CHAPTER ONE

Introduction and Literature Review

1.1 General Introduction and Project Objectives

Over the last twenty to thirty years it has become increasingly recognised that integrated approaches to insect pest management [combining good agricultural practices (GAP), use of natural enemies and use of chemical (synthetic) insecticides] should provide the means for 'control' of insect pest populations within crop storage situations, maintaining them below economically adverse thresholds (Perkins, 1982; Mashaya, 2000; Wang *et al.*, 2004). Such integrated approaches were developed owing to observations that the application of chemical insecticides alone was proving to be unsustainable (Adane *et al.*, 1996; Oduor *et al.*, 2000; Throne and Eubanks, 2002). Other problems associated with the use of chemical insecticides range from their pricing differentials within developing countries, their direct deleterious effects on the environment and problems surrounding their registration (Graves *et al.*, 1999).

Although agrochemical inputs do sustain agricultural industries through conferring a fast remedy against insect pests (Graves *et al.*, 1999; Baumgärtner *et al.*, 2003), their 'side effects' are great cause for concern. Some of the more prominent problems associated with the application of chemical insecticides include i) observations that the availability of new chemicals is decreasing due to difficulties in discovering new synthetic insecticides, ii) the development of insect resistance (tolerance) to the applied product, and iii) their detrimental effects on populations of non-target organisms (Robinson, 1971; Graves *et al.*, 1999; Throne and Eubanks, 2002).

With regards to the ability of insects becoming tolerant to pesticides, it is commonly observed that insect pest populations continue to resurge and persist after pesticide applications (Oduor *et al.*, 2000). In an attempt to alleviate the problem of insect resistance to pesticides, the Global Crop Protection Federation set up resistance management guidelines issued by the Resistance Action Committees to users of

pesticides (insecticides, herbicides, fungicides) in the early 1990s (Russell, 2001). Aside from such initiatives to educate farmers and users of agrochemicals, other research ideas are being investigated to alleviate the problems arising from the sole use of synthetic insecticides. On a global level, there are ongoing and concerted efforts to develop innovative means for insect pest management that are truly effective, affordable to small scale farmers as well as safe to the end user and the environment (Shah and Pell, 2003). These methods include biologically based tactics (Ruberson *et al.*, 1999). For example, investigations into the effects of cinnamaldehyde and methylchavicol as grain protectants have been proposed, where the repelling-effects of these products show potential against insect pests (Ojimelukwe and Adler, 2002). Other studies have used high temperatures to control pests. Adler (2002) demonstrated that high temperatures and adequate exposure times were lethal against the tobacco beetle, *Lasioderma serricorne* (Coleoptera: Anobiidae) and the lesser grain borer, *Rhyzopertha dominica* (Coleoptera: Bostrichidae). These forms of pest control may be termed non toxic, and stand alongside the use of natural enemies as effective alternatives to the ongoing and popular use of chemical insecticides (Boucias and Pendland, 1998; Ruberson *et al.*, 1999).

The need to investigate and develop natural products for management of insect pests stems from various stand points including environmental, social, and economic conditions encountered by agro-producers on a global level (Adane *et al.*, 1996; De La Rosa *et al.*, 2002; Dimbi *et al.*, 2003; Lux *et al.*, 2003; Wang *et al.*, 2004). In this study the use of microbial disease-causing agents is investigated owing to observations made for instance, by ecologists, that insect pathogens have a significant role in regulation of natural insect populations (Steinkraus, 2002). Microorganisms such as entomopathogenic fungi (EPF) are naturally occurring, soil dwelling organisms which possess a natural ability to infect and cause sometimes fatal diseases to insects (Ferron, 1981; Samson *et al.*, 1988; Shah and Pell, 2003; Wang *et al.*, 2004).

It is this capacity to infect insects that microbiologists and other researchers wish to harness, as effective alternatives for insect pest control. The following project was therefore planned to evaluate EPF *in vitro* against prominent insect pests of stored

products. This was conducted through the following literature review and laboratory evaluations comprising bioassays designed to assess the effects of such fungi on selected insect pests. Such evaluations are essential prior to further development and registration of microbial control products proposed for commercial insect pest control and constitutes an important first stage of the process in formulating alternative products (Goettel, 1984; Goettel and Inglis, 1997).

Since the 1980s, considerable efforts have been focused on developing commercial bioinsecticides using the entomopathogenic fungus, *Beauveria bassiana*, and other entomopathogenic hyphomycete fungi (Goettel, 1984; Shah and Pell, 2003). *Beauveria bassiana* has been successfully evaluated *in vitro* and used against several prominent insect pests around the world. Examples include the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Anderson and Roberts, 1983), the diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) (Furlong and Pell, 2001), and the Mexican fruit fly, *Anastrepha ludens* (Loew) (Diptera: Tephritidae) (De La Rosa *et al.*, 2002). A further example involves one of the pests of interest in this study, the maize weevil, *Sitophilus zeamais* (Coleoptera: Curculionidae) which was targeted for evaluation using *B. bassiana* as the disease-causing agent by Adane *et al.* (1996). In these cases, *B. bassiana* was found to be highly pathogenic against the targeted insect pests and its popularity as well as ultimate effectiveness against a wide range of insect pest species makes it an ideal choice for evaluation under controlled conditions. Other studies evaluating *B. bassiana* aimed strictly at assessing genetic variation in populations from different host insects and geographic locations in attempts to holistically understand the biology of the fungus (Castrillo *et al.*, 1999; De Muro *et al.*, 2003). In such cases, the fungus may be viewed as having potential as a biological control agent (BCA), as was the case with Castrillo *et al.* (1999), who investigated genetic relatedness of *B. bassiana* populations associated with the darkling beetle, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), a pest of broiler chickens.

The literature also shows that other entomopathogenic hyphomycete fungi such as *Metarhizium* species have been used against important insect pests such as the African fruit fly species; *Ceratitis capitata* (Weidemann), *C. rosa* (Karsch) and *C. cosyra* (Walker) (Diptera: Tephritidae) (Dimbi *et al.*, 2003; Dimbi *et al.*, 2004) under controlled conditions. Castillo *et al.* (2000) used several species of entomopathogenic hyphomycete fungi, including *M. anisopliae*, *Paecilomyces fumosoroseus*, *Aspergillus ochraceus*, *Verticillium lecanii*, *Penicillium chrysogenum* and *B. bassiana* to target one insect pest species, the fruit fly *Ceratitis capitata* (Weidemann).

For the purposes of this investigation, it was essential to evaluate microbial insect pathogens that are indigenous to South Africa against locally occurring insect pest populations. The entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin (Hyphomycetes) was selected for evaluation against two storage pests, namely the maize weevil, *Sitophilus zeamais* (Motschulski) (Coleoptera: Curculionidae) (Longstaff, 1981), and the tobacco beetle, *Lasioderma serricorne* (Fabricius) (Coleoptera: Anobiidae) (Ashworth, 1993b). *B. bassiana* is a naturally occurring mitosporic fungus with a wide arthropod host range and has been studied because of its potential use as a biopesticide (Castrillo *et al.*, 1999; De Muro *et al.*, 2003).

The current study hoped to determine whether indigenous fungal isolates, in particular, can be used to mitigate the destructive behaviour of these target pests, in comparative bioassays with isolates sourced internationally. The reasons for electing to evaluate EPF against these insect pests stems initially from their prevalence as important pests for stored commodities as mentioned below (section 1.3.1). Furthermore in the case of the maize weevil, the literature shows that this insect has previously been shown to be susceptible to infection by *Beauveria bassiana* isolates and the study by Adane *et al.* (1996) serves as a template for this study. With regards to the tobacco beetle, no information was found on the effects of EPF on the insect; this study will therefore serve as a first account evaluating the effects of *Beauveria bassiana* against this pest (J. Lord, pers. comm.). Moreover, the two coleopteran pests selected for this project have unique indoor life-stages making them ideal as targets for challenging with EPF as they occupy niche environments (Stejskal *et al.*, 2002; Shah and Pell, 2003). The use of EPF to

control storage pests receives little attention because of the belief that storage environments are too hostile for fungal efficacy and survival (Oduor *et al.*, 2000). The onus remains on researchers to develop appropriate technologies that can be effectively used to apply EPF in all environments after determining that targeted insect pests are susceptible (De La Rosa *et al.*, 2002; Dimbi *et al.*, 2003).

The main objectives of the present study were:

i. To source both local and exotic strains of EPF for use in comparative bioassays.
ii. To identify and culture sourced fungal isolates prior to tests designed to determine the effects of various temperatures on radial growth.
iii. To obtain and rear insect cultures of the target pests for evaluation as well as develop means of selecting appropriate numbers for inoculation.
iv. To evaluate the effects of sourced fungal isolates on the target pests – determination of virulence at inundative rates, at different fungal spore concentrations and at various temperatures.
v. To firmly establish that the sourced isolates were indeed responsible for the demise of inoculated pests through comparisons with insects inoculated with spore free inoculum and the occurrence of the process of mycosis.

The following dissertation is structured as follows with the next sections of this chapter providing a literature review covering some of the more important aspects surrounding biological control, the target insects and entomopathogenic fungi in general. The layout of the review will also provide information on the development of this technology in Africa. Chapter 2 presents information on the sourcing of local and exotic isolates of *Beauveria bassiana* as well as the establishment and maintenance of the culture. Chapter 3 presents an experiment designed to assess the effects of various temperatures on the radial growth of all sourced isolates. Chapters 4 and 5 describe the laboratory-based experiments designed to evaluate the effects of *Beauveria bassiana* against the maize weevil and the tobacco beetle respectively, while Chapter 6 briefly summaries the findings and concludes the study.

1.2 Literature Review

1.2.1 Biological Control

The concept of biological control is based on the assumption that naturally occurring biotic mortality factors restrain populations of living organisms (Ruberson *et al.*, 1999). For the purposes of this review, this concept excludes other insect control tactics counted as biologically-based control strategies such as plant host resistance, mating disruption, pheromone trapping, genetic controls and biological toxins (Ruberson *et al.*, 1999). While these methods of pest control are equally essential, they have little in common with biological control or with each other outside the point that they may be integrated and used together (Ruberson *et al.*, 1999). Biological control was first used to control insects, mites and weeds, and today the application has expanded to methods targeted at other invertebrates and plant pathogens (Van Driesche and Bellows, 1996). The subject attracts researchers from many different disciplines, ultimately aiming to develop alternatives to the deleterious effects of chemical pesticides to non-target organisms and the natural environment (Burges and Hussey, 1971; Perkins, 1982; Graves *et al.*, 1999). The most common description for the discipline can be noted as 'the influence of natural predators, parasites and pathogens applied by man against a pest', with distinctions being made between macrobial and microbial control (Burges and Hussey, 1971; Dixon, 2000).

Microbial control agents include bacteria, fungi, nematodes, protozoa and viruses (Evans, 2000). In the case of their control of arthropods (insects and mites) the interactions most relevant are those termed the entomogenous relationships, where the microbes cause a lethal (or fatal) infection of the insect (Boucias and Pendland, 1998). The degree of control obtained through use of any biological control agent (BCA) is however difficult to determine without relevant base-line data (Burges and Hussey, 1971); data that can be obtained by rigorous screening under controlled conditions. *Beauveria bassiana* is one such microbial agent tested for its effects against insect pests for use in biological control.

1.2.2 Strategies of Biological Control

Strategies for biological control which apply to EPF as much as parasitoids and predators of insects (Shah and Pell, 2003) include the following:

1) Classical Biological Control: the use of biological control agents against an introduced pest, which has established itself without its full guild of natural enemies. In this stratagem, surveys are conducted in the centre of origin of the particular pest, aimed at identifying any suitable candidates that are natural enemies in that point of origin. The natural enemy is then released into the colonized area where the pest has made a home for itself (Shah and Pell, 2003).

2) Augmentation: As we are concerned with entomopathogenic fungi, augmentation as a form of biological control that involves the introduction or application, in aqueous suspension of spore and or hyphae/mycelia prepared in a laboratory in combination with a carrier (synthetic matrix) which is typically a formulation component included to enhance fungal infection. This strategy implies the need for mass production of the biological control agent, and two approaches are proposed for agent application – Inoculation and Inundation.

   2.1 Inoculative approach requires that the biological control agent (e.g. entomopathogenic fungi) be applied early in the crop/harvest season with the expectation that the agent will establish epizootics in the pest population and spread over time maintaining pest populations below yield threatening limits.

   2.2 Inundative approach involves application of the biological control agent in large amounts for rapid short-term control with no expectation of epizootics (Shah and Pell, 2003).

3) Autodissemination: Involves the manipulation of a portion of the pest population to facilitate the dispersal of entomopathogenic fungal spores through horizontal transmission to the wider population. This alternative to the conventional methods of pathogen application is receiving considerable attention with the use

of sex pheromone traps as lures to draw male moths to the inoculum pool (Furlong and Pell, 2001; Dimbi *et al.*, 2003; Shah and Pell, 2003).

4) Conservation: Yet another approach noted by Shah and Pell (2003) is very much in line with modern farming doctrine of good agricultural practice. Biological control through the approach of "conservation" seeks to enhance the activity of the EPF populations through modification of farming practices and grower education. Conservation is noted as being more applicable in situations where natural EPF infections are established and have been detected, with the continued task of maintaining the fungal population so that it restricts pest population; continual assessment is required to gauge the status of the fungal population within any given space (Goettel and Hajek, 2001; Shah and Pell, 2003). Lures are used to trap adult insects, which are then analysed for fungal infection (Shah and Pell, 2003).

When appropriate, such approaches and strategies have the potential of managing pest populations in the medium to long term as it is generally considered that entomopathogenic fungi can reduce fitness, rendering pest populations less of a threat to crop yields (Watson and Patersen, 1993; Masuka and Manjonjo, 1996). However, the use of microbial biological control agents *in situ* has the prerequisite that potential agents be evaluated *in vitro* to gauge their impact on the pest life cycle and determine pathogenicity (Goettel and Inglis, 1997). Neuenschwander *et al.* (2003) discusses some of the successes of biological control in Africa, with the following review highlighting some investigations conducted *in vitro* using entomopathogenic fungi.

1.2.3 Overview of Biological Control of Insects

Natural enemies, particularly microbial agents, are the fundamental resource with which biological control success can be achieved (Van Driesche and Bellows, 1996; Evans, 2000; De La Rosa *et al.*, 2002). Microbial BCAs including bacteria, nematodes, fungi, protozoa and viruses are important insect pathogens that play significant roles in the

development of biopesticides (Van Driesche and Bellows, 1996; Goettel and Inglis, 1997; Evans, 2000). These agents do however require specialist knowledge and conditions for successful use (Goettel, 1984; Goettel and Inglis, 1997; Evans, 2000). This section provides some specialist knowledge through a brief historical review into the discoveries and developments of the use of microorganisms (bacterial, viral and fungal agents) to control insect pests. An overview of developments in biological control in Africa is also provided below.

Table 1.1: Major Events in the History of Microbial Pest Control

1873: **Le Conte makes first formal proposal for microbial insect control**
1879–1884: Metchnikoff and Krassilstschik experiment with *M. anisopliae* to establish the first operational production plant for a microbial control agent and apply it to control sugar beet curculio in the Ukraine
1888–1902: *Beauveria bassiana* applications for chinch bug in US Midwest
1906–1942: "Friendly fungi" in Florida
1911: *Bacillus thuringiensis* was first identified when it was discovered that it killed the larvae of flour moths
1921–1940: Development of *Bacillus popilliae*
1929: Glaser and Fox discover *S. glaseri*
1938: Sporéine (Bt), the first commercial product
1958: The 1st international conference on insect pathology in Prague
1959: Journal of Insect Pathology
1964: *Oryctes* virus program begins in South Pacific
1975 *Heliothis* NPV registration
1977: Goldberg and Margalit report the discovery of *B. thuringiensis*
subsp. *Israelensis*
1995: Bt transgenic corn registration

Note: Taken from Lord, (2005).

Bacteria: Insect pathogenic bacteria occur in the families Bacilliaceae, Pseudomonadaceae, Enterobacteriaceae, Streptococcaceae and Micrococcaceae, and while their recognition and use as insect killing agents does not share an equally long history with fungal pathogens, they do have the most successful track record with regards to commercialization (Siegel, 2000; Khetan, 2001). Khetan (2001) notes that the first records of insect pathogenic bacteria were made in 1901, when Ishiwata Shigetane isolated a bacterium from diseased silkworms. In 1911, Berliner isolated a similar

bacterium from a flour moth cadaver and named it *Bacillus thuringiensis* after the place in which he made the isolates, Thuringia in Germany (Khetan, 2001). Later still in 1928, Husz isolated a strain of *Bacillus thuringiensis* from yet another moth species (*Ephestia*), and tested it on the European corn borer (Khetan, 2001). This work constituted the first reported application of *Bacillus thuringiensis* which in turn led to the development of the first commercial product - Sporeine in France (Khetan, 2001; Lord, 2005).

Bacillus thuringiensis (*Bt*) is a Gram-positive spore forming bacterium that produces proteinaceous parasporal inclusions while sporulating, and it is these inclusions that are the important fatal toxin used to target inset pests (Tsuchiya *et al.*, 2002). By the 1960s, various commercial products based on *Bt* insect control were available on markets in North America, the former Soviet Union, France, and Germany, particularly targeting Lepidopteran pests as these strains were only active against this order of insects (Khetan, 2001). Importantly, research work to detect more insect toxic strains went on and in the late 1970s isolates pathogenic against black flies and mosquito larvae were discovered (Khetan, 2001). By 1983 yet another insect pathogenic strain was discovered and in this case the strain was toxic to insects in the Order Coleoptera (Khetan, 2001). Unlike insect pathogenic fungi which can penetrate the insect cuticle directly to cause disease (Samson *et al.*, 1988; Boucias and Pendland, 1998), the mode of action of *Bt* requires that it is ingested by the target (susceptible) pests to achieve infection (Siegel, 2000); however this has not limited the progress of commercialization of products based on this bacterium (Lord, 2005). The financial impetus to develop commercial biopesticides based on *Bt* led to the foundation of new biotechnology companies and by 1993, *Bt* insecticides produced in North America and Western Europe were valued at U$100 million to U$150 million/annum (Khetan, 2001; Siegel, 2000). Today the commercial importance of *Bt* dwarfs that of many of the other insect pathogenic bacterial species/strains or families as illustrated by the number of registered products containing the pathogen's spores and toxin or only the *Bt* toxin (Siegel, 2000). This is also irrespective of the point that so far only pests from three insect Orders (Lepidoptera, Diptera and Coleoptera) can be managed with *Bt* (Tsuchiya *et al.*, 2002).

Yet another type of microbial insect control agent that requires ingestion to kill, are viral insect pathogenic organisms.

Viruses: Among the families of viruses of insects, those containing virus particles occluded within a proteinaceous matrix, have been used with success in microbial control of insects (Evans, 2000). Examples are the baculoviruses (BV), which today may be considered to directly compete with *Bt* for the control of Lepidopteran insect pests (Khetan, 2001), at best within laboratory scenarios. Although insect pathogenic viruses, particularly baculoviruses and their association with occlusion bodies, have a rich 150 year history, penetration of these microbial control agents into the commercial bio pesticide market is slow, and remains so (Evans, 2000). BV control of pest insect populations was originally demonstrated in the 1940s, but the first registered commercial product did not appear for another 30 years. This was the *Helicoverpa zea* nuclear polyhedrosis virus (NPV) (Lord, 2005). Since then, a number of BV based insecticides have been developed, registered and produced commercially. They are all wild-type baculoviruses and, like most microbial insecticides, have had limited success for various reasons (Lord, 2005).

Fungi: Observations that fungi attack and can cause the death of insects date back over two thousand years, with causal agents of honeybee and silkworm diseases being among the first to be identified owing to the insects' economic importance as domesticated-commercial insects (Burges and Hussey 1971; Boucias and Pendland, 1998; Caulder, 1999). In direct relation to the entomopathogenic fungus *B. bassiana*, Agostino Bassi de Lodi, a pioneering Italian bacteriologist, discovered that microorganisms were responsible for certain insect diseases where particular and consistent symptoms were present (Burges and Hussey, 1971; Mahr, 1997). In 1807, he embarked on investigations relating to the silkworm disease *mal de segno* (or muscardine disease), which was causing serious economic losses in Italy (Mahr, 1997). By 1835 he had gone on to correctly note that a fungus was responsible for the demise of silkworms in the highly lucrative silk production sectors in France and Italy; this fungus was subsequently named in his honour *Beauveria bassiana* (Mahr, 1997; Lord, 2005).

Also in the 19th century, Louis Pasteur was commissioned by the French government to investigate a disease that was threatening the French sericulture industry, for which he too identified a fungus as the causal agent (Boucias and Pendland, 1998). In 1879, the first scientifically significant experiments using the entomopathogenic fungus *Metarhizium anisopliae* to infect the larvae of the cereal beetle, *Anisoplia austriaca* (Herbst) (Coleoptera: Scarabaeidae) *in situ* were conducted and recorded by Russian scientist E. Metschikoff (Burges and Hussey, 1971; Possee & King, 1994; Lord, 2005).

In 1969, a departure from the popular notion of dividing the entire living world into plants and animals was made by Whittaker, who recommended a five-kingdom system, placing fungi into their own kingdom (Burges and Hussey, 1971; Roberts and Yendol, 1971). The uniqueness of these organisms was most apparent when comparing entomogenous fungi with other entomogenous microorganisms, with the principle and most significant difference being in the mode of host invasion (Roberts and Yendol, 1971). Entomogenous fungi have thus played an important role in the history of microbial control of insects, some of which mankind considers pests (Roberts and Yendol, 1971). The awareness that insects suffer from disease developed into the science of insect (invertebrate) pathology, with the creation in 1945 of the Laboratory of Insect Pathology at the University of California (Burges and Hussey, 1971).

Today a resurgence of interest in the use of fungi for the biological control of insect pests has developed (Goettel, 1984; Castillo *et al.*, 2000; Furlong and, Pell 2001; Nardini, 2002) with this investigation adding to numerous others and aiming to use entomopathogenic fungi to mitigate the effects of coleopteran storage pests. Although very little is known about the potential risk such fungi have on non-target organisms (Wang *et al.*, 2004), selecting them for use within crop storage facilities constitutes an approach that does not put such organisms at risk. Progress on the development of commercial products that are independently successful is however slow in comparison to products based on insect pathogenic bacteria (Lord, 2005).

African scenario: Traditionally, African agronomic farming models were characterised by a rich and diverse range of crops and crop varieties grown under rotation. These practices helped reduce the problems of insect pest build-up as they were geared and adapted to low human populations (Herren, 2003; Lux *et al.*, 2003). However, in today's modernized agricultural systems of crop production (from the seed beds or nurseries throughout the entire plant growth phases *in situ* or within green houses to crop storage), producers are continually confounded by insect pest infestations causing varying degrees of damage (Oduor *et al.*, 2000). Human population explosions, the introduction of ill-adapted crop varieties, reduced tillage and crop rotations, introductions of new insect pest species, as well as the problem of pest resistance to insecticides, have all led to a shift in the importance of focusing on pest infestations and their management (Metcalf, 1999; Herren, 2003). Historically, biological control in Africa centred on the use of parasites, parasitoids and predators against introduced insect pests species and mimicked developments in Europe and North America (Greathead, 2003; Neuenschwander *et al.*, 2003, Tribe, 2003). The establishment of an East African Station in Uganda in 1962 and a sub station in Ghana in 1969, through collaboration with the Commonwealth Institute of Biological Control (CIBC) and other international organisations, saw the expansion of biological control based research work on the continent (Greathead, 2003). Although microbial control of insects has been incorporated into pest management strategies in Africa for many years, rarely have these approaches developed into commercial products similar to those in the developed world (Langewald *et al.*, 2003).

Following and inspired by the development and success of Green Muscle™ (a *Metarhizium* based bioinsecticide for locust management), as well as the ongoing banning and deregistration of many broad spectrum pesticides targeting hoppers (Graves, 1999; Langewald *et al.*, 2003; Neuenschwander *et al.*, 2003), further attempts to use EPF such as *Beauveria* and *Metarhizium* species to develop a biopesticide were conducted by researchers in Africa for the control of locusts and hoppers. The wingless grasshopper, *Mecostibus pinivorus* (Whellan) a *Pinus patula* (Schiede and Dieppe) defoliator in Zimbabwe, was made the target of laboratory-based evaluations using *Metarhizium flavoviride* and *Beauveria bassiana* by Masuka and Manjonjo (1996). Outbreaks of this

pest may lead to severe damage manifested as complete defoliation and tree death and researchers sought to use EPF to manage the pest. The trees (*Pinus patula*) made up 50% of pine tree stands in the Eastern Highlands of Zimbabwe and it was found that the EPF were highly effective at controlling the hopper (*M. pinivorus*) *in vitro*, particularly when high spore concentrations were used (Masuka and Manjonjo, 1996). More recently Adane *et al.* (2004) aimed to develop a product to control locusts and hoppers in arid environmental conditions and their evaluations centred on producing infectious spores through submerged cultures. Focusing on production and formulation technology for *Metarhizium anisopliae* (var.) *acridum*, Adane *et al.* (2004) used liquid media to mass produce submerged spores which are hydrophilic and highly virulent. This research represents the current shift towards commercialising microbial control agents with investigators going beyond the laboratory based pathogenicity evaluations and seeking means to mass produce the microorganism (Adane *et al.*, 2004). Following successful evaluations, the next step as noted earlier, would be to focus on developing appropriate EPF spore delivery technologies (for example bait lures) that will ensure adequate dispersal of the fungus against targeted pests (Dimbi *et al.*, 2003). By this stage the fungus should already be tested for the environment in which the pest is found for maximum efficiency and effectiveness.

Currently, much of the focus in developing microbial insecticides in Africa is linked to collaborations between international organisations. The different species of mango-infesting fruit flies have also become a target for control with the use of fungal microbial agents (Dimbi *et al.*, 2003; Lux *et al.*, 2003; Dimbi *et al.*, 2004). As a direct result of requests emanating from mango producers eager for cheaper, more effective and environmentally safer control products, the International Centre of Insect Physiology and Ecology (ICIPE) in Kenya embarked on the African Fruit Fly Initiative (AFFI) (Lux *et al.*, 2003). Although parasitoids and predators continue to play the major role as the selected organisms for biological control of this important pest group, the initiative that included twelve African countries also investigated the effects of fungal pathogens (Lux *et al.*, 2003). A review of research into the use of EPF against African fruit fly species by

Dimbi *et al.* (2003) is presented below (section 2.5.3) illustrating the progress of microbial biological control in Africa.

1.3 Insect Pests

1.3.1 Insect pests with emphasis on pests of stored products

The Order Insecta is reputed to contain one million described species and, while they are mostly central to the performance of many ecosystems, it is their nature as herbivores that greatly conflicts with our food security (Boucias and Pendland, 1998; Metcalf, 1999; Shah and Pell, 2003). A huge number of pest species are associated with stored commodities and it is likely that within any particular store, one may expect a permanent occurrence of many pest species (Stejskal *et al.*, 2002). Two major groups (Orders) of insects harbour the mostly economically important post-harvest pests: Coleoptera (beetles and weevils) and Lepidoptera (moths and butterflies) (Ashworth, 1993a; Ashworth, 1993b; Sallam, 1994). Several coleopteran and lepidopteran species attack crops both in the field and in post harvest storage and the Coleoptera alone outnumber all described plant species with more than 300 000 species (Ashworth, 1993b; Metcalf, 1999; Oduor *et al.*, 2000). The ability of insect pests to reproduce in the vicinity of their food sources and/or to rapidly migrate to any such source of food makes them a formidable adversary, with most coleopteran and lepidopteran pests laying their eggs on targeted food sources (Ashworth, 1993a; Ashworth, 1993b; Metcalf, 1999). Losses of stored grains due to insects and other storage problems were estimated to be greater than US$ 1 billion annually in the United States and may exceed 30% in developing countries (Thorne and Eubanks, 2002).

The need to find alternatives to conventional pesticides for stored product pest control has been recognised by the British Government and Industry, in a jointly funded 'LINK' project of four years duration (Wildey *et al.*, 2002). EPF are a major source for agents to be evaluated with findings revealing that most isolates tested conferred 100% mortality within 10 days of application to targeted insects (Wildey *et al.*, 2002). Storage product pests can therefore constitute a good target for biological control agents as they occupy a

niche environment with multiple insect pests (Oduor *et al.*, 2000; Stejskal *et al.*, 2002). Adequate evaluations will therefore suppress negative perceptions on the efficacy of EPF within storage facilities (Oduor *et al.*, 2000).

This project aimed to target two pests of real significance with regards to the damage they cause on harvested agricultural commodities, the maize weevil, *Sitophilus zeamais* and the cigarette or tobacco beetle, *Lasioderma serricorne*. The maize weevil is a common pest throughout sub Saharan Africa (Longstaff, 1981). Along with other prominent post harvest insect pests such as the Angoumois grain moth, *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) and the larger grain borer, *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae), it contributes to estimated post harvest yield losses of between 4 and 5% per annum of maize stored on farms in Africa (Oduor *et al.*, 2000). With regards to the maize weevil's negative impact on stored grain, whether commercially warehoused or on farms, it is one of the more recognised, devastating and hardy of those pests that affect harvested maize (Longstaff, 1981). It can often infest maize while *in situ* prior to harvest, allowing it to enter clean storage facilities undetected. It is thus a highly persistent adversary (Longstaff, 1981; Oduor *et al.*, 2000). The maize weevil can be found in warmer, more humid regions of the globe, and in the more marginal cooler and drier regions, where it is restricted to maize (Sallam, 1994). However, in optimal conditions it is the major pest of stored rice, other grains including wheat and will also attack stored apples (Longstaff, 1981).

In the modern era, the earliest reports suggesting an association of the tobacco beetle with processed tobacco and tobacco products date back to 1848 in Paris and 1886 in America (Ashworth, 1993b; Massey, 1999). Today, the small beetle has a world wide distribution, and although this global distribution is ultimately limited by low temperature and humidity, the pest is common throughout the southern African region, as an economically important pest of cured tobacco (Ashworth, 1993b; Sallam, 1994; Massey, 1999). In Zimbabwe, the beetle can be found throughout all tobacco growing districts and damage is further compounded by restrictions to tobacco trade where infestations are found. These restrictions include financial penalties and delays in movement of produce to sales

floors. In the United States of America, the beetle is now reported as an emerging pest of household pantries, surviving on just about any foodstuff (www.bugspray.com/article/cigarettebeetle.html). The beetle will readily infest books, furniture, stored dried plants, canvas paintings, anything made of straw, just about any spice, cookies, flour, pasta, cotton, medicine, dog food and rat poison (www.bugspray.com/article/cigarettebeetle.html; Sallam, 1994). Moreover, this pest, which may be seeking alternate food sources in the USA, also attacks cigar collections whose proprietors are noted to 'dread the tobacco beetle'. Furthermore, hobbyists who make wreathes and other natural creations are also noted as having to undoubtedly contend with this menace (www.bugspray.com/article/cigarettebeetle.html).

1.3.2 Insect pest taxonomic classification and biology

In the case of crop damage caused by coleopteran pests, both larvae and adults feed and both developmental stages are responsible for direct product degradation (Ashworth, 1993b; Sallam, 1994). Within the Order Coleoptera, destructive post harvest pests include the larger grain borer, *Prostephanus truncates*; the lesser grain borer, *Rhizoperha dominica*; the rice weevil, *Sitophilus oryzae*; and indeed the two pests selected for study here, the maize weevil and the tobacco beetle. Table 1.2 highlights other pests in the Order Coleoptera.

Table 1.2 Taxonomic Chart of Important Insect Pests of Stored Products

COLEOPTERA:	**FAMILY:**	**SPECIES:**
	ANOBIIDAE	***Lasioderma serricorne* (Fabricius)**
	BOSTRICHIDAE	*Rhyzopertha dominica* (F)
	BRUCHIDAE	*Prostephanus truncatus* (Horn)
	CUCUJIDAE	*Acanthoscelides obtectus* (Say)
	CURCULIONIDAE	*Callosobruchus* spp.
	DERMESTIDAE	*Zabrotes subfasciatus* (Boheman)
	SILVANIDAE	*Cryptolestes* spp.
	TENEBRIONIDAE	*Sitophilus oryzae* (L)
		***Sitophilus zeamais* (Motschulsky)**
		Trogoderma granarium (Everts)
		Dermestes spp.
		Oryzuephilus surinamensis (L)

Note: Taken from FAO web site http://www.fao.org/documents/show_cdr.as.htm. Note: bold type denotes species of interest herein. Table also includes other important coleopteran insect pests

The following classifications group the pests of interest in the present study with other related species.

Classification of the maize weevil: Phylum: Arthropoda; Class: Insecta; Order: Coleoptera; Family: Curculionidae – This is a large group of beetles that contains some of the most serious crop and stored grain pests. Morphologically, members of this family are characterised by the form of the snout (rostrum) which is elongated in most species. This family contains the most destructive stored grains pests in the world (Sallam, 1994).

- ***Sitophilus zeamais* (maize weevil)**
- *Sitophilus granaries* (the granary weevil)
- *Pantorhytes plutus* (cocoa weevil)

With reference to the taxonomic history of *S. zeamais* and its smaller relative *S. oryzae*, controversy and up to fifteen years of uncertainty surrounded their correct identification as to whether or not they were indeed a distinct species (Longstaff, 1981). However, in 1961, Kuschel conclusively resolved the matter when he established that the smaller strain (of what was recognised as one species), was actually *S. oryzae*, first described by Linnaeus in 1763 – hence *S. oryzae* (**L.**), and the larger of the weevil strains was *S. zeamais*, originally described by Motschulsky in 1855 (Longstaff, 1981).

Classification of the tobacco beetle: Phylum: Arthropoda; Class: Insecta; Order: Coleoptera; Sub Family: Anobiidae – About 1000 species within Anobiidae are known, most of which are found in the tropics (Sallam, 1994).

- ***Lasioderma serricorne* (tobacco beetle)**
- *Anobium punctatum* (furniture beetle)
- *Stegobium paniceum* (drugstore beetle)

Although the tobacco beetle originated in South America, the literature shows that it was discovered in dried resin in the tomb of the Egyptian Pharaoh Tutankhamun and in linen and plant material filling the mummy of Rameses II (Ashworth, 1993b; Sallam, 1994). The morphological characteristic of the beetle have changed little in the 3000 years that have elapsed since the days of the ancient Egyptians (Ashworth, 1993b). Johann Fabricius was credited for the first taxonomic description of *Lasioderma serricorne* from North America in 1792, hence the notation *L. serricorne* (Fabricius) (Ashworth, 1993b).

While hoping to challenge the selected pests with entomopathogenic fungi, it is essential to gain an understanding of the pest's biology, thus gaining insight to which developmental stage of the pests' life cycle is most suitable to target. An insight into feeding or wandering behaviours of the different developmental stages, for example, may provide information useful in the design of bioassays, where targeting the most appropriate growth phase within the insect life cycle is a critical part of such evaluations *in vitro*.

The maize weevil (*S. zeamais*): Behaviour and Biology

Prior to the introduction of the larger grain borer, *Prostephanus truncatus* (Horn) to the African continent, the maize weevil was reported as the most important pest on stored maize (Longstaff, 1981). The maize weevil, *Sitophilus zeamais* (Motschulsky) (Figure 1.1), is a small snout beetle with adults reaching lengths of between 3 to 3.5 mm (average 3 mm) and it is capable of flight.

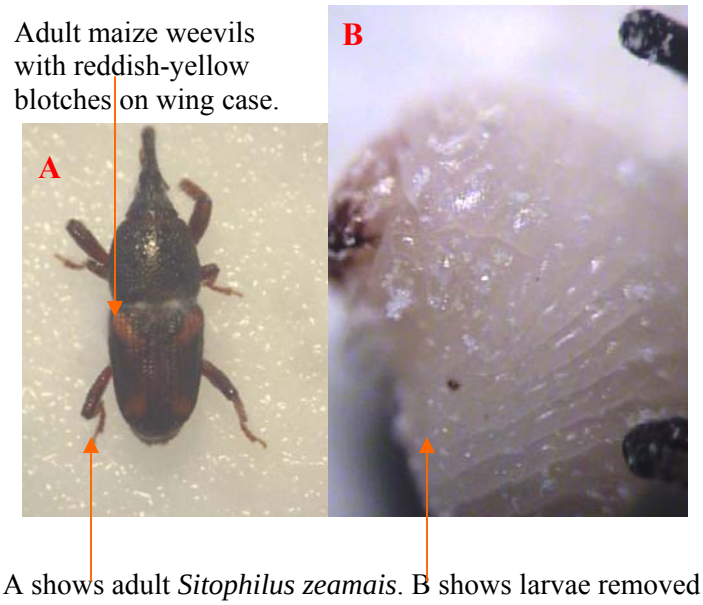


Figure 1.1 – A shows adult *Sitophilus zeamais*. B shows larvae removed from within maize kernel. Larvae of this species lack true legs (Sallam, 1994).

Described as a primary storage pest of maize i.e. with the ability to infest intact kernels (Throne and Eubanks, 2002), adult female weevils excavate shallow pits in the seed coat and lay a single egg inside, sealing it with a waxy plug. Upon hatching, the single fleshy grub develops, growing and feeding within a single grain. The larva changes to a naked white pupa and later the adult eventually emerges via an emergence hole, which may subsequently be enlarged by feeding of the adult inside or by other storage insects (Longstaff, 1981; Jacobs and Calvin, 1988). A minimum of thirty days is required for the cycle (Jacobs and Calvin, 1988). With regards to this pest's host range and the damage it causes, both adult and larvae contribute to reduction in product quality. Within commercial infestations, for instance where grains, including rice, wheat and maize are stored in large quantities within elevators or bins, damage is usually extensive with almost complete destruction of grain.

Damage may be further confounded by heat produced by pest activity, so much so that sprouting of grain occurs (Jacobs and Calvin, 1988). Indeed, as with most pests infesting stored produce, damage is not through direct feeding alone; in the case of this pest, damage to produce also arises from the accumulation of frass, insect cadavers and the spread of post harvest fungal pathogens (Longstaff, 1981; Ashworth, 1993a; Ashworth, 1993b). The maize weevil is a pest that has previously been evaluated with entomopathogenic fungi producing varying degrees of successful results (Adane *et al.*,

1996) (section 1.4.3) and here this pest has been selected as one to be evaluated using entomopathogenic fungal isolates sourced in South Africa (Chapter 4).

The tobacco beetle (*L. serricorne*): Behaviour and Biology

Adult tobacco beetles, *Lasioderma serricorne* (Fabricius) vary in colour and size which depend *inter alia* upon the type of food and atmospheric conditions encountered during development; however, insects are reddish-brown in colour and typically 2 to 4 mm long and weigh between 1.6-4.4 mg (Ashworth, 1993b). The beetle is oval, with head and prothorax bent downwards; the head is deflexed and almost completely obscured from above, thus giving the insect a humped-convex appearance. Both larvae and adults are covered with fine hairs, and adult elytra (wing covers) have no markings (Figure 1.2, below) (Ashworth, 1993b; Sallam, 1994; Massey 1999).

The antennae are serrate, with a saw-like appearance; the segments are practically identical and of the same thickness from base to tip. This feature is used as a diagnostic tool to differentiate this insect from those with similar appearances (Ashworth, 1993b) (Figure 1.2: B).

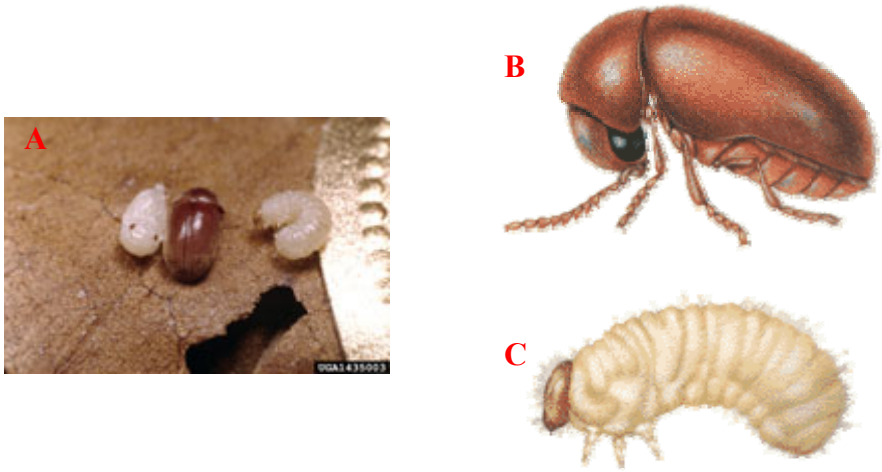


Figure 1.2: Adults (centre: A and B), pupae (far left: A), larvae (right: A and C) of *Lasioderma serricorne***:** (Slide courtesy of ICI Americas, Inc. http://ipmworld.umn.edu/) (Clemson University - USDA Cooperative Extension Slide Series: Pest Control Portal Copyright © 1999 – 2003)

It is reported that in general, this pest only flies during the late afternoon, at dusk and during darkness when the temperature is above 18 ^{O}C, but sometimes in daylight on dull days (Ashworth, 1993b; Massey, 1999). Adult females release the pheromone 4S,6S,7S serricornin to attract mates. After mating and gestation each female lays between 45 and 116 waxy-shelled eggs deposited singly within the infested commodities over a number of days and only hatch in temperatures above 20 ^{O}C after about seven days (Massey, 1999). Larvae are negatively phototrophic when they emerge from their egg shells which they consume, as the shells harbour an external flora of yeast-like symbionts that colonize the insect gut and provide vitamins; for this reason this pest can survive on nutritionally poor food sources (Ashworth, 1993b; Massey, 1999). They grow through four larval stages or instars. During the first instar, larvae are most active (mobile) and suffer the highest mortality rates (Ashworth, 1993b). Larvae are creamy or garish white in colour and are covered with fine hairs, and as they grow older they penetrate the food source causing damage; in the case of cured tobacco, the pest eats small cylindrical galleries through bails (Ashworth, 1993b; Massey, 1999). Damage is further exaggerated by frass, droppings and cadavers (Ashworth, 1993a). Older fourth instar larvae are less mobile but still capable of wandering, and once fully grown, approximately 4.5 mm long, they become less mobile, stop feeding and pupate in a cocoon of food or waste particles (Ashworth, 1993b). Temperatures below 19 ^{O}C see the cessation of activity and the beetle overwinters in the larval stage (Ashworth, 1993b). The prepupal stage, where the larvae constructs its cell, varies depending on atmospheric conditions, however it was reported by Runner (1919) and Howe (1957) cited in Ashworth (1993b), that this stage can withstand low temperatures for a considerable time. After about five to 14 days, pupae moult to become adults and the beetles emerge, with the total development period being between 8 to13 weeks (Massey, 1999).

With regards to this pest's host range and the damage it causes, the larval developmental stages are more significant in terms of being economically destructive. The tobacco beetle is the primary pest of cured tobacco, attacking any processed tobacco, however the beetle will also develop as a secondary pest on a large number of other processed agricultural produce such as dry vegetable products which include herbs, oilseed cake,

rice, cocoa, groundnuts, figs, dates and paprika (http://www.pestcontrolportal.com; Ashworth, 1993b; Massey, 1999). As noted above, this pest causes more then actual feeding damage, with frass and droppings adding to the reduction in the quality of the infested commodity. Such damage is a typical diagnostic tool for officials at the tobacco sales floors in Harare, Zimbabwe. To date, no reports evaluating entomopathogenic fungi against the tobacco beetle have been found (J. Lord, pers. comm.); however Tsuchiya *et al.* (2002) published a report assessing the efficacy of the bacterium *Bacillus thuringiensis* on the tobacco beetle and more on their evaluation is included in section 5.1

1.3.3 Conventional control measure for storage pests

The term conventional is used in the heading with reference to the popular form of insect pest control used regionally, that being the application of synthetic insecticides. Control of storage pests within storage facilities is achieved by an integrated approach mainly through:

- Preventative measures such as general cleaning
- Curative measures such as surface treatment with insecticides or
- Fumigation of produce

(TRB Hand Book: Section I)

This approach is generally successful, but ultimately, prevention in the case of coleopteran pests is very difficult to achieve because pests that fall within this Order can fly, seeking out food sources. The ability of the maize weevil to fly and infest maize prior to harvest makes it one that can enter clean storage facilities undetected, thus a highly persistent adversary (Longstaff, 1981).

Curative measures generally entail the use of broad-spectrum synthetic insecticides and fumigants and these may need to be applied at such a time that some damage may have already occurred and produce quality degraded (Mashaya, 2000). Listed below are recommendations published by the Tobacco Research Board (TRB) in Zimbabwe for the management of pests of stored tobacco, and these recommendations read similar to those of several other storage pests.

Recommendations for Prevention and Management of Tobacco Storage Insect Pests.

- Hygiene

In Zimbabwe, to prevent the establishment and spread of the tobacco moth, it is legislated that tobacco handling and storage facilities should be cleared of tobacco, tobacco scrap and waste and cleaned to the satisfaction of the government pest inspector by 31 October each year. On farms, care should be taken never to store any other product that can be fed on by the moth in the same building as that used to store tobacco. Tobacco dust and scraps should not be allowed to collect in corners or crevices, as these would provide ideal breeding grounds for the pests. Cleaning creates a "dead period" for food sources and this is important in breaking the pests' life cycles. Packing materials such as hessian sacks should also be inspected and fumigated if necessary before use.

- Use of insecticides

Insecticides are used to target pests directly by spraying and as surface treatments whereby surfaces within the storage houses, such as the roof and walls are treated with deltamethrine 2.5% SC (Cislin®) at the recommended rate of 60 ml in five litres of water and applied over 100 m^2. However, this treatment will only kill insects that come into contact with the pesticide and will not control established infestations within the stored tobacco (TRB Hand Book: Section I). In much of sub Saharan Africa, contact insecticides available for the control for storage pests include broad-spectrum synthetic pyrethroids such as deltamethrine and dichloro-diphenyl-trichloroethane (DDT) which are applied as a spray (Massey, 1999; Oduor *et al.*, 2000; Tsuchiya *et al.*, 2002).

- Fumigation

In case of established infestations of the tobacco moth and/or beetle within tobacco, control can be achieved through fumigation with phosphine gas. This fumigant is commonly available as aluminium or magnesium phosphide preparation in a wide range of forms such as sachets, tablets, pellets or plates. The gas is evolved following exposure to atmospheric moisture. Extreme care is required when fumigating with this gas, as it is extremely dangerous and should only be attempted under professional supervision. This not only ensures that the best results are achieved but also contains the danger to a minimum (Mashaya, 2000; TRB Hand Book: Section I).

In Zimbabwe these fumigants and insecticides are 'purple' label products; marketed with all the safety features (Tobacco Research Board (TRB) Hand Book). Purple represents the most toxic grade of agro-chemicals sold for insect control and registered for use on tobacco by the TRB. Phosphine gas is reported to be 100% effective when correctly used against storage pests; only that its killing power will decimate most life forms including humans, it is therefore recommended that only fully trained staff apply this fumigant (Massey, 1999).

Measures applied for the control of the maize weevil within commercial storage facilities across Southern Africa also follow those of prominent storage pests, where combinations of good agricultural practise (GAP) and the use of chemical insecticides are used in an integrated approach. Odour *et al.*, (2000) reported that in Kenya, control measures for maize pests including the maize weevil and the larger grain borer entail the utilization of dust formulations containing mixtures of an organophosphate and a pyrethroid, however the later pest is noted as being likely to develop resistance to pyrethroids and is already tolerant to organophosphates, while pyrethroids are not particularly effective in controlling *Sitophilus* species (Odour *et al.*, 2000; Tsuchiya *et al.*, 2002).

Internationally, research into genetically modified maize and crop varieties resistant to insect attack are at advanced stages, with Throne and Eubanks (2002) noting that some Mexican land-races of maize (*Zea mays* L.) were resistant to maize weevils, and that the resistance was correlated with phenolic acid content of the maize. Notwithstanding this, use of synthetic pesticides is still preferred regionally (Baumgärtner *et al.*, 2003).

With regards to agrochemicals, particularly pesticides, it is still thought that there is no firm grasp on their mode of action in terms of insect physiological responses (let alone to non-target organisms) (Perkins, 1982; Bell, 1992). Cognizance of the importance of the relationship between time, concentration and temperature is lacking, at least at the farmer level. The phenomenon of resistance to the fumigants is always likely where incorrect rates are applied (Bell, 1992). Less complex, non restrictive approaches are required for the modern farmers of this region, who have a firm and growing enthusiasm for new

technologies that will aid them safeguard their stored produce while adhering to concepts of good agricultural practise and maintaining economic thresholds (Bell, 1992). It appears that the advantages of preventive over curative methods are rarely recognised in Africa, with more emphasis being given to the marketed 'silver bullet' in the form of chemical insecticides (Baumgärtner *et al.*, 2003), than to integrated, long term approaches to insect "management" as suggested herein.

1.3.4 Resistance to insecticides

Biological material is seldom uniform and with regards to insects, a wide variation in sensitivity to pesticides often exits in target populations (Robinson, 1971). Insects can rapidly develop tolerance to synthetic insecticides and this is true for the species under study here as mentioned above. These variations can be brought about, arise or be caused by three factors: 1) Genetic or inborn responses; 2) Environmental influence; and 3) Ontogenic error (Robinson, 1971; Hollomon and Butters, 1994).

Larval developmental stages (instars) express different levels of tolerance to synthetic pesticides when applied at rates or for periods of time that do not achieve kill (Bell, 1992). All susceptible individuals will die at the recommended application rates, while the resistant individuals and/or those out of reach of a full dose, survive to reproduce among themselves and as the resistance is governed genetically, the resultant offspring will be resistant (Robinson, 1971), rendering the mortality levels of the population much lower when the recommended rate of a synthetic insecticide is applied.

1.4 Entomopathogenic Fungi (EPF)

1.4.1 Taxonomic distribution and description of EPF

Although fungi with any association or relationship with insects are termed entomogenous fungi, with associations ranging from saprotrophic to fully pathogenic (Boucias and Pendland, 1998), the specific interactions under investigation are those

where a fatal infection takes place or can be induced between entomopathogenic fungi and insects.

Taxonomic Classification: EPF can be found in all Phyla within the fungal Kingdom the Mycota; for purposes of illustration, examples from three Phyla are tabulated below (Table 1.3) from the estimated 750 species of entomopathogenic fungi (Boucias and Pendland, 1998; Shah and Pell 2003).

Table 1.3: Outline Classification of EPF and Examples of Insect Hosts

Phylum/ Class/ Order	Genus	Examples of Insect hosts
1) Zygomycota: Zygomycetes Entomophthorales:	*Conidiobolus*, *Erynia*, *Neozygites*, *Pandora*, *Entomophthora* *Zoophthora*	Aphids, flies, caterpillars
2) Ascomycota:	*Cordyceps*, *Hypocrella*, *Torrubiella*	Scale insects, butterflies, moths and beetles
3) Mitospora: Hyphomycetes Moniliales:	*Aschersonia*, *Beauveria* *Hirsutella*, *Metarhizium* *Tolypocladium*, *Nomuraea* *Verticillium*	Scale insects, whiteflies aphids, hoppers, moths, butterflies and beetles

Note: Table adapted from Shah and Pell (2003).

Entomopathogenic fungi can attack a wide range of insects from Coleoptera, Homoptera, Hemiptera to Diptera and Lepidoptera (Barson *et al.*, 1994; Possee and King, 1994; Boucias and Pendland, 1998; De Muro *et al.*, 2003; Dimbi, 2003; Shah and Pell, 2003), and most, if not all, have a life cycle that shadows that of their insect hosts in the natural environment (Shah and Pell, 2003). Such fungi affect insects in varying degrees, for example fungi in the Entomophthorales are considered to have evolved into higher parasitic forms, having biotrophic relationships with their hosts with little or no saprotrophism (Furlong and Pell, 2001; Shah and Pell, 2003). In this case host demise is due to tissue colonisation with little or no use of toxins (Shah and Pell, 2003). The entomo-fungus *Strongwellsea castrans* (Entomophthorales) is a good example of a highly evolved or specialist fungus affecting flies. After infection this fungus does not

immediately overcome its host but discharges conidia from cavities in the insect abdomen. As this fungus does not interfere with its host feeding or movement, spores are continually dispersed over a long period of time until the insect dies (Shah and Pell, 2003). In cases such as these, the potential for uses of entomopathogenic fungi as commercial biopesticides may be limited as these fungi have a narrow host range, however they would be suitable in situations where a host specific agent is required.

On the other hand entomopathogenic fungi belonging to the Mitospora (Deuteromycota) such as hyphomycete fungi are considered to be opportunistic pathogens, affecting a wide range of insect hosts and death of the host is associated with toxin production overwhelming the insect (Samson *et al.*, 1988; Goettel and Inglis, 1997; Shah and Pell, 2003). Hyphomycete species exist as separate sexual states, denoted anamorph (asexual form) and teleomorph (sexual forms). Species of *Beauveria* and *Metarhizium* produce exposed conidiophores, and these conidia may be solitary or form either synnematal or sporodochial conidiomata (Figure 1.3) (http://helios.bto.ed.ac.uk/bto/FungalBiology). The attachment of such conidia to their insect hosts is a prerequisite of infection and in the case of air-borne spores the process occurs at random (Boucias and Pendland, 1998; Shah and Pell, 2003). The broad-spectrum nature of this class of insect killing fungi makes them important agents in the search for microbial control agents through *in vitro* investigation. Owing to this, the entomopathogenic fungal species *Beauveria bassiana* is of key interest in this study. *B. bassiana* is a ubiquitous, naturally occurring mitosporic fungus with a wide arthropod host range and many isolates have been successfully studied for their potential in the development of biopesticides (Castrillo *et al.*, 2003; De Muro *et al.*, 2003; Shah and Pell, 2003).

1.4.2 Biology and mode of action of EPF

Hyphomycete fungi such as *Beauveria bassiana* are filamentous fungi that reproduce by conidia generally formed aerially on conidiophores arising from the nutrient source (Figure 1.3: a) (Goettel and Inglis, 1997). *B. bassiana* is a white muscardine fungus with its conidia counted as the infectious propagule (Boucias and Pendland, 1998; Castrillo *et*.

al., 1999). Upon first binding to the host cuticle, conidia of this entomopathogen initiate infection of insects at the outer integument, penetrating directly (Goettel and Inglis, 1997; Furlong and Pell, 2001). In the case of spore production for Hyphomycete insect pathogens, the narrow apex of the conidiogenous cell extends sympodially, each new apex becoming converted into a blastic conidium or spore and then the next apex growing out from behind and to one side of it (Figure 1.3) (http://helios.bto.ed.ac.uk/bto/FungalBiology/index.htm#top). The more conidia produced, the longer the conidiogenous cell becomes. Owing to numbers produced, spores are readily dispersed throughout the environment where insect hosts are present and these spores are the infectious component, ready to germinate upon contact with host organisms (Goettel and Inglis, 1997; Furlong and Pell, 2001).

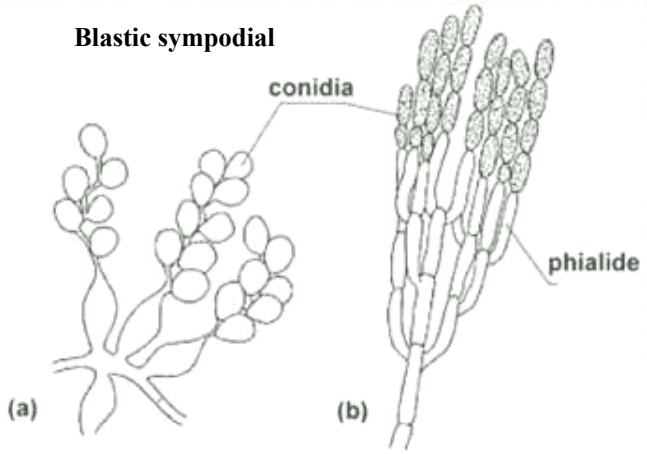


Figure 1.3 - Spore-bearing structures of (a) *Beauveria bassiana*, which produces cream-white conidia alternately on an extending tip of a conidiophore. (b) *Metarhizium anisopliae*, which produces green conidia in chains from phialides. [Courtesy of Jim Deacon, the University of Edinburgh © Jim Deacon] (http://helios.bto.ed.ac.uk/bto/FungalBiology/index.htm#top)

The mode of action with which Hyphomycete species attack a wide range of insects gives them particular interest as broad spectrum bioinsecticides. This wide host range would make such products on par with the killing mode of action of synthetic insecticides but without the damaging impact on the environment, and perfect for pests of stored commodities where at any time several pests may be present (Stejskal *et al.*, 2002). It must be pointed out that the aim of developing bioinsecticides would be for their use in specifically selected locations without risk to beneficial non-targets (Shah and Pell,

2003). Generally EPF penetrate directly through the insect cuticle. Hydrophobic spores adhere to the insect cuticle by non-specific hydrophobic mechanisms (Samson *et al.*, 1988; Goettel and Inglis, 1997; Shah and Pell, 2003; Small and Bidochka, 2005) and use a combination of mechanical pressure, enzymes and toxins to penetrate (Ferron, 1981; Leger *et al.*, 1988; Leger *et al.*, 1993; Possee and King, 1994; Boucias and Pendland, 1998). The enzymes produced partially govern the fungal food/energy sources and much of the evolutionary development of fungi has been along the lines of physiological specialization (Talbot, 1971; Shah and Pell, 2003).

Entomopathogenic Hyphomycetes like *Beauveria* and *Metarhizium* species have both protease and chitinase activity implicated in the pathogenicity determinants involved in host invasion (Leger *et al.*, 1993; Boucias and Pendland, 1998). The role of extracellular chymoelastase and basic proteases are also involved in the virulence of *Metarhizium anisopliae* (Leger *et al.*, 1988; Boucias and Pendland, 1998; Small and Bidochka, 2005). When conidia of these EPF land on the cuticle of a host, they attach and then germinate to produce appressoria (Figure 1.4), initiating cascades of recognition enzyme activating reactions through hyphae which grow to ramify in the insect haemocoel, absorbing nutrients, which eventually result in the demise of the host (Goettel and Inglis, 1997; Shah and Pell, 2003; Small and Bidochka, 2005).

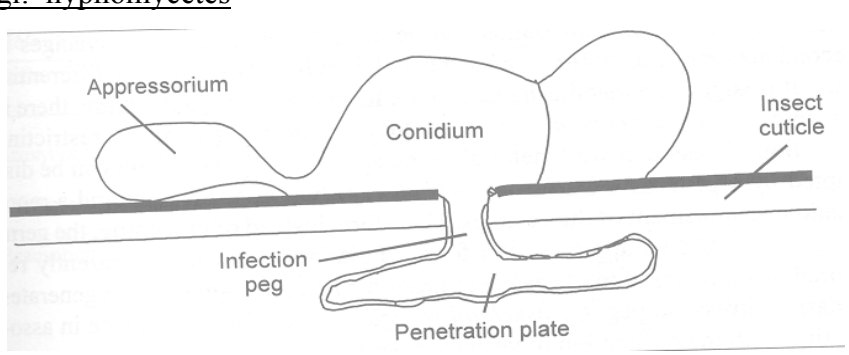


Figure 1.4 - Germination of a fungal hyphomycete, *M. anisopliae* (Boucias and Pendland, 1998).

The insect cuticle consists mostly of chitin fibrils within a protein matrix together with lipids, waxes and small quantities of phenols, inorganics and pigments (Possee and King,

1994). The haemocoel or main body cavity is filled with the pathogen's main nutrient target, after penetration follows insect demise (Figure 1.5) (Boucias and Pendland, 1998).

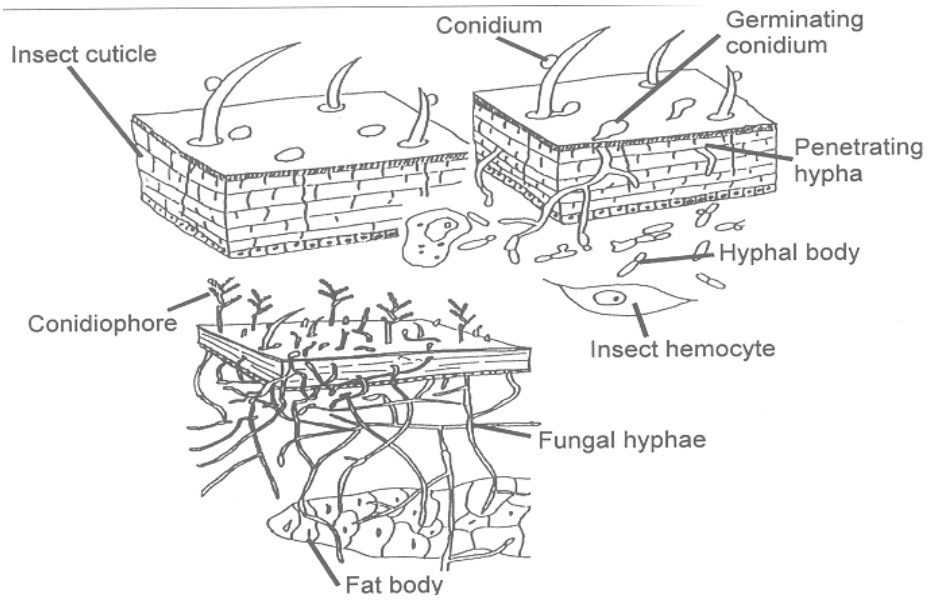


Figure 1.5 - Spore germination and penetration of fungal Hyphomycete (Boucias and Pendland, 1998).

In the case of entomopathogenic Hyphomycetes, host demise is associated with toxin production, conferring a rapid infection period therefore making them perfect broad-spectrum insect killers (Goettel and Inglis, 1997; Boucias and Pendland, 1998). Several authors including Barson (1994), Adane *et al.* (1996), and Dimbi, *et al.* (2004) report lethal times of between two and four days for insects inoculated *in vitro*.

While EPF can penetrate the insect integument and cause the demise of insect hosts without the need to be ingested, infections through the digestive tract are also possible (Goettel and Inglis, 1997). Laboratory examinations on cockchafer larvae, *Melolontha melolontha* (Linnaeus) (Coleoptera), revealed that these fungi can, like plant pathogens, penetrate through natural openings such as the mouth, anus, and between segments of appendages (Goettel and Inglis, 1997). Comparisons between infection sites of laboratory-reared larvae and naturally infested larvae suggest that behaviours such as burrowing in natural populations only allows for certain sites to be more easily penetrable by fungal spores (Ferron, 1981).

1.4.3 EPF as biological control agents

EPF have been evaluated both within laboratory situations and *in situ* for several decades (Boucias and Pendland, 1998; Lord, 2005). This section reviews relevant literature surrounding this technology and draws attention to some important points to bear in mind during the planning of a laboratory evaluation. As the current study focuses on fungal Hyphomycetes, this section will be predominated by reviews highlighting their evaluation.

Emphasis on Hyphomycetes: One of the earliest trials conducted *in situ*, using *Beauveria bassiana*, was in the state of Kansas, USA in 1890 (Roberts and Yendol, 1971; Lord, 2005). The fungus was provided to farmers for the control of the chinch bug, *Blissus leucopterus* (Say) (Hemiptera). After the first two years, results were noted as promising; however, the outlook changed in years to follow when it was observed that the initial and dramatic reduction in bug populations could not be reproduced in the same locality without human intervention (Roberts and Yendol, 1971). The conclusions were that 'the mere presence of the EPF is not adequate to ensure fatality of exposed hosts, owing to the importance of environmental factors in disease induction' (Roberts and Yendol, 1971). Data from these early days resulted in negative dogma eroding interest in entomopathogens, leaving them regarded as biological agents of secondary interest (Lord, 2005). Further to this, commercialisation of chemical insecticides after World War two was highly successful owing to their speedy mode of action.

In the 1950s however several Eastern European countries, unbeknown to specialist western nations, began investigations which resulted in original developments in the prognosis of insect infectious diseases, mode of action in the field, conditions of muscardine development and technology for mass spore production (Ferron, 1981). Today and over the last thirty years or so, *Beauveria bassiana* has continued to dominate literature on microbial control of insects along with *Metarhizium* species (Shah and Pell, 2003). Experimental research evaluating EPF can aim to find many different beneficial

properties conferred by insect pathogens, as well as to determine whether they can work in combination with other insect control products.

The compatibility of *Beauveria bassiana* with insecticides was evaluated by Anderson and Roberts (1983). In an investigation to integrate the use of both biological and synthetic chemical insect killing products against the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera), the aim was to reduce the input of the synthetic component of the strategy (Anderson and Roberts, 1983). For the laboratory based part of their investigation, spore germination was used as the criterion on which compatibility was to be set (Anderson and Roberts, 1983), because of the importance of viable, germinating spores in the infection process. Findings were that synthetic insecticides, and in particular pyrethroids, inhibited germination of *B. bassiana* within 4 hours when applied as a tank mix, although there were some differences in the levels of inhibition (Anderson and Roberts, 1983). Only when applications of the pathogen and insecticides were conducted separately was *B. bassiana* germination not as severely affected, with no discernable inhibition when the pathogen was applied after the insecticide (Anderson and Roberts, 1983). Although it may generally be considered that mixing insecticides with EPF will inhibit the efficacy of the fungus to germinate, Anderson and Roberts (1983) suggest investigating any potential useful formulations. They found for instance that some virulent *B. bassiana* isolates may work better in such mixtures (Anderson and Roberts, 1983).

Strains of the entomopathogenic fungus *B. bassiana* were evaluated against one of the pests of interest herein; the maize weevil (*S. zeamais*) and the investigation of Adane *et al.* (1996) forms part for the bases of the design of this project. Using 10 different fungal isolates from 10 different countries and several insect hosts, Adane *et al.* (1996) found that water-formulated inoculations of infectious spores conferred high levels of mortality against adult insects of certain ages. Experiments were conducted so that different aspects relating to fungal virulence could be obtained from their results. Initially, for what were termed preliminary bioassays, all isolates in laboratory-based experiments were applied as water formulations. Fungal spores were prepared by growing isolates on Sabouraud

Dextrose Agar (SDA) for seven days, with the resultants conidial mats being suspended in Tween 80 (0.01% v/v aqueous solution) in distilled water (Adane *et al.*, 1996). These water formulations were then adjusted to a conidial concentration of 10^8 conidia ml^{-1} and sprayed onto adult weevils. Results collected over an 11 day assessment period indicated that of the ten isolates evaluated, five isolates were highly virulent, three conferred intermediate results and two were weakly virulent against the maize weevil (*S. zeamais*) (Adane *et al.*, 1996). This method of collecting data daily (cumulative data) resulted in percentage mortality data which revealed that the most pathogenic isolates began to kill inoculated pests three days after exposure to the fungus (Adane *et al.*, 1996).

Further to this Adane *et al.* (1996) investigated the effects of six different conidial concentrations formulated as water formulations against the maize weevil. Selecting the most virulent isolate from their preliminary experiments, five replicates each of conidial concentration of 10^4 10^5, 10^6, 10^7, 10^8 and 10^9 conidia ml^{-1} were applied onto 20 weevils. Treatments were applied by spraying 1 ml of the different conidial concentrations onto adult weevils in experiments laid out in complete randomised designs including untreated control plots. In their case results indicated that the fungal isolate was effective at all conidial concentrations, with mortality levels increasing as did the numbers of conidia (Adane *et al.*, 1996). Importantly findings included that the median lethal time for the highest conidial concentration of 10^9 conidia ml^{-1} was three days (Adane *et al.*, 1996).

In yet another of their experiments Adane *et al.* (1996) used dry spores against the maize weevil, comparing the effects of a pathogenic fungal isolate directly with a synthetic insecticide and untreated controls. Observations of treatments blended with grain and laid out in a complete randomised design revealed that there were no statistically significant difference between the fungal pathogen and the insecticide with respect to the proportion of damaged grain, but that the proportion of damaged grains increased with the median lethal time (Adane *et al.*, 1996). Importantly, this experiment also indicated that dry conidia of *B. bassiana* were able to achieve high levels of mortality (> 70%) without the requirement of high humidity (Adane *et al.*, 1996).

Individual species of EPF can be evaluated, developed and used in situations where a specific pest problem has arisen (Shah and Pell, 2003; Lord, 2005). Barson *et al.* (1994) observed that within intensive livestock units for pigs and poultry, infestations by the housefly, *Musca domestica* (Diptera: Muscidae) were causing major discomfort to workers within the enclosed spaces. This led to poor animal management, which in turn led to reductions in animal weight, which meant a reduction in the price per animal (Barson *et al.*, 1994). Investigations went on to evaluate six entomopathogenic fungi cultures from the Hyphomycetes for their control of adult and larval stages of the pest. These included two species from the genus *Beauveria*, (*B. bassiana* and *B. brongniartii*), *M. anisopliae*; *Paecilomyces farinosus*; *Tolypocladium cylindrosporum* and *Verticillium lecanii*. Although targeted for a problem emanating from industry, the resultant laboratory evaluations determined that *M. anisopliae* and *T. cylindrosporum* were the most aggressive against housefly larvae. Adult flies were more susceptible to *M. anisopliae* in an aqueous suspension, conferring 100% mortality in six days. Further screening with oil-based carriers revealed that adult fly mortality could be reduced to three days (Barson, 1994).

Evaluations can also be aimed at pest problems affecting agro-produce destined for export to other countries particularly in cases where the pest causes restrictions in trade (De La Rosa *et al.*, 2002). Prominent insect pest problems encountered *in situ* resulted in laboratory evaluations using *B. bassiana* against the Mexican fruit fly species, *Anastrepha ludens* (Diptera: Tephritidae) by De La Rosa *et al.* (2002), where the fungus was tested as an alternative to chemical insecticides. Tephritid fruit flies are reported to be the major pest of export fruits such as mango and citrus on a global level impacting negatively on yields and are considered to be the most economically important group of phytophagous Diptera (De La Rosa *et al.*, 2002; Dimbi *et al.*, 2003; Dimbi *et al.*, 2004). For their evaluations under laboratory conditions (working under sterile conditions in laminar flow cabinets) De La Rosa *et al.* (2002) propagated the fungus on SDA for 15 days under controlled conditions prior to harvesting and storage at 4 ^{O}C. Different bioassays were designed to determine the effects of conidial concentrations of 10^8 conidia

ml^{-1} of *B. bassiana* against all insect developmental stages of *A. ludens* (De La Rosa *et al.*, 2002).

For evaluations designed to determine the effects of the fungus against pupal and larval developmental stages, specimens were inoculated by dipping for up to 30 seconds in suspensions of 10^8 conidia ml^{-1}. Their findings showed that the evaluated isolates of *B. bassiana* were not pathogenic against the pupae of *A. ludens*; as the inoculated specimens did not die, but went on to develop into adults (De La Rosa *et al.*, 2002). In the case of *A. ludens* larvae, the *B. bassiana* isolates were effective to a limited degree with low levels of mortality observed. Mortality levels of between 1-4% were obtained for treatments incubated on a larval diet and mortalities of between 2-8% for treatments incubated on a soil substrate. Larvae which survived fungus inoculation went on to pupate and after 15 days of pupation adults emerged with no significant differences detected between any of the treatments (larval diet or soil substrate) or control treatments inoculated with spore free inoculations (De La Rosa *et al.*, 2002). De La Rosa *et al.* (2002) concluded that the low levels of pathogenicity of *B. bassiana* against *A. ludens* larvae were due to the extensive sclerotization of the larval cuticle along with other factors which prohibited spore contact and penetration.

For their evaluation of adult flies, insects were inoculated by aqueous sprays of 0.5 ml of the fungus. Results obtained from the 10^8 conidia ml^{-1} inoculations of *B. bassiana* against female adult fruit flies went on to show that all strains were pathogenic causing between 82 and 100% mortality (De La Rosa *et al.*, 2002). Using this information and only targeting adult female flies De La Rosa *et al.*, (2002) went on to determine lethal concentrations (LC_{50}) (defined as the conidial concentration at which 50% of the flies died) and lethal times (LT_{50}) (defined as the time taken for 50% of flies to die) of *B. bassiana* strains against *A. ludens*. In the case of the LC_{50} experiment, four doses of 10^5, 10^6, 10^7 and 10^8 conidia ml^{-1} of three fungal strains were used with the fungus being applied with the spray inoculation technique. Results of treatments incubated at 27 ^{O}C revealed that lethal concentrations were between 10^5 and 10^6 conidia ml^{-1}(De La Rosa *et al.*, 2002). For the LT_{50} evaluation, where all eight isolates were tested, their results

indicated that on average, across all strains, a lethal time of four days was achieved. One strain of *B. bassiana* was lethal against *A. ludens* after two days, with the longest lethal time being found to be five days (De La Rosa *et al.*, 2002).

Knowing that virulent isolates can confer high percentage rates of mortality within three days will therefore be a factor to investigate during the present comparative evaluation of local and exotic strains of entomopathogenic fungi. To confirm an entomopathogenic infection De La Rosa *et al.* (2002) incubated dead insects in environmental chambers and observed development of *B. bassiana* mycelia on adult cadavers. After a 48 hour incubation period all infected individuals became mummified with over 80% fungal growth emerging from the soft parts of insect bodies (De La Rosa *et al.*, 2002). The plethora of information obtained from their laboratory evaluation resulted in conclusions that *B. bassiana* is a suitable candidate to be used as biological control agent against adult *A. ludens in situ* in Mexico following the development of field application technology and research (De La Rosa *et al.*, 2002).

African fruit fly species have also been targeted by scientists hoping to manage pest species with microbial control agents (Dimbi *et al.* 2003; Lux *et al.*, 2003). Dimbi *et al.* (2003) reported that fungal hyphomycetes used in their *in vitro* evaluations to gauge the pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* against three African fruit fly species in Kenya provided lethal times that ranged between three to four days. Using dry spore formulations to infect adult pests, Dimbi *et al.* (2003) observed variations when targeting the pests through devices that worked by attracting insects to stations where they would by contaminated by fungal spores. These autoinoculative devices further illustrate the innovative nature in which the use of EPF can be used to combat insect pests, further highlighting developments in application technology for field application (Furlong and Pell, 2001; De La Rosa *et al.*, 2002; Dimbi *et al.*, 2003). In similar research, investigating the potential of *Metarhizium anisopliae* to control three African fruit fly species, comprehensive bioassays conducted *in vitro*, aimed to evaluate the effects of various temperatures on spore germination and fungal pathogenicity (Dimbi *et al.*, 2004). These experiments used temperatures ranging from 15 ^{O}C to 35 ^{O}C to

determine the ability of *M. anisopliae* to control the target pests with results indicating that the fungus was highly effective at causing high levels of mortality within a wide range of temperatures (Dimbi *et al.*, 2004).

While laboratory-based bioassays may not always yield fungal isolates that confer 100% mortality, there are other criteria to be measured and/or benefits to their use. An example is an evaluation by Kaaya *et al.* (1996) where entomopathogenic fungi were tested for their control of livestock ticks, *Amblyomma variegatum* (Fabricius) (Acari: Ixodidae). In these evaluations, *M. anisopliae* did not achieve high percentage kill rates but did confer or induce reduction in pest engorgement weights, fecundity, and egg hatchability, so much so that fecundity in *A. variegatum* was reduced by up to 94% (Kaaya *et al.*, 1996). Therefore, the possibilities and likely benefits of using fungal insect pathogens goes further than just killing. These fungi can reduce fitness and allow enough time for farmers to include other integrated measures of pest management. The anti-feedant effects of the entomopathogenic fungus on a pest could partly offset its slow speed of kill, and might play an important role in field control maintaining disease within populations (Kaaya *et al.*, 1996; Ekesi, 2001). The potential for entomopathogenic fungi to interrupt reproductive cycles can also be investigated and may be a relevant aspect of assessing effects of fungi inoculated onto a selected pest (Watson and Petersen, 1993; Kaaya *et al.*, 1996; Ekesi, 2001).

A further important aspect that can be investigated *in vitro* involves autodissemination or transmission of spores from mycosed cadavers. Furlong and Pell (2001) investigated the transmission of *Beauveria bassiana* from sporulating (mycosed) cadavers to live larvae of the diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) in comparison with *Zoophthora radicans* (Entomophthorales). Having established that the pest was indeed susceptible to infection by the entomopathogens through a series of preliminary and dose response experiments, they went on to determine whether mycosed fourth instar larvae of *P. xylostella* would have an effect on mortality of second instar larvae of the same pest foraging on whole Chinese cabbage plants. Their results indicated that when measuring the density of *B. bassiana* mycosed cadavers/plant one to three

cadavers caused no differences in transmission frequency of the pathogen, but in the case of *Z. radicans*, there were significant differences in spore transmission between one and three cadavers/plant (Furlong and Pell, 2001). From these trials, comparing the transmission frequencies of the two fungal entomopathogens from mycosed cadavers, Furlong and Pell (2001) concluded that the differences were a direct result of the conidial nature of the two fungi. *Z. radicans* is an obligate insect pathogen adapted to natural dispersal and transmission of its conidia from foliar environments, whereas *B. bassiana* is a facultative, soil borne insect pathogen which causes high levels of mortality to insect species that spend all or a portion of their life cycle in intimate contact with soil (Furlong and Pell, 2001).

Furlong and Pell (2001) also conducted comparative experiments assessing the transmission of fungal spores of *B. bassiana* and *Z. radicans* between adult *P. xylostella* and from adults to larvae. Comparisons of the positive transmission rates showed that conidia of *B. bassiana* were more readily transferred from inoculated live adults to clean (uninoculated) moths than the conidia of *Z. radicans* (Furlong and Pell, 2001). In a discussion of their results, Furlong and Pell (2001) went on to suggest that it was likely that conidia of *B. bassiana* may have been dislodged from inoculated adult insects (owing to the high concentrations of *B. bassiana* conidia applied), making them available to infect clean moths via the substrate in which insects were incubated; rather than suggest infection through high transmission rates between adults, as conidia of both pathogens form rapid and strong attachments upon contact with the host insects (Furlong and Pell, 2001). Their findings did not however show that adult *P. xylostella* could act as efficient vectors of *B. bassiana* conidia *in situ*, as was the case with *Z. radicans* (Furlong and Pell, 2001). Furlong and Pell (2001) concluded that with *P. xylostella*, transmission of *B. bassiana* conidia from sporulating cadavers was likely to be low, however the observation that conidia of *B. bassiana* can be dislodged into the substrate and go on to infect clean insects makes the possibility of using mycosed insect cadavers a vector for fungal spores.

Other entomopathogenic fungal genera: As noted previously, many different characteristics of the insect-pathogen relationships can be evaluated *in vitro*. Using *Erynia neoaphidis* (Zygomycetes: Entomophthorales) a common pathogen of several aphid species including the common nettle aphid, *Microlophium carnosum* (Buckton) (Hemiptera), Hemmati *et al.* (2001) went as far as to evaluate the pattern of conidial discharge. Their investigation discussed the importance of the discharge distance for pathogen spores from aphid cadavers placing emphasis on estimating airborne dose inoculum (Hemmati *et al.*, 2001).

Neozygites floridana (Zygomycetes) has been reported to be highly aggressive against spider mite populations infesting Soya beans in North America. So much so that reports of 84% mortalities were observed two days after fungal inoculation (Chandler, 2005).

The entomopathogenic fungus *Zoophthora radicans* (Entomophthorales) was evaluated with some promise against the diamondback moth, *Plutella xylostella* (L), a prominent and problematic pest of cabbage and other cole crops throughout the world (Pell *et al.*, 1993; Furlong and Pell, 2001; Kfir, 2003). Success of this fungus has led to its application *in situ*, with success against the spotted alfalfa aphid, *Therioaphis trifolii* f. *maculate* (Buckton), when in 1977 the aphid was introduced into Australia and rapidly established itself as a pest (Shah and Pell, 2003). In this case, laboratory-based evaluations resulted in the strategy of classical biological control being employed, where an Israeli isolate of *Zoophthora radicans* was imported for use against the pest. Fungal inoculations were conducted in conjunction with the large-scale release of the parasitic wasp, *Trioxys complanatus* (Quilis) (Hymenoptera). The aphid is no longer reported as a problem and, it was thought likely that the fungus only infected insects closely related to the target host species and not other insects such as the beneficial wasp, which was additionally effective in alleviating the effects of the pest (Shah and Pell, 2003). Similar strains of the same fungus have been associated with the diamondback moth in South Africa; however few studies to investigate this insect pathogen *in vitro* against targeted pests are available (Kfir, 2003).

1.5 Notes on and design of the present study

The literature review above has demonstrated that in the selection and evaluation of potential biological control agents, there are generally different aspects to be investigated when planning to study entomopathogenic fungi *in vitro* and this section briefly summarises some of the more important points incorporated in the present study.

For laboratory bioassays, relatively little infectious material is needed (Goettel, 1984) and the literature shows that when using entomopathogenic hyphomycete fungi, Sabouraud dextrose agar (SDA) is the medium of choice to culture fungi such as *B. bassiana* (Adane *et al.*, 1996; Goettel and Inglis, 1997; Furlong and Pell, 2001; De La Rosa *et al.*, 2002). To firmly establish that sourced isolates of EPF are indeed pathogenic against selected insect pests *in vitro*, researchers conduct preliminary bioassays, which are the first step prior to further virulence evaluations (Adane *et al.*, 1996; Furlong and Pell, 2001; De La Rosa *et al.*, 2002). Typically such bioassays aim to inundate insects with high conidial concentrations of fungal spores applied as aqueous suspensions either by spraying or immersing the insects (Adane *et al.*, 1996; Masuka and Manjonjo, 1996; Goettel and Inglis, 1997; De La Rosa *et al.*, 2002). This ensures that individual insects in each treatment are thoroughly contaminated by the fungus (Goettel and Inglis, 1997; De La Rosa *et al.*, 2002) with upwards of five insects required per replicate, where experiments are laid out in complete randomised blocks of no less than three replicates per treatment (Goettel and Inglis, 1997). Following preliminary bioassays which may confirm isolate pathogenicity and reveal differences between isolates, further experiments may be conducted where researchers may select isolates they view as showing the most potential from their preliminary observations (Adane *et al.*, 1996). Such experiments typically included assessments of fungal virulence under different environmental conditions and conidial concentrations.

De La Rosa *et al.* (2002) evaluated *B. bassiana* against all developmental stages of the Mexican fruit fly species, *A. ludens* with varying results. In the present study, the most suitable developmental stage to evaluate sourced EPF was found to be the adult stage of

insect development. The main reason is that the larvae of both insects are usually buried within the infested commodity making them inaccessible (Longstaff, 1981; Jacobs and Calvin, 1988; Ashworth, 1993b; Massey, 1999), without specialist help from professional insect laboratories. In some cases, scientists are supplied with insects of specific ages or sexes from specialist insect laboratories (De La Rosa *et al.*, 2002; Dimbi *et al.*, 2004). Furthermore, in the case of the maize weevil, only the adult can realistically be selected for evaluation as the larva develops within the grain (Longstaff, 1981; Jacobs and Calvin, 1988) and attempting to collect sufficient numbers for evaluation would be laborious within given time lines. A further reason to test sourced fungi against adult coleopteran pests is that most species use pheromones that can be synthesized and used in lures designed to attract and infect a portion of the adult population as part of *in situ* application technology (Ashworth, 1993b; Massey, 1999; De La Rosa *et al.*, 2002; Dimbi *et al.*, 2004).

The use of control treatments is as an essential component of *in vitro* assessment of the effects of EPF against insect pests (Adane *et al.*, 1996; Furlong and Pell, 2001; De La Rosa *et al.*, 2002). Typically these treatments are spore free equivalents of the fungus treatment where, in the case of aqueous suspensions, the same amount of the control treatment is applied for the same period of time (Adane *et al.*, 1996; De La Rosa *et al.*, 2002). These treatments also aid in confirming that fungal treatments were the direct cause of insect demise, as after incubation, no insects within control plots should have succumbed to the spore free inoculation (Dimbi *et al.*, 2004). Further confirmation that an entomopathogenic infection has taken place can be obtained by allowing the fungus to sporulate and emerge from the insect cadaver (De La Rosa *et al.*, 2002). This process, termed mycosis, whereby the fungus emerges from infected insect cadavers, is stimulated by incubation under conditions of high relative humidity and temperature inside humid chambers (Goettel and Inglis, 1997; De La Rosa *et al.*, 2002); however it is imperative that dead insects are surface sterilized prior to incubation (Goettel and Inglis, 1997). Typically insects allowed to mycose may be assessed within 48 hours and the criterion for mycosis is the appearance of fungal growth and sporulation on the cadaver (Adane *et al.*, 1996; De La Rosa *et al.*, 2002).

For the purpose of the laboratory evaluations set up for this study, the points above were included and laid out for the evaluation of entomopathogenic fungi *in vitro*. Initially, fungal isolates of the entomopathogen *Beauveria bassiana* were sourced and this selection included harnessing local isolates indigenous to South Africa. To better predict the efficacy of the sourced isolates to grow under fluctuating temperatures and obtain an overall optimal growth temperature for all isolates, the effects of environmental conditions were determined using various temperatures as the main parameter (Dimbi *et al.*, 2004). These aspects are covered and presented in Chapter 3. Chapters 4 and 5 present experiments evaluating the effects of sourced fungal isolates against local cultures of two coleopteran pests using adaptaions of some of the afore mentioned methodologies and techniques. Chapter 6 summaries and concludes the findings in brief.

CHAPTER TWO

Establishment of Fungal Cultures

2.1 Sourcing of Fungal Isolates

2.1.1 Introduction

Comprehensive searches for insects naturally infected by fungi makes up one method researchers undertake when seeking entomopathogenic fungi (EPF) *in situ* (Zimmermann, 1986). Although it is often a challenge to determine whether an entomopathogen was responsible for the insect's demise, Boucias and Pendland (1998), propose guidelines in the form of Koch's postulates as follows:

i. The first postulate states that the presence of the etiological agent be correlated with the observed diseased symptoms (many entomopathogenic fungi do not however elicit symptoms while the insect is alive).
ii. The second postulate states that the suspected disease agent be isolated and propagated in pure culture such as supplemented media (*in vitro*).
iii. The third postulate requires that the organism produced in pure culture causes the disease state or symptoms when inoculated to susceptible hosts (a susceptible host may be the same insect species from where the original organism was first isolated to avert any problems).
iv. The fourth postulate states that the organism that was applied to cause insect demise must be re-isolated from the inoculated 'susceptible' host cadaver. (Boucias and Pendland, 1998).

Once insect cadavers exhibiting symptoms of fungal infection are collected *in situ*, they must then be surface sterilised using the following technique; three, 10 second rinses in 3% sodium hypochlorite, one rinse in 70% ethanol and three, 20 second rinses in distilled sterile water (DSW) under sterile conditions (Zimmermann, 1986, Goettel and Inglis, 1997). To further confirm an entomopathogenic infection and to obtain a pure culture, cadavers must be plated onto a medium (either amended potato dextrose agar (PDA) or the widely utilized Sabouraud dextrose agar (SDA)) (Adane *et al.*, 1996; Goettel and

Inglis, 1997; De La Rosa *et al.*, 2002; Dimbi *et al.*, 2003; Dimbi *et al.*, 2004) to be examined two to three days after incubation at room temperature (Goettel and Inglis, 1997). Koch's postulates procedures must then be conducted to verify that the pure culture obtained from cadavers is indeed entomopathogenic.

For isolation of EPF from soils, sampling can be conducted by pushing surface sterilised 15 cm soil cores into the soil and sampling from soil around fungal-infected insect cadavers. Samples are then collected and maintained at 5 ^{O}C prior to the next stages of fungal isolation (Goettel and Inglis, 1997). In comparison with other soil-inhabiting fungi like *Trichoderma* and *Rhizopus* species, EPF are generally slow competitors in soil and are heterogeneously distributed therein (Goettel and Inglis, 1997). However, the Warcup method may be employed using oatmeal agar (OMA) amended with 650 μg/ml of the fungicide dodine (*N*- dodecylguanidine monoacetate), to obtain a pure culture of EPF directly from soil (Goettel and Inglis, 1997). The fungicide is reported to work by suppressing quick growing soil borne fungi such as *Penicillium*, *Rhizopus* and *Trichoderma* species, while supporting the growth of fungal Hyphomycetes (Goettel and Inglis, 1997). These may then be differentiated visually, sub-cultured on to fresh media and viewed under a microscope after further incubation (Goettel and Inglis, 1997); Koch's postulates would then follow.

It was essential to include locally isolated strains of entomopathogenic fungi for evaluation in this investigation, to illustrate the ease by which EPF can be sourced locally for use in the development of bioinsecticides. As mentioned previously, in the search for entomopathogenic fungi, researchers must physically search and collect insect specimens exhibiting signs and/or symptoms of infection *in situ*, then attempt to isolate a pure culture and induce mycosis under laboratory conditions (Zimmermann, 1986; Goettel and Inglis, 1997). While such a survey would provide important scientific data, the planning and conducting of such an exercise may prove costly both with regards to finances and time (Zimmermann, 1986).

An alternative rapid method of obtaining naturally-occurring entomopathogenic fungi particularly Hyphomycetes, is the *Galleria* bait method (Goettel and Inglis, 1997), which is a technique used to harness broad-spectrum (opportunistic) insect infecting fungi directly from the soil (Zimmermann, 1986; Goettel and Inglis, 1997; Taylor, 2002). Although this method was originally devised for trapping entomopathogenic nematodes from within collected soil samples, it has been successfully adapted for the detection of naturally occurring soil borne entomopathogenic fungi by using the larvae of the greater wax moth, *Galleria mellonella* (Linnaeus) (Galleriinae: Pyralidae) (Zimmermann, 1986; Taylor, 2002; Dimbi *et al.*, 2004).

The search method undertaken for this project aimed to isolate specimens from entomopathogenic Hyphomycetes, for example *Beauveria* and *Metarhizium* species, directly from the soil; as this class of fungus has the widest spectra of host range among insect killing fungi (Goettel and Inglis, 1997; Shah and Pell, 2003). To this end the *Galleria* bait method was chosen as the technique most appropriate for the detection of such fungi. Three local strains of *Beauveria bassiana* (Balsamo) Vuillemin were isolated from *Galleria* larvae through soil baiting (section 2.2.1). An out-group entomopathogenic fungal strain of the genus *Metarhizium* was provided by Miss L. Nardini of the Nematode Pathology Laboratory at the University of the Witwatersrand. However, background information (outside it country of origin (Table 2.1)) on this isolate was lacking hence the irregular coding of Ma2pink. A further five exotic cultures of the entomopathogenic fungus *Beauveria bassiana* were provided by Dr. Mariena Aquino De Muro from CABI Biosciences in England, all denoted in Table 2.1 with the IMI code (Appendix 6). Background information with regards to these exotic isolates is also presented in Table 2.1 (section 2.2.1). These five *B. bassiana* isolates (IMI 386 694, IMI 386 696, IMI 386 701, IMI 382 302 and IMI 382 724) were used by De Muro *et al.* (2003) in a broad study investigating the genetic relatedness of 50 *Beauveria* strains sourced from different insect hosts and different geographic points of origin. Importantly, this group of exotic isolates includes three strains (IMI 386 701, IMI 382 302 and IMI 382 724) that were isolated from the maize weevil, one of the pests of interest in this study, and these strains originate from Kenya (De Muro *et al.*, 2003).

In their investigation using amplified fragment length polymorphism (AFLP), De Muro *et al.* (2003) found no obvious correlation between geographic point of origin and genetic similarity. The same was also observed when attempting to link insect hosts with fungal relatedness, which implies that isolates from the same host insect and region may not always necessarily cluster together when analysing their genetic sequences. While isolates of one species from one area and host may not be related, those isolated from different countries may be shown to be closely related genetically with high percentage similarity in genetic material (De Muro *et al.*, 2003).

The following section (2.2) provides descriptions on methods of sourcing, isolating and maintaining entomopathogenic fungi from South African soils through the use of the *Galleria* bait method. Key points in Chapter 3 were to investigate the effects of temperature on sourced isolates in terms of their radial growth rate across a range of temperatures and to determine a general optimal temperature for all isolates, so as to find a common temperature for pathogenicity based bioassays.

2.2 Materials and Methods

2.2.1 Sourcing of indigenous isolates through use of the *Galleria* bait method

The method published by Zimmermann (1986) (described here) was used to source local isolates of EPF from different cultivated fields around the towns of Brits and Rustenburg, in the North West province of South Africa (Figure 2.1, a and b).

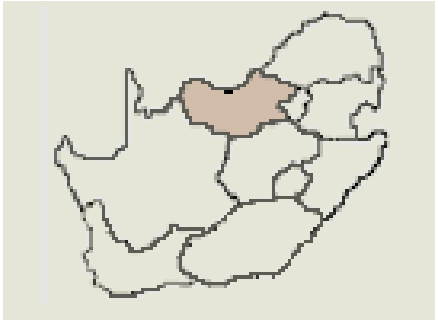


Figure 2.1: a- Map of South Africa highlighting North West Province

Figure 2.1: b- Map of North West Province, South Africa - highlighting towns around which soils were collected. (source: www.cyberprop.com/northwest/ Properties%20north)

Targeting cultivated agricultural lands, three samples per field from four fields (12 samples in total) were collected by shovelling soil to approximately three quarters full level into used clean plastic containers (1 litre). Containers were labelled with the site details *in situ*. In the laboratory, each soil sample was lightly moistened and ten pencil size holes, up to two centimetres deep were pushed into each sample, where individual larvae were to be placed. After insertion of live *Galleria* larvae, holes were lightly covered with soil and the containers incubated at 25 ^{O}C (Zimmermann, 1986). According to Zimmermann (1986) the incubation period required for the onset of visualisation of fungal pathogen symptoms is a one to two week period, however in this case samples were checked after 10 days. Soil in each sample was carefully turned seeking to remove all ten individual larvae, including any live larvae as yet uninfected. All cadavers were collected, and examined for cuticular fungal growth (Goettel and Inglis, 1997), and in two cases cadavers were observed to have such symptoms.

The two cadavers (from different Rustenburg fields) were then surface sterilized using three, 20 second rinses in 1% sodium hypochlorite followed by three, 15 second rinses in DSW (Zimmermann, 1986; Goettel and Inglis, 1997) and labelled MCBG121 and MCBG124. After drying on sterile absorptive paper for 24 hours, the two symptomatic cadavers were subjected to separate methods used to induce mycosis *in vitro*. One of the Rustenburg cadavers (MCBG121) was transferred to the centre of a 90 mm diameter Petri dish containing a semi-selective medium Sabouraud dextrose agar (SDA) + chloramphenicol (Appendix 1); this media suppresses bacterial growth/contaminants (Goettel and Inglis, 1997). The second cadaver (MCBG124) was transferred to the centre of a 90 mm diameter Petri dish containing a supplemented medium SDA + Yeast + Glucose (SDA + Y + G) (Appendix 1); enhancing the medium with carbon sources and other nutrients to aid fungal growth (Goettel and Inglis, 1997)

A third mycosed cadaver (Figure 2.2) obtained from a previous *Galleria* baiting excursion conducted around the town of Brits (North West Province; Figure 2.1) was provided by Professor Vince Gray and the biological control team at the University of the Witwatersrand.

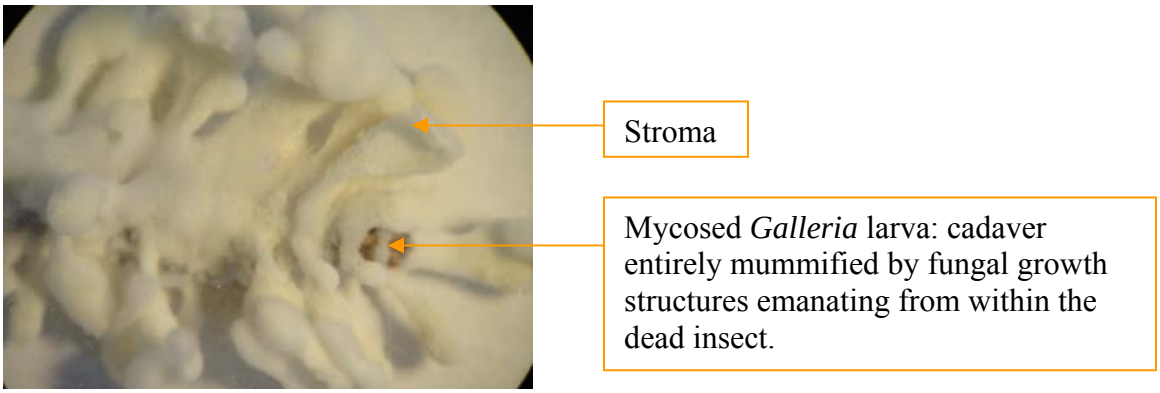


Figure 2.2 - Heavily mycosed *Galleria* larvae cadaver exhibiting stroma. EPF isolate PPRI 04305 (MCBG1).

To obtain a pure culture, spores were aseptically transferred directly from the stroma to fresh semi-selective media (SDA + chloramphenicol) (Appendix 1). All Petri dishes were then incubated at room temperature (20-25 ^{O}C) and observed daily with appropriate sub-culturing to SDA + Y + G commencing on the third day of incubation.

Results from the *Galleria* bait method yielded three indigenous fungal isolates identified by Elna van der Linde at the Biosystematics Division, Plant Protection Research Institute (PPRI) in Pretoria, South Africa as the entomopathogenic fungal species *Beauveria bassiana* (Balsamo) Vuillemin (Mitospora: Hyphomycetes) (Appendix 6). After these confirmations, the MCB codes used *in situ* were replaced with PPRI codes where the isolates were lodged in their Biosystematics Division Culture Collection. Below Table 3.1 shows all isolates used in this study indicating host insect and country of origin and in the case of the three local isolates number 1 represents what was MCBG1, 2 represents what was MCBG121 and 3 represents isolate MCBG124 all coded with the PPRI code.

Table 2.1 Sourced Entomopathogenic Fungi:

No.	Isolate Ref. Number	Host Insect	Country of Origin
1	PPRI 04305	Soil/*Galleria mellonella*	South Africa (Brits)
2	PPRI 04306	Soil/*Galleria mellonella*	South Africa (Rustenburg)
3	PPRI 04307	Soil/*Galleria mellonella*	South Africa (Rustenburg)
4	IMI 386 694	*Popillia japonica*	Portugal
5	IMI 386 696	*Diadrotica* sp.	Brazil
6	IMI 386 701	*Sitophilus zeamais*	Kenya (West 1S)
7	IMI 382 302	*Sitophilus zeamais*	Kenya (Kisii)
8	IMI 382 724	*Sitophilus zeamais*	Kenya (Uasin Gishu)
9	Ma2pink	Unknown	China

Note: Ma2pink- a *Metarhizium* sp. was included as an out-group specimen for the thermal tolerance evaluation. PPRI represents the PPRI code. IMI represents the CABI code Appendix 6. ( ) are regions of origin.

2.2.2 Maintenance of fungal cultures

Fungi were maintained so that both *in vivo* and *in vitro* cultures were stored.

In vivo culture: Koch's postulates pilot surveys provided the opportunity to maintain all isolates on maize weevil and tobacco beetle cadavers. Mummified wax moth larvae however provided the model for this form of culture storage acting as the principle tool for fungal maintenance on insect cadavers (Figure 2.3).

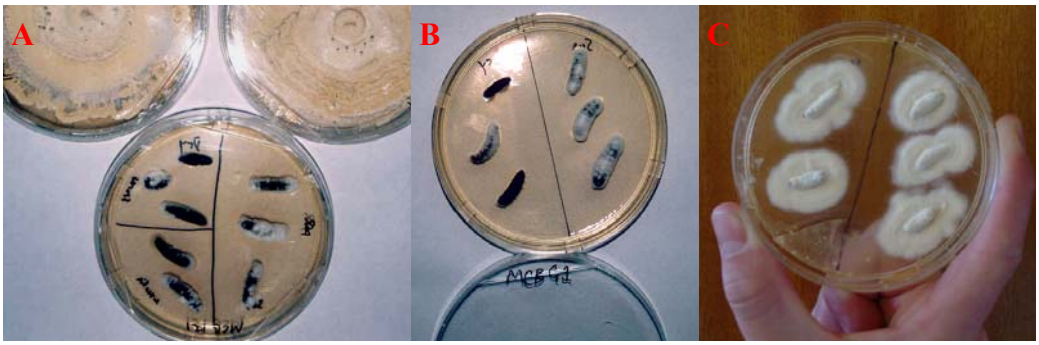


Figure 2.3 - *In vivo* maintenance of fungal cultures on Sabouraud dextrose agar plates using larvae of the greater wax moth, *Galleria mellonella*. A and B show fungus covered cadavers, 48-72 hours after death due to exposure to dry *B. bassiana* spores. C shows mycosed cadavers four weeks after death due to EPF infection (conidial mat spreading out of the mummified larvae on to media).

- Inoculation- live *Galleria* larvae were exposed directly to 15-day-old cultures (Figure 2.3 – A, top) (De La Rosa *et al.*, 2002) of the appropriate fungal isolate within sealed inoculum plates of dry conidia for between 5 and 10 minutes allowing the insects to be fully inundated with spores. After the inoculation period, larvae were transferred to sterile Petri dishes lined with hydrated filter paper.
- Incubation- Dishes were then sealed with Parafilm and incubated at 25 ^{O}C for 24 hours. Once this incubation period had expired larvae were transferred to fresh sterile Petri dishes containing five grams of wax moth larvae feed (Appendix 3) and plates were incubated at room temperature allowing time for disease development and insect demise (Zimmermann, 1986; Goettel and Inglis, 1997).

After the demise of all inoculated larvae, cadavers were surface sterilized using the methods of Zimmermann, (1986) (described in section 2.2.1 above), dried on sterile filter paper then transferred as follows to initially induce mycosis and acquire pure cultures-

i. Larvae transferred to fresh sterile Petri dish lined with damp sterile filter paper not sealed with Parafilm. Upon development of mycosis, plates were sealed and maintained at room temperature.
ii. Larvae transferred to Petri dishes containing SDA + Y + G. These plates were only sealed upon visualisation of cuticular fungal growth, after which time, some plates were maintained at room temperature and others were stored at 4 ^{O}C.
iii. Some cadavers were refrigerated after transfer into a sterile McCartney bottle.

In vitro culture: For maintenance of fungal isolates *in vitro*, fungal growth resulting from the method described above was aseptically transferred to SDA + Y + G plates and incubated at 25 ^{O}C allowing the fungus to cover the media. Some plates were then maintained at room temperature while others were transferred for storage at 4 ^{O}C (Adane *et al.*, 1996; De La Rosa *et al.*, 2002; De Muro *et al.*, 2003, De Muro, pers. comm.).

CHAPTER THREE

Thermal Tolerance Evaluation

3.1 Experiment to Determine the Effects of Temperatures on Radial Growth

3.1.1 Introduction

Environmental factors such as temperature, light radiation and humidity are important in determining fungal entomopathogenicity (Possee and King, 1994; Boucias and Pendland, 1998; Hallsworth and Magan, 1999; Dimbi *et al.*, 2004). Temperature is an abiotic limiting factor that directly affects the rates of sporulation, germination, mycelial growth and the survival of EPF and continually fluctuates throughout the day (Hong *et al.*, 2001; Dimbi *et al.*, 2004). Gauging the thermal tolerance range of fungal isolates thus makes up part of the criteria upon which researchers select fungal biological control agents prior to bioassays designed to challenge fungi with selected pests in order to evaluate the ability of entomopathogenic fungi to control insect populations under different temperatures (Hallsworth and Magan, 1999; Smits *et al.*, 2003; Dimbi *et al.*, 2004). In general, the optimal temperature for most fungi is considered to be in the range of 20 to 25 ^{O}C. The optimal temperature range for the more relevant entomopathogenic fungi is considered to be wider, between 15 and 25 ^{O}C (Smits *et al.*, 2003), with Hallsworth and Magan (1999) and Dimbi *et al.* (2004) reporting that the optimal range for maximum growth rates is between 20 and 30 ^{O}C. The optimal growth temperature for *Beauveria* is reported to be within the range of 23 to 25 ^{O}C and for *Metarhizium* within the range of 27 to 28 ^{O}C (Ferron, 1981; Dimbi *et al.*, 2004). Hallsworth and Magan (1999) reported a growth optima at 25 ^{O}C for isolates of *B. bassiana* used in their evaluations. Based on observations that fungi grow with their hyphae prostate to the nutrient source (Talbot, 1971; Boucias and Pendland, 1998), measuring vegetative radial growth on agar plates (Goettel and Inglis, 1997) was the selected method of assessing effects of various temperatures on vegetative growth using the method described by Dimbi *et al.* (2004).

This section presents results of an experiment designed to determine the effects of different temperatures on radial growth of sourced EPF. The aims were:

i. to determine an optimal temperature for maximum fungal growth rates for all isolates over the temperature range of 15, 20, 25, 28 and 30 ^{O}C
ii. to determine whether the isolates grow over a wide range of temperatures
iii. to gain data comparing growth between strains of *Beauveria* and the out-group *Metarhizium* species and
iv. to gain data on differences between growth rates of *B. bassiana* isolates originating from different geographic sub-regions.

These objectives would aid in further characterizing the fungi and provide information on similarities or differences between local and exotic isolates of *B. bassiana* (De Muro *et al.*, 2003; Dimbi *et al.*, 2004).

3.1.2 Materials & methods

Effects of temperature on growth of EPF are most easily assessed on media such as SDA in Petri dishes using colony diameter (Goettel and Inglis, 1997). The method of Dimbi *et al.* (2004) was adapted for this experiment and all fungal cultures (Table 2.1) were included in this assessment which was laid out in a complete randomised design (CRD) (Goettel and Inglis, 1997). Cultures were revived from 4 ^{O}C storage and to obtain a mycelial mat for each isolate, aliquots (0.25 ml) of conidial suspensions of approximately 0.1ml of 3 x 10^{8} conidia ml^{-1} were prepared and spread onto SDA plates (3 plates each), which were then incubated at 25 ^{O}C for 48 hours (Dimbi *et al.*, 2004). After this incubation period and using a 6 mm cork borer, plugs from each isolate were excised from the two day old culture plates and transferred singly to the centre of labelled fresh plates of SDA (90 mm) which were then sealed with Parafilm (Dimbi *et al.*, 2004). Each isolate was replicated four times, and incubated in a random layout, upside down in complete darkness at 15, 20 25, 28 and 30 ^{O}C (Dimbi *et al.*, 2004). Radial growth was recorded daily in millimetres (mm) commencing 48 hours after placement into incubators, for 11 days using two cardinal diameters through two orthogonal axes

previously drawn on the bottom of each SDA plate (Goettel and Inglis, 1997; Dimbi *et al.*, 2004).

3.1.3 Data analysis

Raw data were regressed (using Microsoft Excel) to obtain a slope for each of the replicates from which growth rates were determined over the 11 day incubation period (Table 3.1). To determine the optimal temperature for overall maximum fungal growth rates for all isolates, one-way ANOVA (SAS Enterprise Guide 3.0 software) was used. For differences between temperatures and for the interactions between isolates and temperature a generalised linear model procedure (two-way ANOVA) was used. In this case post ANOVA data were separated using Students-Newman-Keuls (SNK) multiple comparison test (Dimbi *et al.*, 2004).

3.2 Results and Discussion

Owing to observations that environmental temperatures fluctuate and that different fungi grow optimally within a wide range of temperatures rather than at one specific temperature (Ferron, 1981; Dimbi *et al.*, 2004), means of radial growth were combined (all isolates by temperature) to obtain the optimal temperature at which most isolates grew. It was important to detect one general optimal temperature for fungal growth as this would be the temperature at which pathogenicity bioassays would be conducted.

Fungi evaluated grew at all temperatures and vegetative radial growth rates were significantly affected by temperature ($F = 255.10$; $P = <.0001$) and by isolate ($F = 85.67$; $P = <.0001$) with significant two-way interactions of isolate by temperature ($F = 20.09$; $P = <.0001$) (Appendix 2.1). The rate of growth for all *B. bassiana* isolates was at its slowest at 15 ^{O}C (Table 3.1). The optimal temperature for maximum combined radial growth for all fungi was 25 ^{O}C, although two isolates (PPRI 04307 and IMI 386 701) had faster growth rates (slope) at 28^{O}C and one isolate (IMI 386 696) had a faster growth rate

at 20 ^{O}C than at 25 ^{O}C. Combined average growth was 3.0 mm d^{-1} at 25 ^{O}C which was significantly (F = 30.51; *P* = < .0001) faster than that produced at all other temperatures.

Differences in radial growth rates between local and exotic strains of *B. bassiana* were detected where data reveals significant (*P*< 0.0001) differences through analysis of variance using a post ANOVA test (SNK multiple comparison test) to separate means. These differences do not however suggest commonality between sub-regions and rate of growth as no pattern emerges. De Muro *et al.* (2003) similarly found no patterns when assessing genetic relatedness between isolates from similar geographic origins, although a much smaller sample size was used in this case. Table 3.1 shows the overall growth rates (mm d^{-1}) over the incubation period across all temperature ranges and illustrates that IMI 382 302 had the fastest daily growth rate of 3.02 mm day^{-1}. This rate was significantly (*P*< 0.0001) faster than all other isolates (Table 3.1) across all temperatures. PPRI 04305 had a significantly (*P*< 0.0001) faster growth rate than the other two local isolates and was also significantly (*P*< 0.0001) faster than all other exotic isolates of *B. bassiana* apart from IMI 382 302. No differences were detected between local isolates PPRI 04306 and PPRI 04307 (Table 3.1).

It was found that the out-group fungus grew at a significantly (*P*< 0.0001) faster rate then two of the local *B. bassiana* isolates PPRI 04306 and PPRI 04307 and four of the exotic *B. bassiana* isolates (IMI 382 724, IMI 386 701, IMI 386 696 and IMI 386 694). The *Metarhizium* species grew optimally at 25 ^{O}C with a maximum growth rate of 3.96 mm d^{-1}. Unlike all *Beauveria* isolates, the out-group isolate grew the slowest at the upper end of the temperature range of 30 ^{O}C. This result is inconsistent with reports by Hallsworth and Magan (1999) who found that *M. anisopliae* has a growth optimum at 30 ^{O}C.

Table 3.1: Effect of Temperatures on Radial Growth Rate (mm d^{-1}) of Nine EPF Isolates Cultured on SDA Media.

Rate of EPF Growth (mm d^{-1})						
Temperature	15^{O}C	20^{O}C	25^{O}C	28^{O}C	30^{O}C	
Isolate						
IMI 382 302	2.21 ±0.03	3.29 ±0.07	3.70 ±0.15	2.90 ±0.03	2.97 ±0.04	****3.02 ± 0.12 ^{A}**
PPRI 04305	2.07 ±0.03	2.69 ±0.06	3.65 ±0.15	3.26 ±0.00	2.72 ±0.07	****2.88 ±0.13 ^{B}**
Ma2pink	2.41 ±0.11	3.01 ±0.05	3.96 ±0.05	2.85 ±0.01	1.34 ±0.08	****2.71 ± 0.20 ^{C}**
IMI 382 724	1.23 ±0.05	2.55 ±0.24	3.42 ±0.13	3.07 ±0.18	2.50 ±0.11	****2.38 ± 0.18 ^{D}**
IMI 386 701	1.62 ±0.06	2.04 ±0.13	2.67 ±0.08	3.16 ±0.25	2.41 ±0.13	****2.55 ± 0.13 ^{E}**
IMI 386 696	2.17 ±0.04	2.72 ±0.07	2.31 ±0.05	2.27 ±0.10	2.27 ±0.03	****2.35 ± 0.05 ^{E}**
IMI 386 694	0.88 ±0.06	1.42 ±0.03	2.89 ±0.10	2.70 ±0.06	2.28 ±0.05	****2.04 ± 0.18^{F}**
PPRI 04307	1.21 ± 0.03	1.68 ± 0.06	2.31 ±0.02	2.49 ±0.01	2.26 ±0.04	****1.99 ± 0.11 ^{F}**
PPRI 04306	1.25 ± 0.04	1.71 ± 0.15	2.25 ± 0.11	2.20 ±0.06	2.09 ±0.11	****1.90 ± 0.1 ^{F}**
	***1.7 ±0.1 ^{D}**	***2.3 ± 0.1 ^{C}**	***3.0 ± 0.11 ^{A}**	***2.8 ± 0.07^{B}**	***2.3 ± 0.08^{C}**	**Means**

Values represent means of slope data of daily radial growth (mm d^{-1}). Means with same letter are not significantly different by SNK test ($P< 0.0001$). Data marked * represents combined average growth rate of all isolates by temperature over 11 day period. Data marked ** represents average growth rates of each isolate measured across the range of temperatures in mm d^{-1} over 11 day period. Means (±S.E.).

In summary, the exotic *Beauveria* IMI 382 302 was the fastest growing isolate over the 11 day period, with local strain PPRI 04306 growing the slowest (Table 3.1). Not all local strains shared a similar rate of vegetative radial growth, and the same pattern was observed with three *Beauveria* isolates indigenous to Kenya, where isolate IMI 382 302 grew significantly ($P< 0.0001$) faster than the other two Kenyan isolates, IMI 386 701 and IMI 382 724. In this case, the other two Kenyan isolates grew at significantly ($P< 0.0001$) different rates to each other across all temperature over the 11 day incubation period. Unlike the South African isolates, all three Kenyan strains originated from different geographic locations. While assessing the effects of temperature on vegetative growth of EPF from different origins, Vidal *et al.* (1997), cited in Dimbi *et al.* (2004), demonstrated that the ability of isolates to tolerate low or high temperatures is related to climatic data of their geographic origin. To better assess differences in vegetative growth at various temperatures between fungal isolates more information on climatic data is

required. In this study only information on sub- region and country was available, but it can be concluded from the results that all isolates grew within the range tested and that they grew optimally between 20 ^{O}C and 28 ^{O}C. This range of temperatures is similar to that obtained by other researchers including Ferron, (1981), Hallsworth and Magan, (1999) Dimbi, *et al.* (2004). A larger sample size is also required to assess differences between isolates from different geographical regions. In conclusion, this experiment provides vital information in terms of elucidating the optimal temperature (25 ^{O}C) at which most *Beauveria* isolates grew and this was to be the temperature at which isolates were evaluated at when challenging the EPF against the two selected insect pests (Chapter 4).

CHAPTER FOUR

Pathogenicity of *Beauveria bassiana* Against the Maize Weevil (*Sitophilus zeamais*)

4.1 Introduction

Insight to the biology of the maize weevil indicates that the adult stage of the pest's life cycle is the most appropriate to challenge with entomopathogenic fungi (EPF) (Longstaff, 1981; Adane *et al.*, 1996). Adane *et al.* (1996) used cumulative percentage mortality during their assessment and observed that the most virulent isolates caused the earliest deaths on the third day. Here a four day incubation period was chosen as the time between initial inoculation/incubation and assessments for mortality owing to observations that, highly virulent strains of EPF can cause high levels of mortality commencing as early as three days post inoculation (Adane *et al*., 1996; De La Rosa *et al.*, 2002; Dimbi *et al*., 2004).

Isolates of *Beauveria bassiana* were previously and successfully evaluated *in vitro* against the maize weevil (*S. zeamais*) (Adane *et al.*, 1996). Isolates of this entomopathogenic fungus were also obtained from maize weevil cadavers collected *in situ* (Oduor *et al.*, 2000), illustrating an association of this insect pest with the entomopathogen *B. bassiana* in nature. This chapter outlines procedures, results and brief discussions used to evaluate pathogenicity and virulence of sourced *B. bassiana* isolates (Table 4.1), using water formulations and dry spores against local cultures of the insect pest. The aims of this study dictate evaluations using strains of EPF that are indigenous to South Africa in comparative bioassays with exotic strains. All *B. bassiana* isolates in Table 4.1 (section 4.2.2) were evaluated at 25 ^{O}C, with attempts to first gauge pathogenicity against the pest and then establish patterns with respect to pathogenicity and geographic origin and original host insect.

For virulence assessments under varying conditions inclusive of temperature and different conidial concentrations, bioassays using local isolates were designed to gain

information on their effects on the target pest. In these cases (sections 4.2.4 and 4.2.5) fungi were suspended in water formulations, containing Tween 80 (Sigma), which were used to deliver spores to the pest. Departing from the inoculation techniques used by Adane *et al.* (1996), adult insects in these cases were immersed in the inoculum rather than sprayed on with perfume sprayers. This was to provide more evenly coated spread of spores onto the weevils (De La Rosa *et al.*, 2002). It is considered imperative to deliver as precise a dose as possible under controlled conditions for more accurate, repeatable bioassay results (Goettel and Inglis, 1997; De La Rosa *et al.*, 2002).

In the case of the experiments designed to evaluate the effects of different conidial concentrations to determine dose response, (Adane *et al.*, 1996; De La Rosa *et al.*, 2002) local isolate PPRI 04307 was evaluated (section 4.2.4). For the experiment to determine the effects of various temperatures on fungal virulence (Dimbi *et al*., 2004) local isolate PPRI 04306 was selected for use (section 4.2.5). An evaluation of dry conidia on maize weevil mortality was also included in this study (section 4.2.6), and in this case the potential of two locally isolated strains of *B. bassiana* was gauged in a comparative bioassay with an exotic isolate.

4.2 Materials and Methods

4.2.1 Maintenance and cultures of experimental organisms

Insects - Maize weevil breeding cultures were kindly supplied by Mr. Frikkie Kirsten and his team at the Stored Grain and Oil Seed Research Unit, Plant Protection Research Institute (PPRI) in Roodeplaat, South Africa. Insects were reared on yellow maize seed (Appendix 3) in two litre glass jars sealed with perforated lids for aeration. Growth room temperatures were maintained at 30 ^{O}C with a 12 hour light and dark cycle. These conditions mimicked those from where the culture originated.

To obtain samples of adult insects for inoculation and owing to the pest's loose integration with the food source matrix it was easy to separate and obtain a robust multi-aged random sample by sieving through a 2 mm aperture laboratory test sieve. While

Adane *et al.* (1996) used insects of specific ages, here it was necessary to obtain a mixed sample as non-uniform populations are found *in situ* (D. Moore, pers. comm.). Selections of adult insect samples for inoculation from the main sieved populations (a single jar) were therefore conducted through an innovative approach using a lure designed for these purposes (Figure 4.1). This procedure aimed to select for a random robust sample of fit adult insects capable of wandering (D. Moore, pers. comm.) and the sampling method of sieving allowed for all fit individual insects an equal chance of being selected; this is termed probability sampling.

Figure 4.1 - Innovative Lure dubbed the MNS Tower– for selection of live adult insects from sieved population. A- Assembled tower with sieved insects in 2, the containment zone. B- View from the top showing wandering individuals gathering in 1, the collection chamber.

After sieving, insects were transferred to the containment zone of the MNS Tower (Figure 4.1; A-2) with the collection chamber then being placed over the containment zone as illustrated above. The tower base has six 1cm diameter holes at its base through which fit adults may crawl up through into the collection chamber. Once MNS Tower held sufficient numbers of adults (after a 2-3 hour standing time) the tower was transferred to 4 ^{O}C to immobilize insects for inoculation.

Fungi - The out-group fungus used in the previous chapter was omitted from these pathogenicity evaluations owing to the lack of information on the fungus and because this

isolate did not produce conidia on SDA. *B. bassiana* isolates were revived from *in vivo* storage by aseptic transfer of conidia to fresh media (SDA plates) which were then incubated at 25 ^{O}C in the dark for 8-10 days allowing conidial plugs to develop.

It was necessary to suspend the infectious propagules uniformly by mechanical means because *B. bassiana* possesses hydrophobic cell walls (Goettel and Inglis, 1997). Fungal plugs were excised from the media and macerated in 9 ml distilled sterile water + 0.5% Tween 80 in standard 15 ml McCartney bottles containing glass beads and filtered through a 75 μm sieve (Adane *et al.*, 1996). Aliquots (0.25 ml) of the suspensions were spread plated on to fresh SDA plates (2-3 per isolate) which were incubated upside down in the dark for 15 days (De La Rosa *et al.*, 2002) at 25 ^{O}C. Resultant conidial mats were macerated in 30 ml distilled sterile water + 0.5% Tween 80 in 80 ml graduated bottles containing glass beads, and sieved as above then adjusted to 10^{8} conidia ml^{-1} using a Neubauer haemocytometer (Adane *et al.*, 1996; Goettel and Inglis, 1997). All water formulations were stored at 4 ^{O}C prior to use within three days (Adane *et al.*, 1996; De La Rosa *et al.*, 2002).

Spore viability assessments were conducted prior to bioassays using the procedure of Dimbi *et al.* (2004) (described below); however percentage germination data are not presented owing to observations that all initial checks resulted in between 90 and 100% germinating conidia (Figure 4.2.). To determine spore viability (through germination assessments) (Anderson and Roberts, 1983) aliquots (0.25 ml) of three replicates of each isolate were spread onto fresh SDA + Y + G plates. Three sterile cover slips were then placed onto the media covering the fungal spread. Plates were sealed with Parafilm and incubated in the dark at 25 ^{O}C for 24 hours (Dimbi *et al.*, 2004). Spore viability was determined (for all bioassays) by observing the area under the cover slips using a light microscope at 40x magnification, where germinated spores with germ tubes longer then the spore body were counted as viable (Dimbi *et al.*, 2004; Lazzarini *et al.*, 2006), as illustrated in Figure 4.2.

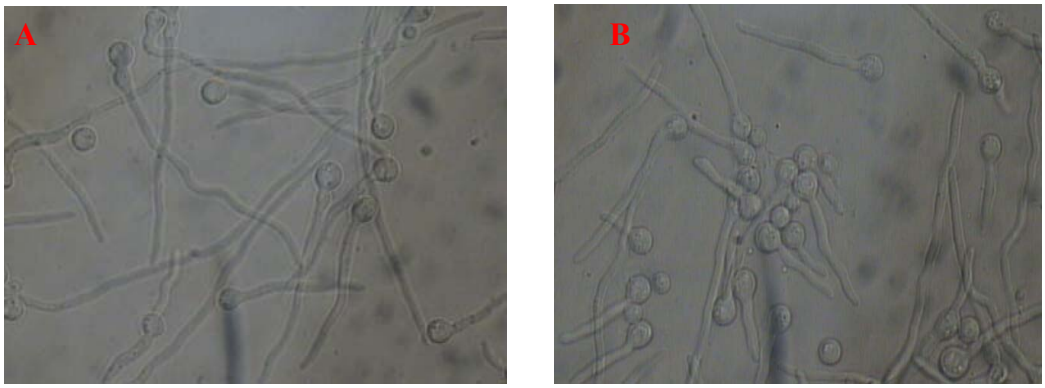


Figure 4.2 - Example of germinated conidia of *B. bassiana* 24 hours post inoculation (spread plate) to SDA and incubation at 25^{O}C for 24 hours. A represents isolate PPRI 04305, and B represents PPRI 04306. (Spores viewed at X400 magnification, light microscope.).

4.2.2 Preliminary bioassays

The use of a standard conidial concentration was employed to inoculate adult maize weevils with all sourced *B. bassiana* isolates for the preliminary bioassays (Adane *et al.*, 1996; De La Rosa *et al.*, 2002). The experiments aimed to inundate the target pest with water formulations of 10^8 conidia ml^{-1} of the fungal pathogens to assess their pathogenicity *in vitro*. Insects within control plots were treated with distilled sterile water (DSW) + 0.5% Tween 80 suspensions containing no fungal spores (Adane *et al.*, 1996; Castillo *et al.*, 2000). Table 4.1 highlights the treatments (isolates) used, their countries of origin and the host insect from where the isolate was obtained.

Treatments were randomly assigned and inoculated so that each treatment contained four replicates of 20 adult insects each. Departing from the procedure of Adane *et al.* (1996) adults were inoculated, under a laminar flow cabinet (De La Rosa *et al.*, 2002), by immersion in 0.25 ml of the appropriate suspensions in 24 Well Cell Culture Cluster packs (Corning Incorporated Costar) for up to 10 seconds. Contents of individual wells were then transferred to appropriate sterile Petri dishes lined with dry-sterile filter paper, sealed with Parafilm and incubated in the dark at 25 ^{O}C for four days maintaining the randomized layout (Goettel and Inglis, 1997).

Table 4.1: *B. bassiana* Isolates Tested Against Adults Maize Weevils

Isolates	Culture Storage Method	Original isolation
1. PPRI 04305	In 4^{O}C on *G. mellonella*	South Africa - *G. mellonella*
2. PPRI 04306	In 4^{O}C on *G. mellonella*	South Africa - *G. mellonella*
3. PPRI 04307	In 4^{O}C on *G. mellonella*	South Africa - *G. mellonella*
4. IMI 382 302	In 4^{O}C on *G. mellonella*	Kenya - *S. zeamais*
5. IMI 382 724	In 4^{O}C on *G. mellonella*	Kenya - *S. zeamais*
6. IMI 386 701	In 4^{O}C on *G. mellonella*	Kenya - *S. zeamais*
7. IMI 386 694	In 4^{O}C on *G. mellonella*	Portugal - *Popillia japonica*
8. IMI 386 696	In 4^{O}C on *G. mellonella*	Brazil - *Diabrotica* sp.

Note: PPRI codes represent isolates identified by the Plant Protection Research Institute. IMI codes represent isolate from CABI Biosciences.

Mortality assessments were conducted on the fourth day post inoculation. After these assessments, and to confirm an entomopathogenic infection, all dead insects were stimulated to mycose after surface sterilization using the modified method of Zimmermann (1986) (section 2.2.1) where cadavers were incubated under conditions of high relative humidity (De La Rosa *et al.*, 2002). The appearance of mycelial growth was the base line criterion used after a 48 hour incubation period (Adane *et al.*, 1996; Castillo *et al.*, 2000; De La Rosa *et al.*, 2002). For all bioassays (including those following below) the criterion for death was gauged by a visual comparison between immobile insects and mobile insects in control plots.

4.2.3 Scanning electron microscopy (SEM)

Resultant mycosed maize weevil cadavers were prepared for SEM by mounting specimens onto single aluminium stubs. Prior to microscopy, samples were coated while on the mount with gold palladium. A JEOL JSM-840 scanning microscope was used which is a thermal emission scanning electron microscope. The accelerating voltages ranged from 1 to 40kv and the measured beam current ranged from 10pA to 600pA.

4.2.4 Effects of different conidial concentrations on fungal virulence

Local isolate PPRI 04307 was selected for this particular experiment owing to its aggressiveness against the maize weevil as observed in results from the preliminary bioassay. For this bioassay, a water formulated inoculum was prepared as above using a 15 day old culture grown on SDA+Y+G and serially diluted to the following conidial concentrations 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8conidia ml^{-1}. The underlining aim was to observe the dose response of different conidial concentrations on insect mortality after a four day incubation period. It would further be possible to determine the concentration at which 50% of the adult maize weevils died after the specified incubation period (De La Rosa *et al.*, 2002).

Treatments were randomly assigned and inoculated so that each treatment contained four replicates of 20 adult insects each (Adane *et al.*, 1996). Adult weevils were inoculated by immersion in aliquots (0.25 ml) of the appropriate conidial concentration in 24 well cell culture cluster packs for up to 10 seconds, with control plots being inoculated with distilled sterile water + 0.5% Tween 80. Contents of individual wells were then transferred to sterile Petri dishes lined with dry-sterile filter paper, sealed with Parafilm under a laminar flow cabinet (De La Rosa *et al.*, 2002) and incubated in the dark at 25 ^{O}C for four days maintaining the complete randomized layout, after which time mortality assessments were conducted. All dead insects were surface sterilized as in section 3.1.2 (Goettel and Inglis, 1997) and stimulated to mycose to further confirm an EPF infection, where the appearance of mycelial growth was the base line criterion used after a 48 hour incubation period (Zimmermann, 1986; Castillo *et al.*, 2000; De La Rosa *et al.*, 2002). For this experiment, micrographs illustrating differences in mycosis patterns resulting from the different conidial concentrations obtained after five days of incubation within moist dishes at 25 ^{O}C (Zimmermann, 1986; De La Rosa *et al.*, 2002) are also presented.

4.2.5 Effects of temperature on fungal virulence

For this bioassay, local isolate PPRI 04306 was used to assess the effects of different temperatures on the ability of the fungal pathogen to infect inoculated adult maize weevils (Dimbi *et al.*, 2004). Although this isolate did not achieve the fastest rates of radial growth (Chapter 3) or confer maximum mortalities in preliminary bioassays, researchers may use a number of different criteria to select isolates for evaluation (Adane *et al.*, 1996; D. Moore, pers. comm.). In this particular case, interest lay in evaluating a local isolate at various temperatures, further more this local isolate grew optimally at 25 ^{O}C. It was postulated that this isolate would confer the highest levels of percentage mortality at the temperature it grew optimally.

Inoculum was prepared as above (section 4.2.1) using a 15 day old culture which was adjusted to 7.6 x 10^7 conidia ml^{-1} prior to inoculations. This conidial concentration was shown to confer high percentage mortality as observed in results from section 4.2.4 presented below in section 4.3.3. Treatments were randomly assigned and inoculated so that each treatment contained four replicates of 20 adult insects each. Adult weevils were inoculated under a laminar flow cabinet (De La Rosa *et al.*, 2002), by immersion in 0.25 ml of the 7.6 x 10^7 conidia ml^{-1} suspension in 24 well cell culture cluster packs for up to 10 seconds. Control plots were treated using the same method where insects were immersed in 0.25 ml of DSW + 0.5% Tween 80, as the spore free inoculum for up to 10 seconds (Adane *et al.*, 1996; Castillo *et al.*, 2000). Contents of individual wells were transferred to sterile Petri dishes lined with sterile-dry filter paper, sealed with Parafilm, and then incubated in the dark in incubators set to 15, 20, 25, 30 and 37 ^{O}C for four days prior to assessments for mortality maintaining a randomized layout. Mortality assessments were conducted after incubation. All dead insects were surface sterilized and allowed to mycose to further confirm an entomopathogenic infection, with the criteria for mycosis being the appearance of mycelial growth on cadavers (Zimmermann, 1986; Adane *et al.*, 1996; Goettel and Inglis, 1997; Castillo *et al.*, 2000; De La Rosa *et al.*, 2002).

4.2.6 Ability of dry spores from cadavers to infect live weevils

Typically, the transfer of fungal spores between live insects is termed autodissemination, where insects are attracted by pheromone or baited lures containing entomopathogens (Dimbi *et al.*, 2003; Shah and Pell, 2003). Insects are then infected within the lure by the EPF, and upon returning to their mates, spread the disease agent (Furlong and Pell, 2001, Dimbi *et al*, 2003; Shah and Pell, 2003). Furlong and Pell (2001) indicated that their test insect (*P. xylostella*) could not work as an effective vector for *B. bassiana* conidia under realistic conditions and that transmission rates of conidia from cadavers were low. However, they also observed that dislodged conidia of the fungus could go on to infect clean, uninoculated individuals within substrates on which insects were incubated (Furlong and Pell, 2001). In this study to evaluate effects of dry spores, mycosed maize weevil cadavers were used as the carrier to deliver infectious propagules to live weevils as described below. This experiment aimed to illustrate that *B. bassiana*-mycosed-cadavers can cause disease where spores emerging from mycosed cadavers are dislodged to infect live, wandering individuals and that mycosed cadavers may be used as carriers for infectious spores (Furlong and Pell, 2001). Although moisture availability is an important factor in the infection process, Adane *et al.* (1996) demonstrated that high mortality could by attained without the requirement of high humidity in their *in vitro* evaluations of *B. bassiana*. Dimbi *et al.* (2003) also demonstrated how dry conidia of EPF could be used to infect adult fruit flies, where flies were contaminated by using velvet carpet material impregnated with conidia.

In this case, mycosed cadavers from the preliminary bioassays were used as the fungal spore carrier and the procedure involved the use of two local (PPRI 04306 and PPRI 04307) and one exotic (IMI 386 701) strain of *B. bassiana*. To determine conidial concentrations of *B. bassiana*-mycosed-weevils, 10 mycosed maize weevil cadavers were macerated and vortexed in 9ml DSW + 0.5% Tween 80 in standard 15 ml McCartney bottles containing glass beads and filtered through a 75 µm sieve (Adane *et al.*, 1996). With the use of a Neubauer haemocytometer, conidial production was found to be 2.0 x 10^6 conidia ml^{-1} for PPRI 04306, 2.1 x 10^6 conidia ml^{-1} for PPRI 04307 and 1.6 x 10^6

conidia ml^{-1} for IMI 386 701 (Goettel and Inglis, 1997; Furlong and Pell, 2001). To verify that spores were viable, resultant suspensions were spread plated on to SDA + Y + G as described above (section 4.2.1) using the method of Dimbi *et al.*, (2004). It was found that all isolates were viable and germinated at over 90%. A complete randomized design of 40 insects per replicate with four replicates per treatment was applied so that each replicate laid out in sterile Petri dishes lined with sterile-dry filter paper contained 10 mycosed weevil cadavers (Figure 4.7; A), 10 grams intact maize seed and 40 live maize weevil adults.

For control plots, adult weevils which had died naturally (collected from the containment zone of the MNS Tower) were used in place of mycosed cadavers. All Petri dishes were sealed with Parafilm and incubated in the dark at 25 ^{O}C for 30 days prior to assessments for mortality and reduction in maize seed weights. Here a 30 day incubation period was chosen owing to an observation by Furlong and Pell, (2001) who reported a low transmission of *B. bassiana* conidia from *Plutella xylostella* larvae. In this case, the criterion of death was gauged by lightly nudging insects with a sterile needle tip for any sign of life as well as comparisons with insects from control plots.

4.2.7 Data Analysis

Data were analyzed using one-way analysis of variance (ANOVA) (Adane *et al.*, 1996; Goettel and Inglis, 1997; Castillo *et al.*, 2000). Analysis was performed on percentage mortality and percentage mycosis data transformed by Acrsin transformations (Goettel and Inglis, 1997; De La Rosa *et al.*, 2002; Dimbi *et al.*, 2003). For the preliminary bioassay (section 4.2.2), analysis was conducted on combined data from two experiments. Except for experiments to determine the effects of temperature on fungal virulence (section 4.2.5) and the ability of dry spores from cadavers to infect live weevils (section 4.2.6), no statistical analysis included control plot data, as these treatments did not result in any insect mortality. Means for mortality and mycosis were separated using the post ANOVA Student-Newman-Keuls (SNK) multiple comparisons test (Dimbi *et al.*, 2004). Analysis was carried out using SAS Enterprise Guide 3.0 software.

4.3 Results and Discussion

4.3.1 Preliminary bioassays

Data from an experiment designed to assess the effects of aqueous suspensions of the entomopathogenic fungus *Beauveria bassiana* against the adult growth stage of the maize weevil, *Sitophilus zeamais* are presented in Table 4.2 below. No mortality occurred in any of the replicates within the control plots from either bioassay and those data were excluded from statistical analysis.

Table 4.2: Maize Weevil Mean Percentage Mortality and Mycosis from Inundative Water Formulation Inoculations of *B. bassiana*.

Isolate Code	Mean Mortality (%)	Mean Mycosis (%)
PPRI 04307	100 ±0 ^{A}	100 ±0 ^{A}
PPRI 04305	98 ±0.06 ^{A}	98 ±0.06 ^{A}
IMI 386 701	98 ±0.06 ^{A}	98 ±0.06 ^{A}
IMI 382 302	96 ±0.08 ^{A}	96 ±0.08 ^{A}
IMI 386 696	90 ±0.15 ^{AB}	90 ±0.15 ^{AB}
IMI 386 694	90 ±0.14 ^{AB}	90 ±0.14 ^{AB}
IMI 382 724	85 ±0.13 ^{AB}	85 ±0.13 ^{AB}
PPRI 04306	81 ±0.09 ^{B}	81 ±0.09 ^{B}

Note: Within column means with the same letter are not significantly different. Post ANOVA Student-Newman-Keuls (SNK) multiple comparison test for arcsin transformed data. Values represent means (± = SE) of 4 replicates of 20 insects each. (Appendix 2.2).

All isolates evaluated showed pathogenicity to adult maize weevils when inoculated as aqueous suspensions of 10^{8}conidia ml^{-1}, after an incubation period of four days in the dark at 25 ^{O}C. For analysis of percentage data, data were Arcsin transformed which produced significant differences ($F = 3.52$; $P = 0.0033$) between means of both percentage mortality and percentage mycosis where the SNK post ANOVA test was used to separate means. Percentage mortality ranged from 81 to 100% with an overall mean of 92% mortality for all isolates. A similar range was obtained for percentage mycosis (Table 4.2). Thus, all dead insects mycosed confirming that the EPF was responsible for the demised of inoculated insects (Adane *et al.*, 1996; De La Rosa *et al.*, 2002).

These results demonstrate that this particular target pest is highly susceptible to *B. bassiana* and that all isolates evaluated here are highly virulent against local cultures of the maize weevil when the above specified conditions are applied. These findings are consistent with those obtained by Adane *et al.* (1996) where similarly high levels of pathogenicity were observed when using a 10^8 conidia ml^{-1} conidial concentration to inoculate the target pest. While fungal Hyphomycetes confer high levels of mortality to insects inoculated *in vitro*, the literature shows that variability between isolates may occur when differences are being evaluated, with these differences being dependent on the targeted insect (Adane *et al.*, 1996; Dimbi *et al.*, 2003). Dimbi *et al.* (2003) observed no differences when evaluating pathogenicity of *M. anisopliae* and *B. bassiana* against the fruit fly, *Ceratitis cosyra*. However, in the same set of experiments targeting different species of fruit fly, *C. capitata* and *C. rosa*, significant difference between evaluated isolates were observed (Dimbi *et al.*, 2003).

In their evaluations, Adane *et al.* (1996) were able to rank their isolates according to levels of virulence against the pest using percentage cumulative mortality over 11 days. Isolates were viewed as conferring low, intermediate or high virulence against the maize weevil (Adane *et al.*, 1996). Although isolates tested here were all highly virulent, conferring an average of 92%, they may also be loosely ranked in a similar manner using mean percentage mortality after a four day incubation period; however, unlike Adane *et al.* (1996) it is not possible to link degree of virulence to the origin of the isolate.

Based on the SNK groupings, local isolate PPRI 04306 from Rustenburg soils, was found to cause the lowest mean percentage mortality of 81%, ($P = 0.0033$), and this isolate may be ranked in the low virulence category. Isolates exotic to South Africa make up the intermediate category of virulence where Brazilian isolate IMI 386 696, Kenyan isolate IMI 382 724 and Portuguese isolate IMI 386 694 conferred 90%, 90%, and 85% mortality respectively (Table 4.2). The category for highly virulent isolates includes two local and two exotic strains; the two remaining Kenyan isolates, IMI 386 701 and IMI 382 302, join local strains PPRI 04305 and PPRI 04307 all conferring between 96 and 100% mortality after a four day incubation period (Table 4.2).

All three local South African isolates were sourced from the same province, with two strains, PPRI 04306 and PPRI 04307, isolated from around the town of Rustenburg, but PPRI 04307 conferred 100% mortality and is ranked highly virulent, while PPRI 04306 is ranked in the low virulence category having produced 81% mortality. PPRI 04305 from the same province but isolated from around the town Brits also falls into the high virulence category. This suggests a lack of any relationship between pathogenicity and geographic origin of the test isolates. The same was observed with the three isolates from Kenya with IMI 386 701 and IMI 382 302 falling in the high virulence category and IMI 382 724 falling in the intermediate category. All three Kenyan isolates were sourced from different geographical regions (De Muro, pers. comm.) (Appendix 6). From the four isolates ranked here as highly virulent, two Kenyan isolates (IMI 382 302 and IMI 386 701) were originally isolated from maize weevil cadavers, but the other Kenyan isolate (IMI 382 724) which is ranked in the intermediate category was originally isolated from *S. zeamais* as well. From the results presented above, there appears to be no pattern linking isolate pathogenicity to either the original host insect or to the country of origin.

No control plot insects died after DSW + Tween 80 (fungus-free) inoculations and the four day incubation period showing that the sample selection procedures devised for this project were adequate and that fungus-treated insects died as a result of fungal infection. Moreover, as noted above, all dead insects mycosed to produce spore-covered cadavers further confirming an entomopathogenic interaction (Adane *et al.*, 1996; Castillo *et al.*, 2000; De La Rosa *et al.*, 2002). Results from the preliminary bioassays showed that local isolates of *B. bassiana* conferred similar and better mortality levels when compared with exotic isolates and for this reason, local isolates were selected for further evaluation (sections 4.3.3, 4.3.4, and 4.3.5).

The following section presents SEM imagery illustrating fungal growth on maize weevil cadavers killed due to infection by *B. bassiana*.

4.3.2 Scanning electron microscopy (SEM)

These images illustrate how the fungus emerges from the insect, confirming that the live individuals met their demise due to infection by *B. bassiana*.

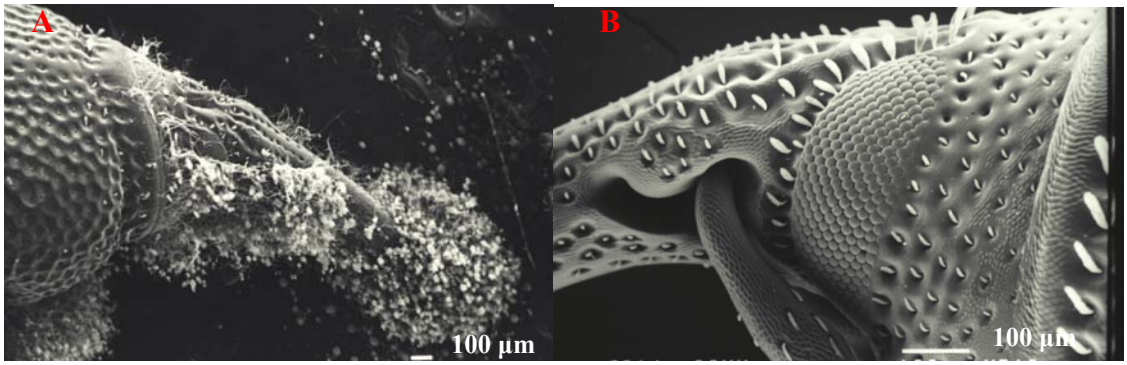


Figure 4.3 – Scanning electron micrographs of the maize weevil (*S. zeamais)* A shows head of mycosed adult weevil infected with *B. bassiana*. B shows head of uninfected adult weevil included for comparison with A to differentiate between fungal growth and insect physiology.

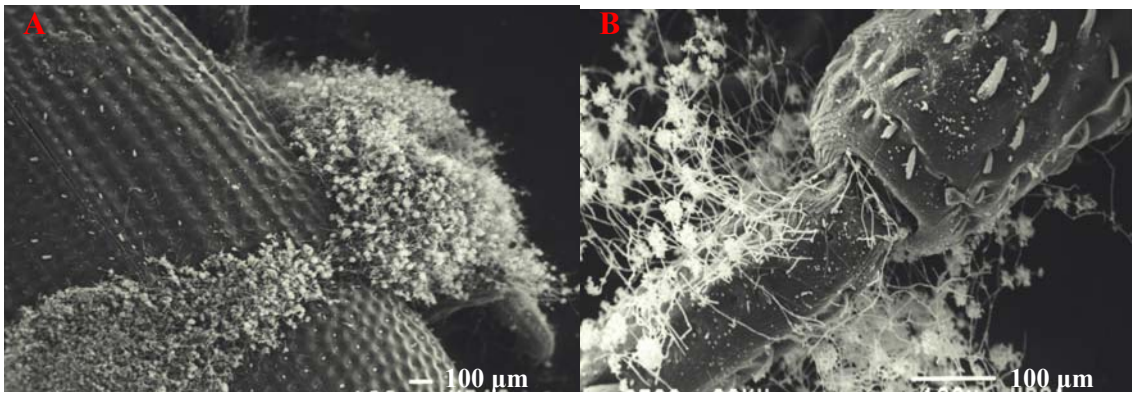


Figure 4.4 - Scanning electron micrographs of the maize weevil (*S. zeamais)* A shows mycosed cadaver of an adult weevil exhibiting fungal through body segments. B shows leg of maize weevil showing emergence of *B. bassiana* growth from joint.

From the images where mycosis has occurred, it is easily seen that the fungus readily emerges from in-between segments (appendages) of the insect. In Figure 4.3 A, fungal growth is pronounced around the tip of the snout (rostrum) of the weevil (Adane *et al.*, 1996). Figure 4.4: A, shows that fungal emergence emanates from insect segments between the thorax and abdomen.

4.3.3 Effects of different conidial concentrations on fungal virulence

To determine the effects of water formulated suspensions containing different conidial concentrations of *B. bassiana* spores, six different concentrations of local isolate PPRI 04307 were prepared and inoculated on to adult maize weevils as described above (section 4.2.4). The following results were obtained (Table 4.3).

Table 4.3: Effects of Various Conidial Concentrations on Fungal Virulence against the Maize Weevil

Conidial Concentration (ml^{-1})	Mean Mortality (%)	Mean Mycosis (%)
10^8	100 ±0 ^{A}	100 ±0 ^{A}
10^7	93 ±3.1 ^{B}	93 ±3.1 ^{B}
10^6	53 ±6.9 ^{C}	51 ±8.8 ^{C}
10^5	32 ±4.3 ^{D}	30 ±2.9 ^{D}
10^4	15 ±3.5 ^{D}	12 ±2.5 ^{DE}
10^3	10 ±4.1 ^{D}	0 ±0 ^{E}

Note: SNK groupings obtained from arcsin transformed data- within column means with the same letter are not significantly ($P < .0001$) different. Values represent means of 4 replicates of 20 insects each. ± = SE. (Appendix 2.3).

Data from control plots were omitted from statistical analysis as no insects died over the four-day incubation period. Results indicate that all conidial concentrations were effective at causing varying degrees of mortality against inoculated adult maize weevils and that generally weevil mortality was dependent on conidial concentration (Adane *et al.* 1996; Castillo *et al.*, 2000). Highly significant (F= 86.17; $P < .0001$) differences between mean numbers of dead insects were observed ranging from an average of two dead insects at the 10^3 conidial ml^{-1} inoculations up to an average of 20 dead insects at 10^8 conidial ml^{-1}. Similarly significant differences for percentage mortality (F= 90.97; P= <.0001) and percentage mycosis (F= 98.76; P= 0.0001) between conidial concentrations were obtained. With regards to percentage mortality, post ANOVA tests (SNK multiple comparison test) show that no differences were observed between means where conidial concentrations of 10^3, 10^4 and 10^5 conidial ml^{-1} were applied.

At the lowest conidial concentrations of 10^3 and 10^4 conidia ml^{-1} mortalities of 10 and 15% were observed respectively after the incubation period. This suggests that such concentrations would not achieve significant levels of kill under the specified conditions. At 10^5conidia ml^{-1} an average of six insects were killed translating to 32% mortality which was significantly ($P < .0001$) lower to the average of 53% mortality which was achieved from the 10^6 conidia ml^{-1} inoculation. At conidial concentrations of 10^6conidia ml^{-1}, it was observed that over 50% of inoculated insects were killed: these finding are consistent with those of De La Rosa *et al.* (2002). At the highest conidial concentrations of 10^7 and 10^8 conidia ml^{-1}, 93% and 100% mortalities were obtained respectively.

In their experiments De La Rosa *et al.* (2002) observed that fruit fly cadavers exhibited fungal growth that covered approximately 80% of the body within 48 hours and that the whole cadaver was covered after 72 hours. In the present experiment to confirm an entomopathogenic interaction, dead insects were surface sterilised, incubated under controlled conditions (section 4.2.4) and allowed to mycose over seven days (Figure 4.5).

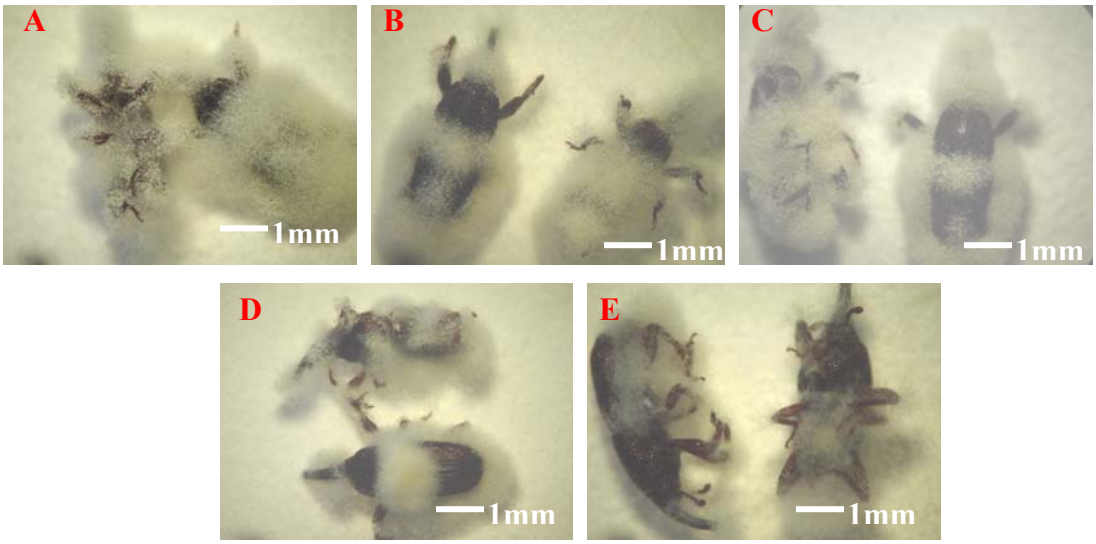


Figure 4.5 – Top- left to right shows mycosed cadavers inoculated with A=10^8, B=10^7 and C= 10^6 conidia ml^{-1}. Bottom- left to right shows cadavers inoculated with D=10^5 and E=10^4 conidia ml^{-1}. Photographs taken seven days after incubation to stimulate mycosis.

Only in cases where the live insects had been inoculated with high conidial concentrations (10^6, 10^7 and 10^8 conidia ml^{-1}) was it possible to observe extensive

mummification of the cadaver by fungal growth (Figure 4.5: A, B and C). Mycosis in these cases was characterised by the presence of thick masses of cream conidial growths (muscardine). Furthermore, only at 10^7 and 10^8 conidia ml^{-1} was it observed that values for percentage mycosis and percentage mortality were the same (Table 4.3). At 10^5 conidia ml^{-1}, growth was visually less extensive, covering less of the cadaver and appearing fluffier with mycelial growth as opposed to conidia (Figure 4.5: D). At the lower inoculation concentration of 10^4 conidia ml^{-1}, mycosis was observed to be sparse with few spore clusters developing within the white mycelial growth (Figure 4.5: E) and not all cadavers mycosed (Table 4.3). No mycosis occurred on the few insects that died when inoculated with the 10^3 conidia ml^{-1} inoculum. These observations indicate that there is a relationship between the concentration of inoculum applied to live insects and the ability of the fungus to subsequently grow and sporulate externally on cadavers. The presence of fungal growth on surface sterilized cadavers allowed to mycose, confirms that a insect pathogen was responsible for the demise of inoculated insects. In all cases however, fungal growth emanated from between insect segments, which confirms the observation of Adane *et al.* (1996) and De La Rosa *et al.* (2002).

4.3.4 Effects of temperature on fungal virulence

Raw data from both control and fungus-treated plots were analysed for this experiment. Maize weevil mortality was significantly affected by treatment ($F = 4.46$, $P = 0.0413$) and by temperature ($F = 23.07$, $P < .0001$), where differences were detected between treatments (fungus and control) and among the different temperatures. Similar observations where made for mean percentage mycosis, which was also significantly affected by treatment ($F = 11.60$, $P = 0.0016$) and by temperature ($F = 3.66$, $P = 0.00136$).

Table 4.4: Effect of Temperature on Mean Insect Mortality and Mycosis

Temperature (^{O}C)	Treatments	Mean Mortality (%)	Mean Mycosis (%)
15	Control	0 ±0	0 ±0
15	**04306**	**1 ±1.3** ^{C}	**0 ±0** ^{B}
20	Control	0 ±0	0 ±0
20	**04306**	**23 ±6.9** ^{CB}	**16 ±4.7** ^{B}
25	Control	0 ±0	0 ±0
25	**04306**	**87** ±5.2 ^{B}	**86** ±5.5 ^{A}
30	Control	1 ±1.3	0 ±0
30	**04306**	**62 ±16.0** ^{CB}	**56 ±19.7** $^{A\,B}$
37	Control	100 ±0	0 ±0
37	**04306**	**100 ±0** ^{A}	**0 ±0** ^{B}

Note: Bold type represents fungus treatments. Student-Newman-Keuls (SNK) multiple comparison test obtained from arcsin transformed data within-column means with the same letter are not significantly different between temperature treatments. Values represent means of 4 replicates of 20 insects each. ± = SE. (Appendix 2.4).

Overall mortality in controls was negligible not exceeding 1% in any of the temperatures except for at 37 ^{O}C, where all insects died during incubation. At this temperature, 100% mortality was obtained from both control and fungus-treated plots and the demise of the incubated insects could not be linked to fungal infection as insects did not mycose once surface sterilised and induced to undergo mycoses under controlled conditions (Zimmermann, 1986; Goettel and Inglis, 1997; De La Rosa *et al.*, 2002). This indicates that the insects died as a result of factors other than an interaction with the fungal pathogen, probably heat and dehydration stress after four days incubated at 37 ^{O}C. Adler (2002) showed how high temperatures above 45 ^{O}C are lethal to stored product arthropods and lethal exposure times decrease with increasing temperature. It is therefore likely that the four day incubation period was a long enough exposure period to cause the demise of all maize weevils incubated at 37 ^{O}C.

Table 4.4 shows a clear pattern in both mortality and mycosis of an increase from 15 ^{O}C to a maximum at 25 ^{O}C followed by a decrease at 30 ^{O}C and 37 ^{O}C. Fungal growth *in vitro* was also greatly retarded at 15 ^{O}C and 37 ^{O}C when compared with growth at 20, 25, and 30 ^{O}C over a four day incubation period and these visual data, (Figure 4.6) amplify those in Table 4.4. At 15 ^{O}C only 1% mortality with no mycosis was obtained and it may not be categorically concluded here that the fungus was responsible as fungal growth is

minimal at this temperature (Figure 4.6: a). Owing to the observation that fungal growth is essential for the infection process (Dimbi *et al.*, 2004) it is likely that this local isolate was not pathogenic at 15 ^{O}C and 37 ^{O}C when applied under the specified conditions used here. Hallsworth and Magan (1999) demonstrated that the growth rate of *B. bassiana* was close to zero at 37 ^{O}C, which is confirmed in Figure 4.6: e where no growth occurred on SDA over four days.

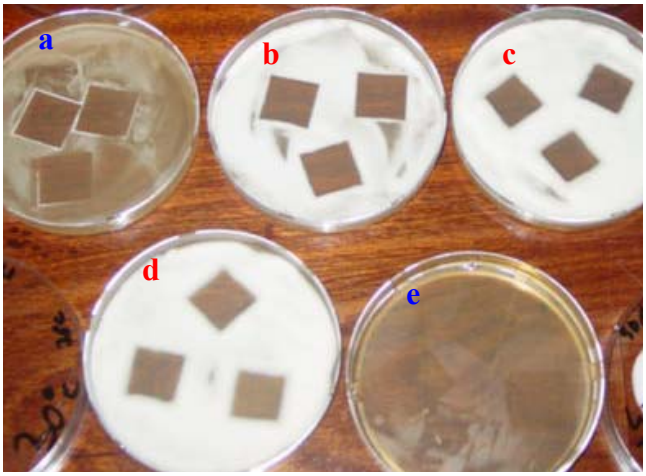


Figure 4.6 – Fungal growth of isolate PPRI 04306 at different temperatures (top a=15, b=20 and c=25^{O}C) (bottom d=30 and e=37 ^{O}C) after a 4 day incubation period.

In the case of 20 ^{O}C incubation however, the fungus clearly shows growth on media (Figure 4.6: b) with improved mortality at this temperature (23%) against adult weevils and resultant mycosis of 16% (Table 4.4). Fungus-treated insects incubated at 25 ^{O}C had the highest percentage mortality of 87% which was concomitant with fungal infection by mycosis of 86% (Table 4.4). At 30 ^{O}C, reduced mortality (62%) and mycosis (56%) was achieved. These data, taken together with data from Chapter 3, show 25 ^{O}C to be the optimal temperature for growth and virulence of this isolate of *B. bassiana*. These results illustrate that optimal temperature assessment for growth of sourced fungal isolates is a critical determinant of virulence (Dimbi *et al.*, 2004). From these results it may be concluded that the optimal temperature for maximum mortality is 25 ^{O}C and that this isolate is effective against adult maize weevils within a temperature range of 20 ^{O}C and 30 ^{O}C, conferring between 23 and 87% mortality. However, while temperature

significantly affects fungal pathogenicity, only in cases where mycosis can be induced and proof of infection shown, can it be fully stated that maize weevil mortality is linked to fungal infection. Dimbi *et al.* (2004) also found a range of temperatures at which their evaluated isolates where most effective against fruit fly species. However this is the first account where such data has been presented evaluating the effects of an EPF against the maize weevil at different temperatures.

4.3.5 Ability of dry spores from cadavers to infect live weevils

The aims of this experiment were satisfied with results reflecting that sporulating maize weevil cadavers transmit fungi to live weevils leading to epizootics within fungus-treated plots over a 30 day incubation period at 25 ^{O}C. Transmission rates were not measured, however the results showed no significant differences detected between fungal isolates in their ability to kill from dry spores dislodged from mycosed cadavers. Mortality levels in the untreated control plots were significantly (P <0.0001) lower than in all plots containing mycosed cadavers (Table 4.5).

Table 4.5: Maize Weevil Mortality from Infection by Dry Conidia

Isolate	Mean Mortality (%)	Mean of Seed Weight (g)
Control	15 ± 3.6 ^{B}	7.8 ± 0.1 ^{A}
IMI 386 701	73 ±3.6 ^{A}	8.6 ±0.4 ^{A}
PPRI 04306	90 ±4.1 ^{A}	8.8 ±0.3 ^{A}
PPRI 04307	80 ±7.3 ^{A}	9.0 ±0.3 ^{A}

Note: SNK multiple comparison test - Means with the same letter are not significantly (P <0.0001) different conducted on arcsin transformed data. ± = SE. Values represent means of 4 replicates of 40 insects each. (Appendix 2.5).

In all plots containing mycosed cadavers, over 70% mortality was observed, with dry spores of PPRI 04306 conferring 90% mortality after the 30 day incubation period. This implies that this isolate (PPRI 04306) may be more aggressive against maize weevils when applied as dry spores than when applied as a water formulation; however different criteria and experimental designs were applied for the experiment.

Observations of all plots at assessment time revealed no evidence of reproduction within fungus-treated plots; however insects in control plot were breeding with larvae found in two of the control plot replicates. Fifteen percent mortality (six insects) was obtained in control plots and this could be attributed to natural levels of mortality within a genetically mixed population of individuals with different vigour traits resulting from the selection method used.

Although there were no significant differences in seed weights between fungus treated and untreated plots at assessment one month after the start of the experiment (Table 4.5), the impact of the presence of fungal spores becomes apparent over time. After a further month's incubation, pest activity and damage in control plots was observed to be accompanied by storage moulds further reducing seed quality. However, fungus treated plots were comparatively free of extensive seed damage and fungal contamination. Figure 4.7: B clearly illustrates these observations where plates containing mycosed cadavers appear cleaner than untreated plates.

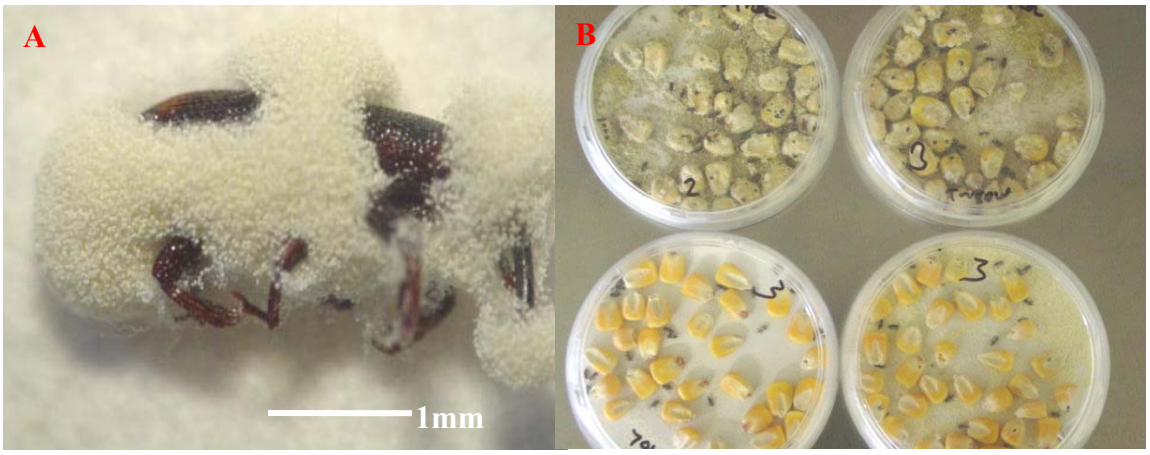


Figure 4.7 – A- example of mycosed weevil cadaver used in dry spore experiment. B - Maize seed damage between untreated and fungus treated plots – top control plots with no EPF infected cadavers, bottom (left IMI 386 701 right PPRI 04307) after two months incubation at 25^{O}C.

These findings are the first account showing that adult maize weevils (*Sitophilus zeamais*) are susceptible to infection by fungal spores emerging from mycosed cadavers of the same pest. Observations at the end of the incubation period revealed that much of

the conidia on cadavers had been dislodged into the substrate. This was likely due to the wandering action of the live adults crawling around and subsequently becoming infected.

The ability of the tested fungal isolates to inhibit the longevity of the targeted pest (compared to the untreated controls), where no added moisture was included, further illustrates that *B. bassiana* over time can impact on insect mortality supporting findings by Adane *et al.* (1996) who achieved high levels of mortality without the requirement of high humidity. Furthermore, results obtained here are consistent with those of Adane *et al.* (1996), where they also obtained low mortality levels in control plots. Moreover Adane *et al.* (1996) found no significant differences in mortality between conidial doses of 0.1, 0.5 and 1 grams (equivalents of 3.54×10^8, 1.77×10^9 and 3.54×10^9 conidia) of the fungus against the maize weevil. Although different conidial doses (2.0×10^6 conidia for PPRI 04306, 2.1×10^6 conidia for PPRI 04307 and 1.6×10^6 conidia for IMI 386 701) were used in this experiment and applied via mycosed cadavers, the same trends were observed here where no differences were detected in percentage mortality between conidial doses.

Dimbi *et al.* (2003) also obtained high levels of mortality from inoculations of fruit fly species with dry spores. In their experiments using several isolates of *M. anisopliae* and *B. bassiana*, fruit flies were contaminated by allowing them to crawl over velvet carpet material impregnated with fungal spores within a cylindrical plastic tube for three minutes. Flies were then transferred to ventilated Plexiglas cages containing a solid insect food source and a cotton bud soaked in water as a water source for the insects (Dimbi *et al.*, 2003). Although a water source was provided for the insects, it is not thought likely that this impacted on increasing humidity as the cages in which the flies were incubated were ventilated, insects were however maintained under conditions of relative humidity of between 40 and 60% (Dimbi *et al.*, 2003). The three minute exposure time of fruit flies to dry conidia was sufficient for an individual fly to pick up between 4.2×10^5 to 1.0×10^6 conidia ml^{-1} and this resulted in high percentage mortalities; however mortality was also influenced by isolate, with some isolates conferring low levels of mortality after a four day incubation period (Dimbi *et al.*, 2003).

The information highlights the opportunistic conidial nature of fungal Hyphomycetes and their ability to attack and cause infection on susceptible insect hosts (Furlong and Pell, 2001). It however remains important to assess the virulence of sourced fungal isolates against target pests to determine the most virulent strains when applying the fungus as dry spores.

In conclusion, this chapter demonstrated the ability of the entomopathogen, *B. bassiana,* to cause lethal infections of the maize weevil (*Sitophilus zeamais*) *in vitro*. Initially eight isolates from different locations (countries and regions, Table 4.1) were used to infect the target pest where the fungus was applied as water formulations containing 10^8 conidia ml^{-1}. A system to rank the isolates according to virulence was adapted from Adane *et al.* (1996); however results showed that both indigenous isolates and exotic strains resulted in an average of 92% mortality. Results also indicated that local isolates of *B. bassiana* caused similar (equal to) and better levels of mortality compared with exotic isolates. Local isolates were found to be effective against the maize weevil under various conditions including different conidial concentrations and temperatures. In experiments using different conidial concentrations, percentage mortality increased with conidial concentration. Furthermore, it was observed that the lethal concentration to kill 50% of inoculated insects was 10^6 conidia ml^{-1}. Evaluating the effects of different temperatures on fungal virulence showed that insect mortality increased from 15 ^{O}C to a maximum at 25 ^{O}C, followed by a decrease at 30 ^{O}C and 37 ^{O}C. Fargues *et al.* (1997) and Ouedraogo *et al.* (1997) cited in Hallsworth and Magan (1999), found that isolates of *B. bassiana* had a thermal threshold of 35 to 37 ^{O}C. Observations of fungal growth (Figure 4.6) indicates that this local isolate has a thermal threshold at 37 ^{O}C where growth was inhibited and this information confirms findings that EPF are not thermophilic in nature (Hallsworth and Magan, 1999). This study is a first account evaluating the effects of an entomopathogenic fungus against the maize weevil at different temperatures and is also a first account reporting the potential of using *B. bassiana*-mycosed cadavers to infect live insects and produce high levels of mortality.

CHAPTER FIVE

Inundative Inoculations of the Tobacco Beetle, *Lasioderma serricorne* With Water Formulations of the Entomopathogenic Fungus, *Beauveria bassiana*

5.1 Introduction

Literature regarding evaluations of entomopathogenic fungi against the tobacco beetle, *Lasioderma serricorne*, was not found. To date, few laboratory trials evaluating the effects of EPF on larvae of the beetle have been conducted; however these trials have as yet to be published (J. Lord, pers. comm.) (Appendix 4). The use of insect pathogenic bacteria was investigated in an assessment of the effects of Japanese *Bacillus thuringiensis* isolates against this pest by Tsuchiya *et al.* (2002). The results indicated that ß-exotoxin related substances of the isolates evaluated are responsible for toxicity against the tobacco beetle (Tsuchiya *et al.*, 2002), indicating that the tobacco beetle is susceptible to the effects of these entomopathogenic bacteria. This chapter outlines attempts to determine whether isolates of *Beauveria bassiana* obtained from different countries including South Africa, are pathogenic against local cultures of the tobacco beetle, a prominent pest of stored products (Ashworth, 1993b; Massey, 1999). Pathogenicity assessments were conducted under controlled laboratory conditions; however, distinct problems were encountered when attempting to rear the beetle *in vitro*, even although similar conditions to those from where the culture was sourced were used. This meant that the whole spectrum of typical pathogenicity and virulence experiments could not be fully conducted as with the maize weevil (Chapter 4).

The previous chapter did however reveal pertinent information, which was used to design bioassays with the following aims:

i. to determine the effects of inundative spore inoculation of *B. bassiana* isolates in the form of water formulation to infect adult tobacco beetles and

ii. to determine the efficacy of fungal cultures of different ages to infect adult tobacco beetles.

This chapter includes details and results from a pilot study which aimed at testing the pest inoculation procedures devised in Chapter 4 and to gauge the effects and responses of adult tobacco beetles to water formulations of four isolates of *B. bassiana* at inundative conidial concentrations of 10^8 conidia ml^{-1}. This was necessary to determine whether the target pest is indeed susceptible to fungal infection by the entomopathogen. Two further experiments are presented where the same inundative rate of fungal inoculations was applied and where more *B. bassiana* isolates where included. Seven isolates were used including all three locally sourced strains. In bioassay 1, a 'fresh' 15 day old culture growing on SDA + Y + G was used, while in bioassay 2, a 'mature', four month old culture growing at room temperature on amended SDA was used. This allowed for the capture of data relevant to the shelf life of the isolates growing in sealed Petri dishes on amended media (SDA + Y + G). It was necessary to include an experiment where stored cultures were evaluated as the ultimate goal in such assessments involves development of bioinsecticides (Hong *et al.*, 2001).

Results from all bioassays are presented below with results indicating that adult tobacco beetles are susceptible to infection by *B. bassiana*. Section 5.2.1 describes the methods and materials used to ascertain there results. Emphasis is also placed on presenting infection results visually, using imagery from scanning electron microscopy (SEM) and digital photography highlighting mycosis on beetle cadavers. The results presented here are the first to demonstrate that the tobacco beetle (*Lasioderma serricorne*) is susceptible to *Beauveria bassiana in vitro*; where both indigenous and exotic strains of the fungus showed high levels of virulence against the insect when applied as water formulations.

5.2 Materials and Methods

5.2.1 Maintenance and culture of experimental organisms

Insects - Insect cultures of the tobacco beetle were supplied by the Plant Protection Research Institute (PPRI) at Roodeplaat. Insect samples were reared under the same

conditions as the maize weevil (section 4.2.1); however the tobacco beetle was fed on an artificial food diet of Pronutro (BOKOMO-Wheat free cereal) supplemented with powdered milk (Cremora: Nestle) (Appendix 3). The diet here is termed artificial as the tobacco beetle has a natural first attraction to infest cured tobacco and cured tobacco products although it is also reported to survive on other food sources (Ashworth, 1993b; Massey, 1999). This food source and the conditions under which the culture was maintained were recommended by fellow scientists at PPRI.

The food matrix coagulated when infested with insects and led to difficulties when attempting to isolate insect samples from the food source. The crude lure device described in Chapter 4 was however useful and allowed for a selection of pests. Use of the lure included removing adults from their food by gently breaking the coagulated substrate and sieving through a 1 mm laboratory test sieve, then transferring the population into the base of the lure device dubbed the MNS Tower. The lure area was baited with fresh filter cigarettes (Figure 5.1) to encourage individuals to crawl up into the collection area. A baiting period of between three and four hours was used to allow enough time for this process to occur.

Figure 5.1 – Innovative sampling device designed to attract adult pests into collection area. A - shows a view from above of the MNS Tower where cigarettes were added as an attractant for fit tobacco beetles. B - illustrates assembled MNS Tower.

After the baiting period the MNS Tower was transferred to a 4 ^{O}C refrigerator to immobilise the pests prior to inoculation.

Fungi – All three local isolates plus four exotic isolates of *B. bassiana* were used in this set of experiments (Table 5.1). Fungal isolates were prepared as in section 4.2.1. For the culture-shelf-life evaluation, isolates were revived in the same manner then maintained on SDA plates at room temperature for four months, ensuring all plates where sealed with laboratory film (Parafilm). Spore viability through germination assessments were conducted for both cultures prior to the bioassays using the procedure of Dimbi *et al.* (2004) as described in sections 4.2.1 above. The mature culture was observed to have developed chlamydospores but still produced adequate percentage germination of between 85 and 95% to be considered as viable.

5.3 Preliminary pilot study

Experimental design entailed using five treatments, four of which were water formulations of 15 day-old *B. bassiana* cultures and the fifth, a DSW + 0.5% Tween 80 inoculation, used as the control treatment (Adane *et al.*, 1996; De La Rosa *et al.*, 2002). The four fungal treatments were formulated from isolates, PPRI 04305, PPRI 04306, PPRI 04307 and IMI 368 696 (highlighted in bold text in Table 5.1 below). A complete randomised design (Goettel and Inglis, 1997) of three replicates of 30 adult insects each was used and inoculum was prepared as described in section 5.2.1 above. Inoculations were conducted under a laminar flow cabinet (De La Rosa *et al.*, 2002) and involved immersing the adult beetles in the different treatments. Adult beetles were immersed in aliquots (0.25 ml) of the different treatments in 24 Well Cell Culture Cluster packs to hold the pests and inoculum for between five and ten seconds as was the case with the maize weevil (Chapter 4).

To further test the procedures devised in the previous chapter, inoculated insects were transferred to labelled sterile Petri dishes lined with sterile-dry filter paper, sealed with Parafilm and incubated in the dark at 25 ^{O}C for four days, after which time mortality assessments were conducted. Following mortality assessments, all dead insects were surface sterilized and allowed to mycose for further confirm of an entomopathogenic fungal infection, with the criteria for mycosis being the appearance of mycelial growth on

cadavers after a 24 to 48 hour incubation period at 25 ^{O}C (Zimmermann, 1986; Goettel and Inglis, 1997; Castillo *et al.*, 2000; De La Rosa *et al.*, 2002).

Table 5.1: Treatments Included in Experimental Bioassays

Isolates/Treatments	**Country of Origin and host insect**
1. PPRI 04305	**South Africa (*G. mellonella*)**
2. PPRI 04306	**South Africa (*G. mellonella*)**
3. PPRI 04307	**South Africa (*G. mellonella*)**
4. IMI 386 696	**Brazil (*Diabrotica* species)**
5. IMI 386 694	Portugal (*Popillia japonica*)
6. IMI 382 302	Kenya (*S. zeamais*)
7. IMI 386 701	Kenya (*S. zeamais*)

Note: Bold type denote pilot study treatments with addition of control.

5.3.1 Inundative bioassays using cultures of two different ages

For both bioassays described here the use of a standard conidial concentration of 10^8 conidia ml^{-1} was employed to evaluate effects of *B. bassiana* isolates (all isolates Table 5.1) against adult tobacco beetles. Treatments were randomly assigned and inoculated so that each treatment contained four replicates of 30 adult insects each. Under a laminar flow cabinet (De La Rosa *et al.*, 2002), adults were inoculated by immersion in 0.25 ml of 10^8 conidial suspensions in 24 Well Cell Culture Cluster packs for up to 10 seconds. Contents of individual wells were transferred to sterile Petri dishes lined with sterile-dry filter paper, sealed with Parafilm and incubated in the dark at 25 ^{O}C for four days maintaining randomized layout, after which time mortality assessments were conducted. Control plot insects were inoculated using the same procedure where insects were immersed in 0.25 ml of DSW + 0.5% Tween 80 as the spore-free inoculum (Adane *et al.*, 1996), and this made up the eighth treatment. In both bioassays the criterion of death was gauged by a comparison between immobile insects and mobile insects in control plots.

After assessments for mortality and to confirm an entomopathogenic infection all dead insects from all treatments were allowed to mycose by using the modified procedure of Goettel and Inglis (1997) (De La Rosa *et al.*, 2002). This procedure involved surface sterilizing dead beetles using three, 10 second rinses in 3% sodium hypochlorite and three, 20 second rinses in distilled sterile water prior to transfer to fresh Petri dishes lined with moistened filter paper under sterile conditions and incubation at 25 ^{O}C for 48 hours (Zimmermann 1986; Goettel and Inglis, 1997).

5.3.2 Scanning electron microscopy (SEM)

Resultant mycosed tobacco beetle cadavers were prepared for SEM by mounting specimens onto single aluminium stubs, and unlike other specimens such as spider mites did not require critical point drying. Samples were then coated while on the mount with gold palladium in preparation of microscopy. A JEOL JSM-840 scanning microscope was used which is a thermal emission scanning electron microscope. The accelerating voltages ranged from 1 to 40kv and the measured beam current ranged from 10pA to 600pA.

5.3.3 Data analysis

Percentage mortality and percentage mycosis data (arcsin transformed) were subjected to one-way ANOVA to determine the effects of *B. bassiana* isolates applied as water formulations of 10^8 conidial ml^{-1} (Adane *et al.*, 1996; Goettel and Inglis, 1997; De La Rosa *et al.*, 2002; Dimbi *et al.*, 2004). For the pilot study resultant means were separated using the post ANOVA Student-Newman-Keuls (SNK) multiple comparisons test (Dimbi *et al.*, 2004) and for the inundative bioassays means were separated using Duncan multiple range test (Adane *et al.*, 1996) to determine differences between the effects of isolate on mortality. Analysis was carried out using SAS Enterprise Guide 3.0 software.

5.4 Results and Discussion

5.4.1 Preliminary pilot study

The pilot study provided information to show that local cultures of the tobacco beetle are highly susceptible to infection by the entomopathogenic fungus *B. bassiana*, when water formulations containing fungal spores (10^8 conidial ml^{-1}) are used to contaminate adult insects (Table 5.2). There were no mortalities observed in control plots and these data were excluded from statistical analysis; however this observation confirms that the presence of fungal spores was the responsible factor for death of the insects. No differences were detected between fungal isolates for mean percentage mortality and mean percentage mycosis and mortalities of over 95% were observed for all fungus treated plots over the incubation period of four days (Table 5.2).

5.2: Results from a Pilot Study Conducted to Determine Tobacco Beetle Susceptibility to *B. bassiana*

Isolate	Mean Percentage Mortality (%)	Mean of Percentage Mycosis (%)
IMI 386 696	95 ±1.11 ^{A}	95 ±1.11 ^{A}
PPRI 04305	97 ±2.22 ^{A}	97 ±2.22 ^{A}
PPRI 04306	97 ±1.11 ^{A}	97 ±1.11 ^{A}
PPRI 04307	98 ±1.11 ^{A}	98 ±1.11 ^{A}

Note: Within column means with the same letter are not significantly different. Means separated using Student-Newman-Keuls (SNK) multiple comparisons test on arcsin transformed data. Values represent means of 3 replicates of 30 insects each. ± = SE.

Table 5.2 also shows that percentage mycosis data is concomitant with that of percentage mortality as all dead insects allowed to mycose, did so. All dead insects were induced to mycose using the modified procedure described in section 5.3.1 to further confirm that an entomopathogenic infection had occurred. Figure 5.2 illustrates examples of mycosed tobacco beetles from the preliminary bioassay.

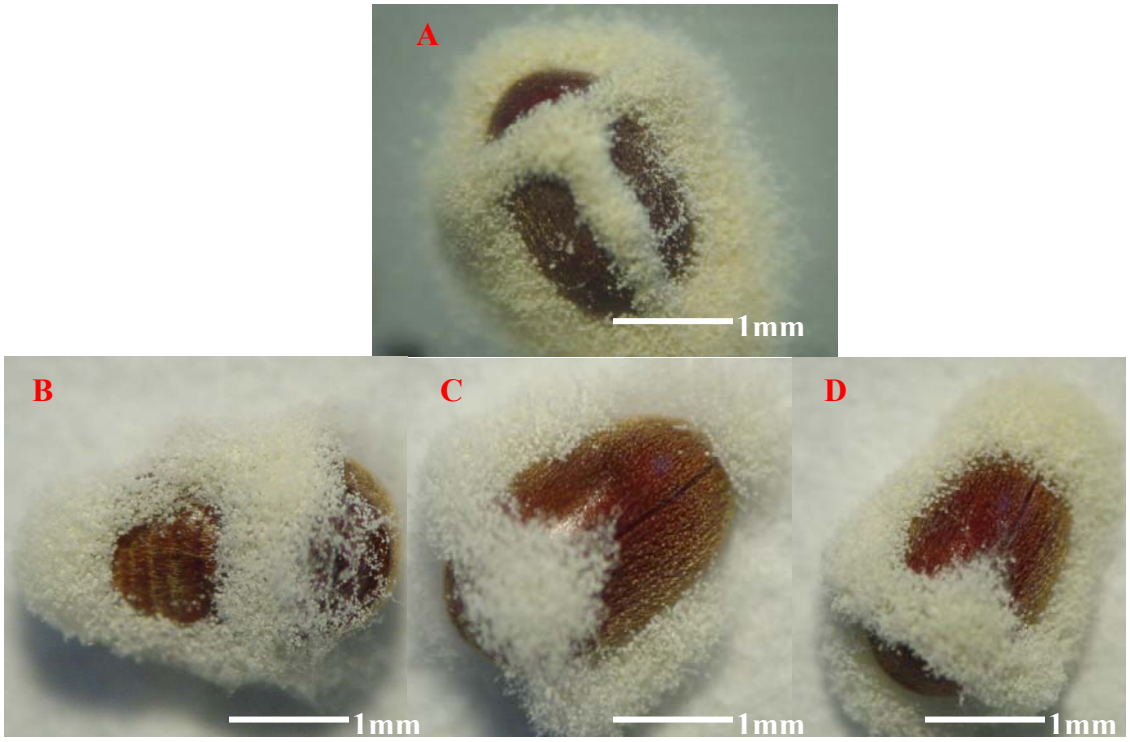


Figure 5.2 – Mycosed tobacco beetle cadavers resulting from inoculations with water formulated *B. bassiana*. A - isolate IMI 386 696; B - isolate PPRI 04305; C - isolate PPRI 04306 and D - isolate PPRI 04307.

Figure 5.2: A, C and D show the dorsal side of mycosed adult tobacco beetles, while B shows the ventral side of a mycosed adult, with fungal growth emerging from insect segments in all cases. The same pattern of sporulation was observed in the case of the maize weevil in the previous chapter. The typical cream coloured *B. bassiana* conidia are also clearly visible within the masses of mycelial growth.

These results confirm that the pest inoculation procedures devised in the previous chapter for the maize weevil were adequate for the more fragile tobacco beetle. Moreover successful infection by *Beauveria bassiana* was accomplished at the conidial concentrations (10^8 conidial ml^{-1}) applied under the specified conditions. Results from this preliminary pilot study confirm that the tobacco beetle is susceptible to infection by *B. bassiana* and the occurrence of mycosis of dead beetles further adds validity to these observations.

5.4.2 Inundative bioassays using cultures of two different ages

Results from both bioassays followed the same trends as observed in the preliminary study, where findings showed that the tobacco beetle is highly susceptible to infection by the entomopathogen *B. bassiana* when the fungus is applied in the form of water formulated spore suspensions containing 10^8 conidia ml^{-1}. Where fresh cultures were used, isolate IMI 386 694 conferred the lowest mean percentage mortality of 85%, and this was significantly (P <0.0001) lower than all other isolates evaluated. The other six isolates evaluated conferred mean percentage mortalities of between 96 and 100%. Isolates IMI 386 696, PPRI 04306, PPRI 04307 and IMI 386 701 all conferred 100% mortality (Table 5.3) with exotic isolate IMI 382 302, and isolate PPRI 04305 conferring 96% mortality. PPRI 04305 mortality was significantly lower (P <0.0001) than the 100% isolates although IMI382 302 was not significantly lower (Table 5.3).

Table 5.3: Tobacco Beetle Mortality Data from Water Formulated Inoculum of Fresh Cultures of *B. bassiana*

Isolate Code	Fresh Culture: Mean Percentage Mortality	Fresh Culture: Mean Percentage Mycosis
IMI 386 696	100 ±0 ^{A}	100 ±0 ^{A}
PPRI 04306	100 ±0 ^{A}	100 ±0 ^{A}
PPRI 04307	100 ±0 ^{A}	100 ±0 ^{A}
IMI 386 701	100 ±0 ^{A}	100 ±0 ^{A}
IMI 382 302	96 ±2.34 ^{AB}	96 ±2.34 ^{AB}
PPRI 04305	96 ±1.4 ^{B}	96 ±1.4 ^{B}
IMI 386 694	85 ±4.4 ^{C}	85 ±4.4 ^{C}

Note: Within column means with the same letter are not significantly different. Means separated using Duncan's Multiple Range test. Values represent means of 4 replicates of 30 insects each. ± = SE. (Appendix 2.8).

As was the case with results obtained in the preliminary study, mean percentage mycosis mirrored mean percentage mortality where all dead insects allowed to mycose did so.
This observation suggests that where 15 day-old cultures of *B. bassiana* are used to contaminate adult tobacco beetles in the form of water formulated spore suspensions, the

fungus is able to reproduce through cadavers and emerge to mummify the insect with spores and mycelia (Figure 5.3; A, C and E). The observation further confirms that an entomopathogenic infection took place and that *B. bassiana* was responsible for the demise of the fungus-treated insects (Adane *et al.*, 1996; Goettel and Inglis, 1997; De La Rosa *et al.*, 2002; Dimbi *et al.*, 2004).

The four month old cultures produced a similar kill profile in terms of percentage mortality to the fresh culture, where mortalities also ranged between 85 and 100%, with isolate IMI 386 694 producing the lowest mean percentage mortality of 85% (P <0.0001). Local isolates PPRI 04305 conferred 99% mortality, three percent higher than what was detected with the use of the fresh inoculum (Table 5.4).

Table 5.4: Tobacco Beetle Mortality Data from Water Formulated Inoculum of Mature (four month old) Cultures of *B. bassiana*

Isolate Code	Mature Culture: Mean Percentage Mortality	Mature Culture: Mean Percentage Mycosis
IMI 386 696	100 ±0 ^{A}	97 ±0.25 ^{AB}
IMI 386 701	100 ±0 ^{A}	73 ±17.9 ^{C}
PPRI 04307	100 ±0 ^{A}	100 ±0 ^{A}
PPRI 04306	99 ±0.83 ^{A}	99 ±0.83 ^{A}
PPRI 04305	99 ±0.83 ^{A}	80 ±10.9 ^{BC}
IMI 382 302	96 ±2.34 ^{A}	96 ±2.34 ^{AB}
IMI 386 694	85 ±4.4 ^{B}	85 ±4.4 ^{BC}

Note: Within column means with the same letter are not significantly different. Means separated using Duncan's Multiple Range test. Values represent means of 4 replicates of 30 insects each. ± = SE. (Appendix 2.8)

This suggests that some isolates became more pathogenic and/or behaved differently when stored over time; however more detailed studies are required such as those by Hong *et al.* (2001). In their study hermetically stored isolates of *B. bassiana* were maintained at different temperatures (10 to 50 ^{O}C) and at six to 11 moisture contents for up to 372 days with evaluations being made for fungal cell germination (Hong *et al.*, 2001). These evaluations testing longevity of the fungus were conducted using concise methods of

storage; however in this study, plates of conidial mats were simply stored at room temperature, which fluctuated over the four month period. In terms of moisture content condensation within the sealed plates was observed and it is therefore considered that humidity was high within the plates.

With regards to post mortality assessments, the criterion for mycosis was the presence of fungal growth emanating from the insect cadaver (De La Rosa *et al.*, 2002) and although both cultures were hydrated during inoculum preparations, visual differences were observed in mycosis patterns of cadavers induced to mycose *in vitro* post incubation. The amounts of emerging spores and mycelia on cadavers from all fungus were diminished for the mature culture inoculations when visually compared to those inoculated with the fresh culture suspensions (Figure 5.3).

These results suggest that older cultures of *B. bassiana* may not be as capable of reproducing through insect cadavers as relatively fresher cultures and the difference in appearance of conidial and mycelia resulting from the different aged cultures are apparent. In the case of mycosis resulting from fresh culture inoculations, spores (chains and clusters) were clearly visible within mycelial masses and all growth had a cream coloration (Figure 5.3: A, C and E). From the mature inoculum, resultant mycosis was comprised mostly of mycelia with little to no spore clusters evident; the predominant colour of the growth in these cases was white with no cream (Figure 5.3: B, D and F). In some cases cadavers did not mycose at all which is reflected in the percentage mycosis values (Table 5.4) which show non-concomitant values in relation to percentage mortality values.

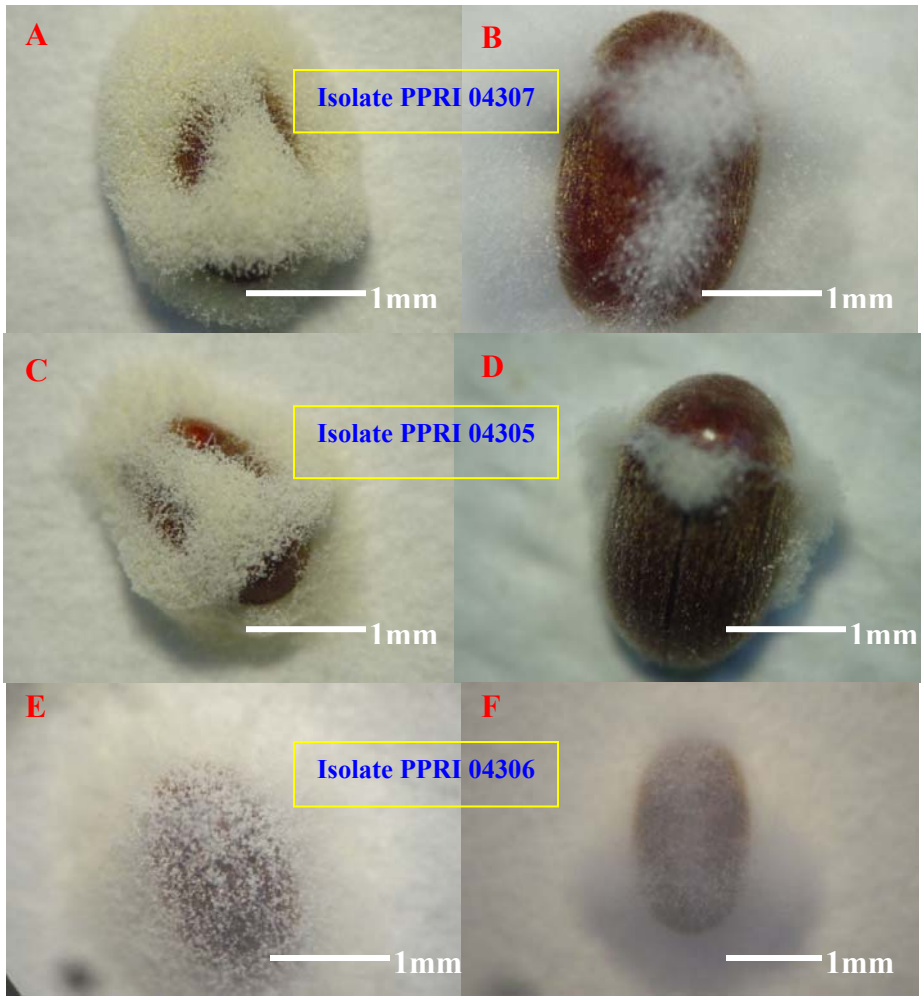


Figure 5.3 – Mycosed tobacco beetle cadavers resulting from; left (A, C and E) fresh inoculum infections and right (B, D and F) mature inoculum infections with 10^8 conidia ml^{-1} water formulations of local isolates (A and B - isolate PPRI 04307, C and D - isolate PPRI 04305 and E and F - isolate PPRI 04306)

These observations are pertinent when considering the development of bioinsecticides, where mycosed insect cadavers are to be used as carriers for infectious spores. If mycosed insect cadavers are to be used as carriers for infectious propagules, the results shown in Tables 5.3 and 5.4, and Figure 5.3 suggest that it is imperative to infect live insects with cultures incubated for up to 15 days (fresh cultures). This should result in cadavers fully mummified with spores as observed in Figure 5.3. Spores are the agents for infection, making mature cultures inadequate for the production of mycosed cadavers for the purposes of using them as carriers. Mycosis was once again observed to emanate

from insect segments (between the head and thorax, between thorax and abdomen, and between wing casings) and subsequently spread.

In summary, a mature culture of four months old has the same virulence as a fresh 15 day old culture when applied as fungal suspensions of 10^8 conidia ml^{-1}, but with reduced ability to reproduce on the dead host. These findings are the first scientific account where adult tobacco beetles have been shown to be susceptible to infection by the entomopathogenic fungus *B. bassiana* under the conditions specified above. The following section presents images produced with the use of SEM, highlighting mycosis of adult tobacco beetles inoculated with water formulated *B. bassiana*, surface sterilized upon death and allowed to mycose.

5.4.3 Scanning electron microscopy (SEM)

Imagery from scanning electron microscopy (SEM) represented by Figures 5.3, 5.5 and 5.6 below illustrates in greater detail the growth of emerging fungal mycelia and spores on tobacco beetle cadavers stimulated to mycose after surface sterilization.

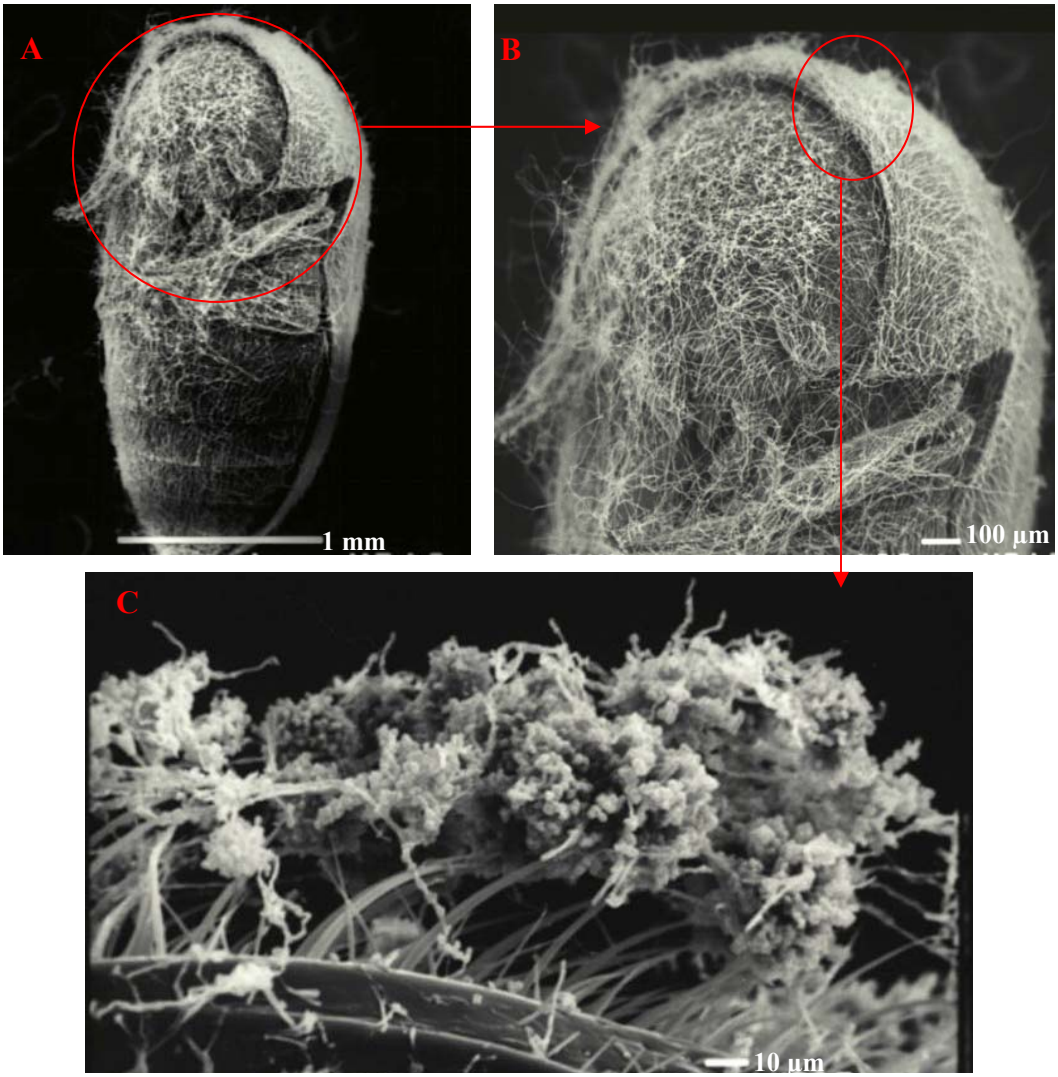


Figure 5.4 - Scanning electron micrographs of a mycosed adult tobacco beetle. A shows whole body of mycosed cadaver. B shows head and thorax of mycosed adult. C shows magnification of fungal spores and mycelia emerging from highlighted area of B.

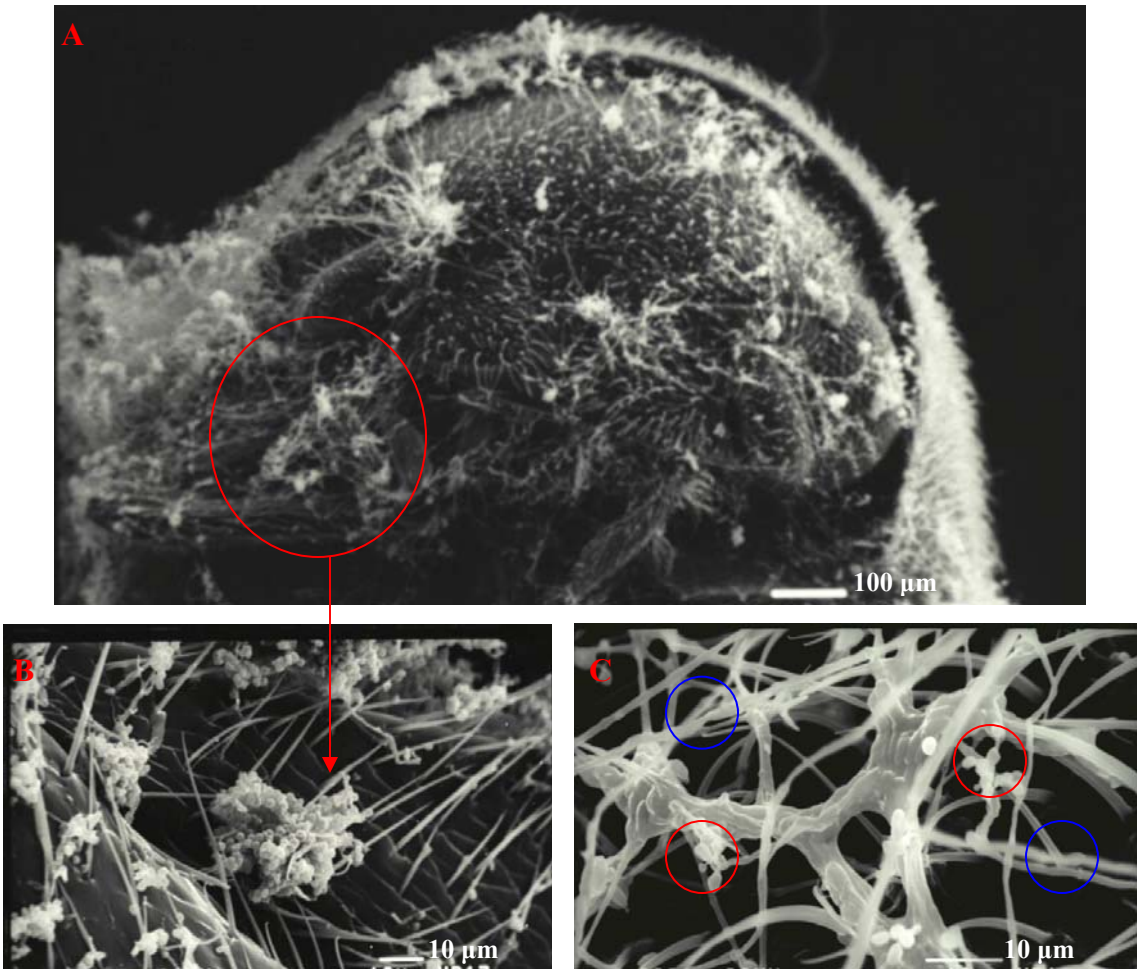


Figure 5.5 - Scanning electron micrographs illustrating mycosis of adult tobacco beetle. A shows the head of a cadaver with sporadic fungal growth. B shows mycelial and spore production of the fungus from the highlighted area of A. C shows spores (red circles) and mycelia (blue circles) within fungal mass emerging from the cadaver.

In figures 5.4 and 5.5, it is evident that the fungal masses contain both mycelia and spores of the EPF. The emerging spores are pathogenic and in nature may be dislodged to infect other insects that come in contact with such cadavers (Furlong and Pell, 2001; Shah and Pell, 2003). Figure 5.6 below shows mycosis on the inner wing casing of an adult beetle clearly showing both spores and mycelia.

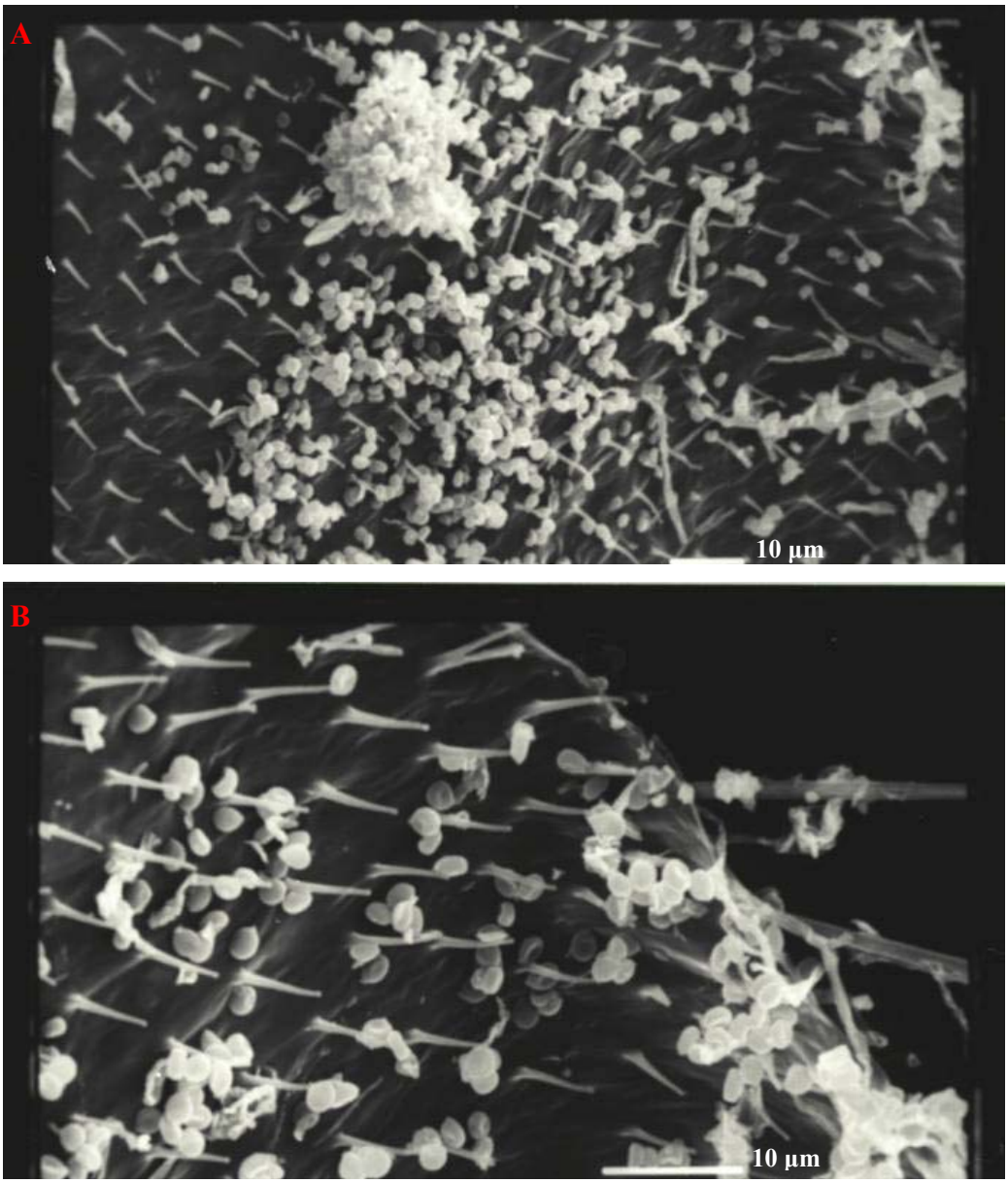


Figure 5.6 - Scanning electron micrographs illustrating fungal growth (mycosis) on the inner wing casing of an adult tobacco beetle resulting from the infection process of *B. bassiana*. A and B show mycelia and spores emerging from an adult tobacco beetle at different magnification.

In conclusion, these are the first experiments where the tobacco beetle has been inoculated *in vitro* with the entomopathogenic fungus *Beauveria bassiana*. The present study used strains indigenous to South Africa and isolates sourced internationally and shows that this entomopathogenic fungus is pathogenic against the tobacco beetle under the experimental condition outlined.

CHAPTER SIX

Summary and Conclusions

6.1 Sourcing, Maintenance and Viability Assessments of EPF

Sourcing local isolates for the evaluation of entomopathogenic fungi was considered an integral part of this study. The short term goals included the collection of baseline data on local isolates compared with exotic strains of EPF. Medium to long term goals would go further to include evaluations *in situ*, research on mass production, design of field application technologies and eventually the development and registration of bioinsecticides for use against insect pests (Goettel, 1984; De La Rosa *et al.*, 2002; Dimbi *et al.*, 2003).

For isolation of native strains to South Africa, the procedure of Zimmermann (1986) was adapted as it is rapid, cost effective and targets broad spectrum EPF (Zimmermann, 1986; Goettel and Inglis, 1997). Dubbed the *Galleria* bait method, the technique has been used to isolate a number of entomopathogenic fungal species including *Metarhizium anisopliae, Paecilomyces farinosus* and *Beauveria bassiana* (Zimmermann, 1986). It was possible to replicate the procedures of Zimmermann (1986) *in vitro* and successfully isolate three strains of EPF from South African soil. These fungi were subsequently identified as *Beauveria bassiana* (section 2.2.1) after which time it was possible to acquire other strains of the same fungal species from international culture collections for comparative assessments. The ease in which indigenous isolates were obtained directly from soil samples illustrates that it may be possible to harness EPF locally without the need to import exotic isolates for the development and use of bioinsecticides for local markets.

Critical evaluations were conducted on the EPF prior to bioassays challenging insect pests with fungal isolates and included viability assessments through percentage germination determination. De La Rosa *et al.* (2002) conducted such assessments and in cases where they obtained 90% germination and above, they considered the isolate to be

viable. In this case the procedure of Dimbi *et al.* (2004) was adapted and successfully used in Chapters 4 and 5. All isolates used in pathogenicity and virulence experiments were found to be viable and germinating between 90 and 100%. These findings show that the culturing and storage techniques employed were adequate for the sourced isolates of *Beauveria bassiana*.

6.1.1 Radial growth at different temperatures

The experiment presented in Chapter 3 aimed to evaluate the effects of various temperatures on radial growth of sourced *B. bassiana* isolates in comparison with each other and to an out-group *Metarhizium* strain. Dimbi *et al.* (2004) analysed their data so that resultant means were compared at each temperature and by each isolate; however this was not the case with results obtained in this study. Averages of radial growth rates were analysed and separated to provide data on the fungal growth across all temperatures and obtain combined average growth rates. This was done owing to the observation that entomopathogenic fungi grow over a wide range of temperatures (Goettel and Inglis, 1997; Hallsworth and Magan, 1999) and for practical applications, determination of an optimal temperature range would be more appropriate. The fungal isolates in this case were found to grow in total darkness (Goettel and Inglis, 1997; Dimbi *et al.*, 2004), at different rates across the range of temperatures; illustrating the ability of these organisms to grow at temperatures between 15 and 30 ^{O}C. This resulted in determining that most isolates grow optimally at 25 ^{O}C, and the range at which they grew optimally was found to be between 20 ^{O}C and 28 ^{O}C. The ability of EPF to grow in darkness makes them ideal for use in dark storage facilities. Comparing the vegetative growth of native isolates with that of exotic strains at different temperatures, it was found that no major differences were observed with most isolates growing optimally at 25 ^{O}C. The literature shows that fungal Hyphomycetes grow optimally within a wide range of temperatures of between 20 ^{O}C and 30 ^{O}C (Ferron, 1981; Smits *et al.*, 2003; Dimbi *et al.*, 2004) and in this study it was found that sourced isolates of *B. bassiana* grew optimally between 20 ^{O}C and 28 ^{O}C. When comparing the growth of *B. bassiana* isolates with a *Metarhizium* species the most obvious differences in radial growth at different temperatures was that the out-group

fungus grew at its slowest at 30 ^{O}C where as the *Beauveria* isolates all grew at their slowest at 15 ^{O}C. No information on the out-group fungus was available and from the results obtained from this experiment, it is not likely that the *Metarhizium* species used was *M. anisopliae*. Hallsworth and Magan (1999) found that isolates of *M. anisopliae* grow optimally at 30 ^{O}C.

6.2 Insect-Pathogen Interactions

6.2.1 Summary on EPF against *Sitophilus zeamais*

The literature shows that the maize weevil, *Sitophilus zeamais* was previously challenged with isolates of the entomopathogen *Beauveria bassiana* owing to its economic importance of a pest of harvested grains (Longstaff, 1981; Adane *et al.*, 1996; Oduor *et al.*, 2000). In this case, successful results similar to those obtained by Adane *et al.*, (1996) were achieved in bioassays conducted *in vitro* under the conditions specified in Chapter 4. Results from preliminary bioassays indicated that the maize weevil was found to be highly susceptible to infection by both local and exotic strains of *B. bassiana* where water formulated aliquots of spore suspensions (10^{8}conidia ml^{-1}) were used to inoculate the pest. Mortality ranged between 81 and 100% for all isolates after an incubation period of four days at 25 ^{O}C and although some isolate grew optimally at 20 ^{O}C and 28 ^{O}C, germination assessments indicated that at 25 ^{O}C all strains germinated at between 90 and 100%.

A further aim of the preliminary experiments was to detect differences between isolates in their ability to kill the targeted pest. This was achieved by ranking the isolates according to percentage mortality, so that they were viewed as conferring low, intermediate or high virulence (Adane *et al.*, 1996). An average of 92% mortality was obtained by all *B. bassiana* isolates in the preliminary bioassays against the maize weevil over a four day incubation period and in this case, two indigenous isolates to South Africa were placed in the highly virulent category and one was placed in the low category. This variation in pathogenicity implies that isolates sourced locally confer a range of mortality values against the maize weevil, with the same being observed for the

exotic isolates. In terms of comparing local and exotic strains, it can be concluded that while differences were apparent, all isolates of *B. bassiana* used in this study were highly pathogenic against the maize weevil. With the high mortality levels obtained here, it is likely that cumulative mortality assessments conducted for instance over an 11 day period as was the case with Adane *et al.* (1996), 100% kill rates would have been obtained for all *B. bassiana* isolates used here. When assessing genetic relatedness of *B. bassiana* isolates, De Muro *et al.* (2003) reported that there were no obvious correlations between isolate, host and geographic origin. The same was observed in this case with no pattern emerging between isolates from the same country and their pathogenicity against the maize weevil.

Assessments to screen the insect-pathogen relationship where different conidial concentrations were applied showed that the maize weevil was susceptible to infection at all rates applied. Insect susceptibility was however dependent on conidial concentration where it was observed that mortality levels increased as fungal spore content within the inoculum increased from 10^3 and 10^8 conidia ml^{-1}. The established lethal concentration of *B. bassiana* (isolate PPRI 04307) to kill 50% of adult maize weevils sourced from a robust and random sample was found to be 10^6 conidia ml^{-1} (section 4.3.3). These findings are consistent with those of De La Rosa *et al.* (2002), who obtained similar trends in their results, although they targeted a different pest. Adane *et al.* (1996) used a different assessment method (cumulative percentage mortality) for their evaluation of the effects of different conidial concentration and obtained between 88 and 100% mortality. In their case, the lowest conidial concentration was a 10^4 conidia ml^{-1} application and 88% mortality was achieved over time, however 100% mortality was observed after 14 days for all concentrations. This suggest that lower conidial concentrations of the disease causing agent may take longer to cause the demise of the inoculated insects, but still manage to kill over time. For this study, between 10 and 100% mortality was achieved over a four day inoculation period as conidial concentration increased from 10^3 to 10^8 conidia ml^{-1}, but if an extended incubation period was used, it is likely that higher kill rates would have been obtained at the lower end of conidial concentrations. Furthermore,

it can be postulated that once an insect is infected, disease development would continue until its demise without the insect developing means to resist future infection.

The results presented in section 4.3.3 are the first account reporting the assessment of a South African isolate of *B. bassiana* against the maize weevil, and to determine the effects of different temperatures on fungal virulence on this pest. To gauge the impact of various temperatures on fungal virulence, it was observed that the isolate used (PPRI 04306) was pathogenic and killed the target pest with mortality varying at the different temperatures. The isolate used was most effective at 25 ^{O}C, where mortality was measured at 87% over a four day incubation period (this kill profile was concomitant with that observed in the preliminary bioassay although a slightly lower conidial concentration was used (7.6 x 10^{7} conidia ml^{-1})). At 15 ^{O}C the effects of the fungus were negligible and at 20 ^{O}C, 23% mortality was achieved. Beyond the most effective temperature for mortality, 62% mortality was achieved at 30 ^{O}C. These results and observations indicate that the rate of disease development increases with temperature until the optimum level is reached after which mortality levels began to decline (Dimbi *et al.*, 2004).

Temperature is an abiotic limiting factor that directly affects the rates of sporulation, germination, mycelial growth and the survival of EPF and continually fluctuates throughout the day (Hong *et al.*, 2001; Dimbi *et al.*, 2004). Temperature not only affects and regulates the physiology of the organisms (fungus and insect) but also the ability of the fungus to infect the insect (Dimbi *et al.*, 2004). This was evident at the highest temperature (37 ^{O}C) and at the lowest temperature (15 ^{O}C) evaluated. At these temperatures, fungal development was retarded to a degree that disease development was prohibited. At 37 ^{O}C, all insects died in both control and fungus treated plots, and mycosis did not occur once insects were incubated to stimulate the process. It may therefore be concluded that 37 ^{O}C is too high a temperature to gauge an interaction between the fungal isolate (PPRI 04306) and the pest, as all maize weevils died prior to infection. At the other end of the temperature scale used (15 ^{O}C), disease development was slow with only 1% mortality obtained, implying that fungal physiology was affected

negatively with regards to infectivity over the four day incubation period. Although exposure to low and high temperatures retarded disease development *in vitro*, insects are not exposed to constant temperature under field conditions, but to diurnal and seasonal fluctuations in temperature (Dimbi *et al.*, 2004).

This experiment clearly showed the importance of temperature to regulate the ability of the entomopathogen to infect the target insect. *In situ*, where temperatures fluctuate around the optimum of maximum infectivity, the control agent should work effectively resulting in the eventual reduction in pest fitness, destructive behaviour, and ultimately death as the disease progresses.

Further assessments to determine whether dry fungal spores emanating from mycosed cadavers could be used to infect live maize weevils showed that it may be possible to use such mummified cadavers as carriers for infections propagules. It was observed that all mycosed cadavers included into Petri dishes containing maize seed and live adults had much less conidial coverage after incubation. These observations indicated that emerging spores were dislodged from the cadavers, into the Petri dishes and were free to infect wandering live insects. Furlong and Pell (2001) found that conidia of *B. bassiana* were transmitted at low rates from cadavers of the diamondback moth (*P. xylostella*), but where fungal conidia were dislodged from inoculated live adult moths, these conidia were free to infect uninoculated moths trapped within close proximity to moths inundated with dry spore inoculations. Although transmission rates were not measured in this study, the results show that percentage mortality in the untreated plots was found to be significantly lower than treatments where mycosed cadavers had been included. This is interpreted to represent disease activity within fungus treated plots. This experiment showed that mycosed cadavers may be used as carriers or vectors for *B. bassiana* conidia, where dry conidia are responsible for infecting live insects. Other researchers (Adane *et al.*, 1996; Furlong and Pell, 2001; Dimbi *et al.*, 2003) have also found that dry spores can infect live insects without the requirement of high humidity. Under realistic conditions, it would only remain to determine whether to apply mycosed cadavers or dry spores to lures or substrates as the means to introduce the disease agent for management of the

maize weevil. This experiment represents a first account reporting the use of *B. bassiana*-mycosed maize weevil cadavers as vectors to transmit infectious fungal spores to live adult weevils resulting in high mortality levels of between 73 and 90%.

High mortality levels ranging between 98 and 100% of *S. zeamais* were achieved by Lourenção *et al.* (1993) cited in Oduor *et al.* (2000), who treated maize grain with conidia of *B. bassiana*, suggesting that there is significant promise in pursuing use of EPF to control storage pests. Furthermore, it may be necessary to investigate whether the live pest itself may be used as a vector of the fungal pathogen. Autodissemination of infectious fungal propagules via inoculated live insects or autoinoculative devices would represent innovative approaches to management of insect pests using EPF (Dimbi *et al.*, 2003).

6.2.2 Summary on EPF against *Lasioderma serricorne*

Results obtained from this investigation into the effects of *Beauveria bassiana* on tobacco beetles represent a first account showing that the fungus is pathogenic against the insect species when tested *in vitro*. In this case, fungal isolates indigenous to South Africa and strains sourced internationally (including isolates from Brazil, Kenya and Portugal) were all found to be pathogenic against adult insects. Results showed that where water formulations of the fungus are applied to the pest at 10^8 conidia mm^{-1} under the specified conditions (Chapter 5), high levels of insect mortality can be achieved.

Due to problems encountered rearing the beetle it was not possible to gauge dose response or the effects of different temperature on pathogenicity. Within laboratory based assessments of fungal pathogenicity however, researchers typically use inundative rates of conidial concentrations to gauge whether the target pest is susceptible to infection (Adane *et al.*, 1996; Furlong and Pell, 2001; De La Rosa *et al.*, 2002). Such tests of the interaction between the fungus and the pest at high conidial concentrations form an essential first step in most investigations and provide base line data for further study (Adane *et al.*, 1996; Masuka and Manjonjo, 1996).

In this study, it was possible to assess the effects of two different cultures of the fungus, where fresh (15 day-old) and mature (four months old) cultures were used against the pest in separate bioassays using inundative rates of 10^8 conidia ml^{-1}. In both cases a similar kill profile was obtained between cultures and in both cases the pest was found to be highly susceptible with average mortality ranging between 85 and 100%. These findings indicate that mature fungal cultures of *B. bassiana* are as effective as fresh cultures in causing high levels of mortality. Differences between the two aged cultures became apparent when assessing resultant cadavers for mycosis, and while mycosis (concomitant with mortality) occurred in both cases, mycosed cadavers from the mature culture inoculations showed diminished fungal growth compared with the mycosis from fresh culture inoculations. These observations suggest that mature cultures of *B. bassiana* possess a reduced capacity to reproduce through the insect upon host death. Further research on this phenomenon is required (J. Lord, pers. comm.).

6.3 Conclusions

Beauveria bassiana is an important biological control agent of a wide range of arthropod pests of agricultural produce for use both *in situ* (Furlong and Pell, 2001; De La Rosa *et al.*, 2002; Dimbi *et al.*, 2003) and in storage (Adane *et al.*, 1996; Oduor *et al.*, 2002). Its potential to be developed as a mycoinsecticide has been realised internationally and ongoing efforts to develop *B. bassiana*-based bioinsecticides further illustrate a concerted drive to source alternatives to chemical insecticides (Castrillo *et al.*, 2003; De Muro, *et al.*, 2003). In this study, base-line data on the effects of *B. bassiana* were evaluated against two important coleopteran insect pests *in vitro*; tests which represent the essential first step in uncovering the potential of sourced fungal isolates and developing bioinsecticide (Goettel, 1984; Goettel and Inglis, 1997). Native strains of *B. bassiana* were found to perform equal to or better than exotic isolates against the maize weevil (*Sitophilus zeamais*) and the tobacco beetle (*Lasioderma serricorne*) in terms of their ability to cause high levels of mortality, particularly where high conidial concentrations were used to infect the insects (Masuka, and Manjonjo, 1996). This point highlights that native strains of *B. bassiana* can be harnessed and used to equal effect against local pest

problems without the need to import exotic EPF. In the case of the maize weevil, local *B. bassiana* isolates performed similarly or better than exotic isolates and were selected for use in experiments to determine the effects of different conidial concentrations and temperatures on pest mortality. From this set of experiments, it can be postulated that high mortality levels may have been achieved at lower concentrations of fungal inoculum if insects were incubated for longer. These observations further imply that at a constant temperature or as temperatures fluctuate around the optimum, *B. bassiana* is highly effective at infecting insects when conidial concentration range between 10^3 to 10^8 conidia ml^{-1} and are applied in the form of water formulations.

Overall from the current study, several aspects and results represent first accounts of their kind in the following respects

i. Isolation of *Beauveria bassiana* from the Northern Province of South Africa using the *Galleria* bait method.
ii. Comparison of vegetative radial growth of *B. bassiana* sourced from South African soils with international isolates including strains from Brazil, Kenya and Portugal.
iii. Demonstration of pathogenicity of the entomopathogen *B. bassiana* sourced in South Africa against two Coleopteran pests (*Sitophilus zeamais* and *Lasioderma serricorne*) also endemic to this country. Comparisons of pathogenicity were also made between local and exotic isolates of the fungus when applied as water formulations of 10^8 conidia ml^{-1}.
iv. Demonstration of the pathogenicity of indigenous isolates of *B. bassiana* against the maize weevil (*Sitophilus zeamais*) at different conidial concentrations and temperatures.
v. Demonstration of ability of *B. bassiana*-mycosed cadavers to operate as carriers, and transmit infectious spores to live insects resulting in high levels of mortality.
vi. Demonstration of pathogenicity of *B. bassiana* (both local and exotic strains) against the tobacco beetle (*Lasioderma serricorne*) using conidial concentrations of 10^8 conidia ml^{-1}.

B. bassiana host specificity was confirmed to encompass the two target pests and the point that local isolates to South Africa produced similar results as those obtained for exotic *B. bassiana* confirms that native strains are as effective and may be used to develop bioinsecticides. The cascade of problems arising from the unsustainable use of synthetic pesticides has provided strong impetus to researchers to develop natural, safer and more cost effective means for insect pest control and this project investigated the potential of entomopathogenic fungi to control two Coleopteran insect pest species. The use of microbial biological control agents by researchers seeking to develop natural methods for crop protection represents a major resurgence in interest in the development of alternative products for use against insect pest infestations (Goettel, 1984; Goettel and Inglis, 1997; De La Rosa *et al.*, 2002; Dimbi *et al.*, 2003; Dimbi *et al.*, 2004). It remains important to merge ideas and aspects from a multi disciplinary stand point, for instance, the ecology of EPF, their use within particular environments and their marketability in the development of commercial biological control agents (Chandler, 2005). Owing to the observation that most crop or commodity storage facilities may be home to several post harvest pest species (Stejskal *et al.*, 2002), the broad host spectrum killing nature exhibited by *Beauveria bassiana* makes this EPF a perfect biological agent within a range of insect pest infestation scenarios. Future research would include aspects discussed by Goettel (1984) such as mass production and by other researchers, such as the development of appropriate technology for dispersal of microbial insect control agents for use *in situ* (De La Rosa *et al.*, 2002; Dimbi *et al.*, 2003). Field application of *B. bassiana* appears to pose no major risk to the environment and the end user as several commercial *B. bassiana*-based mycoinsecticides are available internationally (Castrillo *et al.*, 2003) and it remains up to African scientists to continue the search and evaluation of insect pathogenic fungi for use as alternatives to chemical insecticides.

References

Adane, K., Dietrich, S., Stefan, V. and Gisbert, Z. (2004) Production and processing of *Metarhizium anisopliae* var. *acridum* submerged conidia for locust and grasshopper control. *Mycological Research* **108**: 93-100.

Adane K., Moore, D. and Archer, S. A. (1996) Preliminary study on the use of *Beauveria bassiana* to control *Sitophilus zeamais* (Coleoptera: Curculionidae) in the laboratory. *Journal of Stored Product Research* **32**: 105-113.

Adler, C. (2002) Efficacy of high temperatures to control *Lasioderma serricorne* and *Rhyzopertha dominica* Working Group 'Integrated Protection in Stored Products' Proceedings of the meeting in Lisbon (Portugal), 3 – 5 September, 2001. Edited by: C. Adler, S. Navarro, M. Schöller and L. Stengard-Hansen. IOBC/wprs Bulletin Vol. **25**

Anderson, T. E. and Roberts, D. W. (1983) Compatibility of *Beauveria bassiana* isolates with insecticide formulations used in Colorado potato beetle (Coleoptera: Chrysomelidae) Control. *Journal of Economic Entomology* **76**: 1437-1441.

Ashworth, J. R. (1993a) The biology of *Ephestia elutella*. *Journal of Stored Product Research* **29**: 199-205.

Ashworth, J. R. (1993b) The biology of *Lasioderma serricorne*. *Journal of Stored Product Research* **29**: 291-303.

Barnett, H. L. and Hunter, B. B. (1972) Illustrated Genera of Imperfect Fungi. Third Edition, Burgess Publishing Company.

Barson, G., Renn, N. and Bywater, A.F. (1994) Laboratory evaluation of six species of entomopathogenic fungi for the control of the housefly (*Musca domestica*) a pest of intensive livestock units. *Journal of Invertebrate Pathology* **64**: 107-113.

Baumgärtner, J., Schulthess, F. and Xia, Y. (2003) Integrated arthropod pest management systems for human health improvement in Africa. *Insect Science and its Application* **23**: 85–98.

Bell, C. H. (1992) Time, concentration and temperature relationships for phosphine activity in test on diapausing larvae of *Epestia elutella* (Hübner) (Lepidoptera: Pyralidae). *Pesticide Science* **35**: 255-264.

Boucias, D. and Pendland, J., (1998) Principles of Insect Pathology: Insect-Pathogen Relationships. Chapter 8 General Properties or Fungal Pathogens. Kluwer Academic Publishers.

Burges, H. and Hussey, N. (1971) Microbial Control of Insects & Mites. Academic Press.

Castrillo, L. A., Wiegmann, B. M. and Brooks, M. (1999) Genetic variation in *Beauveria bassiana* populations associated with the Darkling beetle, *Alphitobius diaperinus*. *Journal of Invertebrate Pathology* **73**: 269-275.

Castrillo, L. A., Vanderberg, J. D. and Wraight, S. P. (2003) Strain-specific detection of introduced *Beauveria bassiana* in agricultural fields by use of sequence-characterized amplified region markers. *Journal of Invertebrate Pathology* **82**: 75-83.

Castillo, M-A., Moya, P., Hernandez, E. and Primo-Yufera, E. (2000) Susceptibility of *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) to entomopathogenic fungi and their extracts. *Biological Control* **19**: 274-282.

Caulder, J. (1999) The North American Scenario. In *Biopesticides Use and Delivery. Methods in Biotechnology* (F. R. Hall and J. J.Menn, eds): 13-21. Humana Press.

Chandler, D. (2005) Understanding the evolution and function of entomopathogenic fungi. Evolution of fungal virulence. Warwick HRI, University of Warwick, Wellesbourne, Warwick CV35 9EF, UK. http://www2.warwick.ac.uk/fac/sci/hri2/research/entomopathogenicfungi_/unders1.pdf.pdf

De La Rosa, W., Lopez, F. L. and Liedo, P. (2002) *Beauveria bassiana* as a pathogen of the Mexican fruit fly (Diptera: Tephritidae) under laboratory conditions. *Journal of Economic Entomology* **95**: 36-43.

De Muro, M. A., Mehta, S. and Moore, D. (2003) The use of amplified fragment length polymorphism for molecular analysis of *Beauveria bassiana* isolates from Kenya and other countries, and their correlation with host and geographic origin. *Microbiology Letters* **229**: 249-257.

Dimbi, S., Maniania, N. K., Lux, S. A., Ekesi, S. and Mueke, J. K. (2003) Pathogenicity of *Metarhizium anisopliae* (Metsch.) (Sorokin) and *Beauveria bassiana* (Balsamo) (Vuiillemin) to three adult fruit fly species: *Ceratitis capitata* (Weidemann), *C. rosa* var. *fasciventris* (Karsch) and *C. cosyra* (Walker) (Diptera: Tephritidae). *Mycopathologia* **156**: 375-382.

Dimbi, S., Maniania, N. K., Lux, S. A. and Mueke, J. M. (2004) Effects of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African tephritid fruit flies. *Biological Control* **49**: 83-94.

Dixon, A. F. G. (2000) Insect Predator-Prey Dynamics, Ladybird Beetles & Biological Control. Cambridge University Press.

Ekesi, S. (2001) Pathogenicity and antifeedant activity of entomopathogenic hyphomycetes to the cowpea leaf beetle, *Ootheca mutabilis* (Shalberg) *African Journals On Line Insect Science and its Application. The International Journal of Tropical Insect Science. http://www.inasp.info*.

Evans, H. F. (2000) Viruses: Section IV – Overview of Pathogen Groups. In *Field Manual of Techniques in Invertebrate Pathology.* (L. A. Lacey and H. K. Kaya, eds): 179-208. Kluwer Academic Press.

Ferron, P. (1981) Pest Control by the Fungi *Beauveria* and *Metarhizium*. In *Microbial Control of Pests and Plant Diseases 1970-1980*. (H. D. Burges, ed) Chapter 24: 465-476. Academic Press.

Furlong, M. J. and Pell, J. K. (2001) Horizontal transmission of entomopathogenic fungi by the Diamond back moth. *Biological Control* **22**: 288-299.

Goettel, M. S. (1984) A simple method for mass culturing entomopathogenic Hyphomycete fungi. *Journal of Microbiological Methods* **3**: 15-20.

Goettel, M. S. and Hajek, A.E. (2001) Evaluation of Non-target Effects of Pathogens Used for Management of Arthropods. In *Evaluation Indirect Ecological Effects of Biological Control* (E.Wajnberg, J. K. Scott, and P. C. Quimby, eds): 81-97. CBAI Publishing, France.

Goettel, M. S. and Inglis, D. G. (1997) Fungi: Hyphomycetes. In *Manual of Techniques in Insect Pathology* (L.A. Lacey, ed), Chapter V-3: 213-249 Academic Press.

Graves, J. B., Rogers Leonard, B. and Ottea, J. A. (1999) Chemical Approaches to Managing Arthropod Pests. In *Handbook of Pest Management* (J. R. Ruberson, ed): 449-486. Marcel Dekker, Inc.

Greathead, D. J. (2003) Historical Overview of Biological Control in Africa. In *Biological Control in IMP Systems in Africa.* (P. Neuenschwander, C. Borgemeister, and J. Langewald, eds): 1-26. CABI Publishing.

Hallsworth, J. E. and Magan, N. (1999) Water and temperature relations of growth of the entomogenous fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces farinosus*. *Journal of Invertebrate Pathology* **74**: 261-266.

Hemmati, F., Pell, J. K., McCartney, H. A., Clark, S. J. and Deadman, M. L. (2001) Conidial discharge in the aphid pathogen *Erynia neoaphidis*. *Mycological Research* **105**: 715-722.

Herren, H. R, (2003) Forward. In *Biological Control in IMP Systems in Africa*. (P. Neuenschwander, C. Borgemeister, and J. Langewald, eds): xxi. CABI Publishing.

Hollomon, D. W. and Butters, J. A. (1994) Molecular determinants for resistance to crop protection chemicals. In *Molecular Biology in Crop Protection. Part two*. (G. Marshell, and D. Walters, eds): 98-115. Chapman & Hall.

Hong, T. D., Gunn, J., Ellis, R. H., Jenkins, N. E. and Moore, D. (2001) The effects of storage environment on the longevity of conidia of *Beauveria bassiana*. *Mycological Research* **105**: 597-602.

Jacobs, S. and Calvin, D. (1988) Weevils on Stored Grain. Entomological Note, College of Agricultural Science, Pennsylvania State University US Department of Agriculture 2001.

Kaaya, G. P., Mwangi, E. N. and Ouna, E. A. (1996) Prospects for biological control of livestock ticks *Rhipicephalus appendiculatus* and *Amblyomma variegatum*, using the entomogenous fungi *Beauveria bassiana* and *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* **67**: 15-20.

Kfir, R. (2003) Biological Control of the Diamondback Moth *Plutella xylostella* in Africa. In *Biological Control in IMP Systems in Africa* (P. Neuenschwander, C. Borgemeister, and J. Langewald, eds): 363-376. CABI Publishing.

Khetan, S.K. (2001) Microbial Pest Control. Marcel Dekker Inc.

Langewald, J., Stolz, I., Everts, J. and Peveling, R. (2003) Towards the registration of microbial insecticides in Africa: Non-target Arthropod testing on Green Muscle™, a grasshopper and locust control product based on the fungus *Metarhizium anisopliae* var. *acridum*. In *Biological Control in IPM Systems in* Africa. (P. Neuenschwander, C. Borgemeister, and J. Langewald, eds): 207-225. CABI Publishing.

Lazzarini, G. M., Rocha, L. F. N. and Luz, C. (2006) Impact of moisture on *in vitro* germination of *Metarhizium anisopliae* and *Beauveria bassiana* and their activity on *Triatoma intestans*. *Mycological Research* **110**: 485-492.

Leger, St. R. J., Durrands, P. K., Charnley, A. K. and Cooper, R. M. (1988) Role of extracellular chymeolastase in the virulence of *Metarhizium anisopliae* for *Manduca sexta* (tobacco hornworm). *Journal of Invertebrate Pathology* **52**: 285-293.

Leger, St. R. J., Staples, C. and Roberts, D. W. (1993) Entomopathogenic isolates of *Metarhizium anisopliae*, *Beauveria bassiana* and *Aspergillus flavus* produce multiple extracellular chitinase isozymes. *Journal of Invertebrate Pathology* **61**: 81-84.

Longstaff, B. C. (1981) Biology of the grain pest species of the genus *Sitophilus* (Coleoptera: Curculionidae): A critical review. *Protection Ecology* **2**: 83-130.

Lord, J. C. (2005) From Metchnikof to Monsanto and beyond: The path of microbial control. *Journal of Invertebrate Pathology* **89**: 19–29.

Lux, S. A., Ekesi, S., Dimbi, S., Mohamed, S. and Billah, M. (2003) Mango-infesting Fruit Flies in Africa: Perspectives and Limitations of Biological Approaches to their Management. In *Biological Control in IPM Systems in Africa*. (P. Neuenschwander, C. Borgemeister, and J. Langewald, eds): 277-293. CABI Publishing.

Mahr, S. (1997) University of Wisconsin- Madison – Midwest Biological Control News Online. Volume IV Number 10; (http://www.entomology.wisc.edu/mbcn/mbcn410.html).

Mashaya, N. (2000) Tobacco Moth Situation in Zimbabwe. *Zimbabwe Tobacco* **9:** *No. 9 2000 Journal of the Zimbabwe Tobacco Industry*. Registered At The GPO as a Newspaper 2000.

Masuka, A. J. and Manjonjo, V. (1996) Laboratory screening of *Metarhizium flavoviride* and *Beauveria bassiana* for the control of *Mecostibus pinivorus*, a *Punis patula* defoliator in Zimbabwe. *Journal of Applied Science in Southern Africa* **2**: 91-95.

Massey, E. D. (1999) Stored Tobacco: Insects and their control. In *Tobacco: Production, Chemistry and Technology* (D. L. Dayten, and N. T. Nielsen, eds): 241-249. Blackkwell Science, Oxford, U.K. Eian.

Metcalf, R. L. (1999) Arthropods as Pests of Plants: An Overview. In *Handbook of Pest Management*. (J. R. Ruberson, ed): 377 -394. Marcel Dekker Inc.

© Mycologue publications 2002 http://www.mycolog.com/CHAP4a.htm http://www.mycolog.com/blastic2.gif.

Nardini, L. (2002) Culture and Testing of Entomopathogenic Hyphomycetes Which May be used as Potential Biological Control Agents. BSc Hons. Thesis University of the Witwatersrand.

Neuenschwander, P., Borgemeister, C. and Langewald, J. (2003) Biological Control in IPM Systems in Africa. CABI Publishing.

Oduor, G. I., Smith, S. M., Chandi, E. A., Karanja, L. W., Agano, J. O. and Moore, D. (2000) Occurrence of *Beauveria bassiana* on insect pests of stored maize in Kenya. *Journal of Stored Product Research* **36**: 177-185.

Ojimelukwe, P. C. and Adler, C. (2002) Potentials of cinnamaldehyde and methylchavicol as grain protectants against four insect pests of stored products. Working Group Integrated Protection in Stored Products", Proceedings of the meeting in Lisbon (Portugal), 3 – 5 September, 2001. Edited by: C. Adler, S. Navarro, M. Schöller & L. Stengard-Hansen. IOBC/wprs Bulletin Vol. **25**

Pell, J. K., Wilding, N., Player, A. L. and Clark, S. J. (1993) Selection of an isolate of *Zoophthora radicans* (Zygomycetes: Entomophthorales) for biological control of the diamondback moth *Plutella xylostella* (Lepidoptera: Yponomeutidae). *Journal of Invertebrate Pathology* **61**: 75-80.

Perkins, J. H. (1982) Insects, Experts, and the Insecticide Crisis: The Quest for New Pest Management Strategies. Plenum Press New York.
Pest Control Portal: http://www.pestcontrolportal.com/pests/pestScript1.asp?id=20).

Possee, R. D. and King, L. A. (1994) Molecular Approach to the Design of Biotic Crop Protection Agents. In *Molecular Biology in Crop Protection*. (G. Marshall, and D. Walters, eds): 68. Chapman and Hall.

Roberts, D. W. and Yendol, W. G. (1971) Use of Fungi for Microbial Control of Insects. In *Microbial Control of Insects and Mite*. (H. D. Burges, and N. W Hussey, eds): 125-146. Academic Press.

Robinson R. (1971) International Series of Monographs in Pure and Applied Biology, Zoology Division, Volume 46 – Lepidoptera Genetics, Introduction: 20. Pergamon Press.

Ruberson, J. R., Nechols, J. R. and Tauber, M. J. (1999) Biological Control of Arthropod Pests. In *Handbook of Pest Management*. (J. R. Ruberson ed): 417-448. Marcel Dekker Inc.

Russell, P. (2001) Resistance management and the registration of pesticide products in Europe. *Pesticide Outlook* **April**: 56-59.

Sallam, M. N. (1994) Chapter II Insect Damage: Damage on Post-harvest
Edited by AGSI/FAO: Danilo Mejia (Technical), Beverly Lewis (Language&Style), Carolin Bothe (HTML transfer) Organisation: International Centre of Insect Physiology and Ecology (ICIPE) (http://www.icipe.org/).
http://www.fao.org/inpho/content/compend/text/ch02-01.htm.

Samson, R. A., Evans, H. C. and Latgé, J-P. (1988) Atlas of Entomopathogenic Fungi. Springer-Verlag.

Shah, P. and Pell, J. K. (2003) Entomopathogenic fungi as biological control agents. *Applied Microbiology and Biotechnology* **61**: 413-423.

Siegel, J. P. (2000) Bacteria: Section IV – Overview of Pathogen Groups. In *Field Manual of Techniques in Invertebrate Pathology*. (L. A. Lacey and H. K. Kaya eds): 209-230 Kluwer Academic Press.

Small, C-L. N. and Bidochka, M. J. (2005) Up-regulation of Pr1, a subtilisin-like protease, during conidiation in the insect pathogen *Metarhizium anisopliae*. *Mycological Research* **109**: 307-313.

Smits, N., Brière, J-F. and Fargues, J. (2003) Comparison of non-linear temperature-dependent development rate models applied to *in vitro* growth of entomopathogenic fungi. *Mycological Research* **107**: 1476-1484.

Stejskal, V., Hubert, J. and Luká, J. (2002) Species richness and pest control complexity: Will multispecies infestations always require a multi-bioagent control? Working Group Integrated Protection in Stored Products"Proceedings of the meeting in Lisbon (Portugal), 3 – 5 September, 2001. Edited by: C. Adler, S. Navarro, M. Schöller & L. Stengard-Hansen. IOBC/wprs Bulletin Vol. **25**

Steinkraus, D. C. (2002) Documentation of Naturally-Occurring Pathogens and Their Impact in Agroecosystems. In *Field Manual of Techniques in Invertebrate Pathology*. (L. A. Lacey, and H. K Kaya, eds): 303-320. Kluwer Academic Press.

Talbot, P. H. (1971) Principles of Fungal Taxonomy. The Macmillan Press LTD.

Taylor, S. H. (2002) A study of entomopathogenic nematodes in the greater Johannesburg region. MSc Degree. Thesis University of the Witwatersrand.

Tobacco Research Board: Hand Book for Flue Cured Tobacco: All sections

Throne, J.E. and Eubanks, M.W. (2002) Resistance of Tripsacorn to *Sitophilus zeamais* and *Oryzaephilus surinamensis*. *Journal of Stored Product Research* **38**: 239-245.

Tribe, G. D. (2003) Biological Control of Defoliating and Phloem- or Wood-feeding Insects in Commercial Forestry in South Africa. In *Biological Control in IPM Systems in Africa*. (P. Neuenschwander, C. Borgemeister, and J. Langewald, eds): 113-129. CABI Publishing.

Tsuchiya, S., Kashaishi, Y., Harada, H., Ichimatsu, T., Saitoh, H., Mizuki, E. and Ohba, M. (2002) Assessment of the efficacy of Japanese *Bacillus thuringiensis* isolates against the cigarette beetle, *Lasioderma serricorne* (Coleoptera: Anobiidae) *Journal of Invertebrate Pathology* **81**: 122-126.

Van Driesche, R. G. and Bellows Jr, T. S. (1996) Biological Control. Chapman and Hall.

Wang, C., Fan, M., Li, Z. and Butt, T. M. (2004) Molecular monitoring and evaluation of the application of the insect-pathogenic fungus *Beauveria bassiana* in southeast China. *Journal of Applied Microbiology* **96**: 861-870.

Watson, D. W. and Patersen, J. J. (1993) Sexual activity of male *Musca domestica* (Diptera: Muscidae) infected with *Entomophthora muscae* (Entomophthoraceae: Entomophthorales). *Biological Control* **3**: 22-26.

Wildey, K. B., Cox, P. D., Wakefield, M., Price, N. R., Moore, D. and Bell, B. A. (2002) The use of entomopathogenic fungi for stored product pest control – The "MYCOPEST" project. Working Group Integrated Protection in Stored Products "Proceedings of the meeting in Lisbon (Portugal), 3 – 5 September, 2001. Edited by: C. Adler, S. Navarro, M. Schöller & L. Stengard-Hansen. IOBC/wprs Bulletin Vol. **25**

www.bugspray.com/article/cigarettebeetle.html. All articles copy righted by U-Spray, Inc.4653 Highway 78 Lilburn, Georgia 30047. Phone: (770)985-9388.

Zimmermann, G. (1986) The 'Galleria bait method' for detection of entomopathogenic fungi in soil. *Journal of Applied Entomology* **102**: 213-215.

APPENDICES:

Appendix 1 – Media Preparations

Appendix 1.1 Sabouraud dextrose agar 4% (Biolads) (SDA)
Suspend 60 grams in 1 litre distilled water and stir until well dissolved (heat if necessary to aid complete dissolution). Sterilised by autoclaving at 121^{O}C for 15 minutes. Cool rapidly and pour plates.

Appendix 1.2 Semi-selective media – SDA + chloramphenicol (SDA + C)
Follow preparation procedure for SDA and prior to autoclaving add 400mg chlorophenicol.

Appendix 1.3 Supplemented medium – SDA + yeast extract (Biolads) + glucose (Saarchem) (SDA + Y + G)
Follow preparation procedure for SDA and prior to autoclaving and 2 g yeast extract powder and 20 g glucose.

Appendix 2 – ANOVA Tables (SAS Enterprise guide 3.0)

Appendix 2.1 Effect of varying temperatures on growth rate (mm day^{-1}) of 9 EPF isolates cultured on SDA media. One way ANOVA for effects of temperature on overall fungal growth rates

Dependent Variable: Slope

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	37.94086811	9.48521703	30.51	<.0001
Error	175	54.40398450	0.31087991		
Corrected Total	179	92.34485261			

Two way interaction

SOURCE	DF	TYPE III SS	MEAN SQUARE	F VALUE	PR > F
Isolate	8	25.4824	3.18530596	85.67	<.0001
Temperature	4	37.94086811	9.48521703	255.10	<.0001
Isolate*Temp.	32	23.90201663	0.74693802	20.09	<.0001

Analyzed at the 5% significance level

Appendix 2.1.2 Slope data and regression equations

Please refer to attached spread sheet.

Appendix 2.2 Maize weevil mortality from inundative water formulation inoculations of *B. bassiana* **-** Preliminary Bioassays

Arcsin transformed data for mean percentage mortality and mycosis

Isolate	Mean of MortalityArcSin	Mean of MycosisArcSin
IMI 382 302	1.422±0.08 a	1.422±0.08 a
IMI 386 694	1.320±0.14 ab	1.320±0.14 ab
IMI 386 696	1.323±0.15 ab	1.323±0.15 ab
IMI 386 701	1.475±0.06 a	1.475±0.06 a
IMI 382 724	1.137±0.13 ab	1.137±0.13 ab
PPRI 04305	1.475±0.06 a	1.475±0.06 a
PPRI 04306	0.994±0.09 b	0.994±0.09 b
PPRI 04307	1.571±0 a	1.571±0 a

Note ± = SE. SNK multiple comparison test - within column means with the same letter are not significantly different.

Dependent Variable: Number of dead insects

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	103.1093750	14.7299107	1.83	0.0995
Error	56	450.8750000	8.0513393		
Corrected Total	63	553.9843750			

Dependent Variable: Percentage ArcSinMortality

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	2.06428054	0.29489722	3.52	0.0033
Error	56	4.68937596	0.08373886		
Corrected Total	63	6.75365650			

Dependent Variable: Percentage ArcSinMycosis

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	2.06428054	0.29489722	3.52	0.0033
Error	56	4.68937596	0.08373886		
Corrected Total	63	6.75365650			

Analyzed at the 5% significance level

Appendix 2.3 Effects of different conidial concentrations on fungal virulence against the maize weevil

Arcsin transformed data for mean percentage mortality and mycosis

Conidia/ml	Mean Number of Dead Insects	Mean of MortalityArcSin	Mean of MycosisArcSin
10^8	20±0 a	1.571±0 a	1.571±0 a
10^7	18±0.6 b	1.273±0.11 b	1.273±0.11 b
10^6	10±1.4 c	0.574±0.08 c	0.548±0.10 c
10^5	6±0.9 d	0.332±0.05 d	0.305±0.03 d
10^4	3±0.7 d	0.150±0.04 d	0.125±0.25 de
10^3	2±0.8 d	0.100±0.04 d	0±0 e

Note ± = SE and within column means with the same letter are not significantly different calculated using the SNK multiple comparison test.

Dependent Variable: Dead Insects

Source	DF	Sum of Squares	Mean Square	f value	Pr > F
Conidia/ml	5	1208.833333	241.766667	86.17	<.0001
Error	18	50.500000	2.805556		
Corrected Total	23	1259.333333			

Dependent Variable: Percentage ArcsinMortality

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Conidia/ml	5	7.57064585	1.51412917	90.97	<.0001
Error	18	0.29958164	0.01664342		
Corrected Total	23	7.87022749			

Dependent Variable: Percentage ArcsinMycosis

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Conidia/ml	5	8.24990613	1.64998123	98.76	<.0001
Error	18	0.30073143	0.01670730		
Corrected Total	23	8.55063755			

Analyzed at the 5% significance level

Appendix 2.4 Effect of temperature on mean insect death. One-way ANOVA carried out for effects of treatments (fungus and control) and by temperature.

One-way ANOVA table comparing fungal treatment and control (by isolate)

Means with the same letter are not significantly different.				
SNK Grouping	**Mean ArcSinMortality**	**Mean ArcSinMycosis**	**N**	**Isolate**
A	0.7405±0.139	0.3908±0.11	20	MCBG121
B	0.3167±0.143	0.0000±0	20	Control

Dependent Variable: ArcSinMortality by isolate

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treatment	1	1.79661832	1.79661832	4.46	0.0413
Error	38	15.30873262	0.40286138		
Corrected Total	39	17.10535094			

Dependent Variable: ArcSinMycosis by isolate

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treatment	1	1.52737101	1.52737101	11.60	0.0016
Error	38	5.00447994	0.13169684		
Corrected Total	39	6.53185096			

Dependent Variable: ArcSinMortality by temperature

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Temperature	4	12.40236672	3.10059168	23.07	<.0001
Error	35	4.70298422	0.13437098		
Corrected Total	39	17.10535094			

Dependent Variable: ArcSinMycosis by temperature

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Temperature	4	1.92715675	0.48178919	3.66	0.0136
Error	35	4.60469421	0.13156269		
Corrected Total	39	6.53185096			

Arcsin transformed data for mean percentage mortality and mycosis by temperature

Temperature	Mean of MortalityArcSin PPRI 04306	Mean of MmycosisArcSin PPRI 04306
15	0.006±0.01 c	0±0 b
20	0.120±0.06 cb	0.082±0.04 b
25	0.569±0.23 b	0.558±0.22 a
30	0.376±0.17 cb	0.337±0.18 ab
37	1.571±0 a	0±0 b

SNK multiple comparison test - Means with the same letter are not significantly different. ± = SE
Analyzed at the 5% significance level

Appendix 2.5 Maize weevil mortality from infection by dry conidia

Arcsin transformed data for mean percentage mortality

Treatments	Mean of ArcSinMortality	Mean of Seed Weight (g)
Control	0.16±0.04 b	7.8 ± 0.1 a
IMI 386 701	0.82±0.05 a	8.6 ±0.4 a
PPRI 04306	1.20±0.14 a	8.8 ±0.3 a
PPRI 04307	0.96±0.13 a	9.0 ±0.3 a

SNK multiple comparison test - Means with the same letter are not significantly different. ± = SE

Dependent Variable: ArcSinMortality

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treatments	3	2.40826388	0.80275463	19.83	<.0001
Error	12	0.48585356	0.04048780		
Corrected Total	15	2.89411745			

Dependent Variable: Seed weight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	3.21687500	1.07229167	3.21	0.0618
Error	12	4.00750000	0.33395833		
Corrected Total	15	7.22437500			

Analyzed at the 5% significance level

Appendix 2.6 Tobacco beetle pilot study

One-way Analysis of Variance on % mortality arcsin transformed data

Isolate	Mean of MortalityArcSin	Mean of MycosisArcSin
IMI 386 696	1.28±0.04 a	1.28±0.03 a
PPRI 04305	1.45±0.12 a	1.45±0.12 a
PPRI 04306	1.39±0.09 a	1.39±0.09 a
PPRI 04307	1.48±0.09 a	1.48±0.09 a

SNK multiple comparison test - Means with the same letter are not significantly different. ± = SE

Dependent Variable: Mortality (arcsin transformed data)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model/Isolate	3	0.07470822	0.02490274	1.06	0.4165
Error	8	0.18709895	0.02338737		
Corrected Total	11	0.26180717			

Dependent Variable: Mycosis (arcsin transformed data)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model/Isolate	3	0.07470822	0.02490274	1.06	0.4165
Error	8	0.18709895	0.02338737		
Corrected Total	11	0.26180717			

Analyzed at the 5% significance level

Appendix 2.7 Tobacco Beetle mortality data from inundative inoculation of *B. bassiana* using a fresh culture

Fresh Culture: One-way ANOVA on % mortality and mycosis arcsin transformed data

Isolate	Mean of MortalityArcSin	Mean of MycosisArcSin
IMI 386 696	1.57±0 a	1.57±0 a
PPRI0 4306	1.57±0 a	1.57±0 a
PPRI0 4307	1.57±0 a	1.57±0 a
IMI 386 701	1.57±0 a	1.57±0 a
IMI 382 302	1.39±0.11 ab	1.39±0.11 ab
PPRI 04305	1.35±0.08 b	1.35±0.08 b
IMI 386 694	1.04±0.1 c	1.04±0.1 c

Duncan's multiple range test - Means with the same letter are not significantly different. ± = SE

Fresh Culture Dependent Variable: Percentage Mortality (arcsin data)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	0.95404586	0.15900764	10.06	<.0001
Error	21	0.33192018	0.01580572		
Corrected Total	27	1.28596604			

Fresh Culture Dependent Variable: Percentage Mycosis (arcsin data)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	0.95404586	0.15900764	10.06	<.0001
Error	21	0.33192018	0.01580572		
Corrected Total	27	1.28596604			

Appendix 2.8 Tobacco Beetle mortality data from inundative inoculation of *B. bassiana* using a mature culture

Mature Culture: One-way ANOVA on % mortality and mycosis arcsin transformed data

Isolate	Mean of MortalityArcSin	Mean of MycosisArcSin
IMI 386701	1.57±0 a	0.91±0.24 c
IMI 386 696	1.57±0 a	1.46±0.11 ab
PPRI 04307	1.57±0 a	1.57±0 a
PPRI 04305	1.51±0.06 a	1.07±0.23 bc
PPRI 04306	1.51±0.06 a	1.57±0 a
IMI 382 302	1.4±0.11 a	1.4±0.11 ab
IMI 386 694	1.04±0.1 b	1.04±0.1 bc

Duncan's multiple range test - Means with the same letter are not significantly different. ± = SE

Mature Culture Dependent Variable: MortalityArcSin

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	0.88441251	0.14740208	8.61	<.0001
Error	21	0.35938714	0.01711367		
Corrected Total	27	1.24379964			

Mature culture Dependent Variable: MycosisArcSin

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	1.79910521	0.29985087	3.63	0.0125
Error	21	1.73275264	0.08251203		
Corrected Total	27	3.53185785			

Appendix 3 – Insect Diets

Greater wax moth (*Galleria mellonella*): reared on artificial diet formulated as follows:
500 grams – Pronutro
200 ml – glycerol
200 ml – honey
500 ml sterile water
5 tea spoons yeast extract
1.5 ml 1M hydrochloric acid (HCl)
75 mg – Ascorbic acid
Mix above ingredients thoroughly in autoclave container and autoclave at $121^{O}C$ for 20 minutes and 15 psi. Allow to cool overnight prior to transfer to appropriate container in which to culture the insects (Taylor, 2002).

Maize weevil (*Sitophilus zeamais*): this insect was reared on Brennco yellow maize seed. Hall fill 2 litre glass jar with seed and add live insects. Ensure jar lid is perforated for aeration.

Tobacco beetle (*Lasioderma serricorne)*: this insect was reared on artificial diet formulated as follows:
500 g – wheat free Pronutro
100 g – powdered milk (Cremora)
Mix ingredients well in 2 litre glass jars and add live insects. Ensure jar lid is perforated for aeration.

Appendix 4 – Personal Communications

1. Email communication with Jeff Lord regarding the following.

Dear Maz (author)

I am surprise that you had trouble rearing enough beetles. We get good production on wheat flour with or without brewer's yeast. I should mention that we have treated only larvae with *Beauveria*. As far as I know, there has been no published report on cigarette beetles and fungi to date, although I expect to have a manuscript which includes them before the end of the year. So try to get yours out soon.

I can see no reason that conidia that are similarly pathogenic should cause different mycosis patterns, but older and drier conidia will germinate more slowly than fresh, hydrated conidia and would therefore be slower in initiating infection.

Good luck with your work.

Regards
jeff

From: Masiyiwa Ngoni Sakupwanya [mailto:Masiyiwa@gecko.biol.wits.ac.za]
Sent: Monday, June 05, 2006 12:42 PM
To: Jeff Lord jeffrey.lord@gmprc.ksu.edu
Subject: Hello- second contact.

Good day to you Sir, it is once again a pleasure and honour to write to you. This time may I begin by congratulating you on a great paper (*From Metchk. to Monsanto, Journal of Invertebrate pathology 2005*)

I am writing once again in relation to the tobacco beetle (*Lasioderma serricorne*). The last time we communicated I noted to you that I will be attempting to infect this insect with *B. bassiana in vitro*.

I have since completed that section of my current work (although I only have results from experiments where applications at one conidial concentration were conducted) and I was wondering if the case remains the same that there are no published works evaluating this entomopathogen against this insect.

I was disappointed that I was unable to rear sufficient numbers of the beetle to conduct the full spectrum of typical experimental evaluations but I have made an observation.

I noticed that cultures plates maintained for longer at room tepm remained highly pathogenic, but did not cause similar mycosis patterns when compared to a freshly prepped culture inoculum (water formulations). I am now attempting to make a chapter of these finds.

Please help me with any information you may have with regards to such work. I hope to make notes that at best I am the first to use isolates from South Africa.
I have attached a couple of slides for you viewing and look forward to hearing from you,

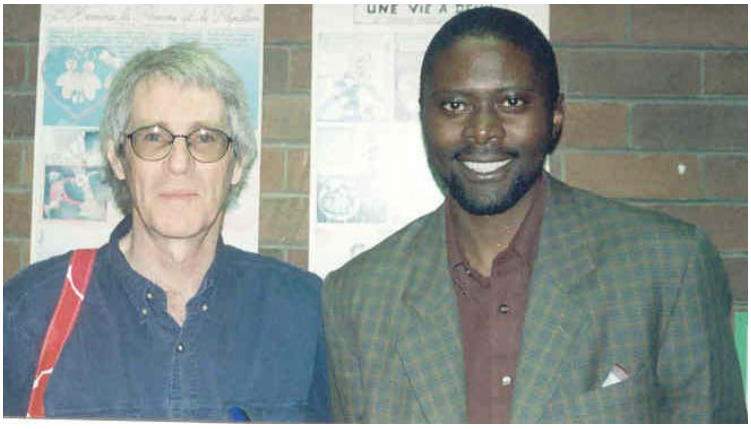
Thank you

Regards
Maz

Mr. M. N. Sakupwanya
MSc - Microbiologist
University of the Witwatersrand
Johannesburg, 2050
South Africa

success is not the position in which you stand but the direction in which you look

2. Personal communication with Doctor Dave Moore on a visit to South Africa in 2004



3. Communication with Mr. Frikkie Kirsten of the Plant Protection Research Institute (PPRI), Stored Grain and Oil Seed Research Unit, South Africa Tel: +27(0)12 8088217

Appendix 5 – Preparation of 1% sodium hypochlorite

A commercial bleach (Jik – Pick and Pay Regular bleach) containing the active ingredient sodium hypochlorite was diluted with distilled water as follows to obtain 1% solution from 3.5%.

Required Volume of 1% (ml)	Volume Jik to be added to distilled water (ml)
50	14.3
100	28.6
150	42.9
200	57.2
250	71.5
300	85.8
500	143.0
750	214.5
1000	286.0

Where 150 ml of a 1% sodium hypochlorite is required pour 42.9 ml Jik to 200ml measuring cylinder and top up to the 150 mark with distilled water. Resultant solution may be used to surface sterilize insects.
(Courtesy of the Plant Pathology Clinic, Kutsaga Research Station, Harare)

Appendix 6 – Native and Exotic Fungal Cultures