Biological control of the red flour beetle, *Tribolium castaneum* using entomopathogenic fungi

Thesis Submitted for the Degree of Doctor of Philosophy

School of Natural and Environmental Sciences

Newcastle University

United Kingdom

By

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September 2019

بسم الله الرحمن الـرحيم

وَيَسْـَٔلُونَكَ عَنِ ٱلرُّوحِ ۖ قُلِ ٱلرُّوحُ مِنْ أَمْرِ رَبِّى وَمَآ أُوتِيتُم مِّنَ ٱلْعِلْمِ إِلَّا قَلِيلًا

(85) And they ask you, [O Muhammad], about the soul. Say, "The soul is of the affair of my Lord. And mankind have not been given of knowledge except a little."

Holy Quran القرآن الكريم

Al-Israa (85) سورة الاسراء

Dedication

I would like to dedicate my thesis to my parents, my husband, my siblings And my loving children Zaid, Ruqaya, Ridha and Kumail

Sienaa Al-Zurfi

September 2019

Declaration

This thesis is submitted to Newcastle University for the degree requirements of Doctor of Philosophy in Biology (Biological control of the red flour beetle, *Tribolium castaneum* using entomopathogenic fungi). The research detailed within was performed during the period of 2015-2019 and was conducted in Newcastle University laboratories under the supervision of Dr Gordon Port and Dr Roy Sanderson.

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

Sienaa M. Al-Zurfi

September 2019

Certificate of Approval

I confirm that, to the best of my knowledge, this thesis is from the student's own work and effort, and all other sources of information used have been acknowledged. This thesis has been submitted with my approval for the PhD degree.

Dr Gordon Port and Dr Roy Sanderson

September 2019

Acknowledgements

In the first place, all praise to Allah for this accomplishment. I am grateful to the Higher Committee for Education Development (HCED), Prime Minister Office in Iraq for awarding me the scholarship to study for a PhD degree at Newcastle University.

I am extremely grateful to my supervisor, Dr Gordon Port, for his unlimited support, guidance and understanding and for always being happy to help; your support exceeds this thesis. Thanks to my second supervisor, Dr Roy Sanderson for his advice on statistical analysis. Thanks to Professor Mark Whittingham and Dr. Richard Bevan for having been my panel throughout my study. A special thanks to Associate Professor Paul Hatcher and Dr Ethan Hack for having been my viva examiners and their valuable feedback.

I would also like to thank:

The EM Research Services Centre in the Medical School at Newcastle University, especially Tracey Davey for helping me prepare samples and take pictures during my experimental period, which was very helpful and convenient, special thanks go to Roselyn Brown and Miriam Earnshaw for their help and assistance in the lab experiments. Gillian Davison for assistance in collecting insects. Mark Bendall for his laboratory safety advice; Roger Furness and Paul Roberts for their help with computer issues; all the staff and students in the school who supported me throughout my study.

I must express my warmest gratitude to Dr Baida for providing me with *T. castanem* treated with RNAi. I must express my warmest gratitude to all my friends and colleagues, Garima Gupta, Siti Md Saad, Dr Taha, Ibrahim, Dr Saddam, Dr Eli Patterson, Dr Rana, Dr Nadeer and all my friends and colleagues for their support throughout the study period.

Finally, my special thanks, I save until last, these go to my husband Dr Ali Kareem. He was always at my side throughout my long journey with higher education. Without his love, patience and encouragement this journey would not have been possible and completed.

I will never forget you!

Publications Related to the Thesis

Oral presentation:

Al- Zurfi, S., Port, G., and Sanderson, R. 2017. Efficacy of entomopathogenic fungi for control of *Tribolium castaneum* (Herbst) (Coleoptera:Tenebrionidae) and other stored products pests. Postgraduate Conference, School of Biology, Newcastle University.

Poster presentation:

Al- Zurfi, S., Port, G., and Sanderson, R. 2016. Effect of infection by fungi on flour beetles. School of Biology poster conference.

Al- Zurfi, S., Port, G., and Sanderson, R. 2017. Effect of infection by fungi on flour beetles, *Tribolium castaneum*. Northern Meeting and Meeting of The Post-Harvest Special Interest Group of the Royal Entomological Society. Stockbridge Technology Centre, Cawood, England.

Al- Zurfi, S., Port, G., and Sanderson, R. 2017. Effect of infection by fungi on flour beetles *Tribolium castaneum.* PG forum in University of Sheffield.

Al- Zurfi, S., Port, G., and Sanderson, R. 2017. Fungi for control of stored products insect pests. Ento 17 Conference. Newcastle University, September 2017.

Al- Zurfi, S., Port, G., and Sanderson, R. 2018. Efficacy of entomopathogenic fungi for control of *Tribolium castaneum* (Herbst) (Coleoptera:Tenebrionidae). XI European Congress of Entomology, 2 to 6 July 2018 at the Stazione Marittima Expo Convention Centre in Napoli, Italy.

Abstract

In this project the factors influencing the efficacy of entomopathogenic fungi on *Tribolium castaneum* (Herbst.) were evaluated. Initially, the efficacy of commercial formulations of *Beauveria bassiana* (Naturalis-L), *Metarhizium anisopliae* (Met52) and *Verticillium lecanii* (Mycotal) was assessed with adults and different instar larvae of *T. castaneum*. Results showed that all fungi tested had a pathogenic impact on *T. castaneum*. *Beauveria bassiana* and *M. anisopliae* were more effective than *V. lecanii* at the concentrations tested. To investigate whether combining dsRNA with fungi would improve the performance against *T. castaneum*, low concentration fungal spore formulations of *B. bassiana* and *M. anisopliae* in combination with the dsRNA of the *T. castaneum* small conductance calcium-activated potassium channel protein (*SK*) and potassium voltage-gated channel protein Shaker (*SH*) genes were evaluated. The combined treatments (fungi and dsRNA) were more successful than fungi or dsRNA treatment alone. To assess the effects of quinones secreted by *T. castaneum* on infection by the fungi the effects of *B. bassiana* and *M. anisopliae* on *T. castaneum* adults were assessed with three different types of flour medium: fresh, naturally conditioned and conditioned by synthetic 2-methyl-1,4-benzoquinone with high or low levels of quinone. Scanning electron microscopy was used to examine the adherence and germination of fungal spores on the adults. Synthetic 2-methyl-1,4-benzoquinone medium was inhibitory to the growth of fungi. These differences may be due to both the number of spores found on the insect and their germination. The effects of fungal application on insect behaviour immediately after treatment were assessed. Adults which were treated with water tended to move larger distances, while adults treated with *B. bassiana* covered smaller distances at slower speeds. The demonstration that *Tribolium castaneum* is susceptible to the commercial formulations of *M. anisopliae, B. bassiana* and *V. lecanii* tested is novel. The fungal strains tested have possible use as a management tool against *T. castaneum* and other stored-product pests.

List of Abbreviations

BCAs	biocontrol agents
BCAs	Biological control agents
Bt	*Bacillus thuringiensis*
CBC	Conservation biological control
cDNA	Complementary DNA
CNS	Central nervous system
dsRNA	double-stranded RNA
dsSH	double-stranded RNA specific for *SH* gene
dsSk	double-stranded RNA specific for *SK* gene
DWV	Deformed wing virus
EBQ	2-ethyl-p-benzoquinone
EPFs	Entomopathogenic fungi
FAO	Food and Agriculture Organization of the United Nations
Fera	The UK Food and Environment Research Agency
GLM	General Linear Model
IPM	Integrated Pest Management
Kana	kanamycin
L:D	light: dark
LC50	Median lethal concentration
MBQ	2-methyl-1,4-benzoquinone
mRNA	Messenger RNA

MST	The median survival time
PCR	Polymerase chain reaction
rh	relative humidity
RNAi	RNA interference
RT-qPCR	Reverse transcription-quantitative PCR
SD	standard deviation
SH	Potassium voltage-gated channel protein Shaker
siRNA	Small interfering RNA
SK	Small conductance calcium-activated potassium channel protein
Tc	*Tribolium castaneum*
v	volume
w	weight
WHO	World Health Organization
SEM	Scanning Electron Microscopy

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Chapter 1. Introduction and literature review

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1.1 Introduction

Agricultural stored products are attacked by approximately 20,000 species of field pests, including 40 rodents, 150 fungi, 355 mites, and, among the insects, 70 species of moth and more than 600 species of beetle, that cause both qualitative and quantitative losses (Rajendran, 2005). This is especially serious in developing countries where post-harvest crop losses due to insect pests represent a risk to food security. Stored-product insects can cause severe post-harvest losses estimated at more than 20% in developing countries and up to 9% in developed countries (Pimentel, 2009). Meanwhile, grain quality deteriorates in storage due to insects (Fig. 1.1), which makes it more susceptible to fungal attack as well as being unsuitable for consumption, planting and trade. There are different insect pests which are associated with stored products, typically from the orders Coleoptera (beetles) and Lepidoptera (butterflies and moths) (Sallam, 2008; Boyer *et al.*, 2012). The main insect pests of stored products are coleopterans (Stejskal *et al.*, 2015) which feed on the product throughout the larval and adult stages resulting in substantial economic losses. A variety of factors govern the effectiveness of using contact insecticides for controlling these and other stored-product insect pests, the most important of which is insecticide resistance (Subramanyam and Hagstrum, 1995; Andrić *et al.*, 2010; Boyer *et al.*, 2012; Opit *et al.*, 2012).

Figure 1.1. The red flour beetle *T. castaneum* feeding on grain. (Source: Google 2019 http://baza.biomap.pl/en/taxon/genus-tribolium/photos_rc. Accessed: 1 May 2019).

The red flour beetle *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) is one of the most important and damaging insect species that attack stored agricultural products in mills and warehouses around the world (Rees, 2004; Andrić *et al.*, 2010; Edde *et al.*, 2012). *Tribolium* species (Fig. 1.2) occur worldwide and have developed insecticide resistance due to several mechanisms (Islam, 2017). In the late 1960s, only a few years after the introduction of the insecticide malathion in the market, data showed that changes in the susceptibility of *T. castaneum* to it had appeared (Champ and Campbell-Brown, 1970; Wool *et al.*, 1982). Populations of malathion-resistant *T. castaneum* were less resistant to diazinon and pyrethrins (Speirs and Zettler, 1969). Currently, insecticides, such as dichlorvos, synergised pyrethrin, pyrethroids (Arthur and Campbell 2008), and the insect growth regulators methoprene (Sutton *et al*., 2011) and pyriproxyfen (Arthur *et al*., 2010), are being used as aerosol insecticides. Pyrethrin is of special interest because of its low mammalian toxicity and efficacy against a broad range of stored-product insects. Commercial formulations used in milling and processing facilities often contain the synergist piperonyl butoxide, which enhances the biological activity of pyrethrins (Brooke 1958). However, the disadvantage associated with the aerosols is that they may not distribute into areas that are obstructed by equipment or into sites that harbour hidden infestations in spillage and accumulated food dust. Nonetheless, the exposed food materials may have some residual effects and insects could be indirectly exposed through treated foods (Arthur *et al*., 2010).

Due to resistance to insecticides and the increased costs associated with chemical control, there is a need to reduce the use of insecticides in line with changing policy. As well as the views of the public and the shift to organic farming (Tomizawa and Casida, 2003), the need for alternative control approaches has become paramount, and the search for safer and more effective methods of control has led to the development of biological control agents (BCAs). Consequently, several natural enemies such as entomopathogenic fungi (EPFs) have been studied as possible BCAs. Over 700 species of EPFs have been recorded (Sandhu *et al.*, 2012; Erper *et al.*, 2016). Biopesticides using EPFs are a possible alternative control method which could be employed to control a wide range of insects inside stores (Moore *et al.*, 2000). In the UK Fera (formerly the Food and Environment Research Agency) researchers have carried out two projects, the first of which (LK0914) established the potential of BCAs for controlling storage pests, using the insect-specific fungus *Beauveria bassiana* for UK storage pests (Cox *et al*., 2004). The second project

(RD - 3079) has made a significant contribution to the improvement of a biopesticide (using an insect-specific fungus *B. bassiana*) through increasing the efficacy of the fungus and also undertaken research to establish that mass production of high quality conidia (asexual spores) is possible, to ensure that formulations have a good shelf-life. This project has sought feedback from stakeholders with regard to this novel control method as a structural treatment for controlling grain storage pests (Wakefield *et al.*, 2013).

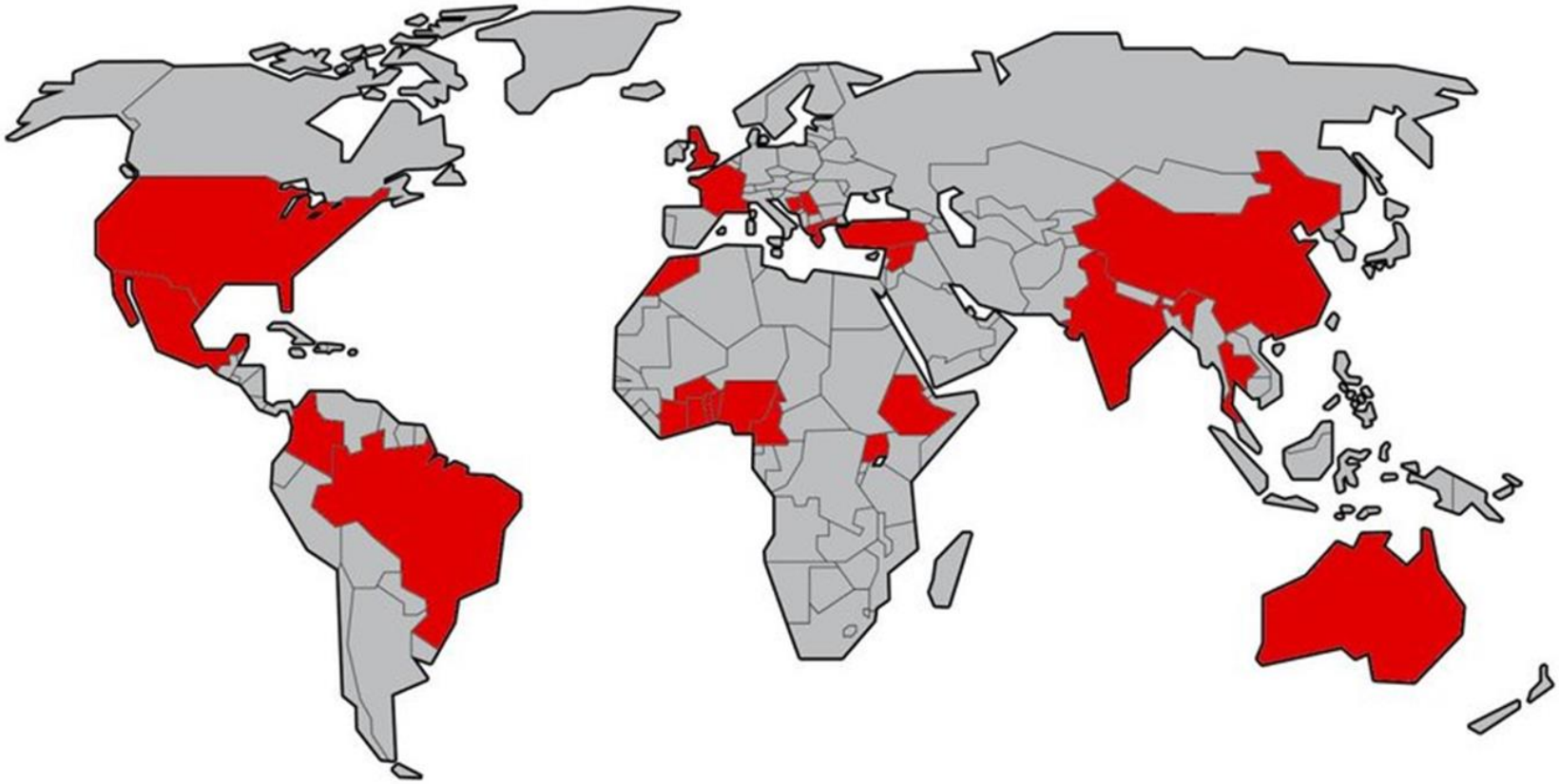


Figure 1.2. Resistance to insecticides of *T. castaneum* worldwide. The red colour depicts countries where *T. castaneum* is resistant to fumigants and insecticides. Source: Islam (2017).

Metarhizium anisopliae and *Beauveria bassiana* are entomopathogenic fungi with a wide species range and have been investigated for use against many important stored-grain pests (Rumbos and Athanassiou, 2017; Batta, 2018). Several studies have revealed the efficiency of *M. anisopliae* and *B. bassiana* for controlling many stored products pests including the red flour beetle *T. castaneum* (Sheeba *et al.*, 2001; Lord, 2007; Golshan *et al.*, 2013; Sewify *et al.*, 2014; Abdu-Allah *et al.*, 2015). Various studies have also considered the possibility of using EPFs inside stores, either alone or in combination with other strategies such as use with diatomaceous earth (DEs), chemical insecticides, natural enemies and natural products, (Lord, 2001; Michalaki *et al.*, 2006; Wakil *et al.*, 2012; Batta and Kavallieratos, 2018).

There is a molecular method recently developed to control insects: RNA interference (RNAi). The RNAi technique is a new approach for the management of crop pests which is considered an eco-friendly method. This technique commonly comes after sequencing of the whole genome of different insect species. This creates useful information for the design of dsRNA to target specific genes within the insect species. Furthermore, it has been observed that *T. castaneum* is very susceptible to this approach; research has shown that RNAi in *T. castaneum* is efficient in conserving the silencing impact for an extended period (Tang *et al.*, 2016; Gao *et al.*, 2017). Also, *T. castaneum* is amenable to systemic RNAi-mediated gene silencing and other genetic tools for functional gene analyses. Despite advances, RNAi has not been marketed yet as a biopesticide due to the cost of production of the RNA, although alternative forms of delivery by bacteria are being investigated.

Tribolium beetles can produce and secrete a quinone mixture, containing about 90% of two different quinones [methy1-1,4-para-benzoquinone (MBQ; 10‑20%), ethyl-1,4-para-benzoquinones (EBQ; 70‑80%)] and about 10% 1-pentadecene (Loconti and Roth, 1953; Villaverde *et al*., 2007). These quinones have a broad antimicrobial function and fend off parasites and predators (Prendeville and Stevens, 2002; Yezerski *et al*., 2007). They act as an external immune defence measure to protect against pathogens. Therefore, it is important to test the efficacy of EPFs in the presence and absence of quinone secretions of beetles.

Movement behaviour can have important consequences for animals living in their environment (Turchin, 1998). For example, *Tribolium castaneum* behaviour is influenced by food odours and aggregation pheromones. Thus, it is also essential to know if there is any effect of the fungal application on beetles' behaviour.

The perception that biological control agents are less effective than traditional chemical control measures may be a barrier in their marketing and use. Such perceptions can be addressed by improving the formulation and supply of the relevant microorganisms. Thus, evidence suggests that some alterations may be necessary to increase the susceptibility of insect pests to pathogenic fungi, in order to improve the efficiency of EPF. Combining EPF and RNAi approaches is a possible future strategy. Combinations of dsRNA and fungi may give potential for cocktail formulations in the control of *T. castaneum* and possibly of other stored-product insects.

It has been claimed that despite the overall improvement in EPF effectiveness achieved through use with different treatments EPFs are still inadequate to control stored-product insects (Batta and Kavallieratos, 2018). Possible reasons for the lack of registration and commercialisation of EPF biopesticides under grain-storage environment could be: (1) the initial or acute killing potential of EPFs is much slower in comparison with chemical pesticides, therefore the control effect of target insects by EPFs is not immediate (Burges 1998), (2) EPFs need high relative humidity for conidial germination and subsequent sporulation (Daoust and Roberts, 1983; Moore *et al*., 1996), therefore the proper formulation should provide enough water for germination and sporulation of these fungi during application, (3) many of the stakeholders in stored grains are not willing to introduce EPFs as biocontrol agents into their facilities because they think that these fungi are as “pathogens” or “moulds” (Batta 2016b). These limitations can probably be overcome in several ways, such as the development of proper formulations that show elevated virulence against the target insect species and ensure both stability of ingredients under the storage environment and their distribution on the surface of the host organism. Therefore, more study is needed to understand the different aspects of using EPFs to control insects in the storage environment, which will help to establish EPFs as a control for stored-product insects. For example, bioassays need to be conducted at a commercial scale under storage conditions using selected formulations to determine the feasibility of the treatment, leading to registration of the most effective formulations as “EPF biopesticides under storage conditions” (Batta, 2018).

1.2 The red flour beetle *Tribolium castaneum* as a pest

In a recent review of post-harvest issues, it is stated that the confused flour beetle *T. confusum* and the red flour beetle *T. castaneum* are "the two most common secondary pests of all plant commodities" (Sallam, 2008). Both *T. castaneum* and *T. confusum* are small, about 3–6 mm in

length, and reddish-brown in colour. The main distinguishing physical difference is the shape of their antennae: antennae of the confused flour beetle increase gradually in size and have four clubs, while red flour beetle antennae have only three. Furthermore, *T. castaneum* has been known to fly short distances, while *T. confusum* does not. The red flour beetle is of Indo-Australian origin (Smith and Whitman, 1992) and is found in temperate areas, but can survive the winter in protected places, particularly where there is central heating (Tripathi *et al*., 2001). In the United States, it is found primarily in the southern states. The confused flour beetle, originally of African origin, has a different distribution in that it occurs worldwide in cooler climates. In the United States it is more abundant in the northern states (Smith and Whitman, 1992).

Tribolium castaneum (Fig. 1.3) feeding causes direct qualitative and quantitative damage in addition to the insect's secretion of quinones, which give the product a characteristically unpleasant odour, and furthermore infested flour has a pinkish colour (Payne, 1925). The life cycle of *T. castaneum* comprises four developmental stages of adult, egg, larva (4-5 mm long) and pupa (Fig. 1.4). *T. castaneum* can develop at temperatures between 22 and 40°C, which has allowed the beetles to spread worldwide. The females can live more than 300 days and can lay over 360 eggs (Howe, 1962). Growth from larvae to pupa and the adult form takes up to 13 weeks (Islam, 2017; Arakane *et al.*, 2008).

Figure 1.3. Adult of red flour beetle *T. castaneum* (Khan *et al.*, 2016).

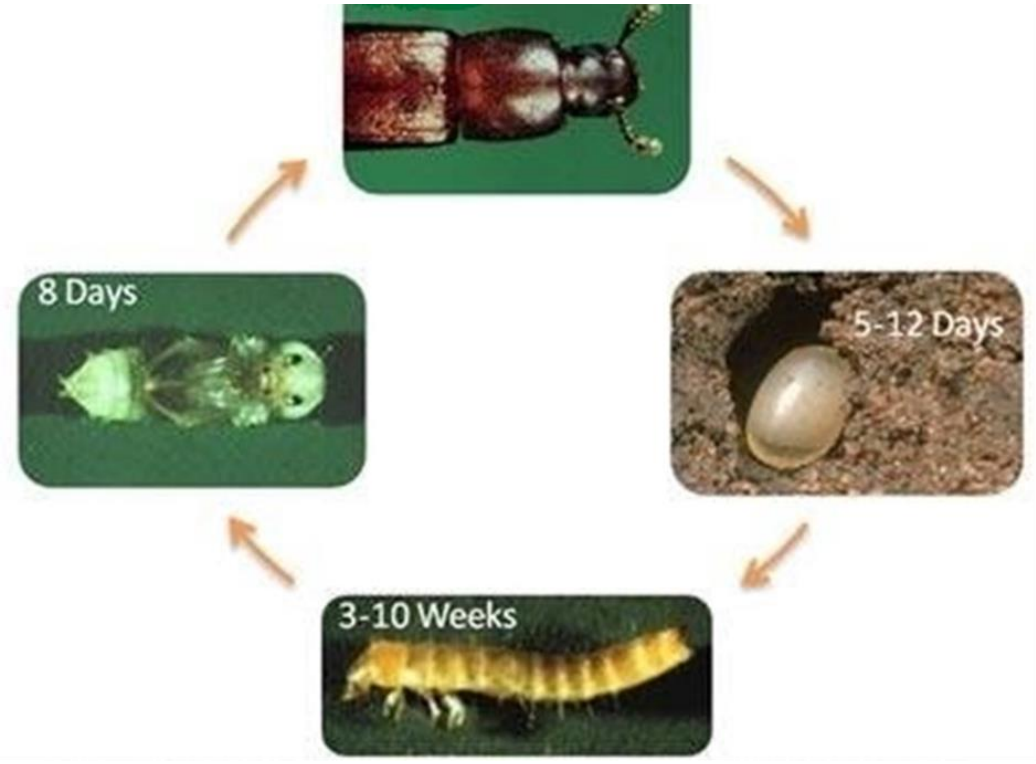


Figure 1.4. The life cycle of *T. castaneum* (Islam, 2017).

1.3 Reasons put forward for biological control alternatives

Since the late 1930s, industries have developed methods for large-scale production of chemicals and chemists have greatly improved their capabilities to synthesise chemicals. Compounds of different chemical groups have been used to control insects (Hajek, 2018). According to Delaplane (1996) the majority of the earlier pesticides were efficient for the rapid control of a wide spectrum of insects and were also easily applied using spraying equipment. Because these compounds were readily available, the potential for successful harvests increased and consequently so did the use of synthetic chemical pesticides (Van Driesche and Bellows, 1996). Although pesticides are still considered the most efficient way to control insects, there are serious issues with their use, which has led to funding for research into alternative methods to reduce insect damage. These issues are briefly discussed below.

1.3.1 Human health and environmental issues

During the 1960s concerns began to be voiced about increases in the harmful effects of chemical pesticides on human health and on wildlife and livestock (Gorell *et al.*, 1998; Bassil *et al.*, 2007; George and Shukla, 2011; Mrema *et al.*, 2013; Hughes *et al.*, 2016; Li *et al.*, 2017; WHO, 2017). Chemical pesticides have a long life, resulting in their residues polluting food, the air and water resources (Carvalho, 2017). Rachel Carson used her book "*Silent Spring*", published in 1962, as a platform to draw attention to the impact of chemical pesticides on the environment and of their residues on human health. It was subsequently widely publicised (Hajek, 2018) that exposure to chemical pesticides could lead to increases in the occurrence of illnesses such as those associated with neurobehavioral deficits, cancer, congenital malformations and other health risks. Also, the accumulation of pesticides and their residues in the groundwater and soil could have a detrimental effect on overall levels of pollution and human health. Extensive use of pesticides in agriculture increases the risk of environment and groundwater contamination, thus exposing many species of fauna and flora to their ill effects (Kim *et al*., 2017).

1.3.2 Effects of chemical pesticides on non-target organisms

It is acknowledged that pesticides are generally effective against pest insects and other invertebrates in agricultural and horticultural crop production systems. However, it has also been found in numerous studies that chemical pesticides have side effects on non-target organisms (Tomizawa and Casida, 2003). Indeed, many species that are natural enemies of the

insect, including parasitoids and predators, are at risk of elimination when insecticides are used to control insects. This is most obvious when a broad-spectrum insecticide is used. It has been found in a database analysis of the impact of worldwide insecticide use upon non-target arthropods that in 45-55% of records, the insecticides caused over 90% mortality rates in non-target species (Murphy *et al.*, 1994). Also, it was noted that when insecticides are used in pest control, those natural enemies which feed on the insect may also decrease in numbers and prevalence. Thus, in cases of reinfection, recolonisation or reinvasion by the same target insects, the densities of their natural enemies will be lower, and the target insect population could thus increase to higher densities than initially, causing an outbreak (Fernandes *et al.*, 2010). Another consequence of the elimination of natural enemies by insecticides is the possibility of secondary crop pests which, contrary to the previous situation, could cause high levels of damage (Dutcher, 2007).

1.3.3 Resistance to chemical pesticides

Very serious problems can arise from the extensive use of chemical pesticides when insects develop resistance to them (Palumbo *et al.*, 2001). The development of resistance to pesticides has been well documented (Ambethgar, 2009). Resistance to one or more pesticides has now been reported in many species of insects and mites (Roush and Tabashnik, 2012). Over 600 species of insect pests have developed resistance to insecticides (Sharma *et al.*, 2007). Pesticide resistance among insect populations occurs when several applications of similar chemical insecticides are used over many generations of the insect pest. Therefore, those individuals which are most susceptible are removed from the population, and the more resistant insects then produce offspring which are harder to control by the application of that insecticide (Siqueira *et al.*, 2000).

Insecticide resistance problems associated with various stored-product insects have been reported in many countries (Benhalima *et al.*, 2004; DARP, 2018) and involve many insecticides, including DDT, malathion, and methyl bromide (DARP, 2018). The insects most likely to have developed resistance to chemical pesticides are beetles, such as *Tribolium* spp. and *Sitophilus* spp. All these species reproduce with multiple generations each year and produce huge numbers of offspring (Sayaboc and Acda, 1990; Zettler and Cuperus, 1990; Talukder and Howse, 1994; Yao and Lo, 1995; Benhalima *et al.*, 2004).

The overall damage caused by post-harvest insect pests worldwide is estimated to be 10-20% of crop yields annually (Rajendran and Sriranjini, 2008). Therefore, there is an urgent need to control stored grain insects in order to improve food supply. Also, it is now considered vital to undertake natural control strategies as part of an integrated pest management (IPM) approach in order to control the species of beetles responsible for most of the economic losses incurred. These strategies provide alternative methods to excessive pesticide use and may contribute significantly to the development of sustainable agriculture (Nicholson and Horne, 2007). For example, the USA has devoted large amounts of money annually to such research, as the importance and value of natural control as part of 'ecosystem services' is acknowledged (Losey and Vaughan, 2006). Also, methods of natural control involve such complex modes of action that it becomes difficult for insect pests to develop resistance to them.

1.4 Introduction to biological control

Biological control has been defined by Eilenberg *et al.* (2001) as: "The use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be". This type of living organism is a natural enemy, or antagonist, of a pest organism and can include predators, parasitoids and pathogens (Hajek, 2018). They have been found in a wide range of taxonomic groups from fish to insects, mites, nematodes and micro-organisms like viruses, bacteria, fungi and protozoa (Hajek, 2018). According to Van Driesche and Bellows (1996) significant strategies for biological insect control fall into three categories. First, 'classical' natural control involves a lengthy period of the introduction of natural enemies into new regions or areas, usually to control exotic insect species without any further need for human intervention. The second type, 'augmentation' relies on the mass rearing of natural enemies which are then released into their usual environment, but in greater than the normal numbers in order to control the target insect. A related method is via 'inundative' release in which the antagonists are introduced but are not expected to build up their populations. When the rapid control of insects is required in a short time, inundative biological control is used. However, inoculative biological control, where populations do build up, provides a longer term and better continuity of control (Hajek, 2018). The aim of the third type of strategy, 'conservation biological control' (CBC) is to encourage naturally occurring enemies by managing the habitat appropriately. This can be accomplished, for example, by the reduction of both pesticide application and ploughing.

1.5 Types of natural enemies used in biological control

There are many different source species which are used for the biological control of invertebrate insects. These can be grouped into predators, parasites and pathogens and taxonomically into mites, nematodes, insects and micro-organisms such as bacteria, viruses, and fungi (Hajek, 2018). Pathogens which are responsible for disease in insects are called entomopathogens, such as entomopathogenic fungi or bacteria. When the biological control agent is a microorganism, the method used is referred to as microbial control, and the terms biopesticide or microbial pesticide are used for products involving microbial agents which have been manufactured specifically for this use. In this case, the active agent in a biopesticide may be a fungus, bacterium or virus. If the active agent is a fungus, the biopesticide is called a mycopesticide or mycoinsecticide (Lomer *et al.*, 1999). Biopesticides or bioagents are normally applied in large quantities for the biological control of insects (Hall and Menn, 1999). Because the present study uses entomopathogenic fungi, this group of microbial agents and specifically the fungi *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales), *Metarhizium anisopliae* (Metchnikoff) Sorokin (Ascomycota: Hypocreales), and *Verticillium lecanii* (Zimmerman) Viegas (Ascomycota: Hypocreales) are discussed below.

Verticillium lecanii has been reclassified as *Lecanicillium lecanii* and *L. muscarium* (Petch) Zare and Gams. The product used in the present work is currently described by the manufacturer, Koppert Biological Systems, as *L. muscarium* (https://www.koppert.co.uk/mycotal/). According to Species Fungorum (http://www.speciesfungorum.org/Names/SynSpecies.asp?RecordID=820861), the current name for *L. muscarium* is *Akanthomyces muscarius*. As the organism tested was previously classified as *Verticillium lecanii*, that name has been used throughout the thesis.

1.6 Entomopathogenic fungi

Overview

Most insect pests have pathogenic micro-organisms naturally associated with them, which is an advantage when choosing control agents. Over 700 species of fungi from almost 90 genera are known to act as insect pathogens (St Leger *et al.*, 2001). Such fungi have been used in the control of a wide range of insect pests (Ferron, 1985; Charnley and Collins, 2007). Fungi are important and unique in that, unlike other pathogens, they invade their host directly through the cuticle rather than needing to be eaten. Consequently, entomopathogenic fungi are likely to be beneficial microbial agents in future natural control methods for insect pests and will hopefully become successful alternatives to chemical pesticides (Sandhu *et al.*, 2012; Erper *et al.*, 2016). Under appropriate conditions, entomopathogenic fungi can cause epizootics (disease) which could eliminate an insect population (Rumbos and Athanassiou, 2017). Many authors have reviewed various aspects of the use of entomopathogenic fungi in history and as microbial control agents (McCoy *et al.*, 1988; Hajek and St. Leger, 1994; Boucias and Pendland, 1998; Roberts and St Leger, 2004). There are several limitations of using entomopathogenic fungi: (1) they need specific environmental conditions to germinate and cause infection, (2) they can be very costly to produce for commercial use, (3) they have a short shelf life, (4) the pest must be present before the pathogen can be usefully applied, thus making preventive treatment difficult, (5) lack of persistence and low rate of infection under challenging environmental conditions, and (6) they are often slow acting and require high application rate and thorough spray coverage (Maina *et al*., 2018).

1.6.1 Beauveria bassiana, Metarhizium anisopliae and Verticillium lecanii

Beauveria bassiana and *M. anisopliae*, in addition to *V. lecanii*, have been used to control pest insects for many years (Zimmermann, 2008). They are anamorphic fungi (reproducing asexually) and have been used with a wide range of host insect species (Clark *et al.*, 1968; Roy *et al.*, 2006). These fungi have been developed commercially as biopesticides; for example, Mycotrol® is produced by Mycotech using a virulent isolate of *B. bassiana*, strain GHA, and Green Muscle® was developed by the LUBILOSA project based on *M. anisopliae* var. *acridum* strain IMI 330189 (Palumbo *et al.*, 2001). *Beauveria bassiana*, *M. anisopliae*, *Verticillium* species and *Paecilomyces fumosoroseus* are the best known entomopathogenic fungi (Zimmermann, 2008; Gurulingappa *et al.*, 2011).

1.6.2 History and general characteristics

Beauveria bassiana is widely used as an entomopathogenic fungus (Clarkson and Charnley, 1996) and it is under intensive test as a recommended biocontrol agent (Charnley and Collins, 2007). About 700 species of insects in almost all taxonomic orders have been recorded as its hosts (Li, 1988; Inglis *et al.*, 2001). This is a well-known fungus which can be isolated from soil, insects, and mites. *Beauveria bassiana* has many advantageous characteristics and has been used as one of the primary micro-organisms in several studies of fungal insect pathology (Vega *et al.*, 2012). Almeida *et al.* (1997) showed that various levels of genetic diversity could be noted in different isolates of *B. bassiana*, and also isolates may vary in their pathogenicity and virulence to different arthropods. White muscardine disease is caused by *B. bassiana*, where the infected hosts are covered with white mould. The main cause of the white mould is the fungal spores germinating inside of the insect's body and then the fungus mycelium growing back out over the cuticle.

Metarhizium anisopliae is one of the most important and studied EPFs that are useful against agricultural insect pests (Alston *et al.*, 2005; Batta and Safieh, 2015). This fungus was previously known as *Entomophthora anisopliae,* and it is widely distributed in soil all over the world. It was first identified as a microbial agent in the 1880s (Genthner and Middaugh, 1995). It was first used as a biocontrol agent by Elie Metchnikoff in 1879 against the wheat grain beetle *Anisoplia austriaca* (Steinhaus, 1949). In 1883 Sorokin renamed the species as *M. anisopliae*. The *Metarhizium* species are known insect pathogens (Vey *et al.*, 1982; Leal *et al.*, 1997), and have been reported in many insect orders such as Dermaptera, Orthoptera, Lepidoptera, Hemiptera, Hymenoptera, Diptera, and Coleoptera (Latch, 1965). The disease caused by *M. anisopliae* is known as green muscardine disease.

Verticillium lecanii is a widely known insect pathogen, which has been commercialised for the management of several insect pests (Goettel *et al.*, 2008). Mycelium of *V. lecanii* grows around the edges of insects that have been infected, and thus it is known as the "white-halo" fungus. *Verticillium* species have been recorded in several geographically distinct regions (O'Garro and Clarkson, 1988). The first report of an entomopathogenic *Verticillium*. was on a coffee scale insect in 1898 in Java (Fatiha *et al.*, 2007). Cox *et al.* (2004) identified the most effective naturally occurring EPFs in the UK for potential application to control pests of storage. They found that different beetle species had different susceptibilities to *B. bassiana*. The most effective isolates gave mortalities close to 100% for beetles and moths, and around 75% for

mites and psocids. Additionally, some healthy grain beetles also succumbed to *B. bassiana* by picking up a lethal dose of spores from dead insects showing signs of external sporulation of the fungus. The potential of PC Floor Traps for disseminating spores was also demonstrated.

1.6.3 Infection process

The attachment of a viable spore to the surface of the cuticle of a susceptible host is a crucial stage for the growth of the fungus and fungal infection. For most EPFs, the attachment of a spore to the insect host cuticle (Fig. 1.5) is a passive activity with the aid of water and wind (McCoy *et al.*, 1988). After attaching to the surface of the cuticle, the conidia absorb moisture and germinate, thus producing germ tubes. Germination is also influenced by the availability of oxygen, nutrients, water, proper pH, and temperature conditions. Fungi with limited host ranges seem to have more specific requirements for germination compared to those that have a wide host range (St. Leger *et al.*, 1991). Fungi naturally penetrate the insect host through the cuticle by both mechanical and enzymatic action (Ferron, 1985), rather than having to be ingested as with other insect pathogen groups. The fungi produce cuticle-degrading enzymes such as proteases, chitinases and lipases, which are essentially chemical factors facilitating penetration (Boucias and Pendland, 1998). The inability to produce the enzymes in some strains may delay the infection process (Bidochka and Khachatourians, 1990), and the mechanical infection process involves hyphae directly puncturing the cuticle (Brodeur *et al.*, 2017).

The fungus enters and circulates in the host insect haemolymph while proliferating by budding in the insect's body, rapidly colonising all tissues and organs and utilising the host`s nutrient resources while producing toxins. This process ultimately leads to the death of the infected host. The success of colonisation is dependent upon the ability of the fungus to overcome the host`s immune system after entering the haemolymph (Gillespie *et al.*, 2000).

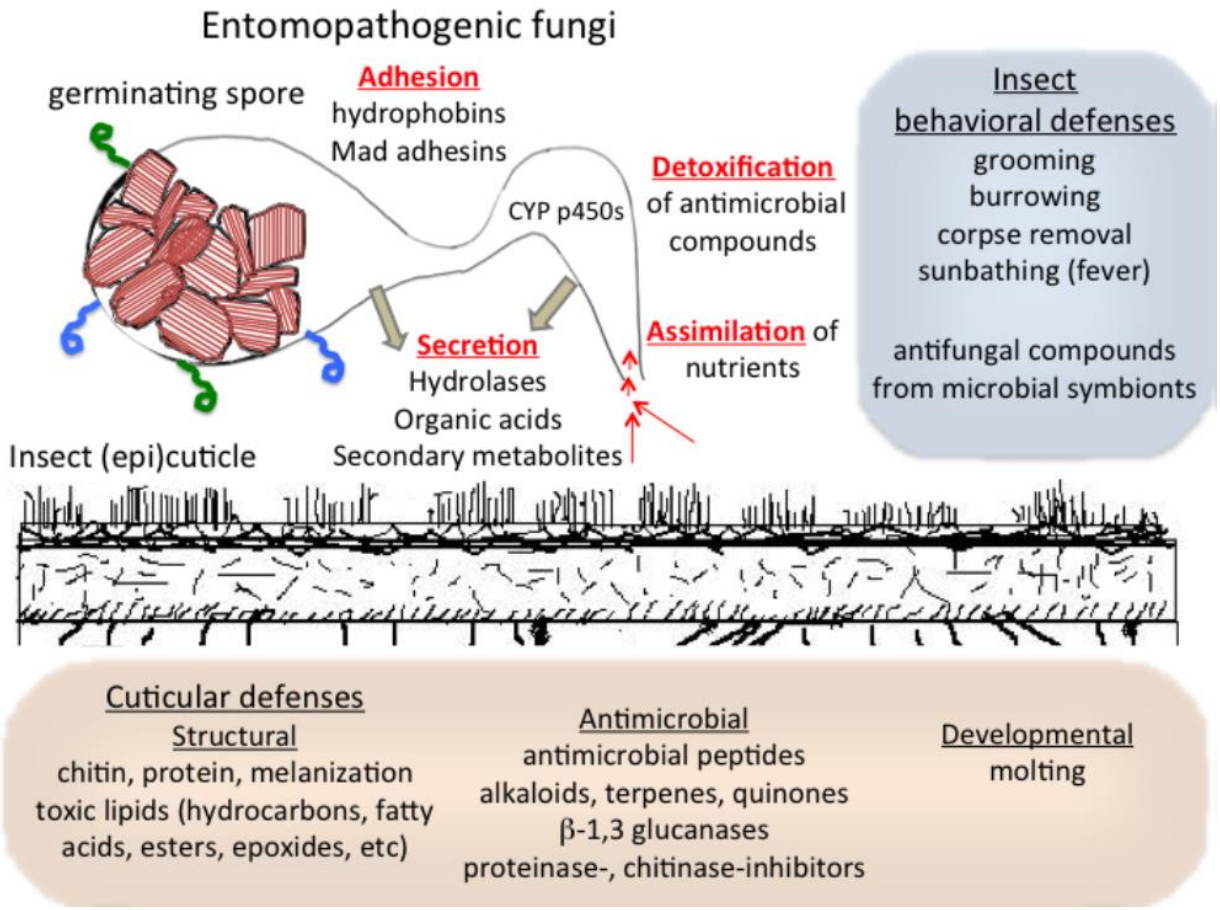


Figure 1.5. The surface interactions between fungi and the insect cuticle and host behaviours (Ortiz-Urquiza and Keyhani, 2013).

1.7 Potassium ion channels as targets for RNAi

Potassium (K^+) channels are present in cell membranes of all species, with the exception of some parasites (Kuo *et al*., 2005); K^+ channels are the largest and most varied of the ion channels. They play crucial roles in controlling the passage of K^+ ions, the secretion of hormones, resting potential, and the excitability of neurons and muscles. Consequently, these channels are important for the activity of excitable tissues such as the brain, heart and nervous system (Lehmann-Horn and Jurkat-Rott, 1999). There are four major classes of potassium channels based on their structure and functional properties: calcium-activated, inwardly rectifying, voltage-gated and two P domain potassium channels (Alshukri *et al*., 2019). Knockdown of expression of genes coding for two potassium channel proteins, SH (voltage-gated) and SK (calbium-activated), by RNAi (Fig. 1.6), has been shown to increase mortality of *Tribolium castaneum* (Alshukri *et al*., 2019).

1.8 Research hypotheses

The hypotheses tested by the research presented in this thesis are as follows. Hypothesis 1 was that the red flour beetle *T. castaneum* could be infected by commercial formulations of *B. bassiana* ATCC 74040, *M. anisopliae* (Met52), and *V. lecanii*. Hypothesis 2 was that the use of entomopathogenic fungi in combination with RNAi in the laboratory would increase the susceptibility of *T. castaneum* to the fungi *B. bassiana* and *M. anisopliae* as part of a strategy for the pest management of *T. castaneum* (Fig. 1.6). Hypothesis 3 was that the efficacy of entomopathogenic fungi (EPFs) could be reduced by the quinone secretions of *T. castaneum* which act as an external immune defence measure to protect against pathogens. Hypothesis 4 was that fungal application could reduce the locomotory activity of *T. castaneum* adults.

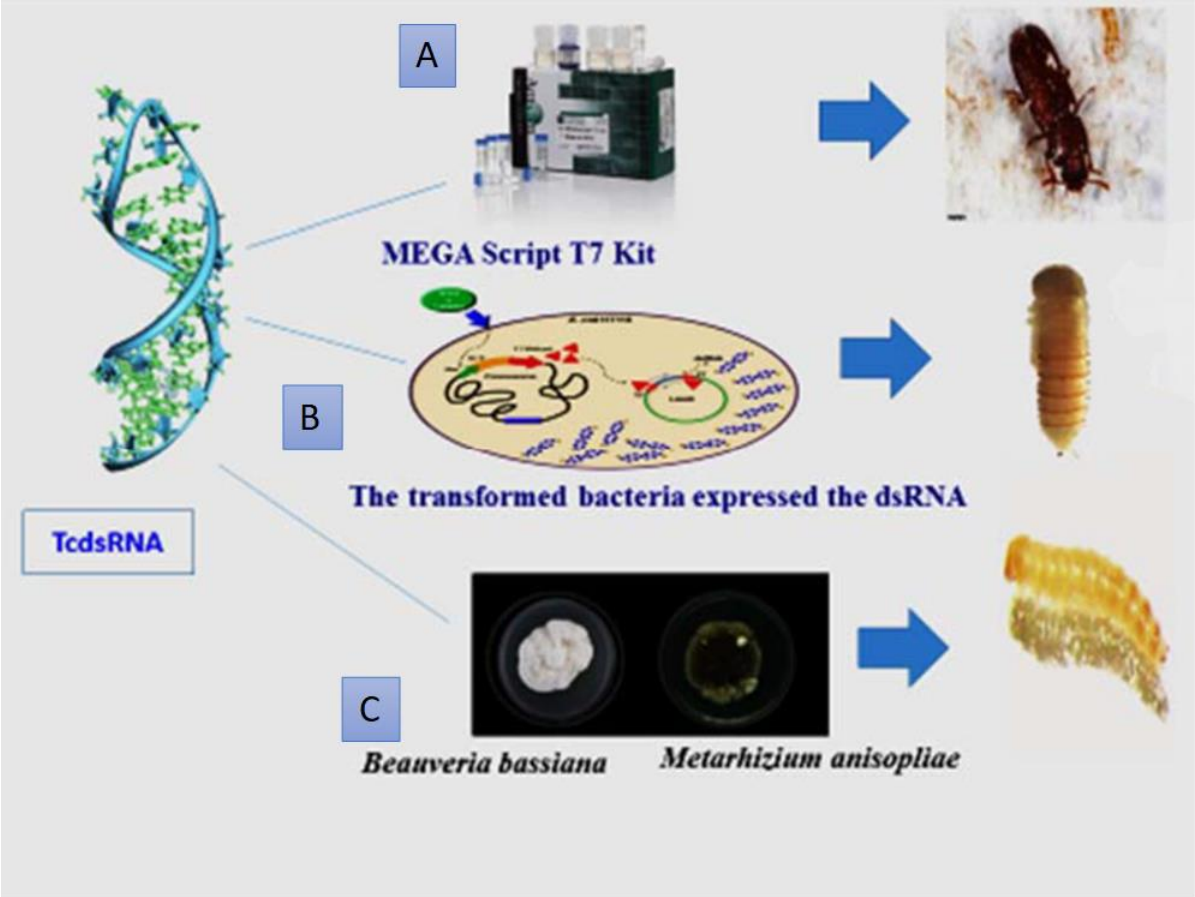


Figure 1.6. RNAi technique in the present study: A) The TcdsRNA produced by a commercial kit was tested for its toxicity against *T. castaneum*; B) transformed bacteria expressing the dsRNA was tested for toxicity against *T. castaneum*; C) TcdsRNA was combined with pathogenic fungi to increase its efficiency (Source: Alshukri, 2018).

1.9 Project aims

In order to test these hypotheses and fulfil the aims of the study, experiments were conducted which are reported in chapters 2-5 of this thesis. The objectives of these experiments were as follows:

1. To determine the effect of the use of the three entomopathogenic fungi *B. bassiana*, *M. anisopliae* and *V. lecanii* on different instar larvae and adults of the flour beetle *T. castaneum* (Chapter 2).

2. To evaluate the potential of the combination of potassium ion channel genes (*SH* and *SK*) with the entomopathogenic fungi *B. bassiana* and *M. anisopliae* as a possible approach to the control of *T. castaneum* (Chapter 3).

3. To understand the relationship between quinone secretion and fungal infection in influencing insect mortality (Chapter 4).

4. To determine the effects of the fungal application by *B. bassiana* on the locomotory activity of *T. castaneum* adults at different times after infection (Chapter 5).

Chapter 2. Effect of infection by *Beauveria bassiana, Metarhizium anisopliae* and *Verticillium lecanii* on different instar larvae and adults of the flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)

2 Chapter 2. Effect of infection by *Beauveria bassiana, Metarhizium anisopliae* and *Verticillium lecanii* on different instar larvae and adults of the flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)

2.1 Abstract

This study aimed to evaluate the pathogenicity of commercial formulations of *Metarhizium anisopliae*, *Beauveria bassiana* and *Verticillium lecanii* applied to different larval instars and adults of *T. castaneum*. Fungal isolates were separately tested in laboratory conditions against the insects using a topical spray. Insects were sprayed with conidial suspensions containing 3×10^8 spores/ml of *B. bassiana* or *M. anisopliae* and 2×10^7 spores/ml of *V. lecanii*. Mortality was recorded daily for seven days while insects were kept on wheat flour. All fungi had a pathogenic impact on *T. castaneum*. *B. bassiana* and *M. anisopliae* were more effective than *V. lecanii* at the concentrations tested. However, susceptibility showed significant differences in percentage mortality at the end of the experiments between first, third, and sixth instar larvae and adults infected by all three fungal species. First and third instar larvae were most susceptible and sixth-instar larvae and adults were least susceptible to infection. In all experiments, temperature had no significant effect on insect mortality caused by *B. bassiana*, *M. anisopliae*, and *V. lecanii*. For each fungus, there was a small difference in the insect mortality level between the different temperatures of 25°C and 30°C. All fungi were slightly but not significantly, more effective at 25°C. The strains of all fungi tested caused significant mortality of *T. castaneum* by contact.

Key words: *Beauveria bassiana*, *Metarhizium anisopliae*, *Verticillium lecanii*, *Tribolium castaneum*, instars, larvae, pathogenicity.

2.2 Introduction

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), is a cosmopolitan insect pest of storage facilities and processing plants. A significant problem with this insect is the massive economic losses it causes to stored wheat grain (Sallam, 2008). It feeds on the flour of many species of grain, including wheat, barley and corn. In particular, stored wheat flour is at risk of invasion by *Tribolium* species (Wakil *et al.,* 2003). This pest causes both qualitative and quantitative damage. Quantitative damage is caused by larval and adult insects feeding on flour and grain, which causes a loss of weight, while qualitative damage is effected by product alteration and loss of nutritional value, and new-generation adults feeding on grains. A comprehensive understanding of the biology of *T. castaneum* is necessary for the management of its population. There have been many studies of the life history of *T. castaneum* in different parts of the world. The crucial most damaging stages in its life cycle are the larval and adult stages when the insects feed most before dispersing. For instance, it has been estimated that, during storage, 5–30 % of the worldwide grain crop is lost yearly mostly because of infestation by *T. castaneum* (Shankar and Abrol, 2012).

According to Ramos-Rodríguez *et al.* (2007), the damage caused by these insects to crops in different countries is worth approximately $1.25 to $2.5 billion annually. Insects are controlled principally using insecticides (Adejumo *et al.*, 2014), but several commonly used chemicals are no longer recommended for use on stored grains because of the potential health hazards to humans. For example, the control of *T. castaneum* in stored products has been achieved using fumigants and residual chemical insecticides. In recent years, around the world, chemical control has been the main method of crop protection using spray applications of chlorpyrifos, deltamethrin, and malathion insecticides, along with indoxacarb, spinosad, buprofezin, polychlorinated petroleum hydrocarbon and azadirachtin (Arthur, 1995). Additional biorational insecticides and other conventional insecticides with different modes of action, such as abamectin, may also be used (Negahban *et al.*, 2007).

Most *Tribolium* species have been reported as having developed resistance to insecticides (Boyer *et al.*, 2012). Furthermore, the need to manage these insects combined with high levels of insecticide resistance increases the risks associated with the application of chemical insecticides to food, and there is a need for safer and more efficient methods of control. Moreover, the costs associated with chemical control, the need to reduce insecticide inputs in

line with changing policy, public opinion and the shift towards less intensive farming (Tomizawa and Casida, 2003) are all contributing to the need for alternative control methods. Therefore, several natural enemies such as entomopathogenic fungi have been investigated as possible biological control agents for the control of many agricultural insect pests (Shah and Pell, 2003).

For the reasons given above, the search for alternative control methods has led to the development of biological agents. Biological control using fungi is a feasible alternative control method (Hidalgo *et al.*, 1998). The possibility of using fungi to control pests in stored products has been studied for many years (Khan and Selman, 1988). The fungi used are specific to insects, and many to particular species, and do not infect plants or other animals. Moreover, fungi are very safe for the environment and therefore are considered by many to be promising alternatives for insect control (Sahayaraj and Tomson, 2010).

There is a large volume of publications describing the possible use of fungal pathogens to control a wide range of insects inside stores, either alone (Searle and Doberski, 1984) or in combination with other control measures such as treatment with diatomaceous earth (Lord, 2001; Michalaki *et al.*, 2006). Entomopathogens have considerable epizootic potential and can spread readily through an insect population and cause its collapse (Bosly and El-Banna, 2015). Finally, in recent years there has been increasing literature in the UK attempting to identify the most effective naturally-occurring fungal pathogens for application to structures for the control of insects in stored products (Wakefield, 2006).

Liu *et al.* (2003) list *B. bassiana* and *M. anisopliae* as safe biopesticides and they are well-known entomopathogens with a broad host range. Moreover, several studies have shown the effectiveness of *B. bassiana*, *M. anisopliae* and *Verticillium lecanii* [reclassified as *Lecanicillium muscarium*] (Gams and Zare, 2001) in controlling pests in stored products such as *Sitophilus oryzae*, *Rhyzopertha dominica*, *Callosobruchus maculatus* and *Tribolium castaneum* (Dal Bello *et al.*, 2000; Sheeba *et al.*, 2001; Cherry *et al.*, 2005; Khashaveh *et al.*, 2011). A significant factor in the loss of inoculum viability of entomopathogenic fungi under field conditions is inactivation caused by UV light (Braga *et al.*, 2001; Benjamin *et al.*, 2002). Most grain storage and processing environments do not have that disadvantage. Many of the critical pests in these environments have proven to be susceptible to *B. bassiana,* but its relatively high production costs and high application rates render it economically impractical

(Hluchý and Samšiňáková, 1989; Adane *et al.*, 1996; Rice and Cogburn, 1999; Bourassa *et al.*, 2001; Lord, 2001; Meikle *et al.*, 2001; Padın *et al.*, 2002). *B. bassiana* and *M. anisopliae* have been reported as naturally occurring pathogens of the red flour beetle in Great Britain and Kenya (Burges, 1973; Oduor *et al.*, 2000). In addition, several fungal isolates have shown potential to control red flour beetles when tested in laboratory conditions and during preliminary field tests (Moore *et al.*, 2000; Akbar *et al.*, 2004; Batta, 2004; Kavallieratos *et al.*, 2006; Michalaki *et al.*, 2006; Kavallieratos *et al.*, 2014; Sabbour, 2014).

This study investigates the effects of using *B. bassiana*, *M. anisopliae*, and *V. lecanii* on *Tribolium* under different environmental conditions which are appropriate for the insect's growth. Different life stages of *T. castaneum* were used as test insects.

Objectives

The major objectives of this experiment were

1. To evaluate the impact of the three fungi *B. bassiana, M.anisopliae*, and *V. lecanii* against *T. castaneum*.
2. To assess the relative susceptibility of adults and different instar larvae to the fungi.
3. To investigate the effect of different temperatures, specifically 25°C and 30°C, which are appropriate for growth of both the insect and the fungi, on the efficacy of *B. bassiana, M. anisopliae*, and *V. lecanii* against *T. castaneum*.
4. To determine the spore concentrations of the entomopathogenic fungi at which 50% of subject insects are killed (LC50) and the time after application at which 50% of subject insects are dead (LT50).

2.3 Materials and methods

2.3.1 Source of insects

Tribolium castaneum adults were mass reared on fresh flour medium (a mixture of brewer's yeast and whole wheat flour mixed 5:100 by weight) in a glass jar (300 ml) covered with a piece of filter paper. The cultures were kept in the dark at a temperature of 30°C which helped

in increasing insect activity. *T. castaneum* has six larval instars. To determine the presence of these larval instars under the laboratory conditions, in order to obtain the required larval stage for each experiment, a study to differentiate between instars was carried out. Using a camel hair brush, adults were put into a container for two days, after which the adults were removed and the containers were left for the eggs to hatch. First, second, third, fourth, fifth and sixth instar larval stages were identified by observing moults after the larvae were put into vented plastic Petri dishes (9 cm × 1.6 cm) which were covered with their lids and kept at 30°C without light. Whenever required, new flour was provided to feed the larvae inside the Petri dishes. The total time from the first instar larvae to the adult stage was 25 days. All adults tested were two weeks after eclosion.

2.3.2 Sources and providers of fungal suspension

Commercial formulations of *M. anisopliae*, *B. bassiana* and *V. lecanii* were used in the experiment. Met52 granules containing *M. anisopliae*, a liquid suspension of *B. bassiana* and a powder of *V. lecanii* were obtained from Fargro Ltd, Toddington Lane, Littlehampton, West Sussex, BN17 7PP UK, Belchim Crop Protection Limited, 1b Fenice Court, Phoenix Park, Eaton Socon, and Koppert BV Ireland respectively. Met52 granules contained 2% w/w *M. anisopliae* var. *anisopliae* strain F52, the liquid suspension was Naturalis-L, an oil dispersion containing 7.16% w/w *B. bassiana* ATCC 74040, and *V. lecanii* was product Mycotal, spore powder containing 16.1% w/w spores with inert ingredients 83.9% w/w. The spore suspension of *B. bassiana* (10 ml) was diluted directly with 100 ml of distilled water in a 500 ml glass beaker. The Met52 granules were prepared into liquid formulation by weighing a certain amount of granules (10 g) using a sensitive balance in the laboratory then granules were mixed with distilled water (100 ml) in a 500 ml glass beaker. To dislodge spores from the granules, the beaker was vortexed. The volume of the mixture was measured, and the spore concentration in the mix was determined using a haemocytometer. The mixture volume and spore level were used to calculate the number of spores per ml of water (Behle and Goett, 2016). The *V. lecanii* spore powder (10 g) was weighed as described above then the powder was mixed with distilled water (100 ml) in a 500 ml glass beaker. The fungal suspension was left for 2-4 h before application to allow the spores to rehydrate and to assist in dispersion. The conidial concentration was estimated using a haemocytometer with a light microscope and was adjusted to 3×10^8 spores/ml for both *B. bassiana* and *M. anisopliae* and 2×10^7 spores/ml for *V. lecanii*.

This suspension was the primary concentration of spores. Five different concentrations were produced from each of the main suspensions of each fungus for use in the experiments. The formula C1V1=C2V2 was used for this purpose, where C1= the concentration in the main suspension; C2= the target concentration required; V1= the volume of the main suspension; and V2= the volume of C2 that must be achieved by dilution.

2.3.3 Contact application of fungi

In three separate experiments, spore suspensions of *B. bassiana, M. anisopliae* and *V. lecanii* formulation in distilled water were sprayed on to the first, third and sixth instar larvae and adults of *T. castaneum*. The bioassay involved treatments with five different spore concentrations made from the original suspension, and water as a control. *Beauveria bassiana* and *M. anisopliae* were used at concentrations 3×10^4, 3×10^5, 3×10^6, 3×10^7, and 3×10^8 spores/ml, and *V. lecanii* at 2×10^3, 2×10^4, 2×10^5, 2×10^6, and 2×10^7 spores/ml. They were applied using a hand-held sprayer.

There were five replicates for each treatment. Each replicate comprised ten insects in one Petri dish 9 cm in diameter. Each group of insects (50 insects) was directly sprayed with 3 ml of the fungal suspension while they stood on a large glass Petri dish (200 cm). Insects were left for 10-15 min to ensure that the fungal suspension had adhered to the insect bodies. After 15 min the treated insects were separated into small (9 cm × 1.6 cm) plastic Petri dishes which acted as replicates to assess mortality. The insects in each replicate were fed with a diet of flour using 5 g fresh flour. The first treatments applied were the low concentration progressing to high and then the water control treatment.

Records of insect mortality started 24 hours after the treatments were applied and continued for seven days. Every day the number of insects that had died in the previous 24 hours was noted, and these were removed. The number of beetles surviving was also recorded daily. The LC50 values (at day 5) of the fungi based on the five conidial concentrations were calculated after treatment. The LT50 (at 3×10^6 spores/ml) for each fungus was also calculated from the survival curve. All treatments for the three experiments were conducted at temperatures of 25°C and 30°C without light and humidity control.

2.3.4 Confirmation of fungal infection in beetles

Mortality caused by fungal infection (fungal treatments) and other causes of mortality (control treatments) was recorded daily to day 7 of the experiment. Dead insects were removed after each assessment. They were then cleaned using cotton wool soaked in 75% ethanol before being placed individually in sterilised Petri dishes which contained damp filter paper. After the initial process, the Petri dishes were put into an incubator at a temperature of 25˚C for 8-10 days in order to retrieve mycelia from the surface of the insect. This was to confirm the fungal infection on the dead insects. After ten days, it was noticed that fungal mycelia were emerging from the bodies of the insects that died from mycosis. Those insects that did not produce mycelia were assumed to have died from fungi also.

2.3.5 Statistical analysis

Minitab® 17.1.0 (© 2010 Minitab Inc.) was used to compare the efficacy of the three entomopathogenic fungi *M. anisopliae, B. bassiana* and *V. lecanii* against different stages of *T. castaneum* (larvae and adults). Data were transformed using logit transformation when necessary to comply with the assumption of normality. A General Linear Model (GLM) was used to test significant differences between treatments, then Tukey's multiple range tests were used to separate means. In addition, LC50 and LT50 values for fungi species were computed by the probit analysis program (Finney, 1971) in Minitab® 17.1.0. The mean and median survival times (MST) for 25˚C and 30˚C combined for each treatment were estimated using the Survival Log Rank Test (Kaplan and Meier, 1958) using SIGMAPLOT program (version 13.0 Systat Software Inc., San Jose, USA).

2.4 Results

Preliminary testing showed that *B. bassiana, M. anisopliae* and *V. lecanii* were able to infect *T. castaneum* by contact, through the consequent death of the beetles. Fungal mycelia emerging from dead beetles were identified according to known morphological characteristics as the fungal species that were initially applied to the insects (Figs. 2.1 & 2.2).

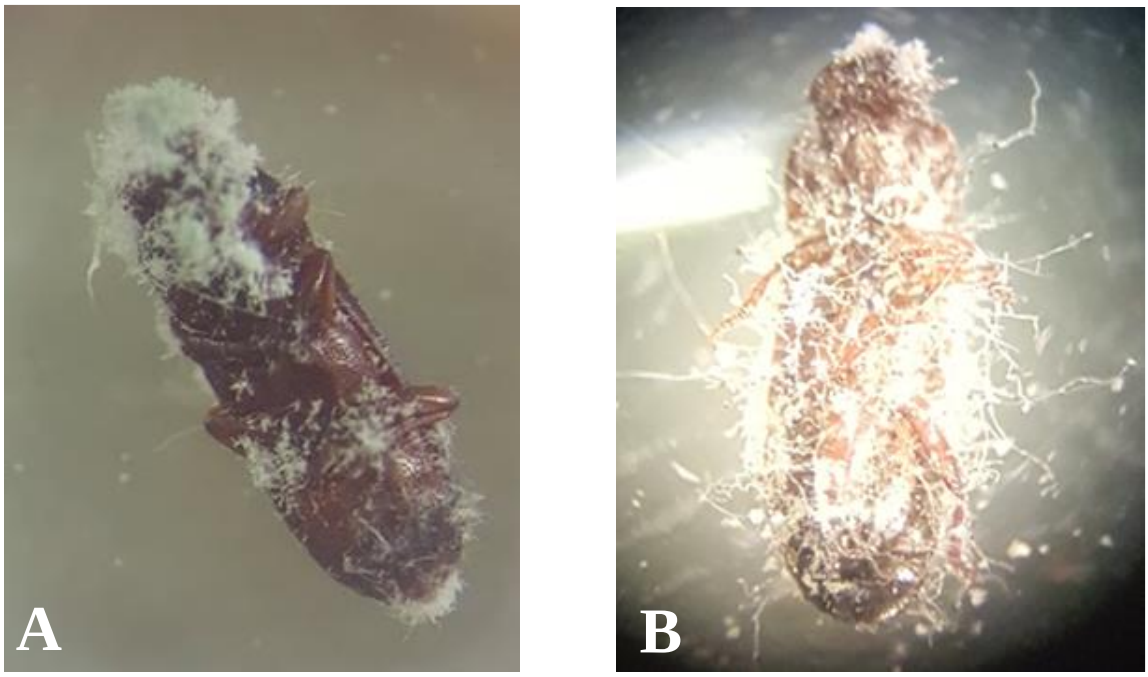


Figure 2.1. (**A**) *Tribolium castaneum* with *M. anisopliae* mycelium. (**B**) *T. castaneum* with *B. bassiana* mycelium.

2.4.1 Experiment 1: The effect of Beauveria bassiana on the mortality of T. castaneum adults and different instar larvae

In the first experiment with the spore suspension of *B. bassiana,* significant differences in *T. castaneum* mortality were noted with different spore concentrations for each of the four different insect stages 7 days after treatment. The effects of the two temperatures and six fungal concentrations (including controls) were 'fixed factors', and dead beetles were treated as a 'response variate' by analysis of variance (1^{st} instar larvae: $F_{(5, 53)} = 464.87$, $P < 0.001$; 3^{rd} instar larvae: $F_{(5, 53)} = 148.50$, $P < 0.001$; 6^{th} instar larvae ($F_{(5, 53)} = 123.96$, $P < 0.001$ and adults: $F_{(5, 53)} = 134.52$, $P < 0.001$). The highest mortality level was noted with the highest fungal dosage of 3×10^8 spores/ml for the four different insect stages (see Fig. 2.3). This means that all the larval stages and adults were susceptible to *B. bassiana* at the higher concentrations. However, susceptibility decreased with the age of the insect, indicating that older larvae and adults were more tolerant to the infection. When incubated at 25°C and 30°C first instar larval mortality rates were respectively 98% and 92%, and 90% and 88% for third instar larvae in comparison with 70% and 68% and 60% and 58% for sixth instar larvae and adults respectively. Furthermore, there was no significant difference between the efficacies at 25°C and 30°C when

the analysis was carried out using results from measurements on days 3 to 7 ($F_{(1,230)} = 0.90$, $P = 0.343$). Mortality did not reach 100% in any of the other treatments. Also, the control mortality of *T. castaneum* larval stages and adults did not exceed 10.0%.

The median survival times (MST) for different insect stages when treated with *B. bassiana* leading to over 50% mortality were five days after treatment at 25˚C and 30°C. Survival times for the four different insect stages are shown in Fig. 2.4.

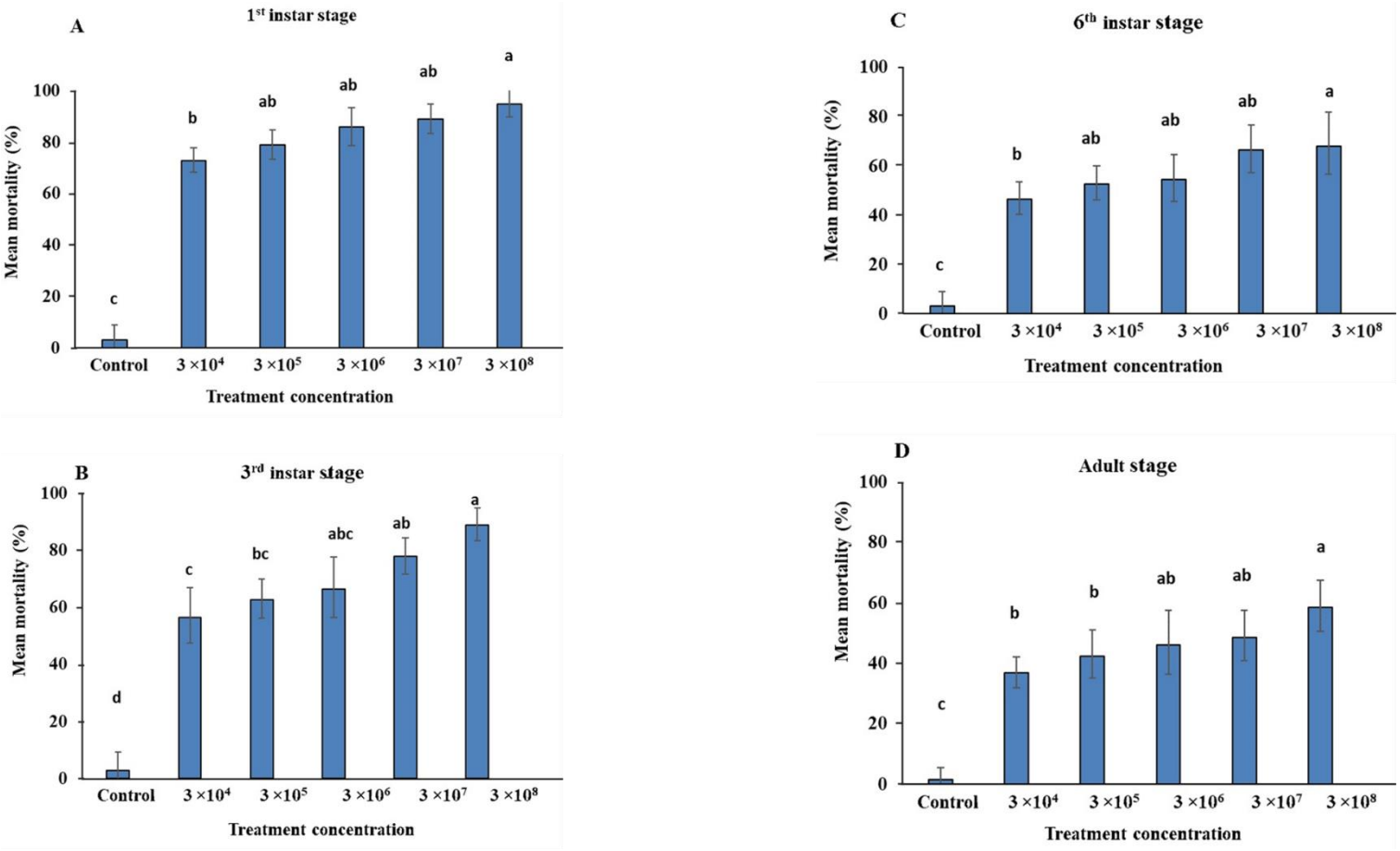


Figure 2.2. Combined mean percentage mortality at 25˚C and 30°C of *T. castaneum* first instar (A), third instar (B), sixth instar larvae (C), and adults (D) after seven days following treatment with different doses (spores ml^{-1}) of *B. bassiana*. Bars with the same superscript letter are not significantly different (P > 0.05 Tukey test). Error bars are ± 1 standard deviation (n = 10).

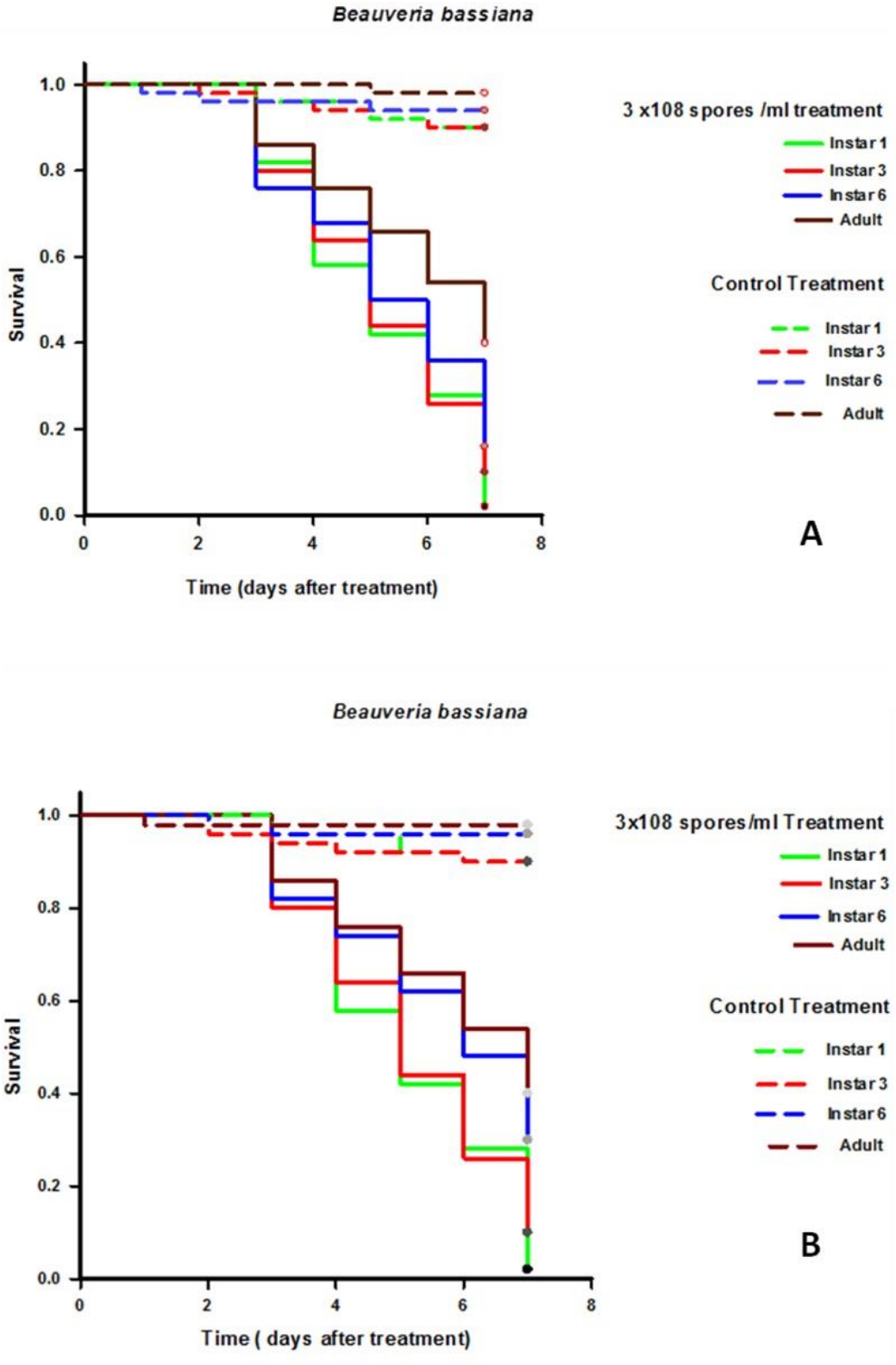


Figure 2.3. Survival curves showing fractional survival of different instar larvae of *T. castaneum* following treatment with 3×10^8 spores/ml of *B. bassiana* at 25°C (A), and 30°C (B).

2.4.2 Experiment 2: The effect of Metarhizium anisopliae on the mortality of T. castaneum adults and different instar larvae

The result of *M. anisopliae* fungus tests showed that high levels of mortality were recorded for first and third instar larvae after seven days of treatment (see Fig. 2.4 A and B) compared with the sixth-instar larval and adult stages (Fig. 2.4 C and D) at the highest fungal dosage. The highest concentration of 3×10^8 spores/ml was more effective at the end of the experiment and significant differences were noted with spore doses for each stage. The effects of the two temperatures and six fungal concentrations (including controls) were 'fixed factors', and dead beetles were treated as a 'response variate' by analysis of variance (1^{st} instar larvae: $F_{(5, 53)} = 110.68$, $P < 0.001$; 3^{rd} instar larvae: $F_{(5, 53)} = 85.72$, $P < 0.001$; 6^{th} instar larvae: $F_{(5, 53)} = 88.76$, $P < 0.001$; and adults: $F_{(5, 53)} = 43.06$, $P < 0.001$). The adults were less susceptible than larvae on the basis of mortality rates. Percentage mortality rates at the temperatures of 25°C and 30°C were 52% and 50% for adults and 68% and 66% for sixth instar larvae. First instar mortality rates were 86% and 84% and rates were 82% and 80% for third instar larvae. These mortality rates mean that the old larvae and adults were more tolerant to fungus than early larvae. For temperatures, there was no significant difference in the mortality rates when the analysis was carried out using results from measurements on days 3 to 7 ($F_{(1, 230)} = 1.30$, $P = 0.255$).

The median survival times (MST) for different insect stages when treated with *M. anisopliae* leading to over 50% mortality were six and seven days after treatment at 25˚C and 30°C. Survival times for the four different insect stages are shown in Fig. 2.5.

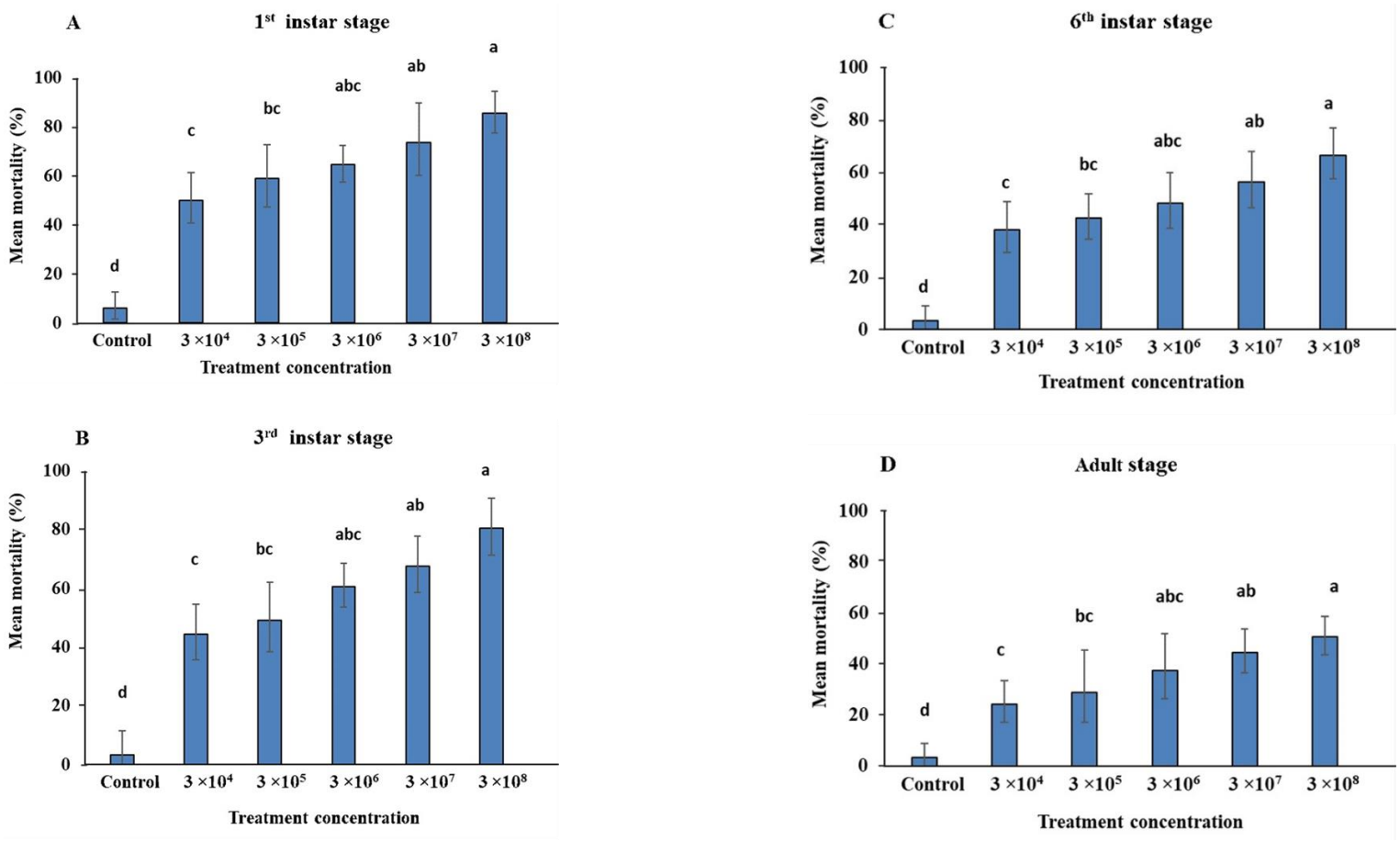


Figure 2.4. Combined mean percentage mortality at 25˚C and 30°C of *T. castaneum* first instar (A), third instar (B), sixth instar larvae (C), and adults (D) after seven days following treatment with different doses (spores ml^{-1}) of *M. anisopliae*. Bars with the same superscript letter are not significantly different (P > 0.05 Tukey test). Error bars are ± 1 standard deviation (n = 10).

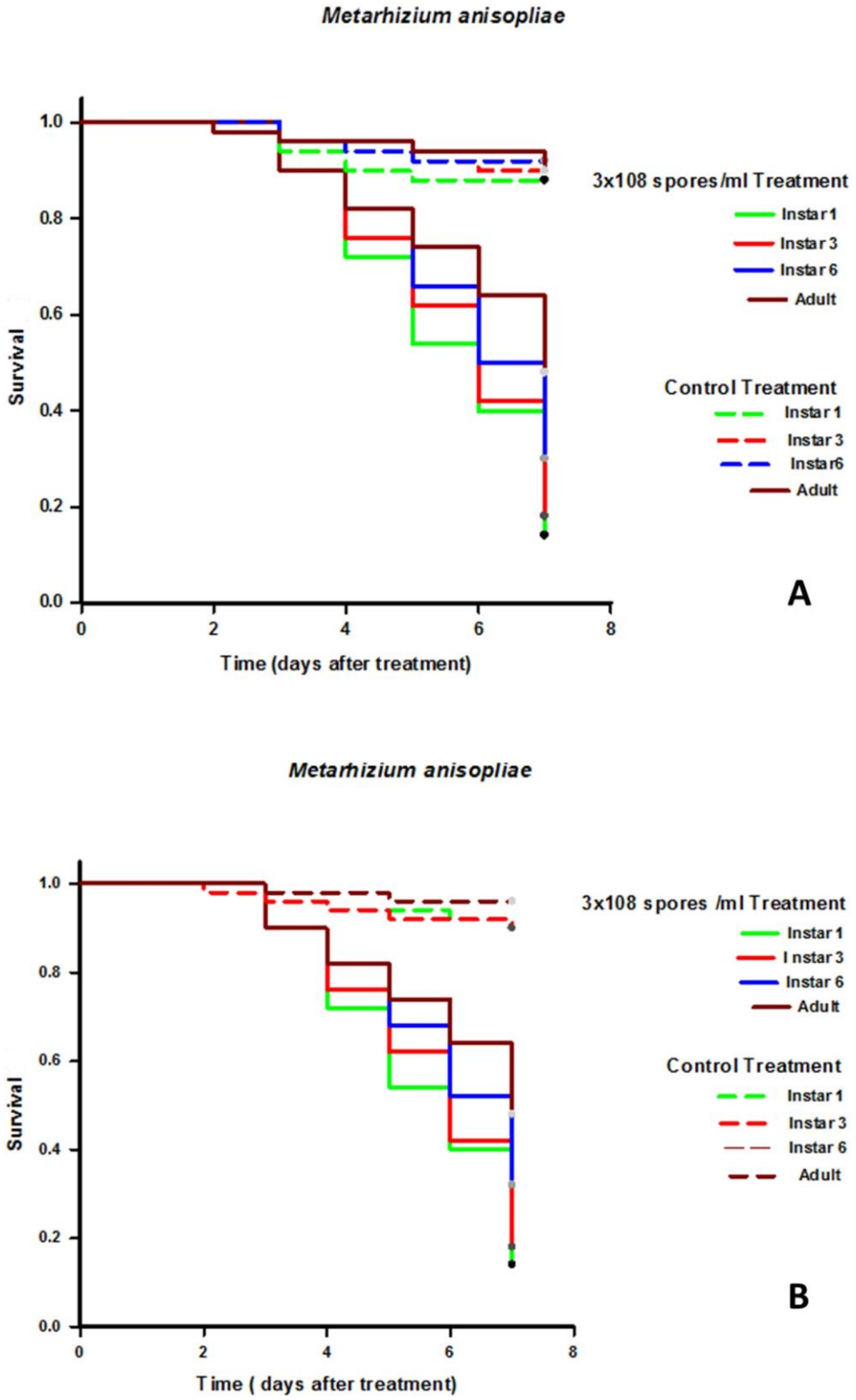


Figure 2.5. Survival curves showing fractional survival of different instar larvae of *T. castaneum* following treatment with 3×10^8 spores/ml of *M. anisopliae* at 25ºC (A), and 30ºC (B).

2.4.3 Experiment 3: The effect of V. lecanii on the mortality of T. castaneum adults and different instar larvae

The highest concentration of 2×10^7 spores/ml gave high mortality at the end of the experiment and significant effects of concentration were found for each stage. The effects of the two temperatures and six fungal concentrations (including controls) were 'fixed factors', and dead beetles were treated as a 'response variate' by analysis of variance (1^{st} instar larvae: $F_{(5,53)} = 108.53$, $P < 0.001$; 3^{rd} instar larvae: $F_{(5,53)} = 79.70$, $P < 0.001$; 6^{th} instar larvae: $F_{(5,53)} = 50.94$, $P < 0.001$; and adults: $F_{(5,53)} = 36.99$, $P < 0.001$) when they were incubated at 25°C and 30°C. For each stage, there was a significant difference between the efficacies of the different *V. lecanii* concentrations. However, there were no significant differences between the efficacies of *V. lecanii* at the two different temperatures. The adults were less susceptible than larvae on the basis of mortality rates: percentage mortality rates at 25°C and 30°C were 48% and 46% for adults and 58% and 56% for sixth instar larvae. Mortality rates were 84% and 82% for first instar and 78% and 76% for third instar larvae, seven days after treatment (see Fig. 2.7 A, B, C and D). The mortality rates above mean that the early larvae stages were less tolerant to fungus than the adults. There was no significant difference between the efficacies at 25°C and 30°C when the analysis was carried out over all four stages ($F_{(1,230)} = 0.66$, $P = 0.418$).

The median survival times (MST) for different beetle stages, when treated with *V. lecanii* at a temperature of 25˚C and 30°C, leading to over 50% mortality, were six and seven days after treatment. Survival times for the four different insect stages are shown in Fig. 2.8.

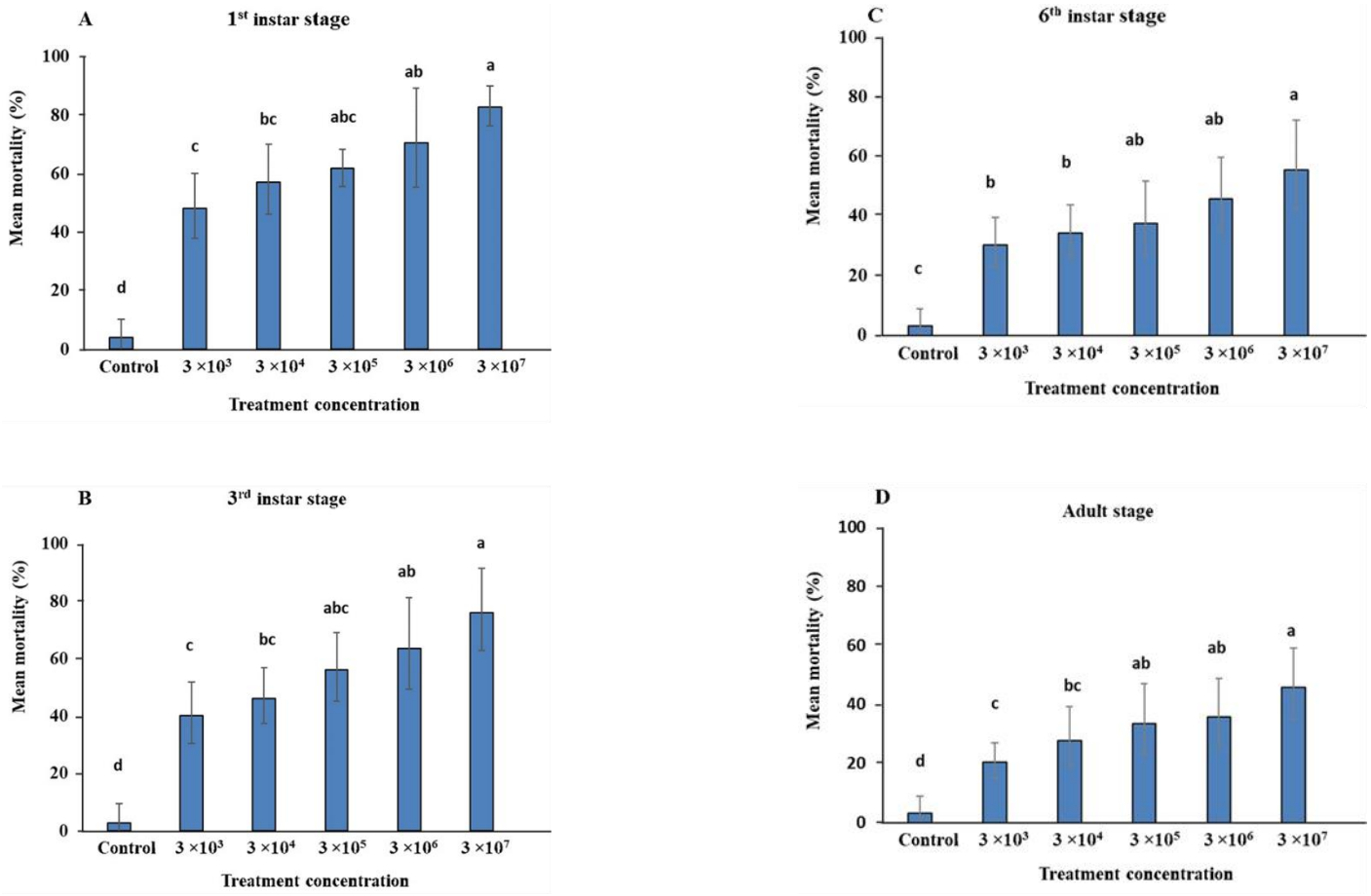


Figure 2.6. Combined mean percentage mortality at 25°C and 30°C of *T. castaneum* first instar (A), third instar (B), sixth instar larvae (C), and adults (D) after seven days following treatment with different doses (spores ml^{-1}) of *V. lecanii*. Bars with the same superscript letter are not significantly different ($P > 0.05$ Tukey test). Error bars are ± 1 standard deviation ($n = 10$).

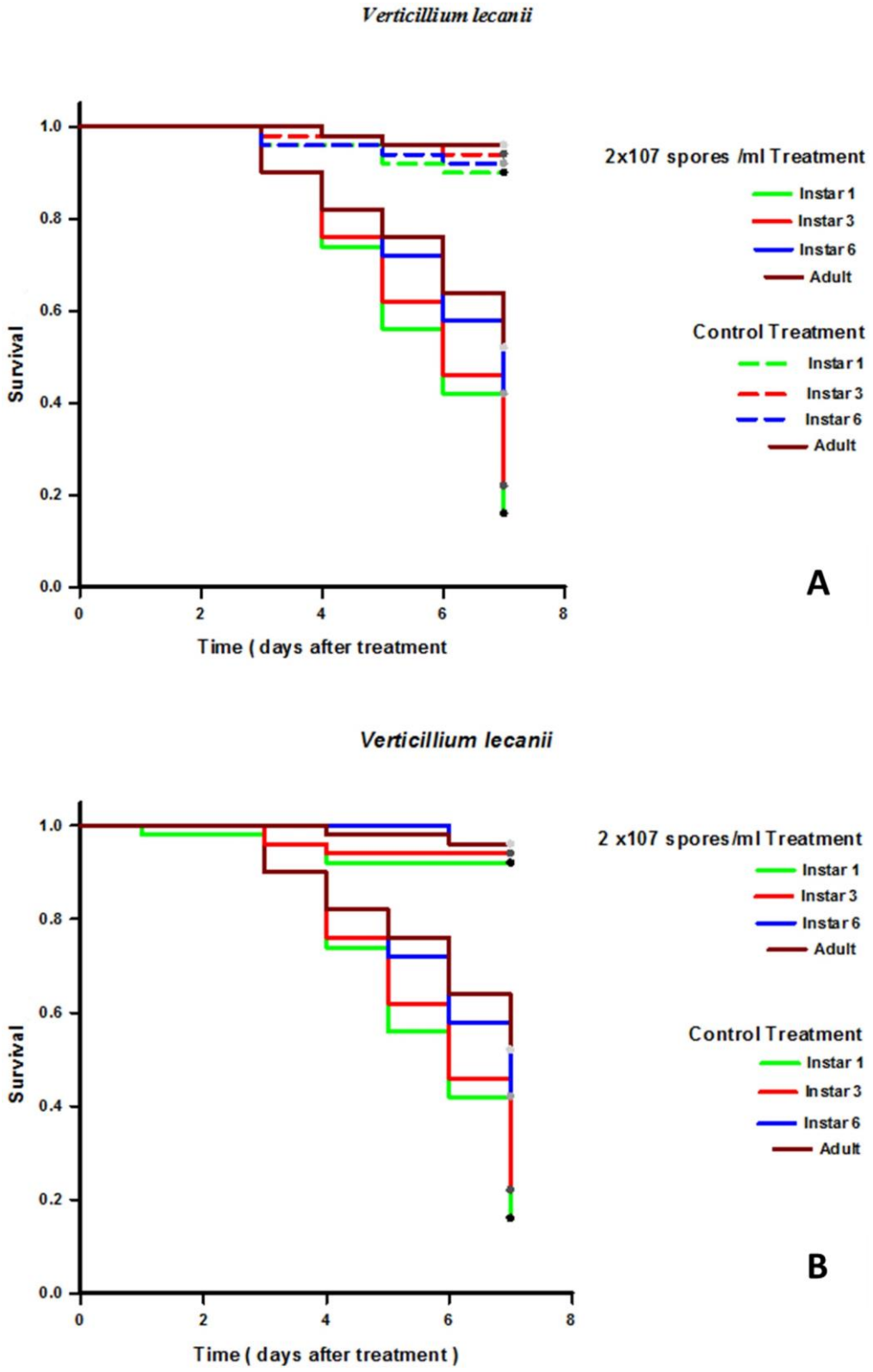


Figure 2.7. Survival curves showing fractional survival of different instar larvae of *T. castaneum* following treatment with 2×10^7 spores/ml of *V. lecanii* at 25ºC (A), and 30ºC (B).

2.4.4 Comparison of fungi

To conclude, first comparing the efficacy of the fungi, *B. bassiana*, *M. anisopliae* and *V. lecanii* were pathogenic against all larval instars and adults. Secondly, comparison of susceptibility between life stages showed significant differences in the percentage mortality between stages for *B. bassiana* ($F_{(3,230)} = 40.42$, $P < 0.001$), *M. anisopliae* ($F_{(3,230)} = 42.62$, $P < 0.001$) and *V. lecanii* ($F_{(3,230)} = 44.65$, $P < 0.001$). Sixth instar larvae and adults were more tolerant to the fungal infection than first and third instar larvae with the highest fungal dosage for the three fungi (Fig. 2.8). Finally, there was no significant interaction between the three factors, insect stage, fungal concentration and temperature for each fungus.

Tribolium castaneum LC50 (spores/ml) values at day 5 confirmed that adults were less susceptible to *B. bassiana*, *M. anisopliae* and *V. lecanii* than the larval stages (Table 2-1). There were differences in the LT50 with 3×10^6 spores/ml. It increased with the age of the insect, indicating that older larvae and adult were more tolerant to the infection (Table 2-1). The LT50 (days) values of the insects treated with *B. bassiana* were shorter than for those insects treated with other fungi. The LT50 values of the adult stage were calculated as 5.7, 6.3 and 6.9 days for *B. bassiana*, *M. anisopliae* and *V. lecanii* respectively, indicating the superiority of *B. bassiana* over the other two.

Finally, in all experiments, as noted above, the results indicated that temperature had no significant effect on insect mortality caused by *B. bassiana*, *M. anisopliae*, and *V. lecanii*.

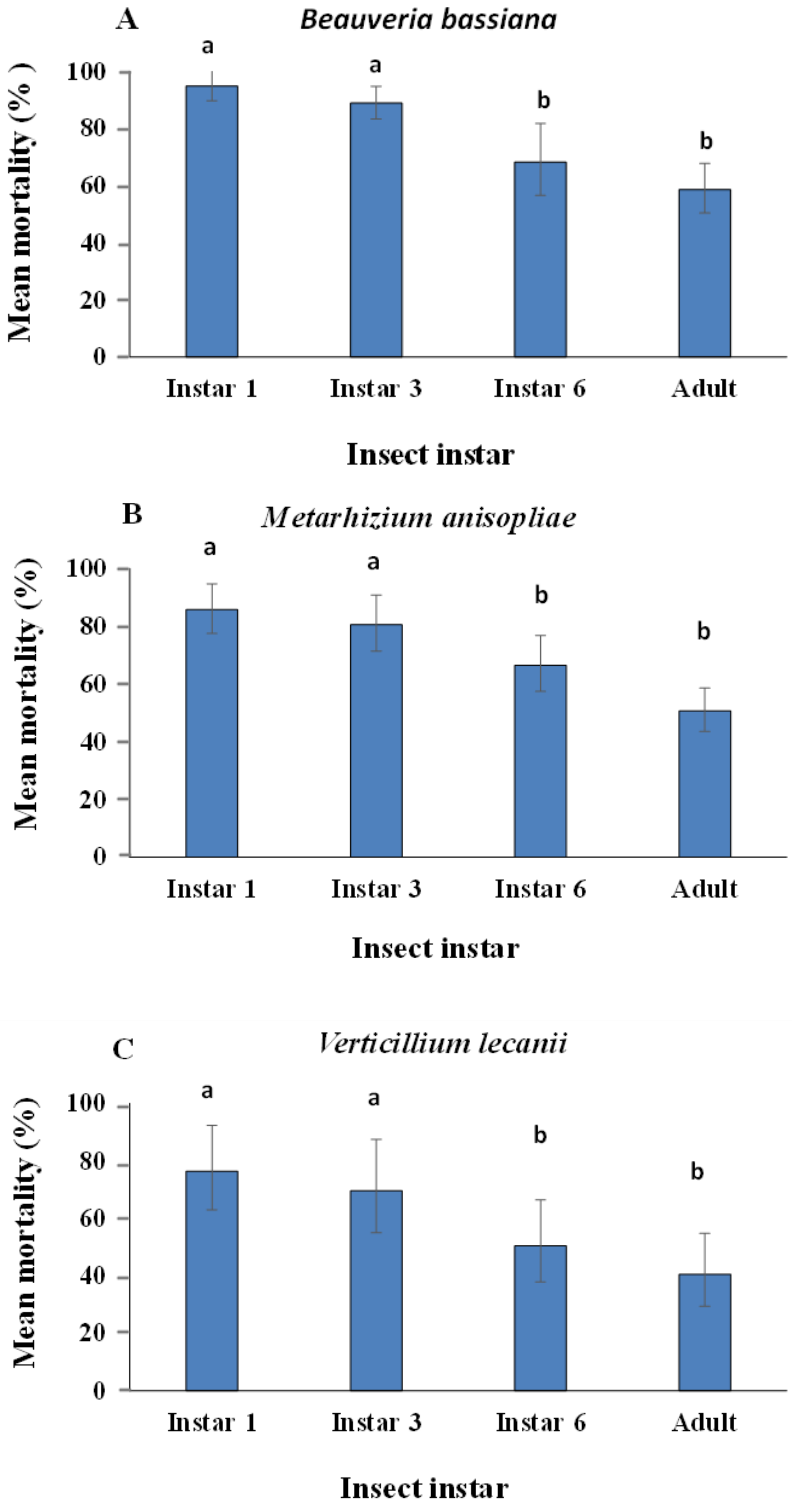


Figure 2.8. Combined mean percentage mortality of *T. castaneum* at 25°C and 30°C after seven days following treatment with 3×10^8 spores/ml of *B. bassiana* (A), *M. anisopliae* (B) and 2×10^7 spores/ml of *V. lecanii* (C). Bars with the same superscript letter are not significantly different ($P > 0.05$ Tukey test). Error bars are $\pm$ 1 standard deviation (n = 10).

Table 2-1. LC50 (spores/ml) (at day 5) and LT50 (days) values (at 3×10^6 spores/ml) of *T. castaneum* treated with *B. bassiana*, *M. anisopliae* and *V. lecanii* fungi.

Life Stages	*B. bassiana*		*M. anisopliae*		*V. lecanii*	
	LC_{50}	**LT_{50}**	**LC_{50}**	**LT_{50}**	**LC_{50}**	**LT_{50}**
First instar	3.5×10^4	3.4	4.7×10^5	4.2	4.1×10^5	4.4
Third instar	4.5×10^5	4.0	5.2×10^5	4.5	4.7×10^5	4.8
Sixth instar	5.6×10^5	4.8	6.3×10^5	5.1	6.4×10^6	5.9
Adult	6.8×10^5	5.7	7.9×10^5	6.3	7.5×10^6	6.9

LC50 value = number of spores per ml needed to reach 50 % mortality.

LT50 = time needed (days) by spores to cause 50 % mortality.

2.5 Discussion

This study has demonstrated that the fungi used are capable of causing infection and mortality in *T. castaneum* through contact. All fungi, *M. anisopliae, B. bassiana* and *V. lecanii*, show potential as control agents for this insect. These findings are similar to those of a study done by Erler and Ates (2015), which demonstrated that commercial formulations of *M. anisopliae* and *B. bassiana* can cause infection and mortality of the June beetle *Cotinis nitida* (Coleoptera: Scarabaeidae). With contact application, *B. bassiana* at the same concentration caused more mortality than the other fungi and took less time to kill the beetles on the basis of LT50 (days) values (see Table 2-1).

At the highest concentration used, the maximum daily mortality caused by *B. bassiana*, *M. anisopliae* and *V. lecanii* occurred on day seven after treatment. The first insect mortality caused by all the concentrations of *B. bassiana, M. anisopliae* and *V. lecanii* was observed three days after treatment. These times are similar to the results of other researchers. Bateman (1997) stated that insect field mortality caused by pathogens rarely occurs earlier than six days after application. In a field trial, the fungal product *Metarhizium anisopliae* var. *acridum* strain IMI 330189 developed by the LUBILOSA project for biological control of grasshoppers and locusts showed the first observable mortality at 7-10 days after application, and the full effects were observed at 14-18 days after application (Lomer *et al.*, 1997).

First and third instar larvae were most susceptible and sixth-instar larvae and adults were least susceptible to infection by all fungi (see Fig. 2.8). This result agrees with the observations by Öztürk *et al.* (2015) that earlier life cycle stages of *Leptinotarsa decemlineata* (Colorado potato beetle) were more susceptible to *B. bassiana* than adults. Entomopathogenic fungi and bioinsecticides in their study were found to be more effective on early larval stages than on fourth larval instars and adults treated by spray methods. Weiser (1953) also found that in laboratory cultures of *T. castaneum*, 98 % of the larvae but only 2-3 % of the adults were infected with a parasitic protist, *Farinocystis tribolii*. The reason for the low percentage mortality due to *F. tribolii* infection of *T. castaneum* adults has still to be further explored.

The relative susceptibility of different developmental stages of a host depends on the host species and the fungal isolate (Pedrini *et al.*, 2007). According to Rohde *et al.* (2006), adults

of *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) were less susceptible to *B. bassiana* and *M. anisopliae* isolates than the larvae. Also, the susceptibility of *Leptinotarsa decemlineata* larvae to *B. bassiana* decreased with age (Fargues *et al.*, 1991). The differential susceptibility levels observed in insects infected by entomopathogenic fungi could be related to moulting by the larval stages, which is relevant when the pathogen is inoculated immediately before ecdysis or when the time interval between sequential moults is short (Ouedraogo *et al.*, 1997; Ekesi and Maniania, 2000; Lopes and Alves, 2011). However, according to the results of the present study high doses and prolonged periods are required for the fungi to cause satisfactory levels of mortality.

Temperatures tested had no significant impact on the efficacy of the fungi. In general, optimum temperatures for germination, growth, sporulation and virulence of entomopathogenic fungi have been reported to range between 20 and 30^{o}C (Ekesi *et al.*, 1999; Tefera and Pringle, 2003; Dimbi *et al.*, 2004; Kiewnick, 2006). Abodarb (2016) proved that a temperature lower than the optimum (20-30°C) could retard the development of the fungus and spore production leading to a reduction of the mortality of insects.

The infection observed in this study occurs in two stages. In the first stage, spores adhere to and interact with the thin (1 to 5 µm) epicuticular layer of the host, which consists of cement and waxy layers containing lipids and other compounds. The spores then start to germinate. Formation of the germinative tube initiates the production of enzymes such as proteases, chitinases, chitobiases, lipases and lipoxygenases that degrade the cuticle, promoting growth of the fungus across the surface of the host and subsequent penetration of the cuticle. Penetration takes place through a combination of enzyme activity and the formation of specialised infection structures, appressoria, that build up mechanical pressure. The insect cuticle is highly heterogeneous. The epicuticle is followed by the procuticle that contains chitin and protein and is typically divided into exo-, meso-, and endo-cuticular layers (Ortiz-Urquiza and Keyhani, 2013). Inside the procuticle are the cells of the epidermis, surrounding the internal structures of the insect. In the later stages of penetration, the pathogen encounters the host immune system. (Ortiz-Urquiza and Keyhani, 2013).

In the present study, *B. bassiana*, *M. anisopliae* and *V. lecanii* were applied. Spores of these fungi connect with the body of the insect host, and germinate, then the fungus penetrates the cuticle, which is the first stage of the fungal infection process. Thus, the second phase of

infection occurs inside the insect, which leads to insect death. The first beetle deaths happened within 3-4 days, but for most it took longer. Afterwards, a mould emerges from the insect (Figs. 2.1 and 2.2) and produces new spores. All three fungal strains have potential as a control agent for this insect.

2.6 Conclusions

1. With all the fungi the mortality of first instar and third instar larvae caused by the fungal doses tested was significantly higher than that of the sixth instar larvae and adults.

2. The temperatures tested had no significant impact on the efficacy of the fungi.

3. Available fungal formulations belonging to the genera *Metarhizium*, *Verticillium* and *Beauveria* could be appropriate biological control agents for the control of *T. castaneum*.

Chapter 3. Combining potassium ion channel genes (SH and SK) with two entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, as a new approach to the control of the red flour beetle, *Tribolium castaneum* (Herbst.)

3 Chapter 3. Combining potassium ion channel genes (*SH* and *SK*) with two entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae,* as a new approach to the control of the red flour beetle, *Tribolium castaneum* (Herbst.)

3.1 Abstract

RNA interference (RNAi) has previously been demonstrated to affect *T. castaneum* through the silencing of genes coding for two small conductance channels, calcium-activated potassium (*SK*) and voltage gated potassium (*SH*). In this study, this approach was used alongside the fungal biological control agents *Beauveria bassiana* and *Metarhizium anisopliae* with the aim of increasing the efficiency of *T. castaneum* control.

The combined treatment (dsRNA and fungus) was more successful than fungus-only or dsRNA only treatments. The mortality levels of *T. castaneum* were significantly higher with the dsRNA combined with *B. bassiana* treatment than with *M. anisopliae* at 25°C. Silencing the *SK* gene was more efficient than silencing the *SH* gene with either *B. bassiana* or *M. anisopliae*. Mortality levels with *SK* dsRNA combined with *B. bassiana* were 100% in the third instar larval stage and 82% in the adult stage, but were lower in both stages using *SH* dsRNA combined with *B. bassiana*. Percentage mortality rates with the *SK* gene combined with *M. anisopliae* were 88% in the third instar larval stage and 76% in the adult stage but were lower in both stages using *SH* dsRNA combined with *M. anisopliae*. In all cases, the effects of combining entomopathogenic fungi with the use of RNAi technology significantly improved *T. castaneum* mortality. These results show that *SK* dsRNA with *B. bassiana* or *M. anisopliae* has potential use in the management of *T. castaneum*.

Key words: *Beauveria bassiana*, *Metarhizium anisopliae*, RNAi, *Tribolium castaneum*, Biological control, entomopathogenic fungi.

3.2 Introduction

RNA interference (RNAi) has been widely investigated in recent years as a non-chemical method for managing coleopteran and lepidopteran pest insects (Baum *et al.*, 2007). The red flour beetle *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) has one of the highest rates of population growth for stored-product insects (Mishra *et al.*, 2012; Knorr *et al.*, 2013). They are among the most problematic of stored-product insects and have been the subject of considerable research. The alternative antioxidant system (superoxide dismutase 2) of *T. castaneum* provides resistance to several insecticides and allows a rapid adaptation to extremes in temperature, periods of drought and prolonged periods of fasting (Tabunoki *et al.*, 2016). Genome sequencing programs have created a wealth of information about RNA interference, and several studies have noted that *T. castaneum* is very susceptible to this technique (Miller *et al.*, 2008).

The efficacy of an RNAi experiment can depend on the mode of delivery of dsRNA. The main dsRNA delivery methods studied in different orders of insects so far include injection, feeding, or spraying. Xiao *et al.* (2014) reported that dsRNA injection directly into the target tissue is regularly used and effective. The major advantage of using this method is that an exact amount of dsRNA can be directly introduced into the targeted tissue with a known concentration, in contrast to delivery by other methods. However, this method has disadvantages including cuticular damage caused during the injection, which stimulates immune functioning and can complicate the interpretation of results (Han *et al.*, 1999; Yu *et al.*, 2013). Injection is also not realistically possible in the field (Scott *et al.*, 2013) and so this delivery method has very limited implementation in pest control planning. Secondly, the delivery of dsRNA by feeding is a practical possibility in insect management and is comparatively simple compared to injection. The feeding of dsRNA has been shown to be an effective gene knockdown method in many insects of the orders Hemiptera, Coleoptera, and Lepidoptera (Nunes and Simões, 2009; Chen *et al.*, 2010; Bolognesi *et al.*, 2012). Also, Whitten *et al.* (2016) reported that ingested recombinant bacteria expressing dsRNA cassettes successfully competed with the wild-type microflora, and sustainably mediated systemic knockdown phenotypes that were horizontally transmissible in *Rhodnius prolixus* and *Frankliniella occidentalis.* This report represents a significant advance in the ability to deliver RNAi, potentially to a large range of non-model insects. However, some studies have demonstrated that the induction of gene silencing by

dsRNA feeding is less effective than dsRNA injection because individuals differ in their consumption and consequently ingest variable amounts of dsRNA (Araujo *et al.*, 2006).

Finally, in the spraying method dsRNA is synthesised in vitro and sprayed onto the plant surface. Recent reports suggest that it is an effective delivery method for dsRNA, protecting potato plants from Colorado potato beetle, *Leptinotarsa decemlineata,* for 28 days under greenhouse conditions (San Miguel and Scott, 2016).

Most of the studies described above have applied RNAi to understand the functions of genes in insects (Gotoh *et al.*, 2016; Noh *et al.*, 2016). Despite the encouraging results, the technique is still not usable because of the significant limitations of the amount and cost of dsRNA required for successful RNAi application in the field (San Miguel and Scott, 2016; Shukla *et al.*, 2016). However, many researchers have been encouraged to find ways to apply RNAi methods to control pests. One such strategy would be to integrate RNAi with biological control agents, and *T. castaneum* could be used in this research.

When used alone RNAi selectively suppresses target genes with high specificity and accuracy. RNA interference can be used to knock down the expression of the *Drosophila melanogaster* DmNav1 homologue in *T. castaneum,* resulting in high insect mortality (Abd El Halim et al., 2016). The *SH* and *SK* genes participate in propagating action potentials via nerve cell ion channels. The *SH* gene codes for voltage-gated potassium channels which are responsible for the repolarisation of the cell (Jegla *et al.*, 1995), while the S*K* gene codes for small conductance-activated calcium potassium channels which play a role in regulation after hyperpolarisation in the neurons of the central nervous system (Faber and Sah, 2007).

We used entomopathogenic fungi as a second approach, combining them with dsRNA for *SK* and *SH* genes to control *Tribolium castaneum.* The possibility of using fungi to control stored-product pests has been studied for many years (Khan and Selman, 1988). The fungi used are very specific to insects and do not infect plants or other animals. Moreover, fungi are very safe for the environment and therefore are considered by many to be promising alternatives for insect control (Sahayaraj and Tomson, 2010). The entomopathogenic fungi *B. bassiana* and *M. anisopliae* develop naturally in soil throughout the world and parasitise various insect species, *B. bassiana* causing white muscardine disease and *M. anisopliae* green muscardine disease (Erler and Ates, 2015; Sree and Varma, 2015). *B. bassiana* and *M. anisopliae* have proven

efficacy in controlling many insect pests of stored grain and grain products either alone or in combination. Combination with other compounds can enhance the efficacy of fungi against *T. castaneum*, for example abrasive powders damage the insect cuticle, thereby increasing spore attachment and making nutrients more available to spores for their germination (Akbar *et al.*, 2004; Lord, 2007). As another example, to improve the control of storage beetles by *B. bassiana* isolate IMI389521 a dry powder co-formulant, kaolin, was used. It has been suggested by Storm *et al.* (2016) that the waxy layer of the adult beetles' integument was abraded and removed by the kaolin, which allowed more spore attachment and fungal penetration through the insect exoskeleton. Furthermore, water loss is increased through the insect cuticle as a consequence of abrasion and absorption of cuticular waxes by inert dusts and may provide suitable conditions for spore germination or increase the insects' stress levels, making them more susceptible to infection by fungi (Storm *et al.*, 2016). We investigated combining dsRNA with *B. bassiana* and *M. anisopliae* to assess the performance against *T. castaneum*.

The experiments reported in this chapter were designed to test the hypothesis that low concentrations (on the basis of the results in Chapter 2) of *B. bassiana* and *M. anisopliae* spores in combination with the dsRNA of *SK* and *SH* genes have a greater effect against *T. castaneum* than either fungal spores or dsRNA alone.

Objective

1. To evaluate the efficacy of *SK* and *SH* dsRNA combined with *B. bassiana* or *M. anisopliae* against *T. castaneum* as a strategy of pest management.

2. To assess the mortality rates of adult and third instar larval stages in combined treatments (*SK* or *SH* dsRNA and fungi) and single treatments with fungus or dsRNA.

3.3 Materials and methods

3.3.1 Test insects

Adults and third instar larvae of *T. castaneum* were used in the test. Adults were cultured in glass jars (300 ml) covered with a piece of filter paper and kept on whole wheat flour plus 5% brewer's yeast (by weight) at a temperature of 25 ^{o}C without light and controlled humidity. Third instar larval stages were identified by observing moults. All adults tested were two weeks after eclosion.

3.3.2 Design and production of dsRNA and PCR (This part of the work was done by Dr Alshukri)

A web application to design optimised RNAi constructs (E-RNAi web tool) (https://www.dkfz.de/signaling/e-rnai3/) selected regions of Tc003580 (the *SH* gene) and Tc014196 (the *SK* gene). These regions had no similarity to other regions in the *T. castaneum* genome. The target sequence for the bacterial kanamycin resistance gene (Km, JN638547 dsRNA) has previously been described by Nakasu (2014) and this dsRNA was used as a control. The cDNA synthesised served as a template to amplify the sequences coding for the SH and SK channels (Abd El Halim *et al.*, 2016). The PCR reaction involved 25 µl of PCR Master Mix, 1 µl of both Forward and Reverse Primers, 1 µl of Template DNA, and finally, the total volume of 50 µl was completed by adding Ambion® Nuclease-Free Water. After gentle vortex mixing, the samples were placed in a thermal cycler (Applied Biosystems, GeneAmp® PCR system 9700). PCR conditions were 95°C for 3 min followed by 35 cycles of 95°C for 30 seconds (denaturation), annealing at 57 °C for 30 s, and the extension temperature of 72 ˚C for 11s for the *SK* gene and 9 s for the *SH* gene, and a final extension step of 72 ˚C for 10 min. The specific primers (Appendix 1) were designed using NCBI/ Primer-BLAST software. All of these primers were used at a final concentration of 10 µM. The primers were designed to amplify PCR products of 181 and 151 bp for the *SK* and *SH* genes respectively. Following electrophoresis, the bands in the gel were purified using a QIAquick MinElute Gel Extraction kit (Qiagen) following the manufacturer's instructions and were then cloned in StrataClone vector pSC-A-amp/kan (Stratagene) following the manufacturer's instructions. The QIAprep Spin Miniprep Kit (Qiagen) protocol was used to purify the plasmid DNA. The method of making dsRNA is described by Alshukri (2018). The dsRNA molecules for both genes *SK* and *SH* are shown in Appendix 2.

3.3.3 Formulations of fungal suspension used for bioassays

Met52 granules containing *M. anisopliae* and liquid *B. bassiana* (Naturalis-L) were obtained from Fargo Ltd, Toddington Lane, Littlehampton, and Belchim Crop Protection Limited, 1b Fenice Court, Phoenix Park, Eaton Socon respectively. The granular product contained 2% w/w *M. anisopliae* var. *anisopliae* strain F52, and the oil dispersion formulation contained 7.16% w/w *B. bassiana* ATCC 74040. Both *M. anisopliae* and *B. bassiana* were prepared as described in Chapter 2. Each formulation was diluted as necessary and applied by spraying the insects with conidial suspension.

3.4 Bioassay

3.4.1 Contact application of fungi

Spore suspensions of *B. bassiana* and *M. anisopliae* in distilled water were sprayed onto the larvae and adults of *T. castaneum* using a handheld sprayer. The bioassay involved treatments made from the primary suspensions of *B. bassiana* and *M. anisopliae* diluted to produce spore concentrations of 3×10^2, 3×10^3, and 3×10^4 spores/ml and water (control).

There were five replicates for each treatment. Each group of insects (50 insects) was sprayed with 3 ml of spore suspension while they were in a 200 cm diameter glass Petri dish. Beetles were left to ensure that the fungal suspension had adhered to the insect bodies. After 15 min the treated insects were separated into small (9 cm × 1.6 cm) plastic Petri dishes which acted as replicates to assess mortality. The insects in each replicate were fed with a diet of flour using 5 g fresh flour. Records of insect mortality started 24 hours after the treatments were applied and continued for ten days. Every day the number of insects that had died in the previous 24 hours was noted, and these were removed daily. All treatments were maintained at a temperature of 25°C without light and humidity control.

3.4.2 Contact application of dsRNA and fungi

The insects were treated with 3 ml for each spore suspension of *B. bassiana* and *M. anisopliae*, which were sprayed on third-instar larvae and adults of *T. castaneum*. Then, after 15 min of fungal treatment each group of insects (50 insects) was treated with dsRNA, which was delivered via flour disks prepared as described by Xie *et al.* (1996). Doses of dsRNA for both

SK and *SH* genes which had caused 50% mortality in the larval and adult stages in previous experiments (Alshukri *et. al*., 2019) were used in this experiment. One disk, treated with dsRNA, was supplied every two days to each insect. 10 µl of flour suspension (flour disks) (dsRNA, 5% brewer`s yeast and flour, nuclease-free water) was prepared in flat bottom wells of a 96 well microtitre plate and dried out at room temperature. Insects were moved to these plates (one insect per well). Third instar larvae were allowed to feed on a diet of flour treated with 65 or 117 ng dsRNA/mg diet (dsRNA for *SK* and *SH* genes respectively) and adults on a diet treated with 290 or 300 ng dsRNA/mg diet (dsRNA for *SK* and *SH* genes respectively). The experiments included four groups of controls: (control 1) only flour without spraying the insects (dry flour), (control 2) nuclease-free water without dsRNA with flour (insects fed on flour dishes treated with RNase free water), (control 3) kanamycin dsRNA with flour at 100 ng Km dsRNA /mg, and (control 4) insects with sprayed only distilled water. Mortality was recorded every 24 hours after the treatments for ten days.

3.4.3 Statistical analysis

Percentage mortality was corrected for percentage untreated control mortality (control 1 only flour) using the Abbott (1925) formula and tested for normality. Insect mortalities were analysed using the Survival Log Rank Test (Kaplan and Meier, 1958) using SIGMAPLOT program (version 13.0 Systat. Software Inc., San Jose, USA).

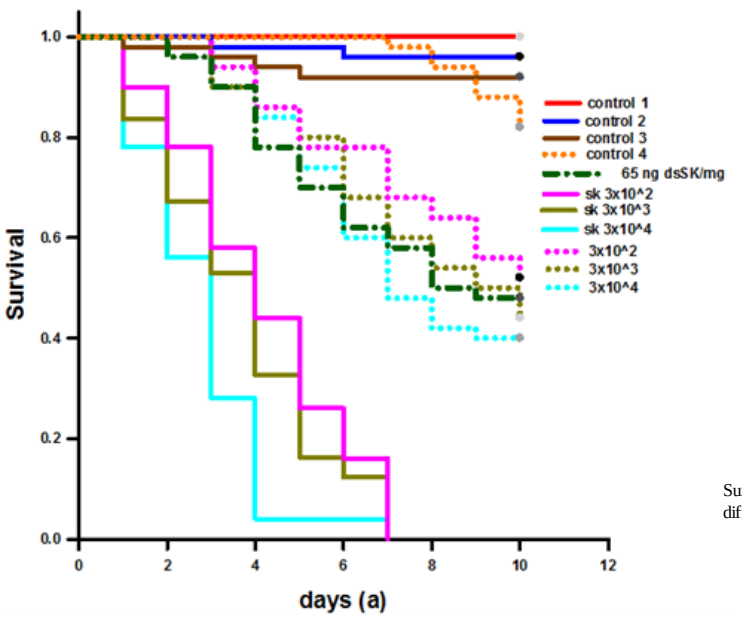
3.5 Results

3.5.1 Effect of Beauveria bassiana or Metarhizium anisopliae combined with dsRNA of SK and SH genes on mortality of 3rd instar larvae of T. castaneum

The results (Fig. 3.1) show that the survival rates of third instar larvae of *T. castaneum* treated with dsRNA (*SK* or *SH* genes) and *B. bassiana* decreased with increasing concentrations of fungus and with the addition of either *SK* or *SH* dsRNA. The combined treatments (fungus and dsRNA) were more successful than the individual treatments (fungus or dsRNA). Larval mortality rates after seven days reached 100% at 3×10^2, 3×10^3, and 3×10^4 spores/ml for *B. bassiana* with 65 ng/mg *SK* dsRNA (Fig. 3.1a). Meanwhile, with *SH* dsRNA at 117 ng/mg with the same concentrations of fungus, rates were 70%, 74%, and 86% after ten days (Fig. 3.1b). The mortality rates after 10 days for either combined treatment (fungus and *SK* or *SH* dsRNA) were greater than control 1 (larvae fed on flour disk), control 2 (larvae fed on RNase free water flour disk), control 3 (larvae fed on Km dsRNA 100 ng/mg flour disk), and control 4 (adults sprayed with distilled water). No mortality occurred in control 1, whereas it was 4% for control 2 and 8% for control 3. The mortality rates of larvae treated with *B. bassiana* only after ten days were 48%, 56% or 54% and 60% at 3×10^2, 3×10^3, and 3×10^4 spores/ml respectively in the different experiments and the mortality rates were 17% for control 4 (larvae treated with distilled water), while *SK* and *SH* dsRNA treatment gave 52% and 50% mortality, respectively. Statistical analysis using the Survival Log Rank test revealed that there were significant differences among treatments in the experiments with fungus and *SK* dsRNA ($\chi^2 = 559.240$, df =10, $P < 0.001$) and with fungus and *SH* dsRNA ($\chi^2 = 221.013$, df =10, $P < 0.001$). Mortality was significantly higher in the combined treatments than in the single treatments with fungus or dsRNA (Fig. 3.1). All treatments caused high rates of mortality for the 3rd instar larvae compared with the four control groups.

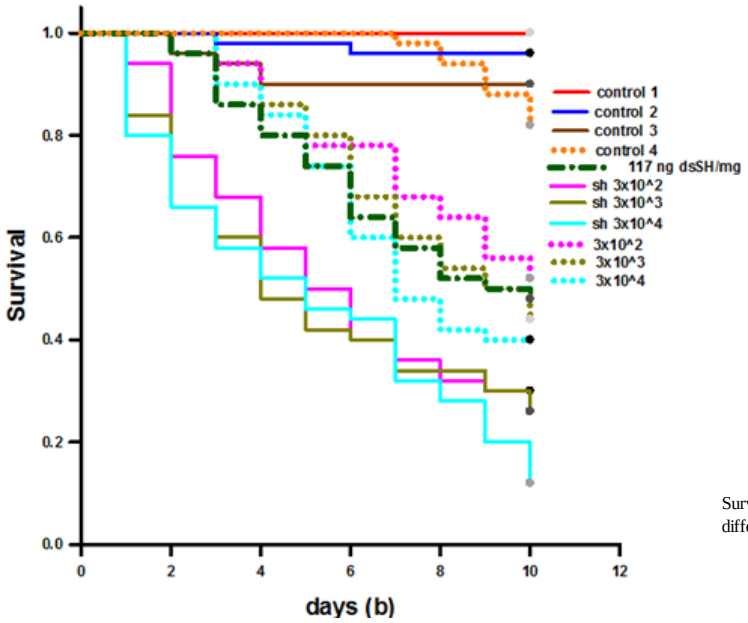
The mortality of larvae treated with *SK* dsRNA at 65 ng/mg and *M. anisopliae* at 3×10^2, 3×10^3, and 3×10^4 spores/ml after ten days reached 74%, 76% and 88% respectively (Fig. 3.2a), and 46%, 54% and 59% with *SH* dsRNA at 117 ng/mg (Fig 3.2b). No mortality occurred in control 1, and the mortality rates were 4% for control 2 and 8% for control 3. The mortality rates of larvae treated with *M. anisopliae* only were 38%, 46% and 52% after ten days at 3×10^2, 3×10^3, and 3×10^4 spores/ml respectively, and the mortality rates were 17% for control 4, whereas larval mortality was 52% and 50% when fed with *SK* and *SH* dsRNA at concentrations of 65 ng/mg and 117 ng/mg respectively. The statistical analysis revealed that

there were significant differences among treatments in the experiments with *SK* RNA (χ^2 = 235.000, df =10, P < 0.001) and with *SH* RNA (χ^2 = 114.803, df =10, P < 0.001). Mortality was significantly higher in the combined treatments with fungus and *SK* dsRNA than in the treatments with fungus or dsRNA alone. However, mortality was not significantly higher in the combined treatments with *SH* dsRNA (Fig. 3.2). All treatments caused greater mortality of the insect larvae than in the four control groups.



Treatment concentration	Survival rates (%) at the end of the exp.
Control 1	100[a]
Control 2	96[a]
Control 3	92[a]
Control 4	83[a]
SK 3 × 10^2	0[c]
SK 3 × 10^3	0[c]
SK 3 × 10^4	0[c]
3 × 10^2	52[b]
3 × 10^3	44[b]
3 × 10^4	40[b]
65 ng/mg SK dsRNA	48[b]

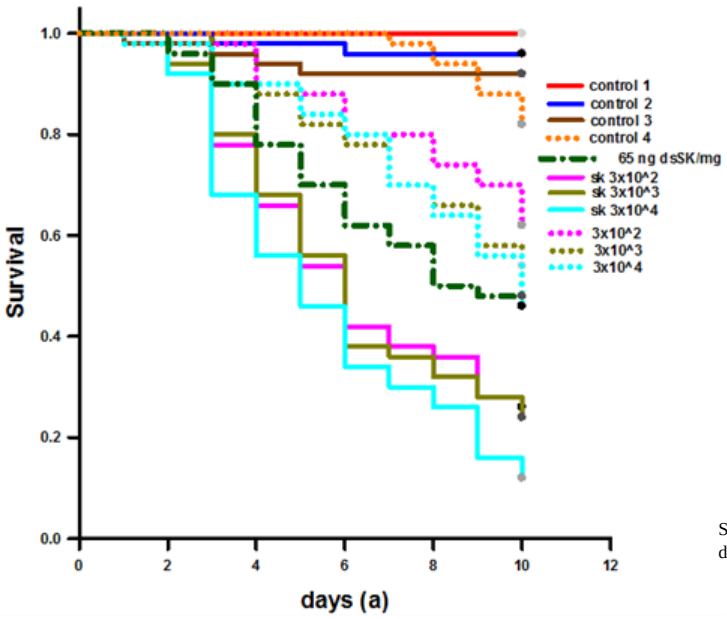
Survival rates followed by the same letter are not significantly different. (N= 50 larvae within 5 replicates).



Treatment concentration	Survival rates (%) at the end of the exp.
Control 1	100[a]
Control 2	96[a]
Control 3	92[a]
Control 4	83[a]
SH 3 × 10^2	30[c]
SH 3 × 10^3	26[c]
SH 3 × 10^4	14[c]
3 × 10^2	52[b]
3 × 10^3	46[b]
3 × 10^4	40[b]
117 ng/mg SH dsRNA	50[b]

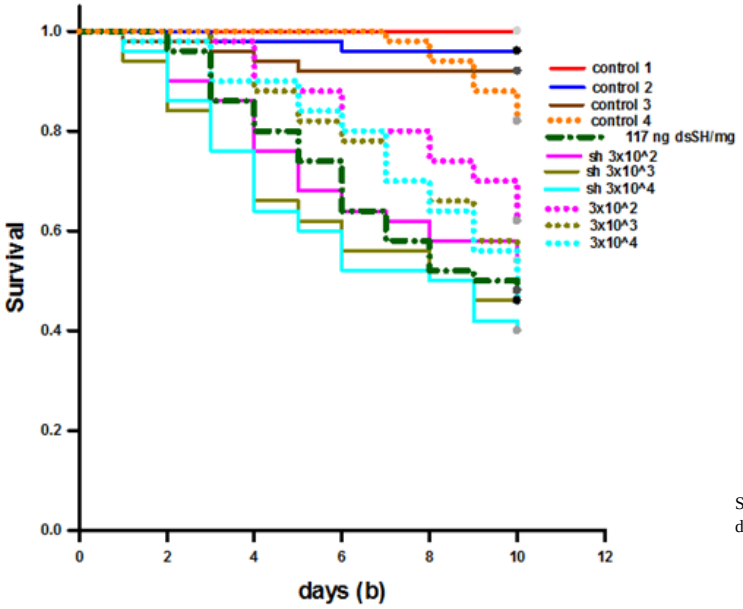
Survival rates followed by the same letter are not significantly different. (N= 50 larvae within 5 replicates).

Figure 3.1. Survival curves of *T. castaneum* larvae fed on dsRNA targeted at the *SK* gene (a) and *SH* gene (b) with *B. bassiana*. Control 1 = untreated larvae, control 2 = larvae fed on flour disks treated with RNase free water, control 3 = larvae fed on dsRNA targeted at the kanamycin gene, control 4 = larvae sprayed with distilled water.



Treatment concentration	Survival rates (%) at the end of the exp.
Control 1	100[a]
Control 2	96[a]
Control 3	92[a]
Control 4	83[a]
SK 3 × 10^2	26[c]
SK 3 × 10^3	24[c]
SK 3 × 10^4	12[c]
3 × 10^2	62[b]
3 × 10^3	54[b]
3 × 10^4	48[b]
65 ng/mg SK dsRNA	48[b]

Survival rates followed by the same letter are not significantly different. (N= 50 larvae within 5 replicates).



Treatment concentration	Survival rates (%) at the end of the exp.
Control 1	100[a]
Control 2	96[a]
Control 3	92[a]
Control 4	83[a]
SH 3 × 10^2	54[c]
SH 3 × 10^3	46[c]
SH 3 × 10^4	41[c]
3 × 10^2	62[c]
3 × 10^3	54[c]
3 × 10^4	48[c]
117 ng/mg SH dsRNA	50[c]

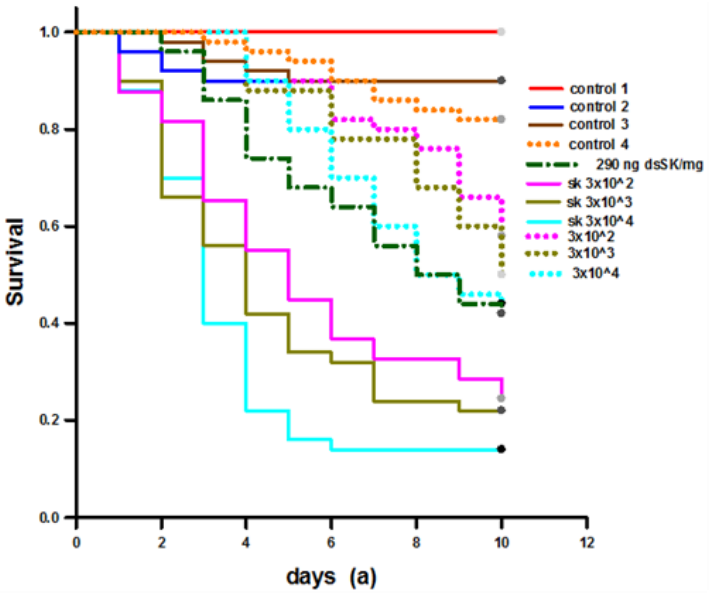
Survival rates followed by the same letter are not significantly different. (N= 50 larvae within 5 replicates).

Figure 3.2. Survival curves of *T. castaneum* larvae fed on dsRNA targeted at the *SK* gene (a) and the *SH* gene (b) with *M. anisopliae*. Control 1 = untreated larvae, control 2 = larvae fed on flour disks treated with RNase free water, control 3 = larvae fed on dsRNA targeted at the kanamycin gene, control 4 = larvae sprayed with distilled water.

3.5.2 Effect of B. bassiana or M. anisopliae combined with dsRNA of SK and SH genes on the mortality of T. castaneum adults

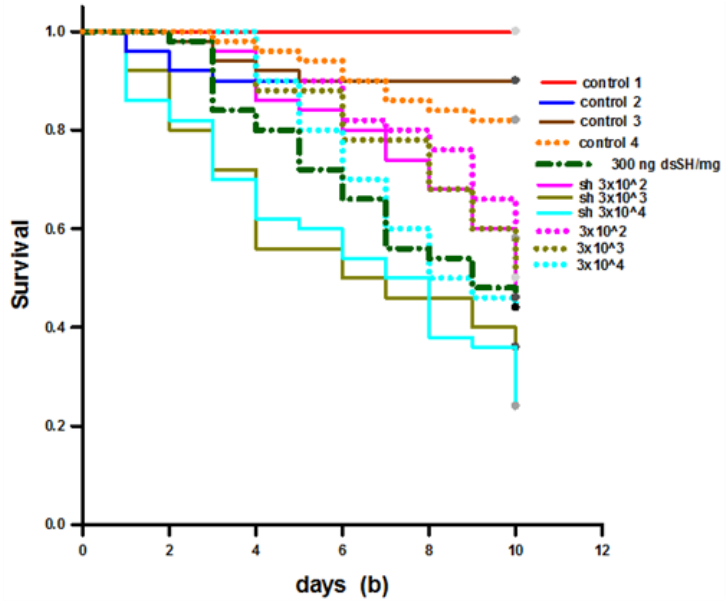
The mortality rates of adults treated with *SK* dsRNA with *B. bassiana* after ten days were 75%, 78% and 82% at 3×10^2, 3×10^3, and 3×10^4 spores/ml respectively (Fig. 3.3 A). However, the rates were 56%, 64% and 76% at 3×10^2, 3×10^3, and 3×10^4 spores/ml respectively for the *SH* gene (Fig. 3.3 B) compared to 0% for control 1 and 10% for controls 2 and 3. For the treatments without dsRNA mortality rates were 41%, 49% and 58% and 41%, 48% and 55% for concentrations of 3×10^2, 3×10^3, and 3×10^4 spores/ml respectively in the different experiments compared to 15% for control 4 (adults sprayed with distilled water). *SK* and *SH* dsRNA treatments without fungus gave 58% and 54% mortality respectively. The statistical analysis revealed that there were significant differences in the survival of adults in the experiments with fungus and *SK* dsRNA ($\chi^2 = 267.824$, df =10, $P < 0.001$) and with fungus and *SH* dsRNA ($\chi^2 = 142.704$, df =10, $P < 0.001$). Mortality was significantly higher in the combined treatments with fungus and *SK* dsRNA than in the single treatments with fungus or dsRNA. With *SH* dsRNA there was an apparent trend towards higher mortality in the combined treatments with the higher spore concentrations, but the differences were not significant (Fig. 3.3). All treatments caused high rates of mortality compared with the four control groups.

The adults' mortality after ten days was 62%, 70% and 76% for *SK* dsRNA with *M. anisopliae* (Fig 3.4a), whereas it was 38%, 46% and 50% for *SH* dsRNA with fungus at 3×10^2, 3×10^3, and 3×10^4 spores/ml respectively (Fig 3.4b). Treatments without dsRNA gave mortality rates of 35%, 41% or 38% and 44% respectively at the same concentrations in the different experiments. All these mortality rates were higher than those of the four control groups: controls 2 and 3 had 10% mortality, with no mortality in control 1, and the mortality rates were 16% for control 4 (adults treated with distilled water). The statistical analysis showed that there were significant differences between treatments in the experiments with fungus and *SK* dsRNA ($\chi^2 = 153.641$, df =10, $P < 0.001$) and with fungus and *SH* dsRNA ($\chi^2 = 77.791$, df =10, $P < 0.001$). Mortality was significantly higher in the combined treatments with fungus and *SK* dsRNA than in the treatments with fungus alone, but not higher than in the treatment with dsRNA alone. Mortality was not significantly higher in the combined treatments with *SH* dsRNA than in the individual treatments (Fig. 3.4). All treatments caused greater mortality for the insect larvae than in the four control groups.



Treatment concentration	Survival rates (%) at the end of the exp.
Control 1	100[a]
Control 2	90[a]
Control 3	90[a]
Control 4	85[a]
SK 3 × 10²	25[c]
SK 3 × 10³	22[c]
SK 3 × 10⁴	18[c]
3 × 10²	59[b]
3 × 10³	51[b]
3 × 10⁴	42[b]
290 ng/mg SK dsRNA	42[b]

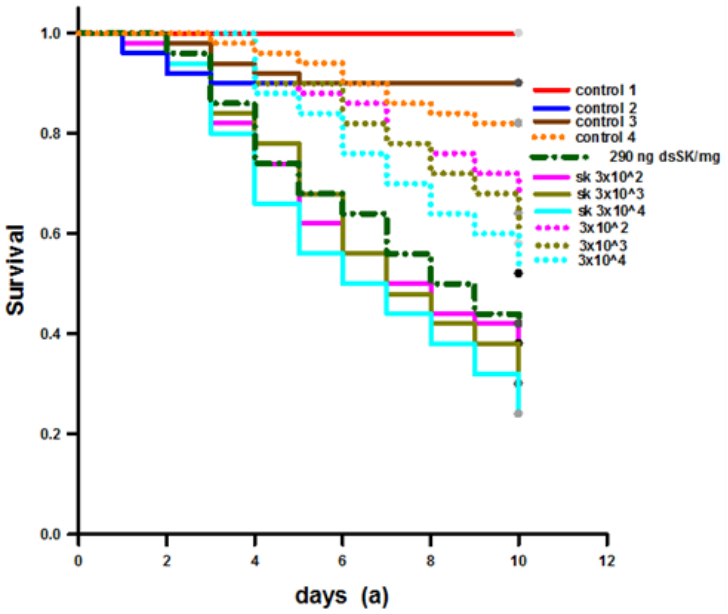
Survival rates followed by the same letter are not significantly different. (N= 50 adult within 5 replicates).



Treatment concentration	Survival rates (%) at the end of the exp.
Control 1	100[a]
Control 2	90[a]
Control 3	90[a]
Control 4	85[a]
SH 3 × 10²	44[c]
SH 3 × 10³	36[c]
SH 3 × 10⁴	24[c]
3 × 10²	59[b]
3 × 10³	52[b]
3 × 10⁴	45[c]
300 ng/mg SH dsRNA	46[c]

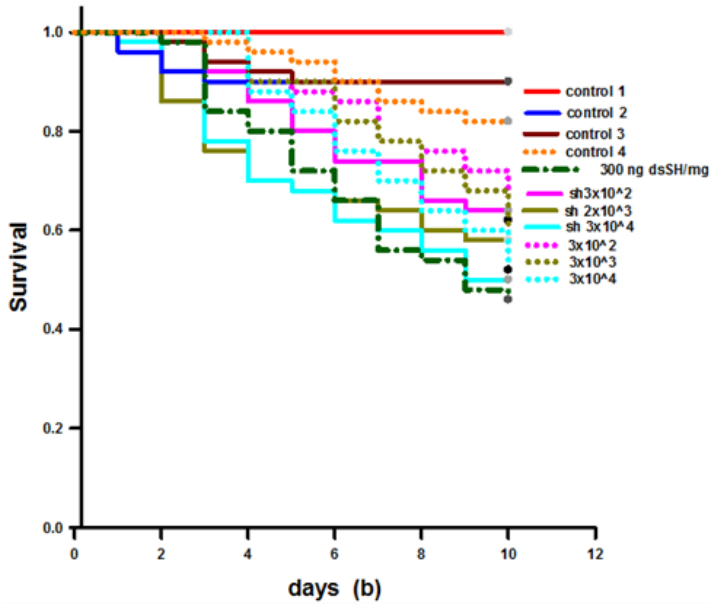
Survival rates followed by the same letter are not significantly different. (N= 50 adult within 5 replicates).

Figure 3.3. Survival curves of *T. castaneum* adults fed on dsRNA targeted at the *SK* gene (A) and *SH* gene (B) with *B. bassiana*. Control 1 = untreated adults, control 2 = adults fed on flour disks treated with RNase-free water, control 3 = adults fed on dsRNA targeted at the kanamycin gene, control 4 = adults sprayed with distilled water.



Treatment concentration	Survival rates (%) at the end of the exp.
Control 1	100[a]
Control 2	90[a]
Control 3	90[a]
Control 4	84[a]
SK 3 × 10^2	38[c]
SK 3 × 10^3	30[c]
SK 3 × 10^4	24[c]
3 × 10^2	65[b]
3 × 10^3	59[b]
3 × 10^4	55[b]
290 ng/mg SK dsRNA	42[c]

Survival rates followed by the same letter are not significantly different. (N= 50 adult within 5 replicates).



Treatment concentration	Survival rates (%) at the end of the exp.
Control 1	100[a]
Control 2	90[a]
Control 3	90[a]
Control 4	84[a]
SH 3 × 10^2	62[b]
SH 3 × 10^3	54[b]
SH 3 × 10^4	50[b]
3 × 10^2	65[b]
3 × 10^3	62[b]
3 × 10^4	55[b]
300 ng/mg SH dsRNA	46[b]

Survival rates followed by the same letter are not significantly different. (N= 50 adult within 5 replicates).

Figure 3.4. Survival curves of *T. castaneum* adults fed on dsRNA targeted at the *SK* gene (a) and *SH* gene (b) with *M. anisopliae*. Control 1 = untreated adults, control 2 = adults fed on flour disks treated with RNase-free water, control 3 = adults fed on dsRNA targeted at the kanamycin gene, control 4 = adults sprayed with distilled water.

3.6 Discussion

The use of RNAi techniques in field conditions for *T. castaneum* control is not practicable yet, owing to the significant limitations of the amount and cost of dsRNA required for successful RNAi application in the field (San Miguel and Scott, 2016; Shukla *et al.*, 2016). To date, the majority of studies have primarily applied RNAi to understand the function of genes in insects (Gotoh *et al.*, 2016; Noh *et al.*, 2016). Therefore, in this work, we tried to overcome some of the challenges involved by combining fungi and low concentrations of dsRNA in an integrated pest management (IPM) approach to provide a safe and effective strategy to control red flour beetle. To my knowledge no previous studies have suggested such a strategy.

Other studies at Newcastle University have targeted potassium channels with RNAi in *T. castaneum,* which has shown a robust RNAi response and knockdown of the *SK* gene (Tc014196) and *SH* gene (Tc003580). Thus, we predicted that the RNA_i knockdown of these genes could be used as part of a control strategy for *T. castaneum.*

The present study demonstrated the efficacy of a combined treatment using *SK* dsRNA with *Beauveria bassiana,* which was more effective than a single treatment of fungus against *T. castaneum* for third instar larvae and adults. Moreover, the double treatment of *SH* dsRNA with *B. bassiana* was also active at the larval stage, but less so than *SK* dsRNA combined with the fungus, on the basis of survival rates of beetles. However, *Metarhizium anisopliae* with *SH* dsRNA did not give higher mortality of adults than the treatment using fungi alone.

In the invertebrate body, there is an interface between the nervous and immune systems. Pavlov and Tracey (2015) described how in mammals the vagus nerve is an essential constituent of a neural reflex mechanism—the inflammatory reflex—that controls immune system responses and inflammation during pathogen infection. The *SK* and *SH* genes play crucial roles in nerve cell potassium ion channels of the red flour beetle. Thus, it can be suggested that the knockdown of these genes has made insects more susceptible to pathogens. However, the control outcomes from combining these biological control agents and the negative impacts on each other are predicted to depend on the time interval between host exposure to the dsRNA and the application of the entomopathogenic fungi as well as the order in which they are applied.

Kawli *et al.* (2010) showed that the nervous system regulates innate immune responses by releasing neurotransmitters (neuropeptides and neurohormones) in the invertebrates. Since *SK*

and *SH* are expressed in the nervous system of *T. castaneum,* it is expected that the knockdown of *SK* and *SH* genes will make the beetle more susceptible to pathogens. The results in this chapter show that combinations of fungal treatment and RNAi are additive or synergistic, according to the survival rates of *T. castaneum* depending on different concentrations of dsRNA (*SK* or *SH* genes). The mortality in this study has two components. Firstly, for the knockdown of genes (Travanty *et al.*, 2004; Nakasu *et al.*, 2014) the current results are consistent with those obtained by Abd El Halim *et al.* (2016), in which feeding *T. castaneum* larvae with dsRNA targeted to the sodium ion channel paralytic A (TcNav) gene at 150 ng/mg induced 51.34% mortality after six days. A similar observation was reported when 40 % mortality of *T. castaneum* larvae resulted from the silencing of TcTre1-4 or TcTre2 (trehalase genes), which play a role in chitin synthesis in the insect cuticle and midgut (Tang *et al.*, 2016). Silencing of TcDad1, which encodes an essential subunit of the oligosaccharyltransferase (OST) complex catalysing the attachment of the N-glycan precursor to a polypeptide in the endoplasmic reticulum, caused 96.4% larval mortality and inhibited larval growth (Walski *et al.*, 2016).

Transgenic plants have allowed RNAi strategies to be applied in the field. Successful oral introduction of RNAi has been achieved through transgenic plants like maize expressing western corn rootworm (WCR) dsRNAs, which led to a significant reduction in damage from western corn rootworm (Baum *et al.*, 2007).

The second component of insect mortality is by pathogenic fungi (Roberts and St Leger, 2004; Maniania *et al.*, 2008; Vega, 2008). In the present study, *B. bassiana* and *M. anisopliae* were applied. The spores of these fungi connect with the body of an insect host, penetrating the cuticle and germinating inside, leading to insect death within 3-4 days. Afterwards, a mould emerges from the insect and new spores are produced. Both fungal formulations show potential as control agents for *T. castaneum*. At the same concentrations, the *B. bassiana* formulation caused higher mortality than *M. anisopliae*, These results are consistent with the findings of Erler and Ates (2015), who demonstrated that *B. bassiana* was more effective than both of the formulations of *M. anisopliae* tested (Metsch and F52), causing mortality rates of 79.8 and 71.6% mortality in younger and older larvae of *Polyphylla fullo* (Scarabaeidae), while *M. anisopliae* caused mortality rates of 74.1 and 67.6% for young and old larvae.

3.7 Conclusions

1- The mortality of third-instar larvae and adults caused by fungal treatment only was lower than the mortality caused by double treatments using *SK* dsRNA and *SH* dsRNA combined with *B. bassiana* and *M. anisopliae.*

2- The double treatment with *SK* dsRNA and either fungus was more successful than *SH* dsRNA and either fungus.

3- The combination of SK dsRNA with *B. bassiana* or *M. anisopliae* has the potential to be used for the management of *T. castaneum.*

Chapter 4. Effects of quinones on infection by entomopathogenic fungi

4 Chapter 4. Effects of quinones on infection by entomopathogenic fungi

4.1 Abstract

The red flour beetle, *Tribolium castaneum*, secretes quinones. These chemicals inhibit the development of various microorganisms and act as an external immune defence that protects against pathogens. Furthermore, quinones also affect population aggregation. These experiments aimed to investigate the impact of quinone secretions on the efficacy of entomopathogenic fungi. *Beauveria bassiana* and *Metarhizium anisopliae* were applied to adults of *T. castaneum* in separate experiments and then the beetles were held in food medium. Three different types of medium were used: fresh medium, naturally conditioned medium, and flour conditioned by synthetic 2-methyl-1,4-benzoquinone (MBQ). For both fungi, the results show high levels of mortality of beetle adults held in fresh medium ten days after treatment (54% and 48% for *B. bassiana* and *M. anisopliae* respectively). The mortality rates of adults held in naturally conditioned media were lower at 42% and 36%, but the difference was not statistically significantly ($P>0.05$). However, the mortality levels for adults held in the flour conditioned by synthetic 2-methyl-1,4-benzoquinone at a concentration of 2.0 mg g^{-1} were 44% and 38%, and at 7.5 and 15.0 mg g^{-1} were 34% and 26% and 30% and 24% for *B. bassiana* and *M. anisopliae* respectively. These levels were significantly lower than mortality rates in fresh and naturally conditioned media. This could be a result of several factors. The infection of a beetle occurs through a series of events. Following adhesion of fungal spores to the cuticle of the beetle, germination and penetration through the body are the main events in the initial stages of infection. In a further part of this study fresh medium and medium conditioned by synthetic 2-methyl-1,4-benzoquinone (MBQ) were used to examine the adherence and germination of entomopathogenic fungal spores using scanning electron microscopy (SEM). There were differences between the types of media regarding both the adhesion of spores on the insect and their germination. Synthetic 2-methyl-1,4-quinone decreased vegetative development and inhibited spore germination of *T. castaneum*. This study gives information on the relative importance of quinone secretions for fungal infection in terms of virulence and understanding the impact of these secretions on insect mortality.

Key words: *Beauveria bassiana*, *Metarhizium anisopliae*, quinone secretion, *Tribolium castaneum*, SEM, entomopathogenic fungi.

4.2 Introduction

The most common pest insects found in milled products and stored grain are flour beetles. Flour beetles, like some other organisms, have developed forms of 'external' defences which complement the usual 'internal' immune defence system, providing greater protection against pathogens (Cotter and Kilner, 2010). This is the case with *Tribolium* flour beetles in their typical habitat of stored grains, where the secretion of defensive chemical compounds can benefit them. These compounds have a broad antimicrobial function (Prendeville and Stevens, 2002; Yezerski *et al.*, 2007; Li *et al.*, 2013) and also they are effective against many helminth parasites (Yan and Phillips, 1996). According to Sokoloff (1974), they are thus said to "keep the beetles environment clean" and so provide an external defence mechanism. If the concentration of quinones in the environment of the beetles becomes too high (e.g. due to increased secretion), their toxic effects will affect the larvae and, above a certain concentration, also the adults. This toxic side effect suggests a potential optimum in the production of defensive secretions with a lower threshold, below which the antimicrobial effect might be insufficient to protect beetles and larvae, and an upper threshold, above which offspring mortality might reduce fitness of the parent producing these secretions. The beetles have glands which secrete quinones such as 2-methyl-1,4-benzoquinone (MBQ), 2-ethyl-1,4-benzoquinone (EBQ), and hydroquinone (Loconti and Roth, 1953; Engelhardt *et al.*, 1965; Ladisch and St. Agatha Suter, 1967; Blum, 1978; Howard, 1987; Alexander and Barton, 1996), which are commonly referred to as benzoquinones. The tenebrionid beetle family is a most prolific source of 2-methyl-1,4-benzoquinone and 2-ethyl-1,4-benzoquinone (Ladisch and McQue, 1953; Omaye *et al.*, 1981; Yezerski *et al.*, 2004; Yezerski *et al.*, 2007) and individual beetles can secrete up to 0.5 mg of quinones into their substrate cereal, grain, or flour over six weeks. The insects rapidly colonise infested food and quinone concentrations may be very high in populations of thousands of individuals. Benzoquinones give grain a characteristically unpleasant odour, and infected flour has a pinkish colour (Payne, 1925).

The current investigation set out to determine the effects of quinones on infection of *T. castaneum* by the entomopathogenic fungi *B. bassiana* and *M. anisopliae* comparing different levels of quinone to determine the impact on the efficacy of the fungi.

To test the effect of the amount of quinone, flour conditioned by synthetic 2-methyl-1,4-benzoquinone was prepared in the laboratory a short time before the start of the experiment.

Flour conditioned by a natural quinone source was used to investigate the impact on the growth of fungi on the insect body. As a control, fresh medium prepared from brewer's yeast and whole wheat flour was tested.

To further understand the relationship between quinone secretion and fungal infection, scanning electron microscopy (SEM) was employed to determine the conidial number and distribution at different cuticular sites of treated adults of *Tribolium castaneum* held in the flour conditioned by synthetic 2-methyl-1,4-benzoquinone (MBQ) at a higher concentration, compared with adults held in fresh medium after exposure to the fungi. The effect of quinone secretions on the spore's attachment to the adult cuticle was determined. The timing of the different phases of the infection process was assessed from SEM observations and compared for the different types of media.

Objectives

The objectives of this work were:

1. To determine the impact of quinone secretion level on the mortality of insects treated with two fungi, *B. bassiana* and *M. anisopliae.*
2. To describe the external development of *B. bassiana* on *T. castaneum* adults held in two different types of medium by scanning electron microscopy (SEM).
3. To determine the duration of various stages of fungal infection.

4.3 Materials and methods

4.3.1 Entomopathogenic fungi and methylquinone used

The entomopathogenic fungi used in the present study were *B. bassiana* and *M. anisopliae*. Commercial formulations of the fungi were obtained from Fargro Ltd, Littlehampton, West Sussex, and Belchim Crop Protection Limited, Eaton Socon, respectively. The LC_{50} concentrations of fungi were used to infect the insects via spraying. The LC_{50} values were determined in the first experiment (Chapter 2) after day five for each treatment. Conditioned flour was prepared from a natural quinone and synthetic quinone sources. The synthetic 2-

methyl-1,4-benzoquinone (MBQ) was a commercial powder formulation produced by Sigma-Aldrich Company Ltd.

4.3.2 Source of insects and experimental methods

The *T. castaneum* adults used were taken from a culture that was kept in the laboratory on whole wheat flour mixed with brewer's yeast at a ratio of 100:5 by weight. The cultures were kept in a controlled environment at 25 °C. All treatments were carried out in the same environmental conditions used for the cultures. The conidial concentrations of solutions for both fungi were estimated using a haemocytometer with a light microscope. A series of dilutions were performed to give concentrations of 10^5 spores/ml. Suspensions were held overnight at room temperature and then checked for conidial germination before use in bioassays, as described by Yeo *et al.* (2003). All adults tested were approximately two weeks post adult eclosion.

4.3.3 Methods of treating the medium

1. To obtain flour conditioned by a natural quinone source, about 600 adults of *T. castaneum* aged between 3 and 5 months without sex differentiation were placed in a glass Kilner jar containing 400 g of fresh flour medium. The jar was covered with filter paper at the top and held for 21 days in an incubator at 25 °C without light. After the scheduled period, the jar was removed from the incubator and beetles were removed from the medium by sieving. The medium was sieved through 500 and 250-micrometre sieves three times over 14 days to isolate and remove eggs and larvae, if any, to avoid their effect on this medium. The medium was used in the experiment 14 days after the beetles were removed.

2. Flour conditioned by synthetic MBQ was prepared as follows. Synthetic quinone was sublimed into the fresh medium by placing a measured amount (2.0, 7.5, or 15.0 mg) in small glass Petri dishes set in big glass jars containing 100 g of fresh medium. Each jar was kept in an oven at 60°C for six hours (Ogden, 1969). When the jar was removed from the oven, the surface layer of the medium was discoloured to brownish pink. The quinone treated medium was then thoroughly mixed before being used in the experiment. Fresh medium for the control was prepared from brewer's yeast and whole wheat flour mixed 5:100 by weight, but without MBQ.

4.3.4 Experimental approach

Experiment 1: The effect of quinone secretion levels on the efficacy of *B. bassiana* and *M. anisopliae*

Adults of *T. castaneum* were treated with the LC_{50} concentrations of *B. bassiana* and *M. anisopliae* (6.8×10^5 and 7.9×10^5 spores/ml, respectively). There were five replicates (giving 50 insects in total) for each treatment. Each group of 50 insects was directly sprayed with 3.3 ml of the spore suspension while they stood in a large glass Petri dish (200 cm diameter). Adults were left for 15 min to ensure that the conidial suspension had adhered to the insect bodies. After 15 min the treated beetles were moved to plastic Petri dishes 9 cm in diameter. The beetles were held in 20 g flour conditioned by the natural quinone source (Treatment 1), or flour conditioned by synthetic MBQ in different concentrations (2.0, 7.5, and 15.0 mg per 100 g flour; Treatment 2, 3 and 4) or finally with the fresh medium for the control (Treatment 5). Records of insect mortality were taken 3, 6, and 9 days after treatment. All treatments were performed at 25 °C and at the same time.

Experiment 2: The effect of quinone secretions on the spores' attachment to the adult cuticle

1. A single type of fungus, *Beauveria bassiana*, was used in this part of the study because it had the strongest effect. Groups of beetles were sprayed with 6.8×10^5 spores/ml suspension of the fungus as above and then maintained either in fresh medium or conditioned medium (flour conditioned by synthetic 2-methyl-1,4-benzoquinone (MBQ) at the highest amount of 15.0 mg) and maintained at 25±1°C. At intervals (24, 48, 72, 96, 120, and 144 h) six beetles were removed from each treatment and killed by immersion in 2% glutaraldehyde in Sorenson's Phosphate Buffer, then rinsed in several changes of Sorenson's Phosphate Buffer (T. Davey, Electron Microscopy Research Services in Newcastle University, pers. comm., Dec. 2017).

2. Beetles from a particular combination of time following treatment and post-treatment medium were kept separate in a tube for that treatment combination.

3. Dehydration was carried out using 25% ethanol, 50% ethanol, and 75% ethanol for 30 min each followed by 100% ethanol (2 x 1 h). Final dehydration with carbon dioxide was

accomplished in a Baltic Critical Point Dryer. The specimens were examined under the SEM to evaluate the level of spore attachment and germination on the body.

The adherence and germination of fungal conidia were examined using low-temperature scanning electron microscopy (cryoSEM). A TESCAN VEGA LMU Scanning Electron Microscope was used, housed within EM Research Services at Newcastle University. Digital images were collected with software supplied by TESCAN. The insects were positioned on an adhesive carbon disc, and the samples were sublimated before sputter coating with gold/palladium. For each beetle, an assessment of the number of conidia present on three different areas of the body was made: the dorsal surface of the elytra, the ventral abdomen, and insect legs (the hind femur). The sample size was 0.0137 mm^2 in the three different areas of the insect body. For each body area three samples were assessed, and the total number of conidia present and the number that had germinated were recorded. One beetle was examined for each of the six time periods post-treatment (beetles collected from 24 to 144 hours after spraying).

4.3.5 Statistical analysis

All data were analysed separately for each fungus and together for both using Minitab® 17.1.0 (© 2010 Minitab Inc.). Percentage mortalities at the end of the experiment were determined. A General Linear Model (GLM) was used to test significant differences between the five treatments, then Tukey's multiple range tests were used to separate means for the experiment with the different types of medium (Experiment 1). The numbers of spores adhering to the cuticle of the ventral abdomen, the dorsal surface of the elytra, and the hind femur were assessed and comparisons between fresh and conditioned media were made using chi-square tests with total numbers of spores. Chi-square tests were also used to compare the proportions of spores that germinated in fresh and conditioned media and the numbers of spores adhering to the beetle's cuticle within three areas of the body (ventral abdomen, elytra, and hind femur) for each treatment (Experiment 2).

4.4 Results

Experiment 1: The effect of quinone secretion levels on the efficacy of *B. bassian*a and *M. anisopliae*

The average mortalities for *B. bassiana* and *M. anisopliae* were always higher if beetles had been held in the fresh medium than in any other medium (Figs. 4.1 a & b). There were significant differences between the mortality of the adults held in the different types of media for each fungus (*B. bassiana* GLM: $F_{(4, 20)} = 11.67$, $P < 0.001$; *M. anisopliae* GLM: $F_{(4, 20)} = 7.67$, $P < 0.001$). Mortality was observed to be consistently higher for *B. bassiana* than *M. anisopliae*. A significant difference was found between the adult mortality caused by the two different fungi when the data for both were analysed together (GLM: $F_{(1, 40)} = 6.51$, $P < 0.05$). High levels of mortality were recorded for both *B. bassiana* and *M. anisopliae* with adults held in the fresh and naturally conditioned media after 10 days of treatment, but significantly lower mortality levels were found for adults held in the flour conditioned by synthetic 2-methyl-1,4-benzoquinone (MBQ) at 15.0 mg (see Figs. 4.1 a & b).

Experiment 2: The effect of quinone secretions on the spore attachment to the adult cuticle

1- Comparing spore numbers on the adults held in a different media

The number of ungerminated *B. bassiana* spores found on the adults held in conditioned medium and fresh medium are shown in Figure 4.2. Examination at three periods post-treatment showed that spores were present in significantly larger numbers on the cuticle of *T. castaneum* adults held in the fresh medium compared to the adults held in the flour conditioned by synthetic 2-methyl-1,4-benzoquinone (chi-square tests: 24 h, $\chi^2_{(1)} = 26.3$, $P < 0.001$; 48 h, $\chi^2_{(1)} = 21.6$, $P = < 0.001$; 72 h, $\chi^2_{(1)} = 5.67$, $P = 0.017$). Numbers adhering in fresh medium were greater than those in conditioned medium for all three body regions examined.

Observations of the number of germinated spores at 24, 48, and 72 h post-treatment showed that many conidia on the cuticle of adults held in the two different media (fresh medium and the flour conditioned by synthetic 2-methyl-1,4-benzoquinone) germinated. Germinated spores were not observed until 48 h post-treatment (Fig. 4.3). At 48 h, the proportion of germinated spores was slightly but not significantly greater with fresh medium than with conditioned medium (chi–square test, $\chi^2_{(1)} = 3.56$, $P = 0.056$). No difference was detected between the

proportions of germinated spores on the adults held in the two treatments for 72 h post-treatment (chi–square test, $\chi^2_{(1)} = 2.41$, $P = 0.121$).

2- Comparing spore numbers on different parts of the insect body

For both treatments (adults held in fresh medium and the flour conditioned by synthetic 2-methyl-1,4-benzoquinone), the density of spores observed on the adult's integument 24 h after inoculation was higher on the hind femur surface than the ventral abdomen and the dorsal surface of the elytra for either treatment (Fig. 4.3). There was a significant difference between the spore numbers on different parts of the insect body with both fresh (chi–square test, $\chi^2_{(2)} = 50.9$, $P < 0.001$) and conditioned medium (chi–square test, $\chi^2_{(2)} = 35.6$, $P < 0.001$).

3- Comparing spore development on the adults held in different media

From the SEM observations, the development of *B. bassiana* is illustrated in Figure 4.4 A-M. Observation of the development of *B. bassiana* on the adults held in the flour conditioned by synthetic 2-methyl-1,4-benzoquinone revealed some differences from the events for the adults held in fresh media. Regarding *B. bassiana* spore adherence to the beetle's integument 24 h after the spraying process, fewer spores were found on *T. castaneum* adults held in flour conditioned by synthetic 2-methyl-1,4-benzoquinone than on adults held in the fresh medium (Fig. 4.4 A, B). The initiation of spore germination happened within 48 h after spraying, and fewer germ tubes of spores appeared to be oriented toward the adult cuticle in the conditioned medium than in fresh medium (Fig. 4.4 C, D). Penetration happened within 72 hours of spraying. Germination tubes in the fresh medium and halo formation around the fungal spore were observed clearly with spore adhesion and germination. The germ tubes in the conditioned medium were much shorter than those observed in fresh medium, and fewer spores showed signs of germination (Fig. 4.4 E, F). In some regions such as the dorsal surface of the elytra, weak growth of germ tubes was seen, perhaps due to the resistance to fungal penetration in these regions.

For both fresh and conditioned media, the host colonising phase took place between 72 and 120 h. Mycelial extrusion occurred between 96 and 120 h after inoculation, particularly in the areas between the wings (Fig. 4.4 G, H). The conidiogenesis process happened between 120 and 144 h after spraying (Fig. 4.4 I, J) in both treatments. After 144 hours, the insect body was completely taken over by *B. bassiana* mycelium (Fig. 4.4 K, M).

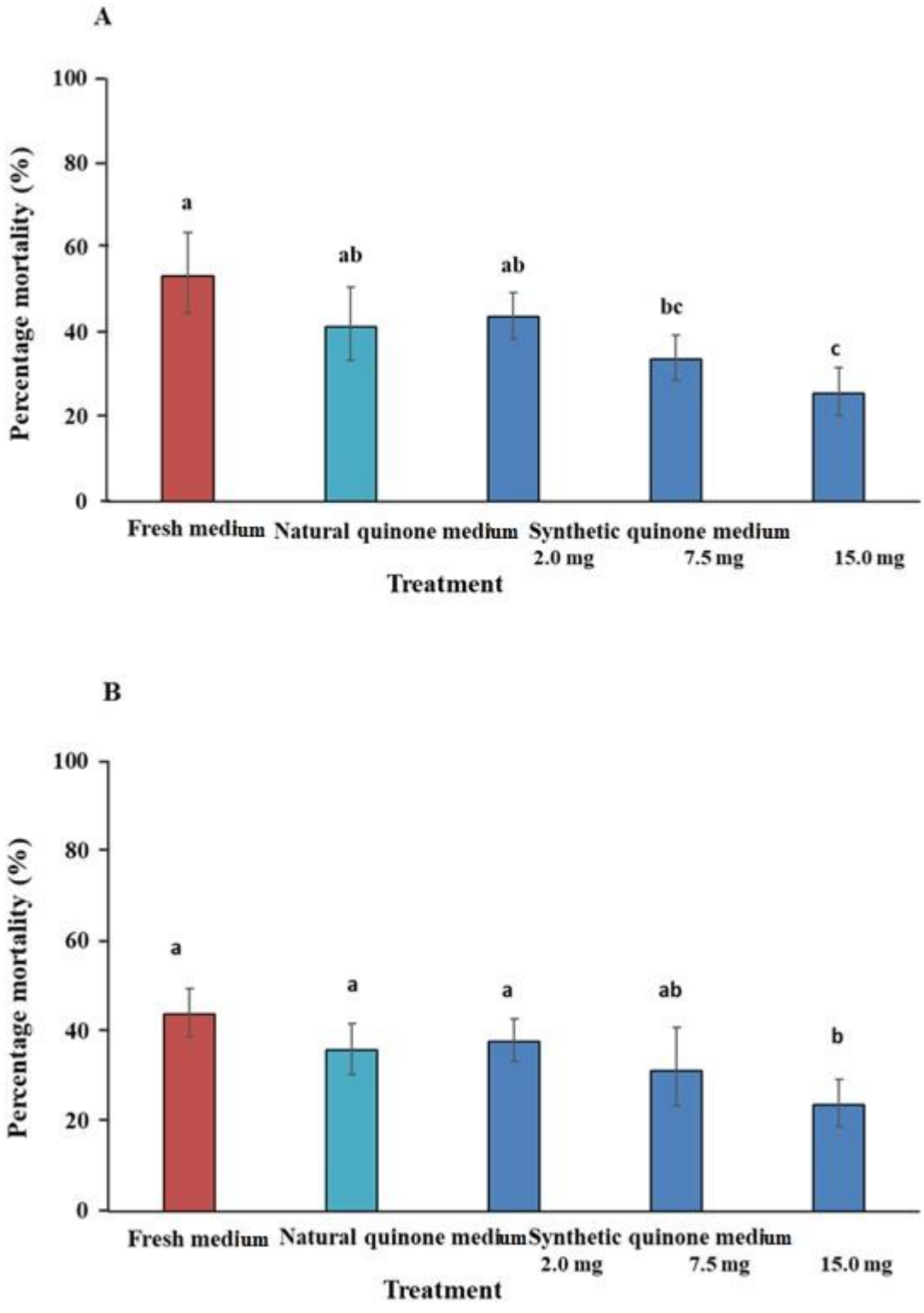


Figure 4.1. Mean percentage mortality of *T. castaneum* adults after 10 days following treatment with LC_{50} (6.8×10^5 and 7.9×10^5 spore/ml) of *B. bassiana* (A), *M. anisopliae* (B), and held in media treated with different doses of naturally produced and synthetic quinones (2.0, 7.5, 15.0 mg) compared to fresh medium at 25ºC. Bars with the same letter do not differ significantly according to Tukey's multiple range test at $P > 0.05$. Error bars show standard error (n = 5).

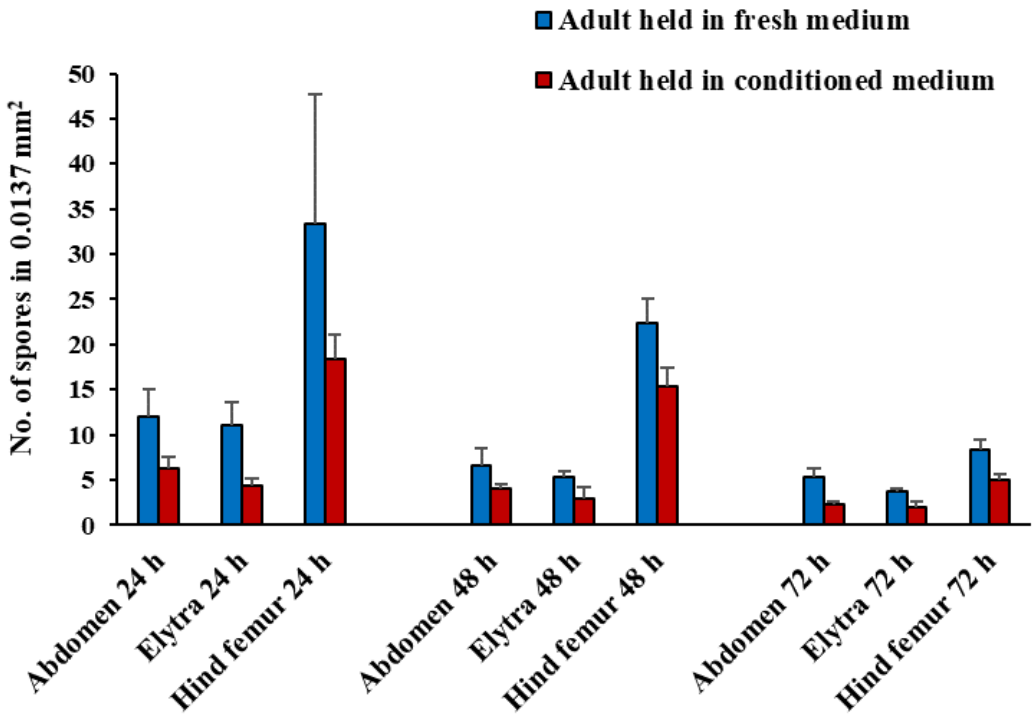


Figure 4.2. The average numbers of ungerminated *B. bassiana* spores attached to the cuticle of *T. castaneum* adults held in fresh and conditioned media at different periods post-treatment. Error bars show standard errors (n = 3).

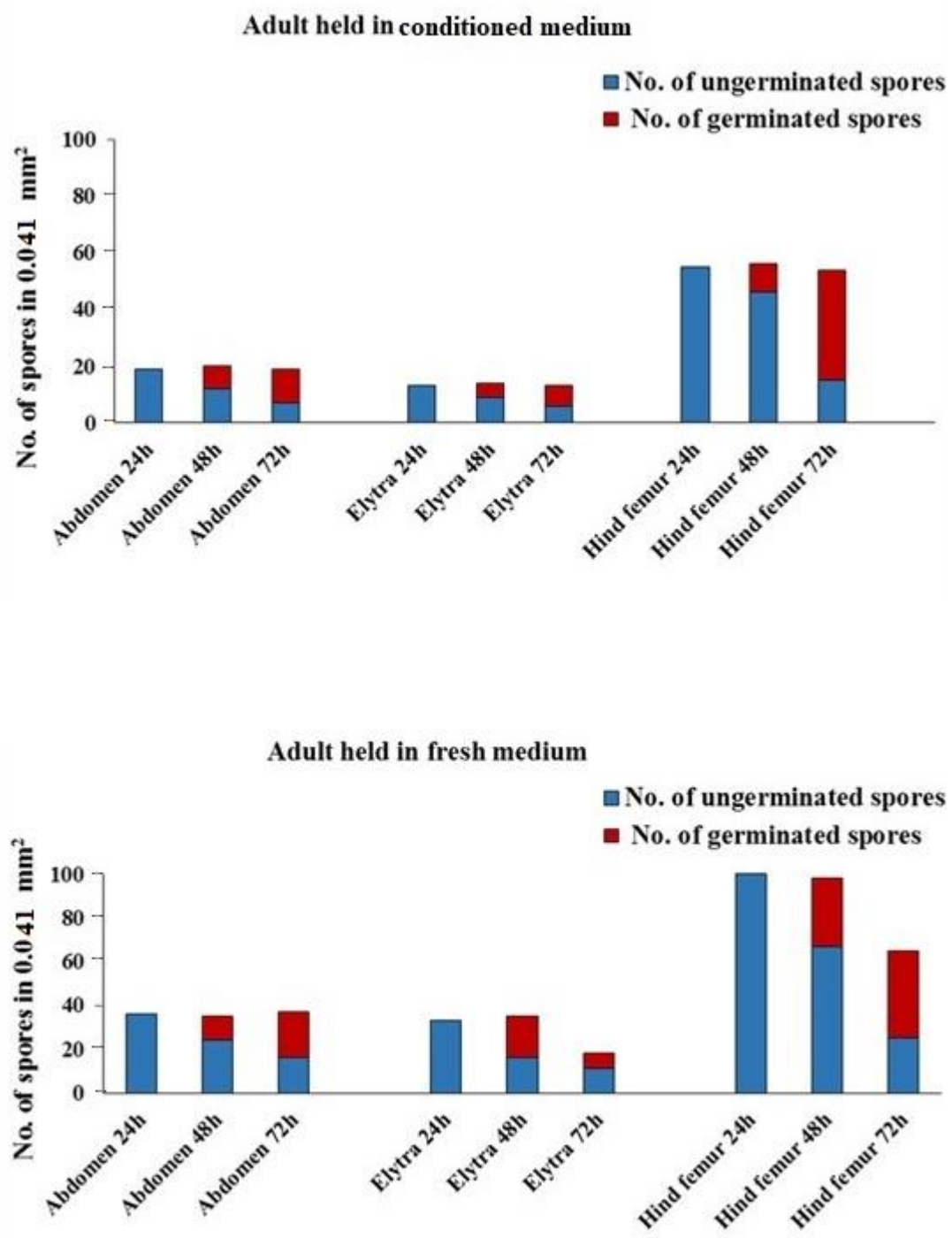
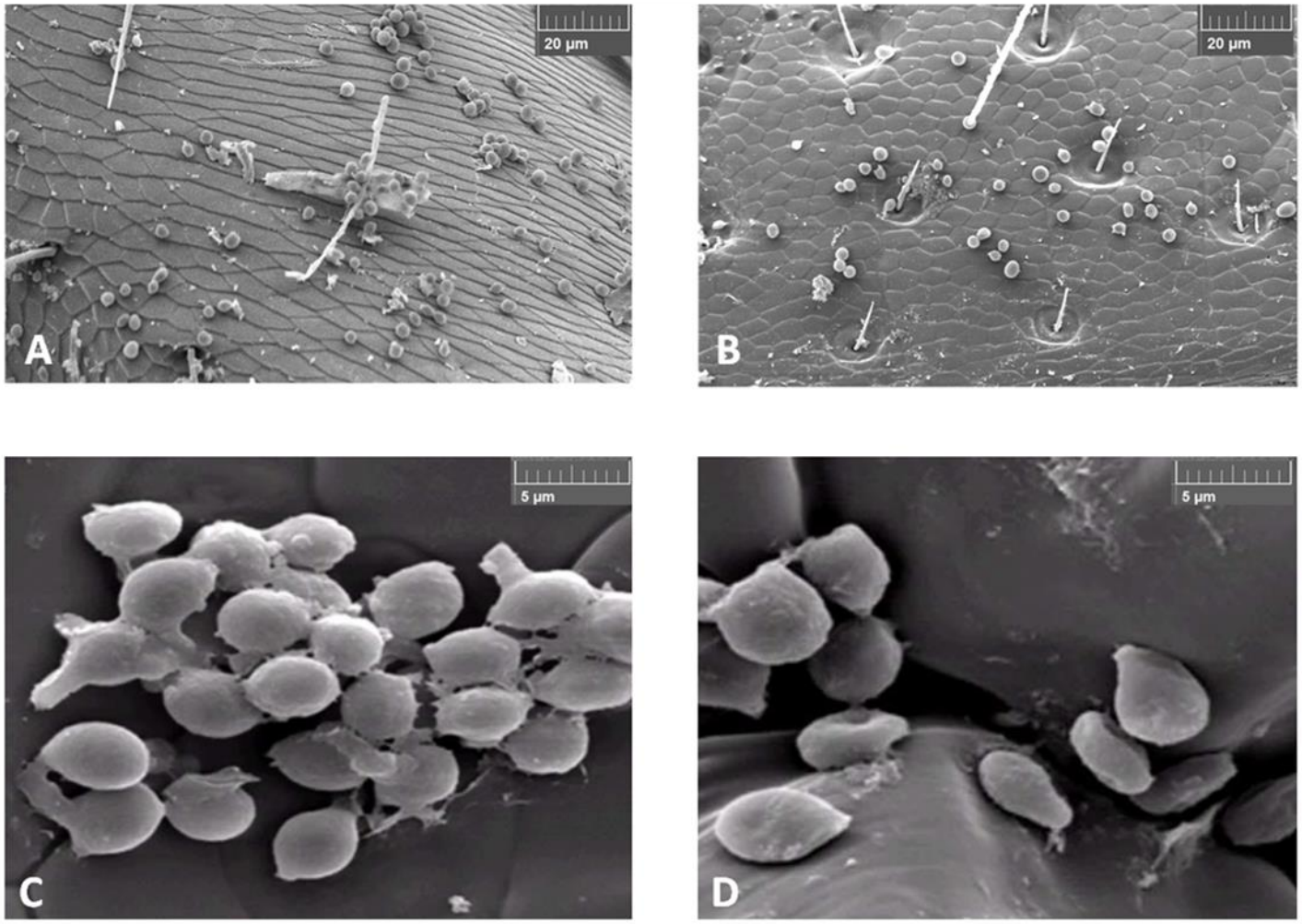
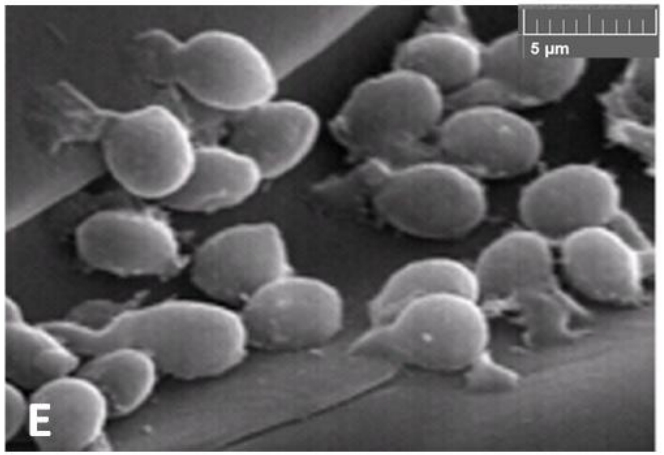
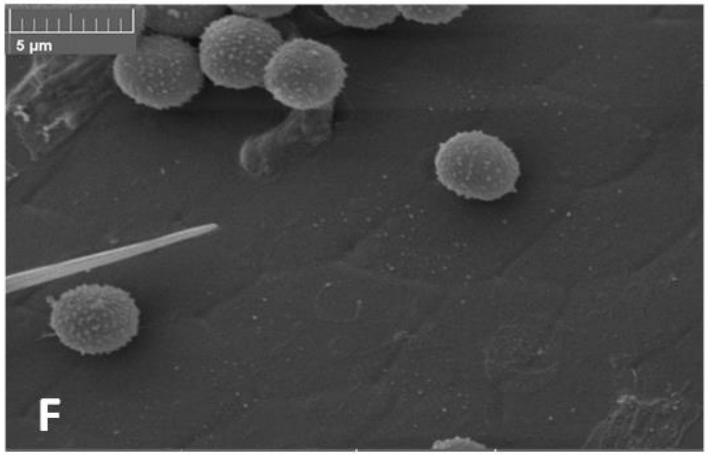


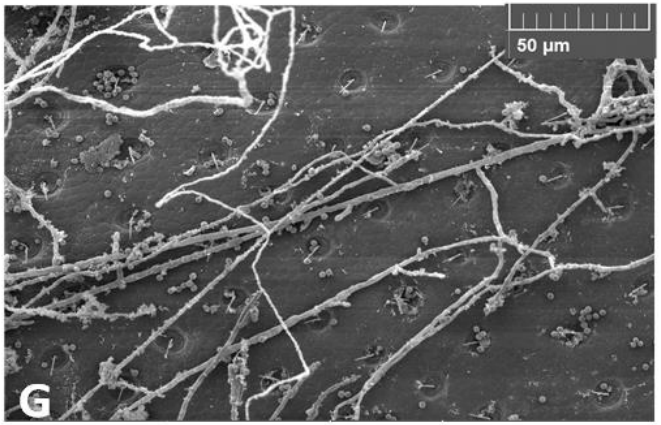
Figure 4.3. The numbers of ungerminated and germinated *B. bassiana* spores attached to the cuticle of *T. castaneum* adults held in fresh and conditioned media at different periods post-treatment. Numbers are totals for three replicate areas, each 0.0137 mm^2.

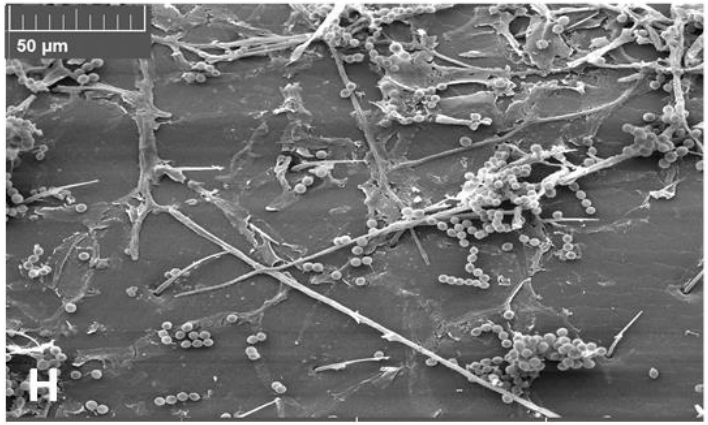
T. castaneum **adults held in fresh medium** ***T. castaneum*** **adults held in conditioned medium**




5 µm
E


5 µm
F


50 µm
G


50 µm
H

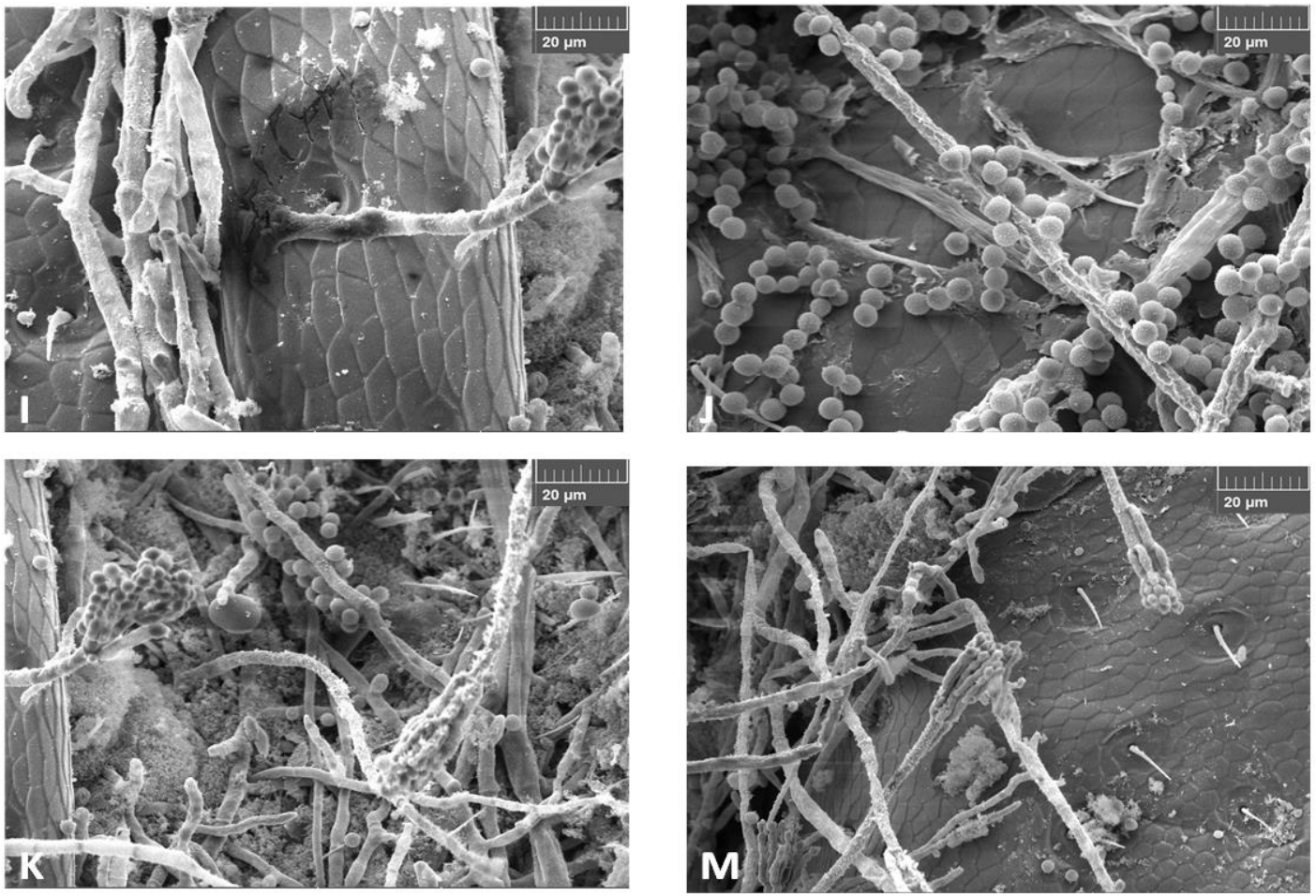


Figure 4.4. Scanning electron micrographs of the development of *B. bassiana* on *T. castaneum* adults held in two different types of media, fresh medium and flour conditioned by synthetic 2-methyl-1,4-benzoquinone. Spores of *B. bassiana* are globose to ovoid and 2 to 4 µm in diameter. A, B) Spores adhered to the tegument (Bar = 20 µm, 24 h after spraying); C, D) Germinating spores in the cuticle (Bar = 5 µm, 48 h); E, F) Penetration of germ-tubes into the cuticle (Bar = 5 µm, 72 h); G, H) Extrusion of the mycelium at intersegmental region (Bar = 50 µm, 96 h); I, J) Conidiophore formation and conidiogenesis on the elytra, with degradation of the cuticle (Bar = 20 µm, 120 h); K and M) Details of the conidiogenesis process (Bar = 20 µm, 120 h).

4.5 Discussion

The results of this series of experiments show that high levels of mortality were recorded for both *B. bassiana* and *M. anisopliae* with adults held in the fresh medium and naturally conditioned medium 10 days after treatment, but much lower mortality levels were found for adults held in the flour conditioned by synthetic 2-methyl-1,4-benzoquinone (MBQ) at 15.0 mg. This level of mortality could mean that MBQ may decrease vegetative development and inhibit spore germination on *Tribolium castaneum*. It is likely that the effects in naturally conditioned medium will be similar, but less dramatic, due to the lower concentrations of benzoquinones. The results are consistent with those from a previous study (Joop *et al*., 2014) which tested fungal inhibition (*Aspergillus niger*) by placing cold-shocked *T. castaneum* beetles on spore-seeded agar plates. In testing the efficacy of the amount of quinones secreted by the beetles against a common mould fungus, lower fungal inhibition was found in beetle lines selected for low quinone secretion compared with unselected control lines, whereas there was increased fungal inhibition in beetle lines selected for high quinone secretion. Ogden (1969) reported that beetle quinones were inhibitory to the growth of *Penicillium* and *Aspergillus* which were cultured on potato dextrose agar.

The electron microscope study showed that quantitative differences could be observed between the two treatments. *T. castaneum* adults held in fresh medium had a higher density of spores adhering to the cuticle, specifically on the hind femur. More germinating spores also were observed on the cuticle of adults held in fresh medium than those held in conditioned medium. There are several possible explanations for this. First, the defensive quinones and cuticular hydrocarbons can either inhibit or promote conidial germination (St Leger *et al.*, 1991). The inhibition of yeast and bacterial growth by defensive secretions from *Tribolium* spp. has previously been demonstrated (Prendeville and Stevens, 2002). From this, it would give the idea that conidia that have not grown and penetrated the cuticle within the first 24-48 hours are unlikely to stay on the epidermis and play a role in the infection process (Wakefield, 2006). Penetration through the cuticle was not easily observed. Even at points where penetration may be expected, such as intersegmental regions, penetration could not be seen. The absence of significant germ tube growth on the cuticles of adults held in the two different types of media (fresh medium and flour conditioned by synthetic 2-methyl-1,4-benzoquinone) may indicate that penetration occurs directly below the point of attachment. *Beauveria bassiana* has been

shown to produce germ tubes that grow over the surface of the insect cuticle until they contact an area of relative weakness where penetration can easily be achieved (Pekrul and Grula, 1979; Butt *et al.*, 1995). In addition, the formation of haloes around fungal spore and germination tubes on the beetle cuticle was observed with spore adhesion and germination. The occurrence of these haloes seems to be associated with the production and excretion of exoenzymes from the entomopathogen during the infection process. The enzymatic action of the fungus *B. bassiana* on the pecan weevil, *Curculio caryae*, has been observed before with SEM (Champlin *et al.*, 1981). Fungal penetration is one of the features which has been linked to the virulence of different isolates of *Paecilomyces fumosoroseus* (Altre and Vandenberg, 2001).

This study has shown that benzoquinones secreted by flour beetles may affect the fungal growth on the insect body. Due to the scarcity of in-depth research investigating the inhibition of fungal growth by defensive secretions in *T. castaneum*, follow-up work is required in this area. Additionally, the possible mechanisms for such effects require further investigation. Increasing susceptibility of insects through improved formulation of the conidia may be possible. Compounds that are known to act on the cuticle could be incorporated, if not detrimental to the conidia.

Chapter 5. Effects of fungal application on insect behaviour

5 Chapter 5. Effects of fungal application on insect behaviour

5.1 Abstract

Fungal application often results in alterations in insect host behaviour. These changes vary in their magnitude, from slight shifts in the time spent by the host performing a given activity to the appearance of new behaviours. The effects of fungal application can change the physiological condition of the host. We tested the locomotory activity of *T. castaneum* adults at different times after treatment with a commercial formulation of the fungus *Beauveria bassiana*. The speed of movement of adults was measured. Adults of *T. castaneum* which were treated only with water tended to move a greater distance, while adults treated with the LC_{50} (at day 5) concentration of *B. bassiana* covered smaller distances and at a lower speed. The treatment with *B. bassiana* affected the locomotory activity of *T. castaneum* adults.

Key words: *Beauveria bassiana*, *Tribolium castaneum*, insect behaviour, entomopathogenic fungi.

5.2 Introduction

Animals adjust their behaviour according to changes in the environment, physiological condition, needs and requirements at the current stage of life and age. This is particularly true in the case of animals such as stored-product insects living in an environment of spatially discrete food patches that vary considerably in quality, size, and persistence. It is also known that parasitic infection leads to changes in host behaviour. These changes caused by a parasite vary in scale, from minor alterations to those where the performance of a given activity results in the emergence of completely new and unpredictable behaviours. The effect of parasites may vary with the age of the host; for example, older individuals with a shorter life expectancy may be more affected than younger individuals. One of the most diverse groups of parasites is the fungi, which display a wide variety of interactions from transient to obligate associations (Oi and Pereira, 1993; Roy *et al.*, 2006; Espadaler and Santamaria, 2012; Csata *et al.*, 2017). Fungi can affect the hosts in many ways, and mechanical behavioural, biochemical, and even physiological changes in insects can be related to the presence of fungal parasites (Kaur and Mukerji, 2006; Ortiz-Urquiza and Keyhani, 2013; Hughes *et al.*, 2016). In ants, infection by fungi causes well-studied alterations in the activity of the insect host (Oi and Pereira, 1993; Heinze and Walter, 2010; Konrad *et al.*, 2012; Tragust *et al.*, 2013). Due to this, fungal infection could be associated with considerable physiological costs for the infected host which could be reflected in altered locomotion, such as in decreased mobility. There has been considerable study of insect movement activity (Turchin, 1998) and the process of insect movement when resources such as shelter and food are distributed inconsistently (Hanski *et al.*, 1997). Nevertheless, studies on how stored product insects and their movement interact with parasites are very limited. To understand stored-product pest ecology, and ultimately to manage pest populations effectively, we need to better understand the impact of fungal infection on the locomotory activity of the insects during stored-product pest management.

Campbell and Hagstrum (2002) examined how a major pest, *T. castaneum,* exploits patches of food. *Tribolium castaneum* is one of the most important pests of processed grain, and it exhibits a high rate of movement among patches (Ziegler, 1976). Earlier studies have observed a variety of factors involved in the movement of *T. castaneum* (Hagstrum, 1973) and dispersal from patches of flour (Hagstrum and Gilbert, 1976; Ziegler, 1976; Lavie *et al.*, 1978). *Tribolium castaneum* responds to the volatiles of food (Seifelnasr *et al.*, 1982; Phillips *et al.*, 1993) and an aggregation pheromone (Obeng-Ofori, 1991) and these may also influence movement

patterns. Other internal and external factors may also affect patch use. What has been lacking so far are studies of *Tribolium* behaviour at smaller scales. Studies of this type could be used to extend studies conducted in the laboratory and make it easier to apply the results of behaviour and ecology research to the management of pests in storage facilities. The experiment described here aims to identify the effects of fungal application on insect locomotory behaviour.

Objectives

The major objective of this experiment was to evaluate the impact of treatment with the fungus *Beauveria bassiana* on the walking distance of *T. castaneum* adults.

5.3 Materials and methods

5.3.1 Source of insects

Adults of *Tribolium castaneum* used in this study had been maintained in the laboratory for more than two years. The beetles were reared on a mixture of wholemeal flour with brewer's yeast (mixed 100:5 by weight) in an incubator kept at 25°C. For the walking speed experiment, 80 adults of mixed sex and age were randomly collected from a stock culture. One day before the test the beetles were placed in a large glass Petri dish 200 cm in diameter, and then the insects were fed with a diet of fresh flour.

5.3.2 Sources of fungal suspension

A commercial formulation of *B. bassiana* fungus was used in the experiment. A liquid suspension of *B. bassiana* was obtained from Belchim Crop Protection Limited, 1b Fenice Court, Phoenix Park, Eaton Socon. The liquid suspension of *B. bassiana* was an oil dispersion containing 7.16% w/w *B. bassiana* ATCC 74040 as described in Chapter 2. The suspension was mixed with 500 ml of distilled water in a glass beaker following the instructions on the package, and then the beaker was vortexed. The volume of the mixture was measured, and the spore concentration in the mix was determined using a haemocytometer. The mixture volume and spore count were used to calculate the number of spores per ml of water (Behle and Goett, 2016). The fungal suspension was left for 2-4 h before application to assist the dispersion of the spores. The conidial concentration was adjusted to 10^6 spores/ml, which represents the dose of LC_{50} (at day 5) of *B. bassiana*.

5.3.3 Experimental methods

In this experiment, each replicate was a 9 cm petri dish, which constrained the beetles' movement. The spore suspension of *B. bassiana* was sprayed onto the adults of *T. castaneum*. In each treatment the insects were divided into four replicates. Each replicate of 10 insects was directly sprayed with 2 ml of spore suspension while they stood in a large glass Petri dish 200 cm in diameter, then adults were directly released into plastic Petri dishes 9 cm in diameter without flour in them, in order to track the walking movement of each beetle. Control insects were sprayed with water in a Petri dish, without the fungus. The distance moved by *T. castaneum* adults in the two treatments was measured by continuous recording of the beetles over a 30 min period. The walking movement of each beetle was recorded using an Inspire four-channel digital security recorder (INS-DVR04V2-250) and a Samsung video camera (SCB_2001). The video clips for each replicate were analysed using the video analysis software application Kinovea©, which is a 2D motion software that enables the measurement of movement parameters (Kinovea, 2018). The distance moved by each beetle during one minute at 5, 10, 15, 20, 25, and 30 min after treatment was measured and analysed through this program. All experiments were run under ambient conditions in the laboratory under uniform low-light conditions.

5.3.4 Statistical analysis

To compare the distance moved by the *T. castaneum* adults, Minitab® 17.1.0 (© 2010 Minitab Inc.) was used. A General Linear Model (GLM) was used to test for significant differences between fungus and control treatments. The LC_{50} value for the fungus was determined in Chapter 2.

5.4 Results

In this experiment, with the LC_{50} spore concentration of *B. bassiana*, significant differences in the distance moved by *T. castaneum* were noted between the two treatments at each time during the 30 min period (5 min, $F_{(1, 75)} = 64.45$, $P < 0.001$; 10 min, $F_{(1, 75)} = 67.85$, $P < 0.001$; 15 min, $F_{(1, 75)} = 59.06$, $P < 0.001$; 20 min, $F_{(1, 75)} = 26.93$, $P < 0.001$; 25 min, $F_{(1, 75)} = 29.22$, $P < 0.001$; and 30 min, $F_{(1, 75)} = 24.16$, $P < 0.001$). The distance moved in each treatment is shown in Figure 5.1 a-f. The results show that the adults treated with the fungal spores walked a shorter distance than the control adults treated with water. However, there was variation between control and fungus treatments for each replicate throughout the times (Appendix 3).

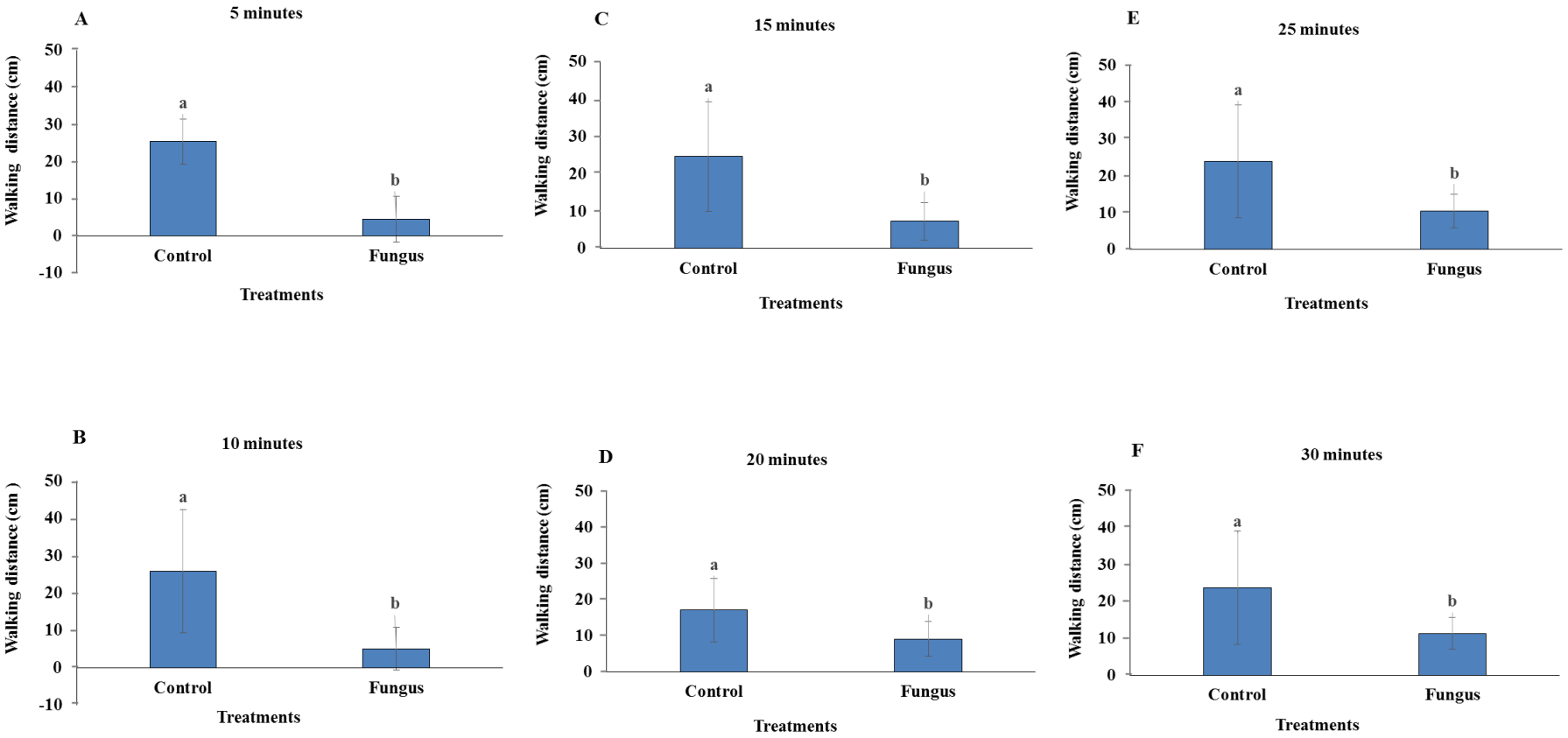


Figure 5.1. Mean walking distance of individual *T. castaneum* adults after 5 min (A), 10 min (B), 15 min (C), 20 min (D), 25 min (E), and 30 min (F) following treatment with the LC_{50} value of *B. bassiana* fungus. Error bars are $\pm$ one standard deviation (n = 40).

5.5 Discussion

Our findings show that *T. castaneum* adults are immediately affected by treatment with a spray of *B. bassiana*. The beetles treated with fungus were usually inactive. These periods of inactivity were often directly after treatment and the beetles tended to be observed in the middle of the arena. However, it was observed that after approximately 20 min the insects returned to their typical activity. In contrast, in the control treatment beetles were usually active directly after treatment. The beetles tended to be observed near the edges of the arena, and this is the normal behaviour of beetles in the absence of food. Campbell and Hagstrum (2002) showed that, when outside food patches, beetles tend to be observed near arena edges. Several factors tended to keep beetles along the edges, including a tendency to be inactive there, to move along edges, and to move more slowly along edges. Our findings indicate that *T. castaneum* adults alter their speed of movement and their reactions after treatment with fungal spores.

Movement behaviour can have important consequences for the animals living in their environment (Turchin, 1998). Wiens *et al.* (1997) showed that the speed of individuals in different environments and the duration of stay in different areas together determine the spatial distribution of the tenebrionid beetle *Eleodes obsoleta*. The extent of insect movement between food spots will also determine the possibility that stored products may become infested in a storage box, processing facility, warehouse, trolley or grocery store, as well as the continued presence of populations of pests within storage facilities and many other aspects relevant to pest management such as the interpretation of catches, and the effectiveness of biological controls, insecticides and insect resistant packaging.

Tribolium castaneum behaviour is influenced by food odours and aggregation pheromones (Seifelnasr *et al.*, 1982; Obeng-Ofori, 1991; Phillips *et al.*, 1993), and in our study, there was an indication that the beetles were strongly affected by the fungus from the first minute of treatment. This may be due to the odour of the fungus and the co-formulants used to maintain the fungus in order for it to function effectively and for a longer time. This may have been sufficient to elicit a strong response to the fungal treatment. In addition, from observation of the recording individual *T. castaneum* adults in the fungal treatment showed similar distributions within arenas. This distribution was an indication of a significant inability to disperse immediately following treatment.

In contrast, from observation of the recording adults in the control treatment exhibited different distributions in the arenas over the whole assessment period following treatment, but they were moving along edges as described in the results. Adults might be searching primarily for food or mating opportunities. All these factors may cause differences in behaviour among the insects in the control treatment in comparison with fungal treatment. What is not clear is what made the insects stop walking immediately after spraying with the spore suspension while the adults in the control treatment showed no response and were active directly after spraying with water.

Further research is needed to understand better how these treatments influence insect behaviour on larger spatial scales in storage facilities. For example, further laboratory investigations on how the beetles respond to surfaces treated with a fungus would be informative. Movement behaviour of treated insects may affect the pest management landscape. The ultimate goal of pest management should be to reduce the movement of stored-product pests from the surrounding environment into the stored commodity in the storage facilities, and that can be done using non-biological methods such as ultraviolet light (UV), low temperature and low humidity.

5.6 Conclusion

The study showed that adults of *T. castaneum* sprayed with a fungal suspension showed less movement activity than adults sprayed with water.

Chapter 6 General discussion and recommendations for future work

6 Chapter 6 General discussion and recommendations for future work

6.1 General discussion

In earlier studies the susceptibility of *Tribolium castaneum* to particular entomopathogenic fungi (EPFs) species and strains has been tested (Wakefield, 2006; Khashaveh *et al.*, 2011; Abdu-Allah *et al.*, 2015; Batta and Safieh, 2015), and the mortality levels of insects have been found to depend on many factors such as the susceptibility of the target stage and the virulence of fungus species/strains. Therefore, the present study tested the possible use of some commercial EPFs, which are used as biological agents against other insect pests in the UK, USA and various countries in Europe, for *T. castaneum* control from four different viewpoints: (i) testing their efficacy against different stages of *T. castaneum* and determining the most virulent species/strains and the most susceptible stage (Chapter 2); (ii) evaluating interactions between fungi and low doses of RNAi by feeding to *T. castaneum* for the potential to increase the efficacy of EPFs against *T. castaneum* (Chapter 3); (iii) studying the relative importance of quinone secretions in affecting efficacy of EPFs, in terms of causing insect mortality (Chapter 4); and (iv) examining the influence of EPFs on the walking distance of *T. castaneum* in order to determine if behaviour is affected by fungal application (Chapter 5).

6.2 The main findings of this study

6.2.1 Chapter 2

- *Beauveria bassiana* ATCC 74040, *Metarhizium anisopliae* (Met52), and *Verticillium. lecanii* had a pathogenic impact on *T. castaneum*.
- All fungi tested caused significant mortality of *T. castaneum* infected by contact.
- There were significant differences between the percentage mortality of first and third instar larvae of *T. castaneum* and the percentage mortality of sixth instar larvae and adults infected by *B. bassiana*, *M. anisopliae* and *V. lecanii*.
- *Beauveria bassiana* was the most virulent in affecting *T. castaneum* followed by *M. anisopliae* then *V. lecanii* at the concentrations tested.

- The first and third larval instars were more susceptible to fungal infection than adults and sixth larval instars.
- Temperature had no significant effect on insect mortality caused by *B. bassiana*, *M. anisopliae*, and *V. lecanii* in tests at 25°C and 30°C.

6.2.2 Chapter 3

- The combination of fungi and dsRNA had a marked effect on *T. castaneum* when the dsRNA of *SK* and *SH* genes was combined with low concentrations (3×10^2, 3×10^3, and 3×10^4 spores/ml) of *B. bassiana* and *M. anisopliae* fungi at 25°C.
- The percentage mortality of third instar larvae and adults of *T. castaneum* in combined treatments with *SK* or *SH* dsRNA and fungi was higher than in single treatments with dsRNA or fungus.
- The combination or treatment with dsRNA and *B. bassiana* led to significantly higher mortality levels of *T. castaneum* than combination with *M. anisopliae* treatment.
- The SK dsRNA with either *B. bassiana* or *M. anisopliae* fungi was more effective than the SH dsRNA.

6.2.3 Chapter 4

- Adults of *T. castaneum* held in flour conditioned with synthetic 2-methyl-1,4-benzoquinone at a high concentration (15 mg/100 g flour) had lower mortality rates than those in fresh and naturally conditioned media with both *B. bassiana* and *M. anisopliae.*
- Mortality levels of *T. castaneum* adults were observed to be consistently higher with *B. bassiana* than *M. anisopliae*.
- There were fewer *Beauveria bassiana* spores adhering to the beetles' integument 24 hours after spraying in scanning electron microscopy (SEM) assessments of *T. castaneum* adults held in flour conditioned by synthetic 2-methyl-1,4-benzoquinone compared to adults held in fresh medium.

6.2.4 Chapter 5

- The locomotory activity of *T. castaneum* adults was affected by treatment with a commercial formulation of *B. bassiana*.
- Adults treated with only water covered a greater distance.
- Adults treated with a sub-lethal dose (LC_{50}) of *B. bassiana* fungus tended to move at lower speed and covered less distance.

6.3 General conclusion

Some commercial EPF formulations are available in the UK, marketed to control insects, including thrips, whiteflies, mites, aphids, and black vine weevil (*Otiorhynchus* spp.), according to their product labels (Copping, 2009). So far, insufficient research has been conducted into the efficacy of these products in controlling stored-product insects in the usual storage environmental conditions. This could be attributed to the possible effect of the beetles' natural environment on the fungal infection process. For example, most EPF conidia need specific conditions such as high relative humidity during application for the germination and subsequent sporulation (Daoust and Roberts, 1983; Moore *et al*., 1996), and these conditions may not be available in the storage environment. Future research could be directed towards the formulation of the most effective strains or isolates of EPFs in order to enhance their efficacy against insects and improve their shelf-life by preserving their viability for longer periods. Oil-based formulations are the most promising and most appropriate for the formulation of effective strains of EPFs because they are able to enhance the efficacy of the introduced EPF conidia and can conserve their viability. Invert (water-in-oil) emulsion formulations with unsaturated oils of plant origin are considered the best oil-based formulations since they contain enough water for germination of fungal conidia during application under hot and dry storage conditions (Batta, 2016a; Batta, 2016b).

A few tests have shown that formulated EPFs are effective against *Tribolium* species (Batta and Kavallieratos, 2018). Therefore, one goal of this study was to determine if the pathogenicity of three commercial formulations of *B. bassiana*, *M. anisopliae*, and *V. lecanii*, which are available in the UK market for the control of whitefly species, could be effective against *T. castaneum* since little is known about the efficacy of these products against this pest (Wakefield, 2006). The results reported in Chapter 2 indicate that these EPFs have the

potential to be important agents for the biological control of *T. castaneum*. The results showed that the pathogenicity of these three commercial formulations was effective with different larval instars and adults of *T. castaneum.* However, adults and older larvae were more tolerant of fungal infection than earlier instars (first and third instar larvae). These results agree with the finding of Öztürk *et al.* (2015), which showed that the earlier instars of *Leptinotarsa decemlineata* (Colorado potato beetle) were more susceptible to *B. bassiana* than older larvae and adults. The differential susceptibility observed in insects infected by fungi means that older larvae may require additional applications than the earlier instars due to their low susceptibility to fungus infection. As one of the most important factors that influences EPF efficacy is the number of applications, it can be recommended to target the most susceptible larval instars (first and third) to achieve a high level of mortality, but in reality, this is not possible. In natural infestations, larvae of different instars will be present. Thus, multiple applications of EPFs must be undertaken targeting to the period when the highest proportion of first and third instar larvae are present, if EPFs are going to be the only method for control.

Combinations of different control methods in protecting stored product commodities have become a vital component of IPM, thus reducing the impact of chemicals on the environment. Many studies have proven the combined efficacy of EPFs with other control treatments such as diatomaceous earth (DE) formulations and chemicals (Kavallieratos *et al.*, 2006; Athanassiou and Steenberg, 2007; Sabbour, 2014; Rumbos and Athanassiou, 2017; Batta and Kavallieratos, 2018). However, prolonged protection is very important when insect management tactics are designed for stored products and alternatives to chemicals should be sought whenever possible. Additionally, for the successful implementation of IPM for the control of *T. castaneum* and other stored product insects, information is not only required about the efficacy of fungi but also on their potential to be combined with other biological control approaches. RNAi, for example, has not yet been applied commercially in the field of pest control.

Consequently, in order to increase the efficacy of biocontrol agents and to reduce costs, *B. bassiana* and *M. anisopliae* could be combined with dsRNA methods such as silencing of the *SH* and *SK* genes. As reported in Chapter 3, low concentrations of *B. bassiana* and *M. anisopliae* in combination with *T. castaneum* SK and SH dsRNA have been tested. The oral application of SH and SK dsRNA at their LC50 values significantly increased the efficacy of

these two EPFs. For both fungal species, the effects with SK dsRNA were greater. This means that reduced amounts of *B. bassiana* and *M. anisopliae* fungi and dsRNA may be necessary for the management of *T. castaneum*, so reducing production costs. However, it has been claimed that, despite the overall improvement in EPF effectiveness achieved through the use of different treatments, such improvements are probably inadequate to control stored-product insects unless applied under the same storage environment conditions against different species of insects (Batta and Kavallieratos, 2018). The possible reasons relate to the limitations described in Chapter 1 (page 5).

It has been reported that *T. castaneum* uses various chemical defences, such as quinone secretions which inhibit the growth of various microorganisms and can be considered as an external immune defence for the protection of beetles against pathogens. Experiments described in Chapter 4 focused on the question of whether or not a relationship exists between the quinone which is secreted by the insect and fungal infection of the insect. The success of the treatments applied was measured in terms of the adherence and germination of fungal conidia on the insect. The results show that mortality levels of adults held in fresh and naturally conditioned medium for both *B. bassiana* and *M. anisopliae* fungi were higher than those for adults held in the flour conditioned by a high concentration of synthetic 2-methyl-1,4-benzoquinone. In terms of both the adhesion of spores and their germination on the insect, synthetic 2-methyl-1,4-quinone inhibits spore germination on *T. castaneum*. These findings are similar to those of an investigation conducted by Joop and Vilcinskas (2016), which demonstrated that quinone secretion inhibits the common mould fungus *Aspergillus niger*.

In cases where insect infestation is at a critical economic level in storage facilities, and especially in the case of infestation with *Tribolium* species, it should be taken into consideration that using biopesticides including EPFs may be inefficient, which may partly be attributed to high levels of quinone secretion by insects, and this is what was found in the results shown in Chapter 4. However, the control of *T. castaneum* populations below the economic threshold level could be achieved using EPFs and other control methods if they are applied early in the stage of *T. castaneum* population increase, as indicated in Chapter 2.

The study of the effects of fungal treatment on insect behaviour, as considered in Chapter 5, is the first attempt to test the locomotory activity of *T. castaneum* adults at different times after treatment with a commercial formulation of the fungus *B. bassiana*. The walking movement of

T. castaneum adults treated with the LC_{50} spore concentration of fungus was more strongly affected than in control adults treated only with water at each time point during the subsequent 30 min. Control individuals exhibited significantly higher locomotory activity than individuals in the fungus treatment. However, the direct response to fungal application in terms of the walking distance of *T. castaneum* observed in this study was not sufficient to understand the response of the beetles to treated surfaces. Hughes *et al.* (2016) indicated that many changes could appear in host behaviour due to parasitic infection. These changes can vary greatly in scale, from minor alterations to those in which the performance of a certain activity results in the appearance of wholly new and unpredictable behaviours. The movement patterns of treated insects may thus affect the pest management landscape. In storage facilities, the movement of insects from the surrounding environment into the stored commodity should be reduced in order to achieve the goals of pest management. Non-biological methods could be used to do that.

No commercial biopesticides based on EPF bioagents have yet been marketed for use against stored-product insects. Furthermore, there is no combination involving any effective strain (formulated or unformulated) of EPF for use in the management of stored-grain insects. Possible reasons for the lack of commercialisation and registration of EPF biopesticides in the grain-storage environment could be due to several limitations described in Chapter 1. These limits may perhaps be overcome using methods such as the improvement of formulations that can show elevated virulence against target insect species and ensure both stability of ingredients under the storage environment and their distribution on the surface of the host organism (Batta and Kavallieratos, 2018). Therefore, the studies undertaken to improve the effectiveness of EPF biopesticides in the storage environment need to address the limitations in the use of EPF for those insects.

On the other hand, based on the results of the present study, it was concluded that the EPF products tested may provide viable alternatives to synthetic insecticides used in the control of *T. castaneum* and other stored product insects. EPF biopesticides could be used as cleaning methods in grain storge facilities by spraying the structure of the building with fungal formulation alone or in combination with other treatments, so any beetles hiding there will be killed. This approach would avoid the possibility of any grain quality deterioration in storage due to fungal growth, which makes products unsuitable for consumption, planting and trade.

Biopesticides as cleaning methods for protection against stored-grain insects involving use of fungi followed by other components could give good control of insects.

6.4 Suggestions for future work

- Further work will be necessary to test if the formulations of the EPFs tested are viable in increasing the mortality levels of *T. castaneum* under normal storage facility conditions. Also, further work is needed to screen for EPF species/strains that are virulent under storage conditions and are tolerant to desiccation and high temperature in order to control *T. castaneum.* It is probable that some work of this nature has been done, but is not yet published (Wakefield, pers. comm.).

- Future research could be oriented towards the formulation of the most effective strains or isolates of EPF for use under prevailing storage conditions in order to enhance their efficacy against stored grain insect pests.

- The possible ways to implement the use of combinations of EPFs with RNAi technology in storage systems need further study.

- Future research will be required to test combinations of EPFs with other non-synthetic control agents, such as diatomaceous earths (DEs), natural products (neem oil) and natural enemies, in particular *Bacillus thuringiensis* (Bt), which is considered so far to be the best organism for use against *T. castaneum*, in order to test the efficacy of EPFs and Bt and to determine if they interact together additively, synergistically or antagonistically.

- The effect of beetle quinone compounds such as 2-methyl-1,4 benzoquinone (MBQ) on spore germination and growth of EPFs was not considered in the present study. Therefore, there is a need for more investigation using *in vitro* tests to evaluate the effects of MBQ at different concentrations in the inhibition of different EPFs.

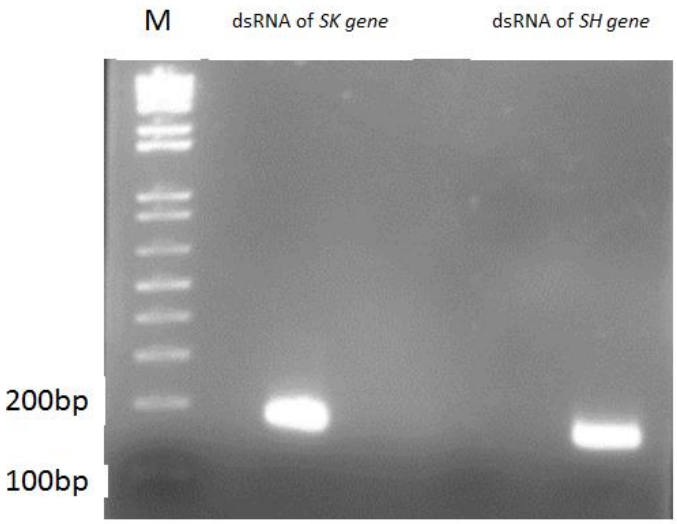
- To clarify the issue of movement behaviour, researchers could investigate the effect of fungal application on insect behaviour, and how the insect responds to surfaces treated with fungi.

7 Appendices

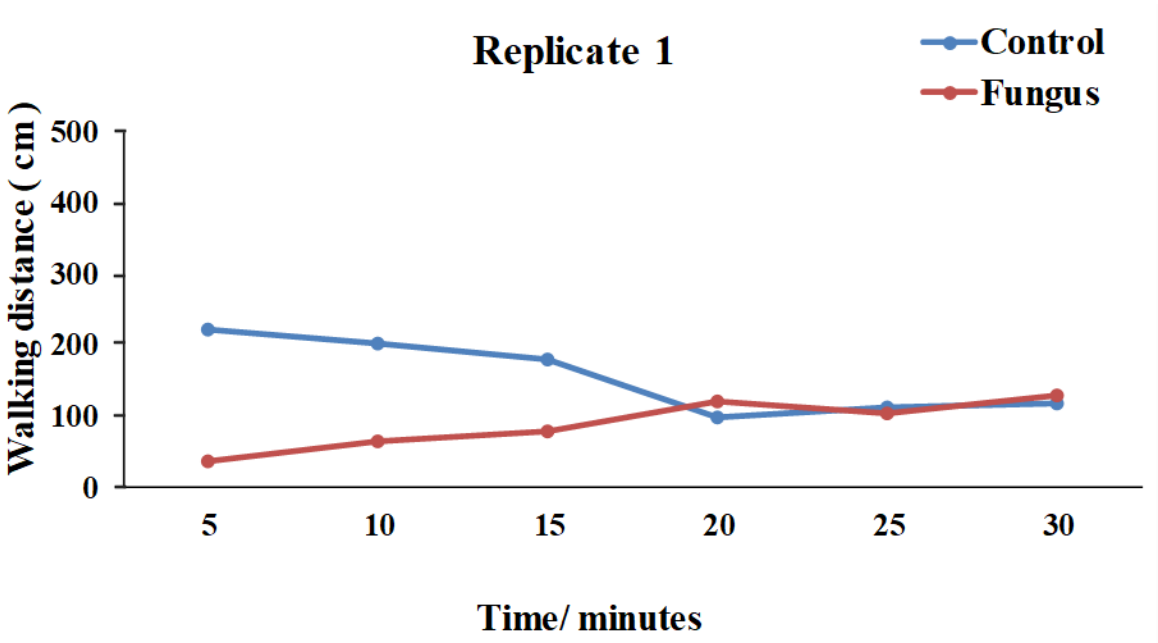
Appendix 1: Primers used for qPCR and PCR analysis in *T. castaneum* for *SK* and *SH* genes. The method of making dsRNA was described by Alshukri (2018).

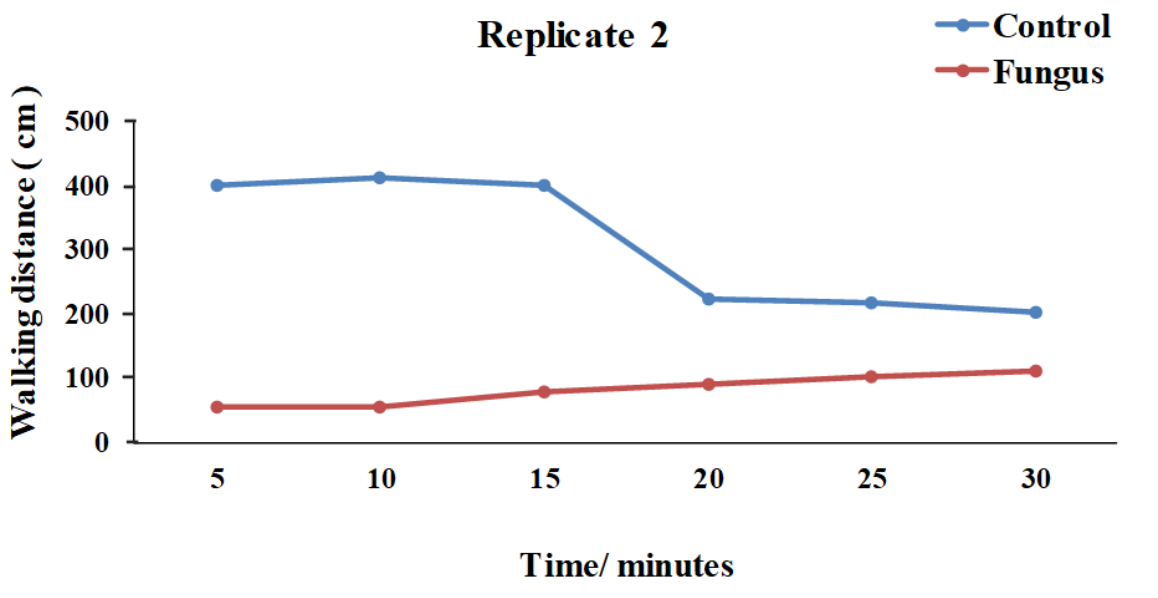
Application	**Primer ID**	**Sequence of primer for *SH* gene**	**Primer ID**	**Sequence of primer for *SK* gene**
qPCR	FA1	CCGGTTCGAGACGCAACTT	FA2	CCTTTGAACTACGAAAGCCC
T. castaneum	RA1	TTGCGAAGCGGGTCGAAGTA	RA2	CAGAGTCCCTGCTCGAGTTC
	FB1	CCGGTTCGAGACGCAACTTAG	FB2	GCGTCGTTCCGTTCGATTTA
	RB1	ATTGCGAAGCGGGTCGAAGTA	RB2	AGAGTCCCTGCTCGAGTTCT
PCR	FC1	GCTGGACGTCTTCTCCGA	FC2	GTGGGACACGTGGACTACCC
T. castaneum	RC1	GTACTCGAACAGCAGCCACA	RC2	ACATCCCCATAACAAGAGCG
dsRNAsy	FD1	TAATACGACTCACTATAGGG-GCTGGACGTCTTCTCCGA	FD2	TAATACGACTCACTATAGGGG-TGGGACACGTGGACTACCC
T. castaneum				
	RD1	TAATACGACTCACTATAGGG-GTACTCGAACAGCAGCCACA	RD2	TAATACGACTCACTATAGGGGA-CATCCCCATAACAAGAGCG
		Kanamycin		**qPCR β-actin**
Reference gene	FG1	TAATACGACTCACTATAGGGC-ATTCGCCGCCAAGTTCTTC	FG2	GATTTGTATGCCAACACTGTCCTT
	RG1	TAATACGACTCACTATAGGGTG-CTCGACGTTGTCACTGAA	RG2	TTGCATTCTATCTGCGATTCCA

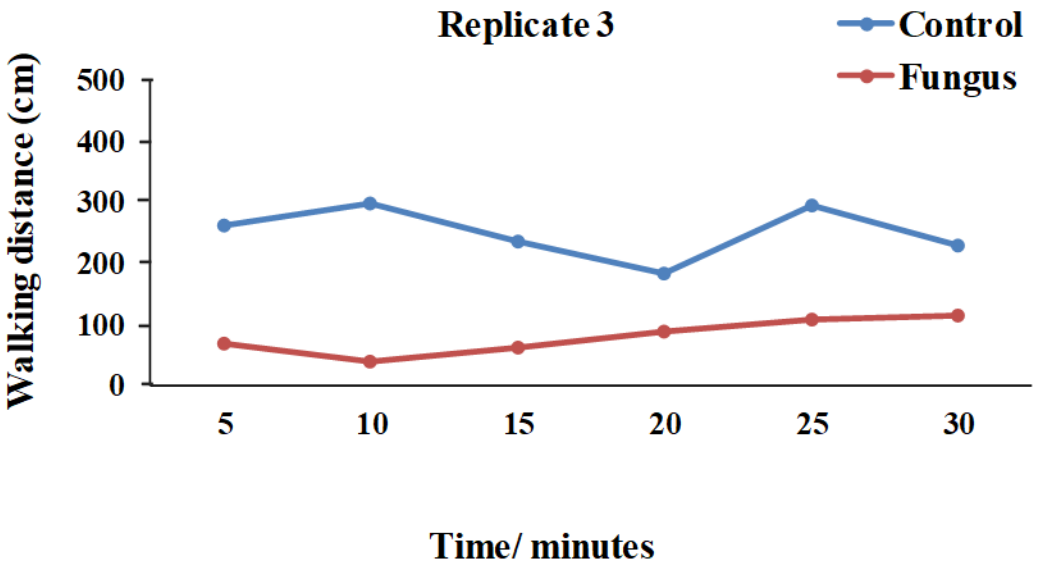
Appendix 2: dsRNA fragments of *SH* (151) and *SK* (181) genes tested by 2% agarose gel electrophoresis, M: 100 bp DNA ladder.

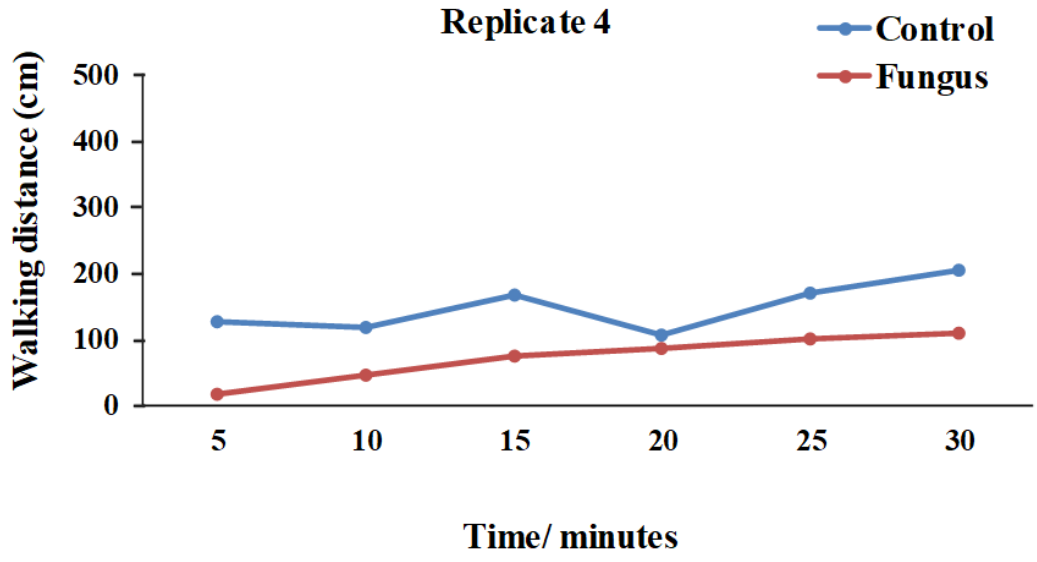


Appendix 3: Walking distance of *T. castaneum* adults after different times in each replicate following treatment with the LC50 value of *B. bassiana* fungus or with water (control treatment).






Replicate 3
Control
Fungus
Walking distance (cm)
500
400
300
200
100
0
5
10
15
20
25
30
Time/ minutes


Replicate 4
Control
Fungus
Walking distance (cm)
500
400
300
200
100
0
5
10
15
20
25
30
Time/ minutes

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