Laboratory and field investigations of the role of entomopathogenic fungi in regulating aphid populations in field brassica crops

Liam Harvey

A thesis in partial fulfilment of the requirements for the degree of:

Doctor of Philosophy

School of Life Sciences, The University of Warwick

February 2018

Declaration

This thesis contains my own work, which has not been submitted for a degree at another university.

Some data gathered for this thesis were presented in the following paper:

Harvey, L. (2013). The role of entomopathogenic fungi in regulating aphid populations in field Brassica crops. *IOBC-WPRS Bulletin*, **107**, 157-161.

Acknowledgments

I would like to thank Dr. Dave Chandler. Your guidance, support and feedback have been invaluable throughout the course of this project.

In addition, I would like to thank the people who have helped me during my PhD. Firstly, Gill Prince for providing help day to day and generally being there through thick and thin. Rosemary Collier for guidance throughout. Judith Pell, for her in depth knowledge on culturing and experimenting with entomopathogenic fungi. Julie Jones and Andrew Mead for statistical support. The horticultural team, without their knowledge of fieldwork and banter the field seasons would have been much more difficult.

I am extremely thankful for my office friends for making the days at Warwick Crop Centre endlessly more enjoyable. I have met a lot of people during my time here and I am grateful to them all for making me the person I am today.

I would like to acknowledge the financial support provided by AHDB Horticulture.

Finally, I thank my mum, dad and two brothers: Lewis and Jake, for their unwavering and unconditional support and encouragement.

CONTENTS PAGE

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Growers of field vegetable crops are under increasing pressure to reduce their reliance on synthetic chemical pesticides and increase their use of alternative pest management tools, including biological control agents. In this project, experiments have been conducted to investigate insect pathogenic fungi as control agents of the cabbage aphid on horticultural brassicas. These fungi contribute to the natural regulation of aphid populations, but they can also be mass-produced and applied to crops by growers as "biopesticides." Field experiments were carried out over two seasons to investigate the association between the population dynamics of cabbage aphids, insect pathogenic fungi, and other natural enemies. This has been backed up by laboratory experiments on one particular fungus, Pandora neoaphidis, which causes natural epizootics in cabbage aphid populations, and which has not been studied against this pest in detail before. Research has focused in particular on developing laboratory methods for characterizing fungal virulence, and on the effect of temperature on fungal infectivity as a key environmental variable affecting Pandora outbreaks. Finally, the susceptibility of cabbage aphid to infection by commercially available fungal biopesticides has been compared against aphid susceptibility to *Pandora neoaphidis*.

CHAPTER 1 INTRODUCTION

Global population increase and climate change have brought to the forefront the need to increase food production whilst at the same time reducing the adverse environmental impacts of agriculture (Vega *et al*., 2009). Crop losses due to pests, disease and weeds represent a major constraint to global food productivity. These losses account for 40% of potential production (Thacker, 2002). Despite a marked increase in the use of pesticides since the 1960s crop losses have not decreased (Bruce, 2010; Vega *et al.*, 2009; Oerke, 2006).

Overall, competition from weed species result in the highest loss of potential yield (34%), with a further 18% and 16% of yield lost as a result of animal damage and to disease, respectively. However, weeds are relatively easy to control and actual losses are greatest for animal pests and pathogens, 13% and 11% respectively (Oerke, 2006). Figures to be treated as a percentage of total crop loses. It is estimated that there are around 67,000 agricultural pest species worldwide, with 10% being classed as major pests (Pimentel, 1997).

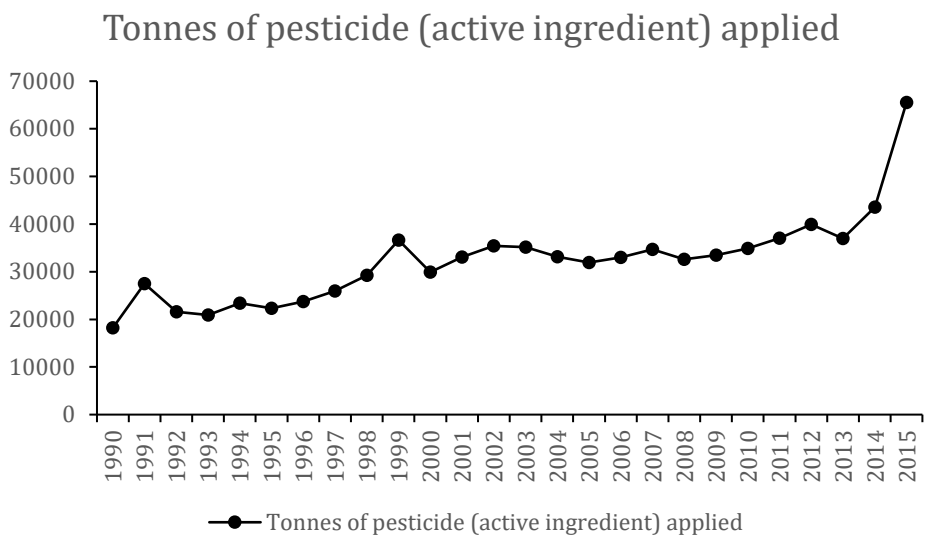


Figure 1.1 Global pesticide usage from 1990 to 2015 expressed as tonnes of active ingredient applied/ year (FAOSTAT, 1998).

Global pesticide usage has increased steadily since the end of the second world war (Figure 1.1) and by 2017, global sales of crop protection chemicals reached USD 54.7 billion (Bloomberg, 2012). However, there has been a great deal of change in the number and composition of actives, from broad spectrum organochlorines such as DDT and organophosphates (IRAC, group 1 acetylcholinesterase inhibitors) in the late 1930s, which irreversibly inhibit acetylcholinesterase enzyme activity. The development of resistance and environmental and human health concerns largely brought about the demise of these actives. Carbamates followed, which also inhibit acetylcholinesterase enzyme activity, but their effects are reversible. Pyrethroids based on the plant defence chemical pyrethrum hailed a different mode of action as sodium channel modulators (IRAC group 3) and their use increased as synthetic analogues were produced. Today there are a variety of synthetic chemical pesticides available, including neonicotinoids (neurotransmitter inhibition), Spinosad (hyperexcitation

of nervous system), Bifenazate (neuronal inhibitor) and the Benzoylureas (chitin synthesise inhibitors).

Most growers in the developed world rely on chemical pesticides and their use has delivered enormous benefits in terms of increased production. However, whilst many of these chemicals have excellent characteristics regarding their efficacy and safety (Thacker, 2002), there are significant concerns relating to their use (Tilman *et al*., 2002; European Commission, 2017). Indeed, growers are under pressure to reduce their reliance on chemicals for a range of concerns: (a) consumer concerns (and by extension retailer concerns) over exposure to chemical residues in produce; (b) detrimental environmental effects, particularly non-target effects (Tilman *et aI*., 1999); (c) effective compounds declining in number as a result of product withdrawals linked to new, more stringent health and safety criteria as part of European pesticides legislation (Directive EC1107/09); and (d) injudicious use of compounds resulting in control failure through the evolution of heritable resistance (IRAG, 2012).

Currently, over 500 species of arthropods worldwide are resistant to one or more insecticide (Hajek, 2004). There is a need for new actives and control strategies, not only to overcome selection pressures and development of resistance but also to reduce negative environmental impacts of large scale crop production. Unfortunately, there has been a net decline in the number of available active ingredients over time. The decline is driven largely by the cost (c.$200 million) and time (c. 10 years) taken to develop new products and more stringent safety criteria and regulation. At the time of writing there are 490 approved active substances, 28 compounds pending approval, and 821 compounds not approved (European Commission, 2017).

In 2014, 71.8 million tonnes of brassicas were produced (FAOSTAT, 1998). In the UK alone brassica production, including oil seed rape (OSR) and leafy salads, was valued at approximately £1.2 billion in 2016, considerably higher than potatoes (excluding seed crops) and tomatoes which were valued at £710 million and £102 million, respectively (DEFRA, 2016). The main species of brassica grown in the UK are OSR (737,000 ha), Brussels sprouts (3029 ha), cabbage (7017 ha) and cauliflower (9440 ha) (DEFRA, 2016). Pesticide applications on brassicas in the UK has fallen from its peak in 2002, but approximately 93, 358kg were applied in 2015 (DEFRA, 2016).

1.2 APHIDS AS CROP PESTS

Aphids (Hemiptera, Aphididae) are one of the most serious pests of vegetable brassica crops (Blackman & Eastop, 1984; Dedryver *et al.* 2010). Among the aphid species colonizing brassica, *Brevicoryne brassicae* and *Myzus persicae* are the most economically important (Blackman & Eastop, 1984). Both species have a worldwide distribution, however, *B. brassicae* is confined to species of the Brassicaceae family, whereas *M. persicae* have many crop host plants including peas, lettuce, turnip, sugar beet, cucumber, corn and mustard (Table 1) (Blackman & Eastop, 1984). Plant damage is caused directly via aphid feeding action on foliage and in the case of *B. brassicae* severe leaf fouling due to its tendency to form dense colonies, or indirectly through the transmission of plant pathogenic viruses, including turnip and cauliflower mosaic virus and cabbage black ring spot virus (Blackman & Eastop, 1984; Flint, 1985). Due to its wide crop host range and

its ability to spread a plethora of plant pathogenic viruses, *M. persicae* is considered the more prolific virus vector of the two species (Table 1).

Annual brassica yield losses due to aphid infestations range from 30% to 80% in developed and developing countries respectively (Razaq *et al.* 2011; Dedryver *et al.* 2010; Isik & Gorur, 2009). Greater crop losses in the developing world are a product of a lack of resources to purchase plant protection products and quality seed, limited agronomic support, more favourable conditions for insect pests and disease vectors, lack of infrastructure and the prevalence of subsistence farmers (Herrera-Estrella, 2000).

1.2.1 *B. brassicae* and *M. persicae* identification

Aphid species and instars can be identified by a variety of distinctive features, the most diversified of which are the head, siphunculi, various abdominal markings and the cauda (Figure 1.2.1). Numerous keys are available for the identification of aphid species and Tatchell (2004) was used throughout this research.

Adults of both species can be alate (winged) or apterous (wingless) morphs. Adult apterous *B. brassicae* are green, however, they are covered in a greyish-white wax. They also possess short black siphunculi (Figure 1.2.1). They are approximately 1.9-2.7 mm in length (Table 1). Alate adults of the species are smaller (1.2-2.3 mm) and lack waxy deposits. They possess a dark head and thorax, with distinctive black markings on the dorsal anterior abdominal segments (Flint, 1985). Adult apterous *M. persicae* are easily distinguishable due to the fact they lack a wax coat and have long siphunculi (Figure 1.2.1). They are also smaller than *B. brassicae* at 1.2-2.1 mm in length. Considerable variation exists in their

colouration, from whitish-green, dark green, and pink to red (Table 1). Alate morphs of *M. persicae* possess a dark spot in the centre of their abdomen (Blackman & Eastop, 1984).

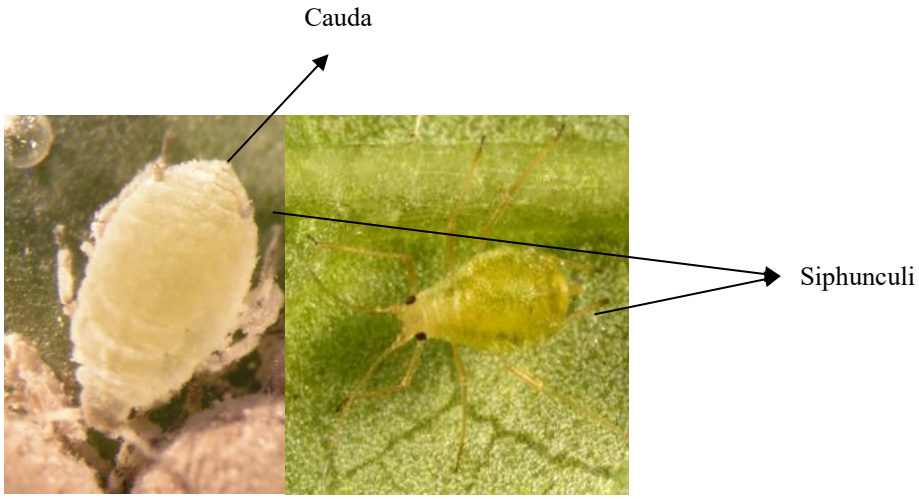


Figure 1.2.1 Left: Image of adult apterous *Brevicoryne brassicae*, right: adult apterous *Myzus persicae*. Picture of *Myzus persicae* by Lyle J. Buss, University of Florida. ljbuss@ufl.edu

1.2.2 Biology and ecology of *B. brassicae* and *M. persicae*

Short reproductive generation times and the ability to produce dispersive alate forms in the face of changing environmental conditions mean aphids are very well adapted to exploit the ephemeral nature of the cropping environment (Wellings, *et al*., 1980). The generation times of *B. brassicae* and *M. persicae* are 7-10 days and 9 days respectively, and each species has five distinct developmental stages or instars (MacGillvray & Anderson, 1958; Dodd, 1976; Gabrys, 2005).

The type of aphid life cycle depends on the temperature during winter. Anholocyclic life cycles occur during mild winters, when the warmer temperatures allow asexual reproduction to continue throughout the year. During mild winters females of *B. brassicae* and *M. persicae* have been observed to continue viviparous parthenogenesis, meaning they give birth to live offspring without the need for

mating, all year round (Blackman, 1974; Pons *et al*., 1995). Individual females of *B. brassicae* and *M. persicae* can produce up to 20 and 46 offspring in a lifetime respectively (Gabrys, 2005).

Alternatively, holocyclic life cycles occur in colder regions where sexual forms are produced. In the case of *B. brassicae* winged males and apterous oviparous females are produced in the autumn in response to the shortening photoperiod and decreasing temperature. *Brevicoryne brassicae* is a monoecious aphid species, completing their lifecycle solely on plants in the family Brassicaceae.

Myzus persicae is a heteroecious species completing its lifecycle on a range of host plants. In the autumn, *M. persicae* virginoparae begin to produce migrants, gynoparae, which seek out the winter host. Winter hosts are predominantly *Prunus* species. These, gynoparae then produce oviparae (mating females) which mate with males on the winter host. The resultant eggs then overwinter in diapause, only hatching in response to increasing temperatures and photoperiod. Summer hosts of *M. persicae* include species in the plant families, Umbelliferae, Brassicaceae, Leguminosae, Solanacecae and Poaceae.

Table 1 Pest status of *Brevicoryne brassicae* and *Myzus persicae*

Species	Appearance	Status[3] & Host	Secondary impacts	Distribution
B. brassicae[1]	Grayish-green, waxy covering gives them a grayish-white to powdery blue appearance. Short dark siphunculi. Length 1.9-2.7 mm.	A problem after mild winters. Oligophagous on *Crucifers.*	Vector of 23 viruses of the *Cruciferae* family.	Native to Europe. Abundant worldwide (Inc. anterior and mid Asia, North America, North Africa, Australia and New Zealand).
M.persicae[2]	Varying shades from yellow, green to pink, red and almost black. Length 1.2-2.1 mm.	A problem spreading viruses during mild winters but less so due to widespread insecticide use. Considered a problem on a range of crops; potatoes, sugar beet, lettuce, brassicas and legumes.	Virus vector responsible for the transmission of over 100 plant viruses. Among the most important are Potato leaf roll virus, Beet western yellows virus and lettuce mosaic virus.	Worldwide.

[1]Flint (1985)

[2]Blackman & Eastop (1984)

[3]Holland & Oakley (2007)

1.3 CURRENT CONTROL METHODS

At present aphid management in brassica crops is heavily reliant on the use of synthetic chemical insecticides; aphicides account for 39% of all insecticide applications (Garthwaite *et al*., 2007). Current chemical control products for aphids include neonicotinoids, pyrethroids, pirimicarb, chlorpyrifos and pymetrozine (IRAG, 2012). Whilst there is currently no evidence to suggest *B. brassicae* is resistant to insecticides, *M. persicae* has three known resistance mechanisms, esterase, MACE and kdr rendering certain organophosphates, carbamates, and pyrethroids ineffective (IRAG, 2012). As a result, there is an urgent requirement to develop alternative aphid management tools and practices.

1.4 ALTERNATIVE CONTROL METHODS

1.4.1 Integrated pest management (IPM)

Wider use of integrated pest management (IPM) strategies is accepted as the most expedient way towards greater sustainability. There are several definitions of IPM, and what is considered IPM. The OECD's (Organisation for Economic Co-operation and Development) definition, for example, focuses on "site- and condition-specific information to manage pest populations", whereas, the FAO (Food and Agriculture Organisation) chooses a broader definition based on "an ecosystem approach to crop production". Additionally, the IOBC (International Organisation for Biological and Integrated Control) highlights reliance on "the use of natural resources" whereas the EU definition states "all available pest control techniques" should be considered to discourage pest development.

Generally, IPM refers to the combined and coordinated use of chemical, cultural and biological control measures to minimise economic injury to crop plants (Garthwaite *et al*., 2007; FAO, 2013). Implementation relies upon close crop monitoring and surveys to determine infestation and economic injury levels for specific crops, which in turn inform action thresholds. Whilst in reality agrochemicals are still the cornerstones of many pest management strategies, IPM considers "…all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms" (FAO, 2013).

Much emphasis is now being placed on other components of IPM such as biological control as a result of the sustainable use directive and European regulations such as EC1107/2009. The sustainable use directive states that each member state must have a national action plan (NAP) setting out a framework of actions to achieve the sustainable use of pesticides, giving preference to biological and other non-chemical control methods (UK NAP, 2013).

1.4.2 Aphid biological control

Definitions of biological control vary depending on which scientific discipline you side with. Despite differences in specific terminology between disciplines a ubiquitous aim is the use of biological control is reducing pest and/or disease pressure through the activity of biological control agents (Eilenberg *et al*., 2001). Biological control agents vary in nature from predators and parasitoids on the

macro scale, to microbial control agents such as bacteria, fungi and viruses, on the micro scale. To encompass the diverse nature of biological control agents and unite research disciplines Eilenberg *et al*. (2001) define biological control as follows:

'The use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging that it would otherwise be.'

There are three main strategies to biological control, classical, augmentation and conservation biological control (CBC). Often augmentation is separated into inundation and inoculation, details of control programs within each strategy and definitions are considered below.

Aphid infestations are predated by a guild of aphidophagous natural enemies, including true predators, parasitoids and pathogens i.e. entomopathogenic fungi (Diaz *et al.* 2010), that have the potential to be exploited for use in biological control strategies. The most abundant native natural enemies in brassica agroecosystems that share aphids as an extraguild prey type and considerably reduce aphid populations are the entomopathogenic fungus *Pandora neoaphidis*, syrphids (*Episyrphus balteatus*), and hymenopteran parasitoids (Aphidiidae and Aphelinidae) (Karley *et al*. 2003; Karley *et al*. 2004). Natural enemies within a guild or community can be used simultaneously as part of a biological control strategy, although, an increase in the richness of natural enemies used for pest control does not necessarily lead to a corresponding increase in efficacy (Diaz *et al.* 2010), largely because intraguild predation and competitive

exclusion will act to decrease the diversity of natural enemies within cropping systems. Natural enemies that co-occur naturally or that are introduced to a cropping system may have additive or synergistic effects should their feeding niches (realized niches) complement each other i.e. minimise exploitation competition (Diaz *et al.* 2010).

Thus, to conserve a diversity of natural enemies in the environment to give additive or synergistic effects, a good understanding of the biology and ecological dynamics of aphid pest populations and their guild of natural enemies is required. Both to develop biological control strategies and to develop individual natural enemy species as useful biocontrol products for augmentation. This understanding is currently lacking in the brassica, *B. brassicae* system. In many situations, natural enemies are present in agroecosystems, but are either too few or active too late to limit crop damage (Bruce, 2010). This provides opportunity to intervene and augment their populations for biocontrol purposes.

CBC and/or augmentation control could be a useful approach for the control of aphids in brassica crops in light of new EU legislation restricting the use of many agrochemicals (Royal Society, 2009).

1.4.3 The aphid population crash

During the growing season (usually July in the UK) many aphid species exhibit a sharp population decline to apparent local extinction (Karley *et al.,* 2003). This mid-season 'crash' occurs in the absence of insecticide in both agricultural (Soroka & Mackay, 1990; Nakata 1995; Parker *et al*., 2000) and natural landscapes (Jarosik & Dixon, 1999; Muller *et al*., 1999) and populations generally remain low or

undetectable for at least 6-8 weeks' post-crash (Karley *et al*. 2003; Karley *et al*. 2004). At present the timing of this crash cannot be predicted accurately.

Many factors have been suggested for the mid-season crash, including plant age (decline in nutritional quality) (Watt, 1979; Douglas, 2003), the action of natural enemies (Jones, 1979; Boiteau, 1986; Holland & Thomas, 1997; Karley *et al.*, 2003) and adverse weather conditions. Extremes of temperature, strong winds and significant rainfall events can negatively affect aphid populations (Ba-Angood & Stewart, 1980; Basky, 1993; Asin & Pons, 2001; Morgan *et al*., 2001). In principle, all the factors above affect population processes including birth, death and emigration. Investigation of the scale and timing of the crash is valuable as the crash might contribute to the overall regulation of aphid populations meaning it could be exploited in pest management strategies. Moreover, a better understanding of the role of natural enemies in aphid population dynamics might enable the mid-season crash to be forecast, which would give growers the option of withholding pesticide sprays. Indeed, data collected in Horticulture Development Company (HDC) sponsored research at the Warwick Crop Centre indicates that natural enemies, particularly entomopathogenic fungi, are associated with the mid-season aphid crash on brassica and lettuce crops.

Steinkraus *et al*. (1991) identified the entomopathogenic fungi, *Neozygites fresenii,* as the causal agent of the population crash in *Aphis gossypii* on cotton in the US. Subsequently, a biological control program was developed exploiting the timing of the natural fungal epizootic which was predicted by a linear model based on the density of diseased insect cadavers (Hollingsworth *et al*., 1995). The model can predict aphid declines in advance of 10 days of their occurrence, dramatically reducing the need for pesticide applications on cotton in the area. Similarly, a

model developed to predict the aphid population crash in the UK on brassica crops would be of analogous benefit. A control strategy based on predicting the timing of the aphid population crash would be particularly useful in organic production where aphid populations are typically high. Organic production in the UK is comparatively small, however, globally the market is markedly growing, particularly in the US. Additional benefits of a predictive model for control of aphid pests become clear when considering the threat of pesticide resistance, consumer/retail demands regarding pesticide residues, worker health and safety, and concerns over the environmental impacts of the injudicious application of pesticides.

1.5 ENTOMOPATHOGENIC FUNGI

The two largest fungal phyla exhibiting entomopathogenicity are the Ascomycota and the Entomophthoromycota (Humber, 2008; Humber, 2012). Within the Entomophthoromycota there are six families, of which five possess obligate entomopathogens (Humber, 2008). The phylum has been resolved into three classes to which the aforementioned six families are assigned:

Class Neozygitomycetes:

Order Neozygitales;

Family Neozygitaceae; *Apterivorax*, *Neozygites*, *Thaxterosporium*

Class Basidiobolomycetes:

Order Basidiobolales;

Family Basidiobolaceae; *Basidiobolus*

Class Entomophthoromycetes:

Order Entomophthorales;

Family *Conidiobolus* ; *Ancylistes;*

Family Entomophthoraceae;

subfamily Entomophthoroideae; *Erynia*, *Batkoa*, *Entomophthora* (in part);

subfamily Erynioideae; *Pandora*, *Zoophthora*, *Furia* (in part).

(Taken from Humber, 2012).

The Ascomycota are considered to be generalist pathogens, causing death via toxin production (Pell *et al*. 2001), whereas, the Entomophthoromycota are considered to have evolved into higher parasite forms leading to narrow host ranges, forming close biotrophic associations with their insect hosts and seldom engaging in saprotrophic growth (Shah & Pell, 2003). In this context, saprotrophic growth refers to the utilisation of dead or decaying matter within the soil as a nutrient source. Epizootics are often caused by Entomophthoromycota because the host is comparatively motile when infected allowing for the spread of the pathogen (Shah & Pell, 2003). Individuals only become incapacitated upon death and sporulation of the fungus. To maximise transmission and therefore fitness many species of the Entomophthoromycota have synchronised with diurnal abiotic conditions to take advantage of the more suitable temperature and humidity conditions present during dusk and throughout the night (Brown & Hasibuan, 1995). Additionally, many Entomophthoromycota have evolved eco-morphological adaptations to closely match the biology and ecology of their hosts,

including actively discharged conidia, the production of resting spores and host behaviour manipulation (Ekesi *et al*., 2005).

The use of pathogens as biocontrol agents has lagged considerably behind that of predators and parasitoids (Lacey *et al*. 2001; Maddox *et al.* 1992). However, these specific eco morphological adaptations mean they are exceptional in their potential as biological control agents (BCAs) for sucking pests i.e. aphids, where the stylet feeding mechanism prevents the transmission of other entomopathogens via ingestion, as they invade through the hosts cuticle or exoskeleton thereby circumventing the need to be ingested (Scorsetti *et al.*, 2010). Most of the research done to date has focused on the use of hypocrealean (ascomycete) entomopathogenic fungi, which are relatively straightforward to mass-produce, and so have been popular choices for development as biopesticides. However, entomopathogenic fungi from the Entomophthorales, which includes *Pandora neoaphidis*, are difficult to mass-produce and so have not been studied in detail. These fungi do, however, produce natural outbreaks in field populations of aphids and thus have potential in conservation biocontrol. There is currently a lack of knowledge on how abiotic factors influence efficacy in field situations, which has hampered their widespread use (Shah & Pell, 2003; Hajek *et al*. 2002).

Below, literature on the successful and unsuccessful use of entomopathogenic fungi (EPF) as BCAs is considered in relation to the three broad biological control approaches:

1.5.1 Classical biological control

Classical biological control states that a pest species is exotic to an area and has been able to establish in the absence of its guild of natural enemies. Eilenberg *et al*. (2001) define it as:

'*The intentional introduction of an exotic, usually co-evolved, biological control agent for permeant establishment and long-term pest control.'*

For example, larvae of the gypsy moth, *Lymantria dispar*, feed on the leaves of many trees including oaks and aspen. It was accidentally introduced to the USA in the 1860s and control with *Entomophaga maimaiga* is now widespread in the United States through a combination of releasing infected cadavers, resting spores from the soil and wind dispersal (Hajek *et al*. 2002; Shah & Pell, 2003).

1.5.2 Augmentation biological control

Natural enemies are generally too few in number within the crop to effectively control pest levels, augmentation biological control aims to enhance their control efficacy through two strategies. Either in an inoculative capacity, defined as:

'The intentional release of a living organism as a biological control agent with the expectation that it will multiply and control the pest for an extended period but not permanently.' (Eilenberg *et al*., 2001)

For example, the EPF *Hirsutella thompsonii* (McCoy, 1981) and *Verticillium* (now *Lecanicillium*) *lecanii* (Hall, 1981) have been utilized via inoculative releases into pest populations where they cause epizootics following repeated applications over a growing season; such epizootics are not expected to persist.

The second of the two strategies is inundation, which uses a similar approach to how agrochemicals are applied and the term 'mycoinsecticide' has been coined for EPF-based products used in this manner (Shah & Pell, 2003). Eilenberg *et al*.'s (2001) definition is as follows:

'The use of living organisms to control pests when control is achieved exclusively by the released organisms themselves'

Mycoinsecticides are microbial biopesticides, pesticides based on living organisms such a fungi, nematodes or bacteria. The term biopesticides can also refer to certain natural materials used in pesticide applications such as canola oil, although, these are often more accurately described as biochemical pesticides. The term biopesticides refers more broadly to pesticides derived from plant materials or organisms.

At present, there are a few commercial augmentation products available in the UK and Europe for the control of aphids based on entomopathogenic fungi, including *Lecanicillium longisporum* and *Lecanicillium muscarium* (marketed as 'Vertalec' and 'Mycotal' respectively) (Table 2.3.1). However, Vertalec is no longer available in UK. There are also products based on *Beauveria bassiana*

(Naturalis L, BotaniGard), *Paecilomyces fumosoroseus* (now classified as *Isaria fumosorosea*) (PreFeRal) and *Metarhizium brunneum* (Met52 and 1 other product from mid-2017) with others in the registration pipeline. A prerequisite of this approach demands that any species that is to be used in this way can be grown in an economic manner in order to produce the large amount of inoculum required during application. As a result, there are currently no examples of the use of Entomophthoralean fungi in an augmentation approach (Shah & Pell, 2003).

1.5.3 Conservation biological control

CBC requires the manipulation of the environment to enhance the activity of natural enemies, including fungal pathogens, adapting farming practices to enhance their control potential in the field. Such manipulations have proved to be successful in systems that are well understood such as *A. gossypii* epizootics caused by *N. fresenii* on cotton in the United States (Shah & Pell, 2003; Steinkraus *et al*, 1995). This example has proved to be a particular success and has been adopted in several cotton-growing regions of the US. The approach has its own dedicated website: (http:// www.uark.edu/misc/aphid/) to keep growers up to date (Shah & Pell, 2003). Similarly, *Pandora neoaphidis* could be used in the UK for CBC of *B. brassicae* on brassica crops. Shah *et al*. 2001 suggest the use of field margins as refugia for *Pandora neoaphidis* by allowing aphids to persist in the environment on secondary hosts within the margin once the crop has been removed.

1.5.4 *Pandora neoaphidis*

P. neoaphidis is the most common entomopathogen causing epizootics in aphid pest species. It has a wide distribution, recorded from Europe, Asia, Africa, North and South America and Australia (Shah & Pell 2003) and is a highly specific, obligate parasite of aphids, presenting no risk to other natural enemies (Diaz *et al.* 2010). Conidia attach to the external surface of a host and under permissible conditions of temperature and humidity the conidia germinate, penetrating the host's cuticle and colonizing the body cavity or hemocoel. Death in some species is attributed to toxin production. Again, under permissible conditions conidiophores develop and in the case of *P. neoaphidis* primary conidia are actively discharged creating the characteristic 'halo' of spores around the infected cadaver. *P. neoaphidis* exists as protoplasts rather than zygospores (Shah & Pell 2003).

Taxonomy of *Pandora neoaphidis*, (Humber, 2012).

	Pandora neoaphidis
Kingdom	Fungi
Phylum	Entomophthoromycota
Class	Entomophthoromycetes
Order	Entomophthorales
Family	Entomophthoraceae
Genus	Pandora
Species	Neoaphidis

There have been many theories as to how *P. neoaphidis* overwinters, from cycling through aphid populations in refugia in field margins to resting spore formation. In 2012, Scorsetti *et al*. (2012) reported the first observation of resting spore formation in the species. Resting spores are significantly different from other

spores in that they are double walled to withstand periods of unsuitable abiotic conditions. Levels of *P. neoaphidis* infection vary year to year because of yearly/seasonal fluctuations in climatic conditions (Baverstock *et al.*, 2008a). Infection levels are also a function of host population density and the amount of inoculum available in the beginning, whether that be resting spores on the surface of the soil, or the size of infected reservoir populations in refugia. Infection levels can be increased via transmission of the pathogen, either between conspecifics or facilitated by other species. Natural enemies who share aphids as an extraguild prey type will facilitate the transmission of *P. neoaphidis* in the most expedient way as they search for the same prey type. Baverstock *et al*. (2008b) showed that transmission of *P. neoaphidis* was increased in the presence of a co-occurring natural enemy, *Aphidius microlophii*. Interestingly, *Coccinella septempunctata* and *Harmonia axyridis* actively avoid aphid individuals that are infected with *P. neoaphidis*, preferentially predating aphids that are uninfected (Baverstock *et al*., 2008b; Ormond *et al*., 2011), thereby improving the potential reduction in aphid populations as intraguild predation and competition are minimised (Roy *et al*., 2008).

In microcosm experiments, although the presence of the predator, *C. septempunctata* and parasitoid, *Aphidius ervi*, has been shown to increase *P. neoaphidis* transmission, there was no extra beneficial decrease in the aphid population (Baverstock *et al*., 2009). This would suggest that *P. neoaphidis* did not serve any additional benefit. Moreover, the reproductive success of *A. ervi* was decreased (Baverstock *et al*., 2009). Presumably the parasitoid can only detect the presence of *P. neoaphidis* once the aphid host is dead and sporulation has occurred (Baverstock *et al*., 2005). The parasitoid wasp's longer development time meant

it was outcompeted by the *P. neoaphidis* within the host. In semi-field, polytunnel trials under more realistic abiotic conditions, *A. ervi* showed no decrease in reproductive success in the presence of *P. neoaphidis*. The fungus lost its competitive advantage over the parasitoid because abiotic conditions were suboptimal in the field (Baverstock *et al*., 2009). The relationships between natural enemies are complex; predators, parasitoids and pathogens can respond differently to the presence of one another impacting the strength and outcome of intraguild competition.

Issues with mass culturing Entomophthorales on a commercial scale mean that for the mean time at least *P. neoaphidis* is best exploited through a CBC approach (Jenkins & Goettel, 1997).

1.6 THERMAL BIOLOGY OF ENTOMOPATHOGENIC FUNGI

Endothermic organisms can generate internal heat and maintain a suitable temperature for reactions and bodily processes to occur, referred to as homeostasis. Ectothermic organisms, such as reptiles or arthropods, on the other hand rely on ambient temperature to heat their bodies and thus their body temperatures and subsequent rate of bodily processes (or activity), vary dramatically, in line with ambient temperature. Entomopathogenic fungi are ectothermic organisms and their activity is therefore dependent on ambient temperature (Blanford & Thomas, 1999; Blandford & Thomas, 2003), It is important to know the optimal temperature for fungal activity as well as the upper and lower limits as this is the range under which the entomopathogenic fungi will be active, i.e. its operational range. Measuring the effect of temperature on insect and EPF physiological function is

relatively straightforward. A graph of the relationship between the rate of a physiological process (e.g. rate of growth, germination etc.) and temperature resembles a normal distribution that is skewed towards the lower temperatures, with a rapid decline in response as temperatures increase past the optimum (Roy *et al*., 2002; Yeo *et al*., 2003; Guzman-Franco, 2008; Bugeme *et al*., 2008; Regniere *et al*., 2012; Thomas & Jenkins, 2014). As a result, even small changes in temperature that occur towards the upper thermal threshold can lead to rapid declines in the response (Davidson *et al*., 2003; Smits *et al*., 2003).

Until recently, entomopathogenic fungi were used mainly as biological control agents of protected crops where temperatures are stable and usually not limiting to fungal activity. However, as these fungi start to be investigated and exploited more as biocontrol agents in outdoor crops, where temperature conditions are more variable, there is an obvious need to understand in detail the effect of temperature on fungal performance (Blanford & Thomas, 1999).

Nonlinear thermal models have been used to model EPF processes such as hyphal growth (Davidson *et al*., 2003; Smits *et al*., 2003) but they have not yet been used to analyse the effect of environmental temperature on the EPF-insect host interaction including host mortality / survivorship. Because nonlinear models are the only way to fully describe the response of EPF, insect, and EPF-insect processes over the full physiological range of temperatures, the absence of a nonlinear modelling approach means that many basic questions about the effect of temperature on these processes remain unanswered. For example, the extent to which the effect of temperature on EPF virulence can be predicted from data on effects of temperature on fungal growth or germination is still not clear.

Aim of PhD project:

Investigate the hypothesis that fungal epizootics are one of the principle factors causing the mid-season crash in populations of aphids on horticultural brassicas.

Specific Objectives:

The three main objectives were to:

i. Monitor populations of healthy and fungus-infected cabbage aphids on sequentially planted brassicas and study abiotic and biotic factors contributing to the mid-season population crash.

ii. Identify insect pathogenic fungi associated with the cabbage aphid *Brevicoryne brassicae* on field brassicas.

iii. Model the effect of temperature on the pathogenicity of fungi to the cabbage aphid providing fundamental insights into host-pathogen interactions.

CHAPTER 2: GENERAL MATERIALS & METHODS

Plant and insect cultures were maintained within controlled environment rooms in the insect rearing unit at Warwick Crop Centre.

2.1 PLANT CULTURES

Brassica oleracea Gemmifera group (Brussels sprout, cultivar 'Trafalgar') (Tozer seeds Ltd, Surrey, UK, KT11 3EH) was used throughout the study. Seeds were shown in moist vermiculite at 20±2°C, 16L: 8D photoperiod until the cotyledons had unfolded (approx. 7-10 days). Seedlings were then transplanted singly into plastic pots (5cm x 5cm x 8cm) filled with F2+S, Levington compost© (Levington, Surrey, UK, GU7 1XE), maintained at 20±2ºC, 16L: 8D photoperiod and watered *ad libitum.*

2.2 INSECT CULTURES

Populations of *Brevicoryne brassicae* (K3 clone, Kirton) and *Myzus persicae* (Mp1S, non-resistant clone) were reared in ventilated Perspex cages (38cm x 38cm x 100cm) on Brussels sprout plants at growth stage 15/16 (BBCH-scale for other brassica vegetables). Colonies were sub-cultured onto new plants as required by transferring 15-20 apterous adults onto fresh plants. Cultures were maintained at 20±2°C, 16L: 8D photoperiod, which ensured the maintenance of an anholocyclic life cycle. Airflow was set at $0.31m^3s^{-1}$.

2.2.1 Production of known-age *B. brassicae* & *M. persicae*

Cohorts of 15-20 adult aphids were removed from culture plants with a fine camel hair paintbrush and placed at the base of Brussels sprout plants at growth stage 15/16 (BBCH-scale). Plants were maintained at 20±2°C, 16L: 8D photoperiod. After 48 hours, all adult aphids were removed, leaving a cohort of similarly aged first instar aphids. Cultures were maintained until the aphids became adults. *M. persicae* became adults after 8 days ±1day and *B. brassicae* became adults after 10days ±1day.

2.3 FUNGUS CULTURES

Seven species of entomopathogenic fungi from five different genera were used in this study (Table 2.3.1 & Table 2.3.3).

2.3.1 *In vitro* culture of strains of hypocrealean entomopathogenic fungi

Stock cultures of the different strains were stored as conidia on porous plastic beads in cryotolerant plastic tubes (Pro-Lab Diagnostics, Bromborough, Wirral, UK, L62 3PW) at minus 80°C (Chandler, 1994). A two-stage system was used to provide material for experiments. Firstly, for each strain, a culture was grown by removing a bead from cryopreservation and placing it on a Sabouraud dextrose agar (SDA) slope in a Universal tube and incubating it in darkness at 20±1°C for 10 days before transfer to cold storage (4±2°C, darkness) (this material was referred to as a “laboratory culture”). Secondly “working cultures” were grown from hyphal material taken from laboratory cultures and grown on SDA in 90mm triple vented Petri dishes (Fisher Scientific, Loughborough, UK, LE11 5RG) in

darkness at 20±1°C for 15- 20 days. Laboratory culture slopes were replaced every 3-4 months in order to minimise the risk of attenuation of fungal strains caused by repeated subculture (Hall, 1980; Nahar *et al.* 2008).

Table 2.3.1 Strains of hypocrealean entomopathogenic fungi used in this study.

Species	Isolate*	Original host	Origin
B. bassiana (ATCC strain)	432.99[(a)]	*Anthonomus grandis*	USA
B. bassiana (GHA strain)	433.99[(b)]	*Bemisia* spp.	USA
L. muscarium	19.79[(c)]	*Trialeurodes vaporariorum*	UK
M. brunneum	275.86[(d)]	*Cydia pomonella*	Germany
I. fumosorosea	409.96[(e)]	*Phenacoccus solani*	USA
L. longisporum	1.72a[(f)]	Vertalec product	-

*Isolate reference for the Warwick Crop Centre culture collection.
[(a)] Isolate forms the active ingredient in the proprietary mycopesticide 'Naturalis L' (Troy Biosciences Inc., 113 South 27th Ave. Phoenix, AZ 850433, USA).
[(b)] Isolate forms the active ingredient in the proprietary mycopesticide 'BotaniGard' (Mycotech, 117 South Parkmont, Butte, MT, 59702-4109, USA).
[(c)] Isolate forms the active ingredient in the proprietary mycopesticide 'Mycotal' (Koppert B.V., Unit 8, 53 Hollands Road, Haverhill, Suffolk, CB9 8PJ, UK).
[(d)] Isolate forms the active ingredient in the proprietary mycopesticide 'Met52' (Novozymes Biologicals Inc., 5400 corporate circle, Salem, VA 24153, USA).
[(e)] Isolate forms the active ingredient in the proprietary mycopesticide 'PFR97' (ThermoTrilogy Corporation, 9145 Guildford Road, Suite 175, Columbia, MD 21046, USA).
[(f)] Isolate forms the active ingredient in the proprietary mycopesticide 'Vertalec' (Koppert B.V., Unit 8, 53 Hollands Road, Haverhill, Suffolk, CB9 8PJ, UK).

2.3.2 Preparation of hypocrealean conidial suspensions

Conidial suspensions were made by agitating the mycelium of a 15-20-day old culture with a 'L-shaped' spreader (Fisher Scientific, Loughborough, UK, LE11 5RG) in 10ml of 0.05% Triton X-100. The suspensions were then passed through glass crucible filters (Fisher Scientific) to remove any hyphal fragments. Suspensions were enumerated using an Improved Neubauer haemocytometer and adjusted to the required concentration using 0.05% Triton X-100.

2.3.3 *In vitro* culture of strains of *Pandora neoaphidis* (=Entomophthoromycota)

Two strains of *Pandora neoaphidis* were used in this study (Table 2.3.3).

Table 2.3.3 *Pandora neoaphidis* strain information.

Species	Isolate*	Original host	Origin
Pandora neoaphidis	NW420 (ARSEF 5372)	*Brevicoryne brassicae*	Denmark
Pandora neoaphidis	WEL1	*Brevicoryne brassicae*	Wellesbourne, UK (2014)

*Isolate reference for the Warwick Crop Centre culture collection.

2.3.3.1 Sabouraud's-Egg-Milk-Agar (SEMA)

Cultures of *P. neoaphidis* were grown on Sabouraud's-Egg-Milk-Agar (SEMA) and prepared as follows: Batches of SDA (20.8g in 320 ml of distilled water contained in a 500ml Duran bottle) and semi-skimmed milk (Dairycrest, Esher, Surrey, KT10 9PN) (34 ml contained within a 100ml Duran bottle) were prepared, autoclaved (121°C, 1.2 bar for a minimum of 15 minutes) and then transferred to a water bath at 50°C and left until cool enough to handle (minimum of 30 minutes). Two medium sized chicken's eggs (53g- 63g) (Sainsbury's Supermarkets, Holdurn, London, EC1N 3DT) were surface sterilised by submerging them in a mixture of 99% 95%-ethyl alcohol and 1% acetone for two hours. Working in a laminar air flow cabinet, the eggs were cracked, the yolks separated from the whites and then transferred to the autoclaved semi-skimmed milk and mixed by shaking the Duran bottle by hand. This was then added to the autoclaved SDA, mixed gently by hand and then decanted to 90mm triple vented Petri dishes. Once the media had solidified the plates were placed in a sterile plastic bag and stored at 4±2°C in darkness for a maximum of 6 weeks.

2.3.3.2 Cryopreservation of *Pandora neoaphidis*

For long-term storage in cryopreservation, *P. neoaphidis* strains were maintained in glycerol contained within sachets. The sachets were made from polypropylene drinking straws (6mm diameter Ø) cut to 200mm lengths and heat-sealed at one end. Ten to fifteen sachets were placed in autoclave bags, sealed and autoclaved at 121°C, 1.2 bar for a minimum of 15 minutes. Working within a Class II sterile air flow cabinet 5-6 plugs of *P. neoaphidis* mycelium (approximately 2mm x

4mm) were taken from the leading edge of a culture grown on SEMA (8 – 10 weeks old, 15°C in darkness) and then placed into the bottom of sachets using a Tungsten-wire probe with the end shaped to a right angle at circa. 3-4mm. Each straw was then filled with 10% glycerol solution (autoclaved, as above) and pipetted to within 1 – 2mm of the top. The sachet was labeled and sealed with a 185mm polythene bag heat sealer (Hulme-Martin, Guilford, London, WCIN 2LD). The seal was tested by squeezing the sachet between fingers. Sachets were then placed within an open-sided straw cane, formed by cutting a drinking straw along its entire length. One end of the cane was weighted with a 3cm paper clip to ensure it did not float in liquid nitrogen. Once the canes were full they were placed in a slow cooling chamber (Figure 2.1) containing 400g of CO_2 ice and 75ml of 95% ethanol and maintained for 75 minutes prior to being placed in liquid nitrogen within a Dewar (35L, Fisher Scientific, Loughborough, UK, LE11 5RG).

2.3.3.3 Removal of *Pandora neoaphidis* from liquid nitrogen and inoculation of SEMA plates

A sachet containing plugs of *P. neoaphidis* mycelium was removed from liquid nitrogen and submerged immediately in a water bath at 37°C for two minutes, after which the sachet was transferred to a class II air flow hood, immersed in 95% ethyl alcohol (for 10 seconds) and allowed to air dry. The sachet was then opened using a sterile pair of scissors and plugs of *P. neoaphidis* were removed using a sterile tungsten wire probe and placed in the centre of 90mm triple vented Petri dishes containing SEMA, one plug per dish. They were then transferred to a plastic box (150mm wide x 300mm long x 60mm high) containing a glass vial (15mm diameter x 30mm long) filled with sterilised distilled water. The plastic box was

sealed and placed at 15°C in darkness until cultures were needed or for up to 10 weeks. This method enabled air movement between the Petri dish and the humid atmosphere within the plastic box, and was necessary for *P. neoaphidis* growth. Fungal cultures placed on SEMA in Petri dishes that were sealed with Parafilm® did not grow as well. Cultures placed on SEMA in unsealed Petri dishes and maintained in an incubator without being placed in a humid atmosphere dried out within three weeks, leading to death of the fungus.

2.3.3.4 Isolation of *P. neoaphidis* from infected aphid hosts via surface sterilisation

Isolation of *P. neoaphidis* by surface sterilisation is only appropriate for insects that are moribund or have died very recently and no rhizoids are visible (J. Pell, 2014, pers. comm., 20th October). In the case of aphids, individuals in this condition are swollen and discoloured, typically orange/yellow.

Moribund aphids, or those that were considered to have died recently from *P. neoaphidis* infection were submerged in 70% ethyl alcohol for 5 seconds to reduce the surface tension and then submerged for five minutes in a 15% sodium hypochlorite solution (Fisher Scientific). The aphids were then immersed in sterile distilled water for five minutes. This was repeated to give a total of three separate washes. Water baths were replaced every fifth individual. Aphid cadavers were then placed on SEMA in the centre of 90mm triple vented Petri dishes. The dishes were sealed using Parafilm® and then incubated at 20°C, 16L: 8D photoperiod and monitored for contamination from bacteria, which sometimes grew out from within aphids. Using this technique *P. neoaphidis* was isolated in 2014 from

Brevicoryne brassicae in a field of Brussels sprouts at Warwick Crop Centre, Wellesbourne, UK (Figure 2.2).

2.4 STATISTICAL ANALYSIS

Data analysis was carried out in R (RStudio©, version 0.98.1091) or SPSS (IBM®, version 22). Details of specific analysis, including packages, are stated in the relevant methods sections.

Figure 2.1 Copper slow cooling chamber for preparation of fungal strains for cryopreservation.

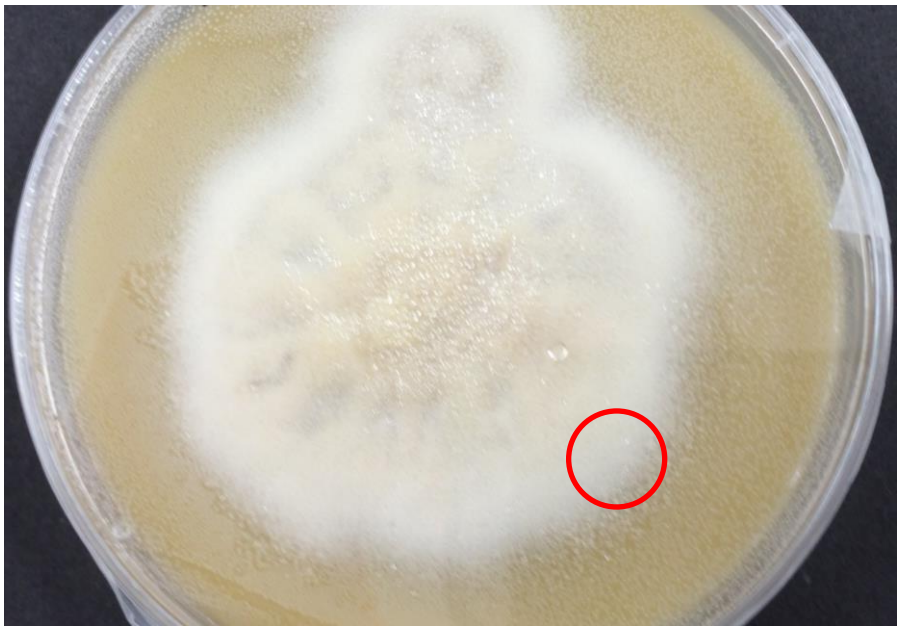


Figure 2.2 Petri dish containing *Pandora neoaphidis* growing on Sabouraud's-Egg-Milk-Agar (SEMA). Highlighted region indicates the area sampled during culturing.

CHAPTER 3: THE APHID POPULATION CRASH IN FIELD BRASSICA CROPS

3.1 INTRODUCTION

Aphids are r-strategist insects that reproduce parthenogenetically in the summer, meaning they are capable of producing significant amounts of biomass in a short period of time (Blackman & Eastop, 1984; Karley *et al*. 2004). A serious concern considering the evolution of resistant biotypes and their role as virus vectors of many plant diseases. However, the exponential growth seen during spring and early summer does not continue. During the growing season (usually July in the UK) many aphid species exhibit a sharp population decline to apparent local extinction (Karley *et al.*, 2003). This mid-season 'crash' occurs in the absence of insecticide in both agricultural (Soroka & Mackay, 1990; Nakata 1995; Parker *et al*., 2000) and natural landscapes (Jarosik & Dixon, 1999; Muller *et al*., 1999) and populations generally remain low or undetectable for at least 6-8 weeks' post-crash (Karley *et al*. 2003; Karley *et al*. 2004). Frequently, aphid populations begin to build again after the crash but seldom reach the levels seen prior to the decline. At present the timing of this crash cannot be predicted accurately.

Many factors have been suggested for the mid-season crash, including plant age (Watt, 1979; Douglas, 2003), the action of natural enemies (Jones, 1979; Boiteau, 1986; Holland & Thomas, 1997; Karley *et al.*, 2003) and adverse weather conditions. Extremes of temperature, strong winds and significant rainfall events can negatively affect aphid populations (Ba-Angood & Stewart, 1980; Basky, 1993; Asin & Pons, 2001; Morgan *et al*., 2001); however, the weather is seldom

as predictable as to cause the aphid population crash at approximately the same time and location each year (Parker *et al*., 2000). Due to this fact, adverse weather conditions are unlikely to be a significant contributory factor in the annual aphid population crash. In principle, all the factors above affect population processes including birth, death and emigration. For example: a decrease in nitrogen content of older plants could result in a decrease in aphid birth rates and increased emigration rates as a result of intraspecific competition, while natural enemies – attracted to large aphid populations – could cause a large increase in mortality (Karley *et al*. 2002; Karley *et al*. 2004). Of the natural enemies, entomopathogenic fungi have been strongly implicated in the crash of aphid populations, highlighted by increased interest in their use as biological control agents, but little is known of their biology (Steinkraus *et al*. 2002; Karley *et al*. 2003; Karley *et al*. 2004; Steinkraus, 2006). Investigation of the scale and timing of the crash is valuable as the crash might contribute to the overall regulation of aphid populations meaning it could be exploited in pest management strategies. Moreover, a better understanding of the role of natural enemies in aphid population dynamics might enable the mid-season crash to be forecast, which would give growers the option of withholding pesticide sprays.

Steinkraus *et al*. (1991) identified the entomopathogenic fungi, *Neozygites fresenii,* as the causal agent of the population crash in *Aphis gossypii* on cotton. Subsequently, a biological control program was developed exploiting the timing of the natural fungal epizootic which was predicted by a linear model based on the density of diseased insect cadavers (Hollingsworth *et al*., 1995). The program, which is operational today, is based on several years of *A. gossypii* population data, which allowed the authors to determine that aphid populations tended to begin a

precipitous decline when the prevalence of fungal infected cadavers reached 15% (Steinkraus & Hollingsworth, 1994; Hollingsworth *et al*., 1995). The model can predict aphid declines 10 days in advance of their occurrence. A similar IPM program was developed by Kish & Allen (1978) for the control of *Anticarsia gemmatalis* on soybean crops using *Neozygites rileyi*, however, due to the use of readily available insecticides and a reduction in soybean growing in Florida the model was never implemented. Particularly effective natural enemy species may also be worth considering as classical or augmentation biocontrol agents (Steinkraus *et al*., 2002).

The aim of this study was to monitor aphid populations and determine the relative contributions of the guild of natural enemies, host plant age (as a proxy for host plant quality) and abiotic factors on their population dynamics.

3.2 MATERIALS & METHODS

3.2.1 Quantifying the effect of host plant age on population development of *B. brassicae* and its natural enemies under field conditions (2013)

The following fieldwork was carried out on plots of Brussels sprout (*Brassica oleracea*) cv. Trafalgar (Tozer Seeds Ltd, Surrey, UK, KT11 3EH) at Warwick Crop Centre, Wellesbourne, CV35 9EF, UK from May to October 2013. The field area was composed of two experiments in 2013; Experiment 3.2.1.1 and 3.2.1.2, both orientated NNE to SSW.

3.2.1.1 Population development of *B. brassicae* and its natural enemies on co-transplanted host plants (2013)

Experiment 3.2.1.1 consisted of 24 plots (12 plots of 'young' plants & 12 plots of 'old' plants). Each plot consisted of 10 experiment plants (arranged 2 rows x 5 plants each, with 50cm spacing) separated by a single guard row of Brussels sprout plants. The plants in each plot were arranged in three beds of equal length (Figure 3.1). Prior to transplanting to the field, the plants were raised in a glasshouse (with a mean temperature of approximately 20°C, with no supplementary lighting) in 160 module propagation trays containing compost (Levington F2 + sand, Everris). The 'young' plants were grown for 4 weeks and the 'old' plants were grown for 8 weeks before transplant. Both sets of plants were transplanted to the field on the same day. The two types of plants (i.e. 'young' versus 'old') were randomly assigned to a plot within each of the beds, i.e. three beds containing an equal number of plots with each plant age treatment. The beds acted as blocks forming a complete randomized block design. All plants were transplanted to the field on June 14th 2013 (growth stage BBCH 13-14). Tracer © (Dow AgroSciences) was applied as a module drench to protect the plants against cabbage root fly damage prior to field transplantation; no additional pesticides were applied. Nitrogen fertilizer pellets were applied at the time of field transplantation (50-80kg/ha). Plants were watered *ad libitum* via drip irrigation laid in between rows. Polypropylene tape (Humming line, Wessex aust PTY Ltd) was crisscrossed over experimental sites between wooden canes, keeping the line taut, in order to scare birds. The experiment was situated in the North-West corner of the 'pump-ground' field. (Figure 3.6). Experimental sites were weeded by hand.

To ensure plants were infested with *B. brassicae*, one week after transplanting all experimental plants in Experiment 3.2.1.1 were inoculated with five *B. brassicae* apterous adults per plant (which were selected from laboratory colonies (see Section 2.2)). These aphids were attached to experimental plants using clip cages (1cm in diameter) for a maximum period of four hours (Figure 3.2).

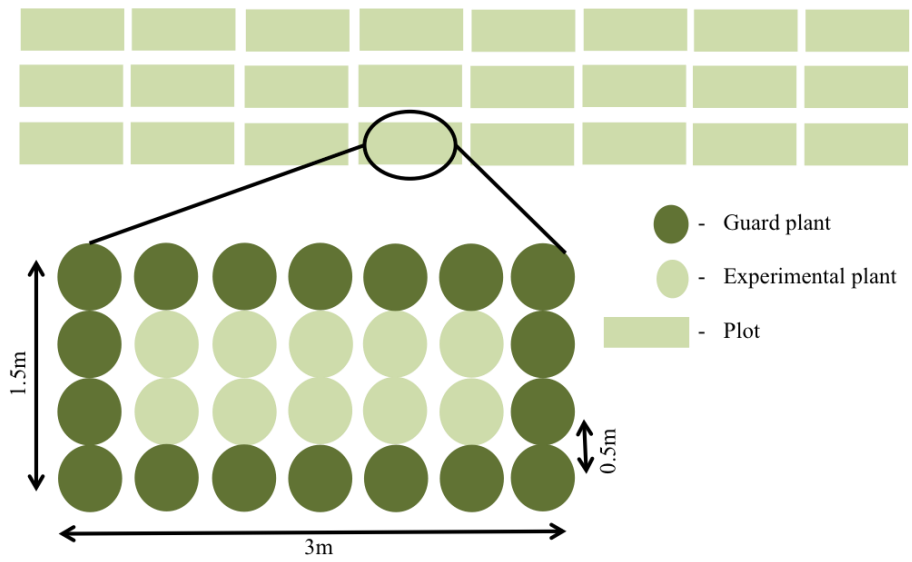


Figure 3.1 Field schematic and close up of a single plot in experiment 3.2.1.1. Eight plots were randomly assigned to 'young' or 'old' plant treatments (4 plots per treatment per bed); plots arranged in three beds of equal length. Each plot contained 10 experimental plants separated by a single guard row totalling approximately 1.5m x 3m in size.

Experimental Brussels sprout plants were inspected every 7-21 days. For the majority of the experiment, inspections were done every seven days, but during periods of the experiment where the change in aphid populations was slow (i.e. at

the start and end of the experiment) some inspections were done at 14 or 21 day intervals. An entire Brussels sprout plant constituted a single sample unit. Plants were examined visually to record the total number of aphids per plant (healthy, infected and parasitized). On occasions that aphid numbers of specific colonies could not legitimately be recorded by counts, colony diameter was used to estimate aphid numbers. Numbers of individuals were estimated using a regression line fitted to a range of actual aphid counts at varying colony diameters (Rosemary Collier pers. comms., 14 July 2013). In total, colony diameter was used to estimate aphid numbers in 25 colonies. In addition to aphid number, fungus-infected cadavers, syrphid larvae, parasitoid mummies, coccinellids and anthocorids were also recorded. No other taxonomic groups of natural enemies were observed. Aphids that were infected with fungi were identified on the basis of having sporulating fungal mycelium on the surface of the cadaver (Figure 3.3). Because counting the number of aphids per plant was time consuming (for example it could take up to 2 hours per plant during periods of peak aphid population size) a minimum of three of the ten experimental plants per plot were chosen at random for inspection on each occasion. The secondary sampling rate was practiced during periods of high aphid density, particularly in June, July and August.

Figure 3.2 Clip cages used in all experiments to inoculate Brussels sprout plants. Plant pictured at standard physiological age at time of transplantation to field.

Temperature and humidity data were collected using iButton loggers (Homechip Ltd, Milton Keynes, MK17 0BH, UK). Temperature data followed data collected from the farm meteorological station, however, humidity data from iButton loggers in the field proved to be unreliable. As a result, temperature and humidity data recorded from the farm meteorological station were used.

Figure 3.3 *Brevicoryne brassicae* infected with *Pandora neoaphidis* on Brussels sprout leaf in 2013. Healthy individual circled to show visual difference in the field.

Figure 3.4 Experiment 3.2.1.2 in May 2013 (Left) and in July 2013 containing May, June & July transplants (Right).

3.2.1.2 Population development of *Brevicoryne brassicae* and its natural enemies on sequentially-transplanted host plants (2013)

Experiment 3.2.1.2 was consisted of 16 plots (4 planting dates x 4 replicates) of 10 experimental plants (arranged in 2 rows x 5 plants each, with 50cm spacing) (Figure 3.4). Plots were separated by 'double' guard rows, made up of experimental plants of the same age, meaning the total size of the experiment covered an approximate area of 8m x 14m (Figure 3.5). Seeds were planted in 160 module propagation trays containing compost (Levington F2 + sand, Everris) and seedlings were transplanted to the field (growth stage BBCH 13-14) on the 2nd May, 3rd June, 3rd July and 5th August 2013 after a month of growth in a glasshouse (with a mean temperature of approximately 20°C, with no supplementary lighting). Each of the four replicates for each transplant date were randomly assigned plots in a 4 x 4 plot grid (complete randomised design). Tracer was applied as a module drench to protect the plants against cabbage root fly damage prior to field transplantation; no additional pesticides were applied. Nitrogen fertilizer pellets were applied (50-80kg/ha) at the time of field transplantation. Plants were watered *ad libitum* via drip irrigation lines laid in between rows. Polypropylene tape (Humming line, Wessex aust PTY Ltd) was crisscrossed over experimental sites between wooden canes, keeping the line taut, in order to scare birds. The experiment was situated in the South-West corner of the 'pump-ground' field. (Figure 3.6). Experimental sites were weeded by hand.

May and June plots were inoculated with *B. brassicae* as in Experiment 3.2.1.1, subsequent transplants (July & August) were colonised naturally. Clip cages (1cm in diameter) were removed after approx. four hours. If aphids did not establish, plants were re-inoculated up to 7 days after the initial inoculation.

Experimental Brussels sprout plants were inspected every 7-21 days. For the majority of the experiment, inspections were done every 7 days, but during periods of the experiment where the change in aphid populations was slow (i.e. at the start and end of the experiment) some inspections were done at 14 or 21 day intervals. An entire Brussels sprout plant constituted a single sample unit. Plants were examined visually to record the total number of aphids per plant (healthy, infected and parasitized). On occasions that aphid numbers in specific colonies could not legitimately be recorded by counts, colony diameter was used to estimate aphid number as in Experiment 3.2.1.1. In total, colony diameter was used to estimate aphid populations in 25 colonies. In addition to aphid number, fungus-infected cadavers, syrphid larvae, parasitoid mummies, coccinellids and anthocorids were also recorded. No other taxonomic groups of natural enemies were observed. Aphids that were infected with fungi were identified on the basis of having sporulating fungal mycelium on the surface of the cadaver (Figure 3.3). Because counting the number of aphids per plant was time consuming, a minimum of three of the ten experimental plants per plot were chosen at random for inspection on each occasion. The secondary sampling rate was practiced during periods of high aphid population density, in June, July and August.

Temperature and humidity data were collected using iButton loggers. Temperature data followed data collected from the farm meteorological station, however, humidity data from iButton loggers used in the field proved to be unreliable. As a result, temperature and humidity data recorded from the farm meteorological station was used. Leaf material was randomly sampled from each of the transplant ages for soluble nitrogen analysis (see section 5.2.3 for details of soluble nitrogen analysis).

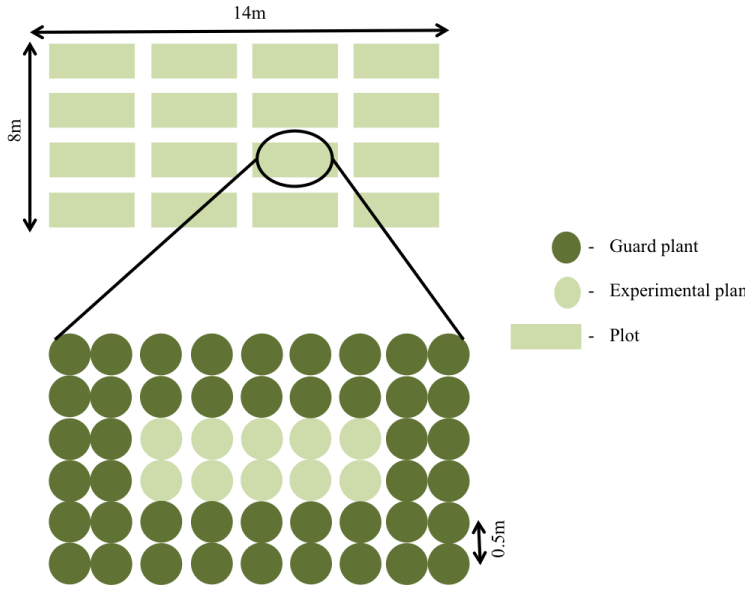


Figure 3.5 Field schematic and close up of a single plot in experiment 3.2.1.2. Sixteen plots arranged in four beds. Each bed was assigned a May, June, July and August transplant treatment, which were randomly located within the bed. Each plot contained 10 experimental plants separated by a double guard row resulting in a field trial approximately 8m x 14m in size.

3.2.2 Population development of *B. brassicae* and its natural enemies on sequentially-transplanted, spatially separated host plants (2014)

Experiment 3.2.1.2 was repeated during May to November 2014 with spatially separated plots. Experiment 3.2.2 consisted of 16 plots (four planting dates x 4 replicates) of 10 experimental plants arranged 2 rows x 5 plants each, with 50cm spacing surrounded by a single guard row. Each experimental replicate was situated in one of four different locations separated by at least 80 meters in order to investigate spatial differences in aphid population dynamics; Little Cherry 'LC', Pump Ground 1 'PG1', Pump Ground 2 'PG2' & Long Meadow West 'LMW' (Figure 3.6). Each experimental replicate included one plot of each of the planting dates (6th May, 3rd June, 3rd July & 1st August) separated by at least 20 meters.

Plants were at growth stage BBCH 13-14 at the time of transplantation. Plants were grown for a month in a glasshouse (with a mean temperature of approximately 20°C, with no supplementary lighting) in 160 module propagation trays containing compost (Levington F2 + sand, Everris). Each of the 4 replicates for each transplant date were randomly assigned positions within locations. Except for Tracer, no other pesticides were applied. Nitrogen fertilizer pellets were applied (50-80kg/ha) at the time of field transplantation. Plants were watered *ad libitum* via drip irrigation laid in between rows. Polypropylene tape was crisscrossed over experimental sites between wooden canes, keeping the line taut, in order to scare birds. All experimental plants were inoculated with *B. brassicae* adults as in section 3.2.1 (Figure 3.2). Survey protocol and data collection remained the same (Section 3.2.1).

Temperature and humidity data were collected (refer to Section 3.2.1.2 for details).

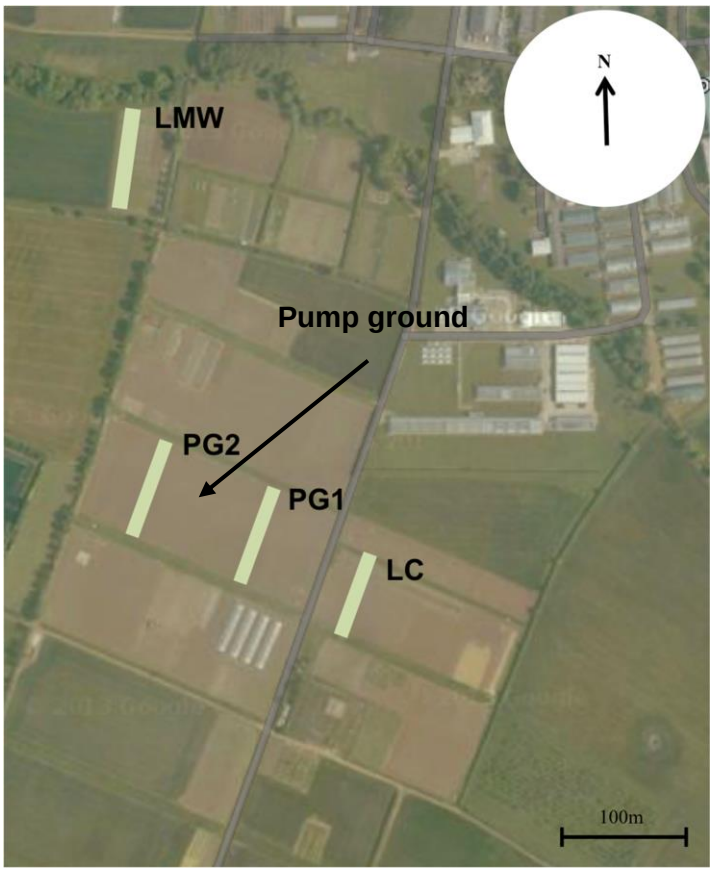


Figure 3.6 A map of the 2014 field trial area showing the locations of each experimental site: Long Meadow West 'LMW', Pump Ground 1 'PG1', Pump Ground 2 'PG2' and Little Cherry 'LC'.

3.2.3 Identification of natural enemies

In 2013 parasitoid mummies, fungal cadavers and true predators (both juvenile and adult stages) were collected from designated monitoring plants in order to taxonomically describe the guild of natural enemies occurring within the crop. Guard row plants were designated for monitoring purposes so as to minimise the influence of removing natural enemies from experimental plants. Samples were of unidentifiable natural enemies (juvenile syrphid and parasitoid mummies) were taken from randomly selected guard plants of experiment 3.2.1.2 when aphid populations were high (10th June 2013).

3.2.3.1 Identification of fungal natural enemies

During the field experiments in 2013 and 2014, the population of *B. brassicae* within the crop became naturally infected by *Pandora neoaphidis* (Entomopthorales). This was the only entomopathogenic fungus witnessed infecting aphid populations. The fungus was initially identified to genus level in 2013 using morphological criteria. This was done with samples of aphid cadavers collected from the field and taken back to the laboratory for examination under the microscope. In 2014, the fungus was isolated onto Grace's liquid insect cell culture medium by placing a 7mm plug taken from the growing edges of cultures that were obtained through surface sterilisation of moribund *B. brassicae* individuals collected from the field (Section 2.3.3.4). Spore morphology and infected cadaver appearance in 2014 indicated that the pathogen was the same as that in 2013, i.e. *P. neoaphidis*. Due to difficulties in obtaining a 'clean' isolate from the field only a single clean culture was obtained from one aphid during this period out of a total

of 32 aphids collected. Genomic DNA was extracted from mycelial mats grown in Grace's liquid insect cell culture medium using a DNeasy Plant Mini Kit (Qiagen Ltd, UK) following the manufacturer's protocol. Prior to using the DNeasy kit the SEMA plug was removed from the centre of the mycelium, the mycelial mat washed in sterile RO water before being freeze dried overnight.

Gene sequencing was then done in order to identify the *Pandora* isolate to species level. PCR was performed on the internal transcribed spacer 1 (ITS 1) using a genus specific forward primer for *Pandora* PnCNf: 5' – TTTGGGTTTAAATAGAAGGTTGA-3' and reverse PnCNr: 5'-AGGCAAAGCCTAGAGCACTT -3' developed in Fekih et al. (2013). The primers were chosen to confirm the identity of the *Pandora* outbreak in populations of *B. brassicae* in the field as morphological data, spore morphology and infected cadaver appearance, had suggested *Pandora neoaphidis* was the agent.

A positive DNA control was included in the form of a strain of *P. neoaphidis* provided by the ARSEF collection of Entomopathogenic Fungal Cultures (Cornell University, Ithaca, NY). *Pandora neoaphidis* NW420 (ARSEF 5372) was originally isolated from *B. brassicae* in Denmark (Table 2.3.3). Negative water controls were also included.

PCR amplifications were performed in 25µl reaction volumes containing 1µmol L^{-1} of water or extracted DNA, 9.5µmol L^{-1} of RO water, 12.5µmol L^{-1} QIAGEN Multiplex PCR master mix solution and 1µmol L^{-1} of each primer. The PCR conditions were denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds, with a final extension at 72°C for 5 minutes.

The size of the PCR amplifications was estimated by electrophoresis on 1.5% agarose gel in 0.5 TBE and the products visualized with a DNA ladder (Gel Logic 200 imaging).

Sequencing was used to verify species identity by combining 5nmol^{-1} of PCR product with 5nmol^{-1} of PnCNf or PnCNr and sending the sample to GATC Biotech (Germany). Prior to sequencing the PCR products were purified with QIAquick PCR purification kit (Qiagen, UK) following the manufacturer's protocol. The final data were received in. fasta format and subject to a sequence similarity search using NCBI BLAST performed in Genbank. Curation and trimming of sequence data was conducted by eye using the 4Peaks© V1.8 (Nucleobytes, NL) software to assess sequence data quality.

A selection of *P. neoaphidis*, *Pandora nouryi* and *Pandora delphacis* sequences were obtained from the NCBI database and used data to build a phylogenetic tree. A total of 17 *P. neoaphidis* isolates (EU267192.1, AF543211.1, AF543207.1, AF543206.1, AF543202.1, AF543205.1, EU267190.1, HQ677587.1, AF543203.1, AF543204.1, AF543208.1, AF543209.1, AF543210.1, EU267189.1, EU267188.1, NW40 & WEL1) and one isolate of *P. nouryi* (HQ677590.1) and *P. delphacis* (AF368321.1) were used (Figure 3.2.3 top). Sequences of all *Pandora* data were aligned using the pairwise ClustalW algorithm in DNASTAR©. The final phylogenetic tree was subject to bootstrap analysis (1000 permutations) in DNASTAR© and drawn using TreeView® in DNASTAR, MegAlign Pro©.

3.2.3.2 Identification of arthropod natural enemies

Parasitoid mummies without evidence of emergence holes were collected in order to obtain the adults developing within. Parasitoid mummies were kept in Petri dishes (triple vented, 90mm diameter) (Fisher Scientific, UK) at room temperature and checked daily for emergence of adults. Adults were stored in ethanol for identification under light microscope using wing venation patterns.

Adult parasitoids that emerged from mummies were identified using an in-house publication supplied by ADAS (Sasha White, pers. Comms 18th October 2013). Syrphidae larva were collected and raised to adulthood for easier identification on infested *B. brassicae* Brussels sprout plants (Stubbs & Falk, 2002). Depending on the instar of the larvae development to pupation took 7-18 days in a controlled environment room set to at 20°C ± 1°C, 16:8 L:D light cycle. Coccinellidae adults were identified in the field with Majerus *et al*. 2006 (FSC, Field Studies Council guide). See section 3.2.3 for sampling information. Too few Anthocorids were collected to be identified to species level.

3.2.4 Statistical treatment

Results were analysed using RStudio© version 0.98.1091. Data were analysed for normality via visual inspection of residual plots and/or Shapiro-Wilk tests.

Kruskal-Wallis was used to determine the effect of plant age on aphid population development and natural enemy dynamics for experiment 3.2.1.1. Non-parametric statistics for interaction terms are unreliable and seldom accurately interpretable. ANOVA is robust against type 1 errors when the data set is large, which is the case for Experiments 3.2.1.2 and 3.2.2. As a result, transplant date

and location effects on aphid populations and grouped natural enemy communities were analysed using ANOVA. Significant results were analysed using Tukey's HSD test. Tukey HSD is also very conservative, has a low Type 1 error rate, and can be used with unequal sample sizes.

Transplant and location effects on the individual groups comprising the natural enemy community in Experiments 3.2.1.1, 3.2.1.2 and 3.2.2, i.e. parasitoid, syrphidae, coccinellidae and fungal cadavers, were analysed using Kruskal-Wallis and significant results were analysed with post-hoc Bonferroni-Dunn tests.

Soluble nitrogen content for Experiment 3.2.1.1 was analysed using Kruskal-Wallis, whereas soluble nitrogen data for Experiment 3.2.1.2 was analysed using ANOVA.

For a direct measure of population growth in Experiments 3.2.1.2 and 3.2.2, instantaneous rate of increase (r_i) was used (Stark & Banks, 2003). The value of the rate varies between -1 and 1; values below 0 indicate that the population is decreasing and vice versa for values above 0 (Stark & Banks, 2003) (Equation3.1)

Equation 3.1

$$r_i = \ln(N_f/N_o)/\Delta T,$$

Where, N_f is the final number of individuals, N_o is the initial number of individuals and ΔT is the change in time (number of days between observations) (Stark & Banks 2003). Analysis was carried out on data spanning the length of field data collection for 2013 and 2014.

Regression analysis was used with instantaneous rate of increase to elucidate the individual and combined effects of the natural enemy guild on aphid population dynamics.

3.3 RESULTS

3.3.1 Population development of *B. brassicae* and its natural enemies on co-transplanted host plants (2013)

On old transplants aphid populations underwent a precipitous decline on the 30th July± 2 days 2013 and did not recover in the following three months. Populations on young transplants underwent more of a gradual decline beginning the previous week (24th July± 1 days 2013), although plant age had no significant effect on aphid population size (Kruskal-Wallis, H= 1.6621, df= 1, *P*= 0.1973) (Figure 3.7). Aphid numbers remained very low in both young and old transplants (c.11/plant). Whilst the plants were of different ages at the time of transplantation to the field and in terms of their BBCH growth stage; 13-14 and 15-16 for young and old respectively, physiologically, in terms of their soluble nitrogen concentration, at the time leaf material was taken (8th August 2013), old and young transplants did not vary significantly (Kruskal-Wallis, H= 0.125, df= 1, *P*= 0.7237), 1364.71 ±581.29 µg of nitrate/g of dry weight and 1214.24 ±498.36 µg of nitrate/g of dry weight respectively (Figure 3.16). Young and old transplants were of the same growth stage (BBCH 19) by the 8th August 2013

Aphid population dynamics were mirrored in the natural enemy community, with peak natural enemy numbers following peak aphid density (Figure 3.7). The most numerous natural enemy were parasitoid mummies and parasitism rates peaked at 60.2% and 32.3% on the 2nd September 2013 for old and young plots respectively (Figure 3.7b.). Fungal infections peaked on the 20th August 2013, with the highest percentage infection on the 5th October 2013 (14.3%) and on the 20th August 2013 (16.2%) for young and old plots respectively (Figure 3.7f.).

In addition to transplant age having no significant effect on aphid population size, it did not significantly affect the number of parasitoid mummies (Kruskal-Wallis, H= 0.53116, df= 1, *P*= 0.4661), Syrpihidae (Kruskal-Wallis, H= 0.5892, df= 1, *P*= 0.4427), Anthocoridae (Kruskal-Wallis, H= 1.8386, df= 1, *P*= 0.1751) or fungal cadavers (Kruskal-Wallis, H= 0.20599, df= 1, *P*= 0.6499) present in the experiment. Coccinellidae numbers did vary between transplant ages (Kruskal-Wallis, H= 5.3527, df= 1, *P*= 0.02069) with significantly more individuals present in the old plots, indeed a general pattern emerged that all natural enemies were in greater abundance on plots containing older plants (Figure 3.7).

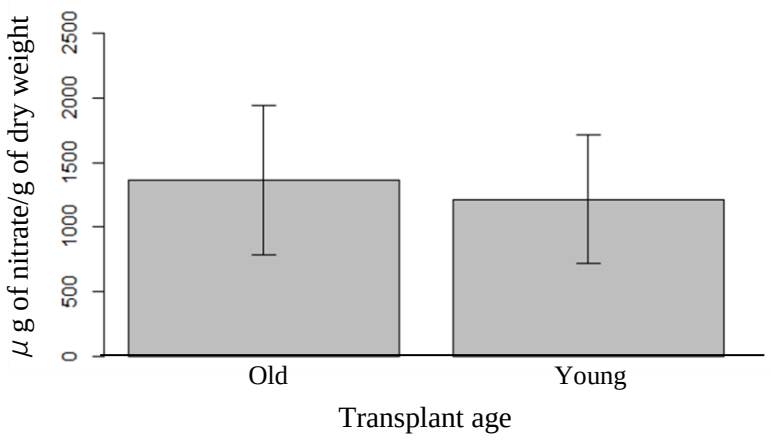


Figure 3.16 Mean soluble nitrogen concentration (±stdev) in leaf material collected from old and young transplants in experiment 3.2.1.1 at approximately the time of the aphid population crash.

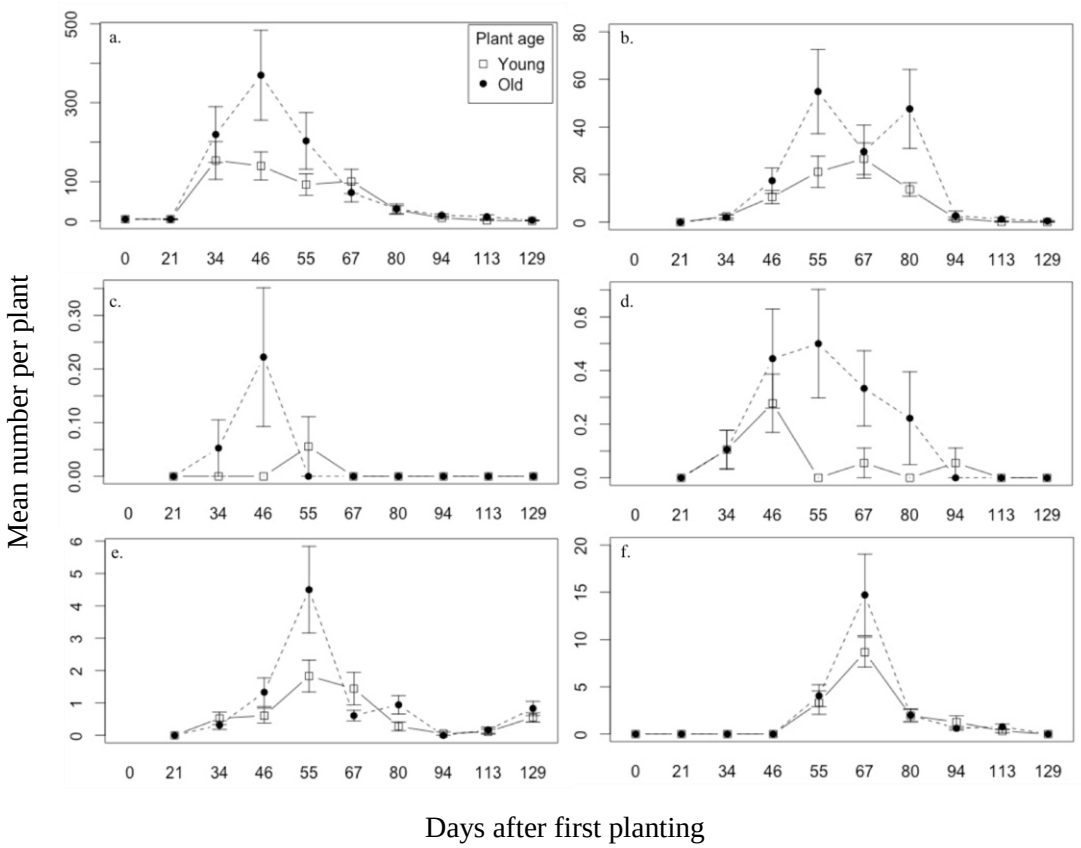


Figure 3.7 Changes in mean number of aphids and recorded natural enemies per plant (±stdev) for young and old transplants in experiment 3.2.1.1. Letters indicate different counts, starting top left: (a) *Brevicoryne brassicae*, (b) Parasitoid mummies, (c) Anthocoridae, (d) Coccinellidae, (e) Syrphidae and (f) fungal cadavers.

3.3.2 Population development of *B. brassicae* and its natural enemies on sequentially-transplanted host plants (2013)

There was significant variation in the size of *B. brassicae* populations on transplants of different ages (based on planting date) (ANOVA, F= 7.337, df= 3, $P < 0.001$), with significantly larger populations on plants transplanted in May than June (Tukey HSD, $P = 0.0105309$) and August (Tukey HSD, $P = 0.0002885$). No other significant differences in aphid populations were detected between transplant dates (Table 3.1). Significant differences were detected between natural enemy communities occurring on plants according to transplant dates (ANOVA, F= 4.838, df= 3, $P = 0.00244$), June transplants varied significantly from May transplants (Tukey HSD, $P = 0.0412734$) and August (Tukey HSD, $P = 0.0020401$). No other significant differences were detected between transplant date and natural enemy communities (Table 3.2). As mentioned, May transplants supported the greatest aphid populations and in turn the greatest number of natural enemies with the exception of Coccinellidae, who were most numerous on June transplants.

Table 3.1 Post hoc Tukey HSD analysis showing significant differences in aphid populations between transplant dates in Experiment 3.2.1.2.

Transplant comparison	***P***
August- July	= 0.330
August- June	= 0.228
August- May	= 0.002
June- July	= 0.999
May- July	= 0.057
May- June	= 0.017

Table 3.2 Post hoc Tukey HSD analysis showing significant differences in the size of natural enemy community between transplant dates in Experiment 3.2.1.2.

Transplant comparison	***P***
August- July	= 0.223
August- June	= 0.002
August- May	= 0.206
June- July	= 0.308
May- July	= 0.989
May- June	= 0.041

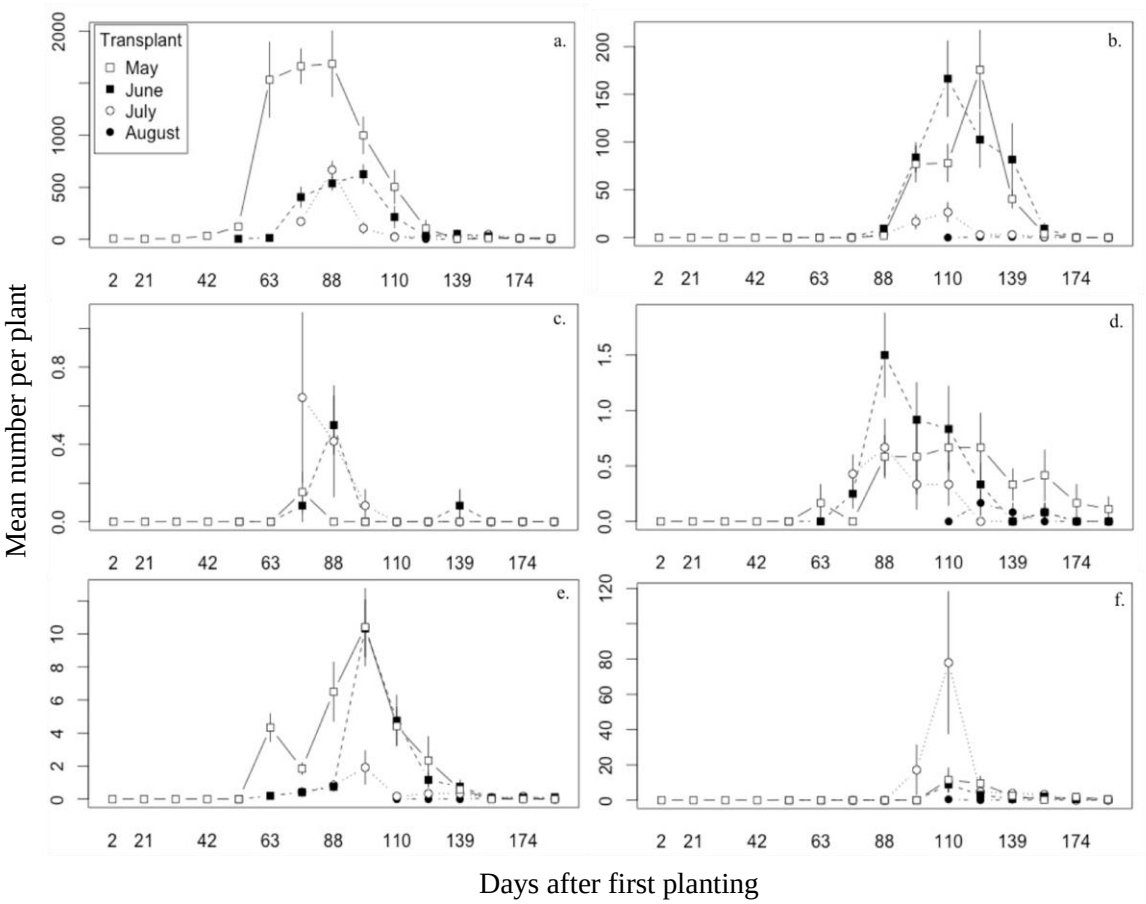


Figure 3.8 Changes in mean number of aphids and recorded natural enemies per plant (±stdev) for May, June, July and August transplants in experiment 3.2.1.2. Letters indicate different counts, starting top left: (a) *Brevicoryne brassicae*, (b) parasitoid mummies, (c) Anthocoridae, (d) Coccinellidae, (e) Syrphidae and (f) fungal cadavers.

Despite the differences in aphid population size the general pattern of their population dynamics over time remained similar (Figure 3.8: b, c, d, e & f). Aphid population dynamics were mirrored in the natural enemy community as in experiment 3.2.1.1 with peak natural enemy numbers following peak aphid density (Figure 3.8). Peak number of natural enemies occurred approximately 18 days after peak aphid numbers, during this time natural enemy numbers began to build

at their fastest rate during the field season. The fastest reacting natural enemy to the high number of aphids were the Coccinellidae and Syrphidae. There was some delay in the rise in numbers of parasitoid mummies and fungal cadavers (Figure 3.8b & 3.8f, respectively).

The number of parasitoid mummies varied significantly with transplant date (Kruskal-Wallis, H= 37.1554, df= 3, $P<0.001$), August varied from June (Bonferroni-Dunn, $P<0.001$) and July (Bonferroni-Dunn, $P<0.001$) but not May (Bonferroni-Dunn, $P=0.69935$). May transplants varied from June (Bonferroni-Dunn, $P=0.00041$) and July transplants (Bonferroni-Dunn, $P<0.001$) but there was no difference between June and July transplants (Bonferroni-Dunn, $P= 1$). There were no significant differences in the size of Coccinellidae populations between locations (Kruskal-Wallis, H= 8.2074, df= 3, $P= 0.04191$). There was a significant effect of transplant date on Syrpihidae populations (Kruskal-Wallis, H= 21.398, df= 3, $P<0.001$). May varied from June (Bonferroni-Dunn, $P<0.001$), July (Bonferroni-Dunn, $P= 0.0029$) and August (Bonferroni-Dunn, $P= 0.0024$) transplants, no other differences were present. Anthocoridae populations varied between transplants also (Kruskal-Wallis, H= 13.649, df= 3, $P= 0.003425$) but only between May and July transplants (Bonferroni-Dunn, $P= 0.020$). Finally, the number of fungal cadavers varied significantly with transplant date (Kruskal-Wallis, H= 58.767, df= 3, $P<0.001$), with significantly more infected cadavers in July than May (Bonferroni-Dunn, $P<0.001$), June (Bonferroni-Dunn, $P<0.001$) and August (Bonferroni-Dunn, $P<0.001$).

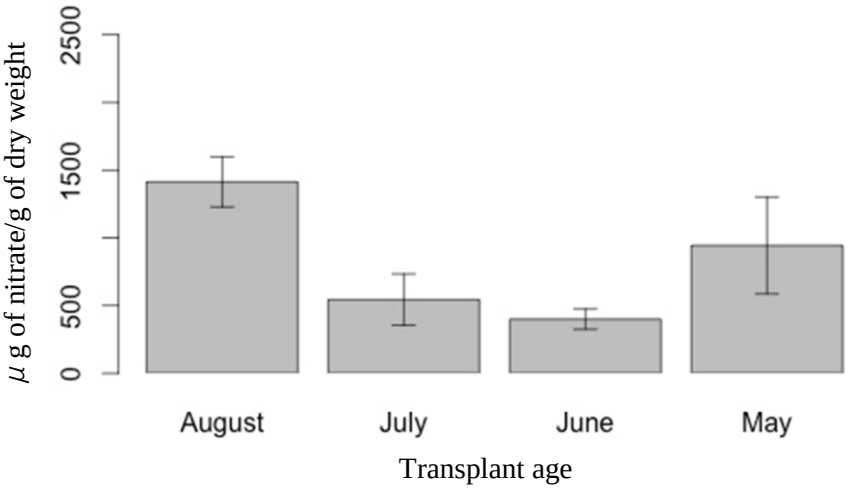


Figure 3.17 Mean soluble nitrogen concentration (±stdev) in leaf material collected from May, June, July and August transplants in experiment 3.2.1.2 on 8th August 2013 which coincided with the time of the aphid population crash.

At the time leaf material was taken (8th August 2013), soluble nitrogen concentration varied among May, June, July and August transplants (ANOVA, F= 4.035, df= 3, $P<0.05$) (Figure 3.17). August had a significantly higher concentration of soluble nitrogen in petiole material than June transplants, 1413.409± 185.55 µg of nitrate/g of dry weight to 399.64± 75.02 µg of nitrate/g of dry weight (TukeyHSD, $P<0.05$). All other transplant comparisons were not significant. Aphid populations crashed regardless of physiological or temporal differences in plant age after their peak circa 30th July± 2 days 2013. Populations began to increase during mid to late September, but failed to reach the densities seen earlier in the season. Populations declined for a second time on June and July transplants at the beginning of October. Populations on May and August transplants continued to increase until the end of the experimental period (29th October).

Parasitoids (as evidenced by aphid mummies) and fungi were the most numerous natural enemies in the field (Figure 3.8), reaching peak populations on approximately 28th August and the 13th August respectively. The number of fungal-infected cadavers and aphid mummies peaked on the 5th September, reaching 34.09% and 89.32% of the total population on each sampling date, respectively.

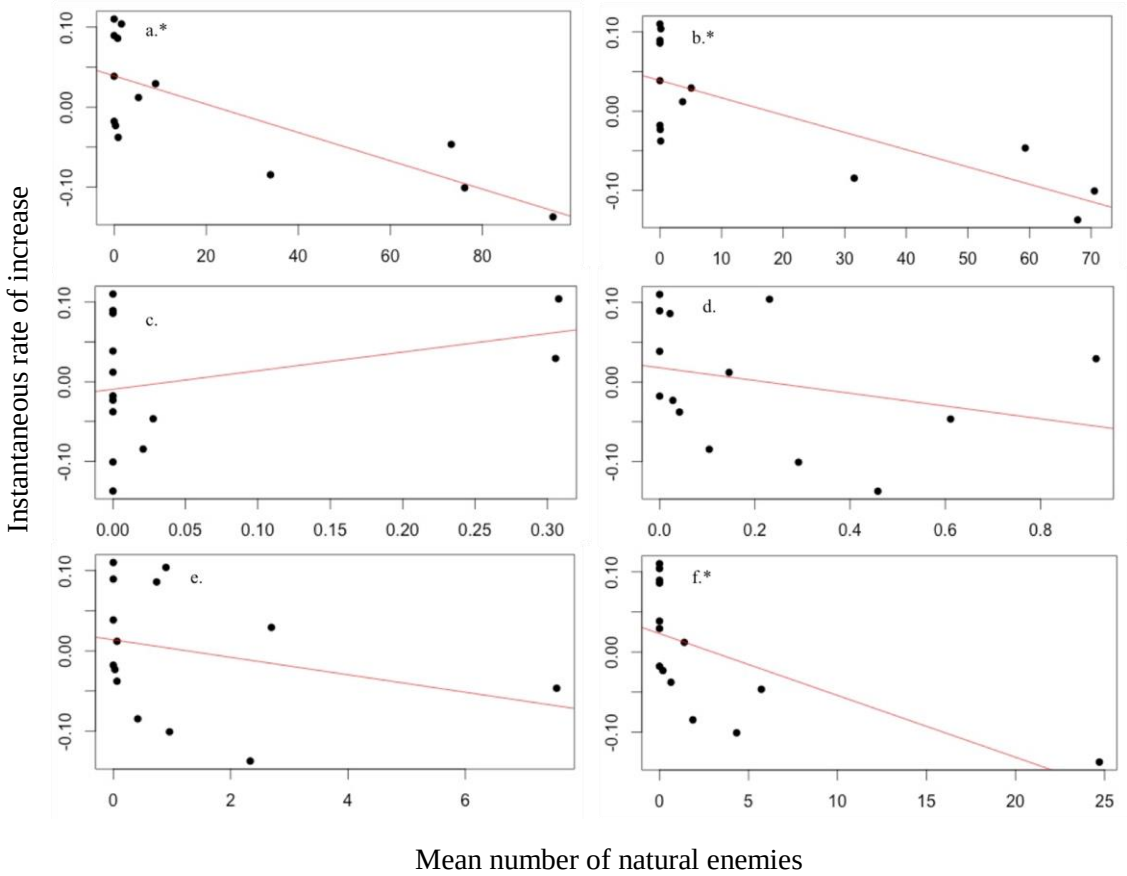


Figure 3.9 Instantaneous rate of increase against mean number of natural enemies, separated for (a) parasitoid mummy and fungal cadaver numbers, (b) Parasitoid mummies, (c) Anthocoridae, (d) Coccinellidae, (e) Syrphidae and (f) fungal cadavers in experiment 3.2.1.2. Red lines indicate linear models and '*' indicate significant fits.

The lowest values of instantaneous rate of increase for *B. brassicae* occurred when the density of fungal-infected cadavers and parasitoid mummies was highest (Figure 3.9a.). Regression analysis indicated a significant negative linear relationship between the number of fungal cadavers combined with the number of parasitoid mummies and the ability of *B. brassicae* populations to increase (r^2= 0.5525, P= 0.01398) (Figure 3.9a.). Further regression analysis on

the separate components of the natural enemy guild revealed that a significant negative relationship was mirrored with the parasitoid mummies alone (r^2= 0.5569, P= 0.00131) (Figure 3.9b.) and fungal natural enemies alone (r^2= 0.3613, P= 0.0136) (Figure 3.9f.). There was no significant relationship for Anthocoridae (r^2= 0.03129, P= 0.256) (Figure 3.9c.), Coccinellidae (r^2= 0.003974, P= 0.325 (Figure 3.9d.) or Syrphidae (r^2= 0.002857, P= 0.329) (Figure 3.9e.).

Weather 2013

Humidity, temperature and rainfall data were obtained from the University of Warwick, Wellesbourne, UK meteorological station. Figure 3.18 shows the mean daily minimum and maximum temperatures (°C) and rainfall (mm) for the duration of experiments 3.2.1.1 and 3.2.1.2 in 2013. The highest mean monthly temperature was 25.28°C in July and the lowest was 6.12°C in May. The highest mean monthly rainfall occurred in May (2.02mm) and the least in June (1.07mm).

Figure 3.19 shows mean relative humidity (%) data for the same period. Mean relative humidity steadily increased from 75.81% in May 2013 to 81.97% in September 2013 (Table 3.7).

Table 3.7 Mean monthly relative humidity (%) for 2013 experimental period

Month (2014)	Mean relative humidity (%)
May	75.81
June	76.9
July	71.55
August	75.48
September	81.97
October	87.03

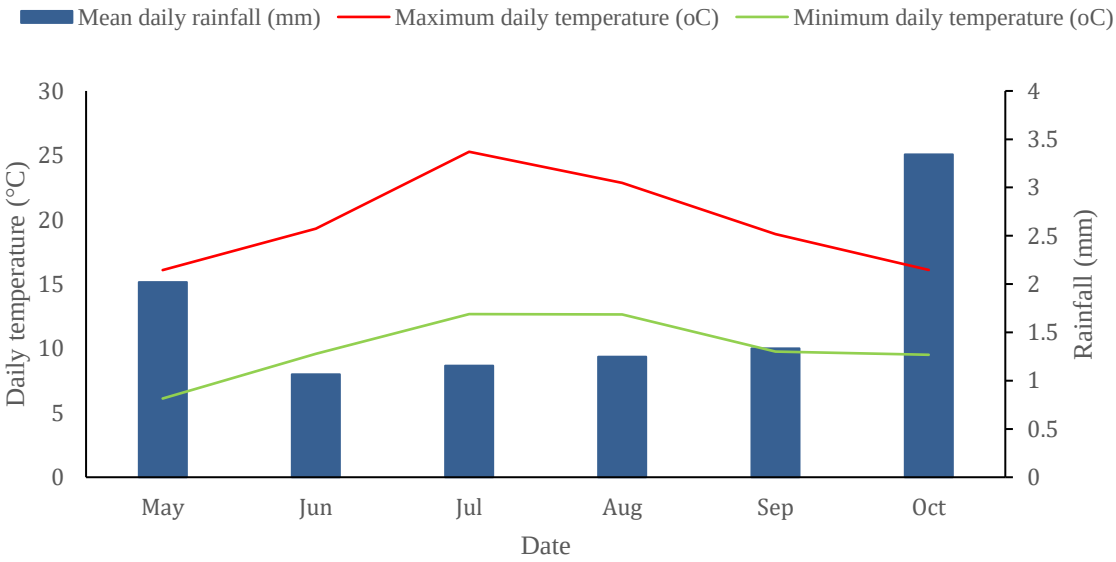


Figure 3.18 Daily maximum and minimum temperatures and daily rainfall recorded in 2013 for the duration of experiments 3.2.1.1 and 3.2.1.2.

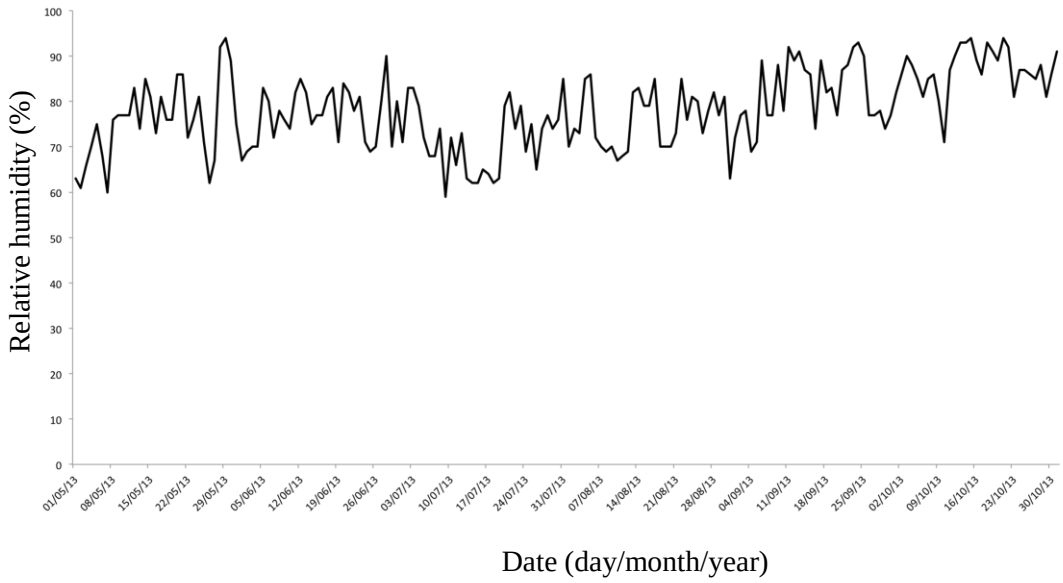


Figure 3.19 Daily relative humidity (%) in 2013 for the duration of experiments 3.2.1.1 and 3.2.1.2.

3.3.3 Population development of *B. brassicae* and its natural enemies on sequentially-transplanted, spatially separated host plants (2014)

Aphid populations crashed at the same time regardless of plant age or location at the end of September/beginning of October (c.5th October 2014). This occurred after populations peaked on (approximately) September 26th, 2014. This differed notably from 2013 in that it occurred much later in the year (Figure 3.14 a.1, b.1, c.1 & d.1). Populations subsequently remained low until the end of the experiment with a slight increase in populations on July-planted plots at the beginning of November.

Transplant date had a significant effect on the size of aphid populations (ANOVA, F= 22.155, df= 3, *P*<0.001) and populations varied between locations (ANOVA, F= 11.380, df= 3, *P*<0.001). However, there was no significant interaction between transplant date and location (ANOVA, F= 1.789, df= 9, *P*=0.0666). Further analysis showed that all transplants were different from each other with the exception of May and June (Tukey's HSD, P= 0.7801451) (Table 3.3) and LC varied from PG1, PG2 and LMW but no other differences were found between any of the other locations (Table 3.4).

Table 3.3 Post hoc Tukey HSD analysis showing significant differences in aphid populations between transplant dates in experiment 3.2.2

Transplant comparison	***P***
August- July	= 0.002
August- June	<0.0001
August- May	<0.0001
June- July	= 0.036
May- July	= 0.007
May- June	= 0.780

Table 3.4 Post hoc Tukey HSD analysis showing significant differences in aphid populations between locations in experiment 3.2.2. Little Cherry 'LC', Pump Ground 1 'PG1', Pump Ground 2 'PG2' & Long Meadow West 'LMW'.

Location comparison	***P***
LC-LMW	<0.0001
LC-PG1	=0.017
LC-PG2	<0.0001
PG1-LMW	= 0.340
PG2-LMW	= 0.879
PG1-PG2	= 0.074

There were significant differences in the total number of natural enemies among the four locations (ANOVA, F= 3.978, df= 3, *P*= 0.00789). Of the four locations, only PG1 and LMW were significantly different from each other (TukeyHSD, *P*= 0.0081440). There were also significant differences between transplant dates (ANOVA, F= 31.195, df= 3, *P*< 0.001); August varied significantly from May, June and July whereas all other transplant dates did not differ significantly from each other (Table 3.5). Unlike aphid populations in 2014 there was a significant interaction between location and transplant date for natural enemy communities (ANOVA, F= 5.686, df= 3, *P*<0.001), with significantly more natural enemies in PG1 on August and July transplants. (Table 3.6).

Peak parasitism rates for both parasitoid wasps and entomopathogenic fungi were achieved within 11 days of each other in all locations (Figure 3.14). Fungal parasitism peaked in locations LMW, PG1 and PG2 on the 28th October 2014 reaching 37.03%, 85.05% and 82.66% respectively. LC reached peak fungal parasitism rate (31.95%) on the 17th October 2014. Parasitoid mummies were most numerous on the 17th October 2014 for LC, LMW and PG2 reaching 16.8%, 14.50% and 20.68% respectively. PG1 parasitoid mummies peaked on the 28th October at 27.87%.

As in both 2013 field experiments, despite the differences in aphid population size, the changes in their relative size over time was similar across all locations and planting dates (Figure 3.14: a.1, b.1, c.1 & d.1.). In addition, natural enemy population dynamics mirrored that of the aphid population (Figure 3.14).

Table 3.5 Post hoc Tukey HSD analysis showing significant differences in natural enemy communities between transplant dates in experiment 3.2.2.

Transplant comparison	*P*
August- July	<0.0001
August- June	<0.0001
August- May	<0.0001
June- July	= 0.307
May- July	= 0.188
May- June	= 0.999

When considered individually, natural enemy groups did not vary with location; parasitoid mummies (Kruskal-Wallis, H= 2.0344, df= 3, *P*= 0.5653) (Figure 3.10), Coccinellidae (Kruskal-Wallis, H= 1.2775, df= 3, *P*= 0.7345) (Figure 3.11), Syrphidae (Kruskal-Wallis, H= 0.97318, df= 3, *P*= 0.8077) (Figure 3.12), and fungal cadavers (Kruskal-Wallis, H= 0.60437, df= 3, *P*= 0.8954) (Figure 3.13). Coccinellidae did not vary with transplant date (Kruskal-Wallis, H= 0.2222, df= 1, *P*= 0.6374). However, parasitoid mummies did (Kruskal-Wallis, H= 13.063, df= 3, *P*= 0.004502) as did Syrphidae (Kruskal-Wallis, H= 10.195, df= 3, *P*= 0.01698), but only between May and August transplants (Bonferroni-Dunn, *P*= 0.0029, Bonferroni-Dunn, *P*= 0.0018 for parasitoid mummies and syrphids respectively). Additionally, the incidence of fungal cadavers varied significantly with transplant date (Kruskal-Wallis, H= 90.27, df= 3, *P*<0.001). All transplant dates were significantly different from each other except between May and June (Bonferroni- Dunn, *P*= 0.24631). No Anthocoridae were found in any plots in experiment 3.2.2 so no further analyses were carried out

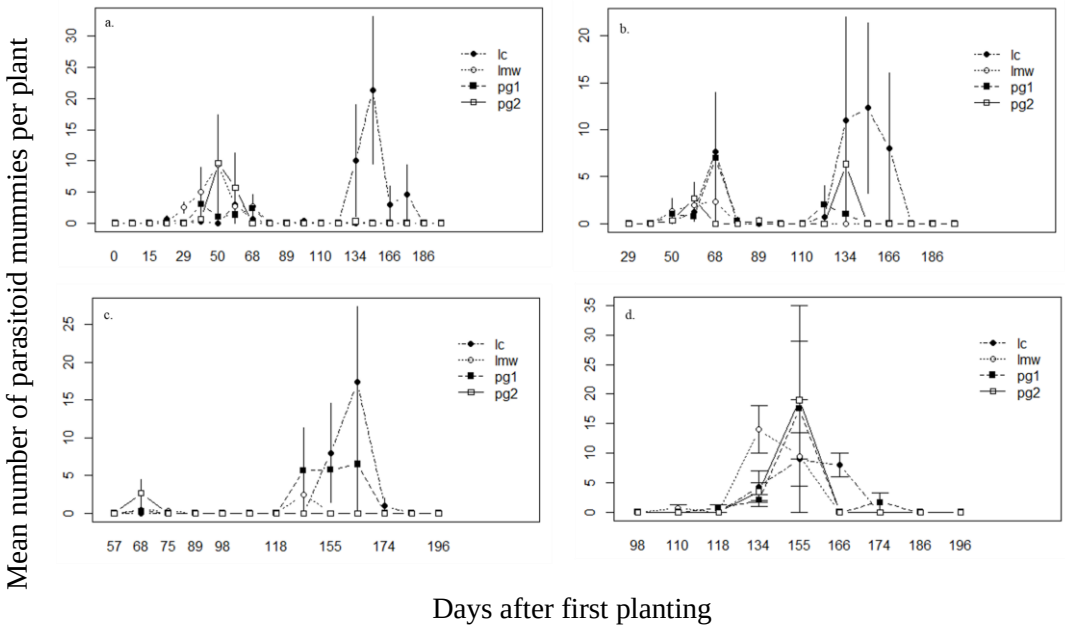


Figure 3.10 Changes in the mean number of parasitoid mummies per plant (±stdev) in each location for May (a.), June (b.), July (c.) and August (d.) transplants in experiment 3.2.2.

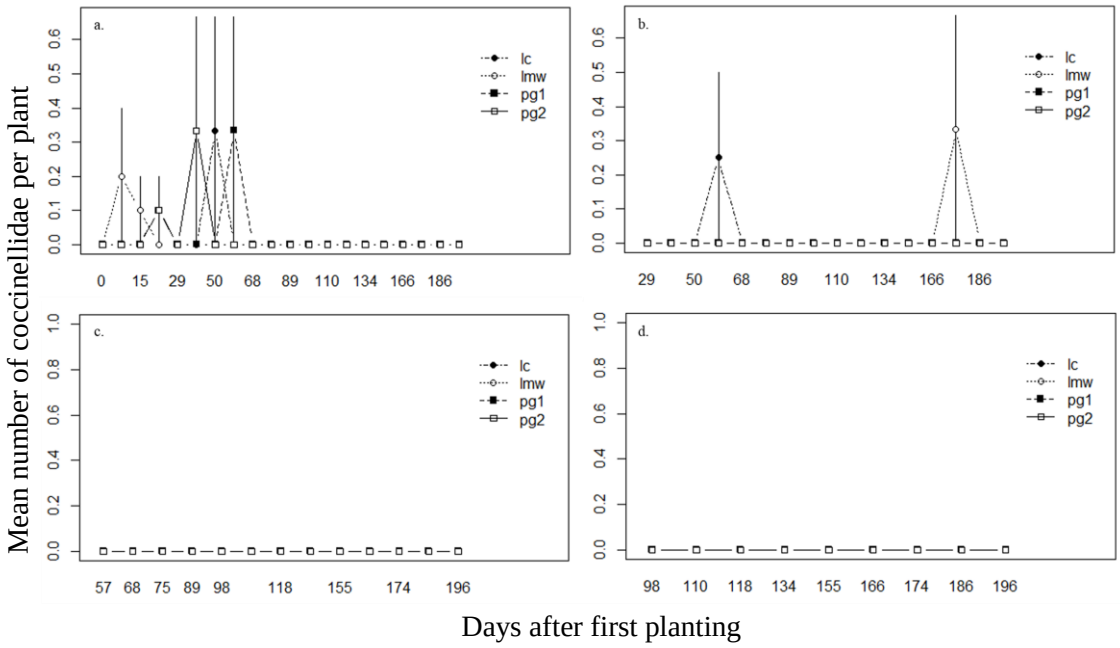


Figure 3.11 Changes in the mean number of Coccinellidae per plant (±stdev) in each location for May (a.), June (b.), July (c.) and August (d.) transplants in experiment 3.2.2.

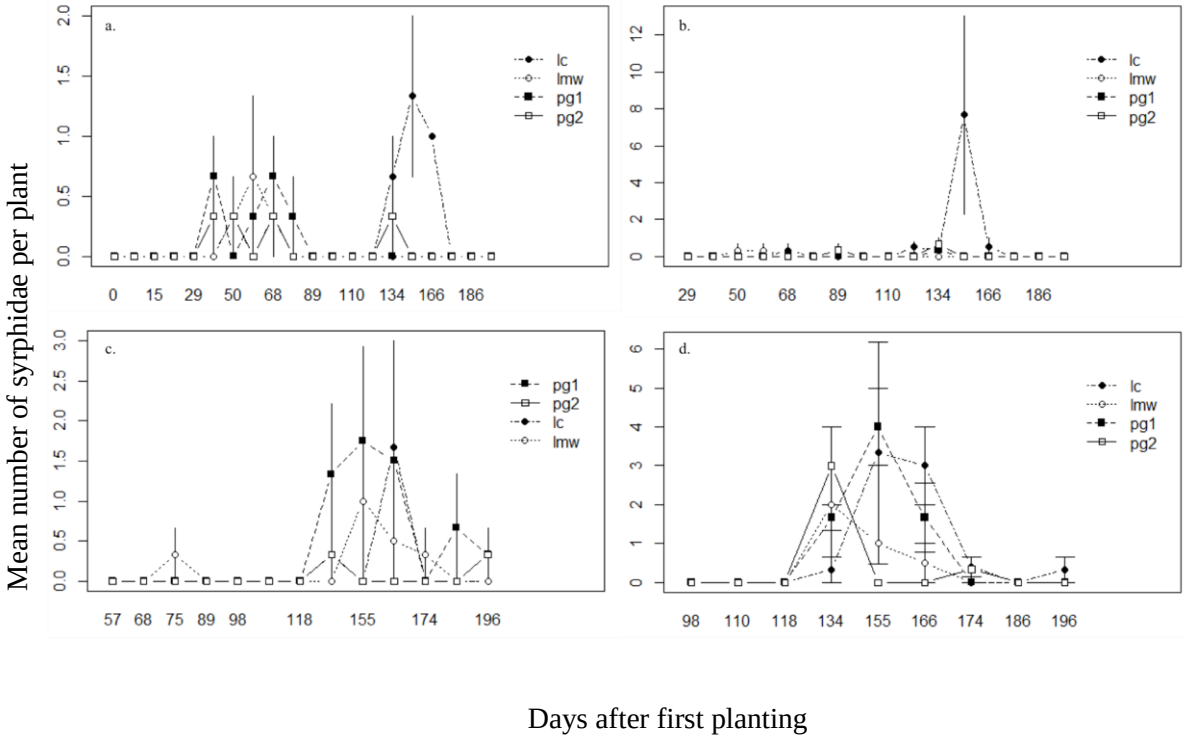


Figure 3.12 Changes in the mean number of Syrphidae per plant (±stdev) in each location for May (a.), June (b.), July (c.) and August (d.) transplants in experiment 3.2.2.

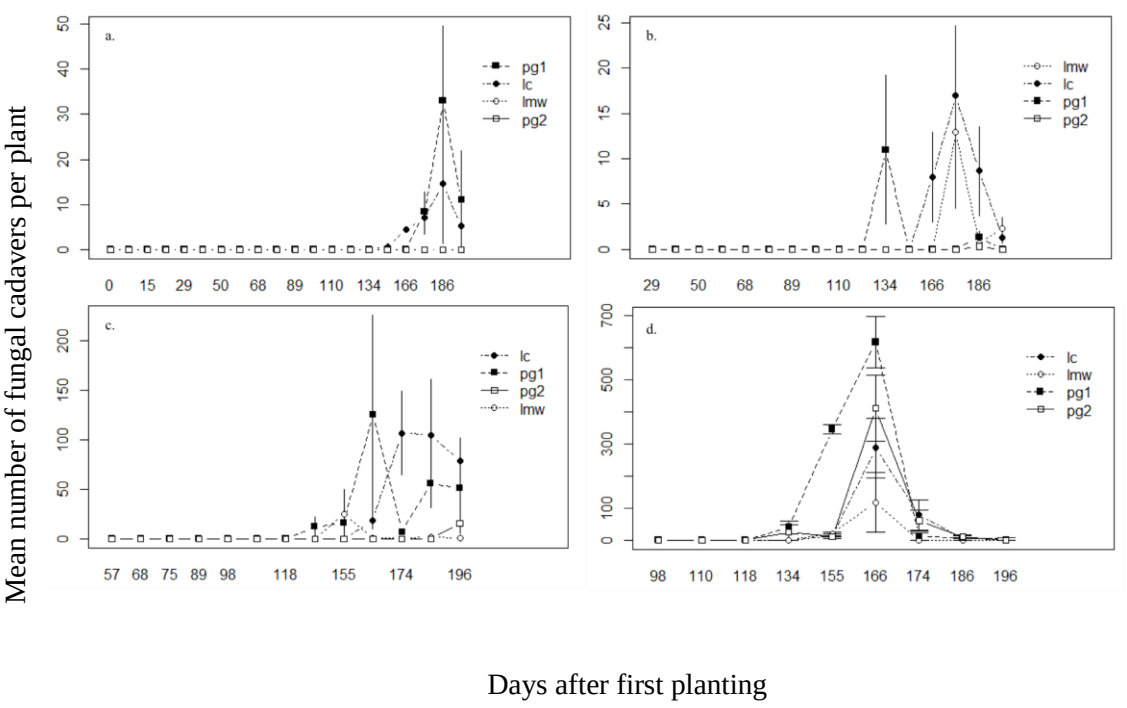


Figure 3.13 Changes in the mean number of fungal cadavers per plant (±stdev) in each location for May (a.), June (b.), July (c.) and August (d.) transplants in experiment 3.2.2.

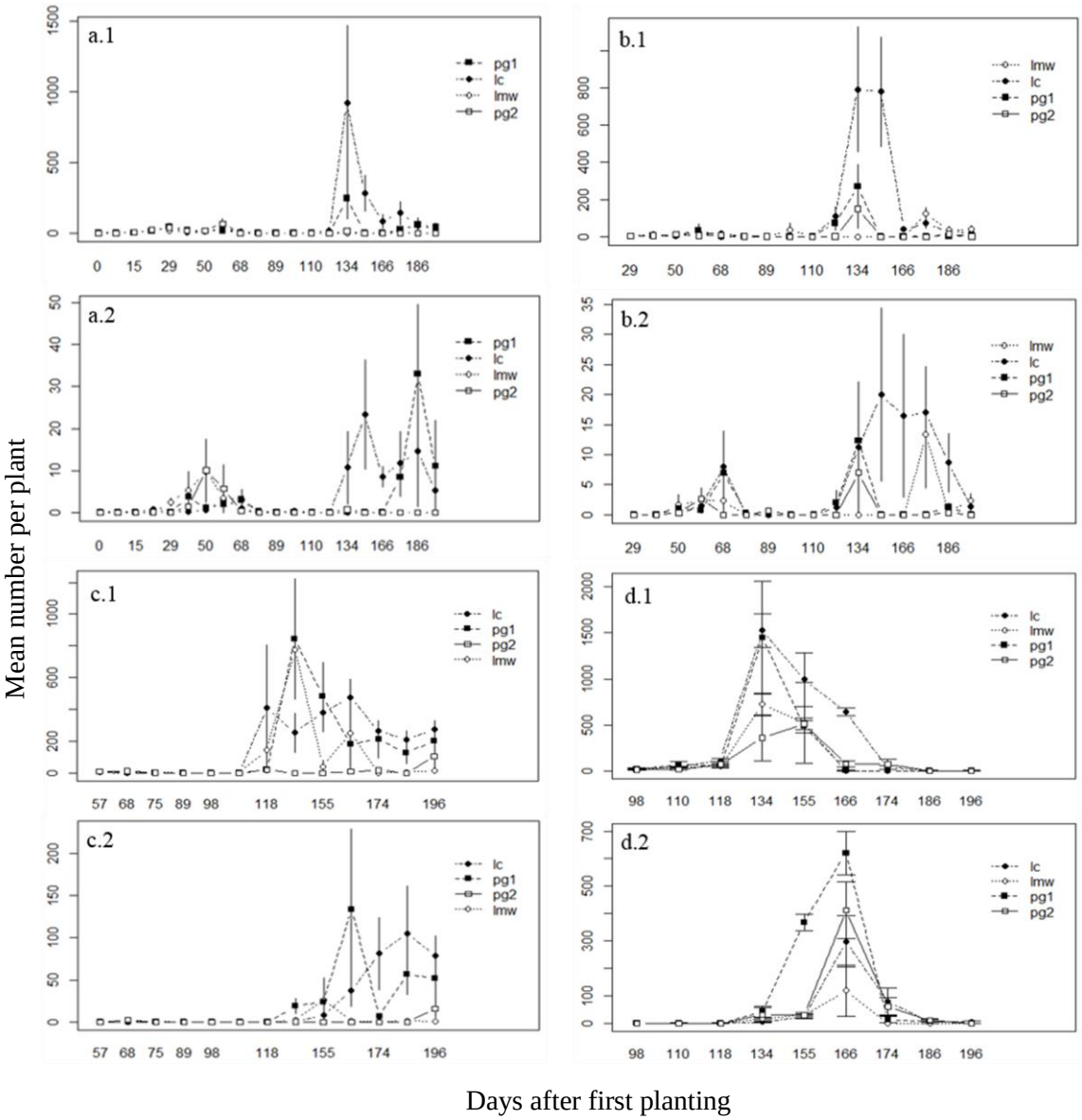


Figure 3.14 Changes in the mean number of aphids and natural enemies per plant (±stdev) in experiment 3.2.2 in each location; Little Cherry 'LC', Pump Ground 1 'PG1', Pump Ground 2 'PG2' & Long Meadow West 'LMW', grouped for transplant date. (a.1) *Brevicoryne brassicae* population May; (a.2) natural enemy population May, (b.1) *B. brassicae* population June; (b.2) natural enemy population June, (c.1) *B. brassicae* population July; (c.2) natural enemy population July & (d.1) *B. brassicae* population August; (d.2) natural enemy population August.

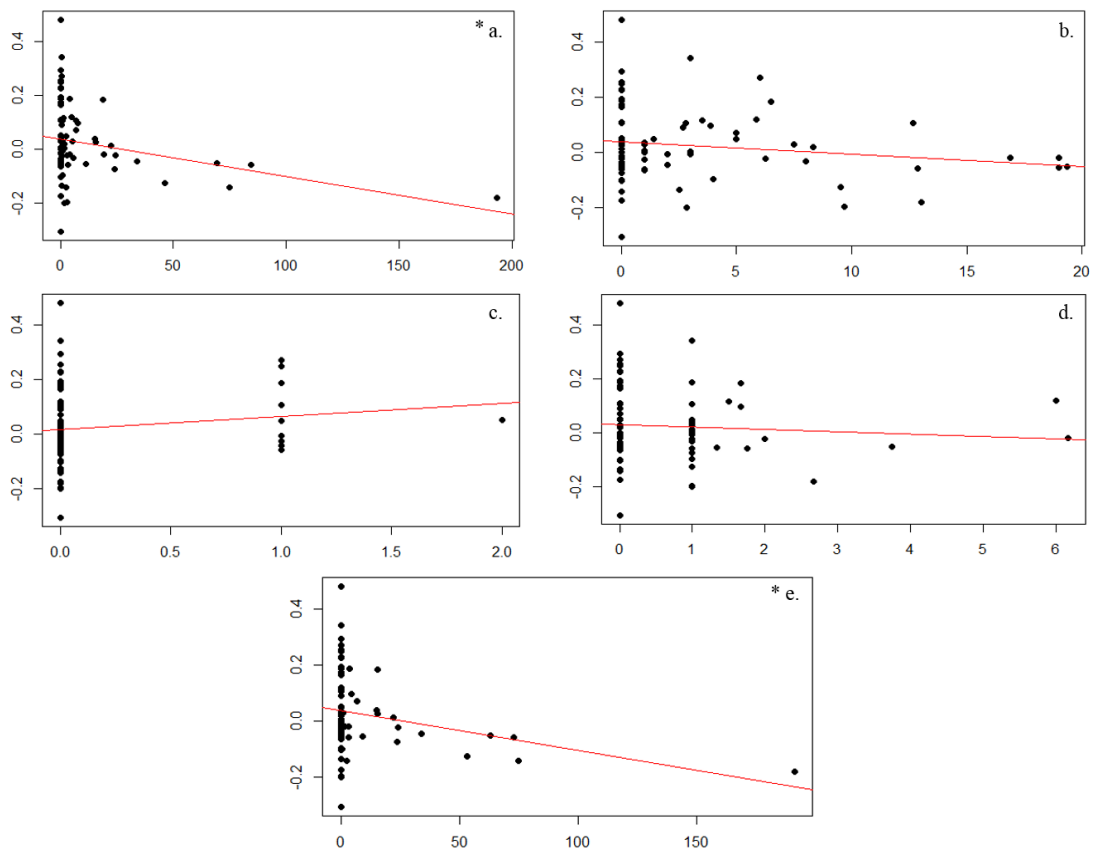


Figure 3.15 Instantaneous rate of aphid population increase against mean number of natural enemies, separated for (a) parasitoid mummies and fungal cadaver numbers (b) parasitoid mummies, (c) Coccinellidae, (d) Syrphidae and (e) fungal cadavers in experiment 3.2.2. Red lines indicate linear models and '*' indicate significant fits.

Regression analysis confirmed a significant negative linear relationship between the number of parasitoid mummies and fungal-infected cadavers and the ability of the aphid population to increase, however the adjusted r^2 value was very low (r^2= 0.06431, P= 0.01538) (Figure 3.15a.). Further regression analysis on the separate components of the natural enemy guild revealed that a significant negative relationship was mirrored with the fungal-infected cadavers alone (r^2= 0.06434, P= 0.01536) (Figure 3.15e.), although the adjusted r^2 value was again very low. However, there was no significant relationship for parasitoids (r^2= -

0.00758, P= 0.5112) (Figure 3.15b.), Coccinellidae (r^2= 0.005957, P= 0.2324 (Figure 3.15c.) or Syrphidae (r^2= 0.002857, P= 0.329) (Figure 3.15d.).

Weather 2014

Humidity, temperature and rainfall data were obtained from the University of Warwick, Wellesbourne, UK meteorological station. Figure 3.20 shows the mean daily minimum and maximum temperatures (°C) and rainfall (mm) for the duration of experiment 3.2.2 in 2014. The highest mean monthly temperature was 24.23°C in July and the lowest was 5.54°C in November. The highest mean monthly rainfall occurred in June (2.73mm) and the least in September (0.4mm).

Figure 3.21 shows mean relative humidity (%) data for the duration of experiment 3.2.2. Mean relative humidity steadily increased from 75.26% in July to 92.1% in November (Table 3.8).

Table 3.8 Mean monthly relative humidity (%) for 2014 experimental period

Month (2014)	**Mean relative humidity (%)**
May	80.58
June	78.7
July	75.26
August	78.52
September	81.7
October	86.42
November	92.1

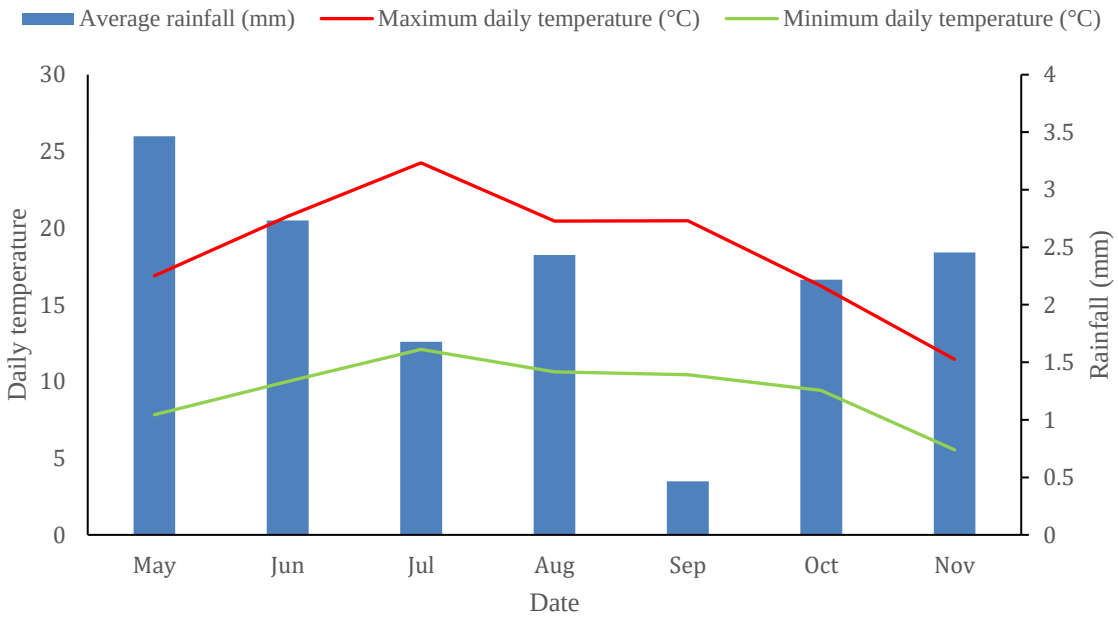


Figure 3.20 Daily maximum and minimum temperatures and daily rainfall recorded in 2014 for the duration on experiment 3.2.2.

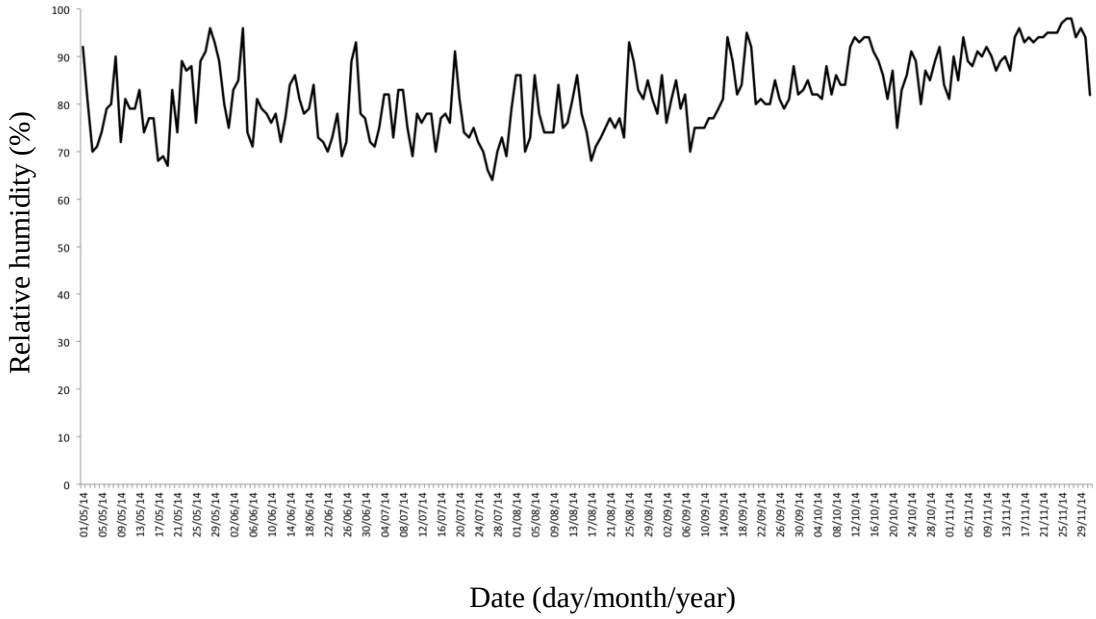


Figure 3.21 Relative humidity (%) data from Wellesbourne airport weather station (circa 1 mile from experimental site) in 2014 for the duration of experiment 3.2.2.

3.3.4 Identification of fungal natural enemies

The genus specific ITS 1 primer set PnCNf/PnCNr (*Pandora neoaphidis*) was compatible with freeze dried mycelium, similar to the results of Fekih *et al*. (2013) (Figure 3.22). BLAST similarity searches of nucleotide sequences in Genbank showed that the WEL1 isolate showed 98 – 100 % homology with all *P. neoaphidis* strains. The lowest sequence similarity was seen between *Pandora delphacis* AF368521.1 and *P. neoaphidis* isolate NW343 (AF543202.1) at 36.6% with a divergence of 130.7. The most similar *P. neoaphidis* isolates to WEL1 were HQ677587.1, EU267189.1, AF543210.1, AF543209.1, AF543208.1, AF543204.1 and AF543203.1 at 99.6%. None of which were shared with *P. neoaphidis* isolate

NW420, whose closest sequence matches were EU267192.1, AF543211.1, AF543206.1 and AF543207.1 at 99.4% (Figure 3.23 bottom).

A consensus tree based on the ITS1 sequence data was constructed with a bootstrap value of 100% supporting the separation of *P. neoaphidis* from out-groups: *P. delphacis* and *P. nouryi*. *Pandora neoaphidis*, *P. delphacis* and *P. nouryi* are clearly separated into three distinct groups. Tree topology within the *P. neoaphidis* group was supported by bootstrap values of >60% with WEL1 and NW420 within the *P. neoaphidis* clade, albeit separated slightly (Figure 3.23 top). The grouping of WEL1 within the *P. neopahidis* clade is supported by sequence similarity data.

(c.) (a.) (a.) (b.)

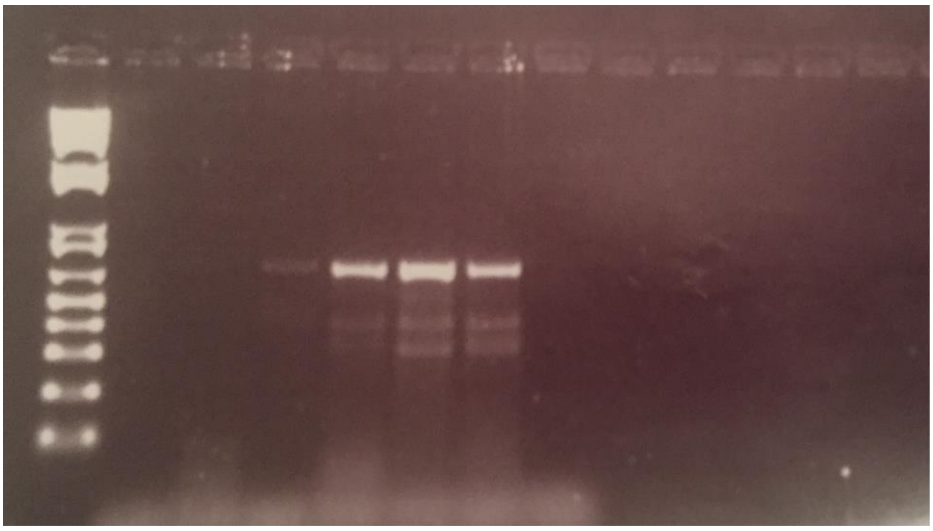


Figure 3.22 Picture of ITS1 photo Amplification of ITS1 region using *Pandora* primers PnCNf/PnCNr using DNA extraction from freeze dried mycelium initially grown on Grace's liquid insect cell culture medium. Letter represent isolates (a.) WELL1 (b.) NW420 (c.) water

Percentage identity (%)

Divergenc

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
1		99.5	99.5	99.9	99.7	99.7	99.5	99.5	99.5	99.7	98.7	99.5	99.5	99.7	99.5	36.6	63.0	99.1	99.1	**1**
2	0.5		100.0	99.6	99.7	99.7	100.0	100.0	100.0	99.7	99.2	100.0	99.5	99.7	100.0	36.7	63.3	99.6	99.1	**2**
3	0.5	0.0		99.6	99.7	99.7	100.0	100.0	100.0	99.7	99.2	100.0	99.5	99.7	100.0	36.7	63.3	99.6	99.1	**3**
4	0.1	0.4	0.4		99.9	99.9	99.6	99.6	99.6	99.9	98.8	99.6	99.6	99.9	99.6	36.6	63.1	99.2	99.2	**4**
5	0.3	0.3	0.3	0.1		100.0	99.7	99.7	99.7	100.0	99.0	99.7	99.7	100.0	99.7	36.7	63.3	99.4	99.4	**5**
6	0.3	0.3	0.3	0.1	0.0		99.7	99.7	99.7	100.0	99.0	99.7	99.7	100.0	99.7	36.7	63.3	99.4	99.4	**6**
7	0.5	0.0	0.0	0.4	0.3	0.3		100.0	100.0	99.7	99.2	100.0	99.5	99.7	100.0	36.7	63.3	99.6	99.1	**7**
8	0.5	0.0	0.0	0.4	0.3	0.3	0.0		100.0	99.7	99.2	100.0	99.5	99.7	100.0	36.7	63.3	99.6	99.1	**8**
9	0.5	0.0	0.0	0.4	0.3	0.3	0.0	0.0		99.7	99.2	100.0	99.5	99.7	100.0	36.7	63.3	99.6	99.1	**9**
10	0.3	0.3	0.3	0.1	0.0	0.0	0.3	0.3	0.3		99.0	99.7	99.7	100.0	99.7	36.7	63.3	99.4	99.4	**10**
11	0.8	0.3	0.3	0.7	0.5	0.5	0.3	0.3	0.3	0.5		99.2	98.7	99.0	99.2	36.7	63.5	98.8	98.3	**11**
12	0.5	0.0	0.0	0.4	0.3	0.3	0.0	0.0	0.0	0.3	0.3		99.5	99.7	100.0	36.7	63.3	99.6	99.1	**12**
13	0.5	0.5	0.5	0.4	0.3	0.3	0.5	0.5	0.5	0.3	0.8	0.5		99.7	99.5	36.7	63.0	99.1	99.1	**13**
14	0.3	0.3	0.3	0.1	0.0	0.0	0.3	0.3	0.3	0.0	0.5	0.3	0.3		99.7	36.7	63.3	99.4	99.4	**14**
15	0.5	0.0	0.0	0.4	0.3	0.3	0.0	0.0	0.0	0.3	0.3	0.0	0.5	0.3		36.7	63.3	99.6	99.1	**15**
16	130.7	129.8	129.8	130.5	129.8	129.8	129.8	129.8	129.8	129.8	128.8	129.8	129.6	129.8	129.8		42.9	36.7	36.5	**16**
17	44.0	43.5	43.5	43.8	43.5	43.5	43.5	43.5	43.5	43.5	42.4	43.5	44.0	43.5	43.5	96.8		63.5	62.6	**17**
18	0.9	0.4	0.4	0.8	0.7	0.7	0.4	0.4	0.4	0.7	0.7	0.4	0.9	0.7	0.4	130.0	43.0		98.7	**18**
19	0.3	0.3	0.3	0.1	0.0	0.0	0.3	0.3	0.3	0.0	0.5	0.3	0.3	0.0	0.3	130.5	43.9	0.7		**19**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	

AF543202.1|Pandora_neoaphidis_NW343
AF543203.1|Pandora_neoaphidis_NW356
AF543204.1|Pandora_neoaphidis_NW195
AF543205.1|Pandora_neoaphidis_NW283
AF543206.1|Pandora_neoaphidis_NW316
AF543207.1|Pandora_neoaphidis_NW327
AF543208.1|Pandora_neoaphidis_NW415
AF543209.1|Pandora_neoaphidis_ARSEF835
AF543210.1|Pandora_neoaphidis_ARSEF1609
AF543211.1|Pandora_neoaphidis_ARSEF5374
EU267188.1|Pandora_neoaphidis_ARSEF1607
EU267189.1|Pandora_neoaphidis_ARSEF7937
EU267190.1|Pandora_neoaphidis_ARSEF7938
EU267192.1|Pandora_neoaphidis_ARSEF7939
HQ677587.1|Pandora_neoaphidis
AF368521.1|Pandora_delphacis
HQ677590.1|Pandora_nouryi_ARSEF366
WELL1_Pandora_neoaphidis_Wellesbourne
NW420_Pandora_neoaphidis_Rothamsted

Figure 3.23 Top: Consensus phylogenetic tree obtained from ITS1 sequence data using pairwise ClustalW analysis. Results are displayed as a phylogram from DNASTAR, MegAlign©. Bootstrap values are shown at nodes. Circled are isolate WEL1 obtained from infected *Brevicoryne brassicae* at Wellesbourne in 2013 and NW420 obtained from ARSEF culture collection (USA), original host *B. brassicae*, which was used in further experiments for comparison.

Bottom: Percentage divergence and similarity of WEL1 and NW420 ITS1 sequence data against *Pandora neoaphidis*, *Pandora delphacis* and *Pandora nouryi* sequences data obtained from Genbank.

3.3.5 Identification of arthropod natural enemies

The natural enemy guild consisted of a range of arthropods. However, characterization down to species level was only conducted for parasitoid wasps, coccinellids and syrphids. Parasitoid mummies identified from field collections in June of 2013 were *Praon volucre*. However, a number of hyper-parasitoids were also found and identified to *Alloxysta brevis.* Syrphid larvae collected and raised to adulthood were identified as *Episyrphus balteatus*. Coccinellidae species seen in field experiments included *Chilocorus renipustulatus, Coccinella 5-punctata and Coccinella 7-punctata* with *Harmonia axyridis* being the most numerous in 2013.

3.4 DISCUSSION

Accurate prediction of the aphid population crash or the factors, that cause them, notably epizootics, could inform novel IPM strategies and avoid unnecessary pesticide applications (Hollingsworth *et al*., 1995).

The aphid population crash occurred much later in 2014 than in 2013, c.30th July 2013 and c.5th October 2014. Climatic conditions varied greatly between the two years at the time of the population crash. During October 2014, nearly twice as much rainfall was recorded than in July 2013, 2.219 mm and 1.15 mm respectively. Additionally, the mean monthly temperature for October 2014 was 12°C and for July 2013 was 25.28°C (the hottest month of the season in 2013). Such differences in the abiotic conditions between the 2013 and 2014 crash periods suggest that the timing of the crash is less reliant on the weather and more on aphid population density and the presence of natural enemies. Abiotic conditions merely need to be permissible (not exceed cardinal limits). Regarding the fungal epizootic, infection could theoretically occur over night (see section 4). The 'mid-season' aphid population crash appears to be a point of collapse at approximately the same time every year as a function of the growth of aphid populations over time and the action of natural enemies. However, if aphid population growth is reduced or natural enemy populations are low, potentially because of reduced aphid population growth, there is no reason to expect the population crash to occur at the same time of year.

Data presented here suggest that the number of natural enemies is related to the size of aphid populations, increasing in line with increasing aphid number. That there was a general trend (non-significant) for a greater abundance of natural enemies on plots containing older plants could be function of the size of the plants

supporting larger aphid populations, rather than a function of plant age. It is difficult to manipulate the timing of the crash to occur earlier in this situation, as vast quantities of natural enemies used as augmentation biological control agents would be required to induce an aphid population crash. It is possible to calculate the additional number of natural enemies needed to reduce aphid populations from linear regression of instantaneous rate of increase and the number of natural enemies (figure 3.9 & 3.15). Regarding, *P. neoaphidis*, point source infections must proliferate within the aphid population until infection reaches a threshold where it can form an epizootic (Hollingsworth *et al*. 1995). At which time the crash occurs over a matter of days, c.7-10 days. The epizootic only occurs when aphid populations reach a certain density, as observed by the different timings of the crashes in 2013 and 2014. At lower pest densities, lateral transmission of the pathogen; from host to host, will be reduced as the physical distances between individuals increases and chances of contacting an infected individual decreases. An epizootic is unlikely to occur as a result of the decline in transmission and a large amount of inoculum would be required to ensure the population crash occurred earlier in the season at lower pest density. Currently, *P. neoaphidis* cannot be massed produced on a large scale (Shah & Pell, 2003). For parasitoid wasps, the requirement for the release of large numbers as augmentative biocontrol agents is mitigated by the fact that individuals can actively search for hosts. However, parasitoids are also more likely to be attracted to patches in the landscape where suitable hosts are most abundant. Thus, large aphid populations are more likely to be attacked by parasitoids than smaller ones. The same is true of predators as this efficient searching behavior is an evolutionary strategy that maximizes reproductive success.

One example of where the aphid population crash is successfully exploited is in cotton against *Aphis gossypii* using *Neozygites fresenii* (Steinkraus *et al*., 1995; Steinkraus *et al*. 2002). The life cycles of *N. fresenii* and *P. neoaphidis* are more or less identical, initiated by germination of a spore on a suitable host, followed by development of infection structures which penetrate the host cuticle; the fungus then grows within the hemocoel eventually producing conidiophores from which primary conidia are actively discharged. Lessons learned from exploiting *Neozygites* could equally apply to *Pandora.* The pathogen *N. fresenii* causes cotton aphid populations to decline by 80% in approximately 5 days over vast areas. Similarly, *P. neoaphidis* in this study reduced aphid populations by 80-90% in 7-10 days during both field seasons. Populations of *A. gossypii* were calculated to begin to decline when fungal prevalence reached 15% of the aphid population (Hollingsworth *et al*., 1995). Average prevalence of *P. neoaphidis* around the timing of the crash was 12% in 2013 and 7% in 2014. This appears to be lower than the infection threshold required to induce a population crash according to Hollingsworth *et al.* (1995). The two cropping systems; cotton and brassica, are vastly different in their structure and growing regime meaning a 15% threshold may not applicable to brassica systems where the crop canopy is much denser. However, the present study reported a guild of aphidophagous natural enemies and when the prevalence of parasitoid mummies is also considered a parasitism threshold of 47% and 19% is reached in 2013 and 2014 respectively. Theoretically, enough to cause a population crash. Theoretical, because this is based on the assumption that the relationship between entomopathogenic fungi and parasitoid wasps is additive. The shortened developmental period of *P. neoaphidis* means the pathogen would outcompete parasitoid wasps. Therefore,

parasitoid wasps would increase their fitness by avoiding colonies or individuals infected with *P. neoaphidis*. This could increase control by minimizing antagonistic interactions between biological control agents. However, *Aphidus ervi*, a common natural enemy sharing aphids as an extraguild host, is only able to detect the presence of *P. neoaphidis* once aphid hosts are dead and sporulating (Baverstock *et al*., 2005). Additive interactions can occur as Coccinellidae predators have shown avoidance behavior of individuals, colonies and surfaces contaminated with entomopathogenic fungi. (Ormond *et al*., 2011). This highlights the importance of considering the entire guild of natural enemies in a biological control program.

The 2013 and 2014 field seasons saw the establishment of a fungal epizootic around the timing of the crash as in 2013. This fungus was successfully isolated and through DNA identification been confirmed as *P. neoaphidis*. Contrary to Diaz et al. (2010), who also monitored aphids and a guild of natural enemies over time, *P. neoaphidis* arrived much later than other more mobile natural enemies. Infected insect cadavers were observed after other aphidophagous natural enemies in both 2013 and 2014. Fungus infected cadavers were seen 3-4 weeks after the first parasitoid mummies were observed and approximately 10 days after the first appearance of coccinellid predators in 2013. Once infection was seen, the epizootic developed over the course of c.7-10 days suggesting once infection was initiated; from a point source, it can spread rapidly through a suitably dense aphid population. Feng *et al*. (2007) proposed adult alate dispersal flights were responsible for the widespread dissemination of entomopathogenic fungi, including *P. neoaphidis.* In both 2013 and 2014 infected aphids were seen a long time after the appearance of alate adults, in fact dispersive morphs were seen

throughout the majority of the field season, and aphid populations in 2013 had been established for a significant amount of time prior to infection. Resting spores have been suggested as background sources of primary infection and resting spores have recently been identified for *P. neoaphidis* (Scorsetti *et al*., 2012); however, suitable abiotic conditions and suitable insect hosts were available prior to the appearance of the epizootic in the field suggesting a foreign point source of primary inoculum. Indeed, Roy *et al*. (2001) found that coccinellids can vector entomopathogenic fungi from infected to uninfected populations of pea aphid in the lab. Additionally, the co-occurrence of *Inachis io, Aphidius ervi* and *Aphidius rhopalosiphi* in lab experiments have been shown to enhance the transmission of entomopathogenic fungi (Baverstock *et al*., 2008). Scaled up, microcosm experiments conducted in poly tunnels also report that the presence of predators and parasitoids enhanced the transmission of *P. neoaphidis* (Baverstock, *et al*., 2009). Field observations during this study suggest that contaminated predators and parasitoids play a more substantial role in pathogen dissemination than previously thought (Feng *et al*., 2007). However, despite suitable hosts being available prior to the crash perhaps they were not available in sufficient number as to produce an epizootic. Host density also plays a vital role in the initiation of the timing of the epizootic, as it is functional tied to disease transmission.

Many aphids show aggregation behavior on the leave surface. *Brevicoryne brassicae* in particular, has a tendency to form dense clusters of populations on the leaf. Conidia of *P. neopahidis*, actively discharged from an individual within a densely populated, highly aggregated population will inevitably land on another suitable host, continuing the infection cycle and rapidly proliferating through the entire cluster and ultimately the population. In bioassays with *P. neoaphidis* the

fungus produced up to 111 conidia/mm^2 in 55 minutes (section 4). Additionally, mortality appeared to be independent of dose, meaning a small number of conidia was sufficient to cause mortality (Section 4). The rapid nature of the spread of disease, expedited by densely packed susceptible hosts, would result in the precipitous decline of an aphid population. This was observed in the field studies carried out on *B. brassicae* in 2013 and 2014, suggesting that entomopathogenic fungi are a casual factor in their decline.

The hypothesis tested during 2013 and 2014 field trials was that natural enemies, specifically naturally occurring entomopathogenic fungi, play a significant role in regulating aphid populations. That is to say that when aphid numbers decline they do so in conjunction with increasing natural enemy numbers.

Older transplants in Experiment 3.2.1.1 had significantly larger aphid populations than younger transplants. However, the population dynamics were generally similar on both age groups. Correspondingly, old transplants had a slightly greater number of natural enemies in all cases. After approximately 2 weeks in the field young and old transplants were of the same BBCH growth stage creating a homogenous crop explaining the non-significant difference between soluble nitrogen content in plant samples taken from each treatment. Plant age/soluble nitrogen affects *B. brassicae* fecundity and mortality in the lab but this relationship is not seen in the field. Aphids are capable of telescoping generations; mothers are born with daughters developing inside. Host plants mothers are raised on will precondition the developing aphid within. Laboratory experiments should mitigate these transgenerational effects, by raising individuals on experimental plants for successive generations prior to recording plant effects on aphid performance (Wang *et al*. 2017). In this study plant, physiological age and soluble

nitrogen were used as proxy for host quality. Nitrogen is a limiting factor for aphids (Karley *et al*. 2003; Karley *et al*. 2004) and this element is affected by plant age (Van Emden & Bashford, 1969; Mattson, 1980; Minkenberg & Ottenhein, 1990). Plant age has been shown to affect aphid population dynamics (Karley *et al*. 2003; Karley *et al*. 2004); however, in this study the decline in aphid populations occurred irrespective of plant age in all experiments. Moreover, in experiment 3.2.1.2 there were significant differences in the soluble nitrogen content of plant samples, yet despite these differences aphid populations declined at the same time. In 2014 aphid populations declined despite physiological differences among transplants indicated by BBCH growth stage.

Experiment 3.2.1.2 had the greatest number of natural enemies perhaps due to proximity of all plants making an aphid reservoir. The fastest reacting natural enemies appeared to be the Coccinellidae and Syrphidae as they had the shortest lag between peak aphid numbers and their peak numbers. The longer reaction times seen in the occurrence of fungal disease and parasitoid mummies are indicative of the incubation period of each natural enemy rather than a slow response to increasing aphid number. Infected aphids took time to show visible symptoms and therefore recordable effects from these natural enemies.

Investigating the role of natural enemies in the aphid population crash is difficult as data do not definitively highlight a cause and effect. The data represent counts and associations between aphid and natural enemy populations. Whilst correlations and relationships can be inferred from such data, one experiment remains to be conducted, an exclusion experiment. Excluding parasitoids and other arthropod natural enemies is straightforward, i.e growing plants in cages, but this

method alters the physical conditions within the cage so they do not directly reflect the physical conditions in the field, and they may not exclude fungal pathogens; primarily fungal pathogens that produce resting spores and are found in/on the soil. Conditions may be improved for entomopathogenic fungi in such experiments, as decreased air movement within the cage would elevate the humidity and temperature, which would favor fungal infection.

Natural enemy abundance is reported to be significantly negatively associated with instantaneous rate of increase of aphid populations suggesting an antagonistic relationship. Of the natural enemy guild in 2013, parasitoid mummies and fungal cadaver abundance were the only two natural enemies to produce a significant negative relationship with the aphid instantaneous rate of increase. Additionally, in 2014 fungal cadaver abundance was the only natural enemy reported to have a significant negative relationship with aphid instantaneous rate of increase. Such observations suggest that these natural enemies are the most important in reducing aphid numbers. Rapid aphid population decline occurred much later in the 2014 field season and this could explain why parasitoid mummy abundance was not significantly negatively correlated with aphid instantaneous rate of increase. Aphid numbers were not sufficient earlier in the season to support great populations of parasitoid wasps, thereby reducing their overall number during the field season. Once aphid numbers began to increase the entomopathogenic fungi; *P. neoaphidis*, was quick to establish further limiting the reproductive success of any parasitoid wasps that may have been active.

It is important to consider in the interpretation of these results that data collected for *P. neoaphidis* and parasitoids provide a measure of the prevalence of parasitism, a direct measure of the effect of these natural enemies on population

size. However, for predators, recorded data refers to a count of individuals not a functional response. Future studies should multiply the numbers of predators observed by the average number of aphids they are likely to consume to get a similar functional variable to pathogens and parasitoids. The relatively small number of predators reported here could have the same if not a greater impact on aphid population instantaneous rate then the parasites.

Table 3.6 Post hoc Tukey HSD analysis showing significant differences in natural enemy communities for each location and transplant date combination in experiment 3.2.2. Little Cherry 'LC', Pump Ground 1 'PG1', Pump Ground 2 'PG2' & Long Meadow West 'LMW', '*' marks significance at the 0.05 level.

Comparison (Location 1: Transplant 1-Location 2:Transplant 2)	***P***
lmw:Aug-lc:Aug	0.7356384
pg1:Aug-lc:Aug	0.0000363*
pg2:Aug-lc:Aug	1
lc:July-lc:Aug	0.9962525
lmw:July-lc:Aug	0.0756765
pg1:July-lc:Aug	0.6898033
pg2:July-lc:Aug	0.0619593
lc:June-lc:Aug	0.1069305
lmw:June-lc:Aug	0.0442593*
pg1:June-lc:Aug	0.0423718*
pg2:June-lc:Aug	0.0367656*
lc:May-lc:Aug	0.0266307*
lmw:May-lc:Aug	0.0165107*
pg1:May-lc:Aug	0.0263529*
pg2:May-lc:Aug	0.0148587*
pg1:Aug-lmw:Aug	0*
pg2:Aug-lmw:Aug	0.4935009
lc:July-lmw:Aug	0.9996434
lmw:July-lmw:Aug	0.9999204
pg1:July-lmw:Aug	1
pg2:July-lmw:Aug	0.9998532
lc:June-lmw:Aug	0.999999
lmw:June-lmw:Aug	0.9997865
pg1:June-lmw:Aug	0.9997896
pg2:June-lmw:Aug	0.9995819
lc:May-lmw:Aug	0.9998759
lmw:May-lmw:Aug	0.9992568
pg1:May-lmw:Aug	0.9998252
pg2:May-lmw:Aug	0.9990307

pg2:Aug-pg1:Aug	0.0003356
lc:July-pg1:Aug	0.00001*
lmw:July-pg1:Aug	0.00001*
pg1:July-pg1:Aug	0.00001*
pg2:July-pg1:Aug	0.00001*
lc:June-pg1:Aug	0.00001*
lmw:June-pg1:Aug	0.00001*
pg1:June-pg1:Aug	0.00001*
pg2:June-pg1:Aug	0.00001*
lc:May-pg1:Aug	0.00001*
lmw:May-pg1:Aug	0.00001*
pg1:May-pg1:Aug	0.00001*
pg2:May-pg1:Aug	0.00001*
lc:July-pg2:Aug	0.9564564
lmw:July-pg2:Aug	0.0264398*
pg1:July-pg2:Aug	0.4191185
pg2:July-pg2:Aug	0.0209476*
lc:June-pg2:Aug	0.0374518*
lmw:June-pg2:Aug	0.0139924*
pg1:June-pg2:Aug	0.0132619*
pg2:June-pg2:Aug	0.0113891*
lc:May-pg2:Aug	0.0074611*
lmw:May-pg2:Aug	0.0044507*
pg1:May-pg2:Aug	0.0074345*
pg2:May-pg2:Aug	0.0039648*
lmw:July-lc:July	0.6824939
pg1:July-lc:July	0.9998596
pg2:July-lc:July	0.6336253
lc:June-lc:July	0.8081648
lmw:June-lc:July	0.5643159
pg1:June-lc:July	0.5567538
pg2:June-lc:July	0.5153764
lc:May-lc:July	0.4838714
lmw:May-lc:July	0.3672784
pg1:May-lc:July	0.4753138

pg2:May-lc:July	0.3455519
pg1:July-lmw:July	0.9938069
pg2:July-lmw:July	1
lc:June-lmw:July	1
lmw:June-lmw:July	1
pg1:June-lmw:July	1
pg2:June-lmw:July	1
lc:May-lmw:July	1
lmw:May-lmw:July	1
pg1:May-lmw:July	1
pg2:May-lmw:July	1
pg2:July-pg1:July	0.9902506
lc:June-pg1:July	0.9994672
lmw:June-pg1:July	0.985029
pg1:June-pg1:July	0.984632
pg2:June-pg1:July	0.9770555
lc:May-pg1:July	0.9824987
lmw:May-pg1:July	0.9512758
pg1:May-pg1:July	0.9797588
pg2:May-pg1:July	0.9427199
lc:June-pg2:July	1
lmw:June-pg2:July	1
pg1:June-pg2:July	1
pg2:June-pg2:July	1
lc:May-pg2:July	1
lmw:May-pg2:July	1
pg1:May-pg2:July	1
pg2:May-pg2:July	1
lmw:June-lc:June	1
pg1:June-lc:June	1
pg2:June-lc:June	1
lc:May-lc:June	1
lmw:May-lc:June	1
pg1:May-lc:June	1
pg2:May-lc:June	0.9999999

pg1:June-lmw:June	1
pg2:June-lmw:June	1
lc:May-lmw:June	1
lmw:May-lmw:June	1
pg1:May-lmw:June	1
pg2:May-lmw:June	1
pg2:June-pg1:June	1
lc:May-pg1:June	1
lmw:May-pg1:June	1
pg1:May-pg1:June	1
pg2:May-pg1:June	1
lc:May-pg2:June	1
lmw:May-pg2:June	1
pg1:May-pg2:June	1
pg2:May-pg2:June	1
lmw:May-lc:May	1
pg1:May-lc:May	1
pg2:May-lc:May	1
pg1:May-lmw:May	1
pg2:May-lmw:May	1
pg2:May-pg1:May	1

CHAPTER 4: THE EFFECT OF ABIOTIC VARIABLES ON THE DEVELOPMENT AND VIRULENCE OF ENTOMOPATHOGENIC FUNGI

4.1 INTRODUCTION

Entomopathogenic fungi and their insect hosts are ectothermic organisms. Therefore, the temperature of the environment determines: (i) the rate of EPF physiological processes such as hyphal growth, spore formation and spore germination together with the biochemical, enzymatically mediated processes that underlie them; and (ii) the rate of physiological and biochemical processes occurring in the insect hosts of EPF. It follows that environmental temperature will also mediate the EPF-host interaction, including fungal infection processes and the rate of host death (Smit *et al*. 2003; Thomas & Blanford, 2003). The response of fungal physiological processes, such as growth and germination to temperature, is sometimes referred to as the thermal profile of the EPF (Thomas and Blanford, 2003), and are commonly-used criteria for the selection of EPF strains to identify microbial control agents (MCAs) that will perform under the temperature conditions that occur within the intended area of use. Understanding how the EPF is affected by temperature is essential not only for the development of MCAs but also for the development of novel biocontrol strategies, such as combining cold and heat tolerant fungal strains together in a single formulation to create a biopesticide product that can perform under a wider range of temperature conditions (Inglis *et al*., 1997; Guetsky *et al*., 2001).

Measuring the effect of temperature on insect and EPF physiological function is relatively straightforward. A graph of the relationship between the rate of a physiological process (e.g. speed of germination, rate of growth, etc.) and temperature resembles a normal distribution that is skewed towards the lower temperatures, with a rapid decline in response as temperatures increase past the optimum. (Thomas & Blanford, 2003). As a result, even small changes in temperature that occur towards the upper thermal threshold can lead to rapid declines in the response (Davidson et al., 2003; Smits et al., 2003). The use of nonlinear models to analyse the thermal biology of EPF and their hosts have not been investigated widely. Nonlinear thermal models have been used to model EPF processes such as hyphal growth (Davidson et al., 2003; Smits et al., 2003) (see section 4.2.5) but they have not yet been used to analyse effects of environmental temperature on the EPF-insect host interaction including host mortality / survivorship. Because nonlinear models are the only way to fully describe the response of EPF, insect, and EPF-insect processes over the full physiological range of temperatures, the absence of a nonlinear modelling approach means that many basic questions about the effect of temperature on these processes remain unanswered. For example, the extent to which the effect of temperature on EPF virulence can be predicted from data on effects of temperature on fungal growth or germination is still not clear (Davidson et al., 2003; Smits et al., 2003).

Furthermore, there may be differences in the response of biophysical processes for the same species. The optimum for growth may be different from germination or speed of kill. Where some disparities lie between the thermal profiles of host and parasite, environmental temperature, through its effect on host body temperature may have antagonistic effects. Antagonistic, through retarded

fungal growth rates or synergistic effects, e.g. greater mortality through increased temperature stress of the host. Discrepancies in the thermal profiles of host and parasite mean that cardinal temperatures will vary between them further complicating the outcome of their interaction. In applied terms, these differences could be the difference in achieving efficacious control or not.

The aim of the study was to build a model that could precisely and accurately describe the relationship between fungal growth, germination and pathogenicity and to test the hypothesis that as a result of co-evolution of the fungal isolates with aphid hosts, the thermal biology of a pathogen should mirror that of the host, i.e. they should have similar cardinal temperature values

4.2 MATERIALS AND METHODS

The following experiments were conducted at Warwick Crop Centre, Wellesbourne, UK in the Crop Centre lab and Insect Rearing Unit.

4.2.1 Effects of temperature on the growth and germination of selected isolates of entomopathogenic fungi

The objective of this part of the research was to characterize and compare effects of temperature on the growth and germination of isolates of two phylogenetically distant orders of entomopathogenic fungi (i) six species from the Hypocreales (phylum Ascomycota, class Sordariomycetes) and (ii) *Pandora neoaphidis* (phylum Entomopthoromycota, class Entomophthoromycetes, order Entomophthorales). The isolates of fungi from the Hypocreales that were selected have all been reported in the literature as being pathogenic to aphids. The

hypothesis was that, as a result of co-evolution of the fungal isolates with aphid hosts, the thermal biology of the pathogen should mirror that of the host, i.e. they should have similar cardinal temperature values. Initially, experiments were done with the isolates of hypocrealean fungi, during which time the basic culture and bioassay methods for *P. neoaphidis* (which is difficult to culture and presents particular problems for bioassay design) were developed. Once these methods were in place, temperature experiments were done with *P. neoaphidis*, using a reference isolate from the Hypocreales when required.

4.2.1.1 Effect of temperature on the growth of six hypocrealean entomopathogenic fungi from four different genera, compared against *Pandora neoaphidis* (Entomopthoromycota, Entomophthorales)

4.2.1.1.1 Effect of temperature on the growth of six hypocrealean entomopathogenic fungi from four different genera

The effect of temperature on the rate of mycelial colony extension was investigated for six isolates of hypocrealean entomopathogenic fungi that are reported to be pathogenic to aphids (Table 2.3.1). These experiments were done at six different temperatures (10, 15, 20, 25, 30 and 33°C) on two different growth media; SDA (Sabouraud dextrose agar; Fisher Scientific) and SEMA (Sabouraud dextrose agar supplemented with milk and egg yolk) (for method see section 2.3.3.1). Fungal isolates for experiments were taken from slope cultures (refer to section 2.3.1) and grown on 90mm Petri dishes (Fisher Scientific) containing SDA in darkness at 20 ^{o}C ± 1°C for 15 days. Conidial suspensions were prepared by agitating the

mycelium with an ‘L-shaped’ spreader (Fisher Scientific) in 0.05% Triton X-100. Suspensions were enumerated using an Improved Neubauer haemocytometer and diluted (in 0.05% Triton) to 1.0 $x10^7/ml^{-1}$. Then 0.1ml of the 1.0 $x10^7/ml^{-1}$ suspension for each species was spread over a clean triple vented 90mm Petri dish containing SDA and left for 48 hours. Plugs (7mm) were taken from these cultures with a flame-sterilised cork borer and inverted in the centre of a 90mm Petri dish containing either SEMA or SDA, two dishes per isolate x temperature combination. The bases of the Petri dishes had been previously marked with an x-y axis to prevent bias when measuring colony growth (Figure 4.1). Petri dishes were then transferred to incubators (Sanyo environmental test chamber, MLR351) set at the range of temperatures above. Assays were read every 3-4 days for a total of 22 days, measuring colony extension along 2 axes (mm). Actual incubator temperature was logged using iButton thermologgers (Homechip Ltd, Milton Keynes, MK17 0BH, UK). The experiment was repeated four times. This method has the advantage of producing a large volume of reliable data in a short amount of time. Practically, temperature effects on colony growth would be experienced at the hypha level and monitoring hyphal growth using a microscope under different temperature regimes would allow a more realistic extrapolation to the ‘real-world’.

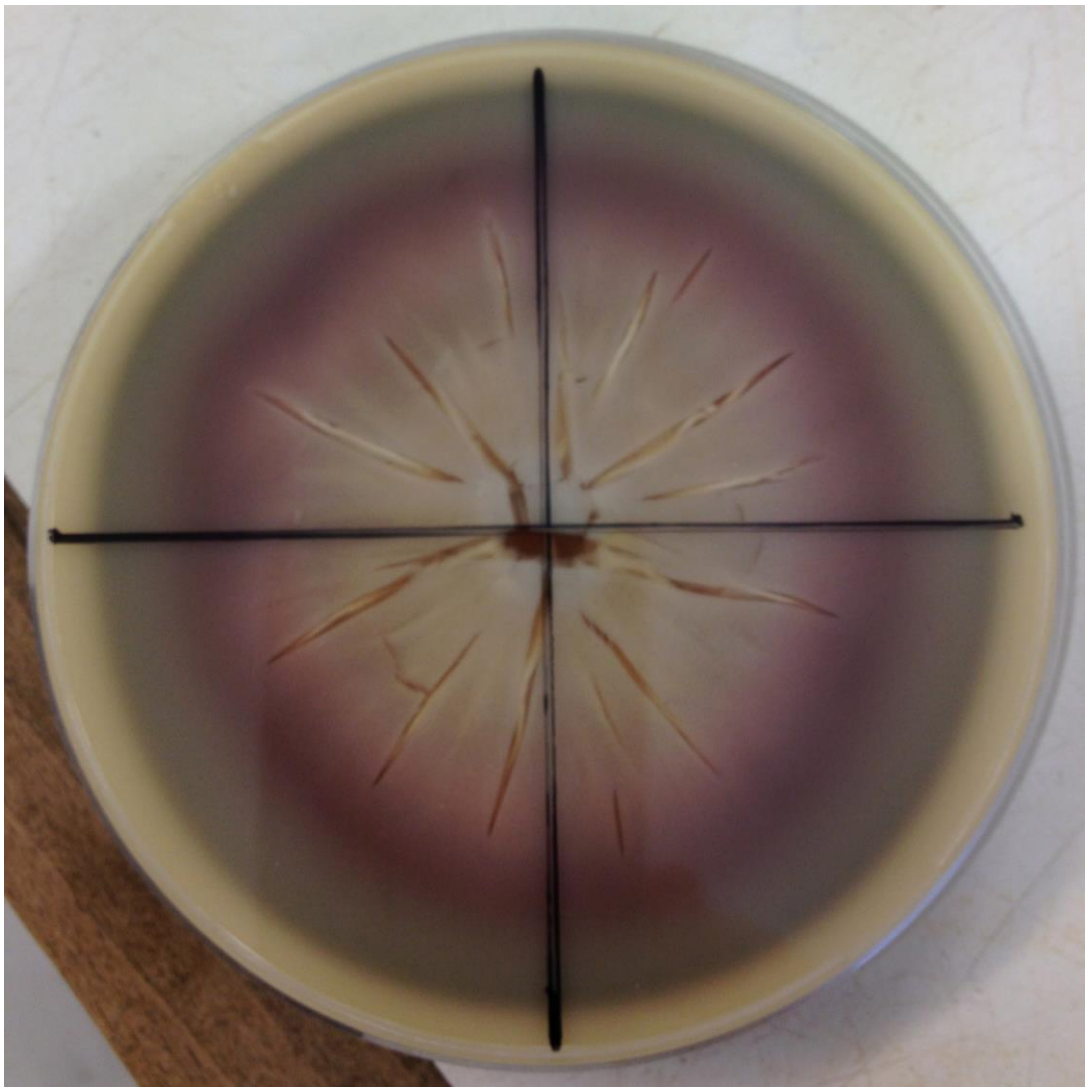


Figure 4.1 The base of a Petri dish containing *Beauveria bassiana on* SEMA marked with an x-y axis during mycelial growth assays (experiment 4.2.1.1).

4.2.1.1.2 Effect of temperature on the growth of *Pandora neoaphidis* (Entomopthoromycota, Entomophthorales) compared against *Beauveria bassiana* 432.99

Following completion of 4.2.1.1.1, a second set of experiments was done to compare effects of temperature on the rate of mycelial colony extension for two *P. neoaphidis* isolates (NW420 and WEL1) and *Beauveria bassiana* strain 432.99 as a reference (see Wraight *et al*. 2010). Assays were carried out as above (4.2.1.1.1) except plugs of actively growing mycelium were taken from the leading edges of *P. neoaphidis* Petri dish cultures that were 4-5 weeks old and only conducted using SEMA growth media. The experiment was repeated three times.

4.2.1.2 Effect of temperature on the germination of six hypocrealean entomopathogenic fungi from four different genera

Hypocreales

Fungal isolates for experimental use were taken from slope cultures (section 2.3.1) and grown in the dark in 90mm triple vented Petri dishes on SDA for 17 days at 20°C ± 1°C. Conidia were harvested in 0.05% Triton X-100 by agitating the mycelium with an 'L-shaped' spreader, enumerated using an Improved Neubauer haemocytometer, and adjusted to a concentration of 1.0 x 10^7 conidia ml^{-1}. Aliquots of 20µl were then pipetted onto 1.5% distilled water agar contained within 45mm diameter Petri dishes (Fisher Scientific), the bases of which had been

previously marked with three circles approximately 1cm in diameter. Plates were sealed with Parafilm® (Sigma-Aldrich) and incubated in the dark at each of six different temperatures; 10, 15, 20, 25, 30 and 33°C ± 1°C. Actual incubator temperature was logged using iButton thermologgers. Sampling was carried out destructively at regular time intervals (every four hours for 48 hours) by pipetting a drop of lactophenol methylene blue inside each circle (Figure 4.2). Plates were sealed with Parafilm® and stored at 4°C before examination under a light microscope (magnification x200) (Figure 4.2). Numbers of germinated and non-germinated conidia were recorded for approximately 100 conidia per circle. Germination was defined as the point when an emerging germ tube was equal to, or larger than, the length of the conidium. The experiment was repeated three times.

***Pandora neoaphidis (*= Entomophthoromycota)**

4.2.1.4 Determining the rate of discharge of primary conidia of *Pandora neoaphidis* (NW420) from fungal mycelium

Conidia of *P. neoaphidis* are actively discharged. Therefore, to obtain inocula of primary conidia for experimental treatment requires knowledge of the rate of sporulation and discharge.

Isolates of *P. neoaphidis* were grown on SEMA in 90mm triple vented Petri dishes for 4-5 weeks at 15°C ± 1°C in darkness adhering to methods in section 2.3. Plugs (1.7mm ø) were taken from the growing edge of these cultures and placed in the lid of 90mm triple vented Petri dishes on moist filter paper in an off-centre position (c. 2cm). Excess growth media was removed from the base of the

plugs. The lid was placed over the base of another Petri dish containing eight 18x18mm^2 coverslips arranged in a circle, the circumference of which ran above the plug of *P. neoaphidis* mycelium. Petri dishes were then stored in darkness at 15 ^{o}C ± 1°C. Every hour for the first eight hours the lid was rotated clockwise to ensure the plug was sporulating over a 'clean' coverslip in the Petri dish below it, and the previous coverslip was removed. For reads at 12, 16, 20, 24, 28, 32, 36, 40, 44, 48 and 72 hours, coverslips (18x18mm^2) were placed under the sporulating plug for one hour prior to removal. All coverslips were fixed and stained with lactophenol methylene blue and enumerated under a light microscope (magnification 400x) with a marked 0.2x0.2mm^2 grid in the eyepiece. From this the density of conidia deposited on cover slips was calculated (as conidia /mm^2). The experiment was repeated three times.

4.2.1.3 Effect of temperature on the germination of two *Pandora neoaphidis* (Entomopthoromycota, Entomophthorales) isolates

Isolates of *Pandora neoaphidis* (NW420 and WEL1) were grown on SEMA in 90mm triple vented Petri dishes for 4-5 weeks at 15 $^\circ$C ± 1°C in darkness as described in section 2.3. To obtain primary conidia, three plugs (6-7mm ø) were taken from *P. neoaphidis* cultures using a cork borer and these were arranged in a triangular formation (approximately 1cm x 1cm x 1cm) on moist filter paper on the lid of a 45mm Petri dish. The base of the Petri dish contained 1.5% distilled water agar. Excess growth media, media that was not immediately in contact with the mycelial mat, was removed from the base of the plugs using a scalpel. These Petri dishes were then stored at 15°C ± 1°C in darkness for 16-20 hours prior to

experimental use to ensure the fungus was sporulating profusely. The lid of each Petri dish was then transferred over the base of a 45mm Petri dish containing fresh (i.e. newly poured) 1.5% distilled water agar and left in place for 60 minutes at a time. Pilot experiments showed that this time allowed sufficient conidia to be deposited onto the agar to enable germination to be counted readily but did not provide so many conidia that it was difficult to clearly assess individual conidia. Once groups of plugs reached 24 hours in age they were discarded. Following conidia showering, the original Petri dish lids were replaced and dishes were incubated at a range of experimental temperatures; 10, 15, 20, 25, 30 and 33ºC ± 1ºC. Temperatures were logged. Sampling was carried out destructively at 8, 12, 16, 24 and 48 hours' post inoculation using lactophenol methylene blue to stain the area where conidia had been deposited. Petri dishes were sealed with Parafilm® and stored at 4ºC for later examination under a light microscope (magnification 400x). Incidence of germination was recorded for approximately 100 conidia per Petri dish in arbitrarily chosen, non-overlapping fields of view ($0.2mm^2$). Germination stage was assessed for each conidium and assigned one of six ordinal categories; (1) Ungerminated, (2) Germ tube visible <50% width of conidium, (3) Germ tube >50% width of conidium, (4) Majority of cytoplasm in germ tube or secondary conidia, fully formed secondary conidia, (5) 'Ghost' conidia, empty primary conidia, and (6) Secondary conidia. Both *P. neoaphidis* isolates were treated in the same way. The experiment was repeated three times.

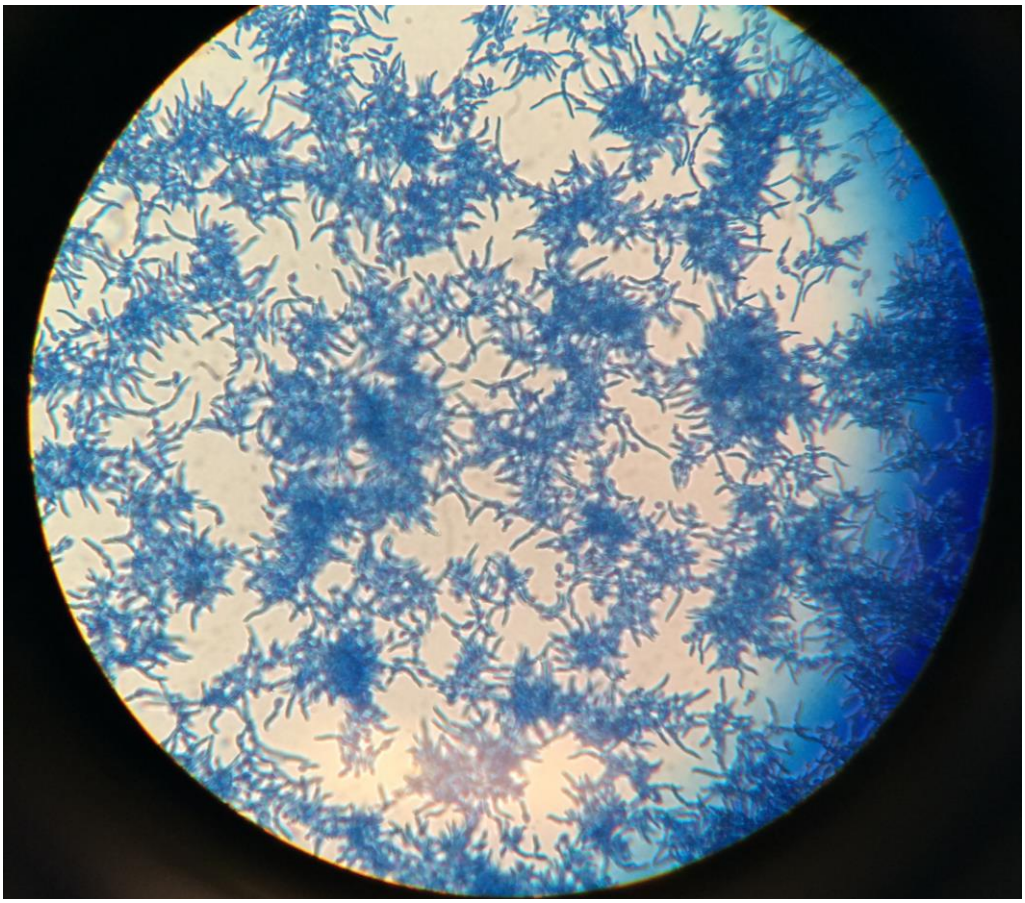


Figure 4.2 Image of *Beauveria bassiana* (432.99) conidia germinating on 1.5% distilled water agar stained with lactophenol methylene blue as seen under a light microscope (x400 magnification). Image taken after incubation at 20 ± 1ºC for 48 hours; 100% germination.

4.2.2 Temperature- Insect interactions

4.2.2.1 The effect of temperature on the time to adult development of *Brevicoryne brassicae*

First instar *B. brassicae* (<16 hours old) were obtained by placing adult aphids (reared according to section 2.2) on 4-5-week-old Brussels sprout plants (BBCH 15/16 -5/6 true leaves cv. 'Trafalgar') and storing them at 20 ºC ± 1ºC for 16 hours, after which adult aphids were removed. Cohorts of a minimum of 20 nymphs from

these plants were then transferred to Brussels sprout plants (BBCH 15/16 -5/6 true leaves cv. 'Trafalgar') using a fine camel hair brush. Infested plants were placed in incubators (Sanyo environmental test chamber, MLR351) at each of nine different temperatures; 10, 11, 12, 15, 17, 20, 22, 24 and 28°C (± 1ºC). Actual incubator temperature was logged using iButton thermologgers. There were not enough incubators to run experiments at all temperatures concurrently; instead the experiment was done according to a randomized design (two temperatures were run in tandem). Aphids were monitored daily to observe moult, development and survival, using a handheld magnifying glass or by light microscope if necessary. When moulting occurred to the final (i.e. adult) stage, the time at which this occurred was taken as the mid-point between the current and previous observation. Adults were identified based on the number and stage of development of antennal segments and the size and shape of the cauda (Dodd, 1976). Exuviae, individuals that had reached adulthood, plus any newly emerged nymphs produced by adults, were removed from experimental plants on the day of each observation.

4.2.2.2 The effect of temperature on the fecundity and survival of adult *B. brassicae*

The effect of temperature on the fecundity and survival of adult *B. brassicae* was analysed using data obtained from control treatments from experiment 4.2.3.5. Full methods are given in 4.2.3.5. In brief: *B. brassicae* adults (10-12 days old) were placed on a single leaf of a 4-5-week-old Brussels sprout plant (BBCH 15/16 cv Trafalgar) encased in a Blackman box (124 x 82 x 22mm) containing moist filter paper. Aphids were maintained at each of five temperatures (Sanyo environmental test chamber, 12, 15, 20, 24 or 28°C ± 1ºC); 16:8 L: D photoperiod.

Actual incubator temperature was logged using iButton thermologgers. Adult survival, and the production of nymphs, was assessed every 24 h. Each assay was performed on three separate occasions.

4.2.3 Temperature- Insect- Fungi interactions

4.2.3.2 The susceptibility of adult *Myzus persicae* and *Brevicoryne brassicae* to the hypocrealean entomopathogenic fungi *Beauveria bassiana, Lecanicillium muscarium and Lecanicillium longisporum*

An experiment was done to quantify and compare the susceptibility of apterous adult *B. brassicae* and *M.s persicae* to *B. bassiana* 432.99, *L. muscarium* 19.79 and *L. longisporum* 1.72. Bioassays were conducted at 20±1°C, 16:8 L: D photoperiod in a controlled environment room. The production of known age aphids was achieved using the rearing method described in section 2.2.1. Time to adulthood for *B. brassicae* and *M. persicae* was 10 and 8 d respectively.

Fungal isolates were taken from slope cultures (section 2.3.1) and grown in the dark in 90mm triple vented Petri dishes on SDA (Sabouraud dextrose agar) for 10 to 12 d at 20°C ± 1°C. Conidia were harvested in 0.05% Triton X-100 by agitating the mycelium with an 'L-shaped' spreader, enumerated using an Improved Neubauer haemocytometer, and adjusted to concentrations of 3.0×10^5 ml^{-1}, 1.0×10^6 $/ml^{-1}$, 1.0×10^7 $/ml^{-1}$ and 1.0×10^8 $/ml^{-1}$ (section 2.3.2 for details). The procedure remained identical for each fungal isolate.

Cohorts of approximately 20 apterous adult aphids were placed on moist filter paper in the lid of a 90 mm Petri dish and sprayed with 2ml of the above

suspensions using a Potter tower (Potter, 1952) set at 34.5 kPa spray pressure. Control aphids were sprayed with 0.01% Triton X-100. After 1 h the 10 most active individuals (less likely to have suffered handling damage) were placed on a single leaf of a 4-5-week-old Brussels sprout plant (BBCH 15/16 cv Trafalgar) and encased in a Blackman box (12.5 cm x 8cm x 2cm) (Blackman, 1971) containing moist filter paper. All plants were then placed in a controlled environment room at a temperature of 20°C, RH c.80-96% and 16:8 L: D photoperiod. Mortality was monitored daily for up to 10 days. Any dead aphids were placed in a marked Petri dish on moist filter paper to encourage sporulation and checked after 24 hours to attribute death to mycosis. Any nymphs produced from adult aphids were removed daily. The experiment was repeated three times.

4.2.3.3 Effect of temperature on the mortality of adult *B. brassicae* treated with *L. longisporum* 1.72 and *B. bassiana* 432.99

In this experiment, a laboratory bioassay was used to quantify effects of temperature on the mortality of apterous adult *B. brassicae* treated with conidia of *L. longisporum* 1.72 and *B. bassiana* 432.99. Aphids were inoculated with a concentration of conidia suspension equivalent to the LD95 value at 20°C calculated from experiment 4.2.3.2. The experiment was done as follows: Fungal culturing, production of aphids, preparation of conidial suspensions; aphid inoculation with fungal conidia was done as described in section 4.2.3.2, with the exception that aphids were treated with the LD95 concentration of each fungal isolate, i.e. 1.72 = 1.14×10^6 and 432.99 = 5.30×10^7 (see results section 4.3.6). Control aphids were sprayed with 0.01% Triton X-100 only. Cohorts of 10 fixed aged (c. 48 hours old) apterous aphids were maintained at the following

experimental temperatures in a Sanyo environmental chamber: 12, 15, 20, 24 or 28°C ± 1°C; 16:8 L: D photoperiod. Actual incubator temperature was logged using iButton thermologgers. Each fungal species x temperature bioassay was repeated three times in a randomized block design. The dose of conidia applied in each bioassay was estimated using glass microscope cover slips (18 x 18 mm) placed in Petri dishes alongside aphids as they were sprayed with conidia. Cover slips were washed by placing them in a 10ml universal tube (Fisher Scientific) with 1 ml of 0.05% Triton X-100 and shaken for 3-5 minutes in a vortex (LabDancer ™, IKA®). Aliquots of these conidial suspensions were then enumerated using an Improved Neubauer haemocytometer. Assessments for moribund, dead and sporulating aphid cadavers were conducted as well as counts of the number of newly emerged nymphs every 24 h for a maximum of 10 days. Dead aphids were identified as individuals that exhibited no movement when touched with the tip of a fine camel-hair brush. Nymphs were removed at each observation. The number of mycosed and unmycosed adult aphids was noted. Dead non-sporulating adult aphids were placed on 1.5% distilled water agar to promote fungal outgrowth and confirm death due to mycosis.

4.2.3.4 Development of a laboratory bioassay method for inoculating *B. brassicae* with primary conidia of *P. neoaphidis* and measuring aphid survival

P. neoaphidis produces infective conidia that are actively discharged, as ballistospores, from mycelium. For experiments, aphids were inoculated by placing them in a Petri dish arena below *P. neoaphidis* mycelium that had been induced to produce ballistospores. These conidia were then ‘showered’ onto the aphids for a defined period of time. A number of different experimental methods

and apparatus were evaluated in order to develop a consistent, reliable bioassay. For brevity, only the main developments are summarized here. The final bioassay system took approximately six months to develop. The principal tasks were to achieve uniform coverage of primary conidia within the arena and to ensure that individual aphids received the same dose. The final method is detailed in section 4.2.3.5.

Production of *P. neoaphidis* ballistospores.

Production of *P. neoaphidis* cultures and treatment to induce production of primary conidia is described in section 4.2.1.2. In brief: Isolates of *P. neoaphidis* were grown on SEMA for 4-5 weeks at 15 °C ±1°C in darkness. Plugs (6-7mm ø) were taken from the leading edge of colonies using a cork borer and placed on moist filter paper on the lid of a 45mm Petri dish. The plugs were induced to produce primary conidia by trimming away all parts of the SEMA plug that had not been colonized fully by *P. neoaphidis* mycelium.

Inoculation of adult apterous aphids with *P. neoaphidis* primary conidia.

This part of the work initially followed the methods of Feng & Johnson (1991), Papierok & Hajek (1997) and Sierotzki et al. (2000). *Brevicoryne brassicae* apeterous adults 10-12 days old were produced as described in section 2.2.1. *Brassica oleracea* cv Trafalgar leaf discs (2cm in diameter) were mounted on 1.5% distilled water agar in 45mm diameter Petri dishes. Leaf discs were placed on 'hand hot' molten agar that was warm enough to not have fully set and cool enough not to cause the leaf discs to curl. This method was tried repeatedly, but it was

found that *B. brassicae* individuals did not settle on the mounted leaf discs. Instead, they had a tendency to walk across the agar surface and come into direct contact with the *P. neoaphidis* mycelium. To prevent this, two different modifications were investigated. Firstly, a nylon gauze (0.1mm mesh size) was added beneath the sporulating plugs of *P. neoaphidis* mycelium. It was held in place within a Perspex ring placed around the leaf disc (25mm in length x 21mm internal diameter) and also acted to suspend the sporulating plug above the aphids. However, aphids climbed on the nylon gauze; while individuals were kept from direct contact with *P.neoaphidis* mycelium, a condidial 'rain shadow' formed directly beneath these aphids that prevented inviduals remaining on the leaf disc from acquiring the same dose of conidia. It was also found that the nylon gauze intercepted *P.neoaphidis* conidia, which were observed adhering to it, thereby affecting the total number of conidia deposited in the arena below. The final iteration of the method consisted of the following: the conidia 'showering' arena consisted of a *B. oleracea* cv Trafalgar leaf disc (2cm diameter) floated on distilled water to prevent aphids 'escaping' from the disc; this also helped maintain high relative humidity within the arena. The leaf disc was pinned to a drawing pin that was anchored to the base of the Petri dish using pressure sensitive putty ('Blu-Tack', Bostic UK Ltd.). Differences were measured in the rate at which conidia were discharged from sporulating plugs, which meant that the use of a single plug (1.7cm diameter) was insuffient to create a uniform spread of conidia across the arena. Instead, three smaller plugs (6-7mm ø) were used, postioned in a triangluar formation (1cm x 1cm x 1cm) above the leaf disc.

4.2.3.5 Effect of temperature on the virulence of *P. neoaphidis* to *B. brassicae*

Each bioassay was set up as follows: three plugs of *P. neoaphidis* mycelium (6-7mm ø) were taken from the leading edge of a fungal culure that had been grown on SEMA at 15 °C ±1°C for 4-5 weeks in darkness (see section 4.2.1.2), trimmed with a scalpel to remove any agar that had not been colonised by the fungus and arranged in a triangular formation (1cm x 1cm x 1cm) on moist filter paper in the lid of a 45mm diameter Petri dish. The plugs were maintained at 15 °C ±1°C in darkness for 16 – 20 hours to induce sporulation. A *B. oleracea* cv Trafalgar leaf disc (2cm in diameter) was suspended in distilled water, abaxial side up, in the centre of a 90mm triple vented Petri dish and held in place with a drawing pin. Bioassays were done using *P. neoaphidis* isolates NW420 and WEL1. Each bioassay was set up as follows: A group of 15-20 *B. brassicae* apterous adults (10-12 days old, fixed age culturing, section 2.2.1) were transferred with a fine camel hair brush to the leaf disc. The *P. neoaphidis* mycelial plugs were then placed on top of a Perspex ring (25 mm in length x 20 mm internal diameter) which was placed around the leaf disc. This formed the conidial showering arena. The arena was left in place for different lengths of time in order to produce different doses of conidia, i.e., 5, 15, 25, 35, 45, 55 and 75 minutes. Control aphids were treated in the same way as the longest condial shower except they were placed in an arena for 75 minutes without being exposed to *P. neoaphidis*.

After innoculation, 10-15 of the most active individual aphids from each showering time were transferred onto a single leaf of a 4-5-week-old Brussels sprout plant (BBCH 15/16 -5/6 true leaves cv. 'Trafalgar') using a fine camel hair brush and encased in a Blackman box (12.5 x 8 x 2 cm) containing moist filter paper to maintain high humidity (RH c.80-96%). All plants were then placed in

controlled environment incubators at 12, 15, 20, 24 or 28°C ± 1°C at a 16:8 L: D photoperiod. Actual incubator temperature was logged using iButton thermologgers. Due to limited availability of incubators, it was not possible to run all treatments at the same time, and so treatments were done according to a randomized design (randomized block design) with three true replicates per temperature. An estimate of dose was obtained for each treatment by placing a cover slip (18x18 mm) under the plugs immediately after aphids had been inoculated and lasting for the same showering time. An accurate representation of the number of conidia, as the rate of discharge remained constant over 20 hours. Coverslips were fixed with lactophenol methylene blue. The number of conidia on each cover slip was counted under a light microscope (magnification 400x) in 20 random, non-overlapping, fields of view and converted to conidia/mm^{-2}. Numbers of moribund, dead and sporulating aphid cadavers were counted every 24 h for a maximum of 10 days, as well as numbers of nymphs. Nymphs were removed at each observation. Dead, non-sporulating aphids were placed on 1.5% distilled water agar to promote fungal outgrowth and confirm death due to mycosis. Assays at each temperature were repeated three times. The method remained the same for both *P. neoaphidis* isolates.

4.2.4 Humidity- Insect- Fungal interaction

4.2.4.1 Effect of relative humidity on the virulence of *P. neoaphidis* to apterous adult *B. brassicae*

Relative humidity is a difficult abiotic factor to manipulate and maintain in insect bioassays (Andersen *et al.* 2006). In order to experimentally manipulate humidity in a reliable way, *B. brassicae* individuals were placed for various durations in a high humidity environment before being moved to a lower humidity environment. The method was as follows:

Cohorts of approximately 15-20 apterous adult *B. brassicae* (10-12 days old, fixed age culturing, section 2.2.1) were inoculated with primary conidia of *P. neoaphidis* (NW420 and WEL1) for a 'showering' time of 50 minutes as described in 4.2.3.5. Fungus inoculated aphids were then transferred, using a fine camel hair brush, to moist filter paper in the lid of 90mm Petri dish, over the top of which was placed a 90 mm Petri dish base containing e 1.5% distilled water agar. Petri dishes were sealed using Parafilm® and maintained at 20 ± 1°C, 16:8 L: D photoperiod for 4, 8, 12, 16, 20 or 24 hours. The water agar treatment was intended to maintain relative humidity at >98% RH. Groups of 10-15 of the most active individuals from each exposure time were then transferred using a fine camel hair brush onto a single leaf of a 4-5-week-old Brussels sprout plant (BBCH 15/16 - 5/6 true leaves cv. 'Trafalgar') and encased in a Blackman box (12.5 x 8 x 2 cm). Control aphids were treated in the same way as the longest high humidity treatment but were not inoculated with the fungus. Plants were held in a controlled environment room at 20°C and a 16:8 L: D photoperiod (temperature was logged

using iButton, HomeChip). To reduce humidity within the box, a hole (10 x 17mm) was made in the back and covered with nylon netting (0.1mm mesh). This maintained humidity at approximately 40-60% RH (humidity was measured in dummy boxes using iButtons from HomeChip capable of measuring relative humidity). Daily assessments for moribund, dead and sporulating cadavers were conducted up to a maximum of 7 days. Nymphs were removed from experimental leaves at each assessment. The number of mycosed and unmycosed individuals was also noted and dead non-sporulating individuals were placed on 1.5% distilled water agar. The experiment was repeated three times.

4.2.5 Statistical analysis

Results were analysed using RStudio© version 0.98.1091. Data were analysed for normality via visual inspection of residual plots and/or Shapiro-Wilk tests.

4.2.5.1 Effect of temperature on fungal radial extension

The radial extension rate of fungal colonies was calculated from the mean of two diameter measurements. Mean diameter measurements were plotted over time for each fungal isolate, temperature and replicate, and extension rate was calculated from fitted linear regression lines. Colony extension rates were analysed using ANOVA and significant results were analysed with Tukey HSD.

Colony extension rates for each fungal isolate and replicate were then plotted over temperature. The resulting curves were analysed with five non-linear models, the details of which are given below in addition to model fit criteria. To

obtain an additional estimate of the theorised minimum threshold (T_0) linear regression was fitted to the linear phase of growth.

Effect of temperature on the germination rate of selected species of entomopathogenic fungi

The percentage of a population of spores was calculated for each experimental temperature at various time points during a 48-hour period. These data were then used to generate GT50 values using the method described below. Subsequently, the GT50 values were plotted for each fungal isolate over temperature to obtain isolate specific thermal profiles.

Data were analysed using the 'drc' package version 2.1-4 (Ritz & Stribig, 2005). Three models that are commonly used to describe dose response survival data are the log-logistic and Weibull 1 and Weibull 2 models. These models can also be used to describe the relationship between percentage population germination and time, using independent data points. Germination is a binomial response, germinated or ungerminated, (and so can be used in the same way as data on insect survival for dose response survival data). Data for *P. neoaphidis* was treated in the same way except, in order to form a binomial response variable, ordinal categories other than that of 'ungerminated' (category 1) were grouped to form a 'germinated' category.

Parameter estimates for *P. neoaphidis* were obtained from graphs of the observed data as the relationship between germination and temperature of isolate NW420 and WEL1 were not conducive to the fit of the models above.

A four-parameter log-logistic model is depicted in figure 4.3 and contains the parameters, upper limit, lower limit, slope and point of inflection. Weibull 1 and 2 models are also pictured in figure 4.3. These models account for asymmetry in the data set around the point of inflection. ED50 refers to the effective dose to kill 50% of the population. In this study ED50 is equivalent to the time taken for 50% of the population of conidia to germinate. In this way GT5, GT50 and GT90 refer to the time taken for 5%, 50% and 90% of the population to germinate respectively.

***Log-logistic* equation-** three parameter

$$f(x) = 0 + \frac{d-0}{1+\exp(b(\log(x)-\log(e)))}$$

Where, b= slope, d= upper limit and e= (LD_{50}) lethal dose which kills 50% of individuals.

Weibull 1 equation- four parameter

$$f(x) = 0 + (d-c) \exp(-\exp(b(\log(x)-e)))$$

Where, b= slope, c= lower limit, d= upper limit and e= the point of inflection

Weibull 2 equation- four parameter

$$f(x) = 0 + (d-c) \exp(-\exp(b(\log(x)-e)))$$

Where, b= slope, c= lower limit d= upper limit and e= the point of inflection. For both Weibull equations c and d can be set at 0 or 1 respectively to reduce the number of parameters.

The upper temperature limit could not be fixed as 100% germination was not reached at the higher temperatures. As a result, three parameter models were used to describe germination data. For models that fit the data Goodness-of-fit was determined by Akaike information criterion (AIC), the lower the value of AIC the better the model fit (see below for details). For germination data in all cases a three parameter Weibull 1 provided the best fit.

Parameters were checked for fit to the data and outputs based on parameters that did not fit the data; $P>0.05$, were not reported. Fungal isolate estimates of GT5, 50 and 90 at each temperature were compared using a t-test.

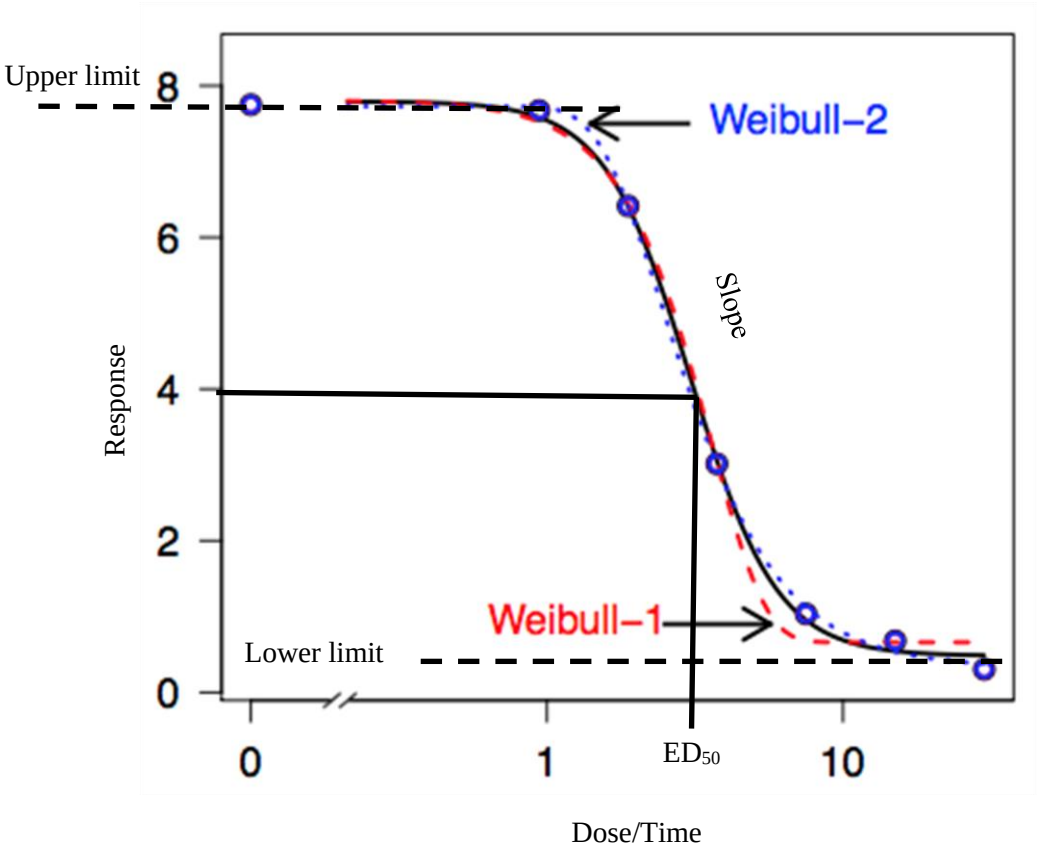


Figure 4.3 Graphical representation of the four-parameters estimated by log-logistic, Weibull 1 and 2. The variable parameters are upper limit, lower limit, slope and ED50; for Weibull models the point of inflection. The Log-logistic dose response model (black) shows a steep slope approaching the lower asymptote; both Weibull models show their compensation for asymmetry around the point of inflection at the lower asymptote (Weibull 1, red) and the upper asymptote (Weibull 2, blue) (adapted from Ritz & Stribig, 2005).

Minimization of dose-response (DRC) model parameters

To obtain the most parsimonious models, model reduction was applied to all analyses. Initially models were fitted to individual temperatures and each had its own discrete set of parameters, termed unconstrained model. Models were then fitted where values for each individual parameter, i.e. the slope or effective dose 50 (ED50), were constrained to a single common value. If the fit of the constrained models were no worse than the unconstrained, the reduced model (constrained)

was selected to describe the data. Constrained and unconstrained models were compared using ANOVA; if models were significantly different the slope and ED50's were individually calculated for each temperature. A single model estimate or slope or ED50 was insufficient to describe the data due to the separation of germination rate under different constant temperatures; as a result, unconstrained models were used in all cases.

Germination rate to 50% germination (1/GT50) for each fungal isolate were plotted over temperature. The resulting curves were then analysed with five non-linear models, the details of which are below in addition to model fit criteria.

Effect of temperature on the development of *B. brassicae*

The number of day degrees taken for *B. brassicae* (K3 clone, Kirton) to reach adulthood was calculated from 1/slope of linear regression fitted to development rate (1/no. of days) over temperature (^{o}C). The minimum development threshold (T_0) was calculated from the same linear regression solving for x=0 (Rahman *et al*. 2011; Raworth, 1984; Hughes, 1963). The non-linear models Taylor (Gaussian), Kontdamis and Briere-1 were fitted to this data, the details of which are below in addition to model fit criteria.

Bioassay data analysis

Dose-response data for hypocrealean fungal species data was analysed using the 'drc' package version 2.1-4 as above except only log-logisitc model was applied to the data (Ritz & Stribig, 2005). The log-logistic model was applied to dose

response data for *P. neoaphidis* data; however there appeared to be no relationship between dose and mortality (section 4.3.9).

The cumulative mortality responses for each fungal isolate across seven days of assessment were analysed using Kaplan-Meier survival analysis (Kaplan & Meier, 1958). This allowed an estimate of lethal time to 50% mortality (LT50) for those temperature*fungal isolate combinations that caused 50% mortality after 7 days and for individuals that survived to the end of the assessment period to be marked as censored. The event was defined as mycosis and mortality data were corrected for control mortality using Abbott's formula (Abbott, 1925).

Insect survival at each temperature was analysed using the Cox proportional hazard model (Cox, 1972). Baseline comparison data was set to target temperature 28°C for comparisons. The Cox proportional hazard is expressed as a hazard ratio, which is a measure of the relative average daily risk of death. The event was defined as death. This was the only analysis in this study to be carried out in SPSS version 22. Aphid fecundity data were compared using ANOVA; significant results were analysed using Tukey's HSD.

Lethal time to 50% mortality (LT50) for each fungal isolate was plotted over temperature. The resulting curves were then analysed with four non-linear models (Taylor, Lactin-1, Kontodimas & Briere-1), the details of which are below in addition to model fit criteria.

Effects of different humidity regimes on the pathogenicity of *P. neoaphidis* to *B. brassicae*

Mortality of *B. brassicae* was analysed using ANOVA on day 5 data. Significant results were analysed with Tukey's HSD.

Temperature dependent Model fit and analysis

The priority of this study was the selection of models that provided a good description of data sets. Models were fit in RStudio© version 0.98.1 using the devRate package version 0.1.1 and the 'nlsLM' function.

Initially, linear models were used. Linear models are suitable for describing developmental rate below the thermal optimum and can estimate the lower developmental temperature threshold. However, at values past the thermal optimum it assumes developmental rate is functionally tied to increasing temperature and the upper developmental threshold is never reached. This results in larger differences between observed and expected developmental rates at temperatures past the optimum; as a result, regression models were fitted to the linear phase of fungal growth (Fragues *et al*. 1997; Shi *et al*. 2010). In order to account for data collected past the optimum and allow estimates of Tmax and Topt, non-linear models were considered. Five models were chosen from the literature on the basis that they were characterised by a strong asymmetric curve and would provide parameters of biological interest (Smit *et al*. 2003). Starting values for each model were gleaned from the literature.

Linear model

Linear models (Equation 1) are the most widely used examples of models describing the effect of temperature on development rate.

$$Rt = mT + b$$

(Equation 1)

Where T is the incubation temperature, m is the slope of the regression line and b is the intercept.

Taylor (Gaussian) model

The first non-linear model tested was Taylor (Gaussian), it is a simple normal function, where, Rm is the maximum rate of development at temperature (T), Tm is the optimal development temperature (Topt) and To is the lower developmental threshold (T0).

$$rT = Rm * \exp(-1/2 * ((T - Tm)/To)^2)$$

(Equation 2)

Lactin-1 model

Lactin-1 is a simplification of the Logan-6 model (Lactin *et* al. 1995; Logan *et al*. 1976),

$$rT \sim \exp(aa * T) - \exp(aa * Tmax - (Tmax - T)/deltaT)$$

(Equation 3)

Where, *aa* is a constant defining the rate at Topt, deltaT is the temperature range during which physiological breakdown becomes the dominant factor, retarding development rate.

Kontodimas

As with all selected models Kontodimas has an asymmetrical curve skewed towards lower temperatures. The equation is below:

$$rT = aa * (T - Tmin) \wedge 2 * (Tmax - T)$$

(Equation 4)

Where, T, Tmin and Tmax are the same as previous models and *aa* is an empirical constant (Kontodamis *et al*.2004).

The final two models based on three; Briere-1, and four; Briere-2, parameters were proposed by Briere *et al*. (1999), again for modelling the effect of temperature on

insects. They were included as their curves exhibit a sharp decline at temperatures past the thermal optimum.

Briere-1

$$rT \sim aa * T * (T - Tmin) * (Tmax - T) \wedge (1/2)$$

(Equation 5)

Briere-2

$$rT \sim aa * T * (T - Tmin) * (Tmax - T) \wedge (1/bb)$$

(Equation 6)

Tmin and Tmax refer to the lower and upper (or lethal) temperature developmental thresholds, *aa* is a parameter that effects the maximal value achieved by the curve and *bb*, found in Briere-2, affects the asymmetrical nature of the curve.

Wherever possible values were compared from model estimates, some models failed to fit the data and thus could not be discussed any further. Where models did not significantly fit the data or parameters could not be acquired, estimates of parameters were made from the estimated curves. Optimal development temperatures (Topt) were calculated for Lacin-1 (equation 10) and both Briere (equation 11) models from estimated curves as follows.

Lactin-1 optimal development temperature calculation

$$\text{Topt} = \text{Tmax} - \text{deltaT}$$

(equation 10) (Roy *et al*. 2002)

Where Tmax is the maximum development threshold and deltaT is as derived from Lactin-1 model, as deltaT is a parameter calculated from Lactin-1.

Briere-1 and -2 optimal development temperature

$$\frac{2bbTmaxTmin + \sqrt{4bb^2}Tmax^2 + (bb+1)^2 - 4bb^2TminTmax}{4bb+2}$$

(Equation 11)

When *bb*=2 equation 11 denotes Topt for Briere-1 (Koda & Nakamura, 2012).

Comparison of model fit

There are many methods for comparing fitted non-linear models, such as the coefficient of determination (R^2), adjusted coefficient of determination (R^2_{adj}),

residual sum of squares (RSS), Akaike information criterion (AIC) and Bayesian information criterion (BIC). Two statistics, the RSS and AIC were used for comparison of model fits (Shi and Ge 2010), however, the number of biological parameters fitted and the of cardinal development limits were also taken into consideration.

RSS

The residual sum of squares is a measure of the distance of the values predicted by a model from an empirical dataset to which it is fit.

$$\text{RSS}=\sum_{i=1}^{N}(v_i - v_{id})^2$$

(Equation 7)

Where, N is the sample size, v_i the observed development rate at the ith temperature and v_{id} the expected development rate at the ith temperature.

AIC

$$\text{AIC}= 2k- 2\ln(L)$$

(Equation 8)

Where, k is the number of parameters including corresponding error and L is the maximised log-likelihood value, calculated from (equation 9):

$$L=-\frac{N}{2}Ln\left(\frac{Rss}{N}\right)$$

(Equation 9)

AIC is an estimation of the information lost when a specific model is used to describe a data set, it is a calculation of the trade-off between the goodness-of-fit and the complexity (number of parameters) of a given model (See equation 8) (Shi & Ge, 2010). As with RSS, preference is given to models with the lowest value, negative AIC values are suitable.

Coefficients of determination (R^2) were not compared between linear and non-linear models because linear models were only fitted to the linear portion of data sets making a direct comparison inappropriate.

4.3 RESULTS

4.3.1 Effect of temperature on the growth of six hypocrealean entomopathogenic fungi from four different genera, compared against *Pandora neoaphidis* (Entomopthoromycota, Entomophthorales)

Growth rates of all isolates followed the same general pattern, with the slowest growth rates observed at temperature extremes. Figure 4.4 illustrates the asymmetrical nature of the curve which is skewed to the left (lower temperatures). Temperature had a significant impact on growth rates (ANOVA, F= 116.26, df= 5, $P<0.001$). Growth rates at all temperatures varied significantly from each other except 15 and 33°C, and 20 and 30°C (Tukey HSD, $P<0.001$). There was no significant difference in the growth rate of *B. bassiana* 432.99 when run as a standard in *P. neoaphidis* growth assays, and in the experimental data for *B. bassiana* 432.99 (ANOVA, $P>0.05$), allowing both datasets to be treated as one.

Growth rates also varied significantly between entomopathogenic fungal isolates (ANOVA, F= 38.576, df= 7, $P<0.001$), with both *P. neoaphidis* isolates (NW420 and WEL1) varying significantly from all isolates except *L. longisporum* (Tukey HSD, $P>0.05$). *P. neoaphidis* isolates did not significantly vary from each other (Tukey HSD, $P= 1$). *Lecanicillium longisporum* in turn varied from all other Hypocrealean isolates (Tukey HSD, $P>0.05$). There were no other significant growth rate*fungal isolate differences.

In addition, colony extension rates varied significantly between SDA and SEMA growth media (ANOVA, F= 144.001, df=1, $P<0.001$), with a general trend to higher growth rates on SEMA (Figure 4.4 and Table 4.1). There was a

significant isolate*growth media interaction (ANOVA, F= 4.047, df= 5, *P*= 0.00129). Both *P. neoaphidis* isolates varied significantly from all Hypocrealean species on SEMA (Tukey HSD, *P*<0.05) except *L. longisporum* (Tukey HSD, *P*>0.05). Both *P. neoaphidis* isolates also varied from *M. brunneum*, *B. bassiana* (strain 432.99) and *B. bassiana* (GHA) on SDA (Tukey HSD, *P*<0.05). *Beauveria bassiana* (strain 432.99) and *B. bassiana* (GHA) grown on SEMA varied significantly from all other Hypocrealean isolates, including themselves, when grown on SDA (Tukey HSD, *P*<0.05). *Lecanicillium muscarium* and *M. brunneum* grew significantly faster on SEMA than *L. longisporum* on SDA and SEMA, *L. muscarium* on SDA and *I. fumosorosea* on SDA (Tukey HSD, *P*<0.05). Finally, *L. longisporum* grew significantly slower than both *B. bassiana* isolates and *I. fumosorosea* on SEMA (Tukey HSD, *P*<0.05).

B. bassiana (strain 432.99) had the fastest growth rate in the study at 4.71mm/day^{-1} (25°C, SEMA) and *M. brunneum* had the fastest growth rate on SDA at 3.55mm/day^{-1} (25°C). Both *P. neoaphidis* isolates grew consistently slower than all hypocrealean fungi on SEMA, peaking at 1.79 mm/day^{-1} at 20°C and 1.50 mm/day^{-1} at 25°C for NW420 and WEL1 respectively. Overall, both *Lecanicillium* species were the slowest growing hypocrealean fungi (Table 4.1 and Appendix 1).

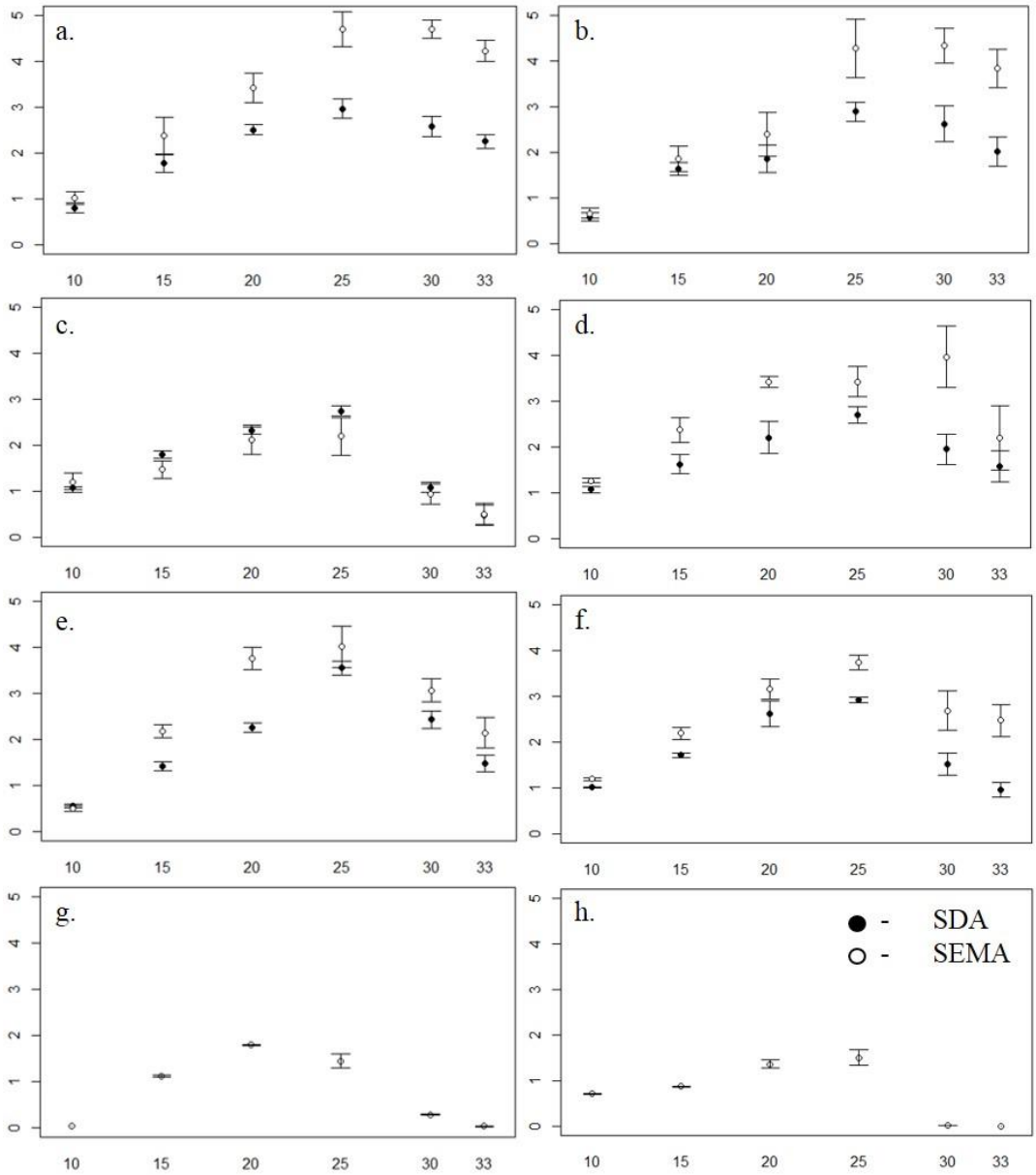


Figure 4.4 Effect of temperature on the rate of colony extension (±se) for a. *Beauveria bassiana* (strain 432.99) b. *Beauveria bassiana* (GHA) c. *Lecanicillium longisporum* d. *Lecanicillium muscarium* e. *Metarhizium brunneum* f. *Isaria fumosorosea* g. *Pandora neoaphidis* (NW420) and h. *Pandora neoaphidis* (WEL1) on SDA (filled circles) and SEMA (open circles).

Table 4.1 Effect of temperature on the growth rate (mm/day^{-1}) of different species of entomopathogenic fungi used in this study.

Fungal isolate	Temperature (ºC)	Growth media	
		SDA (mm/day^{-1})	SEMA (mm/day^{-1})
Beauveria bassiana (strain 432.99)	10	0.79	1.00
	15	1.77	2.37
	20	2.50	3.42
	25	2.96	4.70
	30	2.57	4.69
	33	2.24	4.22
Beauveria bassiana (strain 433.99)	10	0.58	0.66
	15	1.63	1.86
	20	1.85	2.40
	25	2.88	4.27
	30	2.62	4.33
	33	2.01	3.83
Lecanicillium longisporum	10	1.06	1.18
	15	1.79	1.46
	20	2.31	2.11
	25	2.74	2.19
	30	1.07	0.93
	33	0.47	0.50
Lecanicillium muscarium	10	1.06	1.26
	15	1.62	2.37
	20	2.20	3.41
	25	2.28	3.42
	30	1.95	3.96
	33	1.57	2.18
Metarhizium brunneum	10	0.55	0.49
	15	1.41	2.17
	20	2.25	3.75
	25	3.55	4.01
	30	2.43	3.06
	33	1.47	2.14
Isaria fumosorosea	10	1.00	1.17
	15	1.71	2.18
	20	2.60	3.15
	25	2.90	3.74
	30	1.51	2.68

	33	0.96	2.46
Pandora neoaphidis (NW420)	10	-	0.02
	15	-	1.11
	20	-	1.79
	25	-	1.44
	30	-	0.27
	33	-	0.025
Pandora neoaphidis (WEL1)	10	-	0.70
	15	-	0.86
	20	-	1.36
	25	-	1.50
	30	-	0.01
	33	-	0

4.3.2 Determining the rate of discharge of primary conidia of *P. neoaphidis* (NW420) from fungal mycelium

In general, production of conidia decreased over time. However, the rate at which conidia were discharged appeared to be fairly constant (Figure 4.5). In the first 20 hours, conidial counts ranged from 102.1 to 144.7 conidia/mm^2, whereas from 24 hours+ after removal from culture, conidial counts ranged from 44.6 to 118.8 conidia/mm^2. As a result of (i) increased variability, (ii) a drop in the total number of conidia produced, and (iii) to minimise potential differences in the response of conidia produced at different times (Morgan *et al*. 1995), conidia were consistently collected between 16-20 hours after plugs were removed from the culture medium. This finding was used to define the experimental protocols in this study (Figure 4.5). Data were similarly used for *P. neoaphidis* isolate WEL1.

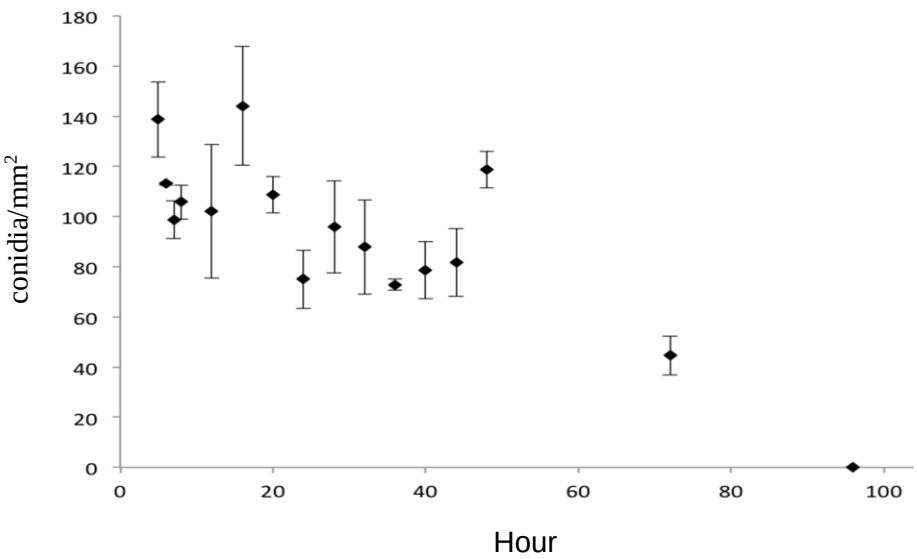


Figure 4.5: Scatter graph of the mean number of conidia (± SE) produced from plugs of *Pandora neoaphidis* (NW420) stored in darkness at 20ºC ± 1ºC at 5, 6, 7, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48 and 96 hours.

4.3.3 Effect of temperature on the germination of six selected hypocrealean fungal species from four different genera and *P. neoaphidis*

In general, the number of ungerminated conidia (category 1) counts decreased over time. However, the rate of germination was affected by temperature. The percentage of conidia in category one remained >95% and >92% at 30ºC and 33ºC for *P. neoaphidis* NW420 and *P. neoaphidis* WEL1 respectively (Table 4.3). Ghost conidia (category 5; empty primary conidia, no cytoplasm and the remains of the conidiophore) had a similar relationship with observation time, increasing with increasing time; however, no ghost conidia were seen at 30ºC and 33ºC for *P. neoaphidis* NW420, and fewer than 3% were seen at 30ºC and 33ºC for *P. neoaphidis* WEL1 (Table 4.3).

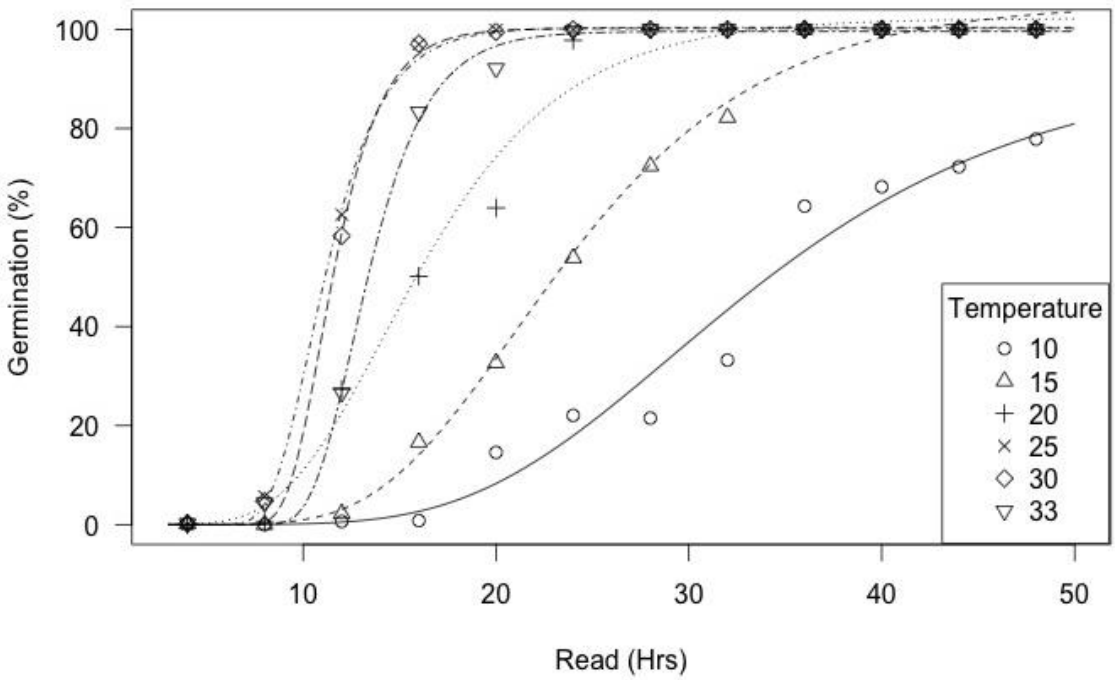


Figure 4.6 An example of the fit of the Weibull 2 model to *Beauveria bassiana* (432.99) germination over time at different constant temperatures. Time to 5, 50 and 90% germination were obtained from these plots for each species.

The time to 50% germination (GT50) was calculated as per the method above (section 4.3.5); figure 4.6 is an example of the plots produced by the process. All obtainable GT50's are shown in table 4.2. For *B. bassiana* 432.99 all GT50-temperature comparisons varied except comparisons between 10ºC and 15ºC, and 25ºC and 30ºC. All temperature comparisons of GT50 varied for *B. bassiana* 433.99 (GHA strain). Estimates of GT50 for *L. longisporum* did not significantly vary between 15ºC and 20ºC, 15ºC and 30ºC, and 20ºC and 30ºC. All other comparisons were significantly different. Additionally, GT50 values at 15 and 30ºC, and 10 and 33ºC for *L. muscarium* did not vary significantly; all other temperature comparisons were significantly different. Temperature did not appear to have a great effect on the GT50 of *M. brunneum* as the only significant differences were between 15 and 20ºC, 15 and 30ºC, 15 and 33ºC, 20 and 30ºC, 20 and 33ºC and 30 and 33ºC (Table 4.2).

Table 4.2 Comparison of GT50 at each temperature for *Beauveria bassiana* (432.99), *Beauveria bassiana* (433.99), *Lecanicillium longisporum*, *Lecanicillium muscarium* and *Metarhizium brunneum*.

Fungal isolate	Temperature comparison: GT50/GT50	*P*
Beauveria bassiana (432.99)	10/15:50/50	0.137
	10/20:50/50	0.0453
	10/25:50/50	0.024
	10/30:50/50	0.0244
	10/33:50/50	0.0306
	15/20:50/50	0
	15/25:50/50	0
	15/30:50/50	0
	15/33:50/50	0
	20/25:50/50	0
	20/30:50/50	0
	20/33:50/50	0
	25/30:50/50	0.621
	25/33:50/50	0
	30/33:50/50	0
Beauveria bassiana (433.99)	10/15:50/50	0.009
	10/20:50/50	0.0001
	10/25:50/50	0
	10/30:50/50	0
	10/33:50/50	0
	15/20:50/50	0
	15/25:50/50	0
	15/30:50/50	0
	15/33:50/50	0
	20/25:50/50	0
	20/30:50/50	0
	20/33:50/50	0.0025
	25/30:50/50	0.177
	25/33:50/50	0
	30/33:50/50	0
Lecanicillium longisporum	10/15:50/50	0
	10/20:50/50	0
	10/25:50/50	0
	10/30:50/50	0
	10/33:50/50	0
	15/20:50/50	0.3592

	15/25:50/50	0.0019
	15/30:50/50	0.3017
	15/33:50/50	0
	20/25:50/50	0
	20/30:50/50	0.6623
	20/33:50/50	0
	25/30:50/50	0
	25/33:50/50	0
	30/33:50/50	0
Lecanicillium muscarium	10/15:50/50	0
	10/20:50/50	0
	10/25:50/50	0
	10/30:50/50	0
	10/33:50/50	0.7635
	15/20:50/50	0
	15/25:50/50	0
	15/30:50/50	0.7475
	15/33:50/50	0.0003
	20/25:50/50	0.0011
	20/30:50/50	0
	20/33:50/50	0
	25/30:50/50	0
	25/33:50/50	0
	30/33:50/50	0.0003
Metarhizium brunneum	10/15:50/50	0.583
	10/20:50/50	0.5683
	10/25:50/50	0.5464
	10/30:50/50	0.5366
	10/33:50/50	0.5216
	15/20:50/50	0.0003
	15/25:50/50	0.2143
	15/30:50/50	0.0002
	15/33:50/50	0
	20/25:50/50	0.2949
	20/30:50/50	0.0029
	20/33:50/50	0
	25/30:50/50	0.8029
	25/33:50/50	0.676
	30/33:50/50	0.043

Table 4.3 The effect of temerature on the germination stage (1-6) of both *Pandora neoaphidis* isolates (NW420 and WEL1). (1) Ungerminated, (2) Germ tube visible <50% width of conidia, (3) Germ tube >50% width of conidia, (4) Majority of cytoplasm in germ tube or secondary conidia, fully formed secondary conidia, (5) 'Ghost' conidia, empty primary conidia and (6) Secondary conidia Data shown is percentage of total conidia in each category.

Fungal isolate	Temperature	Read (Hour)	Germination catergory					
			1	2	3	4	5	6
		8	91.670675	1.255611672	2.808842392	4.264870932	0	0
		12	80.41288984	3.292453092	6.138323026	0.31152648	9.84480756	0
	10	16	50.08298993	4.070668914	11.81656885	0.260416667	3.55790043	30.2114552
		24	31.16943832	26.93272891	9.303997195	17.50617779	8.95102852	6.13662927
		48	17.45760783	8.068737908	8.026061227	7.536701946	53.8978036	5.01308752
		8	83.46237551	0	1.707118019	9.53504601	5.29546046	0
		12	63.2089171	8.862169214	22.54757121	0.41404712	4.96729536	0
	15	16	45.82146249	3.264482431	6.558641975	0.967473884	17.9368471	25.4510921
		24	24.36162871	16.563147	10.54520359	8.902691511	33.4989648	6.12836439
		48	17.49269038	2.278063329	12.97097629	8.065903733	55.9464189	3.24594737
		8	51.60406985	0.262467192	1.935071087	27.46562788	17.6123158	1.12044818
		12	58.04885603	3.742056753	8.734478043	0	29.4746092	0
	20	16	23.72822943	1.929969672	16.31396821	1.23380204	52.444823	4.34920768
		24	11.65557049	7.629953268	7.311525084	3.498922391	58.0504876	11.8535412
		48	8.869687402	5.136214018	3.093477937	4.634167847	69.8946336	8.37181918
Pandora neoaphidis (NW420)		8	93.0330033	0.333333333	0	0.663366337	2.31683168	3.65346535
		12	98.87278583	0.322061192	0	0	0.80515298	0
	25	16	66.3800527	0.366300366	3.382816014	0	25.2850074	4.58582353
		24	27.64453713	0.994477272	4.866180049	0.243309002	52.2013672	14.0501294
		48	6.403265695	0	0	4.33836894	88.3734096	0.88495575
		8	99.34640523	0	0	0	0	0.65359477
		12	100	0	0	0	0	0
	30	16	96.80888267	0	3.191117328	0	0	0
		24	96.17145997	0	0	0	0	3.82854003
		48	100	0	0	0	0	0
		8	100	0	0	0	0	0
		12	99.68253968	0	0.317460317	0	0	0
	33	16	99.71014493	0	0.289855072	0	0	0
		24	100	0	0	0	0	0
		48	100	0	0	0	0	0
		8	99.02912621	0	0.647249191	0	0.3236246	0
		12	53.62153914	9.19300104	29.69599294	0.266666667	7.22280021	0
	10	16	21.83560513	14.01394249	40.58759178	11.12349463	11.5384651	0.9009009
		24	22.09890989	21.92098358	7.507598679	13.7742685	32.4977002	2.20053915
		48	40.48716261	2.867383513	1.660449126	2.962475313	41.0430839	10.9794455
		8	82.54747445	0.856231109	10.19155475	3.098414871	0.18018018	3.12614465
		12	59.98908499	4.174344174	8.958818959	0.567580568	26.3101713	0
	15	16	22.92968469	10.6626625	18.78232062	4.381239675	41.9557567	1.28833585
		24	12.82883783	9.286173597	9.842054497	6.795503347	53.5361765	7.71125426
		48	7.10646817	0.93377065	3.777784877	5.420775385	65.0398981	17.7213029
		8	76.1656535	0	5.864066194	4.315265113	9.72070247	3.93431273
		12	44.13009913	3.10511411	12.86456274	0.731848006	38.9268301	0.24154589
	20	16	22.77558612	4.012071688	9.217274509	0.219298246	52.8839325	10.8918369
		24	8.780525305	2.726586439	4.722487442	7.405654648	52.1944241	24.1703221
		48	5.044670033	0.459770115	0.934579439	0.934579439	76.2470906	16.3793103
Pandora neoaphidis (WEL1)		8	57.84743634	0	2.174450915	4.481176963	7.18472841	28.3122074
		12	96.95514658	0.786224821	0.492011619	0	1.47421932	0.29239766
	25	16	31.0330771	5.637755559	13.16857496	0	48.6057356	1.55485674
		24	48.07429169	11.46601921	3.101508833	3.298583936	18.276714	15.7828823
		48	48.50770826	1.604752376	1.154466626	1.818205992	32.668486	14.2463808
		8	100	0	0	0	0	0
		12	100	0	0	0	0	0
	30	16	95.79976521	0.56998557	1.767676768	0	1.35752195	0.50505051
		24	98.10267857	0.297619048	0	0.297619048	0	1.30208333
		48	92.33763005	0	0	0	6.03635369	1.62601626
		8	100	0	0	0	0	0
		12	100	0	0	0	0	0
	33	16	96.69135331	0	0.252525253	0	3.05612144	0
		24	99.33333333	0.666666667	0	0	0	0
		48	96.44238325	0	0	0	0	3.55761675

Linear regression was not suitable for describing the relationship between GT50 and temperature as data was collected past the optimum temperature. As a result the following non-linear models were used to describe the data; Taylor, Lactin-1, Kontdamis, Briere-1 and Briere-2. Additionally, the nature of the curve for *I. fumosorosea* was such that the models here were not sufficient to accurately describe the data; therefore, further analysis of germination time over temperature was not conducted. Also, despite optimisation of parameter estimates the Briere-2 model did not fit GT50 data for *M. brunneum* or *L. longisporum.* The only model that described a good deal of the relationship between GT50 and temperature for *P. neoaphidis* isolates (NW420 and WEL1) was Kontdamis.

Interestingly, the best model varied, indicated by the lowest AIC and RSS values, between species and even within genus. The model that provided the best description of the data for *B. bassiana* 432.99 was Taylor (RSS = 0.002543), whereas for *B. bassiana* (433.99) Briere-1 was the best (RSS = 0.003927). Similarly, Briere-1 fit *L. longisporum* data the best but Kontdamis best explained *L. muscarium* data. In general, all models fit *L. longisporum* data comparatively poorly compared with other species (Table 4.4). As with *B. bassiana* 432.99 the Taylor model explained the most variation in *M. brunneum.*

Table 4.4 Parameter estimates with their corresponding error and measure of model fit of five temperature-dependent growth models for time to 50% germination (GT50) data.

Fungal isolate	Temperature	Germination time (GT)	T0	TM	Topt	Rmax	aa	DeltaT
B. bassiana 432.99	Taylor	GT50	9.930104 (0.545027)	-	27.597939 (0.390244)	0.088064 (0.001855)	-	-
	Lactin-1	GT50	-	36.94647 (0.88500)	-	-	0.12476 (0.01174)	7.99548 (0.74613)
	Kontdamis	GT50	3.632 (1.095)	40.18 (0.9101)	-	-	0.00001219 (0.000002107)	-
	Briere-1	GT50	0.3760 (3.052)	35.84 (0.5760)	28.709850	-	0.00004116 (0.000006621)	-
	Briere-2	GT50	4.660 (5.842)	38.39 (5.843)	28.193730	-	0.00002108 (0.00003617)	-
B. bassiana 433.99	Taylor	GT50	10.15261 (0.697996)	-	27.071505 (0.587388)	0.092104 (0.002914)	-	-
	Lactin-1	GT50	-	38.76654 (2.32413)	-	-	0.09041 (0.01560)	10.96353 (1.85638)
	Kontdamis	GT50	1.884 (1.413)	40.33 (1.343)	-	-	0.00001088 (0.000002440)	-
	Briere-1	GT50	-1.478 (2.721)	35.53 (0.5017)		-	0.00004152 (0.000005723)	-
	Briere-2	GT50	-88.94 (6.375)	35.77 (2.665)		-	0.00003918 (0.00002517)	-
L. longisporum	Taylor	GT50	13.51819 (13.70017)	-	22.685 (4.76777)	0.1028 (0.02124)	-	-
	Lactin-1	GT50	-	34.85582 (5.37863)	-	-	0.0933 (0.05824)	10.59691 (6.44188)
	Kontdamis	GT50	3.190 (22.84)	59.86 (48.76)	-	-	-0.000003750 (0.00001461)	-
	Briere-1	GT50	-30.06 (11)	32.75 (3.674)	24.341530	-	0.00002815 (0.00006572)	-
	Briere-2	GT50	-	-	-	-	-	-
L. muscarium	Taylor	GT50	8.562574 (0.862162)	-	22.513349 (0.685484)	0.072323 (0.00532)	-	-
	Lactin-1	GT50	-	34.58825 (0.75164)	-	-	0.10899 (0.01514)	9.13492 (1.25284)
	Kontdamis	GT50	-19.28 (2.006)	35.58 (0.7797)	-	-	0.00001045 (0.000002665)	-
	Briere-1	GT50	-30.06 (34.05)	33.72 (0.5488)	25.095960	-	0.00001654 (0.00001124)	-
	Briere-2	GT50	0.08527 (9.335)	35.75 (3.458)	23.572310	-	0.000009633 (.00001523)	-
M. brunneum	Taylor	GT50	12.12148 (6.21832)	-	31.35986 (6.54179)	0.09643 (0.01123)	-	-
	Lactin-1	GT50	-	-	-	-	-	-
	Kontdamis	GT50	9.571 (6.048)	93.25 (83.34)	-	-	-0.000001148 (0.0000033)	-
	Briere-1	GT50	-2.313 (25.02)	40.34 (11.32)	32.048760	-	0.00003069 (0.00004131)	-
	Briere-2	GT50	-	-	-	-	-	-
P. neoaphidis (NW420)	Kontdamis	GT50	-12.05 (8.005)	32.17 (3.151)	-	-	0.000005455 (0.000004303)	-
P. neoaphidis (WEL1)	Kontdamis	GT50	-23.60 (33.30)	37.14 (13.49)	-	-	0.000002456 (0.00005826)	-

4.3.4 Temperature effects on *B. brassicae* development (time to adulthood)

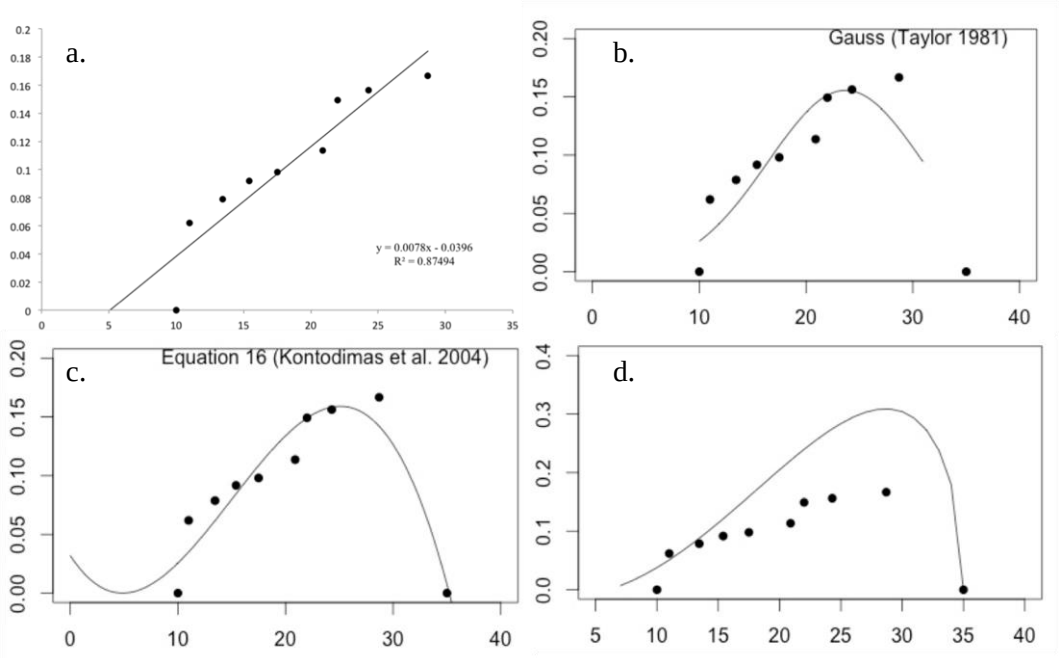


Figure 4.7 Comparison of three temperature-dependent growth models developed by Taylor (b.), Kontodimas (c.) and Briere-1 (d.) adjusted to the development rate of *Brevicoryne brassicae* at recorded incubator temperatures. Liner regression (a.).

No nymphs survivied for longer than 24 hours at 10 ºC. Development rate was fastest at 28.7 ºC (0.1667) and this was the maximum experimental temperature. Taylor provided a poor estimate of R_{max} and T_{opt} as the symmetrical nature of its curve proved insufficient to account for the steep drop off in development rate at high temperatures (Figure 4.7). The maximum development rate (R_{max}) was under-estimated by the Taylor model (0.1555 *vs* 0.1667) (Table 4.10).

The linear regression (R^2 = 0.87) of development rates at the range of experimental temperatures indicates that the accumulated thermal units needed for

B.brassiace K3 clone to develop from nymph to adulthood was 128.21-day degrees (Figure 4.7). The minimum temperature threshold (T_0) was 5.08 ^oC for the simple linear regression model. Estimates of T_0 ranged between 4.86 and 7.25 ^oC, in the Kontodimas and Taylor models respectively, showing low standard error (SE) (Table 4.10).

Briere-1 (Figure 4.7 (d.)) provided a poor fit to the data. An upward inflexion at the lower temperatures skewed the estimate of T_0 for *B. brassicae*, the SE is almost as high as the parameter estimate (Table 4.10).

The Taylor model underestimated the Topt for *B. brassicae* development, whereas Briere-1 provided a good estimate of Topt but resulted in a severely overestimated max development rate (Figure 4.7 (d)).

The values of AIC and RSS gave an evaluation of the portion of data variability accounted for by the models. All AIC values were negative. The greatest AIC and RSS values were for the Briere-1 model. The model that provided the best fit to the data was Kontodimas; AIC and RSS values -43.8912 and 0.0216 respectively, with good estimates of T_0, T_M, and T_{opt} (Figure 4.7).

4.3.5 The effect of temperature on the fecundity and survival of adult *B. brassicae* survival

Analysis indicates that survival was dependent on temperature (Chi-square = 11.501, df = 5, P = 0.42), with significantly more individuals surviving at 12^oC compared to 28^oC (Table 4.5). The lowest levels of survival were at 28^oC and 24^oC (Figure 4.8).

Figure 4.9 indicates the relationship between temperature and hazard. The hazard ratios increase with increasing temperature except at 15 ^{o}C, which had a hazard ratio greater than that of 20°C and 17.5^{o}C. However, the estimated 95% confidence intervals for the hazard ratio of 15°C have the largest spreads suggesting a large amount of variation in the observed responses (Table 4.5). As expected, the pattern is also mirrored in the survival data for 15°C (Figure 4.8).

Table 4.5 Survival analysis results of the time-mortality response of *Brevicoryne brassicae* adults to a range of constant temperatures after 7 days. Data from the control bioassays used in experiment 4.2.3.5.

			95.0% CI for hazard ratio	
Target temperature (^{o}C)	***P* (Hazard ratio)**	**Hazard ratio**	**Lower**	**Upper**
12	0.006	0.39	0.2	0.761
15	0.311	0.766	0.457	1.283
17.5	0.063	0.585	0.332	1.029
20	0.129	0.697	0.437	1.111
24	0.482	0.856	0.555	1.321
28	0.068	-	-	-

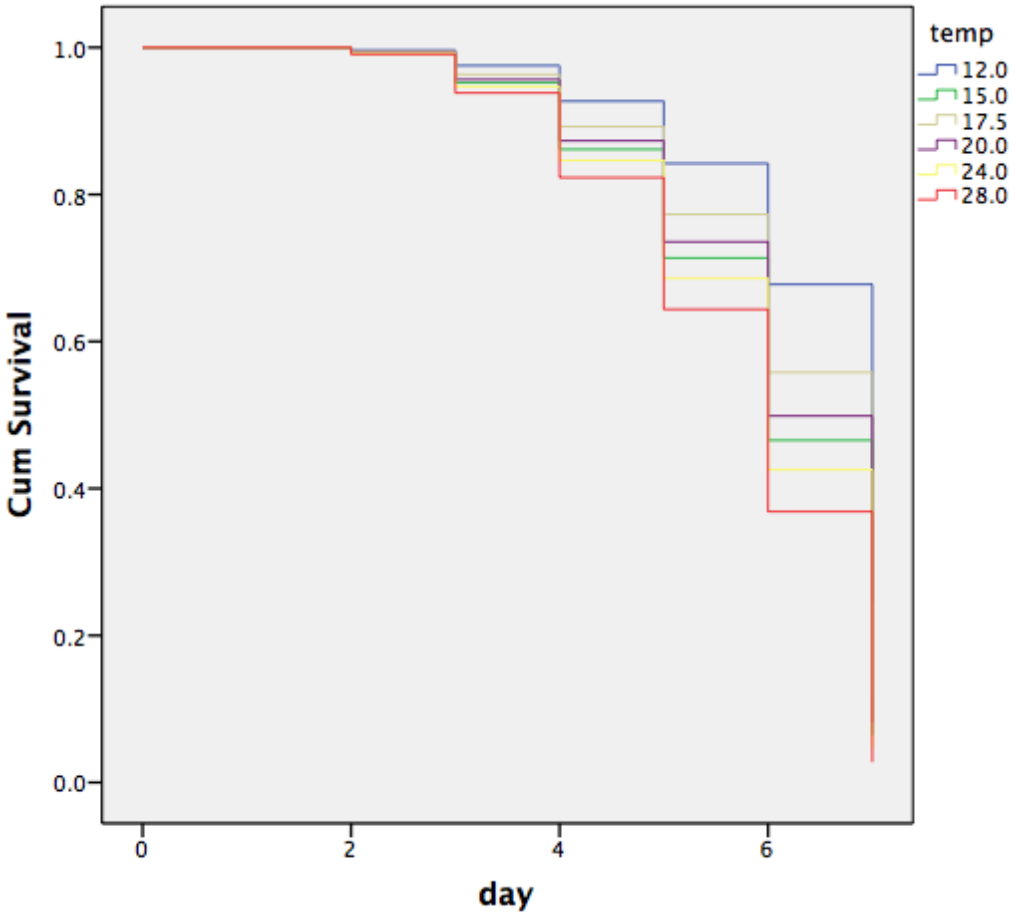


Figure 4.8. Cumulative survival of *Brevicoryne brassicae* controls over the assessment period at target temperatures during experiment 4.2.3.5.

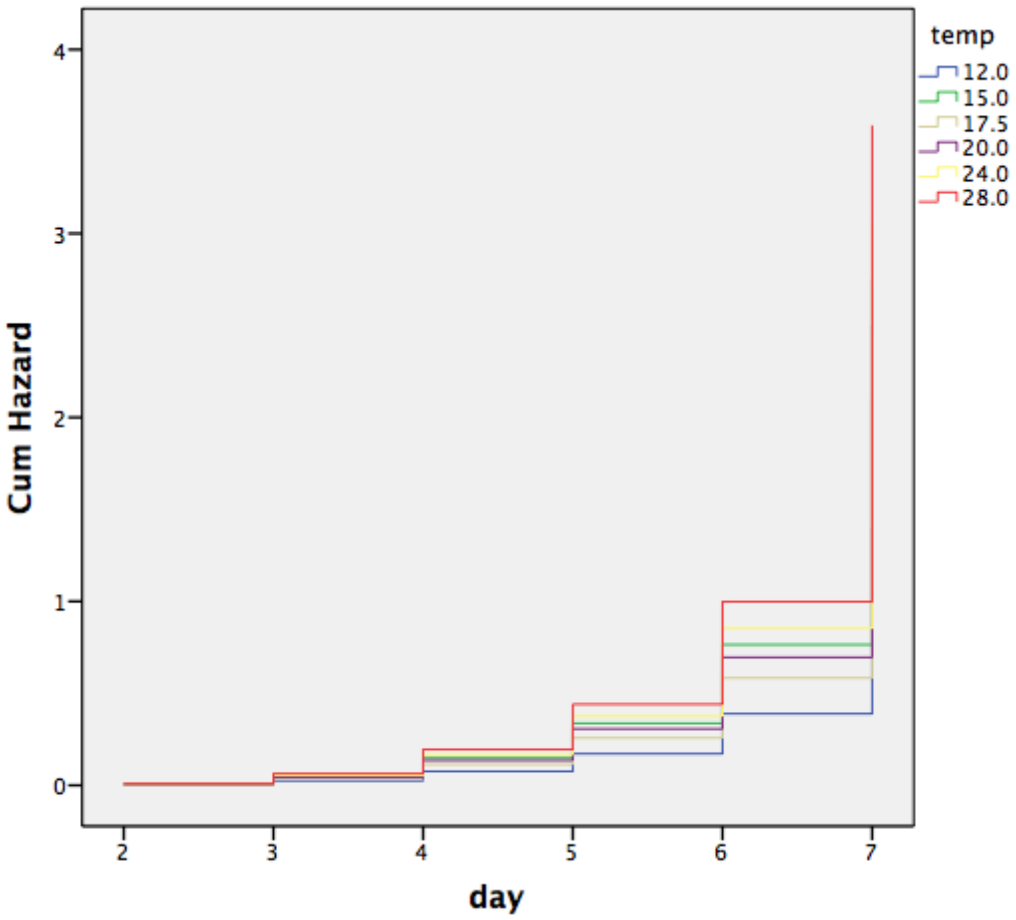


Figure 4.9. Cumulative hazard function for *Brevicoryne brassicae* controls at each target temperature over the assessment period for experiment 4.2.3.5.

Fecundity

At the set of experimental target temperatures, temperature had a significant effect on *per capita* production of *B. brassicae* nymphs after 5 days (ANOVA, F = 5.384, df = 5, *P* = 0.001). Tukey's HSD comparison indicates that significantly more nymphs/capita were produced at 20ºC than at 12ºC, (*P* = 0.007), 15ºC (*P* = 0.02) and 28ºC (*P*<0.001) (Figure 4.10). There were no other significant differences.

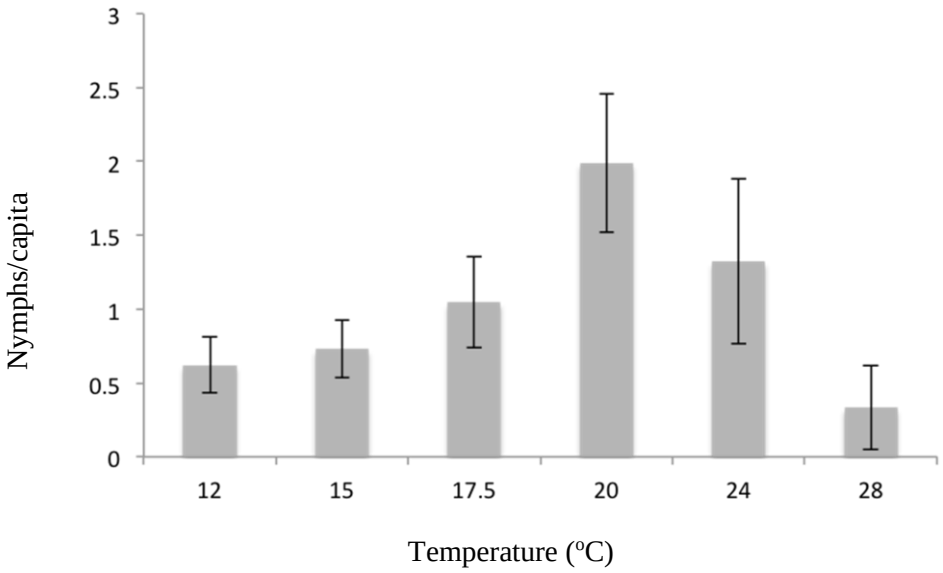


Figure 4.10 Mean *per capita* production (±SE) of *Brevicoryne brassicae* nymphs (control) at day 5 in experiment 4.2.3.5.

4.3.6 Susceptibility of adult *Myzus persicae* and *Brevicoryne brassicae* to the hypocrealean entomopathogenic fungi *Beauveria bassiana, Lecanicillium muscarium and Lecanicillium longisporum*

A two-parameter log-logistic model was applied to dose-response data as it was the only model that significantly fitted all aphid*fungal isolate combinations, thus allowing for a more direct comparison of results (Figure 4.11).

Table 4.6. The differential susceptibility (LD 10, 50 and 95) (±SE) of *Brevicoryne brassicae* and *Myzus persicae* to *Lecanicillium longisporum*, *Lecanicillium muscarium* and *Beauveria bassiana* (432.99). Estimates were produced by a two-parameter log-logistic model.

Aphid species	Fungal Isolate	LD	Estimate	Std. Error
	Lecanicillium longisporum	10	8.52E+05	4.25E+06
	Lecanicillium longisporum	50	9.65E+05	1.08E+06
	Lecanicillium longisporum	95	1.14E+06	4.64E+06
	Lecanicillium muscarium	10	4.10E+04	6.07E+04
Brevicoryne brassicae	*Lecanicillium muscarium*	50	5.65E+05	3.52E+05
	Lecanicillium muscarium	95	1.90E+07	3.65E+07
	Beauveria bassiana 432.99	10	2.17E+02	2.26E+03
	Beauveria bassiana 432.99	50	4.36E+04	1.11E+05
	Beauveria bassiana 432.99	95	5.30E+07	4.95E+08
	Lecanicillium longisporum	10	5.62E+06	1.64E+07
	Lecanicillium longisporum	50	6.24E+07	4.84E+08
	Lecanicillium longisporum	95	1.57E+09	2.26E+10
	Lecanicillium muscarium	10	2.44E+06	7.63E+06
Myzus persicae	*Lecanicillium muscarium*	50	7.92E+07	5.00E+08
	Lecanicillium muscarium	95	8.40E+09	9.43E+10
	Beauveria bassiana 432.99	10	4.25E+05	7.06E+05
	Beauveria bassiana 432.99	50	1.02E+07	1.78E+07
	Beauveria bassiana 432.99	95	7.19E+08	3.42E+09

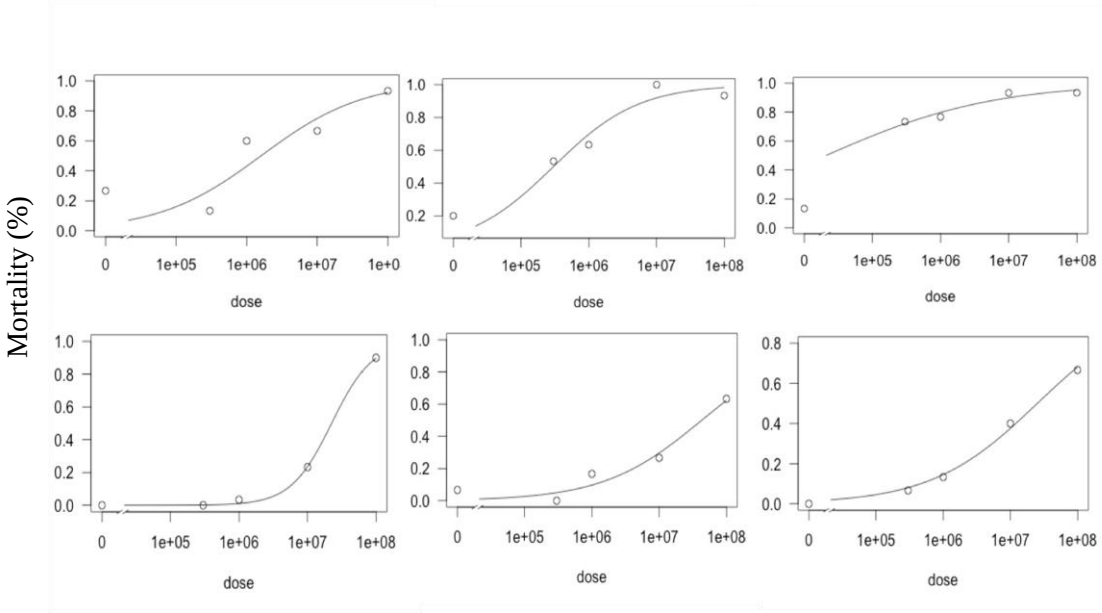


Figure 4.11 Dose response relationship graphs. Top three graphs are for *Brevicoryne brassicae*, from left to right: *Lecanicillium longisporum*, *Lecanicillium muscarium* and *Beauveria bassiana* 432.99. The bottom three graphs are for *Myzus persicae*, from left to right: *Lecanicillium longisporum*, *Lecanicillium muscarium* and *Beauveria bassiana* 432.99. Model fitted, 2 parameter log-logistic.

In all cases, *B. brassicae* was more susceptible than *M. persicae* to the fungal entomopathogens tested (Figure 4.11). Based on the LD-values calculated, both *B. brassicae* and *M. persicae* were more susceptible to *B. bassiana* 432.99. Similarly, both aphid species appeared to be less susceptible to *L. longisporum* based on the concentration of conidia required to kill 10, 50 or 95% of the exposed individuals (Table 4.6).

4.3.7 Effect of temperature on the mortality of *Brevicoryne brassicae* adults treated with *Lecanicillium longisporum* 1.72 and *Beauveria bassiana* 432.99

Generally, *L. longisporum* was more virulent than *B. bassiana* 432.99 based on the calculated LT-values (Table 4.7). However, mortality rate was significantly affected by temperature, as mortality was <50% at two of the experimental temperatures after 7 days, whereas, mortality reached 50% or higher for *B. bassiana* at all experimental temperatures after 7 days.

Table 4.7 The effect of temperature on lethal time to 50% mortality (LT50) for *Beauveria bassiana* (432.99) and *Lecanicillium longisporum* applied at the calculated LD95 for *Brevicoryne brassicae.* Data are based on mycosed individuals and '*' indicates treatments in which 50% mycosis was not reached.

Fungal isolate	Temperature (°C)	LT50	Std. Err
B. bassiana (432.99)	12	9.34563	1.07116
B. bassiana (432.99)	15	6.77474	0.34681
B. bassiana (432.99)	17.5	6.22834	0.44186
B. bassiana (432.99)	20	4.56866	0.21684
B. bassiana (432.99)	24	3.96378	0.16426
B. bassiana (432.99)	28	7.34664	0.85231
L. longisporum	12	7.1146	3.4846
L. longisporum	15	4.1	-
L. longisporum	17.5	3.9895*	-
L. longisporum	20	4.3698	1.3327
L. longisporum	24	3.4341	0.58429
L. longisporum	28	2.9951*	0.019088

Of the four non-linear models (Taylor, Lactin-1, Kontdamis & Briere-1) applied to LT50 data for each isolate (*B. bassiana* 432.99 and *L. longisporum*), the

Lactin-1 model provided the best fit with *B. bassiana* 432.99 data and Briere-1 to *L. longisporum* data. However, the Briere-1 model has notably high standard error for its estimates and the Taylor model dramatically reduced this error with very little effect on model fit parameters (Table 4.11).

4.3.8 Effect of temperature on the virulence of *Pandora neoaphidis* to *Brevicoryne brassicae*

The dose-response relationship for *P. neoaphidis* was less complex than with the hypocrealean fungal isolates in experiment 4.3.6 because mortality due to fungal infection was independent of conidia concentration. This relationship is apparent in Figure 4.12. Analysis of conidial concentration and mortality attributable to fungal infection (i.e. mycosed individuals) on day 5 shows no significant effect of conidia concentration (conidia/mm^2) on aphid mortality from either *P. neoaphidis* isolate, ANOVA, F= 1.415, df= 38, *P*= 0.108 and ANOVA, F= 1.176, df= 37, *P*= 0.271, for WEL1 and NW420 respectively. Analysis excluded control data 0 conidia/mm^2, as there were significant differences between exposure and non-exposure to the pathogen. As a result, analysis of lethal time to 50% mortality (LT50) was conducted on grouped conidia concentration data. Analysis is below, however, in the first instance a temperature relationship can be inferred from table 4.8.

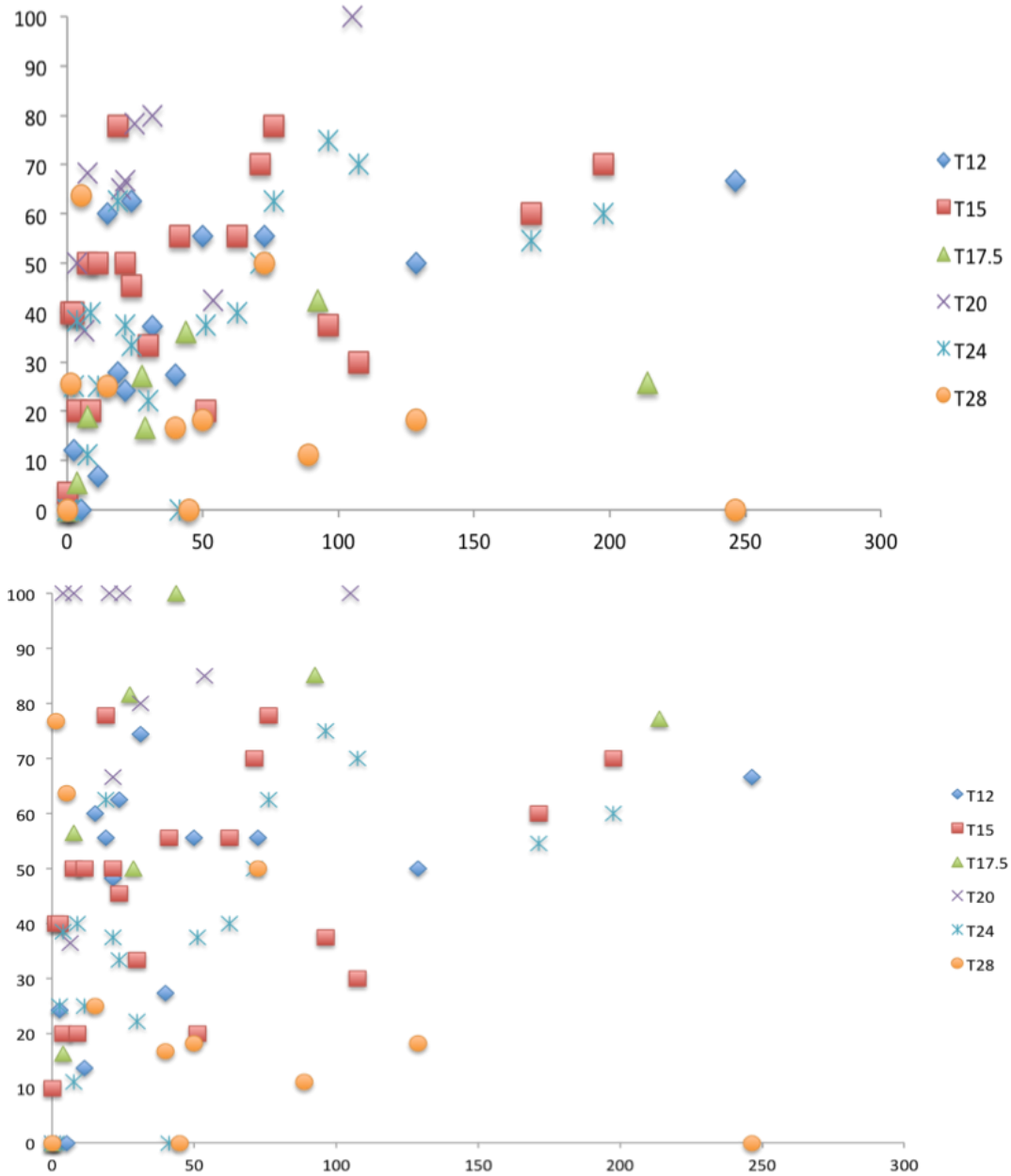


Figure 4.12 Effect of conidia concentration (conidia/mm^2) on percentage mycosis of *Brevicoryne brassicae* adults on day 5 at each experimental temperature. Top graph *Pandora neoaphidis* NW420, bottom *P. neoaphidis* WEL1.

Table 4.8 The effect of temperature on Lethal time to 50% mortality for *Brevicoryne brassicae* (LT50) for both *Pandora neoaphidis* isolates.

Fungal isolate	Temperature	LT50	Std. Err
P. neoaphidis (NW420)	12	11.415	0.00078291
	15	7.1981	0.45534
	17.5	4.9574	-
	20	4.5035	0.086202
	25	5.0366	-
	28	5.3149	-
P. neoaphidis (WEL1)	12	7.5805	0.0019725
	15	4.2592	-
	17.5	4.214	-
	20	5.8932	0.11585
	25	5.5415	-
	28	9.4991	0.31875

Table 4.11. Parameter estimates with their corresponding error and measure of model fit of four temperature-dependent growth models for lethal time to 50% mortality (LT50) of *Brevicoryne brassicae* exposed to selected fungal pathogens.

Fungal isolate	Temperature	Lethal time	T0	TM	Topt	Rmax	aa	DeltaT	RSS	AIC
Beauveria bassiana 432.99	Taylor	LT50	7.04783 (1.20875)	-	21.83957 (0.88368)	0.22981 (0.2198)	-	-	0.030730	-20.921830
	Lactin-1	LT50	-	29.538238 (0.251296)	-	-	0.170775 (0.009753)	5.830149 (0.327198)	0.011710	-32.496900
	Kontdamis	LT50	4.544 (1.984)	31.21 (1.11)	-	-	0.00008278 (0.00003123)	-	0.026130	-22.869360
	Briere-1	LT50	3.170 (2.666)	28.83 (0.2812)	23.404030	-	0.0002185 (0.00004094)	-	0.017140	-27.929320
Lecanicillium longisporum	Taylor	LT50	19.1966 (16.8672)	-	34.9201 (24.8325)	0.3494 (0.1495)	-	-	0.035860	-19.070770
	Lactin-1	LT50	-	43.84620 (27.06680)	-	-	0.08207 (0.05796)	11.87663 (8.17005)	0.036630	-18.818180
	Kontdamis	LT50	11.157996 (1.501945)	23.676140 (0.973985)	-	-	-0.001043 (0.000469)	-	0.181300	0.379075
	Briere-1	LT50	-24.57 (50.29)	53.95 (14.55)	41.266650	-	0.000008381 (0.0001769)	-	0.034740	-19.448370
Pandora neoaphidis (NW420)	Taylor	LT50	-7.71210 (0.01174)	-	22.36991 (0.52859)	0.22964 (0.01174)	-	-	0.015720	-28.963160
	Lactin-1	LT50	-	31.2329 (1.7352)	-	-	0.1390 (0.0306)	7.1378 (1.5335)	0.029850	-21.270550
	Kontdamis	LT50	9.651 (0.6145)	46.53 (2.820)	-	-	-0.00002926 (0.000008346)	-	0.013690	-30.620870
	Briere-1	LT50	-1.416 (7.724)	30.06 (1.162)	23.910470	-	0.0001521 (0.00006776)	-	0.025230	-23.287620
Pandora neoaphidis (WEL1)	Taylor	LT50	-8.00118 (2.09748)	-	18.54569 (1.44540)	0.22009 (0.02682)	-	-	0.045055	-17.594220
	Lactin-1	LT50	-	30.31247 (2.06621)	-	-	0.09655 (0.04051)	10.04400 (3.91328)	0.045150	-16.301200
	Kontdamis	LT50	-3.411 (7.533)	30.94 (2.113)	-	-	0.00003599 (0.00003209)	-	0.042340	-17.074720
	Briere-1	LT50	-64.57 (137.1)	29.13 (1.523)	20.775780	-	0.000005210 (0.0001080)	-	0.043550	-16.738280

Pandora neoaphidis (WEL1) data did not lend itself as much as isolate NW420 data to modelling with these equations as AIC and RSS values were notably lower in all cases. The models that provided the best description of the data were Kontdamis and Taylor for NW420 and WEL1 respectively (Table 4.11).

4.3.9 Effect of relative humidity on the virulence of *P. neoaphidis* to apterous adult *B. brassicae*

There was a general trend for mortality to increase with the amount of time spent in a high humidity environment (Figure 4.13). Mortality significantly varied according to fungal isolate (ANOVA, F = 20.478, df = 2, $P<0.001$) and duration at high humidity (ANOVA, F = 17.262, df = 4, $P<0.001$). Both *P. neoaphidis* isolates varied from each other (Tukey's HSD, $P = 0.0003129$) and from the control, $P = 0.0054890$ and $P>0.001$ for NW420 and WEL1 respectively. Aphids kept at high humidity for 4 and 8 hours suffered significantly less mortality than those kept at high relative humidity for 16, 20 and 24 hours (Table 4.9). The shortest durations, 4 and 8 hours, did not significantly vary from each other (Tukey's HSD, $P = 0.998$). The only significant difference within humidity treatments between *P. neoaphidis* isolates was observed when held at high humidity for 24 hours (NW420-WEL1, Tukey's HSD, $P = 0.0019$)

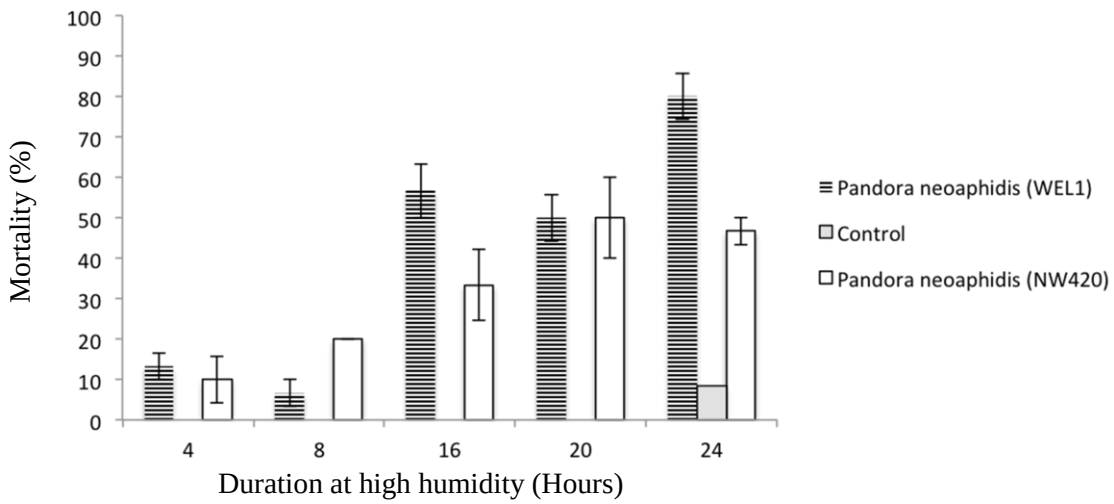


Figure 4.13 The effect of varying durations at high relative humidity on the mortality of *Brevicoryne brassicae* exposed to *Pandora neoaphidis* isolates WEL1 and NW420, 5 days after treatment.

Table 4.9 Post hoc Tukey HSD analysis showing significant differences in *Brevicoryne brassicae* mortality between different experimental durations at high humidity

Comparison	***P***
4-8	0.9998694
4-16	0.0000654
4-20	0.0000006
4-24	0.0000192
8-16	0.0000335
8-20	0.0000003
8-24	0.0000086
20-16	0.8518316

Table 4.10 Parameter estimates (with their standard error) and R^2, residual sum of squares (RSS) and Akaike information criterion (AIC) of a linear and three temperature dependent growth models for the development of *Brevicoryne brassicae*.

Model	T_0	T_M	T_{opt}	R_{max}	aa	R^2	RSS	AIC
Linear	5.0769	-	-	-	-	0.87494	-	-
Taylor	7.25411 (1.15362)	-	23.69841 (1.22089)	0.15555 (0.01819)	-	-	0.03204	-36.00198
Kontodimas	4.863 (1.469)	35.21 (0.618)	-	-	0.00003841 (0.000008282)	-	0.0216	-43.8912
Briere-1	6 (6,254)	35 (0.00039)	28.67012	-	0.000189 (0.00008099)	-	0.09283	-14.72702

4.4 DISCUSSION

The relationship between environmental temperature and the rate of physiological processes in ectothermic organisms is nonlinear. Fitting non-linear models allows subtleties in the response to be discussed, but only when sufficient data are gathered on the response to temperatures past the optimum. Numerous studies have investigated effects of temperature on growth and germination of entomopathogenic fungi, however, analyses have been confined to ANOVA meaning limited inference can be extrapolated from the data. Thomas & Jenkins (2014) attempted to address the nuances of the data by 'forcing' a curve fitting programme to fit to the data although the model had little physiological meaning. This study supports previous work by Eski *et al*. (1999), Yeo *et al*. (2003), Vidal *et al.* (2011), Thomas & Jenkins (2014), and Mohammed & Hatcher, (2016) in that it shows that temperature affects fungal growth, germination and fungus-induced mortality. However, the present study represents the first time that nonlinear temperature-dependent growth models have been applied to data other than fungal mycelial growth (Davidson *et al*. 2003), bacterial growth and insect performance (Godlizadeh *et al*. 2007; Kontodimas, 2012). This study suggests that these models provide a useful analytical method for describing other fungal biological characteristics such as germination and host-pathogen interactions such a speed of kill. Models were fit to mycelial growth, germination, insect development and fungus-induced insect mortality and compared. The ability to use a single model that enables description of different biological processes (growth, germination, insect development, fungal-induced mortality) within a single, unified modelling framework, is conceptually attractive. However, interestingly, no single model provided the best fit across these processes (Appendix 1). The best model fits and

parameter estimations for lethal time was achieved by Kontdamis, for growth Lactin-1 and for germination Briere-1. This illustrates the complexity and challenges of studying temperature-host-pathogen interactions. Choosing a single model would gloss over the intricacies of the biology, although direct comparison is useful and for the most part for models that fit the data well, cardinal estimates did not vary too much. With this in mind, the Briere-1 provided the best overall model for describing all the aforementioned biological processes. Model fit estimates, AIC and RSS, were consistently among the lowest, and it provides a good number of biologically relevant estimates; further, the model only includes three parameters.

The results presented here highlight differences in the cardinal temperature values of *P. neoaphidis* and host, contrary to the main hypothesis of this study that the biological thermal profiles of host and pathogen should match because of co-evolution (Thomas & Blandford, 2003). To produce these thermal biology difference selection may be acting in different ways on the host and pathogen. For generalist fungal pathogens which have a range of host species survival outside of the host might be prioritised leading to difference in their thermal tolerance to incorporate functioning at temperatures during which susceptible hosts are active or available for infection. By this logic, specialist organisms like the obligate parasite *P. neoaphidis* would be expected to more closely match the thermal preferences of their host species. Indeed this is what the results reported here suggest, the thermal profiles of *B. brassicae* and *P. neoaphidis* (for growth and LT50) are matched much more closely than that of the hypocrealean fungi. The optimum temperature for development of *B.brassicae* was calculated at 28.7 ^{o}C and the average estimate of T_{opt} for growth of *P. neoaphidis* was 28.2 ^{o}C, compared

to 27.8 ºC for *B. bassiana* 432.99, and 24.7 ºC for *L. longisporum*. Both *B. bassiana* isolates used in this study originate from the US, whereas the *P. neoaphidis* isolates NW420 and WEL1 originate from Denmark and the UK respectively. Geographic origin has been shown previously to affect temperature tolerances in entomopathogenic fungi (Drauzio *et al*., 2005; Vadim, *et al.*, 2017). Growth estimates of T_{opt} for *M. brunneum* and *L. muscarium* are closer to that of *P. neoaphidis* (section 4.3) supporting previous evidence that geographic origin can affect thermal tolerance in entomopathogenic fungi as these isolates are from Germany and the UK respectively. Denmark, the UK and Germany are European countries that share similar climates i.e. temperate, maritime. Additionally, the hypocrealean fungi in this study were not originally isolated from aphid hosts. The hypothesis of co-evolution assumes that both pathogen and host exist together. The extent to which these pathogens are affected by their origin is unknown, however, all were shown to be pathogenic towards aphids. Further highlighting their generalist nature.

The variation in optimum temperature estimates for growth, germination and virulence, even for species within the same genera, has significant implications for the selection of MCAs. As mentioned, the criteria for choosing MCAs should not be based solely on growth and/or germination performance as these appear to be poor indicators of fungal virulence**.**

Infection is a product of transmission of the pathogen which is a function of host density but also gemination capacity. Insect mortality is a product of the host-pathogen interaction but also growth of the pathogen within the insect host. This chapter has shown that both germination capcity, growth and fungus-induced mortality are all effected by temperature supporting findings on growth (Eski *et*

al. 1999; Yeo *et al*. 2003; Vidal *et al* 2011), germination (Yeo *et al*. 2003) and mortality (Kope *et al*. 2007; Regniere *et al*. 2012; Dimbi *et al*. 2004).

Conidia present on the cutile of insects must first germinate to initiate the infection process. If germination is prevented by unsuitable abiotic conditions such as low humidity or extremes of temperature, infection and therefore death will not occur. Interestingly, fungal species have been shown to be much more tolerant of low ambient humidities at temperatures close to their optimum, suggesting temperature may be the most important abiotic factor limiting fungal activity (Ayerst, 1969). The ability of a *P. neoaphidis* to kill a host has also been linked to humidity in this study. This is in line with previous studies on entomopathogenic fungi that show germination is dependent on suitable humidity regimes (Luz *et al*. 1997; Kope *et al*. 2008). It appears that *P. neoaphidis* requires a long period of high relative humidity in order to germinate. Field work conducted during this research has shown that the aphid population crash occurred in 2013 and 2014 during or immediately after periods of the highest relative humidity (section 3.4). Caution is advised however as the relative humidty directly around the spore- its boundary layer- will be vastly different than that of ambient. It is difficult to measure the relative humidity within this boundary layer as the layer is comprised of still air directly around the spore.

Results presented here support previous findings and suggest that host-pathogen interactions should be examined in future in the context of temperature and humidity regimes. Indeed, some investigations into the interactive effects of humidity and temperature have been undertaken, Fargues & Luz (2000) found that different regimes of relative humidity and temperature produced varying levels of mortality but concluded that humidity was the most important abiotic factor. Zhou

(2011) stated that infection levels in the field varied with temperature and humidity. This study suports the findings of Zhou (2011) and Fargues & Luz (2000) in that the biological processes that contribute to transmission are affected by abiotic factors such as temperature and humidity. Since transmision is key in the spread of fungal disease in the field a closer look at the abiotic factors affecting the biological prcesses that contribute to it is warranted. The results here suggest that the outcome of host-pathogen intereactions and therefore epizootics is a function of humidity and temperature.

Future work should look at using these temperature-dependent models to predict the effect of flutuating temperatures on host-pathogen interactions. Bioassays conducted at specified durations under optimal and sub-optimal conditions could be used to valiadate such predictive models before they were applied in field situations. Studies that have looked directly at fluctuating temperature bioassays have shown reduced infection by entomopathogenic fungi (Shah *et al*., 2002; Fragues & Luz, 2011). Presummably because of the reduced time at which the pathogen is exposed to suitbale abiotic conditons for germination and/or growth.

CHAPTER 5: CULTURAL METHODS AFFECTING THE APHID POPULATION CRASH

5.1 INTRODUCTION

With the withdrawal of many pesticides from the market by EU regulation (79/117/EEC) the need to find sustainable, environmentally friendly alternatives has increased. Of these alternatives, entomopathogenic fungi have received considerable interest because of their mode of action and pest specificity. The advent of integrated pest management (IPM) increases the likelihood that these biological control agents will be used alongside chemical pesticides. Clearly, a pesticide that inhibits the growth and/or germination of an entomopathogen is likely to result in an antagonistic interaction and reduce control efficacy if used in combination (Jaronski, 2010). For IPM to be successful chemical pesticides must be compatible with the entomopathogens that they are used with.

Crop husbandry practices can significantly impact the efficacy of entomopathogenic fungi, both those applied in an inundative capacity and those used in conservation biocontrol. More specifically to this study, cultural practices with field brassicas, such as sequential planting, often mean that there are ranges of different aged host plants with varying nutrient profiles available to pest species in agricultural landscapes. Host plant quality has been suggested as an important factor in aphid population crashes (Karley *et al*. 2003; Karley *et al*. 2004). By understanding the nature of tritrophic (i.e. plant-insect-pathogen) interactions in

the field, it will be easier to clarify triggers leading to aphid population crashes and determine how these may be enhanced to promote aphid control.

This study focuses on nitrogen as a limiting resource and looks at the performance of *Brevicoryne brassicae* on different aged plants and under a pathogen threat. It also considers the compatibility of three fungicides with *Pandora neoaphidis.* Fungicides were chosen on the basis that they are the most relevant in terms of brassica agronomy, i.e. most likely to come into contact with *Pandora* or other mycoinsecticides in the field.

5.2 MATERIALS AND METHODS

5.2.1 Effect of *Brassica oleracea* plant growth stage and leaf age on the reproduction of adult aphids

B. oleracea Gemmifera group (Brussels sprout, cultivar 'Trafalgar') (Tozer seeds Ltd, Surrey, UK, KT11 3EH) were cultured as per section 2.1 and three physiological ages were chosen for experiments in accordance with the BBCH-scale for other brassica vegetables. 'Young' plants were at growth stage BBCH-13/14 (third/fourth true leaf unfolded), 'medium' plants were at growth stage BBCH-16 (sixth/seventh true leaf unfolded) and 'old' plants were at growth stage BBCH-19 (eleventh/twelfth true leaf unfolded) (figure 5.1). Three plants of each age were used during each repetition of the experiment and the experiment was repeated three times. Clip cages containing one, young apterous *B. brassicae* adult (10-12 days) were attached to the abaxial side of the youngest and oldest leaves of each experimental plant. Nymph production and adult mortality were recorded

daily for seven days and any nymphs produced were carefully removed, minimizing disruption to adults. Plants were maintained in a controlled environment room at 20 ± 2°C, 16L: 8D photoperiod. After the experiment, young and old leaves infested with *B. brassicae* from all experimental plants were removed for soluble nitrogen analysis (section 5.2.3).

Figure 5.1 Experimental *Brassica oleracea* Gemmifera group (Brussels sprout, cultivar 'Trafalgar') plants from left to right; 'young' plants (BBCH-13/14 third/fourth true leaf unfolded), 'medium' plants (BBCH-16 sixth/seventh true leaf unfolded) and 'old' plants (BBCH-19 eleventh/twelfth true leaf unfolded).

5.2.2 Effect of host plant growth stage on the susceptibility of *B. brassicae* to the entomopathogenic fungus *P. neoaphidis*

B./ oleracea Gemmifera group (Brussels sprout, cultivar 'Trafalgar') (Tozer seeds Ltd, Surrey, UK, KT11 3EH) were cultured as per section 2.1. Cohorts of 60 young apterous *B. brassicae* adults (c.48 hours old) were placed on *B. oleracea* leaf discs

(2.5cm diameter Ø) with a fine camel hair paintbrush. Leaf discs were floated on distilled water in 90mm triple vented Petri dishes and held in place with drawing pins. Aphids were exposed to *P. neoaphidis* (isolate WEL1) conidia in a showering chamber (section 4). *P. neoaphidis* plugs (2.5mm Ø) were taken from the growing edge of 5-6-week-old cultures using a cork borer. Three plugs were arranged in a triangle formation and mounted in the lid of 45mm Petri dishes on moist filter paper, suspending them over 1.5% distilled water agar in the base. These Petri dishes were then stored at 15°C ± 1°C for 16-20 hours prior to their use to ensure plugs were sporulating profusely. Plugs of *P. neoaphidis* were inverted over aphid cohorts at a height of 2.5cm for 60 minutes. After one hour, 10-15 apterous aphids were transferred to treatment plants (either "young" plants at BBCH-13/14 or "medium" plants at growth stage BBCH-16) using a camel`s hair paintbrush. Plants were double bagged in bread bags for 48 hours in order to maintain high relative humidity (>97%). The concentration of spores applied to the aphids was estimated by placing an 18x18mm^2 coverslip under the plugs post conidial showering for 60 minutes and then staining it with 10% cotton blue in lacto-phenol. Estimates were calculated from counts of twenty 0.2mm^2 non-overlapping fields of view of primary conidia. This gave a concentration of 9.16±0.83 conidia/mm^2. Control aphids were treated in the same way but were not exposed to *P. neoaphidis*.

Following inoculation of the aphids with the fungus, the bags were removed and the plants were held in a controlled environment room at 20°C ± 1°C, 16:8 L: D photoperiod, 40-50% RH and aphids were monitored daily for mortality. Dead individuals were removed and if not sporulating were placed in a Petri dish lined with moist filter paper at held at 15°C ± 1°C to promote production of spores

on the surface of cadavers. Individual replicates lasted 7 days and the experiment was repeated on three occasions using two replicate plants for each *P. neoaphidis*-plant age treatment. At the end of the experiment the oldest leaves were taken for soluble nitrogen analysis (section 5.2.3)

5.2.3 Soluble Nitrogen analysis

Inorganic nitrogen in the form of nitrate was extracted by drying leaf material at 80 ± 1°C for 48 hours, grinding the dried material, adding RO water and then removing debris by pouring the sample into a 'fresh' universal through filter paper. Samples were left for a maximum of 30 minutes prior to analysis. The nitrates present in these samples were reduced to nitrite on a cadmium column and the nitrite was reacted with an indicator (50-50 methyl red and methylene blue), that changed colour. The intensity of the colour change is proportional to the concentration of nitrite and was measured by adsorption of light at a specific wavelength (400nm). Measurements were taken using an FIAstar 5000 flow injection analyser.

5.2.4 The effect of three fungicides on the growth of *P. neoaphidis* isolates on agar based medium

For this experiment, 7mm plugs were taken from the growing edge of 4-5-week-old *P. neoaphidis* (NW420 and WEL1) cultures grown on SEMA at 15°C ± 2°C in darkness. Plugs were inverted and placed in the centre of 90mm triple vented Petri dishes containing cool but unset SEMA, amended to contain 0, 5, 10, 25, 50, 100 and 200% of field rate application (a.i/ha) of the one of the following three

fungicides; azoxystrobin (Amistar®; Syngenta, Cambridge, UK), trifloxystrobin + tebuconazole (Nativo® 75WG; BayerCropScience, Cambridge, UK) and difenoconazole (Plover®; Syngenta) (Table 5.1). Petri dishes were transferred to a polypropylene box (150mm wide x 300mm long x 60mm high) containing a glass vial (15mm diameter x 30mm long) filled with sterilised distilled water and placed in an incubator in darkness at 20°C ± 1°C for 18-20 days. The procedure was the same for both *P. neoaphidis* isolates used in the experiment (Table 2.3.3). Petri dishes had been pre-marked with two perpendicular lines on the base along which hyphal extension was measured and growth in millimeters day^{-1} calculated. The experiment was repeated on three separate occasions using two pseudo-replicates per treatment. Contaminated Petri dishes were discarded and the experiment continued as long as one Petri dish for each treatment remained.

Table 5.1 Recommended rates, chemical components, FRAC activity group and mode of action for fungicides used in experiments 5.2.4, 5.2.5 & 5.2.6.

Product name	Recommended rate	Component	Activity group	Mode of action
Nativo 75WG®	0.4kg a.i. /ha	250g/kg trifloxystrobin + 500g/kg tebuconazole	Group C3, Respiration, FRAC code 11 + Group G1, Sterol biosynthesis in membranes, FRAC code 3	QoI- fungicide: Quinone outside inhibitor + DMI-fungicide: DeMethylation inhibitor
Plover®	0.5l a.i. /ha	250g/l difenoconazole	Group G1, Sterol biosynthesis in membranes, FRAC code 3	DMI-fungicide: DeMethylation inhibitor
Amistar®	1l a.i. /ha	250g/l azoxystrobin	Group C3, Respiration, FRAC code 11	QoI-fungicide: Quinone outside inhibitor

5.2.5 Effect of three fungicides on the germination of primary conidia of two *P. neoaphidis* isolates on an agar based medium

Three 7 mm plugs were taken from the growing edge of 4-5-week-old *P. neoaphidis* cultures grown on SEMA at 15°C ± 2°C in darkness. They were arranged in a triangle on the lid of a 45mm Petri dish on moist filter paper. These Petri dishes were stored at 15°C ± 2°C in darkness 16-20 hours prior to experimentation to ensure plugs were sporulating profusely. The lids were then placed over bases of 45mm Petri dishes filled with 1.5% DSW (distilled water) agar amended to 0, 5, 10, 25, 50,100 & 200% of the field rate of the following

fungicides: azoxystrobin (Amistar), trifloxystrobin + tebuconazole (Nativo 75WG) and difenoconazole (Plover) (Table 5.1). Conidial showering continued for 60 minutes to ensure sufficient primary conidia were deposited on the surface of the DSW agar. Twenty hours after the start of the experiments plates were stained with 10% cotton blue in lacto-phenol and examined microscopically. Germination was assessed by counting 100-200 conidia in arbitrarily chosen non-overlapping fields of view (0.2mm^2) and assigning them to one of 5 ordinal categories; (1) ungerminated conidium, (2) germinated conidium, germ tube <50% of length of primary conidium, (3) germinated conidium, germ tube ≥50% of length of primary conidium, (4) germinated conidium, majority of cytoplasm in secondary conidium or germ tube; secondary conidium formed and not discharged, (5) secondary conidium discharged; empty primary conidium. The experiment was repeated on three occasions.

5.2.6 Effect of selected fungicides on the susceptibility of *B. brassicae* to two *P. neoaphidis* isolates on Brussels sprout plants

B. oleracea Gemmifera group (Brussels sprout, cultivar 'Trafalgar') were cultured as per section 2.1 and experimental plants were at growth stage BBCH-16. Two fungicides were used, Amistar and Plover; these were prepared at the recommended field rate and applied by spraying until runoff (Table 5.1). Both *P. neoaphidis* isolates were used in this study (Table 2.3.3). The fungicides were applied as 'pre-spray' or 'post- spray' treatments:

- For the 'post-spray' treatment, aphids that were feeding on plants were first inoculated with fungus, and then sprayed with fungicide. This meant that

the fungus was directly contacted by the fungicide. The treatment was done as follows: plants were infested prior to the experiment to obtain even-aged cohorts. Individual aphids were removed until groups of 10-15 young apterous *B. brassicae* adults that had settled in nucleated colonies on the abaxial side of one group per plant remained. These groups were then showered with conidia as in section 5.2.2. After aphids, had been inoculated (c. 10-20 mins), the plants were sprayed with fungicide until runoff by means of a handheld bottle sprayer. The treated, infested leaf was then enclosed in a Blackman box (12.5 cm x 8cm x 2cm) (Blackman, 1971) lined with moist filter paper to maintain high relative humidity.

- For the 'pre-spray' treatment, fungus-inoculated aphids were placed on plants that had been sprayed earlier with fungicide. This meant that the fungus would not be directly contacted by the fungicide, but would potentially be exposed either (i) because fungicide had been ingested by aphids feeding on the plant or (ii) through volatile action of the fungicide. The treatment was done as follows: Plants were treated with fungicide in a laminar flow, fume hood 24 hours prior to aphid infestation. Fungicides were applied using a handheld bottle sprayer to the point of runoff. Plants remained in the laminar flow hood (temperature approximately 21 ± 2°C) which was kept running for 24 hours after application. Cohorts of 60 young apterous *B. brassicae* adults (c.48 hours old) were inoculated with each of *P. neoaphidis* isolates in a conidial-showering arena as described above (section 5.2.2). After 60 minutes' cohorts of 10 aphids were placed on the oldest leaf of experimental plants and each leaf was encased within a

Blackman box (12.5 cm x 8cm x 2cm) lined with moist filter paper to maintain high humidity.

Aphids were showered with *P. neoaphidis* conidia for 60 minutes for both treatments. This was considered long enough to deliver sufficient conidia to kill 100% of the population (see chapter 4). The concentration of conidia each replicate received was estimated as above (section 5.2.2). Control treatments consisted of (i) fungicide treated and no pathogen exposure (ii) pathogen exposure and no fungicide treatment and (iii) water spray only. Experimental plants were held in a controlled environment room at 20 ± 2°C, 16L: 8D photoperiod, 50% RH and monitored daily for aphid mortality. Dead individuals were removed and total mortality recorded. Each bioassay was run for 7 days and the experiment was repeated on three occasions using three pseudo-reps.

5.2.7 Statistical treatment

Results were analysed using RStudio© version 0.98.1091. Data were analysed for normality via visual inspection of residual plots and/or Shapiro-Wilk tests.

Plant and leaf age effects on aphid fecundity (experiment 5.2.1) were based on the total number of nymphs produced and were analysed using Kruskal-Wallis rank sum test, as the data could not be normalised (Shapiro-Wilk, $p<0.05$). Significant results were analysed using a post hoc Dunn test applying a Bonferroni-type adjustment to p-values; this was done to account for inflated type 1 errors when using non-parametric tests, facilitating ‘pairwise multiple

comparison of mean ranks (PMCMR)'. Mortality data for this experiment were analysed in the same way as above. The concentration of soluble nitrogen in dried plant material for experiment 5.2.1 was analysed using ANOVA and significant results were compared using Tukey's HSD test.

Tritrophic effects on aphid mortality (experiment 5.2.2) at day 3, 5 and 7 were analysed using ANOVA after mortality data were corrected using Abbott's correction term (Abbott, 1925) and arcsine transformed. Soluble nitrogen concentrations for this experiment were also analysed using ANOVA.

Fungicide growth (experiment 5.2.4) data were analysed using Kruskal-Wallis and significant results were analysed using a post hoc Dunn test applying a Bonferroni-type adjustment to p-values.

Differences between fungicide treatments with respect to germination categories (experiment 5.2.5) were tested by ANOVA following arcsine transformation of the percentage of conidia in each germination stage. Significant results were analysed using Tukey's HSD test. Data on the effect of fungicide exposure on the susceptibility of *B. brassicae* to *P. neoaphidis* (experiment 5.2.6) was analysed using ANOVA and significant results were analysed using Tukey's HSD test. Control mortality during experiment 5.2.6 was low and as such no correction term was required

5.3 RESULTS

5.3.1 Effect of *B. oleracea* plant growth stage and leaf age on the reproduction of adult aphids

The soluble nitrogen content of the youngest and oldest leaves taken from the same plant were not significantly different, irrespective of the growth stage of the plant. (ANOVA, F=0.016, df=1, *P*=0.9). However, significant variation was found in the soluble nitrogen concentration between plants of different growth stages (ANOVA, F= 14.23, df= 2, *P*= 0.0000134) (Figure 5.2). Post hoc analysis revealed significant differences between young and medium-age plants (*P*=0.0018), and young and old plants (*P*= 0.000012), but not between medium and old plants (*P*= 0.323).

Aphid fecundity was significantly affected by plant age (Kruskal-Wallis, H= 35.98, df= 2, *P*= 0.00000001535) but not leaf age (Kruskal-Wallis, H= 1.67, df= 1, *P*=0.196) (Figure 5.3). On average aphids produced 1.27 ± 0.11, 0.68 ± 0.11 and 0.59 ± 0.09 nymphs per day on young, medium and old plants respectively. Post hoc analysis showed that aphid fecundity on young plants was significantly greater than on medium (*P*= 0.0000013), or old (*P*= 0.00000031) plants. Aphid fecundity on medium and old plants were not significantly different (*P*=1).

Adult mortality was significantly affected by plant age (Kruskal-Wallis, H=7.21, df=2, *P*=0.027). Mortality on old plants was significantly higher than on young plants (*P*=0.02) but mortality on young or old plants was not significantly different from that on medium aged plants, *P*=0.132 and *P*=0.538 respectively (Table 5.2).

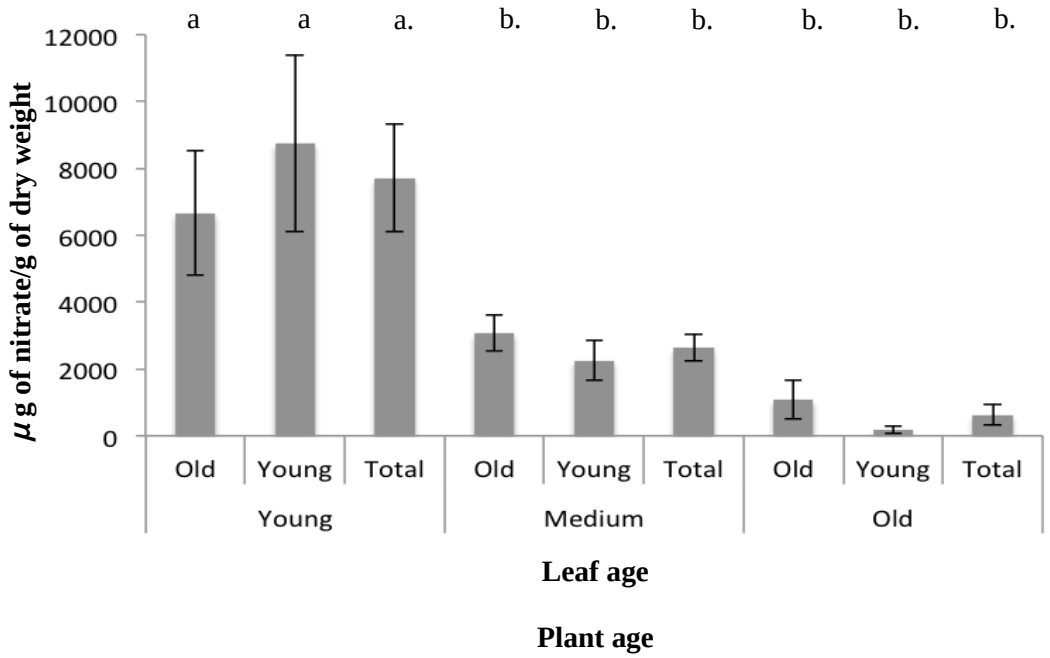


Figure 5.2 Soluble nitrogen concentrations in the youngest and oldest leaves of young (BBCH-13/14), medium (BBCH-16) and old plants (BBCH-19) (±SE). Different letters indicate significant differences between values at the 5% level (Bonferroni-Dunn test).

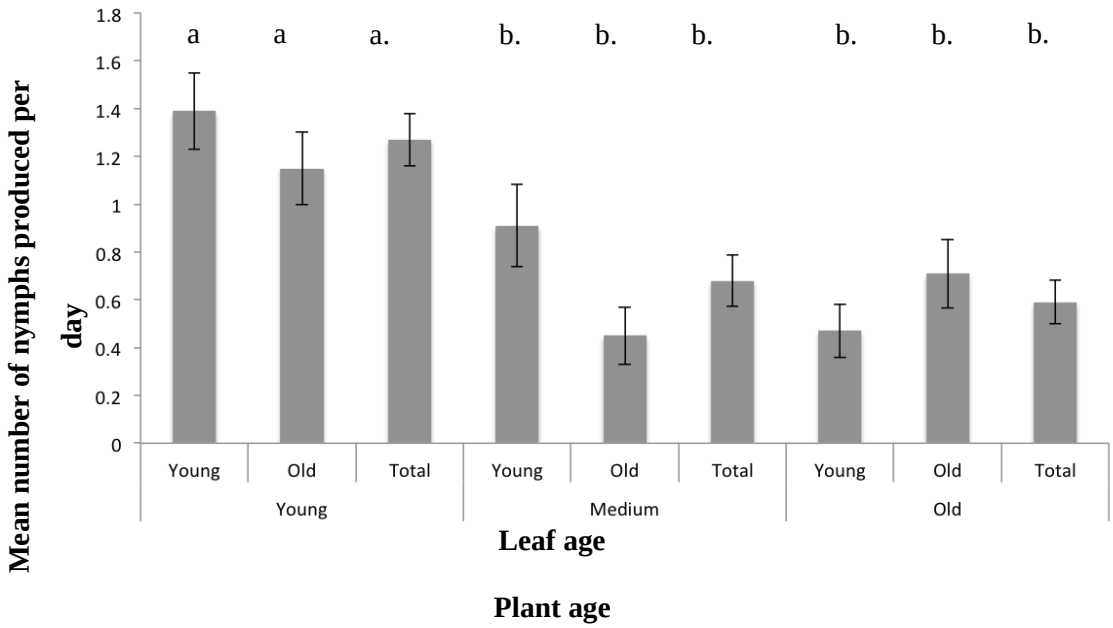


Figure 5.3 Fecundity of *Brevicoryne brassicae* (±SE) raised on the youngest and oldest leaves of different aged *Brassica oleracea* Gemmifera group (Brussels sprout, cultivar 'Trafalgar'); Young plants (BBCH-13/14), medium plants (BBCH-16) and old plants (BBCH-19). Different letters indicate significant differences between values at the 5% level (Bonferroni-Dunn test). Total refers to the mean number of nymphs produced on old, medium and young plants irrespective of leaf age.

Table 5.2 Mortality of *Brevicoryne brassicae* on young (BBCH-13/14), medium (BBCH-16) and old (BBCH-19) plants after seven days. Different letters indicate significant differences in mortality levels at the 5% level (Bonferroni-Dun test).

Plant age	Mortality ±se (%)
Young	16.6 ±11.39[a]
Medium	44.4 ±14.05[ab]
Old	72.2 ±10.24[b]

5.3.2 Effect of host plant growth stage on the susceptibility of *B. brassicae* to the entomopathogenic fungus *P. neoaphidis*

Experimental plants varied significantly in their soluble nitrogen concentrations (ANOVA, F= 7.3, df=1, *P*=0.0355), 6005.19 ± 536.15μg of nitrate/g of dry weight and 2713.38 ± 1094.06μg of nitrate/g of dry weight for young and medium plants respectively (Figure 5.4). Despite this difference aphid mortality was not significantly affected by plant age (ANOVA, F=0.350, df=1, *P*=0.565). Mortality did not vary significantly over time as when plant age is taken into account there was no difference between days three, five and seven post treatment (ANOVA, F=2.243, df=1, *P*=0.149) (Figure 5.5). Peak aphid mortality for both physiological plant age treatments was day five, 15.13± 5.63% and 23.75± 4.27% respectively on young and old plants. In addition, there was no plant age by days after treatment (DAT) interaction (ANOVA, F=0.401, df=1, *P*=0.679).

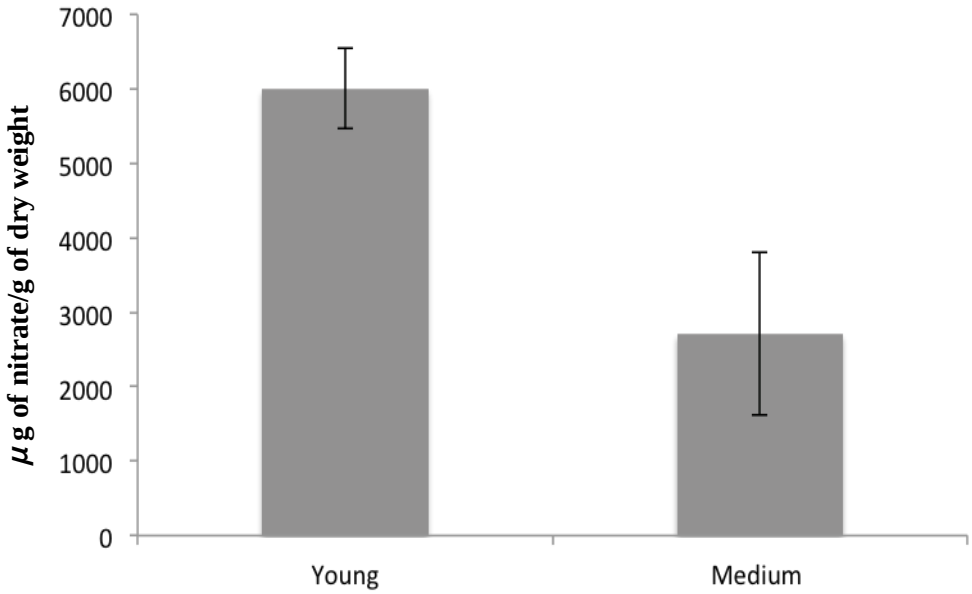


Figure 5.4 Mean soluble nitrogen concentrations in young (BBCH-13/14) and medium (BBCH-16) aged plants (±SE).

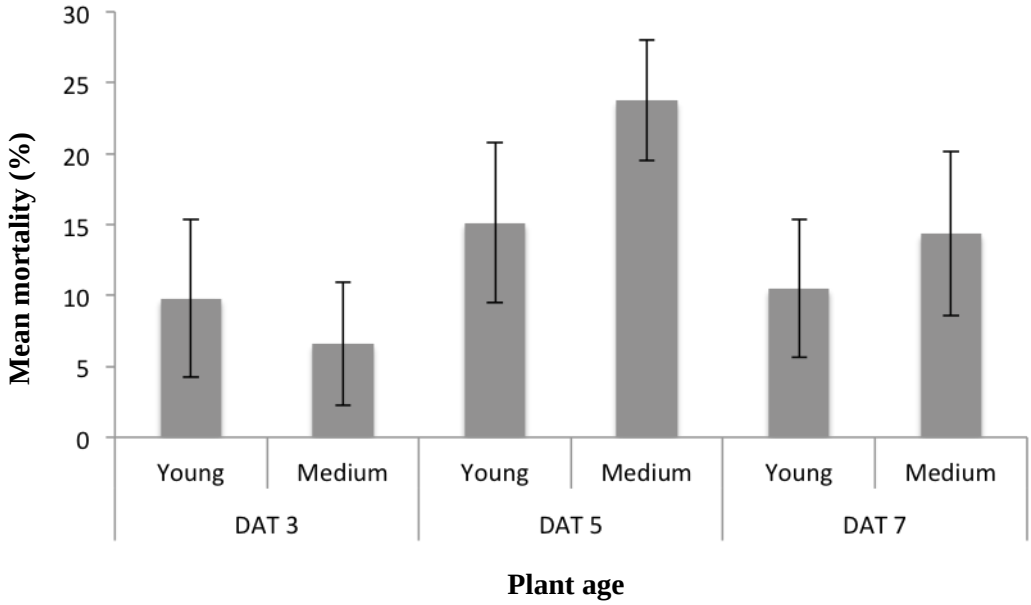


Figure 5.5 Abbott's adjusted mortality of *Brevicoryne brassicae* after exposure to *Pandora neoaphidis* (isolate=WEL1) on young (BBCH-13/14) and medium (BBCH-16) aged plants on the third and fifth day post treatment (±SE).

5.3.3 Effect of three fungicides on the growth of *P. neoaphidis* isolates on agar based medium

There were significant effects of fungicide concentration on extension of *P. neoaphidis* mycelia (Kruskal-Wallis, H= 41.5049, df= 2, $P<0.0001$) (Figure 5.6). However, there were no significant differences in the response of either isolate of *P. neoaphidis* to the fungicides (Kruskal-Wallis, H= 0.5026, df= 1, $P= 0.4784$). Amistar completely inhibited the growth of NW420 and WEL1 at all test concentrations, whereas Nativo 75WG did not affect extension at the 5% of field rate for NW420 and 10% of field rate for WEL1, where growth was 0.0530 ± 0.0303 mm/day^{-1} and 0.0461 ± 0.0009 mm/day^{-1} respectively. Mycelial extension on Plover-infused SEMA differed significantly from Nativo 75WG ($P<0.0001$) and Amistar ($P<0.0001$), but effects of Nativo 75WG and Amistar were not significantly different ($P=0.48$). Plover treated growth media supported growth of both isolates of *P. neoaphidis* up to 100% of the field rate. In general, mycelial extension was greater for isolate WEL1.

Mycelial extension rate varied significantly with fungicide concentration (Kruskal-Wallis, H=19.1012, df= 5, $P=0.00184$) Non-parametric two-way analysis of variance is difficult to interpret, given Plover was (effectively) the only fungicide treatment on which the fungi grew and subsequently produced data that were significantly different (by concentration), and a post hoc Bonferroni-Dunn analysis of dose data allowed the interaction of dose and fungicide to be assessed. Growth rates on treated media at 5% of the field dose varied significantly from that on 100% and 200% field dose, $P=0.033$ and 0.011 respectively. Additionally, growth rates on treated media at 10% of field rate varied significantly only from

growth rates on 200% of field rate, P= 0.039.

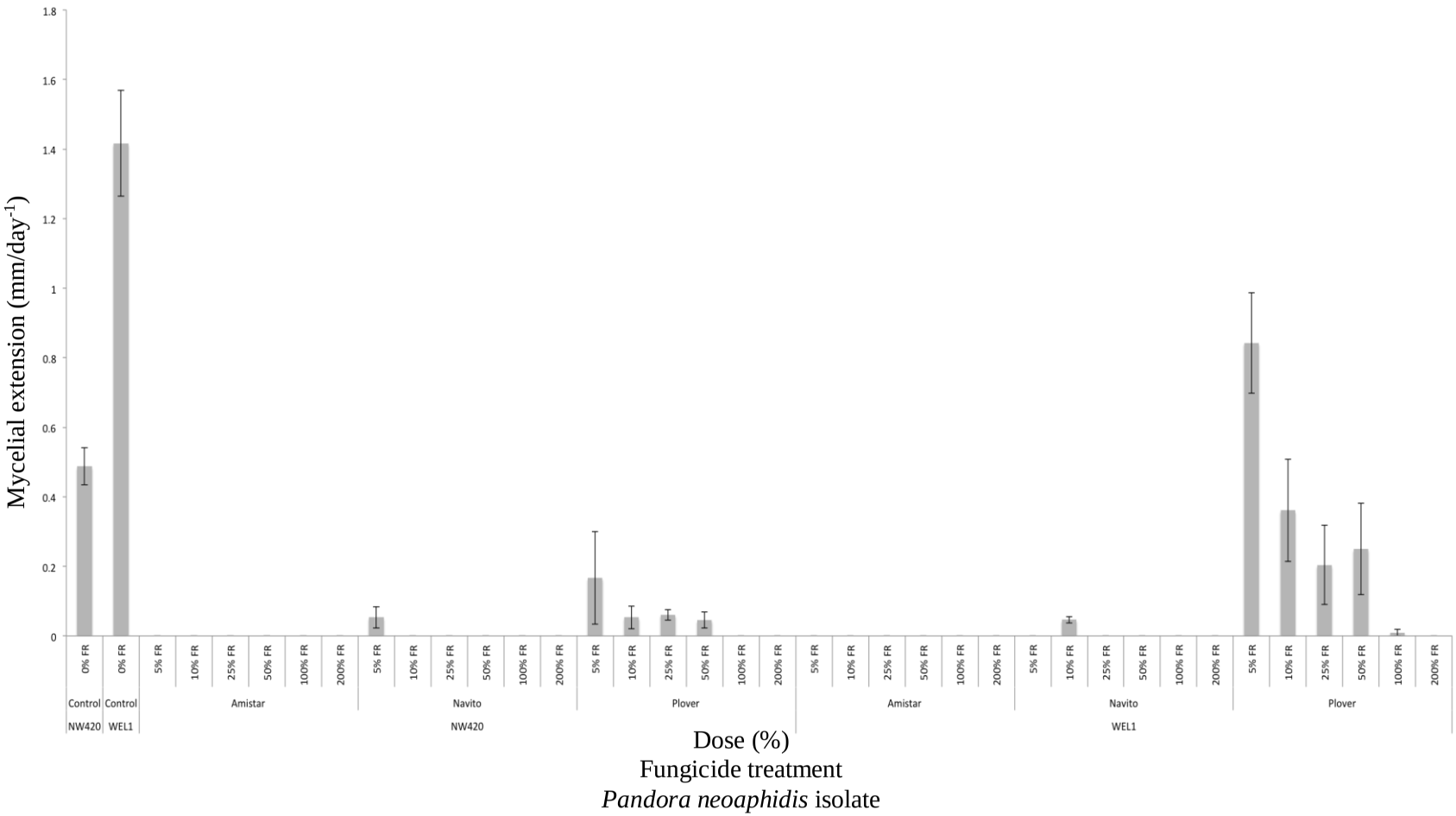


Figure 5.6 Mean mycelial extension rate (mm/day^{-1}) for two *Pandora neoaphidis* isolates (NW420 and WEL1) at 0, 5, 10, 25, 50,100 & 200% of field rate for selected fungicides (±SE).

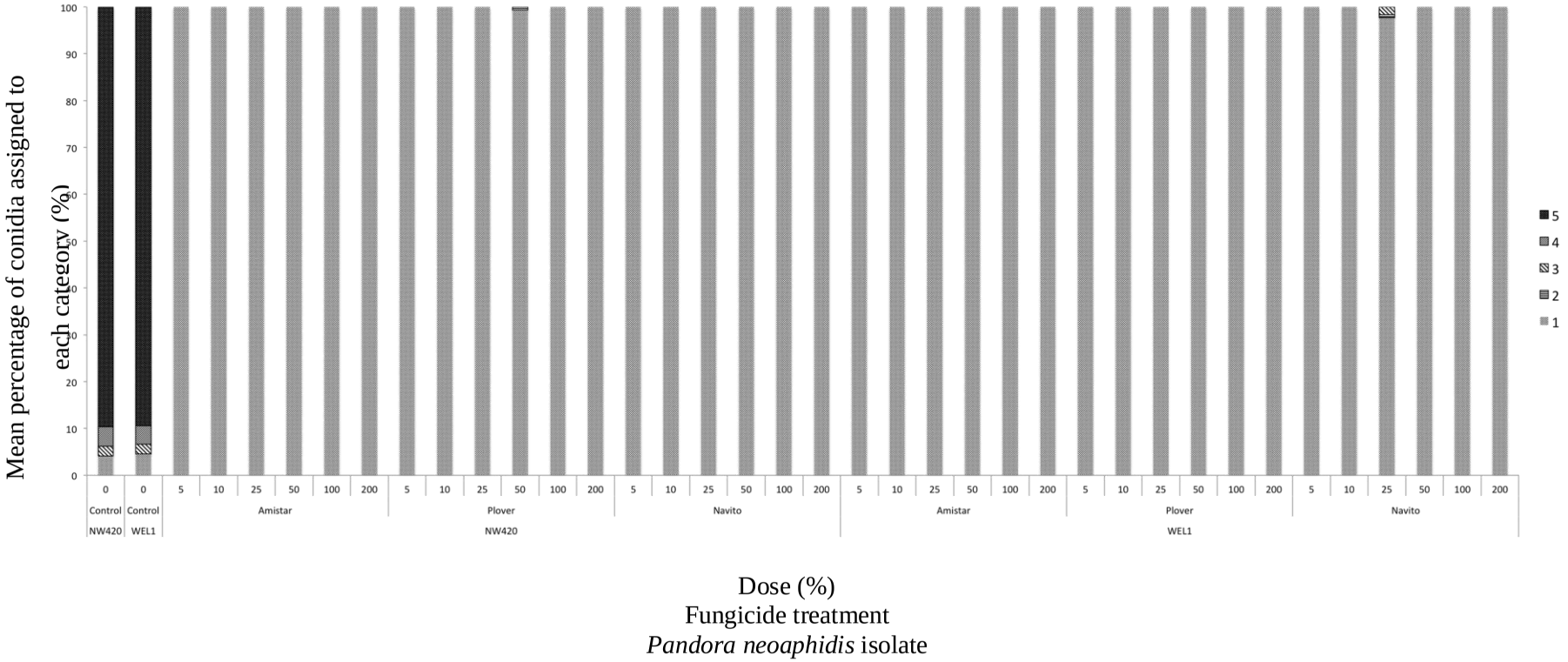
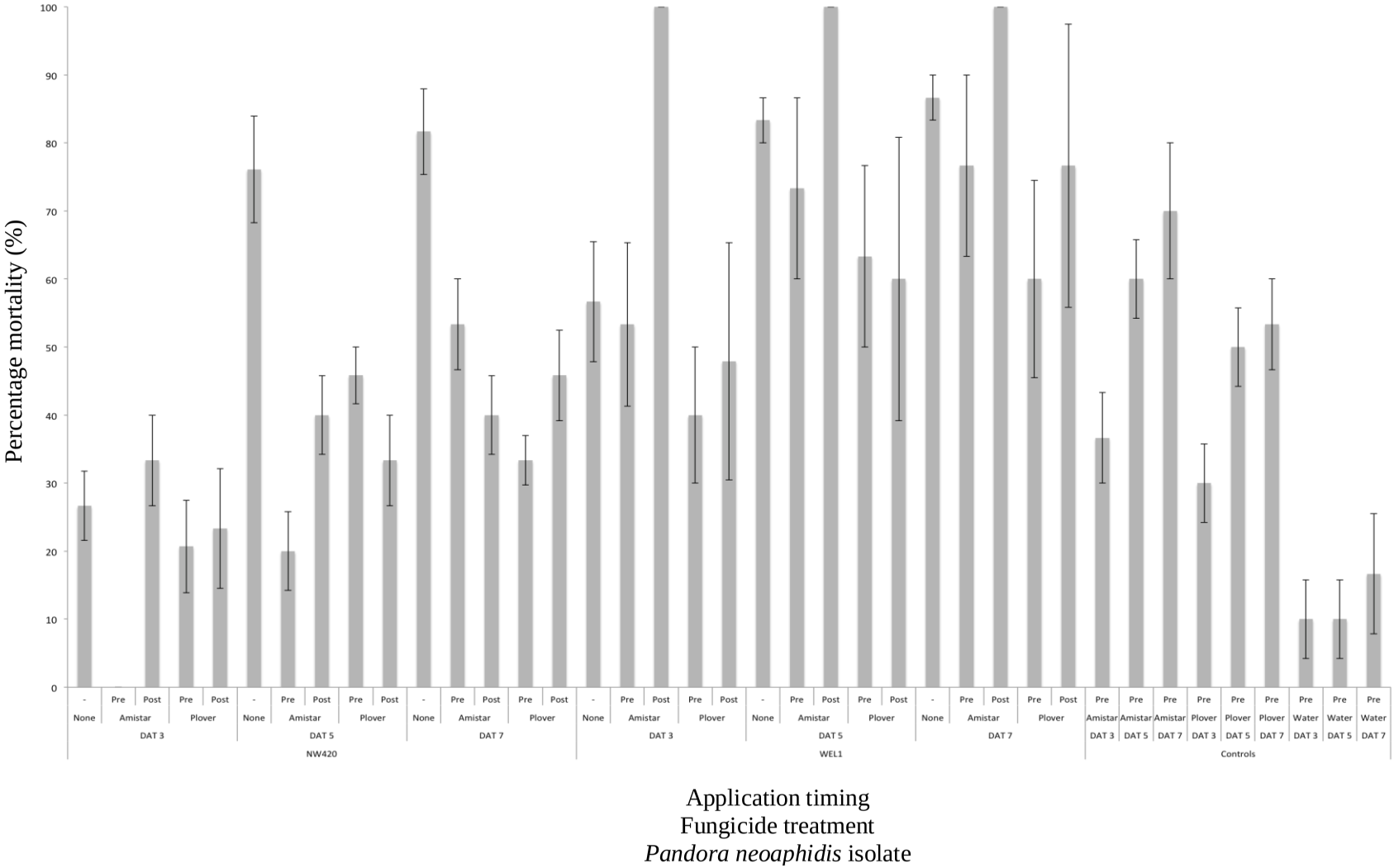


Figure 5.7 Effect of selected fungicides on the germination of two *Pandora neoaphidis* isolates (NW420 and WEL1). Categories; (1) ungerminated, (2) germinated, germ tube <50% of length of primary conidium, (3) germinated, germ tube ≥50% of length of primary conidium, (4) germinated, majority of cytoplasm in secondary conidium or germ tube; secondary conidium formed and not discharged, (5); empty conidium.

Figure 5.8 Mean total mortality of *Brevicoryne brassicae* (±SE) exposed to *Pandora neoaphidis* and one of two selected fungicides on days three, five and seven post treatment.



Application timing
Fungicide treatment
Pandora neoaphidis isolate

5.3.4 Effect of three fungicides on the germination of primary conidia of two *P. neoaphidis* isolates on an agar based medium

There were no significant differences between fungicide treatments (ANOVA, F=0.87, df=2, *P*=0.4179) or concentration (ANOVA, F=12.73, df=2, *P*=0.2745) on the germination of primary conidia. All fungicides used in the study, Amistar, and Nativo 75WG and Plover inhibited the germination of conidia at rates as low as 5% of the field rate (Figure 5.7). Category 1, ungerminated conidia, was significantly different to all other ordinal categories of germination; whereas none of the other categories were different from each other (Table 5.3).

Table 5.3 Post hoc Tukey HSD analysis of the percentage germination of conidia in each ordinal category; (1) ungerminated conidium, (2) germinated conidium, germ tube <50% of length of primary conidium, (3) germinated conidium, germ tube ≥50% of length of primary conidium, (4) germinated conidium, majority of cytoplasm in secondary conidium or germ tube; secondary conidium formed and not discharged, (5) secondary conidium discharged; empty primary conidium.

Germination category comparison	***P*=**
1-2	0
1-3	0
1-4	0
1-5	0
2-3	0.99
2-4	0.98
2-5	0.98
3-4	0.97
3-5	0.97
5-4	1

5.3.5 Effect of selected fungicides on the virulence of two *P. neoaphidis* isolates to *B. brassicae* on Brussels sprout plants

Aphids in both pre- and post-sprayed treatments were showered with *P. neoaphidis* conidia at a rate of >60 conida/mm^2. Based on chapter 4 results, this is sufficient to cause 100% mortality after 7 days.

Isolate WEL1 appeared to be the more virulent isolate to *B. brassicae* across treatments (Figure 5.8 shows total mortality). There were significant differences in the response of *P. neoaphidis* isolates exposed to fungicide treatments (ANOVA, F= 49.94, df= 1, *P*= <0.0001). Both WEL1 and NW420 were significantly affected by fungicide exposure; ANOVA, F= 5.267, df= 2, *P*= 0.00669 & ANOVA, F= 6.643, df= 2, *P*= 0.00195, respectively. Aphid mortality from isolate NW420 at 5 and 7 DAT was significantly reduced in both fungicide treatments and these treatments did not vary from each other. (Table 5.4 & Figure 5.8).

Fungicide treatment had little effect on the efficacy of WEL1 against *B. brassiace* as there were no significant differences in aphid mortality levels between Amistar or Plover and no fungicide application. However, mortality responses did vary significantly between Amistar and Plover (Table 5.5).

Predictably, aphids exposed to water alone suffered significantly lower mortality than any other treatment (Tukey HSD, *P*<0.05, in all cases) (Figure 5.8). Aphid mortality levels in the Amistar- and Plover-only control treatments did not significantly differ from *P. neoaphidis*-only controls. There was no significant difference between Plover and Amistar controls (*P*=0.822).

The timing of the fungicide applications, i.e. ‘pre-sprayed’ and ‘post-sprayed’, had no significant effect on the mortality of *B. brassicae* exposed to NW420

(ANOVA, F= 0.135, df= 1, *P*= 0.714). However, fungicide application timing did have a significant effect on the mortality of *B. brassicae* exposed to WEL1 (ANOVA, =F= 7.084, df= 1, *P*= 0.00907). A greater number of aphids died in the 'post-sprayed' treatment (Figure 5.8).

Table 5.4 Comparison of different fungicide treatments on the mortality of *Brevicoryne brassicae* exposed to *Pandora neoaphidis* isolate NW420. Tukey HSD; experimentwise alpha = 0.05.

***P. neoaphidis* isolate NW420**	**Amistar**	**Plover**
Amistar		*P*=0.6754528
Plover	-	
No fungicide	*P*=0.0015212	*P* =0.0129557

Table 5.5 Comparison of different fungicide treatments on the mortality of *Brevicoryne brassicae* exposed to *Pandora neoaphidis* isolate WEL1. Tukey HSD; experimentwise alpha = 0.05.

***P. neoaphidis* isolate WEL1**	**Amistar**	**Plover**
Amistar		*P*=0.0050389
Plover	-	
No fungicide	*P*=0.6593956	*P* =0.1922533

Significant interactions between fungicide application timing and no fungicide treatment were detected for NW420, F= 4.634, df= 1, *P*= 0.03375. When Amistar was applied to plants prior to fungal treatment ('pre-sprayed'), aphid mortality was significantly lower than that in the 'no fungicide' treatment (*P*= 0.0038240). Aphid

mortality in the Amistar pre-sprayed treatment was 20 ± 5.77% five days after treatment, whereas mortality in the no fungicide treatment was 60 ± 5.77% after 5 days (Figure 5.8). Similarly, when aphids were sprayed with Plover after the fungal treatment had been applied ('post-sprayed'), mortality levels were significantly lower than those in the 'no fungicide' treatment (*P*= 0.0551069). Aphid mortality in the Plover post-sprayed treatment was 33.33 ± 6.66% five days after treatment, whereas mortality in the no fungicide treatment was 50 ± 5.77% after 5 days (Figure 5.8).

For isolate WEL1 significant interactions between application timings and fungicide were apparent, F= 8.189, df= 1, *P*= 0.00513. When Amistar was applied to after the aphids had been exposed to *P. neoaphidis* ('post-sprayed') aphid mortality was significantly higher than in all other fungicide treatments; Plover post-sprayed (*P*= 0.0013086), Plover pre- sprayed (*P*= 0.0021876) and Amistar pre-sprayed (*P*= 0.0051686). When Amistar was applied after fungal treatment, *B. brassicae* mortality reached 100 ± 0% after just three days, whereas the NW420-only treatment caused 40 ± 5.77% mortality after seven days (Figure 5.8). There were no other significant fungicide* application interactions for either isolate. In addition, there was no significant fungal species*fungicide*application timing interaction (ANOVA, F= 1.140, df= 1, *P*=0.287).

There were significant day after treatment effects (ANOVA, F= 45.82, df= 6, *P*<0.001). Although, there was no significant days after treatment*fungal species interaction (ANOVA, F= 1.61, df= 12, *P*= 0.0889), differences in aphid mortality at three and five days after treatment were significant, as were differences between mortality at three and seven days after treatment (*P*= 0.0019105 and *P*<0.0001 respectively). Differences in mortality five and seven days after treatment were not significant (*P*= 0.6115121).

Table 5.6 Summary table of fungicide treatment effects.

Isolate	Did fungicide exposure significantly reduce aphid mortality?	Was there a difference in effects on aphid mortality between the two fungicide treatments?	Was there an application timing effect on aphid mortality?
Pandora neoaphidis NW40	Y No differences between Amistar and Plover in their effects on *B. brassicae* infection and mortality.	N	N
Pandora neoaphidis WEL1	N	Y Plover application reduced aphid mortality; Amistar did not.	Y Increased aphid mortality in post-sprayed treatment.

5.4 DISCUSSION

This study clearly showed that fungicides have variable effects on fungal mycelial extension, germination and fungus-induced insect mortality. Mycelial growth of *P. neoaphidis* (NW420 and WEL1) was completely inhibited by Amistar at all but the 5% of field rate. Nativo allowed some level of intermediate growth at the lowest doses and Plover had no adverse effect on growth at up to 100% of field rate. Germination was the most sensitive biological process and was inhibited down to the 5% of field rate by all three of the fungicides used. This indicates that even at very low concentrations, fungicides could impact infection and disease transmission processes, ultimately resulting in decreased efficacy of the control program. Insect mortality data painted a much more complex and nuanced picture. There was significant variation in the aphid mortality response to both *P. neoaphidis* isolates, and Amistar and Plover treatments reduced mortality of aphids treated with NW420, but not with WEL1. Although, statistically insignificant, Plover treatments did reduce mortality levels of aphids treated with WEL1, whereas Amistar had no effects on aphid mortality at all. The timing of application also affected levels of aphid mortality; when fungicides were applied after aphid treatment with isolate WEL1 ('post-applied'), aphid mortality levels increased.

Activity of the selected fungicide appeared to be fungistatic rather than fungicidal, given the observed effects on germination of *P. neoaphidis* conidia and mycelial growth, but the lack of effects when aphid infection was assessed. Overall, it appears that fungicide compatibility depends on the particular fungal isolate in question, even within the same species, and the timing of the application. In this study application of fungicide after application of an entomopathogenic fungus increased aphid mortality in some cases. Potentially, once conidia have germinated and infected

the insect host, given a ‘compatible’ fungicidal product is applied, growth within the host and death of the host is not inhibited. Additionally, application post entomopathogenic fungi application has the additional benefit of allowing the pathogen an additional layer of protection, that of the insect cuticle, effectively sheltering the fungal pathogen from the fungicide.

Many investigations into the compatibility of agrochemicals with biological control agents such as entomopathogenic fungi and nematodes are carried out *in vitro* (De Nardo & Grewal, 2003; Er & Gocke, 2004; Kouassi *et al*.; 2003; Luke & Batemen, 2006). These studies are valid as preliminary investigations to identify potentially antagonistic interactions, but the results (and those presented here) should be treated with caution as they are representative of a worst-case scenario in the field. In reality, many agrochemicals have poor persistence in the environment meaning concentrations decline rapidly and some plants are known to compartmentalize chemicals within their tissues (Jaronski, 2010; Inglis *et al*., 2001). A more realistic method to determine the effects of fungicide exposure on entomopathogenic fungi would be to monitor conidia under a light microscope after exposure. The level at which the effect would interact in the environment.

The performance of entomopathogenic fungi, particularly in conservation biocontrol is a function of fungal activity and host density. This study suggests that the performance of the host *B. brassicae* is significantly affected by plant age or plant quality, with a dramatic reduction in performance on older plants. This could have been a product of the transgenerational effects; individual aphids are capable of telescoping generations. Grandmothers are born with mothers and daughters within. Built up reserves and progeny are directly connected to the host plants individuals were reared on (Wang *et al*., 2017). Nonetheless, reduced aphid performance has

important implications for the efficacy of entomopathogenic fungi used as biological control agents in conservation biological control programs, by reducing the number of available hosts.

CHAPTER 6: GENERAL DISCUSSION

Over the last decade, there has been a significant and widespread reduction in the availability of fully effective synthetic chemical pesticides. This has been driven by a combination of: (1) more stringent regulations on pesticide approvals with respect to human and environmental safety, with the result that many products based on 'old' chemistry have been withdrawn from sale; (2) the evolution of heritable resistance in target pests; (3) a lack of new active pesticide substances in the development pipeline; (4) consumer concerns about pesticide residues in food, which has resulted in food retailers placing additional restrictions on the use of synthetic chemical pesticides in food production (European Commission, 2017; Garthwaite *et al*., 2007; Hajek, 2004; IRAG, 2012; Tilman *et aI*., 1999; Tilman *et al*., 2002). As a result, farmers and growers are under pressure to find alternative pest control measures that are suitable for use in IPM (Garthwaite *et al*., 2007; UK NAP, 2013).

The challenge of sustainably controlling pests in brassicas and other field vegetables with reduced insecticide inputs is particularly difficult, as these crops do not undergo processing before sale, which limits opportunities to reduce pesticide residues. Furthermore, retailers have very high quality standards for fresh produce and frequently have zero tolerance for pest damage. Other crops grown to such high specifications tend to be produced in protected environments (e.g. greenhouse vegetables, baby greens, soft fruit), where the use of inundative biological control agents is more reliable (Guetsky *et al*., 2001). On the other hand, the fact that brassicas are grown in open fields creates opportunities for using a wider range of IPM tools that might not be possible in protected crops. In the case of biocontrol, this includes the use of conservation biocontrol strategies (i.e. exploiting naturally occurring biocontrol agents) in addition to augmentation biocontrol (i.e. the deliberate application of biocontrol products).

Conservation biological control on brassicas and other field vegetables has only been

investigated to a limited extent. Research to date has focused primarily on the role of arthropod natural enemies rather than on microbial agents (Landis *et al*., 2000; Maniania *et al.*, 2008; Orre *et al*., 2009; Pell *et al.*, 2009). Much of the work on entomopathogenic fungi and arthropod interactions, predators and parasitoids, has been conducted on a laboratory or semi-field scale (Baverstock *et al*., 2005; Baverstock *et al*., 2009; Ormond *et al*., 2011; Roy *et al*., 2008). Diaz *et al*. (2010) investigated natural enemy guild interactions at a field scale in lettuce in Spain but these studies were limited to entomopathogenic fungi (*Pandora neoaphidis*) and aphidophagous hoverflies. The research presented here is one of the first studies to investigate the role of naturally occurring insect pathogenic fungi, predators and parasitoids in the regulation of aphid pests of vegetable brassica crops.

The aphid population 'crash' in *B. brassica*e was observed in two successive years of field experiments (2013 and 2014) and was associated with an increase in the population size of a guild of natural enemies, including *P. neoaphidis*, Coccinellidae, Syrphidae and parasitoid wasps in the genus *Aphidius*. The observation of a fungal epizootic on *B. brassicae* is important as it has not been previously reported in the literature. Natural enemy abundance was significantly negatively correlated with the instantaneous rate of increase of aphid populations, the activity of *P. neoaphidis* and the number of aphid mummies, suggesting an antagonistic relationship. A significant relationship was not found for Coccinellidae or Syrphidae larvae. However, field observations of *P. neoaphidis* and aphid mummies provide a direct measure of their incidence or activity (a one to one congruence of natural enemy activity and aphid parasitism), whereas one predator may kill a large number of aphids but will only be recorded as one individual. Predator natural enemy abundance cannot be extrapolated to natural enemy activity.

These field observations show an association of natural enemy presence and aphid population dynamics but they do not demonstrate a causal relationship. This would require an exclusion experiment to remove natural enemies using field cages, although this method has its own limitations and can be difficult to interpret (Section 3.4). Exclusion experiments

require netting to prevent the incursion of arthropod natural enemies into the experimental plot. However, netting artificially increases the humidity within the enclosed area, lending entomopathogenic fungi a competitive advantage underneath. Additionally, it is difficult to exclude entomopathogenic fungi from field plots as fungicides will not completely sanitize the area. The fungicides may also have an unintended impact on aphid populations or natural enemy communities.

Nevertheless, the association evidence in this research strongly suggests that natural enemies play a significant role in the regulation of *B. brassicae* populations, particularly *P. neoaphidis* and parasitoids. When the crash occurs, aphid populations are reduced to low levels within 7 – 10 days. In 2013, parasitized mummies were more numerous than *P. neoaphidis*-infected mummies, while the opposite was true in 2014. Once observed in the field, parasitoid mummies persisted and increased proportionately within the aphid populations which continued to grow. It was not until fungal cadavers were recorded that a precipitous decline in aphid numbers was recorded. It could be inferred that the presence of *P. neoaphidis* catalyzed a rapid decrease in an aphid population already stressed by several other natural enemies. The relationships between natural enemies are complex; predators, parasitoids and pathogens can respond differently to the presence of one another impacting the strength and outcome of intraguild competition (Baverstock *et al*., 2005). Because *P. neoaphidis* is an obligate parasite of aphids it represents very low intraguild competition for other natural enemies (Pell *et al*., 2001; Pell *et al*., 2009). The fungus exerts its competitive pressure only through its colonization of the aphid as a shared resource. Competition does exist however, as parasitoid wasps laying eggs in aphids infected by *P. neoaphidis* will be outcompeted due to the pathogen's superior development time (Baverstock *et al*., 2005). In the same way, *P. neoaphidis* will be detrimentally affected by predators unable to distinguish between infected and uninfected aphids which subsequently consume fungus-infected individuals (Baverstock *et al*., 2005; Baverstock *et al*., 2008b; Baverstock *et al*., 2009; Ormond *et al*., 2011; Roy *et al*., 2008).

Previous work (Watt, 1979; Douglas, 2003; Karley *et al*. 2003; Karley *et al*. 2004) has provided evidence that the aphid crash is caused by reductions in the soluble nitrogen content of host plants (host plant quality). However there was no evidence that this was the case in this study. While host plant age/soluble nitrogen affected *B. brassicae* fecundity and mortality under controlled laboratory conditions, this relationship was not seen in the field. Although the aphid population decline occurred at a much later stage in the 2014 season than in 2013, in both cases plant age did not apparently affect the timing of the crash. Conclusions cannot be drawn about the direct relationship between the timing of the crash and nitrogen levels, as leaf samples for nitrogen analysis were not collected at the time of the population crash. There was no significant effect of location in 2014, suggesting little spatial heterogeneity in the timing of the crash. In both cases, the crash occurred at the same time as the fungal epizootic; Contrary to the hypothesis of natural enemies vectoring the pathogen, the lack of spatial heterogeneity in the timing of the crash would suggest that the pathogen (*P. neoaphidis*) is ubiquitous, and simply requires the right conditions (population density, environmental or a combination of the two) before infection outbreak (i.e. an epizootic) occurs.

Temperature had a significant effect on fungal growth, germination and host-pathogen interactions, in keeping with previously published research (Shah *et al*., 2002; Kiewnick, 2007; Vidal *et al*., 2011; Rivas *et al*., 2013; Borisade *et al*., 2014). This highlights the importance of temperature in the biology of entomopathogenic fungi and its consequences for pest management strategies. There have been few attempts to use non-linear statistical regression models to understand effects of temperature on the biology of EPF (Davidson *et* al., 2003; Golizadeh *et al*., 2007), mainly because these models are more challenging to develop than simple linear models, plus the fact that collecting enough data to run the models is time consuming. Sufficient independent data points are required over the entire range of cardinal temperatures to fit these types of models and be certain of their interpretation. Nonlinear models have been used to describe the effect of temperature on EPF growth before

(Davidson *et al*., 2003; Smits *et al*., 2003) but they have not been used prior to this study to investigate the effect of temperature on fungal virulence or the germination of conidia. Model estimates of cardinal temperatures for germination and growth of the fungal strains used in this study differed between species. Differences in cardinal temperature estimates were also apparent between fungal processes, i.e., growth, germination and virulence, of the same species. Thus, studies that have previously used growth and germination experiments as a proxy for the effect of temperature on fungal efficacy are probably not very reliable. Insect-pathogen bioassays provide more robust data when investigating effects of temperature on the virulence of EPF, e.g. in screening programmes. The models used in this study fitted both hypocrealean species and *P. neoaphidis* (Entomophthoromycota), and provide a more accurate estimate of cardinal limits including temperature optima. Therefore, they are more informative than previous approaches based on simple qualitative line fitting or linear regression models (Thomas & Jenkins, 2014).

The genera *Beauveria* and *Metarhizium* are not aphid specialists and it was evident that the thermal biology of the isolates investigated in this study did not match that of *B. brassicae*. Members of both these genera infect a wide range of insect species, which themselves are likely to vary in their thermal profiles, while they can also grow in soil as rhizosphere colonists or as endophytes (Vega, 2008). The selection pressure and trade-offs involved in having such a generalist biology and ecology is likely to affect their adaptation and response to temperature. The thermal profiles of the host, *B. brassicae*, and that of the generalist hypocrealean entomopathogenic fungal species differed by as much as 3-4°C in some cases. Co-evolution of host and pathogen has driven the thermal biology of more specialized aphid pathogens to be 'matched' as hypothesized. *Lecanicillium longisporum*, for example, is much more of an aphid specialist (Kim *et al*., 2007) and it could be argued therefore that this was reflected in its thermal profile. *Pandora neoaphidis* is an aphid specialist and the data presented here (Chapter 4) strongly suggests that the thermal profile of strains NW420 and WEL1 (both of which were obtained from *B. brassicae*) match that of the

aphid, particularly with respect to the optimum temperature for infection. The thermal optimum of *P. neoaphidis* most closely matched that of *B. brassicae* at (23°C), 22°C and 24°C for strains WEL1 and NW420 respectively. Of the more generalist, hypocrealean species *L. longisporum* was the only species to be within 1.5°C of the thermal optimum of *B. brassicae*. The average thermal maximum for *P. neoaphidis* was substantially lower than that of *B. brassicae*, 32°C and 35°C respectively, and it was the only fungal species to have a thermal maximum below 35°C. *Pandora neoaphidis* is an obligate parasite of *B. brassicae* and does infect other aphid species; the selective pressures for this may have altered the upper thermal limit of this fungal species. Matching the thermal profiles of a microbial control agent to a specific pest/environment could make the difference between success or failure in pest control and therefore it requires a greater level of investigation by biopesticide developers than has been the case thus far.

Relative humidity has not always been shown to affect the pathogenicity of entomopathogenic fungi to aphids (Hsiao *et al*., 1992). However, field observations on the timing of the epizootic suggest that a long period of high ambient relative humidity (RH) (2-3 weeks) was required for infection to occur. This observation was corroborated in laboratory experiments, which showed that longer incubation periods at high humidity significantly increased aphid mortality. Specifically, *B. brassicae* exposed to *P. neoaphidis* and kept at >98% RH for 24 hours were significantly more likely to die from infection than those kept at high RH for shorter periods. Longer periods of incubation at high relative humidity has also been shown to increase the virulence of *B. bassiana* infecting *Rhodnius prolixus* (Luz & Fargues, 1999; Ramoska, 1984; Mishra *et al.*, 2013).

Fungicides have variable effects on fungal growth, germination and fungus-induced aphid mortality. Mycelial extension of *P. neoaphidis* was inhibited by exposure to Amistar and Nativo fungicides, whereas Plover (difenoconazole) had limited effect when tested up to 100% of field rate. Germination did not occur in the presence of any of the fungicides at any of the rates tested. Both Amistar and Plover reduced aphid mortality from *P. neoaphidis* isolate

NW420 but not WEL1. Isolate WEL1 was isolated from an agricultural field at Warwick Crop Centre where the application of fungicides occurs regularly. It is possible that this exposure exerted selection pressures on the fungus, causing a shift in the fungal population to one that is more resistant to these fungicides (Shapiro-IIan *et al*., 2011). Unfortunately, information on the precise location where NW420 was isolated in not available. These results would suggest the most compatible fungicide is Plover; however, germination of conidia was inhibited by this fungicide. Germination is a vital process in the infection cycle. Without it, the fungus cannot enter the host and proliferate within it. Fungicide application will inhibit biological control with entomopathogenic fungi through their inhibition of the germination of conidia, even if they have no effect on mycelial extension compatibility.

Fungal activity, as a whole (growth, germination and virulence) is seldom taken into consideration when investigating the compatibility of chemical control agents in IPM strategies (De Nardo & Grewal, 2003; Er & Gocke, 2004; Kouassi *et al.*, 2003; Luke & Batemen, 2006; Koch *et al*., 2010). The results presented here highlight the importance of investigating the range of fungal responses to chemical exposure. Fungicide applications could prevent naturally occurring entomopathogenic fungi from helping to control aphid populations in field crops. The development of more selective fungicides would help to mitigate this effect, as would the more judicious timing of applications, for example, applying after *P. neoaphidis* has caused an epizootic, and avoiding spray drift into areas acting as reservoirs for the fungus i.e. field margins or other natural habitat close to the field where susceptible aphid populations may be acting as disease reservoirs. Interestingly, there is a drive for more selective insecticides to minimize non-target effects on natural enemies, the same should apply to fungicides.

The fact that the aphid population crash occurred at very different times of the year in 2013 and 2014 but under similar abiotic conditions, suggests that the aphid population crash is a function of aphid population density, natural enemy presence and prevailing climatic conditions. The density at which the population crash occurred, irrespective of plant

developmental stage, in both years was between 170-320 (uninfected) individuals per plant. On both occasions, *P. neoaphidis* was prevalent. This would suggest that the threshold for the aphid population crash and an epizootic outbreak are similar. Instantaneous rate of increase heads to 0 (i.e. no aphid population growth) when 10 infected aphids are recorded per plant. The density of uninfected aphids immediately prior to the crash was c.300. This suggests that an estimated infection threshold of c.3% of the population in enough to initiate an epizootic and cause the aphid population crash (Figure 3.9 & 3.15).

The temperature development models can only be used for the speed at which an aphid population will decline *after* infected cadavers have been seen and populations have potentially already begun to decline. The threshold density, i.e., observed population density immediately prior to the crashes in 2013 and 2014, can be predicted *before* infected cadavers have been recorded and aphid populations begin to decline. This provides a useful tool whereby the aphid population crash could be exploited in IPM strategies. Organic growers are the most obvious beneficiaries of such management strategies given their limited access to other control options. However, conventional growers might also be able to benefit from the aphid crash if they were able to adapt their crop management practices, to be compatible with the development and timing of the population crash. Organic production in the UK is comparatively small, however, globally the market is growing, particularly in the US (Greene, 2017). The benefits of being able to predict and utilize the aphid crash are likely to become more evidence with increasing awareness by growers, policy makers, consumer groups etc. of the threat of pesticide resistance, pesticide residues and environmental impacts of injudicious pesticides application. However, the high aphid densities currently necessary before the crash occurs are likely to be unacceptable on conventionally grown vegetable crops despite the fact brassica crops are particularly hardy plants and recover well from such high *B. brassicae* densities. The exploitation of the aphid population crash is probably best suited to brassica crops that are harvested in late autumn, can tolerate high aphid numbers in the summer and will subsequently recover over time, i.e. Brussels sprouts. This might be a low risk strategy

for Brussels sprouts growers and could have added benefits in terms of preserving local populations of arthropod natural enemies as a consequence of reduced insecticide applications to the crop. Of course, *P. neoaphidis* must be present in the area. *Pandora neoaphidis* can overwinter on the soil surface providing a source of inoculum for the following year; additional inoculum could even be introduced with infected aphids (Ekesi *et al*., 2005; Pell *et al*., 2001; Pell *et al*., 2009; Zhou, 2011).

Attempting to make the aphid population crash occur earlier in the season would be difficult, not least because the crash appears to be a function of high aphid densities. To initiate an epizootic in the low aphid populations seen earlier in the season would require large amounts of inoculum. This is a particular problem for *P. neoaphidis* because of the difficulty with its mass production (Shah & Pell, 2003). An alternative would be to infect aphids in the laboratory and introduce live infected or sporulating cadavers into the crop in order to provide the increased levels of inoculum needed for sufficient levels of infection at low host densities.

The biology of entomopathogenic fungi is moderated by abiotic conditions, notably temperature. Global climate change and predicted higher mean environmental temperatures are likely to impact on biological control strategies using pathogens and beneficial arthropods, as well as pest populations, because they are ectotherms. The results presented in this study suggest that *P. neoaphidis* will be more susceptible to higher mean temperatures since modelling estimates its Tmax at 32°C. The Hypocreales appear more able to deal with increased temperature under climate change since they have much higher Tmax estimates, 35-38°C. The effect of increased mean temperatures is likely to affect the host, *B. brassicae*, in a similar way to the hypocrealean fungi as Tmax estimates for the species are between 35 and 35.2°C. This is based on fungal activity across growth, germination and pathogenicity, however, the fungus may be able to survive such conditions, in a dormant state, to re-infect hosts when conditions become more suitable. Future work should also consider the survivability of fungal species at temperatures that exceed their fungal activity cardinal limits.

Other areas for future research include the following:

The temperature dependent models used in this study could be extended to predict the rate of aphid population decline in an epizootic under the conditions of diurnally fluctuating temperature that typically occur in the field. A simple day degree model, like the models applied to insect development, based on accumulated thermal time units could be fitted to the growth, germination and virulence of fungal pathogens. It could be used for fungal isolate selection in a biopesticide screening programmes (e.g. to identify fungal isolates that are best able to kill under fluctuating temperatures) as well as giving more detailed information about the mechanisms of the *P. neoaphidis* associated aphid crash.

New research is also needed to understand why no relationship was observed in the field between host plant age and the timing of the aphid crash, and yet an association was observed under controlled laboratory conditions between plant age/soluble nitrogen content and aphid fecundity and mortality. This field experiment would require measurements of soluble nitrogen within plants of different ages over the course of the season, to be compared with aphid population development rates and the timing of the crash. Investigation of the effect of within-plant variation in soluble nitrogen content on aphid development and susceptibility to *P. neoaphidis* is also warranted. The amino acid content of leaves can vary markedly in brassicas, dependent upon the fertilizer regime used to grow the crop, with young leaves having higher soluble nitrogen content than older leaves on the same plant (van Emden & Bashford, 1969; Minkenberg & Ottenhein, 1990; Tilsner *et al*., 2005). Because *B. brassicae* were found to colonise every part of the plant, it could be hypothesized that differences in the levels of available nitrogen in young versus older leaves on the same plant could result in different rates of aphid growth (Awmack & Leather, 2002). It is not known whether the rate of growth of *P. neoaphidis* is also dependent upon nitrogen availability within the aphid hosts. There is little information available on the effect of host insect quality on the virulence of entomopathogenic fungi, although dietary protein quality has been shown to impact on the antibacterial immune response in *Spodoptera littoralis* (Lepidoptera) (Lee *et al*., 2008).

Therefore, it would be worthwhile investigating whether the nitrogen content of the plant impacts on the nitrogen content of the aphid, which in turn affects the virulence of *P. neoaphidis*.

A conservation biological control strategy based around the aphid population crash would need to be tested in practice on Brussels sprouts. This would require yield assessments as aphid numbers are allowed to build to significantly higher levels than in conventional systems. It is important to consider the approach in terms of the entire system of crop management, where conventional products are treated as a last resort as opposed to banned outright. The systems approach advocates practices to be considered in unison not isolation, reducing the potential for antagonistic effects on the CBC program.

REFERENCES

Abbott, W.S. (1925). A method for computing the effectiveness of an insecticide. *Journal of Economical Entomology*, **18**, 265–267

Awmack, C.S. & Leather, S.R. (2002). Host plant quality and fecundity in herbivorous insects. *Annual Review of Entomolgy*, **47**, 817-844.

Andersen, M., Magan, N., Mead, A. & Chandler, D. (2006). Development of a population-based threshold model of conidial germination for analysing the effects of physiological manipulation on the stress tolerance and infectivity of insect pathogenic fungi. *Environmental Microbiology*, **8**, 1625–1634.

Asin, L. & Pons, X. (2001). Effect of high temperature on the growth and reproduction of corn aphids (Homoptera: Aphididae) and implications for their population dynamics on the northeastern Iberian peninsula. *Environmental Entomology*, **30**, 1127-1134.

Ayerst, G. (1969) The effects of moisture and temperature on growth and spore germination in some fungi. *Journal of Stored Products Research*, **5**, 127-141.

Ba-Angood, S.A. & Stewart, R.K. (1980). Occurrence, development and distribution of cereal aphids on early and late cultivars of wheat, barley and oats in southwestern Quebec. *Canadian Entomologist*, **112,** 615-620.

Basky, Z. (1993). Incidence and population fluctuation of *Diuraphis noxia* in Hungary. *Crop Protection*, **12**, 605-609.

Bugeme, D.M., Maniania, N.K., Knapp, M. & Boga, H.I. (2008). Effect of temperature on virulence of *Beauveria bassiana* and *Metarhizium anisopliae* isolates to *Tetranychus evansi*. *Experimental and Applied Acarology*. **46**, 275 – 285.

Baverstock, J., Alderson, P.G. & Pell, J.K. (2005). Influence of the pathogen *Pandora neoaphidis* on the foraging behaviour of the aphid parasitoid *Aphidius ervi*. *Ecological entomology*, **30**, 665-672.

Baverstock, J., Clark, S.J. & Pell. J.K. (2008a). Effect of seasonal abiotic conditions and field margin habitat on the activity of *Pandora neoaphidis* inoculum on soil. *Journal of Invertebrate Pathology*, **97**, 282-290.

Baverstock, J., Baverstock, K.E., Clark, S.J. & Pell, J.K. (2008b). Transmission of *Pandora neoaphidis* in the presence of co-occurring arthropods. *Journal of Invertebrate Pathology*, **98**¸ 356-359.

Baverstock, J., Clark, Anderson, P.G., S.J. & Pell. J.K. (2009). Intraguild interactions between the entomopathogenic fungus *Pandora neoaphidis* and an aphid predator and parasitoid at the population scale. *Journal of Invertebrate Pathology*, **102**, 167-172.

Blackman, R.L. (1974). Lice-cycle variation of *Myzus persicae* (Sulz.) (Hom., Aphididae) in different parts of the world, in relation to genotype and environment. *Bulletin of Entomological Research*, **63**, 595-607.

Blackman, R., L. (1971). Variation in the photoperiodic response within natural populations of *Myzus persicae*. *Bulletin of Entomological Research,* **60**, 533-546.

Blackman, R.L. & Eastop, V.F. (1984). Aphids on the world's crops. *An identification guide*. Wiley & Sons, USA.

Blanford, S. & Thomas, M. B. (1999). Host thermal biology: the key to understanding host-pathogen interaction and microbial pest control. *Agricultural and Forest Entomology*, **1**, 195-202.

Bloomberg. (2012). Bloomberg professional. [online]. Available at: Subscription service (Accessed: 13 September 2017).

Boiteau, G. (1986). Native predators and the control of potato aphids. *Canadian Entomologist*, **118**, 1177-1183.

Borisade, O. a. & Magan, N. (2014). Growth and sporulation of entomopathogenic *Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria farinosa* and *Isaria fumosorosea* strains in relation to water activity and temperature interactions. *Biocontrol Science and Technology*, **24,** 999–1011.

Briere, J. F., P. Pracros, Le Roux, A. Y. & Pierre, J. S. (1999.) A novel rate model of temperature-dependent development for arthropods. *Environmental Entomology*, **28**, 22-29.

Brown, G.C., Hasibuan, R. (1995). Conidial discharge and transmission efficiency of *Neozygites floridana*, an entomopathogenic fungus infecting two-spotted spider mites under laboratory conditions. *Journal of Invertebrate Pathology*, **65**, 10 – 16.

Bruce, T.J.A. (2010). Tackling the threat to food security caused by crop pests in the new millennium. *Food Security*, **2**, 133-141.

Chandler, D. (1994). Cryopreservation of fungal spores using porous beads. *Mycological Research*, **98**, 525-526.

Commonwealth mycological institute. (1979). *Erynia neoaphidis* factsheet.

Cox, D., R. (1972). Regression models and life tables. *Journal of Statistical Society*. **43**, 187-220.

Danish Ministry of the Environment. (2013). miljoestyrelsen.dk. http://www.miljoestyrelsen.dk/common/Udgivramme/Frame.asp?http://www.miljoestyrelsen.dk/udgiv/publications/2001/87-7944-343-5/html/kap01_eng.htm accessed 22/05/2013.

Davidson, G., Phelps, K., Sunderland, K.D., Pell, J.K., Ball, B.V., Shaw, K.E. & Chandler, D. (2003). Study of temperature-growth interactions of entomopathogenic fungi with potential for control of *Varroa destructor* (Acari: Mesostigmata) using a nonlinear model of poikilotherm development. *Journal of Applied Microbiology*, **94**, 816–825.

Dedryver, C.A., Ralec, A.L. & Fabre, F. (2010). The conflicting relationships between aphids and men: A review of aphid damage and control strategies. *Comptes Rendus Biologies*, **333**, 539-553.

DEFRA (2016). The Department for Food and Rural Affairs, 2016. DEFRA Statistics Database.

De Nardo, E.A.B & Grewal, P. S. (2003). Compatibility of *Steinernema feltiae* (Nematoda: Steinernematidae) with pesticides and plant growth regulators used in glasshouse plant production. *Biocontrol Science and Technology*, **13**, 441- 448.

Diaz, B.M., Legarrea, S., Marcos-Garcia. M.A. & Fereres, A. (2010). The spatio-temporal relationships among aphids, the entomophthoran fungus, *Pandora neoaphidis*, and aphidophagous hoverflies in outdoor lettuce. *Biological Control*, **53**, 304-311.

Dimbi, S., Maniania, N.K., Lux, S. a. & Mueke, J.M. (2004). Effect of constant temperatures on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African tephritid fruit flies. *BioControl*, **49,** 83–94.

Dodd, D., G, 1976. Key for the identification of the instars of the Cabbage aphid (*Brevicoryne brassicae* (L.)). *Plant Pathology*, **25**, pp. 84-86.

Douglas, A.E. (2003). Nutritional physiology of aphids. *Advances in Insect Physiology*, **31**, 73-140.

Er, M.K. & Gokce, A. (2004). Effects of selected pesticides used against glasshouse tomato pests on colony growth and conidial germination of *Paecilomyces fumosoroseus*. *Biological Control*, **31**, 398-404.

Ekesi, S., Maniania, N.K. & Ampong-Nyarko, K. (1999). Effect of Temperature on Germination, Radial Growth and Virulence of Metarhizium anisopliae and Beauveria bassiana on Megalurothrips sjostedti. *Biocontrol Science and Technology*, **9**, 177–185.

Ekesi, S., Shah, P.A., Clark, S.J., Pell, J.K. (2005). Conservation biological control with the fungal pathogen *Pandora neoaphidis*: implications of aphid species, host plant and predator foraging. *Agriculture and Forest Entomology*. **7**, 21 – 30.

European Commission. (2017). EU Pesticides Database [online] Available at: http://ec.europa.eu/food/plant/pesticides/eu-pesticides database/ (Accessed 13 September 2017).

FAO. (2013). http://www.fao.org/agriculture/crops/core-themes/theme/pests/ipm/en/ accessed 11/09/2013.

FAOSTAT. (1998). Food and Agriculture Organisation of the United Nations, 1998. FAO Statistics Database.

Fargues, J., Goettel, M.S., Smits, N., Oudraogo, A. and Rougier, M. (1997). Effect of temperature on vegetative growth of *Beauveria bassiana* isolates from different origins. *Mycologia*, **89**, 383–392.

Fargues, J. & Luz, C. (2011). Effects of Fluctuating Moisture and Temperature Regimes on the Infection Potential of *Beauveria bassiana* for *Rhodnius prolixus*. **211**, 202–211.

Fekih, I. Ben, Boukhris-Bouhachem, S., Eilenberg, J., Allagui, M.B. & Jensen, A.B. (2013). The occurrence of two species of entomophthorales (entomophthoromycota), pathogens of *Sitobion avenae* and *Myzus persicae* (Hemiptera: Aphididae), in Tunisia. BioMed Research International.

Feng, M., Chen, C., Shang, S., Ying, S., Shen, Z. & Chen, X. (2007). Aphid dispersal flight disseminates fungal pathogens and parasitoids as natural control agents of aphids. *Ecological Entomology*, **32**, 97-104.

Feng, M.-G. & Johnson, J.B. (1991). Bioassay of four entomophthoralean fungi (Entomophthorales) against *Diuraphis noxia* and *Metopolophium dirhodum* (Hompoptera: Aphididae). *Environmental Entomology*, **20,** pp. 338–345.

Flint, M.L. (1985). Cabbage Aphid, *Brevicoryne brassicae*. 36-39. In: *Integrated pest management for Cole crops and Lettuce*. University of California publication.

Garthwaite, D.G., Thomas, M.R., Heywood, E., Battersby, A., Parrish, G. & Smith, L. (2007). Pesticide usage survey report 219: Outdoor vegetable crops in GB. FERA.

Golizadeh, A., Kamali, K., Fathipour, Y. & Abbasipour, H. (2007). Temperature dependent development of diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae) on two brassicaceous host plants. *Insect Science*, **14**, 309–316.

Greene, C. (2017). https://www.ers.usda.gov/topics/natural-resources-environment/organic-agriculture/organic-market-overview.aspx. Accessed 27/04/2017.

Guetsky, R., Shtienberg, D., Elad, Y. & Dinoor, A. (2001). Combining biocontrol agents to reduce the variability of biological control. *Phytopathology*, **91**, 621-627.

Guzman-Franco, A.W., Clark, S.J., Alderson, P.G. & Pell, J.K. (2008). Effect of temperature on the *in vitro* radial growth of *Zoophthora radicans* and *Pandora blunckii*, two co-occurring fungal pathogens of the diamondback moth *Plutella xylostella*. *Biocontrol*, **53**, 501 – 516.

Hajek, A. (2004). Natural enemies: an introduction to biological control. Cambridge University Press, Cambridge, UK.

Hajek, A.E., McNeil, J.J. & Filotas, M.J. (2002). Using Entomophthorales for control: maturation, dormancy and survival of laboratory produced *Entomophaga maimaiga*. *Proceedings of the XXXIV annual meeting of the society for invertebrate pathology*, Noordwijkerhout, The Netherlands.

Hall, R.A. (1980). Effect of repeated subculturing on agar and passaging through an insect host on pathogenicity, morphology, and growth rate of *Verticillium lecanii*. *Journal of Invertebrate Pathology*, **36**, 216–222.

Hall, R.A. (1981). The fungus *Verticillium lecanii* as a microbial insecticide against aphids and scales. 483-498. In: *Microbial control of pests and plant diseases 1970-1980*. Academic Press, London/New York.

Herrera-Estrella, L., R. (2000). Genetically modified crops and developing countries. *Plant Physiology*, **124**, 923.

Holland, J.M. & Thomas, S.R. (1997). Quantifying the impact of polyphagous invertebrate predators in controlling cereal aphids and in preventing wheat yield and quality reductions. *Annals of Applied Biology*, **131**, 375-397.

Hollingsworth, R.G., Steinkraus, D.C. & Slaymaker, P.H. (1995). Sampling to predict fungal epizootics on cotton aphids (Homoptera: Aphididae). *Environmental Entomology*, **24**, 1414-1421.

Holland, J.M. & Oakley. J. (2007). Importance of arthropod pests and their natural enemies in relation to recent farming practice changes in the UK. *HGCA research review*, **6**, 1-105.

Hsiao, W., F., Bidochka, M.J. & Khachatourians, G.G. (1992). Effect of temperature and relative humidity on the virulence of the entomopathogenic fungus, *Verticillium lecanii*, toward the oat-bird berry aphid, *Rhopalosiphum padi* (Hom. Aphididae). Journal of Applied Entomology, **114**, 484-490.

Humber, R.A. (2008). Evolution of entomopathogenicity in fungi. *Journal of Invertebrate Pathology*, **98**, 262-266.

Humber, R.A. (2012). Entomophthoromycota: a new phylum and reclassification for entomophthoroid fungi. *Mycotaxon*, **120**,477-492.

Inglis, G.D., Goettel, M.S., Butt, T.M. & Strasser, H. (2001). Use of hyphomycetous fungi for managing insect pests. In: Butt TM, Jackson C, Magan N (eds) *Fungi as biocontrol agents: progress problems and potential*. CABI Publishing, Wallingford, 23–70.

Inglis, G.F., Johnson, D.L. & Goettel, M.S. (1997). Field and laboratory evaluation of two conidial batches of *Beauveria bassiana* (Balsamo) vuillemin against grasshoppers. *The Canadian Entomologist*, **129**, 171-186.

IRAG (Insecticide Resistance Action Group). (2012). Guidelines for controlling aphids in brassicae crops and combating insecticide resistance in the peach-potato aphid, *Myzus persicae*. 1-8.

Isik, M. & Gorur, G. (2009). Aphidicidial activity of seven essential oils against the cabbage, *Brevicoryne brassicae* L. (Hemiptera: Aphididae). *Munis Entomology and Zoology*, **4**, 424-431.

Jaronski, S. T. (2010). Ecological factors in the inundative release of fungal entomopathogens. *BioControl*, **55**, 159 – 185.

Jarosik, V. & Dixon, A.F.G. (1999). Population dynamics of a tree-dwelling aphid: regulation and density-independent processes. *Journal of Animal Ecology*, **68**, 726-732.

Jenkins, N.E. & Goettel, M.S. (1997). Methods for mass-production of microbial control agents of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada*, **171**, 37 – 48.

Jones, M.G. (1979). Abundance of aphids on cereals from before 1973 to 1977. *Journal of Applied Ecology*, **16**,1-22.

Karley, A. J., Douglas, A.E. & Parker, W.E. (2002). Amino acid composition and nutritional quality of potato leaf phloem sap for aphids. *The Journal of Experimental Biology*, **205**, 3009-3018.

Karley, A.J., Parker, W.E., Pitchford, J.W. & Douglas, A.E. (2004). The mid-season crash in aphid populations: why and how does it occur? *Ecological Entomology*, **29**, 383-388.

Karley, A.J., Pitchford, J.W., Douglas, A.E., Parker, W.E. & Howard, J.J. (2003). The causes and processes of the mid-summer crash of the patio aphids *Macrosiphum euphorbiae* and *Myzus persicae* (Hemiptera: Aphididae). *Bulletin of Entomological Research*, **93**, 425-437.

Kaplan, E. L. & Meier, P. (1958). Nonparametric estimate for incomplete observations. *Journal of the American Statistical Association*, **53**, 457-481.

Kiewnick, S. (2007). Effect of temperature on growth, germination, germ-tube extension and survival of *Paecilomyces lilacinus* strain 251. *Biocontrol Science and Technology*, **16,** 535–546.

Kim, J.J., Goettel, M. S. & Gillespie, D.R. (2007). Potential of *Lecanicillium* species for dual microbial control of aphids and the cucumber powdery mildew fungus, *Sphaerotheca fuliginea*. *Biological Control*, **40**, 327-332.

Kish, L.P. & Allen, G.E. (1978). The biology and ecology of *Nomuraea rileyi* and a program for predicting its incidence on *Anticarsia gemmatalis* in soybean. *University of Fla. Agricultural Experimental Station Technical Bulletin*, **795**.

Koch, K. A., Potter, B.D. & Ragsdale, D.W. (2010). Non-target impacts of soybean rust fungicides on the fungal entomopathogens of soybean aphid. *Journal of Invertebrate Pathology*, **103,** 156–164.

Koda, K. & Nakamura, H. (2012). Effects of temperature on the development and survival of an endangered butterfly, Lycaeides argyrognomon (Lepidoptera: Lycaenidae) with estimation of optimal and threshold temperatures using linear and nonlinear models. *Entomological Science*, **15,** 162–170.

Kontodimas, D.C.H. (2012). Nonlinear models for describing development and fecundity of the pseudococcid predators *Nephus includens* and *Nephus bisignatus*. **21**, 13–24.

Kope, H.H., Alfaro, R.I. & Lavallée, R. (2007). Effects of temperature and water activity on Lecanicillium spp. conidia germination and growth, and mycosis of *Pissodes strobi*. *BioControl*, **53**, 489–500.

Kope, H.H., Alfaro, R.I., Levallée, R. (2008). Effects of temperature and water activity on *Lecanicillium* spp. conidia germination and growth, and mycosis of *Pissodes strobi*. *BioControl* **53**, 489–500.

Kouassi M, Coderre D, Todorova, S.I. (2003). Effect of plant type on the persistence of *Beauveria bassiana*. *Biocontrol Science and Technology*, **13**, 415–427

Lacey, L.A., Frutos, R., Kaya, H.K. & Vail, P. (2001). Insect pathogens as biological control agents: do they have a future? *Biological Control*, **11**, 230-248.

Lactin, D. J., N. J. Holliday, D. L. Johnson, and R. Craigen. (1995). Improved rate model of temperature-dependent development by arthropods. *Environmental Entomol*ogy, **24**, 68-75.

Landis, D.A., Wratten, S.D. & Gurr, G.M. (2000). Habitat management to conserve natural enemies of arthropod pests in agriculture. *Annual Review of Entomology*, **45**, 175-201.

Lee, K.P., Simpson, S.J. & Wilson, K. (2008). Dietary protein-quality influences melanisation and immune function in an insect. *Functional Ecology*, **22**, 1052-1061.

Logan, J.A., Wollkind, D.J., Hoyt, S.C., Tanigoshi, L.K. (1976). An analytic model for description of temperature dependent rate phenomena in arthropods. *Environmental Entomology*, **5**, 1133–1140.

Luke, B. M. & Bateman, R. P. (2006). Effects of chemical and botanical insecticides used for locust control on *Metarhizium anisopliae* var. *acridum* conidia after short- to medium-term storage at 30°C. *Biocontrol Science and Technology*, **16**, 761 – 766.

Luz, C. & Fargues, J. (1999). Dependence of the entomopathogenic fungus, *Beauveria bassiana*, on high humidity for infection of *Rhodnius prolixus*. *Mycopathologia*, **146**, 33-41.

Luz, C., Fargues, J. (1997). Temperature and moisture requirements for conidial germination of an isolate of *Beauveria bassiana*, pathogenic to *Rhodnius prolixus*. *Mycopathologia* **138**, 117–125.

MacGullivray, M.E. & Anderson, G.B. (1958). Development of four species of aphids (Homoptera) on potato. *Canadian Entomologist*, **90**, 148-155.

Maddock, J.V., McManus, M.L., Jeffords, M.R. &Webb, R.E. (1992). Exotic insect pathogens as classical biological control agents with an emphasis on regulatory considerations. 27-39. In: *Selection criteria and ecological consequences of importing natural enemies*. Thomas Say publications entomology society, USA, Lanham, MD.

Maniania, N.K., Bugeme, D.M., Wekesa, V.W., Delalibera, I. & Knapp, M. (2008). Role of entomopathogenic fungi in the control of *Tetranychus evansi* and *Tetranychus urticae* (Acari: Tetranychidae), pests of horticultural crops. *Experimental & applied acarology*, **46**, 259–74.

Mattson., W., J. (1980). Herbivory in relation to plant nitrogen content. *Annual Review of Ecology and Systematics*, **11**, 1-152.

McCoy, C.W. (1981). Pest control by the fungus *Hirsutella thompsonii*. 499-512. In: *Microbial control of pests and plant diseases 1970-1980*. Academic Press, London/New York.

Minkenberg., O., P., J. & Ottenhein., J., G., W. (1990). Effect of nitrogen content of tomato plants on preference and performance of leafmining fly. *Oecologia*, **83**, 291-298.

Mishra, S., Kumar, P. & Malik, A. (2013). Effect of temperature and humidity on the pathogenicity of native *Beauveria bassiana* isolate against *Musca domestica* L. *Journal of Parasitic Diseases*, **39**, 697-704.

Mohammed, A.A. & Hatcher, P.E. (2016). Effect of temperature, relative humidity and aphid developmental stage on the efficacy of the mycoinsecticide Mycotal ® against *Myzus persicae*. *Biocontrol Science and Technology*, **3157**, 1–32.

Morgan, L., W., Boddy, L., Clark, S., J. & Wilding N. (1995). Influence of temperature on germination of primary and secondary conidia of *Erynia neoaphidis. Journal of Invertebrate Pathology*, **65**, 132-138.

Morgan, D., Walters, K.F.A. & Aegerter, J.N. (2001). Effect of temperature and cultivar on pea aphid, *Acyrthosiphon pisum* (Hemiptera: Aphididae) life history. *Bulletin of Entomological Research*, **91**, 47-52.

Muller, C.B., Adriaanse, I.C.T., Belshaw, R. & Godfray, H.C.J. (1999). The structure of an aphid-parasitoid community. *Journal of Animal Ecology*, **68**, 346-370.

Nahar, P.B., Kulkarni, S.A., Kulye, M.S., Chavan, S.B., Kulkarni, G., Rajendran, A., Yadav, P.D., Shouche, Y. & Deshpande, M. V. (2008). Effect of repeated in vitro sub-culturing on the virulence of *Metarhizium anisopliae* against *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Biocontrol Science and Technology*, **18,** 337–355.

Nakata, T. (1995). Population fluctuations of aphids and their natural enemies on potato in Hokkaido. *Applied Entomology and Zoology*, **30**,129-138.

Oerke, E.C. (2006). Crop losses to pests. *Journal of Agricultural Science*, **144**, 31-43.

Ormond, E.L., Thomas, A.P.M., Pell, J.K., Freeman, S.N. & Roy, H.E. (2011) Avoidance of a generalist entomopathogenic fungus by the ladybird, *Coccinella septempunctata*. *Microbiology Ecology*, **77**, 229-237.

Orre, G.U.S., Wratten, S.D., Jonsson, M. & Hale, R.J. (2009). Effects of a herbivore-induced plant volatile on arthropods from three trophic levels in brassicas. *Biological Control*, **53**, 62-67.

Parker, W.E., Howard, J.J. & Karley, A.J. & Douglas, A.E. (2000). Crop growth stage and phenology of aphid populations on potato. *The Brighton Crop and Pests Conference 2000*. 955-960.

Papierok, B. & A. Hajek. (1997). Fungi: Entomophthorales. In: L. Lacey (ed), Manual of Techniques in Insect Pathology. Academic Press Inc., San Diego, pp. 187–212.

Pell, J.K., Eilenberg, J., Hajek, A.E. & Straus, D.C. (2001). Fungi as Biocontrol Agents. Chapter 4. Biology,Ecology and Pest Management Potential of Entomophthorales.: CAB International.

Pell, J.K., Hannam, J.J. & Steinkraus, D.C. (2009). Conservation biological control using fungal entomopathogens. *BioControl*, **55,** 187–198.

Pimentel, D. (1997). Techniques for reducing pesticides: environmental and economic benefits. John Wiley and Sons, Chichester, UK.

Pons, X., Comas, J. & Hardie, J. (1995). Occurrence of holocyclic and anholocyclic populations of *Rhopalosiphum padi* and *Sitobion avenae* (Hom., Aphididae) in the northeast of Spain. *Journal of Applied Entomology*, **119**, 171-175.

Potter, C., 1952. An improved laboratory spray apparatus for applying direct sprays and surface films, with data on the electrostatic charge of atomized spray fluids. *Annuals of Applied Biology*, **39**, 1-28.

Ramoska, W.A. (1984) The influence of relative humidity on *Beauveria bassiana* infectivity and replication in the chinch bug, *Blissus leucopterus*. *Journal of invertebrate Pathology*, **43**, 389-394.

Rangel, D.E.N., Brage, G.U.L., Anderson, A.J. & Roberts, D.W. (2005) Variability in conidial thermotolerance of *Metarhizium anisopliae* isolates from different geographic origins. *Journal of Invertebrate Pathology*, **88**, 116-125.

Razaq, M., Aslam, M., Amer, M. & Shad, S.A. (2011). Insect pest status of aphids on oilseed brassica crops and need for chemical control. *Crop and Environment*, **2**, 60-63.

Régnière, J., Powell, J., Bentz, B. & Nealis, V. (2012). Effects of temperature on development, survival and reproduction of insects: experimental design, data analysis and modeling. *Journal of insect physiology*, **58,** 634–47.

Rehner, S.A. & Buckley, E. (2005). A *Beauveria* phylogeny inferred from nuclear ITS and EFI-alpha sequences: evidence fro cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia*, **97**,84-98.

Ritz, C. & Streibig. J., C. (2012). Dose reposne curives and other nonlinear curves in Weed Science and Ecotoxicology with the add-on packaged *drc* in **R**.

Rivas, F., Nuñez, P., Jackson, T. & Altier, N, (2013). Effect of temperature and water activity on mycelia radial growth, conidial production and germination of *Lecanicillium* spp. isolates and their virulence against *Trialeurodes vaporariorum* on tomato plants. *BioControl*, **59,** 99–109.

Royal Society Report, (2009). *Reaping the benefits: Science and the sustainable intensification of global agriculture.*

Roy, H.E., Baverstock, J., Ware, R.L., Clark S.J., Majerus, M.E.N., Baverstock, K.E. & Pell, J.K. (2008). Intraguild predation of the aphid pathogenic fungus *Pandora neoaphidis* by the invasive coccinellid *Harmonia axyridis*. *Ecological entomology*, **33**, 175-182.

Roy, M., Brodeur, J. & Cloutier, C. (2002). Relationship Between Temperature and Developmental Rate of *Stethorus punctillum* (Coleoptera: Coccinellidae) and Its Prey *Tetranychus mcdanieli* (Acarina: Tetranychidae). *Environmental Entomology*, **31,** 177–187.

Roy, H.E., Pell, J.K. & Alderson, P.G. (2010). Targeted dispersal of aphid pathogenic fungus *Erynia neoaphidis* by the aphid predator *Coccinella septempunctata*. *Biocontrol Science and Technology*, **11**, 99-110.

Scorsetti, A.C., Jensen, A.B., Lopez Lastra, C. & Humber, R.A. (2012). First report of *Pandora neoaphidis* resting spore formation in vivo in aphid hosts. *Fungal Biology*, **116**, pp. 196-203.

Scorsetti, A.C., Maciá, A., Steinkraus, D.C. & López Lastra, C.C. (2010). Prevalence of Pandora neoaphidis (Zygomycetes: Entomophthorales) infecting *Nasonovia ribisnigri* (Hemiptera: Aphididae) on lettuce crops in Argentina. *Biological Control*, **52**, 46-50.

Shah, P. A., Aebi, M. & Tuor, U. (2002). Effects of constant and fluctuating temperatures on sporulation and infection by the aphid-pathogenic fungus *Pandora neoaphidis*. *Entomologia Experimentalis et Applicata*, **103,** 257–266.

Shah, P.A. & Pell, J.K. (2003). Entomopathogenic fungi as biological control agents. *Applied Microbiological Biotechnology*, **61**, 413-423.

Shah, P.A., Tymon, A. & Pell, J.K. (2001). Conservation biocontrol with mycopathogens: *Erynia neoaphidis* and agricultural field margins. *Proceedings of the XXXIV annual meeting of the society for invertebrate pathology*, Noordwijkerhout, The Netherlands.

Shapiro-IIan, D.I., Reilly, C.C. & Hotchkiss, M.W. (2011). Comparative impact of artificial selection for fungicide resistance on *Beauveria bassiana* and *Metarhizium brunneum*. *Environmental Entomology*, **40**, 59-65.

Shi, P. & Ge, F. (2010). A comparison of different thermal performance functions describing temperature-dependent development rates. *Journal of Thermal Biology*, **35,** 225–231.

Shi, P., Ge, F., Sun, Y. & Chen, C. (2011). Journal of Asia-Paci fi c Entomology A simple model for describing the effect of temperature on insect developmental rate. *Journal of Asia-Pacific Entomology*, **14,** 15–20.

Sierotzki, H., Camastral, F., Shah, P.A., Aebi, M. & Tuor, U. (2000). Biological characteristics of selected *Erynia neoaphidis* isolates. *Mycoogical Research*, **104**, 213-219.

Smits, N., Briere, J.-F. & Fargues, J. (2003). Comparison of non-linear temperature-dependent development rate models applied to in vitro growth of entomopathogenic fungi. *Mycological Research*, **107,** 1476–1484.

Soroka., J.J. & Mackay, P.A. (1990). Growth of pea aphid, *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae), populations on caged plants of six cultivars of field peas and the effects of pea aphids on harvest components of caged field peas. *Canadian Entomologist*, **122**, 1193-1199.

Stark, J.D. & Banks, J.E. (2003). Population-level effects of pesticides and other toxicants on arthropods. *Annual review of entomology*, **48,** 505–19.

Steinkraus, D.C., Hollingsworth, R.G.& Slaymaker, P.H. (1995). Prevalence of *Neozygites fresenii* (Entomophthorales: Neozygitaceae) on cotton aphids (Homoptera: Aphididae) in Arkansas cotton. *Environmental Entomology*, **24**, 465-474.

Steinkraus, D.C. (2006). Factors affecting the transmission of fungal pathogens of aphids. *Journal of Invertebrate Pathology*, **92**, 125-131.

Steinkraus, D.C., Boys, G.O. & Rosenheim, J.A. (2002). Classical biological control of *Aphis gossypii* (Homoptera: Aphididae) with *Neozygites fresenii* (Entomophthorales: Neozygitaceae) in California cotton. *Biological Control*, **25**, 297-304.

Steinkraus, D.C. & Hollingsworth, R.G. (1994). Predicting fungal epizootics on cotton aphids. *Arkansas Farm Research*, **43**, 10-11.

Steinkraus, D.C., Kring, T.J. & Tugwell, N.P. (1991). *Neozygites fresenii* in *Aphis gossypii* on cotton. *Southwestern Entomologist*, **16**, 118-122.

Stubbs., A. & Falk., S., J. (2002). British hoverflies: an illustrated guide. British entomological & natural history society. 2nd Ed.

Sullivan, D.J. & Völkl, W. (1999). Hyperparasitism: Multitrophic ecology and behaviour. *Annual Review of Entomology*, **44**, 291-315.

Tatchell, M. (2004). Guide to the identification of aphids found commonly on Lettuce, Brassicas and Potatoes (unpublished).

Thacker, J.R.M. (2002). An introduction to arthropod pest control. Cambridge University Press, Cambridge, UK.

Tilman, D., Cassman, K.G., Matson, P.A., Naylor, R. & Polasky, S. (2002). Insight. *Nature*, **418**, 671 – 677.

Tilman, D. (1999). Global environmental impacts of agricultural expansion.: the need for sustainable and efficient practices. *Proc. Natl Acad. Sci. USA*, **96**, 5995-6000.

Tilsner, J., Kassner, N., Struck, C. & Lohaus, G. (2005). Amino acid contents and transport in oilseed rape (*Brassica napus* L.) under different nitrogen conditions. *Planta*, **221**, 328-338.

Thomas, M.B. & Blanford, S. (2003). Thermal biology in insect-parasite interactions. *Trends in Ecology & Evolution*, **18,** 344–350.

Thomas, M.B. & Jenkins, N.E. (2014). Effects of temperature on growth of *Metarhizium flavoviride* and virulence to the variegated grasshopper, *Zonocerus variegatus*,. *Mycological Research*, **101**, 1469–1474.

UK, NAP. (2013). UK National Action Plan for the Sustainable Use of Pesticides (Plant (Protection Protducts) [available online: http://c-ipm.org/fileadmin/c-ipm.org/British_NAP__in_EN_.pdf]: Last accessed 10/01/2016.

van Emden, H, F. & Bashford, M. A. (1969). A comparison of the reproduction of *Brevicoryne brassicae* and *Myzus persicae* in relation to soluble nitrogen and leaf age (leaf position) in the Brussels sprout plant. *Entomologia Experimentalis et Applicata*, **12**, 351-364.

Vadim, K., Olga, Y., Tyurin, M., Yu, A., Evgeniy, E, Ting-Chi, W, Oksana, T. & Victor, Y.G. (2017). Ecological preferences of *Metarhizium* species from Russia and neighboring territories and their activity against Colorado potato beetle larvae. *Journal of Invertebrate Pathology*, **149**, 1-7.

Vega, F. E. (2008). Insect pathology and fungal endophytes. *Journal of Invertebrate Pathology*, **98**, 277-279.

Vega, F.E., Goettel, M.S., Blackwell, M., Chandler, D., Jackson, M.A., Keller, S., Koike, M., Maniania, N.K., Monzon, A., Ownley, B.H., Pell, J.K., Rangel, D.E. & Roy, H.E. (2009). Fungal entomopathogens: new insights on their ecology. *Fungal Ecology*, 2, pp. 149-159.

Vidal, C., Fargues, J. & Lacey, L.A. (2011). Intraspecific Variability of *Paecilomyces fumosoroseus* : Effect of Temperature on Vegetative Growth. **26,** 18–26.

Wang, S.Y., Qi, Y.F., Desneux, N., Shi.X.Y., Biondi, A. & Gao, X. (2017). Sublethal and transgenerational effects of short-term and chronic exposures to the

neonicotinoid nitenpyram on the cotton aphid *Aphis gossypii*. *Journal of Pest Science*, **90**, 389-396.

Watt, A.D. (1979). The effect of cereal growth stages on the reproductive activity of *Sitobion avenae* and *Metopolophium dirhodium*. *Annals of Applied Biology*, **91**,145-157.

Wellings, P.W., Leather, S.R. & Dixon, A.F.G. (1980). Seasonal variation in reproductive potential: a programmed feature of aphid life cycles. *Journal of Animal Ecology*, **49**, 975-985.

White, S. (2013). Entomology Fellowship Training, Boxworth, UK. ADAS in-house training, 1-18.

Wiliding, N. & Brobyn. (1980). Effects of fungicides on the development of *Entomophthora aphidis*. Transactions of the British Mycological Society, **75**, 297-302.

Wraight, S.P., Ramos, M.E., Avery, P.B., Jaronski, S.T. & Vandenberg, J.D. (2010). Comparative virulence of *Beauveria bassiana* isolates against lepidopteran pests of vegetable crops. *Journal of Invertebrate Pathology*, **103,** 186–199.

Yeo, H., Pell, J.K., Alderson, P.G., Clark, S.J. & Pye, B.J. (2003). Laboratory evaluation of temperature effects on the germination and growth of entomopathogenic fungi and on their pathogenicity to two aphid species. *Pest management science*, **59**, 156–65.

Zhou, X., 2011. Winter prevalence of obligate aphid pathogen *Pandora neoaphidis* mycosis in the host *Myzus persicae* populations in southern China: modeling description and biocontrol implication. *Brazilian Journal of microbiology*, **43**, pp. 325-331.

APPENDICES

Appendix 1 Parameter estimates with their corresponding error and measure of model fit of linear and five temperature-dependent growth models for mycelial extension rates of all fungal isolates used in this study.

Fungal isolate	Model	Media	T0	TM	Topt	Rmax	aa	DeltaT	bb	*R2*	RSS	AIC
Beauveria bassiana (ATCC strain)	Observed	SDA	5	38	25	3.3039	-	-	-	-	-	-
		SEMA	7	38	28	5.6538	-	-	-	-	-	-
	Linear	SDA	3.5986159	-	-	-	-	-	-	0.98	-	-
		SEMA	5.6567901	-	-	-	-	-	-	0.99	-	-
	Taylor	SDA	9.6153 (0.7599)	-	25.2182 (0.5467)	2.9715 (0.1294)	-	-	-	-	0.4748	69.61541
		SEMA	-10.3553 (1.0881)	-	27.8119 (0.9999)	4.7598 (0.1916)	-	-	-	-	0.6957	80.91013
	Lactin-1	SDA	-	35.31593 (0.65484)	27.83516	-	0.11717 (0.01172)	7.48077 (0.43856)	-	-	0.5042	75.37627
		SEMA	-	35.86951 (0.83222)	29.11399	-	0.13032 (0.01415)	6.75552 (0.40785)	-	-	0.7055	81.91212
	Kontdamis	SDA	7.767 (0.7195)	59.31 (4.086)	-	-	-0.0001347 (0.00003861)	-	-	-	0.494	73.42326
		SEMA	2.388 (1.65)	41.04 (1.988)	-	-	0.0005533 (0.000161)	-	-	-	0.6932	80.64584
	Briere-1	SDA	-4.8748028 (4.6615993)	34.2586853 (0.5538865)	26.95989	-	0.0012902 (0.0002649)	-	-	-	0.4824	71.13716

		SEMA	0.8573 (3.305)	35.44 (0.9403)	28.43904	-	0.002343 (0.0004748)	-	-	-	0.6868	79.97854
	Briere-2	SDA	3.295 (3.944)	39 (6.331)	25.70771	-	0.0002897 (0.0006526)	-	0.9008 (0.5795)	-	0.4784	71.26358
		SEMA	0.348658 (6.871072)	35.095923 (3.751434)	28.50005	-	0.002524 (0.002013)	-	2.147150 (1.698334)	-	0.6973	81.96966
Beauveria bassiana (GHA strain)	Observed	SDA	7	36	25	3.6266	-	-	-	-	-	-
		SEMA	7	38	26	6.6941	-	-	-	-	-	-
	Linear	SDA	5.28371768	-	-	-	-	-	-	0.95	-	-
		SEMA	7.39558	-	-	-	-	-	-	0.95	-	-
	Taylor	SDA	9.5683 (1.4083)	-	26.1499 (1.1036)	2.7258 (0.2118)	-	-	-	-	0.704	90.31716
		SEMA	-9.549 (1.449)	-	28.5289 (1.3808)	4.3588 (0.2759)	-	-	-	-	1.147	144.9721
	Lactin-1	SDA	-	35.6566 (0.8554)	28.49	-	0.1289 (0.0171)	7.1666 (0.6844)	-	-	0.6942	89.19406
		SEMA	-	36.24838 (1.08911)	29.77812	-	0.14464 (0.02085)	6.47026 (0.68619)	-	-	1.153	145.3893
	Kontdamis	SDA	2.703 (2.141)	38.63 (1.670)	-	-	0.0004005 (0.0001371)	-	-	-	0.6876	88.43485

		SEMA	4.480 (2.340)	41.18 (2.824)	-	-	0.0005884 (0.0002513)	-	-	-	1.152	145.3704
	Briere-1	SDA	0.8195 (4.817)	34.13 (0.8391)	27.38719	-	0.001495 (0.0004183)	-	-	-	0.692	88.94504
		SEMA	4.984 (3.601)	35.56 (1.522)	28.99327	-	0.002449 (0.0006311)	-	-	-	1.13	143.5919
	Briere-2	SDA	-1.6726034 (11.4267407)	33.4135002 (1.9219190)	24.7253	-	0.0017621 (0.0007767)	-	2.5462280 (1.39792846)	-	0.7008	90.85557
		SEMA	1.160860 (9.160968)	33.063606 (1.709689)	29.63327	-	0.004004 (0.001211)	-	4.235276 (4.239717)	-	1.137	145.0505
Lecanicillium longisporum	Observed	SDA	1	34	24	3.3125	-	-	-	-	-	-
		SEMA	0	36	25	3.2962	-	-	-	-	-	-
	Linear	SDA	-0.25246	-	-	-	-	-	-	0.98	-	-
		SEMA	-2.23861171	-	-	-	-	-	-	0.93	-	-
	Taylor	SDA	7.1895 (0.4368)	-	21.2984 (0.3872)	2.6839 (0.1454)	-	-	-	-	0.4727	67.85265
		SEMA	8.6139 (1.2704)	-	21.0839 (0.9716)	2.1956 (0.2401)	-	-	-	-	0.851	125.6297
	Lactin-1	SDA	-	32.599189 (0.170470)	25.160527	-	0.111962 (0.008328)	7.438662 (0.230555)	-	-	0.3854	48.66296

		SEMA	-	33.73584 (0.87176)	25.22648	-	0.08606 (0.02555)	8.50936 (0.59961)	-	-	0.8524	125.7891
	Kontdamis	SDA	1.693 (0.9311)	33.06 (0.2461)	-	-	0.0005809 (0.00007388)	-	-	-	0.3799	47.3081
		SEMA	6.4393109 (1.7550346)	45.1627117 (3.7545204)	-	-	-0.0002380 (0.000112)	-	-	-	0.8708	127.8404
	Briere-1	SDA	-0.3359 (0.1717)	32.37 (0.5447)	25.86263	-	0.0005956 (0.0001904)	-	-	-	0.432	59.37988
		SEMA	-88.55 (14.92)	32.82 (0.4971)	23.21047	-	0.0002827 (0.0003933)	-	-	-	0.7671	101.8029
	Briere-2	SDA	0.7836 (3.257)	32.71 (0.3269)	23.00104	-	0.0007280 (0.0001572)	-	1.164 (0.1708)	-	0.3792	48.04287
		SEMA	-1.3178619 (11.4039237)	34.4825994 (3.0065241)	22.30145	-	0.0003293 (0.0004866)	-	0.9414039 (0.5724420)	-	0.7524	101.0945
Lecanicillium muscarium	Observed	SDA	5	36	23	3.132	-	-	-	-	-	-
		SEMA	6	35	26	6.242	-	-	-	-	-	-
	Linear	SDA	0.32097649	-	-	-	-	-	-	0..85	-	-
		SEMA	-2.20858896	-	-	-		-	-	0.89	-	-
	Taylor	SDA	11.2704 (2.0770)	-	23.6504 (1.2285)	2.2256 (0.2026)	-	-	-	-	0.7467	110.8286

		SEMA	9.8331 (1.5626)	-	24.4729 (1.0653)	3.7543 (0.3381)	-	-	-	-	1.229	157.6691
	Lactin-1	SDA	-	36.08223 (1.71871)	27.24782	-	0.08589 (0.02392)	8.83441 (0.85547)	-	-	0.752	111.4914
		SEMA	-	34.74582 (0.94993)	27.58906	-	0.11280 (0.02356)	7.15676 (0.48366)	-	-	1.218	156.78
	Kontdamis	SDA	5.611 (2.104)	56.88 (7.525)	-	-	-0.0001067 (0.00006374)	-	-	-	0.7503	111.28
		SEMA	0.5871 (2.865)	37.39 (1.8)	-	-	0.0005129 (0.0002145)	-	-	-	1.22	156.9834
	Briere-1	SDA	-49.11 (72.78)	34.79 (1.721)		-	0.0003828 (0.0004269)	-	-	-	0.7472	110.8823
		SEMA	-6.0492358 (9.1442316)	33.6033835 (0.7168937)		-	0.0017002 (0.0006352)	-	-	-	1.206	155.8767
	Briere-2	SDA	-8.0721298 (39.7648982)	40.2448877 (21.4710363)		-	0.0001127 (0.0008087)	-	0.8602495 (1.7957740)	-	0.7552	112.8092
		SEMA	-30.020191 (63.514732)	32.558556 (0.559016)		-	0.001613 (0.001399)	-	3.82082 (2.912174)	-		
Metarhizium brunneum	Observed	SDA	9	36	-	3.9673	-	-	-	-		
		SEMA	9	36	-	5.5795	-	-	-	-		
	Linear	SDA	7.614837398	-	-	-	-	-	-	0.99	-	-

		SEMA	6.754631536	-	-	-	-	-	-	0.93	-	-
	Taylor	SDA	6.5944 (0.3421)	-	24.599 (0.2732)	3.3854 (0.1451)	-	-	-	-	0.4543	65.37673
		SEMA	7.2049 (0.4783)	-	23.8970 (0.3695)	4.3724 (0.2372)	-	-	-	-	0.759	114.6478
	Lactin-1	SDA	-	33.304692 (0.197772)	27.236871	-	0.156992 (0.008348)	6.067821 (0.253365)	-	-	0.444	63.18127
		SEMA	-	33.70045 (0.38368)	27.16298	-	0.13611 (0.01385)	6.53747 (0.36763)	-	-	0.8754	128.3489
	Kontdamis	SDA	6.721 (0.7536)	34.52 (0.3524)	-	-	0.001030 (0.0001375)	-	-	-	0.4427	62.90286
		SEMA	5.134886 (1.053795)	34.821610 (0.494616)	-	-	0.001101 (0.000194)	-	-	-	0.7557	114.2316
	Briere-1	SDA			-	-		-	-	-		
		SEMA			-	-		-	-	-		
	Briere-2	SDA			-	-		-		-		
		SEMA			-	-		-		-		
Isaria fumosorosea	Observed	SDA	7	35	25	4.4959	-	-	-	-	-	-
		SEMA	6	37	25	4.2763	-	-	-	-	-	-

	Linear	SDA	1.916667	-	-	-	-	-	-	0.97	-	-
		SEMA	2.707035755	-	-	-		-	-	0.99	-	-
	Taylor	SDA	7.2999 (0.4492)	-	22.3143 (0.3676)	2.9479 (0.1554)	-	-	-	-	0.5086	76.21718
		SEMA	9.0410 (0.7889)	-	24.0958 (0.5452)	3.6497 (0.2042)	-	-	-	-	0.72	109.5851
	Lactin-1	SDA	-	33.14114 (0.25734)	25.8535	-	0.11728 (0.01024)	7.28764 (0.30395)	-	-	0.4985	74.28708
		SEMA	-	34.74520 (0.63356)	27.42727	-	0.10932 (0.01528)	7.31793 (0.32392)	-	-	0.7505	113.5638
	Kontdamis	SDA	2.430 (1.073)	33.89 (0.3628)	-	-	0.0006212 (0.00009583)	-	-	-	0.4776	70.17191
		SEMA	1.235 (1.642)	36.73 (0.9525)	-	-	0.0005455 (0.00013258)	-	-	-	0.7216	109.7906
	Briere-1	SDA			-	-		-	-	-		
		SEMA			-	-		-	-	-		
	Briere-2	SDA			-	-		-		-		
		SEMA			-	-		-		-		

Pandora neoaphidis (NW420)	Observed	SEMA	9	32	21	1.819	-	-	-	-	-	-
	Linear	SEMA	6.401015228	-	-	-	-	-	-	0.99	-	-
	Taylor	SEMA	-4.9051 (0.21540)	-	21.07302 (0.22074)	1.9912 (0.08367)	-	-	-	-	0.1481	-12.96151
	Lactin-1	SEMA	-	32.02155 (0.71320)	28.4495	-	0.01369 (0.01335)	3.57205 (3.14853)	-	-	0.5992	37.36138
	Kontdamis	SEMA	10.2681433 (0.3116083)	36.0849142 (0.9833010)	-	-	-0.0006601 (0.0001039)	-	-	-	0.254	6.458489
	Briere-1	SEMA	-	-	-	-	-	-	-	-	-	-
	Briere-2	SEMA	-	-	-	-	-	-	-	-	-	-
Pandora neoaphidis (WEL1)	Observed	SEMA	7	32	20	1.8402	-	-	-	-	-	-
	Linear	SEMA	-1.789565217	-	-	-	-	-	-	0.86	-	-
	Taylor	SEMA	-5.9844 (0.8070)	-	20.4467 (0.8141)	1.1536 (0.8111)	-	-	-	-	2.1	20.40944
	Lactin-1	SEMA	-	31.80081 (0.53165)	27.93391	-	0.01026 (0.01053)	3.86690 (2.52093)	-	-	0.4094	23.64594

	Kontdamis	SEMA	0.9826 (2.217)	31.64 (0.431)	-	-	0.0003244 (0.0000898)	-	-	-	0.3063	13.20297
	Briere-1	SEMA	-	-	-	-	-	-	-	-	-	-
	Briere-2	SEMA	-	-	-	-	-	-	-	-	-	-