Project title:	The role of entomopathogenic fungi in regulating aphid populations in field Brassicas
Project number:	CP092
Project leader:	Dr. Dave Chandler, Warwick Crop Centre, University of Warwick, CV35 9EF
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Location of project:	Warwick Crop Centre, University of Warwick, CV35 9EF
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Date project commenced:	01/02/2013
Date project completed (or expected completion date):	30/04/2016

DISCLAIMER

[The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.]

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

Populations of cabbage aphid, feeding on brassica plants in a field experiment, exhibited a sharp increase in numbers followed by a precipitous decline. This population decline, or "crash", occurred at the same time (in October) on plants of different physiological age and was associated with an increase in the population of natural enemies feeding on the aphids. The "crash" has been observed in experiments in the previous year of this project, but it occurred in mid-summer. Laboratory experiments showed that the rate of population increase of aphids on brassica plants is affected by the age of the plant and its soluble nitrogen content; however this does not translate into observable effects on the timing of the population crash in the field. The entomopathogenic fungus *Pandora neoaphidis* is one of the key natural enemies affecting brassica aphid populations in field experiments, and detailed laboratory experiments have characterised how environmental temperature influences the speed of kill of the fungus against aphids.

Background

This HDC PhD studentship is investigating the precipitous decline in populations of brassica aphids that occurs most years, which is referred to hereafter as the "aphid crash". The project focuses on the role of naturally occurring entomopathogenic fungi in the aphid crash, but includes studies of a range of factors that may impact on the timing and size of the crash including the presence of other natural enemy species and the role of plant age.

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). 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). 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). At present, aphid management in brassica crops is heavily reliant on the use of synthetic chemical insecticides and aphicides account for 39% of all insecticide applications (Garthwaite *et al.* 2007). Current chemical control methods of aphids include neonicotinoids, pyrethroids, pirimicarb, chlorpyrifos and pymetrozine (IRAG, 2012). However,

growers are under pressure to reduce their reliance on insecticides for a number of reasons: (a) consumer concerns (and by extension retailer concerns) over pesticide residues in food; (b) effective insecticides 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 (c) excessive use of insecticides resulting in control failure through the evolution of heritable resistance (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). Reliance on a small number of insecticdes for *B. brassicae* control increases the chances of this pest evolving insecticide resistance. Because of the need to prevent over-reliance on synthetic chemical pesticides, there is a requirement to develop alternative forms of aphid management that can be used as part of Integrated Pest Management

Aphid population dynamics

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). However, the exponential growth seen during spring and early summer does not continue. During the growing season (usually July) 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 and natural landscapes 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, the action of natural enemies and adverse weather conditions. These factors could 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.* 2004). Of the natural enemies, entomopathogenic fungi have been strongly implicated in the crash of aphid populations but little is known of their biology (Karley *et al.* 2003; Karley *et al.* 2004). 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. Particularly effective natural enemy species may also be worth considering as augmentation biocontrol agents.

Thermal biology of entomopathogenic fungi

The activity of entomopathogenic fungi is dependent on temperature (Blanford & Thomas, 1999). However, there have been relatively few detailed studies of the effect of temperature on fungal infectivity to aphid hosts. Most of the research on the thermal biology of entomopathogenic fungi has looked at the effect of temperature on processes such as fungal growth and germination in the absence of the insect host. 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).

As a result the aims and objectives of this project are:

Aim:

This project is investigating a hypothesis that fungal epizootics are one of the principle factors causing the mid-season crash in populations of aphids on horticultural brassicas. There are three main objectives as follows:

Objectives:

i. Monitor populations of healthy and fungus-infected cabbage aphids on sequentially planted brassicas and study the 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 and moisture on the pathogenicity of fungi to the cabbage aphid to forecast the outbreak of fungal epizootics.

Summary

Below, summaries and main findings are briefly discussed.

Spatial heterogeneity in population fluctuations of Brevicoryne brassicae and their natural enemies on sequentially planted Brussels sprout plants in 2014

A field experiment was done over 2014 to monitor populations of cabbage aphid and its natural enemies on plots of brassica plants at different stages of growth (= physiological age). It was found that the aphid population crashed in mid October (figure 1). This was later than the crash observed in a similar experiment in 2013, which occurred at the end of July. However, in both years , plant age was shown to have no effect on the timing of the crash, that is all transplants responded in the same way at the same time (figure 1). Moreover, both years saw the establishment of a fungal epizootic and an increase in the population of other natural enemy species coinciding with the time of the crash

Quantification of the effect of density of apterous adult aphids on production of alate forms on brassica plants

One explanation put forward for the aphid crash is that an increase in the population of apterous (wingless) aphids on a particular brassica plant results in a sudden switch to the production of alate (winged) forms for emigration, resulting in a sharp population decline on the same plant. An experiment was done to monitor the production of alate forms in relation to the density of apterous forms. From this data it will be possible to calculate the density at which alate production begins. It should then be possible to manipulate aphid density and investigate its effect on natural enemy activity, in particular the epidemiology of *P.neoaphidis*. With this information it will be possible to build a simple epidemiological model to predict levels of infection/control in the field.

Preliminary investigation suggests the threshold for alate production to be approximately 100-150 individuals per plant. Based on observations in field experiments, a population of this size would not significantly damage brassica plants, and it is unlikely that the cue for alate production is related to a decrease in host plant quality caused by a large aphid population. **Effect of *B.oleracea* plant growth stage and leaf age on the reproduction of adult aphids (Experiment 5.2.1)**

That soluble nitrogen affects fecundity in aphids is well documented (van Emden & Bashford, 1969). As a result it is not surprising that there is no significant difference in nymph production between plants of 'medium' and 'old' physiological ages as there was no significant different in soluble nitrogen. Nymph production does declines significantly between the youngest and two older plant ages, but it is highly unlikely by itself to cause the sudden decline in aphid populations you observe in the field. For this to happen there would have to be no births. Field data also indicates an increase in mortality because the crash occurs over the course of a

week. Plant age could be a contributory factor to the aphid population crash but is likely masked by other factors in the field.

Field experiments set up at Wellesbourne during the 2014 growing season in order to monitor aphid populations on brassicas and study the link between the mid-season crash and epizootics of insect pathogenic fungi (objective 1) saw the establishment of a field epizootic which acted to reduced aphid infestations as in 2013. Attempts were made to isolate the fungi and were successful. Morphological data, as in 2013, suggests that the epizootic was caused by *Pandora neoaphidis* (Commonwealth Mycological Institute, 1979). DNA identification as confirmed that the pathogen isolated from *Brevicoryne brassicae* at Wellesbourne is *Pandora neoaphidis*.

The effect of temperature on the growth, germination and pathogenicity of entomopathogenic fungi

Experiments were conducted to elucidate the effect temperature has on the growth, germination and pathogenicity of 7 species on entomopathogenic fungus. The details of which can be found in table 2.3.1 and 2.3.3. The effect of various durations of high relative humidity on the ability of *P.neoaphidis* to kill *B.brassicae* was also investigated. Additionally, the effect of temperature on *Brevicoryne brassicae* was explored to gain a deeper understanding of host-pathogen interactions. Experiments conducted were:

- The effect of temperature on the growth of 6 selected Ascomycetes from four different genera and *Pandora neoaphidis*
- The effect of temperature the germination of 6 selected Ascomyctetes from four different genera and *Pandora neoaphidis*
- Temperature effects on Brevicoryne brassicae development (time to adulthood)
- Temperature effects on the performance of Brevicoryne brassicae (fecundity & mortality)
- Influence of various constant temperatures on the virulence of three biopesticides to Brevicoryne brassicae
- Influence of various constant temperatures on the virulence of Pandora neoaphidis to Brevicoryne brassicae
- Effect of relative humidity on the virulence of *Pandora neoaphidis* to *Brevicoryne brassicae*

The effect of temperature on the rate of colony extension of entomopathogenic fungal species from the phylum Ascomycota and Pandora neoaphidis = Entomophthoromycota.
. Analysis suggests the optimal temperature for isolate *P. neoaphidis* NW420 is approximately 22°C which is lower than the optima calculated for the Ascomycetes *B. bassiana* ATCC &

GHA, *M. brunneum, I. fumosoros* & *L. longisporum* (25°C) but higher than that of *L.muscarium* (Harvey, 2013).

Laboratory evaluation of the effect of temperature on the germination of entomopathogenic fungal species from the phylum Ascomycota and Pandora neoaphidis = Entomophthoromycota.

Germination times varied greatly depending on temperature with the slowest germination times for all isolates at 15°C and increasing with increasing temperature.

Temperature dependence of the pathogenicity of entomopathogenic fungal species from the phylum Ascomycota and P.neoaphidis = Entomophthoromycota.

A series of bioassays to measure the effect temperature has on the speed of kill of entomopathogenic fungi against *Brevicoryne brassica* were carried out at a range of temperatures from 12 to 28°C. Various spore showering times from 5-75 minutes were used for *Pandora neoaphidis* isolates, whereas LD95's were chosen for the Hypocrealean fungal species used in bioassays. Death could be attributed to *P.neoaphidis* at all temperature because spores were produced on cadavers i.e. individuals were mycosed. Dead but unmycosed individuals were only observed at 24°C and 28°C inferring that heat stress could be another source of mortality.

Temperature clearly affects the ability of the fungus to kill individuals, not surprising because ectothermic organisms need suitable environmental conditions to germinate and grow. These findings have important implications not only for pest management strategies involving the use of biopesticides i.e. spray windows, but also any conservation biocontrol strategy where the activity of enzootic fungal pathogens will be limited by the temperature of the environment.

Financial Benefits

It is difficult to comment on the financial benefits given that this work is in its infancy. However any new method that would allow growers to reduce their reliance on synthetic chemical would clearly be financially beneficial.

Action Points

The research eludes to the role entomopathogenic fungi and other natural enemies play in the crash of aphid populations. Using the knowledge of temperature- insect-pathogen interactions accumulated in this project work can begin on a predictive model to determine to timing of the aphid population crash.

SCIENCE SECTION

1.1 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).

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). 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). 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). At present, aphid management in brassica crops is heavily reliant on the use of synthetic chemical insecticides and aphicides account for 39% of all insecticide applications (Garthwaite *et al.* 2007). Current chemical control methods of aphids include neonicotinoids, pyrethroids, pirimicarb, chlorpyrifos and pymetrozine (IRAG, 2012). However, growers are under pressure to reduce their reliance on insecticides for a number of reasons: (a) consumer concerns (and by extension retailer concerns) over pesticide residues in food; (b) effective insecticides 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 (c) excessive use of insecticides resulting in control failure through the evolution of heritable resistance (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 forms of aphid management.

Table 1. Pest status of B.brassicae & M.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 1.8-2.3 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 1.2-2.3 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. Amoung 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 Aphid population dynamics

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). However, the exponential growth seen during spring and early summer does not continue. During the growing season (usually July) 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 and

natural landscapes 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, the action of natural enemies and adverse weather conditions. These factors could all 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.* 2004). Of the natural enemies, entomopathogenic fungi have been strongly implicated in the crash of aphid populations but little is known of their biology (Karley *et al.* 2003; Karley *et al.* 2004). 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. Particularly effective natural enemy species may also be worth considering as augmentation biocontrol agents.

1.4 Current control methods

This section repeats text from 1.2 and 1.3 so needs to be corrected. At present aphid management in brassica crops is heavily reliant on the use of synthetic chemical insecticides with aphicides account for 39% of all insecticide applications (Garthwaite *et al.*, 2007). Current chemical control methods of aphids include neonicotinoids, pyrethroids, pirimicarb, chlorpyrifos and pymetrozine (IRAG, 2012). However, growers are under pressure to reduce their reliance on insecticides: (a) consumer concerns (and by extension retailer concerns) over pesticide residues in food; (b) effective insecticides 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 (c) excessive? use of insecticides resulting in control failure through the evolution of heritable resistance (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 forms of aphid management making it more sustainable by reducing reliance on synthetic chemical pesticides.

1.5 Alternative control methods

1.5.1 Integrated pest management (IPM)

Integrated pest management (IPM) is accepted as the most expedient way to make crop protection more sustainable. 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 aims to minimise "possible disruption to agroecosystems and encourage natural pest control mechanisms" (FAO, 2013).

With this in mind, much emphasis is now being placed on other components of IPM such as biological control.

1.5.2 Aphid biological control

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). This guild of natural enemy species has the potential to be exploited for use in three broad biological control strategies: classical, augmentation and conservation biological control (CBC). A good understanding of the 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. 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)

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 can be used simultaneously as part of a CBC strategy, although, an increase in the richness of natural enemies used for pest control does not necessarily lead to a corresponding increase in CBC 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, in order to conserve a diversity of natural enemies to give additive or synergistic effects, a good understanding of their ecology is required. 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 augment their contribution to biocontrol.

1.6 Entomopathogenic fungi

The two largest fungal phyla exhibiting entomopathogenicity are the Ascomycota and the Entomophthoromycota (Humber, 2008; 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 utlisation 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.

Little is known about the role entomopathogenic fungi play in natural population regulation of aphids, although data collected in HDC sponsored research at the Warwick Crop Centre indicates that they are associated with the mid season aphid crash on brassica and lettuce crops. 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, entomopathogenic fungi exhibit certain ecomorphological adaptations 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).

1.6.1 Thermal biology of entomopathogenic fungi

The activity of entomopathogenic fungi is dependent on temperature (Blanford & Thomas, 1999). However, there have been relatively few detailed studies of the effect of temperature on fungal infectivity to aphid hosts. Most of the research on the thermal biology of entomopathogenic fungi has looked at the effect of temperature on processes such as fungal growth and germination in the absence of the insect host. 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).

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:

1.6.2 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. 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; control with *Entomopthora maimaiga* is now widespread in the United States through a combination of releasing infected cadavers, collecting resting spores from the soil and through wind dispersal. Work is currently underway investigating mass production methodologies to reduce the labour requirements associated with using *E. maimaiga* in the above strategies (Hajek *et al.* 2002; Shah & Pell, 2003).

1.6.3 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 as with the EPF *Hirsutella thompsonii* (McCoy, 1981) or *Verticillium lecanii* (Hall, 1981), often inoculative releases are repeated during a season as it is not expected that the epizootic will persist, or in an inundation capacity, in a similar approach to how agrochemicals are applied (Shah & Pell, 2003). Indeed, the term 'mycoinsecticide' has been coined (Shah & Pell, 2003). At present there are a few commercial augmentation products available for the control of aphids including *Lecanicillium longisporum* and *Lecanicillium muscarium* (marketed as 'Vertalec' and 'Mycotal' respectively) (Table 2.3.1). Vertalec no longer available in UK. Also there are products based on Beauveria bassiana (2 products) and Metarhizium brunneum (2 products 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).

1.6.4 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 with *Neozygites fresenii* and *Aphis gossypii* on cotton in the United States (Shah & Pell, 2003; Steinkraus *et al,* 1995). This example has proved to be a particular success and now covers a large portion of the USA and has it own dedicated website: (http://www.uark.edu/misc/aphid/) to keep growers up to date (Shah & Pell, 2003). 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.

1.6.5 Pandora neoaphidis

Pandora neoaphidis is the commonest 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 hosts 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. Its lifecycle is summarised in figure 1a, *P.neoaphidis* exists as protoplasts rather than zygospores (Shah & Pell 2003). 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.

Figure 1a. Generalised lifecycle of Entomophthoralean fungi
(Danish Ministry of the Environment, 2013.

As result the aims and objectives of this project are:

Aim:

This project is investigating a hypothesis that fungal epizootics are one of the principle factors causing the mid-season crash in populations of aphids on horticultural brassicas. There are three main objectives as follows:

Objectives:

i. Monitor populations of healthy and fungus-infected cabbage aphids on sequentially planted brassicas and study the 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 and moisture on the pathogenicity of fungi to the cabbage aphid to forecast the outbreak of fungal epizootics.

2 general materials & methods used in the study

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±2°C, 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 *Brevicoryne brassicae* & *Myzus 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
Beauveria bassiana (ATCC strain)	432.99[a]	*Anthonomus grandis*	USA
Beauveria bassiana (GHA strain)	433.99[b]	*Bemisia* spp.	USA
Lecanicillium muscarium	19.79[c]	*Trialeurodes vaporariorum*	UK
Metarhizium brunneum	275.86[d]	*Cydia pomonella*	Germany
Isaria fumosorosea	409.96[e]	*Phenacoccus solani*	USA
Lecanicillium 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 autoclavable 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 labelled 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 *Pandora 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.

3: The aphid population crash in field brassica crops

3.2 Materials & Methods

3.2.1 Population fluctuations of *Brevicoryne brassicae* and their natural enemies on sequentially planted Brussels sprout plants in 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 Aphid population dynamics and that of their natural enemies on two different ages of *Brassica oleracea* cv. Trafalgar transplanted to the field at the same time

Experiment 3.2.1.1 consisted of 24 plots (12 'young' plots & 12 'old' plots) of 10 experiment plants (arranged 2 plants x 5 plants, with 50cm spacing) separated by a single guard row ordered in three beds of equal length (Figure 3.1). 'Young' plants were grown for 4 weeks and 'old' plants were grown for 8 weeks prior to transplanting in Hassy trays in a glasshouse and randomly assigned a plot in each of the beds (BBCH 13-14). All plants were transplanted to the field on the 14th June 2013 after experimental treatment. Tracer © (Dow AgroSciences) was used as a module drench to protect the plants against cabbage root fly damage prior to field transplantation; no additional agrochemicals were applied.

To ensure a population of *B.brassicae* all experimental plants in experiment 3.2.1.1 were inoculated with 5 *B.brassicae* apterous adults per plant after a week in the field using clip cages (Figure 3.2). Colonies of *B.brassicae* for this initial infestation of field plots were maintained as in section 2.2.
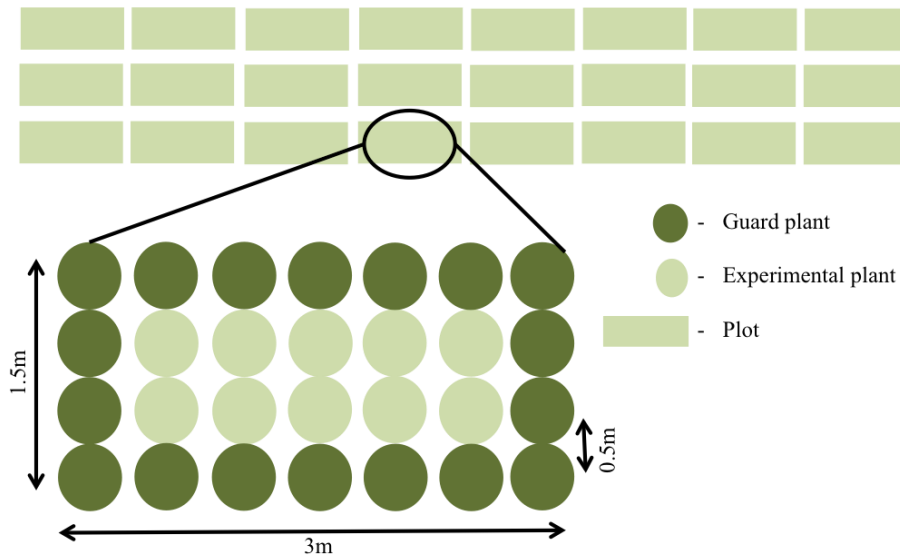
Figure 3.1 Field schematic and close up of a single plot in experiment 3.2.1.1. Eight plots randomly assigned four young and four old plots arranged in three beds of equal length. Each plot contained 10 experimental plants separated by a single guard row totaling approximately 1.5m x 3m in size.

Experimental Brussels sprout plants were inspected every 7-21 days. An entire Brussels sprout plant constituted a single sample unit. Plants were examined visually to record aphid density, fungus-infected cadavers, syrphid larvae, parasitoid mummies, coccinellidae and anthocorids. Fungi infected individuals were identified as sporulating cadavers (Figure 3.3). Due to time constraints as aphid population density grew, a minimum of three of the ten experimental plants per plot were randomly inspected.

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 and leaf material was randomly sampled from each of the transplant ages for soluble nitrogen analysis.


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 Aphid population dynamics and that of their natural enemies on sequentially planted *Brassica oleracea* plants

Experiment 3.2.1.2 was composed of 16 plots (4 planting occasions x 4 replicates) of 10 experimental plants (arranged 2 plants x 5 plants, with 50cm spacing) (Figure 3.4). Plots were separated by 'double' guard rows meaning the total size of the experiment covered an approximate area of 8m x 14m (Figure 3.5). Plots of Brussels sprout plants were transplanted sequentially: on 2nd May, 3rd June, 3rd July and 5th August after a month of growth in Hassy trays in a glasshouse (BBCH 13-14). Each of the 4 replicates for each transplant date were randomly assigned plots in a 4 x 4 plot grid. Tracer © (Dow AgroSciences) was used as a module drench to protect the plants against cabbage root fly damage prior to field transplantation; no additional agrochemicals were applied.

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 were removed after approx. four hours. If aphids were found not to have established re-inoculation took place up until 7 days post initial inoculation.

Experimental Brussels sprout plants were inspected every 7-21 days. As above, an entire Brussels sprout plant constituted a single sample unit. Plants were examined visually to record aphid density, fungus-infected cadavers, syrphid larvae, parasitoid mummies, coccinellidae and anthocorids. Fungi infected individuals were identified as sporulating cadavers. Due to

time constraints as aphid population density grew, a minimum of three out of the ten experimental plants per plot were randomly inspected.

Temperature and humidity data were collected and leaf material was randomly sampled from each of the transplant ages for soluble nitrogen analysis.
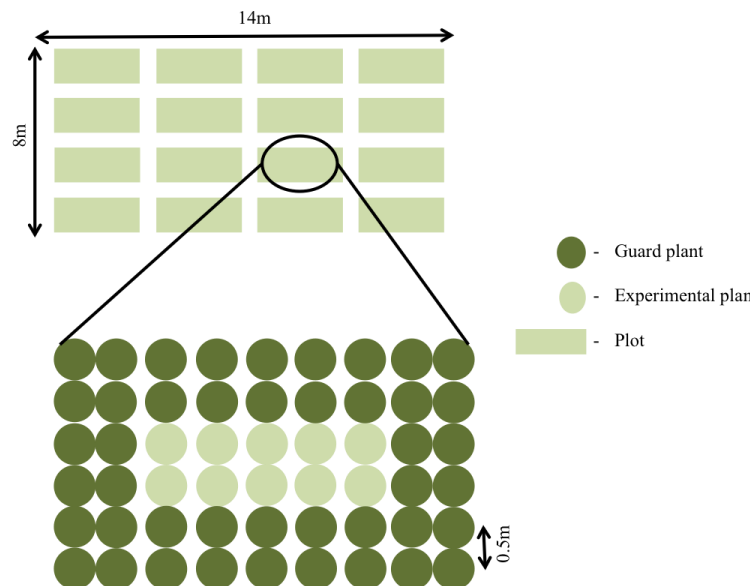
Figure 3.5 Field schematic and close up of a single plot in experiment 3.2.1.2. Sixteen plots arranged in four beds. Each bed was assigned a May, June, July and August transplant, which were randomly located within the bed. Each plot contained 10 experimental plants separated by a double guard row resulting in a field trial approximately 8m x 14m in size.

3.2.2 Spatial heterogeneity in population fluctuations of *Brevicoryne brassicae* and their natural enemies on sequentially planted Brussels sprout plants in 2014

Experiment 3.2.1.2 was repeated in 2014 with spatially distinct plots during May to November. Experiment 3.2.2 consisted of 16 plots (four planting occasions x 4 replicates) of 10 experimental plants arranged 2 plants x 5 plants, with 50cm spacing surrounded with a single guard row. There were four different locations separated by at least 80 meters, Little Cherry 'LC', Pump Ground 1 'PG1', Pump Ground 2 'PG2' & Long Meadow West 'LMW' (Figure 3.6, appendix 1). In each location there was one of each of the planting occasions 6th May, 3rd June, 3rd July & 1st August separated by at least 20 meters. Plants were grown for a month in Hassy trays in a glasshouse (BBCH 13-14). Each of the 4 replicates for each transplant date were randomly assigned positions within locations. Except for Tracer © (Dow AgroSciences) no other agrochemicals were applied. 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 and leaf material was randomly sampled from each of the transplant ages for soluble nitrogen analysis.

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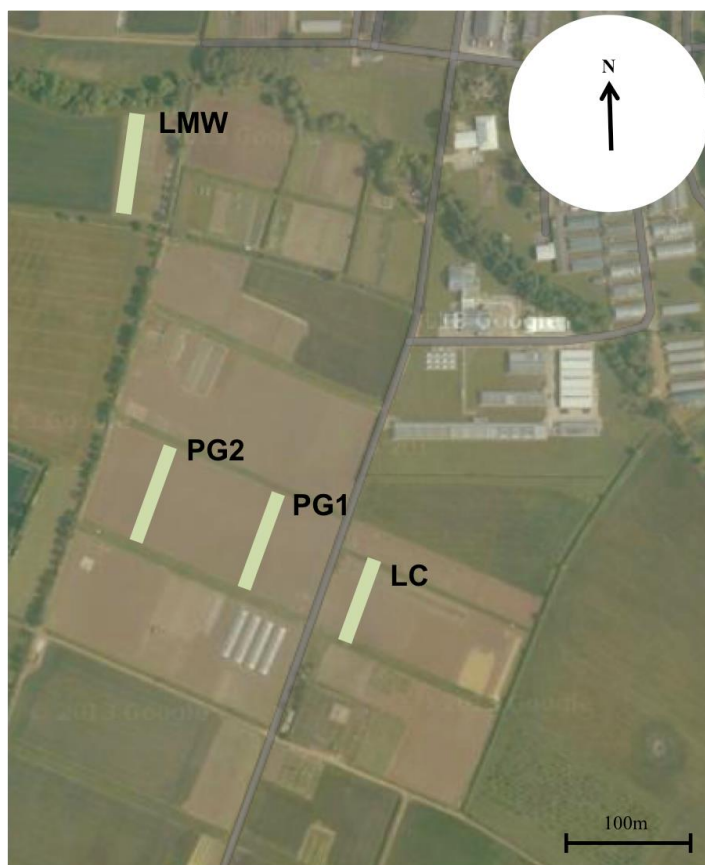
Long Meadow West 'LMW', Pump Ground 1 'PG1', Pump Ground 2 'PG2' and Little Cherry 'LC'.

3.2.3 Identification of natural enemies

In 2013 & 2014 parasitoid mummies, fungal cadavers and natural enemies at juvenile and adult stages were collected from designated monitoring plants in order to identify the guild of natural enemies.

3.2.3.1 Identification of fungal natural enemies

Genomic DNA from entomopathogenic fungi isolated from field experiments conducted at Wellesbourne, UK using surface sterilisation of moribund *Brevicoryne brassicae* individuals (Section 2.3.3.4) and grown on Grace's liquid insect cell culture medium (ThermoFisher, UK) was extracted using DNeasy Plant Mini Kit's (Qiagen Ltd, UK) following the manufacturers 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.

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 detect and confirm the identity of fungi infecting populations of *B.brassicae* in the field as morphological data, spore morphology and infected cadaver appearance, had suggested *Pandora neoaphidis* was the culprit.

Positive DNA controls were provided by the ARSEF collection of Entomopathogenic Fungal Cultures: NW420 (ARSEF 5372): *Pandora neoaphidis* isolated from *B.brassicae* in Denmark (Table 2.3.3), and negative water controls were 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} RedTaq Ready Mix PCR reaction Mix (sigma-Aldrich) 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 manufacturers protocol. The final data was subject to a sequence similarity search using NCBI BLAST performed in Genbank. A selection of *P.neoaphidis*, *Pandora nouryi* and *Pandora delphacis* sequences were used with obtained sequence data to build a phylogenetic tree.

3.2.3.2 Identification of arthropod natural enemies

Parasitoid mummies were collected without the presence of emergence holes in order to collect the adults developing within and identified with an in house publication supplied by ADAS (Sasha White, pers. comms). Syrphidae larva were collected and raised to adulthood on infested *Brevicoryne brassicae* Brussels sprout plant material in order to more easily identify individuals. Coccinellidae adults were identified in the field with FSC, Field Studies Council guide. Surveying was carried out a number of occasions throughout the field season in order to capture the entire guild.

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 HSD. Tukey HSD is also very conservative, 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 experiment 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. should change this in line with the changes you made to your thesis for this section 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. It has been used in a variety of contexts and is calculated by the natural log of the population size at time point b divided by the population at time point a divided by the amount of time elapsed between time point a and b. The value of the rate varies between -1 and 1, values below 0 and the population is said to be decreasing and vice versa for values above 0. See equation below:

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

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).

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 of field trials in 2013 & 2014

3.3.1 Aphid population dynamics and that of their natural enemies on two different ages of *Brassica oleracea* cv. Trafalgar transplanted to the field at the same time

Aphid populations crashed regardless of plant age on the 30th July± 2 days 2013 and did not recover in the following three months, remaining very low in both young and old transplants. Whilst the plants varied temporally in their 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).

Aphid population dynamics were mirrored in the natural enemy community, with peak natural enemy numbers following peak aphid density, resulting in a predator prey cycling dynamic (Figure 3.7). The most numerous natural enemy were parasitoid mummies and parasitism rates peaked at 60.823% and 32.632% 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 (14.634%) and on the 20th August 2013 (16.922%) for young and old plots respectively (Figure 3.7f.).

There was no significant difference between the size of aphid populations on young and old plants in experiment 3.2.1.1 (Kruskal-Wallis, H= 1.6621, df= 1, *P*= 0.1973) (Figure 3.7). Additionally, transplant age 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 on the old plots, indeed a general pattern emerged that all natural enemies were in greater abundance on old plots (Figure 3.7).
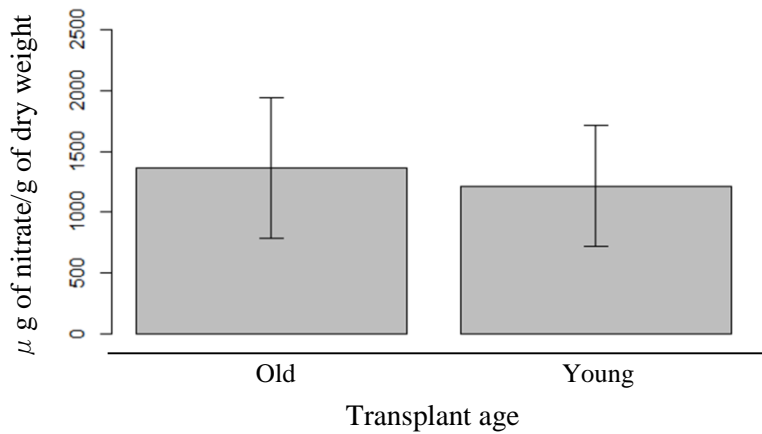
Figure 3.16. Mean soluble nitrogen concentration (±stdev) in leaf material collected from old and young transplants in experiment 3.2.1.1 at approximately the time of the aphid population crash.
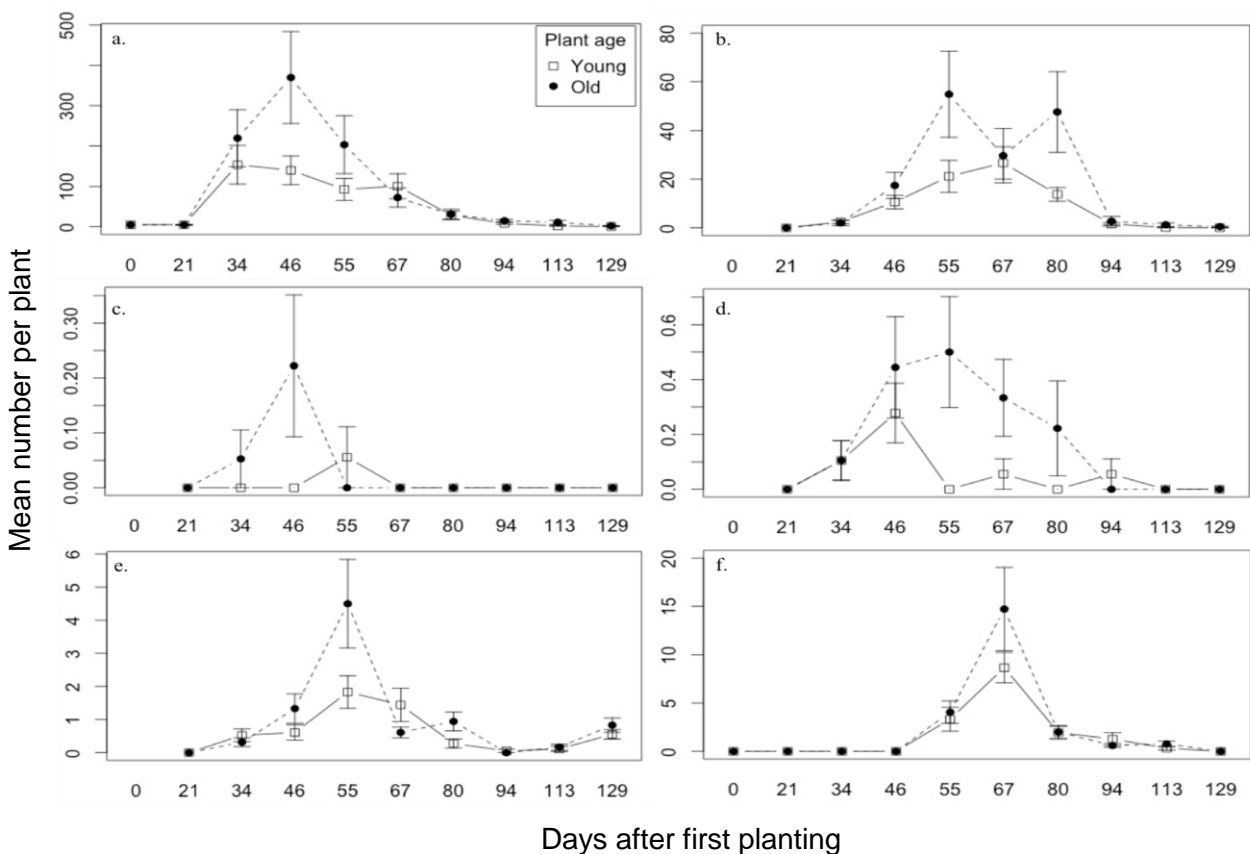

Figure 3.7 Changes in mean number of aphids and recorded natural enemies per plant (±stdev) for young and old transplants in experiment 3.2.1.1. Letters indicate different counts, starting top left: (a) *Brevicoryne brassicae*, (b) Parasitoid mummies, (c) Anthocoridae, (d) Coccinellidae, (e) Syrphidae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

3.3.2 Aphid population dynamics and that of their natural enemies on sequentially planted *Brassica oleracea* plants

There was significant variation in the size of *Brevicoryne brassicae* populations on transplants of different ages (ANOVA, F= 7.337, df= 3, $P< 0.001$), with May transplants having significantly larger populations than June (Tukey HSD, $P= 0.0105309$) and August (Tukey HSD, $P= 0.0002885$). No other significant differences existed in aphid populations between transplants (Table 3.1). Moreover, there were significant differences in the size of natural enemy communities between 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 existed between transplant date and natural enemy community (Table 3.2).

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	= 0.3303202
August- June	= 0.2281357
August- May	= 0.0002885
June- July	= 0.9999142
May- July	= 0.0573656
May- June	= 0.0165384

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	= 0.2233075
August- June	= 0.0020401
August- May	= 0.2061138
June- July	= 0.3077934
May- July	= 0.9891841
May- June	= 0.0412734
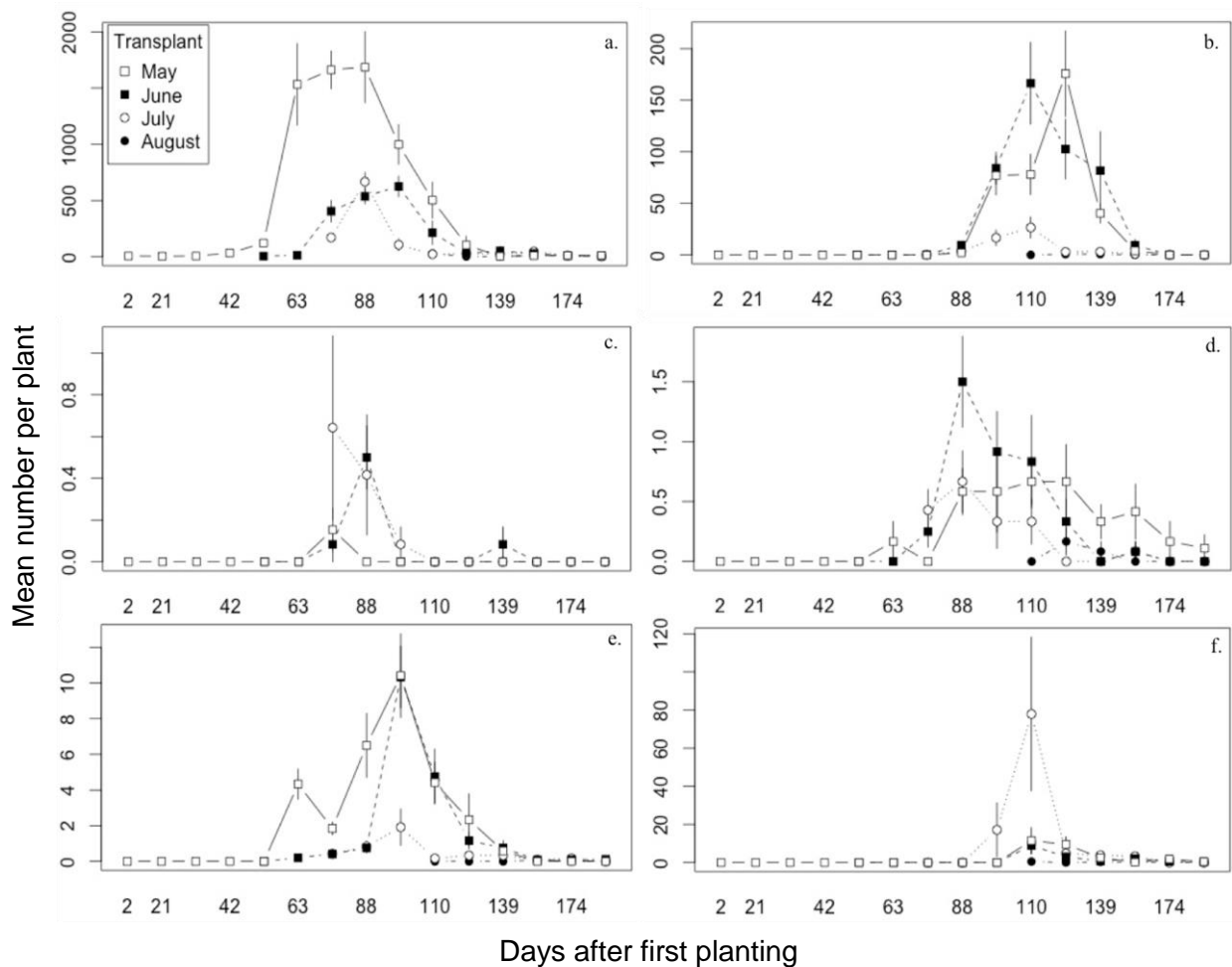
Figure 3.8 Changes in mean number of aphids and recorded natural enemies per plant (±stdev) for May, June, July and August transplants in experiment 3.2.1.2. Letters indicate different counts, starting top left: (a) *Brevicoryne brassicae*, (b) Parasitoid mummies, (c) Anthocoridae, (d) Coccinellidae, (e) Syrphidae and (f) fungal cadavers.

Despite the differences in aphid population size the changes in their relative size over time, or their dynamics, 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).

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 July having significantly more entomopathogenic fungi infected cadavers than May (Bonferroni-Dunn, *P*<0.001), June (Bonferroni-Dunn, *P*<0.001) and August (Bonferroni-Dunn, *P*<0.001).
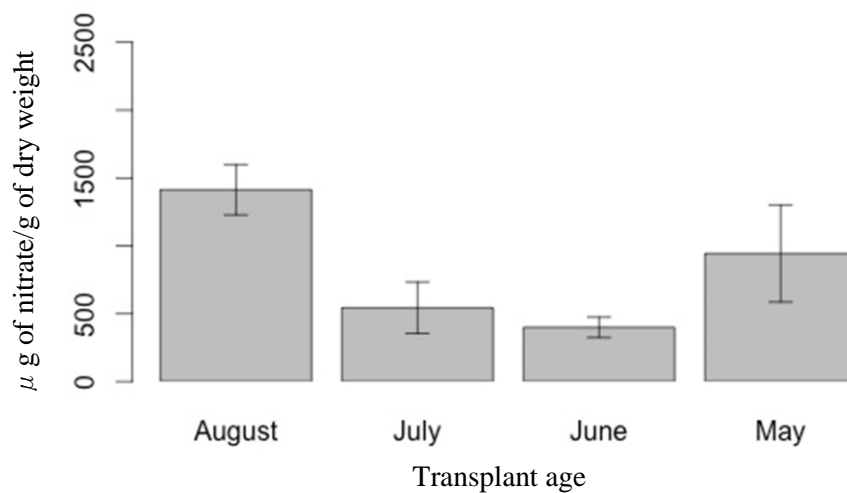
Figure 3.17 Mean soluble nitrogen concentration (±stdev) in leaf material collected from May, June, July and August transplants in experiment 3.2.1.2 at approximately the time of the aphid population crash.

Physiologically, in terms of soluble nitrogen concentration, at the time leaf material was taken (8th August 2013) May, June, July and August transplants varied significantly from each other (ANOVA, F= 4.035, df= 3, *P*= 0.045) (Figure 3.17). August had a significantly higher concentration of soluble nitrogen in petiole material than June transplants, 1413.409359± 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 did not vary. 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 again during mid to late September, but failed to reach the densities seen earlier in the season, until they declined for a second time on June and July transplants at the beginning of October. May and August *B.brassicae* populations continued to increase until the end of the experimental period (29th October).

Parasitoid mummies and fungal cadavers were the most numerous natural enemies in the field (Figure 3.8), reaching peak populations on approximately 28th August and the 13th August respectively. Peak parasitism rates of entomopathogenic fungi and parasitoid wasps were achieved on the 5th September, reaching 34.09% and 89.32% respectively.
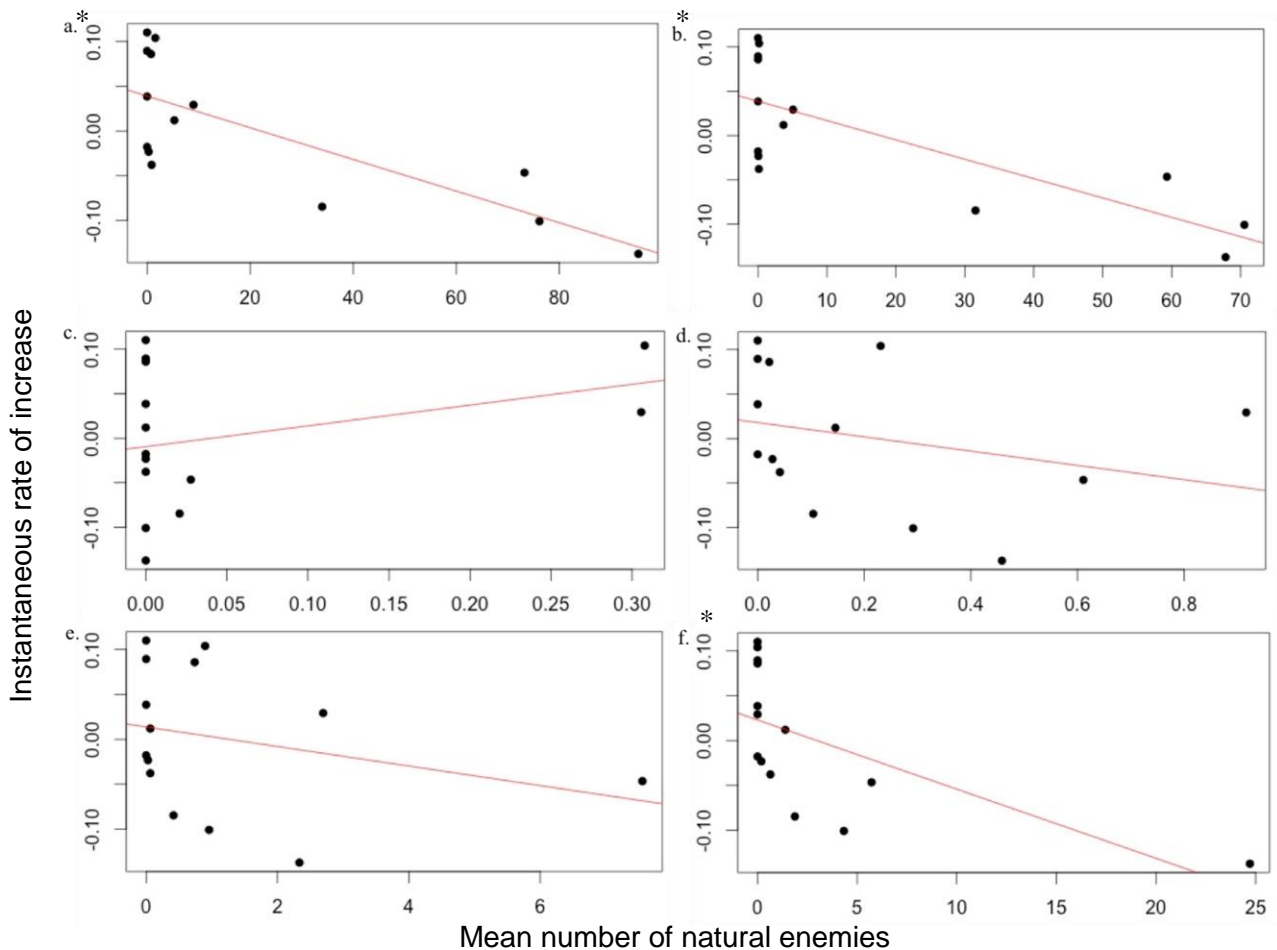
Figure 3.9 Instantaneous rate of increase against mean number of natural enemies, separated for (a) Total number of natural enemies, (b) Parasitoid mummies, (c) Anthocoridae, (d) Coccinellidae, (e) Syrphidae and (f) fungal cadavers in experiment 3.2.1.2. Red lines indicate linear models and '*' indicate significant fits.

The lowest values of instantaneous rate for *Brevicoryne brassicae* occurred at the highest densities of natural enemies and vice versa (Figure 3.9a.). Regression analysis confirmed a significant negative linear relationship between the guild of natural enemies 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 (r^2= 0.5569, *P*= 0.00131) (Figure 3.9b.) and fungal natural enemies (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
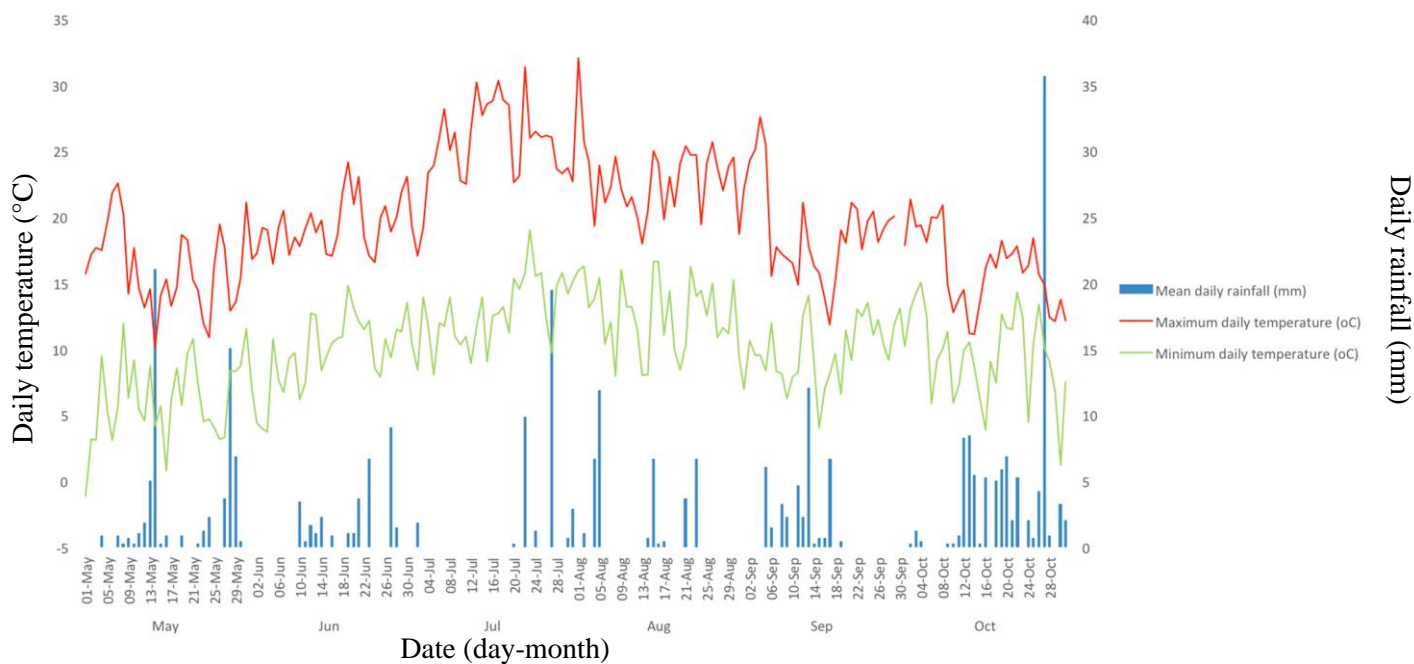
Figure 3.18 Daily maximum and minimum temperatures and daily rainfall recorded in 2013 for the duration on experiments 3.2.1.1 and 3.2.1.2.
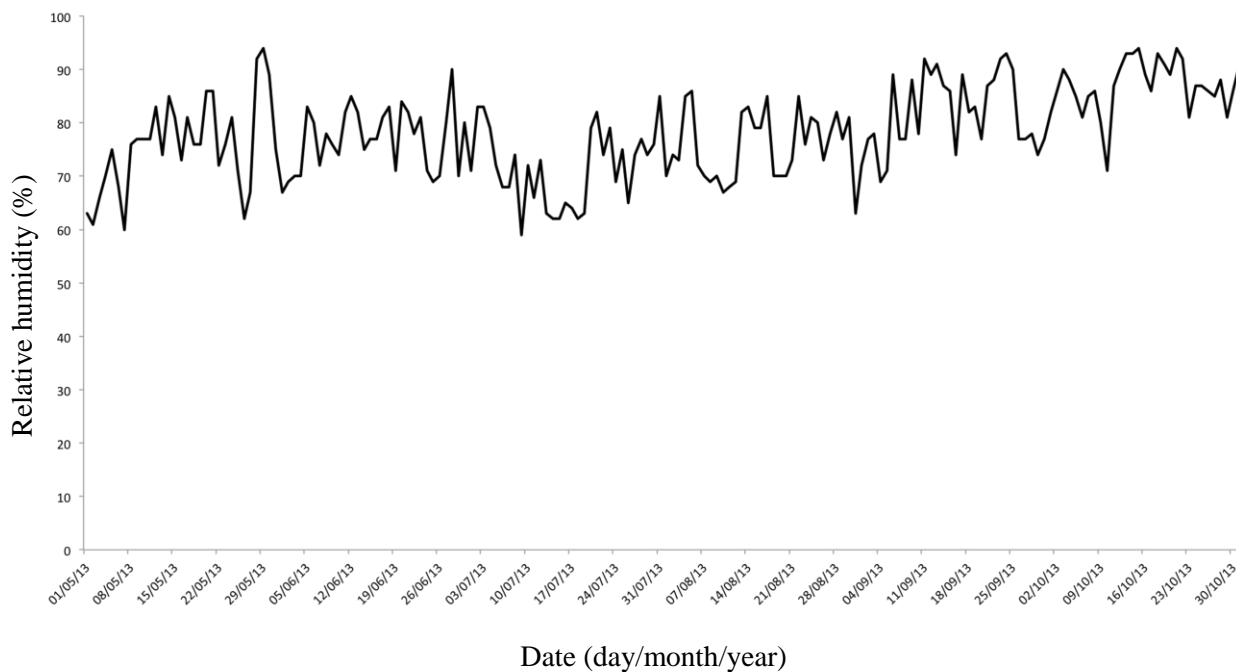
Figure 3.19 Daily relative humidity (%) in 2013 for the duration of experiments 3.2.1.1 and 3.2.1.2.

3.3.3 Spatial heterogeneity in population fluctuations of *Brevicoryne brassicae* and their natural enemies on sequentially planted Brussels sprout plants in 2014

Aphid population crashed at the same time regardless of plant age or location at the end of September, beginning of October after having reached a peak population approximately on the 26th September. This differed notably from 2014 in that it was 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 plots at the beginning of November.

Transplant date significantly affected 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 accepted May and June (TukeyHSD, P= 0.7801451) (Table 3.3) and LC varied from PG1, PG2 and LMW but no other differrences were found between any of the other locations (Table 3.4).

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

Transplant comparison	*P*
August- July	= 0.0002851
August- June	<0.0001
August- May	<0.0001
June- July	= 0.0357151
May- July	= 0.00077
May- June	= 0.7801451

Table 3.4 Post hoc TukeyHSD 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	=0.0172099
LC-PG2	<0.0001
PG1-LMW	= 0.3402969
PG2-LMW	= 0.8796005
PG1-PG2	= 0.0742289

There were significant differences in the total number of natural enemies between 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 transplants did not vary 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) (Table 3.6, appendix 1).

Peak parasitism rates for both parasitoid wasps and fungal cadavers were achieved within 11 days of each other in all locations (Figure 3.14). Fugal peak parasitism in locations LMW, PG1 and PG2 occurred on the 28th October 2014 reaching 37.03%, 85.05% and 82.66% respectively. LC reached peak fungal parasitism rate on the 17th October 2014 achieving 31.95%. Parasitoid mummies were most numerous on the 17th October 2014 for LMW and PG2 reaching 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 remained similar (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 TukeyHSD analysis showing significant differences in natural enemy community 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	= 0.3068785
May- July	= 0.1876731
May- June	= 0.9994886

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), syrphidae (Kruskal-Wallis, H= 0.97318, df= 3, *P*= 0.8077) (Figure 3.12), coccinellidae (Kruskal-Wallis, H= 1.2775, df= 3, *P*= 0.7345) (Figure 3.11) 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 syrphidae respectively. Additionally, fungal cadavers varied significantly with transplant date (Kruskal-Wallis, H= 90.27, df= 3, *P*<0.001) with all transplants being significantly different except between May and June (Bonferroni- Dunn, *P*= 0.24631). No anthocoridae were found on any plots in experiment 3.2.2 so no further analysis was carried out.
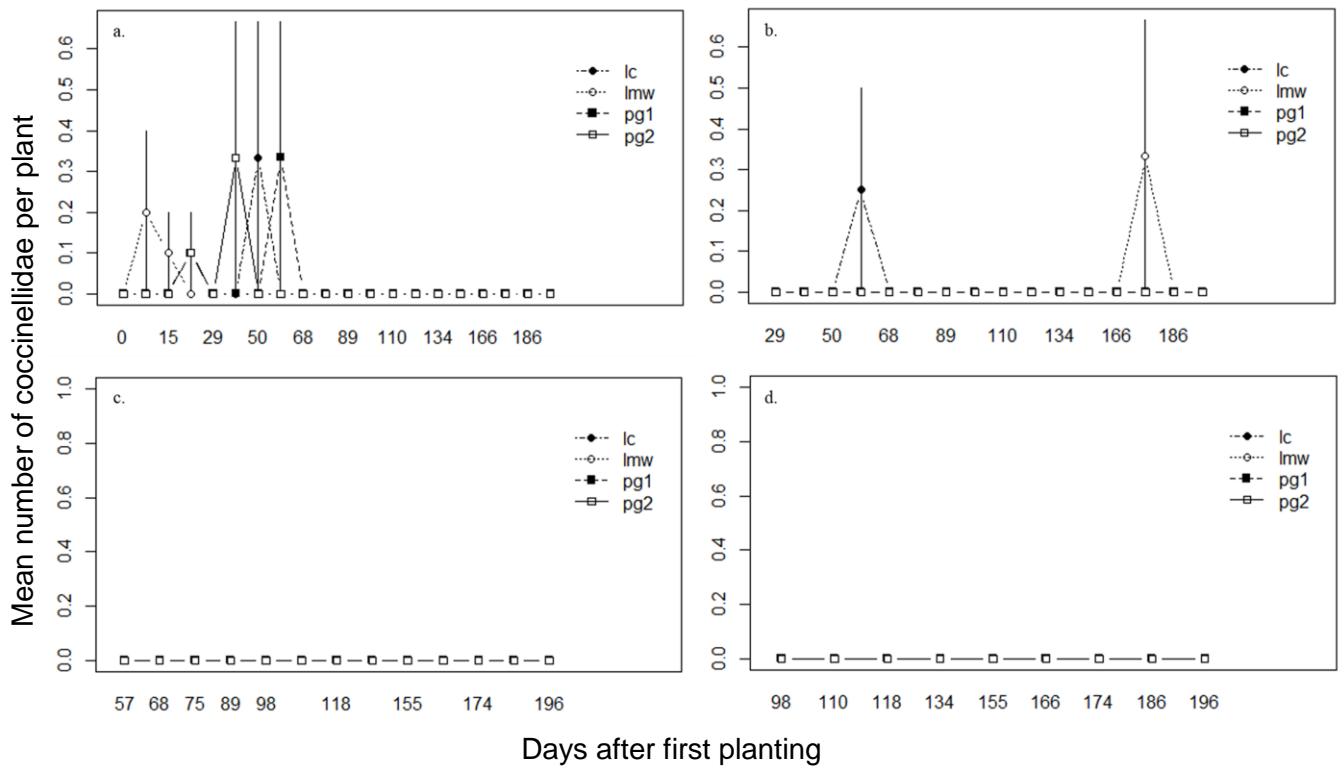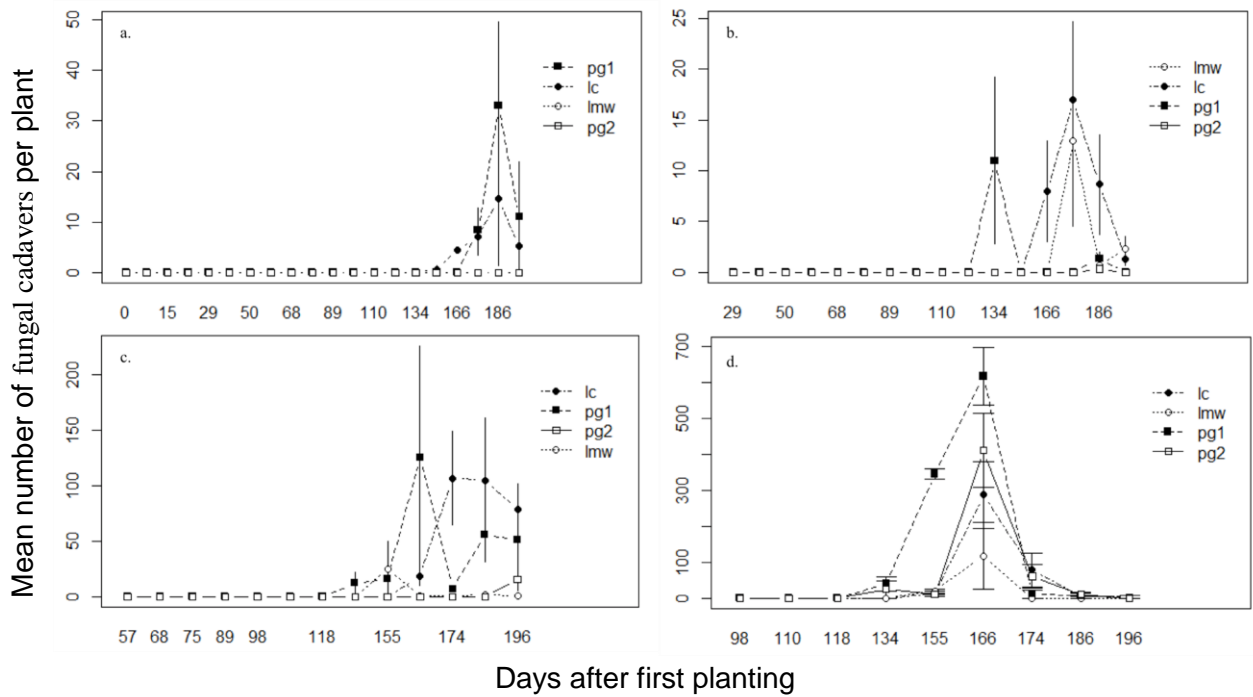
Figure 3.10 Changes in mean number of coccinellidae per plant (±stdev) in each location for May (a.), June (b.), July (c.) and August (d.) transplants in experiment 3.2.2.
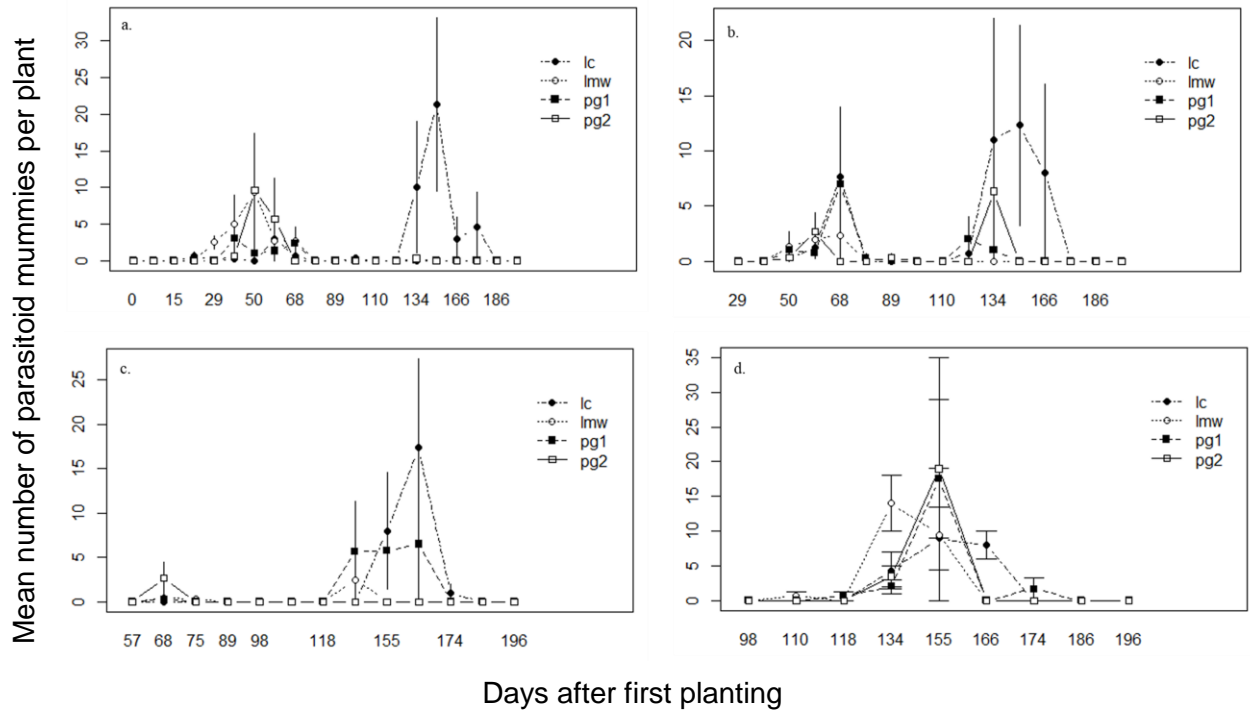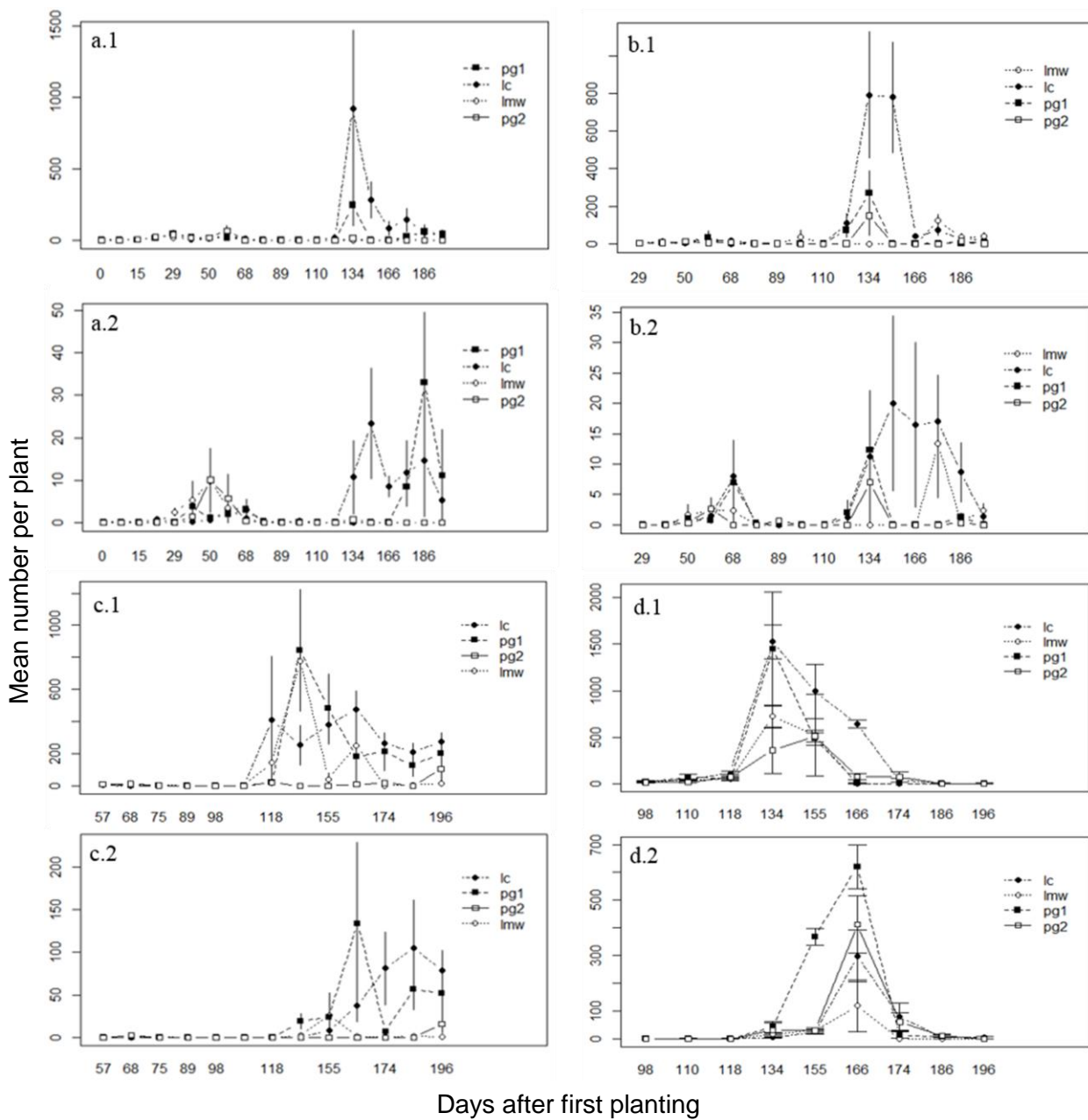
**Figure 3.11** Changes in mean number of coccinellidae per plant (±stdev) in each location for May (a.), June (b.), July (c.) and August (d.) transplants in experiment 3.2.2.
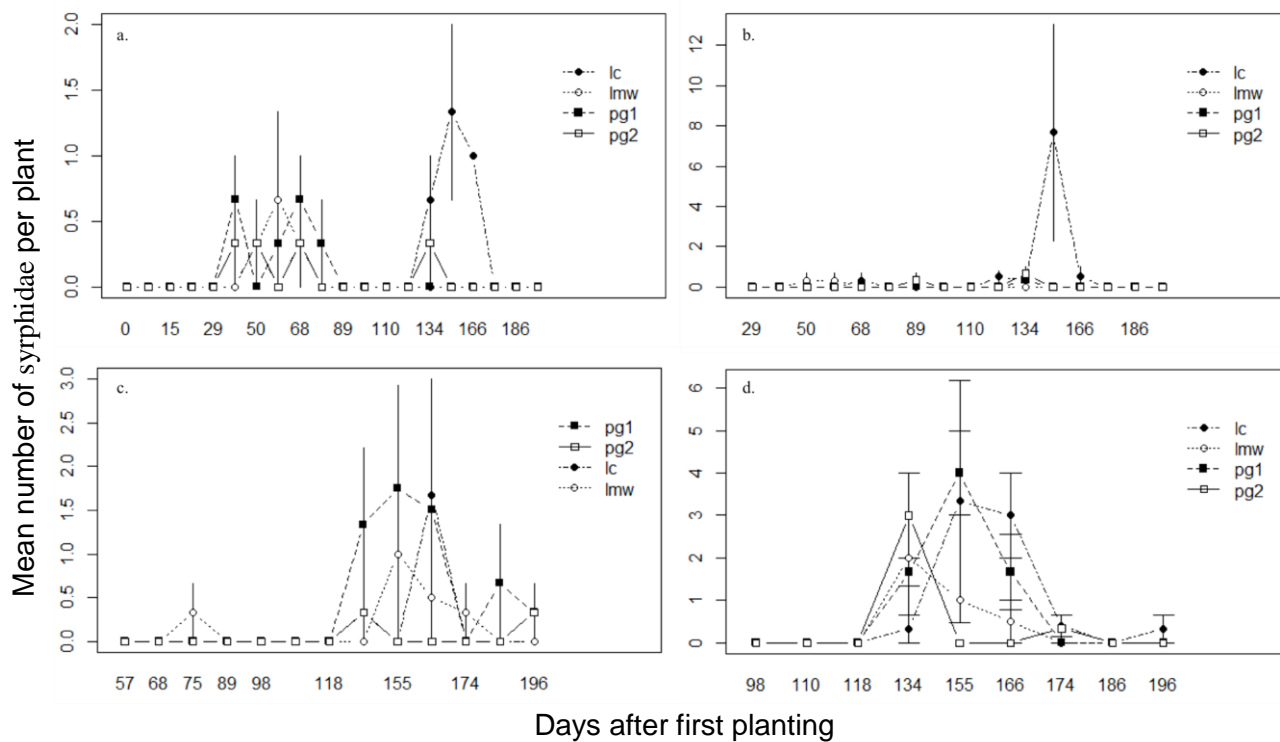
Figure 3.12 Changes in mean number of syrphidae per plant (±stdev) in each location for May (a.), June (b.), July (c.) and August (d.) transplants in experiment 3.2.2.

Figure 3.13 Changes in 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 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.
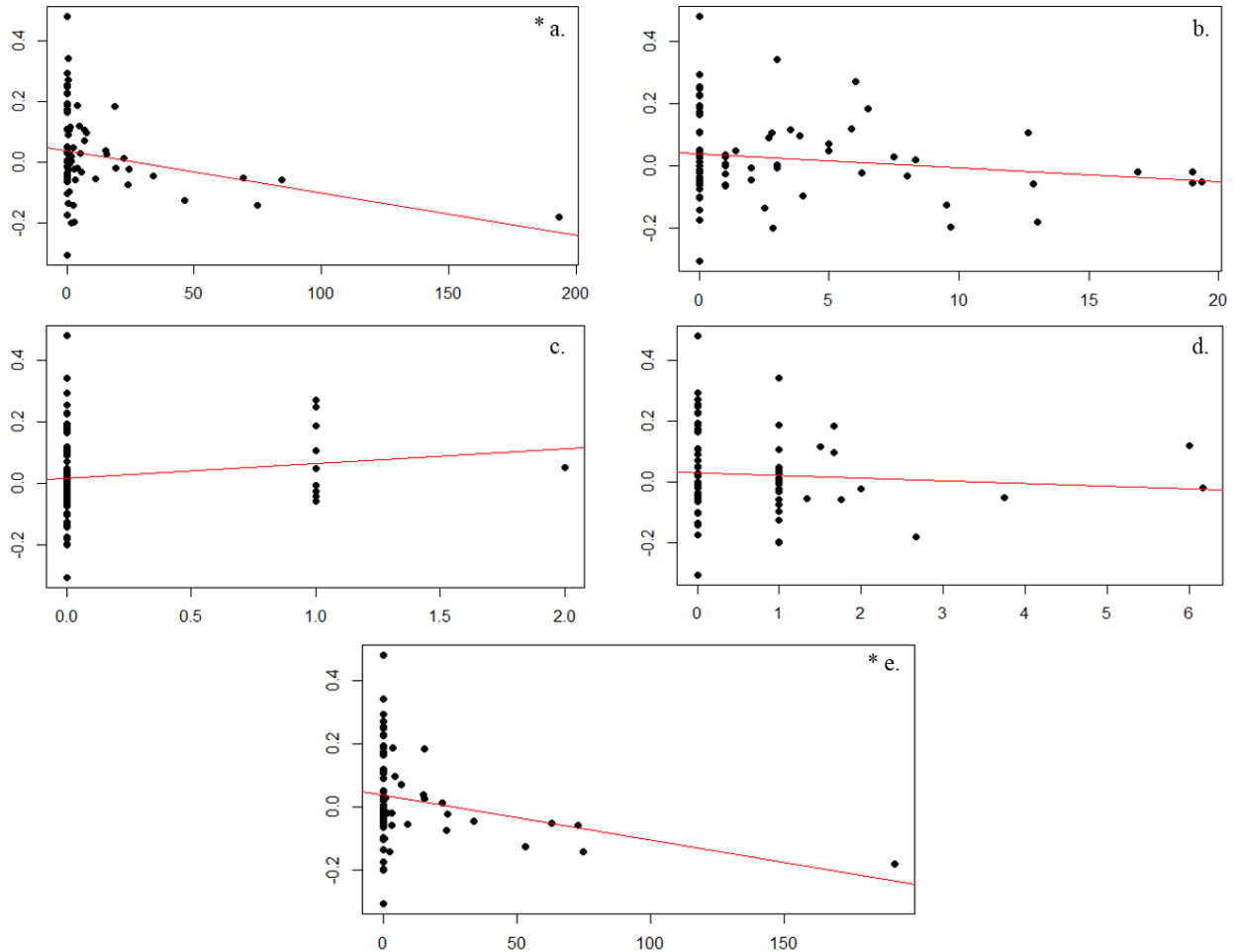
Figure 3.15 Instantaneous rate of increase against mean number of natural enemies, separated for (a) Total number of natural enemies, (b) Parasitoid mummies, (c) Coccinellidae, (d) Syrphidae and (e) fungal cadavers in experiment 3.2.2. Red lines indicate linear models and '*' indicate significant fits.

Regression analysis confirmed a significant negative linear relationship between the guild of natural enemies and the ability of the aphid population to increase, however the adjusted r^2 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 cadavers (r^2= 0.06434, P= 0.01536) (Figure 3.15e.), although the adjusted r^2 was again very low. 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).
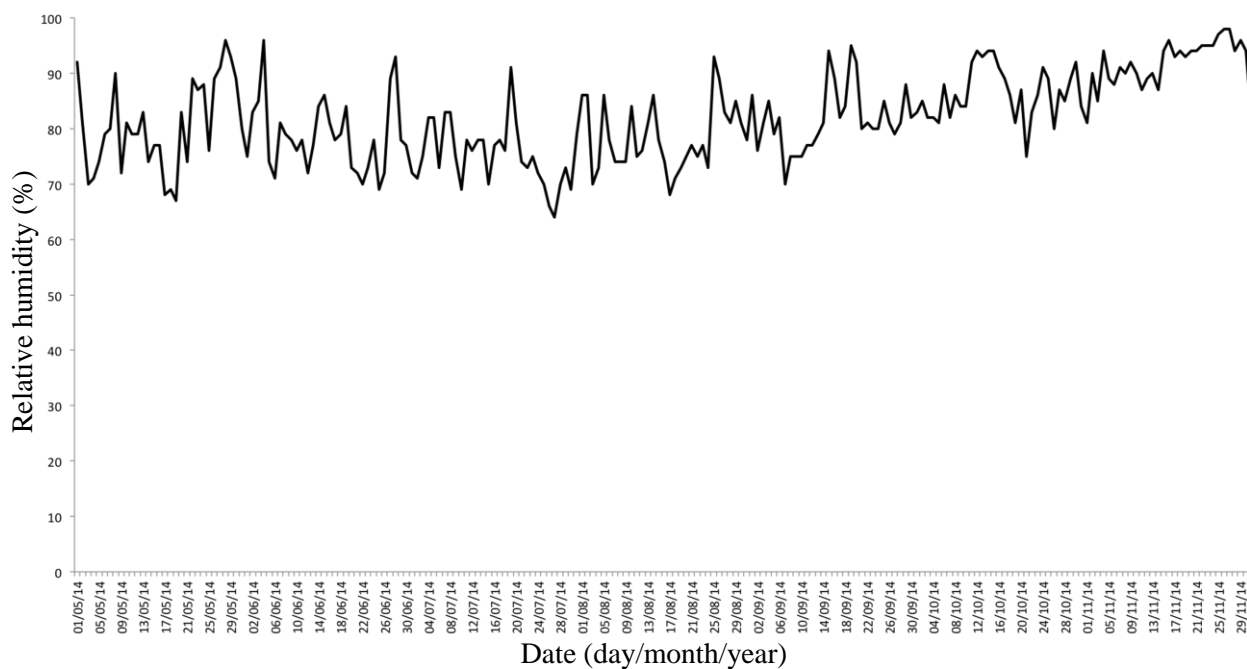
Figure 3.21 shows mean relative humidity (%) data for the duration of experiment 3.2.2. Mean relative humidity steadily increased from 75.26% in July to 92.1% in November (Table 3.8).

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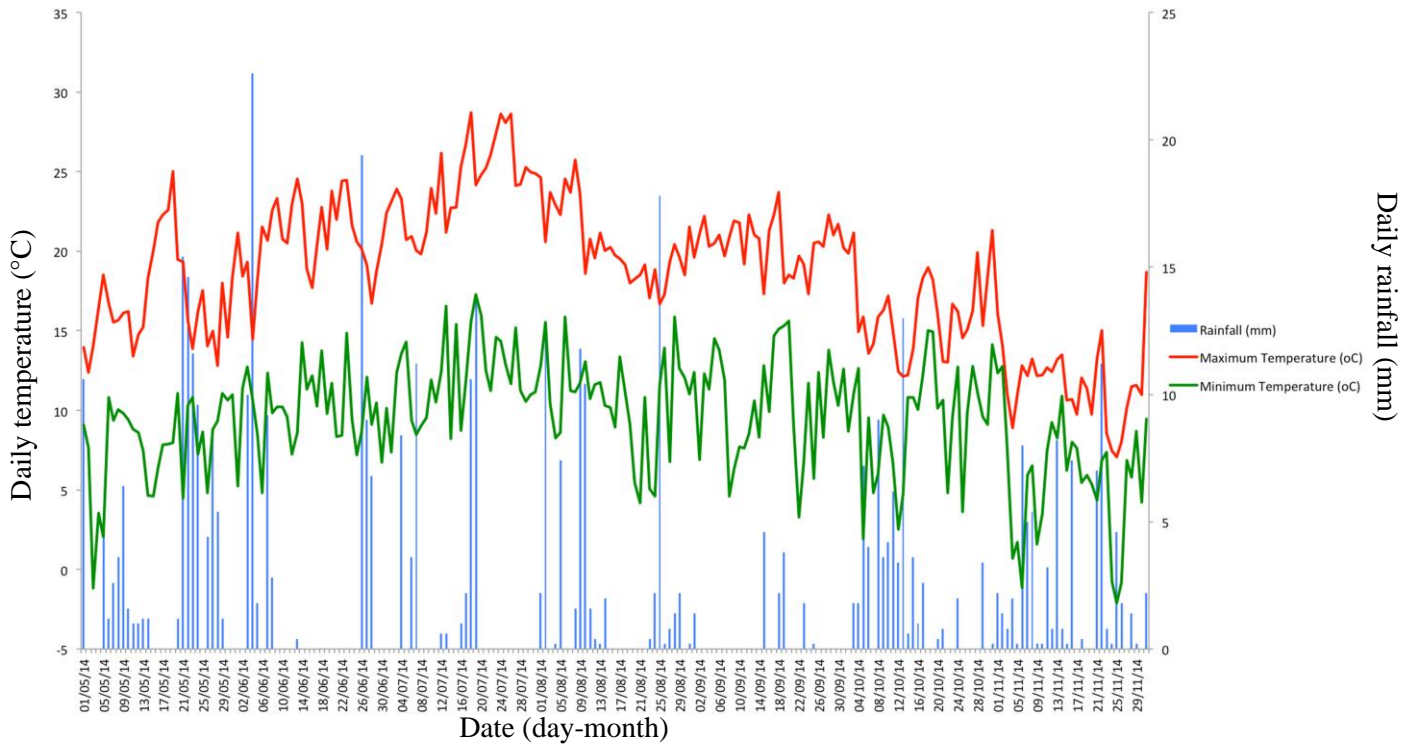
Figure 3.20 Daily maximum and minimum temperatures and daily rainfall recorded in 2014 for the duration on experiment 3.2.2.

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*) worked well on freeze dried mycelium, similar to Fekih *et al.* 2013 (Figure 3.22). BLAST similarity searches of nucleotide sequences in Genbank showed that the WEL1 isolate was virtually identical to many *P.neoaphidis* isolates with homologies of 98-100 percent. The lowest sequence similarity was seen between *Pandora delphacis* and *Pandora neoaphidis* isolate NW343 at 36.6% with divergence of 130.7. The most similar *P.neoaphidis* isolates to WEL1 were HQ677587.1, ARSEF7937, ARSEF1609, ARSEF835, NW415, NW195 and NW356 at 99.6%. None of which were shared with *P.neoaphidis* isolate NW420, whose closest sequence matches were ARSEF7939, ARSEF5374, NW316 and NW327 at 99.4% (Figure 3.23 bottom).
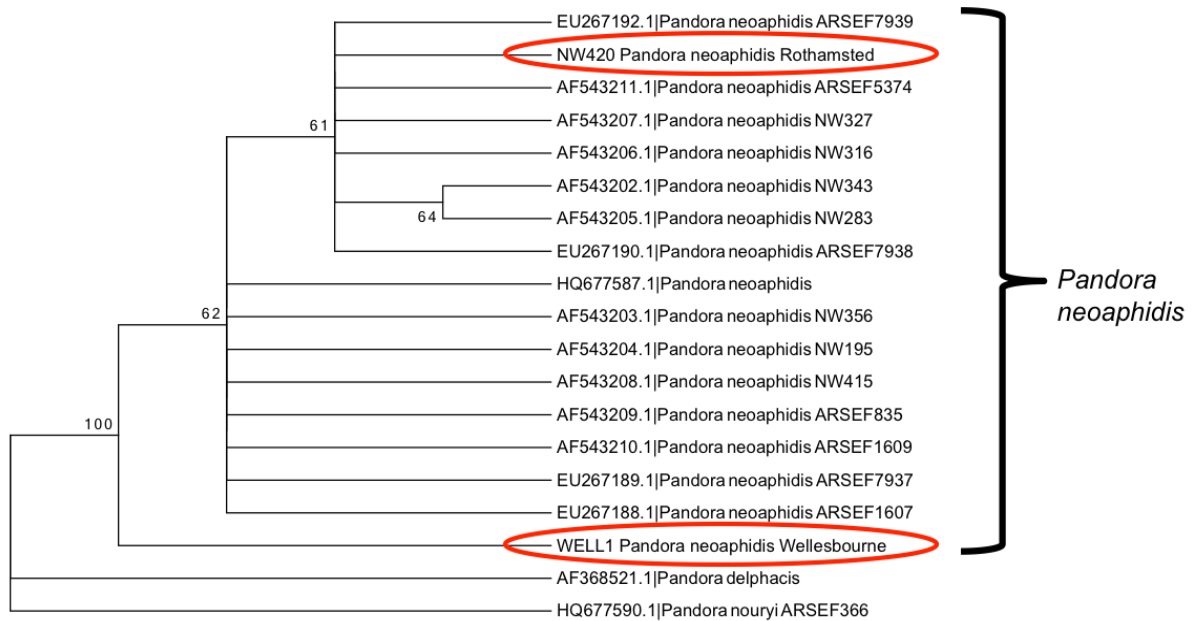
A consensus tree based on the ITS1 sequence data was constructed with a bootstrap value of 100% supporting the separation of *P.neoaphidis* from out-groups: *P.delphacis* and *P.nouryi*. *P.neoaphidis, P.delphacis and Pandora nouryi* are clearly separated into three distinct groups. Tree topology within the *P.neoaphidis* group was supported by bootstrap values of >60% with WEL1 and NW420 within the *P.neoaphidis* clade, all be it separated slightly (Figure 2.23 top). The grouping of WEL1 within the *P.neopahidis* clade is supporting by sequence similarity data.

(c.)   (a.)   (a.)   (b.)
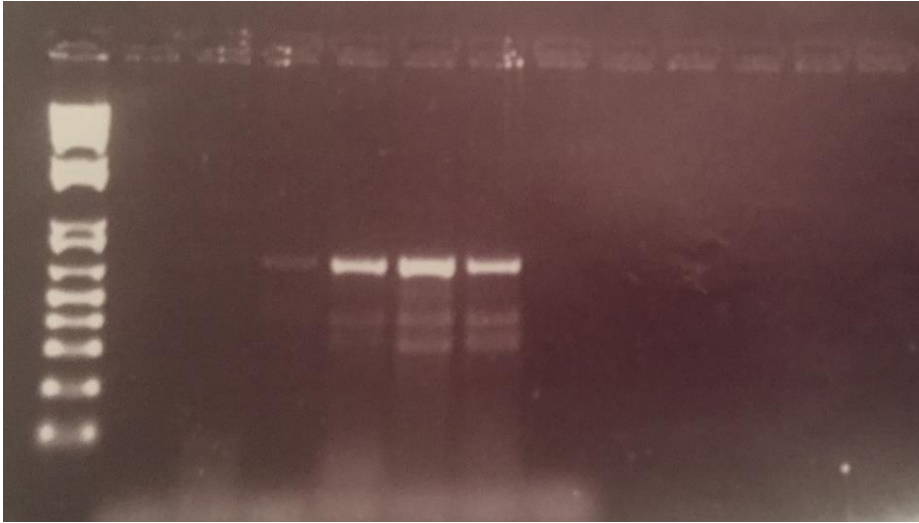
Figure 3.22 Picture of ITS1 photo

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

Figure 3.23 (top) — Consensus phylogenetic tree (ITS1, pairwise Clustal V)

- EU267192.1|Pandora neoaphidis ARSEF7939
- NW420 Pandora neoaphidis Rothamsted *(circled)*
- AF543211.1|Pandora neoaphidis ARSEF5374
- AF543207.1|Pandora neoaphidis NW327
- AF543206.1|Pandora neoaphidis NW316
- AF543202.1|Pandora neoaphidis NW343
- AF543205.1|Pandora neoaphidis NW283
- EU267190.1|Pandora neoaphidis ARSEF7938
- HQ677587.1|Pandora neoaphidis
- AF543203.1|Pandora neoaphidis NW356
- AF543204.1|Pandora neoaphidis NW195
- AF543208.1|Pandora neoaphidis NW415
- AF543209.1|Pandora neoaphidis ARSEF835
- AF543210.1|Pandora neoaphidis ARSEF1609
- EU267189.1|Pandora neoaphidis ARSEF7937
- EU267188.1|Pandora neoaphidis ARSEF1607
- WELL1 Pandora neoaphidis Wellesbourne *(circled)*
- AF368521.1|Pandora delphacis
- HQ677590.1|Pandora nouryi ARSEF366

Bracketed group: Pandora neoaphidis
Bootstrap values at nodes: 61, 64, 62, 100.

Percentage identity (%) *(upper right = % identity; lower left = Divergence)*

	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	AF543202.1|Pandora_neoaphidis_NW343
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	AF543203.1|Pandora_neoaphidis_NW356
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	AF543204.1|Pandora_neoaphidis_NW195
4	0.1	0.4	0.4	■	99.9	99.9	99.6	99.6	99.6	99.9	98.8	99.6	99.9	99.6	36.6	63.1	99.2	99.2		4	AF543205.1|Pandora_neoaphidis_NW283
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	AF543206.1|Pandora_neoaphidis_NW316
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	AF543207.1|Pandora_neoaphidis_NW327
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	AF543208.1|Pandora_neoaphidis_NW415
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	AF543209.1|Pandora_neoaphidis_ARSEF835
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	AF543210.1|Pandora_neoaphidis_ARSEF1609
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	AF543211.1|Pandora_neoaphidis_ARSEF5374
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	EU267188.1|Pandora_neoaphidis_ARSEF1607
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	EU267189.1|Pandora_neoaphidis_ARSEF7937
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	EU267190.1|Pandora_neoaphidis_ARSEF7938
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	EU267192.1|Pandora_neoaphidis_ARSEF7939
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	HQ677587.1|Pandora_neoaphidis
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	AF368521.1|Pandora_delphacis
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	HQ677590.1|Pandora_nouryi_ARSEF366
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	WELL1_Pandora_neoaphidis_Wellesbourne
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	NW420_Pandora_neoaphidis_Rothamsted
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		

(Row label axis: Divergence)

Figure 3.23 top: Consensus phylogenetic tree obtained from ITS1 sequence data using pairwise Clustal V 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, which was used in further experiments for comparison.

Figure 3.23 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, charaterisation down to species level was only conducted for parasitoid wasps, coccinellidae and syrphidae. Parasitoid mummies identified from field collections in June of 2013 were predominately *Praon volucre*. However, a number of hyper-parasitoids were also found and identified to *Alloxysta brevis*. Syrphidae larvae collected and raised to adulthood in August of 2013 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 and 2014.

4: The effect of temperature on the growth, germination and pathogenicity of entomopathogenic fungi and the effect of humidity on host-pathogen interactions

The effect of temperature on the rate of colony extension of fungal isolates
Results for Ascomycota were reported in the annual report for 2013 (Harvey, 2013). Results for *P.neoaphidis* isolate NW420 are reported below, Data on isolate WELL1 are currently being collected.

$$y = -0.0127x^2 + 0.5356x - 4.0302$$
$$R^2 = 0.9386$$
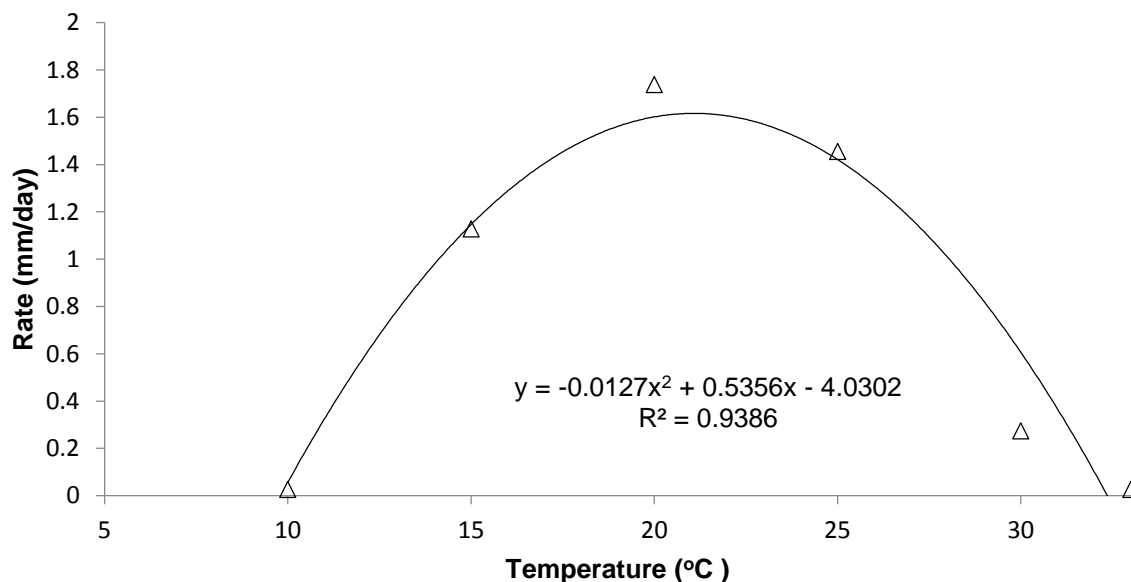
Figure 7. The effect of temperature on the growth rate of *Pandora neoaphidis* (NW420). Model fitted, polynominal quadratic (*P*=<0.05).

Preliminary analysis suggests the optimal temperature for growth of isolate NW420 is c.22°C. This is lower than all the Ascomycetes tested in 2013 (Harvey, 2013) with the exception of *Lecanicillium muscarium*.

*Laboratory evaluation of the effect of temperature on the germination of fungal species from the phylum ascomycota*

Isolate	Temperature (^{o}C)	GT10	GT50	GT90	Model fit (lack of fit test)
432.99	15	15.88	23.05	33.47	
433.99	15	13.40	21.54	34.63	
409.96	15	31.13	34.31	37.81	0.31
19.79	15	16.84	21.66	27.86	
275.86	15	19.23	23.33	28.3	
432.99	20	10.42	15.25	22.32	
433.99	20	9.33	14.20	21.60	
409.96	20	20.29	25.48	31.99	0.20
19.79	20	9.72	14.86	22.69	
275.86	20	19.13	19.74	20.36	
432.99	25	8.80	11.31	14.54	
433.99	25	8.35	10.99	14.46	
409.96	25	13.92	16.53	19.64	0.99
19.79	25	8.76	13.91	19.85	
275.86	25	10.38	11.25	12.19	

Table 5. Relative germination times in hours for various Ascomycota. GT10 (time to 10% germination), GT50 (time to 50% germination) and GT90 (time to 90% germination).

There are substantial differences in germination times between species of Ascomycota but also between isolates 432.99 and 433.99 that are both Beauveria *bassiana* (table 5). Isolate 433.99 has GT50 times of 21.54, 14.20 & 10.99 at 15, 20 & 25 ^{o}C respectively, lower than the other isolates at these temperatures. Model fitted to data was log logistic. All models fitted the data significantly (table 5).

*Temperature dependence of the pathogenicity of P.neoaphidis*

The effect of exposure to *P.neoaphidis* is discussed in relation to lethal time; no reference is made about dose in this report other than Fig 8 as analysis is on going. The analysis below is preliminary and should be treated as such. Fecundity data is currently being analysed. Data on lethal time 50 is presented for a showering time, 35 minutes.
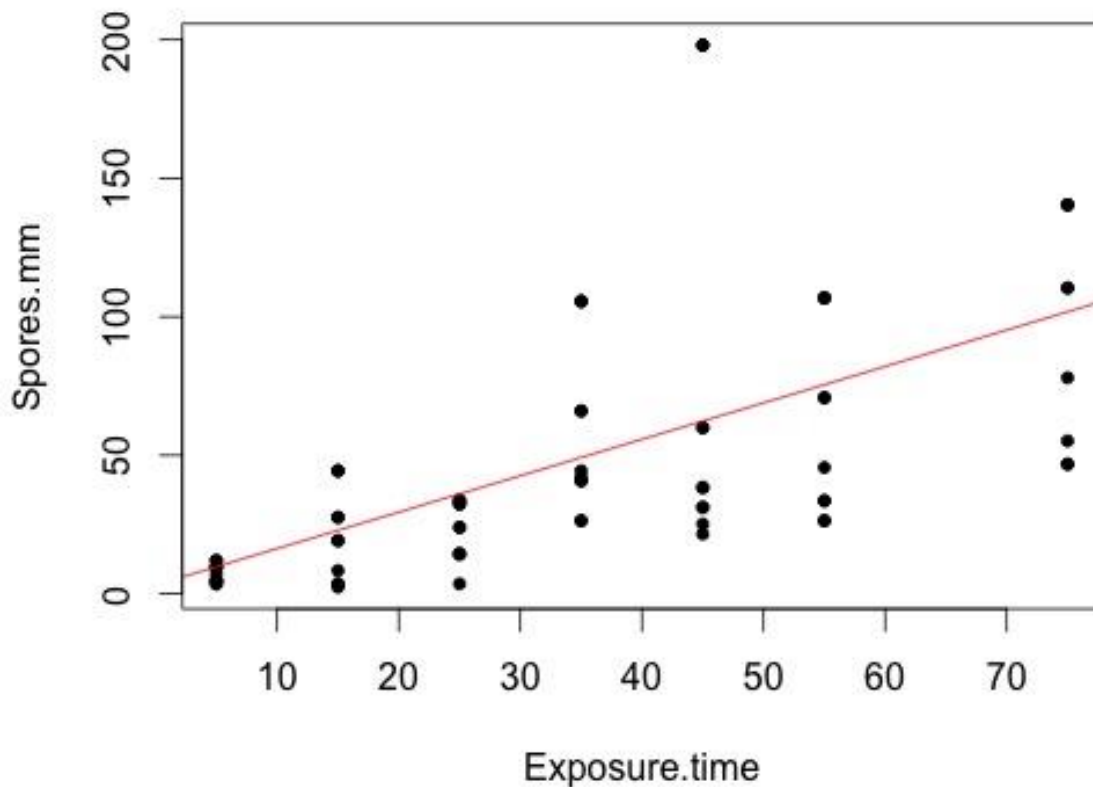
Figure 8. Exposure or showering time against the number of spores/mm^{-2} experimental populations of aphids would have received (linear regression, *P*=<0.001).

Exposure time can be used as a proxy for dose as although there is variation there is a significant linear relationship between exposure time and the number spores received by the aphids (figure 8). As such a single exposure time was used in the following analysis, 35 minutes.
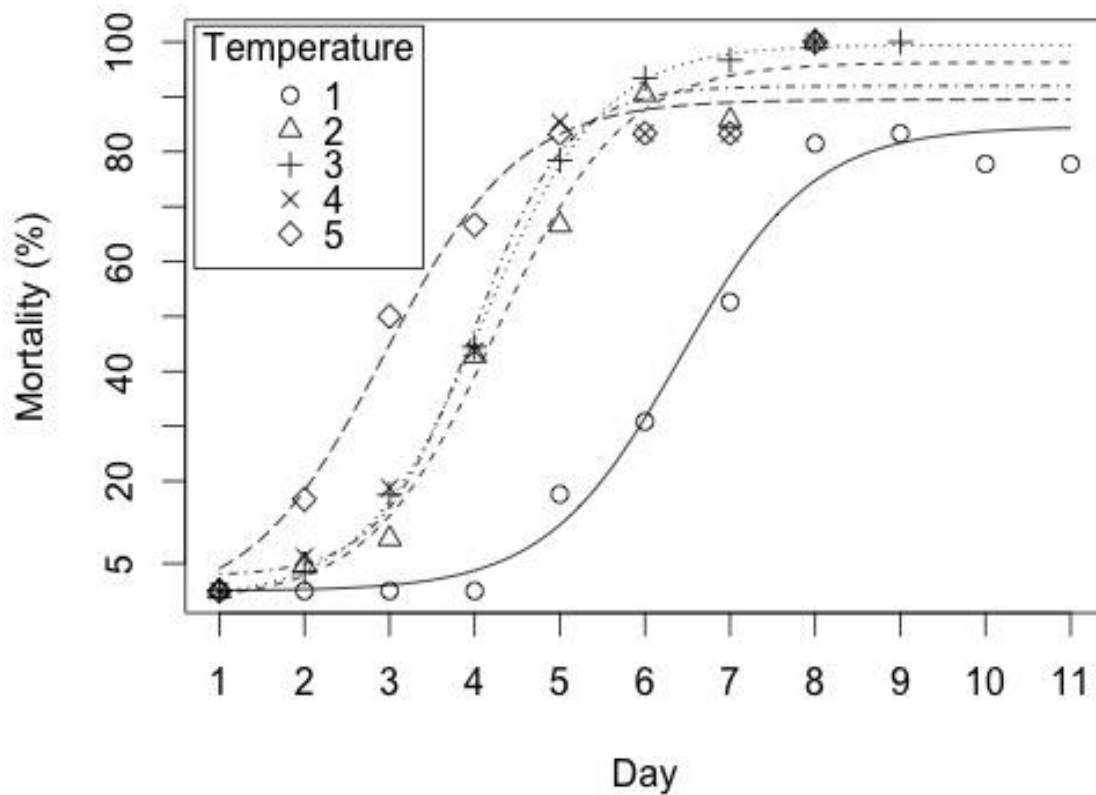
Figure 9. Percentage mortality over time (day) for each temperature 1=12 °C, 2=15 °C, 3=20 °C, 4=24 °C, 5=28 °C at a 35 minute spore showering duration. Model fitted to data was log logistic, lack-of-fit test *P* has to be greater than 0.05 (*P*=1.00).

Table 6. Lethal time to 50% mortality at experimental temperatures and associated standard error for exposure time 35 minutes giving an average dose of 60.9 spores/mm-2.
*Numbers 1 to 6 refer to their respective temperatures in the Fig 9.

Temperature (°C)*	LT50 (days)	St.err.
(1) 12	6.4	0.34
(2) 15	4.3	0.35
(3) 20	4.1	0.26
(4) 24	4.0	0.30
(5) 28	3.0	0.63

Analysis shows the lowest lethal time 50 (LT50) was at 28°C and the highest was at 12°C for an exposure time of 35 minutes.

5: Cultural methods affecting the aphid population crash

5.1 Introduction

This work considers cultural practices that may impact the aphid population decline such as fungicide application and sequential planting.

5.2 Materials & methods

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

Brassica 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 *Brevicoryne brassicae* adult (10-12 days) were attached to the abaxial side of the youngest and oldest leaves of each experimental plant. Nymph production and mortality were monitored daily for seven days and any nymphs produced were removed. 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 The effect of host plant age on the susceptibility of *Brevicoryne brassicae* to the entomopathogenic fungus *Pandora neoaphidis*

Brassica 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) (Table 5.2) conidia in a showering chamber. *P.neoaphidis* plugs (2.5mm diameter 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 to suspend 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. This gave a dose of 9.16±0.83 conidia/mm^2. Since the rate of discharge changed very little over this period the dose each replicate received was estimated by staining an 18x18mm^2 coverslip placed under the plugs post conidial showering 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. 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 hair paintbrush and plants were double bagged in bread bags for 48 hours in order to maintain high relative humidity (>97%). Control aphids were treated in the same way except for exposure to the pathogen, *P.neoaphidis*. Plants were stored in a controlled environment room at 20°C±1°C, 16:8 L: D photoperiod and monitored daily for mortality, dead individuals were removed and if not sporulating were placed in a Petri dish lined with moist filter paper at 15°C±1°C to monitor for infection. Individual reps 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 leaf samples 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 and removing debris with filter paper and RO water. The nitrates present in the samples were reduced to nitrite on a cadmium column and the nitrite was reacted with an indicator 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 *in vitro* effect of three fungicides on the growth of *Pandora neoaphidis* isolates

For *in vitro* experiments 7mm 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. Plugs were then inverted and placed in the centre of 90mm triple vented Petri dishes (Fisher Scientific, Loughborough, UK, LE11 5RG) containing SEMA amended to contain 0, 5, 10, 25, 50, 100 and 200% of field rate application of the one of the following three fungicides; azoxystrobin (Amistar; Syngenta, Cambridge, UK), trifloxystrobin + tebuconazole (Navito 75WG; BayerCropScience, Cambridge, UK) and difenoconazole (Plover; Syngenta) (Table 5.1). Petri dishes 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 and placed in an incubator in darkness at 20°C±1°C for 18-20 days. The procedure remained the same for both isolates of *P.neoaphidis* used in the experiment (table 5.2). 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-reps per treatment. Contaminated petri dishes were discarded and the experiment continued as long as one petri dish for treatment remained.

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

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

Table 5.2 Isolate information for *Pandora neoaphidis* used in experiments.

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.

5.2.5 The *in vitro* effect of three fungicides on the germination of primary conidia of two *Pandora neoaphidis* isolates

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 of these Petri's 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 field rate of the following fungicides: azoxystrobin (Amistar ©; Syngenta), trifloxystrobin + tebuconazole (Navito 75WG ©; BayerCropScience) and difenoconazole (Plover ©; Syngenta) (Table 5.1). Conidial showering continued for 60 minuets to ensure sufficient numbers of primary conidia were deposited for counting 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. The germination of conidia was assessed by counting 100-200 conidia in arbitrarily chosen non-overlapping fields of view ($0.2mm^2$) and assigned them to one of 5 ordinal categories; (1) ungerminated conidium, (2) germinated conidium, germ tube <50% of length of primary conidia, (3) germinated conidium, germ tube ≥50% of length of primary conidia, (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 *Brevicoryne brassicae* to two *Pandora neoaphidis* isolates on Brussels sprout plants

Brassica oleracea Gemmifera group (Brussels sprout, cultivar 'Trafalgar') (Tozer seeds Ltd, Surrey, UK, KT11 3EH) were cultured as per section 2.1 and experimental plants were at growth stage BBCH-16. Two fungicides were used, Amistar ©; Syngenta and Plover ©;

Syngenta, and applied at field rate until runoff (Table 5.2). Both isolates of *P.neoaphidis* were used in this study (Table 5.2). There were two fungicide application timings referred to as 'pre-sprayed' and 'post- sprayed'. Pre-sprayed plants were treated with fungicide in a laminar flow hood 24 hours prior to aphid infestation, whereas, post-sprayed plants were treated after aphid infestation and *P.neoaphidis* exposure. Cohorts of 60 young apterous *B.brassicae* adults (c.48 hours old) in the 'pre-sprayed' treatment were individually exposed to isolates of *P.neoaphidis* as above (section 5.2.2) in a conidial-showering arena. After approximately 60 minutes aphids were placed on the oldest leaf of experimental plants and encased within a Perspex box lined with moist filter paper to maintain high humidity. Aphids on infested plants were removed until groups of 10-15 young apterous *B.brassicae* adults that had settled in nucleated colonies on the abaxial side of leaves remained. These groups formed the 'post- sprayed' treatment and were showered with conidia as in section 5.2.2 with the exception that individuals were settled on a leaf not on a leaf disc over distilled water. After showering, exposed aphid colonies were encased in a Perspex box lined with moist filter paper. Showering time was 60 minutes for both treatments, as this was considered long enough to receive sufficient conidium to kill 100% of the population. The dose each replicate received was estimated as above (section 5.2.2). Control treatments consisted of fungicide treated and no pathogen exposure and pathogen exposure and no fungicide treatment. Experimental plants were stored in a control environment room at 20±2°C, 16L: 8D photoperiod and monitored daily for mortality. Dead individuals were removed. Individual reps lasted up to 7 days and the experiment was repeated on three occasions.

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 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 Bonferroni-type adjustment to p-values; to account for inflated type 1 errors when using non-parametric tests, in the package 'pairwise multiple comparison of mean ranks (PMCMR)'. Mortality data for this experiment was 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 analysed using Tukey HSD.

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 and arcsine

transformed. Soluble nitrogen concentrations for this experiment were also analysed using ANOVA.

Fungicide growth (experiment 5.2.4) data was analysed using Kruskal-Wallis and significant results were analysed using a post hoc Dunn test applying 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 at each germination stage. Significant results were analysed using Tukey HSD. Data on the effect of fungicide exposure on the susceptibility of *Brevicoryne brassicae* to *Pandora neoaphidis* (experiment 5.2.6) was analysed using ANOVA and significant results were analysed using Tukey HSD. Control mortality during experiment 5.2.6 was low and as such no correction term was required.

5.3 RESULTS

### 5.3.1 Plant mediated performance of *Brevicoryne brassicae*

Soluble nitrogen concentration did not vary between the youngest and oldest leaves of experimental plants (ANOVA, F=0.016, df=1, P=0.9). However, significant variation was found in the soluble nitrogen concentration between plants of different ages (ANOVA, F= 14.23, df= 2, P= 0.0000134) (Figure 5.2). Post hoc analysis revealed significant differences between young and medium 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 that of aphids on medium (P= 0.0000013) or old (P= 0.00000031) plants. Aphid fecundity on medium and old plants was not significantly different (P=1).

Moreover, aphid 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 that of young plants (P=0.02) but neither young nor old plants varied significantly from medium aged plants, P=0.132 and P=0.538 respectively (Table 5.3).
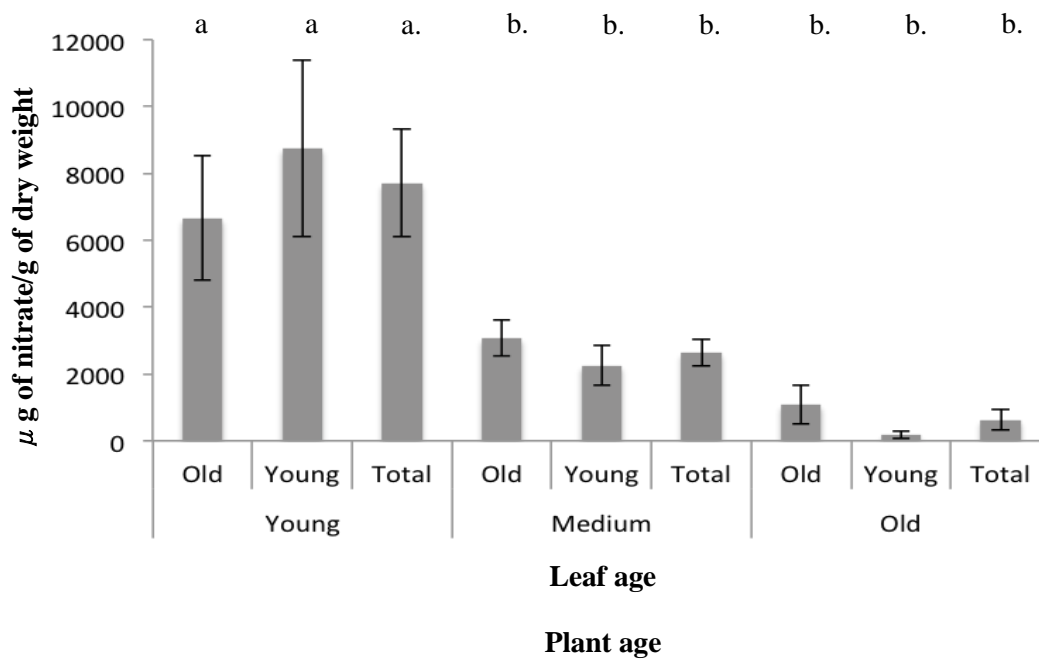
Figure 5.2 Soluble nitrogen concentrations in the youngest and oldest leaves of young (BBCH-13/14), medium (BBCH-16) and old plants (BBCH-19) (±se). Different levels indicate significant differences at the 5% level (Bonferroni-Dunn test).
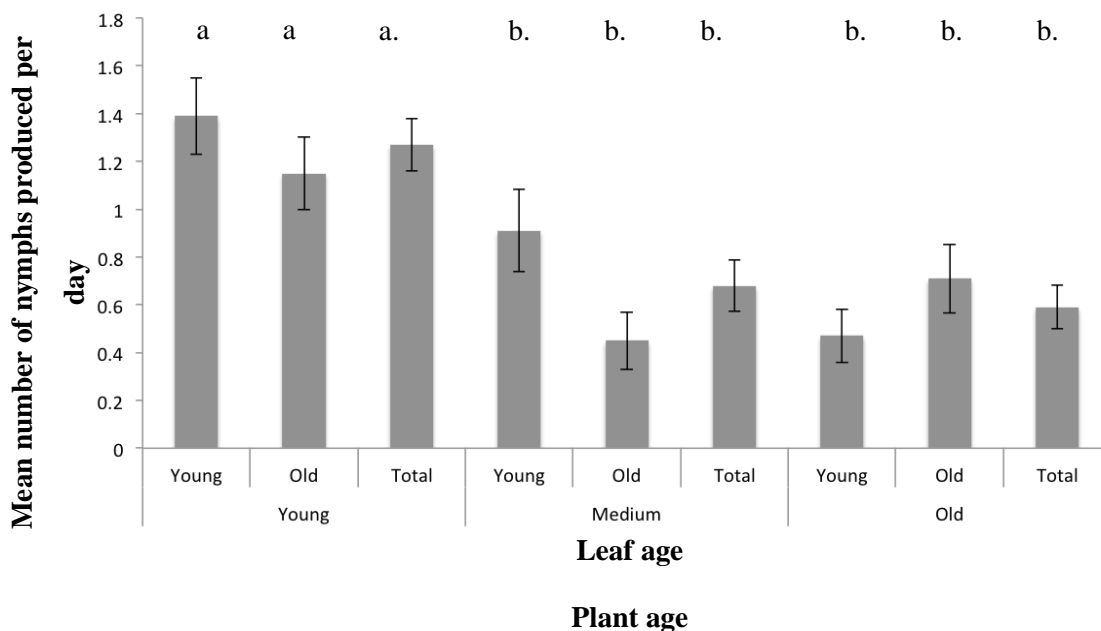
Figure 5.3 Fecundity of *Brevicoryne brassicae* (±se) raised on the youngest and oldest leaves of different aged *Brassica oleracea* Gemmifera group (Brussels sprout, cultivar 'Trafalgar'); Young plants (BBCH-13/14), medium plants (BBCH-16) and old plants (BBCH-19). Different letters indicate significant differences at the 5% level (Bonferroni-Dunn test).

Table 5.3 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 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 The effect of host plant age on the susceptibility of *Brevicoryne brassicae* to the entomopathogenic fungus *Pandora 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 motility for both physiological age treatments was day

five, 15.13± 5.63% and 23.75± 4.27% respectively for 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).
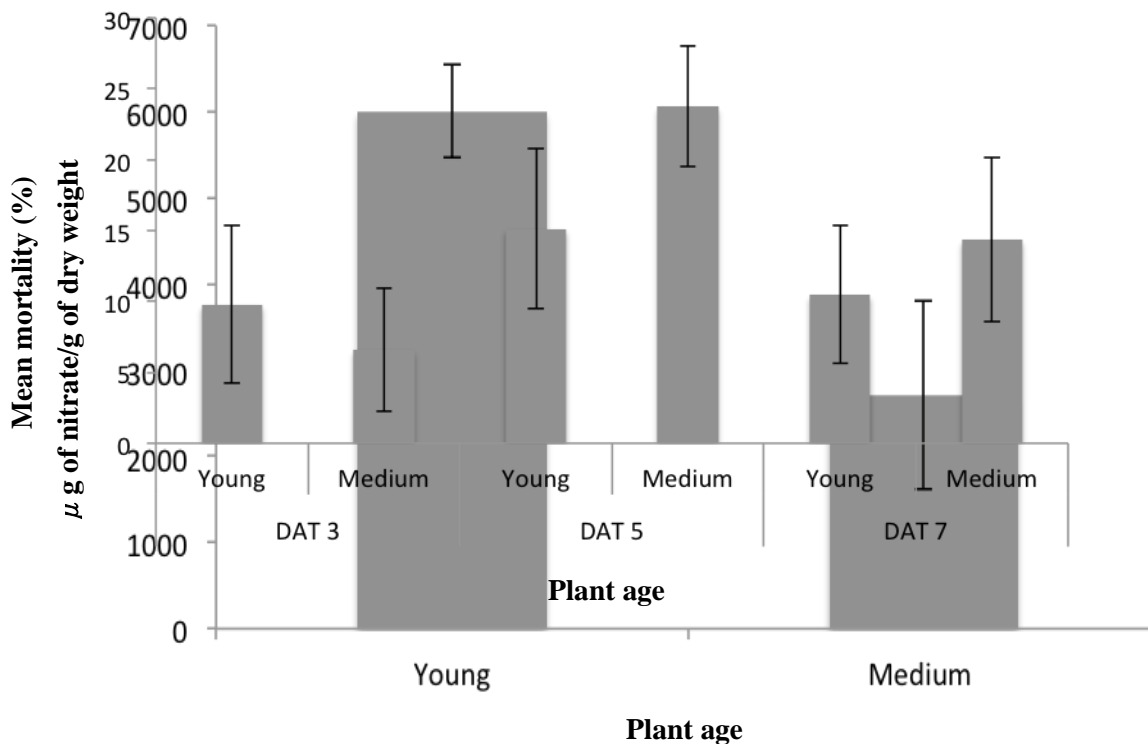
Figure 5.5 Abbotts adjusted mortality of *Brevicoryne brassicae* after exposure to *Pandora neoaphidis* (isolate=WEL1) on young (BBCH-13/14) and medium (BBCH-16) aged plants on the third and fifth day post treatment (±se).
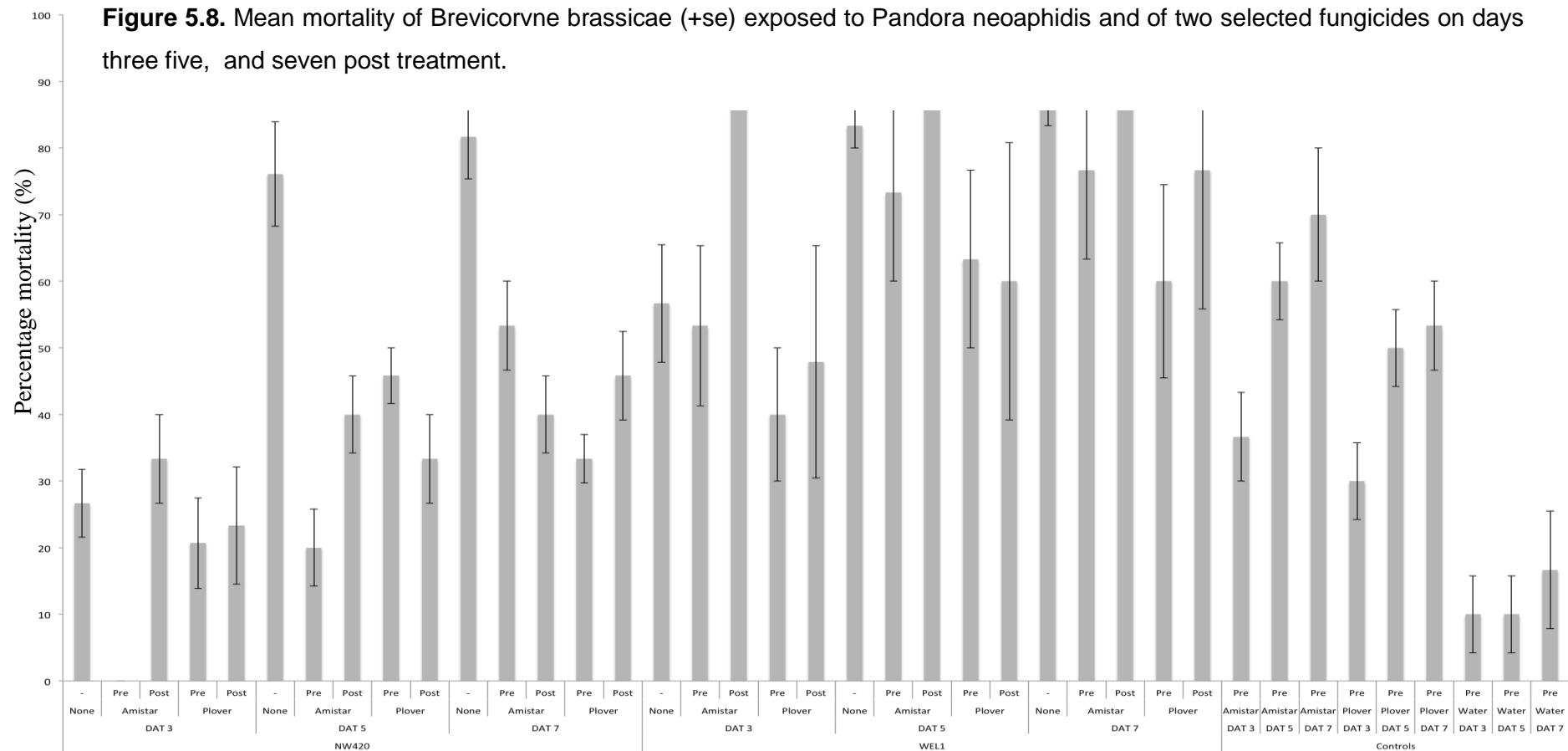
5.3.3 The *in vitro* effect of three fungicides on the growth of *Pandora neoaphidis* isolates

There were significant effects of fungicide exposure on mycelial extension of *P.neoaphidis* (Kruskal-Wallis, H= 41.5049, df= 2, *P*<0.0001). However, there were no significant differences in the response of either isolate of *P.neoaphidis* to fungicide exposure (Kruskal-Wallis, H= 0.5026, df= 1, *P*= 0.4784). Amistar © (Syngenta) completely inhibited the growth of NW420 and WEL1, where as Navito 75WG © (BayerCropScience) did not at 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 after treatment with Plover © (Syngenta) varied significantly from Navito 75WG © (*P*<0.0001) and Amistar © (*P*<0.0001), but Navito 75WG © and Amistar © did not vary significantly (*P*=0.48).

Mycelial extension rate varied significantly with dose (Kruskal-Wallis, H=19.1012, df= 5, *P*=0.00184) Non-parametric two-way analysis of variance is enigmatic, given Plover © was the only significantly different fungicide treatment post hoc Bonferroni-Dunn analysis of dose data was sufficient in determining the interaction of dose and fungicide. Growth rates at 5% of field dose varied significantly from that of 100% and 200% field dose, *P*=0.033 and 0.011 respectively. Additionally, growth rates at 10% of field rate varied significantly only from growth rates at 200% of field rate, *P*= 0.039.
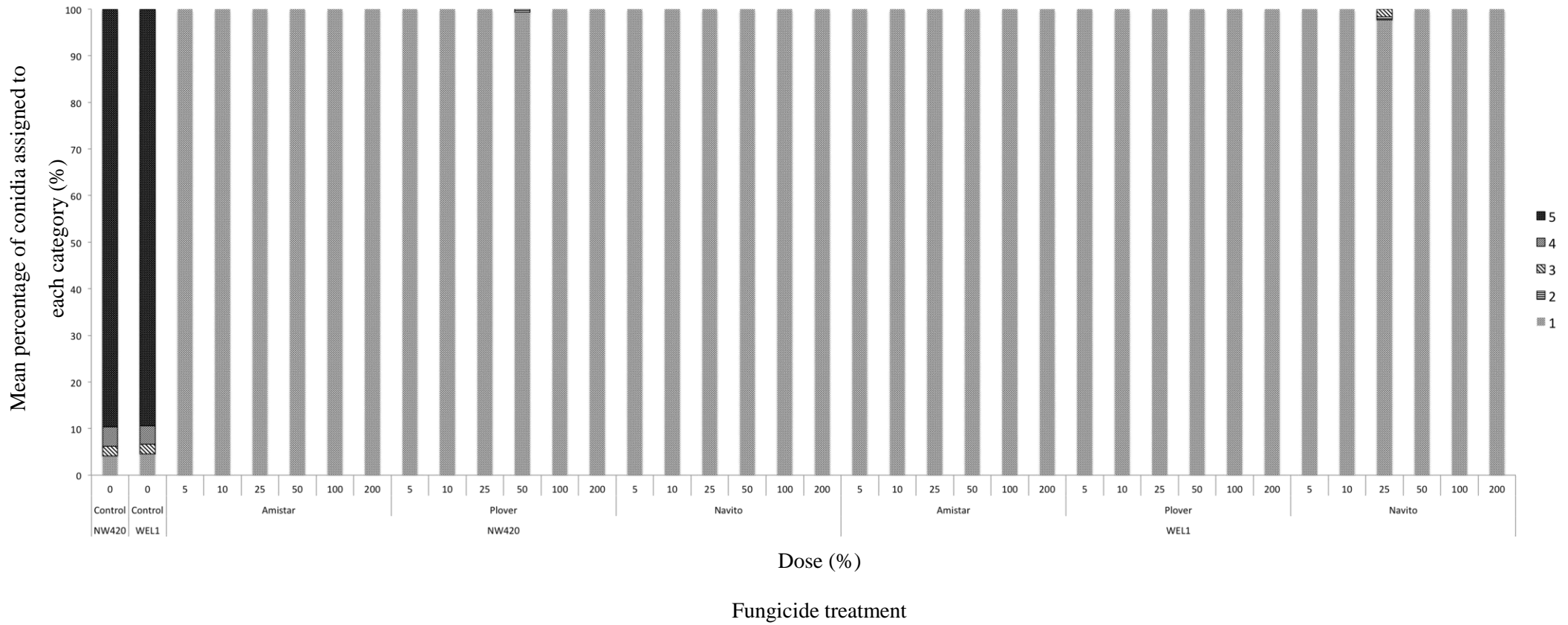
Figure 5.8. Mean mortality of Brevicorvne brassicae (+se) exposed to Pandora neoaphidis and of two selected fungicides on days three five, and seven post treatment.
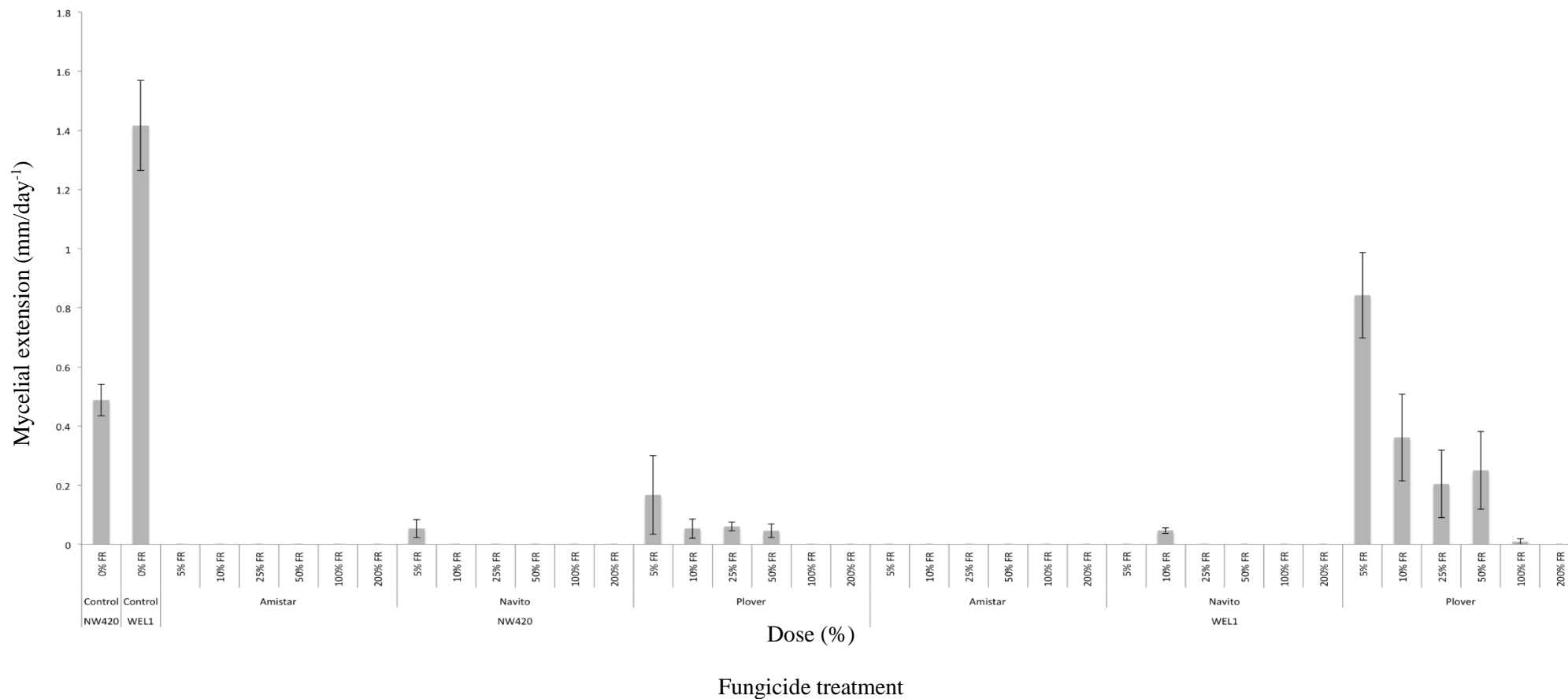
Figure 5.7 The effect of selected fungicide exposure on the germination of two isolates of *Pandora neoaphidis* (NW420 & WEL1)

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

5.3.4 The *in vitro* effect of three fungicides on the germination of primary conidia of two *Pandora neoaphidis* isolates

There were no significant differences between fungicide treatments (ANOVA, F=0.87, df=2, P=0.4179) or dose (ANOVA, F=12.73, df=2, P=0.2745) on the germination of primary conidia. All fungicides used in the study; Amistar © (Syngenta) and Navito 75WG © (BayerCropScience) and Plover © (Syngenta) inhibited the germination of conidia down to 5% of field rate (Figure 5.7). Category 1, ungerminated conidia, was significantly different to all other ordinal categories of germination; where as none of the other categories were different from each other (Table 5.4).

Table 5.4 Post hoc TukeyHSD analysis of the percentage of each conidia in ordinal germination categories; (1) ungerminated conidium, (2) germinated conidium, germ tube <50% of length of primary conidia, (3) germinated conidium, germ tube ≥50% of length of primary conidia, (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.

5.3.5 Effect of selected fungicides on the virulence of two Pandora neoaphidis isolates to Brevicoryne brassicae on Brussels sprout plants

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

There were significant differences in the response of *Pandora neoaphidis* isolates exposed to fungicide treatments (ANOVA, F= 49.94, df= 1, *P*= <0.0001). Isolate WEL1 appeared to be the more virulent isolate to *Brevicoryne brassicae* across treatments (Figure 5.8). 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. The mortality of *B.brassicae* exposed to NW420 was significantly different for both fungicide treatments compared to no fungicide treatment, however fungicide treatments did not vary significantly from each other (Table 5.4). Conversely, fungicide treatments varied significantly from each other for isolate WEL1 but there were no significant differences between either of the fungicides applied to no fungicide application (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). Amistar © and Plover © only control treatments did not significantly vary from their *P.neoaphidis* exposed counterparts, suggesting no additive benefits to the mortality of *B.brassicae* when exposed to an entomopathogenic fungi and a fungicide. There was no significant difference between Plover © and Amistar © controls *P*=0.822.

Fungicide application timing; 'pre-sprayed' & 'post-sprayed', had no significant effect on the mortality of *Brevicoryne 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).

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 and application were present for both NW420 & WEL1, these were, F= 4.634, df= 1, *P*= 0.03375 and F= 8.189, df= 1, *P*= 0.00513, respectively. For isolate NW420, Amistar © pre- sprayed treatment was significantly different from no fungicide treatment (*P*= 0.0038240). Amistar pre- sprayed caused 20± 5.77% five days after treatment, whereas no fungicide treatment caused 60± 5.77% after the same time period (Figure 5.8). Moreover, Plover © post-sprayed was significantly different from no fungicide treatment (*P*= 0.0551069), causing 33.33± 6.66% and 50± 5.77% mortality after five days respectively (Figure 5.8).

For isolate WEL1, Amistar © post- sprayed varied from all other fungicide treatments; Plover © post- sprayed (*P*= 0.0013086), Plover pre- sprayed (*P*= 0.0021876) and Amistar pre-sprayed (*P*= 0.0051686). Amistar © post- sprayed resulted in 100± 0% mortality of *B.brassicae* after just three days for isolate WEL1, whereas for the same treatment, isolate NW420 only reached 40± 5.77% 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). There was no significant days after treatment* fungal species interaction (ANOVA, F= 1.61, df= 12, *P*= 0.0889). Days three and five and days three and seven after treatment varied significantly, *P*= 0.0019105 and *P*<0.0001 respectively. Days five and seven after treatment did not vary significantly, *P*= 0.6115121.

6. CONCLUSIONS

- Although the aphid population crash occurred at a much later stage in the season in 2014 than in 2013 plant age was once again shown not to affect the timing of the crash. Moreover, there were no significant location variations in 2014 suggesting little spatial heterogeneity in the timing of the crash.

- Natural enemy abundance is reported to be significantly negatively associated with instantaneous rate of increase of aphid populations suggesting an antagonistic relationship.

- The 2014 field season also saw the establishment of a fungal epizootic around the timing of the crash as in 2013. This fungus was successfully isolated and through DNA identificationbeen confirmed as *Pandora neoaphidis*.

- Alate production is linked to aphid population density.

- Plant age/soluble nitrogen effects *Brevicoryne brassicae* fecundity and mortality in the lab but this relationship is not seen in the field.

- Temperature has a significant impact on growth, germination and virulence further highlighting its importance in the biology of entomopathogenic fungi and consequences for any pest management strategy.

7. KNOWLEDGE AND TECHNOLOGY TRANSFER

- The Royal Entomological Society Aphid Special Interest Group. "Exploring new ways to manage aphids in crops" Leamington Spa, UK. 11th September 2013. Oral presentation.
- IOBC (International Organisation for Biological and Integrated Control)/ WPRS (West Palaearctic Regional Section). "Integrated protection in field vegetables" Bergerac, France. 21st – 26th September 2013. Oral presentation.
- HDC (Horticultural Development Company) R&D Technical Conference. Lincolnshire, UK. 9th October 2013. Oral presentation.
- AAB (Association of Applied Biologists). "IPM: Pushing back the frontiers" Lincolnshire, UK. 15th- 16th October 2013. Oral presentation.
- IOBC (International Organisation for Biological and Integrated Control)/ WPRS (West Palaearctic Regional Section). "The role of naturally enemies in cotrolling aphid populations in field Brassicas." Article publication.
- BGA (British Growers Association) Conference. Lincolnshire, UK. 21st January 2014.

- SIP (Society of Invertebrate Pathology), Mainz, Germany. 2nd-7th August 2014. Paper presentation.
- Biopesticide conference. Swansea, 7th-9th September 2015. Poster presentation.
- AHDB studentship conferences. Droitwich, 16-17th September 2015. Poster & oral presentation.
- AAB (Assocaition of Applied Biologists). "IPM: The 10 year plan" Lincolnshire, UK. 18th-19th November 2015. Oral presentation.

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APPENDICES

Appendix 1

Table 3.6 Post hoc TukeyHSD analysis showing significant differences in natural enemy community 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