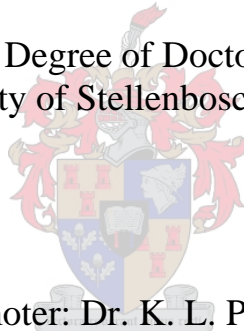

Biological control potential of the spotted stem borer *Chilo partellus*
(Swinhoe) (Lepidoptera: Crambidae) with the entomopathogenic fungi
Beauveria bassiana and *Metarhizium anisopliae*

By

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Dissertation Presented for the Degree of Doctor of Philosophy in Agriculture
at the University of Stellenbosch, South Africa



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Department of Entomology & Nematology
Faculty of Agricultural & Forestry Sciences
University of Stellenbosch

April 2004

Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted to any university for a degree.

Signature: _____

Date: _____



ABSTRACT

Biological control studies were conducted with isolates of entomopathogenic fungi *Beauveria bassiana* and *Metarrhizium anisopliae* from Ethiopia and South Africa against the spotted stem borer *Chilo partellus*. The study was conducted from April 2002 to April 2003, at the department of Entomology and Nematology, University of Stellenbosch, South Africa. The objectives were to screen these isolates for pathogenicity and to determine the susceptibility of different larval instars; to study the effect of temperature on fungal development and virulence; to investigate food consumption of fungus treated larvae; to determine compatibility of fungal isolates with insecticides; to study the effect of exposure methods and diets on larval mortality; and to evaluate promising isolates under greenhouse conditions using artificially infested maize plants.

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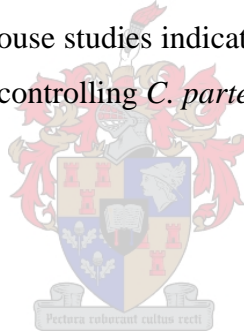
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In *in-vitro* studies, five concentrations (0.1 ppm, 1 ppm, 5 ppm, 10 ppm, and 100 ppm active ingredients) of the insecticides benfuracarb and endosulfan were tested with the isolates PPRC-4, PPRC-19, PPRC-16, EE-01 and BB-01. Increasing the concentration of the insecticides adversely affected germination, radial growth and sporulation of the isolates. In *in-vivo* studies combining the fungi, PPRC-4 and BB-01,

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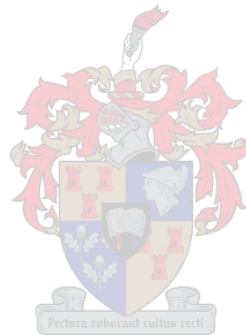
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Dedication

To my late grandmother, Kibnesh Dibaba, for her great dedication in educating me



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Second to none, I am thankful to almighty God through which all things are made possible to me.

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Publications from this dissertation

1. Tefera, T. & Pringle, K.L. 2003. Germination, radial growth, and sporulation of *Beauveria bassiana* and *Metarhizium anisopliae* isolates and their virulence to *Chilo partellus* (Lepidoptera: Pyralidae) at different temperatures. *Biocontrol Science and Technology* 13, 699-704.
2. Tefera, T. & Pringle, K.L. 2003. Effect of exposure method to *Beauveria bassiana* and conidia concentration on mortality, mycosis and sporulation in cadavers of *Chilo partellus* (Lepidoptera: Pyralidae). *Journal of Invertebrate Pathology* 84, 90-95.
3. Tefera, T. & Pringle, K.L. 2004. Mortality and maize leaf consumption of *Chilo partellus* (Lepidoptera: Pyralidae) larvae infected by *Beauveria bassiana* and *Metarhizium anisopliae*. *International Journal of Pest Management* 50, 29-34.
4. Tefera, T. & Pringle, K.L. 2003. Food consumption by *Chilo partellus* (Lepidoptera: Pyralidae) larvae infected by *Beauveria bassiana* and *Metarhizium anisopliae* and effect natural versus artificial diet on mortality and mycosis. *Journal of Invertebrate Pathology* 84, 220-225.
5. Tefera, T. & Pringle, K.L. 2004. Evaluation of *Beauveria bassiana* and *Metarhizium anisopliae* isolates from Ethiopia for controlling *Chilo partellus* (Lepidoptera: Pyralidae) in maize. *Biocontrol Science and Technology*. Accepted.
6. Tefera, T. & Pringle, K.L. 2004. Susceptibility of stem borer *Chilo partellus* (Lepidoptera: Pyralidae) to entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae*. Submitted.
7. Tefera, T. & Pringle, K.L. 2004. *In-vitro* and *in-vivo* compatibility of *Beauveria bassiana* and *Metarhizium anisopliae* with benfuracarb and endosulfan against *Chilo partellus* (Lepidoptera: Pyralidae). Submitted.

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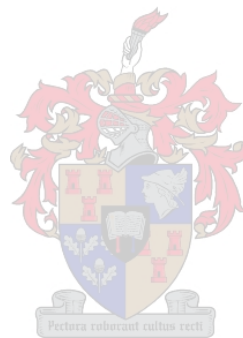
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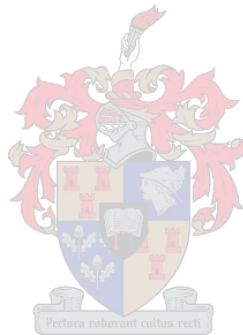
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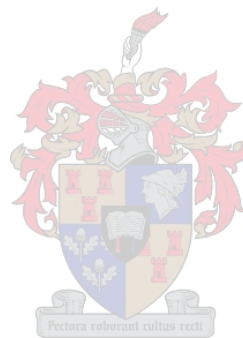
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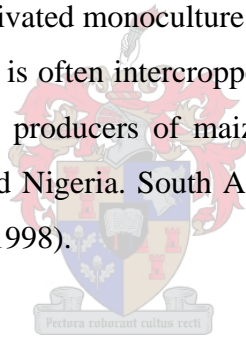


CHAPTER 1

INTRODUCTION

1.1. Maize

Maize (*Zea mays* L.) originated in Central America and was introduced to Africa by the 17th century (Seshu Reddy, 1998). In Africa maize is used as both human and animal food, eaten directly as grilled cobs or as various products of maize flour. It is easily stored after drying or milling (Polaszek & Khan, 1998). In Eastern Africa, for instance, 3.9% of the cultivated land is under maize production with grain yields of 700 to 1800 kg ha⁻¹ as opposed to 7437 kg ha⁻¹ in the USA (FAO, 1991). In general, maize in Africa is grown on a small-scale by farmers for local consumption, and yields tend to be low, averaging less than half that of Asia and Latin America (FAO, 1993). In some countries of Africa such as South Africa and some parts of Senegal, maize is an extensively cultivated monoculture and irrigated (Kfir, 1998; Polaszek & Khan, 1998). On small farms, maize is often intercropped with legumes, such as groundnuts, cowpeas or haricot beans. Principal producers of maize in sub-Saharan Africa are Kenya, South Africa, Tanzania, Ethiopia and Nigeria. South Africa is the only sub-Saharan country exporting maize (Polaszek & Khan, 1998).



1.2. Stem borers (stalk borers)

Lepidopterous borers are among the most economically important pests of maize in Africa (Bosque-Perez & Schulthess, 1998) causing significant yield reductions. These are the maize stalk borer, *Busseola fusca* Fuller (Noctuidae), the pink stalk borer, *Sesamia calamistis* Hampson (Noctuidae); the African sugar-cane borer, *Eldana saccharina* Walker (Pyralidae), the ear borer *Mussidia nigrivenella* Rogonot (Pyralidae), and the spotted stalk borer, *Chilo partellus* (Swinhoe) (Crambidae). The first four are African in origin and are present in most countries of sub-Saharan Africa (Harris, 1962; Appert, 1970; Girling, 1978). *Chilo partellus* originated in Asia and was accidentally introduced to eastern Africa some 60 years ago (Bowden, 1954). In addition to the above five species, other lepidopterous borers of minor economic importance on maize in Africa include *Sesamia botanophaga* (Tams and Bowden) (Noctuidae), *Chilo aleniellus* (Strand) (Crambidae), *Chilo orichalcociliellus* (Strand) (Crambidae), *Chilo agamemnon* (Bleszynski) (Crambidae), *Chilo diffusilineus* (de Joannis) (Crambidae), and *Coniesta igneffusalis* (Hampson) (Crambidae) (Harris, 1962; Endrody-Younga, 1968, Appert, 1970; Bleszynski, 1970; Bonzi, 1982, Moyal & Tran, 1992).

1.3. Bioecology of *Chilo partellus*

The bioecology of *C. partellus* was studied in South Africa by Van Hamburg (1980) and Kfir (1992). Adults emerge from pupae during late afternoon and early evening and are active at night. During daytime they are inactive and rest on plants. After mating, 1-3 days after emergence, females lay eggs in batches, 10-80 overlapping eggs, parallel to the long axis of the underside of leaves. The eggs hatch and larvae disperse to adjacent plants before they move up to the leaf whorl to feed on the young leaves. Later, they penetrate into the stem tissues to feed, producing extensive tunnels in stems and in maize ears. They then pupate in the tunnels, after excavating emergence windows to facilitate the exit of moths. There are three overlapping generations per year. The fifth and sixth instars of the third generations enter diapause in dry stalks. The first three instars feed on leaf whorls while the last three instars bore into the stem (Van Hamburg, 1987; Kfir, 1992).

Haile & Hofsvang (2001) found *C. partellus* at altitudes of 1400 m above sea level in Eritrea while Warui & Kuria (1983) found this pest up to an altitude of 1500 m in Kenya. Ingram (1958) reported that *C. partellus* does not survive temperatures below 15.5 °C

1.4. Economic importance of *C. partellus*

1.4.1. Damage

Most stem borers produce similar symptoms on infested maize plants. Newly hatched larvae feed initially by scrapping in leaf whorls of growing plants, producing characteristic ‘window-paning’ and ‘pin-holes’ (Seshu Reddy, 1998). Later, the larvae tunnel into the stems and may kill the central leaves and growing points, producing ‘deadhearts’. The larvae also bore into maize cobs and feed on the developing grains. Affected plants thus have poor growth and reduced yield and they are more susceptible to wind damage and secondary infections.

1.4.2. Yield loss

Yield loss of 18 % in maize due to *C. partellus* and *C. orichalcociliellus* were reported in Kenya (Warui & Kuria, 1983). Seshu Reddy & Sum (1991) reported that there was a maximum grain yield reduction and stalk damage in maize due to *C. partellus* on a 20-day-old crop, while there was an insignificant effect on yields by larval feeding on a 60-day-old crop. They have also defined economic injury levels of *C. partellus* as 3.2 and 3.9 larvae per plant in maize 20 and 40 days after plant emergence, respectively. Yield losses of more than 50 %

are common in southern Mozambique (Sithole, 1990). Larvae of the third generation were reported to infest 87 % cobs of late-planted maize resulting in 70 % grain damage (Berger, 1981).

In South Africa, pest control alone may amount to 56 % of the gross margin above cost for an average yield (Van Hamburg, 1987). In general, yield losses vary from place to place, season to season and with crop growth stage.

1.5. Management of *C. partellus*

1.5.1. Host-plant resistance

A wide range of mechanism are involved in *C. partellus* resistance in maize and sorghum, including non-preference (antixenosis) for oviposition, reduced feeding, reduced tunneling, tolerance of plants to leaf damage, deadheart and stem tunneling and antibiosis (Seshu Reddy, 1998). In addition, morphological, physical, chemical, and non-plant factors, including photo- and geotactic stimuli, were involved (Van Rensburg & Malan, 1992; Kumar & Saxen, 1992; Kumar, 1993).

1.5.2. Cultural control

Cultural practices include appropriate disposal of crop residues, time of planting, tillage and mulching, spacing, intercropping, removal and destruction of volunteer and alternative hosts, removal of borer-infested plants, fertilizer application and crop rotation (Seshu Reddy, 1998). Destruction of crop residues and stubble to reduce stem borer infestations has been recommended (Unnithan & Seshu Reddy, 1989). Early planting has been found to lower stem borer infestations (Abu, 1986). Intercropping sorghum with cowpea delayed *C. partellus* larval population build up (Minja, 1990).

1.5.3. Chemical control

Several insecticides have been screened for the control of maize and sorghum stem borers in different regions in Africa. Those insecticides which have been found effective as spray or dust treatments include carbofuran, carbaryl, deltamethrin, endosulfan, trichlorfon and synthetic pyrethroids (Sithole, 1990; Van Rensburg & Vandenberg, 1992). However, in Africa, control of stem borers exclusively by insecticides by small-scale farmers is uneconomical and often unpractical (Seshu Reddy, 1998).

1.5.4. Biological control

1.5.4.1. Parasitoids

Parasitoids of interest in Africa include the egg parasitoids, such as *Trichogramma* spp., larval parasitoids, including *Cotesia sesamiae* (Cameron) (Hymenoptera: Braconidae) and *C. flavipes* (Cameron) (Hymenoptera: Braconidae) and pupal parasitoids, *Pediobius furvus* *Pediobius furvus* Gahan (Hymenoptera: Eulophidae) (Ajayi, 1989; Getu *et al.*, 2003). However, the overall rate of parasitism of stem borers was low (10 -14%) (Seshu Reddy, 1998).

1.5.4.2. Predators

Earwigs, ants, spiders, and ladybird beetles have been found to feed on the eggs, larvae and pupae of *C. partellus* (Seshu Reddy, 1998). In Uganda, Mohyuddin & Greathead (1970) reported the ants *Tetramorium guineense* Bernard (Hymenoptera: Formicidae) and *Pheidole megacephala* Fabricius (Hymenoptera: Formicidae) destroyed almost 90 % of eggs and first instar larvae of *C. partellus*.

1.5.4.3. Microbial pathogens

Pathogenic organisms are often encountered in the field attacking *C. partellus* in Africa (Van Rensburg *et al.*, 1988; Odindo *et al.*, 1989; Maniania, 1991; Hoekstra & Kfir, 1997).



1.5.4.3.1. Nematodes

Most studies of nematodes attacking *C. partellus* in Africa have been simply distribution records. Two genera of nematode, *Hexameris* and *Panagrolaimus* belonging to the families, Mermithidae and Panagrolaimidae have been reported from Kenya (Otieno, 1986).

1.5.4.3.2. Protozoa

Nosema partelli Walters & Kfir, is endemic to South Africa and is a widespread disease in field and laboratory populations of *C. partellus* in the region (Walters & Kfir, 1993). However, it was only infective in laboratory cultures and less active under field conditions. *Nosema* sp, has a great potential as both a cheap and effective control agent in Kenya (Odindo *et al.*, 1993).

1.5.4.3.3 Bacteria

Attempts at using *Bacillus thuringiensis* Berliner against *C. partellus* have been reported by Berger (1981) in Mozambique and by Brownbridge (1991) in Kenya. In Mozambique, various combinations of *B. thuringiensis* with chemical insecticides were used. However, it was concluded that this type of treatment was too expensive. In South Africa, Hoekstra & Kfir (1997), reported *B. thuringiensis*, *Streptococcus* sp. and *Serratia* sp. infecting a field-collected population of *C. partellus*.

1.5.4.3.4. Viruses

Studies made in Kenya and South Africa identified granulosis viruses, polyhedral inclusion bodies, cytoplasmic polyhedrosis virus and entomopox virus (Odindo *et al.*, 1989; Hoekstra & Kfir, 1997). In Egypt, infection by nuclear polyhedrosis virus of *Chilo agamemnon* Bleszynski (Lepidoptera: Crambidae) (Abbas, 1987), had a detrimental effect on the development of a larval parasitoid, *Habrobracon brevicornis* Wesmael (Hymenoptera: Braconidae). In India, granulosis viruses have been used with some success for the control of *Chilo sacchariphagus* Stramineelus (Caradza) (Lepidoptera: Pyralidae) and *Chilo infuscatellus* Snellen (Lepidoptera: Pyralidae) (David & Easwaramoorthy, 1990).

1.5.4.3.5. Fungi

Metarhizium anisopliae (Metschnikoff) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin have been isolated from infected *C. partellus* in South Africa and Kenya (Maniania, 1991; Odindo, 1989; Hoekstra & Kfir, 1997). *Entomophthora* sp. was the most common fungal pathogen of *C. partellus* in South Africa (Hoekstra & Kfir, 1997). The fungus was present throughout the growing season and its incidence was high mainly after irrigation or rainfall. However, little attention has been given to microbial pathogens of *C. partellus* in South Africa (Hoekstra & Kfir, 1997). Studies carried out in India, showed that *B. bassiana* could cause up to 60 % mortality to *C. infuscatellus* larvae in sugar-cane (Easwaramoorthy & Santhalakshmi, 1987). Studies in Kenya, using both indigenous and exotic fungi to control *C. partellus* have shown reduction of the larval populations (Maniania, 1992; 1993).

1.6. PRESENT STUDY

Control of *C. partellus* has largely been through the use of insecticides (Warui & Kuria, 1983; Vandenberg & Van Rensburg, 1996). However, this stem borer is difficult to control with insecticides because of a prolonged emergence pattern, multiple generations and its cryptic feeding behaviour (Kfir, 1992). In addition, chemical pesticides may cause ecological

problems and are also expensive for African farmers (Dedat, 1994; Du Toit, 1995). As an alternative to chemical control, biological control with the use of the entomopathogenic fungi *B. bassiana* and *M. anisopliae* may play a role in managing *C. partellus*. However, empirical information on this approach is still scanty. This study was designed to do the basic research on identifying pathogenic isolates of fungi and determining the optimum conditions for their future use.

Therefore, the objectives of the current study were to:

1. identify locally available pathogenic isolates of *B. bassiana* and *M. anisopliae*
2. test isolates of *B. bassiana* and *M. anisopliae* against second, third, fourth, fifth and sixth instar *C. partellus* larvae and to determine conidial concentration-mortality response of the most pathogenic isolates;
3. investigate the effect of temperature on conidia germination, radial growth and sporulation of *B. bassiana* and *M. anisopliae* and on their virulence to *C. partellus*;
4. study food consumption by *C. partellus* larvae infected by *B. bassiana* and *M. anisopliae* and effects of diets on susceptibility to the fungal isolates.
5. determine the *in-vitro* and *in-vivo* compatibility of *B. bassiana* and *M. anisopliae* with the insecticides endosulfan and benfuracarb;
6. study the effect of exposure method, conidial concentrations and temperature on larval mortality, mycosis and sporulation of *B. bassiana* in cadavers, and
7. evaluate the controlling effects of *B. bassiana* and *M. anisopliae* against artificial infestations of *C. partellus* larvae on maize in a greenhouse.

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CHAPTER 2

Susceptibility of the spotted stem borer, *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) larval instars to the entomopathogenic fungi *Beauveria bassiana* Balsamo (Vuillemin) and *Metarhizium anisopliae* (Metschnikoff) Sorokin (Deuteromycotina:Hyphomycetes)

ABSTRACT

The pathogenicity of Ethiopian and South African fungal isolates of *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin to second, third, fourth, fifth and sixth instar *Chilo partellus* (Swinhoe) larvae was studied. The isolates originated from different arthropod hosts including *C. partellus*. A single concentration (1×10^8 conidia/ml) of each isolate was assayed against second instar larvae. Of the ten isolates tested, *B. bassiana* (BB-01) and *M. anisopliae* (PPRC-4, PPRC-19, PPRC-61, EE-01) were the most virulent inducing 93 to 100 % mortality. A single concentration of the five pathogenic isolates was further tested against third, fourth, fifth and sixth instar larvae. Second and sixth instar larvae were the most susceptible stages suffering 97 % and 98 % mortality, respectively. The LT_{50} values were low for second instar (2 days) and sixth (4.8 days) instar larvae. Multiple concentration assays (1.25×10^6 , 2.5×10^7 , 5×10^7 , and 1×10^8 conidia /ml) were conducted against second instar larvae with three of the most pathogenic isolates (PPRC-4, BB-01, EE-01). The LC_{50} was 1.44×10^3 , 1.53×10^4 , and 3.12×10^4 for BB-01, PPRC-4 and EE-01, respectively.

2.1. INTRODUCTION

Maize and sorghum growers rely almost entirely on the application of chemical insecticides for controlling *Chilo partellus* Swinhoe, (Seshu Reddy, 1998). Chemical insecticides can result in adverse effects on non-target organisms and the presence of residues in food and water. Entomopathogenic fungi offer an environmentally safe alternative to chemical insecticides (Maniania, 1992). Preliminary studies demonstrated the potential of *Beauveria bassiana* (Balsamo) Vuillemin, *Metarrhizium anisopliae* (Metschnikoff) Sorokin and *Paecilomyces fumosoroseus* (Wize) Brown & Smith for controlling *C. partellus* (Odindo *et al.*, 1989; Maniania, 1991; Hoekstra & Kfir, 1997).

An important consideration in developing the use of entomopathogenic fungi as mycoinsecticides is the selection of effective isolates (McCoy, 1990; Maniania, 1991; Moorehouse *et al.*, 1993; Ekesi, 2001). This can initially be carried out under laboratory conditions. *Metarrhizium anisopliae* and *B. bassiana*, for example, have a wide range of natural hosts and their pathogenicity also varies according to their hosts (Hall & Papierok, 1982).

The pathogenicity of *M. anisopliae* and *B. bassiana* was affected by host age (Boucias & Pendland, 1998; Butt & Goettel, 2000). *C. partellus* has six larval instars (Van Hamburg, 1980; Kfir, 1988). However, the effects of *M. anisopliae* and *B. bassiana* against these developmental stages has not been studied. Thus, the goals of the present study were to:

1. determine the mortality of second, third, fourth, fifth and sixth instar larvae of *C. partellus* treated with ten of isolates of *B. bassiana* and *M. anisopliae*;
and
2. quantify the concentration-mortality response of the three most virulent isolates against second instar larvae.

2.2. MATERIALS AND METHODS

2.2.1. Insects

A laboratory colony of *C. partellus* larvae was obtained from the Agricultural Research Council, Plant Protection Research Institute (PPRI), Pretoria, South Africa. The larvae

were reared on an artificial diet according to Kfir (1992). In order to condition the larvae to natural diet, they were allowed to feed on 4-week old maize leaves (variety CRN 3414) for 2-3 days before application of the fungi. The maize plants were grown in growing cabinet at 25°C. No fertilizers were added and plants were watered as required.

2.2.2. Fungi

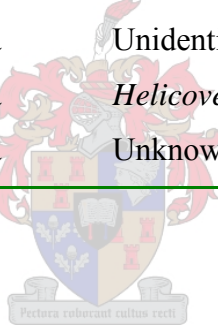
Four *B. bassiana* and six *M. anisopliae* isolates were screened (Table 2.1). The isolates POCH-01 and POCH-02 were isolated from field collected dead larvae of *C. partellus* and *Busseola fusca*, respectively, from South Africa, Pochefstroom. BCP-01, is a commercial product donated by Biological Control Products, Pine Town, South Africa. The other isolates were originated from Ethiopia (Plant Protection Research Center, Alemaya University and Addis Ababa University). The isolates were isolated between 1995 and 2002. Fungal cultures were maintained at 25°C in darkness on Sabouraud dextrose agar (SDA) containing 10 g peptone, 40 g dextrose, and 10 g agar in 1 liter water.

2.2.3. Conidial preparation

Conidia were obtained from 3 week old sporulating cultures. The conidia of each isolate were harvested by brushing the surface of the culture with a sterile camel hairbrush into a 500 ml glass beaker containing 50 ml sterile distilled water with Tween 80 (0.1 % v/v) (Difco™). The conidial suspension was prepared by mixing the solution with a magnetic stirrer for five minutes. It was then adjusted to the desired concentration using Neubauer haemocytometer.

Table 2.1. Host and country of origin of *Beauveria bassiana* and *Metarrhizium anisopliae* isolates used in bioassays against *Chilo partellus*.

Fungal species	Isolate	Origin	Host
<i>B. bassiana</i>	BB-01	Ethiopia	<i>Helicoverpa armigera</i> Hübner (Lepidoptera: Noctuidae)
	BCP-01	South Africa	Unknown (commercial product)
	POCH-01	South Africa	<i>Chilo partellus</i> (Swinhoe) (Lepidoptera: Crambidae)
	POCH-02	South Africa	<i>Busseola fusca</i> Fuller (Lepidoptera: Noctuidae)
<i>M. anisopliae</i>	PPRC-4	Ethiopia	<i>Pachnoda interrupta</i> Olivier (Coleoptera: Scarabaeidae)
	PPRC-19	Ethiopia	<i>P. interrupta</i> (Coleoptera: Scarabaeidae)
	PPRC-61	Ethiopia	<i>P. interrupta</i> (Coleoptera: Scarabaeidae)
	EE-01	Ethiopia	Unidentified Crustacea (Isopoda?)
	MA-01	Ethiopia	<i>Helicoverpa armigera</i> (Lepidoptera: Noctuidae)
	MA-02	Ethiopia	Unknown



2.2.4. Conidial germination

About 1 ml of 1×10^6 conidia ml^{-1} aqueous conidial suspension of each isolate was spread-plated on SDA in Petri dishes in a laminar flow cabinet. The plates were allowed to dry in the cabinet for 10 minutes. Two sterile microscope coverslips were placed on each plate. The plates were sealed with masking tape and incubated at 25 °C in complete darkness. After 24 h of incubation, 1 ml of formaldehyde (0.5 %) was transferred to each plate in order to halt germination. Germination rates were determined by randomly examining 100 conidia per plate using a compound microscope (400X). A conidium was considered to have germinated if the germ tube was at least as long as the width of the conidium. Each plate served as a replicate. There were four replicates per isolate.

2.1.4.1. Statistical analysis

Data on percent conidial germination was angular-transformed ($\arcsine \sqrt{\textit{proportion}}$) before being subjected to a one-way analysis of variance using SPSS-11 for windows. Student-Newman-Keuls Test was used for mean separation (Newman, 1939; Keuls, 1952).

2.3. BIOASSAY

2.3.1. Single concentration assays

Twenty second-instar *C. partellus* larvae were placed in a 9 cm diameter Petri dish. The larvae were then treated with three ml of each fungal suspension at 1×10^8 conidia /ml using a Potter's precision laboratory spray tower (Burkard Manufacturing Ltd., England). Initially, ten fungal isolates (Table 2.1) were tested. against second instar larvae. From the results of this assay, five isolates (BB-01, PPRC-4, PPRC-19, PPRC-61 and EE-01) were selected for further assays. The selected isolates were tested against third, fourth, fifth and sixth instar larvae following the same procedure as described above for the second instar larvae. Twenty larvae of each instar treated with distilled water containing Tween 80 (0.1 % v/v) served as controls. First instar larvae were not included due to high mortality encountered during shipping from the supplier (PPRI) to the University of Stellenbosch where the experiments were conducted.

Petri dishes containing treated and control insects were sealed with masking tape and incubated at 25 °C. All treatments and their controls were replicated four times with 20 larvae per replication. They were arranged in a completely randomized design. The treated insects and controls were provided with maize leaves daily after frass and leaf debris had been removed. Mortality was recorded daily. Dead insects were removed and placed in Petri dishes lined with moist filter paper. Fungal infection was confirmed after observing mycosed cadavers under stereo-microscope .

2.3.1.1. Statistical analysis

Mortality data were corrected for control mortality (Abbott, 1925). The data were then angular-transformed in order to stabilize the variances. Mortality data for second instar

larvae were subjected to a one-way analysis of variance using SPSS-11 for windows. Means were separated using the Student-Newman-Keuls test. A factorial analysis with five fungal isolates (PPRC-4, PPRC-19, PPRC-61, EE-01 & BB-01) and four larval instars (third, fourth, fifth & sixth) as main effects was performed on the angular transformed mortality data using SPSS-11 for windows. Student-Newman-Keuls test was used to separate the means. The LT_{50} (lethal time required to kill 50 % of the treated insect population) was determined using probit analysis of correlated data for each replicate (Throne *et al.*, 1995). A factorial analysis with the five fungal isolates and four larval instars as main effects was performed for LT_{50} data. Student-Newman-Keuls test was used to separate the means.

2.3.2. Multiple concentration assays

An experiment was conducted to determine the concentration-mortality response of some of the most virulent isolates, BB-01, PPRC-4 and EE-01. Concentrations of 1.25×10^6 , 2.5×10^7 , 5×10^7 , and 1×10^8 conidia ml^{-1} were applied to second instar larvae following the same procedure as described above for single concentration assays. Twenty larvae were used for each concentration of each isolate and the control, which was treated with distilled water containing Tween 80 (0.1 % v/v).

Petri dishes containing treated and control insects were sealed with masking tape and incubated at 25 °C. All treatments and their controls were replicated four times with 20 larvae per replication. They were arranged in a completely randomized design. The treated insects and controls were provided with maize leaves daily after frass and leaf debris had been removed. Mortality was recorded daily. Dead insects were removed and placed in Petri dishes lined with moist filter paper. Fungal infection was confirmed after observing mycosed cadavers under stereo-microscope.

2.3.2.1. Statistical analysis

The LC_{50} (lethal concentration of conidia required to kill 50 % of the treated insect population) was determined using probit analysis with the POLO-PC program (LeOra software, Berkeley, CA, USA).

2.4. RESULTS

2.4.1. Conidial germination

The percentage germination of conidia differed significantly ($F = 31.3$; $df = 9, 30$; $P < 0.001$) among isolates (Figure 2.1). Conidial germination of all isolates ranged from 78 to 98 % with the least germination in PPRC-61.

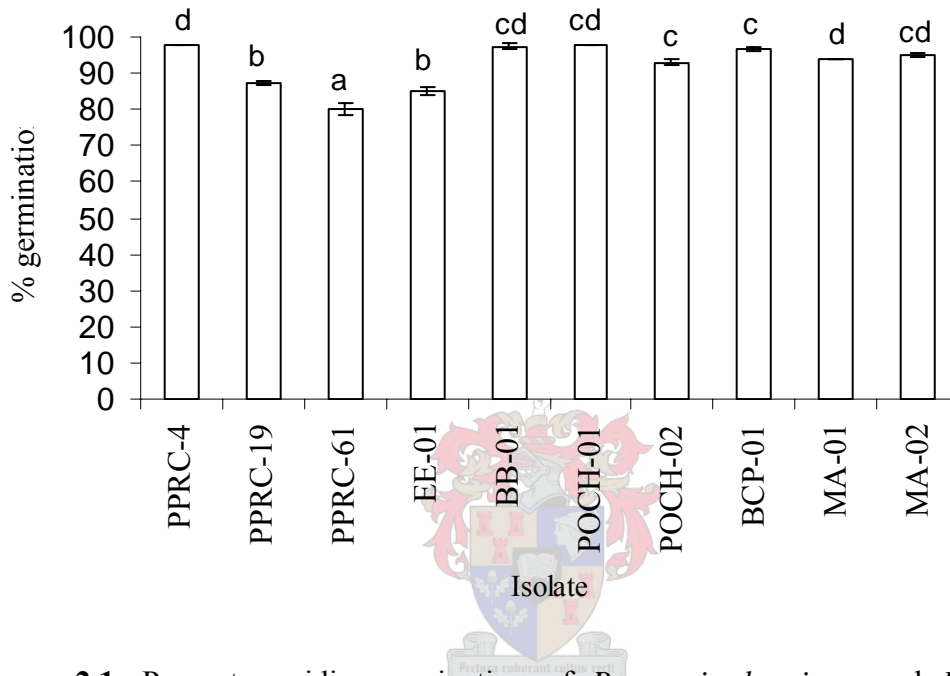


Figure 2.1. Percent conidia germination of *Beauveria bassiana* and *Metarrhizium anisopliae* isolates. Vertical lines represent standard errors (SE). Bars with the same letter are not significant at $P > 0.05$ using Student-Newman-Keuls test.

2.4.2. Single concentration assays

Control mortality was less than 10 %. There were significant differences between isolates in causing mortality to second instar larvae ($F = 48.1$; $df = 9, 30$; $P < 0.001$) (Table 2.2). *B. bassiana* (BB-01) and *M. anisopliae* (PPRC-4, PPRC-19, PPRC-61 & EE-01) induced the highest mortality (93 to 100 %). The LT_{50} values for BB-01, PPRC-4, PPRC-19, PPRC-61 and EE-01 were also shorter (1.7 to 2.6) than for the other isolates (Table 2.2).

Table 2.2. Percent corrected mortality after 7 days and lethal time for 50 % mortality (LT₅₀) of second instar *Chilo partellus* larvae treated with isolates of *Beauveria bassiana* and *Metarrhizium anisopliae* at the rate of 1x10⁸ conidia /ml.

Isolate	%mortality ± SE* (after 7 days)	LT ₅₀ ± SE* (days)	Intercept ± SE	Slope ± SE	χ ²	P-value
PPRC-4	100 ± 0.00c	1.70 ± 0.20 a	-1.04 ± 0.33	4.75 ± 1.04	3.93	0.25
BB-01	98.3 ± 1.67 c	1.99 ± 0.20 a	-0.83 ± 0.32	2.94 ± 0.71	9.99	0.01
PPRC-19	98.3 ± 1.67 c	2.31 ± 0.64 a	-1.07 ± 0.34	2.95 ± 0.66	6.82	0.11
PPRC-61	96.7 ± 3.33 c	2.03 ± 0.17 a	-0.52 ± 0.29	1.77 ± 0.50	15.2	0.33
EE-01	93.3 ± 6.67 c	2.61 ± 0.44 a	-1.26 ± 0.371	3.02 ± 0.73	12.4	0.56
MA-02	50.4 ± 15.3 b	10.23 ± 3.33 ab	-2.34 ± 0.64	0.64 ± 2.42	6.41	0.30
POCH-01	23.3 ± 9.83** a	12.8 ± 2.06 ab	-1.90 ± 0.75	0.75 ± 1.81	9.74	0.16
BCP-01	12.5 ± 5.81 a	23.0 ± 15.24 c	-1.55 ± 0.59	0.59 ± 0.90	1.50	0.13
POCH-02	15.4 ± 2.57 a	15.8 ± 6.61 b	-2.64 ± 0.95	0.95 ± 2.33	2.80	0.13
MA-01	13.3 ± 9.43 a	13.4 ± 0.72 ab	-4.17 ± 2.62	3.81 ± 3.44	3.04	0.09

*Means ± SE followed by the same letter with in a column are not significant at P > 0.05 using Student-Newman-Keuls Test.

** Probit analysis was used to determine the LT₅₀ when mortality was less than 50% (Throne *et al.*, 1995).

Table 2.3. Factorial analysis of mortality and LT₅₀ of third, fourth, fifth and sixth instar *Chilo partellus* larvae treated with *Beauveria bassiana* and *Metarrhizium anisopliae* at the rate of 1x10⁸ conidia/ml.

Factor	Mortality				LT ₅₀			
	Mean square	df	F	P	Mean square	df	F	P
Isolate (Iso)	0.27	4	5.77	<0.001	21.7	4	3.3	0.02
Instar (Ins)	2.37	3	49.5	<0.001	186.9	3	28.4	<0.001
Iso*Ins	0.0048	12	1.0	0.45	26.6	12	4.1	<0.001
Error	0.0048	75			6.57	60		

There were no interactions between isolate and instar for mortality (Table 2.3). However, there were differences in mortality between third, fourth, fifth and sixth instar larvae (Table 2.4). Sixth instar larvae suffered the highest mortality (97.5 %), while fifth instar larvae suffered the least (41.2 %) (Table 2.4). PPRC-4 and BB-01 induced higher mortality (83 %) than the other isolates (64 to 66 %) (Table 2.4).

There were interactions between isolate and instar for LT₅₀ (Table 2.3). The LT₅₀ also varied between isolates and larval instars. The LT₅₀ values were shortest in third and sixth instar larvae (Table 2.5). The LT₅₀ values for PPRC-4, PPRC-19 and BB-01 were shorter (6.9 to 7.5 days) than those for PPRC-61 and EE-01 (9.1 to 9.2 days) (Table 2.5). There was an increase in the LT₅₀ from the third instar to the fifth instar and a decrease in the LT₅₀ in sixth instar larvae for PPRC-4 and EE-01 (Table 2.5). However, in the case of PPRC-19, PPRC-61 and BB-01, the LT₅₀ increased in the fifth instar (Table 2.5). This discrepancy in sensitivity of the fifth instar larvae to the different isolates (Table 2.5) resulted in the interactions between isolate and instar (Table 2.3).

Table 2.4. Mean mortality (\pm SE) of *Chilo partellus* third, fourth, fifth and sixth instar larvae treated with *Beauveria bassiana* and *Metarrhizium anisopliae* at the rate of 1×10^8 conidia/ml 15 days after treatment.

Isolate	Larval instars				Isolate mean*
	Third	Fourth	Fifth	Sixth	
PPRC-4	91.8 \pm 4.8	82.5 \pm 10.3	62.2 \pm 9.1	97.5 \pm 5.0	83.5 \pm 7.5 b
PPRC-19	77.9 \pm 7.5	58.2 \pm 11.2	33.2 \pm 9.4	95.0 \pm 5.7	66.1 \pm 7.1 a
PPRC-61	82.2 \pm 11	45.1 \pm 10.6	35.8 \pm 13.1	100 \pm 0.00	65.7 \pm 8.2 a
EE-01	78.0 \pm 7.7	62.6 \pm 9.2	21.7 \pm 7.8	97.5 \pm 5.0	64.9 \pm 7.8 a
BB-01	94.4 \pm 5.5	85.6 \pm 5.7	53.1 \pm 7.7	97.5 \pm 5.0	82.6 \pm 5.2 b
Instar mean*	84.9 \pm 8.5 c	66.8 \pm 6.2 b	41.2 \pm 9.3 a	97.5 \pm 6.3 d	

*Means \pm SE followed by the same letter within a column (isolate mean) and a row (instar mean) are not significant at $P > 0.05$ using Student-Newman-Keuls Test.

Table 2.5. Mean LT_{50} (\pm SE) of *Chilo partellus* third, fourth, fifth and sixth instar larvae treated with *Beauveria bassiana* and *Metarrhizium anisopliae* at the rate of 1×10^8 conidia/ml 15 days after treatment.

Isolate	Larval instars				Isolate mean
	Third	Fourth	Fifth	Sixth	
PPRC-4	5.4 \pm 0.8	8.4 \pm 0.7	8.9 \pm 1.2	5.2 \pm 1.4	6.9 \pm 1.3 a
PPRC-19	6.8 \pm 0.6	10.8 \pm 3.2	7.8 \pm 2.9	4.7 \pm 0.2	7.5 \pm 2.1 a
PPRC-61	6.0 \pm 1.5	15.1 \pm 2.3	11.3 \pm 4.2	4.6 \pm 0.9	9.2 \pm 1.1 b
EE-01	7.7 \pm 0.6	10.2 \pm 3.7	13.5 \pm 1.5	4.9 \pm 0.2	9.1 \pm 2.3 b
BB-01	6.1 \pm 1.5	5.6 \pm 0.9	11.2 \pm 1.6	4.8 \pm 0.1	6.9 \pm 1.4 a
Instar mean	6.4 \pm 0.3 b	10.0 \pm 1.9 c	10.2 \pm 0.6 c	4.8 \pm 0.3 a	

*Means \pm SE followed by the same letter within a column (isolate mean) and a row (instar mean) are not significant at $P > 0.05$ using Student-Newman-Keuls Test.

2.4.3. Multiple concentration assays

The probit mortality on log concentration regression lines for the concentration of conidia were the same. The common probit regression line was described by $Y = 3.45 + 0.412(x)$ ($\chi^2 = 16.4$; $df = 11$; $P = 0.12$). The LC_{50} for BB-01 (1443 conidia/ml) was lower than for PPRC-4 (15293 conidia/ml) and EE-01 (31241 conidia/ml) (Table 2.6). However, there was overlap in fiducial limits between BB-01, PPRC-4 and EE-01 (Table 2.6). Therefore the differences in the LC_{50} values were not significant. This was expected as the slopes and intercepts of the probit regression lines were the same, as mentioned above.

Table 2.6. Concentration-mortality of *Chilo partellus* second instar larvae exposed to *Beauveria bassiana* and *Metarrhizium anisopliae* isolates.

Isolate	LC_{50}	SE	90 % Fiducial limit
BB-01	1.443×10^3	0.959	$6.5685 \times 10^{-5} - 9.5794 \times 10^4$
PPRC-4	1.5293×10^4	0.961	$1.0669 \times 10^{-1} - 3.8204 \times 10^5$
EE-01	3.1241×10^4	0.965	$8.6707 \times 10^{-1} - 6.0107 \times 10^5$

2.5. DISCUSSION

The isolates originating from *C. partellus* (POCH-01) and *Busseola fusca* (POCH-02) were less pathogenic than isolates from *Helicoverpa armigera* (BB-01), *Pachnoda interrupta* (PPRC-4, PPRC-19, PPRC-61) and crustacea (EE-01). In most cases, however, fungal isolates are pathogenic to their original host or to a closely related species (Poprawski *et al.*, 1985; Samuels *et al.*, 1989). Thus, results from this study indicate that screening of potential isolates should not be limited to those isolated from the original host. There is increasing evidence that habitat selection, not insect host selection, drives the virulence of *B. bassiana* and *M. anisopliae* (Bidochka *et al.*, 2001; 2002).

There were differences in pathogenicity among the isolates of *B. bassiana* and *M. anisopliae*. For example, *B. bassiana* (BB-01) was more pathogenic than BCP-01, POCH-01 and POCH-02. The *M. anisopliae* isolates, PPRC-4, PPRC-19, PPRC-61 and EE-01 were more pathogenic than MA-01 and MA-02. Interspecific and intraspecific

variation in pathogenicity is common among isolates of entomopathogenic fungi including *B. bassiana* and *M. anisopliae* (Feng & Johnson, 1990; Maniania, 1992; Ekesi, 2001). *Beauveria bassiana* infects over 200 species of insects in nine orders (Maniania, 1992), although some isolates have a high degree of specificity. A variety of host determinants, such as physiological and nutritional status, properties of the arthropod's tegument and cellular defense could affect the expression of pathogenicity by entomopathogenic fungi (Askary *et al.*, 1998).

The isolates BB-01, PPRC-4 and EE-01, had low LC₅₀ values to second instar larvae. The LC₅₀ ranged from 1.44x10³ to 3.12x10⁴ conidia/ml. The LC₅₀ for European corn borer *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae) larvae treated with *B. bassiana* isolates varied from 9.25x10² to 2.89x10⁶ CFU/cm² (Feng *et al.*, 1985). Ekesi *et al.* (2002) also reported LC₅₀ of *B. bassiana* and *M. anisopliae* against tephritid fruit fly adults to range from 5.5x10⁵ to 7.7x10⁶ conidia/ml.

The germination of conidia of the isolates was more than 80 %. Similar results have been obtained in laboratory bioassays and field experiments (Lomer *et al.*, 1997; Ekesi, 2001).

Second and sixth instar larvae of *C. partellus* were the most susceptible stages to infection by *B. bassiana* and *M. anisopliae* isolates. Early instars of *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) were more susceptible than late instar larvae to infection by *Nomuraea rileyi* Farlow and *P. fumosoroseus* (Fargues & Rodriguez-Rueda, 1980), while first and fifth instar larvae were the most susceptible stages of *O. nubilalis* to *B. bassiana* infection (Feng *et al.*, 1985). In contrast, first and second instar larvae of *Heliothis zea* Boddie (Lepidoptera: Noctuidae) were less susceptible to *N. rileyi* than the third and fourth instars (Mohamed *et al.*, 1977). The sixth instar larvae of *C. partellus* is the diapausing stage (Kfir, 1988). Thus, the sixth instar will not cast the conidia through molting before infection occurs. Vey and Fargues (1977) and Vandenberg *et al.* (1998) reported that Colorado potato beetle *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) escaped *B. bassiana* infection by casting the conidia with their exoskeleton at the time of molting.

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CHAPTER 3

Effect of temperature on germination, radial growth, sporulation and pathogenicity of *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin isolates to *Chilo partellus* Swinhoe (Lepidoptera: Crambidae)

ABSTRACT

The effect of temperature on conidia germination, radial growth and pathogenicity of *Beauveria bassiana* (BB-01) and *Metarhizium anisopliae* (PPRC-4, PPRC-19, PPRC-61 and EE-01) infecting the spotted stalk borer *Chilo partellus* was investigated. Conidia germination, radial growth and sporulation of all isolates were delayed at 15 and 35 °C. A suitable temperature range for the isolates was from 25 to 30 °C. There was more than 95 % germination for all isolates when incubated at 25 and 30 °C for 24 h. Conidia germination was less tolerant of low temperature (15 °C) than was radial growth. Radial growth and sporulation reacted differently to temperature. The optimum temperatures for sporulation and radial growth of isolate BB-01 were 20 and 25 °C, respectively, while optimum sporulation and radial growth of isolates PPRC-4, PPRC-19, and EE-01 occurred at 25 and 30 °C, respectively. For PPRC-61 the optimum temperatures for radial growth and sporulation were 20 and 25 °C, respectively. At both 25 and 30 °C, all isolates induced 100 % mortality to larvae within four to six days. The LT₅₀ of the isolates differed at 15 °C with the shortest (3.5 days) for BB-01 and longest (14.4 days) for PPRC-61. The shortest mean LT₅₀ (1.7 days) was recorded at 25 °C for all isolates.

3.1. INTRODUCTION

Infection, growth and sporulation of several entomopathogenic fungi are influenced by environmental factors, especially temperature and humidity (Tanada & Kaya, 1993; Tang & Hou, 2001). Temperature is one of the environmental factors that influences fungal growth and disease development in insects (Roberts & Campbell, 1977). The process of conidia germination and growth on the insect cuticle is also dependent on temperature. Germination, mycelia growth, and infectivity at high temperatures (25-35 °C) are a prerequisite for successful biological control of tropical insect pests (Welling *et al.*, 1994). Temperature-dependent growth and infectivity have been demonstrated for many hyphomycetous fungi, including *B. bassiana* and *M. anisopliae* (Metschnikoff) Sorokin (Walstad *et al.*, 1970; Doberski, 1981; Soares *et al.*, 1983; Carruthers *et al.*, 1985; Moorhouse *et al.*, 1994; Welling *et al.*, 1994; Vestergaard *et al.*, 1995; Fargues *et al.*, 1997; Ekesi *et al.*, 1999; Tang & Hou, 2001). Many workers have reported variability in temperature dependence (Roberts & Campbell, 1977; Soares *et al.*, 1983; Maniania & Fargues, 1992; Ekesi *et al.*, 1999; Milner *et al.*, 2002).

Selection of fungal pathogens tolerant to the temperature range found in the agricultural ecosystem involved is essential in order to use insect pathogens in pest management programs (Ferron *et al.*, 1991; McCammon & Rath, 1994). Differences in the effects of temperature on isolates of *B. bassiana* and *M. anisopliae* also emphasise the importance of selecting isolates for use in specific climates (Moorhouse *et al.*, 1994). Therefore, it is necessary to assess the influence of environmental factors on conidia germination, mycelial growth and sporulation as well as disease development in the host (Tang & Hou, 2001).

Chilo partellus (Swinhoe) is reported to occur in the temperature range of 15-35 °C (Ingram, 1958; Gebre-Amlak, 1985; Haile & Hofsvang, 2001). However, the virulence of entomopathogenic fungi to *C. partellus* at this range of temperatures has not been determined. In order to use fungi for the control of this stalk borer, screening effective and climatically adapted isolates is essential.

The aims of the present study were to:

1. investigate the effect of temperature on conidia germination, radial growth and sporulation of one *B. bassiana* and four *M. anisopliae* isolates;
2. determine the virulence of these isolates to second instar *C. partellus* larvae at a range of temperatures.

3.2. MATERIALS AND METHODS

3.2.1. Larvae, fungal isolates and conidia preparation

A laboratory colony of *C. partellus* larvae was obtained from the Agricultural Research Council, Plant Protection Research Institute (PPRI), Pretoria, South Africa (see Chapter 2).

The isolates were obtained from Ethiopia (Plant Protection Research Center and Alemaya University) (see Chapter 2)).

Conidia were obtained from 3 week old sporulating cultures. The conidia of each isolate were harvested by brushing the surface of the culture with a sterile camel hairbrush into a 500 ml glass beaker containing 50 ml sterile distilled water with Tween 80 (0.1 % v/v) (Difco™). The conidial suspension was prepared by mixing the solution with a magnetic stirrer for five minutes. It was then adjusted to the desired concentration using Neubauer haemocytometer.

3.2.2. Effect of temperature on conidial germination

Aqueous conidial suspensions (1×10^6 conidia/ml) of 1 ml of each isolate were spread-plated on Sabouraud dextrose agar (SDA) in Petri dishes and left in a laminar flow cabinet for 10 minutes to allow excess moisture to dry. The plates were then sealed with masking tape and incubated at 15, 20, 25, 30, and 35 °C in complete darkness. After an incubation period of 18 and 24 h, 1-ml formaldehyde (0.5 %) was transferred to each plate in order to halt germination. Two sterile microscope cover slips were placed on each plate. Germination rates were determined by randomly examining 100 conidia per plate using a compound microscope (400X). A conidium was considered germinated if the germ tube was at least as long as the width of a conidium. Each plate served as replicate, and there were four replicates per temperature and per incubation period.

3.2.2.1. Statistical analysis

Percent conidial germination was angular-transformed ($\arcsine \sqrt{\text{proportion}}$). A factorial analysis with isolate and temperature as main effects was conducted on the transformed data using SPSS-11 for windows. Since there were significant interactions ($P < 0.05$) between isolate and temperature, the data were analyzed for each individual temperature using a one-way

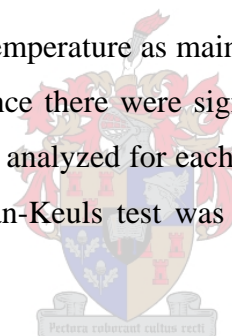
analysis of variance. Student-Newman-Keuls test was used to identify significant differences between means.

3.2.3. Effect of temperature on radial growth

A 3 ml conidial suspension (1×10^8 conidia ml^{-1}) of each isolate was spread-plated on SDA plates. The plates were then sealed with masking tape and incubated at 25 °C in complete darkness for 72 h. From these plates, mycelium disks were cut using a cork borer 6 mm in diameter and were placed in the center of freshly prepared SDA plates. The inoculated plates were sealed with masking tape and incubated at 15, 20, 25, 30, and 35 °C in complete darkness. The diameter of the growing colony was measured daily for 11 days on a pre-marked line with a ruler. Each plate served as replicate. There were five replicates per treatment.

3.2.3.1. Statistical analysis

A factorial analysis with isolate and temperature as main effects was conducted on radial growth data using SPSS-11 for windows. Since there were significant interactions ($P < 0.05$) between isolate and temperature, the data were analyzed for each individual temperature using a one-way analysis of variance. Student-Newman-Keuls test was used to indicate significant differences between means.



3.2.4. Effect of temperature on sporulation

To determine the effect of temperature on sporulation, the above plates (from radial growth) were used. Conidia were harvested by scraping the surface of the culture with a sterile camels' hairbrush into a glass beaker of 500 ml containing 50 ml sterile distilled water containing Tween 80 (0.1 % v/v). The conidial suspension was prepared by mixing the solution with a magnetic stirrer for five minutes. The conidia were then counted using a compound microscope (400X) using Neubauer haemocytometer. Each plate served as a replicate and there were four replicates per treatment.

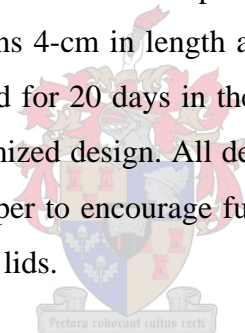
3.2.4.1. Statistical analysis

Sporulation data were Log₁₀-transformed. A factorial analysis with isolate and temperature as main effects was conducted on transformed data using SPSS-11 for windows. Since there were

significant interactions ($P < 0.05$) between isolate and temperature, the data were analyzed for each individual temperature using a one-way analysis of variance. Student-Newman-Keuls test was used to indicate significant differences between means.

3.2.5. Effect of temperature on larval mortality and LT_{50} after treatment with the fungi

Batches of 10 second instar *C. partellus* larvae were placed in sterile 9-cm diameter Petri dishes. An aqueous suspension of 1×10^8 conidia/ml of each fungal isolate was prepared in sterile water containing Tween 80 (0.1 % v/v) as described under conidial preparation in Chapter 2. Each batch was treated with 3 ml of each of the fungal suspension using a Potter's precision laboratory spray tower. Control groups were treated with sterile distilled water containing Tween 80 (0.1 % v/v). The Petri dishes containing treated insects were sealed and incubated at 15, 20, 25, and 30 °C. There were separate controls for each temperature treatment. Each temperature treatment and control was replicated four times with 10 larvae per replication. The treated insects were provided daily with maize leaf sections 4-cm in length and 1-cm wide. Mortality was recorded daily for 6 days for 25 and 30 °C, and for 20 days in the case of 15 and 20 °C. The treatments were arranged in a completely randomized design. All dead insects were removed and placed in Petri dishes lined with moist filter paper to encourage fungal mycelial growth. The Petri dishes were not sealed but covered with their lids.



3.2.5.1. Statistical analysis

Percent cumulative mortality data were angular-transformed ($\arcsine \sqrt{\text{proportion}}$). A factorial analysis with isolate and temperature as main effects was conducted on transformed data using SPSS-11 for windows. Since there were significant interactions ($P < 0.05$) between isolate and temperature, the data were analyzed for each individual temperature using one-way analysis of variance. Student-Newman-Keuls test was used to indicate significant differences between means. The LT_{50} (median lethal times required by the isolates to kill 50 % of the treated insect population) was determined using probit analysis of correlated data for each replicate (Throne *et al.*, 1995). The data were analyzed for each individual temperature using a one-way analysis of variance. Student-Newman-Keuls test was used to indicate significant differences between means.

3.3. RESULTS

3.3.1. Effect of temperature on conidial germination

There were interactions between temperature and isolate for conidia germination (Table 3.1). There were differences in germination of conidia between the fungal isolates at all the temperatures (15, 20, 25, 30 & 35 °C) and incubation time (18 and 24 hrs) ($P < 0.001$ for all the temperatures, Table 3.2). Germination of PPRC-4 and PPRC-19 was higher than that of the other isolates at 20 and 25 °C (Table 3.2). In general, germination of conidia was inhibited at 15 °C and 35 °C. However, germination was lower at 15 than at 35 °C (Table 3.2). PPRC-4, PPRC-61 and EE-01 conidia failed to germinate at 15 °C and BB-01 failed to germinate at 35 °C. All isolates had more than 95 % mean germination when incubated at 25 and 30 °C for 24 h (Table 3.2). When compared to the other isolates, the germination of PPRC-61 was low (42 %) at 25 °C after 18 h. However, it doubled (87 %) when the incubation time was extended to 24 h (Table 3.2).

3.3.2. Effect of temperature on radial growth

Isolate by temperature interactions were significant for radial growth (Table 3.1). There were also differences in mean radial growth between the isolates at all of the temperatures ($P < 0.001$ for all of the temperatures) (Table 3.3). The isolates EE-01, PPRC-4 and PPRC-19 had the highest radial growth at 30 °C; while BB-01 and PPRC-61 had the highest growth at 25 °C and 20 °C, respectively. These differences in optimum temperature for the different isolates resulted in the isolate by temperature interactions. Lowest radial growth occurred at 15 °C and 35 °C for all isolates except BB-01, which ceased to grow at 35 °C (Table 3.3).

3.3.3. Effect of temperature on sporulation

The interaction between isolate and temperature was significant for sporulation (Table 3.1). There were also differences in sporulation (number of conidia/ml) between the isolates at all of the temperatures ($P < 0.001$ for all of the temperatures) (Table 3.4). The optimum temperature for sporulation was 20 °C for BB-01 and 25 °C for the remaining isolates. These differences in optimum temperature between isolates resulted in the isolate by temperature interactions.

3.3.4. Effect of temperature on mortality and LT₅₀ of larvae after treatment with the fungi

There were interactions between the isolate and temperature for mortality (Table 3.1). There were significant differences between isolates in causing mortality to larvae at 15 °C ($F = 3.61$; $df = 4, 15$; $P < 0.05$), with PPRC-61 causing the least mortality (Figures 3.1). There were no differences in mortality between isolates at 20, 25 and 30 °C. However, at both 25 and 30 °C, all isolates induced 100 % mortality within four to six days.

The LT₅₀ for BB-01 was shorter (3.5 days) than the LT₅₀ for the other isolates (6.9 to 14.4 days) at 15 °C (Table 3.5). The mean LT₅₀ was very short (1.7 days) for all isolates at 25 °C.

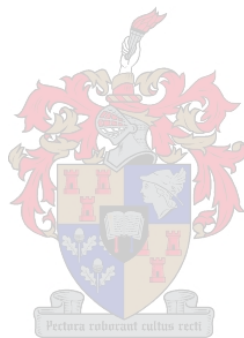


Table 3.1. Factorial analysis of effect of temperature on conidia germination, radial growth, sporulation and pathogenicity of *Beauveria bassiana* and *Metarhizium anisopliae* isolates.

Parameter	Germination			Radial growth			Sporulation			Mortality		
	F	df	P	F	df	P	F	df	P	F	df	P
Isolate (I)	231.9	4	<0.001	307.9	4	<0.001	22.6	4	<0.001	3.5	4	<0.01
Temperature (T)	1882	4	<0.001	2714.6	4	<0.001	239.3	4	<0.001	36.9	3	<0.001
I*T	95	16	<0.001	156.5	16	<0.001	19.2	16	<0.001	3.3	12	<0.001
Error		75			100			75			60	



Table 3.2. Percent conidia germination (mean \pm SE) of *Beauveria bassiana* and *Metarhizium anisopliae* isolates after 18 and 24 hours of incubation at different temperatures.

Isolate	Time (h)	Temperature ($^{\circ}$ C)				
		15	20	25	30	35
18						
BB-01		4.5 \pm 1.8* b	57.5 \pm 4.6 c	93.4 \pm 1.6 b	92.3 \pm 1.3 b	0.0 \pm 0.0 a
PPRC-4		0.0 \pm 0.0 a	98.0 \pm 0.0 d	100 \pm 0.0 c	98.8 \pm 1.2 b	51.3 \pm 0.4 c
PPRC-19		0.8 \pm 0.4 a	96.3 \pm 0.2 d	100 \pm 0.0 c	91.5 \pm 2.3 b	62.5 \pm 4.2 d
PPRC-61		0.0 \pm 0.0 a	32.0 \pm 2.9 b	42.5 \pm 3.3 a	78.0 \pm 4.0 a	12.0 \pm 4.3 b
EE-01		0.0 \pm 0.0 a	16.0 \pm 2.0 a	93.0 \pm 1.0 b	76.5 \pm 0.8 a	58.0 \pm 4.6 dc
SEM		0.00018	0.00021	0.00014	0.00045	0.0003
F-probability		<0.001	<0.001	<0.001	<0.001	<0.001
24						
BB-01		4.0 \pm 0.5 b	59.8 \pm 2.1 b	99.3 \pm 0.7 b	99.3 \pm 0.4 a	0.0 \pm 0.0 a
PPRC-4		0.0 \pm 0.0 a	98.8 \pm 0.2 c	100 \pm 0.0 b	99.5 \pm 0.2 a	85.0 \pm 4.0 d
PPRC-19		3.2 \pm 0.7 b	99.0 \pm 0.4 c	100 \pm 0.0 b	98.7 \pm 0.7 a	88.5 \pm 1.7 d
PPRC-61		0.0 \pm 0.0 a	36.3 \pm 3.4 a	87.8 \pm 1.4 a	87.3 \pm 2.2 a	48.3 \pm 2.0 b
EE-01		0.0 \pm 0.0 a	24.0 \pm 1.2 a	99.0 \pm 0.4 b	90.7 \pm 2.4 a	70.0 \pm 3.3 c
SEM		0.0017	0.0017	0.0002	0.0081	0.00029
F-probability		<0.001	<0.001	<0.001	<0.001	<0.001

*Means \pm SE followed by the same letter in a column are not significant at $P > 0.05$ using Student-Newman-Keuls test.

Table 3.3. Radial growth (mm) of *Beauveria bassiana* and *Metarhizium anisopliae* isolates after 11 days of incubation at different temperatures

Isolate	Temperature (°C)				
	15	20	25	30	35
BB-01	13.6 ± 0.7* d	19.6 ± 0.4 a	27.0 ± 0.4 c	22.6 ± 0.5 a	0.0 ± 0.0 a
PPRC-4	7.4 ± 0.4 a	17.2 ± 0.3 a	18.9 ± 0.6 a	35.4 ± 0.4 c	11.2 ± 0.3 d
PPRC-19	8.2 ± 0.4 ab	18.6 ± 0.8 a	20.2 ± 0.2 a	32.7 ± 0.2 b	9.0 ± 0.6 c
PPRC-61	11.4 ± 0.4 c	25.2 ± 1.0 c	23.7 ± 0.4 b	23.9 ± 0.7 a	11.6 ± 0.4 d
EE-01	9.2 ± 0.3 b	21.2 ± 1.0 b	24.0 ± 0.7 b	38.3 ± 0.5 d	7.4 ± 0.2 b
SEM	0.06	0.15	0.07	0.07	0.03
F-probability	<0.001	<0.001	<0.001	<0.001	<0.001

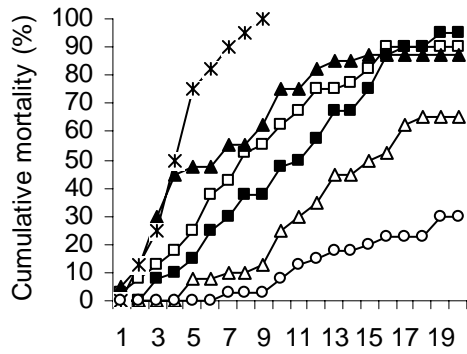
*Means ± SE followed by the same letter in a column are not significant at P > 0.05 using Student-Newman-Keuls test.

Table 3.4. Sporulation intensity (conidia ml⁻¹) (mean ± SE) of *Beauveria bassiana* and *Metarhizium anisopliae* isolates after incubation for 11 days at five temperatures.

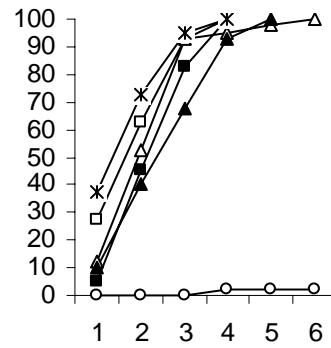
Isolate	Temperature (°C)				
	15	20	25	30	35
BB-01	1.24x10 ⁷ ± 0.7x10 ⁶ *b	2.25x10 ⁷ ± 0.27x10 ⁶ d	1.78 x10 ⁷ ± 0.12 x10 ⁷ b	7.5 x10 ⁵ ± 0.1 x10 ⁵ a	0 ± 0 a
PPRC-4	9.12 x10 ⁵ ± 0.55 x10 ⁵ a	1.77x10 ⁶ ± 0.14 x10 ⁶ b	3.93 x10 ⁶ ± 0.22 x10 ⁶ a	2.77 x10 ⁶ ± 0.17 x10 ⁶ ab	7.5 x10 ⁴ ± 0.3 x10 ⁵ b
PPRC-19	3.5 x10 ⁵ ± 0.88 x10 ⁵ a	1.42 x10 ⁶ ± 0.2 x10 ⁶ b	3.88 x10 ⁶ ± 0.33 x10 ⁶ a	3.25 x10 ⁶ ± 0.2 x10 ⁷ ab	4.37 x10 ⁴ ± 0.62 x10 ⁴ b
PPRC-61	6 x10 ⁵ ± 0.4 x10 ⁵ a	6.37 x10 ⁵ ± 0.74 x10 ⁵ a	4 x10 ⁶ ± 0.4 x10 ⁶ a	5 x10 ⁴ ± 0 a	0 ± 0 a
EE-01	1.27 x10 ⁷ ± 0.17 x10 ⁶ a	2.78 x10 ⁶ ± 0.12 x10 ⁶ c	1.58 x10 ⁷ ± 0.9 x10 ⁶ b	5.61 x10 ⁶ ± 0.36 x10 ⁶ b	1.25 x10 ⁴ ± 0.72 x10 ⁴ b
SEM	0.0008	0.00038	0.00028	0.0027	0.088
F-probability	<0.001	<0.001	<0.001	<0.001	<0.001

Means ± SE followed by the same letter in a column are not significant at P > 0.05 using Student-Newman-Keuls test.

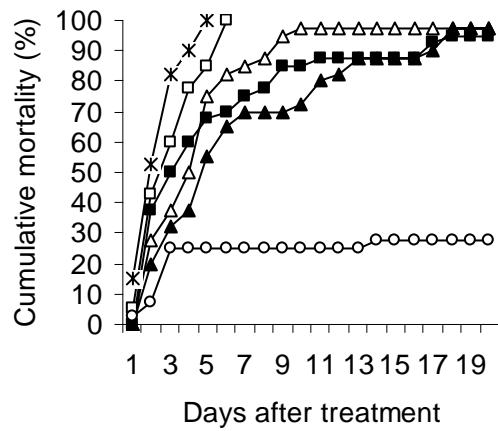
15 °C



25 °C



20 °C



30 °C

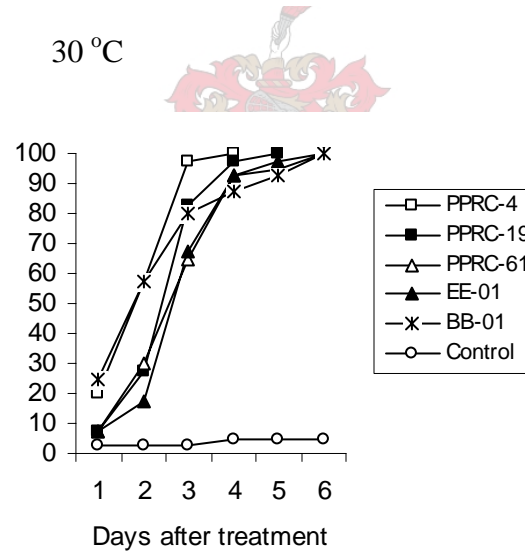


Figure 3.1. Mortality of *Chilo partellus* second instar larvae after being treated with *Beauveria bassiana* and *Metarhizium anisopliae* isolates and incubated for 6 to 20 days at different temperatures.

Table 3.5. Effect of temperature on days, median lethal time (LT₅₀) (mean ± SE) for *Beauveria bassiana* and *Metarhizium anisopliae* isolates against *Chilo partellus* second instar larvae.

Isolate	Temperature (°C)			
	15	20	25	30
BB-01	3.5 ± 0.4* a	3.2 ± 1.2 a	1.3 ± 0.1 a	1.6 ± 0.4 a
PPRC-4	6.9 ± 1.3 b	3.0 ± 0.6 a	1.4 ± 0.1 a	2.1 ± 0.3 a
PPRC-19	8.3 ± 0.7 b	4.4 ± 1.3 a	2.0 ± 0.2 a	2.1 ± 0.1 a
PPRC-61	14.4 ± 1.2 b	3.3 ± 0.6 a	1.9 ± 0.1 a	2.3 ± 0.3 a
EE-01	8.0 ± 4.5 b	5.4 ± 1.1 a	2.2 ± 0.2 a	2.2 ± 0.1 a
SEM	1.34	0.29	0.01	0.02
F-probability	0.04	0.44	0.34	0.57

*Means ± SE followed by the same letter in a column are not significant at P > 0.05 using Student-Newman-Keuls test.

3.4. DISCUSSION

Conidial germination, radial growth and sporulation of all isolates were retarded at 15 and 35 °C. Low temperature (15 °C) was, however, more detrimental to conidial germination than radial growth. The isolates PPRC-4 and PPRC-19, had more than 90 % germination between 20-30 °C. Most workers reported that *M. anisopliae* isolates were mesophilic with an optimum temperature for germination and growth of 25-30 °C, a maximum temperature tolerance of 32-35 °C and a minimum temperature tolerance of about 10-12 °C (Roberts & Campbell, 1977; Hywel-Jones & Gillespie, 1990; Glare & Milner, 1991; Welling *et al.*, 1994). Conidia of *M. anisopliae* lost activity at 49-60 °C, but could persist for 2 years at the optimal temperatures (Daoust & Roberts, 1983). Welling *et al.* (1994) showed that only the most thermophilic isolates of *Metarhizium* spp. were highly infective at the warmer regimes of 25-30 °C. The *B. bassiana* isolate, BB-01 also had an optimal germination temperature of about 25 °C. This is in line with the findings of Walstad *et al.* (1970) and Ekesi *et al.* (1999).

Radial growth occurred at both 15 and 35 °C for most of the isolates but to a more limited extent than at the other temperature. However, BB-01 had the highest radial growth and sporulation at 15 °C when compared to the other isolates. Complete cessation of growth of this isolate occurred at 35 °C, indicating that it thrived best at low to moderate temperatures. PPRC-4, PPRC-19 and EE-01 had peak radial growth at 30 °C and peak sporulation at 25 °C. It was therefore apparent that radial growth and sporulation have different optimum temperatures. High temperature (30 °C) may favour vegetative growth more than sporulation. Seven out of eight *M. anisopliae* isolates originating from tropical areas were capable of growing at 35 °C, while a strain of *Metarhizium flavoviride* Gams & Rozsypal of temperate origin stopped growing at 32 °C (Fargues *et al.*, 1992). Milner *et al.* (2002) reported that isolates of *M. anisopliae* had no mycelial growth at 35 °C while rapid growth occurred from 20-30 °C with the best growth at 30 °C. Ouedraogo *et al.*, (1997) reported that *M. anisopliae* isolates have an optimum growth rate at 28 °C.

All isolates used in this study were tropical in origin, and they appeared to be less pathogenic at 15 and 20 °C except BB-01 which was pathogenic at these temperatures. They were, however, highly pathogenic at 25 and 30 °C, inducing 100 % mortality. *Beauveria bassiana* and *M. anisopliae* infection to a number of insects vary from 15 to 30 °C (Liu *et al.*, 1990; Rath *et al.* 1995; Ekesi *et al.*, 1999). Inglis *et al.* (1997) found a poor relationship between

conidia germination, vegetative growth and mortality of grasshopper *Melanoplus sanguinipes* Fabricius (Orthoptera: Acrididae) treated with a Brazilian isolate of *M. flavoviride*. Samuels *et al.* (1989) demonstrated that rapid germination and growth of *M. anisopliae* was correlated with high pathogenicity toward the planthopper *Nilaparvata lugens* Stal (Hemiptera: Delphacidae). We found no consistent relationship between virulence, conidial germination, vegetative growth and sporulation indicating that there may be other factors governing pathogenicity of the isolates.

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CHAPTER 4

Maize leaf consumption by *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) larvae treated with *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin and effects on mortality and mycosis of feeding on natural versus artificial diets

ABSTRACT

Second and third instar *Chilo partellus* larvae were treated with *Beauveria bassiana* and *Metarhizium anisopliae* and measurements were made of daily consumption of maize leaf. Treatment with the fungi was associated with a reduction in mean cumulative daily food consumption. Four concentrations 1×10^8 , 1×10^7 , 1×10^6 , and 1×10^5 conidia/ml, were tested against second instar larvae. At the highest concentrations of 1×10^8 and 1×10^7 conidia/ml, there was a marked reduction in daily food consumption when compared with the low concentrations of 1×10^6 and 1×10^5 conidia/ml. Reduction in food consumption by *C. partellus* larvae treated with *B. bassiana* and *M. anisopliae* may offset the long time to mortality induced by the fungi. The effect of artificial versus natural diet on mortality and mycoses of second instar larvae treated with *B. bassiana* and *M. anisopliae* at 1×10^8 conidia/ml was determined. Larvae provided with an artificial diet suffered little mortality (10 to 20 %) and had fewer mycosed cadavers (20 to 40 %) compared with larvae provided with maize leaves. The LT_{50} was also longer (7 to 19 days) in the case of larvae fed on artificial diet than for those fed on maize leaves (1 to 4.2 days).

4.1. INTRODUCTION

Insect pathogens often require several days to kill their hosts following infection. During this incubation period, the insects may continue feeding, adding to crop damage before their death (Hajek, 1989; Fargues *et al.*, 1994). For this reason microbial pathogens have been criticized as alternatives to chemical insecticides. However, food consumption by insects can be reduced when they become infected (Hajek, 1989). Therefore, infection with pathogens can result in a degree of control in terms of reduced food consumption (Thomas *et al.*, 1997). Thus, effective control need not be determined by mortality alone.

Infection with the entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin, decreased food consumption by some insect pests. Examples include reduction of feeding by Colorado potato beetle larvae *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) and adult cowpea leaf beetle *Ootheca mutabilis* Shalberg (Coleoptera: Chrysomelidae) infected with *B. bassiana* (Fargues *et al.*, 1994; Ekesi, 2001) and the desert locust *Schistocerca gregaria* Forskal (Orthoptera: Acrididae) and variegated grasshopper *Zonocerus variegatus* Linné (Orthoptera: Acrididae) infected by *Metarhizium flavoviride* Gams & Rozypal (Moore *et al.*, 1992; Thomas *et al.*, 1997). However, infection by *M. anisopliae* had little impact on food consumption rates of the subterranean pasture beetle *Adoryphorus couloni* Burmeister (Coleoptera: Scarabaeidae) (Rath & Worledge, 1995). The same was true for *B. bassiana* infecting *Ceratomyia arcuata* Olivier (Coleoptera: Chrysomelidae) (Lord *et al.*, 1987). The influence of entomopathogenic fungi on food consumption by *C. partellum* (Swinhoe) has not been investigated.

Development of fungal pathogens within hosts can be influenced not only by immune reactions of the hosts but also indirectly by the host's diet. For instance, chalkbrood alfalfa leafcutter bees *Megachile rotundata* Fabricius (Hymenoptera: Megachilidae) reared on a natural diet were less susceptible to *Ascosphaera aggregata* Skou than were bees reared on artificial diet (Goettel *et al.*, 1993). Adult chinch bugs *Blissus leucopterus* Leonard (Hemiptera: Lygaeidae) inoculated with *B. bassiana* demonstrated higher mortality when fed wheat, barley, or artificial diet than when fed corn or sorghum (Ramoska & Todd, 1985). The effect of diet composition on the susceptibility of *Chilo partellus* to *B. bassiana* and *M. anisopliae* has not been determined.

Therefore, the objectives of the present study were to:

1. determine maize leaf consumption by second and third instar *C. partellus* larvae treated with the entomopathogenic fungi, *B. bassiana* (BB-01) and *M. anisopliae* (PPRC-4).
2. study how diet (artificial and natural) influences the susceptibility of second instar larvae to infection by *B. bassiana* (BB-01) and *M. anisopliae* (PPRC-4).

4.2. MATERIALS AND METHODS

4.2.1. Insect and fungal cultures and conidial preparation

A laboratory colony of *C. partellus* larvae was obtained from the Agricultural Research Council, Plant Protection Research Institute (PPRI), Pretoria, South Africa (see chapter 2).

The isolates were obtained from Ethiopia (Plant Protection Research Center and Alemaya University) (see chapter 2)).

Conidia were obtained from 3 week old sporulating cultures. The conidia of each isolate were harvested by brushing the surface of the culture with a sterile camel hairbrush into a 500 ml glass beaker containing 50 ml sterile distilled water with Tween 80 (0.1 % v/v) (Difco™). The conidial suspension was prepared by mixing the solution with a magnetic stirrer for five minutes. It was then adjusted to the desired concentration using Neubauer haemocytometer.

4.2.2. Food consumption by *C. partellus* larvae treated with *B. bassiana* and *M. anisopliae*

The following experiments were conducted to determine the effect of *B. bassiana* and *M. anisopliae* treatment on food consumption of *C. partellus*.

Experiment 1: Second instar larvae were treated with 1×10^8 conidia/ml of *B. bassiana* and *M. anisopliae*.

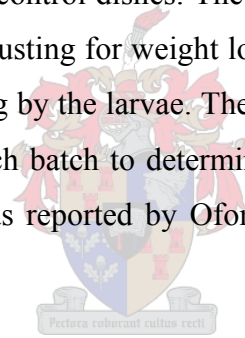
Experiment 2: Third instar larvae were treated with 1×10^8 conidia/ml of the fungi.

Experiment 3: Four conidial concentrations, 1×10^5 , 1×10^6 , 1×10^7 , and 1×10^8 conidia/ml, of *B. bassiana* and *M. anisopliae* were prepared and used to treat second instar larvae.

In each experiment, a batch of 10 *C. partellus* larvae was placed in a sterile Petri dish (9 cm diameter) and treated with a fungal suspension using a Potter's spray tower. Each fungal isolate was assayed by spraying a 3-ml conidial suspension. Control groups in all experiments were treated with distilled water containing Tween 80 (0.1 % v/v). Each experiment had its own

control group. All experiments, with their controls, were replicated four times with 10 larvae per replicate. All experiments were arranged in a completely randomized design and maintained at $25 \text{ }^{\circ}\text{C} \pm 1 \text{ }^{\circ}\text{C}$ for 7-15 days. Dead insects were removed daily.

In order to determine food consumption, maize (variety CRN 3414) was planted in 5 L plastic pots (5 seedlings/pot) and maintained in a growth cabinet at $25 \pm 2 \text{ }^{\circ}\text{C}$. Leaves (from 4-week old seedlings) were washed in a solution of commercial bleach (3% sodium hypochlorite) for 2 to 3 min and then dried for 5 to 10 min. About 62.5 ml of the bleach was diluted in 5-liter water before washing the leaves. Pre-weighed pieces of leaf, 4 cm long and 1 cm wide, were offered to the larvae in a Petri dish which was then sealed with masking tape. Larvae were allowed to feed for one day before replacing the leaf with a new one. The unconsumed leaf tissue was weighed after larval frass was carefully removed. Pre-weighed pieces of control leaf were kept in separate Petri dishes alongside the experimental dishes to determine the weight loss by evaporation. There were no larvae in control dishes. The difference between the initial and final weights of the pieces of leaf, after adjusting for weight loss due to evaporation from leaves, was used to estimate the amount of feeding by the larvae. The total weight consumed was divided by the number of surviving larvae in each batch to determine the average daily food consumption per individual larva. This method was reported by Ofomata *et al.* (2000) in determining food consumption by *Chilo* species.



4.2.2.1. Statistical analysis

Two-sample *t*-tests were used to compare cumulative food consumption between treated and control larvae in Experiments 1 and 2. Cumulative food consumption data from Experiment 3 were analyzed using Dunnett's *t*-test after performing an analysis of variance to determine if the mean food consumption in each treatment differed from the control. SPSS-11 for windows was used for the analysis. A non-linear regression of the form $Y = ae^{bx}$ was used to describe the relationship between log concentration of conidia and percent reduction in consumption; where $Y = \%$ reduction in consumption, a and b are regression coefficients, e is the base of the natural log and $x = \log$ concentration.

4.2.3. Effect of diet on mortality and mycoses of *C. partellus* treated with *B. bassiana* and *M. anisopliae*

4.2.3.1. Diet

The first instar larvae were initially reared on an artificial diet (Kfir, 1992). The composition of the diet was, chickpea powder 720 g, brewers yeast 96 g, glucose 50 g, methyl 4-hydroxybenzoate 6 g, ascorbic acid 8.6 g, sorbic acid 4 g, 3 ml ethyl alcohol 100 % and 2000 ml distilled water. About 500 larvae were maintained on 250 g diet in a plastic bottle (5 cm x 11 cm). The newly molted second instar larvae from the artificial diet were provided with pieces of maize leaves collected from 4-week-old plants (1 cm x 4 cm) 0, 1, 2, 3 and 4 days before application of the fungal isolates. After treatment with the fungi, the larvae were given fresh maize leaves daily for 7 days. Another group of second instar larvae were maintained on the artificial diet for 7 days before and 10 days after treatment with the isolates.

4.2.3.2. Bioassay

A batch of 10 larvae was placed in a sterile Petri dish (9 cm diameter) and treated with 3 ml of conidial suspension, 1×10^8 conidia/ml, using the spray tower. Control groups were treated with distilled water containing Tween 80 (0.1 % v/v). There were six treatments for each isolate. In treatment 1, larvae were sprayed with fungal isolates before feeding on the leaves, and were provided with leaves after treatment (Day-0); larvae in the other treatments were fed leaves for one day (Day-1), two days (Day-2), three days (Day-3), and four days (Day-4) before treatment with the isolates and for 10 days after treatment. In the final treatment, larvae were provided only with the artificial diet before and after application of the fungi (artificial diet only). Twenty grams of the diet were placed in a sterile Petri dish and ten treated larvae were then placed in the Petri dish. The Petri dishes containing the treated insects were sealed with masking tape. Each treatment had its own control. All treatments with their controls were replicated five times with 10 larvae per replication. All experiments were arranged in a completely randomized design and maintained at $25^\circ\text{C} \pm 1^\circ\text{C}$. Mortality was recorded daily for ten days. Dead insects were removed daily and kept in Petri dishes lined with moist filter paper. These were maintained at $25^\circ\text{C} \pm 1^\circ\text{C}$

for seven days to encourage mycoses in the cadavers. Mycosis was verified by examining the cadavers using stereo-microscope.

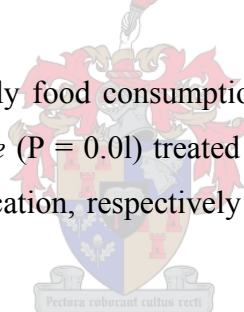
4.2.3.3. Statistical analysis

Mortality data were adjusted for control mortality (Abbott, 1925). Mortality and mycoses data were angular-transformed ($\arcsine\sqrt{\textit{proportion}}$) before subjecting them to an analysis of variance using SPSS-11 for windows. LT_{50} (lethal time required to kill 50 % of the treated insect population) for each replicate was determined using probit analysis for correlated data (Throne *et al.*, 1995). Student-Newman-Keuls test was used for mean separation after subjecting to one-way ANOVA.

4.3. RESULTS

4.3.1. Food (leaf) consumption (by *C. partellus* larvae treated with *B. bassiana* and *M. anisopliae*)

Experiment 1: Mean cumulative daily food consumption differed between the control and *B. bassiana* ($P = 0.04$) and *M. anisopliae* ($P = 0.01$) treated second instar larvae from two and one days until six days after fungal application, respectively (Figure 4.1 a & c). The treated larvae consumed less food than the control.



Experiment 2: There were significant differences in food consumption between the control and third instar larvae treated with *B. bassiana* ($P = 0.01$) and *M. anisopliae* ($P = 0.01$) between six and 15 days after treatment with *B. bassiana* (Figure 4.1 b) and between 11 and 15 days after treatment with *M. anisopliae* (Figure 4.1 d).

Experiment 3: There were differences ($P = 0.02$) in food consumption between the control and *B. bassiana* treated second instar larvae at a concentration 1×10^8 conidia/ml, five days after treatment (Table 4.1). However, the differences ($P = 0.02$) in consumption between the controls and larvae treated with 1×10^8 *M. anisopliae* conidia/ml were apparent two days after treatment (Table 4.2). Larvae treated with *B. bassiana* at 1×10^7 conidia/ml differed ($P = 0.03$) from the control eight days after treatments (Table 4.1). There were no differences between the controls

and larvae treated with 1×10^6 and 1×10^5 *B. bassiana* conidia/ml (Table 4.1). In the case of *M. anisopliae*, differences in food consumption between the controls and larvae treated with 1×10^5 ($P = 0.04$), 1×10^6 ($P = 0.01$) and 1×10^7 ($P = 0.02$) conidia/ml occurred seven days after treatment (Table 4.2). With increasing conidial concentrations, mean cumulative daily leaf consumption decreased. At 1×10^8 conidia/ml there was about 80 % reduction in food consumption (Figure 4.2 a & b).

4.3.2. Effect of diet on mortality, LT_{50} and mycosis in *C. partellus* treated with *B. bassiana* and *M. anisopliae*

There were differences in mortality ($F = 43.5$; $df = 5, 24$; $P < 0.001$) and LT_{50} ($F = 26.9$; $df = 5, 24$; $P < 0.001$) of *C. partellus* larvae treated with *B. bassiana* and fed on the different diets (Table 4.3). The same was true for larvae treated with *M. anisopliae* (Differences in mortality: $F = 56.9$; $df = 5, 24$; $P < 0.001$. Differences in LT_{50} : $F = 35.6$; $df = 5, 24$; $P < 0.001$) (Table 4.4). Larvae fed the artificial diet before and after being treated with the fungi suffered little mortality (10 to 20 %), while larvae provided with maize leaves always suffered high mortality (92 to 100 %).

There was an extended LT_{50} (7 to 19 days) in the case of larvae provided with the artificial diet but the LT_{50} was short (1 to 4.2 days) for larvae provided with maize leaves (Tables 4.3 & 4.4).

Mycoses in cadavers varied between diets for *B. bassiana* ($F = 24.3$; $df = 5, 24$; $P < 0.001$) and *M. anisopliae* ($F = 4.57$; $df = 5, 24$; $P < 0.001$) treated larvae. The least (20 to 40 %) mycosed cadavers occurred among larvae provided with the artificial diet before and after application of the fungi (Figure 4.3 a & b).

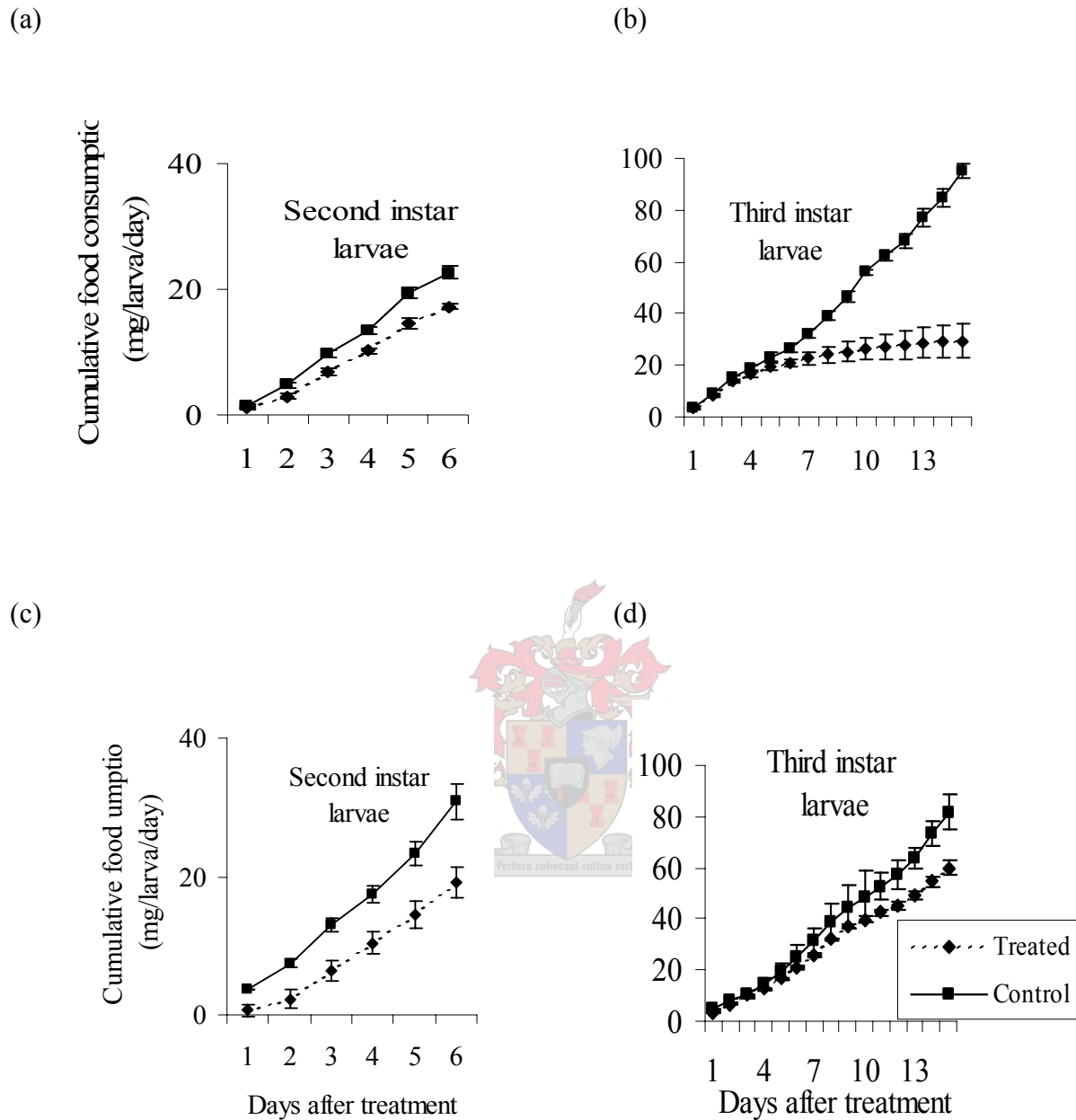


Figure 4.1. Cumulative daily maize leaf consumption by *Chilo partellus* second (a & c) and third (b & d) instar larvae after being treated with *Beauveria bassiana* (a & b) and *Metarhizium anisopliae* (c & d). Vertical lines represent standard errors.

Table 4.1. Mean (\pm SE) cumulative daily maize leaf consumption (mg/larva/day) by second instar *Chilo partellus* larvae treated with different conidial concentrations (conidia/ml) of *Beauveria bassiana*.

Days after treatment	Conidial concentration (conidia/ml)				Control
	1×10^5	1×10^6	1×10^7	1×10^8	
1	0.9 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.2 ± 0.4
2	7.0 ± 0.2	6.5 ± 0.3	6.4 ± 0.3	6.0 ± 1.0	5.8 ± 0.1
3	10.8 ± 0.5	10.3 ± 0.6	9.6 ± 0.5	8.0 ± 1.4	9.3 ± 0.1
4	16.1 ± 0.3	16.0 ± 0.8	13.2 ± 0.9	10.2 ± 2.0	14.0 ± 0.6
5	19.5 ± 0.5	20.3 ± 1.3	16.6 ± 1.2	$11.8 \pm 2.7^*$	18.7 ± 0.8
6	22.2 ± 0.9	22.9 ± 1.5	18.6 ± 1.1	$11.8 \pm 2.7^*$	22.1 ± 0.9
7	26.3 ± 1.5	26.2 ± 1.8	22.1 ± 1.8	^a	26.3 ± 1.3
8	30.0 ± 2.2	30.5 ± 2.3	$23.0 \pm 2.3^*$	-	30.4 ± 1.7
9	35.0 ± 2.8	35.3 ± 2.9	$24.9 \pm 2.9^*$	-	36.0 ± 2.2
10	38.9 ± 2.9	40.2 ± 3.2	$26.8 \pm 3.5^*$	-	40.4 ± 2.7
11	44.1 ± 3.1	45.5 ± 4.1	$29.8 \pm 4.4^*$	-	46.4 ± 3.3
12	47.1 ± 3.3	48.8 ± 4.3	$31.0 \pm 4.9^*$	-	50.6 ± 3.5
13	50.1 ± 3.7	51.8 ± 4.8	$32.6 \pm 5.5^*$	-	55.7 ± 4.4
14	56.1 ± 4.5	56.8 ± 5.5	$34.9 \pm 6.3^*$	-	75.6 ± 6.5
15	62.2 ± 5.1	61.8 ± 5.9	$37.3 \pm 7.1^*$	-	81.4 ± 5.7

*Means significantly different ($P < 0.05$) from the control using Dunnett's t-test.

^aNo consumption as all treated insects died.

Table 4.2. Mean (\pm SE) cumulative daily maize leaf consumption (mg/larva/day) by second instar *Chilo partellus* larvae treated with different conidial concentrations (conidia/ml) of *Metarhizium anisopliae*.

Days after treatment	Conidial concentration (conidia/ml)				Control
	1×10^5	1×10^6	1×10^7	1×10^8	
1	2.8 \pm 0.8	2.3 \pm 0.4	2.3 \pm 0.3	2.1 \pm 0.3	4.0 \pm 0.7
2	8.0 \pm 1.0	6.9 \pm 0.6	7.6 \pm 0.3	6.1 \pm 0.4*	9.0 \pm 0.6
3	12.2 \pm 0.9	11.3 \pm 0.5	12.1 \pm 0.4	9.6 \pm 0.7*	12.9 \pm 0.7
4	16.3 \pm 0.8	14.9 \pm 0.8	15.8 \pm 0.6	12.8 \pm 0.8*	17.6 \pm 0.9
5	19.8 \pm 1.2	18.4 \pm 0.9	19.2 \pm 0.7	14.2 \pm 1.3*	23.3 \pm 1.3
6	23.7 \pm 2.1	22.0 \pm 1.0	23.0 \pm 0.8	15.0 \pm 1.9*	28.9 \pm 1.8
7	27.4 \pm 2.6*	25.4 \pm 1.0*	26.5 \pm 1.0*	- ^a	34.2 \pm 1.9
8	31.9 \pm 3.2*	30.7 \pm 1.0*	30.4 \pm 1.7*	-	39.6 \pm 1.6
9	35.6 \pm 3.3*	34.6 \pm 1.3*	33.8 \pm 2.2*	-	44.4 \pm 1.4
10	39.7 \pm 3.5*	38.8 \pm 1.6*	36.9 \pm 2.1*	-	49.8 \pm 1.8
11	44.2 \pm 3.7*	41.6 \pm 2.3*	40.5 \pm 2.5*	-	54.4 \pm 1.8
12	50.5 \pm 4.9*	45.0 \pm 2.6*	44.6 \pm 2.6*	-	60.9 \pm 2.5
13	55.3 \pm 5.9*	49.2 \pm 2.3*	48.7 \pm 2.8*	-	68.1 \pm 2.0
14	60.8 \pm 6.7*	53.9 \pm 2.2*	53.5 \pm 3.1*	-	75.3 \pm 1.4
15	66.7 \pm 7.4*	59.4 \pm 2.6*	58.8 \pm 3.8*	-	83.0 \pm 1.5

*Means significantly different ($P < 0.05$) from the control using Dunnett's t-test.

^aNo consumption as all treated insects died.

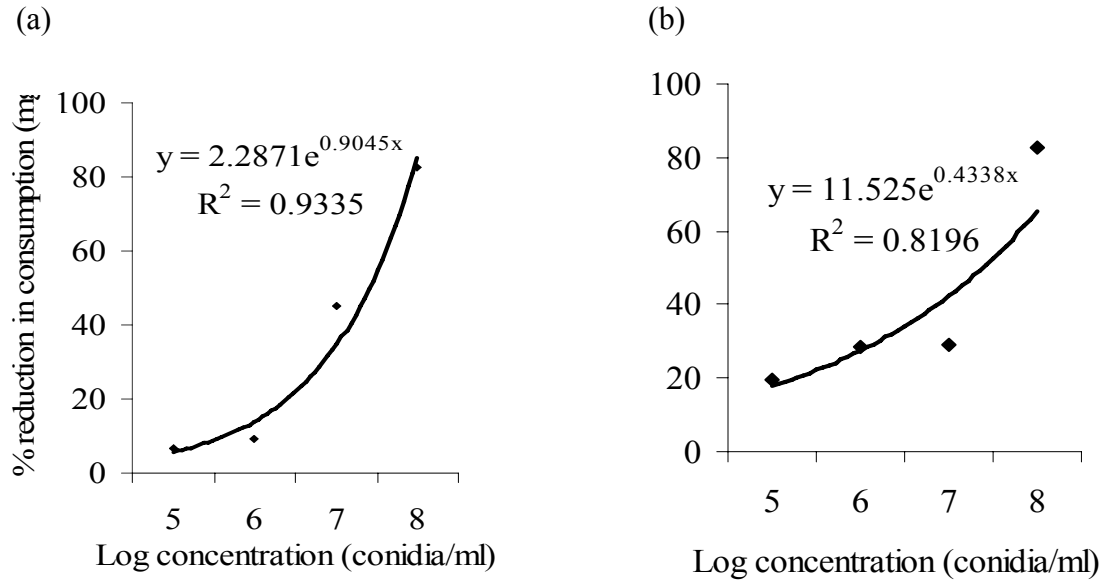


Figure 4.2. Percent reduction in leaf consumption (mg/larva) of *Chilo partellus* second instar larvae treated with (a) *Beauveria bassiana* and (b) *Metarhizium anisopliae* at conidial concentrations of 1×10^5 , 1×10^6 , 1×10^7 and 1×10^8 conidia/ml.

Table 4.3. Mortality and LT_{50} of *Chilo partellus* second instar larvae provided with natural (maize leaf) and artificial diets before and after treatment with *Beauveria bassiana*. The Chi-square (χ^2) and P-values indicate the goodness of fit of the regression model.

Days	%mortality \pm SE*	$LT_{50} \pm$ SE*	Intercept \pm SE	Slope \pm SE	χ^2	P-value
0	100 \pm 0.0 b	1.5 \pm 0.1 a	-1.39 \pm 0.25	6.8 \pm 2.5	1.0	0.01
1	98 \pm 2.0 b	3.3 \pm 0.8 a	-2.17 \pm 0.37	7.7 \pm 3.1	1.0	0.33
2	96 \pm 4.0 b	2.9 \pm 0.4 a	-1.74 \pm 0.25	3.9 \pm 1.2	2.0	0.01
3	98 \pm 2.0 b	4.0 \pm 0.2 a	-2.87 \pm 0.37	8.7 \pm 2.7	2.3	0.54
4	100 \pm 0.0 b	2.4 \pm 0.3 a	-1.57 \pm 0.25	5.4 \pm 2.0	1.1	0.35
Artificial diet only	20 \pm 5.5 a	19.3 \pm 6.6 b	-2.6 \pm 0.56	0.3 \pm 0.3	1.1	0.01

*Means \pm SE followed by the same letter with in a column are not significant at $P > 0.05$ using Student-Newman-Keuls. **Number of days larvae fed on maize leaves before treatment.

Table 4.4. Mortality and LT₅₀ of *Chilo partellus* second instar larvae provided with natural (maize leaf) and artificial diets before and after treatment with *Metarhizium anisopliae*. The Chi-square (χ^2) and P –values indicate the goodness of fit of the regression model.

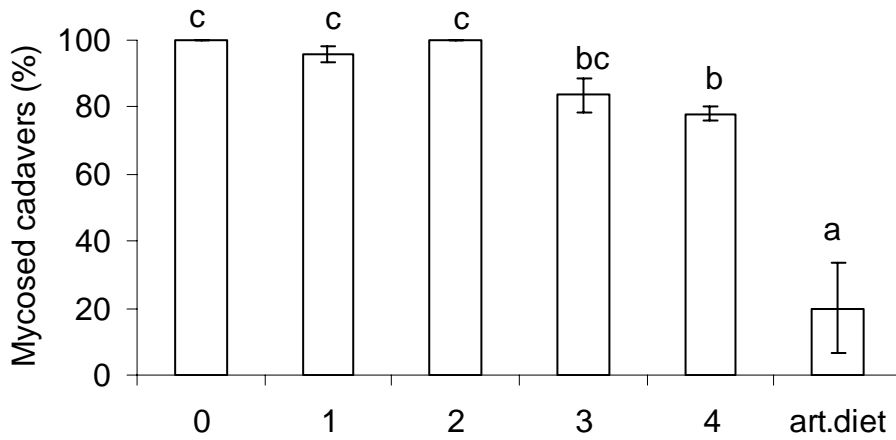
Days **	%mortality ± SE*	LT ₅₀ ± SE*	Intercept ± SE	Slope ± SE	χ^2	P- value
0	100 ± 0.0 b	1.0 ± 0.0 a	0.24 ± 0.18	2.7 ± 1.4	1.0	0.01
1	98 ± 2.0 b	2.7 ± 0.4 b	-1.52 ± 0.23	4.8 ± 1.4	1.9	0.99
2	92 ± 3.7 b	4.2 ± 0.4 bc	-2.54 ± 0.23	7.6 ± 2.2	6.4	0.40
3	100 ± 0.0 b	3.4 ± 0.1 bc	-2.97 ± 0.40	5.1 ± 1.6	4.8	0.22
4	98 ± 2.0 b	3.5 ± 0.3 bc	-2.56 ± 0.39	6.1 ± 1.8	1.5	0.58
Artificial only	diet 10 ± 4.5 a	7.0 ± 0.6 d	-1.52 ± 0.28	1.7 ± 1.7	1.1	0.01

*Means ± SE followed by the same letter with in a column are not significant at P > 0.05 using Student-Newman-Keuls Test.

** Number of days larvae fed on maize leaves before treatment.



(a)



(b)

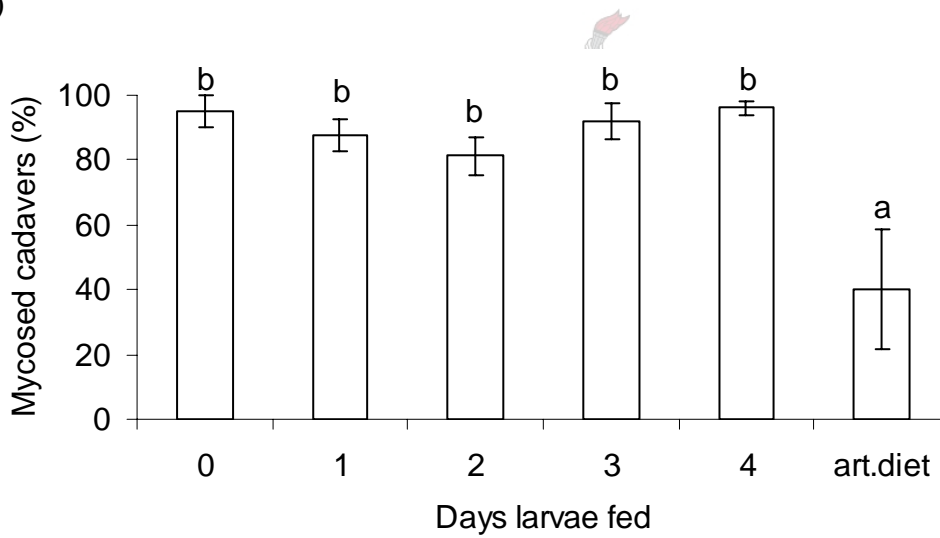


Figure 4.3. The effect of maize leaves and artificial diet consumed by second instar *Chilo partellus* larvae on mycosis in cadavers caused by (a) *Beauveria bassiana* and (b) *Metarhizium anisopliae*. Vertical bars represent SE. Bars with the same letters do not differ at $P > 0.05$ using Student-Newman-Keuls test.

4.4. DISCUSSION

Daily food consumption of *C. partellus* second instar larvae decreased 1 and 2 days after treatment with *M. anisopliae* and *B. bassiana*, respectively. The most pronounced effect was observed with the highest concentration (1×10^8 conidia/ml) of conidia. Infection of *O. mutabilis* by *M. anisopliae* and *B. bassiana* caused a reduction in food intake 1 day after application of the fungus (Ekesi, 2001). In addition, studies by Moore *et al.* (1992), Seyoum *et al.* (1994) and Thomas *et al.* (1997) have all shown significant reductions in feeding as early as 1 to 4 days after treatment with *M. flavoviride*, in the desert locust, *S. gregaria* and the variegated grasshopper, *Z. variegatus*, respectively. *Beauveria bassiana* infection in the Colorado potato beetle caused a decrease in food consumption 3 days after treatment (Fargues *et al.*, 1994).

Our studies revealed that treated larvae consumed less food than untreated larvae. However, Cheung & Gula (1982) reported the continued feeding of infected corn earworm larvae *Heliothis zea* Boddie (Lepidoptera: Noctuidae) until death, which may indicate that infection caused starvation due to biochemical changes in the hemolymph of infected larvae. Reduction in feeding may be attributed to production of toxic substances by the fungi. The destructive effects of proteases produced by the pathogen on insect cuticle have been reported (St. Leger *et al.*, 1991). Secondary metabolites produced by *M. anisopliae* act on insect tissues including the mid-gut (Samuels *et al.*, 1988). The production of these metabolites may cause loss of appetite.

Reduction in food consumption of third instar larvae was evident 4 to 5 days after infection with the fungi. Fargues *et al.* (1994) reported higher susceptibility and reduction in consumption by first instar than final instar larvae of the Colorado potato beetle infected with *B. bassiana*.

Bioassays of *C. partellus* larvae fed artificial versus natural diet revealed susceptibility to the fungal isolates was influenced by diet types. Larvae provided with the artificial diet before and after being treated with the fungus suffered less mortality and fewer mycoses than did larvae with natural provisions. The artificial diet contained antimicrobial substances (methyl 4-hydroxybenzoate, ascorbic acid, sorbic acid and ethyl alcohol) (Kfir, 1992), which probably inhibited infectivity of the fungal isolates. Goettel *et al.* (1993) reported larvae of *Megachile rotundata* Fabricius (Hymenoptera: Megachilidae) reared on an artificial diet had decreased number of sporulating cadavers.

The high rate of mortality and mycosis resulting from larvae fed on maize leaves (natural diet) implied that bioassay of *C. partellus* larvae fed on the artificial diet would underestimate the pathogenicity of the isolates. Thus, an appropriate diet for larvae being used for bioassay of fungal pathogens against *C. partellus* would be a natural diet. Hajek & St. Leger (1994) reported that disease development in *Lymantria dispar* Linnaeus (Lepidoptera: Lymantriidae) infected with *Entomophaga* sp. was linked to the effects of plant nutritional quality, suggesting that host-plant factors inhibitory to fungal growth were absent in larval hemolymph. In contrast, few cadavers of chinch bugs that had eaten corn or sorghum produced conidia, demonstrating an inhibitory effect of these foods, presumably from fungistatic secondary plant chemicals (Ramosaka & Todd, 1985).

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CHAPTER 5

***In-vitro* and *in-vivo* compatibility of *Beauveria bassiana* Balsamo (Vuillemin) and *Metarhizium anisopliae* (Metschnikoff) Sorokin (Deuteromycotina: Hyphomycetes) with benfuracarb and endosulfan against *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae)**

ABSTRACT

In-vitro and *in-vivo* studies were conducted to determine the compatibility of *Beauveria bassiana* (BB-01) and *Metarhizium anisopliae* (PPRC-4, PPRC-19 & EE-01) with the insecticides, benfuracarb and endosulfan. In *in-vitro* studies, five concentrations of the insecticides: 0.1 ppm, 1 ppm, 5 ppm, 10 ppm, and 100 ppm active ingredients in agar were tested against the isolates. The effects of the insecticides on conidia germination, radial growth and sporulation varied among the isolates. The response of the isolates also varied with the type of insecticide. Increasing concentration of the insecticides adversely affected conidia germination, radial growth and sporulation. In *in-vivo* studies, BB-01 and PPRC-4 (both at 1×10^7 conidia /ml) and 1 ppm and 5 ppm concentration of the insecticides were applied in combination and alone against third instar *Chilo partellus* larvae. Combining benfuracarb at 1 ppm and 5 ppm and endosulfan at 5 ppm with BB-01 and PPRC-4 resulted in an additive effect on *C. partellus* larval mortality. Therefore, combining the fungi with low concentrations (1 ppm and 5 ppm) of the insecticides increased the effectiveness of the former.

5.1. INTRODUCTION

The most commonly recommended and applied insecticides against *Chilo partellus* (Swinhoe) include pyrethroids, monocrotophos and endosulfan (Vandenberg & Van Rensburg, 1993). The persistent nature of this insecticide increases its efficacy against the stem borer (Vandenberg & Nur, 1998). In South Africa, benfuracarb is recommended against *C. partellus* when mixed with other insecticides (Nel *et al.*, 1999).

In the integrated management of stem borers a combination of cultural practices, resistant varieties, natural enemies and insecticides is used (Seshu Reddy, 1998; Vandenberg & Nur, 1998). The entomogenous fungus *B. bassiana* has shown considerable promise as a microbial control agent of *C. partellus* (Maniania, 1993). Although entomopathogenic fungi have potential to control stem borers, the extended time required by the fungi to control insects may necessitate their integration with chemical insecticides. In addition, insecticide-pathogen combinations delayed resistance development to insecticides by Colorado potato beetle (Anderson *et al.*, 1989).

There are conflicting reports regarding the compatibility of *B. bassiana* and *M. anisopliae* with insecticides. These range from synergistic (Quintela & McCoy, 1998; Furlong & Groden, 2001), antagonistic (Anderson & Roberts, 1983; James & Elzen, 2001) to additive (Anderson *et al.*, 1989). Therefore, the integration of fungal isolates with insecticides requires a thorough knowledge of the compatibility of the two agents (Gardner *et al.*, 1979; Vanninen & Hokkanen, 1988).

In-vitro and *in-vivo* laboratory studies on the compatibility of fungi with insecticides are necessary before conducting field applications. Information on the effects of insecticides on conidial germination, mycelial growth and sporulation of *B. bassiana* and *M. anisopliae* are essential if these pathogens are going to be combined with insecticides in an integrated programme against stem borer. Compatibility studies will also assist in the determination of insecticide concentrations that will enhance the effectiveness of *B. bassiana* and *M. anisopliae*. Although compatibility studies between *B. bassiana* and *M. anisopliae* with insecticides have been conducted for a few agricultural pests, this has not yet been done on *C. partellus*. The current investigations were undertaken to:

1. study *in-vitro* effects of benfuracarb and endosulfan at 0.1 ppm, 1 ppm, 5 ppm, 10 ppm and 100 ppm (active ingredients) in agar on conidial germination, radial growth and sporulation of *B. bassiana* (BB-01) and *M. anisopliae* (PPRC-4, PRC-19 and EE-01).

2. study *in-vivo* effects of benfuracarb and endosulfan at 1 ppm and 5 ppm with *B. bassiana* (BB-01) and *M. anisopliae* (PPRC-4) when used against *C. partellus* third instar larvae.

5.2. MATERIALS AND METHODS

5.2.1. Insecticides

Commercial formulations of the insecticides, benfuracarb (trade name, Oncol), emulsifiable concentrate, 200g/litre active ingredient (a.i.) and endosulfan (trade name, endosulfan), emulsifiable concentrate, 350g/liter a.i.), were provided by Sanachem (Pvt), Ltd, South Africa. The concentrations, 1 ppm (1×10^{-6} µl/l), 10 ppm (1×10^{-5} µl/l), 50 ppm (5×10^{-5} µl/l), 100 ppm (1×10^{-4} µl/l) (parts per million a.i.) and 1 ppt (1×10^{-3} µl/l) (part per thousand a.i.) were prepared for each insecticide. The desired insecticide concentrations were prepared by serial dilution of the commercial formulation in sterile distilled water. These insecticides were selected because of their availability.

5.2.2. Fungal cultures and media preparation

Beauveria bassiana (BB-01) and *M. anisopliae* (PPRC-4, PPRC-61 & EE-01) were used. Selection of these isolates was based on their pathogenicity to second instar *C. partellus* larvae (Chapter 2). Fungal cultures were produced on Saboraud dextrose agar (SDA). The medium was prepared by mixing 32.5 g SDA in 500 ml distilled water in glass bottles. After autoclaving in a pressure cooker for 30 minutes, the bottles containing the medium were left on a laboratory bench to cool for about 35 minutes. When the medium had cooled to about 30 °C, the different concentrations of the insecticides were added to the bottles containing the medium in a 1:9 ratio of insecticide to medium. This reduced the insecticide concentration in the agar by 1/10 to 0.1 ppm (1×10^{-7} µl/l), 1 ppm (1×10^{-6} µl/l), 5 ppm (5×10^{-6} µl/l), 10 ppm (1×10^{-5} µl/l) and 100 ppm (1×10^{-4} µl/l). The bottles containing the insecticides mixed with SDA were then hand shaken and rolled on a clean bench for five minutes to ensure uniform mixing of the insecticides with the medium. Approximately 14.4 ml of the medium mixed with 1.6 ml of the insecticide was poured into sterile Petri dishes (6.5 cm in diameter) and allowed to solidify for 15 minutes in a laminar flow cabinet.

5.2.3. Preparation of conidia and mycelia mats

Conidia from 3-week-old sporulating cultures were brushed into beakers filled with 20 ml sterile distilled water containing Tween 20 (0.1 % v/v) using a sterile camels hair brush. The conidial suspension was then stirred using a magnetic stirrer for five minutes to enhance uniform dispersion of the conidia. About 0.5 ml of the conidial suspension (1×10^6 conidia/ml) was spread-plated onto SDA and incubated for three days at 25 °C in darkness in order to obtain mycelia mats. The concentration of conidia was quantified using haemocytometer.

5.2.4. *In-vitro* effects of benfuracarb and endosulfan on conidia germination, radial growth and sporulation of *B. bassiana* and *M. anisopliae*

5.2.4.1. Conidial germination

The effect of insecticides on conidial germination was tested by spread-plating 0.5 ml of conidial suspensions containing 1×10^6 conidia /ml on the SDA plates mixed with benfuracarb and endosulfan. Conidial suspensions spread on SDA plates with no benfuracarb or endosulfan served as controls. Inoculated plates were sealed with masking tape and incubated at 25 °C in complete darkness. After 24 hours of incubation, sterile coverslips were placed on the plate. Percentage germination was then assessed by randomly counting 100 conidia in a plate at 400X magnification using a compound microscope. A conidium was counted as germinated if the length of the germ tube was equal to at least the width of the conidium. Each plate served as a replicate with four replicates per treatment.

5.2.4.2. Radial growth

Round plugs of mycelial mats were cut from each culture plate using a 6 mm diameter sterile cork borer. Each agar plug was inverted onto the center of a SDA plate containing benfuracarb or endosulfan. The plate was sealed with masking tape and incubated at 25 °C. Agar plugs placed on SDA plates with no benfuracarb or endosulfan served as a control. Each plate served as a replicate with four replicates per treatment. Fungal radial growth was measured from the edge of the agar plug daily for 12 days. Measurements were made in millimeters using a ruler along the same two pre-marked radial lines on each day.

5.2.4.3. Sporulation

Conidia from the above plates were collected by scraping them with a sterile camel's hair brush into a beaker containing 50 ml sterile distilled water containing Tween 20 (0.1 % v/v).

The conidia suspensions were agitated using a magnetic stirrer for five minutes. Final conidia production was determined using haemocytometer. Each plate served as a replicate with four replicates per treatment.

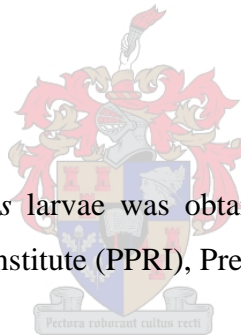
5.2.4.4. Statistical analysis

Dummy variable regressions analysis (Gujarati, 1970) was used to test for differences between the regressions of percent germination, radial growth and sporulation on log-concentration of insecticides. Details are explained in the appendix (see appendix). The structure of the reduced dummy variable models was determined by inspecting the regression coefficients of the full model and using backward stepwise regression. The data were analyzed using Statistica 6.5 computer software.

5.2.5. *In-vivo* compatibility of benfurcarb and endosulfan with *B. bassiana* and *M. anisopliae* against *C. partellus*

5.2.5.1. Insects used

A laboratory colony of *C. partellus* larvae was obtained from the Agricultural Research Council, Plant Protection Research Institute (PPRI), Pretoria, South Africa (see chapter 2).



5.2.5.2. Fungus and insecticide treatment combination

Based on results from the *in-vitro* compatibility tests (5.2.5), 1 ppm and 5 ppm (a.i.) concentrations of the insecticides were prepared in sterile distilled water. One milliliter of the suspension of each insecticide was sprayed onto fresh pieces of maize leaf (1 cm x 4 cm) in a sterile Petri dish using a Potter's spray tower. A batch of 10 *C. partellus* third instar larvae was placed in a sterile Petri dish (9-cm diameter) and then provided with insecticide-treated leaves. After feeding on these leaves for 24 hrs, the larvae were transferred to a sterile Petri dish and sprayed with a 3 ml conidial suspension (1×10^7 conidia/ml) of BB-01 and PPRC-4. The larvae were then transferred to untreated leaves in a new Petri dish (9-cm in diameter) and then sealed with masking tape. The leaves were replaced daily. Control larvae were sprayed with 3 ml sterile distilled water containing Tween 80 (0.1 % v/v). There were separate controls for 1 ppm and 5 ppm treatments. *B. bassiana* (BB-01) and *M. anisopliae* (PPRC-4) were mixed with benfurcarb and endosulfan at 1 ppm and 5 ppm to produce the

following treatments. BB-01 + benfuracarb, BB-01 + endosulfan, BB-01 alone, PPRC-4 + benfuracarb, PPRC-4 + endosulfan, PPRC-4 alone, endosulfan alone, and benfuracarb alone. Each treatment and control was replicated four times with 10 larvae per replication. They were maintained at 25 °C. Mortality was recorded daily for 15 days.

5.2.5.3 Statistical analysis

Mortality was corrected for control mortality (Abbott, 1925). Mortality was angular transformed ($\arcsine\sqrt{\text{proportion}}$). The transformed data were subjected to a one-way ANOVA using SPSS-11 for windows. Student-Newman-Keuls test was used to determine whether or not there were significant differences between means.

5.3. RESULTS

5.3.1. *In-vitro* effects of benfuracarb and endosulfan on conidial germination, radial growth and sporulation of *B. bassiana* and *M. anisopliae*

All the basic slope coefficients were significant (PPRC-4) and negative (b_4 values in Table 5.1). By examining the regression coefficients in Table 5.1, appropriate reduced regression models were tested to determine the effects of insecticides on germination, radial growth and sporulation of the isolates. The results are given in Tables 5.2 & 5.3. In addition, the multiple correlation coefficients (R values in Tables 5.2 & 5.3) were all highly significant ($P < 0.001$). Most of the slopes were negative. Therefore, increasing concentrations of both insecticides adversely affected the germination, radial growth and sporulation by the fungal isolates. Exceptions were the effects of benfuracarb on sporulation of BB-01 (Table 5.2) and endosulfan on the radial growth of PPRC-19. In these two cases the slopes were positive, thus indicating enhancement of sporulation of BB-01 by benfuracarb and of radial growth of PPRC-19 by endosulfan.

Table 5.1. Regression coefficients for the full model with their standard errors (SE) and significance levels (P) for percent germination, radial growth and sporulation regressed on the concentration of benfuracarb and endosulfan. Degrees of freedom = 7.

Coefficient			Benfuracarb			Endosulfan		
			Coefficient value	SE	P	Coefficient value	SE	P
Germination								
b ₀	PPRC-4	Intercept	89.6	4.8	<0.001	78.4	2.3	<0.001
b ₁	PPRC-19	Intercept	0.5	6.8	0.87	10.7	3.2	<0.001
b ₂	EE-01	Intercept	-48.5	6.8	<0.001	10.1	3.2	<0.001
b ₃	BB-01	Intercept	-2.4	6.8	0.94	10.1	3.2	<0.001
b ₄	PPRC-4	Slope	-20.8	4.2	<0.001	-3.8	1.9	0.05
b ₅	PPRC-19	Slope	2.2	5.9	0.71	-16.7	2.8	<0.001
b ₆	EE-01	Slope	-0.8	5.9	0.89	0.3	2.8	0.90
b ₇	BB-01	Slope	-3.2	5.9	0.58	-1.2	2.8	0.66
Radial growth								
b ₀	PPRC-4	Intercept	9.9	0.3	<0.001	14.1	0.2	<0.001
b ₁	PPRC-19	Intercept	0.3	0.4	0.47	-2.0	0.3	<0.001
b ₂	EE-01	Intercept	2.1	0.4	<0.001	1.0	0.3	<0.001
b ₃	BB-01	Intercept	4.5	0.4	<0.001	2.4	0.3	<0.001
b ₄	PPRC-4	Slope	-1.2	0.2	<0.001	-1.0	0.2	<0.001
b ₅	PPRC-19	Slope	-0.4	0.3	0.23	0.5	0.2	0.02
b ₆	EE-01	Slope	-1.4	0.3	<0.001	0.2	0.2	0.28
b ₇	BB-01	Slope	-1.0	0.3	<0.001	-0.004	0.2	0.98
Sporulation								
b ₀	PPRC-4	Intercept	3638348	465705	<0.001	6621418	195575	<0.001
b ₁	PPRC-19	Intercept	1899511	658606	<0.001	-813389	276585	<0.001
b ₂	EE-01	Intercept	13162740	658606	<0.001	-242364	276585	0.38
b ₃	BB-01	Intercept	2364053	658606	<0.001	-5769607	276585	<0.001
b ₄	PPRC-4	Slope	-1594768	408810	<0.001	-373139	171682	0.03
b ₅	PPRC-19	Slope	-786431	578145	0.18	-618960	242795	0.01
b ₆	EE-01	Slope	-4835622	578145	<0.001	-2742038	242795	<0.001
b ₇	BB-01	Slope	1145710	578145	0.05	110425	242795	0.65

5.3.2. *In-vivo* compatibility of benfuracarb and endosulfan with *B. bassiana* and *M. anisopliae*

There were significant differences in mortality of larvae between treatments at both 1 ppm ($F = 22.6$, $df = 7, 24$; $P < 0.001$) and 5 ppm ($F = 13.8$, $df = 7, 24$; $P < 0.001$) (Figure 5.1 a & b). When benfuracarb was combined with BB-01 or PPRC-4 at 1 ppm there was higher mortality than when either benfuracarb, BB-01 or PPRC-4 were used alone. Similar trends of increased mortality were observed when BB-01 was mixed with 5 ppm of benfuracarb. BB-01 mixed with endosulfan at 5 ppm resulted in higher mortality of larvae than when BB-01 or endosulfan were used alone.

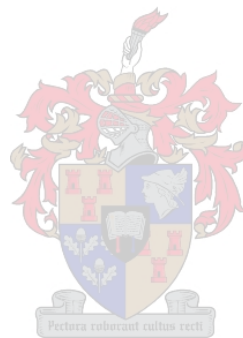


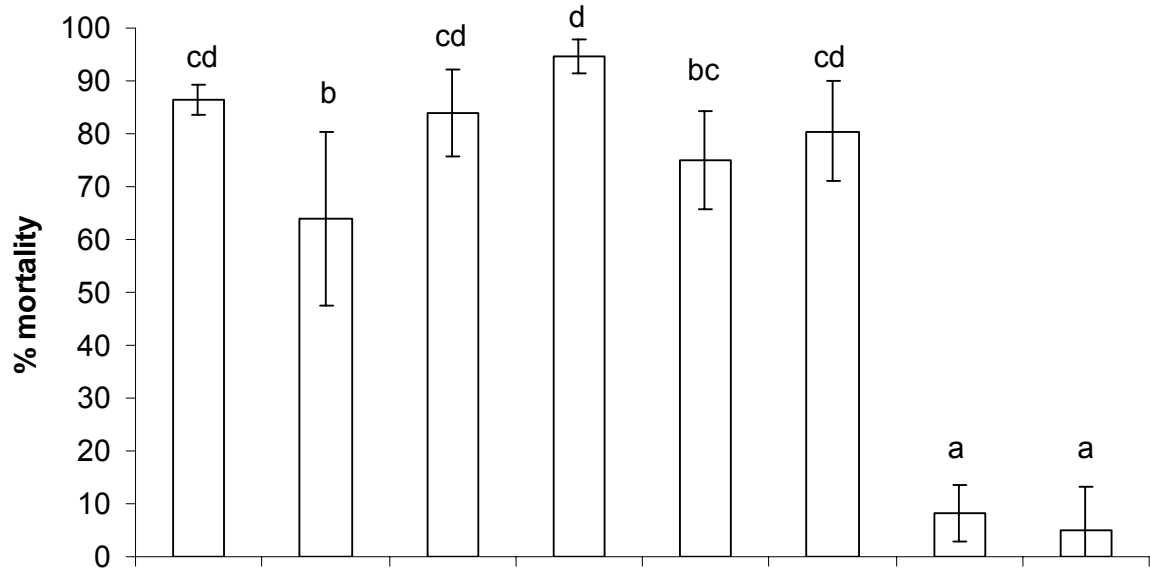
Table 5.2. Reduced regression models with multiple correlation coefficients (R), F-values and P-values of the reduced versus the full models for the effects of benfuracarb on conidia germination, radial growth and sporulation of the isolates.

Parameter	Isolates	Regression	R	Reduced vs. Full model	
				F	P
Germination	PPRC-4	$Y = 89.0007 - 21.2747x$	0.85	0.37	0.88
	PPRC-19	$Y = 89.0007 - 21.2747x$			
	EE-01	$Y = -48.1167 - 21.2747x$			
	BB-01	$Y = 89.0007 - 21.2747x$			
Radial growth	PPRC-4	$Y = 10.0213 - 1.4194x$	0.93	0.74	0.44
	PPRC-19	$Y = 10.0213 - 1.4194x$			
	EE-01	$Y = 1.9962 - 1.1786x$			
	BB-01	$Y = 4.3176 - 0.8384x$			
Sporulation	PPRC-4	$Y = 3850602 - 1987984x$	0.95	1.85	0.51
	PPRC-19	$Y = 1475000 - 1987984x$			
	EE-01	$Y = 12950484 - 4442407x$			
	BB-01	$Y = 2151797 + 1538925x$			

Table 5.3. Reduced regression models with multiple correlation coefficients (R), F-values and P-values of the reduced versus the full models for the effects of endosulfan on conidia germination, radial growth and sporulation of the isolates.

Parameter	Isolates	Regression	R	Reduced vs. Full model	
				F	P
Germination	PPRC-4	$Y = 78.5206 - 4.1137x$	0.81	0.17	0.96
	PPRC-19	$Y = 10.5678 - 16.4281x$			
	EE-01	$Y = 10.2500 - 4.1137x$			
	BB-01	$Y = 9.4000 - 4.1137x$			
Radial growth	PPRC-4	$Y = 14.0464 - 0.9196x$	0.92	0.79	0.66
	PPRC-19	$Y = -1.9986 + 0.4605x$			
	EE-01	$Y = 1.1500 - 0.9196x$			
	BB-01	$Y = 2.3500 - 0.9196x$			
Sporulation	PPRC-4	$Y = 6491655 - 294507x$	0.96	0.42	0.83
	PPRC-19	$Y = -683626 - 697591x$			
	EE-01	$Y = 6491655 - 2867507x$			
	BB-01	$Y = -5622681 - 294507x$			

(a) 1 ppm



(b) 5 ppm

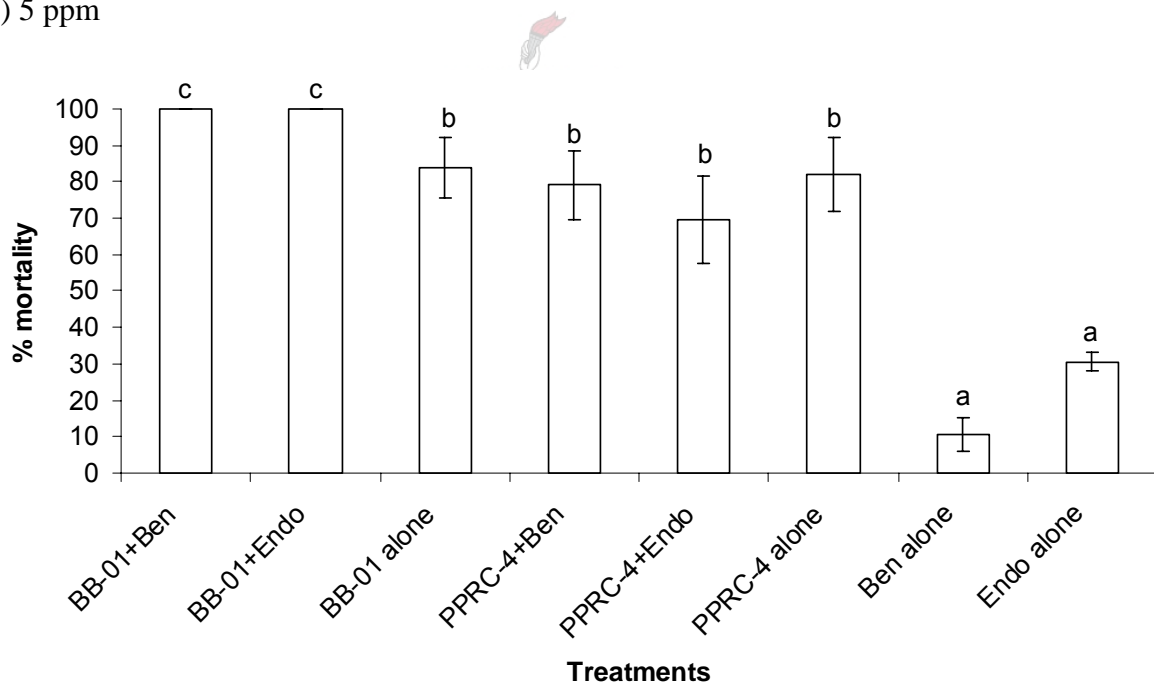


Figure 5.1. Effect of combining *Beauveria bassiana* and *Metarhizium anisopliae* with (a) 1 ppm and (b) 5 ppm active ingredients of benfuracarb and endosulfan on mortality of *Chilo partellus* third instar larvae. Vertical bars represent SE. Bars with the same letter are not significant at $P > 0.05$ using Student-Newman-Keuls test. Ben = benfuracarb; Endo = endosulfan.

5.4. DISCUSSION

The differences in slopes and intercepts between the lines of the fungal isolates indicated that there were differential effects of the insecticides on the germination, radial growth and sporulation of the different isolates. The line for PPRC-4 had high and positive intercepts and low slopes values when mixed with endosulfan (Table 5.3). Thus, this isolate appeared to be less susceptible to endosulfan than the other isolates. Large variation in response to agricultural pesticides by species and strains of entomopathogenic fungi has been reported (Vanninen & Hokkanen, 1988; Anderson *et al.*, 1989; Majchrowicz & Poprawski, 1993; Li & Holdom, 1994). However, pesticides that are inhibitory in the laboratory do not always exhibit the same inhibitory action in the field (Inglis *et al.*, 2001). This can be due to pesticide concentration or the fact that the entomopathogen may not come in contact with the pesticides. For example, Keller *et al.* (1997) observed that one-third of the fungicides tested *in-vitro* completely inhibited conidial germination and mycelial growth of *Beauveria brongniartii* Sacc. However, most of the fungicides tested did not have an adverse effect on *B. brongniartii* when applied to soils. The different means of exposing fungi and insects to chemical pesticides reported have different results (Anderson & Roberts, 1983; Quintela & McCoy, 1998). In this study, exposure of larvae to fungal isolates after being treated with the insecticides might have improved the effectiveness of the fungi by avoiding simultaneous exposure to the insecticides.

The response of the isolates varied with the type of the insecticide used. For example, the slopes of the lines obtained when the isolates were mixed with benfuracarb indicated that increasing the concentration of benfuracarb had more of an inhibitory effect on germination, radial growth and sporulation of isolates than did endosulfan (compare Tables 5.2 with 5.3). Inhibition of *M. anisopliae* growth at concentrations of 50 to 500 ppm of chlorpyrifos and propetamphos was reported by Pachamuthu *et al.* (1999), Samuels *et al.* (1989) and Li & Holdon (1994). While Moorhouse *et al.* (1992) reported that the majority of the pesticides tested did not affect conidial germination but did affect radial growth. In most of the compatibility studies by Urs *et al.* (1967), Gardener *et al.* (1979) and Samuels *et al.* (1989), sporulation of the entomopathogenic fungi was affected more by insecticides than did growth and germination. In the current study, germination, growth and sporulation were affected by the insecticides but to varying degrees.

B. bassiana (BB-01) and *M. anisopliae* (PPRC-4) had an additive effect on *C. partellus* larval mortality when combined with benfuracarb and endosulfan at 1 ppm and 5 ppm. In the studies by Ferron (1971), Anderson *et al.* (1989), the efficacy of *B. bassiana* was improved when combined with sub-lethal doses of insecticides. In addition, Lu (1988) reported the ultra low volume spray of *B. bassiana* conidial suspension with insecticides resulted in 85-99 % mortality in pine caterpillar in China. By contrast, *B. bassiana* was found to be incompatible with diflubenzuron, carbaryl, methomyl, and methyl parathion (Gardner *et al.*, 1979). The combined activity of *M. anisopliae* against the German cockroach *Blattella germanica* Linnaeus (Dictyoptera: Blattellidae) with chlorpyrifos and propetamphos has been reported (Pachamuthu *et al.*, 1999). *Metarhizium flavoviride* Gams & Rozsypal sprayed with cyflutherin and cypermethrin increased mortality of locust *Locusta migratoria migratorioides* Linnaeus (Orthoptera: Acrididae) (Sanyang & Van Emden, 1996).

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APPENDIX: Dummy variable models

The full dummy variable regression model with separate slopes and intercepts for the isolates was:

$$Y = b_0 + b_1Z_1 + b_2Z_2 + b_3Z_3 + b_4X + b_5Z_1X + b_6Z_2X + b_7Z_3X + e$$

Where,

$Z_1 = 1$ if isolate is PPRC-19, = 0 otherwise,

$Z_2 = 1$ if isolate is EE-01, = 0 otherwise,

$Z_3 = 1$ if isolate is BB-01, = 0 otherwise.

PPRC-4 used as a central model against which the other isolates compared.

b_0 = intercept for PPRC-4

b_1 = intercept for PPRC-19

b_2 = intercept for EE-01

b_3 = intercept for BB-01

b_4 = slope for PPRC-4

b_5 = slope for PPRC-19

b_6 = slope for EE-01

b_7 = slope for BB-01

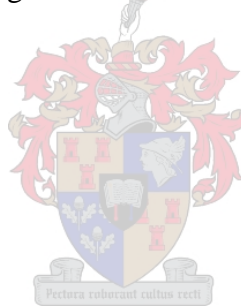
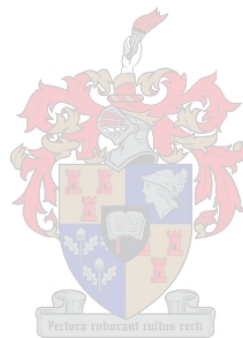


Table 5.4. Data matrix used for input in the full dummy variable model for percent conidia germination, radial growth and sporulation.

Isolate	Z ₁	Z ₂	Z ₃	X (log-concentration)	Z ₁ X	Z ₂ X	Z ₃ X
PPRC-19	1	0	0	X	X	0	0
EE-01	0	1	0	X	0	X	0
BB-01	0	0	1	X	0	0	X
PPRC-4	0	0	0	X	0	0	0



CHAPTER 6

Effect of exposure method, temperature and conidial concentration of *Beauveria bassiana* (Balsamo) Vuillemin on mortality, mycosis and sporulation in cadavers of *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae)

ABSTRACT

The effect of exposure method and conidial concentration on mortality of *Chilo partellus* second instar larvae treated with *Beauveria bassiana* was investigated in laboratory studies. Larvae sprayed directly with conidia, exposed to conidia-treated leaves and larvae dipped into conidial suspensions resulted in high mortality (98 to 100 %). The longest LT₅₀ (3.5 days) and days to onset of mortality (2.6 days) were observed in exposure on treated leaves. The shortest LT₅₀ (1 day) and days to first mortality (1 day) were recorded when exposure was by dipping. As conidial concentration increased, the LT₅₀ and days to mortality decreased. The effect of exposure method, conidial concentration and temperature on mycosis and sporulation in second instar *C. partellus* cadavers were also determined. Larvae exposed to treated leaves and larvae sprayed directly with conidia produced high mycoses in cadavers. Exposure of larvae to treated leaves yielded high sporulation. At the low conidial concentration (1.25×10^7 conidia/ml), mycosis and sporulation were high. The optimum temperature was 20 °C for mycosis and 15 °C for sporulation.

6.1. INTRODUCTION

Second instar *Chilo partellus* (Swinhoe) larvae sprayed with isolates of *Beauveria bassiana* (Balsamo) Vuillemin and or *Metarhizium anisopliae* (Metschnikoff) Sorokin conidial suspensions resulted in more than 90 % mortality (Chapter 2). However, the level of mortality may depend on the method of exposure to the pathogen. For instance, direct spraying of larvae with a conidial suspension, treating leaves with a conidial suspension and dipping the larvae into a conidial suspension affected the rate of mortality in the Colorado potato beetle (Silva *et al.*, 2001). However, it is not yet known how the exposure method affects mortality of *C. partellus* larvae.

Sporulation, or the production of conidia, is a vital part in the reproduction of entomopathogenic fungi (Arthurs & Thomas, 2001). It enables the conidia to multiply and to disseminate so that future generations and new populations of insects come into contact with the pathogen (Roberts, 1989). The epizootic potential of *B. bassiana* depends not only on its infectivity but also on the intensity of sporulation on the host cadavers and on the subsequent ability of the fungus to spread to healthy insects (Carruthers & Soper, 1987; Ferron *et al.*, 1991). Following applications of *B. bassiana*, secondary cycling of the fungus may contribute significantly to biological control, thereby reducing the frequency of application required to maintain pest populations below a given threshold (Thomas *et al.*, 1999).

Climatic factors, especially temperature and humidity, affect sporulation and mycosis (Benz, 1987; Ferron *et al.*, 1991; Glare & Milner, 1991; Hajek & St. Leger, 1994; Arthurs & Thomas, 2001). *In-vivo* temperature-dependent development of *B. bassiana* mycosis was reported for the European corn borer (Carruthers *et al.*, 1985). Other workers have demonstrated a relationship between temperature and *in-vitro* development of *B. bassiana* (Hywel-Jones & Gillespie, 1990; Ekesi *et al.*, 1999). The ability of *B. bassiana* to produce further inoculum following the death of the host has not been determined for *C. partellum*.

The aims of this study were to:

1. determine mortality of *C. partellus* second instar larvae using direct spraying, exposure to treated leaf surfaces and dipping larvae in *B. bassiana* conidial suspension.
2. determine mycosis and sporulation in cadavers following the different exposure methods at different conidial concentrations and temperatures.

6.2. MATERIALS AND METHODS

6.2.1. Insect culture, fungal culture, and conidial preparation

A laboratory colony of *C. partellus* larvae was obtained from the Agricultural Research Council, Plant Protection Research Institute (PPRI), Pretoria, South Africa (see Chapter 2).

The isolates were obtained from Ethiopia (Plant Protection Research Center and Alemaya University) (see Chapter 2)).

Conidia were obtained from 3 week old sporulating cultures. The conidia of each isolate were harvested by brushing the surface of the culture with a sterile camel hairbrush into a 500 ml glass beaker containing 50 ml sterile distilled water with Tween 80 (0.1 % v/v) (Difco™). The conidial suspension was prepared by mixing the solution with a magnetic stirrer for five minutes. It was then adjusted to the desired concentration using Neubauer haemocytometer.

6.3. BIOASSAYS

6.3.1 Effect of exposure method and conidial concentration on mortality, LT₅₀ and days to mortality

Three exposure methods and four conidial concentrations were used.

Direct spray. A batch of 10 second instar *C. partellus* larvae were placed in a sterile Petri dish, 9 cm in diameter. This was sprayed with a 3 ml conidial suspension using a Potter's spray tower. Four concentrations of conidia, 1.25×10^7 , 2.5×10^7 , 5×10^7 , and 1×10^8 conidia/ml were used. The larvae were then allowed to feed on untreated pieces of leaf (4cm x 1 cm) excised from 4-week old maize (variety CRN 3414).

Leaf exposure. Three to four pieces of 1 cm x 4 cm leaf were placed in a Petri dish and sprayed with a conidial suspension using the spray tower. Three ml of conidial suspension (1.25×10^7 , 2.5×10^7 , 5×10^7 , and 1×10^8 conidia/ml) were sprayed on both sides of the leaf surfaces. The Petri dish containing the sprayed leaf material was then left on a laboratory bench to allow to dry for 20-30 min. After transferring the treated leaves to a sterile Petri dishes, ten larvae were then placed on the conidia treated leaves and allowed to feed for 24 h, after which the remains of the leaf material and frass were carefully removed. The larvae were then provided with unsprayed leaves.

Dipping. A group of 10 larvae were dipped in a 3 ml conidial suspension (1.25×10^7 , 2.5×10^7 , 5×10^7 , and 1×10^8 conidia/ml) for 30 seconds in a Petri dish. The larvae were dipped in conidial suspension using sterile camel hair brush. The treated larvae were placed in a sterile Petri dish and provided with untreated maize leaves.

Ten control larvae per exposure method were either sprayed, dipped or provided with leaves treated with sterile distilled water containing Tween 80 (0.1 % v/v). The treatments and controls were replicated six times. The treatments were arranged in factorial design with the three exposure methods and four conidial concentrations as main effects. The materials were kept at 25 °C and photoperiod of 12h: 12h light and darkness. Fresh leaf material was provided daily. Mortality was recorded daily for seven days from the same batch of insects. The number of days (time) to death was recorded for each treatment. Dead insects were removed and placed in a Petri dish lined with moist filter paper in order to encourage fungal growth. The Petri dishes containing dead insects were sealed and kept at 25 °C and a photoperiod of 12h: 12h light and darkness.

6.3.1.1. Statistical analysis

Mortality data were corrected for control mortality (Abbott, 1925). Mortality was angular-transformed ($\arcsine \sqrt{\text{proportion}}$) before subjecting them to an analysis of variance using SPSS-11 for windows. LT_{50} (lethal time to 50% mortality of the treated insects) was determined for each replicate using probit analysis for correlated data (Throne *et al.*, 1995). A factorial analysis with two main effects, the three exposure methods and four conidial concentrations was conducted for mortality, LT_{50} and days to onset of mortality. Differences between means were determined using Student- Newman-Keuls Test ($P < 0.05$).

6.3.2. Effect of exposure method, conidial concentration and temperature on mycosis and sporulation in cadavers

Following the same exposure methods and conidial concentrations approximately 1500 second instar larvae were treated with each exposure method and conidial concentration. Dead larvae were collected daily and placed in a sterile Petri dish. The Petri dish containing dead larvae was sealed and kept at 6 °C for a week. This was done to avoid fungal growth at room temperature before starting the experiment (Luz & Fargues, 1998). Ten dead larvae kept at 6 °C, were placed in a sterile Petri dish lined with moist filter paper. The Petri dishes containing dead insects were sealed and incubated at 15, 20, 27 and 30 °C for ten days.

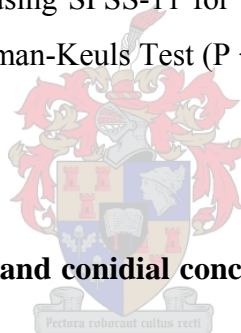
The treatments were replicated four times with 10 larval cadavers per replicate. The treatments were arranged in a factorial design with the three exposure methods, four conidial concentrations and four temperature regimes as main effects. Mycosis, or cadavers showing external mycelial growth, was determined by examining each cadaver using a stereomicroscope. Ten cadavers (each replicate) were placed in a beaker containing 25 ml distilled water with Tween-80 (0.1 % v/v). The beaker containing the cadavers was then stirred for 5 min using a magnetic stirrer. Sporulation, number of conidia/ml, was estimated using a compound microscope (400X) and haemocytometer.

6.3.2.1. Statistical analysis

Data on mycosis and sporulation were angular-transformed ($\arcsine\sqrt{proportion}$) and Log10-transformed, respectively. A factorial analysis with three main effects, namely the three exposure methods, four temperature regimes and four conidial concentrations, was performed on the transformed data using SPSS-11 for windows. Differences between means were determined using Student-Newman-Keuls Test ($P < 0.05$).

6.4. RESULTS

6.4.1. Effect of exposure methods and conidial concentrations on mortality, time (days) to mortality and LT_{50}



There were no interactions between exposure methods and conidial concentrations or differences in mortality between exposure methods or conidial concentrations (Table 6.1 & Figure 6.1). However, there were differences in days to onset of mortality between the exposure methods as well as between the different conidial concentrations. There were also interactions between these two factors (Tables 6.1 & 6.2). These interactions were caused by the fact that time to mortality decreased with increasing conidial concentrations using treated leaves and direct spraying but not using dipping (Table 6.2).

There were differences in LT_{50} between exposure methods and conidial concentrations. There were also interactions between these two factors (Tables 6.1 & 6.3). The interactions were due to a decrease in LT_{50} with increasing conidial concentrations using spray and dipping but not using treated leaves (Table 6.3). The mean LT_{50} was long (3.5 days) when exposure was on treated leaves while it was short (1 day) using dipping.

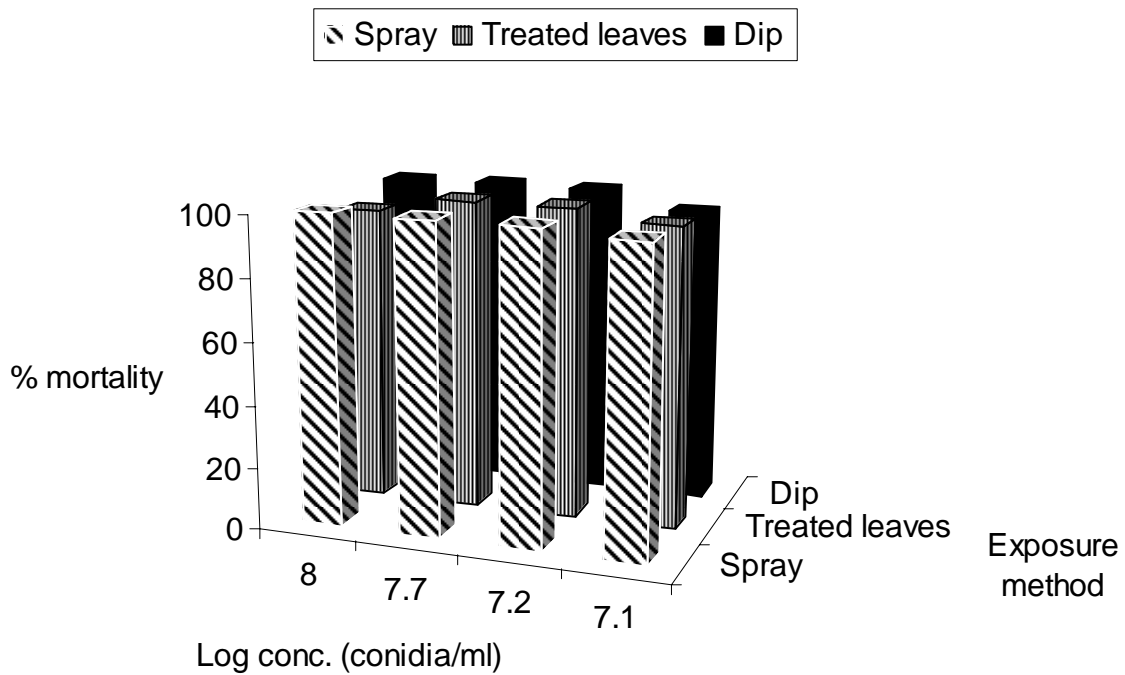


Figure 6.1. The effect of exposure method and conidial concentration on mortality of second instar *Chilo partellus* larvae seven days after treatment.

6.4.2. Effect of exposure methods, conidial concentrations and temperature on mycosis in cadavers

There were higher order interactions between exposure method, conidial concentration and temperature (Table 6.4). The higher order interactions were due to variation in mycosis for the three exposure methods at the different temperatures (Figure 6.2 b) and variation in mycosis for the four conidial concentrations at the different temperatures (Figure 6.2 c). Using direct spraying the level of mycosis was highest at 20 and 27 °C (Figure 6.2 b).

There were differences in mycosis between the levels of all three main effects (exposure methods, conidial concentrations and temperature). There was a high degree of mycosis using leaf and spray methods (Figure 6.3 a), low conidial concentration (Figure 6.3 b) and at 20 and 27 °C (Figure 6.3 c).

6.4.3. Effect of exposure methods, conidial concentrations and temperature on sporulation in cadavers

There were higher order interactions between exposure method, conidial concentration and temperature (Tables 6.4). Exposure of larvae to treated leaves, by direct spraying and dipping produced high, intermediate and low numbers of conidia, respectively, except at 2.5×10^7 conidia/ml (Figure 6.4 a) and 27°C (Figure 6.4 b). There was a sharp decline in sporulation with increasing temperature at 1.25×10^7 and 2.5×10^7 but not at 5×10^7 and 1×10^8 conidia/ml (Figure 6.4 c). The higher order interactions were due to variation in sporulation for the three exposure methods at the different conidial concentrations (Figure 6.4 a) and temperatures (Figure 6.4 b), and variation in sporulation for the four conidial concentrations at the different temperatures (Figure 6.4 c).

There were differences in sporulation between all levels of the main effects (exposure method, conidial concentration and temperature) (Table 6.4). Exposure of larvae to treated leaves yielded high numbers of conidia (Figure 6.5 a). Sporulation in cadavers was high when low concentrations of conidia were used (Figure 6.5 b). Incubation of cadavers at low temperature (15°C) produced higher numbers of conidia than at a high temperature (30°C) (Figure 6.5 c). Therefore, sporulation in cadavers increased using exposure of larvae to treated leaves, decreasing conidial concentrations and decreasing temperature.

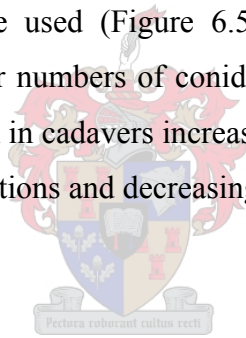


Table 6.1. Factorial analyses of exposure methods and conidial concentrations on mortality, days to onset of mortality and LT₅₀ of second instar *Chilo partellus* larvae infected by *Beauveria bassiana*.

Factor	Mortality			Days to onset of mortality			LT ₅₀ (days)		
	F	df	P	F	df	P	F	df	P
Exposure (E)	1.15	2	0.322	13.58	2	< 0.001	27.00	2	< 0.001
Concentration (C)	2.29	3	0.087	4.44	3	< 0.001	8.50	3	< 0.001
E x C	1.14	6	0.350	3.00	6	0.010	2.75	6	0.026
Error		60			60			60	

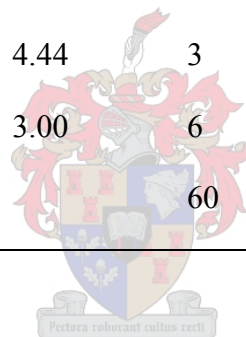


Table 6.2. Mean days to mortality (\pm standard deviation) of second instar *Chilo partellus* larvae infected by *Beauveria bassiana* using three exposure methods at four conidial concentrations.

Exposure method	conidial concentration/ml				Average time to mortality
	1.25x10 ⁷	2.5 x10 ⁷	5 x10 ⁷	1 x10 ⁸	
Leaf	3.0 \pm 1.3	2.8 \pm 0.7	2.6 \pm 0.7	2.1 \pm 1.0	2.6 \pm 1.0 c
Spray	2.8 \pm 0.7	1.8 \pm 0.4	2.1 \pm 0.9	1.0 \pm 0.0	1.9 \pm 0.9 b
Dip	1.0 \pm 0.0	1.0 \pm 0.0	1.0 \pm 0.0	1.0 \pm 0.0	1.0 \pm 0.0 a
Average time to mortality	2.2 \pm 1.0 b	1.8 \pm 0.6 a	1.9 \pm 1.0 a	1.3 \pm 1.1 a	

Means \pm SD followed by the same letter within a column (exposure mean) and a row (concentration mean) are not significant at $P > 0.05$ using Student-Newman-Keuls Test.

Table 6.3. Mean LT₅₀ (days) (\pm standard deviation) of second instar *Chilo partellus* larvae infected by *Beauveria bassiana* using three exposure methods at four conidial concentrations.

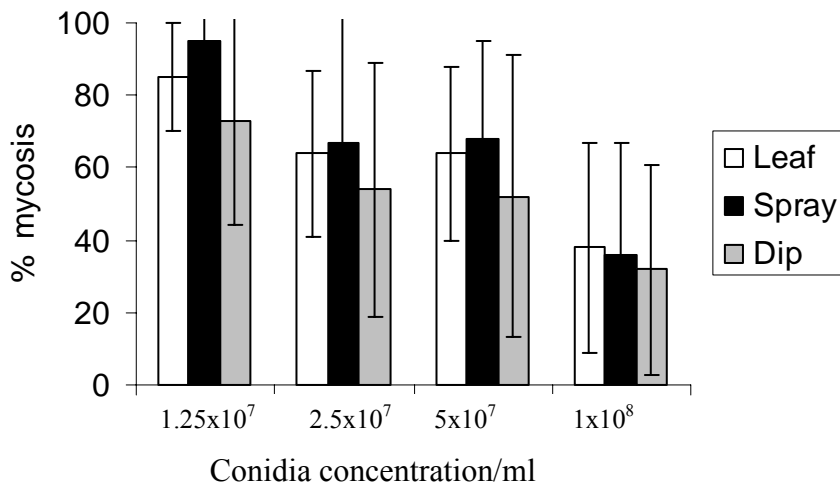
Exposure method	conidial concentration/ml				Mean
	1.25x10 ⁷	2.5 x10 ⁷	5 x10 ⁷	1 x10 ⁸	
Leaf	3.9 \pm 0.7	2.9 \pm 0.8	3.9 \pm 0.6	3.5 \pm 0.7	3.6 \pm 0.8 c
Spray	4.2 \pm 0.7	2.8 \pm 0.4	2.9 \pm 0.4	2.3 \pm 0.3	3.0 \pm 0.9 b
Dip	2.5 \pm 0.8	2.1 \pm 0.8	2.0 \pm 0.8	1.0 \pm 0.0	1.0 \pm 0.0 a
Mean	3.5 \pm 1.1 c	2.6 \pm 0.8 b	2.9 \pm 1.0 ab	1.3 \pm 1.1 a	

Means \pm SD followed by the same letter within a column (exposure mean) and a row (concentration mean) are not significant at $P > 0.05$ using Student-Newman-Keuls Test.

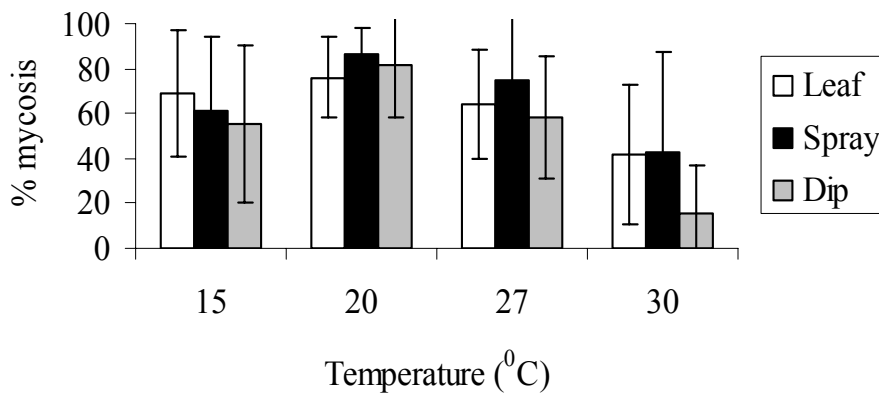
Table 6.4. Factorial analyses of the effect of temperature, conidial concentrations and exposure methods on mycosis and sporulation of *Beauveria bassiana* on second instar *Chilo partellus* larval cadavers.

Factor	Mycosis				Sporulation			
	Mean square	F	df	P	Mean square	F	df	P
Exposure (E)	0.621	7.458	2	<0.001	3.603	162.447	2	<0.001
Conidial concentrations (C)	3.692	44.311	3	<0.001	8.905	401.519	3	<0.001
Temperature (T)	3.538	42.469	3	<0.001	3.936	177.464	3	<0.001
E x C	0.008	0.982	6	0.440	0.497	22.430	6	<0.001
E x T	0.218	2.621	6	0.019	0.944	42.549	6	<0.001
C x T	0.157	1.890	9	0.058	0.550	24.803	9	<0.001
E x C x T	0.179	2.143	18	<0.001	0.465	20.976	18	<0.001
Error	0.008		144		0.0022		144	

(a)



(b)



(c)

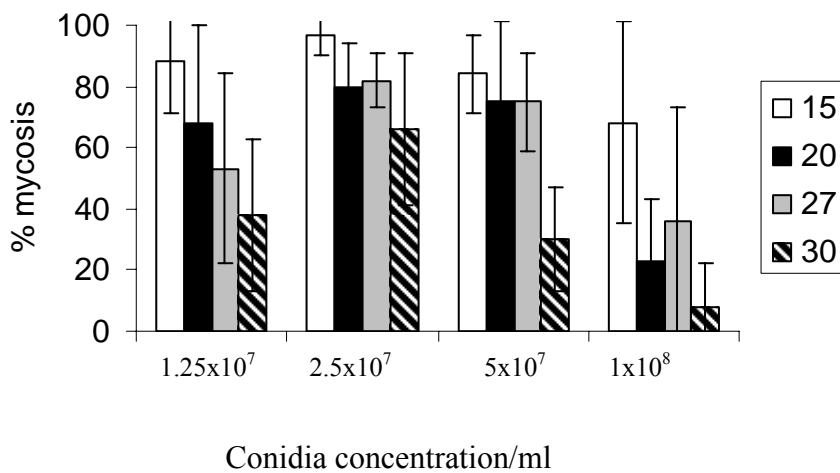
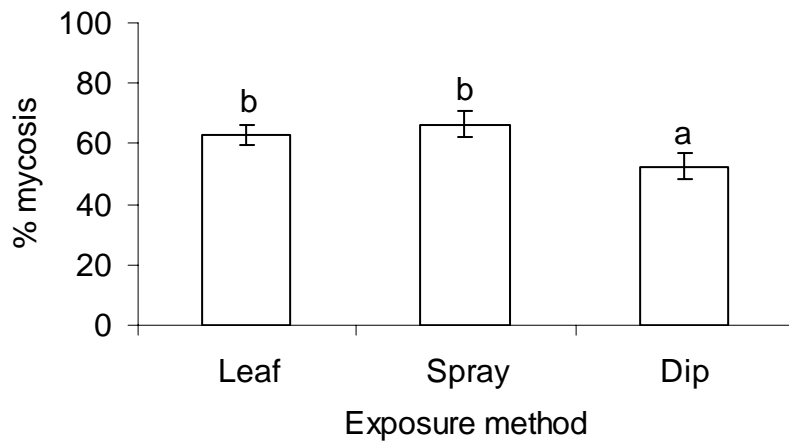
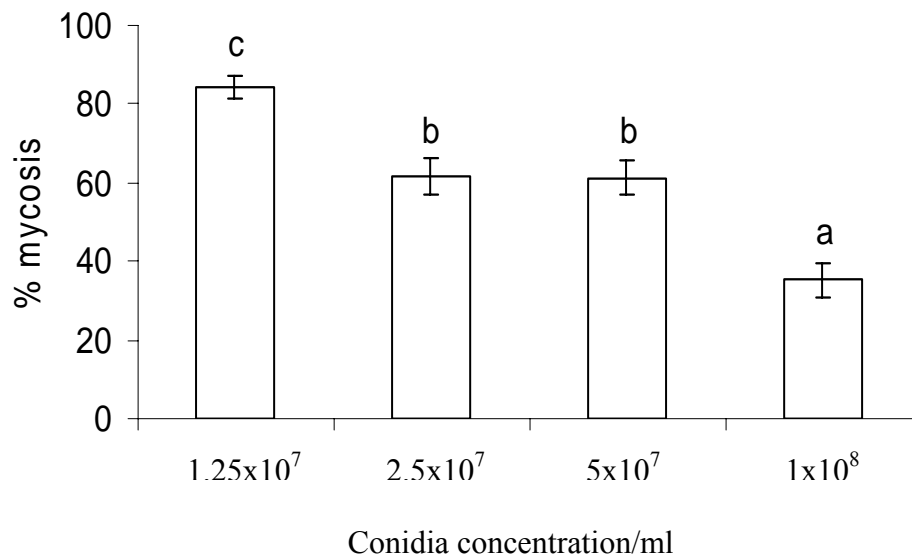


Figure 6.2. Interactions between (a) exposure method and conidial concentration (b) exposure method and temperature, and (c) temperature and conidial concentration on mycosis of *Beauveria bassiana* in cadavers of second instar *Chilo partellus* larvae. Vertical lines represent Standard deviations.

(a)



(b)



(c)

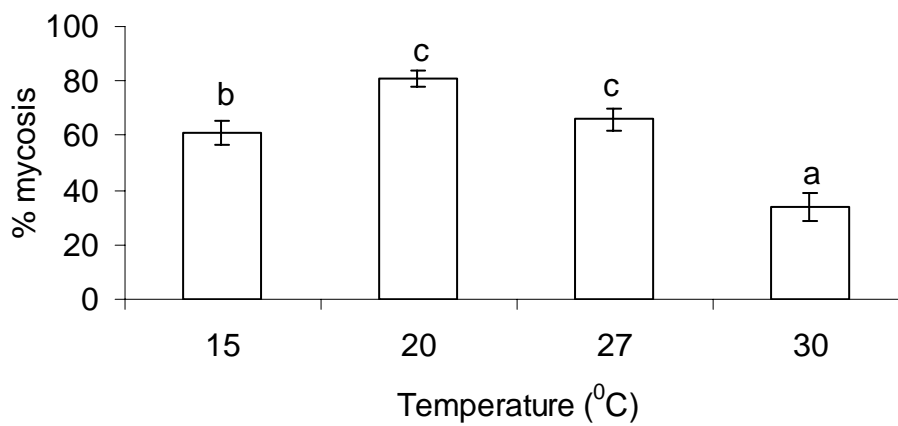


Figure 6.3. The effect of (a) exposure methods (b) conidial concentrations and (c) temperature on mycosis in cadavers of *Chilo partellus* second instar larvae. Vertical bars indicate SE. Bars with the same letters are not significant ($P > 0.05$) using Student-Newman-Keuls test.

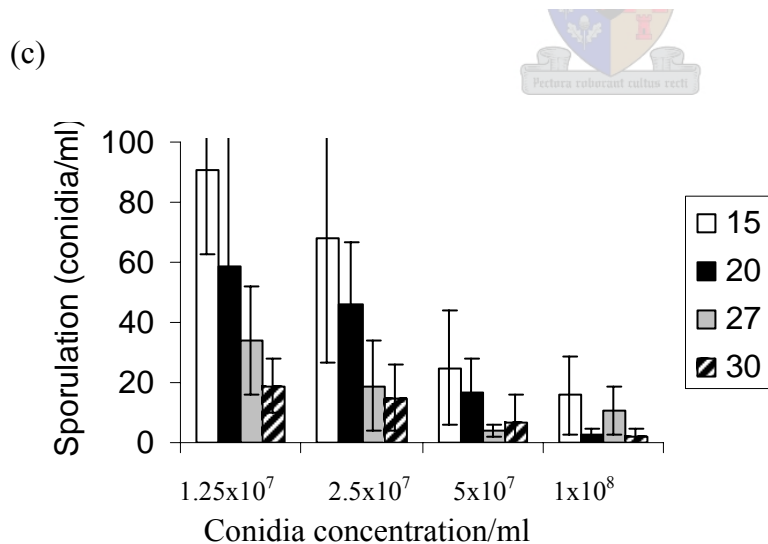
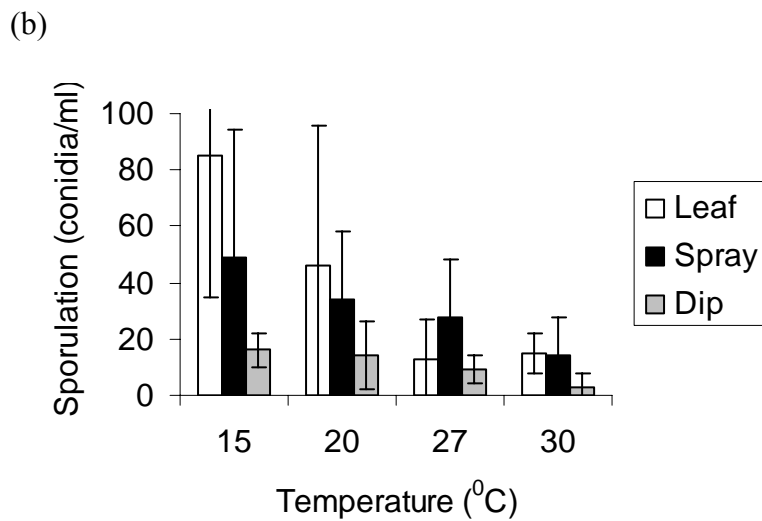
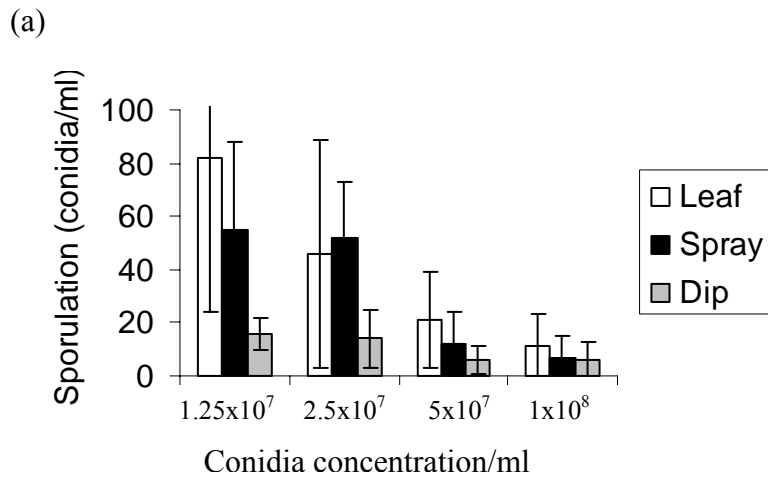
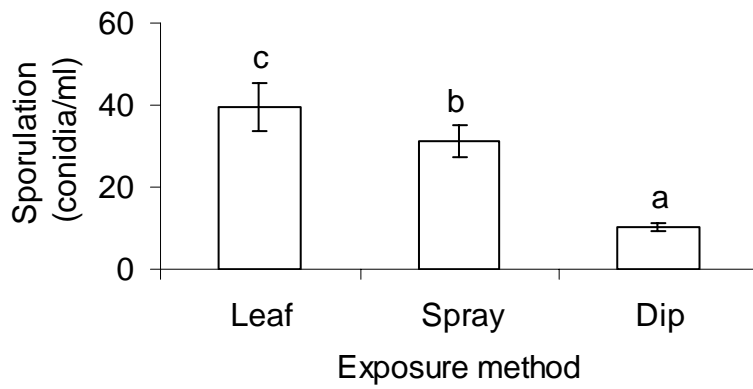
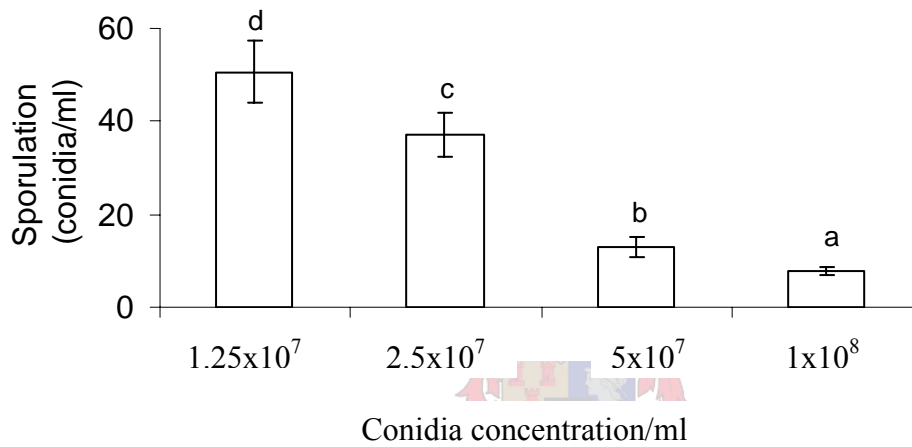


Figure 6.4. Interactions between (a) exposure method and conidial concentration (b) exposure method and temperature, and (c) temperature and conidial concentration on sporulation of *Beauveria bassiana* in cadavers of second instar *Chilo partellus* larvae. Vertical lines represent standard deviations.

(a)



(b)



(c)

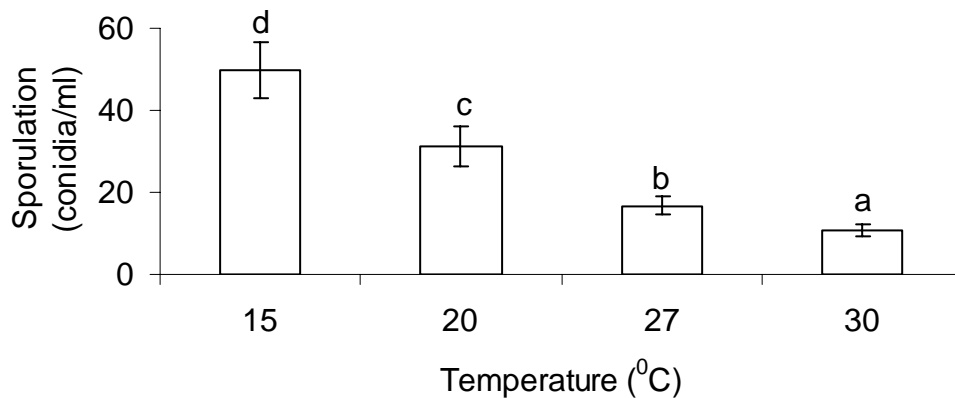


Figure 6.5. The effect of (a) exposure methods (b) conidial concentrations and (c) temperature on sporulation in cadavers of *Chilo partellus* second instar larvae. Vertical bars indicate SE. Bars with the same letters are not significant ($P > 0.05$) using Student-Newman-Keuls test.

6.5. DISCUSSION

All exposure methods and conidial concentrations used in the current study resulted in high mortality. Colorado potato beetle (CPB) larvae sprayed directly with *B. bassiana* suffered higher mortality than larvae feeding on conidia-treated foliage (Silva *et al.*, 2001). The effectiveness of exposure on treated leaves in the present study may be attributed to the survival of conidia on leaf surfaces until contacting the insect. Conidial germination was inhibited by the presence of phyto-chemicals found on potato foliage (Silva *et al.*, 2001). However, *B. bassiana* conidia survived for three days on maize leaves in field cage studies on western corn rootworm (Mulock & Chandler, 2000). In addition, larvae feeding on treated leaves may acquire infection via the gut. Allee *et al.* (1990) found penetration of *B. bassiana* conidia through the gut in larvae of CPB after consumption of treated leaves. Miranpuri & Khachatourians (1991) found germinated *B. bassiana* conidia in the gut of the larvae of the mosquito *Aedes aegypti* Linnaeus (Diptera: Culicidae) after 24-48 h of exposure. Apart from feeding on treated leaves, movement on treated leaves during the course of feeding may facilitate contact with conidia.

The LT_{50} and time to mortality were shorter when larvae were exposed using direct spraying and dipping, particularly at high conidial concentrations (1×10^8 conidia/ml). In the case of direct spraying, the dorsal side of the larvae was exposed to the conidial suspension. In the dipping, however, both the dorsal and ventral sides were exposed. The early death of larvae treated by the dipping method may have been due to the fact that larvae collect high quantities of conidia during immersion. Boucias *et al.* (1988) and Silva *et al.* (2001) reported that direct spraying enhanced conidia lodging within cuticular folds thereby facilitating attachment, germination and penetration.

Mycosis and sporulation in cadavers depended on method of exposure, conidial concentration and temperature. Exposure using treated leaves, low conidial concentration (1.25×10^7) and temperature ranges of 15-20 °C, enhanced mycosis and sporulation. The high rate of mycosis and sporulation in cadavers following exposure using treated leaves and low concentration of conidia may be due to the relatively slow death of the larvae. In the current study, the LT_{50} and days to mortality were long when exposure was by treated leaves at a low conidial concentration. This could be attributed to the fact that the host may reach sufficient size to provide the fungus with resources sufficient for mycosis and sporulation. At high concentrations many larvae died rapidly and the fungus sporulated in few cadavers and produced few conidia. A mechanism of self-inhibition at high concentrations of conidia was

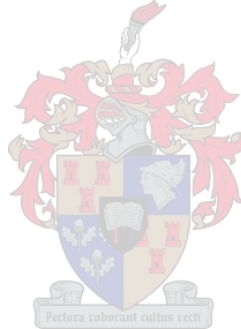
reported for other species of fungi (Garraway & Evans, 1984). Sporulation of *B. bassiana* on cadavers of *Rhodnius prolixus* Stål (Hemiptera: Reduviidae) was highest when incubated at 25 °C, declined at 28-30 °C and was zero at 35 °C (Luz & Fargues, 1998). The chalkbrood fungus, *Ascospaera aggregata* Skou has been shown to have a higher rate of sporulation on cadavers of the alfalfa leafcutting bee, *Megachile rotundata* Fabricius (Hymenoptera: Megachilidae) after application of intermediate concentrations of conidia than at both lower and higher concentrations (Vandenberg, 1992). In conclusion, the development of *B. bassiana* on cadavers of *C. partellus* can be influenced by temperature, conidial concentration and method of exposure.

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CHAPTER 7

Greenhouse evaluation of *Beauveria bassiana* *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin isolates for controlling *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) in maize

ABSTRACT

Maize (variety Katumani) was planted in a greenhouse and plants were infested with 20 *Chilo partellus* second instar larvae 3 to 4 weeks after plant emergence. One isolate of *Beauveria bassiana* (BB-01) and four isolates of *Metarhizium anisopliae* (PPRC-4, PPRC-19, PPRC-61 & EE-01) were sprayed onto the leaf whorl at 2×10^8 conidia/ml 24 h after infestation. Leaf damage by the larvae was greatly reduced by the treatments. The growth of infested unsprayed plants was less than that of fungi treated plants. Stem tunnelling, deadheart, number of attacked nodes and exit/entry holes were also reduced in plants sprayed with conidial suspensions. Both stem and root, fresh and dry biomasses were higher in the treated plants than in the untreated plants. The isolates PPRC-4, PPRC-19 and PPRC-61 seemed to be the best candidates for further testing under field conditions.



7.1. INTRODUCTION

The spotted stem borer, *Chilo partellus* (Swinhoe), is economically the most important pest of maize and sorghum in Africa and Asia (Bosque-Perez & Schulthess, 1998). Control of this pest has been based on application of chemical insecticides (Warui & Kuria, 1983; Vandenberg & Van Rensburg, 1993). However, in the long term this approach is not desirable because of the ecological problems associated with the application of insecticides (Deedat, 1994). For example, predator-species complex occurring on maize and sorghum in commercial farming in South Africa were adversely affected by endosulfan, pyrethroids and monocrotophos (Du Toit, 1995). Adoption of an integrated pest management strategy, whereby a variety of control options are utilized to obtain desirable levels of pest suppression, is being encouraged. Insect pathogens, including bacteria, viruses and fungi, have great potential for inclusion in a stem borer IPM programme (Odindo, 1992; Maniania, 1993; Brownbridge, 2001). The entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* occur naturally in the environment and have been isolated from diseased *C. partellus* and *Busseola fusca* larvae (Van Rensburg *et al.*, 1988; Odindo *et al.*, 1989; Maniania, 1991; Hoekstra & Kfir, 1997).

Several local entomopathogenic fungal isolates of *Beauveria bassiana* and *Metarhizium anisopliae* have been tested against *C. partellus* and some of them were found to be virulent (Chapter 2). The objective of the current trial was to obtain information on the relative efficacy of these new isolates for controlling *C. partellus* under greenhouse conditions using artificially infested plants. This would facilitate the selection of the best strains for further testing under field conditions.

7.2. MATERIALS AND METHODS

6.2.1. Insect and fungal cultures

A laboratory colony of *C. partellus* larvae was obtained from the Agricultural Research Council, Plant Protection Research Institute (PPRI), Pretoria, South Africa (see Chapter 2).

The isolates were obtained from Ethiopia (Plant Protection Research Center and Alemaya University) (see Chapter 2)).

7.2.2. Conidial Preparation

Conidia were obtained from 3 week old sporulating cultures. The conidia of each isolate were harvested by brushing the surface of the culture with a sterile camel hairbrush into a 500 ml glass beaker containing 50 ml sterile distilled water with Tween 80 (0.1 % v/v) (Difco™). The conidial suspension was prepared by mixing the solution with a magnetic stirrer for five minutes. It was then adjusted to the desired concentration using Neubauer haemocytometer

7.2.3. Maize

Seeds of the *C. partellus* susceptible maize variety (Katumani), obtained from the maize research programme, Alemaya University, Ethiopia, were used. Two seeds were planted in plastic pots (16 x by 30 cm) filled with approximately 5 kg of topsoil collected from arable land in Stellenbosch, South Africa. The pots containing the seedlings were kept on greenhouse benches at a photoperiod of 12 h : 12 h light and dark. No fertilizers were added. The plants were watered as needed. Three weeks after plant emergence, the seedlings (3 to 4 leaf growth stage) were thinned to one plant per pot and the leaf whorl infested with 20 second instar larvae per plant. Twenty-four hours after infestation, a 5 ml conidial suspension (2×10^8 conidia/ml) of each isolate was applied to the leaf whorl of each seedling using a hand sprayer (MATABI 1.5 L). There were seven treatments consisting of five fungal isolates and two controls. The fungal isolates were PPRC-4, PPRC-19, PPRC-61, EE-01 and BB-01 (Table 2.1). The two controls were, firstly, infested unsprayed plants (infested-control) and, secondly, uninfested unsprayed plants (uninfested-control). The pots containing the seedlings were arranged in a randomized complete block design with seven replicates per treatment. The plants were sprayed only once. The experiment was conducted twice. However, in the first experiment plants were infested 3 weeks after emergence, while in the second experiment, they were infested 4 weeks after emergence. The experiment was conducted at the University of Stellenbosch, Forestry greenhouse, from February to April, 2003.

7.2.4. Greenhouse temperature and relative humidity

Temperature and humidity in the greenhouse were measured daily using a thermo-hygrograph.

7.2.5. Foliar damage

One week after treatment and every week thereafter, foliar damage assessments were made on a scale from 0 to 5 (Odindo, 1992). A score of 0 = damage free; 1 = a lesion area covering 1 % of the leaf; 2 = a lesion area of up to 5 % of the leaf; 3 = a lesion covering 5 - 10 %; 4 = 10 - 15 %; and 5 = lesions covering > 15 % of the leaf area.

7.2.5.1. Statistical analysis

Foliar damage were categorical data and subjected to Kruskal-Wallis non-parametric analyses. Multiple comparisons using rank sums were used to determine significant differences between means at $P < 0.05$ (Dunn, 1964). Since there was no foliar damage recorded from uninfested control plants, they were excluded from the analyses.

7.2.6. Damage assessment

Stem height (measured from soil level to the tip of the central leaf), root length (from stem base to root tip), plants with deadheart, number of exit/entry holes, number of nodes per plant, and number of attacked nodes per plant were recorded 6 weeks after plants were treated. After dissecting the stems with a knife, the number of larvae, pupae and tunnel length per plant were recorded. Since the total number of larvae and pupae recovered was low, they were combined. Root length was measured after gently washing off the soil particles.

7.2.6.1. Statistical analysis

Tunnel length was expressed as proportion of stem height, and deadheart as percentage of a total sample. Percent deadheart and percent tunnel length were angular-transformed ($\arcsine \sqrt{\text{proportion}}$) in order to stabilize the variances. Number of attacked nodes per plant, holes per plant, larvae and pupae per plant were Log10-transformed. Stem height and root length were not transformed. A split plot analysis with experiment 1 and experiment 2 as main plots and isolates (treatments) as subplots was conducted on stem damage parameters using Statistica 6.5 computer software. Since there were interactions between experiment and treatment, the data were analysed using a one-way analysis of variance for each experiment. Student-Newman-Keuls Test was used to determine whether or not there were significant differences between means.

7.2.7. Plant biomass

Stem and root fresh weights were measured 6 weeks after the plants were treated. The dry weight of stems and roots were determined after placing them in paper bags and incubating them at 120 °C for 24 h.

7.2.7.1. Statistical analysis

Plant biomass data were not transformed. Split-plot analyses with experiment 1 and experiment 2 as main plots and isolates (treatments) as subplots were conducted on data from plant biomasses (Table 7.4). Since there were significant ($P < 0.05$) interactions between experiment and treatment, the data were analysed using a one-way analysis of variance for each experiment. Student-Newman-Keuls Test was used to determine whether or not there were significant differences between means.

7.3. RESULTS

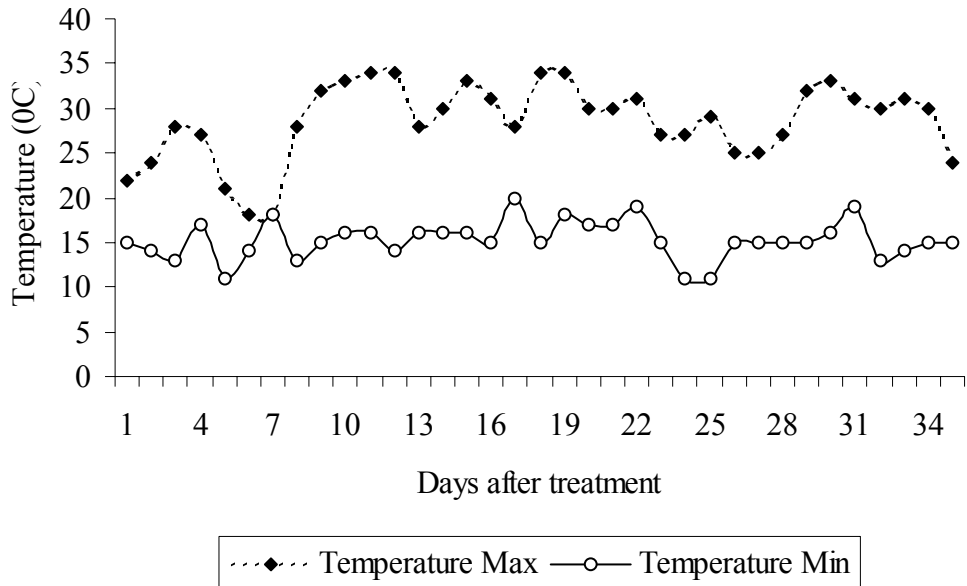
7.3.1. Greenhouse temperature and relative humidity

The mean daily temperature and relative humidity in the greenhouse ranged from 10 to 35 °C and 30 to 90 %, respectively (Figure 7.1 a & b).

7.3.2. Foliar damage

Application of aqueous conidial suspensions effectively suppressed foliar damage (Tables 7.1 and 7.2). Significant differences in foliar damage between infested-control and fungi-treated plants became apparent two weeks after treatment in both experiments. Foliar damage was reduced to low levels in treated plants while it increased to high levels in infested control plants (Tables 7.1 and 7.2).

(a)



(b)

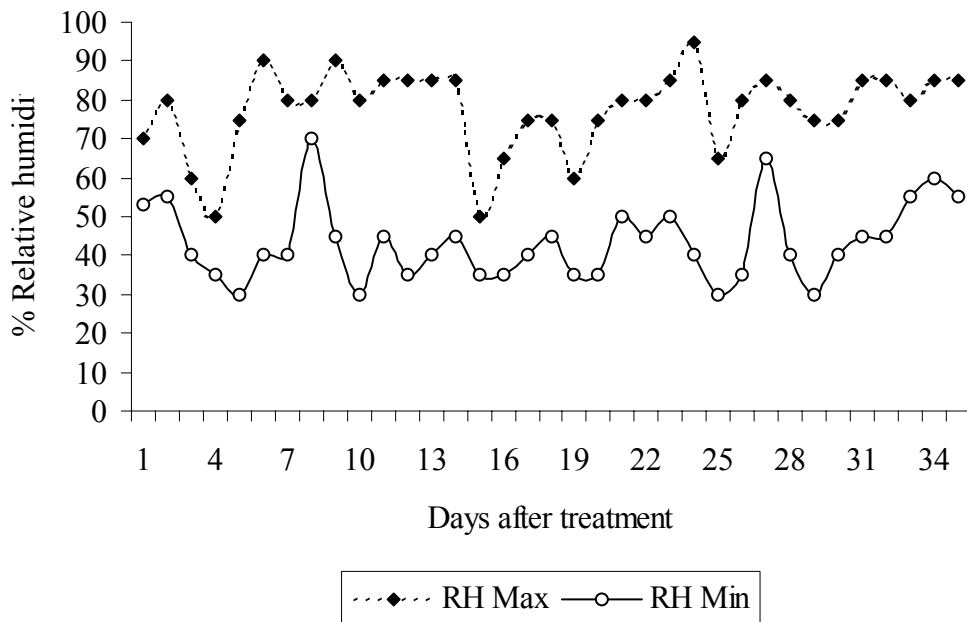


Figure 7.1. Mean minimum and maximum temperatures (a) and relative humidities (b) of the greenhouse.

7.3.3. Stem damage and root length

There were no differences between experiments in percent stem tunnelled, deadheart, number of attacked nodes per plant, holes per plant and larvae and pupae per plant (Table 7.3). However, there were differences in stem height and root length between the two experiments (Table 7.3). In addition, there were interactions between experiment and treatment for all of the stem damage parameters except in percent stem tunnelled and number of holes per plant (Table 7.3).

There were differences between treatments in both experiments in all of the stem damage parameters except in number of attacked nodes per plant in experiment 1 (Tables 7.4 and 7.5). There were no differences in root length between treatments in the two experiments. The infested-control plants were shorter (52 to 60 cm) than plants treated with the fungal isolates (69 to 87 cm) and uninfested control (72 to 90 cm). The extent of stem tunnelling and deadheart was less in the fungi-treated plants than in the infested-control plants. The number of holes per plant and larvae and pupae per plant was higher in the infested-control than in the fungi treatments.

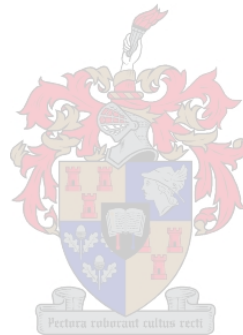


Table 7.1. Kruskal-Wallis non-parametric analysis of foliar damage in 3-week old maize plants (experiment 1) infested with 20 second instar *Chilo partellus* larvae and then treated with conidial suspension (2×10^8 conidia ml⁻¹) of *Beauveria bassiana* and *Metarhizium anisopliae* 24 h after infestation.

Weeks after treatment	Isolate	Mean Rank*	χ^2	df	P-value
1	PPRC-4	12.0 a	9.8	5	0.08
	PPRC-19	12.0 a			
	PPRC-61	18.7 a			
	EE-01	25.3 a			
	BB-01	20.2 a			
	Infested control	22.8 a			
	Mean difference	14.9			
2	PPRC-4	8.0 a	15.9	5	< 0.001
	PPRC-19	11.8 ab			
	PPRC-61	20.7 ab			
	EE-01	20.7 ab			
	BB-01	21.6 ab			
	Infested control	28.0 b			
	Mean difference	14.9			
3	PPRC-4	8.0 a	21.7	5	< 0.001
	PPRC-19	12.4 a			
	PPRC-61	15.7 a			
	EE-01	20.2 ab			
	BB-01	22.5 ab			
	Infested control	32.0 b			
	Mean difference	14.9			

* Mean ranks with same letter did not differ at $P > 0.05$.

Table 7.2. Kruskal-Wallis non-parametric analysis of foliar damage in 4-week old maize plants infested with 20 *Chilo partellus* second instar larvae and then treated with conidial suspension (2×10^8 conidia ml^{-1}) of *Beauveria bassiana* and *Metarhizium anisopliae* 24 h after infestation.

Weeks after treatment	Isolate	Mean rank	χ^2	df	P-value
1	PPRC-4	18.1 a	9.56	5	0.09
	PPRC-19	21.0 a			
	PPRC-61	21.0 a			
	EE-01	21.0 a			
	BB-01	21.0 a			
	Infested control	26.9 a			
	Mean difference	19.2			
2	PPRC-4	16.8 a	22.76	5	< 0.001
	PPRC-19	12.6 a			
	PPRC-61	16.8 a			
	EE-01	20.3 ab			
	BB-01	21.0 ab			
	Infested control	38.4 b			
	Mean difference	19.2			
3	PPRC-4	13.5 a	30.21	5	< 0.001
	PPRC-19	11.9 a			
	PPRC-61	16.1 a			
	EE-01	18.8 a			
	BB-01	18.3 a			
	Infested control	38.4 b			
	Mean difference	19.2			

* Mean ranks with same letter did not differ at $P > 0.05$.

Table 7.3. Split plot analysis of experiments as main plots and treatments as subplots for maize stem damage by *Chilo partellus* recorded 6 weeks after treatment application.

Parameter	Source	df	SS	MS	F	P
Stem height	Experiment (E)	1	1552	1552	6.67	0.04
	Error	6	1391	232		
	Isolate (I)	6	7057	1176	14.2	<0.001
	E x I	6	1391	232	2.79	0.02
	Error	84	6996	83		
Percent stem tunnelled	Experiment (E)	1	0.05	0.05	1.97	0.22
	Error	5	0.13	0.02		
	Isolate (I)	5	0.54	0.11	5.72	<0.001
	E x I	5	0.13	0.02	1.39	0.23
	Error	72	1.35	0.01		
Percent deadheart	Experiment (E)	1	4.23	4.23	4.81	0.8
	Error	5	4.39	0.87		
	Isolate (I)	5	7.67	1.53	123.7	<0.001
	E x I	5	4.39	0.87	70.9	<0.001
	Error	72	0.89	0.01		
No. attacked nodes/plant	Experiment (E)	1	0.17	0.17	2.57	0.16
	Error	5	0.33	0.06		
	Isolate (I)	5	0.82	0.16	5.75	<0.001
	E x I	5	0.33	0.06	2.32	0.05
	Error	72	2.08	0.02		
No. holes/plant	Experiment (E)	1	0.01	0.01	0.85	0.39
	Error	5	0.11	0.02		
	Isolate (I)	5	2.29	0.45	8.59	<0.001
	E x I	5	0.11	0.02	0.41	0.83
	Error	72	3.84	0.05		
No. larvae & pupae/plant	Experiment (E)	1	0.55	0.55	2.94	0.15
	Error	5	0.93	0.18		
	Isolate (I)	5	2.54	0.50	19.14	<0.001
	E x I	5	0.93	0.18	7.06	<0.001
	Error	72	1.91	0.02		
Root length	Experiment (E)	1	21295	21295	204	<0.001
	Error	6	627	105		
	Isolate (I)	6	375	62	1.0	0.41
	E x I	6	627	105	1.7	0.01
	Error	84	5126	61		

Table 7.4. Maize stem damage (mean \pm SE) six weeks after treatment in three-week old plants (experiment 1) infested with 20 *Chilo partellus* second instar larvae and then treated with conidial suspension (2×10^8 conidia ml⁻¹) of *Beauveria bassiana* and *Metarhizium anisopliae* 24 h after infestation.

Treatment	Stem height (cm)	Percent stem tunnelled	Percent deadheart	Number of nodes attacked /plant	Number of holes/plant	Number of larvae & pupae/plant	Root length (cm)
PPRC-4	87.1 \pm 4.2 b	1.4 \pm 0.7 a	0.0 \pm 0.0 a	1.1 \pm 0.3 a	0.8 \pm 0.2 a	0.2 \pm 0.1 a	27.5 \pm 0.7 a
PPRC-19	86.2 \pm 2.7 b	1.1 \pm 0.5 a	16.7 \pm 0.0 b	0.6 \pm 0.3 a	0.4 \pm 0.2 a	0.2 \pm 0.1 a	30.7 \pm 0.6 a
PPRC-61	78.5 \pm 4.2 b	2.3 \pm 0.6 a	33.3 \pm 0.0 c	2.0 \pm 0.5 a	2.1 \pm 0.7 ab	1.0 \pm 0.3 ab	29.1 \pm 0.5 a
EE-01	69.0 \pm 3.2 ab	5.0 \pm 2.7 b	0.0 \pm 0.0 a	1.1 \pm 0.4 a	1.5 \pm 0.6 ab	0.7 \pm 0.3 ab	26.5 \pm 1.8 a
BB-01	68.5 \pm 2.9 ab	5.2 \pm 1.9 b	28.5 \pm 4.7 c	1.5 \pm 0.3 a	2.0 \pm 0.5 ab	3.0 \pm 1.2 b	28.2 \pm 1.2 a
Infested-control	60.0 \pm 1.3 a	5.1 \pm 0.7 b	100 \pm 0.0 d	2.2 \pm 0.5 a	3.0 \pm 0.3 b	6.3 \pm 0.7 c	25.7 \pm 1.6 a
Uninfested-control	90.2 \pm 1.6 b	-**	-	-	-	-	30.1 \pm 0.5 a

* Means \pm SE followed by the same letter within a column are not significant at $P > 0.05$ using Student-Newman-Keuls Test.

** No data collected.

Table 7.5. Maize stem damage (mean \pm SE) six weeks after treatment in four-week old plants (experiment 2) infested with 20 *Chilo partellus* second instar larvae and then treated with conidial suspension (2×10^8 conidia ml⁻¹) of *Beauveria bassiana* and *Metarhizium anisopliae* 24 h after infestation.

Treatment	Stem height (cm)	Percent stem tunnelled	Percent deadheart	Number of attacked nodes /plant	Number of holes/plant	Number of larvae & pupae/plant	Root length (cm)
PPRC-4	70.2 \pm 5.5 b	1.0 \pm 0.4 a	2.0 \pm 1.3 a	0.6 \pm 0.2 a	0.5 \pm 0.1 a	0.1 \pm 0.0 a	57.4 \pm 4.1 a
PPRC-19	73.5 \pm 3.9 b	0.6 \pm 0.3 a	8.3 \pm 2.3 a	0.3 \pm 0.1 a	0.2 \pm 0.1 a	0.1 \pm 0.0 a	57.5 \pm 1.6 a
PPRC-61	78.0 \pm 3.8 b	1.2 \pm 0.4 a	16.6 \pm 4.6 a	1.0 \pm 0.3 a	1.6 \pm 0.6 a	0.5 \pm 0.2 a	59.1 \pm 5.2 a
EE-01	68.9 \pm 3.2 b	4.6 \pm 1.8 a	0.0 \pm 0.0 a	1.0 \pm 0.3 a	1.4 \pm 0.4 a	0.6 \pm 0.2 a	66.6 \pm 5.0 a
BB-01	69.5 \pm 3.4 b	3.8 \pm 1.5 a	15.2 \pm 4.4 a	0.9 \pm 0.2 a	2.0 \pm 0.5 a	1.5 \pm 0.7 a	56.7 \pm 3.8 a
Infested-control	51.6 \pm 2.2 a	22.4 \pm 12 b	55.0 \pm 12.5 b	2.5 \pm 0.4 b	3.3 \pm 0.4 b	4.1 \pm 0.7 b	55.0 \pm 4.7 a
Uninfested-control	72.3 \pm 3.1 b	-**	-	-	-	-	52.0 \pm 1.6 a

* Means \pm SE followed by the same letter within a column are not significant at $P > 0.05$ using Student-Newman-Keuls Test.

** No data collected.



7.3.4. Plant biomass

There were differences in fresh root and dry root weights between the experiments (Table 7.6). The interactions between experiment and treatment were also significant for all fresh and dry plant biomass except in fresh stem weight (Table 7.6).

There were differences between treatments within the two experiments in plant biomass (Tables 7.7 and 7.8). Fresh and dry plant biomasses were lower in infested control plants than in fungal treatments and uninfested control in both experiments. However, total fresh and dry plant biomass of plants treated with EE-01 and BB-01 was as low as the infested control in experiment 1 (Table 7.7). Treatment of plants with PPRC-4 yielded similar fresh and dry total biomass to the uninfested control (Tables 7.7 and 7.8).

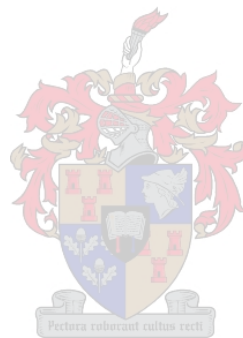


Table 7.6. Split plot analysis of experiments as main plots and treatments as subplots for maize biomass recorded 6 weeks after treatment application.

Plant biomass	Source	df	SS	MS	F	P
Fresh stem weight	Experiment (E)	1	1.10	1.10	0.003	0.95
	Error	6	2068	344		
	Isolate (I)	6	42911	7151	34.7	<0.001
	E x I	6	2068	344	1.67	0.13
	Error	84	17297	205		
Fresh root weight	Experiment (E)	1	4096	4096	13.7	0.01
	Error	6	1786	297		
	Isolate (I)	6	20351	3391	29.8	<0.001
	E x I	6	1786	297	2.6	0.02
	Error	84	9547	113		
Fresh total weight	Experiment (E)	1	3955	3955	3.5	0.92
	Error	6	6653	1108		
	Isolate (I)	6	115292	19215	52.0	<0.001
	E x I	6	6653	1108	3.0	0.01
	Error	84	31028	369		
Dry shoot weight	Experiment (E)	1	3.3	3.3	0.9	0.33
	Error	6	104	17.2		
	Isolate (I)	6	1332	222	41.8	<0.001
	E x I	6	104	17.2	3.2	<0.001
	Error	84	445	5.3		
Dry root weight	Experiment (E)	1	46	46	12.6	0.01
	Error	6	22	4		
	Isolate (I)	6	171	28.4	14.0	<0.001
	E x I	6	22	3.6	1.8	0.01
	Error	84	171	2.0		
Dry total weight	Experiment (E)	1	74	74	2.3	0.17
	Error	6	189	32		
	Isolate (I)	6	2273	379	41.1	<0.001
	E x I	6	190	32	3.4	<0.001
	Error	84	773	9.2		

Table 7.7. Maize fresh and dry biomasses (mean \pm SE) six weeks after treatment application. Three-week- old plants (experiment 1) were infested with 20 *Chilo partellus* second instar larvae and then treated with conidial suspension (2×10^8 conidia ml⁻¹) of *Beauveria bassiana* and *Metarhizium anisopliae* 24 h after infestation.

Treatment	Fresh stem weight	Fresh root weight	Fresh total weight	Dry stem weight	Dry root weight	Dry total weight
PPRC-4	77.2 \pm 9.1 cd	71.6 \pm 7.9 c	148.9 \pm 12.8 c	17.6 \pm 1.4 cd	13.2 \pm 0.9 c	30.9 \pm 1.7 c
PPRC-19	59.6 \pm 8.4 bc	52.6 \pm 2.5 bc	112.2 \pm 8.9 b	14.5 \pm 1.4 bc	10.5 \pm 0.4 bc	25.0 \pm 1.5 b
PPRC-61	48.1 \pm 9.1 b	58.7 \pm 7.0 c	106.9 \pm 11.2 b	12.9 \pm 1.4 b	10.8 \pm 0.7 bc	23.7 \pm 1.7 b
EE-01	33.6 \pm 6.8 ab	20.2 \pm 3.4 a	53.9 \pm 9.9 a	7.2 \pm 0.3 a	10.5 \pm 1.0 bc	17.8 \pm 1.3 a
BB-01	35.3 \pm 5.9 ab	35.7 \pm 2.5 ab	71.1 \pm 6.3 a	10.9 \pm 0.9 ab	9.4 \pm 0.6 ab	20.4 \pm 0.8 ab
Infested-control	12.4 \pm 1.5 a	35.1 \pm 8.4 ab	47.5 \pm 9.1 a	8.5 \pm 0.4 a	7.5 \pm 0.7 a	16.0 \pm 1.0 a
Uninfested-control	88.3 \pm 8.9 d	71.9 \pm 6.6 c	160.2 \pm 12.2 c	20.7 \pm 1.9 d	12.4 \pm 0.7 bc	33.2 \pm 2.5 c

Means \pm SE followed by the same letter in within a column are not significant at $P > 0.05$ using Student-Newman-Keuls Test.

Table 7.8. Maize fresh and dry biomasses (mean \pm SE) six weeks after treatment application. Four-week-old plants (experiment 2) were infested with 20 *Chilo partellus* second instar larvae and then treated with conidial suspension (2×10^8 conidia ml⁻¹) of *Beauveria bassiana* and *Metarhizium anisopliae* 24 h after infestation.

Treatment	Fresh stem weight	Fresh root weight	Fresh total weight	Dry stem weight	Dry root weight	Dry total weight
PPRC-4	64.1 \pm 5.2 d	46.0 \pm 3.2 cd	110.1 \pm 6.4 c	15.3 \pm 1.0 cd	10.2 \pm 0.4 b	25.5 \pm 1.2 cd
PPRC-19	54.1 \pm 4.2 cd	37.2 \pm 1.5 bc	91.3 \pm 5.4 c	13.2 \pm 0.7 bc	9.2 \pm 0.2 b	22.4 \pm 0.9 bc
PPRC-61	59.5 \pm 5.4 cd	46.7 \pm 3.3 cd	106.2 \pm 6.2 c	14.3 \pm 0.5 bc	10.1 \pm 0.4 b	24.5 \pm 0.8 cd
EE-01	34.7 \pm 5.8 ab	20.7 \pm 2.9 a	55.4 \pm 8.5 a	9.0 \pm 0.6 a	8.9 \pm 0.5 b	17.9 \pm 1.1 a
BB-01	44.3 \pm 4.2 bc	29.1 \pm 2.9 ab	73.4 \pm 5.7 b	12.3 \pm 0.5 ab	8.7 \pm 0.4 b	21.0 \pm 0.7 b
Infested-control	21.1 \pm 2.2 a	16.7 \pm 3.0 a	47.9 \pm 2.7 a	9.0 \pm 0.2 a	7.4 \pm 0.2 a	16.4 \pm 0.3 a
Uninfested-control	78.4 \pm 3.1 e	50.0 \pm 2.4 d	128.4 \pm 4.2 d	17.2 \pm 0.7 d	10.2 \pm 0.2 b	27.4 \pm 0.9 d

* Means \pm SE followed by the same letter in within a column are not significant at $P > 0.05$ using Student-Newman-Keuls Test.

7.4. DISCUSSION

The fungal isolates used in these experiments appeared to have potential for stalk borer control. All the isolates used protected the infested maize plants from foliar and stem damage by *C. partellus* larvae. Plants were infested at a very susceptible stage (Odindo, 1994; Brownbridge, 2001) in their development and the infestation rates (20 larvae/plant) used were sufficient to cause considerable plant damage and reduction in plant biomass as can be seen in the infested-control. Although plants in both infested-control and fungi-treated pots were at the same stage of foliar damage at the time of the application of the pathogen, further damage by the borers ceased immediately after treatment of the plants with the fungi. Fungal treatments were applied while larvae were causing external damage to the leaves before boring into plants. Therefore, they became infected with conidia which they took with them into the plants. In addition, fungal infection can reduce food consumption by larvae (Tefera & Pringle, 2004).

Application of these fungi may play a strategic role in the management of *C. partellus*, provided applications are made sufficiently early to target young larvae feeding in the leaf whorl. The critical period for control of *C. partellus* is the first two weeks after hatching when the first, second and third instar larvae feed externally on maize leaves (Odindo, 1994). Once inside the stem (fourth, fifth and sixth instar larvae) the larvae may not be infected following pathogen applications. However, further mortality may occur through previous infection by pathogens consumed on the leaves, or inoculation via infected cadavers inside the stem. Thus, adult and larval activity will require intensive scouting to determine the correct timing of spray applications. Previous studies using the microsporidian *Nosema* sp. against *C. partellus* showed that plants were protected when the pathogen was applied within seven days after larval infestations (Odindo, 1994). *Beauveria bassiana* applied to maize foliage can effectively control European corn-borers (Bing & Lewis, 1992). *Beauveria bassiana* has recently been shown to form an endophytic relationship with maize (Lewis *et al.*, 1996). The maize plants treated with liquid or granular formulations of *B. bassiana* conidia at the whorl stage of development became internally colonized by the fungus (Lewis *et al.*, 1996). The effect of endophytic *Beauveria* against *C. partellus* in maize needs further investigation.

The temperature and relative humidity in the greenhouse ranged from 10 to 35 °C and 30 to 90 %, respectively. Tefera & Pringle (2003) reported mortality of *C. partellus* larvae infected with *B. bassiana* and *M. anisopliae* between 15 and 30 °C.

A high relative humidity is essential for the development of fungal epizootics (Roberts & Campbell, 1977; Benz, 1982) but is not a limiting factor in fungal infection (Ferron, 1977; Marcandier & Khachatourian, 1987). Thus, the microenvironment in the greenhouse might have favoured fungal infection. At the end of the experiment insect cadavers which had been killed due to infection by the fungi were not collected. However, insect cadavers were seen on and in maize plants during scoring of leaf damage, stem dissections, and measurements of tunnelling. Rapid decomposition of young larvae killed by fungus has been reported in the case of *Ostrinia nubilalis* Hubner (Lepidoptera: Pyralidae) (Feng *et al.*, 1988). In the present study the experiment was terminated 42 days after treatment, by which time most of the dead insects had decomposed.

The isolates PPRC-4, PPRC-19 and PPRC-61 were superior to BB-01 and EE-01 in reducing most of the damage symptoms. Further studies under field conditions are required. Maniania (1993) reported that granular formulations of *B. bassiana* were effective against *C. partellus*. In the current study, unformulated suspension of *M. anisopliae* isolates (PPRC-4, PPRC-19 & PPRC-61) in water substantially reduced larval feeding and improved plant biomass. *B. bassiana* has been developed as a mycoinsecticide for the control of the European corn borer, *O. nubilalis* (Hussey & Tinsley, 1981).

In the present study, infested-control plants were stunted, had highly tunnelled stems, deadhearts, and low biomasses. Bosque-Perez & Mareck (1991) reported that after the larvae have penetrated the stems, they created tunnels by eating through the vascular bundles, leading to reduced translocation of nutrients and assimilates. Stem tunnelling also reduced plant vitality and impeded the grain filling process, and promoted lodging of plants as they matured. Vandenberg & Van Rensburg (1991) found considerable yield reduction in sorghum due to stem tunnelling by *C. partellus*.

7.5. REFERENCES

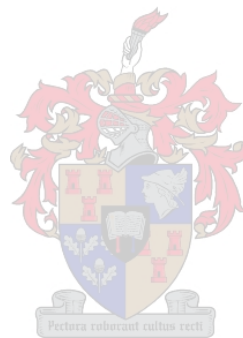
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CHAPTER 8

CONCLUSION AND DISCUSSION

The present study achieved its goals by demonstrating:

1. The susceptibility of *Chilo partellus* larval instars to isolates of *B. bassiana* and *M. anisopliae*,
2. effect of temperature on fungal development and pathogenicity,
3. food consumption by fungal treated larvae and effects of diets on mortality and mycosis,
4. compatibility of fungal isolates with selected insecticides,
5. exposure methods of larvae to fungal isolates; and
6. greenhouse evaluation of fungal isolates against *C. partellus* in maize.

The pathogenicity of four isolates of *B. bassiana* and six isolates of *M. anisopliae* to second instar *C. partellus* larvae were tested. Four *M. anisopliae* isolates, PPRC-4, PPRC-19, PPRC-61 and EE-01 and one *B. bassiana* isolate (BB-01) were the most pathogenic to *C. partellus* second, third, fourth, fifth and sixth instar larvae. The isolates were also constantly pathogenic to all larval stages tested. In addition, these isolates originated from diverse hosts and not necessarily from *C. partellus*.

The variation within isolates of the same species fungi could be attributed production of toxic substances. The production of proteases by entomopathogenic fungi on insect cuticle have been reported (St. Leger *et al.*, 1991). Secondary metabolites produced by *M. anisopliae* act on insect tissues including the mid-gut (Samuels *et al.*, 1988). The production of these metabolites may cause variation among the isolates.

There was a decreasing pattern of susceptibility from the second instar to the fifth instar and an increase in susceptibility in the sixth instar *Chilo partellus* larvae treated with PPRC-4, PPRC-19, PPRC-61, EE-01 and BB-01. Therefore, second and sixth instar larvae were the most susceptible developmental stage to the fungal isolates. There was an increase in the LT₅₀ from the second instar to fifth instar. The LT₅₀ decreased in sixth instar larvae. Control measures should be aimed at the most vulnerable stages in the life cycle of the pest.

In the case of *C. partellus*, the second instar larvae is the stage when young larvae feed in plant leaf whorls. If fungi are applied during the whorl feeding period, there will be high probability that larvae will be exposed to infection by the fungi. Although the sixth instar larvae were the most susceptible, practical application of these isolates against sixth instar larvae will hardly be possible because they are inside the maize stems at this stage. However, mortality may occur due to previous infection during second and third instar stages.

Conidia germination, radial growth, sporulation and pathogenicity of *B. bassiana* (BB-01) and *M. anisopliae* (PPRC-4, PPRC-19, PPRC-61 & EE-01) were affected by temperature as well as the isolate used. The optimum temperature for conidia germination, radial growth and sporulation differed between isolates. The optimum temperature for germination of the isolates was between 25-30 °C. PPRC-4, PPRC-19 and EE-01 had optimum growth and sporulation between 25 and 30 °C, and BB-01 and PPRC-61 between 20 and 25 °C. Therefore, PPRC-4, PPRC-19 and EE-01 were tolerant to higher temperature than BB-01 and PPRC-61. At 25 and 30 °C, larval mortality was high and the LT₅₀ was short. This indicated that pathogenicity of the isolates was temperature dependent.

Chilo partellus (Swinhoe) is reported to occur in the temperature range of 15-35 °C (Gebre-Amlak, 1985; Haile & Hofsvang, 2001). The virulence of entomopathogenic fungi to *C. partellus* at this range of temperatures has been determined in this study. In order to use fungi for the control of this stalk borer, the effectiveness of the isolates could be between 25 and 30 °C. However, mortality can occur between 15 and 20 °C but with extended duration.

Treatment of second and third instar larvae with *B. bassiana* (BB-01) and *M. anisopliae* (PPRC-4) reduced mean daily food consumption by the larvae. This is of advantage to pest management. At high conidial concentration (1×10^8 conidia/ml), there was high reduction in food consumption. Apart from direct mortality effect, reduction in food consumption of *B. bassiana* and *M. anisopliae* to treated *C. partellus* would enhance the integration of the fungi with other control options. Not all control options of *C. partellus* require instant knock-down effect as most of the cultural practices of the stalk borers can be done in non-crop habitats such as wild hosts, crop residue, where control is preventive rather than curative. Under these circumstances, slow speed of kill need not be a limitation, as feeding may be reduced some time before death.

Mortality and mycosis in larvae were influenced by the type of diets larvae fed before and after treatment with the fungal isolates. Larvae feeding on artificial diet reduced the effectiveness of the fungi. In bioassays of *B. bassiana* and *M. anisopliae* against *C. partellus*, therefore, natural diet is preferred to artificial diets. This is because artificial diets contain

antimicrobial substances (Kfir, 1992) which can adversely affect the pathogenicity and sporulation of the isolates.

There was a relationship between conidial germination, radial growth and sporulation of fungal isolates and the insecticides benfuracarb and endosulfan. In *in-vitro* studies increasing the concentration of the insecticides adversely affected conidial germination, radial growth and sporulation of the isolates. The insecticides will impose limitations on the use of the two agents if the concentrations of the insecticides are high. In *in-vivo* laboratory studies larval mortality was high when 1 ppm and 5 ppm active ingredients of the insecticides were mixed with fungal suspensions. These concentrations were less than field application rates for benfuracarb (10 ml/litre water/ha) and endosulfan (2 ml/litre water/ha) (Nel *et al.*, 1999). Therefore, 1 ppm and 5 ppm active ingredients were appropriate concentrations of insecticides for use with fungal isolates.

Bioassay exposure methods such as direct spraying, dipping and treated leaves can be used to determine the mortality, mycosis and sporulation of *B. bassiana*. All methods resulted in high mortality indicating the possible use of any of these methods in bioassay of *B. bassiana* against *C. partellus*. The optimum temperature for mycosis in cadavers was between 20 and 27 °C and 15 °C for sporulation. Mycosis and sporulation of the fungus in larval cadavers may increase the chance of contact with larvae, thereby helping the fungi to disperse horizontally and establish in the crop ecosystem.

Spraying conidial suspensions of the fungal isolates, PPRC-4, PPRC-19, PPRC-61, EE-01 and BB-01, on maize seedlings (1x10⁹ conidia/ seedling) infested with second instar larvae substantially reduced foliar damage caused by larval feeding. Fungal treatments also reduced stem tunneling, deadheart formation, and improved plant height and biomass. The application of *B. bassiana* and *M. anisopliae*, for the control of *C. partellus*, should be therefore synchronized with the occurrence of the second instar larvae. Most chemical control applications against the stalk borer *C. partellus* are target at early instars (first, second and third). Because the late instars (fourth, fifth and sixth) bore into the stem they are inaccessible to chemical control (Seshu Rddy, 1989; Van Humburg, 1980).

Results obtained under greenhouse conditions may not necessarily guarantee the same level of efficacy of the isolates in the field. Our studies, however, confirmed that both *B. bassiana* and *M. anisopliae* have high potential as biological control agents against *C. partellus*. Therefore the isolates, PPRC-4, PPRC-19, PPRC-61, EE-01 and BB-01 are promising candidates. However, their efficacy should be further evaluated under field conditions.

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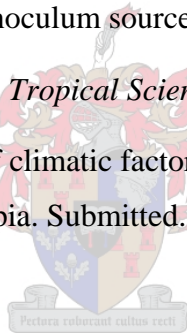
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