# Enhanced biocontrol options for the Australian sugar industry: a proteomic approach

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# Table of contents

## Abstract

The filamentous fungus *Metarhizium anisopliae* is a naturally occurring biological control agent of many insects including the greyback canegrub (*Dermolepida albohirtum*), a sugarcane pest in Australia. While there have been some gene-based approaches into identifying determinants for biological control and developing improved strains, this study provides a new comparative proteomics approach into identifying key proteins produced by *M. anisopliae* during infection of greyback canegrubs.

Pathogenicity-related proteins have been identified by both liquid and solid culture approaches using proteomic technologies. Proteome maps of healthy canegrubs, canegrubs infected with *Metarhizium* and fungus only were produced and analysed. Comparative proteome analysis of proteins produced in solid culture provided a view into cellular reactions triggered in the canegrub in response to *Metarhizium* infection. Some of the proteins identified included cytoskeleton proteins, proteases, peptidases, metalloproteins and proteins involved in signal transduction. Liquid culture approach was used to display secreted proteins of *Metarhizium* growing on the whole greyback canegrubs and their isolated cuticles. Proteins identified included 64-kDa serine carboxypeptidase, 1,3 β-exoglucanase, dynamin GTPase, THZ kinase, calcineurin like phosphoesterase and phosphatidylinositol kinase. These proteins have not been previously identified from the culture supernatant of *M. anisopliae* during infection. To our knowledge, this is the first proteomic map established to study the extracellular proteins secreted by *M. ansiopliae* (FI-1045), a strain currently used for biological control of greyback canegrubs.

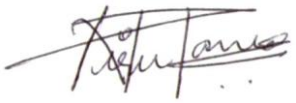
*Metarhizium anisopliae* strain FI-1045 was further subjected to UV mutagenesis to select mutants that can tolerate better environmental conditions such as varying temperature and pH

ranges. *M. anisopliae* mutant strain (NM10) was isolated and bioassays against greyback canegrubs proved that the mutant strain was more virulent than the parental strain. Two-dimensional electrophoresis was employed to display secreted proteins of the *M. anisopliae* mutant strain (NM10) growing on the whole greyback canegrubs and their isolated cuticles, in order to identify various proteins involved in infection of canegrubs. Eighty six secreted proteins were identified in this approach, amongst them six proteins that have not been previously identified from the culture supernatant of *M. anisopliae* during infection. These included the 56-kDa aspartyl aminopeptidase, 29-kDa secreted aspartyl protease, cyclin-dependent protein kinase, thymidylate kinase, septin and adenylate kinase. Finally, mutant strain NM10, generated by UV-mutagenesis was stably transformed and found to be highly resistant to benomyl, a commonly used fungicide in agriculture. Benomyl resistant transformants were found to tolerate 40 times higher concentration of benomyl than then amount that inhibits the parental strain. Laboratory bioassays proved that four transformants resistant to benomyl retained virulence characteristics against greyback canegrubs.

The proteomic methods established, developed and applied in this thesis proved their strength and suitability in the visualisation, detection and identification of proteins produced by the fungus during infection of greyback canegrubs. Genetic manipulation techniques such as mutagenesis and transformation methods described in this thesis demonstrated successful steps in improving the *Metarhizium* strain while retaining pathogenicity against the greyback canegrubs. Combining the proteomics data obtained in this work with other 'omics' data such as genomics, transcriptomics, metabolomics and bioinformatics, will lead to a more complete understanding of the biology of canegrub infection by *Metarhizium* at the molecular level.

## Declaration

This research thesis contains original work, which was performed by me. Many features of this research work have been conducted in partnership with others. These people have been acknowledged and their contributions have been recognised in the section where help was received. To the best of my knowledge it contains no material previously published and all information adapted has been promptly recognised in the text. No part of this thesis has been presented to any other institution for any other award. I consent to this thesis being made available for photocopy or for loan.



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## List of manuscripts for publication

This thesis is prepared according to journal style format and on the following articles, referred to in the text by the Roman numerals listed below. Manuscripts of these articles have been sent to different journals for publication.

**Manuscript I:**

Manalil NS, Te'o VSJ, Braithwaite K, Brumbley S, Samson P, Grinyer J, Nevalainen H (2008) Proteomic map of the greyback canegrub, *Dermolepida albohirtum* (Waterhouse).
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**Manuscript II:**

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**Manuscript III:**

Manalil NS, Te'o VSJ, Braithwaite K, Brumbley S, Samson P, Nevalainen H (2009) Comparative analysis of the *Metarhizium anisopliae* secretome in response to exposure to the greyback canegrubs and grub cuticles.
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**Manuscript IV:**

Manalil NS, Te'o VSJ, Braithwaite K, Brumbley S, Samson P, Nevalainen H (2009) Proteomic analysis of the secretome of *Metarhizium anisopliae* in response to exposure to the greyback canegrubs and grub cuticles.
*Under review in Current Genetics, submitted in September 2009.*

# Abbreviations

Abbreviations frequently used in the text are:

| | |
|---|---|
| 2D | Two-dimensional |
| 2-DE | Two-dimensional electrophoresis |
| 3D | Three-dimensional |
| AMP's | Antimicrobial peptides |
| BCA | Biological control agent |
| BHC | Benzene hexachloride |
| BT | *Bacillus thuringiensis* |
| BV | Baculoviridae |
| C7BzO | 3-(4-Heptyl)phenyl 3-hydroxy propyl dimethyl ammonio propane sulfonate |
| CBB | Coomassie brillitant blue |
| CHAPS | 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane sulfonate |
| CID | Collision-induced dissociation |
| CSI | Cross species identification |
| DIGE | Differential gel electrophoresis |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| DTX | Destruxin |
| EPF | Entomopathogenic fungi |
| eV | Collision energy |
| FS | Fluorescent stains |
| GV | Granulosis viruses |
| IEF | Isoelectric focusing |
| IPG | Immobilised pH gradient |
| IPM | Integrated pest management |
| kDa | Kilodaltons |
| LC | Liquid chromatography |
| MALDI | Matrix assisted laser desorption ionisation |
| MCA | Microbial control agent |
| MS | Mass spectrometry |
| MS/MS | Mass spectrometry/mass spectrometry |
| m/z | Mass to charge ratio |

| | |
|---|---|
| MudPIT | Multi-dimensional protein identification technology |
| MW | Molecular weight |
| NAG | N-acetylglucosaminidase |
| NMR | Nuclear magnetic resonance |
| NVP | Nuclear polyhedrosis viruse |
| PAGE | Polyacrylamide gel electrophoresis |
| PEG | Polyethylene glycol |
| PCR | Polymerase chain reaction |
| pI | Isoelectric point |
| PMF | Peptide mass fingerprinting |
| PR | Protease |
| PTM | Post-translational modification |
| QqQ | Triple quadrupole |
| QTOF | Quadrupole time of flight |
| SDS | Sodium dodecyl sulphate |
| TBP | Tributylphosphine |
| TOF | Time of flight |

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# 1. General Introduction

In modern agriculture, extensive monoculture plantings generate conditions that facilitate the rapid growth of insect pest populations. The current insect pest management programs heavily rely on the use of chemical insecticides or pesticides and herbicides. There is increasing concern about the continuous use of chemical pesticides at high dosage rates against key agricultural pests is neither sustainable nor desirable from an economic, human health or environmental perspective (Lacey and Goettel, 1995). The aspiration of maintaining high levels of agricultural yield and profit while reducing pesticide use presents a significant challenge. Research and development strategies should focus on increasing the implementation of integrated pest management (IPM) practices on all crops, emphasizing the benefit of culture strategies and biological control as the first line of defence against pests (Kogan, 1998). The main motivation behind using chemical insecticides is because they are readily available, rapid acting, highly reliable and a single application may control several different pest species.

Biological control refers to the use of biological control agents (BCA) such as fungi, bacteria, viruses and protozoa for the management of pests. Amongst BCA, fungi are the predominant pathogens found in insect populations. They are generally harmless to beneficial insects and non-target organisms and pose minimal risk to humans and the environment (Roy and Pell, 2000) and have other desirable traits like they leave no toxic residues on crops. Furthermore, their host-specific nature and unique ability to infect their hosts through the external cuticle boosts their potential role in management of pests. The initial step in tapping this unique potential of fungi is the isolation and identification of a virulent pathogen (Zimmerman, 2006). To ascertain usefulness, the pathogen must remain viable in the formulated form after application in the field. Genetic improvements may expand their potential as BCA by increasing virulence and resistance to environmental extremes (Jackson, 1999).

## 1.1. Entomopathogens used as bio-insecticides

Entomopathogens such as bacteria, viruses, nematodes or fungi contribute to the natural regulation of many populations of arthropod pests. Much of the research in this area relates to the causal agents of insect diseases and their exploitation for biological pest control. Many entomopathogens have been mass produced, formulated, and applied to pest populations in a manner analogous to chemical pesticides.

### 1.1.1. Entomopathogenic bacteria

Even though there are about 100 species of insect pathogenic bacteria that have been documented, only specific *Bacillus* species have revealed their potential to control important agricultural pests (Martin and Travers, 1989). Of these, *Bacillus thuringiensis*, *B. moritai*, *B. sphaericus*, *B. lentimorbus* and *Paenibacillus popilliae (*previously known *B. popilliae*) have been used as commercial insecticides. *B. thuringiensis* (*Bt*) has the longest history and is one of the oldest bacteria used as a microbial pest control agent that has been commercialized world wide on a large scale. *B. thuringiensis* is an aerobic, spore forming bacterium, easy to grow on various media as well as cheap waste products from various food processing industries. During sporulation the bacterium produces a spore as well as a large proteinaceous crystal. This protein crystal, when ingested by an appropriate insect pest, damages the gut lining leading to paralysis. The affected insect stops feeding and dies from the combined effects of starvation and tissue damage. Toxic proteins vary depending on the different subspecies of *Bt*, which yields a variance in *Bt* toxicity to a variety of insect species (Attathom et al. 1995). *B. thuringiensis* var. *kurstaki* is ideal for controlling Lepidoptera, including hornworms, armyworms, diamondback moth, spruce budworm, bagworms and Indian meal moth larvae (Miller, 1990). *B. thuringiensis* var. *israelensis* is a highly specific biological pesticide used against Diptera, including mosquitoes (traded as Mosquito Dunks), black fly and fungus gnat larvae (Ghosh et al. 1988). *B. thuringiensis* var. *sandiego* and *B.*

*thuringiensis* var. *tenebrionis* are toxic against leaf-eating beetle species and elm leaf beetles. *P. popilliae* and *B. lentimorbus* have been mass-produced for the control of Japanese beetle larvae in turfs (Redmond and Potter, 1995). The disease on beetles caused by bacteria is known as "milky disease". Moreover, several toxin genes from *B. thuringiensis* have been used in transgenic crops such as cotton, corn and potatoes (Payne and Kim, 1992).

### 1.1.2. Entomopathogenic viruses

The insect-specific viruses, the Baculoviridae (BV) family have been recognized previously as an environmentally safe, potential alternative to chemical pesticides. These viruses are highly host specific and non-pathogenic to beneficial insects and other non-target organisms including mammals, thus making them attractive candidates for integrated pest management (IPM) (Carbonell and Miller, 1987). A characteristic trait of these viruses is that they produce significantly large, highly refractile particles (virions) called occlusion bodies or polyhedra within the host cell. The Baculoviridae are a family of large rod-shaped viruses that can be divided into three groups depending on the type of viral occlusion bodies they form. Group A contains nuclear polyhedrosis viruses (NPV's) which produce intranuclear occlusions containing multiple virion particles. Group B contains granulosis viruses (GV's) which produce intracytoplasmic occlusions containing one virion particle. Group C represents a small group of baculoviruses that have one nucleocapsid per envelope and do not produce occlusion bodies (Boucias et al. 1989). NPV's and GV's are arthropod-specific pathogens and the majority of the baculoviruses are infectious only for insect species within the order Lepidoptera, with no adverse effect on members of other orders (Wood and Granados, 1991). Exploitation of NPV's of *Autographa californica* (Alfalfa looper), *Anagrapha falcifera* (Celery looper) and *Bombyx mori* (Silk worm) for the control of mulberry silk worm is of great economic importance to silk farmers and has escalated the use of baculovirus for biocontrol (Acharya et al. 2002).

### 1.1.3. Entomopathogenic nematodes

Nematodes are plain, colorless, unsegmented roundworms visible to the naked eye and may be free living, parasitic or predatory in nature. Parasitic nematodes cause significant diseases in plants, animals and humans (Kaya and Gaugler, 1993). Some species are capable of infecting important agricultural pests, but only a few can cause insect death. Entomopathogenic nematodes of the families *Steinernematidae* and *Heterorhabditidae* are obligate parasites of insects and are associated with their symbiotic bacteria (*Xenorhabdus* for *Steinernematidae* and *Photorhabdus* for *Heterorhabditidae*), which represents a unique mode of biological control (Georgis et al. 1991). Nematodes parasitize their hosts by penetrating the cuticle or enter through natural openings. Once inside the body of the insect, symbiotic bacterium are released from the nematode gut, quickly multiplying within the insect body and causing insect death within 48 hours (Boemare et al. 1996). Bacteria produce a variety of hydrolytic enzymes and a wide range of toxins to break down the insect body (Kaya and Gaugler, 1993). Many species of nematodes are currently used as biological insecticides. *Steinernema carpocapsae* is effective against lepidopterous larvae, including webworms, cutworms, armyworms and wood-borers. *S. feltiae* attacks dipterous insects, including mushroom flies, fungus gnats, and tipulids. *S. kushidai* and *S. glaseri* are known to parasitize scarab larvae. *S. riobravis* is effective against a wide range of insects but is currently marketed for suppression of plant parasitic nematodes infesting turfgrass. *S. scapterisci* is the only species used as a biocontrol agent to suppress adult crickets. *Heterorhabditis bacteriophora* and *H. megidis* appear most useful against root weevils, particularly black vine weevil (Georgis and Hague, 1991).

### 1.1.4. Entomopathogenic fungi

Entomopathogenic fungi (EPF) are attracting attention as biological control agents against insects and as one component within integrated pest management systems. There are more

than 750 species of insect pathogenic fungi and approximately 100 genera that are highly pathogenic to insects, which are widely distributed throughout the fungal kingdom. The majority of them occur in the Deuteromycotina and Zygymycotina (Clarkson and Charnley, 1996), recently assigned to Sordariomycetes. Some insect pathogenic fungi have restricted host ranges, for example, *Erynia neoaphidis* infects only aphids in temperate regions (Morgan et al. 1995), while other fungal species have a wide host range, for example *Metarhizium anisopliae* and *Beauveria bassiana* infect over 200 species of insects in nine orders (Roy and Pell, 2000). The potential of fungal pathogens for the control of insect pests has been recognised since the late part of the 19$^{th}$ century when *M. anisopliae* was tested against the wheat cockchafer *Anisoplia austriaca* and sugar beet curculionid *Cleonus punctuventris*. Over a decade, more than 100 microbial products are available worldwide for use in horticulture, agriculture and forestry. Some of the currently registered biopesticides based on entomopathogenic fungi and their targeted hosts are shown in Table 1.

**Table 1**. **Currently registered biopesticides based on entomopathogenic fungi**

| **Fungus** | **Product name** | **Targets** | **Country** |
|---|---|---|---|
| *Beauveria bassiana* | Mycotrol | Whiteflies, thrips | USA |
| | Bassiana | Caterpillers | Columbia |
| | Biorin | Thrips | India |
| *Metarhizium anisopliae* | BIO 1020 | Vine weevil | Germany |
| | BioCane | Greyback canegrubs | Australia |
| | Green guard | Locust, grasshoppers | Australia |
| | Green muscle | Locust, grasshoppers | South Africa |
| *Metarhizium flavoviride* | BioGreen | Red headed cockchafer | Australia |
| *Paecilomyces fumosoroseus* | PFR-97™ | Whiteflies, thrips | USA, Europe |
| | AGO Biocontrol *Paecilomyces* | Nematodes | Colombia |
| *Verticillium lecanii* | Vertalec/Mycotal | Aphids, thrips | UK |

## 1.2. The entomopathogenic Sordariomycete, *Metarhizium anisopliae*

*Metarhizium anisopliae*, formerly known as *Entomophthora anisopliae*, is a fungus that occurs naturally in soils throughout the world and causes disease in various insects by acting as a parasite; it thus belongs to the entomopathogenic fungi (Driver et al. 2000). The disease caused by the fungus is called "green muscardine" disease because of the green coloured spores. Different strains of *M. anisopliae* are highly adapted to particular host insects (Clarkson and Charnley, 1996). *M. anisopliae* strains have been isolated from a variety of insects worldwide. *M. anisopliae* hosts a various order of insects such as Coleoptera, Lepidoptera, Orthoptera and Hemiptera. However, this broad range disguises the fact that individual strains of the same fungal species have varying host ranges and specificities. The target pests are termites, locusts, grasshoppers, cockroaches, spittlebugs, noctuids, scarabs, curculionids, white flies, thrips, mosquitoes and ticks. Spores of *M. anisopliae* are long-lived in the soil and provide long term suppression of pests (Zimmermann, 2006). Details of *Metarhizium* taxonomy can be sourced at http://www.uniprot.org/taxonomy/5530.

### 1.2.1. The lifecycle of *Metarhizium anisopliae*

*M. anisopliae* is a dimorphic fungus with invasive hyphal stages as well as a yeast-like vegetative stage (blastospore). Each phenotype has unique characteristics that contribute to pathogenicity of the fungus towards insects. In *M. anisopliae*, switching from one morphological stage to another both *in vivo* and *in vitro* is not usually well coordinated. Thus the hyphal and blastospore stages often occur simultaneously during growth of the fungus (Dillon and Charnely, 1985). In the absence of a suitable insect host, *Metarhizium* enters into a vegetative lifecycle that involves germination, filamentous growth and the formation of conidiophores, which produce mature primary conidia (Arruda et al. 2005). In the presence of a susceptible insect host, *Metarhizium* switches to the pathogenic lifecycle. In the life cycle of *Metarhizium* (Figure 1B), the spores (conidia) must land on the surface of a suitable host

where they attach and then germinate. Generally a limited amount of surface growth occurs before the fungus produces an appressorium (a swelling at the end of a germ tube) (Figure 1A and 1C). These events herald the start of invasion or infection process (Clarkson and Charnley, 1996, St. Leger et al. 1989 and 1991). Detailed description of the events in the infection process is provided below.

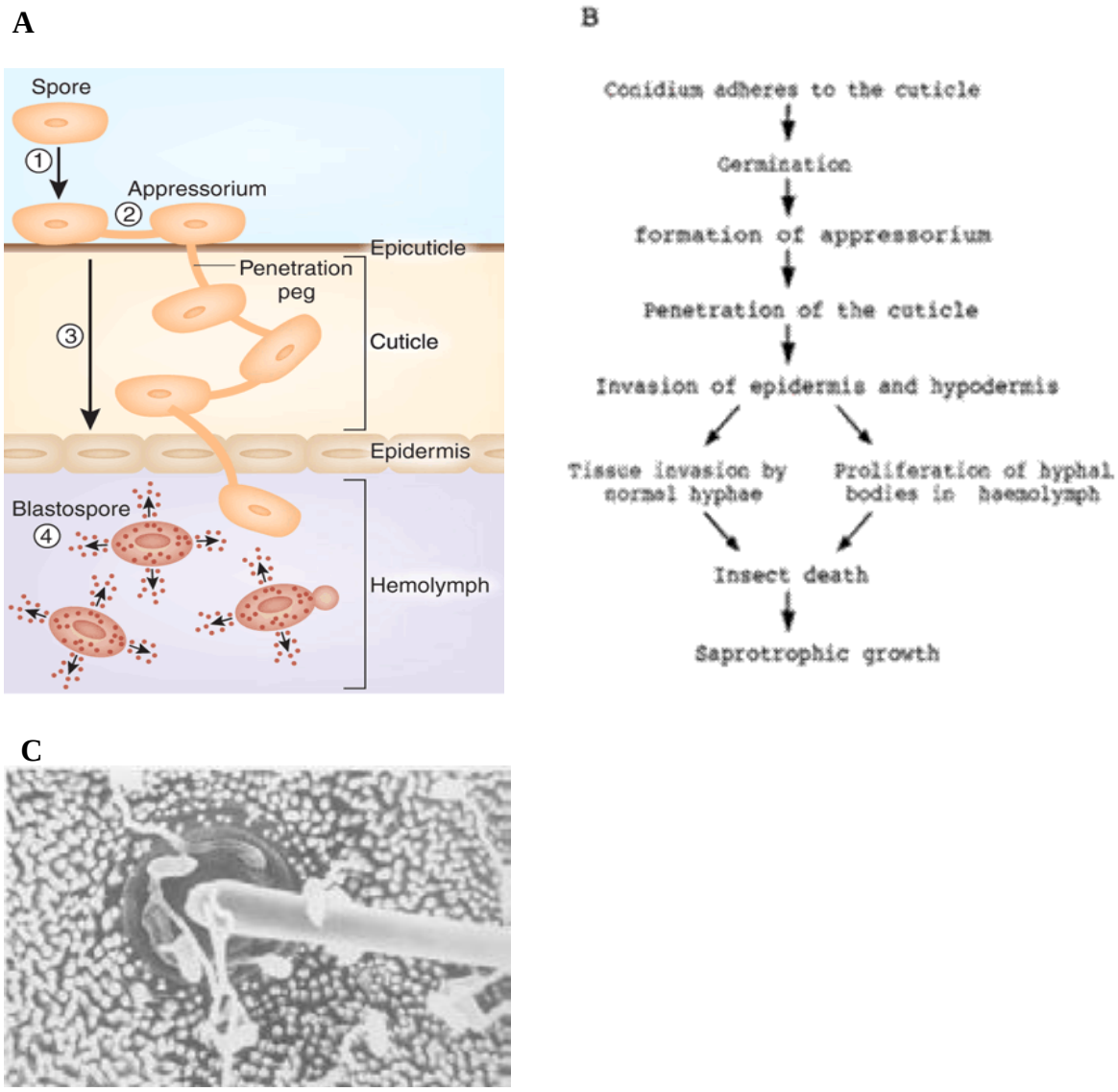


**Figure 1: The infection process of *M. anisopliae***

(A) Structure of the insect cuticle and mode of penetration. Picture adapted from Thomas and Read (2007). (B) Flow diagram of the infection steps. Picture adapted from Clarkson and Charnley (1996). (C) Scanning electron micrograph showing that the appressorium has formed at the base of the caterpillar hair socket. Picture adapted from Clarkson and Charnley (1996).

#### 1.2.1.1. Adhesion and germination of conidia

Contact between fungal conidia and the insect host is the prerequisite for the establishment of an infection process. Conidia are initially attached via a passive process with the aid of hydrophobic forces. Based upon the physical nature of the conidial outer walls, the entomopathogenic conidia have been divided into two categories, wet and dry conidia (Boucias and Pendland, 1991). The outer walls of the dry aerial conidia of *Metarhizium* comprise of rodlets that are organized in a firm interlaced fascicles, responsible for the adhesion on the surface of the host cuticle. The distinctive nature of these rodlets is that they are found only in the conidial stage. The rodlet layer is very robust and can be only disrupted with a sonicator or with chemical treatments. Boucias et al. (1988) demonstrated that the resistance of the rodlet layers varies among conidia of various entomopathogenic fungi. Conidia should possess surface hydrophobic properties as well as specific spore surface antigens that allow for specific binding to the cuticle. Lectins, a type of carbohydrate binding glycoproteins have been detected on the condial wall of *Metarhizium*. Lectins could therefore mediate specific recognition between fungal conidia and a sugar residue on the host cuticle that would result in the initial recognition event (Rath et al. 1995). Distribution and germination of conidia on the insect cuticle may be subjected to the availability of nutrients, oxygen, water, pH and temperature. Cuticular fatty acids have a profound effect on the conidial germination and differentiation as they are toxic, fungistatic or stimulatory in nature (Butt et al. 1995). Entomopathogenic fungi with a relatively broad host range produce conidia which germinate in response to nonspecific sources of carbon and nitrogen, such as amino acids, carbohydrates, proteins and a range of cuticular lipids. Entomopathogenic fungi with a restricted host range develop specific requirements for germination (St. Leger, 1993). Finally, conidia with faster germination rates are capable of enhancing infection by reducing the chances for desiccation and competition with other microorganisms (Dillon and Charnley, 1985).

### 1.2.1.2. Germ tube development and appressorium formation

Entomopathogenic fungi invade their hosts by direct penetration of the host cuticle through a combination of enzymatic and mechanical processes. Fungal proliferation on the host cuticle requires the formation of specific infection structures, which include germ tube development followed by appressorium formation as shown in Figure 1C (St. Leger, 1989). The hyphal tip of *M. anisopliae* secretes a fluid which enhances the  of germ tubes to the epicuticular surface of the host (St. Leger et al. 1988). Studies have demonstrated that *Metarhizium* appressoria are induced *in vitro* on hard hydrophobic surface with limiting nutrient conditions (Butt et al. 1995). Nutrient availability and topographical sensing or thigmotropic signal sensing are often required for fungal induction.

### 1.2.1.3. Penetration of the cuticle

Pathogenic fungi need to penetrate through the cuticle into the insect body to obtain nutrients for their growth and reproduction. During this process many entomopathogenic fungi, in particular *Metarhizium* spp. and *Beauveria* spp., secrete an array of degradative extracellular enzymes that facilitate infection in the host (Paterson et al. 1994). Synthesis of extracellular enzymes such as proteases, lipases and chitinases on host cuticles often determines important fungal traits such as pathogenicity or virulence factors (St. Leger et al. 1986). Amino and carboxyl peptidases, endoproteases (termed PR1, PR2, PR3 and PR4) and esterases are produced during the initial phase of penetration, concurrent with appressorial formation. Initially, N-acetylglucosaminidases (NAG), chitinases and lipases are produced at low levels and later they accumulate to high levels at the site of proteolytic degradation (St. Leger et al. 1987 and 1993). The most studied penetration protein from *M. anisopliae* is PR1, a serine protease. PR1 solubilises the proteinaceous insect cuticle and is produced in large amounts by infection structures. In addition, PR1 is known to have toxic effects in the host hemolymph through activation of the prophenoloxidase system (St. Leger et al. 1996). Protease deficient

mutants of *M. anisopliae* demonstrate reduced virulence emphasizing the importance of extracellular enzymes in fungal pathogenicity (Wang et al. 2002).

#### 1.2.1.4. Production of toxins

Many of the biologically active mycotoxins that have been studied are produced by entomopathogens (Kaijiang and Roberts, 1986). Successful infection of insects by *Metarhizium* is enhanced by the production of toxins known as destruxins (DTX's), a group of cyclic peptides containing ester linkages. Over 20 toxins have been isolated from *Metarhizium*, among which A, B and E predominate, and E is the most toxic to insects. DTX's are insecticidal and various application strategies are adopted for testing the effect of DTX on various insect species (Dumas et al. 1994). The most widely used method is injection or forced feeding and toxicity is most acute among Lepidopteran larvae and Dipteran adults. DTX's causes a variety of physiological effects such as degeneration of muscle membranes, including paralysis by directly or indirectly activating calcium channels (Samuels et al. 1988). *Metarhizium* exhibits both inter/intra-specific variation in destruxin production and this characteristic is useful in isolating a virulent strain. However, destruxin production may not be the only strategy used by *Metarhizium* (Amiri-Besheli et al. 2000, Kershaw et al. 1999).

### 1.2.2. Host defence systems

Insects are mostly resistant to microbial infections even though they do not have an acquired immune system capable of recognizing and eliminating foreign antigens. The impervious and hard cuticle, the biochemical environment of the midgut fluid, peritrophic matrix and tracheal system of the midgut together form an effective mechanical and physiological barrier for protecting the insect's body cavity against fungal invasion (Butt et al. 1995). Relatively low humidity in the gut is a limiting factor for the germination of spores and proliferation of the fungus inside the respiratory tract. The insect cuticle functions both as an exoskeleton and as

barrier between living tissues and the environment. The cuticle consists of a waxy layer containing fatty acids, lipids, sterols and phenol stabilized proteins which have a potent antifungal action. The cuticle is damaged enzymatically by the growing hyphae, allowing access to the hemolymph. The dynamic response to fungal invasion involves localized melanisation on the cuticle around the penetrating hyphae, followed by cellular and humoral immune reactions of the host to the presence of fungi in the hemolymph and production of protease inhibitors (Gillespie et al. 1997).

Melanisation is a process by which phenolic compounds are oxidised to dihydrophenylalanine, characterised by the production of black or brown pigments in insects in response to fungal infection. Prophenoloxidase is a key enzyme in melanisation reactions and investigation in other insects signifies that phenoloxidase is present in the hemolymph as an inactive precursor (Zou et al. 2005). The activation of phenoloxidase has vital roles in wound healing and detection of foreign materials in the hemolymph. In mosquitoes, resistance to malarial infection is linked with encapsulation and melanisation of invading parasites, and six prophenoloxidase genes have been recognised in *Anopheles gambiae* (Lee at al. 1998). Cuticular melanisation provides an efficient strategy for defence only against weak and slow growing fungi and is ineffective against virulent strains.

The cellular response of insects to the presence of fungi in the hemolymph includes phagocytosis, nodule formation and encapsulation. These processes are mediated by hemocytes. Inhibition of phagocytic activity in *Galleria mellonella* by *M. anisopliae* and *B. bassiana* is primarily through ingestion of blastospores by plasmatocytes during the initial stage of infection (Vilcinskas et al. 1997). Complex processes such as cellular encapsulation and nodule formation occur when foreign pathogens are too large or too abundant to be ingested by phagocytotic cells. Insect hemocytes such as plasmatocytes and granular cells can

differentiate between self and non-self structures. The ability to recognise non-self structures may be mediated by surface molecules lectins, which bind to polysaccharides such as β-1-3 glucans, a major component of fungal cell walls. Numerous β-1-3 glucan binding proteins with varying molecular weights have been identified in a variety of insects (Matha et al. 1990; Ma and Kanost, 2000). Inoculation of *M. anisopliae* or *B. bassiana* blastospores elicits cellular responses in insects. However, encapsulated blastospores of these fungi are not inactivated completely in the insect hemolymph. Moreover, formation of nodules is inadequate to hinder the development of these pathogens (Hoffmann, 1995).

The humoral response in insects is indicated by a rapid synthesis of antimicrobial peptides or immune proteins by the fat body. Antimicrobial peptides have broad and overlapping specificities which restrict the progression of microorganisms. Numerous peptides which are directly or indirectly involved in inhibiting microbial growth have been identified, characterised and their genes have been cloned from many insects (Meister et al. 1997). At least two major pathways are involved in controlling humoral response, Toll pathway and Imd pathway. The Toll pathway plays a role in the proliferation of hemocytes which controls the defence against fungal molecules. It is known to involve several extracellular proteolytic cascades leading to localised melanisation and coagulation of hemocytes and synthesis of potent antimicrobial peptides (AMP's) by the fat body (De Gregorio et al. 2002). However, the Imd pathway appears to control the synthesis of antibacterial peptides.

Once the fungus proliferates vegetatively in the hemolymph it impairs the function of the host immune system. Fungal reliance on extracellular catabolic processes to supply nutrients for invading fungal cells elevates the probability of the host developing ways to hinder or postpone mycosis by opposing proteolytic enzymes. Protease inhibitors have been observed and detected in various insect tissue homogenates as well as in the hemolymph (Polanowski et

al. 1992). Work conducted by Jiang and Kanost (1997) revealed that 45 kDa serpins present in the *M. sexta* hemolymph are able to inhibit the PR1 and PR2 enzymes from *M. anisopliae*. An inducible metalloprotease inhibitor, an 8.4 kDa thermostable, glycosylated protein, was detected in the hemolymph of a *G. mellonella* larva during *M. anisopliae* infection (Wedde et al. 1998). This protein inactivates thermolysin-like metalloprotease released by *M. anisopliae* during the infection process. The metalloprotease inhibitor contributes in defence reactions in two ways. Firstly, it protects hosts against cell and tissue damage caused by invading fungal proteases. Secondly, it prevents unnecessary activation of phenoloxidase by fungal proteases (St. Leger et al. 1994).

Most work has been concentrated on inducible fungal protease inhibitors and there is less evidence for the presence of specific antifungal proteins. The majority of work has been conducted on *Drosophila*, where a major antifungal protein, drosomycin, has been isolated (Ferrandon et al. 1998). This protein is not only produced by the fat body but also expressed in a number of epithelial tissues. Another novel inducible proline-rich peptide, Metchnikowin, has been isolated from *Drosophila* with both antifungal and antibacterial (gram positive bacteria) properties (Lemaitre et al. 1995). Other antifungal proteins isolated from various host insects are cecropins, defensins and lysozymes. These proteins have a profound effect on fungal cell membranes as they interfere with a variety of metabolic processes including glycolysis and respiration. In addition, these proteins are also capable of detoxifying fungal secondary metabolites (Vilcinskas and Gotz, 1999).

## 1.3. Canegrubs and the Australian sugar industry

Sugarcane (*Saccharum* spp.) is a giant member of the grass family, which has been cultivated in Australia for more than 160 years. Sugarcane is currently grown from Mossman in far North Queensland to Grafton in northern New South Wales, with the majority grown in

Queensland. Sugar is Australia's largest cash crop with approximately four million tons of raw sugar exported each year. Sugarcane industries worldwide have faced a major crisis of pest and disease resurgence at one time or another, leading to considerable losses in sugar production. The Australian sugar industry spends billions of dollars in controlling the major pests and diseases of sugarcane (Anon, 2004). The major pests and diseases that cause losses in sugarcane production include canegrubs, vertebrates such as native rats and feral pigs, and various bacteria, fungi and viruses.

Sugarcane white grubs or canegrubs, larvae of endemic melolonthine beetles have been pests of Australian sugarcane ever since the crop was cultivated (Griggs, 2005). There are 19 species of canegrubs placed in four genera, *Dermolepida*, *Lepidiota*, *Antitrogus* and *Rhopaea*. Due to their diversity in life cycle, distribution, behavior and ability to damage the underground root system of the plant, canegrubs are the most significant pest of the Australian sugar industry (Figure 2). Canegrubs cost the sugar industry over a billion dollars a year in damage and control costs (Allsopp and Chandler, 1989).

**Figure 2: Damage caused by Greyback canegrubs in the Burdekin river area**

Picture of sugarcane fields highlighting patchy and brown areas damaged by canegrubs. Undamaged areas are seen as dark green patches. Picture adapted with permission from BSES Photo library – Greyback canegrub Management.

The greyback cane beetle, *Dermolepida albohirtum* (Waterhouse), is a native Australian beetle and a pest of the sugarcane in northern Queensland (Figure 3) and has been detected on a wide range of soil types since the 1870s (Robertson et al. 1995). Adult beetles eat the leaves of sugarcane but actual damage is the done by their larvae, which are known as greyback canegrubs (Figure 4).


**Figure 3: Adult Greyback cane beetle**

*Dermolepida albohirtum* (Waterhouse), commonly known as 'greyback' because white scales on the wing coverings of the beetle (adult stage) gives a greyish apprearence. Picture adapted with permission from CSIRO, AICN Collection: http://www.ento.csiro.au/aicn/name_c/a_1933.htm

**Figure 4: Greyback canegrub larvae**

The larvae have a large, fleshy, white to translucent body with a brown head capsule and large jaws. They have conspicuous large brown legs on the thorax and back posterior end. They usually curl into a C-shape when exposed. Picture adapted with permission from the BSES Photo library – Greyback canegrub Management.

Damage results from the larvae feeding on the roots of the sugarcane plant, leading to retarded growth, reduced yield, lodging, loss of stools at harvest, poor ratooning and in extreme cases plant death (Figure 5). Damage resulting from Greyback canegrubs often occurs in patches and this is attributed to the different soil types in cane growing areas. This variability is evident within fields as well as in different parts of the cane-growing area. Up to one million tonnes of cane ($38 million) are lost to damage each year by Greyback canegrubs (Hunt et al. 2002). Factors which trigger outbreaks or suppress population densities of canegrubs are poorly understood.

**Figure 5: Greyback canegrub damage to sugarcane plant**

Canegrubs destroy root systems by their feeding and tunneling activities. Damaged stools are deprived of roots, lose anchorage and are easily pulled out of the ground. Picture adapted with permission from BSES Photo library – Greyback canegrub Management.

### 1.3.1. Life cycle of greyback canegrubs

Greyback canegrubs have a one year life cycle (Figure 6) (Griggs, 2005). Adult beetles emerge after good rain periods between October to February. They fly after dark to feed on trees, particularly figs, wattles, palms, jackfruit and bananas. After feeding for 14 days, they mate and female cane beetles lay 20-30 eggs in a clutch, 20-45 cm deep among the roots of the sugarcane plants. After two weeks, the eggs hatch and first instar grubs feed for four weeks on organic matter and roots, usually close to the surface of the soil. Second instar

grubs gather under the cane stools and feed on the roots for the next five weeks. Third instar grubs feed heavily on the roots and stools and grow rapidly during February to May when they cause the most damage. After three to four weeks, fully fed grubs burrow down and form chambers in the soil. Grubs turn into pupae between July and October and beetles develop within one month. They remain in the chamber until suitable weather conditions trigger their emergence.

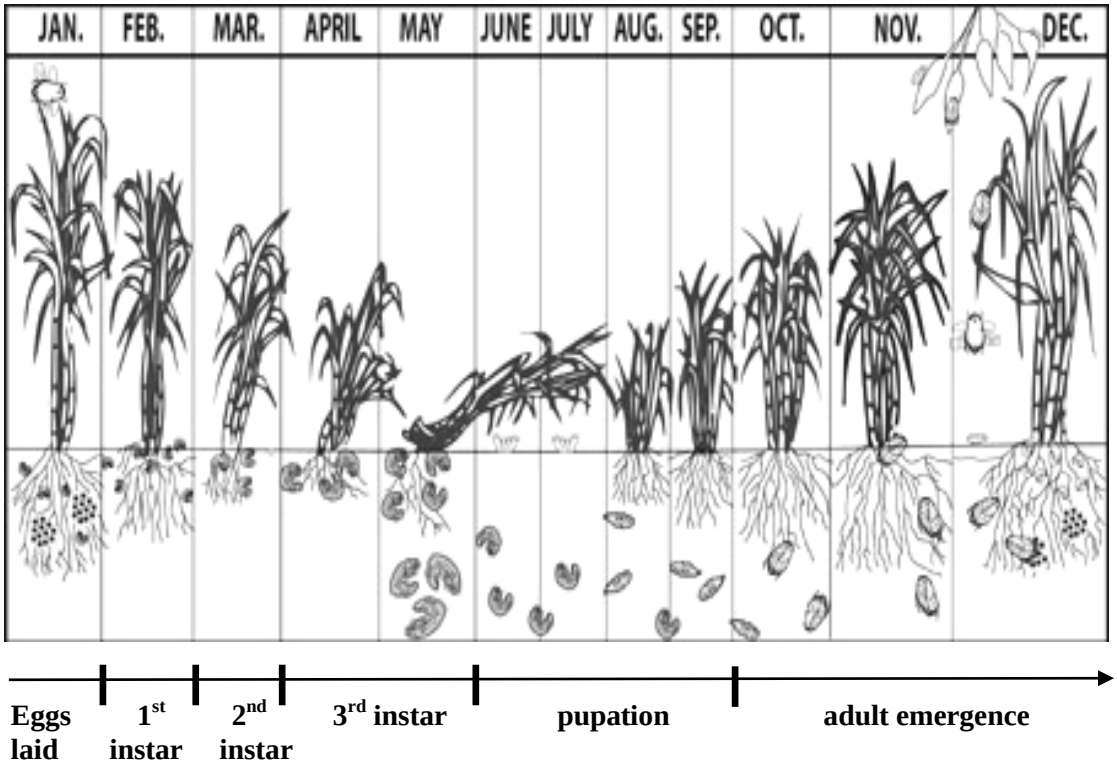


**Figure 6: Life cycle of greyback canegrubs**

Typical one year life cycle of greyback canegrubs and clear evidence showing reduction of cane roots during the development of canegrubs. Picture adapted with permission from the BSES Photo library – Greyback canegrub Management.

### 1.3.2. Control strategies for greyback canegrubs

Controlling the population densities of canegrubs does not rely on a single control strategy. A combination of cultural, chemical, biological and microbial control strategies is beneficial for a productive, profitable and sustainable sugar industry.

### 1.3.2.1. Cultural control strategies

Cultivation and fallowing (land which has been plowed but not sown with seeds) have been used to control canegrubs, mainly in summer when the larvae are in the upper soil levels (Allsopp and Chandler, 1989). Fallowing as a control measure has limited success because canegrub populations are not completely destroyed. Similarly, delayed irrigation and inter-row cultivation of infested ratoons are ineffective because canegrubs are concentrated under the living cane plants (Robertson et al. 1995). Green-harvesting of cane and retention of trash may help with canegrub management, as observations suggested that greyback canegrub population was much higher where trash was burnt compared to retaining all trash each year (Anon, 1993). Early attempts to control canegrubs in Queensland included the use of light-traps, hand collection of beetles from feeding trees and hand collection of larvae after cultivation. Despite collection and destruction of *D. albohirtum* beetles, the species remained a pest in the cane growing areas (Robertson et al. 1995). Cultural control of canegrubs may be possible in low-rainfall areas where irrigation is practiced. Delayed irrigation during summer when larvae are small and at shallow depths may kill the larvae through desiccation. Waterlogging after drought may increase the mortality of eggs and young larvae. The effects of manipulating irrigation on canegrubs and the crops need to be monitored through the different trials of research.

### 1.3.2.2. Chemical control strategies

In early 1947, the organochlorine insecticide benzene hexachloride (BHC) was first introduced for controlling canegrubs (Robertson et al. 1995). Other organochlorine insecticides used against greyback canegrubs were heptachlor, dieldrin and aldrin. These products were later withdrawn from use as residues were found in exported foodstuffs although they were not found in sugar (Robertson et al. 1995). Currently there are three types of chemical insecticides registered for greyback canegrub control in Australia: suSCon Blue

and suSCon Plus (controlled release formulations of the organophosphate chlorpyrifos), suSCon Maxi (controlled release formulation of imidacloprid), and Confidor Guard and related products (liquid formulations of imidacloprid). SuSCon Blue can give control for one or more years but results may be inconsistent due to inappropriate application of granules and accelerated loss of active ingredient in certain soil types. Chlorpyrifos is relatively immobile in soil and does not move far from the granules so placement of granules is critical for optimum control (Robertson et al. 1998). A formulation of sulphur-coated controlled release insecticide, suSCon Plus, provided grub control in alkaline soils, and new imidacloprid-based insecticides are now being used extensively in the industry. The possible development of resistance in greyback canegrubs to insecticides is a major drawback for chemical methods.

#### 1.3.2.3. Biological control strategies by natural enemies

All species of Australian canegrubs are native and have natural enemies such as predators and parasites. Native insect predators of canegrubs include scoliid wasps, dexiid and tachinid flies and wireworms (Robertson et al. 1995). There are three important approaches in biological control of pests. Importation of a biological agent is the first approach. Giant cane toads (*Bufo marinus*) were imported from Central America and introduced to North Queensland to reduce canegrub population. It turned out that cane toads did not have any impact on canegrubs, but in turn have become a major pest spreading throughout Queensland and threatening the survival of many native animal species (Anon, 1938). The second approach to biological control is augmentation, which is the manipulation and intensification of existing natural enemies to increase their effectiveness. This can be accomplished by mass production and periodic release of natural enemies in affected areas. The third approach is conservation which involves detection and modification of factors that limit the effectiveness of natural enemies. This approach is best suited for small scale processing but practically unfeasible for large scale processing.

### 1.3.2.4. Microbial control strategies

Microbial pesticides are naturally-occurring or genetically altered bacteria, fungi, algae, viruses or protozoans. They suppress pests by producing toxins, instigating a disease or inhibiting growth of other microorganisms through competition. Success of microbial control depends on highly specific interactions between the pathogen and insects where the pathogen can overcome the host defences. These pathogens are naturally occurring with the ability to persist in the soil and recycle through the host populations (Samson et al. 2005). Selection of hypervirulent strains with environmental competence is a key step in microbial control of soil dwelling pests. Screening of isolates, formulation, standardisation, mass production, quality control and field evaluation are important phases in successfully releasing microbial pesticides. The only microbial pesticide used in Australia is based on *Metarhizum anisopliae* (Griggs, 2005) and BioCane$^{TM}$ (FI-1045) is a *Metarhizium* based product used for the control of Greyback canegrubs in Australia (Samson et al. 1999) and BioCane$^{TM}$ infected greyback is shown below (Figure 7).

**Figure 7: Greyback canegrub infected with *Metarhizium anisopliae***

Third instar greyback canegrub infected with *M. anisopliae* spores (BioCane$^{TM}$). Picture adapted with permission from the BSES Photo library – Greyback canegrub Management.

BioCane$^{TM}$ is a rice based granule covered with *Metarhizium* spores and is applied directly to the sugarcane plants. It has been shown that the devastating effects caused by greyback

canegrubs were greatly reduced with the application of BioCane$^{TM}$ which explains the use of microbial pesticides in agriculture. The main difficulty in the use of microbial pesticides arises in maintaining spore viability when exposed on the soil surface, due to combined effects of heat, desiccation and ultraviolet light. In addition, soil is a highly complex, competitive environment where survival of pathogens is uncertain (Samson et al. 2001). The above mentioned problems pose particular challenges for successful biological control of canegrubs in Australia.

## 1.4. Prospects of exploiting entomopathogenic fungi as bio-control agents

In the past, significant amounts of chemical pesticides including persistent organic compounds were used for pest control. In recent years there is increasing concern over reliance of chemical pesticides because of their effects on man, wildlife and environment. These concerns have brought back the interest on the search for biotic agents that can control important pests of crops. In recent years, some of the most significant progress has come from studies into insect pathogenic organisms, particularly those of entomopathogenic fungi. Entomopathogenic fungi are potentially the most versatile biocontrol agents. The exploitation of mycoinsecticides such as entomopathogenic fungi is still in an early phase, although a relatively large number of pathogens have been assessed and few have been commerialised or are currently on the market. Some of the beneficial characteristics and constraints of using entomopathogenic fungi are listed below.

Beneficial characteristics of using entomopathogenic fungi as microbial insecticides include:

1. The microbial control agents (MCA's) serve as alternatives to chemical insecticides.
2. The toxic action of microbial insecticides is often specific to a single group or species of insects and this specificity means that most microbial insecticides do not directly affect beneficial insects (including predators or parasites of pests) in treated areas.

3. The high degree of host specificity makes them ecologically and environmentally desirable.
4. The MCA's are harmless to humans and leave no toxic residues in the environment.
5. The insect populations do not develop resistance to microbial insecticides as they can with chemical insecticides.
6. The microbial pathogens are capable of reproduction and have the ability to multiply rapidly in the field and also cycle through pest populations.
7. The MCA's have high persistence in the environment and help in the regulation of pest resurgence.
8. The microbial pathogens can be used in conjunction with chemical pesticides because in majority of the cases, microbial products are not deactivated or damaged by chemical residues in the field.
9. The MCA's are amenable to genetic engineering, increasing the number of microbial strains for possible development as insecticides.

Constraints of using entomopathogenic fungi as microbial insecticides include:

1. The narrow host range prevents their use to control multiple pests that cycle through same field.
2. The speed of kill is a major limiting factor as microbial insecticides require more than 2-3 weeks to kill the insects whereas chemical insecticides may have an instant effect or 2-3 days to knock down the pests completely.
3. The challenging environmental conditions such as low relative humidity, extremes of temperatures, low host numbers and application of antagonistic fungicides may affect pathogen performance and application.
4. The microbial insecticides usually involve high manufacturing costs such as infrastructure costs, production costs when compared to chemical insecticides.

5. Finding correct formulation which could overcome difficulties such as lack of environmental persistence and infection dynamics.
6. The restrictions involved in the process of registration and release of recombinant entomopathogens.

Entomopathogenic fungi, often not realised by man, are doing extraordinary jobs in controlling certain pests without any interference. This part of the "balance of nature" is being inadvertently destroyed by continuous use of chemical insecticides. A factor that may be holding back the improvement of biological control using entomopathogenic fungi is the lack of research on the evolution and functional ecology of fungi with biocontrol potential. Identifying both beneficial and negative traits of the fungus will enrich our understanding of how entomopathogenic fungi infect their hosts and it will certainly increase their acceptance as means of pest control.

## 1.5. Genetic engineering to improve strains of entomopathogenic fungi

Biocontrol experiments with pathogenic fungi are often effective in the laboratory but the level of control achieved in the field is sometimes unpredictable and this has often hindered commercial development of bioinsecticides. The recent development of molecular techniques for genetic engineering of filamentous fungi provides new opportunities for the study of fungi used in biological control. The isolation of genes encoding pathogenicity or virulence determinants will allow rigorous testing of their role in pathogenesis and provide a rational basis for strain improvement by direct genetic manipulation. Entomopathogenic fungi represent a huge untapped source of pesticide genes and insecticidal toxins and genetic engineering of entomopathogenic fungi offers enormous potential to provide alternative control methods for insect pests (Zimmermann, 2006). Extensive use of these fungi for

biocontrol depends upon advancement of wild type and mutant strains by genetically altering the characteristics of different strains (Heale et al. 2006).

Many different strains of *Metarhizium* spp. and *Beauveria* spp. have been registered worldwide and offer particular promise as suppressive agents for many soil insect pests; thus making them an ideal candidates for genetic improvement. Insect pathogens secrete a plethora of enzymes/toxins and these enzymes/toxins are encoded by single genes that are highly amenable to genetic manipulation (Zimmermann, 2006). The addition and expression of pesticidal genes in *M. anisopliae* is quite straightforward. One best-studied example is insertion of additional copies of the gene encoding a cuticle-degrading protease (Pr1) from *M. anisopliae* into the *Metarhizium* genome and over-expression of this protease in the hemolymph of *M. sexta*, activating the prophenoloxidase system (St. Leger, 1996). The infected insects were rapidly melanised and resulted in reduced food consumption by 40% compared to the wild type fungus.

Molecular biology also offers necessary tools for improving the efficacy, survivability and expansion of the host range of recombinant strains. Genetic transformation is usually achieved through a vector system which contains a selection marker and allows the selection of cells that have been successfully transformed. Three different methods employed to transform *M. anisopliae* using benomyl resistance as a selectable marker are the use of protoplasts with polyethylene glycol (PEG), electroporation and biolistic transformation (Bogo et al. 1996). Genetic engineering is expected to produce hypervirulent strains against target insect pests by increasing the speed of kill, improving formulations for ease of application, increasing environmental persistence and shelf life, and improving interaction with the environment and other IPM components. Engineered strains will be evaluated for their virulence and those

accepted will be released for commercial use in agriculture for insect pest control (Hu and St. Leger, 2002; St. Leger, 2008).

## 1.6. Microbial proteomics

The term proteome was first coined by Dr. Marc Wilkins at Macquarie University in 1994 and described as the 'entire complement of proteins expressed by a genome, cell, tissue or organism' (Graves and Haystead, 2002). More specifically, it is the set expressed proteins at a given time under defined conditions (Anderson and Anderson, 1998*).* The proteome varies between organisms depending on the genome and on external and internal conditions such as the physiological state, health, disease and stress. Compared with the genome, the complexity of the proteome is much greater due to protein processing and modification. Proteomics involves the systematic study of proteins in order to provide a comprehensive view of the structure, function and regulation of biological systems (Asthon et al. 2001). Even though proteomics as a field is fairly new, the methodologies in proteomics have been under development for decades. Proteomics is a scientific discipline that bridges the gap between genome sequence and cell behavior by studies into dynamic range of protein products of the genome, protein interactions, protein modifications, protein functions and protein localisation.

The proteome is highly dynamic, changing constantly with time and external conditions. In a single genome the proteomes of different tissues, body fluid and cell types vary. For example, the proteome of an organelle varies from that of the proteome of cell surface in the same organism (Patterson and Aebersold, 2003). Moreover, proteins interact to create functional networks which are made up of various species of interacting molecules. The diversity of the proteome is also enhanced by identifying post-translational modifications (PTM) of proteins such as phosphorylation, glycosylation, acylation and methylation (Johnson and Gaskell, 2006). Such modifications are important for the proper functioning of many proteins and also

in regulating numerous cellular processes. The rapid advancement of proteomics in the modern era of biotechnology is greatly driven by a combination of sophisticated techniques (Figure 8) including two dimensional electrophoresis (2-DE), image analysis, mass spectrometry, amino acid sequencing and bioinformatics to resolve, quantify and characterise proteins (Blackstock and Weir, 1999). The proteomics field is equally powered by bioinformatic tools that have improved the characterisation and reliability of tandem mass spectrometry (Huang et al. 2002).

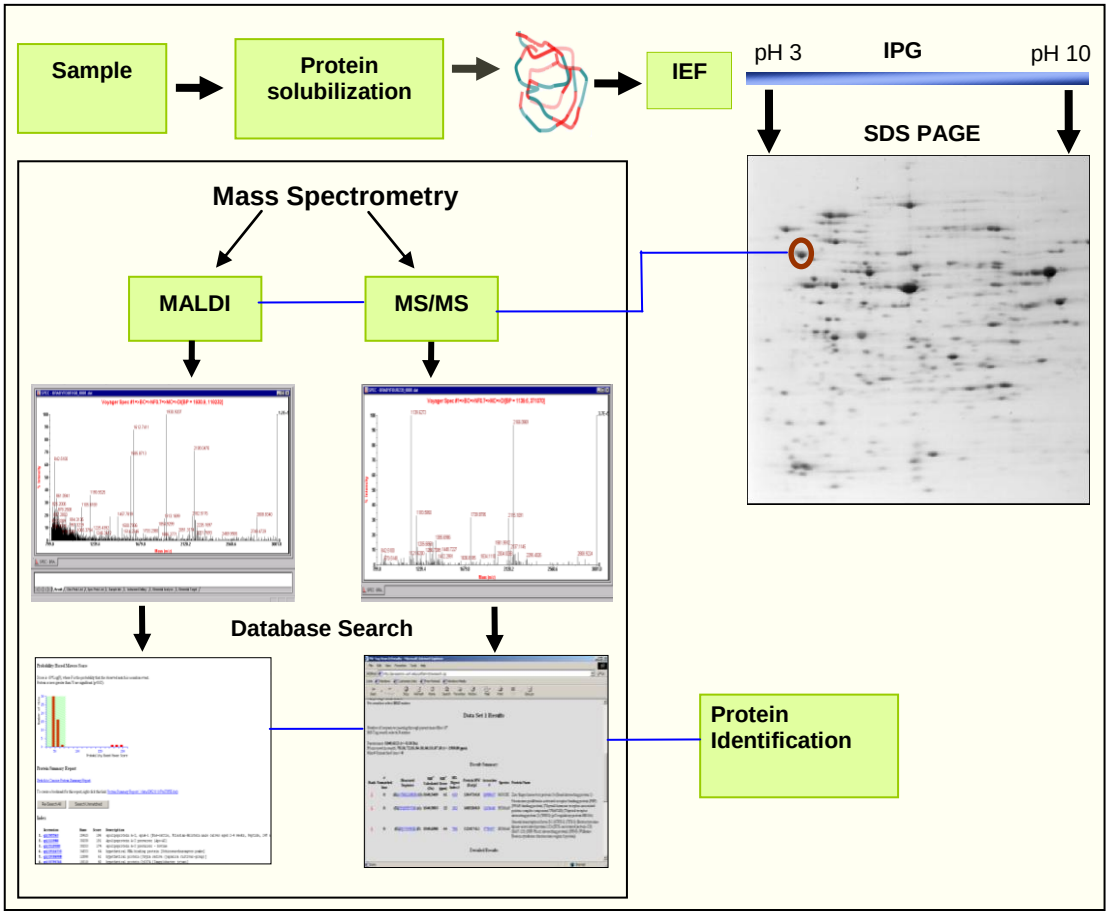


**Figure 8: Workflow of proteomic technologies involving proteome analysis by2-DE gels**

Proteins extracted from the cell are first solubilised and then separated according to their pI and subsequently according to their MW. Protein spots of interest are identified by MALDI or tandem MS. The peptide mass fingerprint or amino acid sequence is then compared against databases to identify the protein.

In the context of this study, proteomics portrays an excellent approach to study the host genome in action through the evaluation of the host proteome through the host-pathogen (canegrub-*Metarhizium*) interaction process. Inadequate proteomic information is available regarding the biochemical and physiological interactions in many host–pathogen systems (Thomas et al. 2005) but using proteomic tools, host proteome responses in reaction to pathogen invasion can be detected and identified (Asthon et al. 2001; Barrett et al. 2000). Similarly, proteomic analysis of the pathogen (filamentous fungus, *Metarhizium*) helps in identifying pathogenicity related proteins. This is achieved by studying the whole cell proteome as well as the secretome (proteome of the secreted proteins) of *M. anisopliae* grown in media containing either whole canegrubs or isolated cuticles of canegrubs. Gel sets showing protein maps can be investigated for qualitative and quantitative differences such as differences between spot intensities, up/down regulated proteins, post-translational modifications and detection of unique proteins synthesised during host-pathogen interaction. A review of the most relevant proteomic applications used in this study addressing canegrub-*Metarhizium* interactions is presented.

## 1.7. Types of proteomics

Proteins are complex organic macromolecules that can be studied in various different frameworks such as sequence, structure, interactions, expression, localisation and modification (Blackstock and Weir, 1999). Therefore, proteomics can be divided into three types: profiling or expression proteomics, structural proteomics and functional proteomics.

### 1.7.1. Profiling or expression proteomics

The study focuses on the description of the whole proteome (physical cell maps) in a given tissue, body fluid, cell type or organelle (Asthon et al. 2001). Information from this approach can be used to identify disease specific proteins and novel proteins, for example in signal

transduction. In recent times, studies on insect immunity have concentrated on profiling/expression proteomics in response to microbial infections. Examples include analysis of whole cell proteomes of *Drosophila melanogaster* (Vierstraete et al. 2003 and 2005; Levy et al. 2004; Karlsson et al. 2004), *Anopheles gambiae* (Prevot et al. 2003; Kalume et al. 2005), *Aedes aegypti* (Biron et al. 2005) and *Bombyx mori* (Wang et al. 2004). Similarly, this approach has been successfully adopted by many researchers to study the difference between whole-cell proteomes as well as the secretomes in filamentous fungi. Grinyer et al. (2004) were the first to provide a whole-cell proteome of the mycoparasite *T. harzianum* as well as the secretome of *T. atroviride* (Grinyer et al. 2005) grown in media containing either *R. solani* cell walls or glucose. Of late, several fungal proteomic studies have begun to appear in literature for many genera including *Aspergillus*, *Botrytis*, *Beauveria*, *Saccharomyces* and *Magnaporthe* (Kim et al. 2007).

### 1.7.2. Structural proteomics

Structural proteomics also known as structural genomics, attempts to identify all proteins within a protein complex or organelle, verify their location and characterise all protein-protein interactions (Norin and Sundström, 2002). Information from this approach will help to assemble the overall design of the cells and elucidate how expression of some proteins gives a cell its unique characteristics (Peng and Gygi, 2001). This approach relies on the wealth of genomic and protein sequences present in the sequence databases like GenBank (http://www.ncbi.nlm.nih.gov/Genbank/submit.html), EMBL (The European Molecular Biology Laboratory Nucleotide Sequence Database, http://www.embl-heidelberg.de/), DDBJ (DNA Data Base of Japan, http://www.ddbj.nig.ac.jp/), Swiss-Prot (Swiss Institute of Bioinformatics, http://au.expasy.org/sprot/) and TrEMBL (Translated European Molecular Biology Laboratory Nucleotide Sequence Database, http://www.ebi.ac.uk/uniprot/). The study of three dimensional (3D) protein structures is supported by technologies such as X-ray

crystallography and nuclear magnetic resonance (NMR) and bioinformatics software's (Hochstrasser, 1998).

### 1.7.3. Functional proteomics

Functional proteomic studies aim to define the function of every protein in a given organism in a particular cellular state (MacBeath, 2002). The study also concentrates on the changes in protein abundance and modification during the differentiation, proliferation and signaling of cells, in both qualitative and quantitative terms. It also includes studies of the coordinated expression of proteins, as well as the elucidation of the sequence of regulatory events during all stages which a cell or an organism undergoes during its entire lifespan (Graves and Haystead, 2002). A functional proteomics approach is being used routinely to study various host-pathogen interactions and identify virulence-associated proteins or proteomic alterations in host response. The recent advancement of 'proteome chips' where purified proteins expressed from a genome can be immobilized at high density on flat surfaces to give 'protein chips', which will aid high-throughput characterization of the biochemical and functional properties of cellular proteins (Blackstock and Weir, 1999). This approach mainly focuses on the exploration of pathogenicity mechanisms and has been extensively used for many filamentous fungi such as *Aspergillus fumigatus* (Kim et al. 2008), *Trichoderma harzianum* (Ambrosino et al. 2004), and the yeasts *Candida albicans* (Rupp, 2004) and *Saccharomyces cerevisiae* (Ito et al. 2001). A functional proteomics approach has been used to study insect immune responses to microbial challenges. Most of these studies have been carried out in *Drosophila melanogaster* (Benting et al. 2000; Vierstraete et al. 2003), *Tribolium castaneum* (Richards et al. 2008), *Anopheles gambiae* (Koutsos et al. 2007) and *Bombyx mori* (Ote et al. 2004).

## 1.8. Two-dimensional electrophoresis (2-DE)

The two-dimensional electrophoresis (2-DE) technique developed by Klose (1975) and O'Farrel (1975) is considered as the backbone of proteomics. Two-dimensional electrophoresis is a powerful tool used routinely for separating complex protein mixtures into their individual components. In this approach, proteins are separated in the first dimension according to their charge or isoelectric point (pI) by isoelectric focusing (IEF) using immobilized pH gradient (IPG) strips. During IEF, an electric field is applied over the IPG strip and the proteins migrate along the pH gradient in the strip until they reach the pH at which their overall charge is neutral (pI of the protein). IPG strips are regularly used to separate µg to mg quantities of protein mixtures. The development of IPG technology for the first dimension of 2-DE has revolutionised the reproducibility and resolving power of this procedure, and is now the system of choice for various applications (Gorg et al. 2000). Some of the features and benefits of IPG technology are: (i) using narrow and wide range pH gradient strips with overlap options which are available in different lengths and allow optimal resolution of most protein samples, (ii) labeling the strips for polarity to ensure proper orientation, (iii) reducing preparation time as well as reagent waste, and (iv) controlling the manufacture of strips to ensure reproducible performance and providing maximum protein loading capabilities. The IPG strip is equilibrated with a buffer to reduce electro endo-osmotic effects (Görg et al. 1988) which are held responsible for reduced protein transfer from the first to the second dimension.

In the second dimension, the proteins are separated by molecular weight (MW) or size in a standard sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The combination of these two separation techniques unravel proteins into a map of protein spots where each spot corresponds to a specific pI and MW. The map of proteins can be considered as the "protein fingerprint" of that sample. The advantage of this method is the visualisation

of a large number (3000-5000) of protein spots on large format gels and around 1000-2000 protein spots for standard sized gels commonly used in the laboratory (Yarmush and Jayaraman, 2002). This technology is still the most widely used protein separation technology to examine the pathogen and host genome in action, through the evaluation of the host proteome during the host-pathogen interaction process (Shi and Paskewitz, 2006).

In addition to its remarkable separation capabilities, another reason for the choice of 2-DE technology over liquid chromatography (LC) based approaches for protein separation is that a reproducible 2-DE proteome reference map is a fixed visual entity. Fully annotated 2-DE proteome reference maps obtained from interaction studies are valuable as they can save time and money when hunting for differentially expressed proteins in response to infection or a particular treatment. For example, two *Drosophila* serpins (inhibitors of serine proteases) that have important roles in the control of innate immunity and in development (Levy et al. 2004) were detected on the proteome map of *Drosophila* challenged with fungi. Although there a number of limitations to this technology such as under-representation of high and low molecular weight proteins or difficulties in detection of membrane proteins (Graves and Haystead, 2002; Unlu et al. 1997), this time and labor intensive 2-DE method is currently one of the preferred approaches for quantitative characterization of complex protein samples. Furthermore, the use of 2-DE will continue with technical developments such as advancement in protein sample preparation, improvement in staining techniques and expansion in protein identification methods including data analysis (Graves and Haystead, 2002). Moreover, over the past decade, there have been several improvements in the design and automation of 2-DE instrumentation and 2-DE gel analysis software such as Delta2D, PDQuest and Progenesis.

### 1.8.1. A bottle neck of 2-DE: protein sample preparation of biological samples

Despite improvements in 2-DE technology, sample preparation is still one of the major hurdles for most biological studies. Extraction and solubilisation of proteins from the biological samples is crucial to obtain a complete picture of the proteome or secretome. The most common method for increasing cellular proteins in proteomic studies is to lyse the tissue/cells of interest, either chemically or mechanically. Mechanical cell lysis methods such as using a sonicator, grinding with (or without) liquid nitrogen, use of ultrasonic probes, application of ultra-quite cell disrupter (Ho et al. 2006), bead mill technology and pressure cycling technology (Ringham et al. 2007; Smejkal et al. 2006) have resulted in a higher yield of detectable proteins. One of the common bottle necks for efficient 2-DE lies in maintaining protein solubility during the IEF step. It is particularly difficult to solubilise hydrophobic and membrane bound proteins. However, many new reagents developed in recent years along with 2-DE suited chemicals have improved the solubilisation capabilities (Rabilloud et al. 1997; Galvini et al. 2001; Herbert, 1998; Molloy, 2000). These reagents include chaotropes such as thiourea, surfactants which have greater compatibility with chaotropes, such as CHAPS (3-{3-Cholamidopropyl dimethylammonio}-1-propanesulfonate), and reducing agents such as tributylphosphine and dithiothreitol. Protease inhibitors play a vital role in preventing the degradation of proteins during their isolation and characterization. There are number of commercially available protease inhibitors for targeting the four main classes of proteases (serine, cysteine, metallo and aspartic proteases). Broad spectrum protease inhibitors and cocktails have been developed to help protect the integrity of proteins. Work conducted by Grinyer et al. (2004) has shown that addition of a protease inhibitor prevents the degradation of fungal proteins in a solubilised sample. Generally, the denaturing properties of most sample buffers are adequate to prevent the action of proteases. This is true to a certain extent but the addition of protease inhibitors prevents massive degradation (Graves and Haystead, 2002).

### 1.8.2. Protein detection and quantitation

Protein quantitation and detection is an area of proteomics that has changed remarkably over the last decade. The accessibility of enhanced staining and detection methodologies has led to the visualization of dynamic range of proteins on 2D gels. Even though there are several methods for detecting proteins in the gel (Smejkal et al. 2004; Graves and Haystead, 2002; Westemeier, 2006), Coomassie Brilliant Blue (CBB) and silver staining are the most preferred methods (Patterson, 1994). An improvement in the CBB stain, Colloidal CBB or G-250 is reported to be have enhanced sensitivity over the classic CBB or R-250 and is generally recommended for proteomic applications (Candiano et al. 2004). Silver staining is considered to be more sensitive than CBB, with a detection range of 0.1-1ng (Graves and Haystead, 2002; Patterson, 1994). However, problems associated with silver stains include poor reproducibility, poor linear dynamic range and non-quantitative negative staining of modified proteins (Görg et al. 2000; Yarmush and Jayaraman, 2002) which cause problems for quantitation and spot matching. Availability of a wide range of fluorescent stains (FS) such as SyproRuby$^{TM}$, Deep Purple$^{TM}$, Ruthenium II, Pro-Q Diamond and Pro-Q Emerald specific for phosphoproteins and glycoproteins, have resulted in increased detection range of proteins (Graves and Haystead, 2002). Although fluorescent stains are more sensitive, but more expensive than CBB and silver stains. Moreover, fluorescent stained gels have to be countered-stained with CBB or silver stain to accurately determine spot location and visualize the picture of an organism's proteome or sectretome.

Protein spot quantitation rests on the detection and resolution of each spot using image analysis software like MELANIE, PDQUEST, Delta2D and Progenesis (Yarmush and Jayaraman, 2002). The advantage of using these softwares include automated spot detection which minimizes the time needed for matching gel images to each other and 100% spot matching giving complete expression profiles for every protein. This provides a chance to do

cross-checking, e.g. dual channel images to assess the overlapping of protein spots, or direct display of the spot background image to assess quantitation, handles large number of raw images and results in accurate statistical data in less time (Anderson and Anderson, 1998).

### 1.8.3. Mass spectrometry

Traditionally, proteins have been identified by *de novo* peptide sequencing where sequencing is performed using Edman degradation without prior knowledge of the amino acid sequence (Yates, 2000). Presently, this technique has been replaced by mass spectrometry (MS) which plays a key role in proteomics for the identification of protein spots of interest. MS permits a complex mixture of proteins to be analysed by the production of ions in the gas phase and subsequently separate and detect them based on their mass to charge ratio (m/z). The main components of a conventional mass spectrometer (Figure 9) are the ion source, the mass analyser and the detector (Aebersold and Mann, 2003).

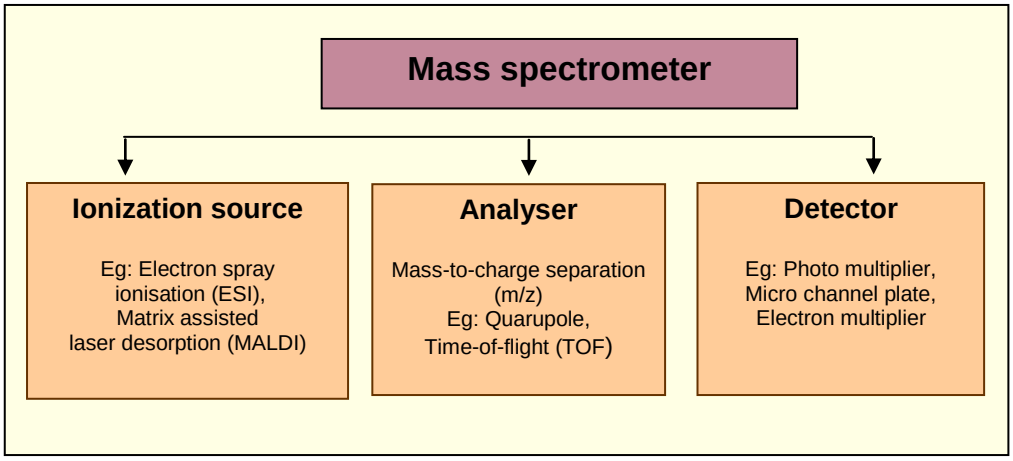


**Figure 9: Simplified schematic of a mass spectrometer**

Mass spectrometer contains three main components: an ion source, a mass analyser and an ion detector to analyse complex mixture of proteins.

The signals generated from the detector are analysed by the computer to obtain spectra which are the compared against databases to identify proteins. Two main approaches to MS spot characterization used throughout the work presented here include peptide mass fingerprinting

(PMF) and tandem mass spectrometry (MS/MS) (Figure 10). The protein spots of interest are either picked manually or with automated spot picker and digested with a proteolytic enzyme, such as trypsin to produce a set of short peptides or tryptic fragments unique to each protein. The masses of the peptides obtained in this work were analysed by Matrix-Assisted Laser Desorption Ionisation (MALDI), a soft ionisation technique which allows molecules to remain relatively intact during the ionisation process (Yates, 1998).

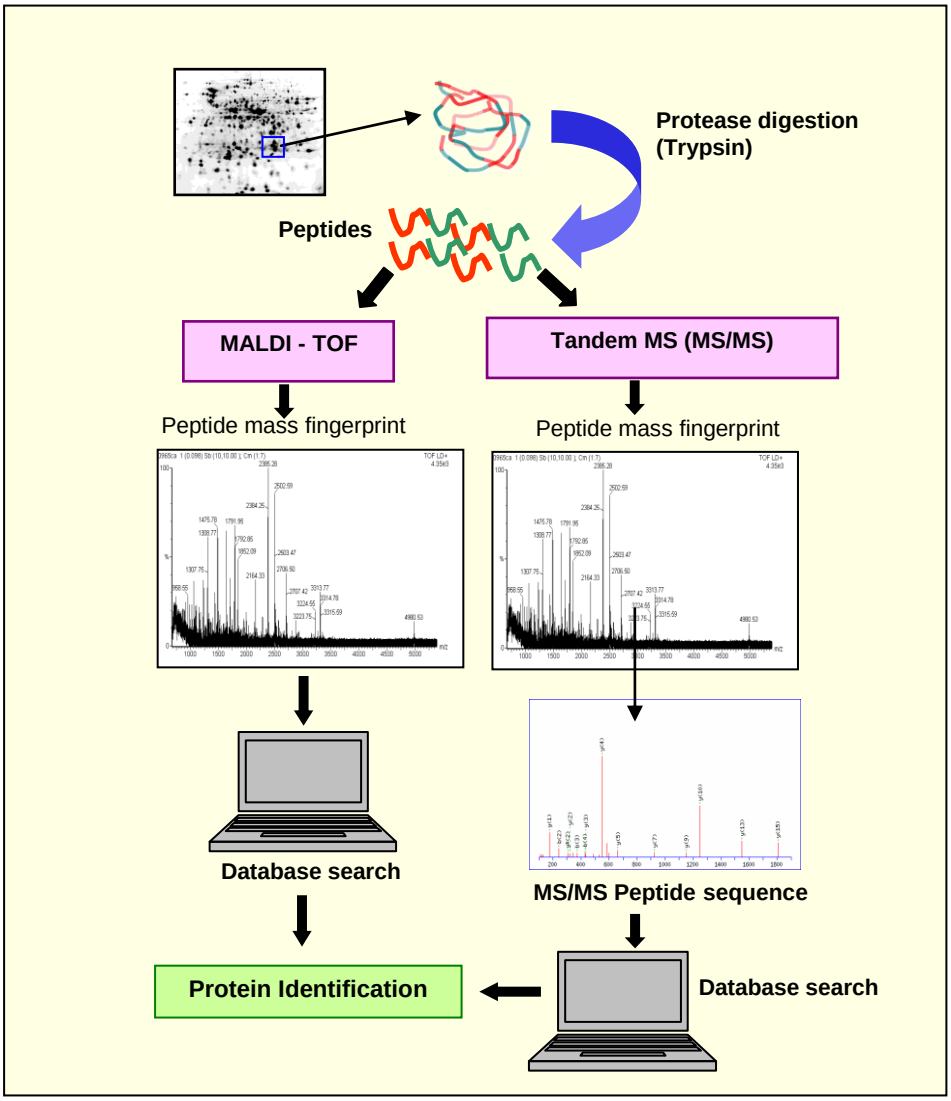


**Figure 10: Schematic of typical mass spectrometry for protein identification**

Peptide masses obtained from mass spectrometry are analysed using MALDI-TOF and Tandem mass spectrometry (MS/MS) methods.

#### 1.8.3.1. Matrix assisted laser desorption ionisation time-of-flight mass spectrometry

Matrix assisted laser desorption ionisation time-of-flight mass spectrometry **(**MALDI-TOF MS) is a soft ionisation technique used in mass spectrometry, allowing the analysis of intact biomolecules (biopolymers such as proteins, peptides and sugars) and large organic molecules (such as polymers and macromolecules) and is the workhorse for proteomics. For MALDI-TOF-MS analysis, protein spots from a 2D gel are excised, destained, dried and digested with trypsin overnight. Tryptic peptides can be applied directly to the MALDI target plate, or can undergo a recommended clean-up and concentration step in C18 zip-tip columns. A C18 column (Millipore) desalts and concentrates the peptides, enhancing peptide signal in the mass spectrometer (Rappsilber et al. 2003). In this MS method (Figure 11) a solid matrix is used, which absorbs light at the wavelength the laser produces (nitrogen laser at 337 nm). The sample is mixed with a matrix solution (a-cyano-4-hydroxycinnamic acid) and allowed to air-dry, forming a co-crystalline sample/matrix complex on a target plate. When the laser is fired at the target the matrix absorbs the laser light energy which vaporizes it (it desorbs from the surface) and this carries some of the sample with it. At the time that the laser is pulsed a voltage is applied to the target plate to accelerate the ionized sample towards a time-of-flight (TOF) mass analyzer. The TOF can be operated in either linear or reflectron mode and can easily separate proteins up to 500 kDa (Vorm and Mann, 1994). The precision is considered to be approx 10 ppm. Once a good quality signal has been obtained by optimising the parameters, 20-30 laser shots are accumulated to produce a mass/charge (*m/z*) spectrum. The spectrum can be compared to lists generated from the theoretical digestion of known proteins for identification (Beavis and Chait, 1990).

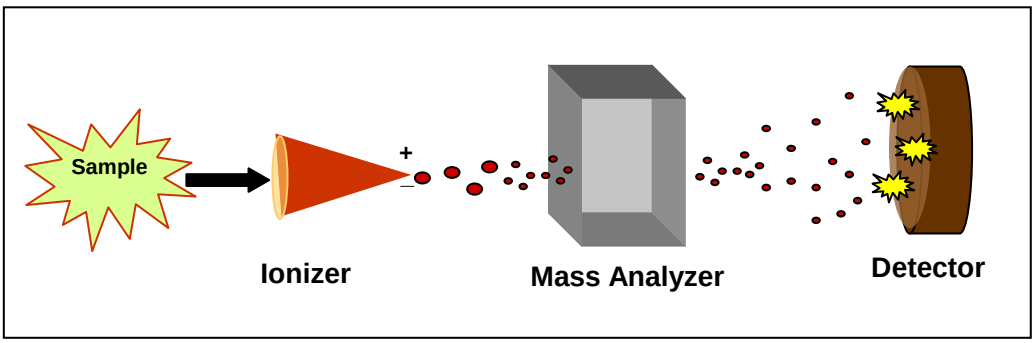


**Figure 11: Schematic of soft ionisation technique, MALDI-TOF MS**

Sample is mixed with a matrix and coated on to a plate and subjected to a collimated focused laser beam causing ionisation and desorption. The ions finally arrive at the detector and digitised data generated from successive laser shots are summed yielding a TOF mass spectrum.

Some of the advantageous features of MALDI-TOF-MS include analysis of broad range of biomolecules, simplicity of use, high sensitivity and better tolerance to impurities such as buffers, detergents, salts and additives than the other MS techniques (Herbert et al. 1998; Galvini et al. 2001). Altogether, these characteristics make MALDI-TOF MS very suitable for the direct analysis of biological tissues or fluids. MALDI-TOF MS methodology is routinely used to study host-pathogen interactions eg. insect-fungus interactions and has lead to the discovery of protein biomarkers (Uttenweiler-Joseph et al. 1998; Shi and Paskewitz, 2006; Asthon et al. 2001; Barrett et al. 2000).

### 1.8.3.2. Tandem mass spectrometry

Tandem mass spectrometry (Tandem MS) commonly known as MS/MS is used to generate structural information about a compound by fragmenting specific sample ions inside the mass spectrometer and identifying the resulting fragment ions (Figure 12). This information can then be pieced together to generate structural information regarding the intact molecule. Tandem mass spectrometry also enables specific compounds to be detected in complex mixtures on account of their specific and characteristic fragmentation patterns (Biemann and Scoble, 1987). During MS/MS analysis, MALDI-TOF-TOF instrument offers the opportunity

to finely control fragmentation conditions and concurrently provides both low-energy and high-energy collision induced spectra. The degree of fragmentation and structural information acquired are linked to regulation in laser intensity, collision gas pressure and collision energy (eV). Collision energy  less than 10 eV in the collision induced dissociation (CID) cell results in the production of low mass internal fragments, ions from amino acid side chain fragmentations, ions specific of particular amino acids increasing the confidence in the peptide sequence interpretation and identification of multiple proteins from complex mixtures.

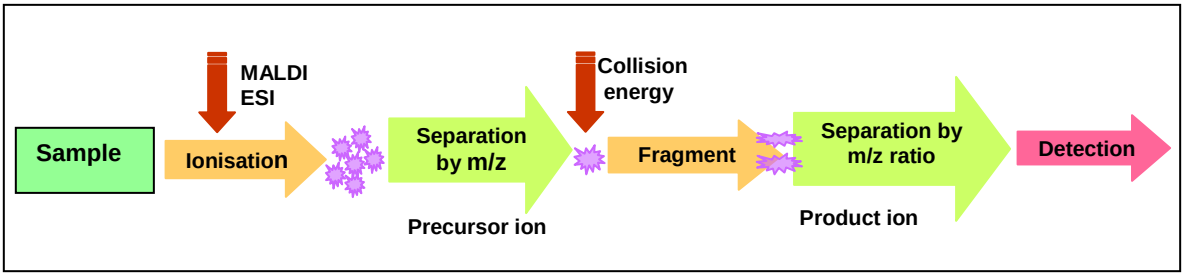


**Figure 12: Schematic of Tandem mass spectrometry**

Tandem MS has two mass analysers are used to measure precursor ions and product ions. In the first mass analyser the sample undergoes dissociation and generates precursor ions. These are then analysed by second mass analyser where dissociation of ions is accomplished by target gas to generate product ions. A third analyser is then used to separate these fragments by mass and finally detected by a detector to generate a spectrum.

There are various tandem mass spectrometry experiments (Griffin and Aebersold, 2001) that can be prepared with a variety of instruments that are either in tandem-in-space or tandem-in-time.  Tandem MS in space involves the physical separation of the instrument components (triple quadrupole [QqQ] or quadrupole-TOF [QTOF]), tandem MS in time involves the use of an ion trap. As a result of its simplicity, excellent mass accuracy, high resolution and sensitivity, MS/MS has become one of the most important analytical techniques for the identification of proteins and for the analysis of posttranslational modifications (PTM) (Canas et al. 2006).

### 1.8.3.3. Liquid chromatography - mass spectrometry

Liquid chromatography coupled to mass spectrometry (LC-MS and LC-MS/MS) is a broadly used and powerful technique for the analysis of proteins and peptides (Wysocki et al. 2005). Proteomic samples are usually complex even after pre-fractionation steps. Firstly, proteins are separated by 2-DE and individual protein spots are digested with enzymes such as trypsin and an acid such as trifluoroacetic acid is added to extract the peptides. Digest peptides are concentrated and desalted on a reversed phase C18 column (Covey et al. 1986). Digested peptides bind to the C18 column and peptides are eluted from the column are directly inserted into the triple quadrupole/linear ion trap mass spectrometer (Q TRAP LC/MS/MS System) and their mass values are determined. Peptide ions will simultaneously be isolated and fragmented within the spectrometer, producing daughter ion spectra from which sequence information on individual peptides can be obtained. This information together with the peptide mass values are then used to search protein databases or software such as SEQUEST (Eng et al. 1994), leading to identification of protein components.

Certain improvements have transformed LC-MS/MS into a routine laboratory procedure. Recently, a higher resolution and higher capacity 2D separation has been achieved with multi-dimensional protein identification technology (MudPIT). The success of MudPIT for proteomics is an outcome of the two-dimensional resolution of peptides and the power of database hunting programs to identify proteins with one or more peptides. By using peptides for identification, unbiased identification of proteins can be accomplished even for relatively low abundance proteins, extremes in pI and high or low molecular weight proteins can be identified (Aebersold and Mann, 2003). MudPIT has been proven robust for the resolution of complex mixtures of peptides; improvements in the resolution of peptides are desirable to identify proteins present at widely varying expression levels and to provide increased protein coverage (Wysocki et al. 2005). Although there are many advantages to using MuDPIT, it

does not solve all of the problems associated with complex protein mixtures such as plasma where they are large differences in protein abundances (Aebersold and Goodlett, 2001). Similarly, determining relative ratios of modifications on proteins is complicated using the MuDPIT approach (Wolters et al. 2001). Nevertheless, it is obvious that prospects of combining 2-DE with MS and LC-MS/MS approaches will generate superior results than using individual approach (Wysocki et al. 2005).

### 1.8.4. Cross-species protein identification and databases

The ultimate goal in most proteomic studies is to confidently identify all the proteins enclosed within a sample. However the proteomic approach has limited capacity in identifying proteins whose sequences remain unknown (i.e. not present in a database), or heavily modified proteins (Graves and Haystead, 2002; Yarmush and Jayaraman, 2002). Using MS and available protein sequences, cross-species identification is accomplished by partially aligning the protein of interest from an organism with an unsequenced genome to a database sequence from a related species. Due to the absence of genome sequence data for both the fungus (*M. anisopliae*) as well as the insect (*D. albohirtum*, greyback canegrub) studied in this work, we have used cross-species identification (CSI) approach to identify the proteins. This approach heavily relies on the indentification of proteins from databases of closely related species with peptide mass fingerprinting (PMF) data and tandem mass spectrometric (MS/MS) data.

#### 1.8.4.1. Identification of proteins by peptide mass fingerprint

When ions are passed into a mass spectrometer, they are separated according to their mass resulting in a mass fingerprint of the peptides present in the mixture (Figure 10). These peptides are the result of the cleavage of a particular protein using a sequence-specific protease, such as trypsin. The set of masses obtained by the mass spectrometer are compared with virtual fingerprints obtained by theoretical cleavage of protein sequences stored in

protein databases and the top scoring proteins are retrieved as possible candidate proteins. Peptide mass fingerprint (PMF) allows for cross species identification of proteins if the analyzed protein and reference database entry have more than 80% sequence identity (Wilkins and Williams, 1997). The high mass accuracy of modern TOF instruments increases confidence in cross-species peptide mapping and generates sequence identity where less peptide masses would be needed for a confident match (Lester and Hubbard, 2002). There are numerous databases on the world-wide web which are publicly accessible. One of the most popular is the ExPASy biological server (http://www.expasy.org) which was also used in this study as it has an extensive collection of proteomics tools [eg. Mascot-search peptide mass fingerprint from Matrix Science; ProFound-search known protein sequences with peptide mass information from Rockefeller and NY Universities; ProteinProspector-search sequence databases in conjunction with mass spectrometry data (MS-Fit, MS-Pattern, MS-Digest)] and links to other protein characterization tools (eg. Compute the theoretical isoelectric point (pI) and molecular weight (Mw) from a UniProt; ProtParam-tool which allows the computation of various physical and chemical parameters for a given protein stored in Swiss-Prot or TrEMBL).

#### 1.8.4.2. Identification of proteins by MS/MS

The tandem mass spectra produced by the collision-induced dissociation (CID) of selected peptides generates b and y ions, which indicate fragmentation at the amide bond with charge retention on the N/C/terminus, respectively (Wilkins and Williams, 1997). The tandem mass spectrum relies on measuring the mass differences between adjacent fragment ion peaks of one of the major ion series (Figure 10). The experimental MS/MS spectrum is matched against a calculated spectrum for all peptides in the database (e.g. MASCOT, SEQUEST and MS BLAST). A score is calculated which reflects the quality of the match between the experimental spectrum and the theoretical one (Lester and Hubbard, 2002). Since tandem

mass spectra data contain information on the sequence of the peptides, these searches are generally more specific and discriminating than peptide mass fingerprints. For proteins with a lower sequence identity, MS/MS analysis of peptides gives confident cross-species identification with a few peptide sequences, depending on the length of amino acid composition (Liska and Shevchenko, 2003).

## 1.9. Literature Cited

Acharya A, Sriram S, Sehrawat S, Rahman M (2002) *Bombyx mori* nucleopolyhedrovirus: molecular biology and biotechnological applications for large-scale synthesis of recombinant proteins. J Curr Sci 83: 455-465.

Aebersold R, Goodlett DR (2001) Mass spectrometry in proteomics. Chem Rev 101: 269-295.

Aebersold R, Mann M (2003) Mass spectrometry-based proteomics. Nature 422: 198-207.

Allsopp PG, Chandler KJ (1989) Canegrub research and control in Australia since 1970. Proc Int Soc Sugar Cane Technol 20: 810-817.

Ambrosino P, Scala V, Marra R, Vinale F, Soriente I, Ferraioli S, Carbone V, Ruocco M, Woo S L, Lorito ML (2004) Extracellular proteome of *Trichoderma harzianum* to identify proteins with biotechnological value. J Zhejiang Uni (Agri and Life Sci) 30: 452-456.

Amiri-Besheli B, Khambay B, Cameron S, Deadman ML, Butt TM (2000) Inter- and intra-specific variation in destruxin production by insect pathogenic *Metarhizium* spp., and its significance to pathogenesis. Mycol Res 104: 447-452.

Anderson NL, Anderson NG (1998) Proteome and proteomics: new technologies, new concepts, and new words. Electrophoresis 19: 1853-1861.

Anon (1938) Cane pests. Queensland Agricul J 50: 523-528.

Anon (1993) Greyback canegrubs are on the increase in the North. Aus Canegrower 15: 24.

Anon (2004) The Biology and Ecology of Sugarcane *(Saccharum* spp. hybrids) in Australia. (http://www.health.gov.au/internet/ogtr/publishing.nsf/Content/sugarcane3/$FILE/biologysugarcane.pdf ) 22–25.

Arruda W, Lu Beck I, Schrank A, Vainstein MH (2005) Morphological alterations of *Metarhizium anisopliae* during penetration of *Boophilus microplus* ticks. Exp App Acarol 37: 231- 244.

Asthon PD, Curwen RS, Wilson RA (2001) Linking proteome and genome: how to identify parasite proteins. Trends Parasitol 17: 198-202.

Attathom T, Chongrattanameteekul W, Chanpaisang J, Siriyan R (1995) Morphological diversity and toxicity of delta-endotoxin produced by various strains of *Bacillus thuringiensis*. Bull Entomol Res 85: 167-173.

Barrett J, Jefferies JR, Brophy PM (2000) Parasite proteomics. Parasitol Today 16: 400-403.

Beavis RC, Chait BT (1990) High-accuracy molecular mass determination of proteins using matrix-assisted laser desorption mass spectrometry. Anal Chem 62: 1836-1840.

Benting J, Lecat S, Zacchetti D, Simons K (2000) Protein expression in *Drosophila* Schneider cells. Anal Biochem 278: 59-68.

Biemann K and Scoble H (1987) Characterization by tandem mass spectrometry of structural modifications in proteins. Science 237: 992-998.

Biron DG, Agnew P, Marche L, Renault L, Sidobre C, Michalakis Y (2005) Proteome of *Aedes aegypti* larvae in response to infection by the intracellular parasite *Vavraia culicis*. Int J Parasitol 5: 1-13.

Blackstock WP, Weir MP (1999) Proteomics: quantitative and physical mapping of cellular proteins. Trends Biotechnol 17: 121-127.

Boemare N, Laumond C, Mauleon H (1996) The entomopathogenic nematode-bacterium complex: biology, life cycle and vertebrate safety. Biocontrol Sci Technol 6: 333-346.

Bogo MR, Vainstein MH, Aragao FJ, Rech E, Schrank A (1996) High frequency gene conversion among benomyl resistant transformants in the entomopathogenic fungus *Metarhizium anisopliae*. FEMS Microbiol Lett 142: 123-127.

Boucias DG, Pendland JC, Latge JP (1988) Non-specific factors involved in the attachment of entomopathogenic deuteromycetes to host insect cuticle. Appl Environ Microbiol 54: 1795–1805.

Boucias DG, Maruniak JE, Pendland JC (1989) Characterization of a non-occluded baculovirus (subgroup C) from the field cricket, *Gryllus rubens*. Arch Virol 106: 93-102.

Boucias DG, Pendland JC (1991) Attachment of mycopathogens to cuticle: Initial event of mycosis in arthropod hosts. In, The fungal spore and disease initiation in plants and animals. Edited by, G.T. Cole and H.C. Hoch. Plenum, New York. pp 101-127.

Butt TM, Ibrahim L, Clark SJ, Beckett A (1995) The germination behavior of M. anisopliae on the surface of aphid and flea beetle cuticles. Mycol Res 99: 945-950.

Canas B, Lopez-Ferrer D, Ramos-Fernandez A, Camafeita E, Calvo E (2006) Mass spectrometry technologies for proteomics. Brief Funct Genomic Proteomic 4: 295-320.

Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG (2004) Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25: 1327-1333.

Carbonell LF, Miller LK (1987) Baculovirus interaction with non-target organisms: a virus-borne reporter gene is not expressed in two mammalian cell lines. J Appl Environ Microbiology 53: 1412–1417.

Clarkson JM, Charnley AK (1996) New insights into the mechanisms of fungal pathogenesis in insects. Trends Microbiol 4: 197-203.

Covey TR, Lee ED, Henion JD (1986) High-speed liquid chromatography/tandem mass spectrometry for the determination of drugs in biological samples. Anal Chem 58: 2453-2460.

De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B (2002) The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. J Embo 21: 2568-79.

Dillon RJ, Charnley AK (1985) A technique for accelerating and synchronizing germination of conidia of the entomopathogenic fungus *Metarhizium anisopliae*. Arch Microbiol 142: 204-206.

Dumas C, Rober P, Pais M, Vey A, Quiot JM (1994) Insecticidal and cytotoxic effects of natural and hemi synthetic destruxins. Comp Biochem Physiol 108: 195-203.

Eng JK, McCormack AL, Yates III JR (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J American Soc Mass Spectrom 5: 976-989.

Ferrandon D, Jung AC, Criqui MC, Lemaitre B, Uttenweiler-Joseph S, Michaut L, Reichhart JM, Hoffmann JA (1998) A drosomycin-GFP reporter transgene reveals a local immune response in *Drosophila* that is not dependent on the Toll pathway. J Embo17: 1217–1227.

Galvani M, Rovatti L, Hamdan M, Herbert B, Righetti PG (2001) Protein alkylation in the presence/absence of thiourea in proteome analysis: a matrix assisted laser desorption/ionization-time of flight-mass spectrometry investigation. Electrophoresis 22: 2066-2074.

Georgis R, Hague NGM (1991) Nematodes as biological pesticides. Pestic Out 2: 29-32.

Georgis R, Kaya HK, Gaugler R (1991) Effect of steinernematid and heterorhabditid nematodes on nontarget arthropods. Environ Entomol 20:815-22.

Ghosh D, Majumder T, Bhattacharya T (1988) Efficacy of *Bacillus thuringiensis israelensis* as larvicide of *Culex* at laboratory condition. Envir Ecol 6: 509-511.

Gillespie JP, Kanost MR, Trenczek T (1997) Biological mediators of insect immunity. Ann Rev Entomol 42: 611-643.

Graves PR, Haystead TAJ (2002) Molecular biologists guide to proteomics. Microbiol Mol Biol Revs 66: 39-63.

Griffin TJ, Aebersold R (2001) Advances in Proteome Analysis by Mass spectrometry. J Biol Chem 276: 45497-45500.

Griggs P (2005) Entomology in the service of the State: Queensland scientists and the campaign against cane beetles, 1895-1950. Histor Rec Aust Sci 16: 1-29.

Grinyer J, McKay M, Nevalainen H, Herbert BR (2004) Fungal proteomics: Initial mapping of biological control strain *Trichoderma harzianum*. Curr Genet 45: 163-169.

Grinyer J, Hunt S, McKay M, Herbert BR, Nevalainen H (2005) Proteomic response of the biological control fungus *Trichoderma atroviride* to growth on the cell walls of *Rhizoctonia solani*. Curr Genet 47: 381-388.

Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W (2000) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21: 1037-1053.

Görg A, Postel W, Gunther S (1998) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 9: 531-546.

Heale JB, Isaac JE, Chandler D (2006) Prospects for strain improvement in entomopathogenic fungi. Pestic Sci 26: 79-92.

Herbert BR, Molloy MP, Gooley AA, Walsh BJ, Bryson WG, Williams KL (1998) Improved protein solubility in two-dimensional electrophoresis using tributylphosphine as reducing agent. Electrophoresis 19:845-851.

Ho CH, Chew TK, Ling TC, Kamaruddin S, Tan WS, Tey BT (2006) Efficient mechanical disruption of *Escherichia coli* by an ultrasonicator and recovery of intracellular hepatitis B core antigen. Proc Biochem 41: 1829-1834.

Hochstrasser DF (1998) Proteome in perspective. Clin Chem Lab Med 36: 825-828.

Hoffmann JA (1995) Innate immunity of insects. Curr Opin Immunol 7: 4-10.

Hu G, St. Leger RJ (2002) Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveals that it is rhizospere competent. App Environ Microbiol 68: 6383-6387.

Huang SH, Triche T, Jong AY (2002) Infectomics: genomics and proteomics of microbial infections. Funct Integr Genomics 1: 331-344.

Hunt WD, Chander KJ, Horsfield A, Cocco R, Sgarbossa PJ (2002) Developing and extending 'GrubPlan' for management of Greyback canegrub damage in Queensland sugarcane. Proc Aust Soc Sugar Cane Technol 24: 156-163.

Ito T, Chiba T, Ozawa R, Yoshida M, Hattori M, Sakaki Y (2001) A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc Natl Acad Sci USA 98: 4569-4574.

Jackson TA (1999) Factors in the success and failure of microbial control agents for soil dwelling pests. Integrated Pest Manag Rev 4: 281-285.

Jiang HB, Kanost MR (1997) Characterisation and functional analysis of twelve naturally occurring reactive site variants of serpin-1 from *Manduca sexta*. J Biol Chem 272: 1082-1087.

Johnson H, Gaskell SJ (2006) Proteomics gets faster and smarter. Genome Biol 7: 331-333.

Kaijiang L, Roberts DW (1986) The production of destruxins by the entomogenous fungus, *Metarrhizium anisopliae* var. *major*. J Invert Pathol 47: 120-22.

Kalume DE, Okulate M, Zhong J, Reddy R, Suresh S, Deshpande N, Kumar N, Pandey A (2005) A proteomic analysis of salivary glands of female *Anopheles gambiae* mosquito. Proteomics 5: 3765-3777.

Karlsson C, Korayem AM, Scherfer C, Loseva O, Dushay MS, Theopold U (2004) Proteomic analysis of the *Drosophila* larval hemolymph clot. J Biol Chem 279: 52033-52041.

Kaya HK, Gaugler R (1993) Entomopathogenic nematodes. Ann Rev Entomol 38: 181-206.

Kershaw MJ, Moorhouse ER, Bateman RP, Reynolds SE, Charnley AK (1999) The role of destruxins in the pathogenicity of *Metarhizium anisopliae* for three species of insect. J Invertebr Pathol 74: 213-223.

Kim Y, Nandakumar MP, Marten MR (2007) Proteomics of filamentous fungi. Trends Biotechnol 25: 395-400.

Kim Y, Nandakumar MP, Marten MR (2008) The state of proteome profiling in the fungal genus *Aspergillus*. Brief Funct Genom Proteom 7: 87-94.

Klose J (1975) Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. Humangenetik 26: 231-243.

Kogan M (1998) Integrated pest management: Historical perspectives and contemporary developments. Ann Rev Entomol 43: 243-270.

Koutsos AC, Blass C, Meister S, Schmidt S, MacCallum RM, Soares MB, Collins FH, Benes V, Zdobnov E, Kafatos FC (2007) Life cycle transcriptome of the malaria mosquito *Anopheles gambiae* and comparison with the fruit fly *Drosophila melanogaster*. PNAS 104: 11304-11309.

Lacey LA, Goettel MS (1995) Current developments in microbial control of insect pests and prospects for the early 21st century. Entomophaga 40: 1- 25.

Lee SY, Cho MY, Hyun JH, Lee KM, Homma KI, Natori S, Kawabata SI, Iwanaga S, Lee BL (1998) Molecular cloning of cDNA for pro-phenol-oxidase-activating factor I, a serine protease is induced by lipopolysaccharide or 1,3-ß-glucan in coleopteran insect, *Holotricha diomphalia* larvae. Eur J Biochem 257: 615-621.

Lemaitre B, Kromer-Metzger E, Michaut L, Nicholas E, Meister M, Georgel P, Reichhart JM, Hoffmann JA (1995) A recessive mutation, immune deficiency (Imd), defines two distinct control pathways in the Drosophila host defence. Proc Natl Acad Sci 92: 9465-9469.

Lester PJ, Hubbard SJ (2002) Comparative bioinformatic analysis of complete proteomes and protein parameters for cross-species identification in proteomics. Proteomics 2:1392-1405.

Levy F, Bulet P, Ehret-Sabatier L (2004) Proteomic analysis of the systemic immune response of *Drosophila*. Mol Cell Proteomics 3: 156-166.

Liska AJ, Shevchenko A (2003) Expanding the organismal scope of proteomics: cross-species identification by mass spectrometry and its implications. Proteomics 3: 19-28.

Ma C and Kanost MR (2000) A β-1-3 glucan recognition protein from an insect, *Manduca sexta*, agglutinates microorganisms and activates the phenoloxidase cascade. J Biol Chem 273: 7505-7514.

MacBeath G (2002) Protein microarrays and proteomics. Nat Genet. 32 Suppl: 526-532.

Martin PAW, Travers RS (1989) Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl Envir Microb 55: 2437-2442.

Matha V,Grubhoffer L, Weyda F, Hermanova L (1990) Detection of β-1-3 glucan-specific lectin on the surface of plasmatocytes, immunocompetent cells of greater wax moth, *Galleria mellonella*. Cytobios 64: 35-42.

Meister M, Lemaitre B, Hoffmann JA (1997) Antimicrobial peptide defence in *Drosophila* Bioassays 19: 1019-1026.

Miller JC (1990) Effects of a microbial insecticide, *Bacillus thuringiensis kurstaki*, on non target Lepidoptera in a spruce budworm-infested forest. J Res Lepidop 20: 267- 276.

Molloy MP, Herbert BR, Slade MB, Rabilloud T, Nouwens AS, Williams KL, Gooley AA (2000) Proteomic analysis of the *Escherichia coli* outer membrane. Eur J Biochem 267: 2871-2881.

Morgan LW, Boddy L, Clark SJ, Wilding N (1995) Influence of temperature on germination of primary and secondary conidia of *Erynia neoaphidis* (Zygomycetes: Entomophthorales). J Invertebr Pathol 65: 132-138.

Norin M, Sundström M (2002) Structural proteomics: developments in structure-to-function predictions. Trends Biotechnol 20: 79-84.

O'Farrel PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250: 4007-4021.

Ote M, Mita K, Kawasaki H, Seki M, Nohata J, Kobayashi M, Shimada T (2004) Microarray analysis of gene expression profiles in wing discs of *Bombyx mori* during pupal ecdysis. Insect Biochem Mol Biol 34: 775-784.

Paterson IC, Charnley AK, Cooper RM, Clarkson JM (1994) Partial characterization of specific inducers of a cuticle-degrading protease of the insect pathogenic fungus, *M. anisopliae*. Microbiol 140: 3153-3159.

Patterson SD, Aebersold RH (2003) Proteomics: The first decade & beyond. Nat Genet 33: 311-323.

Payne J, Kim L (1992) *Bacillus thuringiensis*: insects and beyond. Biotechnol 10: 271- 275.

Peng J, Gygi SP (2001) Proteomics: the move to mixtures. J Mass Spectrom 36: 1083-1091.

Polanowski A, Wilusz T, Blum M, Escoubas P, Schmidt J, Travis J (1992) Serine proteinase inhibitor profiles in the haemolymph of a wide range of insect species. Comp Biochem Physiol 102: 757-760.

Prevot GI, Laurent-Winter C, Rodhain F, Bourgouin C (2003) Sex specific and blood meal-induced proteins of *Anopheles gambiae* midguts: analysis by two-dimensional gel electrophoresis. Malaria J 2: 1-7.

Rabilloud T, Adessi C, Giraudel A, Lunardi J (1997) Improvement of the solubilisation of proteins in two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 18:307-316.

Rappsilber J, Ishihama Y, Mann M (2003) Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal Chem 75: 663-670.

Rath AC, Guy PL, Webb WR (1995) *Metarhizium* spore surface antigens are correlated with pathogenicity. Mycol Res 100: 57-62.

Redmond CT, Potter DA (1995) Lack of efficacy of in-vivo and putatively in-vitro produced *Bacillus popilliae* against field populations of Japanese beetle (Coleoptera: Scarabaeidae) grubs in Kentucky. J Econ Entomol 88: 846-854.

Richards S, Hauser F, Cazzamali G, Williamson M, Grimmelikhuijzen CJP (2008) The genome sequence of the model beetle and pest *Tribolium castaneum*. Nature 452: 949-955.

Ringham H, Bell RL, Smejkal GB, Behnke J, Witzmann FA (2007) Application of pressure cycling technology to tissue sample preparation for 2-DE. Electrophoresis 28: 1022-1024.

Robertson LN, Allsopp PG, Chandler KJ, Mullins RT (1995) Integrated management of canegrubs in Australia: Current situation and future research directions. Aust J Agric Res 46: 1-16.

Robertson LN, Chandler KJ, Stickley BDA, Cocco RF, Ahmetagic M (1998) Enhanced microbial degradation implicated in rapid loss of chlorpyrifos from the controlled-release formulation suSCon® Blue in soil. Crop Protection 17: 29-33.

Roy HE, Pell JK (2000) Interactions between entomopathogenic fungi and other natural enemies: implications for biological control. Biocontrol Sci Technol 10: 737-752.

Rupp S (2004) Proteomics on its way to study host-pathogen interaction in *Candida albicans*. Curr Opin Microbiol 7:330–335.

Samson PR, Milner RJ, Bullard GK (1999) Development of *Metarhizium*-based biopesticides for sugarcane pest management- current progress and future prospects. Proc Aust Soc Sugar Cane Technol 21: 156-163.

Samson PR, Robertson L, Bakker P, Cocco R, Horsfield A, Logan D, Kettle C, Harris B, Allsopp P, McGill N, Milner RJ, Bullard GK (2001) Development of *Metarhizium*-based biopesticides for use against sugarcane white grubs in Australia. Proc Int Soc Sugar Cane Technol 24: 354-360.

Samson PR, Milner RJ, Sander ED, Bullard GK (2005) Effect of fungicides and insecticides during planting of sugarcane on viability *of Metarhizium anisopliae* and its efficacy against white grubs. BioControl 50: 151-163.

Samuels RI, Reynolds SE, Charnley AK (1988) Calcium channel activation of insect muscle by destruxins, insecticidal compounds produced by the entomopathogenic fungus, *Metarhizium anisopliae*. Comp Biochem Physiol 90: 403-412.

Shi L, Paskewitz SM (2006) Proteomics and insect immunity. Invertebr Survival J 3: 4-17.

Smejkal GB (2004) The Coomassie chronicles: past, present and future perspectives in polyacrylamide gel staining. Expert Rev Proteomics 1: 381-387.

Smejkal GB, Robinson MH, Lawrence NP, Tao F, Saravis CA, Schumacher RT (2006) Increased protein yields from *Escherichia coli* using pressure-cycling technology. J Biomol Tech 17: 173-175.

St. Leger RJ, Charnley AK, Cooper RM (1986) Cuticle-degrading enzymes of entomopathogenic fungi: synthesis in culture on cuticle. J Inveterbr Pathol 48: 85-95.

St. Leger RJ, Charnley AK, Cooper RM (1987) Characterization of cuticle-degrading proteases produced by the entomopathogen *Metarhizium anisopliae*. Arch Biochem Biophys 253: 221–232.

St. Leger RJ, Durrands PK, Charnley AK, Cooper RM (1988) Regulation of production of proteolytic enzymes by the entomopathogenic fungus *Metarhizium anisopliae*. Arch Microb 150: 413-416.

St. Leger RJ, Butt TM, Staples RC, Roberts DW (1989) Production in vitro of appressoria by the entomopathogenic fungus *Metarhizium anisopliae*. Exp Mycol 13: 274–288.

St. Leger RJ, Goettel MS, Roberts DW, Staples RC (1991) Prepenetration events during infection of host cuticle by *Metarhizium anisopliae*. J Invertebr Pathol 58: 168-179.

St. Leger RJ, Frank DC, Roberts DW, Staples RC (1993) Molecular cloning and regulatory analysis of the cuticle degrading protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*. Eur J Biochem 204: 991–1001.

St. Leger RJ, Bidochka MJ, Roberts DW (1994) Isoforms of the cuticle-degrading proteinase and production of a metalloproteinase by *Metarhizium anisopliae.* Arch Biochem Biophys 313: 1-7.

St. Leger RJ, Joshi L, Bidochka MJ, Roberts DW (1996) Construction of an improved mycoinsecticide over expressing a toxic protease. Proc Natl Acad Sci 93: 6349–6354.

St. Leger RJ (2008) Studies on adaptations of *Metarhizium anisopliae* to life in the soil. J Invertebr Pathol 98: 271-276.

Thomas F, Adamo S, Moore J (2005) Parasitic manipulation: where are we and where should we go? Behav Process 68: 185-199.

Thomas MB, Read AF (2007) Fungal bioinsecticides with a sting. Nature Biotechnol 25: 1367-1368.

Unlu M, Morgan ME, Minden JS (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18: 2071-2077.

Uttenweiler-Joseph S, Moniatte M, Lagueux M, Dorsselaer AV, Hoffmann JA, Bulet P (1998) Differential display of peptides induced during the immune response of *Drosophila*: A

matrix-assisted laser desorption ionization time-of-flight mass spectrometry study. Proc Natl Acad Sci USA 95: 11342-11347.

Vierstraete E, Cerstiaens A, Baggerman G, Van den Bergh G, De Loof A, Schoofs L (2003) Proteomics in *Drosophila melanogaster*: first 2D database of larval hemolymph proteins. Biochem Biophys Res Commun 304: 831-838.

Vilcinskas A, Matha V, Gotz P (1997) Inhibition of phagocytic activity of plasmatocytes isolated from *Galleria mellonella* by entomogenous fungi and their secondary metabolites. J Insect Physiol 43: 475-483.

Vilcinskas A, Gotz P (1999) Parasitic fungi and their interactions with insect immune system. Advan Parasitol 43: 267-313.

Vorm O, Mann M (1994) Improved mass accuracy in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of peptides. J Am Soc Mass Spectrom 5: 955-958.

Wang C, Typas MA, Butt TM (2002) Detection and characterisation of *pr1* virulent gene deficiencies in the insect pathogenic fungus *Metarhizium anisopliae*. FEMS Microbiol Lett 213: 251-255.

Wang Y, Zhang P, Fujii H, Banno Y, Yamamoto K, Aso Y (2004) Proteomic studies of lipopolysaccharide-induced polypeptides in the silkworm, *Bombyx mori*. Biosci Biotechnol Biochem 68: 1821-1823.

Wedde M, Weise C, Kopecek P, Franke P, Vilcinskas A (1998) Purification and characterization of an inducible metalloproteinase inhibitor from the haemolymph of greater wax moth larvae, *Galleria mellonella*. Eur J Biochem 255: 535-543.

Westemeier R (2006) Sensitive, quantitative and fast modifications for Coomassie Blue staining of polyacrylamide gels. Proteomics 6: 61-64.

Wilkins MR, Williams KL (1997) Cross-species protein identification using amino acid composition, peptide mass fingerprinting, isoelectric point and molecular mass: a theoretical evaluation. J Theor Biol 186: 7-15.

Wolters DA, Washburn MP, Yates JR 3rd. (2001) An automated multidimensional protein identification technology for shotgun proteomics. Anal Chem 73: 5683-5690.

Wood H A, Granados RR (1991) Genetically engineered Baculoviruses as agents for pest control. Annu Rev Microbiol 45: 69–87.

Wysocki VH, Resing KA, Zhang Q, Cheng G (2005). Mass spectrometry of peptides and proteins. Methods 35: 211-222.

Yarmush ML, Jayaraman A (2002) Advances in proteomic technologies. Ann Rev Biomed Eng 4: 349-373.

Yates JR. 3rd (1998) Mass spectrometry and the age of the proteome. J Mass Spectrom 33:1-19.

Yates JR. 3rd (2000) Mass spectrometry - from genomics to proteomics. Trends Genetics 16: 5-8.

Zimmermann G (2006) The entomopathogenic fungus *Metarhizium anisopliae* and its potential as a biocontrol agent. Pest Sci 37: 375-379.

Zou Z, Wang Y, Jiang H (2005) *Manduca sexta* prophenoloxidase activating proteinase-1 (PAP-1) gene: organization, expression, and regulation by immune and hormonal signals. Insect Biochem Mol Biol 35: 627-636.

## 1.10. Aims of the thesis

The research of this thesis was undertaken with a *Metarhizium anisopliae* var. *anisopliae*, strain FI-1045, which is highly pathogenic for the greyback canegrubs (*Dermolepida albohirtum*).

The specific goals of the present study were:

1) To adapt and evaluate proteomic methods to analyse and identify proteins from greyback canegrubs. The work included differential protein extraction, solubilisation and separation by two-dimensional gel electrophoresis.

2) To identify proteins from two-dimensional electrophoresis gels to produce a reference map of the greyback canegrub proteome for future investigations.

3) To employ comparative proteomic analysis for the identification of differentially expressed proteins from healthy canegrubs, infected canegrubs and the infecting fungus during the infection process.

4) To identify proteins from two-dimensional electrophoresis gels to investigate and discover novel proteins from the interacting proteomes during *Metarhizium* infection of canegrubs.

5) To examine the production of various proteins by *M. anisopliae* (strain FI-1045) in the presence of greyback canegrubs and isolated canegrub cuticles.

6) To display different secretome profiles and identify novel proteins involved in the biocontrol response of *M. anisopliae*.

7) To improve virulence of *Metarhizium* using UV mutagenesis and assess the secretion of various proteins by *M. anisopliae* mutant strains in the presence of greyback canegrubs and isolated canegrub cuticles.

8) To test the transformation efficiency of *Metarhizium* using a benomyl resistance gene introduced via biolistic bombardment.

9) To conduct bioassays to evaluate the virulence characteristics of the mutant strains and benomyl resistance transformants.

The overall aim and aspirations of this project was to identify novel proteins and develop tools to improve the isolate FI-1045 (*M. anisopliae* var. *anisopliae*), currently used as a biopesticide for canegrub control. This aim was approached by using proteomic techniques to identify key proteins expressed during infection of canegrubs using solid culture and liquid culture approaches. UV mutagenesis and screening was used as a traditional genetic method for strain improvement and biolistic transformation technique was used to test the transformation efficiency of *Metarhizium*. The *Metarhizium* mutant strains and transformants that showed promising results in laboratory bioassays will ultimately be subjected to greenhouse and small plot field trials.

## 2. Proteomic map of the greyback canegrub, *Dermolepida albohirtum* (Waterhouse) – Manuscript I

This section describes the differential protein extraction and solubilisation methods used to produce a 2D reference map of greyback canegrub *Dermolepida albohirtum*. In this chapter, figures have been included in the body of the text for easy reading and tables are presented at the end of the chapter.

The manuscript has been submitted to the Australian Journal of Entomology in November 2008 and is currently under editorial review. The paper is presented in the format required for publication in the Australian Journal of Entomology.

I would like to thank Jasmine Grinyer for introducing proteomic techniques such as 2-DE. I would also like to express thanks to Peter Samson for continuous supply of canegrubs and my supervisors, Helena Nevalainen and Kathy Braithwaite for the contributions made in reviewing this section. I would also like to thank Junior Te'o and Stevens Brumbley for checking the manuscript. I also thank the Australian Proteome Analysis Facility (APAF) for mass spectrometry analysis.

*Note*: There is a repetition in materials and methods section in Chapters 2, 3, 4 and 6 due to the nature of work presented in these chapters. At the time of preparation and submission of the thesis none of the papers were published for the purpose of being able to be referred to in the other manuscripts.

# Proteomic map of the greyback canegrub, *Dermolepida albohirtum* (Waterhouse)

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**Total number of figures – 2**

**Total number of tables – 1**

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## 2.1. Abstract

The greyback canegrub is a native pest causing regular damage to sugarcane throughout northern and central Queensland thus crippling the production of sugarcane in Australia. A proteomic approach was used to produce a 2D reference map for the greyback canegrub (*Dermolepida albohirtum*). Differential protein extraction and solubilisation methods were employed to obtain sufficient amounts of protein for the 2D analysis. Approximately 156 protein spots were detected on the 2D gel of the whole grub of which 54 proteins were identified using cross species identification. The majority of the resolved and identified proteins were muscle actin along with tropomyosin and myosin, which reflects the grub physiology. The proteome map presented here serves as a reference for future investigations into how canegrubs respond to different physiological conditions at the protein level.

Keywords: *Proteins, proteome map, greyback canegrub, actin*

## 2.2. Introduction

Greyback canegrubs, *Dermolepida albohirtum* (Waterhouse) are economically disastrous pest insects of sugarcane fields in Australia, currently costing the industry more than $12 million annually in insecticides and lost production. These insects spend four months in the larval stage feeding below ground on the root system of the sugarcane plants. The greyback canegrub commonly known as "white grub" belongs to the typical holometabolous insect group Coleoptera. The damage from greyback canegrub leads to lodging, loss of stools at harvest, poor rationing, retarded growth, root and stem breakage and in extreme cases plant death (Ward 2003). The larval stage of these holometabolous insects typically represents a period of developmental growth and often inhabits niches distinct from the adults. The larva develops near food sources where the worm like body form is practical (Barret *et al*. 2002). During the developmental stage of insects there is a dynamic alteration of protein expression which is strictly regulated both temporally and spatially. Gene expression during insect development has been extensively studied in insects such as fruit fly (*Drosophila melanogaster*; White *et al*. 1999), honey bee (*Apis mellifera*; Evans & Wheeler 1999) and termites (*Retculitemes flavipes*; Scharf *et al*. 2003).

Proteomics is potentially a powerful method for gaining insights to many types of physiological changes in insects such as developmental changes from larval to adult form and responses to stress and parasitisation. Proteomic analyses have been carried out with several insects such as mosquito (*Anopheles gambiae*; Johnson *et al*. 2004), cattle tick (*Boophilus microplus*; Madden *et al*. 2002), and tobacco hornworm (*Manduca sexta*; Jiang *et al*. 1996). Proteomics has been applied in comparing the protein profiles of both the biocontrol agents (bacteria, fungi and virus) as well as the target pests which are of agricultural importance. For example, comparative proteomics was used to identify *Bacillus thuringiensis cry9Aa2* gene that encodes a 129 kDa protein with insecticidal activity against larval stages of potato tuber

moth (PTM), *Phthorimaea operculella*, which causes serious damage to potatoes in storage, as well as plants in the field (Cooper 1994). Current integrated pest management (IPM) strategies for greyback cane grubs include sound horticulture practices, beetle light traps, naturally occurring predators and parasites, adult beetle monitoring, use of organophosphates and a combination of suppressive tactics to control larvae and adults (Robertson *et al*. 1995).

To date there are no reported data about genes or proteins identified from the greyback canegrubs. The objective of this work was to display the proteome of the larval stage of *Dermolepida albohirtum* to pave the way for identification of potential targets for biological control of these pests (Manalil *et al*., in preparation).

## 2.3. Materials and methods

### 2.3.1 Insect larvae

Greyback canegrubs, *D. albohirtum* were dug from the soil below sugarcane stools in commercial fields around Townsville and each grub was packed in a single plastic tube for transportation to Sydney. After arrival, the cane grubs were removed from the plastic tube and transferred into 100 ml sterile transparent plastic tubs (Technoplas, Australia) filled with 30 g of sterilized garden peat (Killarney peat moss, Australia). The cane grubs were fed with fresh pieces of carrot and held at room temperature (RT) for one month before conducting protein extractions.

### 2.3.2. Preparation of protein samples

Around ten healthy canegrubs were immobilized by cotton dipped in chloroform (Ajax Finechem, Sydney) to depress the nervous system. Canegrubs were then rinsed in sterile distilled water to remove peat particles attached to the body and transferred into 15 ml tubes, freeze dried and ground into a fine powder using mortar and pestle. Protein extraction was

performed as described in Grinyer *et al*. (2004). Briefly, 0.5 g of freeze-dried material was resuspended in either 10 mL of extraction solution I (7 M urea, 2 M thiourea, 1% C7BzO, 80 mM citric acid, 5 mM tributylphosphine, 1 mM PMSF, 0.1% complete Mini EDTA-free Protease Inhibitor Cocktail Tablet [Roche Applied Science, NSW, Australia]) or extraction solution II (7 M urea, 2 M thiourea, 4% CHAPS, 80 mM citric acid, 5 mM tributylphosphine, 1 mM PMSF, 0.1% complete Mini EDTA-free Protease Inhibitor Cocktail Tablet) and left on ice for 1 h. The samples were lysed using the Digital Branson Sonifier® 450 (Branson Ultrasonics Corporation, Danbury, CT, USA) at an output level of 60% intensity for 6 cycles of 30 s and kept on ice for 1 min between sonication cycles. The debris was centrifuged for 30 min at 21000 *g* at 20°C. The supernatant was collected and proteins were precipitated by adding nine volumes of acetone. The samples were then incubated for 15 min at RT. The proteins were centrifuged for 15 min at 2500 *g* at 20°C, acetone was poured off and the pellet dried for 5 min at RT. The pellet was resuspended in either 4 mL of sample buffer I (7 M urea, 2 M thiourea, 1% C7BzO, 40 mM tris, 5 mM tributlyphosphine, 10 mM acrylamide, 1 mM PMSF, 0.1% complete Mini EDTA-free Protease Inhibitor Cocktail Tablet) or sample buffer II (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM tris, 5 mM tributlyphosphine, 10 mM acrylamide, 1 mM PMSF, 0.1% complete Mini EDTA-free Protease Inhibitor Cocktail Tablet) and incubated at RT for 90 min to completely reduce and alkylate the proteins. The reduction and alkylation reaction was quenched by adding 10 mM dithiothreitol (DTT). Insoluble material was removed by centrifugation at 21000 *g* for 10 min. Samples were prepared at RT to avoid urea from precipitating out from solution. Protein concentration was quantified using the Bradford method (Bio-Rad Protein Assay Kit, CA, USA). Samples were passed through 2-D Clean-Up Kit (Amersham Biosciences, NJ, USA) to remove salts from the sample buffer. Finally, protein pellets were dissolved in a buffer containing 7 M urea, 2 M thiourea and 1% C7BzO or 4% CHAPS depending on the detergent used for extraction. The

sample (representing an average of 10 cane grubs) was either used immediately to rehydrate IPG strips or stored at – 20°C until required. All samples were prepared in triplicate.

### 2.3.3. Isoelectric focusing (IEF) and 2D-SDS-PAGE

First, the samples were used directly to passively rehydrate pH 3-10 immobilized pH gradient (IPG) strips (11 cm; Amersham Biosciences Fairfield, CT, USA) by applying 200 µL of each sample (containing upto 100 µg of protein) and most of the proteins were found to be within the pH range of 4-7. Henceforth, samples (100 µg protein) were used directly to rehydrate pH 4-7 IPG strips (11 cm; Amersham Biosciences Fairfield, CT, USA) by applying 200 µL of sample per IPG strip. IEF was performed on an IsoelectrIQ$^{2TM}$ system (Proteome Systems, Sydney, Australia) at 14°C. The focusing program included a linear ramp from 100 V – 10,000 V over 8 h and hold at 10,000 V for 8 h. IPG strips were equilibrated for 20 min in the ProteomIQ equilibration buffer. The IPGs were placed on 8-16% linear gradient GelChips (11cm x 1mm; Proteome Systems, Sydney, Australia) for second dimension electrophoresis by SDS-PAGE with ElectrophoretIQ$^{3}$™ system (Proteome Systems, Sydney, Australia). The gels were run at a constant 30 mA until the indicator dye reached the bottom of the gel. Gels were fixed in 10% methanol and 7% acetic acid solution for 30 min and then stained overnight with Sypro Ruby (Molecular Probes, OR, USA) as instructed. Gels were destained in the fixing solution before proteins were visualised under UV light using ChemiImager 4400 Imaging system (Alpha Innotech Corporation, CA, USA). Gels were then counter stained with Coomassie colloidal blue G250 (Proteome Systems, Sydney, Australia) and scanned at the Australian Proteome Analysis Facility (APAF) with an FX Molecular Imager (Bio-Rad, CA, USA), to produce a digital image. The image analysis program used was Progenesis image analysis software, version 2005 (Nonlinear Dynamics, Newcastle, UK).

### 2.3.4. Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI – TOF-MS)

Protein spots were digested with trypsin for 16 h at 37ºC. Peptides were desalted and concentrated on an Eppendorf C18 zip tip column before being eluted in 1 µl matrix (α-cyano-4-hydroxycinnamic acid, 5 mg/mL in 70% (v/v) AcN (acetonitrile), 1% (v/v) TFA (trifluoroacetic acid) and spotted onto a target plate. MALDI mass spectrometry was performed with an Applied Biosystems 4700 Proteomics Analyser (Applied Biosystems, Foster City, CA, USA) with TOF/TOF optics in MS mode. A Nd:YAG laser (355 nm) was used to irradiate the sample. The spectra were acquired in reflectron mode in the mass range of 750 to 3500 Da. The instrument was then switched to MS/MS (TOF/TOF) mode where the eight strongest peptides from the MS scan were isolated and fragmented, then re-accelerated to measure their mass and intensities. A near point external calibration was applied and will give a typical mass accuracy of less than 50 ppm. To identify the protein, all MS/MS spectra of tryptic peptides derived from a protein spot were searched against metazoan protein sequences from NCBInr and Swiss-Prot databases using the MASCOT search program (www.matrixscience.com). The top scoring proteins were compared to the true protein using BLAST program. The following parameters were used in all searches: the maximum number of missed cleavage allowed = 1, the mass tolerance = 1 Da, peptide tolerance = ± 50 ppm, MS/MS tolerance = ± 0.8 Da, peptide charge = "1+", minimum peptides required to match = 4. The monoisotopic masses of observation peaks were used to match the calculated monoisotopic fragment masses for protein identification. Possible variable modifications considered in this search procedure were oxidation of methionine and propionamide of cysteine.

## 2.4. Results

Whole grub extract was used to construct a 2D map of *D. albohirtum* proteins. We were able to separate a total of 156 protein spots across a pH range of 4-7, as seen in Fig. 1A and Fig. 1B. Protein extraction and solubilisation of *D. albohirtum* proteins were compared using either C7BzO or CHAPS detergent (extraction solution I and II). Protein extraction with C7BzO resulted in better protein separation (Fig. 1A) across the gel to that obtained by solubilisation with CHAPS (data not shown) as the intensity and size of every protein spot in the lower part of the gel increased markedly. A thin immiscible layer was observed in both extraction solutions during sample preparation which could be due to the fat bodies of the grub. This resulted in streaking on the gels which could not be completely avoided even after the use of surfactants and acetone precipitation.

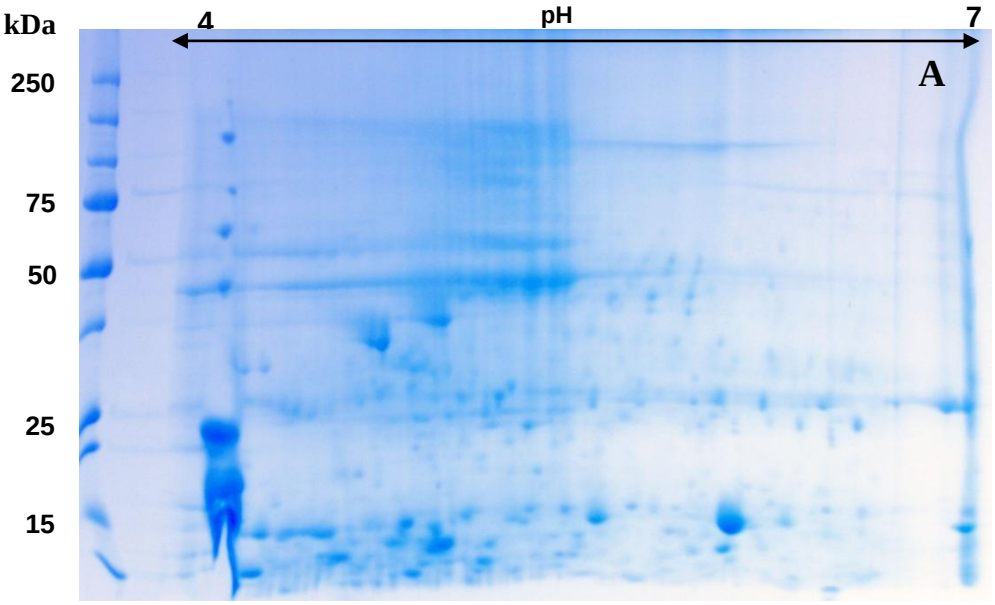


***Fig.1A.*** Coomassie stained 2D-PAGE map of larval proteins of the greyback canegrub, *Dermolepida albohirtum* (Waterhouse). Proteins (100 µg) were separated by IEF using 11 cm IPG strips (pH 4-7) in the first dimension and followed by 8-16% gradient SDS-PAGE in the second dimension.

All 156 protein spots separated by 2D-SDS-PAGE were isolated and analyzed by MALDI-TOF-MS (Fig. 1B). Proteins were identified by searching against all Metazoan species and results are tabulated in Table 1 at the end of this chapter. From the 156 protein spots cut for

MALDI analysis, 54 were identified successfully giving a 34% identification rate. Of the 54 protein spots identified, 25 proteins were found to be actin from muscles (contractile proteins) and five protein spots were found to be tropomyosin (muscle regulating proteins). Other proteins identified included arginine kinase, myosin, putative kelch-like protein, thioredoxin, elongation factor, triose phosphate isomerase, kinesin light chain, formate hydrogenlyase, RNA polymerase II subunit, Trans activation response (TAR) RNA binding protein1, epsilon-globin and huntingtin-interacting protein1.

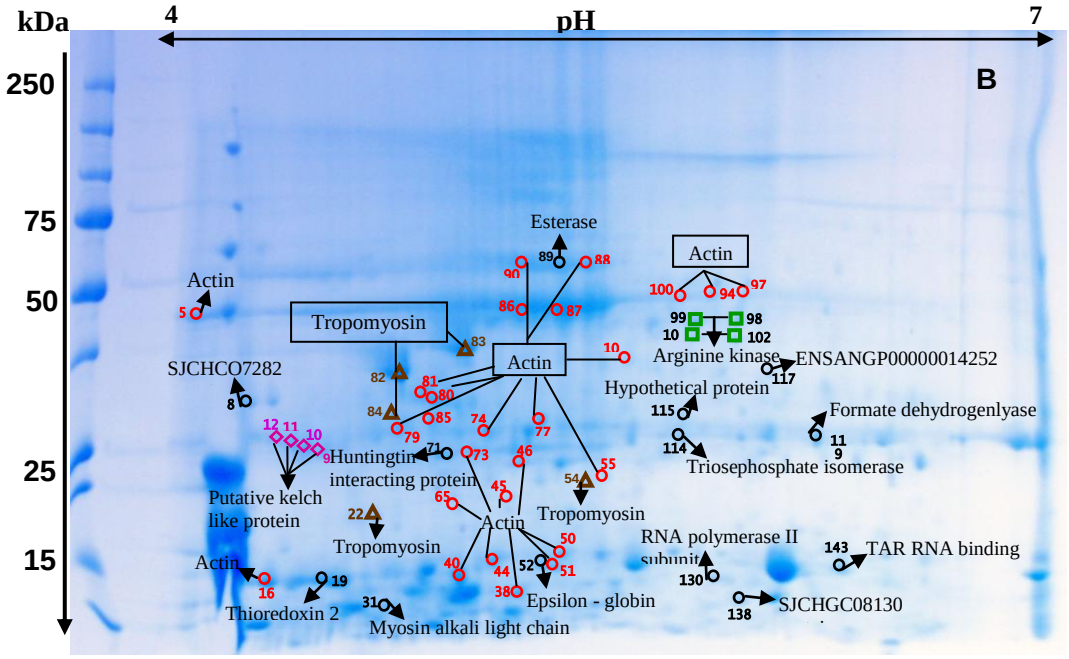


***Fig.1B.*** Reference map of *Dermolepida albohirtum* (Waterhouse) containing protein identifications after mass spectrometry. The whole protein extract *Dermolepida albohirtum* is displayed across an IPG (pH 4-7) in the first dimension and 8-16% SDS PAGE in the second dimension. Additional information about these proteins can be found in Table 1.

## 2.5. Discussion

Holometabolous larvae are an ancient and highly successful group of organisms occupying different ecological niches. A better understanding of the molecular basis of insect development may help find out why these organisms are so successful and to formulate novel

strategies for biological control of these pests. Here we present the first published protein map for the greyback canegrub (*Dermolepida albohirtum*). To establish the best possible 2-D gel conditions, a pilot experiment was conducted where protein sample was extracted and separated first on broad range (pH 3-10) IPG strips for the first dimension and 8-16% linear gradient GelChips for the second dimension. The majority of the protein spots were detected in the centre of the gel (data not shown), indicating that a narrower pH range for the IPG strip was necessary for increased resolution. However, proteins were well separated from top to bottom by their molecular weight. Thus, pH 4-7 range was used for subsequent experiments. A total of 156 protein spots ranging from 10 kDa to 150 kDa were resolved across the pH range of 4-7 using 2D-SDS-PAGE of which fifty four proteins were identified (Table 1).

The primary technical limitations in invertebrate proteomics are complexities inherent in protein extraction, protein purification and availability of sufficient quantity of canegrub material. The number of protein spots detected in this study seems considerably less when compared to larval proteomic studies involving fruit flies (*Drosophila melanogaster*; Levy *et al*. 2004) and mosquitoes (*Anopheles gambie*; Zdobnov *et al*. 2002). The number of protein spots resolved in fruit flies and mosquitoes have been around 300-400 (Liu *et al*. 2002), whereas 166 protein spots were observed in the larvae of parasitic organisms like *Fasciola hepatica* (Jefferies *et al*. 2000). Differences in the number of protein spots between different invertebrates could be attributed mainly to factors such as solubilization of the sample under different extraction conditions, developmental events occurring during metamorphosis and also the size of insect. The choice of precipitation of the sample with acetone employed in generating a 2D map of *Fasciola hepatica* is comparable to our extraction and precipitation method. This suggests that extraction and precipitation procedures have a profound effect on the profile, number and concentration of protein spots seen on 2D-SDS-PAGE gels. Two hundred and eighty six protein spots displayed on 2D-SDS-PAGE were successfully isolated

from *Caenorhabditis elegans*, following an increased number of protein separation and prefractionation steps (Krijgsveld *et al*. 2003). Gross changes in protein quantities have been observed during larval development and among the pupal and moth stages of holometabolous insects like *Manduca sexta* (Ziese & Dorn 2002). For example, Yang and colleagues demonstrated that there was significant fluctuation in the protein content during the developmental stage of southern armyworm (Yang *et al*. 1973). The lower number of proteins isolated from *D. albohirtum* compared to *C. elegans* and *M. sexta* could be explained by timing and developmental age of the canegrubs used in this study.

Despite the absence of genome data for *Dermolepida albohirtum*, we have shown that it is possible to identify a significant number of proteins from this organism using cross species identification (Liska & Shevchenko 2003a). In our study, we discovered matches to proteins from related Metazoans such as *Drosophila* and *C. elegans*. Positive protein matches obtained revealed minor differences in the theoretical pI and MW values which occur commonly when using different databases and different molecular weight standards for protein estimation. In this work, a 34% protein identification rate was achieved when compared to 16% - 25% protein identification rates using comparable 2D approaches (Grinyer *et al*. 2004; Khan *et al*. 2003).

The majority of proteins identified from the greyback canegrub were muscle specific actin and tropomyosin. The peptide mass fingerprinting (PMF) data showed that the identified actin and tropomyosin share homology to those of fruit flies, silkworm and mosquitoes in which actin and tropomyosin genes have been investigated in detail (Zdobnov *et al*. 2002). Filamentous actin outlines the core of all muscle thin filaments and is an integral part of the acto-myosin motor system that powers cell growth and movement. Differential expression of six actin genes and two tropomyosin genes during development have been described in two

holometabolous insects, *D. melanogaster* and *Bombyx mori* (Mounier & Sparrow 1993). In vertebrates and insects, different actin isoforms have been grouped according to their amino acid sequence and tissue-specific gene expression profiles into muscle and nonmuscle actins with high electrophoretic mobilities, indicating that the different actins may have functional significance (Mounier *et al*. 1991). Furthermore, proteomic data indicate the existence of several actin encoding genes in the *D. albohirtum* genome.

The kelch-repeat superfamily is evolutionarily widespread and comprises a unique kind of actin binding proteins. It is described by tandemly arranged motifs of about 50 amino acids. In our analysis, four kelch-like proteins were identified. These proteins were found in more than one position on the gel, possibly due to post-translational modification or proteolysis (Fig.1A). The first Kelch protein was isolated from *Drosophila* (Xue & Cooley 1993). Electron microscopy (Kelso *et al*. 2002) demonstrated that phosphorylation of Kelch is critical for the proper morphogenesis of actin during ring canal growth. Previous studies showed that most members of the kelch-repeat family were cytoskeletal proteins implicated in various cellular processes, such as actin cytoskeleton interaction, cytoplasmic sequestration of transcription factors and cell morphology. It has been identified that mutant forms of Kelch result in a dramatic reduction in the dynamics of actin-binding proteins involved with actin polymerization or depolymerization.

We also identified five spots corresponding to arginine kinase in the greyback canegrub. Arginine kinase (AK) belongs to a class of kinases that play a major role in muscle contraction and maintenance of ATP levels by the phosphorylation of "phosphagens" which then serve as a high energy source from which ATP can be rapidly replenished. In a wide range of invertebrates phosphoarginine is the most important phosphagen. It is associated

with actin binding region (Reddy *et al*. 1992) and higher levels of AK are often associated with higher locomotory performance in insects.

Proteomic data signifies the presence of thioredoxin in the *D. albohirtum* genome. Thioredoxins belong to a widely distributed group of small proteins with strong reducing activities mediated by a consensus redox-active dithiol (Cys-Gly-Pro-Cys). Thioredoxins have been found in all cell types and play a variety of functions such as DNA synthesis and repair, antioxidant defence, regulation of transcription factor activity, protein biosynthesis in the fat bodies and redox-regulatory processes via a pair of conserved cysteine residues. Thioredoxin 2 identified in this study is a mitochondrial specific member and has a crucial role in the signal transduction for apoptosis and scavenging ROS (reactive oxygen species) in mitochondria (Arner & Holmgren, 2000; Kanzok *et al*. 2001). This regulatory cascade may be part of a long distance signaling system needed in systemic responses. In dipteran insects like *Drosophila* and *Anopheles*, which lack a genuine glutathione reductase, thioredoxins fuel the glutathione system with reducing equivalents (Rasmusson *et al*. 1994). Therefore, features distinguishing thioredoxins of dipteran insects to that of thioredoxins of glutathione reductase -containing organisms are of great interest with regard to mechanisms of redox reactions and their control. They also provide information on novel targets for insect control.

In this study we have yet to identify 100 proteins and these proteins will be identified as the greyback canegrub genome structure becomes better understood. Though this paper describes the first attempt at greyback canegrub, *Dermolepida albohirtum* proteomics, it is anticipated that these data will eventually facilitate identification of potential targets for the biological control of these pests in sugarcane fields.

## 2.6. References

Arner ES & Holmgren A. 2000. Physiological functions of thioredoxin and thioredoxin reductase. *European Journal of Biochemistry* **267**, 6102–6109.

Barret J, Jefferies JR & Brophy, PM. 2002. Parasite proteomics. *Parasitology* **16**, 400-403.

Cooper D. 1994. *Bacillus thuringiensis* toxins and mode of action. *Agriculture, ecosystems & environment* **49**, 21-26.

Evans JD & Wheeler DE. 1999. Differential gene expression between developing queens and workers in the honey bee, *Apis mellifera*. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 5575-5580.

Grinyer J, McKay M, Nevalainen H & Herbert BR. 2004. Fungal proteomics: initial mapping of biological control strain *Trichoderma harzianum*. *Current Genetics* **45**, 163-169.

Jefferies JR, Brophy PM & Barrett J. 2000. Investigation of *Fasciola hepatica* sample preparation for two-dimensional electrophoresis. *Electrophoresis* **21**, 3724-3729.

Jiang H, Wang Y & Kanost MR. 1996. Primary structure of ribosomal proteins S3 and S7 from *Manduca sexta*. *Insect Molecular Biology* **5**, 31-38.

Johnson JR, Florens L, Carucci DJ & Yates JR. 2004. Proteomics in malaria. *Journal of Proteome Research* **3**, 296-306.

Kanzok SM, Fechner A, Bauer H, Ulschmid JK, Muller HM, Botella-Munoz J, Schneuwly S, Schirmer RH & Becker K. 2001. Substitution of the thioredoxin system for glutathione reductase in *Drosophila melanogaster*. *Science* **291**, 643-646.

Kelso RJ, Hudson AM & Cooley L. 2002. *Drosophila* kelch regulates actin organization via Src64-dependent tyrosine phosphorylation. *Journal of Cell Biology* **156**, 703-713.

Khan A, Williams K, Molloy MP & Nevalainen H. 2003. Purification and characterization of a serine protease and chitinases from *Paecilomyces lilacinus* and detection of chitinase activity on 2D gels. *Protein Expression and Purification* **32,** 210-220.

Krijgsveld J, Ketting RF, Mahmoudi T, Johansen J, Artal-Sanz M, Verrijzer CP, Plasterk RHA & Heck AJR. 2003. Metabolic labelling of *C. elegans* and *D. melanogaster* for quantitative proteomics. *Nature Biotechnology* **21**, 927-931.

Liska AJ & Shevchenko A. 2003. Expanding organismal scope of proteomics: cross-species protein identification by mass spectrometry and its implications. *Proteomics* **3**, 19-28.

Levy F, Bulet P & Ehret-Sabatier L. 2004. Proteomic analysis of the systemic immune response of *Drosophila*. *Molecular and Cellular Proteomics* **3**, 156-166.

Liu H, Lin D & Yates JR. 2002. Multidimensional separations for protein/peptide analysis in the post-genomic era. *Biotechniques* **32**, 898-900.

Madden RD, Sauer JR & Dillwith JW. 2002. A proteomics approach to characterizing tick salivary secretions. *Experimental and Applied Acarology* **28,** 77-87.

Miyazaki J, Yahata K, Makioka T & Hirabayashi T. 2005. Tissue specificity of arthropod tropomyosin. *Journal of Experimental Zoology* **267**, 501-509.

Mounier N, Gouy M, Mouchiroud D & Prudhomme JC. 1991. Insect muscle actins differ distinctly from invertebrate and vertebrate cytoplasmic actins. *Journal of Molecular Evolution* **34**, 406-415.

Mounier N & Sparrow JC. 1993. Muscle actin genes in insects. *Comparative biochemistry and physiology. B, Comparative Biochemistry* **105**: 231-238.

Prior C, Carey M, Abraham YJ, Moore D, Bateman RP (1995) Development of a bioassay method for the selection of entomopathogenic fungi virulent to the desert locust *Schistocerca gregaria* (Forskal). *Journal of Applied Entomology* 119: 567 – 572.

Rasmusson K, Serr M, Gepner J, Gibbons IR & Hays TS. 1994. A family of dynein genes in *Drosophila melanogaster*. *Molecular Biology of the Cell* **5**, 45-55.

Reddy SRR, Houmeida A, Benyamin Y & Roustan C. 1992. Interaction *in vitro* of scallop muscle arginine kinase with filamentous actin. *European Journal of Biochemistry* **206**, 251-257.

Robertson LN, Allsopp PG, Chandler KJ & Mullins RT. 1995. Integrated management of canegrubs in Australia: current situation and future research directions. *Australian Journal of Agricultural Research* **46**, 1-16.

Scharf WD, Scharf ME, Pittendrigh BR & Bennett GW. 2003. Expressed sequence tags from a polyphenic *Reticulitermes flavipes* cDNA library. *Sociobiology* **41**, 479-490.

Ward AL. 2003. Does soil texture influence the distribution of the greyback canegrub, *Dermolepida albohirtum* (Waterhouse) (Coleoptera: Scarabaeidae), in the Burdekin River sugarcane growing area? *Australian Journal of Agricultural Research* **54**, 861-868.

White KP, Rifkin SA, Hurban P & Hogness DS. 1999. Microarray analysis of *Drosophila* development during metamorphosis. *Science* **286**, 2179-2184.

Xue F & Cooley L. 1993. *kelch* encodes a component of intercellular bridges in *Drosophila* egg chambers. *Cell* **72**, 681-693.

Yang RSH, Pelliccia JG & Wilkinson CF. 1973. Age-Dependent arylsulphatase and sulphotransferase activities in the southern armyworm: a possible insect endocrine regulatory mechanism? *Journal of Biochemistry* **136**, 817-820.

Zdobnov EM, vonMering C, Letunic I, Torrents D, Suyama M, Copley RR, Christophides GK, Thomasova D, Holt RA, Subramanian GM, Mueller HM, Dimopoulos G, Law JH, Wells MA, Birney E, Charlab R, Halpern AL, Kokoza E, Kraft CL & Lai Z. 2002. Comparative genome and proteome analysis of *Anopheles gambiae* and *Drosophila melanogaster*. *Science* **298**, 149-159.

Ziese S & Dorn A. 2002. Embryonic integument and "molts" in *Manduca sexta* (insecta, Lepidoptera). *Journal of Morphology* **255**,146-161.

| **Table 1: Proteins identified from greyback canegrub *D. albohirtum*** | | | | | | |
|---|---|---|---|---|---|---|
| **Spot #** | **Protein** | **Accession #** | **pI** | **Molecular mass (Da)** | **Coverage or number of peptides matched** | **Species** |
| 5 | Actin | CAA29661 | 5.29 | 41776 | 23% | *Bombyx mori* |
| 8 | SJCHCO7282 protein | AAW24686 | 6.32 | 31090 | 19% | *Schistosoma japonicum* |
| 9 | (Putative kelch-like protein) | XP792995 | 5.37 | 25357 | 34% | *Strongylocentrotus purpuratus* |
| 10 | (Putative kelch-like protein) | XP792995 | 5.37 | 25357 | 38% | *Strongylocentrotus purpuratus* |
| 11 | (Putative kelch-like protein) | XP792995 | 5.37 | 25357 | 28% | *Strongylocentrotus purpuratus* |
| 12 | (Putative kelch-like protein) | XP792995 | 5.37 | 25357 | 38% | *Strongylocentrotus purpuratus* |
| 16 | Actin, muscle A2 | P07837 | 5.29 | 41776 | 22% | *Bombyx mori* |
| 19 | Thioredoxin 2 | AAW27316 | 9.1 | 17169 | 46% | *Schistosoma japonicum* |
| 22 | Tropomyosin 2 | P09491 | 4.77 | 32961 | 16% | *Drosophila melanogaster* |
| 31 | Myosin alkali light chain | AAA53447 | 5.8 | 9468 | 24% | *Drosophilia melanogaster* |
| 35 | Elongation factor 1 alpha | AAW73091 | 5.93 | 16753 | 24% | *Pseudococcus callitris* |
| 38 | Actin | Q11212 | 5.76 | 18032 | 19% | *Spodoptera littoralis* |
| 40 | Actin, cytoplasmic 1D | P84185 | 5.3 | 41795 | 22% | *Anopheles gambiae* |
| 44 | Actin, muscle A2 | P07837 | 5.29 | 41776 | 23% | *Bombyx mori* |
| 45 | Actin, muscle A2 | P07837 | 5.29 | 41776 | 19% | *Bombyx mori* |
| 46 | Actin 2 | AAC78682 | 5.11 | 41822 | 4 peptides | *Panaeus monodon* |
| 50 | Actin, muscle A2 | P07837 | 5.29 | 41776 | 3 peptides | *Bombyx mori* |
| 51 | Actin, muscle A2 | P07837 | 5.29 | 41776 | 5 peptides | *Bombyx mori* |
| 52 | Epsilon-globin | AAA36948 | 8.7 | 16305 | 38% | *Saimiri sciureus* |
| 54 | Tropomyosin | CAC84593 | 4.8 | 28085 | 4 peptides | *Lepisma saccharina* |
| 55 | Actin, larval muscle (Actin-79B) | P02574 | 5.3 | 41760 | 17% | *Drosophila melanogaster* |
| 65 | Actin, muscle A2 | P07837 | 5.29 | 41776 | 5 peptides | *Bombyx mori* |
| 71 | Huntingtin-interactingprotein | XP798478 | 6.12 | 20461 | 29% | *Strongylocentrotus purpuratus* |
| 73 | Actin fragment | XP623736 | 5.56 | 41394 | 25% | *Apis mellifera* |
| 74 | Actin | CAB72311 | 5.2 | 27426 | 19% | *Daphnia pulex* |
| 77 | Actin, muscle A2 | P07837 | 5.29 | 41776 | 22% | *Bombyx mori* |
| 79 | Actin | EAA09799 | 5.29 | 41632 | 15% | *Anopheles gambiae* |
| 80 | Actin | AAY88916 | 5.3 | 41783 | 14% | *Culex pipiens pipiens* |
| 81 | Actin, muscle A2 | P07837 | 5.29 | 41776 | 17% | *Bombyx mori* |
| 82 | Tropomyosin 2 | P09491 | 4.77 | 32961 | 6 peptides | *Drosophila melanogaster* |
| 83 | Tropomyosin | P31816 | 4.7 | 32420 | 28% | *Locusta migratoria* |

| | | | | | | |
|---|---|---|---|---|---|---|
| 84 | Tropomyosin 2 | P09491 | 4.77 | 32961 | 5 peptides | *Drosophila melanogaster* |
| 85 | Actin indirect flight muscle | P83969 | 5.29 | 41673 | 6 peptides | *Bactrocera dorsalis* |
| 86 | Actin, muscle A2 | P07837 | 5.29 | 41776 | 22% | *Bombyx mori* |
| 87 | Actin, muscle A2 | P07837 | 5.29 | 41776 | 22% | *Bombyx mori* |
| 88 | Actin, indirect flight muscle | P83969 | 5.29 | 41673 | 5 peptides | *Bactrocera dorsalis* |
| 89 | Esterase | CAC83739 | 6.01 | 61254 | 18% | *Culex pipiens* |
| 90 | Actin, indirect flight muscle | P83969 | 5.29 | 41673 | 22% | *Bactrocera dorsalis* |
| 94 | Actin, larval muscle (Actin-79B) | P02574 | 5.3 | 41760 | 20% | *Drosophila melanogaster* |
| 97 | Actin fragment | XP623736 | 5.56 | 41394 | 15% | *Apis mellifera* |
| 98 | Arginine kinase | AAF43438 | 6.34 | 40343 | 16% | *Chasmagnathus granulata* |
| 99 | Putative arginine kinase | AAU95198 | 5.8 | 39958 | 7 peptides | *Oncometopia nigricans* |
| 100 | Actin, muscle A2 | P07837 | 5.29 | 41776 | 22% | *Bombyx mori* |
| 101 | Putative arginine kinase | AAU95198 | 5.8 | 39958 | 19% | *Oncometopia nigricans* |
| 102 | Arginine kinase | P91798 | 5.87 | 39991 | 6 peptides | *Schistocerca americana* |
| 103 | Putative arginine kinase | AAU95198 | 5.8 | 39958 | 23% | *Oncometopia nigricans* |
| 105 | Actin, larval muscle (Actin-79B) | P02574 | 5.3 | 41760 | 17% | *Drosophila melanogaster* |
| 114 | Triosephosphate isomerase | AAT06243 | 5.67 | 23304 | 17% | *Nucula proxima* |
| 115 | Hypothetical protein | CAE59816 | 5.52 | 25544 | 22% | *Caenorhabditis briggsae* |
| 117 | ENSANGP00000014252 | EAA13940 | 5.61 | 53683 | 23% | *Anopheles gambiae* |
| 119 | Formate hydrogenlyase | AAB86206 | 5.28 | 15680 | 48% | *Methanothermobacter thermautotrophicus* |
| 130 | RNA polymerase II largest subunit | AAK11885 | 5.89 | 15624 | 25% | *Craterostigmus tasmanianus* |
| 138 | SJCHGC08130 protein | AAX30844 | 6.05 | 9763 | 40% | *Schistosoma japonicum* |
| 143 | Similar to TAR RNA binding protein | XP623778 | 6.36 | 18440 | 35% | *Apis mellifera* |

## 3. A proteomic view into infection of greyback canegrubs (*Dermolepida albohirtum*) by *Metarhizium anisopliae* - Manuscript II

This section describes the *Metarhizium* infection in greyback canegrubs and to identify the differentially expressed proteins in *Metarhizium* infected canegrubs. The proteins identified in this study have provided an insight into the role of various immune related proteins of greyback canegrubs during the infection process. In this chapter, figures have been included in the body of the text for easy reading and tables are presented at the end of the chapter.

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# A proteomic view into infection of greyback canegrubs (*Dermolepida albohirtum*) by *Metarhizium anisopliae*

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## 3.1. Abstract

*Metarhizium anisopliae* is a naturally occurring cosmopolitan fungus infecting greyback cane grubs (*Dermolepida albohirtum*). The main molecular factors involved in the complex interactions occurring between the greyback cane grubs and *M. anisopliae* (FI-1045) were investigated by comparing the proteomes of healthy cane grubs, cane grubs infected with *Metarhizium* and fungus only. Differentially expressed proteins from the infected cane grubs were subjected to mass spectrometry to search for pathogenicity related proteins. Immune related proteins of cane grubs identified in this study include cytoskeletal proteins (actin), cell communication proteins, proteases and peptidases. Fungal proteins identified include metalloproteins, acyl-CoA, cyclin proteins and chorismate mutase. Comparative proteome analysis has provided a view into cellular reactions triggered in the cane grub in response to the fungal infection at the onset of biological control.

Keywords: *Greyback canegrub, Metarhizium anisopliae, biological control, differential proteins, 2DE.*

## 3.2. Introduction

Greyback canegrubs (*Dermolepida albohirtum*, Waterhouse, Coleoptera: Scarabaeidae) cause extensive damage throughout central and north Queensland's valuable sugarcane crop every year by feeding on the roots, severely affecting plant growth. Current control of cane grubs is based on the use of insecticides such as Suscon® Blue (140 g/kg chlorpyrifos in a controlled-release formulation), Mocap (100 g/kg ethoprophos) and Rugby (100 g/kg cadusafos) in conventional clay-based granules. However, chemical insecticides do not work well as cane grubs develop resistance (Chandler and Erbacher, 1997) and active ingredients are degraded in the soil when exposed to high environmental temperatures. Therefore, efficient biological control would provide an attractive alternative.

The entomopathogenic fungus *Metarhizium anisopliae* [(Metschn) Sorokin (Ascomycota: Hypocreales)] is primarily a pathogen of soil-inhabiting scarabaeid larvae (Milner et al. 2003). It has been developed as a biopesticide against many insects (Faria and Wraight, 2007) including soil grubs of pasture (Milner et al. 1998; Milner and Jenkins, 1996). In general, different strains of *M. anisopliae* have shown to be species specific in infection (Wang and St. Leger, 2007), i.e. a particular *M. anisopliae* strain found to infect one insect species will not necessarily infect others. While this specificity limits the use of *Metarhizium* for general pest control, it makes it safer for non-target organisms (Bidochka et al. 1997). Therefore, further development of biological control agents would require a good understanding of the cellular and molecular basis of fungal infection. The life cycle of entomopathogenic fungi usually involves the adhesion, germination, penetration, invasion, colonisation of the host tissues and sporulation. The fungus multiplies within the soft tissues of the host, and death usually occurs within three to ten days after infection (for small insects such as fruit fly), due to water loss, nutrient deprivation, gross mechanical

damage and the action of toxins (Ferron, 1978; Hajek and St. Leger, 1994; Wahlman and Davidson, 1993). Under favorable conditions, the fungus sporulates extensively on the cadaver to facilitate further infection of the host population to continue the disease cycle. The timing of each of these infection stages is variable depending on the size of the host and environmental conditions (Hajek and St. Leger, 1994). Greyback cane grubs make a good host for fungal pathogens because they are generally soft bodied and inhabit environments with humid microclimates which favor infection and transmission of infection to the healthy grubs (Logan and Kettle, 2002). There are a number of publications which describe the efforts made to recognise different modes of infection by entomopathogenic fungi (Clarkson and Charnley, 1996; St. Leger et al. 1988 and 1989; Patterson et al. 1994). *M. anisopliae* has been administered by a number of methods such as direct spraying (Samson et al. 2005; Freimoser et al. 2003), dipping in fungal spore suspension (Wang and St. Leger, 2006), injecting insects with fungal spores (Frobius et al. 2000), direct application of the fungal spores to plant material (Samson et al. 2005) and fungal spores dispersed on different parts of the insect body after dissection (Freimoser et al. 2003).

Proteomic analysis has been employed to identify a great variety of proteins associated with fungal virulence (Murad et al. 2006) and *Metarhizium* microarrays have been applied to assess gene expression (Freimoser et al. 2005) upon infection. Expressed sequence tag (EST) database which contains a large number of pathogenicity related genes has been established from *Metarhizium* strains (Freimoser et al. 2003). *M. anisopliae* produces several cuticle degrading enzymes including four different classes of proteases, chitinases and lipases (Freimoser et al. 2003). Biochemical and pharmacological studies have indicated that *M. anisopliae* also produces a class of insecticidal metabolites called destruxins which are usually secreted into the culture

medium during growth (Liu et al. 2004). Most of the above compounds/molecules related to pathogenesis have been identified from liquid culture experiments.

However, infection never happens in liquid culture in nature potentially leading to a biased interpretation about the mechanisms by which *M. anisopliae* regulates protein synthesis and secretion in a natural infection process. There are a number of articles describing various proteins secreted by entomopathogenic fungi in response to ground insect cuticles or whole insects (Freimoser et al. 2005; Gillespie et al. 1998; St. Leger et al. 1994), but to date there is very little information about the diverse range of proteins exhibited when the insect is infected by entomopathogenic fungi in a natural process. Our study differs from previous proteomic studies in two important aspects. Firstly, a solid culture approach was employed by dipping the cane grubs in fungal spore solution to find the proteins that are expressed during the early phases of infection. Secondly, a three way comparison of the expressed proteins from healthy grubs, infected grubs and the infecting fungus was used to investigate changes in the interacting proteomes during infection.

## 3.3. Materials and methods

### 3.3.1. Insect larvae

Third instar greyback cane grubs, *Dermolepida albohirtum* (Waterhouse) were handpicked from sugarcane fields around Townsville [Queensland, Australia, (http://www.ga.gov.au/bin/gazd01?rec=155794)] and transported to Sydney. Upon arrival, the cane grubs were instantly immobilised using a cotton ball dipped in chloroform (Ajax Finechem, Sydney, Australia) to depress the nervous system and then surface sterilised in 5 % (vol/vol) sodium hypochlorite solution (Prior et al. 1995) and 75 % (vol/vol) ethanol solution and then

rinsed with plenty of sterile distilled water. Cane grubs were then transferred into 100 ml sterile transparent plastic tubs (Technoplas, Australia) filled with 30 g of sterilised garden peat (Killarney peat moss, Australia). Garden peat was sterilised by heating and drying (150 °C, 4 h), then mixed with distilled water. The cane grubs were fed with fresh pieces of carrot and held at room temperature (RT) for seven days until the grubs were used for experiment because only healthy cane grubs were selected for further experiment.

### 3.3.2. Fungal strain and cultivation conditions

*Metarhizium anisopliae* strain FI-1045 was received from BSES Limited (Bureau of Sugar Experiment Stations, Queensland, Australia). *M. anisopliae* was sporulated and maintained on potato dextrose agar (PDA, Difco Laboratories, Detroit, Michigan). Spores were then collected in 10 ml of 0.9 % (wt/vol) sodium chloride and 0.01 % (vol/vol) Tween-80 (Sigma Chemical Co., St. Louis, MO) and filtered through a sterile cotton plug. Spore concentration was adjusted to about $1\times10^8$ spores/ml using a hemocytometer. Spore viability was determined by plating 100 µl of the spore suspension on PDA and counting colonies after 48 h incubation at 28 °C. After incubation, three droplets of lactophenol cotton blue stain (0.5% cotton blue) were added to each petri plate to fix and stain the conidia, and to prevent any further germination. The droplets were covered with a glass slide and evaluated using 400× phase-contrast magnification. The numbers of conidia that germinated in the first 100 spores observed in each droplet were counted, and thus, 300 conidia were read for each petri plate. A conidium was considered to be viable if it germinated (the length of the germ tube was visible and greater than or equal to the width of the conidium). Viability estimates for all treatments were based on the proportion of conidia that had germinated after incubation.

### 3.3.3. Infection of canegrubs

Greyback cane grubs assigned for treatment as well as cane grubs used as control were first immobilized using a cotton ball dipped in chloroform to depress the nervous system and then surface sterilized in 5 % (vol/vol) sodium hypochlorite solution (Prior et al. 1995) and 75 % (vol/vol) ethanol solution and then rinsed with plenty of sterile distilled water. Cane grubs assigned for infection were individually dipped and gently shaken for two minutes in 5 ml of fungal spore suspension ($5x10^8$ spores/ml) and they were put into sterile tubs containing sterilised peat. Each cane grub was fed with a fresh piece of carrot and visually monitored for infection for two weeks. A totally static cane grub which did not consume the carrot piece was considered 'positive' in terms of infection, while a mobile healthy cane grub feeding normally on carrot pieces was 'negative'. Approximately 14 d post-infection all cane grubs were dead. Dead grubs were removed periodically from the tubs and rinsed in sterile distilled water to remove peat particles attached to the body and transferred into 15 ml tubes and freeze dried. On day fourteen, remaining static/dead cane grubs that were infected by *M. anisopliae* as well as 10 healthy control cane grubs were removed from the tubs and rinsed in sterile distilled water to remove peat particles attached to the body and transferred into 15 ml tubes and freeze dried. Freeze dried grubs (ten infected and ten healthy ones) were ground into a fine powder separately using a mortar and pestle.

### 3.3.4. Preparation of protein samples from *D. albohirtum* larvae and *M. anisopliae*

Protein extraction was performed as described by Grinyer et al. (2004). Briefly, 0.5 g of freeze-dried material of infected whole grub (IWG), healthy whole grub (HWG) and the fungus (MY) were resuspended in 10 ml of extraction solution {7 M urea, 2 M thiourea, 1 % (wt/vol) C7BzO, 80 mM citric acid, 5 mM tributylphosphine, 1 mM PMSF, 0.1 % (wt/vol) complete Mini EDTA-free Protease Inhibitor Cocktail Tablet (Roche Applied Science, NSW, Australia)} and left on ice

for 1 h. The samples were lysed using the Digital Branson Sonifier® 450 (Branson Ultrasonics Corporation, Danbury, CT, USA) at an output level of 60 % intensity for 6 cycles of 30 s and kept on ice for 1 min between sonication cycles. The debris was spun down for 30 min at 21000 g at 20 °C. The supernatant was collected and proteins were precipitated by adding nine volumes of acetone. The samples were then incubated for 15 min at RT. The proteins were spun down for 15 min at 2500 g, 20 °C, acetone was poured off and the pellet dried for 5 min at RT. The pellet was resuspended in 4 ml of sample buffer (7 M urea, 2 M thiourea, 1 % (wt/vol) C7BzO, 40 mM tris, 5 mM tributlyphosphine, 10 mM acrylamide, 1 mM PMSF, 0.1 % (wt/vol) complete Mini EDTA-free Protease Inhibitor Cocktail Tablet) and incubated at RT for 90 min to completely reduce and alkylate the proteins. The reduction and alkylation reaction was quenched by adding 10 mM dithiothreitol (DTT). Insoluble material was removed by spinning at 21000 g for 10 min. Samples were prepared at RT to avoid urea from precipitating out from solution. Protein concentration was quantified using the Bradford method (Bio-Rad Protein Assay Kit, Catalog # 500-0002). The sample was passed through 2-D Clean-Up Kit (Amersham Biosciences, Catalog # 80-6484-51) to remove salts from sample buffer. Finally, protein pellets were dissolved in a buffer containing 7 M urea, 2 M thiourea and 1 % (wt/vol) C7BzO. The sample was directly used to rehydrate immobilized pH gradient (IPG) strips or stored at –20 °C until required. All samples were prepared in triplicates.

### 3.3.5. Isoelectric focusing (IEF) and 2D-SDS-PAGE

Protein samples extracted from infected larvae, healthy larvae or fungus after extraction were used directly to passively rehydrate IPG strips (11 cm, pH 4-7; Amersham Biosciences Fairfield, CT, USA) by applying 200 µl (~ 100 µg protein) of each sample. IEF was performed on an IsoelectrIQ$^{2TM}$ system (Proteome Systems, Sydney, Australia) at 14 °C. The focusing program

included a linear ramp from 100 V – 10,000 V over 8 h and focusing program was held at 10,000 V for 8 h. IPG strips were equilibrated for 20 min in the ProteomIQ equilibration buffer. The IPGs were placed on 8-16 % linear gradient GelChips (11 cm x 1 mm; Proteome Systems, Sydney, Australia) for second dimension electrophoresis by SDS-PAGE with ElectrophoreticIQ$^{3TM}$ system (Proteome Systems, Sydney, Australia). The gels were run at a constant 30 mA until the indicator dye reached the bottom of the gel and fixed in 10 % (vol/vol) methanol and 7 % (vol/vol) acetic acid solution for 30 min and then stained overnight with Sypro Ruby solution (Molecular probes) as instructed. Gels were destained in the fixing solution before proteins were visualised under UV light using ChemiImager 4400 Imaging system (Alpha Innotech Corporation, San Leandro, CA, USA) and counter stained with Coomassie colloidal blue G250 (Proteome Systems, Sydney, Australia).

### 3.3.6. Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI – TOF-MS)

Selected protein spots underwent a 16 h tryptic digest at 37ºC. Peptides were desalted and concentrated on an eppendorf C18 zip tip column before being eluted in 1 µl matrix (alpha-cyano-4-hydroxycinnamic acid, 5 mg/ml in 70 % (vol/vol) AcN, 1 % (vol/vol) TFA) and spotted onto a target plate. MALDI mass spectrometry was performed with an Applied Biosystems 4700 Proteomics Analyser (Applied Biosystems, Foster City, CA, USA) with TOF/TOF optics in MS mode. A Nd:YAG laser (355 nm) was used to irradiate the sample. The spectra were acquired in reflectron mode in the mass range 750 to 3500 Da. The instrument was then switched to MS/MS (TOF/TOF) mode where the eight strongest peptides from the MS scan were isolated and fragmented, then re-accelerated to measure their mass and intensities. To identify the protein, all MS/MS spectra of tryptic peptides derived from a protein spot were searched against protein sequences from all fungal and metazoan species in NCBInr and Swiss-Prot databases using the

MASCOT search program (www.matrixscience.com), where a modified MOWSE scoring algorithm was used to rank results (http://www.matrix-science.com/help/scoring_help.html). Top scoring proteins were selected using protein BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) from NCBI. The monoisotopic masses of observation peaks were used to match the calculated monoisotopic fragment masses for protein identification. Possible variable modifications considered in this search procedure were oxidation of methionine and propionamide of cysteine.

### 3.3.7. Gel image analysis and statistics

For image analysis, all gels were scanned at APAF (Australian Proteome Analysis Facility, Sydney, Australia) using FX Molecular Imager (Bio-Rad Laboratories, Hercules, CA, USA) to produce a digital image and analysed with Progenesis™ v1.5 software program (Nonlinear dynamics, Newcastle upon Tyne, UK). The software allowed background subtraction, automatic spot detection, wrapping, matching and reference gel modifications. Spot volumes were normalised against the total volume of all the spots in the gel. Automatic spot detection in each gel was verified by visual inspection in order to obtain an image pattern as similar as possible with the original gel. The fold difference in normalised volume indicates the fold increase or decrease in normalised volume in HWG gel relative to the protein spots on the IWG gel. Differential proteomic analysis between the HWG sample and IWG sample was first carried out to investigate potential changes in the grub proteome as response to fungal infection. Then, the IWG sample and the MY sample were compared to identify fungal proteins in infection. Figure 1 used the statistical functions and automatic wrapping properties of the Progenesis software package (Rosengren et al. 2003). In this study differences were considered significant when the protein spot intensities increased or decreased by 2.5 fold limit or greater.

## 3.4. Results

*Metarhizium anisopliae* infection of greyback cane grubs resulted in 100 % mortality after two weeks. The first fatality was recorded after six days, most of the cane grubs died around fourteen days post-infection. The dead grubs displayed a hard and solid structure due to saprophytic development of the fungus internally. Proteins extracted from healthy cane grubs, cane grubs infected with *Metarhizium* and fungus only were separated on 2D gels over a pH 4-7 gradient. Proteins separated in this pH range were distributed evenly across the pH gradient, which made it easier to detect the up-regulated, down-regulated and unique proteins during image analysis.

### 3.4.1. Comparison of protein expression profiles and differences between healthy whole grub and infected whole grub

The expression patterns of proteins of the healthy whole grub were compared with the proteins of an infected whole grub. Automated spot detection revealed a total of 136 protein spots in the healthy whole grubs sample and 185 protein spots in the infected whole grub sample. Quantitative changes during fungal infection were determined by overlapping of the HWG gel and IWG gel. A total of 39 protein spots were determined to be unique to the HWG sample, 85 protein spots were unique to the IWG sample (Figure 3) and 30 proteins spots were common to both HWG and IWG sample. A total of 20 protein spots were found to be up-regulated and 10 protein spots were down-regulated by 2.5 fold or greater in the IWG sample compared to the HWG sample (Figure 1). Ten protein spots were found to be up-regulated and 20 protein spots were down-regulated by 2.5 fold or greater in the HWG sample compared to the IWG sample (Figure 2).

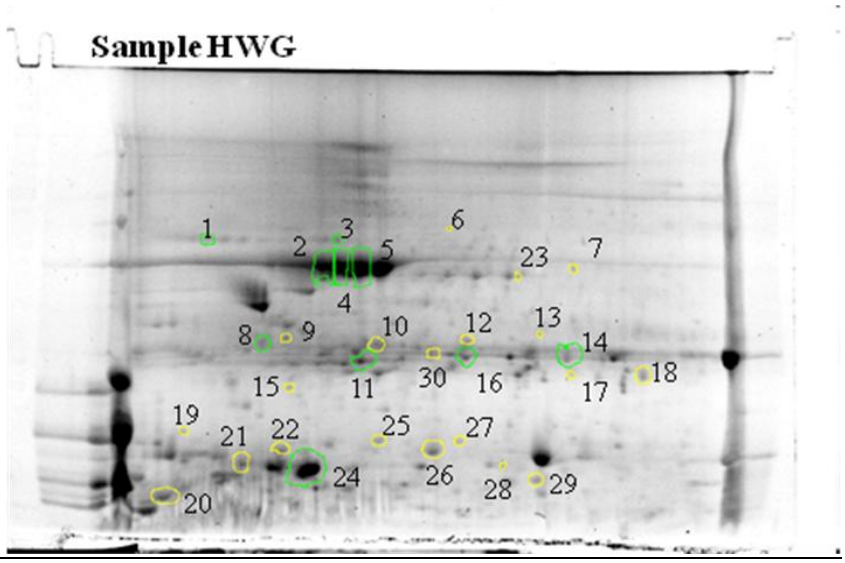


**Figure 1:** Protein spots up- or down-regulated by 2.5 fold or greater in HWG compared to IWG, analysed by Progenesis image analysis software. Yellow coloured rings around the proteins spots indicate proteins spots down-regulated by 2.5 fold or greater. Green coloured rings around the protein spots indicate proteins spots up-regulated by 2.5 fold or greater.

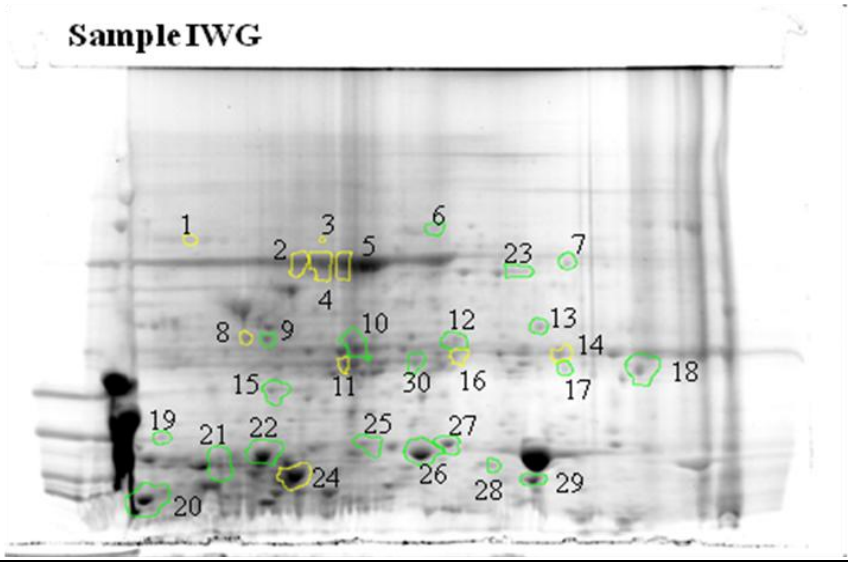


**Figure 2:** Protein spots up- or down-regulated by 2.5 fold or greater in IWG compared to HWG analysed by Progenesis image analysis software. Yellow coloured rings around the proteins spots indicate proteins spots down-regulated by 2.5 fold or greater. Green coloured rings around the protein spots indicate proteins spots up-regulated by 2.5 fold or greater.

### 3.4.2. Comparison of protein profiles of infected whole grubs and the fungus only

The second analysis was comparing the protein profiles of infected whole grub samples and fungus only samples. A total of 236 protein spots in the MY gel were detected by automated spot detection. Automated wrapping of the MY gel and the IWG gel was also applied for detecting fungal proteins in IWG. A total of 11 protein spots were found to be common for both samples of which eight proteins (protein spots 5, 6, 25, 30, 32, 48, 60, 64, Figure 3) were successfully identified (Table 1). Common proteins identified were ubiquitin dependent protein, two-component histidine kinase, actin proteins, 14-3-3 like protein, acyl CoA binding protein, DNAJ-like CSL protein and CALM KLULA.

### 3.4.3. Analysis of unique protein spots on 2D gel of infected whole grub

Eighty five unique proteins from the IWG gel (Figure 3) were cut and analysed by MALDI-TOF-MS. In total, fifty seven proteins were successfully identified (Table 1, presented at the end of this chapter) from IWG which also included 18 up-regulated proteins and four down-regulated proteins from overlapping of HWG/IWG gels, and eight common proteins from overlapping of IWG/MY gels. Unique protein spots identified in this study contained proteins from fungal (Table 1) and metazoan species. Types of proteins that were identified were cytoskeletal proteins (actin), electron transporters, binding proteins, cell signaling proteins and proteases.

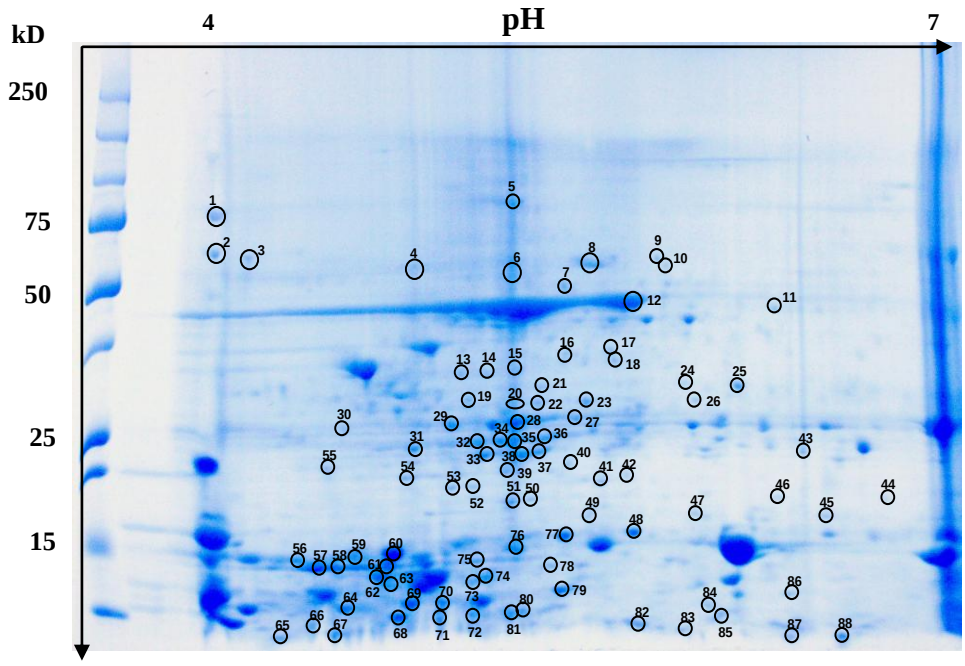


**Figure 3:** Coomassie stained 2D map of proteins of infected whole grub (IWG). Proteins (100 µg) were separated by IEF using 11 cm IPG strips (pH 4-7) in the first dimension, followed by 8-16 % gradient SDS-PAGE in the second dimension. Circled spots indicate the unique spots on IWG.

## 3.5. Discussion

*Metarhizium anisopliae* FI-1045 is currently used as a biopesticide in Australia for the control of sugarcane cane grubs. Earlier research has mainly focused on the long term efficacy of the biopesticide production, strain improvement and application techniques. However, protein expression profiling between the fungus, cane grubs infected with the fungus and healthy greyback cane grubs has not been carried out earlier. The aim of this work was to explore targets or biomarkers that are expressed around two weeks post-*Metarhizium* infection in greyback cane grubs. In order to identify key proteins from *Metarhizium* infected cane grubs, a three way comparison of expressed proteins from HWG, IWG and fungal mycelia were analysed using Progenesis image analysis software. A number of cane grub proteins that were affected by fungal

challenge were identified and bestow a framework for future investigations. Protein expression profiling of insects challenged with entomopathogenic fungi have been studied in the tobacco hornworm larvae (*Manduca sexta*) (Bidochka et al. 1997), the tropical cockroach (*Blaberus giganteus*) (Bidochka et al. 1997), the beet armyworm (*Spodoptera exigua*) (Boucias et al. 1994) and other insects (Hajek and St. Leger, 1994; Ferron, 1978). However, there are considerable methodological differences between the above mentioned proteomics studies to provide enough guidance for the most efficient approach.

In this study, development of *Metarhizium* infection in cane grubs was monitored visually. Infected grubs were found to be motionless/ immobile on day four and the first mortality was observed after day six post-infection. Dead grubs were collected periodically and transferred to individual tubes and then freeze dried. All grubs used for experiment were dead by two weeks post-infection. Field studies conducted by Samson et al. (2005) have also indicated that *M. anisopliae* initiates infection in cane grubs around six days post-infection and it takes around 2-7 weeks before the cane grubs die.

**Analysis of up-regulated proteins:** The total number of protein spots detected on HWG was 136 whereas 185 protein spots were detected in IWG which suggests that there is an increase in the number of proteins after fungal infection either by inclusion of fungal proteins or changes in the grub proteins caused by fungal infection. Twenty proteins were up-regulated by at least 2.5 fold in IWG (protein spots 6, 7, 9, 10, 12, 13, 15, 17, 18, 19, 20, 21, 22, 23, 25, 26, 27, 28, 29, 30, in Figure 2) indicating that these proteins are associated with the greyback cane grub immune response to fungal infection. The majority of the up-regulated proteins were actin present in larval muscle. The peptide mass fingerprinting data showed that the identified muscle specific actins shared homology to fruit fly actin. From the IWG gel (Figure 3) it is interesting to see that

actin proteins were up-regulated by 2.5 fold and an increased number of actin spots appeared suggesting depolymerisation of actin by fungal infection. We speculate that depolymerisation of muscle actin in the greyback canegrubs may be attributed to *Metarhizium* toxins known as destruxins and cytochalasins (Wahlman and Davidson 1993; Kershaw et al. 1999). Destruxins are produced as the mycelium grows inside the insect and depolarise muscle membranes by activating calcium ion channels, which leads to inertness and paralysis as also seen in our study. Cytochalasins have the ability to bind to actin filaments and block polymerisation as well as the elongation of actin (Kershaw et al. 1999).

**Immune response and defence proteins:** In this study, Glutathione S-transferase and thioredoxin-related proteins, which are involved in both immune response and detoxification defence mechanisms, were found to be up-regulated during infection. Glutathione S-transferase (GST) (protein spot 26) plays an important role in detoxification of lipids as well as a protection against oxidative stress. Several studies have indicated that oxidative stress is linked with immune response, including insect's innate immune response (Vierstraete et al. 2003; Guedes et al. 2005; Levy et al. 2004). Up-regulated GST levels detected in the hemolymph of fungus challenged *Drosophila* larvae may have a defensive role against harmful effects of oxidative stress (Vierstraete et al. 2003). Thioredoxin (protein spot 61 and 75) may control the activity of enzymes, receptors and transcription factors via its protein disulphide reductase activity. In cells, thioredoxin is present predominantly in the reduced form, but under oxidative stress conditions, the oxidised form may dominate, possibly generating disulphides. Thioredoxins help in the regulation of transcription factor DNA-binding activity, antioxidant defence, modulation of apoptosis and immune response (Powis and Montfort 2001). In *Drosophila*, thioredoxin system is responsible for restoration of intracellular redox homeostatis (Kanoz *et al*., 2001). Putative coproporphyrinogen oxidase (protein spot 78) and cytochrome P450, a haemoprotein (protein

spot 86) are present in the hemolymph of insect larvae and are induced upon immune challenge. These proteins serve as electron transporters involved in haem-binding as well as storage proteins (Engström et al. 2004). Nitric oxide synthase (NOS) (protein spot 76, Figure 3) is a haem-containing enzyme which forms nitric oxide (NO) from L-arginine. NOS are multifunctional neuro-signaling molecules which play an important role in diverse physiological processes such as olfaction, locomotion, memory and learning and in host defence mechanisms (Jacklet 1997). Interestingly, Weiske and Weisner (1999) reported that NOS activity increased by three-fold in the hemocytes upon infection with pathogenic bacteria and inhibition of NOS leads to the disintegration of nervous system. Work conducted by Rivero (2006) highlights the defence role of NOS against bacterial, fungal and protozoan infections in invertebrates.

**Fungal proteins:** Nineteen fungal proteins were successfully identified in IWG (Table 1). CALM KLULA, a $Ca^{2+}$ binding protein (protein spot 64, in Fig. 3) and 14-3-3 like protein (protein spot 32) have an important role in cell communication or signal transduction mechanisms. Signaling cascades in fungi play a pivotal role in filamentous growth, cell differentiation, mating and virulence (Lengeler et al. 2000). Chorismate mutase (CM) (protein spot 37 and 39) is a key enzyme in the shikimate pathway, which is responsible for the production of tyrosine and phenylalanine. CM is the only characterised enzyme that catalyses a percyclic process and as a result has generated considerable interest in the bioorganic circles (Lee et al. 1995). Since CM is located at the branch point of the shikimate pathway, this enzyme in many organisms (bacteria, fungi and higher plants) is an important point of regulation for maintaining the correct balance of aromatic amino acids in the cell (Strater et al. 1997). Acyl-Co A-binding protein (protein spot 48) involved in lipid metabolism was identified in the IWG gel. Entomopathogenic fungi have a unique ability to degrade a series of hydrocarbons structures that are similar to those of their insect hosts, utilising them for energy production and finally

degrading the host's cellular components (Ferron 1978; Napolitano and Juarez 1997). Although the insect cuticle is made up of proteins and chitin, it contains a thin layer of lipids usually composed of complex mixture of hydrocarbon, fatty acids, wax esters and glycerides (Merzendorfer et al. 2003; Anderson et al. 1995; Anderson, 1979). Since these lipid layers participate in chemical communication process as well as in the regulation of microbial activity, an alkaline adaptation increases the fungal ability to invade the host (Hajek and St. Leger 1994) and in that process Acyl Co-A is the first enzyme involved in fatty acid beta-oxidation system.

**Proteolytic enzymes from insects:** Proteolytic enzymes released during biological response play an important role in protein quality control by eliminating short-lived regulatory proteins, as well as proteins that are misfolded and damaged, thus maintaining cellular homeostasis in insects (Engström et al. 2004). Scolexin B, a serine type endopeptidase (S1 family; protein spot 29, in Fig. 3) from *Manduca sexta* was found to be up-regulated upon infection. Scolexin is an immune related serine protease with coagulation-inducing properties on insect's hemolymph and is associated with hemocytic nodule formation in response to bacterial injection (Finnerty and Granados 1997). Scolexin associated with response to fungal challenge has been found in larvae of tobacco horn-worm and adult tropical cockroach (Bidochka et al. 1997). Protein spot 24 (Figure 3) corresponds to a chymotrypsin-like serine protease from *D. melanogaster* which was found to be down-regulated post infection. This protein is one of the main digestive serine proteases present in the midgut of insects and during infection this enzyme may have a significant effect on the survival of ingested microbes (Shen et al. 2000). ATP dependent Clp protease (protein spot 34) is a serine endopeptidase (from *D. melanogaster*) that has a pivotal role in cytoplasmic protein quality control in insects (Yu and Houry 2007).

**Proteolytic enzyme from fungi:** Proteolytic enzymes are important in the invasion and utilization of host tissues by entomopathogenic fungi such as *Metarhizium*, *Beauveria* and *Isaria*. These fungi produce a range of cuticle degrading enzymes *in vitro* when insect cuticle is supplied as the sole carbon source (St. Leger et al. 1994; Samuels and Paterson 1995; Hajek and St. Leger 1994). In this study, we have identified a putative metallopeptidase protein (protein spot 46) in the IWG gel. Studies have indicated that metalloproteins play a dominant role during pathogenesis and affect vascular permeability, hemorrhages in the internal organs and sepsis (Maeda 1996). Metalloproteinases have also been found to be linked with diverse groups of entomopathogens such as *Metarhizium*, *Beauveria* and *Verticillum* and credited to assist their development within the infected insect host or to intervene with its immune system. For example, a thermolysin-like metalloproteinase identified among other enzymes released from *M. anisopliae* was found to be extremely toxic when injected into *G. mellonella* (St. Leger et al. 1994). Further studies conducted by St. Leger et al. (1997) have revealed five metalloproteases over the pH range 6 to 8 when *M. anisopliae* was grown on a cockroach cuticle. Furthermore, subtilisin-like serine proteases (Pr1a and Pr1b), trypsin-like proteases (Pr2) and metalloproteases all work synergistically to break nearly all types of peptide bonds in the insect cuticle. Metalloproteins may also function as a back-up system if Pr1 proteins are inhibited by serine proteinase inhibitors present in the insect hemolymph and cuticles (St. Leger et al. 1994). In this study, Pr1 and Pr2 proteins could not be detected in infected whole grubs as the grubs were harvested around two weeks post-infection, which is at an early stage of infection. Interestingly, Pr1 and Pr2 proteases were also not detected during early hemolymph colonization of *M. sexta* and *S. gregaria* infected with *Metarhizium* (Gillespie et al. 2000; St. Leger et al. 1987). Work conducted by St. Leger's group clearly indicates that Pr1 and Pr2 proteases are produced at very low levels during the initial phase of *Metarhizium* infection in liquid culture and their levels intensify during the later phase (St. Leger et al. 1994).

**Analysis of down-regulated proteins:** Ten proteins were down-regulated by at least 2.5 fold in IWG (Figure 2) and four of them were successfully identified: protein spots 5, 14 and 16 from Figure 3, correspond to actin proteins and protein spot 24 (Figure 3) correspond to CG16705-PA protein, a chymotrypsin serine protease belonging to S1 peptidase family from *Drosophila melanogaster*. Genes encoding serine proteases (SPs) and their homologs constitute the second largest family of genes in the genome of *Drosophila* (Engström et al. 2004). Several proteomic studies have shown the up-regulation or down-regulation of SPs in *Drosophila*, *M. sexta*, *Anopheles gambiae* and *Bombyx mori* challenged with microbial infections (Engström et al. 2004; Bidochka et al. 1997; Vierstraete et al. 2003; Mounier et al. 1997; Ferron 1978). In this study, we speculate that during fungal challenge, serine protease of the cane grubs and dominant muscle specific actin proteins were suppressed by the invading pathogen to inactivate the insect's defence responses and cytoskeletal proteins. By doing so the fungus can proliferate by utilising the cane grub's hemolymph and soft tissues until death occurs.

Currently the genome data of *M. anisopliae* and other *Metarhizium* sp. are not publicly available, including the EST database. In order to enhance the size of the protein database accessible for peptide mass fingerprint searching, we included all proteins across the fungal and metazoan kingdoms. From the data reported in this work and on the basis of evidence from the literature, we can conclude that three way comparisons of expressed proteins from healthy canegrubs, infected canegrubs and the infecting fungus have highlighted the role of various immune related proteins of the greyback canegrubs during early *Metarhizium* infection. This study also underlines some of the fungal proteins such as metalloproteases that are involved in the infection process. Future work will involve differential proteomic analysis of infected canegrubs harvested at different stages of *Metarhizium* infection; such studies will help understand the molecular interplay at various stages of infection.

## 3.6. References

Andersen SO (1979) Biochemistry of the insect cuticle. Annu Rev Entomol 24: 29-61.

Andersen SO, HØjrup P, Roepstorff P (1995) Insect cuticular proteins. Insect Biochem Mol Biol 25: 153 -176.

Bidochka MJ, St. Leger RJ, Roberts DW (1997) Induction of novel proteins in *Manduca sexta* and *Blaberus giganteus* as a response to fungal challenge. J Invertebr Pathol 70: 184-189.

Boucias DG, Hung SY, Mazet I, Azbell J (1994) Effect of the fungal pathogen, *Beauveria bassiana*, on the lysozyme activity in *Spodoptera exigua* larvae. J Insect Physiol 40: 385-391.

Chandler KJ, Erbacher JP (1997) Susceptibility of canegrubs to the insecticide chloropyrifos. Proc Soc Sugarcane Technol 19: 118-126.

Clarkson JM, Charnley AK (1996) New insights into the mechanisms of fungal pathogenesis in insects. Tren Microbiol 4: 197-203.

Engström Y, Loseva O, Theopold U (2004) Proteomics of the *Drosophila* immune response. Trend Biotech 22: 600-605.

Faria M, Wraight SP (2007) Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biocontrol 43: 237-256.

Ferron P (1978) Biological control of insect pests by entomogenous fungi. Annu Rev Entomol 23: 409-442.

Finnerty CM and Granados RR (1997) The plasma protein scolexin from *Manduca sexta* is induced by baculovirus infection and other immune challenges. Insect Biochem Mol Biol 27: 1-7.

Freimoser FM, Screen S, Bagga S, Hu G, St. Leger RJ (2003) Expressed sequence tag (EST) analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insects hosts. Microbiology 149: 239-247.

Freimoser FM, Hu G, St Leger RJ (2005). Variation in gene expression patterns as the insect pathogen *Metarhizium anisopliae* adapts to different host cuticles or nutrient deprivation *in vitro*. Microbiology 151: 361-371.

Frobius AC, Kanost MR, Gotz P, Vilcinskas A (2000) Isolation and characterization of novel serine protease inhibitors from larval hemolymph of greater wax moth *Galleria mellonella*. Eur J Biochem 267: 2046-2053.

Gillespie JP, Bateman R, Charnley AK (1998) The role for cuticle degrading proteases in the virulence of *Metarhizium* spp. for the desert locust, *Schistocerca gregaria*. J Invertebr Pathol 71: 128-137.

Grinyer J, McKay M, Nevalainen H, Herbert BR (2004) Fungal proteomics: initial mapping of biological control strain *Trichoderma harzianum*. Curr Genet 45: 163-169.

Guedes SDM, Vitorino R, Domingues R, Tomer K, Correia AJ, Amado F, Domingues P (2005) Proteomics of immune challenged *Drosophila melanogaster* larvae hemolymph. Biochem Biophys Res Commun 328: 106-115.

Hajek AE, St. Leger RJ (1994) Interaction between fungal pathogens and insect hosts. Annu Rev Entomol 39: 293-322.

Jacklet, JW (1997) Nitric oxide signaling in invertebrates. Invertebr Neurosci 3: 1-14.

Kanzok SM, Fechner A, Bauer H, Ulschmid JK, Müller H, Botella-Munoz J, Schneuwly S, Schirmer RH, Becker K (2001) Substitution of the thioredoxin system for glutathione reductase in *Drosophila melanogaster*. Science 291: 643-646.

Kershaw MJ, Moorhouse ER, Bateman RP, Reynolds SE, Charnley AK (1999) The role of destruxins in the pathogenicity of *Metarhizium anisopliae* for three species of insect. J Invertebr Pathol 74: 213–223.

Lee AY, Stewart JD, Clardy J, Ganem B (1995) New insight into the catalytic mechanism of chorismate mutases from structural studies. Chem Biol 2: 195-203.

Lengeler KB, Davidson RC, D'souza C, Harashima T, Shen W, Wang P, Pan X, Waugh M, Heitman J (2000) Signal transduction cascades regulating fungal development and virulence. Microbiol Mol Biol Rev 64: 746-785.

Levy F, Bulet P, Ehret-Sabatier L (2004) Proteomic analysis of the systemic immune response of *Drosophila*. Mol Cell Proteomics 3: 156-166.

Liu CM, Huang SS, Tzeng YM (2004) Analysis of destruxins produced from *Metarhizium anisopliae* by capillary electrophoresis. J Chromatograph Sci 42: 140-145.

Logan DP, Kettle CG (2002) Effect of food and larval density on survival and growth of early instar greyback canegrub, *Dermolepida albohirtum* (Waterhouse) (Coleoptera: Scarabaeidae). Aus J Entomol 41: 253-261.

Maeda H (1996) Role of metalloproteinases in pathogenesis. Microbiol Immunol 40: 685-699.

Merzendorfer H, Zimoch L (2003) Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. J Experiment Biol 206: 4393-4412.

Milner RJ, Jenkins K (1996). *Metarhizium*: a versatile mycoinsecticide of the future. Professional Pest Management 1: 32-36.

Milner RJ, Staples JA, Lutton GG (1998) The selection of isolates of the hyphomycete fungus, *Metarhizium anisopliae* for control of termites in Australia. Biol Control 11: 240-247.

Milner RJ, Samson P, Morton R (2003) Persistance of conidia of *Metarhizium anisopliae* in sugarcane fields: Effecet of isolate and formulation on persistence over 3.5 years. Biocontrol Sci Technol 13: 507-516.

Mounier N, Perriard JC, Gabbiani G, Chaponnier C (1997) Transfected muscle and nonmuscle actins are differentially sorted by cultured smooth muscle and non muscle cells. J Cell Sci 110: 839-846.

Murad AM, Laumann RA, Lima TA, Sarmento RB, Noronha EF, Rocha TL, Valadares-Inglis MC, Franco OL (2006) Screening of entomopathogenic *Metarhizium anisopliae* isolates and proteomic analysis of secretion synthesized in response to cowpea weevil (*Callosobruchus maculatus*) exoskeleton. Comp Biochem Pysiol C Toxicol Pharmacol 142: 365- 370.

Napolitano R, Juarez MP (1997) Entomopathogenous fungi degrade epicuticular hydrocarbons of *T. infestans*. Arch Biochem Biophys 344: 208-214.

Paterson IC, Charnley AK, Cooper RM, Clarkson JM (1994) Partial characterization of specific inducers of a cuticle-degrading protease of the insect pathogenic fungus, *Metarhizium anisopliae*. Microbiol 140: 3153-3159.

Powis G and Montfort WR (2001) Properties and biological activities of thioredoxins. Annu Rev Biophys Biomol Struct 30: 421-455.

Prior C, Carey M, Abraham YJ, Moore D, Bateman RP (1995) Development of a bioassay method for the selection of entomopathogenic fungi virulent to the desert locust *Schistocerca gregaria* (Forskal). J Appl Entomol 119: 567 – 572.

Rivero A (2006) Nitric oxide: an antiparasitic molecule of invertebrates. Trends in Parasitology 22: 219-225.

Rosengren AT, Salmi JM, Aittokallio T, Westerholm J, Lahesmaa R, Nyman TA, Nevalainen OS (2003) Comparison of PDQuest and Progenesis software packages in the analysis of two-dimensional electrophoresis gels. Proteomics 3: 1936-1946.

Samson PR, Milner RJ, Sander ED, Bullard GK (2005) Effect of fungicides and insecticides applied during planting of sugarcane on viability of *Metarhizium anisopliae* and its efficacy against white grubs. Biocontrol 50: 151-163.

Samuels RI, Paterson IC (1995) Cuticle-degrading proteases from insect moulting fluid and culture filtrates of entomopathogenic fungi. Comp Biochem Physiol 110: 661-669.

Shen Z, Edwards MJ, Jacobs-Lorena M (2000) A gut-specific serine protease from the malaria vector *Anopheles gambiae* is down regulated after blood ingestion. Insect Mol Biol 9: 223-229.

St. Leger RJ, Charnley AK, Cooper RM (1987) Production of cuticle-degrading enzymes by the entomopathogen, *Metarhizium anisopliae* during infection of cuticles from *Calliphora vomitoria* and *Manduca sexta*. J Gen Microbiol 133: 1371-1382.

St. Leger RJ, Durrands PK, Charnley AK, Cooper RM (1988) Regulation of production of proteolytic enzymes by the entomopathogenic fungus *Metarhizium anisopliae*. Arch Microb 150: 413-416.

St. Leger RJ, Butt TM, Staples RC, Roberts DW (1989) Production *in vitro* of appressoria by the entomopathogenic fungus *Metarhizium anisopliae*. Exp Mycol 13: 274–288.

St. Leger, Bodichka MJ, Roberts DW (1994) Isoforms of the cuticle degrading Pr 1 proteinase and production of a metalloproteinase by *Metarhizium anisopliae*. Arch Biochem Biophys 313: 1-7.

St. Leger RJ, Joshi L, Roberts D (1997) Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hydrophobin by *Metarhizium anisopliae*. Appl Environ Microbiol 64: 709-713.

Strater N, Schnappauf G, Braus G, Lipscomb WN (1997) Mechanisms of catalysis and allosteric regulation of yeast chorismate mutase from crystal structures. Structure 5: 1437-1452.

Vierstraete E, Verleyen P, Baggerman G, D'Hertog W, Van den Bergh G, Arckens L, De Loof A, Schoofs L (2003) A proteomic approach for the analysis of instantly released wound and immune proteins in *Drosophila melanogaster* hemolymph. Proc Natl Acad Sci USA 101: 470-475.

Wahlman M and Davidson BS (1993) New destruxins from the entomopathogenic fungus *Metarhizium anisopliae*. J Nat Prod 56: 643-647.

Wang C, St. Leger RJ (2006) A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses. Proc Natl Acad Sci USA 103: 6647-6652.

Wang C, St. Leger RJ (2007) A scorpion neurotoxin increases the potency of a fungal insecticide. Nature Biotechnol 25: 1455-1456.

Weiske LJ, Weisner A (1999) Stimulation of NO synthase activity in the immune-competent lepidopteran *Estigmene acraea* haemocyte line. Nitic Oxide 3: 1.

Yu AY, Houry WA (2007) ClpP: a distinctive family of cylindrical energy-dependent serine proteases. FEBS Lett 581: 3749-3757.

## Table. 1. Unique proteins identified in infected whole grub

| Spot # | Protein | Species | Accession number | pI | Molecular mass (Da) | Score | Coverage or Peptides matched |
|---|---|---|---|---|---|---|---|
| **Fungal proteins** | | | | | | | |
| **Cytoskeletal proteins** | | | | | | | |
| 5 | Actin, gamma [*D] | *Penicillium chrysogenum* | Q9URSO | 5.45 | 41730 | 130 | 2 peptides |
| 30 | Actin [*U] | *Metschnikowia aff. chrysoperlae* | gi\|74035798 | 5.59 | 36483 | 66 | 26% |
| **Proteolytic enzymes** | | | | | | | |
| 46 | Putative metallopeptidase | *Aspergillus oryzae* | Q2UQH9 | 6.96 | 15806 | 80 | 40% |
| **Signal transduction** | | | | | | | |
| 6 | Two-component histidine kinase [*U] | *Cochliobolus heterostrophus* | gi\|32400392 | 5.64 | 61907 | 58 | 23% |
| 32 | 14-3-3-like protein | *Pracoccidioides brasiliensis* | gi\|38569374 | 4.68 | 29624 | 63 | 20% |
| 45 | Sybindin-like protein | *Yarrowia lipolytica* | gi\|50547499 | 6.81 | 15283 | 77 | 31% |
| **Cell regulation** | | | | | | | |
| 25 | Ubiquitin dependent protein [*U] | *Ashbya gossypii* | gi\|44984865 | 6.14 | 28019 | 76 | 22% |
| 31 | Spindle pole associated protein | *Aspergillus nidulans FGSC A4* | gi\|67526533 | 4.79 | 25169 | 57 | 25% |
| 37 | Chorismate mutase | *Aspergillus fumigatus* | gi\|66850977 | 5.48 | 30445 | 76 | 20% |
| 39 | Chorismate mutase | *Aspergillus fumigatus* | gi\|66850977 | 5.48 | 30445 | 84 | 29% |
| 42 | Cyclin domain protein | *Aspergillus fumigates* | gi\|66851456 | 5.61 | 32990 | 61 | 23% |
| 47 | Putative oxidoreductase | *Cryptococcus neoformans* | gi\|57229117 | 5.83 | 30553 | 63 | 20% |
| 51 | Putative oxidoreductase | *Cryptococcus neoformans* | gi\|57229117 | 5.83 | 30553 | 84 | 25% |
| 60 | DNAJ-like CSL protein | *Candida albicans* | gi\|68472551 | 4.75 | 16757 | 78 | 32% |
| 64 | CALM KLULA | *Kluyveromyces lactis* | gi\|50303999 | 4.25 | 16053 | 61 | 29% |
| 83 | Ubiquitin | *Neurospora crassa* | gi\|136671 | 5.76 | 8593 | 68 | 26% |
| **Lipid metabolism** | | | | | | | |
| 48 | Acyl CoA binding protein family | *Aspergillus fumigates* | gi\|66853077 | 5.71 | 17299 | 59 | 24% |
| **Other proteins** | | | | | | | |
| 54 | Predicted protein | *Neurospora crassa* | gi\|32422205 | 5.35 | 26748 | 56 | 21% |
| 58 | Hypothetical protein FG10160.1 | *Gibberealla zeae PH-1* | gi\|42545154 | 5.26 | 13227 | 53 | 38% |
| **Metazoan proteins** | | | | | | | |
| **Cytoskeletal proteins** | | | | | | | |
| 12 | Actin, muscle A2 [*U] | *Bombyx mori* | PO7837 | 5.29 | 41776 | 366 | 5 peptides |
| 13 | Beta actin [*U] | *Cherax quadricarinatus* | gi\|40887063 | 5.11 | 41899 | 99 | 2 peptides |
| 14 | Putative muscle actin [*D] | *Oncometopia nigricans* | PO7837 | 5.29 | 41759 | 210 | 4 peptides |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 15 | Actin, muscle A2 *U | *Bombyx mori* | PO7837 | 5.29 | 41776 | 76 | 2 peptides |
| 16 | Actin *D | *Chasmagnathus granulata* | gi\|28435512 | 5.42 | 31197 | 69 | 31% |
| 18 | Actin, muscle A2 *U | *Bombyx mori* | PO7837 | 5.29 | 41776 | 112 | 1 peptide |
| 19 | Actin, larval muscle *U | *Drosophila melanogaster* | PO2574 | 5.3 | 41760 | 87 | 1 peptide |
| 20 | Actin 5, muscle specific *U | *Bactrocera dorsalis* | P45887 | 5.3 | 41744 | 229 | 4 peptides |
| 21 | Actin, muscle A2 *U | *Bombyx mori* | PO7837 | 5.29 | 41776 | 255 | 4 peptides |
| 22 | Actin, larval muscle *U | *Drosophila melanogaster* | PO2574 | 5.3 | 41760 | 313 | 4 peptides |
| 23 | Actin, larval muscle *U | *Drosophila melanogaster* | PO2574 | 5.3 | 41760 | 367 | 5 peptides |
| 27 | Actin, larval muscle *U | *Drosophila melanogaster* | PO2574 | 5.3 | 41760 | 315 | 5 peptides |
| 28 | Actin, larval muscle *U | *Drosophila melanogaster* | PO2574 | 5.3 | 41760 | 225 | 2 peptides |
| 33 | Actin T2 | *Liptopenaeus vanname* | gi\|49473508 | 5.05 | 41969 | 84 | 25% |
| 35 | Actin, larval muscle | *Drosophila melanogaster* | PO2574 | 5.3 | 41760 | 250 | 2 peptides |
| 40 | Actin 3, muscle specific | *Bactrocera dorsalis* | P45886 | 5.3 | 41789 | 88 | 1 peptide |
| 50 | Actin, muscle A2 | *Bombyx mori* | PO7837 | 5.29 | 41776 | 148 | 2 peptides |
| 52 | Actin, muscle A2 | *Bombyx mori* | PO7837 | 5.29 | 41776 | 111 | 1 peptide |
| 68 | Actin | *Ostertagia ostertagia* | gi\|2981073 | 4.71 | 11492 | 72 | 43% |
| 73 | Actin, muscle | *Manduca sexta* | P49871 | 5.22 | 41750 | 109 | 3 peptides |
| 74 | Actin | *Spodoptera littoralis* | Q11212 | 5.76 | 18032 | 83 | 1 peptide |
| 41 | Kelch like protein | *Strongylocentrotus purpurtus* | gi\|72166985 | 5.37 | 25357 | 55 | 26% |
| **Developmental protein** | | | | | | | |
| 17 | Neoplasma protein *U | *Drosophila melanogaster* | Q9W1I2 | 6.71 | 139189 | 70 | 2 peptides |
| 38 | Similar to N-acetylserotonin O-methyltransferase - like protein | *Strongylocentrotus purpurtus* | gi\|72012760 | 5.11 | 20390 | 57 | 27% |
| **Proteolytic enzymes** | | | | | | | |
| 9 | Ubiquitin carboxyl-terminal hydrolase *U | *Drosophila melanogaster* | Q9VKZ8 | 5.93 | 53707 | 61 | 24% |
| 10 | CG3731-PA- metalloendopeptidase *U | *Drosophila melanogaster* | Q9VFF0 | 5.67 | 51874 | 59 | 23% |
| 24 | CG16705-PA, Chymotrypsin serine protease *D | *Drosophila melanogaster* | Q9VCJ8 | 5.63 | 28446 | 115 | 22% |
| 29 | Scolexin B *U | *Manduca sexta* | gi\|4262359 | 5.39 | 30021 | 74 | 23% |
| 34 | ATP dependent Clp protease | *Drosophila melanogaster* | gi\|24654090 | 5.07 | 24423 | 71 | 27% |
| **Defence proteins** | | | | | | | |
| 26 | Glutathione S-transferase *U | *Drosophila melanogaster* | gi\|68482789 | 5.73 | 31347 | 122 | 28% |
| 76 | Nitric oxide synthase | *Anopheles gambiae* | gi\|38196155 | 5.44 | 17825 | 67 | 32% |
| **Immune proteins** | | | | | | | |
| 61 | Thioredoxin related | *Anopheles gambiae str. PEST* | gi\|55247106 | 5.01 | 11669 | 66 | 40% |
| 75 | Thioredoxin related | *Anopheles gambiae str. PEST* | gi\|55247106 | 5.01 | 11669 | 72 | 47% |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 78 | Putative coproporphyrinogen oxidase | *Drosophila melanogaster* | gi\|26185795 | 5.69 | 18610 | 56 | 29% |
| **Regulatory protein** | | | | | | | |
| 7 | Troponin T (TnT) [*U] | *Periplaneta americana* | Q9XZ71 | 4.97 | 45880 | 77 | 3 peptides |
| 82 | Polyubiquitin GmUblast | *Galleria mellonella* | Q9BMJ2 | 6.56 | 8673 | 62 | 25% |
| **Membrane proteins** | | | | | | | |
| 86 | Family 4 cytochrome P4501V2 | *Reticulitermes flavipes* | gi\|82622286 | 6.02 | 14392 | 54 | 22% |
| **Other proteins** | | | | | | | |
| 36 | Hypothetical protein with Saposin A type domain | *Schistosoma japonicum* | gi\|29840898 | 5.37 | 24305 | 55 | 26% |
| 55 | Similar to CG31997-PA | *Apis mellifera* | gi\|66560574 | 4.88 | 16902 | 60 | 23% |

[*D] Down-regulated proteins

[*U] Up-regulated proteins

# 4. Comparative analysis of the *Metarhizium anisopliae* secretome in response to exposure to the greyback canegrubs and grub cuticles – Manuscript III

This section gives an insight into the extracellular proteins secreted by *Metarhizium anisopliae* strain FI-1045, in response to exposure to the greyback canegrubs and their isolated cuticles. Different proteolytic enzymes produced during the infection process were identified amongst other proteins such as subtilisin-like serine protease and trypsin-like protease. In this work we have identified six extracellular proteins from *Metarhizium* that have not been previously reported to be produced during infection of various insect hosts. Figures have been included in the body of the text for easy reading and tables are presented at the end of the chapter.

The manuscript has been accepted for publication in Mycological Research, with minor modifications in September 2009. The paper is presented in the format required for publication in Mycological Research.

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# Comparative analysis of the *Metarhizium anisopliae* secretome in response to exposure to the greyback canegrubs and grub cuticles

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## 4.1. Abstract

*Metarhizium anisopliae* is a well-characterized biocontrol agent of a wide range of insects including canegrubs. In this study, two-dimensional electrophoresis was used to display secreted proteins of *M. anisopliae* strain FI-1045 growing on the whole greyback canegrubs and their isolated cuticles. Hydrolytic enzymes secreted by *M. anisopliae* play a key role in insect cuticle degradation and initiation of the infection process. We have identified all the 101 protein spots displayed by cross species identification from the kingdom fungi, thus achieving a 100% identification rate. Among the identified proteins were the 64-kDa serine carboxypeptidase, 1,3 beta exoglucanase, Dynamin GTPase, THZ kinase, calcineurin like phosphoesterase and phosphatidylinositol kinase secreted by *M. ansiopliae* (FI-1045) in response to exposure to the greyback canegrubs and their isolated cuticles. These proteins have not been previously identified from the culture supernatant of *M. anisopliae* during infection. To our knowledge, this is the first proteomic map established to study the extracellular proteins secreted by *M. ansiopliae* (FI-1045) during infection of greyback canegrubs and its cuticles.

Keywords: *Greyback canegrub, Metarhizium, novel proteins, hydrolytic enzymes, electrophoresis*

## 4.2. Introduction

The greyback canegrub, *Dermolepida albohirtum* (Waterhouse) (Coleoptera: Scarabaeidae) is considered the most serious pest of sugarcane in tropical areas of Queensland, Australia (Robertson et al., 1997). Damage is caused by the larvae feeding on the roots of the sugarcane plant leading to retarded growth and in extreme cases, plant death. The fungus *Metarhizium anisopliae* grows naturally in soils throughout the world and causes disease in various insects. In Australia, it is applied as a biopesticide for use against various canegrub species (Milner, 2000) and is the active ingredient in the biopesticide 'BioCane$^{TM}$' (Milner et al. 2002).

Entomopathogenic fungi exhibit many characteristics that determine virulence towards their hosts, including the production of degradative enzymes. Hydrolytic enzymes such as proteases and chitinases are produced during fungal penetration through the cuticle. Among them, fungal proteases are considered to play a significant role in cuticle degradation and are essential for the initiation of the infection process (St. Leger et al. 1987; St. Leger et al. 1996). One of the best studied protease of which the function in host invasion has been clearly established is subtilisin-like serine protease (Pr1) of *Metarhizium anisopliae* (St. Leger et al. 1986a). During the early stages of pathogenesis, Pr1 degrades insect cuticular proteins (St. Leger et al. 1996; Freimoser et al. 2003) and has been ultrastrucutrally localised in the host cuticle during early phase of penetration (St. Leger et al. 1996). A trypsin-like serine protease (Pr2) also appears during the early stages of colonisation, suggesting that it has some role in cuticle degradation complementary to that of Pr1 (St. Leger et al. 1987). Pr1 and Pr2 proteases have been identified in various entomopathogenic fungi including *Metarhizium anisopliae*, *M. flavoviride*, *Beauveria bassiana* and *Paecilomyces fumosoroseus* (Bidochka and Meltzer, 2000; Joshi et al. 1997; St. Leger, 1995; Shah and Pell, 2003). Inhibition of protease activity or use of protease deficient

mutants resulted in decreased virulence against insects (Bidochka and Khachatourians, 1990). Several authors have described Pr1 and Pr2 activity in *Metarhizium* growing in liquid minimal medium supplemented with different insect cuticles (Bidochka and Khachatourians, 1994; Pinto et al. 2002; St. Leger et al. 1986b). The synthesis of extracellular proteases (Pr1 and Pr2) is controlled by several regulatory paths that include repression and induction by the carbon sources (St. Leger et al. 1987; Bidochka and Khachatourians, 1988). In addition, *Metarhizium anisopliae* produces several chitinolytic enzymes which act after the proteases have considerably digested the cuticular proteins thereby exposing the chitin part of the cuticle (Shah and Pell, 2003).

Proteins represent more than 50% of the weight of the insect cuticle and insect larvae have a soft thin cuticle (epicuticle) over most of their body. The major portion of the insect larvae is made up of muscle which contains approximately 70 to 75% water, 20 to 22 % protein, 4 to 8% lipid, 1% ash and no carbohydrates. Two-dimensional gel electrophoresis has uncovered over hundred different cuticular proteins that differ in their molecular weight and isoelectric point (St. Leger et al. 1996; Freimoser et al. 2003; Andersen et al. 1995). However, there are differences in the ability of entomopathogenic fungal proteases to degrade different types of insect cuticle because these cuticle types differ in their protein composition. In this study, we have examined the production of various proteins by *Metarhizium anisopliae* in the presence of greyback canegrubs and isolated canegrub cuticles, extending our knowledge about protein production by this fungus with a view of establishing novel biotechnological tools to use for greyback canegrub control.

## 4.3. Materials and methods

### 4.3.1. Insect larvae

Greyback canegrubs, *D. albohirtum* were dug from the soil below sugarcane stools in commercial fields around Townsville and each grub was packed in a single plastic tube for

transportation to Sydney in a ventilated container. After arrival, the canegrubs were removed from the plastic tube and transferred into 100 ml sterile transparent plastic tubs (Technoplas, Australia) filled with 30 g of sterilised garden peat (Killarney peat moss, Australia). Garden peat was sterilised by heating and drying (150 °C, 4 h), then mixed with distilled water. The canegrubs were fed with fresh pieces of carrot and held at room temperature (RT). Around ten healthy canegrubs were immobilized by cotton dipped in chloroform (Ajax Finechem, Sydney) to depress the nervous system. Canegrubs were then rinsed in sterile distilled water to remove peat particles attached to the body and transferred into sterile 15 ml tubes, freeze dried and pulverised using mortar and pestle. Cuticles were isolated by extracting the soft tissue from homogenised insects with potassium tetra borate (St. Leger et al. 1986b). Clean cuticle samples from the greyback canegrubs were prepared as described previously (St. Leger et al. 1987).

### 4.3.2. Fungal strain and cultivation conditions

*Metarhizium anisopliae* (FI-1045) was conidiated and maintained on potato dextrose agar plates (Difco Laboratories, MI, USA). Conidia were collected using 5 ml of 0.9% (w/v) sodium chloride, 0.01% (v/v) Tween 80 and 5 x $10^8$ conidia were used to inoculate 50 ml of minimal medium (MM; 110 mM potassium phosphate, 38 mM ammonium sulphate, 2.4 mM magnesium sulphate, 4.1 mM calcium chloride, 2.9 mM manganese sulphate, 7.2 mM iron sulphate, 0.35 mM zinc sulphate, 0.71 mM cobalt sulphate, pH 5.5) supplemented with 2% (w/v) glucose in a 250-ml Erlenmeyer flask, similarly to the method used by Grinyer et al. (2005). Cultures were grown at 28 °C on a shaker at 250 rpm for 48 h (preculture). The mycelia from each flask were collected, washed three times with 50 ml of Milli-Q water by invertion and centrifugation at 4,000 *g* for 10 min at 15 °C and re-inoculated in fresh MM with 1% (w/v) pulverised greyback cane grub cuticles (Culture-A) or 1% (w/v) pulverised whole greyback cane grubs (Culture-B). Control flasks contained only fresh MM with 1% (w/v) pulverised greyback cane grub cuticles or

fresh MM with 1% (w/v) pulverised whole greyback cane grubs. Cultures were grown for 48 h on a shaker at 250 rpm at 28 °C. Culture supernatants and mycelia were harvested at both 24 h and 48 h time points. The experiment was set up in triplicate but materials from only two cultivations were used. Fungal and yeast protease inhibitor cocktail (0.05% v/v) was added to the culture and allowed to incubate at RT for 20 min. Culture supernatants containing protease inhibitors were collected by centrifugation at 4,000 *g* for 10 min at 15 °C and stored at – 20 °C until required. The mycelia were washed three times with 50 ml of Milli-Q water by invertion and centrifugation at 4,000 *g* for 10 min at 15 °C, collected and stored at – 20 °C until use.

### 4.3.3. Precipitation of proteins from culture supernatant

The *M. anisopliae* (FI-1045) culture supernatant was thawed and 13 ml were taken and spun at 21,000 *g* for 15 min at 10 °C. Ammonium sulphate was added to the supernatant to give an 80% saturated solution that was stirred overnight at 4 °C to allow protein precipitation. The precipitated proteins were pelleted by centrifugation at 21,000 *g* for 15 min at 4 °C. Precipitated proteins were resuspended in 1 ml of re-suspension solution {7 M urea, 2 M thiourea, 4% (w/v) CHAPS (3-[(3- cholamidopropyl) dimethylammonio]-1-propanesulfonate) (detergent C-3023: Sigma), 40 mM Tris, 5 mM tributylphosphine, 10 mM acrylamide, 1 mM PMSF (phenylmethylsulphonyl fluoride), 0.1% protease inhibitor tablet} and incubated at RT for 90 min to allow complete reduction and alkylation of proteins. The reduction and alkylation reactions were quenched with 10 mM dithiothreitol before insoluble material was removed by spinning at 21,000 *g* for 10 min. The solution was desalted (buffer exchanged) further by ultra-filtration with 7 M urea, 2 M thiourea, 4% (w/v) CHAPS in Ultrafree™ 5 kDa cut-off centrifugal concentrators (Millipore, USA) at 2,000 *g* at 20 °C until the volume reached 500 µl. Protein samples were then passed through the 2D Clean-Up Kit from GE Healthcare (formerly Amersham Biosciences, USA) to remove further impurities. The conductivity of the sample was measured with a Twin

Cond conductivity meter (Horiba, Japan). If the conductivity was higher than 300 µS/cm then the desalting step was repeated. The desalted protein solution was rehydrated into immobiline pH gradient strips.

### 4.3.4. Protein assay

Proteins from culture supernatants were assayed using the Bradford's reagent (Bio-Rad, Germany) following the manufactures instructions (Bradford, 1976). Absorbances were read on a Versamax microplate reader (Molecular devices, USA) and analysed using the softmax PRO ver 3.1.2. Software.

### 4.3.5. Isoelectric focusing (IEF) and 2D-SDS-PAGE

The samples were used directly to passively rehydrate pH 4-7 and pH 6-11, 11 cm IPG strips (Amersham Pharmacia, Uppsala, Sweden) by applying 180 µl of each sample (containing upto 230 µg of protein). IPG's were focused to a total of 80,000 Volt hours (Vh) using a three-step focusing program. The focusing program included a rapid ramp to 300 V for 4 h, a linear ramp to 10,000 V over 8 h, and a 10,000 V step until 80,000 Vh were reached. IPGs were equilibrated for 20 min in 6 M urea, 2 % (w/v) SDS, 50 mM Tris–HCl, pH 8.8, 0.1% (w/v) bromophenol blue. The IPGs were then placed on top of Proteome Systems 6–15% Gelchips (Proteome Systems, Australia) and ran at 30 mA constant until the bromophenol blue dye reached the bottom of the gel. Gels were fixed in 10% (v/v) methanol, 7% (v/v) acetic acid solution for 30 min, then stained with Sypro Ruby solution (Molecular Probes) for 16 h. Gels were destained in the fixing solution before scanning on a Fluorescence scanner (Alpha Innotech Corporation, California). Gels were restained for 16 h with Coomassie Colloidal Blue G250 (17% (w/v) ammonium sulphate, 34% (v/v) methanol, 3.6% (v/v) orthophosphoric acid, 0.1% (w/v) Coomassie G-250) and destained with 1% (v/v) acetic acid for further analysis as required. Each sample was run in triplicate.

### 4.3.6. Mass spectrometry and identifications

Protein spots were excised using the XciseTM apparatus (Shimadzu Biotech, Japan). Gel pieces were destained and dried. Trypsin was added to each gel piece and they were incubated at 37 °C for 16 h for protein digestion. Each peptide solution was desalted and concentrated using ZipTips$^{TM}$ from Millipore (USA) and spotted onto the target plate with 1.0 µl matrix solution (4 mg ml$^{-1}$ alpha-cyano-4-hydroxy-cinnamic acid in 70% (v/v) acetonitrile, 1% (v/v) trifluoroacetic acid). Peptide mass fingerprints of tryptic peptides were generated by matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) using an Applied Biosystems 4700 Proteomics Analyser with TOF/TOF optics in the MS mode. A Nd:YAG laser (355 nm) was used to irradiate the sample. The spectra were acquired in reflectron mode in the mass range 750 to 3,500 Da. The instrument was then switched to MS/MS (TOF/TOF) mode where the eight strongest peptides from the MS scan were fragmented by collision-induced dissociation. A near point external calibration was applied to give a mass accuracy within 50 ppm. Mass spectrometry data was searched against proteins from all fungal species using Mascot Peptide Mass Fingerprint where a modified MOWSE scoring algorithm was used to rank results (http://www.matrixscience.com/help/scoring_help.html).

## 4.4. Results

Extracellular proteins extracted from *M. anisopliae* (FI-1045) when grown on canegrub cuticles were separated on 2D gels over pH range 4-7 (Sample-A; Fig.1) and pH range 6-11 (Sample-A; Fig.2). Proteins extracted from the fungus grown on whole canegrubs (Sample-B; Fig. 3) were separated on 2D gels over pH range 4-7. In this study, majority of the proteins were separated in the pH range 4-7 (Fig. 1 and 3) and only few proteins from Sample-A were separated in pH range 6-11 (Fig. 2). A total of 167 proteins were separated in pH range 4-7 from Sample-A, compared

to 113 proteins from Culture-B. The protein pattern established at the 24 h time point from Cultures A and B was overlapped with the protein pattern established at the 48 h time point from Samples A and B. Most of the proteins from the 24 h time point gels from samples A and B coincided with proteins from the 48 h time point gels (data not shown). No unique spots were seen in the 24 h time point gels from Samples A and B.

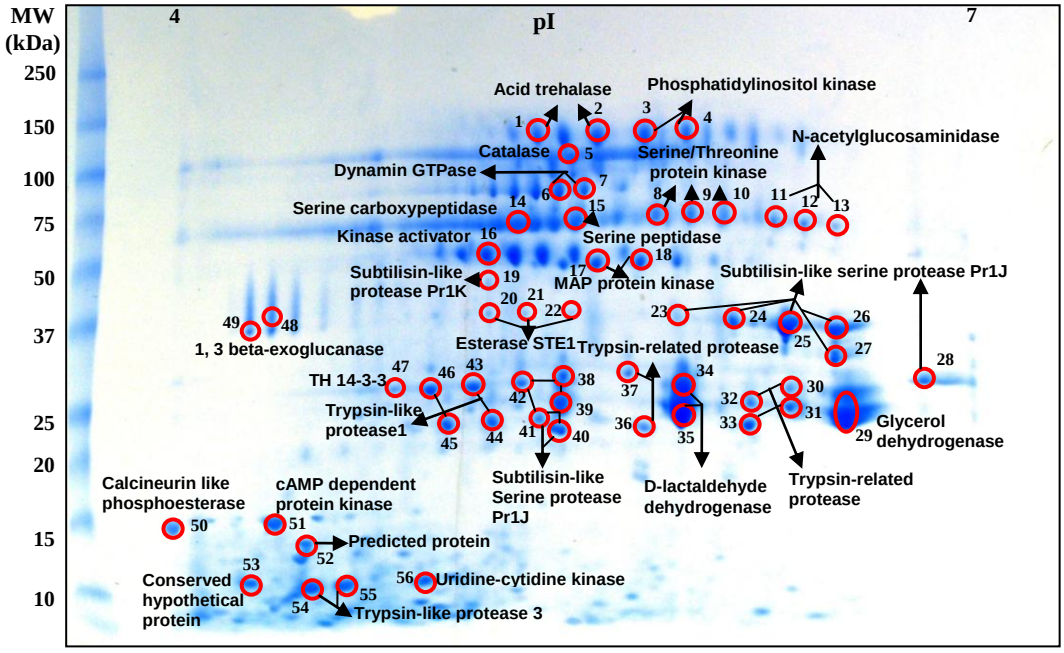


**Fig. 1:** Secreted proteins from the culture supernatant of *M. anisopliae* (FI-1045) when grown on greyback canegrub cuticles (Sample-A). Circled spots contain protein identifications by mass spectrometry. The gels were run on 11 cm, 4-7 IPG strips in the first dimension and 8-16% SDS-PAGE in the second dimension.

Figure 1 displays proteins ranging from 150-kDa to 5-kDa across the pH range 4-7 (from Sample-A) whereas Sample-B displays proteins ranging from 200-kDa to 15-kDa across the pH range 4-7. Fifty six spots from the 48 h time point gel run in the pH 4-7 range (Sample-A), 10 spots from the 48 h time point gel separated in the pH 6-11 range (Sample-A) and 35 spots from 48 h time point gel run in pH 4-7 range (Sample-B) were excised and processed for mass

spectrometry analysis and protein identification. The use of cross-species identification (CSI) increased the size of the protein database searched to include proteins from all fungal species, or selected closely related species. Identified proteins are highlighted and numbered on the protein maps (Fig. 1, 2 and 3) and additional information about these proteins can be found in Table 1 presented at the end of this chapter. All the proteins cut for MALDI analysis were successfully identified, giving a 100% identification rate.

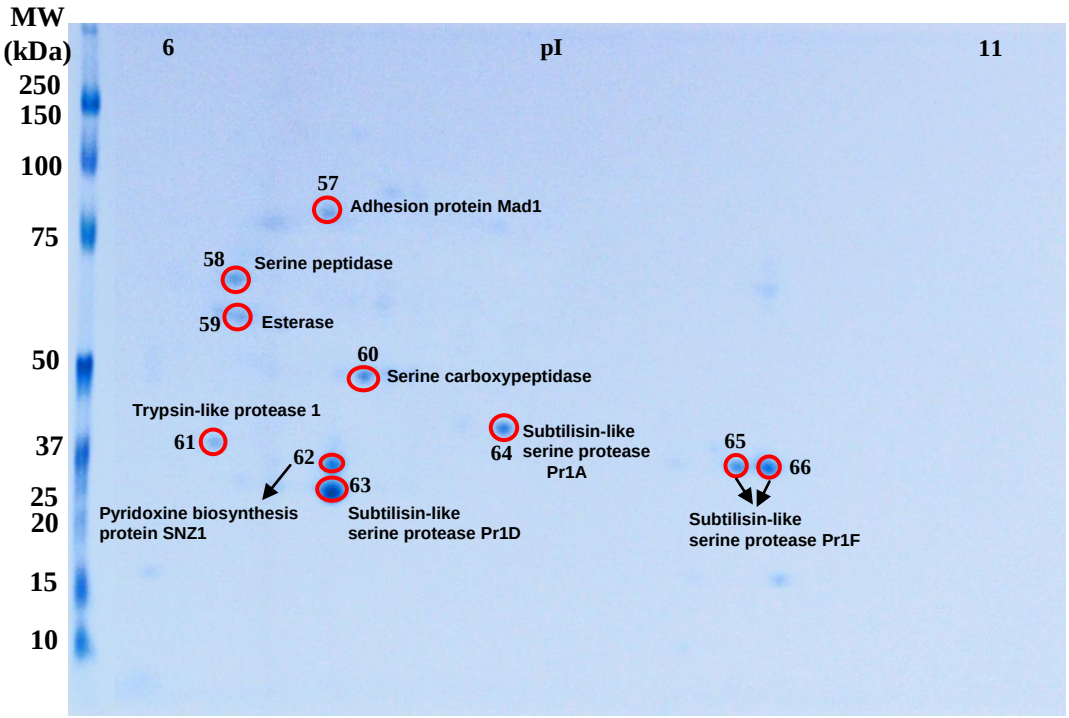


**Fig.2:** Secreted proteins from the culture supernatant of *M. anisopliae* (FI-1045) when grown on greyback canegrub cuticles (Sample-A). Circled spots contain protein identifications by mass spectrometry. The gels were run on 11 cm, 6-11 IPG strips in the first dimension and 8-16% SDS-PAGE in the second dimension.

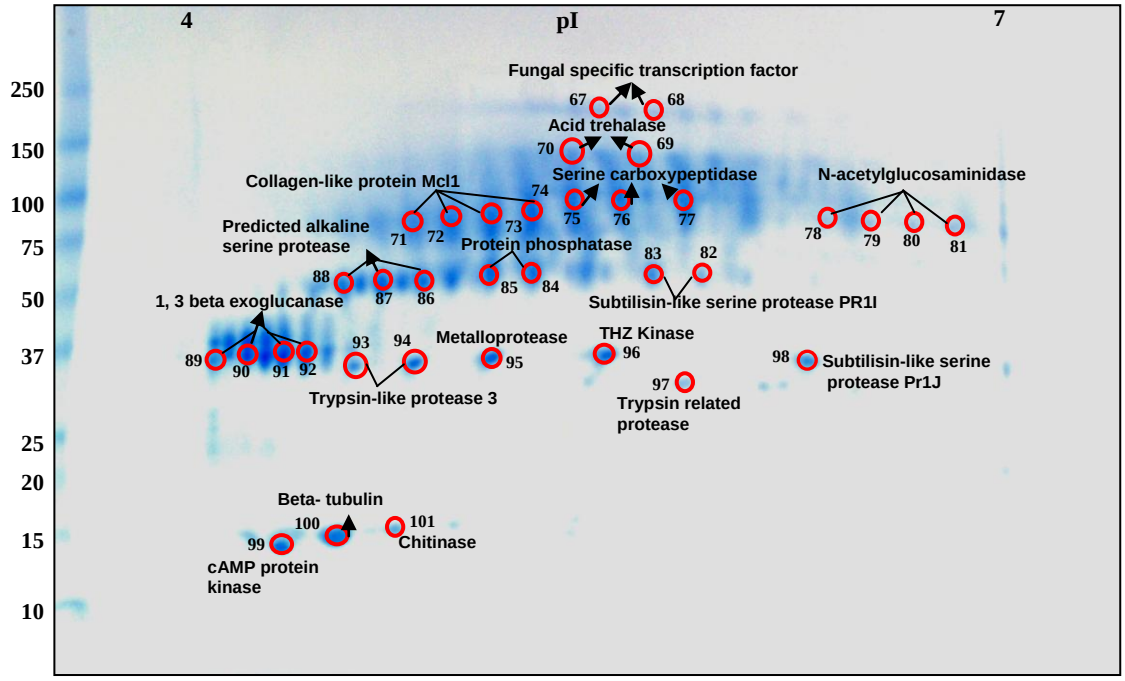


**Fig. 3:** Secreted proteins from the culture supernatant of *M. anisopliae* (FI-1045) when grown on greyback canegrubs (Sample-B). Circled spots contain protein identifications by mass spectrometry. The gels were run on 11 cm, 4-7 IPG strips in the first dimension and 8-16% SDS-PAGE in the second dimension.

Among the identified proteins, sixteen corresponded to various isoforms of cuticle degrading subtilisin-like serine proteases (Pr1) and thirteen protein spots corresponded to different isoforms of trypsin-related proteases (Pr2). Some of the other proteins identified from Sample A and B were N-acetylglucosaminidase (three spots), serine protein kinase (three spots), serine peptidase (two spots), serine carboxypeptidase (two spots), esterase (four spots), 1,3 β-exoglucanase (two spots), catalase (one spot), GTPase (two spots), kinase activator (one spot), MAP protein kinase (one spot), acid trehalase (two spots), D-lactaldehyde dehydrogenase (two spots), glycerol dehydrogenase (one spot), uridine-cytidine kinase (one spot) and adhesion protein (one spot); remaining proteins are tabulated in Table 1. Identified proteins from Sample-B included, fungal specific transcription factor (two spots), acid trehalase (two spots), collagen-like protein (four

spots), serine carboxypeptidase (three spots), N-acetylglucosaminidase (four spots), alkaline serine protease (three spots), 1,3 beta exoglucanase (four spots), subtilisin-like serine protease (Pr1) (two spots), trypsin protease (Pr2) (two spots), metalloprotease (one spot), chitinase (one spot), beta-tubulin (one spot), protein phosphatase (two spots), cAMP protein kinase (one spot), as shown in the annotated map in Figure 3. Secretion of Pr1, Pr2 and other hydrolytic enzymes indicate that a strong cellular response was occurring leading to formation of infection structures and pathogenicity.

## 4.5. Discussion

Fungal pathogenesis is a complex and multi-factorial phenomenon with particular virulence factors coming into play at various stages of infection and death of the target. Like most fungal pathogens, *M. anisopliae* uses a combination of enzymes to penetrate the cuticle and access the nutrient rich host.

The proteomic comparison of extracellular proteins secreted *M. ansiopliae* (FI-1045) grown on canegrub cuticles and whole grubs has highlighted many differentially expressed proteins. A wide variety of proteins were expressed when *M. anisopliae* was grown on the insect cuticles, whereas fewer proteins were expressed when grown on whole grubs. The number of identified proteases is more in the culture containing insect cuticle because it contains 100% insect cuticle which is predominantly composed of proteins and chitin and it is known that *M. anisopliae* produces a variety of proteases to digest the cuticular proteins which are an effective barrier against most microbial infections. Moreover, in the culture containing grub homogenate, the percentage of cuticle component is comparatively less and this might be the reason why there are less proteases identified in the culture containing grub homogenate. In our study, we observed

that *M. anisopliae* (FI-1045) produced five different isoforms of subtilisin-like serine protease (Pr1 J, K, A, D and F) when grown on canegrub cuticles, whereas only two isoforms of Pr1 (Pr1 J and I) were observed when grown on ground whole canegrubs. The enhancing effect of cuticle on Pr1 production suggests that this protease type may be specifically induced by cuticular components. Pr1 is the most extensively studied and best understood protein involved in entomopathogenicity and to date, eleven subtilisins have been identified in *M. anisopliae* during growth on insect cuticles (Freimoser et al. 2003; Bagga et al. 2004). In this study we noted that three isoforms of Pr1 (K, D, and J) were separated from other proteases by narrow-range isoelectric focusing (IEF) and detected in the acidic region; Pr1 A was detected at pH 7 and Pr1F was detected at the alkaline region. Charge differences among Pr1 isoforms probably affect their cuticle degrading ability, since St. Leger et al. (1994a) showed that electrostatic binding of Pr1 was a prerequisite for cuticle hydrolysis. St. Leger's group also demonstrated that cuticle-degrading enzymes were synthesized only at the pH at which they function effectively (St. Leger et al. 1998).

In this study, we identified twelve trypsin-related proteases (Pr2) when the fungus was grown on canegrub cuticles and only three Pr2 proteins were detected when the fungus was grown on whole canegrubs. Similar observations were reported by St. Leger et al. (1996) where thirteen Pr2 proteins were synthesized by *M. anisopliae* in response to exposure to cockroach cuticles. Studies from a number of laboratories have highlighted that Pr2 proteins do not have the ability to degrade an intact cuticle but assist in proteolytic degradation of the cuticular barrier (St. Leger et al. 1996; Freimoser et al. 2003; Paterson et al. 1994). Both types of proteases (Pr1 and Pr2) seem to form a part of a cascade of reactions facilitating the fungal penetration of host cuticles.

A 32-kDa metalloprotease was detected when *Metarhizium* was grown on pulverized whole canegrubs (Fig. 3). The metalloprotease is present as isoenzymes (probably three) and is active against wide range of proteins, including insect cuticle. A metalloprotease activity was described from *M. anisopliae* that was reported to act in concert with several serine proteases to bring about effective destruction of insect cuticle or it may be serving as a back-up for Pr1 activities (St. Leger et al. 1995). Exopeptidases (serine peptidases and serine carboxypeptidases) were observed in the secreted proteome of *M. anisopliae* (FI-1045) (Figs. 1, 2, 3). Serine carboxypeptidases are widely distributed in fungi and they probably function to degrade peptides released by endoproteases, producing free amino acids needed for nutrition and metabolism (Freimoser et al. 2003; St. Leger et al. 1994b). These authors also showed that serine carboxypeptidase produced by *M. anisopliae* during growth on cockroach cuticles indicated that the binding specificity of the carboxypeptidase complement Pr1 activity role in cuticle-degradation. The CSI approach used to detect proteins in our study indicated that serine carboxypeptidase was identified from two different fungal species: *Ustilago maydis* and *M. anisopliae*. Protein spots 14 (Fig. 1), 75, 76 and 77 (Fig. 3) corresponded to a 64-kDa serine carboxypeptidase, identified from a pathogenic plant fungus *Ustilago maydis* and protein spot 60 (Fig. 2) corresponded to a 45-kDa serine carboxypeptidase, identified from *M. anisopliae*. This enzyme from *Ustilago maydis* usually has subunit molecular weights of 45-kDa to 75-kDa, whereas *M. anisopliae* enzyme is a single unit protein with molecular mass of 45-kDa (St. Leger et al. 1994b). Secretion of a high molecular weight serine carboxypeptidase has not been previously reported in *M. anisopliae* during infection process. Therefore, the 64-kDa serine carboxypeptidase identified here seems to be a novel *Metarhizium* protein.

In this work an esterase protein was identified only when the fungus was grown on insect cuticles (Fig.1 and 2). During fungal infection, insect cuticle is the first barrier, which comprises waxes

and cutin. Formation of a penetration peg and development of an appressorium by the fungus on the cuticle are associated with secretion of esterases (Devi et al. 2003). Previous studies have shown that 25 distinct esterase-type isoenzymes were produced by M*etarhizium* in a culture containing insect cuticles (Freimoser et al. 2003). Adhesion protein *Mad1* (Fig. 2: Protein spot 57) secreted by *Metarhizium* indicates that this protein plays a vital role in anchoring to insect surfaces that enables *Metarhizium* to proliferate and colonize during the infection process. Attachment and  to host surface are the key initial steps in proliferation and colonisation. Studies have indicated that deletion of *Mad1* gene in *Metarhizium* mutants resulted in reduced germination, suppressed blastospore formation and reduced virulence activity against target pests (Wang and St. Leger, 2007).

Of the previously identified proteins, N-acetyl glucosaminidase, β-1, 3-glucanase and chitinase have been found to play a vital role in fungal biocontrol. Enzymes N-acetyl glucosaminidase and chitinase are secreted from *M. anisopliae* and are utilized in cuticle penetration (St. Leger et al. 1986b). N-acetyl glucosaminidase (chitobiase) is a secreted protein which is induced by fungal cell walls or found to be a product of chitin degradation (Kang et al. 1999). In this work, the 60-kDa N-acetyl glucosaminidase was detected in the secretome of *M. anisopliae* (FI-1045) when grown on ground canegrub cuticles (Protein spots 11, 12, 13; Fig. 1) or ground whole grubs (Protein spots 78, 79, 80, 81; Fig. 3). The beta-linked polysaccharide, β-1,3-glucan is the major structural component of the cell wall and secretory constituent of all pathogenic fungi giving shape and osmotic support (Lengeler et al. 2000). Contextual to entomopathogenic fungi, a fungal pathogen must evade activation of host immune system which leads to the destruction of the invading pathogens, it has been speculated that pathogens may avoid immune recognition by camouflaging or modifying their β-glucan (Wang and St. Leger, 2006). Recently, examination of thin sections of *B. bassiana* cells extracted from infected *M. sexta* (Tobacco hornworm), *T. ni*

(Cabbage looper) and *S. exigua* (Beet armyworm) demonstrated the presence of β-1,3 glucans (Tartar et al. 2005). Cho et al. (2006) have also reported that *B. bassaina* possesses β-1,3 exoglucanase activity when grown on chitin media. In our study, β-1,3 exoglucanase (Fig. 1: Protein spots 48, 49; Fig. 3: Protein spots 89, 90, 91, 92) was secreted by *M. anisopliae* (FI-1045) growing on ground canegrub cuticles as well as whole grubs. These two proteins (β-1,3 glucans and β-1,3 exoglucanase) have not been reported in *Metarhizium* protein databases and are therefore novel *Metarhizium* proteins. A 13-kDa chitinase (Protein spot 101) was identified in the secretome of FI-1045 when grown on ground whole grubs (Fig. 3). Unlike proteases, chitinases are not detected in the early phases of host insect infection (Kang et al. 1999) because the cuticular chitin is composed of proteins which have to be first degraded by proteases before the chitin is exposed to chitinases. Action of chitinase on procuticle was found to occur approximately fourty hours post-inoculation which suggested that release of the chitinase was reliant on the accessibility of the substrate (St. Leger et al. 1996). Insects have a high concentration of trehalose (disaccharide) in the hemolymph and entomopathogenic fungi are capable of utilizing trehalose as a fuel for their proliferation (Zhao et al. 2006). Secretion of trehalose-hydrolysing enzymes may be a prerequisite for successful exploitation of this resource by the pathogen. Acid trehalases (α-glucosidases) are extracellular enzymes or vacuolar glycoproteins that hydrolyse extracellular trehalose. Using $C^{14}$ labeled trehalose, Xia et al. (2002) have shown that *Metarhizium* acquires host trehalose through secretion of hydrolases and absorption of glucose breakdown products. In this work we found that an acid trehalase was secreted by *M. anisopliae* (FI-1045) when grown on canegrub cuticles (Fig. 1: Protein spots 1, 2) as well as on ground whole grubs (Fig. 3: Protein spots 69, 70).

Investigation of signal transduction protein cascades that regulate fungal development and virulence have been reported in various plant, animal and human pathogenic fungi (Lengeler et

al. 2000). Signaling proteins identified in this study include mitogen-activated protein (MAP) kinase (Fig. 1: Protein spots 17, 18) signaling for cell integrity, cell wall construction, mating and osmo-regulation. Cyclic AMP-dependent protein kinase (Protein spot 51 in Fig.1; Protein spot 99 in Fig. 3) and serine/threonine protein kinases (Fig. 1: Protein spots 8, 9, 10) function as regulatory components which sense and transfer stress signals from the host environment to the cell machinery that controls virulence and pathogenicity factors, thus establishing infection and allowing survival of the pathogen (Engebrecht, 2003). Dynamin GTPases (Fig. 1: Protein spots 6, 7) and THZ kinase (4-methyl-5-beta-hydroxyethylthiazole kinase) play a role in transmitting growth regulation signals from membrane localised receptors to different pathways (Lengeler et al. 2000). In fungi, Calcineurin like phosphoesterase (Fig. 1: Protein spot 50) is present only in *Candida albicans* and it plays an important role in hyphal elongation and activation of calcineurin during stress conditions and thereby protects itself against harsh environment of the host (Engebrecht, 2003). Phosphatidylinositol kinase (Fig. 1: Protein spots 3, 4) controls cellular functions necessary for cell growth and fungal proliferation in response to host nutrients (Lengeler et al. 2000). Dynamin GTPases, THZ kinase, Calcineurin like phosphoesterase and Phosphatidylinositol kinase proteins have not previously been recognised from *M. anisopliae* and are therefore novel identifications.

Identification of proteins involved in signaling and modulating various cellular processes provides further support that exposure of *M. anisopliae* (FI-1045) to greyback cane grubs and their isolated cuticles has successfully triggered a biocontrol-like response leading to utilisation of the available nutrients. In this work, we have identified six extracellular proteins (64-kDa serine carboxypeptidase, 1,3-β-exoglucanase, Dynamin GTPases, THZ kinase, calcineurin like phosphoesterase and phosphatidylinositol kinase) secreted by *M. ansiopliae* (FI-1045) that have not been reported previously. These proteins may provide novel or additional targets for further

development of *M. ansiopliae* as a biocontrol organism. The genes encoding six novel proteins will be isolated in the future in order to establish their involvement in the biocontrol response of *M. anisopliae*.

## 4.6. References

Andersen SO, Højrup P, Roepstorff P (1995) Insect cuticular proteins. Insect Biochem Mol Biol 25: 153-176.

Bagga S, Hu G, Screen SE, St. Leger RJ (2004) Reconstructing the diversification of subtilisins in the pathogenic fungus *Metarhizium anisopliae*. Gene 324: 159-169.

Bidochka MJ, Meltzer MJ (2000) Genetic polymorphisms in three subtilisin-like protease isoforms (Pr1, Pr1B and PrC) from *Metarhizium* strains. Can J Microbiol 46: 1138-1144.

Bidochka MJ, Khachatourians GG (1988) Regulation of extracellular protease in the entomopathogenic fungus *Beauveria bassiana*. Appl Environ Microbiol 53: 1679-1684.

Bidochka MJ, Khachatourians GG (1990) Identification of *Beauveria bassiana* extracellular protease as a virulence factor in pathogenicity toward the migratory grasshopper, *Melanoplus sanguinipes*. J Invertebr Pathol 56: 362-370.

Bidochka MJ, Khachatourians GG (1994) Protein hydrolysis in grasshopper cuticles by entomopathogenic fungal extracellular proteases. J Invertbr Pathol 63: 7-13.

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal biochem 72: 248-254.

Cho EM, Liu L, Farmerie W, Keyhani NO (2006) EST analysis of cDNA libraries from the entomopathogenic fungus *Beauveria* (*Cordyceps*) *bassiana*. I. Evidence for stage-specific gene expression in aerial conidia, *in vitro* blastospores and submerged conidia. Microbiol 152: 2843-2854.

Devi UK, Reddy NNR, Sridevi D, Mohan CM (2003) Esterase-mediated tolerance to a formulation of the organophosphate insecticide monocrotophos in the entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuill: a promising biopesticide. Pest Manag Sci 60:408–412.

Engebrecht J (2003) Cell signaling in yeast sporulation. Biochem Biophys Res Commum 306: 325-328.

Freimoser, F. M., Screen, S., Bagga, S., Hu, G. & St Leger, R. J. (2003) Expressed sequence tag (EST) analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts. Microbiology 149: 239–247.

Grinyer J, McKay M, Nevalainen H, Herbert BR (2004) Fungal proteomics: initial mapping of biological control strain *Trichoderma harzianum*. Curr Genet 45: 163-169.

Joshi L, St. Leger RJ, Roberts DW (1997) Isolation of a cDNA encoding a novel subtilisin-like protease (PrIB) from the entomopathogenic fungus, *Metarhizium anisopliae* using differential display RT-PCR. Gene 197: 1-8.

Kang SC, Park S, Lee DG (1999) Purification and characterization of a novel chitinase from the entomopathogenic fungus, *Metarhizium anisopliae*. J Invertebr Pathol 73: 276-281.

Lengeler KB, Davidson RC, D'Souza C, Harashima T, Shen WC, Wang P, Pan X, Waugh M, Heitman J (2000) Signal transduction cascades regulating fungal development and virulence. Microbial Mol Biol Rev 64: 746-785.

Milner RJ (2000) Current status of *Metarhizium* for insect control in Australia. Biocontrol News Inf 21: 47-50.

Milner RJ, Samson P and Bullard G (2002) A profile of a commercially useful isolate of *Metarhizium anisopliae* var. *anisopliae*. Biocontrol Sci Technol 12: 43–58.

Paterson IC, Charnley AK, Cooper RM, Clarkson JM (1994) Specific induction of a cuticle-degrading protease of the insect pathogenic fungus *Metarhizium anisopliae*. Microbiol 140: 185-189.

Pinto FGS, Fungaro MHP, Ferreira JM, Valadares-Inglis MC, Furlaneto MC (2002) Genetic variation in the cuticle-degrading protease activity of the entomopathogen *Metarhizium flavoviride*. Gen Mol Biol 25: 231-234.

Robertson LN, Kettle CG, Bakker P (1997) Field evaluation of *Metarhizium anisopliae* for control of greyback canegrub (*Dermolepida albohirtum*) in north Queensland sugarcane. Proc Aust Soc Sugar Cane Technol 19: 111–117.

Shah PA, Pell JK (2003) Entomopathogenic fungi as biological control agents. Appl Microbiol Biotechnol 61: 413-423.

St. Leger RJ, Charnley AK, Cooper RM (1986a) Cuticle-degrading enzymes of entomopathogenic fungi: mechanisms of interaction between pathogen enzymes and insect cuticle. J Invertebr Pathol 47:295–302.

St. Leger RJ, Charnley AK, Cooper RM (1986b) Cuticle-degrading enzymes of entomopathogenic fungi: synthesis in culture on cuticle. J Invertebr Pathol 48:85–95.

St. Leger RJ, Charnley AK, Cooper RM (1987) Characterization of cuticle-degrading proteases by the entomopathogen *Metarhizium anisopliae*. Arch Biochem Biophys 253: 221–232.

St. Leger RJ, Bidochka M, Roberts DW (1994a) Isoforms of the cuticle-degrading Pr1 proteinse and production of metalloproteinase by *M. anisopliae*. Arch Biochem Biophys 313: 1-7.

St. Leger RJ, Bidochka M, Roberts DW (1994b) Characterisation of a novel carboxypeptidase by the entomopathogenic fungus *Metarhizium anisopliae*. Arch Biochem Biophys 314: 392-398.

St. Leger RJ (1995) The role of cuticle-degrading proteases in fungal pathogenesis of insects. Can J Bot 73: 1119-1125.

St. Leger RJ, Joshi L, Bidochka MJ, Roberts DW (1995) Protein synthesis in *Metarhizium anisopliae* growing on host cuticle. Mycol Res 99: 1034-1040.

St. Leger RJ, Joshi L, Bidochka M, Rizzo MJ, Roberts DW (1996) Characterization and ultrastructural localization of chitinases from *Metarhizium anisopliae*, *M. flavoviride* and *Beauveria bassiana* during fungal invasion of host (*Manduca sexta*) cuticle. Appl Environ Microbiol 62:907–912.

St. Leger RJ, Joshi L, Roberts DW (1998) Ambient pH is a major determinant in the expression of cuticle degrading enzymes and hydrophobin by *Metarhizium anisopliae*. Appl Environ Microbiol 64: 709-713.

Tartar A, Shapiro AM, Scharf DW, Boucias DG (2005) Differential expression of chitin synthase (*CHS*) and glucan synthase (*FKS*) genes correlates with the formation of a modified, thinner cell wall in *in vivo*-produced *Beauveria bassiana* cells. Mycopathologia 160: 303–314.

Wang C, St. Leger RJ (2006) A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses. Proc Natl Acad Sci USA 103: 6647-6652.

Wang C, St. Leger RJ (2007) The MAD1 adhesin of *Metarhizium anisopliae* links adhesion with blastospore production and virulence to insects, and the MAD2 adhesin enables attachment to plants. Eukaryotic Cell 6: 808-816.

Xia Y, Clarkson JM, Charnley AK (2002) Trehalose-hydrolysing enzymes of *Metarhizium anisopliae* and their role in pathogenesis of the tobacco hornworm, *Manduca sexta*. J Invertebr Pathol 80: 139-147.

Zhao H, Charnley AK, Wang Z, Yin Y, Li Z, Li Y, Cao Y, Peng G, Xia Y (2006) Identification of an extracellular acid trehalase and its gene involved in fungal pathogenesis of *Metarizium anisopliae*. J Biochem 140: 319-327.

**Table 1. Extracellular proteins produced from *M. anisopliae* (FI-1045)**

| Spot # | Protein name | Accession # | pI | Mol wt | Coverage or number of peptides matched | Species |
|---|---|---|---|---|---|---|
| ***Metarhizium* proteins secreted when grown on canegrub cuticles (Sample-A)** | | | | | | |
| 1 | Acid trehalase | A9XE63 | 5.31 | 116252 | 4 peptides | *Metarhizium anisopliae* |
| 2 | Acid trehalase | A9XE64 | 5.31 | 116252 | 4 peptides | *Metarhizium anisopliae* |
| 3 | Phosphatidylinositol kinase | gi\|50545769 | 5.47 | 108071 | 35% | *Yarrowia lipolytica* |
| 4 | Phosphatidylinositol kinase | gi\|50545769 | 5.47 | 108071 | 29% | *Yarrowia lipolytica* |
| 5 | Catalase | gi\|88766403 | 5.62 | 78747 | 3 peptides | *Metarhizium anisopliae* |
| 6 | Dynamin GTPase | gi\|70999089 | 5.98 | 80366 | 36% | *Aspergillus fumigatus Af293* |
| 7 | Dynamin GTPase | gi\|70999090 | 5.98 | 80366 | 36% | *Aspergillus fumigatus Af294* |
| 8 | Serine/Threonine protein kinases | gi\|6324444 | 7.18 | 43178 | 32% | *Saccharomyces cerevisiae* |
| 9 | Serine/Threonine protein kinases | gi\|6324445 | 7.18 | 43178 | 32% | *Saccharomyces cerevisiae* |
| 10 | Serine/Threonine protein kinases | gi\|6324446 | 7.18 | 43178 | 32% | *Saccharomyces cerevisiae* |
| 11 | N-acetylglucosaminidase | Q52JJ1 | 6.07 | 69118 | 4 peptides | *Metarhizium anisopliae* |
| 12 | N-acetylglucosaminidase | Q52JJ1 | 6.07 | 69118 | 2 peptides | *Metarhizium anisopliae* |
| 13 | N-acetylglucosaminidase | Q52JJ1 | 6.07 | 69118 | 2 peptides | *Metarhizium anisopliae* |
| 14 | Serine carboxypeptidase | gi\|71006740 | 5.21 | 64911 | 5 peptides | *Ustilago maydis 521* |
| 15 | Serine peptidase | gi\|70988815 | 6.04 | 55653 | 34% | *Aspergillus fumigatus Af293* |
| 16 | Kinase activator Atg17 | gi\|71002124 | 4.99 | 55674 | 5 peptides | *Aspergillus fumigatus Af293* |
| 17 | MAP Protein kinase, Putative | gi\|57223234 | 6.54 | 64051 | 41% | *Cryptococcus neoformans JEC21* |
| 18 | MAP Protein kinase, Putative | gi\|57223234 | 6.54 | 64051 | 33% | *Cryptococcus neoformans JEC21* |
| 19 | Subtilisin-like protease Pr1K | Q9HFL7 | 5.06 | 38474 | 6 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| 20 | Esterase STE1 | Q9UUR6 | 5.4 | 39207 | 44% | *Metarhizium anisopliae* var.*anisopliae* |
| 21 | Esterase STE1 | Q9UUR6 | 5.4 | 39207 | 44% | *Metarhizium anisopliae* var.*anisopliae* |
| 22 | Esterase STE1 | Q9UUR6 | 5.4 | 39207 | 44% | *Metarhizium anisopliae* var.*anisopliae* |
| 23 | Subtilisin-like serine protease Pr1J | gi\|6624952 | 6.04 | 42304 | 28% | *Metarhizium anisopliae* var.*anisopliae* |
| 24 | Subtilisin-like serine protease Pr1J | gi\|6624953 | 6.04 | 42304 | 31% | *Metarhizium anisopliae* var.*anisopliae* |
| 25 | Subtilisin-like serine protease Pr1J | gi\|6624954 | 6.04 | 42304 | 19 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| 26 | Subtilisin-like serine protease Pr1J | gi\|6624955 | 6.04 | 42304 | 14 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| 27 | Subtilisin-like serine protease Pr1J | gi\|6624956 | 6.04 | 42304 | 9 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| 28 | Subtilisin-like serine protease Pr1J | gi\|6624957 | 6.04 | 42304 | 10 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| 29 | Glycerol dehydrogenase GCY1 | gi\|70995191 | 7.1 | 33472 | 72% | *Aspergillus fumigatus Af293* |

| | | | | | | |
|---|---|---|---|---|---|---|
| 30 | Trypsin-related protease | gi\|4768909 | 5.68 | 26273 | 1 peptide | *Metarhizium anisopliae* |
| 31 | Trypsin-related protease | gi\|4768909 | 5.68 | 26273 | 2 peptides | *Metarhizium anisopliae* |
| 32 | Trypsin-related protease | gi\|4768909 | 5.68 | 26273 | 1 peptide | *Metarhizium anisopliae* |
| 33 | Trypsin-related protease | gi\|4768909 | 5.68 | 26273 | 1 peptide | *Metarhizium anisopliae* |
| 34 | D-lactate dehydrogenase Putative | gi\|57227156 | 6.15 | 38160 | 55% | *Cryptococcus neoformans JEC21* |
| 35 | D-lactate dehydrogenase Putative | gi\|57227156 | 6.15 | 38160 | 62% | *Cryptococcus neoformans JEC21* |
| 36 | Trypsin-related protease | gi\|4768909 | 5.68 | 26273 | 49% | *Metarhizium anisopliae* |
| 37 | Trypsin-related protease | gi\|4768909 | 5.68 | 26273 | 37% | *Metarhizium anisopliae* |
| 38 | Subtilisin-like serine protease Pr1J | gi\|6624952 | 6.04 | 42304 | 3 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| 39 | Subtilisin-like serine protease Pr1J | gi\|6624952 | 6.04 | 42304 | 5 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| 40 | Subtilisin-like serine protease Pr1J | gi\|6624952 | 6.04 | 42304 | 8 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| 41 | Subtilisin-like serine protease Pr1J | gi\|6624952 | 6.04 | 42304 | 6 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| 42 | Subtilisin-like serine protease Pr1J | gi\|6624952 | 6.04 | 42304 | 3 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| 43 | Trypin-like protease1 | gi\|556657 | 5.45 | 26100 | 2 peptides | *Metarhizium anisopliae* |
| 44 | Trypin-like protease1 | gi\|556657 | 5.45 | 26100 | 4 peptides | *Metarhizium anisopliae* |
| 45 | Trypin-like protease1 | gi\|556657 | 5.45 | 26100 | 4 peptides | *Metarhizium anisopliae* |
| 46 | Trypin-like protease1 | gi\|556657 | 5.45 | 26100 | 1 peptide | *Metarhizium anisopliae* |
| 47 | TH14-3-3 | Q27JQ9 | 4.86 | 29865 | 21% | *Metarhizium anisopliae* |
| 48 | 1,3 beta-exoglucanase | Q7Z9L3 | 4.56 | 44372 | 23% | *Aspergillus oryzae* |
| 49 | 1,3 beta-exoglucanase | Q7Z9L3 | 4.56 | 44372 | 23% | *Aspergillus oryzae* |
| 50 | Calcineurin like phosphoesterase | gi\|68491579 | 5.21 | 35876 | 30% | *Candida albicans SC5314* |
| 51 | cAMP dependent protein kinase | gi\|71024243 | 5.14 | 26990 | 22% | *Ustilago maydis 521* |
| 52 | Predicted protein | gi\|88177108 | 4.57 | 30418 | 21% | *Chaetomium globosum* |
| 53 | Conserved hypothetical protein | gi\|57226280 | 4.64 | 10913 | 25% | *Cryptococcus neoformans JEC21* |
| 54 | Trypsin-like protease 3 | gi\|5042250 | 4.9 | 26184 | 2 peptides | *Metarhizium anisopliae* |
| 55 | Trypsin-like protease 3 | gi\|5042250 | 4.9 | 26184 | 2 peptides | *Metarhizium anisopliae* |
| 56 | Uridine-cytidine kinase | gi\|50555015 | 5.67 | 27750 | 20% | *Yarrowia lipolytica* |
| ***Metarhizium* proteins secreted when grown on canegrub cuticles (Sample-B)** | | | | | | |
| 57 | Adhesion protein Mad1 | Q2LC49 | 6.13 | 74569 | 54% | *Metarhizium anisopliae* |
| 58 | Serine peptidase | gi\|70988815 | 6.04 | 55653 | 20% | *Aspergillus fumigatus Af293* |
| 59 | Esterase STE1 | Q9UUR6 | 5.4 | 39207 | 44% | *Metarhizium anisopliae* var.*anisopliae* |
| 60 | Serine carboxypeptidase | B5LXD6 | 6.63 | 45776 | 39% | *Metarhizium anisopliae* |
| 61 | Trypsin-like protease1 | gi\|556657 | 5.45 | 26100 | 28% | *Metarhizium anisopliae* |
| 62 | Pyridoxine biosynthesis protein | gi\|50549493 | 6.1 | 32111 | 43% | *Yarrowia lipolytica* |
| 63 | Subtilisin-like protease Pr1D | gi\|7688246 | 5.81 | 42694 | 7 peptides | *Metarhizium anisopliae* var.*anisopliae* |

| | | | | | | |
|---|---|---|---|---|---|---|
| 64 | Subtilisin like serine protease Pr1A | gi\|16215677 | 7.14 | 40303 | 2 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| 65 | Subtilisin-like protease Pr1F | Q874T3 | 8.36 | 35265 | 4 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| 66 | Subtilisin-like protease Pr1F | Q874T3 | 8.36 | 35265 | 4 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| ***Metarhizium* proteins secreted when grown on whole canegrubs (Sample-C)** | | | | | | |
| 67 | Fungal specific transcription factor | gi\|67537746 | 6.31 | 100048 | 34% | *Aspergillus nidulans FGSC A4* |
| 68 | Fungal specific transcription factor | gi\|67537747 | 6.31 | 100048 | 30% | *Aspergillus nidulans FGSC A5* |
| 69 | Acid trehalase | A9XE63 | 5.31 | 116252 | 41% | *Metarhizium anisopliae* |
| 70 | Acid trehalase | A9XE63 | 5.31 | 116252 | 35% | *Metarhizium anisopliae* |
| 71 | Collagen-like protein Mcl1 | Q307M9 | 5.04 | 60404 | 2 peptides | *Metarhizium anisopliae* |
| 72 | Collagen-like protein Mcl1 | Q307M10 | 5.04 | 60404 | 4 peptides | *Metarhizium anisopliae* |
| 73 | Collagen-like protein Mcl1 | Q307M11 | 5.04 | 60404 | 4 peptides | *Metarhizium anisopliae* |
| 74 | Collagen-like protein Mcl1 | Q307M11 | 5.04 | 60404 | 3 peptides | *Metarhizium anisopliae* |
| 75 | Serine carboxypeptidase | gi\|71006740 | 5.21 | 64911 | 30% | *Ustilago maydis* 521 |
| 76 | Serine carboxypeptidase | gi\|71006740 | 5.21 | 64911 | 30% | *Ustilago maydis* 521 |
| 77 | Serine carboxypeptidase | gi\|71006740 | 5.21 | 64911 | 30% | *Ustilago maydis* 521 |
| 78 | N-acetylglucosaminidase | Q52JJ1 | 6.07 | 69118 | 35% | *Metarhizium anisopliae* |
| 79 | N-acetylglucosaminidase | Q52JJ1 | 6.07 | 69118 | 31% | *Metarhizium anisopliae* |
| 80 | N-acetylglucosaminidase | Q52JJ1 | 6.07 | 69118 | 29% | *Metarhizium anisopliae* |
| 81 | N-acetylglucosaminidase | Q52JJ1 | 6.07 | 69118 | 33% | *Metarhizium anisopliae* |
| 82 | Subtilisin-like serine protease Pr1I | Q96UG0 | 6.14 | 40248 | 41% | *Metarhizium anisopliae* var.*anisopliae* |
| 83 | Subtilisin-like serine protease Pr1I | Q96UG0 | 6.14 | 40248 | 3 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| 84 | Protein phosphatase | gi\|50420691 | 5.43 | 64124 | 43% | *Debaryomyces hansenii CBS767* |
| 85 | Protein phosphatase | gi\|50420691 | 5.43 | 64124 | 43% | *Debaryomyces hansenii CBS767* |
| 86 | Predicted alkaline serine protease | gi\|83764459 | 4.95 | 40594 | 2 peptides | *Aspergillus oryzae* |
| 87 | Predicted alkaline serine protease | gi\|83764459 | 4.95 | 40594 | 2 peptides | *Aspergillus oryzae* |
| 88 | Predicted alkaline serine protease | gi\|83764459 | 4.95 | 40594 | 2 peptides | *Aspergillus oryzae* |
| 89 | 1,3 beta-exoglucanase | Q7Z9L3 | 4.56 | 44372 | 6 peptides | *Aspergillus oryzae* |
| 90 | 1,3 beta-exoglucanase | Q7Z9L3 | 4.56 | 44372 | 3 peptides | *Aspergillus oryzae* |
| 91 | 1,3 beta-exoglucanase | Q7Z9L3 | 4.56 | 44372 | 5 peptides | *Aspergillus oryzae* |
| 92 | 1,3 beta-exoglucanase | Q7Z9L3 | 4.56 | 44372 | 3 peptides | *Aspergillus oryzae* |
| 93 | Trypsin-like protease 3 | gi\|5042250 | 4.9 | 26184 | 1 peptide | *Metarhizium anisopliae* |
| 94 | Trypsin-like protease 3 | gi\|5042250 | 4.9 | 26184 | 2 peptides | *Metarhizium anisopliae* |
| 95 | Metalloprotease | Q9UUR6 | 5.45 | 32046 | 5 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| 96 | THZ Kinase | gi\|46125773 | 6.12 | 53165 | 17% | *Gibberella zeae PH-1* |
| 97 | Trypsin related protease | gi\|4768909 | 5.68 | 26273 | 28% | *Metarhizium anisopliae* |

| | | | | | | |
|---|---|---|---|---|---|---|
| 98 | Subtilisin-like serine protease Pr1J | gi\|6624952 | 6.04 | 42304 | 3 peptides | *Metarhizium anisopliae* var.*anisopliae* |
| 99 | cAMP dependent protein kinase | gi\|71024243 | 5.14 | 26990 | 28% | *Ustilago maydis 521* |
| 100 | Beta-tubulin | Q8TGB1 | 4.74 | 18023 | 2 peptides | *Metarhizium flavoviride* |
| 101 | Chitinase | Q12631 | 4.82 | 13869 | 24% | *Metarhizium anisopliae* |

# 5. A mutant strain of *Metarhizium anisopliae* var. *anisopliae* with increased virulence against greyback canegrubs

## 5.1. Introduction

In recent years, the focus on management of pest diseases has shifted from chemical control to the eco-friendly method of biological control. Several fungi have been developed as mycoinsecticides to control important agricultural pests. Among them, the hypomycete fungus *Metarhizium anisopliae* is of considerable interest in Australia, and has been used against canegrubs in the sugarcane fields. Currently, *Metarhizium anisopliae* isolate FI-1045 is applied to cane plants as a granular product, BioCane™ for controlling greyback canegrubs.

Field trials over the years have shown that the greyback canegrubs were killed up to 60% in the first 4-6 months when BioCane™ was applied during planting (Samson et al. 2006). Despite this success, greyback canegrubs are still a threat to the sugar industry. Work conducted by Samuels et al. (1990) has indicated that environmental factors such as temperature and pH of the soil govern the persistence of the fungus (BioCane™) in the field. Therefore, an increased understanding of the ecology of *M. anisopliae* and the impact of environmental factors on the performance of this fungus is important not only with respect to the development of successful biocontrol strategies, but also to increase knowledge on its persistence in the environment. Genetic manipulation of the fungus through induced mutagenesis and molecular cloning of key virulence genes have been considered as important tools in improving the biocontrol potential of *Metarhizium* spp (Prior et al. 1995; Lacey and Goettel, 1995; St. Leger, 1993). As a part of further improvement of the currently applied strain, the conidia of the *M. anisopliae* FI-1045

isolate were irradiated with UV light to create mutant strains more tolerant to different environmental conditions and retaining their virulence against greyback canegrubs.

In previous studies, secretion of a dominant enzyme has been used as an indicator to monitor changes in protein secretion after mutagenic treatment (Champlin et al. 1981; Morley-Davies et al. 1995; Nevalainen et al. 1980). In the current work, proteolytic activity was used as an indicator for general protein secretion after mutagenesis. *M. anisopliae* produces families of catalytically distinct extracellular subtilisin-like proteases (Pr1), trypsin-like proteases (Pr2), metalloproteases and several families of exo-acting peptidases that are involved in insect cuticle degradation (St. Leger et al. 1986). In this work we also tested mutants for chitinase activity because *M. anisopliae* produces a series of chitinases some of which act synergistically with proteases to degrade insect cuticle (St. Leger et al. 1989). Five best mutants showing increased proteolytic activity which also coincided with high chitinase activity were further subjected to cultivation on different temperature and pH ranges and the best mutant (NM10) was subjected to a bioassay to evaluate its virulence against greyback canegrubs. This work was conducted with a view of applying the fungus to sugarcane fields in various regions with varying range of environmental conditions.

## 5.2. Materials and methods

### 5.2.1. Fungal strain and UV mutagenesis

The parental strain *M. anisopliae* FI-1045 [received from BSES Limited (Bureau of Sugar Experiment Stations, Queensland, Australia)] was grown on potato dextrose agar (PDA, Difco Laboratories, Detroit, Michigan) at 28 °C for 14 days. Conidia were then collected in 10 ml of 0.9 % (w/v) sodium chloride and 0.01 % (v/v) Tween-80 (Sigma Chemical Co., St. Louis, MO)

and filtered through a sterile cotton plug. Spore concentration was adjusted to about $1\times10^8$ spores/ml using a hemocytometer. Spore viability was determined by plating 100 µl of the spore suspension on PDA and counting colonies after 48 h incubation at 28 °C. Around 20 ml of spore suspension containing $10^8$ conidia/ml were pipetted in 20 cm glass petri dish with a flea and placed on a magnetic stirrer at about 15 cm distance from the UV source. The spore suspension was irradiated under UV light of 30W and wavelength of 254 nm for 2, 5, 10, 15, 20 and 25 minutes. The irradiated (mutagenised spores) and non-irradiated (untreated control) spore suspensions were cultured on PDA plates at 28 °C for four days to allow colony formation. Survival rate was calculated at each time point, compared to the control and colonies from the time point with a survival rate of 2-10 % were streaked onto screening plates to test for protease and chitinase activity.

### 5.2.2. Enzyme screening

Enzyme secretion of the mutant strains was evaluated in solid minimal medium ($NaNO_3$ 6.0 g/L, KCl 0.52 g/L, $MgSO_4.7\ H_2O$, 0.52 g/L, $KH_2PO_4$ 1.52 g/L, 15 g/L agar and 0.001 g/L of $FeSO_4.7H_2O$ and $ZnSO_4$, pH 6.9), supplemented with 0.1% (w/v) yeast extract and one of the following carbon sources: 1 % (w/v) dry skim milk (Nestlé, Australia Ltd) for protease and 5 % (w/v) purified crab chitin (Sigma Chemical Co., St. Louis, MO) for chitinase assay. The plates were incubated at 28 °C for 7 days. A clearing halo appeared around each colony due to substrate degradation by enzyme activity. Clearing zones were measured and compared to those of the non-mutagenised parental strain as in Morley-Davies et al. 1995. In order to test the stability of mutants, isolated colonies showing large clearing zones were subcultured twenty times on PDA, protease and chitin screening plates. Five stable mutant strains (NM10, NM4, NM18, NM23 and NM 42) showing consistently large clearing zones were selected for further testing.

### 5.2.3. Effect of temperature and pH on radial growth of *Metarhizium* strains

The five mutant strains (NM10, NM4, NM18, NM23 and NM 42) were sporulated and maintained on potato dextrose agar (PDA, Difco Laboratories, Detroit, Michigan). Conidia were then collected in 10 mL of 0.9 % (w/v) sodium chloride and 0.01 % (v/v) Tween-80 (Sigma Chemical Co., St. Louis, MO) and filtered through a sterile cotton plug. Conidial concentration was adjusted to about $1\times10^8$ conidia/mL using a hemocytometer. Growth was tested by spread-plating 100 µL of spore suspension of each mutant strain on a minimal medium ($NaNO_3$ 6.0 g/L, KCl 0.52 g/L, $MgSO_4.7H_2O$, 0.52 g/L, $KH_2PO_4$ 1.52 g/L, glucose 10.0 g/L, agar 15 g/L, $FeSO_4.7H_2O$ 0.001 g/L and $ZnSO_4$ 0.001 g/L, pH 6.9). The plates were incubated at 15, 20, 25, 28, 30, 32, 35, 38 and 40 °C for 7 days and mointered daily. Radial growth of *Metarhizium* strains were scored on day seven. The mutant strains were then tested for growth over a pH range of 2 to 12. Growth was tested by spread-plating 100 µL of conidial suspension of each mutant strain on a minimal medium described above. The pH of the medium was adjusted with 0.1 M HCl or 0.1 M NaOH to obtain pH values of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. The plates were incubated at 28 °C for 7 days. Germination of spores of the mutant strains tested for temperature tolerance was checked at regular intervals for 7 days using phase-contrast microscope with 400 x magnification. A spore was considered to have germinated if a germ tube was formed and was continuing to grow to form hyphae. The radial growth of the fungus was measured in centimeters using a transparent ruler and growth was scored on day seven. Parental strain FI-1045 was used as control. All experiments were carried out in triplicate and mean value was scored.

### 5.2.4. Insect larvae

The third instar greyback canegrubs, *Dermolepida albohirtum* (Waterhouse) were hand picked from the sugarcane fields around Townsville [Queensland, Australia

(http://www.ga.gov.au/bin/gazd01?rec=155794)] and transported to Sydney. Upon arrival, the canegrubs were instantly immobilised using a cotton ball dipped in chloroform (Ajax Finechem, Sydney, Australia) to anaesthetise the grubs and then surface sterilised in 5 % (v/v) sodium hypochlorite solution (Prior et al. 1995) and 75 % (v/v) ethanol solution and then rinsed with plenty of sterile distilled water. Canegrubs were then transferred into 100 ml sterile transparent plastic tubs (Technoplas, Australia) filled with 30 grams of sterilised garden peat (Killarney peat moss, Australia). Garden peat was sterilised by heating and drying (150 °C, 4 h), then mixed with distilled water. The canegrubs were fed with fresh pieces of carrot and held at room temperature (RT) for seven days until the grubs were used for experiment because only healthy canegrubs were selected for further experiment.

### 5.2.5. Infection of canegrubs

Greyback canegrubs assigned for the treatment with the mutant strain NM10 as well as canegrubs used as control were first immobilised using a cotton ball dipped in chloroform to anaesthetise the grubs and then surface sterilised in 5 % (v/v) sodium hypochlorite solution (Prior et al. 1995) and 75 % (v/v) ethanol solution and then rinsed with plenty of sterile distilled water. Canegrubs assigned for infection were individually dipped and gently shaken for two minutes in 5 ml of fungal spore suspension ($5x10^8$ spores/mL) and they were put into sterile tubs containing sterilised peat. Each canegrub was fed with a fresh piece of carrot and visually monitored for infection for two weeks. A totally static canegrub which did not consume the carrot piece was considered as dead or positive in terms of infection, while a mobile healthy canegrub feeding normally on carrot pieces was negative. Thirty canegrubs were treated with the mutant strain NM10 and another thirty canegrubs were treated with the parental strain FI-1045. The number of dead grubs was noted periodically over two weeks time.

## 5.3. Results and discussion

### 5.3.1. Mutant isolation

Twenty minutes exposure to ultraviolet light killed more than 90 % of *M. anisopliae* FI-1045 conidia. Approximately 150 colonies growing on PDA plates were examined after mutagenesis by growing them first on protease screening plates. Sixty four colonies showing clearing zones around the colony on protease screening plates were also tested on chitinase screening plates. Sixty colonies showing clearing zones around the colony on chitinase screening plates were selected and tested for stability as described in Section 5.3.3. Among them five colonies with more than 4cm width clearing zone on protease and chitin screening media were chosen and plated on PDA plates. These five mutant strains (NM10, NM4, NM18, NM23 and NM42) were further tested for their tolerance to varying temperature and pH ranges. Clearing zone of the wild type was around 2.9cm in width. UV radiation has been carried out with several genera of entomopathogenic fungi such as *Metarhizium*, *Beauveria* and *Paecilomyces* (Morley-Davies et al. 1995; Heale et al. 2006; Lacey and Goettel, 1995) and studies have shown that different strains and isolates of entomopathogenic fungi respond differently to UV light (Morley-Davies et al. 1995).

### 5.3.2. Effect of temperature on radial growth of *Metarhizium* strains

Under the conditions of this study, three mutant strains NM10, NM18 and NM4 grew at all tested temperatures ranging from 15 °C to 40 °C. Mutant strains NM23 and NM42 grew at temperatures between 15 °C to 38 °C but no growth was observed at 40 °C. Maximum growth was observed at 28 °C for all mutants and the parental strain tested (Figure 1). Parental strain did not grow at very low and high temperatures, but only between 20 °C to 35 °C. Mutant strain NM10 showed the best growth rate among all other strains tested. The investigations into radial growth of the

parental strain and mutants showed that all displayed typical symmetric fungal growth curves (Thomas and Jenkins, 1997) over a range of temperatures. The growth temperatures for the isolates investigated in this study ranged from 15 °C to 40 °C, three mutant strains grew in all temperatures tested and the mutant strain NM10 showed the best growth at all temperatures tested. The temperature range used in this study was based on the average soil/surface temperature where the fungus will be applied in Queensland. Previous studies have shown that temperature can affect the germination, growth and viability of the entomopathogenic fungi in the laboratory as well as in the field (Hywel-Jones and Gillespie, 1990). Work conducted by Hall and Papierok (1982) suggested that laboratory studies which simulate varying field conditions can provide quantitative data that may be useful to predict field results.

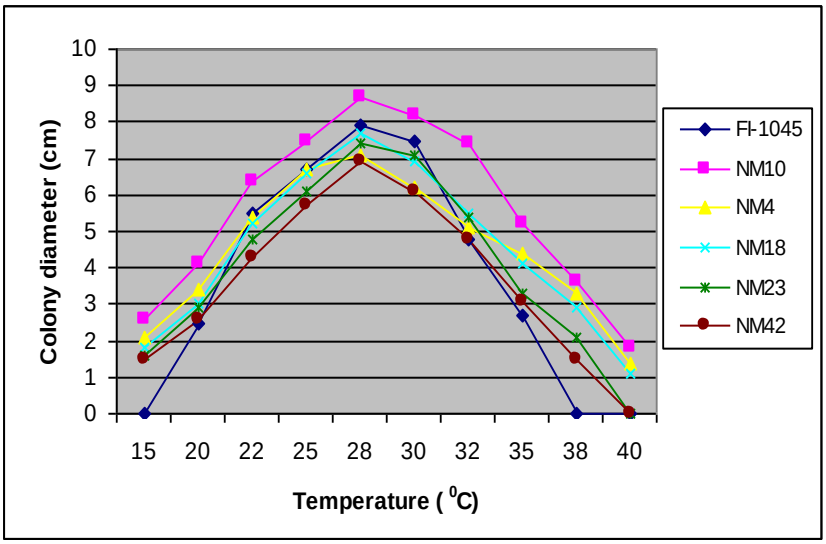


**Figure 1: Effect of temperature on radial growth of *Metarhizium* strains**

Radial growth (measured in centimeters) of *M. anisopliae* mutants NM10, NM4, NM18, NM23 and NM42 and the parental strain FI-1045 on PDA in relation to temperature. Values are means of three independent assays.

### 5.3.3. Effect of pH on radial growth of *Metarhizium* strains

Under the conditions of this study, growth was observed for the mutant strains NM10, NM4, NM18, NM23 and NM42 on PDA plates from pH 2 to pH 12 at 28 °C. The growth of each

mutant strain was recorded from pH 4 to pH 10 whereas the growth range for the parental strain was from pH 4 to 8 (Figure 2). No growth was observed at pH 10 and pH 12 for the parental strain whereas all tested mutant strains showed some growth at high pH. Mutant strain NM10 showed the highest radial growth from pH 4 to pH 12 at 28 °C and this strain was selected for the bioassay to establish its virulence against greyback canegrubs.

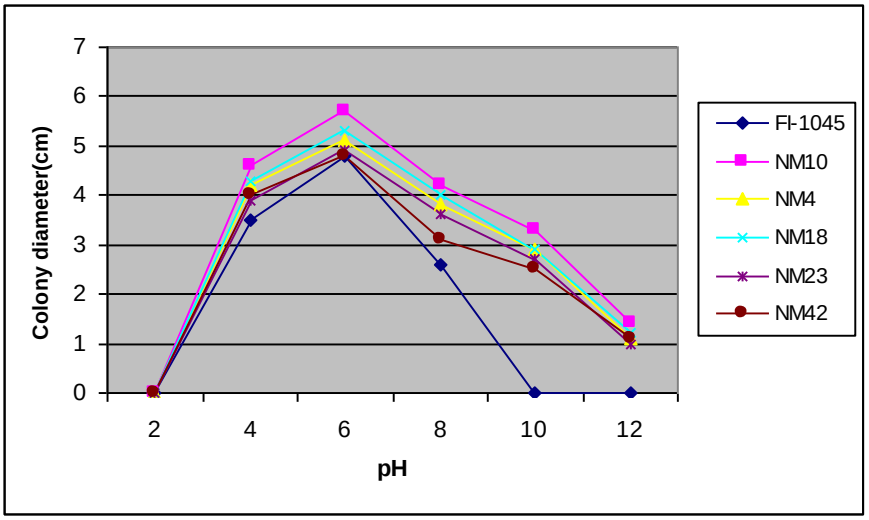


**Figure 2: Effect of pH on radial growth of *Metarhizium* strains**

Radial growth (measured in centimeters) of *M. anisopliae* mutants NM10, NM4, NM18, NM23 and NM42 and the parental strain FI-1045 on PDA in relation to pH. Values are means of three independent assays.

Soil pH is acknowledged as one of the many factors that can influence the persistence and efficacy of entomopathogenic fungi (Sharma et al. 1992). Of the effect of several of the abiotic factors on entomopathogenic fungi, the role of pH or ionic conductivity is least understood (Inglis et al. 2001). In this study, pH tolerance was recorded by measuring the radial growth of isolates in an acidic as well as alkaline pH. All mutants grew from pH 4 to 12 whereas the parental strain grew from pH 4 to 8. All strains seemed to peak at pH 6. Mutant strain NM10 showed the maximum growth rate at all pH range tested.

The broad pH range used in this study was chosen on the following two factors. Firstly, greyback canegrubs are reported to be common in a range of soils with varying pH ranges (Ward, 2003). Secondly, research into the management of canegrubs with the current product did not yield consistent results with differing soil pH especially above pH 6.2 (Milner et al. 2003; Rath et al. 1995). These authors also noted that fungistasis against *Metarhizium* in some soils was caused solely by pH of the soil. Work conducted by St. Leger et al. (1998) has shown that *Metarhizium* infection process was influenced by pH in the micro environmental niche on the host cuticle. The various proteins produced from the mutant strain (NM10) during infection of greyback canegrubs and their isolated cuticles were separated on 2DE gels over a pH range of 4-7 (detailed description in Chapter 7). Therefore selection of fungal isolates tolerant to a wide pH range is useful in mycopesticide formulations.

### 5.3.4. Infection of canegrubs

*M. anisopliae* mutant strain NM10 and the parental strain FI-1045 were capable of infection of greyback canegrubs (*Dermolepida albohirtum*) in laboratory conditions (Section 5.2.5). Fifty percent of infected canegrubs with mutant strain NM10 were dead on day seven and 50 % of infected canegrubs with parental strain FI-1045 were dead on day twelve. Finally, infection of canegrubs with the mutant strain (NM10) resulted in 100 % mortality of canegrubs on day eleven whereas the parental strain FI-1045 took sixteen days for 100 % mortality of canegrubs. A canegrub was deemed dead when it was totally static. Bioassay results showed that the mutant strain NM10 imposed a rather rapid action (68.75 % faster) on the greyback canegrubs than the parental strain (*M. anisopliae* FI-1045) currently used in the BioCane™ product.

In this study, it is evident from graphs that growth differences between the mutants were not great compared to the wild type (FI-1045) they were great. Therefore, we conclude that the

*Metarhizium* UV mutant NM10 isolated in this work demonstrated enhanced enzyme secretion on solid medium, improved tolerance to varying temperature and pH conditions and instigated faster infection in greyback canegrubs under laboratory conditions. The secretion of hydrolytic enzymes by *Metarhizium* mutant NM10 was investigated in Chapter 6/ Manuscript IV. These results suggest that NM10 has good potential for the management of greyback canegrubs in the field. The NM10 mutant strain will be handed over to BSES Limited for further testing in greenhouse as well as small plot trails. The data showing increasing temperature and pH tolerance for each of the mutant strains compared to the wild type open avenues for further research. Future work can focus on analysing the secretion ability of *Metarhizium* mutant strains at different pH and temperature ranges in response to canegrub cuticles and grub homogenate.

## 5.4. References

Champlin FR, Cheung PYK, Pekrul S, Smith RJ, Burton RL, Grula EA (1981) Virulence of *Beauveria bassiana* mutants for the pecan weevil. J Econ Entomol 74: 617-621.

Hall RA, Papierok B (1982) Fungi as biological control agents of arthropods of agricultural and medical importance. Parasitol 8: 205-240.

Heale JB, Isaac JE, Chandler D (2006) Prospects for strain improvement in entomopathogenic fungi. Pestic Sci 26: 79-92.

Hywel-Jones NL, Gillespie AT (1990) Effect of temperature on spore germination in *Metarhizium anisopliae* and *Beauveria bassiana*. Mycol Res 94: 389-392.

Inglis DG, Goettel SM, Butt MT, Strasser H (2001) Use of hypomycetous fungi for managing insect pests. *In Fungi as biocontrol agents, progress, problems and potential*. eds. Butt, T.M., Jackson, C. & Magan, N. pp. 23-69.

Lacey LA, Goettel MS (1995) Current developments in microbial control of insect pests and prospects for the early 21st century. Entomophaga 40: 1- 25.

Milner RJ, Samson P, Morton R (2003) Persistence of conidia of *Metarhizium anisopliae* in sugarcane fields: effect of isolate and formulation on persistence over 3.5 years. Biocontrol Sci Technol 13: 507-516.

Morley-Davies J, Moore D, Prior CJ (1995) Screening of *Metarhizium* and *Beauveria spp*. conidia with exposure to simulated sunlight and a range of temperatures. Mycol Res 100: 31-38.

Nevalainen KMH, Palva ET, Bailey MJ (1980) A high cellulase producing mutant strain of *Trichoderma reesei*. Enzyme and Microbial Technol 2: 59-60.

Prior C, Carey M, Abraham YJ, Moore D, Bateman RP (1995) Development of a bioassay method for the selection of entomopathogenic fungi virulent to the desert locust *Schistocerca gregaria* (Forskal). J Appl Entomol 119: 567 – 572.

Rath AC, Guy PL, Webb WR (1995) *Metarhizium* spore surface antigens are correlated with pathogenicity. Mycol Res 100: 57-62.

Samson PR, Staier TN, Bull JI (2006) Evaluation of an application procedure for *Metarhizium anisopliae* in sugarcane ratoons for control of the white grub *Dermolepida albohirtum*. Crop Protection 25: 741-747.

Samuels KDZ, Pinnock DE, Bull RM (1990) Scarabeid larvae control in sugarcane using *Metarhizium anisopliae*. J Invertebr Pathol 55: 135-137.

Sharma S, Agarwal GP, Rajak RC (1992) Effect of temperature, pH and light on toxin production by *Beauveris bassiana* (Bal) *Vuill*. Indian J Exp Biol 30: 918-919.

St. Leger RJ, Charnley AK, Cooper RM (1986) Cuticle-degrading enzymes of entomopathogenic fungi: synthesis in culture on cuticle. J Inveterbr Pathol 48: 85-95.

St. Leger RJ, Butt TM, Staples RC, Roberts DW (1989) Production in vitro of appressoria by the entomopathogenic fungus *Metarhizium anisopliae*. Exp Mycol 13: 274–288.

St. Leger RJ (1993) Biology and mechanisms of insect cuticle invasion by deuteromycete fungal pathogens. In Parasites and Pathogens of Insects. Edited by N. C. Beckage, S. N. Thompson & B. A. Federici. San Diego, CA: Academic Press. pp. 211–229.

St. Leger RJ, Joshi L, Roberts DW (1998) Ambient pH is a major determinant in the expression of cuticle degrading enzymes and hydrophobin by *Metarhizium anisopliae*. App Environ Microbiol 64: 709–713.

Thomas MB, Jenkins NE (1997) Effects of temperature on growth of *M. flavoviride* and virulence to the variegated grasshopper, *Zonocerus variegates*. Mycol Res 101: 1469-1474.

Ward AL (2003) Does soil texture influence the distribution of the greyback canegrub, *Dermolepida albohirtum* (Waterhouse) (Coleoptera: Scarabaeidae), in the Burdekin river sugarcane growing area? Aust J Agricul Res 54: 861-868.

# 6. Proteomic analysis of the secretome of *Metarhizium anisopliae* in response to exposure to the greyback canegrubs and grub cuticles – Manuscript IV

This section gives an insight into the extracellular proteins secreted by *M. anisopliae* stain NM10, in response to the exposure to greyback canegrubs and their isolated cuticles. The *Metarhizium* strain used is a UV mutant strain produced in this work as described in Chapter 5 and the conducted laboratory bioassays showed that the strain was highly virulent against greyback canegrubs. With the proteomic study, we were able to identify various proteolytic enzymes produced during infection process, amongst them six extracellular proteins that have not been previously reported to be produced during infection of various insect hosts by *Metarhizium*. In this chapter, figures have been included in the body of the text for easy reading and tables are presented at the end of the chapter.

The manuscript has been submitted to Current Genetics in September 2009 and is currently under editorial review. The paper is presented in the format required for publication in Current Genetics.

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# Proteomic analysis of the secretome of a highly virulent *Metarhizium anisopliae* mutant strain in response to exposure to the greyback canegrubs and grub cuticles

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**Total number of tables – 1**

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## 6.1. Abstract

*Metarhizium anisopliae* is a well known bio-control agent of several insect pests including canegrubs. Hydrolytic enzymes produced by the fungus are key factors for cuticle degradation and initiation of the infection process. To identify various proteins involved in the infection of canegrubs, two-dimensional electrophoresis was employed to display proteins secreted by *M. anisopliae* strain NM10 growing on the whole greyback canegrubs and their isolated cuticles. A total of 86 protein spots were analysed using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. All 86 protein spots were identified by cross species identification from the kingdom fungi, thus achieving a 100% identification rate. Among the identified proteins were 56-kDa aspartyl aminopeptidase, 29-kDa secreted aspartyl protease, cyclin-dependent protein kinase, thymidylate kinase, septin and adenylate kinase. These proteins have not been previously identified from the culture supernatant of *M. anisopliae* during infection.

Keywords: *canegrubs, Metarhizium, proteins, hydrolytic enzymes, electrophoresis*

## 6.2. Introduction

Greyback canegrub, *Dermolepida albohirtum* (Waterhouse) (Coleoptera: Scarabaeidae) is a serious pest of sugarcane in northern Queensland, Australia (Robertson et al. 1997). Damage results from the larvae feeding on the roots of the sugarcane plant leading to retarded growth and reduced sugar yields. The entomopathogenic fungus *Metarhizium anisopliae* grows naturally in soils and has been described as one of the promising species for the development of a biological control agent against canegrubs (Milner, 2000). Consequently, a *Metarhizium* based product has been registered for the control of canegrubs (Milner et al. 2002).

The first physical barrier to infection is the insect cuticle which is composed of various cuticular proteins and chitin. Entomopathogenic fungi break this barrier by producing a wide array of degradative enzymes such as proteases and chitinases. One of the best known fungal pathogenicity determinants is subtilisin-like serine protease (Pr1) of *M. anisopliae* (St. Leger et al. 1986a). This enzyme is adapted to extensively degrade cuticular proteins (St. Leger et al. 1996; Freimoser et al. 2003) and has been ultrastrucutrally localised in the host cuticle during initial stages of infection (St. Leger et al. 1996). A trypsin-like serine protease (Pr2) also appears during the early stages of colonisation, suggesting that it has a role in cuticle degradation complementary to that of Pr1 (St. Leger et al. 1987). Inhibition of protease activity or using *Metarhizium* mutants deficient in protease production (Bidochka and Khachatourians, 1990) resulted in decreased virulence against insects. *M. anisopliae* also produces chitinolytic enzymes that act after the proteases have digested the cuticle protein components to expose the chitin fibrils present in the cuticle. In fungi, chitinases have a physiological role in hyphal growth and morphogenesis (St. Leger et al. 1991 and 1987) and the role of the chitinases in the host infection process is not yet completely understood.

*M. anisopliae* synthesises and secretes various extracellular proteases into its growth medium when an exogenous protein such as an insect cuticle serves as source of nitrogen and carbon (St. Leger et al. 1987). The insect cuticle contains several hundreds of proteins (St. Leger et al. 1996; Freimoser et al. 2003; Andersen et al. 1995) and it has been established that there are differences in the suspectibility of these proteins to fungal proteases (Shah and Pell, 2003). In this study, we have examined the various proteins secreted by a highly virulent *M. anisopliae* mutant strain NM10 developed in our laboratory (Manalil et al. unpublished data) in the presence of greyback canegrubs and isolated canegrub cuticles, with a view of finding out if the efficiency of canegrub infection by this strain corresponds to the amount and type of secreted enzymes and finding new enzymatic tools to use for greyback canegrub control.

## 6.3. Materials and methods

### 6.3.1. Insect larvae

Third instar greyback canegrubs, *Dermolepida albohirtum* (Waterhouse) were handpicked from the sugarcane fields around Townsville region [Queensland, Australia (http://www.ga.gov.au/bin/gazd01?rec=155794)] and transported to Sydney. Upon arrival, the cane grubs were instantly immobilized using a cotton ball dipped in chloroform (Ajax Finechem, Sydney, Australia) to anaesthetise the grubs and then surface sterilized in 5 % (vol/vol) sodium hypochlorite solution (Prior et al. 1995) and 75 % (vol/vol) ethanol solution and then rinsed with plenty of sterile distilled water. Cane grubs were then transferred into 100 ml sterile transparent plastic tubs (Technoplas, Australia) filled with 30 g of sterilised garden peat (Killarney peat moss, Australia). Garden peat was sterilised by heating and drying (150 °C, 4 h), then mixed with distilled water. The cane grubs were fed with fresh pieces of carrot and held at room temperature (RT) for seven days until the grubs were used for experiment because only healthy canegrubs

were selected for further experiments. Ten healthy canegrubs were immobilized by cotton dipped in chloroform (Ajax Finechem, Sydney) to depress the nervous system. Canegrubs were then rinsed in sterile distilled water to remove peat particles attached to the body and transferred into sterile 15 ml tubes, freeze dried and pulverised using a mortar and pestle. Cuticles were isolated by extracting the soft tissue from homogenised insects with potassium tetra borate (St. Leger et al. 1986a). Clean cuticle samples from the greyback canegrubs were prepared as described previously (St. Leger et al. 1987).

### 6.3.2. Fungal strain and cultivation conditions

*Metarhizium anisopliae* (NM10) a UV mutant strain from parental strain (FI-1045) showing increased virulence against greyback canegrubs was conidiated and maintained on potato dextrose agar plates (Difco Laboratories, MI, USA). Conidia were collected in 5 ml of 0.9% (w/v) sodium chloride, 0.01% (v/v) Tween 80 and 5 x $10^8$ conidia were used to inoculate 50 ml of minimal medium (St. Leger et al. 1986b) supplemented with 2% (w/v) glucose in a 250-ml Erlenmeyer flask. Cultures were grown at 28 °C on a shaker at 250 rpm for 48 h (preculture). The mycelia from each flask were collected, washed three times with 50 ml of Milli-Q water by invertion and centrifugation at 4,000 *g* for 10 min at 15 °C and re-inoculated in fresh minimal medium with 1% (w/v) pulverised greyback canegrub cuticles (Culture-A) or 1% (w/v) pulverised whole greyback canegrubs (Culture-B). Control flasks contained only fresh MM with 1% (w/v) pulverised greyback cane grub cuticles or fresh MM with 1% (w/v) pulverised whole greyback cane grubs. Cultures were grown for 48 h on a shaker at 250 rpm at 28 °C. Culture supernatants and mycelia were harvested at the 48 h time point. Fungal and yeast protease inhibitor cocktail (0.05% v/v) was added to the culture supernatant and allowed to incubate at RT for 20 min. Culture supernatants containing protease inhibitors were collected by centrifugation

at 4,000 *g* for 10 min at 15 °C and stored at – 20 °C until required. The mycelia were washed three times with 50 ml of Milli-Q water and stored at – 20 °C until use.

### 6.3.3. Precipitation of proteins from culture supernatant

The *M. anisopliae* (NM10) culture supernatant was thawed and 13 ml were taken and spun at 21,000 *g* for 15 min at 10 °C. Ammonium sulphate was added to the supernatant to give an 80% saturated solution which was stirred overnight at 4 °C to allow protein precipitation. The precipitated proteins were pelleted by centrifugation at 21,000 *g* for 15 min at 4 °C. Precipitated proteins were resuspended in 1ml of re-suspension solution {7 M urea, 2 M thiourea, 4% (w/v) CHAPS (3-[(3- cholamidopropyl) dimethylammonio]-1-propanesulfonate)(detergent C-3023: Sigma), 40 mM Tris, 5 mM tributylphosphine, 10 mM acrylamide, 1 mM PMSF (phenylmethylsulphonyl fluoride), 0.1% (w/v) protease inhibitor tablet (Roche)} and incubated at RT for 90 min to allow complete reduction and alkylation of proteins. The reduction and alkylation reactions were quenched with 10 mM dithiothreitol before insoluble material was removed by spinning at 21,000 *g* for 10 min. The solution was desalted (buffer exchanged) further by ultra-filtration with 7 M urea, 2 M thiourea, 4% (w/v) CHAPS in Ultrafree™ 5 kDa cut-off centrifugal concentrators (Millipore, USA) at 2,000 *g* at 20 °C until the volume reached 500 µl. Protein samples were then passed through the 2D Clean-Up Kit from GE Healthcare (formerly Amersham Biosciences, USA) to remove further impurities. The conductivity of the sample was measured with a Twin Cond conductivity meter (Horiba, Japan). If the conductivity was higher than 300 µS/cm then the desalting step was repeated.

### 6.3.4. Protein assay

Proteins from the culture supernatants were assayed using the Bradford's reagent following the manufactures instructions. Absorbances were read on a Versamax microplate reader (Molecular devices, USA) and analysed using the softmax PRO ver 3.1.2. Software.

### 6.3.5. Isoelectric focusing (IEF) and 2D-SDS-PAGE

The samples were used directly to passively rehydrate pH 4-7, 11 cm Immobilized pH gradient (IPG) strips (Amersham Pharmacia, Uppsala, Sweden) by applying 180 µl of each sample (equivalent to 230 µg of protein). IPG's were focused to a total of 80,000 Volt hours (Vh) using a three-step focusing program. The focusing program included a rapid ramp to 300 V for 4 h, a linear ramp to 10,000 V over 8 h, and a 10,000 V step until 80,000 Vh were reached. IPGs were equilibrated for 20 min in 6 M urea, 2 % (w/v) SDS, 50 mM Tris–HCl, pH 8.8, 0.1% (w/v) bromophenol blue. The IPGs were then placed on top of Proteome Systems 6–15% (w/v) Gel chips (Proteome Systems, Australia) and ran at 30 mA constant until the bromophenol blue dye reached the bottom of the gel. Gels were fixed in 10% (v/v) methanol, 7% (v/v) acetic acid solution for 30 min, then stained with Sypro Ruby solution (Molecular Probes) for 16 h. Gels were destained in the fixing solution before scanned on a Fluorescence scanner (Alpha Innotech Corporation, California). Gels were restained for 16 h with Coomassie Colloidal Blue G250 (17% (w/v) ammonium sulphate, 34% (v/v) methanol, 3.6% (v/v) orthophosphoric acid, 0.1% (w/v) Coomassie G-250) and destained with 1% (v/v) acetic acid for further analysis as required. Each sample was run in triplicate.

### 6.3.6. Mass spectrometry and identifications

Protein spots were excised using the XciseTM apparatus (Shimadzu Biotech, Japan). Gel pieces were destained and dried. Trypsin was added to each gel piece and they were incubated at 37 °C

for 16 h for protein digestion. Each peptide solution was desalted and concentrated using ZipTips$^{TM}$ from Millipore (USA) and spotted onto the target plate with 1.0 µl matrix solution (4 mg ml$^{-1}$ alpha-cyano-4-hydroxy-cinnamic acid in 70% (v/v) acetonitrile, 1% (v/v) trifluoroacetic acid). Peptide mass fingerprints of tryptic peptides were generated by matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) using an Applied Biosystems 4700 Proteomics Analyser with TOF/TOF optics in the MS mode. A Nd:YAG laser (355 nm) was used to irradiate the sample. The spectra were acquired in reflectron mode in the mass range of 750 to 3,500 Da. The instrument was then switched to MS/MS (TOF/TOF) mode where the eight strongest peptides from the MS scan were fragmented by collision-induced dissociation. A near point external calibration was applied to give a mass accuracy within 50 ppm. Mass spectrometry data was searched against proteins from all fungal species using Mascot Peptide Mass Fingerprint where a modified MOWSE scoring algorithm was used to rank results (http://www.matrixscience.com/help/scoring_help.html).

## 6.4. Results

Extracellular proteins from *M. anisopliae* (NM10) grown on canegrub cuticles (Sample-A; Fig.1) and proteins from the fungus grown on whole canegrubs (Sample-B; Fig. 2) were separated on 2D gels over pH range 4-7. Figure 1 displays proteins ranging from 120-kDa to 10-kDa across the pH range 4-7 (from Sample-A) and Figure 2 displays proteins ranging from 80-kDa to 10-kDa across the pH range 4-7(from Sample-B) after 48 h cultivation. Forty nine protein spots from Sample-A and 37 spots from Sample-B were excised and processed for mass spectrometry analysis and protein identification. The use of cross-species identification (CSI) enhanced number of proteins that could be matched to data from proteins from all fungal species or selected closely related species. Identified proteins are highlighted and numbered on the 2D

protein maps (Figs. 1 and 2) and additional information about these proteins can be found in Table 1. All the proteins cut for MALDI analysis were successfully identified, giving a 100% identification rate.

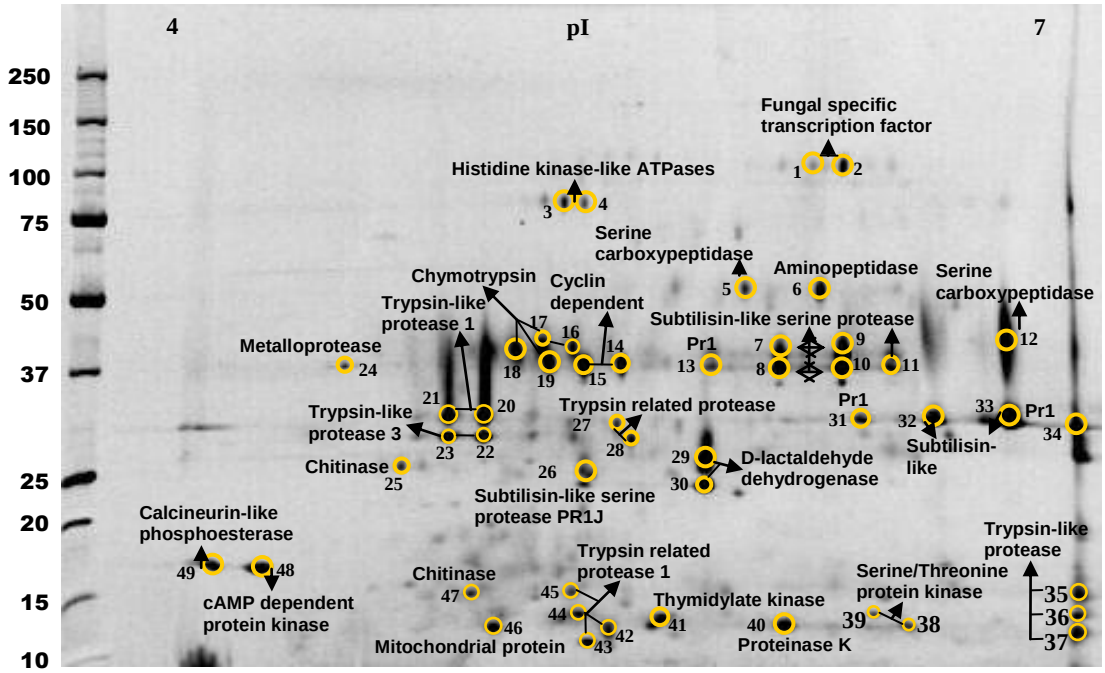


**Fig. 1: Proteins from the culture supernatant of *M. anisopliae* (NM10) when grown on greyback canegrubs cuticles (Sample-A)**

Circled spots contain protein identifications by mass spectrometry. The gels were run on 11 cm, 4-7 IPG strips in the first dimension and 8-16% SDS-PAGE in the second dimension.

Among the identified proteins from Sample-A (Fig. 1), eleven protein spots corresponded to various isoforms of cuticle degrading subtilisin-like serine protease (Pr1), thirteen protein spots corresponded to different isoforms of trypsin-related protease (Pr2) and nine protein spots corresponded to a variety of protein kinases. Some of the other proteins identified from Sample A were fungal specific transcription factor (two spots), chitinase (two spots), chymotrypsin (four spots), aminopeptidase (one spot), metalloprotease (one spot), serine carboxypeptidase (two

spots), D-lactaldehyde dehydrogenase (two spots), novel yeast mitochondrial protein (one spot) and calcineurin-like phosphoesterase (one spot).

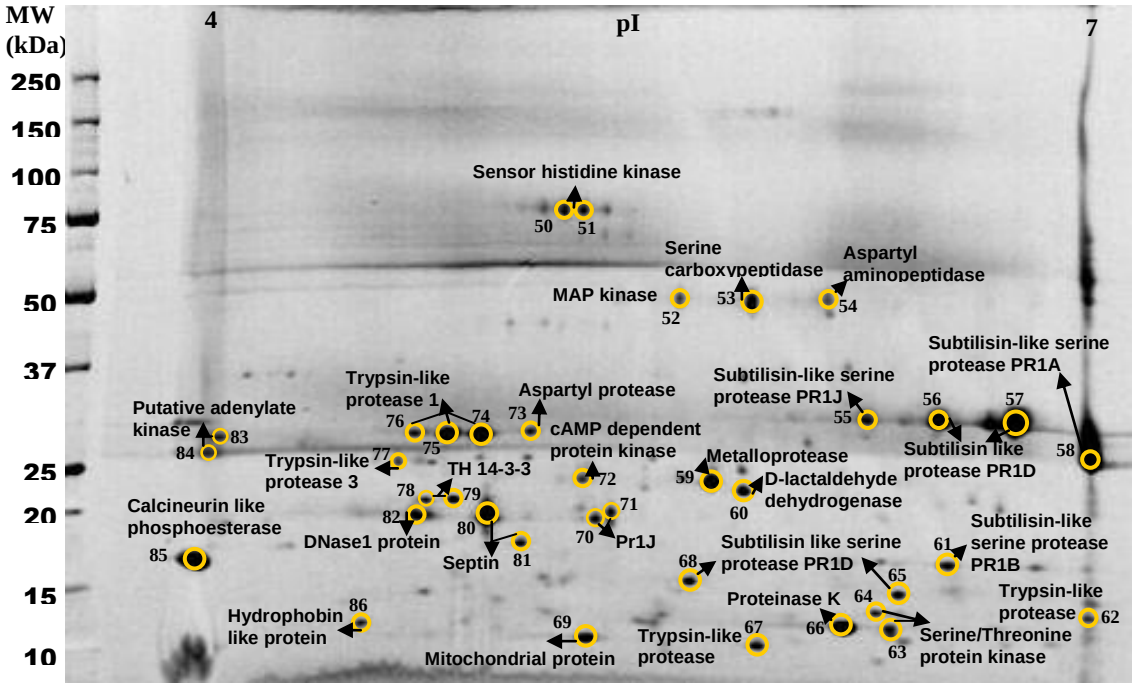


**Fig. 2: Proteins from the culture supernatant of *M. anisopliae* (NM10) when grown on greyback canegrubs (Sample-B)**

Circled spots contain protein identifications by mass spectrometry. The gels were run on 11 cm, 4-7 IPG strips in the first dimension and 8-16% SDS-PAGE in the second dimension.

Amongst the proteins identified from Sample-B (Fig. 2) nine corresponded to various isoforms of cuticle degrading subtilisin-like serine protease (Pr1), six protein spots corresponded to different isoforms of trypsin-related protease (Pr2) and nine protein spots corresponded to a variety of protein kinases. Other proteins identified were serine carboxypeptidase (one spot), aspartyl aminopeptidase (one spot), metalloprotease (one spot), D-lactaldehyde dehydrogenase (one spot), secreted aspartyl protease (one spot), TH14-3-3 proteins (two spots), septin (two spots), DNase1 protein (one spot), calcineurin-like phosphoesterase (one spot) and hydrophobin-like protein (one

spot), as shown in Figure 2. Secretion of Pr1, Pr2 and other hydrolytic enzymes indicated a strong cellular response to the presence of whole pulverized canegrubs.

## 6.5. Discussion

Fungal pathogenesis is a complex phenomenon and involves the interplay of many virulence factors at various stages of infection. Similarly to most fungal pathogens, *Metarhizium* produces extracellular enzymes for the degradation of the host cuticle during infection, assisting penetration and providing nutrients for further growth (St. Leger et al. 1987). Here we have profiled the extracellular enzymes produced by a *M. anisopliae* mutant strain with an improved ability of killing canegrubs in laboratory bioassays.

Several changes in enzyme profiles have been described during growth of *Metarhizium* on different insect cuticles (St. Leger et al. 1986b; Paterson et al. 1994) and on whole grubs (Chandler and Davidson, 2005; Kabaluk et al. 2005; Krueger et al. 1992). In the current study, a wide array of proteins (49 proteins) was expressed when *M. anisopliae* was grown on the insect cuticle, whereas fewer proteins (37 propteins) were expressed when the fungus was grown on whole grubs. A number of studies have indicated enhanced enzyme production on various insect cuticles (St. Leger et al. 1988; 1994a; 1995; Paterson et al. 1994) beause proteins are the main component of insect cuticle (Andersen et al. 1995) and thus the most important barrier to fungal infection. Low protein secretion was reported in a number of studies when whole grubs were subjected to infection because the whole grub contains only 25 % protein content from the cuticle and 75% body fat (Krieger de Moraes et al. 2003; St. Leger et al. 1986a).

**Proteolytic enzymes:** *M. anisopliae* secretes a great variety of proteases such as exopeptidases and endopeptidases to digest the peptide bonds in cuticular proteins (St. Leger et al. 1994; Shah and Pell, 2003), some of which have been associated with virulence.

**Endopeptidases**: Endoproteases/endopeptidases identified in this study include serine proteases, trypsin proteases, aspartic proteases, chymotrypsin and metalloproteases (Figs. 1 and 2, Table 1). *M. anisopliae* (NM10) produced four different isoforms of subtilisin-like serine protease (Pr1 J, E, B and A) when grown on canegrub cuticles and four different isoforms of Pr1 (Pr1 J, D, B and A) when grown on ground whole canegrubs. In total eleven protein spots corresponding to Pr1 were seen in Figure 1 and nine protein spots corresponding to Pr1 protein seen in Figure 2. Pr1 is the best understood protein involved in entomopathogenicity and to date, eleven subtilisins have been identified in *M. anisopliae* during growth on insect cuticles (Freimoser et al. 2003; Bagga et al. 2004). The Pr1 isoforms in Figure 1 and 2 varied in their isoelectric point as well as in their molecular weight. A similar phenomenon has been described by St. Leger et al. (1994a) who also showed that electrostatic binding of Pr1 was a prerequisite for cuticle hydrolysis.

We identified twelve trypsin-related proteases (Pr2) and four chymotrypsins when the fungus was grown on canegrub cuticles (Fig. 1). Six Pr2 proteins were detected when the fungus was grown on whole canegrubs (Fig. 2). Similar observations have been reported by St. Leger et al. (1996) where thirteen Pr2 proteins were synthesized by *M. anisopliae* in response to the exposure to cockroach cuticles. Studies from a number of laboratories have highlighted that Pr2 proteins do not have the ability to degrade an intact cuticle but can assist in proteolytic degradation of the cuticular barrier (St. Leger et al. 1996; Freimoser et al. 2003; Paterson et al. 1994). Chymotrypsins secreted from *M. anisopliae*, identified in this study (Fig. 1, protein spots 16, 17,

18 and 19) function by scavenging nutrients and penetrate host barriers (Screen and St. Leger, 2000; Mohanty et al. 2008). Secretion of metalloproteases was observed when *Metarhizium* was grown on canegrub cuticles (Fig. 1; protein spot 24) and whole canegrubs (Fig. 2; protein spot 59). During *Metarhizium* infection metalloproteases act in concert with several serine proteases to bring about the destruction of the insect cuticle; they may also serve as a back-up for Pr1 activity (St. Leger et al. 1995). Thus all four types of proteases (Pr1, Pr2, chymotrypsin and metalloprotease) seem to mediate a cascade of reactions facilitating the fungal penetration of host cuticles.

A 29-kDa secreted aspartyl protease (Fig. 2; protein spot 73) identified from *Candida dubliniensis* was detected when *Metarhizium* was grown on pulverized canegrubs. Secreted aspartyl proteins (SAP) execute a number of specialized functions during the infection process, which include the simple role of digesting molecules for nutrient acquisition, digesting or distorting host cell membranes to facilitate adhesion and tissue invasion, and digesting cells and molecules of the host immune system to avoid or resist antimicrobial attack by the host (Pereira et al. 2007). Secretion of a 45-kDa SAP protein has been previously reported in *M. anisopliae* (St. Leger et al. 1998). However, secretion of a 29-kDa SAP protein, identified in this study has not been previously reported in *M. anisopliae* and therefore we speculate that it is a novel *Metarhizium* protein. In this study, the presence of a hydrophobin-like protein (Fig. 2: protein spot 86) indicated that this protein plays a vital role in fungal pathogenesis. Fungal hydrophobin proteins provide another example besides proteases which are differentially regulated during early infection (St. Leger et al. 1996). Attachment of conidia to insect cuticle is mediated by conidial surface proteins called hydrophobins (Bidochka et al. 2001).

**Exopeptidases:** Exoproteases/exopeptidases such as serine carboxypeptidases and aminopeptidases were observed in the secreted proteome of *M. anisopliae* (NM10) (Fig. 1 and 2; Table 1). During infection process, *Metarhizium* secretes exopeptidases to degrade the peptides released by endopeptidases (such as Pr1 and Pr2) producing free amino acids for uptake and provide nutrition for the fungus (St. Leger et al. 1994b). In this study, protein spot 5 (Fig. 1) and protein spot 53 (Fig. 2) both corresponded to a 64-kDa serine carboxypeptidase, identified from a pathogenic plant fungus *Ustilago maydis*. Secretion of a high molecular weight serine carboxypeptidase has not been previously reported in *M. anisopliae* during the infection process. However, this enzyme was first detected in the secretome of the parental strain (FI-1045) in response to the greyback canegrub and their isolated cuticles (Manalil et al. unpublished data). In this study, a 56-kDa aspartyl aminopeptidase which is a zinc binding metalloprotease, identified from *Aspergillus fischerianus*, was observed when *Metarhizium* was grown on pulverized whole canegrubs (Fig. 2; protein spot 54). Secretion of this protein has not been previously reported in *M. anisopliae* and therefore the 56-kDa aspartyl aminopeptidase detected in this study seems to be a novel *Metarhizium* protein.

**Chitinases:** Two chitinase proteins were identified in the secretome of the mutant strain NM10 when grown on ground canegrub cuticles (Fig. 1; protein spots 25 and 47). Unlike proteases, chitinases are not detected in the early phases of host insect infection (Kang et al. 1999), most probably because the cuticular proteins have to be first degraded by proteases before the chitin is exposed for chitinases to act upon. Action of a chitinase on a procuticle has been found to occur 40 h post-inoculation which suggests that release of the chitinase is reliant on the accessibility of the substrate (St. Leger et al. 1996).

**Signal transduction proteins:** Investigation of signal transduction protein cascades that regulate fungal development and virulence have been reported in various plant, animal and human pathogenic fungi (Lengeler et al. 2000). Signaling proteins identified in this study include mitogen-activated protein (MAP) kinase (Fig. 2: protein spot 52) signaling for cell integrity, cell wall construction, mating and osmo-regulation; cyclic AMP-dependent protein kinase (protein spot 48 in Fig. 1; protein spot 72 in Fig. 2) and serine/threonine protein kinases (protein spots 38, 39 in Fig. 1; protein spots 63, 64 in Fig. 2). These proteins function as regulatory components which sense and transfer stress signals from the host environment to the cell machinery that controls virulence and pathogenicity factors, thus establishing the infection process (Engebrecht, 2003). Histidine kinase-like ATPases (Fig. 1; protein spots 3, 4) and sensor histidine kinase (Fig. 2; protein spots 50, 51) play a central role in transmitting a variety of cellular responses including sporulation, osmoregulation and microbial pathogenesis (Wang and St. Leger, 2007; Bilwes et al. 1999). In fungi, calcineurin-like phosphoesterase (Fig. 1; protein spot 49; Fig. 2; protein spot 85) plays an important role in hyphal elongation and activation of calcineurin during stress conditions such as infection (Engebrecht, 2003). Cyclin-dependent protein kinase (Fig. 1; protein spots 14, 15) functions to help integrate environmental signals with developmental decisions to allow ordered differentiation of specific cell types under varying growth conditions (Bussink and Osmani, 1998). Thymidylate kinase (Fig. 1; protein spot 41) is involved in nucleotide metabolism and cell cycle regulation in fungi (Jong et al. 1994). Septin proteins (Fig. 2; protein spots 80, 81) are GTPases that form filaments during cytokinesis and cell division in fungi. Septins are also involved in cell morphogenesis during filamentous growth, chitin deposition, cell cycle regulation, cell compartmentalization, membrane trafficking, spore wall formation and organisation of the cytoskeleton (Field and Kellogg, 1999). In fungi, putative adenylate kinase (Fig. 2; protein spots 83, 84) is a critical player in metabolic monitoring and systemic integration

of different signaling pathways to ensure cellular energy homeostasis and tolerable response to a broad range of functional, environmental and stress challenges (Dzeja et al. 1998). Mitochondrial protein (Fig. 1; protein spot 46; Fig. 2; protein spot 69) mediates protein import and/or protein folding in the mitochondrial matrix (Herrmann and Neupert, 2000). Adenylate kinase (AK) (Fig. 2; protein spots 84, 83) is an important enzyme in the regulation of the cellular energy balance in the signaling cascade. Cyclin-dependent protein kinase, thymidylate kinase, septin and adenylate kinase proteins play a critical role in signal transduction cascades that regulate fungal development and virulence. These proteins have not previously been identified from *M. anisopliae*.

Identification of proteins involved in the signaling and modulation of various cellular processes provides further support that exposure of *M. anisopliae* (NM10) to greyback canegrubs and their isolated cuticles has successfully triggered a biocontrol-like response leading to utilisation of the available nutrients. In this work, we have identified six extracellular enzymes (56-kDa aspartyl aminopeptidase, 29-kDa secreted aspartyl protease, cyclin-dependent protein kinase, thymidylate kinase, septin and adenylate kinase) secreted by *M. ansiopliae* (NM10) that have not been reported previously. The variability of enzymes secreted during the infection of canegrub cuticles and whole canegrubs increases the range of potential tools naturally available to eradicate insect pests. Novel proteins identified in this study may provide novel targets for further development of *M. ansiopliae* as a biocontrol organism against greyback canegrubs. The genes encoding the six novel proteins will be isolated in the future in order to establish their involvement in the biocontrol response of *M. anisopliae*.

## 6.6. References

Andersen SO, Højrup P, Roepstorff P (1995) Insect cuticular proteins. Insect Biochem Mol Biol 25: 153-176.

Bagga S, Hu G, Screen SE, St. Leger RJ (2004) Reconstructing the diversification of subtilisins in the pathogenic fungus *Metarhizium anisopliae*. Gene 324: 159-169.

Bidochka MJ, Khachatourians GG (1990) Identification of *Beauveria bassiana* extracellular protease as a virulence factor in pathogenicity toward the migratory grasshopper, *Melanoplus sanguinipes*. J Invertebr Pathol 56: 362-370.

Bidochka MJ, De Koning J, St Leger RJ (2001) Analysis of a genomic clone of hydrophobin (*ssgA*) from the entomopathogenic fungus *Metarhizium anisopliae*. Mycol Res 105: 360–364.

Bilwes AM, Alex LA, Crane BR, Simon MI (1999) Structure of CheA, a signal-transducing histidine kinase. Cell 96: 131-139.

Brakhage AA (2005) Systemic fungal infections caused by *Aspergillus* species: epidemiology, infection process and virulence determinants. Curr Drug Targets 6: 875–886.

Bussink HJ, Osmani SA (1998) A cyclin-dependent kinase family member (PHOA) is required to link developmental fate to environmental conditions in *Aspergillus nidulans*. EMBO J 17: 3990-4003.

Chandler D, Davidson G (2005) Evaluation of entomopathogenic fungus *Metarhizium anisopliae* against soil-dwelling stages of cabbage maggot (Diptera: Anthomyiidae) in glasshouse and field experiments and effect of fungicides on fungal activity. J Econom Entomol 98: 1856–1862.

Dzeja PP, Zelenznikar RJ, Goldberg ND (1998) Adenylate kinase: kinetic behaviour in intact cells indicates it is integral to multiple cellular processes. Mol Cell Biochem 184: 169–182.

Engebrecht J (2003) Cell signaling in yeast sporulation. Biochem Biophys Res Commum 306: 325-328.

Field CM, Kellogg D (1999) Septins, cytoskeletal polymers or signalling GTPases? Trends Cell Biol 9: 387-394.

Freimoser FM, Screen S, Bagga S, Hu G, St. Leger RJ (2003) Expressed sequence tag (EST) analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts. Microbiol 149: 239–247.

Herrmann JM, Neupert W (2000) Protein import into mitochondria. Curr Opin Microbiol 3: 210–214.

Jong AY, Kuo CL, Campbell JL (1994) The CDC8 gene of yeast encodes thymidylate kinase. J Biol Chem 259: 11052-11059.

Kabaluk T, Goettel M, Erlandson M, Ericsson J, Duke G, Vernon R (2005) *Metarhizium anisopliae* as a biological control for wireworms and a report of some other naturally occurring parasites. IOBC/WPRS Bulletin 28: 109–115.

Kang SC, Park S, Lee DG (1999) Purification and characterization of a novel chitinase from the entomopathogenic fungus, *Metarhizium anisopliae*. J Invertebr Pathol 73: 276-281.

Krieger de Moraes C, Schrank A, Vainstein MH (2003) Regulation of extracellular chitinases and proteases in the entomopathogen and acaricide *Metarhizium anisopliae*. Curr Microbiol 46: 205–210.

Krueger SR, Villani MG, Martins AS, Roberts DW (1992) Efficacy of soil applications of *Metarhizium anisopliae* (Metsch.) Sorokin conidia and standard lyophilized mycelial particles against scarab grubs. J Invertebr Pathol 59: 54–60.

Lengeler KB, Davidson RC, D'Souza C, Harashima T, Shen WC, Wang P, Pan X, Waugh M, Heitman J (2000) Signal transduction cascades regulating fungal development and virulence. Microbial Mol Biol Rev 64: 746-785.

Milner RJ (2000) Current status of *Metarhizium* for insect control in Australia. Biocontrol News Inf 21: 47-50.

Milner RJ, Samson P and Bullard G (2002) A profile of a commercially useful isolate of *Metarhizium anisopliae* var. *anisopliae*. Biocontrol Sci Technol 12: 43–58.

Mohanty SS, Raghavendra K, Dash AP (2008) Induction of chymoelastase (Pr1) of *Metarhizium anisopliae* and its role in causing mortality to mosquito larvae. World J Microbiol Biotechnol 24: 2283–2288.

Paterson IC, Charnley AK, Cooper RM, Clarkson JM (1994) Specific induction of a cuticle-degrading protease of the insect pathogenic fungus *Metarhizium anisopliae*. Microbiol 140: 185-189.

Pereira JL, Noronha EF, Miller RNG, Franco O L (2007) Novel insights in the use of hydrolytic enzymes secreted by fungi with biotechnological potential. Lett App Microbiol 44: 573-81.

Prior C, Carey M, Abraham YJ, Moore D, Bateman RP (1995) Development of a bioassay method for the selection of entomopathogenic fungi virulent to the desert locust *Schistocerca gregaria* (Forskal). J Appl Entomol 119: 567 – 572.

Robertson LN, Kettle CG, Bakker P (1997) Field evaluation of *Metarhizium anisopliae* for control of greyback canegrub (*Dermolepida albohirtum*) in north Queensland sugarcane. Proc Aust Soc Sugar Cane Technol 19: 111–117.

Screen SE, St. Leger RJ (2000) Cloning, expression and substrate specificity of a fungal chymotrypsin. Evidence for lateral gene transfer from an actinomycete bacterium. J Biol Chem 275: 6689–6694.

Shah PA, Pell JK (2003) Entomopathogenic fungi as biological control agents. Appl Microbiol Biotechnol 61: 413-423.

St. Leger RJ, Charnley AK, Cooper RM (1986a) Cuticle-degrading enzymes of entomopathogenic fungi: mechanisms of interaction between pathogen enzymes and insect cuticle. J Invertebr Pathol 47: 295–302.

St. Leger RJ, Charnley AK, Cooper RM (1986b) Cuticle-degrading enzymes of entomopathogenic fungi: synthesis in culture on cuticle. J Invertebr Pathol 48:85–95.

St. Leger RJ, Charnley AK, Cooper RM (1987) Characterization of cuticle-degrading proteases by the entomopathogen *Metarhizium anisopliae*. Arch Biochem Biophys 253: 221–232.

St. Leger RJ, Durrands PK, Cooper RM, Charnley AK (1988) Regulation of production of proteolytic enzymes by the entomopathogenic fungus *Metarhizium anisopliae*. Arch Microbiol 150: 413–416.

St. Leger RJ, Charnley AK, Cooper RM (1991) Kinetics of the digestion of insect cuticles by a protease (Pr1) from *Metarhizium anisopliae.* J Invertebr Pathol 57: 146–147.

St. Leger RJ, Bidochka M, Roberts DW (1994a) Isoforms of the cuticle-degrading Pr1 proteinse and production of metalloproteinase by *M.anisopliae*. Arch Biochem Biophys 313: 1-7.

St. Leger RJ, Bidochka M, Roberts DW (1994b) Characterisation of a novel carboxypeptidase by the entomopathogenic fungus *Metarhizium anisopliae*. Arch Biochem Biophys 314: 392-398.

St. Leger RJ (1995) The role of cuticle-degrading proteases in fungal pathogenesis of insects. Can J Bot 73: 1119-1125.

St. Leger RJ, Joshi L, Bidochka MJ, Roberts DW (1995) Protein synthesis in *Metarhizium anisopliae* growing on host cuticle. Mycol Res 99: 1034-1040.

St. Leger RJ, Joshi L, Bidochka M, Rizzo MJ, Roberts DW (1996) Characterization and ultrastructural localization of chitinases from *Metarhizium anisopliae*, *M. flavoviride* and *Beauveria bassiana* during fungal invasion of host (*Manduca sexta*) cuticle. Appl Environ Microbiol 62:907–912.

St. Leger RJ, Joshi L, Roberts DW (1998) Ambient pH is a major determinant in the expression of cuticle degrading enzymes and hydrophobin by *Metarhizium anisopliae*. Appl Environ Microbiol 64: 709-713.

Wang C, St. Leger RJ (2007) The MAD1 adhesin of *Metarhizium anisopliae* links adhesion with blastospore production and virulence to insects, and the MAD2 adhesin enables attachment to plants. Eukaryotic Cell 6: 808-816.

| **Table 1: Extracellular proteins produced from *M. anisopliae* mutant strain ( NM10)** | | | | | | |
|---|---|---|---|---|---|---|
| **Spot #** | **Protein name** | **Accession #** | **pI** | **Mol wt** | **Coverage or number of peptides matched** | **Species** |
| ***Metarhizium* proteins secreted when grown on canegrub cuticles** | | | | | | |
| 1 | Fungal specific transcription factor | gi\|67537746 | 6.31 | 100048 | 33.00% | *Aspergillus nidulans FGSC A4* |
| 2 | Fungal specific transcription factor | gi\|67537746 | 6.31 | 100048 | 28.00% | *Aspergillus nidulans FGSC A4* |
| 3 | Histidine kinase-like ATPases | gi\|83769600 | 6.52 | 61657 | 26.00% | *Aspergillus oryzae* |
| 4 | Histidine kinase-like ATPases | gi\|83769600 | 6.52 | 61657 | 22.00% | *Aspergillus oryzae* |
| 5 | Serine carboxypeptidase | gi\|71006740 | 5.21 | 64911 | 30.00% | *Ustilago maydis 521* |
| 6 | Aminopeptidase | gi\|50293527 | 6.63 | 56476 | 44.00% | *Candida glabrata* |
| 7 | Subtilisin like serine protease PR1J | gi\|6624952 | 6.04 | 42304 | 4 peptides | *Metarhizium anisopliae var.anisopliae* |
| 8 | Subtilisin like serine protease PR1J | gi\|6624953 | 6.04 | 42304 | 4 peptides | *Metarhizium anisopliae var.anisopliae* |
| 9 | Subtilisin like serine protease PR1J | gi\|6624954 | 6.04 | 42304 | 3 peptides | *Metarhizium anisopliae var.anisopliae* |
| 10 | Subtilisin like serine protease PR1J | gi\|6624955 | 6.04 | 42304 | 5 peptides | *Metarhizium anisopliae var.anisopliae* |
| 11 | Subtilisin like serine protease PR1J | gi\|6624956 | 6.04 | 42304 | 3 peptides | *Metarhizium anisopliae var.anisopliae* |
| 12 | Serine carboxypeptidase | B5LXD6 | 6.63 | 45776 | 2 peptides | *Metarhizium anisopliae* |
| 13 | Subtilisin like serine protease PR1E | Q874T4 | 5.91 | 41917 | 1 peptide | *Metarhizium anisopliae var.anisopliae* |
| 14 | Cyclin-dependent protein kinase | gi\|33772776 | 6.2 | 34394 | 38.00% | *Emericella nidulans* |
| 15 | Cyclin-dependent protein kinase | gi\|33772776 | 6.2 | 34394 | 31.00% | *Emericella nidulans* |
| 16 | Chymotrypsin | Q9Y843 | 5.07 | 38278 | 1 peptide | *Metarhizium anisopliae* |
| 17 | Chymotrypsin | Q9Y843 | 5.07 | 38278 | 1 peptide | *Metarhizium anisopliae* |
| 18 | Chymotrypsin | Q9Y843 | 5.07 | 38278 | 2 peptides | *Metarhizium anisopliae* |
| 19 | Chymotrypsin | Q9Y843 | 5.07 | 38278 | 2 peptides | *Metarhizium anisopliae* |
| 20 | Trypsin-like protease 1 | gi\|556657 | 5.45 | 26100 | 33.00% | *Metarhizium anisopliae* |
| 21 | Trypsin-like protease 1 | gi\|556657 | 5.45 | 26100 | 33.00% | *Metarhizium anisopliae* |
| 22 | Trypsin-like protease 3 | gi\|5042250 | 4.9 | 26184 | 48.00% | *Metarhizium anisopliae* |
| 23 | Trypsin-like protease 3 | gi\|5042250 | 4.9 | 26184 | 37.00% | *Metarhizium anisopliae* |
| 24 | Metalloprotease | A2QPS1 | 4.52 | 35435 | 43.00% | *Metarhizium anisopliae var.anisopliae* |
| 25 | Chitinase | Q6QDR4 | 4.93 | 31762 | 36.00% | *Metarhizium anisopliae* |
| 26 | Subtilisin like serine protease PR1J | gi\|6624956 | 6.04 | 42304 | 3 peptides | *Metarhizium anisopliae var.anisopliae* |
| 27 | Trypsin related protease | gi\|4768909 | 5.68 | 26289 | 48.00% | *Metarhizium anisopliae* |
| 28 | Trypsin related protease | gi\|4768909 | 5.68 | 26289 | 48.00% | *Metarhizium anisopliae* |
| 29 | D-lactaldehyde dehydrogenase | gi\|57227156 | 6.15 | 38160 | 34.00% | *Cryptococcus neoformans var. neoformans* |

| | | | | | | |
|---|---|---|---|---|---|---|
| 30 | D-lactaldehyde dehydrogenase | gi\|57227156 | 6.15 | 38160 | 27.00% | *Cryptococcus neoformans var. neoformans* |
| 31 | Subtilisin like serine protease PR1B | Q9P3Y1 | 6.7 | 39959 | 3 peptides | *Metarhizium anisopliae var.anisopliae* |
| 32 | Subtilisin like protease | gi\|7688246 | 5.81 | 42694 | 40.00% | *Metarhizium anisopliae var.anisopliae* |
| 33 | Subtilisin like protease | gi\|7688246 | 5.81 | 42694 | 38.00% | *Metarhizium anisopliae var.anisopliae* |
| 34 | Subtilisin like serine protease PR1A | gi\|16215677 | 7.14 | 40278 | 1 peptide | *Metarhizium anisopliae var.anisopliae* |
| 35 | Trypsin-like protease | Q6SV39 | 7.1 | 15973 | 32.00% | *Metarhizium anisopliae* |
| 36 | Trypsin-like protease | Q6SV40 | 7.1 | 15973 | 44.00% | *Metarhizium anisopliae* |
| 37 | Trypsin-like protease | Q6SV41 | 7.1 | 15973 | 37.00% | *Metarhizium anisopliae* |
| 38 | Serine/Threonine protein kinases | Q6C2A3 | 6.01 | 27803 | 28.00% | *Yarrowia lipolytica* |
| 39 | Serine/Threonine protein kinases | Q6C2A4 | 6.01 | 27803 | 25.00% | *Yarrowia lipolytica* |
| 40 | Proteinase K [fragment] | gi\|295946 | 6.6 | 40275 | 24.00% | *Tritirachium album* |
| 41 | Thymidylate kinase | P00572 | 5.51 | 24687 | 27.00% | *Saccharomyces cerevisiae* |
| 42 | Trypsin related protease 1 | QO1136 | 5.45 | 26116 | 2 peptides | *Metarhizium anisopliae* |
| 43 | Trypsin related protease 1 | QO1136 | 5.45 | 26116 | 2 peptides | *Metarhizium anisopliae* |
| 44 | Trypsin related protease 1 | QO1136 | 5.45 | 26116 | 3 peptides | *Metarhizium anisopliae* |
| 45 | Trypsin related protease 1 | QO1136 | 5.45 | 26116 | 1 peptide | *Metarhizium anisopliae* |
| 46 | Mitochondrial protein | QO5676 | 5.68 | 13897 | 34.00% | *Saccharomyces cerevisiae* |
| 47 | Chitinase | Q12631 | 4.82 | 13869 | 42.00% | *Metarhizium anisopliae var.anisopliae* |
| 48 | cAMP dependent protein kinase | gi\|71024243 | 5.14 | 26990 | 28.00% | *Ustilago maydis 521* |
| 49 | Calcineurin like phosphoesterase | gi\|68491579 | 5.21 | 35876 | 29.00% | *Candida albicans SC5314* |
| ***Metarhizium* proteins secreted when grown on whole canegrubs** | | | | | | |
| 50 | Sensor histidine kinase | BOYF45 | 5.26 | 88376 | 36.00% | *Aspergillus fumigatus* |
| 51 | Sensor histidine kinase | Q4WMD9 | 5.93 | 78977 | 31.00% | *Aspergillus fumigatus* |
| 52 | MAP kinase | Q9P8P0 | 5 | 59726 | 26.00% | *Kluyveromyces lactis* |
| 53 | Serine carboxypeptidase | gi\|71006740 | 5.21 | 64911 | 30.00% | *Ustilago maydis 521* |
| 54 | Aspartyl aminopeptidase | A1CZR9 | 6.38 | 56165 | 27.00% | *Aspergillus fischerianus* |
| 55 | Subtilisin like serine protease PR1J | gi\|6624956 | 6.04 | 42304 | 3 peptides | *Metarhizium anisopliae var.anisopliae* |
| 56 | Subtilisin like protease PR1D | gi\|7688246 | 5.81 | 421694 | 4 peptides | *Metarhizium anisopliae var.anisopliae* |
| 57 | Subtilisin like protease PR1D | gi\|7688246 | 5.81 | 421694 | 3 peptides | *Metarhizium anisopliae var.anisopliae* |
| 58 | Subtilisin like serine protease PR1A | gi\|16215677 | 7.14 | 40278 | 2 peptides | *Metarhizium anisopliae var.anisopliae* |
| 59 | Metalloprotease | Q9UUR7 | 5.45 | 32046 | 35.00% | *Metarhizium anisopliae var.anisopliae* |
| 60 | D-lactaldehyde dehydrogenase | gi\|57227156 | 6.15 | 38160 | 27.00% | *Cryptococcus neoformans var. neoformans* |
| 61 | Subtilisin like serine protease PR1B | gi\|16215666 | 6.7 | 39959 | 3 peptides | *Metarhizium anisopliae var.anisopliae* |

| | | | | | | |
|---|---|---|---|---|---|---|
| 62 | Trypsin-like protease | Q6SV39 | 7.1 | 15973 | 28.00% | *Metarhizium anisopliae* |
| 63 | Serine/Threonine protein kinases | Q6C2A3 | 6.01 | 27803 | 34.00% | *Yarrowia lipolytica* |
| 64 | Serine/Threonine protein kinases | Q6C2A4 | 6.01 | 27803 | 28.00% | *Yarrowia lipolytica* |
| 65 | Subtilisin like serine protease PR1D | gi\|16305048 | 5.81 | 42763 | 2 peptides | *Metarhizium anisopliae var.anisopliae* |
| 66 | Proteinase K [fragment] | gi\|295946 | 6.6 | 40275 | 24.00% | *Tritirachium album* |
| 67 | Trypsin-like protease | Q6SV41 | 5.9 | 14216 | 28.00% | *Metarhizium anisopliae* |
| 68 | Subtilisin like serine protease PR1D | gi\|16305049 | 5.81 | 42763 | 2 peptides | *Metarhizium anisopliae var.anisopliae* |
| 69 | Mitochondrial protein | QO5676 | 5.68 | 13897 | 34.00% | *Saccharomyces cerevisiae* |
| 70 | Subtilisin like serine protease PR1J | gi\|6624952 | 6.04 | 42304 | 3 peptides | *Metarhizium anisopliae var.anisopliae* |
| 71 | Subtilisin like serine protease PR1J | gi\|6624952 | 6.04 | 42304 | 3 peptides | *Metarhizium anisopliae var.anisopliae* |
| 72 | cAMP dependent protein kinase | gi\|71024243 | 5.14 | 26990 | 28.00% | *Ustilago maydis 521* |
| 73 | Secreted aspartyl protease | B8YPG8 | 5.57 | 29544 | 31.00% | *Candida dubliniensis* |
| 74 | Trypsin-like protease 1 | gi\|556657 | 5.45 | 26100 | 2 peptides | *Metarhizium anisopliae* |
| 75 | Trypsin-like protease 1 | gi\|556657 | 5.45 | 26100 | 2 peptides | *Metarhizium anisopliae* |
| 76 | Trypsin-like protease 1 | gi\|556657 | 5.45 | 26100 | 2 peptides | *Metarhizium anisopliae* |
| 77 | Trypsin-like protease 3 | gi\|5042250 | 4.9 | 26184 | 42.00% | *Metarhizium anisopliae* |
| 78 | TH 14-3-3 | Q27JQ9 | 4.86 | 29865 | 19.00% | *Metarhizium anisopliae* |
| 79 | TH 14-3-3 | Q27JQ9 | 4.86 | 29865 | 22.00% | *Metarhizium anisopliae* |
| 80 | Septin | gi\|21435778 | 5.8 | 42822 | 34.00% | *Candida albicans* |
| 81 | Septin | gi\|21435778 | 5.8 | 42822 | 28.00% | *Candida albicans* |
| 82 | DNase1 protein | P52752 | 4.27 | 9920 | 26.00% | *Metarhizium anisopliae* |
| 83 | Putative adenylate kinase | Q8TG40 | 4.07 | 28668 | 21.00% | *Candida albicans* |
| 84 | Putative adenylate kinase | Q8TG40 | 4.07 | 28668 | 24.00% | *Candida albicans* |
| 85 | Calcineurin like phosphoesterase | gi\|68491579 | 5.21 | 35876 | 29.00% | *Candida albicans SC5314* |
| 86 | Hydrophobin like protein | P527S2 | 4.27 | 9920 | 1 peptide | *Metarhizium anisopliae* |

# 7. Biolistic transformation of the entomopathogenic fungus *Metarhizium anisopliae* resulting in high resistance to benomyl

## 7.1. Introduction

Modification of entomopathogenic fungi has been progressing rapidly over the past decade. A number of studies have reported successful insertion of foreign genes into entomopathogenic fungi. A key to manipulation of entomopathogenic fungi using molecular techniques has been the establishment of genetic transformation systems. These techniques enable gene disruption methods to be applied, which can lead to greater understanding of the genetics of e.g. disease processes and can provide ways to introduce new genes into fungi and overexpress selected genes in entomopathogenic fungi (Zimmerman, 1993; Milner and Jenkins, 1996; Faria and Wraight, 2007).

The first transformation of an entomopathogenic fungus was reported by Goettel et al. (1990) and since then, there have been other reports on transformation of the Deuteromycete entomopathogens using a variety of methods. St. Leger et al. (1995) used electroporation and biolistic delivery methods to transform *M. anisopliae* with plasmids containing β-glucuronidase and benomyl resistance genes. The cotransformants showed normal growth rates and retained their pathogenicity towards *B. mori*. Polyethylene glycol (PEG) - mediated transformation of protopasts is another method for transformation of entomopathogenic fungi, as used with the *P. fumosoroseus* and *P. lilacinus* (Inglis et al. 1999) using benomyl as selective agent.

One important strategy for the biocontrol of insects by fungi is to develop fungal strains resistant to common agricultural fungicides, such as benomyl (Goettel et al. 1990). The recombinant

strains could be then used to control insect pests in an environment where fungicides are applied to control fungal pathogens of plants. Since benomyl was banned in Australia, the benomyl resistant transformants may not be used for biological control. However, transformants resistant to other fungicides can be prepared using the protocol describes here.

To develop recombinant strains of *M. anisopliae*, we have adapted transformation protocols applied to other filamentous fungi (Fincham, 1989), using the *ben*A3 gene from *Aspergillus nidulans* (St. Leger et al. 1995) encoding the major β-tubulin. Benomyl binds to β-tubulin and inhibits formation of microtubules and mutations in the *A. nidulans ben*A3 gene, which encodes the major β-tubulin expressed in the hyphae, can confer resistance to benomyl (Goettel et al. 1990). Using the plasmid pBENA3 containing the *ben*A3 allele, we have genetically transformed *M. anisopliae* mutant strain NM10 (Chapter 5) to benomyl resistance to enable an integrated approach into the control of canegrubs.

## 7.2. Materials and methods

### 7.2.1. Plasmid

The 10 kb plasmid pBENA3 (Fig.1) constructed by Jung and Oakley (1990) which carries the *ben*A3 allele of *Aspergillus nidulans* was obtained from the Fungal Genetics Stock Center, University of Missouri, Kansas City, USA. The plasmid also contains the bacterial a*mp* resistance gene for ampicillin resistance originating from the plasmid pUC19 and the *pyr*4 gene of *Neurospora crassa* as an auxotrophic selection marker. Propagation of the plasmid DNA in *E. coli* was carried out using standard protocols described in Sambrook et al. (1989). Plasmid DNA was purified using Qiagen (Hilden, Germany) columns prior to *M. anisopliae* transformation according to the manufacturer's protocol.

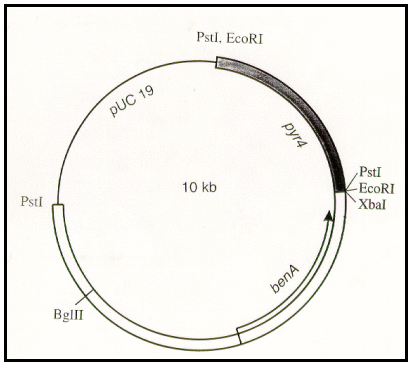


**Figure1:** Pictorial presentation of the plasmid pBENA3 used in the biolistic transformation. *benA* refers to the benomyl resistance gene that can also be used in the selection of transformants and *pyr4* is an auxotrophic selection marker.

### 7.2.2. Fungal strain and cultivation conditions

*M. anisopliae* strain NM10 used in this experiment is a UV mutant strain (as described in Chapter 5). The strain was maintained on potato dextrose agar (PDA, Difco Laboratories, Detroit, Michigan) at 28 °C for 14 days. Conidia were then collected in 10 ml of 0.9 % (w/v) sodium chloride and 0.01 % (v/v) Tween-80 (Sigma Chemical Co., St. Louis, MO) and filtered through a sterile cotton plug. Conidial concentration was adjusted to about $1.5\times10^7$ conidia/mL using a hemocytometer.

### 7.2.3. Biolistic transformation, mitotic stability and enzyme production

High velocity biolistic transformation of *M. anisopliae* was performed using the Bio-Rad PDS-1000/He system. Fourteen days old spores ($1.5 \times 10^7$ spores in 0.9 % NaCl, 0.01 % Tween 80 solution) were plated on the centre of standard PDA plates to align with the seven barrels of the Hepta Adaptor, and left to dry. Precipitation of 5 µg pBENA3 DNA onto gold particles (0.6 micron particles, INBIO GOLD) was carried out as described in Te'o et al. (2002).

For bombardment, 10 µL of DNA-coated gold particles were loaded onto seven macrocarrier disks sitting in the seven slots of the Hepta Adaptor holder and left to dry. A single rupture disk was placed on the top to temporarily block the helium gas from entering the seven barrels of the Hepta Adaptor instrument. A rupture disk of 900 psi was used in the experiment. When dried, the Hepta Adaptor holder containing DNA-coated gold particles was placed inside the PDS-1000/He system chamber directly below the Hepta Adaptor device. PDA plates containing conidia for bombardment were placed at a target distance of 6 cm. PDA plates containing conidia were bombarded in triplicate unless stated otherwise. After bombardment, PDA plates were incubated at 28 °C for 18 h before overlaying with 10 mL of PDA containing 10 µg/mL of benomyl for transformant selection and returned to 28 °C for a further 3 to 5 days of growth. A stock solution of benomyl 10 mg/mL in dimethylsulphoxide was kept at -20 °C and filter-sterilised aliquots were added after the medium was autoclaved. A control plate was included where *M. anisopliae* conidia were bombarded with gold particles without DNA. Potato dextrose agar was supplemented with 10 µg/mL of benomyl (PS222, SUPELCO; Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate) to select the transformants. Benomyl resistant transformants were first subcultured on fresh PDA plates containing 10 µg/mL of benomyl and further tested on PDA containing a range of benomyl concentrations (20, 30, 40 and 50 µg/mL). Transformants which grew on benomyl plates (50 µg/mL of benomyl) after the second selection were considered as true transformants. Resulting transformants were then tested by subculturing on PDA containing benomyl concentrations upto 200 µg/mL (50, 100, 150, 200 µg/mL). Mitotic stability of the transformants was tested by transferring the colonies to PDA without benomyl as a nonselective medium. After every fifth transfer, these transformants were tested for benomyl resistance by growth on PDA/benomyl (200 µg/mL of benomyl). Selected benomyl resistant transformants

were then tested for protease secretion to ensure that this trait was unaffected in the NM10 transformants.

Enzyme secretion of the transformants was tested by inoculating 100 µL of spore suspension of each transformant on minimal medium plates ($NaNO_3$ 6.0 g/L, KCl 0.52 g/L, $MgSO_4.7\ H_2O$, 0.52 g/L, $KH_2PO_4$ 1.52 g/L, 15 g/L agar and 0.001 g/L of $FeSO_4.7H_2O$ and $ZnSO_4$, pH 6.9), supplemented with 0.1% (w/v) yeast extract and 1 % (w/v) dry skim milk (Nestlé, Australia Ltd) for screening of protease secretion. The plates were incubated at 28 °C for 7 days. A clearing halo appeared around each colony due to substrate degradation. Clearing zones were measured in centimeters using a transparent ruler and compared to the non-transformed strain NM10. Four transformants (T7, T14, T27 and T31) showing 4 cm clearing zone around the colony, similar to those of NM 10 were selected for virulence testing against greyback canegrubs.

### 7.2.4. Genomic DNA isolation

The four transformants (T7, T14, T27 and T31) tested for protease secretion was also chosen for genomic DNA isolation. Genomic DNA was isolated directly from the mycelial mass grown on PDA (with 10 µg/mL of benomyl) for 5 to 7 days, following the method described by Raeder and Broda (1985). Isolated genomic DNA's were checked using standard agarose electrophoresis, and usually diluted 10-fold (10 ng) for use in PCR reactions.

### 7.2.5. PCR analysis

Two oligonucleotide primers (*benA* forward: 5′- GCC AGC GGT AAC AAG TACG -3′ and *benA* reverse: 5′-CTC GTT GTC AAT ACA GAA AG-3′) were designed for DNA-amplification from the transformants. Genomic DNA isolated from a non-transformed *M. anisopliae* NM10

strain was used as a negative control. PCR reactions were carried out using the Amplitaq Gold Polymerase (Perkin Elmer, USA) under following conditions: [1×] 94 °C, 10 min; [35×] 94 °C, 30 s; 50 °C, 30 s; 72 °C, 30 s. Electrophoresis was performed according to standard procedure as described by Sambrook et al. (1989). Reaction products were analysed on standard 1% agarose gels and the expected size of the product was 455 base pairs.

### 7.2.6. Insect larvae and Infection of canegrubs

The third instar greyback canegrubs, *Dermolepida albohirtum* (Waterhouse) used in this experiment were nurtured as described in Chapter 5 (section 5.2.4) and canegrubs assigned for infections with the selected transformants were carried out as described in Chapter 5 (section 5.2.5). Ten greyback canegrubs were treated with each transformant (T7, T14, T27 and T31) and ten canegrubs with the non-transformant strain NM10 for control.

## 7.3. Results and discussion

Spores of *M. anisopliae* mutant strain NM10 were bombarded with the plasmid pBENA3 to generate benomyl resistant transformants. The NM10 mutant strain used in the experiment had not been genetically transformed before and there are no published reports on transformation of *M. anisopliae* by biolistic bombardment using the seven barrel system. Three days after bombardment, small but well-separated/single transformant colonies became obvious. Due to the filamentous nature of the fungus, the individual colonies can start to merge into each other after 7 days of growth, resulting in difficulty with counting. No colonies were found on control plates where no DNA was precipitated onto gold particles.

A relatively high transformation frequency, 56 colonies per µg of plasmid DNA was obtained. This transformation frequency is about 3.5 fold higher compared to that reported by St. Leger et al. (1995). Studies conducted by Goettel et al. (1990) and Inglis et al. (2000) have reported low transformation efficiencies (9-15 transformants per µg of plasmid DNA) for *M. anisopliae* using single barrel particle delivery. In contrast, Valadares-Inglis and Inglis (1997) reported high transformation frequencies for *M. flavoviride* (upto 84 transformants per µg of plasmid DNA), which is unusual for entomopathogenic fungi.

The method allowed isolation of transformants of *M. anisopliae* highly resistant to benomyl. A total of 50 randomly selected benomyl resistant transformants were tested by sub-culturing onto PDA containing various concentrations of benomyl (10, 20, 30, 40, 50 µg/ml). Fifteen transformants were selected after second round selection on benomyl and only 10 transformants (T1, T2, T6, T7, T14, T21, T24, T27, T31 and T40) tolerated higher concentrations of benomyl (200 µg/mL). The stability of the transformants was determined after five subsequent transfers onto PDA as a nonselective medium. Four transformants (T7, T14, T27 and T31) were still found to be highly resistant to benomyl after twenty successive transfers on benomyl-free medium. Integration of the plasmid pBENA3 was determined by PCR analysis (Figure 1) and all four transformants showed the integration of the plasmid as the PCR products from genomic DNA were at the expected size of 455 bp. High mitotic stability has been described in earlier studies for benomyl transformants of *M. anisopliae* (Goettel et al. 1990). All four transformants sporulated well on PDA with the presence or absence of benomyl, which was considered to be an important feature since high amounts of fungal spores are commonly required for commercial mycoinsecticide-formulated products.

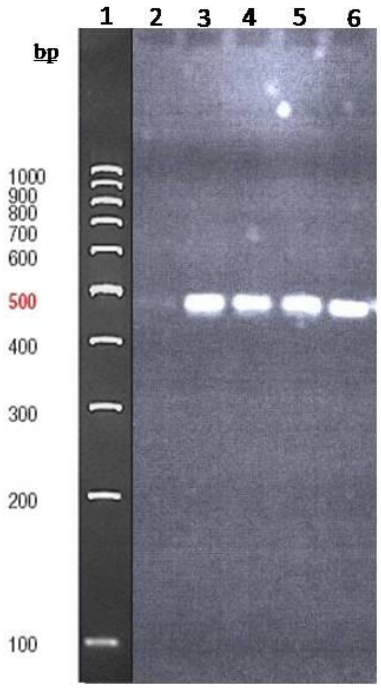


**Figure 1: PCR analysis of the gDNA of *M. anisopliae* transformants for the integration of the *benA* gene**

Lane 1: DNA ladder (Fermentas), Lane 2: *M. anisopliae* NM 10 strain, Lane 3: T7, Lane 4: T14, Lane 5: T27 and Lane 6: T31.

Secretion of proteolytic enzymes from entomopathogenic fungi is considered to be one the most important pathogenicity factors during the infection process (St. Leger et al. 1987; Gillespie et al. 1998). The production of protease enzyme by the four stable transformants (T7, T14, T27 and T31) was visualised on minimal medium containing 1 % (w/v) skim milk in the presence or absence of benomyl (50 µg/mL), following the procedure described by Thomas and Jenkins (1997). All four transformants showed wide clearing zones indicated high secretion of protease activity similar to the NM10 mutant strain. High protease secreting mutants have been shown to display enhanced virulence characteristics in insects (Gillespie et al. 1998).

Selected transformants (T7, T14, T27 and T31) and the control strain (NM10) killed 100 % of the target grubs within eleven days post-infection. This indicated that all four transformants (T7,

T14, T27 and T31) were equally virulent towards canegrubs similarly to that of the non-transformant strain NM10. The ability to infect insects faster has also been reported by benomyl resistant *Metarhizium* transformants developed against grasshopper (*R. schistocercoides*) (Furlaneto et al. 1999; Inglis et al. 2000), locust (*L. migratoria*) (Cao et al. 2007) and tobacco hornworm (*M. sexta*) (Goettel et al. 1990).

Given the current renewed interest in the use of entomopathogenic fungi as mycoinsecticides, genetic manipulation studies with promising strains will attain greater importance. We have herein demonstrated that four transformants (T7, T14, T27 and T31) obtained in this study displayed high mitotic stability and high enzyme production comparable to the non-transformant strain NM 10, and retention of pathogenicity to greyback canegrubs. The study also indicated the potential use of these transformants to control canegrubs when benomyl is used in the field for the control of plant pathogens. Further studies will enable the development of transgenic strains with improved virulence to insects and provide compatibility with other components of pest control such as fungicides.

## 7.4. References

Cao Y, Peng G, He Z, Wang Z, Yin Y, Xia Y (2007) Transformation of *Metarhizium anisopliae* with benomyl resistance and green fluorescent protein genes provides a tag for genetically engineered strains. Biotechnol Lett 29: 907-911.

Faria M, Wraight SP (2007) Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biocontrol 43: 237-256.

Fincham JR (1989) Transformation in fungi. Microbiol Rev 53: 148-170.

FurlanetoMC, Paião FG, Pinto FG da S, Fungaro MHP (1999) Transformation of the entomopathogenic fungus *Metarhizium flavoviride* to high resistance to benomyl. Can J Microbiol 45: 875-878.

Gillespie JP, Bateman R, Charnley AK (1998) Role of cuticle degrading proteases in the virulence of *Metarhizium* spp. for the desert locust, *Schistocerca gregaria*. J Inverterbr Pathol 71: 128-137.

Goettel MS, St. Leger RJ, Bhairi S, Jung MK, Oakely BR, Roberts DW, Staples RC (1990) Pathogenicity and growth of *Metarhizium anisopliae* stably transformed to benomyl resistance. Curr Genet 17: 129-132.

Inglis PW, Tigano MS, Valadares-Inglis MC (1999) Transformation of the entomopathogenic fungi, *Paecilomyces fumosoroseus* and *Paecilomyces lilacinus* (Deuteromycotina: Hypomycetes) to benomyl resistance. Genet Mol Biol 22: 119-123.

Inglis PW, Aragao FJL, Frazao H, Magalhaes BP, Valadares-Inglis MC (2000) Biolistic co-transformation of *Metarhizium anisopliae* var. *acridum* strain CG423 with green fluorescent protein and resistance to glufosinate ammonium. FEMS Microbiol Lett 191: 249-254.

Jung MK, Oakley BR (1990) Identification of an amino acid substitution in the *ben A*, beta-tubulin gene of *Aspergillus nidulans* that confers thiabendazole resistance and benomyl super sensitivity. Cell Motil Cytoskeleton 17: 87–94.

Milner RJ, Jenkins K (1996) *Metarhizium*: a versatile mycoinsecticide of the future. Professional Pest Management 1: 32-36.

Raeder U, Broda P (1985) Rapid preparation of DNA from filamentous fungi. Lett Appl Microbiol 1: 17-20.

Sambrook KJ, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd Eds. Cold Spring Harbor Laboratory Press, NY.

St. Leger RJ, Charnley AK, Cooper RM (1987) Characterization of cuticle-degrading proteases produced by the entomopathogen *Metarhizium anisopliae*. Arch Biochem Biophys 253: 221–232.

St. Leger RJ, Shimizu S, Joshi L, Bidochka MJ, Roberts DW (1995) Co-transformation of *Metarhizium anisopliae* by electroporation or using gene gun to produce stable GUS transformants. FEMS Microbiol Lett 131: 289-294.

Te'o VSJ, Berquist PL, Nevalainen KMH (2002) Biolistic transformation of *Trichoderma reesei* using the Bio-Rad seven barrels Hepta Adaptor system. J Microbiol Methods 51: 393-399.

Thomas MB, Jenkins NE (1997) Effects of temperature on growth of *M. flavoviride* and virulence to the variegated grasshopper, *Zonocerus variegates*. Mycol Res 101: 1469-1474.

Valadares-Inglis MC, Inglis PW (1997) Transformation of the entomopathogenic fungus, *Metarhizium flavoviride* strain CG423 to benomyl resistance. FEMS Microbiol Lett 155: 199-202.

Zimmerman G (1993) The entomopathogenic fungus *Metarhizium anisopliae* and its potential as a biocontrol agent. Pestic Sci 37: 375–379.

## 8. Summary of main findings and conclusions

There is a steadily growing interest into the use microbial pesticides to replace harmful chemicals to combat important agricultural pests. Amongst the fungi developed for biological control of insects, *Metarhizium* based products are gaining popularity; this particular fungus represents a huge untapped source of pesticide genes and insecticidal toxins. Greyback canegrubs are soil dwelling larvae which chew the roots of sugarcane plants causing considerable economic losses to sugarcane growers as well as the sugar industry. Control of canegrubs is heavily reliant on synthetic pesticides such as suSCon® Blue and the biopesticide BioCane™, a *Metarhizium* based product to specifically target greyback canegrubs. Extensive research into the control of canegrubs has shown that neither chemical nor microbial pesticides are fully effective alone in districts such as Burdekin (QLD). The area has alkaline soil facilitating alkaline hydrolysis and microbial degradation of the bioactive ingredient in suSCon® Blue. In addition, alkalinity of the soil has an impact on the growth of *Metarhizium*. Following from the above, environmental factors have a great impact on the success of pest control.

This work illustrates the development and application of proteomic methods such as 2-DE, and other methods such as UV-mutagenesis and transformation of *Metarhizium* for increasing knowledge and improving the biological control properties of the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae* (FI-1045). Proteomics and bioinformatics methods were established and applied to study the proteomes of third star greyback canegrubs (*Dermolepida albohirtum*) (Chapter 1), the entomopathogenic fungus *Metarhizium anisopliae* (isolate FI-1045) (Chapter 2), as well as the secretome maps of *M. anisopliae* (Chapter 4) and *Metarhizium* mutant strain (NM10) developed in this work (Chapter 6) under different biological conditions. The combination of proteomic and secretomic approaches resulted in a 2D map of proteins which

provided a valuable tool for understanding the protein functions and networking in *Metarhizium* infecting the greyback canegrubs. UV mutagenesis method (Chapter 5) was used to improve the characteristics of the *M. anisopliae* FI-1045 strain currently used for biological control in Australia. Further on, genetic transformation of *Metarhizium* mutant strain NM10 was achieved via biolistic bombardment (Chapter 7) by using a benomyl resistance gene as a selection marker. *Metarhizium* strains along with improved entomopathogenic properties can be used with other components of pest control such as application of fungicides.

Some of the key findings and achievements in each of the chapters presented in this thesis work are summarised below:

**1)** Proteomic methods were established and applied to study the proteome of greyback canegrub (*Dermolepida albohirtum*). Differential protein extraction and solubilisation methods were employed to obtain sufficient amounts of protein for the 2D analysis. Proteomic reference map for the greyback canegrub (Section 2.7, Fig 1b) was produced using MALDI-TOF-MS (Section 2.3.4). Proteins identified in this work will eventually facilitate identification of potential targets for enhancing biological control of the greyback canegrubs. Here we have presented the first protein map for the greyback canegrub (*Dermolepida albohirtum*).

**2)** A proteome map of *Metarhizium* infected canegrub was established (Section 3.4.3, Fig 3). Three way comparison of proteins that were expressed in healthy canegrubs, *Metarhizium* infected canegrubs and the infecting fungus was used to investigate the changes in the interacting proteomes during infection (Section 3.3.7). Canegrubs were infected by dipping in fungal spore suspension. Comparative analysis highlighted the role of various immune-related proteins of the

greyback canegrubs during infection process. Detection of fungal proteins such as chymotrypsin serine protease indicated participation of proteases in the infection process.

**3)** Secretome maps of *M. anisopliae* strain FI-1045 in response to greyback canegrubs (Section 4.7, Fig. 3) and their isolated cuticles (Section 4.7, Fig 1 and 2) were established using two-dimensional electrophoresis and MALDI-TOF-MS (Section 4.7; Section 4.3.5; Section 4.3.6). High numbers of extracellular proteins were secreted into the culture medium containing grub cuticles compared to whole canegrubs indicating the importance of cuticle degrading proteases during early stages of infection. When fungi grow in liquid culture we cannot rule out the possibility 100% that fungal mycelia hadn't ruptured or they are stressed in the cuture. Six secreted proteins (64-kDa serine carboxypeptidase, 1,3-beta exoglucanase, Dynamin GTPase, THZ kinase, calcineurin like phosphoesterase and phosphatidylinositol kinase) discovered in this study (Section 4.8, Table 1), have not been previously identified from the culture supernatant of *M. anisopliae* during infection. Proteins identified in this work can become a vital tool in the future for developing improved strains of *Metarhizium* against greyback canegrubs through isolation of the genes encoding the proteins identified above and transforming them into the fungal host for gene over-expression or targeted inactivation. In this study, although the proteins were identified from fungal database, we cannot exclude the possibility that certain soluble insect proteins might have participated with fungal proteins.

**4)** A mutagenesis method using ultraviolet light was employed to improve *M. anisopliae* strain FI-1045 (Section 5.3.2). Mutants were first selected based on enzyme (protease and chitinase) production which is an essential criterion for virulence against insect hosts. Mutants with increased proteinase and chitinase secretions were subjected to tests at different temperatures and

pH range because the end application requires enhanced survival traits at high temperatures (above 35°C) and alkaline soil pH (above 6.2) to successfully control canegrubs. The best mutant designated NM10 was tested for its virulence against greyback canegrubs under laboratory conditions. Results indicated that NM10 displayed faster infection rate compared to the parental strain *M. anisopliae* FI-1045. The extracelluar proteins secreted by this mutant strain in response to greyback canegrubs were analysed by 2DE (Chapter 7).

**5)** Secretome maps of *M. anisopliae* strain NM10 (a UV mutant strain from Chapter 5) in response to exposure to greyback canegrubs (Section 7.7, Fig 2) and their isolated cuticles (Section 4.7, Fig 1) were established using two-dimensional electrophoresis and MALDI-TOF-MS (Section 7.7; Section 7.3.5; Section 7.3.6). Extracellular proteins identified in this study include a wide array of proteolytic enzymes which are considered essential for the initiation of infection process. Six secreted proteins (56-kDa aspartyl aminopeptidase, 29-kDa secreted aspartyl protease, cyclin-dependent protein kinase, thymidylate kinase, septin and adenylate kinase) have not been previously identified from the culture supernatant of *M. anisopliae* during infection (Section 7.8, Table 1). Proteins identified in this work may be used to isolate the corresponding genes for detailed molecular studies into canegrub control.

**6)** Transformation of the *Metarhizium* UV mutant strain NM10 was conducted via biolistic bombardment (Section 7.2.3) using a benomyl resistance gene. Before 2006 benomyl was used as a common fungicide in agriculture to control plant pathogenic fungi. Since this experiment was conducted before 2006 we thought that incorporating such a gene into an insect pathogen will be useful for integrated pest control. Here transformants generated could tolerate forty times more concentration of benomyl compared to the control. This may relate to the copy numbers of the

*benA* gene which was not explored in this study. Transformants were mitotically stable after ten successive transfers on a non-selective medium. Laboratory bioassays proved that the transformants tested could infect greyback canegrubs at the same rate as the non-transformant strain NM 10.

**7)** Highlighting the important differences between the secretome maps of *M. anisopliae* (FI-1045) and mutant strain of *M. anisopliae* (NM10):

Comparative secretome analysis of *M. anisopliae* (FI-1045; parental strain) and the mutant strain (NM10) in response to exposure to greyback canegrubs and their isolated cuticles showed that both the parental strain and the mutant strain secrete a larger number of proteins when grown on canegrub cuticles compared to canegrub homogenate. This may be expected since several studies have demonstrated improved enzyme production on various insect cuticles (Paterson et al. 1994; St. Leger et al. 1994, 1995); the insect cuticle is mainly comprised of proteins (Andersen et al. 1995) and is the most important barrier to fungal infection. Low protein secretion was described in many studies when whole grubs were used for infection and this is because protein content of the cuticle is only 25% where as 75% is body fat (St. Leger et al. 1986, Krieger de Moraes et al. 2003), therefore, the relative amount of cuticle material in the whole grub homogenate is comparatively less.

*M. anisopliae* secretes a wide variety of proteolytic enzymes to digest the insect cuticular proteins, some of which have been associated with virulence. For example, subtilisin-like serine protease (Pr1) which is the best understood protein involved in entomopathogenicity. So far, eleven subtilisins have been identified in *M. anisopliae* during growth on insect cuticles

(Freimoser et al 2003; St. Leger et al. 1996). The parental strain (FI-1045) produced sixteen different isoforms of subtilisin-like serine protease (Pr1) (Chapter 4) and the mutant strain (NM10) produced eleven different isoforms of Pr1 proteins (Chapter 6). The Pr1 isoforms in parental strain and the mutant NM10 differed in their isolectric point as well as in their molecular weight. A similar observation has been described by St. Leger et al. (1994) who also demonstrated that electrostatic binding of Pr1 is a prerequisite for cuticle hydrolysis. Both the parental strain (FI-1045) as well as the mutant strain (NM10) produced thirteen different isoforms of trypsin-related proteases (Pr2). A similar observation has been reported by St. Leger et al. (1996) where thirteen Pr2 proteins were synthesised by *M. anisopliae* in response to cockroach cuticles. A number of studies have highlighted that trypsin-like proteases assist in proteolytic degradation of the cuticle barrier since they do not have the ability to degrade an intact cuticle alone (Freimoser et al. 2003; St. Leger et al. 1996). In the current work, the mutant strain produced four chymotrypsins when the fungus was grown on canegrub cuticles. Chymotrypsins function by scavenging the nutrients and penetrate host barriers (Screen and St. Leger, 2000). The mutant strain (NM10) also secreted two metalloproteases and a zinc binding metalloprotease (56-kDa aspartyl aminopeptidase) when grown on canegrub cuticles as well as on whole grubs, but secretion of metalloprotease was observed in the parental strain (FI-1045) only in response to whole canegrubs. During *Metarhizium* infection, metalloproteases act in concert with several serine proteases to bring about the destruction of the insect cuticle and also serve as a back-up for Pr1 activity (St. Leger et al. 1995). Secretion of 56-kDa aspartyl aminopeptidase protein has not been previously reported in *M. anisopliae* and it therefore seems to be a novel *Metarhizium* protein. The mutant strain secreted a 29-kDa aspartyl protease when grown on whole canegrubs and secretion of this protein has been previously reported in *M. anisopliae* by St. Leger et al. (1998) at 45-kDa. Therefore, we speculate that it is a novel *Metarhizium* protein. Aspartyl

proteases carry out a number of specialised functions such as digesting molecules for nutrient acquisition and digesting host cell membranes to facilitate adhesion and tissue invasion during infection process (Pereira et al. 2007). All these proteases seem to mediate a cascade of reactions facilitating the fungal penetration of host cuticles.

Chitinase proteins were secreted by the mutant strain (NM10) when grown on canegrub cuticles as well as on whole canegrubs, but the parental strain (FI-1045) secreted chitinase only when grown on whole canegrubs. These enzymes come into action mainly after the proteases have degraded cuticular proteins and chitin has been exposed. Work conducted by St. Leger et al. (1996) has indicated that chitinase acts on the insect procuticle around 40 h post-inoculation which suggests that release of chitinase is reliant on the accessibility of the substrate. This coincides with our findings as the chitinase was detected in 48 h cultures. Hydrophobin-like protein was secreted by the mutant strain (NM10) when grown on whole canegrubs and this protein has been reported to play a vital role in fungal pathogenesis in mediating attachment of conidia to the insect cuticle (Bidochka et al. 2001). Fungal hydrophobins provide another example of proteins, in addition to proteases, that are differentially regulated during early infection (St. Leger et al. 1996).

Both the parental strain and the mutant strain secreted a variety of signal transduction proteins that regulate fungal development and virulence. Some examples of the signaling proteins secreted by the mutant strain (NM10) are cyclin-dependent protein kinase that functions to help integrate environmental signals with developmental activity (Bussink and Osmani, 1998), and thymidylate kinase that is involved in nucleotide metabolism and cell cycle regulation in fungi (Jong et al. 1994). Other signaling proteins include septins that are involved in cell morphogenesis during

filamentous growth, chitin deposition, cell cycle regulation, cell compartmentalisation, membrane trafficking, spore wall formation and organisation of the cytoskeleton (Field and Kellogg, 1999), and adenylate kinase which is an important enzyme in the regulation of the cellular energy balance in the signaling cascade. Cyclin-dependent protein kinase, thymidylate kinase, septin and adenylate kinase proteins play a critical role in signal transduction cascades that regulate fungal development and virulence. These proteins were not secreted by the parental strain (FI-1045) and have not been reported previously from *M. anisopliae*.

*Metarhizium* mutant strain (NM10) secreted a wide variety of proteins below 50-kDa and less proteins above 50-kDa across the pH range of 4-7 when grown on canegrub cuticles and whole canegrubs. However, the parental strain (FI-1045) secreted a larger number of high molecular weight proteins when grown on canegrub cuticles as well as on whole canegrubs and a lower number of proteins below 25-kDa. The secreted proteins were scattered more evenly across the pH range of 4-7 in the secretome maps of the mutant strain (NM10) compared to the secretome of the parental strain (FI-1045). Also, the amount of proteins secreted by the mutant strain in response to canegrub cuticles and whole cane grubs was almost the same. In the parental strain, more proteins were secreted in response to canegrub cuticles and very few proteins were secreted in response to whole cane grubs. Striking differences between the secretome maps of the mutant strain and the parental strain may be attributed to genomic alterations (deletions, insertions, single nucleotide changes, duplications, phenotypic differences) by UV mutagenesis (Bouws et al. 2008).

Finally, the overall variability of enzymes produced by the mutant strain (NM10) in response to canegrub cuticles and grub homogenate was greater compared to the parental strain (FI-1045).

Bioassay results discussed in Chapter 5 (Section 5.3.4; Infection of canegrubs) also indicate that the mutant strain (NM10) imposed a rather rapid action (68.75 % faster) on the greyback canegrubs compared to the parental strain (*M. anisopliae* FI-1045) in laboratory conditions.

In summary, we have identified six new extracellular proteins (64-kDa serine carboxypeptidase, 1,3-β-exoglucanase, Dynamin GTPases, THZ kinase, calcineurin like phosphoesterase and phosphatidylinositol kinase) secreted by *M. ansiopliae* (FI-1045) during growth on isolated canegrub cuticles and canegrub homogenate. Another six new extracellular proteins (56-kDa aspartyl aminopeptidase, 29-kDa secreted aspartyl protease, cyclin-dependent protein kinase, thymidylate kinase, septin and adenylate kinase) secreted by *M. ansiopliae* mutant strain (NM10) were identified during growth on isolated canegrub cuticles and canegrub homogenate. Novel proteins identified in this study may provide novel targets for further development of *M. ansiopliae* as a biocontrol organism against greyback canegrubs. The genes encoding the six novel proteins will be isolated in the future in order to establish their involvement in the biocontrol response of *M. anisopliae*. In conclusion, the work discussed above presents a new multipronged approach into studies addressing fungal biological control.

## 8.1. References

Andersen SO, Højrup P, Roepstorff P (1995) Insect cuticular proteins. Insect Biochem Mol Biol 25: 153-176.

Bidochka MJ, De Koning J, St Leger RJ (2001) Analysis of a genomic clone of hydrophobin (*ssgA*) from the entomopathogenic fungus *Metarhizium anisopliae*. Mycol Res 105: 360–364.

Bussink HJ, Osmani SA (1998) A cyclin-dependent kinase family member (PHOA) is required to link developmental fate to environmental conditions in *Aspergillus nidulans*. EMBO J 17: 3990-4003.

Bouws H, Wattenberg A, Zorn H (2008) Fungal secretomes-nature's toolbox for white biotechnology. Appl Microbiol Biotechnol 80: 381-388.

Field CM, Kellogg D (1999) Septins, cytoskeletal polymers or signalling GTPases? Trends Cell Biol 9: 387-394.

Freimoser FM, Screen S, Bagga S, Hu G, St. Leger RJ (2003) Expressed sequence tag (EST) analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts. Microbiol 149: 239–247.

Jong AY, Kuo CL, Campbell JL (1994) The CDC8 gene of yeast encodes thymidylate kinase. J Biol Chem 259: 11052-11059.

Krieger de Moraes C, Schrank A, Vainstein MH (2003) Regulation of extracellular chitinases and proteases in the entomopathogen and acaricide *Metarhizium anisopliae*. Curr Microbiol 46: 205–210.

Paterson IC, Charnley AK, Cooper RM, Clarkson JM (1994) Specific induction of a cuticle-degrading protease of the insect pathogenic fungus *Metarhizium anisopliae*. Microbiol 140: 185-189.

Pereira JL, Noronha EF, Miller RNG, Franco O L (2007) Novel insights in the use of hydrolytic enzymes secreted by fungi with biotechnological potential. Lett App Microbiol 44: 573-81.

Screen SE, St. Leger RJ (2000) Cloning, expression and substrate specificity of a fungal chymotrypsin. Evidence for lateral gene transfer from an actinomycete bacterium. J Biol Chem 275: 6689–6694.

St. Leger RJ, Charnley AK, Cooper RM (1986) Cuticle-degrading enzymes of entomopathogenic fungi: mechanisms of interaction between pathogen enzymes and insect cuticle. J Invertebr Pathol 47: 295–302.

St. Leger RJ, Bidochka M, Roberts DW (1994) Isoforms of the cuticle-degrading Pr1 proteinse and production of metalloproteinase by *M.anisopliae*. Arch Biochem Biophys 313: 1-7.

St. Leger RJ, Joshi L, Bidochka MJ, Roberts DW (1995) Protein synthesis in *Metarhizium anisopliae* growing on host cuticle. Mycol Res 99: 1034-1040.

St. Leger RJ, Joshi L, Bidochka M, Rizzo MJ, Roberts DW (1996) Characterization and ultrastructural localization of chitinases from *Metarhizium anisopliae*, *M. flavoviride* and *Beauveria bassiana* during fungal invasion of host (*Manduca sexta*) cuticle. Appl Environ Microbiol 62:907–912.

St. Leger RJ, Joshi L, Roberts DW (1998) Ambient pH is a major determinant in the expression of cuticle degrading enzymes and hydrophobin by *Metarhizium anisopliae*. Appl Environ Microbiol 64: 709-713.

## 9. Future work

The major limitation of a proteomic approach relating to studying entomopathogenic fungi is the lack of protein and genomic data in publicly available databases that are needed for protein identification. For example, the NCBI database contains a combined total of 492 proteins for the genus *Metarhizium* compared to Swiss Prot and Tremble database which contains a combined total of 315 proteins for the genus *Metarhizium*. The Entomopathogenic Genome Resource (TEGR) at the University of Maryland has started a program featuring *Metarhizium* genome sequencing. However, the information in TEGR is not publicly available and therefore the information required for protein identification has to be obtained from other filamentous fungi and yeasts, for which a complete genome sequence has been produced. In the future, completion of the *Metarhizium* genome sequencing and making it publicly accessible will enhance protein identification and help isolation of genes encoding major determinants in biological control.

In this study, genes encoding some of the secreted *Metarhizium* proteins such as aspartyl protease, 1,3, β-exoglucanase, N-acetylglucosaminidase, serine carboxypeptidase produced during the infection of canegrubs represent good candidates to be isolated and overexpressed in *Metarhizium* to improve biocontrol performance. While the emphasis in this work was on the discovery of proteins participating canegrub infection, the tools developed will serve for achieving broader goals. Transformants and mutant strains will eventually be subjected to pot trails in a glasshouse and then small plot field trials. The genes encoding key fungal proteins discovered in this work will be considered for *Metarhizium* strain improvement and working out the molecular basis for infection. The combination of proteomic techniques, mutagenesis and screening and fungal transformation approaches would probably produce the best results in the development of novel methods for canegrub control.