



**EVALUATION OF ENTOMOPATHOGENIC FUNGI (ASCOMYCOTA) FOR THE
CONTROL OF CYDIA POMONELLA (LEPIDOPTERA: TORTRICIDAE)**

BY

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Title page

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DECLARATION

I, Asomiba Rita Abaajeh, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed

Date

Dedication

- To God almighty for keeping me healthy and guiding me throughout this study
- To Dr. P. Igue
- To my parents; Mr & Mrs Abaajeh.

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I want to thank:

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ABSTRACT

Codling moth (*Cydia pomonella* (Lepidoptera: Tortricidae)) infest pomes resulting in high production losses to fruit farmers in South Africa and globally. Many farmers are adopting biocontrol methods including the use of entomopathogens as alternatives to synthetic insecticides for sustainable management of Arthropod pests. Research activities on screening and application of entomopathogenic fungi (EPF) have intensified in recent years. This study was carried out to isolate and identify virulent indigenous entomopathogenic fungal strains from soils of selected locations in the Western Cape region of South Africa that are pathogenic against codling moth (*Cydia pomonella* (Linn) [Lepidoptera: Tortricidae]) and to evaluate the possibility of combining semiochemicals and fungal inoculums in a simulated attract-and-fungus contamination system for codling moth control.

Soil samples were collected from 10 locations in the Western Cape, South Africa for the isolation of entomopathogenic fungi (EPF) by baiting the soil samples with 5th instar larvae of codling moths. Fungal strains were identified and characterized using light microscopy and DNA analysis (internal transcribed spacer region) and elongation factor 1- α (EF 1- α) genes of fungal cultures. Thirty-nine (39) isolates belonging to six species were obtained; *Purpureocillium lilacinum* (8 isolates), *Fusarium oxysporum* (five isolates), *Fusarium polyphialidicum* (two isolates), *Beauveria pseudo-bassiana* (one isolate), *Aspergillus flavus* (three isolates) and *Metarhizium robertii* (20 isolates). Generally, *M. robertii* was the most frequently encountered species representing 51% of the total number of isolates collected from the soil samples. This is the first report of the isolation of *M. robertii* in southern Africa. A screening test to identify the most virulent fungal strains against codling moth was carried out on 39 isolates by immersing 5th instar larvae of *C. pomonella* into aqueous spore suspension 1×10^8 conidia ml⁻¹. Mortality data from the immersion bioassay indicated that the 39 fungal isolates were pathogenic against fifth instar larvae of codling moth inducing 47-85% insect mortality at an infective concentration of 1×10^8 spores' ml⁻¹, 5 days post-treatment. Two fungal strains

MTL151 and GW461 induced over 85% larval mortality and were selected for further evaluations. The effect of MTL151 and GW461 on egg hatchability of 0-day old eggs was evaluated by exposing freshly laid eggs on wax papers that were pre-treated with fungal spores ranging from 10^3 - 10^8 spores/ml. Egg hatchability reduced significantly from 93-71% (GW461) and 95-66% (MTL151) as spore concentration increases from 1×10^3 to 1×10^8 spores ml^{-1} , respectively. The highest spore concentration significantly inhibited egg hatchability resulting in lower egg hatchability compared to that obtained with the cypermethrin containing commercial insecticide (Fruitfly [registered by Kombat (PTY) Ltd]) tested at the recommended dose of 0.25 ml/250 ml of water. The potential of the two *M. robertsii* strains to protect apples from infestation by codling moth neonates was assessed in an apple fruit bioassay under laboratory and field bioassay. Codling moth neonates were exposed to apples that were sprayed topically with varied conidia concentrations (10^3 - 10^8 spores ml^{-1}). The mean number of participating apple fruits having developing/developed larvae in the core/flesh significantly reduced from 5.3 to 1 and 7.6 to 1 for MTL151 and GW461, respectively as spore concentrations increased from 1×10^3 to 1×10^8 spores/ ml^{-1} . A concurrent decrease in apple fruit rot as conidia concentration increased was observed. Up to 90% of apples treated with 1×10^8 spores/ ml^{-1} had no larva present in their cores and this result compared favourably with the commercial pesticide (Fruitfly) used at a recommended dose of 0.25g/250 ml of water. No significant difference was found between the EC_{50} values of 1.2×10^3 ($\text{CI}=1.2 \times 10^2$ - 1×10^5) (MTL151) and 1.1×10^5 ($\text{CI} = 7 \times 10^3$ - 5.6×10^5) (GW461) spores ml^{-1} . However, neither the two *M. robertsii* strains nor the insecticide deterred the neonates from feeding on the surface of the fruits.

The attraction responses of male and female adult codling moths to butyl hexanoate and codlemone blend; butyl hexanoate (0.025 mg / ml of hexane) and codlemone (0.1 mg / ml of hexane) in a ratio of 1:1 (0.5 ml: 0.5 ml from both solutions) were assessed in a one-choice wind tunnel olfactory bioassay during scoto-phase in the absence of fungal spores. The blend attracted more females (69%) than males (66.7%), however these responses were not significantly different ($P > 0.5$). The prospect of integrated use of entomopathogenic fungal conidia and semiochemical blend for control of *C. pomonella* adults was assessed in a wind tunnel containing the combo lure and *M. robertsii* (MTL151) spores formulated as a powder (250 mg) or aqueous spore suspension (3.1×10^8 spores $\text{m}^{-1} \pm 7 \times 10^2$ spores ml^{-1}). Insect attraction, mean

number of spores picked per insect and insect mortality was assessed. No significant difference ($P > 0.5$) was observed in the number of inoculi picked by unsexed adult moths in the two conidial formulations tested; spore powder ($3.1 \times 10^3 \pm 1 \times 10^3$ spores ml^{-1}) and aqueous spore suspension ($3.1 \times 10^3 \pm 7 \times 10^2$ spores ml^{-1}). No mortality was recorded among fungus or control-treated moths. Both *M. robertsii* (MTL151) spore formulations did not significantly ($P > 0.05$) affect the attraction responses of the insects to the combo lure; powder ($73.3 \pm 3.3\%$) and aqueous ($68.6 \pm 2.9\%$) compared to the control treatment (without the fungal spores) ($70 \pm 0.5\%$). These results suggest that the fungus did not inhibit the attraction of moths by semiochemicals. Despite the successful autoinoculation of moths with fungal spores, the level of contamination was too low to induce mortality among adult moths. This study opens up opportunities for research in attract-and-fungus contamination delivery systems.

This is the first report of the occurrence of *M. robertsii* in the southern Africa. Further, results from this study indicates that *C. pomonella* is susceptible to indigenous South African entomopathogenic fungi and 2 indigenous *M. anisopliae* strains, MTL151 and GW461 provided adequate protections against codling moth larval infestations of apple fruits. Future research endeavours to improve adherence of conidia to adult moth cuticle is recommended.

Key words: Entomopathogenic fungi (EPF), codling moth, *B. pseudobassiana*, *M. robertsii*, larvae, pathogenicity and biological control, coddlemone, butyl hexanoate, attraction, semiochemicals.

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List of abbreviations

SDA	Sabouraud dextrose agar
PDA	Potatoes dextrose agar
OA	Oatmeal agar
CYA	Czapek yeast agar
MEA	Malt extract agar
EPF	Entomopathogenic fungus/i
SP	Scotophase

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1: Introduction

Insect pests have been man`s greatest concern for many centuries and as a result, man has devoted a lot of effort in trying to manage insect pest population numbers. Insects are known to infest and transmit diseases to plants and animals. For example, *Cydia pomonella* (Linn) (Lepidoptera: Tortricidae) is among the most economically important pests of orchard fruits (pear, apple and cherry) in South Africa and globally (Moran, 1983; Barnes, 1991). It is ranked the fifth plant pest in South Africa (Moran, 1983); this is taught to be the country with the highest infestation rate (Pringle *et al.*, 2003).

Presently, farmers in South Africa rely on chemical insecticides such as organophosphates for control of codling moth (Riedl *et al.*, 1998). However, Bakri, (n.d) reported that, new legislation has reduced acceptable residue levels and even banned some synthetic insecticides that are considered to be highly toxic. These synthetic chemicals have raised serious concerns over environment contamination, human safety, and insecticide resistance in codling.

These setbacks associated with the current pest control strategy have favoured the search for alternative control methods. For example, the use of biocontrol agents such as entomopathogens which include entomopathogenic nematodes, entomopathogenic bacteria, entomopathogenic viruses and entomopathogenic fungi (Osborne & Landa, 1992; Scholte *et al.*, 2004; Goble, 2009). These organisms have been suggested as ideal candidates to be incorporated into integrated control strategies that are currently being developed (De Waal *et al.*, 2008).

Currently, commercially available entomopathogenic products in the markets include, “Cryptogram™”, coelemone and Bb plus®.

Some of the well-studied entomopathogenic fungi (EPF) include *Baeuveria bassiana* and *Metarhizium anisopliae* [Ascomycota: Hypocreales (Meyling & Eilenberg, 2007)]. These fungi have several special characteristics that make them ideal microbial biological control agents against Arthropods. They usually attach themselves to the external body surface of insects in the form of microscopic bodies (Osborne & Landa, 1992). Experiments by Goble (2009) demonstrated that these organisms could be used to control a number of insects including false codling moth and other phytophagous pests. In a separate study, *M. anisopliae* reduced cattle tick numbers under experimental field conditions (Nchu *et al.*, 2010).

Although *B. bassiana* and *M. anisopliae* have been extensively tested with success on many lepidopteran pests (RangaRao & Reddy, 1997; Ansarii & Butt, 2012; Chouvinc *et al.*, 2012), no literature was found indicating that these fungi have been tested on codling moth (*C. pomonella*). It would therefore be of importance to test the pathogenicity of these fungi against codling moth. Despite the recognized potential of entomopathogenic fungi in controlling Arthropod pests and the notion that entomopathogenic fungi generally have a cosmopolitan distribution (Ali-Shtayeh *et al.*, 2002), very few studies have focused on the isolation of indigenous entomopathogenic species in South Africa (Goble, 2009). In this study, indigenous entomopathogenic fungal strains were isolated from soils using insect bait method and subsequently screened for pathogenicity against eggs and larvae of *C. pomonella*.

Furthermore, because the larvae of *C. pomonella* spends more time inside than outside the fruits of host plants, technologies that will optimize contact between *C. pomonella* and EPF are critically needed. Codlemone and butyl hexanoate are known to attract male and female of *C. pomonella* (Witzgall *et al.*, 2001; Hern & Dorn 2004) respectively. Attraction of adults of codling moths to semiochemicals; codlemone and butyle hexanoate, were evaluated in a specially designed one-way olfactory wind tunnel bioassay. This was then followed by the

integration of semiochemical blend that elicited optimum attraction of adults of *C. pomonella* and an entomopathogenic fungus spores in a semiochemical-baited-fungus-trap which could reduce the need for inundative application of conidia. Inundative spray application of EPF spores has some disadvantages; non-targeted species may be contaminated and spores exposed to unfavourable climatic conditions (U.S. Geological Survey, 1999).

The findings from this research project may lead to the identification of indigenous strains that are pathogenic to *C. pomonella* and the development of efficient delivery methods for entomopathogenic fungi for the control of *C. pomonella*.

1.2: Literature review

This study was focused on the isolation, pathogenicity of indigenous entomopathogenic fungi on *C. pomonella* and the integration of semiochemicals-baited trap and entomopathogenic fungal inoculums. The following aspects are discussed; the origin of codling moth and its distribution, host range, pest status, biology, monitoring and control strategies. Also, entomopathogenic fungi: their host range, mode of infection, distribution and their uses as myco-insecticides; and all the factors influencing their successful application are tackled. Finally, semiochemicals (codlemone and butyl hexanoate); their uses in Integrated pest management are reviewed.

1.2.1: Codling moth



Fig 1.1: The codling moth (source: www.gemplers.com/tech/icodmoth.htm. view; 11/08/2013)

1.2.1.1: Origin and dissemination

Cydia pomonella (Linn) (Lepidoptera: Tortricidae) is a plant pest of high economic importance in the world and South Africa in particular (Bakri, n.d.; Barnes, 1991). It was named “codling moth” by Wilkies in 1747 and was first recorded in Netherlands by Jean Goedaerdt in 1635 who termed it “pear eater” (Barnes, 1991).

C. pomonella is reported to have originated from Eurasia and spread throughout the northern and southern hemispheres but for Japan and Western Australia where the insect is said to have been eradicated (Barnes, 1991). Its distribution is influenced by food factors and the climatic conditions surrounding these regions (Shel-deshova, 1967).

The insect is spread by transportation of infected fruits and packaging materials. It is reported that the first account of the insect in South Africa was recorded in Graaff-Reinett in 1898 and was said to have been brought in by a tourist in infested apples (Luonsbury, 1898). Presently, it is a key pest in deciduous fruits orchards (Giliomee & Riedl, 1998)

1.2.1.2: Host range

C. pomonella is a pest to a variety of fruits including, nectarines, pears, quinces, walnuts, apricots, plums and peaches (Reidl, 1983; Barnes, 1991). However, apples are the primary host of codling moth (Barnes, 1991).

1.2.1.3: Pest status

Codling moth (Fig. 1.1) is ranked the fifth most important plant pest species in South Africa which is said to be the country with the highest infestation rate (Moran, 1983); and it was rated the third most important crop pest in 1994 (Bell & McGoech, 1996). Codling moth is presently regarded as the key pest of major concern in most deciduous fruits orchards in South Africa (Pringle *et al.*, 2003).

1.2.1.4.: Biology

The females lay 50-100 single eggs each, directly on the developing fruits or on leaves. When the larvae hatch from the egg, 5-18 days later, the caterpillar undergoes a wandering stage that takes 2 to 5 days (2.1). After a couple of bites, it penetrates a fruit where a second fruit or a leaf is touching (Bloemfield, 2003; Knight & Light, 2012). While in the fruit, the larvae pass through five stages during a period of 3-5 weeks, feeding on the immature seeds and the inside of the fruit (Bloemfield, 2003).

The last in-star comes out of the fruit, crawls down the tree, and hides in a cracks in the bark or a dark place to complete its growth (Higbee *et al.*, 2001). Its larvae build cocoons in their hiding places. It either pupates immediately or falls into diapauses. The pupating larvae then results in another generation of adults within the same season (Audema, 1991). He further states that, *C. pomonella* can have up to 4 generations per year, if the climatic conditions are favourable.

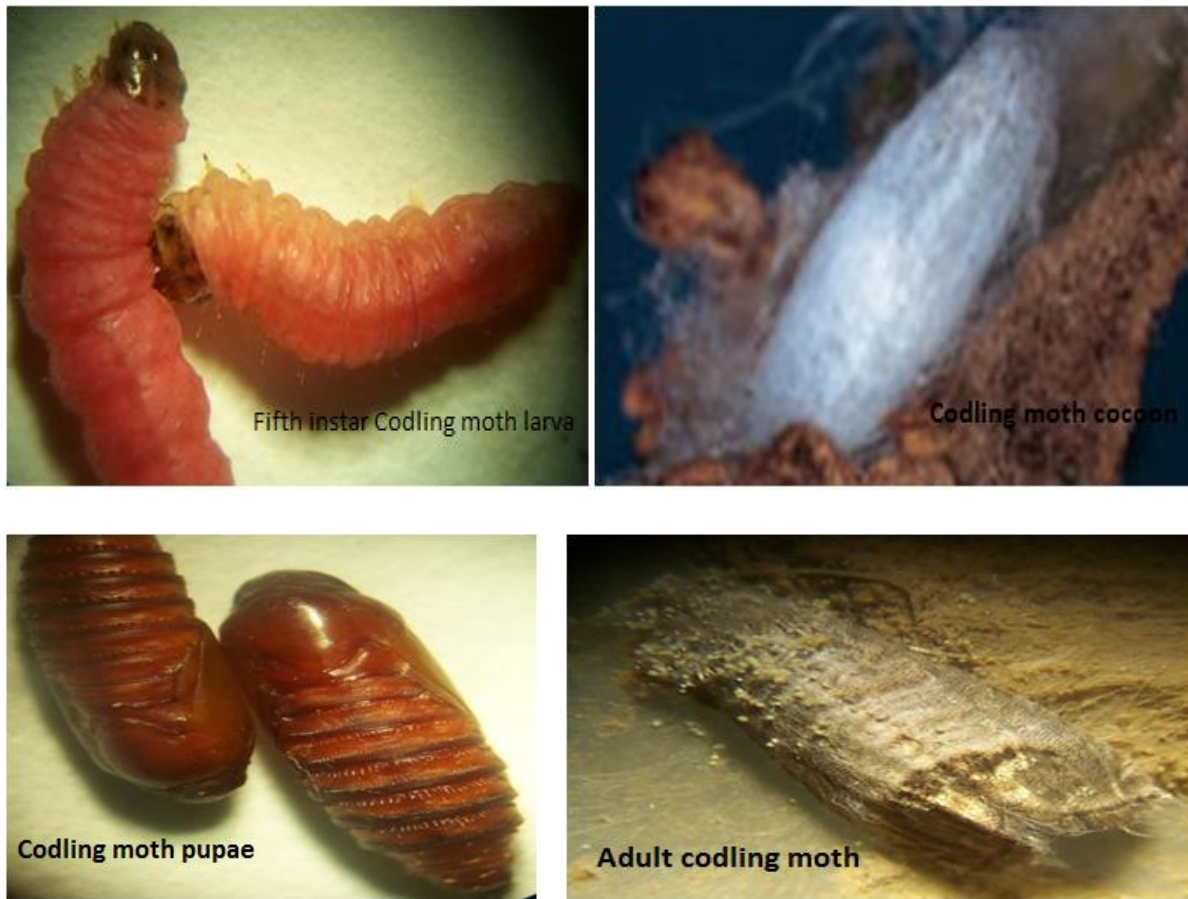


Fig. 2.1: stages of codling moth

1.2.2: Damage

Signs of codling moth damage on the fruit are distinctive entry holes (Fig. 3.1), which are filled with frass and surrounded by a dark reddish ring. Fruits infested by the first generation drops prematurely and those attacked later are damaged by nibbling on the surface, tunneling and soiling with frass and therefore, unmarketable. Bakri, (n.d) reported that, more than 95% of the crop may be damaged if trees are not protected from this insect.



Fig.3. 1: signs of codling moth damage on apple fruit (source: [http:// davesgarden.com / guides / articles / view](http://davesgarden.com/guides/articles/view); 08/10/2012)

1.2.3: Monitoring

Regular observations of Codling moth population numbers throughout the season can provide information for potential damage (Riedl *et al.*, 1998). This can be done by using pheromone baited and light traps to disrupt insect`s mating. Interrupting the mating process is the best way to preventing the cycle from being established. It can be understood that there would be a certain number of pupae in crakes of every apple tree in an orchard, which will hatch every spring since moths, cannot come into an orchard from other areas, as most pupae hatch close to where they fed as larvae.

Hence, disturbing this first hatching can have a remarkable effect on how many codling moths will be able to develop over the year. It is therefore important that, traps be put-up before any over wintering pupa hatches. Placing traps at the Wright times will cut down on the amount of eggs laid. Therefore, putting up traps early and replacing them during the season should be of crucial importance.

Fruits infestation assessment between second and fifth season and during seven seasons post-harvest is imperative (Bloemfield, 2003). Thus, information gathered from fruits infestation assessment is very important for the accurate indication of the extent of codling moth infestation in an orchard.

Although pheromone baited traps can serve as good indicator of the insect population in the orchard, farmers need to understand that, these traps cannot replace or completely control moth populations in the orchard. Therefore, it is crucial to integrate this strategy with other control programs.

1.2.4: Control

Currently, commercial farmers are reliant on chemical spraying such as organophosphates, which are the most used in South Africa for the control of codling moth (Riedl *et al.*, 1998). However, new legislation is reducing acceptable residue levels or even banning essential insecticides (Bakri, n.d). These synthetic chemicals have also raised some concerns for the environment, human safety and spreading of resistant population of codling moth (Bloemfield, 2003). Furthermore, Bakri, (n.d), reported that, the development and use of new and eco-friendly technologies for the control of insect is the only method of obtaining uncontaminated fruits presently.

Consequently, these concerns have moved researchers to develop alternative technologies for the control of *Cydia pomonella*, which are eco-friendly (Bloemfield, 2003). These alternative control methods include; the use of pheromone-base mating disruption, which is so far the most, used (Barnes, 1991; Pringle *et al.*, 2003). Unfortunately, this method is only effective when insect's population levels are low (Vickers & Rothschild, 1991). Problems of resistance and inadequate control are starting to arise in areas where chemicals have been replaced with other control techniques (Bakri, n.d). In addition, the sterile insect technique is only effective when

applied on an area-wide basis or integrated with other biocontrol methods (Bakri, n.d), though it is still in its growth stage in South Africa (Addison, 2005). The use of entomopathogens such as entomopathogenic nematodes, entomopathogenic viruses and entomopathogenic bacterial and entomopathogenic fungi (Osborne & Landa, 1992, Scholte *et al.*, 2004; Goble, 2009) have been suggested as ideal candidates to be incorporated into control strategies currently being developed (Bakri, n.d).

1.2.5: Entomopathogenic fungi

Entomopathogenic fungi (EPF) are fungal species that are parasitic to insects; these include *Beauveria* spp (Balsamo) and *Metarhizium* spp (Metschnikoff).

1.2.5.1: Mode of infection

Most EPF are soil borne fungal species and all have the same mode of infection (Goettel, 1995). When the fungal conidia encounter the host, they attach themselves to the cuticle through hydrophobic mechanisms and germinate to form germ tubes in favourable conditions (Inglis *et al.*, 2001). The germ tube penetrates the cuticle aided by the action of other enzymes such as, metalloid proteases and amino peptidases (Bidochka & Small, 2005). Once the fungi are in the insect, the mycelia grow in the host haemocoel and produces external conidia when the host dies (Inglis *et al.*, 2001).

Some EPF species, such as *Metarhizium* and *Beauveria*, have been reported to produce insecticidal peptides called destruxins and a toxic metabolite called oosporein (Goettel *et al.*, 1989; Inglis *et al.*, 2001). These strains are sought after for the development of bioinsecticides because they have a double action in killing their host while those that do not produce these only kill their host through hypha growth in the host, and take longer to kill (Bidochka & Small, 2005).

The insect's cuticle is its primary defense mechanism and plays a vital role in determining fungal specificity. If the fungus breaks this defense, infection will only occur if the fungus overcomes the natural immune response of the insect (Hoffman *et al.*, 1999). This response is in both cellular and hormonal behavior to fungal infection. Fungi can overcome host immune response by developing secret growth forms that are hidden from the insect's defense responses, or by producing substances that suppress the host defense system (Bidochka & Small, 2005).

1.2.6: EPF as myco-insecticides agents

The majority of fungi that naturally adjust insect populations in the field are of the orders Hypocreales and Entomophthorales (Dolinski & Lacey, 2007).

The use of microbial control agents particularly EPF, have been investigated for the control of a wide range of orchard and veterinary pests (Cross *et al.*, 1999; Puterka 1999; Alves *et al.* 2005; Castrillo *et al.*, 2005; Dolinski & Lacey, 2007; Lacey & Shapiro-Ilan, 2008; Nchu *et al.*, 2010). Current commercial entomopathogenic products in the markets being used to control a number of insects world wide include, "Cryptogram™" and Bb plus®, *Metarhizium* 50® (AgoBiocontrol), Biogreen® and Green Guard®, BIO 1020® and Green Muscle® (Bidochka & Small, 2005).

The use of fungi as insecticides has several advantages which include; their wide host range, eco-friendliness, they pose less threat to humans and other vertebrates than chemicals (Prior, 1996). some can be mass produced and applied by conventional methods and are compactable with most chemical pesticides as well as providing persistent control by means of multiplying in the pest population (viruses) (copping & Menn, 2000). Zimmermann, (2007), reported that the use of EPF as alternative control is safe for fish, reptiles, birds, amphibians and humans. In

addition, they do not contaminate underground water, as is the case with chemicals and are not pathogenic to non-targeted organisms (Thungrabeab & Tongma, 2007).

In addition to the environmental benefits, there are opportunities to optimize efficacy of entomopathogens; results from numerous studies have indicated that integration of entomopathogens and semiochemicals can optimize efficacy of entomopathogenic fungi (Huffaker, 1985). Thus, the use of technologies that will optimize insect's contact with virulent EPF is critical. Furthermore, selecting EPF strains, which are highly virulent, can withstand environmental temperature fluctuations, and produce destruxin and oosporein such as *B. bassiana* and *M. anisopliae* is an important consideration in the development of a commercial product. Inglis *et al.* (2001) stated that, EPF strains that come from hotter areas thrive better in hot weathers while those from cooler regions thrive better in cooler seasons. Hence, utilization of indigenous strains is preferable as they are adapted to the climatic conditions of the region. It is also important to select fungal strains that are compatible with other pesticides and can survive in the cropping systems for which they are required. The genera *Beauveria* and *Metarhizium*, which are also used in this study, are two of the most important genera of EPF widely used as fungal biological control agents and are from the order Hypocreales that as mentioned before, is one of the orders that naturally control insects in nature.

1.2.7: Genus *Beauveria*

Beauveria (Balsamo) Vuillemin (Ascomycota: Hypocreales) is an international genus of soil borne EPF, capable of infecting more than 700 species of arthropods. It was discovered in Europe as a disease of silkworms in the 18th and 19th centuries, called the white muscardine disease (Rehner, 2005) where its investigation was aimed at protecting cultivated insects such as silkworms and honey bees, (Lord, 2005).

Forty-nine species have been classified in the genus of which twenty-two are currently considered valued (Rehner, 2005).

1.2.7.1: Distribution

The genus is widely distributed making it easy to recognize. *B. bassiana* is thought to be sensitive to soil disturbance resulting from cultivation practices, and is therefore restricted to natural habitats such as the forest (Vänninen, 1995; Sánchez-Peña *et al.*, 2011) it can be easily isolated from insect cadavers or from the soil using simple media, antibiotics and selective agents (Gottel & Inglis, 1997). Furthermore, *Beauveria* can be easily mass-produced and formulated as a myco-insecticide (Rehner, 2005).

1.2.7.2: Identification

In spite of the fact that this genus has been extensively researched, and several complete taxonomic studies carried out, there are still problems with its nomenclature (Rehner & Buckley, 2005).

The genus can easily be distinguished morphologically; its most characteristic features are the whorled clusters of short globose to flask-shaped conidiogenous cells that create a chain of one-celled, sessile and hyaline conidia on an elongating “zigzag” rachis (Domsch *et al.*, 2007).

Beauveria species normally produce white colored mycelium and conidia in culture, though some isolates may produce yellow pigment in the older, central parts of the colony (Domsch *et al.*, 2007). Colony is fast growing, and the texture of the mycelium is characteristically woolly (Rehner, 2005). *Beauveria* conidial production can often be profuse, frequently creating a powdery appearance on the colony surface and some isolates may emit a red pigment into the culture medium (Rehner, 2005).

The conidial forms which vary from globose, ellipsoid, cylindrical and vermiform; and sizes from 1.8 to 6.0 μm are the only morphological features of the genus that has proven helpful for species description (Domsch *et al.*, 2007).

1.2.7.3: Host range

This genus is pathogenic to many economically important pests and its wide variation in virulence towards different insect hosts makes it one of the ideal candidates of EPF for the biological control of insect pests (Rehner, 2005).

1.2.7.4: Genus *Metarhizium*

Metarhizium (Metschnikoff) (Ascomycota: Hypocreales) is also a cosmopolitan genus of soil borne EPF. It was first isolated in the 1880s by Elie Metchnikoff, a Russian microbiologist, from his grain beetle research and named it the green muscardine fungus, *Entomophthora anisopliae* (Lord, 2005).

1.2.7.4.1: Identification

The genus is morphologically diverse based on the arrangement of the phialides, which bear chains, and columns of green, dry and slightly ovoid conidia (Zimmermann, 2007). *M. anisopliae* species complex include; *M. robertsii*, *M. guizhouense* and *M. pingshaense*. These species are morphologically identical and have been synonymised with *M. anisopliae* in the past, therefore difficult to differentiate based on morphological characteristics (Bischoff *et al.*, 2009). However, they are currently considered separate species and can be separated based on their elongation 1-alpha gene sequence.

Metarhizium robertsii belongs to a diverse class of filamentous ascomycetes known as pyrenomycete fungi that includes insect and plant pathogens (Moorhouse *et al.*, 1993; Freeman *et al.*, 2000). Sasan & Bidochka, (2012) reported that this species is not randomly distributed in soils but is associated with the plant root surface and endophytically colonizes switchgrass. *M. robertsii* has been studied and used as an insect pathogen for biocontrol (Milner, 1997).

1.2.7.4.2: Distribution

Metarhizium spp. are known to persevere in cultivated soils and is more frequently isolated from intensely disturbed soils (Rath *et al.*, 1992; Quesada-Moraga *et al.*, 2007; Sánchez-Peña *et al.*, 2011).

1.2.7.4.3: Host range

Metarhizium species have a wide range of virulence and are known to infect more than 200 different insect species, many of which are major agricultural pests, such as sugarcane stem-borers, scarab grubs and grasshoppers (Bidochka & Small, 2005). Furthermore, *Metarhizium* is capable of infecting insects from over 14 different orders including; Ephemeroptera and Dermaptera (Zimmermann, 2007).

1.2.8: Genus Aspergillus

Aspergillus flavus is used to describe a species as well as a group of closely related species. It causes infections in insects (Campbell, 1994), and diseases in agricultural crops, such as maize and peanuts (Hedayati *et al.*, 2007). This species is abundant in the air and is said to be the leading cause of invasive *aspergillus* that causes superficial infections in humans after *A. fumigatus*. *A. flavus* produces aflatoxins, and is said to be the most toxic and potent hepatocarcinogenic natural compounds ever characterized (Hedayati *et al.*, 2007). The *A. flavus* complex currently includes 23 species including two sexual species. The primary branches are up to 10 mm in length, and the secondary up to 5 mm in length. Conidia are typically globose to subglobose, conspicuously echinulate, varying from 3.5 to 4.5 mm diameter. However, due to their common morphological and biochemical characteristics, identification of species within the *A. flavus* species has been difficult and much taxonomic and genetics work needs to be done.

1.2.9: Genus *Purpureocillium*

Purpureocillium lilacinum (Thom) (previously *Paecilomyces lilacinus*) is a hyphomycete with an omnipresent distribution. It has been abundantly isolated from cultivated and uncultivated soils, nematode eggs, forest, deserts, sewage sludge, insects (Luangsa-ard *et al.*, 2011) and decaying vegetable matter as a saprobe as well as rhizosphere of some crops (De Hoog *et al.*, 2000). *P. lilacinum* is the only species in this genus (Luangsa-ard *et al.*, 2011); it causes a wide spectrum of clinical symptom in immunocompromised and immunocompetent individuals (Antas *et al.*, 2012) and insects (Luangsa-ard *et al.*, 2011).

1.2.9.1: Distribution and identification

The species forms dense mycelium, which is white at first, then changes to shades of vinaceous. The conidiophores bears phialides from whose tips chains of spores are formed. The hyphae are smooth-walled and translucent. The conidia are in divergent chains, ellipsoid to fusiform in shape. The walls are smooth to rough (Luangsa-ard *et al.*, 2011). The species is closely related to *Nomuraea atypicola* and *Isaria takamisusanensis* (sung *et al.*, 2007)

1.2.10: Factors influencing EPF application

Fungal genera with broad host ranges, such as *Beauveria* and *Metarhizium* are always present in the soil, are facultative and are principal candidates for inundative biological control of insect pests (Goettel, 1995). However, the application of EPF as bio-insecticides is influenced by a variety of factors including; fungal host range and the specificity of fungal pathogens, for example: the fungal strain, the host physiological state, nutrition, host defense mechanisms (Inglis *et al.*, 2001). Further, epizootics development is controlled by a variety of environmental factors and is influenced by the distribution of both the host and EPF populations (Goettel, 1995). One of the most important concepts however, is that, stressed insects are more susceptible to fungi than non-stressed insects (Inglis *et al.*, 2001). Another important factor is the

developmental stage of an insect pest as not all stages in an insect's life cycle are equally susceptible to fungal infection. Variation in susceptibility by insect growth stages is probably related to loss of infection through ecdysis, as the insect molts during instar development, so it may lose fungal inoculi as the cuticle is removed (Vestergaard *et al.*, 1995)

Insect density is also very important for the development of epizootics, as insect density increases there are higher chances of insects being exposed to infected individuals. The behavior of insects can also greatly affect the persistence of fungal disease, for example, grooming behavior in termites can auto-transfer the conidia of EPF to other individuals (Inglis *et al.*, 2001), but this behaviour can also remove inoculums and limit the spread of disease and instill resistance in some social insect groups (Yanagawa *et al.*, 2009).

1.2.10.1: The environment

Environmental factors such as solar radiation, temperature, rainfall and wind interact with each other and have remarkable effects on the efficacy and stability of EPF in the field.

1.2.10.1.1: Solar radiation

The effect of irradiance is one of the most important environmental factors for the persistence of EPF fungal spores; exposure to sunlight for even a few hours, particularly the UV-B portion of the spectrum (285 - 315 nm) can completely inactivate the infectious conidia of *Metarhizium* (Braga *et al.*, 2001). However, there are significant differences in susceptibility to irradiation among fungal strains and species, and mechanisms for resistance against irradiation should be considered when selecting candidate EPF for product formulation (Inglis *et al.*, 2001). Much research has focused on enhancing conidial persistence in the field by using solar blockers or UV-B absorbing substances in the form of Sunscreens such as, clay and optical brighteners such as Tinopal™ or stilbene (Ibargutxi *et al.*, 2008).

1.2.10.1.2: Temperature

Ambient temperature influences the rate of insect infection and may restrict the use of EPF in biological control. Most EPF can grow at a range of temperatures but grow optimally at temperatures between 25 and 35°C. Species such as *Beauveria bassiana* can grow at a wide range of temperatures (8 to 35°C) with a maximum threshold of 37°C (Fernandes *et al.*, 2008). Selecting EPF fungal strains that can withstand environmental temperature fluctuations is an important consideration in the development of a commercial product and often, as mentioned before, strains that come from hotter areas perform better at higher temperatures and likewise those that come from cold climates perform better at lower temperatures (Inglis *et al.*, 2001). Thus, selecting commercial fungal strains that obtain better infection at higher temperatures or the other way round should be considered.

1.2.10.1.3: Relative humidity

Moisture is a very important factor for the persistence and germination of conidia in nature. Conidiogenesis on insect surfaces requires high moisture (Inglis *et al.*, 2001), if this is low, conidia production slows and the horizontal transmission of spores is reduced, slowing the transfer of infection. Studies have shown that dry condition after the application of EPF are less harmful; and that if there was sufficient moisture within the microhabitats, the spore will be able to germinate and effectively infect insects at low ambient humidity, as was the case with *Beauveria* and *Metarhizium* strains (Inglis *et al.*, 2001). The requirement for high environmental moisture was lessened with the breakthrough technology of formulating fungal spores in oil (Bateman & Alves, 2000). The conidia remain active inside the oil droplets and research now concludes that oil enhances the insect infectivity at low humidity levels (Inglis *et al.*, 2001).

1.2.10.1.4: Rainfall

Precipitation plays a vital role in the dispersal of conidia from substrates, but there is strong evidence to suggest that significant amounts of fungal conidia may be removed from the insect

cuticle during rainfall despite the hydrophobic mechanisms of conidial adhesion (Inglis *et al.*, 2000).

1.2.11: Compatibility of fungi with agricultural chemicals

It is important for farmers to be aware that EPF will not replace chemical pesticides in all commercial crop production systems because EPF may not target all insect pests, in which case chemical might be needed to speedily reduce expanding insect populations (Inglis *et al.*, 2001).

1.12: Semiochemicals

In order to surmount the challenges associated with the use of EPF as bio-insecticides, the development of strategies that increase contamination, reduce time interval before contact and reduce effects of varying climatic ranges is crucial. Fortunately, semiochemicals, particularly, can enhance efficacies of entomopathogens by attracting targeted insect pests to infective conidia (Thungrabeab & Tongma, 2007).

Semiochemicals, from *semeion* (in Greek) or signal, are chemicals released by living organisms (plants, insects, humans, etc.) that induce a behavioural or a physiological response in other individuals. These compounds can be classified in two groups considering whether they act as intraspecific (pheromones) or interspecific (allelochemicals) mediators. Allelochemicals include allomones (emitting species benefits), kairomones (receptor species benefits) and synomones (both species benefit). However, a single chemical signal may act as both pheromone and allelochemical. There are different types of pheromones according to the response they induce on the perceiving individuals (Cork, 2004). The most common are female sex pheromones, produced by females of a species in order to attract males of the same species for mating.

Kairomones and pheromones are some of the semiochemicals being exploited, extensively for control of *C. pomonella* under field conditions. Experiments by Huang *et al.* (2010) demonstrated that combining appropriate blend(s) of codlemone produced by female codling moth and buytl hexanoate produced by fruits (apple) *could* attract male and females as well as

the immature stage (larvae) of codling moth to control traps. The larvae of *C. pomonella* spends a relatively short period outside the host (2 - 3 days) (Bloemfield, 2003), thus the use of technologies that will shorten contact time among insect and virulent EPF is critical.

1.3: Aims of the study

The aims of the study were to isolate and identify virulent entomopathogenic fungal strains from soils obtained from selected locations in the Western Cape region of South Africa and to evaluate the prospects of integrating semiochemical and entomopathogenic fungal spores for control of *C. pomonella* (codling moth).

1.4: The specific objectives of the study were:

- Isolate and culture strains of entomopathogenic fungi from soils originating from selected locations of Western Cape region, South Africa
- Determine mortalities induced by different EPF isolates on larvae of *C. pomonella* *in vitro*
- Evaluate the effect of a virulent EPF strain on hatchability of eggs of *C. pomonella*
- Establish the attraction responses of adults of *C. pomonella* to individual semiochemical (codlemone and butyl hexanoate) blend in a one-choice olfactory wind tunnel bioassay.
- Evaluate the effects (spore contamination, repellency and mortality) on adults of *C. pomonella* following the integrated use of fungal spores with a semiochemical blend in wind tunnel bioassay.
- Assessing the effect of fungus treatment on *C. pomonella* larval infestation of apple fruits *in vitro*

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CHAPTER TWO

Isolation and pathogenicity of South African strains of entomopathogenic fungi (Ascomycota) on *Cydia pomonella* (Lepidoptera: Tortricidae)

ABSTRACT

There is a growing interest in isolating entomopathogenic fungi from different parts of the world where they are climatically adapted, suited to and have potentials to control insects in those areas. The current chapter focuses on the isolation and identification of virulent entomopathogenic fungal strains against *Cydia pomonella* (Lepidoptera: Tortricidae) in the Western Cape region of South Africa. Soil samples were collected from 10 locations in the Western Cape, South Africa and sampled for the presence of entomopathogenic fungi (EPF) by baiting the soil samples with 5th instar larvae of codling moths. Fungal strains were identified and characterized using light microscopy and DNA analysis (internal transcribed spacer region) and elongation factor 1- α (EF 1- α) genes of fungal cultures. Thirty-nine (39) isolates belonging to six species were obtained; *Purpureocillium lilacinum* (8 isolates), *Fusarium oxysporum* (five isolates), *Fusarium polyphialidicum* (two isolates), *Beauveria pseudo-bassiana* (one isolate), *Aspergillus flavus* (three isolates) and *Metarhizium robertsii* (20 isolates). Generally, *M. robertsii* was the most frequently encountered species representing 51% of the total number of isolates collected from the soil samples. This is the first report of the isolation of *M. robertsii* in southern Africa. A screening test to identify the most virulent fungal strains against codling moth was carried out on 39 isolates by immersing 5th instar larvae of *C. pomonella* into aqueous spore suspension 1×10^8 conidia ml⁻¹. Mortality data from the immersion bioassay indicated that the 39 fungal isolates were pathogenic against 5th instar larvae of codling moth inducing 47-85% insect mortality at an infective concentration of 1×10^8 spores ml⁻¹, 5 days post-treatment. Two fungal strains MTL151 and GW 461 induced over 85% larval mortality and were selected for further evaluations. The effect of MTL151 and GW461 on egg hatchability of 0-day old eggs

was evaluated by exposing freshly laid eggs on wax papers that were pre-treated with fungal spores ranging from 10^3 - 10^8 spores ml^{-1} . Egg hatchability reduced significantly from 93-71% (GW461) and 95-66% (MTL151) as spore concentration increases from 1×10^3 to 1×10^8 spores ml^{-1} , respectively. The highest spore concentration significantly inhibited egg hatchability resulting in lower egg hatchability compared to that obtained with the cypermethrin containing commercial insecticide (Fruitfly [registered by Kombat (PTY) Ltd]) tested at the recommended dose of 0.25 ml / 250 ml of water. The potential of the two *M. robertsii* strains to protect apples from infestation by codling moth neonates was assessed in an apple fruit bioassay under laboratory and field bioassay. Codling moth neonates were exposed to apples that were sprayed topically with varied conidia concentrations (10^3 - 10^8 spores ml^{-1}). The mean number of participating apple fruits having developing/developed larvae in the core/flesh significantly reduced from 5.3 to 1 and 7.6 to 1 for MTL151 and GW461, respectively as spore concentrations increased from 1×10^3 to 1×10^8 spores ml^{-1} . A concurrent decrease in apple fruit rot as conidia concentration increased was observed. Up to 90% of apples treated with 1×10^8 spores ml^{-1} had no larva present in their cores and this result compared favourably with the commercial pesticide (Fruitfly) used at a recommended dose of 0.25g / 250 ml of water. No significant difference was found between the EC_{50} values of 1.2×10^3 ($\text{CI}=1.2 \times 10^2$ - 1×10^5) (MTL151) and 1.1×10^5 ($\text{CI}=7 \times 10^3$ - 5.6×10^5) (GW461) spores ml^{-1} . However, neither the two *M. robertsii* strains nor the insecticide deterred the neonates from feeding on the surface of the fruits.

This study demonstrated that fungal strains occurring in the Western Cape region may protect apple fruit from codling moth infestations.

Keywords: Entomopathogenic fungi, *Metarhizium robertsii*, codling moth larvae, biological control, and indigenous.

2.1: Introduction

Codling moth (*Cydia pomonella*) (Lepidoptera: Tortricidae) infest pomes resulting in high production losses to fruit farmers in South Africa and globally. Its infestation in South Africa is the highest in the world (Pringle *et al.*, 2003). Newly hatched first-instar larvae penetrate and tunnel into the core of fruits where they feed, develop and damage the fruits in the process and exit the fruits as 5th instar larvae to seek a location to pupate. Farmers are heavily reliant on chemical pesticides for the control of this insect (Riedl *et al.*, 1998). Fruit-seeking 1st and 5th larval instars of *C. pomonella* are the prime targets for suppression because once a larva has penetrated an apple fruit, it is protected from pesticides. Increased risks of pesticide resistance and environmental contamination associated with the current methods of control of Arthropod pests have resulted in the increased search for alternative control methods, including biocontrol agents such as entomopathogenic fungi (EPF) (Osborne & Landa, 1992; Sauphanor *et al.*, 1998; Reuveny & Cohen, 2004; Scholte *et al.*, 2004; Goble, 2009).

Entomopathogenic fungi occur in vast arrays of ecosystems, however, very few studies have focused on the isolation, characterization and ecology of fungal strains that are pathogenic against common agricultural and horticultural pests in South Africa (Goble, 2009). Entomopathogenic fungi may regulate and suppress insect populations (Ali-Shtayeh *et al.*, 2002). Members of the genera *Metarhizium* and *Beauveria* have shown great potential for control of Arthropod pests in large-scale field experiments (Hatting *et al.*, 2010; Stafford & Allan, 2010). The facultative ability of some EPF makes them ideal candidates for bio-pesticides; they can adapt and persist in the soil until host becomes available in case of host scarcity in a particular habitat. They possess other qualities that make them ideal microbial biological control agents which include; wide host range, eco-friendliness, can be applied even when crop is almost ready and it is believed they pose less threat to humans and other vertebrates than chemicals pesticides (Prior, 1996). Entomopathogenic fungi are available commercially around the globe in different trade names, for examples; Green muscle® and *Metarhizium* 50® from *Metarhizium Anisopliae* (Ascomycota: Hypocreales), Naturalist®, Mycotal®, Bb plus® and Botanigard® based on *B. bassiana* and Vertalec® (*Lecanicillium longisporum*) (Ascomycota: Hypocreales) (Bidochka & Small, 2005; Zimmermann, 2007; Khan *et al.*, 2012). There has been an increase in research activities that focus on the use of

entomopathogenic fungi (*Beauveria* spp. and *Metarhizium* spp. [Hypocreales]) against arthropod pests (Khan *et al.*, 2012). The search for virulent fungal strains with potential to control codling moth infestations in orchards are needed to reduce growers` use of chemical insecticides.

Hypocreales fungi adapt well to their local climatic conditions. Often, strains that come from hotter areas perform better at higher temperatures and likewise those that come from cold climates perform better at lower temperatures (Inglis *et al.*, 2001). Consequently, there is growing interest in isolating entomopathogenic fungi from different parts of the world where they are climatically adapted and suited to and have potentials to control insects in those areas. Although it is important to identify very infective and virulent strains of EPF to control pests, it is more important to look for isolates that are also capable of surviving and persisting in the environments and habitats that are being targeted for insect control. Hence, the objectives of this study were to isolate indigenous soil-borne entomopathogenic fungal strains from soil samples of the Western Cape region of South Africa and to assess their pathogenicity on immature stages (larvae and freshly laid eggs) of *C. pomonella* under laboratory conditions.

2.2: Materials and methods

2.2.1: Soil collection sites

Soil samples were collected from disturbed sites such as roadsides and farm margins. Three (3) points from each location were sampled. Garmin Global Positioning System, Geographic Information Systems (GIS) and Google Earth Maps used to locate sites. Soils were sampled from the following locations (Fig. 1) during winter. The soil samples were transported to the laboratory for fungal isolation using the insect bait technique.

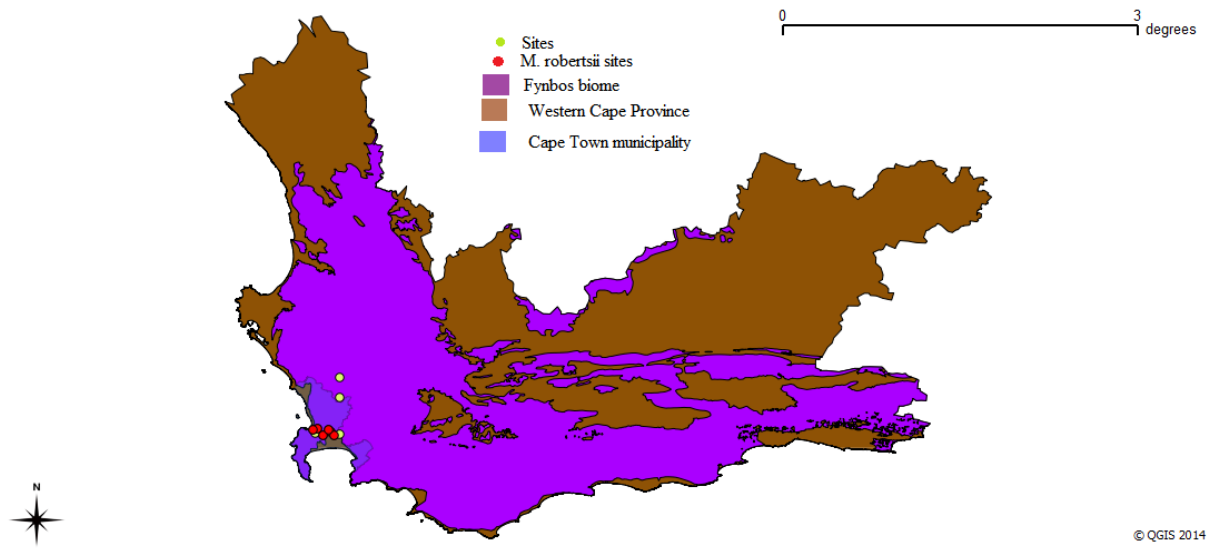


Fig. 1.2: Soil collection sites

2.2.2: Insects

Insects (1st and 5th instars larvae of *C. pomonella*) were collected from Entomon PTY Ltd. Larvae were delivered on artificial diet mixture and were stored in the laboratory at 4°C until their use. The diet mixture was based on the work described by Stenekamp (2011). (Water 76-78%, Agar Agar (food grade yelling agent) 0.64-068%, Carrageennan (food grade yelling agent) 0.48-051%, Whole Wheat or Wheat flour 9.5-14% (relative to sawdust/woodchips), Wheat Germ 3.56-3.67%, Brewer's Yeast 3.8-3.9%, Benzoic Acid 0.24-0.25%, Ascorbic Acid 0.51%, Methyl Paraben (Nipigen) 0.18-0.19%, Formaldehyde 0.16%, Calco Red (die) + Canola Oil 0.08%, Sawdust/Woodchips (*Pinus* sp.) 0-4.75%).

2.2.3: Insect bait technique

Soil sampling and isolation of fungus were based on the methods of Goettel & Inglis (1997) and Meyling (2007). Soils (500 g) were sieved through a metal sieve with a mesh size of 4 mm.

Samples were subsequently transferred into transparent plastic pots with perforated lids labeled with soil collection sites. 5th instar larvae of codling moth were placed on the surface of each soil sample container and incubated in the dark at 25°C. Containers were turned upside down daily for the first week to increase contact between insects and soil particles. Samples were checked for dead larvae every 3-4 days for three weeks. Dead larvae were surface-sterilized with 70% ethanol followed by incubation in a moisture chamber (Petri dish with moistened filter paper) at 25°C. Sporulating larvae were placed on a selective media; Sabouraud Dextrose Agar [(SDA), Merck] supplemented with “Penstrep” (10,000 units of Penicillin / 10 mg of streptomycin) at 2 ml / L of media.

2.2.4: Isolation and identification of fungi

Monocultures were made on the selective media; Sabouraud Dextrose Agar [(SDA), Merck] supplemented with “Penstrep” (10,000 units of penicillin / 10 mg of streptomycin) at 2 ml / L of media. Initially, the fungal cultures were examined for morphological characteristics (conidia and colony characteristics) in the Plant Science Laboratory, Cape Peninsula University of Technology using light microscopy technique. Thirty-nine (39) fungal cultures were transferred to the Molecular Biology Laboratory in the Department of Microbiology, Stellenbosch University for a more precise morphological and molecular characterization of the fungal strains. All strains were plated on Malt Extract agar (MEA), Czapek Yeast agar (CYA) and Oatmeal agar (OA) for initial grouping. Representative strains were selected from each group and used for taxonomic characterization. The morphology was determined from structures mounted in lactic acid, using a Nikon Eclipse E800 light microscope. For molecular characterization, DNA was extracted from fresh cells using the ZR Fungal/bacterial DNA kit (Zymo Research,

California, USA) and the presence of genomic DNA was checked on a 1 % agarose gel, stained with ethidium bromide. PCR reactions were done using a GeneAmp PCR System 9700 (Applied Biosystems, USA). The reaction mixture contained 0.5 μ l (\pm 50 ng/ μ l) of the purified genomic DNA, 500 nM of each primer and 5 μ l of 2X Kapa Taq Ready mix (Kapa Biosystems, South Africa) in a total volume of 10 μ l. Gene regions used for the comparisons included the internal transcribed spacer region (ITS) (ITS1 [forward] ITS4 [reverse]) (White *et al.*, 1990), calmodulin (CMD5 [forward] CMD6 [reverse]) (Hong *et al.*, 2006) and elongation factor 1- α (EF 1- α) genes (EFF [forward] EFR [reverse] (Jacobs *et al.*, 2004). The PCR conditions consisted of initial denaturing step at 94° C for 10 min, followed by 36 cycles of 94° C for 30 s, 56° C (ITS)/ 53° C (EF) for 30 s and 72° C for 1 min. The reaction was completed with a final extension for 7 min at 72° C and then cooled and held at 4° C. PCR samples were separated on a 1% agarose gel, stained with ethidium bromide and visualized using ultraviolet light. The amplicons from the PCR reactions were run on an ABI 3010xl Genetic Analyser Sequences were blasted against the NCBI Genbank database and trees were compiled using ClustalX for the alignment and PAUP for the analysis. Distance analyses using the neighbour joining were performed and the strength of the branches was calculated with a 1000 bootstrap repetitions. Fungal cultures are kept in the germplasm of CPUT and Department of Microbiology, University of Stellenbosch.

Spores from fungal monoculture of each of the fungal strains were harvested and surfaced-cultured on PDA (Potato dextrose agar) in 9 cm diameter Petri dishes at $25 \pm 2^\circ\text{C}$; $70 \pm 2\%$ RH for 3-4 weeks. The viability of conidia was determined by spread-plating 0.1 ml of conidia suspension, titrated to 3×10^6 conidia ml^{-1} on PDA plates. Two replicated sterile microscope cover slips were placed on each plate and incubated at $26 \pm 2^\circ\text{C}$. Plates were then examined after 24 h and percentage germination determined from 100-spore counts under each cover slip. The germination percentage was 90% and 95%.

2.2.5: Immersion Bioassay

A dipping bioassay was used to quickly assess the virulence of each of 39 fungal isolates collected from the sampled soils in order to identify the most virulent strains for further evaluations. All isolates were tested at a standard infective concentration of 1.0×10^8 conidia ml^{-1} in 0.05% Tween 80 and sterile distilled water. Insects (5th instar larvae) were dipped in 2 ml of aqueous conidia formulation for 30 seconds. Controls were treated with sterile distilled water containing 0.05% Tween 80. Ten (10) treated insects were placed in 9 cm diameter Petri-dishes with perforated sides and fed on artificial diet; and incubated at $22 \pm 2^\circ\text{C}$, $60 \pm 2\%$ RH and 10:14 (L: D) each treatment was replicated 6 times. Laval mortality was recorded at 5 days post-treatment and mortality data was Abbott-corrected (Abbott, 1925). In order to determine the cause of mortality, dead insects were surfaced sterilized in 70% ethanol and incubated at $22 \pm 2^\circ\text{C}$ and $90 \pm 5\%$ RH. Mycosis was later checked under a dissecting microscope

2.2.6: Effects of EPF strains (MTL151 and GW461) on freshly-laid eggs and 1st instar larvae of *C. pomonella*

2.2.6.1: Anti-hatchability bioassay

The anti-hatchability effect of *M. robertsii* strains MTL 151 and GW 461 on 0-day old egg. A modified bioassay described by [24] was used [24]. Five (5) millilitres of aqueous spore suspensions 1.0×10^3 , 1.0×10^5 , or 1.0×10^7 conidia ml^{-1} prepared using sterile distilled water, 0.05% Tween 80 and spores from the *M. robertsii* strains (MTL151 and GW461). In the control, wax papers were treated with 5 ml of sterile distilled water containing 0.05% Tween 80. The commercial contact and stomach insecticide (Fruitfly®) registered by Kombat PTY Ltd., which contains the active ingredient cypermethrin (20 g/l) and supplied by Starke Ayres PTY Ltd. was used as a positive control, and was tested at the recommended dose of 0.25 ml per 250 ml of water containing 0.05% Tween 80. The treated wax papers were allowed to dry at 20°C and $55 \pm 5\%$ RH for 1 hour, and were transferred into plastic tubs (10 cm [diameter] by 15 cm [height]). The fungus or control- treated wax paper was laid at the bottom of the tub with the treated

surface facing up. Five (5) unmated males and five (5) female moths were released in each plastic tub lined with fungus-treated wax paper for 1 day and insects were removed from the tubs and the number of eggs on wax paper strips was determined using a dissecting microscope. Thereafter, the wax papers were transferred into clean tubs and maintained at room temperature for 6 days to allow the eggs to hatch. Each tub was covered with a perforated plastic lid. There were 10 replicates per treatment. The number of eggs that successfully hatched was recorded under a dissecting microscope. Percentage hatchability were determined and compared among treatments.

2.2.6.2: Feeding deterrent and larval development activities of *M. robertsii* strains MTL151 and GW 461 on *C. pomonella* in whole apple fruit bioassay

M. robertsii strains (MTL151 and GW461) at a concentration of 1×10^8 conidia m^{-1} in water containing 0.05% Tween 80 was sprayed on fresh ripe “granny-smith” apple fruits. The circumference of participating apple fruits ranged from (18.5-20 cm). The stalk held each fruit and using a pressurized hand-held garden sprayer, 4 ml of spore suspension was applied on the surface of the fruit while ensuring complete spray coverage of the fruit was attained. In the negative control treatment, whole apple fruits were treated with sterile distilled water containing 0.05% Tween 80. The commercial contact and stomach insecticide (Fruitfly® registered by Kombat (PTY) Ltd.), which contains the active ingredient cypermethrin (20 g/l) and supplied by Starke Ayres PTY Ltd. was used as a positive control, and was tested at the recommended dose of 0.25 ml per 250 ml of water containing 0.05% Tween 80. Each of the control or fungus-treated fruits was placed separately in a perforated plastic tub for air circulation. Each fruit was placed adjacent to a piece of wax paper (surface area; 2-4 cm^2) containing ten (10) 3-day old codling moth eggs in a plastic tub. The number of eggs on wax paper strips was determined using a dissecting microscope. The apple fruit lures newly hatched *C. pomonella* larvae (Sutherland, 1972). The plastic tubs were housed in a climate-controlled room under the following conditions; $28 \pm 2^\circ C$, $70 \pm 2\%$ RH and 10:14 (L: D). Data was recorded at 21 days post-treatment. Feeding deterrent activity was assessed by counting fruits with surface wounds and stings. Anti-larval development activity was assessed by cutting open the fruit through the middle and the presence or absence of developing/developed larva (e) in the flesh or core of

apples exposed to the various treatments was captured as the number of fruits with larva (e). The number of apples with fruit rot disease in each treatment was also recorded at 21 days after treatment. For each treatment, 10 fruits corresponding to ten replicates were used. The experiment was repeated three times.

Statistical analysis

Percentage larval mortality in the quick screening bioassay was Abbott-corrected (Abbott, 1925) and the data were analyzed using one-way analysis of variance (ANOVA). Egg hatchability (%) among treatments was compared using one-way ANOVA. The effective concentrations (EC₅₀) needed to inhibit 50% larval development of 1st instar larval neonates in treated apple fruits were determined using probit analysis (EPA). The numbers of infested apples in test and control treatments were assessed using one-way ANOVA. Means were separated by Tukey's pairwise comparisons test. The statistical level of significance was fixed at P = 0.05 and analysis of variance was performed using Statistica (StataSoft, 2013).

2.3: Results

2.3.1: Isolation of fungi

Thirty-nine (39) indigenous isolates belonging to 6 entomopathogenic fungal species were obtained from sampled soils using the insect bait technique. The most prevalent species collected from the soil using the insect-bait method was *M. robertsii* (20 isolates) followed by *Purpureocillium lilacinum* (8 isolates), *Fusarium oxysporum* (5 isolates), *Aspergillus flavus* (3 isolates), *Fusarium polyphialidicum* (2 isolates) and 1 *Beauveria pseudo-bassiana* isolate.

Generally, there was a significant difference (DF =5; $X^2=36$; $P<0.001$) in the number of isolates collected among the 6 fungal species (Table 1).

Table 2.1: Sampling locations of soils that were baited with 5th instar larvae of *C. pomonella* for isolation of entomopathogenic fungi

Location	Coordinates <i>Lon; lat.</i> (Decimal degree)	<i>M.</i> <i>robertsii</i>	<i>F.</i> <i>oxysporum</i>	<i>P.</i> <i>lilacinum</i>	<i>A.</i> <i>flavus</i>	<i>B.</i> <i>pseudobassiana</i>	<i>F.</i> <i>polyphialidicum</i>
CPUT Bellville	18.64;-33.93	4	0	2	0	0	1
Kuilsriver	18.67;-33.96	5	1	0	0	0	0
Stellenbosch	18.72;-33.95	0	0	1	1	0	0
Nootridargh	18.58;-33.96	2	0	1	0	0	0
Pinelands	18.51;-33.94	0	0	0	1	0	0
Bellville	18.63;-33.91	4	1	0	0	0	0
Goodwood	18.53;-33.90	2	0	1	1	0	1
Maitland	18.49;-33.92	3	1	0	0	0	0
Malmesbury	18.72;-33.47	0	0	3	0	1	0
Swartland	18.72;-33.64	0	2	0	0	0	0

There was a significant difference (DF =5; $X^2 = 46.3$; $P < 0.001$) in the number of isolates collected among the 6 fungal species

2.3.2: Immersion bioassay

Results from the immersion bioassay showed that all the isolates were pathogenic against codling moth 5th instar larvae inducing significantly (DF = 38, F = 12.12, P <0.001) varied mortalities (46 – 85%) among the fungus treated insects (Table 2). However, strains of *M. robertsii* (GW461 and MTL151), *A. flavus* (GW417) and *P. lilacinum* (GW482) were the most pathogenic causing 85, 81 and 82% mortalities among the larvae, respectively while the least pathogenic strain was *M. robertsii* (B537), which induced 46% mortality among 5th instar larvae at 5 days post treatment (Table 2.2).



Fig 2.2: Mycosis on fifth instar codling moth larval cadaver

Table 2.2: Abbott-corrected mean mortality \pm SE (%) of 5th instar larvae induced by indigenous isolates of entomopathogenic fungi following immersion in aqueous conidial suspension (1×10^8 spores ml⁻¹) at 5 days post treatment.

Collection site	Isolate number	Species	Abbott-corrected mean mortality (\pm SE)(%)
Maitland	MTL 111	<i>Metarhizium robertsii</i>	84 (0.37)
Maitland	MTL 151	<i>Metarhizium robertsii</i>	85 (0.37)
Maitland	MTL 121	<i>Metarhizium robertsii</i>	82 (0.32)
Maitland	MTL 211	<i>Fusarium oxysporum</i>	57 (0.52)
CPUT Bellville	CPUT 271	<i>Purpureocillium lilacinum</i>	54 (0.62)
CPUT Bellville	CPUT 221	<i>Metarhizium robertsii</i>	58 (0.42)
CPUT Bellville	CPUT 274	<i>Metarhizium robertsii</i>	62(0.43)
CPUT Bellville	CPUT 232	<i>Metarhizium robertsii</i>	57(0.32)
CPUT Bellville	CPUT 273	<i>Fusarium polyphialidicum</i>	67 (0.32)
CPUT Bellville	CPUT 911	<i>Purpureocillium lilacinum</i>	70 (0.40)
CPUT Bellville	CPUT 278	<i>Metarhizium robertsii</i>	56 (0.52)
Goodwood	GW 417	<i>Aspergillus flavus</i>	81(0.35)
Goodwood	GW 416	<i>Metarhizium robertsii</i>	71(0.67)
Goodwood	GW 461	<i>Metarhizium robertsii</i>	85(0.29)
Goodwood	GW 482	<i>Purpureocillium lilacinum</i>	82 (1.27)
Goodwood	GW 483	<i>Fusarium polyphialidicum</i>	78 (0. 30)
Malmesbury	MALMES421	<i>Beauveria pseudobassiana</i>	56 (0.33)
Malmesbury	MALMES 211	<i>Purpureocillium lilacinum</i>	57 (0.32)
Malmesbury	MALMES 231	<i>Purpureocillium lilacinum</i>	65 (0.29)

Table 2.2 cont: Abbott-corrected mean mortality \pm SE (%) of 5th instar larvae induced by indigenous isolates of entomopathogenic fungi following immersion in aqueous conidial suspension (1×10^8 spores ml⁻¹) at 5 days post treatment.

Collection site	Isolate number	Species	Abbott-corrected mean mortality (\pm SE)(%)
Malmesbury	MALMES 411	<i>Purpureocillium lilacinum</i>	64 (0.37)
Pinelands	PL1312	<i>Aspergillus flavus</i>	77 (0.27)
Swartland	SWTL 111	<i>Fusarium oxysporum</i>	77 (0.36)
Swartland	SWTL 331	<i>Fusarium oxysporum</i>	77 (0.40)
Bellville	B 711	<i>Metarhizium robertsii</i>	73 (0.40)
Bellville	B 742	<i>Metarhizium robertsii</i>	63 (0.40)
Bellville	B 537	<i>Metarhizium robertsii</i>	46 (0.28)
Bellville	B 721	<i>Metarhizium robertsii</i>	71(0.20)
Bellville	B 241	<i>Fusarium oxysporum</i>	75 (0.37)
Nootrodargh	NT 881	<i>Purpureocillium lilacinum</i>	65 (0.29)
Kuilsriver	KR 111	<i>Metarhizium robertsii</i>	75 (0.37)
Kuilsriver	KR 511	<i>Fusarium oxysporum</i>	76 (0.28)
Kuilsriver	KR 133	<i>Metarhizium robertsii</i>	82 (0.36)
Kuilsriver	KR 121	<i>Metarhizium robertsii</i>	61 (0.38)
Kuilsriver	KR 151	<i>Metarhizium robertsii</i>	47 (0.40)
Kuilsriver	KR161	<i>Metarhizium robertsii</i>	71 (0.35)
Nootrodargh	NT 311	<i>Metarhizium robertsii</i>	84 (0.33)
Nootrodargh	NT 151	<i>Metarhizium robertsii</i>	77 (0.40)
Stellenbosch	STL 126	<i>Aspergillus flavus</i>	66 (0.44)
Stellenbosch	STL 174	<i>Purpureocillium lilacinum</i>	71 (0.37)

C. pomonella larval mortalities induced by different fungal strains varied significantly (DF = 1, 38, F = 12.12, $P < 0.001$). Abundance of entomopathogenic fungal species in sampled sites was significantly different (DF = 5; $X^2 = 36$; $P < 0.001$).

2.2.4: Anti-egg hatchability effect of EPF strains (MTL151 and GW 461) on 0-day eggs of codling moth in vitro

Egg hatchabilities observed among fungi treatments ranged from 71 to 93% (GW461) and 66 to 95% (MTL151). Treatments significantly reduced egg hatchability among freshly laid eggs that were exposed to pre-treated wax paper strips at higher spore concentrations 1×10^7 and 1×10^8 spores ml^{-1} compared to lower spore concentrations and the positive control (Table 3). Differences in hatchability was not significantly different (DF =1, 18; F=1.4; P =0.2) at 1×10^8 spores ml^{-1} in both strains.

Table 2.3: Egg hatchabilities following exposure of freshly-laid (0-day) eggs of *C. pomonella* to pre-treated wax paper strips with varying concentrations of aqueous conidia formulation of *M. robertsii* strains (MTL151 and GW 461), control or Kombat fruitfly (positive control) recorded at 6 days post treatment.

Concentration	Egg hatchability (%)	
	GW461	MTL151
1x10 ³	77.5 ± 3AB	95.1±1.6D
1x10 ⁴	83.9 ± 1ABC	90.5±1.3CD
1x10 ⁵	80.4 ± 2.7ABC	81.4±3.6BC
1x10 ⁶	71.2±4.3A	86.3±3.1CD
1x10 ⁷	75.7±2.4A	72.7±5.8AB
1x10 ⁸	71.7±4.3A	66.4±1.2A
Control	93±1.3 C	93.1±1.3CD
Positive control	90.2±2.9BC	90.2±2.9CD

The same uppercase letters in the same column are not significantly different at P = 0.05 (Tukey's pairwise comparisons). Anova: MTL151 (DF = 1, 7; F, 13.9; P < 0.000); GW461 (DF 1, 7; F 7.13; P < 0.000)

2.2.5: Feeding deterrent and larval development activities of *M. robertsii* strains (MTL151 and GW 461) on *C. pomonella* in whole apple fruit bioassay

The fungus and control treatments did not deter feeding of the 1st instar larvae; stings were observed in 100% of participating apples in negative control or test treatments 21 days post treatment (Table 4). On the other hand, the commercial insecticide used in this study showed modest deterrent effects (mean number of fruits with surface wounds was 4.3). At 3 weeks post treatment, larval development in fungus treated apples was significantly inhibited compared to control-treated apples. The mean number of apples with larval infestations (presence of developing or developed larva(e) in their core or flesh) following exposure of 1st instar neonates to fungus-treated apples dropped significantly from 5.3 to 1 and 7.6 to 1 for MTL151 and GW461, respectively as spore concentrations increased from 1×10^3 to 1×10^8 spores ml⁻¹. The protection offered by both strains at 1×10^8 spore ml⁻¹ was slightly better than the commercial insecticide. Dosage-larval infestation (presence of developing/developed larva (e) inside fruits) curves were determined for 1st instar larvae. And they yielded EC₅₀ values of 1.2×10^3 (CI= 1.2×10^2 - 1×10^5) and 1.1×10^5 (CI = 7×10^3 - 5.6×10^5) spores ml⁻¹ for MTL151 and GW461 respectively, which on the basis of the corresponding confidence intervals (CI) were not statistically different (Table 4). Fruit rot increased linearly with rate of larval infestations; GW461 ($R^2 = 0.7$, $y = 0.795x - 0.211$) and MTL151 ($R^2 = 0.98$, $y = 1.04x - 0.67$). Despite the presence of symptoms of external feeding among fungus-treated apples, both strains (MTL151 and GW461) were effective in reducing larval development inside apple fruits.

Table 2.4: Protective effects induced by varying spore concentrations of *M. anisopliae* strains (MTL151 and GW461) applied to whole apple fruits and tested against *C. pomonella* under laboratory conditions. Parameters assessed were; mean no. of fruits \pm SE with surface wounds, larval development and fruit rot

Treatment	Isolates					
	MTL 151			GW461		
Sporesml-1	Mean no. of fruits \pm SE with:			Mean no. of fruits \pm SE with:		
	Surface wounds	Mature/Developing larvae	Fruit rot	Surface wounds	Mature/Developing larvae	Fruit rot
0 (-ve control)	10 \pm 0.0B	7.6 \pm 0.3C	7.3 \pm 1.2C	10.0 \pm 0.0B	9 \pm 0.6D	7.3 \pm 1.2C
1 x 10 ³	10 \pm 0.0B	5.3 \pm 0.9BC	5 \pm 0.6BC	9.7 \pm 0.3B	7.6 \pm 1.5	7.6 \pm 0.7C
1 x 10 ⁴	10 \pm 0.0B	3.6 \pm 0.3AB	2.6 \pm 0.8AB	10.0 \pm 0.0B	7.3 \pm 1.5CD	4.3 \pm 0.3BC
1 x 10 ⁵	10 \pm 0.0B	2.6 \pm 0.3AB	2.3 \pm 0.3AB	10.0 \pm 0.0B	5 \pm 1.5BCD	6.0 \pm 1C
1 x 10 ⁶	10 \pm 0.0B	2.3 \pm 0.8A	2.0 \pm 1.1AB	9.7 \pm 0.6B	6.3 \pm 0.9BC	2.0 \pm 0.6AB
1x 10 ⁷	10 \pm 0.0B	1 \pm 0.6A	0.6 \pm 0.6A	9.0 \pm 0.3B	1.6 \pm 0.9AB	1.0 \pm 0.6AB
1 x 10 ⁸	10 \pm 0.0B	1 \pm 0.6A	0.3 \pm 0.3A	8.7 \pm 0.3B	1 \pm 0.6A	0.7 \pm 0.3A
+ve control	4.3 \pm 0.7A	2.3 \pm 0.3A	1.3 \pm 0.3A	4.3 \pm 0.7A	2.3 \pm 0.3ABC	1.3 \pm 0.3AB
EC50	1.2 x 10 ³ (CI=1.2 x 10 ² -1 x 10 ⁵) spores ml-1			1.1 x 10 ⁵ (CI =7 x10 ³ – 5.6x10 ⁵) spores ml-1		

The same uppercase letters in each column denotes means of egg hatchability among treatments were not significantly different at $P > 0.05$. (Tukey's pairwise comparisons at $P = 0.05$)

2.3: DISCUSSION

Fungal strains belonging to six species were obtained using the insect bait technique in this study, and *M. robertsii* was the most abundant species representing 51% of the total number of isolates. These results suggest that the diversity of entomopathogenic fungi in the Western Cape region of South Africa might be high. In a previous study carried out in the Eastern Cape region of South Africa, 62 EPF isolates belonging to four genera were recovered from 288 soil samples. Most of which were *B. bassiana*, species (15.63%) followed by *M. anisopliae* var. *anisopliae*, which was found to occur in fewer soil samples at a lower frequency of 3.82% (Goble *et al.*, 2009). Even though the use of codling moth larvae as bait may not isolate all the entomopathogens in the sampled soil, insect bait method is still a very simple and efficient method for isolating entomopathogenic fungi from soils (Tuininga *et al.*, 2009).

This is the first report of the occurrence of *M. robertsii* in southern Africa. This species is morphologically identical to *M. anisopliae* and as a result, both species are difficult to separate based on morphological characteristics (Fig. 3) (Bischoff *et al.*, 2009). However, they are currently considered separate species and can be separated based on their elongation 1-alpha gene sequences. Phylogenetically, *M. robertsii* forms part of the *M. anisopliae* species complex that contains the species *M. anisopliae*, *M. robertsii*, *M. guizhouense* and *M. pingshaense* (Fig. 4). The use of DNA extraction, amplification, and sequencing to complement morphological identification of fungi has greatly improved the reliability of entomopathogenic fungal identification.

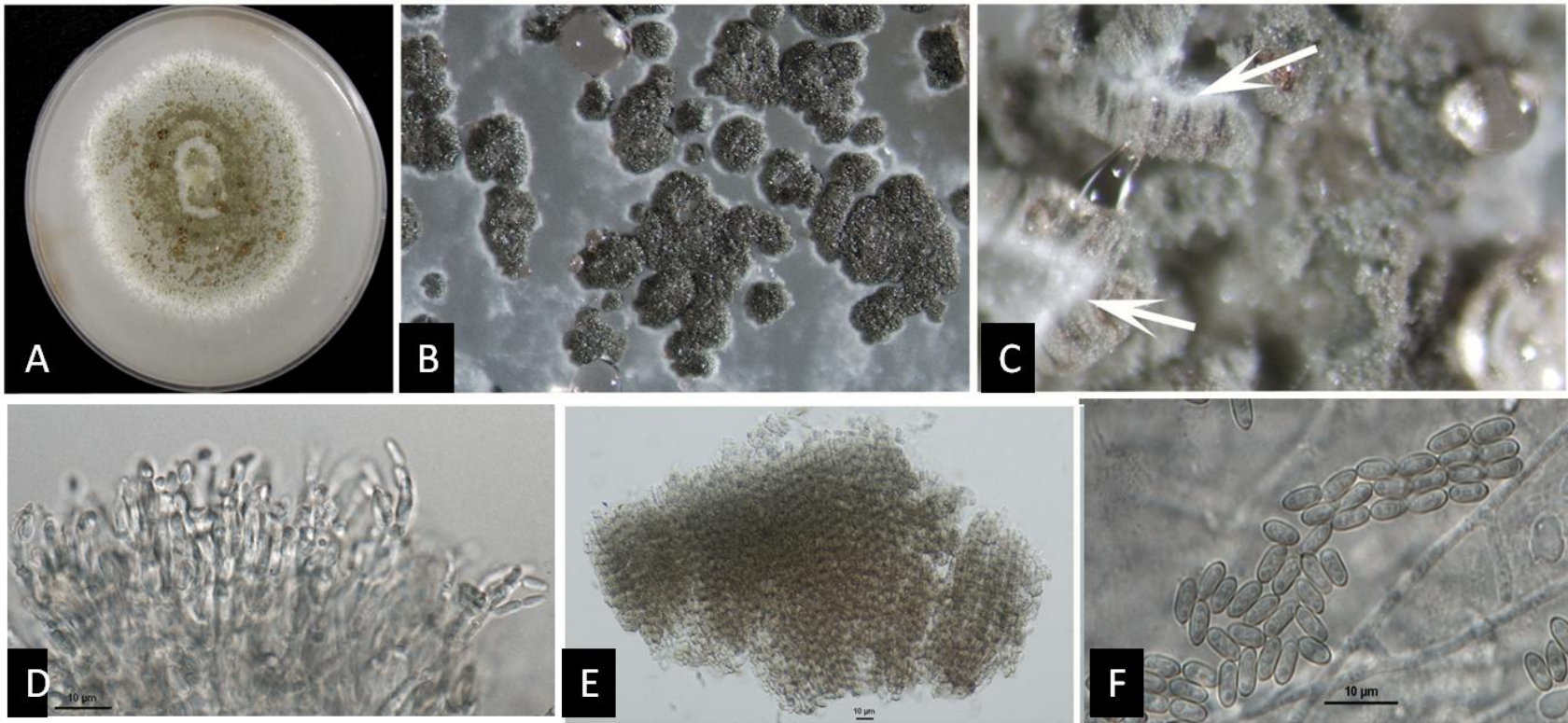


Fig. 2.3: Main morphological characters of *M. robertsii*. A. Sporodochial structures with columns of conidia. B. conidiogenous cells (arrows) with columns of olive-grey conidia. C. - D. 13 Conidiogenous cells bearing chains of conidia. E. Conidia forming columns. F. Chains of 14 conidia

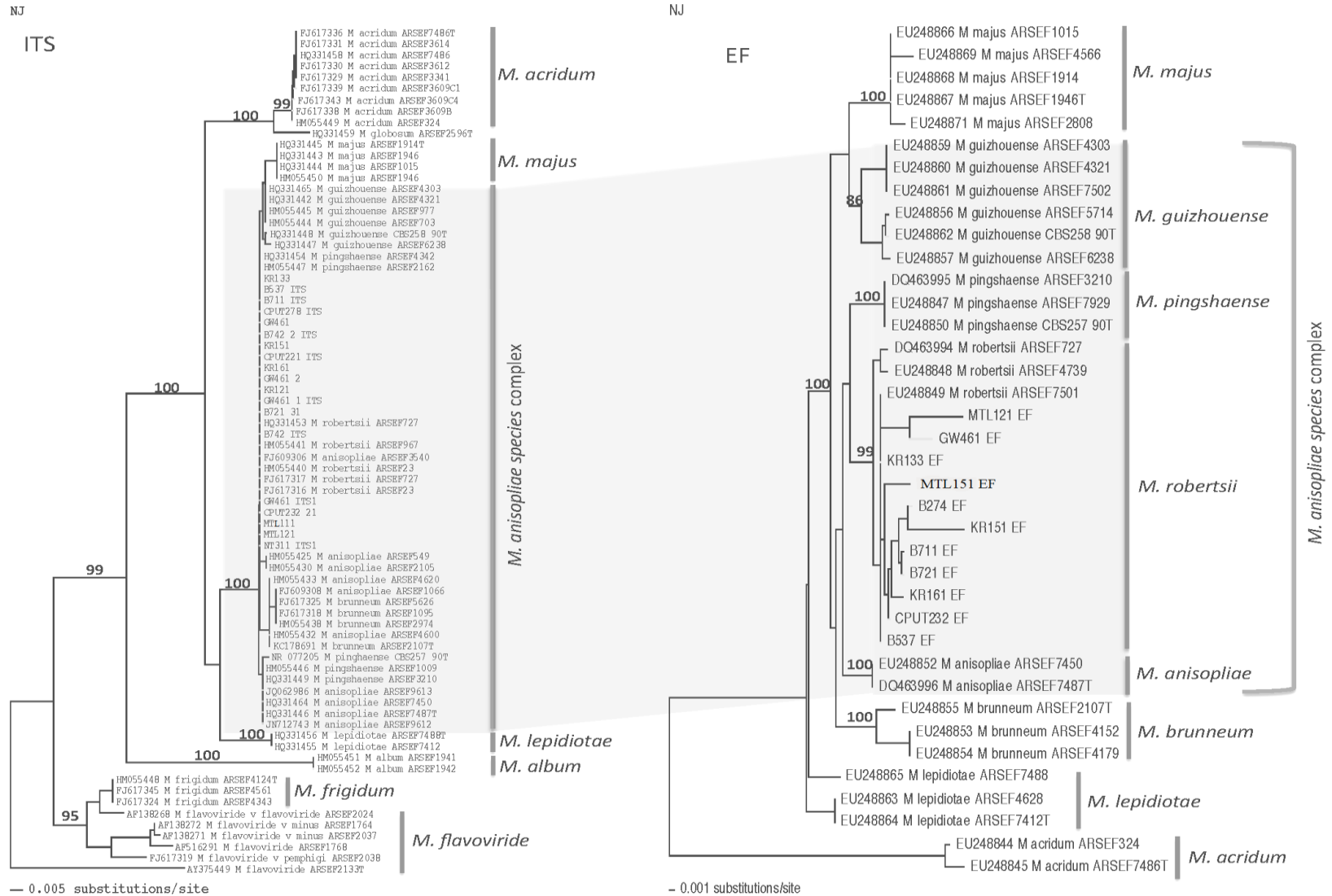


Fig. 2.4: Fig. 1. Main morphological characters of *M. robertsii*. A. Sporodochial structures with columns of conidia. B. conidiogenous cells (arrows) with columns of olive-grey conidia. C.- D. Conidiogenous cells bearing chains of conidia. E. Conidia forming columns. F. Chains of conidia.

Although *M. robertsii* species are thought to have a global distribution, it is recognized that knowledge on *Metarhizium* from the African continent is scarce (Bischoff *et al.*, 2009). In this study, seven *Fusarium* isolates belonging to two species (*F. oxysporum* and *F. polyphialidicum*) were obtained using the insect bait technique. The genus *Fusarium* has a host range that includes *Coleoptera*, *Diptera*, *Hemiptera*, *Hymenoptera* and *Lepidoptera* (Teetor-Barsch & Roberts 1983; Humber, 1992; Pelizza, 2011). Only one (1) isolate of *B. pseudo-bassiana* was obtained from the soil samples. According to Rehner *et al.* (2011) *B. pseudo-bassiana*, is morphologically similar to *B. bassiana* but both phylogenetically distinct (Fig. 5 and 6). For example, two South African strains that were initially identified as *B. bassiana* were re-designated as *B. caledonica* and *B. sp. aff. Caledonica* (Morar-Bhana *et al.*, 2011).

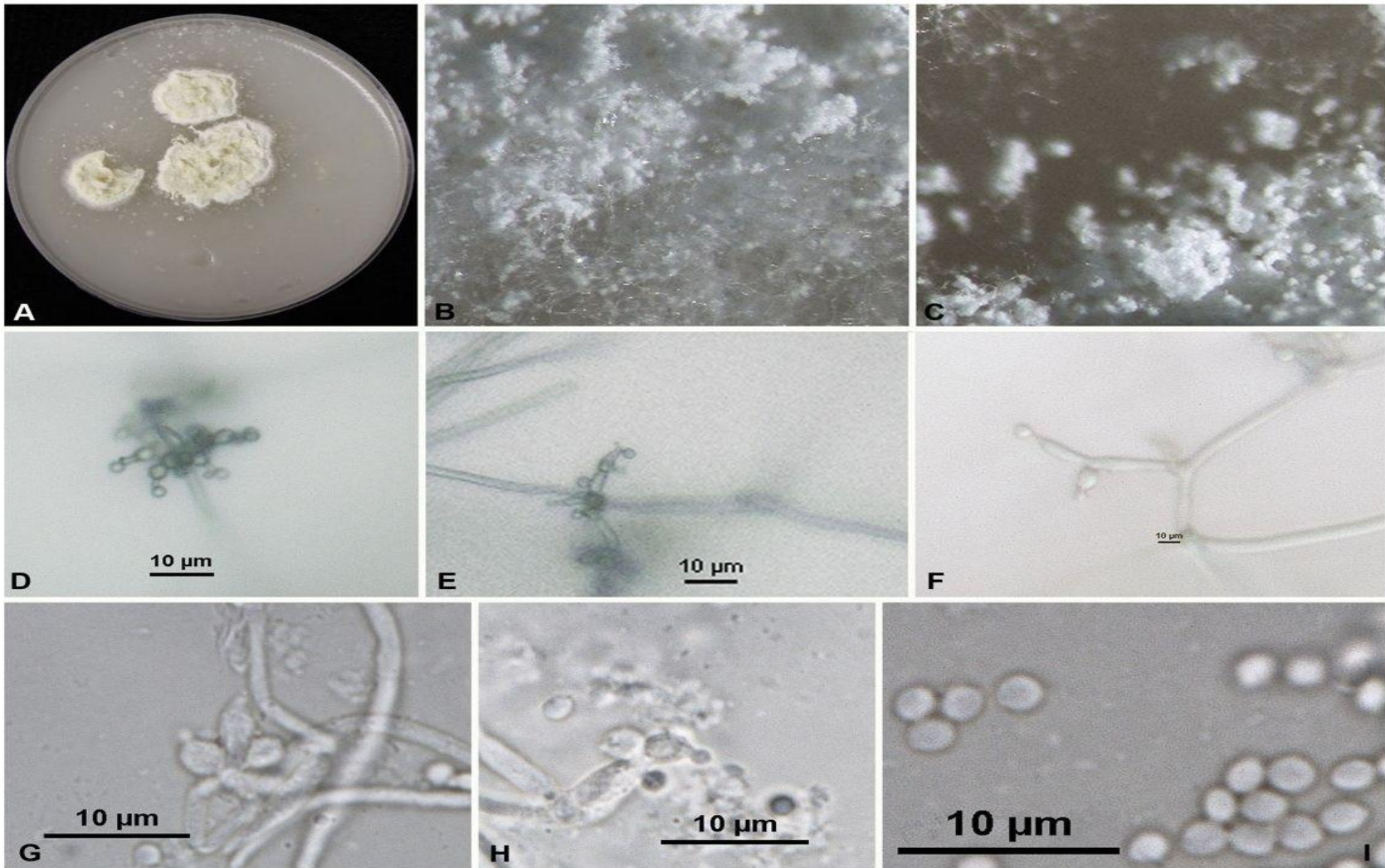


Fig. 2.5: A transmission electron micrograph showing conidiogenous cells on the hyphae of *Beauveria* spp. A. Cream colored, velvety colonies on OA. B and C. close up of colony showing clumps of conidia. D - H. conidiophores with conidia; I. Globose conidia

NJ

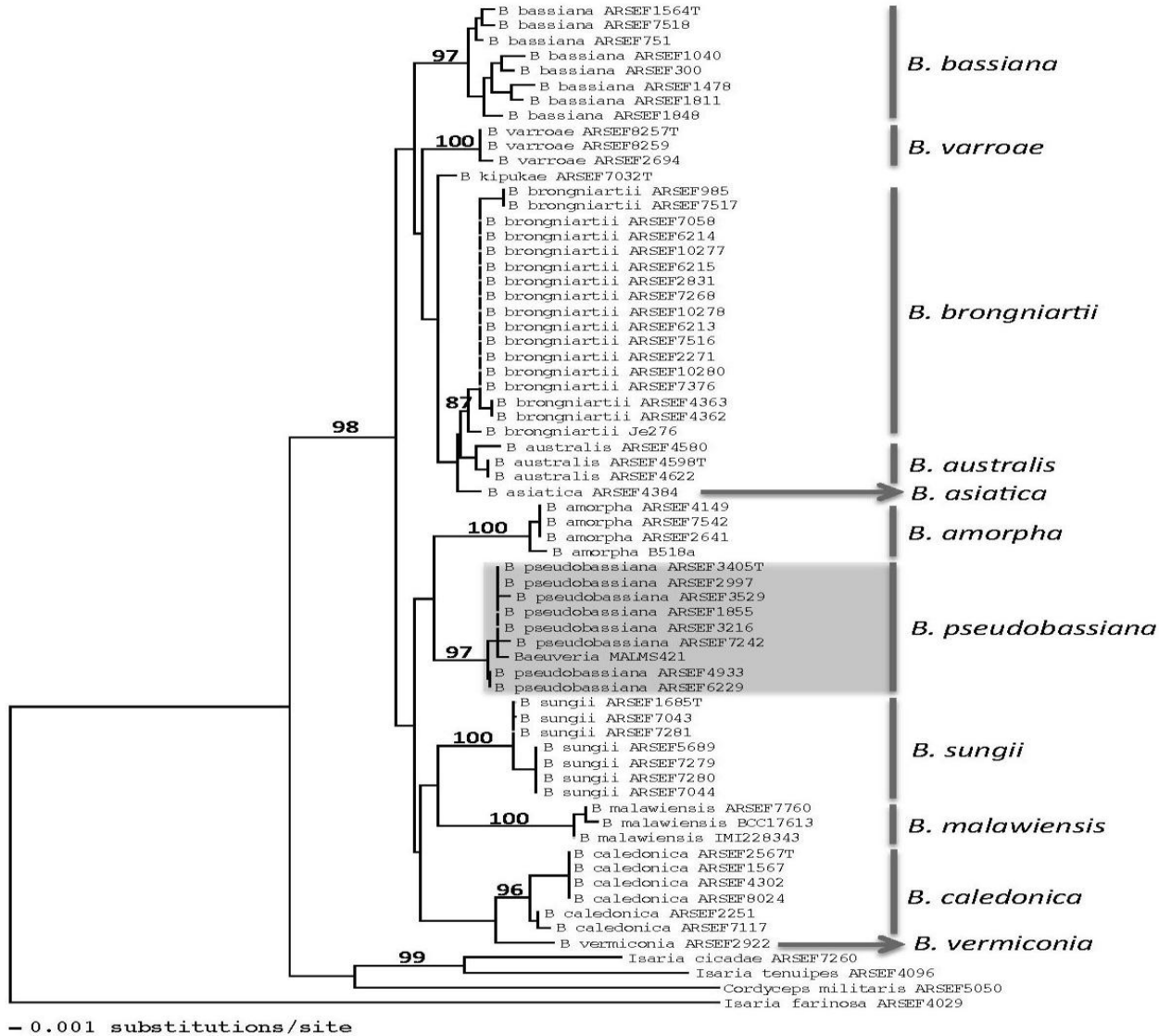


Fig. 2.6: The phylogenetic relationships of the genus *Beauveria* based on several independent Bayesian and parsimony analyses

In the current study, results from the immersion bioassay showed that all the isolates of *M. robertsii* induced mortalities among 5th instars larvae of codling moth ranging from 46 - 85% (Table 2). These results are in agreement with those obtained from other laboratory studies that indicate some *M. robertsii* strains are highly pathogenic against insect pests (Lomer *et al.*, 1997; Milner & Pereira, 2000; Maniania *et al.*, 2002; Shah & Pell, 2003). In the current study, conidial suspension of the strain of *F. oxysporum* used in the immersion bioassay induced up to 77% mortality among 5th instar larvae of codling moth. An isolate of *Fusarium verticillioides* (Saccardo) Nirenberg (Ascomycota: Hypocreales), the most common fungus reported on infected corn kernels and vegetative tissues was pathogenic to two grasshoppers species (*Tropidacris collaris* (Stoll) (Orthoptera: Acridoidea: Romaleidae) and *Ronderosiabergi* (Stål) (Acridoidea: Acrididae: Melanoplinae) (Pelizza *et al.*, 2011). The extracellular metabolites of *F. oxysporum* are lethal against the larvae of *A. stephensi* and *C. quinque fasciatus* (Prakash *et al.*, 2010). In India, 5 fungi species (*B. bassiana*, *Pacilomyces* spp., *Aspergillus flavus*, *Fusarium* spp. and *M. anisopliae*) were successfully isolated using the insect bait method. During 1991 and 1992, *Fusarium polyphialidicum* was isolated from cadavers of Gypsy moth, *Lymantria dispar* and caused larval mortality in laboratory bioassays. Generally, *F. polyphialidicum* is a rare species that was first described in 1984 from plant debris collected in South Africa (Marasas, 1986). There is no case of human or animal infection caused by this fungus (Guarro *et al.*, 2003). *Beauveria* strains can cause mortality among lepidopteran larvae and 56% mortality of larvae was induced by the *B. pseudo-bassiana* strain tested in this study. Field survey of cadavers of *Zonocerus variegatus* revealed high incidences of *Aspergillus flavus* (10%) and *B. bassiana* (18%) in Ibadan, Nigeria (Balogun and Fagede, 2004). In the current study, the incidence of *A. flavus* was 30% (occurred in three of the 10 locations) and induced 66-81% mortality among codling moth

larvae in the immersion bioassay. This study opens up research opportunities in entomopathogenic fungal ecology in the Fynbos region of South Africa.

Two *M. robertsii* strains, MTL151 and GW461 were selected for further evaluation based on the promising results with these against 5th instar larvae of *C. pomonella* in the immersion bioassay. Both fungal strains significantly reduced hatchability of freshly laid *C. pomonella* eggs that were exposed to wax paper strips that were pre-treated with fungal spore suspensions at high concentrations (1×10^7 and 1×10^8 sporesml⁻¹) compared to control and the commercial insecticide treatments. Generally, the antihatchability activities of both strains against *C. pomonella* eggs could be described as bioactive; at least 66% of eggs successfully hatched at the highest spore concentration tested. These results are in agreement with those of other authors who have successfully reduced the number of codling moth eggs hatched with chitin inhibiting chemicals (Hoying & Riedl, 1980; Gökçe *et al.*, 2009; Kim *et al.*, 2011). These findings suggest that the application of conidia on egg laying sites in time (just before codling moth females lay their eggs) may reduce the number of eggs hatched.

In the whole apple fruit bioassay, fungus treatment of apple did not deter 1st instar larvae from feeding on the surface of fruits. Entomopathogenic fungi are slow acting and require many days to kill their insect hosts (Lacey *et al.*, 2001). It is likely that the contaminated larvae would have had sufficient time to begin feeding on the surface of the fruit before succumbing to the fungal infection. Both *M. robertsii* strains (MTL151 and GW461) offered protection against larval development and establishment of larval infestations inside apple fruits when used at high doses. In addition, both strains offered protection against fruit rot caused by secondary fungal infection. Efficacies of both strains compared favourably against the commercial insecticide tested.

In conclusion, indigenous fungal isolates were collected from soils in the Western Cape region of South Africa and two virulent fungal strains with potential to control *C. pomonella* were identified. This study opens up the possibility for inclusion of indigenous fungal strains for control of codling moth in South Africa.

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CHAPTER THREE

ATTRACTION RESPONSES OF ADULTS OF CYDIA POMONELLA (CODLING MOTH) (LEPIDOPTERA: TORTRICIDAE) TO SEMIOCHEMICALS UNDER LABORATORY CONDITIONS

ABSTRACT

The objective of this chapter was to assess the possibility of integrating semiochemicals and entomopathogenic fungi in codling moth control. Firstly, the attraction responses of male and female adult codling moths to butyl hexanoate and codlemone blend; butyl hexanoate (0.025 mg/ml of hexane) and codlemone (0.1 mg/ml of hexane) in a ratio of 1:1 (0.5ml: 0.5ml from both solutions) were assessed in a one-choice wind tunnel olfactory bioassay during scotophase in the absence of fungal spores. The blend attracted more females (69%) than males (66.7%), however these responses were not significantly different ($P > 0.5$). Secondly, the prospect of an integrated use of entomopathogenic fungal conidia and semiochemical blend for control of *C. pomonella* adults was assessed in a wind tunnel containing the combo lure and *M. robertsii* (MTL151) spores formulated as a powder (250 mg) or aqueous spore suspension (3.1×10^8 spores $m^{-1} \pm 7 \times 10^2$ spores ml^{-1}). Insect attraction, mean number of spores picked per insect and insect mortality was assessed. No significant difference ($P > 0.5$) was observed in the number of inoculums picked by unsexed adult moths in the two conidial formulations tested; spore powder ($31 \times 10^3 \pm 1 \times 10^3$ spores ml^{-1}) and aqueous spore suspension ($3.1 \times 10^3 \pm 7 \times 10^2$ spores ml^{-1}). Both *M. robertsii* (MTL151) spore formulations did not significantly ($P > 0.05$) affect the attraction responses of the insects to the combo lure; powder (73.3 ± 3.3) and aqueous (68.6 ± 2.9) compared to the control treatment (without the fungal spores) (70 ± 0.5 %). However, no mortality was recorded among fungus or control-treated moths. These results suggest that the fungus did not inhibit the attraction of moths by the semiochemicals and despite the observed autoinoculation of moths with fungal spores, the level of contamination seemed too low to induce mortality among adult moths.

3.1: Introduction

Earlier (in chapter 2), it was demonstrated that entomopathogenic fungi can potentially control immature stages of *C. pomonella*. Adult codling moths are usually difficult to control. They are mobile and travel long distances. Semiochemical could enhance efficacy of a pesticide. Results from many previous field studies showed that Arthropod pests' populations could be reduced when insect behavioural modulating semiochemicals are used in combination with fungi in an attract-and-kill system (Maranga *et al.*, 2006; Nchu, 2009). Crucially, semiochemicals can minimizing some of the setbacks associated with EPF use in Arthropod pest control; low viability, attenuation and high susceptibility to climatic variations (Inglis *et al.*, 2000; Braga *et al.*, 2001; Fernandes *et al.*, 2008) by increasing contact rate between a targeted insect species and conidia. Horizontal transfer has been suggested as a better way of bypassing some of the difficulties linked to poor efficacies of EPF under field conditions (Scholte *et al.*, 2004). For example, in a study carried out by Dimbi *et al.* (2013), adult fruit flies were able to pick and transmit lethal dosages of fungal spores for up to three mating lines of flies resulting in 72 – 100% insect mortality. The use of semiochemicals reduces the need for inundative spray application of fungal spores, which can unintentionally contaminate non-targeted Arthropod pests.

Females of codling moth are attracted to plant volatiles (pear esters or butyl hexanoate) (Landolt & Guédot 2008; Hern & Dorn, 2004). The attraction of insects including codling moth is greatly influenced by the concentration of a specific semiochemical and the interactions between many semiochemicals. For example, butyl hexanoate attracted mated codling moth females over a dosage range of 0.00125, 0.36 and 1.25µg (Hern & Dorn, 2004). The use of pheromone blend for mating disruption in codling moth has received considerable attention. The sex pheromone secreted by the females of codling moths contains thirteen minor compounds (Witzgall *et al.*, 2001) including; dodecanol (12OH), tetradecanol (14OH), and codlemone. Codling moth females have been identified to be attracted to the kariumone butyl hexanoate, produced by apple fruits (Trona *et al.*, 2010).

The objective of this chapter was to assess the prospect of integrating semiochemicals and entomopathogenic fungi in codling moth control.

3.2: Materials and Methods

3.2.1: Semiochemicals

The semiochemicals used were codlemone (98.3% chemical purity) butyl hexanoate (98% chemical purity) and Hexane (100% chemical purity), supplied by Sigma ®. Solutions of 1mg of codlemone or 0.00125mg of butyl hexanoate per 1L of hexane were prepared and 0.5 ml from each of these solutions were mixed together to obtain 1 ml of semiochemical blend, ready for use in bioassays.

3.2.2: Insects

Late stage pupae of *C. pomonella* supplied by Entomon Technologies (Pty), Ltd. (Stellenbosch) were maintained at 25 ± 2 °C; $60 \pm 5\%$ RH until they molted into adult male or female insects. 1-3 day old emerging adult moths were used in the bioassays.

3.2.3: Entomopathogenic fungus

The entomopathogenic fungus used in this experiment was a strain of *M. robertsii* (MTL151) originally isolated from a soil sample collected from Maitland, Cape Town, South Africa in 2012. Morphological and molecular techniques were employed in the identification of the fungus and cultures of the strain are kept in fungal culture collection of the Department of Microbiology, University of Stellenbosch and Plant Sciences Laboratory, Department of Horticultural Sciences, Cape Peninsula University of Technology. The fungus was cultured on Potato Dextrose Agar (PDA). Petri-dish cultures were incubated at 25 ± 2 °C and $85 \pm 5\%$ RH. Four (4) weeks old, conidia from cultures were harvested by scraping with scalpel and used for testing. Viability tests were carried out using the technique described by Nchu *et al.* (2010); conidial suspension (0.1 ml) titrated to 3×10^6 conidia ml⁻¹ was spread-plated on 9-cm Petri dishes containing PDA and after incubation at 25 ± 2 °C for 24 h. The percentage germination was determined by counting the number of germinated conidia / 100 conidia in two separate areas per plate at 200X under a microscope. Two replicate plates were used. Viability was 89 and 90%.

3.2.3: Olfactory experiment

3.2.3.1: Wind tunnel

Specially designed pulling-air type wind tunnels (figure 1) made of Plexiglass were used in the olfactometry bioassay. Each wind tunnel had three compartments (A, B and C). Compartment A was the insect release chamber, wherein the insects were released and were expected to move upwind towards the semiochemical(s) source in Compartment C through Compartment B, which is designed to be the contamination chamber. The tunnel was designed to ensure unidirectional movement of the insect, i.e. insects are expected to move upwind towards the semiochemical. Humidified air was pulled through the tunnel (from compartments C to A) at a rate of 25 cm/s.

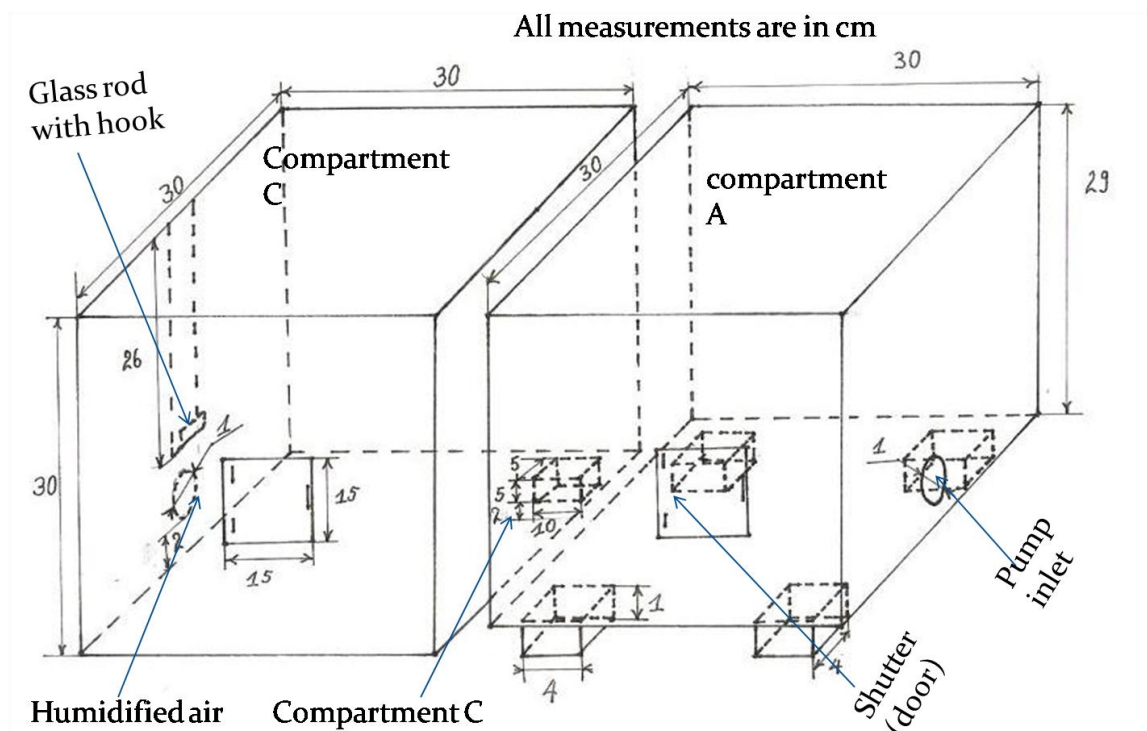


Fig. 3. 1: Schematic diagram of the wind tunnel used in the wind tunnel bioassay

3.2.3.2: One-choice olfactory bioassay

A blend of (codlemone [0.1 ml (McNally & Barnes, 1980)] + butyl hexanoate [0.00125 mg (Hern & Dorn, 2004; Fernandes *et al.*, 2010) were placed on a strip of filter paper (2x2 cm) hanging at 2 cm-height above the tunnel floor and held by a glass hook attached to the inner wall of Compartment (C). In the control group, the filter paper strips were treated with sterile distilled water. Naïve moths were released in Compartment A at the same height as the dispensers (lure) in Compartment C. The tunnels were housed in a temperature controlled room (25 ± 2 °C) and illuminated by a fluorescent bulb (40 W) mounted at 22 cm producing a light intensity of 1500 lux inside the tunnels and was dimmable to 15 lux by means of a variable sensor, which allowed for induced scotophase.

One (1) male or female adult *C. pomonella* was released in compartment “A” of the tunnel during scotophase (3 hrs. before sunset) at 22 ± 1 °C, 52-58% RH. After 10 min, the insect position was recorded as attracted or un-attracted and the bioassay was stopped. Insects that moved upwind to chamber C, which contained either the control or semiochemicals were considered attracted. Each experiment was performed as an independent assay with 10 replicates (corresponding to 1 trial) and repeated three times on separate days. Wind tunnels were cleaned with 70% ethanol and distilled water then, air-dried after each experiment. The experiment was conducted in the Plant Sciences Laboratory, Cape Peninsula University of Technology.

3.2.4: Evaluating the rate of fungal contamination of codling moth adults in a simulated semiochemical-baited-fungus-trap

In order to assess the potential of combining fungal spores and olfaction trap for control of codling moth, a simulated semiochemical-baited-fungus-trap consisting of a wind tunnel containing the combo lure and *M. robertsii* stain (MTL151) conidial spores was used. The olfactory wind tunnel described above was used to assess the rate of contamination of unsexed adult insects with formulated spores placed in Compartment B (inoculation chamber). Unsexed adult insects were released in Compartment A and were expected to move upwind through the inoculation chamber to the semiochemical source in Compartment

C. Two fungal spore formulations were used. Formulation A consisted of sterile distilled water (100 ml), 0.05% Tween 80 and conidia (1×10^{10} conidia ml^{-1}) and was sprayed in the inoculation chamber and allowed to dry for 1 h. Formulation B; dried spore powder (250 mg) were dusted on the floor of compartment B of the tunnel. Insects were allowed to walk on the spores and the quantity of spores picked per insect was determined. Moths that reached compartment C were considered attracted. The treatments were replicated 10 times and repeated 3 times on separate days. Comparison in attraction response was made between control and fungus-treated insects. In order to assess the number of inoculums picked by moths, moths that were attracted to combo blend in the attract-and-fungus contamination system were freeze-killed and placed individually in a 2-ml plastic vial containing 1ml of a mixture of water and 0.05% Tween 80 and the tubes were vortex-mixed for 5 min to extricate conidia from the insects` bodies. The number of spores was estimated using a Neubauer hemocytometer. In total, eight adult moths that had successfully gone through formulated fungal spores were inspected for the number of inoculums picked per formulation. A subsample of eight adult moths from each treatment was checked for mortality after 5 days. In order to determine the cause of mortality, dead insects were surfaced sterilized in 70% ethanol and incubated at $25 \pm 5^\circ\text{C}$; $60 \pm 5\%$ RH. Mycosis was later checked under a dissecting microscope.

3.3: STATISTICS

Insect attraction response data is presented as percentage (%) attraction and significant difference in attraction responses between insects exposed to control and test treatments was determined using Mann-Whitney test. Differences in attraction responses to semiochemicals between males and females and among insects exposed to different treatments were compared using one-way ANOVA of the PAST 2001 version (Hammer et al., 2001). The efficacy of attraction was calculated using the formula: efficacy of attraction = $([\text{number of insects not attracted in the control} - \text{number of insects not attracted in the test-treatment}] / \text{number of insects not attracted in the control}) \times 100$; $[(C - T / C)] \times 100$.

3.4: Results

3.4.1: Male and female attraction response to the semiochemical blend

The male attraction response ($66.7 \pm 0\%$) elicited by a blend of codlemone (1 ml) plus butyl hexanoate (0.0125 mg) in a 1:1 ratio (Table 1) was lower than that of the female (69 ± 10.1). However, there was no significant difference ($P > 0.05$) between in both mean attraction responses. The calculated efficacies of attraction (%) elicited by combo blend in the one-choice wind tunnel olfactory bioassay during scotophase were; male (66.7%) and female (69%).

Table 3.1: Attraction efficacies (mean \pm SE) of combo lure (codlemone 1 mg + butyl hexanoate 0.0125 mg) for male and female codling moths in a one-way wind olfactory bioassay during scotophase

Attraction efficacies (%)		
Replicate	(male)	(female)
1	66.7	55.6
2	66.7	88.9
3	66.7	62.5
Mean \pm SE	66.7 ± 0	69 ± 10.1

The responses recorded among male and female codling moths to the attractant were not statistically different ($N = 3$; $P > 0.05$) (Mann-Whitney). Each replicate represents 10 replications. Means are pooled data from replicates 1, 2 and 3.

3.4.2: Integration of codlemone-butyl hexanoate blend with fungal spores

No significant difference ($P > 0.5$) was observed in the inoculums picked by unsexed adult insects in the two conidial formulations; spore powder ($31 \times 10^3 \pm 1 \times 10^3$) and aqueous spore suspension ($3.1 \times 10^3 \pm 7 \times 10^2$) in the attract-and-fungus contamination bioassay (Table 3.2). No spore contamination was observed among moths in the control treatment that was formulated without fungal spores. The fungal spores and spore formulations did not significantly affect the attraction responses of the insects to the semiochemical combo; 68.6 ± 2.9 , 72.6 ± 3.3 and 70% attractions were recorded for dried spore powder, aqueous spore formulation and control, respectively. The fungus did not induce mortality.

Table 3.2: Mean number of spores picked by insects in aqueous and powder spore formulations and mean % of insects attracted in different treatments; control (no fungus) and formulations of *Metarhizium robertsii* spores in a simulated semiochemical-based-fungus-trap.

	Mean \pm SE % attraction	Mean \pm SE spores picked
Spore powder	73.3 ± 3.3	$3.1 \times 10^3 \pm 1 \times 10^3$
Aqueous formulation	68.6 ± 2.9	$3.1 \times 10^3 \pm 7 \times 10^2$
Control	70 ± 0.5	0

No significant difference ($P > 0.05$) in number of spore picked up by adult insects among the tested spore formulations

3.5: Discussion

Results from the wind tunnel bioassay (Tables 3.1) showed that a mixture of codlemone (1 mg/ cm²) and butyl hexanoate (0.00125 mg/cm²) could attract both sexes of codling moths; males (66.7 ± 0%) and females (69 ± 10.1%). The above results are in agreement with the findings of Trona *et al.* (2010) and Witzgall *et al.* (2003). Trona *et al.* (2010) reported that codlemone blended with plant volatiles induced a high attraction in both male and female Lepidopteran species.

In the current study, adult insects in test treatments successfully picked-up conidia as they walk through the fungus-contaminated chamber; and were not repelled by the conidial formulations resulting in 73.3% (powder) and 68.6% (aqueous) attraction responses to semiochemicals in the wind tunnel bioassay. Previously, Mburu *et al.* (2011) demonstrated that EPF could induce repellency behavior among insects (termites). The spores in the attraction-and-fungus contamination bioassay induced no mortality after 5 days post-contamination. It is likely that the number of spores picked were not adequate to cause infection in the insects. In addition, morphologically, *C. pomonella* are scaly (Fujii & Shimada, 2007); which could prevent spore adhesion or/and penetration. Sticking agents including electrostatically charged wax powder (Entostat powder) enhanced pheromone adhesion on cuticle of *C. pomonella in-vitro* (Huang *et al.*, 2010). Future research on developing formulations that optimize conidial adherence to the cuticle of codling moth is recommended.

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CAPTER FOUR

GENERAL DISCUSSION

The increasing interest in environmentally sustainable farming globally will certainly increase the demand for environmentally benign pest control methods in the future. Many entomopathogenic fungi species have a cosmopolitan distribution and occur in a wide range of habitats including cultivated soils. Results from this research project indicate that many entomopathogenic fungal species including *M. robertsii* inhabit soils of the Western Cape region of South Africa. This is the first report of the occurrence of *M. robertsii* in southern Africa. This finding is significant especially to those interested in the field of Arthropod pathology. Currently, scant information is available on the distribution of the species in Africa.

In total, 39 isolates belonging to six species (*Purpureocillium lilacinum*, *Fusarium oxysporum*, *Fusarium polyphialidicum*, *Beauveria pseudobassiana*, *Aspergillus flavus* and *M. robertsii*) were isolated using the insect bait technique and spores from cultures of these fungal strains induced varied mortalities among 5th instars larvae of codling. These results are in agreement with previous studies (Ranga Rao & Reddy, 1997; Goble, 2009; Chouvenec, *et al.*, 2012). However, assessing pathogenicity of fungal strains against *C. pomonella* in dipping bioassay may have some limitations (Nchu *et al.*, 2010).

A further evaluation of two of the identified virulent isolates of *M. robertsii* in egg hatchability bioassay revealed that the fungal inoculums might reduce hatchability of eggs of *C. pomonella* if egg-laying surfaces are pretreated before the female moths lay the eggs. This information may be important to organic orchard farmers. Plants would need fungal treatment just before oviposition by female codling moth. However, for this to be effective, important information such as degree-days and efficient monitoring would be required. Based on results obtained from the apple fruit bioassay, it appears that spraying fungus on fruit did not deter codling moth larvae from feeding on the surface of apple fruit. Spray application of fungus on apple fruits was effective in protecting fruits against larval infestations, especially at high doses of conidia.

In Chapter 3, we assessed the combination of semiochemicals and fungus in a simulated semiochemical-based-fungus-trap bioassay on the potential for effective delivery of fungal spores. The mean numbers of inoculi picked by unsexed adult insects in the two conidial formulations tested were not significantly different ($P > 0.5$); spore powder ($3.1 \times 10^3 \pm 1 \times 10^3$) and aqueous spore suspension ($3.1 \times 10^3 \pm 7 \times 10^2$) spores ml^{-1} unsexed adult in the semiochemical blend-and-fungus bioassay. However, the number of spores picked by insects was not enough to cause infection in both treatments, probably due to the scaly nature of codling moth (Fujii & Shimada, 2007) which might have prevented adhesion/penetration. It is a possibility that sticking agents may enhance the adhesion of fungal spores to the cuticle of the moth. Huang *et al.* (2010) demonstrated that sticking agent [electrostatically charged wax powder (Entostat powder)] enhanced pheromone adhesion on cuticle of *C. pomonella in-vitro*).

These results hinted high diversity of entomopathogenic fungal species occur in the Western Cape region of South Africa and this is the first report on the occurrence of *M. robertsii* in southern Africa. Further, immature stages of codling moths are susceptible to these indigenous EPF strains. Based on the results obtained in the simulated attract-and-contamination system, *M. robertsii* spores did not inhibit attraction of adult moths to the semiochemical blend tested (codlemone and butyl hexanoate) and the autoinoculation of adult codling moth with fungal spores is possible. There are prospects for incorporation of EPF in IPM programmes for the control of codling moths in South Africa, however, future research work on the fungal ecology and adhesion of spores to codling moth adults are recommended.

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