Elucidating the natural function of cordycepin, a secondary metabolite of the fungus *Cordyceps militaris*, and its potential as a novel biopesticide in Integrated Pest Management

By

Victoria Clare Woolley

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Life Sciences

University of Warwick, School of Life Sciences

September 2018

Table of Contents

List of Figures

List of Tables

Abbreviations

AMP	Anti-microbial peptide
ANOVA	Analysis of variance
CFU	Colony forming unit
DEPC	Diethyl pyrocarbonate
DHI	5, 6-dihydroxyindole
DIF	Dorsal-related immunity factor
DMSO	Dimethyl sulfoxide
EC_{50}	The concentration at which 50% of the maximum effect occurs
ED_{50}	The dose at which 50% of the maximum effect occurs
EPF	Entomopathogenic fungi
IKK	IκB kinase
IMD pathway	Immune deficiency pathway
IMPI	Insect metalloprotease inhibitor
IPM	Integrated pest management
ITS	Internal transcribed spacer
JAK	Janus kinase
LC_{50}	Concentration at which 50% mortality occurs
LD_{50}	Dose at which 50% mortality occurs
LPS	Lipopolysaccharide
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NRP	Non-ribosomal peptides
OUT	Operational taxonomic unit
NRPS	Non ribosomal peptide synthase
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PK	Polyketide
PO	Phenol-oxidase
PPO	Prophenol-oxidase
qPCR	Quantitative PCR

RT-qPCR	Quantitative reverse transcription polymerase chain reaction
ROS	Reactive oxygen species
SDA	Sabouraud dextrose agar
SE	Standard error
SEM	Standard error of the mean
STAT	Signal transducer and activator of transcription
TLR	Toll-like receptor

Acknowledgements

Firstly, I would like to thank my supervisors Graham Teakle and Dave Chandler for their guidance, help and support. I have really appreciated their enthusiasm throughout the project and whenever I had a problem they always helped me 'see the wood for the trees'. I am also grateful to Cornelia de Moor, her lab group and Sonia Lopez de Quinto for their assistance and guidance during cell culture experiments.

I am very grateful to everyone in the Chandler lab group for teaching me laboratory techniques, helping me develop new experiments and encouraging me throughout my Ph.D., particularly Gill Prince, Jess Fannon, Liam Harvey and Laura Reyes. I would also like to thank all the friends I've made at Wellesbourne for keeping me sane for four years and only sometimes making fun of how my experiments were not working, especially Spencer Collins, Dan Wilson, Lawrence Bramham, Dec Perry, Scott Dwyer and all past members of our not-very-successful pub quiz team!

I would like to acknowledge financial support from MIBTP and BBSRC for funding this research.

I would also like to thank my family-Mum, Dad, Issy and Jo- for their support and encouragement during my Ph.D. Finally, I would like to thank Mike for believing in me and for getting me to stop stressing when things weren't going to plan (or at least trying to!).

Declarations

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work presented (including data generated and data analysis) was carried out by myself unless otherwise stated.

Abstract

Pest insects cause significant losses of crops in terms of both yield and quality. Currently chemical pesticides are heavily relied upon for pest control, however the use of these chemicals is being restricted, primarily due to health and environmental concerns. Therefore, it is important to develop alternative control techniques and integrated pest management (IPM) approaches. Entomopathogenic fungi (EPF) infect and kill insects and have a large potential for use in IPM. *Cordyceps militaris* (Ascomycota, Hypocreales) is an EPF that naturally infects Lepidoptera pupae. It synthesizes the secondary metabolite cordycepin (3'-deoxyadenosine), which has been investigated for its anti-inflammatory and anti-cancer properties in human medicine, however, little is known about its natural function. The first aim of this project was to elucidate the natural function of cordycepin and the second aim was to develop it as a potential biopesticide. A series of bioassays and RT-qPCR analyses were carried out in *Galleria mellonella* and *Drosophila melanogaster* S2r+ cells to determine the impact of application of cordycepin and EPF, both alone and in combination, on the expression of immune-related genes. It was found that cordycepin inhibits the immune response by reducing the expression of immune-related genes, including anti-microbial peptides (AMPs). It was hypothesised that cordycepin has a role in facilitating the natural infection of insects by *C. militaris* through inhibition of the host immune response. Bioassays were also performed to quantify the effect of foliar applications of cordycepin, both alone and in combination with EPF, against the Lepidopteran pest *Plutella xylostella*. Cordycepin alone was found to cause mortality of this insect, while at some doses EPF and cordycepin were found to interact synergistically to increase mortality. This suggests that there is potential to develop cordycepin as a biopesticide for use in IPM.

Chapter 1- Introduction

1. 1 The importance of effective crop protection for global food production

The development of more sustainable methods of crop production is a major challenge for human society. By 2050, global food production may need to increase by as much as 70% above current levels in order to support the needs of an expanding population (United Nations 2015, Crist *et al*., 2017). At the same time, the crop production systems used in modern farming to achieve high yields are also a significant cause of global environmental harm, including loss of biodiversity and greenhouse gas emissions (Cole *et al*., 1997; Altieri, 1999; Robinson and Sutherland, 2002). Therefore, it is necessary that agriculture becomes more sustainable, whereby it does not have a negative impact on the environment, society or human health (Pretty and Bharucha 2014). Some experts have called for the 'sustainable intensification' of agriculture, with the aim of increasing food production without causing further environmental damage (Crist *et al*., 2017), although the precise mechanisms by which this could be achieved are still a matter of debate (Tilman *et al*., 2002; Loos *et al*., 2014; Pretty and Bharucha, 2014).

One area of agriculture where there is significant potential for improvement is in the protection of crops from arthropod (*i.e*. insect and mite) pests. It is estimated that globally arthropod pests cause 18-26% reduction in crop yields each year (Culliney, 2014), despite the use of large amounts of plant protection products and other tools by farmers and growers (Popp *et al*., 2013). Furthermore, it is predicted that crop yield losses due to insects will increase dramatically as a result of global warming (Deutsch *et al*., 2018). Since the start of the Green Revolution in the 1960s (Pimentel, 1996; Tilman, 1998), the standard method of crop protection against arthropod pests has been the application of synthetic chemical pesticides. For example, in the U.K it is estimated that since 1990 at least 4.5 million ha of crops have been treated annually with insecticides (FERA, 2016). However, the excessive use of synthetic chemical pesticides can be harmful to the environment and non-target beneficial organisms (Wood and Goulson, 2017), as well as a threat to the health of farmworkers exposed

to the undiluted pesticide (Pimentel, 1996). Historically, chemical pesticides have been developed in succession, with newer products increasing in efficacy and improving human safety (Sugavanam, 1996). 'Old' insecticide chemistries such as organochlorines and organophosphates have gradually been replaced by newer compounds such as pyrethroids that offer many benefits (Miyamoto, 1976; Maroni *et al*., 2000; Tanabe, 2002), including reduced mammalian toxicity and shorter environmental persistence (Maroni *et al*., 2000). Concerns remain about adverse impacts on the environment and human health, particularly in less developed countries where 'old' insecticides are still being used and farm workers do not have access to adequate safety equipment, storage facilities and training (Ecobichon, 2001). Problems also occur through the evolution of heritable resistance to pesticides in target pest populations. Since the first documented case of insecticide resistance in 1914, the number of species exhibiting insecticide resistance had increased to 586 species in 2014 (Sparks and Nauen, 2015). Additionally, there are concerns about the evolution of pest populations to all chemical pesticides, for example, the peach potato aphid (*Myzus persicae*) has developed high levels of insecticide resistance through multiple mechanisms (overexpression of esterase, super-Kdr genotypes and MACE), which reduces the number of pesticides available for its control (Criniti *et al*., 2008; Bass *et al*., 2014).

A combination of health concerns, environmental impacts and insecticide resistance has led to the increasing withdrawal of pesticide products from the market (Chandler *et al*., 2011). In 2009 the European Union introduced regulation (EC 1107/2009; European Parliament, 2009) that applied more stringent requirements for pesticide registration, where they have to meet more exacting toxicological, environmental and safety standards (Jess *et al*., 2014). It was estimated that this legislation would lead to the withdrawal of 20% of active ingredients in pesticides by 2015 (Hillocks, 2012), however the overall sales of pesticides remained the same between 2011-2015 (European Environment Agency, 2017). There is not yet data available to determine whether sales of harmful pesticides were replaced with those less harmful (European Environment Agency, 2017). It is likely that, with the globalisation of food standards, other regions will also place more restrictions on the use of conventional chemical

pesticides. A more sustainable solution for crop protection is needed as a matter of priority (Pimentel *et al*., 1992; Pimentel, 1996; Tilman *et al*., 2011).

1.2 Crop protection and Integrated Pest Management

Most experts agree that the most effective way to make pest control more sustainable is through Integrated Pest Management (IPM) (Perrin, 1997; Tilman *et al*., 2002). IPM is the coordinated use of different pest management tools to minimise negative impacts on society and the environment, whilst providing maximum benefit to the producers (Kogan, 1998). Chandler *et al*., (2011) describe IPM as the combined use of complementary crop protection methods including:

- Choosing chemical pesticides that have minimal health and environmental impacts and using them sparingly to reduce the risk of resistance emerging.
- Selective breeding of crops for pest resistance.
- Maintaining good cultivation practices such as crop rotations.
- Physical controls.
- Using natural products as pest control agents, in some cases these natural products can be the basis for synthetic insecticides (Gerwick and Sparks, 2014) *e.g*. spinosad and pyrethrum (Dayan *et al*., 2009).
- Using biological control, such as pathogenic microorganisms or natural enemies.
- Decision support tools, such as pest forecasts, to determine the best time to use control methods.

IPM strategies have been adopted in many countries with varying levels of success. Of the 62 IPM initiatives studied by Pretty (2008), greater than 80% showed reduced pesticide usage and approximately 60% showed a reduction in pesticide use and increase in crop yield. Farmers may not yet have adopted IPM strategies due to a lack of understanding or unfavourable attitude towards changing farming practices (Tscharntke *et al*., 2012; Barzman *et al*., 2015), but the government incentives described in Section 1.1 and increasing research may change this.

1.3 The use of entomopathogens as biopesticides in IPM

Biopesticides are an important aspect of IPM. While the definition of a biopesticide has not been formally agreed, it generally refers to microbial organisms or natural products that are used to control pests (Chandler *et al.,* 2011). Others may extend it to include genetically transformed crops, insect pheromones and natural enemies (Copping and Menn, 2000). An increasingly important aspect of IPM is the use of microbial biopesticides based on entomopathogenic microorganisms including bacteria, fungi and viruses. These microbial pathogens cause lethal infections in insects and other arthropods and can be used as pest control agents (Table 1.1). The global biopesticide industry is rapidly growing, between 2013-2015 the biological inputs industry grew on average 16.7% each year, with biochemical and microbial products growing on average 40.9% and 20.9% each year respectively (IBMA, 2016). There are currently commercially available biopesticides with activity against insects, nematodes and fungi (Table 1.1).

Most microbial biopesticides are applied to crops as part of an inundation biological control strategy, whereby a high concentration/volume is applied to kill a maximum number of the target pest. Other common biological control strategies include classical, inoculation and conservation control. Classical control is the release of a non-native biological control agent with the aim that it establishes and gives long-term pest control. Inoculation control is similar, but the biological control agent does not establish or give permanent control (Eilenberg *et al*., 2001). Finally, conservation control protects or enhances the population of existing natural enemies to reduce the pest population, such as through modification of the environment to favour these species (Eilenberg *et al*., 2001).

Microbial biopesticides can be used in conjunction with other pest control techniques such as conventional chemical pesticides, to give a greater reduction in pest numbers (Glare *et al*., 2012). As an example, codling moth (*Cydia pomonella*; Lepidoptera) an economically important pest of apples, can be managed successfully using a combination of pheromone mediated mating disruption and *Cydia pomonella* granulosis virus (CpGV) (Lacey *et al*., 2008). This has been used successfully in North American organic farms (Lacey *et al*., 2008).

Table 1.1- Examples of commercially available biopesticides [1].

Category of biopesticide	Type of biopesticide	Active ingredient	Activity against	Type of active ingredient	Target
Microorganism	Insecticide	*Bacillus thuringiensis subsp. Kurstaki*	Insects	Bacteria	Caterpillars
	Insecticide	*Bacillus sphaeicus*	Insects	Bacteria	Mosquito larvae
	Insecticide	*Chromobacterium subtsugae*	Insects	Bacteria	Broad spectrum
	Nematicide	*Pasteuria* spp.	Nematodes	Bacteria	Plant parasitic nematodes
	Fungicide	*Bacillus subtilis*	Fungi	Bacteria	*Botrytis* spp.
	Insecticide	*Beauveria bassiana*	Insects	Fungus	Thrips, aphids and whiteflies
	Insecticide	*Metarhizium brunneum*	Insects	Fungus	Thrips, whiteflies and weevils
	Nematicide	*Purpureocillium lilacinum*	Nematodes	Fungus	Plant parasitic nematodes
	Fungicide	*Coniothyrium minitans*	Fungi	Fungus	*Sclerotinia* spp.
	Insecticide	Cydia pomonella granulosis virus	Insects	Virus	Codling moth
	Insecticide	Spodoptera exigua nucleopolyhedrovirus	Insects	Virus	Beet armyworm
Biochemical	Insecticide	4-allyanisol (basil oil)	Insects	Natural biochemical	Beetles
	Fungicide	Reynoutria sachalinensis	Fungi	Natural biochemical	Powdery mildew, downy mildew and *Botrytis*.
	Nematicide	*Quillaja saponaria*	Nematodes	Natural biochemical	Plant-parasitic nematodes

[1] Biopesticides that are commercially available worldwide, based on Chandler *et al*., (2011), Seiber *et al*., (2014) and Arthurs and Dara, (2018).

One of the most widely used biopesticides is *Bacillus thuringiensis* (Bt), an entomopathogenic bacteria for control of a number of Lepidoptera, Coleoptera and Diptera pests including: Colorado potato beetle, beet armyworm, tobacco hornworm, mosquitos and European corn borer (MacIntosh *et al*., 1990). *Bacillus thuringiensis* produces a protein crystal (the Bt ∂-endotoxin) during spore formation that, following ingestion by a susceptible insect, disrupts the integrity of the midgut resulting in rapid

death (Gill *et al*., 1992). Spores of *B. thuringiensis*, or purified ∂-endotoxin, can be applied to crops as a spray Chandler *et al*., (2011) or plants can be genetically transformed to produce the toxin (Wünn *et al*., 1996). Resistance to Bt toxin began to emerge in pest species, particularly diamondback moth (*Plutella xylostella*), in the 1990s (Tabashnik, 1994). This highlights the need for IPM techniques, where the use of multiple control methods could reduce the risk of resistance emerging (Phillips *et al*., 1989).

1.4 Entomopathogenic fungi and their use as biopesticides

Fungal pathogens of arthropods, commonly referred to as entomopathogenic fungi (EPF) are natural regulators of a wide range of insect and mite species (Quesada-Moraga *et al*., 2007). There are estimated to be 750-1000 known species (Vega *et al*., 2012), but there is also significant genetic variation within species, with most species consisting of a wide range of strains that vary in pathogenicity related characteristics. EPF are predominantly found within two distinct taxa of the fungal kingdom; the order Hypocreales (Ascomycota, Sordariomycetes) and the Entomophthorales (Entomophthoromycota) (Figure 1.1). EPF have been used in classical, augmentation and inundation biocontrol strategies (Section 1.3) (Shah and Pell, 2003).

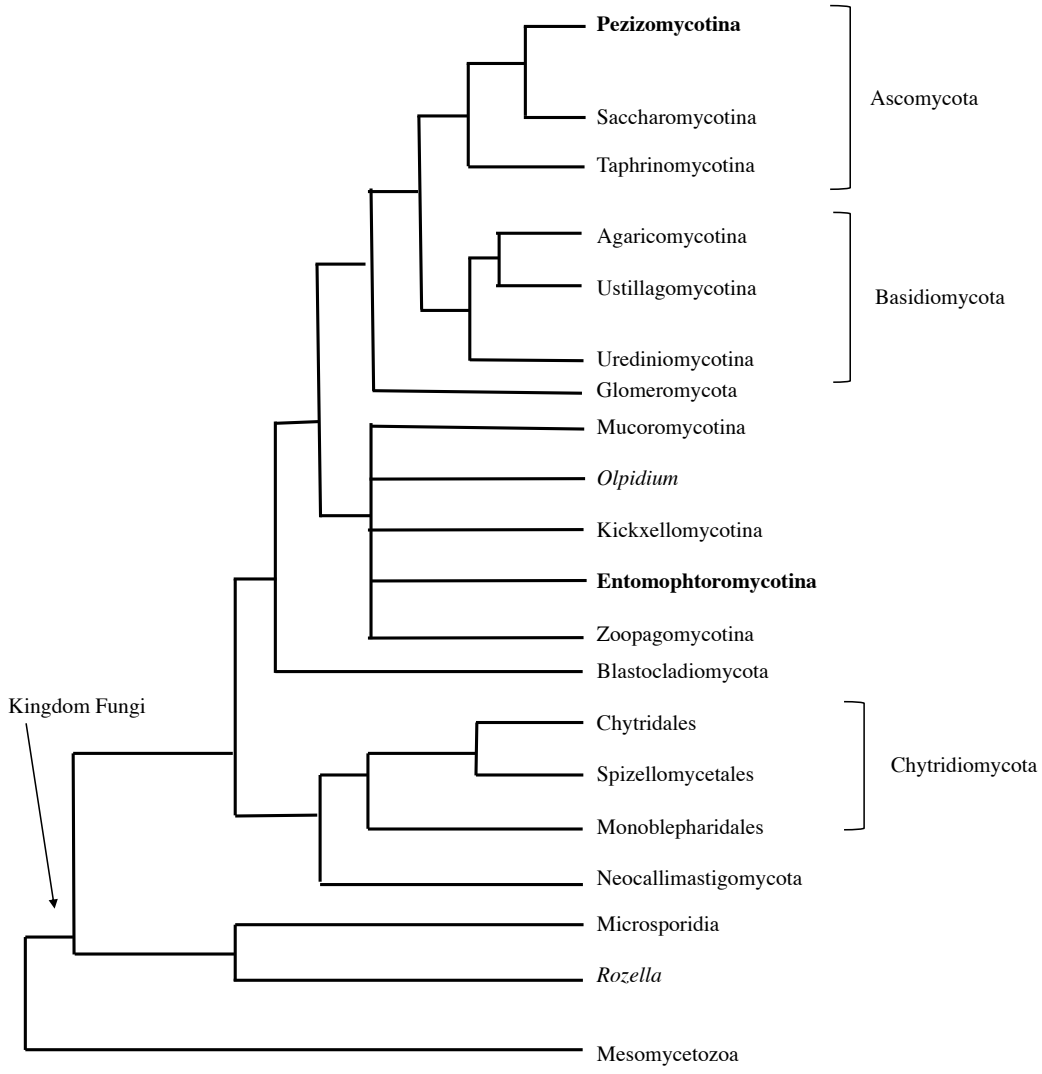


Figure 1.1-Phylogenetic tree showing major groups of fungi. Based on the classification by Hibbett *et al*., (2007) from Roy *et al*. (2010). Groups containing EPF species are highlighted in bold.

The Entomophthorales cause natural epizootics in insect and mite populations but they are not used as biopesticides because they are generally difficult to grow in culture and so are not economically viable to produce (Jaronski, 2014). Strategies to exploit entomophthoralean EPF for biological pest control have involved conservation of natural populations (Ekesi *et al*., 2005), the development of forecasts of natural epizootics (Hollingsworth *et al*., 1995), and inoculative applications such as lure and kill methods. For example, forecasting methods have been developed by Hollingsworth *et al*., (1995) to predict the prevalence of *Neozygites fresenii* (Entomophthorales, Neozygitacaea) in cotton aphids (*Aphis gossypii*), to inform pesticide application timings. An example of the lure and kill method has been developed by Furlong *et al*., (1995) in which a pheromone trap delivered *Zoophthora radicans* (Entomophthorales, Zygomycetes) conidia to *Plutella xylostella* (diamond back moth); infected *P. xylostella* then transferred conidia to other individuals in the field.

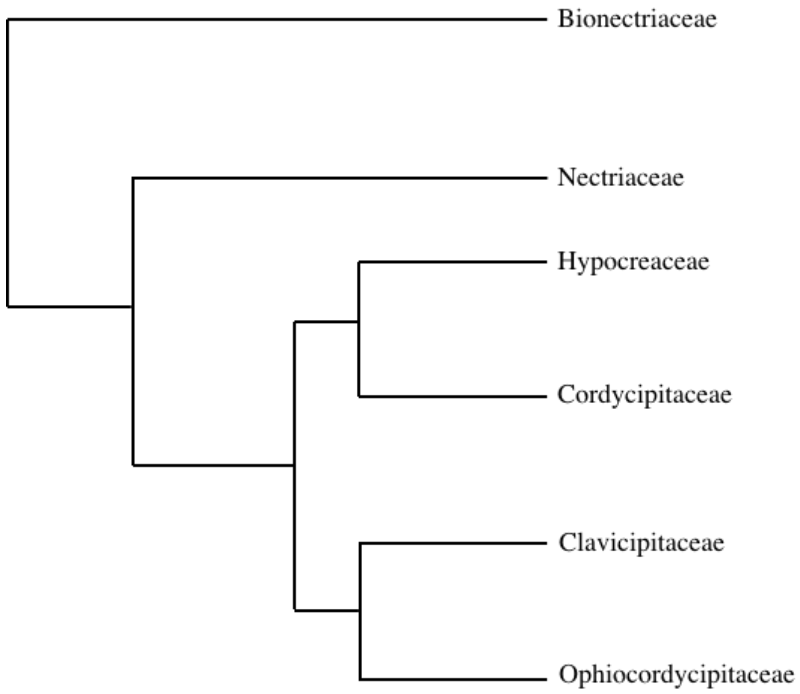


Figure 1.2- Phylogenetic tree showing Fungal families in the order Hypocreales. From Roy *et al*., (2010).

The majority of known species of EPF occur within three families in the Hypocreales within the phylum Ascomycota (Figure 1.1), these are Cordycipitaceae, Clavicipitaceae and Ophiocordycipitaceae (Figure 1.2). These families contain species

that are in their teleomorphic or anamorphic states. Teleomorphs reproduce sexually, whereas anamorphs reproduce asexually and a particular species can have both a teleomorphic and anamorphic state (Lacey, 2016). Currently teleomorphs and anamorphs of the same species may be classified as separate species, but this is being resolved through molecular phylogenetic techniques (Kepler *et al*., 2014). For example, *Metarhizium anisopliae sensu lato* (in the broad sense) was reclassified in 2009 into nine separate species through phylogenetic analysis (Bischoff *et al*., 2009). The authors sequenced multiple housekeeping genes and investigated morphology to identify differences within the complex (Bischoff *et al*., 2009). The commercially important strain F52 was formerly classified as *M. anisopliae*, but was reclassified to *M. brunneum*. The families *Clavicipitaceae* and *Ophiocordycipitaceae* contain species that can use animals, plants or other fungi as a nutrition source, whereas species in the family *Cordycipitaceae* can only obtain nutrition from arthropods (Humber, 2008). The main entomopathogenic species in these families include (Lacey, 2016):

'***Clavicipitaceae***. Teleomorphs: *Hypocrella*, *Metacordyceps*, *Regiocrella*, *Torrubiella*. Anamorphs: *Aschersonia*, *Metarhizium*, *Nomuraea*, some *Paecilomyces*-like fungi excluded from *Isaria s.s*.'

'***Cordycipitaceae***. Teleomorphs: *Cordyceps*, *Torrubiella*. Anamorphs: *Beauveria*, *Microhilum*, *Engyodentium*, *Isaria*, *Mariannaea*-like species, *Lecanicillium*, *Simplicillium*.'

'***Ophiocordycipitaceae***. Teleomorphs: *Ophiocordyceps*, *Elaphocordyceps*. Anamorphs: *Haptocillium*, *Harposporium*, *Hirsutella*, *Hymenostilbe*, some *Paecilomyces*-like species, *Paraisaria*, *Sorosporella*, *Synglíocladium*, *Tolypocladium*.'

Many of the anamorphic entomopathogenic Hypocreales can be readily mass produced and they are also amenable to formulation and application using conventional spray technologies, so they are popular choices for biopesticides (Feng *et al*., 1994b; Kassa *et al*., 2008). In addition, their conidia can persist in the environment and may infect insects at a later time (Lomer *et al*., 2001). Furthermore, it has been shown that secondary infection by infected insects is an important aspect of pest control with biopesticides (Thomas *et al*., 1995). Because EPF infect through the host cuticle they are also useful as control agents of sap feeding pests that would not acquire pathogens that infect via ingestion. At present, all commercial

biopesticides based on EPF come from a relatively small number of genera within this group: Beauveria, Metarhizium, Isaria, Lecanicillium (de Faria and Wraight, 2007). Strains are selected as biopesticides if they have characteristics such as high virulence against the target pest and the ability to be mass produced (Feng *et al*., 1994).

Most of the teleomorphic species within the EPF Hypocreales cannot yet be grown in culture easily, in part due to their restricted host range (Hesketh *et al*., 2010) and so are not used as biopesticides. One exception is *Cordyceps militaris*, which can be grown in culture relatively easily (see Section 1.6 for more details). However, they are increasingly being studied because of their role as natural regulators of insect populations, and in particular because of their adaptations to their host life cycles, which can also involve manipulation of host behaviour to improve fungal infection and transmission. For example, in tropical rainforests *Ophiocordyceps unilateralis* infects and manipulates the behaviour of ants and it is being used as a model to understand the diversity of fungal interactions in tropical forests (Evans *et al*., 2011).

1.4.1 Use of anamorphic hypocrealean EPF as biopesticides in IPM

Anamorphic hypocrealean EPF are employed as biopesticides as part of IPM, providing pest control with minimal risk to the environment. They are not thought to pose a high risk to beneficial organisms (Garrido-Jurado *et al*., 2011), although low-level infection of non-target species has been observed in the field (Zimmermann, 2007a; Zimmermann, 2007b). Different fungal strains have different host specificities, so strain selection impacts on the risk posed to non-target species (Zimmermann, 2007a).

In 2007 *Beauveria bassiana* accounted for 33.9% of commercial EPF biopesticides while *M. anisopliae s.l.* accounted for 35.7% (de Faria and Wraight, 2007). EPF products are predominantly used for control of greenhouse pests, but have also been successfully used in the field. For example, *M. anisopliae s.l.* has been used to control the leaf sugarcane spittlebug (*Mahanarva posticata*) in Brazil giving between 30-60% control (Li *et al*., 2010). There are currently four biopesticide products based on EPF that are registered for use in the U.K (Table 1.2), these are based on either *B. bassiana*,

M. brunneum or *Lecanicillium muscarium*. Most are used predominantly in protected environments against a limited range of pests including glasshouse whitefly (*Trialeurodes vaporarum*) and western flower thrips (*Frankliniella occidentalis*).

Table 1.2- EPF authorized as biopesticides in the U.K. Strains of EPF registered for use in the U.K, from the Pesticides Register Database, Health and Safety executive (2018). Information on the target species obtained from the product label.

Strain	Product	Marketing Company	Target	Examples of Crops
Beauveria bassiana ATCC-74040	Naturalis-L	Fargro Ltd (U.K)	Glasshouse whitefly (*Trialeurodes vaporariorum*) and western flower thrips (*Frankliniella occidentalis*)	Protected edibles and protected ornamentals
Beauveria bassiana GHA	Botanigard WP	Certis (USA)	Glasshouse whitefly (*Trialeurodes vaporariorum*)	Protected ornamentals, protected aubergine, protected cucumber and protected squash
Metarhizium brunneum	Met 52	Fargro Ltd (U.K)	Black vine weevil (*Otiorhynchus* spp.)	Ornamentals, blackberry, blackcurrant, strawberry, protected cucumber, garlic, onion and protected aubergine
Lecanicillium muscarium	Mycotal	Koppert BV (The Netherlands)	Glasshouse whitefly (*Trialeurodes vaporariorum*), western flower thrips (*Frankliniella occidentalis*) and two-spotted spider mite (*Tetranychus urticae*)	Protected aubergine, protected cucumber, protected lettuce and protected broad bean

EPF are more sensitive to environmental conditions (temperature, water availability, UV light) than traditional synthetic pesticides, which has been found to impact their efficacy in the field and this has limited their adoption for pest control. The influence of temperature on EPF has been widely investigated and it has been found to impact their germination and growth in a strain-dependent manner (Fargues *et al*., 1997; Ouedraogo *et al*., 1997; Yeo *et al*., 2003). EPF have an optimum temperature for

growth, with growth rates reducing above or below this value and growth being inhibited at extreme temperatures (Fargues *et al*., 1997; Ouedraogo *et al*., 1997; Yeo *et al*., 2003). Temperature also affects the susceptibility of the host insect and the condition of the plant being fed upon, which may further impact EPF virulence (Thomas and Blanford, 2003). Humidity is another factor that has been found to affect EPF, with conidia requiring high humidity for germination (Gillespie and Crawford, 1986). UV radiation affects EPF germination in the field, with different strains having different UV tolerances (Braga *et al*., 2001a; Braga *et al*., 2001b; Fernandes *et al*., 2007). There are opportunities to select or breed strains of EPF with good tolerance to these environmental stressors and there has also been progress in genetic engineering strains for this purpose (Fang *et al*., 2005; Fang *et al*., 2009; Shang *et al*., 2012).

When using EPF for pest management there is often a lag between treatment and population reduction (Johnson and Goettel, 1993; Jaros-Su *et al*., 1999; Benjamin *et al*., 2002; Peng *et al*., 2008). This lag and a lower lethality in comparison to chemical pesticides are current limitations of their use as biopesticides. However, their use in conjunction with other treatments as part of an IPM could help give a high level of control through synergistic interactions (expanded on in Chapter 6). The potential of these synergistic interactions has been explored in some cases, but more detailed research is needed. For example, imidacloprid was found to act synergistically with *B. bassiana* and *M. anisopliae s.l.* to increase mortality of citrus root weevil (*Diaprepes abbreviatus*), a pest of citrus fruit (Quintela and McCoy, 1998). But other biopesticides have been found to act antagonistically, for example Shapiro-Ilan *et al*., (2004) observed antagonistic relationships between entomopathogenic nematodes and entomopathogenic fungi on mortality of pecan weevil (*Curculio caryae*).

1.5 Hypocrealean Entomopathogenic fungi; fungal life cycle and mechanisms of infection of arthropod hosts

Hypocrealean EPF are important for use as biopesticides in IPM, as such, it is important to understand their biology. Broadly the life cycle of these EPF is as follows (Figure 1.3); firstly, conidia adhere to the insect and then in favourable conditions they germinate and penetrate the host cuticle using an infection peg. The EPF then proliferates within the host, eventually killing the insect. Following this, the EPF grows externally, producing conidia which may then disperse thorough wind, rain or insect activity (Meyling and Eilenberg, 2007). The EPF may also exhibit endophytic interactions that could aid in the infection of insects. This has been best characterised for *B. bassiana* and *M. anisopliae s.l.* (Section 1.5.4).

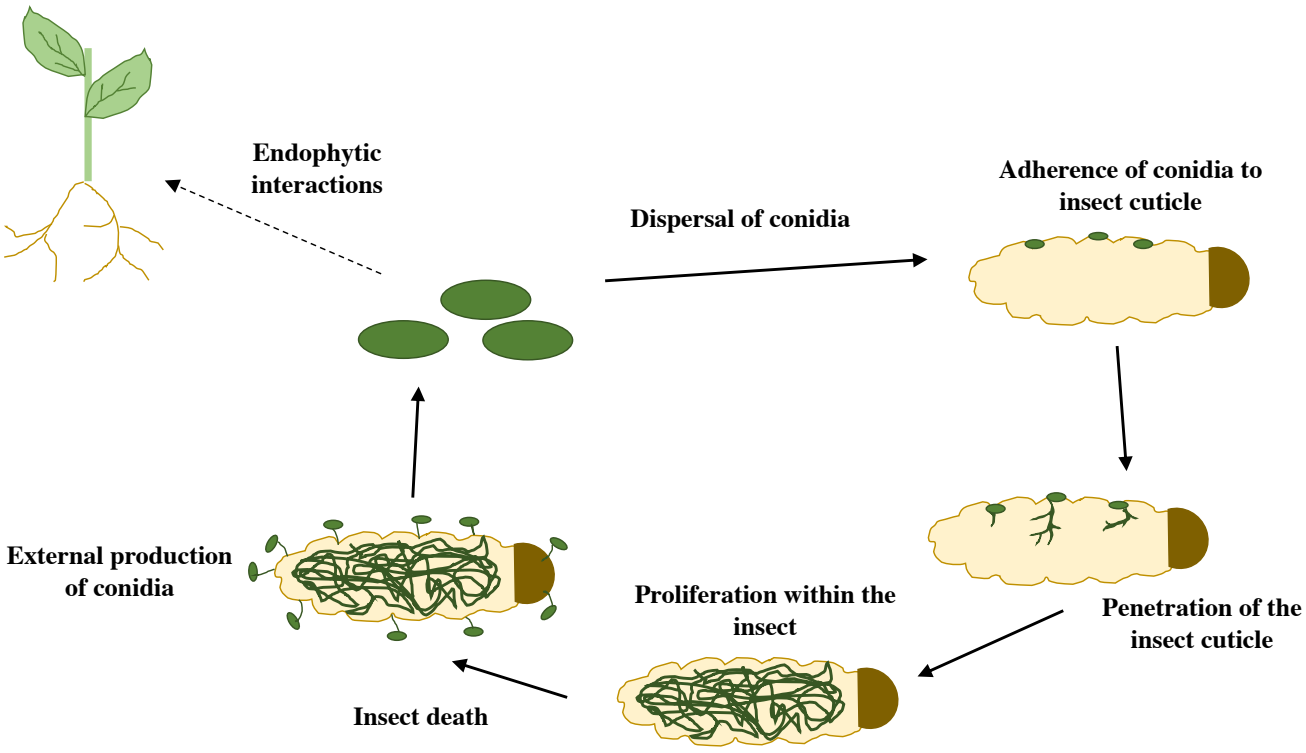


Figure 1.3- Life cycle of EPF. The lifecycle of a typical hypocrealean EPF beginning with adherence of fungal conidia to the host insect, followed by germination of the conidia and penetration of the cuticle. The EPF then proliferate in the insect, depleting the nutrients in the host ultimately causing its death. Some EPF such as *Metarhizium anisopliae s.l.* and *Beauveria bassiana* also exhibit endophytic interactions (Section 1.5.4).

1.5.1 Attachment of conidia to the host integument and penetration of the cuticle

Insects are infected by EPF following adherence of conidia (asexual spores) to the cuticle (Hajek and St. Leger, 1994). This cuticle is a major barrier to infection composed of the epicuticle and procuticle which contains multiple layers of chitin and proteins, it is also coated with a waxy lipid-rich outer layer (Andersen, 1979; Vincent and Wegst 2004). In order to infect an insect, the EPF must first pass through the cuticle which is achieved using a combination of mechanical and chemical processes.

Under favourable conditions EPF conidia will produce structures to penetrate the host cuticle and secrete degrative enzymes. Many EPF such as *Metarhizium* spp. develop appressoria, which are specialised cells that adhere to the cuticle. These cells produce infection pegs that use high turgor pressure to penetrate the cuticle (Howard *et al*., 1991) and allow focused release of cuticle-degrading enzymes (Hajek and St. Leger, 1994). Chitinases and proteases are particularly important for degrading the cuticle (Ortiz-Urquiza and Keyhani, 2013) and have been shown to be important virulence determinants in *B. bassiana* and *M. anisopliae s.l.* For example, these EPF have been engineered to overexpress or express more effective proteases or chitinases and this increased their virulence (Fan *et al*., 2007; Zhang *et al*., 2008; Boldo *et al*., 2009; Fang *et al*., 2009). Proteases are believed to be more important than chitinases because chitin in the cuticle is surrounded by protein which must first be degraded to permit chitin degradation (Blackwell and Weih, 1980; Fang *et al*., 2009).

1.5.2 Growth of EPF within the host insect

Following penetration of the cuticle, EPF need to evade or subvert the cellular immune response. To do this they change morphology in the haemocoel to become blastospores. These cells lack cell wall components such as β-1,3-glucan, which may help prevent recognition by the insect immune system (Jiang *et al*., 2010). In the case of *M. anisopliae s.l.* it was found that the MC1 protein allows immune subversion, as blastospores lacking this protein are rapidly attacked by haemocytes. It is thought that MC1 prevents adhesion of haemocytes as it hides the cell wall structure (Wang and St. Leger, 2006). In some cases, EPF blastospores may still be recognised by the insect immune system and encased in nodules or phagocytosed. It has been reported that *B.*

bassiana blastospores are still be able to germinate within *Spodoptera exigua* haemocytes, whereas fungi that are not pathogenic to insects (*Candida albicans*) are not able to grow as rapidly under these conditions (Hung *et al*., 1993).

EPF also produce a number of compounds in the insect that interfere with its immune system, particularly secondary metabolites, some of which are thought to aid in the infection process (Zimmermann, 2007a). The function of secondary metabolites is discussed in detail in Section 1.8, but in general they are thought to facilitate host infection and colonisation while repressing the growth of other microorganisms (Zimmermann, 2007a). Insects produce anti-microbial peptides (AMPs) in response to infection (Section 1.7.2.4), but EPF may be resistant to their activity. For example, while cecropins inhibit the growth of *Sacchromyces cerevisiae*, *Dipodascopsis uninucleate* and *Geotrichum candidum*, some *B. bassiana* strains are entirely resistant to this AMP (Ekengren and Hultmark, 1999). Also, destruxin A (a secondary metabolite produced by *Metarhizium* spp.) suppresses the expression of some AMPs in *Drosophila melanogaster* (Pal *et al*., 2007), which may allow *Metarhizium* spp. to colonise the host more rapidly. There is also evidence the EPF can directly sense and respond to AMPs during infection; in culture *Metarhizium robertsii* has been observed to synthesise proteolytic enzymes at higher levels when the anti-fungal AMP Metchnikowin is present in the culture medium, compared to control medium (Mukherjee and Vilcinskas, 2018).

1.5.3 Host death and EPF transmission

Host death following EPF infection is caused by the EPF consuming nutrients from the insect's haemolymph or fat body and producing toxins (Zimmermann, 2007a). The time taken for insect death to occur depends on multiple factors including: species of the host, condition of the host, environmental conditions and strain of the fungus (Zimmermann, 2007b). Following host death, the EPF grows outside the host and produces conidia or ascospores (sexual spores) that can infect other insects. Insects can come into contact with the conidia directly from the infected insect cadaver or in the environment if the conidia have dispersed *e.g*. on plant surfaces or in the soil (Hesketh *et al*., 2010).

Hypocrealean EPF are semelparous parasites that must kill the host insect in order for transmission to occur (Ebert and Weisser, 1997; Chouvenc and Su, 2012), because of this there are complex interactions between the host and parasite during infection. It is thought that there is a trade-off between virulence and transmission of EPF. A higher virulence would be beneficial for initial infection, but would cause premature death of the host insect (Boomsma *et al*., 2014). A longer period of infection would result in a greater accumulation of EPF biomass and hence more conidia being produced after host death (Ebert and Weisser, 1997).

1.5.4 Endophytic interactions of EPF

Some EPF such as *M. anisopliae s.l.* and *B. bassiana* have been found to be associated with plants. Infection of plants with *B. bassiana* can help protect the plant against herbivorous insects (Bing and Lewis, 1991; Meyling and Eilenberg, 2007) and this fungal species has been observed as an endophyte in many species including cocoa (Posada and Vega, 2005), poppy (Quesada-Moraga *et al*., 2006), tomato and cotton (Ownley *et al*., 2008). *Metarhizium anisopliae s.l.* has also been found to be endophytic and is particularly associated with plant roots (Meyling and Eilenberg, 2007). This has been observed in tomato (Sasan and Bidochka, 2012), oilseed rape (Batta, 2013) and broad bean (Jaber and Enkerli, 2016). In light of the discovery of these endophytic interactions, it has been suggested that association with plants is an important part of the EPF lifecycle, especially for *B. bassiana* and M. *anisopliae s.l.* (Meyling and Eilenberg, 2007).

1.6 Biology of *Cordyceps militaris*

C. militaris is an EPF in the family *Cordycipitaceae* (Figure 1.2) that is predominantly a pathogen of Lepidoptera (Shrestha *et al*. 2012b). It is distributed throughout the northern hemisphere (Zheng *et al*., 2011), but most notably occurs in East Asia. Here it is used as a traditional herbal medicine, purportedly having lung and kidney benefits (Das *et al*., 2010b). More recently, *C. militaris* and its chemical constituents have been investigated for their medicinal properties (summarised in Table 1.3). The focus of this project is cordycepin (3'-deoxyadenosine, Section 1.8.4). Due to its relevance to the pharmaceutical industry a number of mass production techniques have been developed to culture large quantities of *C. militaris* relatively cheaply (Kim *et al*., 2003; Masuda *et al*., 2007; Das *et al*., 2008; Das *et al*., 2010a).

Table 1.3- Medicinal effects of *Cordyceps militaris* and its products.

Medicinal effect	Effective *C. militaris* constituent	Reference
Anti- angiogenic	Whole fungal extract	Yoo *et al*., (2004)
Anti-ageing	Extracted polysaccharide	Yu *et al*., (2007)
Anti-bacterial	Nucleoside analogue (Cordycepin)	Ahn *et al*., (2000)
Anti-fungal	Peptide (Cordymin)	Wong *et al*., (2011)
Anti-tumour	Whole fungal extract	Zhao-Long *et al*., (2000)
Hepatoprotective	Whole fungal extract	Jung *et al*., (2004)
Immunomodulatory	Whole fungal extract	Shin *et al*., (2010)

C. militaris is able to produce both sexual (ascospores) and asexual (conidia) spores. Conidia are produced from phialides (specialised cells that produce conidia) and ascospores from the club-shaped fruiting body (Shrestha *et al*., 2012b). This fungus has been observed to be both heterothallic and homothallic, although heterothallism is thought to predominate (Shrestha *et al*., 2012b). Heterothallic individuals require a partner of a different mating type to reproduce sexually, whereas a homothallic individual can reproduce sexually without a partner. The *C. militaris* genome is haploid and there are different mating types, of which only certain combinations are compatible (Shrestha *et al*., 2004; Yokoyama *et al*., 2006; Zheng *et al*., 2011). Homothallic strains have been occasionally observed (Shrestha *et al*., 2004; Shrestha *et al*., 2012b), which may be due to mating type switching, the production of

heterokaryotic cells, production of diploids or two compatible mating types appearing on the chromosomes (Shrestha *et al*., 2012b).

1.7 The role of the insect immune system in defence against microbial infection

Extensive homologies exist between the innate immune response in vertebrates and the humoral response in invertebrates (Kimbrell and Beutler, 2001). Because of this, insects have been used to elucidate human immune pathways and to assess the impact of human pathogens on the innate immune system (Kimbrell and Beutler, 2001; Ramarao *et al*., 2012).

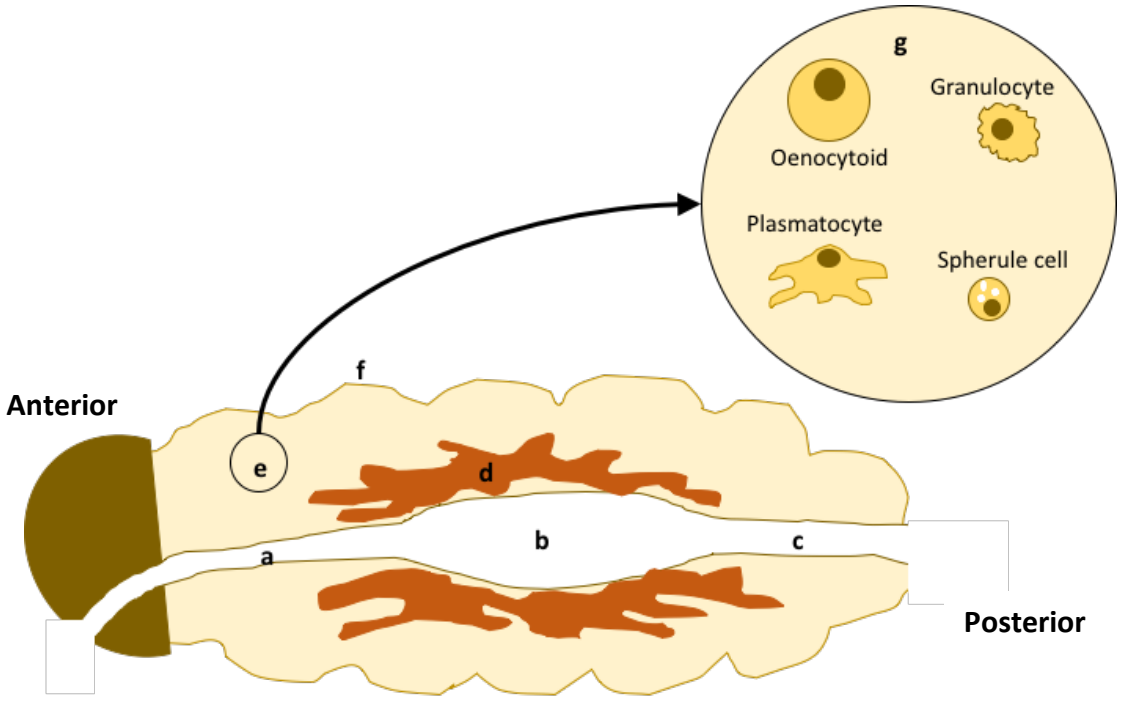


Figure 1.4- Outline of insect anatomy. Overview Lepidoptera physiology showing the a) foregut, b) midgut, c) hindgut, d) fat body, e) haemolymph, f) cuticle and g) haemocytes. Adapted from Strand, (2008).

Insects defend themselves against infection by microorganisms present in the environment and have evolved complex immune responses to do this. These responses can be broadly classified into two categories, the humoral response and the cellular response. Although the broad principles of the immune response to EPF are known (Pal *et al*., 2007; Wojda *et al*., 2009) the details are yet to be elucidated. These include differences in the immune signalling pathways and effectors between different insect

groups (Strand, 2008). One of the main knowledge gaps concerns how the EPF is able to overcome the insect immune system.

1.7.1 The cellular immune response of insects

The cellular immune response is mediated by haemocytes; blood cells that reside in the insect's haemolymph (Lemaitre and Hoffmann, 2007). In *Drosophila melanogaster* there are predominantly three types of haemocytes: plasmatocytes are the most numerous (~90%), followed by crystal cells (<5%) and lamellocytes (rarely observed in healthy insects) (Lanot *et al*., 2001). The main role of plasmatocytes is to phagocytose dead cells and pathogens (Lemaitre and Hoffmann, 2007) and if a pathogen or object is too large to be phagocytosed it can be encapsulated by lamellocytes. Crystal cells are involved in the melanisation pathway (Lemaitre and Hoffmann, 2007), which is described in detail in Section 1.7.2.6.

In Lepidoptera there are four types of haemocytes (Figure 1.4): granulocytes, oenocytoids, spherule cells and plasmatocytes (Strand, 2008). Granulocytes are the most numerous (Strand, 2008) and are thought to be responsible for phagocytosis of pathogens (Strand *et al*., 2006). Oenocytoids are thought to be involved in the melanisation pathway (Lavine and Strand, 2002), while spherule cells may transport integument peptides to the cuticle (Sass *et al*., 1994). The main role of plasmatocytes in Lepidoptera is to encapsulate pathogens and foreign objects (Lavine and Strand, 2002) which contrasts with their role in *D. melanogaster* where they mainly phagocytose pathogens.

1.7.2 The humoral immune response in insects

The humoral response in insects involves the release of soluble effector proteins into the haemocoel in response to infection (Strand, 2008). These include anti-microbial peptides (AMPs), small peptides mainly produced in the fat body (Lemaitre and Hoffmann, 2007) following activation of either the Toll or IMD pathways by fungi or bacteria (Section 1.7.2.1). The prophenol-oxidase cascade (Section 1.7.2.6) can also be categorised as part of the humoral response as during this reaction, enzymes are released into the haemocoel to drive melanisation and clotting. Finally, the Jak-

STAT pathway is stimulated by stress, tissue damage and viral infection, it results in the production of anti-viral proteins and cell signalling molecules (Section 1.7.2.5).

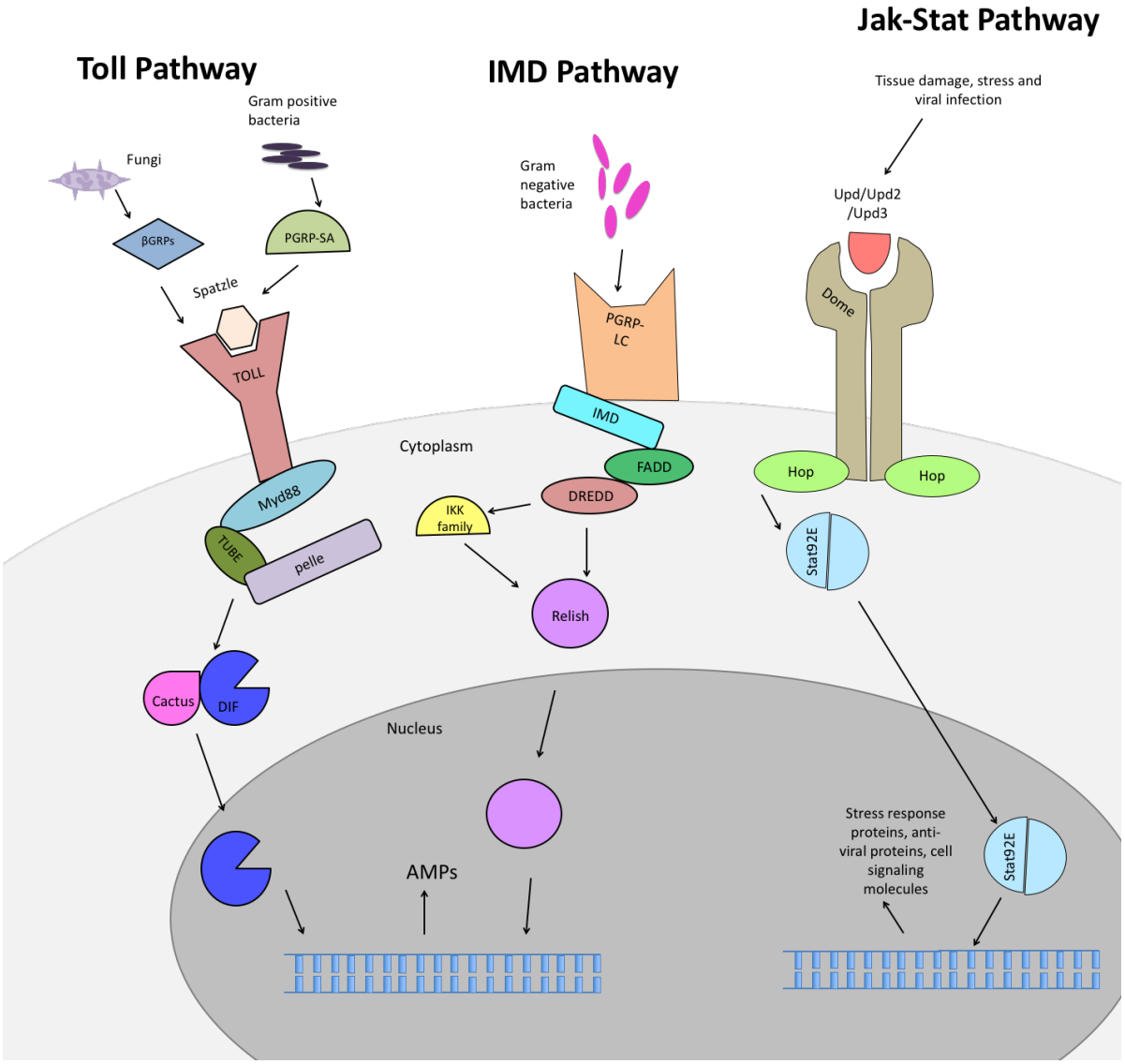


Figure 1.5- Diagram illustrating the humoral immune response in insects. Fungi and gram-positive bacteria are detected by the Toll pathway. Fungal PAMPs are detected by β-GRPs and gram-positive bacteria PAMPs by PGRP-SAs. These then bind to the Toll receptor via the protein Spatzle. This activates a protein cascade in the cytoplasm that ends in DIF being released from the inhibitory protein Cactus. DIF then diffuses into the nucleus where it acts as a transcription factor activating AMPs. Gram-negative bacteria are detected by the PGRP-LC receptor, which triggers a protein cascade in the cytoplasm that ends in the activation of the transcription factor Relish. Relish then diffuses into the nucleus and activates transcription of AMPs. The Jak-Stat pathway detects Upd/Upd2/Upd3, which are expressed in response to tissue damage or viral infection. This triggers a cascade that activates the transcription factor Stat92E which causes expression of stress response proteins, anti-viral proteins or cell signaling molecules. Adapted from Lemaitre and Hoffmann, (2007) and Myllymäki and Rämet, (2014).

1.7.2.1 The Toll pathway

The Toll pathway (Figure 1.5) responds to fungi and gram-positive bacteria to trigger the transcription of AMPs (these are described in more detail in Section 1.7.2.4) and other immune-related genes (Ganesan *et al*., 2010). This pathway has been best characterised in *D. melanogaster* which has been adopted as a model organism to elucidate insect immune responses and improve understanding of the innate immune response in humans. This pathway is activated through recognition of peptidoglycans (PGN) by PGRP-SA (Michel *et al*., 2001), PGRP-SD (Leone *et al*., 2008) or GNBP1 proteins (Rolff and Reynolds, 2009; Ganesan *et al*., 2010). It is also activated by fungal cell wall components; for example the receptor GNBP3 recognises β-(1,3)-glucans (Gottar *et al*., 2006). EPF avoid recognition by GNBP3, possibly by evasion of this receptor or inhibition of the Toll pathway, but are thought to trigger the Toll pathway through recognition of virulence factors (Gottar *et al*., 2006; El Chamy *et al*., 2008). For example, the enzyme PR1A, produced during penetration of the host cuticle by *M. anisopliae s.l.* (Leger *et al*., 1989), activates the Toll pathway independently of GNBP3 (Gottar *et al*., 2006).

Activation of the Toll pathway following pathogen associated molecular pattern (PAMP) recognition triggers a cascade that results in the degradation of Cactus, a protein that prevents the transport of the transcription factors DIF and Dorsal (in the NFκB transcription factor family) into the nucleus (Ganesan *et al*., 2010). Following Cactus degradation DIF or Dorsal can dimerise and activate the transcription of immune-related genes in the nucleus. In *D. melanogaster* DIF and Dorsal have redundant functions in the immune response (Manfruelli *et al*., 1999; Meng *et al*., 1999). In adult *D. melanogaster*, DIF alone appears to respond to fungal or gram-positive bacterial infection (Rutschmann *et al*., 2000). In other insect species there appears to be a loss of one of these genes; *Bombyx mori* and *Anopheles gambiae* have only one gene from the *dorsal/DIF* gene family (Tanaka *et al*., 2008).

1.7.2.2 The IMD pathway

The IMD pathway (Figure 1.5) responds mainly to gram-negative bacteria through the detection of PGNs containing diaminopimelic acid (DAP). It is detected by the receptors PGRP-LC and PGRP-LE (Choe *et al*., 2002; Takehana *et al*., 2004), which trigger a cascade that results in activation of the IKK signalling complex and Dredd (Lemaitre and Hoffmann, 2007). IKK is thought to phosphorylate Relish (a transcription factor in the NFκB family), which may trigger its cleavage by the caspase-like protein Dredd (Stöven *et al*., 2003). This cleavage removes an N-terminal domain from Relish, which enables it to dimerise (Han and Ip, 1999) and be transported to the nucleus to drive the transcription of immune-related genes, in association with other co-activator proteins (Goto *et al*., 2008).

1.7.2.3 Cross-talk between immune pathways

The humoral immune response in insects has been found to be more complicated than initially believed, as cross-talk has been identified between different pathways. This cross-talk is not fully understood but has been shown to occur in response to certain bacteria or fungi. For example, the fungus *Geotrichum candidum* activates the IMD pathway (normally activated by gram-negative bacteria) in *D. melanogaster* to trigger AMP expression (Hedengren-Olcott *et al*., 2004). Furthermore, the expression of some AMPs may be controlled by both the Toll and IMD pathways, for example *cecropin* (an anti-bacterial AMP) expression may be triggered by either pathway (Han and Ip, 1999).

There is also evidence that DIF, Dorsal and Relish can form heterodimers with each other (Han and Ip, 1999; Tanji *et al*., 2010). DIF-Relish heterodimers have been detected in the *D. melanogaster* fat body and haemocytes (Tanji *et al*., 2010). It is thought that DIF-Relish dimers can efficiently drive *drosomycin* (anti-fungal AMP) transcription and Dorsal-Relish dimers can drive *defensin* (AMP active against gram-positive bacteria) transcription (Han and Ip, 1999). However, the regulation of these heterodimers is not clear. For example, it has not been resolved whether both Toll and

IMD pathways need to be activated simultaneously to allow Cactus degradation and Relish inhibitory domain cleavage (Tanji *et al*., 2010).

1.7.2.4 The role of AMPs in insect immunity

AMPs are well characterised effector molecules in the immune response to pathogens. They are small peptides (typically less than 10kDa) with anti-bacterial or anti-fungal activity (Lemaitre and Hoffmann, 2007). AMPs vary greatly between insect taxa as there has been rapid evolution of these genes, with different taxa often having unique AMPs (Juneja and Lazzaro, 2009).

In *D. melanogaster* 20 AMPs have been identified, which are classified into seven groups (Table 1.4). The other species used in this project (*Galleria mellonella* and *Plutella xylostella*) also produce AMPs (Table 1.4), some of which show homology to those in *D. melanogaster*, *e.g*. Cecropins and Defensins. *G. mellonella* and *P. xylostella* are pest species in the order Lepidoptera and are described in detail in later chapters. Although some of these AMPs are homologous, they may have activity against different pathogens (Table 1.4). AMPs are predominantly produced in the insect fat body then secreted into the haemolymph where they exert their antimicrobial activity (Meister *et al*., 1997).

Table 1.4- AMPs of model insect species. The groups of AMPs present in different insect species and the microbes that they are active against.

Species	AMP	Activity	Reference
D. melanogaster	Attacin	Gram-negative bacteria, fungi	Lemaitre and Hoffmann, (2007) De Gregorio *et al*., (2002)
D. melanogaster	Cecropin	Gram-negative bacteria	Lemaitre and Hoffmann, (2007)
D. melanogaster	Defensin	Gram-positive bacteria	Lemaitre and Hoffmann, (2007)
D. melanogaster	Diptericin	Gram-negative bacteria	Lemaitre and Hoffmann, (2007)
D. melanogaster	Drosocin	Gram-negative bacteria	Lemaitre and Hoffmann, (2007)
D. melanogaster	Drosomycin	Fungi	Lemaitre and Hoffmann, (2007)
D. melanogaster	Metchnikowin	Fungi	Lemaitre and Hoffmann, (2007)
G. mellonella	Cecropin	Gram-negative bacteria and Gram-positive bacteria	Wojda, (2016)
G. mellonella	Defensin	Fungi	Wojda, (2016)
G. mellonella	Galiomicin	Fungi	Lee *et al*., (2004)
G. mellonella	Gallerimycin	Fungi	Langen *et al*., (2006)
G. mellonella	Gloverin	Gram-negative bacteria and Gram-positive bacteria	Cheng *et al*., (2006)
G. mellonella	Moricin-like peptide	Fungi, Gram-negative bacteria and Gram-positive bacteria	Wojda, (2016)
P. xylostella	Cecropin	Gram-negative bacteria	Xiaofeng *et al*., (2015)
P. xylostella	Gloverin	Gram-positive bacteria	Xiaofeng *et al*., (2015)
P. xylostella	Moricin	Gram-negative bacteria	Xiaofeng *et al*., (2015)

1.7.2.5 The Jak-Stat pathway

The Jak-Stat pathway (Figure 1.5) has been best characterised in *D. melanogaster*, however some aspects of its function remain unknown. It is activated by Upd, Upd2 or Upd3 and the expression of these proteins is induced by tissue damage and viral infection (Myllymäki and Rämet, 2014). These ligands bind to the external domain of a Dome dimer, which interacts with Hop (in the Jak family). This then phosphorylates and activates the transcription factor STAT92E, (Agaisse and Perrimon, 2004) which upregulates the expression of genes including *tep1* and *totA* (Agaisse and Perrimon, 2004).

The expression of *totA* is increased by septic injury through activation of the Jak-Stat pathway and it appears to protect against heat stress (Agaisse *et al*., 2003). Tep1 is involved in promoting phagocytic activity in mosquito (*Anopheles gambiae*) haemocytes (Levashina *et al*., 2001) and this may be its function in other insects. Another feature of Jak-Stat activation is that it promotes differentiation of lamellocytes (Agaisse and Perrimon, 2004), although the mechanism behind this is unknown. This pathway also responds to viral infection, although the mechanism behind this is not known, it along with other immune pathways induce *vir-1* expression (Dostert *et al*., 2005). VIR-1 is a protein induced by viral infection, but its function is also not known (Myllymäki and Rämet, 2014).

1.7.2.6 The prophenol-oxidase cascade

Phenol-oxidase (PO) is initially synthesised as the inactive precursor prophenol-oxidase (PPO). It is converted to its active form through the PPO cascade, which is primarily mediated by crystal cells in *D. melanogaster* and oenocytoids in Lepidoptera. Release of PPO from crystal cells/oenocytoids is triggered by recognition of PAMPs (Cerenius *et al*., 2008) or tissue damage (Galko and Krasnow, 2004). In the haemolymph PPO is cleaved to PO by a serine protease cascade and active PO drives the reactions that cause melanisation. This is the formation of melanin around a foreign body or wound, which appears as a dark pigment in the affected insect (Gillespie and *et al*., 1997) (Figure 1.6).

It is thought that the function of melanisation is to facilitate wound healing by stimulating clot formation and this also isolates pathogens (Gillespie and *et al*., 1997). PO produces other toxic compounds that may adversely affect the pathogen (Cerenius *et al*., 2008). These anti-microbial chemicals include 5, 6-dihydroxyindole (DHI) and quinones (Zhao *et al*., 2007). DHI has anti-bacterial, anti-fungal and antiviral activity and also has activity against parasitic wasps (Zhao *et al*., 2011). In addition, the PPO cascade produces peroxinectin, an opsonin that binds to pathogens to promote phagocytosis by haemocytes (Cerenius *et al*., 2008).

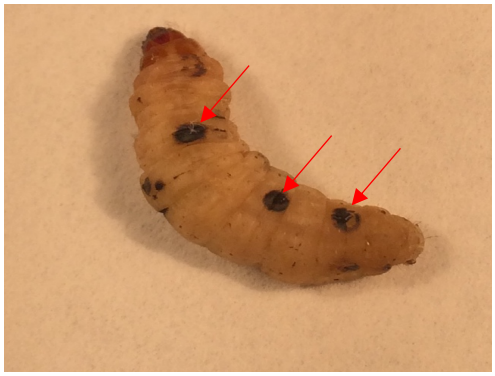


Figure 1.6- Melanisation in *Galleria mellonella*. Arrows indicate patches of melanisation in *G. mellonella* caused by penetration of *B. bassiana* conidia.

1.7.3 The role of lysozyme in insect immunity

Lysozyme is an enzyme that cleaves the β-1,4-glycosidic bonds in the peptidoglycan cell wall of bacteria (Jollès and Jollès, 1984) and is a key component of the immune response in certain insects. It is also thought to have anti-fungal properties, but the mechanism behind this is less clear. High concentrations of human lysozyme kills *Candida albicans*, possibly through cell wall interactions (Wu *et al*., 1999), and insect lysozymes may display a similar activity. Evidence of the anti-fungal activity of insect lysozyme was found by Wojda *et al*., (2009). They monitored the activity of this enzyme in *G. mellonella* in response to *B. bassiana* infection and found that increasing fungal concentration caused increased lysozyme activity (Wojda *et al*., 2009). The mechanism of lysozyme activity against fungi is not well understood, but it is thought

to promote apoptosis of fungal cells in *G. mellonella* (Sowa-Jasiłek *et al*., 2016). There is also evidence that lysozyme from *G. mellonella* may work synergistically with AMPs such as cecropins to kill pathogens (Cytryńska *et al*., 2001).

In contrast to other insects, lysozyme expression in *Drosophila* spp. is not thought to be associated with immunity. Daffre *et al*., (1994) monitored expression of *lysozyme* in *D. melanogaster* and determined that its expression is localised to the digestive system, in particular the gut and salivary glands. In addition to this it was not found to be expressed in the fat body or by haemocytes, therefore in *D. melanogaster* lysozyme is thought to be mainly involved in digestion of bacteria (Daffre *et al*., 1994). In fact there is evidence that *lysozyme* genes have diverged in Diptera to function predominantly as digestive enzymes (Regel *et al*., 1998).

1.7.4 The wounding response in insects

The response to wounding in insects integrates multiple pathways to prevent infection and promote wound healing. Understanding this response is particularly important as insect bioassays often introduce bacteria or fungi through injection and the immune response to wounding can complicate interpretation of the bioassay results (Erler *et al*., 2011).

A principal response to wounding is the activation of the PPO cascade (Section 1.7.2.6) and components of this pathway are involved in the formation of a scab over the wound. The role of the PPO cascade in wound healing varies between species, in *D. melanogaster* the PPO response is localised whereas in *G. mellonella* PPO activation is more systemic (Bidla *et al*., 2009). The JAK-STAT pathway (Section 1.7.2.5) also responds to wounding. Sterile or septic injury increases *totA* expression (Agaisse *et al*., 2003); TotA is part of the general stress response and its expression is also triggered by bacterial infection, heat stress and UV light (Ekengren and Hultmark, 2001). Finally, wounding triggers the production of AMPs (Section 1.7.2.4). In the bumblebee *Bombus terrestris* wounding induced the expression of three AMPs, but at lower levels than that caused by a bacterial challenge (Erler *et al*., 2011). This was also the case for *D. melanogaster*, where injury increased expression of five AMPs at

lower levels than those following bacterial and fungal challenge (Lemaitre *et al*., 1997).

1.7.5 The immune response to EPF in insects

There has been limited investigation into the insect cellular response to EPF infection and most has focused on the response of haemocytes, in particular their phagocytic activity and morphological changes. For example, *B. bassiana* blastospores were seen to evade detection by *Spodoptera exigua* haemocytes (Pendland *et al*., 1993) and *G. mellonella* haemocytes became round and developed swollen nuclei upon *M. anisopliae s.l.* infection (Gillespie *et al*., 2000). Additionally, studies into the humoral signalling pathway in response to fungal infection in insects have not often been done with EPF. Early studies to elucidate the components of the Toll and IMD pathways often used either bacteria or non-insect pathogenic fungi such as *Escherichia coli* or *Aspergillus fumigatus* to elicit an immune response (Lemaitre *et al*., 1996; Ferrandon *et al*., 1998). Since these studies, more research has been done to understand the impact of EPF on humoral immunity. For example, Wojda *et al*. (2009) observed the impact of *B. bassiana* on AMP expression in *G. mellonella* and Vilcinskas and Matha, (1997) observed the impact of this EPF on the lysozyme and antibacterial activity of *G. mellonella* haemolymph. Some EPF secondary metabolites are thought to play a key role in insect infection (Vilcinskas *et al*., 1997c), although this is not well understood. A major knowledge gap is how these metabolites facilitate infection and whether they do this through interaction with the immune system (discussed further in Section 1.8).

1.8 Metabolites of entomopathogenic fungi: role in insect infection and use as novel natural products

1.8.1 Classes of EPF secondary metabolites

Keller *et al*., (2005) describe four classes of secondary metabolites produced by fungi: polyketides (PKs), non-ribosomal peptides (NRPs), terpenes and indole alkaloids. The largest class of metabolites are the PKs and they are synthesised by polyketide synthases (PKSs) (Molnár *et al*., 2010). Oosporein (Figure 1.7) is an example of a nonreduced PK produced by *Beauveria* spp. (Molnár *et al*., 2010), that is thought to disturb membrane integrity (Jeffs and Khachatourians, 1997). The second group of metabolites is the NRPs, which are synthesised by multienzyme complexes called non-ribosomal peptide synthetases (NRPSs) (Moffitt and Neilan, 2000). Destruxins (a group of metabolites of *Metarhizium* spp.) and beauverolides (a group of metabolites of *Beauveria* spp.) are in a subclass of NRPs called cyclic nonribosomal peptides (Molnár *et al*., 2010). They are thought to be involved in the virulence of EPF (Vilcinskas *et al*., 1997c; Vilcinskas *et al*., 1999).

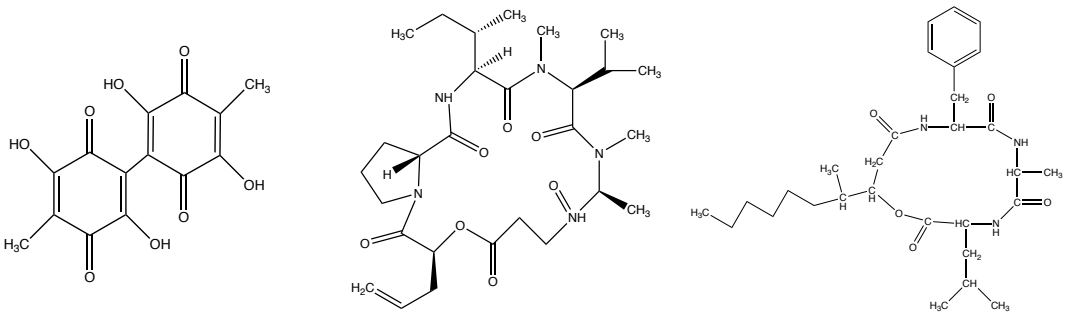


Figure 1.7-Chemical structures of EPF secondary metabolites. Oosporein (left), destruxin A (centre) and beauverolide L (right). Adapted from Suzuki *et al*., (1966), Eyal *et al*., (1994) and Jegorov *et al*., (1994). Made using ChemDraw Professional Ver 16 (PerkinElmer Inc).

Fungal terpenes and indole alkaloids are smaller classes of secondary metabolites and have been less researched than PKs and NRPs. Common terpenes include diterpenes and carotenoids (Keller *et al*., 2005). The most widely investigated indole alkaloid is ergotamine which was first discovered due to its toxicity towards humans, but has since been developed as a medicine for conditions such as migraines (Tfelt-Hansen *et al*., 2000).

Fungal genes encoding for secondary metabolites are often co-located within the genome (Sbaraini *et al*., 2016); the members of these biosynthetic gene clusters (BGCs) are normally co-regulated (Keller *et al*., 2005) and contain different functional classes including PKSs and NRPSs (Sbaraini *et al*., 2016). Clusters can be identified using bioinformatics, particularly through the identification of putative NRPS and PKS genes (Keller *et al*., 2005). This identification has been facilitated by the whole genome sequencing of *B. bassiana* (Xiao *et al*., 2012), *C. militaris* (Zheng *et al*., 2011) and *M. anisopliae* (Gao *et al*., 2011). In *B. bassiana* 13 NRPS genes and 12 PKS genes have been identified (Xiao *et al*., 2012), *M. anisopliae* was found to have 14 NRPS and 24 PKS genes (Gao *et al*., 2011), while 5 NRPS and 9 PKS genes have been identified in *C. militaris* (Zheng *et al*., 2011). This large number of biosynthetic gene clusters suggests that only a small fraction of secondary metabolites from these EPF have been identified so far.

1.8.2 The role of EPF metabolites in insect infection

The current levels of understanding of the natural function of EPF metabolites is poor and it is likely that many metabolites have yet to be discovered. It is difficult to identify secondary metabolites necessary for infection as they may be transiently produced or only produced in low quantities during infection (Soukup *et al*., 2016). Experimentally it is also difficult to determine the effect of individual metabolites, as increasing expression or knocking out the genes to produce one metabolite may have effects on other downstream pathways or there may be redundancy in virulence factors (Stergiopoulos and de Wit, 2009).

Broadly there have been two categories of experiments that have been used to determine the natural function of EPF metabolites. Firstly, there are those which observe the effect of introduction of a metabolite (usually at a high concentration) into either a whole insect or a specific insect tissue/cell type. Mortality, behaviour, morbidity or changes in cell structure can then be monitored to determine the impact of the metabolite. Although this type of experiment does begin to unravel the function of a metabolite, the high concentrations used are unlikely to be representative of what would occur in nature. It is also possible that any results seen are secondary effects

due to the impact of the metabolite on other pathways. The second type of experiment relies on the metabolite synthesis genes having been discovered. These genes can then be knocked out or overexpressed through genetic manipulation and the impact on virulence assessed. This has the advantage of being able to determine whether a metabolite is necessary for virulence but does not establish how the metabolite acts to confer virulence.

The EPF metabolites that have been investigated most thoroughly include those produced by *B. bassiana* and *M. anisopliae s.l.* The *B. bassiana* metabolites bassianolide and beauvericin have been found to be virulence factors that facilitate infection of *G. mellonella*, Corn Earworm (*Helicoverpa zea*) and Beet Armyworm (*Spodoptera exigua*) (Xu *et al.*, 2008; Xu *et al.*, 2009). The genes responsible for the production of these metabolites were knocked out in a strain of *B. bassiana* and resulted in a significant reduction in virulence, although a low level of virulence remained (Xu *et al.*, 2008; Xu *et al.*, 2009). The conclusions drawn from this were that bassianolide and beauvericin contribute to *B. bassiana* virulence in nature, however they may also have other functions. Previous experiments have identified bassianolide and beauvericin as being insecticidal if administered alone (Hamill *et al.*, 1969; Suzuki *et al.*, 1977). Therefore, they could be important in endophytic interactions, as production of insecticidal chemicals by EPF following colonisation of a plant may reduce insect herbivory. Another *B. bassiana* metabolite that has begun to be characterised is beauverolide, which has been found to inhibit the phagocytic activity of *G. mellonella* plasmatoytes, but not cause significant mortality of these larvae (Vilcinskas *et al.*, 1999).

M. anisopliae s.l. also produces secondary metabolites that appear to facilitate infection, the best understood being destruxins. Destruxins have been reported to have multiple activities in insects (Samuels *et al.,* 1988; James *et al.,* 1993; Vilcinskas *et al.*, 1997c; Pal *et al.,* 2007) including effects on the immune system. For example, destruxins A and E have been found to inhibit phagocytic activity of plasmatocytes in *G. mellonella*, and at high doses they cause paralysis and eventual death of larvae (Vilcinskas *et al.*, 1997c). Additionally, these toxins have been found to inhibit attachment and spreading of plasmatocytes (Vilcinskas *et al.*, 1997b). Destruxins A,

B and E also facilitated infection of *Phaedon cochleariae* by *M. anisopliae s.l.* (Amiri *et al.,* 1999). However, this was thought to be due to destruxins causing increased stress in insects, as opposed to a direct impact on the insect immune system. A subsequent study on destruxin A countered this hypothesis as it determined that destruxin A inhibits the IMD immune pathway in *D. melanogaster* (Pal *et al.,* 2007).

1.8.3 Medicinal uses of EPF metabolites

Whilst the natural function of EPF metabolites is not well understood, more research has been carried out into their potential for use as pharmaceuticals. An example of this is cyclosporine, a secondary metabolite produced by *Tolypocladium niveum* (Weber *et al*., 1994), which is used following organ transplant as an immunosuppressant to reduce the risk of organ rejection (Merion *et al*., 1984; Ponticelli *et al*., 1988). Metabolites of EPF that are used commercially for biopesticides have also been investigated for their medicinal potential. Destruxin B (produced by *Metarhizium* spp.) suppresses the expression of a surface antigen of the hepatitis B virus (HBV) and may have potential to be developed into an anti-HBV drug (Chen *et al*., 1997). Beauvericin (produced by *Beauveria* spp.) stimulates the influx of calcium ions into human leukaemia cells, which results in the death of these cells (Chen *et al*., 2006). Therefore the research that has previously been carried out into the medicinal function of metabolites can potentially inform understanding of their natural function.

1.8.4 Cordycepin: A secondary metabolite of *C. militaris*

The nucleoside analogue cordycepin (3'-deoxyadenosine; Figure 1.8) is a secondary metabolite produced by *C. militaris*, which has been found to have a wide range of biological properties (Table 1.5). The majority of work published to date concerns fundamental studies on the effect of cordycepin on transcription in eukaryotes, or on its potential as a novel human medicine. One of the first investigations of cordycepin concerned inhibition of transcription (Chu and Edelman, 1972). This inhibition occurs as cordycepin cannot be distinguished from adenosine by certain enzymes (Figure 1.8). Therefore, during transcription cordycepin is incorporated into the poly(A) tail of mRNA, preventing addition of further adenosine residues and reducing the stability

of the transcript (Kondrashov *et al*., 2012). This poly (A) chain termination appears to primarily affect inflammatory mRNAs rather than housekeeping mRNAs (Kondrashov *et al*., 2012). Although the reason for this is not clear, it may be linked to the relative turnover rates of the mRNAs or the affinity of different classes of poly(A) polymerases for cordycepin (Kondrashov *et al*., 2012).

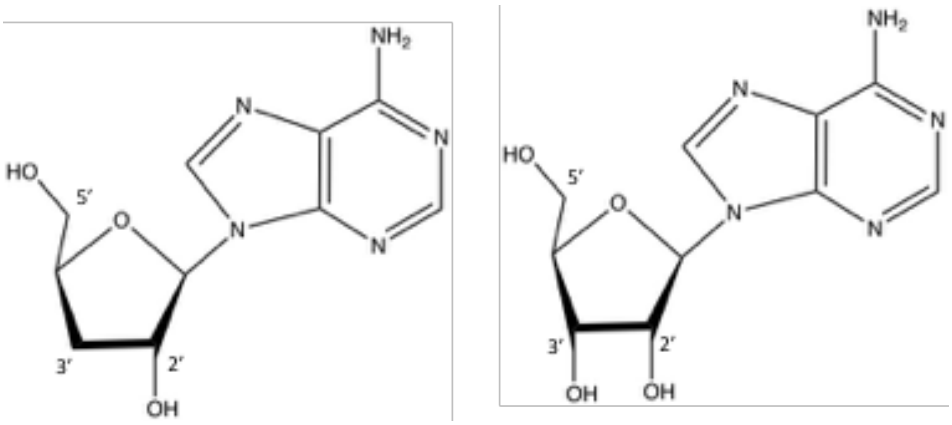


Figure 1.8- Structure of cordycepin and adenosine. The molecular structure of cordycepin (3'-deoxyadenosine) (left) and adenosine (right).

Another notable aspect of cordycepin is its anti-cancer properties. It has been found to promote apoptosis in cancerous cell lines, including human colorectal cancer cells, breast cancer cells and neuroblastoma cells (He *et al*., 2010; Choi *et al*., 2011; Baik *et al*., 2012), which reduces the proliferation of these cells. It also exerts the same effect by halting the cell cycle at key checkpoints (Wu *et al*., 2007, Lee *et al*., 2009, Jung *et al*., 2012). Cordycepin has been found to have anti-metastatic activity by reducing the ability of cells to move through the extracellular matrix and by inhibiting angiogenesis (Tuli *et al*., 2013).

The anti-inflammatory properties of cordycepin are thought to contribute to many of its medicinal benefits. Jeong *et al*., (2010) found that cordycepin inhibits the production of inflammatory molecules such as nitric oxide and pro-inflammatory cytokines in mouse microglial cells. They hypothesised that this was due to inhibition of the NFκB, AKT and MAPK signalling pathways. The NFκB and AKT pathways were also found to be inhibited by cordycepin in macrophage cells (Kim *et al*. 2006).

Cordycepin also possesses anti-microbial properties. It has been observed to have activity against *Candida* spp., reducing mortality of mice treated with these fungi if co-administered with pentostatin, a chemical that prevents the breakdown of cordycepin (Sugar and McCaffrey, 1998). Cordycepin also inhibits the growth of *B. subtilis*, which is thought to be due to prevention of purine synthesis (Rottman and Guarino, 1964).

While the medicinal properties of cordycepin are beginning to be investigated, there has been little research into its natural function, *i.e.* its role in the infection cycle of *C. militaris*. As discussed previously, some EPF secondary metabolites may have immuno-suppressive functions in insects, but this has not been confirmed for cordycepin. A number of pathways for the synthesis of cordycepin have been suggested (Zheng *et al.*, 2011; Xiang *et al.*, 2014; Kato *et al.*, 2017), but it is only recently that the genes involved in its production have been elucidated (Xia *et al.*, 2017). Three enzymes involved in cordycepin biosynthesis and one putative transporter have been identified, while the production of cordycepin has been shown to be coupled to the production of pentostatin. This is an inhibitor of adenosine deaminase, an enzyme that converts cordycepin to inosine. Other fungal species have been shown to also produce cordycepin including *Cordyceps kyusyuensis* and *Aspergillus nidulans* (Xia *et al.*, 2017). It has also been shown that *Ophiocordyceps sinensis*, which was previously thought to produce cordycepin (Huang *et al.*, 2003; Li *et al.*, 2004), did not in fact produce this molecule (Xia *et al.*, 2017). This may be due to the misidentification of *O. sinensis* in other studies or differences between strains, reflecting the importance of verifying the identity of organisms under study.

Table 1.5- Activity of cordycepin. The activity of cordycepin in different organisms or tissues.

Activity of cordycepin	Organism/Tissue	Reference
Anti-angiogenic	Human HepG2 cells	Lu *et al*., (2014)
Anti-tumour	Mice	Yoshikawa *et al*., (2004)
	Mouse melanoma cells	Nakamura *et al*., (2006)
	Mouse Lewis lung carcinoma cells	Nakamura *et al*., (2006)
Anti-metastasis	Human bladder cancer cells	Lee *et al*., (2010)
	Mouse melanoma cells	Nakamura *et al*., (2005)
Induction of apoptosis	Mouse tumour cells	Jen *et al*., (2011)
	Human colorectal cancer cells	He *et al*., (2010)
	Human breast cancer cells	Choi *et al*., (2011)
	Human leukemia cells	Jeong *et al*., (2011)
Anti-malarial	*Plasmodium knowlesi in vitro*, *Plasmodium berghei in vivo*	Trigg *et al*., (1971)
Anti-fungal	Mice	Sugar and McCaffrey, (1998)
Anti-bacterial	*Bacillus subtilis*	Rottman and Guarino, (1964)
Anti-inflammatory	Mouse microglial cells	Jeong *et al*. (2010)
	Mouse RAW cell line	Kim *et al*., (2006b)
	Rats	Zhang *et al*., (2014)
	Mice	Yang *et al*., (2015)
Anti-depressant	Mice	Tianzhu *et al*., (2014)
Anti-viral	Epstein-Barr virus	Ryu *et al*., (2014)
	HIV	Mueller *et al*., (1991)
Anti-diabetic	Mice	Ma *et al*., (2015)
Anti-arthritic	Human chondrocytes	Hu *et al*., (2014)
	Human chondrocytes	Ying *et al*., (2014)
Anti-ageing	Rats	Ramesh *et al*., (2012)
Insecticidal	*Plutella xylostella*	Kim *et al*., (2002)

1.8.5 The potential of cordycepin as a novel pest control agent

To date, very little research has been done on the natural function of cordycepin, *i.e.* its role in the *C. militaris* infection process, in particular its interaction with the insect immune system. If cordycepin operates as an EPF virulence factor, then it may have potential as a novel plant protection product (PPP), given that:

- Cordycepin is known to inhibit the mammalian innate immune system (Table 1.5), and therefore is likely to impact on insect immune function, because core functions of innate signalling pathways are conserved across insects and mammals.
- It is possible that cordycepin would be low risk for human health, since *C. militaris* is widely used as a herbal medicine (Das *et al.*, 2010b) with no reports of ill effects, and cordycepin itself is being studied as a novel human medicine (Table 1.5).

The potential of cordycepin as a novel biopesticide has been explored by Kim *et al.* (2002), who found that feeding *P. xylostella* larvae cordycepin caused significant mortality. Leaves were dipped in cordycepin solution, hence the exact dose administered could not be determined and the mode of action of cordycepin was not elucidated, although it was thought to be gut-acting. It is important to determine its mode of action and interactions with other biopesticides before developing cordycepin further as a biopesticide. If this research was performed, cordycepin could be incorporated into IPM, for example it could be coapplied with EPF biopesticides that do not themselves produce cordycepin such as *B. bassiana* and *M. brunneum*. This has potential to increase the speed of kill by these EPF-based biopesticides, which is a significant limiting factor in their use (Section 1.4.1).

1.9 Aims and Objectives

The main aims of this project were to improve understanding of the natural function of cordycepin and identify whether it has potential as a biopesticide, either alone or in conjunction with EPF. In general, there has been a lack of research into the natural functions of EPF secondary metabolites and it is likely that many remain to be discovered. These compounds have potential to be used as biopesticides and this project also aims to provide techniques and experimental protocols by which their natural function and potential as biopesticides can be assessed. These aims were achieved through the following objectives in each chapter:

- Assess the impact of EPF and cordycepin individually on the model insect *G. mellonella*.
- Monitor immune-related gene expression in *G. mellonella* in response to EPF. This was necessary to develop experimental protocols and improve understanding of the natural infection process of EPF.
- Measure the impact of cordycepin on immune-related gene expression in *G. mellonella*. Identify interactions between EPF and cordycepin to evaluate its potential as a biopesticide.
- Examine the effect of cordycepin on immune-related gene expression in a *D. melanogaster* cell culture. This was done because *D. melanogaster* is a well-studied model organism with thoroughly characterized immune pathways.
- Assess the potential of cordycepin as a biopesticide in the pest species *P. xylostella*, both on its own and in conjunction with EPF.

Chapter 2 - Determining the effect of entomopathogenic fungi and cordycepin on the survival of *Galleria mellonella*

2.1 Background

2.1.1 The model insect *G. mellonella*

Galleria mellonella (Lepidoptera, Pyralidae) is becoming increasingly popular as a laboratory model for investigating microbial infections in animals (Mylonakis *et al*., 2005; Bergin *et al*., 2006; Ramarao *et al*., 2012; Harding *et al*., 2013). In nature it is a pest of beehives where the larvae feed on pollen and wax (Hussein *et al*., 2012), which can lead to weak colonies being completely destroyed (Shimanuki, 1981). It can be reared between 20-30 ˚C and infection studies are performed over a wide range of environmentally relevant temperatures (15 ˚C-37 ˚C) (Ramarao *et al*., 2012). The large size of the larvae facilitates injection, which permits known doses of chemicals or microorganisms to be delivered into the insect (Glavis-Bloom *et al*., 2012). Recently a draft *G. mellonella* genome sequence was published (Lange *et al*., 2018), which will enable genetic manipulation and allow comparisons to other species. *G. mellonella* is particularly useful for studying the innate immune response against human pathogens (Mylonakis *et al*., 2005; Mukherjee *et al*., 2010; Harding *et al*., 2013), partly because of the conserved nature of the vertebrate and invertebrate innate immune systems (Vilmos and Kurucz, 1998) and also because studies can be performed at 37 ˚C. *G. mellonella* could also act as a useful tool for development of new control methods for Lepidopteran pests since the Pyralidae family contains a large number of species that are pests of a range of agricultural crops.

G. mellonella is very susceptible to EPF infection, possibly because beehives are a very clean environment due to the production of antimicrobial chemicals such as propolis by bees (Grange and Davey, 1990). As a result, there may not have been selection pressure for *G. mellonella* larvae to possess a robust immune system.

Because of this, they can also be used as 'bait' to acquire new isolates of entomopathogenic fungi and nematodes from soil (Chandler *et al*., 1997).

The immune response of *G. mellonella* after infection with EPF has been reasonably well studied. Plasmatocytes isolated from *G. mellonella* treated with *M. anisopliae s.l.* were found to exhibit morphological and cytoskeletal changes upon infection (Vilcinskas *et al*., 1997b), in addition to impaired phagocytic activity (Vilcinskas *et al*., 1997c). *Beauveria bassiana* infection has also been studied in *G. mellonella,* particularly the effect of this EPF on the systemic antifungal immune response (Vilcinskas and Matha, 1997; Wojda *et al*., 2009). This is discussed in more detail in later chapters.

2.1.2 Laboratory bioassays investigating the infection of insects by EPF

Due to the use of EPF as biological control agents their interaction with insects has been studied extensively using laboratory bioassays. In particular, *B. bassiana* and *M. brunneum* have been widely investigated (Copping and Menn, 2000), with isolates of both having been licenced for use as pest control agents (Section 1.4). In the U.K, two commercial products based on these species are *B. bassiana* in BotaniGard (Certis, USA) and *M. brunneum* in Met52 (Fargro, U.K).

In a bioassay EPF can be applied to insects through injection or surface application, with either conidia or blastospores. Conidia are the infectious stage of the fungal lifecycle commonly found in nature (Figure 1.3), whereas blastospores are vegetative cells that are produced during fungal growth in the insect haemolymph (Pendland and Boucias, 1997). Previous studies have treated *G. mellonella* by injection of *B. bassiana* blastospores into the haemolymph to monitor its impact on the expression of immune-related genes using RT-qPCR or to monitor its impact on the cellular immune response (Vilcinskas and Matha, 1997; Wojda *et al*., 2009). Injection allows treatment of insects with an accurate dose of pathogen, but is not necessarily informative about EPF virulence as the ability to penetrate the cuticle is a major virulence determinant (Lacey, 2012).

Various techniques have been used for the surface application of conidia onto insects. Firstly, insects can be infected by direct contact with the fungus. For example,

Vertyporokh and Wojda, (2017) infected *G. mellonella* with *B. bassiana* by rolling the larvae onto the sporulating fungus in an agar plate. Alternatively, insects can be immersed in a suspension of conidia (Lacey, 2012). A disadvantage of these techniques is that the dose of EPF received by an insect cannot be measured precisely. EPF can also be applied topically using an apparatus such as a Potter tower air atomising sprayer (Bateman, 1999), where a rotary atomiser is used to apply uniform droplets to insects. The final technique to apply conidia to the insect surface is to place a droplet of spore suspension onto the insect (Lacey, 2012). This enables a precise dose to be administered, but is only possible for use with some larger insects.

2.1.3 *Galleria mellonella* as a tool for studying the effect of fungal secondary metabolites on insects

In addition to its use to study EPF-insect interactions, *G. mellonella* has also been used as a model to study the impact of secondary metabolites on insects. Beauverolides have been found to have contrasting effects on the *G. mellonella* immune system following injection (Vilcinskas *et al*., 1999), having immunosuppressive and immunostimulatory effects depending on the quantification method. Injection of this metabolite stimulated lysozyme and antibacterial activity *in vivo* but inhibited phagocytic activity of plasmatocytes *in vitro* (Vilcinskas *et al*., 1999). Another study (Fiolka, 2008) found that cyclosporins (a secondary metabolite of *Tolypocladium inflatum*) have a strong immunosuppressive activity in *G. mellonella*.

Cordycepin, a secondary metabolite of the EPF *C. militaris*, is lethal when fed to *P. xylostella* larvae (Kim *et al*., 2002). It has also been reported to cause deformity when injected into *G. mellonella* pupae (Roberts, 1981). However, detailed studies on its mode of action have not been carried out and the reason for mortality and deformity have not been determined.

2.1.4 The insect gut as a barrier to infection

The cuticle of the external integument is a major barrier to EPF infection. One way in which pathogenic microorganisms may bypass this barrier is by entering the insect

through the gut following feeding. Lepidoptera have defensive mechanisms to prevent this route of infection, including a very low gut pH, digestive enzymes to prevent the growth of microorganisms, and the production of reactive oxygen species (ROS) (Vallet-Gely *et al*., 2008). If a potentially pathogenic microorganism is able to persist, AMP production may be triggered in the gut epithelial cells via the Toll (Ferreira *et al*., 2014) or IMD pathways (Tzou *et al*., 2000). AMP production has been found to be critical in resisting oral infection, for example *D. melanogaster* lacking an IMD pathway are more susceptible to oral infection by bacteria than wild-type flies (Liehl *et al*., 2006).

Commensal bacteria also reside in the insect gut and are thought to have beneficial functions such as aiding in the digestion of some materials (Breznak and Switzer, 1986; Prem Anand *et al*., 2010), modulating development/ growth (Shin *et al*., 2011) and protecting against pathogenic microorganisms (Dillon and Charnley, 2002; Engel and Moran, 2013). The reason that these commensals are not targeted by the immune system in the gut is not clear (Vallet-Gely *et al*., 2008). In *D. melanogaster* it is thought that in gut epithelial cells the transcription factor Caudal acts as a master regulator for AMP expression, preventing overexpression of AMPs (Ryu *et al*., 2008) and allowing commensal organisms to survive. Bacteria have been isolated from the guts of healthy *G. mellonella*, which suggests that there are either commensal bacteria or pathogens that are kept in check by the innate immune system (Bucher and Williams, 1967).

2.1.5 Aims

The first aim of this component of the Ph.D. was to quantify survival of *G. mellonella* larvae in response to treatment with EPF and the secondary metabolite cordycepin. In order to do this, it was necessary to develop an assay that could be used to reliably infect *G. mellonella* and monitor their survival. The second aim was to elucidate the cause of larval death when treated with cordycepin.

2.2 Materials and methods

2.2.1 Insect material

G. mellonella larvae were purchased from Wazp Brand UK Ltd. (Yorkshire, U.K) where larvae were cold-treated to slow down their development. Final instar larvae were maintained at 15 ˚C in darkness without feeding prior to experiments. Larvae were used for experiments within seven days of delivery and weighed between 200 and 300 mg.

2.2.2 Fungal material

The fungal isolates used are detailed in Table 2.1. The isolates of *B. bassiana* and *M. brunneum* were from the Warwick Crop Centre culture collection, where they were maintained for long term storage on porous cryotolerant plastic beads in liquid nitrogen. The isolate of *C. militaris* was obtained from the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARESF Agriculture, 2016). For this project all fungi were maintained on Sabouraud dextrose agar (SDA; Oxoid, USA) slopes at 5 ˚C. Prior to experiments, fungi were subcultured onto SDA in 9 cm Petri dishes (SARSTEDT, Germany) and incubated at 23 ˚C in darkness for 10-14 days.

Table 2.1- Origins of fungal isolates used during this project

Species	Accession number in Crop Centre Collection	Source	Origin	Commercial use
Beauveria bassiana	433.99	*Bemisia* sp. (Hemiptera: Aleyrodidae)	USA	Active ingredient in 'BotaniGard' (Mycotech Corporation, PO Box 4109, Butte, MT 59702, USA)
Metarhizium brunnuem	275.86	*Cydia pomonella* (Lepidoptera: Tortricidae)	Germany	Active ingredient in 'Met52' (Novozymes, Krogshoejvej 36, 2880 Bagsvaerd, Denmark)
Cordyceps militaris	ARSEF 11703	Lepidoptera, unknown species	China	-

In order to observe spore morphology, chitin was stained with lactophenol cotton blue (Pro-Lab Diagnostics, Canada). Approximately 20 µl of spore suspension was placed onto a glass microscope slide, stained with 20 µl of lactophenol cotton blue then sealed with a cover slip and clear nail varnish. The slide was visualized under a Leica DMi inverted microscope imaging system (Leica microsystems, Germany) at 100x magnification and photographed using a 5-megapixel camera (Leica MC170 HD).

2.2.3 Preparing conidial suspensions

Conidial suspensions were prepared (Figure 2.1) by pipetting 10 ml of 0.01% Triton X-100 (Merck, Germany) onto fungal plates and agitating with an 'L'-shaped spreader (Greiner bio-one, Austria) for approximately 2 min. This suspension was then filtered through two layers of sterile milk filter paper (Goat Nutrition Ltd, U.K) to remove mycelial fragments. The suspension was diluted 1 in 10 and the number of conidia counted using an Improved Neubauer haemacytometer. Following this, 10 ml aliquots were made using 0.01% Triton X-100 ranging from $1x10^7$ to $1x10^3$ conidia ml^{-1}.

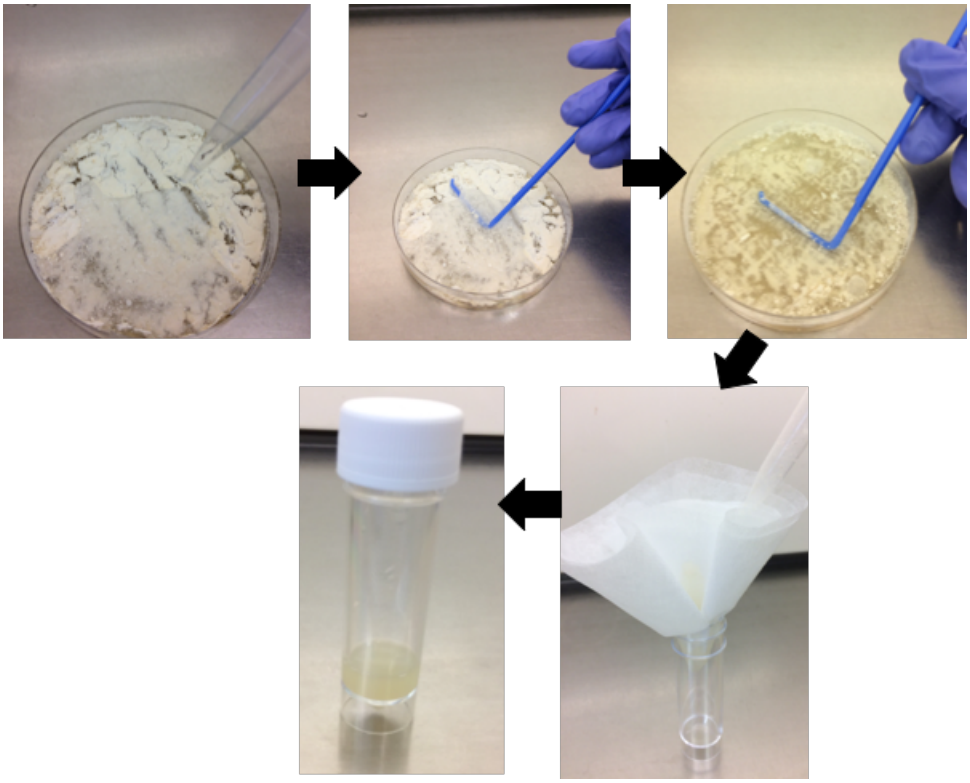


Figure 2.1-Preparation of conidial suspension. Preparation of a *Beauveria bassiana* 433.99 conidia suspension. Firstly, Triton X-100 is pipetted onto conidia, then agitated with an 'L' shaped spreader. This is filtered through two sterile milk filters.

2.2.4 Confirmation of fungal species identity using nucleotide sequences

Mycelia were scraped off fungal SDA plates grown in the dark for approximately 10 days using a sterile scalpel and ground in liquid nitrogen using a sterile mortar and pestle. DNA was extracted using a Qiagen DNeasy plant mini kit (Qiagen, The Netherlands) following the manufacturer's guidelines and the quality and quantity of DNA was assessed using a NanoDrop ® ND-100 Spectrophotometer (ThermoFisher Scientific, USA). The 20 µl PCR reactions were prepared using 1 µl of template DNA, RedTaq Ready Mix (Sigma-Aldrich, USA) and forwards and reverse primers (10 µM each) for the ITS region, large ribosomal subunit rRNA (LR) or translation elongation factor (EF) 1α gene (Table 2.2) following the manufacturer's guidelines and ran on a GeneAmp ® PCR System 9700 (Applied Biosystems, USA). The PCR conditions for ITS primers were: initial denaturation of 94 ˚C for 1 min followed by 35 cycles of 94 ˚C for 45s, 55 ˚C for 30s and 72 ˚C for 1 min. All PCRs were followed by a final amplification of 7 min at 72 ˚C unless otherwise stated. The conditions for LR primers

were: initial denaturation of 94 ˚C for 1 min followed by 30 cycles of 94 ˚C for 1 min, 50 ˚C for 45s and 72 ˚C for 1 min. The conditions for EF primers were: initial denaturation of 94 ˚C for 30s followed by 35 cycles of 94 ˚C for 30s, 56 ˚C for 30s and 72 ˚C for 1 min, then a final elongation for 10 min at 72 ˚C.

The PCR products were separated on a 1.2% agarose gel (Sigma-Aldrich, USA) prepared with Tris-Acetate EDTA (Fisher Scientific, USA) containing 2 µl GelRed ® (Biotium, USA) per 100 ml. This was run in Tris-Acetate EDTA at 90 V for 1 h in a submerged horizontal electrophoresis cell (BIO-RAD, USA) and visualised using a UV transilluminator (G:BOX, Synegene, U.K). The PCR product was purified using a QIAquick PCR purification kit (Qiagen, The Netherlands) and sequenced by GATC Biotech using the forward primer (5 µM) for each gene.

Table 2.2- Primers used for fungal identification.

Gene	Primer name	Primer sequence (5'-3')	Reference
Large ribosomal subunit	LROR	ACCCGCTGAACTTAAGC	Vilgalys and Hester (1990)
	LR5	TCCTGAGGGAAACTTCG	
Elongation factor 1- alpha	EF1-983F	GCYCCYGGHCAYCGTGAYTTYAT	Rehner and Buckley (2005)
	EF1-1567R	ACHGTRCCRATACCACCRATCTT	
ITS	ITS1	TCCGTAGGTGAACCTGCGG	White *et al*., (1990)
	ITS4	TCCTCCGCTTATTGATATGC	

A phylogenetic tree was constructed in MEGA6 (Tamura *et al*., 2013) including 18 reference sequences for type species (Appendix A.1) obtained from the National Center for Biotechnology Information (NCBI, 2017). The maximum likelihood method was used to construct the tree, based on the Kimura 2-parameter model with gamma distribution for ITS and EF1α genes. A Tamura-Nei 93 model with gamma distribution was used for the LR gene and a bootstrap value of 1000 was used for all three genes.

2.2.5 *Galleria mellonella* bioassay- fungal injection

Conidial suspensions of three EPF isolates (Table 2.1) were prepared as described in Section 2.2.3 at the following concentrations: $1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$ and $1x10^3$

conidia ml^{-1}. Final instar *G. mellonella* larvae were cooled on ice for 5 min prior to injection of 30 µl of fungal suspension into the right front proleg using a 0.3 ml microfine insulin syringe (BD, USA). Control larvae were injected with 30 µl of 0.01% Triton X-100. The injection platform was assembled using filter paper and a 1000 µl pipette tip (Figure 2.2). Ten larvae were treated at each dose and the experiment replicated on three occasions. Larvae injected with the same dose were placed together in a 9 cm Petri dish lined with filter paper (Whatman, GE Healthcare, U.K). They were maintained in an environmental test chamber (Sanyo, Japan) at 20 ˚C and 16:8 h light:dark cycle and their survival was monitored for seven days. A model was fitted to the survival on day seven using the DRC package (Ritz *et al*., 2015) in R (RStudio, 2017) and used to calculate the LC_{50} (concentration that causes 50% mortality) for each EPF.

2.2.6 *Galleria mellonella* bioassay- fungal topical application

Conidial suspensions of three EPF isolates (Table 2.1) were prepared as described in Section 3.2.3 at the following concentrations: $1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$ and $1x10^3$ conidia ml^{-1}. Final instar *G. mellonella* were cooled on ice for 5 min. Groups of 10 larvae per concentration for each isolate were placed on filter paper (Whatman, GE Healthcare, U.K) in a 9 cm Petri dish lid and sprayed with 4 ml of conidia suspension using a Potter tower air atomising sprayer (Potter, 1952) at 34.5 KPa. Controls were sprayed with 0.01% Triton X-100 prior to any fungal application to prevent contamination. The experiment was replicated on three occasions. Larvae were allowed to recover at room temperature for 30 min before being transferred to a new 9 cm Petri dish lined with damp filter paper and sealed with Parafilm 'M' ® (Bemis, USA) to maintain a humid environment. The Petri dishes were maintained in an environmental test chamber (Sanyo, Japan) at 20 ˚C and 16:8 h light:dark cycle and the survival was recorded over seven days. A model was fitted to the survival on day seven using the DRC package as described in Section 2.2.5.

2.2.7 *Galleria mellonella* bioassay- cordycepin injection

A stock solution of cordycepin was prepared by dissolving 100 mg of cordycepin (Sigma-Aldrich, USA) in 1 ml of DMSO (Fisher Scientific, USA). This was diluted using DEPC-treated water (Fisher Scientific, USA) to the following concentrations: 1, 1.82, 3.3, 6 and 11 mg ml^{-1}. These concentrations were selected as they are equidistant on a log2 scale. Final instar *G. mellonella* were cooled on ice for 5 min prior to injection with 30 µl of the solution in the right front proleg (Figure 2.2) using a 0.3 ml microfine insulin syringe (BD, USA). Controls were injected with a 0.01% Triton X-100/DMSO mixture. Ten larvae were treated at each dose and the experiment was replicated on three occasions. Larvae injected with the same dose were placed together in a 9 cm Petri dish lined with filter paper (Whatman, GE Healthcare, U.K). Dishes were maintained in an environmental test chamber (Sanyo, Japan) at 20 ˚C and 16:8 h light:dark cycle and the survival was monitored daily for seven days. Survival was analysed in SPSS Statistics (Version 24, IBM) using a Kaplan-Meier estimator. A log-rank test was carried out during this analysis to determine whether there was any significant difference between replicates (Kleinbaum and Klein, 2012).

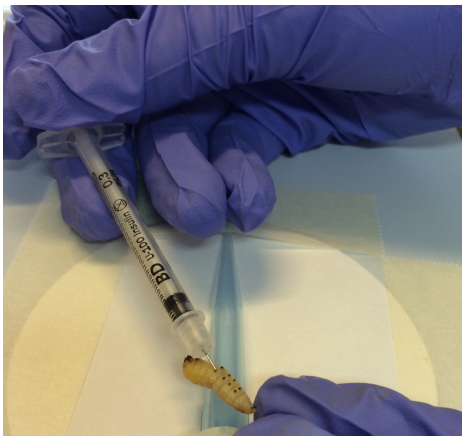


Figure 2.2- Injection of final instar *Galleria mellonella* larvae. Injection platform constructed from filter paper and a 1000 µl pipet tip to allow immobilization of larvae and injection with a 30 µl syringe.

2.2.8 Identification of bacteria isolated from *G. mellonella* cadavers

Unexplained death was observed in the cordycepin injection bioassays (Section 2.2.7). In order to identify the causal agent, final instar *G. mellonella* were injected with 11

mg ml^{-1} cordycepin or a control solution as described in Section 2.2.7. Haemolymph was extracted from larvae on day seven by piercing the abdomen and squeezing the larvae until a droplet formed. The droplet was collected using a 2 µl pipette and 1 µl of the haemolymph was diluted 1000-fold in sterile distilled water. A 100 µl aliquot of this was spread onto a 9 cm Petri dish containing 1% nutrient agar using an 'L'-shaped spreader (Greiner bio-one, Austria) and the plates were incubated for 24 h at 25 ˚C in darkness. Individual colonies were isolated and spread onto new 1% nutrient agar plates as previously described and incubated for a further 24 h. Bacterial DNA was extracted using the Qiagen DNeasy Blood and Tissue DNA extraction kit, following the gram-positive bacteria DNA extraction protocol. DNA (1 µl) was used as a template for PCR (GeneAmp ® PCR System 9700, Applied Biosystems, USA) using RedTaq Ready Mix (Sigma-Aldrich, USA) and the 16S rRNA primers and conditions described in Weisburg *et al.,* (1991). Following amplification PCR products were ran on a gel as described in Section 2.2.4 and purified using the QIAquick PCR purification kit (Qiagen, The Netherlands). Purified DNA was sequenced by GATC Biotech (Germany) using the forward primer (5 µM). A phylogenetic tree was constructed in MEGA6 (Tamura *et al*., 2013) including reference sequences (Appendix A.2) from the National Center for Biotechnology Information (NCBI, 2017) using the maximum likelihood method based on the Jukes-Cantor model and a bootstrap value of 1000.

2.3 Results

2.3.1 Confirmation of fungal species identity

The identity of EPF isolates was confirmed using lactophenol cotton blue staining of conidia, observation of EPF physiology and gene sequencing. Physiology of all isolates cultured on SDA was consistent with description by Brady (1979). Following lactophenol cotton blue staining *M. brunneum* 275.86 conidia appeared to be elongated and oval, approximately 10 μm long and 3 μm in diameter (Figure 2.3), which is consistent with the description from Petch (1935). The conidia of *C. militaris* 11703 ranged from spherical to 'pear shaped' and were approximately 4 μm in diameter, this is consistent with the description from Brady (1979). *B. bassiana* 433.99 conidia appeared spherical and 3 μm in diameter, which is consistent with those described by de Hoog (2000).

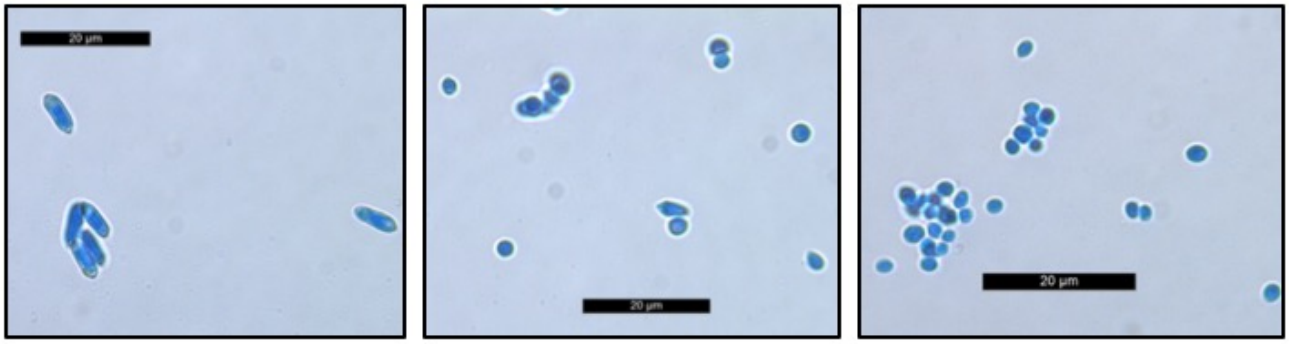


Figure 2.3- Microscopic visualization of EPF conidia stained with lactophenol cotton blue. A) Conidia of *Metarhizium brunneum* 275.86, B) *Cordyceps militaris* 11703 and C) *Beauveria bassiana* 433.99 stained with lactophenol blue. Black bars are 20 μm.

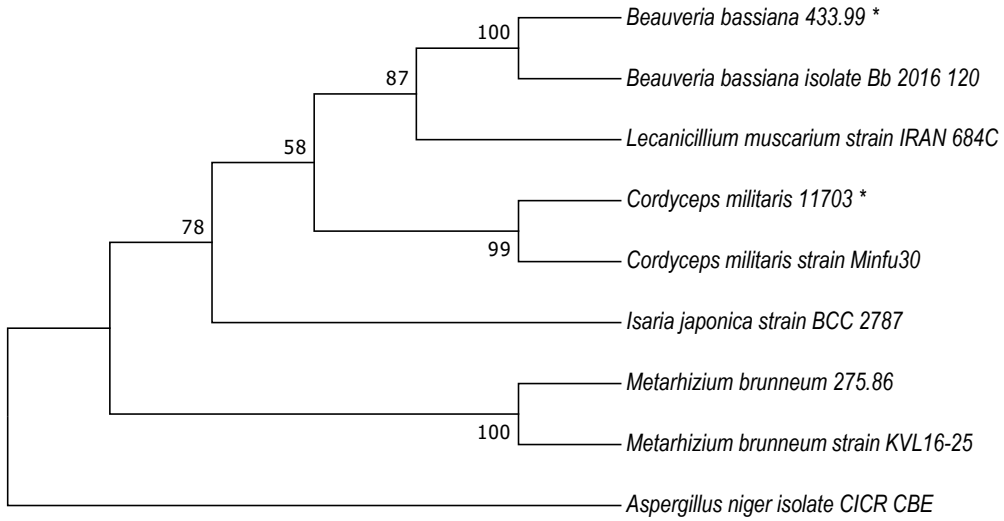


Figure 2.4 - Phylogenetic tree constructed using ITS gene. Tree constructed using ITS gene sequences and the Kimura 2-parameter model with gamma distribution. Bootstrap values are 1000. Isolates marked with an * are sequences from the isolates used in this project, all other sequences were obtained from the National Center for Biotechnology (NCBI, 2017). Constructed using MEGA6 software.

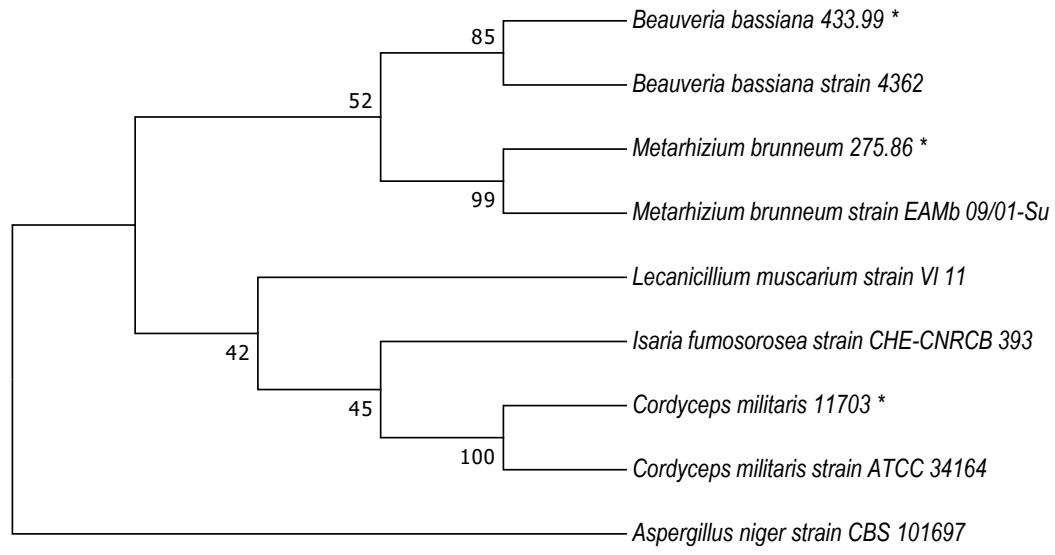


Figure 2.5- Phylogenetic tree constructed using elongation factor 1 alpha gene. Tree constructed using elongation factor 1 alpha gene sequences and the Kimura 2-parameter model with invariant sites. Bootstrap values are 1000. Isolates marked with an * are sequences from the isolates used in this project, all other sequences were obtained from the National Center for Biotechnology Information (NCBI, 2017). Constructed using MEGA6 software.

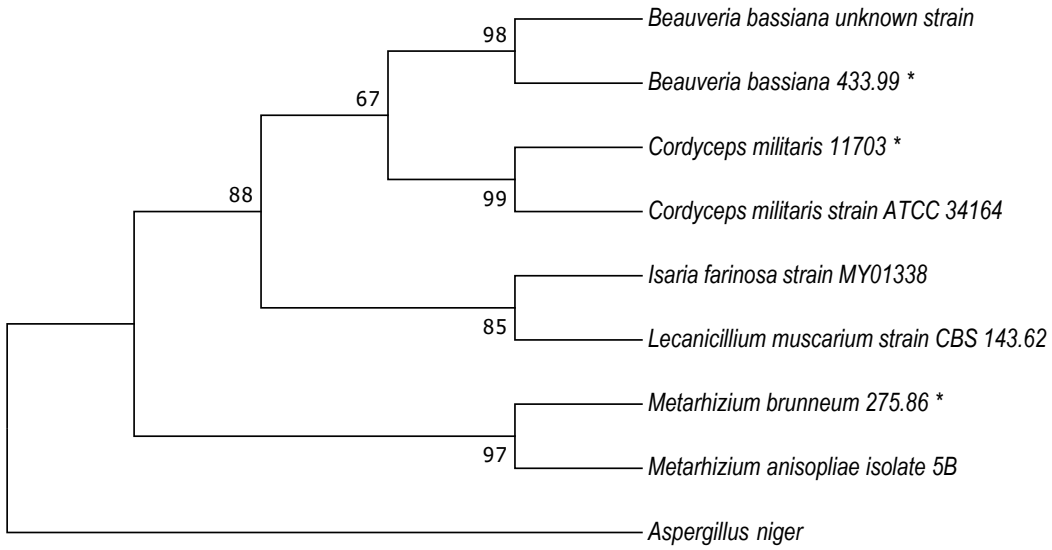


Figure 2.6- Phylogenetic tree constructed using large ribosomal subunit rRNA gene. Tree constructed using large ribosomal subunit rRNA gene sequences and the Tamura-Nei 93 model with gamma distribution. Bootstrap values are 1000. Isolates marked with an * are sequences from the isolates used in this project, all other sequences were obtained from the National Center for Biotechnology Information (NCBI, 2017). Constructed using MEGA6 software.

All three genes tested from the three EPF isolates used during this project clustered with expected sequences (Figures 2.4, 2.5 and 2.6), confirming the identity of these isolates. However, the three trees (Figures 2.4, 2.5 and 2.6) were not congruent with each other which could indicate differences in the evolution of genes or errors in the identity of sequences in the NCBI database. In particular this could be the case for *Isaria* spp. and *Lecanicillium* spp., which have been reclassified on multiple occasions (Sung *et al.,* 2007). There were a low number of average substitutions per site in comparison to reference sequences for all of the genes investigated, genetically confirming the identification of the isolates. The differences between the ITS genes was a mean of 0.003, <0.001 and <0.001 base substitutions per site for *C. militaris* 11703, *B. bassiana* 433.99 and *M. brunneum* 275.86, respectively. The differences between the elongation factor 1 alpha genes was a mean of <0.001, 0.022 and 0.009 base substitutions per site for *C. militaris* 11703, *B. bassiana* 433.99 and *M. brunneum* 275.86, respectively. The differences between the large ribosomal subunit rRNA

genes was a mean of <0.001, 0.003 and 0.001 base substitutions per site for *C. militaris* 11703, *B. bassiana* 433.99 and *M. brunneum* 275.86, respectively.

2.3.2 *G. mellonella* bioassay techniques

During the bioassays described in Sections 2.25 to 2.27, a number of problems were encountered. Table 2.3 summarises these problems and the solutions found to overcome them.

Table 2.3- Problems encountered during the development of *Galleria mellonella* bioassays and the techniques used to overcome them.

Problem	Cause	Solution
High control mortality	Bacterial infection Fungal infection	• Surface sterilise larvae with 70% ethanol before treatment.
	Internal injury from injection	• Inject larvae in the front right proleg • Inject no more than 30 μl into each larva
Low mortality following EPF spraying	Low humidity	• Maintain larvae in Petri dish lined with damp filter paper and seal with parafilm
Inconsistent mortality following EPF spraying	Insects moving during spraying	• Chill larvae on ice for approximately five minutes before spraying

2.3.3 *G. mellonella* bioassay- fungal injection

Dose responses were observed when *G. mellonella* were injected with EPF and the best fitting model for all EPF was a two-parameter log-logistic model (Figure 2.7). This was used to calculate the LC_{50} on day six (Table 2.4) as being $3.57x10^3$, $4.03x10^4$ and $6.96x10^3$ conidia ml^{-1} for *B. bassiana* 433.99, *C. militaris* 11703 and *M. brunneum* 275.86, respectively. This is equivalent to LD_{50} values of $1.1x10^2$, $1.2x10^3$ and $2.1x10^2$ conidia per larva for *B. bassiana* 433.99, *C. militaris* 11703 and *M. brunneum* 275.86, respectively. The LC_{50} values of all three isolates were significantly different

(p<0.001 for *B. bassiana* 433.99 v *C. militaris* 11703, p=0.006 for *B. bassiana* 433.99 v *M. brunneum* 275.86 and p=0.009 for *M. brunneum* 275.86 v *C. militaris* 11703).

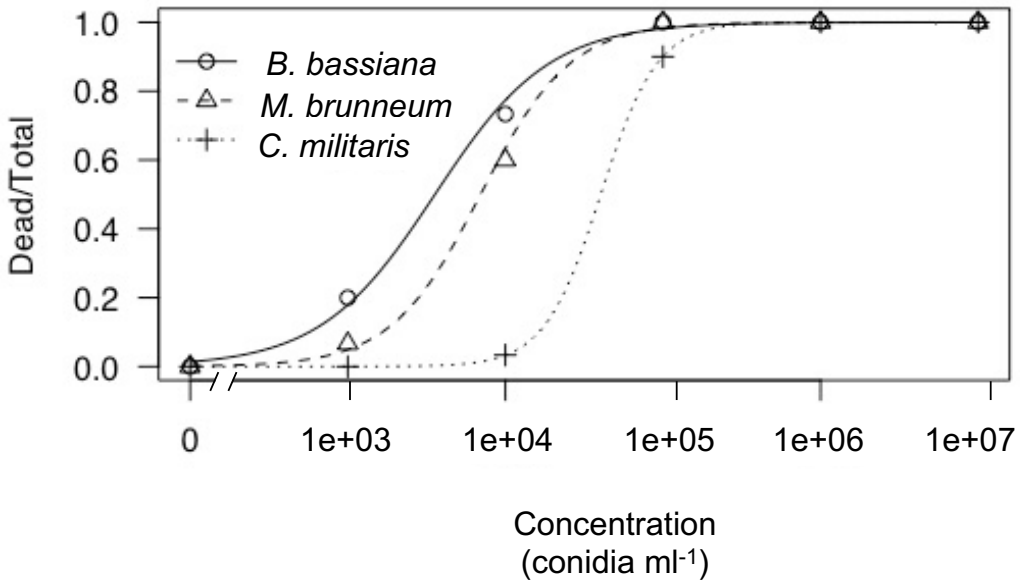


Figure 2.7 - The proportion mortality of *Galleria mellonella* injected with EPF. The proportion mortality of *G. mellonella* larvae (n=30) injected with *B. bassiana* 433.99, *M. brunneum* 275.86 or *C. militaris* 11703 conidia at six concentrations on day six after treatment. A two-parameter log-logistic model is fitted to the responses.

Table 2.4 - Estimates of the LC_{50} following injection of EPF [1,2].

Fungus	LC_{50} (conidia ml^{-1})	95% confidence limits	
		Lower bound	Upper bound
B. bassiana 433.99	$3.57x10^3$ a	$1.72x10^3$	$5.42x10^3$
C. militaris 11703	$4.03x10^4$ b	$2.05x10^4$	$5.81x10^4$
M. brunneum 275.86	$6.96x10^3$c	$4.04x10^3$	$9.98x10^3$

[1] Estimate of the LC_{50} following injection of *G. mellonella* with *C. militaris* 11703, *B. bassiana* 433.99 and *M. brunneum* 275.86 conidia.

[2] The LC_{50} was calculated by fitting an LL.2 model in RStudio and significant differences determined using a T-test. Significant differences between LC_{50} are denoted with letters (p<0.05).

2.3.4 Topical application of EPF conidia onto *G. mellonella*

Dose responses were also observed when EPF were topically applied to *G. mellonella* larvae and the best fitting model for *G. mellonella* mortality was a two-parameter Weibull function (Figure 2.8). This was used to calculate the LC_{50} on day six (Table 2.5) as being $9.57x10^4$, $1.73x10^6$ and $1.03x10^5$ conidia ml^{-1} for *B. bassiana* 433.99, *C. militaris* 11703and *M. brunneum* 275.86, respectively. The LC_{50} of *C. militaris* 11703 was significantly higher than for the other isolates tested ($p<0.001$ in comparison to *B. bassiana* 433.99 and $p=0.008$ in comparison to *M. brunneum* 275.86).

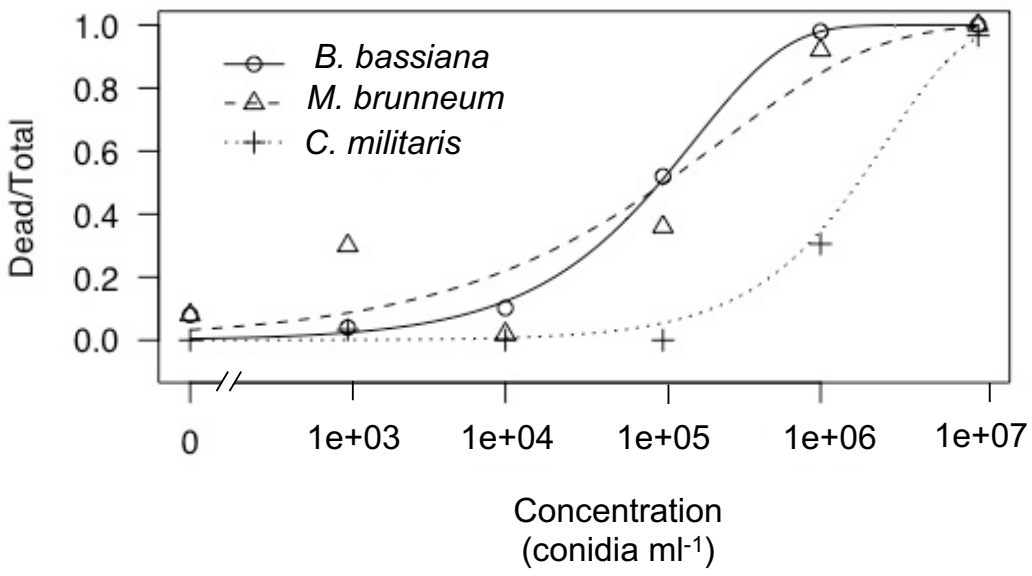


Figure 2.8 - The proportion mortality of *Galleria mellonella* sprayed with EPF. The proportion mortality of *G. mellonella* larvae (n=30) sprayed with *B. bassiana* 433.99, *M. brunneum* 275.86 or *C. militaris* 11703 conidia at six concentrations on day six after treatment. A two-parameter Weibull function is fitted to the responses.

Table 2.5- Estimates of the LC_{50} following topical application of EPF [1,2].

Fungus	LC_{50} (conidia ml^{-1})	95% confidence limits	
		Lower bound	Upper bound
B. bassiana 433.99	$9.57x10^4$ a	$5.78x10^4$	$1.33x10^5$
C. militaris 11703	$1.73x10^6$ b	$9.43x10^5$	$2.53x10^6$
M. brunneum 275.86	$1.03x10^5$ a	$4.86x10^4$	$1.58x10^5$

[1] Estimate of the LC_{50} following spraying of *G. mellonella* with *C. militaris* 11703, *B. bassiana* 433.99 and *M. brunneum* 275.86 conidia at day six after treatment.

[2] The LC_{50} was calculated by fitting a model in RStudio and significant differences determined using a T-test. Significant differences between LC_{50} are denoted with letters ($p<0.05$).

2.3.5 Effect of cordycepin on survival of *G. mellonella*

In order to assess the impact of cordycepin on the survival of *G. mellonella,* final instar larvae were injected with cordycepin and the survival of three replicates was monitored (Figure 2.9). At 11 mg ml^{-1} and 6 mg ml^{-1} cordycepin (330 µg and 180 µg per larva, respectively), a significant difference was observed in the survival of larvae between three replicates. At 11 mg ml^{-1} in replicate two there was 100% mortality after three days, but for replicates one and three there was 50% and 70% mortality, respectively, at day seven. A similar variation was observed at 6 mg ml^{-1}, where in replicate two there was 100% mortality after four days, but for replicates one and three there was 50% and 40% mortality, respectively, after day seven. At all other concentrations of cordycepin (3.3, 1.82, 1 and 0 mg ml^{-1}) there was no significant difference in survival of *G. mellonella* larvae between replicates. It was therefore not possible to calculate a dose response for cordycepin as the mean of the replicates obtained at 6 mg ml^{-1} and 11 mg ml^{-1} would be unreliable. The survival curves for all cordycepin doses are shown in Figure 2.9. The mean survival of larvae on day seven was 87%, 93%, 100% and 97% following treatment with 3.3, 1.82, 1 and 0 mg ml^{-1}, respectively.

Where death occurred, particularly at higher doses of cordycepin, larvae exhibited distinctive symptoms prior to death and had atypical cadavers. Between 24-48 h prior

to death insects became grey and movement was reduced. After death, cadavers were black and their abdomen appeared fluid-filled (Figure 2.10).

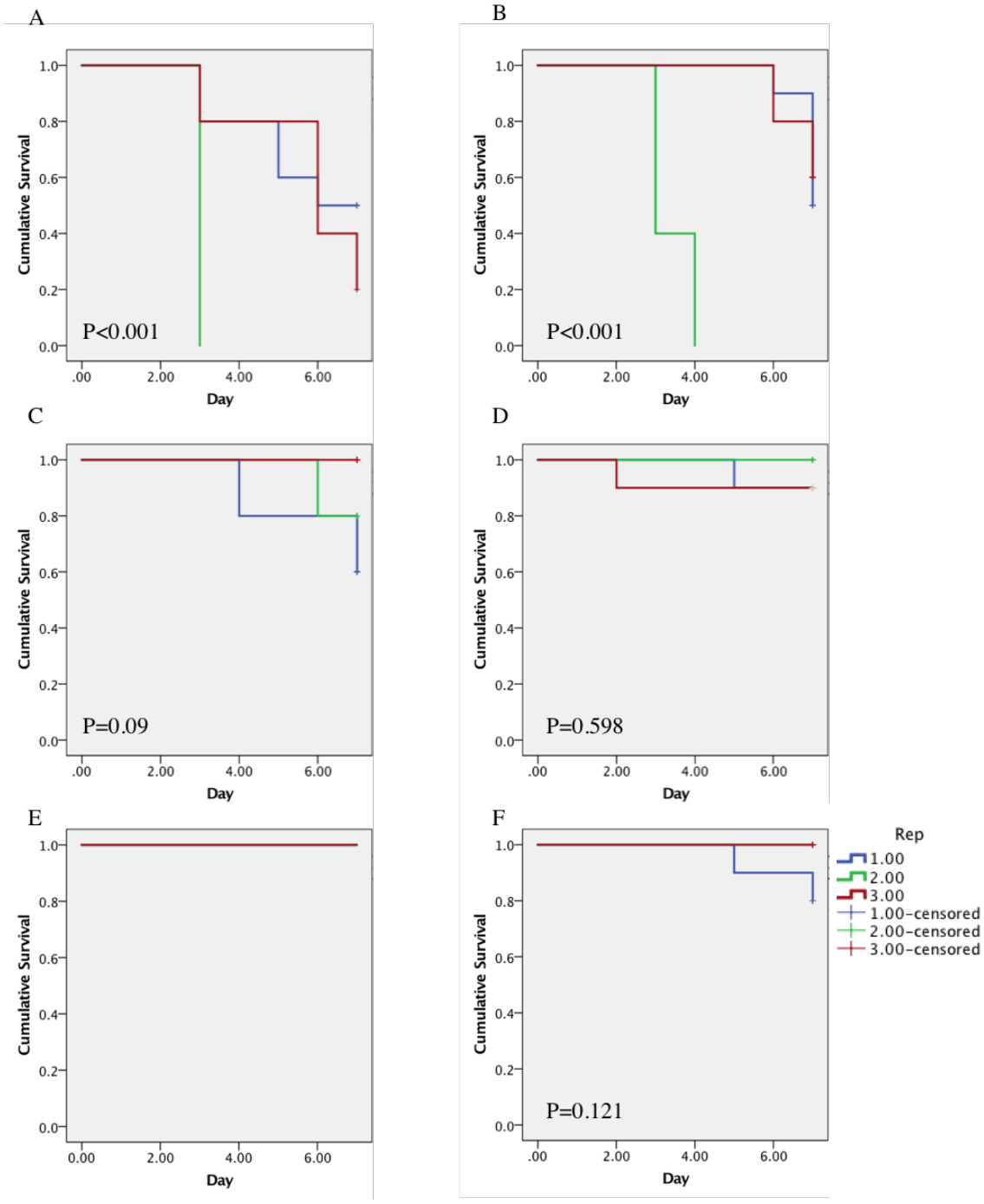


Figure 2.9- Kaplan-Meier plots showing the effect of cordycepin on *Galleria mellonella* survival. Cumulative survival of *G. mellonella* following treatment with A) 11, B) 6, C) 3.3, D) 1.82, E) 1 and F) 0 mg ml^{-1} of cordycepin. Survival of each replicate is shown for each treatment, with ten individuals treated per treatment per replicate. A log-rank test was done for each replicate and the p-values are shown. Figure was produced using SPSS Statistics (Version 24, IBM).

Figure 2.10- Photograph of *Galleria mellonella* cadavers. *G. mellonella* cadavers that had died following treatment with cordycepin.

2.2.6 Identification of bacteria isolated from *G. mellonella*

Bacteria were isolated from the haemolymph (Figure 2.11) of ten *G. mellonella* larvae that were treated with cordycepin following surface sterilisation with ethanol. One species was isolated from each of eight larvae and two species from two individuals. No bacteria were isolated from control individuals as haemolymph is sterile in healthy individuals.

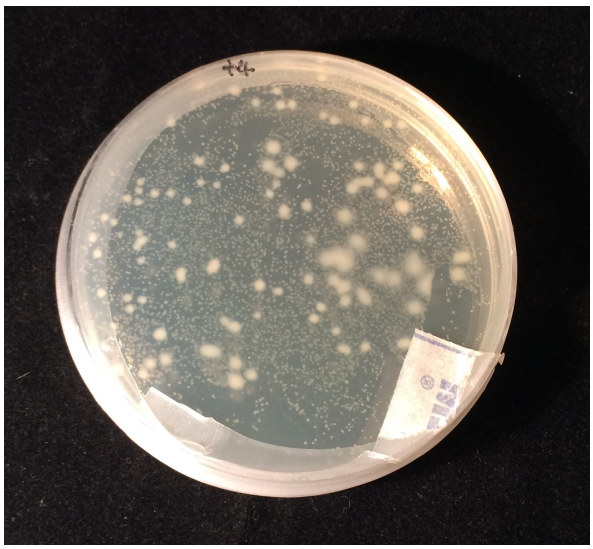


Figure 2.11- Bacteria isolated from *Galleria mellonella* haemolymph. Example of the bacterial colonies obtained from the haemolymph of *G. mellonella* that had been treated with 11 mg ml^{-1} cordycepin.

The 16S rRNA sequences from all 12 species of bacteria isolated from *G. mellonella* cadavers showed homology to sequenced bacteria and clustered into multiple groups (Table 2.6; Figure 2.12). The differences between isolates from cordycepin-treated *G. mellonella* and isolates from the NCBI database ranged from 1-7% (Table 2.6).

Table 2.6- Bacteria isolated from *Galleria mellonella* treated with cordycepin. The similarity of 16S sequences of bacteria isolated from *G. mellonella* treated with 11 mg ml $^{-1}$ cordycepin to the top BLAST hit in the NCBI database.

Bacterial Isolate	Top NCBI BLAST hit/ Accession number	Percentage similarity/ range (bp)
1	*Pantoea agglomerans*/MH158730	99% / 1012
2	*Pantoea vagans*/ MH141452	99% /1053
3	*Bacillus cereus*/ KX069360	99% /799
4	*Bacillus thuringiensis*/ MF083059	98%/ 751
5	*Enterobacter xiangfangensis*/ CP024908	99%/ 1021
6	*Enterococcus faecalis*/ MH150804	99% /540
7	*Enterobacter hormaechei*/ MH396771	98% /774
8	*Enterobacter cloacae*/ MF471480	99% / 804
9	*Serratia liquefaciens*/ KY458800	99% / 637
10	*Enterobacter cloacae*/ KX450937	93%/ 756
11	*Serratia liquefaciens*/ KY458800	99% / 756
12	*Enterobacter asburiae* / EU239468	95% / 772

Both gram-positive (nine isolates) and gram-negative (three isolates) bacteria were identified. The most common genera were *Enterobacter*, followed by *Pantoea*, *Serratia* and *Bacillus*. These are in the phyla Proteobacter or Firmicutes, both of which contain gut-resident bacteria that have previously been isolated from insects (Yun *et al.*, 2014).

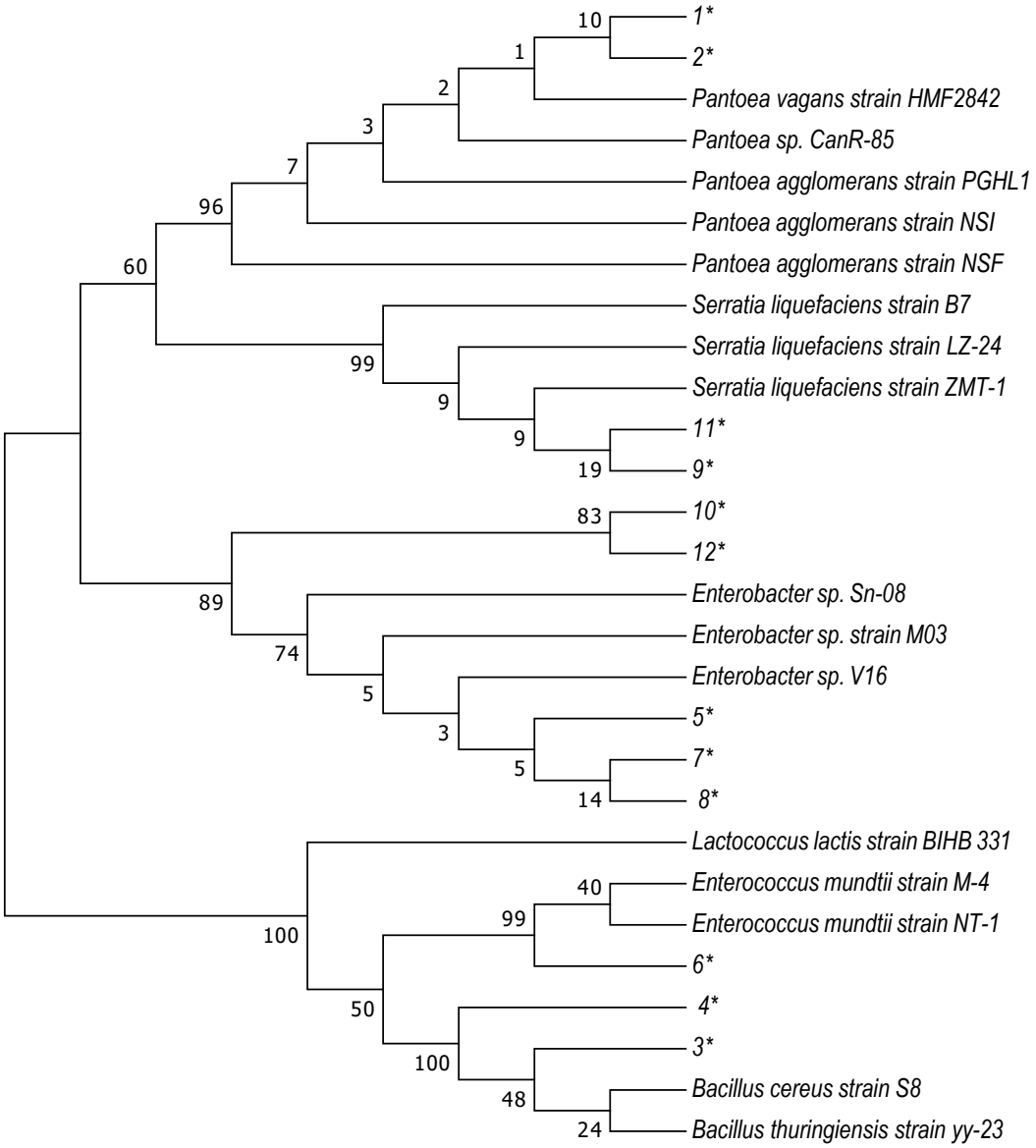


Figure 2.12- Phylogenetic analysis of 16S rRNA gene sequences to identify haemolymph bacteria. Phylogenetic tree constructed using 16S rRNA gene sequences and the Jukes-Cantor model, bootstrap values are 1000. Isolates marked with * were extracted from *G. mellonella* that had been treated with cordycepin, all other sequences were obtained from the National Center for Biotechnology Information (NCBI, 2017). Constructed using MEGA6 software.

2.4 Discussion

The development of a good bioassay system for testing EPF is difficult, as results must be reproducible and insects need to be easy to observe. Bioassays with *G. mellonella* have been performed by Wojda *et al.* (2009), Mukherjee *et al.* (2010) and Harding *et al.* (2013) who treated larvae by injection of pathogens (Mukherjee *et al.*, 2010; Harding *et al.*, 2013) or topical application of EPF (Wojda *et al.*, 2009). *G. mellonella* is related to several key crop pest species, therefore it is a useful model to investigate the impact of pesticides and other pest control methods. The innate immune system of *G. mellonella* is similar to that of mammals and larvae can be maintained at human body temperature (37 ˚C), so it is also useful for the study of human pathogens.

The bioassay system developed in this chapter provided a reproducible means of treating *G. mellonella* that minimised control mortality, was quick to set up and facilitated observation of larvae. The inoculation bioassay was similar to that described by Harding *et al.* (2013), but larvae were surface sterilised more thoroughly, inoculated with a different volume, weighed prior to inoculation and maintained at a lower temperature. An important finding during this bioassay development process was the requirement to maintain a clean environment when injecting and spraying as *G. mellonella* were highly susceptible to infection by opportunistic fungi and bacteria. As *G. mellonella* larvae naturally occur in the relatively sterile environment of beehives (Dickman, 1933), they may have lost some of their immune response capability compared with other Lepidoptera.

Mortality caused by EPF varied depending on the mode of application, with larvae inoculated by injection of EPF appearing to have a higher rate of mortality than when EPF were applied topically. However, direct comparison between the mortality of topically treated or injected larvae was not possible as the number of conidia in each larva was not quantified following topical application. It is likely that any differences were because conidia must penetrate the insect cuticle when applied topically (Andersen, 1979), but would bypass this if inoculated. Penetration of the cuticle is a time consuming and energy-expensive process due to the production of cuticle-degrading enzymes (Ortiz-Urquiza and Keyhani, 2013) and appressoria (Howard *et al.*, 1991) by EPF. The mortality of *G. mellonella* following *B. bassiana*, *C. militaris*

and *M. brunneum* infection has been assessed by other studies. Wojda *et al*., (2009) found that injection of *G. mellonella* with $1x10^4$ *B. bassiana* isolate 80.2 blastopores caused 100% mortality of larvae after three days, whereas the work described here found that injection with a similar dose ($3x10^4$ conidia per larva, 30 µl of $1x10^6$conidia ml^{-1}) caused 100% mortality after five days. The differences in mortality could be due to isolate variation (Ekesi *et al*., 1999) or differences in virulence between blastospores and aerial conidia (Hegedus *et al*., 1992). Most other studies into EPF virulence in *G. mellonella* applied EPF conidia topically (Ment *et al*., 2012; Kryukov *et al*., 2015; Ríos-Moreno *et al*., 2017), so it is difficult to compare the results of these studies to the results in this chapter due to the different methods for conidia application.

G. mellonella were less susceptible to infection by *C. militaris* 11703 than either *B. bassiana* 433.99 or *M. brunneum* 275.86. Fungal virulence is a result of complex interactions between insect host and the EPF and can be influenced by EPF isolate, host species, temperature (Thomas and Blanford, 2003), humidity (Gillespie and Crawford, 1986) and UV light exposure (Braga *et al*. 2001a). It has been reported that the optimum temperature for *C. militaris* is in the range of 20 ˚C to 28 ˚C (Lin and Chiang, 2008), 20 to 30 ˚C for *B. bassiana* (Fargues *et al*., 1997) and 25 to 30 ˚C for *M. brunneum* (Ekesi *et al*., 1999). All experiments were carried out at 20˚C, which is close to the optimum for these species, although slightly low for *M. brunneum*. So the variation in EPF virulence could be due to the origin of the isolate and the climate to which it is adapted. Alternatively, the reason for *C. militaris* 11703 virulence being lower than other EPF may be its lifestyle. In nature it predominantly infects Lepidoptera pupae (Kryukov *et al*., 2011) and pupation generally lasts longer than each larval instar in Lepidoptera (Briere and Pracros, 1998; Liu *et al*., 2002; Mironidis and Savopoulou-Soultani, 2014). This gives *C. militaris* a longer period of time for infection, as moulting between larval stages sheds EPF conidia on the cuticle, therefore *C. militaris* may not have evolved to colonise the host rapidly. In contrast, *M. brunneum* and *B. bassiana* are generalists (Li, 1988; Roberts and St Leger, 2004; Wraight *et al*., 2010), due to the higher abundance of potential hosts they may have evolved to be more virulent to permit infection of a higher number of insects.

Bioassays were successfully developed to quantify the effect of cordycepin on *G. mellonella* survival. Concentrations of cordycepin greater than 6 mg ml^{-1} (180 μg per larva) caused mortality of larvae, however this was variable between replicates. Variability in mortality and dark brown *G. mellonella* cadavers suggest that cordycepin was not directly responsible for mortality. The dark brown/ black cadavers with liquefied interiors suggested that mortality was caused by bacterial infection (Tanada and Kaya, 2012). Similar symptoms were observed by Kim *et al*., (2002), who found that *P. xylostella* fed on cordycepin became dark brown and lysed. However, these authors linked these symptoms to a direct effect to cordycepin and did not suggest a mode of action.

In support of the hypothesis that cordycepin was facilitating bacterial infection, bacteria were isolated from the haemolymph of cadavers, but not of control larvae. The bacteria isolated from the haemolymph were identified as belonging to the phyla Proteobacter and Firmicutes. The 16S rRNA sequences of all bacteria isolated showed a high similarity to isolates in the NCBI database, with sequence differences between 1-7%. This could indicate that new strains were isolated in this project, however further studies would be needed to confirm strain/species differences (Fox *et al*., 1992). This is consistent with the hypothesis that cordycepin facilitates opportunistic infection of *G. mellonella* by gut-resident bacteria which are common in insects. Yun *et al*. (2014) isolated bacteria from the gut of 305 insects (218 species) and identified the following proportions of phyla: 62.1% Proteobacteria, 20.7% Firmicutes, 6.4% Bacteroidetes, 4.8% Actinobacteria, 1.9% Tenericutes and 3% unclassified. They also found that the range of bacteria present in the insect gut is dependent on the species of the host, host habitat, diet and developmental stage. Bucher and Williams, (1967) identified commonly occurring bacteria in *G. mellonella* including: *Alcaligenes faecalis*, *Bacillus* spp., *Staphylococcus aureus*, *Micrococcus* spp., *Enterobacter* spp., *Pseudomonas* spp., *Lactobacillus* sp. These are consistent with at least 9 of the 12 bacteria (*Enterobacter* spp. and *Bacillus* spp.) isolated from *G. mellonella* cadavers in this project.

The presence of gut-resident bacteria in the haemolymph may be due to disruption of the insect gut by cordycepin, or inhibition of the insect immune system. Both of these

hypotheses would explain how the opportunistic bacteria were able to leave the gut and colonise the haemolymph. In mammalian systems it has been found that cordycepin reduces the expression of inflammatory genes (Kim *et al*., 2006a; Lee *et al*., 2010) through inhibition of the NFκB pathway. Therefore, it is possible that cordycepin inhibits a homolog of NFκB in the insect immune system, which stops the immune system from being able to prevent colonisation by gut bacteria. This will be expanded on in later chapters.

2.5 Conclusions

Injection and spraying bioassays were developed in *G. mellonella* that were reliable and reproducible with minimal control mortality and could be readily adapted for different application methods of EPF or other pathogens. Characterisation of the virulence of EPF and cordycepin in *G. mellonella* showed that the response to EPF was dose-dependent, regardless of the fungal species, but the impact of cordycepin was more complex. Cordycepin may facilitate colonisation of *G. mellonella* by gut-resident bacteria, which could be due to the inhibition of the immune system by cordycepin, as has been previously described in mammals.

Chapter 3- Determining the effect of entomopathogenic fungi on the expression of genes associated with immune reactions in *Galleria mellonella*

3.1 Background

3.1.1 The *G. mellonella* humoral immune response

Galleria mellonella is increasingly used as a model organism to investigate the insect immune response to infection (Mylonakis *et al*., 2005; Bergin *et al*., 2006; Ramarao *et al*., 2012; Cook and McArthur, 2013; Harding *et al*., 2013), particularly the humoral response which has a critical role in the insect immune system by regulating the production of anti-microbial peptides (AMPs) and other effectors in response to infection (Leclerc and Reichhart, 2004). AMPs are peptides that kill microorganisms (Rolff and Schmid-Hempel, 2016), although they can have additional functions. They have been observed to have roles as immunomodulators, regulating inflammatory pathways, particularly in vertebrates (Mansour *et al*., 2014). In addition, they have been found to have an important role in mediating interactions between the host and commensal bacteria in invertebrates (Rolff and Schmid-Hempel, 2016). For example, the expression of the AMP *attacin* is thought to reduce the survival of trypanosomes in the gut of *Glossina* spp. (Tsetse flies; Hu and Aksoy, 2006). Six AMPs have been identified from *G. mellonella* to date (Schuhmann *et al*., 2003; Lee *et al*., 2004; Wedde *et al*., 1998; as described in Section 1.7.2.4) and have been used in studies of its response to infection (Mowlds *et al*., 2008; Mowlds and Kavanagh, 2008; Wojda *et al*., 2009). However, little is known about whether insect pathogens utilise counter measures against AMPs and other insect immune defence molecules. Since cordycepin is known to have anti-inflammatory effects in mammalian systems (Kondrashov *et al*., 2012) this raises the question about whether it functions as an immune inhibitor during infection by *C. militaris*.

Two AMPs (gallerimycin and galiomicin) and two other insect defence molecules (IMPI and lysozyme) were selected in the present study to characterise the humoral response of *G. mellonella* to infection, as they have been used previously to quantify the immune response of this insect (Altincicek and Vilcinskas, 2006; Wojda *et al*., 2009). Schuhmann *et al*. (2003) first cloned the AMP gallerimycin from *G. mellonella* and found it to have anti-fungal properties; it inhibited the growth of *M. anisopliae s.l*., but not *S. cerevisiae*. It was later classified as a defensin, a class of AMPs present in both plants and animals (Langen *et al*., 2006) which have a conserved structure, although their targets and modes of action differ (Aerts *et al*., 2008; Yi *et al*., 2014). Some defensins such as defensin A and B in northern blowfly (*Phormia terranovae*) target gram-positive bacteria (Lambert *et al*., 1989) while others such as the defensin in the European bumblebee (*Bombus pascuorum*) inhibit growth of gram-positive bacteria, gram-negative bacteria and fungi (Rees *et al*., 1997). Galiomicin is another antifungal defensin from *G. mellonella* (Mak and Zdybicka-barabas, 2010), it has been found to kill filamentous fungi such as *Fusarium oxysporum* and yeasts including *Candida albicans* (Lee *et al*., 2004). Its expression also increases following challenge with the bacteria *Escherichia coli* and *Bacillus subtillis*, although it does not inhibit their growth (Lee *et al*., 2004).

Metalloproteases are reported to play an important role as virulence factors in insect pathogens (Vallet-Gely *et al*., 2008). For example, the bacteria *Bacillus thuringiensis* and *Pseudomonas entomophila* both produce metalloproteases to facilitate infection of insects. It is hypothesised that they either degrade AMPs or destroy tissues to facilitate the spread of the pathogen in the insect (Vallet-Gely *et al*., 2008). Metalloproteases are also produced by EPF such as *Metarhizium anisopliae s.l*., where they have been conjectured to have a role in degradation of the insect cuticle or defence molecules (Qazi and Khachatourians, 2007; Mukherjee and Vilcinskas, 2018). *G. mellonella* produces an insect metalloprotease inhibitor (IMPI); this is the only metalloprotease inhibitor discovered so far in insects and plays a role in the innate immune response (Wedde *et al*., 2007). Its expression is stimulated by bacterial and fungal elicitors and it may confer resistance to metalloproteases (Wedde *et al*., 1998).

Another key component of the insect immune system is lysozyme (Section 1.7.3), which cleaves the β-1,4-glycosidic bonds in the peptidoglycan cell wall of bacteria (Jollès and Jollès, 1984). In Lepidoptera, including *G. mellonella*, it has been found to have activity against both gram-negative bacteria and gram-positive bacteria (Yu *et al*., 2002). Its production can also be activated by fungal elicitors such as zymosan, a polysaccharide fungal cell wall component, and by living yeast cells, suggesting that it may also have activity against fungi (Vilcinskas and Matha, 1997). The mechanism of lysozyme activity against fungi is not well understood, but is thought to promote apoptosis of fungal cells (Sowa-Jasiłek *et al*., 2016).

3.1.2 The immune response of *G. mellonella* to fungal infection

The immune response of *G. mellonella* to fungal infection has been characterised using EPF and non-pathogenic fungi, primarily through monitoring the expression of immune-related genes. Following infection of *G. mellonella* with *C. albicans*, an opportunistic human pathogen, the expression of *galiomicin*, *IMPI* and *gallerimycin* was observed to increase, peaking at 24 h after infection (Mowlds and Kavanagh, 2008). However, as no sham injection control was included it is not possible to draw reliable conclusions from this work. This was subsequently addressed by Rossoni *et al*. (2017) who used appropriate controls and confirmed that the expression of *gallerimycin* and *galiomicin* increased following *C. albicans* treatment.

Similarly, expression of *IMPI*, *galiomicin* and *gallerimycin* was significantly upregulated between 48 h and 72 h after either topical application or injection of *B. bassiana* conidia into *G. mellonella* (Wojda *et al*., 2009; Vertyporokh and Wojda 2017). However, these experiments only measured expression in the fat body and, although this is the primary site of AMP production (Hoffmann and Reichhart, 2002), other cells may also contribute to the immune response (Ryu *et al*., 2006). In addition, these experiments used a limited number of insects, so may not be statistically robust.

3.1.3 Monitoring the progression on EPF infection

As described previously, the EPF infection process is complex, but understanding it is important for evaluating and improving the efficacy of biopesticides. A number of classical approaches have been employed to quantify fungal biomass following treatment of insects with EPF as fungal infection progresses (Lacey, 2012). Dilution plating can be used to quantify the number of CFUs (colony forming units) from either the surface of insects or from macerated insects (Lacey, 2012). A disadvantage of this technique is that it cannot differentiate whether a colony grew from a conidia or mycelial fragment (Bell *et al*., 2009). The number of blastospores in insect haemolymph can also be determined microscopically (Ouedraogo *et al*., 2003), but this does not distinguish between viable and non-viable cells.

More recently, qPCR has been used to monitor the progression of EPF infection in insects. One of the first studies to do this was in the mosquito *Anopheles stephensi* (Bell *et al*., 2009). A qPCR bioassay was designed with species-specific fluorescent probes to quantify the number of conidial genomes within each mosquito for seven days following *B. bassiana* treatment. A gradual increase in the number of conidial genomes was observed until 3-5 days after treatment (depending on dose), after which there was a rapid increase (10-fold change) in the number of conidial genomes. This increase in conidial units correlated with an increase in insect mortality.

A similar increase in the number of fungal genomes following infection was observed for *M. brunneum* (Ríos-Moreno *et al*., 2017). The authors topically infected *G. mellonella* with this EPF and used a SYBR Green-based qPCR assay to quantify the number of *M. brunneum* genomes in the larvae, while also monitoring the amount of destruxin A produced using HPLC. The increase in the number of fungal genomes varied between two *M. brunneum* isolates. One increased until day four after treatment then reduced until the end of the experiment, while the other showed an initial increase in the number of fungal genomes up to day two, followed by a decrease until day four, then another increase until the end of the experiment. There was also a large variation in destruxin production between isolates. In the studies by both Bell *et al*. (2009) and Ríos-Moreno *et al*. (2017) insects were treated with high concentrations of conidia,

which would not be representative of natural infection. These experiments would therefore be informative for studies of biopesticide application, but would need to be adapted to investigate the natural progression of infection.

3.1.4 Aims and objectives

The overall aim of the research described in this chapter was to characterise the immune response of *G. mellonella* to EPF in order to develop a system that could be used later to quantify the impact of cordycepin on the expression of these genes. This was achieved through two objectives, firstly the expression of immune-related genes was quantified and secondly the progression of EPF infection was monitored.

The expression of four immune-related genes (*gallerimycin*, *galiomicin*, *IMPI* and *lysozyme*) was monitored as they had been previously reported to respond to EPF infection (Wojda *et al.*, 2009; Vertyporokh and Wojda, 2017). The expression levels of these immune-related effector genes are directly related to the activity of the *G. mellonella* immune system in response to fungal infection. The response to a non-pathogenic fungus was also included to allow comparison to EPF. In addition, the progression of EPF infection was quantified with the objective of relating this to immune-related gene expression.

3.2 Materials and methods

3.2.1 Insect material

All experiments used final instar *G. mellonella* larvae, which were purchased and maintained as described in Section 2.2.1.

3.2.2 Fungal material

Two isolates of EPF were used for these experiments, *B. bassiana* 433.99 and *C. militaris* 11703 (Table 2.1). These isolates showed different levels of virulence in *G. mellonella* bioassays (Chapter 2) and *B. bassiana* 433.99 (GHA strain) has been widely investigated as a biopesticide (Wraight *et al*., 2000; Liu and Bauer, 2006; Castrillo *et al*., 2010). The maintenance, sub culturing and preparation of conidial suspensions was carried out as in Section 2.2.2. Aliquots (10 ml) of conidia were initially prepared at a concentration of $1x10^7$ conidia ml^{-1} and diluted as required for each experiment.

3.2.3 Timecourse experiments

3.2.3.1 Treatment of *G. mellonella* with *S. cerevisiae*

The expression of immune-related genes in *G. mellonella* was monitored following treatment with the non-pathogenic fungus *Saccharomyces cerevisiae*, to establish their activity during a successful immune response. A suspension of *S. cerevisiae* (Type II, Sigma Aldrich) was prepared by dilution in sterile distilled water and the cell density determined using an improved Neubauer haemocytometer. This suspension was further diluted with sterile distilled water to give a final density of approximately 3333 cells ml^{-1}. *G. mellonella* larvae were injected with 30 µl of this cell suspension as described in Section 2.2.5, to give an estimated dose of 100 cells per larva. Five larvae were treated for each treatment at each timepoint and two replicates were performed. Larvae were placed in 9 cm Petri dishes lined with filter paper (Whatman, GE Healthcare, U.K), and were maintained at 20 ˚C in a 16:8 h light:dark cycle throughout

the experiment. Larvae were sampled at 8, 48 and 72 h after treatment by snap freezing in liquid nitrogen. These times were chosen based on published immune-gene responses to *B. bassiana* injection (Wojda *et al*., 2009). RNA extraction and RT-qPCR were performed as described in Section 3.2.3.3.

3.2.3.2 Treatment of *G. mellonella* with *B. bassiana* 433.99 conidia

G. mellonella larvae were either sprayed or injected with *B. bassiana* 433.99 conidia as described in Sections 2.2.5 and 2.2.6. Final instar larvae were injected with approximately 100 conidia per larva using a 0.3 ml MicroFine insulin syringe (BD, U.K) or 4 ml of $1x10^6$ conidia ml^{-1} was applied topically using a Potter tower air atomising sprayer at 34.5 KPa. These concentrations were selected as levels of mortality were similar between treatments. Within each replicate, larvae were kept on filter paper (damp filter paper following spray application) in a group with all other individuals to be sampled at the same timepoint. The dishes were sealed with Parafilm 'M' ® (Bemis, USA) and maintained at 20 °C and 16:8 h light:dark cycle throughout the experiment. For all experiments larvae were sampled by snap freezing in liquid nitrogen and stored at -80 °C until RNA extraction. RNA extraction, cDNA synthesis and qPCR are described in Section 3.2.3.3.

Two timecourse experiments were set up to elucidate the impact of injection of *B. bassiana* 433.99 conidia on *G. mellonella* immune-related gene expression. In the first timecourse larvae were injected with *B. bassiana* 433.99 conidia or 0.01% Triton X-100, then sampled at 15 min, 2, 4, 8, 16, 24, 48 and 72 h after treatment. Five larvae were sampled for each treatment at each timepoint in two independent replicates. An additional timecourse was also performed where larvae were injected with *B. bassiana* 433.99, 0.01% Triton X-100 or remained untreated, then sampled at 24, 48 and 72 h after treatment. This was repeated on three occasions and within each replicate five larvae were sampled for each treatment at each timepoint.

A third timecourse experiment was performed to assess the impact of topical application of *B. bassiana* 433.99 conidia on immune-related gene expression in *G. mellonella*. Larvae were sampled at 24, 48, 72 and 96 h after treatment. The

experiment was replicated on two occasions, with five larvae being sampled for each treatment on each timepoint in each replicate.

3.2.3.3 RNA extraction from *G. mellonella*

Previously published experiments have extracted RNA from either just the *G. mellonella* fat body (Wojda *et al*., 2009) or the whole larvae (Vogel *et al*., 2011), so pilot experiments were done to determine the most effective means of extracting RNA for this work. RNA was extracted from three different types of larval tissue: fat body, haemolymph and whole larvae. Haemolymph was extracted from larvae that had been chilled on ice for 5 min by piercing the front right proleg, applying pressure to the abdomen and collecting liquid in a chilled 1.5 ml Eppendorf tube. The maximum volume of haemolymph obtained by this method was approximately 10 µl. To extract the fat body, larvae were chilled on ice for 5 min and incised ventrally along the abdomen. Pressure was applied until the fat body was forced out of the larvae and haemolymph was removed by absorption with tissue. The fat body was excised and placed in a chilled 1.5 ml Eppendorf tube. All tissues were frozen in liquid nitrogen and ground using an autoclaved mortar and pestle (9 cm diameter). Following these experiments, the whole insect extraction method was selected.

The phenol: chloroform method, as described in the Sigma-Aldrich (USA) Tri-reagent product information, was used for RNA extraction from tissues. Briefly, 1 ml of Tri-reagent (Sigma-Aldrich, USA) was added to the ground tissue, mixed by pipetting and centrifuged at 4 ˚C and 12,000 g for 10 min. Following this, the supernatant was removed and incubated for 5 min at room temperature, after which 200 µl of chloroform was added and the mixture shaken for 15 s by hand. This was centrifuged at 4 ˚C, 12,000 g for 15 min and the colourless, upper aqueous phase containing RNA was removed. Following this, 500 µl of isopropanol (Scientific Laboratory Supplies Ltd., U.K) was added, mixed and centrifuged for 10 min at 12,000 g at 4 ˚C. The resulting RNA pellet was washed in 70% ethanol and re-suspended in 50 µl DEPC-treated water (Fisher Scientific, USA). To remove any DNA contamination, the RNA was treated with DNase I (ThermoFisher Scientific, USA) following the manufacturer's instructions. The concentration of RNA was determined using a

NanoDrop ® ND-100 spectrophotometer (ThermoFisher Scientific, USA) and stored at -80 ˚C until use.

3.2.3.4 cDNA preparation

RNA (5 μg) was reverse transcribed using Superscript II (ThermoFisher Scientific, USA) according to the manufacturer's guidelines (ThermoFisher Scientific, USA). The cDNA was diluted 1 in 5 in DEPC-treated water (ThermoFisher Scientific, USA) and stored at -20˚C.

3.2.3.5 Quantitative PCR

Expression of *gallerimycin*, *galiomicin*, *IMPI* and *lysozyme* was analysed using qPCR and normalised to the expression of the ribosomal protein *S7e* (a housekeeping gene). Primers for all genes are detailed in Table 3.1.

Table 3.1- Primers used for qPCR. A list of published primers used for RT-qPCR analysis of *Galleria mellonella* immunity-related genes.

Gene	Primer sequence (5'-3')	Reference
gallerimycin	TATCATTGGCCTTCTTGGCTG GCACTCGTAAAATACACATCCGG	Wojda and Jakubowicz, 2007
galiomicin	TCGTATCGTCACCGCAAAATG GCCGCAATGACCACCTTTATA	Wojda *et al*., 2009
lysozyme	TCCCAACTCTTGACCGACGA AGTGGTTGCGCCATCCATAC	Altincicek and Vilcinskas, 2006
Insect metalloprotease inhibitor (IMPI)	AGATGGCTATGCAAGGGATG AGGACCTGTGCAGCATTTCT	Altincicek and Vilcinskas, 2006
S7e	ATGTGCCAATGCCCAAGTTG GTGGCTAGGCTTGGGAAGAAT	Wojda and Jakubowicz, 2007

QPCR was performed using a Lightcycler 480 (Roche Holding AG, Switzerland). Each reaction was composed of the following: 5 µl SensiFAST SYBR No-ROX (Bioline, USA), 2 µl DEPC-treated water, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM) (Table 3.1) and 1 µl cDNA prepared in Section 3.2.3.4. The PCR conditions were adapted from Wojda and Jakubowicz, (2007): initial denaturation at 95 ˚C for 2 min, followed by 40 cycles of 95 ˚C for 5 s, 60 ˚C for 10 s and 72 ˚C for 20 s. The Ct (cycle threshold) values were normalised against the housekeeping gene *S7e*, then the expression on day zero to give the ΔΔCt values, $2^{-\Delta\Delta Ct}$ values (Livak and Schmittgen, 2001) were analysed using a Kruskal-Wallis test in SPSS Statistics (Version 24, IBM).

3.2.4 Quantification of *B. bassiana* 433.99 conidia on the surface of *G. mellonella* following topical application

The number of conidia adhering to the surface of *G. mellonella* larvae was quantified following topical application of 4 ml of $1x10^6$ conidia ml^{-1} *B. bassiana* 433.99 conidia using a Potter tower air atomising sprayer (Section 2.2.6). This concentration was selected as it was used during the timecourse experiment described in Section 3.2.3 due to mortality being similar to that following inoculation with 100 *B. bassiana* 433.99 conidia. It was desirable to determine the number of conidia on the surface of the *G. mellonella* larvae to allow further comparison between inoculation and spraying treatments. Groups of ten larvae were either sprayed with 4 ml of $1x10^6$ conidia ml^{-1} *B. bassiana* 433.99 conidia or 0.01% Triton X-100 and allowed to air dry. They were then placed individually into a 1.5 ml Eppendorf tubes containing 1 ml of 0.01% Triton X-100 and vortexed for 1 min. Larvae were removed and 100 µl of the suspension was spread onto each of two 9 cm Petri dishes containing SDA with 0.06% (v/v) dodine, a semi-selective medium to select for *B. bassiana* (adapted from Inglis *et al.*, 2012). The plates were incubated for approximately five days at 23 ˚C in darkness (LMS Incubator, U.K). When colonies became visible they were counted. This experiment was replicated on three occasions and the mean number of conidia per larva calculated.

3.2.5 Development of primers for the quantification of *B. bassiana* 433.99 and *C. militaris* 11703 conidia from infected *G. mellonella*

In order to relate the progression of fungal infection to the immune response, a bioassay was carried out in which *G. mellonella* larvae were injected or sprayed with *B. bassiana* 433.99 or *C. militaris* 11703 conidia and the progression of infection monitored. Injection and spraying were carried out as described in Sections 2.2.5 and 2.2.6 at 100 conidia per larva for injection and $1x10^6$ conidia ml^{-1} for spraying. Three larvae were snap frozen in liquid nitrogen every 24 h for seven days and stored at -80 ˚C. Larvae were then individually weighed and freeze-dried using a Modulyo ® freeze drier (Edwards, U.K). Larvae were individually weighed again and ground in liquid nitrogen using a mortar and pestle. DNA was extracted from 50 mg of tissue using a Qiagen DNeasy plant mini kit following the manufacturer's instructions.

In addition, DNA was extracted from a known concentration ($5x10^7$) of both *B. bassiana* 433.99 and *C. militaris* 11703 conidia for use as a standard in qPCR analysis. A conidial suspension was prepared for both EPF from 10-day old plates as described previously and the concentration adjusted to $1x10^7$ conidia ml^{-1}. Aliquots (5 ml) were centrifuged at 12,000 g for 2 min and supernatant discarded. Ten glass beads and 0.5 g of silica beads were added to the tube and this was shaken in a FastPrep-24 (MP Biomedicals, USA) for three cycles of 20 s at a speed setting at 4.5. DNA was extracted using a Qiagen DNeasy plant mini kit (Qiagen, The Netherlands) following the manufacturer's guidelines.

Primers were designed for the genes in Table 3.2 and checked for specificity using the NCBI primer designing tool (Ye *et al.*, 2012). Initially they were used to amplify EPF DNA in a conventional PCR, either extracted from *G. mellonella* during infection or from pure conidia, using the method described in Section 2.2.4. The PCR products were visualised by electrophoresis using a 2% agarose gel and GelRed (Section 2.2.4). Primers that resulted in good amplification were selected for qPCR.

Table 3.2 - Primers designed for biomass assessment of entomopathogenic fungi. A list of primers developed in this project to quantify the number of *Beauveria bassiana* 433.99 and *Cordyceps militaris* 11703 genomes in insects.

Fungus	Gene	Primer name	Primer sequence (5'-3')
B. bassiana 433.99	Actin	BACT.F	CTCGACTCTGGTGATGGTGT
		BACT.R	CACGCTCAGCAAGGATCTTC
	Elongation Factor 1-alpha	BEF.F	CTCGAACCAGCAATCTTCCG
		BEF.R	GCAAGCGATCAAGGTCCATT
	Beta-tubulin	BET.F	CCATTGCTCTTGTTGACCCC
		BET.R	CCTCGGACCCATTTCAGACT
	28S	B28.F	AAAGCCAACACTACCAAGAG
		B28.R	TCGCAGAACCCACTACCATT
C. militaris 11703	Actin	CACT.F	TGCAAAAGGAGTTGACTGGC
		CACT.R	GCCAGCCTCATCATACTCCT
	Elongation Factor 1-alpha	CEF.F	CCGTGTCGAGACTGGTATCA
		CEF.R	CACGACGGATTTCCTTGACG
	Beta-tubulin	CET.F	CATGTCCAACACTACCAAGAG
		CET.R	ACGAGCTGATGTACGGAGAG
	28S	C28.F	CATTTCAACCCTCGACGTCC
		C28.R	TGATCCGAGGTCAACGTTCA

3.2.6 Quantification of *C. militaris* 11703 and *B. bassiana* 433.99 conidia from infected *G. mellonella*

The 28S primers (Table 3.2) were selected from Section 3.2.5 to quantify *C. militaris* 11703 and *B. bassiana* 433.99 DNA from infected *G. mellonella* (Section 3.2.5). DNA was quantified using SensiFast No-ROX SYBR Green (Bioline, USA) and the conditions described in Section 3.2.3.5. The efficacy of amplification was assessed by observing the melt curve produced during heating to 97 ˚C and cooling. Due to the high copy number of this gene it is likely that differences in pipetting could introduce a high level of variation at low concentrations of DNA, therefore the detectable limit for the number of genomes was set at 1000 (Bell *et al*., 2009).

3.3 Results

3.3.1 RNA extraction from *G. mellonella* larvae

The concentration of RNA obtained from *G. mellonella* haemolymph was too low to be detected using the NanoDrop ® ND-100, so this technique was not used in later experiments. On the other hand, it was possible to extract a high concentration of RNA from the *G. mellonella* fat body and whole larvae, both gave successful amplification during qPCR. However, extraction of RNA from the fat body was found to be laborious and time consuming, so all extractions for gene expression analysis were from whole larvae, enabling a greater number of larvae to be processed at any one occasion.

3.3.2 The response of *G. mellonella* immune-related genes to *S. cerevisiae* treatment

RT-qPCR was used to monitor the expression of immune-related genes to injection with *S. cerevisiae* cells, to quantify a successful immune response. In some cases, RT-qPCR was unsuccessful (there was low/no amplification), which may have been due to error in RNA extraction or cDNA synthesis. These samples were discarded, leaving 9-10 samples per timepoint. The expression of the immune-related genes *gallerimycin*, *galiomicin*, *IMPI* and *lysozyme* were monitored following treatment with *S. cerevisiae*. There were no significant differences in expression of *gallerimycin* or *galiomicin* between larvae that were uninjected, injected with water or injected with *S. cerevisiae* (Figure 3.1). There was a significant increase (~4.5-fold) in *lysozyme* expression in larvae injected with water compared to the uninjected control at 48 h ($p=0.012$). There was also a significant increase in *lysozyme* expression 72 h after treatment in yeast-injected larvae compared to uninjected larvae ($p=0.018$), with expression of treated larvae being ~3.5-fold greater than the uninjected control. However, in both of these cases there was no significant difference between the larvae injected with yeast or water.

Similarly, there was a significant increase in *IMPI* expression in larvae injected with water or yeast 48 h after treatment, with expression of both being ~3.5 times greater

than the control (p=0.002). At 72 h there was a significant difference between expression in the uninjected control compared to the larvae injected with yeast (p=0.018), but neither of them was significantly different to the control injected with water.

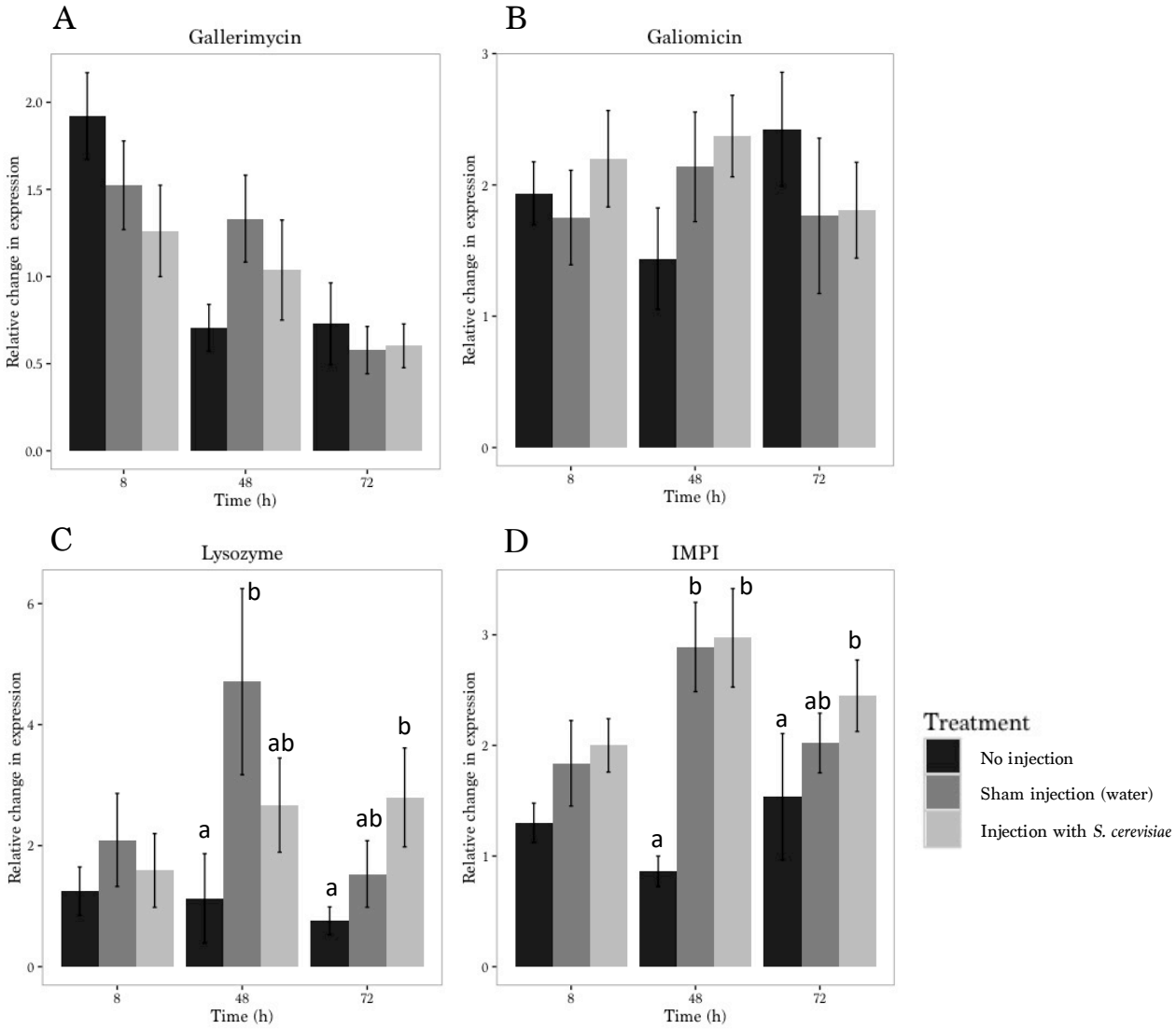


Figure 3.1- Expression of immune-related genes in *Galleria mellonella* following injection of *Saccharomyces cerevisiae*. Change in expression of A) *gallerimycin*, B) *galiomicin*, C) *lysozyme* and D) *IMPI* in *G. mellonella* (n=10 per treatment) following injection with 100 *S. cerevisiae* cells (pale grey), injection with water (mid grey) and no injection (dark grey). Expression was normalised against *S7e* expression and expression at day 0. A Kruskall-Wallis test was carried out at each time point to identify significant differences in expression, different letters indicate significant differences ($p<0.05$). Error bars show ±SEM.

3.3.3 The response of *G. mellonella* immune-related genes to injection with *B. bassiana* 433.99

RT-qPCR was employed to monitor the expression of immune-related genes in *G. mellonella* in response to injection with *B. bassiana* 433.99 conidia. In some cases, qRT-PCR was unsuccessful (there was low/no amplification), which may have been due to error in RNA extraction or cDNA synthesis. These samples were discarded, leaving 8-10 samples per timepoint for the first injection timecourse and 13-15 for the second.

Immune-related gene expression was initially monitored at: 0.25, 2, 4, 8, 16, 24, 48 and 72 h after treatment with *B. bassiana* 433.99 conidia. Larvae injected with Triton X-100 were compared to those injected with conidia (Figure 3.2). There was a significant increase (~2.5-fold) in the expression of *gallerimycin* in larvae treated with *B. bassiana* 433.99 compared to sham-injected larvae at 72 h after treatment ($p=0.007$). There was also an ~2-fold increase in *galiomicin* expression in larvae treated with *B. bassiana* 433.99 compared to sham-injected larvae at 4 h ($p=0.003$) and ~3.5-fold increase 8 h after treatment ($p=0.004$). There was a significant increase in *IMPI* expression in larvae treated with *B. bassiana* 433.99 relative to the sham-injected control at 2, 4 and 72 h after treatment of ~2-fold ($p=0.028$), ~2.5-fold ($p=0.004$), ~4-fold ($p=0.011$), respectively.

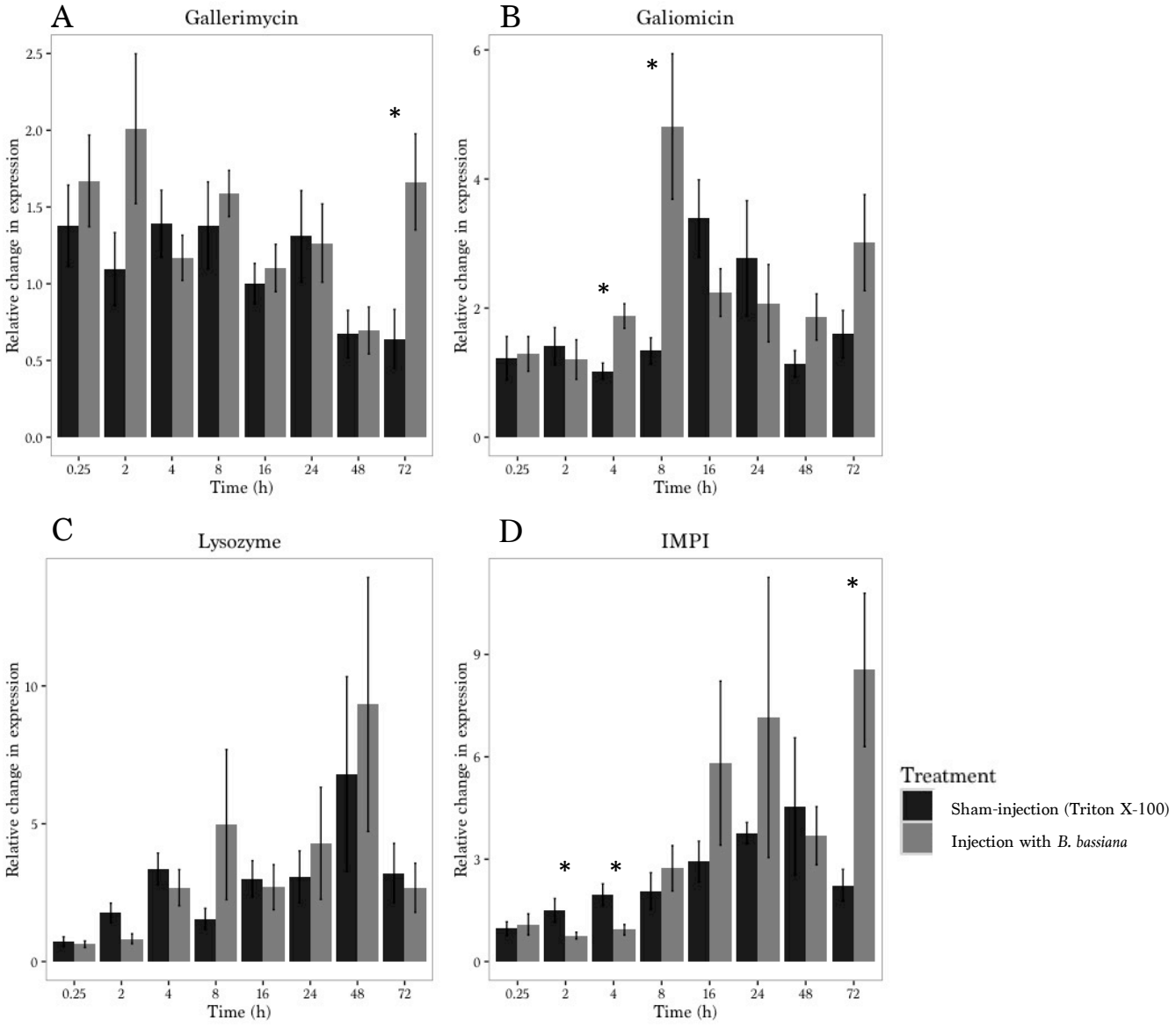


Figure 3.2- Expression of immune related genes following injection with *Beauveria bassiana* 433.99 conidia. Change in expression of A) *gallerimycin*, B) *galiomicin*, C) *lysozyme* and D) *IMPI* in *G. mellonella* (n=10 per treatment) following injection with 100 *B. bassiana* 433.99 conidia (mid-grey) and injection with Triton X-100 (dark grey). Expression was normalised against S7e expression and expression at day 0. A Kruskall-Wallis test was carried out at each time point to identify significant differences in expression, * indicates significant differences ($p<0.05$). Error bars show ±SEM.

Following the experiment in Figure 3.2, it became clear that an uninjected control was necessary at all time points due to the wounding response caused by Triton X-100 injection. Therefore, immune-related gene expression was monitored again at 24, 48

and 72 h post-injection (Figure 3.3). *Gallerimycin* showed ~2-fold greater expression in infected larvae relative to the uninjected control at 48 h after treatment ($p=0.013$), but the increase was not significantly different from the injected control. At the same timepoint, *galiomicin* expression increased ~2.5-fold in infected larvae compared to the uninjected control ($p=0.001$), but again this was not significantly different from the injected control.

At 72 h after treatment the expression of *galiomicin* was ~2-fold higher in infected larvae than either the uninjected ($p=0.016$) or the sham-injected control larvae ($p=0.021$). The expression of *IMPI* at 72 h in infected larvae was ~2-fold greater than uninjected controls ($p=0.048$), but not significantly different from the injected controls. At 24 h after treatment *lysozyme* expression in infected larvae was ~9-fold higher than in uninjected larvae and at 72 h it was ~5-fold higher (at 24 h $p<0.001$, at 72 h $p=0.01$). However, expression was not significantly different from the injected control. At 48 h after treatment, the expression of *lysozyme* in larvae injected with only Triton X-100 was significantly higher than the uninjected control ($p=0.001$), but not significantly different from infected larvae. The expression of immune-related genes appeared greater following the sham injection than the uninjected control for most treatments, although this was mostly not statistically significant. This suggests that there is a wounding response following injection.

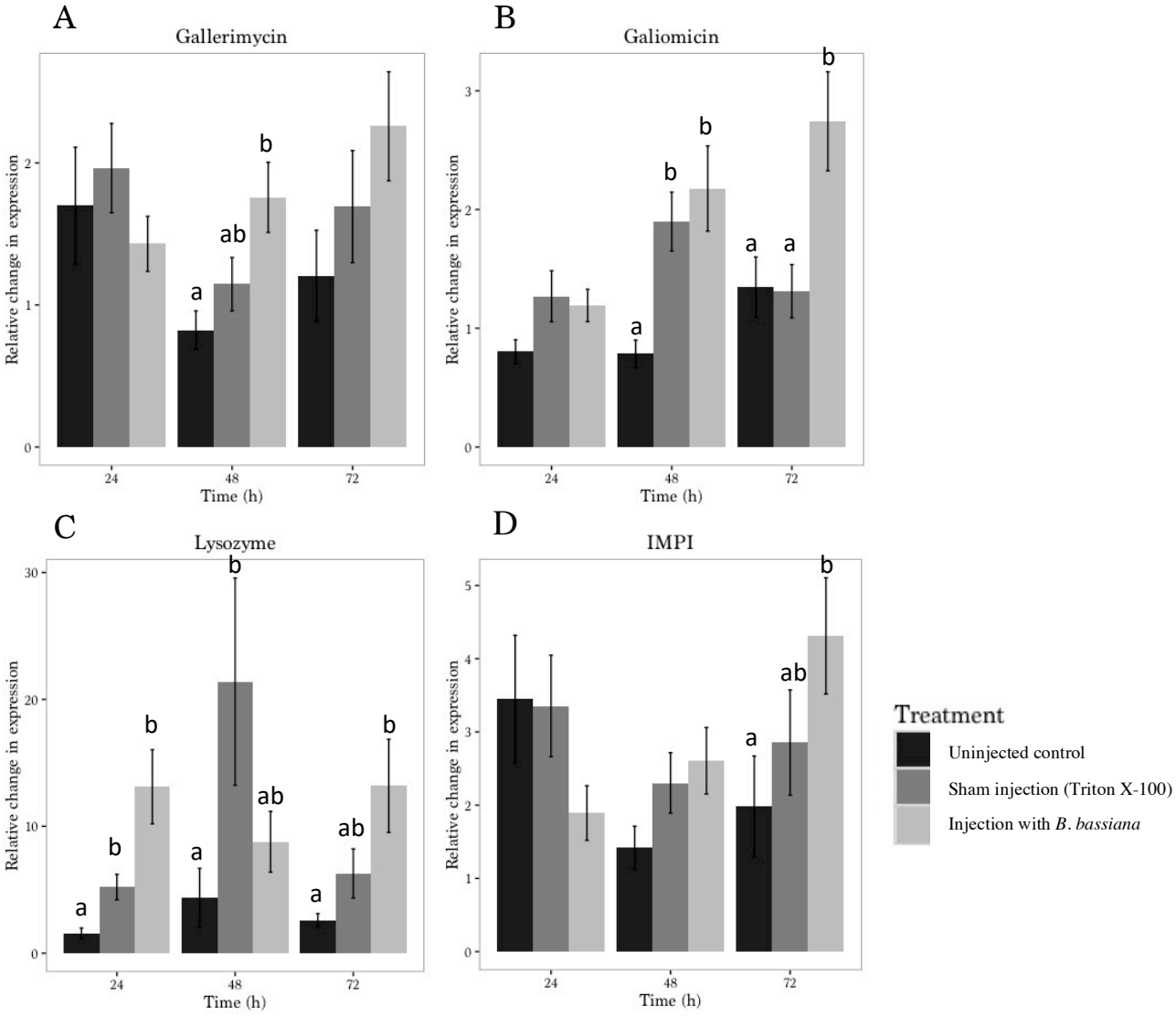


Figure 3.3-Expression of immune-related genes in *Galleria mellonella* following injection with *Beauveria bassiana* 433.99 conidia. Change in expression of A) *gallerimycin*, B) *galiomicin*, C) *lysozyme* and D) *IMPI* in *G. mellonella* (n=12-15 per treatment) following injection with 100 *B. bassiana* conidia (pale grey), injection with Triton X-100 (mid grey) and no injection (dark grey). Expression was normalised against S7e expression and expression at day 0. A Kruskall-Wallis test was carried out at each time point to identify significant differences in expression, different letters indicate significant differences ($p<0.05$). Error bars show ±SEM.

3.3.4 The response of the *G. mellonella* immune system to topical application of *B. bassiana* 433.99

In order to circumvent the issues of the immune response being triggered by wounding (injection), *B. bassiana* 433.99 conidia were applied topically then the response of immune-related genes monitored using RT-qPCR. In some cases, qRT-PCR was

unsuccessful (there was low/no amplification), which may have been due to error in RNA extraction or cDNA synthesis. These samples were discarded, leaving 8-10 samples per timepoint. When *B. bassiana* 433.99 was applied topically to final instar *G. mellonella* no change in gene expression was observed until 72 h, except for expression of *lysozyme* (Figure 3.4, Table 3.3). Expression of *gallerimycin* was ~2.5-fold higher than the control at 72 h and over 15-fold greater than the control at 96 h. Expression of *galiomicin* was ~1.5-fold higher than the control at 72 h and 2.5-fold higher at 96 h. At 72 h after treatment expression of IMPI was ~2.5-fold higher than the control, then at 96 h expression was ~6-fold higher than the control. Finally, expression of lysozyme showed the greatest response to infection, with expression in infected larvae at 72 h being ~9.5-fold higher than the control and at 96 h it was over 80-fold higher than the control. Although the expression of *lysozyme* was found to be significantly less in infected larvae than in the control larvae at 24 h following spraying, this result is probably not meaningful as the difference only amounts to a very small relative change. There was no change in expression of any genes other than *lysozyme* in controls sprayed with Triton X-100 throughout the experiment.

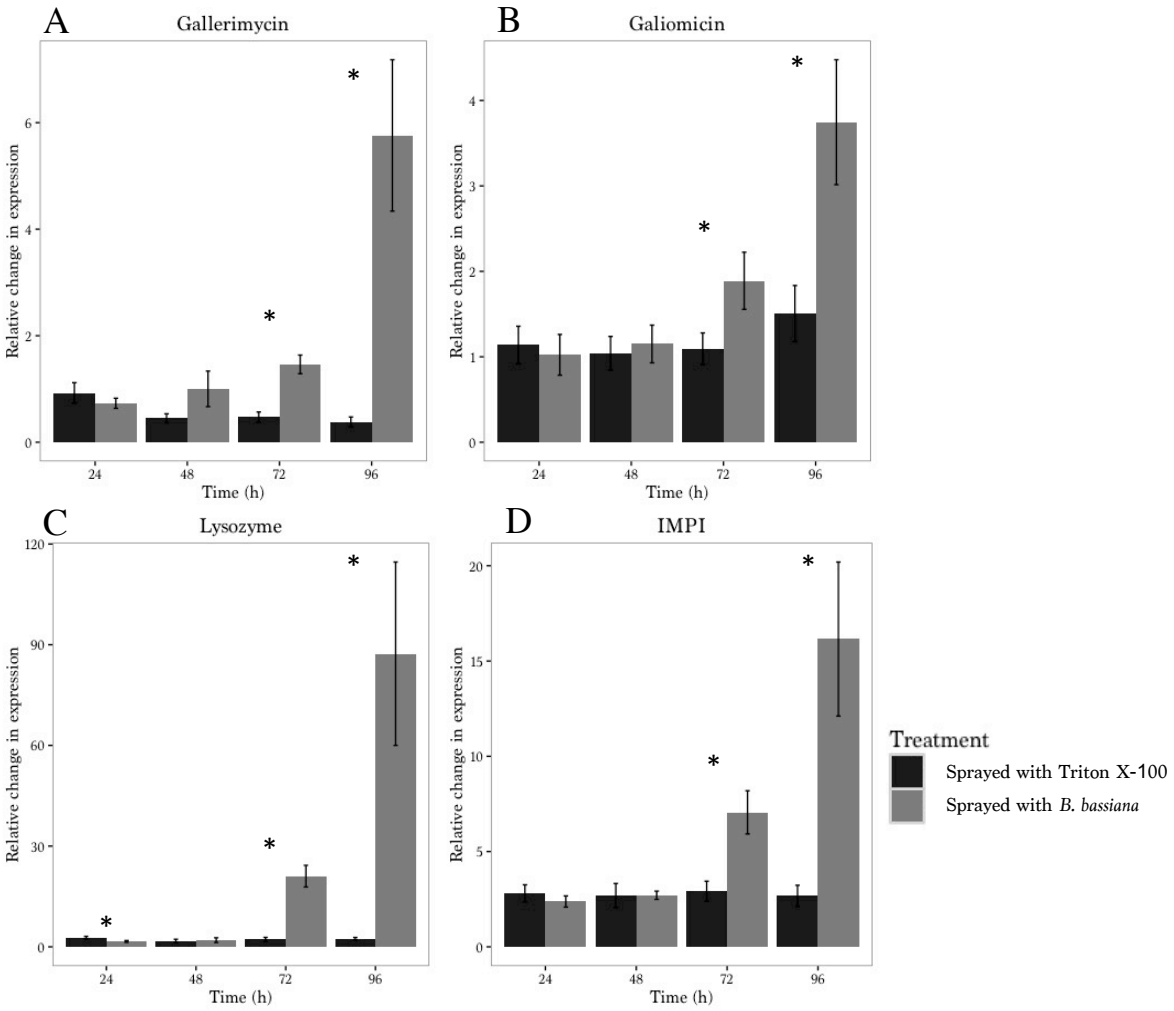


Figure 3.4-Experssion of immune-related genes in *Galleria mellonella* following spraying with *Beauveria bassiana* 433.99 conidia. Change in expression of A) *gallerimycin*, B) *galiomicin*, C) *lysozyme* and D) *insect metalloprotease inhibitor* (*IMPI*) in *G. mellonella* larvae (n=8-10 per treatment) following spraying with 4 ml of $1x10^6$ *B. bassiana* 433.99 conidia ml^{-1} (mid-grey) and spraying with Triton X-100 (dark-grey). Expression was normalised against *S7e* expression and expression at day 0. A Kruskall-Wallis test was carried out at each time point to identify significant differences in expression, asterisks indicate significant differences ($p<0.05$).

Table 3.3- Significance of differences [1] in immune related gene expression following spraying of *Galleria mellonella* with *Beauveria bassiana* 433.99 conidia in comparison to Triton X-100.

Gene	Time (h)	p-value
Gallerimycin	24	0.529
	48	0.353
	72	<0.001*
	96	0.001*
Galiomicin	24	0.796
	48	0.739
	72	0.035*
	96	0.009*
Lysozyme	24	0.029*
	48	0.853
	72	<0.001*
	96	0.003*
Insect metalloprotease inhibitor	24	0.853
	48	0.529
	72	0.002*
	96	<0.001*

[1] A Kruskall-Wallis test was carried out at each time point to identify significant differences in expression, asterisks indicate significant differences (p>0.05).

3.3.5 Quantification of *B. bassiana* 433.99 conidia on the *G. mellonella* surface following topical application

In order to quantify the number of *B. bassiana* 433.99 conidia on the surface of *G. mellonella* larvae following topical application, conidia were washed off larvae, cultured on selective media and the number of colonies counted. The mean number of viable conidia recovered from larvae was 437 (± 50.7), 1 (± 0.48) and 4 (± 2.10) for larvae topically treated with either B. *bassiana* 433.99, Triton X-100 or left untreated, respectively (Table 3.4). The recovery of conidia on the control larvae could indicate the existence of low levels of *B. bassiana* conidia in the environment. However, it is also possible that the Potter tower sprayer was contaminated despite efforts to

decontaminate it after each use, or the conidia of other species were able to germinate on this selective media, although the observed colonies were morphologically identical to *B. bassiana* 433.99.

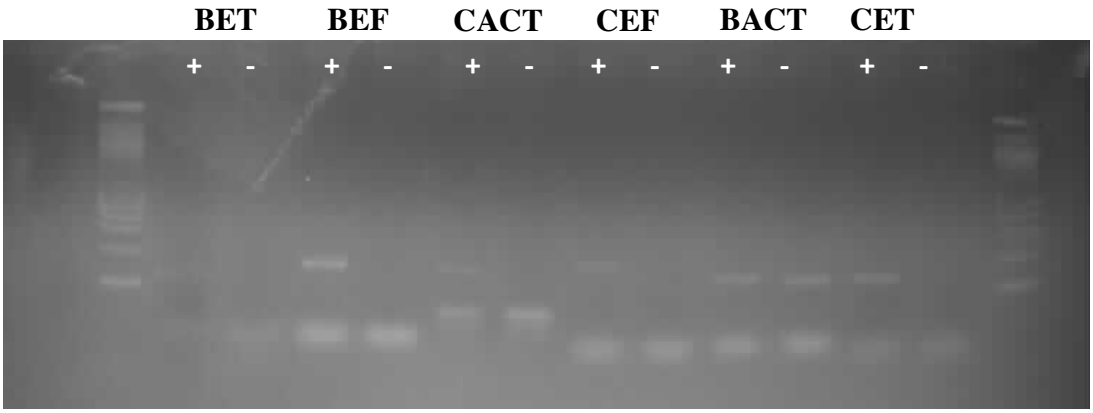
Table 3.4- The number of conidia on *Galleria mellonella* following topical application [1].

Treatment	Mean number of conidia ± SEM
B. bassiana 433.99	436.8 ± 50.7
Triton X-100	1.33 ± 0.48
Unsprayed	4.00 ± 2.10

[1]The mean number of conidia on the surface of each *G. mellonella* larva sprayed with 4 ml of *Beauveria bassiana* 433.99 conidia ($1x10^6$ conidia ml^{-1}) or Triton X-100 using a Potter tower.

3.3.6 Development of primers for the quantification of *B. bassiana* 433.99 and *C. militaris* 11703 conidia from infected *G. mellonella*

Primers were designed to amplify DNA from both *C. militaris* 11703 and *B. bassiana* 433.99, to be used in later qPCR experiments quantifying EPF biomass during infection. The quality of primers was initially assessed by amplification of fungal DNA by PCR, followed by visualisation of the PCR products by agarose gel electrophoresis. Figure 3.5 illustrates these gels for a selection of primers, and shows that B28 and C28 gave the best amplification with minimal primer dimers and so were selected for further analysis.



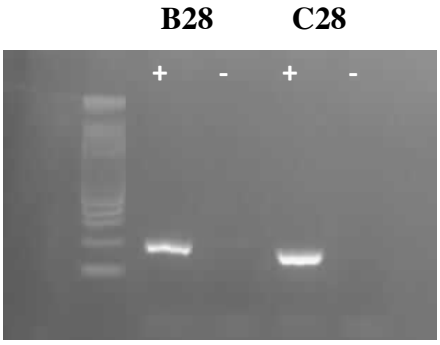


Figure 3.5- Agarose gel showing amplification of *Beauveria bassiana* 433.99 or *Cordyceps militaris* 11703 DNA using primers developed in this project. Agarose gel showing the products of PCR following amplification with primers targeting actin (ACT), elongation factor 1-alpha (EF), beta-tubulin (ET) and 28S (28). Positive controls amplified DNA from either *B. bassiana* (B prefix) or *C. militaris* conidia (C prefix) and negative controls, containing no DNA, were included.

3.3.7 Quantification of *C. militaris* 11703 and *B. bassiana* 433.99 conidia from infected *G. mellonella*

Preliminary qPCR tests showed that at low concentrations of fungal DNA, B28 primers exhibited non-specific binding. The melt curve produced at the end of the qPCR run showed multiple peaks inconsistent with the positive control (Figure 3.6). Non-specific binding was not seen in the negative control but was seen in control larvae that had not been treated with EPF, indicating that the primers had bound to *G. mellonella* DNA to give some low-level amplification that was not detected in a conventional PCR. However, the 28S primers for *C. militaris* 11703 successfully amplified DNA extracted from *G. mellonella* (Figure 3.6). A standard curve was constructed using DNA from a known concentration of *C. militaris* 11703 conidia ($5x10^7$-$5x10^1$ conidia). It was assumed that this would be equivalent to the number of genomes present in the sample *i.e.* DNA from 1000 conidia is equivalent to 1000 genomes.

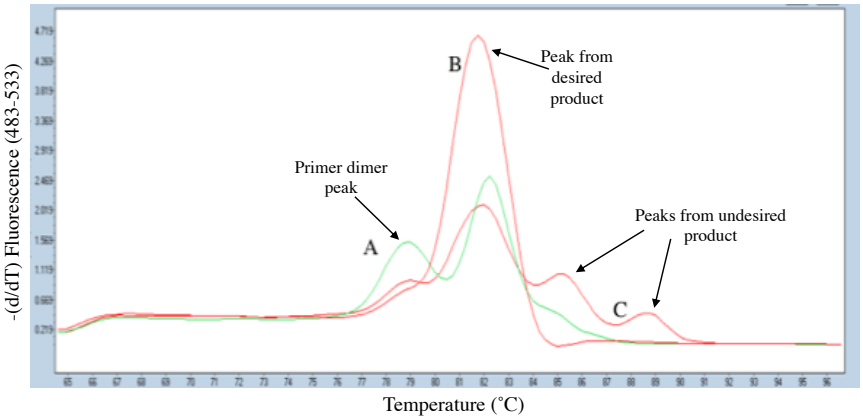


Figure 3.6-Melt curves produced following amplification of fungal DNA with primers designed in this project. Melt curves produced by Lightcycler 480 (Roche) from heating PCR products to 97°C and then cooling. Curves A and C are examples curves produced using B28 primers and *Beauveria bassiana* DNA extracted from infected *G. mellonella*, curve B is produced using C28 primers from *Cordyceps militaris* 11703 DNA from infected larvae.

When *C. militaris* 11703 conidia were introduced by injection, their presence was first detected on day four, but then fell back below detectable levels on days five and six. However, by day seven there was a very large increase in the number of *C. militaris* genomes from <1000 genomes per larva to 5.36 x10^4 genomes per larva, which was more than a 500-fold increase on initially injected dose of conidia. On day seven there was a detectable amount of DNA in some untreated control larvae, which may be due contamination during the injection process or during qPCR (Figure 3.7).

When *G. mellonella* larvae were sprayed with *C. militaris* 11703 conidia an increase in the number of fungal genomes was first detected two days after treatment and rose to approximately 2.00 x 10^7 genomes per larva on day seven. The largest increase in the number of *C. militaris* genomes was between days six and seven, where the number of genomes detected increased approximately 400-fold. There was a low level of *C. militaris* DNA detected on days one and seven after treatment in the control. The number of *C. militaris* genomes detected was higher in larvae that had been sprayed with *C. militaris* 11703 than those injected, but both had a similar pattern of fungal genome accumulation and the rate of mortality was similar. The number of conidia applied during each treatment was also comparable, 100 conidia were delivered by injection and approximately 400 by topical application. There was a slow increase in the number of *C. militaris* genomes between days one to six which was below

detectable levels when this fungus was injected, followed by a rapid increase in the number of fungal genomes between days six and seven.

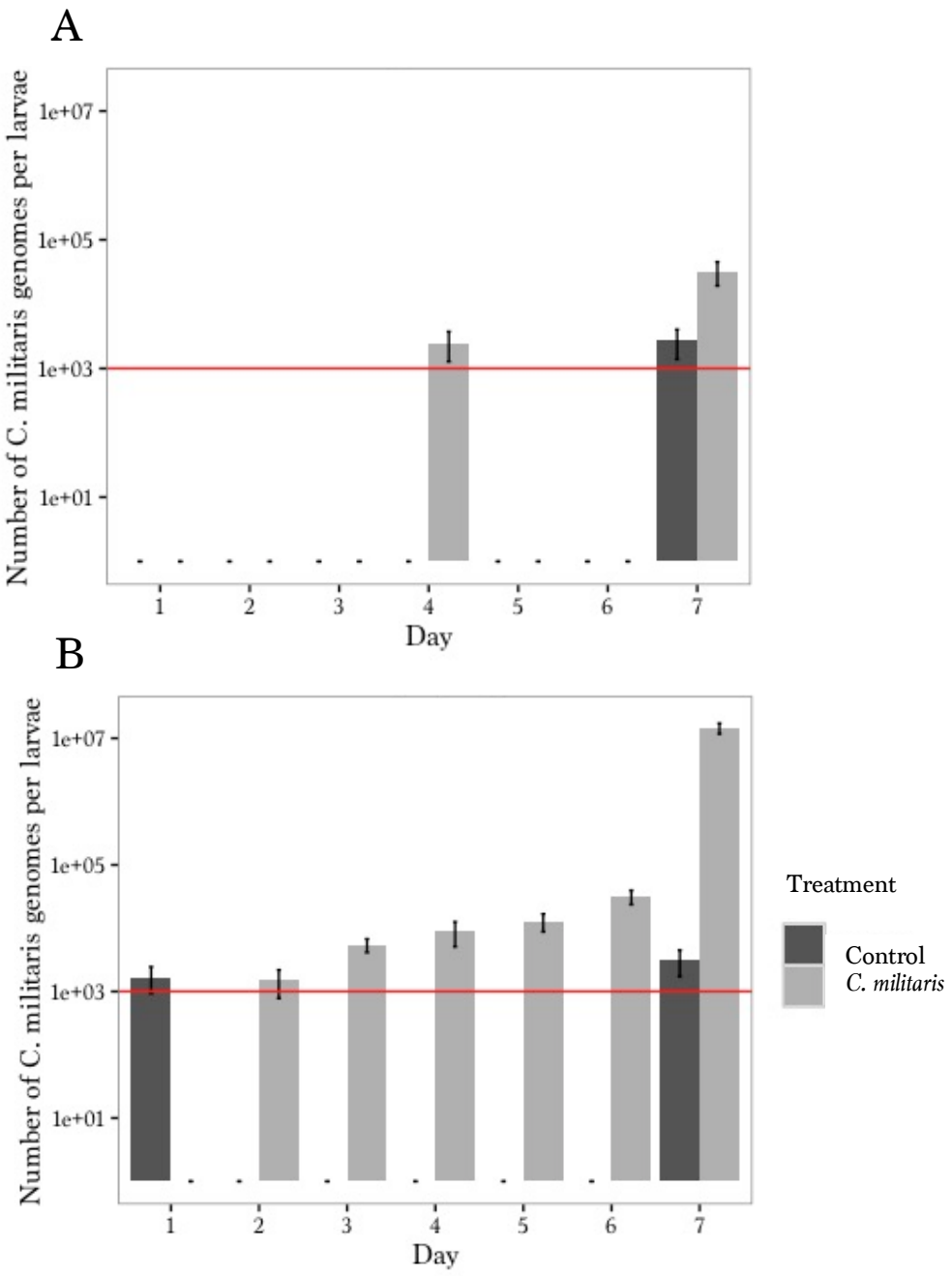


Figure 3.7- The mean number of *Cordyceps militaris* genomes present in *Galleria mellonella* following infection by either spraying or injection with *C. militaris* 11703 conidia. The mean number of *C. militaris* genomes detected using qPCR per *G. mellonella* larvae when injected with 100 conidia per larva (A) or sprayed with 4 ml of $1x10^6$ conidia (B). The cut off for detection was 1000 genomes per larva (red line).

3.4 Discussion

Insects respond to infection through the activation of immune pathways, however, EPF evade or overcome these immune responses to successfully colonise a host. The aim of the work in this chapter was to develop an RT-qPCR system for quantifying the expression of immune-associated genes of *G. mellonella* in response to EPF infection. Initial experiments focused on quantifying the immune response of *G. mellonella* to injection of *B. bassiana* 433.99 conidia over time to determine which timepoints should be investigated in more detail. The response to EPF was compared to a control treatment of larvae injected with Triton X-100. Three of the immune genes tested (*galiomicin*, *gallerimycin* and *IMPI*) showed an increase in expression on at least one time point throughout the experiment relative to the control, illustrating that *B. bassiana* 433.99 conidia are detected by the *G. mellonella* immune system which responds by production of these immune effector proteins. However, a key finding was that sham injection also stimulated an immune response, which made interpretation of immune-related gene expression more difficult. This response to wounding was also observed when *G. mellonella* were injected with water in Section 3.3.2. A general response to wounding has been characterised in other insects (Section 1.7.4), although only a small number of studies have been done to date. Upregulation of AMPs has been observed in *Bombus terrestris* in response to wounding (Erler *et al*., 2011). It was thought that this response may have evolved to prime the immune system for infection as most wounds in nature result in infection. An experiment by Bidla *et al*., (2009) found that the *D. melanogaster* phenoloxidase response is also induced by wounding, leading to melanisation localised around the injection site. Another factor that may have contributed to the response to wounding identified in this chapter could be the presence of pyrogens in the sterile, distilled water used to prepare Triton X-100 for sham injections. Future experiments could be performed using pyrogen free water to quantify the response of immune-related gene expression to wounding in *G. mellonella*.

S. cerevisiae is reported to be non-pathogenic to *G. mellonella* larvae and is a commonly used negative control when assessing the impact of pathogenic yeasts on this insect (Vilcinskas and Matha, 1997; Brennan *et al*., 2002; Reeves *et al*., 2004). The expression of the selected *G. mellonella* immune-related genes to challenge by *S.*

cerevisiae therefore demonstrates what to expect for a successful immune response. In the present study no significant difference was observed in the expression of *gallerimycin* and *galiomicin* between treated and control larvae, suggesting that infection with *S. cerevisiae* is not cleared through production of anti-fungal AMPs. The expression of these AMPs is likely to be driven primarily by the Toll pathway (Lemaitre and Hoffmann, 2007), which is activated by yeasts such as *C. albicans* and *S. cerevisiae* in *G. mellonella* (Bergin *et al*., 2006). It is possible that the Toll pathway was only activated weakly by the low numbers of S. *cerevisiae* used in this study, so was undetectable through RT-qPCR. Alternatively, an early immune response may clear the *S. cerevisiae* infection, so any upregulation in *gallerimycin* or *galiomicin* could have occurred prior to 8 h after treatment (when sampling in this experiment began), or it may be cleared by the cellular immune response. In particular, melanisation and associated reactions are an immediate response to infection (Lemaitre and Hoffmann, 2007) and may clear *S. cerevisiae* rapidly through encapsulation and the production of toxins (Section 1.7.1).

Expression of *lysozyme* was upregulated in larvae treated with *S. cerevisiae* relative to the uninjected control, but was not significantly different from the injected control. Therefore, it is not possible to conclude whether the increase in expression of *lysozyme* was due to challenge with *S. cerevisiae*. *IMPI* expression was also upregulated in response to both wounding and treatment with *S. cerevisiae*. In particular at 48 h after treatment, larvae injected with water or *S. cerevisiae* had significantly higher expression of *IMPI* than the uninjected control. It has previously been reported that IMPI activity increases in *G. mellonella* upon injection with bacterial (LPS) and fungal (zymosan) elicitors up to 48 h after treatment (Wedde *et al*., 1998). However, the authors did not quantify the expression of *IMPI* in response to sham injection, so it is not clear whether the response was caused by the elicitors or wounding. IMPI is thought to be active against bacterial metalloproteases and endogenous matrix metalloproteases, which may be involved in the degradation of AMPs or insect tissues (Wedde *et al*., 2007; Vallet-Gely *et al*., 2008). IMPI is thought to aid in the innate immune response and possibly play a role in metamorphosis. It may have evolved through host-parasite coevolution, driven by the evolution of metalloproteases in *M. anisopliae s.l.* (Joop and Vilcinskas, 2016). The experiments in this chapter suggest

that IMPI plays a role in immunity, although in injection experiments it can be difficult to resolve the response to fungi from the wounding response.

When an untreated control was included, injection of *G. mellonella* larvae with *B. bassiana* 433.99 conidia increased expression of all four of the immune-related genes tested (*gallerimycin*, *galiomicin*, *IMPI* and *lysozyme*) relative to the uninjected control at 48 and 72 h post-injection. However, only *galiomicin* expression was significantly higher than in the sham injected larvae, confirming that expression of this gene is significantly upregulated in response to *B. bassiana* 433.99 infection. This contrasts with the response to *S. cerevisiae*, where any increase in immune-related expression could not be separated from the sham-injection control. This indicates that *B. bassiana* 433.99 induced a stronger immune response in *G. mellonella* than *S. cerevisiae* at the timepoints studied here, which could be due to activation of the Toll pathway by a different mechanism. The Toll pathway is reported to detect yeasts through the GNBP3 receptor, whereas *B. bassiana* is reported to activate the Toll response through the recognition of virulence factors, such as proteases which may give responses of different magnitudes (Gottar *et al*., 2006; Issa *et al*., 2018). Alternatively, *B. bassiana* could elicit a stronger immune response by growing within the larvae, unlike *S. cerevisiae*, making it more likely to be detected by the immune system. Although, the results from Section 3.3.7 suggest that significant *B. bassiana* proliferation does not occur until late in the infection process, there may be slow growth of *B. bassiana* during early infection leading to an increased immune response. The response of *lysozyme* expression to *B. bassiana* 433.99 injection seen in this chapter contrasts with the findings of Vilcinskas and Matha, (1997a), who observed that treatment of *G. mellonella* with *B. bassiana* reduced the activity of lysozyme in comparison to treatment with yeast. This could be due to differences between *B. bassiana* strains or EPF affecting lysozyme activity post-transcription.

A recent study by Vertyporokh and Wojda (2017) characterised the response of *G. mellonella* to *B. bassiana* by injecting *G. mellonella* larvae with 1000 *B. bassiana* conidia and monitoring gene expression using RT-qPCR. The authors identified peaks in expression for all genes tested (*gallerimycin*, *galiomicin* and *IMPI*) at 48 h post injection, which was significantly higher than the uninjected controls. However, they

did not compare this expression to injected controls for all time points, only at 24 h post-injection. These results are similar to the experiments described here, where high expression was identified at 48 h post-injection for *gallerimycin* and *galiomicin*. In addition, this project found that the expression of *galiomicin* increased at 72 h post-injection which is different to the findings of Vertyporokh and Wojda (2017). The difference may be because Vertyporokh and Wojda (2017) monitored expression in the fat body, which is the main site of AMP production, or there was variability in gene expression between individual larva.

In order to better elucidate the response of *G. mellonella* to *B. bassiana* 433.99, conidia were topically applied to larvae to permit natural infection. This eliminated the wounding response, allowing only the response to the fungi to be characterised. There was a strong induction of immune-related gene expression, beginning at 72 h post-spraying and becoming highest at 96 h. All genes tested (*galiomicin*, *gallerimycin*, *IMPI* and *lysozyme*) had significantly increased expression relative to the control. Galiomicin and gallerimycin are anti-fungal AMPs (Schuhmann *et al*., 2003; Mak and Zdybicka-barabas, 2010), therefore their expression would be expected to be upregulated in response to *B. bassiana*. Both IMPI and lysozyme are thought to be anti-microbial, so their increased expression in response to infection would also be expected (Vilcinskas and Matha, 1997; Wedde *et al*., 2007). It was determined that the number of conidia on the surface of each *G. mellonella* larvae following topical application of *B. bassiana* 433.99 conidia was a mean of 400, which is comparable with the number of conidia (100 per larvae) applied during injection gene expression studies. Expression levels of immune-related genes following either topical application or injection of conidia were similar 72 h after treatment. However, identification of significant results was facilitated by topical application of conidia as sham injection triggered an immune reaction that could not always be separated from the response to *B. bassiana* 433.99 conidia.

Vertyporokh and Wojda (2017) also characterised expression of *gallerimycin*, *galiomicin* and *IMPI* over 96 h following topical application of *B. bassiana* conidia. As in this project, they found that *gallerimycin* expression increased over 96 h following topical application. However, they found that *galiomicin* expression

reduced after 48 h, whereas in this experiment expression continued to increase up to 96 h. This may be due to changes being detected more rapidly when analysing only the fat body which is the main site of AMP production, *B. bassiana* strain differences, different doses of conidia or because they analysed five insects per timepoint so variation between individuals could have a bigger impact on the average value reported. The expression pattern of *IMPI* was also different between these studies. Vertyporokh and Wojda (2017) only saw a significant increase in *IMPI* expression after 48 h, in contrast, the results here show an approximately 5-fold increase in expression relative to the control after 96 h. IMPI is a protein unique to *G. mellonella*, homologs have not been identified in other insects so little is known about its production. These results could suggest that cells outside the fat body, such as free haemocytes in the haemolymph, are also responsible for *IMPI* expression. This is supported by a study by Dubovskiy *et al*., (2013) who found that *IMPI* is expressed by cells in both the fat body and integument.

In summary these results indicate that *B. bassiana* is detected by the *G. mellonella* immune system during infection and this triggers the expression of immune-related genes. This may be through the recognition of virulence factors, such as degradative enzymes (Gottar *et al*., 2006; Issa *et al*., 2018). The immune-related genes monitored in this experiment are likely to be induced by different immune pathways, which illustrates that different aspects of the *G. mellonella* immune system work in concert against infection. Expression of *galiomicin* and *gallerimycin* are likely to be controlled by the Toll pathway, as these are antifungal AMPs (Schuhmann *et al*., 2003; Lee *et al*., 2004). Gallerimycin and galiomicin are both defensins (Schuhmann *et al*., 2003; Bergin *et al*., 2006) and the expression of defensins are controlled by the Toll pathway in *D. melanogaster* (Meng *et al*., 1999). On the other hand, lysozyme is constitutively expressed but is upregulated in response to fungi and the method of IMPI induction is not known at this time. Therefore, multiple pathways are integrated in the immune response to *B. bassiana* to drive the expression of immune-related genes.

In order to better understand the progression of EPF infection and relate it to the expression of immune-related genes, experiments were done to quantify EPF growth during infection. The most promising primers amplified the 28S rRNA gene from *C.*

militaris 11703 and *B. bassiana* 433.99. There are likely to be multiple copies of this gene in *B. bassiana* 433.99 and *C. militaris* 11703 because multiple copies of rRNA genes have been observed in other filamentous fungi (Black *et al.*, 2013). The qPCR assay based on 28S rRNA primers for *C. militaris* 11703 allowed detection above approximately 1000 genomes per sample. This cannot be assumed to be equivalent to approximately 1000 fungal cells per sample, because although *C. militaris* has not been reported to produce multinucleated mycelia this is common in other filamentous fungi (Roper *et al.*, 2011). When 28S primers for *B. bassiana* 433.99 were used for qPCR there appeared to be primer dimers and non-specific primer binding, which may have been due to binding to insect DNA or other fungi present.

Low concentrations of *C. militaris* DNA were detected in three negative controls. This may be due to contamination of *G. mellonella* larvae during injection and spraying or contamination of DNA during the DNA extraction process. The number of *C. militaris* 11703 genomes increased throughout the experiment gradually until day seven, where there was a large increase in the number of *C. militaris* genomes per larva. This may be similar to the progression of *B. bassiana* 433.99 infection, but this could not be established during this project due to the non-specific binding of *B. bassiana* primers. This builds on the findings of Ríos-Moreno *et al.* (2017) and Bell *et al.* (2009), who quantified *B. bassiana* and *M. brunneum* DNA from insects. In particular these findings are similar to those of Bell *et al.* (2009), who observed a gradual increase in the number of *B. bassiana* genomes until days 3-5 after treatment followed by a rapid increase in the number of conidial genomes. Bell *et al.* (2009) identified an increase in the number of fungal genomes shortly before larval death, which is similar to the experiment described in this chapter. The concentrations of *C. militaris* 11703 used in this experiment cause mortality after approximately days 6-7 for topical application and later than day 7 when injected (Chapter 2), the rapid increase in the number of *C. militaris* genomes was observed shortly prior to this. The large increase in the number of fungal genomes late in the infection process shown in this and previous studies supports the hypothesis that EPF must first overcome the insect immune response before they are able to proliferate rapidly. It also suggests that the interactions between an EPF and its host constitutes a major part of the infection process, which has implications for selection of EPF biopesticides and strain improvement. In particular

strains that are virulent and kill the insect more rapidly would be desirable, so it is important to identify strains that are able to overcome this insect immune system more rapidly. It also raises a question about how EPF are able to overcome the insect immune system prior to their rapid growth within the host, in particular whether EPF produce chemicals that inhibit host immunity. For example, destruxin A is thought to play a role in suppression of the insect immune response during *Metarhzium anisopliae s.l.* infection (Pal *et al.*, 2007).

3.5 Conclusions

This study has developed methodologies to monitor the expression of immune-related genes in *G. mellonella*, which will permit the effect of cordycepin on the insect immune system to be measured. The experiments in this chapter have demonstrated that there is an increase in the expression of immune-related genes (*galiomicin*, *gallerimycin*, *IMPI* and *lysozyme*) in *G. mellonella* following *B. bassiana* 433.99 topical infection. However, the immune response to injection of *B. bassiana* 433.99 conidia was difficult to separate from the wounding response, therefore topical application of conidia allowed a better understanding of the immune response to infection. Although an attempt was made to link the increase in the number of *B. bassiana* 433.99 genomes to the immune response of larvae, it was unsuccessful. The increase in the number of *C. militaris* 11703 genomes suggested that the growth of EPF is initially impaired by the insect immune response, but at later stages of infection the number of fungal genomes increases rapidly.

Chapter 4- Determining the effect of cordycepin on *Galleria mellonella* survival and the expression of immune-related genes

4.1 Background

4.1.1 The impact of fungal secondary metabolites on the insect immune system

The infection process by hypocrealean EPF results in the death of the host and the production of a large number of conidia on the host cadaver. To achieve this, most of the host soft tissue is converted to fungal biomass (Duan *et al*., 2017). Research presented in the previous chapter and performed by Bell *et al*. (2009), with *C. militaris* and *B. bassiana*, showed that fungal growth remains at low levels within the insect for a large duration of the pre-mortality period. The number of fungal genomes then increases rapidly shortly before host death, one explanation for this is that in order to successfully colonise a host insect in this way, EPF need to subvert or overcome the host immune response. Secondary metabolites are thought to play a role in this process (Section 1.8; Pal *et al*., 2007; Xu *et al*., 2008; Xu *et al*., 2009), although little is known about their exact function and mode of action. There are two approaches used to research the natural function of EPF secondary metabolites (Section 1.8.2): artificially introducing the metabolite into an insect and observing the response, or creating mutant EPF strains that lack genes to produce the metabolite and testing their virulence. In this chapter the first approach is employed to characterise the impact of cordycepin on *G. mellonella*, which was intended to provide baseline data that would act as a foundation for gene knockouts in the future. With the long-term aim of developing both a fundamental understanding of the mode of action of cordycepin during *C. militaris* infection and its potential as a biopesticide. Similar studies have previously been done to research the action of destruxins (Figure 1.7), a group of secondary metabolites produced by *Metarhizium* spp. (Vilcinskas *et al*., 1997c). Destruxins A and E inhibit the phagocytic activity of *G. mellonella* plasmatocytes *in vitro* and at high doses cause paralysis and death of larvae (Vilcinskas *et al*., 1997c).

Subsequently this group of toxins were seen to inhibit attachment and spreading of *G. mellonella* plasmatocytes (Vilcinskas *et al*., 1997b). Furthermore, Pal *et al*. (2007) found that destruxin A inhibits the IMD immune pathway in *D. melanogaster*. The authors challenged *D. melanogaster* with *E. coli* and/or destruxin A, then used RT-qPCR to measure AMP expression and a microarray to monitor expression of over 400 other immune-related genes. They found that genes involved in the IMD pathway, which is known to regulate the anti-bacterial humoral response in insects (Lemaitre and Hoffmann, 2007), were downregulated by destruxin A following bacterial immune challenge. The success of these experiments illustrates the ability of bioassays and RT-qPCR to assess the function of secondary metabolites.

4.1.2 Understanding the effects of different biopesticides applied together as part of an IPM strategy

EPF metabolites that act as virulence factors could have potential for use as biopesticides, most probably to be applied together with other pest control agents as part of an IPM strategy. IPM is the coordinated use of pest management strategies to minimise negative impacts on society and the environment, whilst providing maximum benefit to the producers (Kogan, 1998). In order to optimise this strategy, it is critical to assess the interactions between IPM components (Section 1.2), to understand if different crop protection agents interact when used together, and the types of interactions that occur. Understanding the type of interaction is also important for ecotoxicological assessments as there is concern that combined applications of pesticides may result in synergistic effects on non-target organisms (Cedergreen *et al*., 2014) and the same might be true for biopesticides.

There have been a limited number of studies looking at the effects of combining EPF with conventional pesticides (Appendix B.1), with the aim of achieving enhanced levels of pest control and reduced pesticide inputs. No studies have been published to date about using EPF metabolites as novel biopesticides in conjunction with EPF. An example of combining EPF with insecticides is the use of *B. bassiana* and *M. anisopliae s.l*. to treat citrus root weevil (*Diaprepes abbreviatus*) larvae in conjunction with a sub-lethal dose of the insecticide imidacloprid which found a synergistic

interaction leading to an increase in larval mortality (Quintela and McCoy, 1998). This type of study has been replicated with these EPF and a range of low dose insecticide applications in different pest species (Appendix B.1) (Hiromori and Nishigaki, 2001; Purwar and Sachan, 2006; Farenhorst *et al*., 2010), with most studies having been based around measuring the effects of applying a fixed dose of each agent. Whether the effect of co-application of EPF and insecticide is synergistic, additive or antagonistic depends on the mode of action of the insecticide and the characteristic of the EPF (Purwar and Sachan, 2006).

The effect of co-application of two different agents has been more thoroughly characterised in the field of pharmacology because it is important to know if drugs interact within the body to cause side effects. In this field, four main ways of identifying the outcome of a combination treatment are used (Williamson, 2001):

1. Identify whether the effect (E) of a combination of fixed doses of two compounds (A and B) is greater than the sum of their individual effects, *i.e*. $E_{A+B} > E_A + E_B$.
2. Measure the effect of a fixed dose of one compound on the dose response of the other.
3. Identify whether the effect (E) of the combination of treatments (A and B) is greater than the effect of each treatment individually, *i.e*. $E_{A+B} > E_A$ and $E_{A+B} > E_B$.
4. Determine the effect of a range of different doses of one compound on a range of doses of another (Figure 4.1). This is known as the isobole method.

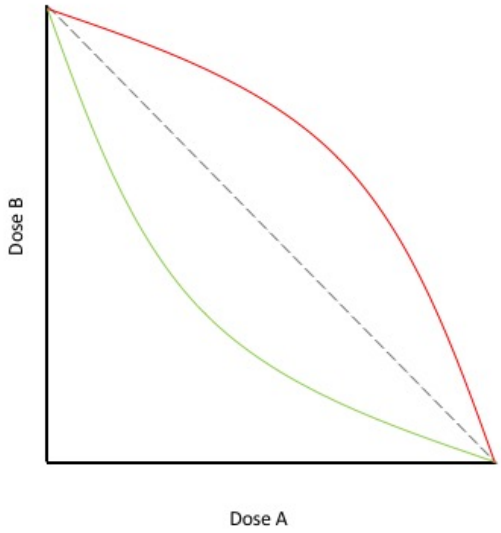


Figure 4.1- An example of an isobologram. The responses to doses of chemical A are plotted against responses to chemical B. The red line illustrates a typical antagonistic response, green a synergistic response and the dashed blue line shows no interaction. (Adapted from Williamson 2001).

In pharmacology the isobole method is regarded as the most reliable for identifying interactions between drugs (Williamson, 2001). However, there does not appear to be a consensus reached in terms of the best way to investigate the effects of application of two different agents in pest control techniques (Appendix B.1). In some cases, approach 3 (see the above list) is used, where low concentrations of the pesticide and biopesticide are applied and the combined effect is compared to their effects individually using an ANOVA (Hiromori and Nishigaki, 2001; Sun *et al*., 2011; Seyed-Talebi *et al*., 2014). Other studies have used approach 2, where the effect of a fixed dose of insecticide is measured in conjunction with multiple doses of a biopesticide (Furlong and Groden, 2001). Chi-squared tests are also used to assess whether combinations of treatments interact synergistically (Purwar and Sachan, 2006; Mohan *et al*., 2007), this will be discussed further in Chapter 6.

A review by Cedergreen *et al*. (2014) that assessed environmental toxicity of chemicals characterised synergy of independently acting chemicals as:
$E_{mix} = E_A + E_B - E_A E_B$. Where E_{mix} is the probability of dying from the chemical mixture, E_A is the probability of dying from the first chemical and E_B is the probability of dying from the second chemical. The predominant criticism of the use of this equation to determine synergy is that chemicals rarely act independently- even if their modes of action differ, there may be more subtle interactions that have not been discovered (Williamson, 2001). Additionally, this method does not account for the shape of the dose effect curve for each agent used in combination.

4.1.3 Aims and objectives

The first aim of this chapter was to identify the impact of cordycepin on the expression of immune-related genes in *G. mellonella* to better understand its natural function. The second aim was to identify whether EPF and cordycepin would be compatible for use as biopesticides in IPM. These aims were achieved through the following objectives:

1. Identify whether cordycepin inhibits EPF germination.
2. Determine the type of outcome (synergistic, additive or antagonistic) of the combined application of cordycepin and EPF in *G. mellonella*.
3. Use RT-qPCR to monitor expression of immune-related genes in response to cordycepin treatment in *G. mellonella*.

4.2 Materials and Methods

4.2.1 Insect material

Final instar *G. mellonella* larvae were purchased and maintained as described in Section 2.2.1.

4.2.2 Fungal material

B. bassiana 433.99, *C. militaris* 11703 and *M. brunneum* 275.86 were used for experiments (Table 2.2.2). They were selected as *B. bassiana* and *M. brunneum* are commercially available biopesticides and *C. militaris* produces cordycepin. The maintenance, subculturing and preparation of conidia suspensions was carried out as described in Section 2.2.3. Aliquots (10 ml) of conidia were prepared at a concentration of $1x10^7$ conidia ml^{-1} and were diluted to the required concentration for each experiment.

4.2.3 The impact of cordycepin on germination of EPF conidia

The impact of cordycepin on fungal germination was determined through incubation of *B. bassiana* 433.99, *C. militaris* 11703 and *M. brunneum* 275.86 conidia with two different concentrations of cordycepin (Sigma-Aldrich, USA), 6 mg ml^{-1} and 3 mg ml^{-1}. These concentrations were selected as 3 mg ml^{-1} was found to be sub-lethal when applied to *G. mellonella* larvae, whereas 6 mg ml^{-1} caused mortality of these larvae (Chapter 2). Cordycepin was prepared by diluting a 100 mg ml^{-1} stock solution in DMSO (Fisher Scientific, USA) with 0.01% Triton X-100. The aim of this was to establish whether it would be appropriate to coapply EPF and cordycepin in later experiments.

A preparation was made containing 100 µl conidial suspension ($1x10^7$ conidia ml^{-1}), 0, 30, 60 µl of cordycepin (100 mg ml^{-1}), 2.7 µl pentostatin (2'-deoxycoformycin; Sigma-Aldrich, USA; 0.1 mg ml^{-1}) and made up to 1 ml using Sabouraud's Dextrose Broth (Sigma-Aldrich, USA). This gave final concentrations of: $1x10^6$ conidia ml^{-1},

30, 60 mg ml^{-1} (12 mM and 24 mM respectively) cordycepin and 1 μM pentostatin. The purpose of adding pentostatin was to inhibit adenosine deaminase (Johnston, 2011) to reduce the breakdown of cordycepin.

This mixture was incubated in darkness for 24 h at 23 ˚C and the number of germinated and ungerminated conidia was counted. Briefly, the mixture was vortexed for approximately 2 min and 20 μl pipetted onto an improved Neubauer haemocytometer and visualised using an Olympus BH-2 microscope (Olympus U.K & Ireland, U.K). The germination of approximately 100 conidia was recorded, a conidium was considered germinated if the germ tube was longer than the length of the conidium. Percentage germination was calculated and the experiment was performed on three separate occasions. Percentage germination was transformed using the LOGIT transformation (Warton and Hui, 2011) and analysed using a one-way ANOVA in SPSS (Version 24, IBM). A post-hoc Bonferroni test was used to identify pairwise significant differences between the percentage germination of EPF at different concentrations of cordycepin. During the LOGIT transformation process, a constant (1%) was subtracted from germination values so that 100% germination could be included in the analysis.

4.2.4 Identifying the influence of co-administration of cordycepin and EPF on *G. mellonella* survival

The interactions between cordycepin and EPF (*B. bassiana* 433.99, *M. brunneum* 275.86 and *C. militaris* 11703) were assessed using *G. mellonella* bioassays. Conidial suspensions were prepared as described in Section 2.2.2 and diluted in 0.01% Triton X-100 to give concentrations of approximately 666 and 6666 conidia ml^{-1}. A stock solution of cordycepin was diluted with DEPC-treated water (Fisher Scientific, USA) to 6 mg ml^{-1}. Mixtures for injection were prepared by adding equal volumes of conidial suspension to cordycepin solution, giving final concentrations of approximately 333 or 3333 conidia ml^{-1}, 3 mg ml^{-1} cordycepin and 3% DMSO. The cordycepin solution also contained pentostatin at a final concentration of 1 μM. The dose of cordycepin used (3 mg ml^{-1}) had been found during preliminary experiments to be sub-lethal to *G. mellonella*.

Three independent replicates of ten larvae per treatment were injected with 30 µl of each mixture into the right front proleg using a 0.3 ml microfine insulin syringe (BD, USA) or 30 µl of a 0.01% Triton X-100/DMSO mixture as a control (Section 2.2.5). This gave a final dose of approximately 10 or 100 conidia and 90 µg of cordycepin per larva. Larvae were maintained in 9 cm petri dishes lined with filter paper at 20 ˚C and 16:8 h light:dark cycle and survival was monitored for eight days. A Kaplan-Meier estimator was carried out in SPSS (Version 24, IBM) and the significance of differences in survival were assessed using a log-rank test.

4.2.5 Assessing the impact of cordycepin on immune-related gene expression in *G. mellonella* using RT-qPCR

To quantify the effect of cordycepin on the *G. mellonella* humoral response, larvae were injected with *B. bassiana* 433.99 or *C. militaris* 11703 conidia (100 conidia per larva) and cordycepin (90 µg per larva), as described in Section 2.2.5. Injection was selected to administer EPF and cordycepin as cordycepin does not have activity when applied topically (Kim *et al*., 2002) and final instar *G. mellonella* do not feed. Also, *B. bassiana* 433.99 was chosen as it has been used previously to characterise the *G. mellonella* immune response and *C. militaris* 11703 was used as it produces cordycepin. Larvae were snap frozen in liquid nitrogen at 48 and 72 h after injection and stored at -80 ˚C until use. Fifteen larvae were sampled for each treatment at each time point from three independent experiments.

RNA was extracted from whole larvae using the phenol:chloroform method as described in Section 3.2.3.3 and reverse transcribed using SuperScript II (ThermoFisher Scientific, USA) to give cDNA (Section 3.2.3.4). The expression of *galiomicin*, *gallerimycin*, *lysozyme* and *IMPI* was measured with a Lightcycler 480 (Roche Holding AG, Switzerland) using primers and conditions described in Section 3.2.3.5. To determine whether cordycepin had an impact on *S7e* expression, a Mann-Whitney test was performed to compare the Ct (cycle threshold) values between *G. mellonella* that were not treated with cordycepin (n=105) and those that were (n=60). To assess the impact of cordycepin on immune-related gene expression Ct values were

normalised against the housekeeping gene *S7e*, then the expression on day zero to give $\Delta\Delta$Ct values and $2^{-\Delta\Delta Ct}$ values (Livak and Schmittgen, 2001) were analysed using a Kruskal-Wallis test in SPSS Statistics (Version 24, IBM).

4.3 Results

4.3.1 The impact of cordycepin on fungal germination

EPF conidia were incubated with cordycepin to determine the impact of this metabolite on germination. *B. bassiana* 433.99 and *M. brunneum* 275.86 germination was significantly reduced (back-transformed data in Figure 4.2) to 57.6% and 25.4% (back-transformed means), respectively when treated with 6 mg ml^{-1} cordycepin compared to 79.3% and 72.5% at 3 mg ml^{-1} ($p=0.001$ for both), and 74.7% and 81.2% at 0 mg ml^{-1} ($p=0.004$ for *B. bassiana* and $p<0.001$ *M. brunneum*). Differences in germination of *C. militaris* 11703 conidia between cordycepin treatments were found to be significant using an ANOVA ($p=0.038$), but pairwise differences were not found to be significant in a Bonferroni post-hoc test. Germination at 0, 3 and 6 mg ml^{-1} cordycepin was 81.5%, 79.4% and 63.2%, respectively (back-transformed means).

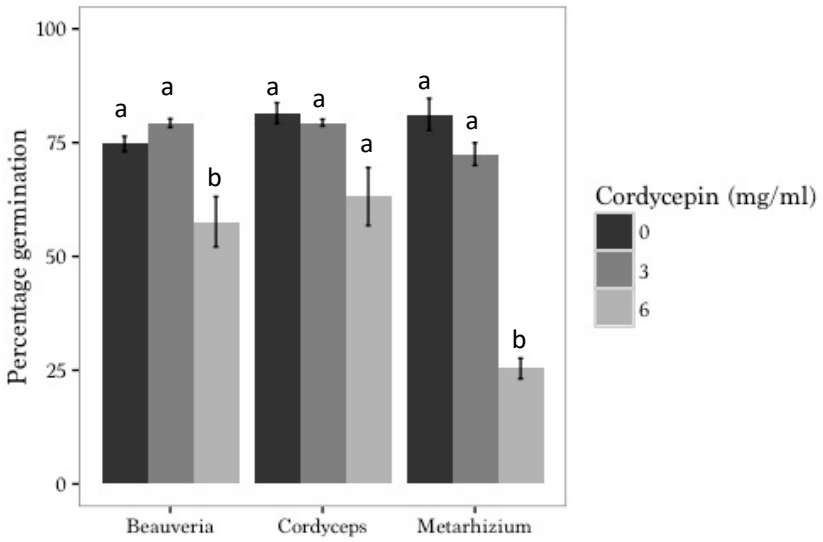


Figure 4.2 - The impact of cordycepin on EPF germination. Mean (back-transformed) percentage germination of *Beauveria bassiana* 433.99, *Cordyceps militaris* 11703, and *Metarhizium brunneum* 275.86 conidia ±SE following incubation for 24 h with 0 (dark grey), 3 (mid grey) and 6 (pale grey) mg ml^{-1} of cordycepin. Different letters represent significant differences ($p<0.05$) between cordycepin treatments within each EPF species determined by ANOVA and Bonferroni post-hoc test.

4.3.2 Determining the influence of co-administration of cordycepin and EPF on *G. mellonella* survival

In order to characterise the effect of co-application of EPF and cordycepin, *G. mellonella* larvae were injected with a sub-lethal dose of cordycepin and EPF and their survival monitored. Survival of larvae injected with only cordycepin was not significantly different from those injected with DMSO only ($p=0.178$; Figure 4.3.D). For each of *B. bassiana* 433.99, *C. militaris* 11703 and *M. brunneum* 275.86 there was a significant difference between survival of *G. mellonella* when cordycepin was applied with EPF (Figure 4.3) compared to EPF alone. When treated with 100 *B. bassiana* 433.99 conidia, cordycepin reduced the time to mortality by approximately 1 day ($p=0.001$). When treated with 10 *B. bassiana* 433.99 conidia, cordycepin reduced the time to mortality by 2-3 days ($p<0.001$). Mortality caused by *C. militaris* 11703 appeared to be the most affected by cordycepin treatment, with the time to mortality reducing by at least 2 days following treatment with 10 ($p<0.001$) or 100 ($p<0.001$) conidia. Mortality caused by treatment with *M. brunneum* 275.86 conidia appeared to be the least affected by treatment with cordycepin. At 100 conidia per larva the time to mortality appeared to only differ by 12 h following cordycepin treatment ($p=0.011$), at 10 conidia per larva cordycepin reduced the time to mortality by 1-2 days ($p<0.001$). This suggests that cordycepin reduced the time to mortality when co-injected with EPF and this effect was more apparent when a lower dose of EPF was applied.

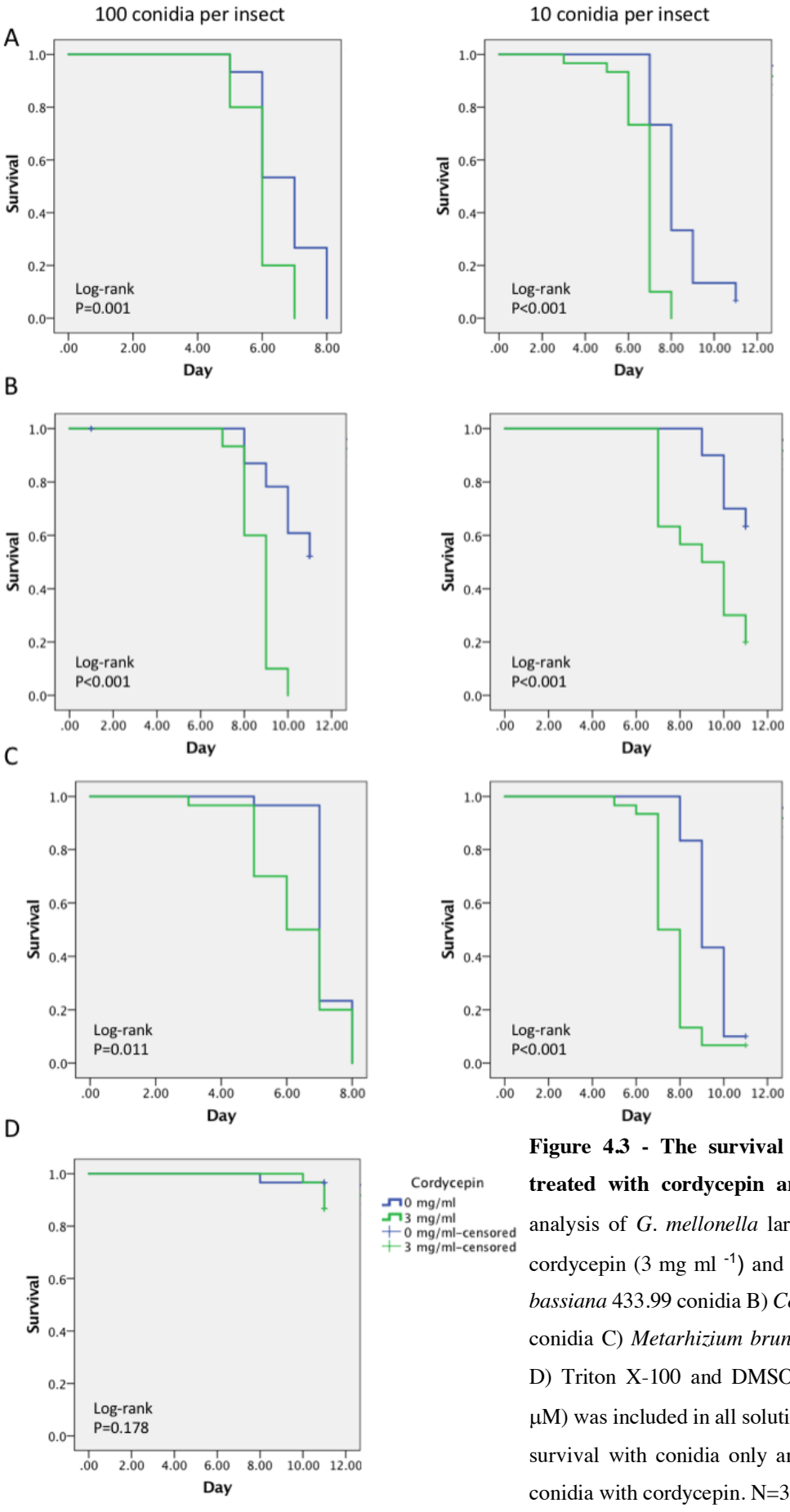


Figure 4.3 - The survival of *Galleria mellonella* treated with cordycepin and EPF. Kaplan-Meier analysis of *G. mellonella* larvae injected with 30 µl cordycepin (3 mg ml $^{-1}$) and 100 or 10 A) *Beauveria bassiana* 433.99 conidia B) *Cordyceps militaris* 11703 conidia C) *Metarhizium brunneum* 275.86 conidia or D) Triton X-100 and DMSO control. Pentostatin (1 µM) was included in all solutions. The blue line shows survival with conidia only and the green line shows conidia with cordycepin. N=30 per treatment.

4.3.3 Assessing the impact of cordycepin on the expression of immune-related genes in *G. mellonella* larvae using RT-qPCR

To confirm whether cordycepin impacts the expression of immune-related genes, *G. mellonella* larvae were injected with cordycepin and *B. bassiana* 433.99 conidia, then immune-related gene expression monitored using RT-qPCR. RNA extraction and cDNA synthesis were successful for 14 of the 15 larvae sampled. The Ct values for *S7e* expression in *G. mellonella* injected with cordycepin were compared to controls using a Mann-Whitney test. There was found to be no significant difference between them ($p=0.581$), which shows that cordycepin did not alter expression of this housekeeping gene. Therefore, it was appropriate to normalise expression of the genes of interest (*galiomicin*, *gallerimycin*, *IMPI* and *lysozyme*) against *S7e*.

Figure 4.4 shows the expression of *galiomicin*, *gallerimycin*, *IMPI* and *lysozyme* 48 h and 72 h after treatment with 100 *B. bassiana* 433.99 conidia with and without cordycepin and the response to a sham injection (Triton X-100). In all cases the expression of these genes increased following treatment with *B. bassiana* 433.99 or a sham injection. It appeared that injection with *B. bassiana* 433.99 resulted in a larger increase in expression than sham-injection, however they were not significantly different (Appendices B.2-B.9). At 48 h after injection cordycepin appeared to reduce the expression of all immune-related genes, whether their expression was induced by wounding or *B. bassiana* 433.99 injection. The p-values from a Kruskal-Wallis test followed by Bonferroni correction are in Appendices B.2-B.9. When the immune response was stimulated with *B. bassiana* 433.99, cordycepin significantly reduced the expression of all immune-related genes. Expression of *galiomicin* was reduced by ~3.5 times ($p=0.004$) following cordycepin treatment in conjunction with *B. bassiana* 433.99 treatment, *gallerimycin* by ~3 times ($p=0.044$), IMPI by ~7.5 times ($p<0.001$) and *lysozyme* by ~6.5 times ($p<0.001$). The expression of immune-related genes was similar 72 h after treatment, although the response to wounding (sham injection) appeared to be lower than at 48 h. Like at 48 h, the expression of all genes (*galiomicin*, *gallerimycin*, *IMPI* and *lysozyme*) in response to *B. bassiana* 433.99 infection was significantly reduced by co-application of cordycepin. Expression of *galiomicin* was reduced by ~3 times ($p=0.006$) following cordycepin treatment in addition to *B. bassiana* 433.99, *gallerimycin* by ~3 times ($p=0.012$), *IMPI* by ~5 times ($p<0.001$)

and *lysozyme* by ~3.5 times (p<0001). The expression of these genes following sham injection was also significantly reduced by treatment with cordycepin, particularly at 48 h after treatment (Appendices B.2-B.9).

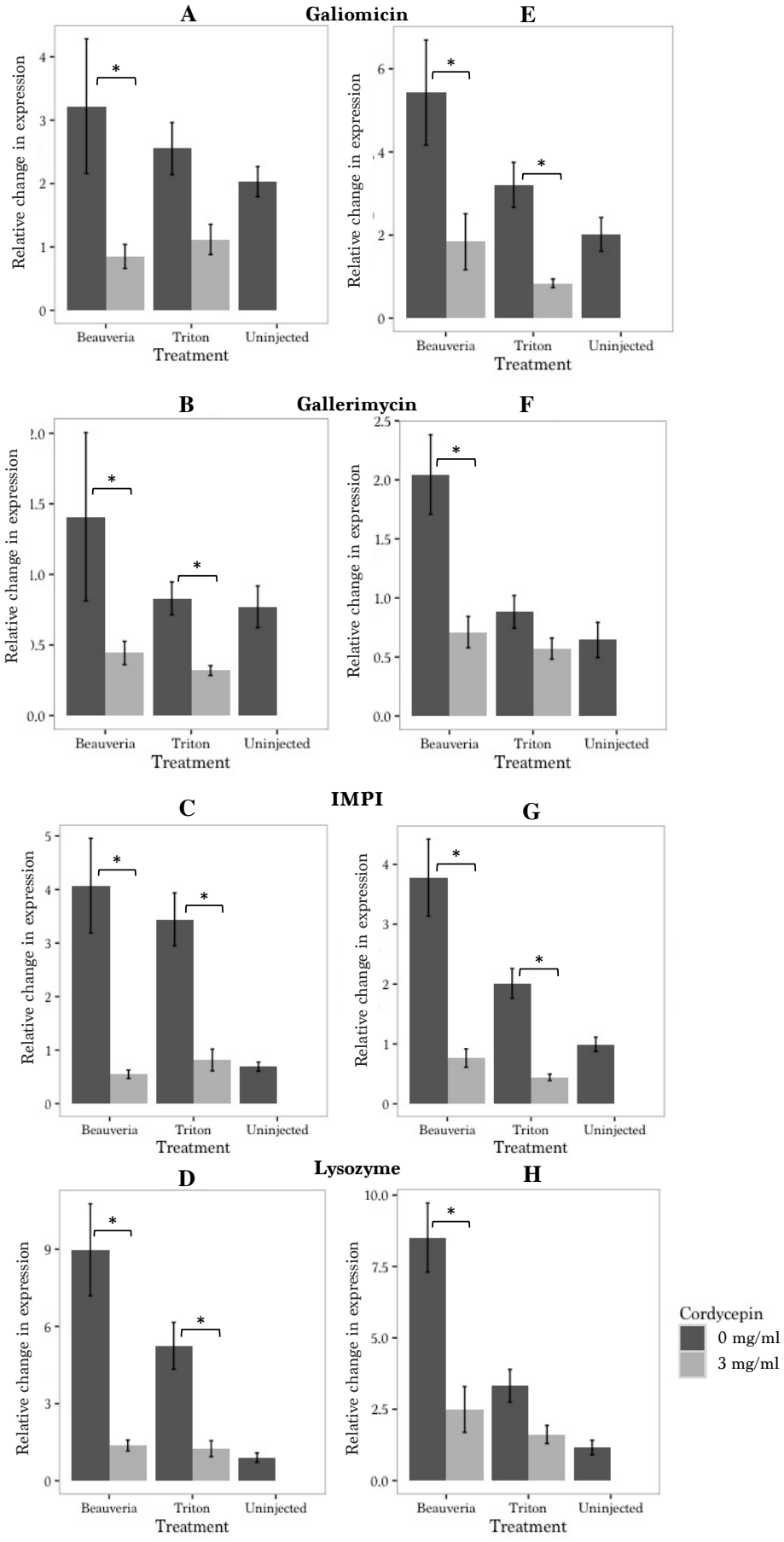


Figure 4.4 - Expression of immune-related genes in response to cordycepin and *Beauveria bassiana* 48 h and 72 h after treatment. The expression of *galiomicin*, *gallerimycin*, insect metalloprotease inhibitor (*IMPI)* and *lysozyme* in response to stimulation by *B. bassiana* injection and sham (Triton X-100) injection 48 h (A-D) and 72 h (E-H). Expression is normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. Asterisks indicate significant differences (p<0.05) between treatments with and without cordycepin, other p-values are in Appendices B.2-B.9.

G. mellonella were also treated with 100 *C. militaris* 11703 conidia and cordycepin or conidia alone (Figure 4.5), a sham injection (Triton X-100) was also included. A similar pattern of expression was seen to that when insects were injected with *B. bassiana* 433.99 and cordycepin. The expression of immune-related genes increased following treatment with *C. militaris* 11703 or sham injection. In most cases it appeared that injection with *C. militaris* 11703 resulted in a larger increase in expression than sham-injection, however they were not significantly different (Appendices B.10-B.17). At both timepoints immune-related gene (*galiomicin*, *gallerimycin*, *IMPI* and *lysozyme*) expression when treated with *C. militaris* 11703 alone was significantly higher than when it was co-administered with cordycepin (p-values in Appendices B.10-B.17). At 48 h after treatment expression of *galiomicin* was reduced by ~5 times ($p<0.001$) following cordycepin treatment in addition to *C. militaris* 11703, *gallerimycin* by ~ 2 times ($p=0.005$), *IMPI* by ~4 times ($p=0.005$) and *lysozyme* by ~6.5 times ($p=0.002$). At 72 h after treatment, expression of *galiomicin* was reduced by ~4.5 times ($p<0.001$) following cordycepin treatment in addition to *C. militaris* 11703, *gallerimycin* by ~2 times ($p=0.007$), IMPI by ~6 times ($p<0.001$) and *lysozyme* by ~4 times ($p<0.001$). The expression of immune-related genes in response to *C. militaris* 11703 appeared to be lower than the response to *B. bassiana* 433.99, however these cannot be directly compared as they were separate experiments.

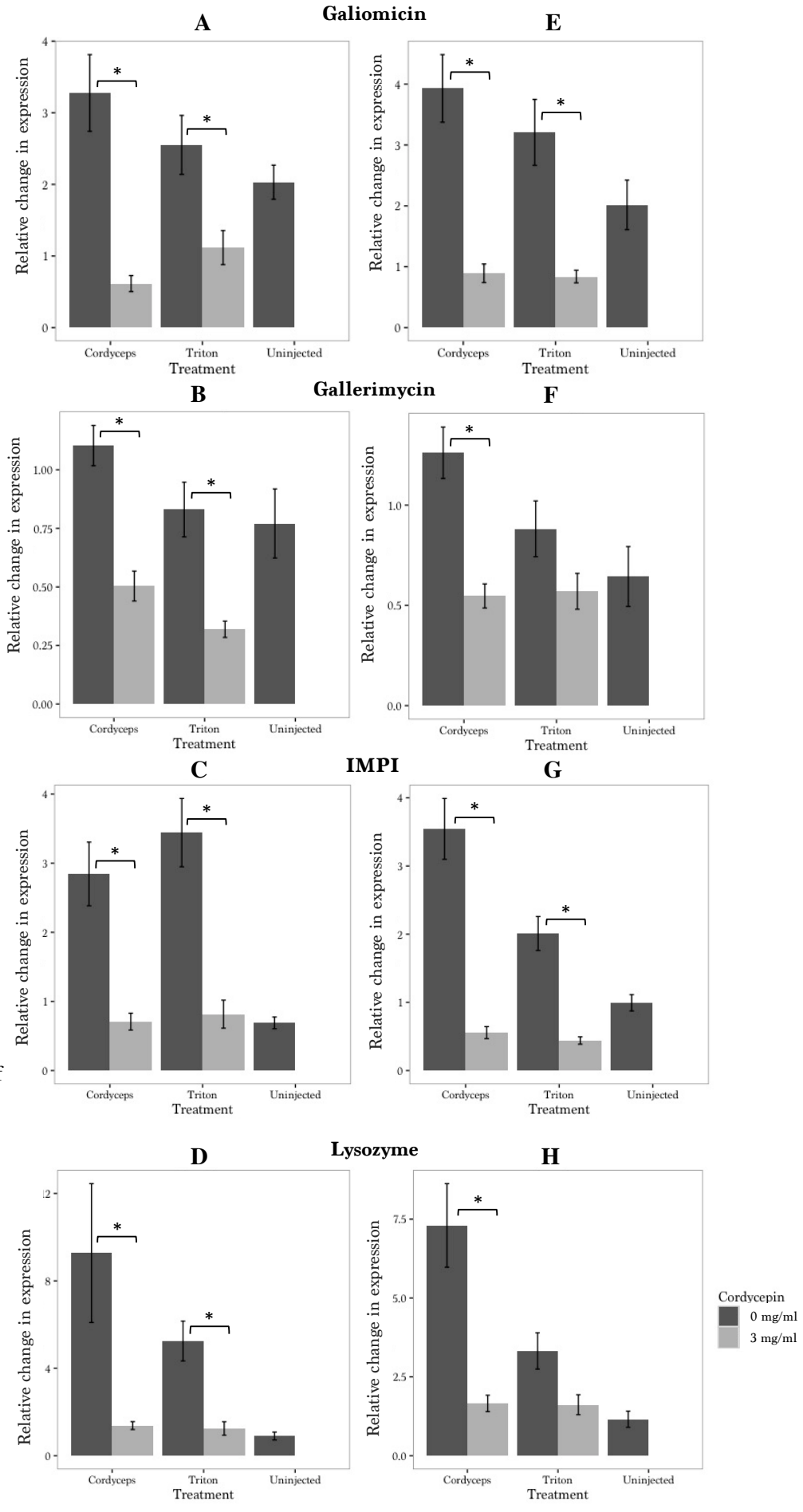


Figure 4.5- Expression of immune-related genes in response to cordycepin and *Cordyceps militaris* 48 h and 72 h after treatment. The expression of *galiomicin*, *gallerimycin*, *insect metalloprotease inhibitor (IMPI)* and *lysozyme* in response to stimulation by *C. militaris* injection and sham (Triton X-100) injection 48 h (A-D) and 72 h (E-H). Expression is normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. Asterisks indicate significant differences ($p<0.05$) between treatments with and without cordycepin, other p-values are in Appendices B.10-B.17.

4.4 Discussion

An aim of this chapter was to identify whether EPF and cordycepin would be compatible for IPM, by assessing the effect of cordycepin on EPF germination. A concentration of cordycepin of 6 mg ml^{-1} was found to inhibit germination of *M. brunneum* 275.86 and *B. bassiana* 433.99 relative to conidia treated with DMSO and Triton X-100, whereas a lower concentration (3 mg ml^{-1}) did not. The mode of action of this inhibition is not known, however one hypothesis is that it could be due to cordycepin preventing mRNA processing of genes critical to germination and survival, in particular it has been seen to reduce the expression of rapidly expressed genes such as those involved in the anti-inflammatory response (Kim *et al.,* 2006). The switch of fungal conidia from a resting state to a germinating state likely requires a large, rapid upregulation of genes, as has been documented in *Fusarium graminearum* (Seong *et al*., 2008). A GeneChip was used to monitor expression levels in *F. graminearum* and it was found that less than two hours after initiation of germination, 1805 genes were upregulated including those involved with metabolism, protein synthesis, the cell cycle, nucleotide metabolism and amino acid metabolism. A similar switch in expression likely occurs in EPF so cordycepin may inhibit the expression of these rapidly upregulated genes and prevent germination.

C. militaris 11703 conidia were the least affected by cordycepin treatment, possibly because *C. militaris* produces cordycepin and has evolved to tolerate high concentrations. This tolerance could be due to a putative cordycepin transporter that has been identified in *C. militaris* and is thought to be responsible for the transport of cordycepin out of cells (Xia *et al*., 2017). *M. brunneum* 275.86 seemed to be more affected by cordycepin then *B. bassiana* 433.99, despite neither of them being known to produce cordycepin. Using LC-MS analysis Xia *et al*. (2017) showed that cordycepin cannot be detected from *B. bassiana* or *Metarhizium robertsii* (closely related to *M. brunneum*). *Beauveria* spp. are more closely related to *Cordyceps* spp. than *Metarhizium* spp. (Section 1.4) as they are in the same family, so *B. bassiana* may share genes with *C. militaris* that confer some resistance to cordycepin. Alternatively, there may be a structural feature of *M. brunneum* conidia that makes them more susceptible to cordycepin. This suggests that if cordycepin and EPF were

used in combination for IPM, it would be important to select a concentration of cordycepin that does not inhibit EPF germination. Furthermore, it appears that *B. bassiana* 433.99 would be more appropriate for use with cordycepin as germination was less affected than *M. brunneum* 275.86.

As 3 mg ml^{-1} cordycepin did not inhibit EPF germination, this concentration was selected to be co-administered to *G. mellonella* with EPF. Although this dose was not lethal to *G. mellonella* larvae if administered alone, it did reduce the time to mortality of larvae when co-injected with either 10 or 100 conidia of all three of the EPF tested. The hypothesis developed from this research is that cordycepin inhibits the immune response of larvae thus enabling the EPF to colonise the larvae more rapidly. This is supported by the finding in Chapter 2, where it was speculated that cordycepin facilitated opportunistic infection by bacteria through inhibition of the insect immune system. Alternatively, this may be due to cordycepin preventing mRNA processing or halting the cell cycle (Wu *et al*., 2007; Jung *et al*., 2012; Kondrashov *et al*., 2012) in larval cells, resulting in stress that increases the susceptibility of the insect to infection (Adamo and Parsons, 2006). The impact of cordycepin on the progress of mortality during EPF infection appeared more pronounced at 10 conidia per larva than at 100 conidia per larva, as a higher number of conidia would presumably overwhelm the humoral and cellular immune responses more rapidly and make the impact of cordycepin less noticeable. The effect of cordycepin was greatest for *C. militaris* 11703, but least for *M. brunneum* 275.86. *C. militaris* 11703 is less susceptible to the toxic effects of cordycepin than *M. brunneum* 275.86 (Section 4.3.1) and this could account for the differences in their interactions within *G. mellonella*.

The experiments in Section 4.3.2 used a sub-lethal dose of cordycepin to characterise its interactions with EPF. A sub-lethal dose simplifies the calculation used to characterise interactions between chemicals, where E_A would be the effect of EPF application and E_B the effect of cordycepin application (Section 4.1.4), as E_B and E_AE_B would both be equal to zero and any increase in mortality above E_A would be due to potentiation by cordycepin. Therefore, the results in Section 4.3.2 indicate that cordycepin acts to enhance the effect of EPF to increase the rate of *G. mellonella* mortality. However, a condition for the use of the equation in Section 4.1.4 is that the

chemicals being tested act independently which may not be true for EPF and cordycepin. EPF interact with the insect immune response at different levels, including the cellular and humoral responses, in order to successfully infect an insect (Hajek and St. Leger, 1994). For example, plasmatocytes isolated from *G. mellonella* treated with EPF proteases have reduced phagocytic activity (Griesch and Vilcinskas, 1998), suggesting that *in vivo* these proteases inhibit the cellular immune response. *B. bassiana* infection triggers the expression of AMPs in *G. mellonella* (Vertyporokh and Wojda, 2017), which illustrates that EPF also interact with the humoral response. Cordycepin may also impact these immune pathways, which is supported by the results in Sections 4.3.3 and 2.2.6. Consequently, cordycepin and EPF may act upon the same immune pathways and identification of potentiation using $E_{mix} = E_A + E_B - E_A E_B$ may not be reliable, although it does not negate the possibility that cordycepin potentiates the effect of EPF. A more reliable means of assessing interactions between chemicals would be to use the isobole method (Williamson, 2001). This would require a range of doses of cordycepin to be administered to *G. mellonella* with a range of doses of EPF and mortality monitored. This has not been commonly used for assessment of pesticide interactions, probably because it would require a high number of bioassays, which would increase the cost and time taken for the experiments. However, in the future it would be beneficial to use the isobole approach to investigate the effect of the dose of cordycepin on interactions with EPF, in particular at concentrations of this metabolite that would be realistic in the field if it were developed as a biopesticide.

In order to test how cordycepin affects the *G. mellonella* immune system when co-injected with EPF, the expression of the four immune-related genes *gallerimycin*, *galiomicin*, *IMPI* and *lysozyme* were monitored using RT-qPCR. When *B. bassiana* 433.99 conidia were injected into larvae with cordycepin, expression of *gallerimycin*, *galiomicin*, *lysozyme* and *IMPI* was reduced at 48 and 72 h post-injection. In all cases, levels were reduced to that of uninjected larvae which suggests that cordycepin could be associated with effects on the insect immune system, particularly reducing the expression of immune-related genes. Similar results were seen when the immune response of *G. mellonella* was stimulated through injection of *C. militaris* 11703 conidia (Figure 4.5). This was unexpected as *C. militaris* 11703 produces cordycepin (Huang *et al.*, 2003), which might be anticipated to inhibit the immune response of

larvae irrespective of additional cordycepin. However, it is possible that the dose of cordycepin introduced during this experiment was higher than that produced during the early stages of natural infection. The amount of cordycepin found to be produced by *C. militaris* mycelia varies depending on cultivation method, strain and extraction method, but is between 750-16500 μg g^{-1} (Huang *et al*., 2003; Li *et al*., 2004; Yang and Li, 2008). The amount injected in Section 4.2.5 was 90 μg per insect, which based on the above estimates would be produced by 5.5 mg-120 mg of *C. militaris*. This level of biomass would likely not accumulate until the late stages of the *G. mellonella* infection. To quantify the concentration of cordycepin produced by *C. militaris* early in infection LC/MS or HPLC (Huang *et al*., 2003; Li *et al*., 2004) could be used, however analysis of these low concentrations would likely be complicated by the structural similarity of cordycepin to adenosine (Figure 1.8). As discussed in Chapter 3, sham injection increases expression of *galiomicin*, *gallerimycin*, *IMPI* and *lysozyme*. Cordycepin significantly reduced the expression of these genes at either 48 or 72 h after treatment, despite their expression being induced by wounding rather than infection, illustrating that cordycepin reduces immune-related gene expression regardless of how expression was stimulated.

There has been limited previous research into the effect of EPF secondary metabolites on the insect immune system and the majority has focused on their impact on the activity or structure of haemocytes (Vilcinskas and Matha, 1997a; Vilcinskas *et al*., 1997b; Vilcinskas *et al*., 1997c). There has been one previous study in which immune-related gene expression has been monitored following treatment with an EPF metabolite. In *D. melanogaster* destruxin A was found using microarray data to cause significant down regulation of humoral response genes in comparison to other genes (Pal *et al*., 2007). The same study also found that destruxin reduced AMP expression, specifically AMPs controlled by the IMD pathway (Pal *et al*., 2007). This is an antibacterial response, which contrasts with the effect on the Toll response observed in this chapter. However, Pal *et al*., (2007) did not carry out experiments suitable to identify a specific response to the Toll pathway as they used bacteria to stimulate the IMD pathway. In the current study, the Toll response was focused on as it would be more relevant to natural infection by EPF. In the future, other immune pathways could be investigated which would be facilitated by the whole genome sequence of *G.*

mellonella recently becoming available (Lange *et al*., 2018). In *G. mellonella* beauverolides (a metabolite of *Beauveria* spp.) also have an effect on the immune system (Vilcinskas *et al*., 1999), but the response is less clear. When administered at sub-lethal concentrations beauverolides increased nodulation and melanisation but inhibited phagocytic activity. This could indicate that the response to cordycepin, like destruxins and beauverolides, is part of a larger more complex response that needs further characterisation.

There is a large amount of cross talk between immune response pathways (Han and Ip, 1999; De Gregorio *et al*., 2002; Hedengren-Olcott *et al*., 2004), so cordycepin may be affecting multiple immune pathways individually or a single process impacting multiple pathways. Although the genes investigated during this project are all immune-related, their transcription is not likely to be controlled by the same immune-response pathway, which supports the hypothesis that cordycepin acts on multiple immune pathways. Gallerimycin is reported to be antifungal (Schuhmann *et al*., 2003) and galiomicin is reported to be anti-bacterial and anti-fungal (Lee *et al*., 2004). It is likely that their expression is controlled, at least in part, by the Toll pathway as this pathway predominantly responds to fungi. Expression of lysozyme and IMPI is likely to be controlled by separate pathways, but there may be feedback from the Toll pathway as their expression increases following exposure to fungi (Chapter 3). Cordycepin has been reported to particularly reduce the expression of inflammatory genes through the inhibition of polyadenylation (Kondrashov *et al.,* 2012), although it has also been reported to reduce the polyadenylation of other genes. For example, it reduces the mRNA levels of genes involved in the cell cycle including *c-myc* (White; Ioannidis *et al*., 1999) and *met* (Chen *et al*., 2008). Therefore, through inhibition of polyadenylation, cordycepin may impact multiple pathways within insects, including immune pathways.

4.5 Conclusions

Cordycepin appears to facilitate infection of *G. mellonella* by multiple EPF (*B. bassiana* 433.99, *C. militaris* 11703 and *M. brunneum* 275.86), although it could not be conclusively determined whether this was an additive or synergistic effect because

the way in which both treatments interact with larvae is not known. However, taken together with the results from Chapter 2, where cordycepin permitted opportunistic bacterial infection, the data presented indicates that cordycepin inhibits the *G. mellonella* immune system. In order to elucidate the mechanism behind cordycepin's impact on the *G. mellonella* immune response, immune-related gene expression was monitored. It was found that cordycepin reduced expression of *galiomicin*, *gallerimycin*, *IMPI* and *lysozyme* whether their expression was stimulated by the wounding or treatment with EPF. This suggests that the natural function of cordycepin is to inhibit the insect immune response thereby facilitating the infection and colonisation by *C. militaris*.

Chapter 5- Investigating the effect of cordycepin on the expression of immune-related genes in *Drosophila melanogaster* S2r+ cells

5.1 Background

5.1.1 Immunity of *D. melanogaster*

The *D. melanogaster* immune system is one of the best characterised in insects (Section 1.7; Rolff and Reynolds, 2009). In particular, it has been used to elucidate the Toll and IMD pathways of the humoral response (Lemaitre *et al*., 1995; O'Neill, 2006) which are activated by detection of PAMPs, virulence factors and stress signals. Upon activation they upregulate the transcription of immune-related genes (Gottar *et al*., 2006; Erler *et al*., 2011). Additionally, the Toll pathway is involved in dorsal-ventral patterning during the development of the *D. melanogaster* embryo (Belvin and Anderson, 1996).

There are seven groups of AMPs (Section 1.7.2.4) in *D. melanogaster* (Table 1.4; Section 1.7) and four were selected for the present study: Attacin, Diptericin, Drosomycin and Metchnikowin. Attacin is identified in the literature as having antibacterial activity, although its expression also increases following *B. bassiana* infection despite it not being known to have antifungal activity (Lemaitre *et al*., 1997). Similarly, Diptericin is reported to have activity against gram-negative bacteria (Lemaitre and Hoffmann, 2007) and its expression also increases in response to *B. bassiana* infection, although it too has not been found to have antifungal activity (Lemaitre *et al*., 1997). Metchnikowin is reported to have activity against gram-positive bacteria and fungi, inhibiting the growth of both *Micrococcus luteus* and *Neurospora crassa* (Levashina *et al*., 1995). Whereas Drosomycin is reported to be primarily active against fungi, inhibiting the growth of *N. crassa*, *Botrytis cinerea* and *Fusarium oxysporum*, but has no activity against bacteria (Fehlbaum *et al*., 1994). Two additional proteins, Relish and DIF are transcription factors in the NFκB family

that control AMP transcription in the IMD and Toll pathways, respectively. They have a key role in activating transcription of immune-related genes in these pathways (Rolff and Reynolds, 2009; Ganesan *et al*., 2010).

Cellular immunity (Section 1.7.1) is another important aspect of the *D. melanogaster* immune response. Circulating haemocytes can phagocytose or encapsulate invading microorganisms, but also have key roles in wound healing and the melanisation response (Williams, 2007). Additionally, there is evidence that haemocytes are involved in activating the humoral immune response. Mutant *D. melanogaster* larvae that do not produce haemocytes cannot synthesise AMPs in response to bacterial infection (Basset *et al*., 2000). It was hypothesised that this is due to the need for haemocytes to produce a second signal following detection of pathogens to trigger AMP production (Basset *et al*., 2000). A second signal has been identified as being necessary for the Toll pathway, as haemocytes produce the cytokine Spätzle following detection of infection to trigger this pathway (Shia *et al*., 2009).

The impact of a selection of fungal secondary metabolites on the survival and immune response of insects has been previously investigated using *D. melanogaster* (Paterson *et al*., 1987; Pal *et al*., 2007). For instance, metabolites derived from *Penicillium* spp. were fed to *D. melanogaster* larvae and their effect on mortality and feeding quantified to evaluate their potential for use as biopesticides (Paterson *et al*., 1987). Additionally, Pal *et al*. (2007) carried out a series of experiments to further understand the activity of destruxin A in *D. melanogaster*. RT-qPCR was used to show that this metabolite inhibits the expression of the AMPs *diptericin*, *attacin* and *metchnikowin* (Pal *et al*., 2007). This is the only study to specifically investigate the impact of an EPF secondary metabolite on immune-related gene expression in insects, but its success suggests that similar techniques may be applied to determine whether cordycepin can also affect the *D. melanogaster* immune system.

5.1.2 *Drosophila melanogaster* cell lines

D. melanogaster is the primary model organism for studying genetics (Jennings, 2011; Gramates *et al*., 2017), as well as researching innate immune pathways and improving

understanding of the innate immune system in humans (Buchon *et al*., 2014). There are a range of *D. melanogaster* cell lines available including S2 and Kc, which can be transformed using plasmid expression vectors (Cherbas *et al*., 1994) or RNAi and have been used to study signalling pathways (Clemens *et al*., 2000). This is potentially of use for future work into understanding the natural function of cordycepin as it will permit use of techniques including gene knockouts and reporter gene assays.

The S2 cell line was developed by Schneider (1972) from late embryonic stage *D. melanogaster* cells and it has since been found to have macrophage-like characteristics, such as the ability to phagocytose bacteria (Rämet *et al*., 2001). This has led to it being used as a tool to characterise the *D. melanogaster* immune response (Georgel *et al*., 2001; Cheng and Portnoy, 2003; Kallio *et al*., 2005; Wang *et al*., 2006), especially monitoring gene expression in response to immune stimulation (Tauszig *et al*., 2000; Kallio *et al*., 2005). The present study uses the *D. melanogaster* S2r+ cell line, a line derived from S2 cells, which differs from the S2 line in its ability to respond to Wingless signalling (Yanagawa *et al*., 1998). The Wingless pathway is important in the regulation of tissue development (Swarup and Verheyen, 2012).

5.1.3 Mammalian innate immunity vs invertebrate immunity

The innate immune response is an early response to pathogens found in many multicellular organisms (Kimbrell and Beutler, 2001). There are extensive homologies between the innate immune response in vertebrates and the humoral response in invertebrates. These homologies extend to immune receptors, immune effectors and signalling pathway components (Kimbrell and Beutler, 2001). Comparing human and invertebrate responses can be particularly informative when elucidating immune pathways or for determining the effect of chemicals on the functioning of the immune system. Most of the research published to date on cordycepin has focused on its impact on the human inflammatory response. However, because of the conservation of the innate immune response between vertebrates and invertebrates, this can inform research into its function during infection by *C. militaris*. The Toll-like receptor (TLR) pathway (Figure 5.1) in mammals shows homology with the Toll and IMD pathways of invertebrates (Kimbrell and Beutler, 2001). It controls the release of inflammatory

cytokines (Takeda and Akira, 2004) and can be initiated by recognition of fungi (Bourgeois and Kuchler, 2012), bacteria and viruses (Medzhitov, 2001). The recognition of a PAMP by a TLR triggers a signalling cascade (Figure 5.1), involving Myd88 and IRAK, which are homologous to Myd88 and Pelle, respectively, in the *D. melanogaster* Toll pathway (Kimbrell and Beutler, 2001).

NFκB is a key transcription factor in the mammalian TLR pathway that is homologous to DIF and Relish in the insect Toll and IMD pathways, respectively and its activation occurs via a similar mechanism to these insect transcription factors. NFκB is held in an inactive form in the cytoplasm by association with the inhibitory protein IκB (Tak and Firestein, 2001). Upon activation, the TLR signalling cascade activates IκB kinase (IKK) which phosphorylates IκB triggering its degradation (Figure 5.1). This frees NFκB to dimerise and enter the nucleus (Tak and Firestein, 2001), where it binds to the promoters of immune responsive genes and activates their expression. In insects, DIF remains in the cytoplasm due to association with Cactus, a homologue of IκB (Karin, 1999). Following activation of the Toll pathway Cactus is phosphorylated which triggers its degradation, although the precise mechanism is not known. DIF then dimerises and enters the nucleus to drive transcription of target genes (Valanne *et al*., 2011). Activation of Relish is slightly different to that of DIF and NFκB as it contains an IκB-like module that holds it in the cytoplasm. The IMD signalling cascade activates a kinase in the IKK family that phosphorylates the IκB-like module of Relish causing it to be cleaved, freeing Relish to dimerise and enter the nucleus (Valanne *et al*., 2011). The similarities between the insect and mammalian NFκB pathways show that they have been evolutionarily conserved and understanding the pathway in one organism can improve understanding of that in other organisms.

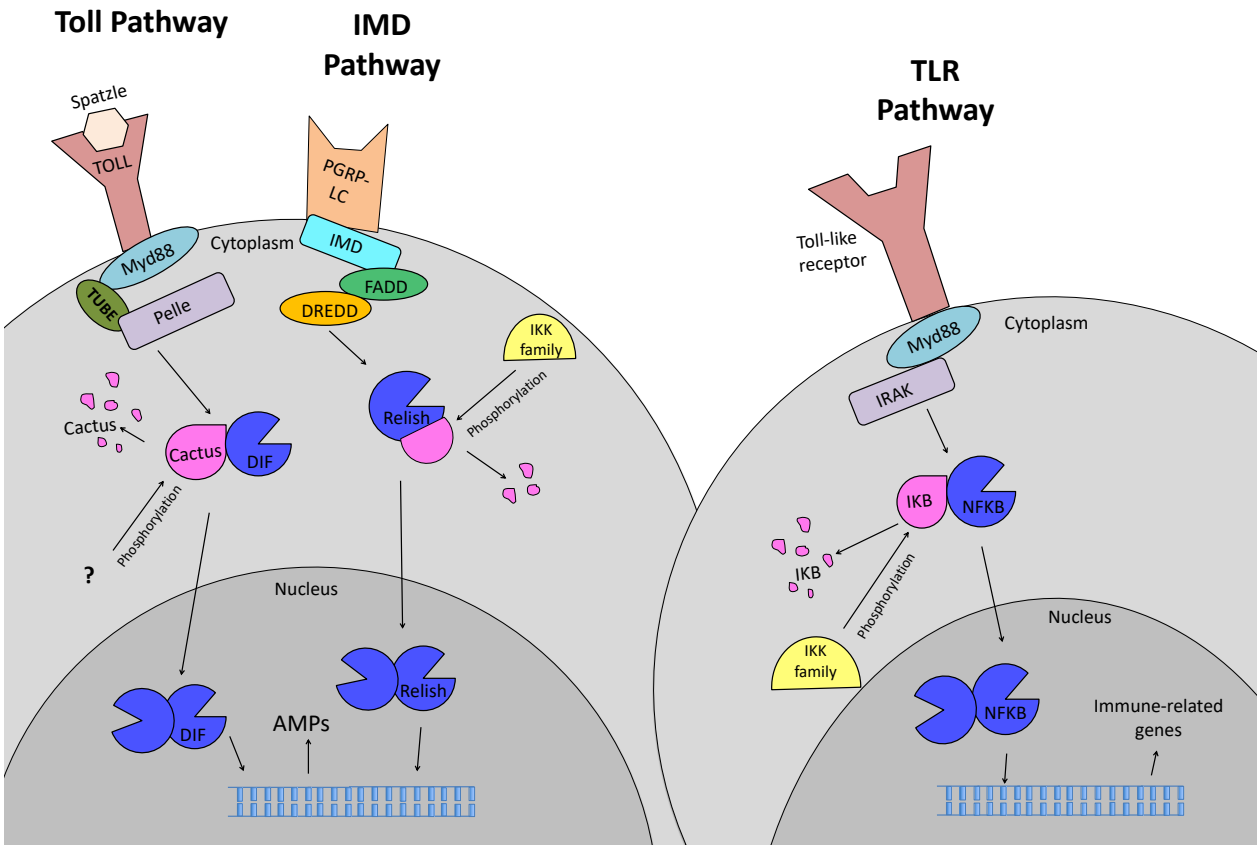


Figure 5.1 - The Toll and IMD pathways in *Drosophila melanogaster* cells (left) and the Toll-like receptor (TLR) pathway in mammalian cells (right). A diagram comparing the Toll and IMD pathways in *D. melanogaster* cells to the TLR pathway in mammalian cells, homologous proteins have the same shape and colour. The TLR pathway responds to bacteria, viruses and fungi to trigger an inflammatory immune response. A family of TLR receptors bind to PAMPs, which triggers a signalling a cascade within the cell that includes the proteins Myd88 and IRAK (which are homologous to Myd88 and Pelle in the Toll pathway, respectively). Following the cascade, a kinase in the IKK family phosphorylates IκB (homologous to Cactus), which leads to its degradation. NFκB (homologous to DIF and Relish) is then free to dimerise and enter the nucleus. Fungi and gram-positive bacteria are detected by the Toll pathway. Fungal PAMPS are detected by β-GRPs and gram positive bacteria by PGRP-SAs. These then bind to the Toll receptor via the protein Spatzle. This activates a protein cascade in the cytoplasm that ends in DIF being released from the inhibitory protein cactus. DIF then diffuses into the nucleus where it acts as a transcription factor activating AMPs. Gram negative bacteria are detected by the PGRP-LC receptor, which triggers a protein cascade in the cytoplasm that ends in the activation of the transcription factor Relish. Relish then diffuses into the nucleus and activates transcription of AMPs. Adapted from Lemaitre and Hoffmann, (2007), Takeda and Akira, (2004) and Myllymäki and Rämet, (2014).

5.1.4 Aims

The overall aim of this chapter was to identify the impact of cordycepin on the expression of immune-related genes in *D. melanogaster* S2r+ cells to improve understanding of the natural function of cordycepin. This builds on the findings from Chapter 4, where cordycepin was seen to reduce immune-related gene expression in *G. mellonella*. For this chapter, a cell line was used to circumvent the problems seen previously with the wounding response that occurred when *G. mellonella* were injected with EPF and cordycepin, which complicated the analysis of immune-related gene expression. Furthermore, because the *D. melanogaster* immune response has been well characterised the results obtained should produce new fundamental information of the mechanism of action of cordycepin.

5.2 Materials and Methods

5.2.1 Maintenance of *Drosophila melanogaster* S2r+ cell culture

D. melanogaster S2r+ cells (Yanagawa *et al*., 1998) were provided by Dr Lopez de Quinto (Cardiff University, U.K). Cells were cultured in 15 ml cell culture flasks (VWR, USA) with 12 ml of Schneider's Drosophila Insect Medium (ThermoFisher Scientific, USA) supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich, USA) and 10% penicillin-streptomycin (Sigma-Aldrich, USA). The cell cultures were maintained at 25 ˚C in an environmental test chamber (Sanyo, Japan) and were split into new 15 ml cell culture flasks when their density reached approximately $1x10^7$ cells ml^{-1}. All cells used in experiments were between passages 5-15.

Cells to be used in RT-qPCR experiments were seeded into 6-well plates (TPP, Switzerland) 24 h prior to experiments. In order to do this, the density of cells was determined following trypan blue staining and used to calculate the total number of cells present in each flask. Briefly, cells were loosened from the flask by aspirating with a serological pipette and 100 µl of cell suspension was mixed with 100 µl of 0.4% trypan blue. Living cells exclude this dye, whereas dead cells are stained blue, which allows their differentiation. Cells were visualized using an improved Neubauer Haemocytometer and the number of viable cells per ml calculated using the following equation: $\frac{Total\ No.\ viable\ cells\ in\ a\ large\ square}{16}\ x\ 10000\ x\ 2$. Cells were collected in a 15 or 50 ml universal tube (Fisher Scientific, USA) by centrifugation at 1500 g for 5 min at 4˚C. The media was removed and replaced with fresh Schneider's Drosophila Insect Medium (ThermoFisher Scientific, USA) supplemented with 10% FBS (Sigma-Aldrich, USA). This was aspirated to disrupt the pellet and 3 ml pipetted into each well of a 6-well plate (TPP, Switzerland) at a density of approximately $8x10^5$ cells ml^{-1}. Plates were incubated at 25 ˚C in an environmental test chamber (Sanyo, Japan).

5.2.2 The effect of cordycepin on *D. melanogaster* S2r+ survival

The density of S2r+ cells was determined by staining with 0.4% trypan blue solution (Sigma-Aldrich, USA) as described in Section 5.2.1. The total number of cells required for each experiment was calculated and the desired number were seeded into

new media. This was done by centrifuging the cell suspension for 5 min at 1500 g (Tabletop centrifuge, Fisher Scientific, USA) to pellet the cells and resuspending in Schneider's Insect Medium (ThermoFisher Scientific, USA) supplemented with 10% FBS (Sigma-Aldrich, USA) to the volume required for the experiment.

Cordycepin (Sigma-Aldrich, USA) was diluted in DMSO (Fisher Scientific, USA) to give the following concentrations: 50, 100, 200, 400, 800 and 1600 mM. Then 3 ml of cell suspension was pipetted into eight wells of two 6-well plates (TPP, Switzerland) and 3 µl of each cordycepin dilution was added to each well giving final cordycepin concentrations of 50, 100, 200, 400, 800 and 1600 µM. Two controls were included in the experiment, one remained untreated whilst the other contained 3 µl of DMSO. These plates were incubated at 25 ˚C in an environmental test chamber (Sanyo, Japan) for 48 h and the number of viable and non-viable cells were counted daily using trypan blue staining, as described previously. All cells within the larger 4x4 haemacytometer grid were counted, in each replicate three aliquots were counted from each treatment and the mean calculated. The experiment was repeated on three occasions. A model was fitted to the data using the DRC package (Ritz *et al*., 2015) in R (RStudio, 2017). Initially five models were fitted to the data; two, three and four parameter log-logistic models, Weibull-1 model and Weibull-2 model. These were compared using a lack-of-fit test and the model with the highest p-value selected and used to calculate the LC_{50} (concentration that causes 50% mortality) for cordycepin after 24 and 48 h exposure to cordycepin.

5.2.3 Development of a *D. melanogaster* S2r+ bioassay system

A series of pilot experiments were performed to determine the optimum conditions for later RT-qPCR experiments. For all pilot experiments one biological replicate was performed. In order to determine the most appropriate time for sampling, cells were treated with sterile distilled water, LPS (Lipopolysaccharides from *Escherichia coli* O127:B8 purified by phenol extraction, Sigma-Aldrich, USA) or curdlan (Carbosynth Ltd, Newbury U.K) to a final concentration of 1 µg ml^{-1} 24 h after seeding. LPS stimulates an anti-bacterial response as it is a bacterial cell wall component and curdlan stimulates an anti-fungal immune response as it is structurally similar to fungal cell wall polysaccharides, although it is produced by the bacteria *Alcaligenes faecalis*

(Harada *et al*., 1968). Cells were incubated at 25 ˚C for 1, 2 or 4 h.

In order to identify conditions to achieve maximum expression of immune-related genes, varying concentrations of LPS or curdlan (10 or 20 μg ml^{-1}) were applied 24 h after cell seeding. A control treated only with sterile RO water was also included. Cells were incubated at 25 ˚C for 4 h. Finally, to identify the optimum concentration of cordycepin to use in future experiments, S2r+ cells were treated with 20 μg ml^{-1} of stimulant (LPS or curdlan) and 20, 50 or 100 μM of cordycepin (Sigma-Aldrich, USA) dissolved in DMSO (Fisher Scientific, USA). Controls containing DMSO only were also included. Cells were then incubated at 25 ˚C for 4 h. In all experiments, following incubation of cells, RNA was extracted and used as a template for RT-qPCR of a selection of genes (*diptericin*, *drosomycin*, *RP49* and *attacin*), which is described in detail in Section 5.2.4.

5.2.4 Monitoring the impact of cordycepin on the expression of immune-related genes in *D. melanogaster* S2r+ cells

At 24 h after seeding, S2r+ cells were stimulated with 20 μg ml^{-1} curdlan (Carbosynth Ltd, Newbury U.K), 20 μg ml^{-1} LPS (Sigma-Aldrich, USA) or sterile RO water. At the same time, they were treated with either 100 μM cordycepin or the same volume of DMSO. In all experiments, an untreated control was also included. The cells were incubated at 25 ˚C for 4 h, then placed on ice. The cell suspension was gently pipetted up and down to loosen cells adhering to the plate, then transferred to a 15 ml universal tube and centrifuged at 4 ˚C for 5 min at 1500 g to pellet cells. The medium was removed and the pellet washed with ice cold PBS and centrifuged as before. RNA was extracted using the ReliaPrep™ RNA Cell Miniprep system (Promega, USA) following the manufacturer's instructions, the concentration measured using a NanoDrop ® ND-100 spectrophotometer (ThermoFisher Scientific, USA) and stored at -80˚C. Three independent biological replicates of this experiment were performed.

SuperScript III (ThermoFisher Scientific, USA) was used to carry out reverse transcription reactions on 100 μg of RNA from each sample. The manufacturer's instructions were followed, except 0.5 μl (100 units) of Superscript III was added to each reaction instead of the 1 μl (200 units) recommended. The cDNA produced from

this reaction was diluted 1 in 5 in DEPC-treated water.

QPCR was performed using a Lightcycler 480 (Roche Holding AG, Switzerland) and SensiFAST SYBR No-ROX (Bioline, USA) as described in Section 3.2.3.5. The PCR primers used for gene amplification are listed in Table 5.1. Reactions and cycle conditions were the same as those described in Section 3.2.3.5. The raw Ct values for *Rp49* (rRNA gene) expression with and without cordycepin were compared using a 2-tailed t-test, to determine whether it was appropriate to normalise expression of genes of interest against this gene. Following this test, it was found that cordycepin did not affect *RP49* expression, so Ct values were normalised against *RP49* and the expression of untreated control using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). These $2^{-\Delta\Delta Ct}$ values were assessed for normality using a Shapiro-Wilk test and analysed in SPSS Statistics (Version 24, IBM) using either an independent 1-tailed t-test if they were normally distributed or a 1-tailed Mann-Whitney U test if they were not normally distributed. This was based on the hypothesis developed in Chapter 4 that treatment with cordycepin would result in lower levels of gene expression compared to those treated with an immune challenge only.

Table 5.1 -Primers used for analysis of immune-related gene expression in *Drosophila melanogaster* S2r+ cells.

Gene	Primer sequence (5'-3')	Reference
attacin A	AGGTTCCTTAACCTCCAATC CATGACCAGCATTGTTGTAG	Jin *et al*., (2008)
RpL32	AGTCGGATCGATATGCTAAGCTGT TAACCGATGTTGGGCATCAGATACT	Jin *et al*., (2008)
diptericin	ATGCAGTTCACCATTGCCGTC TCCAGCTCGGTTCTGAGTTG	Jin *et al*., (2008)
drosomycin	CTTGTTCGCCCTCTTCGCTGTC AGCACTTCAGACTGGGGCTGCA	Jin *et al*., (2008)
RP49	GACGCTTCAAGGGACAGTATCTG AAACGCGGTTCTGCATGAG	Gobert *et al*., (2003)
relish	GGCATCATACACACCGCCAAGAAG GTAGCTGTTTGTGGGACAACTCGC	Petersen *et al*., (2013)
dif	CAGTTTGCTACGACCGGAGAGCTA GAATATCCGCCAGTTGCAGAGTGC	Petersen *et al*., (2013)
metchnikowin	TCTTGGAGCGATTTTTCTGG AATAAATTGGACCCGGTCTTG	Castillo *et al*., (2013)

5.3 Results

5.3.1 The effect of cordycepin on *D. melanogaster* S2r+ cell survival

D. melanogaster S2r+ cells were incubated with a range of cordycepin concentrations and survival was quantified at 24 and 48 h after treatment. To generate dose-response curves, the DRC package in R was used to fit models to survival. No model tested was found to give a good fit to the survival 24 h after treatment, as the p-values obtained from lack-of-fit tests were less than 0.05. This may be due to percentage mortality not exceeding 45%. The best fitting model was the W2.2 model (Weibull type 2 model with a lower limit of 0 and upper limit of 1; Figure 5.2), which gave a p-value of 0.012. The LL.2 model (2 parameter log-logistic model with EC_{50} as a parameter, upper limit of 1 and lower limit of 0) was found to best fit the data at 48 h after treatment using a lack-of-fit test (p=0.70). This model was used to estimate the LC_{50} for cordycepin treatment as being 383.55 μM. At both timepoints, the fitted models indicate that the mortality of S2r+ cells increased approximately linearly with log[concentration of cordycepin].

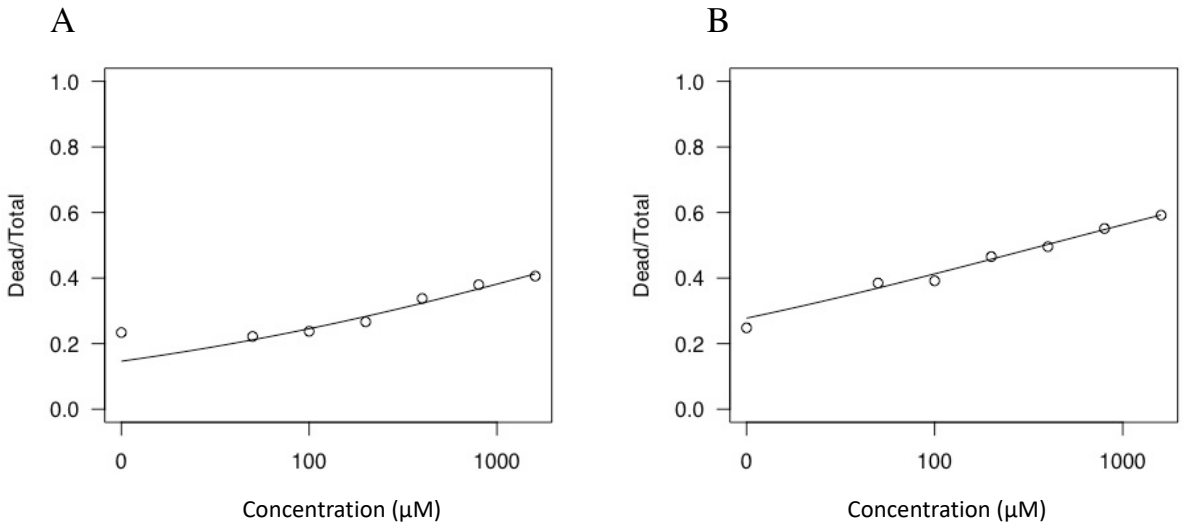


Figure 5.2 - Survival of *Drosophila melanogaster* S2r+ cells in response to cordycepin treatment. Dose response curves produced in R showing the survival of *D. melanogaster* S2r+ cells in response to treatment with a range of cordycepin doses (50, 100, 200, 400, 800 and 1600 μM) after incubation for A) 24 h and B) 48 h. A W2.2 model is fitted to survival after 24 h and LL.2 model to survival after 48 h. Points are the mean of three replicates.

5.3.2 Development of a *D. melanogaster* S2r+ bioassay system

To determine a suitable timepoint for S2r+ cell sampling following treatment with LPS or curdlan, an expression timecourse was performed. The concentrations of LPS and curdlan used did not cause a large stimulation of AMP expression, but the highest increase in expression appeared 4 h after treatment (Figure 5.3). Therefore 4 h was selected as the sampling time for later experiments and it was determined that higher concentrations of LPS and curdlan would be necessary.

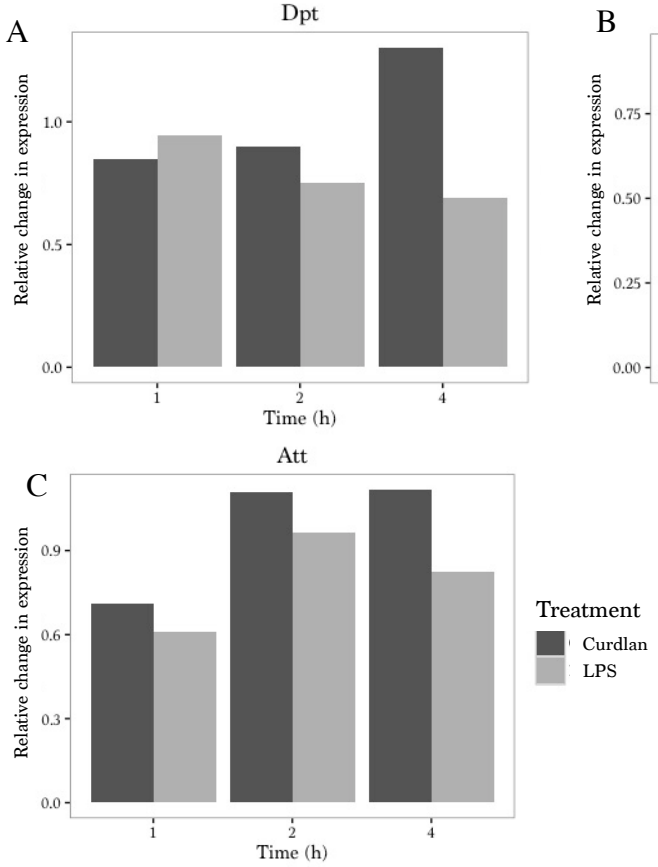


Figure 5.3- Expression of immune-related genes in *Drosophila melanogaster* S2r+ cells 1, 2 and 4 h after curdlan or LPS stimulation. The expression of A) *diptericin* (Dpt), B) *drosomycin* (Drm) and C) *attacin* (Att) in a preliminary experiment, following stimulation with LPS or curdlan (1 mg ml^{-1}). Expression was normalized against the housekeeping gene *RP49* and the expression of unstimulated cells at each timepoint. A single biological replicate was performed.

In order to establish the concentrations of stimulant necessary to give a large increase in expression of immune-related genes, S2r+ cells were treated with either 10 or 20 μg ml^{-1} of LPS or curdlan and expression measured 4 h after treatment. Expression of all immune-related genes increased following LPS or curdlan treatment relative to unstimulated cells. As 20 μg ml^{-1} gave the greatest increase in expression (Figure 5.4) and was used for later experiments. LPS and curdlan did not appear to affect

expression of *RpL32,* a housekeeping gene, hence this concentration was used in future experiments. Finally, an expression timecourse was performed to identify the concentration of cordycepin that affected immune-related gene expression in *D. melanogaster* S2r+ cells (data not presented). Treatment with 100 μM cordycepin gave the greatest reduction in AMP gene expression, without appearing to effect *RpL32* expression, therefore this was selected for later experiments.

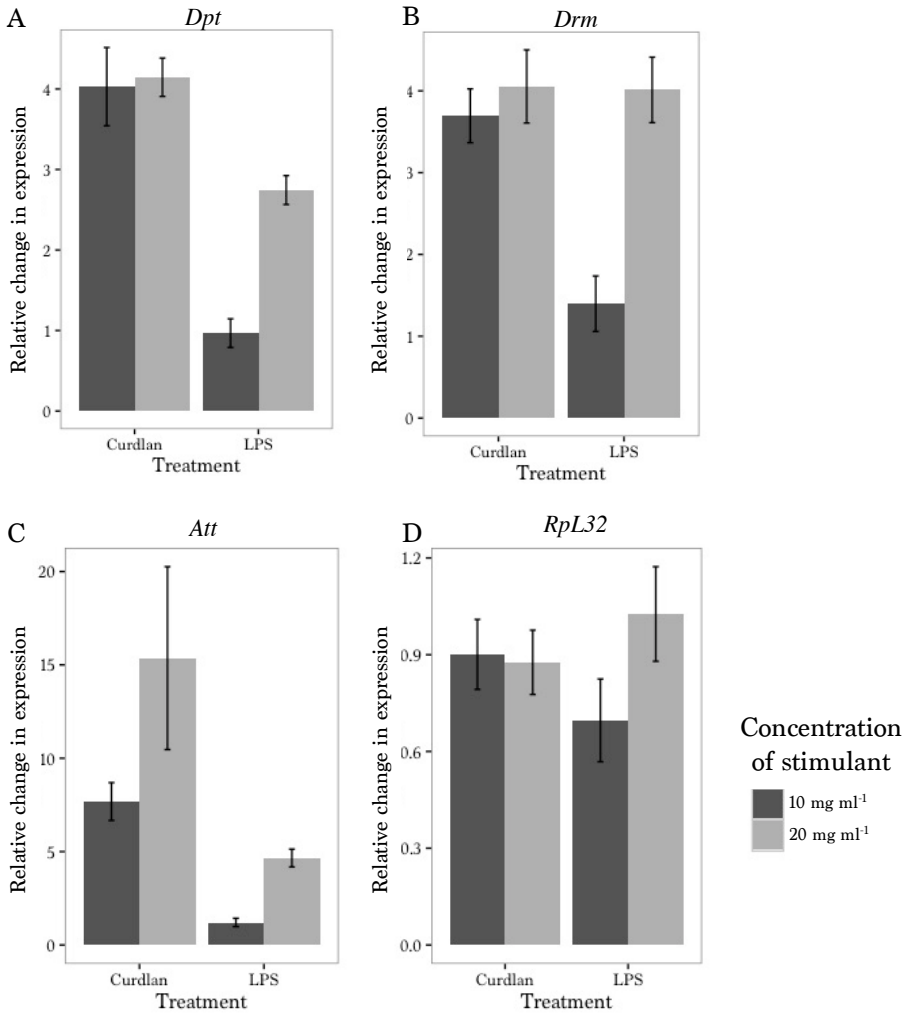


Figure 5.4- Expression of AMP genes in *Drosophila melanogaster* S2r+ cells in response to LPS or curdlan stimulation. The expression of A) *diptericin* (*Dpt*), B) *drosomycin* (*Drm*), C) *attacin* (*Att*) and D) *RpL32* in response to stimulation with 10 or 20 mg ml^{-1} of LPS or curdlan 4 h after treatment. The expression was normalized against the housekeeping gene *RP49* and the expression in untreated cells. A single biological replicate and three technical replicates were performed. Error bars show ±SEM.

5.3.3 Determining the effect of cordycepin on the expression of immune-related effector genes in *D. melanogaster* S2r+ cells

D. melanogaster S2r+ cells were incubated with 100 μM cordycepin and the expression of immune-related genes determined with RT-qPCR (Figures 5.5 and 5.6). Expression of *diptericin* and *drosomycin* appeared to increase slightly in response to curdlan treatment, but cordycepin had no significant effect on expression of these genes. Both genes also showed no response to LPS treatment. However, the expression of both *attacin* and *metchnikowin* increased following curdlan or LPS treatment, but this increase was prevented by the presence of cordycepin. The increases in expression of *attacin* and *metchnikowin* in response to curdlan were significantly reduced by the addition of cordycepin by ~3-fold and ~2.5-fold, respectively ($p=0.025$ and $p=0.049$ respectively). This was determined using a one-tailed Mann-Whitney U test for *attacin* expression, which was not normally distributed and a one-tailed t test for *metchnikowin* which was normally distributed.

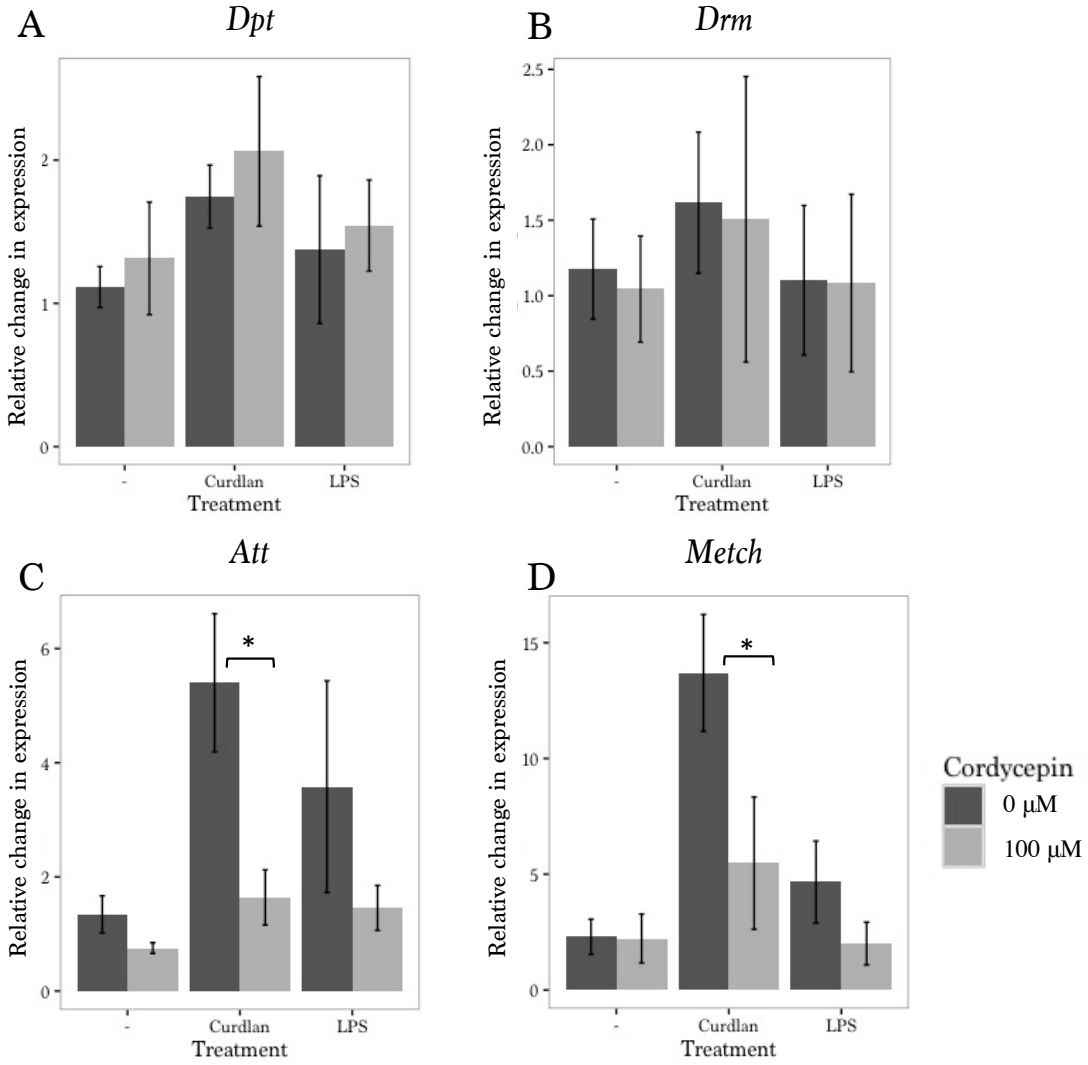


Figure 5.5- Expression of AMPs in *Drosophila melanogaster* S2r+ cells in response to cordycepin treatment. S2r+ cells (three biological replicates and three technical replicates) were treated with 20 μg ml^{-1} LPS or curdlan and treated with either DMSO or 100 μM cordycepin then sampled 4 h after treatment. Expression was normalized against *RP49* and the expression of untreated cells. Relative change of expression of A) *diptericin* (*Dpt*), B) *drosomycin* (*Drm*), C) *attacin* (*Att*) and D) *metchnikowin* (*Metch*) is shown. Error bars show ± SEM and asterisks indicate significant differences ($p<0.05$) that were determined using either a one-tail t test (*diptericin*, *drosomycin*, and *metchnikowin*) or one-tailed Mann-Whitney test (*attacin*).

In order to investigate the humoral immune pathways of *D. melanogaster* in more detail and to determine the cause of the reduction in AMP expression by cordycepin, the response of the transcription factors *dif* and *relish* were compared to the housekeeping gene *RpL32*. The relative expression of *RpL32* remained at approximately 1.0 throughout treatments, however this increased to 1.4 following

curdlan treatment. Furthermore, *RpL32* expression was significantly reduced by the addition of cordycepin in both the control (p=0.025) and curdlan-treated cells (p=0.025). *Dif* and *relish* expression fluctuated slightly between treatments but relative expression levels remained close to 1.0. The large standard error of some treatments may indicate that there was variation in sample quality or there is natural variation in the expression of these genes. The significance of the impact of cordycepin treatment was assessed using an independent one tailed t test where $2^{-\Delta\Delta Ct}$ values were normally distributed (*relish*, *dif*) or one tailed Mann-Whitney U test where $2^{-\Delta\Delta Ct}$ values were not normally distributed (*RpL32*).

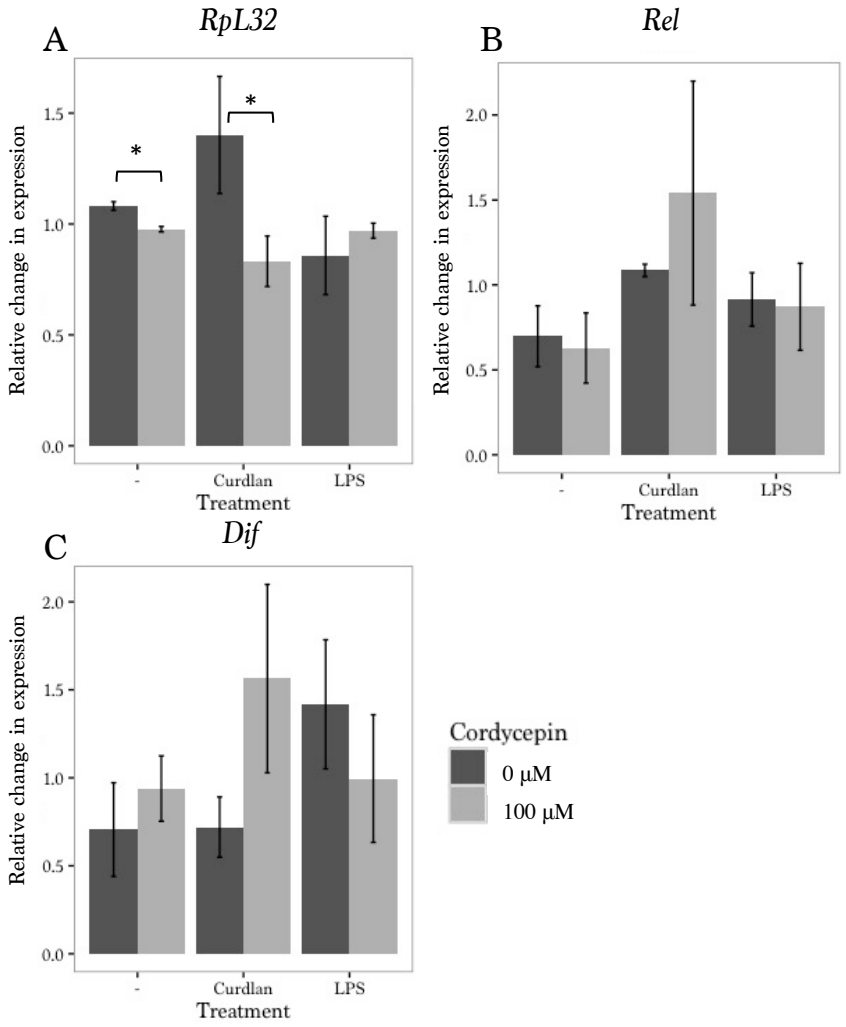


Figure 5.6-Expression of *Drosophila melanogaster* immune-related genes in response to cordycepin treatment. S2r+ cells (3 biological replicates and 3 technical replicates) treated with 20 μg ml^{-1} LPS or curdlan together with either DMSO or 100 μM cordycepin and sampled 4 h after treatment. Expression was normalized against RP49 expression and the expression of untreated cells. Expression of A) *RpL32*, B) *relish* (*Rel*) and C) *Dif* is shown. Error bars show ± SEM and asterisks indicate significant differences ($p<0.05$) that were determined using either a one-tailed t test (*relish* and *dif*) or one-tailed Mann-Whitney test (*RpL32*).

5.4 Discussion

The aim of the research presented in this chapter was to improve understanding of the natural function of cordycepin, in particular its impact on the insect immune system, building on the findings from Chapter 4. Initial experiments observed the impact of cordycepin on *D. melanogaster* S2r+ cell survival to assess whether it would be appropriate to treat cells with this metabolite in future experiments. At 48 h after treatment, cordycepin (50-1600 μM) caused mortality in *D. melanogaster* S2r+ cells compared to the control, the mortality increased linearly with log[concentration of cordycepin]. A similar experiment has been performed in a human multiple myeloma cell line (MM.1S) by Chen *et al*. (2008), who found that cordycepin (>10 μM) inhibited cell growth significantly after 24 h and induced apoptosis of these cells. This was thought to be due to cordycepin reducing the expression of *MET*, a survival factor in multiple myeloma cells (Chen *et al.,* 2008). MET is a receptor associated with tumours and is thought to be a key determinant of malignancy (Shinomiya *et al*., 2004). MET mRNA has a very short half-life (Moghul *et al*., 1994) *i.e*. it is rapidly turned over within cells. Rapidly expressed genes e.g. inflammatory genes, are targets of cordycepin (Kondrashov *et al*., 2012), which may be the reason for reduction in *MET* expression (Chen *et al*., 2008). By extension, inhibition of genes that produce mRNA with a short half-life may be the cause of *D. melanogaster* cell death observed in this chapter. It may take longer for death to occur in S2r+ cells than cancerous cells as they divide at a slower rate and so may have a slower mRNA turnover.

Further studies have found that cordycepin induces apoptosis in cell lines by other mechanisms, such as increasing the expression of pro-apoptotic molecules, causing cell cycle arrest, activating caspases and generating ROS (He *et al*., 2010; Choi *et al*., 2011; Jeong *et al*., 2011). In a human oral cancer cell line, concentrations of cordycepin greater than 10 μM cause cell cycle arrest at the G2/M transition (Wu *et al*., 2007). In breast cancer cells cordycepin stimulates the production of Bax, a proapoptotic molecule that results in cell death (Choi *et al*., 2011). In human leukaemia cells cordycepin treatment causes the production of ROS to activate caspases that drive apoptosis (Jeong *et al*., 2011). This illustrates that the mechanisms of cordycepin-induced apoptosis vary between cell types. The concentrations of

cordycepin used in this chapter may have inhibited the cell cycle by one of these mechanisms and induced apoptosis in *D. melanogaster* S2r+ cells. It would be possible to test this hypothesis using flow cytometry, which can quantify the number of cells in each phase of the cell cycle following cordycepin treatment (Wu *et al.*, 2007; Chen *et al.*, 2008), and this technique has been previously used for cell cycle analysis in *D. melanogaster* tissue culture cells (Tapon *et al.*, 2001).

In this chapter the expression of immune-related genes was monitored in S2r+ cells to determine the impact of cordycepin on the insect immune response, whilst eliminating the effect of wounding observed in Chapter 4. The expression of *attacin* and *metchnikowin* appeared to increase following treatment with curdlan and LPS, whereas *drosomycin* and *diptericin* expression did not, although the significance of these changes was not assessed. The *attacin* and *metchnikowin* genes are expressed by *D. melanogaster* in response to fungi, with *attacin* having been found to be expressed in response to *B. bassiana* and *metchnikowin* in response to *Micrococcus luteus* and *Neurospora crassa* (De Gregorio *et al.*, 2002; Lemaitre and Hoffmann, 2007). Additionally, *attacin* has been found to be expressed at high levels in response to gram-negative bacteria (Lemaitre and Hoffmann, 2007). Therefore, the increase in *attacin* and *metchnikowin* expression in response to curdlan treatment observed in this chapter was expected, as well as an increase in *attacin* expression following LPS stimulation. Other studies have found that *drosomycin* and *diptericin* genes show an increased expression in response to immune challenges of adult *D. melanogaster* with fungi (*Aspergillus luteus*) or bacteria (*Micrococcus luteus* or *Erwinia carotovora*) respectively (Pili-Floury *et al.*, 2004), but they were not upregulated in the experiments in this chapter. This may be due to differences in the experimental conditions between the two pieces of work. In a study by Pal *et al.* (2007), adult *D. melanogaster* were treated by injection with *E. coli* cells, which caused increased *drosomycin* expression 8 h after treatment and increased *attacin* and *metchnikowin* expression 4 h after treatment. This is concurrent with the findings of this chapter. However, Pal *et al.* (2007) also saw increased *diptericin* expression after 4 h which was not seen in this chapter, but may have been a response to wounding in their work.

There were significant differences between the expression of *attacin* and *metchnikowin* with and without cordycepin treatment, although there were no differences identified between *diptericin* and *drosomycin* expression following cordycepin treatment. Expression of *attacin* and *metchnikowin* was increased by stimulation with curdlan and significantly reduced by co-application with cordycepin, which supports the findings from Chapter 4 that suggested cordycepin reduces expression of insect immune-related genes. Cordycepin had little effect on *drosomycin* and *diptericin* expression. This is likely because *drosomycin* and *diptericin* expression was not stimulated by either curdlan or LPS treatment in this experiment, therefore any impact of cordycepin was not noticeable. There were significant differences in the expression of the housekeeping gene *RpL32* following the co-application of cordycepin in both the control and curdlan treatments. There also appeared to be an increase in *RpL32* expression following treatment with curdlan alone. The increased expression of this gene in response to curdlan could be due to a general increase in the metabolic rate of insect cell cultures in response to infection (Palomares *et al*., 2004), although if this were the case an increase in LPS expression may have also been expected. Therefore, it could also be an anomalous result and further replicates would need to be performed to determine this. Additionally, the reduction in *RpL32* expression caused by cordycepin could be due to a reduction in overall transcription because of the impact of cordycepin on polyadenylation (Ioannidis *et al*., 1999; Chen *et al*., 2008; Kondrashov *et al*., 2012). However, cordycepin has been reported to not have a large effect on polyadenylation of housekeeping genes (Kondrashov *et al*., 2012) and this project did not see an impact of cordycepin on the expression of another rRNA gene, *Rp49*. In addition to this, the relative difference between the expression of *RpL32* with and without cordycepin was less than 1-fold, hence further replicates would need to be performed to validate these results.

In human cells, the impact of cordycepin on inflammatory gene expression is thought to be due to its interaction with the NFκB pathway (Kim *et al*., 2006b; Lee *et al*., 2010; Choi *et al*., 2014), in particular it has been observed to inhibit NFκB activation, by preventing its transport to the nucleus (Lee *et al*., 2009; Jeong *et al*., 2010; Ren *et al*., 2012). In this chapter the expression of *relish* and *dif* (NFκB homologs) was monitored to determine whether cordycepin inhibits the Toll/IMD pathways by reducing the

transcription of these genes. Their expression was found to vary slightly between treatments, but changes were neither significant nor did they follow the pattern of expression seen for the AMPs *attacin* and *metchnikowin*. This suggests that cordycepin does not inhibit the expression of these transcription factors, so it is possibly interacting with them directly or affecting the expression or activity of other proteins upstream in the Toll pathway to inhibit Relish/Dif activity post-translation.

The mechanism by which cordycepin reduces expression of *attacin* and *metchnikowin* has not been determined in the present study, but there are a range of possibilities based on evidence from studies with mammalian cells. In particular, studies using mammalian cells have found that cordycepin prevents the translocation of NFκB to the nucleus through the interaction with IκB and IKK (Jeong *et al*., 2010; Ren *et al*., 2012). In unstimulated cells NFκB remains in the cytoplasm in complex with the inhibitory protein IκB (Baeuerle and Henkel, 1994). Activation of the TLR pathway causes IκB to be degraded following phosphorylation, freeing NFκB to form dimers and enter the nucleus to drive gene expression (Gilmore, 2006). Cordycepin has been found to prevent phosphorylation of IκB in human cells, possibly by reducing the activity of kinases that phosphorylate this protein through direct interactions with IKK as oppose to inhibition of IκB/IKK transcription, although the nature of these interactions is not known (Ren *et al*., 2012). Cactus is an IκB homolog found in *D. melanogaster* that inhibits the transport of DIF into the nucleus (Karin, 1999). Cordycepin may prevent degradation of Cactus in a similar way to IκB, which would prevent DIF from dimerising and entering the nucleus, reducing the expression of AMPs. Unlike DIF, Relish contains its own IκB-like domain that holds it in the cytoplasm. This domain is cleaved following IMD activation (Stöven *et al*., 2000), which requires kinase activity (Stöven *et al*., 2003). Cordycepin may interact with the kinases that phosphorylate the IκB-like domain by a similar mechanism that it does in human cells (Ren *et al*., 2012). The prevention of transport of DIF and Relish to the nucleus would account for the reduction of *attacin* and *metchnikowin* expression following cordycepin treatment. Alternatively, cordycepin could affect transcription of AMPs directly by inhibiting polyadenylation of their mRNA, through its incorporation into the poly(A) tail, reducing the stability of these transcripts (Kondrashov *et al*., 2012).

In order to better understand the mechanism behind the effect of cordycepin, future experiments could monitor the activity of Relish and DIF in more detail. Western blotting has been used to monitor the localisation of NFκB transcription factors in human airway smooth muscle cells (Kondrashov *et al*., 2012) and this could be applied to *D. melanogaster* cells. Additionally, luciferase reporter assays could be performed to monitor NFκB activity following treatment with cordycepin (Ren *et al*., 2012), the impact of knockouts introduced using RNAi, or the impact of cordycepin in cells overexpressing key proteins such as IKK (Ren *et al*., 2012). Western blots can also monitor degradation of proteins, therefore this technique could be used to follow the degradation of IκB-like proteins in *D. melanogaster* cells, using a similar method to that employed by Ren *et al*., (2012) to monitor the degradation of IκB in human embryonic kidney cells.

5.5 Conclusions

Cordycepin (50-1600 μM) was lethal to *D. melanogaster* S2r+ cells after 48 h. In human cell lines this lethality is due to it arresting the cell cycle and inducing apoptosis, so a similar response may occur in S2r+ cells. Cordycepin also caused a reduction in expression of the AMPs *attacin* and *metchnikowin*, which supports the findings from Chapter 4. However, the expression of the housekeeping gene *RpL32* was also reduced, which may be an anomalous result or indicate that cordycepin has a general impact on transcription. The impact of cordycepin on AMP expression may be due to it preventing Relish and DIF from entering the nucleus as similar effects have been observed in human cells, however further work needs to be done to elucidate this mechanism.

Chapter 6- Investigating the potential of cordycepin as a biopesticide against *Plutella xylostella*

6.1 Background

6.1.1 Diamondback moth (*Plutella xylostella*)

Diamondback moth (*P. xylostella*) is a major lepidopteran pest of *Brassica* worldwide, costing the world economy US$ 4-5 billion annually in control methods and crop losses (Zalucki *et al*., 2012). Its larvae (Figure 6.1) feed on the foliage of important food crops (Figure 6.2) including: canola, cauliflower, cabbage, mustard and turnip (Sarfraz *et al*., 2005).

Figure 6.1- Fourth instar *Plutella xylostella* larva.

P. xylostella has a wide temperature range (10 ˚C-32.5 ˚C) (Golizadeh *et al*., 2007; Marchioro and Foerster, 2011) and is thought to be the most widely distributed Lepidoptera pest (Shelton, 2004). Multiple generations of *P. xylostella* can occur in a year depending on climatic conditions and between 4 and 20 generations per year have been reported (Harcout, 1986; Feng *et al*., 2011). The larvae have four instars and the mean time taken to develop from egg to adult at 20 ˚C is 21.34 days on cauliflower and 23.56 days on cabbage (Golizadeh *et al*., 2007).

P. xylostella has developed resistance to all major classes of conventional chemical insecticides (Furlong *et al*., 2013), which is likely due to its short life cycle. In 2018

the Arthropod Pesticide Resistance Database had documented reports of resistance to 95 pesticides in *P. xylostella* populations in the field (APRD, 2018), including resistance to *B. thuringiensis* (Ferré *et al.*, 1991). There is little plant genetic resistance to this pest, so new non-chemical control methods are urgently needed.

Figure 6.2- Damage to *Brassica oleracea* var. *botrytis* caused by *Plutella xylostella*. Characteristic 'window pane' damage to *Brassica oleracea var. botrytis* from a culture containing multiple developmental stages of *P. xylostella* larvae.

6.1.2 Biological control of *P. xylostella*

Due to the prevalence of insecticide resistance in *P. xylostella*, IPM techniques have been widely investigated for its control and the use of parasitoids in particular has been explored. Globally there are over 135 insect species that parasitize *P. xylostella* (Sarfraz *et al.*, 2005) and some have been used successfully to control it as part of IPM programmes (Vos, 1953; Goodwin, 1979). For example, three species of parasitoid wasp (*Diadegma cerophaga*, *Thyraeela collaris* and *Diadegma rapi*) were released in Australia and their effect on *P. xylostella* population monitored over two years. These parasitoids became established and gave an average of 49% parasitism over the course of the study (Goodwin, 1979). IPM is important to minimise the use of chemical pesticides when parasitoids are being considered to control pest populations, as many pesticides can cause parasitoid mortality (Haseeb *et al.*, 2004) and reduce the rate of parasitism (Haseeb *et al.*, 2000).

Inhibition of moulting is an activity of some pesticides and has been suggested for use in IPM. For example the chemical azadiractin is an extract from the plant Neem (*Azadirachta indica*) that disrupts moulting and growth (Khater, 2012). Additionally, microbial biopesticides have been investigated for the control of *P. xylostella*. As mentioned in Section 1.3, *B. thuringiensis* spores and the purified Bt ∂-endotoxin are widely used biopesticides and have been used to control *P. xylostella*. However, resistance has emerged in field populations (Ferré *et al*., 1991) which makes this control method less reliable and highlights the need for novel control methods. EPF have also been explored to control *P. xylostella*, in particular *Beauveria bassiana* and *Metarhizium anisopliae s.l*. The virulence of these EPF against *P. xylostella* has been studied in both laboratory experiments (Vandenberg *et al*., 1998b; Yoon *et al*., 1999; Godonou *et al*., 2009; Wraight *et al*., 2010; Batta, 2013) and field trials (Vandenberg *et al*., 1998a; Shi and Feng, 2006; Godonou *et al*., 2009).

In laboratory experiments, *B. bassiana* has been found to significantly increase the mortality of *P. xylostella* larvae, particularly when applied at high concentrations (Vandenberg *et al*., 1998b; Yoon *et al*., 1999). A large variation in strain virulence has also been observed (Wraight *et al*., 2010), which would impact strain selection for use in biopesticides. Results from field and glasshouse trials have suggested that *B. bassiana* may be useful to control *P. xylostella* commercially. Vandenberg *et al*. (1998a) carried out short-term field and glasshouse experiments by introducing laboratory-reared *P. xylostella* to broccoli or cabbage and treating crops with *B. bassiana* conidia, a significantly increased larval mortality was identified in both trials. Godonou *et al*. (2009) carried out field trials over two years to assess the efficacy of *B. bassiana* treatment on cabbage and found that it was more effective at controlling *P. xylostella* than the traditional insecticide bifenthrin. However, both Godonou *et al*. (2009) and Vandenberg *et al*. (1998a) concluded that the use of EPF for pest control needed more research and development. A problem with using EPF in the field is that in general there is a lag between treatment and population reduction (Johnson and Goettel, 1993; Jaros-Su *et al*., 1999; Benjamin *et al*., 2002; Peng *et al*., 2008). EPF also cause a lower level of mortality than most traditional chemical insecticides and are more sensitive to environmental conditions (discussed in Section 1.4.1), which reduces their efficacy. The use of other biopesticides or novel

compounds, such as cordycepin, in conjunction with EPF may increase the rate of insect mortality and efficacy to EPF, allowing them to be more widely used in IPM. This would be a novel approach for using EPF within insect control programs.

6.1.3 The impact of cordycepin on *P. xylostella*

There has been one published study into the impact of cordycepin on *P. xylostella* larvae (Kim *et al*., 2002). In this work 3rd instar larvae were fed on leaf disks dipped in cordycepin solutions ranging in concentration from 25-500 mg ml^{-1} and survival monitored. Up to 100% mortality was observed when larvae were fed 500 mg l^{-1} of cordycepin, with lower mortality at 400, 300, 200, 100, 50 and 25 mg l^{-1}. It was observed that prior to death, larvae became dark brown and then body lysis occurred. It was also found that topical application of cordycepin did not cause mortality and therefore it was hypothesised that cordycepin was acting on the larval gut (Kim *et al*., 2002).

6.1.4 Aims and objectives

The overall aim of this chapter was to identify the potential of cordycepin as a biopesticide against *P. xylostella*. This was done by achieving the following objectives:

- Assess the impact of feeding cordycepin on *P. xylostella* survival.
- Identify synergistic/antagonistic/additive effects of combined applications of cordycepin and *B. bassiana* when applied to *P. xylostella* larvae to determine whether they would be compatible in an IPM strategy against this pest.

6.2 Materials and Methods

6.2.1 Plant material

All plants were grown in 7x7x8 cm plant pots (Desch Plantpak, The Netherlands) in Levington F2 +S compost (Levington Horticulture Ltd., U.K) and maintained at 20 ˚C at 16:8 h light:dark cycle in a controlled environment room.

6.2.2 Insect material

P. xylostella used in this project were taken from a culture maintained at Warwick Crop Centre (The University of Warwick, Wellesbourne, U.K) originally collected in 1995 at Wellesbourne, Warwickshire, U.K. Adult *P. xylostella* were kept in a 30 x 30 x 30 cm BugDorm cage (Watkins and Doncaster Ltd, Herefordshire, U.K) with a single Brussels sprout cv. Doric plant (*Brassica oleracea* var. *gemmifera*) and allowed to oviposit. After 24-48 h these plants were removed and placed in a 47.5x 47.5 x 47.5 cm BugDorm cage (Watkins and Doncaster Ltd, Herefordshire, U.K) with cauliflower cv. Skywalker plants (*Brassica oleracea* var. *botrytis*). After larvae had emerged, new plants (10-20 weeks old) were added and old plants removed as necessary. Adult *P. xylostella* were transferred between cages using a mechanical pooter (Watkins and Doncaster Ltd., Herefordshire, U.K). All cages were maintained at 18 ˚C with 16:8 h light:dark cycle in a controlled environment room.

Second instar *P. xylostella* larvae were collected for use in experiments by production of a fixed age culture. One Brussels sprout plant was placed in a 30 x 30 x 30 cm BugDorm cage with adult *P. xylostella* to allow oviposition for 24 h. This plant was then refrigerated at 4 ˚C while the process was repeated with another plant. The following day, both plants were placed in a 47.5x 47.5 x 47.5 cm BugDorm cage with a cauliflower plant (<10 weeks old). Early second instar larvae (approximately six days after oviposition) were used for experiments and starved for 3 h prior to use.

6.2.3 Fungal material

B. bassiana 433.99 (Table 2.1) was used for these experiments because it is commercially available as a biopesticide and it is effective in laboratory studies for the control of *P. xylostella* (Vandenberg *et al.*, 1998b; Yoon *et al.*, 1999). The maintenance, subculturing and preparation of conidial suspensions was carried out as in Section 2.2.3. Aliquots (10 ml) of conidia were prepared at a concentration of $1x10^7$ conidia ml^{-1} and this was diluted to the concentration desired in each experiment.

6.2.4 Response of *P. xylostella* larvae to cordycepin treatment

Two experiments were carried out to determine the impact of cordycepin on survival of *P. xylostella* larvae (Figure 6.3). In the first, cordycepin was applied as a one-off treatment and in the other it was administered continuously.

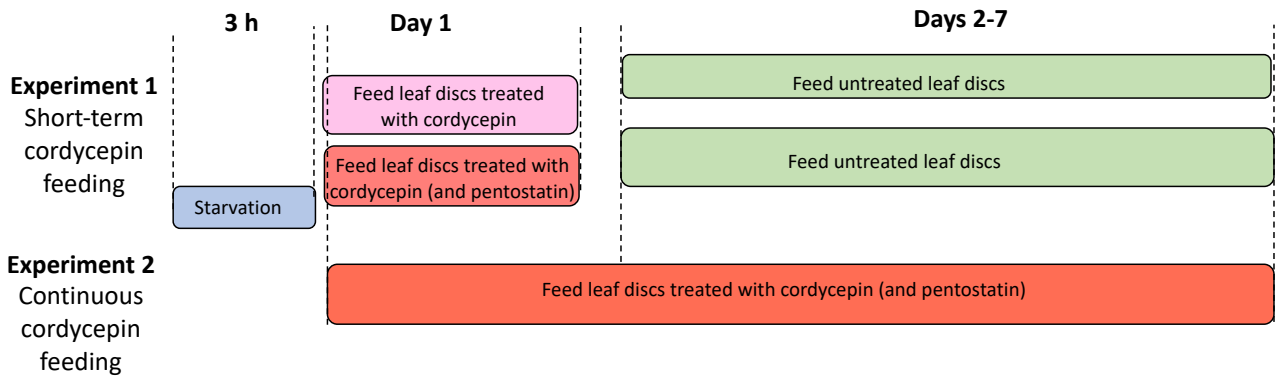


Figure 6.3- Short term and long term cordycepin feeding experiments in Chapter 6. Time taken for each section of short-term exposure to cordycepin experiments (experiment 1; Section 6.2.4.1) and prolonged exposure to cordycepin experiments (experiment 2; Section 6.2.4.2).

6.2.4.1 Response of *P. xylostella* larvae to short-term cordycepin treatment

Leaf discs were cut from cauliflower leaves using a size 16 cork borer (23.75 mm diameter). Dilutions of cordycepin at 6, 3, 1.5, 0.75 and 0.375 mg ml^{-1} were prepared by diluting a 100 mg ml^{-1} cordycepin stock solution (prepared in DMSO) in sterile 0.01% Triton X-100 (Merck, Germany). These solutions contained either pentostatin at a final concentration of 1 µM or the same volume of DMSO. Then 100 µl aliquots

of the solution were spread onto the underside of each leaf disc with an 'L' shaped spreader (Greiner bio-one, Austria). Two controls were incorporated that contained DMSO in 0.01% Triton X-100, or DMSO in 0.01% Triton X-100 with 1 μM pentostatin. The leaf discs were dried for at least 2 h in a laminar flow hood before use in experiments.

A single leaf disc was placed in the centre of a 9 cm Petri dish (SARSTEDT, Germany) (Figure 6.4) containing 1.2% water agar (Agar Technical No. 3, Oxoid, USA). To allow air flow into the Petri dish and minimise condensation, the lid contained holes covered with perforated plastic (micro-perforated polypropylene bag, Cryovac ®). Up to 12 second instar *P. xylostella* larvae were placed on the leaf disc and the Petri dish was sealed with Parafilm 'M' ® (Bemis, USA). The dishes were maintained at 20 ˚C (16:8 h light:dark) in an environmental test chamber (Sanyo, Japan). Every 24 h for seven days surviving larvae were transferred to an untreated size 16 (23.75 mm diameter) leaf disc and the old leaf disc was photographed. The experiment was replicated on three separate occasions. The area of leaf disc eaten was determined using ImageJ (Schneider *et al.*, 2012). Regression models (linear, second degree polynomial or third degree polynomial) were fitted to mortality on day seven in R (RStudio, 2017) and the fit of the models was compared using an ANOVA to determine whether they gave significant improvement over a linear regression model. Where no significant improvement was seen, a linear regression was used and models were visualised using the ggplot2 package in R (Wickham, 2016). This was also done to visualise the mean percentage of leaf disc eaten per individual larva throughout the experiment.

The significance of the effect of co-administering pentostatin was determined using a chi-squared test. The expected mortality was calculated using the following equation: $M_E = M_P + M_C(1 - \frac{M_P}{100})$, where M_P is the percentage mortality caused by pentostatin alone and M_C is the percentage mortality caused by cordycepin alone. Chi-squared values were calculated using the following equation: $X^2 = (M_O - M_E)^2/M_E$, where M_O is the observed percentage mortality for combined treatments. These values were compared to the chi-squared value for 1 degree of freedom at 0.05 significance level

(3.841). Any chi-squared value greater than this indicated synergy (or antagonism) and anything lower suggested an additive effect.

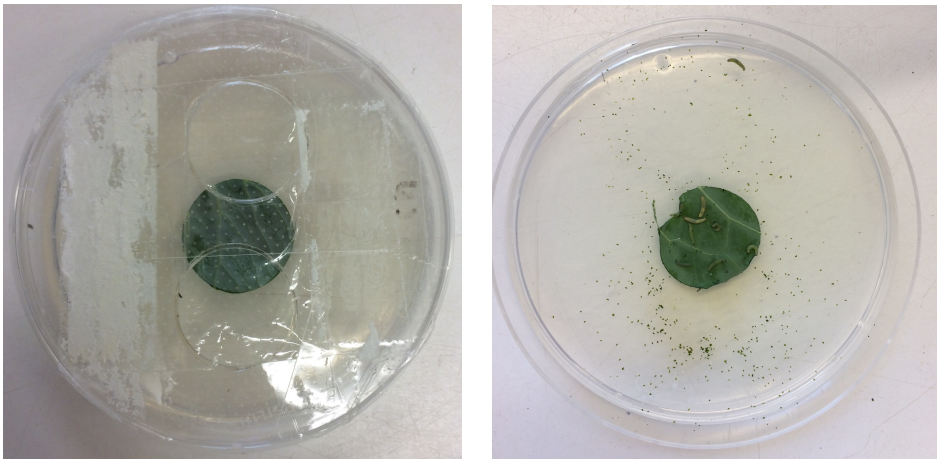


Figure 6.4- *Plutella xylostella* larvae in a leaf disc feeding bioassay. Size 16 leaf disc (*Brassica oleracea* var. *botrytis*) in the centre of a 9 cm Petri dish containing water agar with *P. xylostella* larvae that had been sprayed with *Beauveria bassiana* 433.99 conidia ($1x10^5$ conidia ml^{-1}). Holes were made in the Petri dish lid and covered in perforated plastic (left) to prevent condensation.

6.2.4.2 Response of *P. xylostella* to prolonged exposure to cordycepin

Leaf discs were cut from cauliflower leaves using a size 16 cork borer (23.75 mm diameter). The following dilutions of cordycepin (Sigma-Aldrich, USA) were made from a 100 μM stock solution (in DMSO) using Triton X-100: 1.5, 0.75, 0.375, 0.185 and 0.093 mg ml^{-1}. These solutions also contained pentostatin at a final concentration of 1 μM. A control was included that contained DMSO in 0.01% Triton and 1 μM pentostatin. Aliquots (100 μl) were spread onto each leaf disc as described in Section 6.2.4.

A single leaf disc was placed in the centre of a 9 cm Petri dish (Figure 6.4) containing water agar (Agar Technical No. 3, Oxoid, USA), as described in Section 6.2.4.1. Up to 12 second instar *P. xylostella* larvae were placed on the leaf disc and the Petri dish was sealed with Parafilm 'M' ® (Bemis, USA). Dishes were maintained at 20 ˚C (16:8 h light:dark) in an environmental test chamber (Sanyo, Japan). After 24 h, surviving

larvae were counted and transferred to another freshly treated leaf disc and the old leaf disc was photographed. This was repeated every 24 h for seven days and the experiment was repeated on three occasions. The area of leaf disc eaten was determined using ImageJ (Schneider *et al*., 2012). Regression models (linear, second degree polynomial or third degree polynomial) were fitted to mortality at day seven in R (RStudio, 2017) and the fit of the models was compared using an ANOVA to determine whether they gave significant improvement over a linear regression model. Where no significant improvement was seen, a linear regression was used and models were visualised using the ggplot2 package in R (Wickham, 2016). This was also done to visualise the mean percentage of leaf disc eaten per individual larva throughout the experiment.

6.2.5 Effect of combined applications of cordycepin and *B. bassiana* 433.99 on the mortality of *P. xylostella*

Two experiments were carried out to quantify the effect of combined application of *B. bassiana* 433.99 conidia and cordycepin. In the first, cordycepin was given as one short-term dose for 24 h (1.5 mg ml^{-1}) and in the other a low concentration (0.1 mg ml^{-1}) was administered continuously. These concentrations were selected as 0.1 mg ml^{-1} was found to be sub-lethal and 1.5 mg ml^{-1} to cause a high level of mortality (but less than 80% mortality) and reduce feeding activity in previous experiments. Cordycepin was applied to cauliflower leaf discs as described in Section 6.2.4. In the first experiment larvae were fed 1.5 mg ml^{-1} cordycepin for 24 h following *B. bassiana* 433.99 application, then fed untreated leaf discs for the remainder of the experiment. In the second experiment, larvae were fed leaf discs treated with 0.1 mg ml^{-1} of cordycepin following *B. bassiana* 433.99 application, which were replaced with fresh cordycepin-treated leaf discs daily.

Conidial suspensions of *B. bassiana* 433.99 were prepared at the concentrations: $1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$ and $1x10^3$ conidia ml^{-1} (Section 2.2.3) and 0.01% Triton X-100 was used as a control. Groups of ten larvae, which had been cooled on ice for five minutes, were placed onto filter paper (Whatman, GE healthcare, U.K) within a 9 cm Petri dish lid. Larvae were treated with 4 ml of a conidial suspension using a

Potter tower air atomising sprayer (Potter, 1952) at 34.5 KPa. Larvae were allowed to recover at room temperature for 30 min and surviving larvae were transferred onto a cordycepin-treated leaf disc or a control leaf disc (treated with DMSO in 0.01% Triton X-100) in a 9 cm Petri dish containing 1.2% water agar (Agar Technical No. 3, Oxoid, USA) (similar to Figure 6.4). Dishes were maintained at 20 ˚C 16:8 h light:dark cycle in an environmental test chamber (Sanyo, Japan) and treated or untreated leaf discs were replaced daily. Survival was monitored daily for seven days, larvae that died immediately following topical application (due to handling) were not included in the overall mortality. This experiment was replicated on three occasions for each dose of cordycepin (0.1 mg ml^{-1} or 1.5 mg ml^{-1}).

The mortality of *P. xylostella* on days five, six and seven were used to perform a chi-squared test to compare observed mortality and expected mortality at different concentrations of *B. bassiana* 433.99. This method has been used previously to identify synergy between EPF and other compounds (Koppenhöfer and Fuzy, 2003; Morales-Rodriguez and Peck, 2009). The expected mortality was calculated using the following equation: $M_E = M_C + M_B(1 - \frac{M_C}{100})$, where M_C is the percentage mortality caused by cordycepin alone and M_B is the percentage mortality caused by *B. bassiana* 433.99 alone. Chi-squared values were calculated using the following equation: $X^2 = (M_O - M_E)^2/M_E$, where M_O is the observed percentage mortality for combined treatments. These values were compared to the chi-squared value for 1 degree of freedom at 0.05 significance level (3.841). Any chi-squared value greater than this indicated synergy (or antagonism) and anything lower suggested an additive effect.

6.2.6 Choice experiments to determine the antifeedant effect of cordycepin

Choice experiments were carried out to identify whether cordycepin has an anti-feedant effect. The doses chosen were 0.1 or 1.5 mg ml^{-1}, because they were used in Section 6.2.5 and caused low mortality after 48 h of feeding. Cauliflower leaf discs (0.5 cm diameter) were treated with either 0, 0.1 or 1.5 mg ml^{-1} of cordycepin solution (with 1 μM pentostatin) as described in Section 6.2.4. Two treated leaf discs were placed on a 9 cm Petri dish containing water agar (Section 6.2.4.1) in the following combinations: 0 mg ml^{-1}/0 mg ml^{-1}, 0 mg ml^{-1}/0.1 mg ml^{-1}, and 0 mg ml^{-1}/1.5 mg ml^{-1}

[1] (Figure 6.5). A single second instar *P. xylostella* larva was placed equidistant between them and the Petri dish was sealed with Parafilm 'M' ® (Bemis, USA). On each occasion there were ten individuals per treatment combination and the experiment was replicated three times. Dishes were maintained at 20 ˚C and 16:8 h light:dark in an experimental test chamber (Sanyo, Japan).

After 24 and 48 h leaf discs were photographed and ImageJ (Schneider *et al*., 2012) was used to calculate the area of leaf disc eaten. If a larva had died or had not consumed any leaf disc, it was excluded from analysis. The anti-feedant index (AI) was calculated using the following equation described by Ortego *et al*., (1995): $AI=\frac{C-T}{C+T}x100$. Where C is the area of the untreated leaf disc consumed and T is the area of the treated leaf disc consumed. The mean AI was determined for three replicates and analysed in SPSS Statistics (Version 24, IBM). Firstly, normality was assessed using a Shapiro-Wilk test, then a one-sample Wilcoxon signed rank test was performed, with a hypothesis that the median was equal to 0.

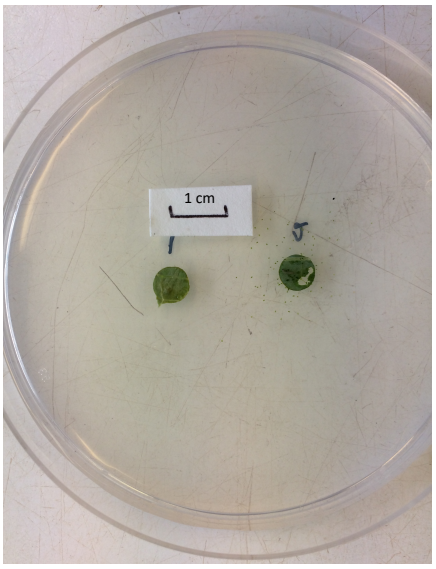


Figure 6.5- *Plutella xylostella* choice experiment after 24 h. A second instar *P. xylostella* larva given the choice between two leaf discs (0.5 mm diameter), both treated with a control solution (DMSO, Triton X-100 and pentostatin).

6.3 Results

6.3.1 Response of *P. xylostella* larvae to cordycepin treatment

6.3.1.1 Response of *P. xylostella* to short-term cordycepin treatment

Cordycepin (without pentostatin) reduced the survival of *P. xylostella* larvae when it was administered by feeding for 24 h at the beginning of the experiment (Figure 6.6). Often, cadavers appeared to be dark grey or black (Figure 6.7) following cordycepin feeding, which may be a symptom of bacterial infection. At day seven after treatment there appeared to be a dose response, with 1.5-3 mg ml^{-1} causing the highest mortality in all three replicates and mortality decreasing at higher concentrations (Figure 6.6). At 1.5 mg ml^{-1} and 3 mg ml^{-1} of cordycepin the mean mortality was 51.2% and 62.3%, respectively on day seven and the mean mortality of control larvae was 6%. A second order polynomial regression was found to best fit the data (adjusted R-squared= 0.5031) and the regression analysis showed that the correlation between concentration of cordycepin and mortality was significant (p=0.002; Appendix C.1).

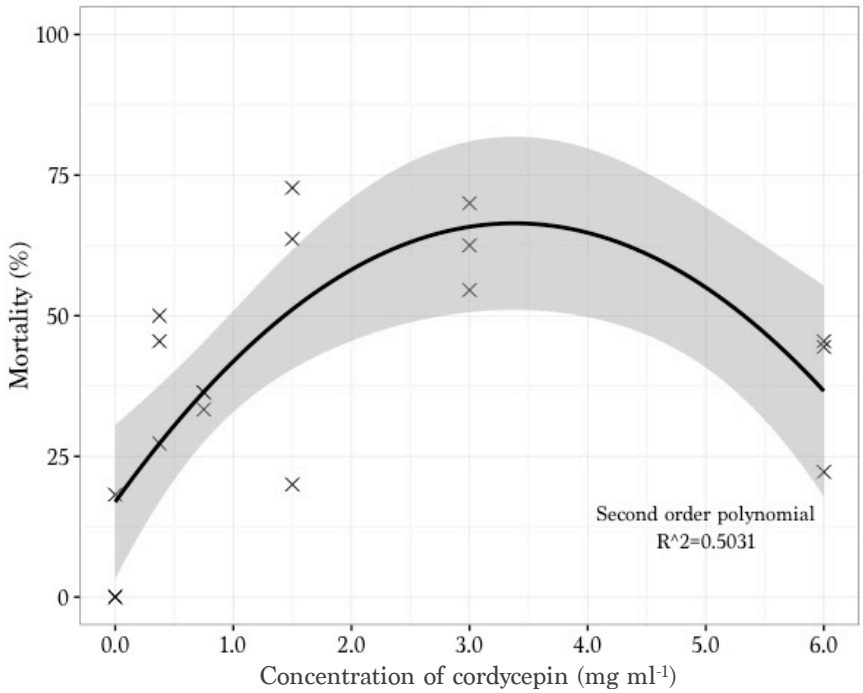


Figure 6.6- Mortality of *Plutella xylostella* larvae following 24 h of cordycepin feeding. The percentage mortality on day seven of *P. xylostella* larvae (n=10 for each dose in each replicate) following 24 h of feeding with cordycepin at: 0, 0.375, 0.75, 1.5, 3 and 6 mg ml^{-1}. A second order polynomial regression was fitted in R (R Studio 2017), which gave an adjusted R-squared value of 0.5031. Shaded area represents the standard error of the model.

Figure 6.7-*Plutella xylostella* larvae cadavers following treatment with cordycepin. Examples of *P. xylostella* cadavers following feeding with 0.375 mg ml^{-1} cordycepin (left) or 3 mg ml-1 cordycepin (right). Cadavers are circled in red.

When larvae were treated with a mixture of cordycepin and pentostatin, there also appeared to be a dose response after seven days, however it was variable between replicates (Figure 6.8). Treatment with 1.5 mg ml^{-1} and 3 mg ml^{-1} of cordycepin appeared to cause the highest mortality on day seven (71.6 % and 71.5 % mean mortality, respectively) and the mean percentage mortality of the control was 21.9 %. The high mortality at 0.375 mg ml^{-1} may be an anomaly as it is higher than survival at 0.75 mg ml^{-1} and does not fit the dose response pattern. A second order polynomial regression was found to best fit the data (adjusted R-squared=0.2741) and the regression analysis showed that there was a significant correlation between concentration of cordycepin and mortality (p=0.03; Appendix C.1).

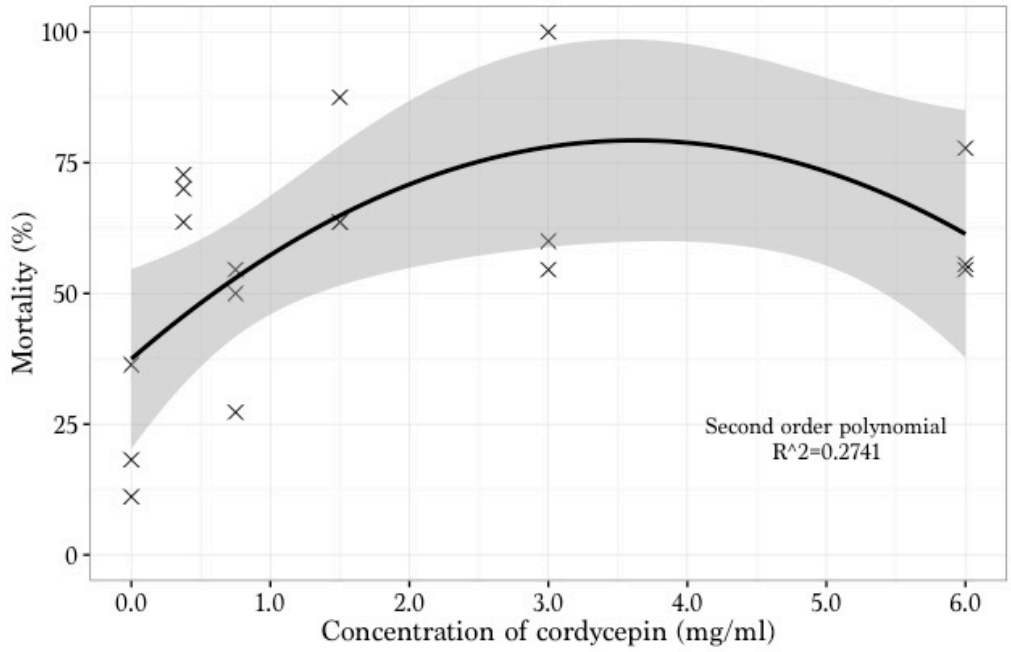


Figure 6.8- Mortality of *Plutella xylostella* larvae following 24 h of cordycepin (with pentostatin) feeding. The percentage mortality on day seven of *P. xylostella* larvae (n=10 for each dose in each replicate) following 24 h of feeding with cordycepin (and 1 μM pentostatin) at: 0, 0.375, 0.75, 1.5, 3 and 6 mg ml^{-1}. A second order polynomial regression was fitted in R (R Studio 2017) and gave the adjusted R-squared value of 0.2741. Shaded area represents the standard error of the model.

A chi-squared test illustrated that co-application of pentostatin with cordycepin had either an additive or synergistic effect on day six after treatment, depending on the concentration of cordycepin (Table 6.1). Similar results were obtained on day five and day seven after treatment (Appendix C.1). However, these results may not be reliable due to the variation between replicates.

Table 6.1- Effect of co-application of a range of cordycepin concentrations and pentostatin on *Plutella xylostella* survival. Mortality of *P. xylostella* larvae on day six after treatment following feeding of cordycepin with or without pentostatin (1 μM) for 24 h. Interactions between treatments were identified as additive or synergistic using a chi-squared test, chi-squared values are also presented.

Concentration of cordycepin (mg ml^{-1})	Measurement	Percentage Mortality (+-SEM)	Chi-squared	Effect
0.375	Observed	62.42	7.08	Synergistic
	Expected	44.64		
0.75	Observed	40.91	0.31	Additive
	Expected	44.64		
1.5	Observed	68.56	2.88	Additive
	Expected	55.88		
3	Observed	68.18	0.02	Additive
	Expected	69.20		
6	Observed	48.48	0.00	Additive
	Expected	48.77		

Feeding was monitored throughout the experiments, the anti-feedant effect could not be calculated as mortality occurred at all concentrations of cordycepin. Whether cordycepin was fed alone or with pentostatin it appeared to cause a reduction of feeding (Figures 6.9 and 6.10), the effect appeared greatest at 3 mg ml^{-1} and 6 mg ml^{-1}, although there was a large variation between replicates. This is illustrated by low adjusted R-squared values and high p-values obtained during regression analyses (Appendices C.2 and C.3).

The mean percentage of leaf disc eaten per individual *P. xylostella* larva when fed only cordycepin is shown in Figure 6.9. Feeding activity was low on days one to four, which prevented regressions being fitted to the data, hence only days five to seven are presented. The mean percentage of control leaf disc eaten per individual on day seven when fed only cordycepin was 10.4%. This was reduced by the addition of cordycepin to 3.7% and 1.8% when treated with 3 mg ml^{-1} and 6 mg ml^{-1}, respectively. Linear regression analysis best described the data on days five to seven (Figure 6.9; adjusted R-squared values in Appendix C.2), indicating that feeding reduced as the concentration of cordycepin increased. However, in all cases the regression analysis did not show that this relationship was significant ($p>0.05$; Appendix C.2), which

could be due to variance between replicates. Larvae in replicate one appeared to consume a greater area following cordycepin treatment than the other two replicates, possibly due to biological variation between individuals or differences in the amount of cordycepin ingested on day one.

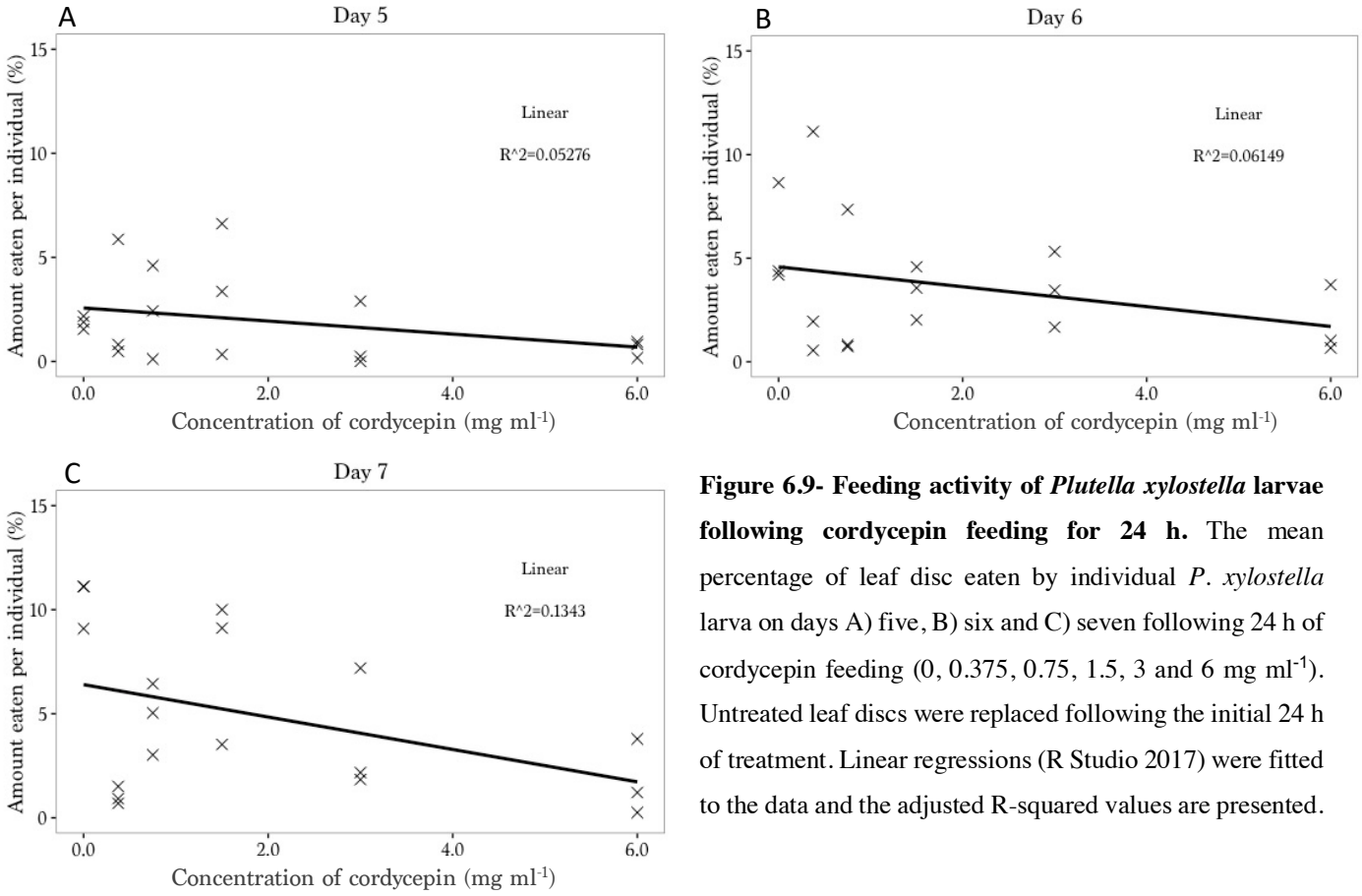


Figure 6.9- Feeding activity of *Plutella xylostella* larvae following cordycepin feeding for 24 h. The mean percentage of leaf disc eaten by individual *P. xylostella* larva on days A) five, B) six and C) seven following 24 h of cordycepin feeding (0, 0.375, 0.75, 1.5, 3 and 6 mg ml^{-1}). Untreated leaf discs were replaced following the initial 24 h of treatment. Linear regressions (R Studio 2017) were fitted to the data and the adjusted R-squared values are presented.

When pentostatin was co-applied with cordycepin the mean percentage of leaf disc eaten per individual was 10.5% for controls on day seven. It was reduced to 2.4% and 1.6% for by the addition of 3 mg ml^{-1} or 6 mg ml^{-1} of cordycepin, respectively. The best fitting regression models were linear for days five to seven (Figure 6.10; adjusted R-squared values in Appendix C.3), however regression analyses only identified a significant relationship between feeding and concentration of cordycepin on day seven after treatment (p=0.0303; Appendix C.3). The lack of significance may be due to variation between replicates. There was not an effect of pentostatin alone on the feeding activity of larvae, the mean percentage eaten per individual without pentostatin was 10.4% on day seven and with pentostatin was 10.5%.

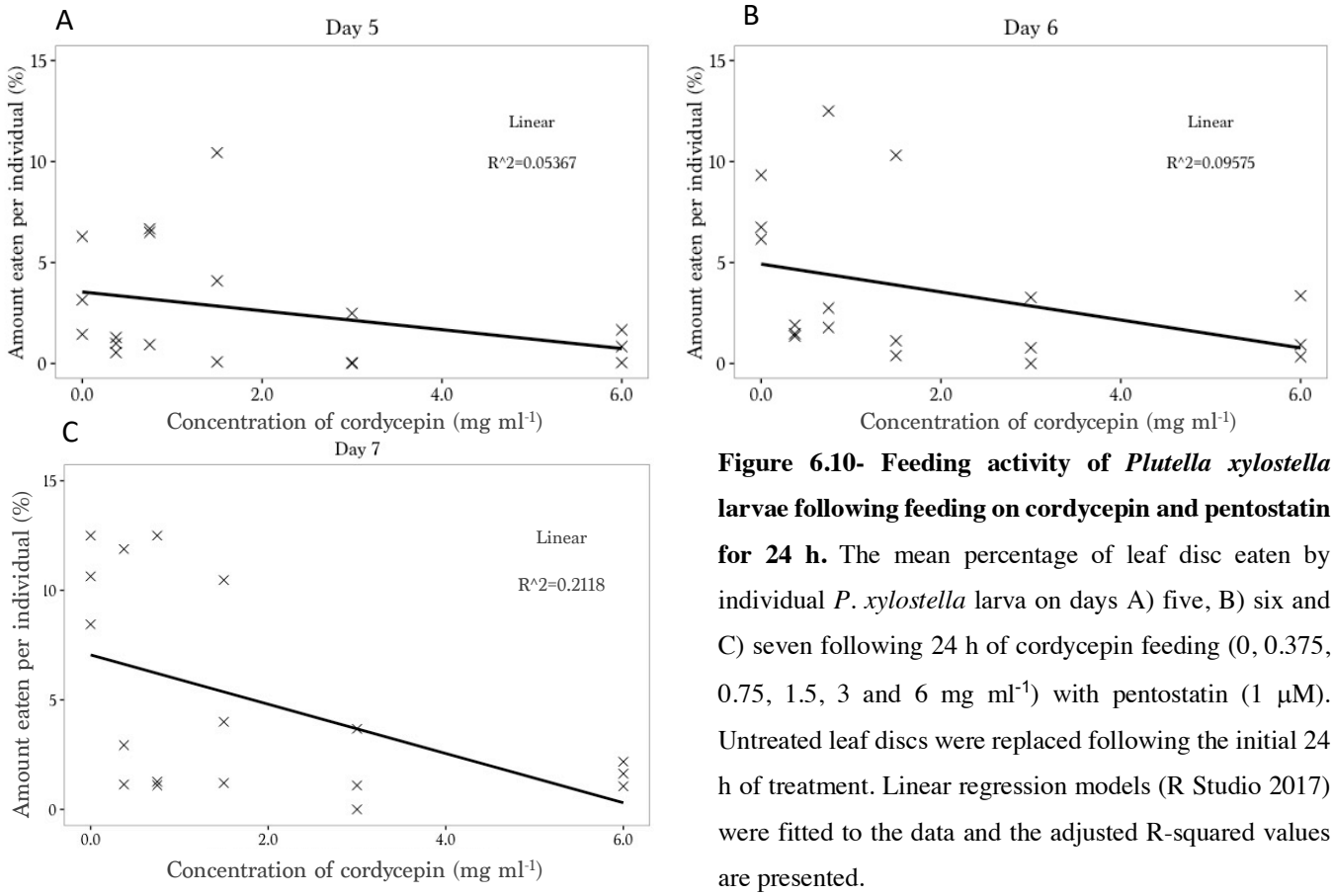


Figure 6.10- Feeding activity of *Plutella xylostella* larvae following feeding on cordycepin and pentostatin for 24 h. The mean percentage of leaf disc eaten by individual *P. xylostella* larva on days A) five, B) six and C) seven following 24 h of cordycepin feeding (0, 0.375, 0.75, 1.5, 3 and 6 mg ml^{-1}) with pentostatin (1 μM). Untreated leaf discs were replaced following the initial 24 h of treatment. Linear regression models (R Studio 2017) were fitted to the data and the adjusted R-squared values are presented.

6.3.1.2 Response of *P. xylostella* to prolonged exposure to cordycepin

P. xylostella larvae were continuously fed on leaf discs treated with a range of low concentrations of cordycepin. The percentage mortality increased linearly as the concentration of cordycepin increased (Figure 6.11; adjusted R-squared value in Appendix C.1) and regression analysis illustrated that this relationship was significant ($p<0.001$). On day seven the two concentrations of cordycepin that caused the highest mortality were 0.75 mg ml^{-1} and 1.5 mg ml^{-1}, giving a mean mortality of 56.7% and 70%, respectively, with control larvae having a mean mortality of 13.7%.

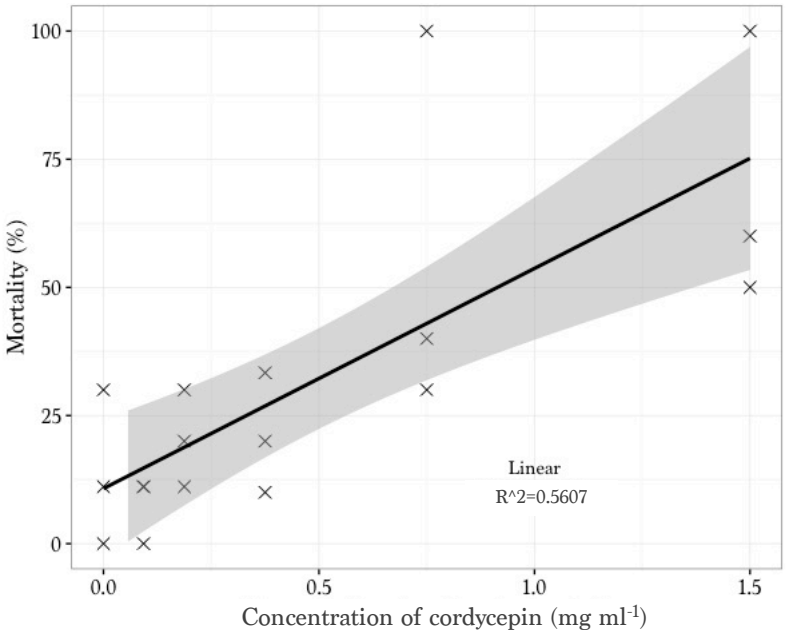


Figure 6.11- Mortality of *Plutella xylostella* larvae continuously fed cordycepin. The percentage mortality on day seven of *P. xylostella* larvae (n=10 for each dose in each replicate) while being fed cordycepin (and 1 μM pentostatin) at: 0, 0.093, 0.1875, 0.375, 0.75 and 1.5 mg ml^{-1}. A linear regression model was fitted in gglot2 (R Studio 2017) and the adjusted R-squared value is presented. Shaded area represents the standard error of the model.

The percentage of leaf disc eaten per individual when cordycepin was fed continuously is shown in Figure 6.12. Cordycepin reduced feeding of *P. xylostella* larvae, particularly at 0.75 mg ml^{-1} and 1.5 mg ml^{-1} and regression analyses (adjusted R-squared values in Appendix C.4) identified a significant relationship between the concentration of cordycepin and percentage of leaf disc eaten per individual larva ($p<0.001$ for days five and six, $p=0.001$ for day seven). On day seven the mean percentage of leaf disc eaten by control larvae was 7.1%, for larvae treated with 0.75 mg ml^{-1} of cordycepin was 0.1% and for those treated with 1.5 mg ml^{-1} of cordycepin was 0.2%.

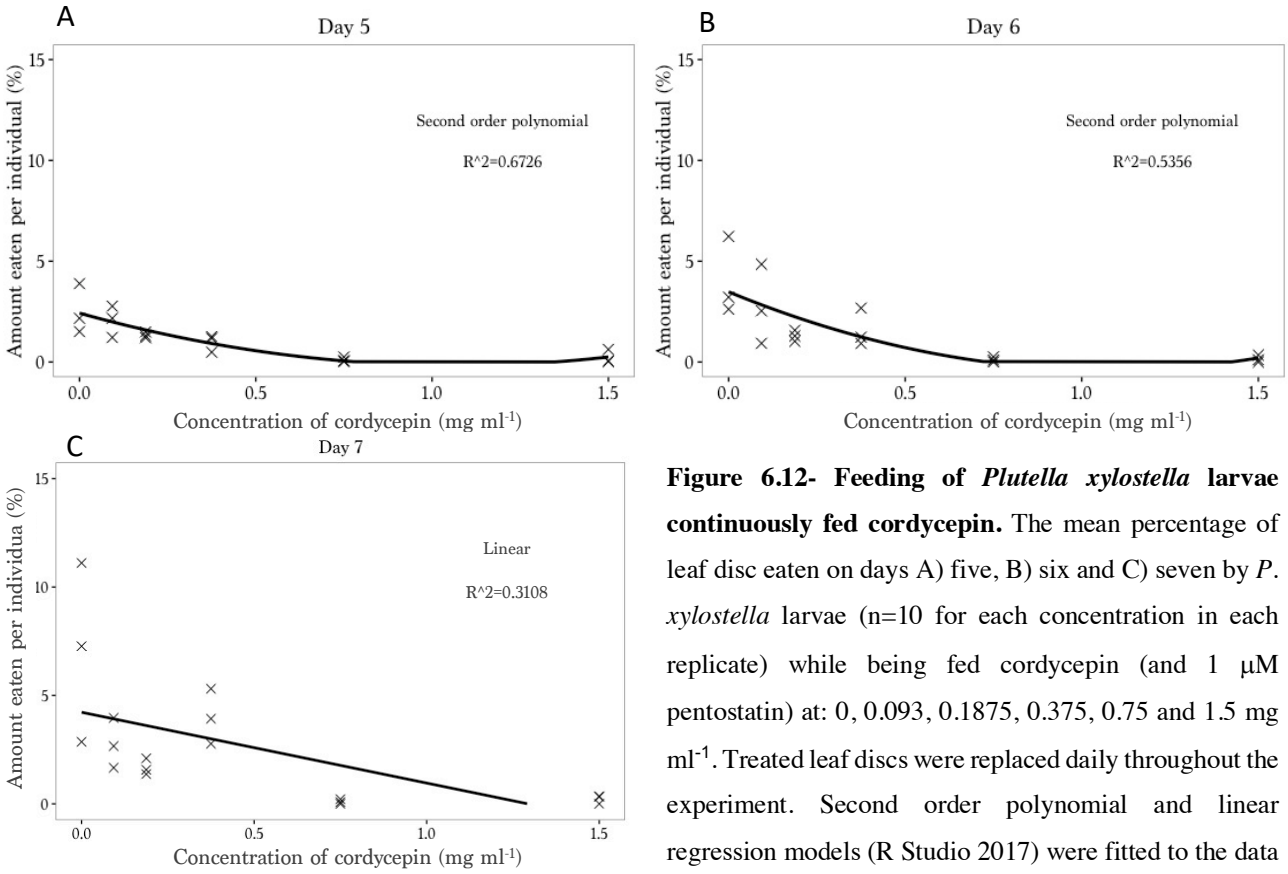


Figure 6.12- Feeding of *Plutella xylostella* larvae continuously fed cordycepin. The mean percentage of leaf disc eaten on days A) five, B) six and C) seven by *P. xylostella* larvae (n=10 for each concentration in each replicate) while being fed cordycepin (and 1 µM pentostatin) at: 0, 0.093, 0.1875, 0.375, 0.75 and 1.5 mg ml^{-1}. Treated leaf discs were replaced daily throughout the experiment. Second order polynomial and linear regression models (R Studio 2017) were fitted to the data and the adjusted R-squared values are presented.

6.3.2 Effect of combined applications of cordycepin and *B. bassiana* 433.99 on the mortality of *P. xylostella*

P. xylostella larvae were fed cordycepin after being sprayed with *B. bassiana* 433.99 conidia and insect survival was monitored over seven days (Figure 6.13). Initially larvae were fed 1.5 mg ml^{-1} of cordycepin for 24 h and then fed untreated leaf discs for the remainder of the experiment. This treatment was selected as in the previous experiments it had been shown to cause mortality and reduce feeding of *P. xylostella* larvae. When larvae were fed 1.5 mg ml^{-1} of cordycepin and treated with less than $1x10^6$ *B. bassiana* 433.99 conidia ml^{-1}, the percentage mortality of larvae ranged from 32.1% to 59.1% on day seven ($1x10^3$-$1x10^6$ conidia ml^{-1}, respectively; Table 6.2). These were not found to vary from expected values suggesting co-application of ≤ $1x10^6$ *B. bassiana* 433.99 conidia ml^{-1} and cordycepin had an additive effect. At a

higher concentration of *B. bassiana* 433.99 ($1x10^7$ conidia ml^{-1}) the observed mortality of larvae on day seven was 100%, this was higher than the expected mortality of 77% (Appendix C.5), which suggests that the interactions were synergistic (chi-squared=6.85). This is supported by the results on days five and six (Appendices C.6 and C.7), where additive effects were observed at $1x10^3$-$1x10^6$ conidia ml^{-1} of *B. bassiana* 433.99 and interactions were synergistic at $1x10^7$ conidia ml^{-1} (chi-squared values of 24.79 and 11.19 for days five and six respectively).

Figure 6.13- *Beauveria bassiana* 433.99 growing from *Plutella xylostella* cadaver following infection by this EPF.

Table 6.2- Effects of co-application of a range of *Beauveria bassiana* 433.99 concentrations and short-term cordycepin treatment. Mortality of *Plutella xylostella* larvae on day seven after treatment following spraying with *B. bassiana* 433.99 conidia and feeding 1.5 mg ml^{-1} cordycepin (with 1 μM pentostatin) for 24 h. Chi-squared values are presented and the effects of treatments identified as additive or synergistic using a chi-squared test.

Concentration of *B. bassiana* (conidia ml^{-1})	Measurement	Percentage mortality (±SEM)	Chi-squared	Effect
1.00E+03	Observed	32.1 ± 17.5	1.17	Additive
	Expected	38.9		
1.00E+04	Observed	39.4 ± 10.6	0.30	Additive
	Expected	36.1		
1.00E+05	Observed	53.9 ±13.3	1.96	Additive
	Expected	44.6		
1.00E+06	Observed	59.1 ±21.5	0.04	Additive
	Expected	57.7		
1.00E+07	Observed	100 ±0	6.85	Synergistic
	Expected	77		

Following the short-term treatment of *P. xylostella* larvae with cordycepin and *B. bassiana* 433.99, cordycepin (0.1 mg ml^{-1}) was fed continuously to *P. xylostella* larvae following topical application of *B. bassiana* 433.99 conidia. This concentration of cordycepin was selected as it was found to be sub lethal. On day seven the percentage mortality ranged from 25.2% to 70% ($1x10^5$ conidia ml^{-1} and $1x10^7$ conidia ml^{-1}, respectively; Table 6.3; Appendix C.8). The effect of co-application was additive at $1x10^3$, $1x10^6$ and $1x10^7$ conidia ml^{-1}, but antagonistic at $1x10^4$ and $1x10^5$ conidia ml^{-1} (chi-squared values of 4.79 and 12.06 respectively). The effect varied between days five and six (Appendices C.9 and C.10), with synergistic, additive and antagonistic effects being identified.

Table 6.3- Effects of co-application of a range of *Beauveria bassiana* concentrations and prolonged cordycepin treatment. Mortality of *Plutella xylostella* larvae on day seven after treatment following spraying with *B. bassiana* 433.99 conidia and feeding 0.1 mg ml^{-1} cordycepin (with 1 μM pentostatin) throughout the experiment. Chi-squared values are presented and the effects of treatments identified as additive or synergistic using a chi-squared test.

Concentration of *B. bassiana* (conidia ml^{-1})	Measurement	Percentage mortality (±SEM)	Chi-squared	Effect
1.00E+03	Observed	31.7 ±10.8	0.11	Additive
	Expected	33.6		
1.00E+04	Observed	27.8 ±7.9	4.79	Antagonistic
	Expected	42		
1.00E+05	Observed	25.2 ±5.1	12.06	Antagonistic
	Expected	49.7		
1.00E+06	Observed	44 ± 15.6	0.21	Additive
	Expected	41		
1.00E+07	Observed	70 ± 21.9	0.51	Additive
	Expected	64.3		

6.3.3 *P. xylostella* choice experiment

In order to determine the mechanism behind cordycepin causing mortality in *P. xylostella*, a choice experiment was carried out (Figure 6.14). The mean (± SEM) area of leaf disc eaten on day one for control treatments was 1.67 (±0.51): 2.26 (±0.44) mm^2 (0 mg ml^{-1}: 0 mg ml^{-1}cordycepin), for treatment with the lowest concentration of cordycepin was 2.27 (±0.44) : 1.36 (±0.38) mm^2 (0 mg ml^{-1}: 0.1 mg ml^{-1} cordycepin) and for treatment with the highest concentration of cordycepin was 1.00 (±0.42) : 2.30 (±0.40) mm^2 (0 mg ml^{-1}: 1.5 mg ml^{-1} cordycepin). On day two the mean area of leaf disc eaten for control treatments was 2.57 (±0.73) : 4.08 (±0.85) mm^2 (0 mg ml^{-1}: 0 mg ml^{-1} cordycepin), for treatment with the lowest concentration of cordycepin was 3.55 (±0.89) : 4.08 (±0.81) mm^2 (0 mg ml^{-1}: 0.1 mg ml^{-1} cordycepin) and for treatment with the highest concentration of cordycepin was 1.98 (±0.72) : 4.81 (±0.69) mm^2 (0 mg ml^{-1}: 1.5 mg ml^{-1} cordycepin). This suggests that cordycepin (1.5 mg ml^{-1}) promotes feeding by *P. xylostella* larvae, although there was a large variation in the total area consumed by each larva.

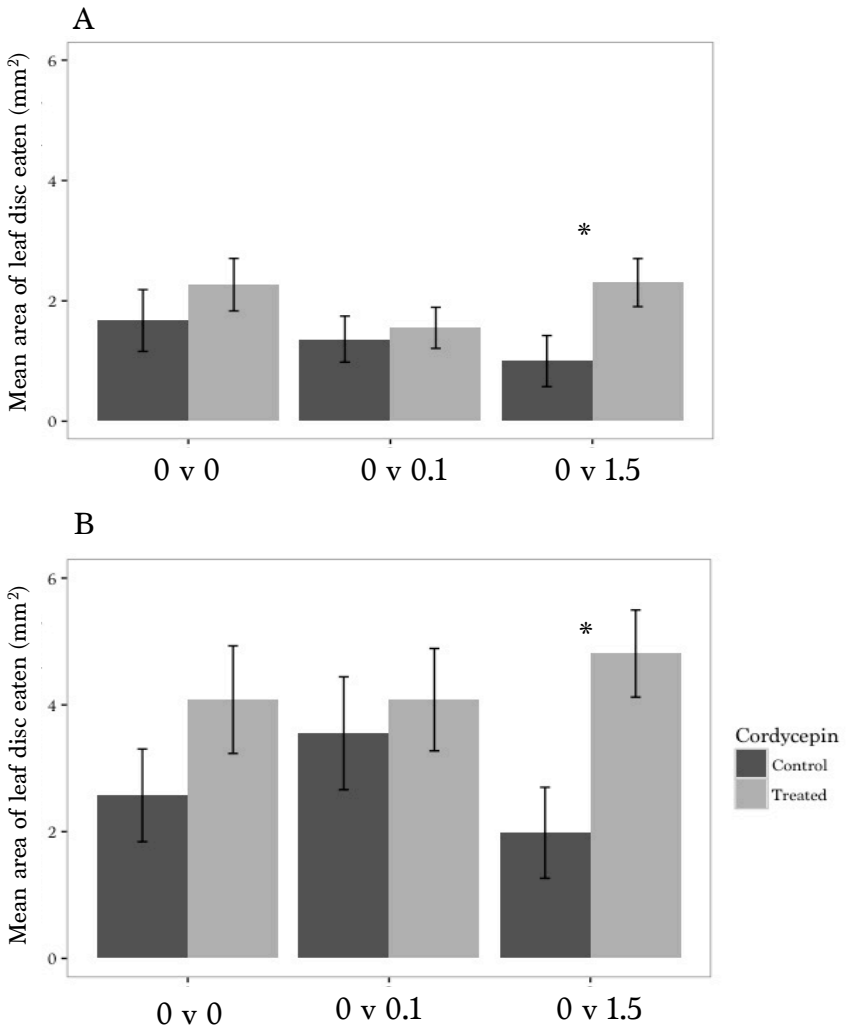


Figure 6.14- The area of leaf discs consumed by *Plutella xylostella* during choice experiments. The mean area of leaf disc consumed after (A) 24 h and (B) 48 h by *P. xylostella* larvae during choice experiments, where larvae were given a choice between: two untreated leaf discs (0v0), an untreated leaf disc (dark grey) v a leaf disc treated with 0.1 mg ml^{-1} of cordycepin (light grey; 0v0.1) and an untreated leaf disc (dark grey) v a leaf disc treated with 1.5 mg ml^{-1} of cordycepin (light grey; 0v1.5). Asterisks indicate significant differences ($p<0.05$) identified using the anti-feedant index analysis displayed in Table 6.4.

The anti-feedant index, $AI=\frac{C-T}{C+T}x100$, was calculated to determine the significance of variation in feeding caused by cordycepin treatment (Table 6.4). No significant effect on feeding was seen with leaves treated with 0.1 mg ml^{-1} of cordycepin, but treatment with 1.5 mg ml^{-1} significantly promoted feeding after 24 h ($p=0.034$) and 48 h ($p=0.005$) of feeding.

Table 6.4- Choice experiment to determine anti-feedant effect of cordycepin on *Plutella xylostella*. Mean antifeedant index ± SEM of second instar larvae fed with leaf discs treated with combinations of 0.1 mg ml^{-1}, 1.5 mg ml^{-1} or 0 mg ml^{-1} of cordycepin. Asterisks show significant differences from a median of 0, determined by a Wilcoxon signed-rank test.

Cordycepin (mg ml^{-1})	Time (h)	Anti-feedant index (%) (±SEM)
0	24	-21 ± 19
0.1	24	-23± 19
1.5	24	-42 ± 19 *
0	48	-19 ± 20
0.1	48	-18 ± 19
1.5	48	-55 ± 15 *

6.4 Discussion

The aim of this chapter was to identify the potential of cordycepin as a novel biopesticide by assessing its effect on *P. xylostella* survival and feeding. Cordycepin caused mortality of second instar *P. xylostella* larvae when administered for a short period (24 h of feeding) or when administered continuously (every day for seven days). This is consistent with the findings of Kim *et al*. (2002) who established that cordycepin caused significant mortality of third instar *P. xylostella* larvae when it was fed continuously. They suggested that cordycepin was gut-acting and this hypothesis is supported by the findings in this chapter. Mortality was induced by 24 h of feeding on cordycepin (>0.75 mg ml^{-1}), which suggests that cordycepin is toxic and mortality is not due to an anti-feedant effect.

Intermediate concentrations of cordycepin (1.5-3 mg ml^{-1}) caused the highest mortality, within the range of concentrations tested, when administered as a one-off treatment (*i.e*. a single application by feeding cordycepin-treated leaf discs for 24 h). Feeding larvae on leaf discs coated in 1.5 mg ml^{-1} and 3 mg ml^{-1} of cordycepin caused the highest mortality, with 6 mg ml^{-1} causing a lower mortality. The curves that best fit this response were second order polynomial curves, which suggests the dose response to cordycepin is not a classic sigmoidal curve commonly seen in response to pesticides (Nauen *et al*., 2003) or EPF (Yoon *et al*., 1999; Ansari *et al*., 2004). In a sigmoidal dose response, mortality increases as dose increases until maximum mortality occurs (Figure 6.15). In contrast, cordycepin appeared to give a bell-shaped dose response curve in which mortality increased with the concentration of cordycepin initially, then decreased at higher concentrations (Figure 6.15). This might be due to high concentrations of cordycepin having an antifeedant effect, which would result in larvae fed an intermediate concentration receiving a higher overall dose as they ate more. However, in choice experiments 1.5 mg ml^{-1} cordycepin appeared to significantly promote feeding of larvae. This was unexpected, as when the amount of leaf disc consumed was monitored over seven days (Section 6.3.1), there was a negative relationship between increasing concentrations of cordycepin and amount of leaf disc consumed. This suggests that at 1.5 mg ml^{-1} cordycepin initially promotes feeding, but the toxicity of the compound then reduces feeding over time. Future

experiments could build on this research and use higher concentrations of cordycepin in feeding choice experiments. Other studies have also found that the deterrent properties of compounds depend on dose (Fischer *et al*., 1990; Isman *et al*., 1990). For example, neem oil was found to deter feeding by *Peridroma saucia* (cutworm) larvae in a choice bioassay (Isman *et al*., 1990). Like the choice bioassays developed during this project, neem oil was applied to leaf discs and larvae were given a choice between treated or untreated leaf discs. In this case, the larvae were found to prefer untreated leaf discs, but the response varied depending on the concentration of the active chemical (azadirachtin) in the neem oil. Furthermore, if larvae only had access to treated diet, they consumed less and their weight reduced (Isman *et al*., 1990).

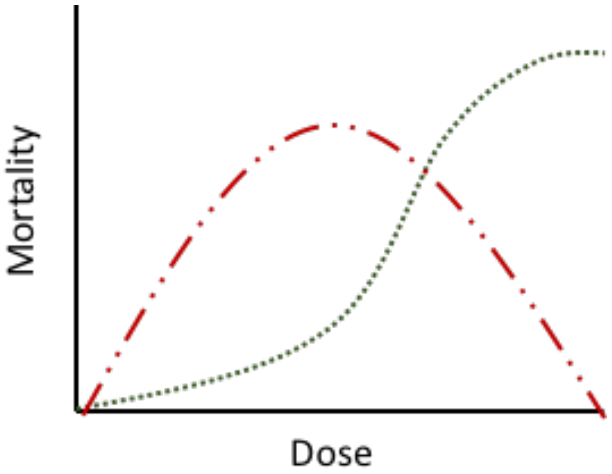


Figure 6.15 - Typical dose response curves. A classic sigmoidal dose response curve (green) and a bell-shaped dose response curve (red).

When administered continuously (*i.e*. insects were fed with a new cordycepin-treated leaf disc everyday), cordycepin appeared to cause a similar level of mortality to when it was administered as a one-off treatment. However, responses were less variable between replicates when cordycepin was administered continuously and there was a linear dose response relationship between concentration of cordycepin applied and mortality. This linear relationship is likely due to lower cordycepin concentrations being used for this experiment than when cordycepin was only fed to larvae for 24 h. In particular, a comparison can be made between treatment with 0.375, 0.75 and 1.5 mg ml^{-1} of cordycepin as these doses were applied in both experiments. The mean mortality of larvae treated with cordycepin and pentostatin for 24 h on day seven was

68.8%, 43.9% and 71.6% for 0.375, 0.75 and 1.5 mg ml^{-1} cordycepin, respectively. The mean mortality of larvae treated continuously with cordycepin on day seven was 20.8%, 55.0% and 72.5% for 0.375, 0.75 and 1.5 mg ml^{-1}, respectively. The survival of larvae treated with 0.75 and 1.5 mg ml^{-1} of cordycepin was similar between experiments, however survival after treatment with 0.375 mg ml^{-1} was different between the two experiments (Figures 6.6 and 6.8). Therefore, mortality following short-term cordycepin (plus pentostatin) treatment at 0.375 mg ml^{-1} may be anomalous. This could be due to a degradation of cordycepin on the leaf disc (Johns and Adamson, 1976) or differences between batches of *P. xylostella* larvae. In Chapter 2 it was postulated that cordycepin promotes colonisation of larvae by opportunistic gut bacteria and *P. xylostella* cadavers exhibited a similar morphology to the *G. mellonella* cadavers observed in Chapter 2. Different batches of *P. xylostella* could have different bacterial loads or contain species of bacteria that are more/less pathogenic. Microbial diversity in the midgut of *P. xylostella* has been characterised by sequencing (Xia *et al.*, 2013). Differences were seen between the number of operational taxonomic units (OTUs; closely related bacteria) identified from different *P. xylostella* strains, but samples were pooled so differences between individuals were not determined. Another reason for the differences between replicates may be that larvae in one replicate consumed less cordycepin during the 24 h of exposure by chance, so were less affected by toxicity.

Not only did cordycepin appear to cause mortality of *P. xylostella* larvae, it also reduced their feeding activity. When fed cordycepin for 24 h and then fed untreated leaf discs for the remainder of the experiment, increasing the concentration of cordycepin appeared to reduce feeding activity of *P. xylostella* larvae. However, this relationship was not significant, which was likely to due to the variation between replicates. Similarly, when larvae were fed cordycepin daily, feeding activity decreased as the concentration of cordycepin increased, but in this case the relationship was statistically significant. However, as this project found that cordycepin initially promotes feeding, a hypothesis can be developed that cordycepin is toxic to larvae and may act on the gut over time to reduce appetite.

It was determined that the effect of applying *B. bassiana* 433.99 and cordycepin varied depending on the concentrations applied. At a higher concentration of cordycepin and *B. bassiana* 433.99 the interaction was synergistic, whereas low concentrations of *B. bassiana* 433.99 had an additive effect with a high concentration of cordycepin. At the lower concentration of cordycepin, *B. bassiana* 433.99 had either an additive or antagonistic effect, therefore cordycepin may need to reach a critical dose within the larva to have an effect on the immune system and facilitate infection by this EPF. Or this effect could be because a low concentration cordycepin may be turned over more rapidly in the larva or on the leaf surface by adenosine deaminase (Johns and Adamson, 1976). Importantly, the effect of co-application of $1x10^7$ conidia ml^{-1} of *B. bassiana* 433.99 with both concentrations of cordycepin was either additive or synergistic. This is similar to the recommended field concentration of *B. bassiana*, which is $2.75x10^7$ conidia ml^{-1} for Botanigard WP (product label, Certis, U.K). Therefore, *B. bassiana* and cordycepin may be compatible for use in the field, although glasshouse and field trials would need to be performed in the future to determine this.

Interactions between *B. bassiana* and insecticides have been previously documented (Appendix B.1). Additive effects have been identified between this EPF and the insecticides: abamectin, triflumuron, thuringiensin and carbaryl, when assessed in laboratory and field cage studies against Colorado Potato Beetle (Anderson *et al.*, 1989). Synergistic, additive and antagonistic effects have been identified between *B. bassiana* and Cry toxins or *B. thuringiensis* (Wraight and Ramos, 2005; Ma *et al.*, 2008). Synergy has also been observed between *B. bassiana* and the insecticides deltamethrin (Bahiense and Bittencourt, 2004), imidacloprid (Furlong and Groden, 2001; Purwar and Sachan, 2006), oxydemeton methyl (Purwar and Sachan, 2006) and neem (Mohan *et al.*, 2007). However, identification of synergy varies between experimental conditions (Furlong and Groden, 2001). In fact, antagonistic effects have been observed between *B. bassiana* and imidacloprid in silverleaf whitefly (*Bemisia argentifolii*) (James and Elzen, 2001), whilst they have synergistic interactions in Colorado Potato beetle (Furlong and Groden, 2001). This illustrates that there can be considerable variation in the interactions of *B. bassiana* with chemicals and this can vary between experimental conditions, as such it is important to understand the mechanism behind any interactions.

The reason for synergistic interactions between cordycepin and *B. bassiana* 433.99 at higher doses may be that this chemical interacts with *P. xylostella* in a way that facilitates infection or colonization by *B. bassiana*. Cordycepin inhibits transcription of certain genes, including those that are involved in the immune and inflammatory responses (Kondrashov *et al.*, 2012), so it may inhibit the immune response in insects (Chapters 4 and 5), facilitating growth of *B. bassiana*. Additionally, *P. xylostella* larvae treated with cordycepin showed reduced food consumption over time (although it did not act as an antifeedant at the concentrations tested) which could stress the *P. xylostella* larvae, potentially increasing their susceptibility to infection. Starvation of the Colorado Potato beetle has been found to increase susceptibility to *B. bassiana* infection (Furlong and Groden, 2003). Likewise, starvation of the green lacewing (*Chrysoperla carnea*) significantly increases mortality caused by *B. bassiana* infection (Donegan and Lighthart, 1989). This is postulated to be due to starvation increasing the moulting period, which would give *B. bassiana* longer to penetrate the insect cuticle. It may also be due to starved insects having lower energy reserves, which might impede the immune response (Furlong and Groden, 2003). Immunosuppression following mechanical stress has been observed in crickets (*Gryllus texensis*) (Adamo and Parsons, 2006), suggesting that stress itself can increase susceptibility to infection and this may be the mechanism by which higher concentrations of cordycepin interact synergistically with high concentrations of *B. bassiana* 433.99 conidia to increase mortality.

Future work would be needed to determine the mechanisms by which cordycepin interacts with *P. xylostella* to cause mortality, including whether it causes mortality through interactions with the larval gut or whether it is transported out of the gut to affect other tissues. Furthermore, glasshouse and field trials would need to be performed to determine its potential, either alone or with EPF, for IPM on a large scale. A particular area for development would be the formulation of cordycepin for application onto leaves. It is degraded by adenosine deaminase (Johns and Adamson, 1976), which is produced by plants, mammals, microorganisms and invertebrates, hence any formulation would need to reduce the activity of this enzyme (Cristalli *et al.*, 2001). Additionally, cordycepin is likely to be sensitive to UV light as nucleoside-5'-monophosphates have a similar structure and breakdown when exposed to UV light

(Cavaluzzi and Borer, 2004). However, neem oil, a non-traditional insecticide, is sensitive to degradation by UV light, but different formulations can significantly increase its stability (da Costa *et al.*, 2014). This suggests that similar techniques could be developed to increase the stability of cordycepin.

6.4.1 Conclusions

This chapter aimed to identify the potential of cordycepin as a biopesticide either alone or in conjunction with EPF. It was found that cordycepin is toxic to *P. xylostella* larvae, both administered over a long period of time or as a short-term treatment. High concentrations of cordycepin and *B. bassiana* 433.99 have either an additive and synergistic effect when applied together to cause mortality in *P. xylostella*, which suggests that they have potential to be used together in an IPM strategy to improve control by EPF alone. These effects may be due to cordycepin interacting with the *P. xylostella* immune system. However, lower concentrations of cordycepin and *B. bassiana* had either antagonistic or additive effects, which suggests that more research needs to be performed into the potential of cordycepin as a biopesticide.

Chapter 7- General discussion

7.1 Conclusions

This project has begun to elucidate the natural function of cordycepin and characterise its potential as a biopesticide. It has developed a platform to further investigate cordycepin and other secondary metabolites of entomopathogenic fungi by establishing experimental procedures to characterise their interactions with insects. There is currently a lack of understanding about the natural function of EPF secondary metabolites, particularly those involved in the infection process as they may be produced transiently or in low quantities (Soukup *et al.*, 2016). Improving understanding of secondary metabolites will increase understanding of EPF biology and inform potential medicinal or agricultural uses for these metabolites in the future. In particular it is becoming increasingly important to develop new techniques for pest control, as it is predicted that losses of crop yields due to insects will increase dramatically as a result of global warming (Deutsch *et al.*, 2018).

The main findings of this project were:

- Cordycepin has insecticidal activity:
  - Concentrations of cordycepin greater than 6 mg ml^{-1} (120 μg per larva) caused death in *G. mellonella* larvae when administered by injection, possibly by increasing susceptibility to bacterial infection.
  - Concentrations of cordycepin greater than 50 μM (approximately 12.6 μg ml^{-1}) caused death of *D. melanogaster* S2r+ cells after 48 h.
  - Concentrations of cordycepin greater than approximately 0.75 mg ml^{-1} applied onto leaves caused mortality in *P. xylostella* larvae when administered by feeding.
- Cordycepin reduces the expression of the immune-related genes *gallerimycin*, *galiomicin*, *IMPI* and *lysozyme* in *G. mellonella* and *metchnikowin* and *attacin* in *D. melanogaster* S2r+ cells. It is hypothesised that this is its natural function during *C. militaris* infection.
- High concentrations of cordycepin can interact synergistically with when co-applied, suggesting that they have potential to be used together as biopesticides

in a novel strategy. However, this response was dose-dependent and further research is required.

- Cordycepin (3 mg ml^{-1}; 90µg per larva) potentiated the effect of *B. bassiana* 433.99, *C. militaris* 11703 and *M. brunneum* 275.86 when applied against *G. mellonella* larvae.
- Synergism was identified between *B. bassiana* 433.99 and cordycepin when applied together against *P. xylostella*, although the precise nature of the effect of co-application was dependant on the concentration of cordycepin and the duration of cordycepin exposure.

This project has developed procedures to begin to elucidate the natural function of cordycepin, which could be applied to other EPF secondary metabolites. There have been few previous studies into the impact of cordycepin on insects (Kim *et al*., 2002), with most research focusing on its medicinal potential (Nakamura *et al*., 2005; Nakamura *et al*., 2006; Jeong *et al*., 2011; Lu *et al*., 2014) rather than natural function. The *G. mellonella* bioassays in this project built on those described by Harding *et al*. (2013) and were adapted for use with EPF, allowing both larval mortality and gene expression to be monitored following topical application or injection of an EPF/secondary metabolite. There are many benefits to using *G. mellonella* as a system to study the impact of chemicals on insects and the insect immune system. Their large size allows injection of known doses of a test agent (Ramarao *et al*., 2012), although in this project the wounding response resulting from injection was found to complicate analysis of immune-related gene expression. *G. mellonella* are also small enough that it is possible to homogenise a whole larva to monitor gene expression in all tissues (Altincicek and Vilcinskas, 2006). Additionally, they are highly susceptible to infection by EPF, which can be therefore be applied topically, eliminating the issues arising from the wounding response following injection (Chandler *et al*., 1997; Wojda *et al*., 2009). Another key experimental procedure developed in this project was the use of a *D. melanogaster* S2r+ cell system to elucidate the function of secondary metabolites. This provides a high-throughput means of assessing the toxicity of metabolites and allow identification of their activity at a cellular level.

The natural function of cordycepin was initially investigated through the treatment of *G. mellonella* larvae, *P. xylostella* larvae and *D. melanogaster* S2r+ cells with this metabolite. Injection of *G. mellonella* larvae with concentrations of cordycepin greater than 6 mg ml^{-1} (90 μg per larva) caused indirect mortality which is postulated to be caused by opportunistic bacterial infection. The types of bacteria isolated from *G. mellonella* cadavers following cordycepin treatment were consistent with those isolated from the guts (from the crop to the hindgut) of insects by Yun *et al*. (2014). This suggests that cordycepin is able to prevent the normal functioning of the insect gut as a barrier to infection by bacteria that reside within the gut lumen. Mortality was also observed in *P. xylostella* larvae fed cordycepin, cadavers appeared dark black or brown which is consistent with the symptoms of bacterial infection, although this was not confirmed by culturing bacteria from the haemolymph or using molecular identification. This further supports the hypothesis that cordycepin has indirect toxicity by inhibiting the insect immune response, allowing infection by opportunistic microorganisms. However, in a sterile system, cordycepin also caused *D. melanogaster* S2r+ cell death, which suggests that it can be directly toxic depending on the concentration of cordycepin applied. Overall these results indicate that cordycepin may act as a virulence factor, that impairs the insect immune system to increase susceptibility to infection. However, it could also function to deter feeding on the fruiting body by animals or other insects due to its direct toxicity, so could have multiple effects.

In this project it was hypothesised that cordycepin affects the expression of immune-related genes in insects to facilitate infection by *C. militaris*, because it has been found to reduce inflammatory gene expression in mammals (Kondrashov *et al*., 2012). In order to investigate the interactions of cordycepin with the insect immune system, the expression of immune-related genes was monitored using RT-qPCR in *G. mellonella* larvae and *D. melanogaster* S2r+ cells. Both experiments found that expression of immune-related effector genes was reduced by treatment with sub-lethal doses of cordycepin. These genes are likely to be controlled by different immune pathways, for example *gallerimycin* and *galiomicin* are controlled by the Toll pathway (Schuhmann *et al*., 2003; Lee *et al*., 2004), whereas lysozyme production is likely to be controlled by additional pathways since it has been reported to be constitutively expressed in

Lepidoptera (Kong *et al*., 2016) and *IMPI* expression is likely to be regulated separately as its expression increases following detection of metalloproteases directly (Altincicek and Vilcinskas, 2006). Therefore, this implies that cordycepin impacts multiple immune pathways and naturally it may act as a virulence factor to facilitate infection of insects by *C. militaris*. To investigate how cordycepin interacts with the insect immune system in more detail, the expression of immune pathway-activating transcription factors DIF and Relish was monitored in *D. melanogaster* S2r+ cells using RT-qPCR. Their expression was not affected by cordycepin, which is consistent with its activity in mammalian cells. In human cells, cordycepin prevents the phosphorylation of IκB, halting activation of NFκB so it can no longer enter the nucleus and drive transcription of inflammatory genes (Ren *et al*., 2012). However, it is not known to alter the expression of NFκB itself. Therefore, cordycepin may impact Relish and DIF via a post-translational mechanism in insect cells, through a similar mechanism to that in human cells, but further research needs to be done to determine this (Section 7.1.2). Furthermore, cordycepin may directly impact mRNA stability through incorporation into the poly(A) tail during transcription (Kondrashov *et al*., 2012), therefore it could impact mRNA transcription directly or act through multiple mechanisms.

This project attempted to quantify *B. bassiana* 433.99 and *C. militaris* 11703 growth during infection of *G. mellonella* larvae in order to relate the progression of infection to the production of AMPs by the host. Although this was only successful for *C. militaris* 11703, the progression of infection appeared to follow a similar pattern to that for *B. bassiana* observed in another study (Bell *et al*., 2009). There was a slow increase in the number of *C. militaris* genomes early in infection, which then increased rapidly shortly prior to host death (Figure 7.1). Furthermore, expression of the immune-related genes *gallerimycin*, *IMPI* and *lysozyme* were seen to increase significantly in *G. mellonella* larvae at 48 or 72 h following injection with *C. militaris* 11703 conidia (Chapter 4). Taken together with the findings of Chapter 3, there appears to be a lag time for the expression of immune-related genes following EPF infection. Based on these observations, hypotheses can be developed regarding the importance of cordycepin and other secondary metabolites during EPF infection (Figure 7.1). Firstly, if the natural function of cordycepin is to reduce the immune-

related gene expression of the host, it may be produced at high concentrations following the initial stages in infection. It could then reduce expression of immune-related effector genes and once the levels of immune effectors become low enough, rapid *C. militaris* growth can occur. However, it may take time for concentrations of cordycepin to accumulate to levels that are able to reduce immune-related gene expression, since *C. militaris* biomass is initially low and adenosine deaminase may break down cordycepin in the haemolymph. Alternatively, cordycepin may not play a large role in the reduction of immune-related gene expression and *C. militaris* may overcome these effectors by another mechanism. For example, *M. anisopliae* produces metalloproteases that are thought to degrade AMPs (Qazi and Khachatourians, 2007; Mukherjee and Vilcinskas, 2018), *C. militaris* may similarly degrade AMPs or other immune-related effector proteins. Insect cellular immunity also has a key role in preventing infection and limiting the growth of pathogens (Figure 7.1; Lavine and Strand 2002), additionally it is necessary for coordinating the humoral response to infection (Basset *et al.,* 2000; Shia *et al.,* 2009). EPF including *B. bassiana* and *M. anisopliae s.l.* have been found to inhibit the insect cellular immune response (Vlicinskas *et al.,* 1997b,c) and *C. militaris* may have a similar activity during infection. This illustrates that *C. militaris* is likely to interact with the insect immune response through multiple mechanisms and further research is needed to determine the function of cordycepin in these interactions.

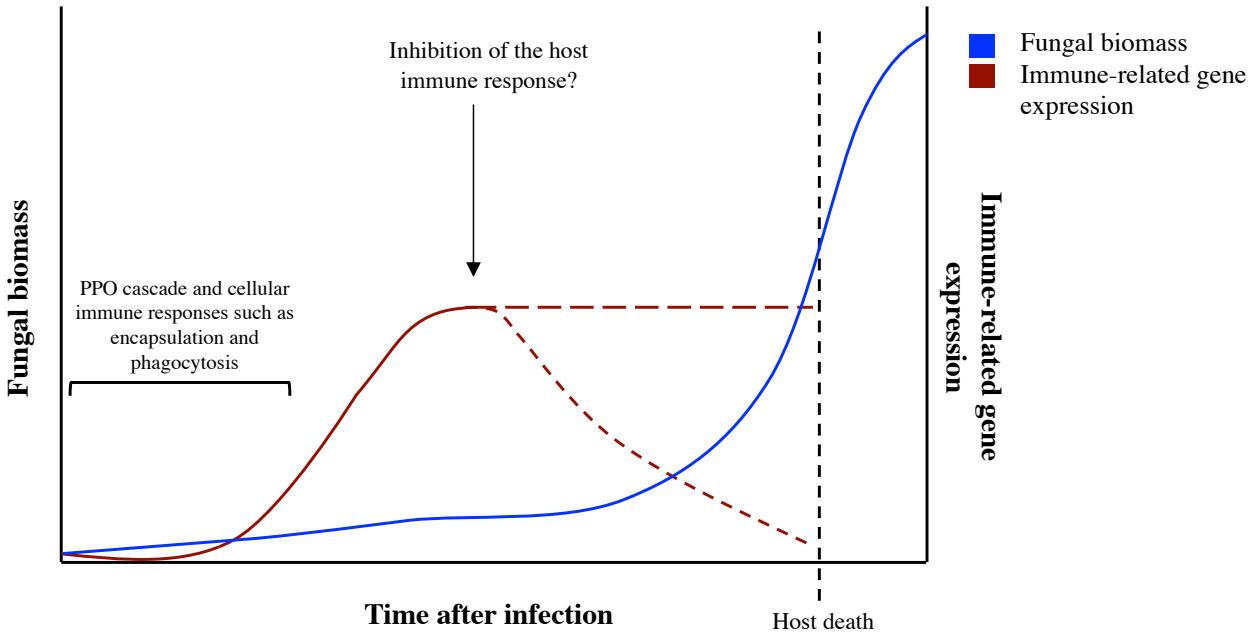


Figure 7.1 - Diagram illustrating the progression of EPF infection and the expression of immune-related genes. The increase of EPF biomass (blue) compared to the expression of immune-related genes by the host insect (red). If the EPF reduces the expression of immune related effector genes *e.g.* through the production of cordycepin, immune-related gene expression will decrease (short dashes), if not it will remain at a high level (long dash).

Not only is cordycepin insecticidal, but at 6 mg ml^{-1} it reduces germination of the EPF *B. bassiana* 433.99 and *M. brunneum* 275.86. This is consistent with work by Sugar and McCaffrey, (1998), who found that cordycepin had anti-fungal activity against *Candida* spp. It has also been found to have anti-bacterial properties (Rottman and Guarino, 1964), particularly against *B. subtilis*. The anti-microbial activity of cordycepin might prevent *C. militaris* being out-competed within an insect, or prevent infection of its fruiting body and mycelia in the environment. However, since high concentrations of cordycepin were required to inhibit growth of other EPF, this could indicate that this is not its primary function.

Treatment with cordycepin appeared to facilitate infection of *G. mellonella* larvae (Chapter 4) and *P. xylostella* larvae (Chapter 6) by EPF. This supports the hypothesis that cordycepin interacts with the insect immune system, increasing susceptibility to infection. Interactions between EPF and cordycepin varied between EPF species. In

particular, interactions between cordycepin and *B. bassiana* 433.99 were investigated against *P. xylostella* as this EPF isolate is a commercially available biopesticide in the U.K (Botanigard, Certis, U.K). These experiments showed that synergistic interactions between cordycepin and *B. bassiana* 433.99 were dose-dependent. EPF are currently used as commercial biopesticides, but their lag time and lower lethality relative to synthetic pesticides has limited their uptake for use in pest control (Johnson and Goettel, 1993; Jaros-Su *et al*., 1999; Benjamin *et al*., 2002; Peng *et al*., 2008). The combination of cordycepin with these biopesticides could potentially increase the rate of mortality, and hence efficacy, in the field facilitating the use of EPF in IPM. However, further work would need to be done to determine the optimum dose of cordycepin and EPF to apply and the formulation of these mixtures. The toxicity of cordycepin alone against *P. xylostella* seen in this project confirms the findings of Kim *et al*. (2002), who observed that cordycepin caused mortality in *P. xylostella* when administered by feeding and recognised it as a potential biopesticide. Furthermore, this project has found that this may be due to the indirect effect of cordycepin inhibiting the insect immune response, facilitating infection by opportunistic bacteria. This might be a concern for the future development of cordycepin as a biopesticide, particularly whether any of these opportunistic bacteria pose a risk to human health, hence this would need to be investigated further.

7.1.2 Future work

In order to better understand the natural function of cordycepin, the experiments described in this project could be taken further. In particular *D. melanogaster* S2r+ cell experiments (Chapter 5) would need to be repeated to confirm differences identified in *RpL32* expression following cordycepin treatment. Furthermore, these could be expanded on to identify the mechanism of cordycepin activity, in particular to test the hypothesis that cordycepin affects the stability of the NFκB homologs DIF and Relish or their transport to the nucleus. Western blotting could be used to monitor the degradation and localisation of DIF/Relish and other proteins that interact with them such as Cactus (IκB homolog) and IKK (Kondrashov *et al*., 2012; Ren *et al*., 2012). For example, nuclei of *D. melanogaster* cells can be separated from other organelles by subcellular fractionation (Zhang *et al*., 2000), then western blotting

could be used to determine in which cellular fraction DIF/Relish is localised following cordycepin treatment. Western blotting could also monitor the progression of Cactus degradation, which may be expected to reduce in the presence of cordycepin, as this has been observed in human cells (Kondrashov *et al*., 2012). RT-qPCR and other transcriptomic experiments in *G. mellonella* could be expanded on to monitor the expression of other immune-related genes as its whole genome sequence has recently become available (Lange *et al*., 2018). It would be especially important to examine the potential impact of cordycepin on other immune-pathways such as the Jak-Stat and IMD pathways. It could also be informative to determine the impact of cordycepin on the cellular immune response, such as expression of genes involved in the PPO cascade.

This project used three insect species to elucidate the natural function of cordycepin and its potential as a biopesticide: *D. melanogaster*, *G. mellonella* and *P. xylostella*. In the future it would be beneficial to select one species for all experiments, although there are challenges that must be overcome to develop this system. Final instar *G. mellonella* were selected for experiments as they could be injected (Harding *et al*., 2013) and it was possible to purchase large numbers at the same age. However, final instar *G. mellonella* do not feed and are not an agricultural pest, hence they were not suitable for assessing the potential of cordycepin as a biopesticide. In the future, rearing *G. mellonella* would allow earlier instars to be used for feeding experiments although it may still be beneficial to select an agricultural pest for experiments as this would be more relevant in the field. Furthermore, a *D. melanogaster* cell line was chosen to assess the effect of cordycepin on insect cells in this project as it has been reported to possess macrophage-like characteristics (Rämet *et al*., 2001), making it appropriate to investigate the immune response. Cell lines able to respond to immune system stimuli have not yet been developed from *P. xylostella* or *G. mellonella* tissue, although other cell lines from these insects are available (Lynn, 2007). Therefore, in the future it would be beneficial if a macrophage-like cell line could be developed from these insects.

This project focused on introducing cordycepin into insects to begin to understand its natural function during *C. militaris* infection. Whilst this has indicated a potential role

of cordycepin as a virulence factor due to its impact on the insect immune system, the doses of cordycepin administered to insects during this project were likely higher than that produced during natural infection. When grown in culture *C. militaris* mycelia produce cordycepin at 750-16500 μg g^{-1} (Huang *et al.*, 2003; Li *et al.*, 2004; Yang and Li, 2008), suggesting that the 90 μg applied during *G. mellonella* RT-qPCR experiments would be produced by 5.5 mg-120 mg of *C. militaris*. However, it is likely that during infection cordycepin would be secreted locally by growing mycelium and the concentration produced at this small scale is not known at this time. In order to develop stronger proof of the role of cordycepin as a virulence factor it would be necessary to engineer *C. militaris* strains that do not produce this metabolite and observe its impact on virulence. Although, it is possible that a significant reduction in virulence of *C. militaris* would not be observed following knockout of cordycepin genes, even if it is a virulence factor, due to a redundancy with other virulence factors. For example, a study has been performed to elucidate the natural function of bassianolide (Xu *et al.*, 2009) and beauvericin (Xu *et al.*, 2008) in *B. bassiana*, by developing knockout strains that did not produce these secondary metabolites. The result was that knocking out either metabolite caused a significant reduction in virulence of *B. bassiana*, but knocking out both did not reduce virulence more than when a single gene was knocked out (Xu *et al.*, 2008; Xu *et al.*, 2009). This suggests that there are complex interactions between secondary metabolites, which can make it difficult to ascertain their natural function. The genes that are thought to be responsible for the production of cordycepin (*cns1-4*) have been knocked out of a strain of *C. militaris* (CGMCC 3.14242) previously, but virulence of this strain was not tested (Xia *et al.*, 2017). Future work could consist of knocking out *cns1-4* from a strain of *C. militaris* using *Agrobacterium*-mediated transformation (Feng *et al.*, 2015; Xia *et al.*, 2017), then assessing the virulence of this strain through the insect bioassays developed in this project.

In addition to investigating the natural function of cordycepin, this project also explored the potential of cordycepin as a biopesticide using *P. xylostella*. This work could be developed further to investigate the use of cordycepin alone and in conjunction with EPF on a larger scale and to control other insect pests. Cordycepin is degraded by adenosine deaminase (Johns and Adamson, 1976), an enzyme produced

by plants, mammals, microorganisms and invertebrates (Cristalli *et al*., 2001) and is also likely to be sensitive to UV light, similarly to nucleoside-5'-monophosphates (Cavaluzzi and Borer, 2004). Therefore, the first challenge to its use on a large scale would be the formulation to slow its breakdown. Following this, trials could be done using cordycepin against *P. xylostella* in glasshouses or in the field (Vandenberg *et al*., 1998a; Godonou *et al*., 2009). Additionally, cordycepin could be considered for use against other insects, particularly chewing pests such as Coleoptera or Lepidoptera, because it needs to be ingested to cause mortality (Kim *et al*., 2002). The potential of co-application of cordycepin and other microbial biopesticides, such as EPF and *B. thuringiensis* could also be explored further in laboratory bioassays and field trials.

As *C. militaris* is a teleomorph the ability to perform crosses between isolates (Shrestha *et al*., 2012a; Zhang and Liang, 2013) would facilitate genetic manipulation. Crosses have been performed between isolates to investigate fruiting body formation, particularly to identify the interaction between different mating types (Zheng *et al*., 2011; Shrestha *et al*., 2012a; Zhang and Liang, 2013). In these studies, *C. militaris* isolates of different mating types were crossed, then the effect on fruiting body formation and cordycepin production assessed. A similar technique could be used to determine whether certain alleles are important to virulence or other characteristics. For example, crosses could be done between a highly virulent *C. militaris* strain and a less virulent strain. The virulence of the progeny could be determined through bioassays and genetic differences characterised.

The experimental pipelines developed in this project can be used to identify the natural function of secondary metabolites from other entomopathogenic species and determine whether they have potential as biopesticides. However, in order for this approach to be used, a metabolite would need to be extracted from the organism in large quantities, which may not always be possible if they are produced transiently or in low quantities. There are many fungal secondary metabolites that are presumed to exist due to the presence of biosynthetic gene clusters in EPF genomes, particularly polyketide synthases and nonribosomal peptides synthases (Keller *et al*., 2005). These genes could be identified as important virulence factors through proteomics or

transcriptomics (Doyle, 2011; Gao *et al*., 2011). Additionally, a biosynthetic gene cluster could be transformed into *S. cerevisiae* allowing production of these metabolites on a larger scale (Xia *et al*., 2017). Once the secondary metabolite is obtained, procedures described in this project could be used to elucidate their natural function and potential as biopesticides.

Bibliography

ADAMO, S. & PARSONS, N. 2006. The emergency life-history stage and immunity in the cricket, *Gryllus texensis*. *Animal behaviour,* 72**,** 235-244.

AERTS, A., FRANÇOIS, I., CAMMUE, B. & THEVISSEN, K. 2008. The mode of antifungal action of plant, insect and human defensins. *Cellular and Molecular Life Sciences,* 65**,** 2069-2079.

AGAISSE, H. & PERRIMON, N. 2004. The roles of JAK/STAT signaling in Drosophila immune responses. *Immunological reviews,* 198**,** 72-82.

AGAISSE, H., PETERSEN, U.-M., BOUTROS, M., MATHEY-PREVOT, B. & PERRIMON, N. 2003. Signaling role of hemocytes in Drosophila JAK/STAT-dependent response to septic injury. *Developmental cell,* 5**,** 441-450.

AHN, Y. J., PARK, S. J., LEE, S. G., SHIN, S. C. & CHOI, D. H. 2000. Cordycepin: Selective growth inhibitor derived from liquid culture of *Cordyceps militaris* against Clostridium spp. *Journal of Agricultural and Food Chemistry,* 48**,** 2744-2748.

ALTIERI, M. A. 1999. The ecological role of biodiversity in agroecosystems. *Agriculture, Ecosystems & Environment,* 74, 19-31

ALTINCICEK, B. & VILCINSKAS, A. 2006. Comparative Metamorphosis and collagen-IV-fragments stimulate innate immune response in the greater wax moth , *Galleria mellonella*. *Developmental & Comparative Immunology* 30**,** 1108-1118.

ANBESSE, S. A., ADGE, B. J. & GEBRU, W. M. 2008. Laboratory screening for virulent entomopathogenic nematodes (*Heterorhabditis bacteriophora* and *Steinernema yirgalemense*) and fungi (*Metarhizium anisopliae* and *Beauveria bassiana*) and assessment of possible synergistic effects of combined use against grubs of the barley chafer *Coptognathus curtipennis*. *Nematology,* 10**,** 701-709.

ANDERSEN, S. O. 1979. Biochemistry of insect cuticle. *Annual review of entomology,* 24**,** 29-59.

ANDERSON, T., HAJEK, A., ROBERTS, D. W., PREISLER, H. & ROBERTSON, J. 1989. Colorado potato beetle (Coleoptera: Chrysomelidae): effects of combinations of *Beauveria bassiana* with insecticides. *Journal of Economic Entomology,* 82**,** 83-89.

ANSARI, M., VESTERGAARD, S., TIRRY, L. & MOENS, M. 2004. Selection of a highly virulent fungal isolate, *Metarhizium anisopliae* CLO 53, for controlling *Hoplia philanthus*. *Journal of invertebrate pathology,* 85**,** 89-96.

APRD. 2018. *Arthropod Resistance Database*. *East Lansing: Michigan State University*. [Online]. https://www.pesticideresistance.org/index.php. [Accessed JUN 2018].

ARTHURS, S. & DARA, S. K. 2018 (in press). Microbial biopesticides for invertebrate pests and their markets in the United States. *Journal of invertebrate pathology*. https://doi.org/10.1016/j.jip.2018.01.008

BADALI, H., FAKHIM, H., ZAREI, F., NABILI, M., VAEZI, A., POORZAD, N., DOLATABADI, S. & MIRHENDI, H. 2016. In vitro activities of five antifungal drugs against opportunistic agents of *Aspergillus nigri* complex. *Mycopathologia,* 181**,** 235-240.

BAEUERLE, P. A. & HENKEL, T. 1994. Function and activation of NF-kappaB in the immune system. *Annual review of immunology,* 12**,** 141-179.

BAHIENSE, T. C. & BITTENCOURT, V. R. 2004. Laboratory evaluation of the compatibility and the synergism between the entomopathogenic fungus *Beauveria bassiana* and deltamethrin to resistant strains of *Boophilus microplus*. *Annals of the New York Academy of Sciences,* 1026**,** 319-322.

BAIK, J. S., KWON, H. Y. & KIM, K. S. 2012. Cordycepin induces apoptosis in human neuroblastoma SK-N-BE (2)-C and melanoma SK-MEL-2 cells. *Indian Journal of Biochemistry and Biophysics*, 49, 86-91.

BARZMAN, M., BÀRBERI, P., BIRCH, A. N. E., BOONEKAMP, P., DACHBRODT-SAAYDEH, S., GRAF, B., HOMMEL, B., JENSEN, J. E., KISS, J. & KUDSK, P. 2015. Eight principles of integrated pest management. *Agronomy for sustainable development,* 35**,** 1199-1215.

BASS, C., PUINEAN, A. M., ZIMMER, C. T., DENHOLM, I., FIELD, L. M., FOSTER, S. P., GUTBROD, O., NAUEN, R., SLATER, R. & WILLIAMSON, M. S. 2014. The evolution of insecticide resistance in the peach potato aphid, *Myzus persicae*. *Insect biochemistry and molecular biology,* 51**,** 41-51.

BASSET, A., KHUSH, R. S., BRAUN, A., GARDAN, L., BOCCARD, F., HOFFMANN, J. A. & LEMAITRE, B. 2000. The phytopathogenic bacteria *Erwinia carotovora* infects Drosophila and activates an immune response. *Proceedings of the National Academy of Sciences,* 97**,** 3376-3381.

BATEMAN, R. 1999. Delivery systems and protocols for biopesticides. Hall, F. R., Menn, J., (Eds.). *Biopesticides: Use and Delivery***,** Publ. Humana Press, New Jersey. p 509-528.

BATTA, Y. A. 2013. Efficacy of endophytic and applied *Metarhizium anisopliae* (Metch.) Sorokin (Ascomycota: Hypocreales) against larvae of *Plutella xylostella* L.(Yponomeutidae: Lepidoptera) infesting *Brassica napus* plants. *Crop protection,* 44**,** 128-134.

BELL, A. S., BLANFORD, S., JENKINS, N., THOMAS, M. B. & READ, A. F. 2009. Real-time quantitative PCR for analysis of candidate fungal biopesticides against malaria: technique validation and first applications. *Journal of invertebrate pathology,* 100**,** 160-168.

BELVIN, M. P. & ANDERSON, K. V. 1996. A conserved signaling pathway: the Drosophila toll-dorsal pathway. *Annual review of cell and developmental biology,* 12**,** 393-416.

BENJAMIN, M. A., ZHIOUA, E. & OSTFELD, R. S. 2002. Laboratory and field evaluation of the entomopathogenic fungus *Metarhizium anisopliae* (Deuteromycetes) for controlling questing adult *Ixodes scapularis* (Acari: Ixodidae). *Journal of Medical Entomology,* 39**,** 723-728.

BERGIN, D., MURPHY, L., KEENAN, J., CLYNES, M. & KAVANAGH, K. 2006. Pre-exposure to yeast protects larvae of *Galleria mellonella* from a subsequent lethal infection by *Candida albicans* and is mediated by the increased expression of antimicrobial peptides. *Microbes and infection,* 8**,** 2105-2112.

BIDLA, G., HAULING, T., DUSHAY, M. S. & THEOPOLD, U. 2009. Activation of insect phenoloxidase after injury: endogenous versus foreign elicitors. *Journal of Innate Immunity,* 1**,** 301-308.

BING, L. A. & LEWIS, L. C. 1991. Suppression of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) by endophytic *Beauveria bassiana* (Balsamo) Vuillemin. *Environmental entomology,* 20**,** 1207-1211.

BISCHOFF, J. F., REHNER, S. A. & HUMBER, R. A. 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia,* 101**,** 512-530.

BLACK, J., DEAN, T., BYFIELD, G., FOARDE, K. & MENETREZ, M. 2013. Determining fungi rRNA copy number by PCR. *Journal of Biomolecular Techniques: JBT,* 24**,** 32-38.

BLACKWELL, J. & WEIH, M. 1980. Structure of chitin-protein complexes: ovipositor of the ichneumon fly Megarhyssa. *Journal of molecular biology,* 137**,** 49-60.

BOLDO, J. T., JUNGES, A., DO AMARAL, K. B., STAATS, C. C., VAINSTEIN, M. H. & SCHRANK, A. 2009. Endochitinase CHI2 of the biocontrol fungus *Metarhizium anisopliae* affects its virulence toward the cotton stainer bug *Dysdercus peruvianus*. *Current genetics,* 55**,** 551-560.

BOOMSMA, J. J., JENSEN, A. B., MEYLING, N. V. & EILENBERG, J. 2014. Evolutionary interaction networks of insect pathogenic fungi. *Annual Review of Entomology,* 59**,** 467-485.

BOURGEOIS, C. & KUCHLER, K. 2012. Fungal pathogens—a sweet and sour treat for toll-like receptors. *Frontiers in cellular and infection microbiology,* 2**,** 142.

BRADY, B. L. K. 1979. *Beauveria bassiana*, *Cordyceps militaris* & *Metarhizium anisopliae*. CMI Descriptions of Pathogenic Fungi and Bacteria. No's. 602, 605 & 609. Commonwealth Mycological Institute: Kew, Surrey, England.

BRAGA, G. U., FLINT, S. D., MILLER, C. D., ANDERSON, A. J. & ROBERTS, D. W. 2001a. Both solar UVA and UVB radiation impair conidial culturability and delay germination in the entomopathogenic fungus *Metarhizium anisopliae*. *Photochemistry and Photobiology,* 74**,** 734-739.

BRAGA, G. U., FLINT, S. D., MILLER, C. D., ANDERSON, A. J. & ROBERTS, D. W. 2001b. Variability in response to UV-B among species and strains of Metarhizium isolated from sites at latitudes from 61 N to 54 S. *Journal of Invertebrate Pathology,* 78**,** 98-108.

BRENNAN, M., THOMAS, D. Y., WHITEWAY, M. & KAVANAGH, K. 2002. Correlation between virulence of *Candida albicans* mutants in mice and *Galleria mellonella* larvae. *Pathogens and Disease,* 34**,** 153-157.

BREZNAK, J. A. & SWITZER, J. M. 1986. Acetate synthesis from H2 plus CO2 by termite gut microbes. *Applied and Environmental Microbiology,* 52**,** 623-630.

BRIERE, J.-F. & PRACROS, P. 1998. Comparison of temperature-dependent growth models with the development of *Lobesia botrana* (Lepidoptera: Tortricidae). *Environmental Entomology,* 27**,** 94-101.

BUCHER, G. & WILLIAMS, R. 1967. The microbial flora of laboratory cultures of the greater wax moth and its effect on rearing parasites. *Journal of invertebrate pathology,* 9**,** 467-473.

BUCHON, N., SILVERMAN, N. & CHERRY, S. 2014. Immunity in *Drosophila melanogaster—*from microbial recognition to whole-organism physiology. *Nature reviews immunology,* 14**,** 796-810.

CASTILLO, J., SHOKAL, U. & ELEFTHERIANOS, I. 2013. Immune gene transcription in Drosophila adult flies infected by entomopathogenic nematodes and their mutualistic bacteria. *Journal of insect physiology,* 59**,** 179-185.

CASTRILLO, L. A., GRIGGS, M. H., LIU, H., BAUER, L. S. & VANDENBERG, J. D. 2010. Assessing deposition and persistence of *Beauveria bassiana* GHA (Ascomycota: Hypocreales) applied for control of the emerald ash borer, *Agrilus planipennis* (Coleoptera: Buprestidae), in a commercial tree nursery. *Biological Control,* 54**,** 61-67.

CAVALUZZI, M. J. & BORER, P. N. 2004. Revised UV extinction coefficients for nucleoside-5'-monophosphates and unpaired DNA and RNA. *Nucleic acids research,* 32**,** e13-e13.

CEDERGREEN, N., LIBERATI, A., TETZLAFF, J., ALTMAN, D. G., WANG, Y. H. & BAUN, A. 2014. Quantifying Synergy: A Systematic Review of Mixture Toxicity Studies within Environmental Toxicology. *PLoS ONE,* 9**,** e96580-e96580.

CERENIUS, L., LEE, B. L. & SÖDERHÄLL, K. 2008. The proPO-system: pros and cons for its role in invertebrate immunity. *Trends in immunology,* 29**,** 263-271.

CHANDLER, D., BAILEY, A. S., TATCHELL, G. M., DAVIDSON, G., GREAVES, J. & GRANT, W. P. 2011. The development, regulation and use of biopesticides for integrated pest management. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 366**,** 1987-1998.

CHANDLER, D., HAY, D. & REID, A. 1997. Sampling and occurrence of entomopathogenic fungi and nematodes in UK soils. *Applied Soil Ecology,* 5**,** 133-141.

CHEN, B.-F., TSAI, M.-C. & JOW, G.-M. 2006. Induction of calcium influx from extracellular fluid by beauvericin in human leukemia cells. *Biochemical and biophysical research communications,* 340**,** 134-139.

CHEN, H.-C., CHOU, C.-K., SUN, C.-M. & YEH, S. F. 1997. Suppressive effects of destruxin B on hepatitis B virus surface antigen gene expression in human hepatoma cells. *Antiviral research,* 34**,** 137-144.

CHEN, L. S., STELLRECHT, C. M. & GANDHI, V. 2008. RNA-directed agent, cordycepin, induces cell death in multiple myeloma cells. *British Journal of Haematology,* 140**,** 682-391.

CHENG, L. W. & PORTNOY, D. A. 2003. Drosophila S2 cells: an alternative infection model for *Listeria monocytogenes*. *Cellular microbiology,* 5**,** 875-885.

CHERBAS, L., MOSS, R. & CHERBAS, P. 1994. Transformation techniques for Drosophila cell lines. *Methods in cell biology,* 44, 161-179.

CHOE, K.-M., WERNER, T., STÖVEN, S., HULTMARK, D. & ANDERSON, K. V. 2002. Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in Drosophila. *Science,* 296**,** 359-362.

CHOI, S., LIM, M.-H., KIM, K. M., JEON, B. H., SONG, W. O. & KIM, T. W. 2011. Cordycepin-induced apoptosis and autophagy in breast cancer cells are independent of the estrogen receptor. *Toxicology and applied pharmacology,* 257**,** 165-173.

CHOI, Y. H., KIM, G.-Y. & LEE, H. H. 2014. Anti-inflammatory effects of cordycepin in lipopolysaccharide-stimulated RAW 264.7 macrophages through Toll-like receptor 4-mediated suppression of mitogen-activated protein kinases and NF-κB signaling pathways. *Drug design, development and therapy,* 8**,** 1941-1953.

CHOUVENC, T. & SU, N.-Y. 2012. When subterranean termites challenge the rules of fungal epizootics. *PLoS One,* 7**,** e34484.

CHU, L. L. & EDELMAN, I. S. 1972. Cordycepin and α-amanitin: Inhibitors of transcription as probes of aldosterone action. *The Journal of membrane biology,* 10**,** 291-310.

CLEMENS, J. C., WORBY, C. A., SIMONSON-LEFF, N., MUDA, M., MAEHAMA, T., HEMMINGS, B. A. & DIXON, J. E. 2000. Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. *Proceedings of the National Academy of Sciences,* 97**,** 6499-6503.

COLE, C., DUXBURY, J., FRENEY, J., HEINEMEYER, O., MINAMI, K., MOSIER, A., PAUSTIAN, K., ROSENBERG, N., SAMPSON, N. & SAUERBECK, D. 1997. Global estimates of potential mitigation of greenhouse gas emissions by agriculture. *Nutrient cycling in Agroecosystems,* 49**,** 221-228.

COOK, S. M. & MCARTHUR, J. D. 2013. Developing *Galleria mellonella* as a model host for human pathogens. *Virulence,* 4**,** 350-353.

COPPING, L. G. & MENN, J. J. 2000. Biopesticides: a review of their action, applications and efficacy. *Pest Management Science,* 56**,** 651-676.

CRINITI, A., MAZZONI, E., CASSANELLI, S., CRAVEDI, P., TONDELLI, A., BIZZARO, D. & MANICARDI, G. C. 2008. Biochemical and molecular diagnosis of insecticide resistance conferred by esterase, MACE, kdr and super-kdr based mechanisms in Italian strains of the peach potato aphid, Myzus persicae (Sulzer). *Pesticide Biochemistry and Physiology,* 90**,** 168-174.

CRIST, E., MORA, C. & ENGELMAN, R. 2017. The interaction of human population, food production, and biodiversity protection. *Science,* 356**,** 260-264.

CRISTALLI, G., COSTANZI, S., LAMBERTUCCI, C., LUPIDI, G., VITTORI, S., VOLPINI, R. & CAMAIONI, E. 2001. Adenosine deaminase: functional implications and different classes of inhibitors. *Medicinal research reviews,* 21**,** 105-128.

CULLINEY, T. W. 2014. Crop losses to arthropods. D. Pimental, R. Peshin (Eds.). *Integrated pest management*. Publ. Springer, New York. p. 201-225.

CYTRYŃSKA, M., ZDYBICKA-BARABAS, A., JABŁOŃSKI, P. & JAKUBOWICZ, T. 2001. Detection of antibacterial polypeptide activity in situ after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. *Analytical biochemistry,* 299**,** 274-276.

DA COSTA, J. T., FORIM, M. R., COSTA, E. S., DE SOUZA, J. R., MONDEGO, J. M. & JUNIOR, A. L. B. 2014. Effects of different formulations of neem oil-based products on control *Zabrotes subfasciatus* (Boheman, 1833)(Coleoptera: Bruchidae) on beans. *Journal of stored products research,* 56**,** 49-53.

DAFFRE, S., KYLSTEN, P., SAMAKOVLIS, C. & HULTMARK, D. 1994. The lysozyme locus in *Drosophila melanogaster*: an expanded gene family adapted for expression in the digestive tract. *Molecular and General Genetics MGG,* 242**,** 152-162.

DAS, S. K., MASUDA, M., HATASHITA, M., SAKURAI, A. & SAKAKIBARA, M. 2008. A new approach for improving cordycepin productivity in surface liquid culture of *Cordyceps militaris* using high-energy ion beam irradiation. *Letters in Applied Microbiology,* 47**,** 534-538.

DAS, S. K., MASUDA, M., HATASHITA, M., SAKURAI, A. & SAKAKIBARA, M. 2010a. Optimization of culture medium for cordycepin production using *Cordyceps militaris* mutant obtained by ion beam irradiation. *Process Biochemistry,* 45**,** 129-132.

DAS, S. K., MASUDA, M., SAKURAI, A. & SAKAKIBARA, M. 2010b. Medicinal uses of the mushroom *Cordyceps militaris*: Current state and prospects. *Fitoterapia,* 81**,** 961-968.

DAYAN, F. E., CANTRELL, C. L. & DUKE, S. O. 2009. Natural products in crop protection. *Bioorganic & medicinal chemistry,* 17**,** 4022-4034.

DE FARIA, M. R. & WRAIGHT, S. P. 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. *Biological Control,* 43**,** 237-256.

DE GREGORIO, E., SPELLMAN, P. T., TZOU, P., RUBIN, G. M. & LEMAITRE, B. 2002. The Toll and Imd pathways are the major regulators of the immune response in Drosophila. *The EMBO journal,* 21**,** 2568-2579.

DE HOOG, G. S. 2000. *Atlas of clinical fungi* 2nd Ed. Publ. Centraalbureau voor Schimmelcultures , Utrecht, The Netherlands.

DELGADO, F. X., BRITTON, J. H., ONSAGER, J. A. & SWEARINGEN, W. 1999. Field Assessment of *Beauveria bassiana* (Balsamo) Vuillemin and Potential Synergism with Diflubenzuron for Control of Savanna Grasshopper Complex (Orthoptera) in Mali. *Journal of invertebrate Pathology,* 73**,** 34-39.

DEUTSCH, C. A., TEWKSBURY, J. J., TIGCHELAAR, M., BATTISTI, D. S., MERRILL, S. C., HUEY, R. B. & NAYLOR, R. L. 2018. Increase in crop losses to insect pests in a warming climate. *Science,* 361**,** 916-919.

DICKMAN, A. 1933. Studies on the waxmoth, *Galleria mellonella*, with particular reference to the digestion of wax by the larvae. *Journal of Cellular Physiology,* 3**,** 223-246.

DILLON, R., CHARNLEY, K., 2002. Mutualism between the desert locust *Schistocerca gregaria* and its gut microbiota. *Research in Microbiology,* 153(8), 503-509.

DONEGAN, K. & LIGHTHART, B. 1989. Effect of several stress factors on the susceptibility of the predatory insect, *Chrysoperla carnea* (Neuroptera: Chrysopidae), to the fungal pathogen *Beauveria bassiana*. *Journal of Invertebrate Pathology,* 54**,** 79-84.

DONG, T., ZHANG, B., JIANG, Y. & HU, Q. 2016. Isolation and classification of fungal whitefly entomopathogens from soils of Qinghai-Tibet Plateau and Gansu Corridor in China. *PloS one,* 11(5)**,** e0156087.

DOSTERT, C., JOUANGUY, E., IRVING, P., TROXLER, L., GALIANA-ARNOUX, D., HETRU, C., HOFFMANN, J. A. & IMLER, J.-L. 2005. The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. *Nature immunology,* 6**,** 946-953.

DOYLE, S. 2011. Fungal proteomics: from identification to function. *FEMS microbiology letters,* 321**,** 1-9.

DUAN, Y., WU, H., MA, Z., YANG, L. & MA, D. 2017. Scanning electron microscopy and histopathological observations of *Beauveria bassiana* infection of Colorado potato beetle larvae. *Microbial pathogenesis,* 111**,** 435-439.

DUBOVSKIY, I. M., WHITTEN, M. M., YAROSLAVTSEVA, O. N., GREIG, C., KRYUKOV, V. Y., GRIZANOVA, E. V., MUKHERJEE, K., VILCINSKAS, A., GLUPOV, V. V. & BUTT, T. M. 2013. Can insects develop resistance to insect pathogenic fungi? *PloS one,* 8**,** e60248.

EBERT, D. & WEISSER, W. W. 1997. Optimal killing for obligate killers: the evolution of life histories and virulence of semelparous parasites. *Proceedings of the Royal Society of London B: Biological Sciences,* 264**,** 985-991.

ECOBICHON, D. J. 2001. Pesticide use in developing countries. *Toxicology,* 160**,** 27-33.

European Environment Agency 2017. Environmental indicator report 2017 - In support to the monitoring of the 7 Environmental Action Programme, EEA report No21/2017. [Online] Available: https://www.eea.europa.eu/publications/environmental-indicator-report-2017#tab-news-and-articles [Accessed AUG 2018].

EILENBERG, J., HAJEK, A. & LOMER, C. 2001. Suggestions for unifying the terminology in biological control. *BioControl,* 46**,** 387-400.

EKENGREN, S. & HULTMARK, D. 1999. Drosophila cecropin as an antifungal agent. *Insect biochemistry and molecular biology*. 29**,** 965-972.

EKENGREN, S. & HULTMARK, D. 2001. A family of Turandot-related genes in the humoral stress response of Drosophila. *Biochemical and biophysical research communications,* 284**,** 998-1003.

EKESI, S., MANIANIA, N. & AMPONG-NYARKO, K. 1999. Effect of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti*. *Biocontrol Science and Technology,* 9**,** 177-185.

EKESI, S., SHAH, P., CLARK, S. & PELL, J. 2005. Conservation biological control with the fungal pathogen *Pandora neoaphidis*: implications of aphid species, host plant and predator foraging. *Agricultural and forest entomology,* 7**,** 21-30.

EL CHAMY, L., LECLERC, V., CALDELARI, I. & REICHHART, J.-M. 2008. Sensing of'danger signals' and pathogen-associated molecular patterns defines binary signaling pathways' upstream'of Toll. *Nature immunology,* 9**,** 1165-1170.

ENGEL, P. & MORAN, N. A. 2013. The gut microbiota of insects - diversity in structure and function. *FEMS Microbiology Reviews,* 37**,** 699-735.

ERLER, S., POPP, M. & LATTORFF, H. M. G. 2011. Dynamics of immune system gene expression upon bacterial challenge and wounding in a social insect (*Bombus terrestris*). *PLoS One,* 6(3)**,** e18126.

EUROPEAN PARLIAMENT. 2009. Regulation (EC) 1107/2009 of the European Parliament of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. *Official Journal of the European Union* , L 309/1

EVANS, H. C., ELLIOT, S. L. & HUGHES, D. P. 2011. *Ophiocordyceps unilateralis*: A keystone species for unraveling ecosystem functioning and biodiversity of fungi in tropical forests? *Communicative & integrative biology,* 4**,** 598-602.

EYAL, J., MABUD, M. A., FISCHBEIN, K., WALTER, J., OSBORNE, L. & LANDA, Z. 1994. Assessment of *Beauveria bassiana* Nov. EO-1 strain, which produces a red pigment for microbial control. *Applied Biochemistry and Biotechnology,* 44**,** 65-80.

FAN, Y., FANG, W., GUO, S., PEI, X., ZHANG, Y., XIAO, Y., LI, D., JIN, K., BIDOCHKA, M. J. & PEI, Y. 2007. Increased insect virulence in *Beauveria bassiana* strains overexpressing an engineered chitinase. *Applied and Environmental Microbiology,* 73**,** 295-302.

FANG, W., FENG, J., FAN, Y., ZHANG, Y., BIDOCHKA, M. J., LEGER, R. J. S. & PEI, Y. 2009. Expressing a fusion protein with protease and chitinase activities increases the virulence of the insect pathogen *Beauveria bassiana*. *Journal of invertebrate pathology,* 102**,** 155-159.

FANG, W., LENG, B., XIAO, Y., JIN, K., MA, J., FAN, Y., FENG, J., YANG, X., ZHANG, Y. & PEI, Y. 2005. Cloning of *Beauveria bassiana* chitinase gene Bbchit1 and its application to improve fungal strain virulence. *Applied and environmental microbiology,* 71**,** 363-370.

FARENHORST, M., KNOLS, B. G., THOMAS, M. B., HOWARD, A. F., TAKKEN, W., ROWLAND, M. & N'GUESSAN, R. 2010. Synergy in efficacy of fungal entomopathogens and permethrin against West African insecticide-resistant *Anopheles gambiae* mosquitoes. *PLoS One,* 5**,** e12081.

FARGUES, J., GOETTEL, M., SMITS, N., OUEDRAOGO, A. & ROUGIER, M. 1997. Effect of temperature on vegetative growth of *Beauveria bassiana* isolates from different origins. *Mycologia*, 89(3),383-392.

FEHLBAUM, P., BULET, P., MICHAUT, L., LAGUEUX, M., BROEKAERT, W. F., HETRU, C. & HOFFMANN, J. A. 1994. Insect immunity. Septic injury of Drosophila induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. *Journal of Biological Chemistry,* 269, 33159-33163.

FENG, M., POPRAWSKI, T. & KHACHATOURIANS, G. G. 1994a. Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control: current status. *Biocontrol science and technology,* 4, 3-34.

FENG, P., SHANG, Y., CEN, K. & WANG, C. 2015. Fungal biosynthesis of the bibenzoquinone oosporein to evade insect immunity. *Proceedings of the National Academy of Sciences,* 112, 11365-11370.

FENG, X., LI, Z., WU, Q., CHEN, A., WU, Y., HOU, Y., HE, Y., LI, J., XIE, S. & ZHANG, J. 2011. Research progress of the resistance management and sustainable control of diamondback moth (*Plutella xylostella*) in China. *Chinese Journal of Applied Entomology,* 48, 247-253.

FERA. 2016. *PUS STAT: Pesticide Usage Surveys*. [Online]. Available: https://secure.fera.defra.gov.uk/pusstats/myindex.cfm [Accessed AUG 2018].

FERNANDES, E. K., RANGEL, D. E., MORAES, A. M., BITTENCOURT, V. R. & ROBERTS, D. W. 2007. Variability in tolerance to UV-B radiation among *Beauveria* spp. isolates. *Journal of invertebrate pathology,* 96, 237-243.

FERRANDON, D., JUNG, A., CRIQUI, M. C., LEMAITRE, B., UTTENWEILER-JOSEPH, S., MICHAUT, L., REICHHART, J. M. & HOFFMANN, J. 1998. A drosomycin–GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway. *The EMBO journal,* 17, 1217-1227.

FERRÉ, J., REAL, M. D., VAN RIE, J., JANSENS, S. & PEFEROEN, M. 1991. Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. *Proceedings of the National Academy of Sciences,* 88, 5119-5123.

FERREIRA, Á. G., NAYLOR, H., ESTEVES, S. S., PAIS, I. S., MARTINS, N. E. & TEIXEIRA, L. 2014. The Toll-dorsal pathway is required for resistance to viral oral infection in Drosophila. *PLoS pathogens,* 10, e1004507.

FIOLKA, M. J. 2008. Immunosuppressive effect of cyclosporin A on insect humoral immune response. *Journal of invertebrate pathology,* 98, 287-292.

FISCHER, D. C., KOGAN, M. & PAXTON, J. 1990. Effect of glyceollin, a soybean phytoalexin, on feeding by three phytophagous beetles (Coleoptera: Coccinellidae and Chrysomelidae): dose versus response. *Environmental Entomology,* 19, 1278-1282.

FOX, G. E., WISOTZKEY, J. D. & JURTSHUK JR, P. 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *International Journal of Systematic and Evolutionary Microbiology,* 42**,** 166-170.

FURLONG, M. & GRODEN, E. 2001. Evaluation of synergistic interactions between the Colorado potato beetle (Coleoptera: Chrysomelidae) pathogen *Beauveria bassiana* and the insecticides, imidacloprid, and cyromazine. *Journal of Economic Entomology,* 94**,** 344-356.

FURLONG, M. J. & GRODEN, E. 2003. Starvation induced stress and the susceptibility of the Colorado potato beetle, *Leptinotarsa decemlineata*, to infection by *Beauveria bassiana*. *Journal of invertebrate pathology,* 83**,** 127-138.

FURLONG, M. J., PELL, J. K., CHOO, O. P. & RAHMAN, S. A. 1995. Field and laboratory evaluation of a sex pheromone trap for the autodissemination of the fungal entomopathogen *Zoophthora radicans* (Entomophthorales) by the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae). *Bulletin of Entomological research,* 85**,** 331-337.

FURLONG, M. J., WRIGHT, D. J. & DOSDALL, L. M. 2013. Diamondback moth ecology and management: problems, progress, and prospects. *Annual Review of Entomology,* 58**,** 517-41.

GALKO, M. J. & KRASNOW, M. A. 2004. Cellular and genetic analysis of wound healing in Drosophila larvae. *PLoS biology,* 2**,** e239.

GALLOU, A., SERNA-DOMÍNGUEZ, M. G., BERLANGA-PADILLA, A. M., AYALA-ZERMEÑO, M. A., MELLÍN-ROSAS, M. A., MONTESINOS-MATÍAS, R. & ARREDONDO-BERNAL, H. C. 2016. Species clarification of Isaria isolates used as biocontrol agents against *Diaphorina citri* (Hemiptera: Liviidae) in Mexico. *Fungal biology,* 120**,** 414-423.

GANESAN, S., AGGARWAL, K., PAQUETTE, N. & SILVERMAN, N. 2010. NF-κB/Rel proteins and the humoral immune responses of *Drosophila melanogaster*. *Current Topics in Microbiology and Immunology*, 349, 25-60.

GAO, Q., JIN, K., YING, S.-H., ZHANG, Y., XIAO, G., SHANG, Y., DUAN, Z., HU, X., XIE, X.-Q. & ZHOU, G. 2011. Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. *PLoS genetics,* 7**,** e1001264.

GARRIDO-JURADO, I., RUANO, F., CAMPOS, M. & QUESADA-MORAGA, E. 2011. Effects of soil treatments with entomopathogenic fungi on soil dwelling non-target arthropods at a commercial olive orchard. *Biological Control,* 59**,** 239-244.

GEORGEL, P., NAITZA, S., KAPPLER, C., FERRANDON, D., ZACHARY, D., SWIMMER, C., KOPCZYNSKI, C., DUYK, G., REICHHART, J.-M. & HOFFMANN, J. A. 2001. Drosophila immune deficiency (IMD) is a death domain protein that activates antibacterial defense and can promote apoptosis. *Developmental cell,* 1**,** 503-514.

GERWICK, B. C. & SPARKS, T. C. 2014. Natural products for pest control: an analysis of their role, value and future. *Pest management science,* 70**,** 1169-1185.

GILL, S. S., COWLES, E. A. & PIETRANTONIO, P. V. 1992. The mode of action of *Bacillus thuringiensis* endotoxins. *Annual review of entomology,* 37**,** 615-634.

GILLESPIE, A. & CRAWFORD, E. 1986. Effect of water activity on conidial germination and mycelial growth of *Beauveria bassiana*, *Metarhizium anisopliae*, *Paecilomyces* spp. and *Verticillium lecanii*. R. A. Samson, J. M. Vlak & D. Peters (Eds.). *Fundamental and applied aspects of invertebrate pathology*. Publ. Society of Invertebrate Pathology, Wageningen, The Netherlands. p. 254.

GILLESPIE J. P., & KANOST, M. R. & TRENCZEK, T. 1997. Biological mediators of insect immunity. *Annual review of entomology,* 42**,** 611-643.

GILLESPIE, J. P., BAILEY, A. M., COBB, B. & VILCINSKAS, A. 2000. Fungi as elicitors of insect immune responses. *Archives of Insect Biochemistry and Physiology,* 44**,** 49-68.

GILMORE, T. D. 2006. Introduction to NF-κB: players, pathways, perspectives. *Oncogene,* 25**,** 6680-6684.

GLARE, T., CARADUS, J., GELERNTER, W., JACKSON, T., KEYHANI, N., KÖHL, J., MARRONE, P., MORIN, L. & STEWART, A. 2012. Have biopesticides come of age? *Trends in biotechnology,* 30**,** 250-258.

GLAVIS-BLOOM, J., MUHAMMED, M. & MYLONAKIS, E. 2012. Of Model Hosts and Man: Using *Caenorhabditis elegans, Drosophila melanogaster* and *Galleria mellonella* as Model Hosts for Infectious Disease Research. *Advances in Experimental Medicine and Biology*, 710, 11-17.

GOBERT, V., GOTTAR, M., MATSKEVICH, A. A., RUTSCHMANN, S., ROYET, J., BELVIN, M., HOFFMANN, J. A. & FERRANDON, D. 2003. Dual activation of the Drosophila toll pathway by two pattern recognition receptors. *Science,* 302**,** 2126-2130.

GODONOU, I., JAMES, B., ATCHA-AHOWÉ, C., VODOUHE, S., KOOYMAN, C., AHANCHÉDÉ, A. & KORIE, S. 2009. Potential of *Beauveria bassiana* and *Metarhizium anisopliae* isolates from Benin to control *Plutella xylostella* L.(Lepidoptera: Plutellidae). *Crop Protection,* 28**,** 220-224.

GOLIZADEH, A., KAMALI, K., FATHIPOUR, Y. & ABBASIPOUR, H. 2007. Temperature-dependent development of diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae) on two brassicaceous host plants. *Insect Science,* 14**,** 309-316.

GOODWIN, S. 1979. Changes in Numbers in the Parasitoid Complex Associated With the Diamond-Back Moth, *Plutella xylostella* (L.)(Lepidoptera), in Victoria. *Australian Journal of Zoology,* 27**,** 981-989.

GOTO, A., MATSUSHITA, K., GESELLCHEN, V., EL CHAMY, L., KUTTENKEULER, D., TAKEUCHI, O., HOFFMANN, J. A., AKIRA, S., BOUTROS, M. & REICHHART, J.-M. 2008. Akirins are highly conserved nuclear proteins required for NF-κB-dependent gene expression in Drosophila and mice. *Nature immunology,* 9**,** 97-104.

GOTTAR, M., GOBERT, V., MATSKEVICH, A. A., REICHHART, J.-M., WANG, C., BUTT, T. M., BELVIN, M., HOFFMANN, J. A. & FERRANDON, D. 2006. Dual detection of fungal infections in Drosophila via recognition of glucans and sensing of virulence factors. *Cell,* 127**,** 1425-1437.

GRAMATES, L., MARYGOLD, S,. DOS SANTOS, G., URBANO, J-M., ANTONAZZO, G., MATTHEWS, B., REY, A., TABONE, C., CROSBY, M., EMMERT, D., FALLS, K., GOODMAN, J., HU, Y., PONTING, L., SCHROEDER, A., STRELETS, V., THURMOND, J., ZHOU, P. & THE FLYBASE CONSORTIUM. 2017. Flybase at 25: looking to the future. *Nucleic acids research,* 45 (D1), D663-D671.

GRANGE, J. & DAVEY, R. 1990. Antibacterial properties of propolis (bee glue). *Journal of the Royal Society of Medicine,* 83**,** 159-160.

GRIESCH, J. & VILCINSKAS, A. 1998. Proteases Released by Entomopathogenic Fungi Impair Phagocytic Activity, Attachment and Spreading of Plasmatocytes Isolated from Haemolymph of the Greater Wax Moth *Galleria mellonella*. *Biocontrol Science and Technology,* 8**,** 517-531.

HAJEK, A. & ST. LEGER, R. 1994. Interactions between fungal pathogens and insect hosts. *Annual review of entomology,* 39**,** 293-322.

HAMILL, R. L., HIGGENS, C., BOAZ, H. & GORMAN, M. 1969. The structure of beauvericin, a new depsipeptide antibiotic toxic to *Artemia salina*. *Tetrahedron Letters,* 10**,** 4255-4258.

HAN, Z. S. & IP, Y. T. 1999. Interaction and specificity of Rel-related proteins in regulating Drosophila immunity gene expression. *Journal of Biological Chemistry,* 274**,** 21355-21361.

HARADA, T., MISAKI, A. & SAITO, H. 1968. Curdlan: a bacterial gel-forming β-1, 3-glucan. *Archives of Biochemistry and Biophysics,* 124**,** 292-298.

HARCOUT, D. 1986. Population dynamics of the diamondback moth in southern Ontario. *Diamondback moth and other cruciferous pests. Proceedings of the First International Workshop, 11-15 March*. N. Talekar (Ed). Asian Vegetable Research and Development Center, Taiwan.

HARDING, C. R., SCHROEDER, G. N., COLLINS, J. W. & FRANKEL, G. 2013. Use of *Galleria mellonella* as a model organism to study *Legionella pneumophila* infection. *Journal of visualized experiments: JoVE*, 81, e50964.

HASEEB, M., AMANO, H. & NEMOTO, H. 2000. Pesticidal effects on mortality and parasitism rates of *Diadegma semiclausum*, a parasitoid of the diamondback moth. *BioControl,* 45**,** 165-178.

HASEEB, M., LIU, T.-X. & JONES, W. 2004. Effects of selected insecticides on *Cotesia plutellae*, endoparasitoid of *Plutella xylostella*. *BioControl,* 49**,** 33-46.

HE, W., ZHANG, M.-F., YE, J., JIANG, T.-T., FANG, X. & SONG, Y. 2010. Cordycepin induces apoptosis by enhancing JNK and p38 kinase activity and increasing the protein expression of Bcl-2 pro-apoptotic molecules. *Journal of Zhejiang University Science B,* 11**,** 654-660.

HEALTH AND SEFETY EXECUTIVE. 2018. *Pesticides Register Database* [Online]. Available: http://www.hse.gov.uk/pesticides/index.htm [Accessed JUN 2018].

HEDENGREN-OLCOTT, M., OLCOTT, M. C., MOONEY, D. T., EKENGREN, S., GELLER, B. L. & TAYLOR, B. J. 2004. Differential activation of the NF-κB-like factors Relish and Dif in *Drosophila melanogaster* by fungi and Gram-positive bacteria. *Journal of biological chemistry,* 279**,** 21121-21127.

HEGEDUS, D. D., BIDOCHKA, M. J., MIRANPURI, G. S. & KHACHATOURIANS, G. G. 1992. A comparison of the virulence, stability and cell-wall-surface characteristics of three spore types produced by the entomopathogenic fungus *Beauveria bassiana*. *Applied microbiology and biotechnology,* 36**,** 785-789.

HESKETH, H., ROY, H., EILENBERG, J., PELL, J. & HAILS, R. 2010. Challenges in modelling complexity of fungal entomopathogens in semi-natural populations of insects. *BioControl,* 55**,** 55-73.

HIBBETT, D. S., BINDER, M., BISCHOFF, J. F., BLACKWELL, M., CANNON, P. F., ERIKSSON, O. E., HUHNDORF, S., JAMES, T., KIRK, P. M. & LÜCKING, R. 2007. A higher-level phylogenetic classification of the Fungi. *Mycological research,* 111**,** 509-547.

HILLOCKS, R. J. 2012. Farming with fewer pesticides: EU pesticide review and resulting challenges for UK agriculture. *Crop Protection,* 31**,** 85-93.

HIROMORI, H. & NISHIGAKI, J. 2001. Factor analysis of synergistic effect between the entomopathogenic fungus *Metarhizium anisopliae* and synthetic insecticides. *Applied Entomology and Zoology,* 36**,** 231-236.

HOFFMANN, J. A. & REICHHART, J.-M. 2002. Drosophila innate immunity: an evolutionary perspective. *Nature Immunology,* 3**,** 121-126.

HOLLINGSWORTH, R., STEINKRAUS, D. & MCNEWZ, R. 1995. Sampling to predict fungal epizootics in cotton aphids (Homoptera: Aphididae). *Environmental entomology,* 24**,** 1414-1421.

HOWARD, R. J., FERRARI, M. A., ROACH, D. H. & MONEY, N. P. 1991. Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proceedings of the National Academy of Sciences,* 88**,** 11281-11284.

HU, C. & AKSOY, S. 2006. Innate immune responses regulate trypanosome parasite infection of the tsetse fly *Glossina morsitans*. *Molecular microbiology,* 60**,** 1194-1204.

HU, P., CHEN, W., BAO, J., JIANG, L. & WU, L. 2014. Cordycepin modulates inflammatory and catabolic gene expression in interleukin-1 beta-induced human chondrocytes from advanced-stage osteoarthritis: an in vitro study. *International journal of clinical and experimental pathology,* 7**,** 6575-6584.

HUANG, L.-F., LIANG, Y.-Z., GUO, F.-Q., ZHOU, Z.-F. & CHENG, B.-M. 2003. Simultaneous separation and determination of active components in *Cordyceps sinensis* and *Cordyceps militaris* by LC/ESI-MS. *Journal of Pharmaceutical and Biomedical Analysis,* 33**,** 1155-1162.

HUMBER, R. A. 2008. Evolution of entomopathogenicity in fungi. *Journal of invertebrate pathology,* 98**,** 262-266.

HUNG, S.-Y., BOUCIAS, D. G. & VEY, A. J. 1993. Effect of *Beauveria bassiana* and *Candida albicans* on the cellular defense response of *Spodoptera exigua*. *Journal of Invertebrate Pathology,* 61**,** 179-187.

HUSSEIN, K. A., ABDEL-RAHMAN, M. A., ABDEL-MALLEK, A. Y., EL-MARAGHY, S. S. & JOO, J. H. 2012. Pathogenicity of *Beauveria bassiana* and *Metarhizium anisopliae* against *Galleria mellonella*. *Phytoparasitica,* 40**,** 117-126.

IBMA 2016. International Biocontrol Manufacturer's Association Biocontrol Survey Report [Online] http://www.ibma-global.org/en/news/ibma-biocontrol-survey-report. [Accessed JUN 2018]

INGLIS, G. D., ENKERLI, J. & GOETTEL, M. S. 2012. Laboratory techniques used for entomopathogenic fungi: Hypocreales. L.L. Lacey (Ed.). *Manual of Techniques in Invertebrate Pathology* (2nd Ed.). Publ. Elsevier Ltd, London.

IOANNIDIS, P., COURTIS, N., HAVREDAKI, M., MICHAILAKIS, E., TSIAPALIS, C. M. & TRANGAS, T. 1999. The polyadenylation inhibitor cordycepin (3'dA) causes a decline in c-MYC mRNA levels without affecting c-MYC protein levels. *Oncogene,* 18**,** 117-125.

ISMAN, M. B., KOUL, O., LUCZYNSKI, A. & KAMINSKI, J. 1990. Insecticidal and antifeedant bioactivities of neem oils and their relationship to azadirachtin content. *Journal of Agricultural and Food Chemistry,* 38**,** 1406-1411.

ISSA, N., GUILLAUMOT, N., LAURET, E., MATT, N., SCHAEFFER-REISS, C., VAN DORSSELAER, A., REICHHART, J.-M. & VEILLARD, F. 2018. The circulating protease Persephone is an immune sensor for microbial proteolytic activities upstream of the Drosophila Toll pathway. *Molecular cell,* 69**,** 539-550.

JABER, L. R. & ENKERLI, J. 2016. Effect of seed treatment duration on growth and colonization of Vicia faba by endophytic *Beauveria bassiana* and *Metarhizium brunneum*. *Biological Control,* 103**,** 187-195.

JAMES, P., KERSHAW, M., REYNOLDS., CHARNLEY., A. 1993. Inhibition of desert locust (*Schistocerca gregaria*) Malpighian tubule fluid secretion by destruxins, cyclic peptide toxins from the insect pathogenic fungus *Metarhizium anisopliae*. *Journal of Insect Physiology*, 39, 797-804.

JAMES, R. & ELZEN, G. 2001. Antagonism between *Beauveria bassiana* and imidacloprid when combined for *Bemisia argentifolii* (Homoptera: Aleyrodidae) control. *Journal of economic entomology,* 94**,** 357-361.

JARONSKI, S. T. 2013. Mass production of entomopathogenic fungi: state of the art. J. Morales-Ramos, M. Guagelupe Rojas & D. Shapiro-Ilan (Eds.). *Mass production of beneficial organisms*. Publ. Elsevier, London, 357-413.

JAROS-SU, J., GRODEN, E. & ZHANG, J. 1999. Effects of Selected Fungicides and the Timing of Fungicide Application on *Beauveria bassiana*-Induced Mortality of the Colorado Potato Beetle (Coleoptera: Chrysomelidae). *Biological Control,* 15**,** 259-269.

JEFFS, L. B. & KHACHATOURIANS, G. G. 1997. Toxic properties of Beauveria pigments on erythrocyte membranes. *Toxicon,* 35**,** 1351-1356.

JEGOROV, A., SEDMERA, P., MAŤHA, V., ŠIMEK, P., ZAHRADNÍČKOVÁ, H., LANDA, Z. & EYAL, J. 1994. Beauverolides L and La from *Beauveria tenella* and *Paecilomyces fumosoroseus*. *Phytochemistry,* 37**,** 1301-1303.

JEN, C.-Y., LIN, C.-Y., HUANG, B.-M. & LEU, S.-F. 2011. Cordycepin induced MA-10 mouse Leydig tumor cell apoptosis through caspase-9 pathway. *Evidence-Based Complementary and Alternative Medicine,* 2011.

JENNINGS, B. H. 2011. Drosophila–a versatile model in biology & medicine. *Materials Today,* 14**,** 190-195.

JEONG, J.-W., JIN, C.-Y., KIM, G.-Y., LEE, J.-D., PARK, C., KIM, G.-D., KIM, W.-J., JUNG, W.-K., SEO, S. K. & CHOI, I.-W. 2010. Anti-inflammatory effects of cordycepin via suppression of inflammatory mediators in BV2 microglial cells. *International immunopharmacology,* 10**,** 1580-1586.

JEONG, J.-W., JIN, C.-Y., PARK, C., HONG, S. H., KIM, G.-Y., JEONG, Y. K., LEE, J.-D., YOO, Y. H. & CHOI, Y. H. 2011. Induction of apoptosis by cordycepin via reactive oxygen species generation in human leukemia cells. *Toxicology in vitro,* 25**,** 817-824.

JESS, S., KILDEA, S., MOODY, A., RENNICK, G., MURCHIE, A. K. & COOKE, L. R. 2014. European Union policy on pesticides: implications for agriculture in Ireland. *Pest management science,* 70**,** 1646-1654.

JIANG, H., VILCINSKAS, A. & KANOST, M. R. 2010. Immunity in Lepidopteran Insects. K. Söderhäll (Ed.). *Invertebrate Immunity*. Publ. Springer, New York, 181-204.

JIN, L. H., SHIM, J., YOON, J. S., KIM, B., KIM, J., KIM-HA, J., KIM, Y.-J., G. M. 2008. Identification and Functional Analysis of Antifungal Immune Response Genes in Drosophila. *PLoS Pathogens,* 4**,** e1000168-e1000168.

JOHNS, D. G. & ADAMSON, R. H. 1976. Enhancement of the biological activity of cordycepin (3'-deoxyadenosine) by the adenosine deaminase inhibitor 2'-deoxycoformycin. *Biochemical pharmacology,* 25**,** 1441-1444.

JOHNSON, D. L. & GOETTEL, M. S. 1993. Reduction of grasshopper populations following field application of the fungus *Beauveria bassiana*. *Biocontrol Science and Technology,* 3**,** 165-175.

JOHNSTON, J. B. 2011. Mechanism of action of pentostatin and cladribine in hairy cell leukemia. *Leukemia & lymphoma,* 52**,** 43-45.

JOLLÈS, P. & JOLLÈS, J. 1984. What's new in lysozyme research? *Molecular and cellular biochemistry,* 63**,** 165-189.

JOOP, G. & VILCINSKAS, A. 2016. Coevolution of parasitic fungi and insect hosts. *Zoology,* 119**,** 350-358.

JUNEJA, P. & LAZZARO, B. P. 2009. Population genetics of insect immune responses. J. Rolff, S. Reynolds (Eds.) *Insect infection and immunity: evolution, ecology and mechanisms*. Publ. Oxford University Press, Oxford**,** 206-224.

JUNG, K., KIM, I.-H. & HAN, D. 2004. Effect of medicinal plant extracts on forced swimming capacity in mice. *Journal of ethnopharmacology,* 93**,** 75-81.

JUNG, S.-M., PARK, S.-S., KIM, W.-J. & MOON, S.-K. 2012. Ras/ERK1 pathway regulation of p27KIP1-mediated G1-phase cell-cycle arrest in cordycepin-induced inhibition of the proliferation of vascular smooth muscle cells. *European journal of pharmacology,* 681**,** 15-22.

KAAKEH, W., REID, B. L., BOHNERT, T. J. & BENNETT, G. W. 1997. Toxicity of imidacloprid in the German cockroach (Dictyoptera: Blattellidae), and the synergism between imidacloprid and *Metarhizium anisopliae* (Imperfect Fungi: Hyphomycetes). *Journal of Economic Entomology,* 90**,** 473-482.

KALLIO, J., LEINONEN, A., ULVILA, J., VALANNE, S., EZEKOWITZ, R. A. & RÄMET, M. 2005. Functional analysis of immune response genes in Drosophila identifies JNK pathway as a regulator of antimicrobial peptide gene expression in S2 cells. *Microbes and Infection,* 7**,** 811-819.

KARIN, M. 1999. How NF-κB is activated: the role of the IκB kinase (IKK) complex. *Oncogene,* 18**,** 6867-6874.

KASSA, A., BROWNBRIDGE, M., PARKER, B. L., SKINNER, M., GOULI, V., GOULI, S., GUO, M., LEE, F. & HATA, T. 2008. Whey for mass production of *Beauveria bassiana* and *Metarhizium anisopliae*. *Mycological Research,* 112**,** 583-591.

KATO, T., AHMAD, S. & PARK, E. Y. 2017. Functional Analysis of Ribonucleotide Reductase from *Cordyceps militaris* Expressed in *Escherichia coli*. *Applied Biochemistry and Biotechnology*, 182, 1307-1317.

KELLER, N. P., TURNER, G. & BENNETT, J. W. 2005. Fungal secondary metabolism—from biochemistry to genomics. *Nature Reviews Microbiology,* 3**,** 937-947.

KEPLER, R. M., HUMBER, R. A., BISCHOFF, J. F. & REHNER, S. A. 2014. Clarification of generic and species boundaries for Metarhizium and related fungi through multigene phylogenetics. *Mycologia,* 106**,** 811-829.

KHATER, H. F. 2012. Prospects of botanical biopesticides in insect pest management. *Pharmacologia,* 3**,** 641-656.

KIM, H. G., SHRESTHA, B., LIM, S. Y., YOON, D. H., CHANG, W. C., SHIN, D. J., HAN, S. K., PARK, S. M., PARK, J. H., PARK, H. I., SUNG, J. M., JANG, Y., CHUNG, N., HWANG, K. C. & KIM, T. W. 2006a. Cordycepin inhibits lipopolysaccharide-induced inflammation by the suppression of NF-kappaB through Akt and p38 inhibition in RAW 264.7 macrophage cells. *European Journal of Pharmacology,* 545**,** 192-9.

KIM, J. R., YEON, S. H., KIM, H. S. & AHN, Y. J. 2002. Larvicidal activity against *Plutella xylostella* of cordycepin from the fruiting body of *Cordyceps militaris*. *Pest management science,* 58**,** 713-717.

KIM, S. W., HWANG, H. J., XU, C. P., SUNG, J. M., CHOI, J. W. & YUN, J. W. 2003. Optimization of submerged culture process for the production of mycelial biomass and exo-polysaccharides by *Cordyceps militaris* C738. *Journal of applied microbiology,* 94**,** 120-126.

KIMBRELL, D. A. & BEUTLER, B. 2001. The evolution and genetics of innate immunity. *Nature Reviews Genetics,* 2**,** 256-267.

KLEINBAUM, D. G. & KLEIN, M. 2012. Kaplan-Meier survival curves and the log-rank test. D. G. Kleinbaum (Ed.). *Survival analysis*. Publ. Springer, New York.

KOGAN, M. 1998. Integrated pest management: historical perspectives and contemporary developments. *Annual review of entomology,* 43**,** 243-270.

KONDRASHOV, A., MEIJER, H. A., BARTHET-BARATEIG, A., PARKER, H. N., KHURSHID, A., TESSIER, S., SICARD, M., KNOX, A. J., PANG, L. & DE MOOR, C. H. 2012. Inhibition of polyadenylation reduces inflammatory gene induction. *RNA,* 18**,** 2236-2250.

KONG, H., LV, M., MAO, N., WANG, C., CHENG, Y., ZHANG, L., JIANG, X. & LUO, L. 2016. Molecular Characterization of a Lysozyme Gene and Its Altered Expression Profile in Crowded Beet Webworm (*Loxostege sticticalis*). *PloS one,* 11**,** e0161384.

KOPPENHÖFER, A. M. & FUZY, E. M. 2003. *Steinernema scarabaei* for the control of white grubs. *Biological Control,* 28**,** 47-59.

KRAMER, G. J. & NODWELL, J. R. 2017. Chromosome level assembly and secondary metabolite potential of the parasitic fungus *Cordyceps militaris*. *BMC genomics,* 18**,** 912.

KRYUKOV, V. Y., YAROSLAVTSEVA, O., LEDNEV, G. & BORISOV, B. 2011. Local epizootics caused by teleomorphic cordycipitoid fungi (Ascomycota: Hypocreales) in populations of forest lepidopterans and sawflies of the summer-autumn complex in Siberia. *Microbiology,* 80**,** 286-295.

KRYUKOV, V. Y., YAROSLAVTSEVA, O. N., SURINA, E. V., TYURIN, M. V., DUBOVSKIY, I. M. & GLUPOV, V. V. 2015. Immune reactions of the greater wax moth, *Galleria mellonella* L. (lepidoptera, pyralidae) larvae under combined treatment of the entomopathogens *Cordyceps militaris* (L.: Fr.) Link and *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycota, Hypocreales). *Entomological Review,* 95**,** 693-698.

LACEY, L. A. 2012. *Manual of techniques in invertebrate pathology*, Academic Press, San Diego.

LACEY, L. A. 2016. *Microbial control of insect and mite pests: From theory to practice*, Academic Press, San Diego.

LACEY, L. A., THOMSON, D., VINCENT, C. & ARTHURS, S. P. 2008. Codling moth granulovirus: a comprehensive review. *Biocontrol Science and Technology,* 18**,** 639-663.

LAKSHMI, B., SRI, P. R., DEVI, K. A. & HEMALATHA, K. J. 2014. Screening, optimization of production and partial characterization of alkaline protease from haloalkaliphilic *Bacillus* sp. *International Journal of Research in Engineering and Technology*, 03, 435-443.

LAMBERT, J., KEPPI, E., DIMARCQ, J.-L., WICKER, C., REICHHART, J.-M., DUNBAR, B., LEPAGE, P., VAN DORSSELAER, A., HOFFMANN, J. & FOTHERGILL, J. 1989. Insect immunity: isolation from immune blood of the dipteran *Phormia terranovae* of two insect antibacterial peptides with sequence homology to rabbit lung macrophage bactericidal peptides. *Proceedings of the National Academy of Sciences,* 86**,** 262-266.

LANGE, A., BEIER, S., HUSON, D. H., PARUSEL, R., IGLAUER, F. & FRICK, J.-S. 2018. Genome sequence of *Galleria mellonella* (greater wax moth). *Genome announcements,* 6**,** e01220-17.

LANGEN, G., IMANI, J., ALTINCICEK, B., KIESERITZKY, G., KOGEL, K.-H. & VILCINSKAS, A. 2006. Transgenic expression of gallerimycin, a novel antifungal insect defensin from the greater wax moth *Galleria mellonella*, confers resistance to pathogenic fungi in tobacco. *Biological chemistry,* 387**,** 549-557.

LANOT, R., ZACHARY, D., HOLDER, F. & MEISTER, M. 2001. Postembryonic hematopoiesis in Drosophila. *Developmental biology,* 230**,** 243-257.

LAVINE, M. & STRAND, M. 2002. Insect hemocytes and their role in immunity. *Insect biochemistry and molecular biology,* 32**,** 1295-1309.

LECLERC, V. & REICHHART, J. M. 2004. The immune response *of Drosophila melanogaster*. *Immunological reviews,* 198**,** 59-71.

LEE, E.-J., KIM, W.-J. & MOON, S.-K. 2010. Cordycepin suppresses TNF-alpha-induced invasion, migration and matrix metalloproteinase-9 expression in human bladder cancer cells. *Phytotherapy Research,* 24**,** 1755-1761.

LEE, Y., YUN, E., JANG, W., KIM, I., LEE, J., PARK, S., RYU, K., SEO, S., KIM, C. & LEE, I. 2004. Purification, cDNA cloning and expression of an insect defensin from the great wax moth, *Galleria mellonella*. *Insect molecular biology,* 13**,** 65-72.

LEE, Y.-R., NOH, E.-M., JEONG, E.-Y., YUN, S.-K., JEONG, Y.-J., KIM, J.-H., KWON, K.-B., KIM, B.-S., LEE, S.-H. & PARK, C.-S. 2009. Cordycepin inhibits UVB-induced matrix metalloproteinase expression by suppressing the NF-κB pathway in human dermal fibroblasts. *Experimental & molecular medicine,* 41**,** 548.

LEGER, R. J. S., BUTT, T. M., STAPLES, R. C. & ROBERTS, D. W. 1989. Synthesis of proteins including a cuticle-degrading protease during differentiation of the entomopathogenic fungus *Metarhizium anisopliae*. *Experimental Mycology,* 13**,** 253-262.

LEMAITRE, B. & HOFFMANN, J. 2007. The Host Defense of *Drosophila melanogaster*. *Annual Review of Immunology,* 25**,** 697-743.

LEMAITRE, B., KROMER-METZGER, E., MICHAUT, L., NICOLAS, E., MEISTER, M., GEORGEL, P., REICHHART, J.-M. & HOFFMANN, J. A. 1995. A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense. *Proceedings of the National Academy of Sciences,* 92**,** 9465-9469.

LEMAITRE, B., NICOLAS, E., MICHAUT, L., REICHHART, J.-M. & HOFFMANN, J. A. 1996. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell,* 86**,** 973-983.

LEMAITRE, B., REICHHART, J.-M. & HOFFMANN, J. A. 1997. Drosophila host defense: differential induction of antimicrobial peptide genes after infection by

various classes of microorganisms. *Proceedings of the National Academy of Sciences,* 94**,** 14614-14619.

LEONE, P., BISCHOFF, V., KELLENBERGER, C., HETRU, C., ROYET, J. & ROUSSEL, A. 2008. Crystal structure of Drosophila PGRP-SD suggests binding to DAP-type but not lysine-type peptidoglycan. *Molecular immunology,* 45**,** 2521-2530.

LEVASHINA, E. A., MOITA, L. F., BLANDIN, S., VRIEND, G., LAGUEUX, M. & KAFATOS, F. C. 2001. Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*. *Cell,* 104**,** 709-718.

LEVASHINA, E. A., OHRESSER, S., BULET, P., REICHHART, J. M., HETRU, C. & HOFFMANN, J. A. 1995. Metchnikowin, a novel immune-inducible proline-rich peptide from Drosophila with antibacterial and antifungal properties. *European journal of biochemistry,* 233**,** 694-700.

LI, S., LI, P., LAI, C., GONG, Y., KAN, K. K., DONG, T. T., TSIM, K. W. & WANG, Y. 2004. Simultaneous determination of ergosterol, nucleosides and their bases from natural and cultured Cordyceps by pressurised liquid extraction and high-performance liquid chromatography. *Journal of Chromatography A,* 1036**,** 239-243.

LI, Z. 1988. List on the Insect Hosts of *Beauveria bassiana*. *Study and application of entomogenous fungi in China,* 1**,** 241-255.

LI, Z., ALVES, S. B., ROBERTS, D. W., FAN, M., DELALIBERA JR, I., TANG, J., LOPES, R. B., FARIA, M. & RANGEL, D. E. 2010. Biological control of insects in Brazil and China: history, current programs and reasons for their successes using entomopathogenic fungi. *Biocontrol Science and Technology,* 20**,** 117-136.

LIEHL, P., BLIGHT, M., VODOVAR, N., BOCCARD, F. & LEMAITRE, B. 2006. Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model. *PLoS pathogens,* 2**,** e56.

LIN, Y.-W. & CHIANG, B.-H. 2008. Anti-tumor activity of the fermentation broth of *Cordyceps militaris* cultured in the medium of Radix astragali. *Process Biochemistry,* 43**,** 244-250.

LIU, H. & BAUER, L. S. 2006. Susceptibility of *Agrilus planipennis* (Coleoptera: Buprestidae) to *Beauveria bassiana* and *Metarhizium anisopliae*. *Journal of Economic Entomology,* 99**,** 1096-1103.

LIU, S.-S., CHEN, F.-Z. & ZALUCKI, M. P. 2002. Development and survival of the diamondback moth (Lepidoptera: Plutellidae) at constant and alternating temperatures. *Environmental Entomology,* 31**,** 221-231.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods,* 25**,** 402-408.

LOMER, C., BATEMAN, R., JOHNSON, D., LANGEWALD, J. & THOMAS, M. 2001. Biological control of locusts and grasshoppers. *Annual review of entomology,* 46**,** 667-702.

LOOS, J., ABSON, D. J., CHAPPELL, M. J., HANSPACH, J., MIKULCAK, F., TICHIT, M. & FISCHER, J. 2014. Putting meaning back into "sustainable intensification". *Frontiers in Ecology and the Environment,* 12**,** 356-361.

LU, H., LI, X., ZHANG, J., SHI, H., ZHU, X. & HE, X. 2014. Effects of cordycepin on HepG2 and EA. hy926 cells: Potential antiproliferative, antimetastatic and anti-angiogenic effects on hepatocellular carcinoma. *Oncology letters,* 7**,** 1556-1562.

LYNN, D. E. 2007. Available lepidopteran insect cell lines. D. Murhammer (Ed.). *Methods in molecular biology series Baculovirus and insect cell expression protocols*. Publ. Springer, New York, 117-137.

MA, L., ZHANG, S. & DU, M. 2015. Cordycepin from *Cordyceps militaris* prevents hyperglycemia in alloxan-induced diabetic mice. *Nutrition Research,* 35**,** 431-439.

MA, X.-M., LIU, X.-X., NING, X., ZHANG, B., HAN, F., GUAN, X.-M., TAN, Y.-F. & ZHANG, Q.-W. 2008. Effects of *Bacillus thuringiensis* toxin Cry1Ac and *Beauveria bassiana* on Asiatic corn borer (Lepidoptera: Crambidae). *Journal of invertebrate pathology,* 99**,** 123-128.

MACINTOSH, S. C., STONE, T. B., SIMS, S. R., HUNST, P. L., GREENPLATE, J. T., MARRONE, P. G., PERLAK, F. J., FISCHHOFF, D. A. & FUCHS, R. L. 1990. Specificity and efficacy of purified *Bacillus thuringiensis* proteins against agronomically important insects. *Journal of invertebrate pathology,* 56**,** 258-266.

MAK, P. & ZDYBICKA-BARABAS, A. 2010. A different repertoire of *Galleria mellonella* antimicrobial peptides in larvae challenged with bacteria and fungi. 34**,** 1129-1136.

MANFRUELLI, P., REICHHART, J. M., STEWARD, R., HOFFMANN, J. A. & LEMAITRE, B. 1999. A mosaic analysis in Drosophila fat body cells of the control of antimicrobial peptide genes by the Rel proteins Dorsal and DIF. *The EMBO journal,* 18**,** 3380-3391.

MANSOUR, S. C., PENA, O. M. & HANCOCK, R. E. 2014. Host defense peptides: front-line immunomodulators. *Trends in immunology,* 35**,** 443-450.

MARCHIORO, C. & FOERSTER, L. 2011. Development and survival of the diamondback moth, *Plutella xylostella* (L.)(Lepidoptera: Yponomeutidae) as a function of temperature: effect on the number of generations in tropical and subtropical regions. *Neotropical entomology,* 40**,** 533-541.

MARONI, M., COLOSIO, C., FERIOLI, A. & FAIT, A. 2000. Biological monitoring of pesticide exposure: a review. *Toxicology,* 143**,** 1-118.

MASUDA, M., URABE, E., HONDA, H., SAKURAI, A. & SAKAKIBARA, M. 2007. Enhanced production of cordycepin by surface culture using the medicinal mushroom *Cordyceps militaris*. *Enzyme and Microbial Technology,* 40**,** 1199-1205.

MEDZHITOV, R. 2001. Toll-like receptors and innate immunity. *Nature Reviews Immunology,* 1**,** 135-145.

MEISTER, M., LEMAITRE, B. & HOFFMANN, J. A. 1997. Antimicrobial peptide defense in Drosophila. *Bioessays,* 19**,** 1019-1026.

MENG, X., KHANUJA, B. S. & IP, Y. T. 1999. Toll receptor-mediated Drosophila immune response requires Dif, an NF-κB factor. *Genes & development,* 13**,** 792-797.

MENT, D., CHURCHILL, A. C., GINDIN, G., BELAUSOV, E., GLAZER, I., REHNER, S. A., ROT, A., DONZELLI, B. G. & SAMISH, M. 2012. Resistant ticks inhibit Metarhizium infection prior to haemocoel invasion by reducing fungal viability on the cuticle surface. *Environmental microbiology,* 14**,** 1570-1583.

MERION, R. M., WHITE, D. J., THIRU, S., EVANS, D. B. & CALNE, R. Y. 1984. Cyclosporine: five years' experience in cadaveric renal transplantation. *New England Journal of Medicine,* 310**,** 148-154.

MEYLING, N. V. & EILENBERG, J. 2007. Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: potential for conservation biological control. *Biological control,* 43**,** 145-155.

MICHEL, T., REICHHART, J.-M., HOFFMANN, J. A. & ROYET, J. 2001. Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature,* 414**,** 756-759.

MIRONIDIS, G. & SAVOPOULOU-SOULTANI, M. 2014. Development, survivorship, and reproduction of *Helicoverpa armigera* (Lepidoptera: Noctuidae) under constant and alternating temperatures. *Environmental Entomology,* 37**,** 16-28.

MITINA, G., KAZARTSEV, I., VASILEVA, A. & YLI-MATTILA, T. 2017. Multilocus genotyping based species identification of entomopathogenic fungi of the genus Lecanicillium (= *Verticillium lecanii* s.l.). *Journal of basic microbiology,* 57**,** 950-961.

MIYAMOTO, J. 1976. Degradation, metabolism and toxicity of synthetic pyrethroids. *Environmental Health Perspectives,* 14**,** 15-28.

MOFFITT, M. C. & NEILAN, B. A. 2000. The expansion of mechanistic and organismic diversity associated with non-ribosomal peptides. *FEMS microbiology letters,* 191**,** 159-167.

MOGHUL, A., LIN, L., BEEDLE, A., KANBOUR-SHAKIR, A., DEFRANCES, M. C., LIU, Y. & ZARNEGAR, R. 1994. Modulation of c-MET proto-oncogene (HGF receptor) mRNA abundance by cytokines and hormones: evidence for rapid decay of the 8 kb c-MET transcript. *Oncogene,* 9**,** 2045-2052.

MOHAN, M. C., REDDY, N. P., DEVI, U. K., KONGARA, R. & SHARMA, H. C. 2007. Growth and insect assays of *Beauveria bassiana* with neem to test their compatibility and synergism. *Biocontrol Science and Technology,* 17**,** 1059-1069.

MOLNÁR, I., GIBSON, D. M. & KRASNOFF, S. B. 2010. Secondary metabolites from entomopathogenic Hypocrealean fungi. *Natural product reports,* 27**,** 1241-1275.

MORALES-RODRIGUEZ, A. & PECK, D. C. 2009. Synergies between biological and neonicotinoid insecticides for the curative control of the white grubs *Amphimallon majale* and *Popillia japonica*. *Biological control,* 51**,** 169-180.

MORALES-VALENZUELA, G., SILVA-ROJAS, H. & OCHOA-MARTÍNEZ, D. 2017. First report of *Pantoea agglomerans* causing leaf blight and vascular wilt in maize and sorghum in Mexico. *Plant Disease,* 91, 1365

MOWLDS, P., BARRON, A. & KAVANAGH, K. 2008. Physical stress primes the immune response of *Galleria mellonella* larvae to infection by *Candida albicans*. *Microbes and Infection,* 10**,** 628-634.

MOWLDS, P. & KAVANAGH, K. 2008. Effect of pre-incubation temperature on susceptibility of *Galleria mellonella* larvae to infection by *Candida albicans*. *Mycopathologia,* 165**,** 5-12.

MUELLER, W. E., WEILER, B. E., CHARUBALA, R., PFLEIDERER, W., LESERMAN, L., SOBOL, R. W., SUHADOLNIK, R. J. & SCHROEDER, H. C. 1991. Cordycepin analogs of 2', 5'-oligoadenylate inhibit human immunodeficiency virus infection via inhibition of reverse transcriptase. *Biochemistry,* 30**,** 2027-2033.

MUKHERJEE, K., ALTINCICEK, B., HAIN, T., DOMANN, E., VILCINSKAS, A. & CHAKRABORTY, T. 2010. *Galleria mellonella* as a model system for studying *Listeria pathogenesis*. *Applied and environmental microbiology,* 76**,** 310-317.

MUKHERJEE, K. & VILCINSKAS, A. 2018. The entomopathogenic fungus *Metarhizium robertsii* communicates with the insect host *Galleria mellonella* during infection. *Virulence,* 9**,** 402-413.

MYLLYMÄKI, H. & RÄMET, M. 2014. JAK/STAT pathway in Drosophila immunity. *Scandinavian journal of immunology,* 79**,** 377-385.

MYLONAKIS, E., AUSUBEL, F. M., GILMORE, M. & CASADEVALL, A. (Eds.) *Recent Advances on Model Hosts*. Publ. Springer New York.

MYLONAKIS, E., MORENO, R., EL KHOURY, J. B., IDNURM, A., HEITMAN, J., CALDERWOOD, S. B., AUSUBEL, F. M. & DIENER, A. 2005. *Galleria mellonella* as a model system to study *Cryptococcus neoformans* pathogenesis. *Infection and immunity,* 73**,** 3842-3850.

NAKAMURA, K., KONOHA, K., YOSHIKAWA, N., YAMAGUCHI, Y., KAGOTA, S., SHINOZUKA, K. & KUNITOMO, M. 2005. Effect of cordycepin (3'-deoxyadenosine) on hematogenic lung metastatic model mice. *In vivo,* 19**,** 137-141.

NAKAMURA, K., YOSHIKAWA, N., YAMAGUCHI, Y., KAGOTA, S., SHINOZUKA, K. & KUNITOMO, M. 2006. Antitumor effect of cordycepin (3'-deoxyadenosine) on mouse melanoma and lung carcinoma cells involves adenosine A3 receptor stimulation. *Anticancer research,* 26**,** 43-47.

NAUEN, R., EBBINGHAUS-KINTSCHER, U., SALGADO, V. L. & KAUSSMANN, M. 2003. Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants. *Pesticide Biochemistry and Physiology,* 76**,** 55-69.

NCBI, R. C. 2017. Database Resources of the National Center for Biotechnology Information. *Nucleic acids research,* 45**,** D12.

NIKOH, N. & FUKATSU, T. 2000. Interkingdom host jumping underground: phylogenetic analysis of entomoparasitic fungi of the genus Cordyceps. *Molecular Biology and Evolution,* 17**,** 629-638.

O'NEILL, L. A. 2006. How Toll-like receptors signal: what we know and what we don't know. *Current opinion in immunology,* 18**,** 3-9.

ORTEGO, F., RODRÍGUEZ, B. & CASTAÑERA, P. 1995. Effects of neo-clerodane diterpenes from Teucrium on feeding behavior of colorado potato beetle larvae. *Journal of chemical ecology,* 21**,** 1375-1386.

ORTIZ-URQUIZA, A. & KEYHANI, N. O. 2013. Action on the surface: entomopathogenic fungi versus the insect cuticle. *Insects,* 4**,** 357-374.

OUEDRAOGO, A., FARGUES, J., GOETTEL, M. & LOMER, C. 1997. Effect of temperature on vegetative growth among isolates of *Metarhizium anisopliae* and *M. flavoviride*. *Mycopathologia,* 137**,** 37-43.

OUEDRAOGO, R. M., CUSSON, M., GOETTEL, M. S. & BRODEUR, J. 2003. Inhibition of fungal growth in thermoregulating locusts, *Locusta migratoria*, infected by the fungus *Metarhizium anisopliae* var acridum. *Journal of invertebrate pathology,* 82**,** 103-109.

OWNLEY, B. H., GRIFFIN, M. R., KLINGEMAN, W. E., GWINN, K. D., MOULTON, J. K. & PEREIRA, R. M. 2008. *Beauveria bassiana*: endophytic colonization and plant disease control. *Journal of invertebrate pathology,* 98**,** 267-270.

PAL, S., ST LEGER, R. J. & WU, L. P. 2007. Fungal peptide Destruxin A plays a specific role in suppressing the innate immune response in *Drosophila melanogaster*. *The Journal of biological chemistry,* 282**,** 8969-77.

PALOMARES, L. A., LÓPEZ, S. & RAMÍREZ, O. T. 2004. Utilization of oxygen uptake rate to assess the role of glucose and glutamine in the metabolism of infected insect cell cultures. *Biochemical Engineering Journal,* 19**,** 87-93.

PATERSON, R., SIMMONDS, M. & BLANEY, W. 1987. Mycopesticidal effects of characterized extracts of Penicillium isolates and purified secondary metabolites (including mycotoxins) on *Drosophila melanogaster* and *Spodoptora littoralis*. *Journal of invertebrate pathology,* 50**,** 124-133.

PEL, H. J., DE WINDE, J. H., ARCHER, D. B., DYER, P. S., HOFMANN, G., SCHAAP, P. J., TURNER, G., DE VRIES, R. P., ALBANG, R. & ALBERMANN, K. 2007. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nature biotechnology,* 25**,** 221-231.

PENDLAND, J. & BOUCIAS, D. 1997. In vitro growth of the entomopathogenic hyphomycete *Nomuraea rileyi*. *Mycologia***,** 66-71.

PENDLAND, J., HUNG, S. & BOUCIAS, D. 1993. Evasion of host defense by in vivo-produced protoplast-like cells of the insect mycopathogen *Beauveria bassiana*. *Journal of bacteriology,* 175**,** 5962-5969.

PENG, G., WANG, Z., YIN, Y., ZENG, D. & XIA, Y. 2008. Field trials of *Metarhizium anisopliae* var. acridum (Ascomycota: Hypocreales) against oriental migratory locusts, *Locusta migratoria manilensis* (Meyen) in Northern China. *Crop Protection,* 27**,** 1244-1250.

PERRIN, R. 1997. Crop protection: taking stock for the new millennium. *Crop protection,* 16**,** 449-456.

PETCH, T. 1935. Notes on entomogenous fungi. *Transactions of the British mycological Society,* 19**,** 161-194.

PETERSEN, A. J., KATZENBERGER, R. J. & WASSARMAN, D. A. 2013. The innate immune response transcription factor relish is necessary for neurodegeneration in a Drosophila model of ataxia-telangiectasia. *Genetics,* 194**,** 133-142.

PHILLIPS, J., GRAVES, J. & LUTTRELL, R. 1989. Insecticide resistance management: relationship to integrated pest management. *Pest Management Science,* 27**,** 459-464.

PILI-FLOURY, S., LEULIER, F., TAKAHASHI, K., SAIGO, K., SAMAIN, E., UEDA, R. & LEMAITRE, B. 2004. In vivo RNAi analysis reveals an unexpected role for GNBP1 in the defense against Gram-positive bacterial infection in Drosophila adults. *Journal of Biological Chemistry*, 279, 12848-12853.

PIMENTEL, D. 1996. Green revolution agriculture and chemical hazards. *Science of the total environment,* 188**,** S86-S98.

PIMENTEL, D., STACHOW, U., TAKACS, D. A., BRUBAKER, H. W., DUMAS, A. R., MEANEY, J. J., ONSI, D. E. & CORZILIUS, D. B. 1992. Conserving biological diversity in agricultural/forestry systems. *BioScience,* 42**,** 354-362.

PONTICELLI, C., MINETTI, L., DI, F. P., VEGETO, A., BELLI, L., CORBETTA, G., TARANTINO, A. & CIVATI, G. 1988. The Milan clinical trial with cyclosporine in cadaveric renal transplantation. A three-year follow-up. *Transplantation,* 45**,** 908-913.

POPP, J., PETŐ, K. & NAGY, J. 2013. Pesticide productivity and food security. A review. *Agronomy for sustainable development,* 33**,** 243-255.

POSADA, F. & VEGA, F. E. 2005. Establishment of the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) as an endophyte in cocoa seedlings (*Theobroma cacao*). *Mycologia,* 97**,** 1195-1200.

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

PREM ANAND, A. A., VENNISON, S. J., SANKAR, S. G., GILWAX PRABHU, D. I., VASAN, P. T., RAGHURAMAN, T., JEROME GEOFFREY, C. & VENDAN, S. E. 2010. Isolation and characterization of bacteria from the gut of *Bombyx mori* that degrade cellulose, xylan, pectin and starch and their impact on digestion. *Journal of Insect Science,* 10**,** 107.

PRETTY, J. 2008. Agricultural sustainability: concepts, principles and evidence. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* 363**,** 447-465.

PRETTY, J. & BHARUCHA, Z. P. 2014. Sustainable intensification in agricultural systems. *Annals of botany,* 114**,** 1571-1596.

PURWAR, J. & SACHAN, G. 2006. Synergistic effect of entomogenous fungi on some insecticides against Bihar hairy caterpillar *Spilarctia obliqua* (Lepidoptera: Arctiidae). *Microbiological Research,* 161**,** 38-42.

QAZI, S. S. & KHACHATOURIANS, G. G. 2007. Hydrated conidia of *Metarhizium anisopliae* release a family of metalloproteases. *Journal of invertebrate pathology,* 95**,** 48-59.

QUESADA-MORAGA, E., CARRASCO-DIAZ, J. A. & SANTIAGO-ALVAREZ, C. 2006. Insecticidal and antifeedant activities of proteins secreted by entomopathogenic fungi against *Spodoptera littoralis* (Lep., Noctuidae). *Journal of Applied Entomology,* 130**,** 442-452.

QUESADA-MORAGA, E., NAVAS-CORTÉS, J. A., MARANHAO, E. A., ORTIZ-URQUIZA, A. & SANTIAGO-ÁLVAREZ, C. 2007. Factors affecting the occurrence and distribution of entomopathogenic fungi in natural and cultivated soils. *Mycological research,* 111**,** 947-966.

QUINTELA, E. D. & MCCOY, C. W. 1998. Synergistic effect of imidacloprid and two entomopathogenic fungi on the behavior and survival of larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in soil. *Journal of Economic Entomology,* 91**,** 110-122.

RAMARAO, N., NIELSEN-LEROUX, C. & LERECLUS, D. 2012. The insect *Galleria mellonella* as a powerful infection model to investigate bacterial pathogenesis. *Journal of visualized experiments: JoVE*, e4392.

RAMESH, T., YOO, S.-K., KIM, S.-W., HWANG, S.-Y., SOHN, S.-H., KIM, I.-W. & KIM, S.-K. 2012. Cordycepin (3'-deoxyadenosine) attenuates age-related oxidative stress and ameliorates antioxidant capacity in rats. *Experimental gerontology,* 47**,** 979-987.

RÄMET, M., PEARSON, A., MANFRUELLI, P., LI, X., KOZIEL, H., GÖBEL, V., CHUNG, E., KRIEGER, M. & EZEKOWITZ, R. A. B. 2001. Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria. *Immunity,* 15**,** 1027-1038.

REES, J. A., MONIATTE, M. & BULET, P. 1997. Novel antibacterial peptides isolated from a European bumblebee, *Bombus pascuorum* (Hymenoptera, Apoidea). *Insect biochemistry and molecular biology,* 27**,** 413-422.

REEVES, E. P., MESSINA, C., DOYLE, S. & KAVANAGH, K. 2004. Correlation between gliotoxin production and virulence of *Aspergillus fumigatus* in *Galleria mellonella*. *Mycopathologia,* 158**,** 73-79.

REGEL, R., MATIOLI, S. R. & TERRA, W. R. 1998. Molecular adaptation of *Drosophila melanogaster* lysozymes to a digestive function. *Insect biochemistry and molecular biology,* 28**,** 309-319.

REHNER, S. A. & BUCKLEY, E. 2005. A Beauveria phylogeny inferred from nuclear ITS and EF1-α sequences: evidence for cryptic diversification and links to Cordyceps teleomorphs. *Mycologia,* 97**,** 84-98.

REN, Z., CUI, J., HUO, Z., XUE, J., CUI, H., LUO, B., JIANG, L. & YANG, R. 2012. Cordycepin suppresses TNF-α-induced NF-κB activation by reducing p65 transcriptional activity, inhibiting IκBα phosphorylation, and blocking IKKγ ubiquitination. *International immunopharmacology,* 14**,** 698-703.

RÍOS-MORENO, A., GARRIDO-JURADO, I., RAYA-ORTEGA, M. & QUESADA-MORAGA, E. 2017. Quantification of fungal growth and destruxin A during infection of *Galleria mellonella* larvae by *Metarhizium brunneum*. *Journal of invertebrate pathology,* 149**,** 29-35.

RITZ, C., BATY, F., STREIBIG, J. C. & GERHARD, D. 2015. Dose-response analysis using R. *PloS one,* 10**,** e0146021.

ROBERTS, D. W. 1981. Toxins of entomopathogenic fungi. Microbial control of pests and plant diseases 1970-1980. *Academic Press, London***,** 441-465.

ROBERTS, D. W. & ST LEGER, R. J. 2004. *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: mycological aspects. *Advances in applied microbiology,* 54**,** 1-70.

ROBINSON, R. A. & SUTHERLAND, W. J. 2002. Post-war changes in arable farming and biodiversity in Great Britain. *Journal of applied Ecology,* 39**,** 157-176.

ROLFF, J. & REYNOLDS, S. 2009. (Eds) *.Insect infection and immunity: evolution, ecology, and mechanisms*, (No. 25) Publ. Oxford university press, Oxford, UK.

ROLFF, J. & SCHMID-HEMPEL, P. 2016. Perspectives on the evolutionary ecology of arthropod antimicrobial peptides. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* 371**,** 20150297.

ROPER, M., ELLISON, C., TAYLOR, J. W. & GLASS, N. L. 2011. Nuclear and genome dynamics in multinucleate ascomycete fungi. *Current biology,* 21**,** R786-R793.

ROSSONI, R. D., FUCHS, B. B., DE BARROS, P. P., DOS SANTOS VELLOSO, M., JORGE, A. O. C., JUNQUEIRA, J. C. & MYLONAKIS, E. 2017. *Lactobacillus paracasei* modulates the immune system of *Galleria mellonella* and protects against *Candida albicans* infection. *PloS one,* 12**,** e0173332.

ROTTMAN, F. & GUARINO, A. J. 1964. Studies on the inhibition of *Bacillus subtilis* growth by cordycepin. *Biochimica et Biophysica Acta (BBA)-Specialized Section on Nucleic Acids and Related Subjects,* 80**,** 632-639.

ROY, H. E., VEGA, F. E., CHANDLER, D., GOETTEL, M. S., PELL, J. & WAJNBERG, E. (Eds.) 2010. *The ecology of fungal entomopathogens*, Publ. Springer, New York.

RSTUDIO, T. 2017. RStudio: Integrated Development for R.(2015). *RStudio, Inc., Boston, MA. URL http://www. rstudio. com*. [Accessed, JAN 2017].

RUTSCHMANN, S., JUNG, A. C., HETRU, C., REICHHART, J.-M., HOFFMANN, J. A. & FERRANDON, D. 2000. The Rel protein DIF mediates the antifungal but not the antibacterial host defense in Drosophila. *Immunity,* 12**,** 569-580.

RYU, E., SON, M., LEE, M., LEE, K., CHO, J. Y., CHO, S., LEE, S. K., LEE, Y. M., CHO, H. & SUNG, G.-H. 2014. Cordycepin is a novel chemical suppressor of Epstein-Barr virus replication. *Oncoscience,* 1**,** 866-881.

RYU, J.-H., KIM, S.-H., LEE, H.-Y., BAI, J. Y., NAM, Y.-D., BAE, J.-W., LEE, D. G., SHIN, S. C., HA, E.-M. & LEE, W.-J. 2008. Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in Drosophila. *Science,* 319**,** 777-782.

RYU, J. H., HA, E. M., OH, C. T., SEOL, J. H., BREY, P. T., JIN, I., LEE, D. G., KIM, J., LEE, D. & LEE, W. J. 2006. An essential complementary role of NF-κB pathway to microbicidal oxidants in Drosophila gut immunity. *The EMBO journal,* 25**,** 3693-3701.

SAMUELS, R, REYNOLDS,S & CHARNLEY, A. 1988. Calcium channel activation of insect muscle by destruxins, insecticidal compounds produced by the entomopathogenic fungus *Metarhizium anisopliae*. *Comparative biochemistry and physiology*, 90(2) 403-412.

SARFRAZ, M., KEDDIE, A. B. & DOSDALL, L. M. 2005. Biological control of the diamondback moth, *Plutella xylostella*: a review. *Biocontrol Science and Technology,* 15**,** 763-789.

SASAN, R. K. & BIDOCHKA, M. J. 2012. The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. *American journal of botany,* 99**,** 101-107.

SASS, M., KISS, A. & LOCKE, M. 1994. Integument and hemocyte peptides. *Journal of insect physiology,* 40**,** 407-421.

SBARAINI, N., GUEDES, R. L. M., ANDREIS, F. C., JUNGES, Â., DE MORAIS, G. L., VAINSTEIN, M. H., DE VASCONCELOS, A. T. R. & SCHRANK, A. 2016. Secondary metabolite gene clusters in the entomopathogen fungus *Metarhizium anisopliae*: genome identification and patterns of expression in a cuticle infection model. *BMC genomics,* 17**,** 736.

SCHNEIDER, C. A., RASBAND, W. S. & ELICEIRI, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature methods,* 9**,** 671-675.

SCHNEIDER, I. 1972. Cell lines derived from late embryonic stages of *Drosophila melanogaster*. *Development,* 27**,** 353-365.

SCHOCH, C. L., SEIFERT, K. A., HUHNDORF, S., ROBERT, V., SPOUGE, J. L., LEVESQUE, C. A., CHEN, W., BOLCHACOVA, E., VOIGT, K. & CROUS, P. W. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences,* 109**,** 6241-6246.

SCHUHMANN, B., SEITZ, V., VILCINSKAS, A. & PODSIADLOWSKI, L. 2003. Cloning and expression of gallerimycin, an antifungal peptide expressed in immune response of greater wax moth larvae, *Galleria mellonella*. *Archives of insect biochemistry and physiology,* 53**,** 125-133.

SEIBER, J. N., COATS, J., DUKE, S. O. & GROSS, A. D. 2014. Biopesticides : State of the Art and Future Opportunities. *Journal of agricultural and food chemistry,* 62**,** 11613–11619.

SEONG, K.-Y., ZHAO, X., XU, J.-R., GÜLDENER, U. & KISTLER, H. C. 2008. Conidial germination in the filamentous fungus *Fusarium graminearum*. *Fungal Genetics and Biology,* 45**,** 389-399.

SEYED-TALEBI, F.-S., KHERADMAND, K., TALAEI-HASSANLOUI, R. & TALEBI-JAHROMI, K. 2014. Synergistic effect of *Beauveria bassiana* and spirodiclofen on the two-spotted spider mite (*Tetranychus urticae*). *Phytoparasitica,* 42**,** 405-412.

SHAH, P. A. & PELL, J. K. 2003. Entomopathogenic fungi as biological control agents. *Applied Microbiology and Biotechnology,* 61**,** 413-423.

SHANG, Y., DUAN, Z., HUANG, W., GAO, Q. & WANG, C. 2012. Improving UV resistance and virulence of *Beauveria bassiana* by genetic engineering with an exogenous tyrosinase gene. *Journal of invertebrate pathology,* 109**,** 105-109.

SHAPIRO-ILAN, D. I., JACKSON, M., REILLY, C. C. & HOTCHKISS, M. W. 2004. Effects of combining an entomopathogenic fungi or bacterium with entomopathogenic nematodes on mortality of *Curculio caryae* (Coleoptera: Curculionidae). *Biological Control,* 30**,** 119-126.

SHELTON, A. 2004. Management of the diamondback moth: déjà vu all over again. N. Endersby & P. Ridland (Eds.). *Proceedings: The management of diamondback moth and other crucifer pests*. The Fourth International Workshop, 26-29 November 2001, Melbourne, Australia. p. 3-8.

SHI, W.-B. & FENG, M.-G. 2006. Field efficacy of application *of Beauveria bassiana* formulation and low rate pyridaben for sustainable control of citrus red mite *Panonychus citri* (Acari: Tetranychidae) in orchards. *Biological Control,* 39**,** 210-217.

SHIA, A. K., GLITTENBERG, M., THOMPSON, G., WEBER, A. N., REICHHART, J.-M. & LIGOXYGAKIS, P. 2009. Toll-dependent antimicrobial responses in Drosophila larval fat body require Spätzle secreted by haemocytes. *Journal of Cell Science,* 122**,** 4505-4515.

SHIMANUKI, H. 1981. *Controlling the greater wax moth: a pest of honeycombs* (No 2217) , US Department of Agriculture Farmers' Bulletin 2217, Washington DC, p. 12.

SHIN, S., PARK, Y., KIM, S., OH, H.-E., KO, Y.-W., HAN, S., LEE, S., LEE, C.-K., CHO, K. & KIM, K. 2010. *Cordyceps militaris* enhances MHC-restricted antigen presentation via the induced expression of MHC molecules and production of cytokines. *Immune network,* 10**,** 135-143.

SHIN, S. C., KIM, S.-H., YOU, H., KIM, B., KIM, A. C., LEE, K.-A., YOON, J.-H., RYU, J.-H. & LEE, W.-J. 2011. Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science,* 334**,** 670-674.

SHINOMIYA, N., GAO, C. F., XIE, Q., GUSTAFSON, M., WATERS, D. J., ZHANG, Y.-W. & WOUDE, G. F. V. 2004. RNA interference reveals that ligand-independent met activity is required for tumor cell signaling and survival. *Cancer research,* 64**,** 7962-7970.

SHRESTHA, B., HAN, S.-K., SUNG, J.-M. & SUNG, G.-H. 2012a. Fruiting body formation of *Cordyceps militaris* from multi-ascospore isolates and their single ascospore progeny strains. *Mycobiology,* 40**,** 100-106.

SHRESTHA, B., KIM, H.-K., SUNG, G.-H., SPATAFORA, J. W. & SUNG, J.-M. 2004. Bipolar heterothallism, a principal mating system of *Cordyceps militaris* in vitro. *Biotechnology and Bioprocess Engineering,* 9**,** 440-446.

SHRESTHA, B., ZHANG, W. & ZHANG, Y. 2012b. The medicinal fungus *Cordyceps militaris* : research and development. *Mycological Progress*, 11, 599-614.

SOUKUP, A. A., KELLER, N. P. & WIEMANN, P. 2016. Enhancing nonribosomal peptide biosynthesis in filamentous fungi. *Methods in Molecular Biology,* 1401, 149-160.

SOWA-JASIŁEK, A., ZDYBICKA-BARABAS, A., STĄCZEK, S., WYDRYCH, J., SKRZYPIEC, K., MAK, P., DERYŁO, K., TCHÓRZEWSKI, M. & CYTRYŃSKA, M. 2016. *Galleria mellonella* lysozyme induces apoptotic changes in *Candida albicans* cells. *Microbiological research,* 193**,** 121-131.

SPARKS, T. C. & NAUEN, R. 2015. IRAC: Mode of action classification and insecticide resistance management. *Pesticide Biochemistry and Physiology,* 121**,** 122-128.

STERGIOPOULOS, I. & DE WIT, P. J. 2009. Fungal effector proteins. *Annual review of phytopathology,* 47**,** 233-263.

STÖVEN, S., ANDO, I., KADALAYIL, L., ENGSTRÖM, Y. & HULTMARK, D. 2000. Activation of the Drosophila NF-κB factor Relish by rapid endoproteolytic cleavage. *EMBO reports,* 1**,** 347-352.

STÖVEN, S., SILVERMAN, N., JUNELL, A., HEDENGREN-OLCOTT, M., ERTURK, D., ENGSTRÖM, Y., MANIATIS, T. & HULTMARK, D. 2003. Caspase-mediated processing of the Drosophila NF-κB factor Relish. *Proceedings of the National Academy of Sciences,* 100**,** 5991-5996.

STRAND, M. R. 2008. The insect cellular immune response. *Insect Science,* 15**,** 1-14.

STRAND, M. R., BECK, M. H., LAVINE, M. D. & CLARK, K. D. 2006. Microplitis demolitor bracovirus inhibits phagocytosis by hemocytes from *Pseudoplusia includens*. *Archives of insect biochemistry and physiology,* 61**,** 134-145.

SUGAR, A. M. & MCCAFFREY, R. P. 1998. Antifungal activity of 3'-deoxyadenosine (cordycepin). *Antimicrobial agents and chemotherapy,* 42**,** 1424-1427.

SUGAVANAM, B. 1996. Risk reduction in pesticide development in developing countries-challenges and opportunities. *Journal of Environmental Science & Health Part B,* 31**,** 307-323.

SUN, M., REN, Q., LIU, Z., GUAN, G., GOU, H., MA, M., LI, Y., LIU, A., YANG, J. & YIN, H. 2011. *Beauveria bassiana*: Synergistic effect with acaricides against the tick *Hyalomma anatolicum anatolicum* (Acari: Ixodidae). *Experimental parasitology,* 128**,** 192-195.

SUNG, G-H., HYWEL-JONES, N., SUNG, J-M., LUANGSA-ARD, J., SHRESTHA, B. & SPATAFORA, J. 2007. Phylogenetic classification of Cordyceps and the clavicipitaceous fungi. *Studies in Mycology,* 57, 5-59.

SUZUKI, A., KANAOKA, M., ISOGAI, A., TAMURA, S., MURAKOSHI, S. & ICHINOE, M. 1977. Bassianolide, a new insecticidal cyclodepsipeptide from *Beauveria bassiana* and *Verticillium lecanii*. *Tetrahedron Letters,* 18**,** 2167-2170.

SUZUKI, A., KUYAMA, S., KDAIRA, Y. & TAMURA, S. 1966. Structural elucidation of destruxin A. *Agricultural and Biological Chemistry,* 30**,** 517-518.

SWARUP, S. & VERHEYEN, E. M. 2012. Wnt/wingless signaling in Drosophila. *Cold Spring Harbor perspectives in biology***,** 4.

TABASHNIK, B. E. 1994. Evolution of resistance to *Bacillus thuringiensis*. *Annual review of entomology,* 39**,** 47-79.

TAK, P. P. & FIRESTEIN, G. S. 2001. NF-κB: a key role in inflammatory diseases. *The Journal of clinical investigation,* 107**,** 7-11.

TAKEDA, K. & AKIRA, S. 2004. TLR signaling pathways. *Seminars in immunology*, 16, 3-9.

TAKEHANA, A., YANO, T., MITA, S., KOTANI, A., OSHIMA, Y. & KURATA, S. 2004. Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in Drosophila immunity. *The EMBO journal,* 23**,** 4690-4700.

TAMURA, K., STECHER, G., PETERSON, D., FILIPSKI, A. & KUMAR, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution,* 30**,** 2725-2729.

TANABE, S. 2002. Contamination and toxic effects of persistent endocrine disrupters in marine mammals and birds. *Marine pollution bulletin,* 45**,** 69-77.

TANADA, Y. & KAYA, H. K. 2012. *Insect pathology*, Academic press, San Diego, USA.

TANAKA, H., ISHIBASHI, J., FUJITA, K., NAKAJIMA, Y. & SAGISAKA, A. 2008. A genome-wide analysis of genes and gene families involved in innate immunity of *Bombyx mori*. *Insect Biochemistry and Molecular Biology,* 38**,** 1087-1110.

TANJI, T., YUN, E.-Y. & IP, Y. T. 2010. Heterodimers of NF-κB transcription factors DIF and Relish regulate antimicrobial peptide genes in Drosophila. *Proceedings of the National Academy of Sciences,* 107**,** 14715-14720.

TAPON, N., ITO, N., DICKSON, B. J., TREISMAN, J. E. & HARIHARAN, I. K. 2001. The Drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. *Cell,* 105**,** 345-355.

TAUSZIG, S., JOUANGUY, E., HOFFMANN, J. A. & IMLER, J.-L. 2000. Toll-related receptors and the control of antimicrobial peptide expression in Drosophila. *Proceedings of the National Academy of Sciences,* 97**,** 10520-10525.

TFELT-HANSEN, P., SAXENA, P., DAHLÖF, C., PASCUAL, J., LAINEZ, M., HENRY, P., DIENER, H.-C., SCHOENEN, J., FERRARI, M. & GOADSBY, P. 2000. Ergotamine in the acute treatment of migraine: a review and European consensus. *Brain,* 123**,** 9-18.

THOMAS, M. B. & BLANFORD, S. 2003. Thermal biology in insect-parasite interactions. *Trends in Ecology & Evolution,* 18**,** 344-350.

THOMAS, M. B., WOOD, S. N. & LOMER, C. J. 1995. Biological control of locusts and grasshoppers using a fungal pathogen: the importance of secondary cycling. *Proceedings of the Royal Society B: Biological Sciences,* 259**,** 265-270.

TIANZHU, Z., SHIHAI, Y. & JUAN, D. 2014. Antidepressant-like effects of cordycepin in a mice model of chronic unpredictable mild stress. *Evidence-Based Complementary and Alternative Medicine,* 438506.

TILMAN, D. 1998. The greening of the green revolution. *Nature,* 396**,** 211-212.

TILMAN, D., BALZER, C., HILL, J. & BEFORT, B. L. 2011. Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences,* 108**,** 20260-20264.

TILMAN, D., CASSMAN, K. G., MATSON, P. A., NAYLOR, R. & POLASKY, S. 2002. Agricultural sustainability and intensive production practices. *Nature,* 418**,** 671.

TRIGG, P., GUTTERIDGE, W. & WILLIAMSON, J. 1971. The effects of cordycepin on malaria parasites. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 65**,** 514-520.

TSCHARNTKE, T., CLOUGH, Y., WANGER, T. C., JACKSON, L., MOTZKE, I., PERFECTO, I., VANDERMEER, J. & WHITBREAD, A. 2012. Global food security, biodiversity conservation and the future of agricultural intensification. *Biological conservation,* 151**,** 53-59.

TULI, H., SHARMA, A., SANDHU, S., KASHYAP., D. 2013. Cordycepin: a bioactive metabolite with therapeutic potential. *Life Sciences,* 93(23), 863-869.

TZOU, P., OHRESSER, S., FERRANDON, D., CAPOVILLA, M., REICHHART, J.-M., LEMAITRE, B., HOFFMANN, J. A. & IMLER, J.-L. 2000. Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia. *Immunity,* 13**,** 737-748.

UNITED NATIONS. 2015. World Population Prospects, the 2015 revision. https://population.un.org/wpp/. [Accessed August 2018]

U.S DEPARTMENT OF AGRICULTURE 2016. Agricultural Research Service, Biological Integrated Pest management Research Unit. *ARS Collection of Entomopathogenic Fungal Cultures (ARSEF)*. U.S. Department of Agriculture, Agricultural Research Service

VALANNE, S., WANG, J. H. & RAMET, M. 2011. The Drosophila Toll Signaling Pathway. *The Journal of Immunology,* 186**,** 649-656.

VALLET-GELY, I., LEMAITRE, B. & BOCCARD, F. 2008. Bacterial strategies to overcome insect defences. *Nature Reviews Microbiology,* 6**,** 302-313.

VANDENBERG, J., SHELTON, A., WILSEY, W. & RAMOS, M. 1998a. Assessment of *Beauveria bassiana* sprays for control of diamondback moth (Lepidoptera: Plutellidae) on crucifers. *Journal of Economic Entomology,* 91**,** 624-630.

VANDENBERG, J. D., RAMOS, M. & ALTRE, J. A. 1998b. Dose–response and age-and temperature-related susceptibility of the diamondback moth (Lepidoptera: Plutellidae) to two isolates of *Beauveria bassiana* (Hyphomycetes: Moniliaceae). *Environmental Entomology,* 27**,** 1017-1021.

VEGA, F. E., MEYLING, N. V., LUANGSA-ARD, J. J. & BLACKWELL, M. 2012. Fungal entomopathogens. F. Vega & H. Kaya (Eds.). *Insect Pathology*. Publ. Academic, San Diego, USA.

VERTYPOROKH, L. & WOJDA, I. 2017. Expression of the insect metalloproteinase inhibitor IMPI in the fat body of *Galleria mellonella* exposed to infection with *Beauveria bassiana*. *Acta Biochimica Polonica,* 64**,** 273-278.

VILCINSKAS, A., JEGOROV, A., LANDA, Z., GÖTZ, P. & MATHA, V. 1999. Effects of beauverolide L and cyclosporin A on humoral and cellular immune response of the greater wax moth, *Galleria mellonella*. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology,* 122**,** 83-92.

VILCINSKAS, A. & MATHA, V. 1997a. Effect of the entomopathogenic fungus *Beauveria bassiana* on the humoral immune response of *Galleria mellonella* larvae (Lepidoptera: Pyralidae). *European Journal of Entomology,* 94**,** 461-472.

VILCINSKAS, A., MATHA, V. & GÖTZ, P. 1997b. Effects of the entomopathogenic fungus *Metarhizium anisopliae* and its secondary metabolites on morphology and cytoskeleton of plasmatocytes isolated from the greater wax moth, *Galleria mellonella*. *Journal of Insect Physiology,* 43**,** 1149-1159.

VILCINSKAS, A., MATHA, V. & GÖTZ, P. 1997c. Inhibition of phagocytic activity of plasmatocytes isolated from *Galleria mellonella* by entomogenous fungi and their secondary metabolites. *Journal of Insect Physiology,* 43**,** 475-483.

VILGALYS, R. & HESTER, M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. *Journal of bacteriology,* 172**,** 4238-4246.

VILMOS, P. & KURUCZ, E. 1998. Insect immunity: evolutionary roots of the mammalian innate immune system. *Immunology letters,* 62**,** 59-66.

VINCENT, J. & WEGST, U. 2004. Design and mechanical properties of the insect cuticle. *Arthropod strucutre & development*, 33(3), 187-199.

VOGEL, H., ALTINCICEK, B., GLÖCKNER, G. & VILCINSKAS, A. 2011. A comprehensive transcriptome and immune- gene repertoire of the lepidopteran model host *Galleria mellonella*. *BMC Genomics,* 12, 308

VOS, H. 1953. Introduction in Indonesia of *Angitia cerophaga* Grav., a parasitoid of *Plutella maculipennis*. *Contributions of the General Agricultural Research Station, Bogor, Indonesia,* 134, 1-32.

WANG, C. & LEGER, R. J. S. 2006. A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses. *Proceedings of the National Academy of Sciences,* 103**,** 6647-6652.

WANG, X.-H., ALIYARI, R., LI, W.-X., LI, H.-W., KIM, K., CARTHEW, R., ATKINSON, P. & DING, S.-W. 2006. RNA interference directs innate immunity against viruses in adult Drosophila. *Science,* 312**,** 452-454.

WARTON, D. I. & HUI, F. K. 2011. The arcsine is asinine: the analysis of proportions in ecology. *Ecology,* 92**,** 3-10.

WEBER, G., SCHÖRGENDORFER, K., SCHNEIDER-SCHERZER, E. & LEITNER, E. 1994. The peptide synthetase catalyzing cyclosporine production in *Tolypocladium niveum* is encoded by a giant 45.8-kilobase open reading frame. *Current genetics,* 26**,** 120-125.

WEDDE, M., WEISE, C., KOPACEK, P., FRANKE, P. & VILCINSKAS, A. 1998. Purification and characterization of an inducible metalloprotease inhibitor from the hemolymph of greater wax moth larvae, *Galleria mellonella*. *The FEBS Journal,* 255**,** 535-543.

WEDDE, M., WEISE, C., NUCK, R., ALTINCICEK, B. & VILCINSKAS, A. 2007. The insect metalloproteinase inhibitor gene of the lepidopteran *Galleria mellonella* encodes two distinct inhibitors. *Biological Chemistry,* 388**,** 119-127.

WEISBURG, W., BARNS, S., PELLETIER, D. & LANE, D. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology,* 173, 697-703.

WHITE, T. J., BRUNS, T., LEE, S. & TAYLOR, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications,* 18**,** 315-322.

WICKHAM, H. 2016. *ggplot2: elegant graphics for data analysis*. Publ. Springer, New York.

WILLIAMS, M. J. 2007. Drosophila hemopoiesis and cellular immunity. *The Journal of Immunology,* 178**,** 4711-4716.

WILLIAMSON, E. M. 2001. Synergy and other interactions in phytomedicines. *Phytomedicine : international journal of phytotherapy and phytopharmacology,* 8**,** 401-409.

WOJDA, I. & JAKUBOWICZ, T. 2007. Humoral immune response upon mild heat-shock conditions in *Galleria mellonella* larvae. *Journal of insect physiology,* 53**,** 1134-1144.

WOJDA, I., KOWALSKI, P. & JAKUBOWICZ, T. 2009. Humoral immune response of *Galleria mellonella* larvae after infection by *Beauveria bassiana* under optimal and heat-shock conditions. 55**,** 525-531.

WONG, J. H., NG, T. B., WANG, H., SZE, S. C. W., ZHANG, K. Y., LI, Q. & LU, X. 2011. Cordymin, an antifungal peptide from the medicinal fungus *Cordyceps militaris*. *Phytomedicine,* 18**,** 387-392.

WOOD, T. J. & GOULSON, D. 2017. The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013. *Environmental Science and Pollution Research,* 24**,** 17285-17325.

WRAIGHT, S., CARRUTHERS, R., JARONSKI, S., BRADLEY, C., GARZA, C. & GALAINI-WRAIGHT, S. 2000. Evaluation of the entomopathogenic fungi *Beauveria bassiana* and *Paecilomyces fumosoroseus* for microbial control of the silverleaf whitefly, *Bemisia argentifolii*. *Biological Control,* 17**,** 203-217.

WRAIGHT, S. & RAMOS, M. 2005. Synergistic interaction between *Beauveria bassiana* and *Bacillus thuringiensis* tenebrionis based biopesticides applied against field populations of Colorado potato beetle larvae. *Journal of Invertebrate Pathology,* 90**,** 139-150.

WRAIGHT, S., RAMOS, M., AVERY, P., JARONSKI, S. & VANDENBERG, J. 2010. Comparative virulence of *Beauveria bassiana* isolates against lepidopteran pests of vegetable crops. *Journal of Invertebrate Pathology,* 103**,** 186-199.

WU, T., SAMARANAYAKE, L., LEUNG, W. & SULLIVAN, P. 1999. Inhibition of growth and secreted aspartyl proteinase production in *Candida albicans* by lysozyme. *Journal of medical microbiology,* 48**,** 721-730.

WU, W.-C., HSIAO, J.-R., LIAN, Y.-Y., LIN, C.-Y. & HUANG, B.-M. 2007. The apoptotic effect of cordycepin on human OEC-M1 oral cancer cell line. *Cancer Chemotherapy and Pharmacology,* 60**,** 103-111.

WÜNN, J., KLÖTI, A., BURKHARDT, P. K., BISWAS, G. C. G., LAUNIS, K., IGLESIAS, V. A. & POTRYKUS, I. 1996. Transgenic indica rice breeding line IR58 expressing a synthetic crylA (b) gene from *Bacillus thuringiensis* provides effective insect pest control. *Nature Biotechnology,* 14**,** 171-176.

XIA, X., YU, L., XUE, M., YU, X., VASSEUR, L., GURR, G. M., BAXTER, S. W., LIN, H., LIN, J. & YOU, M. 2015. Genome-wide characterization and expression profiling of immune genes in the diamondback moth, *Plutella xylostella* (L.). *Scientific Reports,* 5**,** 9877-9877.

XIA, X., ZHENG, D., ZHONG, H., QIN, B., GURR, G. M., VASSEUR, L., LIN, H., BAI, J., HE, W. & YOU, M. 2013. DNA sequencing reveals the midgut microbiota of diamondback moth, *Plutella xylostella* (L.) and a possible relationship with insecticide resistance. *PLoS One,* 8**,** e68852.

XIA, Y., LUO, F., SHANG, Y., CHEN, P., LU, Y. & WANG, C. 2017. Fungal Cordycepin Biosynthesis Is Coupled with the Production of the Safeguard Molecule Pentostatin. *Cell chemical biology,* 24**,** 1479-1489. e4.

XIANG, L., LI, Y., ZHU, Y., LUO, H., LI, C., XU, X., SUN, C., SONG, J., SHI, L., HE, L., SUN, W. & CHEN, S. 2014. Transcriptome analysis of the *Ophiocordyceps sinensis* fruiting body reveals putative genes involved in fruiting body development and cordycepin biosynthesis. *Genomics,* 103**,** 154-159.

XIAO, G., YING, S.-H., ZHENG, P., WANG, Z.-L., ZHANG, S., XIE, X.-Q., SHANG, Y., LEGER, R. J. S., ZHAO, G.-P. & WANG, C. 2012. Genomic perspectives on the evolution of fungal entomopathogenicity in *Beauveria bassiana*. *Scientific reports,* 2**,** 483.

XU, Y., OROZCO, R., WIJERATNE, E. K., ESPINOSA-ARTILES, P., GUNATILAKA, A. L., STOCK, S. P. & MOLNÁR, I. 2009. Biosynthesis of the cyclooligomer depsipeptide bassianolide, an insecticidal virulence factor of *Beauveria bassiana*. *Fungal Genetics and Biology,* 46**,** 353-364.

XU, Y., OROZCO, R., WIJERATNE, E. K., GUNATILAKA, A. L., STOCK, S. P. & MOLNÁR, I. 2008. Biosynthesis of the cyclooligomer depsipeptide beauvericin, a virulence factor of the entomopathogenic fungus *Beauveria bassiana*. *Chemistry & biology,* 15**,** 898-907.

YANAGAWA, S.-I., LEE, J.-S. & ISHIMOTO, A. 1998. Identification and Characterization of a Novel Line of Drosophila Schneider S2 Cells That Respond to Wingless Signaling. *Journal of Biological Chemistry,* 273**,** 32353-32359.

YANG, F. & LI, S. 2008. Effects of sample preparation methods on the quantification of nucleosides in natural and cultured Cordyceps. *Journal of pharmaceutical and biomedical analysis,* 48**,** 231-235.

YANG, X., LI, Y., HE, Y., LI, T., WANG, W., ZHANG, J., WEI, J., DENG, Y. & LIN, R. 2015. Cordycepin alleviates airway hyperreactivity in a murine model of asthma by attenuating the inflammatory process. *International immunopharmacology,* 26**,** 401-408.

YE, J., COULOURIS, G., ZARETSKAYA, I., CUTCUTACHE, I., ROZEN, S. & MADDEN, T. L. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC bioinformatics,* 13**,** 134.

YEO, H., PELL, J. K., ALDERSON, P. G., CLARK, S. J. & PYE, B. J. 2003. Laboratory evaluation of temperature effects on the germination and growth of entomopathogenic fungi and on their pathogenicity to two aphid species. *Pest Management Science,* 59**,** 156-165.

YI, H. Y., CHOWDHURY, M., HUANG, Y. D. & YU, X. Q. 2014. Insect antimicrobial peptides and their applications. *Applied microbiology and biotechnology*, 98, 5807-5822.

YING, X., PENG, L., CHEN, H., SHEN, Y., YU, K. & CHENG, S. 2014. Cordycepin prevented IL-β-induced expression of inflammatory mediators in human osteoarthritis chondrocytes. *International orthopaedics,* 38**,** 1519-1526.

YOKOYAMA, E., ARAKAWA, M., YAMAGISHI, K. & HARA, A. 2006. Phylogenetic and structural analyses of the mating-type loci in Clavicipitaceae. *FEMS microbiology letters,* 264**,** 182-191.

YOO, H.-S., SHIN, J.-W., CHO, J.-H., SON, C.-G., LEE, Y.-W., PARK, S.-Y. & CHO, C.-K. 2004. Effects of *Cordyceps militaris* extract on angiogenesis and tumor growth. *Acta Pharmacologica Sinica,* 25**,** 657-665.

YOON, C. S., SUNG, G. H., PARK, H. S., LEE, S. G. & LEE, J. O. 1999. Potential of the entomopathogenic fungus, *Beauveria bassiana* strain CS-1 as a biological control agent of *Plutella xylostella* (Lep., Yponomeutidae). *Journal of Applied Entomology,* 123**,** 423-425.

YOSHIKAWA, N., NAKAMURA, K., YAMAGUCHI, Y., KAGOTA, S., SHINOZUKA, K. & KUNITOMO, M. 2004. Antitumour activity of cordycepin in mice. *Clinical and Experimental Pharmacology and Physiology,* 31, S51-S53.

YOUSEF, M., GARRIDO-JURADO, I. & QUESADA-MORAGA, E. 2014. One *Metarhizium brunneum* strain, two uses to control *Ceratitis capitata* (Diptera: Tephritidae). *Journal of Economic Entomology,* 107**,** 1736-1744.

YU, K. H., KIM, K. N., LEE, J. H., LEE, H. S., KIM, S. H., CHO, K. Y., NAM, M. H. & LEE, I. H. 2002. Comparative study on characteristics of lysozymes from the hemolymph of three lepidopteran larvae, *Galleria mellonella*, *Bombyx mori*, *Agrius convolvuli*. *Developmental & Comparative Immunology,* 26**,** 707-713.

YU, R., YANG, W., SONG, L., YAN, C., ZHANG, Z. & ZHAO, Y. 2007. Structural characterization and antioxidant activity of a polysaccharide from the fruiting bodies of cultured *Cordyceps militaris*. *Carbohydrate Polymers,* 70**,** 430-436.

YUN, J.-H., ROH, S. W., WHON, T. W., JUNG, M.-J., KIM, M.-S., PARK, D.-S., YOON, C., NAM, Y.-D., KIM, Y.-J. & CHOI, J.-H. 2014. Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host. *Applied and Environmental Microbiology,* 80**,** 5254-5264.

ZALUCKI, M. P., SHABBIR, A., SILVA, R., ADAMSON, D., SHU-SHENG, L. & FURLONG, M. J. 2012. Estimating the economic cost of one of the world's major insect pests, *Plutella xylostella* (Lepidoptera: Plutellidae): just how long is a piece of string? *Journal of Economic Entomology,* 105**,** 1115-1129.

ZARE, R. & GAMS, W. 2008. A revision of the *Verticillium fungicola* species complex and its affinity with the genus Lecanicillium. *Mycological research,* 112**,** 811-824.

ZHANG, D.-W., WANG, Z.-L., QI, W., LEI, W. & ZHAO, G.-Y. 2014. Cordycepin (3'-deoxyadenosine) down-regulates the proinflammatory cytokines in inflammation-induced osteoporosis model. *Inflammation,* 37**,** 1044-1049.

ZHANG, G. & LIANG, Y. 2013. Improvement of fruiting body production in *Cordyceps militaris* by molecular assessment. *Archives of microbiology,* 195**,** 579-585.

ZHANG, H., HUANG, Q., KE, N., MATSUYAMA, S., HAMMOCK, B., GODZIK, A. & REED, J. C. 2000. Drosophila pro-apoptotic Bcl-2/Bax homologue reveals evolutionary conservation of cell death mechanisms. *Journal of Biological Chemistry,* 275**,** 27303-27306.

ZHANG, Y.-J., FENG, M.-G., FAN, Y.-H., LUO, Z.-B., YANG, X.-Y., WU, D. & PEI, Y. 2008. A cuticle-degrading protease (CDEP-1) of *Beauveria bassiana* enhances virulence. *Biocontrol Science and Technology,* 18**,** 543-555.

ZHAO, P., LI, J., WANG, Y. & JIANG, H. 2007. Broad-spectrum antimicrobial activity of the reactive compounds generated in vitro by *Manduca sexta* phenoloxidase. *Insect biochemistry and molecular biology,* 37**,** 952-959.

ZHAO, P., LU, Z., STRAND, M. R. & JIANG, H. 2011. Antiviral, anti-parasitic, and cytotoxic effects of 5, 6-dihydroxyindole (DHI), a reactive compound generated by phenoloxidase during insect immune response. *Insect biochemistry and molecular biology,* 41**,** 645-652.

ZHAO-LONG, W., XIAO-XIA, W. & WEI-YING, C. 2000. Inhibitory effect of *Cordyceps sinensis* and *Cordyceps militaris* on human glomerular mesangial cell proliferation induced by native LDL. *Cell Biochemistry and Function: Cellular biochemistry and its modulation by active agents or disease,* 18**,** 93-97.

ZHENG, P., XIA, Y., XIAO, G., XIONG, C., HU, X., ZHANG, S., ZHENG, H., HUANG, Y., ZHOU, Y., WANG, S., ZHAO, G.-P., LIU, X., ST LEGER, R. J. & WANG, C. 2011. Genome sequence of the insect pathogenic fungus *Cordyceps militaris,* a valued traditional chinese medicine. *Genome Biology,* 12, R116.

ZIMMERMANN, G. 2007a. Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Science and Technology,* 17**,** 553-596.

ZIMMERMANN, G. 2007b. Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Science and Technology,* 17**,** 879-920.

Appendix A

Appendix A.1- Reference sequences for phylogenetic tree construction. Reference sequences from the National Center for Biotechnology Information (Harding *et al*., 2013; NCBI, 2017) used for construction of phylogenetic trees based on elongation factor 1-alpha, ITS and large ribosomal rRNA sequences.

Gene	Species	Strain	Accession number	Reference
Elongation Factor 1 alpha	*Beauveria bassiana*	4362	AY531934	Rehner and Buckley, (2005)
Elongation Factor 1 alpha	*Cordyceps militaris*	ATCC 34164	CP023324	Kramer and Nodwell, (2017)
Elongation Factor 1 alpha	*Metarhizium brunneum*	EAMb 09/01-Su	KJ158746	Yousef *et al*., (2014)
Elongation Factor 1 alpha	*Lecanicillium muscarium*	VI 11	MF359590	Mitina *et al*., (2017)
Elongation Factor 1 alpha	*Isaria fumosorosea*	CHE-CNRCB 393	KT225597	Gallou *et al*., (2016)
Elongation Factor 1 alpha	*Aspergillus niger*	CBS 101697	KT965677	Badali *et al*., (2016)
ITS	*Beauveria bassiana*	Bb 2016-120	KY697196	Balachandran *et al*. unpublished
ITS	*Cordyceps militaris*	Minfu30	MF379267	Fu *et al*. unpublished
ITS	*Metarhizium brunneum*	KVL16-25	MF063329	Thapa *et al*. unpublished
ITS	*Lecanicillium muscarium*	IRAN 684C	EF641892	Zare and Gams, (2008)
ITS	*Isaria fumosorosea*	IfTS01	KX057375	Dong *et al*., (2016)
ITS	*Aspergillus niger*	CICR CBE	KX290301	Chakrabarty *et al*. unpublished
Large ribosomal subunit	*Beauveria bassiana*	not specified	AB027382	Nikoh and Fukatsu, (2000)
Large ribosomal subunit	*Cordyceps militaris*	ATCC 34164	CP023322	Kramer and Nodwell, (2017)
Large ribosomal subunit	*Metarhizium anisopliae*	Isolate 5B	KX255642	Gowrilakshmi and Sundari, unpublished
Large ribosomal subunit	*Lecanicillium muscarium*	CBS 143.62	KM283798	Park and Shin, unpublished
Large ribosomal subunit	*Isaria farinosa*	MY01338	JN940901	Schoch *et al*., (2012)
Large ribosomal subunit	*Aspergillus niger*	CBS 513.88	AM270052	Pel *et al*., (2007)

Appendix A.2- Reference sequences for phylogenetic tree construction. Reference sequences from the National Center for Biotechnology Information (Harding *et al*., 2013; NCBI, 2017) used for construction of phylogenetic trees based on 16S rRNA sequences.

Gene	Species	Strain	Accession number	Reference
16S	*Enterococcus mundtii*	NT-1	KT723001	Wang, unpublished
16S	*Enterococcus mundtii*	M-4	KT722993	Wang, unpublished
16S	*Pantoea vagans*	HMF2842	KP099965	Kang *et al*. unpublished
16S	*Bacillus cereus*	S8	KT241012	Lakshmi *et al*., (2014)
16S	*Bacillus thuringiensis*	yy-23	FJ463780	Zhang *et al*. unpublished
16S	*Pantoea agglomerans*	PGHL1	EF050808	Morales-Rodriguez and Peck, (2009)
16S	*Pantoea agglomerans*	NSI	KT075174	Yang and Zhao, unpublished
16S	*Pantoea agglomerans*	NSF	KT075171	Yang and Zhao, unpublished
16S	*Pantoea sp*.	CanR-85	KT580675	Chen, unpublished
16S	*Enterobacter sp*.	M03	KT964804	Zhang, unpuplished
16S	*Pantoea agglomerans*	PGHL1	EF050808	Morales-Valenzuela *et al*., (2017)
16S	*Serratia liquefaciens*	LZ-24	KU950364	Han, unpublished
16S	*Serratia liquefaciens*	B7	KJ781948	Lou *et al*. unpublished
16S	*Enterobacter sp*.	V16	GU064368	Garcia-Bartolomei *et al*. unpublished
16S	*Enterobacter sp*.	Sn-08	KJ716535	Liu *et al*. unpublished
16S	*Serratia liquefaciens*	ZMT-1	KU999993	Zheng *et al*. unpublished
16S	*Enterobacter sp*.	M03	KT964804	Zhang, unpublished

Appendix B

Appendix B.1- Studies in which the EPF *B. bassiana* or *M. anisopliae* s.l. have been assessed for synergistic interactions with pesticides or biopesticides.

Fungus	Chemical/ Treatment	Insect	Interaction	Methodology	Reference
B. bassiana	Imidacloprid	*Diaprepes abbreviatus*	Synergy	Soil treated with three doses of *B. bassiana* and a sub lethal dose of imidacloprid.	Quintela and McCoy, (1998)
B. bassiana	Permethrin	*Anopheles gambiae*	Synergy	Insects treated with different sequences of insecticide *and B. bassiana*, then survival assessed. Synergy determined by combined mortality being greater than the sum of individual treatments.	Farenhorst *et al*., (2010)
B. bassiana	Deltamethrin	*Hyalomma anatolicum*	Synergy (not identified using statistical method)	Tics treated with a range of acaricide doses and *B. bassiana*. Survival monitored over time.	Sun *et al*., (2011)
B. bassiana	Spriodiclofen	*Tetranychus urticae*	Synergy	Mites treated with acaricide and *B. bassiana*. Synergy determined by combined mortality being greater than the sum of individual treatments.	Seyed-Talebi *et al*., (2014)

Fungus	Chemical/ Treatment	Insect	Interaction	Methodology	Reference
B. bassiana	Neem	*Spodoptera litura*	Synergy	Insects treated with a single concentration of Neem and *B. bassiana*. Synergy assessed using a chi-square test.	Mohan *et al*., (2007)
B. bassiana	Bacillus thuringiensis	*Leptinotarsa decemlineata*	Synergy	Insects treated with *Bacillus thuringiensis* and *B. bassiana* in the field. Synergy assessed using an ANOVA to compare mortality of combined treatments with individual treatments.	Wraight and Ramos, (2005)
B. bassiana	Imidacloprod	*Bemisia argentifolii*	Antagonism	Insects sprayed with pesticide and *B. bassiana*. Mortality monitored and ANOVA used to identify synergy.	James and Elzen, (2001)
B. bassiana	Endosulfan, imidacloprid, lufenuron,diflubenzuron, dimethoate, oxydemeton	*Spilarctia obliqua*	Additive or no interaction	LC50 of *B. bassiana* administered with range of pesticide doses. Chi-squared test used to identify significance of synergy.	Purwar and Sachan, (2006)
B. bassiana	Triflumeron, thuringiensin, abamectin, carbaryl	*Leptinotarsa decemlineata*	Additive	Combinations of *B. bassiana* and insecticide were given and mortality monitored.	Anderson *et al*., (1989)
B. bassiana	Cry toxin	*Ostrinia furnacalis*	Additive or antagonistic	Insects were treated with a range doses of Cry toxin and *B. bassiana*. Survival was assessed on one day and the CTF (cotoxicity factor) calculation was used to assess synergy.	Ma *et al*., (2008)

Fungus	Chemical/ Treatment	Insect	Interaction	Methodology	Reference
B. bassiana	Diflubenzurin	*Orthoptera* spp.	Additive	Insects exposed in the field to *B. bassiana* and pesticide. Survival monitored up to 2 weeks after treatment	Delgado *et al*., (1999)
M. anisopliae s.l.	Imidacloprid	*Diaprepes abbreviatus*	Synergy	Soil treated with three doses of *M. anisopliae s.l.* and a sub lethal dose of imidacloprid.	Quintela and McCoy, (1998)
M. anisopliae s.l.	Fenitrothion, Treflubenzuron	*Anomala cuprea*	Synergy	Soil treated with insecticide and *M. anisopliae s.l.* . Survival, haemocyte activity and phenoloxidase activity assessed for synergy.	Hiromori and Nishigaki, (2001)
M. anisopliae s.l.	Endosulfan, imidacloprid, lufenuron,diflubenzuron, dimethoate, oxydemeton	*Spilarctia obliqua*	Additive or no interaction	LC50 of *M. anisopliae s.l.*administered with range of pesticide doses. Chi-squared test used to identify significance of synergy.	Purwar and Sachan, (2006)
M. anisopliae s.l.	Permethrin	*Anopheles gamibae*	Synergy	Insects treated with different sequences of insecticide and *M. anisopliae s.l.* , then survival assessed. Synergy determined by combined mortality being greater than the sum of individual treatments.	Farenhorst *et al*., (2010)
M. anisopliae s.l.	Imidacloprid	*Blatella germanica*	Synergy (not identified using statistical method)	Insects fed imidacloprid and treated with *M. anisopliae s.l* . Mortality monitored over time.	Kaakeh *et al*., (1997)

Fungus	Chemical/ Treatment	Insect	Interaction	Methodology	Reference
M. anisopliae s.l.	Entomopathogenic nematodes	*Coptognathus curtipennis*	Synergy	Insects exposed to *M. anisopliae s.l.* for a long period, then treated with nematodes	Anbesse *et al*., (2008)
M. anisopliae s.l.	Entomopathogenic nematode	*Otiorhynchus sulcatus*	Synergy	Insects were treated with nematodes and *M. anisopliae s.l.* in glasshouse trials. Mortality was monitored and chi-squared test used to identify synergy.	Ansari *et al*., (2004)

Appendix B.2- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *galiomicin* following treatment with *B. bassiana* 433.99 conidia or Triton X-100 at 48 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant ($p<0.05$).

Galiomicin 48h		Uninjected	*B. bassiana*		Triton X-100	
		0 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}
Uninjected	0 mg ml^{-1}		1	0.002*	0.034*	1
B. bassiana	0 mg ml^{-1}			0.004*	0.06	1
	3 mg ml^{-1}				1	0.017*
Triton X-100	0 mg ml^{-1}					0.204
	3 mg ml^{-1}					

Appendix B.3- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *gallerimycin* following treatment with *B. bassiana* 433.99 conidia or Triton X-100 at 48 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant ($p<0.05$).

Gallerimycin 48h		Uninjected	*B. bassiana*		Triton X-100	
		0 mg ml^{1}	0 mg ml^{1}	3 mg ml^{1}	0 mg ml^{1}	3 mg ml^{-1}
Uninjected	0 mg ml^{-1}		1	0.951	0.202	1
B. bassiana	0 mg ml^{-1}			0.044*	1	0.226
	3 mg ml^{-1}				<0.001*	1
Triton X-100	0 mg ml^{-1}					<0.001*
	3 mg ml^{-1}					

Appendix B.4- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *IMPI* following treatment with *B. bassiana* 433.99 conidia or Triton X-100 at 48 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant (p<0.05).

IMPI 48h		Uninjected	*B. bassiana*		Triton X-100	
		0 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}
Uninjected	0 mg ml^{-1}		<0.001*	1	<0.001*	1
B. bassiana	0 mg ml^{-1}			<0.001*	1	<0.001*
	3 mg ml^{-1}				<0.001*	1
Triton X-100	0 mg ml^{-1}					<0.001*
	3 mg ml^{-1}					

Appendix B.5- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *lysozyme* following treatment with *B. bassiana* 433.99 conidia or Triton X-100 at 48 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant (p<0.05).

Lysozyme 48h		Uninjected	*B. bassiana*		Triton X-100	
		0 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}
Uninjected	0 mg ml^{-1}		<0.001*	1	<0.001*	1
B. bassiana	0 mg ml^{-1}			<0.001*	1	<0.001*
	3 mg ml^{-1}				0.002*	1
Triton X-100	0 mg ml^{-1}					<0.001*
	3 mg ml^{-1}					

Appendix B.6- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *galiomicin* following treatment with *B. bassiana* 433.99 conidia or Triton X-100 at 72 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant ($p<0.05$).

***Galiomicin* 72h**		**Uninjected**	***B. bassiana***		**Triton X-100**	
		0 mg ml^{-1}	**0 mg ml^{-1}**	**3 mg ml^{-1}**	**0 mg ml^{-1}**	**3 mg ml^{-1}**
Uninjected	**0 mg ml^{-1}**		0.254	1	1	0.409
B. bassiana	**0 mg ml^{-1}**			0.006*	1	<0.001*
	3 mg ml^{-1}				0.107	1
Triton X-100	**0 mg ml^{-1}**					0.003*
	3 mg ml^{-1}					

Appendix B.7- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *gallerimycin* following treatment with *B. bassiana* 433.99 conidia or Triton X-100 at 72 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant ($p<0.05$).

***Gallerimycin* 72h**		**Uninjected**	***B. bassiana***		**Triton X-100**	
		0 mg ml^{-1}	**0 mg ml^{-1}**	**3 mg ml^{-1}**	**0 mg ml^{-1}**	**3 mg ml^{-1}**
Uninjected	**0 mg ml^{-1}**		0.001*	1	1	1
B. bassiana	**0 mg ml^{-1}**			0.012*	0.182	0.001*
	3 mg ml^{-1}				1	1
Triton X-100	**0 mg ml^{-1}**					1
	3 mg ml^{-1}					

Appendix B.8- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *IMPI* following treatment with *B. bassiana* 433.99 conidia or Triton X-100 at 72 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant (p<0.05).

***IMPI* 72h**		**Uninjected**	***B. bassiana***		**Triton X-100**	
		0 mg ml^{-1}	**0 mg ml^{-1}**	**3 mg ml^{-1}**	**0 mg ml^{-1}**	**3 mg ml^{-1}**
Uninjected	**0 mg ml^{-1}**		0.001*	1	0.205	0.151
B. bassiana	**0 mg ml^{-1}**			<0.001*	1	<0.001*
	3 mg ml^{-1}				0.006*	1
Triton X-100	**0 mg ml^{-1}**					<0.001*
	3 mg ml^{-1}					

Appendix B.9- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *lysozyme* following treatment with *B. bassiana* 433.99 conidia or Triton X-100 at 72 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant (p<0.05).

***Lysozyme* 72h**		**Uninjected**	***B. bassiana***		**Triton X-100**	
		0 mg ml^{-1}	**0 mg ml^{-1}**	**3 mg ml^{-1}**	**0 mg ml^{-1}**	**3 mg ml^{-1}**
Uninjected	**0 mg ml^{-1}**		<0.001*	1	0.021*	1
B. bassiana	**0 mg ml^{-1}**			<0.001*	0.121	<0.001*
	3 mg ml^{-1}				1	1
Triton X-100	**0 mg ml^{-1}**					0.267
	3 mg ml^{-1}					

Appendix B.10- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *gallerimycin* following treatment with *C. militaris* 11703 conidia or Triton X-100 at 48 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant (p<0.05).

Gallerimycin 48h		Uninjected	*C. militaris*		Triton X-100	
		0 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}
Uninjected	0 mg ml^{-1}		0.135	1	1	0.068
C. militaris	0 mg ml^{-1}			0.005*	0.933	<0.001*
	3 mg ml^{-1}				0.654	0.953
Triton X-100	0 mg ml^{-1}					0.004*
	3 mg ml^{-1}					

Appendix B.11- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *galiomicin* following treatment with *C. militaris* 11703 conidia or Triton X-100 at 48 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant (p<0.05).

Galiomicin 48h		Uninjected	*C. militaris*		Triton X-100	
		0 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}
Uninjected	0 mg ml^{-1}		1	0.003*	1	0.239
C. militaris	0 mg ml^{-1}			<0.001*	1	0.002*
	3 mg ml^{-1}				<0.001*	1
Triton X-100	0 mg ml^{-1}					0.038*
	3 mg ml^{-1}					

Appendix B.12- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *IMPI* following treatment with *C. militaris* 11703 conidia or Triton X-100 at 48 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant (p<0.05).

IMPI 48h		Uninjected	*C. militaris*		Triton X-100	
		0 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}
Uninjected	0 mg ml^{-1}		0.042*	1	<0.001*	1
C. militaris	0 mg ml^{-1}			0.005*	0.965	0.012
	3 mg ml^{-1}				<0.001*	1
Triton X-100	0 mg ml^{-1}					<0.001*
	3 mg ml^{-1}					

Appendix B.13- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *lysozyme* following treatment with *C. militaris* 11703 conidia or Triton X-100 at 48 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant (p<0.05).

Lysozyme 48h		Uninjected	*C. militaris*		Triton X-100	
		0 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}
Uninjected	0 mg ml^{-1}		<0.001*	1	<0.001*	1
C. militaris	0 mg ml^{-1}			0.002*	1	<0.001*
	3 mg ml^{-1}				0.001*	1
Triton X-100	0 mg ml^{-1}					<0.001*
	3 mg ml^{-1}					

Appendix B.14- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *gallerimycin* following treatment with *C. militaris* 11703 conidia or Triton X-100 at 72 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant (p<0.05).

Gallerimycin 72h		Uninjected	*C. militaris*		Triton X-100	
		0 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}
Uninjected	0 mg ml^{-1}		0.007*	1	1	1
C. militaris	0 mg ml^{-1}			0.007*	0.535	0.013*
	3 mg ml^{-1}				1	1
Triton X-100	0 mg ml^{-1}					1
	3 mg ml^{-1}					

Appendix B.15- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *galiomicin* following treatment with *C. militaris* 11703 conidia or Triton X-100 at 72 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant (p<0.05).

Galiomicin 72h		Uninjected	*C. militaris*		Triton X-100	
		0 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}
Uninjected	0 mg ml^{-1}		0.162	0.224	1	0.227
C. militaris	0 mg ml^{-1}			<0.001*	1	<0.001*
	3 mg ml^{-1}				0.001*	1
Triton X-100	0 mg ml^{-1}					0.001*
	3 mg ml^{-1}					

Appendix B.16- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *IMPI* following treatment with *C. militaris* 11703 conidia or Triton X-100 at 72 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant ($p<0.05$).

IMPI 72h		Uninjected	*C. militaris*		Triton X-100	
		0 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}
Uninjected	0 mg ml^{-1}		0.002*	0.567	0.227	0.136
C. militaris	0 mg ml^{-1}			<0.001*	1	<0.001*
	3 mg ml^{-1}				<0.001*	1
Triton X-100	0 mg ml^{-1}					<0.001*
	3 mg ml^{-1}					

Appendix B.17- P-values for comparisons between expression ($2^{-\Delta\Delta Ct}$ values) of *lysozyme* following treatment with *C. militaris* 11703 conidia or Triton X-100 at 72 h after treatment. Expression was normalized against *S7e* expression and expression of each gene prior to injection. Stimulation without cordycepin is shown by dark grey bars and with cordycepin by light grey bars, expression in uninjected insects is also included. N=13-15 per treatment. P-values marked with asterisks are statistically significant ($p<0.05$).

Lysozyme 72h		Uninjected	*C. militaris*		Triton X-100	
		0 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}	0 mg ml^{-1}	3 mg ml^{-1}
Uninjected	0 mg ml^{-1}		<0.001*	1	0.013*	1
C. militaris	0 mg ml^{-1}			<0.001*	0.6	<0.001*
	3 mg ml^{-1}				0.439	1
Triton X-100	0 mg ml^{-1}					0.205
	3 mg ml^{-1}					

Appendix C

Appendix C.1- Models fitted to P. xylostella survival following treatment with cordycepin. Regression models fitted to percentage mortality on day seven of *P. xylostella* larvae (n=10 for each dose in each of three replicates) following A) 24 h of feeding with cordycepin at: 0, 0.375, 0.75, 1.5, 3 and 6 mg ml^{-1}, B) 24 h of feeding with cordycepin (and 1 μM pentostatin) at: 0, 0.375, 0.75, 1.5, 3 and 6 mg ml^{-1}C) daily feeding cordycepin (and 1 μM pentostatin) at: 0, 0.093, 0.1875, 0.375, 0.75 and 1.5 mg ml^{-1}. Regression models were fitted in R, adjusted R-squared value and p-values are shown.

Treatment	Model	Adjusted R-squared	P-value
A) Cordycepin without pentostatin	Second order polynomial	0.503	0.002
B) Cordycepin with pentostatin	Second order polynomial	0.274	0.035
C) Continuous feeding of cordycepin	Second order polynomial	0.561	<0.001

Appendix C.2- Models fitted to *P. xylostella* feeding following 24 h of feeding with cordycepin. Regression models fitted to percentage of leaf disc eaten per individual *P. xylostella* larva on days five, six and seven of (n=10 for each dose in each of three replicates) with cordycepin at: 0, 0.375, 0.75, 1.5, 3 and 6 mg ml^{-1}. Regression models were fitted in R, adjusted R-squared value and p-values are shown.

Day	Model	Adjusted R-squared	P-value
5	Linear	0.053	0.182
6	Linear	0.062	0.165
7	Linear	0.134	0.075

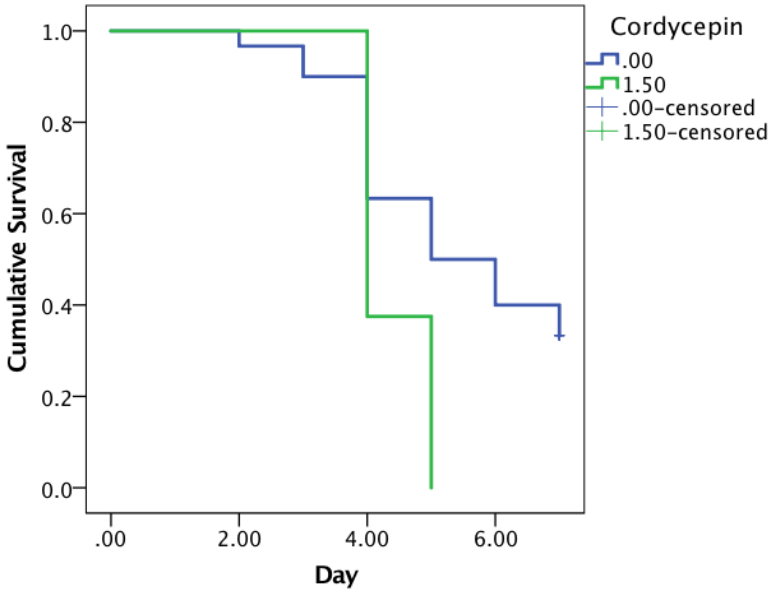
Appendix C.3- Models fitted to *P. xylostella* feeding following 24 h of feeding with cordycepin (plus pentostatin). Regression models fitted to percentage of leaf disc eaten per individual *P. xylostella* larva on days five, six and seven of (n=10 for each dose in each of three replicates) following 24 h of feeding with cordycepin at: 0, 0.375, 0.75, 1.5, 3 and 6 mg ml^{-1}. Regression models were fitted in R, adjusted R-squared value and p-values are shown.

Day	Model	Adjusted R-squared	P-value
5	Linear	0.053	0.180
6	Linear	0.096	0.114
7	Linear	0.212	0.031

Appendix C.4- Models fitted to *P. xylostella* feeding whilst being fed cordycepin (plus pentostatin) daily. Regression models fitted to percentage of leaf disc eaten per individual *P. xylostella* larva on days five, six and seven of (n=10 for each dose in each of three replicates) when fed cordycepin (and 1 μM pentostatin) at: 0, 0.093, 0.1875, 0.375, 0.75 and 1.5 mg ml^{-1}. Regression models were fitted in R, adjusted R-squared value and p-values are shown.

Day	Model	Adjusted R-squared	P-value
5	Second order polynomial	0.690	<0.001
6	Second order polynomial	0.536	0.001
7	Linear	0.311	0.010

Appendix C.5- Survival of *P. xylostella* larvae when fed 1.5 mg ml^{-1}of cordycepin for 24 h and treated with 1x10^{7} B. bassiana 433.99 conidia ml^{-1}. The cumulative survival of *P. xylostella* larvae (n=30 per treatment) when fed leaf discs coated in 1.5 mg ml^{-1} of cordycepin or a Triton X-100 for 24 h following topical application of 1x10^{7} B. bassiana 433.99 conidia.



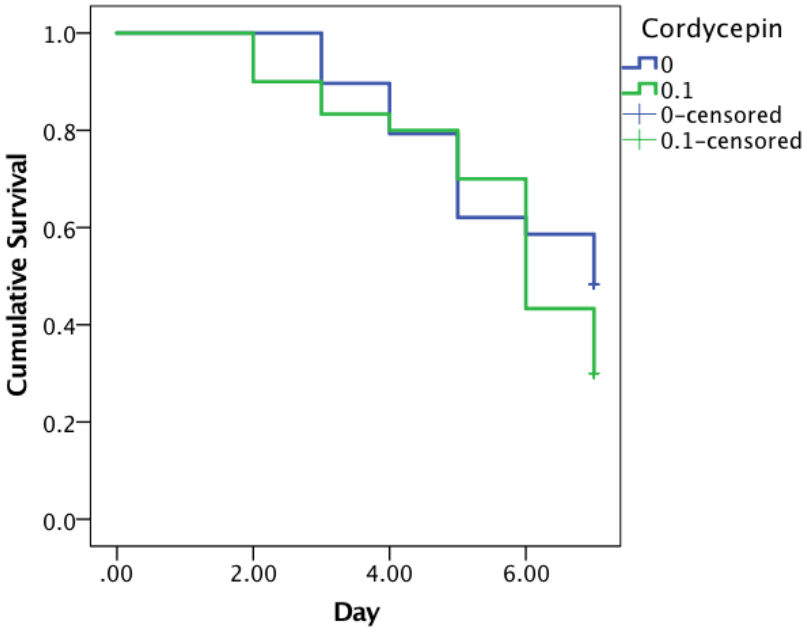
Appendix C.6- Interactions between a range of *B. bassiana* concentrations and short-term cordycepin treatment on day five after treatment. Mortality of *P. xylostella* larvae on day five after treatment following spraying with *B. bassiana* 433.99 conidia and feeding 1.5 mg ml^{-1}cordycepin (with 1 μM pentostatin) for 24 h. Chi-squared values are presented and the effects of treatments identified as additive or synergistic using a chi-squared test.

Concentration of *B. bassiana* 433.99 (conidia ml^{-1})	Measurement	Mortality (±SEM)	Chi-squared	Effect
1.00E+03	Observed	23 ±18.7	1.82	Additive
	Expected	30.5		
1.00E+04	Observed	29.7±10.3	0.21	Additive
	Expected	27.3		
1.00E+05	Observed	25.5 ± 7.3	1.35	Additive
	Expected	32		
1.00E+06	Observed	39.4 ±20.3	0.65	Additive
	Expected	44.8		
1.00E+07	Observed	100 ±0	24.79	Synergistic
	Expected	61.1		

Appendix C.7- Interactions between a range of *B. bassiana* concentrations and short-term cordycepin treatment on day six after treatment. Mortality of *P. xylostella* larvae on day six after treatment following spraying with *B. bassiana* 433.99 conidia and feeding 1.5 mg ml^{-1} cordycepin (with 1 μM pentostatin) for 24 h. Chi-squared values are presented and the effects of treatments identified as additive or synergistic using a chi-squared test.

Concentration of *B. bassiana* 433.99 (conidia ml^{-1})	Measurement	Mortality (±SEM)	Chi-squared	Effect
1.00E+03	Observed	29.1 ±17.3	0.65	Additive
	Expected	33.8		
1.00E+04	Observed	33.0 ±12.3	0.19	Additive
	Expected	35.6		
1.00E+05	Observed	41.4 ±14.2	0.08	Additive
	Expected	39.7		
1.00E+06	Observed	52.4 ±19.1	0.02	Additive
	Expected	51.5		
1.00E+07	Observed	100 ± 0	11.19	Synergistic
	Expected	71.7		

Appendix C.8- Survival of *P. xylostella* larvae when fed 0.1 mg ml^{-1} of cordycepin daily and treated with 1x10^{7} B. bassiana 433.99 conidia ml^{-1}. The cumulative survival of *P. xylostella* larvae (n=30 per treatment) when fed leaf discs coated in 0.1 mg ml-1 of cordycepin or a Triton X-100 control daily for seven days following topical application of 1x10^{7} B. bassiana 433.99 conidia.



Appendix C.9- Interactions between a range of *B. bassiana* concentrations and prolonged cordycepin treatment on day five after treatment. Mortality of *P. xylostella* larvae on day five after treatment following spraying with *B. bassiana* 433.99 conidia and feeding 0.1 mg ml^{-1} cordycepin (with 1 μM pentostatin) throughout the experiment. Chi-squared values are presented and the effects of treatments identified as additive or synergistic using a chi-squared test.

Concentration of *B. bassiana* 433.99 (conidia ml^{-1})	Measurement	Mortality (±SEM)	Chi-squared	Effect
1.00E+03	Observed	23.3 ±7.4	3.25	Additive
	Expected	16.1		
1.00E+04	Observed	12 ±7.2	5.31	Antagonistic
	Expected	23.1		
1.00E+05	Observed	22.2 ±8.3	0.13	Additive
	Expected	23.9		
1.00E+06	Observed	24.1 ± 14.5	1.28	Additive
	Expected	19.1		
1.00E+07	Observed	30 ±11.5	4.61	Antagonistic
	Expected	44.3		

Appendix C.10- Interactions between a range of *B. bassiana* concentrations and prolonged cordycepin treatment on day six after treatment. Mortality of *P. xylostella* larvae on day six after treatment following spraying with *B. bassiana* 433.99 conidia and feeding 0.1 mg ml^{-1} cordycepin (with 1 μM pentostatin) throughout the experiment. Chi-squared values are presented and the effects of treatments identified as additive or synergistic using a chi-squared test.

Concentration of *B. bassiana* 433.99 (conidia ml^{-1})	Measurement	Mortality (±SEM)	Chi-squared	Effect
1.00E+03	Observed	30.8 ± 10.8	1.30	Additive
	Expected	25.1		
1.00E+04	Observed	15.7 ±7.9	4.03	Antagonistic
	Expected	26.0		
1.00E+05	Observed	28.3 ± 5.1	1.27	Additive
	Expected	34.9		
1.00E+06	Observed	31.9 ± 15.6	0.68	Additive
	Expected	27.6		
1.00E+07	Observed	56.7 ± 21.9	0.63	Additive
	Expected	51.0		