

**Field evaluation of the use of select
entomopathogenic fungal isolates as
microbial control agents of the soil-dwelling
life stages of a key South African citrus
pest, *Thaumatotibia leucotreta* (Meyrick)
(Lepidoptera: Tortricidae)**

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Abstract

The control of false codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick, 1912) (Lepidoptera: Tortricidae), in citrus orchards is strongly reliant on the use of integrated pest management as key export markets impose stringent chemical restrictions on exported fruit and have a strict no entry policy towards this phytosanitary pest. Most current, registered control methods target the above-ground life stages of FCM, not the soil-dwelling life stages. As such, entomopathogenic fungi which are ubiquitous, percutaneously infective soil-borne microbes that have been used successfully as control agents worldwide, present ideal candidates as additional control agents. Following an initial identification of 62 fungal entomopathogens isolated from soil collected from citrus orchards in the Eastern Cape Province, South Africa, further laboratory research has highlighted three isolates as having the greatest control potential against FCM subterranean life stages: *Metarhizium anisopliae* G 11 3 L6 (Ma1), *M. anisopliae* FCM Ar 23 B3 (Ma2) and *Beauveria bassiana* G Ar 17 B3 (Bb1). These isolates are capable of causing above 80% laboratory-induced mycosis of FCM fifth instars. Whether this level of efficacy was obtainable under sub-optimal and fluctuating field conditions was unknown. Thus, this thesis aimed to address the following issues with regards to the three most laboratory-virulent fungal isolates: field efficacy, field persistence, optimal application rate, application timing, environmental dependency, compatibility with fungicides and the use of different wetting agents to promote field efficacy. Following fungal application to one hectare treatment blocks in the field, FCM infestation within fruit was reduced by 28.3% to 81.7%. Isolate Bb1 performed best under moderate to high soil moisture whilst Ma2 was more effective under low soil moisture conditions. All isolates, with the exception of Ma2 at one site, were recorded in the soil five months post-application. None of the wetting agents tested were found to be highly toxic to fungal germination and similar physical suspension characteristics were observed. Fungicide toxicity varied amongst isolates and test conditions. However, only Dithane (a.i. mancozeb) was considered incompatible with isolate Ma2. The implication of these results and the way forward is discussed. This study is the first report of the field efficacy of three laboratory-virulent fungal isolates applied to the soil of conventional citrus orchards against FCM soil-dwelling life stages. As such, it provides a foundation on which future research can build to ensure the development and commercialisation of a cost-effective and consistently reliable product.

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Table of Contents

Abstract.....	i
Acknowledgements.....	ii
Table of Contents.....	iii
List of Tables.....	ix
List of Figures.....	xi
List of Abbreviations.....	xvii

CHAPTER 1 – GENERAL INTRODUCTION.....1

1.1 SOUTH AFRICAN CITRUS.....	1
1.1.1 The taxonomy of citrus.....	1
1.1.2 Citrus production and export.....	2
1.1.3 Citrus pests and their control.....	3
1.2 TARGET PEST: <i>THAUMATOTIBIA LEUCOTRETA</i>	5
1.2.1 Classification.....	5
1.2.2 Distribution and host range.....	5
1.2.3 Morphology of life stages.....	7
1.2.3.1 <i>The egg</i>	7
1.2.3.2 <i>The larva</i>	7
1.2.3.3 <i>The pupa</i>	8
1.2.3.4 <i>The adult</i>	9
1.2.4 Life cycle.....	10
1.2.5 Economic importance.....	11
1.2.6 Control options.....	12

1.2.6.1	<i>Monitoring</i>	12
1.2.6.2	<i>Cultural control</i>	13
1.2.6.3	<i>Chemical control</i>	14
1.2.6.4	<i>Behavioural control</i>	15
1.2.6.5	<i>Sterile insect technique</i>	15
1.2.6.6	<i>Biological control</i>	16
1.2.6.7	<i>Post-harvest treatment</i>	18
1.3	ENTOMOPATHOGENIC FUNGI (EPF).....	18
1.3.1	General biology.....	18
1.3.2	EPF as control agents.....	20
1.3.3	Efficacy affecting factors.....	22
1.3.3.1	<i>Soil temperature</i>	22
1.3.3.2	<i>Soil moisture</i>	23
1.3.3.3	<i>Soil amendments</i>	23
1.3.3.4	<i>Soil structure and texture</i>	25
1.3.3.5	<i>Soil biota</i>	25
1.3.4	Genera <i>Beauveria</i> and <i>Metarhizium</i>	26
1.3.5	EPF against FCM in South African citrus.....	28
1.4	RESEARCH AIMS.....	31
 CHAPTER 2 – THE EFFECT OF EPF ON FCM FIFTH INSTAR MORTALITY DURING FIELD-CAGE TRIALS.....		33
2.1	INTRODUCTION.....	33

2.2 MATERIALS AND METHODS.....	36
2.2.1 Site details.....	36
2.2.2 Fungal isolates.....	36
2.2.3 Insect culture.....	37
2.2.4 Treatments.....	37
2.2.5 Experimental procedure.....	37
2.2.6 Fungal concentration in cages.....	38
2.3 RESULTS.....	41
2.3.1 Fungal efficacy.....	41
2.3.2 Fungal concentration in cages.....	43
2.4 DISCUSSION.....	44
CHAPTER 3 – SOIL APPLICATION OF EPF IN CONVENTIONAL CITRUS ORCHARDS: EFFICACY AGAINST FCM.....	48
3.1 INTRODUCTION.....	48
3.2 MATERIALS AND METHODS.....	49
3.2.1 Fungal isolates.....	49
3.2.2 Field sites.....	50
3.2.3 Fungal application.....	51
3.2.4 Treatments.....	54
3.2.5 Monitoring fungal efficacy.....	56
3.3 RESULTS.....	58
3.3.1 Early application trials (Atmar 1, Atmar 2 and Oranjelus).....	58
3.3.2 Late application trials (Marwell and Stenhope).....	63

3.4 DISCUSSION.....66

CHAPTER 4 – FIELD PERSISTENCE OF SELECT EPF IN CONVENTIONAL CITRUS ORCHARD SOIL.....73

4.1 INTRODUCTION.....73

4.2 MATERIALS AND METHODS.....74

4.2.1 Fungal isolates.....74

4.2.2 Monitoring fungal persistence.....75

4.2.2.1 *Soil sampling*.....75

4.2.2.2 *CFU analysis*.....76

4.3 RESULTS.....77

4.4 DISCUSSION.....80

CHAPTER 5 – PHYSICAL SUSPENSION CHARACTERISTICS AND TOXICITY OF FIVE ADJUVANTS ON THE SPORES OF FUNGAL ISOLATES Bb1 AND Ma2.....87

5.1 INTRODUCTION.....87

5.2 MATERIALS AND METHODS.....88

5.2.1 Fungal isolates.....88

5.2.2 Adjuvants.....89

5.2.3 Physical suspension characteristics of each suspension concentrate
.....89

5.2.4 Toxicity of adjuvants to Ma2 and Bb1 after 24 h exposure.....91

5.3 RESULTS.....	91
5.3.1 Physical suspension characteristics of each suspension concentrate	91
5.3.2 Toxicity of adjuvants to Ma2 and Bb1 after 24 h exposure.....	102
5.4 DISCUSSION.....	104

CHAPTER 6 – *IN VITRO* IMPACT OF FUNGICIDES ON THE GROWTH, VIABILITY AND SPORULATION OF TWO FUNGAL ISOLATES.....108

6.1 INTRODUCTION.....	108
6.2 MATERIALS AND METHODS.....	110
6.2.1 Fungicides.....	110
6.2.2 Fungal suspensions.....	110
6.2.3 Agar medium.....	111
6.2.4 Experimental treatments.....	111
6.2.4.1 <i>Vegetative growth</i>	112
6.2.4.2 <i>Viability</i>	112
6.2.4.3 <i>Sporulation</i>	113
6.2.5 Compatibility of Ma2 and Bb1 with tested fungicides: Alves’s model.....	113
6.3 RESULTS.....	113
6.3.1 <i>Beauveria bassiana</i> isolate Bb1.....	113
6.3.2 <i>Metarhizium anisopliae</i> isolate Ma2.....	114
6.3.3 Alves’s compatibility model.....	118
6.4 DISCUSSION.....	119

CHAPTER 7 – GENERAL DISCUSSION.....	124
7.1 THESIS SUMMARY.....	124
7.2 IMPORTANT CONSIDERATIONS FOR FUTURE DEVELOPMENT OF EPF AGAINST FCM.....	127
7.2.1 Optimal application rate.....	127
7.2.2 Formulation.....	130
7.2.3 Compatibility.....	134
7.2.3.1 <i>Other control strategies and biological control agents...</i>	134
7.2.3.2 <i>Agrichemicals</i>	137
7.2.4 Field efficacy.....	139
7.3 CONCLUDING REMARKS.....	141
 CHAPTER 8 – REFERENCES.....	 142
 APPENDIX 1: Monthly average soil temperature and moisture recorded at all field sites.....	 168
APPENDIX 2: False codling moth trap catch data at each field site.....	171
APPENDIX 3: Chemicals applied at Atmar 1, Atmar 2 and Oranjelus during early season trials.....	175

List of Tables

CHAPTER 1

Table 1.1: The area, in hectares, covered by each citrus type in each of six South African provinces and Swaziland (Citrus Growers' Association 2014).....	2
Table 1.2: The percentage of fruit of each citrus type exported from South Africa to the various world regions (Citrus Growers' Association 2014).....	3
Table 1.3: Five FCM larval instars are recognised based on the size of their head capsules (Daiber 1979b).....	8
Table 1.4: Chemicals registered for the control of FCM in citrus orchards, South Africa (Moore 2012, S.D. Moore, pers. comm.).....	14

CHAPTER 2

Table 2.1: Information regarding the collection and laboratory assessment of the three entomopathogenic fungal isolates of interest in this study (Goble <i>et al.</i> 2011; Coombes <i>et al.</i> 2015).....	35
--	----

CHAPTER 3

Table 3.1: Soil physical properties determined for each site/treatment block.....	53
Table 3.2: Mean (\pm standard error) weekly fruit drop and FCM infested fruit per 12 trees recorded 15 and 30 weeks after fungal application of Bb1 and Ma2 (over a period of 5 and 20 weeks, respectively) for the control of FCM at Oranjelus. All application rates presented pertain to the quantity of spores applied per hectare.....	63

CHAPTER 4

Table 4.1: Comparison between the initial fungal titre recorded in October 2013 and 19 months post-application (May 2015) for all three isolates (Ma1, Ma2 and Bb1) applied at Atmar 1.....	80
--	----

CHAPTER 6

Table 6.1: Fungicides used for the <i>in vitro</i> compatibility assessment with the entomopathogenic fungi <i>B. bassiana</i> (Bb1) and <i>M. anisopliae</i> (Ma2).....	110
---	-----

Table 6.2: Toxicity of each fungicide on the viability, vegetative growth and sporulation of <i>B. bassiana</i> isolate Bb1 when grown on fungicide amended media and non-amended media after one hour exposure to each fungicide. Means (\pm standard errors) are presented. The percentage expression of each trait relative to the control is presented underneath in bold. Percentages range between 0% and 100%, with 100% representing a response identical to or greater than the control.....	116
---	-----

Table 6.3: Toxicity of each fungicide on the viability, vegetative growth and sporulation of <i>M. anisopliae</i> isolate Ma2 when grown on fungicide amended media and non-amended media after one hour exposure to each fungicide. Means (\pm standard errors) are presented. The percentage expression of each trait relative to the control is presented underneath in bold. Percentages range between 0% and 100%, with 100% representing a response identical to or greater than the control.....	117
---	-----

Table 6.4: Compatibility (T) of tested fungicides with <i>Beauveria bassiana</i> Bb1 and <i>Metarhizium anisopliae</i> Ma2 on amended and non-amended media following brief exposure, according to Alves's model (Alves <i>et al.</i> 1998).....	118
---	-----

List of Figures

CHAPTER 1

- Figure 1.1:** Distribution of FCM within Africa and surrounding islands (Stibick *et al.* 2010).....6
- Figure 1.2:** (A) *Thaumatotibia leucotreta* eggs (indicated by the black arrows) on the rind of an orange (Photo credit: J.H. Hofmeyr, http://idtools.org/id/leps/tortai/Thaumatotibia_leucotreta.htm) and (B) egg development from a newly laid egg (upper right) to just prior to hatching (lower left) as indicated by a clearly visible neonate larva.....7
- Figure 1.3:** (A) Appearance of neonate larva and (B) final (fifth) instar larva.....8
- Figure 1.4:** (A) FCM pupae in soil-silk pupal cocoons and (B) with cocoon removed; male top, female bottom (Photo credit: J.H. Hofmeyr). (→) indicates the position of the knobs and has been magnified in (C) and highlighted by the circle.....9
- Figure 1.5:** Adult *T. leucotreta* male (A) and female (B). Males are distinguished from females at this stage by the presence of a tuft of black hairs on their hind legs (⇔) and scent organ near the anal angle of each wing (→) (Photo credit: T.M. Gilligan & M.E. Epstein, <http://idtools.org/id/leps/tortai/index.html>).....9
- Figure 1.6:** (A) Granular frass left behind by FCM larva after feeding and (B) FCM larvae feeding within a Navel orange.....11
- Figure 1.7:** (A) Spherical conidia of *Beauveria* produced on a denticulate rachis arising from flask-shaped conidiogenous cells, and (B) elongate conidia of *Metarhizium* produced as basipetal chains from phialides (Photo credit: J. Deacon, University of Edinburgh, <http://archive.bio.ed.ac.uk/jdeacon/FungalBiology/chap15im.htm>).....28
- Figure 1.8:** Simplified illustration of the phases and steps necessary for the commercialisation of a biopesticide (adapted from Ravensberg 2011). Criteria underlined are those which have already been met in studies preceding this one (Goble *et al.* 2010, 2011; Coombes *et al.* 2013, 2015). Criteria written in uppercase and bold are under investigation in this study.....30

CHAPTER 2

Figure 2.1: 5 L cages with mesh inserts were buried within the upper soil surface and filled with sand (A and B). Cages were buried underneath the canopy of citrus trees approximately 1 m from an irrigation sprinkler (C). Cages are indicated by the yellow arrows.....40

Figure 2.2: Eclosed FCM adults (A) and pupal casings (B) as seen in cages during counting. Both the number of adults and pupal casings within each cage were counted.....40

Figure 2.3: Median percentage, of eight replicates, of eclosed FCM adults recorded for each treatment. Vertical bars represent the interquartile range. Different letters indicate a significant difference between treatments (multiple comparison of mean ranks, $P < 0.05$). 0.5×, 1× and 2× refer to application rates equivalent to 0.5×10^{14} , 1×10^{14} and 2×10^{14} spores/ha, respectively. Treatments to which mulch was applied are represented by the letter M.....42

Figure 2.4: Median percentage, of eight replicates, of Abbott-corrected FCM mortality calculated for each treatment to which fungus was applied. Vertical bars denote the interquartile range. Significant letters indicate significantly different results (multiple comparison of mean ranks, $P < 0.05$). 0.5×, 1× and 2× refer to application rates equivalent to 0.5×10^{14} , 1×10^{14} and 2×10^{14} spores/ha, respectively. Treatments to which mulch was applied are represented by the letter M.....43

Figure 2.5: Median CFUs/g of dry sample, of eight replicates, determined within the soil, or mulch, of each treatment. Vertical bars denote the interquartile range. Different letters indicate significantly different results (multiple comparison of mean ranks, $P < 0.05$). 0.5×, 1× and 2× refer to application rates equivalent to 0.5×10^{14} , 1×10^{14} and 2×10^{14} spores/ha, respectively. Treatments to which mulch was applied are represented by the letter M.....44

CHAPTER 3

Figure 3.1: The five field trials used to assess the efficacy of the applied entomopathogens, located within close proximity to each other, in Sunday's River Valley area, Eastern Cape, South Africa.....50

Figure 3.2: Fungi were applied as an aqueous suspension using a spray machine and associated hand-held spray guns to the soil surface underneath the canopy of the citrus trees.....52

Figure 3.3: Location of treatment blocks and control blocks at early application sites (A) Atmar 1 and 2 and (B) Oranjelus. No treatment was applied to the controls and at Atmar, fungi were only applied at one rate, 1×10^{14} spores/ha. All application rates presented pertain to the quantity of spores applied per hectare.....55

Figure 3.4: Location of treatment and control blocks at late application sites (A) Marwell and (B) Stenhope. No treatment was applied to the controls and at Stenhope, Bb1 was only applied at 1×10^{14} spores/ha. All application rates presented pertain to the quantity of spores applied per hectare.....56

Figure 3.5: Mean (\pm standard error) weekly fruit drop per 12 trees recorded 15 (■) and 30 (■) weeks after fungal application, over a period of eight and 21 weeks respectively, for the control of FCM at Atmar 1. No significant differences were found (ANOVA, $P > 0.05$). The percentage reduction in fruit drop calculated for each treatment relative to control 2 is presented.....59

Figure 3.6: Mean (\pm standard error) weekly FCM infested fruit per 12 trees recorded 15 (■) and 30 (■) weeks after fungal application, over a period of eight and 21 weeks respectively, for the control of FCM at Atmar 1. A significant difference was found (Tukey’s HSD test, $P < 0.05$) and indicated by the asterisk. The percentage reduction in FCM infestation calculated for each treatment relative to control 2 is presented.....60

Figure 3.7: Mean (\pm standard error) weekly fruit drop per 12 trees recorded 15 (■) and 30 (■) weeks after fungal application, over a period of five and 18 weeks respectively, for the control of FCM at Atmar 2. The percentage reduction in fruit drop calculated for each treatment relative to the control is presented. Different letters denote statistically significant results (Tukey’s HSD test, $P < 0.05$).....61

Figure 3.8: Mean (\pm standard error) weekly FCM infested fruit per 12 trees recorded 15 (■) and 30 (■) weeks after fungal application, over a period of five and 18 weeks respectively, for the control of FCM at Atmar 2. The percentage reduction in FCM infestation calculated for each treatment relative to the control is presented. Different letters denote statistically significant results (Tukey’s HSD test, $P < 0.05$).....62

Figure 3.9: Mean (\pm standard error) weekly fruit drop per 12 trees recorded nine weeks after fungal application, over a period of six weeks, for the control of FCM at Marwell. No significant differences were recorded (ANOVA, $P = 0.90$). The percentage reduction in fruit drop calculated for each treatment relative to the control is presented above the bars. All application rates presented pertain to the quantity of spores applied per hectare.....64

Figure 3.10: Mean weekly (\pm standard error) FCM infested fruit per 12 trees recorded nine weeks after fungal application, over a period of six weeks, for the control of FCM at Marwell. No significant differences were found (ANOVA, $P = 0.48$). The percentage reduction in FCM infestation calculated for each treatment relative to the control is presented above the bars. All application rates presented pertain to the quantity of spores applied per hectare.....64

Figure 3.11: Mean (\pm standard error) fruit drop per 10 trees recorded 10 weeks after fungal application, over a period of six weeks, for the control of FCM at Stenhope. The percentage reduction in fruit drop relative to the control is presented above the bar. A statistical significant difference was found between treatments, denoted by the different letters (T-test, $P = 0.02$).....65

Figure 3.12: Mean (\pm standard error) weekly FCM infested fruit per 10 trees recorded 10 weeks after fungal application, over a period of six weeks, for the control of FCM at Stenhope. No significant difference was found (T-test, $P = 0.20$). The percentage reduction in FCM infestation relative to the control is presented above the bar.....66

CHAPTER 4

Figure 4.1: Monthly persistence of isolate Bb1 at Atmar under micro-sprinkler (Atmar 1) and drip (Atmar 2) irrigation, and at Oranjelus under micro-sprinkler irrigation. Monthly persistence is represented by the median with vertical bars denoting the interquartile range for Atmar 1 and 2; mean and standard error for Oranjelus. For each treatment, different letters are used to indicate statistically significant differences amongst months (Tukey’s HSD test or multiple comparison of mean ranks, $P < 0.05$).....78

Figure 4.2: Monthly persistence of isolate Ma2 at Atmar under micro-sprinkler (Atmar 1) and drip (Atmar 2) irrigation and at Oranjelus under micro-sprinkler irrigation and of Ma1 at

Atmar 1. Monthly persistence is represented by the median with vertical bars denoting the interquartile range for Atmar 1 and 2; mean and standard error for Oranjelus. For each treatment, different letters are used to indicate statistically significant differences amongst months (Tukey's HSD test or multiple comparison of mean ranks, $P < 0.05$).....79

CHAPTER 5

Figure 5.1: Illustration of the degree of suspension and uniformity of the suspension as based on visual examination of isolate Ma2 (A): Cylinders 1, 2 and 3 represent a uniform suspension, non-uniform suspension (most spores settled out) and no noticeable immediate suspension prior to mixing, respectively. (B) A representation of the degree of suspension when uniformity is not achieved as in A1. The more intense (=darker) colour is indicative of more spores. The same visual observation can be observed for isolate Bb1, with the exception that spores held in suspension turn the water milky white, rather than green due to the colour difference of the spores between the two isolates.....90

Figure 5.2: Immediate suspension prior to mixing of each of the five adjuvants for Bb1 (A) and Ma2 (B). The spores had been mixed with the wetting agent prior to them being poured into the water. Wetting agents from left to right: S240, S233, OE 446, Advance and oil.....94

Figure 5.3: Mean percentage foam produced by each adjuvant suspension over time (0, 0.5, 2 and 24 h post-inversion) for both isolates Bb1 (A) and Ma2 (B). 0 hours refers to the percentage foaming immediately after inversion. Vertical bars represent standard errors.....95

Figure 5.4: OE 446 and Advance mix often remained adhered to the sides and bottom of the cylinder after hand inversion. This was recorded for both Bb1 (A) and Ma2 (B).....96

Figure 5.5: Spores still held in suspension after 24 h incubation at 4°C for each adjuvant and each isolate, Bb1 (A) and Ma2 (B). Adjuvants from left to right: S240, S233, OE 446, Advance and oil.....97

Figure 5.6: Spores still held in suspension after 24 h incubation at 25°C for each adjuvant and each isolate, Bb1 (A) and Ma2 (B). Adjuvants from left to right: S240, S233, OE 446, Advance and oil.....98

Figure 5.7: Spores still held in suspension after 24 h incubation at 30°C for each adjuvant and each isolate, Bb1 (A) and Ma2 (B). Adjuvants from left to right: S240, S233, OE 446, Advance and oil.....99

Figure 5.8: At 30°C, two distinct layers form. The lower layer (0-20 ml) contains what appears to be most of the spore/adjuvant mix; the upper (20-100 ml) the water with a few spores still in suspension. This was only apparent for the wetting agent S240 and was observed in both isolates, Bb1 (A) and Ma2 (B).....100

Figure 5.9: For Bb1, the oil/spore mix settles to the top (A) whereas for Ma2, the oil/spore mix appears to originally settle to the top, but over time the spores move out of the oil to settle at the bottom (B).....101

Figure 5.10: When BP Medium Oil was used as the wetting agent, re-suspension was not possible and attempts resulted in flocculation (=aggregation of spores to form clumps, indicated by the arrows) followed by immediate separation out of solution. This was observed for both Bb1 (A) and Ma2 (B).....102

Figure 5.11: Spore viability (=germination) for Bb1 recorded after 24 h exposure to each adjuvant at each of the three temperatures tested and the control. The number of germinated and non-germinated spores was determined 24 h after incubation in complete darkness at 26°C.....103

Figure 5.12: Spore viability (=germination) for Ma2 recorded after 24 h exposure to each adjuvant at each of the three temperatures tested and the control. The number of germinated and non-germinated spores was determined 48 h after incubation in complete darkness at 26°C.....104

List of Abbreviations

%	percentage
°	degree(s)
°C	degree(s) Celsius
'	minute(s)
"	second(s)
µm	micrometre
a.i	active ingredient
CBS	citrus black spot
CrleGV	<i>Cryptophlebia leucotreta</i> granulovirus
CFU	colony forming unit
DAFF	South African Department of Agriculture, Forestry and Fisheries
E	east
e.g.	example
EPF	entomopathogenic fungi
et al.	<i>et alia</i> (and others)
F₁	first generation offspring
FCM	false codling moth
G	gram
Gy	gamma radiation
h	hour(s)
Ha	hectare(s)

IPM	integrated pest management
L	litre
LC₅₀	median lethal concentration
LT₅₀	median lethal exposure time
mg/L	milligrams per litre
ml	millilitre
S	south
SE	standard error
SIT	sterile insect technique
USA	United States of America
UV	ultra-violet
ZAR	South African currency
WAT	weeks after treatment

Chapter 1

General introduction

PROBLEM STATEMENT: In previous studies, native isolates of selected entomopathogenic fungal isolates have been shown to be effective in controlling the soil-dwelling (fifth instar) life stages of false codling moth. This research was, for the most part, restricted to laboratory trials. The field efficacy of these isolates remains unknown, a problem addressed herein.

1.1 SOUTH AFRICAN CITRUS

1.1.1. The taxonomy of *Citrus*

Citrus (Sapindales: Rutaceae) is an ancient crop generally accepted to have originated in the tropics and sub-tropics of Southeast Asia (Nicolosi *et al.* 2000). The classification of *Citrus* is problematic; attributed largely to the high frequency of genetic mutation, both natural and anthropogenic, and the sexually compatible nature of species within *Citrus* and those in closely related genera (Nicolosi *et al.* 2000). In the mid to late 20th century, two systems, used to define the species of *Citrus*, were proposed and varied substantially in the number of species described: Swingle (1943) described only 16 species whilst Tanaka (1977) described 162 species. With the increasing developments in molecular techniques, only three citrus species are considered to be true species: the citron (*C. medica* L.), the pummelo (*C. maxima* Merrill) and the mandarin (*C. reticulata* Blanco). All other types are believed to be derived from these three species either through anthropogenic hybridization or natural genetic mutation coupled with human selection (Scora 1975; Barret & Rhodes 1976; Moore 2001).

In South Africa, a number of citrus cultivars of all citrus types are grown (Citrus Growers' Association 2014). These different cultivars mature at different times of the year, have

different physical and chemical properties (including size, ease of peeling, taste, number of seeds) and can vary in their susceptibility to attack by insects (Novelli *et al.* 2006; Love *et al.* 2014). Variety provides the citrus industry with a means to satisfy the needs of the consumer, the main force driving the development and planting of new varieties. This allows the industry to supply citrus to the northern hemisphere export markets throughout their winter (out-of-season) months (Mather 2003).

1.1.2. Citrus production and export

Based on the 2013 citrus season, citrus trees are planted on an estimated 63 132 hectares of land distributed over six of the nine provinces of South Africa and Swaziland (Citrus Growers' Association 2014). Each citrus producing region is characterised by its own unique suite of climatic conditions and as such, different regions tend to focus production on different varieties of citrus (Bedford 1998; Mather 2003) (Table 1.1). Globally, South Africa is the 13th largest producer of fresh citrus, producing approximately 2 255 thousand tons of fruit over the 2011/2012 period (Citrus Growers' Association 2013).

Table 1.1: The area, in hectares, covered by each citrus type in each of the six South African provinces and Swaziland (Citrus Growers' Association 2014).

PRODUCING REGION	CITRUS GROUP					TOTAL PER REGION
	GRAPEFRUIT AND PUMMELOS	LEMONS AND LIMES	NAVELS	SOFT CITRUS	VALENCIAS AND MIDSEASONS	
LIMPOPO	4 145	1 397	3 110	752	14 566	23 970
EASTERN CAPE	261	2 726	5 791	2 144	3 848	14 770
MPUMALANGA	2 566	572	1 817	642	3 777	9 375
WESTERN CAPE	45	636	3 848	2 554	2 149	9 232
KWAZULU NATAL	1 463	377	560	49	743	3 192
NORTHERN CAPE	363	120	414	159	395	1 451
SWAZILAND	540	13	30	52	508	1 142
TOTAL PER CITRUS GROUP	9 383	5 841	15 570	6 352	25 986	63 132

South Africa is ranked the second largest exporter of citrus in the world after Spain, exporting an estimated 1 490 thousand tons of fresh citrus annually (Citrus Growers' Association 2013). This accounts for approximately 74% of all South Africa's citrus production. The remaining 26% is either sent for processing (18%) or is sold to the local market (8%) (Citrus Growers' Association 2013). Valencia oranges are the most exported citrus type (45%) followed by Navel oranges (25%) and grapefruit (15%). Most citrus is exported to northern Europe (23%), Middle East (20%), Russia (12%), Far East (10%) and United Kingdom (10%) (Citrus Growers' Association 2013). The quantity of each citrus type exported to the different destinations varies (Table 1.2). More detailed information with regards to the export of each citrus type and their cultivars can be found in the Citrus Growers' Association Key Industry Statistics (2013) and Annual Report (2014) (2013, 2014).

Table 1.2: The percentage of fruit of each citrus type exported from South Africa to the various world regions (Citrus Growers' Association 2014).

WORLD REGION	CITRUS GROUP			
	ORANGES	GRAPEFRUIT	SOFT CITRUS	LEMONS
MEDITERRANEAN	5	6	-	3
ASIA	8	5	2	2
UNITED STATES	4	-	3	-
CANADA	3	3	5	3
SOUTHERN EUROPE	3	-	-	-
NORTHERN EUROPE	24	33	25	11
MIDDLE EAST	24	-	7	43
RUSSIA	12	12	10	18
FAR EAST	4	28	5	10
UNITED KINGDOM	8	5	39	6
OTHER	4	7	4	4

1.1.3. Citrus pests and their control

Numerous pests have been recorded on citrus and vary in their degree of economic importance based on the extent and type of damage they inflict. The degree of importance may also vary in different regions, particularly regions with distinctly different climates. Pests may also only be considered economically important at a specific time during the growing season, for example at flowering or during fruiting. In addition, the type or variety of

citrus grown may also affect pest status. For example, although false codling moth (FCM) is classified as a key economic pest of citrus, Navel oranges appear to be the most susceptible to FCM damage, whilst lemons are considered not susceptible (Newton 1998; Smith & Peña 2002; Moore *et al.* 2015a).

Pests may damage the fruit directly. This may be a result of feeding on the fruit, causing it to rot and abscise from the tree or by reducing plant vitality and hence the quality and quantity of fruit produced. Pests may also cause damage indirectly through the transmission of pathogens. For example, citrus psylla, *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae), transmits the bacterium *Candidatus liberibacter* spp., the causal agent of citrus greening, a disease which can severely stunt the growth of trees (Grout & Moore 2015). Damaged fruit is also more susceptible to entry by foreign pathogens such as blue and green mould (Annecke & Moran 1982; Smith & Peña 2002). Some pests do not cause direct or indirect damage to the trees or fruit, but can reduce the appearance of fruit (cosmetic damage). As blemished fruit is typically not considered suitable for export owing to high consumer demand, the monetary worth of this fruit is reduced (Pimental *et al.* 1993; Newton 1998; Smith & Peña 2002).

Initial control of citrus pests in South Africa was strongly associated with chemical spraying regimes. However, several factors have caused the control of these pests to now rely strongly on integrated pest management. These factors include an increase in pest resistance development, increased pressure to become more environmentally safe and the imposition of stringent regulations concerning the use of chemicals by many of the export markets, especially the European and American markets (Bedford 1998; Urquhart 1999; Moore & Hattingh 2012). Integrated pest management (IPM) does not aim to eradicate the pest population, but rather maintain pest levels below an economically feasible level or as close to zero as possible. This is achieved by using a variety of complementary control strategies concurrently. A strong emphasis is placed on the use of natural enemies and other environmentally safe techniques (e.g. mating disruption and sterile insect technique) including cultural techniques (e.g. orchard sanitation) whilst limiting the use of harmful chemical pesticides. If implemented and managed correctly, IPM can be very successful (Smith & Peña 2002).

In South African citrus, seven key citrus pests are recognised; 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), citrus mealybug, *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae), and the species of interest in this study, false codling moth *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) (Smith & Peña 2002; *S.D. Moore, pers. comm.). Other pests recorded on South African citrus, and the thresholds and guidelines for their control, are presented in Grout & Moore (2015).

1.2. TARGET PEST: *THAUMATOTIBIA LEUCOTRETA*

1.2.1. Classification

Thaumatotibia leucotreta Meyrick (1912) (Lepidoptera: Tortricidae), more commonly known as false codling moth (FCM), was first reported on citrus in the KwaZulu-Natal region of South Africa by Fuller (1901) and placed in the genus *Carpocapsa*. It was then later described as *Argyroploce leucotreta* (Eucosmidae: Olethreutidae) by Meyrick in 1912 and soon thereafter, was transferred to the genus *Cryptophlebia* by Clarke in 1958. The species *leucotreta* was subsequently removed from this genus by Komai (1999) and placed into the more suited genus *Thaumatotibia* where it currently resides (Newton 1998; van den Berg 2001; Venette *et al.* 2003).

1.2.2. Distribution and host range

FCM is endemic to sub-Saharan Africa and has been recorded in most African countries, surrounding islands and Swaziland (Figure 1.1). FCM is host to a wide range of non-cultivated and cultivated plants including cotton, sorghum and maize in central Africa, Macadamia nuts and citrus in South Africa and Malawi and as a pest of Macadamia nuts in Israel (Wysoki 1986; Newton 1998; Kirkman & Moore 2007).

In South Africa, FCM has developed a preference for citrus and can be found in all citrus-producing regions of the country (Newton 1998). Citrus produced in the very northern regions tends to experience less FCM damage than citrus produced in the southern regions, likely a result of the drier climate (Newton 1998; Moore 2012). Amongst all the citrus types, Navel oranges are preferred, soft citrus and grapefruit less so with lemons the least as larval development is seldom completed due to lemons' high acidity level (Newton 1998; Moore *et al.* 2015a). A recent laboratory study by Love *et al.* (2014) has also highlighted the variability in susceptibility to FCM oviposition and larval penetration within the Navel orange variety.

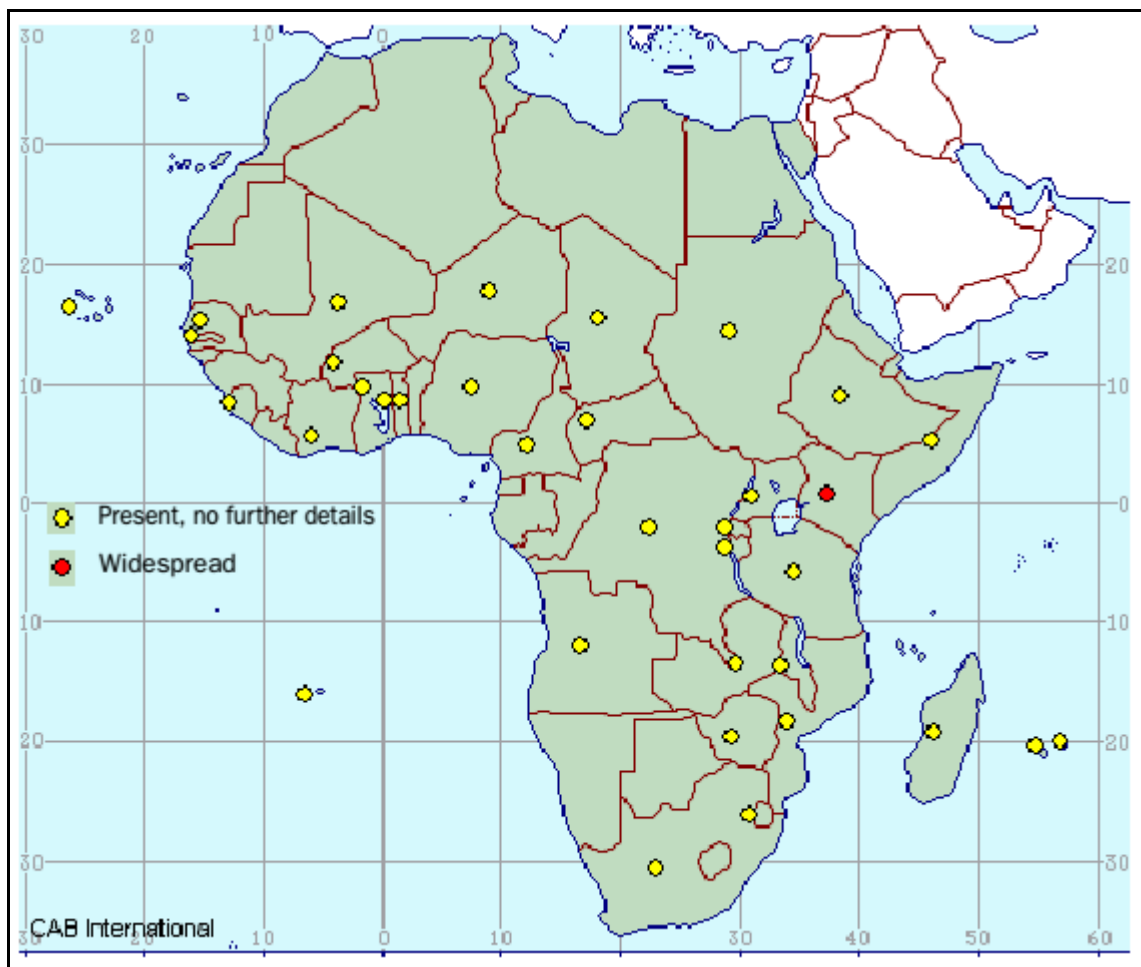


Figure 1.1: Distribution of FCM within Africa and surrounding islands (Stibick *et al.* 2010).

1.2.3. Morphology of life stages

1.2.3.1 The egg

The eggs of FCM are small, measuring approximately 1 mm in diameter. Although they are visible to the naked eye, they can be missed during orchard inspections. Newly oviposited eggs are pale and translucent. As development proceeds, the eggs become red. Just prior to hatching, they become black (Figure 1.2) (Daiber 1979a; Newton 1998; van den Berg 2001; Grout & Moore 2015).

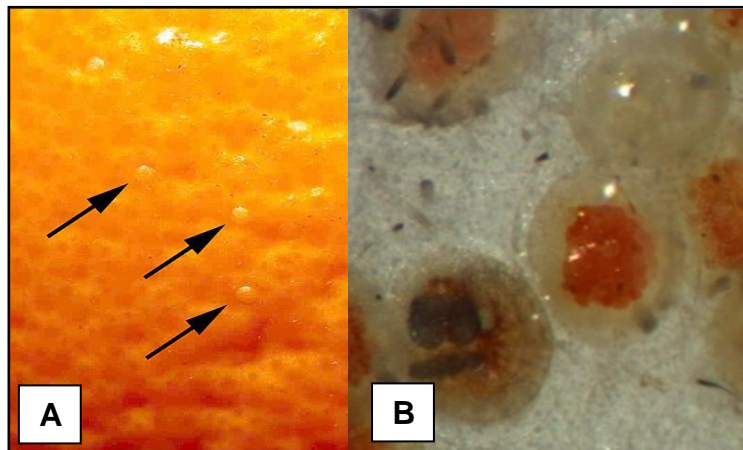


Figure 1.2: (A) *Thaumatotibia leucotreta* eggs (indicated by the black arrows) on the rind of an orange (Photo credit: J.H. Hofmeyr, http://idtools.org/id/leps/tortai/Thaumatotibia_leucotreta.htm) and (B) egg development from a newly laid egg (upper right) to just prior to hatching (lower left) as indicated by a clearly visible neonate larva.

1.2.3.2 The larva

Five larval instars are recognised based on the width of the head capsule (Table 1.3) (Daiber 1979b). First instars or neonates are small measuring approximately 1.5 mm in length whilst final fifth instars can reach lengths of between 12 to 15 mm. As the larvae develop, a colour change from creamy-white to dark pink can be observed (Figure 1.3) (Newton 1998; van den Berg 2001; Grout & Moore 2015). A more detailed description of the morphology, for identification purposes, is presented in Rentel (2013).

Table 1.3: Five FCM larval instars are recognised based on the size of their head capsules (Daiber 1979b).

Instar	Average head capsule width (mm)	95% confidence interval
First	0.21	0.17-0.25
Second	0.37	0.32-0.43
Third	0.61	0.50-0.72
Fourth	0.94	0.82-1.07
Fifth	1.37	1.25-1.49

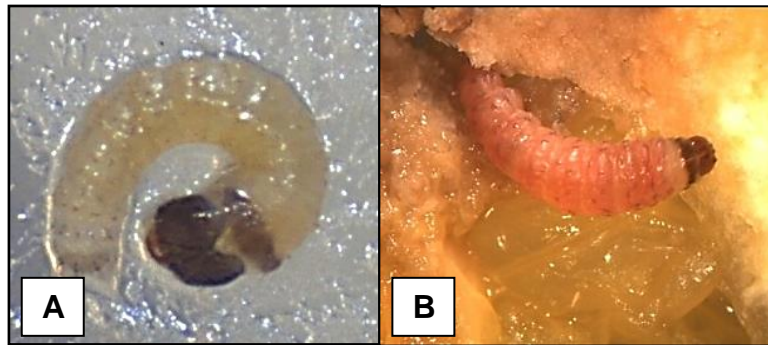


Figure 1.3: (A) Appearance of neonate larva and (B) final (fifth) instar larva.

1.2.3.3 *The pupa*

FCM pupae are formed in the soil and as a result encase themselves in a cocoon formed from a combination of self-spun silk and surrounding soil particles and debris (Newton 1998; van den Berg 2001) (Figure 1.4A). The pupae themselves are dark brown and can be sexed; males have two, small centrally located knobs on the ventral side of the IX abdominal segment, flanking the genital opening. This is absent in females. The IX abdominal segment of females is also larger (Daiber 1979c) (Figure 1.4B, C).

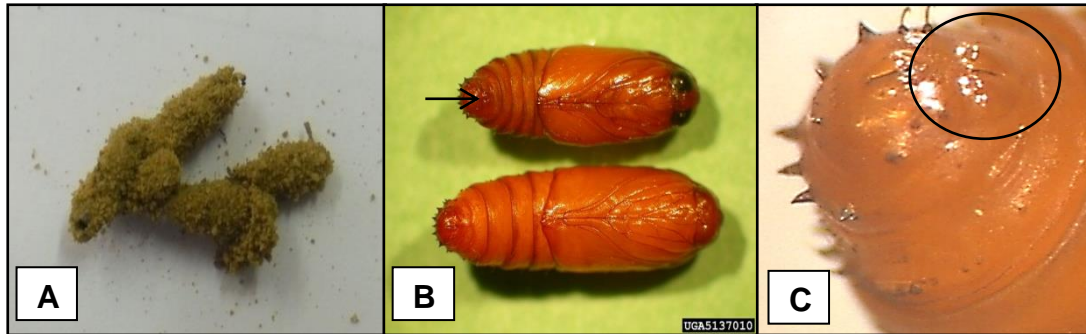


Figure 1.4: (A) FCM pupae in soil-silk pupal cocoons and (B) with cocoon removed; male top, female bottom (Photo credit: J.H. Hofmeyr). (→) indicates the position of the knobs and has been magnified in (C) and highlighted by the circle.

1.2.3.4 The adult

Adult FCM are small with a wingspan of between 16-20 mm. The hind-wings are paler than the mottled brown-grey fore-wings. Males tend to be smaller than females and can be distinguished by the presence of an anal tuft and a scent organ located near the anal angle of each hindwing which the females lack (Newton 1998; van den Berg 2001; Grout & Moore 2015) (Figure 1.5).

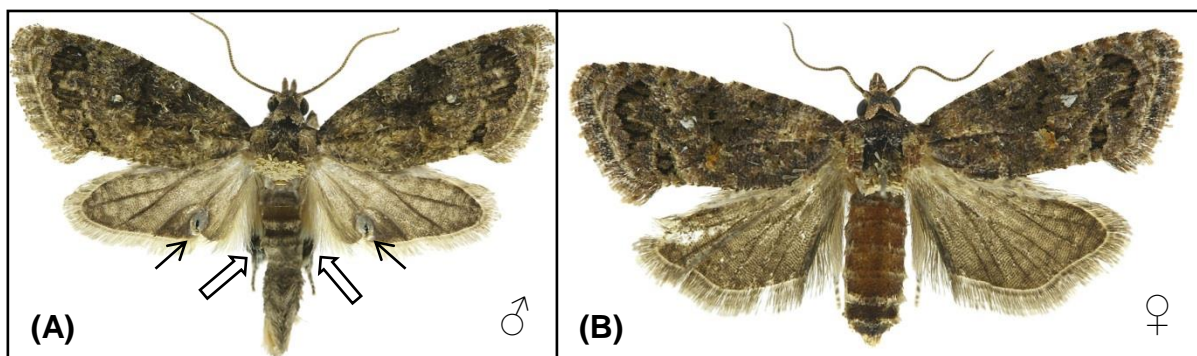


Figure 1.5: Adult *T. leucotreta* male (A) and female (B). Males are distinguished from females at this stage by the presence of a tuft of black hairs on their hind legs (⇔) and scent organ near the anal angle of each wing (→) (Photo credit: T.M. Gilligan & M.E. Epstein, <http://idtools.org/id/leps/tortai/index.html>).

Adult FCM may be confused with other moths within the same family, Tortricidae, owing to similar morphology, overlapping distribution, and fruit damage. These moths include codling moth, *Cydia pomonella* (L.) litchi moth, *Cryptophlebia peltastica* (Meyrick) and the macadamia nut borer moth, *Cryptophlebia batrachopa* (Meyrick) (Venette *et al.* 2003).

1.2.4. Life cycle

The duration of the FCM lifecycle is dependent on both temperature and food quality. In the warmer summer months, development from egg to adult takes between 1.5 and 2 months whilst in the cooler winter months, development from egg to adult takes longer, requiring between 2.5 and 4 months (Stibick *et al.* 2010). FCM does not undergo diapause and is therefore a continual pest with 5 to 6 overlapping generations (Terblanche *et al.* 2014).

Shortly after eclosion, mating occurs and soon thereafter, eggs are oviposited on the rind of fruit; usually singly and between the hours of 17:00 and 23:00. Egg development takes between 6 to 12 days depending on temperature (Daiber 1979a, 1980). In FCM, fecundity tends to be high and, given optimum conditions (high food quality and warm temperatures), a single female can oviposit an average of 450 eggs in its life span (Stibick *et al.* 2010).

After hatching, neonate larvae burrow into the fruit where they remain, feeding on the fruit pith, until fifth instar is reached. Entry often occurs at a point of damage or, in Navel oranges, at the Navel end. Larval development takes approximately 25 to 67 days (Daiber 1979c; Newton 1998). Development may be influenced by the quality of the fruit as well as the citrus cultivar and variety (Daiber 1979c; Newton 1998; Love *et al.* 2014).

When nearing pupation, fifth instar larvae burrow out of the fruit and drop to the ground below where they pupate. Pupation usually occurs within the upper layers of the soil surface (Georgala 1969; Newton 1988; Grout & Moore 2015; Love 2015). The subterranean life stage of FCM lasts approximately 21 to 40 days (Newton 1998). Eclosed adults have a short lifespan of between 1 to 3 weeks and as a result do not feed, but water uptake is essential

(Catling & Aschenborn 1974). A sex ratio of 1:1 is usually found in the field (Newton 1998; van den Berg 2001). The dispersal of FCM at a local scale has been shown, through genetic analysis and mark-recapture studies, to be limited (Newton 1998; Timm *et al.* 2010).

1.2.5. Economic importance

FCM causes damage pre- and post-harvest resulting in an estimated loss of ZAR 100 million per annum (Kirkman & Moore 2007). Development of larvae within the pith of the fruit (Figure 1.6) results in fruit decay, premature ripening and early abscission from trees. Damaged and abscised fruit is unusable and thus a financial loss to the grower and industry. FCM damage can also allow for the entry of other foreign pathogens such as *Penicillium italicum* Wehmer and *P. digitatum* Sacc., the causal agents of citrus blue and green mould respectively (Newton 1988; Newton 1998). Infestation may become apparent from as early as November, peaking in December to March in the Eastern Cape and February to May in the Western Cape (Newton 1998).

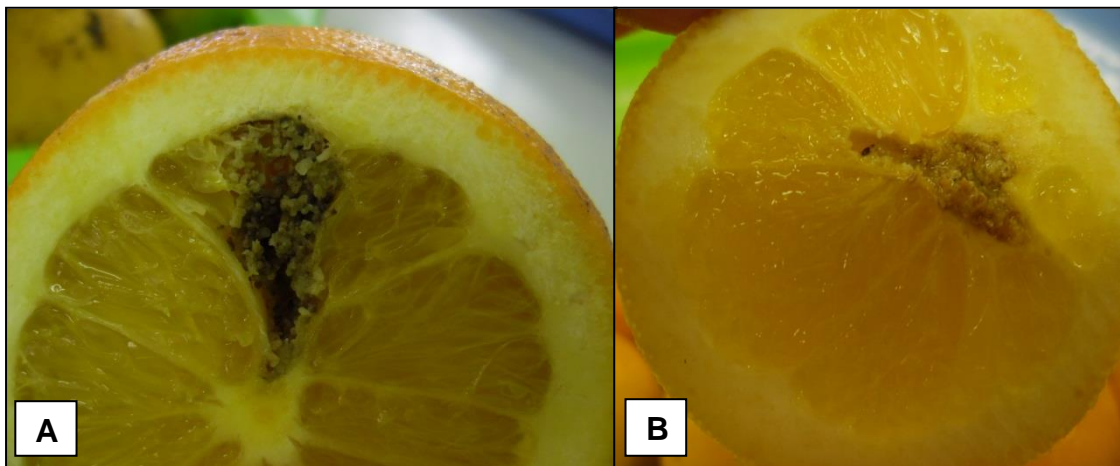


Figure 1.6: (A) Granular frass left behind by FCM larva after feeding and (B) FCM larvae feeding within a Navel orange.

FCM is also classified as a phytosanitary pest by certain lucrative export markets, such as the USA, China and South Korea, which have a strict no entry policy towards FCM (Venette *et al.* 2003; Grout & Moore 2015). Detection of a single FCM larva in cartons bound for export

may result in the rejection of the entire consignment (Moore 2012). Since the citrus industry in South Africa exports the bulk of its production, any rejection in consignments can have serious negative implications for the economy of the South African citrus industry. For this reason alone, it is imperative to maintain FCM populations within orchards to as close to zero as possible (Grout & Moore 2015).

1.2.6. Control options

A number of options are available for reducing FCM infestation in citrus orchards in South Africa. The use of these control options are commonly used in conjunction with one another as part of an IPM programme. Implemented successfully, reductions in FCM populations by 97% or more with currently available control options is possible (Moore & Hattingh 2012). In order to ensure that these options are used correctly, monitoring of the pest population within the orchard is important (Moore 2012).

1.2.6.1 Monitoring

Although not a control strategy *per se*, monitoring does allow for the determination of moth abundance between seasons and between orchards allowing for the prioritisation and correct timing of control strategies (Moore *et al.* 2008; Moore 2012). Pheromone-baited traps, coupled with sticky liners are used to monitor FCM population abundance within orchards. Three pheromones (Lorelei, FCM PheroLure and Chempac FCM lure) and two trap types (PVC pipe trap and yellow delta trap) are registered for use. Only Lorelei is registered and recommended for use with the PVC pipe trap and is the only system for which an association between trap counts and fruit infestation level is known; PheroLure and Chempac can only be used with the yellow delta traps (Moore 2012). If monitoring is to be successful, it is essential that the recommendations for trap placement within the orchard and collection and interpretation of the data, is adhered to (Moore 2012). Fruit drop surveys also serve as an important means of monitoring infestation levels within orchards, determining the cause of fruit drop, fruit decay, and post-harvest risk. They can also be used to validate trap count data. Fruit drop surveys, whereby dropped fruit are collected from underneath marked trees and dissected weekly, are the only means of accurately determining the precise cause of fruit

drop and hence extent of FCM infestation within the orchard. Guidelines for conducting fruit drop surveys are presented in Moore (2012).

1.2.6.2 Cultural control

One of the most common and important methods of cultural control is orchard sanitation; defined by the regular removal and correct disposal of all abscised and damaged or decaying fruit from the citrus orchard (Newton 1998; Moore 2012). It is recommended that orchard sanitation be initiated at the beginning of November and continued on a weekly basis, bi-weekly during the warmer months, until harvest. After harvest any damaged or decaying fruit should also be removed and disposed of correctly. Although it has been shown that small fruit less than 15 mm in diameter can be baked in the sun to kill any FCM present during hot months, it is preferable for all fruit to either be destroyed via pulping or via burial at least 30 cm below the soil surface (Moore & Kirkman 2009; Moore 2012). Orchard sanitation is not employed solely for the control of FCM (also aids in the reduction of other pest species, blue and green mould, sour rot and *Alternaria*), but has been shown to greatly reduce FCM infestation in citrus orchards (Moore & Kirkman 2009). For example a 10 year study conducted in the Eastern Cape showed that regular, weekly orchard sanitation removed 75% of larvae infesting fruit from December to June (Moore & Kirkman 2009). In addition, if orchard sanitation practices are poor, the efficacy of other control measures against FCM may be compromised (Moore & Kirkman 2009).

Another option for growers may be through the cultivation of more resistant citrus varieties in favour of those which are more susceptible to FCM attack. A recent laboratory study by Love *et al.* (2014) showed that different varieties of Navel oranges vary in their susceptibility to FCM oviposition and larval penetration. For example, the mid-season maturing Palmer Navels which are grown extensively across the Eastern Cape are the most susceptible to larval penetration compared to other mid-season maturing varieties, the early maturing Fischer Navels and the late maturing Cambria and Glen Ora Late cultivars.

1.2.6.3 Chemical control

Currently seven chemicals are registered for the control of FCM in citrus (Table 1.4) and have either ovicidal, ovi-larvicidal or larvicidal modes of action (Moore & Hattingh 2012; S.D. Moore, pers. comm.). Alystin[®] and Nomolt[®] are both insect growth regulators and function by disrupting larval development in the egg stage by inhibiting the production of chitin (Moore 2012). If these chemicals are to be successful, the eggs of FCM must be deposited on the residue. The timing of application is therefore important. Alystin[®] was found to be more effective than Nomolt[®] reducing fruit loss as a result of infestation by up to 86.4% in field trials (Newton 1987). Both chemicals have been reported to have a detrimental impact on certain natural enemies of FCM and are restricted for use on citrus destined for the USA. (Kirkman 2007). Meothrin[®] and Cypermethrin[®] are both pyrethroids and are restricted to a single use per season not later than four weeks before harvest. Coragen[®] and Delegate[®] were only registered for use against FCM in citrus in 2011. Both products incorporate more environmentally safe active ingredients, with low residue levels and are suitable for use in integrated pest management programmes (Moore & Hattingh 2012). In addition, a laboratory study showed that oranges dipped in Coragen[®] and Delegate[®], at the recommended application rate, had significantly fewer eggs oviposited on their surface than oranges not treated, or oranges treated with Alsystin[®] and Meothrin[®] (Fullard & Hill 2013). The seventh chemical, Runner, is the most recent registered addition against FCM in citrus and is another insect growth regulator (S.D. Moore, pers. comm.)

Table 1.4: Chemicals registered for the control of FCM in citrus orchards, South Africa (Moore 2012, S.D. Moore, pers. comm.).

Trade name	Active ingredient	Manufacturer
Meothrin [®]	Fenpropathrin	Sanachem, South Africa
Cypermethrin [®]	Cypermethrin	Agropharm, South Africa
Alystin [®]	Triflumuron	Bayer, Germany
Nomolt [®]	Teflubenzuron	Cyanamid, South Africa
Delegate [®]	Rynaxypyr [™]	Dow AgroSciences, Indianapolis
Coragen [®]	Spinetoram	Dupont, South Africa
Runner [®]	Methoxyfenozide	Dow AgroSciences, Indianapolis

1.2.6.4 Behavioural control

Two types of behavioural control options exist, mating disruption and attract-and-kill technique. Both options make use of a synthetic FCM female pheromone to attract male FCM. In attract-and-kill this pheromone is coupled with an insecticide. Thus, rather than preventing mating by making it difficult for males to locate females, males coming into contact with the insecticide die. Two products are registered for use as mating disruptors against FCM in citrus, Isomate FCM and Checkmate FCM. Only one product, Last Call FCM, is registered for use as attract-and-kill and is only recommended for use in areas where FCM density is low (Moore 2012; Moore & Hattingh 2012).

1.2.6.5 Sterile insect technique (SIT)

SIT was first developed against FCM as a viable genetic control strategy in 2002 in the citrus-producing regions in Citrusdal, South Africa. It was commercialised in 2007 by Xsit (Pty) Ltd following extensive research both in the laboratory and field (Hofmeyr *et al.* 2015). The use of SIT in Citrusdal has been greatly successful since its adoption as an area-wide control strategy coupled with improved moth rearing and optimisation of the SIT programme. Over three consecutive citrus growing seasons (2007 to 2010), FCM infestation within treated areas was progressively reduced by 50%, 81% and 93%, respectively (Hofmeyr *et al.* 2015). This success has been maintained in subsequent years and as such, SIT has since been expanded into citrus producing regions in the Eastern Cape Province; Sunday's River Valley and Gamtoos River Valley (Barnes *et al.* 2015). SIT relies strongly on 'over-flooding' the treated area with irradiated (sterilised at 150 to 200 Gy) males at a ratio of at least 10 sterile males to every one wild male moth to increase the likelihood of sterile males mating with wild females, the outcome of which renders a limited number of viable F₁ progeny (Bloem *et al.* 2003; Hofmeyr *et al.* 2005). As it is impractical to separate adult males and females before sterilization occurs, the radiation dose is aimed to render females infertile (occurs at a lower dose than males) and males mostly infertile without reducing their ability to compete with wild males once released (Bloem *et al.* 2003). SIT must be used in combination with other control measures in order to be successful (e.g. coupled with strict orchard sanitation) and is therefore IPM compatible with limited adverse effects on the development of the egg parasitoid *Trichogrammatoidea cryptophlebiae* (Carpenter *et al.* 2004; Barnes *et al.* 2015).

1.2.6.6 Biological control

Currently only one egg parasitoid, *Trichogrammatoidea cryptophlebiae* Nagaraja (Hymenoptera: Trichogrammatidae) and five microbial products, Cryptex[®] (Andermatt-Biocontrol AG, Switzerland), Cryptogran[®] (River Bioscience, South Africa), Gratham (Chempac Pty Ltd, South Africa), Broadband[®] (BASF, South Africa) and Cryptonem[®] (River Bioscience, South Africa), are registered or used commercially for the control of FCM in citrus (Moore & Hattingh 2012; S. D. Moore, pers. comm.). Moore (2002) lists a further suite of 16 parasitoids, four predators and two microbes which are capable of causing mortality amongst populations of FCM, none of which have been commercialised or used against FCM largely due to their inability to provide sufficient levels of control. The hymenopteran parasitoid *Agathis bishopi* Nixon (Hymenoptera: Braconidae), was investigated as a potential control agent of FCM by Gendall (2007), but due to difficulties during mass rearing, research focused elsewhere. Natural populations of this parasitoid have been recorded to achieve FCM larval parasitism of up to 34% in the Eastern Cape (Sishuba 2004) and recent improvements in the rearing protocol of this parasitoid appear promising for its future use against FCM (Zimba 2015).

Initially, the use of *T. cryptophlebiae* was deemed impractical as a study by Newton & Odendaal (1990) reported that in order to achieve a 60% reduction in FCM infestation within an orchard, more than three million parasitoids would need to be released per hectare over approximately eight months. Later research indicated that if between 25 000 and 250 000 parasitoids are released every month per hectare per season, a 60% reduction in an FCM population is achievable. Variability in these results was reported and has been associated with chemical spraying regimes. Commercial mass rearing and releases at 100 000 parasitoids per season per hectare are conducted by Du Roi IPM and Vital Bugs (Moore & Hattingh 2012).

Of the microbial products, three are virus based and all incorporate the *Cryptophlebia leucotreta* granulovirus (CrleGV) as their active ingredient (Moore *et al.* 2011; Moore 2012; Opoku-Debrah *et al.* 2013; Moore *et al.* 2015b). Granuloviruses offer the guarantee of host-specificity and are well suited for use within IPM programmes (Moore & Hattingh 2012; Knox *et al.* 2015). These products target the neonate life stage, thus application is targeted for

deposition on the fruit. As the neonates burrow into the fruit, viral particles are ingested and begin to replicate using the insect as a nutrient source. Three to five days post-infection the larva will succumb and liquefy releasing viral particles back into the environment (Knox *et al.* 2015). An extensive review on the use of granuloviruses in FCM control, with particular emphasis on Cryptogran[®] is presented in Moore *et al.* (2015b). From a representative sample of 13 field trials conducted in citrus orchards across South Africa between 2001 and 2013, viral application was found to reduce FCM infestation in treated areas between 30% and 92%. As with chemicals, the possibility of FCM developing resistance to these viral products exists, although the risk of resistance development is far less than for chemicals. If the problem does arise, Opoku-Debrah *et al.* (2013) have identified further strains of this virus which may be used as replacements.

Cryptonem[®], utilises the insect pathogenic nematode *Heterorhabditis bacteriophora* (Heterorhabditidae: Rhabditida) as its active ingredient and is formulated with an inert clay carrier. It has only recently being brought onto the market, and although it is registered for use against FCM soil-dwelling life stages, it can be used to control other soil-dwelling pests. Entomopathogenic nematodes are small (0.4 mm – 1.1 mm), transparent roundworms which have a mutualistic relationship with bacteria in the genera *Xenorhabdus* and *Photorhabdus* (Van Zyl & Malan 2014). Infective juveniles (IJs), the life stage which initiates infection, have the advantageous ability to actively target their host and have been reported to be compatible with other control agents e.g. entomopathogenic fungi. As such, they are appealing for use as control agents within IPM programmes (Gaugler 1988; Shapiro-Ilan *et al.* 2006; Malan *et al.* 2011; Van Zyl & Malan 2014). Once a host is infected with IJs, these bacteria will be released from the intestinal lumen of the IJs and replicate profusely resulting in the death of the host within 24 to 48 hours. The IJs then feed on the liquefying host tissues and bacterial cells, mature and mate producing progeny IJs which will emerge from the host cadaver in search of new hosts (Gaugler 1988; Van Zyl & Malan 2014). Other EPN and FCM related research has shown that certain isolates of native EPN strains are virulent towards the soil-dwelling life stages of FCM, more so the burrowing fifth instars than the pupae (Malan *et al.* 2011). A study by Manrakhan *et al.* (2014) also found that natural infection of FCM by EPNs in citrus orchards can reduce FCM infestation by an average of 50% and thus recommended that EPNs be preserved or applied as part of an IPM programme. Cryptonem[®]

is the only commercially available product that is registered for use against the soil-dwelling life stages of FCM, the life stage of interest in this study.

Only one fungal based product is registered for use in citrus, Broadband® (a.i. *Beauveria bassiana* strain R4444), against FCM neonates, not soil-dwelling life stages. The use of EPF as microbial control agents, is discussed in general in section 1.3 below and previous research on the use of EPF against FCM soil-dwelling life stages is discussed in more detail in section 1.3.5.

1.2.6.7 Post-harvest treatment

Cold sterilization, although costly is considered to be the most effective means of post-harvest control and used on fruit exported to markets which regulate FCM as a phytosanitary pest (Boardman *et al.* 2012; Moore & Hattingh 2012). Current regulations by phytosanitary markets mandate that fruit be exposed to sub-zero temperatures for 22 to 24 days to ensure probit 9 mortality of any FCM present within exported fruit (DAFF 2014).

1.3 ENTOMOPATHOGENIC FUNGI (EPF)

1.3.1. General biology

The Kingdom Fungi is considered to comprise of at least 1.5 million species of which less than an eighth have been described (Roy *et al.* 2010). The current higher-level phylogenetic classification of the *Fungi* recognises one kingdom, one subkingdom, seven phyla, ten subphyla, 35 classes, 12 subclasses and 129 orders (Hibbett *et al.* 2007). Most EPF can be found within two major lineages, the phylum Ascomycota and subphylum Entomophthoromycotina (Hibbett *et al.* 2007; Humber 2012; Boomsma *et al.* 2014). Most fungi in the phylum Ascomycota fall within the order Hypocreales (subclass Hypocreomycetidae, class Sordariomycetes, subphylum Pezizomycotina) and include well-known cosmopolitan genera such as *Beauveria*, *Metarhizium*, *Lecanicillium* and *Nomuraea*. Ascomycota and the phylum Basidiomycota are now accepted to fall within a subkingdom, Dikarya (James *et al.* 2006). Originally, the phylum Ascomycota was split into two;

Ascomycota and Deuteromycota. The latter housed species which appeared to have no sexual state and was thus sometimes referred to as the Fungi Imperfecti (Roy *et al.* 2006). Advancements in molecular techniques highlighted the similarities many of these share with species in the Ascomycota, later to be discovered as the anamorphic (asexual) and telomorphic (sexual) state of a species respectively (Shah & Pell 2003; Inglis *et al.* 2001). In the subphylum Entomophthoromycotina, most fungi are found within the order Entomophthorales and include the genera *Entomophaga*, *Entomophthora*, *Pandora* and *Zoophthora* (Shah & Pell 2003; Roy *et al.* 2006). Originally, entomophthoralean fungi were found within the phylum Zygomycota. However, the suspected polyphyletic nature of this taxon has, in more recent times, been confirmed. As such, this phylum is no longer accepted. Species within the Zygomycota are now distributed across one phylum, Glomeromycota (which contains only arbuscular mycorrhizal fungi) and five subphyla which have not yet been assigned to a specific phylum: Mucoromycotina, Kickxellomycotina, Zoopagomycotina and Entomophthoromycotina (Hibbet *et al.* 2007; Humber 2012).

Asexual hypocrealean fungi, the focus of this thesis, are hemibiotrophic and can therefore switch between parasitic and saprophytic behaviour. As such, host ranges tend to be broad, but are known to vary depending on the species as well as the strain (=isolate). Temporal and spatial factors, host physiology, insect cuticle composition and the nutritional requirements of the fungus also play a role in determining host range (Sandhu *et al.* 2012). Hypocrealean fungi tend to be associated with soil-dwelling hosts and are capable of producing resting spores in the presence of unsuitable conditions (e.g. lack of host). These spores can persist in the soil environment until conditions once again become favourable for infection (Shah & Pell 2003; Castrillo *et al.* 2005; Roy *et al.* 2006). In order for infection to occur and proceed, four basic steps need to be met: attachment, penetration, proliferation and sporulation (Inglis *et al.* 2001; Castrillo *et al.* 2005; Sandhu *et al.* 2012; Boomsma *et al.* 2014).

Contact between the target insect and infective propagule (e.g. conidia, blastospores, mycelia, microsclerotia) is essential and must be followed by successful attachment to the insect cuticle (Shah & Pell 2003; Ortiz-Urquiza & Keyhani 2013). The mechanisms behind the attachment process are not clear, but is likely a two-step process beginning with the passive attachment of the conidia to the cuticle as a result of their hydrophobic nature followed by the production of adhesion proteins and other adhesion components such as lectins (Boucias *et*

al. 1988; Holder & Keyhani 2005; Wang & St Leger 2007; Wanchoo *et al.* 2009). Attachment may also be limited by a lack of recognition sites on the insect cuticle and certain insect behaviours and cuticular defenses e.g. grooming and moulting (Boucias *et al.* 1988; Baverstock *et al.* 2010; Ortiz-Urquiza & Keyhani 2013). Following attachment, germination and penetration of the cuticle occurs. Penetration is achieved through a combination of mechanical pressure, through the formation of an appressorium which develops from the germ tube, and cuticle-degrading enzymes secreted by the fungus (Inglis *et al.* 2001; Sandhu *et al.* 2012; Boomsma *et al.* 2014). A modified infection hypha, termed the penetration peg, is formed and grows through the cuticle

Once inside the host body, the hypocrelean fungi typically grow as septate hyphae, evolved as a mechanism to overwhelm the host immune system. In some species, growth may be coupled with toxin production. This aids in the infection process either by facilitating nutrient uptake, insect sedation or increasing the speed of kill (Boomsma *et al.* 2014). Eventually, due to one or a combination of factors including damage to host tissue, nutrient depletion and toxicosis, the host will succumb to infection (Sandhu *et al.* 2012). Fungal mycelia will then grow through the insect cuticle in damaged or less sclerotized areas to allow for sporulation of a large quantity of small (< 8 µm), asexual spores on the surface of the cadaver. Typically, this occurs within three to seven days (Roy *et al.* 2006). These spores are then released passively into the environment (Shah & Pell 2003; Castrillo *et al.* 2005; Sandhu *et al.* 2012; Boomsma *et al.* 2014). Discrepancies between the infection and reproductive cycles of other important EPF taxa, including teleomorphic hypocrelean fungi and entomophthoralean fungi, are discussed and accompanied by informative diagrams in Boomsma *et al.* (2014).

1.3.2. EPF as control agents

The use of EPF to control pest insects has been known since the early 19th century when *M. anisopliae* was discovered infecting *Anisoplia austriaca* Herbst (Coleoptera: Scarabaeidae), a pest of grain in Russia. This led to the establishment of the first mass production site and field trials thus opening up the field of applied entomology and the role that EPF can play within it (Lord 2005). EPF are popular for use as biological control agents for a number of reasons: (1) they are typically host-specific; (2) infect via contact, rather than ingestion making them useful in the control of sap-sucking insects or insect life stages which do not feed; (3) are

considered environmentally safe and non-toxic to both vertebrates and the environment and have been shown to be compatible for use within IPM systems; (4) they tend to be easily cultivated on inexpensive culture media and thus amendable to large scale production; and (5) as with other biological agents, are capable of persisting in the environment for extended periods of time and thus can provide prolonged control. In addition, EPF can be used to target pest species in areas which chemicals often fail to reach e.g. subterranean life stages of many agriculturally important pests (e.g. white grubs) and in forestry (Roy & Pell 2000; Inglis *et al.* 2001; Shah & Pell 2003; Kaya & Lacey 2007; Zimmerman 2007a, b; Scheepmaker & Butt 2010; Sandhu *et al.* 2012).

The downside to the use of EPF, and most microbial agents, is that their speed of kill is slow (3-7 days) particularly when compared to chemical pesticides which can kill within a few hours after contact (Inglis *et al.* 2001; Sandhu *et al.* 2012). In addition, because EPF are biological organisms themselves, their application may not always be successful; numerous abiotic and biotic factors may impact field efficacy resulting in poor and variable results. Insects also develop strategies to combat fungal infection including humoral and behavioural responses (e.g. detection and avoidance behaviour) and difficulties can be faced during the development and registration phase (Inglis *et al.* 2001; Roy *et al.* 2006; Baverstock *et al.* 2010; Jaronski 2010). Despite this, numerous successful applications are reported including the control of gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae) by *Entomophaga maimaiga*, and cotton aphids, *Aphis gossypi*, by *Neozygites fresenii* in the USA; *Melolontha* spp. by *Beauveria brongniartii* in Europe; desert locusts in Africa by *M. acridum* and the control of numerous pests in China and Brazil by *B. bassiana* and *M. anisopliae* (Shah & Pell 2003; Douthwaite 2001; Li *et al.* 2010).

A comprehensive review of the number of mycoinsecticides (biopesticides incorporating EPF as their active ingredient) and mycoacaricides was conducted by Faria & Wraight (2007). Although some of the products are no longer in use, a total of 171 products are listed. The majority of the insects targeted can be found within the orders Hemiptera (56.9%), Coleoptera (40.9%), Lepidoptera (17.5%), Thysanoptera (14.6%) and Orthoptera (9.4%). Most of these products were developed by companies and institutions in South America (42.7%), followed by North America, Europe and Asia. African products contribute only

2.9%, although one of the most well-known and successful mycoinsecticides, Green Muscle[®], was developed in Africa. Entomopathogenic fungi used as mycoinsecticides tend to belong to the order Hypocreales with the most common genera being *Metarhizium*, *Beauveria*, *Lecanicillium* and *Isaria* (Faria & Wraight 2007; Vega *et al.* 2009; Hesketh *et al.* 2010).

1.3.3 Efficacy affecting factors

As previously mentioned, numerous environmental factors, both abiotic and biotic can have an impact on the success or failure of the applied entomopathogenic fungal strain. Under laboratory conditions, these factors can be controlled for and thus optimal results are typically obtained. However, in the field, these factors are variable and the impact they may have on fungal efficacy could be neutral, positive or negative. The influence of these factors is dependent on the isolate itself, the target insect and the environment in which it is to be applied. In the soil environment, the target area of concern in this thesis, soil temperature, soil moisture, soil texture, soil amendments and soil biota are typically the most important influential factors and are discussed in more detail below. Although these factors can influence fungal efficacy alone, their constant interaction with one another, renders it challenging to draw a definitive conclusion as to which factor is the greatest contributor to a change in fungal efficacy (Inglis *et al.* 2001; Klingen & Haukeland 2006; Wraight *et al.* 2007; Jaronski 2010).

1.3.3.1 Soil temperature

In general, soil temperatures in the sub-tropical and tropical regions are suitable for the growth of most EPF. The optimum temperature for the growth and germination of EPF typically falls between 20 and 25°C, but can range between 15 and 30°C. At lower temperatures, the rate of infection and hence death, is reduced, but not inhibited and as temperature increases above 28°C, a rapid decline in growth rate is experienced reaching a value of zero between 34 and 37°C (Inglis *et al.* 2001; Wraight *et al.* 2007; Jaronski 2010). In the field, temperature oscillates daily and therefore it is important to choose an isolate which is active within the temperature range experienced in the environment in which application is to occur. Fluctuations of temperature extremes are less apparent in the below-ground than

above-ground environment (Inglis *et al.* 2001). Consideration should also be given to the target pest as certain insect species have the ability to actively increase their body temperature, a process known as behavioural fever (Roy *et al.* 2006).

1.3.3.2 Soil moisture

The availability of free water is an essential requirement for conidial sporulation and germination with dry conditions often being stated as a reason limiting the success of EPF in insect control (Wraight *et al.* 2007). This is not always the case. Studdert & Kaya (1990) found that in drier soils, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) emergence was far lower than in wetter soils and Kreuger *et al.* (1991) found that chinch bugs, *Blissus leucopterus* Say (Hemiptera: Lygaeidae), suffered higher levels of mortality as a result of *B. bassiana* infection in soils with low relative soil moisture compared to high soil moisture. This can be attributed to the availability of free water within microhabitats such as in folds on the insect cuticle or alternately may be related to the isolate under investigation. Applying EPF at night, early hours of the morning or during periods of elevated moisture, can also contribute to more effective pest control (Wraight *et al.* 2007; Wraight & Ramos 2015). Soil moisture will vary amongst areas and will be impacted by rainfall, crop cover and the type of irrigation system employed (Inglis *et al.* 2001).

1.3.3.3 Soil amendments

Any amendment to the soil environment, whether it be as a result of fertiliser application, chemical run-off from spraying or agricultural practices, can impact fungal efficacy (Inglis *et al.* 2001). Many studies have suggested that soils high in organic matter, which may be influenced by the addition of fertilisers, reduce the persistence and efficacy of EPF against the target host due to high biological activity within these soils and hence greater likelihood of antagonistic interactions (Klingen & Haukeland 2006; Quesada-Moraga *et al.* 2007). Magara *et al.* (2003) found that by adding various types of soil amendments including manure and inorganic fertilizer, the efficacy of *B. bassiana* at controlling the banana weevil, *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae), was reduced. This reduction was suspected to be a result of over colonization of the substrate by two other common soil fungi. The addition of fertiliser is not always negative. Fertilisers increase nutrient availability

within the soil environment which can result in an increase in the host or alternate host population. This in turn can aid in increased fungal persistence and hence efficacy (Inglis *et al.* 2001; Klingen & Haukeland 2006).

The use of chemical products in agricultural systems will always exist and thus many studies have investigated the impact that these agricultural chemicals such as herbicides, fungicides and insecticides have on the efficacy of fungal entomopathogens in pest control (Alizadeh *et al.* 2007; Bruck 2009). Although laboratory results do provide a preliminary indication as to which chemicals are toxic to the applied fungi, under more realistic field conditions, this effect may become negligible (Jaros-Su *et al.* 1999; Samson *et al.* 2005; Bruck 2009). This may be attributed to the lack of interaction between the applied products in both space and time or may result from differences experienced in concentration to which the isolates are exposed in the laboratory compared to that in the field, which is typically lower (Jaronski 2010). The latter may be particularly true when chemicals are applied against above-ground pests and EPF against below-ground pests. In addition, fungal conidia generally remain ungerminated within the soil until contact with a suitable host occurs. Ungerminated conidia may be less susceptible to damage by the chemicals and once attached to the insect cuticle or inside the insect host, germinated conidia may be isolated or protected from any negative effects imposed by the application of these agrichemicals (Jaronski 2010).

Soil management practices, such as tillage, sowing and fumigation vary depending on the crop grown and usually reduce the number of infective propagules from the area in which the target host occurs or alternatively, expose the applied propagules to adverse environmental conditions from which they were previously protected. Sosa-Gomez & Moscaradi (1994) found a higher density of *B. bassiana* in areas which were not tilled compared to areas which were tilled. Tillage was suspected to expose the applied conidia to greater extremes in temperature compared to non-tilled systems which not only tend to have lower soil temperatures, but also retain water more effectively. Other studies have also reported the greater recovery of fungal isolates from soils within refugia surrounding the cultivated areas (Goble *et al.* 2010) whilst others report no difference between refugia and areas of cultivation (Klingen *et al.* 2002). With regard to *B. bassiana* and *M. anisopliae*, research indicates that *B. bassiana* prefers more natural soils than soils from cultivated habitats whereas the prevalence

of *M. anisopliae* is not adversely affected by cultivation and, in some cases, strongly associated with soils from cultivated areas (Quesada-Moraga *et al.* 2007; Goble *et al.* 2010; Medo & Cagáñ 2011).

1.3.3.4 Soil structure and texture

Soil texture (=size range of soil particles) typically affects fungal efficacy by increasing or limiting contact between the host and infective propagule (Jaronski 2010). For example, soil particles of sandy soils range in size between 50 to 2000 µm whereas soil particles of clay soils are less than 2 µm. The vertical movement of the fungal propagules out of the target area, and hence loss of fungal titre, can therefore occur more easily in sandy versus clay soils. This movement in turn is also impacted by the properties of the propagules themselves; smaller conidia for example, can pass more easily through pore spaces than larger conidia (Keller & Zimmerman 1989; Barbercheck 1992). The impact of soil texture is further complicated by soil moisture (Jaronski *et al.* 2005) as well as soil structure. Soil structure can be defined as the arrangement of the soil particles into aggregates and is influenced not only by soil texture, the presence of plant roots and level of compaction, but also the movement of soil biota through the area which can create tunnels through which fungal propagules can potentially be lost (Barbercheck 1992).

1.3.3.5 Soil biota

Soil is composed of numerous micro-, meso- and macro-fauna. The biological make-up of soil will vary from place to place for numerous reasons including nutrient or host availability, soil conditions and cultivation practices (Klingen & Haukeland 2006). The presence of soil organisms can reduce the number of fungal spores in an area either through direct mycophagy, competition for resources, by movement of the spores out of the target area or by inhibiting fungal growth or germination through the secretion of certain compounds (Jaronski 2010). A study conducted by Lingg & Donaldson (1981), showed that the growth of *B. bassiana* was inhibited by a water-soluble compound which was secreted by *Penicillium urticae*, a common soil fungus. Dromph (2001, 2003) found that some species of Collembola were able to carry conidia either on their cuticle or within their gut, facilitating the dispersal and vectoring of the EPF. The number of target hosts and alternate hosts present within the

soil environment will also impact fungal efficacy and persistence (Barbercheck 1992). The greater the number of hosts, and provided that application is adequate, the greater the probability of infection, which in turn increases the likelihood of secondary recycling and hence persistence of the applied fungal strain (e.g. Kessler *et al.* 2004). In general, most soils exhibit some level of fungistasis towards EPF and other fungi, which often results in the initial reduction in fungal titre following application. In addition, soil sterilization experiments suggest that biotic factors are more important in limiting fungal efficacy than physical soil properties (Ho & Ko 1986; Kessler *et al.* 2004). However, since natural epizootics do occur, it is unlikely that fungistasis alone can account for the failure of the applied fungal isolate (Jaronski 2010).

1.3.4. Genera *Beauveria* and *Metarhizium*

Both *Beauveria* (Balsamo) and *Metarhizium* (Metschnikoff) are cosmopolitan genera belonging to the order Hypocreales, phylum Ascomycota and are typically soil-borne, but may persist as endophytes within the tissues of certain plant species either naturally or through artificial introduction (Ownley *et al.* 2010; Vega 2008; Sasan & Bidochka 2012; Batta 2013). Species within these genera are genetically diverse with many strains of a single species identifiable which vary in their pathogenicity and virulence towards insects making them highly versatile and ideal candidates for use as microbial control agents. In addition, both genera are relatively easily cultivated and stored on inexpensive media and are amendable to large scale production (Rehner 2005; Kaya & Lacey 2007). *Beauveria* and *Metarhizium* are known to infect over 700 and 200 insect species respectively including a number of important agricultural pests (Shah & Pell 2003; Li *et al.* 2010).

Beauveria was first discovered as a disease of European silkworms in the 18th and 19th century by Agostini Bassi who described it as white muscardine disease (Rehner 2005). *Beauveria* is easily distinguished morphologically; hyaline, smooth-walled, conidia are produced in succession on a rachis, with a characteristic “zig-zag” structure, attached to conidiogenous cells which are spherical to flask-shaped and arranged in spiralled clusters (Figure 1.7A) (Domsch *et al.* 2007; Zimmerman 2007a). In culture, *Beauveria* produces white mycelia and conidia and because growth is often rapid, may have a powdery

appearance. Some isolates may also produce yellow pigment in the older central regions and others may secrete a red pigment into the culture medium during growth. Morphologically, the size and shape of the conidia are the most useful tools for species identification, but because features may overlap amongst species, genetic tools are often necessary for species confirmation (Rehner 2005; Domsch *et al.* 2007; Rehner *et al.* 2011). The most recent classification, which recognises 12 species of *Beauveria*, can be found in Rehner *et al.* (2011). Within this genus, *Beauveria bassiana* is the most recognised and commonly isolated species. It is also the species on which most mycoinsecticides are based, with different strains being used to control a variety of insect pest species from the orders Lepidoptera, Coleoptera and Hemiptera. *Beauveria brongniartii* is also commonly recognised, but is more restricted to use against scarab larvae (Faria & Wraight 2007). The taxon *Cordyceps* contain the teleomorphic (sexual) species of *Beauveria* (and *Lecanicillium*) anamorphs (asexual) (Boomsma *et al.* 2014).

Metarhizium was first discovered and isolated in the 1880s by Elie Metschnikoff, during his work on grain beetles, naming the green muscardine fungus as *Entomophthora anisopliae* (Lord 2005). *Metarhizium* can be distinguished by the elongated, green conidia which are produced on chains, compacted to form columns arising from phialides (Figure 1.7B). In culture, *Metarhizium* produces white mycelia, and green spores (Domsch *et al.* 2007; Zimmerman 2007b). Species identification within this genera, relies more heavily on genetic tools rather than morphological characteristics as the association between species identification and conidia shape and colour is weak (Zimmerman 2007b). *Metarhizium anisopliae* is the most commonly recognised species of this genus and is now accepted to be a species complex comprised of nine different species: *M. anisopliae*, *M. guizenhouse*, *M. acridum*, *M. pingshaense*, *M. lepidiotae*, *M. majus*, *M. globosum*, *M. robertsii* and *M. brunneum* (Bischoff *et al.* 2009). The taxon *Metacordyceps* contains the teleomorphic (sexual) species of *Metarhizium* anamorphs (asexual) (Boomsma *et al.* 2014).

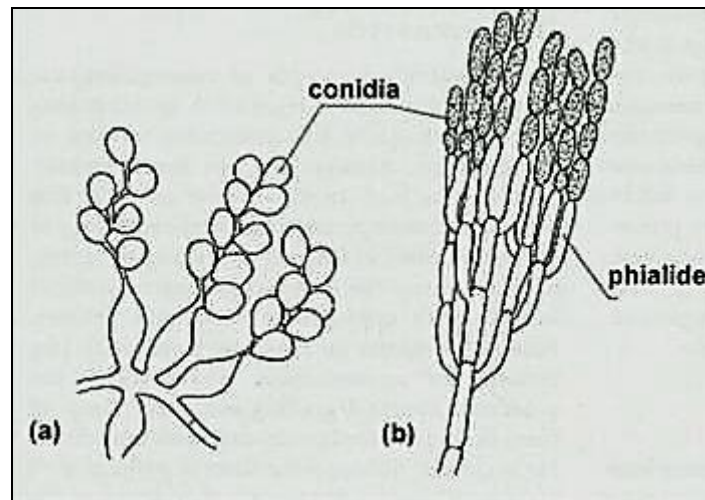


Figure 1.7: (A) Spherical conidia of *Beauveria* produced on a denticulate rachis arising from flask-shaped conidiogenous cells, and (B) elongate conidia of *Metarhizium* produced as basipetal chains from phialides (Photo credit: J. Deacon, University of Edinburgh, <http://archive.bio.ed.ac.uk/jdeacon/FungalBiology/chap15im.htm>).

1.3.5 EPF against FCM in South African citrus

Since 2009, Citrus Research International and the Department of Zoology and Entomology at Rhodes University, have been working towards developing a mycoinsecticide for use against the soil-dwelling life stages of FCM. According to Ravensberg (2011), the development of a biopesticide can be subdivided into four phases: an exploratory phase where the need for the product is investigated (phase 1); a screening phase whereby strains are isolated and selected for, and is often laboratory based (phase 2); the production and product development phase where the requirements for mass production and formulation of the strain are investigated and tested in the field (phase 3); and the implementation phase where the product is optimised, registered and marketed (phase 4) (Figure 1.8). Although this process can essentially be seen as a stepwise progression with certain criteria needing to be met before strain development can advance, certain questions can be addressed simultaneously to hasten the process.

Given the limited products available for use against the soil-dwelling life stages of FCM and the importance of controlling this pest, the decision to investigate soil-borne EPF as an additional biological control agents was validated (phase 1). Phase 2 was initiated in 2009/10 and began with the isolation and identification of a suite of entomopathogenic fungal isolates.

All these isolates were identified from soil collected from various citrus orchards distributed across the Eastern Cape Province (South Africa). In total, 62 isolates were identified using the *Galleria* bait method or a modification thereof (Goble *et al.* 2010). Most isolates were of the species *Beauveria bassiana* followed by *Metarhizium anisopliae* (Goble *et al.* 2010). Twenty-one of these isolates were then screened to determine their virulence towards the soil-dwelling life stages of three key citrus pests: *Ceratitis capitata* Wiedemann, *C. rosa* Karsch (Diptera: Tephritidae) and FCM by exposing final instars to sterile sand inoculated with each isolate at concentration of 1×10^6 conidia/g of soil (Goble *et al.* 2011). Twelve of these isolates achieved fungus-induced mortality greater than 80% against FCM. Further laboratory analyses conducted by Coombes (2013) successfully identified the three most virulent isolates towards FCM final instars and highlighted their ability to persist under semi-field conditions for a period of six months (Coombes *et al.* 2013; Coombes *et al.* 2015).

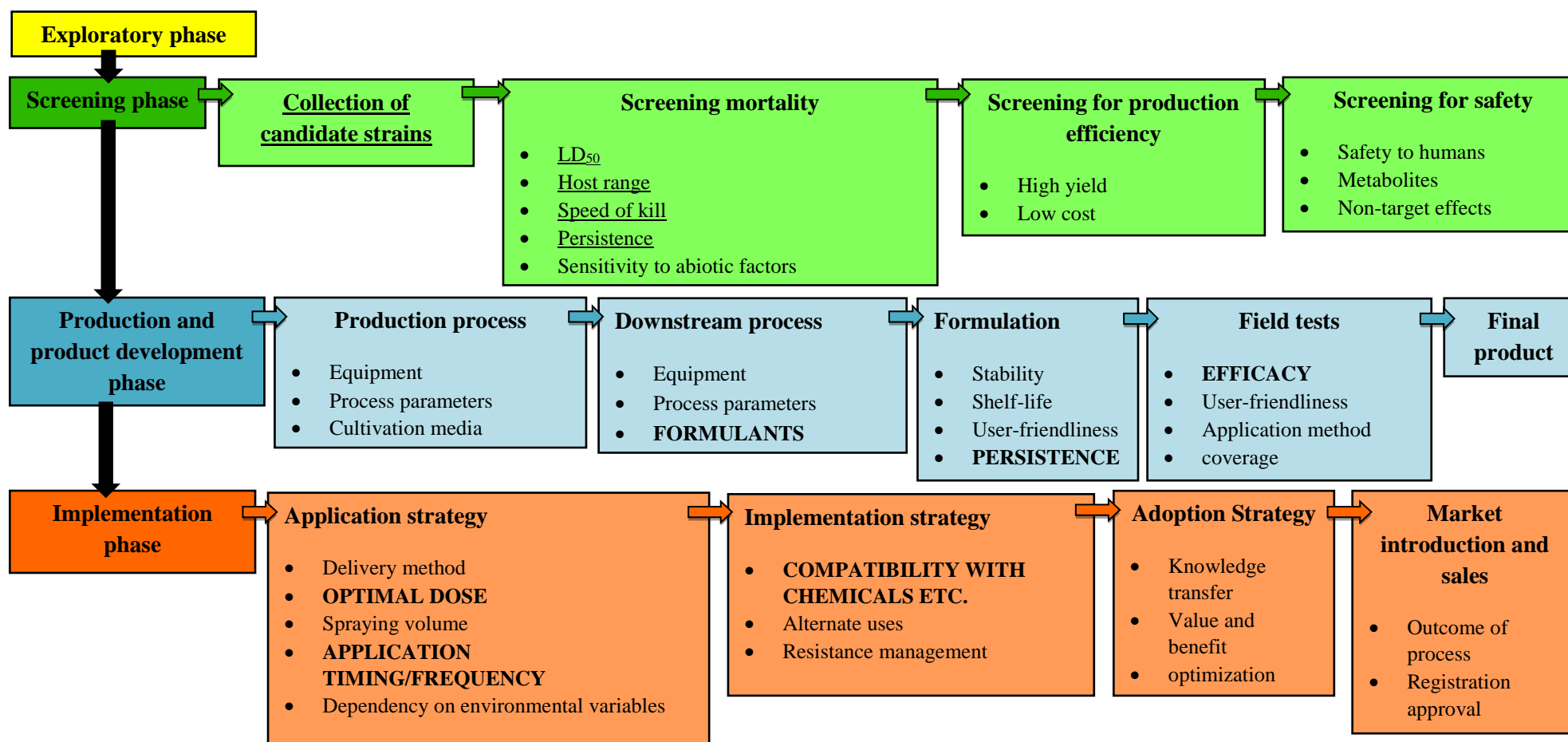


Figure 1.8: Simplified illustration of the phases and steps necessary for the commercialisation of a biopesticide (adapted from Ravensberg 2011). Criteria underlined are those which have already been met in studies preceding this one (Goble *et al.* 2010, 2011; Coombes *et al.* 2013, 2015). Criteria written in uppercase and bold are under investigation in this study.

1.4 RESEARCH AIMS

Much of the research regarding EPF against FCM in citrus is restricted to the screening phase (Figure 1.8). Virulent entomopathogenic fungal isolates have been collected and identified (Goble *et al.* 2010, 2011; Coombes 2013; Coombes *et al.* 2015) and preliminary analysis on field persistence has been undertaken (Coombes *et al.* 2013). Although these results have been promising, there are still many criteria that need to be addressed if the development of one or more of these isolates into a successful mycoinsecticide is to continue (Figure 1.8). During the course of this thesis some of these issues are addressed and will be used to make an informed decision on whether development should proceed, and if so, make recommendations on the way forward. As most of the information regarding these isolates has been obtained under controlled and optimal laboratory conditions, and given that environmental variables can impact fungal efficacy greatly, emphasis is placed on the field performance of the three top performing isolates: *B. bassiana* G Ar 17 B3, *M. anisopliae* G 11 3 L6 and *M. anisopliae* FCM Ar 23 B3.

More specifically, this thesis aims to advance EPF/FCM research by addressing the following issues:

- ❖ Preceding this study, the ability of the top three isolates to perform under field conditions was unknown. By utilising laboratory reared FCM final instars and soil-filled cages inoculated with each isolate at three concentrations, the ability of these isolates to reduce FCM numbers under fluctuating field conditions was determined (Chapter 2).
- ❖ As the ultimate goal of developing these isolates is for use in citrus orchards, the ability of these isolates to reduce FCM infestation within operational citrus orchards over the growing season was evaluated. Here, fungus was applied directly to the soil surface underneath the canopy of citrus trees. During the course of these trials, the efficacy of these isolates in an orchard employing two different types of irrigation systems (drip and micro-sprinkler) was determined and investigations were undertaken to determine optimal dose and application timing (Chapter 3).
- ❖ Although knowledge on the persistence of these three isolates is available, the results are not necessarily reflective of actual field persistence due to the methodology used which limited the influence of soil biota (Coombes *et al.* 2013). Therefore, to obtain a more

accurate reflection of the true persistence of these isolates in a citrus orchard, the fate of these fungi applied directly to the soil in Chapter 3 was monitored over six months by CFU assessment. As irrigation can impact soil moisture, and given that soil moisture can in turn affect fungal persistence, the persistence of these isolates was monitored in orchards under micro-sprinkler and drip irrigation (Chapter 4).

- ❖ For the field trials (Chapter 2 and 3), fungi were applied as aqueous suspensions. As the aerial conidia (=spores) of EPF are highly hydrophobic, the use of a surfactant in aqueous suspensions is essential to promote adherence between the spore and insect cuticle and also to retain the spores in suspension during application. Surfactants vary in their effectiveness in retaining spores in suspension as well their toxicity towards fungal propagules (Jackson *et al.* 2010). The effect of different surfactants on spore viability and retention within a water medium was therefore determined (Chapter 5).
- ❖ Citrus black spot (CBS), caused by the fungus *Phyllosticta citricarpa*, can result in fruit not suitable for export and in extreme cases (if not controlled) can even affect the vigour of trees. Several fungicides are therefore registered for the control of CBS and are applied by growers on a regular basis (Schutte 2009). As fungicides have the greatest potential to harm the applied fungal isolate (Klingen & Haukeland 2006), the compatibility between these fungicides and fungal isolates was investigated (Chapter 6).

Chapter 2

The effect of EPF on FCM fifth instar mortality during field-cage trials

2.1 INTRODUCTION

A number of entomopathogenic fungal isolates have been identified from soil samples collected across six orchards and surrounding refugia in the Eastern Cape Province, South Africa (Goble *et al.* 2010). Subsequent laboratory analysis highlighted three isolates as having the greatest control potential against the wandering pre-pupating fifth instars of FCM (Goble 2009; Goble *et al.* 2011; Coombes 2013; Coombes *et al.* 2013, 2015). Two isolates are of the species *Metarhizium anisopliae*, G 11 3 L6 and FCM Ar 23 B3 designated as Ma1 and Ma2, whilst the remaining isolate, G Ar 17 B3 is of the species *Beauveria bassiana* and will be referred to as Bb1. All three isolates obtained low LC₅₀ values ranging between 1.98×10^5 and 1.92×10^6 conidia/ml and LT₅₀ values of between 4 and 6 days, depending on the exposure dose (Coombes 2013; Coombes *et al.* 2015) (Table 2.1). Based on the calculated LC₅₀ values using a three-dose sand-conidial bioassay, Bb1 was found to be significantly more virulent than Ma1 and Ma2 (Coombes *et al.* 2015). Initial identification of these isolates was based solely on morphological identification (Goble *et al.* 2010). However, molecular analysis, based on sequencing of the internal transcribed spacer region coupled with BLAST GenBank (Altschul *et al.* 1997), has confirmed the identity of Ma2 and Bb1 as *M. anisopliae sensu stricto* and *B. bassiana s.s.*, respectively (Chartier Fitzgerald 2014). Molecular identification of isolate Ma1 as *M. anisopliae s.s.* has not yet been assessed. Therefore, this isolate should be considered as *M. anisopliae sensu lato* as it may represent one of the nine species within the *M. anisopliae* species complex.

Although laboratory assessment of fungal isolate efficacy allows for the determination of the top-performing candidates, this merely forms part of the very initial stages in the

development of a mycoinsecticide (Ravensberg 2011). Moving forward, research needs to begin focusing on the performance of these isolates under more realistic conditions. The inefficacy or reduced efficacy of fungal entomopathogens reported to be highly virulent under laboratory conditions has been reported under field conditions and is often associated with environmental conditions such as temperature and moisture which may be sub-optimal or detrimental for fungal-induced infection (Klingen & Haukeland 2006; Foster *et al.* 2011; Swiergiel *et al.* 2015). The factors which may impede fungal efficacy are discussed in more detail in Chapter 1, section 1.3.3. Semi-field trials are thus the next logical step.

Through the use of cages and artificially introduced fifth instars, the overall aim of this chapter was to determine whether application of the three most laboratory-virulent isolates to citrus orchard soil were capable of causing a reduction in the percentage eclosion of FCM adults in comparison to non-treated cages. The dose-dependent nature of these isolates under more realistic environmental conditions (such as non-sterile soil, fluctuating sub-optimal temperature and soil moisture) was also evaluated and their efficacy compared to a commercially available mycopesticide. As the orchard in which the trial was conducted was mulched regularly with sheep's wool and lucerne hay, the impact that mulching, a type of soil amendment, may have had on fungal efficacy was also determined.

Table 2.1: Information regarding the collection and laboratory assessment of the three entomopathogenic fungal isolates of interest in this study (Goble *et al.* 2011; Coombes *et al.* 2015).

Species	Accession details		In text reference	Fungal isolate location ²	Max mortality ³ (%)	LC ₅₀ (±SE) ⁴	LC ₉₀ (±SE) ⁵	95% Confidence Interval ⁶	
	Rhodes University	PPRI ¹							
<i>Metarhizium anisopliae s.l.</i>	G 11 3 L6	PPRI 9803	Ma1	Mosslands (33°23'54" S; 26°25'41" E) Oakleaf Caledon, April 2008	58.75	6.26×10 ⁵ (1.93×)	1.97×10 ⁷ (1.39×)	1.32×10 ⁶ ; 3.68×10 ⁵	58.75 43.75
<i>Metarhizium anisopliae s.s.</i>	FCM Ar 23 B3	PPRI 9561	Ma2	Arundel (33°30'57" S; 25°39'11" E) Loam, April 2008	43.75	1.92×10 ⁶ (1.07×)	1.67×10 ⁸ (2.23×)	9.58×10 ⁶ ; 8.18×10 ⁵	43.75
<i>Beauveria bassiana s.s.</i>	G Ar 17 B3	PPRI 9679	Bb1	Arundel (33°30'57" S; 25°39'11" E) Loam, May 2008	65.00	1.98×10 ⁵ (0.67×)	1.02×10 ⁷ (0.90×)	4.33×10 ⁵ ; 1.03×10 ⁵	

¹Cultures stored at the Plant Protection Research Institute, National Collection of Fungi (Pretoria, South Africa).

²Location data of original fungal-soil isolations (farm name, co-ordinates, soil type and year of isolation) (extracted from Goble *et al.* 2011).

³Average maximum mortality recorded at 1×10⁶ conidia/ml. Control mortality averaged 7.50%.

⁴Proban generated lethal concentration values (LC₅₀) and standard error (SE) from three dose-bioassays with four replicates; for mortality after 7 d at 26°C.

⁵Lethal concentration 90 (LC₉₀) values are an extrapolation of the data set.

⁶Upper and lower limits of confidence interval.

2.2 MATERIALS AND METHODS

2.2.1 Site details

This trial was conducted at Olifantskop (33°37' S, 25°40' E), in an organic 22 year old Palmer Navel orange citrus orchard. Soil texture was characterised as loam (16% clay, 38% silt and 46% sand) with a soil pH of 7.7. Soil surface temperature and at 10 cm depth ranged from 19.4°C to 23.9°C and 17.5°C to 24.2°C, averaging 21.8°C and 21.3°C respectively. Soil moisture at 10 cm depth ranged from 32.8% to 42.6% with an average of 36.5%. Soil texture and pH were determined from soil samples sent to SGS laboratories (Cape Town, South Africa) for analysis. Soil moisture and temperature were measured with the aid of 80 cm soil probes installed according to manufacturer's instructions (DFM Software Solutions, South Africa).

2.2.2 Fungal isolates

The semi-field efficacy of three entomopathogenic fungal isolates against FCM was evaluated: Ma1, Ma2 and Bb1 (Table 2.1). These isolates were chosen as they were found to be the most effective in controlling late fifth/ early pupating instars during laboratory bioassays (Goble *et al.* 2011; Coombes *et al.* 2015). Fungi were obtained as dry aerial conidia from Citrus Research International, following mass production by Agrauxine (Loches, France). Fungi were applied unformulated as an aqueous suspension – water supplemented with 0.01% Breakthru®S240 – using a 1 L hand-held spray applicator. The addition of Breakthru®S240 was necessary to promote fungal suspension within the water due to the hydrophobicity of the spores. Following application, the viability of the applied fungi were measured in the laboratory by plating 100 µl of the applied suspension onto each of three replicate Sabouraud dextrose agar (SDA) plates supplemented with 1 ml dodine, 1 ml 50 mg/ml rifampicin and 1 ml 50 mg/ml chloramphenicol. Plates were incubated overnight, in complete darkness, at 26°C and the number of germinating and non-germinating spores out of 100 per plate was counted.

2.2.3 Insect culture

FCM fifth instar larvae were acquired in bulk from River Bioscience, Addo, South Africa. Larvae were reared on autoclaved maize-based artificial diet at approximately 27°C and 30% relative humidity in clean 350 ml glass honey jars stoppered with cotton wool in which FCM pupation occurs (Moore *et al.* 2014). All FCM fifth instars used were ready to pupate within 24 h as indicated by their movement upwards into the cotton wool.

2.2.4 Treatments

For each isolate, efficacy was monitored at three different concentrations equivalent to 0.5×10^{14} (low), 1×10^{14} (intermediate) and 2×10^{14} (high) spores/ha, and in the presence of a lucerne hay mulch at the intermediate rate. Mulch was added to each respective cage as a thin complete-cover-layer prior to fungal application. Three controls were included: water supplemented with Breakthru®S240, water supplemented with Breakthru®S240 following mulch application and an untreated control. A commercially available *B. bassiana* based mycoinsecticide, Broadband® (BASF, South Africa), was also applied at the recommended field rate. Each treatment consisted of eight replicates, a replicate been represented by one soil-filled cage to which one of four treatments or three controls, as described above, was applied.

2.2.5 Experimental procedure

Cages (5 L plastic tubs, 20×20×30 cm, with breathable mesh inserts) (Figure 2.1A) were buried in the upper soil layers underneath the canopy of citrus trees. Prior to burial, cages were assigned to a treatment and an associated number. A random number generator was then used to determine the order in which cages should be placed in the field. The effect that soil moisture and sunlight exposure may have on the efficacy of the applied fungi was limited by placing cages on the south-facing side of the tree and 1 m away from an irrigation sprinkler (Figure 2.1C). Buried cages were filled with the soil that was removed during hole-digging (Figure 2.1B). Mulch was then added to the cages pre-designated as a mulch treatment. Each treatment replicate was then treated with 125 ml of the appropriate fungal suspension using a 1 L handheld spray

applicator. A separate applicator was used for each fungal treatment including the commercial treatment and controls to avoid cross contamination. No suspension was applied to the untreated control.

Thirty FCM fifth instars were added to each cage 24 h prior to pupation, by inverting a plastic container (diameter 11.5 cm, depth 5.5 cm) directly above the cages and allowing the larvae to drop to the soil below. The cages were sealed with mesh and monitored weekly for the presence of eclosed adults. The experiment was terminated two weeks after eclosion was first noted (one month after the trial was initiated). Cages were brought back to the laboratory and the number of eclosed adults was counted. Pupal casings, which generally remained intact, were also counted for each cage to limit the possibility of underestimation as a result of predation or disintegration of adults (Figure 2.2). The higher of the two counts was taken as the total number of eclosed FCM adults. Soil samples were taken from each cage and used to assess the concentration of fungus present (section 2.2.6). Statistical differences amongst treatments was determined via non-parametric Kruskal Wallis ANOVA ($P < 0.05$) due to the non-normality of the data even after arcsine transformation. If treatment effects were found, a multiple comparison of mean ranks was performed ($P < 0.05$). In addition to percentage eclosion, the percentage FCM corrected mortality was calculated with the aid of Abbott's formula (Abbott 1925). Treatment effects were determined as for the eclosion data. All statistics were conducted in Statistica ver. 10 (StatSoft Inc., 1984-2011).

2.2.6 Fungal concentration in cages

Following moth counts, 100 g of soil, from the upper 5 cm soil surface, was removed from each cage and sieved. 20 g was then suspended in 100 ml sterile 2% saline solution contained within 250 ml conical flasks. The suspension was mixed well by shaking each flask vigorously by hand for approximately five minutes and repeated. Following 5 s sedimentation, 100 μ l of supernatant was removed and spread onto each of three replicate SDA plates supplemented with 1 ml dodine, 1 ml 50 mg/ml rifampicin and 1 ml 50 mg/ml chloramphenicol. Plates were sealed with Parafilm M[®] and incubated at $26 \pm 1^\circ\text{C}$ on a 12 h photoperiod for two weeks after which the number of

colony forming units (CFUs) was counted and the number of CFUs/g of dry soil calculated using the equation given below.

$$CFUs \text{ per } g \text{ dry soil} = \left(\frac{\text{count}}{\text{sample dry weight}} \right) \times \text{dilution factor}$$

The moisture content of each sample was determined by drying a pre-weighed quantity (± 40 g) of wet soil in an oven for 24 h at 100°C. Moisture content was determined according to the equation below and was used to calculate the percentage sample dry weight.

$$\% \text{ moisture} = \left(\frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \right) \times 100$$

The number of CFUs/g dry sample was also determined for the mulch following the procedure described above. For both plating and moisture assessment, 2 g of mulch was used. Statistical differences were determined by non-parametric Kruskal Wallis ANOVA ($P < 0.05$) due to data non-normality even after log and square root transformation. If treatment effects were found, a multiple comparison of mean ranks was used to determine where this significance lay ($P < 0.05$). All statistics were conducted in Statistica ver. 10 (StatSoft Inc., 1984-2011).

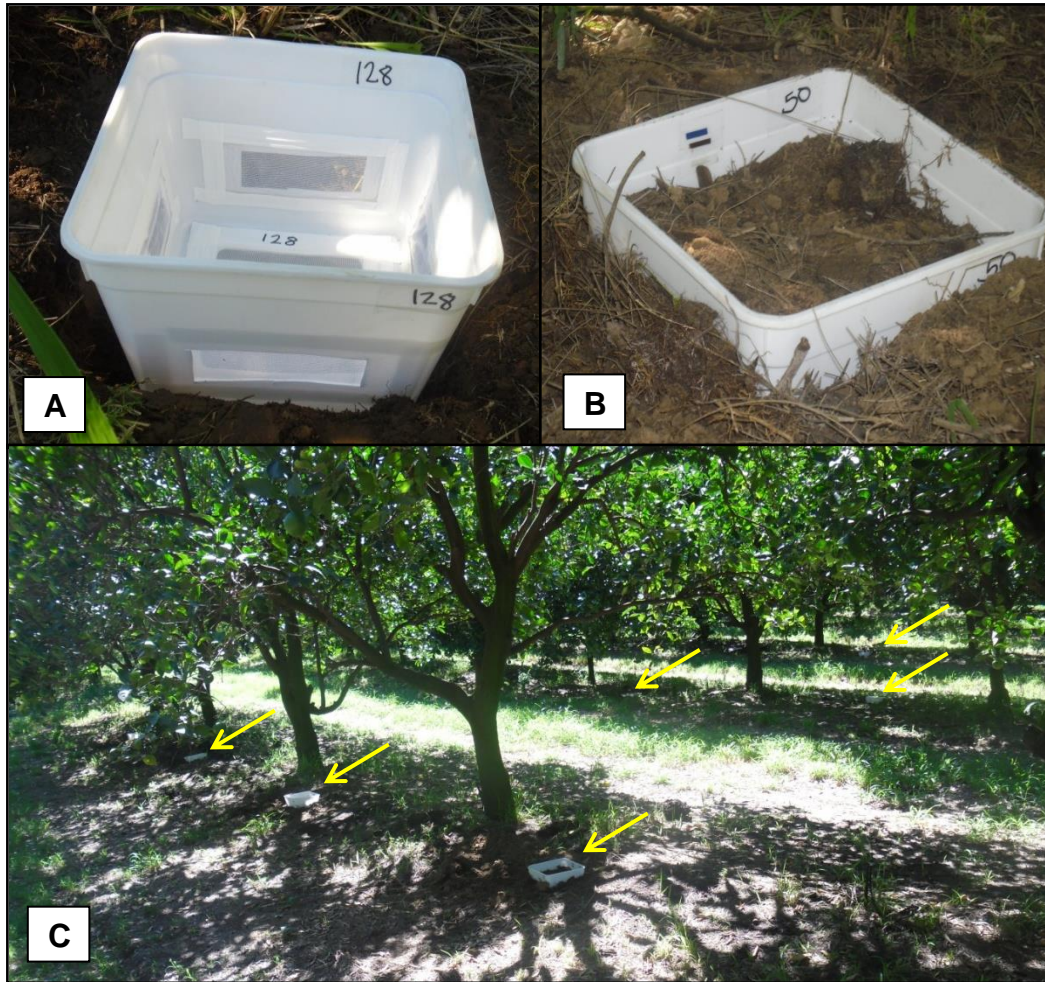


Figure 2.1: 5 L cages with mesh inserts were buried within the upper soil surface and filled with sand (A and B). Cages were buried underneath the canopy of citrus trees approximately 1 m from an irrigation sprinkler (C). Cages are indicated by the yellow arrows.

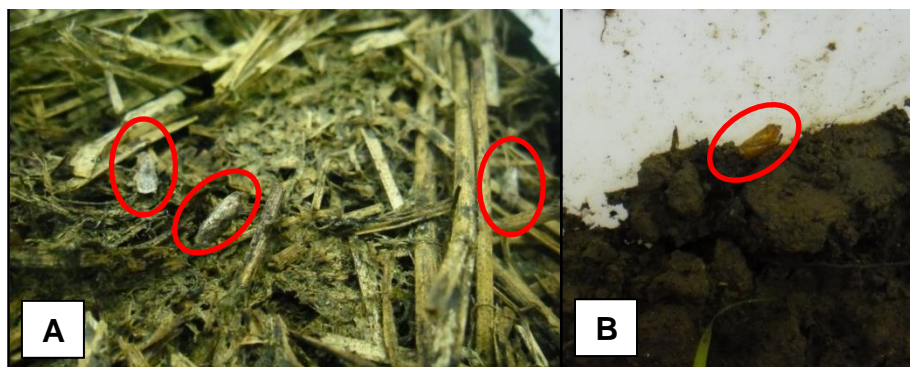


Figure 2.2: Eclosed FCM adults (A) and pupal casings (B) as seen in cages during counting. Both the number of adults and pupal casings within each cage were counted.

2.3 RESULTS

2.3.1. Fungal efficacy

Eclosion percentage: In general, the median number of FCM adults which eclosed was low (maximum = 38.30%) and was greatest in the control treatments (Figure 2.3). Significant differences amongst treatments were found ($H_{15, 128} = 61.20$, $P < 0.0001$). Eclosion percentage was significantly lower in both the Ma2 and Bb1 treatments applied at the highest (2×10^{14} spores/ha) rate compared to Ma1 applied at the lowest (0.5×10^{14} spores/ha) and intermediate rate (1×10^{14} spores/ha), Broadband[®] and all the controls. A significant difference was also found between the untreated control and Ma1 applied at the highest rate and Ma2 applied at the intermediate rate. These results also indicate that as the fungal dose increased, eclosion percentage decreased for Ma2 and Bb1 (Figure 2.3). Fewer adult FCM eclosed from cages treated with all fungi at the highest rate and Ma2 and Bb1 at the intermediate rate, than cages to which Broadband[®] was applied.

Corrected mortality: As natural mortality was high ($> 50\%$), likely the result of predation by ants or spiders which were observed in cages, Abbott's formula was used to determine the corrected mortality of FCM fifth instars within each cage relative to the untreated control (Abbott 1925). Median mortality percentage varied significantly ($H_{12, 104} = 46.31$, $P < 0.0001$) (Figure 2.4). Median mortality was calculated to be greatest in all treatments where fungus was applied at the highest rate; 90%, 90% and 95% for isolate Ma1, Ma2 and Bb1 respectively. Lowest median mortality (5%) was recorded for cages treated with Broadband[®]. Significant differences were only found between the median corrected mortalities of Bb1 and Ma2 applied at the highest rate compared to Ma1 applied at the lowest and intermediate rate and Broadband[®]. Ma2 and Bb1 both exhibited dose-dependent relationships. Mulch cages treated with either Ma2 or Bb1 obtained median corrected mortalities of approximately 25% less than that applied at the equivalent rate in the absence of mulch. However, these differences were not significant. In contrast, a higher median corrected mortality was recorded for Ma1 in the mulch treatment compared to the non-mulch treatment (Figure 2.4). Again, this was not statistically significant. With the exception of the highest rate, Abbott-corrected mortality was higher in cages treated with Ma2 and Bb1 than Ma1.

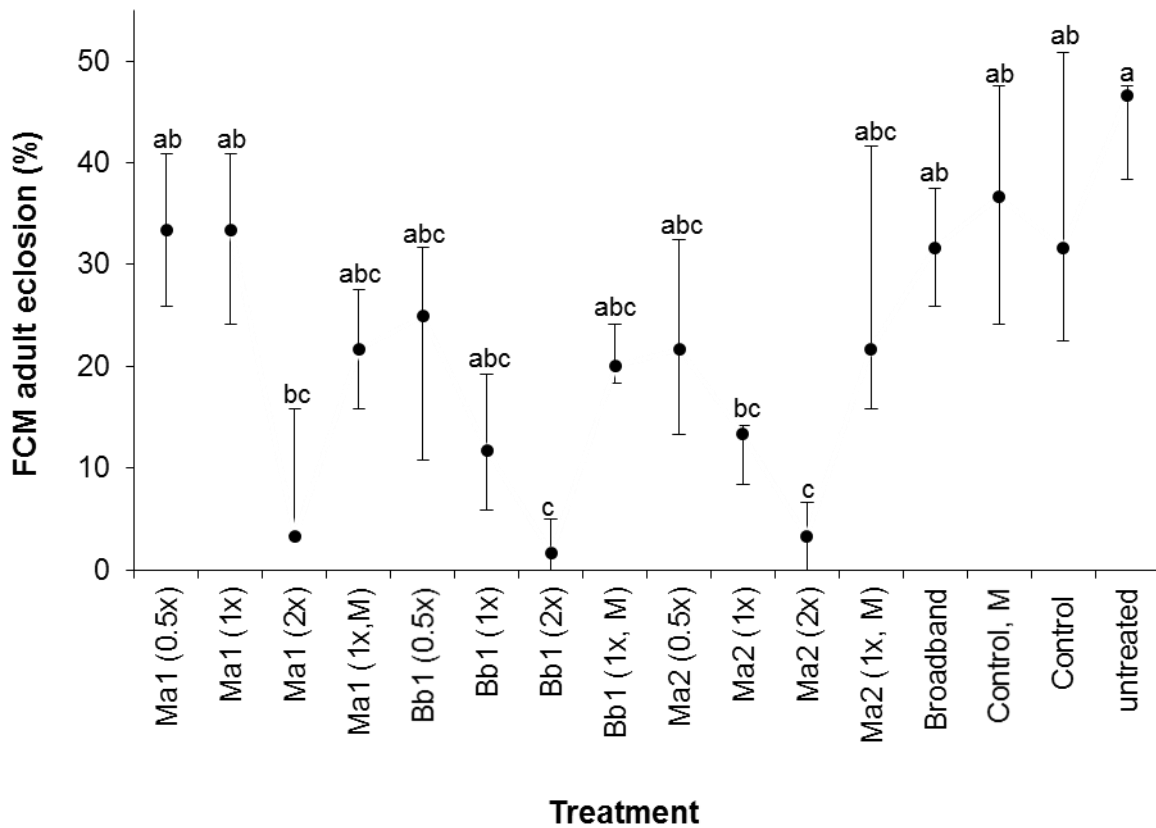


Figure 2.3: Median percentage, of eight replicates, of eclosed FCM adults recorded for each treatment. Vertical bars represent the interquartile range. Different letters indicate a significant difference between treatments (multiple comparison of mean ranks, $P < 0.05$). 0.5×, 1× and 2× refer to application rates equivalent to 0.5×10^{14} , 1×10^{14} and 2×10^{14} spores/ha, respectively. Treatments to which mulch was applied are represented by the letter M.

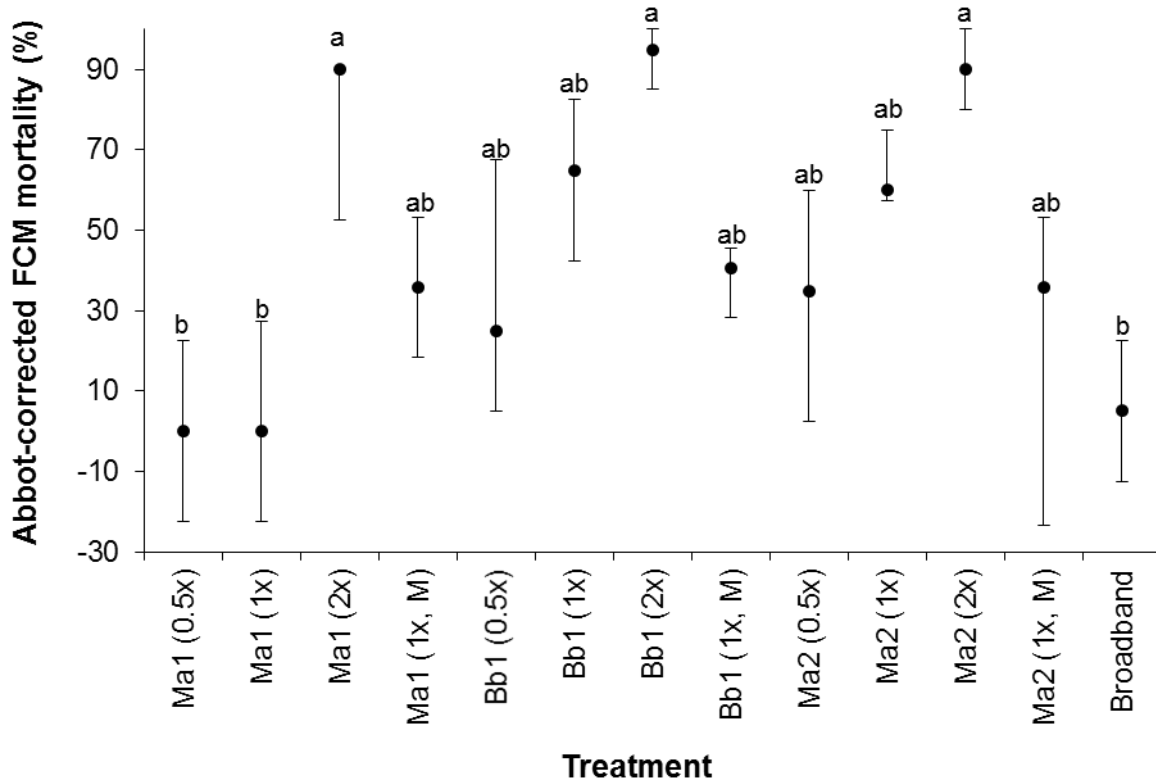


Figure 2.4: Median percentage, of eight replicates, of Abbott-corrected FCM mortality calculated for each treatment to which fungus was applied. Vertical bars denote the interquartile range. Significant letters indicate significantly different results (multiple comparison of mean ranks, $P < 0.05$). 0.5x, 1x and 2x refer to application rates equivalent to 0.5×10^{14} , 1×10^{14} and 2×10^{14} spores/ha, respectively. Treatments to which mulch was applied are represented by the letter M.

2.3.2 Fungal concentration in cages

CFU analysis was only conducted upon completion of the trial. For all isolates, an increase in the number of CFU/g of dry soil is evident with an increase in the fungal application rate. Mulch treatments indicated that median soil CFU counts were similar to application at the lowest rate for each isolate whilst median mulch CFU counts were slightly higher, although highly variable, especially for isolates Ma2 and Bb1 (Figure 2.5). CFUs/g of dry sample were found to be significantly different amongst treatments ($H_{15, 128} = 80.08$, $P < 0.0001$).

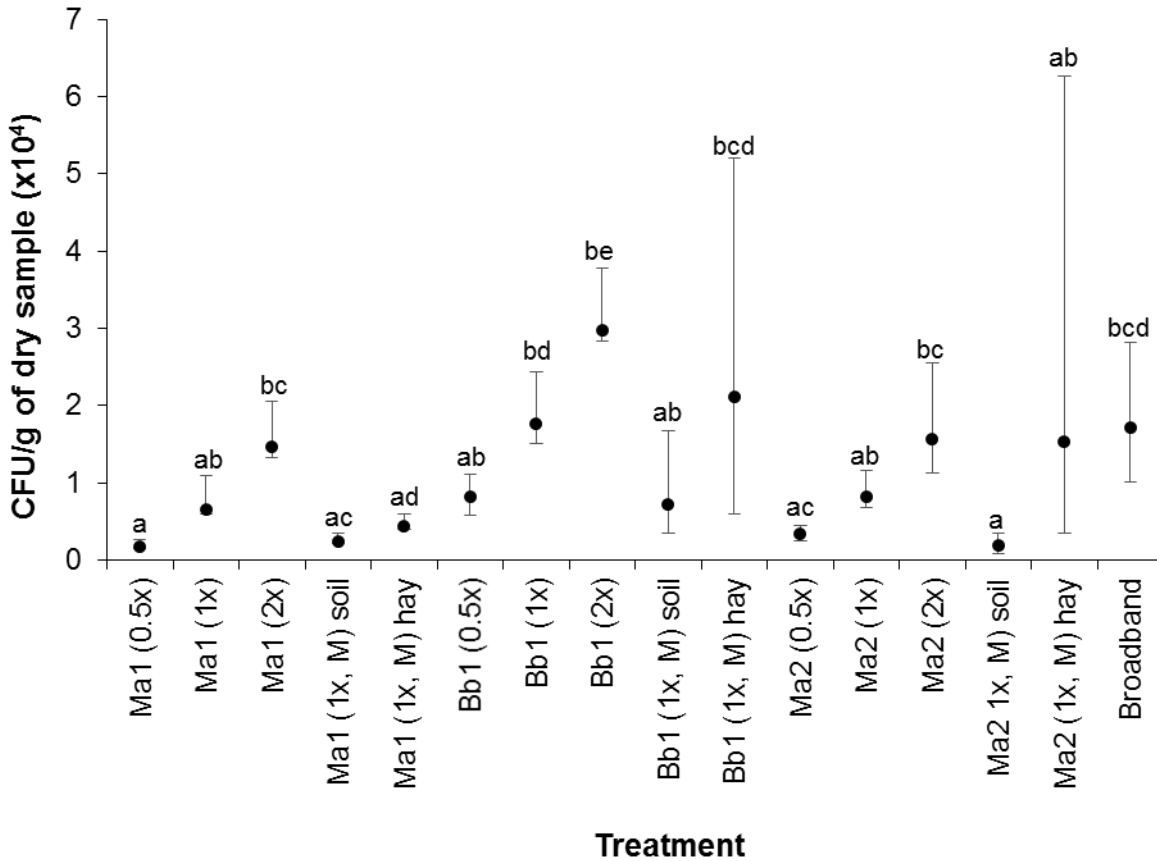


Figure 2.5: Median CFUs/g of dry sample, of eight replicates, determined within the soil, or mulch, of each treatment. Vertical bars denote the interquartile range. Different letters indicate significantly different results (multiple comparison of mean ranks, $P < 0.05$). 0.5 \times , 1 \times and 2 \times refer to application rates equivalent to 0.5×10^{14} , 1×10^{14} and 2×10^{14} spores/ha, respectively. Treatments to which mulch was applied are represented by the letter M.

2.4 DISCUSSION

The inconsistency between laboratory-based trials and field trials is not uncommon and is largely attributed to the effects of environmental variables (Inglis *et al.* 1997; Foster *et al.* 2011). Under laboratory conditions, isolates Ma1, Ma2 and Bb1 are able to cause mortality in fifth instar FCM of 59%, 44% and 65% respectively at 1×10^6 conidia/ml (Coombes *et al.* 2015), and above 80% at 1×10^7 conidia/ml (Goble *et al.* 2011). Abbott-corrected mortality of FCM fifth instars exposed

to fungus applied at the intermediate rate was 0%, 60% and 65% for isolates Ma1, Ma2 and Bb1 respectively. Although not directly comparable with laboratory bioassays due to a difference in concentration used, these results suggest, that at an appropriate dose, isolates Ma2 and Bb1 can reduce FCM populations under potentially sub-optimal and fluctuating field conditions. As no mortality was observed in cages treated with Ma1, the suitability of this isolate for field application is questioned. The failure of Ma1 to induce mortality was not a result of reduced spore viability or concentration. Spore viability for all isolates was estimated to be above 90% and no significant differences in CFU counts were found. The failure of Ma1 to induce mortality may however only be apparent under the conditions measured in this study. Ekesi *et al.* (2003) evaluated the performance of four *M. anisopliae* isolates in inducing infection in puparia of *C. capitata*. Although mortality was higher in drier soil (-0.1 and -0.01 MPa) than wet soil (-0.0055 and -0.0035 MPa), under the latter conditions, two isolates were significantly more effective. Numerous other studies have reported variability amongst entomopathogenic fungal species and isolates in response to temperature, moisture/humidity, pH, soil texture and organic matter (Lingg & Donaldson 1981; Studdert & Kaya 1990; Sun *et al.* 2003; Padmavathi *et al.* 2003; Devi *et al.* 2005; Jaronski *et al.* 2005; Jaronski *et al.* 2007). Therefore, to conclude definitively that Ma1 is not suitable for field application is premature especially given that statistically, differences amongst treatments were limited due to a high level of variability amongst replicates.

Similar to laboratory bioassays, results were dose-dependent. As such, highest Abbott-corrected mortality was recorded in treatments to which fungus was applied at the highest rate, 2×10^{14} spores/ha. At this rate, FCM mortality was 90%, 95% and 90% for Ma1, Bb1 and Ma2 respectively. This is an impressive level of control, but the feasibility of applying spores at this rate to larger areas is improbable due to the cost of conidial production (Mullock & Chandler 2000). However, application at the lowest rate failed to produce satisfactory results. Thus, application at the intermediate rate, 1×10^{14} spores/ha, appears the most appropriate rate to use for future experimental research. Positively, isolates Bb1 and Ma2 at the intermediate rate, were more effective than Broadband[®] in control the soil-dwelling life stages of FCM. As CFUs/g dry soil, measured at the end of the trial, was similar between both isolates and Broadband[®], the results obtained would suggest the greater virulence of isolates Bb1 and Ma2 compared to *B.*

bassiana R4444, the active ingredient of Broadband, against this particular targeted life stage of FCM. This supports the further development of these fungal isolates, specifically isolates Bb1 and Ma2.

It was interesting to note, the apparent reduction (Bb1 and Ma2) and increase (Ma1) in fungal efficacy of the applied isolates in the presence of mulch even though statistically, results were not significant to those obtained at the same rate, but in the absence of mulch. Mulches are typically used in agriculture to maintain soil moisture, prevent soil erosion and to promote soil productivity and plant growth (Mando & Stroosnjider 1999; Li 2003; Ramakrishna *et al.* 2006). They may also increase biological activity. Brévault *et al.* (2007) found an increase in soil arthropods in plots treated with mulch compared to plots not treated with mulch and Tiquia *et al.* (2002) reported increases in the microbial community of soil in the presence of mulching. For EPF, particularly *M. anisopliae* and *B. bassiana*, this increase may be positive if mulching causes an increase in target or alternate susceptible hosts or provides a more suitable microclimate for infection to proceed. However, an increase in biological activity may also result in an increase in antagonistic effects causing a reduction in efficacy. Biological activity was not measured and therefore whether mulching caused an increase in biological activity, and the consequent results thereof, is unknown.

Mulches may also serve as a substrate on which fungi can sporulate and grow, increasing fungal titre within the applied area, particularly as *M. anisopliae* and *B. bassiana* are facultative saprophytes, a trait which makes them amendable to growth on artificial media (Sahayaraj & Namasivayam 2008; Sandhu *et al.* 2012). If an increase in the fungus was apparent, one would expect the sum of the CFUs/g of the soil and mulch to be higher than that recorded for fungal application at the same rate (1×10^{14} spores/ha). This was not observed. However, for Bb1 and Ma2, approximately 3× and 8× more CFUs respectively were recovered from the mulch rather than the soil. In general most pupal casings were located beneath the mulch layer in the upper soil surface. As the success of EPF is dependent on contact between infective propagule and host (Inglis *et al.* 2001), the lower quantity of fungal spores present in the soil, and therefore the

lower probability of contact, may have contributed to the reduction in fungal efficacy. However, a separation in CFUs between mulch and soil was also apparent for isolate Ma1, although this discrepancy was not as pronounced. Regardless, this questions whether the reduction in mortality as a result of mulching can be solely attributed to the higher retention of fungal spores within the mulch. As differences amongst mulch treatments and non-mulch treatments at the same rate were not statistically different, mulching may have had no impact at all. Any observed increases or decreases may merely have been attributed to the high variability of the data set. Nevertheless, it is well known that EPF can be adversely impacted by agricultural amendments and practices and thus, if EPF are to be applied to citrus orchards in which mulch is used, compatibility should be established (Magara *et al.* 2003; Klingen & Haukeland 2006).

In summary, three outcomes of this trial were apparent: (1) All three isolates can cause mortality of FCM soil-dwelling life stages in the field, but (2) Ma2 and Bb1 are perhaps better suited for use in the field, although this warrants further assessment and (3) mulch may have an impact on fungal efficacy, but given the variability amongst replicates, this is unclear.

Chapter 3

Soil application of EPF in conventional citrus orchards: efficacy against FCM

3.1 INTRODUCTION

The results of the semi-field efficacy of isolates Ma1, Ma2 and Bb1 trials reported in Chapter 2 suggested that all three isolates are capable of reducing the percentage of eclosed FCM following infection in the soil environment. These trials were conducted using laboratory-reared FCM introduced into cages immediately following fungal application. As such, contact between the infective propagules and target life stage was ensured and may have resulted in an over estimate of the true efficacy of these isolates. Therefore, the field efficacy of these isolates when applied more realistically to larger areas targeting the native FCM population over a longer time period, was unknown. In addition, environmental factors such as temperature, moisture, agrochemicals and soil biota will vary amongst orchards. These factors have the potential to impede the efficacy of fungal entomopathogens and thus limit their use as microbial control agents (Inglis *et al.* 2001; Klingen & Haukeland 2006). A review of these factors in both the foliar and soil environment is presented by Jaronski (2010) and those pertaining to the soil environment – temperature, moisture, soil amendments and soil texture and biota – are broadly discussed in Chapter 1, section 1.3.3.

Despite the existence of potentially limiting factors, the control of pest species using fungal entomopathogens is a practice employed successfully worldwide as both foliar and soil applications (Inglis *et al.* 2001; Shah & Pell 2003). More than 65% of all commercially available products are based on the species *B. bassiana* and *M. anisopliae*, and are used to control pest species distributed across ten insect orders: Hemiptera, Coleoptera and Lepidoptera being the most common (Faria & Wraight 2007). The good field efficacy of *M. anisopliae* and *B. bassiana*

has been demonstrated on numerous occasions. As examples, in mango orchards, the application of *M. anisopliae* to the soil at a rate of 100 kg fungus colonized substrate/ha (= approximately 1×10^{13} spores/ha) against *Bactrocera dorsalis* Drew, Tsuruta and White (Diptera: Tephritidae) resulted in less infested fruit in treatment blocks compared to control blocks (Ekesi *et al.* 2011) and in coffee plantations, *B. bassiana* has been reported to reduce seed infestation by the coffee berry borer, *Hypothenemus hampei* Ferrari (Coleoptera: Curculionidae), by up to 50% when applied at 1×10^9 spores/tree base (Vera *et al.* 2011). Numerous other examples exist, highlighting the efficacy of EPF in insect control. This includes the control of desert locusts in Africa (Lomer *et al.* 2001), spittle bugs in South America (Li *et al.* 2010) and cockchafers in Europe and Australia (Rath *et al.* 1995; Enkerli *et al.* 2004).

Therefore to determine whether isolates Ma1, Ma2 and Bb1 could be used successfully to reduce native FCM populations when applied on a larger scale during a citrus growing season, five field trials were conducted and were aimed at evaluating the timing of application (pre- and post-peak larval descent into the soil) and the impact that environmental parameters may have on the efficacy of the applied fungal isolates.

3.2 MATERIALS AND METHODS

3.2.1 Fungal isolates

The entomopathogenic fungal isolates whose semi-field efficacy was determined in Chapter 2, were the same isolates used in this study – Ma1, Ma2 and Bb1. Details regarding the isolation, species identity and laboratory-virulence of these isolates were presented in the previous chapter (Table 2.1). Fungi were obtained as dry spores, bulked up on rice granules by Agrauxine (Loches, France) and were stored in air-tight containers in a fridge until use.

3.2.2 Field sites

Five field trials were conducted within the Sunday's River Valley citrus producing region (Eastern Cape Province, South Africa). Two of these sites were located on the same farm, Atmar (33° 28' S, 25° 31' E), referred to hereafter as Atmar 1 and Atmar 2. The remaining three sites were located at the farms Oranjelus (33° 24' S, 25° 22' E), Marwell (33°30' S, 25° 40' E) and Stenhope (33°28' S, 25° 35' E) (Figure 3.1). Trials at Atmar were conducted in 16 and 17 year old Palmer Navel orange orchard blocks under micro-sprinkler irrigation (Atmar 1) and a 15 year old Lane Late Navel orchard block under drip irrigation (Atmar 2). Trials at Oranjelus, Marwell and Stenhope were conducted in 26 to 28 year old McClean Navel, Newhall Navel and 16 year old Palmer Navel orange blocks, respectively. All orchards were under micro-sprinkler irrigation.

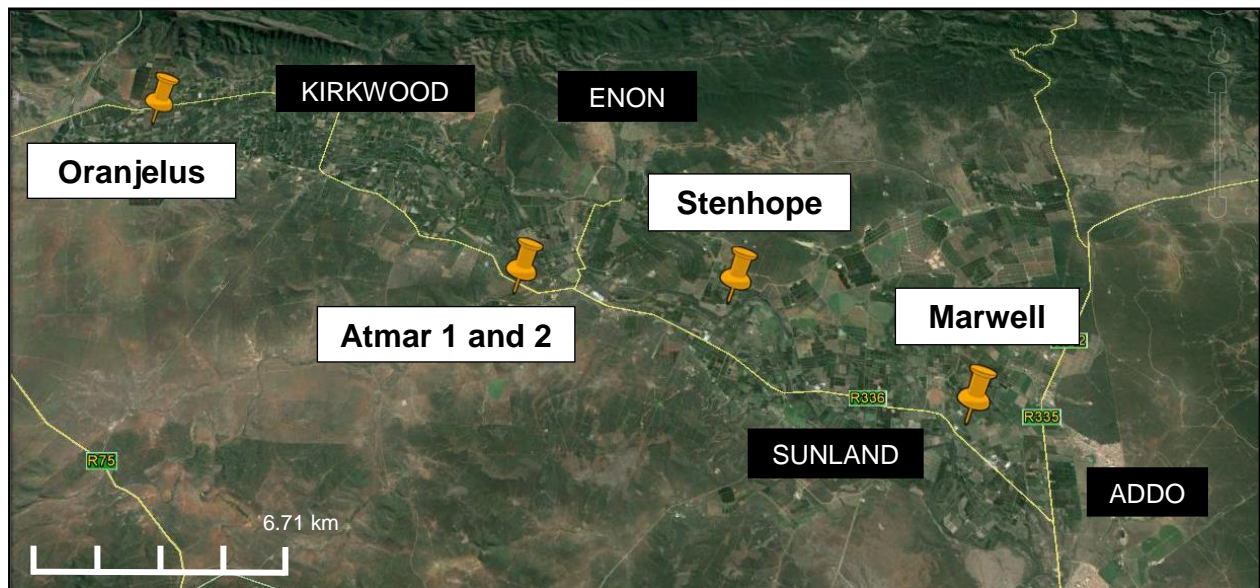


Figure 3.1: The five field trial sites used to assess the efficacy of the applied entomopathogens, located within close proximity to each other, in the Sunday's River Valley, Eastern Cape, South Africa.

For each site, the soil texture (% clay, % silt, % sand) and pH were determined from samples sent to SGS Laboratories (Cape Town, South Africa) for analysis (Table 3.1). Only one sample

was sent for each block used in which treatments were carried out. In some cases, more than one treatment occupied the same orchard block and therefore may be represented by the same sample. Soil moisture at 10 cm depth and soil temperature at the soil surface and 10 cm depth were also monitored throughout the trial using soil probes installed according to manufacturer's instructions (DFM Software Solutions, South Africa). Neither soil temperature nor soil moisture were monitored at the last trial initiated, Stenhope, as all probes were already in use. Average readings and associated minima and maxima, for each site across the entire trial period are presented in Table 3.1. Monthly recorded averages are presented in appendix 1. In some instances, one probe was used to represent the soil temperature and moisture across the entire orchard block due to limited availability. Treatments in the same block may therefore be represented by the same probe.

Trials at Atmar 1 and Marwell were carried out over the 2013/2014 citrus growing season, initiated on 23 October 2013 and 13 March 2014, respectively. Trials at Atmar 2, Oranjelus and Stenhope were carried out the season thereafter, initiated on 23 October 2014, 22 October 2014 and 11 March 2015, respectively. Trials at Atmar 1, Oranjelus, Marwell and Stenhope, were terminated upon fruit harvest (May/June), whilst at Atmar 2, trials were terminated approximately two weeks prior to harvest due to heavy rains around this time which made the orchard difficult to access.

3.2.3 Fungal application

Fungi were not formulated prior to application; they were simply applied as an aqueous suspension. The required mass of dry spores was weighed and mixed with approximately 5 L of water supplemented with 25 ml Break-thru[®]S240 (a.i. polyether modified trisiloxane) (Evonik Industries, South Africa) and shaken vigorously, by hand, to ensure complete suspension before dilution with a further approximate 800 L of water, which had already been added to the spray tank(s). Fungi were applied to the surface underneath the canopy of the citrus trees (Figure 3.2). Application occurred similarly at all sites except Stenhope.

At Atmar 1, fungi were applied with the aid of the grower's motorized spray machine with single 1000 L tank capacity and associated hand held spray guns pulled by a tractor at a slow constant speed of approximately 2.2 km/h. Pressure was set at 20 bars and 2 mm nozzles were used in the guns. At Atmar 2, Marwell and Oranjelus, application occurred with the aid of a CRI-owned Janisch spray machine with a 250 cc Honda motor and two 500 L capacity tanks and associated hand-held spray guns pulled by a motor vehicle at a slow constant speed. Pressure and nozzle size were as described above. At Stenhope, the fungus was applied via the micro-sprinkler irrigation system. The 5 L suspension concentrate was added to the fertigation tank at the pump house and mixed. It took approximately 30 min for the fertigation tank to empty. Once applied, the orchard was irrigated for a further hour to ensure that all the fungus had passed through the sprinkler system. This also provided high moisture conditions to promote fungal germination and infection. At all sites, application occurred only once the target area was shaded (late afternoon into the evening) to prevent any potential inhibitory effects UV radiation may have on spore viability and when wind was minimal to facilitate even application. Control blocks were not treated and the mixing tank was rinsed between sprays to prevent cross-contamination.



Figure 3.2: Fungi were applied as an aqueous suspension using a spray machine and associated hand-held spray guns to the soil surface underneath the canopy of the citrus trees.

Table 3.1: Soil physical properties determined for each site/treatment block.

Field site (and cultivar ¹)	Treatment plot ²	³ Soil texture class	% clay	% silt	% sand	² Soil pH	⁴ Soil temperature (surface) (°C)	⁴ Soil moisture (10 cm depth) (%)
Atmar 1 (Palmer) 23 Oct 2013 – 12 Jun 2014	Ma1 and Bb1	sandy loam	16	18	66	7.5	Ma1: 19.38 (13.41-23.39) Bb1: 20.09 (14.16-23.79)	Ma1: 41.24 (36.41-51.70) Bb1: 25.99 (20.96-33.11)
	Ma2 and Control 2	sandy loam	14	10	76	7.8	19.67 (13.54-24.17)	31.66 (24.83-44.54)
	Control 1	sandy loam	14	10	76	7.7	19.31 (13.44-23.31)	43.64 (33.69-53.54)
Atmar 2 (Lane late) 23 Oct 2014 – 21 May 2015	Bb1, Ma2 and control	sandy loam	16	24	60	7.5	Bb1: 20.22 (16.44-23.23) Ma2: 19.37 (15.50-22.20)	Bb1: 8.33 (6.80-11.26) Ma2: 12.21 (10.70-14.31)
Oranjelus (McClean) 22 Oct 2014 – 21 May 2015	Ma2: 10 ¹² , 10 ¹³ and control	sandy loam	12	16	72	7.8	20.31 (16.41-22.82)	57.23 (53.91-60.20)
	Ma2: 10 ¹⁴	SCL ⁵	20	30	50	7.7	19.60 (15.91-22.41)	42.44 (36.68-49.70)
	Bb1: 10 ¹² and control	sandy loam	18	26	56	7.9	19.72 (15.79-22.38)	57.05 (53.27-58.92)
	Bb1: 10 ¹³ and 10 ¹⁴	SCL	22	22	56	7.8	19.20 (15.29-21.77)	58.24 (49.04-65.03)
Marwell (Newhall) 13 Mar 2014 – 8 May 2014	10 ¹² and 10 ¹⁴	sandy loam	26	16	58	7.6	16.82 (13.30-20.60)	56.75 (42.10-69.51)
	10 ¹³ and control	sandy loam	20	16	64	7.7	17.57 (13.50-22.60)	49.93 (38.24-67.76)
Stenhope (Palmer) 11 Mar 2015 – 21 May 2015	Bb1	Sand	4	6	90	7.2	-	-

¹all cultivars are of the navel orange variety. Details were provided by the growers

²10¹², 10¹³ and 10¹⁴ represent the quantity of spores applied per hectare

³Soil textural class and pH was analysed by SGS Laboratories. If treatment blocks fell in the same orchard block, only one soil sample was analysed

⁴Soil temperature and moisture was recorded by DFM soil probes installed according to the manufacturer's instructions (DFM Software Solutions, Paarl, Cape Town). Average readings as well as the minimum and maximum readings across the trial period are presented in the table. Monthly averages for both soil temperature and moisture can be found in Appendix 1. No records could be taken from Stenhope as no probes were available during the time this trial was conducted.

⁵SCL represents soil textural class, sandy (S) clay (C) loam (L)

3.2.4 Treatments

Atmar 1, Atmar 2 and Oranjelus can be considered early season sprays as fungal application at these sites occurred before the onset of fruit drop and hence before peak larval descent into the soil. At Atmar 1, all three isolates (Ma1, Ma2 and Bb1) were applied at the experimental rate of 1×10^{14} spores/ha. Each isolate was applied to an area of approximately 1 ha (\pm 560 citrus trees – 14 rows \times 40 trees). Two control blocks were included. Control block 1 (C1) was the same size as the treatment blocks, but located across the road (Figure 3.3A). Thus, control block 2 (C2) was included due to its closer proximity to all fungus-treated blocks. C2 was smaller in comparison covering an area of only 0.3 ha (14 rows \times 12 trees). At Atmar 2, only isolates Ma2 and Bb1 were applied at the same rate used at Atmar 1, 1×10^{14} spores/ha. Ma1 was excluded due to space limitation, poorer performance in cage trials compared to both Ma2 and Bb1 and its similar performance to Ma2 at Atmar 1 the season before. Both isolates were applied to an area of approximately 1 ha (14 rows \times 40 trees). A slightly smaller control block (8 rows \times 40 trees) was included within the same orchard block that both fungal treatments took place (Figure 3.3A). At Oranjelus, again only isolates Ma2 and Bb1 were applied to areas of approximately 1 ha. Both isolates were applied at three different rates: 1×10^{14} , 1×10^{13} and 1×10^{12} spores/ha (Figure 3.3B).

Marwell and Stenhope can be considered late treatment sprays as fungal application at these sites occurred after the onset of fruit drop, approximately three months before fruit harvest. As one cause of fruit drop is FCM infestation, it is likely that fungal application at these two sites occurred when a high proportion of FCM were already present in the soil. At Marwell, only isolate Bb1 was applied. This isolate was chosen as it has been determined to be the most laboratory-virulent isolate (Coombes *et al.* 2015) and was performing well at Atmar 1 at the time this trial was initiated. Bb1 was applied at three different rates: 1×10^{12} , 1×10^{13} and 1×10^{14} spores/ha. Treatment blocks were approximately 0.8 ha in area (between 12 – 15 rows, 49 trees per row). A similar sized control block was included (Figure 3.4A). At Stenhope, only isolate Bb1 was applied at a rate of 1×10^{14} spores/ha to an area of approximately 1.1 ha. Bb1 was applied at the highest rate to further support its efficacy as a late treatment spray. A control was included (Figure 3.4B).



Figure 3.3: Location of treatment blocks and control blocks at early application sites (A) Atmar 1 and 2 and (B) Oranjelus. No treatment was applied to the controls and at Atmar, fungi were only applied at one rate, 1×10^{14} spores/ha. All application rates presented pertain to the quantity of spores applied per hectare.



Figure 3.4: Location of treatment and control blocks at late application sites (A) Marwell and (B) Stenhope. No treatment was applied to the controls and at Stenhope, Bb1 was only applied at 1×10^{14} spores/ha. All application rates presented pertain to the quantity of spores applied per hectare.

At all sites, the presence of the applied fungus was confirmed in the soil. Species identity was verified morphologically. Neither *B. bassiana* nor *M. anisopliae* were found in control plots. For Atmar 1, Atmar 2 and Oranjelus, fungal persistence was monitored on a monthly basis as these trials were designed to not only assess fungal efficacy, but also fungal persistence. The sampling procedure and results thereof are presented in the following chapter when fungi were applied only at 1×10^{14} spores/ha (Chapter 4). At Marwell, the presence of isolate Bb1 was confirmed approximately two weeks after the trial was initiated. The sampling procedure used was the same as that outlined in Chapter 4, section 4.2.2. At Stenhope, fungal concentration was recorded one week after application and upon trial completion. The same protocol was used as described in Chapter 4, section 4.2.2 with the exception that this site was divided into three equal quadrats, not eight. Marwell and Stenhope were not designed to assess fungal persistence due to their shorter duration. However, it was important to confirm the presence of the applied isolates in the soil so that any reductions in fruit drop or FCM infestation at these sites could be attributed to the addition of the fungus to the soil.

3.2.5 Monitoring fungal efficacy

For all field trials, the efficacy of the applied isolate was monitored via fruit drop surveys to determine the percentage FCM infestation within each treatment block (Moore 2012). One week prior to the initiation of these trials (approximately 1 to 2 months post application), all

the fruit from underneath 12 centrally located data trees per treatment block (10 for Stenhope), were removed. This included the control blocks. Thereafter, any dropped fruit underneath these trees were collected weekly and dissected to determine whether the cause of fruit drop was a result of FCM infestation. This was continued until the trial was completed.

At Atmar 1, Atmar 2 and Oranjelus, average weekly fruit drop and the number of FCM infested fruit per 12 trees was calculated 15 weeks after treatment (WAT) and upon trial completion, 30 WAT. Any statistical differences amongst treatments at each site, at either 15 or 30 WAT, were determined using one-way analysis of variance (ANOVA) coupled with a Tukey's HSD post-hoc test. Statistical analysis was not conducted on results obtained from Oranjelus. At Marwell and Stenhope, average weekly fruit drop and the number of FCM infested fruit per 12 trees (Marwell) and 10 trees (Stenhope) was calculated once the trial was completed, 8 WAT and 10 WAT, respectively. Again, ANOVA and Tukey's HSD post-hoc test were used to determine any statistical difference amongst treatments at Marwell and a Student's t-test at Stenhope as only two treatments were been compared at this site. All statistics were conducted in Statistica version 10 (StatSoft Inc., 1984-2011) at the alpha level of significance of 0.05.

Fruit drop survey data were also used to calculate the percentage reduction in fruit drop or infestation within each treatment block relative to the control using the calculation presented below. For Atmar 1 and 2 this was calculated 15 and 30 WAT; for Marwell and Stenhope, 8 and 10 WAT (end of the trial), respectively. This was not done for data collected from Oranjelus.

$$\text{Percentage reduction (\%)} = 100 \times \left(\frac{\text{fruit}_{\text{control}} - \text{fruit}_{\text{treatment}}}{\text{fruit}_{\text{control}}} \right)$$

Where $\text{fruit}_{\text{control}}$ and $\text{fruit}_{\text{treatment}}$ refers to the number of dropped fruit found to be infested with FCM over the course of the trial period in the control and treatment blocks, respectively. It should be noted that the significance of differences in total fruit drop amongst treatments in this study is very limited as fruit drop is a consequence of numerous factors, not only FCM infestation (Moore 2012). The only manner in which EPF can have an impact on fruit drop is

through the control of FCM. As such, the data presented on FCM fruit infestation is most relevant (*S.D. Moore, pers. comm.).

FCM population activity was monitored within each treatment block, at each site, with the aid of centrally hung yellow delta traps coupled with a Chempac FCM pheromone lure, which attracted male moths. Lures were changed every three months as recommended. Average moths caught per week during the entire trial period at each site as well as monthly average trap catches at each site is reported in Appendix 2. Differentiation was made between wild and sterile males. Due to the diet on which sterile moths are reared, a bright pink colour is visible when squashed (S.D. Moore, pers. comm.). The presence of sterile males in orchards is a result of an employed control option, sterile insect technique (SIT). A brief introduction to this control measure is presented in Chapter 1 (section 1.2.6.5).

3.3 RESULTS

3.3.1 Early application trials (Atmar 1, Atmar 2 and Oranjelus)

Fruit drop and the number of FCM infested fruit were higher at Atmar 1 than Atmar 2. No significant reductions in fruit drop were recorded at Atmar 1 ($F_{9, 135} = 1.82$, $P = 0.07$), although Bb1 considerably reduced fruit drop throughout the trial period by approximately 75% (Figure 3.5). Bb1 also maintained a high level of FCM suppression throughout the trial period reducing FCM infestation significantly by more than 80% (< 10 FCM infested fruit per week) relative to the control ($F_{9, 135} = 2.62$, $P = 0.008$, Figure 3.6). Ma1 and Ma2 performed similarly and were more effective in reducing fruit drop and FCM infestation when measured 15 weeks after treatment (WAT) measured over a period of eight weeks, than 30 WAT measured over a period of 21 weeks. Neither *M. anisopliae* isolate was capable of reducing FCM infestation by more than 50%. At the end of the trial period (30 WAT), the average number of FCM infested fruit collected weekly was 19 and 21 for Ma1 and Ma2 respectively. This was still lower than both control 1 and control 2 where the average number of FCM infested fruit collected weekly was 25 and 29, respectively.

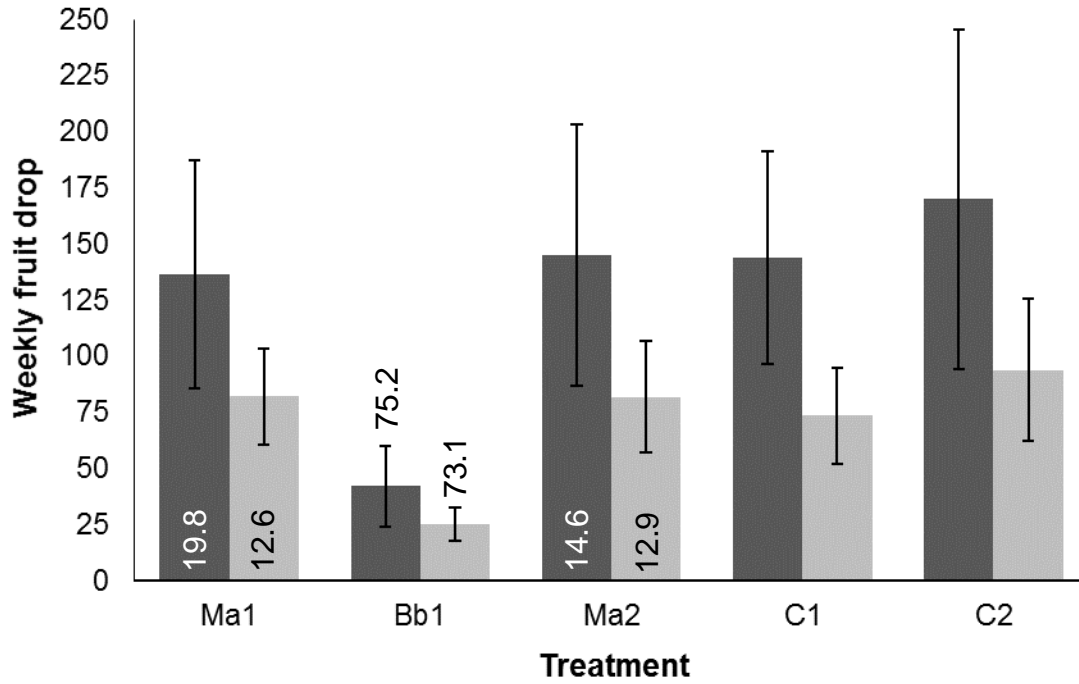


Figure 3.5: Mean (\pm standard error) weekly fruit drop per 12 trees recorded 15 (■) and 30 (▒) weeks after fungal application, over a period of eight and 21 weeks respectively, for the control of FCM at Atmar 1. No significant differences were found (ANOVA, $P > 0.05$). The percentage reduction in fruit drop calculated for each treatment relative to control 2 is presented.

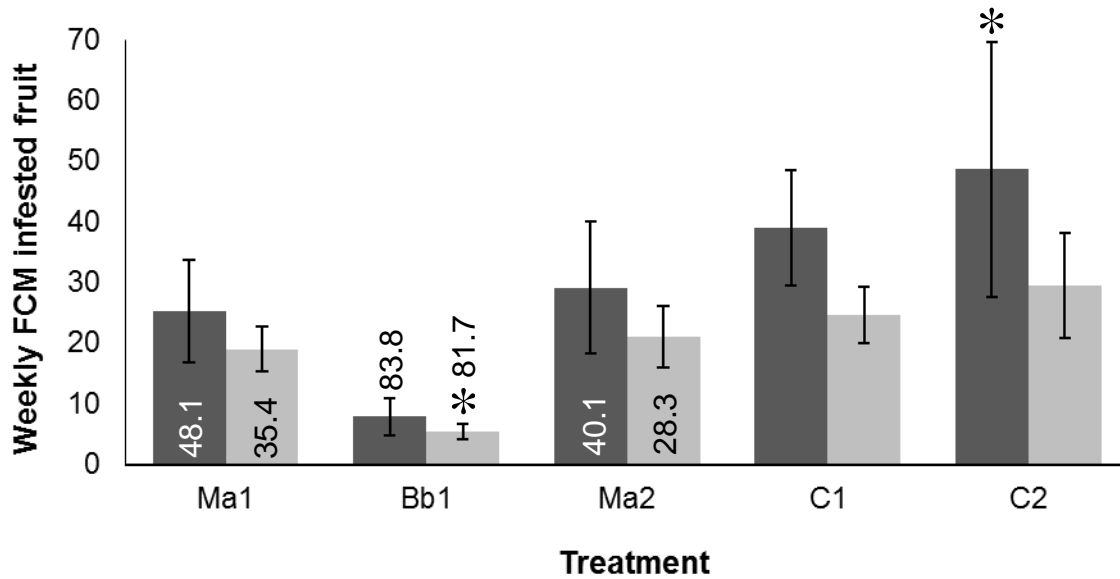


Figure 3.6: Mean (\pm standard error) weekly FCM infested fruit per 12 trees recorded 15 (■) and 30 (▒) weeks after fungal application, over a period of eight and 21 weeks respectively, for the control of FCM at Atmar 1. A significant difference was found (Tukey's HSD test, $P < 0.05$) and indicated by the asterisk. The percentage reduction in FCM infestation calculated for each treatment relative to control 2 is presented.

At Atmar 2, average weekly fruit drop was higher in all treatment blocks including the control 30 WAT (measured over a period of 18 weeks) compared to 15 WAT (measured over a period of five weeks) (Figure 3.7). Significant differences were found ($F_{5, 63} = 4.93$, $P = 0.0007$) with Ma2 at 15 and 30 WAT significantly lower than the control 30 WAT. Interestingly, the percentage reduction in fruit drop relative to the control increased over time and was higher for Ma2 than Bb1. Significantly less FCM infested fruit were recorded in areas to which fungus was applied relative to the control 15 WAT for both isolates and 30 WAT for Ma2 ($F_{5, 63} = 6.19$, $P = 0.0001$). In contrast to Atmar 1, neither isolate maintained continual FCM suppression throughout the trial period and Ma2 was more effective in reducing FCM infestation than Bb1. Reductions of 63.0% and 33.8% were recorded 30 WAT for Ma2 and Bb1, respectively (Figure 3.8).

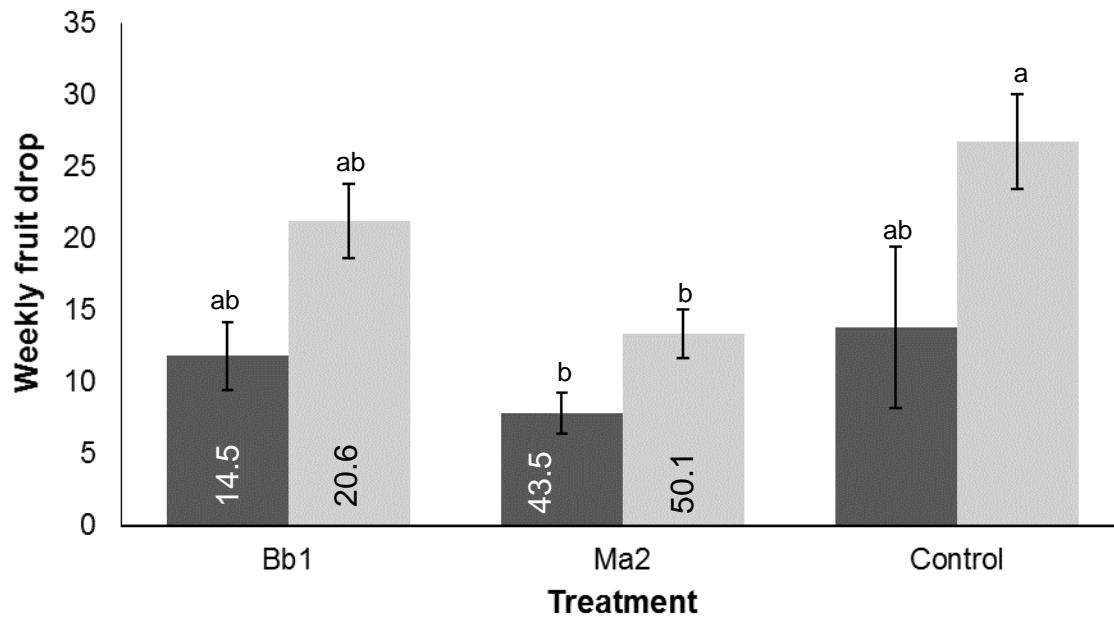


Figure 3.7: Mean (\pm standard error) weekly fruit drop per 12 trees recorded 15 (■) and 30 (▒) weeks after fungal application, over a period of five and 18 weeks respectively, for the control of FCM at Atmar 2. The percentage reduction in fruit drop calculated for each treatment relative to the control is presented. Different letters denote statistically significant results (Tukey's HSD test, $P < 0.05$).

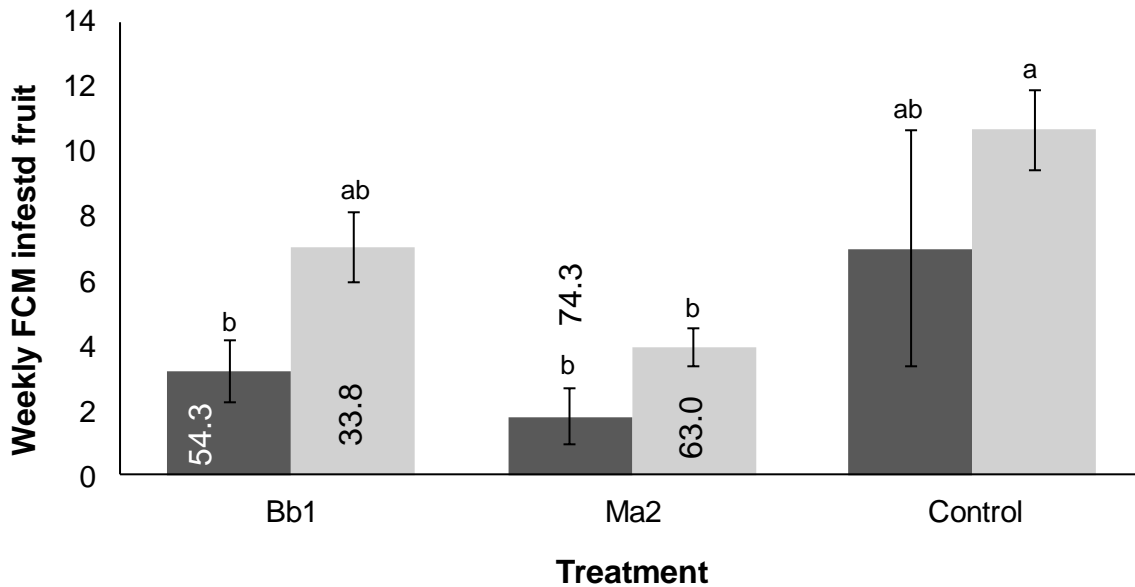


Figure 3.8: Mean (\pm standard error) weekly FCM infested fruit per 12 trees recorded 15 (■) and 30 (■) weeks after fungal application, over a period of five and 18 weeks respectively, for the control of FCM at Atmar 2. The percentage reduction in FCM infestation calculated for each treatment relative to the control is presented. Different letters denote statistically significant results (Tukey's HSD test, $P < 0.05$).

At Oranjelus, fruit drop surveys obtained results which provided no conclusive evidence to suggest the failure or success of the applied fungal isolates, Bb1 and Ma2, at any application rate (Table 3.2). Although fruit drop occurred, varying little amongst treatments, the mean number of FCM infested fruit per 12 trees per week, averaged less than one in all treatment blocks including the controls.

Table 3.2: Mean (\pm standard error) weekly fruit drop and FCM infested fruit per 12 trees recorded 15 and 30 weeks after fungal application of Bb1 and Ma2 (over a period of 5 and 20 weeks, respectively) for the control of FCM at Oranjelus. All application rates presented pertain to the quantity of spores applied per hectare.

Isolate	Treatment	Average weekly fruit drop (\pm SE)		Average weekly FCM infested fruit (\pm SE)	
		15 WAT	30 WAT	15 WAT	30 WAT
Bb1	Control	2.00 (0.55)	4.95 (1.29)	0.00 (0.00)	0.10 (0.07)
Bb1	1×10^{12}	4.40 (0.87)	9.05 (1.93)	0.00 (0.00)	0.40 (0.17)
Bb1	1×10^{13}	1.60 (1.12)	3.60 (0.92)	0.00 (0.00)	0.10 (0.07)
Bb1	1×10^{14}	5.60 (1.36)	6.55 (1.46)	0.00 (0.00)	0.30 (0.13)
Ma2	Control	4.00 (1.22)	7.55 (1.33)	0.00 (0.00)	0.80 (0.30)
Ma2	1×10^{12}	3.80 (1.39)	6.40 (1.19)	0.40 (0.40)	0.80 (0.25)
Ma2	1×10^{13}	3.80 (1.46)	6.20 (1.11)	0.20 (0.20)	0.65 (0.20)
Ma2	1×10^{14}	4.00 (0.55)	9.80 (1.72)	0.40 (0.40)	0.80 (0.22)

3.3.2 Late application trials (Marwell and Stenhope)

In accordance with early application trials, all treatment blocks to which Bb1 was applied, regardless of the application rate, recorded a reduction in both fruit drop (Figures 3.9 and 3.11) and FCM infestation (Figures 3.10 and 3.12) relative to the control. At Marwell, neither mean fruit drop ($F_{3, 20} = 0.20$, $P = 0.90$) nor the mean number of FCM infested fruit ($F_{3, 20} = 0.86$, $P = 0.48$) were significantly different amongst treatments. Bb1 applied at the lowest rate, 1×10^{12} spores/ha, recorded the highest percentage reduction in fruit drop (18.33%), but the lowest reduction in FCM infestation (34.29%) (Figure 3.9 and 3.10). Bb1 applied at the intermediate rate of 1×10^{13} spores/ha, resulted in the greatest reduction in FCM infestation (Figure 3.10). Fungal concentrations recorded from soil samples collected two weeks post-application were calculated to be 4.65×10^2 , 3.37×10^3 and 1.49×10^4 CFUs/g of dry soil in treatment blocks to which fungus was applied at 1×10^{12} , 1×10^{13} and 1×10^{14} spores/ha, respectively.

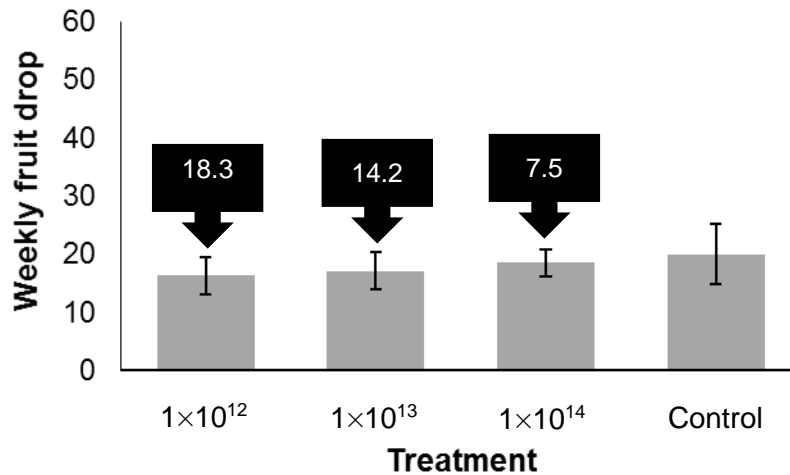


Figure 3.9: Mean (\pm standard error) weekly fruit drop per 12 trees recorded nine weeks after fungal application, over a period of six weeks, for the control of FCM at Marwell. No significant differences were recorded (ANOVA, $P = 0.90$). The percentage reduction in fruit drop calculated for each treatment relative to the control is presented above the bars. All application rates presented pertain to the quantity of spores applied per hectare.

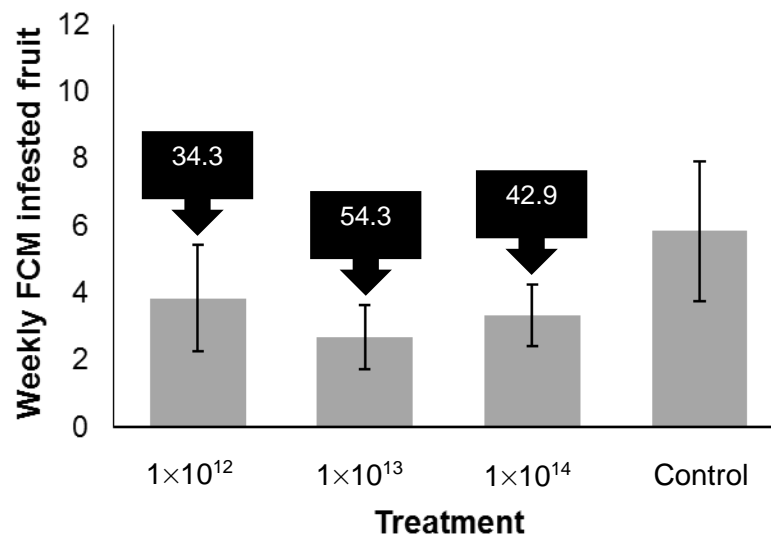


Figure 3.10: Mean (\pm standard error) weekly FCM infested fruit per 12 trees recorded nine weeks after fungal application, over a period of six weeks, for the control of FCM at Marwell. No significant differences were found (ANOVA, $P = 0.48$). The percentage reduction in FCM infestation calculated for each treatment relative to the control is presented above the bars. All application rates presented pertain to the quantity of spores applied per hectare.

At Stenhope, Bb1 reduced both fruit drop and FCM infestation by more than 75%. Statistically, only mean fruit drop was found to be significantly different to the control ($t_{10,6} = -2.72$, $P = 0.02$) (Figure 3.11). The lack of significant difference between Bb1 and the control in mean FCM infested fruit may be a factor of the high weekly variability found in the control (Figure 3.12). An average fungal concentration of 8.64×10^3 and 3.06×10^2 CFUs/g of dry soil was recorded from soil samples collected one week after trial initiation and upon trial completion (six weeks post-application), respectively.

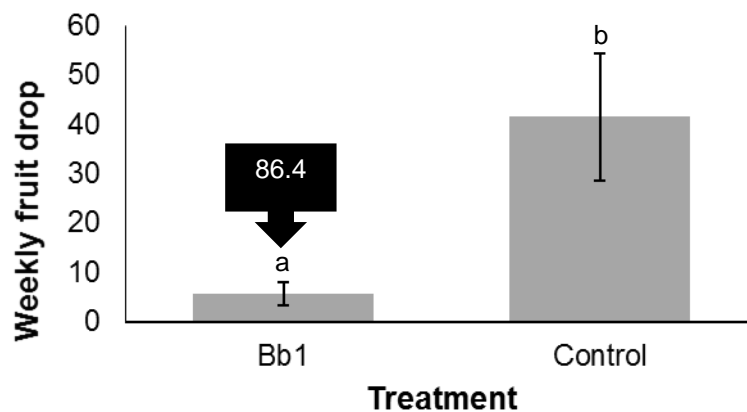


Figure 3.11: Mean (\pm standard error) fruit drop per 10 trees recorded 10 weeks after fungal application, over a period of six weeks, for the control of FCM at Stenhope. The percentage reduction in fruit drop relative to the control is presented above the bar. A statistical significant difference was found between treatments, denoted by the different letters (T-test, $P = 0.02$).

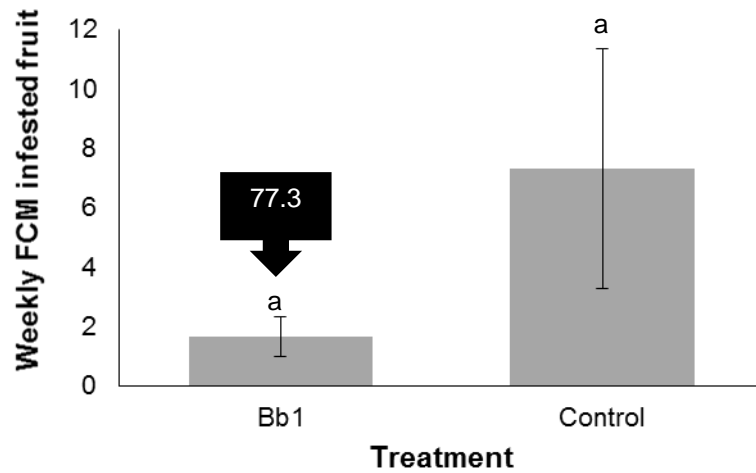


Figure 3.12: Mean (\pm standard error) weekly FCM infested fruit per 10 trees recorded 10 weeks after fungal application, over a period of six weeks, for the control of FCM at Stenhope. No significant difference was found (T-test, $P = 0.20$). The percentage reduction in FCM infestation relative to the control is presented above the bar.

3.4 DISCUSSION

This study is the first report of the performance of selected entomopathogenic fungal isolates applied against the soil-dwelling life stages of FCM under a variety of conditions in conventionally managed citrus orchards (Eastern Cape, South Africa) during the citrus growing season. Positively, the application of these isolates resulted in both a reduction in FCM infestation and fruit drop relative to the control at all field sites, with the exception of Oranjelus. The fact that fungal application not only reduced FCM infestation within the fungus-treated area, but also fruit drop is encouraging; a reduction in fruit drop may have implications on improved yield and profit, particularly where reductions in fruit drop are high. However, the economic implications of this reduction in fruit drop was not quantified and thus requires further attention as this can serve as an added benefit of EPF application.

Trials initiated at Oranjelus produced inconclusive results as FCM infestation within the treatment areas was extremely low, for reasons unknown given that sites were chosen due to their mid to high level of FCM infestation in previous years (S.D. Moore, pers. comm.). On average, less than one FCM infested fruit was found per 12 trees in any fungus-treated or

untreated control blocks. Here, trials were designed to assess the feasibility of using application rates lower than 1×10^{14} spores/ha whilst still maintaining efficacy. As results were inconclusive this is still unknown, but given the results obtained at Marwell, a reduction in the application rate may be possible; fruit drop was lowest when Bb1 was applied at the lowest rate (1×10^{12} spores/ha) whilst the number of FCM infested fruit was lowest in treatment blocks to which Bb1 was applied at the intermediate rate (1×10^{13} spores/ha). The highest experimental rate of 1×10^{14} spores/ha may be considered too high to warrant economically feasible application. This is acknowledged and discussed in the concluding chapter (Chapter 7).

At sites where application was successful (Atmar 1, Atmar 2, Marwell and Stenhope), discrepancies in fungal efficacy were apparent amongst sites and between isolates. The timing of application appeared to have little effect on the efficacy of Bb1 in reducing FCM infestation; a reduction in FCM infestation above 30% was recorded at all sites with fungal efficacy at Atmar 1 (early application spray) similar to Stenhope (late application spray) and fungal efficacy at Atmar 2 (early application spray) similar to Marwell (late application spray). Therefore, provided a susceptible host is present, the efficacy of the applied EPF may be governed more by environmental factors experienced in the area of application rather than whether they are applied before peak larval descent into the soil or after, at least for isolate Bb1. Whether the *M. anisopliae* isolates can reduce FCM infestation when applied closer to fruit harvest, remains to be determined.

Stenhope was the only site which differed substantially in soil texture composition, being mostly sand (90%). Soil texture mediates contact between the infective propagules and target insect (Barbercheck 1992; McCoy *et al.* 1992). It has been suggested that soils with high clay content enhance the abundance of EPF, especially species with small spores such as *B. bassiana* (Studdert *et al.* 1990; Inglis *et al.* 2001; Quesada-Moraga *et al.* 2007). As such, they may improve efficacy. Garrido-Jurado *et al.* (2011a) found no influence of soil texture (including sandy, sandy loam, sandy clay loam and clays soils) on the infectivity of *B. bassiana* or *M. anisopliae* against *C. capitata* puparia. However, Jaronski *et al.* (2005) observed variation in the mortality of third instar sugarbeet root maggot, *Tetanops myopaeformis* von Röder (Diptera: Otitidae), exposed to *M. anisopliae* in five different soil

types. At all field saturations investigated (10, 15 and 30%), mortality was either 98 or 100% in the soil with the highest sand content, whilst in other soil types, mortality varied significantly at different moisture levels. Therefore, whether good performance of Bb1 at Stenhope may solely be attributed to sandy soil is unclear. Stenhope was also the only site to vary in the manner in which the fungus was applied; application occurred via the micro-sprinkler irrigation system, rather than via machine spraying. Whether this facilitated greater contact between the applied infective propagules and pre-pupating FCM is unclear, but warrants further attention. This approach was less labour intensive and more time-effective, but may only be compatible with micro-sprinkler systems, not drip systems due to poorer coverage of the latter.

Results recorded at the two early application sites, Atmar 1 and Atmar 2, were interesting. At Atmar 1, Bb1 was more effective in reducing FCM infestation than Ma2. At Atmar 2, this was reversed: Ma2 was more effective in reducing FCM infestation than Bb1. At these sites, discrepancies between soil temperature, pH, textural class and chemical spray regimes were either not apparent or minimal and therefore unlikely to be the cause of efficacy reversal (Table 4.1, Appendix 1 and 3). Soil moisture within the upper 10 cm soil surface was noticeably different. It is within this area that FCM pupation occurs and hence the area in which individuals will become infected and mycose. The difference in moisture was a result of the type of irrigation system used: micro-sprinkler at Atmar 1, drip at Atmar 2. At Atmar 1, average soil moisture ranged from 20.96% to 33.11% and 24.83% to 44.54% in soils to which Bb1 and Ma2 were applied, respectively. At Atmar 2, average soil moisture ranged from 6.80% to 11.26% and 10.70% to 14.31% for the two fungal isolates, respectively.

The limited efficacy of *B. bassiana* in controlling sawflies, *Hoplocampa testudinea* Klug (Hymenoptera: Tenthredinidae), in apple orchards under drip irrigation has been reported (Swiergiel *et al.* 2015). The authors suggest the conditions were too dry to promote infection and sporulation, but also state that failed application may have been influenced by limited contact between the target species and infective propagules. The failure of *B. bassiana* to be effective under dry conditions has also been reported by Tehri *et al.* (2015) and Wraight & Ramos (2015). However, in both studies, dry conditions were associated with temperatures higher than the low to moderate temperatures (average 20°C) recorded in this study. At 20°C,

Kreuger *et al.* (1991) found that soil at 10, 30 or 50% moisture holding capacity had no significant influence on chinch bug, *Blissus leucopterus* Say (Hemiptera: Lygaeidae), infection by *B. bassiana* and found mean mortality to be highest in the driest soil. This was attributed to the formation of cracks which may have facilitated improved contact between the infective propagule and insect. Soil moisture of the driest soil was still higher than the average soil moisture recorded for Bb1 at Atmar 2. Perhaps moisture less than 10% does not promote infection by *B. bassiana*. Ekesi *et al.* (2003) evaluated the efficacy of *M. anisopliae* against *C. capitata* in soil under controlled temperature and moisture. At 20 to 30°C, mortality caused by all four isolates investigated was higher in drier than wetter soils. This would support the better performance of Ma2 under drier conditions reported in this study. The moisture dependency of isolates Bb1 and Ma2 on factors important for infection and persistence (germination, growth and sporulation) has not yet been determined, but is planned. Field results would suggest that wetter soils are more suitable for *B. bassiana* infection, whilst drier soils are more conducive to *M. anisopliae* infection. However, for both species, soil moisture less than 15% may have implications on persistence (discussed in Chapter 4).

The results obtained in this study typically corroborate efficacies recorded elsewhere against other pest species both in the foliar and soil environment. Shi *et al.* (2008) reported relative efficacies of two *M. anisopliae* and two *B. bassiana* isolates when applied to cotton foliage to control the cotton spider mites, *Tetranychus* spp. (Trombidiformes: Tetranychidae), ranging between 52% and 86% 35 days after treatment. Treatment consisted of two applications, 15 days apart, at concentrations of either 1.05×10^{13} and 1.5×10^{13} spores/ha for the *M. anisopliae* and *B. bassiana* isolates, respectively. In this study, relative efficacies of up to 80% were achieved 30 weeks after treatment following single application (Bb1 at Atmar 1), although a higher concentration was applied. However, this high efficacy was lower than that reported by Poprawski *et al.* (1999). *Beauveria bassiana* applied once to the foliage of citrus trees at 5×10^{13} spores/ha against brown citrus aphid, *Toxoptera citricida* Kirkclady (Homoptera: Aphididae), obtained relative control efficacies of approximately 90% for both field trials initiated. However, in contrast to this study, a formulated product was used. This may have improved efficacy.

Ekesi *et al.* (2005) examined the efficacy of three formulations – aqueous, oil/aqueous (50:50) and granular – of *M. anisopliae* applied to soil at rates equivalent to 4×10^{12} spores/ha in reducing the emergence of three fruit fly species (*C. capitata*, *C. fasciventris* and *C. cosyra*) under semi-field conditions. All formulations significantly reduced fly emergence in the field 9-15 days after treatment. Soil samples brought back from the field 183 and 365 days after treatment and artificially infested with *Ceratitis* spp. puparia in the laboratory, continued to suppress fly emergence for all formulations. However, oil/aqueous and granular formulations were more effective than the aqueous suspension. Only soil samples collected one-year post-application that had been treated with the granular formulation and artificially infested as before maintained significant suppression of all three fly species. In this study, no emphasis was placed on formulation prior to application, but preliminary studies have been conducted to determine whether the use of different surfactants may facilitate better application either by promoting a more uniform application or positively influencing fungal viability (Chapter 5).

Continued suppression of FCM infestation in fungus-treated areas throughout the growing season was only achieved for isolate Bb1 at Atmar 1. A reduction in relative control efficacy was observed for areas treated with Ma1 and Ma2 at Atmar 1 and both isolates (Bb1 and Ma2) at Atmar 2 as the trial progressed (15 WAT versus 30 WAT). Perhaps this could have been counteracted by the addition of another spray, even at a lower rate – Shi *et al.* (2008) reported highest relative control efficacies for all applied isolates 5 to 10 days following the second application – or by the use of an alternate formulation e.g. granules which provided the most effective long-term control against soil-dwelling puparia of *Ceratitis* species (Ekesi *et al.* 2005). Interestingly, persistence results indicated that all isolates applied at Atmar 1 increased over time to levels similar to the initial application (Chapter 4). Assuming these spores to be infective towards FCM, reduced suppression throughout the growing season is puzzling and therefore, may not be counteracted by the use of an alternate formulation or additional sprays. If contact between the propagules and target host was maintained throughout the trial period, reduced suppression may be a result of spores no longer being infective. This was not quantified in this study, but future research should incorporate baiting procedures to address this issue. Alternatively, contact may have been limited for Ma1 and Ma2, but not Bb1. Persistence within the upper 5 cm soil surface was monitored. Love (2015) found that FCM tend to not pupate below 1 cm. Therefore although most spores may be

retained within the upper 5 cm soil surface, the occurrence of both *M. anisopliae* isolates in the very upper layers may be less prominent than that of the *B. bassiana* isolate.

In summary, the application of isolates Ma1, Ma2 and Bb1 reduced FCM infestation and fruit drop at all treated sites. FCM infestation and fruit drop were reduced by between 28.3% to 81.7% and 7.5% to 86.4% respectively as calculated at the end of the trials, relative to an untreated control. Positively, this provides evidence and support for the potential use of these isolates against FCM in citrus. More field trials are necessary to confirm the performance of these isolates not only in citrus orchards in the Eastern Cape Province, but also in other citrus producing regions of South Africa. Further trials will provide invaluable information regarding what type of formulation should be investigated, confirm or reject the suggested influence of environmental factors on field efficacy in this study and ultimately allow for a more informed decision as to which isolate is more suited for field use. Investigating a mixture of these isolates may even be warranted. Reddy *et al.* (2014) found that a combination of *M. brunneum* and *B. bassiana* was the most effective treatment tested for the management of the sweet potato weevil, *Cylas formicarius* F. (Coleoptera: Brentidae). Other treatments included the impact of the fungi alone, chemicals alone and each fungal isolate in combination with each chemical.

Equally important to establish is the most cost-effective application rate, the influence of repeated applications on reducing FCM infestation and fruit drop, the effectiveness of fungal application compared to or in combination with other control options and any potential benefit sub-lethal exposure may have on FCM fecundity, longevity and developmental rate given that no application recorded 100% control. An important consideration for these future trials is replication of the same treatments within the same orchard. The current design assessed a variety of factors e.g. application rate, irrigation type and the timing of application. This improved our understanding of these isolates in the field, but limited the conclusions that could be drawn regarding the reproducibility of these applied fungi (design employs only one replicate per treatment). By limiting the number of different treatments per site whilst increasing the number of the same treatments, the reproducibility of these fungi can be investigated. This will provide insight into the degree of variability of a specific isolate under the same, measured environmental conditions.

Nevertheless the good performance of these isolates following a single application in their unformulated state under a variety of field conditions suggests that through optimisation and continued research, the production of a cost-effective consistent product, is possible.

Chapter 4

Field persistence of select EPF in conventional citrus orchard soil

4.1 INTRODUCTION

The environmental persistence of applied entomopathogenic fungal isolates is considered a key element of successful applications (Jackson 1999). Several of the environmental factors impacting fungal efficacy (refer to Chapter 1, section 1.3.3), also affect fungal persistence. Soil temperature, moisture, pH, texture and biological composition are considered key influential factors within the soil environment (Klingen & Haukeland 2006; Scheepmaker & Butt 2010). The influence of these factors on persistence is strongly dependent on the fungal species or isolate under investigation. A study assessing the occurrence of EPF, and hence their ability to persist, in cultivated and natural habitats of Spain, revealed that *B. bassiana* species were more prevalent in soils of moderate organic matter (1 to 3%), pH range between 8 to 8.5 and clay content of between 10 to 20%, whereas *M. anisopliae* was isolated more frequently from soils high in organic matter content (> 3%), pH between 8 to 8.5 or less than 7 and clay content less than 10% (Quesada-Moraga *et al.* 2007). pH and clay content were considered predictive variables of *B. bassiana* occurrence, whilst only organic matter could be used to predict the occurrence of *M. anisopliae*. A study in Finland evaluated the distribution of four EPF, including *B. bassiana* and *M. anisopliae*. No influence of soil type on the presence of any species was found, whilst cultivation strongly limited the presence of *B. bassiana*, but not *M. anisopliae* (Vänninen 1995). Further, Goble *et al.* (2010) found no significant difference between the percentage occurrence of EPF between organic and conventional farms in soils sampled from citrus orchards in South Africa.

Despite these many factors which may reduce fungal persistence, studies have reported long-term persistence of applied fungal entomopathogens. Rath *et al.* (1995) successfully isolated

formulated *M. anisopliae* DAT F-001 from pasture soils four years post-application in the presence of the host, *Adoryphorus couloni* (Burmeister) (Coleoptera: Scarabaeidae). Enkerli *et al.* (2004) isolated *B. brongniartii* strains from all sampled sites to which the formulated fungus was applied 14 years previously. Similarly Mayerhofer *et al.* (2015) successfully isolated the BIPESCO *B. brongniartii* strain from one site 15 years post application and from all sites to which the product had been applied at least once four years prior to sampling.

The semi-field persistence of the three isolates, Ma1, Ma2 and Bb1, of interest in this study, was previously evaluated (Coombes *et al.* 2013). Mesh bags filled with fungal inoculated soil were buried within the upper 5 cm of the soil surface in a citrus orchard. Thereafter, a representative sample was removed and assessed for the presence of fungus every month. Results indicated that although an initial decline in fungal concentration was apparent, all three isolates persisted six months post-trial initiation. The authors however acknowledged that these results were not necessarily reflective of true field persistence given that the impact of soil biota on the persistence of the fungi was largely eliminated through the use of autoclaved soil and mesh bags.

Therefore, the aim of this chapter was to determine the true persistence of these isolates (Ma1, Ma2 and Bb1), in their unformulated state, after application to the soil environment of an operational citrus orchards. The trial was conducted over a period of five months beginning one week post-application.

4.2 MATERIALS AND METHODS

4.2.1 Fungal isolates

The persistence of Ma2 and Bb1 (refer to Chapter 2, Table 2.1 for fungal details), was monitored during field trials conducted at Atmar 1, Atmar 2 and Oranjelus (refer to Chapter 3 for site and application details). The persistence of Ma1 was only monitored during the field trial at Atmar 1 as this isolate was not applied in field trials conducted at Atmar 2 and

Oranjelus. At Atmar 1 and Oranjelus, a micro-sprinkler irrigation system was employed whilst at Atmar 2, a drip irrigation system was used.

4.2.2 Monitoring fungal persistence

Fungal persistence was monitored by collecting soil samples (section 4.2.2.1) from each site beginning one week post fungal application and ceasing five months thereafter. As the field trial at Atmar 1 was conducted across the 2013/2014 citrus growing season, the presence of all three applied isolates was also determined approximately 19 months post application. Soil samples were collected on the last Thursday of every month. A quadrat design was used to ensure adequate sampling of the entire treatment block. For Atmar 1 and Atmar 2, each 1 ha treatment block to which fungus (Ma1, Ma2 or Bb1) was applied was divided into eight equal quadrats (7 rows, 10 trees per row). For Oranjelus the number of quadrats was reduced to two as this was deemed sufficient at the 90% confidence level based on the results obtained in Atmar 1. As the variation in the data set was not known under drip irrigation, the number of quadrats sampled at Atmar 2 remained at eight. Following soil collection, the number of colony forming units (CFUs) was determined for each quadrat (section 4.2.2.2).

4.2.2.1 Soil sampling

Within each quadrat, 20 soil cores (diameter = 7 cm, depth = 5 cm) were taken from underneath the canopy of 20 different, randomly generated, citrus trees using a spade or soil corer. These sub-samples were combined to form one composite sample per quadrat and the collection apparatus was washed in 70% ethanol between quadrats to prevent cross-contamination. In the laboratory, the samples were sieved through a sterile 1 mm mesh sieve to remove any debris and break up soil clumps before analysis occurred. This also aided in ensuring the homogeneity of the sample. If the soil was too wet to pass through the sieve on the day of collection, it was allowed to dry overnight at room temperature. Sieved samples, approximately 1.5 kg wet weight, were stored at 4°C until use. All samples were analysed within two weeks following collection. The moisture content of each sample was determined by drying a pre-weighed quantity (± 40 g) of wet soil in an oven for 24 hours at 100°C. Moisture content was determined according to the equation below and was used to calculate the number of CFUs/g dry soil.

$$\% \text{ moisture} = \left(\frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \right) \times 100$$

4.2.2.2 CFU analysis

From each well mixed sample, 20 g of wet soil was weighed and added to a 250 ml conical flask containing 100 ml sterile 2% saline solution and shaken vigorously by hand to ensure a homogenous suspension for approximately five minutes and repeated. Following 5 s sedimentation, 100 µl of the soil suspension was removed from the surface layer and spread, using aseptic technique, onto each of three SDA plates supplemented with 1 ml 50 mg/L chloramphenicol, 1 ml 50 mg/L rifampicin and 1 ml dodine. Plates were sealed with Parafilm M[®] and incubated at 26±1°C on a 12 h photoperiod. After 10 days, the number of colony forming units was counted and the number CFUs/g of dry soil calculated using the equation given below. Species identity was confirmed based on morphology (Domsch *et al.* 2007).

$$\text{CFUs per g dry soil} = \left(\frac{\text{count}}{\text{sample dry weight}} \right) \times \text{dilution factor}$$

The CFUs/g dry soil for all quadrats within the same month were combined for analysis purposes (n = 24 for Atmar 1 and Atmar 2, n = 6 for Oranjelus). Mean or median monthly differences amongst treatments were analysed via ANOVA followed by Tukey's HSD post-hoc test or Kruskal Wallis followed by multiple comparison of mean ranks respectively, depending on the normality of the data following square root or log transformation. A Mann-Whitney U test was used to compare fungal density one week after application and 19 months post-application (n = 24). All statistical analysis was conducted in Statistica version 10 (StatSoft Inc., 1984-2011) at P < 0.05.

4.3 RESULTS

No *B. bassiana* or *M. anisopliae* colonies were recorded in untreated control plots at any site.

Isolate Bb1 persisted in all sampled areas for five months post-application (Figure 4.1). At Atmar 1 and 2, there were significant differences in the number of CFUs/g dry soil amongst months under both micro-sprinkler ($H_{5, 144} = 69.02$, $P < 0.00001$) and drip ($H_{5, 144} = 70.18$, $p < 0.00001$) irrigation, as well as at Oranjelus ($F_{5, 30} = 17.30$, $P < 0.00001$). At Atmar 1 (=micro-sprinkler), fungal density showed an initial decline, but then increased approximately three months post-application, with the initial density (2.35×10^3 CFUs/g dry soil), not significantly different to the density calculated upon termination of the trial (3.95×10^3 CFUs/g dry soil). At Atmar 2 (=drip), fungal density remained fairly stable for the first three months following application, with a slight increase recorded in month three. Density declined thereafter with final density lower than the initial fungal count, but not significantly different. At Oranjelus, an initial increase in fungal density was recorded, peaking significantly three months post-application. A sharp decrease followed and continued until trial termination. Fungal density five months post-application (1.92×10^2 CFUs/g dry soil), was not significantly different to the initial density (8.76×10^2 CFUs/g dry soil).

Isolate Ma1 persisted throughout the trial period. As with isolates Bb1 and Ma2, an initial decline in fungal density, followed by an increase three months post-application was recorded (Figure 4.2). Significant differences in monthly persistence were found ($H_{5, 144} = 92.16$, $P < 0.00001$). Final fungal density (1.19×10^3 CFUs/g dry soil) was significantly lower than initial density (5.87×10^3 CFUs/g dry soil), but significantly higher than the lowest recorded counts in month two and three. In contrast to Bb1 and Ma2, fungal density was recorded to decrease between the last two months of monitoring.

Isolate Ma2 persisted throughout the trial period at both Atmar 1 and Oranjelus. At Atmar 2, median CFUs/g dry soil was zero at the end of the trial with fungal density showing a steady significant decrease following application (Figure 4.2) ($H_{5, 144} = 92.16$, $P < 0.00001$). At

Atmar 1, isolate Ma2 behaved as Bb1 and Ma1. Initial density (4.49×10^3 CFUs/g dry soil) and final density (4.33×10^3 CFUs/g dry soil) were not significantly different with final density significantly higher than the lowest density recorded in months two and three. ($H_{5, 144} = 70.18$, $P < 0.00001$) (Figure 4.2). At Oranjelus, Ma2 remained stable for the first three months post application, peaking slightly in month two. Thereafter, density decreased significantly and continued to show a steady decline until trial termination ($F_{5, 30} = 18.42$, $P < 0.00001$) (Figure 4.2).

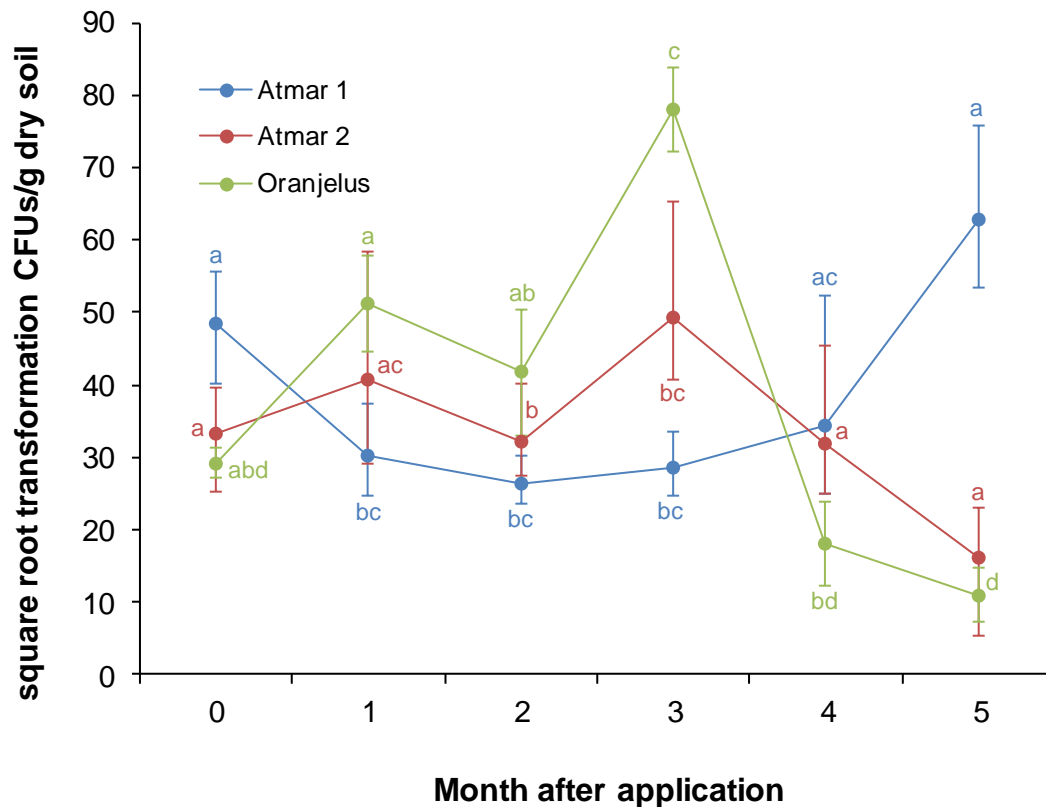


Figure 4.1: Monthly persistence of isolate Bb1 at Atmar under micro-sprinkler (Atmar 1) and drip (Atmar 2) irrigation, and at Oranjelus under micro-sprinkler irrigation. Monthly persistence is represented by the median with vertical bars denoting the interquartile range for Atmar 1 and 2; mean and standard error for Oranjelus. For each treatment, different letters are used to indicate statistically significant differences amongst months (Tukey’s HSD test or multiple comparison of mean ranks, $P < 0.05$).

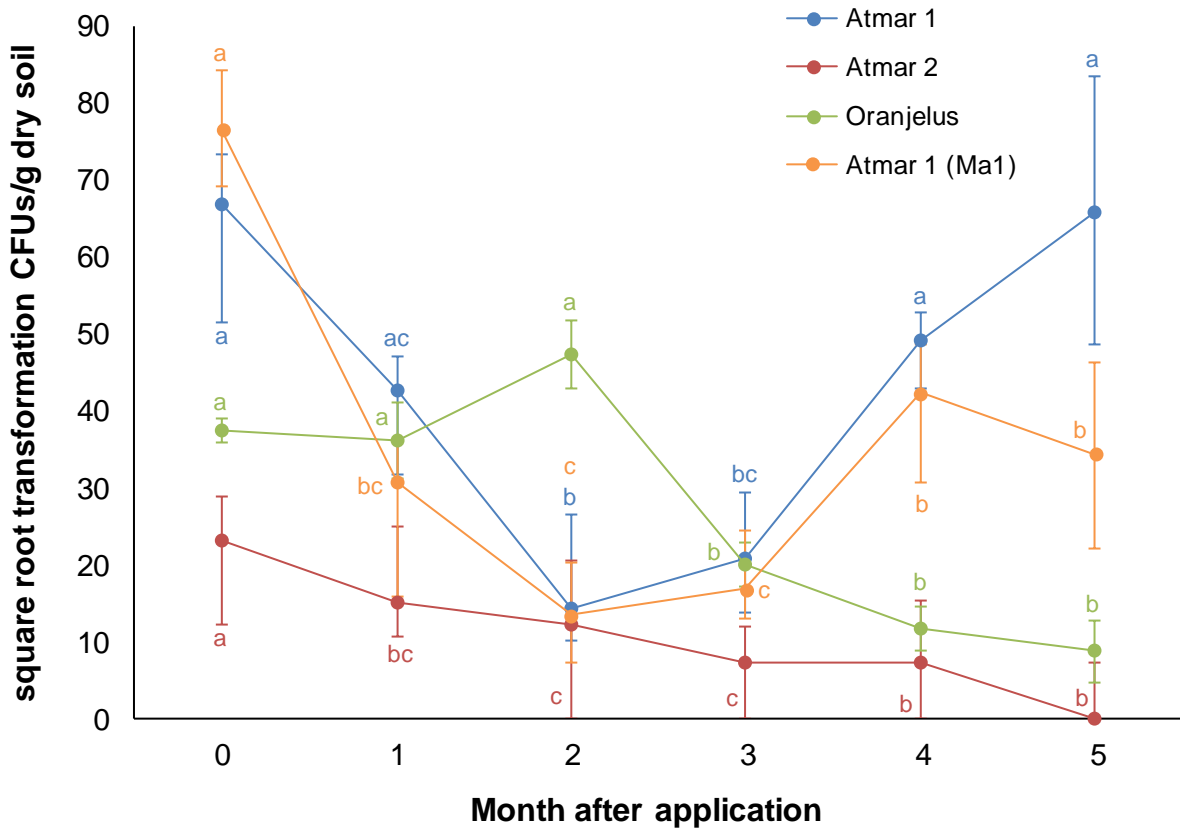


Figure 4.2: Monthly persistence of isolate Ma2 at Atmar under micro-sprinkler (Atmar 1) and drip (Atmar 2) irrigation and at Oranjelus under micro-sprinkler irrigation and of Ma1 at Atmar 1. Monthly persistence is represented by the median with vertical bars denoting the interquartile range for Atmar 1 and 2; mean and standard error for Oranjelus. For each treatment, different letters are used to indicate statistically significant differences amongst months (Tukey's HSD test or multiple comparison of mean ranks, $P < 0.05$).

In May 2015, 19 months post-application, the CFUs/g dry soil for isolate Ma1, Ma2 and Bb1 was determined. For all isolates, a significant reduction in fungal titre compared to initial titre was recorded (Table 4.1). This was most pronounced for *M. anisopliae* isolates Ma1 and Ma2.

Table 4.1: Comparison between the initial fungal titre recorded in October 2013 and 19 months post-application (May 2015) for all three isolates (Ma1, Ma2 and Bb1) applied at Atmar 1.

Isolate	Average fungal titre (\pm standard error) (CFUs/g dry soil)		% reduction	Statistics (n=24) (Mann-Whitney, P = 0.05)
	October 2013 ¹	May 2015 ²		
Ma1	5.88 \times 10 ³ (0.32 \times 10 ³)	4.38 (3.03)	99.9	U = 0.00, p < 0.00001
Ma2	4.18 \times 10 ³ (0.31 \times 10 ³)	1.75 \times 10 ² (1.24 \times 10 ²)	95.8	U= 7.00, p < 0.00001
Bb1	2.38 \times 10 ³ (0.15 \times 10 ³)	3.92 \times 10 ² (1.50 \times 10 ²)	83.5	U = 28.00, p < 0.00001

¹Fungal concentration as determined one week post-application

²Fungal concentration as determined 19 months post-application

4.4 DISCUSSION

Before discussing the implications of the results obtained in this study, it should be noted that species confirmation of the applied isolates was based on morphology, not genetic analysis. Within the same area, studies have reported the co-habitation of unique haplotypes of the same species (Goble *et al.* 2012; Mayerhofer *et al.* 2015) and given that the fungal strains investigated in this study, were isolated from soil samples collected from citrus orchards, it stands to reason that more than one isolate of the same species may be present in the sampling region. However, as no *B. bassiana* or *M. anisopliae* colonies were recorded in untreated control sites and detection of both species declined significantly 19 months post-application at Atmar 1, colonies identified as *M. anisopliae* or *B. bassiana* within the application area, likely represent the applied strain. Nevertheless, identifying molecular markers which can be used to distinguish applied strains from indigenous strains will become important when monitoring the fate of these fungi over a longer time period or in orchards where these species are detectable prior to application (Reineke *et al.* 2014; Biswas *et al.* 2015; Mayerhofer *et al.* 2015).

The lack of *Beauveria* and *Metarhizium* at the control sites is puzzling. Both *Beauveria* and *Metarhizium* are cosmopolitan species and commonly identified from agricultural systems worldwide (Klingen *et al.* 2002; Keller *et al.* 2003; Quesada-Moraga *et al.* 2007). Also, all three isolates investigated in this study were isolated from conventional citrus orchards in the Eastern Cape Province of South Africa (Goble *et al.* 2010). The presence of *Beauveria* and *Metarhizium* species may be expected in the region. Therefore, lack of detection may have been due to the selectivity of the media used. Medo & Cagáň (2011) assessed the occurrence of EPF in soils of Slovakia using two methods, the dodine selective method and *Galleria* bait method. These methods identified three and four fungal species respectively. However, *Beauveria* and *Metarhizium* species were represented by both sampling techniques. Similarly, Keller *et al.* (2003) found that the detection rates of these two methods were similar when used to assess the distribution of EPF in soils of Switzerland. Therefore, it is unlikely that the lack of detection resulted from the sampling method employed. It is more probable that any *Beauveria* or *Metarhizium* within these control sites were present at low concentrations reducing their likelihood of isolation. In addition, the distribution of these unknown *Beauveria* and *Metarhizium* species may have been patchy. Through the mixing of subsamples to form composite samples, this may have further reduced their likelihood of detection.

In this study, all applied isolates, with the exception of Ma2 at Atmar 2, persisted for five months post-application. Persistence varied subtly between isolates, but noticeably between sites. Persistence was greatest at Atmar 1. Although an initial decline in fungal concentration was apparent – a common outcome following application (Scheepmaker & Butt 2010) – an increase was noted two months after application. This increase continued until trial termination. Fungal counts made five months post-application, were comparable to fungal counts made one week post-application and although not significant, was higher in Bb1 and Ma2 treated plots. Based on the average number of FCM infested fruit (25 to 29) recorded per 12 trees every week (over a period of 21 weeks) from the control plots, FCM larval density in the soil was suspected to be higher at Atmar 1 than at Atmar 2 and Oranjelus, which recorded an average FCM infested fruit count of 11 (per 12 trees over a period of 18 weeks) and less than 1 (per 12 trees over a period of 18 weeks), respectively. As host density was high, this likely promoted the opportunity for infection and as such may have led to the

augmentation of the fungi within the applied area due to high numbers of sporulating cadavers two months post-application.

Increases in fungal concentration in response to high host density is not uncommon; Rath *et al.* (1995) reported a 10-fold increase in *M. anisopliae* when mummified cadavers of the target pest *Adoryphorus couloni* Burmeister (Coleoptera: Scarabaeidae) were present and Kessler *et al.* (2004) found that the density of *B. brongniartii* 16 months post-application was significantly higher at infested host sites than non-infested host sites. High host density almost certainly decreased after fruit harvest within the orchard, as host substrate was removed. This likely contributed to the significant reduction of all applied isolates at the end of the following growing season (19 months post-application). Although it is not known what fungal concentrations were at the start of the second growing season, it was unlikely that concentrations were sufficient or uniform enough to warrant infection of FCM. Visual inspection of the fruit revealed high density of FCM infestation. Therefore, in order to provide FCM suppression the following season, re-application of these isolates needs to occur. Mayerhofer *et al.* (2015) state that regular application of fungal isolates is important to maintain high abundance of the applied fungal strain in the soil. Intensive application over a consecutive three year period was suspected to promote the recovery of the applied fungal isolate 15 years post-application. However, persistence for longer than a season or two may not be desirable as this will not only limit product sales (Jaronski 2010), but also potentially increase the risk of non-target effects (Vestergaard *et al.* 2003; Laengle & Strasser 2010). Therefore, the reduction of isolates Ma1, Ma2 and Bb1 19 months post-application may even be considered beneficial. The impact of continuous and repeated applications of these fungi remains to be determined.

Although larval host density in the soil was lower at Atmar 2 than Atmar 1, pest presence can still be considered moderately high. This is possibly a result of the lower susceptibility of the particular Navel orange cultivar (Love *et al.* 2014) and high number of sterile FCM males recorded throughout the trial period (Appendix 2). However, in contrast to Atmar 1, both isolate Bb1 and Ma2 concentrations began to decrease towards the end of the trial. This was more pronounced for Ma2 than Bb1. Isolate Bb1 remained stable for the first three months with a slight increase in month three whereas Ma2 showed a significantly steady decline. The

increase in Bb1 concentration three months post-application, may be a result of fungal augmentation from sporulating cadavers especially since a reduction in FCM infested fruit was recorded (see Chapter 3). However, Bb1 and Ma2 concentration followed a similar pattern at Oranjelus where host density in the soil was low. Therefore increases in fungal concentration cannot be solely explained by host density in the soil and may be a result of microcyclic conidiation (Fargues & Roberts 1985) or may reflect the presence of other susceptible hosts in the soil.

The steady decline in fungal density recorded for Ma2 at Atmar 2 is interesting. Based on fungal efficacy (see Chapter 3), Ma2 reduced FCM infestation more effectively than Bb1 and at Atmar 1. A greater observed efficacy may indicate the presence of more cadavers on which sporulation can occur and as such, an increase in fungal concentration may be anticipated (Kreuger & Roberts 1997). This was not observed. Either the number of sporulating cadavers was too low to allow any increase in concentration to be detectable or environmental conditions limited sporulation. The latter is suspected. At Atmar 2, soil moisture was considerably lower within the upper 10 cm, a result of the irrigation system employed, than soil moisture levels recorded at both Atmar 1 and Oranjelus (see Chapter 3, Table 3.1). The dependency of both Ma2 and Bb1 on soil moisture for germination, infection and sporulation still needs to be determined and has been shown to vary amongst fungal species and isolates (Inglis *et al.* 2001; Hong *et al.* 1997; Devi *et al.* 2005). Typically, drier soils are associated with greater survivability of fungal species. It is for this reason that moisture is removed during storage (Lingg & Donaldson 1981; Hedgecock *et al.* 1995; Hong *et al.* 1997). In dry soils, there should be enough free moisture available to promote germination (Wraight *et al.* 2001). It is unlikely that germination, although possibly reduced, failed at Atmar 2 under dry conditions. Both isolates reduced FCM infestation in comparison to control plots. However, since any increase in fungal concentration was limited for Bb1 and not detectable for Ma2, sporulation may have been impeded at low soil moisture levels (< 15%). The limited ability of *B. bassiana* and *Verticillium lecanii* to sporulate under dry conditions has been reported (Milner & Lutton 1986; Studdert & Kaya 1990). In contradiction, Li & Holdom (1993) reported that *M. anisopliae* sporulated better in moderately dry (-10 to -15 kPa) soils than in wetter (0 to -2.0 kPa) soils. Perhaps under the conditions in this study soil moisture was too low to promote high levels of sporulation of isolate Ma2 under drip irrigation at Atmar 2.

Other abiotic factors which have been reported to reduce persistence include soil temperature, pH, textural class and organic matter (Klingen & Haukeland 2006; Scheepmaker & Butt 2010). Soil temperature, pH and textural class showed minimal variability between sites. Therefore, the impact these factors may have had on the persistence of Bb1 and Ma2 is unclear and remains to be tested. Across all sites, soil temperature was moderate, averaging 20°C and never exceeding 25°C. It is therefore unlikely to have influenced persistence significantly by itself. Cooler temperatures are often associated with increased spore longevity whilst temperatures above 35°C typically kill the spores directly, limiting both persistence and efficacy (Lingg & Donaldson 1981; Hong *et al.* 1997; Inglis *et al.* 2001). The influence of pH toxicity on EPF is dependent on the isolate. In this study pH was neutral. Medo & Cagáñ (2011) found that *M. anisopliae* was more prevalent in soils with a neutral to alkaline pH whilst *B. bassiana* was commonly associated with acidic soils. However, Padmavathi *et al.* (2003) reported the wide pH tolerance (5 to 13) of 29 *B. bassiana* isolates whilst Lingg & Donaldson (1981) reported little effect of pH on the survival of *B. bassiana* spores in soil.

Soil textural class was classified as sandy loam at both Atmar sites and sandy clay loam at Oranjelus. Although soil textural class can influence persistence, moderate clay content (10-30%) has been reported to favour the occurrence of EPF (Quesada-Moraga *et al.* 2007). Clay content at all sites fell within this range and is therefore expected to not have any negative impact on persistence. Soil texture may also influence the percolation of fungal propagules through the soil profile (Barbercheck 1992). This may facilitate the reduction of fungal concentration within the sampled area. Garrido-Jurado *et al.* (2011a) assessed the vertical movement of *B. bassiana* and *M. anisopliae* in 16 soils varying in texture, pH and organic matter. Retention of *B. bassiana* was found to be higher in clay soils whilst for *M. anisopliae*, retention was higher in sandy soils. However, more than 90% of the applied propagules were recovered from soil surface layers (approximately 0 to 5 cm) irrespective of soil properties. Assuming this to be true for this study, it is unlikely that vertical movement can be used to explain any reduction in fungal persistence, as most propagules should have been maintained within the zone sampled (0 to 5 cm).

Organic matter was not measured during these field trials. It has been suggested that soils with high organic matter (> 2%) promote persistence due to the adsorption of spores or because of increased host density (Inglis *et al.* 2001; Klingen & Haukeland 2006). However, *B. bassiana* has been shown to be more adversely affected by increases in organic matter than *M. anisopliae* due to increased susceptibility to soil fungistasis (Lingg & Donaldson 1981; Quesada-Moraga *et al.* 2007). This is contradicted by other studies where no correlation between organic matter and soil fungistasis was found (Grodén & Lockwood 1991).

The application of agrichemicals (insecticides, fungicides, fertilisers) may have impacted fungal persistence. Based on spray programmes obtained from the growers, no major discrepancies were apparent (Appendix 3). No chemicals were applied after January, with application only occurring to the foliar environment. Chemicals applied shortly before or after fungal application (late October), may have contributed to a reduction in fungal persistence. Variability in persistence amongst sites certainly does not suggest this and any impact these applied products may have on persistence are likely negligible when compared to other factors such as soil moisture and host density. Laboratory assessment of the impact of eight fungicides, registered for use in citrus, on the vegetative growth, viability and sporulation of isolates Ma2 and Bb1, is presented and discussed in Chapter 6. Fungicides are applied according to strict mandatory spray programmes in South African citrus orchards due to the prevalence and phytosanitary status of the fungal phytopathogen *Phyllosticta citricarpa*, the causal agent of citrus black spot (CBS) (Schutte 2009, Carstens *et al.* 2012, *S.D. Moore, pers. comm.).

It should also be noted that initial fungal concentration, measured one week post-application, varied amongst isolates and sites even though fungi were applied at the same rate (1×10^{14} spores/ha). This may have had implications on future persistence. Lower initial levels may have decreased the likelihood of contact between target host and infective propagule and thus reduced any potential of fungal augmentation in the treated area. Statistically, a significant difference in initial titre for each isolate amongst sites was only reported for Ma2 where fungal concentration was significantly lower at Atmar 2 than Atmar 1 and Oranjelus ($F_{2, 171} = 10.36$, $P < 0.00006$). Whether this encouraged the steady decline in persistence of Ma2 at Atmar 2, despite FCM infection, is unknown. Rumbos *et al.* (2008) reported no influence of

application rate on the persistence of the nematophagous fungus *Paecilomyces lilacinus* when applied to soil under controlled conditions – a steady decline was noted regardless of whether application occurred at 1×10^6 or 2×10^6 CFUs/g of substrate.

In conclusion, the results of this study suggest that both isolates Bb1 and Ma2, in their unformulated state, can persist for five months post-application with high density of susceptible hosts, coupled with moderate soil moisture appearing to promote fungal persistence.

Chapter 5

Physical suspension characteristics and toxicity of five adjuvants on the spores of fungal isolates Bb1 and Ma2

5.1 INTRODUCTION

Formulation is a critical component in the development of mycopesticides (Jackson *et al.* 2010). According to Ravensberg (2011), formulation serves four important functions: (1) stabilises fungal propagules for long term storage, transport and consistent efficacy, (2) improves the user-friendliness and safety of the product, (3) protects the propagule from harmful environmental variables and thus improves its persistence and (4) aids in maintaining consistency whilst simultaneously improving target delivery. A variety of formulation types as well as additives used within formulations exist. The type of formulation used, and the additives incorporated, are chosen based on several considerations including the target area and the environmental variables associated with it, the target insect and its behaviour, the type of infective propagule under development and, given that EPF are biological organisms, their biocompatibility with the chosen additives (Wraight *et al.* 2001; Jackson *et al.* 2010; Ravensberg 2011). For EPF, most formulations are based on aerial conidia (spores) as these tend to be easily produced in high numbers and are less susceptible to damage by additives than other infective propagules such as blastospores and mycelia. Although the types of formulations are vast, most products based on the aerial conidia of EPF take the form of technical concentrates, wettable powders or oil dispersions (Faria & Wraight 2007).

In this study, aerial conidia of *B. bassiana* and *M. anisopliae* were applied as a wettable powder to the soil environment (Chapter 2 and 3). In wettable powders, dried fungal spores are suspended in water prior to application (Jackson *et al.* 2010). However, aerial conidia are

highly hydrophobic, a result of the arrangement of hydrophobic rodlets in their outer cell wall (Bidochka *et al.* 1995; Holder *et al.* 2007). Therefore wetting agents are used to overcome this hydrophobicity to promote a uniform suspension. As wetting agents also reduce the surface tension of water, they improve the adherence of the suspension to solid surfaces such as leaves and insect cuticles (Jackson *et al.* 2010). Wetting agents, which may be surfactants or oils, differ in their emulsion characteristics and toxicity towards fungal species and isolates. (Jackson *et al.* 2010; Santos *et al.* 2012; Mishra *et al.* 2013). An ideal wetting agent must not hinder the viability of the fungus and should be capable of retaining spores in suspension with minimal agitation during spraying (Jackson *et al.* 2010).

In Chapter 2 and 3, dried spores of the fungal isolates were suspended in water supplemented with the surfactant Breakthru[®] S240. Although the use of Breakthru[®] S240 did not hinder fungal efficacy, other surfactants are available for use. As surfactants can impact the physical (e.g. dispersion and retention within the medium) and biological (e.g. viability) properties of fungi differently (Santos *et al.* 2012), this chapter therefore aimed to investigate four different surfactants, including Breakthru[®] S240 and a mineral oil, to determine if an alternate wetting agent could be used to enhance field application by improving the retention and dispersion of the spores in suspension. The impact of these wetting agents on spore viability was also evaluated.

5.2 MATERIALS AND METHODS

5.2.1 Fungal isolates

Two fungal isolates were used; *M. anisopliae* FCM Ar 23 B3 (Ma2) and *B. bassiana* G Ar 17 B3 (Bb1). Only these isolates were tested as these were the isolates on which most field research was conducted (see Chapter 3 and 4). The dry aerial conidia of both isolates were obtained from Citrus Research International, but were originally mass produced by Agrauxine (Loches, France). Spores were stored in complete darkness at 4°C until use.

5.2.2 Adjuvants

Five adjuvants, for use as potential wetting agents, were tested: four surfactants, Breakthru[®] S240 (a.i. polyether-modified trisiloxane), Breakthru[®] S233 (polyether-modified trisiloxane), Breakthru[®] OE446 (a.i. polyether-modified polysiloxane), Breakthru[®] Advance (a.i. polyether modified trisiloxane/ organo modified polysiloxane) (all from Evonik Industries, South Africa) and a commonly used agricultural mineral oil, BP Medium Oil (BP, South Africa). All adjuvants were stored at room temperature in a cupboard until use. S240 and S233 are classified as wetting agents, OE 446 as an oil enhancer and Advance a spreader and wetter. All wetting agents are registered for use in products destined for agricultural use.

5.2.3 Physical suspension characteristics of each suspension concentrate

The protocol followed was adapted from that outlined for chemical pesticide formulation testing in the CIPAC Handbook Volume F (MT 36 Emulsification characteristics of emulsifiable concentrates) (Dobrat & Martjin 1995) as demonstrated during a formulation training course at CABI (Egham, United Kingdom). A suspension concentrate was prepared by adding 5 ml of each adjuvant to 0.5 g pre-weighed spore powder contained within sterile 25 ml glass universal bottles. The bottles were sealed and shaken vigorously by hand until well mixed. A sterile glass rod was used if hand-shaking was not possible, particularly when the highly viscous adjuvants Breakthru[®] Advance and OE 446 were used. Each suspension concentrate was then added independently to 95 ml of sterile distilled water contained within 100 ml graduated clean and sterile stoppered measuring cylinders to produce an aqueous suspension. Any immediate suspension of the concentrate was noted by visual examination prior to mixing. In suspension, Bb1 turns the water milky white, Ma2 turns it green due to the colour of the spores. The degree of suspension could then be estimated using the volumetric markings on the cylinders and colour intensity of the suspension. This is illustrated in Figure 5.1.

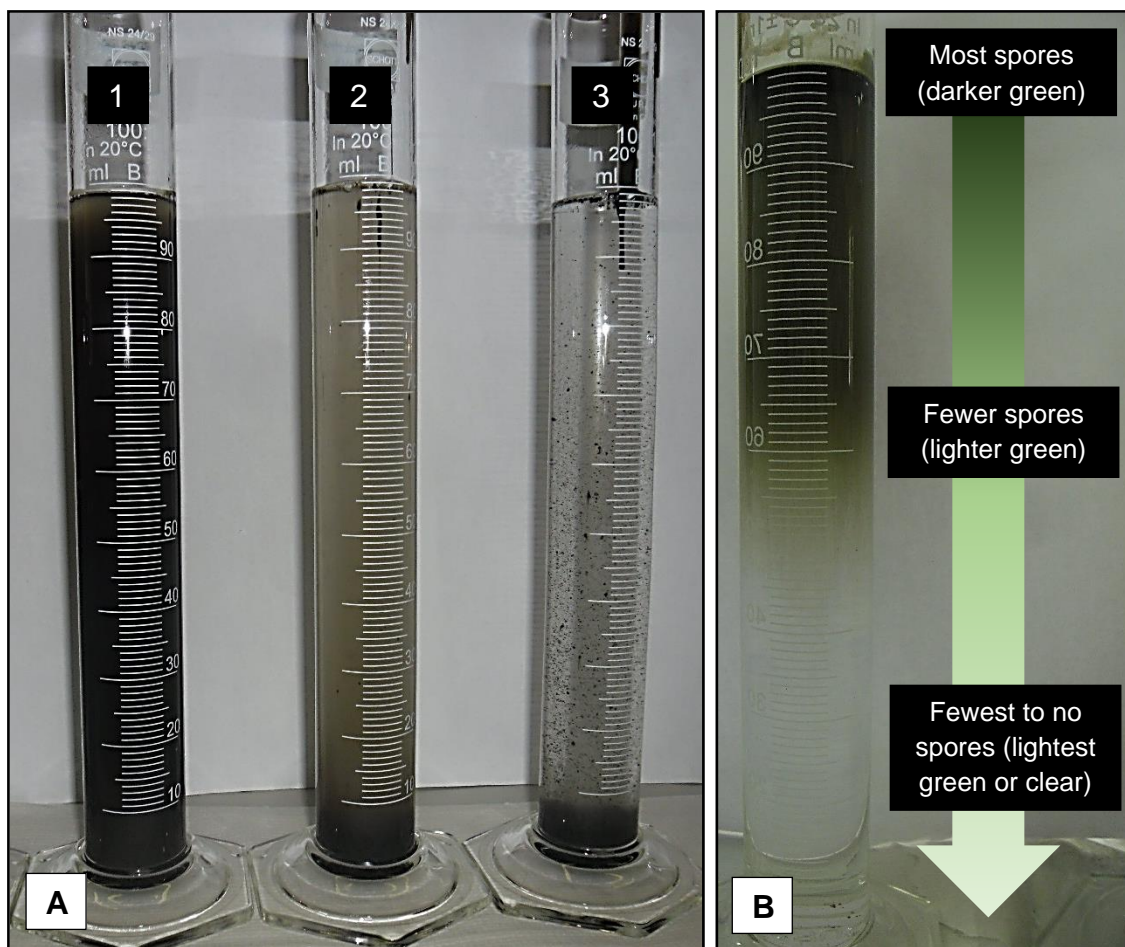


Figure 5.1: Illustration of the degree of suspension and uniformity of the suspension as based on visual examination of isolate Ma2 (A): Cylinders 1, 2 and 3 represent a uniform suspension, non-uniform suspension (most spores settled out) and no noticeable immediate suspension prior to mixing, respectively. (B) A representation of the degree of suspension when uniformity is not achieved as in A1. The more intense (=darker) colour is indicative of more spores. The same visual observation can be observed for isolate Bb1, with the exception that spores held in suspension turn the water milky white, rather than green due to the colour difference between the spores of the two isolates.

The cylinders were carefully inverted 10 times by hand after which the percentage of foaming, degree of suspension and any other points of interest or concern, such as flocculation (grouping of spores to form clumps), were recorded. The cylinders were then incubated at each of three different temperatures: 4°C, 25°C, and 30°C. A water bath was used to maintain temperature at 25°C and 30°C and a fridge at 4°C. The percentage foaming,

degree of suspension and any other points of interest or concern were again recorded after incubation for 0.5 h, 2 h and 24 h to assess the stability of the suspension over a 24 h period. After 24 h, the cylinders were inverted 10 times as before to determine whether spores which may have settled out of suspension were able to re-suspend. The percentage of foaming, degree of suspension and any other points of interest or concern were again recorded. The cylinders were then incubated for an additional 30 min at the temperature under investigation. The percentage foaming, degree of suspension and any other points of interest or concern were again recorded. From each cylinder, a 2 ml sample was removed and used to determine spore viability. For each suspension concentrate at each temperature, three independent observations were made.

5.2.4 Toxicity of adjuvants to Ma2 and Bb1 after 24 h exposure

Aseptic technique was used to spread 100 µl of a 10^{-2} dilution of each 2 ml sample onto a SDA plate supplemented with 50 mg/ml chloramphenicol. Plates were incubated in complete darkness at 26°C. The percentage of germinating and non-germinating spores was determined by counting 300 spores on each plate 24 h after isolation for Bb1 and 48 h for Ma2; the former having a faster germination rate than the latter (personal observation). The viability of the spores not exposed to any of the wetting agents was also evaluated. A fungal suspension using 0.01% Tween 20 (Sigma Aldrich, South Africa) was prepared and viability assessed as previously. As data were non-normal, any differences in spore viability amongst treatments, was evaluated by Kruskal Wallis ANOVA at the 0.05 level of significance. All statistical analyses were conducted in Statistica ver. 10 (StatSoft, Inc. 1984-2011).

5.3 RESULTS

5.3.1 Physical suspension characteristics of each suspension concentrate

Immediate suspension of spores in the water, which always occurred at room temperature, varied amongst wetting agents (Figure 5.2). For both isolates, S240 and S233 performed similarly with spores being suspended throughout the cylinder. No immediate suspension was apparent for Advance, as all spores immediately settled to the bottom of the cylinder. For OE

446, a similar response to Advance was observed, but some suspension did occur as the spores began to settle out of solution. This was more noticeable for Ma2 than Bb1. For the oil, an average immediate suspension of 31.6% and 43.3% was obtained for Bb1 and Ma2 respectively. In addition, a thin oil layer was always visible on the surface of the water. This was apparent regardless of the isolate under investigation.

After inverting the cylinders 10 times, foaming, which dissipated over time, was visible and variable amongst wetting agents (Figure 5.3). For both isolates, a higher percentage of foam was recorded for S233 followed closely by S240. Less foam was produced by Advance and OE 446. The oil produced no foam. S240 and S233, the less viscous surfactants, mixed easier than OE 446 and Advance. The latter two often resulted in a small to moderate amount of mix still adhering to the sides and bottom of the cylinder (Figure 5.4).

Irrespective of temperature, the spores settled out of suspension over time when measured 0.5, 2 and 24 h post-inversion. The results to follow, unless otherwise stated, refer to the state of the suspension after 24 h incubation. At 4°C, spores were still held in suspension, albeit some settling had occurred. Results were similar for both isolates for all four surfactants tested. Spores however appeared to be held in suspension slightly better for Ma2 than Bb1. Bb1 suspended in oil had completely settled out of suspension 24 h post-suspension (Figure 5.5). At 25°C, majority of the spores, irrespective of the adjuvant, had settled out of suspension for Bb1. S233 was observed to retain the most spores in suspension. For Ma2, S233, OE 446, Advance and the oil, all managed to prevent complete settling. Again S233 performed best. For both isolates a layer had formed at the bottom of the cylinder, which showed signs of flocculation in Advance treatments (Figure 5.6). At 30°C, results for both isolates were similar to those recorded at 25°C (Figure 5.7). Again S233 appeared to retain the most spores in suspension after 24 h. In the S240 treatment, for both isolates, two distinct layers were apparent (Figure 5.8). At all three temperatures spores settled out of suspension more rapidly when suspended in the oil compared to the surfactants. This was more pronounced for Ma2 than Bb. In addition, spores appeared to settle out of the oil and move to the bottom of the cylinder and the oil to the top for Ma2, whereas for isolate Bb1, the spores and oil layer both settled at the top (Figure 5.9).

For both isolates, re-suspension (second inversion) of the spores was possible for all Breakthru[®] products but not BP Medium Oil. As before, S240 and S233 allowed the spores to be more easily suspended compared to OE 446 and Advance. The latter two, however, were easier to mix by inversion (less adhered to the sides and bottom of the cylinder) after 24 h incubation, especially at 25°C and 30°C compared to the first inversion. In some cases, re-suspension of Advance resulted in the formation of white clumps (for both Ma2 and Bb1) which could be seen in the suspension. It is unclear what these clumps were. Re-suspension of the oil resulted in flocculation followed by immediate separation out of suspension (Figure 5.10). Foaming and the physical state of the suspension 30 minutes after re-suspension were as before.

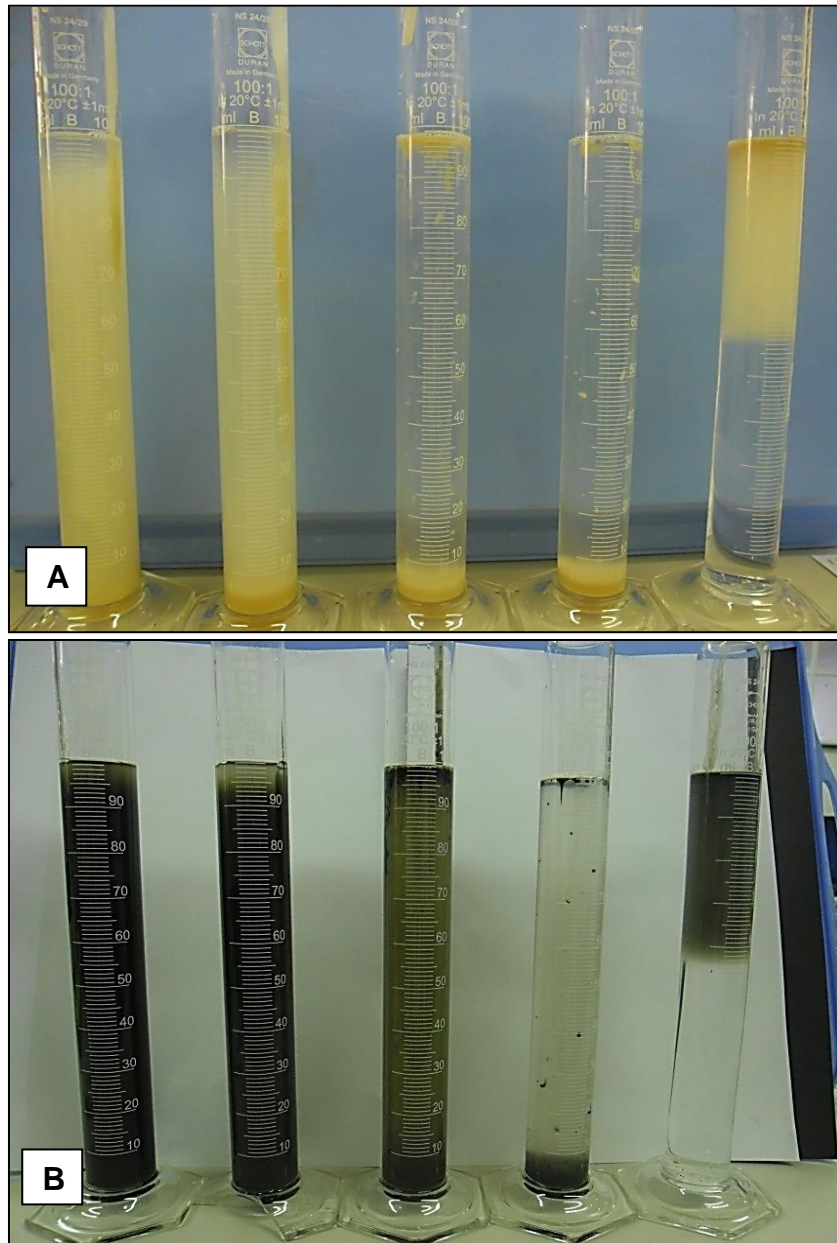


Figure 5.2: Immediate suspension prior to mixing of each of the five adjuvants for Bb1 (A) and Ma2 (B). The spores had been mixed with the adjuvant prior to them being poured into the water. Adjuvants from left to right: S240, S233, OE 446, Advance and oil.

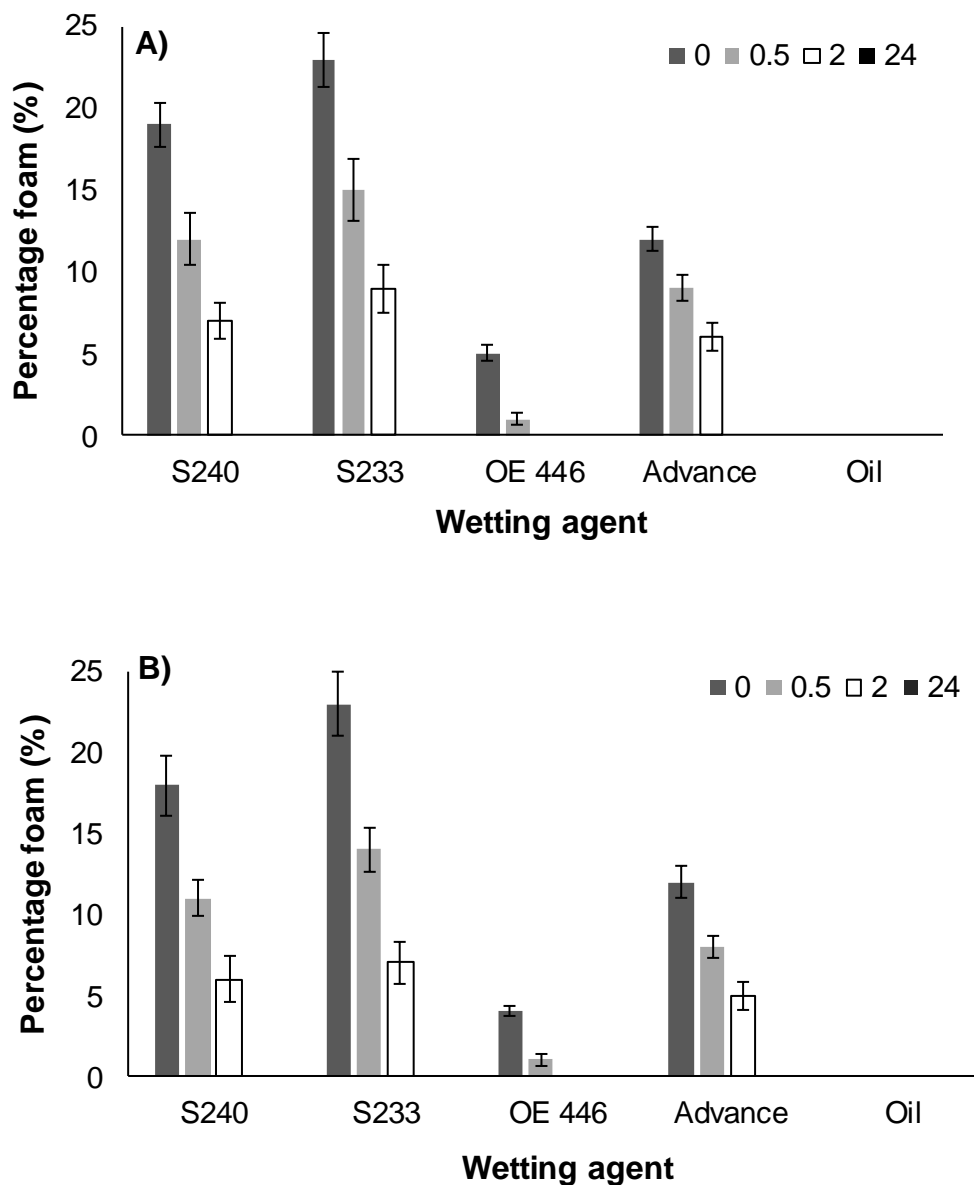


Figure 5.3: Mean percentage foam produced by each adjuvant suspension over time (0, 0.5, 2 and 24 h post-inversion) for both isolates Bb1 (A) and Ma2 (B). 0 hours refers to the percentage foaming immediately after inversion. Vertical bars represent standard errors.

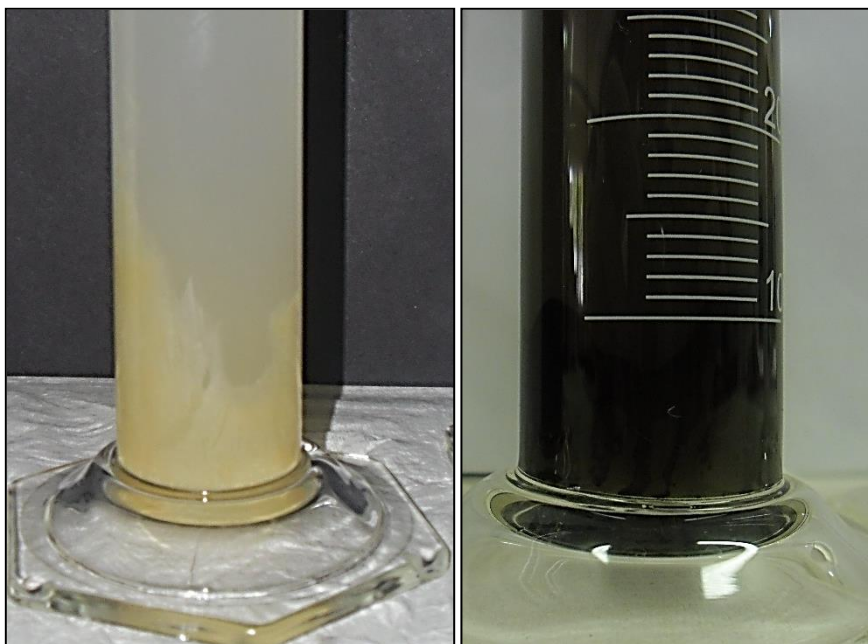


Figure 5.4: OE 446 and Advance mix often remained adhered to the sides and bottom of the cylinder after hand inversion. This was recorded for both Bb1 (A) and Ma2 (B).

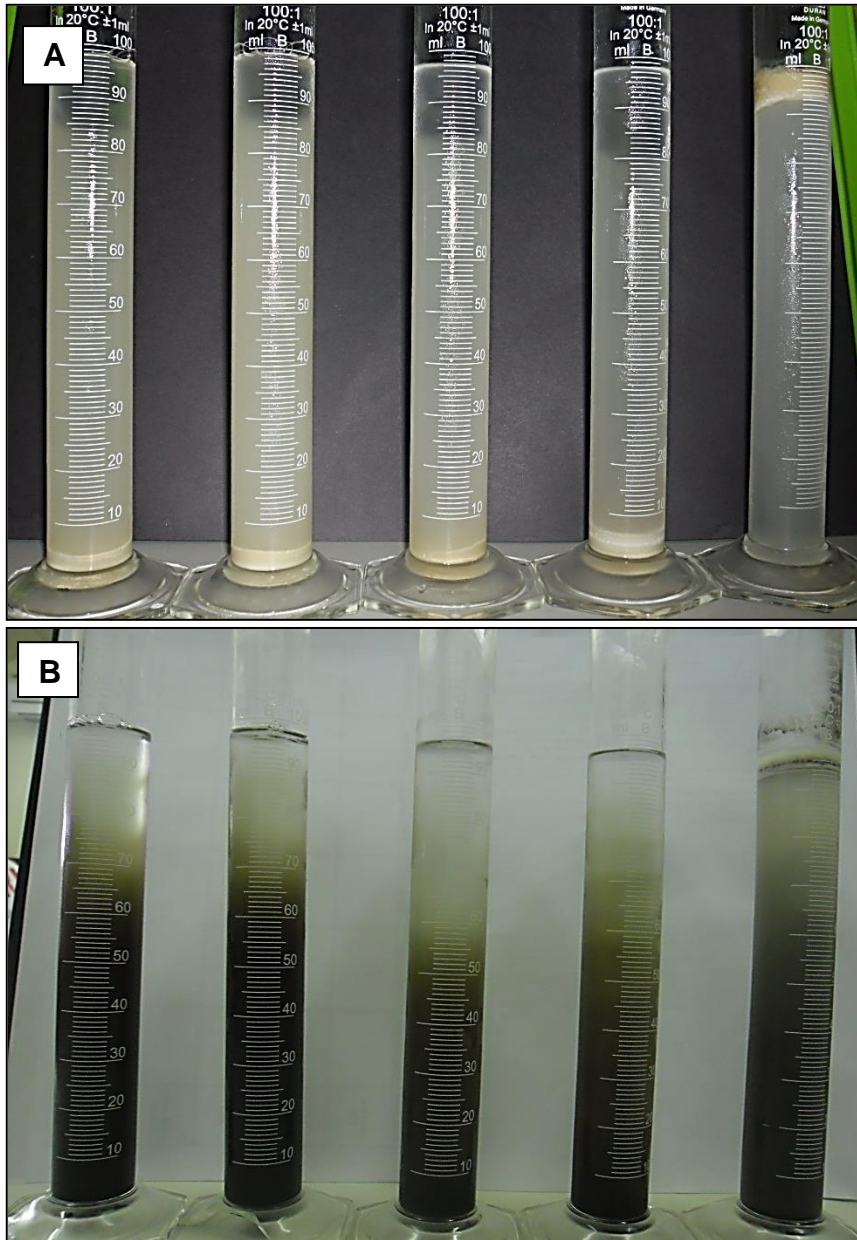


Figure 5.5: Spores still held in suspension after 24 h incubation at 4°C for each adjuvant and each isolate, Bb1 (A) and Ma2 (B). Adjuvants from left to right: S240, S233, OE 446, Advance and oil.

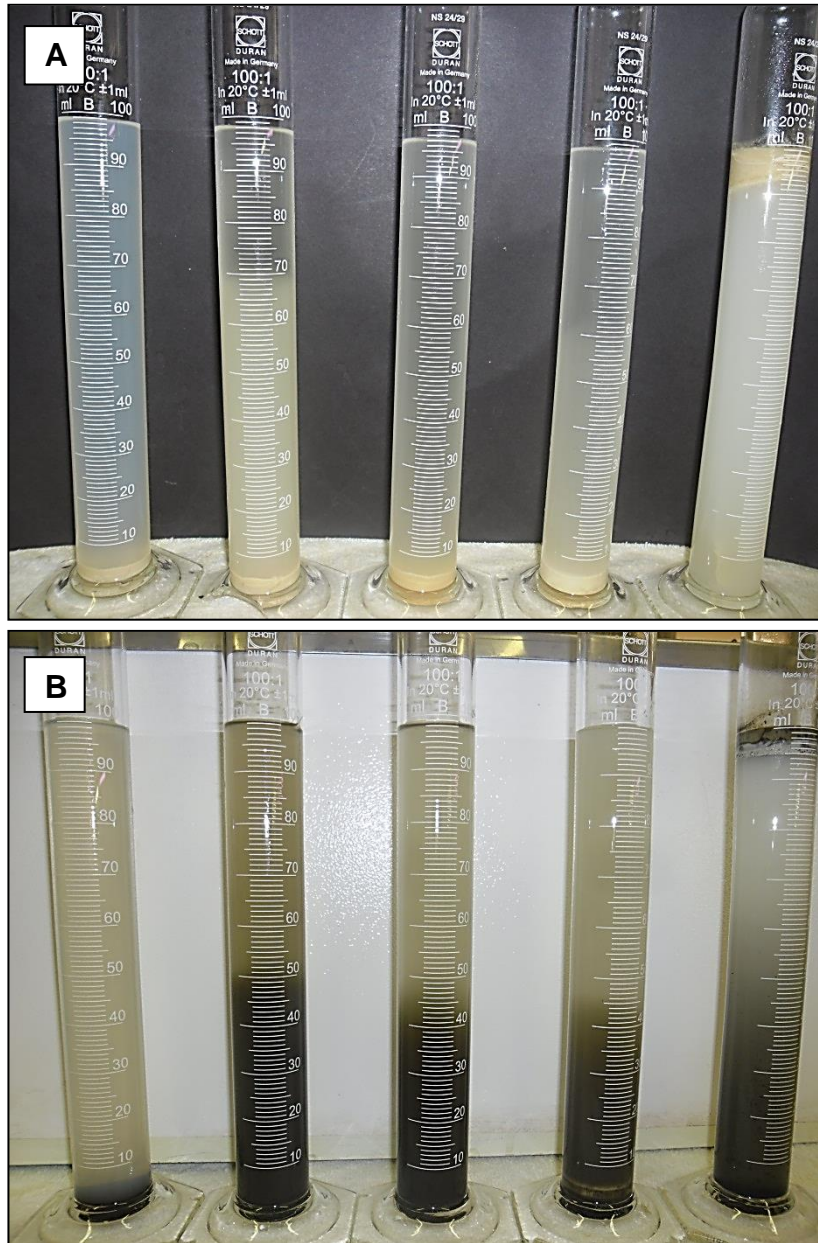


Figure 5.6: Spores still held in suspension after 24 h incubation at 25°C for each adjuvant and each isolate, Bb1 (A) and Ma2 (B). Adjuvants from left to right: S240, S233, OE 446, Advance and oil.

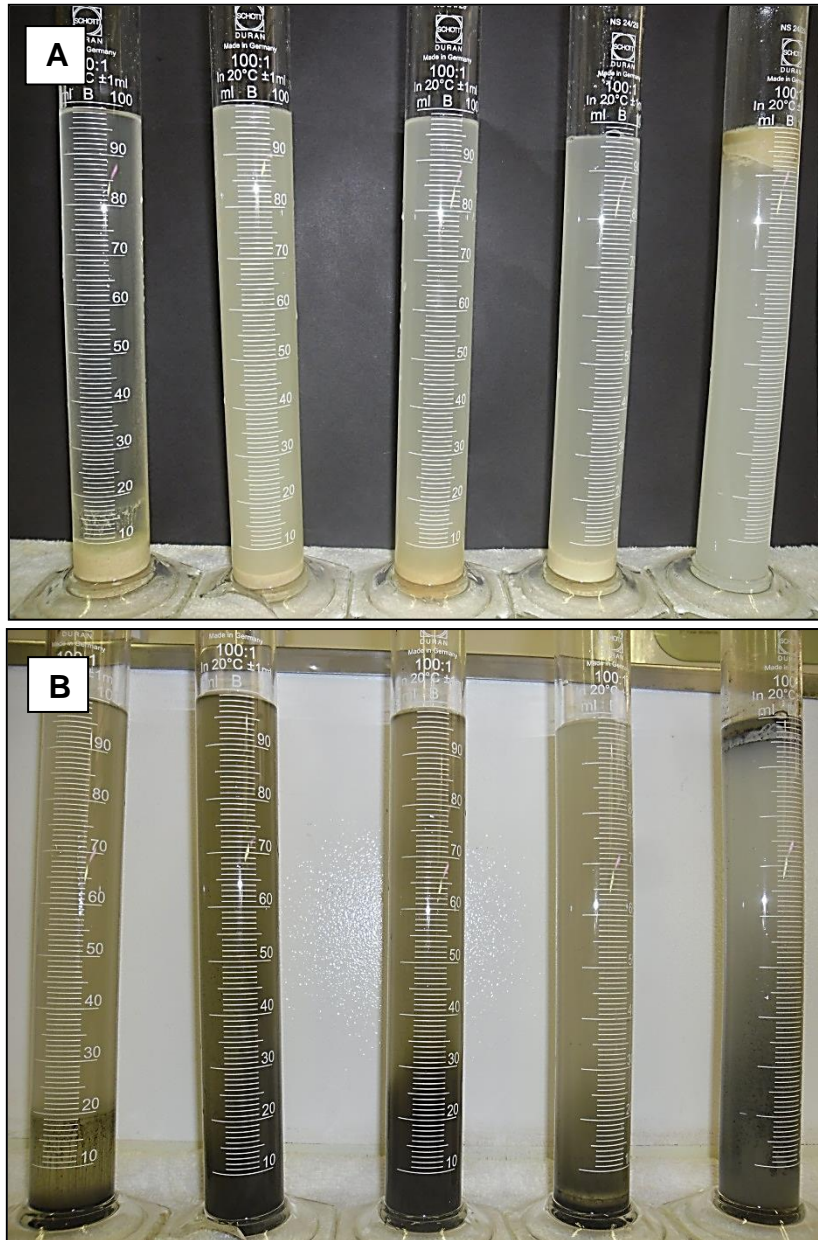


Figure 5.7: Spores still held in suspension after 24 h incubation at 30°C for each adjuvant and each isolate, Bb1 (A) and Ma2 (B). Adjuvants from left to right: S240, S233, OE 446, Advance and oil.

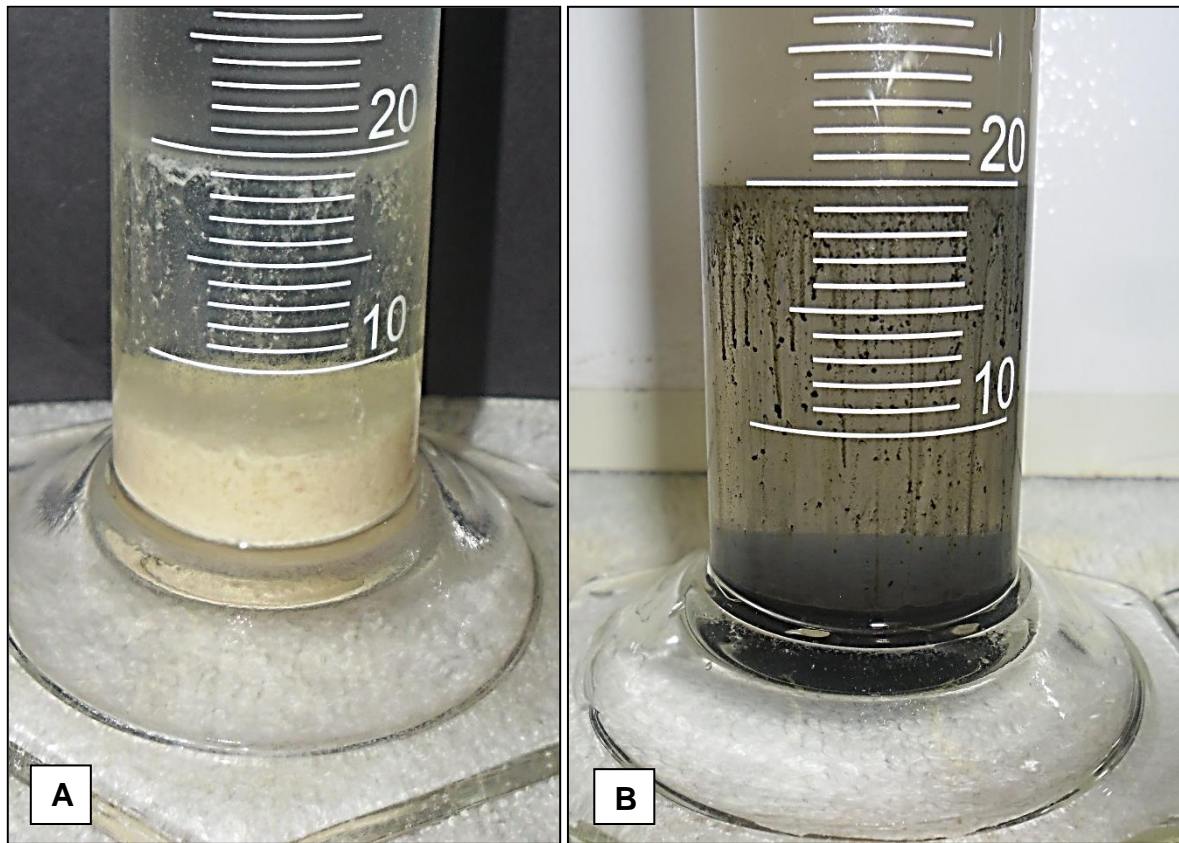


Figure 5.8: At 30°C, two distinct layers form. The lower layer (0-20 ml) contains what appears to be most of the spore/adjuvant mix; the upper (20-100 ml) the water with a few spores still in suspension. This was only apparent for the wetting agent S240 and was observed in both isolates, Bb1 (A) and Ma2 (B).



Figure 5.9: For Bb1, the oil/spore mix settled at the top (A), whereas for Ma2, the oil/spore mix appeared to originally settle at the top, but over time the spores moved out of the oil to settle at the bottom (B).

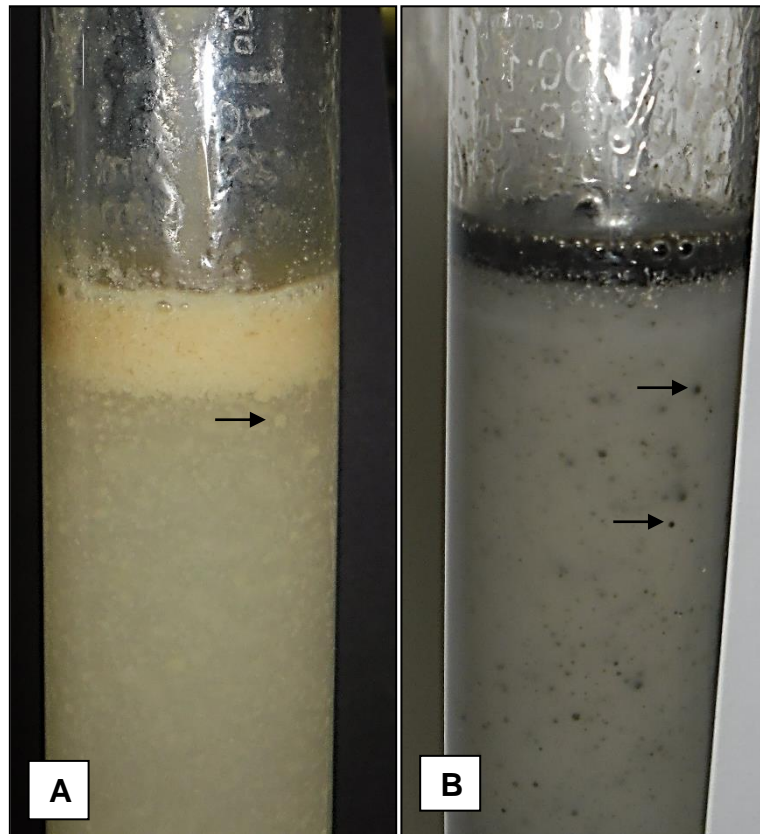


Figure 5.10: When BP Medium Oil was used as the wetting agent, re-suspension was not possible and attempts resulted in flocculation (indicated by the arrows) followed by immediate separation out of solution. This was observed for both Bb1 (A) and Ma2 (B).

5.3.2 Toxicity of adjuvants to Ma2 and Bb1 after 24 h exposure

For Bb1, spore viability was typically not affected by the wetting agent or temperature (Figure 5.11). A significant difference was only found between the viability of the spores exposed to S233 at 4°C compared to spores exposed to no wetting agents ($H_{15, 144} = 31.6702$, $P = 0.0071$). Median spore germination was however above 90% for all treatments.

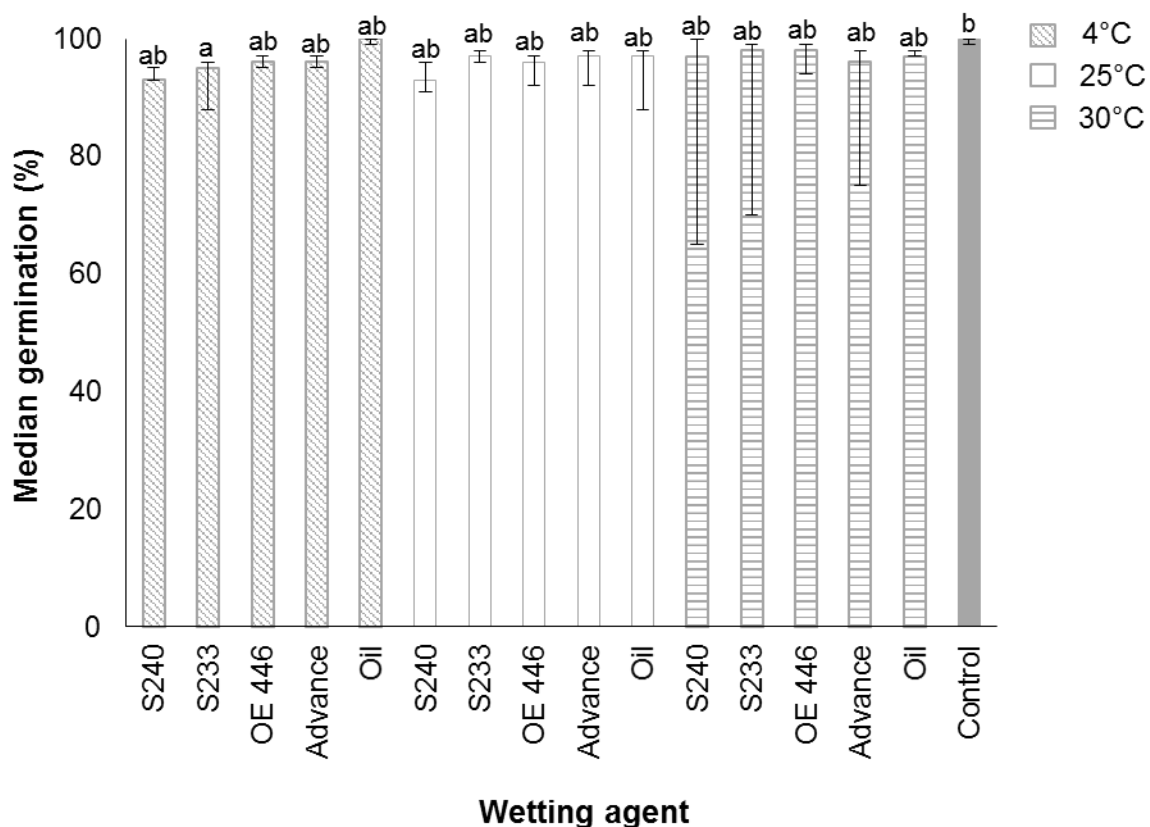


Figure 5.11: Spore viability (=germination) for Bb1 recorded after 24 h exposure to each adjuvant at each of the three temperatures tested and the control. The number of germinated and non-germinated spores was determined 24 h after incubation in complete darkness at 26°C.

For Ma2, spore viability was more variable in comparison to Bb1 (Figure 5.12). Viability tended to be lower for the four surfactants at 25°C and 30°C than at 4°C. Median germination however, was always above 70%. The lowest median germination was recorded for S240 at 25°C and 30°C and OE 446 at 30°C, all of which were significantly different to the control ($H_{15, 144} = 77.52, P < 0.0001$). Viability in the oil remained constant.

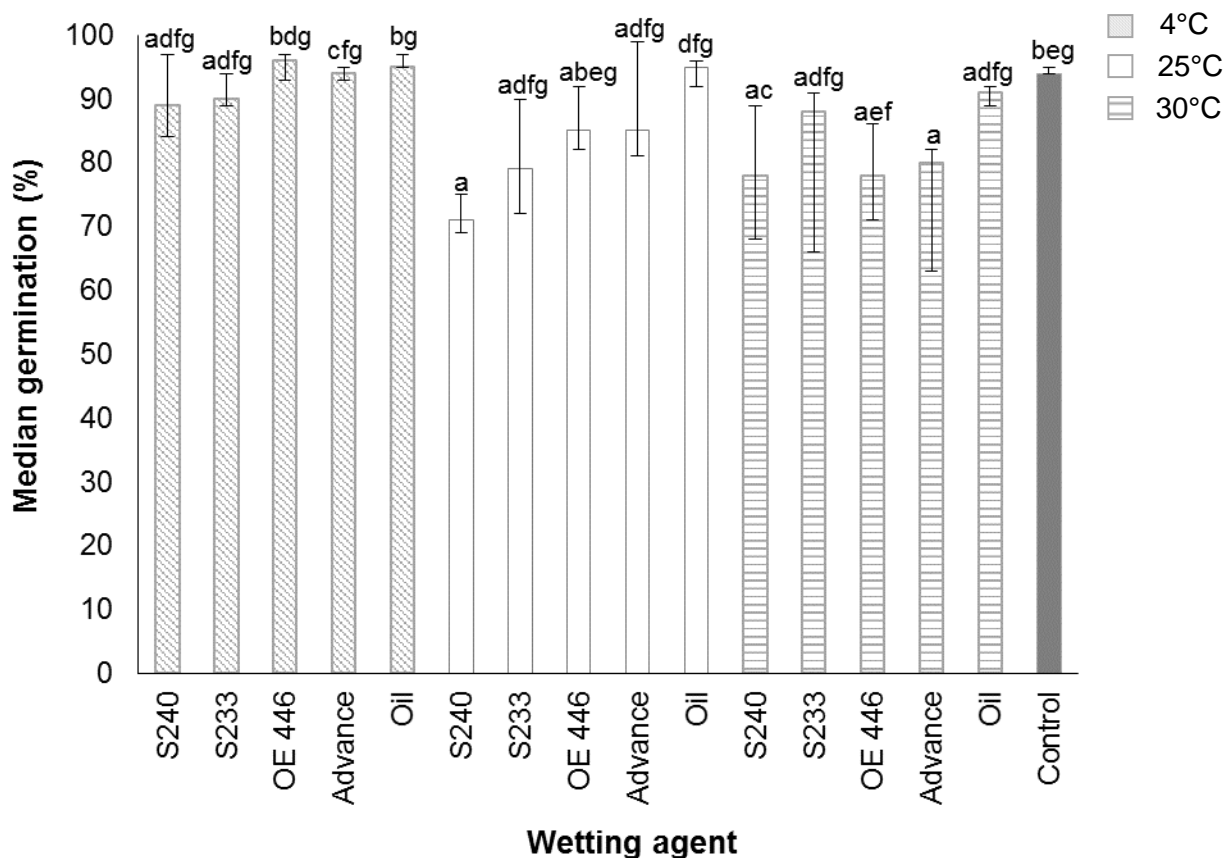


Figure 5.12: Spore viability (germination) for Ma2 recorded after 24 h exposure to each wetting agent at each of the three temperatures tested and the control. The number of germinated and non-germinated spores was determined 48 h after incubation in complete darkness at 26°C.

5.4 DISCUSSION

Similarities and variability amongst the physical suspension characteristics of the wetting agents on the spores of Ma2 and Bb1 were recorded. Similarly, the ability of the wetting agents to retain spores in suspension decreased over time. Continual agitation of the suspension during spraying is therefore necessary to achieve uniform application. This is important to ensure contact between FCM fifth instars entering the soil to pupate and the infective propagule following application. Breakthru® S233 and Breakthru® S240 were easier to suspend than Breakthru® OE 446 and Breakthru® Advance, suggesting that minimal agitation is required for the former two. More vigorous agitation of Breakthru® OE 446 and

Breakthru[®] Advance is necessary to ensure complete suspension of the spores in solution, particularly for initial suspension. However, agitation can generate foam. Foam has the potential to trap spores and as a result, reduce the number of spores applied during spraying (*B. Luke pers. comm.). In addition to hindering optimal fungal application, foaming can also reduce the uniformity of a suspension, resulting in uneven coverage (Jin *et al.* 2008). All the tested wetting agents, with the exception of the oil, produced foam to varying degrees and although the foam dissipated over time, given that continual mixing during application is required, foam will be present throughout the application period. With increased agitation, an increase in foaming would be expected. Although Breakthru[®] S233 and Breakthru[®] S240 produced the most foam, minimal agitation is required during application and as Breakthru[®] OE 446 and Breakthru[®] Advance produced minimal foam, increased agitation may not be of concern.

With the exception of the oil, all other wetting agents allowed for re-suspension after standing for 24 hours regardless of the temperature at which they were stored. The ability of surfactants to allow for re-suspension is important and has implications on storage. Dry spores prove challenging to work with in the field as they are easily dispersed by wind or movement, resulting in not only a loss of product, but also a safety hazard to the operator due to inhalation of the spores (Goettel & Jaronski 1997). The preparation of a stock solution prior to dilution in the field therefore makes for easier handling and limits health concerns for the user. As re-suspension of Ma2 and Bb1 spores was possible for all Breakthru[®] products tested, a stock suspension can be prepared at least 24 hours in advance. For the oil however, re-suspension was not possible. Any re-suspension attempts caused flocculation of the spores. In addition to spore clumping limiting the uniformity of application, flocculation may also clog spray apparatus (B. Luke, pers. comm.). Therefore if the oil is to be used, the suspension to be applied needs to be mixed immediately before application.

Although the oil did not allow for re-suspension after 24 h, its use as an emulsifier should not be ignored. The use of oils in formulations has been shown to be important in preventing spore desiccation and reducing the harmful effects of UV radiation on spore longevity, thus increasing fungal efficacy (Prior *et al.* 1988; Bateman *et al.* 1993; Malsam *et al.* 2002). In this study, spores were applied to the soil surface underneath the canopy of citrus trees and

therefore the use of an oil may not be necessary. Citrus orchards are regularly irrigated and shading provided by the trees reduces UV exposure. However, if these EPF are to be applied against foliar pests, the use of oils as spray carriers into which an emulsifier is incorporated to promote easy suspension in water, warrants further attention.

As wetting agents are chemicals, their antifungal activity towards the applied isolates needs to be determined. Research has indicated that for filamentous fungi, such as Ma2 and Bb1, toxicity of wetting agents appears to be positively correlated with the length of the alkyl chains; the longer the chain, the greater the toxicity (Oros *et al.* 1999; De Jonghe *et al.* 2007; Leal *et al.* 2009). Through chemical modification however, such as alkyl branching, antifungal activity can be reduced (Jackson *et al.* 2010). For Bb1, only Breakthru[®] S233 at 4°C was found to be significantly toxic in comparison to the control. Median viability was however still above 90%, and was found to not be significantly different to the viability recorded for all other treatments. This wetting agent and the others are therefore considered to be non-toxic towards dry spores of Bb1 after 24 h exposure. Temperature had no impact on the viability of Bb1. For Ma2, even though viability never fell below 70%, a loss of viability was observed compared to Bb1 24 h after exposure to certain adjuvants. At 4°C, viability was usually greater than at 25°C and 30°C, suggesting that storage of formulated Ma2 isolates as a stock suspension should be held in the fridge. Viability was however only significantly different between Breakthru[®] S240 at 25°C and 30°C and Breakthru[®] OE 446 at 30°C, in comparison to the control. If either of these surfactants is to be used, storage of Ma2 at these temperatures is therefore not recommended. It should also be noted that although adjuvants can be toxic towards the spores of EPF, adjuvants alone have also been shown to be toxic to the target insect (Srinivasan *et al.* 2008), an aspect not monitored in this study. Although not tested, this may have greater implications for insects which are targeted directly during spraying e.g. foliar insects, rather than soil-dwelling insects.

During the application of Bb1, Ma1 and Ma2 in Chapter 2 and Chapter 3, the surfactant Breakthru[®] S240 was used. In comparison to the other wetting agents, Breakthru[®] S240 performed similarly with respect to its physical properties as a wetting agent. It does however produce a high percentage of foam, the impact of which is unclear given that bioassays using different wetting agents have not been conducted. Based on viability assessment, Breakthru[®]

S240 is compatible with spores of Bb1, but may reduce spore viability of Ma2 at higher temperatures. Use Breakthru[®] S240 as a surfactant may therefore be more suited for use in Bb1 application rather than Ma2 application. Spores which are incapable of germinating cannot cause infection, resulting in reduced efficacy (Inglis *et al.* 2001). However, it should be noted that germination of Ma2 may still have been possible after 48 h. Rather than prohibiting germination, Breakthru[®] S240 may merely have reduced the germination rate. Secondly, the concentration (v/v) used in this experiment was far greater than that used in the field. Compared to the concentration of Breakthru[®] S240 (0.0005%) used in the 5 L suspension concentrate prepared for field trials before further dilution, the concentration of the wetting agent once suspended (5%) in this study, was already 10⁴ times greater. Therefore although the physical properties of the agent are unlikely to change, the biological impact it has on spore viability may be negligible. Santos *et al.* (2012) reported a dose-dependent relationship between germination of *B. bassiana* spores and certain surfactants. A similar finding was also reported by Mishra *et al.* (2013) who, in addition, also reported a decline in germination of fungal spores as the length of exposure time to the surfactants increased. This has implications for medium-term and long-term storage and was not assessed in this study, but is important once a final formulation is determined (Ravensberg 2011).

In conclusion, the use of Breakthru[®] S240 as the surfactant during the field trials is unlikely to have hindered fungal efficacy. In addition, the use of an alternate wetting agent for application to the soil surface is unlikely to improve fungal efficacy given similar physical suspension characteristics and limited toxicity towards the spores of both Bb1 and Ma2.

Chapter 6

In vitro impact of fungicides on the growth, viability and sporulation of two fungal isolates

6.1 INTRODUCTION

Agricultural inputs including fertilisers, insecticides, fungicides and herbicides can influence the efficacy of fungal entomopathogens (Klingen & Haukeland 2006). As such, numerous studies have been designed to assess the impact of these inputs on factors which are important for fungal infection and persistence in the field: germination, growth and sporulation (Oliveira *et al.* 2003; Shah *et al.* 2009; Celar & Kos 2012; Niassy *et al.* 2012). Fungicides are commonly applied in agricultural systems to prevent the growth of plant pathogenic fungi and have been suggested to be the least compatible pesticides with fungal biological control agents (Mochi *et al.* 2005; Demirci *et al.* 2011). Niassy *et al.* (2012) tested the compatibility of six insecticides, two acaricides and three fungicides with *M. anisopliae* ICIPE 69. As expected, the three fungicides reduced vegetative growth and sporulation to a far greater extent than the insecticides. The fungicide carbendazim completely inhibited fungal growth and sporulation, whilst the other two fungicides recorded vegetative growth of 11.1 and 13.1 mm and produced 1.2×10^8 spores/ml. This is much lower than when compared to the vegetative growth (ranging between 16.5 to 29.2 mm) and sporulation (2.9×10^8 to 14.3×10^8 spores/ml) recorded for the other agrochemicals tested and the control (Niassy *et al.* 2012).

The impact of fungicides is dependent on the chemical type, dose and fungal isolate and may vary between *in vitro* and *in vivo* experiments (Samson *et al.* 2005; Luz *et al.* 2007). Shah *et al.* (2009) found that of the 15 fungicides tested, most were inhibitory to *B. bassiana* growth,

but not *Lecanicillium longisporum* (Petch) Zare and Gams. Despite this, the virulence of *B. bassiana* towards the larvae of *Galleria mellonella* was not affected. However, the fungicide tolylfluanid reduced both the growth and virulence of *M. anisopliae*. Even within species, discrepancies are apparent amongst isolates. D'Alessandro *et al.* (2011) examined the influence of eight fungicides on two isolates of *Isaria fumosorosea* (Wize) Brown and Smith (Ascomycota: Hypocreales). Results indicated that although the same fungicides exhibited a similar toxicity pattern between isolates, the degree of toxicity varied. For example, although carbendazim was found to be the second most toxic fungicide, inhibiting the germination of both isolates, germination of isolate CEP 304 was far lower (36.07%) than isolate CEP 315 (62.48%). Other discrepancies between isolates exposed to procimidone and copper oxychloride were also apparent, but not to azoxystrobin, which had the greatest inhibitory effect on germination, which was similar for both isolates.

In citrus in South Africa, the plant pathogenic fungus *Phyllosticta citricarpa*, referred to as citrus black spot (CBS), causes superficial lesions on the fruit of all commercially produced citrus cultivars (Schutte 2009; Truter *et al.* 2010). Fruit showing symptoms of CBS infection are not suitable for export largely due to the phytosanitary status of this organism, rather than the cosmetic damage it causes (Carstens *et al.* 2012; Magarey *et al.* 2015). The USA will only import fruit from CBS-free areas (Western Cape, Northern Cape and Free State) whilst the European Union and Iran will import fruit from only either CBS-free areas or areas where CBS infested fruit has not been detected following official inspection (Carstens *et al.* 2012). As a result, growers follow mandatory fungicide programmes, particularly in orchards where fruit is destined for the EU. This is contrary to research, based on climatic modelling, which indicates that the establishment of CBS across the EU is likely limited (Magarey *et al.* 2015; *S.D. Moore, pers. comm.). As no research has been conducted on the potential impact of these fungicides on the biological traits of the fungal isolates under investigation, this chapter aims to provide initial insight by assessing the *in vitro* compatibility between eight fungicides (registered for use against CBS) and isolates Bb1 and Ma2. Toxicity of the fungicides on fungal viability, growth and sporulation was measured. Only isolates Bb1 and Ma2 were tested as these isolates were used in all field trials and are the isolates on which research is likely to continue.

6.2 MATERIALS AND METHODS

6.2.1 Fungicides

Eight fungicides registered for use in citrus against CBS were investigated (Table 6.1). For all experiments, fungicides were used at the recommended field rate (Schutte 2009) and dissolved in sterile distilled water before use. If more than one field rate was recommended, the highest rate was used.

Table 6.1: Fungicides used for the *in vitro* compatibility assessment with the entomopathogenic fungi *B. bassiana* (Bb1) and *M. anisopliae* (Ma2).

Trade name	Active ingredient	Formulation	¹ Rate /100 L	Manufacturer
Benomyl	benomyl	WP ²	75 g	Arysta LifeScience
Cabrio	pyraclostrobin	EC ³	10 ml	BASF Crop Protection
Copper oxychloride	copper oxychloride	WP	200 g	Villa Crop Protection
Dithane M45	mancozeb	WP	200 g	Dow AgroSciences
Flint	trifloxystrobin	SC ⁴	10 ml	Bayer CropScience
Fungaway	azoxystrobin	SC	20 ml	Villa Crop Protection
Pennfluid	mancozeb	SC	200 ml	Total
Sporekill	didecyldimethyl- ammonium chloride	SC	100 ml	Nufarm

¹highest registered application rate for use against CBS in citrus orchards, South Africa (Schutte 2009);
²wettable powder; ³emulsifiable concentrate; ⁴suspension concentrate

6.2.2 Fungal suspensions

The same dry spores of Bb1 and Ma2 that were used for the field trials were used for the compatibility experiments. These spores were held in air-tight containers in a fridge (4°C) when not in use. Spore suspensions were prepared by suspending a small quantity of dry spores into 20 ml sterile distilled water supplemented with 0.01% Tween 20. The suspension was well mixed by vortexing for five minutes, followed by one minute sonication to ensure

uniformity. The concentration of the stock suspension was determined using a haemocytometer and was used to prepare any dilutions necessary for the vegetative growth, spore viability and spore production experiments detailed below. Suspensions were used immediately after preparation.

6.2.3 Agar medium

Sabouraud dextrose agar (SDA) supplemented with 1 ml chloramphenicol (50 mg/ml) was used as the standard growth medium (non-amended medium). Amended media were prepared by autoclaving the desired quantity of agar in half the volume of distilled water. The same volume of distilled water was autoclaved separately and used to dissolve the fungicide at the desired rate once cooled. The agar and fungicide suspension were then mixed in a 1:1 ratio when the agar was still liquid, but cooled to $40 \pm 5^\circ\text{C}$. Approximately 20 ml was poured into 90 mm petri plates under sterile conditions. All prepared plates (amended or non-amended) were used within one day of preparation.

6.2.4 Experimental treatments

The viability, vegetative growth and sporulation of isolates Bb1 and Ma2 were determined on both amended media (prepared as described in section 6.2.3 above) and on non-amended media following a one hour exposure period to each fungicide at room temperature: 1 ml of each of the isolates at a concentration of 1×10^8 (vegetative growth) or 1×10^5 (viability) spores/ml was added to a 10 ml suspension of each fungicide prepared at this highest recommended field rate using sterile distilled water. A 1×10^5 spores/ml suspension was used for viability assessment as this produced the most accurately-countable colonies: when a 1×10^6 spores/ml suspension was used, a fungal lawn was recorded on control plates. When a 1×10^5 spores/ml suspension was used on control plates produced between 232 to 275 and 103 to 229 countable colonies for Bb1 and Ma2 respectively.

6.2.4.1 *Vegetative growth*

A 5 mm plug of unsporulated hyphae, taken from the growing region of a four day old fungal colony (incubated at 26°C, 60% humidity, D12:L12 photoperiod), was placed in the centre of each fungicide-amended agar plate. For exposure experiments, a 1 µl drop of mix was pipetted onto the centre of a non-amended agar plate after 1 h exposure at room temperature. Controls, grown on non-amended media, were included following the same procedure for amended and exposure experiments. Plates were sealed with Parafilm M[®] and incubated in a constant environment chamber at 26 ± 1°C and relative humidity of approximately 60 ± 5% on a D12:L12 light cycle. Two weeks post-incubation, the diameter of each colony was measured along two previously drawn orthogonal lines and the average radial growth used to compute the growth area in cm². Each treatment, including the control, was replicated five times and the entire procedure repeated on a separate occasion. The results of each repeat were pooled to yield a sample size of 10 for each treatment on which analysis was conducted. Treatment effects were determined by one-way ANOVA followed by Tukey's HSD post-hoc test ($P < 0.05$) if significant effects were found. In addition, the percentage expression in vegetative growth in response to each fungicide relative to the control was calculated.

6.2.4.2 *Viability*

Spore viability of each isolate was assessed using CFU analysis. The procedure followed was similar for both amended and exposure experiments. A 100 µl sample of a pure 1×10^4 conidia/ml suspension or 100 µl of the fungus-fungicide mix was spread using aseptic technique onto each of five replicate plates per treatment. Controls were included. Plates were sealed with Parafilm M[®] and incubated as described above. After four days, the number of viable colonies on each plate was counted and the CFUs/ml calculated. This procedure was repeated on a separate occasion, the results of which were pooled to yield a sample size of 10 for each treatment on which analysis was conducted. Treatment effects were determined by one-way ANOVA followed by Tukey's HSD post-hoc test ($P < 0.05$) if significant effects were found. In addition, the percentage expression in spore viability in response to each fungicide relative to the control was determined. Plates which yielded no viable colonies after four days were held for a further 10 days and assessed thereafter.

6.2.4.3 Sporulation

The plates used in these experiments were the same plates on which vegetative growth was assessed. Treatment plates on which fungi failed to grow were excluded from these experiments – Benomyl amended media for both isolates. Following completion of vegetative growth assessment, a 5 mm plug was taken from a densely sporulated area on each plate and suspended in 1 ml sterile distilled water supplemented with 0.01% Tween 20 housed within sterile 2.0 ml micro-centrifuge tubes. The tubes were vortexed until all the conidia had dislodged from the agar plug. The concentration of spores was then determined, following a dilution if necessary, with the aid of a haemocytometer. Two counts were made per plug and the average count was used to compute the number of spores/ml for each treatment (n=10). Treatment effects were determined by one-way ANOVA followed by Tukey's HSD post-hoc test ($P < 0.05$) if significant effects were found. In addition, the percentage expression in sporulation in response to each fungicide relative to the control treatment was determined.

6.2.5 Compatibility of Ma2 and Bb1 with tested fungicides: Alves's model

Alves *et al.* (1998) listed a model which may be used to classify the compatibility (T) of agrichemicals into categorical groups:

$$T = \frac{(20 \times VG) + (80 \times S)}{100}$$

whereby the values for vegetative growth (VG) and spore production (S) are expressed as percentages relative to the control to generate T values between 0 to 100. Chemicals with T values ranging between 0 to 30, 31 to 45, 46 to 60, 61 to 90 and > 90, are considered very toxic, toxic, moderately toxic, compatible and highly compatible, respectively.

6.3 RESULTS

6.3.1 *Beauveria bassiana* isolate Bb1

All results concerning the impact of each fungicide (amended and exposed) on the viability, vegetative growth and sporulation of isolate Bb1, are represented in Table 6.2.

On amended media, significant treatment effects were recorded for vegetative growth ($F_{8, 81} = 95.38$, $P < 0.00001$), viability ($F_{8, 81} = 202.75$, $P < 0.00001$) and sporulation ($F_{7, 72} = 41.19$, $P < 0.00001$). No viable colonies were recorded on media amended with Benomyl, Cabrio, Dithane and Pennfluid. Statistically, only copper oxychloride did not have a toxic effect on spore viability. All fungicides had an adverse effect on vegetative growth. Benomyl prevented growth completely. Flint was the least toxic, with a colony size half that of the control. All fungicides allowed sporulation to occur, but was significantly reduced in comparison to the control, with the exception of Sporekill. Copper oxychloride and Dithane were most inhibitory to spore production.

When grown on non-amended media after a one hour exposure period to each fungicide, significant treatment effects were recorded for vegetative growth ($F_{8, 81} = 11.39$, $P < 0.00001$) and spore viability ($F_{8, 81} = 168.82$, $P < 0.00001$). In comparison to the results recorded on amended media, the toxicity of most of the fungicides was reduced. No viable colonies were recorded after exposure to Sporekill and limited numbers after exposure to Dithane. Exposure to Benomyl, copper oxychloride, Flint and Fungaway recorded viable counts statistically similar to the control. Cabrio and Pennfluid which produced no viable spores on amended media produced 53.7% and 71.0% of the control count. No fungicide completely inhibited fungal growth. Copper oxychloride and Pennfluid produced average colony sizes not statistically significant to the control. Dithane had the greatest adverse impact on colony size. Statistically, no fungicides had a negative impact on spore production in comparison to the control ($F_{8, 81} = 1.77$, $P = 0.095$). Cabrio and copper oxychloride inhibited spore production the most; Flint and Fungaway the least.

6.3.2 *Metarhizium anisopliae* isolate Ma2

All results concerning the impact of each fungicide (amended and exposed) on the viability, vegetative growth and sporulation of isolate Ma2, are represented in Table 6.3.

On amended media, significant treatment effects were recorded for vegetative growth ($F_{8, 81} = 278.30$, $P < 0.00001$), viability ($F_{8, 81} = 283.79$, $P < 0.00001$) and sporulation ($F_{7, 72} = 33.79$, P

< 0.00001). No viable colonies were recorded on media amended with Benomyl, Cabrio, Dithane and Pennfluid, with limited colonies recorded on media amended with Sporekill. Copper oxychloride and Flint were not inhibitory to viability, whilst Fungaway had a minimal adverse impact on spore viability. Statistically, all fungicides significantly inhibited vegetative growth in comparison to the control. No growth was recorded on media amended with Benomyl, with limited growth recorded on media amended with all other fungicides. Flint and Sporekill were the least toxic. Sporulation was significantly reduced by all fungicides except Sporekill. Copper oxychloride was the least toxic.

When grown on non-amended media after a one hour exposure period to each fungicide, significant treatment effects were found for vegetative growth ($F_{8, 81} = 11.39$, $P < 0.00001$), spore viability ($F_{8, 81} = 168.82$, $P < 0.00001$) and sporulation ($F_{7,72} = 33.79$, $P < 0.00001$). In comparison to the results recorded on amended media, the toxicity of most of the fungicides was reduced. In comparison to the control, only Dithane, Pennfluid and Sporekill significantly hindered viability. Exposure to Benomyl and Flint recorded increases in viable counts, although not significantly different to the control. Only Cabrio and Dithane significantly reduced vegetative growth in comparison to the control. This was more pronounced for Dithane, where colony size was only 6.1% of the control. Dithane was also the only fungicide to significantly reduce spore production whilst exposure to Flint significantly increased the quantity of spores produced.

Table 6.2: Toxicity of each fungicide on the viability, vegetative growth and sporulation of *B. bassiana* isolate Bb1 when grown on fungicide amended media and non-amended media after one hour exposure to each fungicide. Means (\pm standard errors) are presented. The percentage expression of each trait relative to the control is presented underneath in bold. Percentages range between 0% and 100%, with 100% representing a response identical to or greater than the control.

Fungicide	Viability ($\times 10^7$ CFUs/ml)		Vegetative growth (area in cm ²)		Sporulation ($\times 10^7$ spores/ml)	
	Amended	Exposed	Amended	Exposed	Amended	Exposed
Control	2.65 (0.13) a ¹	2.55 (0.04) a	9.29 (0.52) a	9.29 (0.40) ac	13.58 (1.26) a	9.28 (1.57) a
Benomyl	0.00 (0.00) b 0%	2.29 (0.13) a 89.8%	0.00 (0.00) e 0%	5.95 (0.56) bd 64.0%	- ²	8.01 (0.84) a 86.3%
Cabrio	0.00 (0.00) b 0%	1.37 (0.04) b 53.7%	0.85 (0.09) be 9.1%	5.44 (0.45) bd 58.6%	4.03 (0.85) bd 29.7%	6.47 (0.89) a 69.7%
Copper	2.58 (0.10) a 97.4%	2.43 (0.06) a 95.3%	3.10 (0.35) d 33.4%	9.64 (0.27) a 100%	0.13 (0.004) c 0.9%	5.73 (0.79) a 61.8%
Dithane	0.00 (0.00) b 0%	0.17 (0.02) d 6.7%	1.67 (0.38) b 17.9%	4.00 (0.84) b 43.1%	0.52 (0.14) bc 3.8%	8.22 (1.07) a 88.6%
Flint	2.18 (0.05) c 82.3%	2.50 (0.06) a 98.0%	4.56 (0.24) c 49.1%	6.35 (0.59) bd 68.4%	4.12 (0.84) d 30.3%	10.24 (1.05) a 100%
Fungaway	0.99 (0.19) d 37.4%	2.60 (0.05) a 100%	3.61 (0.34) cd 38.8%	6.75 (0.50) cd 72.6%	2.41 (0.63) bcd 17.7%	8.69 (1.28) a 93.6%
Pennfluid	0.00 (0.00) b 0%	1.81 (0.15) c 71.0%	0.90 (0.10) be 9.7%	9.13 (0.26) ac 98.3%	2.24 (0.32) bcd 16.5%	7.65 (0.69) a 82.4%
Sporekill	0.03 (0.01) b 1.1%	0.00 (0.00) d 0%	2.96 (0.07) d 31.9%	5.42 (1.02) bd 58.3%	12.26 (1.28) a 90.3%	7.96 (0.65) a 85.8%

¹ Different letters denote statistically significant differences within each grouping (Tukey's HSD test, $P < 0.05$)

² Spore counts could not be made due to the inability of the fungus to grow when cultured on Benomyl-amended media and was thus excluded from statistical analysis.

Table 6.3: Toxicity of each fungicide on the viability, vegetative growth and sporulation of *M. anisopliae* isolate Ma2 when grown on fungicide amended media and non-amended media after one hour exposure to each fungicide. Means (\pm standard errors) are presented. The percentage expression of each trait relative to the control is presented underneath in bold. Percentages range between 0% and 100%, with 100% representing a response identical to or greater than the control.

Fungicide	Viability ($\times 10^7$ CFUs/ml)		Vegetative growth (area in cm ²)		Sporulation ($\times 10^7$ spores/ml)	
	Amended	Exposed	Amended	Exposed	Amended	Exposed
Control	1.94 (0.11) a ¹	1.66 (0.18) a	38.14 (1.24) a	43.49 (0.54) a	18.80 (2.91) a	6.63 (0.45) ac
	-	-	-	-	-	-
Benomyl	0.00 (0.00) b 0%	1.67 (0.20) a 100%	0.00 (0.02) b 0%	39.33 (2.77) ab 90.4%	- ²	6.51 (0.38) ac 98.2%
Cabrio	0.00 (0.00) b 0%	1.02 (0.17) a 61.4%	0.67 (0.07) b 1.7%	33.35 (0.95) b 76.7%	0.02 (0.005) b 0.1%	5.83 (0.63) ac 87.9%
Copper	1.78 (0.009) ac 91.8%	1.54 (0.17) a 92.8%	6.33 (0.29) c 16.6%	42.62 (0.35) a 97.9%	4.04 (0.59) b 21.5%	5.19 (0.57) ac 78.3%
Dithane	0.00 (0.00) b 0%	0.001 (0.0006) b 0.06%	4.00 (0.25) ce 10.5%	2.64 (1.87) c 6.1%	0.91 (0.22) b 4.8%	2.33 (0.49) b 35.1%
Flint	2.02 (0.006) a 100%	1.67 (0.22) a 100%	12.56 (1.58) d 32.9%	38.60 (0.34) ab 88.6%	0.024 (0.01) b 0.1%	7.16 (0.53) c 100%
Fungaway	1.59 (0.007) c 82.0%	1.62 (0.17) a 97.6%	6.59 (0.22) c 17.3%	38.06 (1.46) ab 87.5%	0.015 (0.004) b 0.08%	4.53 (0.19) ab 68.3%
Pennfluid	0.00 (0.00) b 0%	0.22 (0.003) b 13.2%	2.33 (0.09) be 6.1%	38.22 (1.18) ab 87.9%	0.44 (0.16) b 2.3%	5.26 (0.74) ac 79.3%
Sporekill	0.006 (0.003) b 0.03%	0.006 (0.0008) b 0.36%	13.57 (0.51) d 35.6%	37.95 (2.21) ab 87.3%	16.11 (2.35) a 85.7%	4.82 (0.52) ac 72.7%

¹ Different letters denote statistically significant differences within each grouping (Tukey's HSD test, $P < 0.05$)

² Spore counts could not be made due to the inability of the fungus to grow when cultured on Benomyl-amended media and was thus excluded from statistical analysis.

6.3.3 Alves's compatibility model

On amended media, all fungicides, with the exception of Sporekill, were toxic or very toxic to both Bb1 and Ma2 based on the model provided by Alves *et al.* (1998). Sporekill was classified as compatible with both fungal isolates. The opposite outcome was observed following a one hour exposure to each fungicide: most were compatible, with Flint being highly compatible with both isolates. Only Dithane was classified as very toxic to Ma2 (Table 6.4).

Table 6.4: Compatibility (T) of tested fungicides with *Beauveria bassiana* Bb1 and *Metarhizium anisopliae* Ma2 on amended and non-amended media following brief exposure, according to Alves's model (Alves *et al.* 1998).

Fungicide	Bb1				Ma2			
	Amended		Exposed		Amended		Exposed	
	T	outcome	T	outcome	T	outcome	T	outcome
Benomyl	0	Very toxic	81.8	Compatible	0	Very toxic	96.6	Highly compatible
Cabrio	25.6	Very toxic	67.5	Compatible	0.4	Very toxic	85.7	Compatible
Copper	7.4	Very toxic	69.4	Compatible	20.5	Very toxic	82.2	Compatible
Dithane	6.6	Very toxic	79.5	Compatible	5.9	Very toxic	29.3	Very toxic
Flint	34.1	Toxic	93.7	Highly compatible	6.7	Very toxic	97.7	Highly compatible
Fungaway	21.9	Very toxic	89.4	Compatible	3.5	Very toxic	72.1	Compatible
Pennfluid	15.1	Very toxic	85.6	Compatible	3.1	Very toxic	81.0	Compatible
Sporekill	78.6	Compatible	80.3	Compatible	75.7	Compatible	75.7	Compatible

6.4 DISCUSSION

The variable response of eight fungicides registered for use against CBS on citrus in South Africa on the viability, vegetative growth and sporulation of two fungal isolates under investigation for FCM control, was reported. Inhibition of any of these factors may reduce efficacy and persistence of the applied entomopathogen: germination is needed for infection to occur, whilst vegetative growth is an important pre-requisite for sporulation. Failure to sporulate may significantly reduce persistence (Jaros-su *et al.* 1999; Inglis *et al.* 2001; Shah & Pell 2003). On amended media most fungicides were toxic to both Ma2 and Bb1, limiting viability, vegetative growth and sporulation. Vegetative growth was the most adversely impacted trait recorded for both isolates. These results are similar to a study conducted by Bruck (2009) evaluating the influence of fungicides commonly applied against soil-borne plant pathogens on the viability and mycelial growth of *M. anisopliae* F52 applied for control of black wine weevil, *Otiorhynchus sulcatus* F. (Coleoptera: Curculionidae) in container-grown ornamentals. Of the 17 fungicides which were evaluated *in vitro*, only eight were found to show a significant reduction in the viability of *M. anisopliae* F52 when compared to the untreated control, whilst 14 were found to show a significant reduction on mycelial growth when grown on fungicide-amended media. Those fungicides that impaired germination, also impaired mycelial growth. This included the strobuline fungicides: azoxystrobin, pyraclostrobin and trifloxystrobin investigated in this study. Bruck (2009) found that azoxystrobin reduced germination and mycelial growth by approximately 67% and 92%, respectively relative to the control. Pyraclostrobin completely inhibited germination and reduced mycelial growth by 94%, whilst trifloxystrobin was the least toxic of the three strobulines to germination, reducing spore germination by only 14%. However, mycelial growth was reduced by an approximate 85% relative to the untreated control (Bruck 2009).

In this study, all three strobulines were also found to be highly toxic *in vitro* to the viability and mycelial growth of *M. anisopliae*, pyraclostrobin the most, azoxystrobin the least. Cabrio (a.i. pyraclostrobin) was also the most toxic strobuline to isolate Bb1, more so than Ma2, and was one

of the most toxic fungicides of all eight tested on both amended and non-amended media following exposure. Fungaway (a.i. azoxystrobin) was similarly toxic to Cabrio on all the biological traits assessed, the viability of Ma2 being the exception. However, this similarity was only observed on amended media and in contrast to Cabrio, trait inhibition was never 100%. Azoxystrobin has been reported to completely inhibit the germination of *M. anisopliae* and *L. longisporum* whilst severely limiting the germination of *Isaria fumosorosea*, but not *B. bassiana*, although mycelial growth was reduced (Shah *et al.* 2009). D'Alessandro *et al.* (2011) also observed azoxystrobin to be inhibitory to the germination and growth of *I. fumosorosea*. Overall, Flint (trifloxystrobin) was the least toxic strobilene to both isolates on amended media. Flint and Fungaway were similarly non-toxic to both isolates in exposure experiments. Interestingly, Flint caused an approximate 10% increase in spore production of both Ma2 and Bb1, and increased the viability of Ma2 on both amended media after exposure, suggesting a degree of synergism between this fungicide and the spores of Bb1 and Ma2. An increase in viability was also recorded for Bb1 after brief exposure to Fungaway.

Niassy *et al.* (2012) found that of the three fungicides tested, carbendazim (same chemical family as Benomyl, benzimidazol), fully inhibited the growth of *M. anisopliae* on amended media, whilst Propineb (same chemical family as mancozeb, dithiocarbamate) and copper hydroxide (same chemical family as copper oxychloride, copper-based) significantly reduced growth relative to the control. All fungicides also significantly reduced spore production. In contrast to the results of this study, Propineb was less toxic than copper hydroxide. Similarly, Benomyl-amended media inhibited the production of viable colonies and mycelial growth of Bb1 and Ma2. Lack of growth on Benomyl-amended media was not unexpected. It is well known that continuous exposure to even low concentrations of Benomyl can significantly reduce hyphal growth by inhibiting mitosis (Wraight *et al.* 2001). Carbendazim was also reported as one of the most toxic fungicides, limiting germination and mycelial growth of two isolates of *I. fumosorosea* and unformulated conidia of *M. anisopliae* (Lopes *et al.* 2011; D'Alessandro *et al.* 2011). The dithiocarbamates, Dithane and Pennfluid, were equally toxic on amended media and similar to Benomyl. Mancozeb toxicity towards EPF is well documented (Todorova *et al.* 1998; Majchrowicz & Poprawski 1993; Kouassi *et al.* 2003; Demirci *et al.* 2011) whilst copper-based

fungicides are typically non-toxic (Olmert & Kenneth 1974; Demirci *et al.* 2011; Demirci & Denizhan 2010; D' Alessandro *et al.* 2011). Copper oxychloride was highly inhibitory to vegetative growth and spore production of Bb1 and Ma2 on amended media, but not spore viability. The toxic effects of copper-based fungicides on *B. bassiana* and *M. anisopliae* development have been reported (Kouassi *et al.* 2003; Rachappa *et al.* 2007).

Dithane (a.i. mancozeb) and Sporekill (a.i. didecyldimethyl-ammonium chloride) significantly reduced spore viability on both amended and non-amended media following exposure for both isolates. The lack of viability recorded for these fungicides would suggest their incompatibility with either isolate. For Dithane, this is supported for Ma2 as it is classified as very toxic regardless of whether constant exposure or brief exposure to the fungicide occurred. This was not supported for Bb1 after brief exposure to Dithane, nor after both isolates were exposed to Sporekill either continuously or briefly. According to Alves's model, classification was compatible. Neves *et al.* (2001) have suggested that the vegetative growth component of this model be replaced by germination, particularly considering that most commercial mycopesticides incorporate conidia as their infective propagules rather than mycelia (Faria & Wraight 2007). It is also an essential first step in the infection process (Inglis *et al.* 2001). Germination was not measured in this study, rather spore viability, as only viable colonies are visible following incubation. Germination may still have been possible, but not mycelial growth. Therefore the fungicides which inhibited viability may not necessarily have prevented germination. Since all colonies were capable of mycelial growth on fungicide amended media, except Benomyl, and considering that plates were held for two weeks post-inoculation, the lack of viable colonies suggests that germination was strongly inhibited.

Other studies have classified chemical toxicity based solely on mycelial growth (Celar & Kos 2012; Sterk *et al.* 2003). Based on this classification system, all fungicides tested are moderately harmful to harmful towards Ma2 and Bb1 when grown on amended media, an outcome similar to Alves's model. Accordingly, Dithane was considered harmful to Ma2, whilst all other fungicides were considered harmless when fungicide exposure was brief. Contrastingly, only two fungicides

were considered harmless to Bb1 after brief exposure, copper oxychloride and Pennfluid. All others were considered slightly harmful except Dithane which was classified as moderately harmful. Although Sterk *et al.* (2003) suggest that the results obtained should not be generalised as toxicity was only based on mycelial growth and not spores or any other factors which may restrict efficacy in the field, it is interesting to note the discrepancies and similarities (particularly Dithane toxicity to Ma2) between the systems. It also suggests that using a classification system on which to base a recommendation regarding the compatibility between agrichemicals and beneficial biological control agents, should not be made lightly.

The different response of both isolates to Pennfluid and Dithane found in this study is also interesting to note, although reasons for the discrepancy are unclear. Both fungicides incorporate mancozeb as their active ingredient (at the same concentration) and are applied at the same rate, but differ in formulation: Dithane is a wettable powder, Pennfluid a suspension concentrate formulated in oil. Yet, during exposure experiments, Dithane was far more toxic than Pennfluid. For example, Dithane reduced the viability of Bb1 by approximately 90% whilst Pennfluid only 30%. Similarly the growth of Bb1 and Ma2 was reduced by approximately 55% and 94% respectively after exposure to Dithane, but only 2% and 12%, respectively for Pennfluid. Thus, the mineral oil with which Pennfluid is formulated may have afforded the spores some protection against the toxic effects of the active ingredient, mancozeb.

In essence, the results presented in this study can be viewed as representative of the ‘worst-case’ (amended media) and ‘best-case’ (exposure) responses of isolates Ma2 and Bb1 when exposed to the tested fungicides at the highest recommended field rate. No toxic effects on amended media, where fungi are in constant exposure to the fungicides, would suggest limited adverse impacts in the field, whilst toxicity after only 1 hour exposure would suggest the non-compatibility of the isolate with the fungicide, at that concentration. If this is the case, and based on the model presented by Alves *et al.* (1998) only Dithane is totally incompatible with Ma2 whilst Sporekill possess no threat to either isolate. However, although laboratory studies, like this one, can provide useful information regarding the simultaneous use of fungicides with fungal

entomopathogens, and may help explain failures in the field, basing recommendations solely on *in vitro* assessments of biological traits is not encouraged (Wraight *et al.* 2001). Reasons for this are discussed in Chapter 7, section 7.2.3.2.

In conclusion, this study highlights the variable response of Ma2 and Bb1 to eight fungicides registered for use in citrus orchards across South Africa. On amended media, all fungicides, with the exception of Sporekill on sporulation, were toxic. Toxicity was greatly reduced following brief exposure raising the question as to whether these fungicides, under more realistic conditions, will have an effect on efficacy and persistence. Based on the results of this study, only Dithane appears to be completely incompatible with Ma2, reducing all aspects of the biological traits assessed on both amended media and non-amended media after brief exposure. However, the use of Cabrio, Dithane and Sporekill with Bb1 is cautioned as these fungicides reduced two of the three traits tested, viability and vegetative growth, relative to the control following brief exposure.

Chapter 7

General discussion

7.1 THESIS SUMMARY

FCM is a key economic pest of citrus in South Africa. In addition to pre- and post-harvest fruit damage, some lucrative export markets have a strict no entry policy towards this pest as it is a regulated phytosanitary organism (Moore 2012; Grout & Moore 2015). Until recently, most control measures were targeted against FCM above-ground life stages (eggs, neonates, adults) leaving the below-ground life stages (late fifth, pre-pupae, pupae, eclosing adults) relatively untouched (Grout & Moore 2015). EPF are ubiquitous soil-borne microbes that have been used successfully to control a variety of pest species in an assortment of habitats across the globe (Inglis *et al.* 2001; Shah & Pell 2003; Faria & Wraight 2007). The potential use of EPF to aid as an additional biological control agent of FCM, targeting the soil-dwelling life stages, has been under extensive investigation following the isolation of a suite of potential fungal entomopathogens from soil samples collected from six citrus orchards in the Eastern Cape Province, South Africa (Goble *et al.* 2010). Further laboratory-based bioassays highlighted three fungal isolates, two *Metarhizium anisopliae* and one *Beauveria bassiana*, as the most virulent against FCM fifth/pre-pupating instars and were found to persist well under semi-field conditions (Goble *et al.* 2011, Coombes *et al.* 2013, 2015).

This study has successfully highlighted the field potential of these isolates in reducing FCM infestation within citrus orchards located in the Eastern Cape Province, South Africa. It is now known that these isolates are capable of persisting and performing effectively under field conditions. Based on the results of the cage trial (Chapter 2) and the similar performance of isolate Ma1 to isolate Ma2 during field trials (Chapter 3), the former may be less suited for field application than isolates Ma2 and Bb1. Field efficacy, measured as a reduction in FCM fruit

infestation relative to a control, was variable amongst sites ranging between 28.32% and 81.72% upon trial completion. Highest efficacy was recorded for isolate Bb1 applied to soil under micro-jet irrigation before peak-larval descent into the soil at a rate of 1×10^{14} spores/ha. Field results further suggested that isolate Bb1 was more effective under conditions of moderate soil moisture (range: 20.9% to 51.7%), whilst Ma2 was more effective when soil moisture was low (range: 6.8% to 14.3%). All isolates persisted when soil moisture was moderate and host density high (Atmar 1) showing an increase in fungal concentration towards the end of the trial to reach concentrations similar to those recorded one week post-application. Under conditions of low soil moisture (Atmar 2) persistence was fairly stable for the first few months for isolate Bb1, but declined significantly thereafter despite moderate host density. The fungal concentration of Ma2, under these conditions, decreased steadily following application. This suggested that sporulation on fungal-killed cadavers to be limited. Whether this influenced the lack of continual suppression of FCM at Atmar 2 across the citrus growing season is unclear. An increase in fungal concentration was reported for all three isolates applied at Atmar 1, but only Bb1 maintained continual FCM suppression.

It was considered possible that the application of agrichemicals played a role in decreasing fungal concentration following application. Shortly before or after fungal application, four fungicides tested in this study (Pennfluid, Benomyl, Fungaway and copper oxychloride) were applied to the foliage of the citrus trees within the same orchard block(s) to which fungi were applied to the soil. The results of Chapter 6 would suggest that although all of these fungicides are toxic to fungal viability, mycelial growth and sporulation when grown on fungicide-amended media *in vitro*, toxicity is greatly reduced when exposure is brief. This suggests that the influence of these applied fungicides on fungal efficacy in the field may be limited especially given their spatial separation which may result in a lack of contact or reduce the fungicide dose to which the applied fungi are exposed. Numerous studies have reported the positive influence of reducing the dose of agricultural products on fungal germination and growth, although exceptions are apparent depending on the chemical and fungus (Todovora *et al.* 1998; Oliveira *et al.* 2003; Oliveira & Neves 2004; Alizadeh *et al.* 2007; Shah *et al.* 2009; Aby *et al.* 2015). Despite fungicide application (see Appendix 3) occurring shortly before (October) and after

(December/January) fungi were applied, good field results were obtained at Atmar 1 and 2 (Chapter 3), supporting the likelihood of limited impact of fungicides on fungal efficacy in the field, when applied to the soil. Other agrichemicals were also applied during the field trials at each site and therefore, although their influence on fungal efficacy has not been quantified, it too is unlikely that these applied chemicals had a significant impact in the field, as spray programmes were similar amongst sites, but field efficacy and persistence were variable. Therefore, field efficacy is likely to be governed more by environmental conditions than commonly used agricultural chemicals. This is supported by the lack of informative results obtained at Oranjelus due to low host density and the discrepancy in fungal persistence and efficacy at Atmar 1 and Atmar 2, most likely due to differences in soil moisture.

Isolate Bb1 was previously identified as the most laboratory-virulent isolate (Coombes *et al.* 2015) and in this study, was also the most virulent isolate during field-cage trials and was the isolate which obtained the highest reduction in FCM infestation during field trials after application (80%). Isolate Ma2 was only capable of reducing FCM infestation by a maximum of 75%, measured 15 WAT, with control efficiency decreasing to 63% 30 WAT. Therefore isolate Bb1, which has also shown good control potential of above-ground pest species such as citrus mealybug, red scale and citrus thrips in the laboratory compared to Ma2 (Wiblin & Hill 2013; Chartier Fitzgerald 2014), is preferred for future development. However, the performance of isolate Bb1 at Atmar 2 applied to soil under drip irrigation was not ideal, especially since fungi were applied at a rate which may be considered too high to be economically feasible (see section 7.2.1 below). In this study, fungi were applied unformulated with the aid of the surfactant Breakthru[®] S240 (Evonik Industries, South Africa). It remains to be determined whether the use of a formulated product incorporating Bb1 as the active ingredient will overcome this potential shortcoming. Although an investigation into the influence of select adjuvants, for use as wetting agents, on the spore viability and suspension characteristics of Bb1 and Ma2 was undertaken, results provided minimal input into the enhancement of these isolates in the field. This laboratory-based research suggested that no wetting agents tested would significantly hamper or improve the suspension characteristics of Bb1 or Ma2, as results were similar amongst the adjuvants tested. Unfortunately, no bioassays assessing whether any of these adjuvants promoted

fungal efficacy against FCM soil-dwelling life stages either by weakening the insect cuticle and thus promoting fungal penetration or improving spore attachment to the insect cuticle, have been conducted yet.

The use of these isolates as a mycopesticide has been under development for the past approximately seven years and is progressing well. This study has begun to investigate aspects of commercialisation which are important for product development and implementation; the final phases of commercialisation (see Figure 1.8, Chapter 1, page 3). Although these investigations are still in their infancy, results thus far have been positive and have highlighted aspects of research which require further attention if the development of a cost-effective product capable of providing a consistent level of FCM control after application, is to be achieved. These are discussed below.

7.2 IMPORTANT CONSIDERATIONS FOR FUTURE DEVELOPMENT OF EPF AGAINST FCM

7.2.1 Optimal application rate

In this study, the highest experimental rate used was 2×10^{14} spores/ha (Chapter 2, semi-field cage trials). As previously mentioned, although Abbott-corrected mortality at this rate was highly desirable (90%, 90% and 95% for isolates Ma1, Ma2 and Bb1 respectively), the feasibility of applying fungi at this rate to larger areas becomes impractical due to the cost of conidial production (Mulock & Chandler 2000). However, an acceptable level of control was achieved at the lower application rate of 1×10^{14} spores/ha for two of the three isolates. Thus, this rate was used as the highest experimental rate during field trials. It is acknowledged that if commercialisation of these isolates is to proceed, this application concentration may still be too high to permit cost-effective production. However, this may only be truly possible to discern once a product formulation and production parameters have been finalised.

In a review on the ecological factors which can influence the efficacy of applied fungal entomopathogens, Jaronski (2010) stated that, based on laboratory and field collected data, virulent fungal isolates need to be applied at approximately 10^5 to 10^6 CFUs/g soil to achieve a satisfactory level of pest control. This equates to an application rate of between 10^{14} to 10^{15} spores/ha. Thus, the highest experimental rate used in field trials is validated and acceptable. However, published field trials using EPF as the control agent typically use application rates ranging between 10^{12} to 10^{13} spores/ha, 5×10^{13} spores/ha representing the upper limit (Rath *et al.* 1995; Poprawski *et al.* 1999; Ekesi *et al.* 2005; Shi *et al.* 2008; Filho *et al.* 2011; Gatarayiha *et al.* 2011; Güerri-Agulló *et al.* 2011; Wraight & Ramos 2015). Therefore, 1×10^{14} spores/ha falls marginally outside this typical range. Acceptable efficacy within this application range was often either achieved with the use of a formulated product, multiple sprays or a combination of both. Neither a formulated product of these isolates nor the use of multiple sprays was employed in this study. Given that the efficacy of these isolates in the field were not previously known and coupled with the fact that application of EPF to the soil environment often relies strongly on a high application rate to guarantee that the target pest species comes into contact with enough infectious propagules to ensure fungus-induced mortality, the use of this rate is supported for experimental purposes, but is not economically viable long-term. Based on current production parameters, it was estimated that applying these isolates at 1×10^{14} spores/ha would cost ZAR 35 000 per hectare. This is unaffordable. Application at 1×10^{13} spores/ha becomes more feasible (ZAR 3 500), but is still costly (*S.D. Moore, pers. comm.). Therefore, if these isolates are to be successfully commercialised, it is essential that future research be focused greatly on reducing the quantity of fungal spores required to achieve a good level of FCM control following application.

The efficacy of unformulated conidia of Bb1 and Ma2 applied at rates lower than 1×10^{14} spores/ha was attempted – one field trial evaluated the efficacy of these lower rates when Bb1 and Ma2 were applied prior to peak larval descent into the soil and another the efficacy of only Bb1 applied at lower rates closer to fruit harvest. The former trial rendered inconclusive results due to low FCM infestation within the treated area during the time the trial was conducted. As such, the use of more acceptable application rates in controlling FCM populations in citrus

orchards when applied early in the season remains unknown. The second trial did not assess the performance of Ma2 at reduced rates when applied later in the growing season. However, Bb1 applied at all three rates (1×10^{12} , 1×10^{13} and 1×10^{14} spores/ha) reduced FCM infestation similarly in treated areas relative to the untreated control with the greatest reduction recorded in areas to which Bb1 was applied at 1×10^{13} spores/ha. This suggests that the use of a more cost-effective application rate is possible even when no formulation is considered. Moving forward, defining the most cost-effective application rate is a key area of focus and may only be truly possible once a suitable formulation is identified and once production processes have been optimised.

It is possible to reduce the number of spores applied in order to maintain an efficacious level of pest control, particularly when the target species is restricted to a specific location [e.g. neonate sugarbeet root maggot (Jaronski *et al.* 2005)], can be attracted to auto-dissemination devices [e.g. *Ceratitis capitata* (Navarro-Lopes *et al.* 2015)] or come into contact with treated areas [e.g. rangeland grasshoppers (Lockwood *et al.* 2001)] and be attracted to baits formulated with toxic concentrations of the fungus [e.g. houseflies (Geden *et al.* 1995)]. The life stage of FCM targeted by fungal application to the soil is the late fifth/ early pupating instars, negating the potential use of food attractants or sex-based pheromone attractants. Neither mating nor feeding is important at this time, merely pupation. In addition, a laboratory study has highlighted the lack of a preferential pupation site, limiting the likelihood of targeting specific areas (Love 2015). Thus, the control of FCM soil-dwelling life stages will be dependent on broadcast application, targeting the entire soil arena in which pupation could potentially occur (i.e. the soil area underneath the canopy of the citrus trees). However, targeting a specific area may be possible to a certain extent. Love (2015) found that FCM tend to pupate within close proximity to where they landed upon dropping to the soil below when nearing pupation. Therefore, it is possible to reduce the targetable area to the soil surface only directly underneath the tree canopy, excluding the open area between trees (which was sprayed in this study). This approach is further strengthened by the fact that pupating FCM have been reported to prefer shaded areas (i.e. directly underneath the tree canopy) over unshaded areas for pupation (i.e. area between trees) and that shade offers a

level of protection against the potentially harmful effects of UV radiation on fungal efficacy and persistence (Willoquet *et al.* 1996; Inglis *et al.* 1997; Love 2015).

Another outcome arising from the study by Love (2015), which could potentially be used to reduce the number of spores applied, is that artificially introduced FCM typically pupated on the soil surface and never below 1 cm depth regardless of the abiotic conditions measured: soil texture class, ground cover, shading, soil compaction, ambient air temperature and soil moisture. In this study it is possible that the applied fungal spores percolated to depths greater than 1 cm. Therefore, although application occurred at 1×10^{14} spores/ha, the actual concentration to which FCM would have been exposed may have been reduced. If this is the case, and assuming that the results of the laboratory study by Love (2015) are reflected in the field, then it may be possible to promote the retention of spores within the upper 1 cm soil zone through formulation. Formulation also has the potential to protect the applied fungal propagules from adverse environmental variables or conditions associated with spraying which may reduce spore viability shortly after application (Jackson *et al.* 2010; Ravensberg 2011). A reduction in spore viability is problematic as ideally, fungal application aims to target the bulk of FCM initially entering the soil, as a preventive approach (early season application) rather than curative (late season application). Thus, a formulation which can enhance spore viability within the first month of application may also facilitate a reduction in the rate which needs to be applied to ensure an infectious level of dose-transfer from the soil arena to the cuticle of FCM whilst maintaining the same level of efficacy obtained in this study.

7.2.2 Formulation

Formulation has a variety of functions, including product stability during storage and improved field efficacy (Ravensberg 2011). Numerous formulations exist with the type of formulation chosen dependent largely on the target species and the environmental variables experienced in the area of application (Hynes & Boyetchko 2006; Jackson *et al.* 2010; Gašić & Tanović 2013). The fungal isolates investigated in this study were applied as unformulated conidia. Although application was successful, formulation has been reported to boost efficacy (Wraight *et al.* 2001;

Vega-Aquino *et al.* 2010; Ekesi *et al.* 2011). For example, Luz *et al.* (2011) found that *M. anisopliae* formulated with diatomaceous earth and a vegetable oil, synergised the insecticidal effect of the fungus on *Triatoma infestans* Klug (Hemiptera: Reduviidae) nymphs; formulated, *M. anisopliae* caused 75% mortality compared to 25% when unformulated conidia were used. In some cases, without a suitable formulation, fungal application is not economically viable. For example, the further development of Green Muscle[®] was only possible as a result of the discovery that the use of an oil formulation maintained the viability of the fungus under dry, semi-arid conditions (Prior & Greathead 1989; Bateman *et al.* 1993).

A formulation for the soil application of EPF should be able to increase the efficacy of the applied entomopathogens, be easily applied with conventional agriculture equipment, provide an acceptable shelf-life during storage and ideally facilitate the consistent fungal efficacy following application (Jackson *et al.* 2010; Ravensberg 2011). Granular formulations are commonly used when the soil environment is targeted (Wraight *et al.* 2001). Granules, typically mycelial-based, are favourable as they allow for the addition of a large number of adjuvants to improve field efficacy and provide a nutritive area on which fungal sporulation can occur increasing the initial amount of infectious propagules applied to the soil environment (Wraight *et al.* 2001). Granules have been used successfully to control various soil-dwelling insect pests, including the black vine weevil (Booth & Shanks 1998), cockchafers (Rath *et al.* 1995) and corn rootworms (Kreuger & Roberts 1997). The application of granules appears to be common during pre-planting of annually cropped agricultural crops such as maize, and therefore their use in citrus orchards may be limited due to difficulties which could be faced during application as a result of the tree canopy. Even if granules were shown to enhance field efficacy, its adoption by growers should be carefully considered. It may be far less labour intensive and cheaper to apply the fungus via the micro-sprinkler irrigation system for instance. In addition, while granules allow for sporulation, the dispersal of these spores, which is passive, may be restricted to areas within close proximity to the applied granule. Therefore, even though an insect pest such as FCM entering the soil to pupate may need only to brush against one of these granules to pick up an infectious dose, granule application will still need to occur at a relatively high rate due to the need for uniform application as a result of FCM pupation biology (Love 2015). Another

shortcoming to granular application is that in order for sporulation to occur, moisture is necessary (Wraight *et al.* 2001). Granules may therefore only be suited with micro-sprinkler irrigation than drip irrigation systems. This defeats the purpose of identifying a formulation which promotes fungal virulence in orchards employing either irrigation type.

As previously mentioned, these isolates are also under investigation against other key citrus pests which occur in the foliar environment: red scale, citrus thrips and mealybug (Wiblin & Hill 2013; Chartier Fitzgerald 2014). As such, an oil based formulation may be a good place to begin formulation investigations. Oils have been reported to promote fungal efficacy in the foliar environment largely due to their UV protectant and rainfastness properties. Oils are also ideal candidates in which to suspend hydrophobic fungal spores and have been reported to possess insecticidal properties alone and improve the attachment and distribution of spores on surfaces such as leaves and insect cuticles (Prior *et al.* 1988; Moore & Caudwell 1997; Kooyman & Godonou 1997; Inglis *et al.* 2000; Wraight *et al.* 2001; Malsam *et al.* 2002). For example, Malsam *et al.* (2002) found that *M. anisopliae* conidia formulated in oils were able to induce almost 100% mortality against *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae) and in addition increase the speed of kill. The improved efficacy was attributed to the more uniform distribution of *M. anisopliae* spores on the leaves and insect cuticle. Whilst UV radiation and rain may not be the most efficacy limiting variables in the soil environment, in the foliar environment, they are considered key detrimental variables (Jaronski 2010). The fact that oils possess anti-desiccant properties, a benefit that has been utilised extensively in grasshopper and locust control in areas of low relative humidity in Africa (Moore & Caudwell 1997), is promising and may be exploitable to counteract the poorer performance of Bb1 reported when soil moisture conditions measured were relatively low.

Formulation can also enhance persistence in the field, largely due to their protection of the spores from adverse environmental variables (Wraight *et al.* 2001). The field persistence of the isolates in this study was particularly good (fungal isolates were recovered approximately 30 weeks post-application) and therefore a formulation specifically designed to prolong persistence is unlikely

to be the main focus. However, a formulation which could limit a loss in spore viability shortly after application when fungi are applied to the soil before the bulk entry of FCM, could greatly facilitate the initial reduction in FCM infestation within the orchard. In addition, FCM entering the soil are doing so to pupate. Thus, they will be moulting from fifth instars to pre-pupae and then pupae. Moulting can prevent fungal infection (Ortiz-Urquiza & Keyhani 2013). It is not known how many FCM, if any, escape infection as a result of moulting, but an increase in germination, which can be achieved through formulation by the addition of nutritive materials, may enhance efficacy.

It should be kept in mind that any adjuvant used in a formulation will have an expense and therefore the addition of adjuvants must have a significant benefit and preferably be easily obtainable and cheap. Care must also be given to the type of adjuvant used, as although its use may improve the physical characteristics of a formulation or its delivery in the field, it may significantly reduce the viability of the infective propagule rendering improved application pointless (Ravensberg 2011). Irrespective of whether a formulation can be developed that promotes the efficacy of these isolates in the field or not, it will be important for storage and handling. Liquids are easily measurable and are dispensed prior to application in the field (Wraight *et al.* 2001). Dry spores are extremely dusty. Not only does this make mixing dry spores with tank water prior to application in the field very challenging because these spores are easily dispersed by wind, it also increases the risk of the user developing an allergic response through inhalation or contact with the eyes. The use of liquid formulations, such as an oil formulation, essentially eliminates the safety hazard associated with dry spores, whilst simultaneously improving handling (Goettel & Jaronski 1997). Storage, or shelf-life, is a major obstacle which needs to be overcome to increase the competitiveness of mycopesticides in the market, and on which a lot of emphasis has been placed during formulation developments (Wraight *et al.* 2001). An acceptable shelf-life of a stock suspension, intended for dilution in the field prior to application, should maintain a good level of spore viability for a minimum of one year at room temperature (Jackson *et al.* 2010).

7.2.3 Compatibility

7.2.3.1 Other control strategies and biological control agents

Compatibility amongst control strategies and non-target organisms is an important consideration and an aspect which requires further attention especially since the successful control of FCM in South African citrus orchards is dependent on an IPM approach (Moore & Hattingh 2012; Grout & Moore 2015). In South African citrus, IPM has been reported to reduce FCM infestation by up to 97% or more if control measures are used correctly (Moore & Hattingh 2012). However, as all these measures are aimed at reducing the same pest species, incompatibility between measures for a variety of reasons may exist and should be addressed. For example, trials at Oranjelus produced inconclusive results, suspected to be a result of low host density in the soil. Interestingly, high numbers of sterile FCM, the control agents used in an alternate control strategy, sterile insect technique (SIT), were recorded in yellow delta traps (Appendix 2) on a monthly basis. As already mentioned in Chapter 1, SIT is aimed at reducing the number of viable eggs in the orchard and as such, limits the number of FCM hatching and infesting fruit. The life stage targeted by the applied fungi may therefore be reduced. At Oranjelus, when application occurred at the highest experimental rate (1×10^{14} spores/ha), both applied isolates (Bb1 and Ma2) were recorded within the upper 5 cm soil surface for five months post-application (Chapter 4).

As application was successful at all other sites, it is likely that if FCM entered the soil and came into contact with a lethal quantity of viable spores, infection would occur. Given that high numbers of sterile males were also recorded in traps at Atmar 2, where fungal efficacy was reported, it is unlikely that SIT and EPF are incompatible. However, at Atmar 2, the number of wild FCM males caught in traps and the number of FCM infested fruit was higher than that at Oranjelus (Appendix 2). Therefore, the tandem use of these two control measures may be influenced by pest pressure. Generally, SIT is more effective in areas with moderate to low FCM pressure as this permits a suitable sterile:wild moth ratio from being obtained (Lance & McInnis 2005). Therefore, in areas of high FCM pressure, the application of EPF before peak larval descent into the soil may improve SIT success by reducing a substantial proportion of the pest

population promoting a healthy sterile:wild moth ratio from being obtained. After which, and in citrus orchards where FCM density in the soil may be low, applied EPF may serve rather as a “safety net” for above-ground strategies rather than as the main controlling agent.

Alone, biological control measures do not achieve 100% control efficiency. As such, not all FCM pupating in the soil will succumb to infection, but may be exposed to sub-lethal doses or come into contact with a lethal dose of spores as they eclose. No research on the sub-lethal effects on FCM longevity, developmental rate or fecundity has been conducted. In laboratory studies, research has shown that lepidopteran larvae exposed to sub-lethal doses of the applied fungal entomopathogen, experience an increase in time to eclosion – which may have implications on the timing of above-ground control strategies – and positively reduce the number of eggs oviposited – which can serve as an added benefit promoting the use of EPF in the field (Hafez *et al.* 1997; Guimarães *et al.* 2014; Zhang *et al.* 2015). The virulence of these isolates, if any, on the adult life stages of FCM has not been evaluated, although mycosed FCM adults have been noted during laboratory bioassays, but not quantified (Coombes 2013).

FCM is not the only pest infesting citrus orchards in South Africa (Grout & Moore 2015). Other biological control strategies targeting these other pest species are therefore present in the same arena. Thus the application of a control strategy against one pest should not reduce the efficacy of a control strategy against another or impact the population of beneficial insects in the area of application (non-target effects). Generally, fungal entomopathogens are considered to pose minimal risk to non-target invertebrates including parasitoids and predators of the target species (Goettel *et al.* 2001; Ekesi *et al.* 2005; Babendreier *et al.* 2015). However, there are some reports of incompatibility and non-target effects. For example, Progar *et al.* (2015) reported an increase in the density of the target pest, *Profenusa thomsoni* Konow (Hymenoptera: Tenthredinidae), at certain sites. They suggested that this may be a result of *B. bassiana* application reducing the abundance of natural predators of *P. thomsoni*, although this was not quantified. A parasitoid, *Trichogrammatoidea cryptophlebiae* Nagaraja (Hymenoptera: Trichogrammatidae), is registered for use against FCM in citrus orchards, and although the susceptibility of this parasitoid to the applied fungal isolates under investigation is still unknown, it is likely that even if susceptibility

is reported, that the impacts of EPF applied to the soil will have minimal impact on the parasitoid due to spatial separation.

The only other biological product registered for use against the same life stages of FCM targeted by EPF is based on entomopathogenic nematodes (EPNs). Studies assessing compatibility between EPF and EPN application produced varied results. Shapiro-Ilan *et al.* (2004) reported mostly antagonistic effects of combined application, with one exception. *Heterorhabditis bacteriophora* applied simultaneously with *M. anisopliae* recorded an additive outcome on the mortality of the pecan weevil larvae, *Curculio caryae* Horn (Coleoptera: Curculionidae). Dual inoculation of *H. bacteriophora* with either *B. pseudobassiana* or *M. pingshaense* against *Phyllophaga polyphylla* larvae Bates (Coleoptera: Scarabaeidae) were found to cause slightly higher infection percentages than when applied alone, but interactions between the nematode and fungus were demonstrated to be antagonistic. However, when *B. pseudobassiana* was applied two weeks before *H. bacteriophora*, an additive interaction was observed, a similar finding to that of Ansari *et al.* (2004). *Metarhizium anisopliae* applied at either 2×10^{12} or 2×10^{13} spores/ha, four weeks prior to nematode application, resulted in a synergistic effect on the mortality of third-instar *Hoplia philanthus* Füssly (Coleoptera: Scarabaeidae). Ants, which are naturally found in citrus orchards, have also been reported, under laboratory test conditions, to aid in reducing the survival of soil-dwelling pest life stages, such as FCM, *C. capitata* and *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), in citrus orchards through predation (Bownes *et al.* 2014). Compared to the other pest species investigated, FCM survival was significantly reduced in the presence of the generalist ant species *Anoplolepis custodiens* (Smith) (Formicidae: Formicinae) and *Pheidole megacephala* (Fabricius: Myrmicinae). It is thus recommended that predatory ants be conserved, managed by tree-banding rather than poisoning, in citrus orchards (Bownes *et al.* 2014). The effect of fungal application on ant populations within treated areas is not known and although ants were observed throughout the trial period in all orchards to which fungi were applied, this was not quantified and may be important to determine the extent of damage, if any, on these naturally occurring predators. This area of research should also be expanded to include other soil-dwelling non-target organisms which may come into direct contact with the applied fungal propagules and thus be most at risk to incurring infection.

Given that formulation has the potential to enhance the virulence and infectivity of the applied entomopathogenic fungal isolate, it stands to reason that the host range of this isolate may also alter to include other biological control agents or non-target organisms. Therefore, accurately assessing compatibility may only be possible following the development of the formulated product.

7.2.3.2 Agrichemicals

The compatibility between commonly applied agrichemicals and applied fungal entomopathogens is equally important to consider. Majority of citrus orchards in South Africa are conventional and follow mandatory chemical spray programmes (*S.D. Moore pers. comm.). Compatibility will vary depending on the chemical and isolate applied, the timing of application of both control options and on the area in which application occurs (e.g. tree canopy vs soil) (Klingen & Haukeland 2006). The simultaneous use of certain chemicals at reduced rates and EPF has also been reported to enhance pest mortality compared to fungal application alone (e.g. Feng *et al.* 2004) presumably as biocompatible chemicals weaken the insect, making it more susceptible to fungal infection (Quintela & McCoy 1998; Hiromori & Nishigaki 2001). Preliminary assessment of select fungicides, suggested to be the most damaging agrichemicals (Mochi *et al.* 2005; Demirci *et al.* 2011; Niassy *et al.* 2012), on the vegetative growth, viability and sporulation of isolates Bb1 and Ma2 was undertaken, the results of which are presented in Chapter 6. These results were based on *in vitro* assessment only and may not be reflected in the field.

Samson *et al.* (2005) found no difference in mortality of white grubs between fungicide and control plots 42 days after application of *M. anisopliae* during field trials, despite toxicity being reported *in vitro*. In addition, the trial was considered a severe test of compatibility, as higher than registered rates were used for each fungicide with fungicides applied directly to the *M. anisopliae* covered granules. Jaros-su *et al.* (1999) reported similar findings. In the laboratory, the fungicides chlorothalonil, banol and mancozeb consistently reduced *B. bassiana* induced larval mortality after exposure to leaf discs treated with each fungicide. This was not observed in

the field, and is suggested to be a result of the high rate at which *B. bassiana* was applied, 5×10^{13} spores/ha. No negative impact on the virulence of both *B. bassiana* and *M. anisopliae* against wax moth larvae when exposed to 15 different fungicides applied at the recommended field rate.

Other factors which may influence the lack of adverse impact reported in field studies include the timing of application and formulation. Timing of fungicide application was thoroughly assessed by Kouassi *et al.* (2003). Application of *B. bassiana* two or four days before fungicide application had no negative impact on the mortality of *Lygus lineolaris* adults. Similarly, the application of fungicides two or four days before fungal application recorded higher mortalities than when applied simultaneously. However, mortality was significantly higher when fungus was applied before the fungicides. Lopes *et al.* (2011) investigated the impact of an oil-based formulation on the viability of *M. anisopliae* and *B. bassiana*. Unformulated, *M. anisopliae* germination, after 40 minutes exposure to carbendazim, was only 20%. Under the same conditions, a germination rate of 85% was reported for the formulated *M. anisopliae* spores. This increase in germination promoted a reduction in *Diatraea saccharalis* larval survival time during subsequent bioassays compared to unformulated spores. This may partly explain why *in vitro* studies, which typically use unformulated infective propagules, often suggest fungus-fungicide incompatibility whilst field experiments, which typically use formulated spores (Jaros-su *et al.* 1999; Samson *et al.* 2005), do not.

Although much of the above arguments are specific to fungicides, as these were the chemicals investigated, many of these are also applicable to other commonly applied agricultural inputs such as insecticides, herbicides and fertilisers. As previously mentioned, it does not appear that the application of chemicals sprayed during the course of the field trials (Appendix 3) had a significant impact on fungal efficacy or persistence, likely due to spatial separation. However, if these isolates are to be registered for use of above-ground pest species as well, then compatibility between these fungi and agricultural chemicals may become more of an issue given that direct contact is more probable. The timing of fungal and chemical application may therefore become

an important component of spray regimes, with formulation playing an important role in spore protection against toxicity.

7.2.4 Field efficacy

Five field trials were conducted over two citrus growing seasons, only four (two early application and two late application trials) of which were successful. Far more trials are necessary, not only for registration purposes and for confirmation of the good control efficiency of these isolates, but also to better understand the manner in which environmental variables can influence efficacy. Although application was successful, a positive outcome, control efficiency relative to untreated control blocks varied between sites. A reduction in FCM infestation to which Bb1 was applied ranged between 33.85% and 81.72% whilst Ma2 control efficiency ranged between 28.32% and 63.02%. Inconsistent results are considered one of the limitations and problems associated with biological control products (Mishra *et al.* 2015). In order to address the issues surrounding inconsistency, more field trials need to be conducted to identify the main causal agent or agents of this variability so that this issue can be addressed.

The efficacy of the isolates in this study was monitored following a single application. Examples in the literature exist which show that a second application can further suppress the pest population through the addition of more infective propagules into the arena (Inyang *et al.* 2000; Shi *et al.* 2008; Gatarayiha *et al.* 2011). This may also improve FCM suppression using EPF in citrus orchards. In general, control efficiency and persistence was found to reduce over time. This may have been counteracted by the addition of a second spray, even if the second spray occurred at a lower rate than the initial spray. However, if multiple sprays are to be recommended this will need to occur at rates lower than 1×10^{14} spores/ha to maintain economically feasible application. Comparison between the cost-benefit of multiple spray and single spray applications will need to be undertaken before any recommendations on the number and timing of applications can be made.

The field sites used in this study were located within the Eastern Cape Province of South Africa. The Eastern Cape is not the only province in which citrus is grown (Citrus Growers' Association 2014), and as such the efficacy of these isolates in citrus orchards located elsewhere in the country, particularly areas such as Limpopo and the Western Cape, where FCM infestation is also prominent. In addition, field sites experiencing different environmental variables from each other and to those measured in this study should be identified for use to further assess the environmental dependency of these isolates under field conditions. Unfortunately, discrepancies in soil temperature, soil moisture, soil pH and soil texture varied little amongst field sites used in this study thus limiting any conclusions from been drawn in this regard. Although laboratory studies are important to help improve our understanding of the influence of environmental factors on the efficacy of applied entomopathogens, accurately creating artificial conditions experienced in the field is challenging (Inglis *et al.* 2001; Jaronski 2010). Field trials are consequently the only way to critically evaluate the true influence of these variables on efficacy and given that these variables can differ substantially amongst orchards, many more field trials are necessary to make informed conclusions.

However, having said this, laboratory qualification and quantification of the effects of environmental variables, either alone or in combination, on fungal efficacy are important to support field results and to possibly confirm which variables are the most influential. Quantifying and qualifying the influence of these environmental variables, such as soil moisture, soil temperature, UV radiation, organic matter, pH, soil texture and soil biota, on fungal efficacy is critical to making informed recommendations on the application of these isolates under differing field conditions. It is well known that fungal isolates vary in their response to different environmental variables (e.g. Ekesi *et al.* 2003; Padmavathi *et al.* 2003; Garrido-Jurado *et al.* 2011a, b; Fernandes *et al.* 2015) and as such, recommendations regarding the application of these isolates may vary. For example, it is already suspected that under low soil moisture, the control efficiency of isolate Bb1 and Ma2 is reduced and improved, respectively when compared to areas of higher soil moisture. Thus, Bb1 may be more appropriate for application in citrus orchards employing micro-jet irrigation which creates a moister surface environment, whilst the application of Ma2 may be more appropriate in citrus orchards under drip irrigation where upper

soil surface moisture is low. Laboratory-based experiments designed to assess the influence of these variables on the isolates under question, are planned.

7.3 CONCLUDING REMARKS

Despite much research which still needs to be undertaken, the potential to use the entomopathogenic fungal isolates investigated in this study, particularly *B. bassiana* isolate G Ar 17 B3 (Bb1) and *M. anisopliae* isolate FCM Ar 23 B3 (Ma2), to reduce FCM infestation within citrus orchards is promising with control efficiency of up to 80% obtainable with the use of unformulated conidia. Since it is now known that the laboratory efficacy of these isolates can be translated to the field, future research can focus on aspects which are important for promoting the development of a cost-effective and consistent biological control product for adoption with IPM programmes in citrus orchards of South Africa: production, formulation, application and the cost thereof. This study can therefore be seen as a foundation on which future research can build.

Chapter 8

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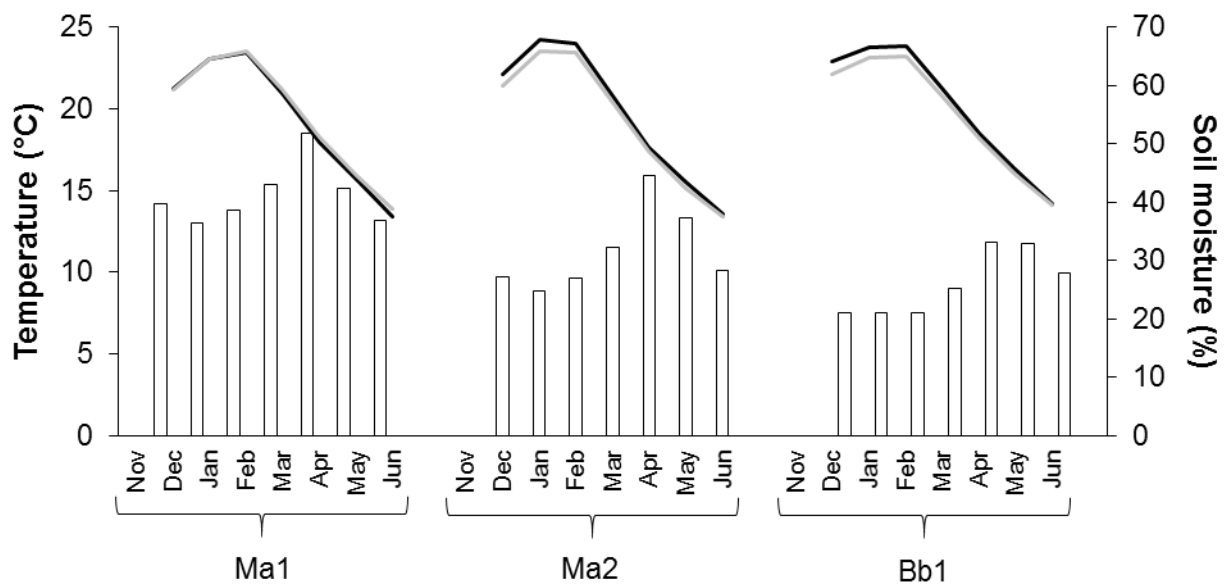
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Appendix 1:

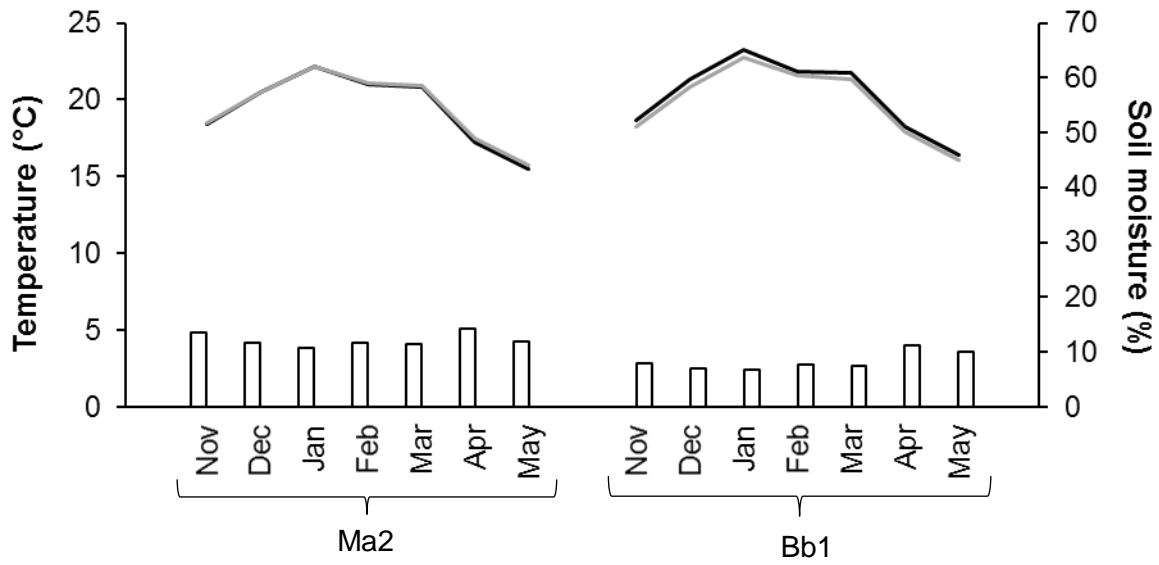
Monthly average soil temperature and moisture recorded at all field sites

- ❖ Soil moisture and soil temperature were monitored using DFM 80 cm probes installed according to the manufacturer's instructions. Average monthly temperatures reported pertain to the surface (—, black line) and 10 cm depth (—, grey line). Average monthly soil moisture recorded at 10 cm depth is reported (□, columns).
- ❖ 10^{12} , 10^{13} and 10^{14} represent 1×10^n spores/ha.

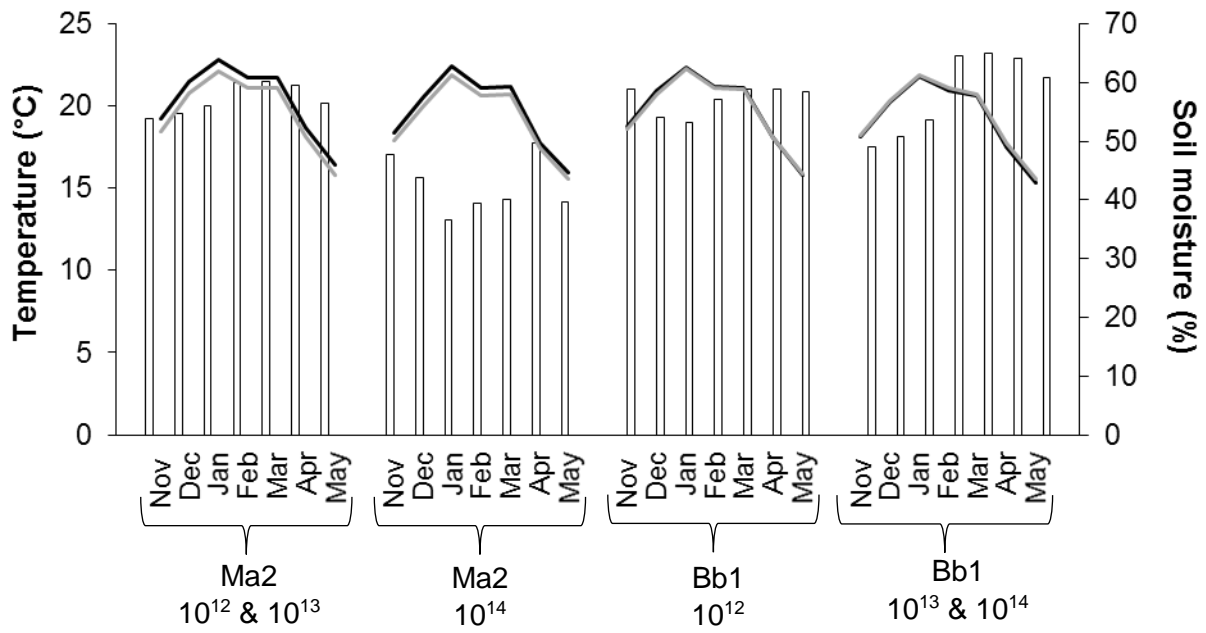
Atmar 1



Atmar 2



Oranjelus



Marwell and Stenhope

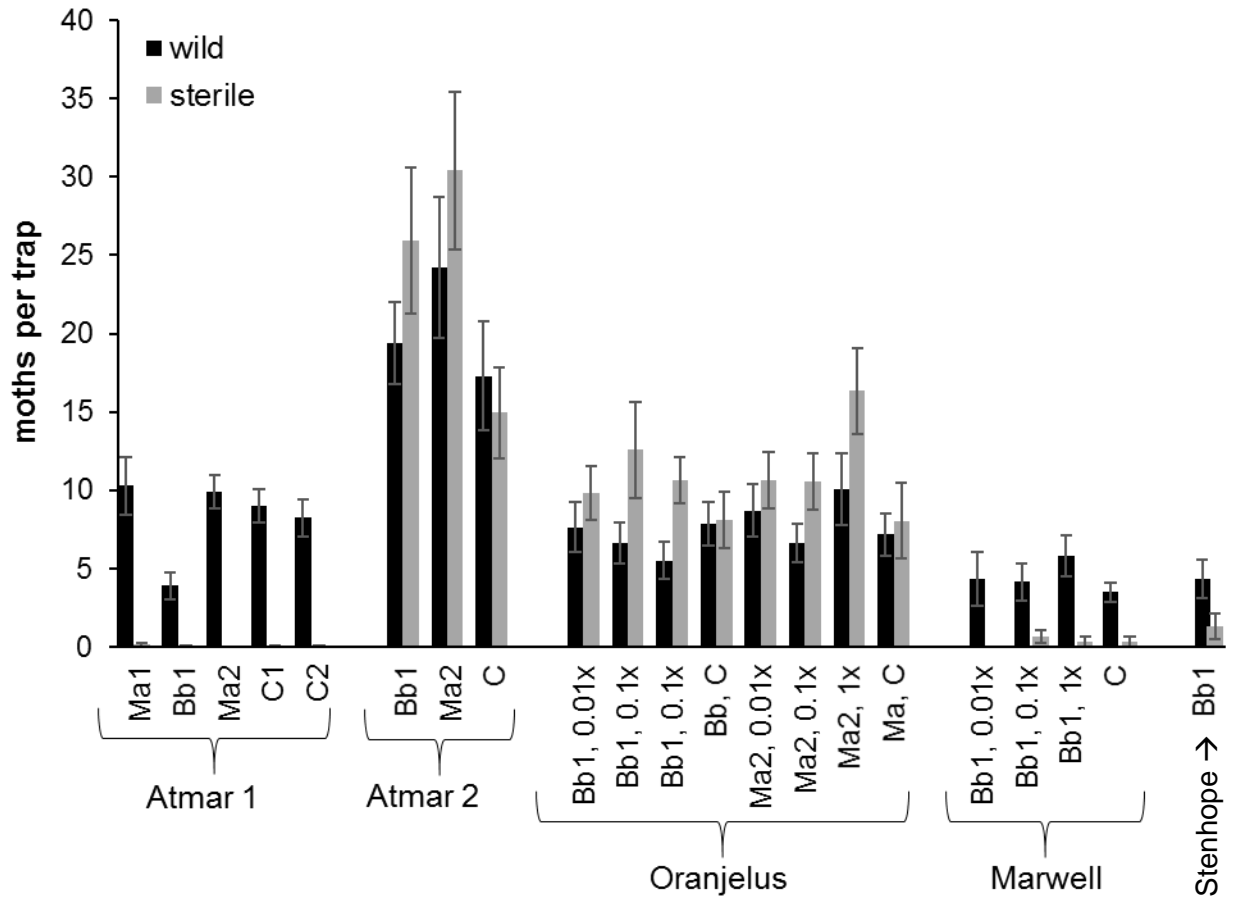
Site	Soil temperature (°C)		Soil moisture (%)
	surface	10 cm depth	
Marwell: Bb1, 10 ¹³	17.57	17.67	49.93
Marwell: Bb1, 10 ¹² & 10 ¹⁴	16.82	17.07	56.75
Stenhope: Bb1, 10 ¹⁴	Not measured. All available probes in use.		

Appendix 2

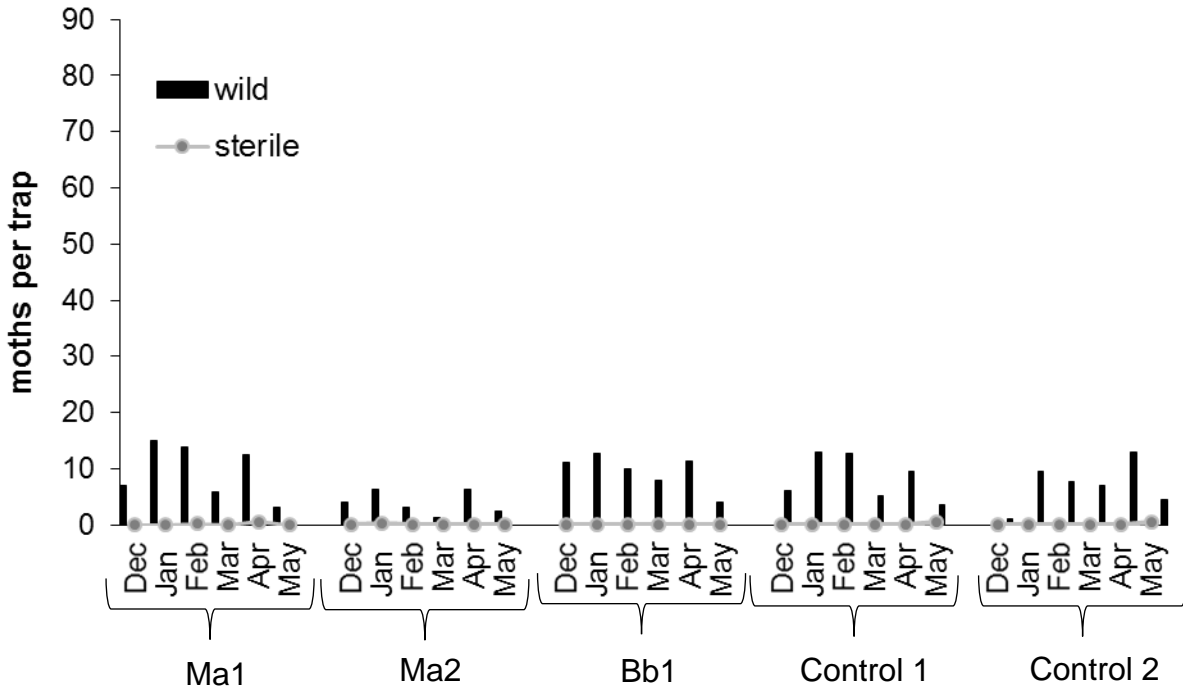
False codling moth trap catch data at each field site

❖ 10^{12} , 10^{13} and 10^{14} represent 1×10^n spores/ha.

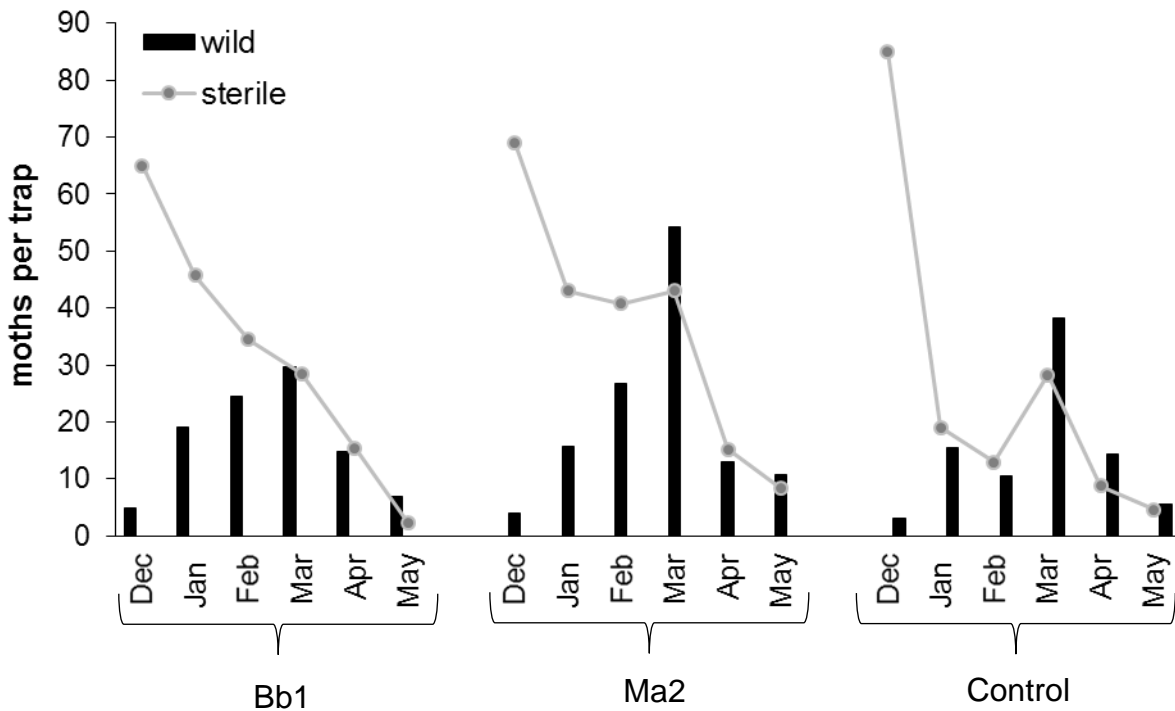
Average (\pm SE) moths per trap caught weekly at all field sites (C = control)



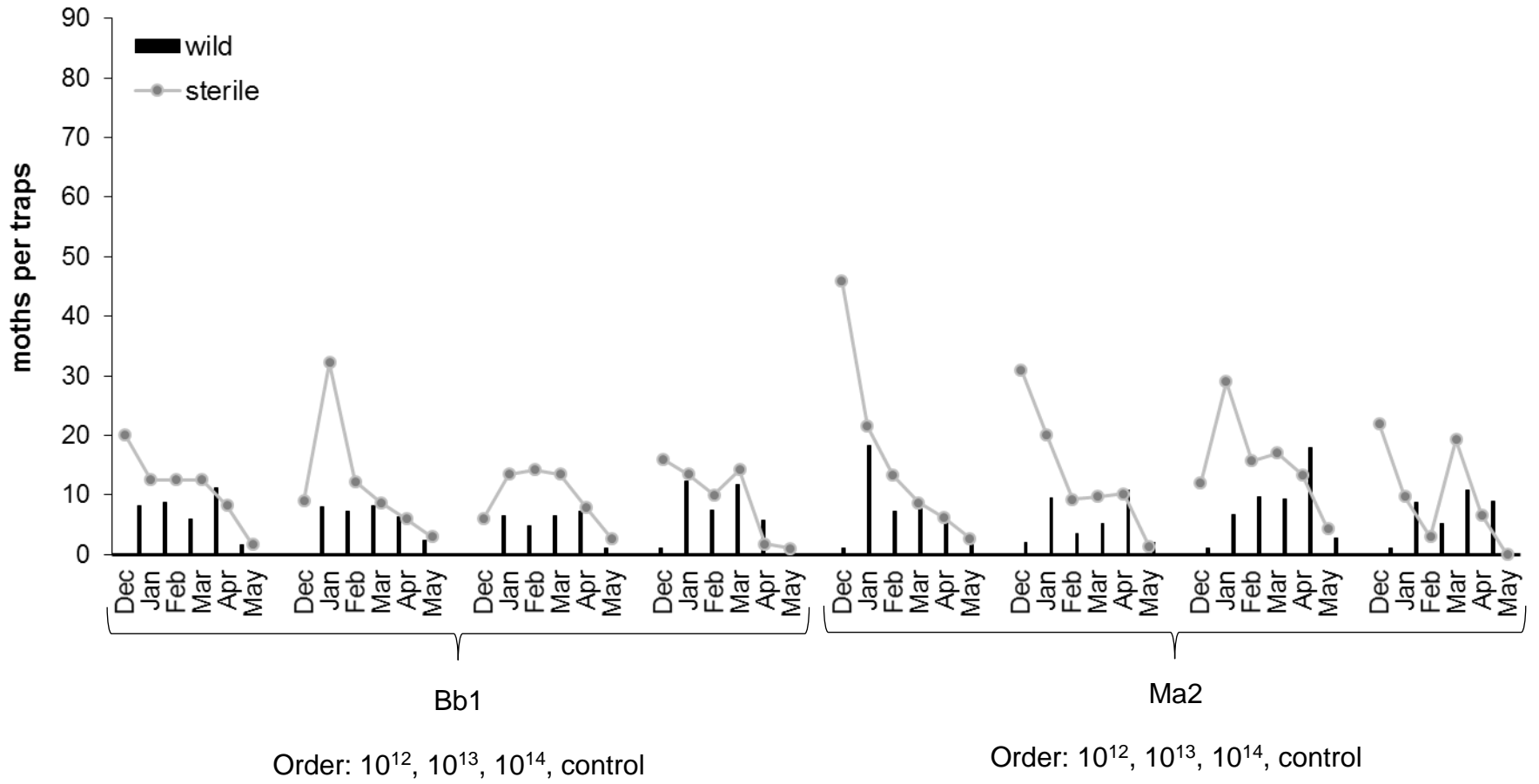
Average moths per trap caught each week monthly at Atmar 1



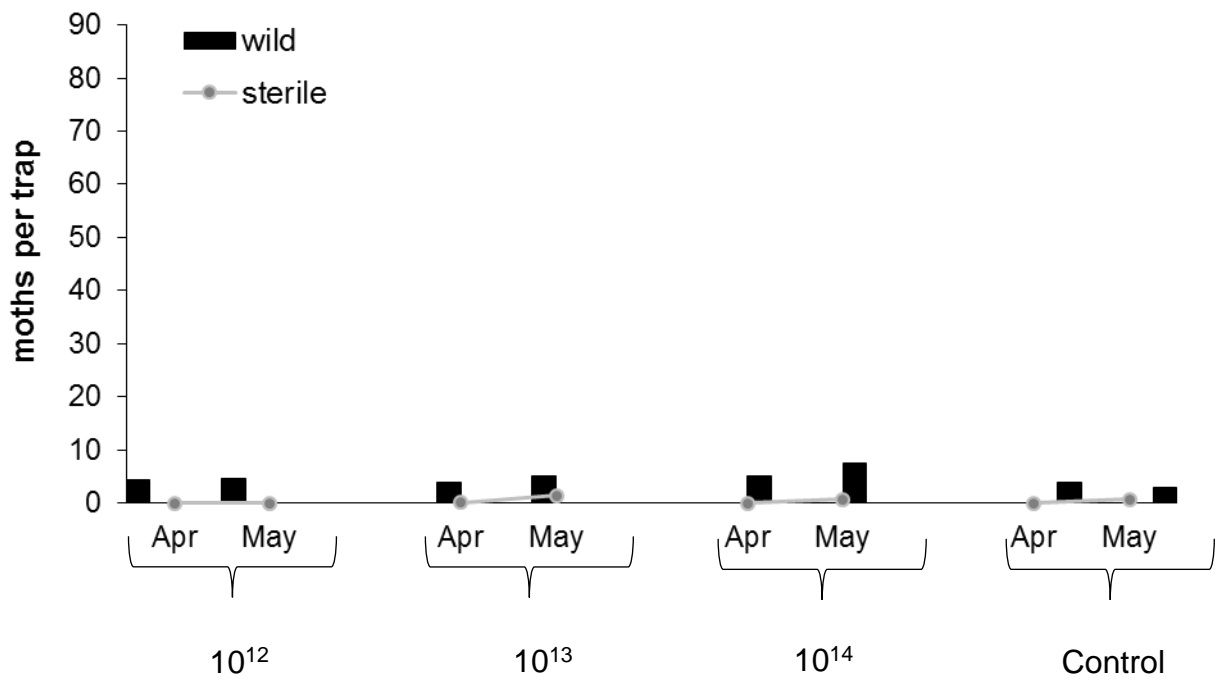
Average moths per trap caught each week monthly at Atmar 2



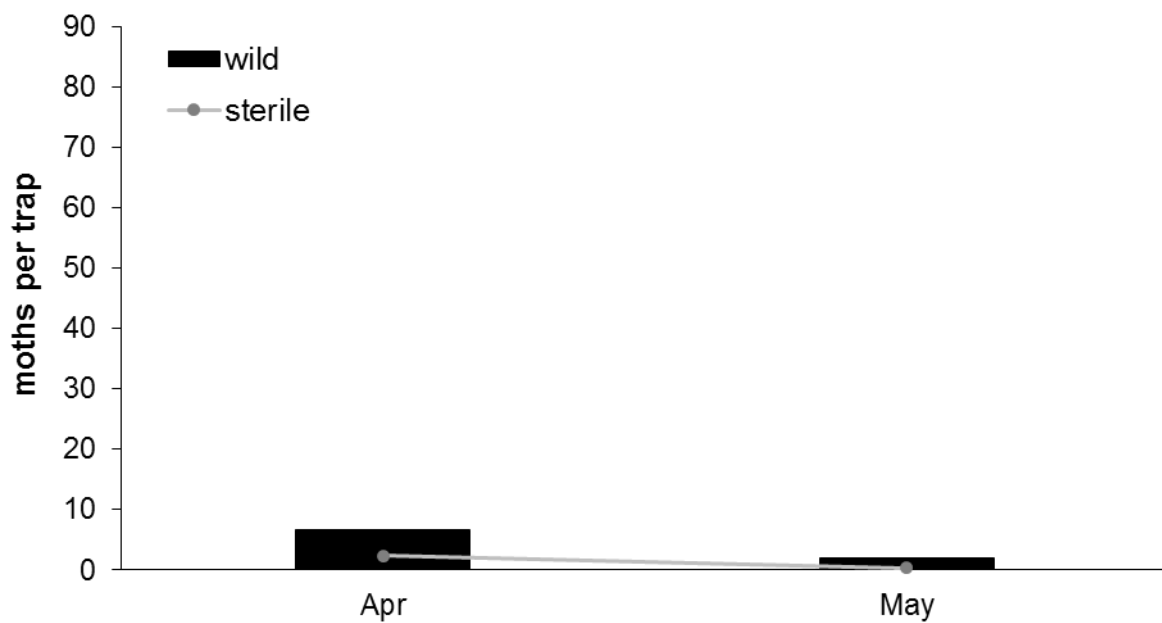
Average moths per trap caught each week monthly at Oranjelus



Average moths per trap caught each week monthly at Marwell



Average moths per trap caught each week monthly at Stenhope in the treatment block. No trap was installed in the control block



Appendix 3

Chemicals applied at Atmar 1, Atmar 2 and Oranjelus during early season trials

❖ Spray programmes were provided by the growers.

Chemicals applied at Oranjelus across the 2014/15 citrus growing season

Month	Chemical	Active ingredient	Type (target)	Dose /100L
Oct, early	Dursban	chlorpyrifos	Insecticide (bollworm)	100 ml
	Bio Boost	plant extract, Mn, Fe	Plant health promoter	20 ml
	Suprathion	methidathion	Insecticide (mealybug)	150 ml
	Tebuzole	tebuconazole	Fungicide (<i>Alternaria</i>)	80 ml
Oct, late	Benomyl	carbendazim	Fungicide (black spot)	50 g
	Pennfluid	mancozeb	Fungicide (black spot)	150 ml
	Klartan	tau-fluvalinate	Insecticide (thrips)	20 ml
Nov, early	Agrimec	avermectin	Insecticide (thrips)	20 ml
Dec, early	Fungaway	azoxystrobin	Fungicide (black spot)	20 ml
	Cryptex	CrleGV	Bioinsecticide (FCM)	3.3
	Dursban	chlorpyrifos	Insecticide (bollworm)	64 g
	Pennfluid	mancozeb	Fungicide (black spot)	150
	Scalex	pyriproxyfen	Insecticide (red scale)	30 ml
Jan, early	Agrimec	avermectin	Insecticide (thrips)	20 ml
	Copper oxychloride	copper	Fungicide (black spot)	150 g
Jan, late	Agrimec	avermectin	Insecticide (thrips)	20 ml
	Cryptex	CrleGV	Bioinsecticide (FCM)	3.3 ml

**Chemicals applied at Atmar 1 across the 2013/14 citrus growing season and
Atmar 2 across the 2014/15 citrus growing season**

Month	Chemical	Active ingredient	Type (target)	Dose /100L
Sep, late ¹	Profenofos	Profenofos	Insecticide (bollworm/mealybug)	75 ml
	Zinnit	nitrogen, zinc	fertilizer	100 ml
Oct, early	Dursban	chlorpyrifos	Insecticide (bollworm)	75
	Progibb	gibberellic acid	Plant growth regulator	2.5 ¹ /10 ² dpm
	Zinnit	nitrogen, zinc	Fertilizer	100 ml
	Klartan	tau-fluvalinate	Insecticide (thrips)	30 ml
	2.4D	2.4D-Amine	Herbicide (fruit fall)	3 ml
Oct, late	Agrimec	Avermectin	Insecticide (thrips)	50
	Pennfluid	mancozeb	Fungicide (black spot)	150 ml
	Scalex	pyriproxyfen	Insecticide (red scale)	30
	Benomyl	carbendazim	Fungicide (black spot)	50 g
Nov, early	Hunter	chlorfenapyr	Insecticide (thrips)	30 ml
Dec, early	Agrimec	Avermectin	Insecticide(thrips)	50 ml
	Buprofenzin	buprofezin	Insecticide (mealybug)	30 g
	Pennfluid	(thiadiazin)	Fungicide (black spot)	150 ml
	Fungaway	mancozeb	Fungicide (black spot)	20 ml
	Crytogran ²	azoxystrobin CrleGV	Bioinsecticide (FCM)	10 ml
Dec, late	Delegate	spinetoram	Insecticide (thrips)	10 g
Jan, early	Agrimec	Avermectin	Insecticide(thrips)	50 ml
	Copper oxychloride	copper	Fungicide (black spot)	150 g
	Fungaway ¹	azoxystrobin	Fungicide (black spot)	20 ml
Jan, late	Agrimec	Avermectin	Insecticide(thrips)	50 ml
	Progibb	gibberellic acid	Plant growth regulator	10 dmp ³

¹only applied at Atmar 2 (orchard no. 53, 2014/15 growing season)

²only applied at Atmar 1 (orchard no. 43 and 50, 2013/14 growing season)