Interactions of entomopathogenic fungi and other control agents: mechanism and field potential against *Hylobius abietis* larvae

A thesis submitted to the National University of Ireland, Maynooth for the degree of Doctor of Philosophy
by

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DECLARATION

This thesis has not been submitted in whole or part to this or any other university for any degree, and is the original work of the author except where otherwise stated.

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Abstract

Biological control is the beneficial application of natural enemies such as pathogens, predators and parasites in managing pests and their damage. Entomopathogenic fungi (EPF) have a crucial role in natural ecosystems and are being developed as alternative control agents for insect pests. Both *Beauvaria bassiana* and *Metarhizium anisopliae* have been proven to be effective biological control agents against a range of pests and are commercially produced. Advantages of using EPF for biological pest control include their degree of specificity, absence of effects on mammals, reduced probability of insects developing resistance and they may persist for long periods in some environments which could provide long term control effects. Disadvantages include that it takes EPF longer to kill insects than their chemical counterparts, application needs to be timed for high relative humidity and low pest numbers, and efficacy varies among different insect species.

If a combination of treatments resulted in a synergistic interaction then the efficacy of these biopesticides would be increased. A number of laboratory and field studies have used combinations of entomopathogenic nematodes (EPN) and EPF against insect pests with resulting interactions ranging from antagonistic to synergistic. In the case of synergism resulting from combined applications it is suggested that EPF may make the host more susceptible through suppressing its immune system. To understand how this putative synergistic interaction between control agents could occur mechanistically, the effect of EPF supernatant was tested on the immune response of the forestry pest, *Hylobius abietis*, to screen for species with immunomodulating properties. The potential of the commonly used model organism, the greater wax moth, *Galleria mellonella,* as a model for the study of the immune response of *H. abietis* to pathogens was also explored.

Hylobius abietis, the large pine weevil, is a major pest of reforestation in Europe. It is estimated that *H. abietis* costs the forestry industry approximately €140 million/year. Current control measures rely heavily on the synthetic chemical cypermethrin. However, due to concerns over its environmental impact cypermethrin is being phased out across Europe. Therefore there is an interest in the use of entomopathogens as biological control agents in integrated pest management. Thus one objective of this work was to assess the efficacy of EPF, EPN and EPF-EPN

combinations for *H. abietis* suppression in the field in order to ultimately determine if the treatments used exert synergistic control over *H. abietis*.

The effect of EPF supernatant on the immune response of insects was assessed through a number of bioassays that investigated the effect of EPF on haemocyte densities and yeast proliferation in the insect haemocoel, as well as testing whether pre-treatment with EPF increases larval susceptibility to subsequent pathogens. The effect of EPF supernatant on the humoral immune response was investigated by subjecting larval haemolymph to label free quantitative (LFQ) proteomic analysis. To enable proteomic investigations into the effects of EPF on the immune response of *H. abietis* and to compensate for the lack of genomic information for *H. abietis*; a *de novo* transcriptome study of *H. abietis* larvae was performed with Beijing Genomics Institute (BGI, Hong Kong).

Bioassays indicated that *M. anisopliae*, *B. bassiana* and *Beauveria caledonica*, demonstrated immunomodulating effects on *H. abietis* larvae, while *M. anisopliae* and *B. caledonica* modulated the immune response of *G. mellonella* making the insect more susceptible to subsequent pathogens. LFQ analysis on larval haemolymph showed that in response to EPF supernatant both insect species displayed altered abundance of proteins involved in antimicrobial defence, the prophenoloxidase cascade, detoxification and detection and sensing. These patterns of alteration may be integral to the modulation of the host immune response by EPF. A major difference observed between the proteomic profiles of *H. abietis* and *G. mellonella* haemolymph was that *H. abietis* injected with *B. caledonica* supernatant had a major alteration in metabolic proteins involved in cellulose cleavage, reflective of its wood based diet. It was concluded *G. mellonella* may have an application as a model for looking for secondary metabolites or natural products that display immunomodulating properties so that EPF isolates could be screened for production of these products. However *G. mellonella* are not a substitute for the target pest for proteomic analysis. Moreover laboratory bioassays with either *G. mellonella* or *H. abietis* are not predictive of whether synergy will occur in the field as there are many more factors involved than just the ability of EPF to modulate the immune response.

To investigate the ability of EPF and EPN to suppress *H. abietis* populations in the field, three field studies were carried out over three consecutive years. Treatments were applied to tree stumps harbouring *H. abietis* developmental stages.

The efficacy of EPF and EPN was investigated alone and in combination through emergence trapping and destructive sampling. Three EPF strains were utilised in these field studies, commercial strains of *M. anisopliae* and *B. bassiana* and a strain of *B. caledonica* native to Ireland. Two EPN species were utilised in these field studies, *Steinernema carpocapsae* and *Heterorhabditis downesi*, the latter is native to Ireland. In this work EPN were found to offer superior control over *H. abietis* in the field than EPF, with all treatments that caused significant reduction in adult emergence being EPN alone or EPN in combination with EPF. Ultimately EPF are not suited to control of *H. abietis* using this strategy. Synergy between EPF-EPN was not achieved in any of the three field studies with all combinations tested giving additive results.

The final aim of this work was to investigate if the little-studied native fungus *B. caledonica* produces immunomodulating compounds active against *H. abietis*. Identification, large scale production and structural determination of an abundant secreted natural product of *B. caledonica* was carried out using High Performance Liquid Chromatography (HPLC) and Nuclear magnetic resonance (NMR) spectroscopy, and the metabolite of interest was found to be oosporein. Subsequently assessment of the potential insecticidal, anti-feedant and immunomodulating effects of oosporein was carried out. Oosporein was identified as an abundant metabolite in *B. caledonica* supernatant that displayed immunomodulating properties in *H. abietis* larvae as well as being a feeding stimulant to *H. abietis* adults.

The work presented throughout this thesis offers the first report of the effect of EPF on the cellular and humoral immune system of *H. abietis*. It presents a critical assessment of G. *mellonella* as a model organism for immune studies of *H. abietis*. It highlights that EPN are a superior biocontrol agent for the control of *H. abietis* in the context of the current approach used. However the native *B. caledonica* isolate tested throughout this thesis presents an interesting avenue for future research into the utilisation of EPF as biocontrol agents in a wider sense than researched in this work.

Peer reviewed publications:

Williams, C.D., Dillon, A.B., Harvey, C.D., Hennessy, R., **Mc Namara, L.** and C.T. Griffin (2013). Control of a major pest of forestry, *Hylobius abietis*, with entomopathogenic nematodes and fungi using eradicant and prophylactic strategies, *Forest Ecology and Management*, 305: 212–222.

Abbreviations

ACN	Acetonitrile
AMP	Antimicrobial peptides
ANOVA	Analysis of variance
APS	Ammonium Persulphate
Bb	*Beauvaria bassiana*
Bc	*Beauvaria caledonica*
βGBP	β-1,3-glucan binding proteins
BGI	Beijing Genomics Institute
BUSCO	Benchmarking Universal Single-Copy Orthologs
$CaCl_2$	Calcium chloride
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EPF	Entomopathogenic fungi
EPN	Entomopathogenic nematodes
ERY	Erythromycin
ESI	Electrospray ionization
EST	Expressed Sequence Tag
GH	Glycoside hydrolase/glycosyl hydrolase
GLM	General linearized model
GST	Glutathione-s-transferase
GO	Gene Ontology
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HSP	Heat Shock Protein

IAA	Iodoacetamide
IEF	Isoelectric Focusing
Imd	Immune Deficiency
IJs	Infective juveniles
JAK-STAT	Janus Kinase-Signal Transducer and Activator of Transcription
JH	Juvenile hormone
JHBP	Juvenile hormone binding protein
kDa	Kilodalton
LC/MS	Liquid chromatography–mass spectrometry
LFQ	Label-free quantification
LPS	Lipopolysaccharides
LTA	Lipoteichoic acids
mAU*s	Milli-Arbitrary Units
MEOH	Methanol
MET	*Metarhizium anisopliae*
MS	Mass spectrometry
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NMR	Nuclear magnetic resonance
NRPS	Nonribosomal peptide synthesis
OA	Orsellinic acid
OE	Organic extract
OpS	Oosporein synthase
P value	Probability
PAMPs	Pathogen-associated molecular patterns
PAPs	ProPO activating proteinases
PBS	Phosphate buffered saline
PCA	Principal component analysis

PCWDE	Plant cell-wall degrading enzymes
PEP	Posterior error probabilities
PGN	Peptidoglycans
PGBP	Peptidoglycan binding protein
PGRP	Peptidoglycan recognition proteins
pI	Isoelectric point
PKS	Polyketide synthesis
PMSF	Phenylmethanesulfonyl fluoride
PO	Phenoloxidase
PPAE	ProPO activating enzyme
PPAF	ProPO activating factor
ProPO	Prophenoloxidase
PRP	Pattern recognition proteins
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RP-HPLC	Reversed-Phase High Performance Liquid Chromatography
RPM	Revolutions per minute
Sab Dex	Sabouraud dextrose
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Serpin	Serine protease inhibitor
SOD	Superoxide dismutase
SODC	Copper & zinc superoxide dismutase
TEMED	Tetramethylethylenediamine
TCA	Trichloroacetic acid

TFA	Trifluoroacetic acid
TLRs	Toll like receptors
G	Gravity
v/v	Volume per volume
w/v	Weight per volume
YEPD	Yeast extract peptone dextrose
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis

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Chapter 1:

General Introduction

1.1 The insect immune response

The evolutionary success of insects is partly attributable to the development of highly efficient defense mechanisms to deal with the inevitable assault from microorganisms (Hoffmann 1995), as well as rapid reproduction. Insects and microorganisms commonly coexist and insects are constantly susceptible to pathogen attack. Consequently insects have evolved means of recognizing and responding to pathogens (Lemaitre and Hoffmann 2007). Both insects and their pathogens must constantly improve their defense and virulence, respectively, to survive (Wojda 2016). Insects, unlike mammals, lack an adaptive immune response, having only an innate immune response (Strand 2008). The insect immune system is composed of the cellular and humoral defenses (Hoffmann 1995). Humoral defenses include antimicrobial peptides (AMPs), production of reactive forms of oxygen and nitrogen, soluble effector molecules and cascades that regulate clotting and melanisation of insect hemolymph (Strand 2008). Cellular defenses encompass haemocyte mediated defenses (Lavine and Strand 2002). There is an overlap between humoral and cellular defenses in the recognition of pathogens; many humoral factors regulate the activity of haemocytes and haemocytes produce many humoral defense molecules such as defense peptides and stress proteins (Strand 2008, Grizanova *et al.* 2014, Wojda 2016).

Initial barriers to infection in insects are physical e.g. the cuticle, and chemical e.g. production of cytotoxic molecules at site of wound and clotting responses (Lavine and Strand 2002). The insect cuticle is composed of several layers including the epicuticle and procuticle. The epicuticle is thin and composed of multiple layers, each with different properties. The lipid layer, the outermost layer of the epicuticle, acts as a water barrier and is largely resistant to degradation by enzymes. This layer helps to keep out cuticle degrading fungal enzymes (Pedrini *et al.* 2007). The insect trachea has a cuticle lining that hardens with age, low humidity and a lack of nutrients, all of which make it difficult for pathogens to colonize (Wojda 2016). The foregut and hindgut are lined with cuticle, a major component of which is chitin, and the pH and presence of digestive enzymes inside the gut also act as a defense against pathogens (Wojda 2016).

1.2 Humoral immune response

Insect humoral defenses are composed of soluble effector molecules found circulating in the haemolymph. These include antimicrobial peptides, complement-like proteins and cascades that regulate melanisation and clotting (Strand 2008). To initiate an immune response a pathogen must be recognized by the host using pattern recognition proteins/receptors (PRPs/PRRs) found on the surface of haemocytes, fat body cells or in the hemolymph (Figure 1.1). Insects can differentiate between major groups of pathogens using these PRRs (Butt *et al.* 2016). These receptors function by recognizing and binding to different ligands and sugar moieties on the surface of the pathogens called pathogen-associated molecular patterns (PAMPs) ultimately initiating an appropriate immune response. These PAMPs include bacterial endotoxins (lipopolysaccharides, LPS), lipoteichoic acids (LTA), mannan and β-1,3-glucans from fungi and viral RNA (Tsakas and Marmaras 2010, Butt *et al.* 2016). Several families of pattern recognition molecules act to detect peptidoglycans (PGN) including Toll like receptors (TLRs) and peptidoglycan recognition proteins (PGRPs) (Kurata 2014). There are three major signaling pathways involved in the detection of pathogens: Toll, JAK-STAT (Janus Kinase-Signal Transducer and Activator of Transcription) and Imd (Immune Deficiency) (Figure 1.1). Each of these pathways recognizes a pathogen and induces the production of peptides that either target the pathogen or act as signaling molecules to amplify the immune response (Stokes *et al.* 2015).

Following recognition of a pathogen by the host, antimicrobial peptides are synthesized and secreted into the haemolymph (Bulet *et al.* 2004). The simultaneous presence of a variety of AMPs will afford the insect greater protection against harmful pathogens (Bulet *et al.* 1999). These AMPs are secreted from the insect fat body into the hemolymph through the activation of two NF-κB signaling pathways: namely the Imd and Toll pathways (Kurata 2014). The Toll pathway is activated mainly in response to fungi and Gram-positive bacteria, inducing AMPs such as dromsomysin (Kurata 2014). For Toll activation in the fat body of *Drosophila*, the serine protease cascade is triggered after PGRP-SA (a sensor PGRP) binds to the PGN of invading Gram-positive bacteria. This converts pro-spatzle into an active ligand for the Toll receptor called Spatzle (Kurata 2014). The Imd pathway is mainly activated in response to Gram-negative and Gram-positive bacteria. In *Drosophila* it requires relish, an NF-κB like transcription factor that activates the expression of

genes encoding AMPs such as diptericin. Relish is sythesised as an NF-κB precursor protein that is cleaved and activated in response to bacterial infection (Silverman and Maniatis 2001). The JAK/STAT pathway is activated by infection or septic injury. Together Toll and Imd pathways compose the humoral immune response that acts through production of AMPs. JAK/STAT signaling induces the production of many molecules necessary for the insect immune response such as antimicrobial molecules and proteins involved in cellular responses such as melanisation and phagocytosis (Stokes *et al.* 2015).

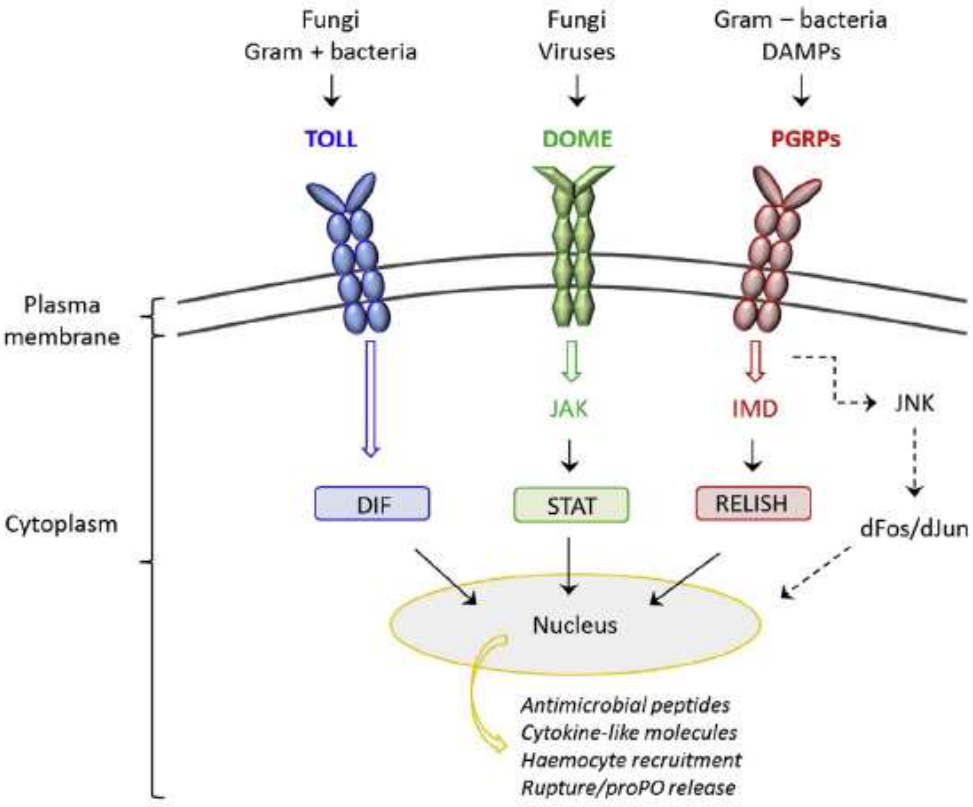


Figure 1.1. Haemocyte signalling cascades involved in pattern recognition (Butt *et al.* 2016).

Pathogen recognition receptors (DOME, Toll and PGRPs) interact with microbial PAMPs or DAMPs to activate the appropriate pathway. Fungal ligands such as β-1,3-glucan, mannan and chitin are recognised by either Toll or DOME leading to the activation of DIF or JAK-STAT which translocate to the nucleus where they induce the production of immune effectors.

More than 150 AMPs have been identified in insects with diverse structures and target organisms, but they can be divided into four main groups: cecropins, cysteine rich peptides (defensins), proline rich peptides (drosocin) and glycine rich peptides (gloverins) (Bulet *et al.* 1999, Tsakas and Marmaras 2010). Insect AMPs are

expressed in the fat body and secreted into the haemolymph in response to infection (van der Weerden *et al.* 2013). The primary characteristics of AMPs are selective toxicity (not toxic to self), their time of action is shorter than the doubling time of the microorganisms they are targeting and they have a broad spectrum of activity (van der Weerden *et al.* 2013). The majority of AMPS work by creating peptide or peptide/lipid lined pores that destabilize cell membranes. Other modes of activity include solubilizing membranes to form micelles or defense molecules entering the microbial cell and disturbing physiological processes e.g. replication (van der Weerden *et al.* 2013, Wojda 2016). Few studies have focused on antifungal peptides compared with antibacterial peptides in insects. Currently characterized insect antifungal peptides include cecropins and defensins.

Cecropins are small (4kDa) AMPs found in Lepidoptera and Diptera that are active against Gram-positive and Gram-negative bacteria, where they form channels and permeabilize the lipid bilayer (Hoffmann 1995). Defensins have been found in every insect screened for their presence to date (van der Weerden *et al.* 2013). They are small AMP that kill bacterial cells by forming voltage-dependent channels in cell walls, causing ions to leak out (Hoffmann 1995). Different defensins vary in their target range, for example drosomycin is a 44 residue defensin that displays potent anti-fungal activity but is not active against yeast or bacteria, while heliomicin is active against filamentous fungi and yeast, but not bacteria (van der Weerden *et al.* 2013).

1.3 Cellular immune response

Haemocytes are blood cells that recognize both foreign pathogens and changes to self. The cellular immune response of insects to invading pathogens involves a number of processes mediated by haemocytes, including: phagocytosis, cell aggregation, nodule formation and encapsulation (Hoffmann 1995). Haemocytes also respond to wounds through clot formation (Lavine and Strand 2002). There are a number of different haemocytes varying in morphology and functionality (Figure 1.2). The most common types are prohaemocytes, granulocytes (granular cells), plasmatocytes, spheruloctyes and oenocytoids (Lavine and Strand 2002). In larval Lepidoptera, 50% of the haemocytes circulating in the haemolymph are made up of granulocytes and plasmatocytes (Lavine and Strand 2002). Granulocytes are most abundant in Lepidoptera and strongly adhere to the surfaces of invading pathogens, spread symmetrically and function as phagocytes (Strand 2008). Plasmatocytes

spread asymmetrically on the surface of invaders and form capsules. Phagocytosis is the engulfment of objects by a cell; haemocytes can phagocytose bacteria, yeast, other apoptotic bodies and also small abiotic objects (Lavine and Strand 2002). Phagocytosis begins when a foreign target binds to its receptor activating a signaling cascade that regulates the formation of a phagosome and ingestion of the pathogen. The phagosome matures via a series of vesicle fusion events, that ultimately kill the target (Strand 2008). In Lepidoptera non-adhesion haemocytes called oenocytoids contain phenoloxidase precursors that may play a role in melanisation of hemolymph (Lavine and Strand 2002). Encapsulation is another important defense mechanism whereby multiple haemocytes bind to a large pathogen such as a nematode that cannot be engulfed by a single cell, rendering it harmless; this may also include coating of the pathogen with melanin (Gillespie and *et al.* 1997, Strand 2008). Nodulation describes the binding of multiple haemocytes to aggregations of bacteria and fungi (Lavine and Strand 2002, Strand 2008).

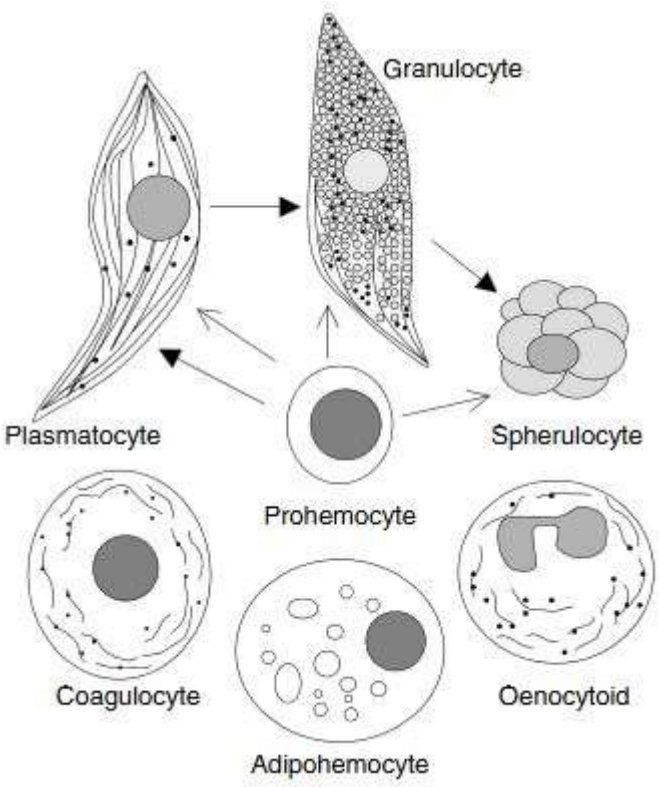


Figure 1.2. An example of insect haemocytes.
Haemocytes shown are those found in *Galleria mellonella* (Kavanagh and Reeves 2004).

1.4 Prophenoloxidase cascade in insects

Often when an insect is wounded, a dark coloration appears around the wound which is a result of the activation of prophenoloxidase (proPO) and subsequent

production of melanin (Gillespie and *et al.* 1997). Melanin is important in the insect defense response, in wound healing and pathogen sequestering (Marmaras *et al.* 1996, Gillespie and *et al.* 1997). Additionally phenoloxidase (PO) can harden the insect cuticle through scleratisation, which may inhibit pathogen invasion as it prevents fungal proteases successfully hydrolyzing the cuticle (Gillespie and *et al.* 1997). Melanin is often deposited around encapsulated bodies, haemocyte nodules and in the cuticle where fungal infections are found (Gillespie and *et al.* 1997). ProPO is activated to PO by the serine protease cascade which involves proPO activating enzymes found in the hemolymph (Figure 1.3) (Kurata 2014). The proPO system is activated by the presence of microbial components, such as LPS and peptidoglycans (PGN) from bacteria and β-1,3-glucans from fungi. The system is composed of pattern recognition proteins (PRPs) including LPS and β-1,3-glucan binding proteins (βGBP) and peptidoglycan binding protein (PGBP), serine protease and their zymogens, proPO and proteinase inhibitors. Proteinase inhibitors are needed to prevent unnecessary activation of the system (Jiravanichpaisal *et al.* 2006). Phenoloxidase catalyzes the oxidation of phenols to quinones, which will then polymerize to melanin. These quinones are reactive intermediates for melanisation, nodulation and phagocytosis (Marmaras and Lampropoulou 2009). The cytotoxic reactive oxygen species and melanin produced during this process are toxic to invading microorganisms (Gillespie and *et al.* 1997, Soderhall and Cerenius 1998, Kurata 2014).

The proPO cascade is tightly regulated by serine proteases that are activated in response to a wound or microbial challenge via cell wall components such as peptidoglycans (Marmaras *et al.* 1996, Marmaras and Lampropoulou 2009). Tight regulation of phenoloxidase activity by serine proteases is crucial as intermediate products of the cascade e.g. quinones and free radicals, display high cytotoxity that may damage the host (Sadd and Siva-Jothy 2006, van der Weerden *et al.* 2013). Insect PO is found in haemocytes, plasma and integuments and there are two main types: tyrosinase type which is found in haemocytes, plasma and integument and the laccase type which is found only in the integument (Marmaras *et al.* 1996). The prophenoloxidase cascade is an example of how the cellular and humoral immune responses work together in insects. The proPO activation cascade links many humoral and cellular defense responses such as melanisation, wound healing, cytotoxic reactions, phagocytosis, encapsulation, nodulation and cuticle scleratisation (Marmaras and Lampropoulou 2009).

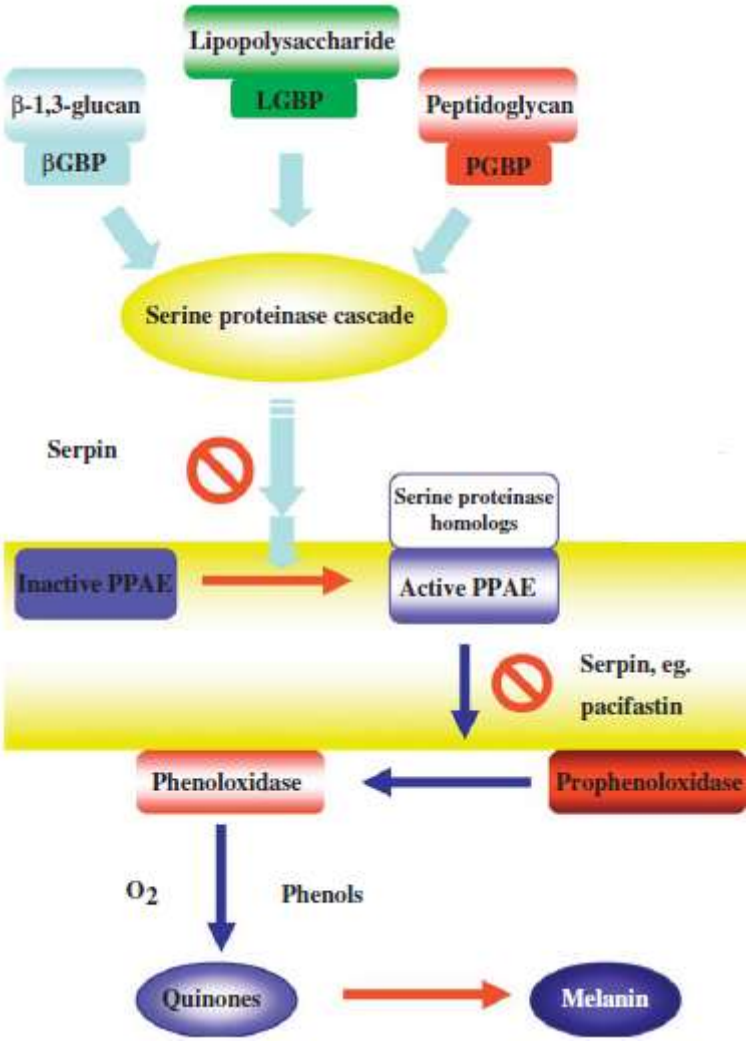


Figure 1.3 Schematic representation of phenoloxidase activation in arthropods (Jiravanichpaisal *et al.* 2006).

The proPO system is activated by the presence of microbial components, such as LPS and peptidoglycans (PGN) from bacteria and β-1,3-glucans from fungi. The system is composed of pattern recognition proteins (PRPs) including LPS and β-1,3-glucan binding proteins (βGBP) and peptidoglycan binding protein (PGBP), serine protease and their zymogens, proPO and proteinase inhibitors that are needed to prevent unnecessary activation of the system.

1.5 *Galleria mellonella* as a model system for the study of microbial pathogens

Invertebrates are regularly used as models in the study of fungal pathogenesis (Fuchs *et al.* 2010). Lepidoptera are useful model systems for the study of the innate immune system in particular for cell biology and immune functionality of haemocytes and analyses of hemolymph proteins. In fact many hemolymph proteins integral to the innate immune response were first discovered in lepidopterans (Jiang

et al. 2010). The greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae), is a pest of beehives where larvae feed on pollen, honey and beeswax. Their lifecycle lasts 7-8 weeks with six larval stages (van der Weerden *et al.* 2013). *G. mellonella* was one of the earliest species used for research into the insect immune response (Jiang *et al.* 2010). *G. mellonella* are regularly employed in the study of fungal pathogenesis and biocontrol agents. *G. mellonella* has been used as a model in the study of many microbes including *Aspergillus flavus*, *Aspergillus fumigatus*, *Candida albicans* and *Metarhizium anisopliae* (Vilcinskas *et al.* 1997a, Cotter *et al.* 2000, A. C. Fröbius *et al.* 2000, Bergin *et al.* 2003, Scully and Bidochka 2005). The use of mammals for studying virulence of microbial pathogens can be costly in terms of time, labor, money and resources. Use of insects such as *Galleria mellonella* as models is growing in popularity as it is cheaper, lacks ethical constraints and results are often comparable to mammals and other insects (Kavanagh and Reeves 2004, Arvanitis *et al.* 2013). Likewise, *Galleria* may have an application as a model for insects that are difficult and labour intensive to source or that are not sold commercially.

1.6 Biological pest control

An arthropod pest is any species of arthropod that can be considered harmful to humans, by being a pest of plants, plant products or animals (Van Emden 2004). Insects attacking crops has been a problem since agriculture began. Crop pests can damage the plant above or below the ground, through chewing, sucking, tunnelling, causing galls, removing parts of the plant or by increasing the susceptibility to viral, bacterial or fungal pathogens (Van Emden 2004). In nature the majority of living species are attacked by natural enemies such as parasites, predators and pathogens which may regulate their population levels (DeBach and Rosen 1991). This is utilised in biological pest control where the application of natural enemies, or their products, is used to reduce the damage caused by pests to tolerable levels (DeBach and Rosen 1991). Biopestides used include microbial pesticides such as bacteria, fungi, viruses and protozoans, entomopathogenic nematodes, carnivorous insects and parasites (Thakore 2006). Utilising natural enemies, and their products, for the reduction of crop and plant damage allows maintenance of biodiversity and protection of both farming and human health (Thakore 2006).

Natural enemies of insects such as entomopathogenic fungi can be used to control pest populations as part of three main biological control strategies: classical

biological control, augmentation or conservation (Shah and Pell 2003, Lacey *et al.* 2015). Classical biological control describes the use of natural enemies against a pest which is not native and is lacking its natural enemies to maintain its population levels (Shah and Pell 2003). The aim of classical biological control is to provide long-term sustainable and economic control of a target insect pest (Shah and Pell 2003). Often natural enemies of the target pest are present at too low a level to limit crop damage, in this instance natural enemies can be augmented by either inoculation or inundation. In the inoculation process the biocontrol treatment is applied in small amounts early in the crops' season, with the intention that the treatment will repeatedly cycle and spread, thus maintain the reduction of the target pest (Shah and Pell 2003). In inundation the natural enemy is applied in a similar way to chemical pesticides; in large quantities, for fast, short-term control of the target pest. In this approach the treatment is often described as a biopesticide (Shah and Pell 2003). In conservation management practices are adopted that enhance the activity of the entomopathogen in the field which may include irrigation and reduction of pesticide use (Shah and Pell 2003). Conservation involves identifying indigenous natural enemies and altering farming practices to improve their activity in the field (Shah and Pell 2003). To improve the activity of entomopathogenic fungi, practices may be altered to increase moisture, for example irrigation, reduction of pesticide use and providing overwintering sites for alternative hosts (Shah and Pell 2003).

The most common strategy for applying microbial agents to control arthropod pests is augmentative biological control using an inudative approach. Augmentation may be appropriate where indigenous natural enemies are unable to suppress pest populations. This may be because they are either too few or active too late, or perhaps they cannot persist over winter (Shah and Pell 2003, Hoy 2008). Augmentative biocontrol may be useful where crops are of high commercial value and natural enemies are affordable. There are two methods of augmentative biological control; inoculation and inundation. As part of the inoculative approach the treatment (e.g. EPF) is applied, in small amounts, early in the season, with the aim that it will cycle in pest populations, so as to keep the pest levels below the economic threshold (Shah and Pell 2003). In inundative augmentation the treatment is applied like a chemical insecticide, in large amounts for rapid short term control (Shah and Pell 2003). There is great interest in EPF as potential inundative augmentative biocontrol agents as their mass production is relatively easy and they can be formulated for used in conventional spraying equipment (Shah and Pell 2003).

In the instance of microbial agents for augmentative control their commercial production involves the following steps: Isolating a suitable pathogen by screening a large number of isolates for activity against the pest of interest, laboratory or greenhouse trials to evaluate efficacy of isolates and to identify environmental issues that may hinder their activity, production of the microbial agent on a commercial scale and finally efficacy trials of the proposed biocontrol agent (Hoy 2008). Several obstacles limit the use of EPF as augmentative biocontrol agents; they can be unpredictable under field conditions, their activity may be too specific in cases where multiple pests are being targeted and they often have a short activity period in the field so it may be necessary to do multiple applications which would increase labour costs. Furthermore they are generally slow acting in comparison to conventionally used chemical pesticides which may result in excessive damage to crops (Hoy 2008). Currently over 50 entomopathogenic fungi, bacteria, viruses and nematodes are being commercially produced and applied as augmentative biological control (Lacey *et al.* 2015).

Microbial pesticides account for approximately 1-2% of pesticides sold worldwide but they have shown greater growth over the last decade than their chemical counterparts which are consistently declining in growth (Lacey *et al.* 2015). One driving force behind this growth is European legislation restricting the use of most synthetic chemical pesticides. Additionally the growing organic sector is aiding the growth of the microbial pesticide market (Lacey *et al.* 2015). The use of entomopathogens in pest management comes with a range of advantages over chemical pesticides; they are safer for applicators and the environment, their specificity reduces their effects on non-target organisms which promotes biodiversity and encourages natural control of pests by indigenous enemies (Lacey *et al.* 2015). However chemical pesticides are often much faster acting. Thus, one option is the use of biological control agents to supplement conventional pesticides in integrated pest management (IPM), therefore reducing the quantities of chemicals used (Thakore 2006). An ideal pesticide is one that would eliminate the target pest without adverse effects to non-target species such as pollinators, it should be biodegradable and have low toxicity towards humans and other mammals (Thakore 2006).

1.7 Entomopathogenic nematodes

The entomopathogenic nematodes (EPN) Steinernematidae and Heterorhabditidae have had the most success as biocontrol agents (Griffin 2015,

Lacey *et al.* 2015). EPN kill insect hosts through a mutualistic symbiosis with bacteria *Xenorhabdus* spp. (in steinernematids) and *Photorhabdus* spp. (in heterorhabditids). For EPN an insect host acts as a food source and a mating environment. EPN locate hosts in the soil using cues such as carbon dioxide and heat (Campos-Herrera 2015). When a nematode reaches the vicinity of a host it must alter its behaviour to enter the host. These changes to behaviour can be energetically expensive and choosing an unsuitable host is fatal to the nematode (Lewis *et al.* 2006). EPN may recognize hosts by reacting to chemical stimuli or physical structures of the host integument. Infective juveniles (IJs), the free living stage, enter hosts through the cuticle or their natural openings; gut via the mouth or anus and tracheal cuticle through the spiracles (Figure 1.4) (Lewis *et al.* 2006, Lacey *et al.* 2015). Depending on the species of EPN and its host different routes will be taken (Lewis *et al.* 2006). When penetrating into the host haemocoel, IJs encounter the host immune response and they may become encapsulated. Nematodes may avoid encapsulation by evading recognition by the host immune defenses, by tolerating the encapsulation response or by actively suppressing encapsulation (Dowds and Peters 2002). Once inside the insect haemocoel, nematodes release their bacteria that kill the host, defend against other invaders and provide the nematodes with nutrients. Nematodes moult and develop to adults, complete up to three generations and then IJs emerge from the cadaver to find new hosts (Lacey *et al.* 2015). A single cadaver can yield thousands of IJs (Dillon *et al.* 2006).

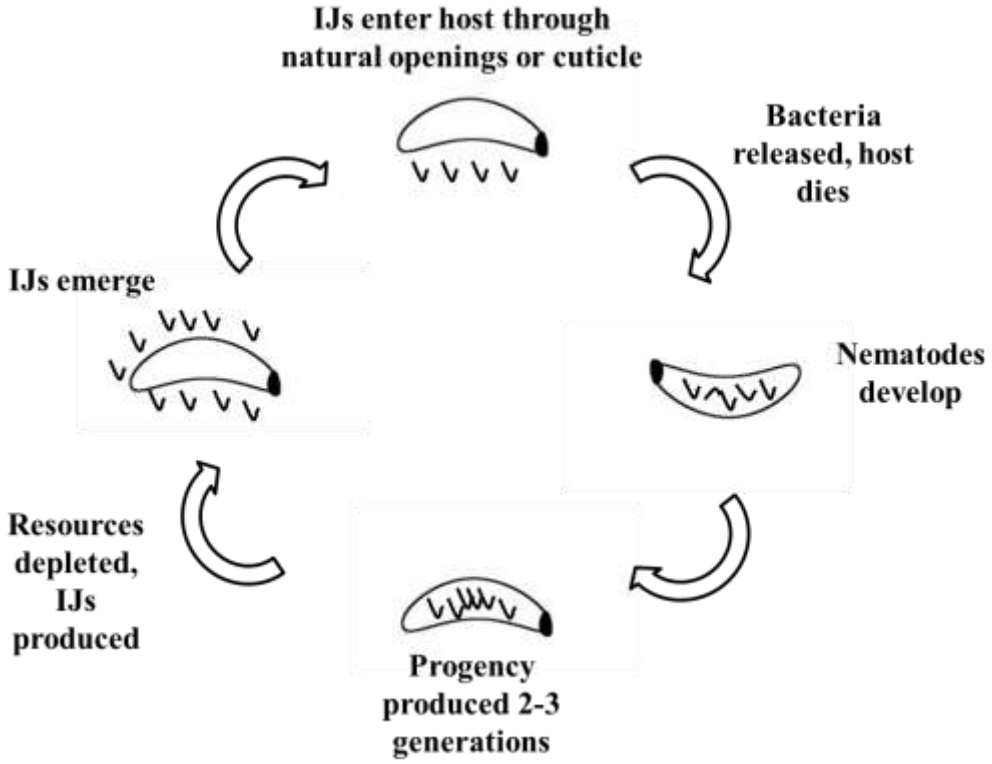


Figure 1.4. Schematic overview of EPN lifecycle

1.8 EPN as biocontrol agents

EPN have many advantages as biocontrol agents; they are safe to humans and are generally safe to non-target organisms and the environment. They actively seek out hosts in the soil, they are fast acting, often killing hosts within 48h, they have limited dispersal and are compatible with a number of herbicides (Campos-Herrera 2015). The majority of EPN have a wide host range with some species being sold commercially against at least 12 insect species. EPN can be mass produced *in vivo* using infected insects and *in vitro* using solid or liquid fermentation (Lacey *et al.* 2015). A range of factors affect the effectiveness of EPN as biocontrol agents; biotic factors include nematode species and rate of application. Environmental factors also affect EPN efficacy in particular nematodes are very sensitive to desiccation and UV light (Shapiro-Ilan *et al.* 2006b). At least 13 different EPN species have been developed as commercial biological pest control agents including *Heterorhabditis bacteriophora*, *Heterorhabditis indica*, *Heterorhabditis marelata*, *Heterorhabditis megidis*, *Heterorhabditis zealandica*, *Steinernema carpocapsae*, *Steinernema feltiae*, *Steinernema glaseri*, *Steinernema kushidai*, *Steinernema kraussei*, *Steinernema longicaudum*, *Steinernema riobrave* and *Steinernema scapterisci* (Lacey *et al.* 2015). EPNs have been cultured commercially for over 25 years; however their

commercialization has had both successes and failures. Success stories include control of the black vine weevil *Otiorhynchus sulcatus*, Diaprepes root weevil *Diaprepes abbreviatus*, billbugs *Sphenophorus* spp., mushroom flies *Lycoriella* spp., the large pine weevil *Hylobius abietis*, filbertworm *Cydia latiferreana* and the pecan weevil *Curculio caryae*, among others (Georgis *et al.* 2006, Shapiro-Ilan *et al.* 2006a, Dillon *et al.* 2007, Williams *et al.* 2013, Lacey *et al.* 2015).

Yet success stories do not always translate to commercial success; the host range of EPN includes over 200 insect species but EPN are only marketed against a small percentage of these insects (Georgis *et al.* 2006). The successful application of EPN as biocontrol agents is hindered by many factors such as cost, shelf life, efficacy, competition and profit margins. Successful market infiltration requires predictable control. EPN are easily killed by desiccation, UV radiation, lack of oxygen and high temperatures (Glazer 2015). Commercially available nematodes are formulated with powder and must be refrigerated until use. Unfortunately the complex interaction between EPN and their surrounding abiotic and biotic factors means that achieving predictable control remains one of the greatest challenges in the biological control sector (Georgis *et al.* 2006). A large number of field trials are instrumental in selecting suitable strains and optimizing protocols to achieve effective and predictable control (Georgis *et al.* 2006).

1.9 Entomopathogenic fungi

Entomopathogenic, insect killing, fungi (EPF) have a crucial role in natural ecosystems and are being developed as alternative control agents for insect pests (Gao *et al.* 2011). More than 700 species of fungi are entomopathogenic, making them the most common insect pathogen (Leger *et al.* 2011, Lacey *et al.* 2015). EPF are found in the divisions Zygomycota, Ascomycota, Deuteromycota, Chytridiomycota and Oomycota (the latter two formerly classified within the Fungi) (Shah and Pell 2003). Some of these fungi have a restricted host range while others have a wide host range with different isolates being more specific to certain pests (Sandhu *et al.* 2012). Species and even isolates within a species can act very differently in terms of their host range, infectivity, rate of germination and optimum temperature (Shah and Pell 2003). They have huge potential as biopesticides as unlike bacteria and viruses they infect hosts through direct penetration of the cuticle, allowing them to act as contact insecticides (Leger *et al.* 2011). The majority of commercially produced EPF include species of *Beauvaria*, *Metarhizium*,

Lecanicillium and *Isaria* (Schrank and Vainstein 2010, Lacey *et al.* 2015). Although there has been much focus on their role as pathogens, EPF have additional roles in nature as plant endophytes, rhizosphere colonists and plant growth promoters (Lacey *et al.* 2015).

1.10 EPF: infection and transmission

EPF can directly penetrate the insect cuticle (Gillespie *et al.* 2000). This is unique to fungi as all other entomopathogenic micro-organisms penetrate the host through the midgut (Samson *et al.* 2013). There are three main phases involved in the development of insect mycosis by EPF (Figure 1.5). The first phase is adhesion and germination of the fungal spore on the host cuticle, the second phase is penetration of the insect integument by a germ tube. The third phase is the development of the EPF inside the insect, ultimately resulting in the death of the host in the majority of cases (Samson *et al.* 2013).

The lifecycle of EPF begins with attachment and germination of conidial (asexual) spores on the host external cuticle (Figure 1.6) (Gillespie *et al.* 2000, Shah and Pell 2003, Schrank and Vainstein 2010, Samson *et al.* 2013). Conidia adhere to the cuticle through hydrophobic interactions and thin mucilaginous material (Schrank and Vainstein 2010). The spore germinates and penetrates the cuticle through hyphal growth. Spore germination may be prevented by fungistatic compounds in the host cuticle and cuticular fatty acids (Gillespie *et al.* 2000). Furthermore successful germination does not always lead to infection (Samson *et al.* 2013). EPF use a combination of physical and enzymatic processes to degrade the cuticle and enter the haemocoel (Gillespie *et al.* 2000). After a period of growth on the host surface, EPF produce apressoria which permanently attaches the EPF to the insect (Gillespie *et al.* 2000, Schrank and Vainstein 2010). Appressoria develop penetration plugs that secrete a range of enzymes that facilitate cuticle penetration. Invasion of the host body and haemolymph occurs when the EPF passes through the cuticle via germ tubes, appressoria and penetration plugs (Gillespie *et al.* 2000). EPF like *Beauvaria* spp. and *Metarhizium* spp. secrete a variety of enzymes to aid this invasion (Gillespie *et al.* 2000).

The next step in the lifecycle is hyphal differentiation into blastospores/hyphal bodies in the haemolymph of the host (Gillespie *et al.* 2000). In EPF, blastospores circulate inside the insect haemolymph producing toxins (Shah and Pell 2003).

Growth of EPF within the host hemolymph is linked with secretion of toxins by the fungus. Host colonization is associated with the ability to overcome the host immune defenses and the extraction of nutrients from the host (Gillespie *et al.* 2000). Furthermore at this stage of the infection cycle, *Metarhizium anisopliae* is known to synthesise enzymes that participate in nutrient uptake from the host. Production of these enzymes is crucial to pathogenicity and virulence of the fungus, as replication relies on overcoming host defences and extracting nutrients from the host body (Gillespie *et al.* 2000). The insect dies from toxicosis or obstruction of organs. Once the hyphae have exhausted the nutrients available in the host environment they penetrate out to the host cadaver surface (Schrank and Vainstein 2010). The EPF then produces conidia on the exterior of the cadaver that are dispersed and the cycle may begin once again if the conidia come into contact with a suitable host (Schrank and Vainstein 2010). In many EPF, fungal conidia are hydrophobic and are passively dispersed from the cadaver (Shah and Pell 2003).

Knowledge of the immune response of insects induced by fungal pathogens contributes to the understanding of both insect defences and the fungal pathogenicity that defeats it. Ultimately uncovering fungal virulence determinants gives rise to opportunities to manipulate these virulence factors to improve the success of biocontrol agents (Gillespie *et al.* 2000). However, it is important to note when considering the use of insect models or before drawing conclusions about the effects of EPF on insects, that different insect species may vary greatly in their susceptibility to fungal toxins (Gillespie *et al.* 2000).

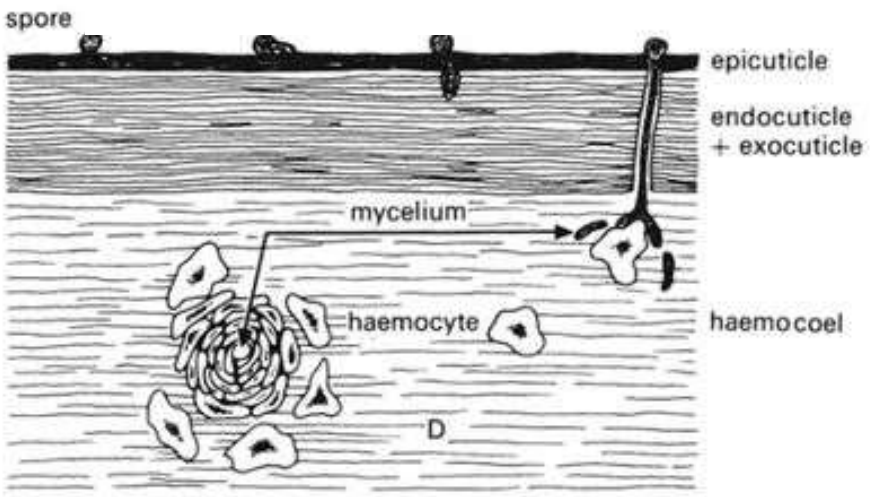


Figure 1.5. A schematic representation of the primary stages involved in the development of insect mycosis by entomopathogenic fungi (Samson *et al.* 2013).

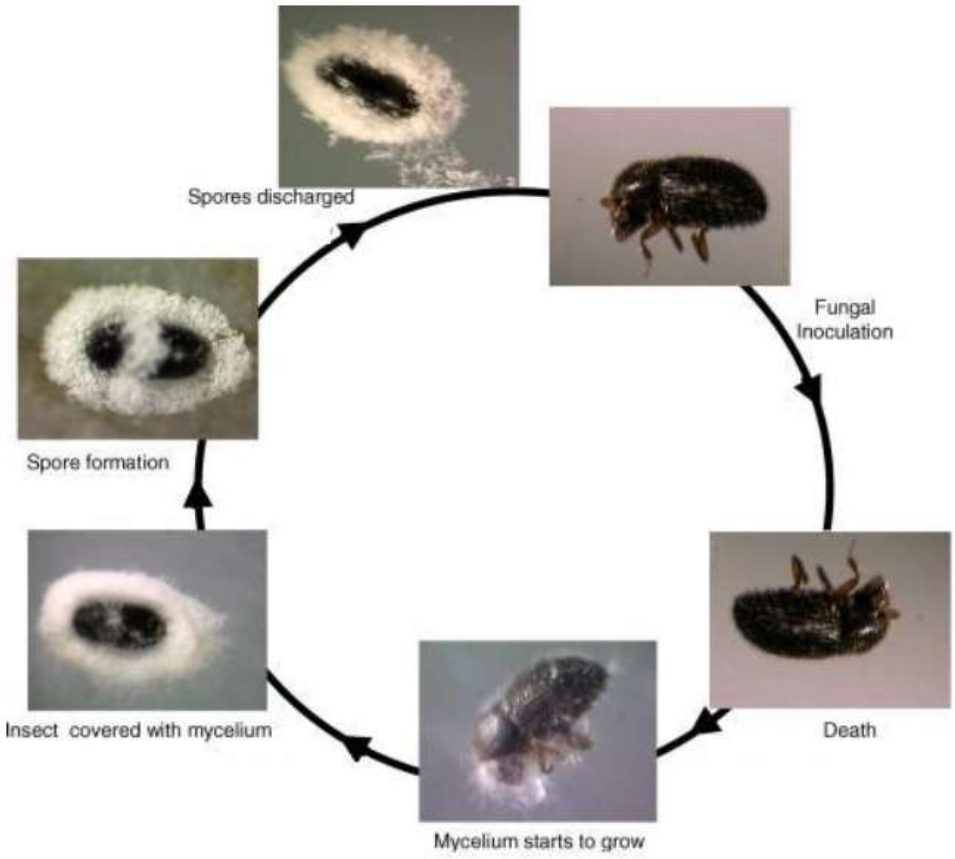


Figure 1.6. Overview of *B. bassiana* lifecycle (Vega *et al.* 2008).

1.11 EPF as biocontrol agents

Some advantages of using EPF for biological pest control include their degree of specificity, absence of effects on mammals, reduced probability of insects developing resistance and they may persist for long periods in some environments which could provide long term control effects (Sandhu *et al.* 2012). Disadvantages of using EPF include that it takes longer to kill insects with fungi than with chemical insecticides and application needs to be timed for high relative humidity and low pest numbers; some EPF display high specificity so more than one control agent may be needed for different pests; production may be expensive and require cold storage and persistence, and efficacy varies among different insect species (Sandhu *et al.* 2012).

Fungal isolates vary in virulence towards different hosts. Virulent strains generally express spore-bound proteases, produce and release exoenzymes during penetration of the cuticle and produce toxins during colonization of target hosts (Lacey *et al.* 2015). Selecting superior strains with these characteristics may be instrumental in overcoming problems with fungi killing pests too slowly.

Furthermore virulence can be enhanced by genetically manipulating fungi; for example genes can be inserted into the fungal genome to promote expression of toxins that may enhance virulence (Lacey *et al.* 2015). Wang and St. Leger (2007) demonstrated that high level expression of an insect specific neurotoxin from the scorpion *Androctonus australis* in the hemolymph of hosts by *M. anisopliae* increased fungal toxicity 22-fold against the tobacco hornworm *Manduca sexta* and ninefold against the yellow fever mosquito *Aedes aegypti*.

Both *Beauvaria bassiana* and *Metarhizium anisopliae* have been proven to be effective biological control agents against a range of pests and are commercially produced (Shah and Pell 2003, Lacey *et al.* 2015). Many EPF are deemed promising for use in biological pest control but fail when they are tested in the field. Environmental conditions and insect behaviour influence fungal activity and these factors need to be taken into account when selecting suitable strains and inoculum for biological control (Lacey *et al.* 2015).

A number of examples of the use of EPF in inundative augmentation include the use of *Verticilllium lecanii* in Europe to control aphids in greenhouses, the use of *Beauvaria bassiana* in North America against a wide range of insect pests and the use of *Metarhizium anisopliae* in Africa against locusts and grasshopper pests (Shah and Pell 2003). An example of the use of EPF in inoculative augmentative biocontrol is the use of *Beauvaria brongniartii* against cockchafer beetles in Europe (Shah and Pell 2003).

1.12 Metarhizium anisopliae

Metarhizium spp. is one of the best characterized and most commonly used fungi in biological pest control, and it is the most abundant EPF isolated from soil (Lomer *et al.* 2001). Furthermore, *Metarhizium anisopliae* is used as a model for studying insect-fungus interactions, evolution, host preference and switching, mechanisms of speciation and is a resource of genes for biotechnology (Gao *et al.* 2011). It is a ubiquitous insect pathogen with more than 200 insect species as suitable hosts (Gao *et al.* 2011). *Metarhizium* spp. produce a diverse range of enzymes and secondary metabolites that are active against insects, fungi, bacteria, viruses and cancer cells (Roberts and St Leger 2004, Gao *et al.* 2011). When the fungus breaches the host cuticle, successful infection lies with it overcoming the host defenses. This is achieved by two main approaches; the use of cryptic forms to hide from host

defenses and production of substances that suppress the host immune response (Schrank and Vainstein 2010).

Metarhizium produces various metabolites that are toxic to a broad range of targets. Destruxins are the most prevalent of the secondary metabolites produced by *Metarhizium* but the various species also produce a variety of other secondary metabolites, for example viridoxin, helvonic acid, hydroxyfungerin and myroridins (Schrank and Vainstein 2010, Liu and Tzeng 2012). Destruxins are cyclic hexadepsipeptides with antiviral, antitumor, insecticidal, cytotoxic, immunosuppressive, phytotoxic and anti-proliferate effects (Kershaw *et al.* 1999, Sowjanya Sree *et al.* 2008, Liu and Tzeng 2012). So far there have been 39 destruxin derivatives identified, most of which have been isolated from cultures of *M. anisopliae* (Liu and Tzeng 2012). These toxins are important in weakening the host immune system, damaging the muscular system and Malpighian tubules, as well as adversely affecting excretion, feeding and mobility (Vilcinskas *et al.* 1997a, Pedras *et al.* 2002, Schrank and Vainstein 2010). Destruxin may act by stimulating apoptosis of haemocytes; eliminating immunocompetent haemocytes appears to be a mechanism to aid in overcoming the immune response of the host (Vilcinskas *et al.* 1997a, Vilcinskas and Götz 1999, Gillespie *et al*. 2000). *Metarhizium* isolates that produce higher amounts of destruxin are generally more virulent (Amiri-Besheli *et al.* 2000, Sowjanya Sree *et al.* 2008). Insecticidal activity has been tested on a wide range of insects, with the toxin being administered topically, through forced ingestion, immersion or injection in both larvae and adults (Kershaw *et al.* 1999, Pedras *et al.* 2002). Destruxins cause an initial tetanic paralysis in insects due to muscle depolarization caused by opening of calcium channels in the membrane; insects can recover from low doses but high doses are lethal (Samuels *et al.* 1988, Pedras *et al.* 2002).

The genus *Metarhizium* contains biologically distinct subtypes that have a wide range of insects hosts and differing abilities to form associations with plants (Leger *et al.* 2011). *M. anisopliae* has been used to effectively control insects such as ticks, black vine weevil, locusts, termites and mosquitos (Kaaya and Hassan 2000, Scholte *et al.* 2003, Wright *et al.* 2005, Peng *et al.* 2008, Shah *et al.* 2008). In 2011 a commercial strain of *M. anisopliae* F52 (sold commercially as Met52 and produced by Novozyme) became available for use in the Irish market and is mainly used for the control of the black vine weevil, *Otiorhynchus sulcatus*.

1.13 Beauvaria bassiana

The majority of species in the genus *Beauvaria* are insect pathogens. *B. bassiana* is a filamentous fungus that grows naturally in soil worldwide (Sandhu et al. 2012). It was identified in 1835 as the cause of white muscardine disease in silkworms *Bombyx mori* (Leger *et al*. 2011). The ubiquitous insect pathogen *Beauvaria bassiana* infects a wide range of insect species (over 700 species) and is used to control both vectors of human disease and plant pests (Vilcinskas and Matha 1997, Glare *et al.* 2008, Xiao *et al.* 2012). Similar to *M. anisopliae*, *B. bassiana* is known to produce cyclic peptides that are cytotoxic and immunosuppressive (Hung *et al.* 1993). *B. bassiana* produces a diverse selection of secondary metabolites including non-peptide pigments and polyketides (oosporein, bassianin and tenellin), non-ribosomally synthesized peptides (beauvericin, bassianolides and beauveriolides) and secreted metabolites that have roles in pathogenesis and virulence (oxalic acid) (Xiao *et al.* 2012). These metabolites have insecticidal properties and can also inhibit growth of other microorganisms (van der Weerden *et al.* 2013). *B. bassiana* products have both realized and proposed applications in industry, pharmaceuticals and agriculture.

As previously stated, for an EPF to successfully invade and propagate in a host it must be able to modulate the host immune responses. It has been demonstrated that antifungal reactions such as phagocytosis and encapsulation are suppressed by EPF (Vilcinskas and Matha 1997). Hung e*t al.* (1993) showed that growth of *B. bassiana* in the beet armyworm *Spodoptera exigua* caused a suppression of phagocytosis and an alteration in haemocyte density; the fungus can grow out of granulocytes and produces progeny cells that are resistant to phagocytosis. It is proposed that metabolites produced by *B. bassiana* initially target the cellular defenses (Hung *et al.* 1993). Insect lysozymes and cecropins have antibacterial and antifungal activity and are often increased within insect hemolymph during fungal infection (Vilcinskas and Matha 1997). However Vilcinskas and Matha (1997) found that *G. mellonella* larvae infected with *B. bassiana* through the integument had no increase in abundance of lysozyme. The lack of any detectable humoral reaction may be due to hyphal bodies without β-1,3-glucans on the surface or the release of secondary metabolites that interfere with the host immune response (Vilcinskas and Matha 1997). Inside the host haemocoel *B. bassiana* grows as blastospores that have a thin cell wall that lacks

galactose residues; this is a mechanism of reducing PAMPs (van der Weerden *et al.* 2013).

B. bassiana is a pathogen to a range of insects including hosts of medical significance that act as vectors for disease such as tsetse fly and hosts of agricultural significance such as the Colorado potato beetle and codling moth (Sandhu *et al.* 2012). It is currently being used as a biological insecticide against termites, white fly and malaria-transmitting mosquitos (Sandhu *et al.* 2012). Like other EPF, *B. bassiana* has many genetically distinct variants with different geographical locations and hosts. As an insecticide it is applied by spraying spores on crops as suspensions or wettable powder (Sandhu *et al.* 2012).

1.14 Beauvaria caledonica

Beauvaria caledonica was found to be a naturally occurring pathogen of the introduced pine bark beetles *Hylastes ater* and *Hylurgus ligniperda* in New Zealand (Glare *et al.* 2008, Reay *et al.* 2008). Bioassays confirmed that it is highly pathogenic to *H. ligniperda* adults, the fungus sporulated on cadavers indicating it can utilize cadavers (Glare *et al.* 2008). *B. caledonica* has also been found as a naturally occurring pathogen of the major forestry pest, the large pine weevil, *Hylobius abietis* in Ireland (Glare *et al.* 2008, Williams *et al.* 2013). Considering *B. caledonica* kills these pests in their natural environment, it makes this fungus a potential biocontrol agent for use against coleopteran forestry pests (Glare *et al.* 2008, Williams *et al.* 2013). *B. caledonica* may have applications beyond its use as a biocontrol agent. *B. caledonica* displays an impressive mineral-transforming ability and high tolerance to toxic metals, through oxalic acid over-excretion, which could have applications in bioremediation, bioleaching and production of oxalic acid (Fomina *et al.* 2005). These kind of properties may in some part contribute to the success of *Beauvaria* species in colonizing a wide range of habitats (Fomina *et al.* 2005).

1.15 The quest for synergy in biological control

As discussed, biocontrol agents are generally slower acting than their chemical counterparts and their high specificity may necessitate the application of more than one agent to control a number of pests. To overcome these obstacles, biocontrol agents may need to be combined in order to make it economical for growers to use them. Combining treatments may result in additive, antagonistic or synergistic

interactions. Synergy can be defined as the interaction of two or more organisms, substances or agents to produce a combined effect greater than the sum of their individual effects. Antagonism describes when the combination of two or more agents results in an overall effect that is less than the sum of their individual effects. Additive effects occur when the effect of two treatments combined is equal to the sum of the effect of the two treatments used separately. If a combination of treatments resulted in a synergistic interaction then the potential cost of using these biopesticides may be reduced. However additive or antagonistic results would be of no benefit to growers.

Synergistic interactions have occurred in some instances of fungal pathogens being combined with sub lethal doses of insecticides. It is thought that this synergism occurs due to the action of the insecticide on the insect's behaviour, either stimulating movement to escape the infected environment thus leading to acquisition of additional fungal inoculum or by negatively impacting on movement and grooming resulting in greater retention of fungal inoculum (Lacey *et al.* 2015). Shah *et al*. (2007) showed that *M. anisopliae* demonstrated effective control over the black vine weevil, *Otiorynchus sulcatus*, alone or in combination with sub-lethal doses of the neonicotinoid imidacloprid or the phenylpyrazole fipronil. Combining *M. anisopliae* with sub-lethal doses of insecticide gave immediate protection against vine weevils because the insecticides prevent the weevils feeding, allowing the slower acting fungi opportunity to kill (Shah *et al.* 2007). Shah *et al*. (2008) found that neem seed cake enhanced the efficacy of *M. anisopliae* for the control of the black vine weevil. Significantly higher conidial attachment occurred in larvae treated with a combination of EPF and neem seed cake, suggesting that neem seed cake increased larval movement and acquisition of conidia. Morales-Rodriguez and Peck (2009) screened numerous combinations of biological control agents with sub-lethal doses of neonicotinoids against the white grubs *Amphimallon majale* and *Popillia japonica*. For *P. japonica* synergistic combinations of *B. bassiana* and *M. anisopliae* with both neonicotinoids was observed in laboratory and greenhouse but not in field trials (Morales-Rodriguez and Peck 2009).

A number of laboratory and field studies have used combinations of EPN and EPF against insect pests with resulting interactions ranging from antagonistic to synergistic. In the case of synergism combined applications may make the host more susceptible through modulating their immune system, prolonging developmental

stages or by the two treatments acting on different components of the host population (Lacey *et al.* 2015). Ansari *et al*. (2006) conducted field trials against the white grub, *Hoplia philanthus* using a combination of *M. anisopliae* and *Heterorhabditis bacteriophora*. Combinations of *M. anisopliae* with *H. bacteriophora* resulted in additive or synergistic effects, causing more than 95% grub mortality when the nematodes were applied four weeks after the application of fungus. Thus they concluded that these pathogen combinations would improve control of *H. philanthus* larvae beyond what is expected from a single pathogen application.

Ansari *et al*. (2008) conducted laboratory and field trials against *O. sulcatus* using a combination of *M. anisopliae* and EPN. In laboratory conditions synergy occurred with *M. anisopliae* and either *H. bacteriophora* or *S. kraussei*. Combinations of *S. feltiae* and *M. anisopliae* resulted in additive control. When greenhouse trials were performed with combination of *M. anisopliae* and EPN at various concentrations, *M. anisopliae* and *H. bacteriophora* combinations resulted in synergistic interactions. Combinations of *M. anisopliae* and *S. feltiae* were synergistic when a high concentration of fungus was used with a low concentration of nematodes, all other combinations gave additive effects (Ansari *et al.* 2008).

Anbesse (2008) carried out laboratory screening of several EPF and EPN to assess potential synergistic effects against grubs of the barley chafer *Coptognathus curtipennis*. They found that combinations of *M. anisopliae* and *H. bacteriophera* increased larval mortality in an additive and synergistic manner. To achieve synergy larvae were exposed to *M. anisopliae* 3 weeks before addition of nematodes (Anbesse *et al.* 2008). Synergism can also occur when different species of EPF are applied together. Inglis (1997) used combinations of *Metarhizium flavoviride* and *B. bassiana* to overcome the constraints of temperature in controlling thermoregulating grasshoppers.

In relation to EPN not only have additive/synergistic interactions been observed when combined with EPF as previously discussed, it has also been seen in combination with chemicals, arthropod predators and even with GM crops (Lacey *et al.* 2015).

1.16 Large pine weevil - *Hylobius abietis*

The large pine weevil *H. abietis* is the most serious pest of reforestation in Europe. The large pine weevil has been recognized as a pest in Europe since the

nineteenth century but reports of damage were uncommon in Ireland and the UK until the twentieth century (Dillon and Griffin 2008). It is estimated that this pest costs the forestry industry approximately €140 million/year. Without controls in place, these weevils could destroy 100% of newly planted trees. In the UK and Ireland approximately 50% of newly planted trees die within the first few years on untreated sites (Evans *et al.* 2015). Conifer plantations account for 7-9% of land area in the UK and Ireland. These plantations are harvested 40-70 years after planting. Clear-felled conifer plantations are the optimum environment for pine weevil. In clear felled forests there is a large increase in concentrations of volatile chemicals released from freshly cut tree stumps. These chemicals attract female pine weevil looking for suitable oviposition sites (S. R. Leather *et al.* 1999). Pine weevils generally have a two year lifecycle but generation time can vary from one to four years. The duration of their lifecycle, timing of adult weevil emergence and number of seasons emergence occurs over has a huge influence on both the economic impact and management strategies for this pest (Wainhouse *et al.* 2004, Inward *et al.* 2012). Their life cycle and dispersal, and thus their economic significance is expected to be influenced by climate change (Inward *et al.* 2012).

Adult weevils are brown with yellow patches on their body (Figure 1.7) and are generally 1.5 cm in length. Female weevils lay their eggs singly, during May to August in the soil around roots of stumps or occasionally in the bark of the stumps if conditions are dry (Nordlander *et al.* 1997). Temperature and moisture affect where the eggs are laid in the soil, if conditions are dry eggs will be laid lower down in the soil. Eggs generally hatch after about a month and larvae make their way to the bark of the stump to begin feeding (Nordlander *et al.* 1997). The majority of the lifecycle is spent below ground where larvae feed and develop in solitary galleries in the bark of conifer roots and stumps of recently felled trees (S. R. Leather *et al.* 1999). Pine weevil development takes longer in spruce than pine, additionally the numbers developing in and emerging from pine are higher than from spruce (Dillon and Griffin 2008, Williams *et al.* 2015).

Figure 1.7.The large pine weevil, *H. abietis*

Only the adults are of economic significance. One adult can damage or kill several young plants, so even small numbers of emerging adults can impact replanted sites (Wainhouse *et al.* 2004, Inward *et al.* 2012). There are two peak periods of damage-spring and autumn (Figure 1.8). It is estimated that >100,000 adults per hectare emerge in just one year on a pine site in Ireland (Dillon *et al.* 2006). Adults generally emerge in late Summer-Autumn to feed before overwintering in soil and remerging in Spring. Clear fell sites are usually replanted, thus when adults emerge from the stumps they feed on the newly planted trees. The main period of feeding occurs in spring on bark of seedlings or twigs of mature trees (Inward *et al.* 2012) (Figure 1.8). Feeding by adults can remove all bark from the stem of young trees causing severe damage or death. Adults then disperse to new clear-fells attracted by the chemicals emitted from the freshly cut trees, where they oviposit in the bark of these stumps or surrounding soil between May and August (Nordlander *et al.* 1997, Inward *et al.* 2012). While the majority of feeding normally occurs during the first year after planting, seedlings may be vulnerable to damage for up to 5 years (Dillon *et al.* 2006). In mature forests, weevils are generally present in low densities as fallen coniferous trees are limited in numbers. In contrast, coniferous in Northern Europe are routinely felled and replanted resulting in a plentiful supply of stumps and roots for weevil development thus increasing the potential for weevil damage (Georgis *et al.* 2006). As replanted clear fell sites are the primary form of commercial tree planting in Ireland, the large pine weevil is of huge economic significance (Evans *et al.* 2015).

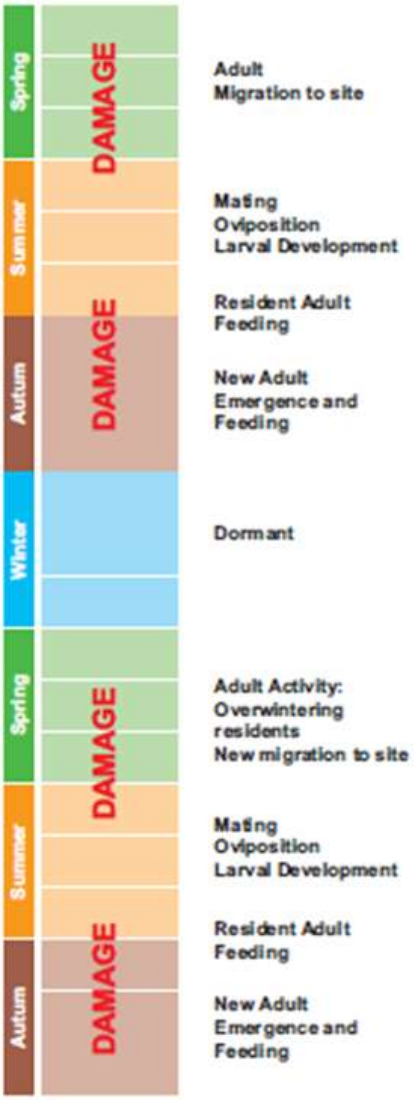


Figure 1.8. Schematic overview of the lifecycle and damage-causing periods of the large pine weevil, *H. abietis* (Evans *et al.* 2015).

1.17 Control of the large pine weevil

In the past, seedlings were treated with the organochlorine insecticide dichlorodiphenyltrichloroethane (DDT) to prevent damage caused by weevil feeding. This practice lasted from the 1950s up to the 1970s when it was banned (Långström and Day 2007). Current practices for controlling pine weevil numbers and damage include chemical, biological and management strategies. Protection in the UK and Ireland generally involves treating young plants prior to planting with synthetic pyrethoids and additional top up sprays. Permethrin was the most widely used pyrethroid in the UK until 2003 when its use in forestry was banned with the EU (Georgis *et al.* 2006). The most effective pesticides, cypermethrin and alpha-cypermethrin are now only available for use in the UK and Ireland under derogation from the Forest Stewardship Council. Additionally while current pesticides repel

pine weevils, thus protecting young seedlings they ultimately have little impact on local populations (Williams *et al.* 2013). Alternatives to chemical pesticides include changes in forestry practices such as mounding, planting later in the season and leaving sites fallow for several years (Williams *et al.* 2013). Various natural enemies kill pine weevil, these include predatory beetles, EPF, and parasitoids including the parasitic wasp *Bracon hylobii* (Dillon *et al.* 2006, Kenis *et al.* 2007).

Field trials have been carried out in Ireland and the UK to identify potential biological control agents for the large pine weevil. Both EPN and EPF have been trialed in the field to control pine weevils. All stages of *H. abietis* are susceptible to EPN (Dillon *et al.* 2006). However targeting the larval/pupal population prevents the damage caused to seedlings when adults emerge. Field trials have shown that the optimum window for EPN application is between May and early July (Georgis *et al.* 2006). An effective biocontrol approach would involve monitoring weevil development to determine appropriate application timing relative to weevil stage. EPN should be applied at least four weeks before adult weevil emergence. EPN have great potential to control pine weevils due to their ability to penetrate through wood and soil to infect larvae under the bark of roots and stumps (Georgis *et al.* 2006).

A preliminary study in the UK in 1994 found that *S. carpocapsae* caused up to 70% reduction in weevil populations (Brixey 1997). Dillon *et al*. (2006) conducted trials across three years to compare the performance of EPN species against immature pine weevils. A mixture of diverse foraging strategies and exotic and native species were trialed. The trials found that *Heterorhabditis downesi* had the greatest potential for controlling weevils in stumps (Dillon *et al.* 2006). Dillon *et al*. (2007) attempted to optimize the application of EPN for the control of pine weevils. They found that *H. downesi* still caused a significant reduction in emergence (75-79%) when application rate was halved. Also a fourfold reduction in application volume did not affect the percentage of weevils parasitized. Additionally there is a wide window for application with EPN applied in April-June significantly reducing weevil numbers (Dillon *et al.* 2007). These findings indicate areas where EPN application may be altered to make the biocontrol agent more cost effective. Further field trials were carried out to assess the establishment, persistence and introgression of EPN in Irish forests. Two exotic species (*Steinernema carpocapsae* and *Heterorhabditis megidis*) and two native species (*Steinernema feltiae and H. downsei*) were investigated and it was found that all species were recovered from the

site of application after three years but only *S. feltiae* was recovered up to five years after application (Dillon *et al.* 2008). Limited horizontal dispersal occurred and a more abundant recovery of *S. feltiae* was observed (Dillon *et al.* 2008). To date *S. carpocapsae* is the only EPN species that has been used to control the large pine weevil at an operational level in Europe (Williams *et al.* 2013).

Ansari and Butt (2012) investigated the potential of *Metarhizium* and *Beauvaria* isolates to control different life stages of the large pine weevil using laboratory susceptibility tests. All life stages were susceptible to *M. robertsii*, *M. brunneum* and *B. bassiana*. Larvae and pupae die more quickly than adults due to *Metarhizium*; this is probably explained by adults possessing thick sclerotinised cuticles that act as barrier to infection while early stages have soft bodies. Ansari and Butt (2012) suggest that EPF offer advantages over EPN because they have a longer shelf life and can be applied against all developmental stages as dry conidia or in a suspension. However the successful use of EPF depends upon selecting the suitable isolate, formulation and application method for the field.

Once the success of EPF and EPN have been shown through field trials the total cost of their use compared to their synthetic counterparts will be important in determining their uptake by commercial foresters.

1.18 Proteomic Analysis

Recent advances in mass spectrometry-based proteomics and the increasing availability of genomic and transcriptomic resources are now making it possible to investigate global systems level changes in an organism's proteome (Ozsolak and Milos 2011). Label-free quantitative (LFQ) mass spectroscopy (MS) aims to quantify peptides and proteins without the use of protein labels. Label free MS measures the relative abundance of proteins across multiple sample groups in single MS runs (Bantscheff *et al.* 2012). Thus it allows the comparison of proteomes of different samples to identify changes to protein expression, molecular processes or biological pathways. In this work larval haemolymph as well as entomopathogenic fungal mycelium was subjected to LFQ analysis and/or 2D-PAGE to compare alterations to their proteomic profile as a result of a number of treatments. 2D-PAGE involves separating proteins in two dimensions, the first based on differing protein isoelectric points and the second by protein mass. The resulting protein spots are then measured densitometrically and compared for differential abundance. Protein separation and

comparison by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), followed by mass spectrometry (MS) or tandem mass spectrometry (MS/MS) identification is the classical method for quantitative analysis of protein samples. Some disadvantages of this approach include: quantification can be ambiguous as spots on a 2D gel may contain more than one protein, also the chosen gel method may have difficulty handling hydrophobic proteins and detecting proteins with extreme molecular weights and pI values (Zhu *et al.* 2009). Numerous gel free quantitative proteomics methods are now available that quantify proteins via mass spectrometry (Bantscheff *et al.* 2012).

LFQ approaches are being adopted in entomological studies for instance Large-Scale Label-Free Quantitative Proteomics was utilised to study the obligate symbiosis between the pea aphid *Acyrthosiphon pisum* and the γ-proteobacterium *Buchnera aphidicola* (Poliakov *et al.* 2011). The mass spectrometry-based identification was performed with a high resolution, high mass accuracy instrument, operating at its highest resolution, thus yielding a data set that can be used to improve aphid genome annotation and provide evidence for predicted *Buchnera* protein models. This study provided excellent proteome coverage of both aphids and *Buchnera* (1900 aphid and 400 *Buchnera* proteins were identified), but also answered important and fundamental questions regarding their symbiotic relationship. A similar proteomic approach to this work was utilised in Surlis (Surlis *et al.* 2016)to observe changes in the expressed proteomes of *Varroa destructor*, either sensitive or resistant to Bayvarol®. Label free shotgun proteomic analysis of Bayvarol® resistant and susceptible *V. destructor* was undertaken to elucidate the determinants and mechanisms of resistance at a protein level.

1.19 Objectives

The overall objectives of this research were;

1. To screen agents for the ability to compromise *H. abietis* cellular and humoral defences
2. To explore the potential of *G. mellonella* as a model for the study of *H. abietis* immune response to pathogens
3. To assess the efficacy of fungus, nematodes and nematode-fungus combinations for *H. abietis* suppression in the field

In addition to the original objectives outlined above the following objective was subsequently incorporated based on findings:

4. To investigate if the native fungus *Beauvaria caledonica* produces immunomodulating compounds active against *Hylobius abietis*

Chapter 2:

Effect of entomopathogenic fungi on the immune response of *Galleria mellonella*

2.1 Introduction

Galleria mellonella are regularly employed in the study of fungal pathogenesis and biocontrol agents. They are also employed in the '*Galleria* bait method' for detecting naturally occurring EPF and EPN in soil (Zimmermann 1986). Use of *G. mellonella* as a model is growing in popularity as it is cheap, commercially available worldwide, lacks ethical constraints, its relatively large size enables precise injection of antibiotics or a number of pathogens, tissue and haemolymph can be easily collected for proteomic approaches, it can be adapted in the laboratory to human physiological temperature (37°C) and results are often comparable to mammals and other insects (Kavanagh and Reeves 2004, Vogel *et al.* 2011, Arvanitis *et al.* 2013, Maguire *et al.* 2016). Due its popular application as a model organism, Vogel *et al.* (2011) subjected the transcriptome of different developmental stages and immune-challenged larvae to next generation sequencing in order to compensate for the lack of genomic information available for *G. mellonella*. *G. mellonella* is believed to be a good model organism to study host interaction with natural insect pathogens like *Bacillus thuringiensis* and *B. bassiana*, which generally are not pathogenic for humans, and so can be used in agriculture for production of bioinsecticides (Wojda 2016).

Here the suitability of *G. mellonella* was investigated as a model for *H. abietis* as the larval stage of *H. abietis* is labour intensive to source and is not sold commercially, so having a viable model would be hugely beneficial for testing potential biocontrol agents. The aim was to investigate the immunomodulating potential of EPF on the insect immune response, as being able to suppress the insect immune system it may have implications for the ability of EPF to act synergistically with other control agents in the field. Immunomodulation refers to any process in which an immune response is altered to a desired level. Microorganisms are capable of modulating the response of the immune system to their presence, in order to establish or consolidate an infection. *G. mellonella* were injected with EPF supernatant to investigate whether natural products produced by the EPF had an effect on the cellular or humoral immune response of larvae. It is known that EPF release secondary metabolites to impair cellular recognition and defenses of host insects (Vilcinskas *et al.* 1997b). Effects of the EPF *M. anisopliae* and *B. bassiana*

on the immune system of *G. mellonella* have been previously investigated, but currently there are no reports of the effect of *B. caledonica* on the immune system of *G. mellonella*.

The cellular response of insects primarily consists of phagocytosis and multicellular encapsulation. Plasmatocytes in the hemolymph of *G. mellonella* are involved in cellular defense and are the main phagocytic cell type (Ratcliffe 1993). Hyphal bodies of EPF are often reported not to be ingested by host phagocytic haemocytes due to the lack of surface epitopes necessary for recognition by the host. Mycosis reduced the phagocytic activity of plasmatocytes against yeast cells and blastospores of both *M. anisopliae* and *B. bassiana*. Sub-lethal concentrations of secondary metabolites from *M. anisopliae* (destruxin A and E, cytochalasin B and D) inhibited phagocytic activity of plasmatocytes. Fungal blastospores coated with secondary metabolites were not ingested (Vilcinskas *et al.* 1997b). Plasmatocytes isolated from *M. anisopliae* infected larvae exhibit impaired attachment, spreading and cytoskeleton formation accompanied with occurrence of blebbing and pycnotic nuclei (Vilcinskas *et al.* 1997a). Plasmatocytes treated with destruxin *in vitro* exhibit similar morphological and cytoskeleton alterations. Cytochalasin affects plasmatocytes in a different manner than destruxin A and E. Thus it is suggested that alterations to plasmatocytes in *M. anisopliae* infected larvae are predominantly caused by destruxins released by the fungus during mycosis (Vilcinskas *et al.* 1997a).

Central to the insect humoral immune response is the synthesis of proteins with potent antibacterial and/or anti-fungal activity. The humoral immune response of larval *G. mellonella* comprises a large increase of lysozyme and cecropin-like molecules in the hemolymph which exhibit anti-fungal activity (Gillespie and *et al.* 1997). EPF utilize proteolytic enzymes to digest cuticle proteins of infected hosts to allow them to infect via the exoskeleton (Clarkson and Charnley 1996). Extracellular proteases produced by *B. bassiana* and *M. anisopliae* appear to participate in suppression of cellular immune responses within the hemolymph of infected *G. mellonella* larvae. The ability to produce suitable proteases in sufficient quantities may determine the virulence of particular fungal strains (Gupta *et al.* 1994). Fröbius (2000) purified three inducible serine protease inhibitors (ISPI-1, 2, 3) from larval hemolymph of *G. mellonella* that are active against various serine proteases including trypsin and toxic proteases released by *M. anisopliae.*

Naturally infecting *G. mellonella* with *B. bassiana* led to an antifungal, but not antibacterial host response, this was observed through the induction of gallerimycin and galiomicin gene expression and, consequently, the appearance of antifungal activity in the hemolymph of infected larvae. The activity of lysozyme increased at the beginning of infection and dropped while infection progressed (Wojda *et al.* 2009). *G. mellonella* larvae injected with *B. bassiana* blastospores, displayed a strong antifungal activity and a significant increase in lysozyme activity in larval hemolymph after 24 h, antibacterial activity was dose dependent (Wojda *et al.* 2009). Similarly, Vilcinskas and Matha (1997) showed that injection of *G. mellonella* larvae with *B. bassiana* blastospores elicits release of AMP within the haemolymph such as lysozyme and cecropin. Injection of *B. bassiana* blastospores 48h before the introduction of microbial provocateurs resulted in weaker humoral responses (Vilcinskas and Matha 1997).

The aim of this chapter was to study the effect of EPF on the cellular and humoral immune response of *G. mellonella* so that the findings can ultimately be compared with the effect of EPF on *H. abietis* larvae. This allows investigation into the immunomodulating potential of natural products of EPF on the insect immune response, alongside the ability to validate the use of *G. mellonella* as a model organism. The effect of two of the EPF used, *M. anisopliae* and *B. bassiana*, have previously been reported in *G. mellonella* but not *H. abietis*. The effect of *B. caledonica* on the insect immune response has not been reported for either insect.

2.1.1 Objectives

1. To assess the effect of EPF supernatant on the cellular immune response of *G. mellonella* larvae.
2. To analyze the effect of EPF supernatant on the humoral immune response of *G. mellonella* larvae using proteomic approaches to study changes in hemolymph proteins.
3. To generate data to be utilised in Chapter Three to evaluate the potential of *G. mellonella* larvae as a model for the study of proposed control agents for the large pine weevil *H. abietis* through comparing their immune response to EPF.

2.2 Materials and Methods

2.2.1 Chemicals and reagents

All reagents used were of molecular grade purity where possible and were sourced from Sigma-Aldrich unless stated otherwise.

2.2.2 EPF source

A commercial strain of *M. anisopliae* (Met52) produced by Novozymes (Denmark) was used. Met52 spores were grown and sold on rice grains. The Novozymes product Met52 was purchased on rice grains from National Agrochemical Distributors, Lusk, Dublin. *B. bassiana* experimental strain 1694, was sourced from Becker Underwood. *B. caledonica* (2c7b) is a native strain isolated from a soil sample from an untreated pine stump in a felled forest in Hortland, Co. Kildare. The soil sample was baited with *G. mellonella* and the fungus on the infected cadaver was identified through DNA sequencing.

2.2.3 Maintaining EPF Stocks

Long term storage of entomopathogenic fungi was achieved in a number of ways. Spores were grown on potato dextrose agar and stored at 4°C. Spores were placed in 50% glycerol (v/v) and stored at -80°C. Spores were stored on potato carrot agar slopes at 4°C. Potato carrot agar was made by grating 20 g of potato and 20 g of carrot into 1L of water and boiled for 1h. The solution was sieved, 20 g of agar was added and this was then sterilized by autoclaving. Slopes were made by pouring agar into 28 ml clear glass screw lid McCartney media vials.

2.2.4 Isolating EPF Supernatant

Each of the EPF from 2.2.2 were cultured in Sabouraud Dextrose liquid medium (Oxoid) for 48 h, 72 h and 96 h in a shaking incubator at 25°C and 250 rpm. After each time point the culture was filtered through 0.45 µm syringe filters and then through 0.2µm syringe filters (Sartstedt). The supernatant was collected and stored at -80°C.

2.2.5 *Candida albicans* culture

Yeast extract peptone dextrose (YEPD) broth was prepared by dissolving glucose (2% w/v), yeast extract (1% w/v) (Oxoid) and bacteriological peptone (2% w/v) (Difco), in deionized water and autoclaving at 121°C for 15 min. To make agar plates 2% (w/v) agar was added and autoclaved. Erythromycin was added to the agar at a concentration of 1mg/L. Agar plates were stored until use at 4°C. *C. albicans* MEN (serotype B, wild-type originally isolated from an eye infection (a gift from Dr. D. Kerridge, Cambridge, UK) was cultured to the stationary phase overnight in YEPD at 30°C and 200 rpm on an orbital shaker. *C. albicans* was placed in 50% glycerol (v/v) and placed at -80°for long term storage.

2.2.6 Phosphate Buffer Saline

One phosphate buffer saline (PBS) tablet (Oxoid) was dissolved in 100 ml of deionized water and autoclaved at 121°C for 15 min. PBS was stored at room temperature.

2.2.7 *G. mellonella* larval storage

Sixth instar larvae of *G. mellonella* (Mealworm Company, Sheffield, England) were stored in the dark at 15°C in wood shavings prior to use. Larvae were stored immediately upon receipt from the supplier.

2.2.8 Inoculation of *G. mellonella*

Larvae that had been stored for 1-3 weeks and weighing 0.24-0.28g were used. Larvae were inoculated with 20 µl of fungal supernatant through the last pro-leg using a Myjector U100 insulin syringe (Terumo Europe, Leuven, Belgium). Light pressure was applied to either side of the larva to aid injecting through the proleg. Larvae were placed in 9 cm petri dishes with 90 mm qualitative filter paper (Fisherbrand) and wood shavings. Control larvae were inoculated with 20 µl of Sabouraud Dextrose liquid medium or 20 µl of PBS depending on the experiment.

2.2.9 Extraction of haemocytes and determination of haemocyte density

Larvae were bled by piercing their anterior region with a 30G BD microlance needle and squeezing out 1-3 drops of haemolymph into a chilled Eppendorf containing ice cold PBS. A 1 in 10 dilution was carried out with 0.37% (v/v)

mercaptoethanol in sterile PBS. Haemocyte density was assessed by counting using a Neubauer haemocytometer.

2.2.10 Determining the effect of EPF supernatant on *G. mellonella* haemocyte densities

EPF were cultured in Sabouraud dextrose liquid medium for 48 h, 72 h and 96 h. The culture was filtered through 0.2µm syringe filters. Larvae were inoculated with 20 µl of fungal supernatant through the last proleg. Control larvae were injected with 20 µl of Sabouraud dextrose liquid medium. Ten larvae were injected for each treatment and time point. Haemocyte density was assessed by bleeding five larvae per treatment and then enumerating using a Neubauer haemocytometer. The experiment was performed on three independent occasions.

2.2.11 Determination of the effect of EPF supernatant on yeast load in *G. mellonella*

C. albicans (MEN) was cultured to the stationary phase overnight in YEPD broth at 30°C and 200 rpm. Larvae were inoculated through the last proleg with 20 µl of each fungal supernatant; controls were injected with Sabouraud dextrose (Figure 2.1). Five larvae were injected for each treatment and time point. Larvae were incubated for 24 h at 20°C. After 24 h larvae were given a second injection through a second proleg with *C. albicans* ($1x10^5$/20 µl). After incubation for a further 24 h or 48 h at 20°C, three larvae from each treatment were homogenized in 3 ml of sterile PBS. After serial dilution in PBS, 100 µl of each sample was spread on YEPD plates containing erythromycin (ERY) (1 mg/ml). The plates were incubated for 48 h at 30°C. The yeast load was subsequently calculated as the yeast cell density per larva. Experiments were performed on three independent occasions.

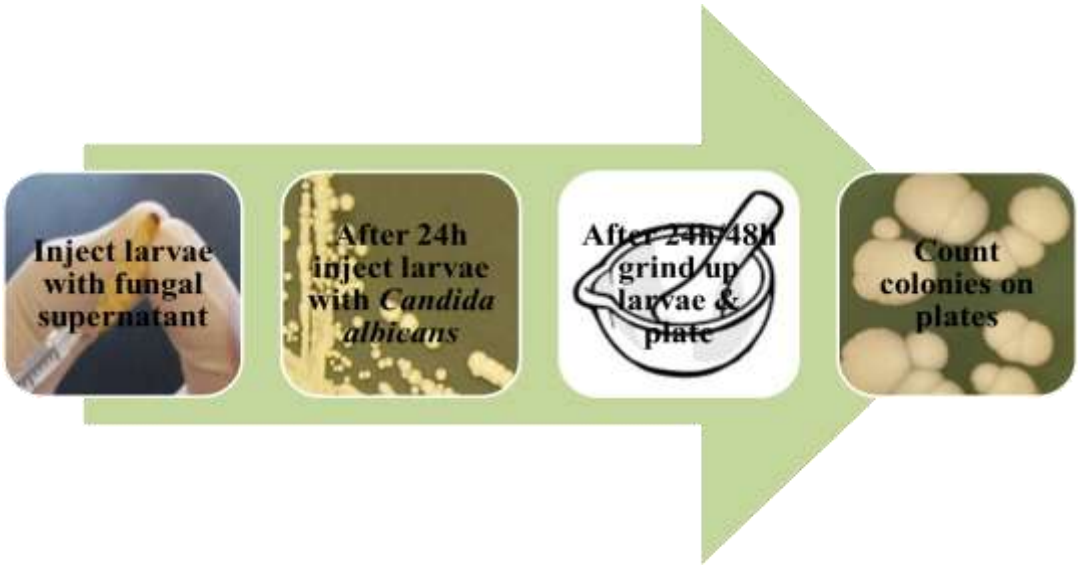


Figure 2.1. Overview of experimental strategy for yeast load determination

2.2.12 Investigating whether EPF supernatant increases susceptibility of *G. mellonella* to a subsequent infection

C. albicans (MEN) was cultured to the stationary phase overnight in YEPD broth at 30°C and 200 rpm. Larvae were inoculated through the last proleg with 20 µl of each fungal supernatant; controls were injected with Sabouraud dextrose. Ten larvae were injected per treatment. Larvae were then incubated for 24 h at 20°C. After 24 h larvae were given a second injection through a second proleg with *C. albicans.* Larvae were incubated at 20°C and mortality was recorded. The experiment was performed on three independent occasions.

2.2.13 1D gel electrophoresis

2.2.14 1.5M Tris-HCl

Tris-HCl (1.5M) was prepared by dissolving 12.1 g trizma base in 175 ml deionized water and adjusted to pH 6.8. Following pH adjustment, solution was autoclaved at 121°C for 15 min. The solution was stored at 4°C.

2.2.15 0.5M Tris-HCl

Tris-HCl (0.5M) was prepared by dissolving 12.1 g trizma base in 175 ml deionized water and adjusted to pH 6.8. Following pH adjustment, solution was autoclaved at 121°C for 15 min. The solution was stored at 4°C.

2.2.16 10% Sodium Dodecyl Sulphate (SDS)

To prepare a 10% (w/v) solution of SDS, 5 g of sodium dodecyl sulphate was dissolved in 50 ml deionized water through vortexing. The solution was stored at room temperature.

2.2.17 10% Ammonium Persulphate (APS)

To prepare a 10% (w/v) solution of APS, 0.1 g of APS was dissolved in 1ml deionized water through vortexing.

2.2.18 10X Electrode Running Buffer

To prepare 10 X running buffer 30 g tris base, 144 g Glycine and 10 g SDS were dissolved in distilled water and made to a volume of 1L. The 10 X buffer was diluted to a working concentration of 1 X in distilled water.

2.2.19 5X Solubilisation buffer for 1D SDS-PAGE

Solubilisation buffer was prepared by mixing 8 ml glycerol, 4 ml deionized water, 1.6 ml 10% (w/v) SDS, 1 ml 0.5 M Tris-HCl, 200 µl bromophenol blue (0.5% w/v) and 400 µl 2-mercaptoethanol. The solution was mixed for 1 h at 4°C, aliquoted and stored at -20°C.

2.2.20 1D SDS-PAGE sample preparation

Protein was extracted from the haemolymph of *G. mellonella* larvae and quantified by a Bradford assay (2.2.24). The volume of sample required to achieve 20 µg/20 µl was ascertained and the sample was made up to 20 µl with 5 X solubilisation buffer. The mixture was then heated at 95°C for 5 min.

2.2.21 1D SDS-PAGE gels

Glass plates were washed and cleaned with 70% (v/v) ethanol prior to use. SDS-PAGE 1D gels were cast using the Mini Protean II gel casting apparatus. Separating gel mixture was prepared using the following components and added to the plates first. To make a stock of 12.5% bis-acrylamide separating gel: 70 ml 1.5 M Tris-HCL (pH 8.9), 93 ml deionized water, 113 ml 30% bis-acrylamide and 2.8 ml 10% SDS were combined. For two 1D gels 50 µl 10% APS and 11.5 µl TEMED were added to 14 ml of stock.

To make a stock of stacking gel the following were mixed: 30 ml 0.5 M Tris-HCL (pH 6.8), 72 ml deionized water, 20 ml 30% bis-acrylamide and 1.2 ml 10% SDS were mixed. For two 1D gels 60 µl 10% APS and 6 µl tetramethylethylenediamine (TEMED) was added to 6 ml of stock, mixed and applied to top of the separating gel. Combs were placed in the gel before it set to create wells for the samples.

2.2.22 1D SDS-PAGE sample loading and voltages

Gels were placed in a Biorad mini gel electrophoresis cell and covered in 1 X running buffer. A current of 40 V was applied to the gels until the protein had moved through the stacking gel, upon which the voltage was increased to 80 V.

2.2.23 2D gel electrophoresis

2.2.24 Bradford Protein Assay

Biorad Bradford protein assay reagent (Biorad Munich Germany) was prepared though a 1/5 dilution in deionized water. To cuvettes (Sarstedt) 980 µl of the reagent and 20 µl of protein were added. A blank was also prepared. The samples were inverted, incubated at room temperature for 5 min and read at an absorbance of 595 nm using an Eppendorf biophotometer.

2.2.25 Acetone precipitation of protein samples

Acetone precipitation was performed to concentrate protein. Following a Bradford protein assay the required volume of protein needed to obtain a concentration of 400 µg was calculated. The required volume of sample was added to a pre-chilled 1.5 ml centrifuge tube and 100 % ice cold acetone was added at ratio of 1:3 (sample: acetone). Protein was left to precipitate at -20°C overnight. The sample was centrifuged at 20,000 x g for 30 min at 4°C to pellet protein. 2D gel electrophoresis was performed as per 2.2.29.

2.2.26 Isoelectric Focusing (IEF) Buffer

The following components were dissolved and mixed in deionized water: 8 M Urea Buffer, 1% (v/v) Triton X-100 (BDH), 4% (w/v) 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS) and 10 mM Tris HCl, 2

mM thiourea. The solution was aliquoted and stored at -20°C. Dithiothreitol (65mM DTT) was added to IEF buffer before use.

2.2.27 Equilibration Buffer

The following components were combined and the pH was adjusted to pH 6.8: 50 mM Tris-base, 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol. The solution was aliquoted and stored at -20°C.

2.2.28 Agarose Sealing Solution

1% (w/v) agarose sealing solution was added to 100 ml 1 X running buffer, heated to mix, and then 0.5% bromophenol blue was added.

2.2.29 2D SDS-PAGE gels

Glass plates were washed in warm water and cleaned with 70% (v/v) ethanol. Acrylamide gels (12.5%) were prepared as follows and poured into the glass plates. The following were combined to make the 2D gels: 60 ml 1.5 M Tris-HCL, 76 ml deionized water, 100 ml 30% (w/v) bis-acrylamide, 2.4 ml 10% (w/v) SDS, 1.5 ml 10% (w/v) APS and 60 µl TEMED.

2.2.30 2D gel electrophoresis to investigate the effect of fungal supernatant on the proteome of *G. mellonella*

Larvae were inoculated with 20 µl of fungal supernatant through the last proleg. Control larvae were injected with 20 µl of Sabouraud dextrose liquid medium. Five larvae were bled per treatment. The experiment was carried out on three independent occasions. Protein was extracted from the hemolymph of *G. mellonella* larvae, quantified using a Bradford assay and 400 µg of protein was acetone precipitated. The acetone precipitate was centrifuged at 20,000 x g for 30 min at 4°C. The pellet was resuspended in 100 µl of IEF buffer and vortexed until dissolved. A further 100 µl of IEF was added with a pipette tip of bromophenol blue. Two µl of Ampholytes (Immobline pH4-7) was added. The sample was placed in a ceramic coffin and a 13 cm Immobline DryStrip 4-7 (GE Healthcare) was placed on top. The strips were covered in Plus One drystrip cover fluid (GE Healthcare) and a lid was placed on top. Focusing was carried out on an Ettan IPGphor II system (Amersham Biosciences, NJ, USA) as follows: Step 1: 50 V Step and Hold 10 h; Step 2: 250 V

Step and Hold 15 min; Step 3: 8000 V Gradient 5 h; Step 4: 8000 V Step and Hold 8 h. After focusing, strips were equilibrated in 10 ml of reducing equilibration buffer (equilibration buffer 2.2.27. with DTT at 0.01 g/ml) for 15 min. Strips were transferred to alkylation buffer (equilibration buffer 2.2.27. with iodoacetamide (IAA) at 0.025 g/ml) for 15 min. After equilibration, strips were rinsed in 1 X running buffer. Strips were placed on top of 2D SDS-PAGE gels and sealed with 1% agarose sealing solution.

The gels were placed in a Proteon Plus^{TM} Dodeca^{TM} Cell gel rig with horizontal electrophoresis cells. The rig was filled with 1 X running buffer, ensuring all acrylamide gel was submerged. Electrophoresis was carried out for 1 h at 1 W/gel, after which it was increased to 1.5 W/gel and run overnight. The following day the power was increased to 2.5 W/gel until the blue tracking line reached the bottom of the gel. The gel rig cooling system was kept at 10°C and the pump was on to ensure even mixing of the running buffer throughout. The gels were transferred to clean dishes for staining.

2.2.31 Colloidal Coomassie Staining

Following 1 D and 2 D electrophoresis, gels were placed in clean dishes on a rotatory shaker and the following steps were performed. Step 1, Fixing: 100 ml (1 D) or 250 ml (2 D) of fixing solution (50% ethanol and 3% phosophoric acid) was added to each gel and incubated for 4 h. Step 2, Washing: 100 ml (1 D) or 250 ml (2 D) of deionized water was added to each gel and incubated for 20 min, this was repeated 3 times. Step 3, Pre-staining: 100 ml (1 D) or 250 ml (2 D) of pre-incubation buffer (34% methanol, 3% phosphoric acid and 17% w/v ammonium sulphate) was added to each gel and incubated for 1 h. After 1 h 1 spatula tip (1D) or 2 spatula tips (2D) of Serva Blue was added to each gel, the gels were incubated for 3-5 days. Step 4, Destaining: 100 ml (1 D) or 250 ml (2 D) of deionized water was added to each gel and incubated for 20 min, this step was repeated three times.

2.2.32 In-gel trypsin digestion and liquid chromatography-mass spectrometry (LC/MS) analysis

2.2.33 Analysis of changes in protein expression

2D gel electrophoresis was performed in triplicate and gels were analyzed using Progenesis Same Spots software (Nonlinear Dynamics) to assess fold changes in

protein expression in treated samples relative to controls. Changes in fold expression were analyzed by ANOVA with p values of <0.05 considered to be statistically significant. The spots corresponding to significantly changed proteins were selected for LC/MS analysis.

2.2.34 Trypsin digestion of protein bands/spots for LC/MS analysis

The method of preparing bands and spots for LC/MS analysis was taken from Shevchenko *et al*. (2007). Scalpels and pipette tips were cut to a variety of lengths and were dipped in acetonitrile prior to use. Spots of interest were excised from 2D gels using pipette tips and were transferred to sterile microcentrifuge tubes. Bands from 1D gels were excised using a scalpel and cut into smaller pieces. Gel pieces were destained by adding 100 µl of 100mM ammonium bicarbonate/acetonitrile in a 1:1 ratio. Gel pieces were incubated at room temperature for 30 min with vortexing every 10 min. Acetonitrile, 500 µl, was added to dehydrate and to shrink gel pieces. After the gel pieces turned white the acetonitrile was removed. Trypsin resuspension buffer was prepared by combining 10 mM ammonium bicarbonate and 10 % (v/v) acetonitrile. Trypsin, 10 ng/µl, was prepared in trypsin resuspension buffer. Gel pieces were covered with 30-50 µl trypsin and placed at 4°C for 30 min to prevent trypsin auto-digestion and allow trypsin to penetrate the gel piece. After 30 min gel pieces were checked to ensure they were completely covered and additional buffer was added if needed. The samples were placed in a 37°C orbital incubator for 24 h to ensure maximum peptide recovery. After digestion, samples were spun down in a microcentrifuge and the supernatant was removed to new 1.5 ml centrifuge tubes. The samples were lyophilized in a vacuum centrifuge to dryness. The samples were stored at -20°C until LC/MS analysis. Samples were resuspended in 20 µl of 0.1 % formic acid and sonicated for 5 min. Samples were transferred to 0.22 µm cellulose acetate spin filter tubes (Spin-X Costar). These were centrifuged at 10,000 x g for 3 min. Samples were transferred to mass spectrometry vials (Agilent). LC/MS analysis was carried out on an Agilent 6340 Ion Trap LC/MS using acetonitrile elution. The LC/MS determines the relative mass to charge ratio from detected ionized particles of the fragmented peptide sample. For the nano pump the column flow rate was 0.6 µl/min with a stop time of 15 minutes, the solvent used were A: 99.9% H_20 and 0.1% Formic acid and B: 90% Acetonitrile, 9.9% H_20 and 0.1% Formic acid. The gradient was 95% solvent A and 5% solvent B. The timetable for the sample run is shown in Table 2.1. For the capillary pump the column flow rate was 4 µl/min. The gradient

was 100% solvent A. The injection volume for the auto sampler was variable depending on the sample.

Table 2.1. LC/MS Timetable	
Time (minutes)	Solvent B
0	5
1	5
7	70
8	100
9	100
10	5

2.2.35 Analysis and identification of LC/MS results

The fragmented protein samples were eluted by LC/MS (Agilent 6340 Ion trap) which determines the relative charge to mass ratio from detected ionized particles. The LC/MS data were analyzed via a Mascot database search (www.matrixscience.com) using a MS/MS Ions Search, which finds identities based on raw MS/MS data from one or more peptides. The database searched against was NCBInr, taxonomy selected was 'other Eukaryota'. The fixed modification selected was Carbamidomethyl and the variable modification was Oxidation. The enzyme selected was trypsin. Mascot scores above 67 were deemed to have a significant match ($p<0.05$). The mass error tolerance was 1 Da allowing for a maximum of no more than two missed cleavages. Spectrum-mill MS Proteomics Workbench (Agilent) was also used to give a more inclusive and accurate list of identities using a 6 Frame *G. mellonella* database. For the MS/MS search Carbamidomethylation and oxidized of methionine were selected as modifications. Verification of protein sequences was confirmed by blasting the protein sequences on the Uniprot (www.uniprot.org) and NCBI (www.ncbi.nlm.nih.gov) websites.

2.2.36 Label Free Quantification of the effect of fungal supernatant on the *G. mellonella* proteome.

Each of the three EPF was grown for 96h, after which supernatant was collected and filtered. Larvae were injected with 20 µl of fungal supernatant and incubated for 48h at 20°C. Five larvae were bled per treatment into a pre-chilled 1.5 ml centrifuge tube and spun at 1,500 x g for 5 min at 4°C. Samples were diluted in PBS and a Bradford assay was carried out (2.2.24). 100 µg of protein was removed and acetone precipitated (2.2.25). The sample was centrifuged at 13,000 x g for 10 min. The acetone was removed and the protein was resuspended in 100 µl of resuspension buffer (5 ml 100 mM ammonium bicarbonate, 4.95 ml deionized water and 50 µl 1 M $CaCl_2$). Samples were sonicated and vortexed to aid resuspension. Protein (75 µg) was reduced with DTT (200 mM) by heating to 95°C for 10 min and was alkylated with IAA (1 M) for 45 min at 25°C in the dark. Alkylation was ended by adding DTT and incubating for 45 min at 25°C. Samples were digested with sequence grade trypsin (Promega, Ireland) at a trypsin:protein ratio of 1:40, overnight at 37 °C.

Tryptic peptides were purified for mass spectrometry using C18 spin filters (Medical Supply Company, Ireland). Samples were mixed with sample buffer (2% trifluoroacetic acid (TFA) in 20% acetonitrile) at a ratio of 3:1 sample to buffer. Activation solution (50% acetonitrile /50% deionized water) was added to the columns and centrifuged at 1,500 x g for 1 min; this was done twice. Equilibration solution (0.5% TFA in 5% acetonitrile) was added to the columns and centrifuged at 1,500 x g for 1 min; this was done twice. Samples were loaded to the top of the resin bed and centrifuged at 1,500 x g for 1 min; flow through was recovered and reapplied to the column. This was repeated a further two times to ensure complete sample binding. Wash buffer (0.5% TFA in 5% acetonitrile) was added to the columns and centrifuged at 1,500 x g for 1 min. This was repeated twice more to remove high levels of contaminants. Columns were placed in fresh 1.5 ml centrifuge tubes and 20 µl of elution buffer (70% acetonitrile) was added to the top of the resin bed and centrifuged at 1,500 x g for 1 min. This was repeated twice more. Samples were dried in a Speedy Vacuum and resuspended in 30 µl of loading buffer (0.05% TFA in 2% acetonitrile) on the day of analysis. Samples were sonicated to aid peptide resuspension and then centrifuged at 13,000 x g for 5 min at room temperature. The samples were transferred to microvials with snap caps (VWR).

Peptide mix, 1 µg, was eluted onto a Q Exactive (ThermoFisher Scientific, U.S.A) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated by an increasing acetonitrile gradient on a Biobasic C18 PicofritTM column (100 mm length, 75 mm ID), using a 120 mins reverse phase gradient at a flow rate of 250 nL /min. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A high resolution MS scan (300-2000 Dalton) was performed using the Orbitrap to select the 15 most intense ions prior to MS/MS.

Protein identification from the MS/MS data was performed using the Andromeda search engine in MaxQuant (version 1.2.2.5; http://maxquant.org/) to correlate the data against a 6-frame translation of the EST contigs for *G. mellonella*. The following search parameters were used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5 ppm with cysteine carbamidomethylation as a fixed modification and N-acetylation of protein and oxidation of methionine as variable modifications and a maximum of 2 missed cleavage sites allowed. Results processing, statistical analyses and graphics generation were conducted using Perseus v. 1.5.0.31. Label free quantification (LFQ) intensities were $\log_2$-transformed and t-tests between the EPF treated larvae and controls were performed using a p-value of 0.05. Proteins were kept in the analysis if they were found in all 4 replicates in at least one group. The Blast2Go suite (www.blast2go.com) of software tools was utilized to assign gene ontology terms (GO terms) relating to biological processes, molecular function and cellular component. Blast was run at NCBI, once complete mapping, annotation and interproscan were run sequentially. Interproscan GOs were merged with annotations. Enzyme code mapping and KEGG was also run. Once all annotation was complete, graphs were made for biological processes, molecular function and cellular components separately for each treatment of the proteins significantly altered in abundance.

2.2.37 Statistical Analysis

Statistical analysis was carried out using Minitab V.16 statistical software and GraphPad Prism V.5. All data were first tested for normality, where data were found not to be normal, the data were transformed before further analysis was carried out. For Section 2.3.1 Data was analysed using two-way ANOVA and Bonferroni post-tests which compared treatments to relevant controls. For Section 2.3.2 data that was not normal was transformed using log or square root transformation. Data were then

analyzed using two-way ANOVA and Bonferroni post hoc tests which compare all treatments to relevant controls. For Section 2.3.3 data were analyzed using MiniTab V.16. Data that were not normal were transformed using arcsine square root or reciprocal 1 x transformations. All data were then tested for significance using paired T-tests.

2.3 Results

2.3.1 The effect of EPF supernatant on haemocyte densities of *G. mellonella* larvae

Larvae treated with *B. caledonica* supernatant showed an alteration in haemocyte densities at both 24h and 48h (Figure 2.2.A). Treatment ($F_{3,16}$=9.58, $p<0.001$) and incubation time ($F_{3,16}$=6.92, $p<0.05$) had a significant effect on haemocyte densities. There was a significant interaction between treatment and incubation time ($F_{3,16}$=4.45, $p<0.05$). After 24h a significant difference was seen in larvae treated with *B. caledonica* fungal supernatant grown for 48h relative to control larvae ($p<0.05$). After 48h incubation a significant difference was seen in larvae treated with supernatant grown for 72h ($p<0.001$) and 96h ($p<0.01$) relative to control larvae.

In larvae treated with *B. bassiana* supernatant, treatment had a significant effect on haemocyte densities ($F_{3,16}$=7.19, $p<0.01$). Incubation time did not have a significant effect but there was a significant interaction between treatment and time ($F_{3,16}$=6.35, $p<0.01$). Larvae treated with *B. bassiana* fungal supernatant showed an alteration in haemocyte densities at both 24h and 48h (Figure 2.2.B). After 48h incubation there was a significant difference in haemocyte densities in larvae treated with supernatant grown for 48h ($p<0.01$), 72h ($p<0.01$) and 96h ($p<0.001$) relative to controls.

In larvae treated with *M. anisopliae* supernatant, treatment had a significant effect on haemocyte densities ($F_{3,16}$=20.77, $p<0.001$). Incubation time did not have a significant effect but there was a significant interaction between treatment and time ($F_{3,16}$=7.54, $p<0.01$). Larvae treated with *M. anisopliae* supernatant showed an alteration in haemocyte densities at both 24h and 48h (Figure 2.2.C). After 24h there was a significant difference in haemocyte densities in larvae treated with supernatant grown for 48h ($p<0.001$) relative to controls. After 48h a there was a significant difference in haemocyte densities in larvae treated with *M. ansioplae* supernatant grown for 48h ($p<0.001$), 72h ($p<0.001$) and 96h ($p<0.001$).

All significant differences in haemocyte densities between treatments and their appropriate controls were in the direction of reduction. The results demonstrated that all three EPF cause a decline in the haemocyte densities of larvae following

inoculation with 72h or 96h fungal supernatant and incubation for 48h, relative to their appropriate controls. There was also a significant interaction between treatment and time in all EPF tested meaning treatments perform differently after different incubation periods.

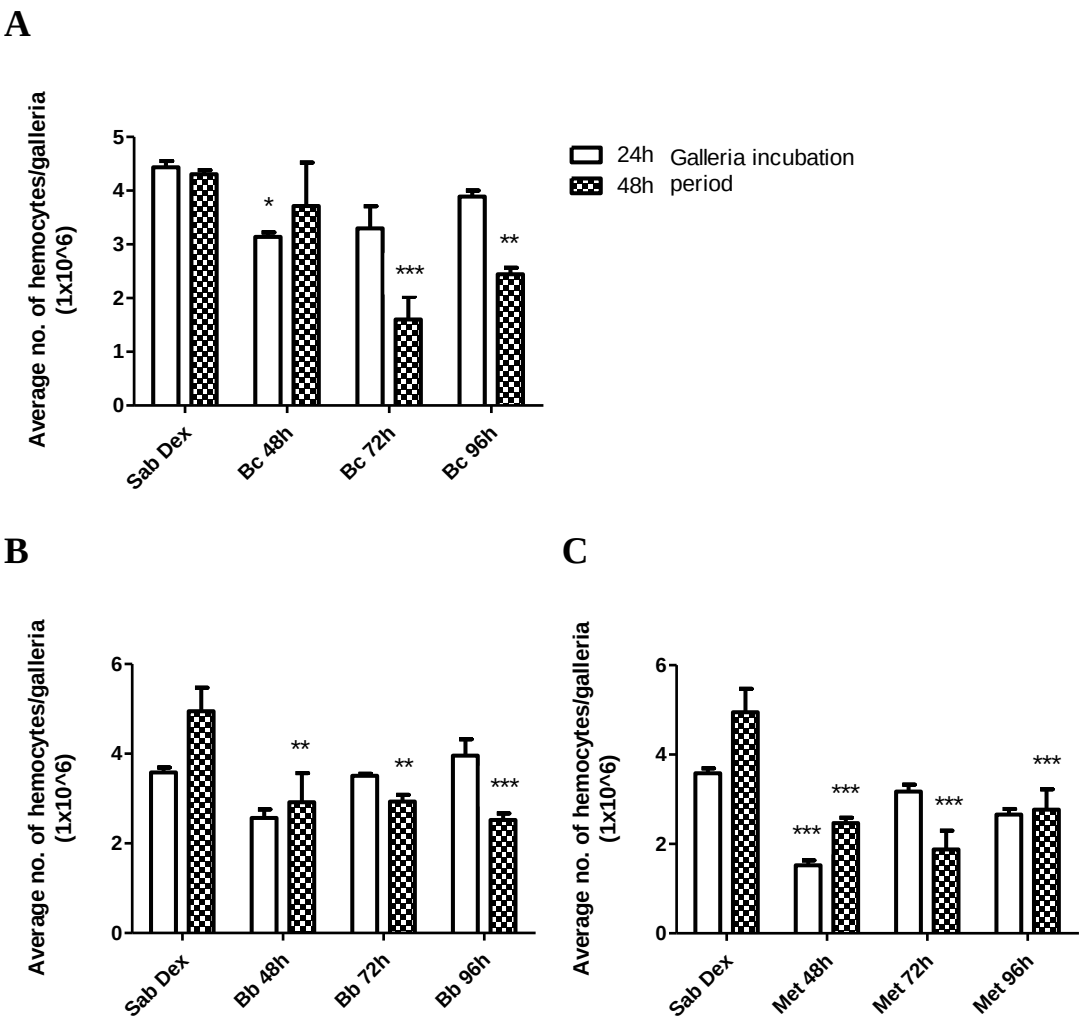


Figure 2.2. Haemocyte densities in larvae treated with EPF supernatant.

Following inoculation with fungal supernatant, larvae where incubated for 24h or 48h at 20°C before bleeding and enumeration. Sab Dex: Control media, Bc: *B. caledonica*, Bb: *B. bassiana* and Met: *M. anisopliae*. X-axis represents length of time EPF was cultured for: 48h, 72h, 96h. Data was analysed using Two Way ANOVA and Bonferroni post-tests which compared treatments to relevant controls. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Larvae treated with **A.** *B. caledonica* supernatant, **B**. *B. bassiana* supernatant, **C.** *M. anisopliae* supernatant.

2.3.2 Determination of the effect of EPF supernatant on yeast load in *G. mellonella*

In larvae treated with *B. caledonica* supernatant, treatment had a significant effect on yeast load ($F_{3,16}$=5.01, $p<0.05$). Incubation time did not have a significant effect and there was no significant interaction between treatment and time. Treatment with *B. caledonica* 96h supernatant induced a significantly alteration ($p<0.01$) in yeast cell density in larva, with a fold increase of three relative to controls (Figure 2.3.A), when the larvae were incubated for 48h following injection with *C. albicans*.

In larvae treated with *M. anisopliae* supernatant, treatment had a significant effect on yeast load ($F_{3,16}$=0.16, $p<0.001$). Incubation time did not have a significant effect and there was not a significant interaction between treatment and time. Treatment with *M. anisopliae* 96h supernatant induced a significantly alteration in yeast load ($p<0.05$) with a fold increase of 3.76 relative to controls, when the larvae were incubated for 48h following injection with *C. albicans* (Figure 2.3.B). Additionally, treatment with *M. anisopliae* 72h supernatant caused a 4.42 fold increase ($p<0.01$) when the larvae were incubated for 24h following injection with *C. albicans* (Figure 2.3.B). In contrast, treatment with *B. bassiana* did not cause a significant alteration in yeast cell density in larvae relative to controls (Figure 2.3.C).

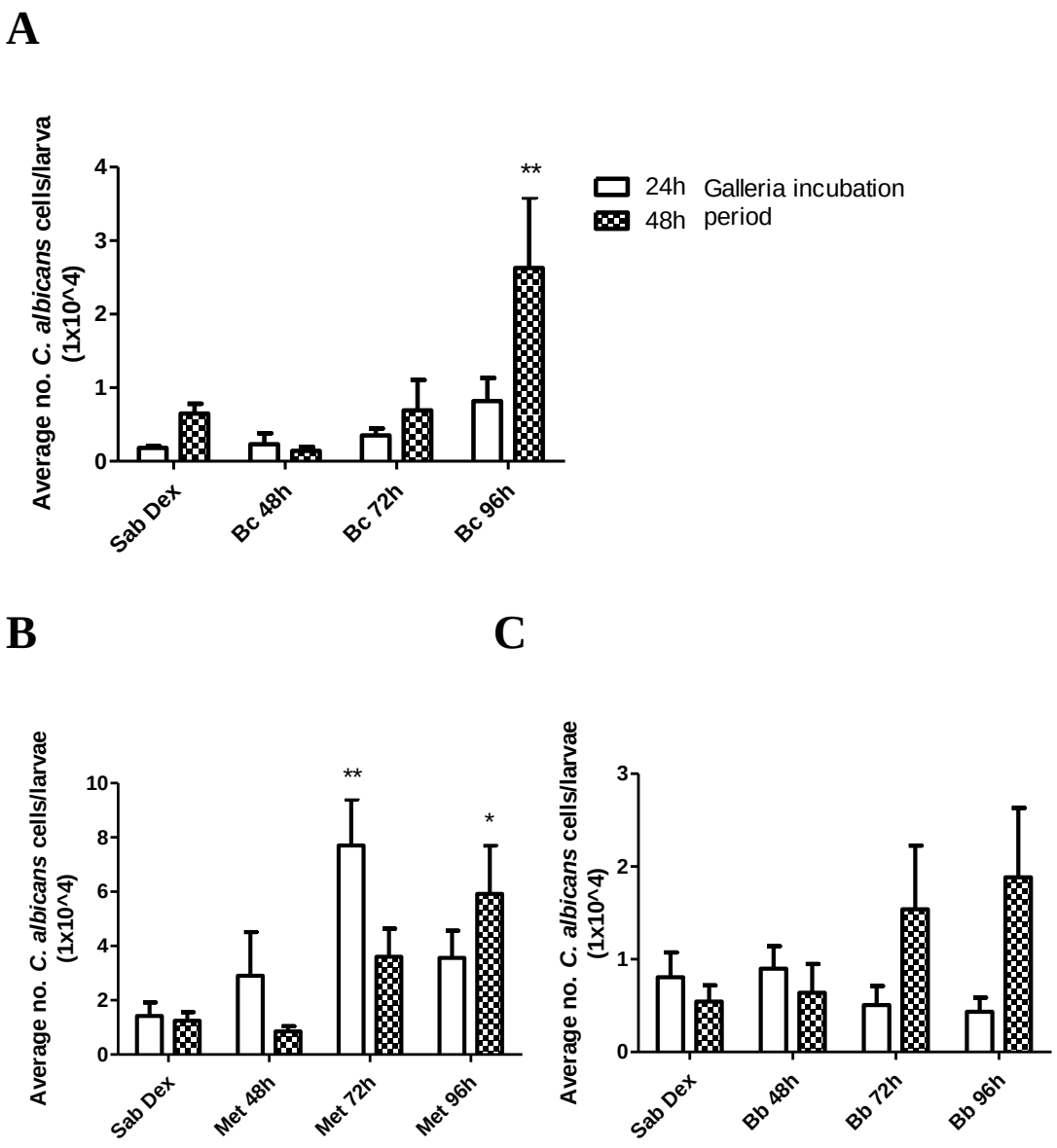


Figure 2.3. Changes in yeast load in *G. mellonella* pre-treated with EPF supernatant.

Number of *C. albicans* cells per larva after incubation for 24h and 48h at 20°C. Larvae were treated with fungal supernatant 24h prior to inoculation with C. *albicans*. Sab Dex: Control media, Bc: *B. caledonica*, Bb: *B. bassiana* and Met: *M. anisopliae*. X-axis represents length of time EPF was cultured for: 48h, 72h and 96h. Data that was not normal was transformed using log or square root transformation. Data was then analyzed using Two Way ANOVA and Bonferroni post-tests which compared treatments to relevant controls. * $p<0.05$, ** $p<0.01$. Larvae treated with **A.** *B. caledonica* supernatant. **B.** *M. anisopliae* supernatant. **C.** *B. bassiana* supernatant

2.3.3 Does EPF supernatant increase susceptibility of *G. mellonella* to infection

To further investigate whether treatment with fungal supernatant makes larvae more susceptible to subsequent pathogens, larvae where injected with fungal supernatant and incubated for 24h at 20°C. After 24h larvae were inoculated with *C. albicans*. Larvae were incubated at 20°C and mortality was recorded over a two week period.

One week after *C. albicans* infection, both *M. anisopliae* ($T= -6.93$, $p<0.05$) and *B. caledonica* ($T=-4.74$, $p<0.05$) showed significantly different mortalities when larvae were treated with a combination of fungal supernatant and *C. albicans* combined compared to single doses (Figure 2.4.A). Two weeks after *C. albicans* infection larvae treated with *M. anisopliae* ($T= 7$, $p<0.05$) and *B. caledonica* ($T= -4.75$, $p<0.05$) had significantly different mortalities when larvae were treated with a combination of fungal supernatant and *C. albicans* combined compared to single doses, with *M. anisopliae* and *C. albicans* combined causing 100% mortality (Figure 2.4.B).

Larvae were treated with *M. anisopliae* and *B. caledonica* supernatant before treatment with *C. albicans*, had higher mortalities than larvae treated with either supernatant or C. albicans alone. This pattern was not seen with *B. bassiana* (Figure 2.4. A+B).

Larvae treated with *M. anisopliae* had a distinctive colouring with darkening around the prolegs, which covered the body as incubation time increased (Figure 2.5). This colouration was not observed in larvae injected with *B. caledonica* or *B. bassiana*. This colouration was also coupled with immediate tetanic paralysis post injection with supernatant. This effect is also seen in the tobacco hornworm, *Manduca sexta*, where injection of destruxin, a toxin produced by *M. anisopliae* resulted in immediate tetanic paralysis due to sustained muscle contraction, followed by flaccid paralysis (Samuels *et al.* 1988).

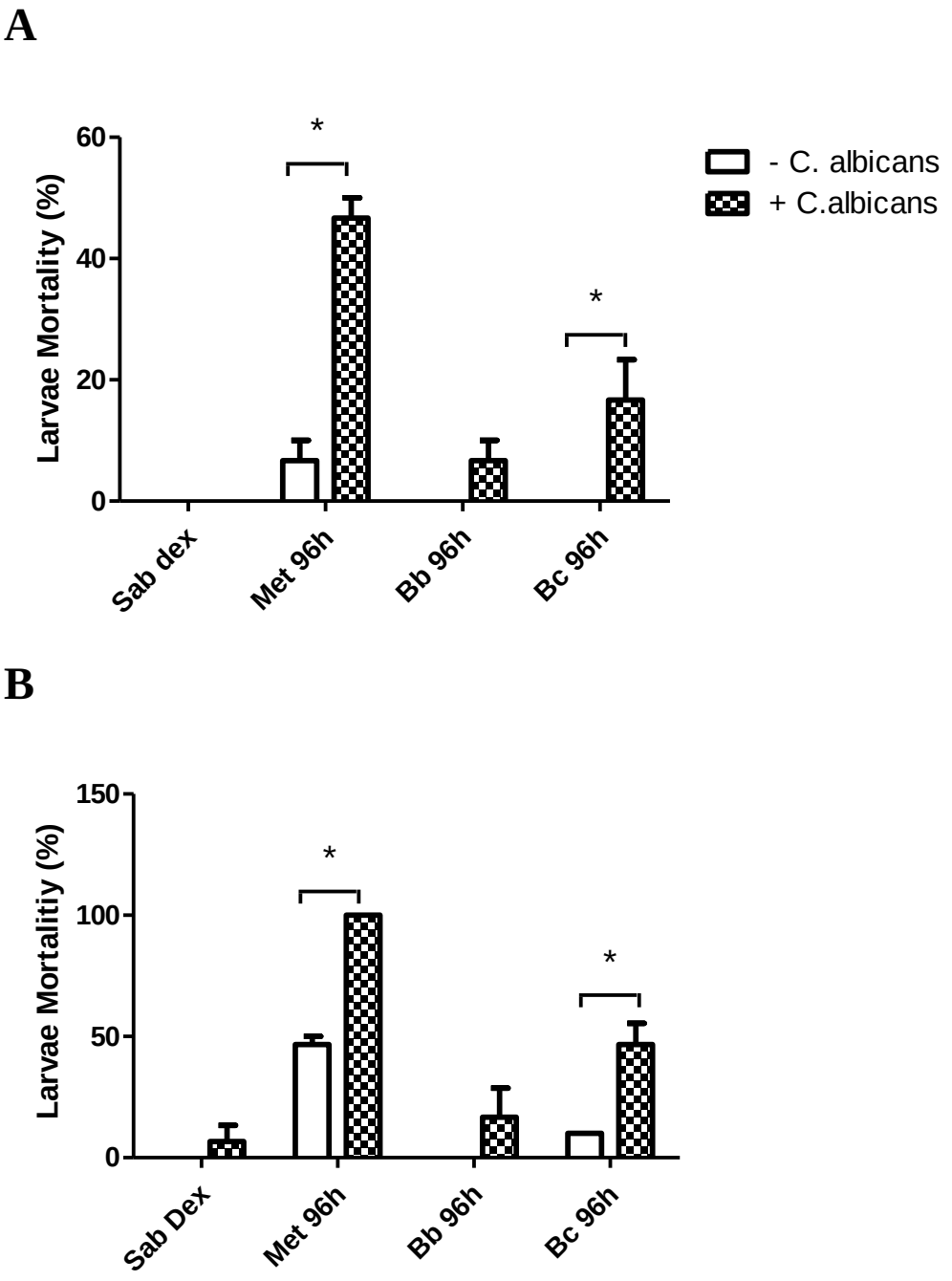


Figure 2.4. Mortality of *G. mellonella* larvae treated with EPF supernatant alone and in combination with *C. albicans*.

+ C. albicans indicates larvae that received a dose of *C. albicans* after 24h, *- C. albicans* indicates larvae that did not. Sab Dex: Control media, Bc: *B. caledonica*, Bb: *B. bassiana* and Met: *M. anisopliae*. X-axis represents length of time EPF was cultured for: 48h, 72h and 96h. Data that were not normal were transformed using arcsine square root or reciprocal 1 x transformations. All data were then tested for significance using paired T-tests. * $p<0.05$, ** $p<0.01$. **A.** Mortality one week after *C. albicans* infection. **B.** Mortality two weeks after *C. albicans* infection.

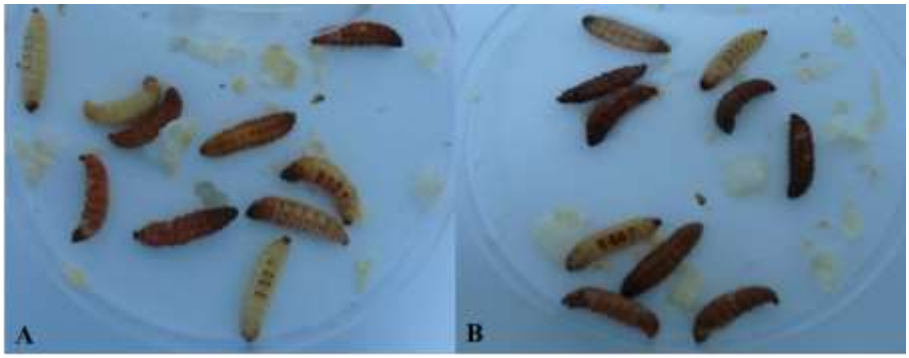


Figure 2.5. *G. mellonella* larvae treated with *M. anisopliae* 96h fungal supernatant. **A.** after 3 days **B.** after 6 days. Larvae had a characteristic colouring, paralysis and darker faeces.

2.3.4 2-Dimensional gel electrophoresis analysis of variations in the proteomic profile of *G. mellonella* larval hemolymph following treatment with EPF supernatant

To investigate the effect of fungal supernatant on the proteomic profile of larval hemolymph, larvae were injected with fungal supernatant from 48h, 72h and 96h cultures. Larvae were incubated for 24h and 48h. Larvae were bled, protein was extracted and resolved by 2D SDS PAGE as described in 2.2.30. Only the results for larvae injected with *M. anisopliae* and *B. bassiana* 96h and incubated for 48h are shown because they caused the most dramatic alteration in the proteome of larval hemolymph.

Larvae incubated for 48h following injection with *M. anisopliae* grown for 96h (Figure 2.6 A+B, Table 2.2) had three spots significantly increased in abundance and three significantly decreased in abundance. Scolexin precursor was found at a higher abundance in treated larvae (Spot 1; 3.8 fold increase, $p<0.01$, Figure 2.6 A+ B). Scolexin is a serine protease homolog that induces coagulation (Finnerty *et al.* 1999). Serpin 3a was identified as being increased in abundance in treated larvae (Spot 4; 1.8 fold increase, $p<0.05$, Figure 2.6 A+B). Serpin 3a is a serine protease inhibitor involved in regulation of the pro-PO activation cascade (Yifei Zhu *et al.* 2003). A hypothetical protein was also increased in abundance in treated larvae (Spot 2: 1.7 fold increase, $p<0.05$, Figure 2.6 A+B). Another member of the serpin superfamily, serine protease inhibitor, was found to be reduced in abundance in treated larvae (Spot 3; 1.3 fold decrease, $p<0.05$, Figure 2.6 A+B). A member of the vWFA superfamily: Predicted inter alpha-trypsin inhibitor heavy chain H4-like (serine protease inhibitor) was also reduced in abundance in the hemolymph of *M.*

anisopliae larvae (Spot 5; -2, -1.9 fold decrease, $p<0.05$ and Spot 6, -2 fold decrease, $p<0.05$, Figure 2.6 A+B).

Larvae incubated for 48h following injection with *B. bassiana* grown for 96h (Figure 2.6 C+D, Table 2.3) had 3 spots significantly increased in abundance and 5 significantly decreased in abundance. 27kDa hemolymph protein was found at a higher abundance in treated larvae (Spot 5; 1.7 fold increase, $p<7.772e-004$ and Spot 6; 1.8 fold increase, $p<0.01$ Figure 2.6 C+D). In contrast a 27kDa hemolymph protein was also found to be decreased in abundance in treated larvae (Spot 3; -2 fold decrease, $p<0.05$). Lopap was found at a higher abundance in treated larvae (Spot 4; 1.4 fold increase, $p<0.05$). Lopap (*L. obliqua* prothrombin activator protease) is a single-chain 69 kDa serine protease implicated in coagulation (Fritzen *et al.* 2005). Apolipophorin III, a protein involved in the insect immune response, pattern recognition, detoxification and lipid transport (Zdybicka-Barabas *et al.* 2012), had a significantly decreased level of expression in treated larvae (Spot 1; 2.7 fold decrease, $p<0.01$ and Spot 2; 1.5 fold decrease and $p<0.01$ Figure 2.6 C+D). A molecule peptide in iron metabolism 32kDa ferritin subunit was found at a lower abundance in treated larvae (Spot 7; 1.5 fold decrease, $p<0.05$), ferritin is an iron storage protein that may also have a role in the immune system (Levy *et al.* 2004, Choi *et al.* 2006). Larvae treated with *B. bassiana* supernatant displayed a reduced abundance in a member of the vWFA superfamily: Predicted inter alpha-trypsin inhibitor heavy chain H4-like protein (Spot 8; 1.4 fold decrease, $p<0.05$).

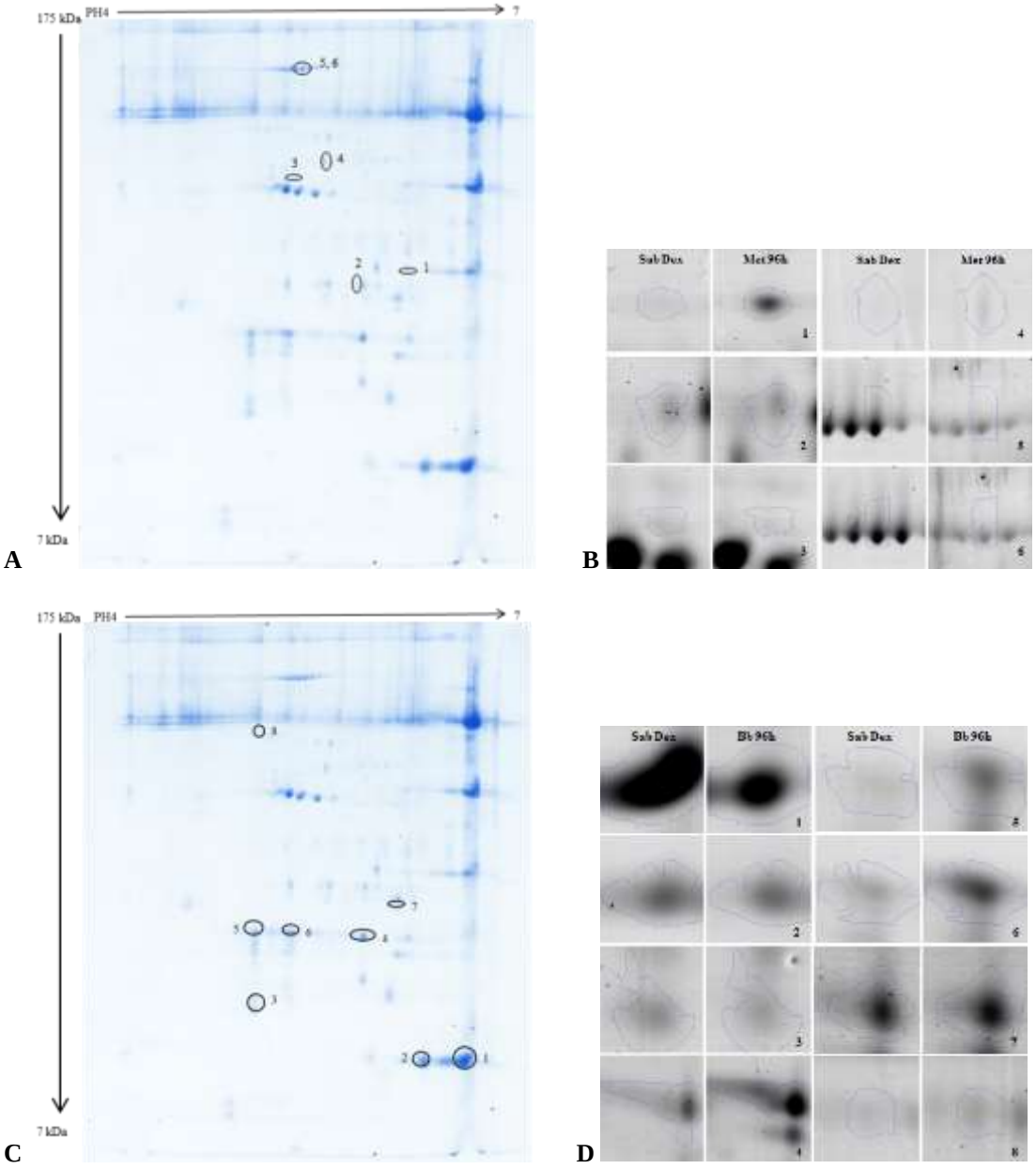


Figure 2.6. 2D SDS PAGE gel images of *G. mellonella* haemolymph following treated with EPF supernatant

A + C: Representive 2D SDS PAGE gel image of *G. mellonella* haemolymph highlighting alterations in proteomic profile between control larvae and those injected with EPF supernatant grown for 96h. **A:** *M. anisopliae* **C.** *B. bassiana*. Changes in protein abundance were identified by Progenesis software, spots were excised, digested and analysed by L/CMS. **B + D:** Progenesis images comparing spots changed significantly in abundance, numbers corresponds to labels on 2D SDS PAGE gel. **B:** *M. anisopliae* **D:** *B. bassiana*.

Table 2.2. Protein identities for excised and digested spots 1-6 (figure 2.6 A+B) identified by LC/MS.

Fold change in protein abundance and corresponding p-value was determined using Progenesis SameSpot Software.

[1] Mr: molecular weight [2] pI: isoelectric point

Spot Number	Protein Identity	Mr[1]	pI[2]	Coverage (%)	Number of Unique Pepitides	Fold Change	P value
1	Tryp-SPc:Scolexin precursor	61044	9.68	18.9	8	+3.8	0.009
2	Hypothetical protein KGM 00684	37400	6.34	34	9	+1.7	0.032
3	Serine protease inhibitor	51104.9	6.01	50.1	18	-1.3	0.05
4	Serpin 3a	116464.7	9.64	6.5	5	+1.8	0.028
5	Predicted inter alpha-trypsin inhibitor heavy chain H4-like	58288.6	5.79	7.8	4	-2	0.002
6	Predicted inter alpha-trypsin inhibitor heavy chain H4-like	58288.6	5.79	17.9	8	-1.9	0.002

Table 2.3. Protein identites for excised and digested spots 1-8 (figure 2.6 C+D) identifed by LC/MS.

Fold change in protein abundance and corresponding p-value was determined using Progenesis SameSpot Software. 27kDa haemolymph protein appeared several times with different fold changes, may be because it is a very abundant protein or is breaking up into fragments. [1] Mr: molecular weight [2] pI: isoelectric point

Spot Number	Protein Identity	Mr[1]	pI[2]	Coverage (%)	Number of Unique Pepitides	Fold Change	P value
1	Apolipophorin III	20499	8.59	50	11	-2.7	0.002
2	Apolipophorin III	20499	8.59	51	4	-1.5	0.005
3	27 kDa haemolymph protein	18279.6	5.58	22.3	2	-2	0.02
4	Lipocalin: Lopap	29926.5	7.14	37.9	9	+1.4	0.046
5	27 kDa haemolymph protein	18279.6	5.58	47.8	4	+1.7	7.772e-004
6	27 kDa haemolymph protein	18279.6	5.58	27.9	4	+1.8	0.005
7	32 kDa ferritin subunit	44434	9.17	18.1	5	-1.5	0.038
8	Predicted inter alpha-trypsin inhibitor heavy chain H4-like	58288.6	5.79	14.9	7	-1.4	0.03

2.3.5 Label free shotgun proteomic analysis of variations in the proteomic profile of *G. mellonella* larval hemolymph following treatment with EPF supernatant

Label-free quantitative (LFQ) mass spectroscopy (MS) aims to quantify peptides and proteins without the use of protein labels. Label free MS measures the relative abundance of proteins across multiple sample groups in single MS runs (Bantscheff *et al.* 2012). Thus it allows the comparison of proteomes of different samples to identify changes to protein expression, molecular processes or biological pathways. Here, label free quantification (LFQ) was used to compare the hemolymph proteome of *G. mellonella* larvae treated with EPF fungal supernatant relative to control larvae (Section 2.2.36). The four sample groups analyzed were larvae treated with *M. anisopliae* fungal supernatant grown for 96h, larvae treated with *B. caledonica* fungal supernatant grown for 96h, larvae treated with *B. bassiana* fungal supernatant grown for 96h and control larvae (treated with Sabouraud dextrose media). Principal component analysis (PCA) was used to emphasize variation and visualize strong patterns in the data. It confirmed a difference between the expression profiles off different treatments and that there were not outliers within the four replicates of each treatment (Figure 2.7). It also showed that the expression profile of larvae treated with *M. anisopliae* was most divergent than the other EPF treatments relative to control larvae.

In total, 100 proteins were identified with two or more peptides. Seventy eight of these proteins were either significantly changed in expression or uniquely detected across the four treatments analyzed. Ten proteins were found to be absent (below the level of detection) in one or more sample groups and present (above the level of detection) in three or less sample groups. These proteins were termed ‘uniquely detected proteins’ (Table 2.4). A data imputation step was performed to replace missing values with values that simulate low abundant proteins. These values were chosen randomly from a distribution specified by a downshift of 2.19 times the mean standard deviation (SD) of all measured values and a width of 0.36 times this SD. A number of uncharacterized, hypothetical or unknown proteins were identified and these were subsequently annotated using their Contig name throughout. To obtain an overall proteomic profile of abundance for all significantly expressed and exclusive

proteins, hierarchical clustering on Z-score normalised intensity values was performed and the relative protein expression values were displayed as a heat map (Figure 2.8).

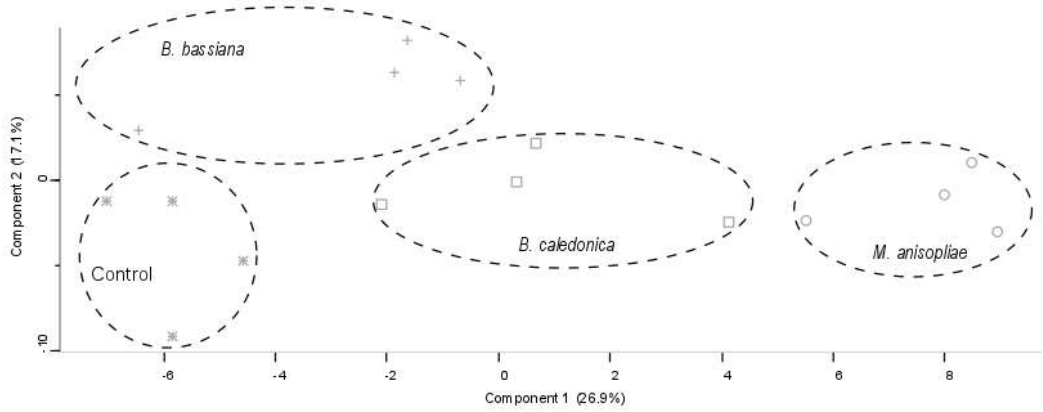


Figure 2.7. Principal component analysis (PCA) of *G. mellonella* haemolymph treated with EPF fungal supernatant versus control (media).

Four replicates of each sample group were included in Perseus analysis. Dashed circles denote sample groups.

Table 2.4. Uniquely detected proteins in the haemolymph of both control larvae and larvae treated with EPF fungal supernatant.

NaN indicates a protein that was absent or below the level of detection. These proteins were termed as being 'uniquely detected. [1]PEP: Posterior Error Probabilities

Protein names	Mean LFQ intensity				[1]PEP	Intensity	MS/MS	Peptides	Sequence Coverage (%)	Mol. Weight (kDa)	Sequence length
	SB	MET	BB	BC							
Apyrase	26.4	26.1	26.7	NaN	8.1^{-39}	7.4^{+08}	41	9	13.9	79.5	714
Cecropin-D precursor	27.5	NaN	27.7	28.4	1.4^{-154}	2.2^{+09}	48	2	14.5	15.4	138
Peptidoglycan recognition-like	26.7	NaN	26	27.9	9.5^{-177}	1.5^{+09}	49	2	16.7	17.8	156
Aminoacylase-1-like	27.3	NaN	NaN	27.5	1.7^{-12}	1.3^{+09}	30	4	16.4	46.7	432
Prophenoloxidase activating factor 3	NaN	29.3	27	29	0	2.7^{+09}	68	5	37.3	25.8	233
Gustatory receptor candidate 25	NaN	29.9	28.8	29	1.5^{-277}	5.4^{+09}	46	2	17	25.8	235
Contig22050_1	NaN	26.2	25.4	26.8	6^{-107}	5.9^{+08}	33	6	20.4	48.4	422
Beta actin	NaN	29.1	28.2	28.3	9.4^{-80}	1.9^{+09}	39	6	38.7	30.1	279
Peptidylprolyl isomerase B precursor	NaN	25.9	NaN	25.8	1.9^{-07}	3.1^{+08}	25	3	14.1	39.1	354
Contig20011_1.r1.	25.9	NaN	NaN	NaN	0	4.4^{+08}	24	2	7.1	57.6	539

Hierarchical clustering resolved four distinct clusters of proteins with similar expression profiles. Cluster A (Table 2.5) is indicated with light blue in Figure 2.8, Cluster B (Table 2.6) in light purple, Cluster C (Table 2.7) in pink and Cluster D (Table 2.8) in green. It appears that Cluster C may have two sub groups but these are not clearly distinguishable based on their mean intensities (Table 2.7) so are combined for future discussion. Cluster A comprises proteins with higher levels of abundance in larvae treated with *M. anisopliae* and *B. caledonica* relative to control larvae and intermediate intensities in *B. bassiana* treated larvae (Table 2.5). Cluster B comprises proteins with lower levels of abundance in larvae treated with *B. bassiana* relative to all other treatments and control larvae (Table 2.6). Cluster C comprises proteins with lower levels of abundance in larvae treated with *M. anisopliae* relative to all other treatments and control larvae (Table 2.7). Cluster D comprises proteins with higher levels of abundance in larvae treated with *B. bassiana* relative to all other treatments and control larvae (Table 2.8).

For LFQ analysis all proteins listed as significantly changed in abundance are those with a p-value less than 0.05 ($p<0.05$) and a relative fold change of 1.5 or above. In larvae treated with *B. caledonica* fungal supernatant 15 proteins were significantly increased in abundance and six proteins were significantly decreased in abundance (Figure 2.9; Table 2.9). There appears to be a number of changes to proteins involved in the regulation and maintenance of PO following treatment with *B. caledonica* fungal supernatant. ProPO activating factor 3 (PPAF 3), proPO activating enzyme 3 (PPAE 3), serpin 3a, serine proteinase, protease inhibitor 1 precursor and scolexin are all increased in expression and may play a role in the proPO pathway. PO activities are vital for early host responses to EPF on the cuticle, during encapsulation responses to EPF in the haemocoel and for the production of melanin and ROS and RNS. Serine proteinases and the molecules they produce can be damaging to both the insect host and the fungal pathogen, affecting nucleic acids, peptides and oxidation of lipids (Jiang *et al.* 2010, Butt *et al.* 2016). Therefore it is important that these pathways are tightly regulated.

Also found upregulated in larvae treated with *B. caledonica* fungal supernatant was gloverin, a glycine-rich polypeptide previously isolated from lepidopteran hemolymph. Gloverins have a mass of 14 kDa and contain 18% of glycine residues. This AMP is effective against Gram-negative bacteria. They act by inhibiting the synthesis of vital outer membrane proteins resulting in an increase in permeability of

the outer membrane of the bacteria (Bulet *et al.* 1999). One implication of an upregulation in AMP active against bacteria is that antibacterial activities can be highly beneficial to EPF as it might help exclude opportunistic infections that would be disadvantageous to the fungus (Butt *et al.* 2016).

In larvae treated with *B. bassiana* fungal supernatant 12 proteins were significantly increased in abundance and 12 proteins were significantly decreased in abundance (Figure 2.10; Table 2.10). In larvae treated with *M. anisopliae* fungal supernatant 20 proteins were significantly increased in abundance (Figure 2.11; Table 2.11). Additionally 23 proteins were significantly decreased in abundance (Figure 2.11). The top ten proteins in highest abundance and top ten proteins in lowest abundance are highlighted with a red box in the volcano plots.

As well as comparing the haemolymph proteome of larvae treated with EPF supernatant to control larvae, the proteomes of different EPF treated larvae were compared against each other using Perseus software. When larvae treated with *B. caledonica* fungal supernatant were compared to those treated with *B. bassiana* nine proteins were significantly increased in abundance and 11 proteins were significantly decreased in abundance (Figure 2.12; Table 2.12). When larvae treated with *M. anisopliae* fungal supernatant were compared to those treated with *B. caledonica* four proteins were significantly increased in abundance and 14 proteins were significantly decreased in abundance (Figure 2.13; Table 2.13). When larvae treated with *M. anisopliae* fungal supernatant were compared to those treated with *B. bassiana* 14 proteins were significantly increased in abundance and 17 proteins were significantly decreased in abundance (Figure 2.14; Table 2.14).

Blast2GO annotation software (www.blast2GO.com) was used to group proteins based on conserved gene ontology (GO) terms in order to identify processes and pathways altered in response to different EPF treatments. However Blast2GO assigned GO terms to only 68 of the 100 proteins identified by LFQ, so this must be taken into account before drawing conclusions about apparent changes in processes and pathways induced by treatment with EPF fungal supernatant. A comparison of changes in proteins annotated by Blast2GO as being involved in cellular processes (Figure 2.15), molecular processes (Figure 2.16) and biological processes (Figure 2.17) is presented.

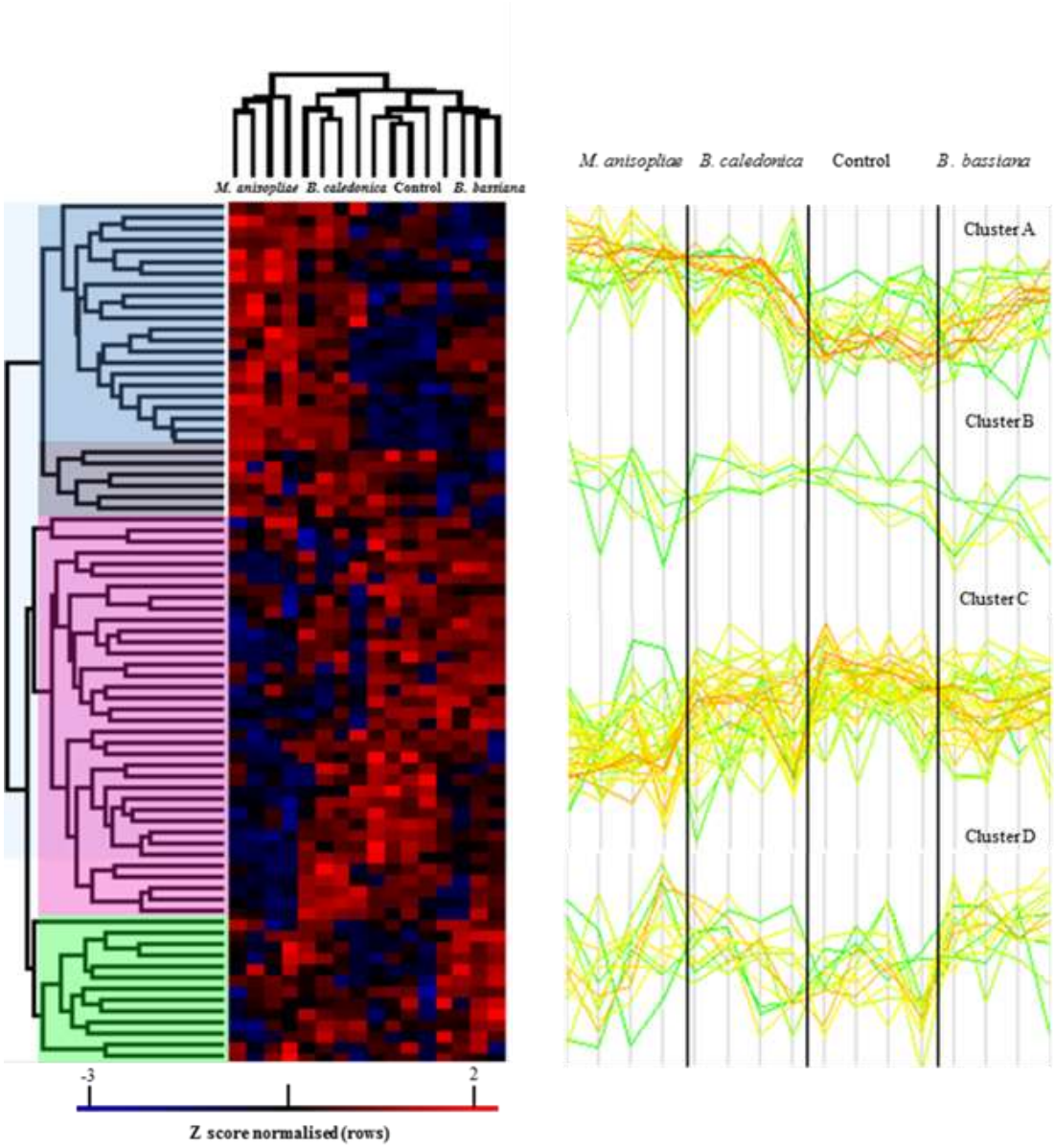


Figure 2.8. Hierarchical clustering of the quantitative differences in the proteomic profile of *G. mellonella* larvae treated with EPF fungal supernatant versus control.

The heat map shows the variation in expression of the proteins from the profiles of *G. mellonella* larvae obtained using Perseus software. The data summarized are for all significantly expressed and uniquely detected proteins. Hierarchical clustering (columns) resolved four distinct clusters. Cluster A is indicated with light blue, Cluster B light purple, Cluster C pink and Cluster D green. Red indicates high level expression and blue indicates low level of expression.

Table 2.5. Proteins of altered abundance in *G. mellonella* from Cluster A

MET and BC treated larvae a higher level of abundance than SB and BB in the proteins in this Cluster.

Cluster A	Average LFQ Intensities			
Protein names	**MET**	**BC**	**SB**	**BB**
Contig_1159.0	27.5	26.8	24.6	25.5
Contig_962.0	29.2	29.3	26.1	27.4
27 kDa hemolymph protein	36.7	35.5	35.4	35.3
Methionine-rich storage protein 2	37.4	37.3	36.8	37.1
Prophenoloxidase activating factor 3	29.3	28.6	24.7	26.0
Fibrohexamerin; 25 kDa silk glycoprotein	30.8	29.1	28.4	28.7
Glyceraldehyde-3-phosphate dehydrogenase	28.3	28.4	27.9	26.0
Putative defence protein Hdd11	29.9	28.6	27.5	26.9
Gustatory receptor candidate 25	28.8	27.9	24.5	28.8
Protease inhibitor 1 precursor	31.4	30.3	28.8	29.5
Contig22050_1	26.1	26.5	24.7	24.5
Twelve cysteine protein 1	30.7	30.3	30.0	30.3
Prophenoloxidase activating enzyme 3	29.5	29.2	27.4	28.2
Alpha-esterase 45; esterase FE4-like isoform	26.9	26.6	26.0	26.0
Heat shock protein 25.4 precursor	32.0	30.9	30.0	31.5
27 kDa hemolymph protein	30.0	29.4	28.4	29.1
Spodoptericin	31.1	30.8	30.7	30.3
Beta actin	29.1	25.6	25.2	25.6
Hemolin	32.2	31.7	31.5	31.1
Scolexin	31.4	30.8	29.7	30.7
Gelsolin	28.9	28.7	26.7	26.4
Gelsolin	33.3	32.7	32.6	33.0

Table 2.6. Proteins of altered abundance in *G. mellonella* from Cluster B.

MET: *M. anisopliae*; BC: *B. caledonica*; SB: Control; BB: *B. bassiana*. BB treated larvae have a lower level of abundance than MET, BC and SB in the proteins in this Cluster. This trend is also indicated by blue in Figure 2.14 which indicates low level of expression.

Cluster B	**Average LFQ Intensities**			
Protein names	**MET**	**BC**	**SB**	**BB**
Ejaculatory bulb-specific 3-like	33.3	33.4	33.5	32.7
Dipetalin; Serine protease inhibitor dipetalogastin	33.2	33.3	33.0	32.7
Peptidylprolyl isomerase B precursor	25.9	25.6	25.0	24.0
Serpin 3a	27.1	28.5	27.0	26.9
Protease inhibitor-like protein	28.3	28.3	29.0	27.4
Contig20011_1	25.7	25.6	25.9	24.8

Table 2.7. Proteins of altered abundance in *G. mellonella* from Cluster C

MET: *M. anisopliae*; BC: *B. caledonica*; SB: Control; BB: *B. bassiana*. MET treated larvae have a lower level of abundance than BB, BC and SB in the proteins in this Cluster. This trend is also indicated by blue in Figure 2.14 which indicates low level of expression.

Cluster C	**Average LFQ Intensities**			
Protein names	**MET**	**BC**	**SB**	**BB**
Beta-1,3-glucan-binding protein	25.7	27.7	26.5	26.0
Cecropin-D-like peptide	25.2	28.0	26.8	26.7
Alpha-esterase 45	30.3	30.3	30.9	31.3
32 kDa ferritin subunit	30.4	31.0	31.6	30.7
Homeobox 2-like isoform X4	30.4	31.6	31.5	31.0
Aarylphorin	33.3	33.4	34.1	33.9
Sensory appendage protein 1	28.8	29.9	29.7	29.3
Nimrod B precursor	29.4	30.5	30.8	30.3
Probable salivary secreted peptide	26.2	26.7	27.1	27.2
Apolipophorins	32.2	32.9	33.8	33.4
Contig22104_1	25.9	27.3	27.4	27.1
Insecticyanin-B	32.1	31.6	32.3	32.2
Peptidoglycan recognition-like protein	24.9	27.9	26.4	26.0
Contig20220_1	30.0	31.1	31.8	30.8
Cationic peptide CP8 precursor	33.0	32.7	34.0	33.1
Carboxylesterase clade H, member 1 precursor	26.8	29.0	29.0	27.9
Apolipophorin-2	33.4	33.2	33.6	33.4
Chitinase EN03	32.7	33.5	33.8	33.3
Gloverin-like protein	24.7	27.1	26.3	25.2
Contig19736_1	28.9	31.2	31.8	30.8
Contig20268_1	28.7	27.4	29.5	28.4

Alpha-esterase 45	30.8	30.8	31.8	31.8
Inter-alpha-trypsin inhibitor heavy chain H4-like	31.3	31.3	31.6	31.9
Juvenile hormone-binding-like	31.8	31.5	32.5	32.4
Anionic antimicrobial peptide 2	30.4	30.9	30.9	31.3
Contig_5861.0	26.4	30.0	28.7	31.2
Abnormal wing disc-like protein; nucleoside diphosphate kinase	25.2	25.6	26.4	26.2
Aminoacylase-1-like	24.7	25.7	27.4	25.4
Multicystatin and procathepsin F precursor	30.4	31.5	31.6	31.0
Putative protease inhibitor 4	30.7	31.2	32.6	31.2
Surface bspA-like	26.7	27.8	28.6	27.2
Adhesion related protein, transmembrane	26.4	26.2	26.7	27.9
Bombyrin precursor; apolipo D-like	31.0	31.5	31.8	31.1
Methionine-rich storage protein	34.5	34.6	35.8	36.0
Hexamerin	33.4	34.0	34.2	34.6
Transferrin	34.2	34.9	34.8	35.1

Table 2.8. Proteins of altered abundance in *G. mellonella* from Cluster D

MET: *M. anisopliae*; BC: *B. caledonica*; SB: Control; BB: *B. bassiana*. BB treated larvae have a higher level of abundance than MET, BC and SB in the proteins in this Cluster. This trend is also indicated by red in Figure 2.14 which indicates low level of expression.

Cluster D	**Average LFQ Intensities**			
Protein names	**MET**	**BC**	**SB**	**BB**
Carboxylesterase	30.0	29.5	29.6	29.8
Serine proteinase	25.9	27.1	25.3	27.5
Ommochrome-binding-like	30.6	30.0	30.0	31.2
Alpha-esterase 45; esterase FE4-like isoform X2	29.2	28.7	28.3	29.6
Carboxylesterase	27.5	28.0	27.4	28.9
Peptidoglycan recognition-like protein B	27.7	30.0	28.9	29.6
PREDICTED: apolipoprotein D	30.6	31.1	30.1	31.4
Integument esterase 2 precursor; esterase E4-like	31.9	31.9	31.4	32.4
Putative serine protease-like protein 2	30.2	30.7	30.2	30.8
Apyrase	26.1	26.1	26.1	26.5
Serpin 1	33.4	33.3	33.1	33.8
26kDa ferritin subunit	32.1	31.0	31.7	31.5
PREDICTED: aldo-keto reductase AKR2E4-like	27.2	27.2	27.3	28.1

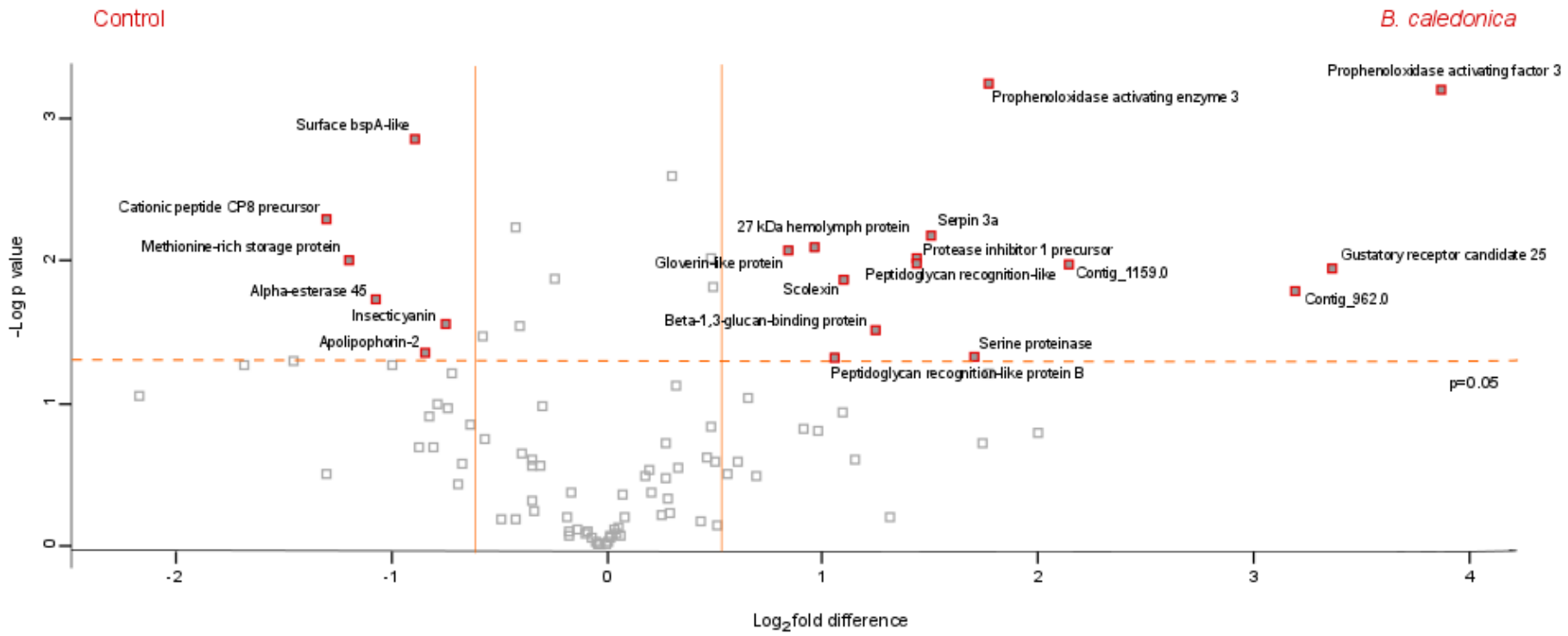


Figure 2.9. Volcano plot highlighting proteins present in highest and lowest abundances between larvae treated with *B. caledonica* supernatant and control larvae.

Image of volcano plot obtained using Perseus software. Proteins above the dashed orange line indicates significance ($p<0.05$) and those to the right and left of the vertical lines indicate fold changes greater than 1.5. Filled grey boxes with red outline are significant, with a fold change greater than 1.5. Proteins present on the right hand side are increased in abundance relative to the control.

Table 2.9. Proteins significantly increased or decreased in abundance in larvae treated with *B. caledonica* supernatant relative to control larvae.

Tables 2.9-2.14: Proteins included have a $p<0.05$ (or a –Log p value above 1.3) and a fold change of above +/-0.58. The average LFQ intensities of the four replicates are given.

			Average LFQ Intensities	
Protein Identity	**P value (-Log)**	**Log Difference**	**BC**	**Control**
Prophenoloxidase activating factor 3	3.2	3.9	28.6	24.7
Gustatory receptor candidate 25	2.0	3.4	27.9	24.5
Contig_962.0	1.8	3.2	29.3	26.1
Contig_1159.0	2.0	2.2	26.8	24.6
Prophenoloxidase activating enzyme 3	3.2	1.8	29.2	27.4
Serine proteinase	1.3	1.7	27.1	25.3
Serpin 3a	2.2	1.5	28.5	27.0
Peptidoglycan recognition like	2.0	1.5	27.9	26.4
Protease inhibitor 1 precursor	2.0	1.5	30.3	28.8
Beta-1,3-glucan-binding protein	1.5	1.3	27.7	26.5
Scolexin	1.9	1.1	30.8	29.7
Peptidoglycan recognition-like protein B	1.3	1.1	30.0	28.9
27 kDa hemolymph protein	2.1	1.0	29.4	28.4
Gloverin-like protein	2.1	0.9	27.1	26.3
Insecticyanin	1.6	-0.7	31.6	32.3
Apolipophorin-2	1.4	-0.8	32.9	33.8
Surface bspA-like	2.8	-0.9	27.8	28.6
Alpha-esterase 45	1.7	-1.0	30.8	31.8
Methionine-rich storage protein	2.0	-1.2	34.6	35.8
Cationic peptide CP8 precursor	2.3	-1.3	32.7	34.0

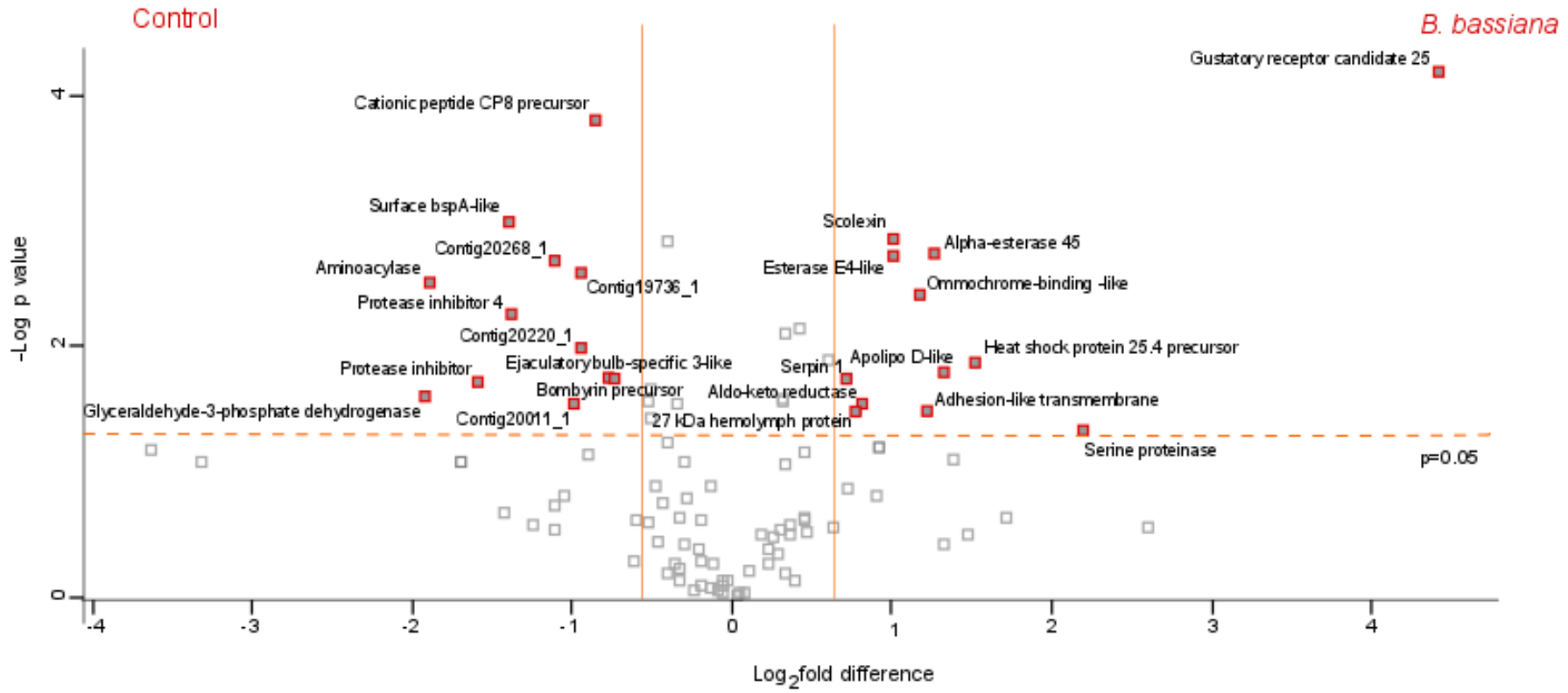


Figure 2.10. Volcano plot highlighting proteins present in highest and lowest abundances between larvae treated with *B. bassiana* supernatant and control larvae.

Image of volcano plot obtained using Perseus software. Proteins above the dashed orange line indicates significance ($p<0.05$) and those to the right and left of the vertical lines indicate fold changes greater than 1.5. Filled grey boxes with red outline are significant, with a fold change above 1.5.

Table 2.10. Proteins significantly increased or decreased in abundance in larvae treated with *B. bassiana* supernatant relative to control larvae.

			Average LFQ Intensities	
Protein Identity	**P value (-Log)**	**Log Difference**	**BB**	**Control**
Gustatory receptor candidate 25	4.2	4.4	28.8	24.5
Serine proteinase	1.3	2.2	27.5	25.3
Heat shock protein 25.4 precursor	1.9	1.5	31.5	30.0
Apolipoprotein D like	1.8	1.3	31.4	30.1
Alpha-esterase 45	2.7	1.2	29.6	28.3
Adhesion like transmembrane	1.5	1.2	27.9	26.7
Ommochrome-binding –like	2.4	1.1	31.2	30.0
Esterase E4 like	2.7	1.0	32.4	31.4
Scolexin	2.8	1.0	30.7	29.7
Aldo-keto reductase	1.5	0.8	28.1	27.3
27 kDa hemolymph protein	1.5	0.7	29.1	28.4
Serpin 1	1.8	0.7	33.8	33.1
Bombyrin precursor	1.7	-0.8	31.1	31.8
Ejaculatory bulb-specific 3-like	1.8	-0.8	32.7	33.5
Cationic peptide CP8 precursor	3.8	-0.9	33.1	34.0
Contig19736_1	2.6	-1.0	30.8	31.8
Contig20220_1	2.0	-1.0	30.8	31.8
Contig20011_1	1.5	-1.0	24.9	25.9
Contig20268_1	2.7	-1.1	28.4	29.5
Protease inhibitor 4	2.3	-1.4	31.2	32.6
Surface bspA-like	3.0	-1.4	27.2	28.6
Protease inhibitor	1.7	-1.6	27.4	29.0
Aminoacylase	2.5	-1.9	25.4	27.4
Glyceraldehyde-3-phosphate dehydrogenase	1.6	-2.0	26.0	27.9

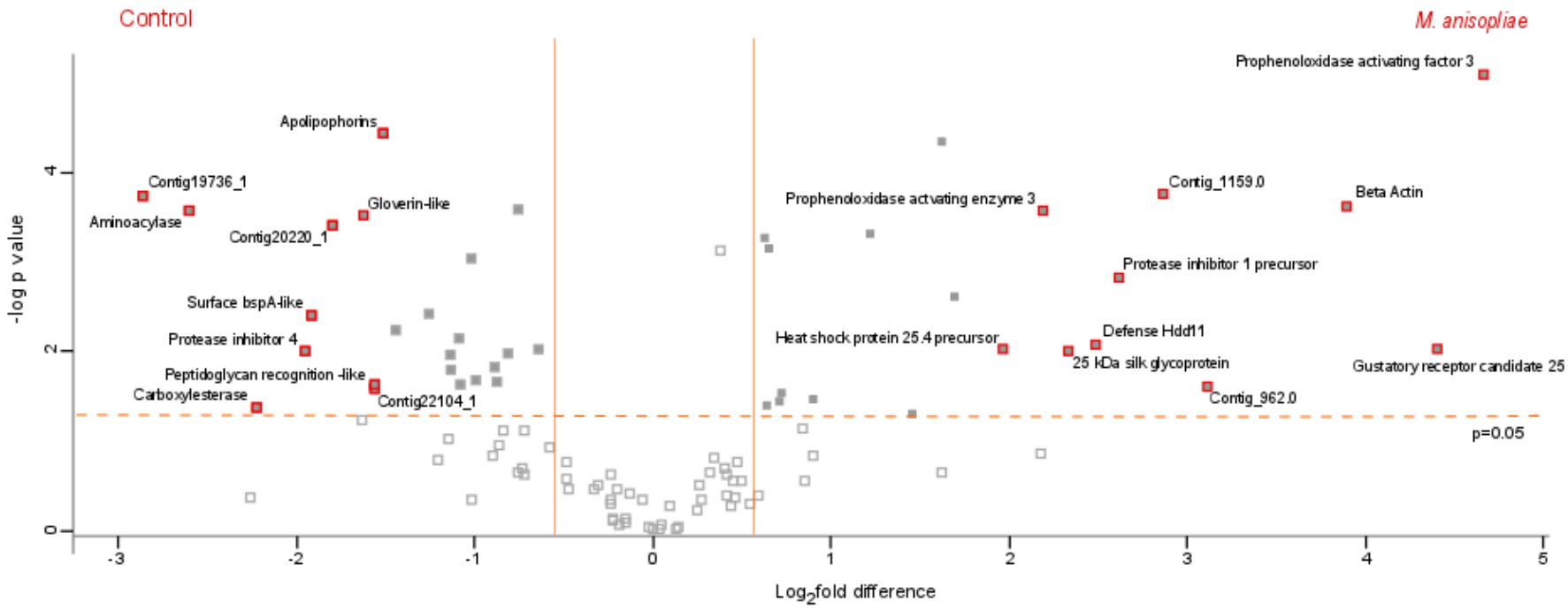


Figure 2.11. Volcano plot highlighting present in highest and lowest abundances between larvae treated with *M. anisopliae* supernatant and control larvae.

Proteins above the dashed orange line are statistically significant ($p<0.05$) and those to the right and left of the vertical lines (log2 fold difference of +/- 0.58) have a relative fold difference of 1.5 or above. The top ten most differentially abundant proteins are annotated for both treatment and control. The top 10 most differentially abundant proteins in both control and *M. anisopliae* are annotated.

Table 2.11. Proteins significantly increased or decreased in abundance in larvae treated with *M. anisopliae* supernatant relative to control larvae.

			Average LFQ Intensities	
Protein Identity	**P value (-Log)**	**Log Difference**	**MET**	**Control**
Prophenoloxidase activating factor 3	5.1	4.7	29.3	24.7
Gustatory receptor candidate 25	2.0	4.4	28.8	24.5
Beta Actin	3.6	3.9	29.1	25.2
Contig_962.0	1.6	3.1	29.2	26.1
Contig_1159.0	3.8	2.9	27.5	24.6
Protease inhibitor 1 precursor	2.8	2.6	31.4	28.8
Defence Hdd11	2.1	2.5	29.9	27.5
25 kDa silk glycoprotein	2.0	2.3	30.8	28.4
Prophenoloxidase activating enzyme 3	3.6	2.2	29.5	27.4
Heat shock protein 25.4 precursor	2.0	2.0	32.0	30.0
Scolexin	2.6	1.7	31.4	29.7
27 kDa hemolymph protein	4.4	1.6	30.0	28.4
Contig22050_1	1.3	1.4	26.1	24.7
27 kDa hemolymph protein	3.3	1.2	36.7	35.4
Peptidylprolyl isomerase B precursor	1.5	0.9	25.9	25.0
Twelve cysteine protein 1	1.6	0.7	30.7	30.0
Hemolin	1.5	0.7	32.2	31.5
Gelsolin	3.2	0.6	33.3	32.6
Ommochrome-binding –like	1.4	0.6	30.6	30.0
Methionine-rich storage protein 2	3.3	0.6	37.4	36.8
Transferrin	2.0	-0.7	34.2	34.8
Hexamerin	3.6	-0.7	33.4	34.2

Aarylphorin	2.0	-0.8	33.3	34.1
Bombyrin	1.7	-0.9	31.0	31.8
Sensory appendage protein 1	1.8	-0.9	28.8	29.7
Cationic peptide CP8 precursor	1.7	-1.0	33.0	34.0
Alpha-esterase 45	3.0	-1.0	30.8	31.8
Imaginal disc growth factor-like protein	1.6	-1.1	32.7	33.8
Homeobox 2-like isoform X4	2.1	-1.1	30.4	31.5
32 kDa ferritin subunit	1.8	-1.1	30.4	31.6
Multicystatin and procathepsin F precursor	2.0	-1.1	30.4	31.6
Methionine-rich storage protein	2.4	-1.3	34.5	35.8
Nimrod B precursor	2.3	-1.5	29.4	30.8
Apolipophorins	4.4	-1.5	32.2	33.8
Contig22104_1	1.6	-1.6	25.9	27.4
Peptidoglycan recognition like	1.6	-1.6	24.9	26.4
Gloverin-like protein	3.5	-1.6	24.7	26.3
Contig20220_1	3.4	-1.8	30.0	31.8
Surface bspA-like	2.4	-1.9	26.7	28.6
Protease inhibitor 4	2.0	-2.0	30.7	32.6
Carboxylesterase	1.4	-2.2	26.8	29.0
Aminoacylase	3.6	-2.6	24.7	27.4
Contig19736_1	3.7	-2.9	28.9	31.8

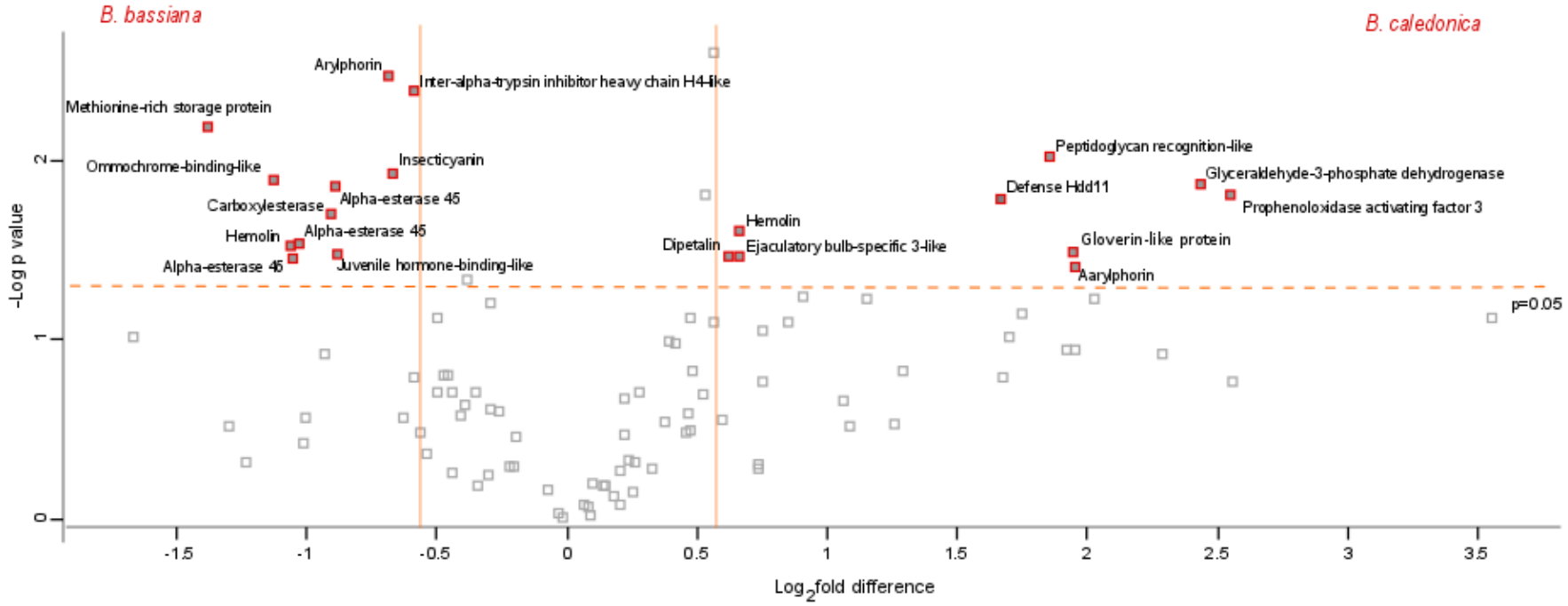


Figure 2.12.Volcano plot highlighting proteins present in highest and lowest abundances between larvae treated with *B. caledonica* and *B. bassiana* supernatant.

Image of volcano plot obtained using Perseus software. Proteins above the dashed orange line indicates significance ($p<0.05$) and those to the right and left of the vertical lines indicate fold changes greater than 1.5. Filled grey boxes with red outline are significant, with a fold change above 1.5 fold. Proteins present on the right hand side are increased in abundance in *B. caledonica* treated larvae relative to *B. bassiana* treated larvae

Table 2.12. Proteins significantly increased or decreased in abundance in larvae treated with *B. caledonica* relative to *B. bassiana* treated larvae.

			Average LFQ Intensities	
Protein Identity	**P value (-Log)**	**Log Difference**	**BC**	**BB**
Prophenoloxidase activating factor 3	1.8	2.5	28.6	26.0
Glyceraldehyde-3-phosphate dehydrogenase	1.9	2.4	28.4	26.0
Aarylphorin	1.4	2.0	26.5	24.5
Gloverin-like protein	1.5	1.9	27.1	25.2
Peptidoglycan recognition-like	2.0	1.9	27.9	26.0
Defence Hdd11	1.8	1.7	28.6	26.9
Ejaculatory bulb-specific 3-like	1.5	0.7	33.4	32.7
Hemolin	1.6	0.7	31.7	31.1
Dipetalin	1.5	0.6	33.3	32.7
Inter-alpha-trypsin inhibitor heavy chain H4-like	2.4	-0.6	31.3	31.9
Insecticyanin-B	1.9	-0.7	31.6	32.2
Arylphorin	2.5	-0.7	35.7	36.4
Juvenile hormone binding-like	1.5	-0.9	31.5	32.4
Alpha-esterase 45	1.9	-0.9	28.7	29.6
Carboxylesterase	1.7	-0.9	28.0	28.9
Alpha-esterase 45	1.5	-1.0	30.3	31.3
Alpha-esterase 45	1.5	-1.0	30.8	31.8
Hemolin	1.5	-1.1	28.2	29.3
Ommochrome-binding –like	1.9	-1.1	30.0	31.2
Methionine-rich storage protein	2.2	-1.4	34.6	36.0

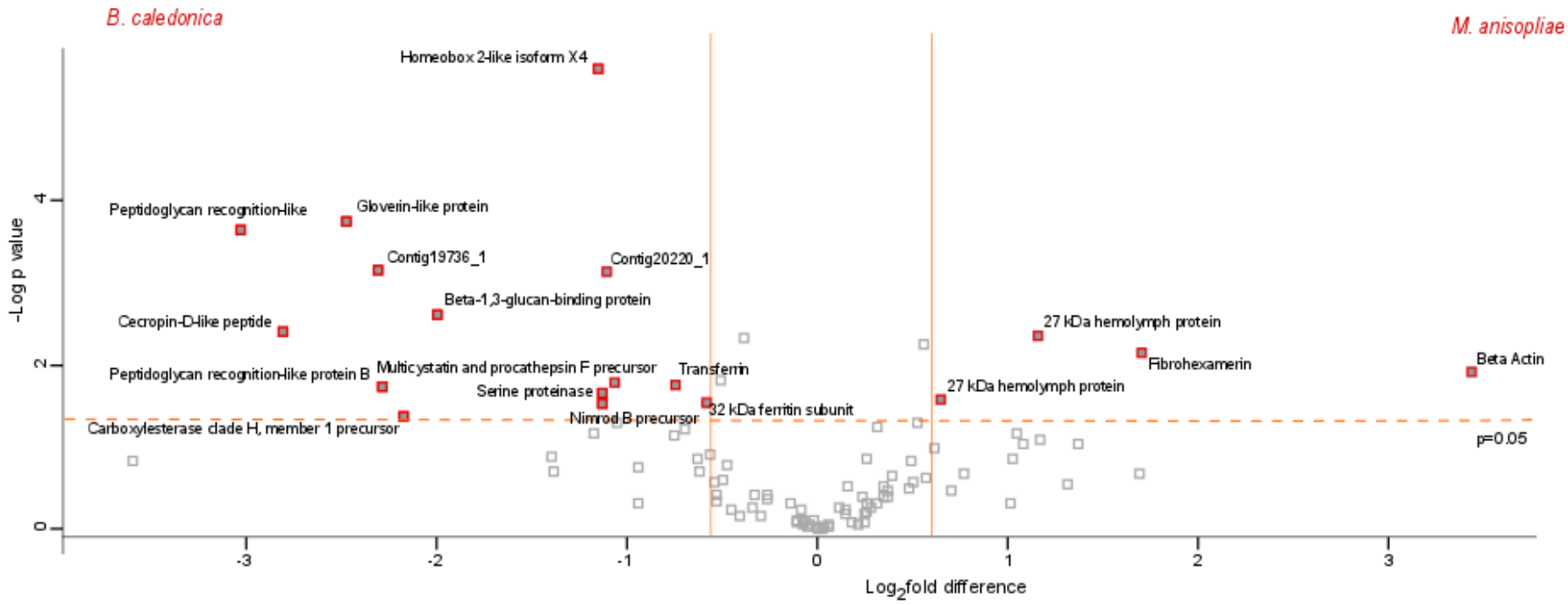


Figure 2.13. Volcano plot highlighting proteins present in highest and lowest abundances between larvae treated with *M. anisopliae* supernatant and *B. caledonica* larvae.

Image of volcano plot obtained using Perseus software. Proteins above the dashed orange line indicates significance ($p<0.05$) and those to the right and left of the vertical lines indicate fold changes greater than 1.5. Filled grey boxes with red outline are significant, with a fold change above 1.5. Proteins present on the right hand side are increased in abundance in *M. anisopliae* treated larvae relative to *B. caledonica* treated larvae.

Table 2.13. Proteins significantly increased or decreased in abundance in larvae treated with *M. anisopliae* supernatant relative to *B. caledonica* treated larvae.

			Average LFQ Intensities	
Protein Identity	**P value (-Log)**	**Log Difference**	**MET**	**BC**
Beta Actin	1.9	3.4	29.1	25.6
Fibrohexamerin (25 kDa silk glycoprotein)	2.1	1.7	30.8	29.1
27 kDa hemolymph protein	2.3	1.1	36.7	35.5
27 kDa hemolymph protein	1.6	0.6	30.0	29.4
32 kDa ferritin subunit	1.5	-0.6	30.4	31.0
Transferrin	1.8	-0.8	34.2	34.9
Multicystatin and procathepsin F precursor	1.8	-1.1	30.4	31.5
Contig20220_1	3.1	-1.1	30.0	31.1
Nimrod B precursor	1.5	-1.1	29.4	30.5
Serine proteinase	1.7	-1.1	25.9	27.1
Homeobox 2-like isoform X4	5.6	-1.2	30.4	31.6
Beta-1,3-glucan-binding protein	2.6	-2.0	25.7	27.7
Carboxylesterase clade H, member 1 precursor	1.4	-2.2	26.8	29.0
Peptidoglycan recognition-like protein B	1.7	-2.3	27.7	30.0
Contig19736_1	3.1	-2.3	28.9	31.2
Gloverin-like protein	3.8	-2.5	24.7	27.1
Cecropin-D-like peptide	2.4	-2.8	25.2	28.0
Peptidoglycan recognition-like	3.6	-3.0	24.9	27.9

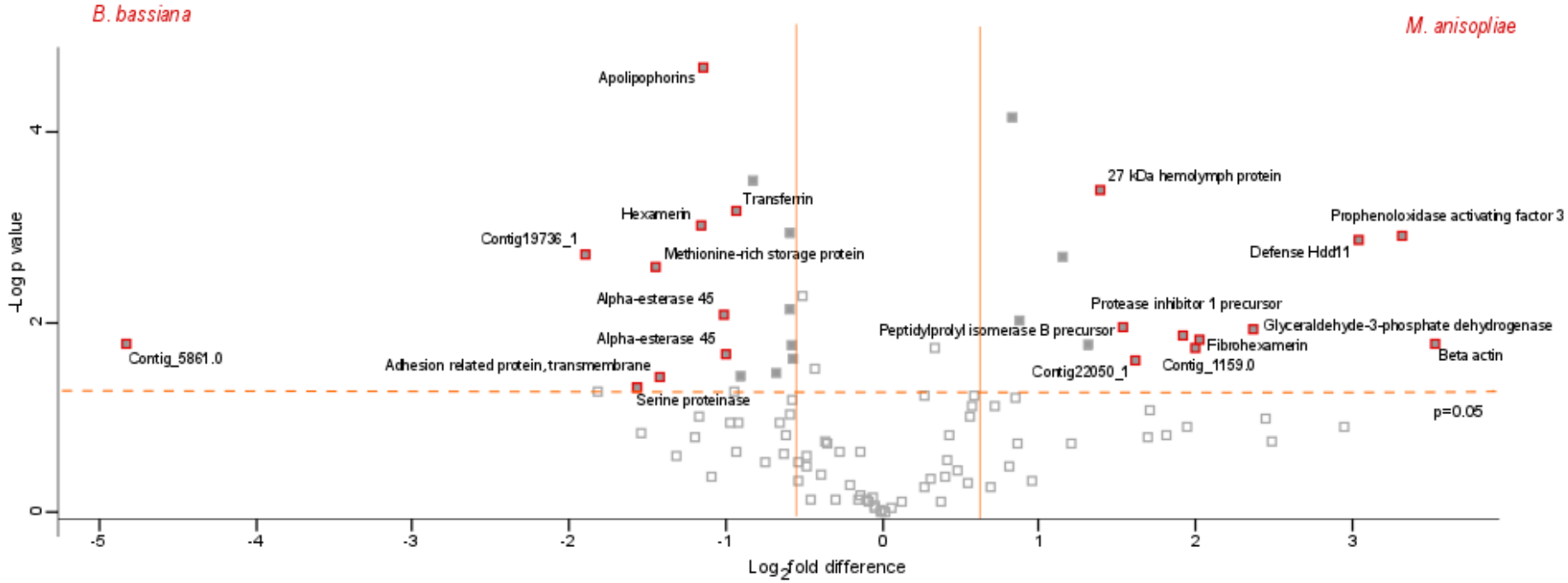


Figure 2.14. Volcano plot highlighting proteins present in highest and lowest abundances between larvae treated with *M. anisopliae* supernatant and *B. bassiana* larvae.

Proteins above the dashed orange line indicates significance ($p<0.05$) and those to the right and left of the vertical lines indicate fold changes greater than 1.5. Filled grey boxes indicate proteins that were significantly changed in abundance that were not in the top 10 changed proteins. Filled grey boxes with red outline are significant, are in the top 10 changed proteins. Proteins present on the right hand side are increased in abundance in *M. anisopliae* treated larvae relative to *B. bassiana* treated larvae.

Table 2.14. Proteins significantly increased or decreased in abundance in larvae treated with *M. anisopliae* supernatant relative to *B. bassiana* treated larvae.

			Average LFQ Intensities	
Protein Identity	**P value (-Log)**	**Log Difference**	**MET**	**BB**
Beta actin	1.8	3.5	29.1	25.6
Prophenoloxidase activating factor 3	2.9	3.3	29.3	26.0
Defence Hdd11	2.9	3.0	29.9	26.9
Glyceraldehyde-3-phosphate dehydrogenase	1.9	2.4	28.3	26.0
Fibrohexamerin (25 kDa silk glycoprotein)	1.8	2.0	30.8	28.7
Contig_1159.0	1.7	2.0	27.5	25.5
Protease inhibitor 1 precursor	1.9	1.9	31.4	29.5
Contig22050_1	1.6	1.6	26.1	24.5
Peptidylprolyl isomerase B precursor	1.9	1.5	25.9	24.4
27 kDa hemolymph protein	3.4	1.4	36.7	35.3
Prophenoloxidase activating enzyme 3	1.8	1.3	29.5	28.2
Hemolin	2.7	1.1	32.2	31.1
27 kDa hemolymph protein	2.0	0.9	30.0	29.1
Spodoptericin	4.1	0.8	31.1	30.3
Putative serine protease-like protein 2	1.8	-0.6	30.2	30.8
Inter-alpha-trypsin inhibitor heavy chain H4-like	1.6	-0.6	31.3	31.9
Homeobox 2-like isoform X4	2.9	-0.6	30.4	31.0
Aarylphorin	2.1	-0.6	33.3	33.9
Aminoacylase	3.5	-0.8	24.7	25.4
Contig20220_1	1.4	-0.9	30.0	30.8

Anionic antimicrobial peptide 2	3.2	-0.9	30.4	31.3
Transferrin	1.7	-1.0	34.2	35.1
Alpha-esterase 45	2.1	-1.0	30.3	31.3
Alpha-esterase 45	4.7	-1.2	30.8	31.8
Apolipophorins	3.0	-1.2	32.2	33.4
Hexamerin	1.4	-1.4	33.4	34.6
Adhesion related protein, transmembrane	2.6	-1.5	26.4	27.9
Methionine-rich storage protein	1.3	-1.6	34.5	36.0
Serine proteinase	2.7	-1.9	25.9	27.5
Contig19736_1	1.8	-4.8	28.9	30.8
Contig_5861.0	3.2	-0.9	26.4	31.2

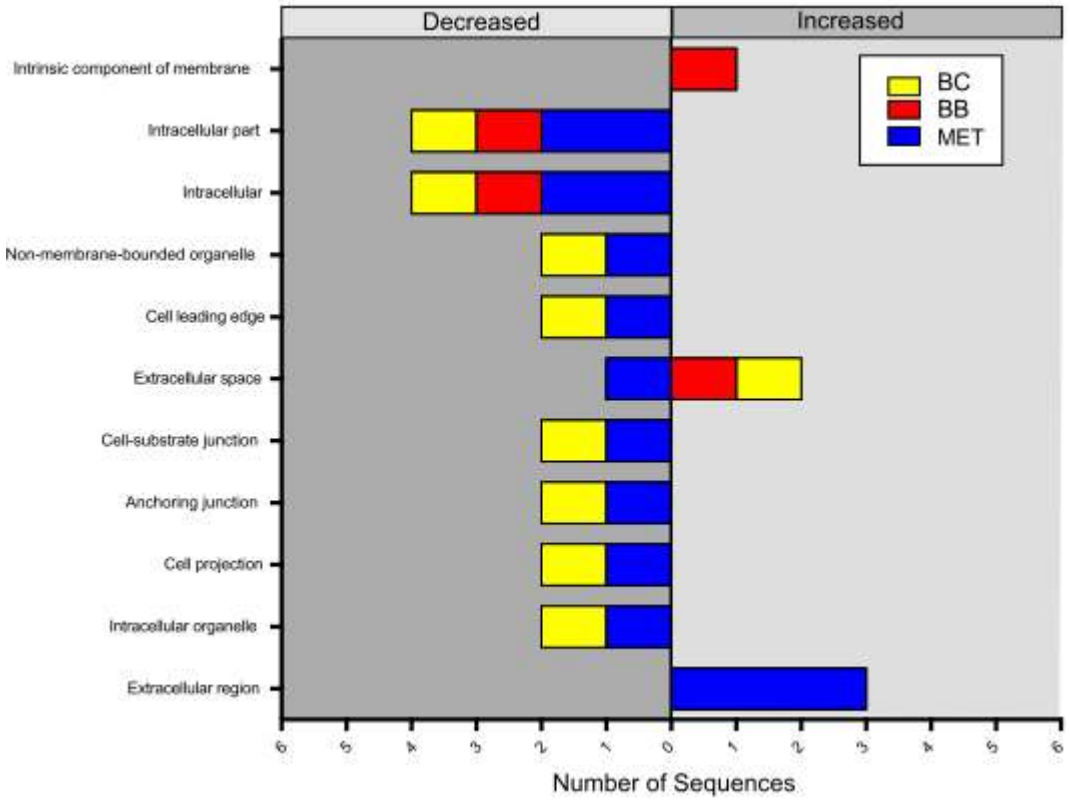


Figure 2.15. Bar chart showing number of proteins changed in various Cellular Processes at level 3 ontolog.

Proteins are grouped based on functional annotation using Blast2Go.

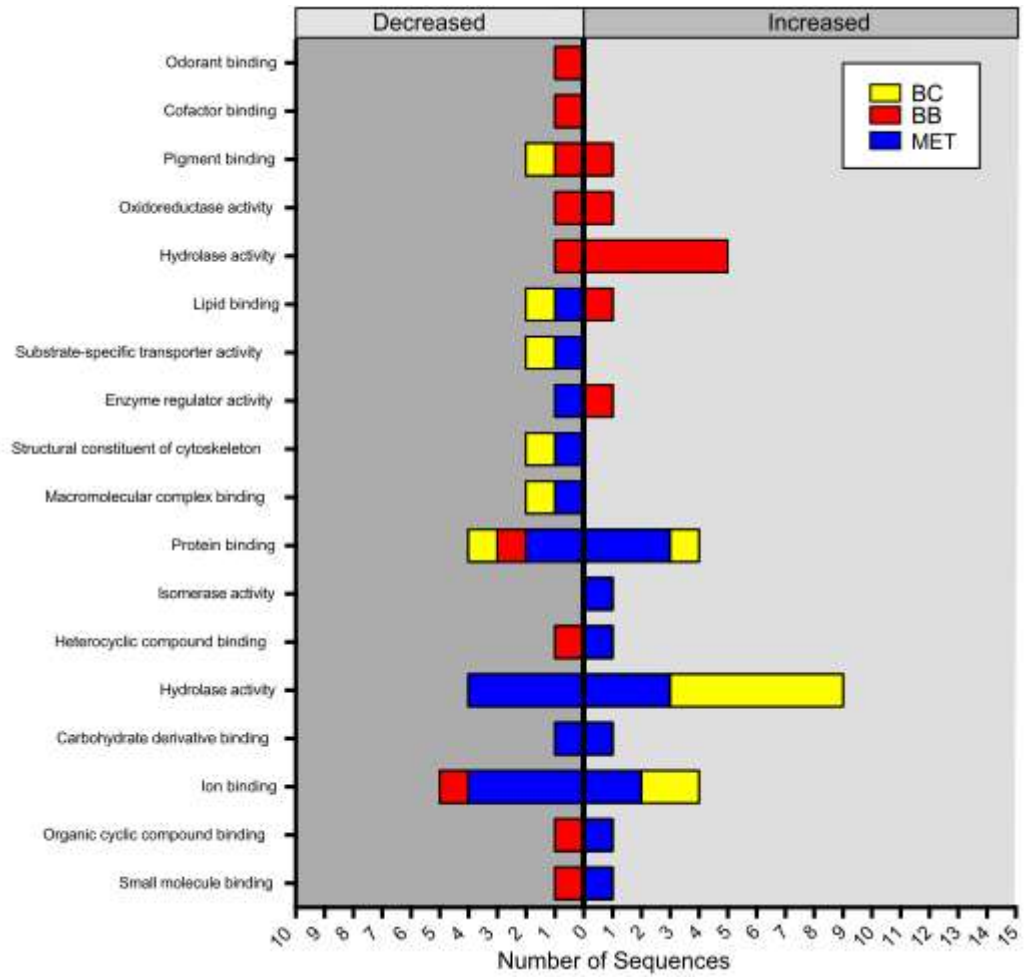


Figure 2.16. Bar chart showing number of proteins changed in various Molecular Processes at level 3 ontolog.

Proteins are grouped based on functional annotation using Blast2Go.

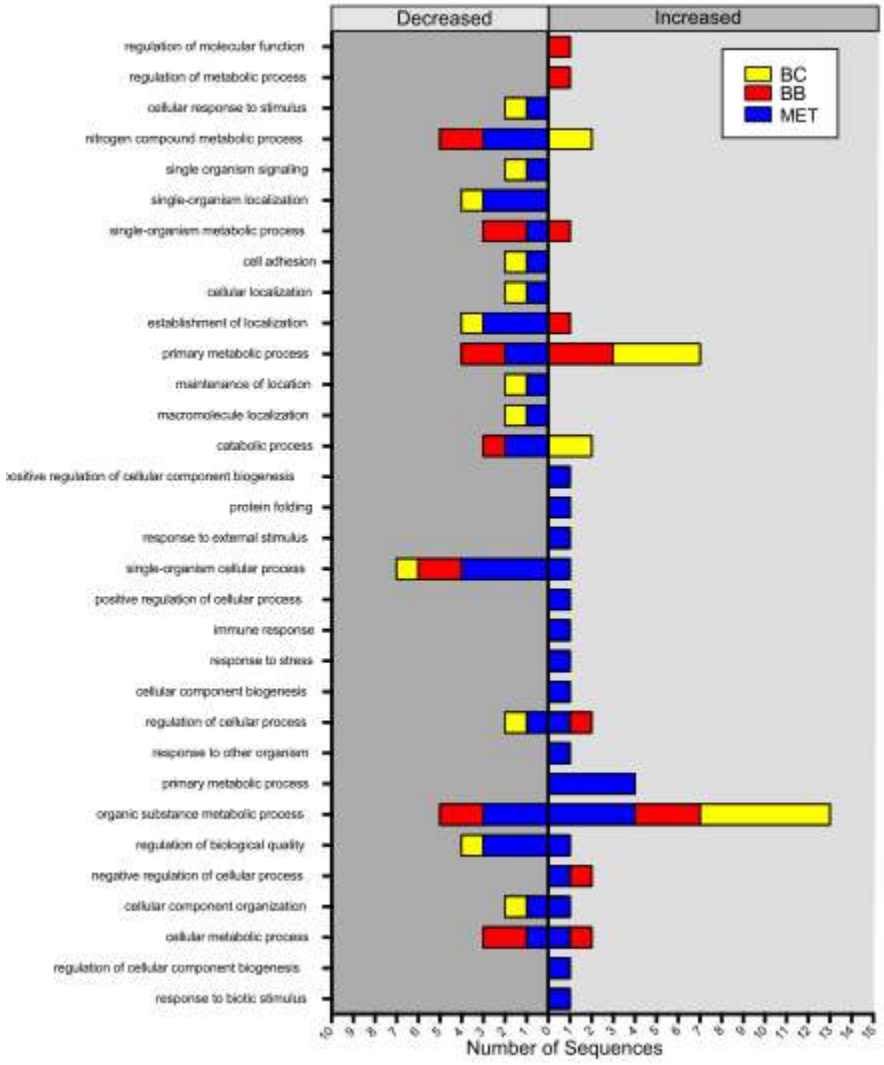


Figure 2.17. Bar chart showing number of proteins changed in various Biological Processes at level 3 ontolog.

Proteins are grouped based on functional annotation using Blast2Go.

2.4 Discussion

A key element of this work was to investigate the use of EPF against *H. abietis* larvae in the field in conjunction with EPN. Combinations of control agents have previously been found to exert synergistic effects, meaning that the effect due to the combination is greater than the sum of the individual effects (Koppenhöfer and Kaya 1997, Anbesse *et al.* 2008, Ansari *et al.* 2008, Morales-Rodriguez and Peck 2009, Ansari *et al.* 2010). This is often attributed to one agent weakening the insect's immune system, making it more susceptible to the second agent. In the case of the EPF-EPN combination, it is suggested that synergy is most likely to be due to EPF rendering the insect more susceptible to EPN (Ansari *et al.* 2004, Ansari *et al.* 2006). Thus the aim of this chapter was to investigate a number of EPF isolates for their ability to modulate the insect immune response rendering them more susceptible to subsequent pathogens. Screening EPF for their ability to modulate the immune response of insects may represent a means of screening EPF for use in the field, particularly if the goal is to use them in combination with other control agents such as EPN or chemical insecticides. Understanding how insects resist and/or detoxify natural products of entomopathogens is critical in developing fungi as effective biological control agents. Likewise identifying the specific biological responses they elicit in susceptible hosts is also critical (Rohlfs and Churchill 2011).

The results presented in this chapter give an insight into the effect that EPF fungal supernatant has on both the cellular and humoral immune response of *G. mellonella* larvae. This provides information regarding the potential immunomodulating abilities of the EPF tested as well as how the insect immune system may be responding to such challenges. The effect of EPF supernatant on the immune response of *G. mellonella* was investigated through a number of bioassays. Based on the results of these bioassays the treatments, *M. anisopliae*, *B. bassiana* and *B. caledonica* fungal supernatant grown for 96h were selected for proteomic analysis as *M. anisopliae* and *B. caledonica* exhibited potential immunomodulating effects on the *G. mellonella* larval immune system. *B. bassiana* was included in the proteomic analysis as a comparison because it did not appear to modulate the immune response to the extent that the insect become more susceptible to subsequent pathogens in these bioassays.

Haemolymph of *G. mellonella* injected with EPF supernatant was subjected to both 2D-PAGE and label free quantitative proteomic analysis. Protein separation and comparison by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), followed by mass spectrometry (MS) or tandem mass spectrometry (MS/MS) identification is the classical method for quantitative analysis of protein samples. Some disadvantages of this approach include: quantification may at times be ambiguous as spots on a 2D gel may contain more than one protein, also the chosen gel method may have difficulty handling hydrophobic proteins and detecting proteins with extreme molecular weights and pI values (Zhu *et al.* 2009). Numerous gel free quantitative proteomics methods are now available that quantify proteins via mass spectrometry (Bantscheff *et al.* 2012). Shotgun proteome digestion approaches generate vastly complex mixtures of (mostly tryptic) peptides that constitute the analytes for both the identification and quantification of proteins. The combination of nanoscale ion pairing reversed-phase LC and electrospray ionization (ESI) MS/MS is still the most popular technology for this purpose. A label-free quantitative method in MS quantifies peptides and proteins without the use of protein labels. Label free proteomics was used in this chapter as it measures the relative abundance of thousands of proteins across multiple sample groups in single mass spectrometry runs (Bantscheff *et al.* 2012). Although these label-free approaches were initially thought of as replacements for gel-based proteomics, they may also be regarded as complements to 2-DE analysis. Both approaches have the ability to resolve hundreds to thousands of features and are both of great value to proteomic studies and may provide complementary information (Abdallah *et al.* 2012) as was seen in this chapter.

In LFQ of *G. mellonella* haemolymph, 100 proteins were identified with two or more peptides. Seventy eight of these proteins were either significantly changed in expression or uniquely detected across the four treatments analyzed. The uniquely detected proteins from Table 2.4 were used in statistical analysis of the total differentially expressed sample groups following a data imputation step which replaced missing values with values that simulate low abundant proteins. These values were chosen randomly from a distribution specified by a downshift of 2.19 times the mean standard deviation (SD) of all measured values and a width of 0.36 times this SD. The overall proteomic profile of expression is shown in Figure 2.8 in a heat map derived from hierarchical clustering that resolved four distinct clusters. These clusters may give an insight into method of action of different EPF or

differential responses of the larval immune system to different fungal supernatants, as the clusters correlate with differences between the effects of EPF observed in the bioassays. Cluster A includes proteins that generally have a higher level of expression induced by the two proposed immunomodulating treatments relative to control and *B. bassiana* treated larvae. Cluster B includes proteins that have a lower level of expression in *B. bassiana* treated larvae than all other treatments. Cluster C contains proteins that have a lower level of expression in *M. anisopliae* treated larvae than any other treatment. Cluster D appears to involve proteins that have a higher level of expression in *B. bassiana* treated larvae than all other treatments.

2.4.1 *B. caledonica, B. bassiana* and *M. anisopliae* supernatants induce variant immune responses in *G. mellonella*

The analysis of the cellular component of the immune response in response to EPF fungal supernatant demonstrated that all three EPF cause a significant decline in the haemocyte density of larvae following inoculation with 72h or 96h fungal supernatant and incubation for 48h. There was also a significant interaction between treatment and incubation time in all EPF tested meaning treatments performed differently after different incubation periods. Reduction in haemocyte densities can be as a result of cell death, prevention of new cells entering circulation or a combination of both. During enumeration of haemocytes cell lysis was observed under the microscope thus it appears cell death occurred in treated larvae. Haemocyte death is important in innate immunity as haemocytes are associated with encapsulation and nodulation (Butt *et al.* 2016). To investigate the potential of EPF fungal supernatant to modulate the larval immune response leaving the insect more susceptible to subsequent pathogens, the effect of fungal supernatant on *C. albicans* proliferation in the haemocoel was assessed. Larvae were injected with fungal supernatant and 24h later were injected with a sub lethal dose of *C. albicans*. Treatment with *B. caledonica* and *M. anisopliae* 96h fungal supernatant induced a significantly increased yeast cell density in larvae, when the larvae were incubated for 48h following injection with *C. albicans* (Figure 2.3). This suggests that *B. caledonica* and *M. anisopliae* supernatant is modulating the immune system of the insect allowing a subsequent pathogen to proliferate. In contrast, treatment with *B. bassiana* fungal supernatant did not cause a significant increase in yeast cells density in larvae relative to controls.

To further investigate whether treatment with fungal supernatant leaves larvae more susceptible to subsequent pathogens, larvae were injected with fungal supernatant and incubated for 24h. After 24h they were inoculated with *C. albicans*. One week after *C. albicans* infection, larvae treated with both *M. anisopliae* and *B. caledonica* showed significantly increased mortality when fungal supernatant and *C. albicans* were combined compared to single doses (Figure 2.4.A). After two weeks mortality caused by *M. anisopliae* and *C. albicans* reached 100% (Figure 2.4.B). It is important to note that the proposed immunomodulation that is rendering the host more susceptible to a subsequent pathogen is being caused by spore free fungal supernatant which is known to contain a diverse mixture of enzymes, proteases and secondary metabolites. Destruxin the most abundantly produced secondary metabolite in *Metarhizium* spp. has previously exhibited a similar pattern in *D. melanogaster* (Pal *et al*. 2007). A direct relationship exists between destruxin production and virulence in *M. anisopliae*; flies co-injected with a sub-lethal dose of destruxin A and a normally non-toxic bacterium *E.coli* showed a greater proliferation of *E. coli* and consequently increased mortality (Pal *et al.* 2007). This suggests that destruxin modulates the immune system making the insect unable to clear a bacterial infection (Rohlfs and Churchill 2011). The majority of generalist *M. anisopliae* strains capable of infecting and killing *G. mellonella* and other insects are in vitro-producers of destruxins which cause tetanic paralysis in these hosts (Rohlfs and Churchill 2011). In this Chapter tetanic paralysis was seen in *G. mellonella* injected with *M. anisopliae* supernatant cultured for 72 and 96h. This strengthens the observation that treatment with *M. anisopliae* fungal supernatant can modulate the larval immune response allowing increased proliferation of *C. albicans* and also increased mortality.

PCA shows that *M. anisopliae* induces the greatest change in proteomic profiles relative to the control. It also indicates that the alterations to the proteome induced by *B. caledonica* are more similar than *B. bassiana* to *M. anisopliae* (Figure 2.7). This links in with the observation that bioassay results of *B. caledonica* and *M. anisopliae* are more similar than *B. bassiana*. Larvae treated with *M. anisopliae* fungal supernatant appeared to undergo the most dramatic changes to their hemolymph proteome (Table 2.11, Figure 2.11). Hierarchical clustering allows comparison between the protein expression induced by different fungal supernatant; the effects they have in common and also how they differ (Figure 2.8). Cluster A demonstrates that for larvae treated with immunomodulating fungal supernatant from *B.*

caledonica and *M. anisopliae* there was a higher level of abundance in proteins involved in the proPO activation cascade for example proPO activating factor 3, proPO activating enzyme 3 and protease inhibitor 1. Cluster C gives an insight into some of the reasons why *M. anisopliae* caused the strongest immunomodulation in treated larvae as it includes several immune relevant proteins that are downregulated in abundance more than in any other treatment (Table 2.7). Cluster C indicates that *M. anisopliae* treated larvae have the lowest level expression of the AMPs cecropin D and gloverin, as well as arylphorin, transferrin and a peptidoglycan recognition-like protein. Arylphorin is a storage protein with proposed immune functionality; it is an effector of the humoral immune response and is capable of causing damage to the outer surface of bacteria (Beresford *et al.* 1997, Zhu *et al.* 2013). Its decreased expression may have implications for the immune response to subsequent pathogens. Both apolipophorin and arylphorin have immune functionality; *G. mellonella* larvae pretreated with non-lethal doses of *A. fumigatus* conidia have increased resistance to a lethal dose later. This increased resistance is also coupled with increased binding of selected hemolymph proteins such as arylphorin, apolipophorin and prophenoloxidase (Fallon *et al.* 2011).

Blast2GO annotation software was used to group proteins based on their conserved gene ontology (GO) terms in order to identify processes and pathways altered to different EPF treatments. However Blast2Go assigned GO terms to only 68 of the 100 proteins identified by label free quantification. Therefore any conclusions made about changes in process and pathway must be made cautiously as they may not be truly representing everything that is going on. In relation to molecular processes (Figure 2.16) *M. anisopliae* appears to be involved in the largest number of process, including protein, lipid and ion binding. *M. anisopliae* has more processes in common with *B. caledonica* than *B. bassiana*. In relation to biological processes (Figure 2.17) *M. anisopliae* again appears to be involved in the largest number of process, including immune response and stress response. Again *M. anisopliae* has more processes in common with *B. caledonica* than *B. bassiana*. The finding that *B. caledonica* induces an immune response in *G. mellonella* more similar to *M. anisopliae* than *B. bassiana* is interesting as *B. caledonica* and *B. bassiana* are more closely related.

LFQ showed that *B. bassiana* treated larvae had a higher level of expression in alpha-esterase and carboxylesterase when compared to *M. anisopliae* and *B.*

caledonica treated larvae (Figure 2.12, Figure 2.14). Insects infected with EPF often upregulate antioxidant genes, fungal infection of insects is associated with increased total esterase and glutathione S-transferase activities in the hemolymph (Butt *et al.* 2016). Increased activity of detoxification enzymes represents the insect immune response to intoxication with metabolites produced by the pathogen or with products of host tissue degradation. Conversely inhibition of detoxification enzymes increases insect death rate from fungal infection (Serebrov *et al.* 2006). Induction of additional esterase isoforms and increased glutathione S-transferase activity in *G. mellonella* larvae with mycoses can decrease their sensitivity to chemical insecticides (Hemingway and Ranson 2000, Serebrov *et al.* 2006). In locusts infected with *M. anisopliae* a lethal dose of fungus was found to increase activity of detoxifying enzymes in the larvae on the third day after infection. Reduction in esterase and GST activity during acute mycosis was linked with effective inhibition of the host defense systems by EPF (Dubovskiy *et al.* 2012). Alpha esterase is decreased in expression in *B. caledonica* treated larvae relative to control larvae, while alpha esterase and carboxylesterase is decreased in expression in *M. anisopliae* treated larvae relative to control. Thus it could be postulated that detoxifying enzymes induced by treatment with *B. bassiana* fungal supernatant may have had some role to play in those larvae not becoming more susceptible to infection.

The 2D SDS PAGE analysis showed that both *M. anisopliae* and *B. bassiana* fungal supernatants cause alterations in the hemolymph proteome of treated larvae and several proteins altered in abundance have proposed immune functionality. However the proteins altered in abundance are different and this may in some part explain the differences in immunomodulation observed in the presented bioassays. The 2D SDS PAGE analysis of the effect of *B. bassiana* on the proteome of *G. mellonella* hemolymph demonstrated that the fungal supernatant influenced the expression of several proteins but had a different effect than that observed by *M. anisopliae*. The only common change observed in both treatments was a down regulation of a member of the vWFA superfamily which has serine type endopeptidase activity. Apolipophorin III was significantly downregulated following treatment with *B. bassiana* fungal supernatant. Apolipophorin III binds to bacteria, stimulates AMP production, enhances phagocytosis, stimulates cellular encapsulation of foreign bodies, and binds and detoxifies LPS and lipoteichoic acid (Whitten *et al.* 2004, Brown and Gordon 2005). Whitten *et al.* (2004) showed that apolipophorin III

from *G. mellonella* binds to conidia of *M. anisopliae* and β-1,3-glucan thus acting as a pattern recognition molecule that recognizes fungi as well as bacteria. They also showed that apolipophorin III has a role in the *in vivo* response to EPF as larvae injected with *M. anisopliae* conidia pre-treated with apolipophorin III had longer survival times than those injected with untreated conidia (Whitten *et al.* 2004). A molecule involved in iron metabolism, 32kDa ferritin subunit was found at a lower abundance in treated larvae. Ferritin is a major iron binding protein so may be a target that microbes can acquire iron from. It has been found that in the case of bacterial infection iron acquisition is vital for growth and pathogenicity, thus withholding iron can be considered an immune strategy (Ong *et al.* 2006). Paskewitz and Shi (2005) demonstrated that in *Anopheles gambiae* there was a disappearance of ferritin from the plasma during infection and light and heavy chains of ferritin decreased after bacterial injections.

2.4.2 *B. caledonica* and *M. anisopliae* supernatants impact upon the ProPO cascade in *G. mellonella*

LFQ and 2D SDS PAGE analysis of the effect of *M. anisopliae* on the proteome of *G. mellonella* hemolymph demonstrated that the fungal supernatant alters the abundance of several proteins implicated in the proPO cascade. In the 2D SDS-PAGE analysis scolexin was increased in response to fungal supernatant and is reported to be a coagulation provoking plasma protein induced in response to bacterial and viral infection in the lepidopteran *Manduca sexta*. Scolexin belongs to a subfamily of chymotrypsin-like serine proteases (Finnerty *et al.* 1999). Conversely serine protease inhibitors (Table 2.2, proteins 3-6) also showed alterations in expression including serpin 3. Several serine protease inhibitors have been identified in insect hemolymph that inhibit PO activation and several serpins have been identified in *M. sexta* including serpin 3 (Kanost 2007). These changes highlight an effect of *M. anisopliae* on a primary component of insect immune response: the prophenoloxidase pathway. Insect melanisation is controlled by a serine protease cascade that ultimately activates the enzyme prophenoloxidase (PPO), which in turn catalyzes the synthesis of melanin. Insect phenoloxidase is present in haemolymph in its inactive form PPO. Inactive PPO is cleaved into active PO by a serine protease known as prophenoloxidase activating enzyme (PPAE). PPAE is also present as an inactive zymogen that is activated by other serine proteases (De Gregorio *et al.* 2002). Experiments with proPO purified from silkworm identified the presence of a

serine protease cascade as an intermediate step between natural proPO activators and the production of active proPO. Serine proteases are hydrolases that cause peptide bond degradation examples include trypsin and chymotrypsin (González-Santoyo and Córdoba-Aguilar 2012). González-Santoyo and Córdoba-Aguilar (2012) found that PO activity doesn't directly correlate with successful pathogen defense and is instead an indication of host condition rather than a sign of resistance to infection. The PO activation system probably has a high cost for the insect for two main reasons. The main compound of the proPO activation pathway is tyrosine, which must be obtained from food. Also melanin which is the final product in the pathway is nitrogen rich and so the pathway may require high levels of nitrogen or protein. Thus the production and maintenance of the proPO activating pathway is dietary dependent (Siva-Jothy and Thompson 2002, González-Tokman *et al.* 2011, González-Santoyo and Córdoba-Aguilar 2012). Alterations in expression of proteins related to phenoloxidase production and maintenance may be an indication of changes to the insect immune defenses or may be an indication of the state of the insect such as the ability to ingest food.

Interestingly treatment with supernatant from the other immunomodulating EPF *B. caledonica* induced alterations in a number of proteins involved in the regulation and maintenance of PO. ProPO activating factor 3 (PPAF 3), proPO activating enzyme 3 (PPAE 3), serpin 3a, serine proteinase, protease inhibitor 1 precursor and scolexin were all increased in expression and may play a role in the proPO pathway. PO activities are vital for early host responses to EPF on the cuticle, during encapsulation responses to EPF in the haemocoel and for the production of melanin and ROS and RNS. Serine proteinases and the molecules they produce can be damaging to both the insect host and the fungal pathogen, affecting nucleic acids, peptides and oxidation of lipids (Jiang *et al.* 2010, Butt *et al.* 2016). Therefore it is important that these pathways are tightly regulated. As previously discussed phenoloxidase is present in the hemolymph in its inactive form proPO and is cleaved into active PO by a serine protease known as prophenoloxidase activating protein, protease, factor or enzyme (ppA/PPAF/PPAE) which appears to be upregulated by this treatment. PPAE is also present as an inactive zymogen that is activated by other serine proteases. Proteinase inhibitors can be present constitutively and may also be produced in response to infection or wounds. Serpin 3 is upregulated in expression following treatment with *B. caledonica*. Many serpins are irreversible inhibitors, key modulators of the proPO cascade and deactivators of microbe-derived proteases

(Jiang *et al.* 2010, Butt *et al.* 2016). Serpin 3 from *M. sexta* has a reactive site loop similar to the proteolytic activation site in proPO. It was found to be constitutively expressed in *M. sexta* at low levels and increased in expression after microbial challenge. Additionally recombinant serpin 3 blocks proPO activation in hemolymph and forms complexes with proPO activating proteinases (PAPs) (Yifei Zhu *et al.* 2003). Thus serpin 3 is a physiological regulator of the proPO activation reaction. Scolexin is also upregulated in expression and as previously discussed belongs to a subfamily of chymotrypsin-like serine proteases.

Together these alterations in the proteome indicate several changes to the proPO pathway (Figure 2.18), some involved in its activation and more in its self-regulation; indicating that the insect is mounting a response to the treatment but may also be attempting to keep the response in check to prevent deleterious effects. It is known that the activation of the phenoloxidase cascade can results in self damage to the host (Sadd and Siva-Jothy 2006), thus regulation of this cascade is crucial. Another explanation may be to do with the EPF strategy. EPF have evolved strategies to evade or tolerate the host immune response and this includes the regression of proteases that activate phenoloxidase (PO). It would not be in the interest of the pathogen to trigger responses that would impede its growth since its ultimate success depends on it generating biomass that it can covert to conidia. Likewise it is in the insects' interest to prevent activation of enzymes that are tightly regulated in other to avoid self-harm. Protease inhibitors (serpins) are utilized by insects as a safety mechanism to regulate PO activation (Butt *et al.* 2016).

Similarities in the proteomic results seen following treatment with *B. caledonica* and *M. anisopliae* fungal supernatant may in part explain their shared immunomodulating potential, in particular changes seen in abundance of proteins involved in the proPO activation pathway. ProPO activating factor 3 is up approximately 3.9 fold in *B. caledonica* treated larvae and approximately 4.7 fold in *M. anisopliae* treated larvae but it was not significantly changed in abundance in *B. bassiana* treated larvae post imputation. Likewise protease inhibitor 1 and proPO activating enzyme 3 were significantly increased in abundance in *M. anisopliae* and *B. caledonica* treated larvae but not *B. bassiana* treated larvae. Scolexin was upregulated in expression due to all three EPF, but at a higher fold change in *M. anisopliae* and *B. caledonica*. Protease inhibitor 4 was downregulated in larvae treated with *M. anisopliae* and *B. bassiana*. As the changes to protein abundance

involved in the activation and regulation of the proPO pathway was also seen in the 2D PAGE analysis of larval hemolymph treated with *M. anisopliae* it confirms our label free proteomic findings. While Cluster A indicates that proPO activating enzyme 3 and proPO activating factor 3 have the highest level of expression in larvae treated with *M. anisopliae* and *B. caledonica*, Cluster B indicates that *B. bassiana* treated larvae had the lowest of expression for serpin 3 and dipetalin a serine protease inhibitor. This highlights that there are differences in how the larvae are responding to the treatments in relation to the proPO cascade.

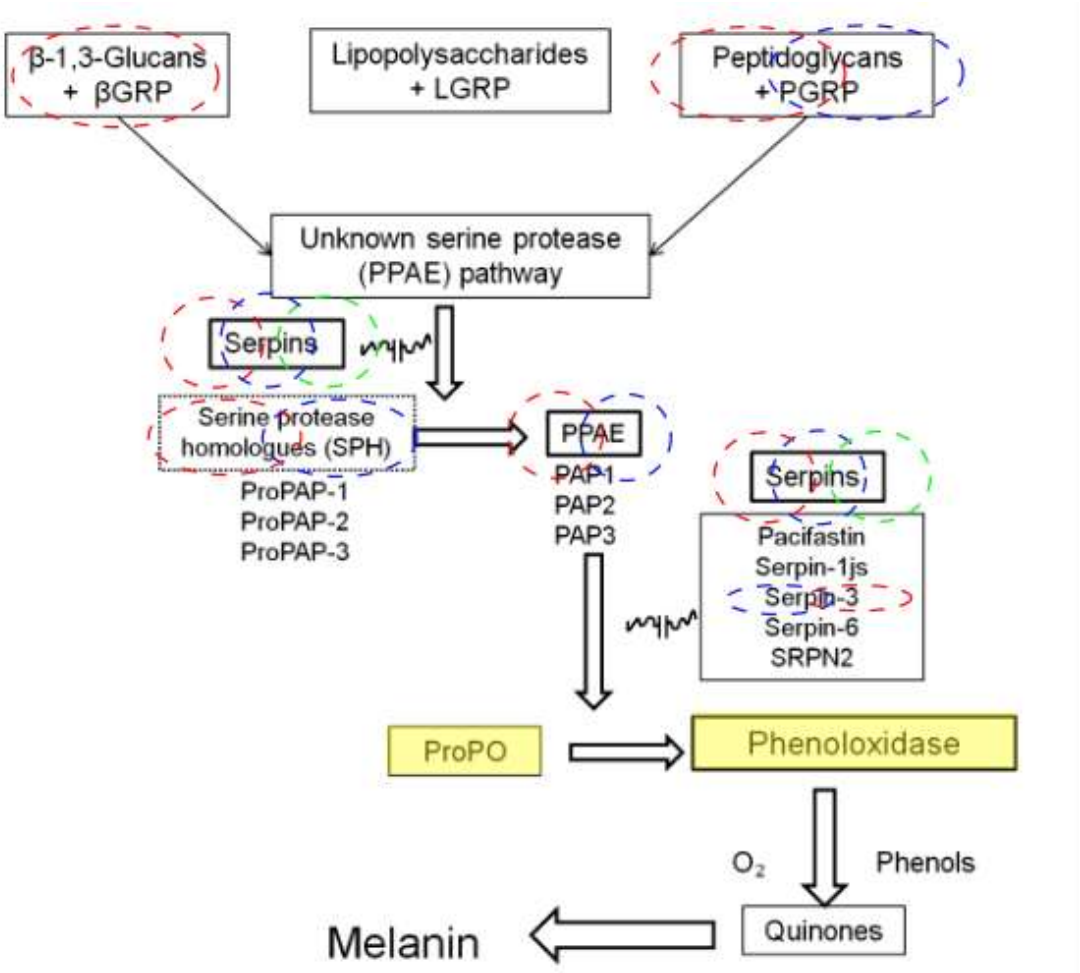


Figure 2.18. Schematic overview of a general model of the proPO activation system in insects.

Circles denote proposed alterations to abundance of proteins involved in the proPO activation system following treatment with EPF supernatant as shown from LFQ (Figure 2.9-2.11). Red circles indicate proteins altered in abundance following treatment with *B. caledonica*, blue circles indicate *M. anisopliae* and green circles represent *B. bassiana*. Image adapted from González-Santoyo and Córdoba-Aguilar (2012). The proPO activation cascade begins when hemolymph recognition proteins (bGRP, LGRP, or PGRP) bind to β-1,3-glucans, lipopolysaccharides, or peptidoglycans on the surface of the pathogen. This

recognition leads to the activation of a serine protease pathway. Subsequently the activation of this pathway causes prophenoloxidase activation. Phenoloxidase catalyzes the oxidation of phenols to form quinines, which then are polymerized to melanin. This pathway is tightly regulated by serine protease inhibitors (serpins) such as serpin-3.

2.4.3 EPF supernatant alters abundance of proteins involved in reception and detection in *G. mellonella*

It is known that the ability to perceive, discriminate and respond to chemical cues by chemoreception strongly impacts on fitness and survival of insects. This process is necessary for the identification of food resources, avoiding intoxication and to communicate and interact with other organisms including fungi. These chemical cues fall into 2 categories; odour produced by volatile compounds detected at low concentrations by olfactory/odorant receptors (ORs) and taste produced by non-volatile compounds at higher concentrations by gustatory receptors (Boucias *et al.* 2012). Insects sense the taste of foods and toxic compounds in their environment through the gustatory system (Sato *et al.* 2011). Gustatory organs are widely distributed over the surface of the body, enabling insects to detect nonvolatile chemosensory information such as foods or toxic compounds. Taste substances are recognized by gustatory sensory neurons that express proteins in the gustatory receptor family (Sato *et al.* 2011). In the proteomic profiles of larvae treated with all three EPF there is increased expression in a gustatory protein. One explanation for this upregulation may be linked to the fact that the production and maintenance of the proPO activating pathway is dietary dependent (Siva-Jothy and Thompson 2002, González-Santoyo and Córdoba-Aguilar 2012) and as there are changes to proteins related to the proPO pathway induced by all three EPF it may be necessary for the larvae to increase their dietary intake to sustain the cascade. The cost of activating and using immune defenses can itself reduce host fitness. The main effect of infection may not be direct damage caused by the pathogen but the cost imposed when the immune system is activated. Hosts may compensate for increased demands by increased resource uptake (Moret and Schmid-Hempel 2000). A different explanation may be found in the work of Lee *et al*. (2010). They demonstrated that *Drosophila* can detect minute concentrations of DEET, the most widely used insect repellent worldwide, through the gustatory response and that it suppressed feeding. This behavior was mediated by direct activation of avoidance gustatory receptor neurons. In a few Orthoptera, a coleopteran, and a number of larval Lepidoptera, compounds that inhibit feeding have been shown to stimulate gustatory receptor cells

in the mouthpart sensilla (Chapman 2003). This work highlights the importance of gustatory receptors in avoiding toxins.

LFQ revealed that EPF supernatant impacts upon proteins instrumental in pathogen recognition, that ultimately lead to activation of an appropriate immune response. LFQ of changes in the proteome of insect hemolymph following treatment with *B. caledonica* supernatant uncovered a number of changes to proteins that appear to be involved in the proPO pathway; both in the activation of the pathway and in its regulation (Figure 2.9). Insects have developed the ability to differentiate between major groups of microbes using PRRs such as peptidoglycan recognition proteins (PGRPs), hemolin and β-1,3-glucan binding protein (βGBPs). PRRs function by binding to PAMPs on microbial cells such as β-1,3-glucan from fungi that acts as a signal to activate the antifungal functions of Toll (Stokes *et al.* 2015). Three proteins involved in recognition were increased in abundance following treatment with *B. caledonica*: β-1,3-glucan binding protein and two peptidoglycan recognition proteins. These receptors are crucial to recognition of pathogens and activation of an appropriate immune response such as the prophenoloxidase pathway. Thus this is in indication of how the insect may be responding to the treatment. Both *M. anisopliae* and *B. caledonica* treated larvae display a downregulation in apolipophorins. Apolipophorins have been found to bind to bacterial and fungal cell walls and thus can be considered pattern recognition receptors (Zdybicka-Barabas and Cytryńska 2013). Furthermore cluster C (Table 2.7) indicates that *M. anisopliae* and *B. caledonica* treated larvae have a lower expression of apolipophorins than larvae treated with *B. bassiana* or controls. Additionally cluster C indicates that *M. anisopliae* treated larvae have the lowest level expression for another PRR peptidoglycan recognition-like protein. Lower levels of PRRs could result in higher susceptibility to further pathogens. Following LFQ of changes in the proteome of insect hemolymph treated with EPF fungal supernatant ten proteins were found to be uniquely detected in one or more sample group (Table 2.4). Of note is the absence of peptidoglycan recognition-like protein in *M. anisopliae* treated larvae only. These receptors are crucial to recognition of pathogens and activation of an appropriate immune response such as the prophenoloxidase pathway. Obviously PRRs respond to whole organisms and their components but in this case they are altered in abundance following treatment with spore free supernatant.

Fungal supernatant appears to be inducing changes in PRR and proPO activation and maintenance. While it is not possible from these results to pinpoint exactly what natural products in the fungal supernatant is inducing these alterations as the repertoire of EPF natural products is diverse, a number of abundant natural products from EPF have been reported to alter the insect immune response for example destruxin, cyclosporine, beauvericin, bassianolide and oosporein. Feng *et al*. (2015) found that oosporein, a natural product of several *Beauvaria* species, blocked PPO cleavage, cleavage of PPO to PO is the hallmark of PPO activation. Inhibiting PPO activity may contribute to fungal virulence by weakening the immune response subsequently facilitating fungal multiplication within the host (Feng *et al.* 2015). In Chapter Five, oosporein was identified as an immunomodulating natural product of the *B. caledonica* isolate used in this work. *G. mellonella* larvae injected with oosporein caused an alteration to a protein involved in the proPO pathway: a serine proteinase inhibitor was below the level of detection in oosporein treated larvae (discussed in section 5.4.2.)

2.4.4 EPF supernatant alters anti-bacterial response in *G. mellonella*

Cecropin-D precursor is below the level of detection in *M. anisopliae* treated larvae only (Table 2.4). Cecropins are a family of antibacterial molecules approximately 4kDa in size found in Lepidoptera and Diptera. They are membrane active antibiotics that act on Gram-positive and Gram-negative bacteria by forming channels that permeabilize the lipid bilayer (Hoffmann 1995, Jiang *et al.* 2010). Some cecropins also demonstrate antifungal activity (Faruck *et al.* 2015). The lower abundance of proteins, such as cecropin and PRR, that are instrumental in the insect's ability to recognize and respond to pathogens are indicative of *M. anisopliae* immunomodulating potential observed in bioassays.

There are similarities between the results of this work on the effect of *M. anisopliae* supernatant on the immune response of *G. mellonella* and the work of Pal *et al*. (2007). The immune responses in this chapter are induced by spore free supernatant which contains many natural products. A direct relationship has previously been established between destruxin production and the virulence of *M. anisopliae*. Pal *et al*. (2007) injected *Drosophila* with destruxin that caused a reduction in expression of AMP genes *Diptericin*, *Cecropin*, *Attacin* and *Metchnikowin.* Co-injection of destruxin with *E. coli* caused a significant decrease in survival of *Drosophila* compared with controls, with over 70% dead within five

days. Flies injected with *E. coli* alone don't demonstrate significant mortality. To test whether the lower expression of AMP genes also resulted in greater bacterial proliferation within the fly, bacterial survival counts were carried out on infected flies using similar methodology to that described in section 2.2.11. Destruxin and *E. coli* co-injected flies had greater *E. coli* proliferation compared with flies injected with *E. coli* alone. It was suggested that the observed correlation between bacterial proliferation and *Drosophila* mortality was consistent with the hypothesis that a lowering in AMP production induced by destruxin allows *E. coli* to proliferate and colonize the fly to accelerate its demise (Pal *et al.* 2007). Through selectively reducing bacterial AMPs with the secretion of destruxin *M. anisopliae* may acting to create an advantageous environment where bacteria can proliferate and, thereby, contribute to accelerating the demise of its insect host (Rohlfs and Churchill 2011). The work of Pal *et al*. (2007) bears striking similarities with the observation in this Chapter that treatment with *M. anisopliae* supernatant causes a reduction in abundance of the AMP cecropin among many other immune relevant proteins in *G. mellonella* and in parallel there is increased yeast proliferation and mortality when *G. mellonella* are injected with fungal supernatant before injection with *C. albicans*. Thus it adds strength to the argument that EPF fungal supernatant and the natural products contained within can modulate the immune response of insects.

Peptidylprolyl isomerase B precursor is present only in insects treated with the two EPF that demonstrate immunomodulating potential *B. caledonica* and *M. anisopliae*. Peptidylprolyl isomerase has previously been shown to be increased in expression in larvae challenged with *C. albicans* (Bergin *et al.* 2006). Expression of peptidylprolyl isomerase B is induced in silkworm following bacterial challenge and is believed to be involved in the silkworm immune system (Zhong *et al.* 2012).

A putative defense protein Hdd11 was upregulated following treatment with *M. anisopliae* only. Homologs of Hdd11 have been identified from several insect species and their expression was strongly induced by bacterial infection. Hdd11 is also believed to be involved in the melanization cascade in *A. mylitta* and *B. mori* (Gandhe *et al.* 2007, Bao *et al.* 2011). Hemolin was significantly increased in expression in *M. anisopliae* treated larvae only. Hemolin is a 47k Da hemolymph protein present at a low constitutive level that is increased upon bacterial challenge. It is thought to have a role in immune recognition and in modulation of defensive responses in *H. cecropia* and *M. sexta*. Hemolin has a role in antibacterial defense

indicated by its structural similarity to cell adhesion molecules and its increased expression induced by bacteria. Hemolin can also bind to the surfaces of bacteria and haemocytes and can stimulate the phagocytic activity of haemocytes (Gillespie and *et al.* 1997, Gillespie *et al.* 2000). A glycine rich AMP peptide gloverin was significantly decreased in expression in *M. anisopliae* treated larvae only. Glycine rich peptides have antifungal activity and are active against yeasts (Faruck *et al.* 2015) so their downregulation in *M. anisopliae* larvae may contribute in part to the increased proliferation of *C. albicans* in larvae pretreated with *M. anisopliae* fungal supernatant.

Also found upregulated in larvae treated with *B. caledonica* fungal supernatant was gloverin, a glycine-rich polypeptide previously isolated from lepidopteran hemolymph. Gloverins have a mass of 14 kDa and contain 18% of glycine residues. This AMP is effective against Gram-negative bacteria. They act by inhibiting the synthesis of vital outer membrane proteins resulting in an increase in permeability of the outer membrane of the bacteria (Bulet *et al.* 1999). One implication of an upregulation in AMP active against bacteria, following treatment with EPF supernatant, is that antibacterial activities can be highly beneficial to EPF as it might help exclude opportunistic infections that would be disadvantageous to the fungus (Butt *et al.* 2016).

2.4.5 Conclusion

Screening EPF for their ability to modulate the immune response of insects may represent a means of screening EPF for use in the field, particularly if the goal is to use them in combination with other control agents such as EPN or chemical insecticides. Additionally EPF isolates could be screened for their ability to produce particular natural products that modulate the immune response of target insects. This aim of this Chapter was to investigate the effect that EPF fungal supernatants have on both the cellular and humoral insect immune response utilizing *G. mellonella* larvae as a model system. The findings of this Chapter will ultimately be compared with the effect of EPF supernatant on *H. abietis* larvae. This allows investigation into the immunomodulating potential of natural products of EPF on the insect immune response, alongside the ability to validate the use of *G. mellonella* as a model organism. It is important to note that the immune responses induced in *G. mellonella* in this Chapter were in response to injection with spore free fungal supernatant, so it is a reflection of the immune response induced by EPF natural products. A number of

fungal natural products are known to be important virulence determinants. It has been previously shown that abundant EPF natural products such as destruxin and oosporein induce changes to immune response of insects affecting AMP and the proPO cascade as well as the cellular immune response. Due to the nature of EPF fungal isolates varying in virulence towards different hosts, the results discussed in this Chapter may be specific to certain insect hosts, thus the importance of validating the use of this insect model organism. Additionally they may be specific to isolates of these fungal species that produce similar natural products. Despite this potential limitation these findings aid in understanding how the desired synergism between biocontrol agents could mechanistically occur e.g. interfering with the proPO cascade and the production of AMP. Understanding how EPF modulate the immune response leaving insects more susceptible to subsequent pathogens may have application in selecting superior strains with these characteristics to overcome problems with fungi killing pests too slowly.

Bioassays allowed assessment of the immunomodulating potential of different treatments and proteomic analysis aided in understanding mechanistically how these variations in immunomodulation may have occurred e.g. alterations to proteins/pathways that may render the insect more susceptible to subsequent pathogens. The results indicate that *M. anisopliae* and *B. caledonica* fungal supernatant exhibit an immunomodulating effect on *G. mellonella* larvae. Together the bioassay results with the PCA analysis, hierarchical clustering, and Perseus comparisons between EPF treated larvae and control larvae as well as comparisons between EPF treated larvae and Blast2GO annotations indicate that *M. anisopliae* fungal supernatant has the greatest impact on the insect immune system. These differences in proteomic profiles for different treatments may be reflective or even partially explanatory for the differences observed in immunomodulating abilities in the bioassays for example higher levels of detoxifying enzymes in *B. bassiana* treated larvae than those treated with *M. anisopliae* and *B. caledonica* may be reflective of their differences in immunomodulation. This proteomic work gives an insight into the mechanisms behind the observed differences in immunomodulation of the tested EPF and is intended to fit into the bigger picture of EPF immunomodulation in insects. The primary aim of this Chapter is to validate the use of *G. mellonella* as a model for the screening of EPF with immunomodulation potential against *H. abietis* and so these findings are to be compared with similar analysis carried out on *H. abietis* larvae discussed in Chapter Three.

Chapter 3:

Effect of entomopathogenic fungi on the immune response of *Hylobius abietis*

3.1 Introduction

The large pine weevil, *Hylobius abietis*, is a major pest of reforestation in Northern Europe. Current practices for controlling *H. abietis* numbers and damage include chemical, biological and management strategies. As a major aim of this work was to assess the efficacy of EPF and EPN in combination for weevil suppression in the field, it was of interest to investigate what mmunomodulation effects EPF may have on the insect immune response. The ability to modulate the immune response of an insect rendering it more susceptible to other pathogens would have great significance for integrated pest management. If a combination of treatments resulted in a synergistic interaction then the potential cost of using these biopesticides may be reduced. In this Chapter the ability of EPF supernatant and the natural products within to modulate the immune system of *H. abietis* larvae was investigated by studying both the cellular and humoral immune response. The larval stage was primarily targeted with EPF and EPN in field trials detailed in Chapter Five, therefore its immune response is of interest in unravelling the potential mechanisms of mmunomodulation and in turn synergy. There are currently no reports of the effects of EPN of the cellular and humoral immune response of *H. abietis*.

Due to the popularity of *G. mellonella* as a model organism Vogel *et al*. (2011) performed a transcriptome characterization of different developmental stages and immune-challenged larvae to develop extensive transcriptomic resources for *G. mellonella*. This transcriptome allows an insight into the genetic makeup of immunity in *G. mellonella*, which will subsequently allow in-depth investigations into the molecular mechanisms underlying immune response to pathogens (Vogel *et al*. 2011). For this Chapter to enable proteomic investigations into the effects of EPF on the immune response of *H. abietis* and to compensate for the lack of genomic information for *H. abietis*; a *de novo* transcriptome study of *H. abietis* larvae was performed with Beijing Genomics Institute (BGI, Hong Kong). To maximize sequence diversity, RNA was extracted from both untreated larvae and larvae injected with *M. anisopliae* fungal supernatant.

The primary aim of this Chapter, alongside Chapter Two was to investigate the mmunomodulation potential of EPF on the insect immune response, as being able to

modulate the insect immune system may have implications for the ability of EPF to act synergistically with other control agents in the field.

3.1.1 Objectives

1. To assess the effect of EPF supernatant on the cellular immune response of *H. abietis* larvae.
2. To perform a transcriptome characterization of *H. abietis* larvae to develop transcriptomic resources that would in turn enable proteomic analysis.
3. To analyze the effect of EPF supernatant on the humoral immune response of *H. abietis* larvae using proteomic approaches to study changes in hemolymph proteins.
4. To evaluate the potential of *G. mellonella* larvae as a model for the study of proposed control agents for the large pine weevil *H. abietis* through comparing their immune response to EPF.

3.2 Materials and methods

3.2.1 Source and storage of *H. abietis* larvae

H. abietis larvae was sourced from a variety of felled forests across Ireland from 2012-2015. Forests were selected that they had been felled 12-18 months previously. Larvae were collected from stumps by removing soil from around the stump and roots. Bark was removed using a chisel. Larvae were collected from their wells in the stump and roots using tweezers. Larvae were placed in 24 well culture plates (Costar) and trays were kept out of direct sunlight to prevent larvae desiccating. Trays were transported back to the laboratory where lightly moistened tissue paper was placed inside the lids of the trays. Trays of larvae were stored in Styrofoam boxes at 4°C for up to three weeks.

3.2.2 Inoculation of *H. abietis* larvae

Larvae were injected with fungal supernatant through an abdominal spiracle (Figure 3.1) using a Myjector U100 insulin syringe (Terumo Europe, Leuven, Belgium). Larvae were placed in 24 well culture plates (Costar) with filter paper rather than petri dishes as *H. abietis* larvae are carnivorous (observations). Control larvae were injected with Sabouraud Dextrose liquid medium or PBS depending on the treatment.

Figure 3.1. *H. abietis* larvae with point of inoculation circled.

3.2.3 Determining the effect of EPF supernatant on *H. abietis* haemocyte densities

EPF was cultured in Sabouraud dextrose liquid medium for 48 h, 72 h and 96 h. The culture was filtered through 0.2 µl syringe filters (Section 2.2.4.). Larvae were injected with 20 µl of fungal supernatant. Control larvae were injected with 20 µl of Sabouraud dextrose liquid medium. Five larvae were injected per treatment and time. Haemocytes were extracted and haemocyte density was determined as per Section 2.2.9. The experiment was performed on three independent occasions.

3.2.4 Determining the effect of EPF supernatant on yeast load in *H. abietis*

C. albicans was cultured as per Section 2.2.5. Larvae were injected through the spiracle with fungal supernatant (Section 3.2.2). Control larvae were injected with Sabouraud dextrose. Five larvae were injected per treatment and time. Larvae were incubated for 24 h at 20°C. After 24 h larvae were given an injection of *C. albicans* ($1x10^4$/20 µl). Determination of the effect of EPF supernatant on yeast load in *H. abietis* was carried out as per 2.2.11. The experiment was performed on three independent occasions.

3.2.5 Investigating whether EPF supernatant leaves *H. abietis* larvae more susceptible to a subsequent infection

C. albicans was cultured as per Section 2.2.5. Larvae were inoculated through the spiracle with fungal supernatant. Control larvae were injected with Sabouraud dextrose. Ten larvae were injected per treatment and time. Larvae were incubated for 24 h at 20°C. After 24 h larvae were injected with *C. albicans* ($1x10^4$/20 µl). Larvae were incubated in multiwall plates at 20°C and mortality was recorded. The experiment was performed on three independent occasions.

3.2.6 RNA extraction of *H. abietis* larvae

Preliminary searches of *H. abietis* haemolymph data against available sequences (*T. castaneum* database and NCBI) resulted in identification of a low number of proteins (results not shown). To account for low sequence coverage, the *H. abietis* transcriptome was sequenced using commercial facilities of BGI. To isolate RNA one larva was crushed to a fine powder in liquid nitrogen using a sterilized pestle and mortar. Trizol (1 ml) was added. Sample was transferred to a 1.5 ml centrifuge tube and homogenized with a power pestle. The liquid was removed to a new 1.5 ml centrifuge tube and spun at 13,000 x g for 10 minutes at 4°C. The supernatant was removed to a new 1.5 ml centrifuge tube, 200 µl of chloroform was added to the sample, vortexed and left at room temperature for 10 minutes. The sample was spun at 12,000 x g for 10 minutes 4°C and the top clear layer was removed to a fresh centrifuge tube. Isopropanol (500 µl) was added to the clear layer and inverted several times. The sample was left for 10 minutes at room temperature. The sample was spun again at 12,000 x g for 10 minutes, and the resulting pellet was washed in 70% ethanol. The sample was spun at 9,000 x g for 5 minutes to remove ethanol, the pellet was allowed to air-dry. The pellet was resuspended in 100 µl of elution buffer (Sigma GenElute Mammalian Total RNA Miniprep Kit). A Sigma GenElute Mammalian Total RNA Miniprep Kit and protocol was used to do complete extraction of the sample. 10 µl of the eluted sample was used for NanoDrop, the remaining 40 µl was frozen at -80°C before sending to BGI. This method was carried out twice: 1. One untreated *H. abietis* larvae and 2. One *H. abietis* larvae injected with cell free *M. anisopliae* supernatant.

3.2.7 *H. abietis* transcriptome

The *H. abietis* transcriptome *de novo* study was completed by Beijing Genomics Institute (BGI, Hong Kong) using Illumina HiSeq 4000 (methodology is described in Appendix B). To assess the completeness of BGIs annotation for the *H. abietis* transcriptome a BUSCO (Benchmarking Universal Single-Copy Orthologs) (Simão *et al.* 2015) assessment was carried out on the predicted protein fasta files provided by BGI for different stages of the annotation, in addition to 34 insect genomes, from a number of insect families, sourced from Insect Base (http://www.insect-genome.com/), an integrated genome and transcriptome resource for insects. This assessment was carried out with Dr. Sarah Connell, Bioinformatics and Molecular Evolution Unit, Maynooth University

3.2.8 LFQ of the effect of EPF supernatant on the *H. abietis* proteome

Larvae were injected with 20 µl of fungal supernatant (Section 3.2.2.), control larvae were injected with Sabouraud dextrose and incubated for 48h at 20°C. Protein was extracted from the haemolymph of *H. abietis* larvae by piercing the anterior region of larvae with a 30GD mircolance needle and squeezing 1-3 drops of hemolymph into a pre-chilled 1.5 ml centrifuge tube and spun at 1,500 x g for 5 min at 4°C. Five larvae were bled per treatment. Extraction, digestion and sample clean up using C18 columns was performed as per Section 2.2.36. Three biological replicates were carried out per treatment. The Q-Exactive mass spectrometry method was carried out as described in Section 2.2.36.

Protein identification from the MS/MS data was performed using the Andromeda search engine in MaxQuant (version 1.2.2.5; http://maxquant.org/) to correlate the data against a predicted protein set derived from the *H. abietis de novo* transcriptome generated in this study. The following search parameters were used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5ppm with cysteine carbamidomethylation as a fixed modification and N-acetylation of protein and oxidation of methionine as variable modifications and a maximum of 2 missed cleavage sites allowed. Results processing, statistical analyses and graphics generation were conducted using Perseus v. 1.5.0.31. LFQ intensities were log_2-transformed and t-tests between the EPF treated larvae and controls were performed using a p-value of 0.05. Proteins were kept in the analysis if they were found in all of the three replicates in at least one group. The Blast2Go suite (www.blast2go.com) of

software tools was utilized to assign gene ontology terms (GO terms) relating to biological processes, molecular function and cellular components (Section 2.2.36).

3.2.9 Statistical analysis

Statistical analysis was carried out using Minitab statistical software V.16 and GraphPad Prism V.5. All data was tested for normality and were either transformed to normality or a suitable non-parametric test was used. For section 3.3.1 Data were not normal and were square root transformed before being analysed using two-way ANOVA and Bonferroni post-tests to compare treatments to relevant controls. For section 3.3.2 Data were not normal and were $\log_2$ transformed before being analyzed using two-way ANOVA and Bonferroni post hoc to compare treatments to relevant controls. For section 3.3.3 Data were normal and were tested for significance using Paired T-Tests.

3.3 Results

3.3.1 The effect of EPF supernatant on haemocyte densities of *H. abietis* larvae

Larvae treated with *B. caledonica* supernatant showed an alteration in haemocyte densities at both 24h and 48h (Figure 3.2.A). Treatment had a significant effect on haemocyte densities ($F_{3,16}$=8.21, $p<0.01$). Incubation time did not have a significant effect and there was no significant interaction between treatment and incubation time. After 24h a significant difference was seen in larvae treated with *B. caledonica* supernatant grown for 96h relative to control larvae ($p<0.01$). After 48h a significant difference was seen in haemocyte densities in larvae treated with *B. caledonica* supernatant grown for 72h ($p<0.05$) and 96h ($p<0.01$) relative to control larvae (Figure 3.2.A).

Larvae treated with *B. bassiana* supernatant showed an alteration in haemocyte densities at both 24h and 48h (Figure 3.2.B). Treatment had a significant effect on haemocyte densities ($F_{3,16}$=49.36, $p<0.001$). Incubation time did not have a significant effect but there was a significant interaction between treatment and time ($F_{3,16}$=4.16, $p<0.05$). After 24h a significant difference in haemocyte densities was seen in larvae treated with *B. bassiana* supernatant grown for 72h ($p<0.001$) and 96h ($p<0.01$) relative to control larvae. After 48h a significant difference in haemocyte densities was seen in larvae treated with *B. bassiana* supernatant grown for 48h ($p<0.01$), 72h ($p<0.001$) and 96h ($p<0.001$) relative to control larvae (Figure 3.2.B)

Larvae treated with *M. anisopliae* supernatant showed an alteration in haemocyte densities at both 24h and 48h (Figure 3.2.C). Treatment ($F_{3,16}$=8.89, $p<0.001$) and incubation time ($F_{3,16}$=12.27, $p<0.01$) had a significant effect on haemocyte densities. There was no significant interaction between treatment and time. After 24h a significant difference in haemocyte densities was seen in larvae treated with *M. ansioplae* supernatant grown for 96h ($p<0.01$). After 48h a significant difference in haemocyte densities was seen in larvae treated with *M. ansioplae* supernatant grown for 96h ($p<0.05$).

All significant differences in haemocyte densities between treatments and their appropriate controls were in the direction of reduction. The results demonstrated that

all three EPF cause a significant decline in the haemocyte density of larvae following inoculation with 96h fungal supernatant and incubation for 48h.

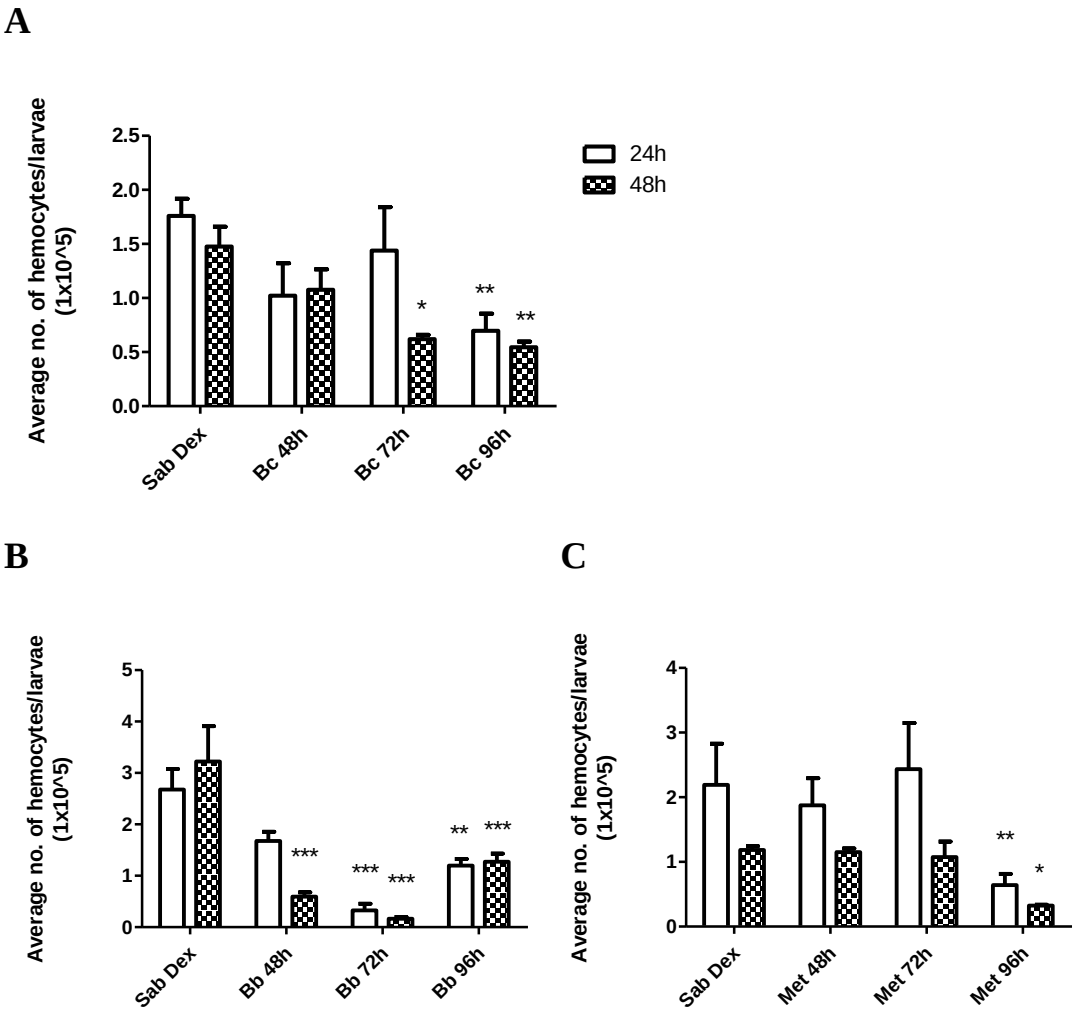


Figure 3.2. Haemocyte densities in larvae treated with EPF supernatant

Following inoculation with fungal supernatant, larvae where incubated for 24h or 48h at 20°C before bleeding and enumeration. Sab Dex: Control media, Bc: *B. caledonica*, Bb: *B. bassiana* and Met: *M. anisopliae*. 48h, 72h, 96h represents length of time EPF was cultured for. Data were analysed using Two Way ANOVA and Bonferroni post-tests which compared treatments to relevant controls. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Larvae treated with **A.** *B. caledonica* supernatant, **B**. *B. bassiana* supernatant, **C.** *M. anisopliae* supernatant.

3.3.2 The effect of EPF supernatant on yeast load in *H. abietis* larvae

In larvae treated with *B. caledonica* supernatant, treatment did not show a significant effect. However larvae treated with *B. caledonica* grown for 96h had a 9.3 fold increase in yeast cells (Figure 3.3.A). Time had a significant effect ($F_{3,16}$=7.93, $p<0.05$), but there was no significant interaction between treatment and time.

In larvae treated with *M. anisopliae* supernatant, both treatment ($F_{3,16}$=36.85, $p<0.001$) and time ($F_{3,16}$=36.43, $p<0.001$) had a significant effect. There was also a significant interaction between treatment and time ($F_{3,16}$=12.96, $p<0.001$). After a 24h incubation, treatment with *M. anisopliae* 48h ($p<0.05$), 72h ($p<0.05$) and 96h ($p<0.001$) supernatant resulted in a significant alteration in yeast load, with a fold increase of 6.2, 2.1 and 16.8 respectively, relative to controls. After a 48h incubation, treatment with *M. anisopliae* grown for 72h ($p<0.001$) and 96h ($p<0.001$) supernatant resulted in a significant alteration in yeast load, with a fold increase of 12.8 and 20.83 respectively, relative to controls (Figure 3.3.B)

In larvae treated with *B. bassiana* supernatant, both treatment ($F_{3,16}$=20.93, $p<0.001$) and time ($F_{3,16}$=77.85, $p<0.001$) had a significant effect. There was also a significant interaction between treatment and time ($F_{3,16}$=6.62, $p<0.01$). After a 48h incubation treatment with *B. bassiana* 72h supernatant induced a significant alteration in yeast load ($p<0.001$), with a fold increase of 44.36 (Figure 3.3.C).

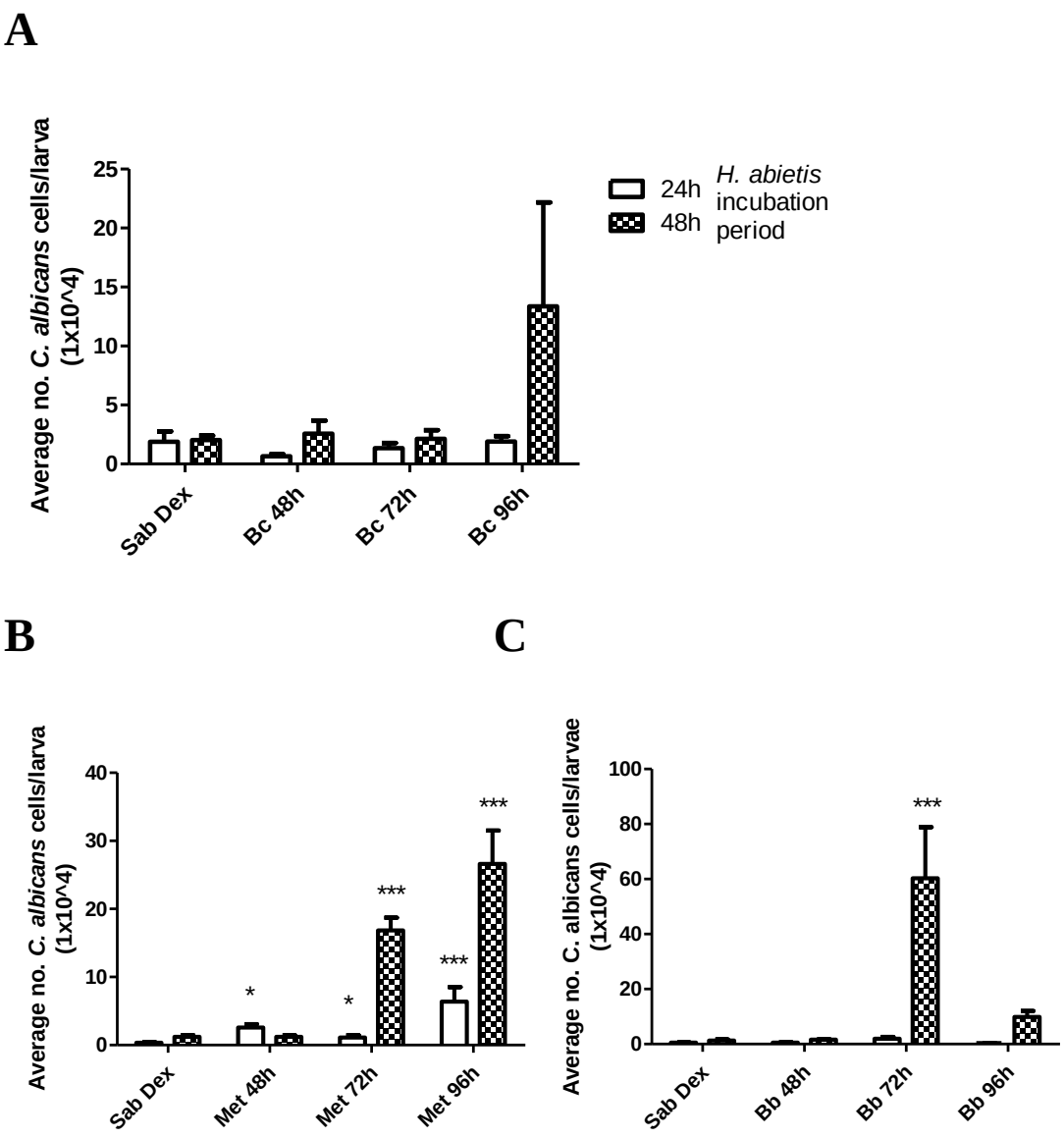


Figure 3.3. Changes in yeast load in *H. abietis* pre-treated with EPF supernatant.
Number of *C. albicans* cells per larva after incubation for 24h and 48h at 20°C. Larvae were treated with fungal supernatant 24h prior to inoculation with C. *albicans*. Sab Dex: Control media, Bc: *B. caledonica*, Bb: *B. bassiana* and Met: *M. anisopliae*. 48h, 72h and 96h represents length of time EPF was cultured for. Data that were not normal was transformed using log transformation. Data were then analyzed using Two Way ANOVA and Bonferroni post-tests which compared treatments to relevant controls. * $p<0.05$, ** $p<0.01$. ***$p<0.001$ A. Larvae treated with *B. caledonica* supernatant. B. *M. anisopliae* supernatant. C. *B. bassiana* supernatant.

3.3.3 Does EPF supernatant increase susceptibility of *H. abietis* to infection

To further investigate whether treatment with fungal supernatant makes larvae more susceptible to subsequent pathogens, larvae were injected with fungal supernatant and incubated for 24h at 20°C. After 24h larvae were inoculated with *C. albicans*. Larvae were incubated at 20°C and mortality was recorded over a one week period. One week after *C. albicans* infection, both *B. bassiana* ($T=-17$, $p<0.01$) and *B. caledonica* ($T=-4.91$, $p<0.05$) showed significantly different mortalities when larvae were treated with a combination of fungal supernatant and *C. albicans* combined compared to single doses (Figure 3.4). Larvae that were treated with EPF supernatant before treatment with *C. albicans* had higher mortality than larvae treated with either supernatant or *C. albicans* alone. However this pattern was only significant in larvae treated with *B. bassiana* and *B. caledonica*.

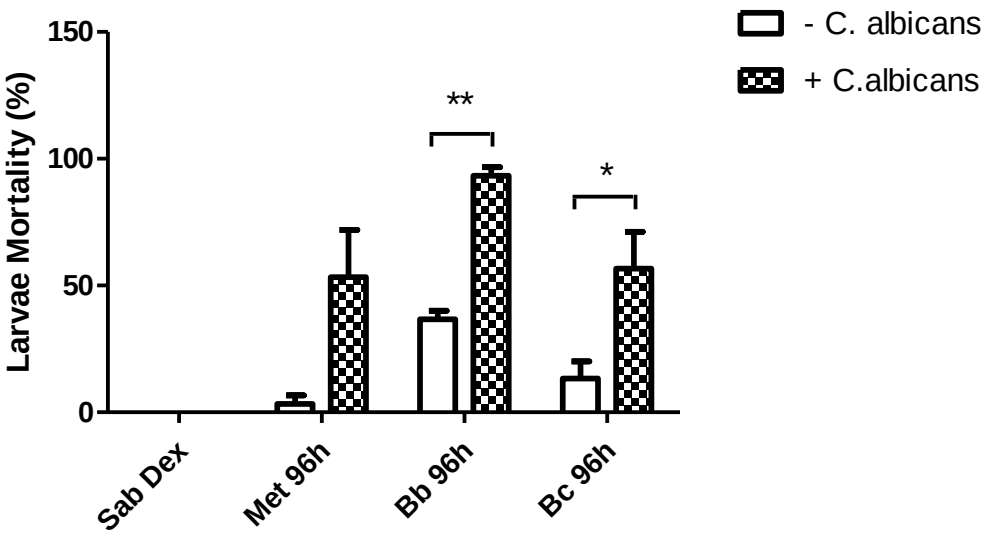


Figure 3.4. Mortality of *H. abietis* larvae treated with EPF supernatant alone and in combination with *C. albicans*.

+ C. albicans indicates larvae that received a dose of *C. albicans* after 24h, *- C. albicans* indicates larvae that did not. Graph shows mortality one week after *C. albicans* infection. Sab Dex: Control media, Bc: *B. caledonica*, Bb: *B. bassiana* and Met: *M. anisopliae*. EPF were cultured for 96h. * indicate difference between larvae without and without *C. albicans*. Paired T-tests were carried out for each EPF separately * $p<0.05$, ** $p<0.01$.

3.3.4 *H. abietis* transcriptome *de novo* study

Preliminary searches of *H. abietis* haemolymph data against using a *T. castaneum* database resulted in identification of six proteins (results not shown), a much higher number was expected as 100 proteins were identified in LFQ of *G. mellonella* larvae haemolymph. To account for the low number of proteins identified the *H. abietis* transcriptome was assembled and characterized by Beijing Genomics Institute (BGI, Hong Kong) and the major characteristics and results are described in Appendix B. The assembled transcriptome was translated into a protein output fasta file which was annotated and used for mass spectrometry searches. In order to determine the comprehensiveness of BGI's assembly and annotation a BUSCO (Benchmarking Universal Single-Copy Orthologs) assessment was carried out on the protein and nucleotide fasta files provided. These were compared with 18 insect proteomes, from a number of insect families, sourced from Insect Base (http://www.insect-genome.com/) (Table 3.1).

All BUSCO analysis was conducted using the single-copy ortholog set of 2,675 conserved genes derived from Arthropoda. The first section of Table 3.1 (*H. abietis transcritpome*) contains results from running BUSCO on the files that BGI provided. 1) Final set of BGI translated protein predictions and 2) Final set of corresponding RNA sequences. 44% (1,188/2,675) of the queried BUSCO orthologs were not present in the final set of BGI's translated protein predictions. Although these conserved genes are expected to be present in the genomes of all Arthropoda, they may not be constitutively expressed. Therefore, this finding is not surprising as not all genes are expected to be detectable in data derived from mRNA sequencing. 1) Final set of BGI translated protein predictions (PFAM_annotated), is the database that was subsequently used for LFQ of *H. abieties* haemolymph (Section 3.3.5). This database contained protein sequences with homology to 1,358/2,675 (or 51%) BUSCOs searched, 386 BUSCOs were duplicated, 129 BUSCOs were fragmented and 1,188 were absent. For comparison, the genome of another Coleoptera, mountain pine beetle, *Dendroctonus ponderosae*, also a major forest pest, had 2,150 BUSCOs, or 80% of the BUSCOs searched, with 290 BUSCOs duplicated.

Table 3.1. BUSCO assesment of transcriptome completeness for *H. abietis*

To assess the completeness of BGIs annotation for the *H. abietis* transcriptome a BUSCO (Benchmarking Universal Single-Copy Orthologs) assessment was carried out on the fasta files provided by BGI for different stages of the annotation, alongside 34 insect genomes, from a number of insect families.

	Organism	**Complete Single-copy BUSCOs**	**Complete Duplicated BUSCOs (subset of Complete Single-copy BUSCOs)**	**Fragmented BUSCOs**	**Missing BUSCOs**	**Total BUSCO groups searched**	**# Organism records searched**
***H. abietis* transcriptome**							
1	BGI *H. abietis* Protein	1,358	386	129	1,188	2,675	27,652
2	BGI *H. abietis* Nucleotide	2,135	586	154	386	2,675	27,653
Insect genomes							
Dendroctonus ponderosae		2,150	290	335	190	2,675	20,996
Aedes aegypti		2,496	652	96	83	2,675	17,143
Anopheles gambiae		2,615	537	36	24	2,675	14,667

Apis florea	2,625	978	41	9	2,675	17,663
Apis mellifera	2,614	242	56	5	2,675	15,314
Bactrocera cucurbitae	2,643	976	13	19	2,675	20,754
Bombus terrestris	2,648	1,294	19	8	2,675	20,321
Bombyx mori	2,024	188	381	270	2,675	14,623
Ceratitis capitate	2,646	1,062	16	13	2,675	23,088
Chilo suppressalis	960	61	480	1,235	2,675	10,221
Culex quinquciatus	2,388	372	149	138	2,675	19,019
Danaus plexippus	2,434	276	216	25	2,675	15,130
Diaphorina citri	1,643	464	548	484	2,675	20,996
Drosophila melanogaster	2,673	1,650	1	1	2,675	30,440
Heliconius Melpomene	1,998	189	385	292	2,675	12,829
Manduca sexta	2,257	1,015	228	190	2,675	27,403
Nilaparvata lugens	1,261	280	355	1,059	2,675	36,724
Plutella xylostella	1,787	489	321	567	2,675	18,073

3.3.5 Label free shotgun proteomic analysis of variations in the proteomic profile of *H. abietis* larval hemolymph following treatment with EPF supernatant

Label free quantification (LFQ) was used to compare the haemolymph proteome of *H. abietis* larvae treated with EPF supernatant relative to control larvae (Section 3.2.8). The groups analyzed were larvae treated with *M. anisopliae, B. caledonica* and *B. bassiana* supernatant grown for 96h and control larvae (treated with Sabouraud dextrose media). PCA confirmed a difference between the expression profiles of different treatments and that there were no outliers within the three replicates of each treatment (Figure 3.5). It also showed that the expression profile of larvae treated with *B. caledonica* was the most divergent EPF treatment relative to control larvae, while larvae treated with *B. bassiana* were most similar to control larvae.

In total, 157 proteins were identified, 155 proteins having two or more peptides. Seventy seven of these proteins were either significantly changed in expression or uniquely detected across the four treatments analyzed. Forty three proteins were found to be absent (below the level of detection) in one or more sample groups and present (above the level of detection) in three or less sample groups. These proteins were termed 'uniquely detected proteins' (Table 3.2 and Appendix C Table 1.1). A data imputation step was performed to replace missing values with values that simulate low abundant proteins. These values were chosen randomly from a distribution specified by a downshift of 1.7 times the mean standard deviation (SD) of all measured values and a width of 0.33 times this SD. To obtain an overall proteomic profile of abundance for all significantly expressed and exclusive proteins, hierarchical clustering on Z-score normalised intensity values was performed and the relative protein expression values were displayed as a heat map (Figure 3.6). Many of the proteins altered in abundance had the same protein name (but different Unigene or CL numbers), belonged to the same families or belonged to families with similar functionality such as glycosyl hydrolases families. The amino acid sequences of all peptides for these proteins were checked manually to ensure they were in fact representatives of different proteins before proceeding with analysis.

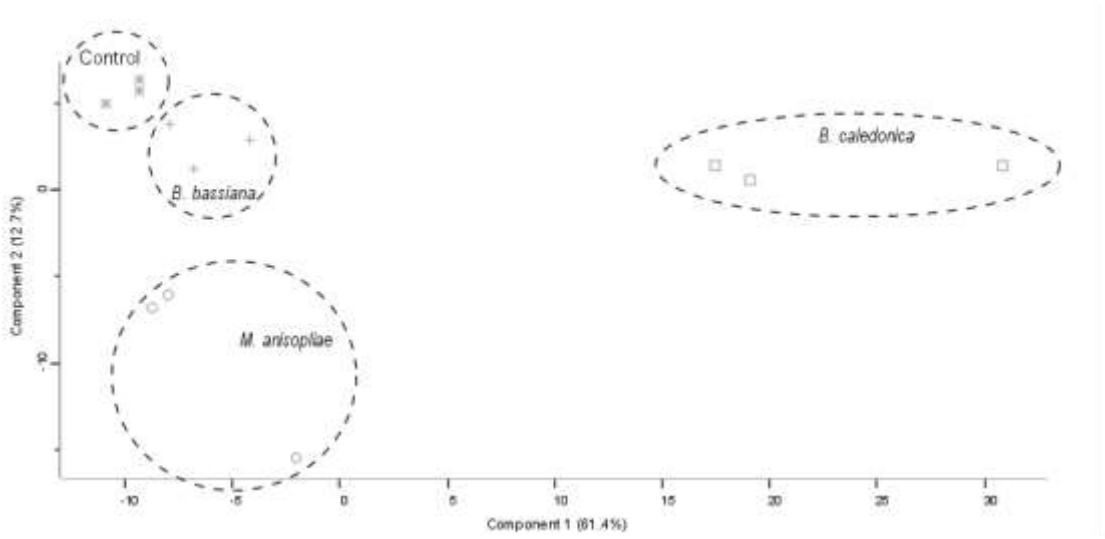


Figure 3.5. PCA of *H. abietis* haemolymph treated with EPF supernatant versus control (media).

Three biological replicates of each sample group were included in Perseus analysis. Each symbol represents a sample, and the shape shows the group to which it belongs. Dashed circles denote sample groups. The two axis account for 74.1 % of the total variation within the dataset.

Table 3.2. Uniquely detected proteins belonging to Glycosyl hydrolase families in the haemolymph of larvae treated with EPF supernatant.

NaN indicates a protein that was absent or below the level of detection. These proteins were termed as being 'uniquely detected'. PEP: Posterior Error Probabilities. Mean LFQ intensity represents average of three biological replicates per sample group.

Protein ID	Annotation	Mean LFQ intensity				PEP[1]	Intensity	MS/MS	Peptides	Mol. Weight (kDa)
		BB	BC	MET	SB					
Unigene28106	Glycosyl hydrolase family 1	NaN	26.5	NaN	NaN	7.1^{-39}	1.3^{+09}	18	7	25.8
Unigene6962	Glycosyl hydrolase family 1	NaN	27.6	NaN	NaN	9.9^{-141}	2.6^{+09}	40	11	56.1
CL1864.Contig4	Glycosyl hydrolase family 2	NaN	25.5	NaN	NaN	6.3^{-89}	4.2^{+08}	31	8	71.7
Unigene12087	Glycosyl hydrolase family 2	NaN	28.3	NaN	NaN	0.0^{+00}	3.5^{+09}	133	32	101.8
Unigene13345	Glycosyl hydrolase family 20	NaN	24.6	NaN	NaN	1.6^{-71}	2.0^{+08}	21	9	61.4
Unigene3946	Glycosyl hydrolase family 28	NaN	27.6	25.9	NaN	7.6^{-128}	2.5^{+09}	53	8	38.0
Unigene13818	Glycosyl hydrolase family 28	24.4	28.7	NaN	NaN	0.0^{+00}	4.7^{+09}	67	10	36.4
Unigene3970	Glycosyl hydrolase family 28	NaN	24.9	NaN	NaN	3.5^{-109}	7.0^{+08}	39	11	35.8
Unigene7925	Glycosyl hydrolase family 31	NaN	29.5	24.2	NaN	0.0^{+00}	5.5^{+09}	175	31	96.6

CL921.Contig2	Glycosyl hydrolase family 31	NaN	26.9	25.2	NaN	4.7^{-296}	1.4^{+09}	69	16	70.7
Unigene12565	Glycosyl hydrolase family 35	NaN	27.4	NaN	NaN	8.2^{-235}	1.7^{+09}	70	17	71.9
Unigene8511	Glycosyl hydrolase family 38	NaN	26.9	NaN	NaN	6.5^{-153}	6.8^{+08}	55	18	111.9
Unigene9634	Glycosyl hydrolase family 45	NaN	28.2	NaN	NaN	5.6^{-78}	2.4^{+09}	18	2	23.8
Unigene3825	Glycosyl hydrolase family 45	NaN	31.0	NaN	NaN	3.7^{-122}	1.5^{+10}	52	3	23.9
Unigene3841	Glycosyl hydrolase family 45	NaN	26.2	NaN	NaN	7.6^{-49}	4.3^{+08}	12	3	25.8
Unigene11986	Glycosyl hydrolase family 48	NaN	31.4	27.4	NaN	0.0^{+00}	2.7^{+10}	230	26	70.5
CL5500.Contig2	Glycosyl hydrolase family 48	NaN	32.3	24.1	NaN	0.0^{+00}	4.5^{+10}	264	29	70.9
Unigene10957	Glycosyl hydrolase family 79	NaN	25.0	NaN	NaN	2.8^{-80}	4.8^{+08}	35	9	52.5

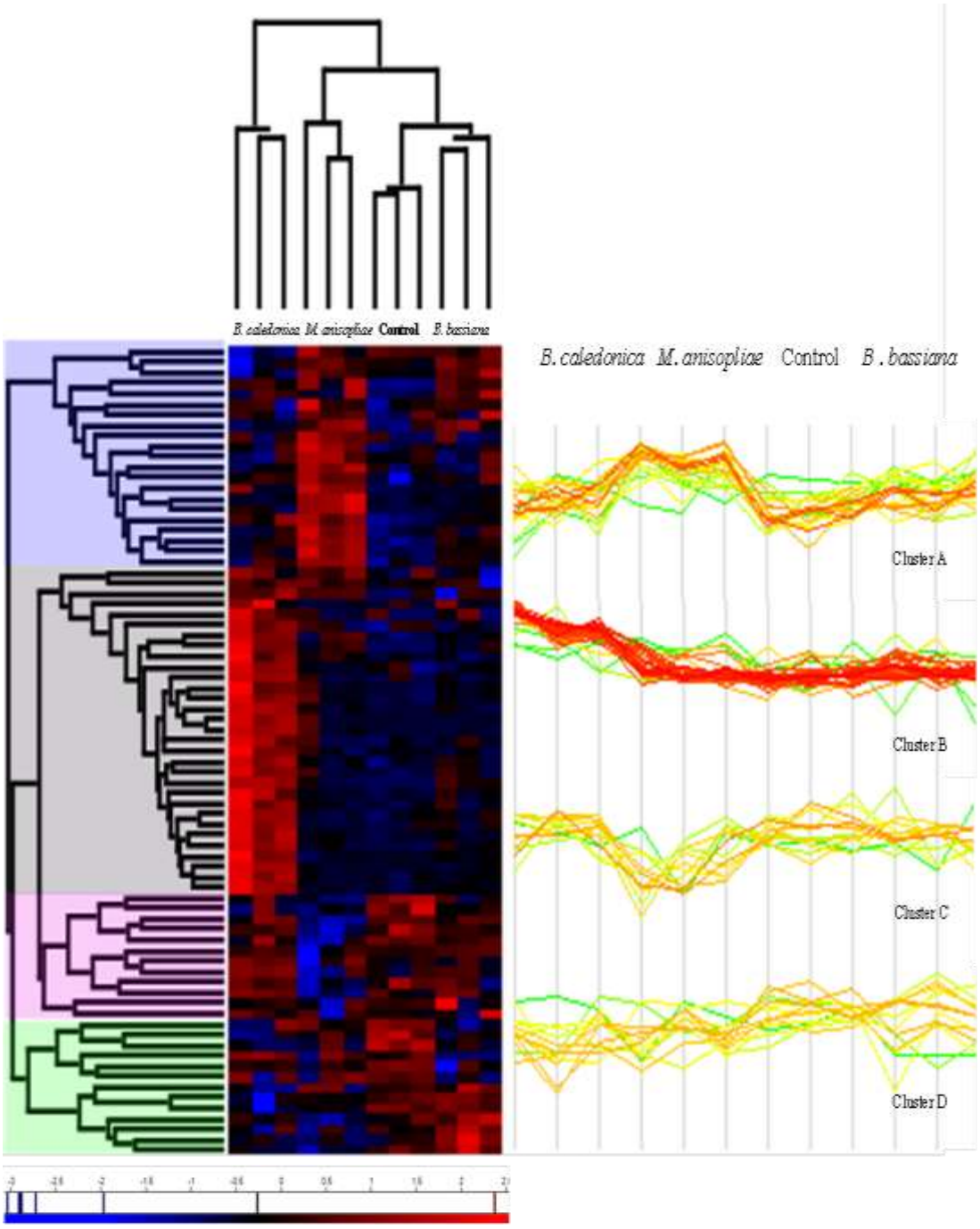


Figure 3.6. Hierarchical clustering of the quantitative differences in the proteomic profile of *H. abietis* larvae treated with EPF supernatant versus control.

The heat map shows the variation in expression of the proteins from the profiles of *H. abietis* larvae obtained using Perseus software. The data summarized are for all significantly expressed and uniquely detected proteins. Hierarchical clustering (columns) resolved four distinct clusters. Cluster A is indicated with light blue, Cluster B light purple, Cluster C pink and Cluster D green. Red indicates high level expression and blue indicates low level of expression. The corresponding cluster tables are in Appendix C Table C 1.2-1.5.

Hierarchical clustering resolved four distinct clusters of proteins with similar expression profiles (Figure 3.6). Cluster A comprises proteins with higher levels of abundance in larvae treated with *M. anisopliae* and *B. caledonica* relative to control larvae (Appendix C Table C.1.2). Cluster B comprises proteins with higher levels of abundance in larvae treated with *B. caledonica* relative to all other treatments and control larvae (Table C.1.3). Cluster C comprises proteins with lower levels of abundance in larvae treated with *M. anisopliae* relative to all other treatments and control larvae (Table C.1.4). Cluster D comprises proteins with highest levels of abundance in control larvae relative to all EPF treated larvae (Table C.1.5).

For LFQ analysis all proteins listed as significantly changed in abundance are those with a p-value <0.05 and a relative fold change of 1.5 or above. In larvae treated with *B. caledonica* fungal supernatant 31 proteins were significantly increased in abundance and six proteins were significantly decreased in abundance (Figure 3.7, Table 3.3). There were many proteins involved in metabolic processes altered in abundance for instance a number of proteins belonging to glycosyl hydrolase and carboxylesterase families. Pathogenesis-related 5, thaumatin- like protein was increased in abundance. Thaumatin is an AMP active against filamentous fungi found in the red flour beetle, *Tribolium castanuem* (Altincicek *et al.* 2008). The AMP defensin was decreased in abundance in *B. caledonica* treated larvae relative to controls, this 4kDa cyclic AMP displays antibacterial activity against Gram positive bacteria (Hoffmann *et al.* 1996).

In larvae treated with *B. bassiana* fungal supernatant 17 proteins were significantly increased in abundance and six proteins were significantly decreased in abundance (Figure 3.8, Table 3.4). A number of proteins belonging to glycosyl hydrolase families were increased in abundance. Pathogenesis-related 5 (thaumatin), melanin-inhibiting protein, heat shock 70 kDa cognate 4 and the AMP attacin C were increased in abundance, all with important immune functionality. Attacin is an antibacterial peptide (Y. Zhu *et al.* 2003). The AMP defensin was decreased in abundance in *B. bassiana* treated larvae relative to controls.

In larvae treated with *M. anisopliae* fungal supernatant 20 proteins were significantly increased in abundance and 13 proteins were significantly decreased in abundance (Figure 3.9, Table 3.5). A number of proteins with serine endopeptidase activity were decreased in abundance: serine protease easter, pro-phenol partial and Kunitz & Bovine pancreatic trypsin inhibitor domain. Heat shock 70 kDa cognate 4

was increased in abundance following injection with all EPF. Heat shock proteins (HSP) are associated with management of a wide range of stress factors and levels of HSP are elevated in response to *B. bassiana* and *M. anisopliae* infection (Butt *et al.* 2016).

Proteins with a Lectin C-type domain are decreased in abundance in *H. abietis* larvae treated with all three EPF. The majority of characterized C-type Lectin domain containing proteins from insects are humoral defense proteins (Zelensky and Gready 2005). Lectins have been reported to specifically bind bacterial LPS and to stimulate phenoloxidase activation. Addition of immulectin, a C-type lectin from the tobacco hornworm, *Manduca sexta*, to hemolymph leads to activation of prophenoloxidase, and this reaction is enhanced when immulectin is combined with bacterial LPS (Yu *et al.* 1999).

As well as comparing the haemolymph proteome of larvae treated with EPF supernatant to control larvae, the proteomes of different EPF treated larvae were compared against each other using Perseus software. When larvae treated with *B. caledonica* fungal supernatant were compared to those treated with *B. bassiana* 30 proteins were significantly increased in abundance and ten proteins were significantly decreased in abundance (Figure 3.10, Table 3.6). When larvae treated with *M. anisopliae* fungal supernatant were compared to those treated with *B. caledonica* 15 proteins were significantly increased in abundance and 30 proteins were significantly decreased in abundance (Figure 3.11, Table 3.7). When larvae treated with *M. anisopliae* fungal supernatant were compared to those treated with *B. bassiana* 13 proteins were significantly increased in abundance and 14 proteins were significantly decreased in abundance (Figure 3.12, Table 3.8).

Blast2GO annotation software (www.blast2GO.com) was used to group proteins based on conserved gene ontology (GO) terms in order to identify processes and pathways altered in response to different EPF treatments. However Blast2GO assigned GO terms to only 115 of the 157 proteins identified by LFQ, so this must be taken into account before drawing conclusions about apparent changes in processes and pathways induced by treatment with EPF fungal supernatant. A comparison of changes in proteins annotated by Blast2GO as being involved in cellular processes (Figure 3.13), molecular processes (Figure 3.14) and biological processes (Figure 3.15) is presented.

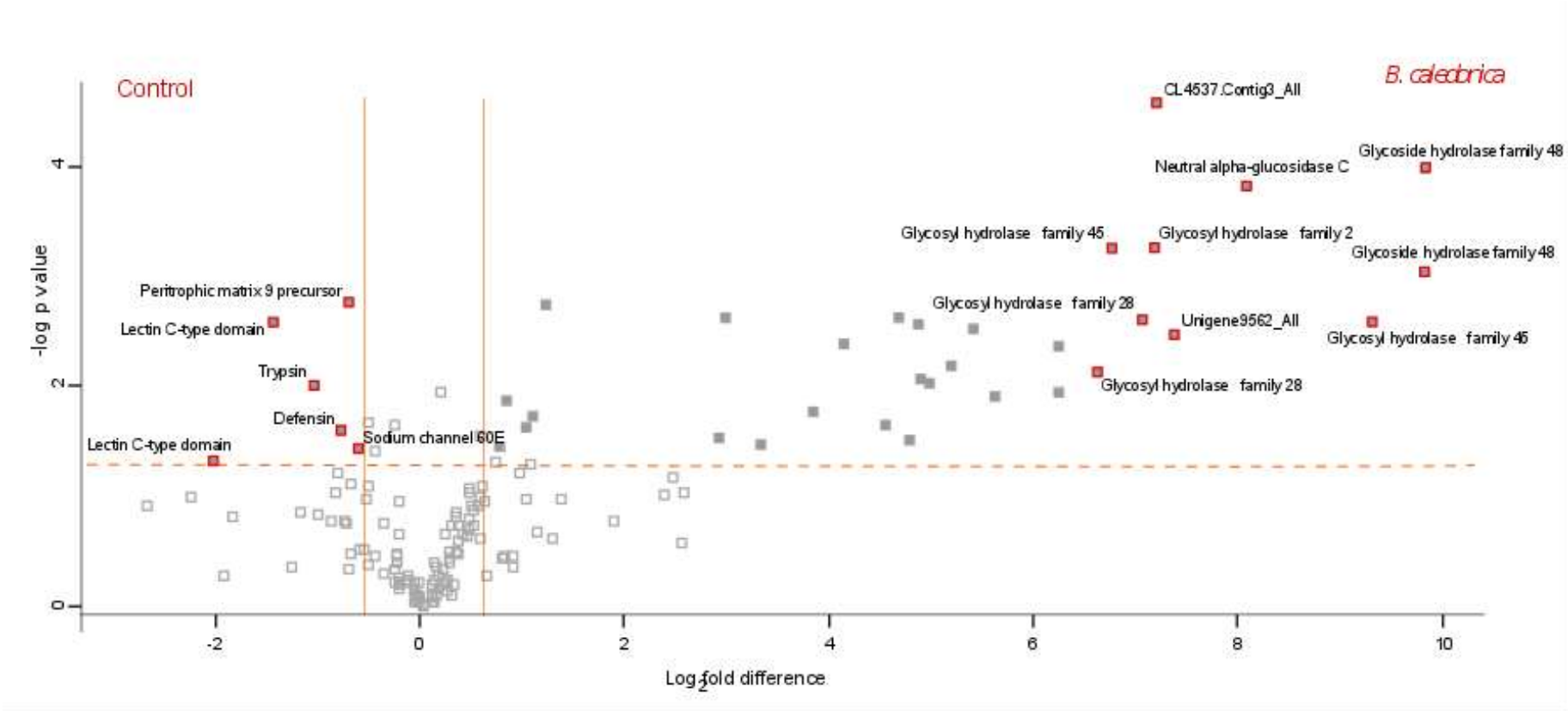


Figure 3.7. Volcano plot highlighting proteins present in highest and lowest abundances between larvae treated with *B. caledonica* supernatant and control larvae.

Image of volcano plot obtained using Perseus software. Proteins above the dashed orange line indicates significance ($p<0.05$) and those to the right and left of the vertical lines indicate fold changes greater than 1.5 fold. Boxes with red outline are significant and are in the top 10. Grey boxes are significant but not in tip 10. Proteins present on the right hand side are increased in abundance relative to the control.

Table 3.3. Proteins significantly increased or decreased in abundance in larvae treated with *B. caledonica* supernatant relative to control larvae.

Tables 3.3-3.8: Proteins included have a $p<0.05$ (or a –Log p value above 1.3) and a fold change of above +/-0.58. The average LFQ intensities of the three biological replicates are given.

				Average LFQ Intensities	
Protein ID	**Annotation**	**P value (-Log)**	**Log_2 Fold Difference**	**BC**	**Control**
Unigene11986	Glycoside hydrolase family 48	3.0	9.8	31.5	21.7
CL5500.Contig2	Glycoside hydrolase family 48	4.0	9.8	32.5	22.7
Unigene3825	Glycosyl hydrolase family 45	2.6	9.3	30.6	21.3
Unigene7925	Neutral alpha-glucosidase C	3.8	8.1	29.7	21.7
Unigene9562	Unigene9562_All	2.5	7.3	29.6	22.2
CL4537.Contig3	CL4537.Contig3_All	4.6	7.2	27.6	20.4
Unigene12087	Glycosyl hydrolase family 2	3.3	7.2	29.0	21.8
Unigene3946	Glycosyl hydrolase family 28	2.6	7.0	28.0	21.0
Unigene9634	Glycosyl hydrolase family 45	3.3	6.7	28.3	21.6
Unigene13818	Glycosyl hydrolase family 28	2.1	6.6	29.1	22.5
Unigene19514	Pectinesterase	2.4	6.2	27.8	21.6
Unigene6962	Glycosyl hydrolase family 1	1.9	6.2	28.0	21.8
CL2640.Contig1	Pathogenesis-related 5	1.9	5.6	28.0	22.4
CL921.Contig2	Glycoside hydrolase family 31	2.5	5.4	27.5	22.1
Unigene12565	Glycosyl hydrolase family 35	2.2	5.2	27.8	22.6
Unigene8511	Glycosyl hydrolase family 38	2.0	4.9	26.5	21.6
Unigene13343	Carboxylesterase family	2.1	4.9	26.9	22.0
Unigene3841	Glycosyl hydrolase family 45	2.6	4.8	26.2	21.4
Unigene28106	Glycosyl hydrolase family 1	1.5	4.7	26.9	22.2
Unigene2311	Alpha-L-fucosidase	2.6	4.7	25.8	21.2

Unigene3970	Glycosyl hydrolase family 28	1.6	4.5	25.8	21.3
Unigene10957	Glycosyl hydrolase family 79	2.4	4.1	25.7	21.6
CL2700.Contig4	Carboxylesterase family	1.8	3.8	26.2	22.3
Unigene3953	Prostatic acid phosphatase	1.5	3.3	25.5	22.2
CL1864.Contig4	Glycosyl hydrolase family 2	2.6	3.0	25.8	22.8
Unigene8018	Chemosensory 6	1.5	2.9	25.8	22.9
Unigene3665	FKBP-type peptidyl-prolyl cis-trans isomerase	2.7	1.2	25.9	24.7
CL416.Contig1	Heat shock 70 kDa cognate 4	1.7	1.1	23.9	22.9
Unigene3489	Serpin	1.6	1.0	26.9	25.9
Unigene1589	Trypsin	1.9	0.8	25.8	25.0
CL797.Contig4	Chymotrypsin-C isoform X1	1.4	0.7	23.8	23.1
CL5549.Contig2	Sodium channel 60E	1.4	-0.6	24.4	25.0
Unigene11176	Peritrophic matrix 9 precursor	2.8	-0.7	25.4	26.1
Unigene5303	Defensin	1.6	-0.8	28.6	29.4
Unigene13338	Trypsin	2.0	-1.1	26.9	27.9
CL2534.Contig1	Lectin C-type domain	2.6	-1.5	22.9	24.4
CL1224.Contig1	Lectin C-type domain	1.3	-2.1	24.8	26.8

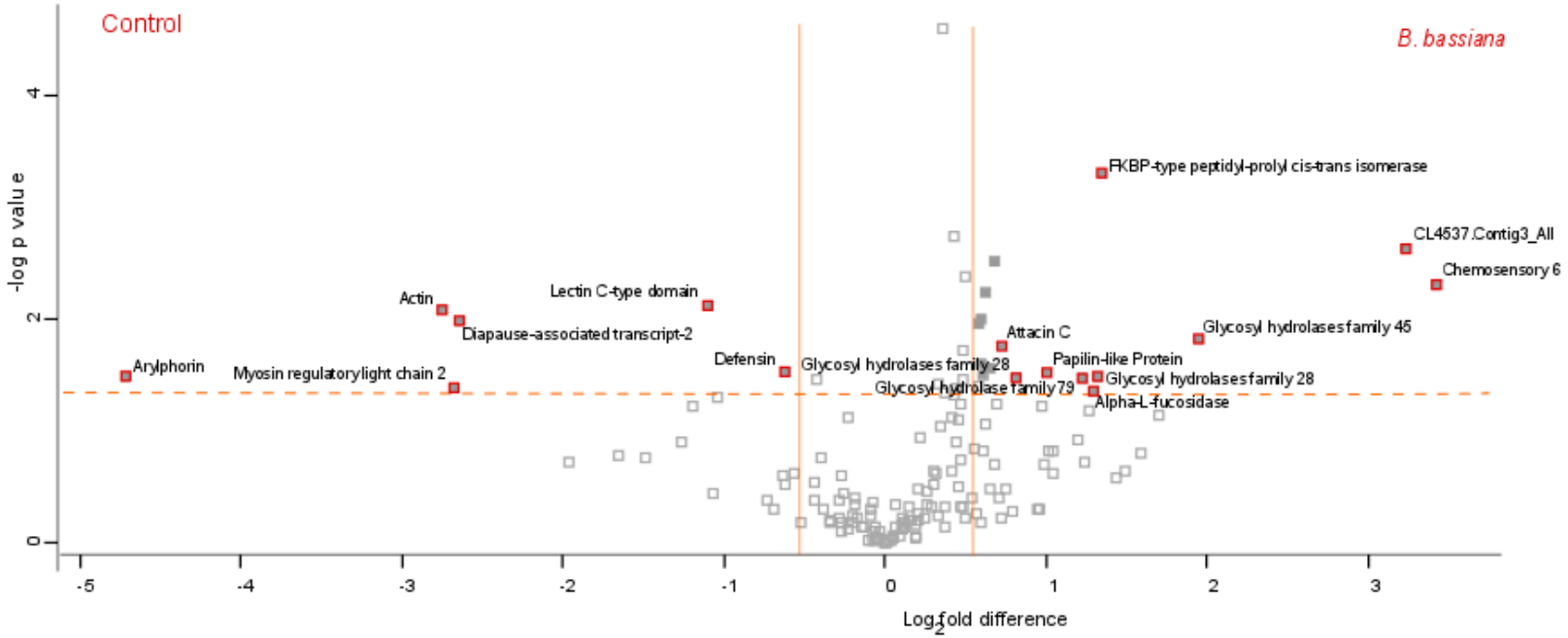


Figure 3.8. Volcano plot highlighting proteins present in highest and lowest abundances between larvae treated with *B. bassiana* supernatant and control larvae.

Image of volcano plot obtained using Perseus software. Proteins above the dashed orange line indicates significance ($p<0.05$) and those to the right and left of the vertical lines indicate fold changes greater than 1.5. Boxes with red outline are significant and are in the top 10. Grey boxes are significant but not in tip 10. Proteins present on the right hand side are increased in abundance relative to the control

Table 3.4. Proteins significantly increased or decreased in abundance in larvae treated with *B. bassiana* supernatant relative to control larvae.

				Average LFQ Intensities	
	Annotation	**P value (-Log)**	**Log_2 Fold Difference**	**BB**	**Control**
Unigene8018	Chemosensory 6	2.3	3.4	26.3	22.9
CL4537.Contig3	CL4537.Contig3_All	2.6	3.2	23.7	20.4
Unigene9634	Glycosyl hydrolase family 45	1.8	2.0	23.6	21.6
Unigene3665	FKBP-type peptidyl-prolyl cis-trans isomerase	3.3	1.4	26.0	24.7
Unigene3946	Glycosyl hydrolase family 28	1.5	1.3	22.3	21.0
Unigene2311	Alpha-L-fucosidase	1.4	1.3	22.5	21.2
Unigene10957	Glycosyl hydrolase family 79	1.5	1.2	22.8	21.6
CL943.Contig7	Papilin-like Protein	1.5	1.0	25.0	24.0
Unigene3970	Glycosyl hydrolase family 28	1.5	0.8	22.2	21.3
Unigene7330	Attacin C	1.8	0.7	31.4	30.7
CL1928.Contig2	Odorant-binding 29	2.5	0.7	31.9	31.2
Unigene9585	Unigene9585_All	1.6	0.6	31.0	30.4
Unigene2445	Regulatory CLIP domain of proteinases	2.2	0.6	26.9	26.2
CL2420.Contig2	Melanin-inhibiting protein	1.5	0.6	29.3	28.7
CL2640.Contig1	Pathogenesis-related 5 (Thaumatin)	1.6	0.6	23.0	22.4
CL416.Contig1	Hsp70 protein	2.0	0.6	23.4	22.9
CL2563.Contig1	Peroxidase isoform X1	2.0	0.6	27.3	26.7
Unigene5303	Defensin	1.5	-0.6	28.8	29.4
CL2534.Contig1	Lectin C-type domain	2.1	-1.1	23.3	24.4
CL515.Contig1	Diapause-associated	2.0	-2.6	21.8	24.4

	transcript-2				
Unigene5426	Myosin regulatory light chain 2	1.4	-2.7	22.9	25.6
CL5881.Contig1	Actin	2.1	-2.7	22.7	25.4
CL61.Contig2	Arylphorin	1.5	-4.7	24.1	28.8

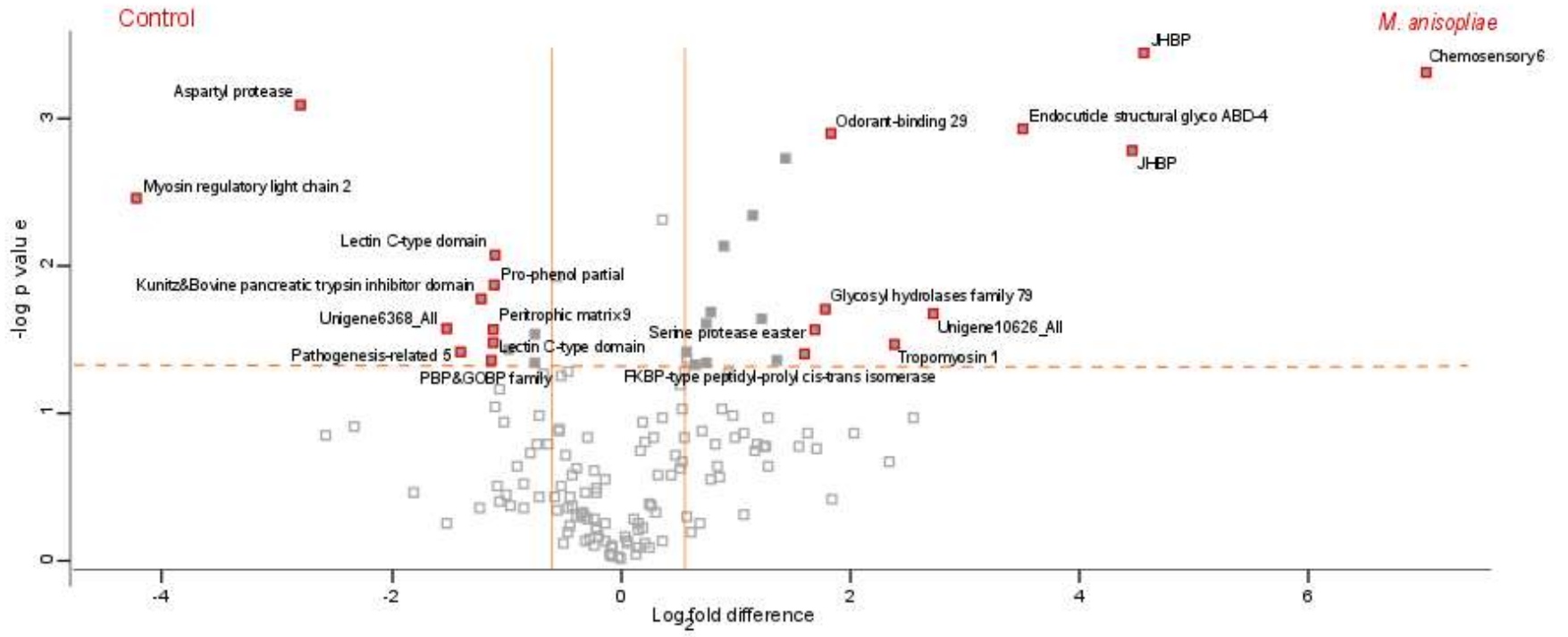


Figure 3.9. Volcano plot highlighting proteins present in highest and lowest abundances between larvae treated with *M. anisopliae* supernatant and control larvae.

Image of volcano plot obtained using Perseus software. Proteins above the dashed orange line indicates significance ($p<0.05$) and those to the right and left of the vertical lines indicate fold changes greater than 1.5. Boxes with red outline are significant and are in the top 10. Grey boxes are significant but not in top 10.

Table 3.5. Proteins significantly increased or decreased in abundance in larvae treated with *M. anisopliae* supernatant relative to control larvae.

				Average LFQ Intensities	
Protein Identity	**Annotation**	**P value (-Log)**	**Log_2 Fold Difference**	**Met**	**Control**
Unigene8018	Chemosensory 6	3.3	7.0	29.9	22.9
Unigene12317	JHBP	3.4	4.6	26.1	21.6
Unigene17410	JHBP	2.8	4.5	26.8	22.3
Unigene8077	Endocuticle structural glyco ABD-4	2.9	3.5	25.2	21.6
Unigene10626	Unigene10626_All	1.7	2.7	25.6	22.9
CL3921.Contig1	Tropomyosin 1	1.5	2.4	24.1	21.7
CL1928.Contig2	Odorant-binding 29	2.9	1.8	33.1	31.2
Unigene10957	Glycosyl hydrolase family 79	1.7	1.8	23.3	21.6
CL466.Contig4	Aerine protease easter (Trypsin)	1.6	1.7	25.5	23.8
Unigene3665	FKBP-type peptidyl-prolyl cis-trans isomerase	1.4	1.6	26.3	24.7
CL416.Contig1	Heat shock 70 kDa cognate 4	2.7	1.4	24.3	22.9
Unigene28106	Glycosyl hydrolase family 1	1.4	1.4	23.5	22.2
Unigene2302	Major royal jelly protein	1.6	1.2	29.7	28.5
Unigene27113	28 kDa desiccation stress	2.3	1.1	29.3	28.2
CL1928.Contig3	Odorant-binding 29	2.1	0.9	33.5	32.6
CL943.Contig7	Papilin-like Protein	1.7	0.8	24.8	24.0
CL3504.Contig2	Beta-1,3-glucan-binding	1.6	0.8	26.8	26.0
CL492.Contig1	Attacin	1.3	0.8	30.3	29.5
CL5881.Contig6	Actin-5C	1.3	0.7	25.6	24.9
Unigene7330	Attacin	1.4	0.6	31.3	30.7
Unigene8093	Copper&zinc superoxide dismutase (SODC)	1.5	-0.7	27.5	28.2

CL3832.Contig2	Peptidoglycan-recognition SC2	1.3	-0.7	26.7	27.4
Unigene3995	Serine protease easter (Trypsin)	1.4	-1.0	27.9	28.8
CL1224.Contig1	Lectin C-type domain	2.1	-1.1	25.8	26.8
CL1617.Contig2	Pro-phenol partial (Trypsin)	1.9	-1.1	27.1	28.2
CL2534.Contig1	Lectin C-type domain	1.5	-1.1	23.3	24.4
Unigene11176	Peritrophic matrix 9 precursor	1.6	-1.1	25.0	26.1
CL2247.Contig3	PBP&GOBP family	1.3	-1.1	34.4	35.6
CL3607.Contig1	Kunitz&Bovine pancreatic trypsin inhibitor domain	1.8	-1.2	32.1	33.3
Unigene4030	Pathogenesis-related 5 (Thaumatin)	1.4	-1.4	29.6	31.0
Unigene6368	Unigene6368_All	1.6	-1.5	22.7	24.2
Unigene417	Aspartyl protease	3.1	-2.8	20.9	23.6
Unigene5426	Myosin regulatory light chain 2	2.5	-4.2	21.4	25.6

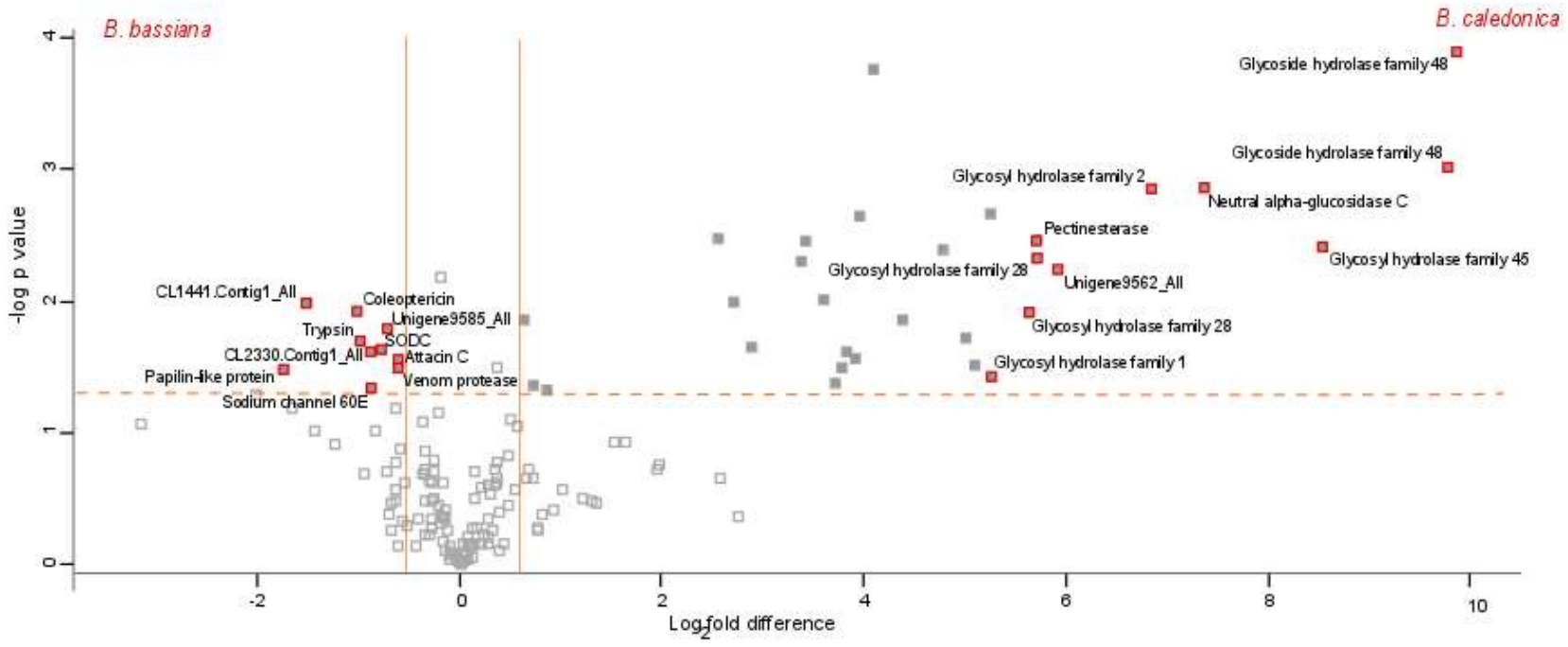


Figure 3.10. Volcano plot highlighting proteins present in highest and lowest abundances between larvae treated with *B. caledonica* and *B. bassiana* supernatant.

Image of volcano plot obtained using Perseus software. Proteins above the dashed orange line indicates significance ($p<0.05$) and those to the right and left of the vertical lines indicate fold changes greater than 1.5. Filled grey boxes with red outline are significant, with a fold change above 1.5 fold. Proteins present on the right hand side are increased in abundance in *B. caledonica* treated larvae relative to *B. bassiana* treated larvae

Table 3.6. Proteins significantly increased or decreased in abundance in larvae treated with *B. caledonica* relative to *B. bassiana* treated larvae

				Average LFQ Intensities	
Protein Identity	**Annotation**	**P value (-Log)**	**Log_2 Fold Difference**	**BC**	**BB**
CL5500.Contig2	Glycoside hydrolase family 48	3.9	9.9	32.5	22.6
Unigene11986	Glycoside hydrolase family 48	3.0	9.8	31.5	21.8
Unigene3825	Glycosyl hydrolase family 45	2.4	8.5	30.6	22.0
Unigene7925	Neutral alpha-glucosidase C	2.9	7.4	29.7	22.4
Unigene12087	Glycosyl hydrolase family 2	2.8	6.8	29.0	22.1
Unigene9562	Unigene9562_All	2.2	5.9	29.6	23.7
Unigene19514	Pectinesterase	2.5	5.7	27.8	22.1
Unigene3946	Glycosyl hydrolase family 28	2.3	5.7	28.0	22.3
Unigene13818	Glycosyl hydrolase family 28	1.9	5.6	29.1	23.4
Unigene6962	Glycosyl hydrolase family 1	1.4	5.3	28.0	22.7
Unigene12565	Glycosyl hydrolase family 35	2.7	5.2	27.8	22.6
Unigene28106	Glycosyl hydrolase family 1	1.5	5.1	26.9	21.8
CL2640.Contig1	Pathogenesis-related 5	1.7	5.0	28.0	23.0
Unigene9634	Glycosyl hydrolase family 45	2.4	4.8	28.3	23.6
CL2700.Contig4	Carboxylesterase family	1.9	4.4	26.2	21.8
CL5881.Contig1	Actin	3.8	4.1	26.8	22.7
CL4537.Contig3	CL4537.Contig3_All	2.6	3.9	27.6	23.7
Unigene8511	Glycosyl hydrolase family 38	1.6	3.9	26.5	22.6
Unigene13343	Carboxylesterase family	1.6	3.8	26.9	23.1
CL921.Contig2	Glycoside hydrolase family 31	1.5	3.8	27.5	23.7
Unigene3970	Glycosyl hydrolase family 28	1.4	3.7	25.8	22.2
Unigene3841	Glycosyl hydrolase family 45	2.0	3.6	26.2	22.6
Unigene3953	Prostatic acid phosphatase	2.5	3.4	25.5	22.1

Unigene2311	Alpha-L-fucosidase	2.3	3.4	25.8	22.5
Unigene10957	Glycosyl hydrolase family 79	1.6	2.9	25.7	22.8
CL3821.Contig1	C-type lection lectoxin-Enh3-like	2.0	2.7	24.6	21.9
CL1864.Contig4	Glycosyl hydrolase family 2	2.5	2.6	25.8	23.2
Unigene1589	Trypsin	1.3	0.8	25.8	25.0
Unigene3489	Serpin	1.4	0.7	26.9	26.2
CL2224.Contig3	Integument esterase	1.8	0.6	31.5	30.9
Unigene5371	Venom protease	1.5	-0.6	24.0	24.7
CL492.Contig1	Attacin C	1.5	-0.6	29.5	30.1
Unigene9585	Unigene9585_All	1.8	-0.7	30.3	31.0
Unigene8093	Copper & zinc superoxide dismutase (SODC)	1.6	-0.8	27.7	28.5
CL2330.Contig1	CL2330.Contig1_All	1.6	-0.9	30.0	30.9
CL5549.Contig2	Sodium channel 60E	1.3	-0.9	24.4	25.3
Unigene13338	Trypsin	1.7	-1.0	26.9	27.9
Unigene1141	Coleoptericin	1.9	-1.0	30.5	31.5
CL1441.Contig1	CL1441.Contig1_All	2.0	-1.5	23.8	25.3
CL943.Contig7	Papilin-like Protein	1.5	-1.8	23.3	25.0

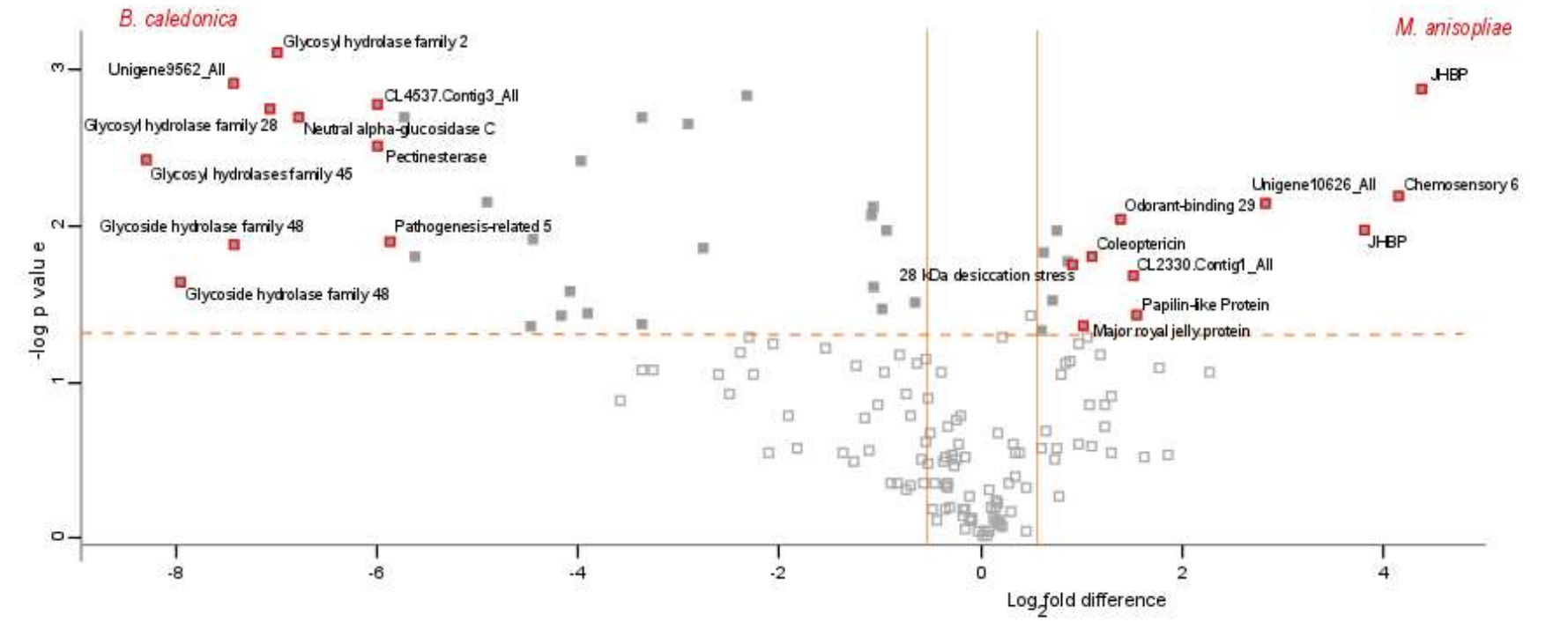


Figure 3.11. Volcano plot highlighting proteins present in highest and lowest abundances between larvae treated with *M. anisopliae* and *B. caledonica* supernatant.

Image of volcano plot obtained using Perseus software. Proteins above the dashed orange line indicates significance ($p<0.05$) and those to the right and left of the vertical lines indicate fold changes greater than 1.5. Filled grey boxes with red outline are significant, with a fold change above 1.5 fold. Proteins present on the right hand side are increased in abundance in *M. anisopliae* treated larvae relative to *B. caledonica* treated larvae.

Table 3.7. Proteins significantly increased or decreased in abundance in larvae treated with *M. anisopliae* relative to *B. caledonica* treated larvae

GH: Glycosyl hydrolase

				Average LFQ Intensities	
Protein Identity	**Annotation**	**P value (-Log)**	**Log_2 Fold Difference**	**MET**	**BC**
Unigene17410	JHBP	2.9	4.4	26.8	22.4
Unigene8018	Chemosensory 6	2.2	4.1	29.9	25.8
Unigene12317	JHBP	2.0	3.8	26.1	22.3
Unigene10626	Unigene10626_All	2.2	2.8	25.6	22.8
CL943.Contig7	Papilin-like Protein	1.4	1.5	24.8	23.3
CL2330.Contig1	CL2330.Contig1_All	1.7	1.5	31.5	30.0
CL1928.Contig2	Odorant-binding 29	2.0	1.4	33.1	31.7
Unigene1141	Coleoptericin	1.8	1.1	31.6	30.5
Unigene2302	Major royal jelly protein	1.4	1.0	29.7	28.7
Unigene27113	28 kDa desiccation stress	1.8	0.9	29.3	28.4
Unigene5371	Venom protease	1.8	0.8	24.9	24.0
CL492.Contig1	Attacin	2.0	0.8	30.3	29.5
CL466.Contig4	Serine protease easter	1.5	0.7	25.5	24.8
Unigene13454	Low-density lipoprotein receptor repeat class B	1.8	0.6	25.2	24.6
CL1928.Contig3	Odorant-binding 29	1.3	0.6	33.5	32.9
CL3832.Contig2	Peptidoglycan-recognition SC2	1.5	-0.7	26.7	27.3
CL3607.Contig1	Kunitz&Bovine pancreatic trypsin inhibitor domain	2.0	-1.0	32.1	33.1
CL1079.Contig2	Cathepsin L protease inhibitor 1	1.5	-1.0	29.1	30.0
Unigene3940	Odorant-binding partial	2.1	-1.1	32.8	33.8
CL2247.Contig3	odorant binding	1.6	-1.1	34.4	35.5

Unigene3698	GH family 18	2.1	-1.1	28.9	30.0
Unigene4030	Pathogenesis-related 5	2.8	-2.3	29.6	32.0
Unigene417	GH family 2	1.9	-2.8	20.9	23.6
CL1864.Contig4	Ecdysteroid-regulated 16 kDa-like	2.7	-2.9	22.9	25.8
Unigene4040	Prostatic acid phosphatase	1.4	-3.4	23.0	26.4
Unigene3953	Carboxylesterase family	2.7	-3.4	22.1	25.5
CL2700.Contig4	GH family 45	1.4	-3.9	22.3	26.2
Unigene3841	GH family 31	2.4	-4.0	22.2	26.2
CL921.Contig2	Carboxylesterase family	1.6	-4.1	23.4	27.5
Unigene13343	GH family 38	1.4	-4.2	22.7	26.9
Unigene8511	GH family 28	1.9	-4.5	22.1	26.5
Unigene3946	GH family 35	1.4	-4.5	23.5	28.0
Unigene12565	GH family 1	2.2	-4.9	22.9	27.8
Unigene6962	GH family 45	1.8	-5.6	22.4	28.0
Unigene9634	Pathogenesis-related 5 (Thaumatin)	2.7	-5.7	22.6	28.3
CL2640.Contig1	Pectinesterase	1.9	-5.9	22.1	28.0
Unigene19514	CL4537.Contig3_All	2.5	-6.0	21.8	27.8
CL4537.Contig3	Neutral alpha-glucosidase C	2.8	-6.0	21.6	27.6
Unigene7925	GH family 2	2.7	-6.8	22.9	29.7
Unigene12087	GH family 28	3.1	-7.0	22.0	29.0
Unigene13818	Unigene9562_All	2.7	-7.1	22.0	29.1
Unigene9562	GH family 48	2.9	-7.4	22.1	29.6
CL5500.Contig2	GH family 48	1.9	-7.4	25.0	32.5
Unigene11986	GH family 45	1.6	-7.9	23.6	31.5

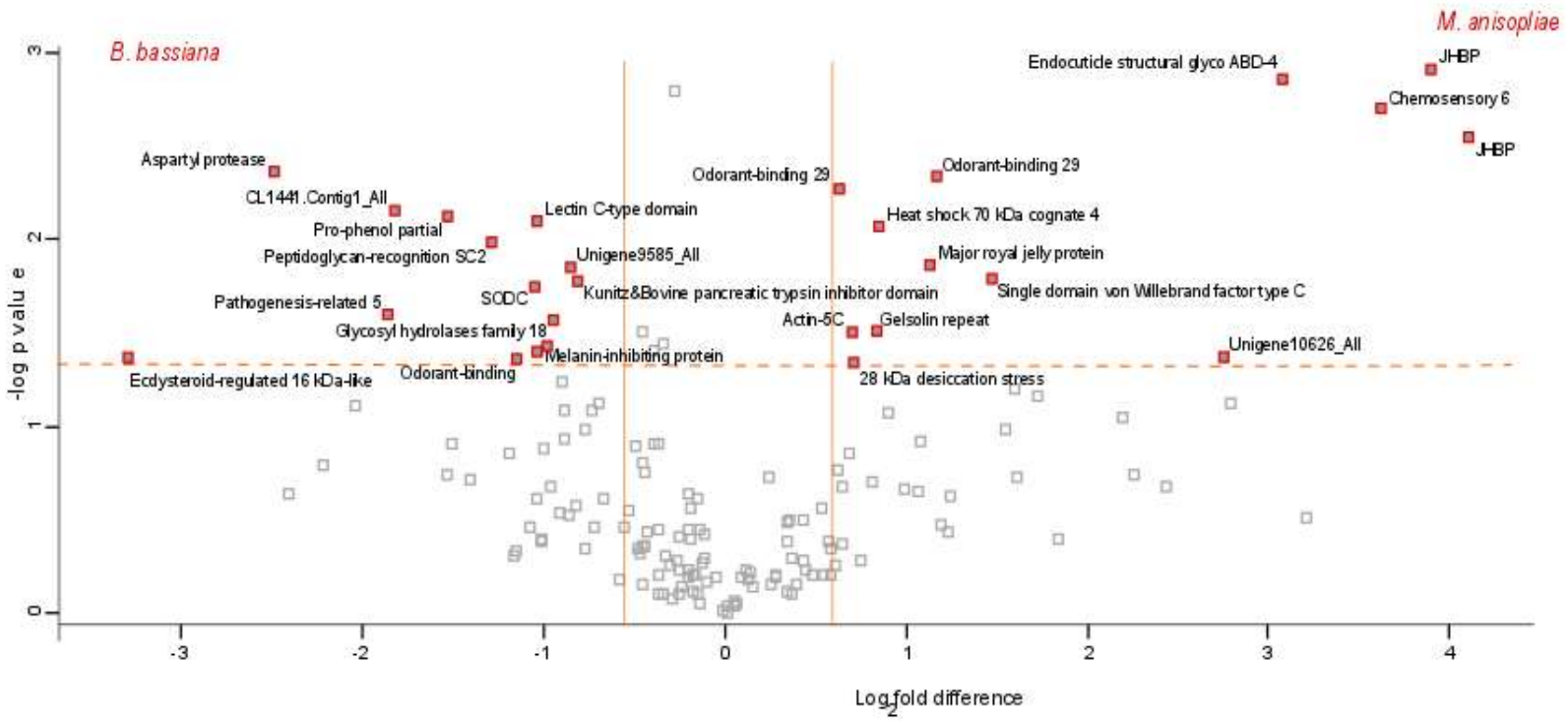


Figure 3.12. Volcano plot highlighting proteins present in highest and lowest abundances between larvae treated with *M. anisopliae* and *B. bassiana* supernatant.

Proteins above the dashed orange line indicates significance ($p<0.05$) and those to the right and left of the vertical lines indicate fold changes greater than 1.5. Boxes with red outline are significant, with a fold change above 1.5 fold. Proteins present on the right hand side are increased in abundance in *M. anisopliae* treated larvae relative to *B. bassiana* treated larvae.

Table 3.8 Proteins significantly increased or decreased in abundance in larvae treated with *M. anisopliae* relative to *B. bassiana* treated larvae

				Average LFQ Intensities	
Protein Identity	**Annotation**	**P value (-Log)**	**Log_2 Fold Difference**	**MET**	**BB**
Unigene17410	JHBP	2.5	4.1	26.8	22.7
Unigene12317	JHBP	2.9	3.9	26.1	22.3
Unigene8018	Chemosensory 6	2.7	3.6	29.9	26.3
Unigene8077	Endocuticle structural glyco ABD-4	2.9	3.1	25.2	22.1
Unigene10626	Unigene10626_All	1.4	2.7	25.6	22.9
Unigene1895	Single domain von Willebrand factor type C	1.8	1.5	29.5	28.0
CL1928.Contig2	Odorant-binding 29	2.3	1.2	33.1	31.9
Unigene2302	Major royal jelly protein	1.9	1.1	29.7	28.6
CL416.Contig1	Heat shock 70 kDa cognate 4	2.1	0.8	24.3	23.4
CL3393.Contig2	Gelsolin repeat	1.5	0.8	31.2	30.4
Unigene27113	28 kDa desiccation stress	1.3	0.7	29.3	28.6
CL5881.Contig5	Actin-5C	1.5	0.7	29.9	29.2
CL1928.Contig3	Odorant-binding 29	2.3	0.6	33.5	32.9
CL3607.Contig1	Kunitz & Bovine pancreatic trypsin inhibitor domain	1.8	-0.8	32.1	32.9
Unigene9585	Unigene9585_All	1.8	-0.9	30.2	31.0
Unigene9233	Glycosyl hydrolases family 18	1.6	-1.0	31.4	32.4
Unigene3698	Glycosyl hydrolases family 18	1.4	-1.0	28.9	29.9
CL2420.Contig2	Melanin-inhibiting protein	1.4	-1.0	28.3	29.3
CL1224.Contig1	Lectin C-type domain	2.1	-1.0	25.8	26.8
Unigene8093	Copper & zinc superoxide dismutase	1.7	-1.1	27.5	28.5
Unigene3940	Odorant-binding partial	1.4	-1.2	32.8	33.9

CL3832.Contig2	Peptidoglycan-recognition SC2	2.0	-1.3	26.7	28.0
CL1617.Contig2	Pro-phenol partial (Trypsin)	2.1	-1.5	27.1	28.7
CL1441.Contig1	CL1441.Contig1_All	2.2	-1.8	23.5	25.3
Unigene4030	Pathogenesis-related 5 (Thaumatin)	1.6	-1.9	29.6	31.5
Unigene417	Aspartyl protease	2.4	-2.5	20.9	23.3
Unigene4040	Ecdysteroid-regulated 16 kDa-like	1.3	-3.3	23.0	26.3

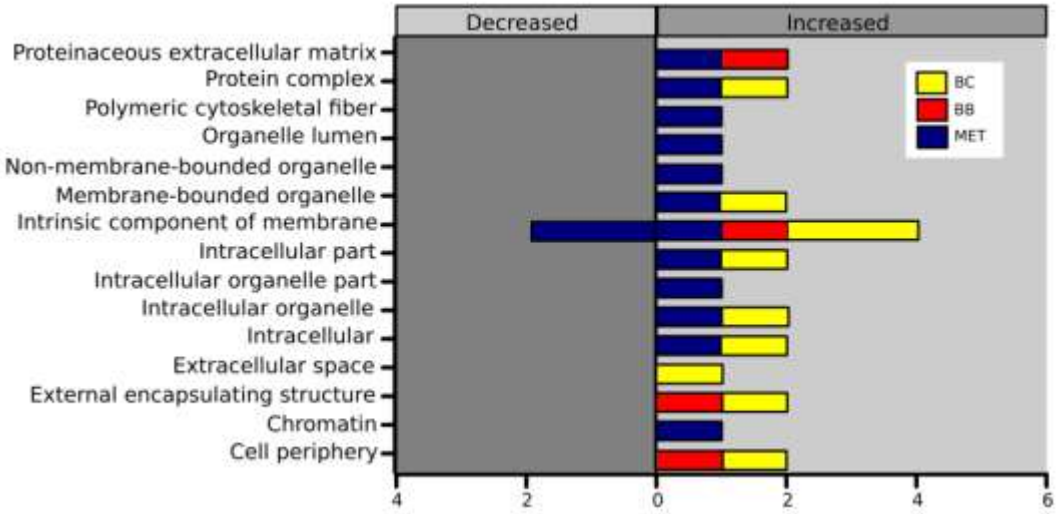


Figure 3.13. Bar chart showing number of proteins changed in various Cellular Processes at level 3 ontology.

Proteins are grouped based on functional annotation using Blast2Go.

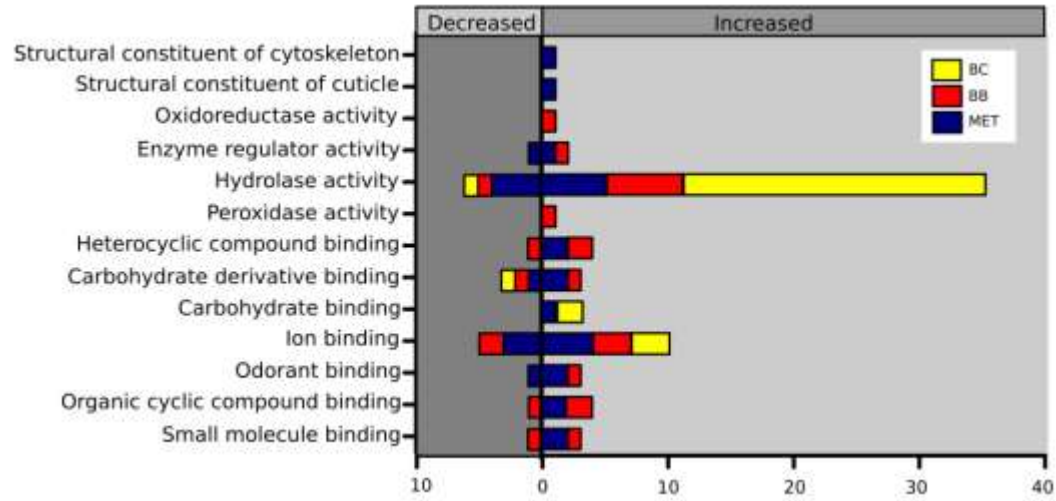
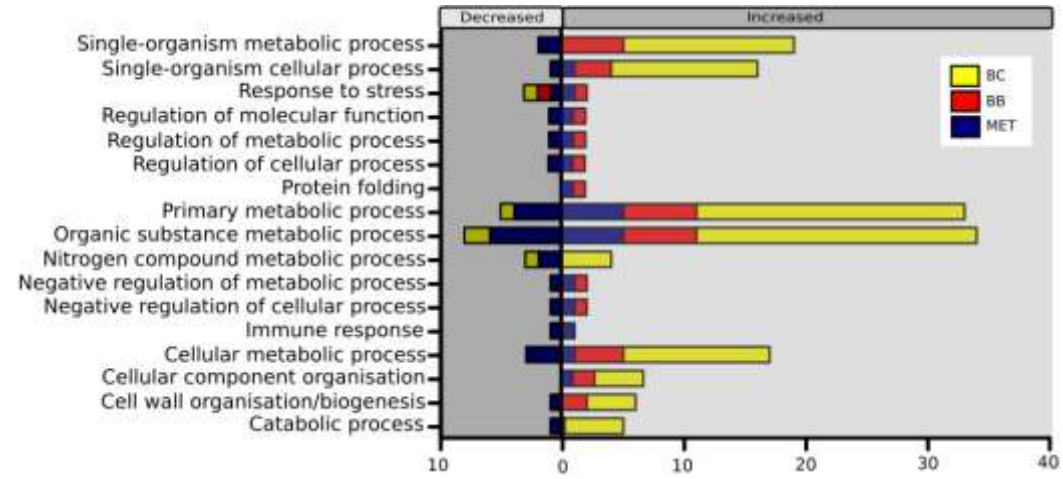


Figure 3.14. Bar chart showing number of proteins changed in various Molecular Functions at level 3 ontology.

Proteins are grouped based on functional annotation using Blast2Go.

A



B

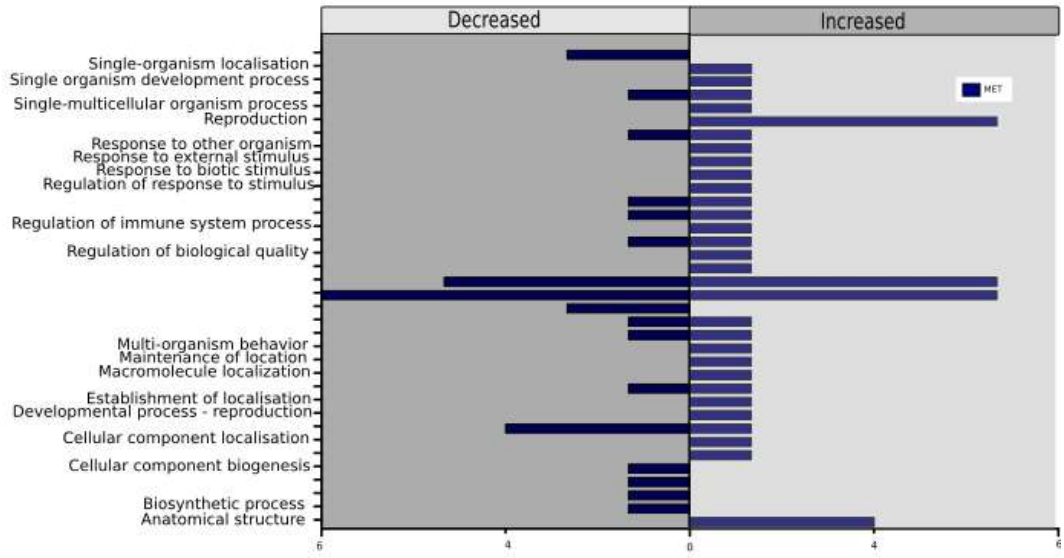


Figure 3.15. Bar chart showing number of proteins changed in various Biological Processes at level 3 ontology.

A Number of proteins changed in biological processes common to larvae treated with all three EPF. **B** Proteins changed in biological processes in larvae injected with *M. anisopliae*. Those labelled are unique to *M. anisopliae*. The Y-axis has labels only for processes that are only changed in *M. anisopliae* larvae treated only relative to control larvae.

3.4 Discussion

As previously discussed in Chapter Two (Section 2.4) a primary aim of this work was to investigate the use of EPF against *H. abietis* larvae in the field in conjunction with EPN. Thus the joint aim of this Chapter and the previous Chapter was to investigate three EPF isolates for their ability to modulate the insect immune response, rendering them more susceptible to subsequent pathogens. Screening EPF for their ability to modulate the immune response of insects may have an application in screening EPF for use in the field, in combination with other control agents such as EPN or chemical insecticides. This Chapter presented results of the same experiments carried out in Chapter Two, but in this instance they were carried out with *H. abietis* larvae rather than *G. mellonella* larvae. EPF are known to have different effects on different target insects, so this allows a greater insight into the effect that EPF fungal supernatant has on the immune response of larvae as the work covers both Lepidoptera (*G. mellonella*) and Coleoptera (*H. abietis*). Additionally the results were compared to validate the use of the routinely used model organism *G. mellonella* as a suitable model for *H. abietis.*

As per Chapter Two the effect of EPF supernatant on the immune response of *H. abietis* was investigated through a number of bioassays and proteomic analysis following injection with *M. anisopliae, B. caledonica* and *B. bassiana* fungal supernatant cultured for 96h. Haemolymph of *H. abietis* injected with EPF supernatant was subjected to label free quantitative proteomic analysis. Numerous gel free quantitative proteomics methods are now available that quantify proteins via mass spectrometry (Bantscheff *et al.* 2012). Shotgun proteome digestion approaches generate vastly complex mixtures of (mostly tryptic) peptides that constitute the analytes for both the identification and quantification of proteins. The combination of nanoscale ion pairing reversed-phase LC and electrospray ionization (ESI) MS/MS is still the most popular technology for this purpose. Label-free quantitative mass spectrometry quantifies peptides and proteins without the use of protein labels and was used in this Chapter as it measures the relative abundance of thousands of proteins across multiple sample groups in single mass spectrometry runs (Bantscheff *et al.* 2012).

3.4.1 *H. abietis* transcriptome *de novo* study

High throughput sequencing of genomes and transcriptomes has opened the way to study the genetic and functional information stored within organisms at an unprecedented scale and speed. These advances facilitate genomics research in species for which genetic or financial resources are limited, including many 'non-model' organisms, which are still of great ecological or evolutionary importance (Haas *et al.* 2013). Many genomic applications have traditionally relied on the availability of a high-quality genome sequences, though they have only been determined for a small portion of known organisms. Sequencing and assembling a genome can be costly due to genome size and repeat content. As the transcriptome is only a fraction of the total genomic sequence, RNA-Sequence data can provide a faster and cheaper approach, for defining a reference transcriptome (Haas *et al.* 2013).

No genome or transcriptome data was available for *H. abietis* to enable proteomic analysis of the immune system. To overcome this obstacle a *de novo* transcriptome was produced for *H. abietis* by Beijing Genomics Institute (BGI, Hong Kong) (Described in appendices B). To maximize sequence diversity and to ensure that a comprehensive suite of immune proteins were represented, RNA was extracted from untreated larvae and larvae injected with *M. anisopliae* fungal supernatant.

The completeness of BGIs transcriptome assembly and protein annotation datasets were measured through comparison with a set of highly conserved single-copy orthologs using BUSCO (Benchmarking Universal Single-Copy Orthologs) (Table 3.1). BUSCO are ideal for quantifications of completeness, as the expectations for these genes to be found in a genome and to be found only in single-copy are evolutionarily sound. Simão *et al.* (2015) used a OrthoDB database of orthologs (www.orthodb.org) to define BUSCO sets for six major phylogenetic clades. Sampling hundreds of genomes, orthologous groups with single-copy orthologs in >90% of species were selected. This threshold allows for the fact that even well-conserved genes can be lost in some lineages, as well as allowing for incomplete gene annotations and rare gene duplications. There are 2,675 BUSCO genes for arthropods for which the *H. abietis* transcriptome was tested against to assess completeness of the BGI annotation. BUSCO assessment allows for informative comparisons, for instance newly sequenced draft genome assemblies to can be compared to high quality model genomes, or it can be used to quantify

improvements to assemblies or annotations (Simão *et al.* 2015). In this work it was used to assess the completeness of the *H. abietis* transcriptome which was intended to be used for proteomic analysis, as well as comparing it to a range of other insect databases currently available. This *H. abietis* transcriptome used for LFQ contained protein sequences with homology to 1,358/2,675 (or 51%) BUSCOs searched, 386 BUSCOs were duplicated, 129 BUSCOs were fragmented and 1,188 were absent. Although these conserved genes are expected to be present in the genomes of all arthropoda, they may not be constitutively expressed. Therefore, this finding is not surprising as not all genes are expected to be detectable in data derived from mRNA sequencing. As well as its applications in this work, the *H. abietis* transcriptome may enable future research into this pest of economic significance; such as its differential immune response to pathogens, identification of novel immune genes or the expansion of gene families. Additionally it supplements the existing genomic and transcriptomic data available for the study of coleoptera.

3.4.2 *B. caledonica, B. bassiana* and *M. anisopliae* supernatants induce variant immune responses in *H. abietis*

Haemocyte densities of *H. abietis* larvae were significantly altered in abundance, relative to control larvae, following injection with 96h fungal supernatant for all three EPF (Figure 3.2). This decline in haemocyte densities was also observed in *G. mellonella* with both 72 h and 96 h fungal supernatant having a significant effect for all EPF (Figure 2.2).

To investigate the potential of EPF fungal supernatant to modulate the larval immune response of *H. abietis*, the effect of fungal supernatant on *C. albicans* proliferation in the haemocoel was assessed (Section 3.2.4). In *H. abietis* treatment with both *M. anisopliae* and *B. bassiana* caused a significant alteration in yeast load in larvae relative to controls (Figure 3.3), while an increase in yeast load was also seen in *G. mellonella* treated with *B. caledonica* and *M. anisopliae* supernatant (Figure 2.3). This suggests that *M. anisopliae* and *B. bassiana* supernatant is modulating the immune system of *H. abietis* larvae allowing a subsequent pathogen to proliferate. *M. anisopliae* supernatant significantly altered yeast densities in both *H. abietis* and *G. mellonella* larvae.

To further investigate whether treatment with fungal supernatant leaves larvae more susceptible to subsequent pathogens, *H. abietis* larvae were injected with fungal supernatant and *C. albicans*. One week after *C. albicans* infection, *H. abietis*

treated with *B. bassiana* or *B. caledonica* showed significantly increased mortality when fungal supernatant and *C. albicans* were combined compared to single doses (Figure 3.4). In *G. mellonella* the ability of fungal supernatant to leave larvae more susceptible to subsequent pathogens was seen with *M. anisopliae* and *B. caledonica* (Figure 2.4). After one week, mortality was higher in *H. abietis* injected with *B. caledonica* than *G. mellonella* but after two weeks mortality reached similar levels in *G. mellonella*. The highest mortality in *G. mellonella* was seen in larvae injected with *M. anisopliae* and *C. albicans*, but in *H. abietis* it was seen in larvae injected with *B. bassiana* and *C. albicans*.

The LFQ analysis of *H. abietis* haemolymph resulted in the identification of 157 proteins of which 155 had two or more peptides. 77 of these proteins were either significantly changed in expression or uniquely detected across the four treatments analyzed. PCA indicated that *B. caledonica* induces the greatest change in proteomic profiles in *H. abietis* larvae relative to control larvae (Figure 3.5); in *G. mellonella* larvae it was *M. anisopliae* that induced the greatest alteration to the proteomic profile. It also indicates that *H. abietis* larvae treated with *B. bassiana* had a proteomic profile most similar to control larvae; this was also seen in *G. mellonella* larvae (Figure 2.7). Hierarchical clustering allows comparison between the protein expression induced by different fungal supernatant; the effects they have in common and also how they differ (Figure 3.6). Cluster A comprises proteins with higher levels of abundance in larvae treated with *M. anisopliae* and *B. caledonica* relative to control larvae (Appendix C Table C1.2). It includes several proteins involved in sensing and recognition: odorant-binding 29, chemosensory 6 and β-1,3-glucan-binding. Cluster B comprises proteins with higher levels of abundance in larvae treated with *B. caledonica* relative to all other treatments and control larvae (Table C1.3). It consists of a number of proteins involved in metabolic process: members of glycosyl hydrolase families 1, 2, 20, 28, 31 35, 45, 48 and 79 and member of the carboxylesterase family. Cluster C comprises proteins with lower levels of abundance in larvae treated with *M. anisopliae* relative to all other treatments and control larvae (Table C1.4). It includes proteins that may be involved in the proPO cascade such as serpin, serine protease easter and serine protease persephone isoform X2. Cluster D comprises proteins with higher levels of abundance in control larvae relative to all EPF treated larvae; it includes the AMP defensin (Table C1.5).

Blast2GO annotation software was used to group proteins based on their conserved gene ontology (GO) terms in order to identify processes and pathways altered to different EPF treatments. In relation to cellular processes *M. anisopliae* appears to be involved in the largest number of process, with *M. anisopliae* and *B. caledonica* having the most processes in common (Figure 3.13). In relation to molecular function *M. anisopliae* appears to be involved in the largest number of functions including ion binding and hydrolase activity. *M. anisopliae* and *B. bassiana* have the most functions in common. While *B. caledonica* is involved in the least functions it has 25 proteins involved in hydrolase activity alone (Figure 3.14). In relation to biological processes *M. anisopliae* appears to be involved in the largest number of processes (Figure 3.15.B). While *B. caledonica* is involved in the least number of processes, it has 23 proteins involved in organic substance metabolic process and 22 proteins involved in primary metabolic process (Figure 3.15A). These BLAST results are reflective of the effect *B. caledonica* has on metabolic processes in *H. abietis*.

All three EPF exhibit immunomodulating effects on *H. abietis* larvae in at least one of the two bioassays used to test for immunomodulating potential. Thus unlike in LFQ of *G. mellonella,* comparisons could not be drawn between the proteomic changes of EPF that do and do not leave larvae more susceptible to subsequent infection in order to determine what changes might confer this weakening of immune defenses. Instead the alterations in proteomic profiles were compared to investigate differences between different immunomodulating EPF strains.

3.4.3 EPF supernatant alters abundance of protease inhibitors in *H. abietis*

Insects possess a variety of protease inhibitors that are involved in development (e.g. insect metalloprotease inhibitor) and regulation of the proPO cascade (e.g. serpin) (Butt *et al.* 2016). Insect hemolymph contains high concentrations of serine proteinase inhibitors belonging to kunitz, kazal, serpin and α-macroglobulin families. These serine proteinase inhibitors are believed to function in protecting the host from infection; some may inhibit fungal or bacterial proteinases while others play a role in regulating endogenous proteinases involved in coagulation, proPO activation, or cytokine activation (Kanost 1999).

H. abietis larvae injected with *M. anisopliae* supernatant had a significant alteration in abundance of a number of proteins with serine-type endopeptidase inhibitor activity: a serine protease easter (Trypsin), papilin-like protein, pro-phenol

partial (Trypsin) and kunitz & bovine pancreatic trypsin inhibitor domain (Figure 3.9, Table 3.5). Inhibitors of the kunitz and kazal families are widespread in insect hemolymph and may be involved in protecting the host from microbial proteinases (Vilcinskas 2010).

Larvae injected with *B. bassiana* supernatant also had an alteration in abundance of papilin-like protein and a melanin-inhibiting protein (Figure 3.8, Table 3.4). *H. abietis* larvae injected with *B. caledonica* supernatant had an alteration in abundance of serpin, trypsin and chymotrypsin-C isoform X1 (Figure 3.7, Table 3.3). Chymotrypsin and trypsin are the major digestive serine proteases of insects from the order Lepidoptera (Dunse *et al.* 2010). Serine proteases, such as trypsins and chymotrypsins, are important digestive enzymes in coleopterans such as *T. castaneum* (Morris *et al.* 2009). The putative effect of EPF fungal supernatant on digestive enzymes is discussed further in Section 3.4.8

Serine proteases and serine protease inhibitors (serpins) were also altered in abundance in the haemolymph of *G. mellonella* larvae injected with *M. anisopliae* and *B. caledonica* fungal supernatant (Section 2.4.2.). The functions of these proteins and their importance in the proPO cascade are discussed in Section 2.4.2. These alterations in abundance of proteins that may be involved in regulation of the proPO pathway in both *G. mellonella* and *H. abietis*, following injection with *M. anisopliae* and *B. caledonica* fungal supernatant highlights the impact these EPF may be having on a primary component of insect immune response: the proPO pathway. It is also possible that a number of the proteases inhibitors altered in abundance in *H. abietis* may be as a response to the presence of microbial proteinases.

3.4.4 EPF supernatant alters abundance of detoxification enzymes in haemolymph

Insects have developed mechanisms to deal with EPF and their secretory products; insects exposed to fungal toxins generally have higher antioxidant enzyme activity (Butt *et al.* 2016). Larvae of the Colorado potato beetle, *Leptinotarsa decemlineata*, had elevated activity of esterases and glutathione-S-transferase when infected with *M. anisopliae* (Dubovskiy *et al.* 2010). *H. abietis* injected with *B. caledonica* supernatant had increased abundance of carboxylesterases in their haemolymph. In *G. mellonella* larvae injected with *B. bassiana* supernatant there was a higher level of expression in alpha-esterase and carboxylesterase when compared to *M. anisopliae* and *B. caledonica* treated larvae (Figure 2.12, Figure 2.14).

Insects have been shown to produce an array of humoral defenses to resist fungal infection including lectins, protease inhibitors, PO, AMPs and reactive oxygen and nitrogen radicals (Butt *et al.* 2016). However these reactive species can damage both the host as well as the pathogen. Thus both possess antioxidant systems, detoxifying enzymes, aimed at neutralizing these reactive species. In insects these enzymes include superoxide dismutase (SOD), catalase, peroxidase and glutathione-S-transferase (Felton and Summers 1995, Butt *et al.* 2016). *H. abietis* larvae treated with *M. anisopliae* or *B. bassiana* supernatant demonstrated alterations in abundance of proteins involved in oxidative stress: copper & zinc SOD and peroxidase isoform X, respectively.

3.4.5 EPF alters the abundance of proteins involved in reception and detection

As discussed in Chapter Two the ability to perceive, discriminate and respond to chemical cues by chemoreception strongly impacts on fitness and survival of insects. This process is necessary for the identification of food resources, avoiding intoxication and to communicate with or detect other organisms including fungi (Boucias *et al.* 2012). Two categories of chemical cues are detected: odour refers to compounds that are volatile and are perceived at relatively low concentrations by olfactory or odorant receptors, whereas taste refers to detection of non-volatile compounds which are perceived at comparatively higher concentrations by gustatory receptors (Boucias *et al.* 2012). Odorant receptors are altered in abundance in response to EPF supernatant in *H. abietis* larvae and gustatory receptors are altered in abundance in response to EPF supernatant in *G. mellonella*.

H. abietis larvae injected with *M. anisopliae* fungal supernatant had altered abundance of proteins involved in reception and detection: chemosensory 6, odorant-binding 29, β-1,3-glucan-binding were increased in abundance, peptidoglycan-recognition SC2 and an odorant binding protein from the PBP&GOBP family were decreased in abundance (Figure 3.9, Table 3.5). In *G. mellonella* larvae treated with *M. anisopliae* a peptidoglycan recognition-like protein falls below the level of detection (Table 2.4). In *G. mellonella* larvae treated with *B. caledonica* three proteins involved in recognition were increased in abundance following treatment with; β-1,3-glucan binding protein and two peptidoglycan recognition proteins (Section 2.4.3). Insects can differentiate between major groups of microbes using PRRs such as PGRPs, hemolin and β-1,3-glucan binding protein. PRRs function by binding to PAMPs on microbial cells such as β-1,3-glucan from fungi

that acts as a signal to activate the antifungal functions of Toll (Stokes *et al.* 2015). These receptors are crucial to recognition of pathogens and activation of an appropriate immune response such as the proPO pathway.

Two major gene families are involved in the perireceptor events of the chemosensory system: the odorant binding protein (OBP) and chemosensory protein (CSP) families (Vieira and Rozas 2011). Chemosensory 6 was increased in abundance in *H. abietis* larvae injected with *B. caledonica* supernatant (Figure 3.7, Table 3.3). Chemosensory and odorant-binding 29 were also increased in abundance in *H. abietis* larvae injected with *B. bassiana* supernatant (Figure 3.8, Table 3.4).

3.4.6 EPF alters the abundance of AMP in the insect haemolymph

Biologically active peptides exhibiting antibacterial, antifungal and antiviral activity are found in abundance in insects. Most insects have high anti-microbial activity against Gram-positive bacteria but less against Gram-negative bacteria, fungi and yeasts (Faruck *et al.* 2015). AMP are expressed in the fat body and secreted into the haemolymph in response to infection. *H. abietis* larvae treated with *M. anisopliae* supernatant had an altered abundance of attacin and pathogenesis-related 5 (thaumatin). Thaumatin-like peptides were identified in red flour beetle, *T. castaneum*, they represent ancient antifungal peptides originally reported from plants, that are absent from the genomes of many other insects such as *Drosophila*, *Anopheles*, and *Apis* (Altincicek *et al.* 2008). They were found to act as an AMP against filamentous fungi in *T. castaneum* (Altincicek *et al.* 2008), so may be indicative of the insect mounting an immune response to EPF. Attacin is a 20 kDa antibacterial protein, originally isolated from haemolymph of *Hyalophora cecropia*, it was produced in response to bacterial infection (Carlsson *et al.* 1998). LPS acts as a receptor for attacin which inhibits growth of Gram-negative bacteria, increases the permeability of the outer membrane and induces synthesis of stress proteins (Carlsson *et al.* 1998). The production of immune effectors is costly for the insect, so production of several in lower concentrations that could work together would be very advantageous (Butt *et al.* 2016). One implication of an upregulation in AMP active against bacteria, following treatment with EPF supernatant, is that antibacterial activity can be highly beneficial to EPF as it might help exclude opportunistic infections that would be disadvantageous to the fungus (Butt *et al.* 2016).

H. abietis larvae treated with *B. caledonica* supernatant had increased abundance of pathogenesis-related 5 and decreased abundance of a defensin. *H. abietis* larvae treated with *B. bassiana* supernatant had increased abundance of attacin C and decreased abundance of a defensin. Insect defensins consist of a diverse family of anti-bacterial peptides highly active against Gram-positive bacteria (Faruck *et al.* 2015). Insect defensins form voltage-dependent channels, leading to rapid leakage of K+ and other ions (Hoffmann 1995). A coleoptericin is increased in abundance in *M. anisopliae* and *B. bassiana* treated *H. abietis* larvae relative to *B. caledonica* treated larvae (Figure 3.10 and Table 3.6; Figure 3.11 and Table 3.7). Antibacterial coleoptericins have been identified in the yellow meal worm beetle, *Tenebrio molitor*, and they were upregulated following bacterial challenge and parasitization (Zhu *et al.* 2014).

G. mellonella larvae treated with EPF supernatant also displayed alterations in abundance of proteins involved in antimicrobial response, although the proteins differ from the ones discussed in this chapter (Section 2.4.4).

3.4.7 EPF supernatant effects the abundance of proteins involved in insect development in *H. abietis*

Susceptibility of insects to infection can depend heavily on their developmental stage, recently moulted insects being particularly vulnerable as the new cuticle is soft and not fully sclerotized (Butt *et al.* 2016). *H. abietis* larvae are more susceptible than adults to both EPF (described in Chapter Five)(Ansari and Butt 2012) and EPN (Williams *et al.* 2015), probably in part due to their differences in cuticle thickness as the cuticle is the first barrier to EPF. *H. abietis* larvae injected with *M. anisopliae* supernatant had an alteration in abundance of proteins involved in development, metamorphosis and structure: JHPB, endocuticle structural glyco ABD-4, tropomyosin 1 and actin 5C were increased in abundance, while myosin regulatory light chain two and a chitin binding protein Peritrophic matrix 9 precursor were decreased in abundance (Figure 3.9, Table 3.5). *H. abietis* larvae injected with *B. bassiana* supernatant had a decrease in abundance in proteins involved in development: diapause-associated transcript-2 and myosin regulatory light chain 2 (Figure 3.8, Table 3.4).

Growth and development in insects, as well as reproduction in adults are regulated by juvenile hormone (JH). Its presence during larval moulting prevents metamorphosis, and it reappears in the adult to regulate female reproductive

maturation (Jindra *et al.* 2013). Insecticides have been developed that mimic the action of the two insect growth and developmental hormones, the steroidal 20-hydroxyecdysone and the sesquiterpenoid JH (Dhadialla *et al.* 1998). The cuticle is the first and most important barrier to EPF and chitin is a major component of the insect cuticle. EPF produce an extensive array of enzymes such as lipases, proteases and chitanases, with some of these cuticle degrading enzymes being considered virulence determinants (Butt *et al.* 2016). Insect growth and morphogenesis are strictly dependent on the capability to remodel chitin-containing structures. Thus insects repeatedly produce chitin synthases and chitin-lytic enzymes.

These alterations in abundance of developmental proteins following injection with natural products of EPF may be indicative of the insect trying to regenerate and protect itself from pathogens or it could be a reflection of EPF natural products (e.g. enzymes or secondary metabolites) within the EPF supernatant having an impact on the insect.

3.4.8 *B. caledonica* supernatant has a significant effect on proteins involved in metabolic processes in *H. abietis*

Cellulose is widely distributed in a number of bacteria, protists, fungi, and in all higher plants. It is the most abundant compound in plant cell walls (Watanabe and Tokuda 2010). Cellulase is a general term for cellulytic enzymes, of which three classes are recognized on the basis of the mode of enzymatic action and their substrate specificities: endoglucanases, exoglucanases and β-glucosidases. Endoglucanases (endo-β-1,4-glucanases or 1,4-β-d-glucan-4-glucanohydrolases) cleave amorphous sites of cellulose chains at random. Exoglucanases (1,4-β-d-glucan cellobiohydrolases or 1,4-β-d-glucan glucohydrolases) act on the non-reducing or reducing termini of cellulose fibers to release either cellobiose (cellobiohydrolases) or glucose (glucohydrolases). β-glucosidases (1,4-β-d-glucosidases or cellobiases) hydrolyze cellobiose or cello-oligomers to glucose from the nonreducing ends. Exoglucanases generally produce cellobiose, whereas most endoglucanases produce cellobiose and smaller amounts of glucose and cellotriose. Cellulases and other carbohydrolases have been grouped into a glycoside hydrolase (GH) family based on amino acid sequence similarities. Members in the same GH family are usually considered to share not only structural motifs and the catalytic machinery, but also an evolutionary origin (Watanabe and Tokuda 2010).

Xylophagous insects, such as *H. abietis*, are well adapted to feeding on wood. Their body plans, including organs, gut structures, digestive enzymes, and symbiotic systems, allow them to thrive on cellulosic substances. Thus xylophagous insects possess efficient systems to convert cellulosic biomass in their bodies (Watanabe and Tokuda 2010). A broad diversity of cellulolytic enzymes are involved in cellulose digestion. The participation of multiple enzymes with a wide spectrum of substrate specificities enables continuous enzymatic actions on wood (Watanabe and Tokuda 2010). Keeling *et al*. (2013) annotated 52 non-redundant plant cell-wall degrading enzymes (PCWDEs) in the mountain pine beetle, *Dendroctonus ponderosae*, a major pest of forestry by comparing protein translations including: glycoside hydrolase family 48 proteins, polysaccharide lyase family 4 proteins, endo-β-1,4-glucanases, pectin methylesterases, and endopolygalacturonases. The identification of PCWDEs in insects has been limited, and has been studied most thoroughly in Coleoptera (Pauchet *et al*. 2010). To date *D. ponderosae* has the largest family of PCWDEs described (Keeling *et al*. 2013). The *H. abietis* transcriptome may be a source for the identification of additional PCWDEs.

Larvae treated with *B. caledonica* supernatant had a higher abundance of proteins involved in metabolic processes, relative to both control larvae and larvae treated with other EPF (Appendix C Figure C1.3.). The amino acid sequences of these proteins were subjected to an NCBI BLAST search to confirm they were coming from the insect host rather than the injected fungal supernatant. GO term mapping was conducted on the proteins altered in abundance in larvae treated with *B. caledonica* using the Blast2GO suite. The GO terms assigned gave an indication of the metabolic process many of these proteins were involved in.

Two proteins from glycoside hydrolase family 48 were increased in abundance (Figure 3.7, Table 3.3.); these are involved in cellulose catabolic process and are enzymes that cleave cellulose or related substances. They showed high sequence similarity to cellulose 1,4-β-cellobiosidase from *Otiorhynchus sulcatus*. Three proteins from glycosyl hydrolases family 45 were increased in abundance; they are involved with starch and sucrose metabolic process and display cellulase activity. The EPF belonging to the Oomcyota divisions have a cell wall mostly composed of glucan-cellulose. However, chitin is a characteristic component of the cell wall of EPF belonging to the Ascomycota (*Metarhizium* and *Beauvaria* spp.) and Basidiomycota divisions and cellulose is rarely found in the cell walls of these EPF

(Lacey and Kaya 2007). Thus it is likely that these enzymes were upregulated in response to nutrient needs as *H. abietis* feed on wood. Cellulose is an important nutritional resource for a number of insect herbivores. Digestion of cellulose and other polysaccharides in plant-based diets requires several types of enzymes including a number of glycoside hydrolase families (Eyun *et al.* 2014).

Neutral alpha-glucosidase C and Unigene9562 are increased in abundance (Figure 3.7, Table 3.3.); both belong to glycosyl hydrolase family 31 which is comprised of key enzymes of carbohydrate metabolism. Two proteins from Glycosyl hydrolases family 2 were increased in abundance; this family contains β-galactosidase, β-mannosidase and β-glucuronidase activities and has a jelly roll fold. They are involved in chlorophyll, carbohydrate and starch metabolic processes. Three proteins from glycosyl hydrolases family 28 were increased in abundance, this family includes polygalacturonase and rhamnogalacturonase, enzymes that are important in cell wall metabolism. Two proteins from glycosyl hydrolase family 1 were increased in abundance; this family consists of β-glucosidase, 6-phospho-β-glucosidase and β-galactosidase that are involved in carbohydrate transport and metabolism. A protein from glycosyl hydrolase family 31 was increased in abundance, this family is comprised of enzymes that are, or similar to, alpha-galactosidases. Members of glycosyl hydrolase families 35, 38 and 75 were also increased in abundance. Additionally two members of the carboxylesterase family, a pectinesterase, α-L-fucosidase and a prostatic acid phosphatase were all increased in abundance following injection with *B. caledonica* supernatant. Pectinesterases are a group of salivary enzymes which act on lipids (Sharma *et al.* 2014).

A number of proteins belonging to glycosyl hydrolase families were also altered in abundance in *B. bassiana* (e.g. GH family 28 and 45) and *M. anisopliae* (e.g. GH family 1) treated *H. abietis* larvae, but not to the same extent as in *B. caledonica* treated larvae (Figure 3.10 and 3.11). These major changes in proteins involved in metabolic processes were not seen in *G. mellonella* and highlights differences between these two insects. Such an abundance of cellulose related enzymes in *H. abietis* larvae relative to *G. mellonella* can be expected as the food source for *H. abietis* is wood. The finding that injection with *B. caledonica* supernatant caused an increase in abundance of proteins involved in cellulose cleavage in *H. abietis* larvae links in with the finding in Chapter Five that *H. abietis* adults that were exposed to oosporein treated food consumed more food than control adults (Section 5.3.11).

Oosporein was identified as an abundant immunomodulating natural product in the *B. caledonica* supernatant used in both Chapters Two and Three (Section 5.3.3). It could be postulated that this increased demand for food is a reflection on the energy demands of mounting and maintaining an appropriate immune response as *B. caledonica* supernatant was shown to have an immunomodulating effect on *H. abietis* larvae after one week (Figure 3.4). *B. caledonica* supernatant did not confer an immunomodulating effect after the same incubation period used in LFQ analysis, thus these alterations to metabolic processes may reflect the insect host's attempt to efficiently deal with immune challenge. Hosts may compensate for increased demands through increased resource uptake (Moret and Schmid-Hempel 2000). Fungal infection of *Bombyx mori* with B. *bassiana* was shown to significantly alter insect energy and nutrient metabolism as well as the immune system (Xu *et al.* 2015). The fungus also demonstrated an anti-feedant effect against *B. mori*. As insect immune responses are energy cost reactions, nutrient deprivation, modulation of the host immune response and toxins produced by the EPF would act together to kill the insect (Xu *et al.* 2015)

As previously discussed evasion of the host immune response by EPF is an important element in the infection process, so that the pathogen can satisfy its demands for nutrients during growth within the host and to fulfil its reproductive potential (Gillespie *et al.* 2000). Once nutrients available in the host environment has been exhausted the fungus penetrates out to the host cadaver surface (Schrank and Vainstein 2010). Perhaps this increase in food consumption and metabolic processes could be a mechanism utilised by the EPF to ensure sufficient nutrients for reproduction.

There is an interest in the discovery of new insect PCWDEs for use in biotechnological applications, such as the degradation of cellulose for conversion of plant cell-wall sugars into biofuels or other bio-products (Oppert *et al.* 2010). Insects are attractive potential candidates to aid in the discovery of novel cellulolytic enzymes, due to the diverse and highly adapted species that feed on very plant tissues (Oppert *et al.* 2010). Thus these results together with the transcriptome of *H. abietis* may warrant further investigation as a source of information for biotechnological applications.

3.4.9 G. *mellonella* as a potential model organism for *H. abietis*

G. mellonella is regularly employed in the study of fungal pathogenesis and biocontrol agents. *G. mellonella* has been used as a model in the study of many microbes including *Aspergillus flavus, Aspergillus fumigatus, Candida albicans* and *Metarhizium anisopliae* (Vilcinskas *et al.* 1997a, Cotter *et al.* 2000, A. C. Fröbius *et al.* 2000, Bergin *et al.* 2003, Scully and Bidochka 2005). In Chapters Two and Three the suitability of *G. mellonella* was investigated as a model for *H. abietis* as the larval stage *of H. abietis* is labour intensive to source and is not sold commercially, so having a viable model would be hugely beneficial for testing potential biocontrol agents. The aim was to investigate the immunomodulating effects of EPF on the insect immune response, as being able to modulate the insect immune system may have implications for the ability of EPF to act synergistically with other control agents in the field.

There were comparable results from bioassays for *M. anisopliae* and *B. caledonica* between *G. mellonella* and *H. abietis* larvae. *M. anisopliae* fungal supernatant significantly altered haemocyte densities and yeast load in both *G. mellonella* (Figure 2.2 and 2.3) and *H. abietis* (Figure 3.2 and 3.3). *M. anisopliae* fungal supernatant left *G. mellonella* more susceptible to subsequent infections, a similar pattern was seen in *H. abietis* larvae but it was not significant. *B. caledonica* significantly altered haemocyte densities and susceptibility to subsequent infections in both *G. mellonella* (Figure 2.2 and 2.4) and *H. abietis* (Figure 3.2 and 3.4). However, there was not as strong a correlation between the results for the effect of fungal supernatant on yeast densities and susceptibility to subsequent pathogens in *H. abietis* (Figure 3.3 and 3.4) as was seen in *G. mellonella* (Figure 2.3 and 2.4). The major difference in bioassays between *G. mellonella* and *H. abietis* larvae is that *B. bassiana* supernatant exhibited immunomodulation effects when injected into *H. abietis* leaving the insect more susceptible to infection, this was not seen in *G. mellonella*. This is indicative of the fact that EPF species and even isolates within a species can act very differently in terms of their host range and infectivity (Shah and Pell 2003).

EPF fungal supernatant induces alterations to the haemolymph proteome of both *G. mellonella* and *H. abietis* larvae. In both insects PCA confirmed that the proteomic profile of larvae treated with *B. bassiana* supernatant was most similar to control larvae. In both insects there was an alteration in the abundance of

proteases/protease inhibitors that may function in regulation of proteolytic pathways such as the proPO cascade. In both insects EPF supernatant altered abundance of proteins involved in detection and sensing, detoxification enzymes and AMP. *H. abietis* larvae differed in that treatment with EPF supernatant altered proteins involved in development in the case of *M. anisopliae*, and numerous proteins involved in metabolic process, in the case of *B. caledonica*. The major alteration in metabolic proteins particularly several proteins from glycosyl hydrolase and carboxylesterase families following treatment with *B. caledonica* supernatant, highlights the differences between these two insects as the high levels of enzymes with cellulose cleavage reflects *H. abietis* wood based diet and also its energy demands following treatment with EPF natural products.

G. mellonella larvae may be a useful model for looking for mechanisms of synergy between EPF and other control agents. Screening EPF supernatant for immunomodulation effects on insects as was carried out in Chapter Two and Three may have an application in screening isolates for the ability to produce secondary metabolites that can modulate the immune response making the host more susceptible to subsequent pathogens. Additionally this may be used to look for ways to enhance isolates by identifying metabolites that suppress the immune response. In terms of screening isolates for the ability to kill insects in the field, testing spores of different isolates against the target insect may be most suitable.

3.4.10 Conclusion

The insect immune response relies on three tightly interconnected reactions: firstly the induction of proteolytic cascades; these are the coagulation cascade which causes localized blood clotting and the proPO cascade. Secondly, the cellular immune response, which consist primarily of the phagocytosis and encapsulation of pathogens. The third reaction is synthesis of AMPs by the fat body; these molecules exhibit a broad spectrum of activity directed against bacteria and/or fungi. They are secreted into the haemolymph where they act synergistically to kill pathogens (Hoffmann *et al.* 1996). EPF supernatant and the natural products contained within were shown to alter all three of these reactions in both *G. mellonella* and *H. abietis*. The key findings from Chapter Two and Three are summarized visually in Figure 3.16 to allow a direct comparison between the alterations to the immune response of *G. mellonella* and *H. abietis* following treatment with each of the EPF supernatant.

G. mellonella larvae may be a useful model for the screening of large numbers of EPF isolates for potential immunomodulation capabilities that may have applications in the field in achieving synergy. *G. mellonella* could be a viable option where it is impractical to source *H. abietis* larvae due to it being labour intensive, time consuming and seasonally dependent. However to fully elucidate the interactions between the *H. abietis* immune system and EPF to better understand the mechanism by which synergy may be achieved through modulating the host immune response, rendering it more susceptible to subsequent pathogens/agents, it is best to study the target insect itself so as not to overlook species specific immune responses. This is discussed further in the Chapter Six.

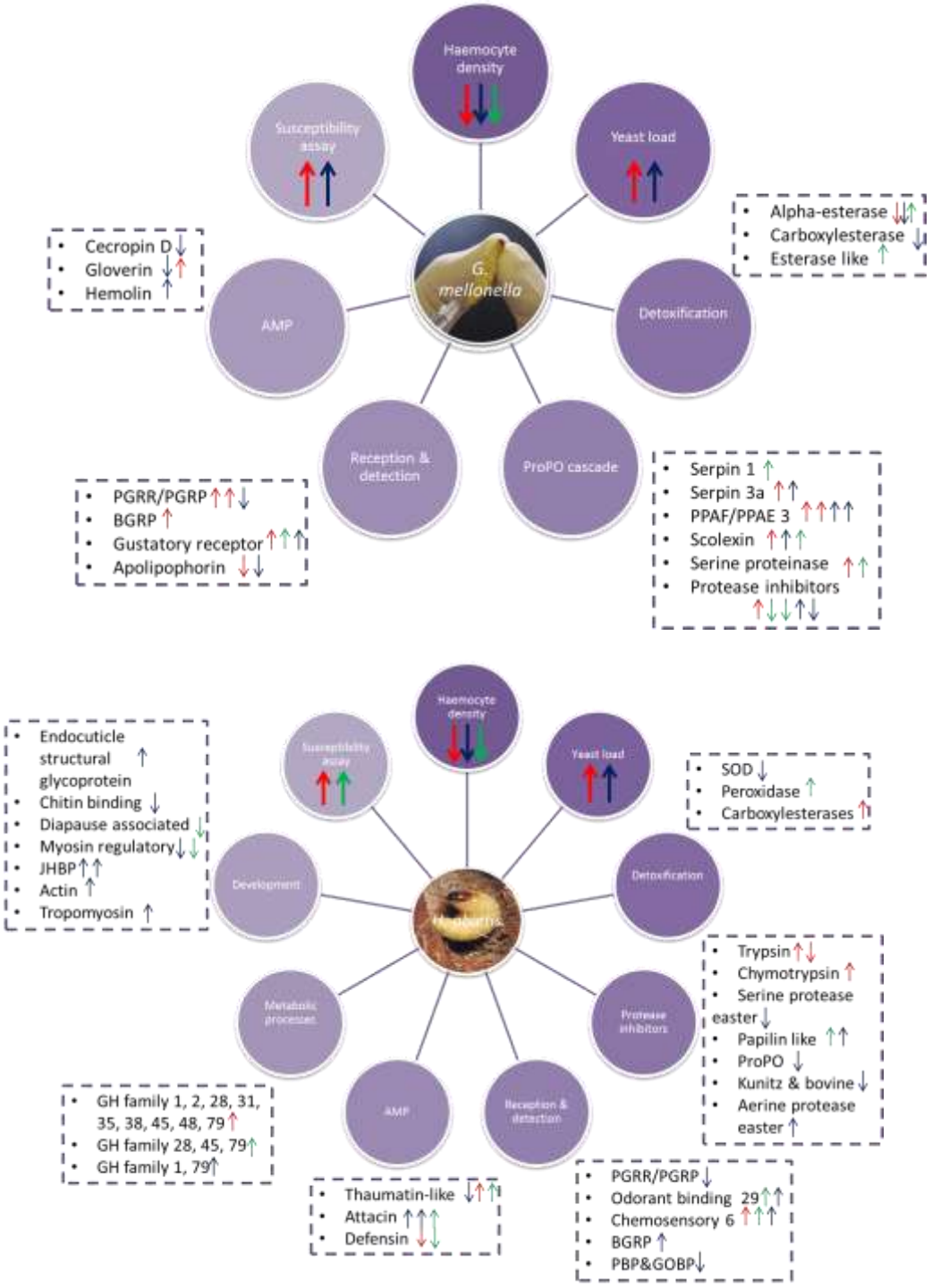


Figure 3.16. Schematic overview of the effect of EPF supernatant on the immune response of *G. mellonella* and *H. abietis* larvae.

The effect of EPF supernatant on the immune system of *G. mellonella* (top) and *H. abietis* (bottom) are summarised to allow comparison. Red arrows denote a change following treatment with *B. caledonica* supernatant, blue *M. anisopliae* and green *B. bassiana*. Arrows pointing upwards represent increases in response/abundance and arrows pointing downwards respresents decreases.

Chapter 4:

Assessing the efficacy of entomopathogenic fungi alone, and in combinations, for the control of *Hylobius abietis* populations in the field

4.1 Introduction

The large pine weevil *Hylobius abietis* (Figure 1.7) Linnaeus (Coleoptera: Curculionidae) is a wide spread pest of plantation forestry across Europe (SR Leather *et al.* 1999) (discussed in section 1.16). Female weevils primarily oviposit in the soil around the roots of recently felled trees (Nordlander *et al.* 1997). It is the adult weevils that are of economic importance. Maturation feeding on conifer bark is vital for the reproductive development of newly emerged weevils. This feeding on young seedlings causes significant damage and cost. A single adult can damage or kill several seedlings, therefore even small numbers of adults can have an economic impact on sites that have been replanted (Wainhouse *et al.* 2004, Inward *et al.* 2012). Although pine weevils generally have a life cycle of two years, it can range from 1-4 years (Inward *et al.* 2012). *Hylobius abietis* is a major forestry pest in 15 European countries, where it is a threat to 3.4 million hectares of forest (Långström and Day 2007). If no chemical control measures were used it is estimated that the resulting damage would reach €140 million for Europe (Långström and Day 2007).Without control measures pine weevils can destroy up to 100% of newly planted trees.

Current control measures include the synthetic chemical cypermethrin. However, due to concerns over its environmental impact cypermethrin is being phased out across Europe (Williams *et al.* 2013). Concerns about the effects of chemical insecticides on non-target species and their environmental impact, has led to an interest in the development of biological methods for the control of pine weevils (Evans *et al.* 2015). Biocontrol agents are slower acting than their chemical counterparts. To overcome this obstacle, agents may need to be combined in order to make it economical for growers to use them. Interactions between two or more pest control agents applied together may be antagonistic, additive or synergistic. Several studies have demonstrated that EPN and EPF act synergistically against several pests (where the effect of the two agents together is greater than that expected based on the effect of each on its own) (Anbesse *et al.* 2008, Ansari *et al.* 2008, Ansari *et al.* 2010) (discussed in section 1.15). If a combination of treatments resulted in a synergistic interaction then the potential cost of using biopesticides may be reduced. *M. anisopliae* combined with EPN have been reported to act synergistically in the control of black vine weevil, *O. sulcatus* (Ansari *et al.* 2008, Ansari *et al.* 2010). The

underlying mechanisms of these synergistic interactions are unclear, but it is suggested that one agent may stress or alter the behaviour (e.g., feeding or movement) of the host, making it more susceptible to another agent. For example, *M. anisopliae* infected insects may be less mobile, allowing EPN more time to penetrate the host (Ansari *et al.* 2004).

At the time of commencement of this project EPF-EPN synergy had not yet been reported against pine weevil. The nematodes used for this were *H. downesi*, an indigenous species that is most effective against pine weevil (Dillon *et al.* 2006) but not yet commercialised, and *S. carpocapsae* which has been used against pine weevils at an operational level by Coillte (Williams *et al.* 2013).

The aim of this chapter was to investigate the ability of EPF and EPN to suppress *H. abietis* populations in the field through carrying out three field studies over three consecutive years. The efficacy of EPF and EPN was investigated alone and in combination through emergence trapping and destructive sampling. Three EPF strains were utilised in these field studies, commercial strains of *M. anisopliae* and *B. bassiana* and a strain of *B. caledonica* native to Ireland. Two EPN species were utilised in these field studies, *S. carpocapsae* and *H. downsei*, the latter is native to Ireland.

4.1.1 Objectives

1. To assess the efficacy of EPF, EPN and EPF-EPN combinations for *H. abietis* suppression in the field.
2. To investigate if application method affects efficacy of EPF.

4.2 Materials and methods

4.2.1 Source of entomopathogenic fungi and nematodes

Steinernema carpocapsae in vermiculite formulation and dry conidiospores of *B. bassiana* (Bals.) Vuill. (Experimental strain 1694) were supplied by Becker Underwood, West Sussex, UK, *Steinernema carpocapsae* was rehydrated, checked for viability and enumerated before use. *B. caledonica* (2c7b) was isolated from a naturally infected pine weevil cadaver in an Irish forest (Williams *et al.* 2013). A commercial product of *M. anisopliae*, Met 52, was used in field studies. The Novozyme product Met 52 was purchased from National Agrochemical Distributors, Lusk, Dublin. *M. anisopliae* was supplied in granular form on rice and conidiospores were shaken off the grains prior to making the suspensions for field studies. Both *B. caledonica* and *B. bassiana* were cultured in the laboratory as per 4.2.3. *Heterorhabditis downesi* (strain K122) was cultured in the lab as per 4.2.2.

4.2.2 Culturing entomopathogenic nematodes for field studies

Heterorhabditis downesi (strain K122) was cultured in *G. mellonella* larvae. Sawdust was removed from boxes (19 cm x 5 cm) of *G. mellonella*, obtained from supplier (Mealworm Company, Sheffield, England) leaving just the larvae. Infective juveniles (IJs) suspension (50 ml, concentration not determined) was poured onto the larvae. Folded tissue paper (10 layers) was placed in the box on top of the larvae. The closed container was inverted a number of times to mix the larvae and IJs. Holes were made in the container to increase ventilation. The container was incubated at 20°C for 2-2.5 weeks. IJs were harvested by placing 10 larvae into a large White trap (15 cm diameter) and checked every 2 days for emergence. White traps consisted of the bottom of a 9 cm Petri dish covered with 9 cm filter paper (Fisher Scientific, Ireland) in a 15 cm Petri dish. The Petri dish was filled to approximately half its depth with tap water. The traps were harvested every two days. To harvest, water from the White traps containing IJs was poured into 2 L conical flasks up to 500 ml. Flasks were filled up to 1.5 L with tap water and mixed. IJs were allowed to settle for 15 minutes and the water was poured off. The concentrated IJs were resuspended in 1.5 L of water. This was done 3 times to wash the IJs. The flasks were then filled to

1.5 L with tap water and stored at 9°C, with aeration from aquarium pumps. The tops of conical flasks were plugged with cotton wool to prevent evaporation.

4.2.3 Culturing *B. caledonica* and *B. bassiana* for field studies

B. caledonica and *B. bassiana* were cultured in Sabouraud dextrose liquid medium (Oxoid) in a shaking incubator for 6 days at 25°C and 250 rpm. A loop of spores was used to inoculate a 50 ml liquid culture. Basmati rice (500 g) was washed twice with water. The rice was covered in water and left submerged overnight. The water was drained off the rice. The drained rice was added to a double-bagged autoclave bag. The opening of the autoclave bag was plugged with a sponge held in place with masking tape and autoclaved at 120°C for 30 minutes. Liquid culture (100 ml) was poured into the bag and mixed with the rice. The rice in the bag was flattened and the bag was sealed by folding the top twice and taping closed. Bags were placed flat on shelves and turned daily for 10-15 days at 25°C. When the rice was ready to use the bags were cut open and allowed to dry in a laminar flow cabinet. EPF inoculated rice was stored at 4°C until the rice was to be used. To remove the spores from the rice, 100 g rice was placed in conical flasks with 100 ml PBST (0.05%) and placed in a shaking incubator at 200 rpm to dislodge spores. The suspension was sieved through a double layer of muslin and the spore suspension was stored in 50 ml centrifuge tubes.

4.2.4 Sites for field studies

There were three field studies carried out over consecutive years in clear-felled lodgepole pine forests, each at a different location (Table 4.1, Site maps and details in Appendix A). The soil at each site was deep peat. At each site, treatments were arranged in a randomized block design, with one stump of each treatment in each block and at least ten blocks. Stumps were marked and colour coded for different treatments before treatment application.

Table 4.1. Field study sites		
Field study	**Location**	**Year of application**
1	Rossnagad, Laois	2012
2	Glendine, Slieve Bloom Laois	2013
3	Cloondara, Longford	2014

4.2.5 Treatments and Application

In all trials 500 ml of the suspension of IJs and/or conidia was applied to the stumps by pouring the suspension onto the soil around the stump (Dillon *et al.* 2006) and the side of the stump. Different application methods were trailed in year three trials. Full EPF treatments were $1x10^9$ spores/stump and half treatments of EPF were $5x10^8$ spores/stump. Full EPN treatments were $3.5x10^6$ IJs/stump and half treatments of EPN were $1.75x10^6$ IJs/stump. Half doses were included to facilitate assessment of interactions between agents in mixed treatments. Control stumps were untreated. *M. anisopliae*, *B. caledonica* and *B. bassiana* were diluted to the desired concentration using 0.05% (v/v) Tween 80 as a surfactant. Treatments were made up in 5 L bottles in the laboratory and were regularly mixed while being transported to the field.

In year one the EPN and EPF used were *B. bassiana* and *S. carpocapsae*. Treatments, dose and number of stumps are listed in Table 4.2. In year two the EPN and EPF used were *B. bassiana*, *B. caledonica*, *M. anisopliae* and *S. carpocapsae*. There were two principle aims in year two field study. One aim was to compare the efficacy of three EPF and this was assessed through destructive sampling and emergence trapping. The second aim was to determine if combinations of *S. carpocapsae* and *M. anisopliae* gave synergistic effects, this was assessed by emergence trapping only. Aim, treatments, dose and number of stumps are listed in Table 4.3.

In Year three the EPN and EPF used were *B. caledonica*, *M. anisopliae* and *H. downesi*. There were two primary aims in year three field study. One aim was to

assess if different methods of applying EPF affected their efficacy. The different methods tested were: treatment poured on ground surrounding stump/side of stump (sides), treatment poured on gap between bark and stump at the top of stump (top) and treatment applied to both top and sides (top + sides). The second was to assess if combinations of *H. downesi* and *M. anisopliae* or *B. caledonica* gave synergistic effects on weevil emergence, for this the treatment was applied to top and sides of the stump. Aim, treatments, dose and number of stumps are listed in Table 4.4.

Table 4.2. Year One Field Study Treatments			
Treatment	Dose	No stumps for emergence trapping	No stumps for destructive sampling
B. bassiana	Full ($1x10^9$ spores/stump)	10	10
B. bassiana	Half ($5x10^8$)	10	10
S. carpocapsae	Full ($3.5x10^6$ IJs/stump)	10	10
S. carpocapsae	Half ($1.75x10^6$)	10	10
Mixed *B. bassiana* & *S. carpocapsae*	Half + Half	10	10
Control	-	10	10

Table 4.3. Year Two Field Study Treatments

[1] Comparison of the efficacy of EPF species. [2] Determine if EPN and EPF combinations gave synergistic effects

Aim	Treatment	Dose	No stumps for emergence trapping	No stumps for destructive sampling
1[1]	*B. bassiana*	Full	10	20
1	*B. caledonica*	Full	10	20
1	*M. anisopliae*	Full	10	20
1	Control	-	0	20
2[2]	*M. anisopliae*	Full	same 10 as aim 1	0
2	*M. anisopliae*	Half	10	0
2	*S. carpocapsae*	Full	10	0
2	*S. carpocapsae*	Half	10	0
2	Mixed *M. anisopliae* & *S. carpocapsae*	Half + Half	10	0
2	Control	-	10	0

Table 4.4. Year Three Study Trial Treatments

1[1] Assess if different application methods affect EPF efficacy. 2[2] Determine if EPN and EPF combinations gave synergistic effects

Aim	Treatment	Application method	Dose	No stumps emergence trapping	No stumps destructive sampling
1[1]	*B. caledonica*	Sides	Full	0	10
1	*B. caledonica*	Top	Full	0	10
1	*B. caledonica*	Top+Sides	Full	0	10
1	*M. anisopliae*	Sides	Full	0	10
1	*M. anisopliae*	Top	Full	0	10
1	*M. anisopliae*	Top+Sides	Full	0	10
1	Control	-	-	0	10
2[2]	*B. caledonica*	Top+Sides	Full	10	0
2	*B. caledonica*	Top+Sides	Half	10	0
2	*M. anisopliae*	Top+Sides	Full	10	0
2	*M. anisopliae*	Top+Sides	Half	10	0
2	*H. downsei*	Top+Sides	Full	10	0
2	*H. downsei*	Top+Sides	Half	10	0
2	Mixed *M. anisopliae* & *H. downsei*	Top+Sides	Half + Half	10	0
2	Mixed *B. caledonica* & *H. downsei*	Top+Sides	Half + Half	10	0
2	Control	-	-	10	0

4.2.6 Assessment of efficacy-destructive sampling and monitoring emergence

Destructive sampling of stumps was carried out using the methods of Dillon *et al.* (2006). Roots were stripped to a distance of 50 cm. One quarter of each stump was destructively sampled (Figure 4.1). Firstly, brash and soil were removed from around the stump and roots. Next a chisel was used to carefully remove the bark from the stump and roots. Pine weevils were carefully removed with a forceps and brought back to the laboratory in 24 well plates. Different forceps were used for different treatments. The stage (larva, pupa, callow adult or adult) and infection status (alive, nematode-killed, fungus-killed and dead due to other causes) of weevils were recorded in the field, along with the location of the individuals (depth above/below soil level and distance from the bole of the stump). Surviving weevils were kept at 20°C for two weeks in the laboratory and checked for the presence of EPF infection.

Emergence traps, modified from a design by Moore (2001) (Figure 4.2 and 4.3), were erected over control and treated stumps after application and were emptied approximately every two weeks throughout the season until weevils ceased emerging.

Figure 4.1. An example of destructively sampled stumps with approximately a quarter of the stump sampled.

Figure 4.2. An example of an emergence trap used to collect *H. abietis*

Figure 4.3. Emergence traps used to collect *H. abietis* in Glendine.

4.2.7 Statistical analysis

Statistical analysis using Minitab V. 16 and GraphPad Prism V.5. was carried out on data from each year separately. For year one field study (Section 4.3.1) differences in the emergence of adult weevils between treatments was analyzed using a one way ANOVA with Dunn's multiple comparison tests. For year two field study (Section 4.3.2) differences in the emergence of adult weevils between treatments was analyzed using a Kruskal-Wallis test. There was no significant difference between treatments so Mann-Whitney U tests were carried out on emergence data to compare each treatment to the control. Differences in proportions of weevils that were fungal infected between treatments during destructive sampling were analyzed using a Kruskal-Wallis test with Dunn's multiple comparison tests. For year three field study (Section 4.3.3) differences in the emergence of adult weevils between treatments was analyzed using a Kruskal-Wallis test with Dunn's multiple comparison tests. Differences in proportions of weevils that were fungal infected between treatments during destructive sampling were analyzed using Kruskal Wallis with Dunn's multiple comparison tests. All post hoc tests were used to compare treated stumps to control stumps.

For emergence data in all field trials the type of interaction (synergistic, additive, or antagonistic) between EPNs and EPF was determined using a procedure described by Ansari *et al*. (2008). The expected additive reduction in emergence RE for the EPN–EPF combinations was calculated by $R_{expected} = R_{nematode} + R_{fungus} (1 - R_{nematode})$, where $R_{nematode}$ and R_{fungus} are the observed reduction caused by EPNs and EPF alone, respectively. Results from a χ^2-test, $\chi^2=(R_{observed} - R_{expecetd})2/R_{expecetd}$, were compared to the χ^2 table value for 1 degree of freedom. If the calculated χ^2-values exceeded the table value, there would be reason to suspect a non-additive effect that is synergistic/antagonistic, between the two agents. If the differences $R_{observed} - R_{expecetd} = D$ had a positive value, a significant interaction was then considered synergistic, and if D had a negative value, a significant interaction was considered antagonistic.

Further statistical analysis was carried out on year two and three field studies using GENSTAT statistical package (Version 14, VSN International, Hemel Hempstead, UK). Analysis of factors influencing weevil infection rates were

performed with generalized linear models, starting from fully saturated models, using where possible empirically estimated scale parameters to account for potential overdispersion, and arriving at the minimum adequate model via backwards model simplification (Crawley 1993). Infection rates among different treatments (either fungi alone in year two or fungi and application method in year three) were compared with logistic analysis and using quasi binomially distributed errors. Infection rates in relation to depth below soil surface and horizontal distance from the bole of the stump were explored with mixed Generalized Linear Models. Fungal species, method of application (year three of trials), depth and distance were introduced as fixed effects whereas each stump was introduced in the analysis as a random effect. Analysis was run separately for both years (years two and three).

4.3 Results

4.3.1 Year one field study

Number of weevils emerging from individual stumps ranged from 163 to zero. Total emergence from control stumps reached 368 weevils. *B. bassiana* treated stumps had the highest total emergence of 549 while full dose *S. carpocapsae* had the lowest total emergence of 140 weevils. Emergence data were not normal and were square root transformed. When a One Way ANOVA was carried out on emergence data, treatment was found to be significant ($F_{5,59}$=4.16, p<0.01). However Dunns' multiple comparison tests did not reveal significant effects (Figure 4.4). The mixed EPF and EPN treatment had an additive effect with an expected reduction in emergence of 65% and an observed reduction of 60% (χ^2=0.004, p=0.95) (Table 4.5) Destructive sampling revealed that the nematode treated stumps had a higher proportion of dead pine weevils than all other stumps (Figure 4.5). In stumps treated with a combination of *B. bassiana* and *S. carpocapsae* a higher proportion of *H. abietis* were killed by EPN than EPF (Figure 4.5).

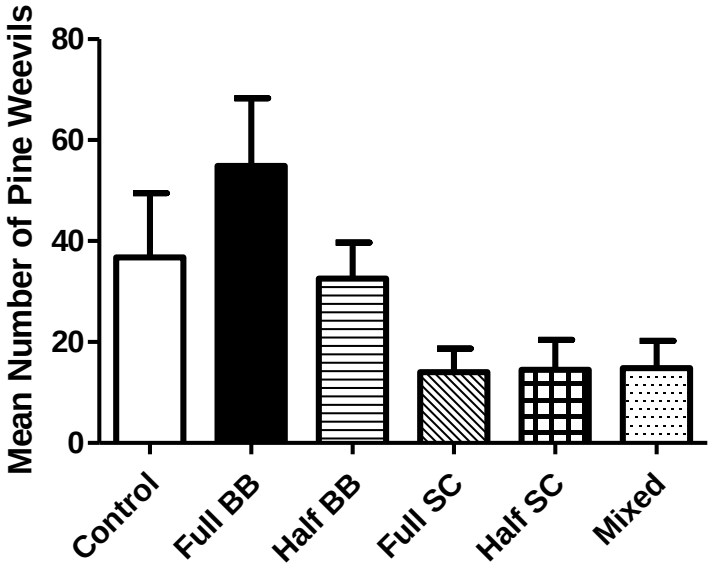


Figure 4.4. Mean number of *H. abietis* emerging in year one field study.
Control: untreated stumps, BB: *B. bassiana*, SC: *S. carpocapsae*, Mixed: half *B. bassiana* and half *S. carpocapsae*. One Way ANOVA showed treatment was significant ($p<0.01$). However Dunns' multiple comparison tests did not reveal significant effects.

Table 4.5 Observed reduction in emergence of *H. abietis* adults in year one field study	
Treatment	**Observed reduction (relative to controls)**
B. bassiana, full dose	-49% (increase instead of reduction)
B. bassiana, half dose	11%
S. carpocapsae, full dose	62%
S. carpocapsae, half dose	61%
Mixed *B. bassiana* and *S. carpocapsae*	60%

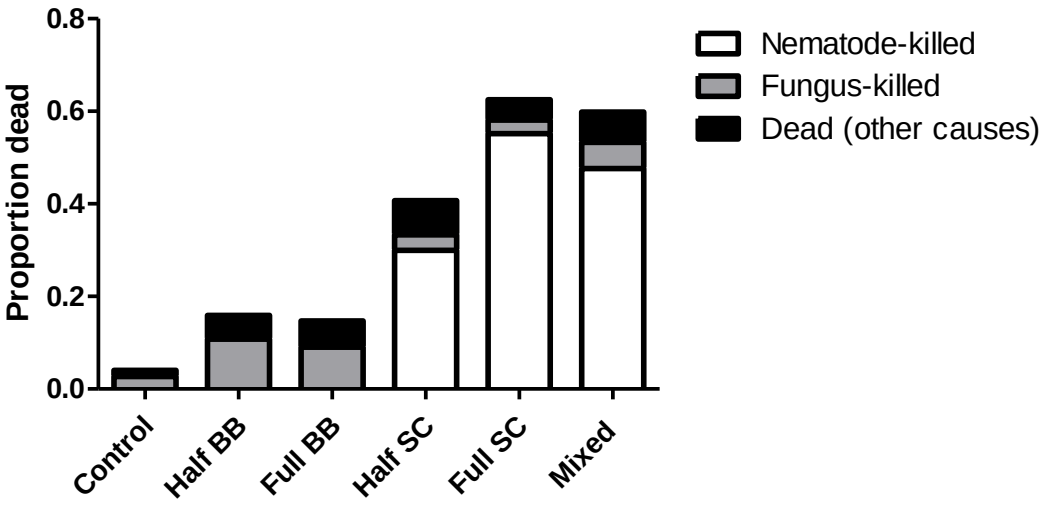


Figure 4.5. Proportion of *H. abietis* nematode killed, fungus killed or dead from unknown causes in year one field study.

Control: untreated stumps, Bb: *B. bassiana*, Sc: *S. carpocapsae*, Mixed: half *B. bassiana* and half *S. carpocapsae*.

4.3.2 Year two field study

The first aim of year two field study was to compare the efficacy of three EPF against pine weevil using destructive sampling and emergence trapping for assessment. The second aim was to investigate the use of *M. anisopliae* alone and in combination with *S. carpocapsae* for the reduction in numbers of adult weevils emerging. When a Kruskal Wallis test was carried out on all emergence data, there was no significant difference between treatments ($H=8.89$, $p>0.05$). Nevertheless, Mann-Whitney U tests were carried out on emergence data comparing each treatment to the control. There was a significant difference between number of weevils emerging in control stumps and full dose *S. carpocapsae* ($W=139$, $p<0.05$), and the mixed treatment ($W=133$, $p<0.05$). Both treatments caused a reduction in number of weevils emerging relative to the control (Figure 4.6). The mixed EPF and EPN treatment had an additive effect with an expected reduction in numbers of weevil emerging of 66% and an observed reduction of 58% ($\chi^2= 0.011$, $p=0.92$) (Table 4.6).

Destructive sampling was carried out on ten blocks four weeks after treatment application and another ten blocks eight weeks after application. The two hacking periods were combined for analysis as they were not significantly different from each other. A Kruskal Wallis ($DF=3$, $P<0.01$) was carried out with Dunn's Multiple Comparison Tests. *B. caledonica* ($p<0.001$) and *M. anisopliae* ($p<0.05$) had a significantly different proportion of fungal infected insects when compared to control stumps (Figure 4.9), with both having a higher proportion of fungal infected insects relative to controls. Different EPF displayed different growth morphology on cadavers: *M. anisopliae* growth doesn't radiate far from the cadaver and the cadaver is covered in spores, *B. bassiana* growth radiates out from the cadaver along the underside of the bark, while *B. caledonica* grows out from the cadaver, exuding out through the bark (Figure 4.7 and 4.8).

Infection rates among different fungal treatments (excluding the untreated controls) were compared with logistic analysis and using quasi binomially distributed errors. Infection rates differed significantly among different fungal species used ($F_{2,55} = 3.29$, $P = 0.045$, Figure 4.10). Infection rates in relation to depth below soil surface and horizontal distance from the bole of the stump were explored with mixed Generalized Linear Models. Fungal species, depth and distance were introduced as

fixed effects whereas each stump was introduced in the analysis as a random effect. Infection rates of weevils were significantly different among fungal species ($F_{2,607}$= 7.83, P< 0.001, Figure 4.11 and 4.12). Infection rates of weevils were negatively influenced by depth ($F_{1,607}$ = 6.16, p= 0.013, Figure 4.12) and distance ($F_{1,607}$=21.85, P <0.001. Figure 4.11).

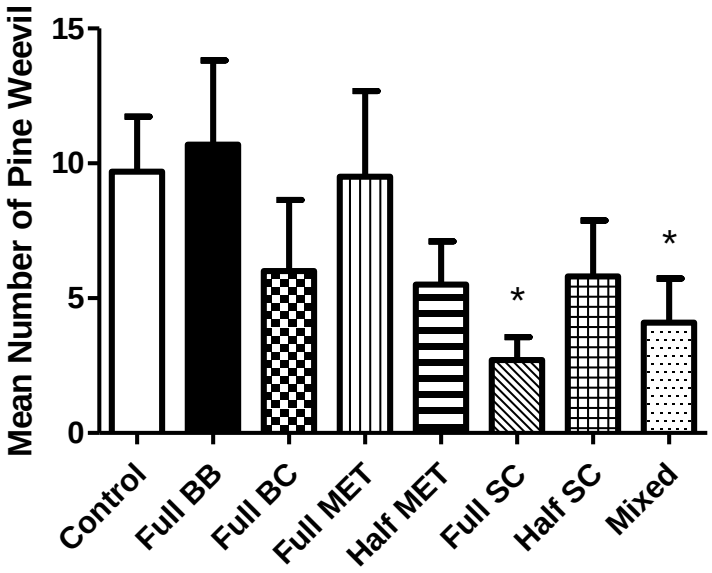


Figure 4.6. Mean number of *H. abietis* emerging in year two field study.
Control: untreated stumps, BB: *B. bassiana* BC: *B. caledonica*, MET: *M. anisopliae*, SC: *S. carpocapsae*, Mixed: half *M. anisopliae* and half *S. carpocapsae*. Mann-Whitney U tests were carried out on emergence data comparing each treatment to the control. There was a significant difference between number of weevils emerging in control stumps and full dose *S. carpocapsae* (W=139, p<0.05), and the mixed treatment (W=133, p<0.05). Both treatments caused a reduction in number of weevils emerging relative to the control. *p<0.05.

Table 4.6. Observed reduction in emergence of *H. abietis* adults in year two field study	
Treatment	**Observed reduction (relative to controls)**
B. bassiana, full dose	-10% (increase instead of reduction)
B. caledonica, full dose	38%
M. anisopliae, full dose	2%
M. anisopliae, half dose	43%
S. carpocapsae, full dose	72%
S. carpocapsae, half dose	40%
Mixed *M. anisopliae* and *S. carpocapsae*	58%

Figure 4.7. *H. abietis* cadavers with EPF on *B. caledonica* treated stumps during destructive sampling of year two field study.

Figure 4.8. *H. abities* cadavers with EPF on *B. bassiana* (left) and *M. anisopliae* (right) treated stumps during destructive sampling of year 2 field study

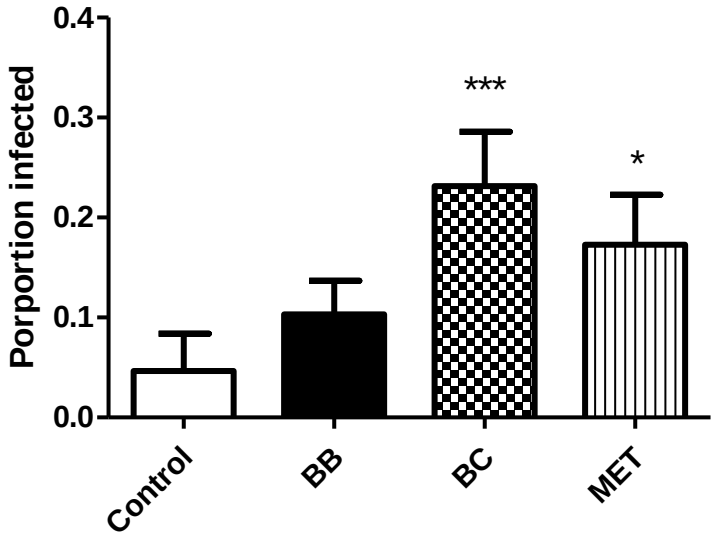


Figure 4.9. Proportion of *H. abietis* infected with EPF four-eight weeks after application in year two field study.

Control: untreated stumps, BB: *B. bassiana* BC: *B. caledonica*, MET: *M. anisopliae*, Kruskal Wallis test (DF=3, P<0.01) was carried out with Dunn's Multiple Comparison Tests. *B. caledonica* (p<0.001) and *M. anisopliae* (p<0.05) had a significantly different proportion of fungal infected insects when compared to control stumps, with both having a higher proportion of fungal infected insects relative to controls. ***p<0.001 *p<0.05

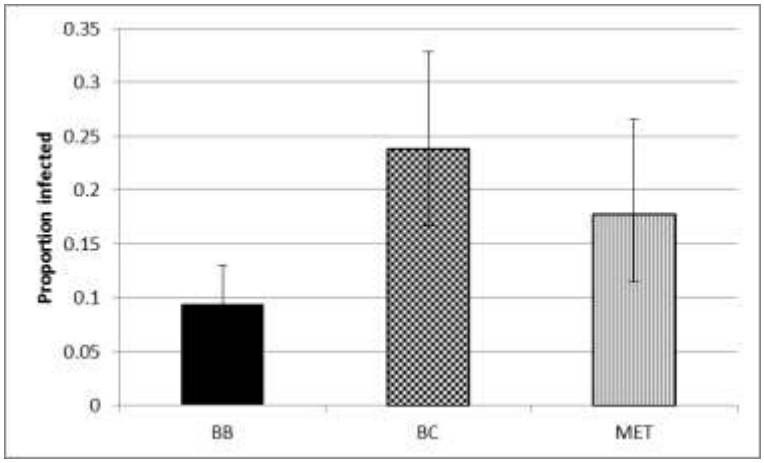


Figure 4.10. Proportion of total *H. abietis* that were fungal infected at time of destructive sampling compared with logistic analysis and using quasi binomially distributed errors.

Infection rates differed significantly among different fungal species ($F_{2,55} = 3.29$, $P = 0.045$).

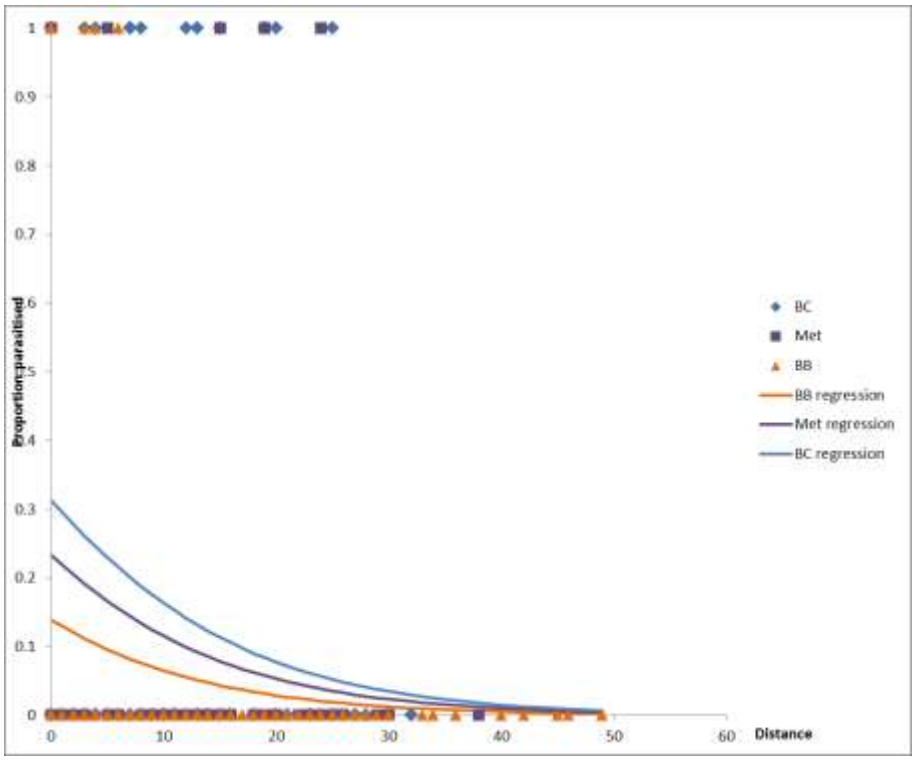


Figure 4.11. Proportion of *H. abietis* infected with EPF, relative to distance (cm) from stump at time of destructive sampling in year 2 field studies.

BB: *B. bassiana* BC: *B. caledonica*, MET: *M. anisopliae.*

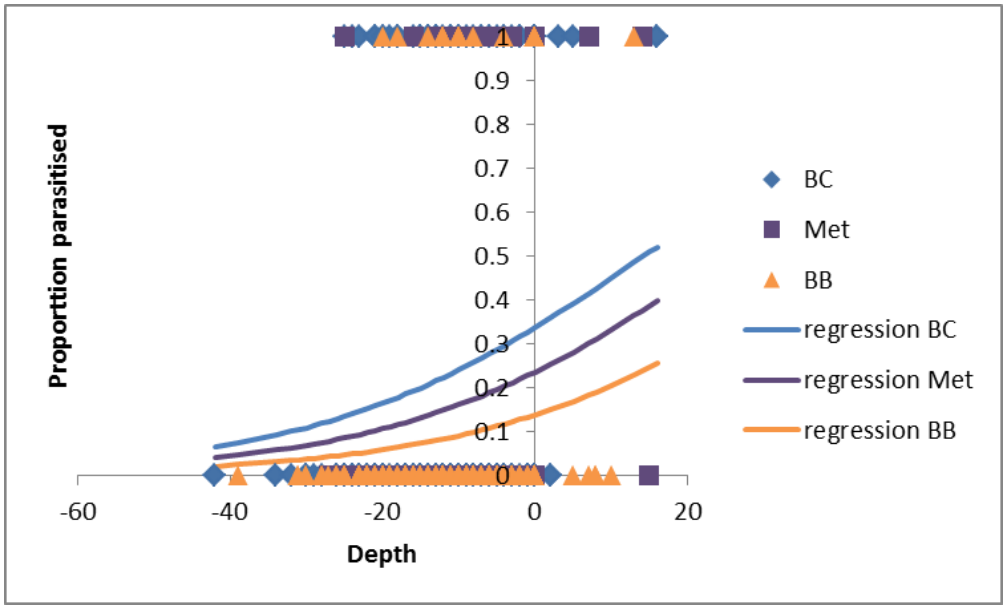


Figure 4.12. Proportion of *H. abietis* infected with EPF, relative to depth (cm) above/below soil level at time of destructive sampling in year two field studies.

BB: *B. bassiana* BC: *B. caledonica*, MET: *M. anisopliae*

4.3.3 Year three field study

One of the aims of the year three field study was to investigate the use of *B. caledonica* and *M. anisopliae* alone and in combination with *H. downesi* for the reduction in numbers of adult weevils emerging (Figure 4.13). There was a significant difference between treated and control stumps (H=22.88, DF=8, $p<0.01$). Full *H. downesi* and the mixed treatment *M. anisopliae* and *H. downesi* were significantly different than control stumps ($p<0.05$), both treatments caused a reduction in pine weevil emergence (Figure 4.13 A). Both of the mixed EPF and EPN treatment had an additive effect. For *M. anisopliae* and *H. downesi* the expected reduction in number of pine weevils emerging was 86% and the observed reduction was 93% (χ^2= 0.86, p= 0.93) (Table 4.7). For *B. caledonica* and *H. downsei* the expected reduction in number of pine weevils emerging was 89.6% and the observed mortality was 86% (χ^2= 0.001, p= 0.97). A second weevil species was collected in the emergence traps and was identified by the Natural History Museum, London as *Otiorhynchus singularis*, the clay coloured weevil (Figure 4.13 B). A Kruskal Wallis test with Dunn's multiple comparison showed that there was no significant difference between numbers of these weevils emerging in treated stumps relative to control stumps, but numbers were lower in all treated stumps (Table 4.7).

Another aim of the year three field study was to compare the effect of different application methods on EPF efficacy against pine weevil. To investigate the effect of application method on treatment efficacy destructive sampling was carried out on five blocks four weeks after application and on another five blocks eight weeks after application (Figure 4.14). There was no significant difference between the two hacking periods ($W=492.5$, $p>0.05$), so they were combined for analysis. A Kruskal Wallis test found that there was no significant difference in proportion of pine weevils infected with EPF between different treatments and application methods ($H= 11.32$, $DF=6$, $p>0.05$) but Dunn's Multiple Comparison Tests found that *B. caledonica* applied to both top and sides of stumps ($p<0.05$) and *M. anisopliae* applied to the sides of stumps ($p<0.05$) had a higher proportion of fungal infected insects than control stumps (Figure 4.14).

Infection rates among different fungal treatments (fungi and application method) (controls excluded) were compared with logistic analysis and using quasi binomially distributed errors. There were not significant differences in weevil infection rates among different treatments (fungi species and application method) ($F_{5,57} = 0.41$, $P = 0.838$) (Figure 4.15). Infection rates in relation to depth below soil surface and horizontal distance from the bole of the stump were explored with mixed Generalized Linear Models. Fungal species, method of application, depth and distance were introduced as fixed effects whereas each stump was introduced in the analysis as a random effect. Fungal species and depth were not significant factors in determining infection rates (Fungus: $F_{1,682}=1.37$, $P=0.242$; depth: $F_{1,682}=0.14$, $P=0.712$) but their interaction was significant ($F_{1,682} = 3.46$, $p = 0.041$, Figure. 4.16). Distance was not significant $F_{1,682} = 0.73$, $p=0.193$).

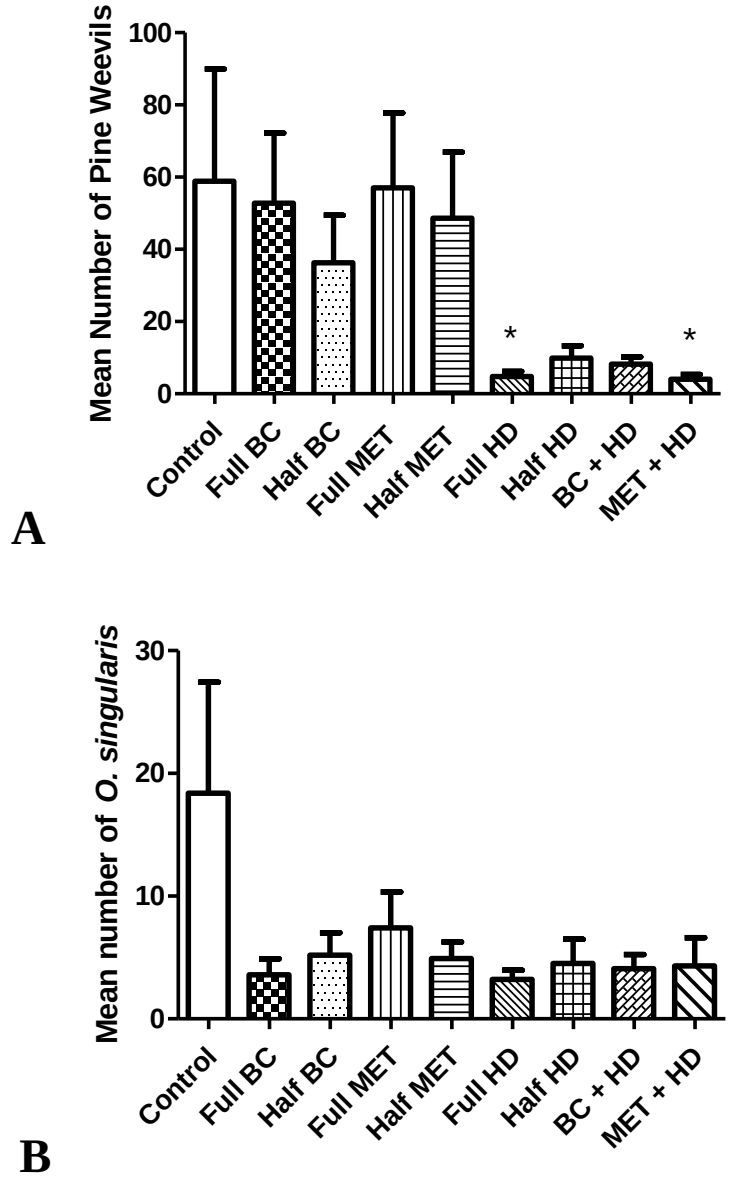


Figure 4.13. Mean number of *H. abietis* emerging in year three field studies.

A. *H. abietis*. **B.** *Otiorhynchus singularis*. **A.** Kruskal Wallis was carried out on emergence data and there was a significant difference between treatment and control (H=22.88, DF=8, P<0.01). Dunn's multiple comparison tests of treated versus control stumps showed that full *H. downsei* and mixed *M. anisopliae* and *H. downesi* were significantly different than control. **B.** There was no significant difference between numbers of weevils in treated stumps relative to controls. Control: untreated stumps, BC: *B. caledonica*, MET: *M. anisopliae*, HD: *H. downsei*. *p<0.05

Table 4.7. Observed reduction in emergence of *H. abietis* and *O. singularis* adults in year three field study	
H. abietis	
Treatment	**Observed reduction (relative to controls)**
B. caledonica, full dose	10%
B. caledonica, half dose	38%
M. anisopliae, full dose	3%
M. anisopliae, half dose	17.5%
H. downesi, full dose	92%
H. downesi, half dose	83%
Mixed *M. anisopliae* and *H. downesi*	93%
Mixed *B. caledonica* and *H. downesi*	86%
O. singularis	
Treatment	**Observed reduction (relative to controls)**
B. caledonica, full dose	80%
B. caledonica, half dose	72%
M. anisopliae, full dose	60%
M. anisopliae, half dose	73%
H. downesi, full dose	83%
H. downesi, half dose	76%
Mixed *M. anisopliae* and *H. downesi*	77%
Mixed *B. caledonica* and *H. downesi*	78%

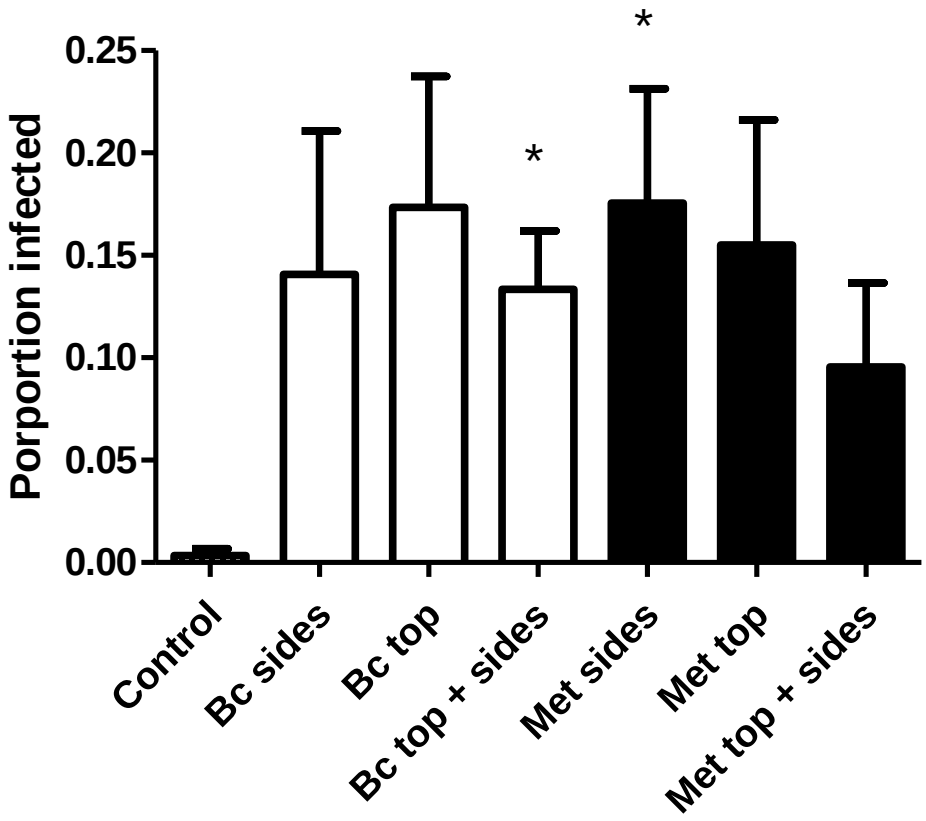


Figure 4.14. Proportion of total *H. abietis* that were infected with EPF at time of destructive sampling in year two field studies.

A Kruskal Wallis test found that there was no significant difference in proportion of pine weevils infected with EPF between different treatments and application methods (H= 11.32, DF=6, $p>0.05$) but Dunn's Multiple Comparison Tests found that *B. caledonica* applied to both top and sides of stumps ($p<0.05$) and *M. anisopliae* ($p<0.05$) applied to the sides of stumps had a significantly different proportion of fungal infected insects relative to control stumps. Control: untreated stumps, BC: *B. caledonica*, MET: *M. anisopliae.**$p<0.05$

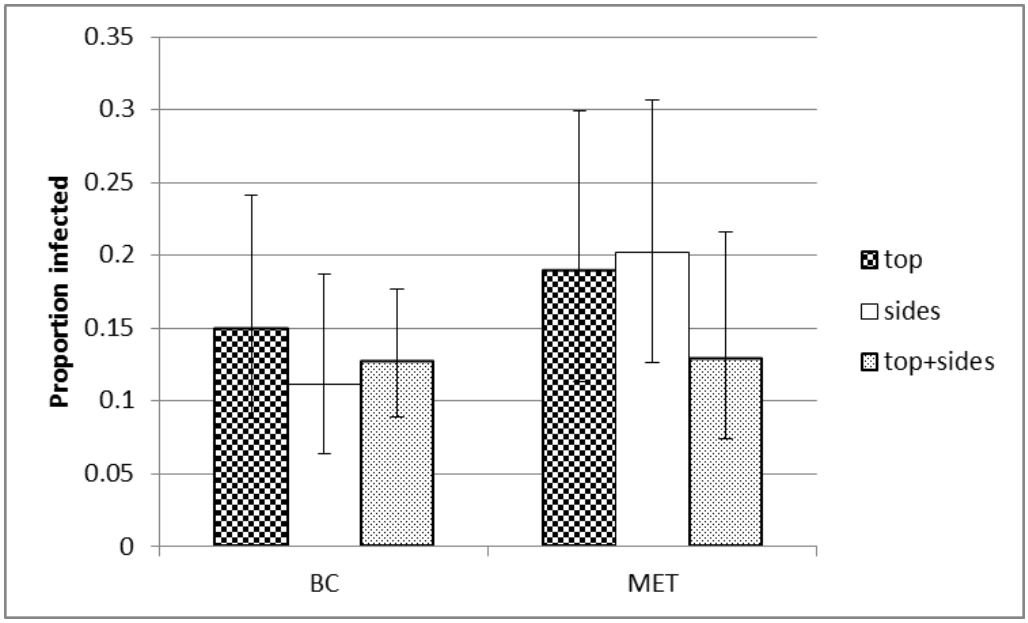


Figure 4.15. Proportion of total *H. abietis* that were fungal infected at time of destructive sampling compared with logistic analysis and using quasi binomially distributed errors.

There were not significant differences in weevil infection rates among different treatments (fungi species and application method) ($F_{5,57}$ = 0.41, P = 0.838).

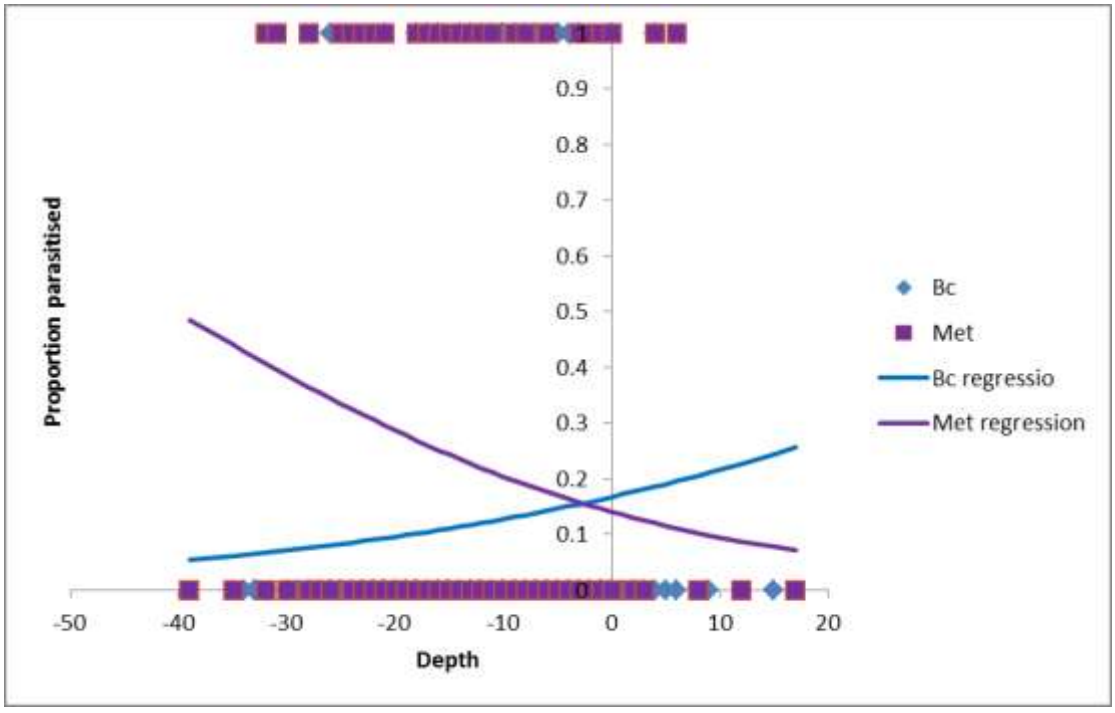


Figure 4.16. Proportion of *H. abietis* infected with EPF, relative to depth (cm) above/below soil level at time of destructive sampling in year three field studies.

BC: *B. caledonica*, MET: *M. anisopliae*

4.4 Discussion

4.4.1 Efficacy of EPF against *H. abietis* in the field

EPF used alone had varying success across both the different field trials and the different fungal species trialed. In year two three fungi *M. anisopliae, B. bassiana* and *B. caledonica* were trialed. When the proportion of fungal infected insects for fungal treated stumps was compared to control stumps both *B. caledonica* (23% infected) and *M. anisopliae* (17% infected) were significantly different than control stumps, with a higher proportion of infected insects (Figure 4.6). Results of destructive sampling and emergence trapping were in agreement, in *B. caledonica* treated stumps 23% of the population were fungal infected, while there was an average reduction in emerging adults of 33%. *B. caledonica* was the most promising EPF candidate for the control of *H. abietis*, outperforming full dose applications of both the commercial fungal strains. The emergence results indicate that half the recommended operational dose of EPF was no less effective than the full dose (Table 4.5, 4.6 and 4.7).

Infection rates among different fungal treatments were also compared with logistic analysis and using quasi binomially distributed errors. Control data were not included in this analysis. Infection rates differed significantly among different fungi species used (Figure 4.10). *B. caledonica* was the most promising EPF used, this was the first time this EPF has been trialed against *H. abietis* and the results highlight its potential application as a biocontrol agent. *B. bassiana* gave disappointing results in both year one and two field studies as well as in additional field trials by Williams *et al*. (2013) as it did not suppress numbers of *H. abietis* emerging relative to controls and so was considered an unsuitable biocontrol agent for use against pine weevils and was not included in future studies.

Based on the findings from year one and two field trials the EPF *M. anisopliae* and *B. caledonica* were selected as the most promising EPF treatments for year three field trials. *B. caledonica* caused an overall observed reduction in emergence of 38% in year two as well as the highest proportion of fungal infected weevils in destructive sampling so was selected as a promising candidate for biocontrol of *H. abietis*. In the year three field study when proportion of fungal infected insects in treated stumps

was compared to control stumps, *B. caledonica* applied to the top and sides of stumps, and *M. anisopliae* applied to the sides of stumps were significantly different to the control, with higher proportions of fungal infected *H. abietis* (Figure 4.14).

While the proportion of fungal infected insects was significantly different between EPF treated stumps and control stumps, there is no significant difference between average numbers of pine weevils emerging in EPF only treated stumps relative to controls. While EPF alone may have caused a reduction in number of *H. abietis* emerging it was not significant as was the case in a number of EPN treatments (both alone and combined with EPF). Also destructive sampling across the field studies revealed that all life cycle stages (larvae, pupae, adult) of *H. abietis* are susceptible to EPF infection (results not shown). Another interesting observation was that the three EPF had distinct growth morphologies in the stump (Figures 4.7 and 4.8). *M. anisopliae* growth doesn't radiate far from the cadaver and sporulates on the cadaver. These spores will be dispersed by water or insects. *B. bassiana* growth radiates out from the cadaver and spreads along the underside of the bark, suggesting it might be better able to recycle, however recycling potential was not examined in this work. *B. caledonica* grows out from the cadaver, exuding out through the bark: this may be useful for infecting insects in the soil.

4.4.2 EPN confer greater control over *H. abietis* numbers than EPF

In year one emergence results indicated that half the recommended operational dose of nematodes was as effective as a full dose; full dose nematodes caused an observed reduction in emergence of 62% while a half dose of nematodes caused an observed reduction of 61% (Table 4.5). The ability to halve the dose of EPN used and still be able to confer a reduction in emergence of *H. abietis* was also observed by Dillon *et al*. (2007) using *H. downesi*. Being able to use a half dose would result in a lower cost of the biocontrol agent, an important consideration in the uptake of biocontrol agents. In year two field trial the EPN *S. carpocapsae* was trailed alone and in combination with *M. anisopliae*. Both full dose *S. carpocapsae* (observed reduction 72%) and the mixed treatment (observed reduction 58%) caused a significant reduction in weevil emergence (Figure 4.6). Full dose *S. carpocapsae* performance is in agreement with year one and previous studies (Dillon *et al.* 2007, Torr *et al.* 2007). In year three the treatments full *H. downesi* (observed reduction 92%) and the mixed treatment *M. anisopliae* and *H. downesi* (observed reduction

93%) caused a reduction in *H. abietis* emergence that was significantly different compared to control emergence.

Ultimately EPN offer superior control over *H. abietis* populations than EPF with the most effective treatments for reducing weevil emergence being EPN alone or in mixed combinations with EPF. These field trials strengthen the use of EPN as a viable control method for pine weevils. *H. downesi* or combinations including *H. downesi* gave the highest level of control against pine weevils with an observed reduction in emergence of 83-93% compared to 60-72% for *S. carpocapsae*. The highest observed reduction in emergence across all treatments and years was a combination of *M. anisopliae* and *H. downesi* (93%). This supports Dillion *et al*. (2007) claim that the native EPN *H. downesi* is the most appropriate species for use against pine weevils in the field. To date *S. carpocapsae* is the only species that has been used to control pine weevils at an operational level in Europe (Williams *et al.* 2013).

4.4.3 Combinations of EPF and EPN confer additive control of *H. abietis*

There was no evidence of synergy in any of the EPF-EPN combinations used across the three field studies: all effects were additive. Year one field study was carried out as part of a larger field study and the results were analyzed in combination with four other field studies, the results of which have already been published (Williams *et al*. 2013). The efficacy of both EPN and EPF were tested alone and in combination in five field studies over three years. In the full field study the EPF *B. bassiana* along with the EPN *S. carpocapsae* and *H. downesi* were tested alone and in combination for ability to reduce *H. abietis* populations in the field. There was no significant effect of EPF and EPN treatments alone or in combination on weevils emerging from stumps.

Mixed applications of EPF and EPN have been reported to act synergistically against a number of coleopteran e.g. combinations of *M. anisopliae* and *H. bacteriophera* resulted in additive or synergistic control of the white grub *H. philanthus* (Ansari *et al*. 2006). In greenhouse trials EPN and *M. anisopliae* worked together synergistically for the control of black vine weevil *O. sulcatus* larvae (Ansari *et al.* 2008). Findings like these were the justification for these field studies to test for synergy between control agents against *H. abietis*. However in all three field studies in this chapter the EPF and EPN combinations used gave additive effects. One consideration for future research might be that when looking for synergy

in treatments a lower nematode dose may be advisable as although a mixed treatment of *M. anisopliae* and *H. downesi* reached an observed reduction in emergence of 93%, which was higher than the expected reduction, it was not possible to achieve synergy as half dose *H. downesi* alone was already reaching a reduction of 83%. Even if the combined dose achieved an observed reduction in emergence of 100% this would still not be significant enough to be determined synergistic. Perhaps when looking for potential for synergy a lower EPN dose might be better suited. Another approach may be to apply EPF earlier in the season rather than when *H. abietis* are already at late instar stage, as done in this work, thus allowing EPF time to colonise the stump so it can confer better control. This recommendation comes from reports of synergistic control of *H. philanthus* by EPF-EPN combinations where the strongest control occurred when the insect was exposed to *M. anisopliae* first followed by the EPN four weeks later indicating that the EPF acts as a stressor making the insect more susceptible to the EPN when subsequently added (Ansari *et al.* 2004).

4.4.4 Application method does not significantly effect EPF efficacy

Similar field studies to those performed in year two were carried out in Wales from 2009-2012 to assess nematode and fungal control agents at a range of doses against pine weevils (Evans *et al.* 2015). *M. anisopliae* was tested alone and in combination with *S. carpocapsae* and results indicated that all treatments were effective even at low doses indicating a potential reduction in cost (Evans et al. 2015). In these studies *S. carpocapsae* had a kill rate above 90% alone or in combination with *M. anisopliae,* much higher than the kill rate observed in my year two study. As the application method in Wales differed to the method used in year one and two field study, an investigation into the effect of the method of application on treatment efficacy was incorporated into the year three field study to see if it accounted for the differences in results. The application method used in Wales was to apply treatments to the gap between the bark and stump at the top, while the application method used in Ireland was to apply treatments to the sides of stumps and surrounding soil. These two application methods were tested, along with a combination of both methods. Infection rates among different fungal treatments (fungi and application method) were compared with logistic analysis and using quasi binomially distributed errors (control stumps were excluded from this analysis). There were not significant differences in weevil infection rates among different

treatments (fungi species and application method) (Figure 4.14). Therefore it can be concluded that the application methods tested do not have a significant effect on how the treatments perform against pine weevils. Thus the difference in efficacy of treatments in the year two field study in comparison to results from wales cannot be explained by application method, nor can success of these biocontrol agents be enhanced by altering application method in this way.

4.4.5 How does depth/distance affect efficacy of EPF?

To gain a greater understanding of how different EPF treatments behaved once applied to the stumps, infection rates in relation to depth below soil surface and horizontal distance from the bole of the stump were explored with mixed Generalized Linear Models. This analysis gives an insight into how the different EPF may be behaving in the field and might help in part to explain their differing efficacy. For the year two field study with *B. caledonica* and *M. anisopliae* there was no interaction between fungal species and depth or distance. Infection rates of *H. abietis* were negatively influenced by depth and distance for all EPF (Figure 4.11 and 4.12), thus it is harder for treatments to target weevils further from their site of application, for instance weevils located deep in the roots of stumps. *B. caledonica* infected the highest proportion of *H. abietis* irrespective of distance and depth. *H. abietis* were found at distances up to 49 cm from the stump bole therefore the ability of the EPF to infect weevils further from where it is applied would allow it to be effective against a greater number of insects. *H. abietis* were found at depths of up to 16 cm above the soil level and 42 cm below the soil level again highlighting the advantage of being able to infect weevils further from where the EPF is applied.

For the year three field study fungal species and depth were not significant factors in determining infection rates but their interaction was significant (Figure 4.16), therefore fungi behave differently at different depths. Distance did not have a significant effect on infection rates. *B. caledonica* and *M. anisopliae* appear to act more effectively at different depths (Figure 4.16); *B. caledonica* infected a higher proportion of weevils above the soil line (at depths >1cm) while *M. anisopliae* infects a higher proportion of weevils below the soil line (at depths >-1cm). This may indicate potential for dual EPF application to control pine weevils, if the EPF are more effective at different depths perhaps together they could target a larger number of weevils conferring greater control. EPF combinations can have promising results for example *M. flavoviride* and *B. bassiana* have been used in combination to

overcome the constraints of temperature in controlling thermoregulating grasshoppers (Inglis *et al.* 1997).

4.4.6 Otiorhynchus singularis

In the year three field study a second weevil was collected in the emergence traps and was identified by the Natural History Museum, London as *Otiorhynchus singularis*, the clay colored weevil (Figure 4.13.B). *O. singularis* is a root weevil that is an important pest of many crops and garden plants in particular raspberries and rhododendron. It is a significant pest in raspberry plantations in Scotland, with most of the damage being caused by adults feeding in the spring (Gordon and Woodford 1986, Bomford and Vernon 2005). *O. singularis* has previously been reported in mixed conifer plantations in Ireland and Germany (Blackith and Blackith 1986). There was no significant difference between numbers of *O. singularis* adults emerging in treated stumps relative to control stumps, but total numbers were lower in all treated stumps (Table 4.7). It is most likely that these weevils were feeding on plants beside the stumps that were also contained within the emergence traps; however these findings may indicate a potential use for these biocontrol agents against weevils in horticulture.

4.4.7 Conclusion

Williams *et al.* (2013) detected EPF-infected insects in stumps to which no EPF was applied during the trials. Almost half of the *Beauveria* isolates from EPF-infected weevils in stumps treated with *B. bassiana* scored as native *B. caledonica*. It highlighted a future avenue of investigation for the use of EPF in the control of *H. abietis*. *B. caledonica* kills *H. abietis* in its cryptic habitat making it an attractive target for further development of biocontrol agents. Its proposed use for the control of pine weevils is strengthened by the fact that studies show that it appears to be restricted to forest coleopterans (Reay *et al.* 2008) which may suggest that the impact of this EPF on non-target insects could be low. Based on these findings a native isolate of *B. caledonica* was incorporated into the year two and three field studies.

While synergy between EPF and EPN was not achieved in these studies, the native EPF *B. caledonica* emerged as a potential candidate for biological pest control, with efficacy rivalling the commercial strains tested. Its use certainly warrants further investigation; different combinations of *B. caledonica* and EPN/EPF or chemical insecticides may have potential for use against coleopteran pests. *B.*

bassiana was ultimately eliminated as a candidate for the control of pine weevils due to its poor performance in these field trials. Emergence trapping and destructive sampling gave comparable results when comparing EPF for ability to control weevils. Both methods were used for these studies as destructive sampling provides an insight into cause of death and how treatments behave once applied, for example at what depths and distances they are most successful, while emergence trapping provides information on reduction in emergence of adults. However if large numbers of EPF isolates were being trialed for ability to reduce *H. abietis* populations, one method would be advisable as they provide comparable results on treatment efficacy. Trapping alone would reduce cost and labour and would still give informative results as reduction in numbers of emerging *H. abietis* is the desired goal.

Chapter 5:

Identification & analysis of a bioactive compound from *B. caledonica*

5.1 Introduction

Secondary metabolites, or natural products, are produced by a range of microorganisms and plants and confer a variety of survival functions on the producing organism. These include protection from abiotic and biotic stresses, such as UV radiation, desiccation, predation from insects and competition with other microbes, as well as participation in metal homeostasis (Keller 2015).

Fungi have successfully adapted to almost all habitats on earth and a primary explanation for their ability to survive in different environment is their diverse range of natural product pathways. These natural products often exhibit antimicrobial, antifungal, immunosuppressive or cytotoxic effects. These properties enable them to establish in environmental niches by allowing them to compete for nutrients, deter predators and communicate with other organisms (Quin *et al.* 2014). The key role natural products play in fungal survival is highlighted by the fact that fungi often have many different secondary metabolite pathways clustered within their genomes. These bioactive products are of considerable interest for industry, agriculture and pharmaceuticals as they can be harnessed for their antimicrobial, anticancer and antiviral properties (Quin *et al.* 2014). There is great interest and work being done in the development of entomopathogenic fungi (EPF) as biocontrol agents. However there is concern that the use of EPF as biocontrol agents may increase levels of toxic microbial metabolites in the environment. For a fungal biocontrol agent to be registered in Europe the Directive 91/414/EEC demands data on the potential risks arising from the applied fungus and the metabolites it produces on the environment (Strasser *et al.* 2000). However, unlike chemical pesticides and antibiotics used in agriculture, no reports of metabolites entering the food chain or accumulating in the environment have been reported as a result of *B. bassiana* metabolite secretion (Vey *et al.* 2001, Amin *et al.* 2010). Thus there is a need for research into the metabolites produced by less well characterized entomopathogenic fungi to aid their implementation as biocontrol agents.

The genus *Beauvaria* contains species that are generally entomopathogenic, these species range from the ubiquitous entomopathogenic fungi *B. bassiana* to rarer species. Several species have been reported as naturally occurring insect pathogens; *B. bassiana*, *B. brongniartii*, *B. velata*, *B. amorpha*, *B. vermiconia* and *B. caledonica*

(Glare *et al.* 2008, Williams *et al.* 2013). *Beauvaria* species are well known for producing a diverse variety of biologically active secondary metabolites including non-peptide pigments and polyketides (oosporein, bassianin and tenellin), nonribosomally synthesized peptides (beauvericin, bassianolides and beauveriolides) and secreted metabolites involved in pathogenesis and virulence (oxalic acid). These metabolites have both potential and realized applications in industry, pharmacology and agriculture (Xiao *et al.* 2012).

B. caledonica was originally isolated and described from soil in Scotland as being both saprotrophic and a significant biological competitor (Bissett and Widden 1988). Glare *et al*. (2008) identified *B. caledonica* as a naturally occurring pathogen of the introduced pine bark beetles, *Hylastes ater* and *Hylurgus ligniperda*, in New Zealand. It was also identified as a naturally occurring pathogen of the economically significant weevil pest *H. abietis* in the UK and Ireland (Glare *et al.* 2008, Williams *et al.* 2013). Thus, *B. caledonica* has potential as a biocontrol agent in forestry for use against coleopteran pests. There is currently limited knowledge about *B. caledonica* in comparison to the commonly used EPF species *M. anisopliae* and *B. bassiana*, with much of what is known about the fungus focusing on its high tolerance to toxic metals. *Beauveria* species are believed to have the capacity to exploit almost all ecophysical niches and can behave in a saprotrophic, necrotrophic and biotrophic manner (Fomina *et al.* 2005). Therefore it is believed they play an important role in biogeochemical cycling of elements. *B. caledonica* is highly tolerant to toxic metals converting them into oxalates. It was found to overexcrete organic acids with strong metal chelating properties such as oxalic and citric acids. Oxalate overexcretion may facilitate the fungal penetration into plants and insects (Fomina *et al.* 2005). Therefore as well as having biocontrol potential, *B. caledonica* may have biotechnological applications in bioremediation and bioleaching. *B. caledonica* overexcretes oxalic acids at comparable levels to *Aspergillus niger* thus it may have applications in the production of oxalic acid. Pairing its commercial potential in the biocontrol and biotechnology sector, further research into the products produced by *B. caledonica* and their bioactivity is justified.

An isolate of *B. caledonica* native to Ireland demonstrated immunemodulation effects on both *G. mellonella* and *H. abietis* larvae, in Chapters Two and Three respectively and infection of *H. abietis* adults in the field as described in Chapter Four. As many natural products of *M. anisopliae* and *B. bassiana*, the other EPF

used in these Chapters, have been characterized, the aim of this Chapter was to investigate the production of natural products in this isolate of *B. caledonica.*

5.1.1 Objectives

1. Detection and isolation of the primary natural products produced by *B. caledonica* in liquid culture.
2. Screening these fractionated natural products for insecticidal properties in order to elucidate the primary toxin secreted by *B. caledonica*.
3. Identification, large scale production and structural determination of oosporein; the primary secreted natural product of *B. caledonica.*
4. Detailed assessment of the insecticidal, anti-feedant and immunomodulation effects of oosporein on both the forestry pest *H. abietis* a natural host of *B. caledonica* and the insect model organism *G. mellonella*.
5. Uncovering a novel feedback induction mechanism regulating oosporein production in *B. caledonica*.

5.2 Materials & Methods

5.2.1 *B. caledonica* Strain and Culturing

Beauveria caledonica (2c7b) was a native strain isolated from a soil sample from an untreated stump in a felled forest in Hortland, Co. Kildare. The soil sample was baited with *Galleria mellonella* and the fungus on the infected cadaver was identified through sequencing. *B. caledonica* was cultured in Sabouraud Dextrose liquid medium (Oxoid) for 96 h in a shaking incubator at 25°C, 250 rpm. The culture supernatant was filtered through 0.45 µm syringe filters and then through 0.2µm syringe filters (Sartstedt). The supernatant was collected and stored at -80°C.

5.2.2 RP-HPLC analysis of entomopathogenic fungi supernatant

Metabolites from all three EPF fungal supernatant (cultured as per Section 2.2.4) were observed by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) on an Agilent Series 1200 HPLC system. RP-HPLC used solvent A: 0.1% (v/v) trifluoroacetic acid (TFA) in HPLC-grade water and solvent B: 0.1% (v/v) TFA in HPLC-grade acetonitrile. 10 µl of organically extracted supernatant was injected onto a C18 column (Agilent Eclipse XDB-C18, 5 µm, 9.4 x 250 mm).

5.2.3 Organic extraction of *B. caledonica* fungal supernatant

Filtered supernatants were organically extracted twice using an equal volume of ethyl acetate. Organic extracts were concentrated by rotary evaporation at 40°C (Stuart RE300DB). The dried extracts were fully resuspended in HPLC grade methanol. The resuspended extract was centrifuged at 13,000 x g for 5 min, transferred to clean 1.5 ml centrifuge tube and stored at – 20°C.

5.2.4 HPLC analysis and fractionation of *B. caledonica* fungal supernatant

Organic extracts (Section 5.2.3) and neat fungal supernatant were analysed by RP-HPLC with UV detection (Agilent 1200 system), using a C18 column (Agilent Eclipse XDB-C18, 5 µm, 9.4 x 250 mm) at a flow rate of 2 ml/min. A mobile phase of water (1% (v/v) trifluoroacetic acid (TFA) in HPLC-grade water) and acetonitrile (0.1% (v/v) TFA in HPLC-grade acetonitrile) was used under various gradient conditions. Injection volume varied from 10 to 100 µl depending on the objective.

Fractionation was carried out under the same mobile phase, gradient and flow rate. Fractions were collected into glass vials (Labquip Limited, Ireland). The fraction at 16.6 minutes was repeatedly collected, pooled and evaporated to form a red particulate that was resuspended in 5 % (v/v) DMSO.

5.2.5 Structural determination of the bioactive Fraction 5 from *B. caledonica* culture supernatants

The red metabolite was fractionated (per Section 5.2.4) until 5 mg of dried metabolite was obtained. Identification of the purified red metabolite of *B. caledonica* was carried out using NMR and Infrared Spectroscopy by Dr. John M. D. Walsh, and Dr. John C. Stephens from the Department of Chemistry, Maynooth University.

5.2.6 Rapid large-scale purification of oosporein using Ion-Exchange Chromatography

Oosporein was concentrated, purified and chromatographed using Q-Sepharose ion exchange resin. The procedure was automated using an AKTA Purifier 100 system (Amersham Biosciences, UK). Q-Sepharose resin (5 ml) was added to 1 L of *B. caledonica* supernatant. The sample was gently agitated at 4° C overnight. The culture supernatant containing Q-Sepharose was packed into an XK 16/20 column and washed with 100 ml binding buffer (20 mM Tris HCL, 10 mM NaCl, pH 8) at a flow rate of 5 ml/min. Fractions were eluted from the column at a flow rate of 2 ml/min with a linear gradient of 1M NaCl in elution buffer (20 mM Tris HCL, 1 mM NaCl, pH 8). Fractions containing red/purple pigment were pooled and analyzed by RP-HPLC.

5.2.7 Spike assay to determine the effect of oosporein on its own regulation in *B. caledonica*

B. caledonica was cultured as per Section 5.2.1. After 24h *B. caledonica* 17.5 µg/ml of oosporein (fractions or organic extract) was added to cultures (50 ml). DMSO or methanol was added to control cultures. After a total growth period of 96h, cultures were filtered through miracloth, dried between tissue and mycelium was snap frozen in liquid nitrogen. Mycelium was used for analysis by label free MS (Section 5.2.8.) and fungal supernatant was analyzed using RP-HPLC (Section 5.2.4). Four biological replicates were carried out per experiment.

5.2.8 Whole cell lysate preparation and digestion of *B. caledonica* mycelia for analysis by Label Free Quantification

B. caledonica was cultured and treated as per Section 5.2.7. Mycelium was harvested through miracloth, dried between tissues and snap-frozen in liquid nitrogen. Mycelium was ground using a pestle and mortar cooled in liquid nitrogen. Four biological replicates were carried out per treatment. Ground mycelium (200 mg, wet weight) was placed in a 1.5 ml centrifuge tube and 400 µl of lysis buffer (pH 7.5, 100 mM Tris HCL, 50 mM NaCl, 20 mM EDTA, 10 ml glycerol, 1 µg/ml pepstatin A, 1 mM PMSF) was added. Mycelium were lysed using a sonication probe MS72 (3 x 10 seconds, 20% power, cycle 6), followed by 1 x 10 seconds using MS73 probe. Lysates were incubated on ice for one hour, followed by centrifugation at 14,000 x g, 10 minutes at 4°C. Supernatants were transferred to new 1.5 ml centrifuge tubes and centrifugation was repeated to obtain clarified lysates. A Bradford assay was carried out to determine the protein concentration in each sample. Samples were brought to 15% trichloroacetic acid (TCA) by addition of the appropriate volume of cold 100% TCA solution. Samples were vortexed and incubated on ice for 30 minutes. Ice cold acetone (500 µl) was added to the TCA/protein suspension, vortexed and incubated at -20°C for one hour. An additional 500 µl of acetone was added and incubated overnight at -20°C. After overnight incubation, samples were centrifuged at 14, 000 x g for 10 minutes at 4°C. Supernatant was discarded; pellet was washed twice with 500 µl ethanol. Acetone was removed and pellet was allowed to dry for a maximum of 5 minutes before resuspension with appropriate volume of 8 M urea (6 M urea, 2 M thiourea, 0.1 M Tris HCL pH 8).

Samples (15 µl: 50 µg) were brought to room temperature to ensure urea was in suspension. Ammonium bicarbonate (78.5 µl: 50 M) was added to the samples. DTT (1 µl; 0.5 M) was added to the samples, followed by incubation at 56°C for 20 minutes. Samples were allowed to return to room temperature before 2.7 µl IAA (0.55 M) was added, followed by incubation at room temperature for 15 minutes in the dark. Protease Max, 1 µl (1% (w/v)) and 1.8 µl of trypsin (1 µg/µl) were added to each sample, followed by incubation at 37°C overnight. After overnight incubation samples were spun briefly and acidified by adding 1 µl of formic acid, vortexed and incubated for five minutes. Samples were centrifuged at 14,000 x g for 10 minutes at room temperature. Samples were stored at -20° until LCMS analysis.

Samples (10 µg) were dried using a Speedy Vacuum and resuspended in 20 µl of resuspension buffer (0.5 % TFA). Samples were sonicated for two minutes to help

resuspend the pellet. ZipTip Pipette Tips (Merck Millipore, Germany) were used to purify samples for mass spectrometry. Zip tips were wetted by aspirating and dispersing 10 µl of wetting solution (0.1 % TCA in 80% acetonitrile) five times. Zip tips were equilibrated by aspirating and dispersing 10 µl of equilibration solution (0.1 % TCA) five times. Samples were bound by aspirating and dispersing 10 µl of resuspended sample 15 times. Zip tips were washed by aspirating and dispersing 10 µl of washing solution (0.1 % TCA) from tip to waste five times. Samples were eluted by aspirating and dispersing 10 µl of elution solution (0.1 % TCA in 60% Acetonitrile) from tip to new 1.5 ml microcentrifuge tubes, this was repeated five times. Samples were dried down and resuspended in 20 µl of loading buffer when ready to carry out mass spectroscopy. Samples were vortexed and centrifuged at 14,000 x g for three minutes.

5.2.9 *B. caledonica* Q-Exactive Mass Spectrometry Method

All peptide mixtures were analysed via a Thermo Fisher Q-Exactive mass spectrometer coupled to a Dionex RSLCnano. LC gradients ran from 4-35 % B over 2 h, and data were collected using a Top15 method for MS/MS scans. Comparative proteome abundance and data analysis was performed using MaxQuant software (Version 1.3.0.5) and Perseus (Version 1.4.1.3) was used to organize the data. Carbamidomethylation of cysteines was set as a fixed modification, while oxidation of methionines and acetylation of N-termini were set as variable modifications. The maximum peptide/protein false discovery rates (FDR) were set to 1% based on comparison to a reverse database. The Label-Free Quantification (LFQ) algorithm was used to generate normalised spectral intensities and infer relative protein abundance. Proteins that matched to a contaminants database or the reverse database were removed and proteins were only retained in final analysis if detected in at least three replicates from at least one sample. Quantitative analysis was performed using a t-test to compare pairs of samples, and proteins with significant change in abundance (p value <0.05) were included in the quantitative results. Qualitative analysis was also performed, to detect proteins that were found in at least three replicates of a particular condition, but undetectable in the other sample condition. Four biological replicates were carried out per treatment for this experiment.

5.2.10 Determining the effect of oosporein on larval haemocyte densities

In the insect bioassays carried out (5.2.10-5.2.13) oosporein was used at a final concentration of 0.125 µg as that was the concentration of oosporein retrospectively calculated in the *B. caledonica* 96 h supernatant treatment used in bioassay and proteomic studies against *G. mellonella* and *H. abietis* in Chapters Two and Three.

B. caledonica organic extract was fractionated as per Section 5.2.4. to collect oosporein. *G. mellonella* larvae were injected with 0.125 µg/20 µl of oosporein through the last proleg (Section 2.2.10). *H. abietis* larvae were injected through their spiracles as per Section 3.2.2. Control larvae were injected with 20 µl of 5 % (v/v) DMSO. Haemocyte density was assessed by bleeding five larvae per treatment and then enumerating using a Neubauer haemocytometer. The experiment was performed on three independent occasions.

5.2.11 Determining the effect of oosporein on yeast load in larvae

C. albicans (MEN) was cultured to the stationary phase overnight in YEPD broth at 30°C and 200 rpm. Larvae were injected through the last proleg or spiracle with 0.125 µg/20 µl of oosporein (Figure 2.6). Control larvae were injected with DMSO. Five larvae were injected per treatment/time/insect species. Larvae were incubated for 24h at 20°C. After 24h larvae were given a second injection with *C. albicans*. After incubation for a further 24 or 48h at 20°C, three larvae from each treatment were homogenized in 3ml of sterile PBS. After serial dilution in PBS, 100 µl of each sample was spread on YEPD ERY plates (Section 2.2.11). The plates were incubated for 48h at 30°C. The yeast load was subsequently calculated as the yeast cell density per larva. Experiments were performed on three independent occasions.

5.2.12 Investigating whether oosporein increases susceptibility of larvae to a subsequent infection

C. albicans (MEN) was cultured to the stationary phase overnight in YEPD broth at 30°C and 200 rpm. Larvae were injected through the last proleg or spiracle with 0.125 µg/20 µl of oosporein. Control larvae were injected with DMSO. Ten larvae were injected per treatment/insect species. Larvae were then incubated for 24h at 20°C. After 24h larvae were given a second injection with *C. albicans*. Larvae were incubated at 20°C and mortality was recorded. The experiment was performed on three independent occasions.

5.2.13 Label Free Quantification of the effect of oosporein on the *G. mellonella* haemolymph proteome.

Larvae were injected with 0.125 µg/20 µl of oosporein (Section 5.2.4.), control larvae were injected with DMSO and incubated for 48h at 20°C. Five larvae were bled per treatment into a pre-chilled 1.5 ml centrifuge tube and spun at 1,500 x g for 5 min at 4°C. Protein extraction, digestion and sample clean up using C18 columns was performed as per Section 2.2.36. Three biological replicates were carried out per treatment. The Q-Exactive mass spectrometry method and subsequent analysis was carried out as described in Section 2.2.36.

5.2.14 Bioassay to investigate if oosporein acts as a contact toxin

Oosporein was dissolved in 5 % (v/v) DMSO to concentrations of 1.5 mg/ml and 125 µg/ml. *H. abietis* adults and *G. mellonella* larvae were dipped in either oosporein or DMSO (control). Ten insects were treated per species and treatment. Insects were incubated for three weeks at 20°C, checked daily and mortalities recorded. The entire experiment was performed on three separate occasions.

5.2.15 Choice-bioassay to investigate if oosporein effects *H. abietis* adult feeding

Oosporein was dissolved in 5 % (v/v) DMSO to concentrations of 1.5 mg/ml and 125 µg/ml. Twigs (2 cm in length) were dipped in either oosporein or DMSO (control). Bioassays were carried out in plastic tubs (6cm x 9.5 cm) lined with a thin layer of moist moss peat. In each tub there was a choice of two twigs treated with oosporein (1.5 mg/ml or125 µg/ml) and two control twigs (Figure 5.1. A). Control tubes contained four twigs dipped in DMSO; this was used as a control for death caused by ingesting oosporein. One adult weevil was placed in each tub. All tubs were placed in a 20°C room with a 16:8 light regime and were checked daily for feeding, which was recorded using a scoring scale (Figure 5.1. B). For the scoring scale the 2 cm twig was visually divided lengthwise into ten sections; when the bark was completely removed from a section it was given a score of one, half removed a score of 0.5 and so on. The twig was turned and scoring repeated to cover the circumference of the twig. Ten insects were tested per treatment and the entire experiment was repeated on three separate occasions.

Figure 5.1. Choice-bioassay to investigate if oosporein affects feeding of *H. abietis* adults.

A. Bioassay choice test set up. **B.** Bioassay feeding scoring.

5.2.16 Statistical analysis

Statistical analysis was carried out using Minitab V.16 statistical software and GraphPad Prism V.5. All data were first tested for normality, where data was found not to be normal, the data was either transformed before further analysis was carried out or a non-parametric alternative was used.

In Section 5.3.2 data were normal and a one way ANOVA was used to compare the effects of different RP-HPLC fractions on yeast load in larvae. In Section 5.3.4 data were not normal and Mann Whitney U tests were carried out to compare treatments. In Section 5.3.6 data that were not normal were log transformed. Two way ANOVAs were then carried out with Bonferroni post-tests to investigate alterations in haemocyte densities. In Section 5.3.7 data were not normal and were log transformed. Two way ANOVAs were carried out with Bonferroni post-tests to investigate alterations in yeast load.

In section 5.3.8 data were not normal. Mann Whitney Tests were used to compare larval mortality in the absence and presence of *C. albicans*. In section 5.3.10 data was not normal and a Kruskal Wallis test was used to assess differences in insect mortality. In section 5.3.11 a general linearized model (GLM) was used to test for the effect of treatment, experiment number and interaction between treatment and experiment number on feeding score. Replicates were combined and a One-Way ANOVA with Tukey's Multiple Comparison test was used to compare total food

eaten in each treatment. Wilcoxon signed rank tests were used to compare the proportion of total food consumed that was treated with oosporein.

5.3 Results

5.3.1 RP-HPLC analysis of EPN supernatant

M. anisopliae, *B. bassiana* and *B. caledonica* were cultured in parallel for 96h, after which they were filtered and subjected to RP-HPLC analysis. RP-HPLC chromatographs are shown for all EPF (Figure 5.2). *B. caledonica* culture filtrate showed one peak with particularly high abundance (Figure 5.2.). The detector maxes out at about 2000 maU so the abundant peak may be substantially higher. Coupled with the culture's pink pigment, its immunomodulating properties (Chapter Two and Three), and its infectivity in the field (Chapter Four) further investigation into *B. caledonica* fungal supernatant was warranted.

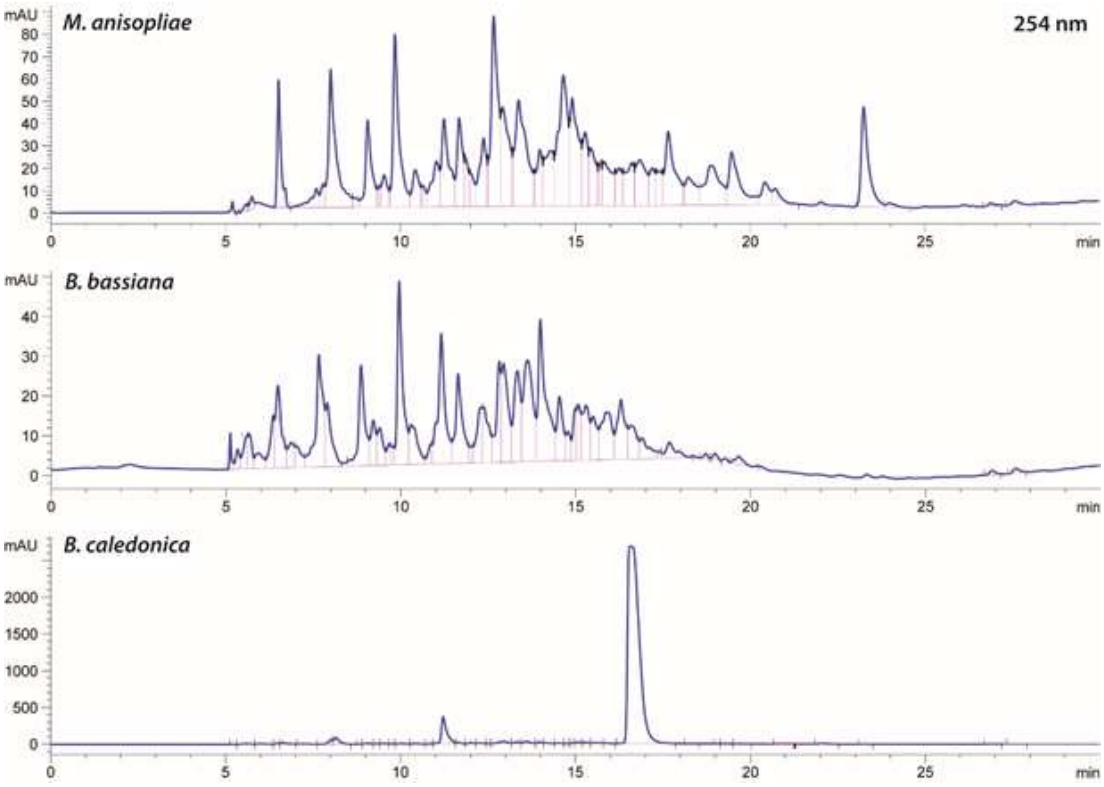


Figure 5.2. RP-HPLC profiles of 96h EPF culture filtrates at 254nm

X-axis represents retention time. Y-axis represents milli-Arbitrary Units (mAU*s)

5.3.2 Fractionation of *B. caledonica* supernatant

Five abundant peaks from organic extracts of *B. caledonica* 96h supernatant were fractionated and collected using RP-HPLC (Figure 5.3). A red pigmented fraction that eluted at approximately 16.6 minutes was selected as a fraction of interest (Figure 5.4). Organic extraction was carried out to concentrate and extract the fraction of interest (Figure 5.5). This fraction was repeatedly collected, dried and pooled until of 5 mg of dried metabolite was obtained and was used to identify the metabolite (Section 5.3.3.). A number of other fractions were injected into *G. mellonella* to investigate if they displayed immunomodulation potential (Figure 5.6) however, pre-treatment with these compounds did not increase yeast load in *G. mellonella* larvae.

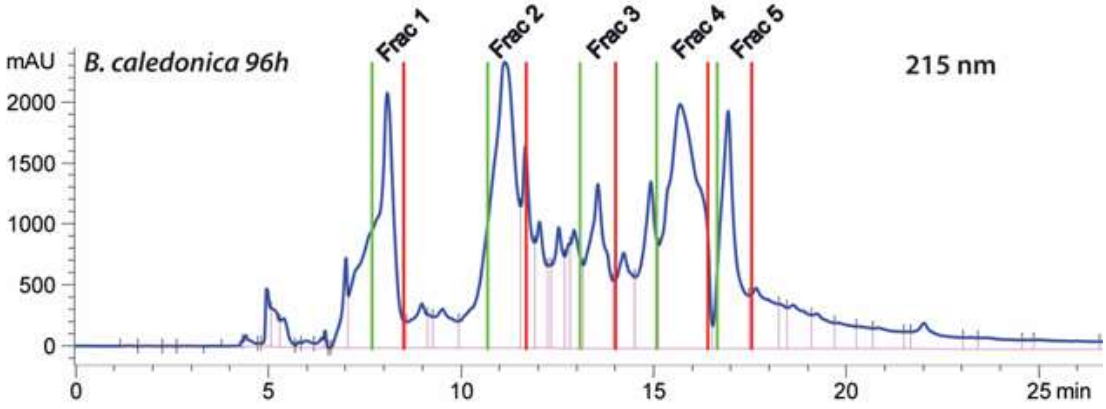
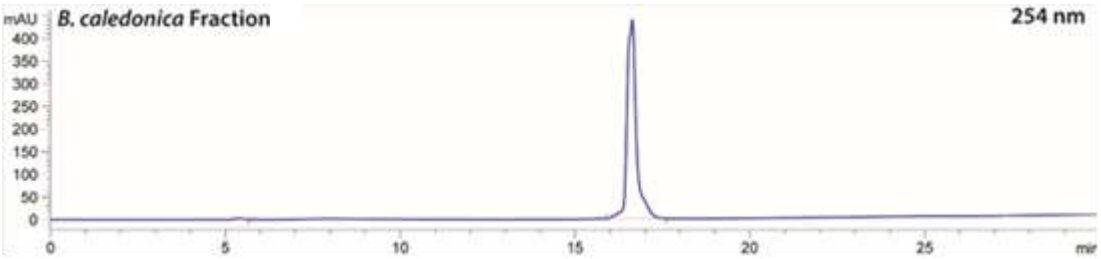


Figure 5.3. RP-HPLC profile of organic extracts of *B. caledonica* 96h culture filtrate at 215 nm indicating where fractions were collected.

A



B

C

Figure 5.4. Fraction of interest from *B. caledonica* 96h culture filtrate.

A. RP-HPLC profile of fraction of interest. **B.** *G. mellonella* larvae injected with fraction of interest. **C.** Microcentrifuge tube containing dried metabolite with distinctive red colour.

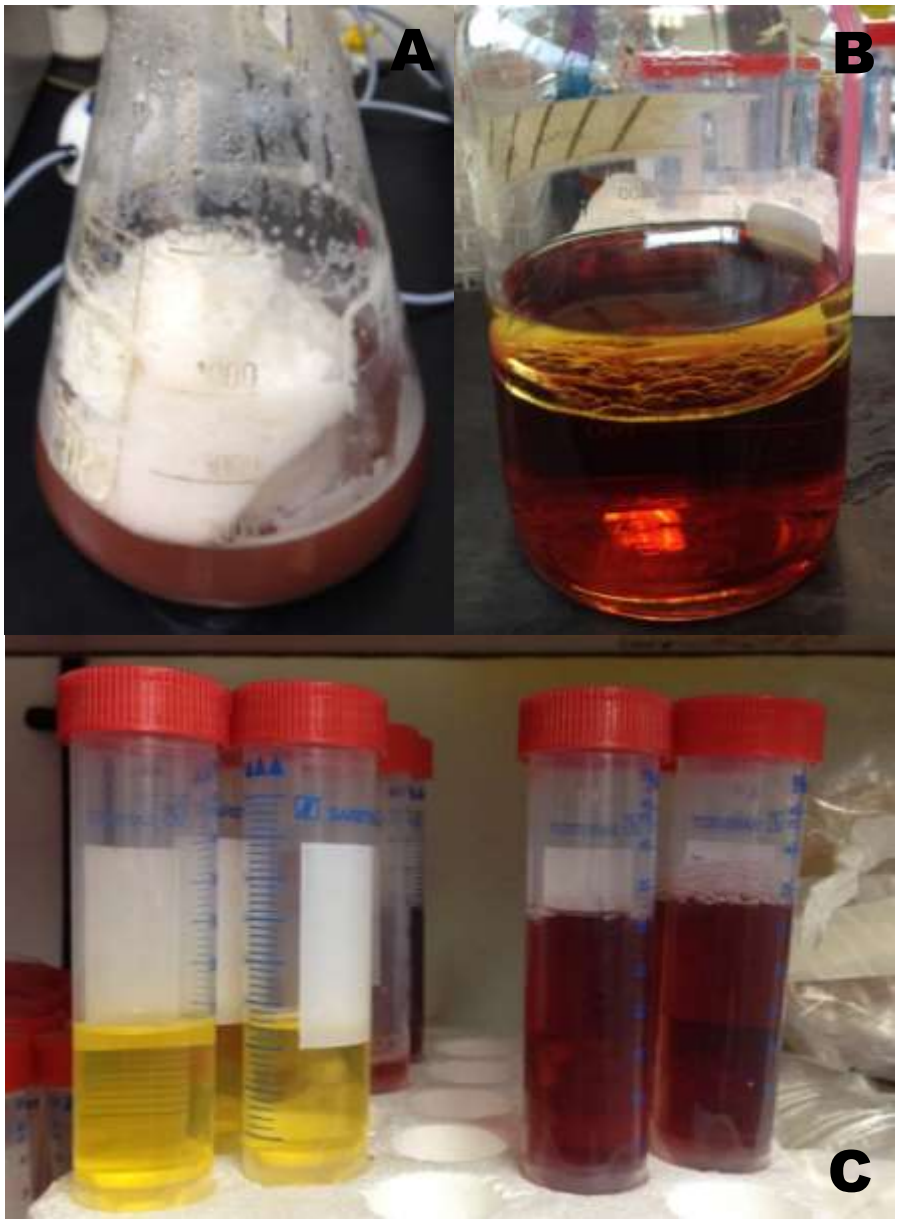


Figure 5.5. Organic extraction of *B. caledonica* 96h culture filtrate.

A. Large scale culturing of *B. caledonica* **B.** Ethyl acetate was added to supernatant in 2:1 ratio. **C.** The solvent layer (yellow) was fractionated using RP-HPLC to obtain the fraction of interest. The tubes on the right contain the aqueous layer.

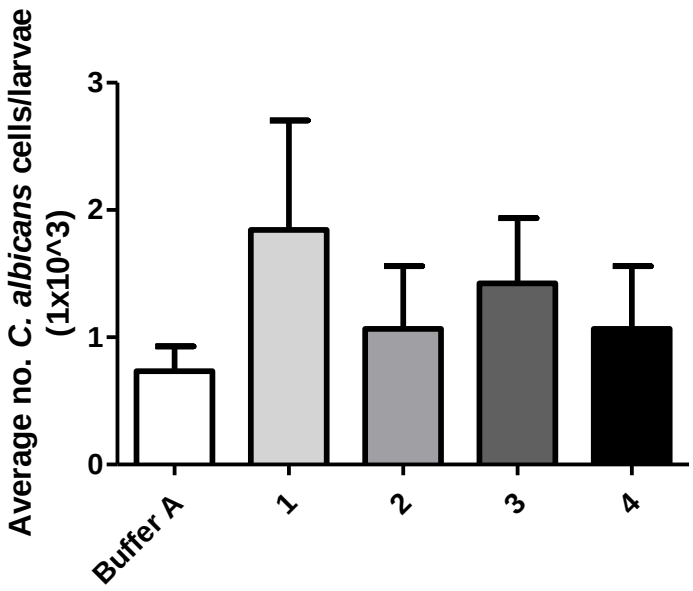


Figure 5.6. Yeast load in *G. mellonella* larvae pre-treated with RP-HPLC fractions 1-4 of *B. caledonica* 96h culture.

Number of *C. albicans* cells per larva after incubation for 24h at 20°C. Larvae were treated with oosporein 24h prior to inoculation with C. *albicans*. RP-HPLC buffer A (0.1% (v/v) trifluoroacetic acid (TFA) in HPLC-grade water and solvent) was used as a control One Way ANOVA showed that pre-treatment with the fractions tested did not significantly increase yeast load in *G. mellonella* larvae.

5.3.3 Identification of red metabolite from *B. caledonica*

Identification of the purified red metabolite of *B. caledonica* was carried out using NMR and Infrared Spectroscopy by Dr. John M. D. Walsh, and Dr. John C. Stephens from the Department of Chemistry, Maynooth University. The purified metabolite from *B. caledonica* appeared as a red/maroon coloured solid with the following characteristics: Melting point of 244-256 °C (lit 250-260 °C)[4], ^{1}H NMR (500 MHz, DMSO) δ 1.81 (s, 1H, CH_3) (Figure 5.7). ^{13}C NMR (126 MHz, DMSO) δ 112.8, 107.4, 7.6 (CH_3) (Figure 5.8). On addition of excess triethylamine the following characteristics were observed; ^{1}H NMR (500 MHz, DMSO & Et_3N) δ 1.67 (s, 6H, CH_3) (Figure 5.9). ^{13}C NMR (126 MHz, DMSO & Et_3N) δ 172.0, 171.2, 108.0, 107.6, 8.01 (CH_3) (Figure 5.10). IR (KBr) 3445 (br s, OH, H-bonded), 3329 (vs, CH), 3309 (vs, CH), 1649 (s), 1624 (s, CO st), 1385 (s), 1301 (s), 1242 (s), 1073 (s) cm^{-1}. HRMS (ESI): m/z calcd. for $C_{14}H_9O_8$: $[M - H]^-$ 305.0303, found 305.0298 [Diff (ppm) = −1.77]. Based on these results the unidentified metabolite was determined to be oosporein (Divekar *et al.* 1959, Cole *et al.* 1974, Nagaoka *et al.* 2004) (Figure 5.11).

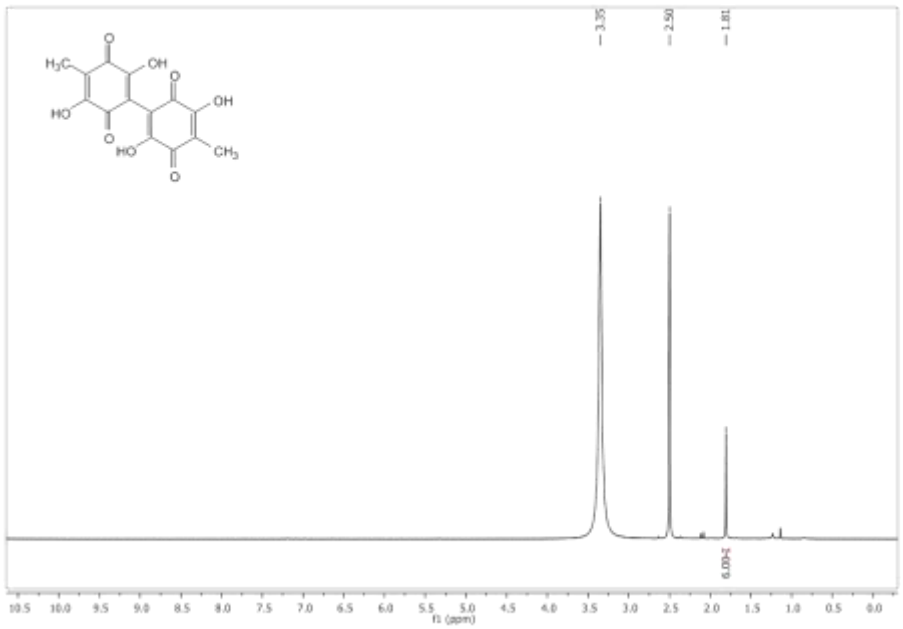


Figure 5.7. ^{1}H NMR spectrum (500 MHz DMSO) of Oosporein

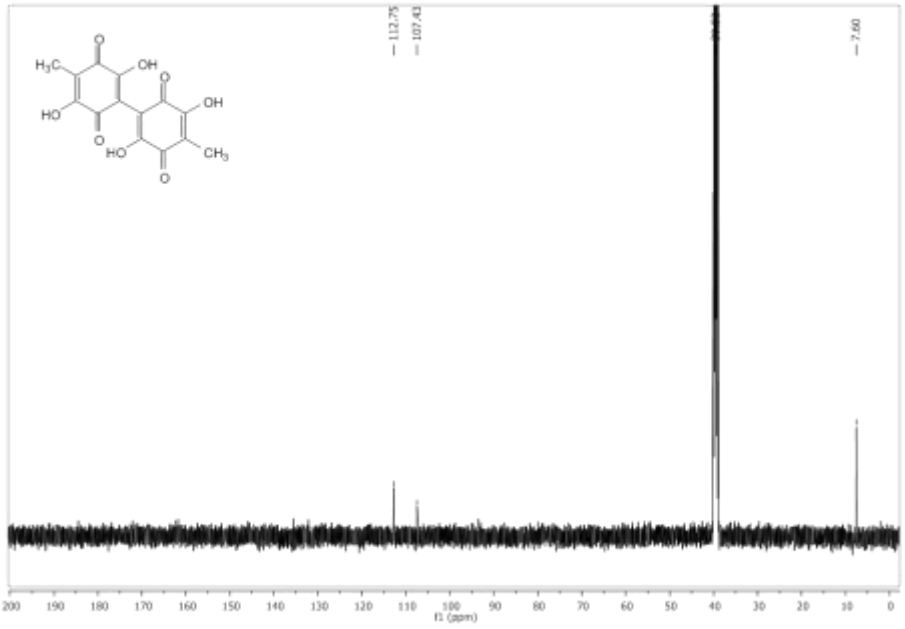


Figure 5.8. ^{13}C NMR spectrum (126 MHz DMSO) of Oosporein

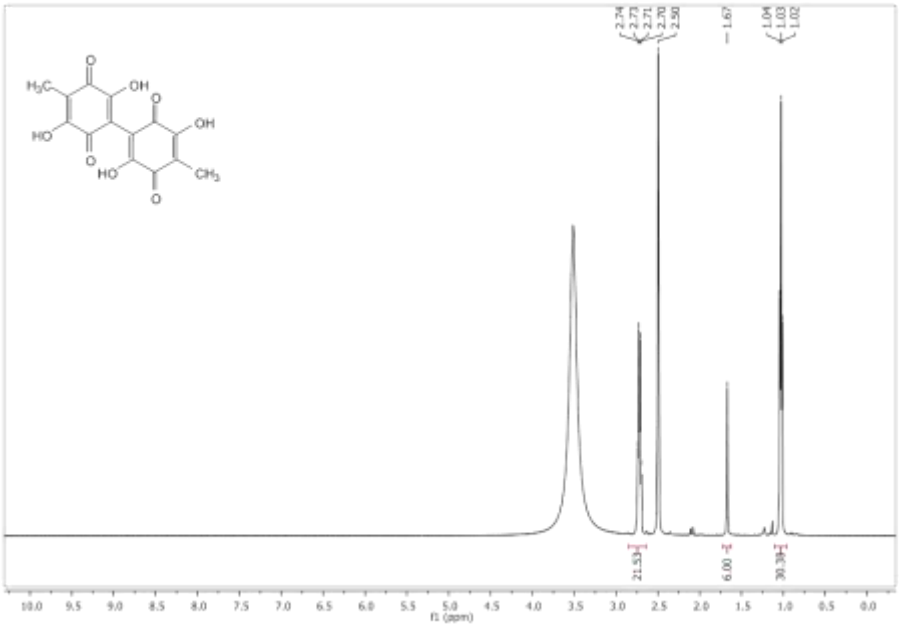


Figure 5.9. ^{1}H NMR spectrum (500 MHz DMSO & Triethylamine) of Oosporein

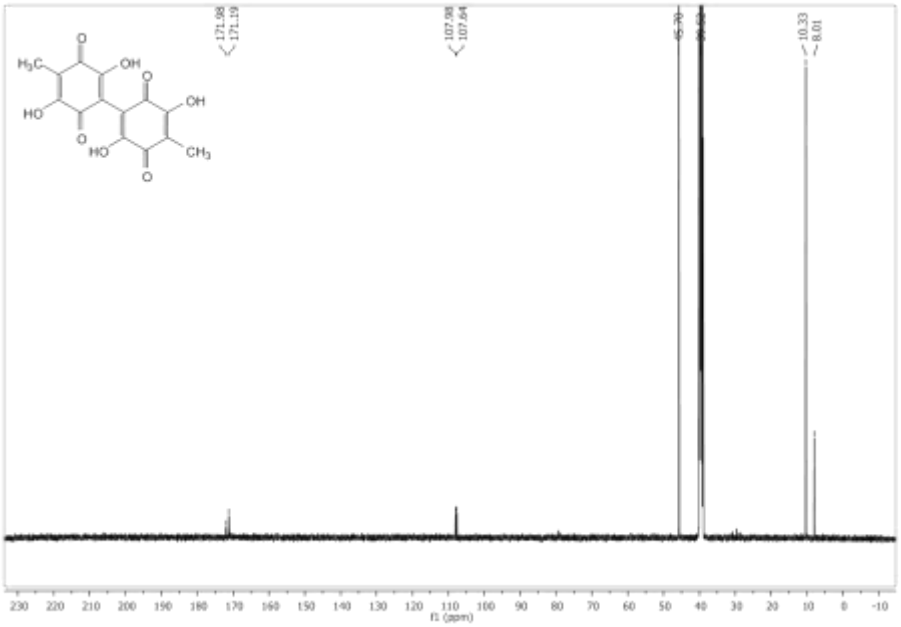


Figure 5.10. ^{13}C NMR spectrum (500 MHz DMSO & Triethylamine) of Oosporein

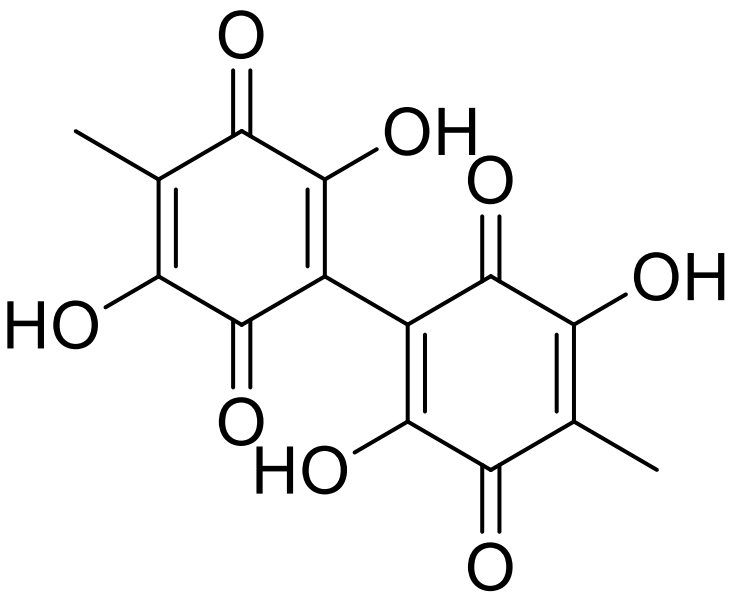


Figure 5.11. 3,3',6,6'-tetrahydroxy-4,4'-dimethyl-[1,1'-bi(cyclohexane)]-3,3',6,6'-tetraene-2,2',5,5'-tetraone (Oosporein)

5.3.4 Uncovering a novel feedback induction mechanism regulating oosporein production in *B. caledonica*.

A number of strategies have been developed in order to enhance microbial natural product production. These include the optimisation of growth media, incubation conditions, co-culture and small molecule elicitation. Considering the potent insecticidal properties of oosporein, strategies to maximize the production and secretion of this compound from *B. caledonica* were investigated.

Cultures were grown for 24 h before a sub-inhibitory concentration (17.5 µg/ml) of oosporein was added (Section 5.2.7). Cultures were grown for 96 h in total before they were filtered and analyzed using RP-HPLC (Figure 5.13). *B. caledonica* spiked with organic extract containing 0.875 mg of oosporein had significantly higher levels of oosporein than media controls spiked with the same concentration (Figure 5.12.A). In order to verify this, both organic extract and purified fractions containing oosporein at the same concentration were spiked into *B. caledonica* cultures (Figure 5.12.C+D). Both treatments resulted in significantly increased levels of oosporein in culture filtrates. This increased oosporein production was visible by eye as dark pink pigmented culture (Figure 5.12 E+F).

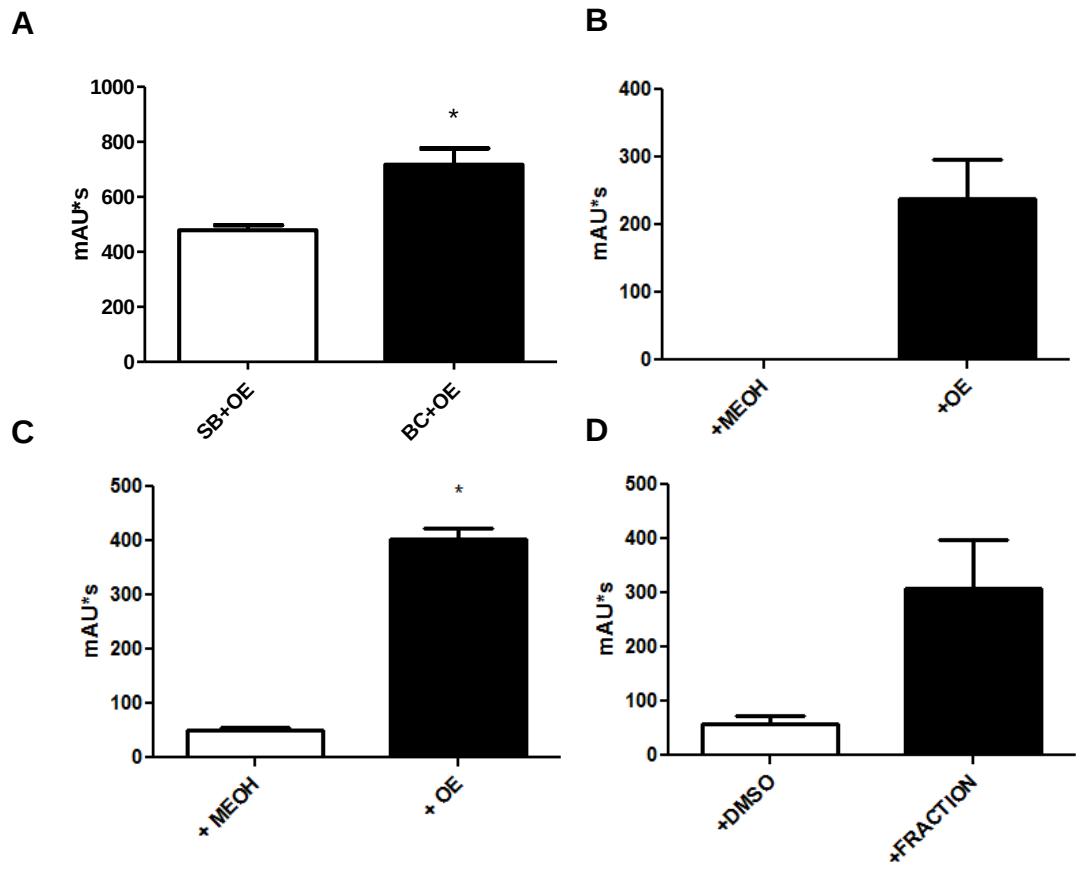


Figure 5.12. Spike assays to investigate the effect of exogenously added oosporein on oosporein biosynthesis in *B. caledonica*.

B. caledonica cultures were grown for 24h before 0.875 mg of Oosporein in 200 µl of solvent was added. Oosporein was either added in DMSO (Fraction) or MEOH (Organic extract, OE) depending on how the oosporein was produced. Cultures were grown for 96h in total before they were filtered and analyzed using HPLC. 40 µl of culture filtrate was injected onto the column. Peak surface area is denoted as milli-Arbitrary Units (mAU*s). Each bioassay was repeated on four occasions. Mann Whitney U tests were carried out to compare treatments, * $p<0.05$. **A.** BC+OE, *B. caledonica* spiked with oosporein; SB+OE media spiked with oosporein. **B**. +MEOH, *B. caledonica* spiked with solvent as a control and +OE *B. caledonica* spiked with oosporein (the average peak surface area for the media control was already subtracted to allow comparison). **C.** The mAU values shown have the

average peak area for the media control subtracted. +OE, *B. caledonica* spiked with oosporein; + MEOH, *B. caledonica* spiked with solvent (control); **D.** +Fraction *B. caledonica* spiked with oosporein; + DMSO, *B. caledonica* spiked with solvent (control). **E.** *B. caledonica* cultures spiked with solvent. **F.** *B. caledonica* cultures spiked with oosporein.

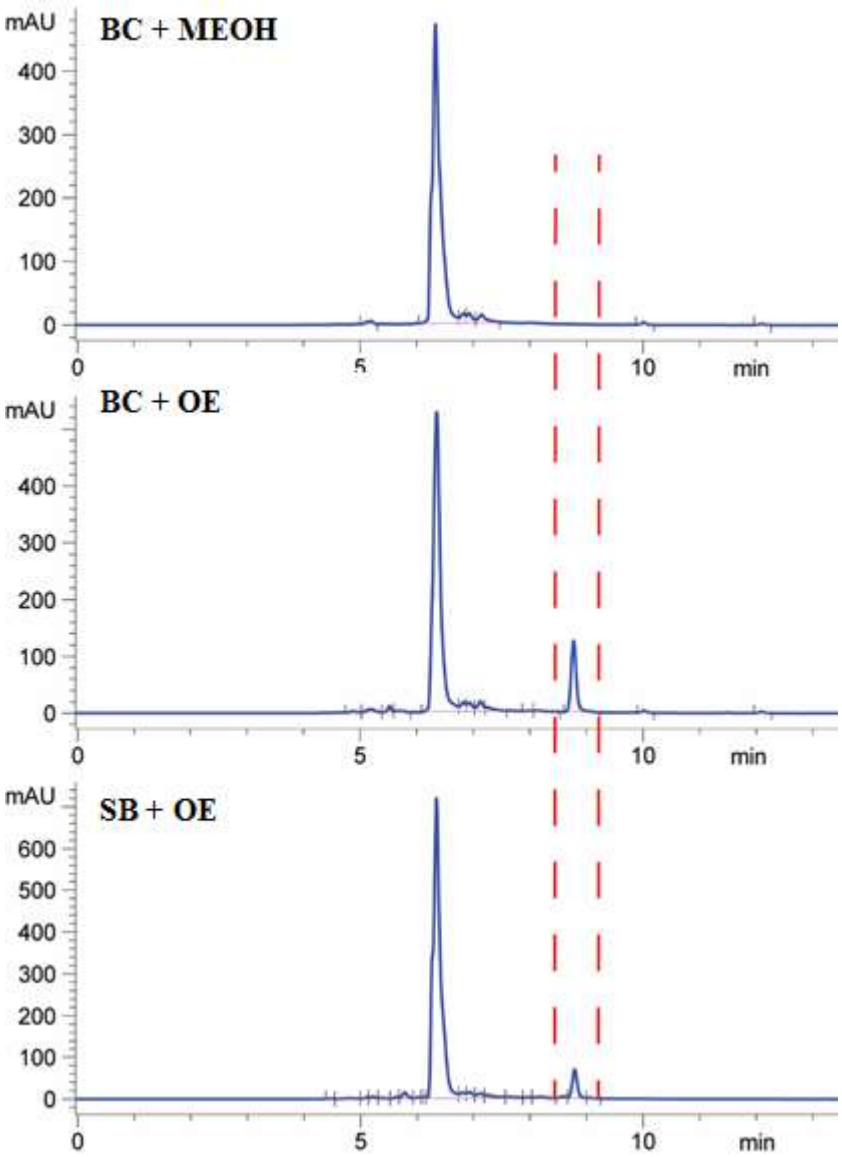


Figure 5.13. RP-HPLC profiles of *B. caledonica* cultures spiked with exogenously added oosporein.

These RP-HPLC profiles represent the results from Figure 5.12 A+B. OE indicates that oosporein was added, MEOH indicates that methanol was added. Red lines indicate retention time of oosporein. A shorter method was used than in Section 5.3.1 and 5.3.2, so oosporein eluted at a retention time of 8.7 minutes rather than 16.6 minutes.

5.3.5 Label Free Quantitative (LFQ) Proteomics of the effect of oosporein on the *B. caledonica* proteome.

Exposure of *B. caledonica* to exogenous oosporein during growth resulted in a dramatic induction of oosporein production in this organism (Figure 5.12 and 5.13). In order to examine this unusual feedback induction mechanism, the proteomic response of *B. caledonica* with and without oosporein exposure was characterized (Section 5.2.9).

The LFQ results presented in this section are preliminary results and are a part of an ongoing collaboration with Prof. Travis Glare (Lincoln University, New Zealand), where they have sequenced the genome of *B. caledonica* and are in the process of annotating the genome. Once complete this genome will enhance the LFQ analysis presented in this Chapter for subsequent publication. Thus the results presented in this section are preliminary and there will not be an in-depth analysis or discussion as they are subject to change. In total 1650 proteins were identified having two or more peptides. Glutathione-s-transferase (GST) was significantly increased in abundance in *B. caledonica* mycelium upon exposure to oosporein (Table 5.1). This protein is encoded by the oosporein cluster (Feng *et al.* 2015), which suggests that exposure to oosporein results in an increase in expression of the oosporein cluster.

Table 5.1. Proteins significantly increased or decreased in abundance in *B. caledonica* mycelia treated with oosporein relative to control mycelia.

Proteins included have a $p<0.05$. The average LFQ intensities of four biological replicates are given.

			Average LFQ intensities	
Protein names	**t-test P value**	**t-test Fold Difference**	**Control**	**Oosporein**
Prenylated Rab acceptor 1 OS=*Beauveria bassiana* (strain ARSEF 2860)	0.001	1.5	29.1	27.6
Cystathionine gamma-synthase OS=*Beauveria bassiana* (strain ARSEF 2860)	0.008	1.4	30.5	29.1
Squalene synthase OS=*Beauveria bassiana* (strain ARSEF 2860)	0.02	1.4	30.6	29.2
Glycerophosphoryl diester phosphodiesterase OS=*Beauveria bassiana*	0.009	1.3	29.6	28.4
Glutathione synthetase OS=*Beauveria bassiana*	0.04	1.2	28.2	27.1
Glutathione-s-transferase OS=*Beauveria bassiana*	0.0008	-1.02	31.8	32.8
Lsm14a protein OS=*Beauveria bassiana*	0.01	-1.1	29.9	30.9
mRNA decapping hydrolase, putative OS=*Beauveria bassiana*	0.007	-1.1	26.6	27.9
Elicitor protein OS=*Beauveria bassiana*	0.006	-1.1	28.5	29.6
RNA recognition domain-containing protein OS=*Beauveria bassiana*	0.001	-1.12	28.0	29.1
Lon protease homolog, mitochondrial OS=*Beauveria bassiana*	0.003	-1.11	27.7	28.8
Memo-like protein OS=*Beauveria bassiana*	0.001	-1.13	26.6	27.7
Ribokinase, bacterial OS=*Beauveria bassiana*	0.02	-1.2	27.7	28.9

Ribosomal L22e family protein OS=*Beauveria bassiana*	0.02	-1.2	32.3	33.5
Actin lateral binding protein OS=*Beauveria bassiana*	0.015	-1.3	30.4	31.7
Eukaryotic translation initiation factor 3 subunit J OS=*Beauveria bassiana*	0.005	-1.3	26.1	27.4
Immunogenic protein OS=*Beauveria bassiana*	0.02	-1.34	29.6	30.9
Ubiquitin family protein OS=*Beauveria bassiana*	0.03	-1.4	27.9	29.3
YCII-related domain protein OS=*Beauveria bassiana*	0.002	-1.5	30.5	32.04
Covalently-linked cell wall protein OS=*Beauveria bassiana*	0.02	-1.6	27.5	29.1
Ribose 5-phosphate isomerase OS=*Beauveria bassiana*	0.04	-1.64	24.6	26.2
ATP synthase delta chain OS=*Beauveria bassiana*	0.0001	-1.7	29.1	30.8
GTPase-activating protein ZNF289 OS=*Beauveria bassiana*	0.0008	-1.7	26.8	28.5
Septation protein SUN4 OS=*Beauveria bassiana*	0.02	-1.8	25.2	27.0
Phospholipase PldA, putative OS=*Beauveria bassiana*	0.02	-2.0	24.2	26.2
Vesicular transport protein OS=*Beauveria bassiana*	0.04	-2.0	24.4	26.4
Peptidase family M48 OS=*Beauveria bassiana*	0.007	-2.0	27.3	29.4
Small nuclear ribonucleoprotein OS=*Beauveria bassiana*	0.04	-2.9	26.1	29.1

5.3.6 The effect of oosporein on haemocyte densities of insect larva

Larvae were injected with oosporein at a concentration of 0.125 µg/20 µl or DMSO (5% v/v) as a control. *G. mellonella* larvae treated with oosporein showed a reduction in haemocyte densities relative to controls at both 24h ($p<0.001$) and 48h ($p<0.01$) (Figure 5.14.A). Treatment ($F_{1,15}=38$, $p<0.001$) and time ($F_{1,15}=53.22$, $p<0.001$) both had a significant effect.

H. abietis larvae treated with oosporein showed a reduction in haemocyte densities at both 24h ($p<0.05$) and 48h ($p<0.01$) (Figure 5.14.B) relative to control larvae. Treatment ($F_{1,15}=16.2$, $p<0.01$) and time ($F_{1,15}=32$, $p<0.001$) both had a significant effect. There was not a significant interaction between treatment and time in either larva.

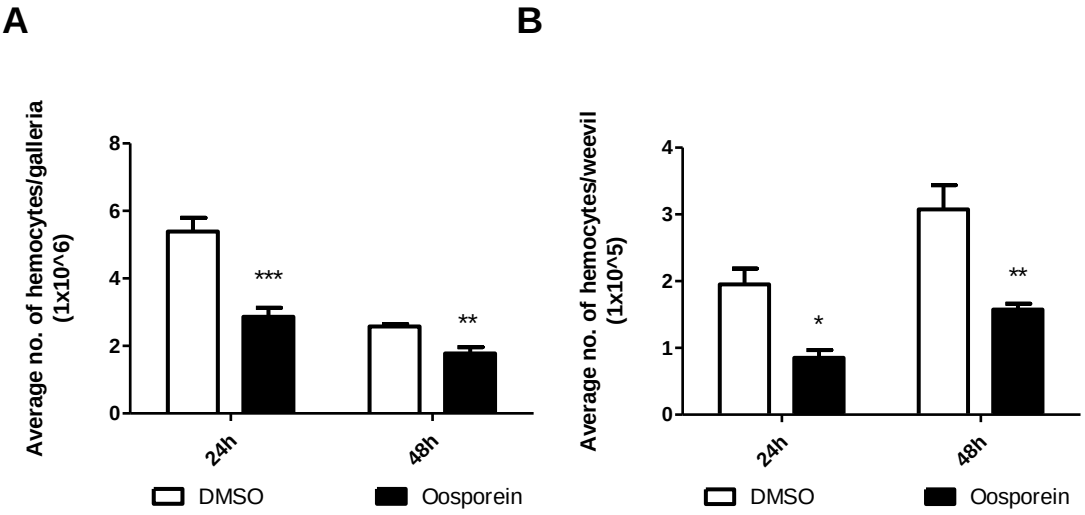


Figure 5.14. Haemocyte densities in larvae treated with oosporein.
Following inoculation with Oosporein or DMSO (Control), larvae were incubated for 24h or 48h at 20°C before bleeding and enumeration **A.** *G. mellonella* larvae. **B**. *H. abietis* larvae. Data that were not normal were log transformed. Two Way ANOVA was carried out with Bonferroni post-tests to compare treatments to relevant controls * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

5.3.7 Determination of oosporein on yeast load in insect larva

In *G. mellonella* larvae treated with oosporein, treatment ($F_{1,11}=18.64$, $p<0.01$) and time ($F_{1,11}=156.6$, $p<0.001$) showed a significant effect. There was no significant interaction between treatment and incubation time of insects. *G. mellonella* larvae treated with oosporein had an increased yeast load ($p<0.05$) relative to control larvae

(Figure 5.15.A), when the larvae were incubated for 48h following injection with *C. albicans*.

In *H. abietis* larvae, treatment ($F_{1,11}$=109.12, p<0.001) showed a significant effect but incubation time of insects did not. There was a significant interaction between treatment and time ($F_{1,11}$=48.05, p<0.001). *H. abietis* larvae treatment with oosporein had an increased yeast load (p<0.001) relative to controls (Figure 5.15.B), when the larvae were incubated for 24h following injection with *C. albicans*.

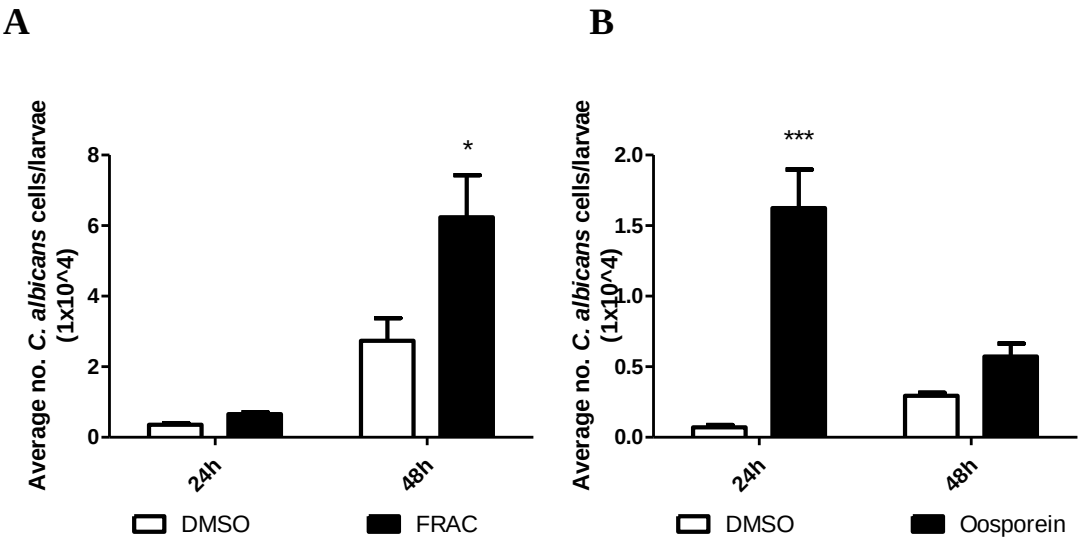


Figure 5.15. Yeast load in larvae pre-treated with oosporein.

Number of *C. albicans* cells per larva after incubation for 24h and 48h at 20°C. Larvae were treated with oosporein 24h prior to injection with C. *albicans*. **A.** *G. mellonella* larvae: treatment with oosporein increased (p<0.05) yeast cell density relative to controls after a 48h incubation. **B.** *H. abietis* larvae**:** treatment with oosporein increased (p<0.05) yeast cell density relative to controls after a 24h incubation. Data was not normal and was log transformed. Two Way ANOVA was carried out with Bonferroni post-tests to compare treatments to relevant controls. * p<0.05, *** p<0.001.

5.3.8 Investigating whether oosporein increases susceptibility of larvae to a subsequent infection

To further investigate whether treatment with oosporein makes larvae more susceptible to subsequent pathogens, larvae were injected with oosporein at a concentration of 0.125 µg/20 µl and incubated for 24h at 20°C. After 24h larvae were inoculated with *C. albicans*. Larvae were incubated at 20°C for one week. *G. mellonella* larvae treated with oosporein and *C. albicans* was the only treatment with mortality but it did not differ significantly to mortality of larvae treated with oosporein alone or a combination of DMSO and *C. albicans* (Figure 5.16.A). *H.*

abietis larvae treated with oosporein and *C. albicans* had a higher mortality compared to larvae treated with oosporein alone or a combination of DMSO and *C. albicans* ($p<0.05$) (Figure 5.16.B). Oosporein increased the susceptibility of *H. abietis* larvae to a subsequent infection.

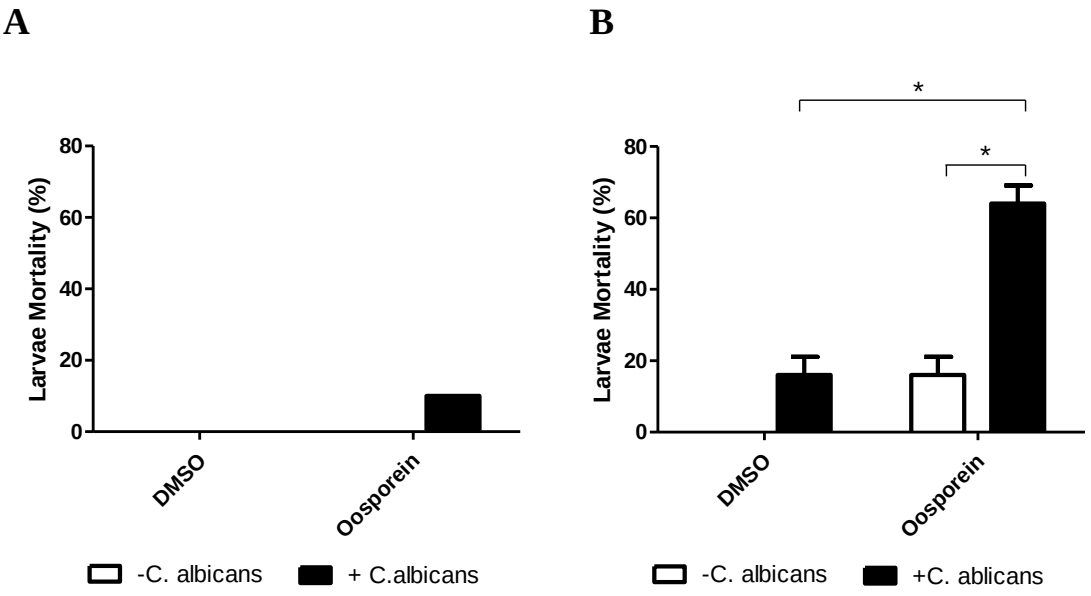


Figure 5.16. Mortality of larvae treated with oosporein alone and in combination with *C. albicans*.

Results shown are larval mortality one week after treatment with *C. albicans*. DMSO (5%) was used as a control. + *C. albicans* indicates larvae that received a dose of *C. albicans* after 24h, - *C. albicans* indicates larvae that did not. Data was not normal, Mann Whitney Tests were used to compare larval mortality between treatments, *$p<0.05$. **A.** *G. mellonella* larvae: there was no significant increase in mortality caused by either oosporein or *C. albicans*. **B.** *H. abietis* larvae: There was a significant difference in mortality between larvae treated with oosporein and *C. albicans* compared to those who only received Oosporein. There was also a significant difference between larvae treated with DMSO and *C. albicans* compared to larvae treated with Oosporein and *C. albicans.*

5.3.9 Label Free Quantitative (LFQ) of the effect of oosporein on the *G. mellonella* proteome.

Label free proteomics was used to compare the haemolymph proteome of *G. mellonella* larvae treated with oosporein at a concentration of 0.125 µg to control larvae treated with DMSO. Principal component analysis (PCA) confirmed a difference between the expression profiles of treated and control larvae (Figure 5.17). In total 288 proteins were identified, 282 having two or more peptides. When these proteins were filtered to contain only proteins found in three replicates of at least one group 142 proteins remained, 27 of these proteins were either significantly changed

in abundance or exclusively detected in one sample group. Three proteins were found to be absent (below the level of detection) in oosporein treated larvae and two proteins were found to be absent in Control larvae (Table 5.4). These proteins were termed 'uniquely detected proteins'. These protein hits were also used in statistical analysis of the total differently expressed sample groups following imputation based on normal distribution. Two proteins were exclusively expressed in oosporein treated *G. mellonella*, an uncharacterized protein and a DEAD-Box RNA helicase. DEAD-Box RNA helicases are a family of proteins that participate in virtually every aspect of RNA metabolism (Cordin *et al.* 2006). Three proteins were below the level of detection in larvae treated with oosporein: fructose-1,6-bisphosphatase, cathepsin B-like cysteine proteinase and serine protease inhibitor 13 precursor. Cathepsin B-like cysteine proteinase is involved in digestive proteolysis by many insects and are often targets of plant defensive cystatins (Koiwa *et al.* 2000). Uniquely detected proteins are also shown before filtering to show only proteins found in three replicates of at least one group (Table 5.2 and 5.3).

A number of proteins identified by LFQ were uncharacterized, hypothetical or unknown and were labelled based on their Contig name. The overall proteomic profile can be seen in Figure 5.18, in a heat map derived from hierarchical clustering following Z-score normalization of LFQ intensities using Perseus software. Proteins with high abundance are represented in red and those with low abundance are represented in blue. In larvae treated with oosporein 19 proteins were significantly ($p<0.05$) increased in abundance at a fold change of >1.5 (Figure 5.19, Table 5.5). Five proteins were significantly ($p<0.05$) decreased in abundance at a fold change of >1.5 (Figure 5.19, Table 5.5). Proteins present on the right hand side are increased in abundance relative to the control. The defensin like AMP Spodoptericin (Seufi *et al.* 2011), is increased in abundance in oosporein treated larvae (Figure 5.19, Table 5.5).

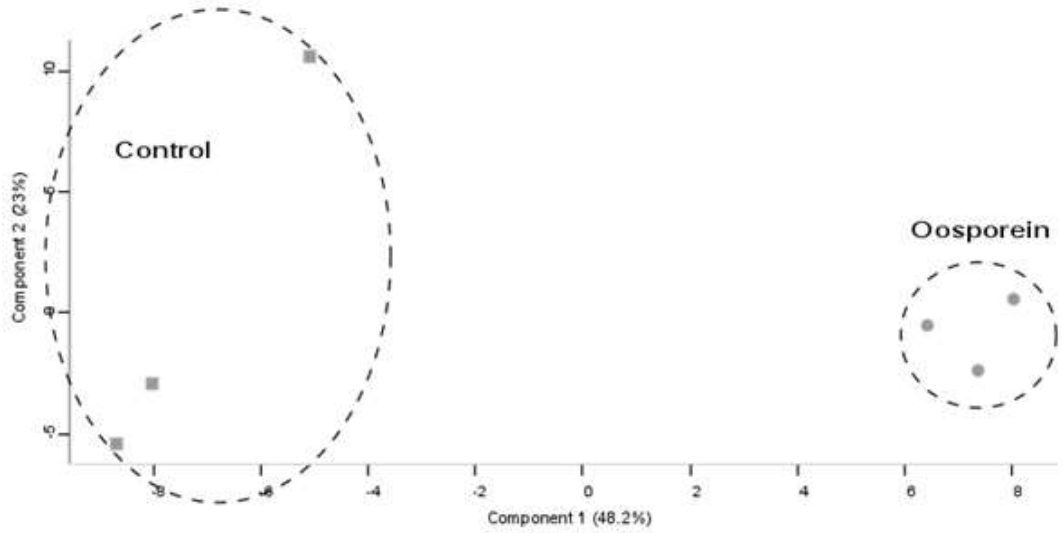


Figure 5.17. Principal component analysis (PCA) of *G. mellonella* haemolymph treated with oosporein versus DMSO control.

Three replicates of each sample group were included in Perseus analysis. Dashed circles denote sample groups.

Table 5.2. Proteins below the level of detection in the hemolymph of control *G. mellonella* larvae.

Proteins were selected based on absence in at least 3 replicates of one sample group. NaN indicates a protein that was absent or below the level of detection. [1]Mean LFQ intensity represents average of three replicates per sample group. [2]PEP: Posterior Error Probabilities.

Protein names	Mean LFQ Intensity						PEP[2]	Intensity	MS/MS	Peptides	Sequence coverage (%)	Mol. Weight (kDa)	Sequence length
	Control[1]			Oosporein[1]									
Thymosin isoform 1	NaN	NaN	NaN	28.1	26.7	NaN	1.9^{-12}	2.9E+08	9	3	25.5	11.9	110
Enolase	NaN	NaN	NaN	27.9	28.1	NaN	2.1^{-62}	5.2E+08	19	6	15.2	63.6	591
Gustatory receptor candidate 25	NaN	NaN	NaN	31.0	NaN	31.0	3.4^{-105}	2.4E+09	33	4	22.1	25.9	235
DEAD-box RNA helicase	NaN	NaN	NaN	29.9	27.93	28.2	6.3^{-88}	6.5E+08	32	8	39	31.9	277
Heat shock protein hsp21.4	NaN	NaN	NaN	28.8	28.3	NaN	5.8^{-39}	5.6E+08	15	5	15	53.4	479

Table 5.3. Proteins below the level of detection in the hemolymph of control *G. mellonella* larvae.

Proteins were selected based on absence in at least 3 replicates of one sample group. NaN indicates a protein that was absent or below the level of detection. . [1]Mean LFQ intensity represents average of three replicates per sample group. [2]PEP: Posterior Error Probabilities.

Protein names	Mean LFQ Intensity						PEP[2]	Intensity	MS/MS	Peptides	Sequence coverage (%)	Mol. Weight (kDa)	Sequence length
	Control[1]			Oosporein[1]									
C-type mannose receptor 2-like	27.7	27.4	NaN	NaN	NaN	NaN	1.2E-19	5.3E+08	22	7	16.7	63.1	557
Dimeric dihydrodiol dehydrogenase	27.5	NaN	28.3	NaN	NaN	NaN	1.3E-29	5.8E+08	15	5	17.5	55.1	497
Apolipophorin-2	29.1	29.0	NaN	NaN	NaN	NaN	1.7E-22	2.3E+09	28	1	13.8	9.8	94
Cathepsin B-like cysteine proteinase	26.0	26.4	25.9	NaN	NaN	NaN	3.2E-23	3.4E+08	18	5	15	46.7	428
Serine protease inhibitor 13 precursor	27.0	27.7	26.7	NaN	NaN	NaN	2.8E-58	8.8E+08	25	6	13.4	49.9	440
Profilin	25.7	25.8	NaN	NaN	NaN	NaN	1.6E-10	1.6E+08	9	3	10.6	35.4	330
Beta-1,3-glucanase	26.9	NaN	26.7	NaN	NaN	NaN	4.5E-45	4.2E+08	14	3	12.9	53.6	482

Contig05588_1	26.4	NaN	26.9	NaN	NaN	NaN	1.5E-50	2.8E+08	12	5	33	32.4	291
Contig04572_1	26.6	NaN	26.5	NaN	NaN	NaN	1.1E-08	3.1E+08	12	4	25.4	22.9	209
Fructose-1,6-bisphosphatase	31.2	30.2	30.1	NaN	NaN	NaN	2.2E-153	6.8E+09	25	3	29.1	8.5	79

Table 5.4. Uniquely detected proteins in the hemolymph of both control *G. mellonella* larvae and larvae treated with oosporein.

Proteins were selected based on abundance in at least 3 replicates of one sample group. NaN indicates a protein that was absent or below the level of detection. These proteins were termed as being 'exclusively expressed'. [1]LFQ intensities are average intensities of three replicates. [2]PEP: Posterior Error Probabilities.

Protein names	Mean LFQ Intensity		PEP[2]	Intensity	MS/MS	Peptides	Sequence coverage (%)	Mol. Weight (kDa)	Sequence length
	Control[1]	Oosporein[1]							
Fructose-1,6-bisphosphatase	30.2	NaN	2.2E-153	6.8E+09	25	3	29.1	8.5	79
Cathepsin B-like cysteine proteinase	26.0	NaN	3.2E-23	3.4E+08	18	5	15	46.7	428
Serine protease inhibitor 13 precursor	27.0	NaN	2.9E-58	8.8E+08	25	6	13.4	49.9	440
DEAD-box RNA helicase	NaN	28.2	6.3E-88	6.5E+08	32	8	39	31.9	277
Contig22050_1	NaN	25.8	7.9E-31	1.2E+08	14	5	13.5	44.4	NaN

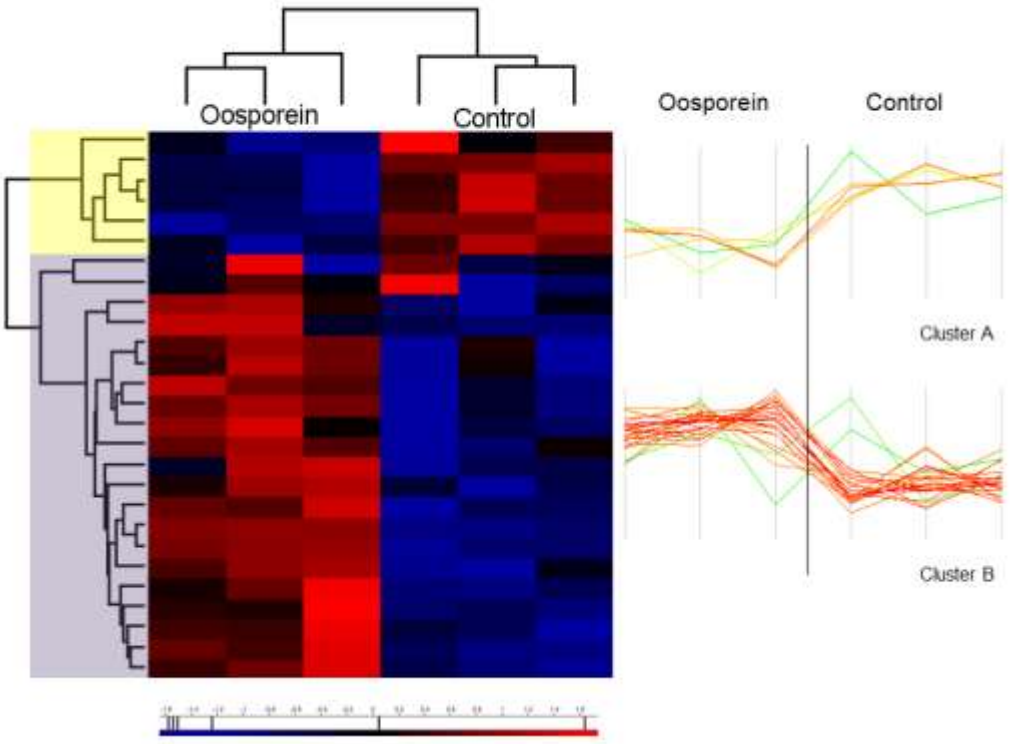


Figure 5.18. Hierarchical clustering of the quantitative differences in the proteomic profile of *G. mellonella* larvae treated with oosporein versus control.

The heat map shows the variation in expression of the proteins from the profiles of *G. mellonella* larvae obtained using Perseus software. The data summarized are for all significantly expressed and exclusive proteins. Hierarchical clustering (columns) resolved two distinct clusters. Cluster A is indicated with light yellow and Cluster B light purple. Red indicates high level of abundance and blue indicates low level of abundance.

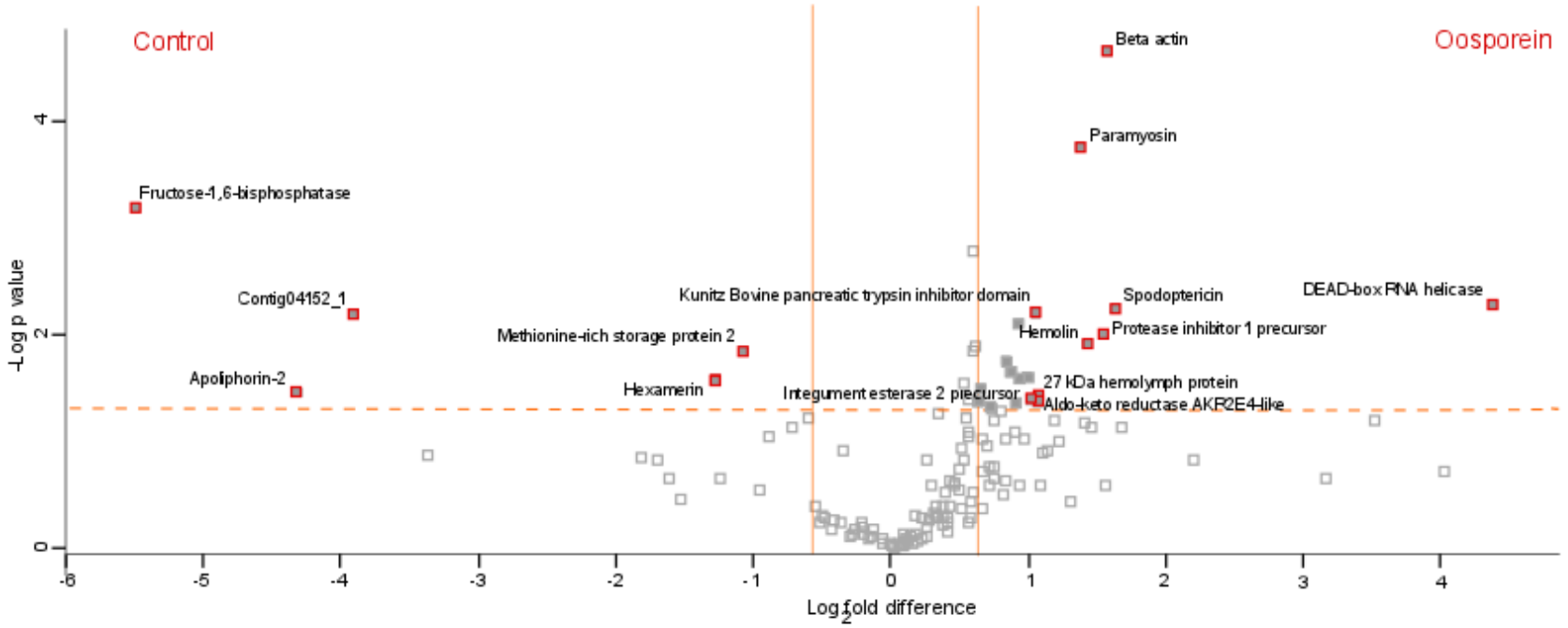


Figure 5.19. Volcano plot highlighting the top proteins present in highest and lowest abundances between *G. mellonella* larvae treated with oosporein and control larvae.

Image obtained using Perseus software. Proteins above the dashed orange line indicates significance ($p<0.05$) and those to the right and left of the vertical lines indicate fold changes greater than 1.5. Filled grey boxes indicate proteins that were significantly changed in abundance that were not in the top 10 changed proteins. Filled grey boxes with red outline are significant and are in the top 10 changed proteins.

Table 5.5. Proteins significantly increased or decreased in abundance in *G. mellonella* larvae treated with oosporein relative to control larvae.

Proteins included have a $p<0.05$ (or a –Log p value above 1.3) and a fold change of above +/-0.58. The average LFQ intensities of three replicates are given.

			Average LFQ intensities	
Protein names	**P value (-Log)**	**Log_2 fold Difference**	**Control**	**Oosporein**
DEAD-box RNA helicase	2.3	4.3	24.3	28.7
Spodoptericin	2.2	1.6	30.2	31.8
Beta actin	4.6	1.5	28.4	29.9
Protease inhibitor 1 precursor	2.0	1.5	28.1	29.6
Hemolin	1.9	1.4	29.4	30.8
Paramyosin, putative	3.8	1.3	27.1	28.4
27 kDa hemolymph protein	1.4	1.0	35.8	36.8
Aldo-keto reductase AKR2E4-like	1.4	1.0	29.8	30.8
Kunitz Bovine pancreatic trypsin inhibitor domain	2.2	1.0	32.2	33.2
Integument esterase 2 precursor	1.4	1.0	29.0	30.0
Arginine kinase	1.6	1.0	29.3	30.3

BCP inhibitor precursor	2.1	0.9	33.3	34.2
Twelve cysteine protein 1	1.6	0.9	30.4	31.3
Carboxylesterase	1.4	0.9	28.4	29.2
Sensory appendage protein 1	1.7	0.8	31.9	32.7
Homeobox 2-like isoform X4	1.8	0.8	33.4	34.2
Contig22104_1	1.3	0.7	31.5	32.2
Kazal-type ase inhibitor	1.5	0.6	31.3	31.9
Cellular retinoic acid binding protein	1.4	0.6	30.3	30.9
Methionine-rich storage protein 2	1.9	-1.1	40.1	39.0
Hexamerin	1.6	-1.3	37.8	36.5
Contig04152_1	2.2	-3.9	29.4	25.4
Apoliphorin-2	1.5	-4.3	33.4	29.0
Fructose-1,6-bisphosphatase	3.2	-5.5	30.5	25.0

5.3.10 Bioassay to investigate if oosporein acts as a contact toxin

H. abietis adults and *G. mellonella* larvae were dipped in oosporein at a concentration of 1.25 mg/ml and 125 µg/ml. DMSO was used as a control (Section 5.2.14). All *G. mellonella* larvae and the majority of *H. abietis* larvae survived for 2 weeks (Figure 5.20.A), with little further mortality occurring after three weeks (Figure 5.20.B). There was no significant difference in mortality caused by treatment for either insect. Thus it appears that oosporein does not act as a contact toxin on *H. abietis* adults and *G. mellonella* larvae at the concentrations tested.

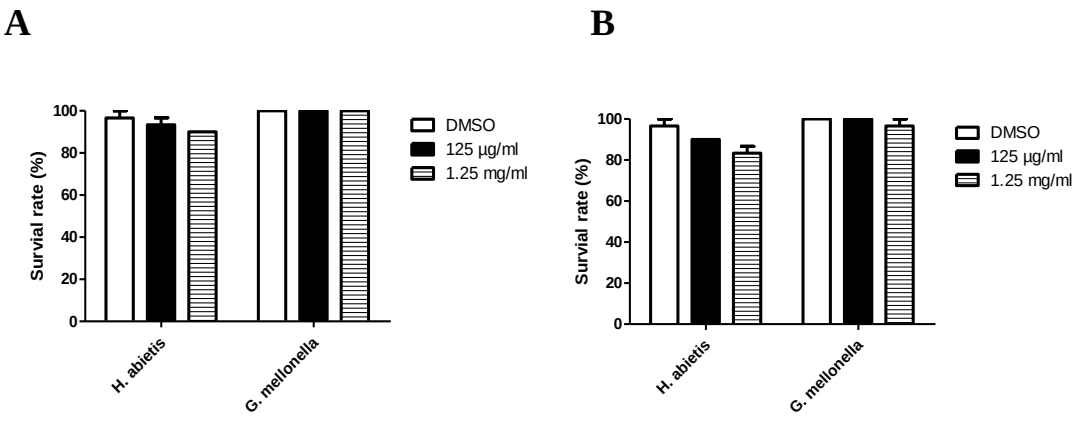


Figure 5.20. Survival rate (%) of *H. abietis* adults and *G. mellonella* larvae following dipping in oosporein.

Larvae were dipped in Oosporein at a concentration of 125 µg/ml or 1.25 mg/ml. DMSO was used as a control. **A.** Survival after incubation for two weeks. B. Survival after incubation for three weeks. Data was not normal and a Kruskal Wallis test was used. There was no significant difference caused by treatment in either insect.

5.3.11 Bioassay to investigate if oosporein acts as a feeding deterrent

After one week of incubation food scores were calculated per insect (Section 5.2.15). A General linearized model (GLM) was used to test for the effect of treatment, experimental run and interaction between treatment and replicate. Treatment was significant ($F_{2,88} = 26.64$, $P<0.01$), experimental run was not significant and there was no interaction between treatment and replicate. Experimental runs were combined and a One-Way ANOVA was used to compare total food eaten (total of four twigs). Overall *H. abietis* adults fed significantly more in tubs that contained oosporein treated twigs (Figure 5.21. A). The most feeding was carried out in tubs containing twigs dipped in 125 µg/ml oosporein. In the tubs

containing oosporein treated twigs, the proportion of total food consumed that were treated with oosporein was assessed. *H. abietis* adults did not feed significantly more or less on twigs treated with oosporein than control twigs, at either concentration (Figure 5.21. B). Oosporein did not display an anti-feedant effect on *H. abietis*. However adults exposed to oosporein treated food fed more overall than those that were only exposed to control food.

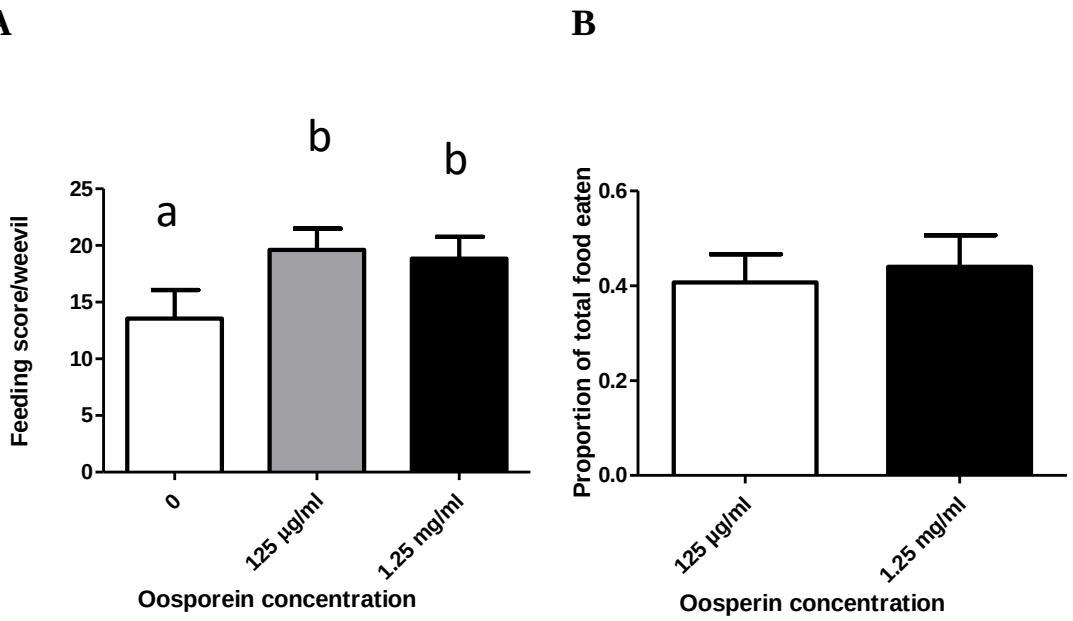


Figure 5.21. The effect of oosporein on *H. abietis* feeding after one week.

A. Total food eaten per tub (total of four twigs) for each treatment. *H. abietis* adults fed more in tubes that contained oosporein treated twigs ($F_{2,88}$=9.06, P<0.001). Tukey's Multiple Comparison test showed that tubes containing 1.25 mg/ml and 125 µg/ml oosporein were significantly different from control tubes but not from each other. **B**. Oosporein treated food: as a proportion of the total food consumed in tubs containing both untreated and oosporein-treated food. Wilcoxon signed rank tests showed that *H. abietis* adults did not feed significantly more or less on twigs treated with oosporein than control twigs, at either concentration.

5.4 Discussion

A wide range of secondary metabolites displaying bioactivity such as insecticidal properties have been identified from *B. bassiana* including the cyclopeptides beauvericin, bassianolide and beauverolide, the yellow pigment pyridines tenellin and bassiatin and the dibenzoquinone oosporein (Xiao *et al.* 2012). A comparative genome survey of the core genes involved in secondary metabolite synthesis in *B. bassiana* found that NPRS, PKS and terpene synthases encoding genes were highly conserved in the four insect pathogens tested (*B. bassiana*, *M. robertsii*, *M. acridium and C. militaris*) but absent in other fungi. This implies that evolution of entomopathogenic fungi may be associated with production of some similar secondary metabolites (Xiao *et al.* 2012). Furthermore RNA-seq transcriptomic analysis demonstrated that *B. bassiana* could sense and adapt to different environments, such as the insect cuticle, haemocoel and plant root exudates by modulating genes involved in signal transduction, secreted proteins and metabolism (Xiao *et al.* 2012).

B. caledonica was chosen for investigation into the production of metabolites that display bioactivity towards insects by using a combined proteomic, metabolomic and immunological approach. To date there has been limited research and publications surrounding *B. caledonica* despite its potential biocontrol and biotechnological applications so it was an ideal candidate.

The unidentified metabolite from *B. caledonica* appeared as an orange/red semi crystalline solid after purification by HPLC. IR and UV data correlated closely with that in literature reports (Divekar *et al.* 1959, Cole *et al.* 1974, Nagaoka *et al.* 2004). The ^{1}H NMR spectrum of the solid in DMSO showed one signal as a singlet at 1.81 ppm which represented the terminal methyl protons. The ^{13}C NMR spectrum showed the corresponding signal for the methyl group at 7.6 ppm. In addition, two signals representing quaternary carbons were observed at 112.8 and 107.4 ppm. On addition of excess triethylamine, as previously reported by Love *et al*. (2009), resolution of the spectrum was achieved with signals resonating at 172.0, 171.2 and also 108.0 and 107.6 ppm. A color change was also observed on addition of the base, with the orange solution turning a dark violet hue. Color changes of this description have been previously reported (Seger *et al.* 2005). Mass spectrometry confirmed the

presence of the oosporein anion. Thus the abundant metabolite produced in *B. caledonica* 96h culture filtrates was identified as oosporein.

Oosporein, the red symmetrical 1,4-bibenzoquinone derivative was first identified from *B. bassiana* (Vining *et al.* 1962, El Basyouni and Vining 1966, Basyouni *et al.* 1968). It is produced by the insect pathogen *B. brongniartii* (Seger *et al.* 2005) as well as several different plant pathogenic and endophytic fungi (Cole *et al.* 1974, Wainwright *et al.* 1986, Nagaoka *et al.* 2004, Alurappa *et al.* 2014). Oosporein exhibits insecticidal activity (Amin *et al.* 2010), antibiotic activity towards Gram-positive, but not Gram-negative, bacteria and antiviral activity against herpes simplex (Terry *et al.* 1992, Feng *et al.* 2015). Oosporein is linked with avian gout in chickens and turkey (Pegram *et al.* 1982), which raises safety concerns for the application of oosporein producing EPF to control insect pests (Feng *et al.* 2015).

5.4.1 The effect of oosporein on its own regulation in *B. caledonica*

In fungi oosporein is synthesized by a polyketide synthase (PKS) pathway including seven genes for quinone biosynthesis (Feng *et al.* 2015). The PKS oosporein synthase 1 (OpS1) produces orsellinic acid that is hydroxylated to benzenetriol by the hydroxylase OpS4. The intermediate is oxidized either non-enzymatically to 5,5′-dideoxy-oosporein or enzymatically to benzenetetrol by the putative dioxygenase OpS7. The latter is further dimerized to oosporein by the catalase OpS5. The transcription factor OpS3 regulates intra-pathway gene expression (Feng *et al.* 2015). The addition of oosporein to *B. caledonica* cultures significantly increased levels of oosporein relative to controls (Figure 5.12), interesting Feng *et al*. (2015) found that the addition of orsellinic acid (OA) to culture that did not produce oosporein due to a loss of OpS1, restored the ability of *Beauvaria* to produce oosporein. To investigate the mechanism underlying the induction of oosporein production as seen in Section 5.3.4, the proteomic response of *B. caledonica* to exogenously added oosporein was characterized (Section 5.2.9).

The LFQ results presented in this section are preliminary results and are a part of an ongoing collaboration with Prof. Travis Glare (Lincoln University, New Zealand), where they have sequenced the genome of *B. caledonica* and are in the process of annotating the genome. Once complete this genome will enhance the LFQ analysis presented in this Chapter for subsequent publication. Thus the results presented in this section are preliminary, as the sequence coverage for the protein identities are relatively low due to a *B. bassiana* genome database being used (Xiao *et al.* 2012).

Glutathione-s-transferase (GST) was significantly increased in abundance in *B. caledonica* mycelium upon exposure to oosporein (Table 5.1). This protein is encoded by the oosporein cluster (Feng *et al.* 2015), which suggests that exposure to oosporein results in an increase in expression of the oosporein cluster. Feng *et al.* (2015) showed that disruption of the gene encoding for GST, lead to oosporein being undetectable in culture extracts. The GST family includes a diverse range of detoxification enzymes best known for their ability to form glutathione conjugates with xenobiotics and leucotrienes and to harness reactive oxygen species and radicals. In gliotoxin biosynthesis, a specialised GST has a role in enzymatic sulfurization (Scharf *et al.* 2011).

These results indicate a direct link between oosporein exposure and induction of oosporein cluster expression. It is expected that planned future analysis of the LFQ data with the *B. caledonica* genome will allow a greater understanding of the effect exogenously added oosporein has on the proteome of *B. caledonica*. However, for now these results are interpreted as an indication that exposure to oosporein effect expression of the oosporein cluster. Further conclusions will not be drawn as these results and their interpretation are subject to change.

5.4.2 Oosporein bioactivity towards insects: immunomodulation and feeding

Oosporein was tested for bioactivity against *H. abietis* and *G. mellonella*. *H. abietis* is a natural host of *B. caledonica* and a significant forestry pest. G. *mellonella* is a well-tested bioassay-organism, that is highly sensitive to a number of secondary metabolites synthesized by EPF (Abendstein *et al.* 2001). In the insect bioassays carried out oosporein was used at a final concentration of 0.125 µg as that was the concentration of oosporein retrospectively calculated in the *B. caledonica* 96 h supernatant treatment used in bioassay and proteomic studies against *G. mellonella* and *H. abietis* in Chapters Two and Three. By ensuring that the same concentration of oosporein was used as detected in *B. caledonica* culture filtrates it allowed for direct comparisons to be drawn between the effects that the full *B. caledonica* supernatant had on the insect immune system (Chapters Two and Three) versus oosporein used alone.

G. mellonella and *H. abietis* larvae treated with oosporein had a significant reduction in haemocyte densities at both 24 h and 48 h relative to control larvae. Oosporein is known to contribute to the evasion of the insect host immune system, strains producing oosporein germinate and escape from haemocyte encapsulation

faster than strains that have lost their ability to produce the toxin (Feng *et al.* 2015). Both *G. mellonella* and *H. abietis* larvae treated with oosporein had significantly increased yeast cell density relative to controls (Figure 5.15). Similar patterns of immunemodulation are seen in both insects following treatment with *B. caledonica* 96h in Chapter Two and Three.

H. abietis larvae treated with oosporein and *C. albicans* had a significantly higher mortality compared to larvae treated with oosporein alone or a combination of DMSO and *C. albicans* (Figure 5.16). Thus oosporein increased the susceptibility of *H. abietis* larvae to a subsequent infection. However, this was not the case for *G. mellonella* larvae (Figure 5.16). In contrast pre-treatment with *B. caledonica* 96h supernatant (containing the same concentration of oosporein) increased the susceptibility of both insects to a subsequent infection in Chapters Two and Three. The ability of red pigmented metabolite produced by a *Beauvaria* species to increase susceptibility to an additional challenge has been reported in the whitefly, *Bemisia tabaci*. Insects treated with *B. bassiana* spores alone had a mortality of 60%, those treated with a red pigment had a mortality of 18%. The greatest mortality was obtained by combining spores and red pigment which achieved a mortality of 92% (Amin *et al.* 2010). They suggested this synergistic action may be as a result of antibacterial activity of the pigment acting as an antagonist of intestinal bacteria in the insect, allowing spores to germinate and proliferate, causing a higher and faster lethal effect on the insect pest (Amin *et al.* 2010). The red pigment used was detected at 96 h in *B. bassiana* cultures and extracted with ethyl acetate, as was oosporein from *B. caledonica* in this Chapter. Together this natural product and *B. caledonica* fungal spores offer great potential for biocontrol, not just in terms of mortality to pests but the antibacterial activity of the metabolite could provide protection to the spore formulation in storage and in the soil upon application (Amin *et al.* 2010).

Following LFQ of changes in the proteome of insect haemolymph treated with oosporein there were a number of proteins significantly altered in abundance, many with immune related functionality. DEAD-Box RNA helicase was ‘uniquely detected’ in *G. mellonella* treated with oosporein. DEAD-Box is the largest family of helicases and they participate in the regulation of virtually all RNA processes from transcription to degradation (Cordin *et al.* 2006). DEAD-box helicases are important in viral defense, with some necessary for sensing presence of viral RNA in infected cells and triggering induction of gene associated antiviral activity (Deddouche *et al.*

2008). Treatment with parasitoid venom suppressed expression of genes related to transcription and translation such as DEAD-box RNA helicases in the lepidopteran *Pieris rapae*. However expression of genes involved in transcription and translation are usually upregulated by immune challenge in insect haemocytes (Fang *et al.* 2010). Treatment with oosporein affects the proPO pathway: a serine proteinase inhibitor was below the level of detection in oosporein treated larvae. Insect melanisation is controlled by a serine protease cascade that ultimately activates the enzyme PPO. Inactive PPO is cleaved into active PO by a serine protease PPAE, PPAE also must be activated by other serine proteases (De Gregorio *et al.* 2002). Proteinase inhibitors can be present constitutively and may also be produced in response to infection or wounds. Serine proteinase inhibitors are reversible inhibitors, key modulators of the proPO cascade and deactivators of microbe derived proteases (Butt *et al.* 2016). Thus the decreased abundance of a serine proteinase inhibitor below the level of detection may be an indication that oosporein is modulating the immune response of larvae as insects use protease inhibitors as a safety mechanism to regulate PO activation (Butt *et al.* 2016). The proPO activation cascade is key to the insect immune response and links many humoral and cellular defense responses such as melanisation, healing, cytotoxic reactions, phagocytosis, encapsulation, nodulation and sclerolization (Marmaras and Lampropoulou 2009). Complementary to these findings injection of oosporein is believed to inhibit PPO activity in *G. mellonella*: Feng *et al*. (2015) found that oosporein blocked PPO cleavage, cleavage of PPO to PO is the hallmark of PPO activation. They also found that oosporein inhibition of PPO activity had a dose dependent trend. Inhibiting PPO activity may contribute to fungal virulence by weakening the immune response subsequently facilitating fungal multiplication within the host (Feng *et al.* 2015). This proposed weakening of the host immune response leaving the insect more susceptible to infection is reflected in the effect of oosporein on yeast load (Figure 5.15) and survival (Figure 5.16). Larvae of both *H. abietis* and *G. mellonella* injected with oosporein had significantly increased yeast cell density relative to controls (Figure 5.15). *H. abietis* larvae injected with oosporein had significantly higher mortality to a subsequent infection of *C. albicans* than control larvae (Figure 5.16 B).

Oosporein has been shown to contribute to fungal virulence in *G. mellonella* as fungal mycosis of insect cadavers is considerably slower in strains that have lost oosporein producing ability (Feng *et al.* 2015). Additionally strains that that did not

produce oosporein due to a loss of OpS1did not turn cadavers the characteristic pink colour seen in wild type strains.

LFQ analysis revealed that oosporein treatment altered abundance of two proteins involved in nutrition: Cathepsin B-like cysteine proteinase (Table 5.3 and 5.4) which is involved in digestive proteolysis by many insects and are often targets of plant defensive cystatins (Koiwa et al. 2000) and a gustatory receptor (Table 5.2). Thus the effect of oosporein on feeding was investigated. Oosporein did not display an anti-feedant effect on *H. abietis*. Oosporein was found not to act as a contact toxin on *H. abietis* adults and *G. mellonella* larvae at the concentrations tested. Oosporein has previously been shown to have no insecticidal, antifeedant or growth inhibitory effects when ingested by lepidopteran larvae of *Capua reticulana*, *Cydia pomonella* and *Mamestra brassicae* (Abendstein *et al.* 2003). When *M. brassicae* larvae feed on food treated with oosporein (0.46 mg) it demonstrated no acute toxicity. In choice bioassays, insects showed no preference between treated (0.325mg oosporein for *M. brassicae* and 0.195 mg for *C. reticulana* and *C. pomonella)* and untreated food. Abendstein (2003) suggest that in relation to concern over the increase in toxic metabolites in the environment due to the use of EPF in biocontrol, their results indicate that oosporein poses no marked risks to insect populations in the soil. Another study investigated whether oosporein exhibits antifeedant, repellant or insecticidal activity against the cockchafer *Melolontha melolontha* and *G. mellonella* larvae. A choice assay with a range of oosporein concentrations (1, 2 and 4 mg/ml) found that oosporein had no repellant or antifeedant effect on *M. melolontha* and it was not toxic when consumed orally (Abendstein *et al.* 2001). More *G. mellonella* were located in control medium and oosporein treated food was less consumed than untreated food. Metamorphosis was unaffected in both insects tested. Thus it is suggested that *M. melolontha* infected by *B. brongniartii,* despite its production of oosporein, are not killed due to toxic properties of the metabolite. It is suggested that alternatively oosporein functions in inhibition of insect microflora, signal transduction during pathogenesis and synergism with other virulence factors (Abendstein *et al.* 2001).

While oosporein did not exhibit anti-feedant or toxic properties, *H. abietis* adults exposed to oosporein treated food fed more overall than those which were only exposed to control food. Exposure to oosporein stimulated feeding: this correlates with the increased abundance in a gustatory receptor following oosporein treatment

(Table 5.2). Perhaps this is a reflection on the energy demands on the immune response to oosporein. Essential immune processes such as prophenoloxidase cascade are energy dependent and thus can be limited by food resources (González-Santoyo and Córdoba-Aguilar 2012). While oosporein is not toxic at the concentrations tested and the adults did not die, bioassays indicate that it modulates the immune system of *H. abietis* making it more susceptible to subsequent pathogens. The increase in food consumption may be linked with an altered immune response; a future recommendation would be to follow this with a treatment of EPN or EPF to see if it leaves the insect more susceptible. These finding link in with findings in Chapter Three that injection with *B. caledonica* supernatant caused an increase in abundance of proteins involved in cellulose cleavage in *H. abietis* larvae, this link is discussed in Section 3.4.8.

5.4.3 Conclusion

Oosporein extracted from *B. caledonica* cultures exhibits immunomodulation properties that may have potential in pest control as it may aid faster and greater mortality in combination with other treatments. Its antibacterial properties may confer protection to fungal spores in storage and upon application in the soil. High yields of oosporein would be desirable if it was to be applied in combination with spores and results indicating that addition of oosporein promotes its own production in culture has implications for optimizing its yields through fermentation. Similarly it may also point to an avenue for improving strains for biocontrol by increasing production of oosporein. Its lack of activity as a contact toxin or toxin through consumption reduces concerns over increased levels of toxic metabolites in the environment impacting on non-target organisms; however this warrants greater investigation with a larger number of target insects to confirm these findings. Weevils exposed to food treated with oosporein over all consumed more, suggesting an altered immune response with high energy demands. An investigation into insect susceptibility to immune challenges following oral consumption of oosporein is warranted due to the known antibacterial properties of oosporein which may hinder the insect gut microflora.

Consistent with work carried out by Feng *et al*. (2015), it appears that oosporein promotes infection rather than directly killing insects. This is the first report of oosporein production by *B. caledonica* and cumulatively these results are a strong

case for future research into the potential use of *B. caledonica* as a biocontrol agent through combinations with oosporein or with enhanced production of oosporein.

Chapter 6:

General Discussion

Hylobius abietis, the large pine weevil, is a major pest of reforestation in Europe. It is estimated that *H. abietis* costs the forestry industry approximately €140 million/year. Without effective controls, these weevils could destroy 100% of newly planted trees. In the UK and Ireland approximately 50% of newly planted trees die within the first few years on untreated sites (Evans *et al.* 2015). Current control measures rely heavily on the synthetic chemical cypermethrin. However, due to concerns over its environmental impact cypermethrin is being phased out across Europe (Williams *et al.* 2013). There is an interest in the use of entomopathogens as biological control agents in integrated pest management as they are safer for applicators and the environment compared to their chemical counterparts (Lacey *et al.* 2015). Additionally their specificity reduces impacts on beneficial and non-target organisms, which promotes biodiversity and natural control of pests (Lacey *et al.* 2015). Both *B. bassiana* and *M. anisopliae* have been proven to be effective biological control agents against a range of pests and are commercially produced (Shah and Pell 2003, Lacey *et al.* 2015). The use of EPF against *H. abietis* in the field has limitations for the approach used in this thesis where spores are applied to stumps above ground level as EPF must by carried passively and/or by phoresis to insect hosts. The justification for investigating EPF as a biocontrol agent against *H. abietis* came from very promising preliminary field trials carried out by a research group in Swansea University where *M. anisopliae* greatly reduced *H. abietis* emergence and the most consistent reduction in weevil numbers came from the combined use of *S. carpocapsae* and *M. anisopliae* (M. Ansari and T. Butt, personal communication)(Evans *et al.* 2015).

Control agents may be combined in order to make it economical for growers to use them. Combining treatments may result in additive, antagonistic or synergistic interactions. Synergy can be defined as the interaction of two or more organisms, substances or agents to produce a combined effect greater than the sum of their individual effects. If a combination of treatments resulted in a synergistic interaction then success rate may be increased and the potential cost of using these biopesticides may be reduced. Ansari *et al.* (2004) hypothesised that synergistic control of *Hoplia philanthus* that occurred when *M. anisopliae* was combined with EPN may be due to the EPF acting as a stressor, increasing long-term control of *H. philanthus* by EPN. A stressor is any stimulus that disrupts the normal homeostasis of an organism.

Furthermore, it was suggested that an explanation for this synergistic interaction may be the weakening of insects by a fungal infection so that their ability to feed or utilize food normally is compromised, resulting in their reduced activity. This weakening causes lethargy that in turn facilitates attachment and penetration by EPN (Ansari *et al.* 2004, Ansari *et al.* 2006). Alternatively it has been suggested that the symbiotic bacteria released by EPN suppress the host immune response, producing proteases that target AMPs, thus making it easier for EPF to colonise the host (Butt *et al.* 2016).

Thus the objectives of this work were to assess the efficacy of EPF, EPN and EPF-EPN combinations for *H. abietis* suppression in the field in order to ultimately determine if the treatments used exert synergistic control over *H. abietis* in the field. To understand how this putative synergistic interaction between control agents could mechanistically occur, the effect of EPF supernatant were tested on the cellular and humoral defences of *H. abietis* larvae to screen isolates for immunomodulation properties. As collection of *H. abietis* larvae is labour intensive and time consuming, and numbers vary with location and season, screening large numbers of EPF isolates on *H. abietis* may prove impractical. Therefore the potential of *G. mellonella* larvae as a model for the study of the immune response of *H. abietis* to pathogens was also explored. Lastly, based on the findings from Chapters Two-Four, *B. caledonica* emerged as an EPF of interest, based on its immunomodulation capabilities and infectivity in the field. Currently there are limited reports on *B. caledonica*, its natural products and effect on insects, in comparison to the well characterised EPF *B. bassiana* and *M. anisopliae*. Thus the final aim of this work was to investigate if the native fungus *B. caledonica* produces immunomodulation compounds active against *H. abietis*.

In the immune investigations described in Chapters Two and Three, spore-free EPF supernatant was used instead of conidia or blastopores, to investigate the effect of their natural products on the immune response. EPF secrete a wide range of natural products, including bassianin, tenellin and oosporein in *Beauveria* species and cyclosporine and 39 varieties of destruxin in *Metarhizium* species. While the natural products produced by *M. anisopliae* and *B. bassiana* are well characterised this is not the case for *B. caledonica*. Some of the natural products produced by EPF have been associated with virulence and specificity. Some are immune modulators that are involved in suppressing the host immune defences. Modulating the immune

response is critical; it allows the fungus to colonise the host, generate biomass and produce conidia needed for dispersal. Suppressing the host immune response means the fungus can invest its energy in growth rather than fighting the insect immune defences (Butt *et al.* 2016). EPF are a source of insecticidal proteins and natural products, which could be utilised for the development of new natural insecticides and biocontrol agents (Degenkolb and Vilcinskas 2015). The crude soluble protein extracts from liquid cultures of fungal isolates belonging to the species *M. anisopliae*, *B. bassiana*, *B. brongniartii* and *Scopulariopsis brevicaulis* were screened for toxicity and some proteins were found to have oral toxicity and anti-feedant activity on larvae of the African cotton leafworm, *Spodoptera littoralis*. (Quesada-Moraga *et al.* 2006). Thus there is an interest in the effects of natural products of EPF as well as the whole organism in a biocontrol setting.

There is an interest in the use of *G. mellonella* as a model to study host-pathogen interactions (Vilcinskas 2010). EPF produce a defined spectrum of molecules considered to be virulence factors such as proteolytic enzymes and natural products which have been identified as fungal toxins. The antifungal immunity of *G. mellonella* has been intensively studied resulting in the identification of defence molecules (e.g. AMPs) that directly kill parasitic fungi, as well as inhibitors of fungal proteinases and proteins that detoxify fungal toxins, which act to inactivate fungal virulence factors (Vilcinskas 2010). In Chapters Two and Three comparable results were observed for *G. mellonella* and *H. abietis* larvae in bioassays testing for immunomodulation capabilities of *M. anisopliae* and *B. caledonica* supernatant (discussed in Section 3.4.9). The major difference in bioassay results between the two insects was that *B. bassiana* supernatant modulated the insect immune response making it more susceptible to subsequent infections when injected into *H. abietis* but this was not seen in *G. mellonella*. This is indicative of the fact that EPF species and even isolates within a species can act very differently in terms of their host range and infectivity (Shah and Pell 2003). This is a limitation of using model organisms to study the efficacy of biological control agents in the laboratory: species specific variability may result in potentially useful isolates being overlooked.

G. mellonella may have an application as a model for looking for secondary metabolites or natural products that display immunomodulation properties so that EPF isolates could be screened for production of these products. Additionally they could be utilised for the identification of novel fungal products that could aid strain

enhancement. However the use of *G. mellonella* as a model or furthermore these type of bioassays are not predictive of whether synergy will occur in the field. All three EPF appeared to have an immunomodulation effect on the *H. abietis* immune response in Chapter Three but no synergy was achieved in the field studies described in Chapter Four. To predict EPF efficacy in the field fungal susceptibility tests are often employed where the insect is dipped in various spore suspensions. However, when *H. abietis* larvae were dipped in various spore concentrations of the three EPF (Appendix D), all EPF killed the larvae. In contrast in the field studies discussed in Chapter Four *B. bassiana* did not reduce *H. abietis* numbers so these types of assays are not reliable either. It is difficult to predict synergy in laboratory trials: previously in the research group interaction assays between EPF and EPN were carried out on *H. abietis* larvae, with a number of combinations displaying synergy, however when the experiments were repeated the results were not consistent, synergy was not repeatable for any experiment under the same conditions (Hennessy 2012). This gap between laboratory and field studies reflects the behaviour of both the host and pathogen in nature. It is perhaps unsurprising that EPF did not achieve as successful control of as *H. abietis* as EPN did. Treatments are applied to stumps above ground level and EPF must be carried passively and/or by phoresis to come into contact with target insects, while EPN can actively seek out hosts in the soil (Section 1.8). These bioassays may be better suited to predict synergy between EPF-EPN against vine weevils, *O. sulcatus*, as spores are mixed with the soil increasing their contact with target insects (Ansari *et al.* 2008, Ansari *et al.* 2010).

To enable proteomic investigations into the effects of EPF on the immune response of *H. abietis* a *de novo* transcriptome study of *H. abietis* larvae was carried out. This transcriptome may enable future research into this pest of economic significance, such as its differential immune response to pathogens, identification of novel immune genes or the expansion of gene families. This is the first reported investigation into the humoral immune response of *H. abietis*. LFQ determined that EPF fungal supernatant induced alterations to the haemolymph proteome of both *G. mellonella* and *H. abietis* larvae. In both insects there was an alteration in the abundance of proteases/protease inhibitors that may function in regulation of proteolytic pathways such as the proPO cascade. In both insects EPF supernatant altered abundance of proteins involved in detection and sensing, detoxification enzymes and AMP. These patterns of alteration may be integral to the observed immunomodulating effect of EPF on the insect immune response and may serve as

indicators, when screening isolates of EPF, for the ability to render a host susceptible to subsequent infections.

A major difference between the proteomic profiles of EPF-injected *G. mellonella* and *H. abietis* was that *H. abietis* larvae treated with EPF supernatant had altered proteins involved in development, in the case of *M. anisopliae*, and numerous proteins involved in metabolic process, in the case of *B. caledonica*. The major alteration in metabolic proteins particularly several proteins from glycosyl hydrolase and carboxylesterase families following treatment with *B. caledonica* supernatant, highlights the differences between these two insects as the high levels of enzymes with cellulose cleavage reflects *H. abietis'* wood based diet.

LFQ analysis suggests that EPF supernatant impacts upon proteins involved in feeding and nutrient assimilation; there was an increase in abundance of gustatory receptors in *G. mellonella* larvae injected with EPF supernatant, and *H. abietis* larvae treated with *B. caledonica* supernatant had an increased abundance of enzymes involved in cellulose metabolism. One explanation for this upregulation may be linked to the fact that maintenance of an appropriate immune response is energy demanding. For instance the production and maintenance of the proPO activating pathway is dietary dependent (González-Santoyo and Córdoba-Aguilar 2012) and as there are changes to proteins related to the proPO pathway induced by immunomodulating EPF it may be necessary for insects to increase their dietary intake to sustain the cascade. The main effect of infection may not be direct damage caused by pathogens but the cost imposed when the immune system is activated and insects may compensate for increased demands by increasing resource uptake (Moret and Schmid-Hempel 2000).

This impact on enzymes involved in cellulose metabolism is reflected in Chapter Five where *H. abietis* adults exposed to oosporein-treated food fed more overall than those that were only exposed to control food. Oosporein did not act as a feeding deterrent or attractant but exposure to oosporein did stimulate feeding. As discussed this may be a reflection of the energy demands imposed by on the immune response to oosporein. While oosporein is not toxic at the concentrations tested and the adults did not die, bioassays indicate that oosporein modulates the immune system of larval *H. abietis* making it more susceptible to subsequent pathogens. The increase in food consumption may be linked with an altered immune response; a future recommendation would be to follow feeding on oosporein with a treatment of EPN

or EPF to see if it leaves the insect more susceptible to subsequent pathogens. As oosporein was found to be an immunomodulating natural product contained in the *B. caledonica* supernatant used in Chapters Two and Three and appears to contribute to the immunomodulation potential of this fungus (discussed in Chapter Five), it warrants future investigation for its biocontrol potential.

Current work within the research group at Maynooth University looks at the effect of applying EPF around seedlings treated with insecticides to investigate whether they result in synergistic control of *H. abietis* adults. There is evidence of synergy from lab trials and a field study is currently underway (J. Van Vlaenderen, personal communication). A possible approach may be to use oosporein or the *B. caledonica* isolate producing oosporein in combination with insecticides against *H. abietis* adults with the hypothesis that oosporein-stimulated feeding may induce insects to feed more on insecticide-treated wood, ultimately accelerating their death. In support of this hypothesis, Wraight and Ramos (2005) suggested that synergistic control of the Colorado potato beetle, *Leptinotarsa decemlineata* observed between *B. thuringiensis* and *B. bassiana* might be explained by enhancement of *B. thuringiensis* efficacy by *B. bassiana*, as Fargues *et al*. (1994) observed a 20% increase in *L. decemlineata* larval feeding during initial exposure to *B. bassiana* conidia; an increase in feeding leads to greater consumption of bacterial toxin associated with treated foliage. However a concern for recommending the use of oosporein to stimulate feeding to accelerate killing by insecticides, is that it may result in excessive damage to plants, which is ultimately how *H. abietis* kill seedlings. Thus this proposed approach would require investigation into optimal concentrations of both agents, to enhance killing and not damage. On the other hand insects infected with *Metarhizium* and *Beauvaria* species often have significantly reduced feeding with feeding decreasing through time until death and it is hypothesised that this reduction may be caused by toxins and mechanical disruption of the host by the fungi (Roy *et al.* 2006).

There are many examples of EPF that have shown 'potential' in the laboratory but have not translated to success in the field. The effect of EPF on insect behaviour such as feeding is an important consideration when trying to translate potential into viable control (Lacey *et al.* 2015). EPF were shown to alter abundance of proteins involved in detection and sensing in both *H. abietis* and *G. mellonella* in Chapters Two and Three. The behavioural response of insects to fungi has a direct effect on

their efficacy as biocontrol agents, with adaptations to detect enemy specific compounds a selective advantage (Baverstock *et al.* 2010). For instance, the common flowerbug, *Anthocoris nemorum*, appears to be able to detect and avoid *B. bassiana* when it forages on host plants (Meyling and Pell 2006). Though virulence of fungal pathogens is generally measured in terms of host mortality, the impact of infection on feeding and reproduction should also be considered. *M. anisopliae* infection has been shown to significantly reduce feeding and oviposition by the pea leaf miner, *Liriomyza huidobrensis* (Migiro *et al.* 2011). Starvation is also believed to play a role in the mechanistic action of synergism in some biocontrol agents (Wraight and Ramos 2005). Fungi and insects have evolved complex relationships and the behavioural response of insects to fungi should be taken into consideration when selecting isolates for biological control of pests (Lacey *et al.* 2015).

In Chapter Four the efficacy of EPF, EPN and EPF-EPN combinations for *H. abietis* suppression in the field was investigated across three consecutive field studies. At the time of commencement of this work EPF-EPN synergy had not yet been reported against *H. abietis* in the field. The effect of application method on efficacy of EPF was also assessed, with application method ultimately determined to not affect EPF efficacy against *H. abietis*. EPN were found to offer superior control over *H. abietis* than EPF, with all treatments that caused significant reduction in adult emergence being EPN alone or EPN in combination with EPF. The native EPN *H. downesi* was also found to give more effective control than *S. carpocapsae*, supporting the work of Dillon *et al*. (2006, 2007). Synergy between EPF-EPN was not achieved in any of the three field studies with all combinations tested giving additive results. Despite this lack of synergy, *B. caledonica* has nevertheless emerged as a potential candidate for biological pest control with infection levels of larvae within the stump rivalling the commercial strains tested. This is the first report testing *B. caledonica* to control *H. abietis*, or any other insect pest, in the field and its use warrants future investigation. Alternative approaches may include applying EPF earlier in the season rather than when *H. abietis* is already at late instar stage, as done in this work; this may give the EPF time to colonise the stump so it can confer better control. This recommendation is strengthened by the observation that the strongest control of *H. philanthus* by EPF-EPN combinations occurred when the insect was exposed to *M. anisopliae* first followed by the EPN four weeks later, indicating that the EPF acts as a stressor making the insect more susceptible to the EPN when subsequently added (Ansari *et al.* 2004).

B. caledonica could also be trialled against *H. abietis* adults alongside insecticides as previously suggested, which could allow lower levels of insecticides to be used. *B. caledonica* has been recovered from mycosed beetles, soil, bark and frass in *Pinus radiata* forests in New Zealand (Reay *et al.* 2008) and may also have an application in biocontrol as a endophyte (Brownbridge *et al.* 2012). Using EPF to control bark beetles by conventional spraying application may prove ineffective and costly (Brownbridge *et al.* 2012) so the potential use of *B. caledonica* to regulate beetles through endophytic colonisation is worth investigating. In laboratory studies *B. caledonica* has been shown to colonise Sitka spruce and lodgepole pine (J. Van Vlaenderen, personal communication). It was also found in the year three field study that *B. caledonica* and *M. anisopliae* appear to act more effectively at different depths. *B. caledonica* infects a higher proportion of weevils above soil level, while *M. anisopliae* infects a higher proportion of weevils below soil level. This may indicate potential for dual EPF application to control pine weevils. If the EPF are more effective at different depths perhaps together they could target a larger number of weevils, conferring greater control.

To conclude, this work has enhanced the understanding of the effect of EPF on the cellular and humoral immune system of *H. abietis*, which has not been previously reported. It highlights that EPN are a superior biocontrol agent over EPF for *H. abietis* in the current approach used, with no synergistic interaction occurring between the two in the field. However the native *B. caledonica* isolate tested has emerged as an interesting avenue for future research into the utilisation of EPF as biological control agents.

References

Abdallah, C., Dumas-Gaudot, E., Renaut, J. and Sergeant, K. (2012) 'Gel-based and gel-free quantitative proteomics approaches at a glance', *International journal of plant genomics*, 2012.

Abendstein, D., Schweigkofler, W. and Strasser, H. (2001) 'Effect of the fungal metabolite oosporein on feeding behaviour and survival of Melolontha melolontha-and Galleria mellonella-larvae', *Laimburg J*, 1(2).

Abendstein, D., Schweigkofler, W. and Strasser, H. (2003) 'Study on insecticidal, antifeedant and growth inhibitory properties of oosporein on selected pest organisms', *IOBC WPRS BULLETIN*, 26(1), 103-106.

Altincicek, B., Knorr, E. and Vilcinskas, A. (2008) 'Beetle immunity: Identification of immune-inducible genes from the model insect Tribolium castaneum', *Developmental & Comparative Immunology*, 32(5), 585-595.

Alurappa, R., Bojegowda, M. R. M., Kumar, V., Mallesh, N. K. and Chowdappa, S. (2014) 'Characterisation and bioactivity of oosporein produced by endophytic fungus Cochliobolus kusanoi isolated from Nerium oleander L', *Natural product research*, 28(23), 2217-2220.

Amin, G. A., Youssef, N. A., Bazaid, S. and Saleh, W. D. (2010) 'Assessment of insecticidal activity of red pigment produced by the fungus Beauveria bassiana', *World Journal of Microbiology and Biotechnology*, 26(12), 2263-2268.

Amiri-Besheli, B., Khambay, B., Cameron, S., Deadman, M. L. and Butt, T. M. (2000) 'Inter-and intra-specific variation in destruxin production by insect pathogenic Metarhizium spp., and its significance to pathogenesis', *Mycological research*, 104(04), 447-452.

Anbesse, S. A., Adge, B. J. and 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(5), 701-709.

Ansari, M. A. and Butt, T. M. (2012) 'Susceptibility of different developmental stages of large pine weevil Hylobius abietis (Coleoptera: Curculionidae) to entomopathogenic fungi and effect of fungal infection to adult weevils by formulation and application methods', *Journal of invertebrate pathology*, 111(1), 33-40.

Ansari, M. A., Shah, F. A. and Butt, T. M. (2008) 'Combined use of entomopathogenic nematodes and Metarhizium anisopliae as a new approach for black vine weevil, Otiorhynchus sulcatus, control', *Entomologia Experimentalis et Applicata*, 129(3), 340-347.

Ansari, M. A., Shah, F. A. and Butt, T. M. (2010) 'The entomopathogenic nematode Steinernema kraussei and Metarhizium anisopliae work synergistically in controlling overwintering larvae of the black vine weevil, Otiorhynchus sulcatus, in strawberry growbags', *Biocontrol Science and Technology*, 20(1), 99-105.

Ansari, M. A., Shah, F. A., Tirry, L. and Moens, M. (2006) 'Field trials against Hoplia philanthus (Coleoptera: Scarabaeidae) with a combination of an entomopathogenic nematode and the fungus Metarhizium anisopliae CLO 53', *Biological Control*, 39(3), 453-459.

Ansari, M. A., Tirry, L. and Moens, M. (2004) 'Interaction between Metarhizium anisopliae CLO 53 and entomopathogenic nematodes for the control of Hoplia philanthus', *Biological Control*, 31(2), 172-180.

Arvanitis, M., Glavis-Bloom, J. and Mylonakis, E. (2013) 'Invertebrate models of fungal infection', *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1832(9), 1378-1383.

Bantscheff, M., Lemeer, S., Savitski, M. M. and Kuster, B. (2012) 'Quantitative mass spectrometry in proteomics: critical review update from 2007 to the present', *Analytical and bioanalytical chemistry*, 404(4), 939-965.

Bao, Y.-Y., Xue, J., Wu, W.-J., Wang, Y., Lv, Z.-Y. and Zhang, C.-X. (2011) 'An immune-induced reeler protein is involved in the Bombyx mori melanization cascade', *Insect biochemistry and molecular biology*, 41(9), 696-706.

Basyouni, S. H. E., Brewer, D. and Vining, L. C. (1968) 'Pigments of the genus Beauveria', *Canadian journal of Botany*, 46(4), 441-448.

Baverstock, J., Roy, H. E. and Pell, J. K. (2010) 'Entomopathogenic fungi and insect behaviour: from unsuspecting hosts to targeted vectors', *Biocontrol*, 55(1), 89-102.

Beresford, P. J., Basinski-Gray, J. M., Chiu, J. K. C., Chadwick, J. S. and Aston, W. P. (1997) 'Characterization of hemolytic and cytotoxic Gallysins: a relationship with arylphorins', *Developmental & Comparative Immunology*, 21(3), 253-266.

Bergin, D., Brennan, M. and Kavanagh, K. (2003) 'Fluctuations in haemocyte density and microbial load may be used as indicators of fungal pathogenicity in larvae of Galleria mellonella', *Microbes and infection*, 5(15), 1389-1395.

Bergin, D., Murphy, L., Keenan, J., Clynes, M. and 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(8), 2105-2112.

Bissett, J. and Widden, P. (1988) 'A new species of Beauveria isolated from Scottish moorland soil', *Canadian journal of botany*, 66(2), 361-362.

Blackith, R. and Blackith, R. (1986) 'Pygostolus sticticus Fab.(Hymenoptera: Braconidae) parasitic on Otiorhynchus singularis (L.)(Coleoptera: Curculionidae) in an Irish pine forest', *The Irish Naturalists' Journal*, 22(2), 69-71.

Bomford, M. K. and Vernon, R. S. (2005) 'Root weevil (Coleoptera: Curculionidae) and ground beetle (Coleoptera: Carabidae) immigration into strawberry plots protected by fence or portable trench barriers', *Environmental entomology*, 34(4), 844-849.

Boucias, D. G., Lietze, V.-U. and Teal, P. (2012) 'Chemical signals that mediate insect-fungal interactions' in *Biocommunication of Fungi*Springer, 305-336.

Brixey, J. (1997) 'The potential for biological control to reduce Hylobius abietis damage', *Research Information Note-Forestry Commission*, (273).

Brown, G. D. and Gordon, S. (2005) 'Immune recognition of fungal β-glucans', *Cellular microbiology*, 7(4), 471-479.

Brownbridge, M., Reay, S. D., Nelson, T. L. and Glare, T. R. (2012) 'Persistence of Beauveria bassiana (Ascomycota: Hypocreales) as an endophyte following inoculation of radiata pine seed and seedlings', *Biological control*, 61(3), 194-200.

Bulet, P., Hetru, C., Dimarcq, J.-L. and Hoffmann, D. (1999) 'Antimicrobial peptides in insects; structure and function', *Developmental & Comparative Immunology*, 23(4), 329-344.

Bulet, P., Stöcklin, R. and Menin, L. (2004) 'Anti-microbial peptides: from invertebrates to vertebrates', *Immunological reviews*, 198(1), 169-184.

Butt, T. M., Coates, C. J., Dubovskiy, I. M. and Ratcliffe, N. A. (2016) 'Entomopathogenic Fungi: New Insights into Host–Pathogen Interactions', *Advances in Genetics*.

Campos-Herrera, R. (2015) *Nematode pathogenesis of insects and other pests: ecology and applied technologies for sustainable plant and crop protection*, Springer.

Carlsson, A., Nyström, T., de Cock, H. and Bennich, H. (1998) 'Attacin-an insect immune protein-binds LPS and triggers the specific inhibition of bacterial outer-membrane protein synthesis', *Microbiology*, 144(8), 2179-2188.

Chapman, R. F. (2003) 'Contact chemoreception in feeding by phytophagous insects', *Annual Review of Entomology*, 48(1), 455-484.

Choi, C. W., Seo, D. H., Yuk, J. E., Park, J. B., Hwang, S. Y., Koh, S. K. and Yun, C. Y. (2006) 'Upregulation of hemolymph and tissue ferritin by dietary HgCl2 in the wax moth Galleria mellonella', *Entomological Research*, 36(4), 185-190.

Clarkson, J. M. and Charnley, A. K. (1996) 'New insights into the mechanisms of fungal pathogenesis in insects', *Trends in microbiology*, 4(5), 197-203.

Cole, R. J., Kirksey, J. W., Cutler, H. G. and Davis, E. E. (1974) 'Toxic effects of oosporein from Chaetomium trilaterale', *Journal of agricultural and food chemistry*, 22(3), 517-520.

Cordin, O., Banroques, J., Tanner, N. K. and Linder, P. (2006) 'The DEAD-box protein family of RNA helicases', *Gene*, 367, 17-37.

Cotter, G., Doyle, S. and Kavanagh, K. (2000) 'Development of an insect model for the in vivo pathogenicity testing of yeasts', *FEMS Immunology & Medical Microbiology*, 27(2), 163-169.

Crawley, M. J. (1993) 'GLIM for ecologists Blackwell Scientific Publications'.

De Gregorio, E., Han, S.-J., Lee, W.-J., Baek, M.-J., Osaki, T., Kawabata, S.-I., Lee, B.-L., Iwanaga, S., Lemaitre, B. and Brey, P. T. (2002) 'An immune-responsive Serpin regulates the melanization cascade in Drosophila', *Developmental cell*, 3(4), 581-592.

DeBach, P. and Rosen, D. (1991) *Biological control by natural enemies*, CUP Archive.

Deddouche, S., Matt, N., Budd, A., Mueller, S., Kemp, C., Galiana-Arnoux, D., Dostert, C., Antoniewski, C., Hoffmann, J. A. and Imler, J.-L. (2008) 'The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in drosophila', *Nature immunology*, 9(12), 1425-1432.

Degenkolb, T. and Vilcinskas, A. (2015) 'Metabolites from nematophagous fungi and nematicidal natural products from fungi as an alternative for biological control. Part I: metabolites from nematophagous ascomycetes', *Applied microbiology and biotechnology*, 1-14.

Dhadialla, T. S., Carlson, G. R. and Le, D. P. (1998) 'New insecticides with ecdysteroidal and juvenile hormone activity', *Annual review of entomology*, 43(1), 545-569.

Dillon, A. and Griffin, C. (2008) 'Controlling the large pine weevil, Hylobius abietis, using natural enemies', *COFORD Connects: Silviculture and Forest Management*, (15), 1-8.

Dillon, A. B., Downes, M. J., Ward, D. and Griffin, C. T. (2007) 'Optimizing application of entomopathogenic nematodes to manage large pine weevil, Hylobius abietis L.(Coleoptera: Curculionidae) populations developing in pine stumps, Pinus sylvestris', *Biological control*, 40(2), 253-263.

Dillon, A. B., Rolston, A. N., Meade, C. V., Downes, M. J. and Griffin, C. T. (2008) 'Establishment, persistence, and introgression of entomopathogenic nematodes in a forest ecosystem', *Ecological Applications*, 18(3), 735-747.

Dillon, A. B., Ward, D., Downes, M. J. and Griffin, C. T. (2006) 'Suppression of the large pine weevil Hylobius abietis (L.)(Coleoptera: Curculionidae) in pine stumps by entomopathogenic nematodes with different foraging strategies', *Biological Control*, 38(2), 217-226.

Divekar, P. V., Haskins, R. H. and Vining, L. C. (1959) 'OOSPOREIN FROM AN ACREMONIUM SP', *Canadian Journal of Chemistry*, 37(12), 2097-2099.

Dowds, B. C. and Peters, A. (2002) '4 Virulence Mechanisms', *Entomopathogenic nematology*, 79.

Dubovskiy, I. M., Kryukov, V., Benkovskaya, G. V., Yaroslavtseva, V. V., Surina, E. V., Glupov, V. V., Äóáîâñêèé, È., Êðþêîâ, Â. and Áåíüêîâñêàÿ, Ã. (2010) 'Activity of the detoxificative enzyme system and encapsulation rate in the Colorado potato beetle Leptinotarsa decemlineata (Say) larvae under organophosphorous insecticide treatment and entomopathogenic fungus Metarhizium anisopliae (Metsch.) infection', *Euroasian Entomol. J*, 9, 577-582.

Dubovskiy, I. M., Slyamova, N. D., Kryukov, V. Y., Yaroslavtseva, O. N., Levchenko, M. V., Belgibaeva, A. B., Adilkhankyzy, A. and Glupov, V. V. (2012) 'The activity of nonspecific esterases and glutathione-S-transferase in Locusta migratoria larvae infected with the fungus Metarhizium anisopliae (Ascomycota, Hypocreales)', *Entomological review*, 92(1), 27-31.

Dunse, K. M., Kaas, Q., Guarino, R. F., Barton, P. A., Craik, D. J. and Anderson, M. A. (2010) 'Molecular basis for the resistance of an insect chymotrypsin to a potato type II proteinase inhibitor', *Proceedings of the National Academy of Sciences*, 107(34), 15016-15021.

El Basyouni, S. H. and Vining, L. C. (1966) 'Biosynthesis of oosporein in Beauveria bassiana (Bals.) Vuill', *Canadian journal of biochemistry*, 44(5), 557-565.

Evans, H., McAllister, F., Saunders, T., Moore, R., Jenkins, T., Butt, T., Ansari, M., Griffin, C., Williams, C., Teck, R. and Sweeney, J. (2015) *The Impact project guide to Hyalobius management.*http://www.impactproject.eu/uploads/impact_hylobius_publication.pdf [accessed.

Eyun, S.-i., Wang, H., Pauchet, Y., Benson, A. K., Valencia-Jiménez, A., Moriyama, E. N. and Siegfried, B. D. (2014) 'Molecular evolution of glycoside hydrolase genes in the western corn rootworm (Diabrotica virgifera virgifera)', *PloS one*, 9(4), e94052.

Fallon, J. P., Troy, N. and Kavanagh, K. (2011) 'Pre-exposure of Galleria mellonella larvae to different doses of Aspergillus fumigatus conidia causes differential activation of cellular and humoral immune responses', *Virulence*, 2(5), 413-421.

Fang, Q., Wang, L., Zhu, J., Li, Y., Song, Q., Stanley, D. W., Akhtar, Z.-r. and Ye, G. (2010) 'Expression of immune-response genes in lepidopteran host is suppressed by venom from an endoparasitoid, Pteromalus puparum', *BMC genomics*, 11(1), 1.

Fargues, J., Delmas, J. C. and Lebrun, R. A. (1994) 'Leaf consumption by larvae of the Colorado potato beetle (Coleoptera: Chrysomelidae) infected with the entomopathogen, Beauveria bassiana', *Journal of economic entomology*, 87(1), 67-71.

Faruck, M. O., Yusof, F. and Chowdhury, S. (2015) 'An overview of antifungal peptides derived from insect', *Peptides*.

Felton, G. W. and Summers, C. B. (1995) 'Antioxidant systems in insects', *Archives of insect biochemistry and physiology*, 29(2), 187-197.

Feng, P., Shang, Y., Cen, K. and Wang, C. (2015) 'Fungal biosynthesis of the bibenzoquinone oosporein to evade insect immunity', *Proceedings of the National Academy of Sciences*, 112(36), 11365-11370.

Finnerty, C. M., Granados, R. R. and Karplus, P. A. (1999) 'The insect immune protein scolexin is a novel serine proteinase homolog', *Protein science*, 8(1), 242-248.

Fomina, M., Hillier, S., Charnock, J. M., Melville, K., Alexander, I. J. and Gadd, G. M. (2005) 'Role of oxalic acid overexcretion in transformations of toxic metal minerals by Beauveria caledonica', *Applied and Environmental Microbiology*, 71(1), 371-381.

Fritzen, M., Flores, M. P. A., Reis, C. V. and Chudzinski-Tavassi, A. M. (2005) 'A prothrombin activator (Lopap) modulating inflammation, coagulation and cell survival mechanisms', *Biochemical and biophysical research communications*, 333(2), 517-523.

Fröbius, A. C., Kanost, M. R., Götz, P. and Vilcinskas, A. (2000) 'Isolation and characterization of novel inducible serine protease inhibitors from larval hemolymph of the greater wax moth Galleria mellonella', *European Journal of Biochemistry*, 267(7), 2046-2053.

Fröbius, A. C., Kanost, M. R., Götz, P. and Vilcinskas, A. (2000) '- Isolation and characterization of novel inducible serine protease inhibitors from larval hemolymph of the greater wax moth Galleria mellonella', - 267(-7), - 2053.

Fuchs, B. B., O'Brien, E., El Khoury, J. B. and Mylonakis, E. (2010) 'Methods for using Galleria mellonella as a model host to study fungal pathogenesis', *Virulence*, 1(6), 475-482.

Gandhe, A. S., John, S. H. and Nagaraju, J. (2007) 'Noduler, a novel immune up-regulated protein mediates nodulation response in insects', *The Journal of Immunology*, 179(10), 6943-6951.

Gao, Q., Jin, K., Ying, S.-H., Zhang, Y., Xiao, G., Shang, Y., Duan, Z., Hu, X., Xie, X.-Q. and Zhou, G. (2011) 'Genome sequencing and

comparative transcriptomics of the model entomopathogenic fungi Metarhizium anisopliae and M. acridum', *PLoS Genet*, 7(1), e1001264.

Georgis, R., Koppenhöfer, A. M., Lacey, L. A., Bélair, G., Duncan, L. W., Grewal, P. S., Samish, M., Tan, L., Torr, P. and Van Tol, R. (2006) 'Successes and failures in the use of parasitic nematodes for pest control', *Biological Control*, 38(1), 103-123.

Gillespie and, J. P., Kanost, M. R. and Trenczek, T. (1997) 'Biological mediators of insect immunity', *Annual review of entomology*, 42(1), 611-643.

Gillespie, J. P., Bailey, A. M., Cobb, B. and Vilcinskas, A. (2000) 'Fungi as elicitors of insect immune responses', *Archives of insect biochemistry and physiology*, 44(2), 49-68.

Glare, T. R., Reay, S. D., Nelson, T. L. and Moore, R. (2008) 'Beauveria caledonica is a naturally occurring pathogen of forest beetles', *Mycological research*, 112(3), 352-360.

Glazer, I. (2015) 'Improvement of Entomopathogenic Nematodes: A Genetic Approach' in *Nematode Pathogenesis of Insects and Other Pests*Springer, 29-55.

González-Tokman, D., Córdoba-Aguilar, A., González-Santoyo, I. and Lanz-Mendoza, H. (2011) 'Infection effects on feeding and territorial behaviour in a predatory insect in the wild', *Animal Behaviour*, 81(6), 1185-1194.

González-Santoyo, I. and Córdoba-Aguilar, A. (2012) 'Phenoloxidase: a key component of the insect immune system', *Entomologia Experimentalis et Applicata*, 142(1), 1-16.

Gordon, S. C. and Woodford, J. A. T. (1986) 'The control of the clay-coloured weevil (Otiorhynchus singularis)(Coleoptera: Curculionidae) in raspberry in Eastern Scotland', *Crop research*, 26(2), 111-119.

Griffin, C. T. (2015) 'Behaviour and population dynamics of entomopathogenic nematodes following application' in *Nematode Pathogenesis of Insects and Other Pests*Springer, 57-95.

Grizanova, E. V., Dubovskiy, I. M., Whitten, M. M. A. and Glupov, V. V. (2014) 'Contributions of cellular and humoral immunity of Galleria mellonella larvae in defence against oral infection by Bacillus thuringiensis', *Journal of invertebrate pathology*, 119, 40-46.

Gupta, S. C., Leathers, T. D., El-Sayed, G. N. and Ignoffo, C. M. (1994) 'Relationships among enzyme activities and virulence parameters in Beauveria bassiana infections of Galleria mellonella and Trichoplusia ni', *Journal of Invertebrate Pathology*, 64(1), 13-17.

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., Couger, M. B., Eccles, D., Li, B. and Lieber, M. (2013) 'De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis', *Nature protocols*, 8(8), 1494-1512.

Hemingway, J. and Ranson, H. (2000) 'Insecticide resistance in insect vectors of human disease', *Annual review of entomology*, 45(1), 371-391.

Hennessy, R. (2012) 'Effects of a dual application of entomopathogenic nematodes and entomopathogenic fungi against a forest pest, Hylobius abietis L.(Coleoptera: Curculionidae) and a horticultural pest, Otiorhynchus sulcatus F.(Coleoptera: Curculionidae)'.

Hoffmann, J. A. (1995) '- Innate immunity of insects', - 7(- 1), - 10.

Hoffmann, J. A., Reichhart, J.-M. and Hetru, C. (1996) 'Innate immunity in higher insects', *Current opinion in immunology*, 8(1), 8-13.

Hoy, M. A. (2008) 'Classical biological control' in *Encyclopedia of Entomology*Springer, 906-923.

Hung, S.-Y., Boucias, D. G. and Vey, A. J. (1993) 'Effect of Beauveria bassiana and Candida albicans on the cellular defense response of Spodoptera exigua', *Journal of invertebrate pathology*, 61(2), 179-187.

Inglis, G. D., Johnson, D. L., Cheng, K. J. and Goettel, M. S. (1997) 'Use of pathogen combinations to overcome the constraints of temperature on entomopathogenic hyphomycetes against grasshoppers', *Biological Control*, 8(2), 143-152.

Inward, D. J., Wainhouse, D. and Peace, A. (2012) 'The effect of temperature on the development and life cycle regulation of the pine weevil Hylobius abietis and the potential impacts of climate change', *Agricultural and Forest Entomology*, 14(4), 348-357.

Jiang, H., Vilcinskas, A. and Kanost, M. R. (2010) 'Immunity in lepidopteran insects' in *Invertebrate Immunity*Springer, 181-204.

Jindra, M., Palli, S. R. and Riddiford, L. M. (2013) 'The juvenile hormone signaling pathway in insect development', *Annual review of entomology*, 58, 181-204.

Jiravanichpaisal, P., Lee, B. L. and Söderhäll, K. (2006) 'Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization', *Immunobiology*, 211(4), 213-236.

Kaaya, G. P. and Hassan, S. (2000) 'Entomogenous fungi as promising biopesticides for tick control', *Experimental & applied acarology*, 24(12), 913-926.

Kanost, M. R. (1999) 'Serine proteinase inhibitors in arthropod immunity', *Developmental & Comparative Immunology*, 23(4), 291-301.

Kanost, M. R. (2007) 'Serpins in a lepidopteran insect, Manduca sexta' in *Molecular and Cellular Aspects of the Serpinopathies and Disorders in Serpin Activity*World Scientific Publishing Co. Hackensack, NJ, 229-241.

Kavanagh, K. and Reeves, E. P. (2004) 'Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens', *FEMS Microbiology Reviews*, 28(1), 101-112.

Keeling, C. I., Yuen, M. M. S., Liao, N. Y., Docking, T. R., Chan, S. K., Taylor, G. A., Palmquist, D. L., Jackman, S. D., Nguyen, A. and Li, M. (2013) 'Draft genome of the mountain pine beetle, Dendroctonus ponderosae Hopkins, a major forest pest', *Genome Biology*, 14(3), 1-20.

Keller, N. P. (2015) 'Translating biosynthetic gene clusters into fungal armor and weaponry', *Nature chemical biology*, 11(9), 671-677.

Kenis, M., Wegensteiner, R. and Griffin, C. T. (2007) 'Parasitoids, predators, nematodes and pathogens associated with bark weevil pests' in *Bark and wood boring insects in living trees in Europe, a synthesis*Springer, 395-414.

Kershaw, M. J., Moorhouse, E. R., Bateman, R., Reynolds, S. E. and Charnley, A. K. (1999) 'The role of destruxins in the pathogenicity of Metarhizium anisopliae for three species of insect', *Journal of invertebrate pathology*, 74(3), 213-223.

Koiwa, H., Shade, R. E., Zhu-Salzman, K., D'Urzo, M. P., Murdock, L. L., Bressan, R. A. and Hasegawa, P. M. (2000) 'A plant defensive cystatin (soyacystatin) targets cathepsin L-like digestive cysteine proteinases (DvCALs) in the larval midgut of western corn rootworm (Diabrotica virgifera virgifera)', *Febs Letters*, 471(1), 67-70.

Koppenhöfer, A. M. and Kaya, H. K. (1997) 'Additive and Synergistic Interaction between Entomopathogenic Nematodes andBacillus thuringiensisfor Scarab Grub Control', *Biological Control*, 8(2), 131-137.

Kurata, S. (2014) 'Peptidoglycan recognition proteins in Drosophila immunity', *Developmental & Comparative Immunology*, 42(1), 36-41.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M. and Goettel, M. S. (2015) 'Insect pathogens as biological control agents: back to the future', *Journal of invertebrate pathology*, 132, 1-41.

Lacey, L. A. and Kaya, H. K. (2007) *Field manual of techniques in invertebrate pathology*, Springer.

Lavine, M. D. and Strand, M. R. (2002) 'Insect hemocytes and their role in immunity', *Insect biochemistry and molecular biology*, 32(10), 1295-1309.

Leather, S., Day, K. and Salisbury, A. (1999) 'The biology and ecology of the large pine weevil, Hylobius abietis (Coleoptera: Curculionidae): a

problem of dispersal?', *Bulletin of Entomological Research*, 89(01), 3-16.

Leather, S. R., Day, K. R. and Salisbury, A. N. (1999) 'The biology and ecology of the large pine weevil, Hylobius abietis (Coleoptera: Curculionidae): a problem of dispersal?', *Bulletin of Entomological Research*, 89(01), 3-16.

Lee, Y., Kim, S. H. and Montell, C. (2010) 'Avoiding DEET through insect gustatory receptors', *Neuron*, 67(4), 555-561.

Leger, R. J. S., Wang, C. and Fang, W. (2011) 'New perspectives on insect pathogens', *Fungal Biology Reviews*, 25(2), 84-88.

Lemaitre, B. and Hoffmann, J. (2007) 'The host defense of Drosophila melanogaster', *Annu. Rev. Immunol.*, 25, 697-743.

Levy, F., Rabel, D., Charlet, M., Bulet, P., Hoffmann, J. A. and Ehret-Sabatier, L. (2004) 'Peptidomic and proteomic analyses of the systemic immune response of Drosophila', *Biochimie*, 86(9), 607-616.

Lewis, E. E., Campbell, J., Griffin, C., Kaya, H. and Peters, A. (2006) 'Behavioral ecology of entomopathogenic nematodes', *Biological Control*, 38(1), 66-79.

Liu, B.-L. and Tzeng, Y.-M. (2012) 'Development and applications of destruxins: A review', *Biotechnology advances*, 30(6), 1242-1254.

Lomer, C. J., Bateman, R. P., Johnson, D. L., Langewald, J. and Thomas, M. (2001) 'Biological control of locusts and grasshoppers', *Annual review of entomology*, 46(1), 667-702.

Love, B. E., Bonner-Stewart, J. and Forrest, L. A. (2009) 'An efficient synthesis of oosporein', *Tetrahedron Letters*, 50(35), 5050-5052.

Långström, B. and Day, K. R. (2007) 'Damage, control and management of weevil pests, especially Hylobius abietis' in *Bark and wood boring insects in living trees in Europe, a synthesis*Springer, 415-444.

Maguire, R., Duggan, O. and Kavanagh, K. (2016) 'Evaluation of Galleria mellonella larvae as an in vivo model for assessing the relative toxicity of food preservative agents', *Cell Biology and Toxicology*, 1-8.

Marmaras, V. J., Charalambidis, N. D. and Zervas, C. G. (1996) 'Immune response in insects: The role of phenoloxidase in defense reactions in relation to melanization and sclerotization', *Archives of Insect Biochemistry and Physiology*, 31(2), 119-133.

Marmaras, V. J. and Lampropoulou, M. (2009) 'Regulators and signalling in insect haemocyte immunity', *Cellular signalling*, 21(2), 186-195.

Meyling, N. V. and Pell, J. K. (2006) 'Detection and avoidance of an entomopathogenic fungus by a generalist insect predator', *Ecological Entomology*, 31(2), 162-171.

Migiro, L. N., Maniania, N. K., Chabi-Olaye, A., Wanjoya, A. and Vandenberg, J. (2011) 'Effect of infection by Metarhizium anisopliae (Hypocreales: Clavicipitaceae) on the feeding and oviposition of the pea leafminer Liriomyza huidobrensis (Diptera: Agromyzidae) on different host plants', *Biological Control*, 56(2), 179-183.

Moore, R. (2001) 'Emergence trap developed to capture adult large pine weevil Hylobius abietis (Coleoptera: Curculionidae) and its parasite Bracon hylobii (Hymenoptera: Braconidae)', *Bulletin of entomological research*, 91(02), 109-115.

Morales-Rodriguez, A. and 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(1), 169-180.

Moret, Y. and Schmid-Hempel, P. (2000) 'Survival for immunity: the price of immune system activation for bumblebee workers', *Science*, 290(5494), 1166-1168.

Morris, K., Lorenzen, M. D., Hiromasa, Y., Tomich, J. M., Oppert, C., Elpidina, E. N., Vinokurov, K., Jurat-Fuentes, J. L., Fabrick, J. and Oppert, B. (2009) 'Tribolium castaneum larval gut transcriptome and proteome: a resource for the study of the coleopteran gut', *Journal of proteome research*, 8(8), 3889-3898.

Nagaoka, T., Nakata, K. and Kouno, K. (2004) 'Antifungal activity of oosporein from an antagonistic fungus against Phytophthora infestans', *Zeitschrift für Naturforschung C*, 59(3-4), 302-304.

Nordlander, G., Nordenhem, H. and Bylund, H. (1997) 'Oviposition patterns of the pine weevil Hylobius abietis', *Entomologia Experimentalis et Applicata*, 85(1), 1-9.

Ong, S. T., Ho, J. Z. S., Ho, B. and Ding, J. L. (2006) 'Iron-withholding strategy in innate immunity', *Immunobiology*, 211(4), 295-314.

Oppert, C., Klingeman, W. E., Willis, J. D., Oppert, B. and Jurat-Fuentes, J. L. (2010) 'Prospecting for cellulolytic activity in insect digestive fluids', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 155(2), 145-154.

Ozsolak, F. and Milos, P. M. (2011) 'RNA sequencing: advances, challenges and opportunities', *Nature reviews genetics*, 12(2), 87-98.

Pal, S., Leger, R. J. S. and Wu, L. P. (2007) 'Fungal peptide Destruxin A plays a specific role in suppressing the innate immune response in Drosophila melanogaster', *Journal of Biological Chemistry*, 282(12), 8969-8977.

Paskewitz, S. M. and Shi, L. (2005) 'The hemolymph proteome of Anopheles gambiae', *Insect biochemistry and molecular biology*, 35(8), 815-824.

Pauchet, Y., Wilkinson, P. and Chauhan, R. (2010) 'Diversity of beetle genes encoding novel plant cell wall degrading enzymes', *PloS one*, 5(12), e15635.

Pedras, M. S. C., Zaharia, L. I. and Ward, D. E. (2002) 'The destruxins: synthesis, biosynthesis, biotransformation, and biological activity', *Phytochemistry*, 59(6), 579-596.

Pedrini, N., Crespo, R. and Juárez, M. P. (2007) 'Biochemistry of insect epicuticle degradation by entomopathogenic fungi', *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 146(1), 124-137.

Pegram, R. A., Wyatt, R. D. and Smith, T. L. (1982) 'Oosporein-toxicosis in the turkey poult', *Avian diseases*, 47-59.

Peng, G., Wang, Z., Yin, Y., Zeng, D. and 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(9), 1244-1250.

Pertea, G., Huang, X., Liang, F., Antonescu, V., Sultana, R., Karamycheva, S., Lee, Y., White, J., Cheung, F. and Parvizi, B. (2003) 'TIGR Gene Indices clustering tools (TGICL): a software system for fast clustering of large EST datasets', *Bioinformatics*, 19(5), 651-652.

Poliakov, A., Russell, C. W., Ponnala, L., Hoops, H. J., Sun, Q., Douglas, A. E. and van Wijk, K. J. (2011) 'Large-scale label-free quantitative proteomics of the pea aphid-Buchnera symbiosis', *Molecular & Cellular Proteomics*, 10(6), M110-007039.

Quesada-Moraga, E., Carrasco-Díaz, J. A. and Santiago-Álvarez, C. (2006) 'Insecticidal and antifeedant activities of proteins secreted by entomopathogenic fungi against Spodoptera littoralis (Lep., Noctuidae)', *Journal of Applied Entomology*, 130(8), 442-452.

Quin, M. B., Flynn, C. M. and Schmidt-Dannert, C. (2014) 'Traversing the fungal terpenome', *Natural product reports*, 31(10), 1449-1473.

Ratcliffe, N. A. (1993) 'Cellular defense responses of insects: unresolved problems', *Parasites and pathogens of insects*, 1, 267-304.

Reay, S. D., Brownbridge, M., Cummings, N. J., Nelson, T. L., Souffre, B., Lignon, C. and Glare, T. R. (2008) 'Isolation and characterization of Beauveria spp. associated with exotic bark beetles in New Zealand Pinusradiata plantation forests', *Biological Control*, 46(3), 484-494.

Roberts, D. W. and St Leger, R. J. (2004) 'Metarhizium spp., cosmopolitan insect-pathogenic fungi: mycological aspects', *Advances in applied microbiology*, 54(1).

Rohlfs, M. and Churchill, A. C. L. (2011) 'Fungal secondary metabolites as modulators of interactions with insects and other arthropods', *Fungal Genetics and Biology*, 48(1), 23-34.

Roy, H. E., Steinkraus, D. C., Eilenberg, J., Hajek, A. E. and Pell, J. K. (2006) 'Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts', *Annu. Rev. Entomol.*, 51, 331-357.

Sadd, B. M. and Siva-Jothy, M. T. (2006) 'Self-harm caused by an insect's innate immunity', *Proceedings of the Royal Society of London B: Biological Sciences*, 273(1600), 2571-2574.

Samson, R. A., Evans, H. C. and Latgé, J.-P. (2013) *Atlas of entomopathogenic fungi*, Springer Science & Business Media.

Samuels, R. I., Reynolds, S. E. and Charnley, A. K. (1988) 'Calcium channel activation of insect muscle by destruxins, insecticidal compounds produced by the entomopathogenic fungus Metarhizium anisopliae', *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology*, 90(2), 403-412.

Sandhu, S. S., Sharma, A. K., Beniwal, V., Goel, G., Batra, P., Kumar, A., Jaglan, S., Sharma, A. K. and Malhotra, S. (2012) 'Myco-biocontrol of insect pests: factors involved, mechanism, and regulation', *Journal of pathogens*, 2012.

Sato, K., Tanaka, K. and Touhara, K. (2011) 'Sugar-regulated cation channel formed by an insect gustatory receptor', *Proceedings of the National Academy of Sciences*, 108(28), 11680-11685.

Scharf, D. H., Remme, N., Habel, A., Chankhamjon, P., Scherlach, K., Heinekamp, T., Hortschansky, P., Brakhage, A. A. and Hertweck, C. (2011) 'A dedicated glutathione S-transferase mediates carbon–sulfur bond formation in gliotoxin biosynthesis', *Journal of the American Chemical Society*, 133(32), 12322-12325.

Scholte, E.-J., Njiru, B. N., Smallegange, R. C., Takken, W. and Knols, B. G. J. (2003) 'Infection of malaria (Anopheles gambiae ss) and filariasis (Culex quinquefasciatus) vectors with the entomopathogenic fungus Metarhizium anisopliae', *Malaria Journal*, 2(1), 29.

Schrank, A. and Vainstein, M. H. (2010) 'Metarhizium anisopliae enzymes and toxins', *Toxicon*, 56(7), 1267-1274.

Scully, L. R. and Bidochka, M. J. (2005) 'Serial passage of the opportunistic pathogen Aspergillus flavus through an insect host yields decreased saprobic capacity', *Canadian journal of microbiology*, 51(2), 185-189.

Seger, C., Erlebach, D., Stuppner, H., Griesser, U. J. and Strasser, H. (2005) 'Physicochemical properties of oosporein, the major secreted metabolite of the entomopathogenic fungus Beauveria brongniartii', *Helvetica chimica acta*, 88(4), 802-810.

Serebrov, V. V., Gerber, O. N., Malyarchuk, A. A., Martemyanov, V. V., Alekseev, A. A. and Glupov, V. V. (2006) 'Effect of entomopathogenic fungi on detoxification enzyme activity in greater wax moth Galleria mellonella L.(Lepidoptera, Pyralidae) and role of detoxification enzymes in development of insect resistance to entomopathogenic fungi', *Biology Bulletin*, 33(6), 581-586.

Seufi, A. M., Hafez, E. E. and Galal, F. H. (2011) 'Identification, phylogenetic analysis and expression profile of an anionic insect defensin gene, with antibacterial activity, from bacterial-challenged cotton leafworm, Spodoptera littoralis', *BMC molecular biology*, 12(1), 1.

Shah, F. A., Ansari, M. A., Prasad, M. and Butt, T. M. (2007) 'Evaluation of black vine weevil (Otiorhynchus sulcatus) control strategies using Metarhizium anisopliae with sublethal doses of insecticides in disparate horticultural growing media', *Biological Control*, 40(2), 246-252.

Shah, F. A., Gaffney, M., Ansari, M. A., Prasad, M. and Butt, T. M. (2008) 'Neem seed cake enhances the efficacy of the insect pathogenic fungus Metarhizium anisopliae for the control of black vine weevil, Otiorhynuchs sulcatus (Coleoptera: Curculionidae)', *Biological Control*, 44(1), 111-115.

Shah, P. A. and Pell, J. K. (2003) 'Entomopathogenic fungi as biological control agents', *Applied microbiology and biotechnology*, 61(5-6), 413-423.

Shapiro-Ilan, D. I., Cottrell, T. E., Brown, I., Gardner, W. A., Hubbard, R. K. and Wood, B. W. (2006a) 'Effect of soil moisture and a surfactant on

entomopathogenic nematode suppression of the pecan weevil, Curculio caryae', *Journal of nematology*, 38(4), 474.

Shapiro-Ilan, D. I., Gouge, D. H., Piggott, S. J. and Fife, J. P. (2006b) 'Application technology and environmental considerations for use of entomopathogenic nematodes in biological control', *Biological Control*, 38(1), 124-133.

Sharma, A., Khan, A. N., Subrahmanyam, S., Raman, A., Taylor, G. S. and Fletcher, M. J. (2014) 'Salivary proteins of plant-feeding hemipteroids–implication in phytophagy', *Bulletin of entomological research*, 104(02), 117-136.

Silverman, N. and Maniatis, T. (2001) 'NF-kappa B signaling pathways in mammalian and insect innate immunity', *Genes & Development*, 15(18), 2321-2342.

Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V. and Zdobnov, E. M. (2015) 'BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs', *Bioinformatics*, 31(19), 3210-3212.

Siva-Jothy, M. T. and Thompson, J. J. W. (2002) 'Short-term nutrient deprivation affects immune function', *Physiological Entomology*, 27(3), 206-212.

Soderhall, K. and Cerenius, L. (1998) 'Role of the prophenoloxidase-activating system in invertebrate immunity', *Current Opinion in Immunology*, 10(1), 23-28.

Sowjanya Sree, K., Padmaja, V. and Murthy, Y. L. N. (2008) 'Insecticidal activity of destruxin, a mycotoxin from Metarhizium anisopliae (Hypocreales), against Spodoptera litura (Lepidoptera: Noctuidae) larval stages', *Pest management science*, 64(2), 119-125.

Stokes, B. A., Yadav, S., Shokal, U., Smith, L. C. and Eleftherianos, I. (2015) 'Bacterial and fungal pattern recognition receptors in homologous innate signaling pathways of insects and mammals', *Frontiers in microbiology*, 6.

Strand, M. R. (2008) 'The insect cellular immune response', *Insect science*, 15(1), 1-14.

Strasser, H., Vey, A. and Butt, T. M. (2000) 'Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of Metarhizium, Tolypocladium and Beauveria species?', *Biocontrol Science and Technology*, 10(6), 717-735.

Surlis, C., Carolan, J. C., Coffey, M. F. and Kavanagh, K. (2016) 'Proteomic analysis of Bayvarol® resistance mechanisms in the honey bee parasite Varroa destructor', *Journal of Apicultural Research*, 1-16.

Terry, B. J., Liu, W.-C., Cianci, C. W., Proszynski, E., Fernandes, P., Bush, K. and Meyers, E. (1992) 'Inhibition of herpes simplex virus type 1 DNA polymerase by the natural product oosporein', *The Journal of antibiotics*, 45(2), 286-288.

Thakore, Y. (2006) 'The biopesticide market for global agricultural use', *Industrial Biotechnology*, 2(3), 194-208.

Torr, P., Heritage, S. and Wilson, M. J. (2007) 'Steinernema kraussei, an indigenous nematode found in coniferous forests: efficacy and field persistence against Hylobius abietis', *Agricultural and Forest Entomology*, 9(3), 181-188.

Tsakas, S. and Marmaras, V. J. (2010) 'Insect immunity and its signalling: an overview', *ISJ*, 7, 228-238.

van der Weerden, N. L., Bleackley, M. R. and Anderson, M. A. (2013) 'Properties and mechanisms of action of naturally occurring antifungal peptides', *Cellular and Molecular Life Sciences*, 70(19), 3545-3570.

Van Emden, H. F. (2004) *Pest and vector control*, Cambridge University Press.

Vega, F. E., Posada, F., Aime, M. C., Pava-Ripoll, M., Infante, F. and Rehner, S. A. (2008) 'Entomopathogenic fungal endophytes', *Biological Control*, 46(1), 72-82.

Vey, A., Hoagland, R. E. and Butt, T. M. (2001) '12 Toxic Metabolites of Fungal Biocontrol Agents', *Fungi as biocontrol agents*, 311.

Vieira, F. G. and Rozas, J. (2011) 'Comparative genomics of the odorant-binding and chemosensory protein gene families across the Arthropoda: origin and evolutionary history of the chemosensory system', *Genome Biology and Evolution*, 3, 476-490.

Vilcinskas, A. (2010) 'Coevolution between pathogen-derived proteinases and proteinase inhibitors of host insects', *Virulence*, 1(3), 206-214.

Vilcinskas, A. and Götz, P. (1999) 'Parasitic fungi and their interactions with the insect immune system', *Advances in parasitology*, 43, 267-313.

Vilcinskas, A. and Matha, V. (1997) '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. and Götz, P. (1997a) '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(12), 1149-1159.

Vilcinskas, A., Matha, V. and Götz, P. (1997b) 'Inhibition of phagocytic activity of plasmatocytes isolated from Galleria mellonella by entomogenous fungi and their secondary metabolites', *Journal of Insect Physiology*, 43(5), 475-483.

Vining, L. C., Kelleher, W. J. and Schwarting, A. E. (1962) 'Oosporein production by a strain of Beauveria bassiana originally identified as Amanita muscaria', *Canadian Journal of Microbiology*, 8(6), 931-933.

Vogel, H., Altincicek, B., Glöckner, G. and Vilcinskas, A. (2011) 'A comprehensive transcriptome and immune-gene repertoire of the lepidopteran model host Galleria mellonella', *BMC genomics*, 12(1), 1.

Wainhouse, D., Boswell, R. and Ashburner, R. (2004) 'Maturation feeding and reproductive development in adult pine weevil, Hylobius abietis

(Coleoptera: Curculionidae)', *Bulletin of entomological research*, 94(01), 81-87.

Wainwright, M., Betts, R. P. and Teale, D. M. (1986) 'Antibiotic activity of oosporein from Verticillium psalliotae', *Transactions of the British Mycological Society*, 86(1), 168-170.

Wang, C. and St Leger, R. J. (2007) 'A scorpion neurotoxin increases the potency of a fungal insecticide', *Nature biotechnology*, 25(12), 1455-1456.

Watanabe, H. and Tokuda, G. (2010) 'Cellulolytic systems in insects', *Annual review of entomology*, 55, 609-632.

Whitten, M. M. A., Tew, I. F., Lee, B. L. and Ratcliffe, N. A. (2004) 'A novel role for an insect apolipoprotein (apolipophorin III) in β-1, 3-glucan pattern recognition and cellular encapsulation reactions', *The Journal of Immunology*, 172(4), 2177-2185.

Williams, C. D., Dillon, A. B., Ennis, D., Hennessy, R. and Griffin, C. T. (2015) 'Differential susceptibility of pine weevil, Hylobius abietis (Coleoptera: Curculionidae), larvae and pupae to entomopathogenic nematodes and death of adults infected as pupae', *BioControl*, 60(4), 537-546.

Williams, C. D., Dillon, A. B., Harvey, C. D., Hennessy, R., Mc Namara, L. and Griffin, C. T. (2013) 'Control of a major pest of forestry, Hylobius abietis, with entomopathogenic nematodes and fungi using eradicant and prophylactic strategies', *Forest Ecology and Management*, 305, 212-222.

Wojda, I. (2016) 'Immunity of the greater wax moth Galleria mellonella', *Insect science*.

Wojda, I., Kowalski, P. and Jakubowicz, T. (2009) 'Humoral immune response of Galleria mellonella larvae after infection by Beauveria bassiana under optimal and heat-shock conditions', *Journal of insect physiology*, 55(6), 525-531.

Wraight, S. P. and Ramos, M. E. (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(3), 139-150.

Wright, M. S., Raina, A. K. and Lax, A. R. (2005) 'A strain of the fungus Metarhizium anisopliae for controlling subterranean termites', *Journal of economic Entomology*, 98(5), 1451-1458.

Xiao, G., Ying, S.-H., Zheng, P., Wang, Z.-L., Zhang, S., Xie, X.-Q., Shang, Y., Leger, R. J. S., Zhao, G.-P. and Wang, C. (2012) 'Genomic perspectives on the evolution of fungal entomopathogenicity in Beauveria bassiana', *Scientific reports*, 2.

Xu, Y.-J., Luo, F., Gao, Q., Shang, Y. and Wang, C. (2015) 'Metabolomics reveals insect metabolic responses associated with fungal infection', *Analytical and bioanalytical chemistry*, 407(16), 4815-4821.

Yu, X.-Q., Gan, H. and Kanost, M. R. (1999) 'Immulectin, an inducible C-type lectin from an insect, Manduca sexta, stimulates activation of plasma prophenol oxidase', *Insect biochemistry and molecular biology*, 29(7), 585-597.

Zdybicka-Barabas, A. and Cytryńska, M. (2013) 'Apolipophorins and insect immune response', *Inv Surv J*, 10, 58-68.

Zdybicka-Barabas, A., Stączek, S., Mak, P., Piersiak, T., Skrzypiec, K. and Cytryńska, M. (2012) 'The effect of Galleria mellonella apolipophorin III on yeasts and filamentous fungi', *Journal of insect physiology*, 58(1), 164-177.

Zelensky, A. N. and Gready, J. E. (2005) 'The C-type lectin-like domain superfamily', *Febs Journal*, 272(24), 6179-6217.

Zhong, X. W., Zhao, P., Zou, Y., Nie, H. Y., Yi, Q. Y., Xia, Q. Y. and Xiang, Z. H. (2012) 'Proteomic analysis of the immune response of the silkworm infected by Escherichia coli and Bacillus bombyseptieus', *Insect Science*, 19(5), 559-569.

Zhu, J.-Y., Wu, G.-X. and Zhang, Z. (2014) 'Upregulation of coleoptericin transcription in Tenebrio molitor parasitized by Scleroderma guani', *Journal of Asia-Pacific Entomology*, 17(3), 339-342.

Zhu, J.-Y., Yang, P., Zhang, Z., Wu, G.-X. and Yang, B. (2013) 'Transcriptomic immune response of Tenebrio molitor pupae to parasitization by Scleroderma guani', *PLoS One*, 8(1), e54411.

Zhu, W., Smith, J. W. and Huang, C.-M. (2009) 'Mass spectrometry-based label-free quantitative proteomics', *BioMed Research International*, 2010.

Zhu, Y., Johnson, T. and Kanost, M. R. (2003) 'Identification of differentially expressed genes in the immune response of the tobacco hornworm, Manduca sexta', *Insect Biochem Mol Biol*, 33, 541-559.

Zhu, Y., Wang, Y., Gorman, M. J., Jiang, H. and Kanost, M. R. (2003) 'Manduca sexta serpin-3 regulates prophenoloxidase activation in response to infection by inhibiting prophenoloxidase-activating proteinases', *Journal of Biological Chemistry*, 278(47), 46556-46564.

Zimmermann, G. (1986) 'The ‘Galleria bait method’for detection of entomopathogenic fungi in soil', *Journal of Applied Entomology*, 102(1-5), 213-215.

Appendices

Appendix A: Sites used for field studies

Figure A.1.1. Year one field study site. Rossnagad, Laois. A clear-felled lodgepole pine forest

Figure A.1.2. Year two field study site. Glendine, Slieve Bloom Laois. A clear-felled lodgepole pine forest

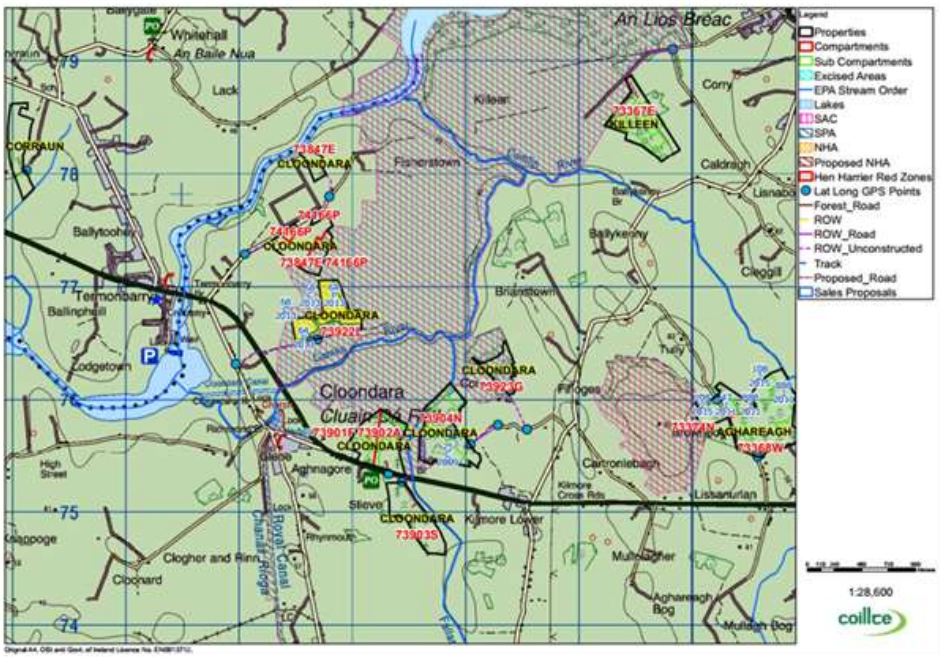


Figure A.1.3. Year three field study site. Cloondara, Laois. A clear-felled lodgepole pine forest

Appendix B: *H. abietis de novo* transcriptome study

B.1. Methodology

The methodology was as follows: After extraction of total RNA and treatment with DNase I, Oligo (dT) adapters were used to isolate mRNA. The mRNA was fragmented by mixing with the fragmentation buffer. cDNA was synthesized using the mRNA fragments as templates. Short fragments were purified and resolved with EB buffer for end reparation and single nucleotide A (adenine) addition. The short fragments were connected with adapters. The suitable fragments were selected for the PCR amplification. During the QC steps, Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System were used in quantification and qualification of the sample library. Then the library was sequenced using Illumina HiSeq 4000. After sequencing, the raw reads were filtered for low-quality, adaptor-polluted and high content of unknown base (N) reads to get clean reads. *De novo* assembly was performed using Trinity with clean reads to get the Unigenes. After that, simple sequence repeats (SSR) detection, Unigene expression analysis, Heterozygous single nucleotide polymorphisms (SNP) detection, and Unigene functional annotation were performed. Then with the functional annotation and expression results, a set of differentially expressed genes (DEG) were detected and functional enrichment

analysis between samples was performed. Unigenes were divided into two classes; clusters with the prefix "CL" (comprising several unigenes with sequence similarity of 70% and above) and singletons with the prefix "Unigene". A schematic overview of the process is shown in Figure B.1.1. and Figure B.1.2.

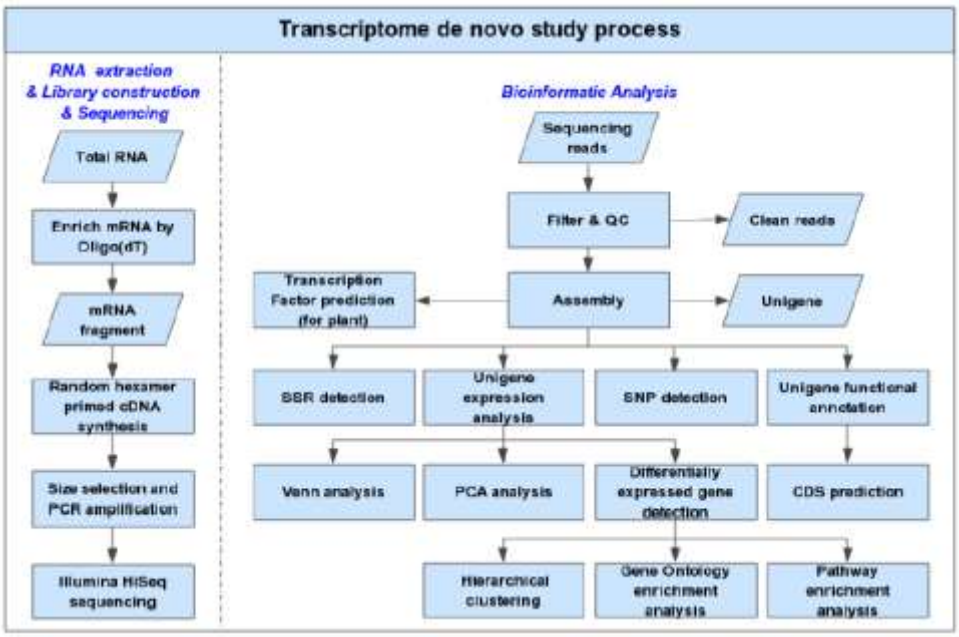


Figure B.1.1. Schematic overview of Transcriptome *de novo* study process.

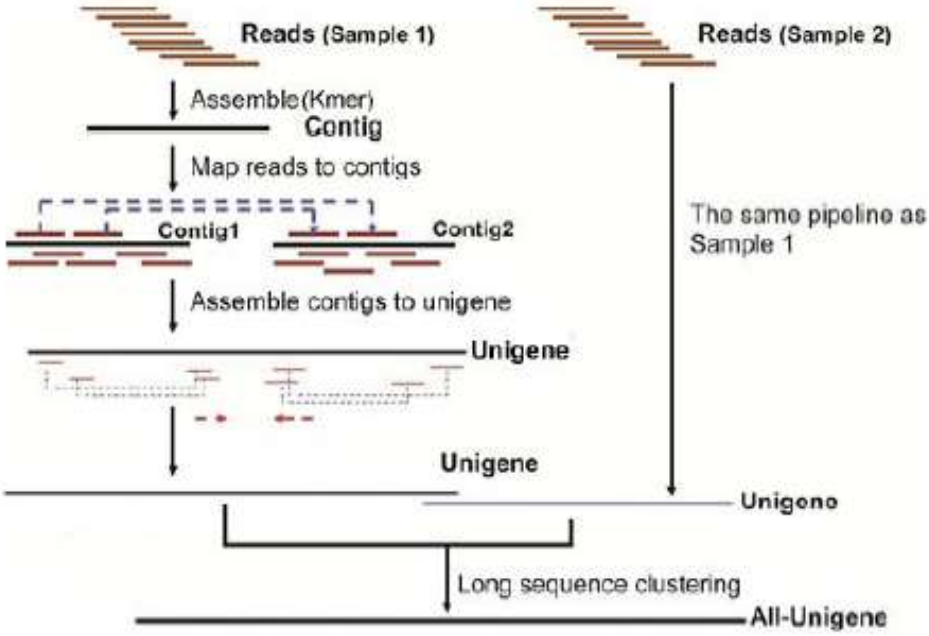


Figure B.1.2. Schematic overview of the assembly process

B.1. Results

Approximately 17.7 Gb bases in total were generated after Illumina Hiseq sequencing. After assembly 49,960 Unigenes were generated, with a total length of

59,001,875bp, an average length of 1,180 bp, N50 of 2,241 bp, and GC content 39.27 %. The unigenes were annotated with 7 functional databases; NR, NT, GO, COG, KEGG, Swissprot and Interpro. With functional annotation 27,653 CDS were detected, and after ESTScan with the remaining Unigenes, a further 2,936 CDS were found. 3,121 SSR distribute were detected on 2,788 Unigenes.

Sequencing reads containing low-quality, adaptor-polluted and high content of unknown base (N) reads were removed before downstream analyses. Reads quality metrics after filtering are shown as Table B.2.1. After reads filtering, Trinity was used to perform *de novo* assembly with clean reads, the assembly quality metrics are in Table B.2.2.. Tgicl was used to cluster transcripts to unigenes and the clustering quality metrics are shown in Table B.2.3. Unigenes were divided into two classes, one are clusters, which have the prefix CL and the other are singeltons, which have the prefix Unigene. Clusters are made up of unigenes with similairty between them of more than 70%. After assembly, functional annotations for Unigenes were performed with seven functional databases (NR, NT, GO, COG, KEGG, Swissprot and Interpro) (Table B.2.4.). The distribution of annotated species is shown in Figure B.2.1. After functional annotation, the segment of Unigenes that best mapped to functional databases was selected as its CDS. For Unigenes that remained annotated, ESTScan was used to predict CDS. Quality metrics of predicted CDS is shown in Table B.2.5.

Trinity was used to perform *de novo* assembly and Tgicl was used to cluster transcripts to Unigenes. Trinity's assembly combines three independent software modules: Inchworm, Chrysalis, and Butterfly that are applied sequentially to process large volumes of reads. Trinity partitions sequence data into many individual de Bruijn graphs, each representing the transcriptional complexity at a given gene, and uses parallel computing to reconstruct transcripts from these graphs (Haas *et al.* 2013). The resulting sequences of Trinity are transcripts. Gene family clustering was performed with TGICL to find Unigenes. TGICL is a pipeline for analysis of large Expressed Sequence Tags (EST) and mRNA databases in which the sequences are first clustered based on stringent pairwise sequence similarity to group those sharing significant regions of near identity, and then assembled by individual clusters to produce longer, more complete consensus sequences (Pertea *et al.* 2003).

Table B.2.1. Summary of sequecning reads after filtering

Q2: the rate of bases whose quality is greater than 20

Sample	Total Raw Reads (Mb)	Total Clean Reads (Mb)	Total Clean Bases (Gb)	Clean Reads Q20(%)	Clean Reads Q30(%)	Clean Reads Ratio (%)
Treated	88.58	88.55	8.85	96.78	92.42	99.97
Untreated	88.58	88.52	8.85	91.62	80.41	99.94

Table B.2.2.Quality metrics of transcripts.

N50: a weighted median statistic that 50% of the total length is contained in transcrpits greather than or equal to this value. GC (%): the percentage of G and C bases in all transcripts

Sample	Total No.	Total Length	Mean Length	N50	N70	N90	GC (%)
Treated	58,747	51,894,975	883	1,841	932	302	39.6
Untreated	72,507	62,439,592	861	1,811	904	290	39.15

B.2.3. Quality metricts of Unigenes.

N50: a weighted median statistic that 50% of the total length is contained in Unigenes greather than or equal to this value. GC (%): the percentage of G and C bases in all Unigenes.

Sample	Total No.	Total Length	Mean Length	N50	N70	N90	GC (%)
Treated	38,151	41,608,255	1,090	2,005	1,145	410	39.75
Untreated	44,326	48,592,227	1,096	2,050	1,166	409	39.30
All Unigene	49,960	59,001,875	1,180	2,241	1,298	445	39.27

Table B.2.4.Summary of functional annotation results.

Overall referes to the number of Unigenes which have been annotated with at least one functional database.

	Annotated								
Values	**Total**	**Nr**	**Nt**	**Swissprot**	**KEGG**	**COG**	**Interpro**	**GO**	**Overall**
No.	49,960	27,702	14,487	18,625	17,491	8,687	19,273	3,818	29,895
%	100	55.45	29	37.28	35.01	17.39	38.58	7.64	59.84

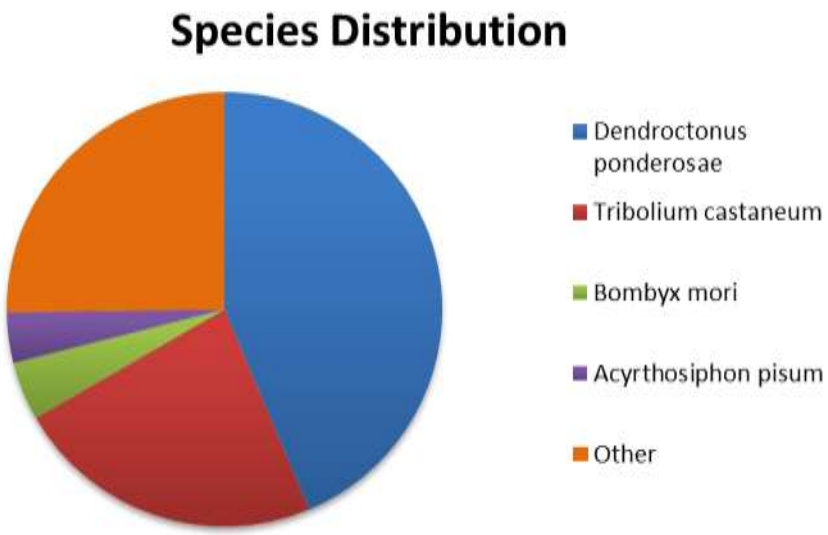


Figure B.2.1. Distrbution of annotated species.

Table B.2.5. Quality metrics of predicted CDS

Software	**Total No.**	**Total Length**	**Mean Length**	**N50**	**N70**	**N90**	**GC(%)**
Blast	27,653	28,694,835	1,037	1,665	1,041	447	43.66
ESTScan	2,936	1,115,403	379	393	282	222	42.66
Overall	30,589	29,810,238	974	1,602	978	402	43.62

APPENDIX C: LFQ of the effect of EPF supernatant on the *H. abietis* proteome

Table C.1.1.Uniquely detected proteins in the haemolymph of control larvae and larvae treated with EPF supernatant.

NaN indicates a protein that was absent or below the level of detection. These proteins were termed as being 'uniquely detected'. PEP: Posterior Error Probabilities. Mean LFQ intensity represents average of three biological replicates per sample group.

Protein ID	Annotation	Mean LFQ intensity				PEP[1]	Intensity	MS/MS	Peptides	Mol. Weight
		BB	BC	MET	SB					
Unigene3953	Prostatic acid phosphatase	NaN	25.1	NaN	NaN	3.9^{-88}	3.0^{+08}	25	7	42.7
CL2640.Contig1	Pathogenesis-related 5 (Thaumatin)	NaN	26.9	NaN	NaN	3.6^{-302}	3.3^{+09}	46	10	27.0
CL2700.Contig4	Carboxylesterase family	NaN	25.3	NaN	NaN	3.7^{-300}	5.6^{+08}	32	12	58.5
Unigene13343	Carboxylesterase family	NaN	27.1	NaN	NaN	3.0^{-95}	1.5^{+09}	32	10	60.4
CL3821.Contig1	C-type lection lectoxin-Enh3-like	NaN	24.5	NaN	NaN	8.0^{-38}	1.5^{+08}	9	2	16.9
CL4537.Contig3	CL4537.Contig3_All	NaN	27.8	NaN	NaN	0.0^{+00}	1.4^{+09}	20	2	12.6
CL820.Contig3	Alpha-L-fucosidase	NaN	24.9	NaN	NaN	3.7^{-55}	2.5^{+08}	30	10	49.1
Unigene2311	Alpha-L-fucosidase	NaN	25.4	NaN	NaN	6.5^{-121}	3.9^{+08}	28	8	49.1
CL2534.Contig1	Lectin C-type domain	23.6	NaN	23.4	24.5	1.5^{-48}	2.7^{+08}	33	6	16.0
CL4420.Contig1	Stabilin-1 isoform X1	23.2	NaN	23.1	22.9	6.3^{-165}	2.5^{+08}	39	11	42.8
CL3921.Contig1	Tropomyosin 1	NaN	NaN	23.9	NaN	1.3^{-240}	6.9^{+08}	38	17	32.7

Unigene10924	Single domain von Willebrand factor type C	NaN	NaN	23.7	NaN	2.5^{-41}	1.1^{+08}	16	5	17.3
Unigene12317	Haemolymph juvenile hormone binding protein	NaN	NaN	25.9	NaN	3.4^{-212}	8.5^{+08}	36	7	28.3
Unigene13634	Insect pheromone-binding family A10&OS-D	NaN	NaN	23.8	NaN	1.1^{-41}	1.9^{+08}	20	6	13.9
Unigene17410	JHBP	NaN	NaN	26.4	NaN	7.2^{-78}	1.3^{+09}	48	15	26.9
Unigene8077	Endocuticle structural glyco ABD-4	NaN	NaN	24.8	NaN	2.7^{-45}	2.1^{+08}	15	3	11.0
CL4960.Contig1	Sodium channel 60E	NaN	22.8	22.8	22.2	9.6^{-27}	7.1^{+07}	17	5	41.8
CL5881.Contig1	Actin	NaN	26.8	26.3	25.0	7.5^{-284}	1.4^{+09}	27	12	39.8
CL515.Contig1	CL515.Contig1_All	NaN	24.7	NaN	24.3	6.5^{-22}	3.2^{+08}	22	5	18.8
Unigene19514	Pectinesterase	NaN	27.7	NaN	22.4	1.5^{-260}	1.9^{+09}	63	12	39.8
Unigene5426	Myosin regulatory light chain 2	NaN	25.3	NaN	25.8	2.0^{-53}	7.0^{+08}	30	6	22.1
Unigene9562	Unigene9562_All	NaN	28.7	22.6	NaN	0.0^{+00}	5.6^{+09}	98	15	40.6
Unigene10626	Unigene10626_All	NaN	NaN	25.9	23.4	3.2^{-39}	5.7^{+08}	33	9	41.4
Unigene417	Aspartyl protease	23.6	24.4	NaN	23.6	1.2^{-25}	2.3^{+08}	25	5	47.7

Table C.1.2. Statistically significant differentially abundant and exclusively expressed proteins of altered abundance in *H. abietis* from Cluster A

MET and BB treated larvae had a higher level of abundance than SB and BC in the proteins in this Cluster.

	Cluster A	Average LFQ Intensities			
Protein ID	**Annotation**	**BC**	**MET**	**SB**	**BB**
CL1928.Contig3	Odorant-binding 29	32.9	33.5	32.6	32.9
CL1928.Contig2	Odorant-binding 29	31.7	33.1	31.2	31.9
CL2563.Contig1	Peroxidase isoform X1	27.0	27.0	26.7	27.3
CL3504.Contig2	Beta-1,3-glucan-binding	25.8	26.8	26.0	25.9
CL3921.Contig1	Tropomyosin 1	22.3	24.1	21.7	21.9
CL416.Contig1	Heat shock 70 kDa cognate 4	23.9	24.3	22.9	23.4
CL4420.Contig1	Stabilin-1 isoform X1	22.5	23.8	22.2	23.2
CL466.Contig4	Serine protease easter	24.8	25.5	23.8	24.8
CL492.Contig1	Attacin C	29.5	30.3	29.5	30.1
CL943.Contig7	Papilin-like Protein	23.3	24.8	24.0	25.0
Unigene10626	Unigene10626_All	22.8	25.6	22.9	22.9
Unigene10924	Single domain von Willebrand factor type C	22.5	23.6	22.4	23.2
Unigene12317	JHBP	22.3	26.1	21.6	22.3
Unigene13338	Trypsin	26.9	27.9	27.9	27.9
Unigene17410	JHBP	22.4	26.8	22.3	22.7
Unigene2302	Major royal jelly protein	28.7	29.7	28.5	28.6
Unigene27113	28 kDa desiccation stress	28.4	29.3	28.2	28.6
Unigene3665	FKBP-type peptidyl-prolyl cis-trans isomerase	25.9	26.3	24.7	26.0
Unigene7330	Attacin C	30.0	31.3	30.7	31.4
Unigene8018	Chemosensory 6	25.8	29.9	22.9	26.3
Unigene8077	Endocuticle structural glyco ABD-4	22.9	25.2	21.6	22.1

Table C.1.3. Statistically significant differentially abundant and exclusively expressed proteins of altered abundance in *H. abietis* from Cluster B

BC treated larvae had a higher level of abundance than SB, MET and BB in the proteins in this Cluster.

	Cluster B	Average LFQ Intensities			
Protein ID	**Annotation**	**BC**	**MET**	**SB**	**BB**
CL1864.Contig4	Glycosyl hydrolases family 2	25.8	22.9	22.8	23.2
CL2640.Contig1	Pathogenesis-related 5	28.0	22.1	22.4	23.0
CL2700.Contig4	Carboxylesterase family	26.2	22.3	22.3	21.8
CL3821.Contig1	C-type lection lectoxin-Enh3-like	24.6	22.6	22.2	21.9
CL4537.Contig3	CL4537.Contig3_All	27.6	21.6	20.4	23.7
CL5500.Contig2	Glycoside hydrolase family 48	32.5	25.0	22.7	22.6
CL5881.Contig6	Actin-5C	25.6	25.6	24.9	24.4
CL797.Contig4	Chymotrypsin-C isoform X1	23.8	23.6	23.1	23.1
CL820.Contig3	Alpha-L-fucosidase-like isoform X1	24.7	22.5	22.9	23.4
CL921.Contig2	Glycoside hydrolase family 31	27.5	23.4	22.1	23.7
Unigene10957	Glycosyl hydrolases family 79	25.7	23.3	21.6	22.8
Unigene11986	Glycoside hydrolase family 48	31.5	23.6	21.7	21.8
Unigene12087	Glycosyl hydrolases family 2	29.0	22.0	21.8	22.1
Unigene12565	Glycosyl hydrolases family 35	27.8	22.9	22.6	22.6
Unigene13343	Carboxylesterase family	26.9	22.7	22.0	23.1
Unigene13345	Glycosyl hydrolases family 20	24.8	22.4	22.3	22.9
Unigene13634	Insect pheromone-binding family A10&OS	23.8	23.8	23.0	22.2
Unigene13818	Glycosyl hydrolases family 28	29.1	22.0	22.5	23.4
Unigene19514	Pectinesterase	27.8	21.8	21.6	22.1
Unigene2311	Glycosyl hydrolases family 2	25.8	23.2	21.2	22.5

Unigene28106	Alpha-L-fucosidase	26.9	23.5	22.2	21.8
Unigene3825	Glycosyl hydrolases family 1	30.6	22.3	21.3	22.0
Unigene3841	Glycosyl hydrolases family 45	26.2	22.2	21.4	22.6
Unigene3946	Glycosyl hydrolases family 45	28.0	23.5	21.0	22.3
Unigene3953	Glycosyl hydrolases family 28	25.5	22.1	22.2	22.1
Unigene3970	Prostatic acid phosphatase	25.8	22.6	21.3	22.2
Unigene6962	Glycosyl hydrolases family 28	28.0	22.4	21.8	22.7
Unigene7925	Glycosyl hydrolases family 1	29.7	22.9	21.7	22.4
Unigene8511	Neutral alpha-glucosidase C	26.5	22.1	21.6	22.6
Unigene9562	Glycosyl hydrolases family 38	29.6	22.1	22.2	23.7
Unigene9634	Unigene9562_All	28.3	22.6	21.6	23.6
CL1864.Contig4	Glycosyl hydrolases family 45	25.8	22.9	22.8	23.2

Table C.1.4. Statistically significant differentially abundant and exclusively expressed proteins of altered abundance in *H. abietis* from Cluster C

MET treated larvae had a lower level of abundance than SB, BB and BC in the proteins in this Cluster.

	Cluster C	**Average LFQ Intensities**			
Protein ID	**Annotation**	**BC**	**MET**	**SB**	**BB**
CL2247.Contig3	Odorant binding	35.5	34.4	35.6	35.1
CL3607.Contig1	Kunitz & Bovine pancreatic trypsin inhibitor	33.1	32.1	33.3	32.9
CL4960.Contig1	Sodium channel 60E	22.8	22.1	22.3	22.7
Unigene1589	Trypsin	25.8	24.7	25.0	25.0
Unigene2445	Regulatory CLIP domain of proteinases	26.6	24.5	26.2	26.9
Unigene3489	Serpin	26.9	25.4	25.9	26.2
Unigene3995	Serine protease easter	28.6	27.9	28.8	28.6
Unigene4030	Pathogenesis-related 5	32.0	29.6	31.0	31.5
Unigene417	Eukaryotic aspartyl protease	23.6	20.9	23.6	23.3
Unigene5359	Serine protease persephone isoform X2	22.9	22.2	23.2	23.3
Unigene5426	Myosin regulatory light chain 2	23.3	21.4	25.6	22.9
Unigene6368	Unigene6368_All	23.0	22.7	24.2	23.2

Table C.1.5. Statistically significant differentially abundant and exclusively expressed proteins of altered abundance in *H. abietis* from Cluster D

Control larvae (SB) had the highest level of abundance in the proteins in this Cluster.

	Cluster D	Average LFQ Intensities			
Protein ID	**Annotation**	**BC**	**MET**	**SB**	**BB**
Unigene8093	Copper & zinc superoxide dismutase	23.0	22.7	24.2	23.2
CL1224.Contig1	Lectin C-type domain	24.8	25.8	26.8	26.8
CL1617.Contig2	Pro-phenol partial (Trypsin)	25.5	27.1	28.2	28.7
CL2420.Contig2	Melanin-inhibiting protein	28.5	28.3	28.7	29.3
CL2534.Contig1	Lectin C-type domain	22.9	23.3	24.4	23.3
CL3832.Contig2	Peptidoglycan-recognition SC2	27.3	26.7	27.4	28.0
CL515.Contig1	Diapause-associated transcript-2	22.6	23.3	24.4	21.8
CL5549.Contig2	Sodium channel 60E	24.4	25.0	25.0	25.3
CL5881.Contig1	Actin	26.8	24.9	25.4	22.7
CL61.Contig2	Arylphorin-like Hexamerin	26.8	27.2	28.8	24.1
Unigene11176	Peritrophic matrix 9 precursor	25.4	25.0	26.1	24.5
Unigene5303	Arthropod defensin	28.6	28.9	29.4	28.8
Unigene9585	Unigene9585_All	30.3	30.2	30.4	31.0

Appendix D. *H. abietis* larvae susceptibility to EPF spores

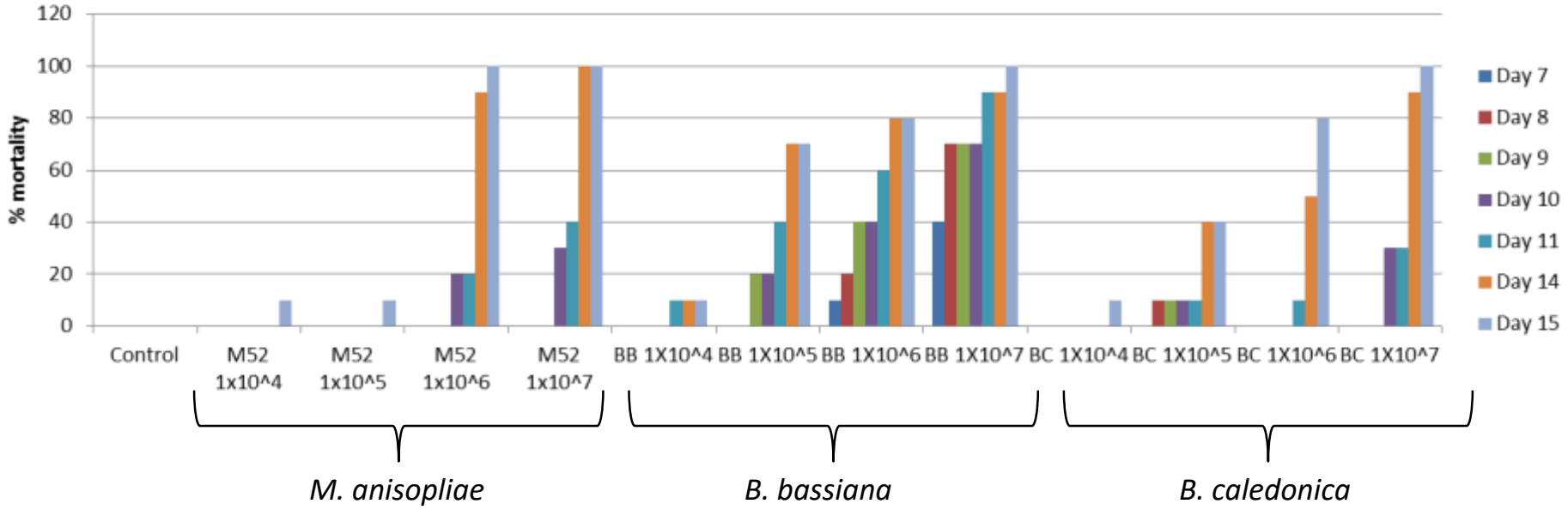


Figure D.1.1. Mortality of *H. abietis* larvae dipped in EPF spore suspensions.

Table D.1.1.Mortality of *H. abietis* larvae dipped in EPF spores after 15 days		
EPF Species	**Dose (spore concentration)**	**Mortality %**
Control	-	0
M. anisopliae	$1x10^4$	10
M. anisopliae	$1x10^5$	10
M. anisopliae	$1x10^6$	100
M. anisopliae	$1x10^7$	100
B. bassiana	$1x10^4$	10
B. bassiana	$1x10^5$	70
B. bassiana	$1x10^6$	80
B. bassiana	$1x10^7$	100
B. caledonica	$1x10^4$	10
B. caledonica	$1x10^5$	40
B. caledonica	$1x10^6$	80
B. caledonica	$1x10^7$	100