Entomopathogenic fungi for control of soil-borne life stages of false codling moth, *Thaumatotibia leucotreta* (Meyrick) (1912) (Lepidoptera: Tortricidae)

A thesis submitted in fulfilment of

the requirements for the degree of

MASTER OF SCIENCE

of

RHODES UNIVERSITY

by

Candice Anne Coombes

December 2012

Abstract

False codling moth (FCM), *Thaumatotibia leucotreta* is an extremely important pest of citrus in South Africa and with the shift away from the use of chemicals, alternate control options are needed. One avenue of control which has only recently been investigated against the soil-borne life stages of FCM is the use of entomopathogenic fungi (EPF). In 2009, 12 entomopathogenic fungal isolates collected from South African citrus orchards showed good control potential during laboratory conducted bioassays. The aim of this study was to further analyse the potential of these isolates through concentration-dose and exposure-time response bioassays. After initial re-screening, concentration-dose response and exposure-time response sand-conidial bioassays, three isolates were identified as exhibiting the greatest control potential against FCM in soil, *Metarhizium anisopliae* var. *anisopliae* (G 11 3 L6 and FCM Ar 23 B3) and *Beauveria bassiana* (G Ar 17 B3). Percentage mycosis was found to be directly related to fungal concentration as well as the amount of time FCM 5th instar larvae were exposed to the fungal conidia. LC_{50} values for the three isolates were not greater than 1.92 x 10^6 conidia.ml^{-1} and at the LC_{50}, FCM 5th instar larvae would need to be exposed to the fungus for a maximum of 13 days to ensure a high mortality level. These isolates along with two commercially available EPF products were subjected to field persistence trials whereby net bags filled with a mixture of autoclaved sand and formulated fungal product were buried in an Eastern Cape citrus orchard. The viability of each isolate was measured on a monthly basis for a period of six months. All isolates were capable of persisting in the soil for six months with the collected isolates persisting far better than the commercially used isolates. Two of the isolates, G 11 3 L6 and G Ar 17 B3, were subjected to small scale laboratory application trials. Two formulations were investigated at two concentrations. For each isolate, each formulation and each concentration, FCM 5th instar larvae were applied and allowed to burrow into the soil to pupate before fungal application or after fungal application. Contact between fungi and FCM host is essential as, in contrast to pre-larval treatments, percentage mortality in post-larval treatments was low for both formulations and both isolates. For isolate G Ar 17 B3, a conidial suspension applied as a spray at a concentration of 1 x 10^7 conidia.ml^{-1} obtained the highest percentage mortality (80 %). For isolate G 11 3 L6 however, both formulations performed equally well at a high, 1 x10^7 conidia.ml^{-1} concentration (conidial suspension: 60 %; granular: 65 %) The results obtained thus far are promising for the control of FCM in citrus, but if these EPFs are to successfully integrate into current FCM control practices more research, some of which is discussed, is essential.

Acknowledgements

Firstly a big thank you to all parties responsible for the funding of this thesis whether this be through living costs or running costs of the project itself – **Citrus Research International**, **National Research Foundation** and **Rhodes University**.

A huge thank you needs to be extended to a number of individuals for their help in one way or another:

Prof. Martin Hill for his supervision, guidance and faith in me during the course of my work. Thank you for all the time you put into reading and correcting my thesis.

Dr Sean Moore for his supervision. Your suggestions and opinions are always appreciated. Thank you for all the time you put in to reading and correcting my thesis.

Dr Jo Dames for her help and knowledge in this field.

Kate Benyon for her financial assistance.

Rob and Steve Moss for allowing me to make use of their citrus orchard for field trials.

River Bioscience for supplying the larvae whenever I needed them. This really made the work much easier and more time-effective.

Tanya Pretorius for always being available to help me with anything and everything. Considering you were my personal driver, I would literally have got nowhere without you.

Again, thank you to anyone involved in this thesis including anyone I have failed to mention. Without you it was not possible.

Table of contents

Abstract…………………………………..………………………… ii

Acknowledgements……………………………………………………… iii

Table of Contents………………………………………………………… iv

List of Figures……………………………………………………………viii

List of Tables……………………………………………………………… xii

List of Abbreviations…………………………………………………… xiii

CHAPTER 1 – GENERAL INTRODUTION……………………….. 1

1.1 CITRUS IN SOUTH AFRICA…………………………………………. 1

1.1.1 Citrus taxonomy……………………………………..….. 1

1.1.2 Citrus production and export…………………………………. 1

1.1.3 Citrus pests: A general overview…………………………….. 4

1.1.4 Control of citrus pests: A general overview………………..... 7

1.2 FALSE CODLING MOTH……………………………………………... 9

1.2.1 Taxonomy……………………………….………………… 9

1.2.2 Distribution & host range…………………………………… 10

1.2.3 Life history…………………………………………………….. 11

1.2.4 Economical importance……………………………………... 13

1.2.5 Control………………………………………………………… 14

1.3 ENTOMOPATHOGENIC FUNGI…………………………………… 17

1.3.1 Classification………………………………………….… 17

1.3.2 General biology……………………………………………… 17

1.3.3 Use as biological control agents.................................. 19

1.4 RESEARCH AIMS... 25

2. CHAPTER 2 – USE OF EPF TO CONTROL FCM: LABORATORY BIOASSAYS..................................... 27

2.1 INTRODUCTION.. 27

2.2 METHODS.. 29

2.2.1 Insect culture.. 29

2.2.2 Fungal cultures.. 29

2.2.3 Preparation of conidial suspensions.............................. 30

2.2.3.1 Determination of concentration.............................. 30

2.2.3.2 Assessment of viability.. 31

2.2.4 Inoculation of FCM... 32

2.2.4.1 Re-screening of isolates....................................... 32

2.2.4.2 Concentration dose-response bioassays.................... 33

2.2.4.3 Exposure time-response bioassays......................... 34

2.2.5 Statistical analysis.. 34

2.3 RESULTS.. 34

2.3.1 Re-screening of previously identified isolates.................. 35

2.3.2 Concentration dose-response bioassays......................... 38

2.3.3 Exposure time-response bioassays............................... 42

2.4 DISCUSSION.. 45

3. CHAPTER 3 – PERSISTENCE OF EPF ISOLATES IN THE FIELD…… 49
3.1 INTRODUCTION…… 49
3.2 METHODS…… 50
3.2.1 Fungi…… 50
3.2.2 Experimental site…… 51
3.2.3 Experimental design…… 51
3.2.4 Statistical analysis…… 52
3.3 RESULTS…… 53
3.4 DISCUSSION…… 61
4. CHAPTER 4 – PRELIMINARY FORMULATION AND APPLICATION TECHNIQUES FOR EPF OF FCM IN CITRUS…… 66
4.1 INTRODUCTION…… 66
4.2 METHODS…… 68
4.2.1 Fungi…… 68
4.2.2 Formulation preparation…… 68
4.2.3 Experimental design…… 69
4.2.4 Statistical analysis…… 71
4.3 RESULTS…… 71

4.4 DISCUSSION…………………………………………………………… 75

5. CHAPTER 5 – GENERAL DISCUSSION…………..……… 81

5.1 SUMMARY OF RESEARCH…………………………………………… 81

5.2 GLOBAL PERSPECTIVE……………………………………………… 83

5.3 INTEGRATION OF EPFs INTO FCM CONTROL………………….…... 84

5.4 DEVELOPING A MYCOPESTICIDE……………………………..…. 88

5.4.1 The LUBILOSA project……………………………………… 88

5.4.2 Issues and considerations arising from the LUBILOSA project……………………………………………………… 92

5.5 CONCLUSION & FUTURE RESEARCH……………………………… 94

6. CHAPTER 6 – REFERENCES………………………………… 96

List of figures

CHAPTER 1

Figure 1.1: The citrus producing areas within the different provinces of South Africa (and Swaziland) including the number of hectares dedicated to the production of various citrus cultivars (Map: CGA Key Industry Statistics 2012, Cultivar production per province: CGA Annual Report 2012)……………………………………………………………………….. 3

Figure 1.2: The geographic distribution of false codling moth, *Thaumatotibia leucotreta*, in Africa. Bullets indicate the countries in which the moth is present (Stibick 2008)……………... 10

Figure 1.3: FCM life stages. (A) eggs; (B) final instar larva; (C) pupae; (D) adult [River Bioscience (A); Varela (2005), ICIPE (B)]………….....………………………………………. 12

Figure 1.4: The damage caused by FCM larvae on citrus. Note the black granular frass-filled exit tunnel and the start of fruit decay (indicated by the arrow) (Ludewig 2003)........................ 13

Figure 1.5: (A) Infection process followed by an entomopathogenic fungus (Goble 2009); (B) Fungal exiting through the less sclerotised areas of a FCM pupa......…………………………... 19

CHAPTER 2

Figure 2.1: (A) Appearance of *Beauveria bassiana* conidia; (B) *Metarhizium anisopliae* conidia (Barnett 1960)…………………………………………………………………...... 28

Figure 2.2: Culture morphology of all 12 isolates investigated. Isolates A-D are *Metarhizium anisopliae*; E-L *Beauveria*……………………………………………………………….…...... 30

Figure 2.3: A Helber bacteria counting chamber with thoma ruling as viewed under a light microscope (400X). Black dots represent conidia. Darkly shaded grey squares represent the corners of the chamber which are counted. Lightly shaded grey squares represent the middle four larger squares of the chamber. Only one of these, chosen at random, is counted in conjunction with the four corners………………………………………………………......... 31

Figure 2.4: (A) Petri dishes each containing 20 5th instar larvae on inoculated soil. Plates were placed on trays containing perlite; (B) Cut, plastic 2L coke bottles served as emergence chambers. These chambers were plugged with cotton wool to prevent both the escape of moths and to allow for the introduction of water to sustain adult moths for the duration of the bioassay………………………………………………………………………………….. 32

Figure 2.5: (A) Pupae showing signs of fungal mycosis (←) after incubation on SDA plates; (B) Pupa showing signs of mycosis via *Metarhizium anisopliae*; (C) Pupa showing signs of mycosis via *B. bassiana*………………………………………………………………… 33

Figure 2.6: Mortality of *T. leucotreta* larvae exposed to four isolates of *M. anisopliae* var. *anisopliae* and eight isolates of *B. bassiana* exposed to conidial concentrations of $1x10^7$ $conidia.ml^{-1}$. Bars represent the mean eclosion of *T. leucotreta* in percentage; whiskers the standard error (±). Different letters are indicative of a significant difference according to the student Newman-Keuls test ($p = 0.05$) (ANOVA: $F_{19,30} = 13.22$; $p < 0.05$). Four of the eight *B. bassiana* isolates and one *M. anisopliae* isolate were repeated. This is indicated by "Re" at the end of the isolate name e.g. FF J&B R5 Re…………………………………………………... 36

Figure 2.7: Mortality of *T. leucotreta* larvae exposed to four isolates of *M. anisopliae* var. *anisopliae* and eight isolates of *B. bassiana* exposed to conidial concentrations of $1x10^7$ $conidia.ml^{-1}$. Bars represent the mean *T. leucotreta* mycosis in percentage; whiskers the standard error (±). Different letters are indicative of a significant difference according to the student Newman-Keuls test ($p = 0.05$) (ANOVA: $F_{19,60} = 10.86$; $p < 0.05$). Four of the eight *B. bassiana* isolates and one *M. anisopliae* isolate were repeated. This is indicated by "Re" at the end of the isolate name e.g. FF J&B R5……………………………………………………... 37

Figure 2.8: Percentage of eclosed FCM recorded upon exposure of 5th instar larvae to three different conidial concentrations. Eight isolates ands two commercial isolates (Eco-Bb® and ICIPE 69) were investigated………………………………………………………………... 39

Figure 2.9: The log-probit regressions of overall (adult and pupal) mortality for all eight isolates (1– 4: *Metarhizium anisopliae*; 5–8: *Beauveria bassiana*) and two commercially available isolates (9 and 10). (Equation of lines: 1. $y = 0.7x + 0.33$; 2. $y = 0.86x – 0.007$; 3. $y = 0.49x + 1.17$; 4. $y = 0.66x + 0.85$; 5. $y = 0.37x + 2.59$; 6. $y = 0.59x + 2.06$; 7. $y = 0.75x + 1.04$; 8. $y = 0.67x + 1.33$; 9. $y = 0.32x +2.94$; 10. $y = 0.44x + 1.72$)…………………………………... 40

Figure 2.10: Percentage FCM mycosis of three fungal isolates after exposure to two different conidial concentrations (LC_{50} and $1 x 10^7$ $conidia.ml^{-1}$). Control bioassays were conducted for each time period. No mortality was observed in the control. Black lines are representative of the low (LC_{50}) concentration; grey the high ($1 x 10^7$ $conidia.ml^{-1}$) concentration………………. 43

Figure 2.11: Adult FCM eclosion of three fungal isolates after exposure to two different conidial concentrations (LC_{50} and $1 x 10^7$ $conidia.ml^{-1}$). Control bioassays were conducted for each time period. No mortality was observed in the control. Black lines are representative of the low (LC_{50}) concentration; grey the high ($1 x 10^7$ $conidia.ml^{-1}$) concentration………………. 44

CHAPTER 3

Figure 3.1: Rice overgrown with (A) *B. bassiana* and (B) *M. anisopliae*……….…………….. 50

Figure 3.2: (A) 0.5 g of formulated product mixed with 100 g autoclaved soil; (B) labelled net bags containing formulated product prior to burial; net bags buried in the soil underneath the canopy of a citrus tree; (C) the location of the bag was identified by the string (string was attached to the bag) and the isolate was identified by the label. The yellow arrows indicate the location of the bags in the soil underneath the tree canopy……………………......................... 52

Figure 3.3: Monthly CFU counts and associated FCM mycosis percentages (percentage of insects mycosed) recorded for isolate G 11 3 L6 over six months. For CFU counts (represented in black) blocks indicate median values, whiskers the interquartile range. For FCM mycosis percentages (represented in grey) blocks indicate means, whiskers standard error. Different letters indicate statistically significantly different results (CFU: Kruskal Wallis ($H_{6,26} = 22.11$, $p = 0.0012$); MYCOSIS: ANOVA ($F_{6,18} = 16.69$, $p < 0.05$)…………………………………... 55

Figure 3.4: Monthly CFU counts and associated FCM mycosis percentages (percentage of insects mycosed) recorded for isolate FCM Ar 23 B3 over six months. For CFU counts (represented in black) blocks indicate median values, whiskers the interquartile range. For FCM mycosis percentages (represented in grey) blocks indicate means, whiskers standard error. Different letters indicate statistically significantly different results (CFU: Kruskal Wallis ($H_{6,25} = 20.27$, $p = 0.0025$); MYCOSIS: ANOVA ($F_{6,18} = 3.54$, $p = 0.02$)…………………… 56

Figure 3.5: Monthly CFU counts and associated FCM mycosis percentages (percentage of insects mycosed) recorded for isolate G AR 17 B3 over six months. For both CFU counts (represented in black) and FCM mycosis percentages (represented in grey) blocks indicate median values, whiskers the interquartile range. Different letters indicate statistically significantly different results (CFU: Kruskal Wallis ($H_{6,27} = 22.75$, $p = 0.0025$); MYCOSIS: Kruskal Wallis ($H_{6,27} = 19.55$, $p = 0.0033$)…………………………………………………… 57

Figure 3.6: Monthly CFU counts and associated FCM mycosis percentages (percentage of insects mycosed) recorded for commercial isolate Eco-Bb® over six months. For CFU counts (represented in black) blocks indicate median values, whiskers the interquartile range. For FCM mycosis percentages (represented in grey) blocks indicate means, whiskers standard error. Different letters indicate statistically significantly different results (CFU: Kruskal Wallis ($H_{6,22} = 19.57$, $p = 0.0033$); MYCOSIS: ANOVA ($F_{6,15} = 5.84$, $p = 0.002$)…………………….. 58

Figure 3.7: Monthly CFU counts and associated FCM mycosis percentages (percentage of insects mycosed) recorded for Kenyan isolate, Ken, over six months. For CFU counts

(represented in black) blocks indicate median values, whiskers the interquartile range. For FCM mycosis percentages (represented in grey) blocks indicate means, whiskers standard error. Different letters indicate statistically significantly different results (CFU: Kruskal Wallis ($H_{6,23} = 18.92$, $p = 0.004$); MYCOSIS: ANOVA ($F_{6,17} = 3.93$, $p = 0.01$)……………………….. 59

Figure 3.8: Correlation data based on linear regression analysis for all five fungal isolates tested. Average percentage mycosis (vertical axis) obtained was correlated with monthly CFU counts (horizontal axis). The equation of each line and R^2correlation efficient is given……….. 60

CHAPTER 4

Figure 4.1: (A) Granular formulation. This was prepared by crushing, using a mortar and pestle, the rice overgrown with fungus; (B) Conidial suspension. This was prepared as outlined in Chapter 2, section 2.2.3………………………………………………………………... 69

Figure 4.2: (A) Treated curry tubs; (B) 5th instar larvae burrowing into soil to pupate after being added to the tubs………………………………………………………………….... 70

Figure 4.3: Percentage of FCM mycosed for the different treatments where *M. anisopliae* isolate G 11 3 L6 was used. Blocks represent the median; whiskers the interquartile range. Black blocks represent pre-larval application treatments; grey blocks post-larval and the white blocks the various controls. Different letters indicate statistically different treatments (Kruskal-Wallis: $H_{15, 160} = 114.49$, $p < 0.05$)……………………………………………………….. 73

Figure 4.4: Percentage of FCM mycosed for the different treatments where *B. bassiana* isolate G AR 17 B3 was used. Blocks represent the median; whiskers the interquartile range. Black blocks represent pre-larval application treatments; grey blocks post-larval and the white blocks the various controls. Different letters indicate statistically different treatments (Kruskal-Wallis: $H_{15, 160} = 109.91$, $p < 0.05$)……………………………………………………………….. 74

CHAPTER 5

Figure 5.1: Commercial development process followed by staff of the LUBILOSA project (Dent 1998 after Baldwin 1986). Highlighted (grey) blocks indicate completed areas in the study of EPFs for FCM control in citrus………………………………………………………… 91

List of Tables

CHAPTER 1

Table 1.1: Pests of citrus in South Africa (Insects) (Annecke & Moran 1982; Bedford 1998; Smith & Peña 2002; S.D. Moore, pers. comm.)………………………………………………… 6

Table 1.2: Pests of citrus in South Africa (Mites) (Annecke & Moran 1982; Bedford 1998; Smith & Peña 2002)……………………………………………………………………….... 7

Table 1.3: Examples of products registered for the control of various pest species worldwide (de Faria & Wraight (2007). A more comprehensive list can be found in de Faria & Wraight (2007)…………………………………………………………………………………….. 21

Table 1.4: Insect pests or outbreak species which are controlled by EPF (Shah & Pell 2003; Scholte *et al* 2004; Er *et al.* 2007; Loc & Chi 2007; Dembilio *et al.* 2010; Pell *et al.* 2010)…… 24

Table 1.5: Examples of sone fungal pathogens which have been used against or are known to cause mortality in citrus pests (Dolinski & Lacey 2007)………………………………….... 25

CHAPTER 2

Table 2.1: Probit data for each of the investigated fungal isolates. The isolates highlighted are those which were investigated further………………………………………………………… 41

Table 2.2: The number of days required for two different fungal concentrations to reach a FCM mortality percentage of 50 % and 90 % …………………………………………………... 45

CHAPTER 3

Table 3.1: Statistical analysis of monthly CFU and mycosis results……………………………. 54

CHAPTER 4

Table 4.1: Treatment details. The same treatments were used for each isolate and both pre-larval and post-larval application………………………………………………………….. 70

List of abbreviations

%	percentage
°C	degrees Celsius
a.i	active ingredient
CFU	Colony forming unit
CGA	Citrus Growers' Association
dH_2O	distilled water
EPF	entomopathogenic fungi
EPN	entomopathogenic nematodes
FCM	false codling moth
g	gram
ha	hectare
IPM	integrated pest management
kg	kilogram
L	litre
LC_{50}	lethal concentration required to kill 50 % of the population
LC_{90}	lethal concentration required to kill 90 % of the population
LT_{50}	time FCM 5th instars need to be exposed to the fungus to kill 50 % of the population
LT_{90}	time FCM 5th instars need to be exposed to the fungus to kill 90 % of the population
mg	milligram
ml	millilitre
mm	millimetre
PPRI	Plant Protection Research Institute
psi	pressure per square inch
SDA	Sabaroud dextrose agar
SIT	sterile insect technique
USA	United States of America
µl	microlitre
χ^2	chi-squared

1

GENERAL INTRODUCTION

1.1 CITRUS IN SOUTH AFRICA

1.1.1 *Citrus* taxonomy

Based on genetic analysis, the genus *Citrus*, family Rutaceae, is now considered to be composed of two subgenera: *Eucitrus* and *Papeda*, the edible and non-edible fruits respectively with *Eucitrus* originating from the Sub-tropical and *Papeda,* the tropical areas of Southeast Asia. It was from these areas that *Citrus* spread to other continents (Nicolosi *et al.* 2000). Most cultivated citrus falls within the subgenus *Eucitrus* and has been derived through extensive selection and hybridization (Mabberly 1997). The classification of *Citrus* is complicated as it is an ancient crop making the determination of its origin challenging and secondly as a result of its reproductive biology (Moore 2001). A large proportion of citrus cultivars reproduce via apomixes (asexual reproduction) through a process known as nuclear embryony. As a result gene exchange tends to be limited allowing for reproductive isolation, a criteria often used to distinguish species. But, within the genus *Citrus,* hybridization is common resulting in a wide variety of phenotypically rather than genetically different fruits suggesting few species with many subspecies. The most commonly followed classification systems are that of Swingle (1944) and Tanaka (1954) (cited by Moore 2001). Through the application of modern techniques using DNA markers, it has been suggested that only three true species exist namely the Citron (*Citrus medica* L.), the Mandarin (*C. reticulata* Blanco) and the Pummelo (*C. maxima* Merril) (Nicolosi *et al.* 2000), with the other cultivars being hybrids.

1.1.2 Citrus production and export

The citrus industry in South Africa is large with approximately 20 million citrus trees planted over approximately 60 355 ha of land distributed over seven of the nine provinces and Swaziland (Bedford 1998; CGA Key Industry Statistics 2012). The main citrus producing

provinces are Limpopo (25 674 ha), Eastern Cape (12 508 ha), Western Cape (8 961 ha) and Mpumalanga (6 817 ha) (Figure 1.1).

These different areas are characterised by different climatic conditions and as a result produce different cultivars which become available at different times of the year. The Western and Eastern Cape areas are located within a cooler climatic zone where production is focused on navel oranges, lemons and easy peeling Mandarin varieties such as Clementines and Satsumas, whereas in the Limpopo, KwaZulu-Natal and Mpumalanga provinces, climatic conditions are warmer favouring the production of grapefruit and Valencia oranges (Mather 2003). In general, the majority of production focuses on Valencia oranges followed by Navel oranges (Figure 1.1). A breakdown of each cultivar (oranges, soft citrus, grapefruit, lemons and limes) and their various varieties is reported in CGA Key Industry Statistics (2012). This breakdown shows the production areas per cultivar, areas planted per cultivar variety as well as various statistics regarding the export of each of the cultivars and their varieties.

The South African citrus industry is the third largest exporter of citrus in the world, slightly behind Turkey with Spain being the largest (CGA Key Industry Statistics 2012). Most citrus produced is exported to overseas markets (70 %) whilst the rest is used for sale to the local market (22 %) or is sent for processing (8 %) (Bedford 1998; CGA Key Industry Statistics 2012). There are ten major export regions with the largest being Northern Europe, constituting 25 % of all exports followed by the Middle East, Far East and Russia constituting 19 %, 13 % and 13 % of total exports respectively (CGA Key Industry Statistics 2012). Exports are the main path through which revenue is produced as even though 70 % of produced fruit is exported, this contributes 85–90 % of the total generated revenue by the industry (Bedford 1998).

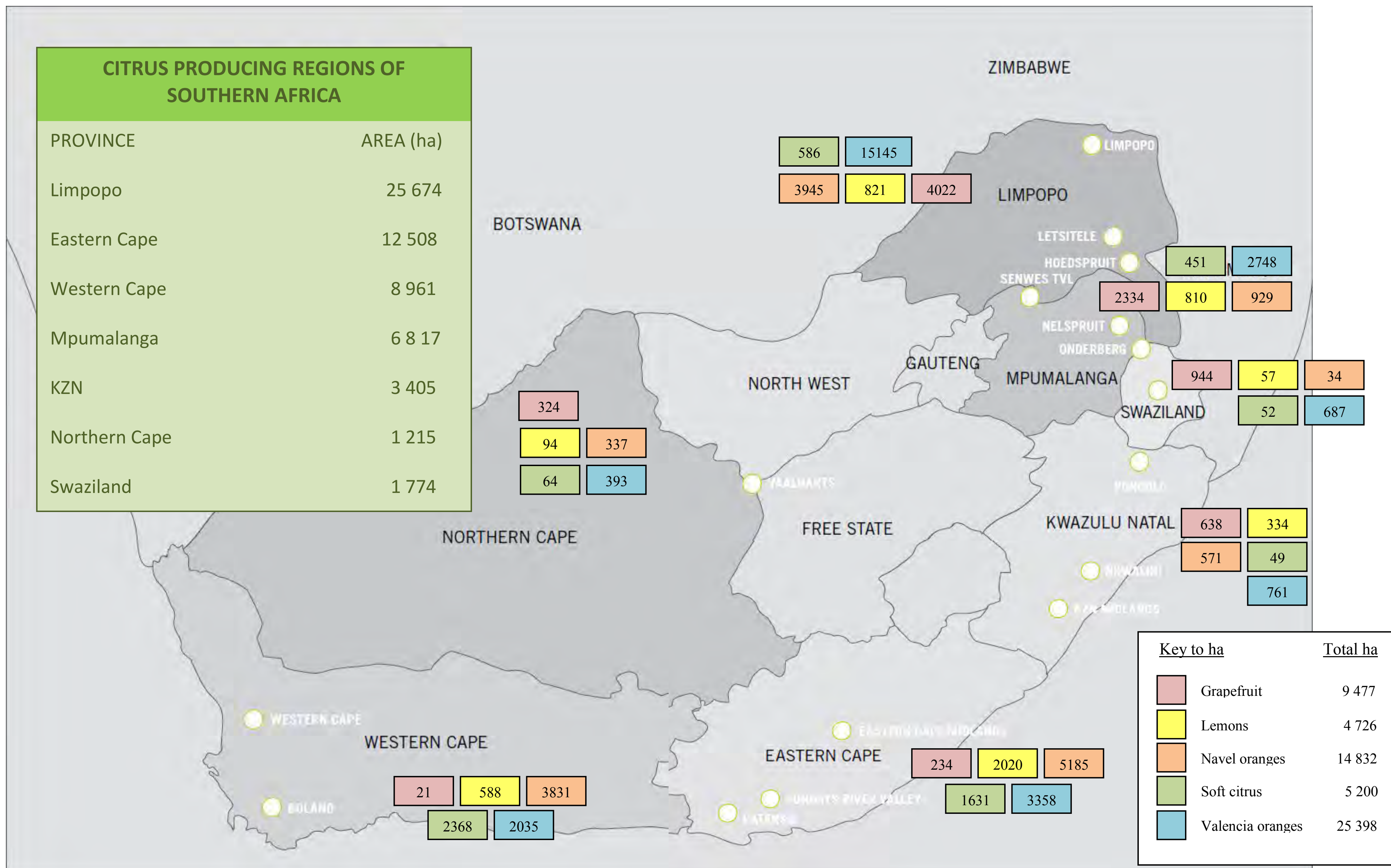

Figure 1.1: The citrus producing areas within the different provinces of South Africa (and Swaziland) including the number of hectares dedicated to the production of various citrus cultivars (Map: CGA Key Industry Statistics 2012, Cultivar production per province: CGA Annual Report 2012).

1.1.3 Citrus pests: A general overview

In South Africa, there are approximately 100 species of citrus pest, some of which are listed (Table 1.1 and 1.2). Of these, only a few are of major importance. As climatic conditions characterise the production distribution of citrus cultivars, citrus pests are also affected by various climatic conditions including temperature, rainfall and humidity. In addition, certain pests may only obtain pest status at certain times of the year when conditions are favourable (Bedford 1998).

Pests are classified as major or minor based on the extent of damage they cause, the frequency of their attacks and the parts of the citrus plant they attack (i.e. the branches, leaves, fruit or roots) (Smith & Peña 2002). The classification of pests however can be seen as graded with key and major pests occurring regularly throughout the season and causing the most damage (reduction in tree health or fruit loss) whilst minor pests are usually only present in small numbers or are easily controllable (Smith & Peña 2002). Some citrus pests, for example many of the scale insects, are regarded as secondary pests since under natural conditions population levels tend to remain low as a result of natural enemy control. If these natural enemies are disrupted e.g. via chemical spraying, an increase in scale pest populations will result (Smith & Peña 2002).

Smith & Peña (2002) listed six key southern African citrus pests, red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diapsidae), citrus thrips, *Scirtothrips aurantii* (Faure) (Thysanoptera: Thripidae) citrus psylla, *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae), Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), Natal fruit fly *Ceratitis rosa* (Karsch) (Diptera: Tephritidae) and false codling moth (Meyrick) (Lepidoptera: Tortricidae), as well as 12 major or occasionally important pests. Citrus mealybug, *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae), is now also considered a key pest of citrus (S.D. Moore*, pers. comm.). A number of minor pests however do exist including various orthopterans, snails, slugs, nematodes and ants which affect citrus indirectly through their cultivation of various scale insects or irritation of workers (Annecke & Moran 1982). Not all of these pests cause direct damage to citrus, but rather indirect damage via the transmission of various pathogens. For example, although citrus psylla does not cause severe damage through its actions, it transmits greening disease, a phloem-restricted bacterial disease which severely stunts the growth of citrus trees. As a

*Dr Sean D. Moore, Programme Manager: IPM, Citrus Research International (Pty) Ltd,
PO Box 20285, Humewood 6013, South Africa

result, these trees tend to produce unusable fruit (Annecke & Moran 1982; Smith & Peña 2002). Other insects, although they can cause damage in large numbers, merely cause cosmetic damage which reduces the quality of the crop and hence its monetary value. This is particularly common among the scale insects (Annecke & Moran 1982).

Table 1.1: Pests of citrus in South Africa (Insects) (Annecke & Moran 1982; Bedford 1998; Smith & Peña 2002; S.D. Moore, pers. comm.).

CLASS INSECTA			
Family	**Common name**	**Scientific name**	**Pest status**
		ORDER HEMIPTERA	
Diapsidae	Red scale	*Aonidiella aurantii* (Maskell)	Key
	Circular purple scale	*Chrysomphalus aonidum* (L.)	Minor
	Citrus mussel scale	*Cornuaspis beckii* (Newman)	Minor*
	Long mussel scale	*Lepidosaphes gloverii* (Packard)	Minor▲*
Coccidae	Soft brown scale	*Coccus hesperidum* (L.)	Minor▲*
	Soft green scale	*Pulvinaria aethiopica* (De Lotto)	Minor▲*
	Citrus wax scale	*Gascardia brevicauda* (Hall)	Minor*
	White wax scale	*Gascardia destructor* (Newstead)	Minor*
Pseudococcidae	Citrus mealybug	*Planococcus citri* (Risso)	Key
	Karoo thorn mealybug	*Nipaecoccus vastator* (Maskell)	Minor
	Oleander mealybug	*Paracoccus burnerae* (Brain)	Major▲
	Long-tailed mealybug	*Pseudococcus longispinus* (Targioni-Tozzetti)	Minor
	Striped mealybug	*Ferrisia virgata* (Cockerell)	Minor
Margarodidae	Australian bug	*Icerya purchasi* (Maskell)	Minor
Aphididae	Black citrus aphid	*Toxoptera citricida* (Kirkaldy)	Minor
	Cotton aphid	*Aphis gossypii* (Glover)	Minor▲
	Brown citrus aphid	*Toxoptera aurantii* (Boyer de Fonscolombe)	Minor
Cicadellidae	Citrus leafhopper	*Penthimiola bella* (Stål)	Minor▲
	Green citrus leafhopper	*Empoasca citrusa* (Theron)	Minor
Aleyrodidae	Citrus blackfly	*Aleurocanthus woglumi* (Ashby)	Minor
Triozidae	Citrus psylla	*Trioza erytreae* (Del Guercio)	Key
		ORDER THYSANOPTERA	
Thripidae	Thrips	*Scirtothrips aurantii* (Faure)	Key
		ORDER LEPIDOPTERA	
Papilionidae	Citrus swallowtail	*Papilio demodocus* (Esper)	Minor°
Tortricidae	False codling moth	*Thaumatotibia leucotreta* (Meyrick)	Key
	Apple leaf roller	*Tortrix capensana* (Walker)	Minor
	Citrus leaf roller	*Archips occidentalis* (Walsingham)	Minor
Geometridae	Citrus looper	*Ascotis selenaria* (Walker)	Minor
Noctuidae	American bollworm	*Helicoverpa armigera* (Hübner)	Minor▲
Pyralidae	Carob moth		Minor
		ORDER DIPTERA	
Tephritidae	Mediterranean fruit fly	*Ceratitis capitata* (Wiedemann)	Key
	Natal fruit fly	*Ceratitis rosa* (Karsch)	Key

* Decreased in importance as a result of successful control, particularly as a result of biological control
▲Occasionally important
° Important on young trees

Table 1.2: Pests of citrus in South Africa (Mites) (Annecke & Moran 1982; Bedford 1998; Smith & Peña 2002).

CLASS ARACHNIDA

Family	**Common name**	**Scientific name**	**Pest status**
	ORDER TROMBIDIFORMES		
Eriophyidae	Citrus bud mite	*Aceria sheldoni* (Ewing)	Major
	Citrus grey mite	*Calacarus citrifolii* (Kiefer)	Minor*
	Citrus rust mite	*Phyllocoptruta oleivora* (Ashmead)	Minor
Tetranychidae	Citrus red mite	*Panonychus citri* (McGregor)	Major
	Red spider mite	*Tetranychus cinnabarinus* (Boisduval)	Minor*▲
	Citrus flat mite	*Brevipalpus californicus* (Banks)	Minor
	Citrus silver mite	*Polyphagotarsonemus latus* (Banks)	Minor
	Oriental spider mite	*Eutetranychus orientalis* (Klein)	Minor▲

* Decreased in importance as a result of successful control, particularly as a result of biological control
▲Occasionally important

1.1.4 Control of citrus pests: A general overview

The control of insect pests can be achieved in a variety of ways. Prior to 1984, control focused solely on the use of chemicals and oil sprays such as resinwash, lime sulphur, nicotine sulphate, oil emulsions and fumigation with hydrogen cyanide (HCN). These were relatively harmless to both man and the pest's natural enemies. A number of these chemicals however were replaced with parathion, a chemical later discovered to be detrimental to the environment (Bedford 1998). With increasing importance placed on chemical control, the cost of control became substantial and outbreaks of certain pest species, in some cases species that were not originally considered economically important, began to occur. This resurgence of pests especially the resurgence of scale insects, particularly red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), was attributed to the decrease in natural enemies as a result of chemical spraying (Bedford 1998). In addition, the extensive use of chemicals resulted in resistance development of many pest species e.g. red scale to organophosphates. Stricter pesticide residue restrictions also began to be placed on exportable fruit, especially by foreign markets (Urquhart 1999). As a result, farmers were forced to seek alternate control options. Integrated pest management (IPM) therefore became an integral component of citrus production in the late 1900's and testing procedures have been

implemented to ensure the compatibility of control agents with IPM programmes (Bedford 1998).

IPM can essentially be defined as making use of a variety of control techniques aimed at negatively impacting the pest population whilst at the same time, minimising harmful effects to the environment. The aim of IPM is not to eliminate the entire population, although this can result in rare cases, but rather to control the pest population below a pre-determined economic threshold. Below this threshold, damage caused by the pest is no longer of economic importance (Pedigo 1996). IPM and the citrus industry are discussed in depth in Urquhart (1999) and Smith & Peña (2002) outline the requirements for a successful IPM programme. A key part of IPM is biological control.

Biological control focuses on the control of pest species using natural enemies. These enemies may be parasitoids, predators or microbes. The Australian bug, *Icerya purchasi* (Maskell) (Homoptera: Margarodidae), was successfully eliminated as a major pest in the South African citrus industry through predation by the vedalia beetle, *Rodolia cardinalis* (Mulsant) (Coleoptera: Coccinellidae). The vedalia beetle alone was responsible for the control of *I. purchasi* for a century until the emergence of new insect growth regulator pesticides were used for the control of red scale. These products reduced vedalia beetle populations which in turn prevented the successful biological control of *I. purchasi* (Bedford 1998). A number of other pest species including the scale insects are no longer major pests as their populations are kept under control by a suite of parasitoids (Bedford 1998). Some other pest species have been successfully controlled with microbial organisms (viruses, bacteria, fungi) (Lacey *et al.* 2001). Some important microbes include the fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin, the bacterium *Bacillus thuringiensis* (Berliner) and viruses belonging to the family Baculoviridae (Dolinski & Lacey 2007; Lacey & Shapiro-Ilan 2008). Microbes are ideal candidates for use in IPM programmes as many tend to be highly specific to a particular species or group and highly pathogenic (Lacey & Shapiro-Ilan 2003).

Other forms of control, incorporated into IPM strategies include behavioural control, genetic control and cultural control. Behavioural control may either involve disrupting the physiological state of the insects or alternatively, modify the behaviour of the insect (Pedigo

1996). In this way the life-cycle of the insect is disrupted and as a result, a reduction in the subsequent generation is generally observed (Pedigo 1996). An example of cultural control is orchard sanitation, a technique which involves the regular removal and correct disposal of fallen or infested fruit around the citrus trees. In false codling moth control, orchard sanitation has been shown to reduce infestation by between 40–75 % (Newton 1998; Moore & Kirkman 2009). Genetic control is largely concerned with sterile insect technique (SIT). In SIT, mass-reared males are sterilised through the use of radiation and released in large numbers into citrus orchards with the expectation that these sterile males will seek out and mate with wild FCM females in the area. The outcome of this mating however generally results in the deposition of non-viable eggs, ultimately leading to a reduction in pest population (Bloem *et al.* 2003).

In all aspects of IPM, monitoring is essential. Monitoring allows for the determination of pest levels and enables economic thresholds to be established. It allows farmers to time applications to coincide with the life cycles of pest species. This allows greater control and reduces the need for overuse of controlling agents (Smith & Peña 2002).

1.2 FALSE CODLING MOTH

1.2.1 Taxonomy

Thaumatotibia leucotreta more commonly known as false codling moth (FCM) belongs to the Order Lepidoptera, Family Tortricidae and has seen a shuffle in taxonomy since its original discovery (Newton 1998). It was first discovered in KwaZulu-Natal by Fuller (1901) who called it the Natal codling moth, *Carpocapsa* sp. (Newton 1998). Shortly afterwards, an orange codling moth was reported from the Transvaal region (Newton 1998). In 1912, Meyrick was the first to describe the moth as *Argyroploce leucotreta* (Eucosmidae, Olethreutidae) using the common name of today, FCM (van den Berg 2001). In 1958, it was transferred to a new genus, *Cryptophlebia* by Clark and 41 years later Komai removed the species *leucotreta* from this genus and subsequently placed it into the genus in which it currently resides, *Thaumatotibia* (Venette *et al.* 2003). The differences and similarities between the genera *Thaumatotibia* and *Cryptophlebia* are reviewed by Venette *et al.* (2003).

1.2.2 Distribution & Host Range

FCM is endemic to Africa south of the Sahara (Figure 1.2) and has been recorded in Congo, Nigeria, Somalia, Kenya, Ivory Coast and Uganda as a pest of cotton and in 1984, as a pest of Macadamia nuts in Israel (Wyoski 1986; Newton 1998). In South Africa it is a pest in all major citrus growing areas (Newton 1998). FCM may be confused with its relatives as a result of overlapping distribution and host range. Two of these relatives include the litchi moth, *Thaumatotibia peltastica* (Meyrick) (Lepidoptera: Tortricidae) and the macadamia nut borer, *Thaumatotibia batrachopa* (Meyrick) (Lepidoptera: Tortricidae) (Newton 1998). FCM may also be confused with another species of tortid namely the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) as a result of similar appearance and damage. Codling moths however, do not attack citrus (Venette *et al.* 2003).

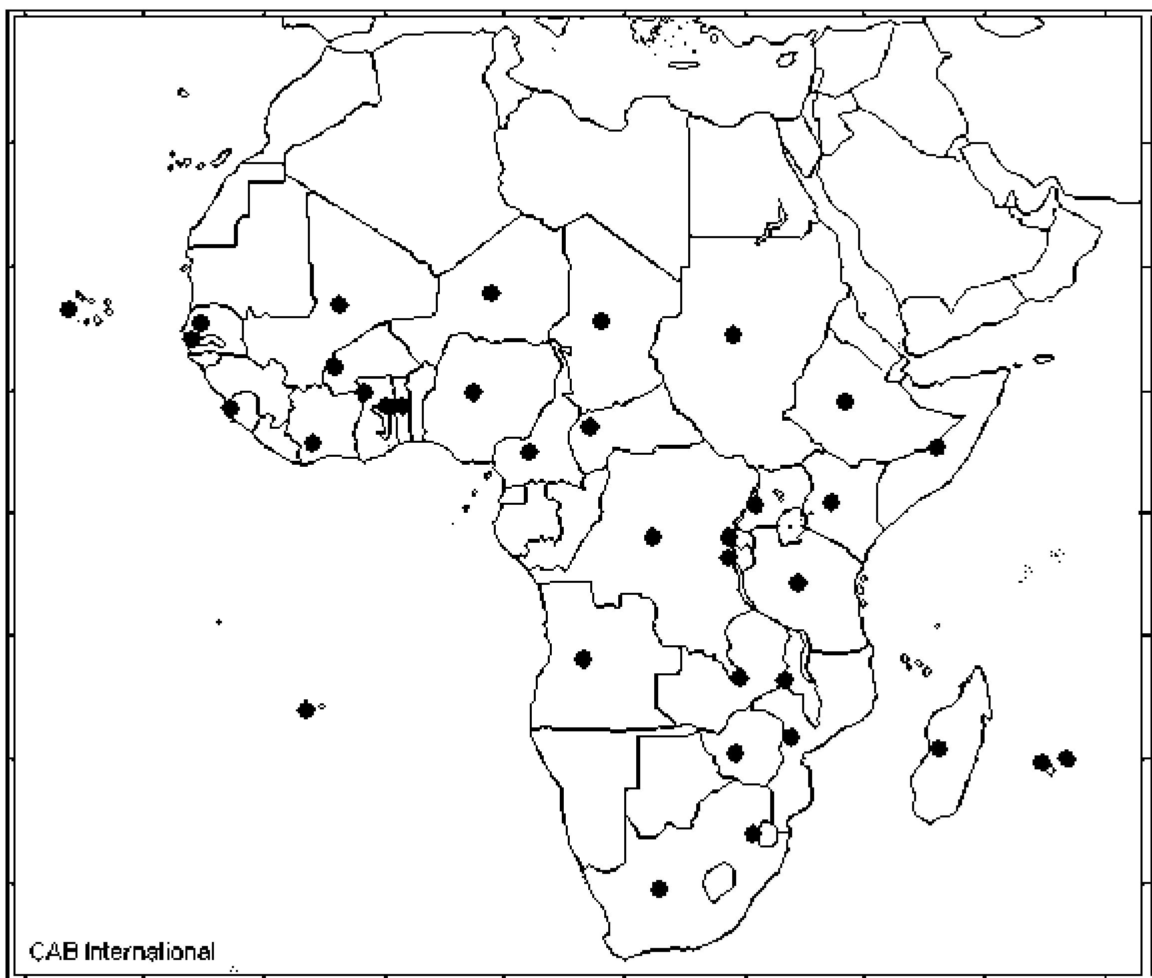

Figure 1.2: The geographic distribution of false codling moth, *Thaumatotibia leucotreta*, in Africa. Bullets indicate the countries in which the moth is present (Stibick 2008).

FCM has an extremely broad host range and has been recorded on approximately 24 cultivated plants and 50 wild plants. The common and scientific names of these plants can be found in Kirkman (2007). However, it must be noted that a number of these are forced associations observed only in the laboratory. In South Africa, citrus is its most preferred host (Annecke & Moran 1982). With the exception of lemons and limes, possibly owing to their higher acidity levels, all citrus cultivars are susceptible to attack with Navel oranges being the most favoured (Newton 1998). The broad host range of FCM enables it to establish and maintain population numbers even when not feeding on citrus (Van den Berg 2001).

1.2.3 Life history

FCM eggs (Figure 1.3A) are small, oval, translucent and pearl-white in appearance measuring approximately 0.77 mm in length and 0.60 mm wide (van den Berg 2001). Prior to hatching, a black spot can be observed and the egg becomes slightly reddish (Newton 1998). The egg stage lasts between 2–22 days. In high densities, more than one egg may be laid on the rind of the fruit, but usually only a single egg is laid. Egg laying usually occurs in the evening between 17:00 and 23:00 (Stibick 2008). As a result of their appearance, the eggs are not easily visible on fruit and are therefore missed during inspection of orchards (Newton 1998).

Based on the width of the head capsule, five larval instars are apparent (Kirkman 2007). The neonate or first instar larvae are relatively small in size (1.5 mm in length) and are creamy-white in appearance. They are fragile and can often suffer high levels of mortality, particularly in response to cold temperatures (Newton 1998). Generally only one larva develops within a single fruit. This is likely attributed to the fact that the moths lay their eggs singly (S. D. Moore, pers. comm.). Cannibalistic behaviour has been reported amongst FCM larvae although this is minimal and rarely occurs (Newton 1998; S.D. Moore, pers. comm.). The final instar larvae (Figure 1.3B) measure 12–15 mm in length and are pink-red in colouration (van den Berg 2001). Shortly after hatching the neonates bore into the fruit where they feed on the softer inner parts and develop until the final instar is reached (Newton 1998). Entry usually occurs where damage is present or at the navel end (Ludewig 2003). When ready to pupate, the final instar larva burrows out of the fruit leaving behind a frass-filled exit-hole through which it drops to the ground burying itself within the soil (van den Berg 2001). Larval development can take between 25–67 days depending on the season and quality

of the fruit. Once in the soil, the larva, using silk and soil particles, spins a cocoon, moulting into a pre-pupa and then a pupa (Figure 1.3C). The length of the pre-pupal stage ranges between 2–27 days and the pupal stage, 11–39 days depending on temperature (Stibick 2008).

The adult moth (Figure 1.3D) can be identified by its mottled grey forewings and paler hindwings with a wingspan of 15–20 mm. The males are generally smaller than the females and can be distinguished phenotypically by the presence of black anal tufts and a scent organ on the anal angle of each hindwing (van den Berg 2001). Females are polyandrous and can oviposit up to 450 eggs during their 1–3 week lifespan (Annecke & Moran 1982; Stibick 2008). Total development time is dependent on a number of environmental factors including temperature and food quality (Stibick 2008), averaging shorter in summer (1½ – 2 months) than in winter (2½ – 4 months). FCM does not undergo diapause during winter and 5–6 generations can overlap each year (Newton 1998).

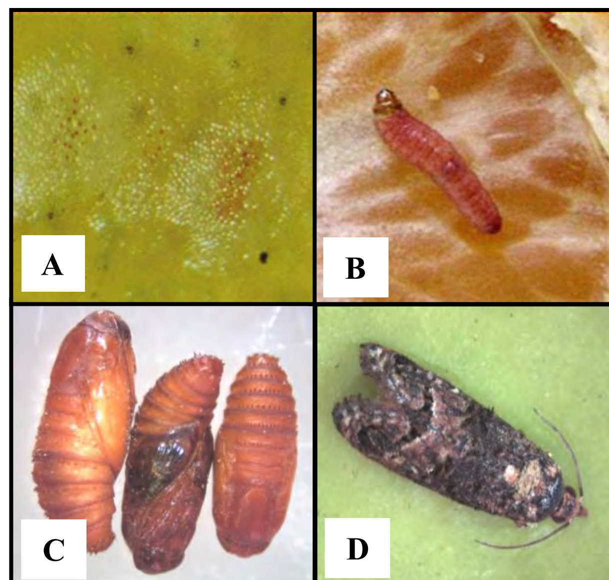

Figure 1.3: FCM life stages. (A) eggs; (B) final instar larva; (C) pupae; (D) adult [River Bioscience (A); Varela (2005), ICIPE (B)].

1.2.4 Economic importance

Shortly after FCM larvae emerge from their eggs they bore into the fruit causing problems in the form of fruit decay, premature ripening and early dropping, ultimately resulting in a reduction in crop yield (Newton 1998) (Figure 1.4). In addition, if this infestation occurs shortly before harvesting, FCM larvae may not be detected prior to packaging and shipping resulting in post-harvest decay (Moore 2002a). Surveys conducted by Newton *et al.* (1986) (cited by Newton 1998), indicated that fruit loss as a result of FCM damage contributed 20–30 % of total fruit drop in Nelspruit and even higher in Citrusdal. Fruit drop due to infestation may be apparent as early as November, but usually peaks in February – May and December – March in the Western and Eastern Cape respectively (Newton 1998; Moore 2002a). Foreign markets also regard FCM as a phytosanitary pest and will reject entire consignments in its presence (Moore 2002a; Kirkman 2007). The USA in particular is concerned with the establishment of FCM due to a similar climate to that of South Africa. If the moth were to establish in the USA, a substantial economic loss would be experienced (Stibick 2008). The citrus industry in South Africa also exports the bulk of its production. The income generated through exports therefore contributes substantially to the total annual income generated by the industry (CGA Key Industry Statistics 2012). The control of FCM is therefore of extreme importance.

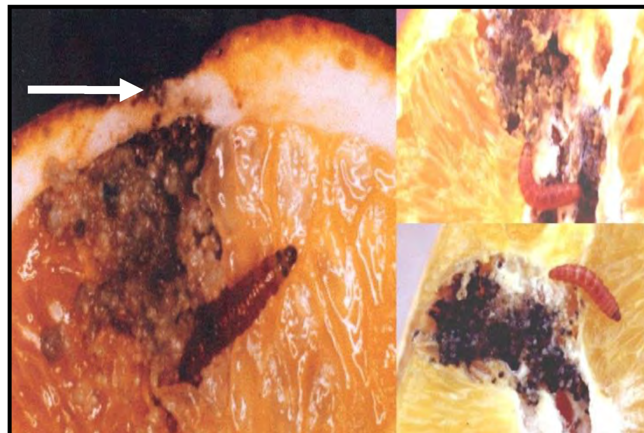

Figure 1.4: The damage caused by FCM larvae on citrus. Note the black granular frass-filled exit tunnel and the start of fruit decay (indicated by the arrow) (Ludewig 2003).

1.2.5 Control

The control of FCM in South Africa takes a variety of forms and incorporates pre-harvest monitoring, cultural control, chemical control, behavioural and genetic control, biological control as well as, in some cases, post-harvest control.

Pre-harvest monitoring essentially serves as an early warning system, rather than a control strategy and generally involves the use of FCM female-sex pheromone and adhesive based traps e.g. Lorelei traps. The pheromone attracts the male moths which subsequently become attached to the trap via the adhesive. As a result, farmers are able to estimate the level of FCM numbers within an orchard or in areas in the near vicinity of the orchard (Moore *et al.* 2008).

One of the earliest and still most important control methods is that of orchard sanitation (Newton 1998). This method involves the regular removal and proper disposal of all infested fruit on both trees and the ground as up to 75 % of all dropped fruit may still be infested with FCM larvae (Moore & Kirkman 2009). Orchard sanitation therefore remains extremely important and forms an integral part of any FCM control programme (Newton 1998).

Currently there are six chemical products registered for the control of FCM in citrus orchards namely Alystin® (a.i. triflumuron) (Bayer, Germany), Nomolt® (a.i. teflubenzuron) (Cyanamid, South Africa), Meothrin (Sanachem, South Africa), cypermethrin (Agropharm, South Africa), Delegate® (Dow AgroSciences, Indianapolis), and recently, Coragen® (a.i Rynaxypyr®) (DuPont, South Africa) (Moore & Hattingh 2012). Alystin® and Nomolt® are both chitin synthesis inhibitors and function by disrupting larval development in the egg stage. These chemicals are however prohibited for use on fruit exported to the US markets and are known to impact certain natural enemies of FCM (Kirkman 2007). Meothrin and cypermethrin are both pyrethroids and can have detrimental effects on a wide range of natural enemies. These two chemicals are therefore not ideal for use in an integrated pest management approach (Moore *et al.* 2004). Delegate® makes use of a chemically modified spinosyn compound as its active ingredient and is considered to have a broad host range, but with low impact on beneficial insects. Coragen® is the most recent chemical addition to FCM control and causes both larvicidal and ovicidal effects via a calcium disruption mechanism (Miletić *et al.* 2011). In recent years however, there has been a need to limit the use of

chemicals in citrus orchards, not only due to their undesirable environmental effects, but also owing to stricter residue restrictions which have been placed on citrus by overseas markets (Inceoglu *et al.* 2001). Biological control has therefore become a favourable option for many citrus growers (Urquhart 1999).

Two behavioural control methods exist, namely mating disruption and the attract and kill technique. These two are similar to each other in that they both attract male moths through the use of female pheromones, ultimately reducing the number of mating opportunities and hence the amount of viable eggs oviposited. However, instead of merely disrupting mating, the attract and kill method causes the death of attracted males via an associated insecticide. Mating disruption has seen success in the deciduous fruit industry of the Western Cape against a close relative, the codling moth (Kirkman 2007) and is thought to be more effective than the attract and kill technique which requires a large proportion of the population to be removed before satisfactory control may be achieved (Kirkman 2007). Two products are registered for mating disruption; Isomate and Checkmate FCM-F whilst only one, Last Call FCM, is registered for attract and kill (Moore & Hattingh 2012).

SIT, a genetic control method discussed previously, is also used for the control of FCM (Kirkman 2007). Initial investigation into the use of SIT for FCM control was initiated by Myburgh in the 1960's and later continued in the 1970's and 1980's by Schwartz and Du Toit respectively (Bloem *et al.* 2003). Hofmeyr *et al.* (2005) have shown the effectiveness of SIT in FCM control using field cage trials. In cages where different ratios of treated (=sterile): untreated males where placed with wild females, the F_1 adult progeny was reduced, the number of larval entries per fruit was lower and the number of undamaged fruit collected was higher than recorded in the control cages indicating that sterile males could successfully compete for wild females. The higher the treated: untreated male ratio, the greater the level of control (Hofmeyr *et al.* 2005). SIT is conducted in the Citrusdal area of the Western Cape and the Sundays River Valley in the Eastern Cape (Kirkman 2007).

The biological control of FCM involves a variety of predatory, parasitic and microbial organisms. The majority of these are insects belonging to the order Hymenoptera with the larval stage most commonly affected (Newton 1998). Moore (2002) lists 17 parasitoids (14 hymenopterans and three tachnid species), four predators (three hemipterans and one mite) and four microbes (two fungi and two viruses). The hymenopteran egg parasitoid,

Trichogrammatoidea cryptophlebia (Nagaraja) (Hymenoptera: Trichogrammatidae) has been shown to reduce FCM population levels by up to 60 %. Success however, is only accomplished when four releases at the application rate of 25 000 insects.ha^{-1} is adhered to (Kirkman 2007). Other key parasitoids include *Apophua leucotreta* (Wilkinson) (Hymenoptera: Ichneumonidae) and *Agathis bishopi* (Nixon) (Hymenoptera: Braconidae) (Annecke & Moran 1982; van den Berg 2001). *Trichogrammatoidea cryptophlebia* is however the only parasitoid currently registered for FCM control (Moore & Hattingh 2012)

Microbial control has become more prominent in biological control strategies and has seen the formulation of granulovirus products, Cryptogran® (River Bioscience, South Africa) and Cryptex® (Andermatt, Switzerland). These viral products make use of the *Cryptophlebia leucotreta* granulovirus, a virus highly virulent and specific to the larvae of FCM which become infected upon ingestion (Moore 2002a; Ludewig 2003; Kirkman 2007). Various fungal entomopathogens, especially *Metarhizium* sp. and *Beauveria bassiana*, have also shown potential against FCM although their formulation into commercially available products has had limited success (van den Berg 2001; Goble *et al.* 2011).

Post-harvest control of FCM generally takes the form of cold sterilization of fruit enroute to the major export markets (Newton 1998; Moore 2002a; Kirkman 2007). Myburgh (1965) showed the effectiveness of cold sterilization against all larval stages when a temperature of -0.6 °C and exposure time of 22 days was used. Recently, Boardman *et al.* (2012) showed that FCM larvae exposed to cold temperature of between -14 °C to -18 °C for more than an hour could not recover (100 % mortality) likely because of freezing which was found to occur between -13.4 °C and -22 °C. Larval mortality was however first recorded at -8 °C and an increase in mortality at this temperature was associated with an increase in exposure time suggesting that at this higher temperature high levels of mortality may be obtained if long exposure times are used (Boardman *et al.* 2012). The process of cold sterilization is however very expensive (Moore 2002a). Nuclear irradiation has been investigated as an alternative to cold sterilization, but must be further developed if implementation is to be practical (Kirkman 2007).

1.3 ENTOMOPATHOGENIC FUNGI

1.3.1 Classification

There are approximately 1.5 million species of entomopathogenic fungi (EPF) worldwide of which approximately only 7.4 % (110 000 species) have been described. Of these, only about 700 species, distributed over nine genera, are considered to be entomopathogens (Roy *et al.* 2010). An entomopathogen can be defined as a pathogenic (disease-causing) organism and includes fungi, bacteria, viruses as well as nematodes which cause harm to insects in particular, although sometimes can infect other arthropods such as spiders (Shah & Pell 2003).

It is likely that EPF arose multiple times during the course of fungal evolution (Humber 2008). Majority of EPF fall within the division Zygomycota and Ascomycota. The latter was previously separated into the Ascomycota and Deuteromycota, also referred to as Fungi Imperfecti as species within this division had no sexual state (Roy *et al.* 2006; Blackwell 2010). However with recent molecular techniques, it has been shown that many of these asexual fungi (anamorphs), shared similarities with the ascomycetes and in addition could be linked to sexual states (telomorphs) (Inglis *et al.* 2001). Within the Ascomycota, the majority of the EPF fall within the order Hypocreales including well known species such as *Beauveria* (Balsamo), *Metarhizium* (Metschnikoff), *Isaria* (=*Paecilomyces*) (Samson) and *Lecanicillium* (=*Verticillium*) (Gams and Zare). Within the Zygomycota, the majority of the fungi are found within the order Entomophthorales (Inglis *et al.* 2001).

1.3.2 General biology

EPF have a cosmopolitan distribution and can be isolated from a wide variety of soil types (Cory & Ericsson 2010). EPF in the division Ascomycota are largely opportunistic pathogens and as a result have a broad host range (Shah & Pell 2003). In addition they exhibit hemibiotrophy whereby initially they are biotrophic, but later switch to necrotrophy (Roy *et al.* 2006). Biotrophy is characterised by the process of obtaining nutrients from living cells ceasing only when nutrients become depleted whereas, in contrast, necrotrophy is the process of obtaining nutrients from dead cells (Vega *et al.* 2009). Different species and different

isolates can have different environmental requirements, environmental tolerances and host ranges (Cory & Ericsson 2010). All EPF, however, have a similar infection process (Roy *et al.* 2006).

Fungi are distinctly different from other entomopathogens in that they do not have to be ingested before infection can occur (Cory & Ericsson 2010). The infection process (Figure 1.5A) is initiated when a fungal propagule adheres to the host cuticle. In some cases, the infection process proceeds no further due to host resistance or lack of recognition between fungus and insect (Castrillo *et al.* 2005). The attachment process in the case of some EPF such as *B. bassiana* and *M. anisopliae* is a passive process resulting from hydrophobic interactions between the insect cuticle and rodlets of hydrophic proteins in the conidia. Infection proceeds with the germination of the conidium to form a germ tube and appressorium which gives rise to a thin penetration peg. This peg is responsible for penetrating the insect cuticle (Inglis *et al.* 2001). Penetration is achieved via turgor pressure as well as through the excretion of chitin-degrading enzymes. Once through the cuticle invasion of the insect circulatory system (haemolymph) and rest of the body occurs. Within the haemolymph, infection is spread by yeast-like cells called blastospores. The host will ultimately succumb to infection 3–7 days post infection. Hyphal growth will then occur and the fungi will exit through the less sclerotised areas of the cuticle (Figure 1.5B) and disperse passively (Shah & Pell 2003). Some EPF also produce secondary metabolites some of which have insecticidal properties such as destruxins (cyclic peptide toxins) which are produced by many *Metarhizium* spp. Other toxins produced largely by *Beauveria* spp. include oosporein, beauvericin and bassianolide (Castrillo *et al.* 2005). These toxins have been shown to increase the rate at which insects succumb to the fungal infection (e.g. McCauley *et al.* 1968).

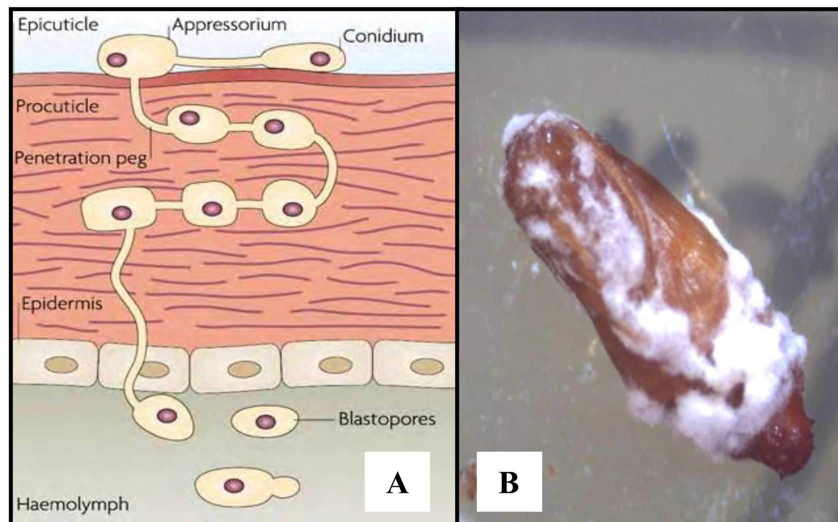

Figure 1.5: (A) Infection process followed by an entomopathogenic fungus (Goble 2009); (B) Fungal exiting through the less sclerotised areas of a FCM pupa.

The infective relationship between insects and EPF is an evolutionary arms race. Fungi evolve adaptations to ensure infection whereas insects evolve adaptations to prevent infection (Roy *et al.* 2006). Insects have both humoral and cellular defences for infection prevention. Preliminary defence mechanisms are activated upon attachment of the conidia and penetration of the cuticle (Castrillo *et al.* 2005). These mechanisms usually involve the encapsulation of germ tubes or upon fungal penetration into the haemocoel, phagocytic activities, nodule formation prevention as well as encapsulation (Castrillo *et al.* 2005). Most fungi however, particularly the virulent strains, have evolved mechanisms to evade or overcome the insect detection and defence system (Baverstock *et al.* 2010). The effect EPF can have on their host is reviewed thoroughly in Roy *et al.* (2006) and includes reduced fecundity, reduced feeding, mate attraction and changes in behaviour.

1.3.3 Use as biological control agents

The use of EPF as control agents began as early as 125 years ago when Metchnikoff suggested the use of a green muscardine fungus, *Entomophthora* (=*Metarhizium*) *anisopliae* for the control of the grain beetle, *Anisoplia austriaca* (Herbst) (Coleoptera: Scarabaeidae) (Lord 2005). To date, approximately 170 products have been developed using at least 12 species of EPF to control a variety of pest species (Vega *et al.* 2009; Jackson *et al.* 2010). A comprehensive list of registered EPF products, what they control and the countries in which

they are registered for commercial use is given in de Faria & Wraight (2007) (Table 1.3). A number of other EPF have not been commercialised into products, but are still used as control agents in certain IPM programmes (Baverstock *et al.* 2010). In nature, most EPF either belong to the Hypocreales or Entomophthorales. However, many of the commercialised fungi belong only to the Hypocreales due to the difficulty in mass producing fungi in the Order Entomophthorales, possibly as they tend to be obligate biotrophs (Dolinski & Lacey 2007; Hesketh *et al.* 2010). Hajek & Dalalibera Jr (2010) investigated fungal pathogens as classical biological control agents and found that EPF have been used in 49.3 % of all programmes making use of various arthropod pathogens with the most commonly introduced species being that of *M. anisopliae.* Along with *M. anisopliae, B. bassiana* is also commonly used as the active ingredient in a number of products (de Faria & Wraight 2007). These two fungal species with regard to their morphology, infection process and safety are discussed in detail in Zimmerman (2007a,b).

Table 1.3: Examples of products registered for the control of various pest species worldwide (de Faria & Wraight (2007). A more comprehensive list can be found in de Faria & Wraight (2007).

Fungal Species	Product	Country*	Controls (Family names)
Beauveria bassiana	Tricho-bass L	Spain	Curculionidae, Scarabaeidae, Castniidae, Pieridae, Aleyrodidae, Thripidae, Tetranychidae
	Bb Plus	South Africa	Aphididae, Tertanychidae
	Bb Weevil	South Africa	Curculionidae
	BioGaurd Rich	India	Curculionidae, Scarabaeidae, Aleyrodidae, Aphididae, Crambidae, Thripidae
	Racer	India	Noctuidae and other unspecified insects
	Biolisa-Madara	Japan	Cerambycidae
	Bea-Sin	Mexico	Curculionidae, Scarabaeidae, Aleyrodidae
	Balence	USA	Muscidae
	Naturalis L	USA, Mexico, Greece, Italy, Spain, Switzerland	Many insect species
	Mirabiol	Nicaragua	Curculionidae
	Bb Moscas	Argentina	Muscidae
	Bioexpert	Colombia	Aleyrodidae, Thripidae
Beauveria brongniartii	Myzel	Switzerland	Scarabaeidae
	Betel	Reunion Island	Scarabaeidae
Conidiobolus thromboides	Vektor 25 SL	Colombia, Honduras, Costa Rica	Aleyrodidae, Ortheziidae
Hirsutella thompsonii	Mycohit	India	India
Isaria fumosorosea	Priority	India	India
	Pae-Sin	Mexico	Mexico
	Fumosil	Colombia	Colombia, Aleyrodidae, Aphididae, Pseudococcidae, Thripidae
Lagenidium giganteum	Laginex AS	USA	Culicidae
Lecanicillium longisporum	Vertalec	Finland, UK, Switzerland, Japan	Aphididae
	Vertirril WP 1300	Brazil	Aleyrodidae, Ortheziidae

Table 1.3 *continued...*

Fungal Species	Product	Country*	Controls (Family names)
Lecanicillium muscarium	Mycotol	Netherlands, Denmark, Finland, France, Italy, Switzerland, UK, Turkey, Japan	Aleyrodidae, Thripidae
Metarhizium anisopliae var. anisopliae	GranMet-P	Austria, Italy	Scarabaeidae, Curculionidae, Nitidulidae
	Bio 1020	Switzerland	Curculionidae
	Bio-Magic	India	Curculionidae, Scarabaeidae, Cercopidae and other plant hoppers
	Biomet Ric	India	Many families within the order Coleoptera and Isoptera
	Biogreen	Australia	Scarabaeidae
	Fitosan-M	Mexico	Scarabaeidae, Orthopteran families
	Tick-EX G	USA	Acari families, Scarabaeidae
	Metadieca	Costa Rica, Panama	Cercopidae
	Metarhisa WP	Nicaragua	Curculionidae, Cercopidae, Crambidae
	Biotech	Brazil, Panama	Cercopidae
	Metanat	Brazil	Cercopidae, Aphididae
	Metariz	Brazil	Cercopidae
	Deep green	Colombia	Scarabaeidae and many hemipterans
	Dextruxin 50 WP	Colombia, Costa Rica, Honduras, Nicaragua	Curculionidae, Scarabaeidae, Miridae, Cercopidae, Delphacidae, Noctuidae
Metarhizium anisopliae var. acridum	Green Muscle OF	Mozambique, Namibia, Tanzania, South Africa, Sudan, Zambia	Acrididae, Pyrgomorphidae
	Green Gaurd	Australia	Acrididae

* The countries mentioned are those where the product is currently being registered, is already registered or is marketed

There are a number of traits which encourage the use of fungi as control agents, including infection occurring via the insect cuticle as opposed to ingestion, safety towards vertebrates, host specificity, environmental safety particularly if indigenous strains are used and also because they leave no toxic residue on crops (Inglis *et al.* 2001; Zimmerman 2007a,b). The downside however is that their control is largely dependent on host (target pest) population numbers which is in contrast to chemical control which is not density dependent and provides almost immediate results (Inglis *et al.* 2001). However, EPF can persist in the environment for long periods of time providing long term control and acting as a barrier preventing the resurgence of target pest populations (Cory & Ericsson 2010).

There are also a number of abiotic (humidity, temperature, soil texture, sunlight, rainfall and various chemicals) and biotic factors (response of target insect (immune and behavioural) as well as soil microbiota) which could influence the activity, fitness, transmission efficiency and persistence of fungal pathogens (Lord 2005; Baverstock *et al.* 2010; Hesketh *et al.* 2010). The factors affecting both foliar applied and soil applied EPF are discussed by Jaronski *et al.* (2010). For this reason, the efficacy of fungal pathogens showing potential for control of pests under laboratory conditions must be tested under more natural (field) conditions. Of the factors mentioned, humidity is considered to be the most important as a low humidity (< 90 %), prevents the successful germination, infection and sporulation of the fungus (Hesketh *et al.* 2010). However other factors, such as temperature, can influence the rate at which infection and hence death occurs (Hesketh *et al.* 2010). For example, studies of two *B. bassiana* and four *M. anisopliae* strains showed that at 15 °C, germination, radial growth and pathogenetic activity were low whereas between 25–30 °C, these were at their optimum (Ekesi *et al.* 1999). Regardless of these factors, EPF have still achieved success and are still showing potential in the successful control of various pest species (Shah & Pell 2003; Dolinski & Lacey 2007) (Table 1.4). A number of EPF have also been used to control various citrus pests (Table 1.5). To date, no EPF have been used to control citrus pests in South Africa even though a number of EPF species have been reported to naturally attack a variety of these pests (Moore 2002b).

Table 1.4: Insect pests or outbreak species which are controlled by EPF (Shah & Pell 2003; Scholte *et al.* 2004; Er *et al.* 2007; Loc & Chi 2007; Dembilio *et al.* 2010; Pell *et al.* 2010).

Pest species	Common name	Fungal control species
Lymantria dispar L. (Lepidoptera: Lymantriidae)	gypsy moth	*Entomophaga maimaiga*
Aphis gossypii Glover (Hemiptera: Aphididae)	cotton aphid	*Neozygites fresenii*
	various aphids	*Pandora neoaphidis, Verticillium lecanii*
Plutella xylostella L. (Lepidoptera: Plutellidae)	diamond back moth	*Beauveria bassiana, Metarhizium anisopliae, Zoophthora radicans*
Melolontha melolontha L. (Coleoptera: Scarabaeidae)	European cockchafer	*Beauveria brongniartii*
Rhynchophorus ferrugineus Olivier (Coleoptera: Curculionidae)	red palm weevil	*Beauveria bassiana*
Thaumetopoea pityocampa Schiff. (Lepidoptera: Thaumatopoeidae)	pine processionary	*Isaria Fumosoroseus*
Anopheles gambiae Giles (Diptera: Culicidae)	mosquito	*Metarhizium anisopliae*
Mononychellus tanajoa Bondar (Acari: Teranychidae)	cassava green mite	*Neozygites tanajoae*
Agrotis segetum Schiff. (Lepidoptera: Noctuidae)	turnip moth	*Tolypocladium cylindrosporum*

Table 1.5: Examples of some fungal pathogens which have been used against or are known to cause mortality in citrus pests (Dolinski & Lacey 2007).

Pest species	Common name	Fungal control species
Phllocoptruta oleivora	citrus rust mite	*Beauveria bassiana* *Hirsutella thompsonii (*Fisher) (Zygomycota: Entomophthorales)
Panonychus citri	citrus red mite	*Beauveria bassiana*
Polyphagotarsonemus latus (Banks) (Acari: Tarsonemidae)	boad mite	*Beauveria bassiana*
Brevipalpus phoenicis (Geijskes) (Acari: Tenuipalpidae)	false spider mite	*Hirsutella thompsonii*
Dialeurodes citri	citrus whitefly	*Aschersonia* spp
Selenaspidus articulatus (Morgan) (Hemiptera: Diaspididae)	rufous scale	*Aschersonia aleyrodis* (Weber) (Ascomycota: Hypocreales)
Chrysomphalus aonidum, Parlatoria ziziphus (Lucas) (Hemiptera: Diaspididae), Cornuaspis beckii,	circular purple scale, black parlatoria scale, citrus mussel scale	*Podonectria coccicola* (Ellis & Everhart) Petch (Ascomycota: Hypocreales) *Pseudomicrocera henningsii* (Koord.) Petch (Ascomycota: Hypocreales) *Sphaerostilbe aurantiicola* (Berk & Br.) Petch (Ascomycota: Hypocreales)
Toxoptera citricida	brown citrus aphid	*Beauveria bassiana*
Ceritatis spp.	fruit flies	*Beauveria bassiana* *Metarhizium anisopliae*

1.4 RESEARCH AIMS

Goble *et al.* (2010, 2011) sampled and screened various citrus orchards and surrounding refugia for fungal isolates which exhibit potential for use in the control of soil-borne life stages of FCM. A total of 62 fungal isolates were identified of which 21 were screened for their control potential. Twelve of these isolates resulted in pupal mycosis of greater than 80

% and adult emergence less than 20 %. Four of these isolates (FCM 10 13 L1, G B Ar 23 B3, G Moss R10 and G 14 2 B5) were further investigated in the form of concentration dose-response and exposure time-response assays. Investigation of the twelve remaining isolates still needed to occur. Testing towards a more natural environment is also needed to determine the efficacy of these isolates under field conditions.

Thus, this thesis aimed to (1) re-screen all twelve isolates (FCM 10 13 L1, G Moss R10, G B Ar 23 B3, G 14 2 B5, G Ar 17 B3, G 11 3 L6, G 14 2 B3, G OL R8, G OL R11, FF J&B R5, FCM Rose R9, FCM Ar 23 B3) to ensure that they were performing as previously; (2) complete concentration dose-response assays to determine the LC_{50} and LC_{90} values of all the fore-mentioned isolates. This however was provided that the respective isolate obtained similar mortality percentages as found by Goble *et al.* (2011). The assays themselves followed the same procedure as outlined in Goble *et al.* (2011). For comparative purposes, two commercially available isolates, *Beauveria bassiana* strain 4222 (Eco-Bb) (PHP, South Africa) and a *Metarhizium anisopliae* isolate ICIPE 69 (Real IPM, Kenya), were also evaluated; (3) complete exposure time-response assays to determine the LT_{50} and LT_{90} values of three of the most promising isolates. The isolates used were chosen based on their performance in the previous bioassay. The assay followed a similar procedure as described by Goble (2009); (4) determine the persistence of these three isolates under field conditions over a period of six months and (5) determine an application method which could be improved upon for use in the field.

2

Use of EPF to control FCM: Laboratory bioassays

2.1 INTRODUCTION

In the early 19th century it was discovered that many natural epizootics are a result of infection by microscopic organisms found in the environment. Since this discovery, these organisms have been studied, manipulated, mass produced and formulated for use in pest control management (Lord 2005). There are well known cases in which EPF have been used to control insect populations. Two such cases include the control of grasshoppers and locusts in Africa using *Metarhizium anisopliae* and larvae of the gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) using *Entomophaga maimaiga* (Humber, Shimazu, Soper and Hajek) (Entomophthorales: Entomophthoraceae) (Shah & Pell 2003). In addition, numerous studies have noted the potential of EPF to control various insect pest species under laboratory conditions such as diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (Loc & Chi 2007), the pine processionary caterpillar, *Thaumetopoea pityocampa* (Schiff.) (Lepidoptera: Thaumatopoeidae) (Er *et al.* 2007), coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae) (Vera *et al.* 2010), citrus rust mite, *Phyllocoptruta oleivora* (Koch) (Acari: Tetranychidae) (Alves *et al.* 2005), red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) (Dembilio *et al.* 2010) and Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (Castillo *et al.* 2000). Since soil acts as a reservoir for most entomopathogenic fungal species, interest has been shown in their use to control the soil-borne life stages of various pest species (Meyling & Eilenberg 2006). FCM 5th instar larvae pupate within the upper centimetres of the soil and therefore EPF could be used as an additional control strategy against this pest (Stibick 2008).

The 12 isolates investigated in this study comprised two species, *Beauveria bassiana* and *Metarhizium anisopliae* var. *anisopliae.* Identification of these species relies mostly on the shape and development of the conidia (fungal spores) rather than their physical appearance on culture media. Morphologically *B. bassiana* is a powdery white-cream becoming yellow

in colour with age, whilst *M. anisopliae* is varying shades of green. The conidia of *B. bassiana* are easily identifiable by their ovoid spores which are held on singular conidiophores often inflated at the base and tapered towards the spore producing end. A characteristic zig-zig shape remains behind after the production of several spores (Figure 2.1A). Conidia of *M. anisopliae* however are produced in basipetal chains and are compacted to form columns. The conidia themselves are cylindrical or long-ovoid in shape (Figure 2.1B) (Barnett 1960). Both these species are considered safe to vertebrates and although they are known to have a wide host range, different strains tend to have restricted host ranges making them suitable for use in biological control programmes (Zimmermann 2007a,b). In addition these isolates are easy and relatively inexpensive to cultivate on artificial media, an advantageous trait if commercialisation is to be considered (Kaya & Lacey 2007).

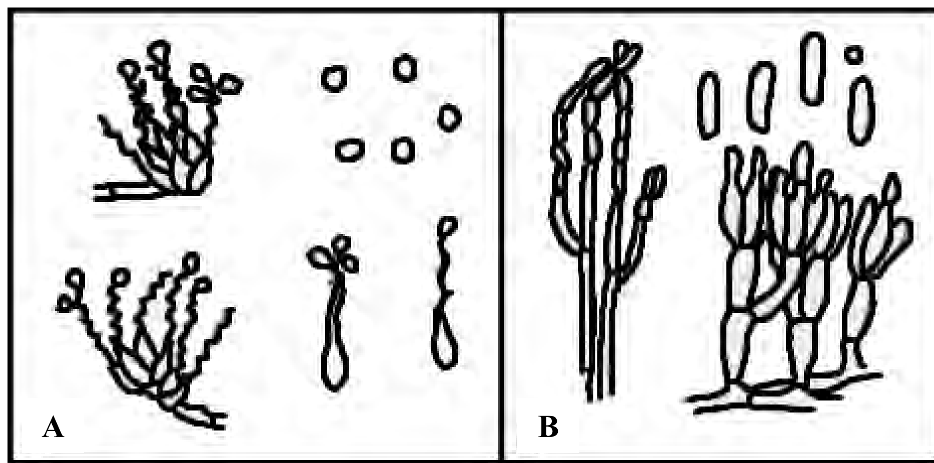

Figure 2.1: (A) Appearance of *Beauveria bassiana* conidia; (B) *Metarhizium anisopliae* conidia (Barnett 1960).

The potential of EPF as biological control agents can be ascertained through biological bioassays. In the case of the hyphomycetes fungi *B. bassiana* and *M. anisopliae,* virulence is largely a factor of the ability of the conidia to penetrate the insect cuticle. Death of the host can then ensue as a result of a combination of effects including toxinosis, general obstruction due to hyphal growth as well as nutrient depletion (Wraight *et al.* 2007). Biological assays are the starting point of any biological investigation where virulence is of importance as they allow for the removal of factors which could impede or reduce virulence towards the target host. This allows for various measures such as the LC_{50} and LC_{90} values to be calculated. As the virulence of a fungus can alter depending on age, growth conditions (e.g. temperature and humidity) and the media on which it is cultured, it is important to ensure that these factors

remain constant throughout the experiment to allow accurate comparisons to be made (Goble *et al.* 2011).

This chapter aimed to (1) re-screen all 12 isolates identified by Goble (2009) as highly pathogenic to the soil-borne life stages of FCM on the basis that fungal virulence may have altered over time, (2) ascertain the LC_{50} and LC_{90} values of all isolates showing overall mortality of 70 % or greater as a result of mycosis, (3) determine the LT_{50} and LT_{90} values using the LC_{50} and a standardised concentration ($1x10^7$ conidia.ml^{-1}) of three of the most promising isolates and finally (4) to compare the results with those obtained by two commercially available isolates, Eco-Bb® (*B. bassiana* strain R444) (PHP, South Africa) and a *M. anisopliae* strain ICIPE 69 (Real IPM, Kenya).

2.2 METHODS

2.2.1 Insect cultures

Late instar FCM larvae were obtained when required from a laboratory culture held at River Bioscience, Addo, Eastern Cape.

2.2.2 Fungal cultures

The 12 isolates (Figure 2.2) identified by Goble *et al.* (2011), four *Metarhizium anisopliae* var. *anisopliae* (G 11 3 L6, FCM Ar 23 B3, G OL R8, G 14 2 B5) and eight *Beauveria bassiana* (FCM 10 13 L1, FCM Rose R9, G 14 2 B3 G Moss R10, FF J&B R5, G OL R 11, G B Ar 23 B3, G AR 17 B3), were investigated as to their virulence towards soil-borne life stages of FCM. Eight of the isolates used were obtained from cultures stored at Rhodes University whilst the remaining four cultures (G OL R8, FCM 10 13 L1, G Moss R10, G 14 2 B5) were obtained from the South African National Collection of Fungi at the Plant Protection Research Institute (PPRI) in Pretoria. All of the isolates used are stored at PPRI. The labelling system used follows that of Goble *et al.* (2011) whereby the first segment represents the bait insect (e.g. G B = *Galleria* bait), the second the farm (e.g. Ar = Arundel) from which the isolate was sampled and third, the orchard or refugia soil number (e.g. 23 B3). Isolates used in this study were grown on sabouraud dextrose agar (SDA) plates supplemented with 1 ml dodine, 50 mg.L^{-1} rifampicin and 50 mg.L^{-1} chloramphenicol and incubated at 26 °C on a D12:L12 photoperiod.

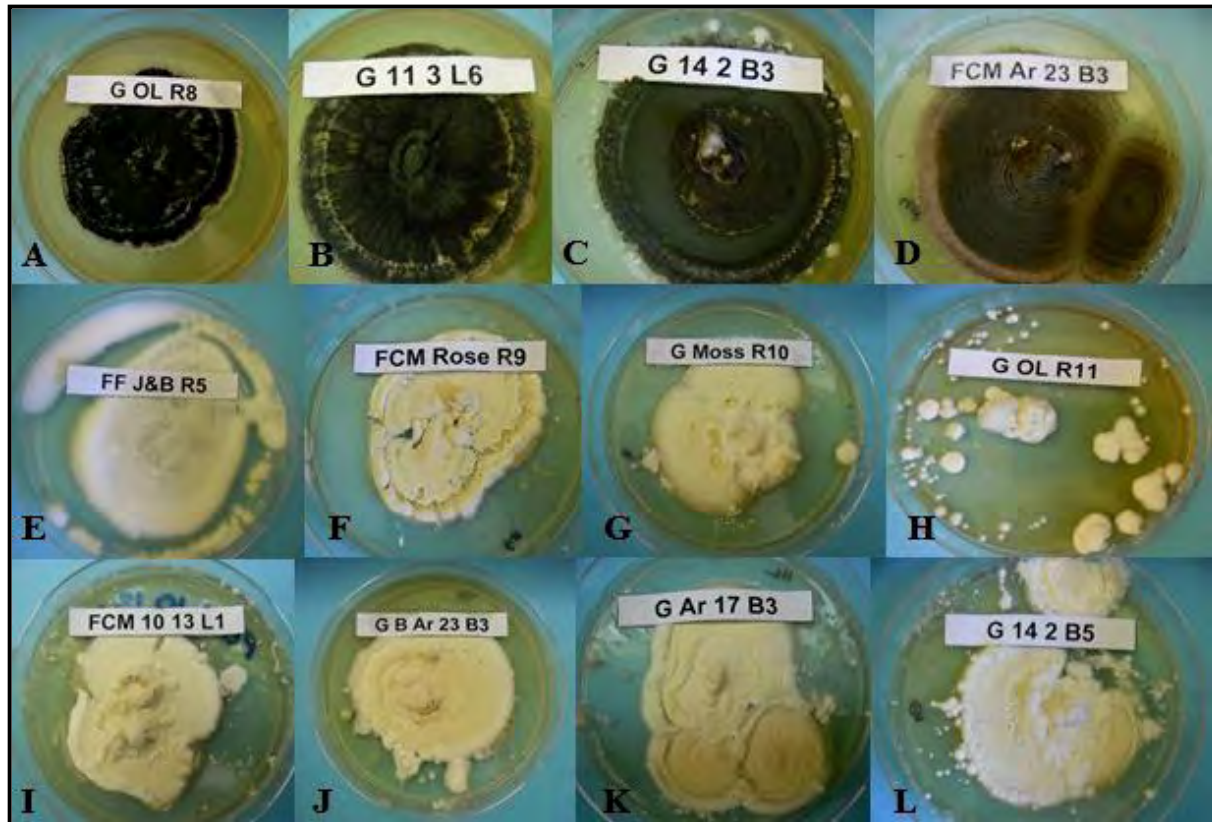

Figure 2.2: Culture morphology of all 12 isolates investigated. Isolates A-D are *Metarhizium anisopliae*; E-L *Beauveria bassiana.*

2.2.3 Preparation of conidial suspensions

Conidia were harvested from 2–3 week old surface cultures by scraping with a glass rod and suspended in approximately 20 ml sterilised distilled water (dH_2O) supplemented with 0.05 % Triton X-100 in sterile McCartney bottles containing 3 mm glass beads. The bottles were sealed and vortexed for approximately two minutes to produce a homogenous suspension. The concentration and viability of the suspensions were determined.

2.2.3.1 Determination of concentration

The concentration of the suspension was determined using a Helber counting chamber with thoma ruling (Figure 2.3). Prior to use, the chamber and cover slips were rinsed thoroughly with 70 % ethanol. Two counts were made for each replicate using a 1/100 dilution. The average count was used in further calculations.

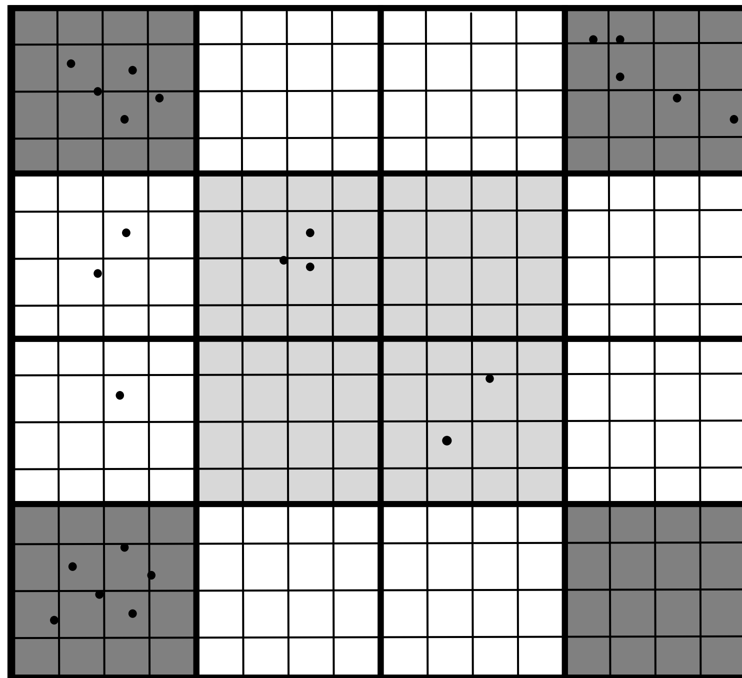

Figure 2.3: A Helber bacteria counting chamber with thoma ruling as viewed under a light microscope (400X). Black dots represent conidia. Darkly shaded grey squares represent the corners of the chamber which are counted. Lightly shaded grey squares represent the middle four larger squares of the chamber. Only one of these, chosen at random, is counted in conjunction with the four corners.

The concentration of the original suspension was then determined using the following formula:

$$\text{conidia.ml}^{-1} = \text{df x d x c}$$

Where: df = dilution factor; d = dilution; c = average number of conidia counted

2.2.3.2 Assessment of viability

A 100 µl of diluted conidial suspension was spread onto each of three SDA plates per isolate. A clean, sterile cover slip was placed in the centre of each plate which was subsequently incubated at 26 °C on a D12:L12 photoperiod. Percentage germination was determined by counting 100 spores under 40X magnification after 24 hours (Ekesi *et al.* 2002).

2.2.4 Inoculation of FCM

2.2.4.1 Re-screening of isolates

Re-screening of the isolates was undertaken to ensure that all fungal isolates previously screened by Goble *et al.* (2011) were producing similar FCM mortality. This was deemed necessary as fungi may exhibit a change in virulence over time (Brownbridge *et al.* 2001). Fungal isolates showing an overall average mortality of less than 70 % were excluded from further investigations.

In a petri dish, 50 g of sieved, autoclaved (120 °C, 15 psi, 20 min) sand was mixed with five ml of a $1x10^7$ conidia.ml^{-1} concentration. Petri dishes were placed on trays filled with autoclaved perlite. A total of 20 larvae ready to pupate within the next 24 hours were placed into each dish and incubated for seven days at 26 °C with a photoperiod of D12:L12 (Figure 2.4A). After seven days, larvae (now pupae) were removed from the petri dishes and placed on new sterile petri dishes containing 50 g of sterilised, sieved sand. Emergence chambers plugged with cotton wool (sterilised coke bottles cut nine cm from the neck of the bottle) were placed over each petri dish (Figure 2.4B) and incubated as before.

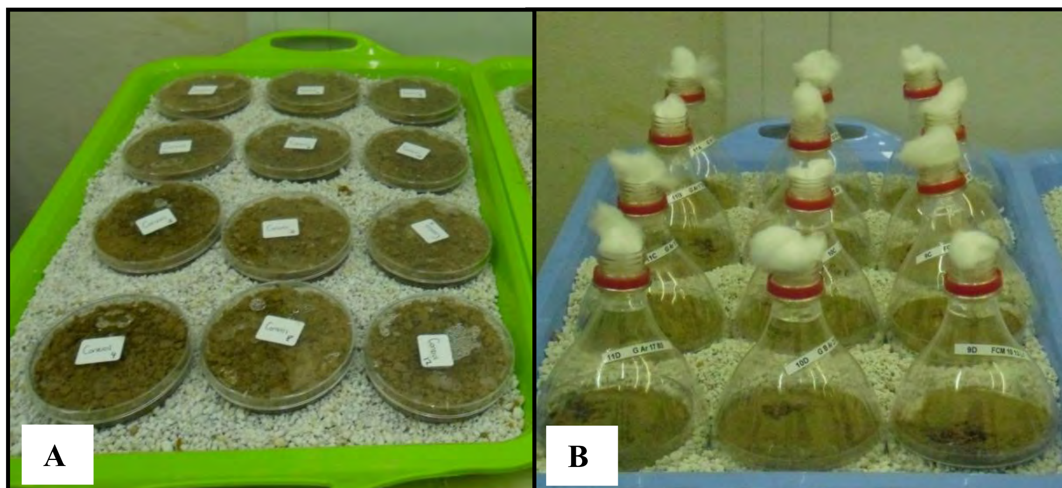

Figure 2.4: (A) Petri dishes each containing 20 5th instar larvae on inoculated soil. Plates were placed on trays containing perlite; (B) Cut, plastic 2 L coke bottles served as emergence chambers. These chambers were plugged with cotton wool to prevent both the escape of moths and to allow for the introduction of water to sustain adult moths for the duration of the bioassay.

Bioassays were ceased 10 days after first emergence. The number of adults which emerged, the number of adults which were dead as well as the number of pupae which failed to eclose was recorded. The latter two were surface sterilised in 70 % ethanol, placed onto fresh SDA plates and incubated at 26 °C on a D12:L12 photoperiod. Mycosis was assessed after three days. Pupae (failed to eclose) and adults (dead) were considered mycosed if fungal sporulation of the isolate occurred (Figure 2.5). The viability of each suspension was determined as described in section 2.2.3.2 and each treatment was replicated four times.

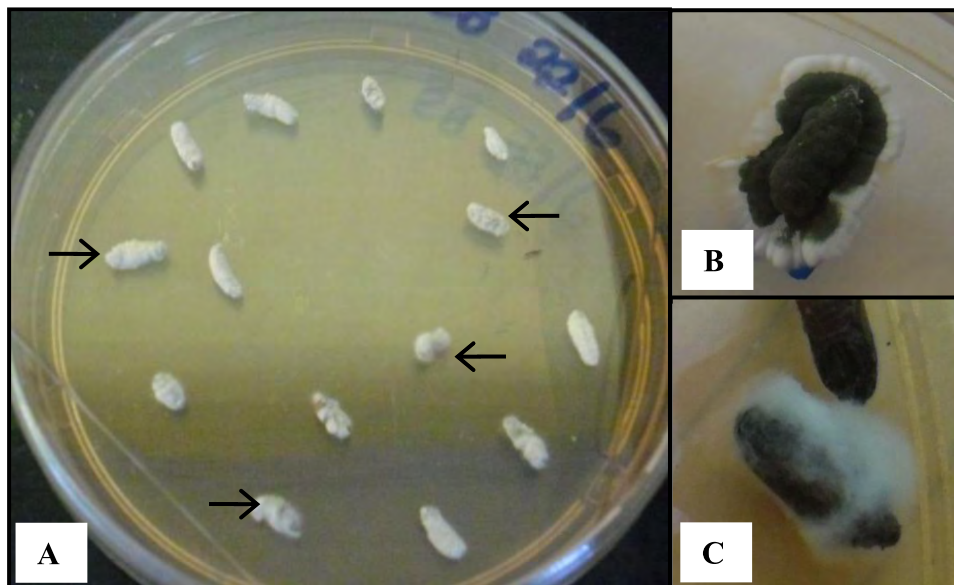

Figure 2.5: (A) Pupae showing signs of fungal mycosis (←) after incubation on SDA plates; (B) Pupa showing signs of mycosis via *Metarhizium anisopliae*; (C) Pupa showing signs of mycosis via *B. bassiana.*

2.2.4.2 Concentration dose-response bioassays

The procedure followed that detailed in section 2.2.4.1 with the exception that three different concentrations (1 x 10^4, 1 x 10^5 and 1 x 10^6 conidia.ml^{-1}) were investigated. Again each treatment was replicated four times and the viability of each suspension was determined as described in section 2.2.3.2. The LC_{50} and LC_{90} values were determined. Mycosis was scored as mentioned above. Eight different isolates were used along with two commercial products, Eco-Bb® (Plant Health Products, South Africa) (a.i. *B. bassiana* strain R444) and *M. anisopliae* ICIPE 69 collected from soil in the Democratic Republic of Congo (Ekesi *et al.* 2002) for comparison. An additional treatment using an Eco-Bb® suspension (prepared

according to the manufacturer's instructions) was also investigated. Again, to maintain consistency, 5 ml of the suspension was mixed with 50 g of autoclaved sand.

2.2.4.3 Exposure time-response bioassays

The most promising isolates, two *M. anisopliae* (G 11 3 L6 and FCM Ar 23 B3) and one *B. bassiana* (G Ar 17 B3), based on screening and concentration-dose response bioassays were used in the exposure-time response bioassays. Four different time inoculation periods were investigated (1, 3, 5 and 7 days) as well as two different concentrations for each isolate (calculated LC_{50} and $1x10^7$ conidia.ml^{-1}). The inoculation procedure followed the same general procedure outlined above in section 2.2.4.1. The LT_{50} and LT_{90} value of each concentration was determined and mycosis was scored as before. Again each treatment was replicated four times and the viability of each conidial suspension was determined as described in section 2.2.3.2.

2.2.5 Statistical analysis

For screening trial results, overall mortality (including both adult moths and pupae which succumbed to mycosis) and eclosion percentage data were arcsine transformed to establish normality and subjected to analysis of variance (ANOVA). If a significant result was found, a Student Newman Keul's test was conducted ($p < 0.05$). All analyses were executed in Statistica ver. 10 (Statsoft, Inc. 1984–2011). In order to control for natural mortality amongst the test population, all data was subjected to Abbott's formula prior to analysis (Abbott 1925). In addition to ANOVA, probit analysis was conducted on concentration-dose response data using PROBAN whereas logit analysis was conducted on exposure time-response data. This allowed for the determination of LC_{50}, LC_{90}, LT_{50} and LT_{90}.

2.3 RESULTS

In all bioassays, the percentage viability of all isolates used was greater than 85 %. When larvae failed to pupate (always a result of mycosis), they were grouped together with mycosed pupae. The percentage mycosis presented in the graphs represents the overall average mycosis shown by each isolate. Overall mycosis includes both that of pupae and adults which showed signs of fungal sporulation upon failure to emerge or death respectively.

In all assays, eclosion in controls was always greater than 85 %. Mortality in controls was minimal and was never a result of mycosis.

2.3.1 Re-screening of previously identified isolates

The isolates identified by Goble *et al.* (2011) showing adult mortality < 20 % and pupal mortality > 80 % were re-screened to determine whether these isolates were still performing as before. A 1 x 10^7 conidia.ml^{-1} suspension for all isolates including the commercial isolates was used. All isolates with the exception of the four *M. anisopliae* isolates, showed eclosion > 20 %, but < 70 % with isolate G OL R8 obtaining the lowest percentage eclosion (Figure 2.6). Eclosion was highest amongst *B. bassiana* isolates. Overall mycosis ranged between 3.1 % (G OL R 11 Re) and 87.4 % (G 11 3 L6). Compared to the results obtained by Goble *et al.* (2011), eight of the tested isolates showed similar virulence results (G OL R8, G 11 3 L6, FCM Ar 23 B3, G 14 2 B3, FCM 10 13 L1, G B Ar 23 B3, G Ar 17 B3 and G 14 2 B5), whilst four showed signs of decreased virulence towards *T. leucotreta* (FF J&B R5, FCM Rose R9, G Moss R10 and G OL R11). Although some of the *B. bassiana* isolates exhibited eclosion levels higher than 20 %, overall mycosis was still above 80 %. This is apparent as emerged adults tended to succumb to the fungus prior to the end of the experiment (Figure 2.7). There were significant differences in eclosion rates between the *M. anisopliae* isolates and the *B. bassiana* isolates (ANOVA: $F_{19,30} = 10.86$; $p < 0.05$). For both species, significant differences were found between isolates exhibiting percentage mycosis above 70 % and those below 60 %.

The isolates originally showing low mycosis percentages (< 60 %) were reassessed to determine whether this was a result of experimental error or a reduction in virulence since initial isolation of the fungus. In addition, an isolate (G 11 3 L6) showing a high mycosis percentage was also reassessed to ensure that if a low percentage was again obtained, it was not a result of experimental error. This was provided that isolate G 11 3 L6 obtained a similar mycosis percentage as before. In all cases, percentage mycosis decreased or remained low. Isolate G 11 3 L6 Re performed as before. These isolates (FF J&B R5, FCM Rose R9, G Moss R10 and G OL R11) were therefore excluded from further analysis.

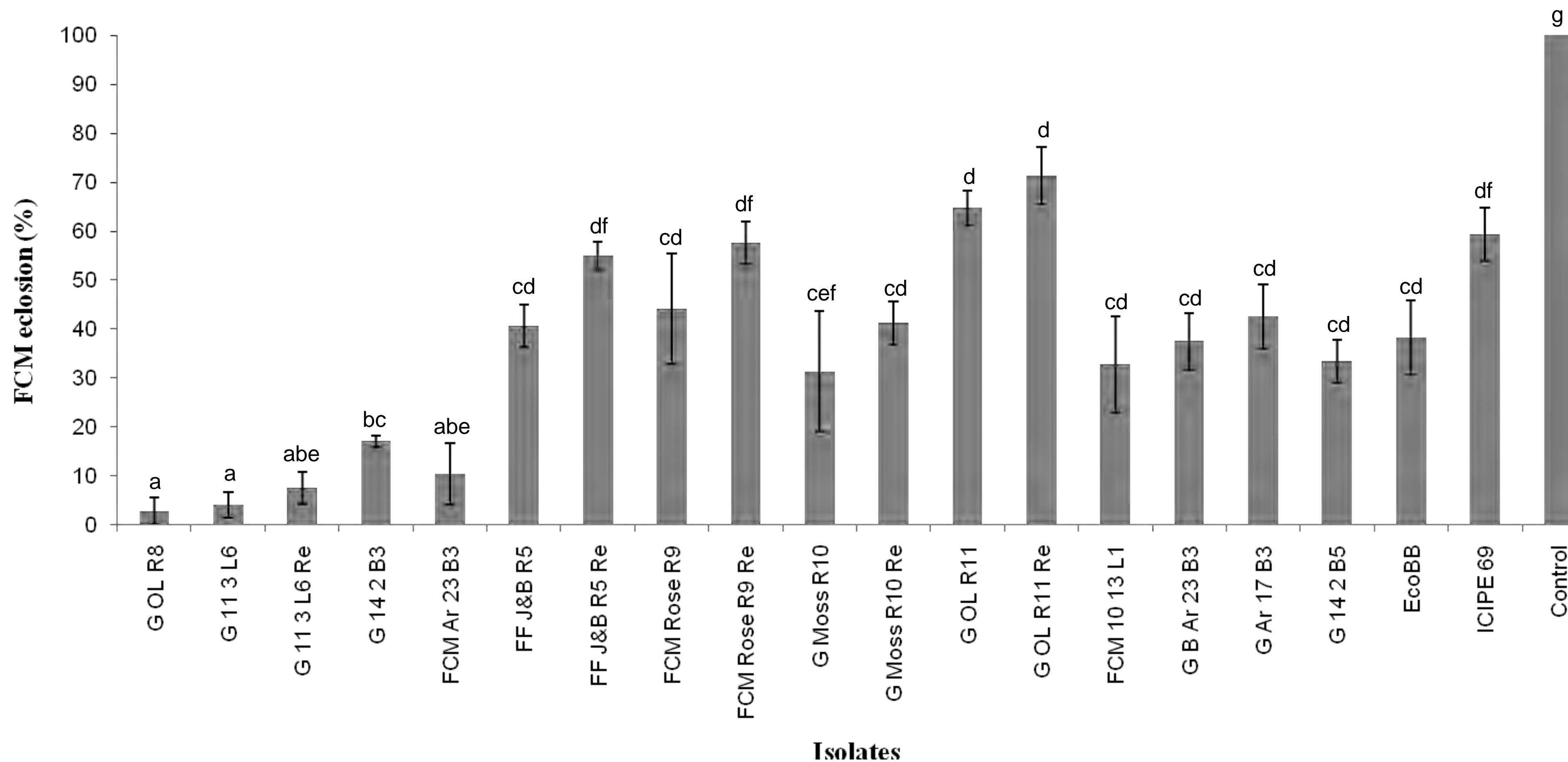

Figure 2.6: Mortality of *T. leucotreta* larvae exposed to four isolates of *M. anisopliae* var. *anisopliae* and eight isolates of *B. bassiana* exposed to conidial concentrations of 1×10^7 conidia.ml^{-1}. Bars represent the mean eclosion of *T. leucotreta* in percentage; whiskers the standard error (±). Different letters are indicative of a significant difference according to the student Newman-Keuls test (p = 0.05) (ANOVA: $F_{19,30} = 13.22$; $p < 0.05$). Four of the eight *B. bassiana* isolates and one *M. anisopliae* isolate were repeated. This is indicated by "Re" at the end of the isolate name e.g. FF J&B R5 Re.

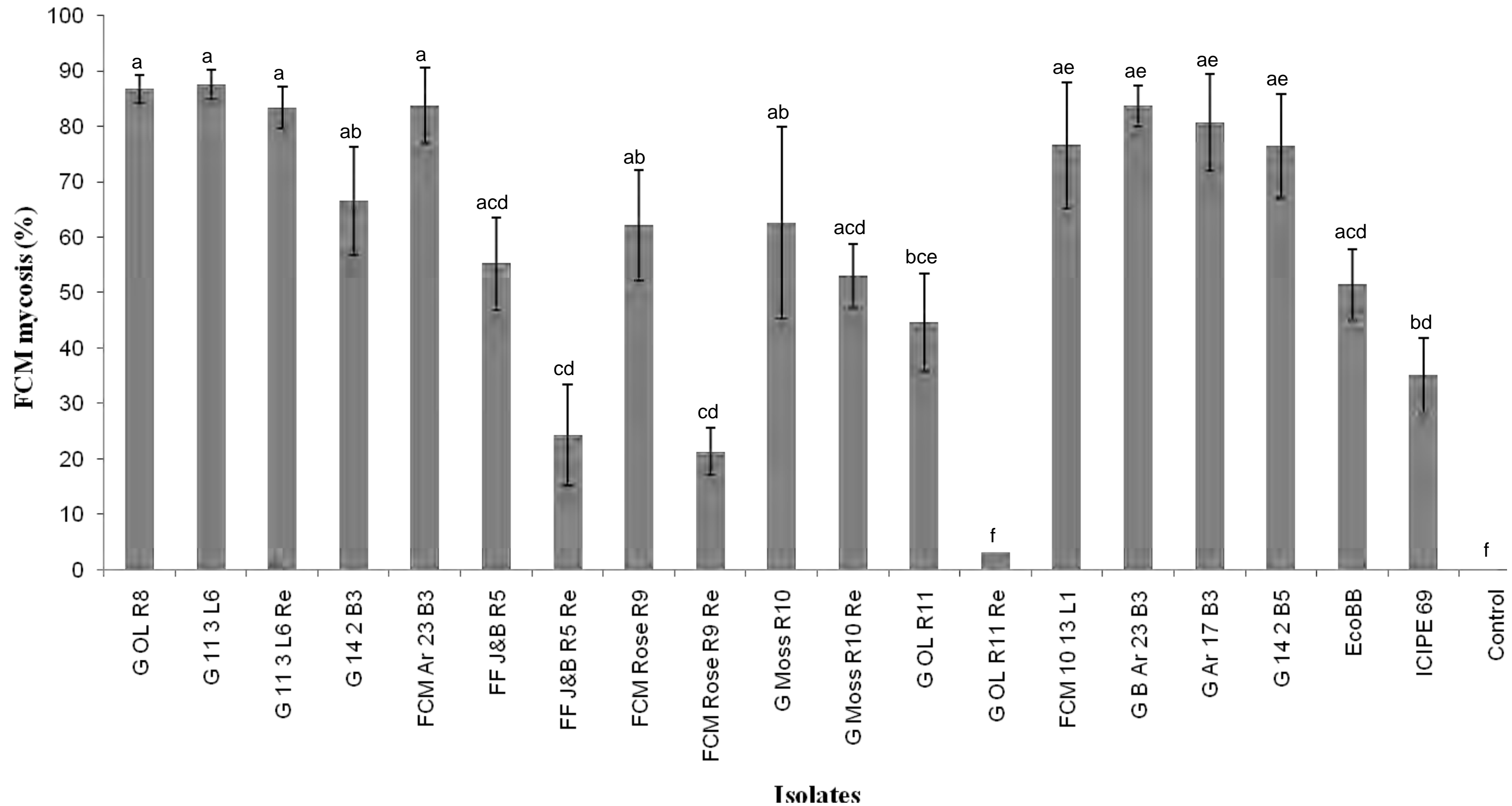

Figure 2.7: Mortality of *T. leucotreta* larvae exposed to four isolates of *M. anisopliae* var. *anisopliae* and eight isolates of *B. bassiana* exposed to conidial concentrations of $1x10^7$ conidia.ml^{-1}. Bars represent the mean *T. leucotreta* mycosis in percentage; whiskers the standard error (±). Different letters are indicative of a significant difference according to the student Newman-Keuls test (p = 0.05) (ANOVA: $F_{19,60} = 10.86$; $p < 0.05$). Four of the eight *B. bassiana* isolates and one *M. anisopliae* isolate were repeated. This is indicated by "Re" at the end of the isolate name e.g. FF J&B R5 Re.

Mycosis percentage with the eight novel isolates was higher than with the two commercially available isolates (Eco-Bb® and ICIPE 69) (Figure 2.7). Eclosion percentages for Eco-Bb® and ICIPE 69 were similar to those obtained for all other *B. bassiana* isolates. Eco-Bb® performed slightly better than ICIPE 69 with an overall mycosis and eclosion percentage of 51.4 % and 38.2 % respectively compared to 35.0 % and 59.3 % respectively. When applying Eco-Bb® according to the manufacturer's instructions (1 $g.L^{-1}$), performance was poor with an overall mycosis percentage of only 34.6 % and eclosion percentage of 56.3 %.

2.3.2 Concentration dose-response bioassays

Although some variation in eclosion did occur, the number of FCM emerged decreased with an increase in conidial concentration. This is clearly evident in isolates G 11 3 L6 and FCM Ar 23 B3 (Figure 2.8). A similar pattern was obtained for FCM mortality, with the exception that mortality increased with increased conidial concentration (Figure 2.9). In general, a steeper slope is indicative of a stronger concentration response opposed to more gentle slopes which tend to indicate a weaker concentration-dose response (Goble 2009). Isolates showing steep slopes include G 11 3 L6, G Ar 17 B3 and FCM Ar 23 B3, whilst those showing gentler slopes include FCM 10 13 L1, Eco-Bb® and ICIPE 69. A comparison of the regression lines showed that residual variances were homogeneous (χ^2 = 0.405, df = 9, p = 1) and slopes parallel (χ^2 = 16.87, df = 9, p = 0.051) thus allowing for elevations to be compared. Line elevations were found to be significantly different (ANOVA: $F_{9,19}$ = 13.41, $p < 0.0001$).

The LC_{50} and LC_{90} was determined (Table 2.1) through probit analysis. Isolates showing lowest LC_{50} and LC_{90} values included G 11 3 L6, G Ar 17 B3, FCM Ar 23 B3 and G 14 2 B5 (Table 2.1). High LC_{50} and LC_{90} values were recorded for isolates G 14 2 B3 and ICIPE 69 (Table 2.1). Based on the data obtained through probit analysis and screening data, three isolates were chosen on which further experiments were conducted. These isolates were G 11 3 L6 (*M. anisopliae*), FCM Ar 23 B3 (*M. anisopliae*) and G Ar 17 B3 (*B. bassiana*). All three isolates showed low FCM eclosion (Figure 2.6), high pupal mortality (Figure 2.7) and low LC_{50} and LC_{90} values (Table 2.1).

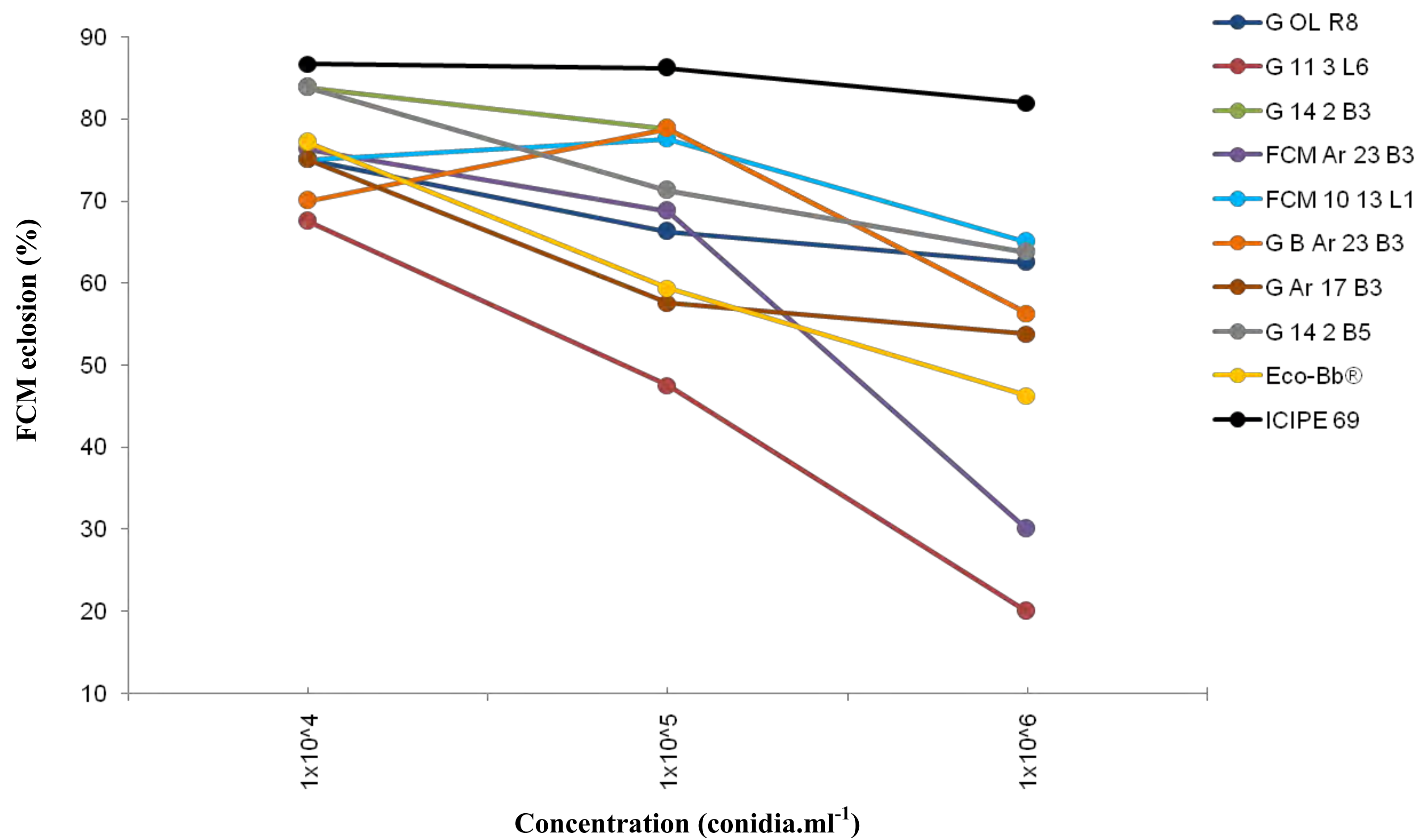

Figure 2.8: Percentage of emerged FCM recorded upon exposure of 5th instar larvae to three different conidial concentrations. Eight isolates and two commercial isolates (Eco-Bb® and ICIPE 69) were investigated.

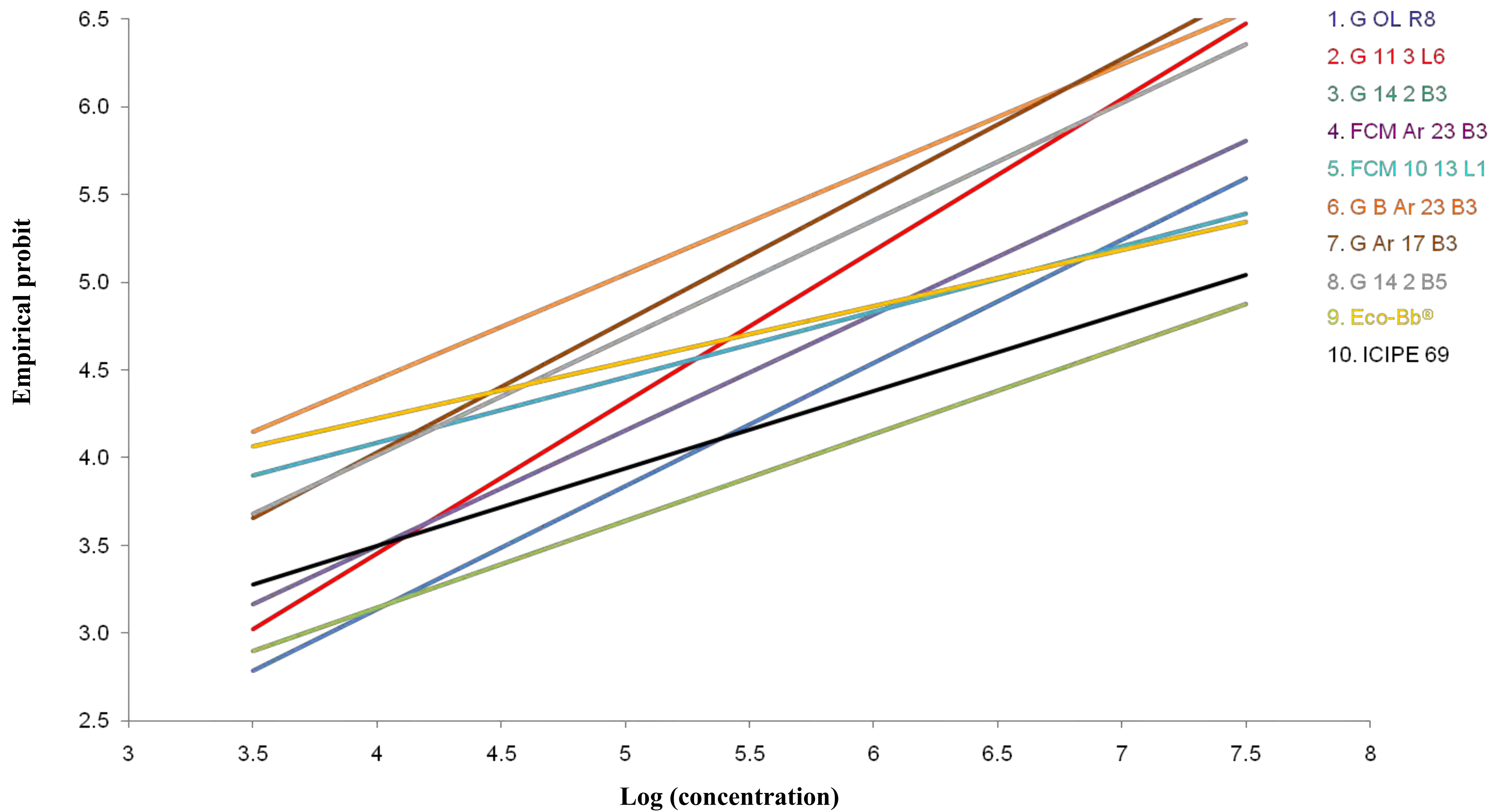

Figure 2.9: The log-probit regressions of overall (adult and pupal) mortality for all eight isolates (1– 4: *Metarhizium anisopliae*; 5–8: *Beauveria bassiana*) and two commercially available isolates (9 and 10). (Equation of lines: 1. $y = 0.7x + 0.33$; 2. $y = 0.86x – 0.007$; 3. $y = 0.49x + 1.17$; 4. $y = 0.66x + 0.85$; 5. $y = 0.37x + 2.59$; 6. $y = 0.59x + 2.06$; 7. $y = 0.75x + 1.04$; 8. $y = 0.67x + 1.33$; 9. $y = 0.32x +2.94$; 10. $y = 0.44x + 1.72$)

Table 2.1: Probit data for each of the investigated fungal isolates. The isolates highlighted are those which were investigated further.

Isolate	**Fit of Line**		**Equation (y = bx + a)**			**Fiducidal**	**Lethal Concentration**			
	χ^2	P	a	b	b (SE)	G	LC_{50}	LC_{50} (SE)	LC_{90}	LC_{90} (SE)
G OL R8	0.336	0.57	0.335	0.700	0.145	0.164	4.58×10^6	3.22x	3.09×10^8	4.72x
G 11 3 L6	0.855	0.36	-0.007	0.863	0.126	0.083	6.26×10^5	1.93x	1.91×10^7	1.39x
G 14 2 B3	0.254	0.62	1.168	0.494	0.152	0.361	5.64×10^7	10.0x	2.20×10^{10}	7.88x
FCM Ar 23 B3	0.402	0.53	0.848	0.661	0.125	0.138	1.92×10^6	1.07x	1.67×10^8	2.23x
FCM 10 13 L1	0.069	0.78	2.593	0.373	0.135	0.501	2.88×10^6	3.65x	0.79×10^{10}	3.16x
G B Ar 23 B3	2.734	0.09	2.058	0.597	0.130	0.183	0.36×10^5	1.95x	0.13×10^8	5.05x
G Ar 17 B3	0.740	0.39	1.036	0.749	0.142	0.137	1.98×10^5	0.67x	1.02×10^7	0.90x
G 14 2 B5	0.016	0.87	1.330	0.670	0.139	0.167	2.99×10^5	1.19x	2.45×10^7	2.78x
Eco-Bb®	0.071	0.78	2.940	0.325	0.106	0.412	2.16×10^6	2.40x	1.92×10^{10}	7.57x
ICIPE 69	1.274	0.26	1.726	0.442	0.128	0.320	2.60×10^7	4.12x	2.08×10^{10}	7.14x

2.3.3 Exposure time-response bioassays

There was a positive relationship between the length of time FCM 5th instar larvae were exposed to both fungal concentrations i.e. as exposure time increased, so too did mortality (Figure 2.10). For all three isolates mortality was greatest when larvae were exposed to the higher concentration. After seven days, mortality obtained at the lower concentration was slightly above 50 % for isolates G 11 3 L6 and G Ar 17 B3 whereas for isolate FCM Ar 23 B3 mortality was recorded to be 70 %. At the higher concentration, mortality percentages fell between 80 % and 85 % for all three isolates (Figure 2.10). Adult FCM eclosion ranged between 12.5 % (after 7 days) and 82.5 % (after 1 day) at the LC_{50} concentration and between 12.5 % (after 7 days) and 70.7 % (after 1 day) at the higher concentration (Figure 2.11). Eclosion increased as exposure time decreased for all three isolates.

Logit analysis indicated that it would require a minimum of five and maximum of 14 days to obtain a LT_{50} and LT_{90} respectively at the LC_{50} concentration whilst at the higher concentration, 1 x 10^7 conidia.ml^{-1}, a minimum exposure time of four and maximum of nine days was required to obtain an LT_{50} and LT_{90} respectively (Table 2.2).

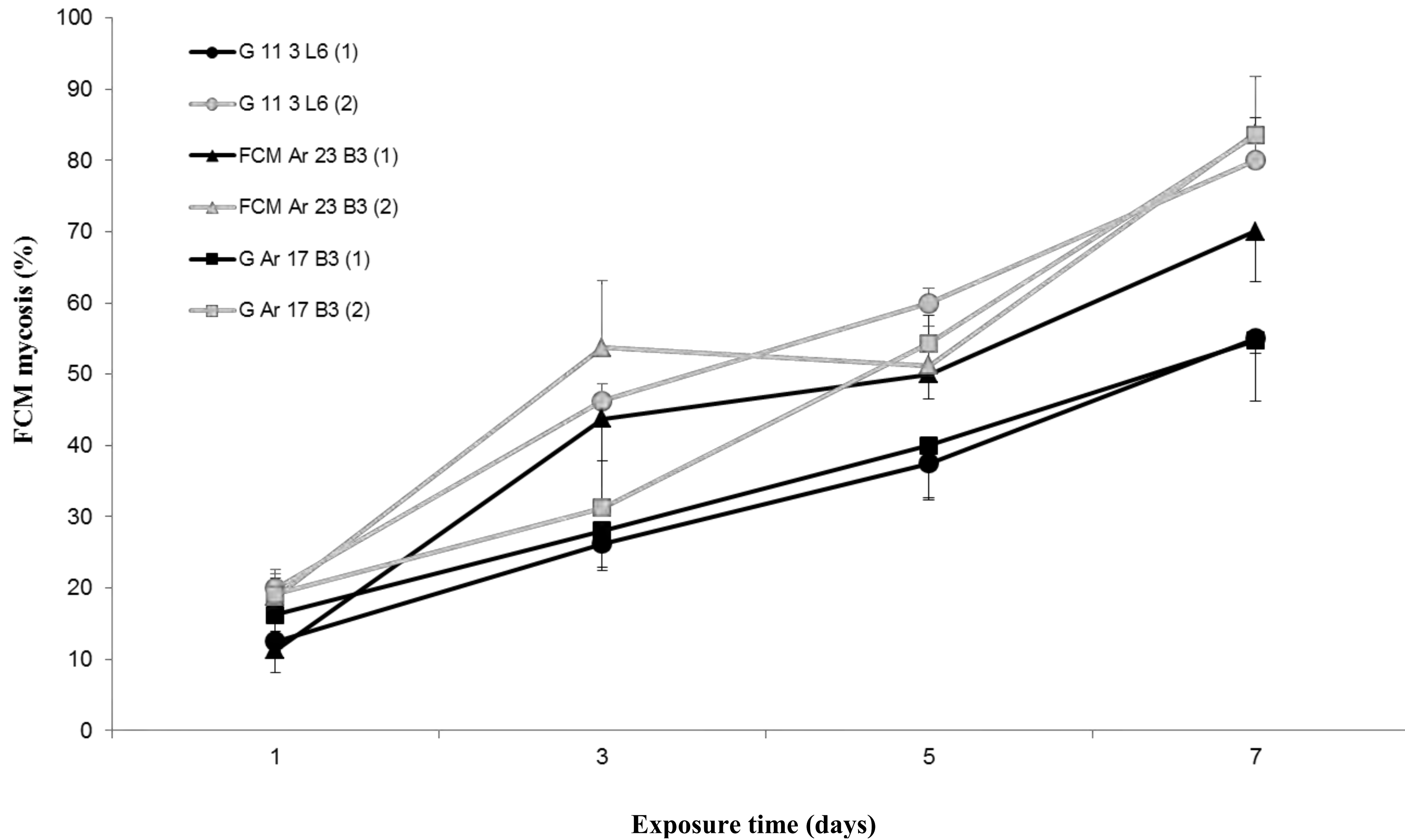

Figure 2.10 Percentage FCM mycosis of three fungal isolates after exposure to two different conidial concentrations (LC_{50} and 1 x 10^7 conidia.ml^{-1}). Control bioassays were conducted for each time period. No mortality was observed in the control. Black lines are representative of the low (LC_{50}) concentration; grey the high (1 x 10^7 conidia.ml^{-1}) concentration.

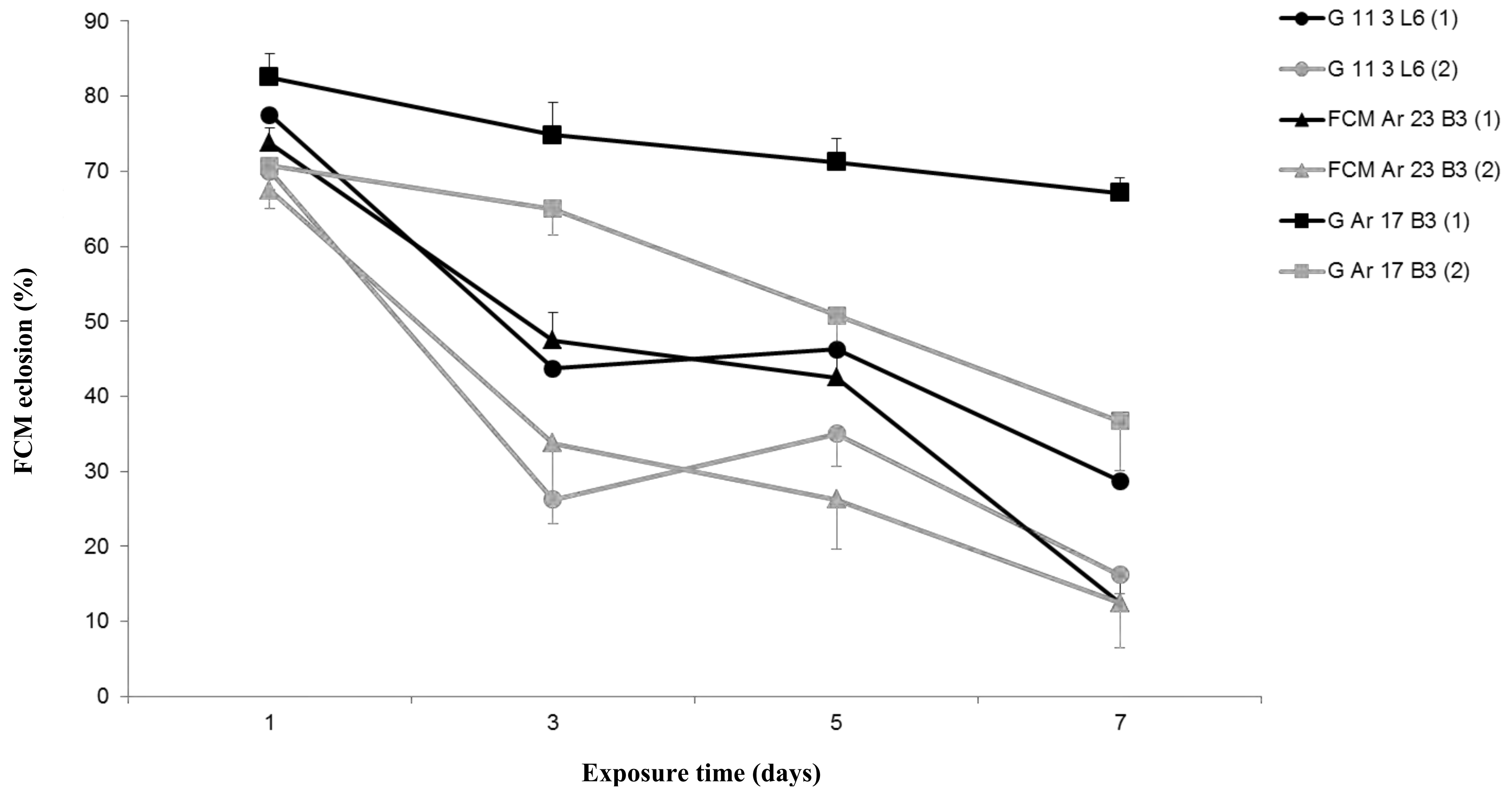

Figure 2.11: Adult FCM eclosion of three fungal isolates after exposure to two different conidial concentrations (LC_{50} and 1 x 10^7 conidia.ml^{-1}). Control bioassays were conducted for each time period. No mortality was observed in the control. Black lines are representative of the low (LC_{50}) concentration; grey the high (1 x 10^7 conidia.ml^{-1}) concentration.

Table 2.2: The number of days required for two different fungal concentrations to reach an FCM mortality percentage of 50 % and 90 %.

Isolate	LC_{50}		$1x10^7$	
	LT_{50} (days)	LT_{90} (days)	LT_{50} (days)	LT_{90} (days)
G 11 3 L6	6	13	4	9
FCM Ar 23 B3	5	10	4	9
G Ar 17 B3	6	14	4	9

2.4 DISCUSSION

Good control potential against FCM was exhibited by eight of the 12 isolates investigated (G OL R8, G 11 3 L6, G 14 2 B3, FCM Ar 23 B3, FCM 10 13 L1, G B Ar 23 B3, G Ar 17 B3 and G 14 2 B5). The remaining four isolates showed reduced virulence. Originally these isolates exhibited good control potential against FCM (Goble *et al.* 2011). The attenuation of microbes over time is not uncommon and has been recorded in a number of studies (Safavi 2011; Srikanth *et al.* 2011). Goble (2009) also noted a decrease in the virulence of isolate FCM 10 13 L1 over time. A study by Shah *et al.* (2007) however showed that this degeneration tends to be strain dependent. They found that *Metarhizium anisopliae* strain V245 degenerated at a faster rate than *Metarhizium anisopliae* V275. This was supported in a study by Ansari & Butt (2011) whereby *M. anisopliae* strain V275 showed no decline in virulence against *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae) even after the 12th subculture. This decrease in virulence as a result of repeated sub-culturing has been linked to a decrease in the production of the cuticle-degrading enzyme Pr1 (Shah *et al.* 2007; Safavi 2011). If commercialisation is to be considered, a fungus, as with any microbial product, which is more stable over time is preferred as in any commercialisation process, sub-culturing is inevitable and in most cases unavoidable. Different isolates however are more susceptible to the effects of sub-culturing than others allowing more sub-cultures before a reduction in virulence is shown (Butt *et al.* 2006). Reduced virulence can however be restored via a technique known as passaging whereby the target host or pseudo-host is infected with the control agent and allowed to sporulate on growth media from which further subcultures are taken. Depending on the control agent in question this process is repeated on a regular basis and might require more than one passage through the host (Butt *et al.* 2006). This loss in virulence may result because of a reduction in the production of enzymes required for host penetration, inability to differentiate into the correct form for growth within

the insect haemocoel or the metabolites that supress host defences may not be produced or if they are, are not produced in high enough quantities or are ineffective (Butt *et al.* 2006). Passaging however is not always successful as was seen in this study with four of the *B. bassiana* isolates and in other studies (Latch 1965; Morrow *et al.* 1989; Wang *et al.* 2003). In this study, passaging of the indigenous isolates through FCM however only occurred twice before they were rejected from further analysis. Some studies have only reported an increase in virulence after 3, 5 or 15 passages through the host insects (Latch 1965; Lord & Roberts 1986). The rejection of these isolates however still remains valid as other isolates, which were performing better after only one passage through the insect host, were available for use.

Out of the eight isolates investigated, three (G 11 3 L6, FCM Ar 23 B3 and G Ar 17 B3) showed good potential performing well in dose-response assays with relatively low LC_{50} and LC_{90} values coupled with low FCM eclosion percentages when exposed to a concentration of 1×10^6 conidia.ml^{-1}. A dose-dependent relationship was found. This relationship has been reported in a number of other studies (Hafez *et al.* 1997; Begemann 2008; Anand *et al.* 2009). For probit analysis, a minimum sample size of three, as used in this study, is required. Larger sample sizes however, do tend to decrease the uncertainty of the response outcome (Burd 2010). Therefore it is possible that with the addition of more samples, the LC_{50} and LC_{90} values calculated may alter. The chi-squared values calculated however did not obtain *p*-values less than the 0.05 level of significance suggesting the suitability of the data for the model.

Anand *et al.* (2009) found that *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) pupae which failed to succumb to infection took approximately 2–5 days longer to emerge than pupae not exposed to fungus. Hafez *et al.* (1997) also found that pupae which successfully emerged despite exposure to *B. bassiana,* showed reduced fecundity. The number of eggs laid per female decreased approximately 2-fold when a 0.26×10^8 conidia.ml^{-1} concentration was used and 5-fold when a 4.12×10^8 conidia.ml^{-1} was used. The indirect effects of EPF on FCM were not the focus of the study, but rather the ability of the fungus to cause death at the soil-inhabiting stage. Control at this stage is far more desirable as it eliminates the problem of egg-laying and thus can potentially greatly reduce FCM populations. However it would be worthwhile to determine whether FCM fecundity is reduced in the presence of the fungal isolates used in this study. Results show that EPF will not kill all FCM pupae within the soil and therefore some eclosion is expected. By definition, biological control alone does not

completely eradicate the problem, but rather suppresses and maintains pest population levels below a pre-determined economical threshold (van Driesche & Bellows 1996). If, however fecundity is reduced, subsequent populations should too show a reduction in size.

Compared to the commercial isolates, the investigated isolates performed better. One possibility as to why the commercial isolates performed poorly in comparison could be related to the external conditions at which the laboratory assays were conducted (Temperature: 26 ± 1 °C, Relative humidity: 70 ± 1 % photoperiod: D12:L12). It has been shown that different temperatures in particular can affect the virulence of a fungus. For example, a study by Sun *et al.* (2003) showed that termite mortality as a result of fungal infection varied depending on the temperature at which the experiment was conducted. Germination of conidia was lower at 35 °C as opposed to 27 °C for both fungal species investigated (*B. bassiana* and *M. anisopliae*). This affected mortality rates with higher germination rates resulting in increased termite mortality. An additional reason for the poor performance of the commercial products could be because the particular strain of fungus used is simply not as virulent towards FCM. As mentioned previously, although *B. bassiana* and *M. anisopliae* tend to affect a wide variety of species, strains of the species tend to be more specific (Zimmerman 2007a,b). Eco-Bb® for example is not registered for use on FCM, but rather it is registered for control against whitefly and red spider mite (Plant Health Products, South Africa). The virulence of *M. anisopliae* strain ICIPE 69 has not been tested against lepidopteran pests, but rather three fruit fly species (*Ceratitis capitata* (Weidemann), *Ceratitis cosyra* (Walker) and *Ceratitis* var. *rosa fasciventris* (Bezzi) (Diptera: Tephritidae)) and a flower thrips, *Megalurothrips sjostedti* (Trybom) (Thysanoptera: Thripidae). Against all four species good control was found (Ekesi *et al.* 1999; Ekesi *et al.* 2002). Goble *et al.* (2011) also found that the isolates isolated from South African citrus orchard soils performed better than the commercial isolate Broadband® (a.i. *B. bassiana*) (Biological Control Products, South Africa). It could be argued that the isolates investigated were more virulent as a result of them having been passaged through the insects prior to use in any of the biological assays. This however is not the case as the commercial isolates were also passaged prior to the initiation of any of the biological assays in which they were used.

Although mortality is observed in all three isolates subjected to exposure time-response experiments, higher FCM mortality percentages were obtained when 5th instar larvae were exposed to the fungus for between 5–7 days. Similarly eclosion percentages were greatly

reduced when 5th instar larvae were exposed to the fungus for between 5–7 days. This pattern was observed in all isolates at both concentrations (exception = G Ar 17 B3 at LC_{50}). Eclosion results suggest that in the case of *B. bassiana* (isolate G Ar 17 B3), a higher concentration may always be needed if it is to be applied in the field to further reduce the number of FCM which emerge.

The LT_{50} and LT_{90} values obtained for the three isolates varied between 5–14 days when the lower concentration (LC_{50}) was used and between 4 –9 days when a higher concentration (1 x 10^7 conidia.ml^{-1}) was used. Therefore provided a concentration not lower than the LC_{50} concentration is used, FCM 5th instar larvae/pupae should only need to be exposed to the fungus for at most approximately 2 weeks if 90 % of the population is to become infected. The pupal stage of FCM ranges between 11–39 days with the prepupal stage lasting between 2–29 days depending on temperature (Stibick 2008). It is therefore possible for the pupating 5th instar larvae to be exposed to the fungus for a sufficient amount of time to ensure greater than 50 % of the population succumb to infection if exposed to the fungus.

The results obtained in this chapter have provided useful information on whether or not EPF can be used to control FCM. However these results have taken place under controlled laboratory conditions. Therefore it remains to be tested as to whether these isolates, particularly the two *M. anisopliae* isolates G 11 3 L6 and FCM Ar 23 B3 as well as the *B. bassiana* isolate, can perform as effectively under semi-field conditions. Interestingly these isolates were three of the seven isolates which Goble (2009) referred to as noteworthy and which warranted further investigation.

3

Persistence of EPF isolates in the field

3.1 INTRODUCTION

The persistence of microbes, such as EPF, in their targeted environment is an extremely important factor if these organisms are to be considered for use in biological control. The longer the microbe is capable of persisting, particularly if it has no non-target effects, the more suited it is for use as a biological control agent. In addition, many microbial organisms infect only certain life stages of the target insect and therefore it is essential that they persist in the environment for a pre-determined length of time to ensure that viable conidia are present at the same time the targeted life stage is (Jackson 1999). Persistence can be defined as the ability of an organism to remain viable in the environment. For entomopathogenic fungi, persistence is generally measured as the number of colony forming units (CFUs) per gram of soil (Madigan & Martinko 2006). Unlike direct spore counts, CFU counts distinguish between viable and non-viable spores as only viable spores are capable of germinating on the artificial growth media and it is these spores which are responsible for initiating infection. Each colony formed is assumed to have arisen from a single fungal spore (Madigan & Martinko 2006).

Since EPF are soil-borne, persistence can be affected by the conditions of the soil in which they reside. Soil is a highly complex environment in terms of both its physical and biological characteristics (Barbercheck 1992). A number of different soil types (e.g. clay, loam, sandy) exist all of which differ in their physical (particle size, moisture levels, temperature, pH) and biological make-up. According to Tugel *et al.* (2000) (cited by Jaronski 2010) a typical gram of soil can contain up to 10^9 bacteria, 20 nematodes, several metres of fungi, 10^5 protozoa and 100 arthropods. In addition farming practices could also influence the success of EPF particularly in conventional farming systems where various agrichemicals, including fungicides, are used. All of these factors can act alone or in combination with one another to impact fungal persistence in the soil (Jaronski 2010).

Jackson (1999) stated that pathogenicity and persistence, often termed environmental competence, are key aspects for a successful microbial insecticide. In Chapter 2, the pathogenicity of a number of fungal isolates was evaluated against the soil-borne life stages of FCM. The environmental competence of these isolates however still remains to be tested. The aim of this chapter was to therefore evaluate the persistence of three of the most promising isolates (G 11 3 L6, FCM Ar 23 B3 and G Ar 17 B3) as well as two commercially available fungal products (ICIPE 69 and Eco-Bb®) within the soil environment of a citrus orchard.

3.2 METHODS

3.2.1 Fungi

All fungal isolates (G 11 3 L6, FCM Ar 23 B3, G Ar 17 B3, Eco-Bb® and ICIPE 69) were grown on rice for approximately 2 weeks. At this point, the rice was overgrown with fungus (Figure 3.1). The rice medium was prepared by soaking 100 g of rice in sterile dH_2O for approximately one hour in a 500 ml conical flask. The rice was then thoroughly drained and allowed to air dry for approximately 15 minutes after which the flask was sealed (plugged with cotton wool and covered with tin foil) and autoclaved for 20 minutes at 120 °C. 1 ml of a 1×10^7 $conidial.ml^{-1}$ suspension was then added to the rice-filled flask and incubated at 26 °C. This was repeated for all five isolates.

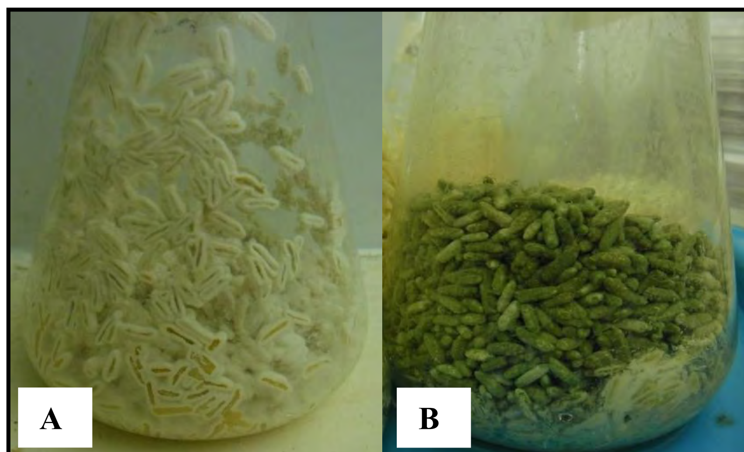

Figure 3.1: Rice overgrown with (A) *B. bassiana* and (B) *M. anisopliae.*

3.2.2 Experimental site

Persistence trials were conducted in a citrus orchard at Mosslands (33°23′54″S; 26°25′41″E); a conventional citrus farm located approximately 18 km outside of Grahamstown.

3.2.3 Experimental design

0.5 g of formulated product (rice overgrown with fungus) was mixed with 100 g of autoclaved soil collected from Mosslands. For each isolate (G 11 3 L6, FCM Ar 23 B3, G Ar 17 B3, Eco-Bb® and ICIPE 69) 28 bags were prepared. Four of these bags were not buried beneath the soil surface and served to establish an initial CFU count. The rest (24) were buried approximately 5 cm below the soil surface. Control bags containing only autoclaved soil were also buried. Holes were created using a soil auger and all holes were dug under the canopy of an orange tree within the irrigation zone in a straight line on the east facing side of the citrus trees. Five holes were dug per tree. The trial ran for six months. Each month four bags of each isolate (identified by labelled tags) were removed from the ground and transported back to the laboratory to determine the number of colony forming units per gram of soil ($CFU.g^{-1}$). From each bag, 1 g of soil was weighed, suspended in 1 ml 2 % saline solution and vortexed. The suspensions were diluted via serial dilution and 100 µl was spread on SDA plates. Three plates were used per sample. After 5 days, the number of noticeable colonies was counted and averaged. The average number of $CFU.g^{-1}$ of soil was then calculated ($CFU.g^{-1}$ = number of colonies x dilution factor x dilution).

Alongside monthly CFU counts, a bioassay was also undertaken. The procedure followed was similar to that outlined in Chapter 2, section 2.2.4.1. To a petri dish, 50 g of soil from the collected bag was added to a petri dish. This is unlike the bioassays conducted in the previous chapter whereby autoclaved soil was mixed with a prepared fungal suspension. A total of 20 late 5th instar FCM larvae were added to each soil-filled petri dish, covered with emergence chambers and incubated at 26 °C. The assay was ended 10 days after first emergence. The number of dead larvae, pupae which failed to emerge or adults which were found dead were recorded, surface sterilised and placed on SDA plates and incubated at 26 °C. After three days on the plates the dead insects were examined for signs of mycosis.

Figure 3.2: (A) 0.5 g of formulated product mixed with 100 g autoclaved soil; (B) labelled net bags containing formulated product prior to burial; net bags buried in the soil underneath the canopy of a citrus tree; (C) the location of the bag was identified by the string (string was attached to the bag) and the isolate was identified by the label. The yellow arrows indicate the location of the bags in the soil underneath the tree canopy.

3.2.4 Statistical analysis

Data found to be non-normal even after transformation using either natural log or arcsine transformation for CFU and mycosis counts respectively, were analysed using the Kruskal-Wallis non-parametric test. If a significant difference was found, a multiple mean rank test was conducted ($p < 0.05$). Data showing a normal distribution were subjected to analysis of variance (ANOVA) followed by the Tukey's post-hoc test if a significant difference was reported. Linear regression analysis was used to determine whether a correlation between the

monthly CFU count and mycosis percentage existed. Prior to analysis the mycosis data was subjected to Abbott's formula to control for natural mortality (Abbott 1925). All analysis was carried out in Statistica ver. 10 (StatSoft, Inc. 1984–2011).

3.3 RESULTS

For all isolates, including the commercial isolates tested, a large initial decrease was recorded in the number of colony forming units (CFU) per gram of soil over the first two months. CFU numbers stabilised thereafter (Figure 3.3–3.7), and after six months in the field, all fungal isolates were still present, although at relatively low numbers (G 11 3 L6 – 1.14 x 10^4 CFU.g^{-1}; FCM Ar 23 B3 – 1.46 x 10^3 CFU.g^{-1}; G Ar 17 B3 – 2.71 x 10^4 CFU.g^{-1}; Eco-Bb® - 2.93 x 10^1 CFU/g and ICIPE 69 9.42 x 10^2 CFU/g). The greatest percentage decrease in CFU.g^{-1} was obtained for Eco-Bb® with the least, G 11 3 L6. For all isolates, significant differences were recorded between CFU counts over time particularly between initial counts and counts taken after the second or third month (Table 3.1).

Average percentage mycosis (number of larvae and pupae which were found mycosed) varied greatly for all isolates over the six month period exhibiting a wide range of percentages (Figure 3.3–3.7). In addition, percentage mycosis showed no correlation to the estimated fungal concentration of the soil with respect to the non-commercial isolates (Figure 3.8), which obtained correlation coefficients not greater than 0.2. In some cases, even though a decrease in the number of CFU.g^{-1} was recorded, the average percentage mycosis still increased. For example, for isolate G 11 3 L6, even though the CFU count recorded for T_1 was lower than that recorded for T_0, FCM percentage mycosis still increased significantly from 59.9 % to 92.3 %. The highest correlation values interestingly were recorded for both the commercial isolates (Eco-Bb® – R^2 = 0.52; ICIPE 69 – R^2 = 0.75) (Figure 3.6 and 3.7).

Table 3.1: Statistical analysis of monthly CFU and mycosis results.

Isolate	**Analysis**		**p-value (α = 0.05)**	
	Mycosis	**CFU**	**Mycosis**	**CFU**
G 11 3 L6	ANOVA: $F_{6,18} = 16.69$	Kruskal-Wallis: $H_{6,26} = 22.11$	$\ll 0.05$	0.0012
FCM Ar 23 B3	ANOVA: $F_{6,18} = 3.54$	Kruskal-Wallis: $H_{6,25} = 20.27$	0.02	0.0025
G Ar 17 B3	Kruskal-Wallis: $H_{6,27} = 19.55$	Kruskal-Wallis: $H_{6,27} = 22.75$	0.0033	0.0009
Eco-Bb®	ANOVA: $F_{6,15} = 5.84$	Kruskal-Wallis: $H_{6,22} = 19.57$	0.002	0.0033
ICIPE 69	ANOVA: $F_{6,17} = 3.93$	Kruskal-Wallis: $H_{6,23} = 18.92$	0.012	0.0043

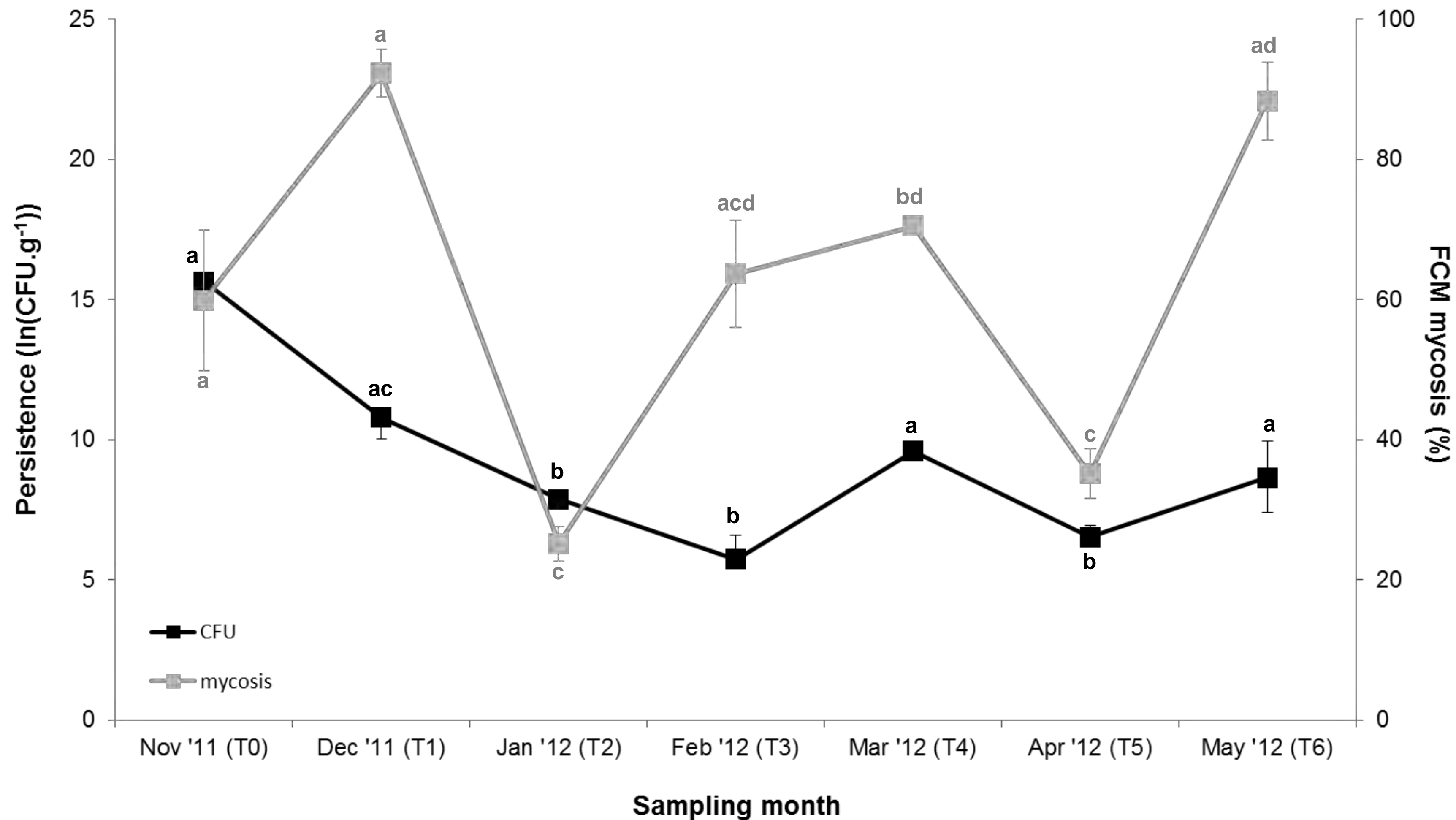

Figure 3.3: Monthly CFU counts and associated FCM mycosis percentages (percentage of insects mycosed) recorded for isolate G 11 3 L6 over six months. For CFU counts (represented in black) blocks indicate median values, whiskers the interquartile range. For FCM mycosis percentages (represented in grey) blocks indicate means, whiskers standard error. Different letters indicate statistically significantly different results (CFU: Kruskal Wallis ($H_{6.26} = 22.11$, $p = 0.0012$); MYCOSIS: ANOVA ($F_{6.18} = 16.69$, $p < 0.05$).

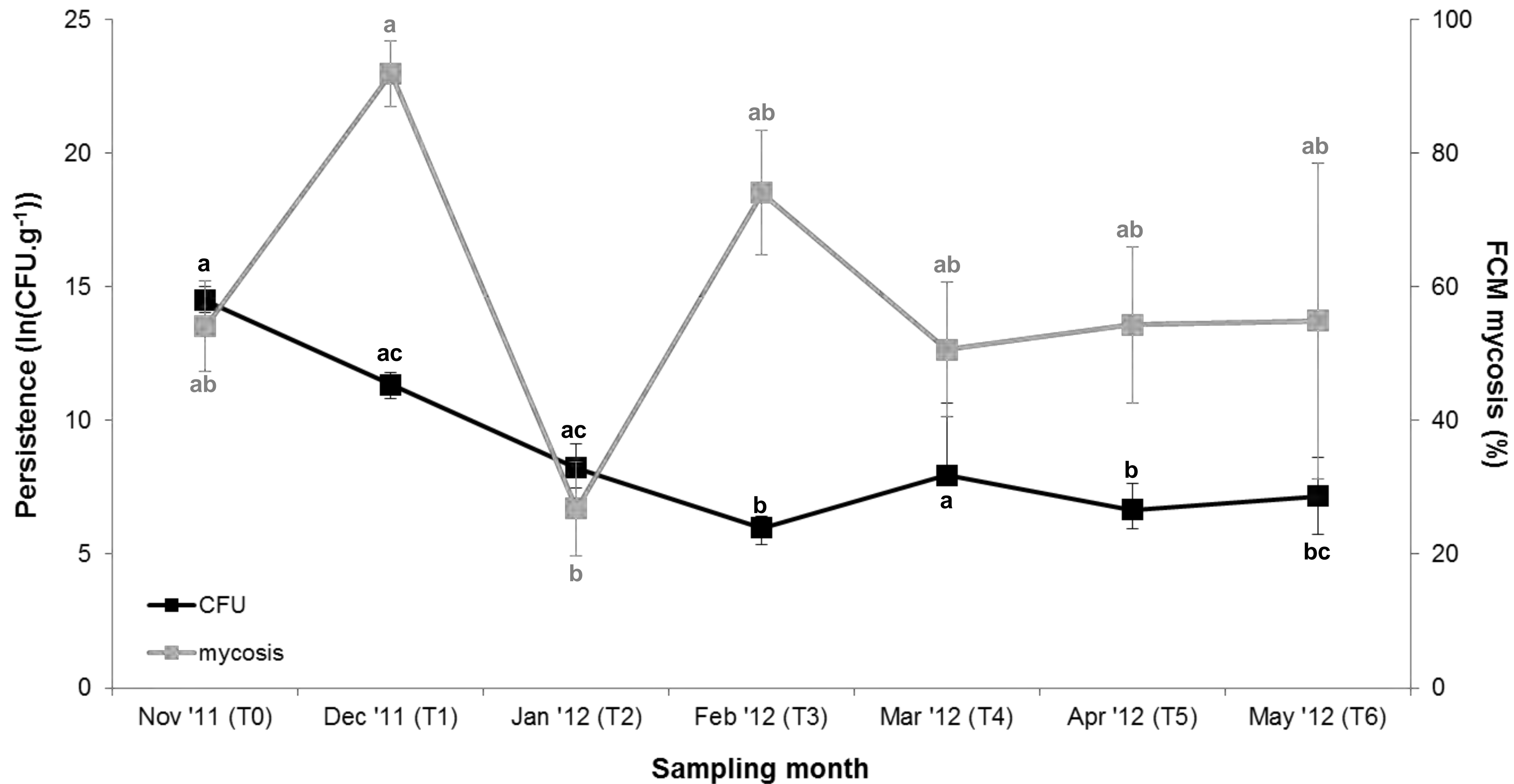

Figure 3.4: Monthly CFU counts and associated FCM mycosis percentages (percentage of insects mycosed) recorded for isolate FCM Ar 23 B3 over six months. For CFU counts (represented in black) blocks indicate median values, whiskers the interquartile range. For FCM mycosis percentages (represented in grey) blocks indicate means, whiskers standard error. Different letters indicate statistically significantly different results (CFU: Kruskal Wallis ($H_{6,25} = 20.27$, $p = 0.0025$); MYCOSIS: ANOVA ($F_{6,18} = 3.54$, $p = 0.02$).

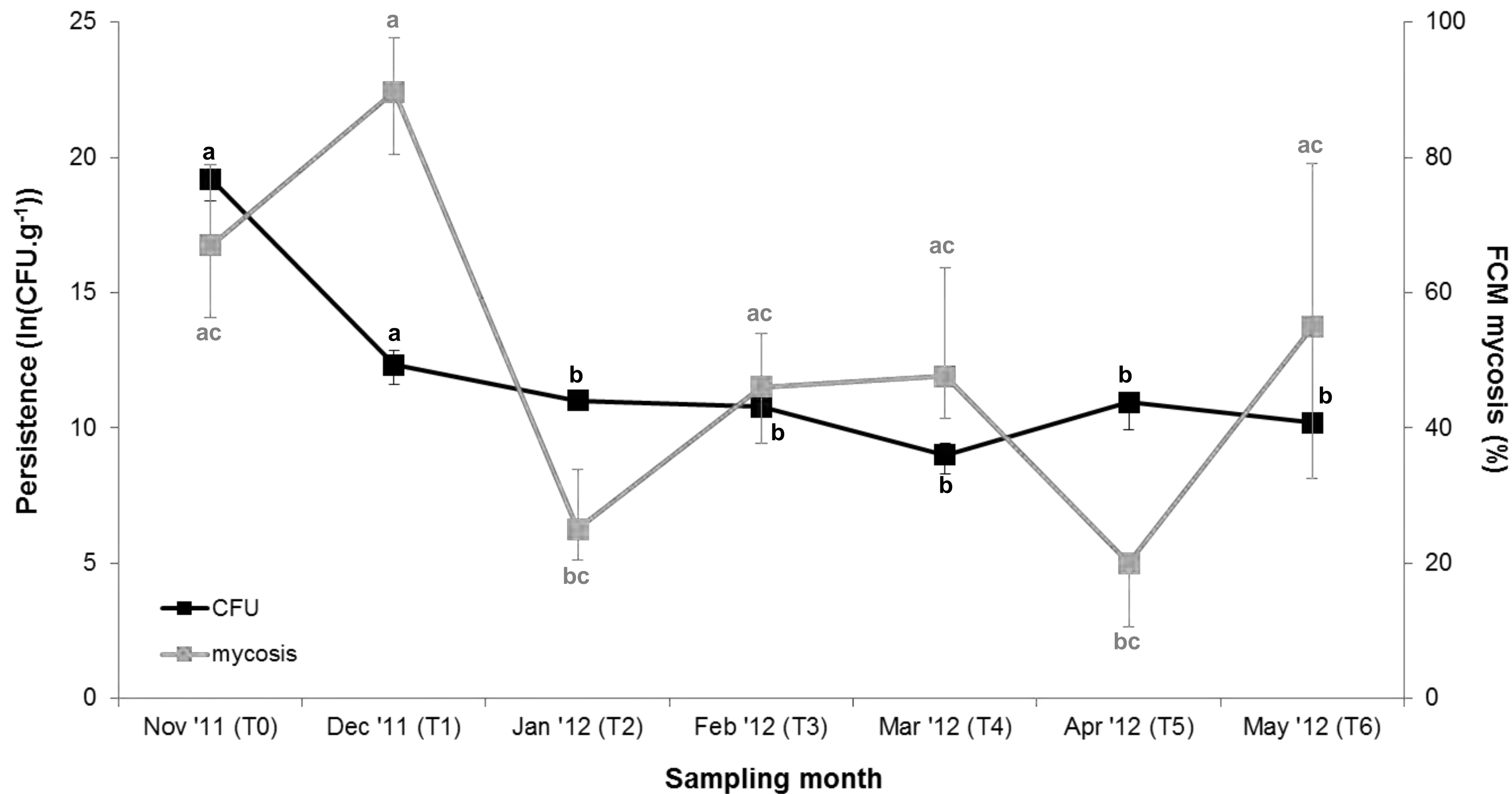

Figure 3.5: Monthly CFU counts and associated FCM mycosis percentages (percentage of insects mycosed) recorded for isolate G AR 17 B3 over six months. For both CFU counts (represented in black) and FCM mycosis percentages (represented in grey) blocks indicate median values, whiskers the interquartile range. Different letters indicate statistically significantly different results (CFU: Kruskal Wallis ($H_{6,27}$ = 22.75, p = 0.0025); MYCOSIS: Kruskal Wallis ($H_{6.27}$ = 19.55, p = 0.0033).

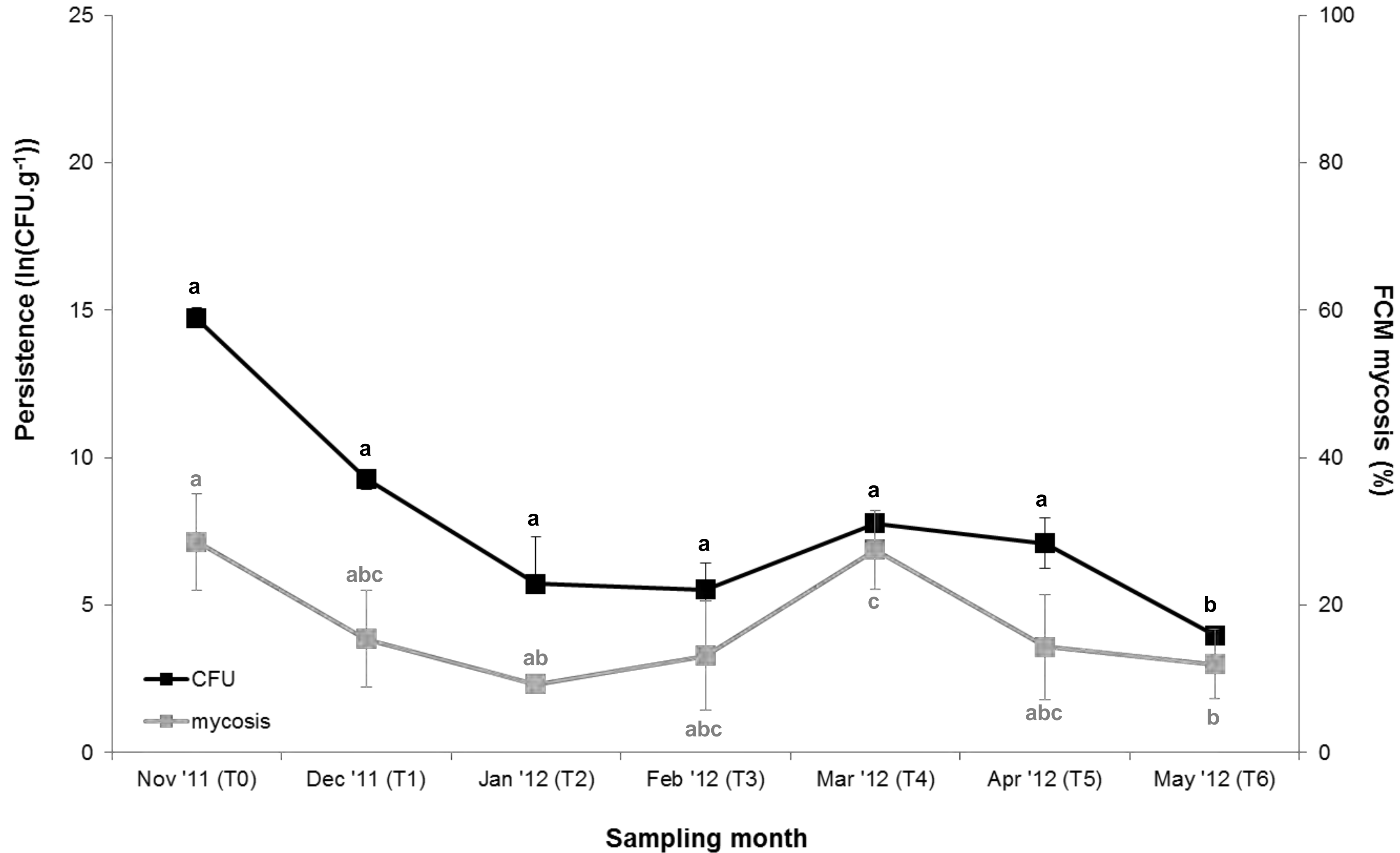

Figure 3.6: Monthly CFU counts and associated FCM mycosis percentages (percentage of insects mycosed) recorded for commercial isolate Eco-Bb® over six months. For CFU counts (represented in black) blocks indicate median values, whiskers the interquartile range. For FCM mycosis percentages (represented in grey) blocks indicate means, whiskers standard error. Different letters indicate statistically significantly different results (CFU: Kruskal Wallis ($H_{6,22} = 19.57$, $p = 0.0033$); MYCOSIS: ANOVA ($F_{6,15} = 5.84$, $p = 0.002$).

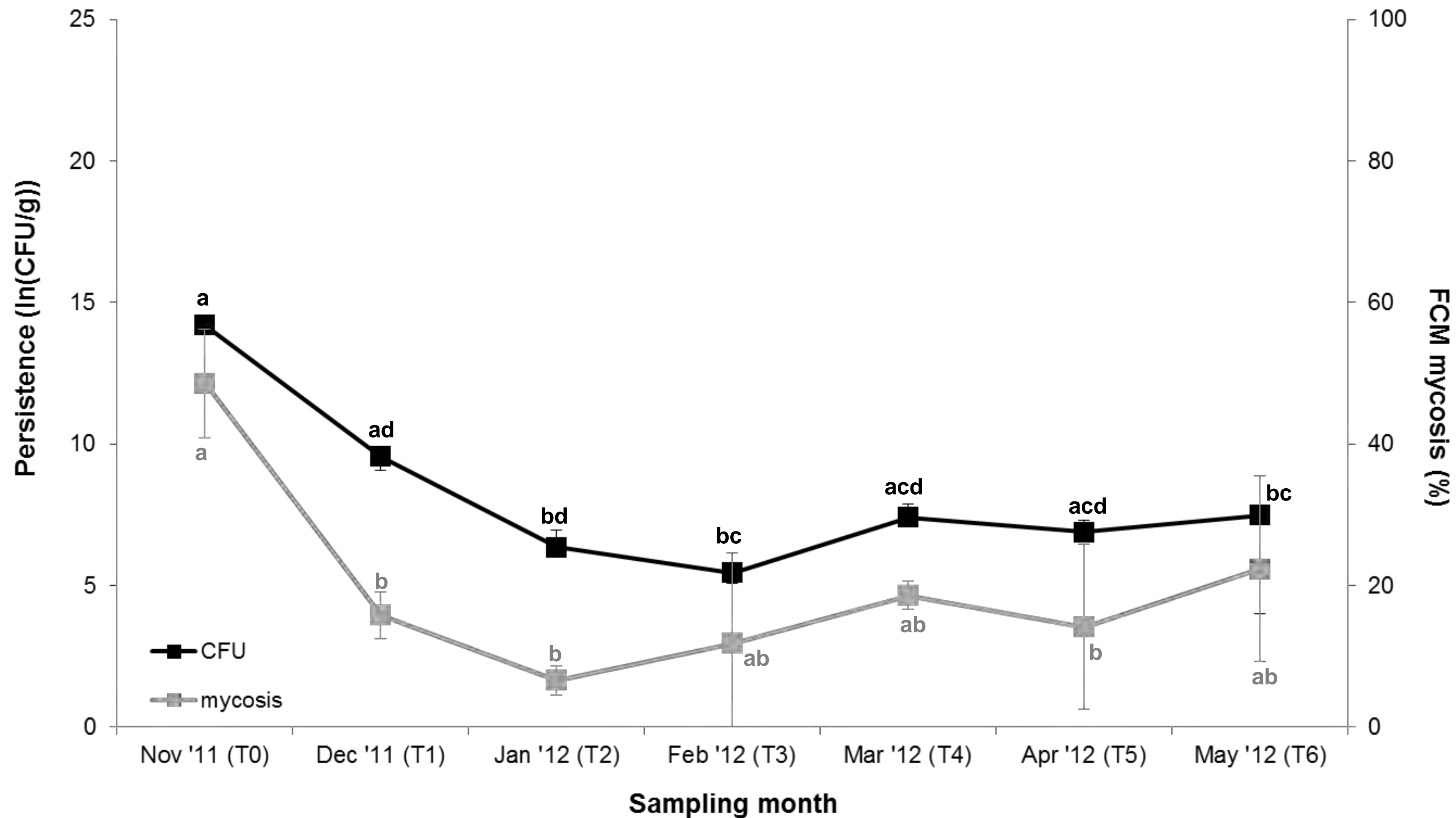

Figure 3.7: Monthly CFU counts and associated FCM mycosis percentages (percentage of insects mycosed) recorded for Kenyan isolate, Ken, over six months. For CFU counts (represented in black) blocks indicate median values, whiskers the interquartile range. For FCM mycosis percentages (represented in grey) blocks indicate means, whiskers standard error. Different letters indicate statistically significantly different results (CFU: Kruskal Wallis ($H_{6,23} = 18.92$, $p = 0.004$); MYCOSIS: ANOVA ($F_{6,17} = 3.93$, $p = 0.01$).

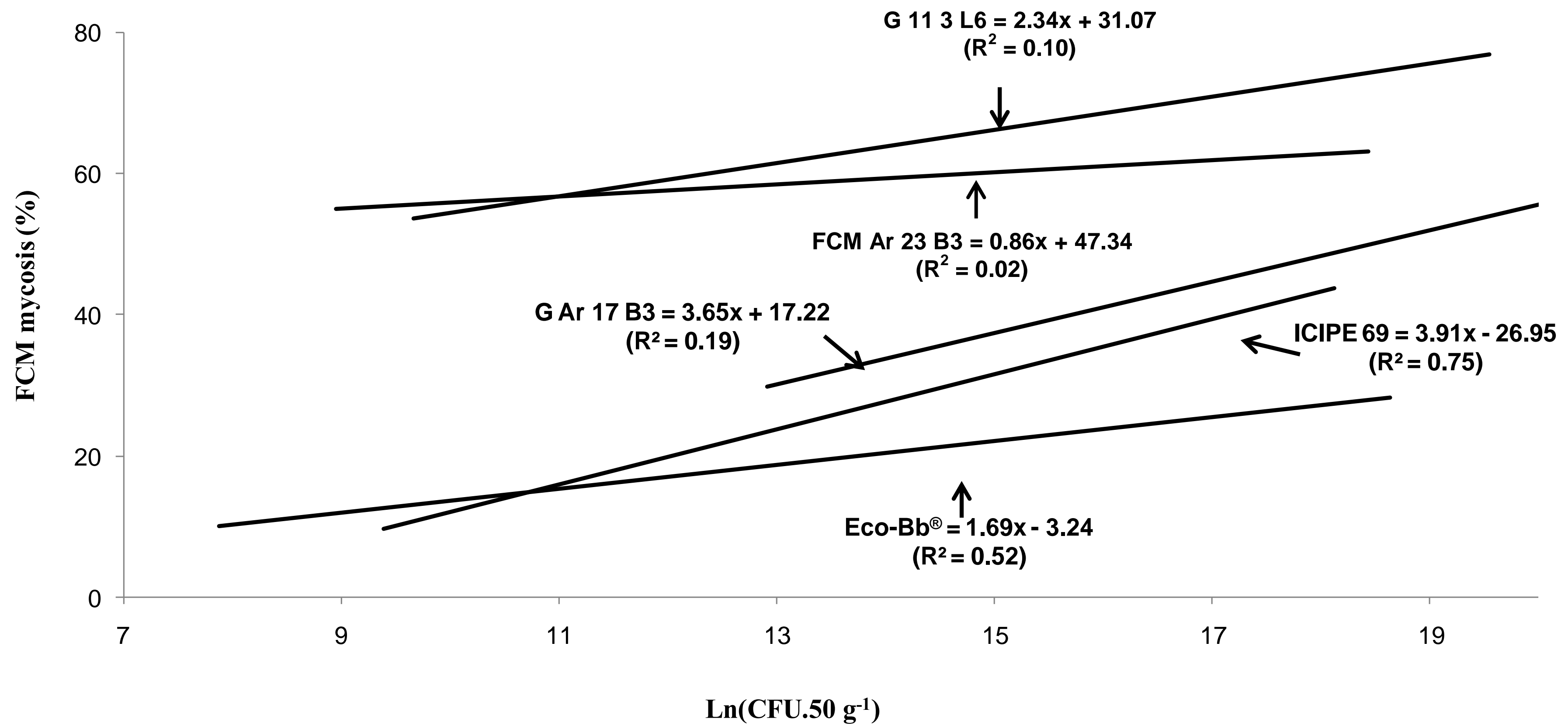

Figure 3.8: Correlation data based on linear regression analysis for all five fungal isolates tested. Average percentage mycosis (vertical axis) obtained was correlated with monthly CFU counts (horizontal axis). The equation of each line and R^2 correlation efficient is given.

3.4 DISCUSSION

The results show that all of the tested isolates including the commercial isolates are capable of persisting for a minimum of six months in the field whilst still initiating infection. The *Metarhizium* isolates collected from South African citrus soils (G 11 3 L6 and FCM Ar 23 B3) did however persist better than both the commercial isolates whilst the *Beauveria* isolate (G Ar 17 B3) only persisted better than one of the commercial isolates (Eco-Bb®) persisting similar to the other (ICIPE 69). The fact that the *M. anisopliae* isolates persisted better than the *B. bassiana* isolates is not uncommon and is consistent with literature as *M. anisopliae* has been suggested to be better adapted at surviving fluctuating environmental conditions (Vänninen *et al.* 2000; Bidochka *et al.* 1998 cited by Asensio *et al.* 2003) and requires less movement through the target insect to maintain fungal titres and virulence (Quesada-Moraga *et al.* 2007). This could explain why both *B. bassiana* isolates investigated showed the greatest reduction in viable conidia over the six month period. The observed decline in CFU count cannot be attributed to the addition of chemicals to the orchard as none were applied during the trial period (R. Moss, pers. comm*).

Monthly percentage mycosis recordings were variable and not correlated to CFU counts. This was surprising as with an increase in CFU, an increase in the percentage mycosis was expected. A larger CFU count is indicative of more viable fungal conidia present in the environment. The more conidia present, the greater the probability that an FCM 5th instar larva would come into contact with an infective fungal propagule and as a result, succumb to fungal infection. Interestingly, mycosis percentages were low for all three isolates in January. This could be coupled with the decline of CFU from December to January.

Reasons as to the variability in mycosis data is unclear, but could potentially be correlated to the combined effects of an optimal 26 °C and soil moisture content during soil bioassays. Ekesi *et al.* (2003) found that with an increase in temperature at the tested soil moisture level, two entomopathogenic fungal isolates tested, ICIPE 18 and ICIPE 20, showed an increase in the number of CFUs when temperature was increased from 15–20 °C to 25–30 °C. Soil moisture, although can reduce EPF persistence, is required for fungal germination (Inglis *et al.* 2001). The soil moisture content of 50 g soil used during the assays however was not measured, but visually appeared relatively high i.e. the soil was wet. High moisture levels are

*Rob Moss, owner of Mosslands Citrus Farm

also likely to be a factor based on previous bioassays conducted in Chapter 2, as higher concentrations were used to obtain, in some cases, similar mycosis percentages. The river sand used in these assays likely had very low soil moisture content. For example even though a relatively low CFU count was recorded (5.70×10^5 per 50 g soil) for the final month of isolate G 11 3 L6, the proportion of FCM succumbing to infection was high (88.34 %). This is in comparison to the laboratory bioassays where a concentration of 1×10^7 conidia.ml^{-1} was required to obtain a similar percentage mycosis. In addition to likely low moisture content, sandy soil has been shown to be the least suited for entomopathogenic fungal activity (Quesada-Moraga *et al.* 2007; Rumbos *et al.* 2008). Due to the saprophytic nature of *B. bassiana* and *M. anisopliae* however, it is possible that inoculum levels can increase in the absence of a host provided the appropriate nutrients for growth are available as these species are capable of growth on artificial media (Meyling & Eilenberg 2007). Conversely, compared to other ubiquitous saprophytic fungi, both *B. bassiana* and *M. anisopliae* are considered to be poor competitors for nutrients. For example, Magara *et al.* (2003) found that in the presence of organic soil amendments (coffee husks, cow manure and an inorganic fertilizer, NPK composite) *B. bassiana* was not capable of achieving high levels weevil mortality as the substrate on which it was applied (maize) was over colonized by two species of common soil fungi, *Aspergillus flavus* (Link) and *Penicillium chrysogenum* (Thom).

Since citrus orchards are irrigated and because of the shade provided by the trees, the soil below tends to remain moist. Moisture level under the tree canopy can be affected by both the type of irrigation system employed, the frequency of irrigation as well as rainfall, factors which are all likely to vary amongst orchards (Fares *et al.* 2000; Fereres *et al.* 2003). For Mosslands, orchards are irrigated when necessary (based on soil profile tests) to maintain the moist, but not saturated soil required for healthy tree development and a microjet irrigation system is employed (R. Moss, pers. comm; Fares *et al.* 2000; Fereres *et al.* 2003). Soil moisture can be both advantageous and detrimental to the use of these isolates against FCM. Moisture is stated as an extremely important factor for EPF germination, but it is also stated as a factor which reduces fungal persistence particularly in the absence of a host. Lingg & Donaldson (1981) showed that of the fungal isolates they investigated, a decrease in conidia survival was generally associated with an increase in soil moisture content. This is a result of conidial germination to form hyphae, which are more susceptible to the effects of environmental conditions surviving for much shorter periods outside a host. Both fungal species however tend to have broad host ranges and therefore could persist within soil-

dwelling insects other than FCM. In addition, due to the biology of FCM, it is likely to be present in the soil albeit at varying population levels throughout the year (van den Berg 2001). In this study, the ability of the fungi to persist in the presence of biological organisms was limited as the netting would have restricted the movement of soil-dwelling organisms into the bag holding the fungus amended soil.

Studies on EPF fungal ecology have found that certain EPF species may also persist in the environment as endophytes. An endophyte generally describes fungi or bacteria which are capable of residing in plant tissue without causing any noticeable symptoms (Carroll 1988; Vega 2008). In citrus soil, root material is abundant thus potentially providing refugia for applied EPF. In addition, studies by St Leger (2008) and Bruck (2010) make reference to the rhizosphere competence of certain entomopathogenic fungal species including *B. bassiana* and *M. anisopliae.* The rhizosphere is a small area of soil which encompasses the roots of the plants into which root exudates, such as amino acids and proteins, are released. These exudates can have profound effects on the microbial and biological communities which occur within the rhizosphere. Rhizosphere competence may however be more important for microbial control when root-feeding insects are of concern rather than insects which are merely pupating in the soil perhaps not even within the rhizosphere.

In general, the commercial isolates did not persist as well as the collected isolates. This could be because the collected isolates used were isolated from soil from Eastern Cape citrus orchards, Arundel (G Ar 17 B3 and FCM Ar 23 B3, soil type – Loam) and Mosslands (G 11 3 L6, soil type – Oakleaf Caledon) and were therefore adapted to the external conditions experienced in these environments. This could also explain the better persistence of isolate G 11 3 L6 as it was in fact isolated from Mosslands where the study was conducted. The active ingredient of Eco-Bb® conversely (*B. bassiana* strain R444), was isolated from Clanwilliam in the Western Cape and according to the Eco-Bb® leaflet, is adapted to hot, dry, semi-desert conditions, conditions which are unlike those experienced within the soil environment of a citrus orchard. Citrus orchards are perennial habitats that shade a large proportion of the soil environment to which a fungal microbial agent would be applied. Shade prevents UV-B rays from penetrating and killing off fungal conidia, whilst at the same time, tends to maintain humidity levels within the soil (McCoy *et al.* 2007 cited by Goble 2009). The *Metarhizium* isolate ICIPE 69 was collected from soil of another country (Democratic Republic of Congo) and therefore perhaps not suited to the soil environment of South Africa.

From the literature, it appears difficult to pinpoint the exact factors reducing fungal persistence in soil. The ability of fungi to persist in the soil appears to be dependent on the species as well as isolate. For example, some studies have found that pH can affect the stability and efficacy of the fungus (Groden & Lockwood 1991; Quesada-Moraga *et al.* 2007) whereas others have not (Lingg & Donaldson 1981; Vänninen *et al.* 2000). The main physical soil characteristic on which consensus does seem to exist is that sandy soils tend to reduce fungal persistence and efficacy (e.g. Rumbos *et al.* 2008) whilst clay soils followed by loamy soils appear to be better suited for EPF. Clay soils have been suggested to be more suited for fungal persistence as the clay particles can act against degradation due to the absorption of fungal conidia to the surface of the particles (Amir & Alabouvette 1993). Sandy soils however are said to be poorly suited possibly because of the low nutritional value associated with them (Rumbos *et al.* 2008). The soil used in this study was Oakleaf Caledon (Goble *et al.* 2010), a soil type which is generally characterised by a high clay content (Jansen van Rensburg *et al.* 2010). Some studies have shown that increasing the organic matter content of the soil, a reduction in conidial survival can result due to increased microbial and invertebrate activity (e.g. Vänninen *et al.* 2000; Meyling & Eilenberg 2006). Other studies however have suggested that an increase in organic matter content can have a positive effect on the persistence of entomopathogenic fungal conidia as with an increase in invertebrate activity, the number of potential hosts increases (Ali-Shtayeh *et al.* 2002; Klingen *et al.* 2002; Rumbos *et al.* 2008). Interestingly studies have shown that an increase in organic matter has a greater negative impact on *Beauveria* species which are more affected by antagonistic organisms than the hardier *Metarhizium* species (Studdert *et al.* 1990; Kessler *et al.* 2003).

The effect of biological factors such as mycophagy, movement of conidia out of the target area by other non-target insects and fungistasis, has not been investigated as extensively as physical soil factors, but should also be of high importance. Lingg & Donaldson (1981) suggested that the persistence of *B. bassiana* was more likely to be dependent on biological factors rather than physical factors. For example, it was found that a ubiquitous soil fungus, *Penicillium urticae* (Bainier) produces a water-soluble compound which inhibits the activity of *Beauveria bassiana.* Another aspect which might warrant consideration is the decay rate of the fungal carrier (rice) which provides a surface on which the fungus can sporulate. Speculating, one might find that carriers which decay more slowly allow the fungus to persist for longer.

This trial made use of EPF isolates which were not formulated. It is known that the persistence of microbial organisms can be improved through formulation either via the use of more hardy or infective propagules or via the addition of adjuvants which prolong persistence protecting the microbe from the potentially harmful effects of its surrounding environment (Jackson *et al.* 2010). In their unformulated state however, these isolates were capable of persisting in the field for six months. This is positive as under field conditions it is likely that these isolates will experience augmentation through the infection and resulting sporulation of FCM succumbing to fungal infection. Studies have reported increases in fungal titres in the presence of sporulating cadavers (Rath *et al.* 1995; Rumbos *et al.* 2008).

The time frame of this study was relatively short if one considers that EPF have been recorded to persist in the soil environment for much longer (Rath *et al.* 1995), in some cases several years (Rath & Bullard 1997 cited by Milner *et al.* 2003). It may therefore be of interest to have recorded persistence over a longer time frame and in the presence of soil-borne organisms. This study does however show that these three identified isolates, in their unformulated state, are a far better option for controlling FCM than some currently registered EPF products and that in the unformulated state, are capable of persisting for at least six months in a citrus orchard. Given the persistence shown here however and based on the life cycle and biology of FCM, it might be necessary to reapply the fungus in the middle of the year to ensure maximum control.

4

Preliminary formulation and application techniques for EPF of FCM in citrus

4.1 INTRODUCTION

To encourage farmers to shift towards the use of products which make use of microbes, such as EPF as their active ingredient, it is important to develop an application mode that is simple and compatible with their current management protocols. The ultimate aim is an integrated pest management approach whereby the use of environmentally undesirable products, such as many of the commonly used chemicals, is reduced and environmentally friendly products, such as natural products, increased (Lacey & Shapiro-Ilan 2003). The mode and timing of application of microbial products is dependent on a number of factors including mass production, formulation, crop phenology and the target insect/life stage (Jackson *et al.* 2010). Timing of application however, will often be governed by the ability of the applied microbial agent to persist in the target area as well as the type of crop (Jaronski 2010). The factors affecting persistence were discussed in more detail in Chapter 3.

EPF can be produced in a number of ways resulting in the production of different infective propagules. For *Metarhizium* and *Beauveria* species, propagules can include hyphae, conidia, blastospores and more recently in the case of *M. anisopliae*, microsclerotia (Jackson *et al.* 2010). Different substrates exhibit different chemical compositions which in turn can either negatively or positively affect the activity of the microbe (Herlinda *et al.* 2008). Each propagule type has its own set of advantages and disadvantages and is produced under different conditions (Jackson *et al.* 2010). The most common production method for *Beauveria* and *Metarhizium* is solid substrate production such as broken maize, rice or bran resulting in the formation of aerial conidia which can then be formulated accordingly. The number of conidia produced is dependent on the substrate used as well as the fungal isolate (Sahayaraj & Namasivayam 2008). *B. bassiana* is capable of producing microcycle conidia under liquid fermentation. These conidia are hydrophobic, less effective than aerial conidia

and require high concentrations of inorganic nitrogen for production. They have thus not been used in commercial mass production (Hegedus *et al.* 1990; Kassa *et al.* 2004). Blastospores, the propagules responsible for growth of the fungus within an insect host, can also be produced via liquid fermentation. Although these propagules tend to show greater infectivity, they are less resistant to environmental conditions and as a result exhibit reduced persistence. Blastospores have not been used for the commercial production of *Beauveria* or *Metarhizium* (de Faria & Wraight 2007). Recently, Jaronski & Jackson (2008) have shown that under specific nutrient conditions during liquid fermentation, *M. anisopliae* can produce dense hyphal networks termed microsclerotia. These propagules have been suggested to be appropriate for soil application.

After mass production, propagules are formulated so that they may be stored and used in the field. Formulations can vary and often incorporate adjuvants which can aid in efficacy enhancement, protection and prolonged persistence. Examples of adjuvants include various oils, nutrients and wetting agents. Common formulations include technical concentrates, wettable powders and oil dispersions. According to de Faria & Wraight (2007), technical concentrates contribute to most mycopesticide formulations worldwide followed by wettable powders and oil dispersions at 29 %, 22 % and 18 % respectively. Technical powders remain relatively unformulated consisting largely of the fungal propagules and production medium e.g. crushed rice. Wettable powders make use of fungal propagules which have been dried and processed into a powder, which when mixed with water forms a conidial suspension for use in spray application. Oil dispersions involve the suspension of fungal propagules in an appropriate oil medium (Jackson *et al*. 2010). Because of the hydrophobic nature of both *M. anisopliae* and *B. bassiana* conidia, oil formulations tend to work best if a spray application is considered and is preferred over a water suspension. It is more favourable as oil suspensions do not require the addition of a wetting agent. Oil-based formulations have however shown varying results with enhanced insecticidal activity reported in some studies (e.g. Lopes *et al.* 2011), but not in others (e.g. Ekesi *et al.* 2005).

For foliar treatment, spray applications are most common. For soil applications however, EPF can be formulated into granules. Granular formulations have an advantage over conidial suspensions as the fungal conidia have a substrate on which to sporulate enhancing the inoculum initially applied to the soil (Ekesi *et al.* 2005). In stored grain, diatomaceous earth (DE), fossilised remains of diatoms, is often an effective component of formulations

(Vassilakos *et al.* 2006; Athanassiou *et al*. 2008). The absorptive nature of DE causes dehydration of the target insect and its characteristic abrasiveness can increase the number of potential entry points for EPF (Riasat *et al.* 2011). The aim of this chapter was to investigate, on a small scale, the effect of different application formulations of two species of EPF (*M. anisopliae,* G 11 3 L6 and *B. bassiana,* G Ar 17 B3) and their ability to infect both 5th instar larvae penetrating the soil and those already present, potentially as pupae, in the upper layer of the soil.

4.2 METHODS

4.2.1 Fungi

For this experiment only two fungal isolates were used, G 11 3 L6 (*M. anisopliae*) and G Ar 17 B3 (*B. bassiana*). Isolates were grown both on a rice medium (Chapter 3, section 3.2.1.1) and SDA (Chapter 2, section 2.2.2). Growth on both media took approximately two weeks.

4.2.2 Formulation preparation

Two formulations were prepared, a granular and conidial suspension (Figure 4.1). Two concentrations per formulation were investigated, a high (1 x 10^7 conidia.ml^{-1}) and a low (1 x 10^5 conidia.ml^{-1}). The suspension was prepared as in Chapter 2, section 2.2.3 using sterile dH_20 containing 0.01 % Tween® 20 and the concentration and viability determined as described in Chapter 2, section 2.2.3.1 and 2.2.3.2. The granular formulation was prepared by crushing the rice overgrown with fungus with a sterile mortar and pestle. The concentration per gram of rice was calculated with the aid of a haemocytometer following the suspension of 1 g of crushed rice in 10 ml of sterile dH_20 containing 0.01 % Tween® 20. The initial concentration measured was then diluted with sterile crushed rice prepared in the same manner as the rice used on which to culture the fungus.

Figure 4.1: (A) Granular formulation. This was prepared by crushing, using a mortar and pestle, the rice overgrown with fungus; (B) Conidial suspension. This was prepared as outlined in Chapter 2, section 2.2.3.

4.2.3 Experimental design

Each treatment was applied to approximately 400 g of autoclaved soil (collected from Mosslands) in sterile plastic curry tubs (Figure 4.2A). The conidial suspension was applied using a standard 25 ml spray applicator whilst the granular formulation was sprinkled on the soil surface using a sterile spoon. Application took place both before the addition of FCM 5th instar larvae (pre-larval treatment) to the tubs and one day prior to larval addition (post-larval treatment).Ten 5th instar larvae, ready to pupate within 24 hours, were added to the surface of the soil in each tub and allowed to pupate (Figure 4.2B). The tubs were sealed with lids into which a hole was cut and covered with mesh to allow for ventilation. Ten replicates were performed per treatment. A total of 16 treatments including four controls were used (Table 4.1).

Tubs were stored at the Waainek Research Station, Rhodes University, Grahamstown, in a constant environment room (temp: 26 °C; humidity: 60 %) for 3– 4 weeks and monitored twice a week (Monday and Friday). The number of FCM (dead larvae and pupae which failed to emerge) was recorded upon completion of the experiment. These dead individuals were then surface sterilised in 70 % ethanol and placed onto SDA plates and incubated at 26 °C. After three days, mycosis was scored as before (Chapter 2, section 2.2.4.1).The number of FCM that eclosed was also recorded.

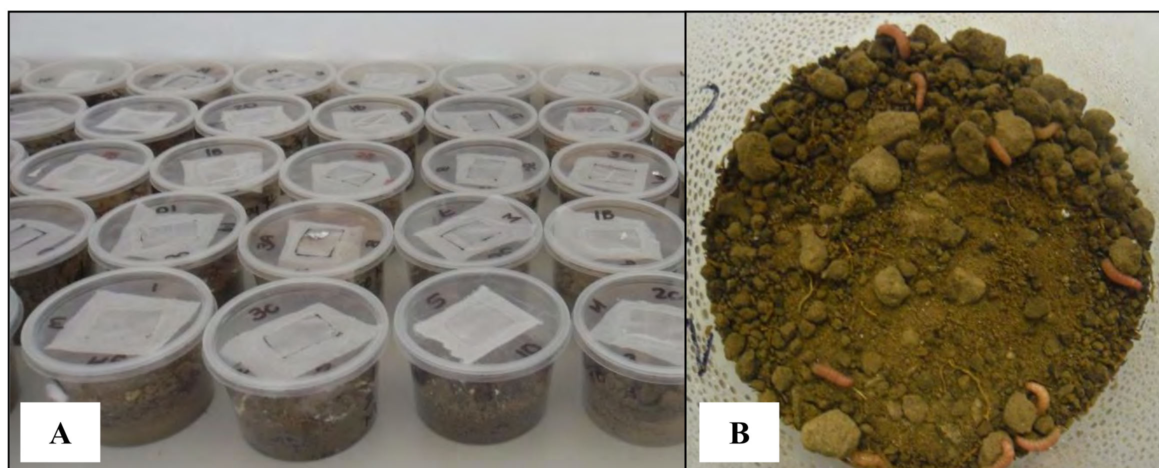

Figure 4.2: (A) Treated curry tubs; (B) 5th instar larvae burrowing into soil to pupate after being added to the tubs.

Table 4.1: Treatment details. The same treatments were used for each isolate and both pre-larval and post-larval application.

Treatment	Description	Details
Spray (H)	conidial suspension (high) (+ Tween®)*	5 ml of a 1 x 10^7 conidia.ml^{-1} concentration was applied using a sterile spray application
Spray (L)	conidial suspension (low) (+ Tween®)	5 ml of a 1 x 10^5 conidia.ml^{-1} concentration was applied using a sterile spray application
Rice (HH)	granular (high), concentration (high)	2.5 g of a 1 x 10^7 conidia.ml^{-1} concentration was sprinkled onto the surface of the soil
Rice (HL)	granular (low), concentration (high)	0.5 g of a 1 x 10^7 conidia.ml^{-1} concentration was sprinkled onto the surface of the soil
Rice (LH)	granular (high), concentration (low)	2.5g of a 1 x 10^5 conidia.ml^{-1} concentration was sprinkled onto the surface of the soil
Rice (LL)	granular (low), concentration (low)	0.5 g of a 1 x 10^5 conidia.ml^{-1} concentration was sprinkled onto the surface of the soil
		Control Treatments
DWA	Spray – dH_2O and Tween®	5 ml of sterile dH_20 + 0.01 % Tween 20® was applied using a sterile spray applicator (autoclaved soil)
DWU	Spray - dH_2O and Tween 20®	5 ml of sterile dH_20 + 0.01 % Tween 20® was applied using a sterile spray applicator (non-autoclaved soil)
GH	App - granular (high)	2.5 g of the granular product (crushed rice) was sprinkled onto the surface of the soil
GL	App- granular (low)	0.5 g of the granular product (crushed rice) was sprinkled onto the surface of the soil

*Tween® is a non-ionic detergent used to obtain a uniform conidial suspension (Sigma Aldrich, South Africa).

4.2.4 Statistical analysis

For both isolates, data failed the test for normality even after arcsine transformation. Comparisons among treatments were therefore made using the non-parametric Kruskal-Wallis ANOVA. If a significant difference was found ($p < 0.05$), a multiple mean test of the ranks was conducted to determine where this difference occurred.

4.3 RESULTS

Pre-larval application indicated that a high conidial spray application was more successful than all other application treatments for isolate G Ar 17 B3 with 84 % mycosis (Figure 4.4). For isolate G 11 3 L6 however, a granular application at a high concentration obtained a mycosis percentage of approximately 70 % (Figure 4.3). No significant differences were, however, recorded between different application treatments during pre-larval application. Overall, a higher FCM mortality was recorded for *B. bassiana* than *M. anisopliae.* FCM mortality ranged between 5–80 % for isolate G Ar 17 B3 and 0–70 % for isolate G 11 3 L6. Lowest recorded values for both isolates were recorded for a low granular formulation concentration, regardless of the amount applied, followed by a low concentration conidial spray. FCM mycosis recorded in control treatments was minimal, with no mycosis recorded when only sterile dH_2O containing Tween® 20 was used (Figure 4.3 and 4.4). Mycosis was much lower for post-larval application treatments with the mycosis percentage never exceeding 19 % for isolate G 11 3 L6 and 22 % for isolate G Ar 17 B3. Again the highest percentages were recorded for the high conidial spray application and the high granular application for G Ar 17 B3 and G 11 3 L6, respectively and the lowest for the low concentration granular formulation.

Significant differences were recorded amongst treatments for both isolates (G 11 3 L6, Kruskal-Wallis: $H_{15,\ 160} = 114.49$, $p < 0.05$ and G Ar 17 B3, Kruskal-Wallis $H_{15,\ 160} = 109.91$, $p < 0.05$). Although pre-larval and post-larval application treatments appeared to be visually different to one another for isolate G 11 3 L6, statistically were not in most cases. This is likely owing to the robustness of the non-parametric Kruskal Wallis test. Pre-larval application treatments were however significantly different to all of the control treatments whereas post-larval application treatments were not. For isolate G Ar 17 B3, however, pre-

larval application treatments tended to be statistically different to both post-larval and control treatments. Again, post-larval application treatments were not significantly different to the control treatments.

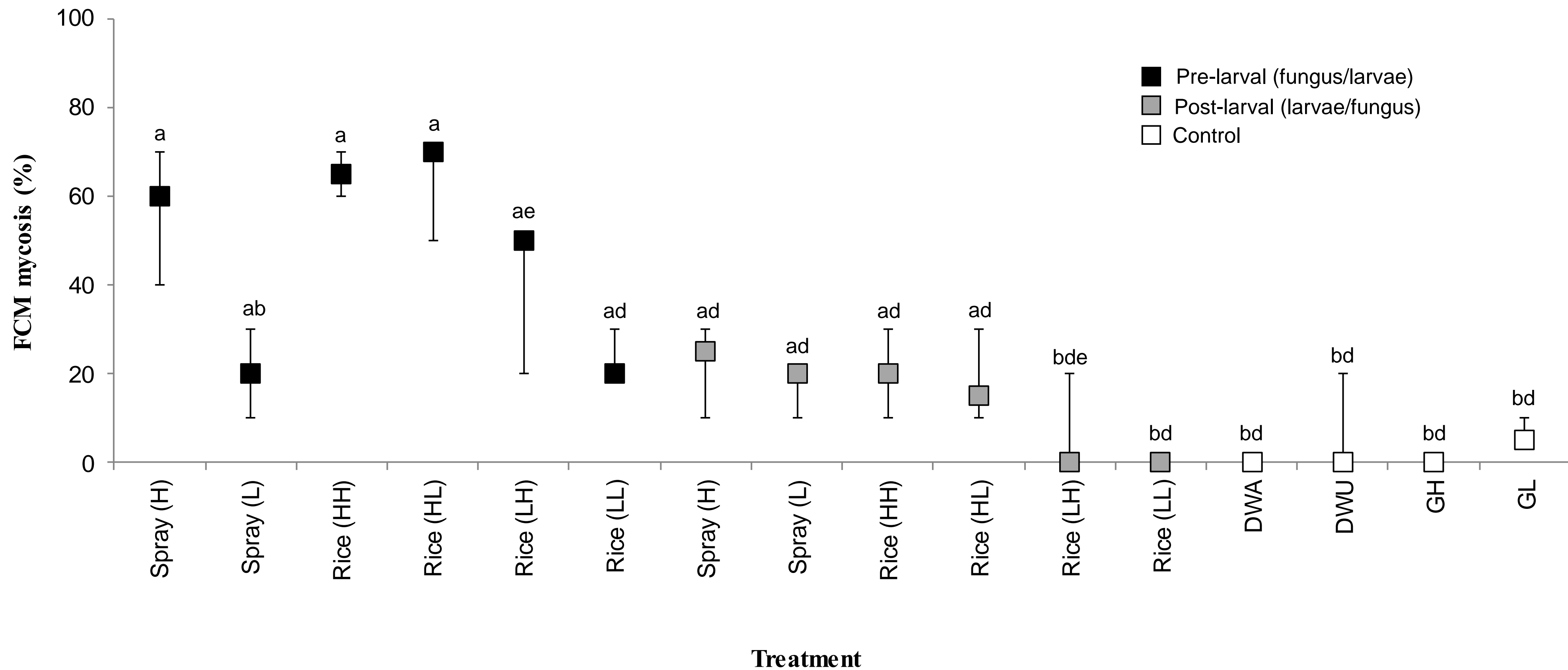

Figure 4.3: Percentage of FCM mycosed for the different treatments where *M. anisopliae* isolate G 11 3 L6 was used. Blocks represent the median; whiskers the interquartile range. Black blocks represent pre-larval application treatments; grey blocks post-larval and the white blocks the various controls. Different letters indicate statistically different treatments (Kruskal-Wallis: $H_{15,\ 160} = 114.49$, $p < 0.05$).

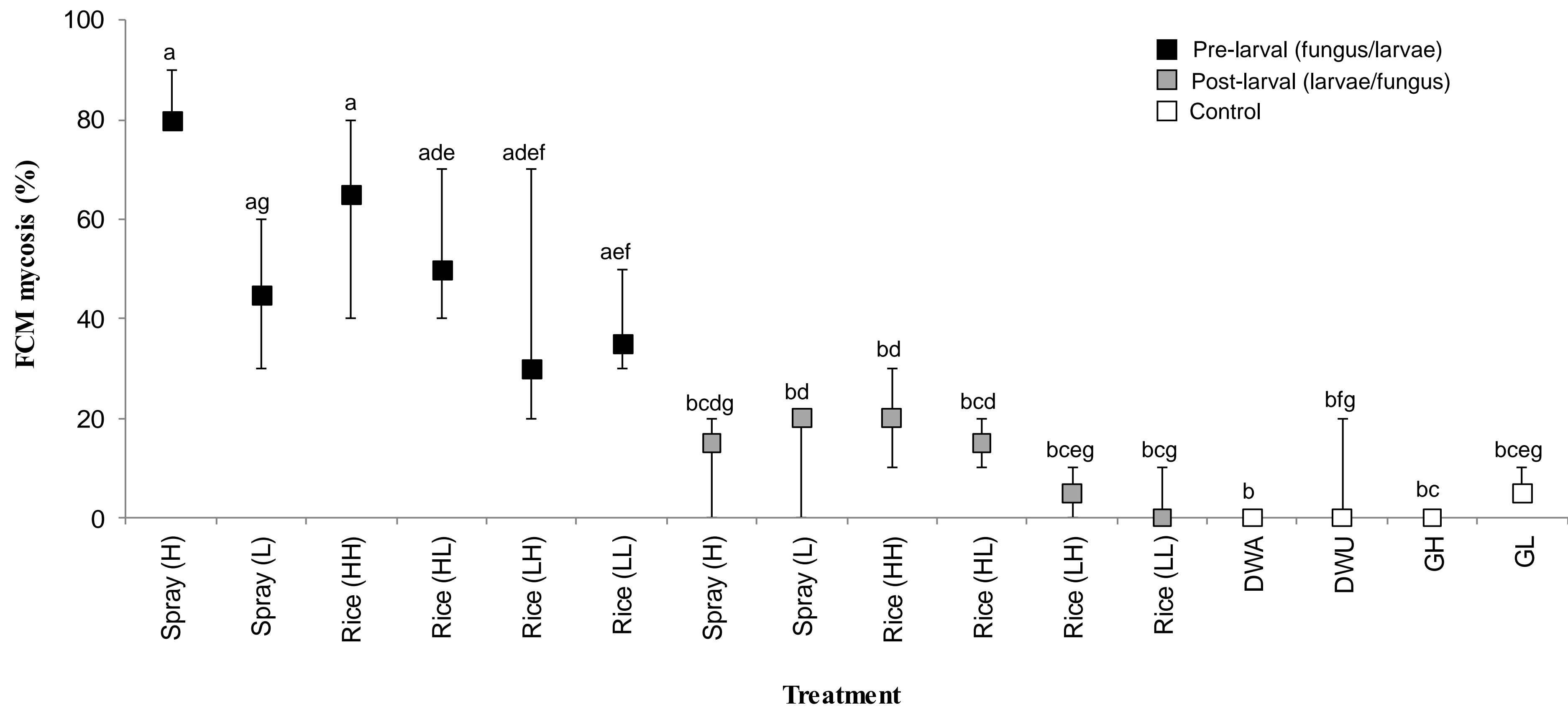

Figure 4.4: Percentage of FCM mycosed for the different treatments where *B. bassiana* isolate G AR 17 B3 was used. Blocks represent the median; whiskers the interquartile range. Black blocks represent pre-larval application treatments; grey blocks post-larval and the white blocks the various controls. Different letters indicate statistically different treatments (Kruskal-Wallis: $H_{15, 160} = 109.91$, $p < 0.05$).

4.4 DISCUSSION

The outcome of these trials indicates that it is essential for the final instar FCM larva to move past a "fungal barrier" as they burrow into the soil surface to pupate. By doing so, conidia are able to adhere to the insect cuticle after which conidial germination and cuticular penetration can occur. It is unfortunate that neither of the fungal isolates was capable of perforating deeper into the soil to infect pupating or already pupated FCM. A trial conducted by Storey & Gardener (1987) investigated the vertical movement of *B. bassiana* in four Georgia soil types (Greenville, Townley, Cecil and Tifton). Results showed that the conidial movement was affected by soil type. Coarser soils, such as the soil used in these trials (Oakleaf Caledon), were more restrictive to vertical movement than finer soils. Vertical movement of fungal spores was however largely restricted to the upper 15 cm of the soil surface with the majority being found within the first 5–10 cm. One of the most important factors influencing the movement of microorganisms through the soil is the flow of water, which was not present in this laboratory study. The flow of water in the soil is affected by particle size. Particle sizes tend to be larger in coarser soils than sandy soils thereby generating larger pore spaces and increased movement of water. As a result microbial movement is usually greater in these coarser soils (Dighton *et al.* 1997). These trials did, however, take place under laboratory conditions and therefore under field conditions, the outcome may well be different.

In the soil environment, numerous other biological organisms exist all of which have the potential to redistribute the applied fungus. Dighton *et al.* (1997) reviewed some of the microorganisms which have been reported to or could potentially play a role in the movement of microorganisms in the soil. The movement of conidia could be both positive and negative. Negatively, organisms could move the conidia out of the target area to which the fungus was initially applied thereby reducing the number of conidia in the area where the majority of the insect pest population is expected to be found. This is problematic as insects need to come into contact with an infective fungal propagule in order to become infected (Shah & Pell 2003). If fungal conidia are being moved out of the area, the likelihood that the insects will come into contact with the spore decreases. This could have implications for FCM mortality. Conversely, the re-distribution of EPF can be positive in that conidia could potentially be brought into contact with uninfected FCM individuals. As a result, an increase in FCM mortality due to fungal infection is possible. A study by Dromph (2001) found that some

collembolan species are capable of carrying conidia of three fungal species including *B. bassiana* and *M. anisopliae* on their cuticle without reducing conidial viability. It was also found that conidia could be carried by the collembolan in the gut, but this had implications on conidial viability for *M. anisopliae.* Dromph (2003) also investigated the ability of three different collembolan species to transfer these fungal species to *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae). All three collembolan species were capable of transmitting sufficient concentrations of conidia to *T. molitor* to ensure infection by the fungus although their ability to do so differed significantly. Oribatid mites have also been shown to be capable of dispersing a number of fungal conidia commonly found in the soil (Renker *et al.* 2005).

The formulations used for the application of each fungal isolate in this study were very simple. The granular formulation involved the crushing of rice that had been overgrown with fungus whilst for the spray application; an aqueous suspension consisting of conidia suspended in dH_2O, supplemented with Tween® 20, was used. For soil application, granules have been shown to be more effective. Pre-larval application trials in this study demonstrated significant differences in FCM mortality between the aqueous formulation and granular formulation for isolate G Ar 17 B3, but not G 11 3 L6. Unlike Ekesi *et al.* (2005), the aqueous formulation at a high concentration, 1 x 10^7 conidia.ml^{-1} worked best for isolate G Ar 17 B3 with an FCM mycosis percentage of 80 %. For the granular formulation of the isolate, FCM mycosis was greatly reduced (65 %). For isolate G 11 3 L6 FCM mycosis percentages were similar for the high aqueous formulation spray application (60 %) and the granular formulation applied at both a high (65 %) and low concentration (50 %). Reasons for this are unclear, but different formulations are known to affect the efficacy of different fungal species and isolates of the same species in varying ways (Jackson *et al.* 2010). This may explain why a large difference, although not statistically different, was found between the high concentration aqueous formulation and the high concentration granular formulation for *B. bassiana*, but not *M. anisopliae*.

As previously mentioned, EPF soil application favours the application of fungal granules. Often this involves application to the soil by hand followed by mixing into the upper layers of the soil surface (e.g. Samson *et al.* 2006; Ekesi *et al.* 2011). Skinner *et al.* (2012) showed a 60 % reduction in plant damage caused by the western flower thrip, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), in treatments where fungal covered millet grains were mixed with potting media. Here, the experimental fungi also proved to be more effective than

an already available commercial product registered for the control of thrips. Ansari & Butt (2012) also achieved good control of the crane fly, *Tipula paludosa* (Meigen) (Diptera: Nematocera) when rice colonized with *Metarhizium robertsii* strain V1005 was added to potting media during greenhouse trials. A number of granular products are also commercially available e.g. BioGreen (Rath *et al.* 1995) and BioCane™ (Samson *et al.* 2006) both of which have been shown to be highly effective in suppressing the target pest population.

Comparative studies have also noted the effect of formulation on fungal efficacy. Compared to spray applications, granular applications tend to achieve better results. Ekesi *et al.* (2005) tested three different formulations of *M. anisopliae* against three fruit fly species. Formulations included an aqueous conidial suspension, a 50:50 oil/aqueous conidial suspension and a granular formulation. The suspensions were applied to the soil at a rate of 1 x 10^{14} conidia.ha^{-1}; the granular 40 kg.ha^{-1}. For all three fruit fly species, a greater reduction in adult eclosion was obtained in treatments to which fungal granules were applied. This was followed by the oil/aqueous suspension. The reduction in adult eclosion however was not statistically different in both aqueous suspension treatments and control treatments. Similar results were found by Maniana (1993). A single granule application of *B. bassiana* achieved similar levels of control as two applications of a conidial suspension applied as a spray against *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae).

Kreuger *et al.* (1992) investigated the control of *Papillio japonica* (Newman) (Colepotera: Scarabaeidae) using dry mycelia particles and conidia. No significant differences were found in insect mortality between the two treatments, but mortality occurred more rapidly in mycelia treatments. The authors speculated that this was because although conidial sprays achieve good coverage of infective units, these infective units are usually found singularly whereas in mycelial application, infective units tend to be aggregated as a hyphal mass increasing the number of infective units the target insects interact with in a single event. The probability of infection is therefore higher. However, Villani *et al.* (1994) found that when a mycelial application was used against the grubs of *P. japonica,* the grubs tended to move away from the site of application. This is not beneficial as in order for EPF to cause infection, contact between the fungus and insect needs to occur (Shah & Pell 2003).

For the control of FCM using EPF in citrus it is suggested that an application which provides the best coverage of the tree canopy will be most effective. The greater the chance that FCM

5th instar larvae have in coming in contact with the fungal spores, the greater the likelihood of infection and therefore mortality. If this is the case, drip irrigation may not be effective, but spray irrigation might. If spray application is to be used then the *B. bassiana* isolate G Ar 17 B3 would be the more appropriate option. The distribution of fungal conidia applied as a spray can be affected by the type of spray apparatus used and nozzle used (Bateman & Chapple 2001) and should therefore be investigated. Spray application however does not appear to be as effective as a granular application in the long term in which case the *M. ansiopliae* isolate G 11 3 L6 would be the more appropriate option. The advantages and disadvantages of each application method therefore need to be thoroughly evaluated before a consensus on the best means of EPF application in citrus is reached. Some studies using entomopathogenic nematodes have investigated the introduction of the control agents via insect cadavers (Shapiro & Lewis 1999). Whether the use of EPF cadavers applied to soil will increase the efficacy of the fungus is not known as it did in the case of EPNs. EPNs are more reliant on their hosts for persistence in the field whereas EPF are not (Susurluk & Ehlers 2008). Passaging, a technique of passing the fungus through the target insect, is routinely used to maintain the virulence of the fungal isolate and therefore EPF introduced via cadavers may be more virulent. This however remains to be tested. The application of EPF in this manner may however be impractical even if a greater virulence is found especially considering that the developed EPF product needs to compete with easy to apply chemicals.

Another important aspect to consider is the compatibility of the microbial agent with other regularly used agrochemicals. Eliminating the use of chemicals entirely is not economically plausible. The probability that chemicals and biological control agents will be applied in the same field, in some cases simultaneously or mixed in the same equipment as agrochemicals, is high (Jaros-Su *et al.* 1999). Compatibility studies have been conducted under both laboratory and field conditions where EPF have been used in combination with a variety of fungicides and pesticides (e.g. Jaros-Su *et al.* 1999; Samson *et al.* 2005). In general, studies conducted in the laboratory indicate that even at lower than registered field rates, particularly fungicides, chemicals can be detrimental to the efficacy of EPF in controlling the target pest. In contrast, field studies often find minimal reduction in EPF efficacy. This is attributed to the lack of contact between the applied chemical and the fungus either spatially, temporally or both. Cavalcanti *et al.* (2002) tested the effect of four pesticides on the germination, vegetative growth, sporulation and pathogenicity of *B. bassiana*; Confidor 700 (a.i. imidacolprid), Actara® 250 WG (a.i. thiamethoxam), Meothrin 300 CE (a.i. fenpropathrin)

and Rovral® 500 SC (a.i. iprodione). The fungicide Rovral® was the least compatible whereas fungal growth was reduced in the presence of Meothrin. Meothrin is used in FCM control and should therefore be evaluated as to the effect it has on the fungal isolates investigated in this study (Moore & Hattingh 2012). Confidor 700 and Actara® however were compatible with *B. bassiana.* These results however were obtained *in vitro*. The compatibility between these EPF isolates under investigation for FCM control in this study would need to be investigated. A similar study was conducted by de Oliveira *et al*. (2003). Here, nine insecticide formulations were investigated for use in IPM in coffee plantations where *B. bassiana* is used to control the coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae). The chemicals Thiamethoxam and Alpha-Cypermethrin were the most compatible. As already mentioned, the isolates used in this thesis were collected from citrus orchards within South Africa and in which chemical spray programmes have been employed to some degree. The orchards which isolate G 11 3 L6 and G Ar 17 B3 were collected from are known to be conventional orchards, not organic and thus chemicals are sprayed intermittently throughout the year when necessary (Goble *et al.* 2010). It is therefore reasonable to assume that these isolates have been exposed to certain agrochemicals and therefore might posses a measure of tolerance to them.

Not only can chemicals have detrimental or neutral effects on EPF, but they can also have synergistic effects. Quintela & McCoy (1998) and Ramakrishnan *et al.* (1999) found that imidacolprid increased the susceptibility of the root weevil larvae, *D. abbreviatus* (L.) (Coleoptera: Cuculionidae) and the termite *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae) respectively. *Reticulitermes flavipes* mortality after 10 days exposure to a combination of *M. anisopliae* and imidacolprid was significantly higher (96.5 %) than when exposed to *M. anisopliae* alone (0.10 %) or the same concentration of imidacolprid alone (20.3 %). This is suspected to be a result of imidacolprid activity which prevents the grooming behaviour of termites and therefore the removal of fungal spores from the cuticle (Boucias *et al.* 1996). Quintela & McCoy (1998) showed that the cuticle of *Diaprepes abbreviatus* larvae treated with imidacolprid had more conidia attached in comparison to untreated larvae. Again, this was attributed to imidacolprid activity which reduced larval mortality. Healthy larvae were found to be able to remove conidia as they moved through the substrate. It was however also found that imidacolprid doses higher than 0.01 % had a negative impact on the ability of *M. anisopliae* to adhere to the insect cuticle. In citrus, imidacolprid is applied as a soil drench restricting this chemical to the base of the tree trunk

(Confidor®70 WG product label, Bayer CropScience, South Africa). The possible synergistic interaction between EPF applied in citrus with imidacolprid may therefore be limited as EPF application would require greater coverage underneath the tree canopy.

Mohamed *et al.* (2011) tested the registered EPF product Green Muscle® (a.i. *M. anisopliae* var. *acridum*) in combination with Nomolt®, an insect growth regulator. The combination of various doses of Nomolt® and Green Muscle® caused 100 % mortality of the desert locust, *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae), within 14 days of exposure. Nomolt® alone caused 100 % mortality after only 21 days. Unfortunately no treatment where only Green Muscle® was used was conducted therefore it is unclear whether this effect is synergistic or additive. Insect growth regulators however have been suggested to weaken insect cuticles increasing the probability of fungal penetration (Joshi *et al.* 1992 cited by Mohamed *et al.* 2011). Nomolt® is a registered chemical for the control of FCM in South Africa and therefore it might be of interest to investigate the combined effects of the isolates used in this study with that of Nomolt®.

In summary, the results of this chapter indicate that for both *B. bassiana* and *M. anisopliae* isolates, aqueous or granular formulations are capable of causing more than 50 % reduction in FCM numbers using a conidial concentration of 1×10^7 $conidia.ml^{-1}$. These trials were however conducted under laboratory controlled conditions. Therefore, in order to better understand the effects that formulation and application can have on the ability of these two isolates against FCM under natural conditions, field trials need to be undertaken. During these trials, the cost and time required for each formulation, ease and cost of application, as well as the compatibility of these isolates with other agricultural products needs to be investigated. It is essential that the benefits of the product outweigh the costs.

5

GENERAL DISCUSSION

5.1 SUMMARY OF RESEARCH

With chemical residue restrictions being placed on exported citrus fruit, particularly fruit exported to the European Union, and the ability of FCM to develop resistance towards currently used chemical insecticides, it has become important to identify alternate control options that as part of an IPM programme, can control the target pest below an economic threshold (Bedford 1998; Hofmeyr & Pringle 1998; Urquhart 1999). One such alternate control strategy against FCM is the use of entomopathogenic fungi to control the soil-borne life stages; stages that are currently not the target of any other biological or chemical control options. It is therefore likely that the integration of EPF into FCM management will complement currently employed control measures.

Initial investigation into the use of EPF against FCM in citrus began with the work conducted by Goble *et al.* (2010) and Goble *et al.* (2011). Here, soil from six citrus orchards within the Eastern Cape was sampled and screened for the presence of entomopathogenic fungal isolates. A total of 288 soil samples were collected from which 62 fungal isolates were isolated. *Beauveria bassiana* and *Metarhizium anisopliae* were the most commonly isolated fungi with an occurrence of 15.63 % and 3.82 % respectively. Other species included *Conidiobolus coronatus* (1 isolate) (Constantin) Batko, *Lecanicillium psalliota* (4 isolates) Treschew and *Metarhizium flavoviride* (1 isolate) Gams and Rozsypal. Of these 62 isolates, 21 were further screened for their control potential against FCM as well as the fruit flies, *Ceratitis capitata* and *Ceratitis rosa.* Four of the most virulent *B. bassiana* isolates (G Moss R10, G 14 2 B5, G B Ar 23 B3 & FCM 10 13 L1) were further subjected to concentration-dose response and exposure-time response bioassays (Goble *et al.* 2011).

This thesis has therefore added to the initial research conducted by Goble *et al.* (2010, 2011). The sole focus of the study was however on FCM. Of the 21 isolates which were screened,

the 12 which recorded the highest percentage mortality against FCM were re-screened. The eight isolates which performed well were subjected to concentration-dose response bioassays (Chapter 2). These isolates belonged to two species, *M. anisopliae* and *B. bassiana*. Only two of these isolates used in the concentration dose-response assays were also evaluated by Goble *et al.* (2011). LC_{50} values varied between 0.36×10^5 conidia.ml^{-1} and 5.64×10^7 conidia.ml^{-1} and like the results recorded by Goble *et al.* (2011), a dose dependant relationship was found (Chapter 2). Based on these trials and the re-screening results, three isolates were chosen and subjected to exposure-time response assays. None of these three isolates were investigated during the exposure-time response trials conducted by Goble *et al.* (2011). In general, a LT_{50} of four days was recorded for all isolates exposed to a concentration of 1×10^7 conidia.ml^{-1}. Again, in accordance with Goble *et al.*'s (2011) findings, percentage mycosis increased with an increase in exposure time (Chapter 2). The ability of all three of these isolates to persist in the field and the effect that application could have on the isolates was investigated. All three isolates were capable of persisting in a citrus orchard for a period of six months whilst still remaining infective against FCM 5th instar larvae (Chapter 3). Laboratory application trials indicated that both a spray (conidial suspension) and granular (crushed rice overgrown with fungus) application is capable of causing infection amongst FCM soil-borne life stages for both fungal isolates. The spray application however achieved higher levels of control for *B. bassiana* isolate G Ar 17 B3 than *M. anisopliae* isolate G 11 3 L6 which obtained a similar mycosis percentage for both the spray and granular application. Unfortunately, the control of FCM already present in the soil was limited under laboratory conditions (Chapter 4). The indigenous isolates performed better than the tested commercial products (Chapter 2 and Chapter 3).

Therefore to date, what is known about the potential of EPF against FCM is that: (1) three fungal isolates have been identified as having exceptionally good control potential against FCM; (2) FCM mycosis is dependent on both fungal concentration as well as exposure time; increases in both fungal concentration as well as exposure time result in increased percentage mycosis; (3) the isolates are capable of persisting in the field for six months on rice granules; (4) regardless of formulation or application mode, FCM 5th instar larvae must be forced to come into contact with the fungi as they burrow into the soil to pupate; and (5) based on the isolates' ability to perform better than the commercial isolates and the improved ability of isolate G 11 3 L6 to persist in its original environment, different isolates may be more beneficial for FCM control in different areas.

5.2. GLOBAL PERSPECTIVE

The use of EPF to control insect pests is not uncommon and has been used worldwide to control pest species attacking a wide variety of crops, both in the open-field and in glasshouses, as well as in natural systems such as forests (Shah & Pell 2003; Augustyniuk-Kram & Kram 2012). EPF have been used in the USA to control a variety of insect pests including the gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) in forests and *Aphis gossypii* (Glover) (Hemiptera: Aphididae) on cotton (Milner *et al.* 1982; Shah & Pell 2003). In North America, three *B. bassiana* based products have been developed and used, Mycotrol® (Mycotech, USA) which is registered for the control of numerous pests including thrips, aphids, grasshoppers and whiteflies; BotaniGard® (Mycotech, USA), registered for use in greenhouses; and Mycotrol® O (Mycotech, USA) which is registered for use in organic farming within the USA. In Europe, *Beauveria brongniartii* (Saccardo) Petch (Ascomycota: Clavicipitaceae) is used for the control of cockchafers and *Lecanicillium lecanii* (Zimmerman) has been developed into a biopesticide for the use against aphids, thrips and whiteflies (Shah & Pell 2003). In Australia two registered *Metarhizium* based products, BioCane™ (Bio-care Pty. Ltd., Australia) and BioGreen™ (Bio-care Pty. Ltd., Australia) are used for the control of the greyback cane grub and grasshoppers respectively (Milner *et al.* 2000; Samson *et al.* 2005). In South Africa, biopesticides incorporating EPF as their active ingredient are registered for the control of whitefly and red spider mite on beans, tomatoes, brinjals and cucumbers (Eco-Bb®) (PHP, South Africa) as wells as thrips and diamondback moth (Broadband®) (Becker Underwood, South Africa). In addition to the already worldwide commercial use of EPF as pest control agents, researchers continue to investigate the potential of EPF in the control against economically damaging agricultural pests and with promising results (Godonou *et al.* 2000; Nankinga & Moore 2000; Jonason *et al.* 2005; Kao 2007; Anand *et al.* 2009; Vera *et al.* 2010; Goble *et al.* 2012; Li *et al.* 2012).

EPF are therefore being used worldwide as pest control agents. This suggests that EPF research is a worthwhile investment and that these organisms are capable of performing effectively against a number of pest species, under a variety of climatic conditions and in a variety of cropping systems. The fungal species and isolate along with the formulation and application technique used differ between all these published studies. Therefore if EPF are capable of successfully being utilised in other cropping systems against other agricultural

pests, it stands to reason that through correct formulation and application, the identified isolates from this study should be capable of achieving some level of control against FCM in citrus orchards in South Africa. Since the control of FCM in citrus of South Africa is becoming increasingly reliant on multiple control measures rather than a single control approach (Moore & Hattingh 2012), compatibility will become an important issue if the fungi are to successfully integrate into current FCM management protocols.

5.3 INTEGRATION OF EPF INTO FCM CONTROL

FCM control has largely focused on either the egg or neonate life stages as a means to prevent larval entry into the fruit or by preventing egg laying either via a reduction in the number of adults (attract and kill) or a reduction in the number of viable eggs deposited by female FCM (SIT & mating disruption) (Chapter 1, section 1.2.5). This is understandable as infested fruits are not usable and are therefore a financial loss. Although in combination with a variety of control tactics a high level of control at the egg/neonate stage can be achieved, control will never be 100 %. Some insects may be resistant to the applied chemicals or missed during application whilst biological control, by definition, is very rarely capable of achieving 100 % control even when a multiple control approach is used (Shah & Pell 2003). Currently, no registered FCM control methods target the soil-borne life stages of FCM (Moore & Hattingh 2012). Incorporating these microorganisms into FCM control practices therefore provides a more complete control approach whereby every life stage is targeted.

Currently, a variety of control options is available for the control of and employed against FCM in citrus orchards (Chapter 1, section 1.2.5). The efficacy of these methods varies across regions as well as amongst citrus types with navel oranges and some mandarin types considered the most susceptible to infestation (Newton 1998). Since chemical control is largely concerned with targeting the egg or neonate life stage, it is applied as a foliar treatment. In contrast, EPF, in this case, would be applied directly to the soil. It is therefore unlikely that the applied chemicals used in FCM control will come into contact with the applied EPF. However chemical run-off can occur thus compatibility between chemicals and EPF should not be overlooked. Chemicals which are more likely to negatively impact the efficacy of EPF include fungicides, those applied directly to the soil e.g. fertilisers or non-compatible chemicals applied using the same equipment as EPF (Jaronski 2010).

Alternate biological control agents of FCM currently registered include the parasitoid, *T. cryptophlebia* and the granuloviruses incorporated into the products Cryptogran® and Cryptex® (Moore & Hattingh 2012). Neither of these two control options are however likely to impact the efficacy of EPF. These viral products are highly specific towards FCM (Moore 2002a) and therefore will not interfere with EPF growth or infectivity whilst *T. cryptophlebia* are egg parasitoids and are thus spatially separated from the soil-dwelling EPF having no discernible reason to interact with the fungus. Because the isolates investigated in this study are from species which are known to have broad host ranges (Zimmerman 2007a,b), it is possible that *T. cryptophlebia* might become infected by the fungi. However, again due to spatial separation between the two organisms and the lack of dispersal ability of the two fungal species, contact is unlikely. In order for an insect to become infected, contact between the insects and fungus is essential (Shah & Pell 2003). However even if contact occurs, infection may not occur if no recognition between the fungus and insects is experienced (Castrillo *et al.* 2005). Non-target effects both in the laboratory and field would nevertheless be worthwhile investigating particularly if the EPF are to be applied in areas currently employing parasitoid control.

The use of entomopathogenic nematodes (EPN) in FCM control is currently under investigation and, like EPF research, has shown promising results (Malan *et al.* 2011). EPNs are also applied to the soil and therefore the likelihood of interaction between these organisms applied at the same time in the same location is high. The interaction between EPF and EPN against FCM is unknown, but can and should be evaluated if both control agents become registered for the control of FCM in citrus orchards. Other studies have found reduced levels of pest control e.g. Barbercheck & Kaya (1991) recorded reduced levels of the pest *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) in treatments where *B. bassiana* was applied in conjunction with the nematodes *Heterorhabditis bacteriophora* (Poinar) (Nematoda: Heterorhabditidae) and *Steinernema carpocapsae* (Weiser) (Nematoda: Steinernematidae) and Ansari *et al.* (2004) found that when *M. anisopliae* CLO 53 was applied 2, 3 and 4 weeks prior to nematode application pest control improved. In contrast Shapiro-Ilan *et al.* (2004) found that the simultaneous application of EPF and nematodes was antagonistic in all combination treatments suggesting that interactions are likely to be dependent on both fungal and nematode species.

Orchard sanitation is exceptionally important in FCM control and is currently recommended to be employed twice a week by farmers (Moore & Kirkman 2009). During the warm summer months, FCM show increased activity and tend to develop at a much faster rate (Newton 1998; Stibick 2008). As a result, FCM have a greater probability of exiting the abscised fruit to pupate in the soil prior to collection and disposal (Moore & Kirkman 2009). This reduces the efficacy of orchard sanitation. The application of EPF however could be a valuable solution to this problem as escaping larvae could become infected with the fungus thus reducing the number of FCM which would have eclosed if EPF were not present. The application of EPF to the soil however does not mean that orchard sanitation can be removed from management practices as the benefits of orchard sanitation are not merely for the purpose of FCM control. It does however mean that if orchard sanitation fails, a safety net is present. This is however provided that the applied EPF are capable of performing effectively within the soil environment of the orchard.

The one component which all these control methods have in common is that they reduce FCM host populations through competition of the same host. In nature, epizootics are generally associated with high pest population numbers as this has important ramifications for fungal recycling and transmission (Inglis *et al.* 2001). EPF may therefore have more noticeable effects when FCM levels exceed the economic threshold. There should however be no expectation of prolonged recycling especially when EPF are applied inundatively, as they would be in this case (Shah & Pell 2003). Any recycling that does occur can be considered an added benefit. Also, it is unclear as to the effects that the identified isolates have on other subterranean citrus pests or their ability to persist as fungal endophytes. Fungal recycling and persistence is therefore not necessarily dependent on the targeted pest population. This study does however suggest that provided application is timed and applied correctly, the fungi are likely to persist in the environment for a long enough period to achieve satisfactory levels of control even in the presence of low FCM levels.

In the same way that other FCM control measures do not guarantee 100 % control, neither do EPF. Laboratory bioassays did not obtain 100 % FCM mortality (Chapter 2) and field mortality is generally accepted to be lower due to various efficacy affecting environmental factors (Jaronski 2010). As a result, a proportion of FCM pupae will inevitably eclose. As the adults exit the soil however, they can potentially come into contact with fungal spores. Some

level of mortality was recorded amongst FCM adults in this study although this was generally low in comparison to pupal mortality. Nevertheless, adults exposed to EPF may show sub-lethal effects e.g. a reduction in the number of eggs oviposited further adding to the benefits of EPF application (Hafez *et al.* 1997; Blanford & Thomas 2001; Baverstock *et al.* 2006). Furthermore, since FCM copulate shortly after eclosing, there is a possibility that fungal spores may be transferred from infected to uninfected individuals. Although even a small level of control in the adult stage is certainly beneficial, the aim of applying EPF to the soil is to substantially reduce the proportion of adults' eclosing. With a reduction in emerging adults, the proportion of fruit becoming infested should be reduced as the total number of eggs oviposited by the now reduced female FCM population should be less. Timing of EPF application will therefore be important and should aim to coincide with high pupal density in the soil.

Fruit drop due to FCM infestation has been reported as early as November peaking in February–May in the Western Cape and December–March in the Eastern Cape (Newton 1998; Moore 2002a). EPF application is expected to be most beneficial immediately prior to the onset of fruit drop, especially when fruit drop peaks, as it is at this stage that most FCM 5th instar larvae will exit the fruit and burrow into the soil to pupate. Therefore in the Western Cape application will likely occur in January, whilst in the Eastern Cape, November. Since peak fruit drop as a result of infestation generally lasts for approximately four months, one application per season might be possible, particularly in highly infested orchards, to achieve satisfactory levels of control. All three isolates investigated where capable of persisting for six months in the field and with the infection of FCM, recycling of the fungus is likely to occur during this time. As a result of this recycling, virulence is maintained as this can be seen as equivalent to passaging in the laboratory. Fungal titres may also increase in the soil (Meyling & Eilenberg 2007). EPF application during the hotter summer months may also favour infection of FCM as these months tend to be extremely humid (Inglis *et al.* 2001). Humidity is a key factor required for conidial germination, the initial step in the fungal infection process (Hesketh *et al.* 2010). The north-eastern areas of South Africa may therefore derive greater benefits from EPF application as these areas tend to be hotter and more humid (van den Berg 2001). However, since these EPF are to be applied to the soil, soil humidity rather than climatic humidity would be expected to have more impact on the

infectivity of the fungal isolates and therefore provided the soil remains moist, humidity is not likely to be the limiting factor in the use of EPF within citrus.

The expectation that these EPF isolates will successfully integrate into FCM control practices is high. It should however be kept in mind, that not only do these EPF isolates need to be compatible with other FCM control options, but also with other chemicals and control agents used in the orchards. Initial testing should focus on chemicals that are either applied using the same equipment as EPF or on chemicals that are applied directly to the soil e.g. fertilisers as this is where interaction between chemical and fungus is likely to be greatest. The need for compatibility testing is therefore essential. Applying EPF to an orchard utilising incompatible chemicals would be futile as no benefits would be derived. Since these EPF were collected from conventionally farmed South African citrus orchards (Goble *et al.* 2010), compatibility might not necessarily be of major concern as similarly to the possibility of the fungi having some level of tolerance to the environmental conditions experienced in a citrus orchard, they might also have some level of tolerance towards the chemicals commonly applied in the orchard. If this does not hold true and because these isolates were obtained from citrus orchards it suggests then that perhaps the interaction between the applied chemicals and fungus is limited.

5.4 DEVELOPING A MYCOPESTICIDE

Since the overall goal of this research is to develop a fungal product for the control of FCM in citrus within South Africa, it seems pertinent to discuss the process of developing such a product. This might be most easily achieved through the use of an example. The LUBILOSA (LUtte BIologique contre les LOcustes et SAuteriaux, biological control of locusts and grasshoppers) project is perhaps the best documented project detailing every stage of development of the mycopesticide, Green Muscle™ (www.lubilosa.org).

5.4.1 The LUBILOSA project

The LUBILOSA project was initiated in 1989 with the ultimate goal of developing an environmentally safe product for the control of grasshoppers and locusts in Africa following the process outlined by Dent (1998, after Baldwin 1986) (Figure 5.1). It was comprised of

four phases: the developmental phase (phase 1), the start-up phase (phase 2), the adaptation phase (phase 3) and the expansion phase (phase 4) (Douthwaite *et al.* 2001). This project only became viable with the discovery made by Prior & Greathead (1989) that by suspending fungal conidia in oil rather than water, the conidia were capable of remaining viable under dry conditions.

Phase 1 (1989–1992) was concerned with problem identification and strain selection. The problem was simple; an environmentally safe method was needed to control outbreaks of grasshopper and locust populations. Fungi of the Deuteromycota phylum were the biological control organisms of choice for a number of reasons, (1) conidia were easy to mass produce, (2) fungi had already been the focus of other studies and thus some isolates were already available along with data on their pathogenicity and data concerning their safety towards vertebrates. Additional indigenous isolates were also screened for by sampling local grasshopper and locust populations (Lomer *et al.* 1997). Research found that, although *B. bassiana* was sampled, *Metarhizium* strains were more common. For this reason, research concerning virulence towards the target pest was focused on isolates of the latter species. Virulence testing identified *Metarhizium anisopliae* var. *acridum* strain IMI330189 as the most promising. Not only was it recorded to be host specific for acridids, but extensive sampling revealed that it was common throughout the Sahel region of Africa. This simplified the licensing process and made meeting phytosanitary requirements easier (Lomer *et al.* 1997).

Phase 2 (1993–1995) was concerned with proving the efficacy of the isolate during small-scale field trials. Focus lay on the efficacy of the oil formulation applied through a variety of spraying equipment. From these results researchers were able to determine an appropriate application rate, the minimum number of spores which would need to be applied to achieve good acridid control as well as an appropriate formulation (Bateman 1997; Douthwaite *et al.* 2001). Over time and with an increase in the size of application equipment, field trials gradually grew larger. This created a problem in that not enough conidia could be produced to cope with these larger trials. As a solution, a production facility was set up and became operational in 1996 (Cherry *et al.* 1999). This facility was capable of producing approximately 325 kg of conidia per year which was determined to provide coverage for a minimum of 3250 ha at a cost of US$17.ha^{-1}. As a result of increased production, more area wide tests could be conducted in collaboration with plant protection agencies, private

companies and non-governmental organisations. Researchers realised early that Green Muscle™ would need to compete with currently used chemicals and so trials comparing the knockdown of grasshoppers and locusts using currently registered chemicals and Green Muscle™ were conducted. Although field trials showed that Green Muscle™ provided better control of grasshoppers and locusts and was capable of persisting over time providing prolonged control, the cost of the product was far greater than the cost of available chemical (de Groote 1997). This was problematic, but surveys conducted suggested that public sector donors at least would be willing to pay the extra cost and in 1997, *M. anisopliae* var. *acridum* was approved for locust control (Lomer 1999).

Phase 3 (1996–1998) saw a continuation of field trials which focused not only on formulation and production, but also on the susceptibility of different acridid species to Green Muscle™ as well as the effects that the fungus had on the behaviour of infected grasshoppers and locusts (Jenkins & Prior 1993; Moore *et al.* 1995; Thomas *et al.* 1996; Langewald *et al.* 1997; Moore & Caudwell 1997; Kooyman & Abdalla 1998; Langewald *et al.* 1999; Blanford & Thomas 2000). It was noted that infected individuals exhibited what is known as behavioural fever were insects increase their normal body temperature as a defensive response against fungal infection. By increasing temperature above conditions the fungus can tolerate, fungal growth and hence infectivity are impaired (Roy *et al.* 2006). As a result, these infected individuals were still capable of ovipositing. However, fecundity was greatly reduced and usually only a single egg pod was oviposited (Blanford *et al.* 1998; Blanford & Thomas 2000). During this phase, ecotoxicological research and socio-economic research was also initiated (Ball *et al.* 1994; Danfa & van der Valk 1999; de Groote 1997). Not only was phase 3 focused on extensive research, but companies were sought after for the mass production and launching of the product. Originally attempts were made to produce conidia via a cheap production system (Cherry *et al.* 1999). This however was problematic as contamination was rife and conidia could not be produced in large enough quantities. A more costly method was therefore invested in, solid-state fermentation. Two companies which were capable of carrying out this mass production method, Biological Control Products in South Africa and Natural Product Protection in France, were approached and agreements were drawn up. Issues pertaining to intellectual property rights, a prerequisite for the commercialisation of Green Muscle™, were also discussed and resolved, and a detailed specification of the product was also drawn up (LUBILOSA 1998,1999).

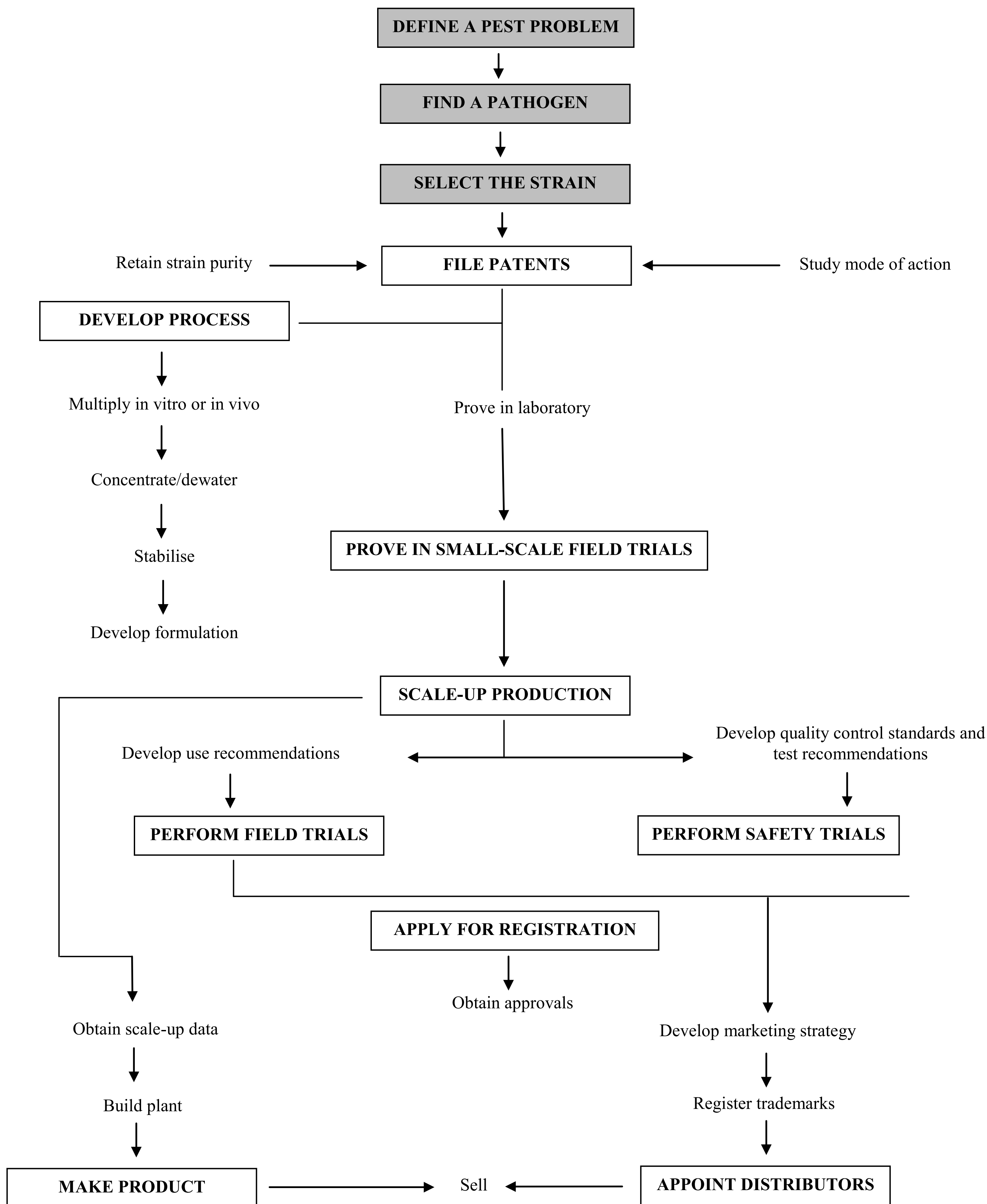

Figure 5.1: Commercial development process followed by staff of the LUBILOSA project (Dent 1998 after Baldwin 1986). Highlighted (grey) blocks indicate completed areas in the study of EPF for FCM control in citrus

In Phase 4 (1999–2002) research continued. At this stage focus shifted to the different application approaches that might be required for the control of different grasshopper and locust species (Lomer *et al.* 1999; Kooyman 2000). Assistance was also given to the two manufacturing companies with respect to quality control, spore extraction and the training of technicians in order to ensure the product continually met the specifications stated (Douthwaite *et al.* 2001). The main priority of phase 4 however, was to increase the demand for the product. This was deemed necessary as although good results had been obtained, cost was still extremely high. This cost could however be reduced if mass production increased. Production however could only be increased if the demand for the product increased (LUBILOSA 1999). Marketing therefore became extremely important and was achieved when aerial spraying of the product was supported in both Mali and Niger (Douthwaite *et al.* 2001).

The LUBILOSA project was terminated in 2002, but the promotion of Green Muscle™ continued along with the undertaking of more field trials. To date, it is still produced by BCP in South Africa and used throughout Africa for the successful control of grasshoppers and locusts.

5.4.2 Issues and considerations arising from the LUBILOSA project

The LUBILOSA project as a case study highlights a number of important aspects for the commercialization of mycopesticides. As stated previously, research conducted in this study is ultimately aimed at utilising these investigated isolates as active ingredients in the development of a mycopesticide product for the control of FCM in citrus orchards. To ensure a successful outcome, the aspects highlighted by LUBILOSA may therefore be important issues to consider.

(1) *The importance of strain selection*. Strain selection should not only be based on the virulence towards the target pest alone, but should also consider aspects such as persistence, ease of production and importantly, consistency.

(2) *The need for extensive research.* Research should include as many aspects of target pest and control agent biology as possible and the interaction between them. Field based

research is extremely important and although should start small to allow for application rates to be determined and to identify a formulation which allows the fungus to obtain optimal results, should gradually increase in size to provide evidence that the product is effective on an operational scale. Other research such as the storage ability of the product and its effects on non-target organisms also becomes important.

(3) *The importance of an appropriate EPF delivery system.* This incorporates not only the production of a stable fungal formulation, but also a mode of application which allows the formulation to still remain virulent towards the target pest. In the case of Green Muscle™ numerous forms of the oil-based formulation were constantly being investigated to identify the one which would provide the most successful cost-effective control.

(4) *The importance of socio-economic analysis.* A recurring problem throughout the development of Green Muscle™ was the cost of the product. If the product is to be successfully adopted into the market, the product needs to be within the monetary range consumers are prepared to pay whilst at the same time, must be capable of obtaining continual quality results. Cost-benefit analysis therefore plays a crucial role in development.

(5) *Financial support and collaboration*. LUBILOSA was well-supported throughout the entirety of the project and this in part insured the development of the product by allowing extensive research to be conducted. LUBILOSA involved a number of organisations including research institutes, non-governmental organisations, private sector companies, as well as the eventual consumer, the farmers. This created the opportunity for support of the project and ensured the availability of expertise when required.

(6) *Product demand.* There must be a high enough demand for the product to warrant production. Often, only if demand is high enough, do production costs become financially feasible. As a result, marketing might become important to ensure the future of the product.

(7) *The process of development is complicated and time consuming*. The LUBILOSA project was a multi-disciplinary project which took over ten years to achieve success. This success is attributed to both the good science that accompanied the project and good financial backing from multiple entities.

(8) *The need for thorough field investigations.* Not only should research be focused on the products ability to perform in the field, but also on its performance ability compared to other currently available, usually chemical, products.

In comparison to the LUBILOSA project, the investigation of EPF against FCM in citrus is in reality only in the initial phases and if one were to follow the four phase design, only phase 1 has being successfully completed (a problem has been identified and promising isolates selected) with research now moving into phase 2 (some level of field-testing has occurred). The LUBILOSA project did however take more than 10 years to achieve success. It is therefore not surprising that this project is still in its infancy as sampling and screening for potential isolates only took place at the beginning of 2008. Evidently, the amount of research and time which still needs to be invested into the development of a mycopesticide for the control of FCM in citrus is abundant (Figure 5.1).

5.5 CONCLUSION & FUTURE RESEARCH

Three fungal isolates collected from citrus orchards within the Eastern Cape have been identified as having good control potential against FCM. At this stage however, it would be premature to state that these isolates will undoubtedly be beneficial for integration into FCM control measures as much research is still needed. However, because EPF would be applied to the soil in this case and target a different life stage than currently registered control options it is likely that EPF will complement available control strategies. The opportunity to conduct both laboratory based and field based studies exists. Field based studies however are extremely important to determine whether or not these isolates are capable of performing effectively in a fully operational citrus orchard where various biotic and abiotic interactions can affect fungal efficacy either positively or negatively.

Field based research should aim to investigate (1) the effect that different formulations and application modes will have on the efficacy of the isolates, (2) the effect of soil type and moisture on both fungal efficacy and persistence, (3) compatibility of the isolates with commonly used agrochemicals particularly those applied directly to the soil, (4) the effect of combining EPF with alternate control strategies e.g. EPNs and granuloviruses and (5) the persistence of the isolates over a longer time frame in their formulated states in the presence and absence of organic matter. Laboratory based research should however not be excluded as other issues concerning (1) cost-effective mass production and formulation methods, (2) the effects of sub-lethal doses of EPF on FCM (3) non-target effects and (4) virulence towards other citrus pests need to be evaluated. The pupation biology of FCM can also be considered an area of future research as knowledge in this area is limited. If 5th instar larvae have a preference for pupation within the soil then it may be possible to apply EPF strategically by targeting the soil-borne life stages and thus increasing their efficacy as control agents, whilst simultaneously reducing the amount applied and therefore the cost of application.

6

REFERENCES

ABBOTT, W.S. 1925. A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology* **18**(2): 265-267.

ALI-SHTAYEH, M., MARA'I, A-B.B.M. & JAMOUS, R.M. 2002. Distribution occurrence and characterization of entomopathogenic fungi in agricultural soil in the Palestinian area. *Mycopathologia* **156**(3): 235-244.

ALVES, S.B., TAMAI, M.A., ROSSI, L.S. & CASTIGLIONI, E. 2005. *Beauveria bassiana* pathogenicity to the citrus rust mite *Phyllocoptruta oleivora*. *Experimental and Applied Acarology* **37**(1-2)**:** 117-122.

AMIR, H. & ALABOUVETTE, C. 1993. Involvement of soil abiotic factors in the mechanisms of soil suppressiveness to *Fusarium* wilts. *Soil Biology and Biochemistry* **25**(2): 157-164.

ANAND, R., PRASAD, B. & TIWARY, B.N. 2009. Relative susceptibility of *Spodoptera litura* pupae to selected entomopathogenic fungi. *BioControl* **54**(1): 85-92.

ANNECKE, D.P. & MORAN, V.C. 1982. *Insects and mites of cultivated plants in South Africa*. 29-62. Butterworth & Co, Durban, South Africa.

ANSARI, M.A. & BUTT, T.M. 2011. Effects of successive subculturing on stability, virulence, conidial yield, germination and shelf-life of entomopathogenic fungi. *Journal of Applied Microbiology* **110**(6): 1460-1469.

ANSARI, M.A. & BUTT, T.M. 2012. Evaluation of entomopathogenic fungi and a nematode against the soil-dwelling stages of the crane fly *Tipula paludosa*. *Pest Management Science* **68**(10): 1337-1344.

ANSARI, M.A., TIRRY, L. & MOENS, M. 2004. Interaction between *Metarhizium anisopliae* CLO 53 and entomopathogenic nematodes for the control of *Hoplia philanthus*. *Biological Control* **31**(2): 172-180.

ASENSIO, L., CARBONELL, T., LÓPEZ-JIMÉNEZ, J.A. & LOPEZ-LLORCA, L.V. 2003. Entomopathogenic fungi in soils from Alicante province. *Spanish Journal of Agricultural Research* **1**(3): 37-45.

ATHANASSIOU, C.G., KAVALLIERATOS, N., VAYIAS, B.J., TSAKIRI, J.B., MIKELI,

N.H., MELETSIS, C.M. & TOMANOVIĆ, Ž. 2008. Persistence and efficacy of *Metarhizium anisopliae* (Metschnikoff) Sorokin (Deuteromycotina: Hyphomycetes) and diatomaceous earth against *Sitophilus oryzae* (L) (Coleoptera: Curculionidae) and *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) on wheat and maize. *Crop Protection* **27**(10): 1303-1311.

AUGUSTYNIUK-KRAM, A. & KRAM, K.J. 2012. Entomopathogenic fungi as an important natural regulator of insect outbreaks in forests (Chapter 12). In: J.A. Blanco & Y-H Lo (Eds.) *Forest Ecosystems – More than Just Trees.* 265-294. InTech, New York, USA. [Available: http://www.intechopen.com/books/forest-ecosystems-more-than-just-trees/entomopathogenic-fungi-as-an-important-natural-regulator-of-insect-outbreaks-in-forests-review-] accessed: 04/12/2012.

BALL, B.V., PYE, B.J., CARRECK, N.L., MOORE, D. & BATEMAN, R.P. 1994. Laboratory testing of a mycopesticide on non-target organisms: the effects of an oil formulation of *Metarhizium flavoviride* applied to *Apis mellifera. Biocontrol Science and Technology* **4**(3): 289-296.

BARBERCHECK, M.E. 1992. Effect of soil physical factors on biological control agents of soil insect pests. *The Florida Entomologist* **75**(4): 539-548.

BARBERCHECK, M.E. & KAYA, H.K. 1991. Competitive interactions between entomopathogenic nematodes and *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) in soilborne larvae of *Spodoptera exigua* (Lepidoptera: Noctuidae). *Environmental Entomology* **20**(2): 707-712.

BARNETT, H. L. 1960. *Illustrated Genera of Imperfect Fungi*. 54-75, 154. Burgess Publishing Company, Minneapolis, USA.

BATEMAN, R.P. 1997. The development of a mycoinsecticide for the control of locusts and grasshoppers.*Outlook on Agriculture* **26**(1): 13-18.

BATEMAN, R. & CHAPPLE A. 2001. The spray application of mycopesticide formulations (Chapter 11). In: T.M. Butt, C.W. Jackson & N. Magan (Eds.) *Fungi as Biocontrol Agents: Progress, Problems & Potential.* 289-310. CABI Publishing, Wallingford, United Kingdom.

BAVERSTOCK, J., ROY, H.E., CLARK, S.J., ALDERSON, P.G. & PELL, J.K. 2006. Effect of fungal infection on the reproductive potential of aphids and their progeny. *Journal of Invertebrate Pathology* **91**(2): 136-139.

BAVERSTOCK, J., ROY, H.E. & PELL, J.K. 2010. Entomopathogenic fungi and insect behaviour: from unsuspecting hosts to targeted vectors. *BioControl* **55**(1): 89-102.

BEDFORD, E.C. 1998. Introduction (Part I) and Methods of controlling citrus pests (Part II), In: E.C.G Bedford, M.A. Van den Berg & E.A. de Villiers (Eds.) *Citrus Pests in the Republic of South Africa*. 1-22. Dynamic Ad, Nelspruit, South Africa.

BEGEMANN, G.J. 2008. The mortality of *Thaumatotibia leucotreta* (Meyrick) final instar larvae exposed to the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin. *African Entomology* **16**(2): 306-308.

BLACKWELL, M. 2010. Fungal evolution and taxonomy. *BioControl* **55**(1): 7-16.

BLANFORD, S. & THOMAS, M.B. 2000. Thermoregulation by two acridid species: effects of habitat and season on thermal behaviour and the potential impact on biocontrol with pathogens. *Environmental Entomology* **29**(5): 1060-1069.

BLANFORD, S. & THOMAS, M.B. 2001. *Schistocerca gregaria* infected with *Metarhizium anisopliae* var. *acridum*: adult survival, maturation and reproduction. *Journal of Invertebrate Pathology* **78**(1): 1-8.

BLANFORD, S., THOMAS, M.B. & LANGEWALD, J. 1998. Behavioural fever in a population of the Senegalese grasshopper, *Oedaleus senegalensis*, and its implications for biological control using pathogens. *Ecological Entomology* **23**(1): 9-14.

BLOEM, S., CARPENTER, J.E. & HOFMEYR, J.H. 2003. Radiation biology and inherited sterility in false codling moth (Lepidoptera: Tortricidae). *Journal of Economic Entomology* **96**(6): 1724-1731.

BOARDMAN, L., GROUT, T.G. & TERBLANCHE, J.S. 2012. False codling moth *Thaumatotibia leucotreta* (Lepidotera: Tortricidae) larvae are chill-susceptible. *Insect Science* **19**(3): 315-328.

BOUCIAS, D. G., STOKES, C., STOREY, G., & PENDLAND, J. C. 1996. The effects of imidacloprid on the termite *Reticulitermes flavipes* and its interaction with the mycopathogen *Beauveria bassiana. Pflanzenshutz-Nachrichten Bayer* **49:** 105-151.

BROWNBRIDGE, M., COSTA, S. & JARONSKI, S.T. 2001. Effects of *in vitro* passage of *Beauveria bassiana* on virulence to *Bemisia argentifolii. Journal of Invertebrate Pathology* **77**(4): 280-283.

BRUCK, D.J. 2010. Fungal entomopathogens in the rhizosphere. *BioControl* **55**(1): 103-112.

BURD, E.M. 2010. Validation of laboratory-developed molecular assays for infectious diseases. *Clinical Microbiology Reviews* **23**(3): 550-576.

BUTT, T.M., WANG, C., SHAH, F.A. & HALL, R. 2006. Degeneration of entomogenous fungi (Chapter 10), In: J. Eilenberg & H.M.T. Hokkanen (Eds.) *An Ecological and Societal Approach to Biological Control*. 213-226. Springer, The Netherlands.

CARROLL, G. 1988. Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. *Ecology* **69**(1): 2-9.

CASTILLO, M-A., MOYA, P., HERNÁNDEZ, E. & PRIMO-YÚFERA, E. 2000. Susceptibility of *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) to entomopathogenic fungi and their extracts. *Biological Control* **19**(3): 274-282.

CASTRILLO, L., ROBERTS, D. & VANDENBERG, J. 2005. The fungal past, present and future: germination, ramification, and reproduction. *Journal of Insect Pathology* **89**: 46-56.

CAVALCANTI, R.S., JUNIOR, A.M., SOUZA, G.C. & ARNOSTI, A. 2002. Efeito dos produtos fitossanitários fenpropatrina, imidaclopride, iprodione e tiametoxam sobre desenvolvimento do fungo *Beauveria bassiana* (bals.) vuill. *Arquivos do Instituto Biolgico* **69**(3): 17-22.

CHERRY, A.J., JENKINS, N.E., HEVIEFO, G., BATEMAN, R. & LOMER, C.J. 1999. Operational and economic analysis of a West African pilot-scale production plant for aerial conidia of *Metarhizium* spp. for use as a mycoinsecticide against locusts and grasshoppers. *Biocontrol Science and Technology* **9**(1): 35-51.

CITRUS GROWERS ASSOCIATION OF SOUTH AFRICA. 2012. Annual Report. [Available: http://www.cga.co.za/site/files/5438/CGA Annual Report 2012e.pdf] accessed: 18/10/2012.

CITRUS GROWERS ASSOCIATION OF SOUTH AFRICA. 2012. Key Industry Statistics for citrus growers. [Available: http://www.cga.co.za/site/files/5438/CGA Stats Book 2012 e.pdf] accessed: 18/10/2012.

CORY, J.S. & ERICSSON, J.D. 2010. Fungal entomopathogens in a tritrophic context. *BioControl* **55** (1): 75-88.

DANFA, A. & VAN DER VALK, C.H.G. 1999. Laboratory testing of *Metrhizium* spp. and *Beauveria bassiana* on Sahelian non-target arthropods. *Biocontrol Science and Technology* **9**(2): 187-198.

DENT, D.R. 1998. Removing the barriers to commercialisation: summary of the process and key issues for a mycoinsecticide for locust and grasshopper control. LUBILOSA, CAB International, Silwood Park, Ascot, UK.

DE FARIA, M.R. & WRAIGHT, S.P. 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. *Biological Control* **43**(3): 237-256.

DE GROOTE, H. 1997. Potential for mycopesticide use in Africa: socioeconomic analysis. LUBILOSA Socio-economic Working Paper Series No. 98/5.

DE OLIVEIRA, C.N., NEVES, P.M.J. & KAWAZOE, L.S. 2003. Compatibility between the entomopathogenic fungi *Beauveria bassiana* and the insecticides used in coffee plantations. *Scientia Agricola* **60**(4): 663-667.

DEMBILIO, O., QUESADA_MORAGA, E., SANTIAGO-ÁLVAREZ, C. & JACA, J.A. 2010. Potential of an indigenous strain of the entomopathogenic fungus *Beauveria bassiana* as a biological control agent against the Red Palm Weevil, *Rhynchophorus ferrugineus*. *Journal of Invertebrate Pathology* **104**(3): 214-221.

DIGHTON, J., JONES, H.E., ROBINSON, C.H. & BECKETT, J. 1997. The role of abiotic factors, cultivation practices and soil fauna in the dispersal of genetically modified organisms in soils. *Applied Soil Ecology* **5**(2): 109-131.

DOLINSKI, C. & LACEY, L. 2007. Microbial control of arthropod pests of tropical trees. *Neotropical Entomology* **36**(2): 161-179.

DOUTHWAITE, B., LANGEWALD, J. & HARRIS, J. 2001. IMPACT: Development and Commercialization of Green Muscle Biopesticide. 1-22. IITA publication.

DROMPH, K.M. 2001. Dispersal of entomopathogenic fungi by collembolans. *Soil Biology and Biochemistry* **33**(15): 2047-2051.

DROMPH, K.M. 2003. Collembolans as vectors of entomopathogenic fungi. *Pedobiologia* **47**(3): 245-256.

EKESI, S., MANIANIA, N.K. & AMPONG-NYARKO, K. 1999. Effect of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti. Biocontrol Science and Technology* **9**(2): 177-185.

EKESI, S., MANIANIA, N. & LUX, S. 2002. Mortality in three tephritid fruit fly puparia and adults caused by the entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*. *Biocontrol Science and Technology* **12**(1): 7-17.

EKESI, S., MANIANIA, N.K. & LUX, S.A. 2003. Effect of soil temperature and moisture on survival and infectivity of *Metarhizium anisopliae* to four tephritid fruit fly puparia. *Journal of Invertebrate Pathology* **83**(2): 157-167.

EKESI, S., MANIANIA, N.K. MOHAMED, S.A. & LUX, S.A. 2005. Effects of soil application of different formulations of *Metarhizium anisopliae* on African tephritid fruit flies and their associated endoparasitoids. *Biological Control* **35**(1): 83-91.

EKESI, S., MANIANIA, N.K. & MOHAMED, S.A. 2011. Efficacy of soil application of

Metarhizium anisopliae and the use of GF-120 spinosad bait spray for suppression of *Bactocera invadens* (Diptera: Tephritidae) in mango orchards. *Biocontrol Science and Technology* **21**(3): 299-316.

ER, M.K., TUNAZ, H. & GÖKÇE, A. 2007. Pathogenicity of entomopathogenic fungi to *Thaumetopoea pityocampa* (Schiff.) (Lepidoptera: Thaumatopoeidae) larvae in laboratory conditions. *Journal of Pest Science* **80**(4): 235-239.

FARES, A. & ALVA, A.K. 2000. Evaluation of capacitance probes for optimal irrigation of citrus through soil moisture monitoring in an entisol profile. *Irrigation Science* **19**(2): 57-64.

FERERES, E. GOLDHAMMER, D.A. & PARSON, L.R. 2003. Irrigation water management of horticultural crops. *HortScience* **38**(5): 1036-1042.

GOBLE, T.A. 2009. Investigation of entomopathogenic fungi for control of false codling moth, *Thaumatotibia leucotreta*, Mediterranean fruit fly, *Ceratitis capitata* and Natal fruit fly, *C. rosa* in South African Citrus. Rhodes University MSc Thesis number TR 10-74, Grahamstown, South Africa.

GOBLE, T.A., COSTET, L., ROBENE, I., NIBOUCHE, S., RUTHERFORD, R.S., CONLONG, D.E. & HILL, M.P. 2012. *Beauveria brongniartii* on white grubs attacking sugarcane in South Africa. *Journal of Invertebrate Pathology* **111**(3): 225-236.

GOBLE, T.A., DAMES, J.F., HILL, M.P. & MOORE, S.D. 2010. The effects of farming system, habitat type and bait type on the isolation of entomopathogenic fungi from citrus soils in the Eastern Cape Province, South Africa. *BioControl* **55**(3): 399-412.

GOBLE, T.A., DAMES, J.F., HILL, M.P. & MOORE, S.D. 2011. Investigation of native isolates of entomopathogenic fungi for the biological control of three citrus pests. *Biocontrol Science and Technology* **21**(10): 1193-1211.

GODONOU, I., GREEN, K. R. , ODURO, K. A., LOMER, C. J. & AFREH-NUAMAH, K. 2000. Field evaluation of selected formulations of *Beauveria bassiana* for the management of the banana weevil (*Cosmopolites sordidus*) on plantain (*Musa* spp., AAB Group).*Biocontrol Science and Technology* **10**(6): 779-788.

GRODEN, E. & LOCKWOOD J.L. 1991. Effects of soil fungistasis on *Beauveria bassiana* and its relationship to disease incidence in the Colorado potato beetle, *Leptinotarsa decemlineata*, in Michigan and Rhode Island soils. *Journal of Invertebrate Pathology* **57**(1): 7-16.

HAFEZ, M., ZAKI, F.N., MOURSY, A. & SABBOUR, M. 1997. Biological effects of the

entomopathogenic fungus, *Beauveria bassiana* on the potato tuber moth *Phthorimaea operculella* (Seller). *Anz. Schlidlingskde.,Pflanzenschutzm Umweltschutz* **70**(1): 158-159.

HAJEK, A.E. & DELALIBERA JR, I. 2010. Fungal pathogens as classical biological control agents against arthropods. *BioControl* **55**(1): 147-158.

HEGEDUS, D.D., BIDOCHKA, M.J. & KHACHATOURIANS, G.G. 1990. *Beauveria bassiana* submerged conidia production in a defined medium containing chitin, two hexosamines or glucose. *Applied Microbiology and Biotechnology* **33**(6): 641-647.

HERLINDA, S., MULYATI, S.I. & SUWANDI. 2008. Selection of isolates of entomopathogenic fungi and the bioefficacy of their liquid production against *Leptocorisa oratorius* Nymphs. *Microbiology* **2**(3): 141-146.

HESKETH, H., ROY, H.E., EILENBERG, J., PELL, J.K. & HAILS, R.S. 2010. Challenges in modelling complexity of fungal entomopathogens in semi-natural populations of insects. *BioControl* **55**(1): 55-73.

HOFMEYR, J.H., CARPENTER, J.E. & BLOEM, S. 2005. Developing the sterile insect technique for *Cryptophlebia leucotreta* (Lepidoptera: Tortricidae): influence of radiation dose and release ratio on fruit damage and population growth in field cages. *Journal of Economic Entomology* **98**(6): 1924-1929.

HOFMEYR, J.H. PRINGLE, K.L. 1998. Resistance of false codling moth, *Cryptophlebia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), to the chitin synthesis inhibitor, triflumuron. *African Entomology* **6**: 373-375.

HUMBER, R.A. 2008. Evolution of entomopathogenicity in fungi. *Journal of Invertebrate Pathology* **98**(3): 262-266.

INCEOGLU, A.B., KAMITA, S.G., HINTON, A.C., HUANG, Q., SEVERSON, T.F., KANG, K. & HAMMOCK, B.D. 2001. Recombinant baculoviruses for insect control. *Pest Management Science* **57**(10): 981-987.

INGLIS, G.D., GOETTEL, M., BUTT, T. & STRASSER, H. 2001. Use of hyphomycetous fungi for managing insect pests. In: T.M. Butt, C.W. Jackson & N. Magan (Eds.) *Fungi as Biocontrol Agents: progress, problems and potential.* 23-70. CABI Publishing, Wallingford, United Kingdom.

JACKSON, T.A. 1999. Factors in the success and failure of microbial control agents for soil dwelling pests. *Integrated Pest Management Reviews* **4**(4): 281-285.

JACKSON, M.A., DUNLAP, C.A. & JARONSKI, S.T. 2010. Ecological considerations in producing and formulating fungal entomopathogens for use in insect biocontrol.

BioControl **55**(1): 129-145.

JANSEN VAN RENSBURG, H.G., CLAASSENS, A.S. & BEUKES, D.J. 2010. Evaluation of the effect of soil acidity amelioration on maize yield and nutrient interrelationships using stepwise regression and nutrient vector analysis. *South African Journal of Plant and Soil* **27:** 117-125.

JARONSKI, S.T. 2010. Ecological factors in the inundative use of fungal entomopathogens. *BioControl* **55**(1): 159-185.

JARONSKI, S.T. & JACKSON, M.A. 2008. Efficacy of *Metarhizium anisopliae* microsclerotial granules. *Biocontrol Science and Technology* **18**(8): 849-863.

JAROS-SU, J., GRODEN, E. & ZHANG, J. 1999. Effects of selected fungicides and the timing of fungicide application on *Beauveria bassiana*-induced mortality of the Colorado potato beetle (Coleoptera: Chrysomelidae). *Biological Control* **15**(3): 259-269.

JENKINS, N.E. & PRIOR, C. 1993. Growth and formation of true conidia by *Metarhizium flavoviride* in a simple liquid medium. *Mycological Research* **97**(12): 1489-1494.

JONASAON, N.B., BOETELL, M.A., EIDE, J.D., CAMPBELL, L.G. & RAO, M.B. 2005. Virulence of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) to Sugarbeet Root Maggot (Diptera:Ulidiidae) Larvae. *Journal of Sugar Beet Research* **42**(3-4): 103-117.

KAO, S-S. 2007. Current Status of Bio-pesticides Development, Farmers' Acceptance and Utilization, and Future Perspective in Taiwan. *Food and Fertilizer Technology Center, Extension Bulletin* **599:** 1-20.

KASSA, A., STEPHAN, D., VIDAL, S. & ZIMMERMAN, G. 2004. Production and processing of *Metarhizium anisopliae* var. *acridum* submerged conidia for locust and grasshopper control. *Mycological Research* **108**(1): 93-100.

KAYA, H.K. & LACEY, L.A. 2007. Introduction to microbial control (Chapter 1). In: L.A. Lacey & H.K. Kaya (Eds.) *Field Manual of Techniques in Invertebrate Pathology* (2nd ed). 3-7. Springer, Dordrecht, The Netherlands.

KESSLER, P., MATZKE, H. & KELLER, S. 2003. The effect of application time and soil factors on the occurrence of *Beauveria brongniartii* applied as a biological control agent in soil. *Journal of Invertebrate Pathology* **84**(1): 15-23.

KIRKMAN, W. 2007. Understanding and improving the residual efficacy of the *Cryptophlebia leucotreta* granulovirus (CRYPTOGRAN). Rhodes University MSc Thesis number TR 08-57, Grahamstown, South Africa.

KLINGEN, I., EILENBERG, J. & MEADOW, R. 2002. Effects of farming system, field margins and bait insect on the occurrence of insect pathogenic fungi in soils. *Agriculture, Ecosystems and Environment* **91:** 191-198.

KOOYMAN, C. 2000. Prospects for the biological control of the Red Locust, *Nomadacris septemfasciata* Audinet-Serville (Orthoptera, Acrididae), in its outbreak areas. *Insect Science and its Application* **19**: 313-323.

KOOYMAN, C. AND ABDALLA, O. M. 1998. Application of *Metarhizium flavoviride* (Deuteromycotina: Hyphomycetes) spores against the Tree Locust, *Anacridium melanorhodon* (Orthoptera: Acrididae), in Sudan. *Biocontrol Science and Technology* **8**(2): 215-219.

KREUGER, S.R., VILLANI, M.G., MARTINS, A.S. & ROBERTS, D.W. 1992. Efficacy of soil applications of *Metarhizium anisopliae* (Metsch.) Sorokin conidia, and standard and lypholized mycelial particles against scarab grubs. *Journal of Invertebrate Pathology* **59:** 54-60.

LACEY, L. & SHAPIRO-ILAN, D. 2003. The potential role for microbial control of orchard insect pests in sustainable agriculture. *Food, Agriculture and Environment* **1**(2): 326-331.

LACEY, L. & SHAPIRO-ILAN, D. 2008. Microbial control of insect pests in temperate orchard systems: Potential for incorporation into IPM. *Annual Review of Entomology* **53**: 121-144.

LACEY, L.A., FRUTOS, R., KAYA, H.K. & VAIL, P. 2001. Insect pathogens as biological control agents: do they have a future? *Biological Control* **21**(3): 230-248.

LANGEWALD, J., KOOYMAN, C., DOURO-KPINDOU, O.-K., LOMER, C., DAHMOUD, A.O. AND MOHAMED H.O. 1997. Field treatment of desert locust (*Schistocerca gregaria* Forskål) hoppers in Mauritania with an oil formulation of the entomopathogenic fungus *Metarhizium flavoviride*. *Biocontrol Science and Technology* **7**(4): 603-611.

LANGEWALD, J., OUAMBAMA Z., MAMADOU, A., PEVELING, R., STOLZ, I. BATEMAN, R., ATTIGNON, S. BLANFORD, S., ARTHURS, S. & LOMER, C. 1999. Comparison of an organophosphate insecticide with a mycoinsecticide for the control of *Oedaleus senegalensis* (Orthoptera: Acrididae) and other Sahelian grasshoppers at an operation scale. *Biocontrol Science and Technology* **9**(2): 199-214.

LATCH, G.C.M. 1965. *Metarhizium anisopliae* (Metschnikoff) Sorokin in New Zealand and their possible use for controlling pasture-inhabiting insects. *New Zealand Journal of*

Agricultural Research **8**(2): 384-396.

LI, M. LIN, H., LI, S., CHENZ, P., JIN, L. & YANG, J. 2012.Virulence of entomopathogenic fungi to adults and eggs of *Nilaparvata lugens* Stal (Homopera: Delphacidae). *African Journal of Agricultural Research* **7**(14): 2183-2190.

LINGG, A.J. & DONALDSON, M.D. 1981. Biotic and abiotic factors affecting stability of *Beauveria bassiana* conidia in soil. *Journal of Invertebrate Pathology* **38**(2): 191-200.

LOC, N.T. & CHI, V.T.B. 2007. Biocontrol potential of *Metarhizium anisopliae* and *Beauveria bassiana* against diamond back moth, *Plutella xylostella. Omonrice* **15:** 86-93.

LOMER, C.J. 1999. Factors in the success and failure of microbial agents for control of migratory pests. *Integrated Pest Management Reviews* **4**(4): 307-312.

LOMER, C.J., BATEMAN, R.P., DENT, D., DE GROOTE, H., DOURO-KPINDOU, O.-K., KOOYMAN, C., LANGEWALD, J., OUAMBAMA, Z., PEVELING, R. & THOMAS, M. 1999. Development of strategies for the incorporation of biological pesticides into the integrated management of locusts and grasshoppers. *Agricultural and Forest Entomology* **1**(1): 71-88.

LOMER, C.J., PRIOR, C. & KOOYMAN C. 1997. Development of *Metarhizium* spp. for the control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada* **129**(S171)**:** 265-268.

LOPES, R.B., PAULI, G., MASCARIN, G.M. & FARIA, M. 2011. Protection of entomopathogenic conidia against chemical fungicides afforded by an oil-based formulation. *Biocontrol Science and Technology* **21**(2): 125-137.

LORD, J.C. 2005. From Metchnikoff to Monsanto and beyond: the path of microbial control. *Journal of Invertebrate Pathology* **89**(1): 19-29.

LORD, J.C. & ROBERTS, D.W. 1986. The effects of culture medium quality and host passage on zoosporogenesis, oosporogenesis, and infectivity of *Lagenidium giganteum* (Oomycetes: Lagenidiales). *Journal of Invertebrate Pathology* **48**(3): 355-361.

LUBILOSA. 1998. The newsletter of Phase 3 of the LUBILOSA programme, Issue no. 5, March. [Available: http://www.lubilosa.org] accessed 13/11/2012.

LUBILOSA 1999. Phase 3 final report. March. [Available: http://www.lubilosa.org] accessed 13/11/2012.

LUDEWIG, M.H. 2003. The establishment of a virus free laboratory colony of *Cryptophlebia leucotreta* (false codling moth) and characterisation of *Cryptophlebia leucotreta*

granulovirus (CrleGV) genes. Rhodes University MSc Thesis number TR 03-56, Grahamstown, South Africa.

MABBERLEY, D.J. 1997. A classification of edible *Citrus* (Rutaceae). *Telopea* **7**(2): 167-172.

MADIGAN, M.T. & MARTINKO, J.M. 2006. Microbial growth (Chapter 6) In: *Brock Biology of Microorganisms* (11th ed.). 135-165. Pearson Prentice Hall, Upper Saddle River New Jersey, USA.

MAGARA, E., NANKINGA, C.M., GOLD, C.S. KYMANYWA, S. TUSHEMEREIRWE, W.K. MOORE, D., GOWEN, S.R. & RAGAMA, P. 2003. Influence of soil amendments in the delivery of *Beauveria bassiana* for control of the banana weevil, *Cosmopolites sordidus* (Germar). *African Crop Science Conference Proceedings* **6:** 205-209.

MALAN, A.P., KNOETZE, R. & MOORE, S.D. 2011. Isolation and identification of entomopathogenic nematodes from citrus orchards in South Africa and their biocontrol potential against false codling moth. *Journal of Invertebrate Pathology* **108**(2): 115-125.

MANIANIA, N.K. 1993. Effectiveness of the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill. for control of the stem borer *Chilo partellus* (Swinhoe) in maize in Kenya. *Crop Protection* **12**(8): 601-604.

MATHER, C. 2003. Regulating South Africa's citrus export commodity chain(s) after liberalisation. *Trade and Industrial Policy Strategies (TIPS) Working Paper Series*. [Available: http://www.tips.org.za/research/papers/showpaper.asp? id=558] accessed: 11/01/2011.

MCCAULEY, V.J.E., ZACHARUK, R.Y. & TINLINE, R.D. 1968. Histopathology of green muscardine in larvae of four species of Elateridae (Coleoptera). *Journal of Invertebrate Pathology* **12**(3): 444-459.

MEYLING, N.V. & EILENBERG, J. 2006. Occurrence and distribution of soil borne entomopathogenic fungi within a single organic agroecosystem. *Agriculture, Ecosystem and Environment* **113**(1-4): 336-341.

MEYLING, N.V. & EILENBERG, J. 2007. Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control. *Biological Control* **43**(2): 145-155.

MILETIĆ, N., TAMAŠ, N. & GRAORA, D. 2011. The control of codling moth (*Cydia*

pomonella L.) in apple trees. *Žemdirbystė=Agriculture* **98**(2): 213-218.

MILNER. R. 2000. *Metarhizium* biopesticides registered in Australia. *Biocontrol News and Information* **21**(2): 47-50.

MILNER, R.J., SAMSON, P. R & MORTON, R. 2003. Persistence of conidia of *Metarhizium anisopliae* in sugarcane fields: effect of isolate and formulation on persistence over 3.5 years. *Biocontrol Science and Technology* **13**(5): 507-516.

MILNER, R.J., SOPER, R.S. & LUTTON, G. 1982. Field release of an Israeli strain of the fungus *Zoophthora radicans* (Brefeld) Batko for biological control of *Therioaphis trifolii* (Monell) *f. maculate. Journal of Australian Entomological Society* **21:** 113-118.

MOHAMED, M.M., ELSHAFIE H.A. & BASHIR, M.O. 2011. Use of teflubenzuron alone and combined with *Metarhizium anisopliae* and Phenylacetonitrile as control agent against the desert locust, *Schistoscera gregaria* (Forskal) (Orhoptera: acrididae). *Agriculture and Biology Journal of North America* **2**(9): 1293-1303.

MOORE, G.A. 2001. Orange and lemons: clues to the taxonomy of *Citrus* from molecular markers. *Trends in Genetics* **17**(9): 536-540.

MOORE, S.D. 2002a. The development and evaluation of *Cryptophlebia leucotreta* granulovirus (CrleGV) as a biological control agent for the management of false codling moth, *Cryptophlebia leucotreta*, on citrus. Rhodes University PhD Thesis number TR 03-137, Grahamstown, South Africa.

MOORE, S.D. 2002b. Entomopathogens & microbial control of citrus pests in South Africa: A review. *South African Fruit Journal* **1**(3): 30-32.

MOORE, D., BATEMAN, R.P., CAREY, M. AND PRIOR, C. 1995. Long term storage of *Metarhizium flavoviride* in oil formulations for the control of locusts and grasshoppers. *Biocontrol Science and Technology* **5**(2): 193-199.

MOORE, D. & CAUDWELL, R.W. 1997. Formulation of entomopathogens for the control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada* **129**(S171): 49-67.

MOORE, S.D. & HATTINGH, V. 2012. A review of current pre-harvest control options for false codling moth in citrus in southern Africa. *SA Fruit Journal* **11**(4): 82-85.

MOORE, S. & KIRKMAN, W. 2009. Citrus orchard sanitation with emphasis on false codling moth control. *South African Fruit Journal* Dec/Jan: 57-60.

MOORE, S.D., KIRKMAN, W. & STEPHEN, P. 2004. The evaluation of the efficacy of a granulovirus (GV) for the control of false codling moth, *CRI Annual Research*

Report: 45.

MOORE, S., GROUT, T., HATTINGH, V. & HOFMEYR, H. 2008. Thresholds and guidelines for intervention against citrus pests. *South African Fruit Journal* Aug/Sept: 77-81.

MORROW, B.J., BOUCIAS, D.G. & HEATH, M.A. 1989. Loss of virulence in an isolate of an entomopathogenic fungus, *Nomuraea rileyi*, after serial in vitro passage. *Journal of Economic Entomology* **82**(2): 404-407.

MYBURG, A.C. 1965. Low temperature sterilization of false codling moth, *Argyroploce leucotreta* Meyr. in export citrus. *Journal of the Entomological Society of Southern Africa* **28**: 277-285.

NANKINGA, C.M. & MOORE, D. 2000. Reduction of banana weevil populations using different formulations of the entomopathogenic fungus *Beauveria bassiana*. *Biocontrol Science and Technology* **10**(5): 645- 657.

NEWTON, P.J. 1998. False codling moth, *Cryptophlebia leucotreta* (Meyrick). In: E.C.G. Bedford, M.A. Van den Berg & E.A. de Villiers (Eds.) *Citrus Pests in the Republic of South Africa*. 192-200. Dynamic Ad: Nelspruit, South Africa.

NICOLOSI, E., DENG, Z.N., GENTILE, A., LA MALFA, S., CONTINELLA, G. & TRIBULATO, E. 2000. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. *Theoretical and Applied Genetics* **100**(8): 1155-1166.

PEDIGO, L. P. 1996. Pest management theory (Chapter 8), Management by modifying insect development and behavior (Chapter 13) and Sterile-insect technique and other pest genetic tactics. In: *Entomology and Pest Management.* 279-299, 455-504. Prentice Hall, New Jersey.

PELL, J.K., HANNAM, J.J. & STEINKRAUS, D.C. 2010. Conservation biological control using fungal entomopathogens. *BioControl* **55**(1): 187-198.

PRIOR, C. & GREATHEAD, D.J. 1989. Biological control of locusts: the potential for the exploitation of pathogens. *FAO Plant Protection Bulletin* **37:** 37-48.

QUESADA-MORAGA, E., NAVAS-CORTÉS, J.A., MARANHAO, E.A.A., ORTIZ-URQUIZA, A. & SANTIAGO-ÁLVAREZ, C. 2007. Factors affecting the occurrence and distribution of entomopathogenic fungi in natural and cultivated soils. *Mycological Research* **111**(8): 947-966.

QUINTELA, E.D. & MCCOY, C.W. 1998. Conidial attachment of *Metarhizium anisopliae* and *Beauveria bassiana* to the larval cuticle of *Diaprepes abbreviatus* (Coleoptera:

Curculionidae) treated with imidacloprid. *Journal of Invertebrate Pathology* **72**(3) 220-230.

RAMAKRISHNAN, R., SUITER, D.R., NAKATSU, C.H., HUMBER, R.A. & BENNETT, G.W. 1999. Imidacloprid-enhanced *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) susceptibility to the entomopathogen *Metarhizium anisopliae. Journal of Economic Entomology* **92**(5): 1125-1132.

RATH, A.C., WORLEDGE, D., KOEN, T.B. & ROWE, B.A. 1995. Long-term field efficacy of the entomogenous fungus *Metarhizium anisopliae* against the subterranean scarab, *Adoryphorus couloni*. *Biocontrol Science and Technology* **5**(4): 439-452.

RENKER, C., OTTO, P., SCHNEIDER, K., ZIMDARS, B., MARAUN, M. & BUSCOT, F. 2005. Oribatid mites as potential vectors for soil microfungi: study of mite-associated fungal species. *Microbial Ecology* **50**(4): 518-528.

RIASAT, T., WAKIL, W., ASHFAQ, M. & SAHI, S.T. 2011. Effect of *Beauveria bassiana* mixed with diatomaceous earth on mortality, mycosis and sporulation of *Rhyzopertha dominica* on stored wheat. *Phytoparasitica* **39**(4): 325-331.

RIVER BIOSCIENCE. 2010. CRYPTOGRAN™ for biological control of the false codling moth. [Available: http://www.riverbioscience.co.za/cycle.html] accessed: 25/01/2011.

ROY, HE., BRODIE, E.L., CHADLER, D., GOETTEL, M.S., PELL, J.K., WAJNBERG, E. & VEGA, F.E. 2010. Deep space and hidden depths: understanding the evolution and ecology of fungal entomopathogens. *BioControl* **55**(1): 1-6.

ROY, H.E., STEINKRAUS, D.C., EILENBERG, J., HAJEK, A.E. & PELL, J.K. 2006. Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. *Annual Review of Entomology* **51:** 331-357.

RUMBOS, C., MENDOZA, A., SIKORA, R. & KIENWNICK, S. 2008. Persistence of the nematophagous fungus *Paecilomyces lilacinus* strain 251 in soil under controlled conditions. *Biocontrol Science and Technology* **18**(10): 1041-1050.

SAFAVI, S.A. 2011. Successive subculturing alters spore-bound Pr1 activity, germination and virulence of the entomopathogenic fungus *Beauveria bassiana. Biocontrol Science and Technology* **21**(8): 883-891.

SAHAYARAJ, K. & NAMASIVAYAM, S.K.R. 2008. Mass production of entomopathogenic fungi using agricultural products and by products. *African Journal of Biotechnology* **7**(12): 1907-1910.

SAMSON, P.R., MILNER, R.J., SANDER, E.D. & BULLARD, G.K. 2005. Effect of fungicides and insecticides applied during planting of sugarcane on viability of

Metarhizium anisopliae and its efficacy against white grubs. *BioControl* **50**(1): 151-163.

SAMSON, P.R., STAIER, T.N. & BULL, J.I. 2006. Evaluation of an application procedure for *Metarhizium anisopliae* in sugarcane ratoons for control of the white grub *Dermolepida albohirtum*. *Crop Protection* **25**(8): 741-749.

SCHOLTE, E.J., KNOLS, B.G.J. & TAKKEN, W. 2004. Autodissemination of the entomopathogenic fungus *Metarhizium anisopliae* amongst adults of the malaria vector *Anopheles gambiae. Malaria Journal* **3:** 45.

SHAH, F.A., ALLEN, N., WRIGHT, C.J. & BUTT, T.M. 2007. Repeated in vitro subculturing alters spore surface properties and virulence of *Metarhizium anisopliae. FEMS Microbiological Letters* **276**(1): 60-66.

SHAH, P.A. & PELL, J.K. 2003. Entomopathogenic fungi as biological control agents. *Applied Microbiology and Biotechnology* **61**(5-6): 413-423.

SHAPIRO, D.I. & LEWIS, E.E. 1999. Comparison of entomopathogenic nematode infectivity from infected hosts versus aqueous suspension. *Biological Control* **28**(5): 907-911.

SHAPIRO-ILAN, D.I. JACKSON, M., REILLY, C.C. & HOTCHKISS M.W. 2004. Effects of combining entomopathogenic fungi or bacterium with entomopathogenic nematodes on mortality of *Curculio caryae* (Coleoptera: Curculionidae). *Biological Control* **30**(1): 119-126.

SKINNER M., KIM, J.S., GOULI, S., FRANK, C.E. & PARKER, B.L. (2012). Management of *Frankliniella occidentalis* (Thysanoptera: Thripidae) with granular formulations of entomopathogenic fungi. *Biological Control* **63**(3): 246-252.

SMITH, D. & PEÑA, J.E. 2002. Tropical Citrus Pests. In: J.E. Peña, J.L. Sharp & M. Wysoki (Eds.) *Tropical Fruit Pests and Pollinators. Biology, Economic Importance, Natural Enemies and Control*. 57- 101. CABI Publishing, Wallingford, United Kingdom.

SRIKANTH, J., SANTHALAKSHMI, G. &NIRMALA, R. 2011. An improved bioassay method for entomopathogenic fungi of sugarcane pests and its evaluation in studies of virulence in subcultures. *Sugar Tech* **13**(2): 156-165.

STIBICK, J. 2008. New pest response guidelines: false codling moth *Thaumatotibia leucotreta*. USDA–APHIS–PPQ–Emergency and Domestic Programs, Riverdale, Maryland.[Available:http://www.aphis.usda.gov/import_export/plants/manuals/emergency/downloads/nprg-fcm.pdf] accessed: 07/09/2010.

ST. LEGER, R.J. 2008. Studies on adaptations of *Metarhizium anisopliae* to life in the soil.

Journal of Invertebrate Pathology **98**(3): 271-276.

STOREY G.K. & GARDNER, W.A. 1987. Vertical movement of commercially formulated *Beauveria bassiana* conidia through four Georgia soil types. *Environmental Entomology* **16**(1): 178-181.

STUDDERT, J., KAYA, H.K. & DUNIWAY, J.M. 1990. Effect of water potential, temperature, and clay-coating on survival of *Beauveria bassiana* conidia in a loam and peat soil. *Journal of Invertebrate Pathology* **55**(3): 417-427.

SUN, J., FUXA, J.R. & HENDERSON, G. 2003. Effects of virulence, sporulation, and temperature on *Metarhizium anisopliae* and *Beauveria bassiana* laboratory transmission in *Coptotermes formosanus*. *Journal of Invertebrate Pathology* **84**(1): 38-46.

SUSURLUK, A. & EHLERS, R.U. 2008. Field persistence of the entomopathogenic nematode *Heterorhabditis bacteriophora* in different crops. *BioControl* **53**(4): 627-641.

THOMAS, M.B., LANGEWALD, J. & WOOD, S.N. 1996. Evaluating the effects of a biopesticide on populations of the variegated grasshopper, *Zonocerus variegatus*. *Journal of Applied Ecology* **33**(6): 1509-1516.

URQUHART, P. 1999. IPM and the citrus industry in South Africa. *Gatekeeper Series No. 86.* International Institute for Environment and Development, Sustainable Agriculture and Rural Livelihoods Programme, London, UK. [Available: http://pubs.iied.org/ 6338IIED.html] accessed 04/12/2012.

VAN DEN BERG, M.A. 2001. *Cryptophlebia leucotreta* (Meyrick). In: M.A. Van den Berg, E.A. de Villiers & P.H. Joubert (Eds.) *Pests and Beneficial Arthropods of Tropical and Non-citrus Subtropical Crops in South Africa.* 320-325. ARC-Institute of Tropical and Subtropical Crops, Nelspruit, South Africa.

VAN DRIESCHE, R.G. & BELLOWS, T.S. 1996. Pest origins, pesticides and the history of biological control (Chapter 1). In: *Biological Control.* 1-20. Chapman & Hall, New York .

VÄNNINEN, I., TYNI-JUSLIN, J. & HOKKANEN, H. 2000. Persistence of augmented *Metarhizium anisopliae* and *Beauveria bassiana* in Finnish agricultural soils. *BioControl* **45**(2): 201-222.

VARELA, A.M. 2005. International Centre of Insect Physiology & Ecology (ICIPE). [Available: http://www.infonet-biovision.org/default/ct/205/crops] accessed: 28/01/2011.

VASSILAKOS, T.N., ATHANASSIOU, C.G., KAVALLIERATOS, N.G. & VAYIAS, B.J. 2006. Influence of temperature on the insecticidal effect of *Beauveria bassiana* in combination with diatomaceous earth against *Rhyzopertha dominica* and *Sitophilus oryzae* on stored wheat. *Biological Control* **38**(2): 270-281.

VEGA, F.E. 2008. Insect pathology and fungal endophytes. *Journal of Invertebrate Pathology* **98**(3): 277-279.

VEGA, F.E., GOETTEL, M.S., BLACKWELL, M., CHANDLER, D., JACKSON, M.A., KELLER, S., KOIKE, M., MANIANIA, N.K., MONZON, A., OWNLEY, B.H., PELL, J.K., RANGEL, D. & ROY, H.E. 2009. Fungal entomopathogens: new insights on their ecology. *Fungal Ecology* **2**(4): 149-159.

VENETTE, R.C., DAVIS, E.E., DA COSTA, M., HEISLER, H. & LARSON, M. 2003. Mini Risk Assessment: false codling moth, *Thaumatotibia* (=*Cryptophlebia*) *leucotreta* (Meyrick) [Lepidoptera: Tortricidae]. University of Minnesota, Department of Entomology, CAPS PRA. 1-30. [Available: http://www.aphis.usda.gov/ppq/ep/pest detection/pra/tleucotretapra.pdf] accessed: 12/02/2010.

VERA, J.T., MONTOYA, E.C., BENAVIDES, P. & GÓNGORA, C.E. 2010. Evaluation of *Beauveria bassiana* (Ascomycota: Hypocreales) as a control of the coffee berry borer *Hypothenemus hampei* (Coleoptera: Curculionidae: Scolytinae) emerging from fallen, infested coffee berries on the ground. *Biocontrol Science and Technology* **21**(1): 1-14.

VILLANI, M.G., KREUGER, S.R., SCHROEDER, P.C., CONSOLIE, F., CONSOLIE, N.H., PRESTON-WILEY, L.M. & ROBERTS, D.W. 1994. Soil application effects of *Metarhizium anisopliae* on Japanese beetle (Coleoptera: Scarabaeidae) behavior and survival in turfgrass microcosms. *Environmental Entomology* **23**(2): 502-513.

WANG, C.S., SKROBEK, A., BUTT, T.M., 2003. Concurrence of losing a chromosome and the ability to produce destruxins in a mutant *of Metarhizium anisopliae*. *FEMS Microbiology Letters* **226**(2): 373-378.

WRAIGHT, S.P., INGLIS, G.D. & GOETTEL, M.S. 2007. Fungi (Chapter 4). In: L.A. Lacey & H.K. Kaya (Eds.) *Field Manual of Techniques in Invertebrate Pathology* (2nd ed). 223-248. Springer, Dordrecht, The Netherlands.

WYSOKI, M. 1986. New records of lepidopterous pests of macadamia in Israel. *Phytoparasitica* **14**(2): 147.

ZIMMERMANN, G. 2007a. Review on the safety of the entomopathogenic fungus *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Science and Technology* **17**(9): 1-44.

ZIMMERMANN, G. 2007b. Review on the safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Science and Technology* **17**(9): 879-920.