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# ANALYSIS OF THE CUTICLE OF TWO SPECIES OF GRAIN STORAGE PEST AND THE INTERACTION WITH GERMINATION AND EARLY GROWTH OF ENTOMOPATHOGENIC FUNGI

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### Master of Applied Science

### Submitted in fulfilment of the requirements for the degree of Master of Applied Science (Research)

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# Keywords

Entomopathogenic fungi, *Metarhizium anisopliae*, *Beauveria bassiana*; grain beetles *Rhyzopertha dominica, Tribolium castaneum*; insect cuticle, cuticular lipids, extraction, wings, elytra, hydrocarbons, host interaction, conidium, germination, appressoria, elytra, wings.

# Table of Contents

# Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

QUT Verified Signature

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# Chapter 1: Introduction

## 1.1. Background and literature review

### 1.1.1. Introductory statement

This thesis investigates the interaction between the entomopathogenic fungi *Metarhizium anisopliae* (Metchnikoff) and *Beauveria bassiana* (Bals) (Hypocreales: Clavicipitaceae) and of the cuticle of two grain beetles, *Tribolium castaneum (*Herbst) (Tenebrionidae: Coleoptera) and *Rhyzopertha dominica* (Fabricius) (Bostrichidae: Coleoptera).

*Tribolium castaneum* and *Rhyzopertha dominica* are the most problematic beetle pest for stored grain and grain products in Australia (Collins et al., 1993; Campbell & Runnion, 2003). They feed on grain products, causing qualitative as well as quantitative damage (Padin et al., 2002). These species have been found in association with a wide range of stored products, including grain, flour, peas, beans, cacao, nuts, and dried fruits (Collins et al., 1993; Campbell & Runnion, 2003).

The use of insecticides is one method of preventing some losses during storage. However, *T. castaneum* and *R. dominica* have developed resistance to most widely used insecticides, including phosphine and methyl bromide, which are used as quarantine and pre-shipment treatments for Australian grain exports, and this poses a significant threat to market access for Australian grain exports (Zettler & Cuperus, 1990; Collins et al., 1993; Runnion, 2003). It is important to develop alternative control methods, such as the use of biopesticides control against stored insect pests.

## 1.2 Biopesticides to control insect pests

Entomopathogenic fungi have been evaluated as biopesticides to control insecticide resistant pests (Copping & Menn, 2000; Butt & Beckett, 1995). The effectiveness of entomopathogenic fungi such as *M. anisopliae* and *B. bassiana* have been reported in several studies for controlling the stored product pests such as *T. castaneum* and *R. dominica (*Moino Jr et al., 2002; Throne & Lord, 2004; Lord, 2007; Gołębiowski et al., 2008; Abdel-Raheem et al., 2015). These fungi have been shown to be safe and useful biological agents in controlling insect pests, and both species are registered as insecticides (Sun et al., 2012; Wilson et al., 2011; Copping and Menn, 2000, Butt & Beckett, 1995; Gołębiowski et al., 2008; Abdel-Raheem et al., 2015).

## 1.3 Fungal infection process

Entomopathogenic fungi infect through the cuticle (Fang & St Leger, 2012). They infect the insect via conidiospores that adhere to the cuticle, germinate and penetrate the cuticle. The fungal conidia attach to the cuticle and germinate to form a germ tube. In this process, the fungus may metabolise components of the insect cuticle to support germination and growth ( St Leger et al.,1987, 1992; Crespo & Juárez, 2000). The fungi then develop appressoria at the hyphal tips of the hyphae, by which the fungus penetrates through the insect cuticle and then into the hemolymph. *B. bassiana* and *M. anisopliae* produce hydrolytic enzymes, including chitinases, protease, lipases/ esterases, catalases, and cytochrome P450 that assist the fungus to penetrate the insect cuticle. These enzymes digest the major constituents of the insect cuticle and are considered essential to the infection process (St Leger et al., 1986; Ortiz-Urquiza & Keyhani, 2013; Van Beilen et al., 2003; Rojo, 2010; Pedrini

et al., 2013).

The fungus then grows as blastospores or vegetative hyphae within the body of the host insect (Hajek & Stleger, 1994). After insect death and under the right environmental conditions, vegetative hyphae emerge from the cadaver and conidia may be produced on the outside of the insect's body.

## 1.4. The insect cuticle composition

The insect cuticle consists of several layers, the epicuticle, the procuticle and the epidermis, and each has a different chemical composition (Pedrini et al., 2013). The epicuticle layer is the first barrier between the pathogen and the host, (Hadley et al., 1981; Pedrini et al., 2013) and is between 1-3mm in thickness (Figure 1). It consists of a cement layer and a thin wax layer (Hadley et al., 1981).

The main constituents of the cement layer are hydrocarbons, protein and lipids (Neville et al., 1976). Immediately below the cement is a wax layer (Hadley et al., 1981) with the important function of limiting water loss and preventing desiccation in insects (Baker et al., 1960; Cherry, 1969). Cuticular waxes of insects play a major role in protecting them from environmental damages (Blomquist & Jackson, 1979; Crespo & Juárez, 2000; Dorset & Ghiradella, 1983; Wertz, 1996).

In most insects, the wax layer that is under the cement layer contains 80% hydrocarbons, a small amount of esters, free primary alcohols, free fatty acids, alcohols, and possibly some triacylglycerols (Jarrold et al., 2007; Lockey & Oraha, 1990) that form a layer approximately 0.25 mm thick (Sun et al., 2012). In some insects, such as cattle ticks, *Boopilus microphilus*, the wax layer is approximately 10% of the epicuticle, with a depth of up to 0.1mm of the 1mm-thick epicuticle (Jarrold et al., 2007).

Studies on insect cuticles have shown that hydrocarbons in the epicuticle are common in all insects (Baker et al., 1978; Blomquist et al., 1980; Blomquist & Jackson, 1979; Brophy et al., 1983; Lockey, 1976; Lockey & Oraha, 1990; Smith & Grula, 1982). Insect cuticular hydrocarbons include a mix of components such as alkanes, n-alkenes and methyl branched chains (Nicolás Pedrini et al., 2007; Saito & Aoki, 1983; Smith & Grula, 1982).

The wax layer may be a barrier to the penetration of microorganisms (Blomquist & Jackson, 1979; Pedrini et al., 2013); it can help inhibit the passage of cuticle degrading fungal enzymes (Alexander & Briscoe, 1944). However, some components, including long chain alkanes, may also be utilised by microorganism such as entomopathogenic fungi (Crespo & Juárez, 2000; Jarrold et al., 2007).,

## 1.5 The interaction between host cuticle and fungal pathogenesis

Infection by fungal conidia occurs in three consecutive stages: firstly, adsorption of the fungi propagules to the cuticular surface, secondly adhesion of the border between the epicuticle and pregerminant propagules, and thirdly growth on the host cuticle, until the appressoria are developed at the start of the penetration stage (Pedrini, et al., 2007; Gołębiowski et al., 2012).

The cuticle appears to influence all stages of the infection process, including temporal differences in adhesion and germination that are important to pathogenicity (Arruda et al., 2005). The biochemistry of cuticular degradation by entomopathogenic fungi has been reviewed by St Leger et al., (1986) and Pedrini et al. (2007, 2010). In one study, cuticular crude polar extracts from locust wings containing fatty acids, fatty acid esters, glucose, amino acids and peptides were shown to be strong promoters of germination in *M. anisopliae* (Jarrold et al., 2007).

Furthermore, fungus used long-chain alkanes and other waxes, for hyphal growth and during the subsequent infection (Jarrold et al., 2007).

Leemon & Jonsson (2012) reported that *M. anisopliae* primarily infects the surface of the insect cuticle in ticks. In the case where fungi grow across the cuticle, they may be utilising the waxes in the cuticle as a source of nutrients and the target insect subsequently dies from dehydration.
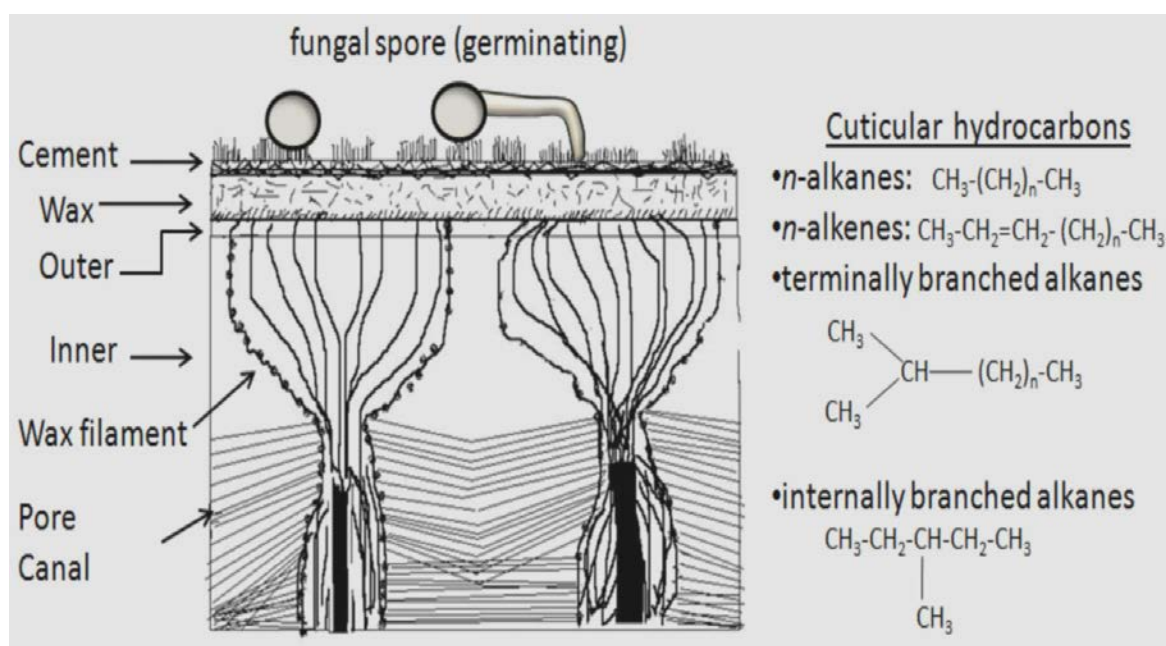


**Figure 1:** The insect cuticle and its hydrocarbon contents. Image taken from (Pedrini et al., 2013). The inner layer, outer layer, wax layer, cement layer, and bloom layers are often considered for insect cuticles.

Epicuticular components play a relevant role in preventing infection as well as affecting insecticide and chemical penetration. Long chain hydrocarbons, fatty alcohols and free fatty acids, some of which can be waxy, are the most abundant components in the epicuticle. (Saito & Aoki, 1983; St. Leger et al., 1987; Nicolás Pedrini et al., 2007).

Multiple studies have shown that the epicuticular lipids in the outer layer may promote or inhibit fungal germination and growth into the insect epicuticle (Lord and Howard, 2004; Jarrold et al., 2007; Pedrini et al., 2007; Pedrini et al., 2013); they have also reported that the barrier properties of the insect cuticle might be essential to enhance the hydrocarbon degradation ability of the insect cuticle. Jarrold et al., (2007) reported that the infection process of fungal degradation may be limited to the cement and wax layers of the insect epicuticle.

It has been reported that entomopathogenic fungi may digest and utilise the outer layer and chemicals in the target insect cuticle to enhance the infection process (Boucias et al., 1988; Leemon & Jonsson, 2012). Several studies have reported that the pathogenic fungi use lipid degrading enzymes, which participate in degrading specific epicuticular lipid components to degrade the barrier of insect waxy layer (Van Beilen et al., 2003; Rojo, 2010; Pedrini et al., 2013). Pedrini et al., (2013) reported that "Alkanes and fatty acids are substrates for a specific subset of fungal cytochrome P450 monooxygenases involved in insect hydrocarbon degradation". Alkanes were found to be highly reduced molecules with a high energy and carbon content, and therefore they can be good carbon and energy sources for microorganisms that are able to metabolise them (Van Beilen et al., 2003; Rojo, 2010; Pedrini et al., 2013). *B. bassiana* contains a range 83 genes coding for Cytochrome P450 enzyme, which has the ability to assimilate n-alkanes and fatty acids in the epicuticular insect as a carbon and energy source for fungal infection (Pedrini et al., 2013).

The chemical composition of the wax layer is complex, but, hydrocarbons are the most common component in this layer (Lecuona et al., 1991). Several studies have reported that the hydrocarbon change during the fungal infection process

(Lecuona et al., 1991; Jarrold et al., 2007), and the differences in the hydrocarbon content of the waxy layer can affect fungal pathogenesis (Pedrini et al., 2013; Van Beilen et al., 2003; Rojo, 2010).

Some hydrocarbons stimulate fungal germination and growth in *B. bassiana* (Lecuona et al., 1991) and in *M. anisopliae* and *B. bassiana* (Boucias et al., 1988), whereas other hydrocarbons including free fatty acids and some carbons inhibit fungal spore germination (Smith and Grula, 1982). Cuticular hydrocarbons, such as fatty acids with ten or fewer carbons, can inhibit fungal spore germination in both *M. anisopliae* and *B. bassiana* conidia adhesion (Saito and Aoki 1983; Lord and Howard, 2004), or enhance the fungal germination process or act as chemical promoters for the production of penetrate germ tubes on insect cuticles (Latge et al., 1987; Pedrini et al., 2013).

Two processes must occur before the fungus reaches and degrades the chitin and proteinaceous components of the insect cuticle. The first process is the adhesion and the interaction between the fungus and the epicuticular layer. The adhesion occurs via two steps, a nonspecific passive adsorption of fungal cells on the surface and then adhesion. Both *M. anisopliae* and *B. bassiana* produce hydrophobic conidia that possess a surface rodlet layer contained of proteins termed hydrophobins. *M. anisopliae* has two genes involved in adhesion (*Mad1* and *Mad2*). These proteins contain single peptide, threonine-proline rich regions, involved in mediating adhesion, and assumed glycosylphosphatidylinositol anchor sites, which would localise the proteins to the plasma membrane. However, the loss of protein *Mad1* may decrease the fungal adhesion and germination process, whereas *Mad2* did not have any effect on adhesion to insect cuticles. In *B. Bassiana,* two hydrocarbons, Hyd1 and Hyd2, are responsible for rodlet layer association, contributing to the

hydrophobic nature of cell surfaces, the adhesion to cell surfaces and virulence (Ortiz-Urquiza & Keyhani, 2013).

It has been reported that the *T. castaneum* has lower susceptibility to *B. bassiana (*Akbar et al., 2004; Lord, 2005) compared to other beetles, including *Acanthoscelides obtectus,* and *Sitophilus oryzae (*Padin et al., 2002*)*. Similar results from the invertebrate microbiology group at QUT have shown that adults of *T. castaneum* are less susceptible to infection by *B. bassiana* and *M. anisopliae* than are adults of *R. dominica* when the fungal conidia are applied directly to the insects' cuticle. If these pathogens are to be used as effective biocontrol, it is important to understand the differences in infection and factors that may cause it. The objectives in this thesis are to examine in detail the initial stages of germination and growth of *B. bassiana* and *M. anisopliae* on the cuticle of *T. castaneum* and *R. dominica*, and to analyse the cuticular lipids that might affect these processes from the wings and elytra of *T. castaneum* and *R dominica* using GCMS. Results from this study offer the first report on the chemical composition of wing and elytra from *T. castaneum* and *R dominica*. The knowledge gained may aid in understanding the role of cuticular lipids in resistance to infection in some species, and be an initial step towards the improving the control of *T. castaneum* and *R. dominica* with entomopathogenic fungi.

# Chapter 2: The interaction between the cuticle of *Tribolium castaneum* and *Rhyzopertha dominica* and the germination of entomopathogenic fungi

**2.1. Abstract**. Two isolates of the entomopathogenic fungi *Metarhizium anisopliae* (Metchnikoff) and *Beauveria bassiana* (Bals) were cultured on cuticles (wings and elytra) of the pest beetles *Tribolium castaneum* (Herbst) and *Rhyzopertha dominica* (Fabricius).

The germination of the isolates and hyphal growth were observed using scanning electron microscopy. At 14 hours there was a significant and consistent reduction in both germination and length of hyphal growth in both species of fungi on elytra of *T. castaneum* compared to elytra of *R. dominica*.

An examination of the number of hyphal tips per conidium and number of appresoria showed few significant differences or consistent patterns between or within species with either fungi. However, there was a significantly higher mean number of appressoria per conidium on elytra of *R. dominica* than on elytra of *T. castaneum*.

The results support a hypothesis that reduced germination, growth of hyphae and formation of appressoria on the elytra of *T. castaneum* indicate a reduced susceptibility to infection by entomopathogenic fungi.

## 2.2. INTRODUCTION

*Tribolium castaneum (*Herbst) (Tenebrionidae, Coleoptera) and *Rhyzopertha dominica* (Fabricius) (Bostrichidae, Coleoptera) are common pests of grains and grain products that cause significant damage to the grain industry. Strains of *T. castaneum* and *R. dominica* are resistant to phosphine and methyl bromide, which are used as quarantine and pre-shipment for Australian grain exports, and this poses a significant threat to market access for wheat exports (Zettler & Cuperus, 1990; Collins et al., 1993; Jagadeesan et al., 2015; Jagadeesan, Nayak, et al., 2015). Alternative controls and options for use in resistance management strategies are urgently needed.

The entomopathogenic Hyphomycetes *Metarhizium anisopliae* (Metchnikoff) and *Beauveria bassiana* (Bals) (*Hypocreales: Clavicipitaceae)* are natural pathogens of insect species, and have been developed as biopesticides against a range of pests (Copping and Menn, 2000; Sun et al., 2012; Wilson et al., 2011). Isolates of *B. bassiana* have been found to be highly effective against several species of stored grain beetles (Lord, 2001; Padin et al., 2002; Throne & Lord, 2004) and have been successfully used to control *T. castaneum* and *R. dominica* in multiple tests (Lord, 2005; Lord, 2007; Moino Jr et al., 2002; Pedrini et al., 2010), either when applied directly to the insects or when mixed with food (Akbar et al., 2004; Padin et al. 2002). However, it was recently shown in this laboratory (Hauxwell, unpublished) that *R. dominica* is more susceptible to infection than *T. castaneum* by both *M.*

*anisopliae* and *B. bassiana*, but that the percentage of mortality following direct application of spores to the insect was low in both species of insect and with both species of fungi.

*M. anisopliae* and *B. bassiana* infect the insect by adhering to and penetrating the host insect's cuticle (Crespo & Juárez, 2000; St. Leger et al.,1987, 1992). The fungal spore attaches to the cuticle and germinates to form a germ tube and then an appressorium, by which the fungus penetrates through the insect cuticle using a combination of mechanical pressure and cuticle-degrading enzymes (Arruda et al., 2005, St. Leger et al., 1992). The fungus then grows as blastopores or vegetative hyphae within the insect (Hajek & St. Leger, 1994). After death of the host insect, and under the suitable conditions of humidity and temperature, vegetative hyphae emerge from the cadaver and conidiospores produced on the outside of the insect's body and are released to infect a new host (Hajek & St. Leger, 1994).

The insect cuticle presents a barrier at all stages of initial infection: adhesion, germination, growth and penetration (Pedrini et al., 2013). However, components of the cuticle, in particular, long chain alkanes, can promote infection as the fungus utilises them during germination and growth (Smith and Grula, 1981; Jarrold et al., 2007).

In beetles, wings are covered by the elytra, the structure of which is thicker and more typical of the cuticle on other body parts. In this study, the germination of conidiospores of the entomopathogenic *M. anisopliae* and *B. bassiana* on the wings and elytra of the two beetle species was observed using scanning electron microscopy. The research objective was to establish whether the fungal activities of *M. anisopliae* and *B. bassiana* conidia on cuticles of different body parts with different structure (wings and elytra) of both insect species could explain the

different susceptibilities of these insects; this was done by identifying differences in germination, germ tube growth (total hyphal length, and hyphal growth units) and appressoria formation.

## 2.3. MATERIALS AND METHODS

### 2.3.1. Insect culture

Adults of two species of grain beetles (*T. castaneum* and *R. dominica*) were obtained from the Department of Agriculture and Fisheries, Queensland. The insects were then reared at the Queensland University of Technology. Adult beetles were reared in jars, on organic flour (*T. castaneum*) and wheat grain (*R. dominica*) maintained at 26°C under a light/dark cycle of 12h (Konopova & Jindra, 2007).

### 2.3.2. Fungal isolates and culture

*M. anisopliae* isolate *M251-P* was obtained from the Queensland Department of Agriculture and Forestry. *B. bassiana* isolate *Bb.spw* was obtained from the QUT collection as single spore clones of an isolate from a sweet potato weevil. Both fungal isolates were used for germination and growth assays.

Fungal cultures for germination assays were grown on Saborauds Dextrose Ager with yeast extract (SDAY) incubated at 26°C under light for 14 days, and spores were collected by tapping them over a clean plastic funnel into 30ml sterilised plastic capped vials. The spores were then air-dried for 12h overnight in a safety cabinet with a sterilised air flow.

The suspension of *Bb.spw* was prepared by adding 2mg of fresh dry spores to 2ml of 0.05% Tween 80 to final concentrations of 2.1 x $10^4$ ml (germination assay) and 2.3 x $10^4$ ml (growth assay). *M. anisopliae* was prepared by adding 16.6 mg of dry spores to 16.6 mL of Tween 80. Suspensions were diluted in Tween 80 to final concentrations of 1.875x $10^6$ conidia ml (germination assay) and 1x $10^6$ conidia ml (growth assay). Final conidia concentration was determined by direct count using a haemocytometer.

### 2.3.3. Germination assays

Adult beetles of both species were removed from the rearing jars, placed in 30ml glass vials, and killed by freezing at -20°C for 12h. Wings and elytra were dissected under light microscopy. The wings and elytra were washed separately three times with sterilised water and sonicated for approximately 30 seconds to remove flour and other contaminants. The water was removed from the samples by pipette, and samples were then dried in a freeze drier (Alpha 1- 4 LD Plus) under vacuum at 0.05 mbar, with the condenser set at -55°C. After drying, the samples were weighed.

The fungal treatments of *M. anisopliae* and *B. bassiana* were used in germination assays. For each fungus, 10 replicates of wings and 10 replicates of elytra were used to assess the germination of fungal spores (5 replicates of wings and 5 replicates of elytra of each insect species). Germination data was collected after time 1 (14h post inoculation) and after time 2 (24 hours post inoculation) with fungi.

In Treatment 1 (*M. anisopliae*), at time zero, 10 replicates of wings and 10 replicates of elytra were placed on water agar plates. The replicates were then inoculated with 10µl of 1.9 x $10^6$ suspensions of *M. anisopliae* spores.

In Treatment 1 (*B. bassiana*), at time zero, 10 replicates of wings and 10 replicates of elytra were inoculated with 2.1 x $10^4$ conidia/ml spore suspension of *B. bassiana*.

Sterile distilled water was applied to five wings and elytra as a control. The treated wings and elytra were maintained at 100% humidity in sealed plastic containers lined with wet paper towels and incubated at 27°C under light for 14 and 24h.

### 2.3.4. Growth by entomopathogenic fungi assays

Ten replicates each of 5 wings and 5 elytra from each beetle species were inoculated as above with 10µl of 1x $10^6$ suspensions of *M. anisopliae* spores and 2.3 x $10^4$ conidia/ml spore suspension of *B. bassiana*, then maintained in a humidity chamber and incubated as above for 14 or 24 hours (25 wings and elytra of each beetle specie per fungal treatment per time point).

### 2.4. Scanning electronic microscopy (SEM)

Wings and elytra were removed at 14 and 24 h post inoculation. Each sample was sputter coated using a Leica Gold Coater and photographed under Zeiss Sigma Scanning electronic microscope under vacuum at 10–15 kv at the Central Analytical Electron Microscopy Facility at Queensland University of Technology.

Germinated and ungerminated spores, appressoria and total hyphal tips were counted either manually or by image processing and analysis software in Java format (Image J, Version 1.49), and hyphal length and branching were measured using image J software.

The total number of spores on each wing and elytron were calculated, followed by counting the germinated spores, and germination was recorded when a germ tube was observed. Germination at each time post inoculation was expressed as a percentage spore germination of total number of spores.

The total length of hyphal from each spore was measured as the sum of the length of the main hypha plus the length of the branches (Reichl et al., 1990).

## 2.5. Statistical analysis

Data were analysed using SPSS Statistics Version 22.

Total percentage germination on each insect body part at each time post inoculation for each fungus was calculated from the total number of germinated spores divided by the total number of spores (including the not-germinated spores). Germination data at 14 hours were first subjected to Arcsin transformed before analysis to check for normality. The data was tested for normality and the assumption of homogeneity of variance on the data of both fungi was tested using Shapiro-Wilk. The test indicated that the data was normally distributed.

Analysis of variance (one-way ANOV) was used to compare the means of total percentage germination for each insect body part. A two-way ANOVA was performed to investigate the combined effects of the two factors, 'insect species' and 'body part', on the percentage germination. The assumption of homogeneity of variances was tested based on Levene's $F$ test. Then, two-way ANOVA was performed to investigate the combined effects of insect species and body part on the percentage of germination in each fungus at 14 post inoculation.

An independent two-sample t-test was performed to compare the mean total hyphal length, and the percentage of hyphal tips that formed appressoria. A Chi-Square Test was performed to investigate the correlation between two categorical variables for the numbers of appressoria and hyphal tips.

## 2.6. RESULTS

### 2.6.1. Percentage germination

Both fungi had 100% germination on both insect body parts at 24 hours. Conidial germination was therefore compared at 14h after inoculation of both fungal isolates. A Levene's test was used to test the homogeneity of variances, and the result showed that the data rejected the null; the assumption of homogeneity of variances was not satisfied based on Levene's $F$ test, $F (3, 295) = 21.863$, ($p < 0.001$). Therefore, Further analysis was applied by transforming the data using ARSIN test, and then the transformed data was tested for homogeneity of variance using a Levene's test. The result indicated that the data was homogeneous $F (7, 28) = 0.815$, ($p = 0.583$).

The mean percentage germination of *M. anisopliae* on the wings of *T. castaneum* was 98% (standard deviation SD = 0.0893), which was significantly higher ($p = 0.001$) than on the elytra (94%, Standard deviation = 0.1734) (Table 2.1). In contrast, the mean percentage germination of *M. anisopliae* conidia on the wings of *R. dominica* was 92% (SD = 0.1960), which was significantly lower ($p = 0.008$) than on the elytra (100% at 14 hours, SD = 0.0168).

When comparing germination on wings and elytra in the two beetle species, the germination of *M. anisopliae* conidia on the wings of *T. castaneum* was significantly higher (98% +- 0.09%) than on the wings of *R. dominica* (92% =/- 0.2%, $p = 0.001$). However, mean percentage germination of *M. anisopliae* conidia

on the elytra of *T. castaneum* was significantly lower (94% +/- 0.17%) than on the elytra of *R. dominica* (100% +/- 0.02%, p = 0.026).

**Table 2.1.** Mean and standard deviation of percentage germination of *M. anisopliae* conidia on wings and elytra of *T. castaneum* and *R. dominica* at 14h post inoculation.

| Insect | Body part | Mean percentage germination (and standard deviation) |
|---|---|---|
| *T. castaneum* | wing | 98 ($\pm$0.09) a |
| | elytra | 94 ($\pm$0.17) b |
| *R. dominica* | wing | 92 ($\pm$0.20) c |
| | elytra | 100 ($\pm$0.02) d |

The mean percentage germination of conidia of *B. bassiana* on the wings of *T. castaneum* was 64% (SD=0.17), which was significantly greater ($p < 0.001$) than on the elytra (46%, SD=0.19) (Table 2.3). In contrast, the mean percentage germination of *B. bassiana* on the wings of *R. dominica* (68%, SD=0.22) was not significantly different to that on the elytra (75%, SD=0.10).

There was no significant difference between the mean percentage of spore germination on the wings of *T. castaneum* and *R. dominica*. In contrast, the germination of *B. bassiana* spores on the elytra of *T. castaneum* was significantly lower (46%, SD=0.19) than on the elytra of *R. dominica* (75%, SD=0.1, $p < 0.001$).

Overall, the germination of *B. bassiana* spores on the elytra of *T. castaneum* was significantly lower than on the wings and elytra of *R. dominica* and on the wings of *T. castaneum*.

**Table 2.3** Mean percentage of germination and standard deviation of *B. bassiana* conidia on wings and elytra of *T. castaneum* and *R. dominica* at 14h post inoculation.

| Insect | Body part | Mean percentage germination (and standard deviation) |
|---|---|---|
| *T. castaneum* | wing | 64 (±0.17) ac |
|  | elytra | 46 (±0.19) b |
| *R. dominica* | wing | 68 (±0.22) ac |
|  | elytra | 75 (±0.10) c |

## 2.6.2. Growth of fungal hyphae

**2.6.2.1. Total hyphal length.** After germination, both fungi produced a single, short germ tube at 14h post inoculation. By 24h post inoculation, both fungi colonised the cuticles with extensive mycelial growth.

**Total hyphal length of *M. anisopliae* at 14h on both insect body parts.**

The mean total hyphal length per conidium is given in Table 2.5.

**Table 2.5.** The mean total hyphal length and standard deviation of *M. anisopliae* wings and elytra of *T. castaneum* and *R. dominica* at 14h post inoculation.

| | Total hyphal length in µm (and standard deviation in µm) | |
|---|---|---|
| Body parts | *Tribolium castaneum* | *Rhyzopertha dominica* |
| Wings | 1460 (±1802) | 983 (±1029) |
| Elytra | 199 (±98) | 792 (±658) |

The mean total hyphal length on the wings of *T. castaneum* were significantly greater than on the elytra by 1261µm (95% CI: 859 – 1664 µm, two- sample t-test, ($p < 0.001$)). In contrast, although the mean length of hyphae on the elytra of R. dominica was also less than on the elytra, the difference was not significant ($p > 0.05$) at 14h post inoculation.

A third independent t-test showed that the mean total hyphal length of *M. anisopliae* spores on *T. castaneum* wings at 14 hours was significantly larger than on the wings of *R. dominica* by 477 µm (95% CI: 18 – 936 µm, two- sample t-test, ($p < 0.05$).

Finally, a fourth independent t-test showed that the mean total hyphal length of *M. anisopliae* on the elytra of *R. dominica* was longer than the mean total length on the elytra of *T. castaneum* by 594 µm (95% CI: 444 - 743µm, two- sample t-test, $p < 0.001$).

Overall, the growth of *M. anisopliae* on the elytra of *T. castaneum* was the shortest for the samples treated, and growth on wings of both species was greater than on elytra.

**Total hyphal length of *B. bassiana* at 14h on both insect body parts.**

The mean total hyphal length is given in Table 2.7.

**Table 2.7.** The mean total hyphal length and standard deviation of *B. bassiana* on wings and elytra of *T. castaneum* and *R. dominica* at 14h post inoculation.

| | Total hyphal length (and standard deviation) in µm | |
|---|---|---|
| Body parts | *T. castaneum* | *R. dominica* |
| Wings | 12 (±15) | 47 (±48) |
| Elytra | 38 (±35) | 109 (±81) |

The mean total hyphal lengths of *B. bassiana* on the elytra of *T. castaneum* were significantly longer than on the wings, by 26µm (95% CI: 17 - 35µm), two-sample t-test, ($p < 0.001$). A second independent sample t-test showed that the mean total hyphal length of *B. bassiana* on *R. dominica* elytra was significantly larger than on the wings, by 62µm (95% CI: 45 - 79µm), two-sample t-test, $p < 0.001$).

The mean total hyphal length of *B. bassiana* spores on *R. dominica* wings at 14 hours was significantly longer than on the wings of *T. castaneum* by 34.75µm (95% CI: 27.19 - 42.31µm, two-sample t-test, ($p < 0.001$).

A fourth independent t-test showed that the mean total hyphal length of *B. bassiana* on the elytra of *R. dominica* was longer than the mean total length on the elytra of *T. castaneum* by 70 µm (95% CI: 53 - 89µm, two-sample t-test, $p < 0.001$).

**Total hyphal length of *B. bassiana* at 24h on both insect body parts.**

The mean total hyphal length in each insect, is given in Table 2.8.

**Table 2.8.** The mean total hyphal length of *B. bassiana* and standard deviation on wings and elytra of *T. castaneum* and *R. dominica* at 24h post inoculation.

| | Total hyphal length in µm | |
|---|---|---|
| Body parts | *T. castaneum* | *R. dominica* |
| Wings | 102 (±103) | 80 (±47) |
| Elytra | 212 (±262) | 164 (±186) |

At 24 hours post inoculation, the mean total hyphal length per spore of *B. bassiana* on the elytra of *T. castaneum* were significantly longer than on the wings, by 110µm (95% CI: 55- 166µm, two-sample t-test, ($p < 0.001$). A second independent t-test also showed that the total hyphal length of *R. dominica* elytra is

significantly longer than the wings by 84µm (95% CI: 45 - 122µm), two- sample t-test, ($p < 0.001$).

In contrast, the mean length of hyphal growth of *B. bassiana* on the elytra of *T. castaneum* was highly variable and was not statistically significant from that on R. dominica ($p = 0.060$).

**Number of tips**

The number of tips formed from each spore is an indication of the pattern of branching in the hyphae.

Pearson Chi-Square test performed on the number of hyphal tips of mycelium per spore of *M. anisopliae* showed that the number of tips formed on the wings is significantly higher than the elytra of *T. castaneum* ($p = 0.002$) In contrast, the number of hyphal tips of *M. anisopliae* was found to be statistically higher on the elytra of *R. dominica* ($p < 0.001$) than the wings.

A Pearson Chi-Square test was performed on the number of hyphal tips of *M. anisopliae at* 14h p.i on the elytra of both insect species. On the elytra of *T. castaneum*, 75.7% of conidia were observed to have one hyphal tip, 24.3% of conidia had two hyphal tips, whereas on the elytra of *R. dominica,* 67.5% of conidia had two hyphal tips, followed by 30% with one hyphal tip and 2.5% of conidia had three hyphal tips. The difference in mean hyphal tip counts for the elytra of the two insects was statistically significant ($p < 0.001$), with greater number of tips per conidium on *R. dominica* that on *T. castaneum*.

The number of hyphal tips per conidium of *B. bassiana* on the wings of *T. castaneum* was significantly greater than on the elytra (Pearson Chi-Square test, $p < 0.001$). The difference between the number of hyphal tips of *B. bassiana* at 14h p.i on wings and

elytra of *R. dominica* was not statistically significant. A total of 90.1% of conidia had one hyphal tip on *R. dominica* wings, and 9.9% had from two to five hyphal tips. Whereas, on the elytra of *R. dominica,* 82.7% of conidia had one hyphal tip, and 17.3% had from two to eight hyphal tips.

Comparing the number of hyphal tips of *B. bassiana* at 14h p.i. on the wings of both insects 99.4% of conidia had one hyphal tip on the wing of *T. castaneum* and none had more than one*,* whereas on the wing of *R. dominica*, the number of conidia that had one hyphal tip was 90.1%, but 9.9%, of conidia had from two to five hyphal tips. The mean number of hyphal tips per conidium of *B. bassiana* on the wings of *R. dominica* 14h p.i. was significantly higher than on *T. castaneum* ($p < 0.001$).

Comparison of the number of tips per conidium on elytra of both species showed no significant difference between insect species at either 14 hours or 24 hours post inoculation, and indeed at 24 hours there was no significant difference in the mean number of tips per conidium of *B. bassiana* in either of the insect species or body parts.

**The formation of fungal appressoria**

Appressorium formation in *M. anisopliae* conidia was seen at 14h post inoculation on both wings and elytra (Figures 2.1a, 2.2c), whereas few were seen in *B. bassiana* conidia until 24h (Figure 2.1b).

At 14h post inoculation, each conidium of *B. bassiana* produced only one germ tube (Figure 2.1a), whereas some conidia of *M. anisopliae* produced long germ tubes with variation in their length prior to appressoria formation (Figure 2.2c, f). The number of fungal appressoria differed for the two isolates on both insect species and their body parts.

One or two appressoria per conidium of *B. bassiana* were produced at 24h at the end of long germ tubes on wings and elytra of both insect species (Figure 2.1). Very few conidia (a total of 3 across all replicates) of *M. anisopliae* were observed on elytra at 24 hours, but those that were seen produced two or more appressoria at the end of each long germ tube on the wings and the elytra of *T. castaneum* (Figure 2.2f and Figure 2.3d).

At 24h pi, *B. bassiana* produced one or two appressoria per spore at the end of germ tubes on the wings and elytra of *T. castaneum* and *R. dominica*, whereas *M. anisopliae* formed two or more appressoria per spore at 24h p.i.

The mean number of appressoria per spore of *M. anisopliae* on *T. castaneum* body parts, on *R. dominica* body parts, and on the elytra of both insect species, was not statistically significant at 14 hrs post inoculation.

The number of appressoria per conidium of *B. bassiana* on *T. castaneum* at 14h was significantly higher on the elytra of *T. castaneum* than on the wings, (Pearson Chi-Square test, $p < 0.001$). The number of appressoria was significantly higher (77.9%) on the wings of *R. dominica* than on the elytra (61.5%) (Pearson Chi-Square test, $p < 0.025$).

On the wings of *T. castaneum*, the majority of the *B. bassiana* conidia at 14h (97.4%) had no appressoria although a small percentage of conidia had one, two, or six appressoria (1.9%, 0.6%, and 0, 6% respectively). In contrast, a significantly greater mean number of appressoria per conidium were observed on the wings of *R. dominica:* 70.3% of conidia had one appressorium, but 1.7% had three appressoria ($p < 0.001$).

Comparing the appressoria of *B. bassiana* on the elytra of both insect species at 14h, 79.1% of conidia has no appressoria on the elytra of *T. castaneum,* 16.4% had

one appressoria per conidium and only 3% of conidia had two appressoria, whereas on the elytra of *R. dominica,* half of the samples had one appressoria (55.8%), but 4.18% had from two to five appressoria. The differences in number of appressoria between the elytra of *T. castaneum* and *R. dominica* was significantly higher on the elytra of *R. dominica than* the elytra of *T. castaneum* ($p < 0.001$).

Enzyme degradation was apparent around both conidia and germ tubes of *B. bassiana* (Figure 2.1d), and *M. anisopliae* (Figure 2.2d). At 24h, both fungi produced evidence of cuticular degradation during the hyphal elongation process on the insect cuticle (Figure 2.2e).
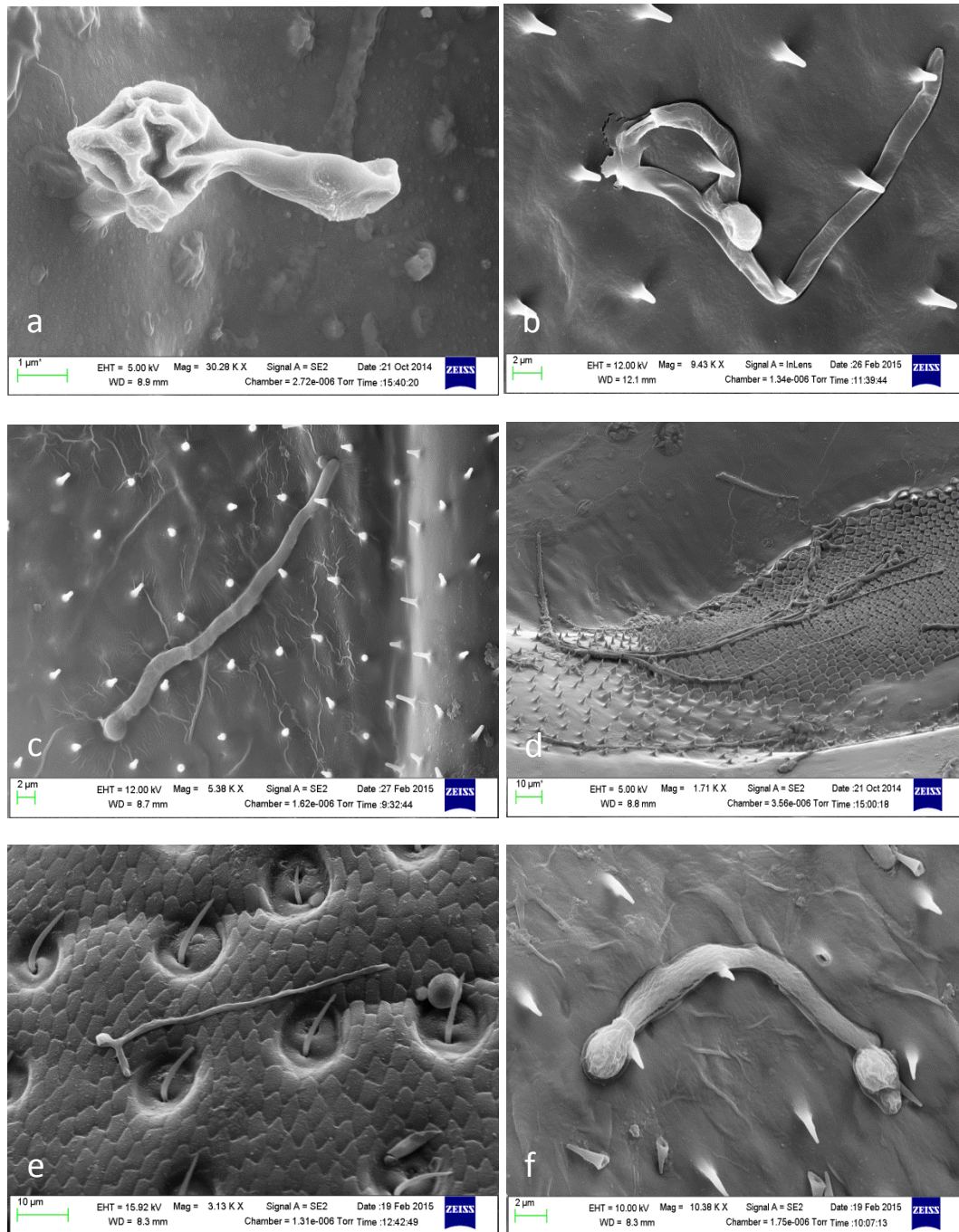
Figure.2.1. (a, b) Conidia of *B. bassiana* on the wing of *R. dominica* 14 and 24h post inoculation respectively. (c) *B. bassiana* 24h on the wing of *R. dominica*. (d) *B. bassiana* on the elytra of *R. dominica* 24h p.i. (e) *B. bassiana* 24h on the elytra of *T. castaneum*. (f) *B. bassiana* and appressorium penetration and enzyme degradation on the wing of *T. castaneum* 24h p.i.
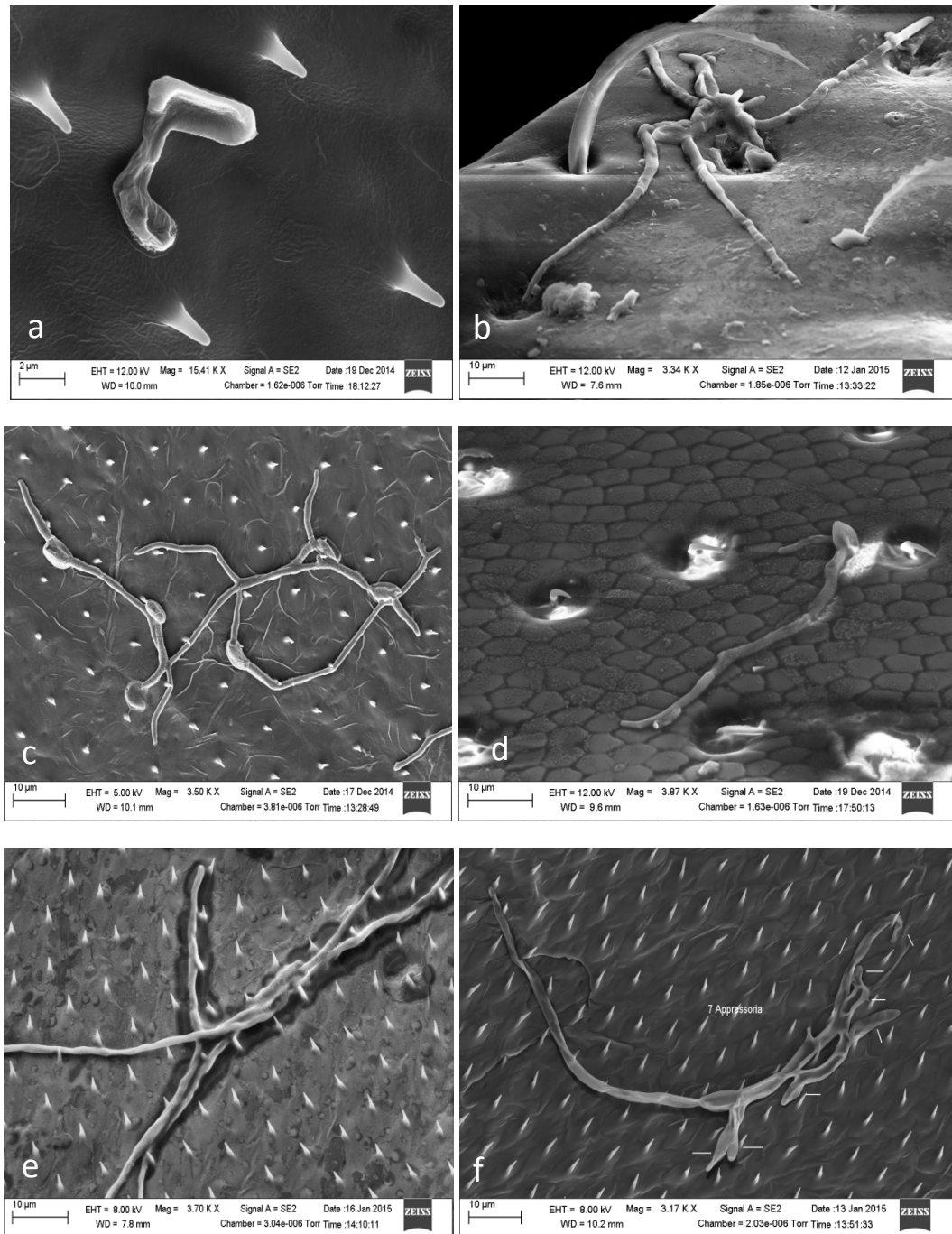
Figure.2.2. (a) Conidia of *M. anisopliae* germinated on *R. dominica* wings 14h post inoculation. (b) Conidia on *R. dominica* elytra 14h post inoculation with *M. anisopliae.* (c) Conidia spore on *T. castaneum* wing 14h post inoculation with of *M. anisopliae.* (d) Conidia spore on *T. castaneum* elytra 14h post inoculation with of *M. anisopliae.* (e) *M. anisopliae* enzyme degradation on the wing of *T. castaneum.* (f*) M. anisopliae* produced two or more appressoria at the end of each long germ tube on the wing of *T. castaneum.*

Figure.2.3. Conidia of *M. anisopliae* development on the elytra of *T. castaneum*, 24h post inoculation. (a) *M. anisopliae* elongation and hyphal growth on the surface. (b) Appressorium formed out of signal germ tube. (c) Appressoria penetrated the cuticle via enzyme degradation. (d) Appressorium degrades the cuticle through enzyme degradation, mechanical pressure.

# DISCUSSION

This study found that the cuticles of both insects have an effect on fungal germination, hyphal growth, and on the formation of appressoria.
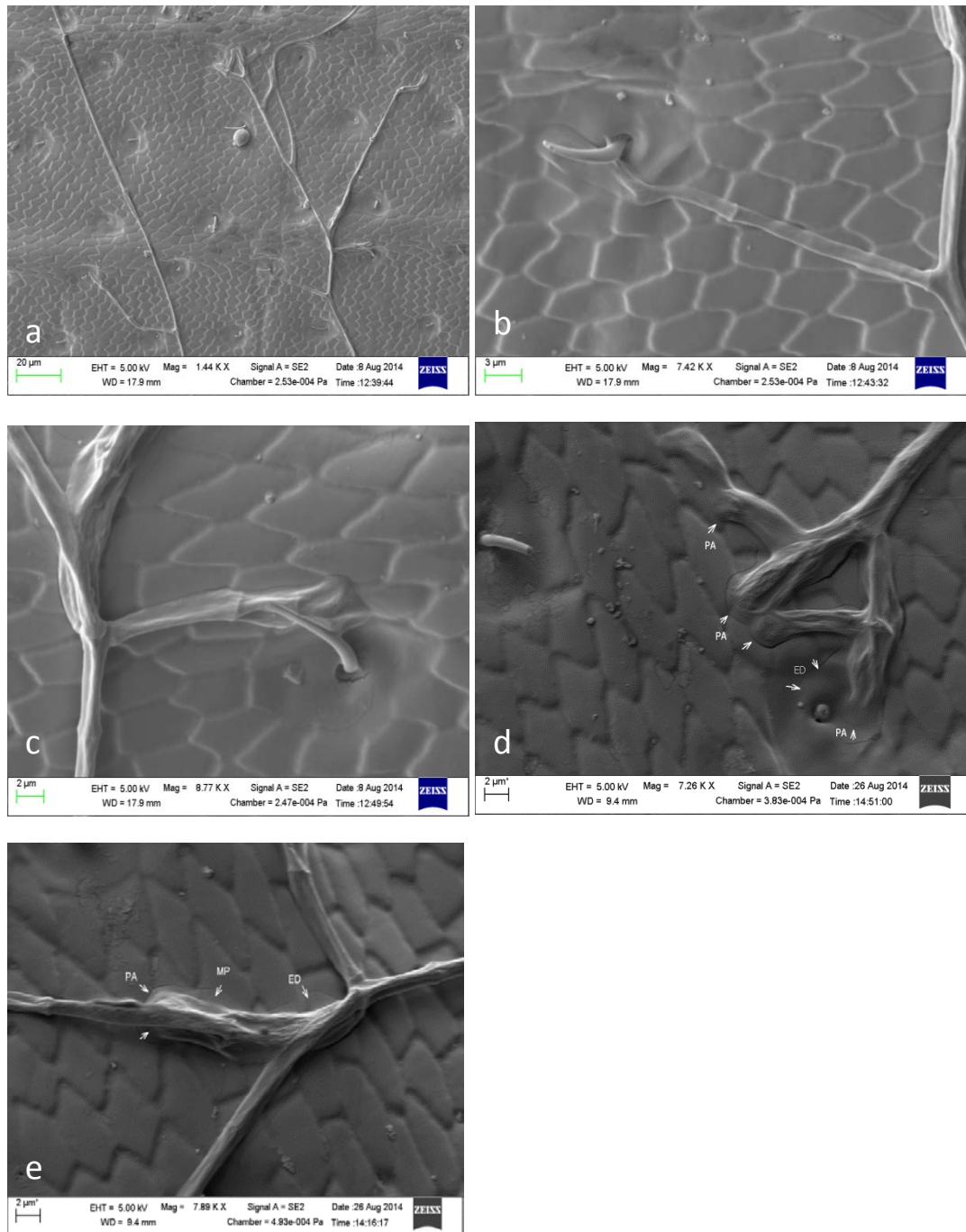
The germination of conidia of both fingal species was significantly less on elytra of *T. castaneum* than on the elytra of *R. dominica* at 14hrs post inoculation, although germination was 100% in both species of fungi and on all body parts at 24hours. At 14 hours post inoculation there was a significant reduction in hyphal length in both species of fungi on elytra of *T. castaneum* than on elytra of *R. dominica*. There were significantly fewer appressoria per conidium observed in *B. bassiana* on the elytra of *T. castaneum* than on elytra of *R. dominica*

A comparison of patterns of germination, growth and formation of appressoria on wings both within and between species was less consistent and frequently not significantly different. However, percentage germination of both fungal species at 14 hours post inoculation was significantly higher on wings in *T. castaneum* than on elytra, and significant higher on elytra than on wings in *R. dominica*. There were differences in hyphal growth between the two species, with longer growth of *B. bassiana* on elytra than on wings of both insect species, but, in contrast, hyphal growth of *M. anisopliae* was longer on wings than on elytra in both insect species at 14 hours post inoculation.

Reduced rate of germination, hyphal growth and formation of appressoria may be an indicator of reduced susceptibility to infection. Reduced germination and appressorium production by conidia of *M. anisopliae* were observed on the cuticle of *Calliphora vomitoria* than on the more susceptible host insects (*Manduca sexta*) (St Leger et al 1987). Similarly, it has been sugested that the formation of appressoria by *M. anisopliae* is the main factor in the virulence of this fungus (Neves and Alves, 2004).

Overall, the results in this chapter support a hypothesis that reduced germination, growth of hyphae and formation of appressoria on the elytra of *T. castaneum* underly a reduced susceptibility to infection by entomopathogenic fungi.

In this study, the conidia of both fungal isolates penetrated the cuticles at 24h post inoculation and *M. anisopliae* induced enzymatic degradation. Fungal production of degradative enzymes such as chitinases, proteases, and lipases, are important to the invasion process on the insect cuticle during the infection stages (Arruda et al., 2005; Da Silva et al., 2010; Leemon & Jonsson, 2012; St. Leger et al. 1987). These enzymes digest the major component of the host insect cuticle during the infection process (St. Leger et al., 1987), and the proteases digest the protein in the insect host cuticle to facilitate passage through the insect cuticle (Leemon & Jonsson, 2012). In particular, it has been suggested that hydrocarbons in the insect cuticle may influence germination and infection (Jarrold *et al* 2007, St Leger *et al* 1988) and the differences in the composition of the cuticle in *T. castaneum* and *R. dominica* were therefor analysed in the next chapter.

# Chapter 3: Comparative chemical analysis of wings and elytra in *Tribolium castaneum* and *Rhyzopertha dominica*

**3.1 Abstract**- Grain beetles are distributed worldwide and include the economically important species *Tribolium castaneum (*Herbst) (Tenebrionidae, Coleoptera) and *Rhyzopertha dominica* (Fabricius) (Bostrichidae, Coleoptera). Both species are resistant to chemical insecticides and, in particular, to phosphine and methyl bromide, which are used as quarantine and pre-shipment treatments for Australian grain exports. Alternative controls and options for resistance management are urgently needed.

Entomopathogenic fungi have potential as biopesticides against these pests, but establishment of infection may be inhibited (or stimulated) by the composition of the cuticle, and in particular by the lipid compositions. Crude extracts of lipids from two different body parts (wings and elytra) of adult *T. castaneum* and *R. dominica* were made using non polar (hexane) and polar (chloroform) solvents and analysed using gas chromatography mass spectrometry. Differences were observed between species and between wings and elytra. Significantly, *T. castaneum* elytra were found to contain fewer long chain alkanes, which are reported to support spore germination and early infection, and to contain alcohols and one free fatty acid (C18), which are reported to inhibit fungal growth, than the elytra of *R. dominica* These differences in composition appear to be correlated with differences in germination and growth, as reported in Chapter 2.

## 3.2 INTRODUCTION

*Tribolium castaneum* (Herbst) (Tenebrionidae, Coleoptera) and *Rhyzopertha dominica* (Fabricius) (Bostrichidae, Coleoptera) are common pests of grains and grain products that cause significant damage to the grain industry (Padin et al., 2002; Campbell & Runnion, 2003). They are resistant to chemical insecticides including malathion, dichlorovs, and to chlorpyrifos-methyl (Zettler & Cuperus, 1990). In Australia, strains of both species are resistant to phosphine and methyl bromide, which are used as quarantine and pre-shipment treatments for Australian grain exports, and thus pose a significant threat to market access for grain exports (Zettler & Cuperus, 1990; Collins et al., 1993). Alternative controls and options for use in resistance management strategies are urgently needed.

The entomopathogenic fungi *M. anisopliae* (Metchnikoff) and *B. bassiana bassiana* (Bals) (*Hypocreales: Clavicipitaceae)* have been developed as biopesticides against a range of pests. (Butt & Beckett, 1995; Copping & Menn, 2000; Moino Jr et al., 2002). These pathogens infect the host following adhesion of conidiospores to the cuticle and subsequent germination and penetration of the host cuticle (St. Leger et al., 1987, 1992; Crespo & Juárez, 2000).

The insect cuticle is covered by a thin layer of lipids consisting of a variety of compounds such as hydrocarbons (Baker et al., 1978; Cvačka et al., 2006), wax esters (Nelson et al., 2000), fatty alcohols and free fatty acids (Gołębiowski et al., 2008; Gołębiowski et al., 2012). Long chain alkanes, have been shown to promote germination and infection (St. Leger et al., 1988). Crude polar extracts from locust wings containing fatty acids, fatty acid esters, glucose, amino acids and peptides were shown to be strong promoters of spore germination in *M. anisopliae,* while non-

polar extracts, including long-chain alkanes and other waxes, promoted hyphal growth and subsequent infection (Jarrold et al., 2007). Some compounds, such as proteases, quinones, and longer chain hydrocarbons may reduce infection by microorganisms through inhibition of cuticle degradation by fungal enzymes (St. Leger et al.,1987, 1992; Crespo & Juárez, 2000; Pedrini et al., 2013).

The cuticular lipids of insects can vary depending on species, age, sex, and developmental stage (Cerkowniak et al., 2013) and can also be affected by environmental conditions such as temperature, humidity and the availability of food (Szafranek et al., 2012; Cerkowniak et al., 2013). There are, in addition, significant differences in composition between structures such as wings and elytra, but the majority of studies have focused on analysis of wings, as these are easily isolated from the insect and have a large cuticle area to mass ratio (St. Leger et al., 1987; Jarrold et al., 2007). Infection by fungi in beetles is more likely to occur on the head, thorax or abdomen, but the cuticle of these is more difficult to isolate from internal tissues. In beetles, the wing is covered by the elytra, the structure of which is thicker and more typical of the cuticle on other body parts and may thus be used as a representative of these structures.

There have been few studies on the composition of the hydrocarbons in *T. castaneum* and *R. dominica*. Baker et al., (1978) studied the cuticular hydrocarbons of *T. castaneum* adults, and Howard & Liang, (1993) analysed larvae of *R. dominica,* using GCMS. In this study, the hydrocarbons extracted from wing and elytra in both *T. castaneum* and *R. dominica* were compared as part of a larger study on the comparative pathology of entomopathogenic fungi in the two species.

### 3.3. METHODS AND MATERIALS

#### *3.3.1. Insect cultures.*

*T. castaneum* and *R dominica* were obtained from The Department of Agriculture and Fisheries, Queensland and subsequently reared in glass jars on either organic flour (*T. castaneum* ) or wheat (*R dominica*) at 30°C under a light/dark cycle of 12h. Adult beetles were harvested by sieving and placed in 30ml glass vials, and killed by freezing at -20°C for 12h. Wings and elytra of adult beetles were dissected under a light microscope and stored at -20°C. The wings and elytra were cleaned by washing three times with sterilised water and sonication for approximately 30 seconds. The water was then removed by pipetting and the insect material freeze-dried overnight and weighed.

#### *3.3.2. Chemical materials.*

Selection of solvents: The wax stearyl stearate was used as a standard and solubility tested in methanol (99.8%), pentane (89.9%), n- Hexane (98.8%), and chloroform (99.8%) (Sigma Aldrich). Two solvents, n- Hexane (98.8%) and chloroform (99.8%), were selected.

*Wings and Elytra Extraction.* Crude preparations were extracted from 1mg of each dried wing or elytrum sample using n-hexane or chloroform. Samples were refluxed with 1.5 ml of hexane or chloroform for 1h at 80°C, over a water bath. Extracts were then cooled to room temp, filtered through a syringe filter (Micro Analytix Pty Ltd-Hydrophilic PTFE, NEW Australia), weighed, and the refluxed samples were then washed with a further 1 ml of solvent. The resulting extract was dried under a stream of nitrogen, reweighed and stored at -80°C until analysis.

### 3.3.3. Derivatisation.

The crude chloroform extracts were subjected to derivatisation by saponification with 1ml of KOH in methanol and heating at 70°C on a dry heating block for 30min, then cooling to room temperature. The fatty acids formed were methylated by adding 2ml of BF3 (boron trifluoride) in methanol and heated for 1h at 70°C. One ml of water and 1ml of chloroform were added. The chloroform layer was removed by pipetting with a glass pipette.

A standard of n-alkanes (C10 – C40; Restek), and FAME mix (F.A.M.E. Mix, C8-C24; Sigma-Aldrich, Australia), was used to calculate the retention index values. The negative control procedure was used to identify the contaminating compounds when identified by Gas Chromotography – Mass Spectrometry (GCMS).

### 3.3.4. Gas Chromatography – Mass Spectrometry (GCMS).

Samples were run on a Shimadzu GCMS QP2010-Ultra, fitted with an Rxi-5MS column (length: 30m, internal diameter: 0.25mm, film thickness 25µm), carrier gas of helium. One microliter of sample was injected into the injector in splitless mode, with a pressure of 81 kPa, and a column flow of 1.4ml/min. The injector temperature was at 280 °C, and the column temperature varied from 50 to 345 °C, increasing at a rate of 10 °C / min. Mass spectra were collected from 4 minutes to the end of the run in scan mode between 35 and 600 m/z, collecting 10 spectra / second. Compounds were identified by comparison of the spectra to the NIST library v11, and by comparison to literature relative retention indexes and a standard of n-alkanes (C10 -40; Restek)

### 3.3.5. Compound identifications and Retention Time Index calculation.

The most common approach to identifying the chemical compound on GCMS is comparison of the recorded mass spectra with standard mass spectral libraries. However, to aid identification, the retention index was calculated. A standard of n-alkanes was used to identify compounds and the retention indices were calculated using the following equation:

$$I = 100 \times \left[ n + (N - n) \frac{t_{r(unknown)} - t_{r(n)}}{t_{r(N)} - t_{r(n)}} \right]$$

Where $I$ = the adjusted Kovats retention index, $n$ = the number of carbon atoms in the smaller n-alkane, $N$ = the number of carbon atoms in the larger n-alkane, $t_r$ = the retention time. The compounds were identified by calculation of retention index and also subsequent verification of the values by comparison with previous literature RI values. This allows the retention index information to be complimented by the GC-MS library search and provide a high level of confidence. Small peaks were not identified due to the small amount of biological materials and resulting low quantity on the GCMS.

## 3.4. RESULTS

The wax standard, stearyl stearate, was found to dissolve in hexane, chloroform and pentane, but not in methanol. Chloroform and hexane were selected as solvents. The compounds identified in the n-hexane extraction varied between species and body parts. The most abundant compounds were n-alkanes, esters, and methyl alkanes.

A total of 9 n-alkanes were identified in the n-hexane extracts, of which 8 were present in extracts from wings and 5 in extracts from elytra. Two alkanes, C20

n-eicosane and C22 n-docosane, were found only on *T. castaneum* wings. The two longest chain n-alkanes, C40 n-Tetracontane and C44 n-Tetratetracontane, were extracted from the wings of both species.

The elytra of *R. dominica* contained 5 n-alkanes, of which one, C26 n-hexacosane, was unique to extracts of elytra from *R. dominica*, while 4 were found in *T. castaneum*, but only in extracts from wings. In contrast, only two n-alkanes, C30 n-Triacontane and C36 n-Hexatriacontane, were found in the extracts of elytra from *T. castaneum*.

Esters were found in both species. Two methyl esters, C17 Hexadecanoic acid, methyl ester and C19 oleic acid, methyl ester were found in extracts from both species and in both wings and elytra. Hexadecanoic acid was found in both wings and elytra of both species, and oleic acid in all samples except wings of *T. castaneum*.

In contrast, two ethyl esters were only found in wings, not in elytra. C20 ethyl 9-octadecenoate /elaidic acid, ethyl ester was detected only in wings of *R. dominica* and C24 docosanoic acid, ethyl ester only in wings of *T. castaneum*.

A small number of methyl alkanes were observed, with differences in presence between species: 2-methylhexacosane was found in both wings and elytra of *R. dominica* but only in the wings of *T. castaneum*; 2-methyl-4-tetradecene was found in both wings and elytra of *R. dominica* but not in *T. castaneum*; 3-methylheneicosane was found only in *T. castaneum* elytra and was the only methyl alkane found in *T. castaneum* elytra.

Unsaturated ketone and amides, fatty acids, esters, and alcohols were identified for the first time in *T. castaneum* and *R. dominica.* Alcohols were detected only in *T. castaneum*: C16 Z,Z-8,10-Hexadecadien-1-ol in wing and C32 1-

octacosanol in elytra. The unsaturated ketone undec-2-en-8-one and the amides 9-octadecenamide and (Z)-13-docosenamide were found only in *R. dominica* and only in wings.

## 3.4. TABLE1. COMPOUND CLASSES WITH CARBON NUMBERS OF WINGS AND ELYTRA OF Tribolium castaneum AND Rhyzopertha dominica EXTRACTED FROM THE REFLUXED HEXANE PROCEDURE

| Chemical class | Compound name | Carbone number | *Tribolium castaneum* | | *Rhyzopertha dominica* | |
|---|---|---|---|---|---|---|
| | | | wing | elytra | wing | elytra |
| Alcohols | 1-Octacosanol | C32H66O | ✓ | | | |
| | Z,Z-8,10-Hexadecadien-1-ol | C16H30O | | ✓ | | |
| Esters | Hexadecanoic acid, methyl ester | C17H34O2 | ✓ | ✓ | ✓ | ✓ |
| | Oleic acid, methyl ester | C19H36O2 | | ✓ | ✓ | ✓ |
| | Ethyl 9-octadecenoate /Elaidic acid, ethyl ester | C20H38O2 | | | ✓ | |
| | Docosanoic acid ethyl ester | C24H48O2 | ✓ | | | |
| Fatty acids | 6-Octadecenoic acid | C18H34O2 | | ✓ | | ✓ |
| n-alkane | n-Eicosane | C20H42 | ✓ | | | |
| | Docosane | C22H46 | ✓ | | | |
| | Tetracosane | C24H50 | ✓ | | | ✓ |
| | n-Hexacosane | C26H54 | | | | ✓ |
| | n-Octacosane | C28H58 | ✓ | | ✓ | ✓ |
| | n-Triacontane | C30H62 | ✓ | ✓ | | ✓ |
| | n-Hexatriacontane | C36H74 | ✓ | ✓ | ✓ | ✓ |
| | n-Tetracontane | C40H82 | ✓ | | ✓ | |
| | n-Tetratetracontane | C44H90 | ✓ | | ✓ | |
| Methyl alkane | 2-Methyl-4-tetradecene | C15H30 | | | ✓ | ✓ |
| | 3-Methylheneicosane | C22H46 | | ✓ | | ✓ |
| | 2-methylhexacosane | C27H56 | ✓ | | ✓ | ✓ |
| unsaturated ketone | undec-2-en-8-one | C11H20O | | | ✓ | |
| unsaturated amides | 9-Octadecenamide | C18H35NO | | | ✓ | |
| | (Z)-13-Docosenamide | C22H43NO | | | ✓ | |

The results from the chloroform derivatisation procedure indicated some similarity and differences between the two body parts of both insect species (Table 2). Two additional n-alkanes were found in extracts from *R. dominica, C32* n-dotriacontanein wing and elytra and C34 n-tetratriacontane only in extracts from wings, but neither were found in *T. castaneum*. Hexatriacontane was not found in any samples from *T. castaneum*, though it was present in both wing and elytra in the hexane extracts. N-triacontane was the only n-alkane identified in derived samples from *T. castaneum*, and, as in the hexane extracts, was detected in both wings and elytra of *T. castaneum* and elytra of *R. dominica*.

Four alcohols (C11, C12, C15, and C19) not found in the hexane extracts were only identified in derived samples, and only in *T. castaneum* extracts table 2. C11 10,11-Epoxy-n-undecan-1-ol, C15 11-dodecen-1-ol, 2,4,6-trimethyl-, C19 10-nonadecanol were found in extracts from elytra, and C12 only in extracts from wings.

Surprisingly, only three esters were detected in derived samples, although methyl esters would be the expected product of derivatisation; C16 derivative hexadecanoic acid, methyl ester and C18 derivative methyl stearate were found in extracts from both species and in both wings and elytra. However, C18 derivative 9,12-octadecadienoic acid (Z,Z)-, methyl ester was present only in the elytra of *T. castaneum*.

| Chemical class | Compound name | Carbone number | *Tribolium castaneum* | | *Rhyzopertha dominica* | |
|---|---|---|---|---|---|---|
| | | | wing | elytra | wing | elytra |
| Alcohols | 10,11-Epoxy-n-undecan-1-ol | C11H22O2 | | ✓ | | |
| | 1-Dodecanol | C12H26O | ✓ | | | |
| | 11-Dodecen-1-ol, 2,4,6-trimethyl- | C15H30O | | ✓ | | |
| | 10-Nonadecanol | C19H40O | | ✓ | | |
| Esters | Hexadecanoic acid, methyl ester | C17H34O2 | ✓ | ✓ | ✓ | ✓ |
| | 9,12-Octadecadienoic acid (Z,Z)-, methyl ester | C19H34O2 | | ✓ | | |
| | Methyl stearate | C19H38O2 | ✓ | ✓ | ✓ | ✓ |
| n-alkane | Tetracosane | C24H50 | ✓ | | | ✓ |
| | n-Hexacosane | C26H54 | | | | ✓ |
| | n-Octacosane | C28H58 | ✓ | | ✓ | ✓ |
| | n-Triacontane | C30H62 | ✓ | ✓ | | ✓ |
| | n-Dotriacontane | C32H66 | | | ✓ | ✓ |
| | n-Tetratriacontane | C34H70 | | | ✓ | |
| | n-Hexatriacontane | C36H74 | ✓ | | ✓ | ✓ |
| Methyl alkane | 2-methylhexacosane | C27H56 | ✓ | | ✓ | ✓ |

***Contaminating compounds***.

One common contaminant was detected; 1,3-benzenedicarboxylic acid, dimethyl ester was present in *T. castaneum* from the derivatisation process. This compound is likely be present from the solvents due to plasticised of the solvent bottle.

It has been suggested by Stein, (1999) that the number of the matching factors that identified above 80 percent matches is reliable, 70 – 79 is often correct,

and 60–69 is very inexact. However, this useful guide depends very much on the particular software algorithm used by the manufacturer. Therefore, the numbers for compounds that drop from the 70s to 60s are usually considered as false positive identifications at the lower match factors (Stein, 1999). The negative control procedure was useful for contaminating compound identifications.

## 3.5. DISCUSSION

Hexane and chloroform are commonly used in studies of composition of insect cuticle (Buckner et al., 2009; Cerkowniak et al., 2013; Lockey, 1976; Lockey and Oraha, 1990). Methanol was also used in a study on composition of locust wings (Jarrold et al., 2007). The observation that the standard, steryl sterate, did not dissolve in methanol, suggests that methanol should be used with caution in future studies.

The cuticular lipids identified fell into seven classes: alcohols, esters, fatty acids, n-alkanes, and methyl alkanes, unsaturated ketone, and unsaturated amides. This compares with other studies in which long chain hydrocarbon, wax esters, fatty alcohols and free or esterified fatty acids are the most common components extracted from insect cuticles (Pedrini et al., 2007; Saito & Aoki, 1983; St Leger, Cooper, & Charnley, 1986). The chemical composition of the wax layer is complex, but hydrocarbons are the most abundant component in this layer (Lecuona et al., 1991).

Akbar et al., (2004) reported that the hydrocarbons represent approximately 40% of cuticular lipids in adult *T. castaneum*, and that components of branched chain hydrocarbons (C27–C29) represent only 30% of the hydrocarbons, whereas short chain account for the remaining 70%.
N-alkanes represent more than 90% of cuticular hydrocarbons in some beetles (Baker et al., 1979). In the present study, both n-alkanes and methyl-branched      alkanes

were found in *R. dominica*. N-alkanes have been found more frequently in *R. dominica* and have only been infrequently reported in *T. castaneum*. N-alkanes, alkadienes and alkenes have been found in the adults of *T. castaneum* (Baker et al., 1978). In contrast, (Howard & Liang, 1993) found that no alkanes were present in the larvae of *R. dominica,* but methyl branched alkanes and dimethyl alkanes were present. However, these studies were on whole insect and would include components from fat body and tissues, not from the cuticle alone.

A number of studies have shown that the epicuticular hydrocarbons in the insect cuticle may promote or inhibit fungal attachment, germination, growth, and penetration (Lord and Howard, 2004; Jarrold et al., 2007; Pedrini et al., 2007; Pedrini et al., 2013). Polar compounds such as (fatty acids, fatty acid esters, glucose, amino acids and peptides) are reported to stimulate and promote fungal germination and growth in *M. anisopliae* (Jarrold et al., 2007). It has been suggested that both n-alkanes and longer methyl-branched alkanes are the sole carbon source for fungal germination in the insect cuticle (Crespo & Juárez, 2000; Jarrold et al., 2007; Lockey & Oraha 1990). Jarrold et al., (2007) reported that fatty acids and fatty acid esters (along with glucose and amino acids) promote fungal germination of *M. anisopliae.* Jarrold et al. (2007) also reported that there was a large decrease or disappearance of a wide range of long-chain fatty acid and ethyl methyl derivatives on locust wings during pre-penetration fungal growth, suggesting that the fungus uses aromatic organic compounds as nutrients during growth stages.

Similarly, *M. anisopliae* successfully germinated and developed appressoria on a medium containing n-alkanes (C>8) as the sole carbon source (St. Leger et al., 1988)., however, the growth of the fungus on solid, long chain alkanes (C > 22) was largely limited to hyphal growth over the wax crystal surface.

Crespo (2000) reported that *B. bassiana* and *M. anisopliae* cultured on a glucose agar containing n-octacosane after 48h, were able to degrade n-octacosane mainly into free fatty acids, acylglycerides, and phospholipids; and that the free fatty acids were the main degradation products from n-octacosane for *B. bassiana* strains. *B. bassiana* also grew on n-tetracosane media. Some hydrocarbons of cuticular components may act as chemical catalysts for the production of penetrating germ tubes on insect cuticles (Latge et al., 1987; Pedrini et al., 2013).

In this study, the diversity of alkanes in *T. castaneum* elytra was very low compared to that of *R. dominica*, and to wings of both species. Both n-octacosane and n-tetracosane were found in *R. dominica* but were absent in *T. castaneum*. These differences may be responsible for differences in the for the differences in growth and germination observed in Chapter 2.

The longer-chain (over 80 carbons) hydrocarbons in the waxy layer can affect fungal pathogenesis by degrading specific components pheromones (Pedrini *et al* 2007, 2013). Similarly, St Leger *et al* (1988) showed that media containing nonane C91 inhibited growth of *M. anisopliae*. Pedrini et al., (2007) reported that "little if any biochemical evidence was available on the ability of microorganisms to utilize very long chain alkanes such as those usually present in the insect epicuticle". However, Smith and Grula, (1981) found that some longer chain fatty acids were utilised in fungal infection of *B. bassiana*.

It has been reported that some alcohols as free alcohols originally extracted from insect lipids, such as blue fly *Lucilia sericata* (*Calliphoridae, Diptera*), act as inhibitory components for fungal attack (Smith and Grula, 1981; Gołębiowski et al., 2012). Gołębiowski et al., (2012) reported that the amount of alcohols in the cuticular lipids of an insect may vary significantly between various species.

Some unsaturated short-chain fatty acids possess strong inhibitory properties in insect epicuticles (Barnes & Moore, 1997; Mieczysława et al., 2010; Cerkowniak et al., 2013; Gutierrez et al., 2015). Saito and Aoki (1983) similarly reported that some hydrocarbons of short- chain fatty acid caprylic acid inhibit both fungal germination and growth of *B. bassiana.* In this study, only one fatty acid (C18) was present in the elytra of *T. castaneum.* Free fatty acids such as pentanoic and hexanoic acids inhibit fungal spore germination of *B. bassiana* (Smith and Grula, 1982). Similarly, Szafranek et al., (2001) reported that sorbic acid as a free acid and pentanoic fatty acids caused complete inhibition of mycelial growth of *B. bassiana* and *Paecilomyces fumosoroseus* on aphids. Pedrini et al., (2007) reported that sorbic acid inhibits fungus from germinating and penetrating into the host insect cuticle. In addition, cuticular hydrocarbons such as fatty acid and ten or fewer carbons can inhibit fungal spore germination in both *M. anisopliae* and *B. bassiana* conidia adhesion (Lord and Howard, 2004). It is possible to suggest that the fatty acid (C18) that was found in the elytra of *T. castaneum* has acted as an inhibitor for *M. anisopliae* and *B. bassiana,* which decreases the number of spore germination on the cuticle (as illustrated in Chapter 2).

(Howard & Liang, 1993) reported that neither aggressive defensive secretions nor very long hydrocarbon chains are detectable in the larvae of *R. dominica.* Low susceptibility to fungal infection was reported to be associated with the short chain fatty acids such as hexanoic and sorbic and pentanoic acids (Pedrini et al., 2007; Szafranek et al., 2001). Szafranek et al., (2001) reported that free fatty acids such as dodecanoic and eicosanoic acids inhibit the mycelial growth and sporulation of *B. bassiana* and *Paecilomyces fumosoroseus.*  Sun & Liu, (2006) reported that neither linoleic acid nor sorbic acid could be utilised as a single carbon source for

entomopathogenic fungi including *B bassiana* and *M. anisopliae*. Both hydrocarbons act as active compounds inhibiting fungal germination of *Isaria fumoroseus* (syn*Paecilomyces fumoso-roseus*) and had no significant effect on conidia germination of *B. bassiana*. According to Smith and Grula, (1982), the lowest sporulation of *Paecilomyces fumosoroseus* was observed in a medium with hexanoic, heptadecanoic, dodecanoic and linoleic acids.

Some free fatty acids were found to be inhibitors for fungal growth. Gołębiowski et al., (2008) reported that some components of fatty acids on *Conidiobolus coronatus* showed that the presence of C16:0 and C18:1, C18:2 or C18:3 in culture media inhibit fungal growth and reduce conidia production. Similar observation in this study found that the C16:0 was present in the elytra of the resistant species of *T. castaneum*. As with the results from Chapter 2, the growth of *M. anisopliae*, and *B. bassiana* was significantly reduced on the elytra of *T. castaneum* when compared to the wings and elytra of *R. castaneum*, but grew well on the wings of *T. castaneum*.

## 3.6. CONCLUSION

Unsaturated ketone and amides, fatty acids, esters, and alcohols were isolated for the first time from both body parts of wings and elytra of *T. castaneum* and *R. dominica*. This study is therefore the first report on the chemical composition from cuticular lipids of these two species.

In this study, both n-octacosane and n-tetracosane were found in *R. dominica* but absent in *T. castaneum*. Both n-tetracosane and n-octacosane were found to be the good carbons for the growth of *B. bassiana* (Crespo 2000), suggesting that these

compounds may be responsible for the greater germination and hyphal growth observed on elytra of *R. dominica* in chapter 2.

A number of potentially inhibitory compounds of alcohol (C16) and a fatty acid (C18) were found in the elytra of *T. castaneum* than in wings, or than wings and elytra of *R. dominica,* which again supports the observed reduction in germination and hyphal growth on elytra of *T. castaneum* compared to elytra of *R. dominica* in chapter 2. The potentially inhibitory alcohols C16 and fatty acids C18 that are found in the elytra of *T. castaneum* might indicate also reduce fungal infection and lead to reduced susceptibility to fungal infection in *T. castaneum.*

# Chapter 4: Conclusions

The insect cuticle is the first barrier against fungal infection, and mediates the initial interaction between entomopathogenic fungi and the host insect. The interaction of fungi with the insect cuticular lipids is poorly understood. This research has examined the biochemistry of hydrocarbon of two economically important grain pests, *T. castaneum* and *R. dominica*, and the interaction between entomopathogenic fungi and their insect cuticular lipids

Two isolates of the entomopathogen *M. anisopliae* and *B. bassiana* were cultured on cuticles (wings and elytra) of *T. castaneum* and *R. dominica* and analysed using electronic microsco*p*y SEM. At 14 hours there was a significant and consistent reduction in both germination and length of hyphal growth in both species of fungi on elytra of *T. castaneum* compared to elytra of *R. dominica*.

An examination of the number of hyphal tips per conidium and number of appresoria showed few significant differences or consistent patterns between or within species with either fungi. However, there was a significantly higher mean number of appressoria per conidium on elytra of *R. dominica* than on elytra of *T. castaneum*.

The results support a hypothesis that reduced germination, growth of hyphae and formation of appressoria on the elytra of *T. castaneum* indicate a reduced susceptibility to infection by entomopathogenic fungi

This study is the first report on the comparative chemical composition of wings and elytra of *T. castaneum* and *R. dominica*. Surface components of *T.*

*castaneum* and *R. dominica* cuticle were analysed using GCMS in order to understand the relationship between insect cuticular components and the entomopathogens. GCMS identified seven chemical classes: alcohols, esters, fatty acids, n-alkanes, methyl alkanes, unsaturated ketones, and unsaturated amides.

Many studies have suggested that a main factor in the interaction between pathogens and host occurs on the cuticular surface. This study suggests that the composition of the insect cuticle plays an important role in the interaction between *T. castaneum* and *R. dominica* and entomopathogenic fungi. The reduced germination and growth of the fungi on elytra of *T. castaneum* correlates with the observed chemical composition: a cuticle rich in alkanes increases fungal developments in *R. dominica,* whereas a lack of such components plus some inhibitors occurring in the elytra of the *T. castaneum* species suppressing germination and growth, and may also underly the reduced formation of appressoria by *B. bassiana*.

The overall results predict that *R. dominica* would be more susceptible to infection by both entomopathogenic fungi than *T. castaneum.* This difference has been confirmed by other researchers at QUT (Hauxwell, unpublished).

There is a need for further research to examine specific cuticular components that promote and/or inhibit entomopathogenic fungi, in order to understand the relationship between the fungi and those carbons that are located in the insect's cuticule and particularly the components that support formation of appressoria. This can contribute to our knowledge of specific carbon sources for entomopathogenic control of some grain beetles that are resistant to fungal infection.

The results obtained in this study contribute to the body of knowledge about the chemical composition and infection of *T. castaneum* and *R. dominica.*

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