plants. Here, I address five different research questions to investigate the effect of asymptomatic pathogen infections on organisms at a higher trophic level. First, I investigated if asymptomatic pathogen infection affected the life history and performance of insect herbivores that fed on the shared host plant and what the consequences were for the other organisms that associated with that insect herbivore. Second, I investigated if the pathogen lifestyle may indirectly affect the host preference behaviour of insect herbivores and their predators and parasitoids. Third, if the life history, performance and behaviour of the insects has been altered by the asymptomatic pathogen infection, I investigate if this type of pathogen infection will alter the density and distribution of the insects in a natural environment. Then, my fourth objective was to investigate how this ecological event may affect the number and identity of the insect herbivore's natural enemies. Lastly, I aimed to quantify the indirect effects of infected plants on healthy neighbours, mediated by insect herbivores.

7.3 Summary by chapter

I untangled the question of the effect of asymptomatic pathogen infection on insect life history, performance and behaviour in Chapters 2 and 3. The experiments in both chapters were done in a controlled environment because it was important to do an investigation looking closely and an individual level, before proceeding to the field. My prediction was that if asymptomatic pathogen infection altered the life history, performance and behaviour of an individual insect, it will then have a consequential effect on the wider system. In Chapter 2, I measured the life history and performance of both insect herbivores and their parasitoids. I found that aphids reared on asymptomatic infected plants were smaller in size and less fecund compared to the aphids fed on uninfected plants. The differences in the aphid's life history might be caused by changes in the quality of the plant. Earlier, I found that infection by *B. cinerea* even if asymptomatic reduced the chlorophyll content and dry weight of the host plants, irrespective of plant variety. As plant quality is a key determinant of the life history and performance of herbivorous insects (Awmack and Leather, 2002; Wetzel et al., 2016) this finding was predicted. I also found that the changes in the insect herbivores life history gave a cascading effect to the life history and performance of their parasitoids. Parasitoids that emerged from hosts reared on asymptomatic plants were smaller and showed reduced starvation resistance. These findings suggest that the reduced quality of plants asymptomatically infected by *B. cinerea* negatively affects the life history of aphids and their parasitoids.

As the asymptomatic plant pathogen infection altered the life history and performance of the insects associated with them, we believed that this would also influence the behaviour of those insects. In Chapter 3, we proved that aphids have an ability to detect the quality differences in host plant caused by the asymptomatic infection of *B. cinerea*, but this result varied between plant varieties. In this context, the chemical cues produced by the uninfected and asymptomatically infected lettuce variety Tom Thumb managed to guide the aphids in making their preference towards uninfected plants. However, this finding does not apply to the lettuce variety Little Gem. Aphids preferred the plant that gave them the best nutrients for growth and survival (Dixon, 1998). If we refer to my first experiment, it is shown that the growth and survival of aphids fed on asymptomatic infected plants are lower compared with ones fed on uninfected plants. Thus, the finding in this second chapter supports the preference behaviour by aphids towards uninfected plants which offered better nutritional value. The results obtained for the preference behaviour of the parasitoid also supported the finding in the parasitoid life history experiment in Chapter 2. The female parasitoids preferred to oviposit in the best quality of host, which can give enough nutrition for the growth and fitness of their offspring (Fellowes et al., 2005). As the parasitoid depends on the cues released by their host or host habitat during the foraging process (Storeck et al., 2000; Stilmant et al., 2008) the differences in the chemical cues released either by the insects or by the plant itself might contribute to this finding. However, I did not measure the type and strength of the chemical cues released by the asymptomatic pathogen infected plant or by the insect herbivore to support my theory. Further studies should be carried out to reveal the actual cause for this behaviour. Contradicting with the preference behaviour of the parasitoid, predatory insects did not show any preference towards aphids that has been fed on uninfected or asymptomatic infected plants. Predatory insects are mobile and can move easily to forage. Unlike parasitoids who need to choose their insect hosts carefully for the sake of their offspring, predatory insects are not tied to one individual for their survival. However, we found that predatory insects could differentiate between plants with aphids and an empty container which shows that the host plant or aphids release a chemical cue to attract the predatory insects. In Chapter 3, we also measured the escape behaviour of aphids towards attack by a predatory ladybird. We found that aphids fed on asymptomatic infected plants are predated more frequently because they drop less compared with aphids on the uninfected plants. This behaviour might relate to the fitness of the aphid itself. Our findings in Chapter 2 show that the off-plant survival for aphids fed on the asymptomatic infected plants are lower, which may explain the reason why aphids choose to remain on the plant despite the increased risk when attacked by their enemies. Chapters 2 and 3 shows that even when host plants are yet to show symptoms, pathogen infection can alter the life histories, performance and behaviour of insects associated with the shared host plant, thus changing the interactions of insect herbivores and their natural enemies.

In chapter 4, I evaluated the effects of pathogen infection on plant traits because these results will present the traits of plants in their natural environment. The results obtained varied between plant varieties. We found that there were no significant differences in plant traits for uninfected and asymptomatic infected Tom Thumb, while if symptomatic Tom Thumb showed reduced chlorophyll content and were smaller compared to uninfected plants. In contrast, symptomatic and asymptomatic Little Gem exhibited a reduction in chlorophyll content, height and shoot:root ratio. As the effect of pathogen infection on plant quality varied, its effect on naturally formed insect assemblages also varied. Little Gem was not affected by asymptomatic pathogen infection in attracting or harbouring aphids, while for Tom Thumb, this asymptomatic infection greatly affected the assemblage of aphids. The diversity of aphids which infested Tom Thumb was also larger compared to that on Little Gem. However, both Tom Thumb and Little Gem, when infected by the asymptomatic pathogens were less attractive to aphid natural enemies. The interactions between the plants, pathogen infection and insects in this system are quite complex. The differences in susceptibility between the two lettuce varieties and the differences in disease severity of the infected plants caused significant changes to the insect communities.

To disentangle the complexity in this system, we further investigated whether asymptomatic pathogen infection alters interactions between the insect herbivores and their natural enemies (Chapter 5). Consistent with other experiments, infection by the pathogen altered the quality of the plants. We found that if natural enemies were excluded, pathogen infection on the plants, whether symptomatic or asymptomatic, changed aphid colony population dynamics. The action of natural enemies reduced the number of aphids, with more predatory insects and parasitoid mummies found on uninfected plants. However, once again these results varied between plant varieties. The results obtained also showed that the natural enemy assemblage in this plant system is indirectly altered by the infection of a fungal pathogen on the host plant. Our findings in this experiment reveal factors that should be investigated in other plant-insect systems to address whether symptomatic and asymptomatic pathogen infections act additively in mediating the assemblage of natural enemies on the plant ecosystem.

My final experiment (Chapter 6) was designed to investigate the effect of asymptomatic pathogen infection on the insects associated with their healthy neighbouring plants. Here I asked if the infection status of a plant can affect the dynamics of an insect herbivore on a neighbouring uninfected plant by influencing natural enemy behaviour, and if this in turn affects the fitness of the neighbouring plant. We found that the pathogen infection status of neighbouring plants significantly affects the plant traits and the structure of insect herbivores on their healthy neighbour. Uninfected plants neighbouring the symptomatic

Botrytis-infected or asymptomatic *Botrytis*-infected had more aphids compared to the uninfected plants neighbouring with other uninfected plants. However, the assemblage of natural enemies was not significantly affected, but the trend shows more predators and parasitoids observed on uninfected plants neighbouring the infected plant compared to neighbouring with uninfected plants. This finding is surprising because there has been little studied about the consequences of mixed health plant status in structuring insect communities. In real environments, it is likely that there will be a mixture of conditions in terms of plant health and quality. Agronomists have only been concerned about symptomatic pathogen infected plants without realising that the asymptomatic pathogen infected plants may also influence the quality of their crop and indirectly alter the abundance of insect herbivores that may damage their crops. This may also influence the success of any biological control programme, such as the use of natural enemies to control herbivore infestation.

In addition to the above I also carried out a further experiment which has not been reported in my thesis. Here, I investigated the ability of aphids to transmit the spore of *B. cinerea*. Aphids are a well-known vector for many plant viruses (Ng and Perry, 2004; Gray, 2008), and it has also been reported that aphids may transfer the spores of fungus from diseased plants to healthy plants (e.g: Kluth et al., 2002). This experiment was carried out in a controlled environment room using the aphid *M. persicae* and both varieties of lettuce, which were either symptomatically infected or uninfected. In this experiment, I found that aphids did not play a role in transferring the *B. cinerea* spores; in fact, I did not detect a single spore on the aphid body.

7.4 Conclusions and suggestions for further research

The findings obtained are important to our understanding of the effect of pathogen infections on plant ecosystems, regardless of whether the infection is obvious or hidden. These results explain the importance of hidden interactions between plants and their pathogens and the consequences for plant-insect interactions. Much research has been carried out to investigate the effect of pathogen infections on plant ecosystems (e.g: Blua et al., 1992; Biere et al., 2002; Alvarez et al., 2007) and it is well known that plant pathogen infection may profoundly impact insect performance, preference, population dynamics and community structure across multiple spatial and temporal scales (Tack and Dicke, 2013). However, instead of considering the effect of asymptomatic pathogen infection, previous research has focused solely on the symptomatic pathogen infection because this type of infection is obvious. Here, my research has shown that the asymptomatic pathogen infection is as important as the symptomatic pathogen infection in influencing the plant ecosystem. However, my findings alone are not enough to explain in detail about the effect of asymptomatic pathogen infections on the higher trophic levels. There is still a knowledge gap to explore and many research questions to answer. Faced with a multitude of friends and enemies, it can be predicted that plant interactions are very complex and that the outcome from such interactions might vary depending on the type of organism that the plant interacts with. Most plant pathologists are concerned solely with the effect of the pathogen infection on the plant, and most plant entomologists are only concerned with the insects associated with the plants. My research suggests that it would be beneficial to examine these relationships across the different scales to glean new insights into what is happening within the system.

Asymptomatic pathogen infections on plants are not uncommon (Malcolm et al., 2013; Stergiopoulos and Gordon, 2014; Shaw et al., 2016). For example, apart from the asymptomatic infection by *B. cinerea,* there are many other fungi that have been found develop the hidden relationship with plants such as *Fusarium oxysporum* that infects and grows asymptomatically within the root cortex of crop plants (Gordon and Martyn, 1997); *Fusarium circinatum*, a pathogen of pine trees that is able to asymptomatically colonize grass (Swett and Gordon, 2012) and corn (Swett and Gordon, 2015); or *Zymoseptoria tritici* the notorious pathogen of wheat that infects the plant without showing any disease symptoms (Ware, 2006). These are just some of the examples of microbial pathogens that were found asymptomatically associated with plants. There are also likely to be many more yet to be discovered. Thus, more research is needed to investigate if there are other important fungi which may asymptomatically infect plants and which may have effects on organisms at higher trophic levels. Different types of pathogens apply different mechanisms to colonize plants, inducing different defence pathways while reacting differently to different insects. Further detailed research is needed to be done on each plant pathogen that asymptomatically infects plants as the result might vary depending on the type of interaction.

Further research should also be carried out to investigate if the pathogen's lifestyle may also affect the plants interaction with other species. Here, I focused on the plants interactions with sap sucking herbivores and their natural enemies. As plants are also attacked by chewing insects, it is worth also studying the effects of asymptomatic pathogen infection on that type of insect. The model insect in this research is a sap sucking insect (aphid) and the asymptomatic infection caused by the necrotrophic pathogen *B. cinerea*. Both organisms induce different defence pathways which then might cause hormone crosstalk (Bostock, 2005; Robert-Seilaniantz et al., 2011). Thus, further research should be done to investigate the effect of asymptomatic pathogen infection caused by *B. cinerea* on chewing insects since both the chewers and *B. cinerea* induce the JA-dependent defence pathway (Glazebrook, 2005; Walling, 2008; Thaler et al., 2010). The similarities in defence pathways induced by those organisms might give a different effect on the interaction between the plant and the organisms in that plant system compared to my study system. If this is the case, the consequent effect of such interactions at higher trophic levels might also be different.

Another area that should be explored in this system is the effect of asymptomatic pathogen infection on the competition between insect herbivores and the consequences of this to the surrounding ecosystem. Future research should cover both interspecific and intraspecific competition since both types of competition are very important in shaping the distribution, density and assemblage of insect herbivores and their natural enemies. As an example, further research needs to be carried out to investigate the competition between different species of aphids, or between sap sucking and chewing insects. In Chapter 4, I found many species of aphids naturally infested the uninfected, symptomatic and asymptomatic infected lettuce plants. Different species of aphid might react differently to the *Botrytis* infection, which has not been explored in my research. There may also be competition between aphid species that naturally infected my plants and it would be interesting to see how the insect community would have changed had I used an alternative host plant. This question is worth exploring so that we can then learn the consequence of insect competition and how this is influenced by the asymptomatic pathogen infection on the preference behaviour or assemblage of insect herbivores and their natural enemies. The findings will be very important for both ecologists and agronomists in understanding the pattern of insect distribution. As an example, when considering biological control programmes that use parasitoids and/or predators, competition between insects driven by asymptomatic pathogen infection may influence the behaviour of the insect herbivore’s natural enemies which then will determine the success of those programmes.

Another aspect to consider is the effect of asymptomatic pathogen infection on mutualistic interactions, such as the mutualism between aphids and ants. Aphids have a mutualistic interaction with ants where they offer honeydew as a nutrient-rich food source and in return the ants protect the aphids by deterring or killing the aphids natural enemies (Matsuura and Yashiro, 2006; Detrain et al., 2010; Yoo and Holway, 2011). Here I found that the asymptomatic pathogen infection altered the quality of the plant. The changes in plant quality affected the life history of the insects, but it is not known whether the alteration in plant quality would also affect the production of honeydew produce by the aphid. If aphids fed on different plant qualities produced a different quality or quantity of honeydew, it could be assumed that these changes will affect the rate of mutualisms between ants and aphids. It would also be interesting to study the effect on the natural enemies of aphids as we know the reward offered by ants may influence the interaction between those insects.

Botrytis is known to infect many flowering plant such as tomato, grapes, apples and berries (Deighton et al., 2001; Dugan et al., 2002; Pezet et al., 2003; Dal Bello et al., 2011). In some conditions the infection is hidden and the sign of infection is only detected after their products, such as fruits, are harvested. As the formation of fruits starts with the formation of flowers, the fungal pathogen might asymptomatically infect the flowers. Another area of interest could be to look at whether the flowers are asymptomatically infected with the pathogen, and if they are, what are the consequences in the interaction between the plants and their pollinators, such as moths or bees. To date, there has been no research that has been done to consider this question. There is currently no record of whether the asymptomatic pathogen infection will affect the volatiles produced by the flowers to attract their pollinators.

Our knowledge in the study of plant-insect-pathogen interactions is still lacking. More research is needed to investigate the effect of pathogen infections on the interaction of species at higher trophic levels. The study of the effect of asymptomatic pathogen infections on plants is still new and has not yet received much interest from researchers as it is not easy to investigate the three-different organisms at the same time. Findings from such further research will give us a better understanding of this complex interaction. Most importantly, this study is likely to unravel new insights into the ecology of plant based communities.

In general, plant disease is a threat to global food security. Thus, it is necessary to define the problem and seek remedies to solve this issue. Understanding these threats and how we can reduce their impact will be key to ensure a robust and secure food supply. Findings from my research may give some information not only to the researchers, but also to the farmers and policy makers in managing pest and disease threat in urban, agriculture and natural areas. To date, plant diseases are likely to be of increasing interest to food and agricultural businesses due to their dependence on pesticides and fungicides. The usage of those chemicals should be avoided or reduce by applying the environmental friendly control methods or by protecting the plant from the attack of those intruders at the first place. The pest control program should carefully selected and applied in a manner that minimizes risks to human health, beneficial and nontarget organisms, and the environment.

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