Screening of entomopathogenic fungi against citrus mealybug (*Planococcus citri* (Risso)) and citrus thrips (*Scirtothrips aurantii* (Faure))

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Abstract

Mealybugs (*Planococcus citri*) and thrips (*Scirtothrips aurantii*) are common and extremely damaging citrus crop pests which have proven difficult to control via conventional methods, such as chemical pesticides and insect growth regulators. The objective of this study was to determine the efficacy of entomopathogenic fungi against these pests in laboratory bioassays. Isolates of *Metarhizium anisopliae* and *Beauveria bassiana* from citrus orchards in the Eastern Cape, South Africa were maintained on Sabouraud Dextrose 4% Agar supplemented with Dodine, chloramphenicol and rifampicin at 25°C. Infectivity of the fungal isolates was initially assessed using 5th instar false codling moth, *Thaumatotibia leucotreta*, larvae. Mealybug bioassays were performed in 24 well plates using 1 x 107 ml-1 conidial suspensions and kept at 26°C for 5 days with a photoperiod of 12 L:12 D. A *Beauveria* commercial product and an un-inoculated control were also screened for comparison. Isolates GAR 17 B3 (*B. bassiana*) and FCM AR 23 B3 (*M. anisopliae*) both resulted in 67.5% mealybug crawler mortality and GB AR 23 13 3 (*B. bassiana*) resulted in 64% crawler mortality. These 3 isolates were further tested in dose-dependent assays. Probit analyses were conducted on the dose-dependent assays data using PROBAN to determine LC_{50} values. For both the mealybug adult and crawlers FCM AR 23 B3 required the lowest concentration to achieve LC_{50} at 4.96 x 10^6 conidia ml^{-1} and 5.29 x 10^5 conidia ml^{-1}, respectively. Bioassays on adult thrips were conducted in munger cells with leaf buds inoculated with the conidial suspensions. Isolate GAR 17 B3 had the highest mortality rate at 70% on thrips while FCM AR 23 B3 resulted in 60% mortality.

Identification of the isolates, FCM AR 23 B3, GAR 17 B3 and GB AR 23 13 3, were confirmed to be correct using both microscopic and molecularly techniques. ITS sequences were compared to other sequences from GenBank and confirmed phylogenetically using MEGA6. Mealybug infection was investigated using scanning electron microscopy, mycosis was confirmed but the infection process could not be followed due to the extensive waxy cuticle. These results indicate that there is potential for the isolates FCM AR 23 B3 and GAR 17 B3 to be developed as biological control agents for the control of citrus mealybug and thrips. Further research would be required to determine their ability to perform under field conditions.

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Chapter 1

General introduction

1.1 Citrus

Citrus trees were introduced to South Africa by the settlers into the Cape in the mid 1600s (Mather and Rowcroft, 2004; CGA Annual Report, 2007). Originally from Southeast Asia, citrus trees have spread to many parts of the world with tropical or subtropical climates (McCoy *et al*., 2007). Most of the domesticated and cultivated citrus trees belong to the genus *Citrus* (Rutaceae) (Federici *et al*., 1998). The exact number of species within this genus is unknown due to the broad morphological diversity and recombination that has occurred within the original basic taxa (Ollitrault and Navarro, 2012). As a result of sexual compatibility between *Citrus* and related genera, spontaneous bud mutations and their long cultivation history, citrus taxonomy and phylogeny is considered to be complicated and confusing (Nicolosi *et al*., 2000). There are four basic taxa of *Citrus*, which are the; pummelos, citrons, andarins and papedas. The more commonly cultivated citrus species such as the sour orange, sweet orange, grapefruit, lemon and lime are all combinations of these 4 basic taxa. In more recent years intergenetic hybrids such as citranges, and citrandarins have been produced between *Citrus* and *Poncirus* (Ollitrault and Navarro, 2012).

1.1.1 Growth conditions

The growth of quality citrus depends on a wide range of specific conditions, such as temperature, relative humidity, rainfall and sunlight. In subtropical areas growth is considerably slower as these plants accumulate carbohydrates at a slower rate due to reduced respiration. In South Africa, citrus

experience mean annual temperatures of 15-20°C rising to 35-40°C inland during the summer with mild to severe winters depending on the exact location (Ladaniya, 2008). Optimum citrus growth temperatures in subtropical areas are between 22°C and 31°C (Wellington, 1960), while optimum growth is negatively affected by temperatures below 12.5°C (Ladaniya, 2008). Large amounts of water are also required by citrus trees to produce high quality fruit. This is most essential during August to November, when blossoming and fruit-setting occurs. In areas where the required amount of rainfall, between 76-102 ml (Wellington, 1960), does not occur, many farmers have relied upon drip irrigation systems (Grout and Stephen, 2005). Citrus also thrives best in soils which are light and well drained, to avoid water logging, such as sandy loam of colluvial origin. These soils are also normally found to be either neutral or alkaline in pH (Wellington, 1960).

1.1.2 Production areas of South Africa

Citrus is the most widely produced and cultivated fruit tree crop in the world (Ollitrault and Navarro, 2012). South Africa is considered to be extremely important within the global citrus industry and is one of the most important fresh citrus exporters in the southern hemisphere. South Africa, due to the varied types of climatic conditions and growing areas, is able to produce a wide range of citrus varieties (Mather, 2003).

The top citrus producing areas within South Africa are the Western Cape, the Eastern Cape, KwaZulu-Natal, Mpumalanga and the Limpopo Province (CGA Annual Report, 2011) (Figure 1.1). The Western and Eastern Cape provide cooler climates which allow for the growth of easy-peelers such as valencia, midseasons and soft citrus, which are heavily in demand by consumers. On the other hand, warmer areas such as Mpumalanga, Limpopo, KwaZulu-Natal and the Northern Cape, produce mainly grapefruits and valencia oranges (Mather, 2003).

Limpopo and Mpumalanga were the producers of most of South Africa's citrus (Mather, 2003), although since 2009 the Eastern Cape has been steadily increasing the amount of hectares dedicated to producing citrus (CGA Annual Report, 2009). This is mostly due to Limpopo and the Eastern Cape having the largest amount of land under cultivation for citrus in comparison to the other provinces (Figure 1.1) (CGA Annual Report, 2011). The amount of land under cultivation for citrus production has been increasing every year. In 2007, 56 623 Ha of land was under cultivation (CGA Annual Report, 2007), in 2009 it rose to 58 598 Ha (CGA Annual Report, 2009) and has been slowly increasing to the 62 238 Ha currently under cultivation (Figure 1.1) (CGA Annual Report, 2013).

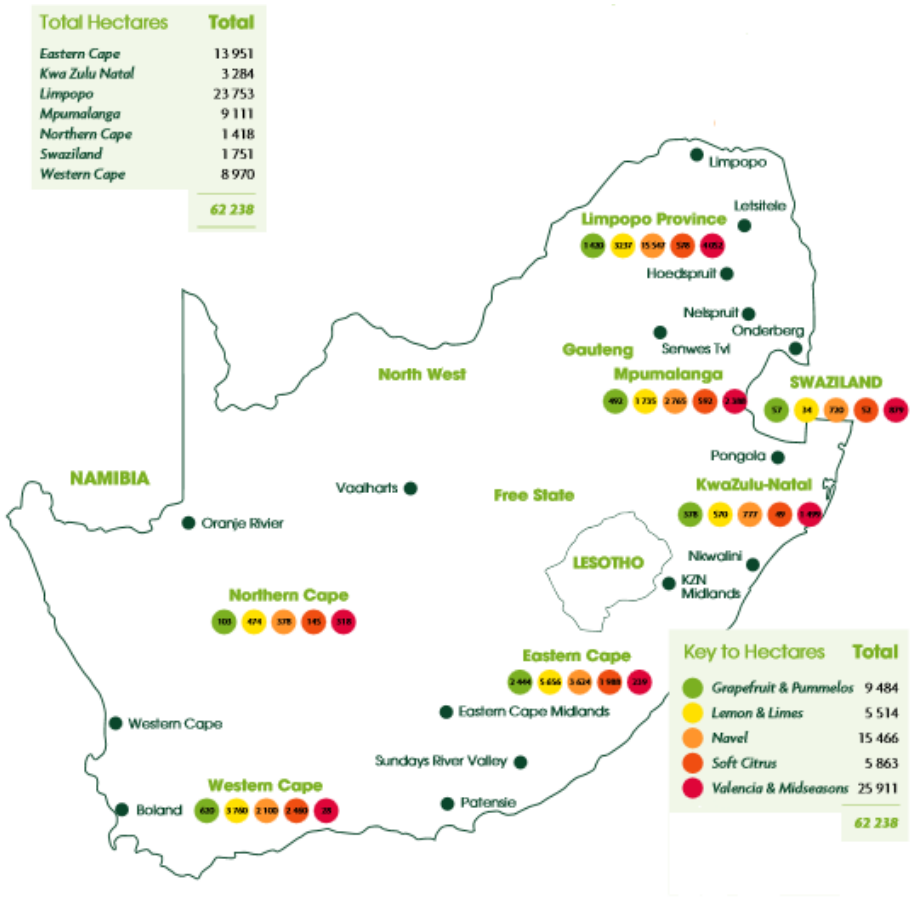


Figure 1.1: Citrus production within South Africa and Swaziland, showing total area planted for citrus and total citrus production per province according to various types of citrus grown (Adapted from CGA Annual Report, 2013).

1.1.3 Export and new markets

The majority of citrus produced within South Africa is exported and in 2012 a total of 103 022 009, 15 Kg crates of citrus were exported. The majority being valencies with 47 289 084 crates, closely followed by navels with 24 554 517 crates, followed by grapefruit, lemons and soft citrus. The largest export majority in 2012 was to Northern Europe, which imported 23% of the oranges, 33% of the grapefruit and 19% of the soft citrus, followed by the Middle East which imported 22% of the exported oranges and 42% of the lemons (CGA Annual Report, 2013).

In May 2012 the South African citrus association was granted access to Thailand's citrus market; the initial application was submitted in 1998. South Korea also changed their status on citrus imports and allowed South African grapefruits and lemons into the country. Previously, South Korea had only

allowed for the export of sweet oranges. With the expansion of these new markets the tolerance for the presence of superficial blemishes and internal and external pests has reduced dramatically, along with the pesticide residue level allowed on citrus (CGA Annual Report, 2011, 2013). Thus, alternative means of citrus pest control must be investigated.

1.2 Target citrus pests

1.2.1 Citrus Mealybugs (Pseudococcidae)

Mealybugs are well known crop pests which feed on a variety of fruit and ornamental crops (Wakgari and Giliomee, 2003). Mealybugs belong to the family Pseudococcidae. Over 60 different mealybug species have been documented on citrus plants (Hattingh *et al.,* 1998; Franco *et al*., 2004). They are widely distributed throughout the world and found in Australia, Africa, South America, the Mediterranean and the USA (Krishnamoorthy and Singh, 1987; Gullan, 2000; Wakgari and Giliomee, 2003; Franco *et al*., 2004). Their origin is not well understood. In South Africa 7 mealybug species have been recorded as either pests or potential pests. These are *Planococcus citri* (Risso), *Nipaecoccus viridis* (Newstead), *Paracoccus burnerae* (Brain), *Delottococcus elisabethae* (Brain), *Pseudococcus longispinus* (Targioni-Tozzetti), *Pseudococcus calceolariae* (Maskell), and *Ferrisia virgata* (Cockerell) (Wakgari and Giliomee, 2003; Pieterse *et al*., 2010).

Planococcus citri are highly polyphagous and the most damaging mealybug species found in nearly all citrus producing countries on a wide range of crops (Hattingh *et al*., 1998; Gullan, 2000; Wakgari and Giliomee, 2003) such as grapes, coffee and mangoes (Krishnamoorthy and Singh, 1987). *Planococcus citri* has the ability to rapidly increase its population size and dominate its habitat, along with displacing any other mealybugs which could be sharing its ecological niche (Wakgari and Giliomee, 2003). They also produce copious amounts of honeydew, which increase the growth of sooty mold. This mold decreases the photosynthetic potential of the plant and if not washed off prevents fruit from being exported. *Planococcus citri* crawlers feed on succulent vegetation and thus can heavily attack areas of new growth and young fruit. Their preferred feeding sites are on developing fruits below calyxes and inside navel openings and in contact areas between two or more fruit. Other common feeding areas are along the exposed sides of the fruit, twigs, below the petioles of leaves, although populations can be found anywhere on the trees. They also cause hyper-pigmentation on navels and grapefruits (Hattingh *et al*., 1998).

Paracoccus burnerae is another common pest in both southern Africa and Australia (Hattingh *et al*., 1998; Gullan, 2000; Wakgari and Giliomee, 2003). It is found in all citrus growing areas and is particularly vigorous in parts of KwaZulu-Natal and Swaziland (Hattingh *et al*., 1998). Like *P. citri,* it is also a polyphagous species and has been documented on 12 different host plants (Hattingh *et al*., 1998; Wakgari and Giliomee, 2003). *Paracoccus burnerae* more readily colonize leaves and are often the cause of extensive leaf curling and also cause hyper-pigmentation when feeding occurs on the fruit themselves (Hattingh *et al*., 1998).

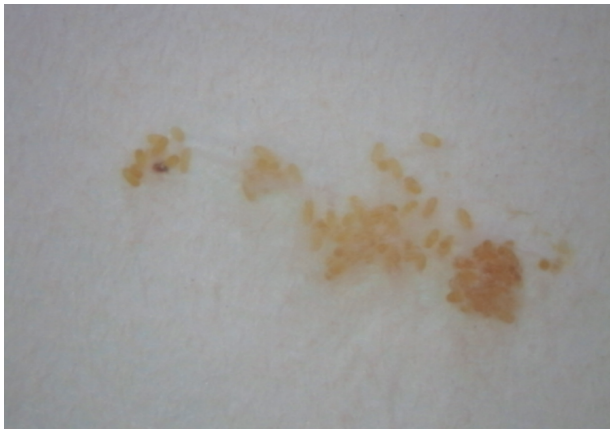
Nipaecoccus viridis is a quarantine pest for exports to USA and South Korea (Wakgari and Giliomee, 2003; Pieterse *et al*., 2010). It is also a polyphagous species, occurring not only on citrus but also on *Acacia karroo* Hayne (Mimosaceae), silky oaks *Grevillea robusta* (Proteaceae) and some *Hibiscus* species, and 25 other host plants. They are easily identified in the field, by the threads of the female's large ovisac which can be drawn out to lengths reaching 150 mm. Depending on the stage of growth, *N. viridis* are found on both young twigs and fruitlets around calyxes, when young, or on older wood in clusters during later stages of growth. The feeding of younger mealybugs on young twigs results in bulbous outgrowths and can stunt the growth of young trees. The feeding on fruitlets results in growth deformations at the ends of the stems and can cause fruits to yellow and then blacken, eventually falling from the tree. During heavy infestations the resulting fruit drop can be as high as 50%. This type of injury only occurs with *N. viridis*. Colonies which form on the face of established green fruit result in raised spots which turn yellow (Hattingh *et al*., 1998).

Pseudococcus longispinus, is known as the long-tailed mealybug, is polyphagous and has been found to have a wide global distribution (Hattingh *et al*., 1998; Wakgari and Giliomee, 2003). It is not only a pest of citrus, but has been known to transmit leafroll virus to grapevines (Wakgari and Giliomee, 2003). It has also been characterized as an export pest for South Korea (Wakgari and Giliomee, 2003; Pieterse *et al*., 2010). In comparison to the other mealybugs mentioned females are viviparous and do not produce ovisacs and; they produce up to 200 crawlers (Hattingh *et al*., 1998).

The remainder of the above-mentioned species are occasional or minor pests but have the potential to become economically damaging. All have been characterized as quarantine pests for export to South Korea, with *D. elisabethae* also an export pest for the U.S.A (Pieterse *et al.,* 2010). *Pseudococcus calceolariae* while presently not a major pest in South Africa currently, is common (Wakgari and Giliomee, 2003) and is classified as a major pest in southern Australia (Gullan, 2000). *Ferrisia virgata* (Cockerell) on the other hand is not a common pest in either country, but has been found in KwaZulu-Natal, Swaziland and Mpumalanga. It is polyphagous and has a host range of 11 known host plants throughout the world (Hattingh *et al.,* 2010).

1.2.1.1 Life cycle (*Planococcus citri)*

The life cycle of a mealybug from egg to egg lasts around 4 weeks in summer and several months in winter. Females lay an average of between 300 to 580 pale yellow eggs in a cottony ovisac which protrudes under and behind the female. Eggs are lain over a period of 1 to 2 weeks and hatch 6 to 10 days later (Hattingh *et al*., 1998). Females have three larval instars while males have four stages, which consist of first, second, prepupal and pupal stages. The first instar larvae are light yellow and highly mobile, and are referred to as crawlers (Hattingh *et al*., 1998; Gullan, 2000). Males and females can be distinguished from each other in their second instar by the eye-spots present on the males. Males undergo significant development within their third and fourth instars, becoming covered in a cottony cocoon. Adult males look significantly different from adult females in that they are small, about 1 mm, yellowish-brown with hyaline wings and two long white anal filaments. Females on the other hand are much larger around 3 mm, yellow, and are covered in a mealy wax secretion layer. They also have 18 pairs of wax filaments which lengthen with time. In the final stages of their life cycle, particularly after ovipositing, adult females usually become sedentary. Each of the larval life stages lasts for about two weeks and females begin to oviposit about two weeks after their final molt (Hattingh *et al*., 1998).



(a)

(b)

(c)

Figure 1.2: *Planococcus citri* life stages, (a) eggs, (b) crawler, (c) adult female. All pictures were taken with a Leica EZ 4D dissecting microscope at ~X16 magnification.

1.2.1.2 Wax production

Mealybug wax is produced through defined sclerotized structures, which act similarly to moulds for the production of structurally different types of wax, and are found on different areas of the mealybug. *Planococcus citri* have 3 forms of wax pores: trilocular and multilocular disc pores and tubular ducts (Cox and Pearce, 1983). Trilocular pores form a raised triangle, with 3 dumb-bell shaped loculi, which act as moulds and produce 3 railway track shaped strands, approximately 3.0 μm across, and are located in a circular depression. They are approximately 3.0 μm along each edge. The loculi are oriented so that the wax strands twist over the edge of the triangular pore in a clockwise direction. This is due to differential thickening which prevents them from coming into contact with each other. These pores are typical of the mealybug family Pseudococcidae and are abundant on the cuticles of all female instars and the first two instars of males (Figure 1.3) (Cox and Pearce, 1983).

Multilocular disc pores are common on adult females and occur predominantly around the vulva, and are also found on second instar males, scattered over the dorsum (Figure 1.3). They are much larger then the trilocular pores at 5.5 μm across, with 6-10 loculi around their circumference. The mouth of each loculus has a bifurate projection, which results in C-shaped wax strands, which cur as they are produced. As these strands curl beneath themselves they break into small segments of about 6 μm in length and 1 μm wide with longitudinal ridges on their outer surfaces (Cox and Pearce, 1983; Kumar *et al*., 1997; Johnson-Cicalese *et al*., 2011).

Tubular ducts are found mainly on adult oviparous females on the abdominal venter and again, also on second instar males. They are circular with raised rims and are 4 μm in diameter. These ducts produce long hollow tubes of wax of 1.5 μm diameter. On *P. citri* they have 8 longitudinal ridges on their external surface which are believed to increase their strength without increasing the bulk. Mealybugs also have an anal ring which is a large tube of heavily matted fine wax filaments supported by six anal ring setae which protrude from the anal ring (Cox and Pearce, 1983; Kumar *et al*., 1997).

The wax filaments also form an integral part of mealybug ovisacs and male cocoons (Johnson-Cicalese *et al*., 2011). The ovisacs are made up of wax filaments, mainly the long strands from tubular ducts and the short curls produced by the multilocular disc pores (Kumar *et al*., 1997). The cocoons produced by second instar males are quite similar and also mainly consist of tubular duct strands and multilocular disc pore curls. Although, these curls are more flexible then those produced by the female. Also instead of breaking up into small curls these cocoon filaments persist as long spirals. Adult males, specifically *P. citri* males have only 2 types of pores, neither of which are found on the females. One is located along the pleura and are approximately 4 μm in diameter with 4-5 loculi, each produce a spiral strand of of wax. The second type are found around the male's pair of caudal cerarii, and are 2 μm in diameter and also have 4-5 loculi, which produce much wider spiral strands. These strands extend along the wax rods produced by the cerarian setae (Cox and Pearce, 1983).

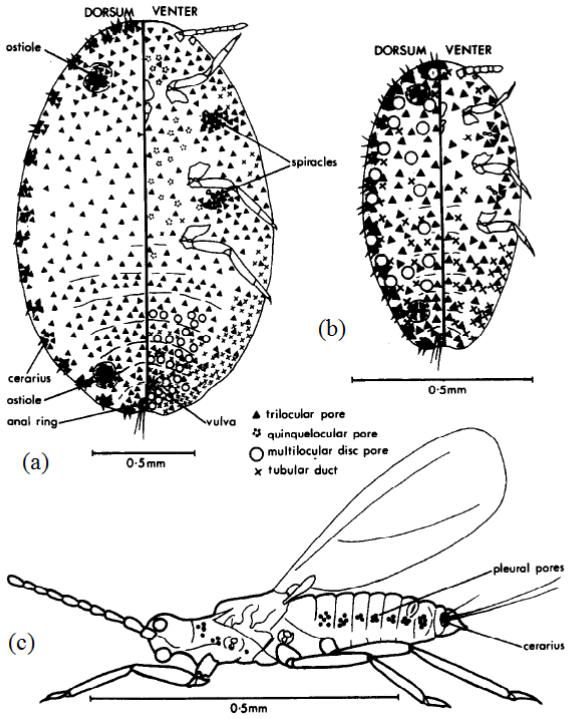


Figure 1.3: Generalised schematic diagrams of 3 different instars of mealybugs with typical positioning of different wax pores and cerarii. (a) adult female, (b) second instar male, (c) adult male. Pores and cerarian setae are shown comparatively larger in relation to the body outline and in fewer numbers than on the actual specimens. All setae except cerarian and anal lobe setae have been omitted (Cox and Pearce, 1983)

1.2.1.3 Economic damage

Within South Africa mealybugs mostly damage mandarins, navel oranges and grapefruit. Mealybugs prefer to colonise fast growing tissues, ingest sap from the phloem and parenchyma of the plant. They also produce large amounts of honeydew. These are all early ripening fruit, thus, the effect of the mealybug's natural enemies is lessened before harvest. At high levels of infestation, mealybugs cause increased fruit drop, damage new growth flushes and result in hyper-pigmentation. Honeydew induced sooty mold results in the reduction of the plant's photosynthetic ability. Thus, causes poor fruit colour development and reduces yield in subsequent crops. Mandarins are especially susceptible to damage after harvest because the sooty mold can not be effectively removed using high pressured sprays which reduces final yield. Mealybug damage to grapefruit is slightly different, as in the early growing season grapefruit are inclined to develop growth deformations such as protrusions or indentations around the calyx at feeding sites (Hattingh *et al.*, 1998).

Mealybugs attain pest status when their population sizes swell to numbers which are economically damaging in a relatively short period of time (Hattingh *et al.*, 1998; Wakgari and Giliomee, 2003). These outbreaks result from consequences of one of two causes; the disruption of their natural enemies (Hattingh *et al.*, 1998)or environmental factors (Franco *et al.*, 2004). In healthy environments this population increase rarely occurs due to the effective presence of their natural enemies. The presence of these natural enemies is often disrupted by pest control methods such as spraying non-selective insecticides for other citrus pests present (Wakgari and Giliomee, 2003). Non-selective insecticides cause mortality differences between mealybugs and their natural enemies and result in indirect negative effects on natural enemies. These insecticides affect the predator- and parasitoid-host interactions and cause positive indirect effects on pests, through changes in the host plant and positive direct effects on pests. They also effect interspecific competition between phytophagous species belonging to different taxa. Outbreaks can also occur at an insect-plant level, due to either the host plant's susceptibility to mealybugs, changes in the host due to water stress or a sudden increase in nutrient levels. At a predator-mealybug level changes in the environment not attributed to pesticides may negatively effect the mealybug's natural enemies (Franco *et al.*, 2004).

1.2.1.4 Control options

Insecticides have been frequently applied to control citrus pests, but mealybugs are difficult to control because of their cryptic nature, waxy coats and the dense colonies they form comprising of multiple overlapping generations (Krishnamoorthy and Singh, 1987; Hattingh *et al.*, 1998). The use of general nonspecific insecticides, or those applied for the control of other citrus pests such as against red scale or citrus thrips, often disrupt the presence of mealybug natural enemies (Hattingh *et al.*, 1998; Wakgari and Giliomee, 2003). For example de Bach and Bartlett (1991) determined that mealybug outbreaks in citrus orchards in California occurred due the presence and use of DDT. In South Africa the use of pyrethroids for the control of citrus thrips *Scirtothrips aurantii* Faure (Thysanoptera: Thripidaez) have been linked to many outbreaks.

Other common causes of mealybug outbreaks are attributed to the application of organophosphates and insect growth regulators (IGR). Outbreaks of *P. citri* and *P. longispinus* in Israel occurred due to the high use of organophosphates against red scale, coccids and citrus flower moths (Franco *et al.*, 2004). The reduction in the use of organophosphates in southern Africa due to the resistance found in target pests, resulted in outbreaks of mealybugs after the additional suppression provided by organophosphates was removed. Many mealybug species have also developed resistance to organophosphates (Hattingh *et al.*, 1998). Resistance also occurred when the IGR juvenoid pyriproxyfen was applied to crops for the control of red scale in both Israel and South Africa. The cause of the

outbreak was shown to be the reduction in fertility and adult emergence in the predator *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) (Franco *et al.*, 2004). Hence alternative means of control for mealybugs are required, the most common approach being biological control.

One of the frequently used biological control option is the use of parasitoids and natural enemies. Some well studied citrus mealybug parasitoids and enemies which have been successfully used in the suppressing of *P. citri* populations are the parasitic wasps *Anagyrus pseudococci* (Girault) (Hymenoptera: Encyrtidae), *Leptomastix dactylopii* Howard (Hymenoptera: Encyrtidae) and *Coccidoxenoides perminutus* (Girault) (Ceballo *et al.*, 2010) and the mealybug destroyer *Cryptolaemus montrouzieri* Mulsat (Coleoptera: Coccinellidae) (Cloyd and Dickinson, 2006; Mansour *et al.*, 2012). Rosas-Garcia *et al.*, (2009) tested different life stages of *C. montrouzieri* against *P. citri* and found that the first instars were the least predatory life stage and that mealybug predation increased with each life stage, as adults were the most predatory. In Japan Teshiba *et al.*, (2012) found that the use of (2,4,4-trimethyl-2cyclohexenyl)-methyl butyrate (cyclolavandulyl butyrate, CLB) induce an indigenous, although "not natural" enemy of the Japanese mealybug, *Planoccocus kraunhiae* (Kuwana) (Hemiptera: Pseudococcidae), the parasitic wasp, *Anagyrus sawadai* Ishii (Hymenoptera: Encurtidae: Anagyrini), to parasitise the mealybug and serve as a biocontrol agent under natural conditions. The use of natural enemies in Integrated Pest Management (IPM) programmes is very common but other microbial alternatives such as viruses or pathogenic fungi are also viable options and should be incorporated.

1.2.2 Citrus Thrips (Thysanoptera)

Thrips are small, often only a few millimeters in length. They are opportunistic, ubiquitous and polyphagous insects (Morse and Hoddle, 2006). Thus, they are well adapted to being crop pests. Unlike mealybugs, Citrus thrips *Scirtothrips aurantii* Faure (Thysanoptera: Thripidae) is indigenous to South Africa (Grout and Richards, 1992, Ansari *et al.*, 2007). Thrips are major pests within South African citrus crops although more so in Mpumalanga and the Northern Cape. The harsher winters in the Western and Eastern Cape reduce the population levels (Ansari *et al.*, 2007). Thrips were first identified as serious economic pests of citrus within South Africa in 1929 and have remained so ever since (Grout *et al.*, 1996). While commonly called citrus thrips, they are also found as pests on other fruit, such as mangoes (Grove *et al.*, 2003).

1.2.2.1 Life cycle

Female thrips lay about 1 to 2 eggs per day within incisions made into plant tissue, such as leaves (Stuart *et al.*, 2011). At first the eggs are small and bean-shaped (Figure 1.4 B) but swell as the embryo develops up to 0.22 mm by 0.11 mm. The embryo within the egg can take anywhere from 6 to 24 days to develop, depending on the season, taking longer in winter. Thrips have two larval stages, the first instar larva are colourless but become yellowish before molting. The second instar grow to a length of around 0.75 mm. They then drop from the tree after sunset and pupate under the trees. These two stages also vary according to season, in summer they take an average of 7 days and 13 days in winter. The prepupa develop wing pads which grow longer in their pupa stage and molt into pupa within the soil or on leaf litter under the citrus trees. Pupa are smaller than the second instar and grow to around 0.56 mm (Figure 1.4 D and F). The prepupal stage lasts around 1 day while the pupa stage lasts around 3 days. Adult thrips are pale orange-yellow. Adult male and female thrips are slightly different with females having regular markings on their abdomens made by darkened antecostal ridges and patches. These markings are not always present on the males and they are the smaller of the two, roughly 0.6-0.7 mm in length. They also have a comb of dark hair on the ventral side of their hind femus along with a dark hooked drepana at the end of the abdomen which identify them as *S. aurantii* males. This species of thrips life cycle lasts about 18 days in summer and 44 days in winter (Gilbert and Bedford, 1998).

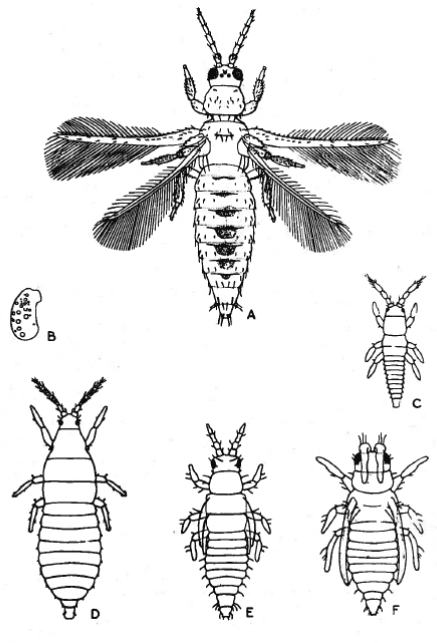


Figure 1.4: *Scirtothrips aurantii* Faure: (a) adult female, (b) egg, (c) first instar larva, (d) second-instar larva, (e) prepupa, (f) pupa (minute dorsal setae are not shown in c to f) (Adapted from Gilbert and Bedford, 1998)

1.2.2.2 Crop damage

Scirtothrips aurantii are economically important species due to the damage they cause to the fruit and to the tree foliage. Their feeding causes permanent superficial scarring of the fruit's epidermis, which if extensive enough prevents the fruit from being exported. Depending on when in the season *S. aurantii* feed, these scars take on different shapes. Early in the growing season thrip feeding results in scars which outline the sepals, as both larvae and adults feed beneath the calyx, or result in ring-shaped scars. The damage increases in size as the fruit grows. In cases of severe infestations the scarring spreads downwards on all sides in an irregular patterns. Later in the growing season thrips feed on twigs, leaves (Grout and Richards, 1992; Grove *et al.,* 2003; Childers and Nakahara, 2006). Thrips browning often occurs if late feeding is too severe. Browning occurs when the pale silvery dead area on a green fruitlet changes into a brown marking as the fruit matures. It often occurs on the lower half of the fruit in a circular area above the navel and is always on the sun exposed side of the fruit. Many blemishes occur on the navel part of the fruit once it has reached 7 mm in diameter and up, as this area allows the thrips direct access to sunlight.

Thrips also feed on the leaves of citrus trees, although not on the mature leaves. Thrips prefer new succulent leaves. They feed along the midrib, usually resulting in 2 parallel lines of damaged tissue on either side. This damaged tissue eventually causes the leaves to curl up and die. When severe infestations occur this can result in apical shoots dying and falling off, which is especially detrimental in young citrus trees. This results in multiple budding occurring from a point originally meant for a single bud, leading to reduced yield in the following season (Gilbert and Bedford, 1998).

1.2.2.3 Current control options

For many years the main control methods were of a preventative nature. Protection can range from light to medium cover sprays, such as bait sprays like tartar emetic mixed with white sugar, organophosphates such as isofenphos and pyrethroids (Gilbert and Bedford, 1998). Each of these applications also have disadvantages. Tartar emetic and sugar for example is considered one of the least harmful to the natural enemies of thrips but it is not able to control high populations of the pest and is easily removed by rain (Grout and Richards, 1992). Grout *et al.,* (1996) found that in two bioassays conducted on *S. aurantii* which had survived exposure to tartar emetic spray, that only 37% mortality occurred from a second exposure, while those not expose to the bait spray for 32 and 97 days had much higher moralities, 85 and 97% respectively. These results showed that a rapid reversion of susceptibility occurred within the thrips population when selection pressures from tartar emetic with sugar were absent. This suggested that this pesticide can not be used continuously because it

increases the farm's cost because of the need to use alternate pesticides. Organophosphates are subjected to strict application criteria and when combined with pyrethroids can be very detrimental to Integrated Pest Management (IPM) (Gilbert and Bedford, 1998).

Depending on the part of the tree that needs protection, the sprays must be applied at different times. For the protection of the fruit, one of the above sprays must be added just after petal fall and continually for another 5 months. For the protection of citrus foliage, specifically new growth flushes, an additional spray in autumn helps keep the number of thrips down over winter (Gilbert and Bedford, 1998).

IPM programmes mainly use natural enemies of thrips as a means of control, such as predacious mites. Many different species of mites have been researched and used for the control of thrips. The majority belonging to the genus *Euseius*. In California the predacious mites *E. hibisci* (Chant) was introduced into citrus orchards to control *S. citrus* (Moulton) and resulted in a 85% reduction in crop damage. In South Africa *E. addoensis* (van der Merwe and Ryke) and *E. citri* are used for the control of *S. aurantii* Faure in citrus orchards, although *E. addoensis* is more common and effective within South Africa (Grout, 1994; Shibao et al., 2004). Other species of mites associated with the reduction of thrips abundance are *Amblyseius womersleyi* Schicha, *A. okinawanus Ehara* and *A. tsugawai* Ehara, which prey on the Japanese thrips species *Thrips palmi* Karny (Shibao et al., 2004). Navarro-Campos *et al.,* (2012) found that the presence of the mite *Gaeolaelaps aculeifer* was connected to a reduction of the thrips *Pezothrips kellyanus* (Bagnall) populations in citrus orchards. While these natural enemies are viable biological control methods they are not the only the only option.

1.3 Entomopathogenic fungi

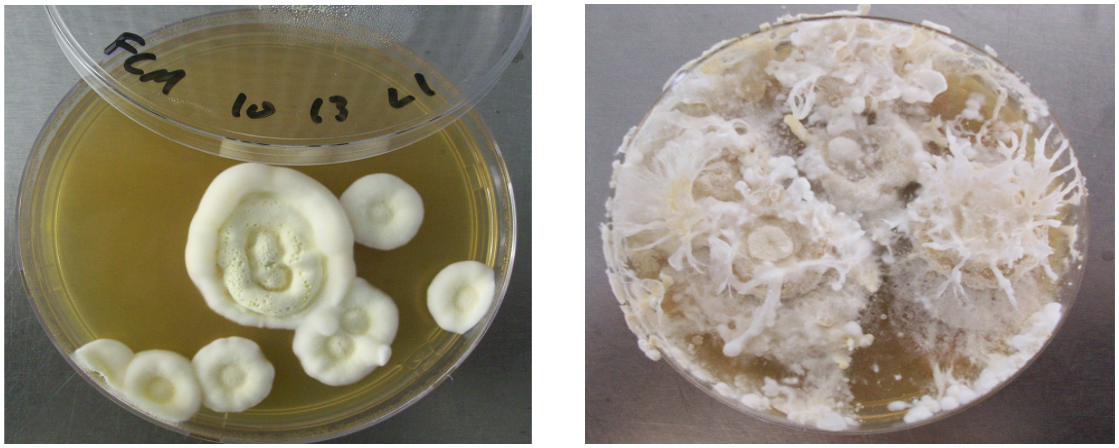
Increasing resistance to chemical pesticides in crop pests has resulted in the search for alternative methods for insect control. One such alternative is microbial control, which consists of integrated control agents such as viruses, bacteria, protozoa and fungi (Inglis *et al.,* 2001). The majority of entomopathogenic (EP) fungi are classified under the order Hypocreales belonging to the division Ascomycota (Inglis *et al.*, 2001; Rehner and Buckley, 2005). Though they are still commonly referred to as belonging to the artificial class Hyphomycetes (Inglis *et al.*, 2001). Entomopathogenic fungi are wide spread and can be found world wide in a large variety of habitats from aquatic to terrestrial environments including forests, agricultural and uncultivated pastures (Ali-Shtayeh *et al.*, 2003). Those found in soil play a vital and important role in the regulation of insect populations because they are natural enemies of a diverse range of arthropods (Quesada-Moraga *et al.*, 2007; Meyling *et al.*, 2009). They have been studied as potential mechanisms for insect pest control for over 100 years,

since the early 1800s where *Beauveria bassiana* was discovered to be the cause of silkworm mortality. Originally EP fungi were very important in controlling insects but since the 1940s chemical insecticides became the preferred and popular choice. In the recent years the market demand for produce acquired without damaging the environment has increased (Vega *et al.*, 2009). Thus, the need for alternate pest control methods such as entomopathogenic fungi has become increasingly importance.

1.3.1 General taxonomy

1.3.1.1 *Beauveria*

The *Beauveria* genus was first described by Agostino Bassi in 1835 when it was found to be the cause of larval silk worms mortalities, commonly referred to then as the white muscardine disease (Vega *et al.*, 2009), although the genus was created in 1912 by Vuillemin (Rehner, 2005; Rehner and Buckley, 2005; Zimmermann, 2007 b). While *Beauveria* species are pathogenic to insects they are also found on plants as endophytes, such as on corn (Rehner, 2009; Devi *et al.*, 2008; Meyling *et al.*, 2009). *Beauveria* is morphologically characterised by its conidia, clusters of globose, flask-shaped conidiophores with zig-zag shaped rachis, which increase in length with the number of conidia produced. In culture *Beauveria* colonies are characterised by white mycelium and conidia (Figure 1.5 a), which may appear yellow as they age. Colonies have a lanate to wooly texture and often produce sunnemalike projections (Figure 1.5 b). Their conidia are the principal morphological feature used in identification. They are hyaline, single-celled, holoblastic and globose to broadly ellipsoidal in shape, and range from between 1.7-5.5 μm in size (Rehner, 2005; Rehner and Buckley, 2005; Zimmermann, 2007 b). Depending on the amount of conidia produced colonies often appear powdery or chalky on the surface (Regner, 2009).



(a) (b)

Figure 1.5: *Beauveria bassiana* isolates; (a) FCM 10 13 L1 with characteristic white wooly mycelial growth and powdery appearance where large amount of conidia have been produced, (b) GB AR 23 B3 older colony beginning to go yellow and with sunnemalike projections, on Sabouraud dextrose 4% agar.

Beauveria bassiana is one of the most abundant and widely distributed species, found worldwide from tropical to temperate regions. *Beauveria* has an extremely wide host range consisting of over 700 species (Zimmermann, 2007 b; Devi *et al.*, 2008; Ghikas *et al.*, 2010). This is a much wider host range compared to most isolates, which exhibit much narrower ranges. For example commercial isolates are developed using an isolate which is specifically virulent against the target pest. Thus, it is viewed as a species complex with morphologically identical strains, and genetically diverse lineages and genotypes (Devi *et al.*, 2008; Morar-Bhana *et al.*, 2011). However, in the 20th century many new species were described which were not morphologically distinct from those already described. This resulted in unreliability in species identification using morphological characteristics especially with *B. bassiana,* because no type specimen exists. Thus, other methods of classification using different molecular markers have been researched (Rehner, 2005). Rehner and Buckley, (2005) resolved *Beauveria* into 6 major clades, each with at least one recognized species, using phylogenetic analysis of nuclear ribosomal internal transcribed spacers (ITS) 1 and 2 and elongation factor-1 alpha (EF-1α) (Figure 1.6).

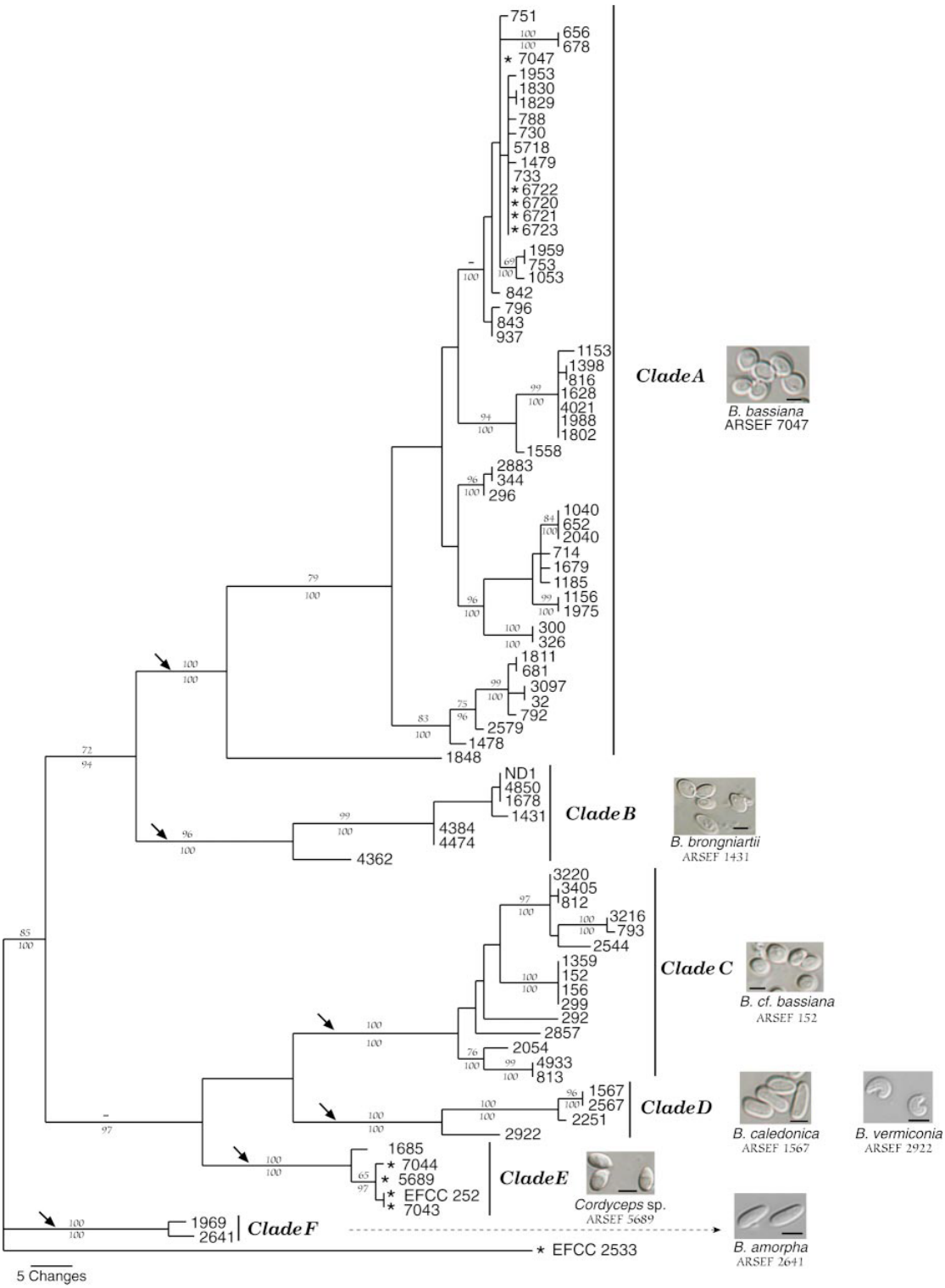


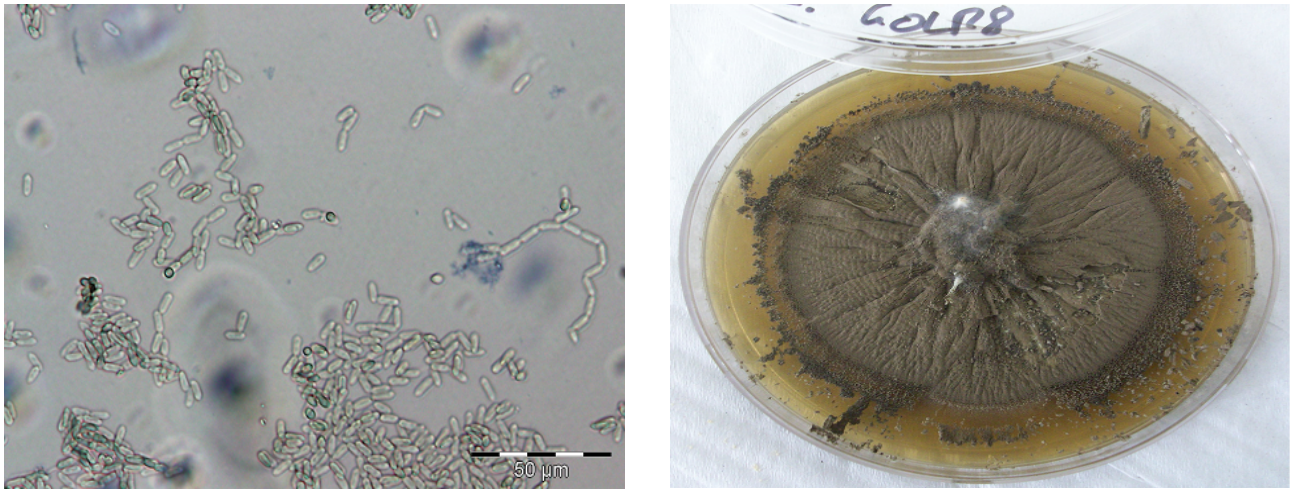
Figure 1.6: Phylogenetic relationships of *Beauveria* from the combined analysis of EF1-α and ITS phylogeny from parsimony and Bayesian likelihood analyses. Bootstrap values > 70% and posterior probabilities > 95% are labeled above and below appropriate internodes and branch termini are labeled according to ARSEF accession numbers of individual isolates. Asterisked accessions indicate *Cordyceps* teleomorphs. Photomicrographs of conidia from representative isolates are illustrated next to each clade. Scale bars are equal to 2 μm (Rehner and Buckley, 2005).

Since its discovery *Beauveria* was believed to reproduce exclusively asexually, but it has since been discovered that *B. bassiana sensu stricto* is linked, both developmentally and phylogenetically, to the Asian sexual species *Cordyceps bassiana.* This is evidence that *B. bassiana s.s* and perhaps others are facultatively sexual (Meyling *et al.*, 2009). The presence of the teleomorph *Cordyceps* species is evidence that there is a direct link between it and *Beauveria* (Rehner and Buckley, 2005; Zimmermann, 2007 b; Ghikas *et al.*, 2010).

1.3.1.2 *Metarhizium*

Metarhizium spp. are haploid anamorph entomopathogenic fungi that are generally green in colour. They are frequently isolated from soils and have a broad host range, although narrower than *B. bassiana's* at ~ 204 insect species. The large majority of these belong to Coleoptera family. They have a wide distribution and have been found throughout the tropics and temperate regions (Zimmermann, 2007 a; Bischoff *et al.*, 2009). *Metarhizium* was first discovered and described by Metschnikoff in 1879, who studied the disease he called green muscardine that was infecting and killing wheat cockchafers (*Anisoplia austriaca).* This isolate later became known as *M. anisopliae.* The genus *Metarhizium* was established by Sorokin in 1883 and was first mass-produced by Krassilstchik in 1888 to control sugar-beet weevils (Zimmermann, 2007 a; Vega *et al.*, 2009). *Metarhizium* is characterised by its sporulating structures on the arrangement of the phialides which bear chains and columns, formed by aggregations of conidial chains. The conidia are usually green, cylindrical or slightly ovoid in shape and differ in size depending on the species or strain (Figure 1.7 a). There are two forms of *M. anisopliae:* those with short spores of about 5-8 μm long and the long form with conidia up to 10-14/16 μm (Zimmermann, 2007 a).

As with *Beauveria, Metarhizium* species were considered asexual until in 1991 Liang *et al.* confirmed that *Metarhizium* was connected to the teleomorph genus *Cordyceps* (Fr.) Link (Clavicipitaceae, Hypocreales). Specifically they linked *C. taii* developmentally to the newly described anamorph species *Metarhizium taii.* Later, *Cordyceps brittlebankisoides* was identified as a teleomorph of *M. anisopliae* var *majus*. The genus is now classified as part of the family Nectriaceae, order Hypocreales, division Ascomycota (Zimmermann, 2007 a). These teleomorphs have so far only been identified in southeastern Asia, which is now considered a likely point of origin for *Metarhizium* (Bidochka and Small, 2005).



(a) (b)

Figure 1.7: (a) Light micrograph of *Metarhizium anisopliae* spores under x4000 magnification, (b) *M. anisopliae* grown on SDA plates at 25°C

1.3.2 The infection process

Unlike other forms of biological arthropod pest control which infect the host via oral means, EP fungi do so via the cuticle (Lacey *et al.*, 2001; Shah and Pell, 2003; Zimmermann, 2007 b; Roy *et al.*, 2009; Vega *et al.*, 2009). *Metarhizium anisopliae* for example penetrates the intersegmental folds or around the mouthparts (Zimmermann, 2007 a). Before penetration, the fungal spore strongly adheres to the insect cuticle via non-specific adhesion mechanisms due to the hydrophobicity of the spore's cell wall (Inglis *et al.*, 2001). Penetration of the external cuticle occurs due to a combination of mechanical pressure and enzyme degradation (Reithinger *et al.*, 1997). Mechanical penetration of the cuticle occurs by the formation of penetration structures; germ tubes and appresorium from which penetration pegs or hyphae are formed (Figure 1.8 1, 2) (Inglis *et al.*, 2001; Shah and Pell, 2003). Enzymatic degradation of the cuticle is mostly carried out by exoproteases but other enzymes such as endoproteases, esterases, lipases, chitinases and chitobiases may also be involved (Inglis *et al.*, 2001).

Even if the fungal spore germinates, penetration does not always occur due to external factors such as climatic conditions (Inglis *et al.*, 2001), or inhibiting factors within the cuticle (Zimmermann, 2007 a). The main climatic conditions which affect penetration are temperature and humidity. Inability to penetrate the cuticle often occurs due to the combined effects of cuticle thickness, tensile strength and the degree to which the cuticle has been hardened by sclerotization (Reithinger *et al.*, 1997). The cuticle could also contain cuticular lipids such as short-chain fatty acids, aldehydes, wax esters

and alcohols, which have antimicrobial activity. The cuticle can also contain substances such as free amino acids or peptides which induce fungal recognition and germination (Zimmermann, 2007 a).

Once the fungus has penetrated the cuticle it enters the insect body cavity and haemolymph (Reithinger *et al.*, 1997; Shah and Pell, 2003; Roy *et al.*, 2009). This is done by proliferating and producing single or multicellular hyphal bodies, known as blastospores (Figure 1.8 3). These are distributed throughout the host passively using the haemolymph (Inglis *et al.*, 2001; Zimmermann, 2007 a). Before proliferation can occur on a large scale the fungus must first overcome the insect's defense responses to the fungal invasion. Most EP fungi do so by producing secondary metabolites as toxins which weaken the host's immune response (Inglis *et al.*, 2001). Some toxins commonly produced are: destruxins, produced by *M. anisopliae*, efrapeptins by *Tolypocladium* species, beauvericin, bassianolide, beauveriolide, by *Beauveria* and *Fusarium* species (Vey *et al.*, 2001).

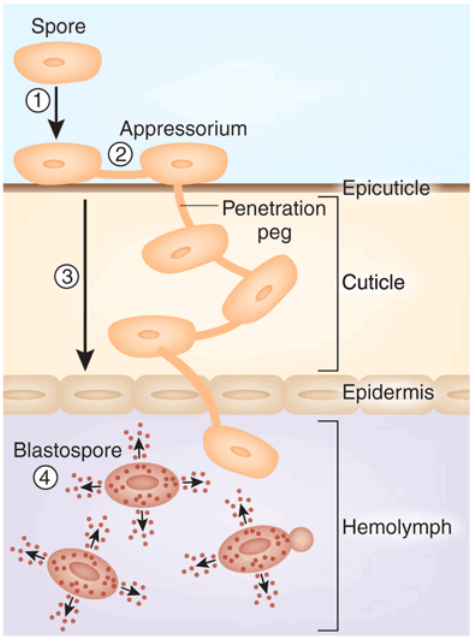


Figure 1.8: Illustration of an EP fungal spore penetrating the host's cuticle (1) recognition of the host by the fungus results in germination of the spore and production of a penetration structure, the appressorium, (2) which grows a penetration peg and a series of hyphal bodies to cross the cuticle and epidermis. (3) Once inside the host, the fungus produces blastospores which bud and spread through the haemolymph. (4) Hemolymph-specific expression restricts release toxin(s) to the period after infection. (Thomas and Read, 2007)

Destruxins are secondary metabolites which were first isolated from *M. anisopliae* in 1961. The name "destruxin" was derived from the species name of *Oospora destructor,* now known as *Metarhizium anisopliae*. It is a cyclic hexadepsipeptide consisting of an α-hydroxy acid and 5 amino residues. Different types of destruxin are produced. Their differences occur in the R group of the hydroxy acid

or as a result of *N*-methylation. These differences in structure bring about differences in effectiveness among the different types, such as the presence of the carboxyl group in destruxin D which decreases its insecticidal activities (Pedras *et al.*, 2002). Destruxin E is the most potent of the many compounds produced (Pedras *et al.*, 2002; Zimmermann, 2007 a). Destruxins cause immediate tetanus paralysis and in high doses can cause titanic paralysis, which decreases insect larval growth (Zimmermann, 2007 a). This results in the disruption of calcium balance within cells, inhibition of vacuolar adenosine triphosphatases (ATPases), deformation of nuclei, degradation of mitochondria and rough endoplasmic reticulum, and interference with haemocyte functioning (Vey *et al.*, 2001; Pedras *et al.*, 2002). Yet, not all insects are equally susceptible to destruxins. Their effects vary depending on the species and specific developmental stage of both the host and the fungus (Vey *et al.*, 2001).

Beauvericin was first identified from *B. bassiana* and is also a cyclic hexadepsipeptide like destruxin. Its insecticidal activity it is also antimicrobial, antiviral and cytotoxic (Wang and Xu, 2012). It is effective across a wide range of insects and is a specific cholesterol acyltransferase inhibitor and can induce cell death similar to apoptosis, which causes cytolysis in insects (Vey *et al.*, 2001; Zimmerman, 2007 b). Efrapeptins on the other hand inhibit intracellular protein transport and mitochondrial ATPases (Vey *et al.*, 2001). Not all secondary metabolites produced by EP fungi are toxic to insects. Some are produced for other necessary reasons, such as fungal survival or competition (Figure 1.9).

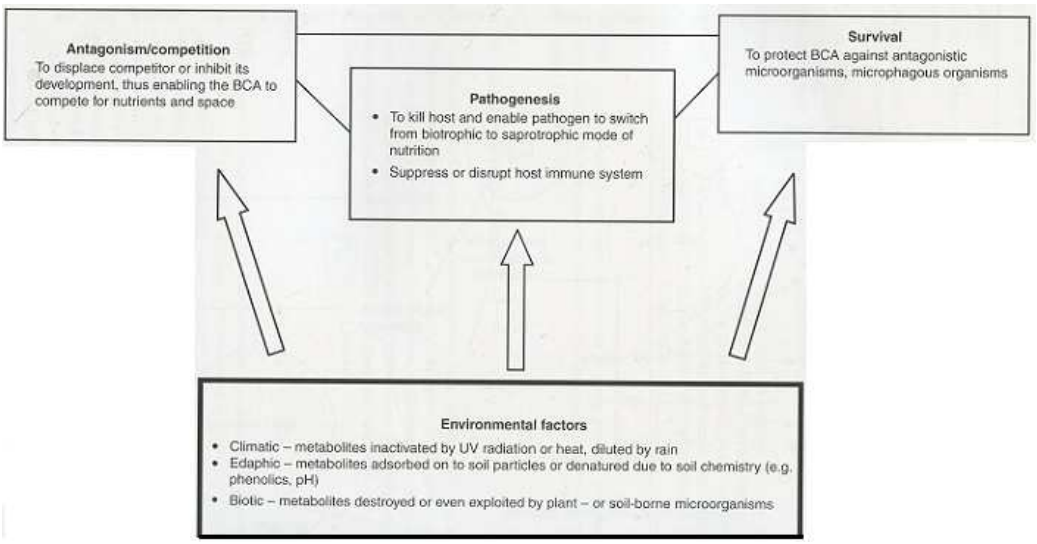


Figure 1.9: Different roles of fungal metabolites and the environmental factors which affect their persistence (Vey *et al.*, 2001)

Once host defense responses have been overcome, extensive vegetative fungal growth occurs within the insect and most tissues are invaded (Zimmermann, 2007 a). Insect death occurs from a combination of fungal actions, the depletion of nutrients from the haemolymph and body fat, physical obstruction of organs and toxicosis (Inglis *et al.*, 2001). EP fungi acquire their nutrients from their

insect hosts in one of three different ways: through biotrophy, necrotrophy or hemibiotrophy and saprotrophically. Biotrophy is the result of acquiring nutrients from only living cells. Necrotrophy on the other hand is when the cells are killed and then utilized for nutrients. Hemibiotrophy is a combination of these two forms. The organism is initially biotrophic and then becomes nectrophic once the host dies (Vega *et al.*, 2009). After the death of the insect host, fungal growth occurs saprotrophically, where it acquires the nutrients from the dead tissue. Under favourable conditions the hyphae emerge from the insect and sporulation occurs on the exterior of the cadaver (Inglis *et al.*, 2001; Shah and Pell, 2003). Under extremely dry conditions, sporulation occurs within the cadaver (Zimmermann, 2007 a). The externally produced spores of the fungus are passively distributed via water, rain splash and wind, hence they are affected by the ambient environment (Ali-Shtayeh *et al.*, 2003).

1.3.3 Use as biological control agents

Many EP fungi have been investigated and implicated over the years as potential biological control agents (BCA). Some fungi are used more commonly then others due to their host range. Many have very narrow host ranges, such as *Aschersonia aleyrodis* which only infects whiteflies (Homoptera: Aleyrodidae),. Others such as *M. anisopliae* and *B. bassiana* have a much wider host range, which covers a large number of arthropod orders. When considering the biological control potential of EP fungi, factors to be considered include; the virulence of the strain being used, the population densities of the target hosts, persistence in the environment and their high dispersion rate. A number of different factors make the target hosts susceptible to the EP fungi, such as population density, behavior, age, nutrition, genetics and exposure to injuries. Any of these factors result in "stressed" insects, which are more susceptible to infection from EP fungi (Inglis *et al.*, 2001).

Some of the most common EP fungi belong to the genera *Metarhizium*, *Beauveria*, *Aspergillus*, *Culicinomyces*, *Hirsutella*, *Nomeuraea*, *Paecilomyces*, *Tolypocladiumi* and *Verticillium (*(Inglis *et al.*, 2001). Many of these have been tested against different species of mealybugs which are pests of other crops such as enset (Lemawork *et al.*, 2011), cotton (Banu *et al.*, 2010), grapevine (Ujjan and Shahzad, 2007), mangoes and pineapples (Dolinsky and Lacey, 2007). Lemawork *et al.*, (2011) tested *B. bassiana* and *M. anisopliae* against adult enset root mealybugs, *Cataenoccus ensete*. Enset is one of the most important food crops in Ethiopia and is threatened by *C. ensete,* which can retard growth and significantly impact crop yield. These EP fungi were tested against enset mealybug in laboratory, pot and under field conditions. In all the various trials mealybug mortality was observed, with *B. bassiana* causing the highest mortality, but both *B. bassiana* and *M. anisopliae* produced levels of mortality in all the trials significant enough for the fungi to be considered for inclusion of IPM programmes for the control of enset mealybug.

Banu *et al.* (2010) also tested these same two fungi and *Verticillium lecanii* against the cotton pests, mealybugs, *Phenococcus solenopsis* and *Paracoccus marginatus*. Under laboratory conditions these three entomopathogenic fungi were tested against nymphal instars and adults. Each of the fungi resulted in mortality in both stages of between 40 and 50% after 48 hour incubation, with *V. lecanii* causing the highest mortality in *P. solenopsis* adults (55%) and 51% mortality in nymphal instars of *P. marginatus*. Similar results were recorded by Devi *et al.* (2008) where different strains of *B. bassiana* were tested under laboratory conditions against the pink hibiscus mealybug, *Maconellicoccus hirsutus*. When compared to the other insects hosts tested in this paper fewer *B. bassiana* strains were highly pathogenic towards the mealybug, with most causing medium mortality (between 34-66% mortality). On the other hand Ujjan and Shahzad (2007) tested 3 different strains of *M. anisopliae* against the pink hibiscus mealybug under laboratory conditions. All of the strains resulted in high mortality in adults, 90% after 8 days, 100% mortality by the fourth day in instars and 60-30% reduction in egg hatching.

Beauveria bassiana has caused high mortality in other mealybugs such as mango pests, *Drosicha mangiferae*. When tested under field conditions *B. bassiana* reduced mealybug populations by up to 100% in some areas after a period of 10 days (Dolinski and Lacey, 2007). Other EP fungi in association with mealybugs are *Hirsutella crytosclerotium* found in association with the mealybug *Rastrococcus invadens* (Roy and Pell, 2000). *Isaria farinose* was used against citrus ground mealybugs, *Rhizoecus kondonis,* over a period of 1-4 months which resulted in 70-100% mortality, and against *Pseudococcus cryptus.* The 4 different strains of *Isaria fumosorosea* were found to cause up to 43% mortality (Zimmermann, 2008).

Entomopathogenic fungi have been found to be potential BCA against other pests such are thrips, fruit flies, many different species of beetle, midges and sandflies. Ansari *et al.* (2007) compared *M. anisopliae* with the ability of chemical pesticides (imidacloprid and fipronil) to kill pupae of western flower thrips. Exposure to *M. anisopliae* resulted in much higher mortality of 70-90% in comparison to the pesticides, which only resulted in a mortality of 20-50% under laboratory conditions, showing its potential for IPM use. Similar results were recorded using *B. bassiana* against onion thrips, *Thrips tabaci,* by Wu *et al.* (2013) where exposure to a conidial concentration of 1 x $10^7 ml^{-1}$ under laboratory conditions resulted in 83-100% mortality in adults. In greenhouses *V. lecanii* has also shown potential for the control of thrips (Inglis *et al.*, 2001).

Granularly formulated *M. anisopliae* was found to significantly reduce the emergence of 3 species of African tephritid fruit flies (*Ceratitis capitata, C. fasciventis* and *C. cosyra*) by 37, 42 and 57% respectively, in field cage trials, and did not have a negative effect on these species non-targeted natural enemies (Ekesi *et al.*, 2005). Goble *et al.* (2011) determined that two indigenous South African *B. bassiana* strains had potential for the control of two fruit fly species, *C. rosa* Karsch, *C. capitata* Wiedemann (Diptera: Tephritidae), and false codling moth, *Thaumatotibia leucotreta*

Meyrick (Lepidoptera: Tortricidae). Cherry *et al.* (2005) tested different strains of *B. bassiana* and *M. anisopliae* against the cowpea seed beetle *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) and found that, of the two most significant strains, *B. bassiana* was consistently more virulent then the *M. anisopliae* strain. It also significantly reduced the beetle population sizes. Entomopathogenic fungi are used for the control of crop pests and for the control of viral vectors of livestock, such as the *Culicoides* (Diptera: Ceratopognidae) biting midges, which transmit bluetongue virus. Many different entomopathogens were tested against adult *C. nubeculosus* by Ansari *et al.* (2011) who found that a *M. anisopliae* strain was the most significantly virulent isolate and exposure to dry conidia resulted in 100% mortality in just 5 days. These virulent strains are sometimes developed into commercial mycoinsecticides, such as "Green Muscle" recommended for the control of locusts and grasshoppers in Africa. This product consists of dried *M. acridum* conidia and is mixed with spray oils before spraying (Shah and Pell, 2003).

1.3.4 Abiotic factors influence infection effectiveness

The ability of entomopathogenic fungal pathogenicity to infect their specific hosts is determined by a large number of different factors such as the state of the host, fungus, and most importantly the environment and its multiple abiotic factors. The ability to persist in a chosen environment is an extremely important consideration when choosing an EP fungus as a biological control agent, especially for the infectious propagules which must persist and remain viable until contact occurs with the host. Some of the most important abiotic factors include temperature, solar radiation and moisture (Inglis *et al.*, 2001; Thompson *et al.*, 2006; Zimmermann, 2007 b; Fernandes *et al.*, 2008).

1.3.4.1 Temperature

Temperature is one of the main factors affecting the efficacy of EP fungi (Inglis *et al.*, 2001; Zimmermann, 2007 a; Fernandes *et al.*, 2008). Temperature impacts germination, the rate of infection, fungal growth both around and in the host and alter the time taken for insect death to occur (Inglis *et al.*, 2001; Zimmermann, 2007 b; Fernandes *et al.*, 2008; Wraight and Hajek; 2009). High temperatures also cause DNA damage, specifically depurination, and if combined with high humidity results in membrane disorganization and protein denaturation (Rangel *et al.*, 2005 a; Li and Feng, 2009). Every EP fungus has its own specific optimum temperature. For the broader genera such as *Beauveria* and *Metarhizium* optimum temperatures vary between species and strains (Zimmermann, 2007 a). Thus, when choosing the best biological control strain the environmental conditions in which it will be used must be considered.

For the majority of EP fungi, optimum growth occurs between 20-35°C (van Driesche and Bellows Jr, 1996; Inglis *et al.*, 2001; Rangel *et al.*, 2005 a; Fernandes *et al.*, 2008). EP fungi are mesophilic and able to grow within a wide range, from 10-40ºC (Fernandes *et al.*, 2008). *Beauveria bassiana* for example experiences optimum growth between 23 and 28ºC yet has a minimum range of 5-10ºC and a maximum range of 30-38ºC, along with a death point between 45-55ºC (Zimmermann, 2007 b; Fernandes *et al.*, 2008). *Metarhizium anisopliae* has a similarly wide range of 5-40ºC with optimum growth occurring between 25 and 30ºC (Zimmermann, 2007 a) and a wide thermal death point range of 49-60ºC (Rangel *et al.*, 2005 a). Dormant conidia have a much higher temperature tolerance. This increased resistance is believed by Fernandes *et al.,* (2008) to be due to an increased amount of saturated fatty acids, mannitol and trehalose. Elevated levels of fatty acids decrease the permeability of the cell membrane, while the mannitol and trehalose protect against protein and membrane denaturation (Fernandes *et al.*, 2008). Moisture content also plays an important role; Rangel *et al.* (2005 a) discovered less conidial damage occurred when exposed to dry heat in comparison to wet heat.

Many *Metarhizium* and *Beauveria* isolates are also cold active or have thermotolerance abilities, which allow them to survive the wide temperatures ranges they are exposed to. For example, African *Beauveria* isolates have been found to have reduced growth and germination at 15 and 35ºC with optimum temperatures ranging from 20-30ºC while isolates collected from the subarctic germinated at 5ºC (Zimmermann, 2007 b). This was also seen in *Metarhizium* isolates collected from the subarctic, which germinated between 2.5 and 10ºC (Zimmermann, 2007 a). Similar results were found by Inglis *et al.* (2001) where EP isolates collected in Europe had optimum temperatures of 20 to 25ºC and a growth range of 8-30ºC, while isolates collected in India were able to grow at increased temperatures of 32-35ºC. While no general relationship has been found in relation to latitude and growth rates, isolates collected from the equator had a higher thermotolerance, while most cold active isolates have been found in northern sites (Rangel *et al.*, 2005 a; Zimmermann, 2007 a; Fernandes *et al.*, 2008). In general it has been observed that *Metarhizium* species are less cold active then *Beauveria* species, although this depends greatly on the specific strain and the natural environment from where it was isolated.

Heat tolerance is extremely important for many isolates, particularly those found in agricultural soils. Entomopathogenic fungi reside within the upper layers of soil, in which can reach up to 65ºC with large amounts of daily fluctuations (Inglis *et al.*, 2001; Rangel *et al.*, 2005 a; Zimmermann, 2007 a). These fluctuations have been shown to affect the fungi in laboratory conditions, thus resulting in a need for thermotolerant species or strains (Inglis *et al.*, 2001). For example Bidochka *et al.* (2002) compared two different *Metarhizium* populations, one from a forest and another from agricultural soils. They found that the population from the forest was cold-active while the second population was heat tolerant and grew at around 37ºC. Similar results with *B. bassiana* were found within populations tested in Finland (Zimmermann, 2007 b).

Thermotolerance is also selected for as a coping mechanism against their host's behaviour. Many insects increase their body temperatures above ambient, usually via habitat selection, sun basking (Fernandes *et al.*, 2008) or behavioural fever (Inglis *et al.*, 2001). In some cases this behaviour has been shown to assist certain hosts to overcome the infection, but it is not a guaranteed method of resistance. Often it only increases length of time until host death or the initial amount of inoculum required to cause infection is increased (Inglis *et al.*, 2001). This indicates that the isolate chosen for biological control must be adapted to the specific environment in which it will be used.

1.3.4.2 Solar radiation

All EP fungal propagules such as conidia, and hyphae of all taxa, are susceptible to solar radiation. As with temperature there is a variance of susceptibility between taxa and strains (Fargues *et al.*, 1996; Inglis *et al.*, 2001; Zimmermann, 2007 b). EP fungi are specifically susceptible to the Ultra Violet ranges UV-B (290-330 nm) and UV-A (330-400 nm). Extended exposure to solar radiation can cause inactivation within a few hours (Zimmermann, 2007 a). In nearly all EP fungal species a few hours of exposure to direct sunlight, especially during midday, is enough to completely inactivate the conidia. Dormant and germinating conidia have different tolerance levels, with germinating conidia being the most sensitive (Santos *et al.*, 2011). The negative effects of UV-B are a result of the formation of cyclobutane pyrimidine dimers within the fungal DNA, which often cause mutagenesis or failures in transcription. UV-A exposure on the other hand produces oxidative stress and causes indirect DNA damage by the production of reactive oxygen species (Fernandes *et al.*, 2007; Santos *et al.*, 2011).

Different strategies have been employed to prevent or reduce UV inactivation of the fungal conidia. The addition of UV protectants as forms of "sunscreen" for the conidia have been tested. The addition of these protectants has been found to be effective in controlled environments, but when tested in field trials, no significant differences in the efficacy of the EP fungi were recorded (Fargues *et al.*, 1996; Inglis *et al.*, 2001; Zimmermann, 2007 a). For example Inglis *et al.* (1995 a) tested *B. bassiana* with a combination of different water and oil compatible compounds, such as Tinopal LPW, clay and oxybenzone, ethyl-cinnamate and many others, in both controlled laboratory conditions and in the field on leaves of crested wheat grass. They found that the oil-soluble protectants significantly increased the survival of the fungus in controlled conditions when exposed to artificial radiation. Although, when tested in the field no significant differences were found. Shah *et al.* (1998) found similar results with *M. acridum* when testing against the grasshopper *Kraussella amabile* as both the control and conidia coated with oxybenzone had an average survival time of 10 days in the field. That being stated some oils such as peanut or shellsol plus Ondina were found to significantly increase the conidial tolerance for a period of 6 hours, thus, fungal sunscreen protectants seem to only add

hours of protection and not days to the survival of the conidia in the field, depending on the isolate (Zimmermann, 2007 a).

While sunscreens may not be able to increase field survival for extended periods under field conditions in direct sunlight, conidia are not always fully exposed to extreme conditions. Protection from direct sunlight exposure can be found in many different microhabitats such as the canopy of trees, or the underside of leaves. While even in these shaded areas indirect sunlight can still inactivate conidia, though conidia have a greater chance remaining viable for an extended enough period of time to allow insect infection (Inglis *et al.*, 2001). Fargues *et al.* (1996) tested the persistence of conidia at different levels within the canopy of alfalfa and crested wheat grass. At the top of canopies conidial populations were reduced by 75 - 99% after 4 days, while in the middle of the canopy they were only reduced by 29 - 85% after 16 days.

Exposure to light during mycelial growth affects a number of different process within the fungi some of these are, primary and secondary metabolism, pigmentation formation in some species and trehalose accumulation (Rangel *et al.*, 2011). The accumulation of trehalose in fungi is believed to indicate and be connected to the process of starvation, along with many other stresses. Trehalose improves the fungal ability to tolerate stress, mainly due to the protection it offers to cellular component from denaturation by stabilising them in their natural state and by preserving the integrity of membranes (Rangel *et al.*, 2006; Rangel *et al.*, 2011). As a viable conidial "sunscreen" has yet to be found many researchers have focused on finding the more resistant isolates or ways to culture them. One of the ways more tolerant species and strains were cultured was by working on the basis that the predisposition to one stress could produce a cross protection to other stresses. On this principle Rangel *et al.* (2008) found that *M. anisopliae* var *anisopliae* conidia had increased UV-B tolerance when produced under osmotic or nutritive stress, especially if produced on a minimal media (MM) with a non-preferred carbon sources such as galactose, fructose or lactose, as found by Rangel *et al.,* (2006).

1.3.4.3 Moisture

High humidity is an important factor required for spore germination and external sporulation on insect cadavers (Inglis *et al.*, 2001; Zimmermann, 2007 a,b). For example *B. bassiana's* optimum relative humidity (RH) range is between 92 and 100% while *M. anisopliae* germination occurs best at 100% RH, it is completely inhibited by humidity less than 85 to 90% (Zimmerman, 2007 a,b). The majority of EP fungi require a minimum of 95% RH on their host's cuticle for germination and subsequent penetration and infection to occur (Inglis *et al.*, 2001; Thompson *et al.*, 2006). This lead to the realisation of the importance of microclimates such as, sufficient moisture levels in the vicinity of

the insect's exoskeleton cuticle folds, germination and sporulation can still occur (Inglis *et al.*, 2001; Thompson *et al.*, 2006; Zimmermann, 2007 a,b). While germination does require high humidity, some taxa, such as *B. bassiana* have been shown to sporulate within the haemocoel of cadavers if external conditions are unfavourable.

Rainfall and irrigation are important sources of moisture. They help to increase humidity and dislodge and disperse fungal conidia. Conidia strongly adhere to their host's cuticle. Rain rarely removes a significant amount of conidia but it is able to remove most of them from the foliage of trees and other plants. Inglis *et al.* (1995 b) tested the effect of simulated rain at two different intensities and durations on the persistence of *B. bassiana* applied using water to the leaves of alfalfa and wheat. After the simulated rain conidial populations were reduced by 28 - 61% and no significant difference was found between the type of plant, rain intensity and duration on conidial retention. On the other hand Thompson *et al.* (2006) tested the effect of high and low levels of irrigation of conidial viability on turfgrass using two different strains of *B. bassiana.* They found that conidial viability was maintained 8 to 12% better with higher irrigation levels. Although, the cause was believed to be higher irrigation levels, which moved the conidia deeper into the turfgrass canopy and soil profile, allowing for increased protection from damaging temperatures and UV exposure.

Research has been conducted into the use of stickers in conidial application. Adding different vegetable and mineral oils such as sunflower and parrafinic oils, to the conidial suspensions of *B. bassiana* was found to determine the retention on plant leaves (Inglis *et al.*, 2000). Retention of conidia was marginally improved. The use of stickers must be closely monitored as they must not prevent the transfer of conidia from the foliage to the targeted insects (Inglis *et al.*, 2001).

The foregoing conditions indicate the great care that must be taken in choosing the correct EP fungal strain. An isolate's effectiveness against a specific pest relies on its persistence within the field. Hence, a balance must be found between effectiveness, resistance and viability under field conditions. While having a strain with high resistance to harmful abiotic factors is helpful, application must also be considered. Where and how the conidia are applied within the canopy is important to increase viability by reducing their risk of exposure to adverse conditions.

1.4 Aims

Many strategies were employed in an attempt to control citrus mealybugs, such as chemical control, ant control, sex pheromones, monitoring techniques, economic thresholds and biological control. Most of the published work conducted for the control of citrus mealybugs with classic biological

control has been with parasitoids. Very little work has been conducted on the use of EP fungi as biological agents to control citrus mealybugs.

In Chapter 2 the susceptibility of the mealybugs to select EP fungi was determined, by achieving the following aims; (1) successfully established a culture of *P. citri* mealybugs on insecticide free butternut for use in bioassays, (2) maintained *Metarhizium* and *Beauveria* isolates from citrus orchard soils. Along with (3) established virulence of isolates through bioassays, (4) morphological and molecular identification of the most virulent entomopathogenic isolates and (5) using those isolates to determine LC_{50} and LC_{90} concentrations. This study also focused on (6) determining the mode of fungal infection on mealybugs using scanning electron microscopy.

In Chapter 3 work was also conducted and determined the susceptibility of citrus thrips to EP fungi by achieving the following aim; (7) performed a smaller bioassay on *S. aurantii* thrips with the most virulent entomopathogenic strains.

In Chapter 4 implications for the results of this study were discussed in relation to the final goal of developing any promising fungi into a mycopesticide product. This includes their use in IPM programmes, especially in citrus, along with future work and final conclusions.

Chapter 2

Screening of entomopathogenic fungi against mealybug

2.1 Introduction

Mealybugs are difficult to control with the use of chemicals, due in part to their cryptic life cycle. As they are found in dense colonies, and in difficult to spray areas such as under leaf axils and fruit calyxes, or between adjacent fruit (Cox and Pearce, 1983; Inglis *et al.*, 2001). They are particularly noted for their intersegmental secretions. Mealybugs produce a fine powdery wax (Cox and Pearce, 1983; Johnson-Cicalese *et al.*, 2011), which gives the "mealybug" its common name. The mealy wax secretions are produced by the many pores and ducts covering the insect. The mealy wax layer helps prevent contamination, reduce the entry of bacteria, fungi or other parasites, and to reduce damage at the points where eggs touch each other (Kumar *et al.*, 1997). It has been suggested that the wax protects the mealybug from its own honeydew excretions (Johnson-Cicalese *et al.*, 2011).

Due to increased difficulties in controlling mealybugs with chemical insecticides and the increasing concern about the impact of these pesticides, alternative control methods such as the use of natural enemies or pathogens are becoming incorporated into, and form major parts of IPM programmes. IPM programmes consider the agrosystem as a whole. They employ a variety of different control techniques both biological and chemical to keep pest populations below economically damaging levels (Orr, 2003). Amoung the commonly considered biological control options investigated are entomopathogenic fungi. Of these *Metarhizium anisopliae* (Metschinikoff) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin are the most commonly used EP fungi for pest control (Chase *et al.*, 1986; Fernandes *et al.*, 2010).

EP fungi used for pest control are commonly isolated from soils via the use of selective media.

Most of these are solid basal mediums such as sabouraud dextrose agar with added addition antibiotics for the inhibition of bacteria and dodine (Chase *et al.*, 1986; Gao *et al.*, 2012). Dodine (*N*-dodecylguanidine acetate) is a fungicide which disrupts the selective permeability in cytoplasmic membranes and severely affects the metabolism of most saprophytic and plant pathogenic fungi. EP fungi have proven less vulnerable to it, hence it is often used in selective media to inhibit the growth of non EP fungal isolates (Rangel *et al.*, 2010; Shapiro-Ilan *et al.*, 2002). Dodine is relied upon heavily in selective media since Beilharz *et al.* (1982) determined its effectiveness in suppressing the growth of non-EP fungi in selective media (Fernandes *et al.*, 2010).

Thus, the aims of this experiment were to (1) isolate an EP fungal strain from either the *Beauveria* or *Metarhizium* genera which was highly pathogenic against mealybugs (2) to determine the LC_{50} and LC_{90} doses and (3) to determine against which life stage this isolate was most pathogenic.

2.2 Methods

2.2.1 Mealybug rearing

A culture of citrus mealybug (*Planococcus citri*) provided by DuRoi Integrated Pest Management (Limpopo, South Africa) was reared by the Rhodes University Entomology Department. The culture was reared on organic butternuts also provided by DuRoi IPM. The butternut were surface sterilised using sporekill (Hydrotech South Africa ACT29GNR) and sodium hypochloride 3.5% and kept at 26°C and at a relative humidity of 30%. Adult mealybugs were brushed onto empty cardboard egg cartons, onto which the butternuts were placed. They were then covered with lightly moistened shredded paper to create humid and a slightly enclosed environment. This induced the crawlers to move onto the butternut. When the butternuts were approximately 30% covered with crawlers they were placed into another enclosure lined with damp newspaper. There they were kept under constant illumination and checked twice a week. When the crawlers had matured into adults they were brushed off the butternut onto the egg cartons and the cycle was repeated. A full cycle from egg to egg, took approximately a month to complete. Females began to oviposit after 25 days and crawlers began to move around 5 to 7 days later (adapted from DuRoi IPM rearing protocol).

2.2.2 Entomopathogenic fungal cultures

Sixteen entomopathogenic fungal isolates belonging to the *Beauveria* and *Metarhizium* genera from the existing Rhodes University fungal collection were used. These were isolated in 2008 from the

soil of 3 conventional and 3 organic citrus farms by Goble *et al.*, (2010). They were isolated from the soil using a modified version of the "*Galleria* bait method" using final instar larvae of greater wax moth (*Galleria mellonella),* false codling moth (FCM) *(Thaumatotibia leucotreta)* and Mediterranean fruit fly (*Ceratitis capitata).* A number of the EP fungal isolates isolated by Goble *et al.* (2010) were submitted to the Plant Protection Research Institute Fungal Collection for identification and given PPRI accession numbers.

The 16 isolates were cultured on Sabouraud Dextrose 4% Agar (SDA) (Merck HG0000C5) supplemented with 50 mg L^{-1} of dodine (Syllit® 400 SC, CA701648), filter sterilised, 50 mg L^{-1} of Ampicillin (Sigma-Aldrich, A9518) and 50 mg L^{-1} of Rifampicin (Sigma-Aldrich, R3501). They were incubated at 26ºC in the dark (adapted from Goble *et al.,* 2011). To confirm pure cultures and pathogenicity, 5th instar FCM larvae, provided by the Rhodes University Entomology Department, were exposed to the isolates and reisolated from the larvae after death. This was achieved by collecting fungal conidia from the SDA plates using a sterile loop and placed in sterile 1.5 µl microcentrifuge tubes containing 1 ml of water and 5% Tween 20 (Merck SAAR 6164500KF). Tubes were vortexed to produce a homogenous solution and adjusted to a concentration of 1 x 10^7 ml^{-1} determined using a hemocytometer (Section 2.2.4). Ten FCM larvae per isolate were immersed in the suspension for 5 seconds each and then placed into petri dishes containing moist filter paper and incubated at 26°C. The larvae were examined daily for the next 7 days. Dead larvae were placed onto SDA plates to encourage external fungal sporulation, then subcultured onto fresh SDA media. All subsequent experiments were conducted using these cultures (Table 2.1).

2.2.3 Morphological identification

Fungal morphology was determined using light microscopy. Tape mounts of all 16 isolates were made, using 0.05% trypan blue in lactoglycerol (Lactic acid: Glycerol: Water 13:12:16) (Smith and Dickson, 1991) as a stain. Under a compound microscope (Nikon YS100) the fungi were examined for identifying characteristics specific for *Beauveria bassiana* and *Metarhizium anisopliae* (Figure 2.1).

Table 2.1: The EP fungal isolates (Goble *et al.*, 2010) and used in this study

Isolate * ^ #	**Genus**	**Origin (farm)**	**PPRI**
GB AR 23 13 3 * ^	*Beauveria*	Arundel	-
FCM 10 13 L1 *	*Beauveria*	Mosslands	9680
GOLR 11 *	*Beauveria*	Olifantskop	9690
G11 BL6 *	*Beauveria*	-	-
GAR 17 B3 * ^ #	*Beauveria*	Arundel	9679
G14 2 B5 *	*Beauveria*	Mosslands	9555
J10 GB AR 23 13 3 *	*Beauveria*	-	-
F8 FCM Rose R9 *	*Beauveria*	Rosedale	9685
G14 2 13 5	*Beauveria*	-	-
FF JB R5	*Beauveria*	J & B Citrus	9556
G12 GMO	*Beauveria*	-	-
L6 G14 2 B 5	*Beauveria*	-	-
FCM AR 23 B3 * ^ #	*Metarhizium*	Arundel	9561
G11 3L6 *	*Metarhizium*	Mosslands	9803
D	*Metarhizium*	-	-
GOLR 8	*Metarhizium*	Olifantskop	9801

PPRI = Plant Protection Research Institute Fungal Collection accession number. * Isolates used in mealybug screening. ^ Isolates used in dose-dependent bioassay. #Isolates used in thrips bioassay.

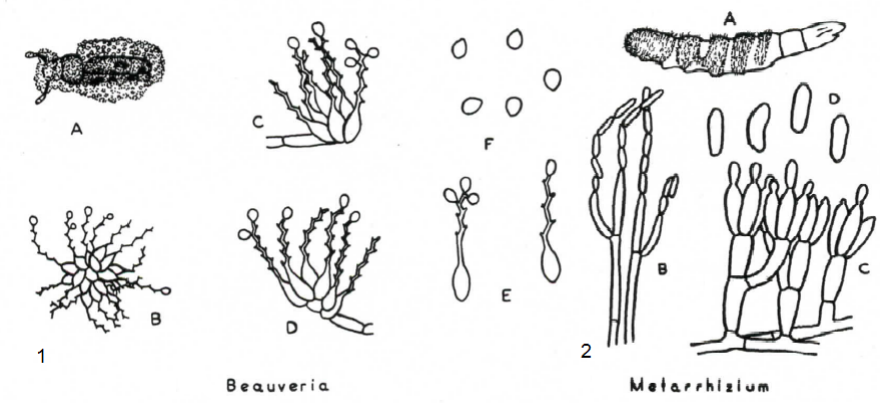


Figure 2.1: (1) *Beauveria bassiana* structures; (a) infected beetle, (b,c,d) clusters of conidiophores, (e) single conidiophores and (f) single conidia. (2) *Metarhizium anisopliae* structures; (a) Infected insect larva, (b) loosely arranged aerial conidiophores, (c) compact conidiophores and (d) loose conidia (Modified from Barnett, 1962)

2.2.4 Preparation of conidial suspensions

Conidia were collected from 2-4 week old fungal cultures by removing the conidia from the mycelium with a sterile loop. The conidia were suspended in MacCartney bottles with 20 ml of sterile water, 5% Tween 20 and glass beads. The bottles were then vortexed for two minutes to produce a homogenous suspension. From this, initial suspension a 1:100 dilution was made and from this conidial concentrations were then determined using a Neubauer hemocytometer (Thoma CE) (Figure 2.2).

The conidial concentrations were determined by aliquoting 5 µl of the dilution onto the hemocytometer grid. The grid was then examined under a light microscope using the dark field light setting and the conidia in 5 squares (Figure 2.2) were counted (top left, top right, bottom right, bottom left, and one of the middle squares). This count represented the final conidia amount. The count was repeated 4 times for each isolate. From these 4 sets of counts an average conidial count was determined (Equation 1).

Equation 1:
DF x V x Mean conidia = Conidia/ ml
DF = Dilution factor = 100
V = Volume of large corner square = 10^4*
Mean Conidia = Average conidial count out of 4 counts
* 10^4= 1mm^2 x 0.1 mm = 0.1 cm x 0.1 cm x 0.1 cm = 10^{-4}cm^3 or 10^{-4}ml

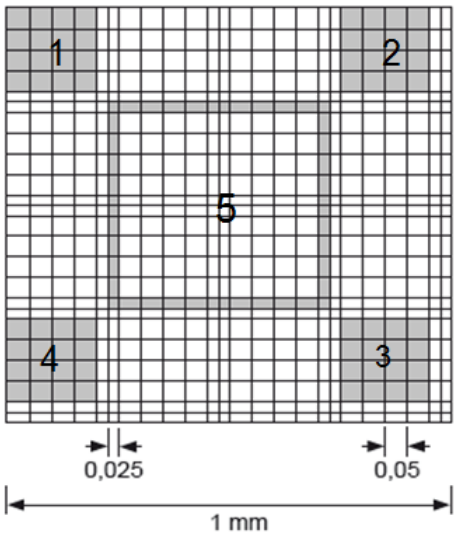


Figure 2.2: Hemocytometer grid layout indicating the 5 squares (shaded) that are counted for determination of the average conidial count (square 5 contains 4 squares one of which must be counted). (Modified from Lasec Product leaflet)

In the dose-dependent bioassays, conidia from several plates were added to the 20 ml water to increase initial stock concentration up to 1 x 10^8ml^{-1}. The amount of initial stock solution required to

make up the lower concentration, 1 x $10^5 ml^{-1}$ to 1 x 10^7 ml^{-1}, was determined using the $C_1V_1 = C_2V_2$ calculation (Equation 2). The 1 x $10^9 ml^{-1}$ concentrations were made by determining the initial stock suspension concentration and then centrifuging (Hangzhou Allsheng Instruments CO. LTD., Superminicentrifuge) the dilution at 10000 rpm for one minute, removing the supernatant, adding another 990 µl of sterile water and another 10 µl of stock solution and centrifuging again. This process was repeated, depending on the stock solution concentration, 5 to 10 times. The final pellet was then resuspended in 990 µl of water and the concentration was then checked using the hemocytometer.

The viability of the spores within the prepared solutions were determined by spread plating 100 µl onto SDA plates and determining the percentage of conidia germinated out of a 100 conidia after 48 hours incubation at 26 °C (Lemawork *et al*., 2011).

Equation 2:
$C_1V_1 = C_2V_2$
C_1 = initial suspension concentration
$V_1 = X$
C_2 = desired concentration (i.e 1 x $10^6 ml^{-1}$)
V_2 = 1 ml

2.2.5 Bioassays

2.2.5.1 Mealybug screening

Twenty-four well, flat bottomed sterile plates (Lasec 662160) were used to conduct both the pilot and initial screening tests. In each plate mealybugs were placed into each of 16 wells, while the remaining 8 wells were filled with 1 ml of sterile water (Figure 2.3), to maintain the humidity within the plate. Into each of the mealybug wells an 11 x 11 mm square of filter paper was placed along with an approximately similarly sized piece of citrus leaf. The leaves were obtained from the Rhodes University, Botha House garden. The leaves were surface sterilised with 70% alcohol, cut and then washed in sterile water. Pilot tests revealed that crawlers were able to escape the wells. To counter this, sterile petroleum jelly was placed at the top of the wells and along the lid to seal the well. However due to the high rate of mortality which occurred as a result of the crawlers getting caught in the petroleum jelly, this method was used only in the adult plates as an extra precaution. Instead, folded, sterilised filter paper was placed in between the lid and the tops of the wells and the lids were secured tightly with elastic bands.

The pilots tests were run with all 16 isolates along with comparative positive controls; a commercial *Beauveria bassiana* product (Bb Plus, BASF (KwaZulu-Natal, South Africa)) and a commercial

Metarhizium product (Real IPM (Real Metarhizium) (Kenya)) and a negative control of sterile water and Tween. Use of the commercial *Metarhizium* product was discontinued as no results were determined in the pilot assays. When plated out onto SDA plates no *Metarhizium* was isolated, indicating that the product was no longer viable, despite the product being kept refrigerated and still within its alloted shelf life.

From the pilot assays, 10 of the most effective isolates were selected (Table 2.1 *) and used in the full screening. In the full screening assay conidial suspensions of 1 x $10^7 ml^{-1}$concentrations were used. Conidial concentrations were pipetted onto the mealybugs, 10 µl were applied to crawlers and 14 µl to adults. For each isolate and the controls, 5 replicate plates were prepared, using a total of 80 crawlers and 80 adults per isolate. Negative and positive controls of sterile water and 5% Tween 20, and the commercial *Beauveria* product (*Beauveria* control) were used. The assays were run at 26°C with a photoperiod of 12 h L: 12 h D, for a period of 5 days in a controlled environment room (Entomology Department). After 5 days the mealybug from each plate were observed under a dissecting microscope (Leica S4E). Dead crawlers and adults were placed onto SDA plates and incubated at 26°C and observed for mycosis, while the mortality rate was recorded.

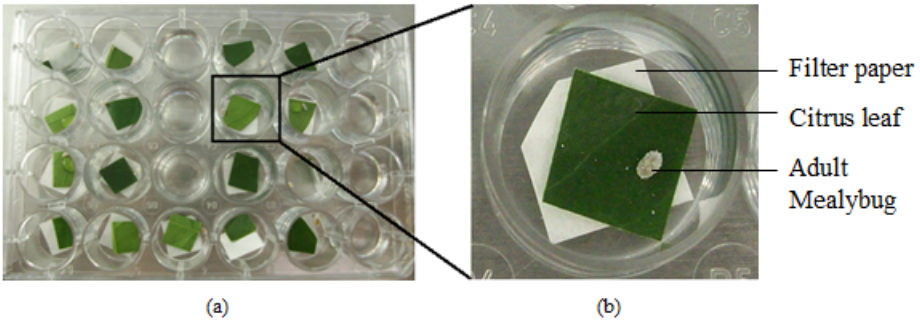


Figure 2.3: (a) Well preparation for both adults and crawlers, (b) Magnification showing an individual well with an adult *Planococcus citri*

2.2.5.2 Dose-dependent assays

To determine LC_{90} and LC_{50}, the top three most virulent isolates (Table 2.1 ^) from the mealybug screening were identified and used in a range of concentrations along with the *Beauveria* control and negative control. Conidial concentrations ranging from 1 x 10^5 to 1 x10^9 conidia ml^{-1} were used, prepared as in Section 2.2.4. For each concentration, five replicate plates, as described in section 2.2.5.1, for both adults and crawlers for each isolate were used. LC_{90} and LC_{50} concentrations were determined using PROBAN software. PROBAN performed regression probit analysis to determine the functional relationship between the log concentration of the fungal isolate and mortality. It also calculates a G-value, used to calculate the amount of response variation in during bioassay procedures

for each isolate. The confidence or fiducial limits for LC_{50} were also calculated and recorded for each isoalte against crawlers and adults (van Ark, 1995).

2.2.6 Molecular identification

2.2.6.1 DNA extraction, amplification and sequencing

As these isolates were previously identified only morphologically (Goble *et al.*, 2010) the identity of the most virulent isolates was confirmed using molecular techniques.

The most virulent isolates were determined by the dose-dependent bioassay screening (Table 2.1 ^). DNA was first extracted using the ZR Bacterial/Fungal DNA Extraction Kit (Zymo Research D6005) following the manufacturer's instructions. For each sample, conidia and hyphae from 4 week old SDA fungal plates of the relevant isolates were scraped using a sterile blade and placed into the ZR BashingBead^{TM} lysis tubes, to which 750 µl of lysis solution was added. The lysis solution and beads break and lysis the fungal cells. The tubes were then vortexed for 5 minutes to assist with the lysis process and to create a homogenous solution, then the BashingBead tubes were then centrifuged for one minute at 10 000 rpm, to pellet the bashing beads and cell debris. Four hundred µl of the supernatant was then transferred into a Zymo-Spin^{TM}IV Spin Filter and collection tube, which was then centrifuged at 7000 rpm for one minute. This step allows for a final filtration and removal of any cell debris or nucleause matter not pelleted out by the initial centrifugation. To the filtrate within the collection tube, 1200 µl of DNA Binding Buffer was added. Eight hundred µl of this solution was then added to a Zymo-Spin^{TM} IIC Column within a collection tube and centrifuged at 10000 rpm for one minute. The flow-through was then discarded and the remaining 800 µl was also centrifuged. The process of centrifugation bound the DNA to the membrane within the Zymo-Spin column. Once all the filtrate and binding buffer was passed through the column 200 µl of DNA Pre-Wash Buffer was added and the column was centrifuged again at 10000 rpm for one minute, the process was then repeated with 500 µl of DNA Wash Buffer. This procedure removed any unwanted components and "washed" DNA bound to the Zymo-Spin column's membrane. The Zymo-Spin column was then transferred into a sterile 1.5 ml microcentrifuge tube and 100 µl of DNA Elution Buffer was added and the tubes were then centrifuged at 10000 rpm for 30 seconds. This buffer releases the DNA from the column membrane

The 10 µl of DNA was then visualised on a 1% Agarose (Promega V3121) gel, at 100 V for 45 minutes, stained with ethidium bromide (Sigma FW394.3), photographed using a UV Transilluminator (Uvipro chemi) and compared to a 100 bp DNA ladder (Promega G210A).

The Internal Transcribed Spacer region (ITS) of these isolate's rDNA was then amplified using a Polymerase Chain Reaction (PCR) (Applied Biosystems 2720 thermal cycler) using the primers

ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Figure 2.4) (White *et al*., 1990). The PCR reaction solution had a final total volume of 50 µl and consisted of: 25 µl of KAPA ready mix from Kapa Biosystems (Lasec KM 1002) containing recombinant *Taq* DNA polymerase, reaction buffer with Mg^{2+} and 0.4 mM of each dNTP, 2 µl of each primer, 2.5 µl of DNA and 18.5 µl of sterile water, which was also used as the DNA substitute in the negative control. The PCR amplification followed the parameters described in (Table 2.2).

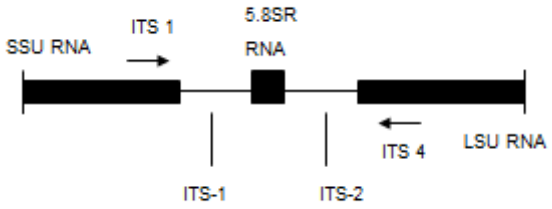


Figure 2.4: Internal Transcribed Regions with the forward and reverse primers used to amplify the ITS region (Adapted from Vilgalys, http://biology.duke.edu/fungi/mycolab/primers.htm Accessed: 07/08/2012)

The sizes of the amplified ITS regions were determined by visualisation on a 1% ethidium bromide stained agarose gel and compared to a 100 bp DNA ladder. The PCR products were then purified for sequencing using the Wizard SV gel and PCR clean up kit (Promega ADA9281) following the instructions provided by the manufacturers. Three times the final volume of the membrane binding solution was added to the DNA which underwent PCR. This solution was then mixed and added to the SV Minicolumn. It was then incubated at room temperature to allow the DNA to bind to the membrane within the minicolumn. The tube was then centrifuged at 16000 rpm for a minute to remove all other reagents present, which were subsequently discarded as supernatant. Membrane Wash Solution (MWS) (700 µl) was then added to the minicolumn and again centrifuged at 16000 rpm for one minute to ensure only DNA was bound to the membrane. This was then repeated with 500 µl of the MWS centrifuged for 5 minutes. The supernatant was then discarded and the minicolumn set up was centrifuged for one minute, to allow for the evaporation of residual ethanol. To remove the washed DNA from the membrane the minicolumn was placed into a sterile 1.5 ml microcentrifuge tube and 50 µl of nuclease free water was added and incubated in the column for a minute after which the tube was centrifuged again at 16000 rpm for one minute.

PCR product was sent to Inqaba Biotechnology Industries (Pty) Ltd. Pretoria for sequencing. The resulting sequences were viewed and edited using Chromas Lite, then compared to other sequences using BLAST GenBank (www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al.*, 1997) to confirm the isolate's genus and species. After identification the sequences were then submitted to GenBank.

Table 2.2: Cycling parameters for PCR amplification of ITS regions

Conditions	Temperature (°C)	Time (seconds)	Cycles
Initial denaturation	94	300	1
Denaturation	94	30	30
Annealing	47	45	
Extension	72	60	
Final extension	72	420	2

2.2.6.2 Phylogenetic analysis

The sequences amplified in this study were reverse complemented using Bioedit (Hall, 1999). Phylogenetic analysis was conducted by comparing ITS sequences of GenBank sequences for both the *Beauveria* and *Metarhizium* genera. These sequences were checked for multiple forward sequence alignment using the online program, Mafft (Katoh and Standley, 2013). The sequences were aligned using MEGA6 (Tamura *et al*, 2013) and used to form a Neighbour-Joining tree (Saitou and Nei, 1987) by applying the Jukes-Cantor model (Jukes and Cantor, 1969) with a bootstrap phylogeny test of 1000 bootstrap replications (Felsenstein, 1985).

2.2.7 Scanning electron microscopy investigation of mode of fungal infection

Mealybug crawlers and adults were inoculated with suspensions of 1 x 10^9conidia ml^{-1} concentration (Section 2.2.4). These were placed in the 24 well plates as in section 2.2.5.1. After 6 days the dead, non-active, insects were placed onto SDA plates and allowed to begin sporulating. After external sporulation had commenced the mealybug were removed from the SDA plates and fixed in 2.5% Glutaraldehyde 0.1 M $P0_4$ buffer for approximately two weeks. They were then washed twice in 0.1 M Phosphate sodium buffer for 10 minutes at a time. The mealybug were then dehydrated in a series of increasing ethanol concentrations (30%, 50%, 70%, 80%, 90% and absolute ethanol), soaking for 5 minutes in each concentration. After dehydration the samples were Critically Point Dried (Polaron) and then mounted on stubs and gold sputter-coated (Quorum QISORS) and viewed using a Scanning Electron Microscope (Tescan Vega LMV) (Cross, 2001).

2.2.8 Statistical analysis

Percentage mortality data from mealybug bioassays and dose-dependent assays was analysed for normality using a Chi-Squared test and found to be non-parametric, thus the data was subjected to a Kruskal-Wallis Analysis of Variance (ANOVA) with the mortality data as the dependent variable and the replicates of each isolate as the independent variable. After which a multiple comparison of mean ranks was performed. For both assays the Kruskal-Wallis ANOVA was performed on the adult and crawler data separately. An arcsine transformation was performed on the adult mealybug dose-dependent assay percentage mortality data so as to render them non-parametric (Agresti and Franklin, 2007). All of these tests were performed using STATISTICA (version 10) software.

2.3 Results

The starter culture of *Planococcus citri* provided by DuRoi IPM was successfully established on organic butternut (Figure 2.5) and the population levels increased and were maintained so that the culture was able to provide the large numbers of adults and crawlers required for the assays.

Figure 2.5: Organic butternut infested with both crawlers and adult mealybug

2.3.1 Entomopathogenic fungal cultures

Pathogenicity of the isolates from the Rhodes University collection was established for 14 of 16 isolates when assayed through 5th instar FCM larvae (Figure 2.6). EP isolates, GOLR 8, and L6 G14 2 B5, were isolated using *G. mellonella* larvae thus were not pathogenic against FCM larvae. Isolate GOLR 8 was pathogenic against mealybug in the pilot tests and was therefore used in the mealybug bioassay (Table 2.1). F8 FCM Rose R9 was no longer pathogenic against FCM. L6 G14 2 B5 was discarded after the initial pilot tests because it was not pathogenic against mealybug.

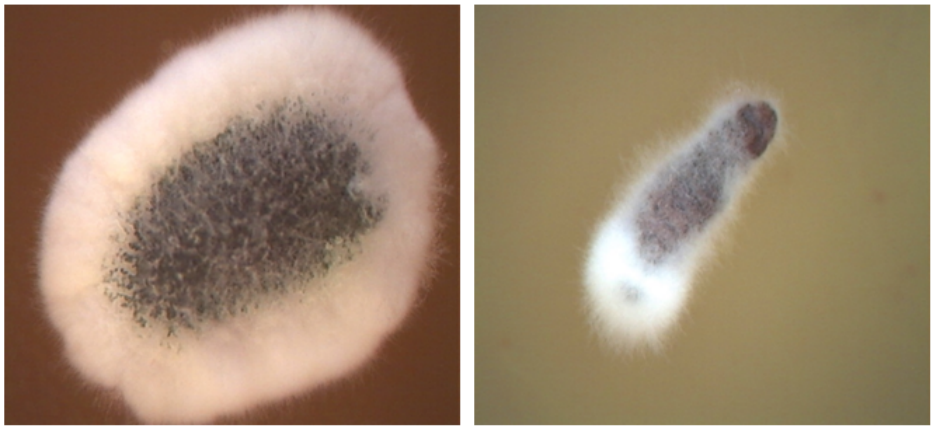
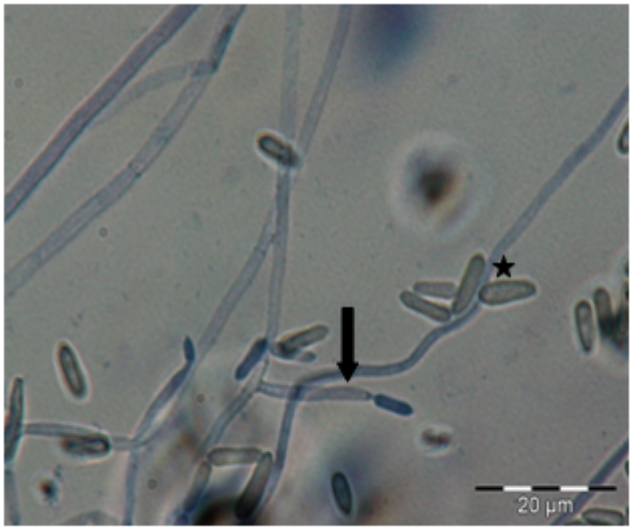


Figure 2.6: FCM 5th instar larvae infected with (a) *Metarhizium anisopliae* isolate G11 3L6, (b) *Beauveria bassiana* isolate GB AR 23 13 3. Both images were take with a Leica EZ 4D dissecting microscope at ~X16 magnification

2.3.2 Morphological identification

Morphological examination of the 16 EP fungal isolates confirmed that 4 of the isolates were indeed *M. anisopliae* and that the remaining 12 were different strains of *B. bassiana. Metarhizium* isolate's mycelial growth was characteristically olive-green in colour (Figure 2.7 b). The different growth morphologies and sectoring, of *M. anisopliae* was also seen (Figure 2.7 c). Conidia were long and ovoid in shape with rounded ends while the conidiophores were mostly only seen as singular loose branches (Figure 2.7 a).

All of the *B. bassiana* isolates were white or off white in colour and fluffy and/or powdery in appearance (Figure 2.8 b). The conidia were much smaller then the *M. anisopliae* conidia with a more rounded ovoid shape. They were usually produced singularly on rachids which were slightly zig-zag shaped (Figure 2.8 a) .

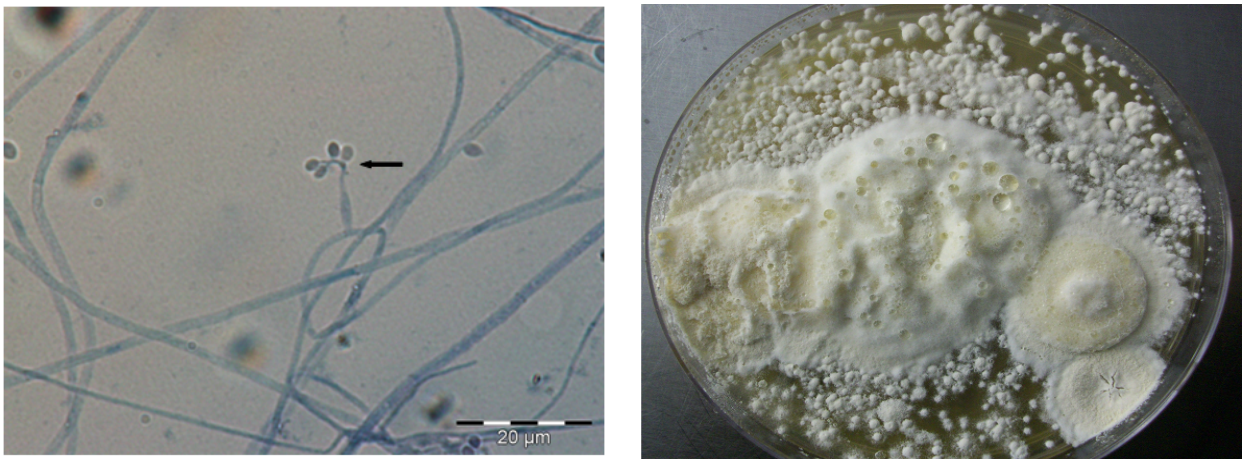


(a)

(b)

(c)

Figure 2.7: (a) Light micrograph of the morphology of *Metarhizium anisopliae;* a single conidiophore with an attached spore and loose spores. (b) SDA plate showing characteristic olive-green mycelial growth of *Metarhizium anisopliae,* (c) sectored growth. Arrows indicate the conidiophores while star indicates the loose conidia. Images were taken with Olympus light microscope at X400 magnification



(a) (b)

Figure 2.8: (a) Light micrograph of *Beauveria bassiana* conidiophore with characteristic zig-zag shape and multiple conidia, arrow indicates conidiophore, (b) SDA plate of characteristic white and fluffy mycelial growth of *Beauveria bassiana*.

2.3.3 Preparation of conidial suspensions

All of the conidial suspensions were determined to be viable after examination of the SDA plates and 100 spore count. All had percentages of conidia germinated of 85% and above.

2.3.4 Bioassays

2.3.4.1 Mealybug screening

The initial screening was carried out with the 10 most virulent isolates (Table 2.1 *) from the pilot tests. The adult and crawlers results were statistically analysed separately. The percentage mortality of crawlers is shown in figure 2.9 a. The average control percentage mortality in the crawlers was 13.75% with percentage mortality of the fungal isolates varying from 45.0% to 67.5%. The Kruskal-Wallis mean separation test showed that *Beauveria* control (the positive control), FCM AR 23 B3 and GAR 17 B3 were significantly the most virulent against crawlers with average percentage mortality of 77.0%, 67.5%, and 67.5% respectively. These isolates were then used in the dose-dependent assays, section 2.3.4.2. As the positive *Beauveria* control was one of these, isolate GB AR 23 13 3 was also used in the dose-dependent assays because it had the 3rd highest average percentage mortality, 63.75%.

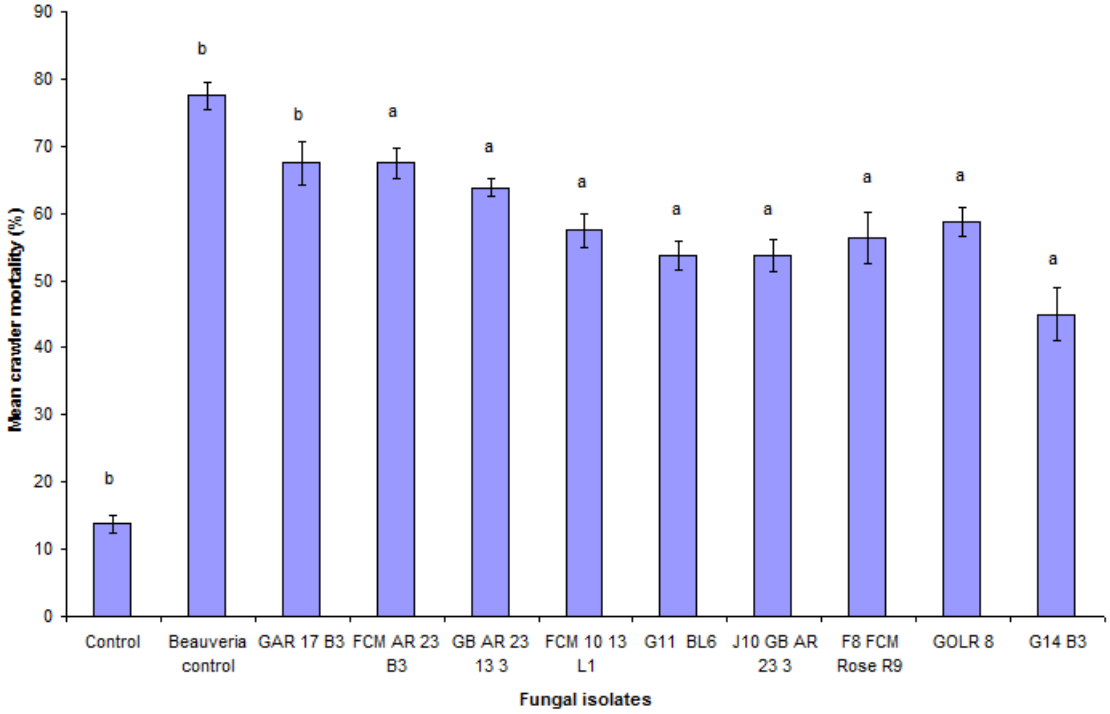
The adult average percentage mortalities are shown in figure 2.9 b. The percentage mortality amongst the adults was much lower in comparison to the crawlers with average percentage mortality ranging from 37.5% to 53.8% and the control average mortality at 11.3%. This is believed to be due to the waxy layer female adults have over their cuticle. The Kruskal-Wallis mean separation test showed very different results to the crawlers. The statistically most virulent isolates against adults were F8 FCM Rose R9 and GOLR 8 with 52.5% and 53.8% mortality respectively. These mortality levels were much lower then those of the crawlers. Hence these isolates were not used in the dose-dependent assays. All of the dead mealybug were placed on SDA and observed to have mycosed within a week.

2.3.4.2 Dose-dependent assays

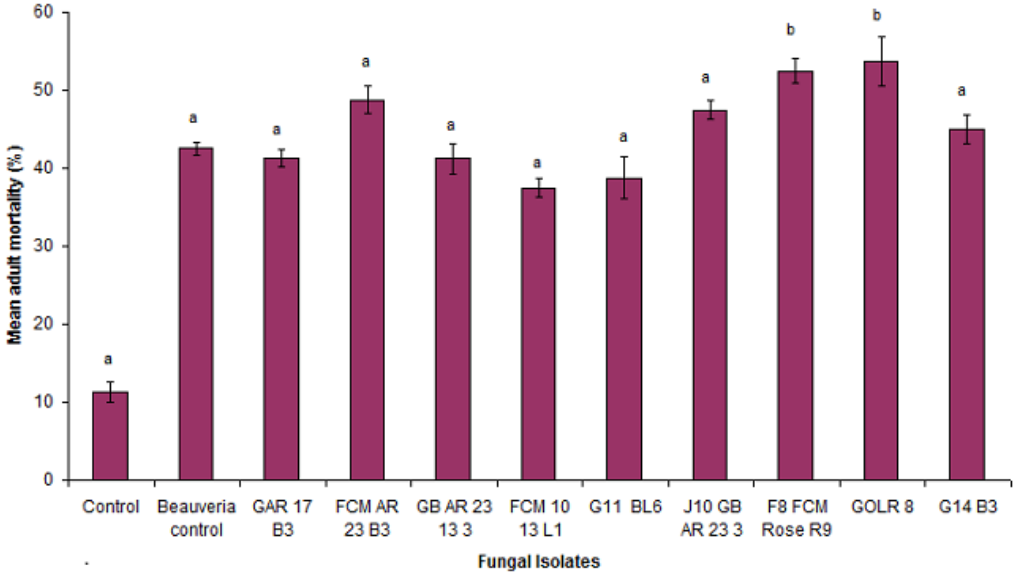
LC_{50} for both crawlers and adults was determined for each fungal isolate used over a wide range of conidial concentrations. The LC_{50} concentrations differed greatly between the crawlers and adults.

Figure 2.10 (a) shows the average percentage mortality for each concentration tested against the crawlers, and the approximate LC_{50} concentration for each isolate. The positive *Beauveria* control and *Metarhizium* isolate, FCM AR 32 B3 both required only low conidial concentrations of 9.94 x 10^5 ml^{-1} and 5.29 x 10^5 ml^{-1} respectively for 50% mortality, while the two *Beauveria* isolates GAR 17 B3 required 4.25 x 10^6 ml^{-1} and GB AR 23 13 3, 6.65 x 10^7 ml^{-1} for 50% mortality (Table 2.4). This trend is continued in the extrapolated LC_{90} conidial concentrations required for each isolate (Table 2.4).

In figure 2.10 (b) the approximate LC_{50} concentrations and average percentage mortality for each concentration tested against adult mealybug is shown. It was not possible to calculate the LC_{50} or LC_{90} concentrations for GB AR 23 13 3, as the highest average percentage mortality was below 50%. The effect that the waxy layer on the cuticle of adult female mealybug has on conidial establishment and thus effectiveness is well represented in this figure. It is clear that much higher conidial concentrations were required to achieve approximate LC_{50} concentrations in comparison to figure 2.10 a. GAR 17 B3 required the highest conidial concentration of 3.50 x 10^8 ml^{-1}, while the positive *Beauveria* control required the second highest conidial concentration of 1.67 x 10^7 ml^{-1}. The LC_{50} concentration for FCM AR 23 B3 only increased to 4.96 x 10^6 ml^{-1} (Table 2.4). This trend is continued in the extrapolated LC_{90} conidial concentrations required for each isolate (Table 2.4). EP fungal concentrations which significantly affected virulence and thus mortality against adults and or crawlers are summarised in table 2.3.

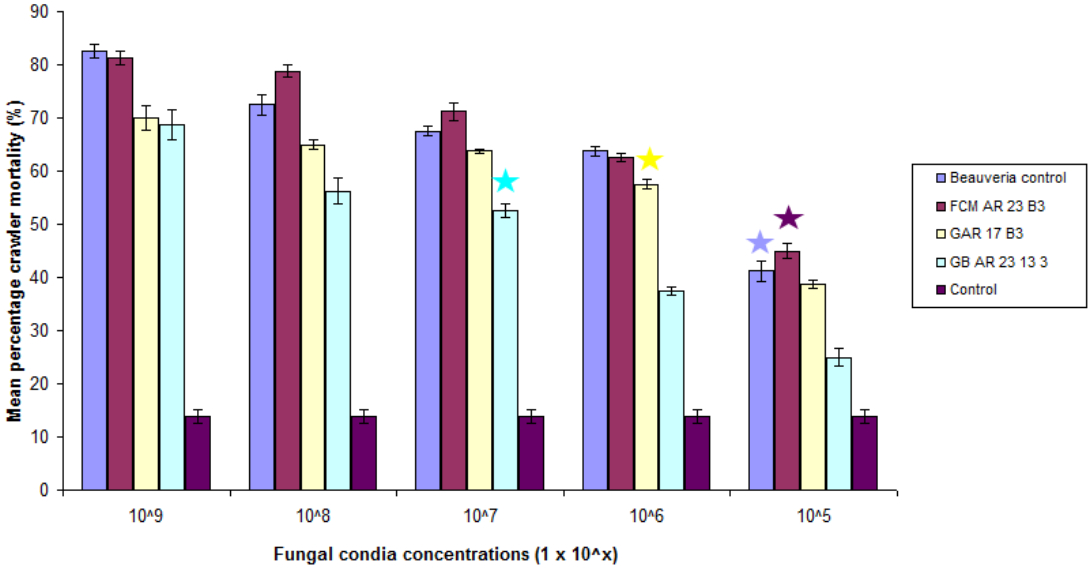


(a)

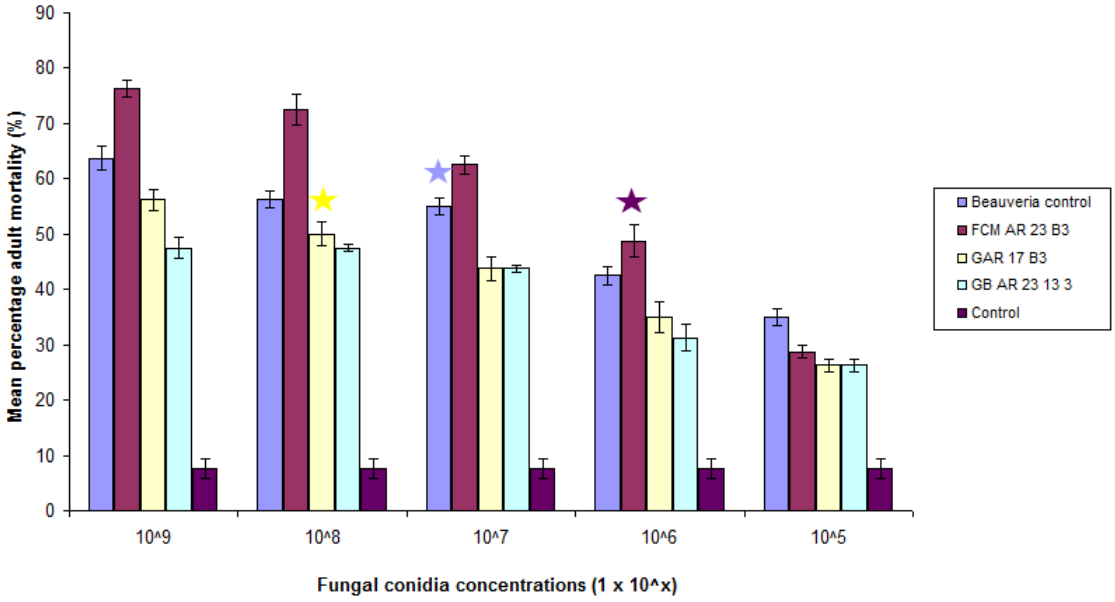


(b)

Figure 2.9: Mean mortality percentages using selected entomopathogenic fungal isolates at a concentration of 1 x 10^7conidia ml^{-1}: (a) crawler mortality (Kruskal-Wallis test H (10, N=55) = 22.78476 p=0.0116), (b) adult mortality (Kruskal-Wallis test; H (10,N=55) =21.42390 p=0.0183). Columns with the same letters are not significantly different from each other. Bars represent standard deviation



(a)



(b)

Figure 2.10: Mortality of the mealybug dose-dependent bioassay at different fungal concentrations (a) crawler mortality (Kruskal-Wallis test: H (20, N=105) = 82.46991 p=0.00001), (b) adult mortality (Kruskal-Wallis test; H (20, N=105) =72.29002 p=0.00001). Stars indicate approximate 50% mortality.

Table 2.3: Summary of significance of isolates and concentrations after Kruskal-Wallis mean separation test: crawler (H (20, N=105)=82.46991 p=0.00001) and adults (H (20, N=105) =71.29002 p=0.00001). + indicate the concentration's significance in comparison to the other concentrations tested

Isolates	**Concentration (Conidia/ml)**	**Significance**	
		Crawlers	**Adults**
Control	1×10^0	-	-
Beauveria control	1×10^9	+	+
	1×10^8	-	-
	1×10^7	+	-
	1×10^6	-	-
	1×10^5	-	-
FCM AR 23 B3	1×10^9	+	+
	1×10^8	+	+
	1×10^7	-	+
	1×10^6	-	-
	1×10^5	-	-
GAR 17 B3	1×10^9	-	-
	1×10^8	-	-
	1×10^7	+	-
	1×10^6	-	-
	1×10^5	-	-
GB AR 23 13 3	1×10^9	-	-
	1×10^8	-	-
	1×10^7	-	-
	1×10^6	-	-

2.3.5 Molecular identification

2.3.5.1 DNA extraction, amplification and sequencing

The three most virulent isolates used in the dose-dependent assays were molecularly analysed via the amplification and sequencing of their ITS regions to confirm the morphological identification. The PCR amplified ITS regions were shown to be approximately 550 bp (Figure 2.11), although this included the primers. Edited sequences were closer to 500 bp.

Table 2.4: Summarised PROBAN data for crawler and adult mortality data for each of the fungal isolates tested

Life stage	**Isolate**	**Significant concentration (Conidia/ml)**	**Lethal concentration**				**Fiducial limits**		
			LC_{50}	**LC_{50} (SE)**	**LC_{90}**	**LC_{90} (SE)**	**G**	**Upper (LC_{50})**	**Lower(LC_{50})**
Crawlers	*Beauveria* Control	$10^9$and 10^7	9.94 x 10^5	7.27 x 10^5	3.50 x 10^{10}	5.86 x 10^{10}	0.13	3.40 x 10^6	1.43 x 10^5
	FCM AR 23 B3	$10^9$and 10^8	5.29 x 10^5	4.27 x 10^6	2.14 x 10^{10}	3.49 x 10^{10}	0.14	1.97 x 10^6	5.68 x 10^4
	GAR 17 B3	10^7	4.25 x 10^6	3.63 x 10^6	8.75 x 10^{12}	3.11 x 10^{13}	0.25	2.20 x 10^7	4.36 x 10^5
	GB AR 23 13 3	-	6.65 x 10^7	3.89 x 10^7	4.98 x 10^{11}	9.35 x 10^{11}	0.12	2.70 x 10^8	2.28 x 10^7
Adults	*Beauveria* Control	10^9	1.67 x 10^7	1.29 x 10^7	5.47 x 10^{12}	1.77 x 10^{13}	0.22	1.04 x 10^8	3.27 x 10^6
	FCM AR 23 B3	10^9, $10^8$and 10^7	4.96 x 10^6	2.38 x 10^6	2.27 x 10^{10}	2.82 x 10^{10}	0.08	1.24 x 10^7	1.71 x 10^6
	GAR 17 B3	-	3.50 x 10^8	3.70 x 10^8	2.20 x 10^{14}	8.75 x 10^{14}	0.21	1.00 x 10^{10}	6.86 x 10^7
	GB AR 23 13 3	-	-	-	-	-	-	-	-

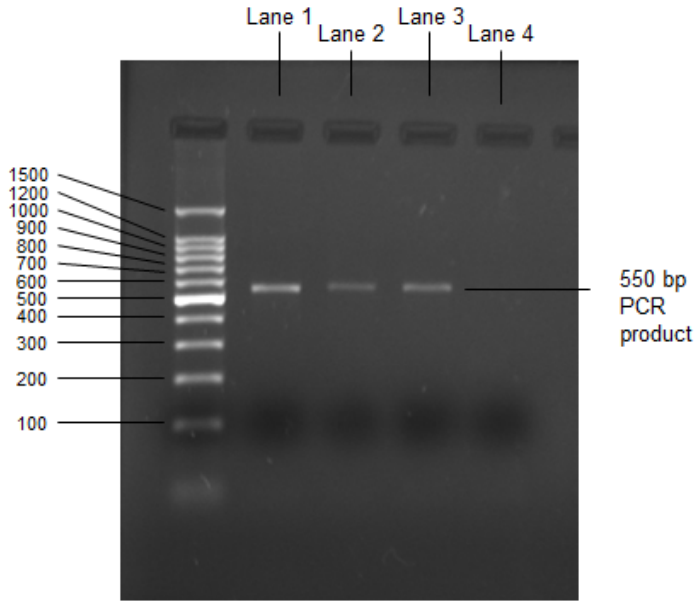


Figure 2.11: 0.1% ethidium bromide stained agarose gel contained the amplified ITS region of the different fungal isolates. Lane 1: FCM AR 23 B3, Lane 2: GAR 17 B3, Lane 3: GB AR 23 13 3 and Lane 4 is the negative control, 100 bp ladder to the far left.

The BLAST analysis of the ITS sequences showed that they correlated with the morphological data and that they were strains of EP fungi belonging to the genera *Metarhizium* and *Beauveria.* Specifically, that they were strains of the two most commonly used and experimented on EP fungal species, *Metarhizium anisopliae* and *Beauveria bassiana* (Table 2.5). Aligned sequences in Appendix.

Table 2.5: BLAST result of the top three most virulent EP fungal isolates.

Isolate	Accession numbers	Species	% Identification	E-value	Query coverage
FCM AR 23 B3	KF834188	*Metarhizium anisopliae*	99	0.0	100%
GAR 17 B3	KF834186	*Beauveria bassiana*	100	0.0	99%
GB AR 23 13 3	KF834187	*Beauveria bassiana*	100	0.0	100%

2.3.5.2 Phylogenetic analysis

Phylogenetic trees were created to determine the relationship of the most virulent isolates in comparison to other species within their genus, and to confirm that the isolates were *B. bassiana* and *M.*

anisopliae (Figures 2.12 and 2.13).

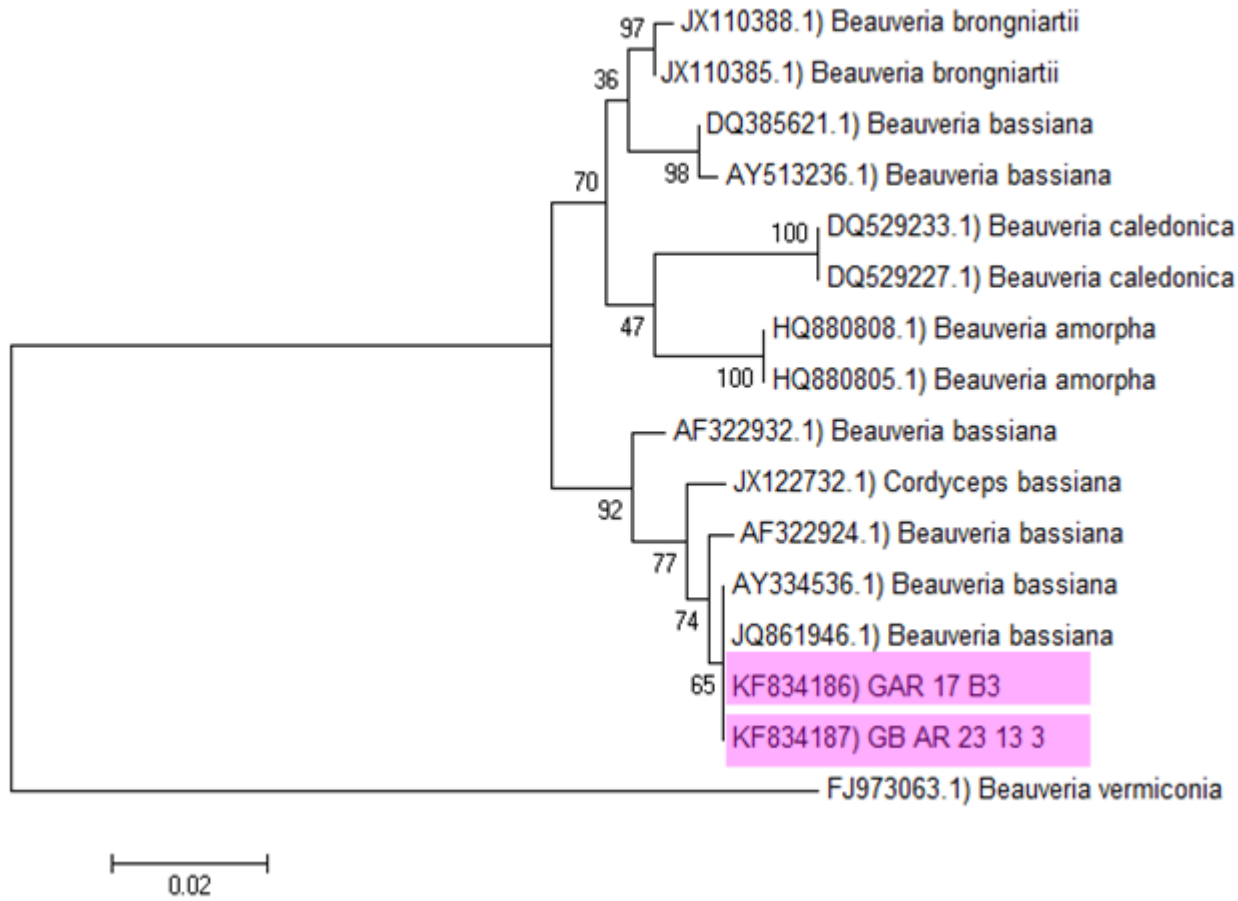


Figure 2.12: Evolutionary relationships of the *Beauveria bassiana* isolates used in this study in relation to other *Beauveria* species and teleomorphs from GenBank. The optimal tree with the sum of branch length = 0.27555847 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

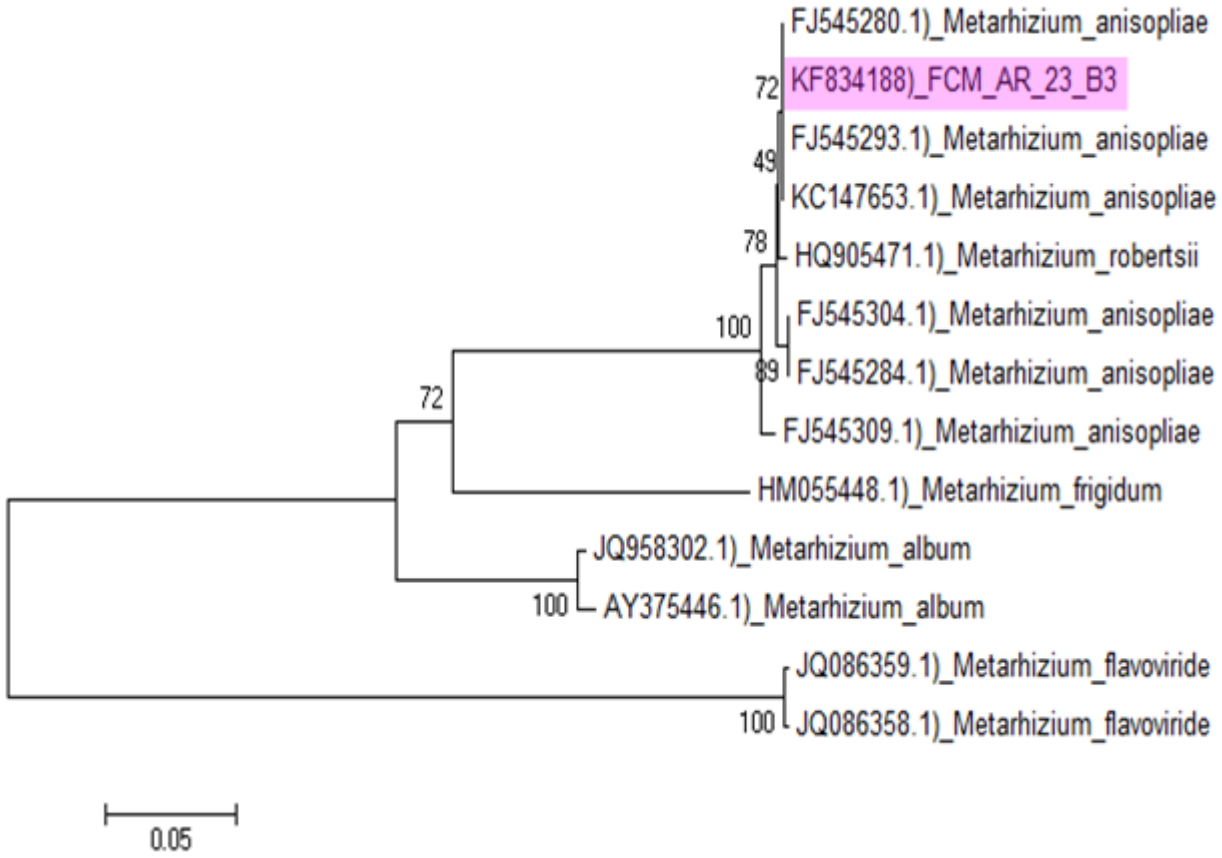


Figure 2.13: Evolutionary relationships of *Metarhizium anisopliae* isolates used in this study in relation to other *Beauveria* species and teleomorphs from GenBank The optimal tree with the sum of branch length = 0.80310577 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 407 positions in the final dataset.

2.3.6 Scanning Electron Microscopy

Because of the waxy layer which covers mealybug it was not possible to locate conidia germinating and penetrating the cuticle. The removal of wax was tested using chloroform but it was found that the conidia were removed along with the wax. Mealybug cadavers were therefore placed onto SDA to allow the EP fungi to begin to externally sporulate before critical point drying and mounting. This resulted in the observation of extensive fungal growth (Figure 2.14 a, c) on all of the mealybug after 6 days. While it was not possible to actually see the hyphae penetrating the cuticle areas due to the

wax filaments covering both the dorsal and basal sides of the mealybug, areas where the hyphae were emerging from the wax layer were identified, such as in figure 2.14 (d). The uninoculated control (not shown) when examined showed no hyphal growth.

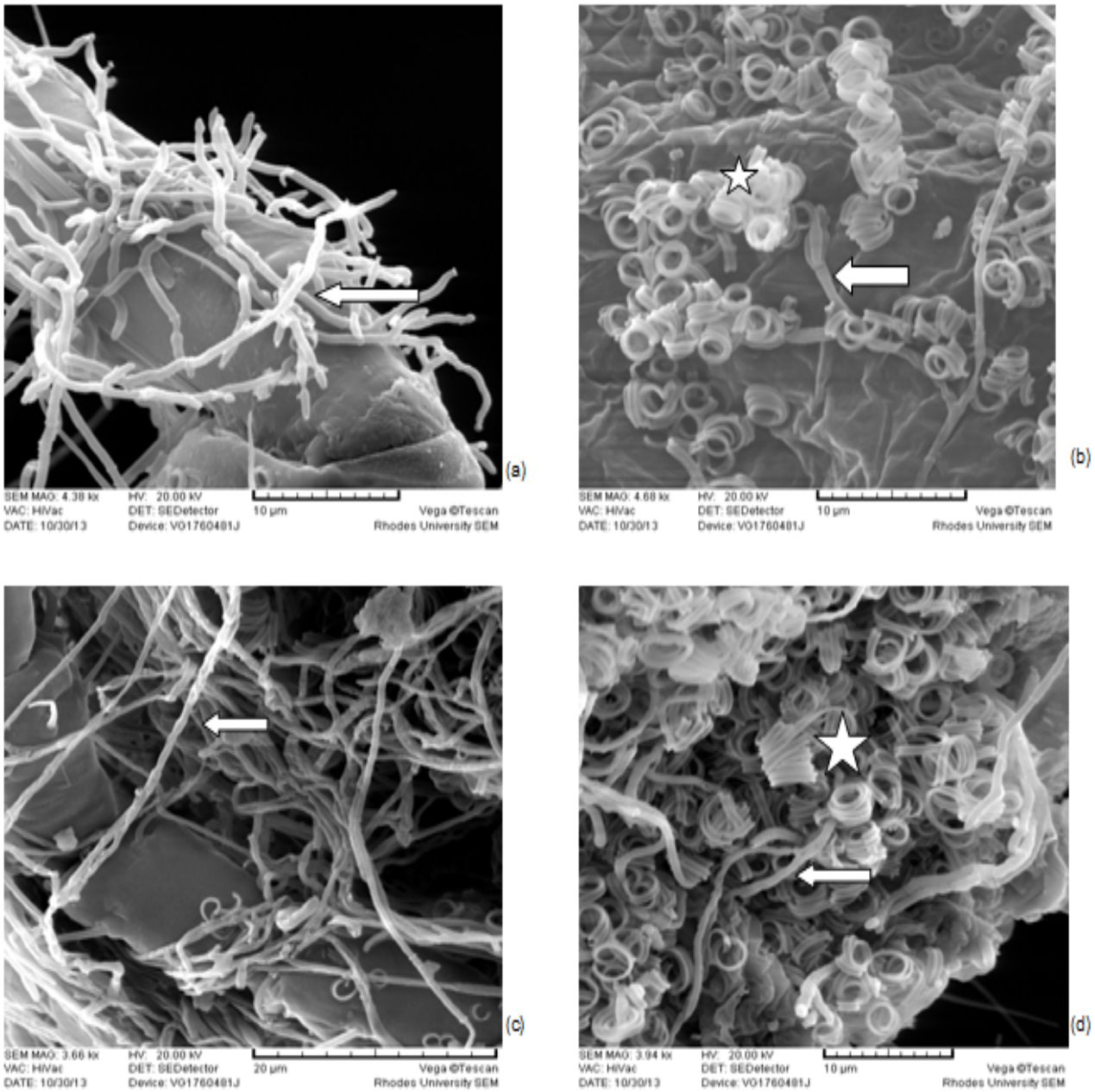


Figure 2.14: SEM of adult *Planococcus citri* mealybug. Arrows indicate EP fungal hyphae and stars indicate the wax segments produced by the mealybug. (a and b) *Beauveria bassiana* hyphal networks, (a) extensive hyphal growth on a leg of the mealybug, (b) hyphae and conidiophore on the basal side of the mealybug. (c and d) *Metarhizium anisopliae* hyphae, (c) extensive growth of hyphae on and around a leg of the mealybug, (d) hyphae growing out of the wax covered cuticle.

2.4 Discussion

In the initial mealybug bioassay only 10 of the original 16 isolates were tested (Table 2.1). The other isolates were shown not to be pathogenic against mealybug in the pilot tests. Different *M. anisopliae* and *B. bassiana* isolates were tested, as both of these species are well known to have wide host ranges. Specific strains have more restricted host ranges, infection levels, germination rates, optimum temperatures (Shah and Pell, 2003; Zimmermann, 2007 b; Vega *et al.*, 2009) and UV-B resistance (Fernandes *et al.*, 2007). These two species are now recognised as being "species complexes" containing a diverse accumulation of genotypes (Cherry *et al.*, 2005; Morar-Bhana *et al.*, 2011). This is best exemplified by some insect species being most vulnerable to EP fungal strains isolated from the same or closely related species compared to those isolated from non-related species (Cherry *et al.*, 2005). For example 8 different strains of *M. anisopliae* were tested against nine different species of Coleoptera, and the majority of those insects were only susceptible to the strains isolated from the same species (Zimmermann, 2007 a).

The bioassays were conducted in 24-well tissue culture plates with eight wells filled only with water (Figure 2.3) to increase humidity and with dampened folded sterile tissue paper in between the lid and wells for crawlers, with petroleum jelly sealing the wells for the adults. In the pilot studies all of the mealybug initially died of desiccation due to the extremely low humidity levels. This was resolved by filling 8 of 24 wells with 1 ml of water. The dampened filter paper also helped to maintain the relative humidity. Due to the size of the crawlers and the way the 24-well plates are built, the crawlers initially escaped from their respective wells. This resulted in high levels of unnatural mortality, which also occurred with the addition of the petroleum jelly. The situation was resolved by using folded tissue paper and rubber bands securing the plates together. The tissue paper prevented crawlers from escaping their wells without harming them. Female adults are far more sedentary then crawlers (Gullan, 2000). The petroleum jelly was used as a precaution against escape, which resulted in little to no mortality. Deaths in the controls occurred from natural causes such as adults reaching the end of their life cycle, possible stress from relocation into the 24-well plates and in the case of crawlers perhaps drowning in the conidial solution if it was not all properly absorbed by the filter paper within the well.

In comparison to the dose dependent 1 x 10^7 m^{-1}, mortality of the crawlers from the four isolates used in the initial bioassay was much higher (Table 2.1 ^) are much higher. With the exception of FCM AR 23 B3, all of the dose-dependent assay isolate's average percentage mortality decreased in the dose-dependent assays (Figures 2.9 a and 2.10 a). The *Beauveria* control and GB AR 23 13 3 both had mortalities decrease by approximately 10%, while GAR 17 B3's was only approximately 5%. This decrease could have been due to a loss of viability by the isolates, as a period of 6 months passed between the initial and dose-dependent bioassays being preformed. Viability depends heavily on the

amount of time an isolate is stored for and at what temperature. Isolates can remain viable for up to three years if stored at low positive temperatures such as + 8ºC in comparison to room temperature, 25ºC (Polovinko, 2013). It is also commonly accepted that virulence decreases slightly with each subculture on artificial media, even if not visible physiological or biochemical changes which affect viability could have occurred (Ignoffo *et al.*, 1982). Large amounts of subculturing between assays did occur making loss of virulence a viable possibility. Quesada-Moraga and Vey (2003) found that the virulence of the *B. bassiana* isolate they were using was significantly reduced after 2 subcultures on SDA in comparison to malt agar. Most studies state the use of supplemented SDA when culturing EP fungal isolates, as we did (Shapiro-Ilan *et al.*, 2002; Ansari *et al.*, 2007; Bischoff *et al.*, 2009; Lemawork *et al.*, 2011; Gao *et al.*, 2012).

Alternatively, the reduction in virulence could be due to the presence of a mycovirus. Mycoviruses have been described in many fungal species including entomopathogenic fungi such as *Metarhizium* and *Beauveria* (Dalzoto *et al.*, 2006). A large amount of EP fungal isolates are infected by these viruses, (Herrero *et al.,* 2012) tested 73 different isolates of *B. bassiana,* 54% of those isolates were infected with mycoviruses. When infected and virus-free EP fungal isolates were compared it was found that the virus-free isolates had increased virulence. Frazzon *et al.* (2000) compared infected and virus-free isolates of *M. anisopliae* against the cattle tick *Boophilus microplus* and found that the virus-free isolates were more effective. Similarly Melzer and Bidochka, (1998) found that the virus-free *M. anisopliae* isolates had significantly increased growth rates, conidiophore production and virulence against both crickets, *Gryllus domesticus,* and waxworm larvae, *Galleria mellonella.* de la Paz Gimenez *et al.* (2002) found that virus-free isolates had higher endochitinase secretion. Although, mycoviruses were not visually detected in the isolates used, it is still possible that molecular work could reveal their presence.

FCM AR 23 B3's average percentage mortality against crawlers increased by approximately 4% (Figures 2.9 a and 2.10 a). Similarly while all of the other isolates percentage mortality against adults were low, below 45%, the dose-dependent assay 1 x 10^7 ml^{-1}concentration results were higher then those of the mealybug screening (Figures 2.9 b and 2.10 b). FCM AR 23 B3 and the *Beauveria* control both had an increase in mortality in comparison to their initial screening mortality rates of close to 10%, while the other two *B. bassiana* isolates only had an increase of approximately 2%. This increase could be due to a number of factors. For FCM AR 23 B3, older cultures were used as the newer subcultures had stored growth which produced few spores. Thus, it is likely that these older cultures were more virulent as they had not been subcultured as many times. For the increase in mortality against adults it is likely that they were stored in cooler temperatures due to winter lowering the ambient room temperature to much lower then normal. This may have kept the isolates more viable. Also, the the amount of conidia required to achieve the different concentrations required that older cultures were also used along with the younger more recently subcultured ones.

The dose-dependent assays illustrated that the *Metarhizium* isolate FCM AR 23 B3 was the most virulent isolate against mealybug, as it had the lowest LC_{50} and LC_{90} concentrations, against both adults and crawlers (Table 2.4). FCM AR 23 B3 and the *Beauveria* control performed on par with each other against crawlers. For the most part, alternating highest mortality percentage at each concentration (Figure 2.10 a). FCM AR 23 B3 performed better against adults (Figure 2.10 b). Neither of the *B. bassiana* isolates (GAR 17 B3 and GB AR 23 13 3), were as virulent as the *Beauveria* control, both having higher crawler LC_{50}conidial concentrations. It was not too surprising that the isolate GB AR 23 13 3 had the highest crawler LC_{50} concentration. It did not cause 50% mortality in adults, as it was not one of the statistically virulent isolates from the mealybug screening (Figure 2.9 a). It is also possible that its virulence was decreased when tested against adults due to one of the reasons above.

The approximate conidial concentrations required for 90% mortality for both adults and crawlers are quite high, i.e. 1 x 10^{10} ml^{-1} and above (Table 2.4). While it must be taken into account that those values are extrapolated from data with is not present, it does provide a general idea of the amount of conidia which would be needed to produce 90% mortalities in mealybug. To achieve these concentrations the processes for conidial production would have to be scaled up. One commonly used method is solid-state fermentation where conidia are produced on cereal grains, most commonly on parboiled white rice (Ye *et al.*, 2006; Mascarin *et al.*, 2013; Taylor *et al.*, 2013). Sorghum has also been shown to have great potential, especially for the growth of *M. anisopliae* (Mar *et al.*, 2012). Cereal grains are commonly used as substrates due to their larger surface area per unit volume in comparison to agar (Ye *et al.*, 2006). Successful fungal mass production relies on a few important factors; the isolate itself, nutrients, the density of the inoculum used and the environmental conditions they are grown under such as temperature and humidity (Mascarin *et al.*, 2010). Diphasic solid-state fermentation, which uses a liquid-culture inoculum instead of dry conidia for the solid substrate. Rice, is considered to be the most viable vehicle for conidial mass-production (Derakhshan *et al.*, 2008; Sahayaraj and Namasicayam, 2008). Solid-state fermentation produces aerial conidia similar to those produced on insect cadavers, which are considered to be better able to tolerate desiccation and UV exposure than those produced while submerged. Submerged conidia have much thinner walls and thus are unable to tolerate these environmental stresses (Ye *et al.*, 2006; Mascarin v 2010). It is also advantageous over liquid fermentation as raw materials are cheaper and the process is simpler (Mar *et al.*, 2012).

For example in 2006 Ye *et al.* developed a fermentation chamber for the bulk production of fungal conidia on rice. This chamber contained 25 bottom-meshed metal trays each with the capacity to hold 2 kg of rice each. With clear walls to allow in natural light, the chambers were kept at 25ºC with high relative moisture levels. The rice used in these chambers was parboiled and then steamed in an autoclave to allow it to retain some of its elasticity. It was then inoculated with a liquid culture of *B. bassiana* with a mycelial biomass of approximately 18 mg ml^{-1}. This method produced on

average 2.4 x 10^{12} conidia Kg^{-1} of rice after 3 days. A similar method was used for the formulation of Green Guard®, a commercially available product using *M. anisopliae* strain FI-985. The conidia are produced on parboiled and autoclaved white rice, which is kept in culture bags at 25ºC, for the extended period of 14 to 18 days. This yielded 5-6 x 10^9conidia Kg^{-1} (Milner and Hunter, 2001). From these two production systems it is possible to see that if FCM AR 23 B3 were to be mass produced the optimum number of days required for fermentation must be determined, to make sure optimum conidial yield is achieved.

The increased concentrations required for all the isolates against adult mealybug (Table 2.4) is believed to be in part due to the difficulties in penetrating the mealy wax layer which covers adult females. Similar results were found by Banu *et al.* (2010) who exposed crawlers and adult *Phenacoccus solenopsis* and *Paracoccus marginatus* mealybugs to both pesticides, acephate, chlorpyriphos, neem oil and nirma powder, Nirma powder and biopesticides *V. lecanii*, *B. bassiana*, *M. anisopliae*. They found that crawlers were more susceptible to both forms of pest control. Larry *et al.* (2002) and Curkovie *et al.* (2007) both also found that mealybug crawlers were more suscpetible than adults. All of these papers speculated the cause to be due to the increased amounts of wax present on the adults and to the smaller size of the crawlers. The wax layer is believed to reduce the entry of bacteria, fungi or other parasites, and to avoid damage at the points where eggs touch (Kumar *et al.*, 1997). The amount of wax present on the mealybug is well represented in figure 2.14. SEM analysis shows large amounts of wax present on the mealybugs used in the study. Thus, if a product were to be made from EP fungi against mealybug, a lipid degrader should be considered as an additive to an EP fungus product produced for use against mealybug, or alternatively combined with BCAs which are ingested such as *Bacillus thuringiensis* toxins, or viruses.

In biological control intra- and inter-specific competition often occurs and these interactions can change the pathogen's ability to infect the host. Amoung EP fungi two models exist: coinfection and superinfection (Cruz *et al.*, 2006). Coinfection, occurs when cadavers produce conidia from each of the EP fungi it was infected with, be they different strains, genera or species. Superinfection is the result of the most virulent strain or genotype overcoming and displacing the less virulent strains. This can happen inside or outside the host (Cruz *et al.*, 2006; Guzmán-Franco *et al.*, 2011). Intra-specific competition between conidia is the most likely cause for the results seen in table 2.3, in which the significant concentrations of *Beauveria* control against crawlers are 1 x 10^7 and 1 x 10^9 conidia ml^{-1}, while 1 x 10^8conidia ml^{-1} is not. As superinfection's suppressive activities can also occur between conidia of the same strain. If the conidial density present on a host are well above normal, competition is often seen, especially in *in vitro* experiments such as these (Hughes *et al.,* 2004; Cruz *et al.,* 2006). Competition between conidia of the same strain was also found by (Hughes *et al.,* 2004) where the relationship between the conidial concentration of *M. anisopliae* and ant mortality was not linear but rather sigmoidal, as after 1 x 10^7conidia ml^{-1} the average percentage mortality did not increase.

This result and that of GAR 17 B3 were the only significant concentration against crawlers was 1 x 10^7conidia ml^{-1} (Table 2.3) suggests the possibility of threshold concentrations, where beyond a specific concentration the competition between conidia becomes deleterious rather then advantageous. It also suggests that the amount of competition between conidia of a same strain differs from strain to strain, since this result did not occur with any of the other isolates tested. Further research must be conducted to investigate further.

The *Beauveria* genus comprises of 12 well supported terminal lineages (Rehner *et al.*, 2011). Of which 5: *B. bassiana, B. brongniartii, B. caledonica, B. amorpha* and *B. vermiconia,* are represented in figure 2.12. The evolutionary relationship of GAR 17 B3 and GB AR 23 13 3 in relation to select isolates from each of these species is shown to be monophylogenetic. Both of this study's *B. bassiana* isolates grouped into a relatively well supported *B. bassiana* clade with a bootstrap value of 77%, supporting the identification of both EP fungal isolates as *B. bassiana.* Figure 2.12 produced similar results to a phylogenitic study performed by Rehner and Buckley (2005), but on a smaller scale. Rehner and Buckley (2005) found that the majority of the *Beauveria* fungal isolates they studied organised themselves into clades previously defined by morphology as seen in figure 1.6. They described these species groups as clades A-F. Clade A consists of globally distributed *B. bassiana* strains. While clade C consists of *B. bassiana* strains, labeled as *B. cf. bassiana,* which are phylogenetically different from those in clade A. Similar results are seen in this study as two seperate *B. bassiana* clades are seen. Morar-Bhana *et al.,* (2011) also found that the majority of *B. bassiana* strains formed one large clade with a smaller subset of *B. cf. bassiana* strains. They suggest that *B. cf. bassiana* and *B. brongniartii* are sister taxa because they form subsets of the the same clade, also seen in figure 2.12 although only supported with a low bootstrap value of 36%. Further analysis is difficult due to the smaller sample size. The global distribution of the GenBank isolates could account for the relatively weaker bootstrap values associated within the tree. The GenBank strains used in this study originated from a variety of places throughout the world such as Columbia (Cruz *et al.,* 2006), China (unpublished) and the U.S.A (Coates *et al.,* 2002).

Similar results were obtained in the *Metarhizium* phylogenetic tree in that the majority of recognised *Metarhizium* species were separated out into clades according to species (Figure 2.13). *Metarhizium anisopliae* is shown to be a monophylogenetic species as all of the strains belonging to this species form a clade which has a strong bootstrap value of 100 suggesting that while currently genetically diversified, they most likely originated from the same ancestoral isolate. This also suggests that the *M. robertsii* isolate that is found in this clade was most likely misidentified. Often noticed in phylogenetic analyses of *Metarhizium* is the separation of *M. anisopliae* into two separate clades, *M. anisopliae* and *M. anisopliae* var. *anisopliae.* Though the relationship within the *M. anisopliae* and *M. anisopliae* var. *anisopliae* is unresolved due to lack of informative sites within the species ITS

region (Driver *et al.,* 2000).

It is also important to note that the host specificity of an isolate is more pronounced in the field than under laboratory conditions (Zimmermann, 2007 a). Thus, while finding the most virulent isolate is important, finding a virulent isolate which is well adapted to the environment in which it will be used in is equally important. Its ability to survive under harsh field conditions and in combination with other chemicalpesticides and natural mealybug enemies must be determined. For which pot and field tests must be undertaken. All of these should be conducted with FCM AR 23 B3 and GAR 17 B3,most importantly FCM AR 23 B3 as it is the more virulent isolate.

The same isolates used in this study were also used in studies by Goble *et al.* (2011) and Coombes *et al.* (2013). Goble *et al.* (2011) determined that the *M. anisopliae* isolates G11 3 L6 and FCM AR 23 B3 and the *B. bassiana* isolate GAR 17 B3 had the most potential against 5th (final) instar FCM larvae in the soil, at a conidial concentration of 1 x 10^7 ml^{-1}. Coombes *et al*. (2013) continued with these isolates and investigated their persistence in soil in comparison to commercially available EP fungal products. All isolates were found to persist in the soil for a minimum of 6 months while still infecting 5th instar FCM larvae. All performed better then the commercially available isolates. These studies show that if FCM AR 23 B3 or GAR 17 B3 were to be developed as mycopesticides for use in citrus orchards they would have the advantage of being able to target multiple damaging citrus pests, reducing the amount of different products needed to control citrus pests.

Chapter 3

Screening of entomopathogenic fungi against citrus thrips

3.1 Introduction

South African citrus thrips *Scirtothrips aurantii* is indigenous to South Africa and is considered one of the most serious citrus and horticultural crop pests within the country (Grout and Richards, 1992; Childers and Nakahara, 2006; Garms *et al*., 2013). It was first described by Faure in 1929 and has been considered a major pest since its first description, due to the large amount of damage it causes to citrus and other horticultural crops (Grout *et al*., 1996; Gilbert and Bedford, 1998) such as mangoes and avocados (Dennill, 1992; Rafter and Walter, 2013). Due to their wide distribution and low susceptibility to many pesticides, many species of thrips have become serious pests throughout the world (Murai and Loomans, 2001; Zahn and Morse, 2013). Within southern Africa, *S. aurantii* is highly polyphagous (Mound, 2005; Garms *et al*., 2013). Of the approximate 100 species of *Scirtothrips,* it is the only species which attacks citrus (Gilbert and Bedford, 1998; Mound and Stiller, 2011).

Scirtothrips aurantii has also been recorded in Australia since 2002, but it does not exhibit the same polyphagous activity (Mound, 2005; Garms *et al.*, 2013; Rafter and Walter, 2013). *Scirtothrips aurantii* are restricted to certain species of Crassulaceae, especially *Bryophyllum delagoense,* but under laboratory conditions breed on various other plants (Mound and Stiller, 2011; Garms *et al.*, 2013).

The control of thrips has always has been problematical as there are no “thrips-specific” insecticides available and thrips often become resistant to generalist insecticides (Reitz *et al.*, 2011). In California,

Scirtothrips citri became resistant to the tartar emetic and sugar bait spray within two years of its use. In South Africa it is not considered a viable option for the control of large thrips populations along with the fact that thrips have developed resistance to this bait spray (Grout *et al.*, 1996). In other instances the incorrect use of insecticides has resulted in the increase of thrips populations, due to the stimulation of reproduction as a result of sub-lethal doses. This was evident in populations of *S. citri* when exposed to 4 pesticides; dicofol, esfenvalerate, formate and malathion residues. In the presence of 21-, 34-, 41- and 64-day old insecticide residues on citrus leaves, oviposition and thus fecundity significantly increased. In the presence of 1-hour old residues, fecundity was decreased and again then elevated at intermediate levels (Morse and Zareh, 1991).

Along with resistance, nearly all of the treatments used for the control of thrips, such as bait sprays, pyrethroids, organophosphates and insecticides, are harmful to the natural enemies of thrips and other citrus pests present (Gilbert and Bedford, 1998; Grout *et al.*, 1996; Grout and Stephen, 2005). These effects on the natural enemies result in a decrease in the natural biological control of citrus pests, and the extent of the effect differs depending on the treatment used (Grout and Richards, 1992; Grout and Stephen, 2005), thus causing problems in IPM programmes (Grout *et al.*, 1996).

This reliance on insecticides for the control of thrips has resulted in a long history of insecticide failure over the past 50 years. Thus, alternatives such as entomopathogenic fungi are essential. EP fungi are promising because it is possible to find a strain which could be specific for thrips, or even a species with a broader host range to attack other citrus pests along with the thrips in orchards (Reitz *et al.*, 2011). Thus the aim of this study was to test the EP fungal isolates most effective against citrus mealybug against adult *S. aurantii* collected from citrus orchards.

3.2 Methods

3.2.1 Thrips collection

Due to the delicate constitution of *S. aurantii*, it was not possible to rear the insects in culture. Thus, adult thrips were collected from the field in late May from Vereenigd farm in the Sunday's River Valley. Collections were made from orchards 3 and 4, (33°29'58.13" S and 25°39'25.10" E) which were three year old Nadorcott Mandarins and used immediately in the bioassay (Section 3.2.4). Thrips were collected by beating young leaves or new growth against plastic trays covered in black paper to provide a colour contrast. The thrips were collected from the trays using aspirators, which contained folded tissue paper and leaf buds (Figure 3.1). They were transported in cooler boxes which had a

layer of cardboard separating the ice packs from the insects to prevent the thrips from becoming too cold and detrimentally affected (Grout *et al.,* 1996; Morse *et al.,* 1986).

The collection of nymphs was first attempted but due to high mortality due to transport they were not collected again. Also the lateness of the season resulted in the majority of the thrips being found was adults. The tissue paper and leaves were also added to the aspirators as later collection trips also resulted in high mortalities without them.

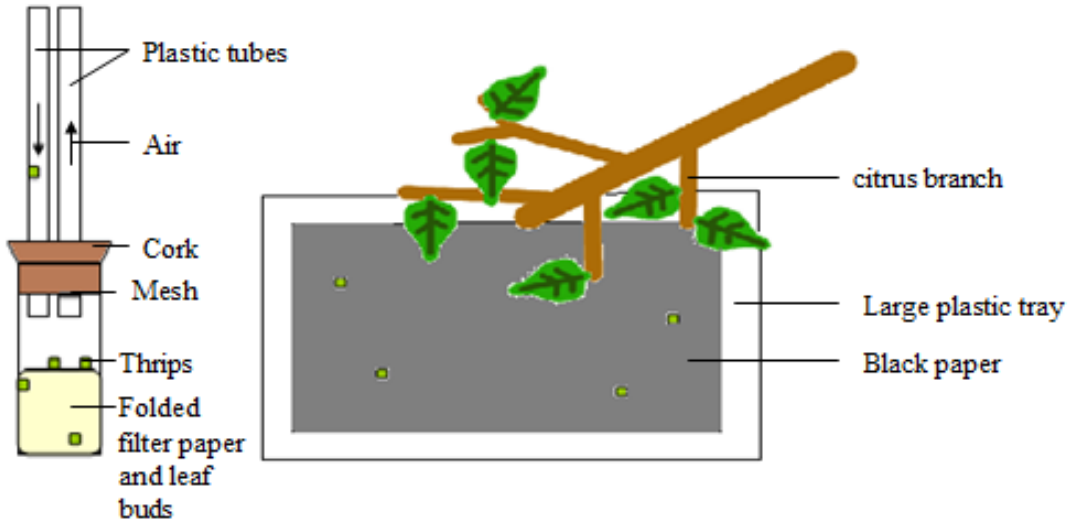


Figure 3.1: Diagram of system used to collect thrips in orchards.

3.2.2 EP fungal cultures

The fungi were maintained on supplemented SDA as described in section 2.2.2. The fungal isolates tested against the thrips are FCM AR 23 B3 and GAR 17 B3 as indicated in table 2.1 [#].

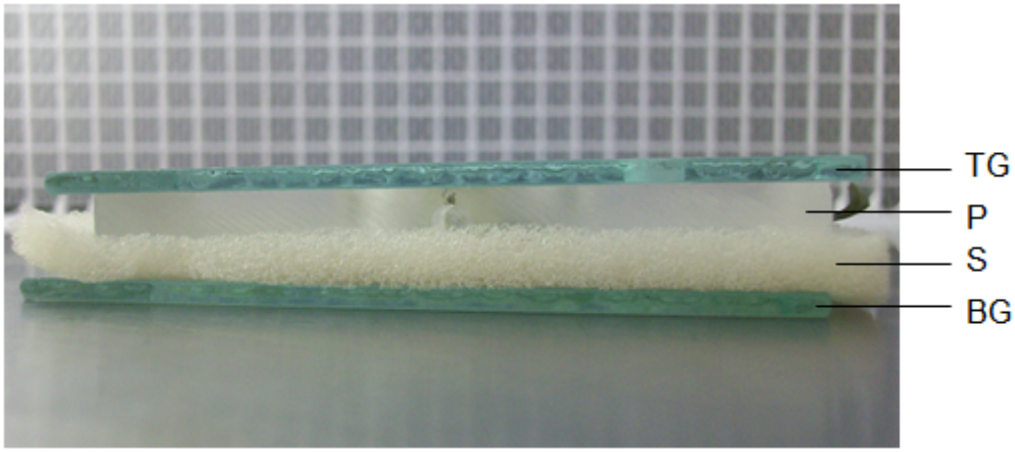
3.2.3 Preparation of conidial suspensions

The conidial suspensions applied to the leaf buds were made up to a 1 x 10^7 conidial ml^{-1} concentration as described in section 2.2.4.

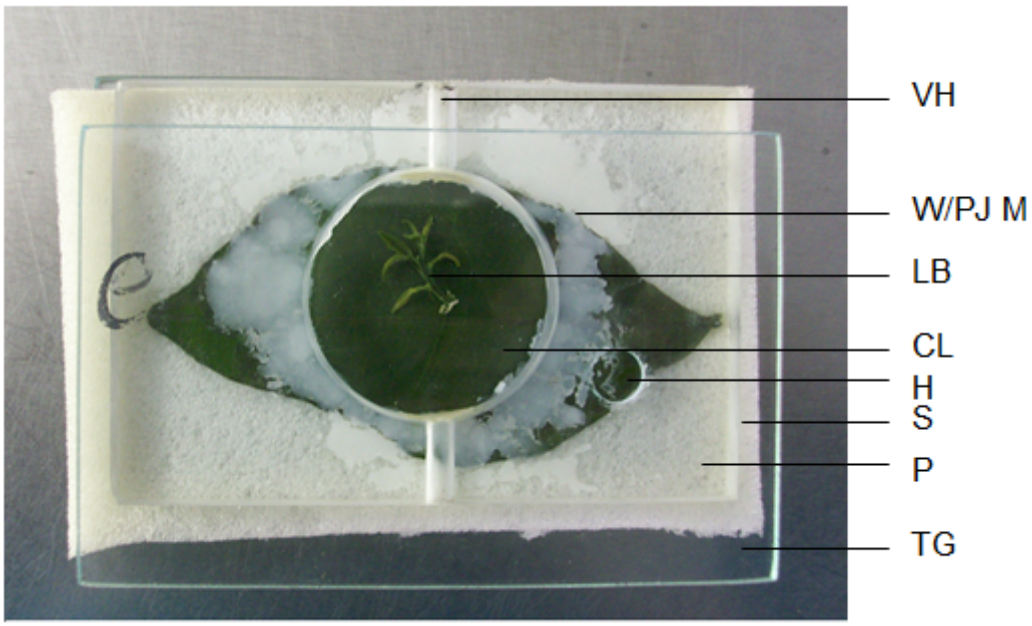
3.2.4 Thrips assay

Due to the difficulties in working with thrips, they were not placed in 24 well plates as with the mealybug, but rather in Munger cells (Figure 3.2). These cells were made up of 4 individual pieces, as indicated in Figure 3.2 (a). The top and bottom layers consisted of 65 x 100 mm glass rectangles, with a small hole (7 mm diameter) drilled through the top layer in the upper third of the rectangle. The centre of the cell consisted of a piece of perspex with a 36 mm diameter hole in the middle. Additional 5 mm holes were drilled perpendicular to the central hole, blocked off with fine gauze to provide ventilation. The thin layer of sponge was soaked with water, to provide moisture before the cells were assembled. To create a protective layer between the thrips and the sponge a large sterilised citrus leaf was placed, abaxial surface up, above the sponge. The leaves were sterilised by washing in 70% alcohol. A mixture of autoclaved candle wax and petroleum jelly (30:70) was placed in a ring on the underside of the perspex around the central hole to create a waterproof barrier between the leaf and the perspex (Figure 3.2b). The cells were bound together with large elastic bands for the duration of the experiment (Modified from Grout *et al*., 1996).

Conidial suspensions were applied to leaf buds, also collected from the field, by dipping the buds into the appropriate suspension. The buds were then placed within the large central hole of the perspex and air dried in a laminar flow cabinet. The adult thrips were placed into the cells using a fine paintbrush. Due to the difficulties in collecting the thrips, only the significantly virulent isolates FCM AR 23 B3 and GAR 17 B3, were used along with an uninoculated control and comparative commercial *Beauveria* control. Each isolate and control had 10 replicates. The Munger cells were incubated at 24°C in full light for 3 days. After 3 days each cell was observed under a dissection microscope to determine the condition of the thrips and the results recorded. Dead thrips were recorded as such and placed onto SDA plates and incubated at 26°C to allow mycosis to occur.



(a)



(b)

Figure 3.2: Munger cell used for thrips bioassays: (a) Side view indicating 4 separate components; TG = top glass piece, P = central perspex, S = sponge, BG = bottom glass piece. (b) Top view of the cell indicating the multiple components; VH = ventilation holes sealed with metal mesh, W/PJ M = wax and petroleum jelly mixture barrier, LB = conidia dipped citrus leaf bud, CL = central citrus Leaf, H = hole drilled through TG, S = sponge, P = perspex, TG = the top glass layer.

3.2.5 Statistical analysis

The mortality percentage data from the thrips bioassay was subjected to a Chi-Squared test using STATISTICA (Version 10) software, with which the expected and observed mortality percentages

were compared (Agresti and Franklin, 2007). The P-value was calculated using a P-value calculator from the website http://www.easycalculation.com/statistics/chi-squared-p-value.php.

3.3 Results

Adult thrips was successfully collected from the field with minimal mortality during transport due to the addition of folded paper and leaf buds in their collection tubes.

3.3.1 Thrips bioassay

A Chi-Squared analysis was performed on the percentage thrips mortality from the bioassay. Table 3.1 shows that the isolates significantly affected mortality rate of adult thrips. The *B. bassiana* isolate, GAR 17 B3, was the most virulent against adults thrips, with a higher mortality than the *Beauveria* control. Both the commercial isolate and FCM AR 23 B3 had over 50% mortality. Mycosis of all dead thrips was recorded.

Table 3.1: Thrips bioassay percentage mortality Chi-Squared test; X^2 (df=3, n=4)= 0.0062, p=0.033

EP fungal isolate	Mortality (%)
Control	10
Beauveria control	60
FCM AR 23 B3	60
GAR 17 B3	70

3.4 Discussion

This study was performed with a small sample group because the thrips were collected from the field and experimented on directly, due to the difficulties in rearing thrips in culture. Since they were collected so late in the season it was not possible to collect large numbers of adult thrips. Methods for rearing thrips in culture are considered “tedious and time-consuming” (Laughling, 1971). Rearing

large populations over an extended period of time is difficult and expensive to maintain, and also highly labour intensive. These methods require constant supply of fresh plant material, which increases the chances of contamination from fungi and other pathogenic organisms (Steiner and Goodwin, 1998; Murai and Loomans, 2001; Garms *et al.*, 2013). In addition to maintaining the quality of the fresh plant material, these methods require precise ventilation control. Thrips also require precise moisture control. If there is too little ventilation, fluctuating temperatures occur which result in high mortality. Too much ventilation decreases the relative humidity, resulting in desiccated plant material and thrips mortalities (Laughlin, 1971).

While rearing thrips is difficult, collection from the field is not without its own challenges, because collection from the field can be extremely stressful for the thrips (Garms *et al.*, 2013). It is quite possible that the transport stress is the cause of the 10% of the natural death which occurred in the control of the bioassay (Table 3.1). To decrease the amount of stress experienced by the thrips, they were collected using aspirators filled with tissue paper and fresh leaf buds for the thrips to feed off of and settle on. Originally they were collected into empty pill bottles, the tissue and leaves help soften the landing and provided a food source for the extended period between collection in the field and the time taken to return to the lab. A similar apparatus was used by Zahn *et al.* (2013) as they also placed leaves in the bottom of the aspirator vials to allow the thrips to settle and feed. This was found to be the most beneficial method of keeping the thrips alive during transport. Without the tissue paper and leaves the thrips overheated and without the presence of the cardboard layer in the cooler box the thrips died from overexposure to cold.

Due to the limited replication for each fungal isolate, this study yields only an overall indication of the effect of South African *M. anisopliae* and *B. bassiana* entomopathogenic fungal isolates against *S. aurantii* and a protocol for successfully testing EP fungi against thrips. *Metarhizium anisopliae* and *B. bassiana* have been tested against many other species of thrips before. Both have shown good potential as biological control agents against thrips for both organic crops and as part of an IPM programme (Morse and Hoddle, 2006; Reitz *et al.*, 2011; Zahn *et al.*, 2013; Zahn and Morse, 2013). *Beauveria bassiana* was tested for its effectiveness in controlling western flowering thrips (WFT), *Frankliniella occidentalis* (Pergande), by Gao *et al.* (2012) where it was found to cause increased levels of mortality (69-96%), with 1 x 10^4- 1 x 10^7 conidia ml^{-1}after 10 days. Similar results were found by Wu *et al.* (2013) against onion thrips, *Thrips tabaci*, which resulted in 83-100% mortality with a concentration 1 x 10^7 conidia ml^{-1}after 4-7 days. This was also found by Zahn *et al.* (2013) against *S. citri,* where thrips populations were reduced 3 days after fungal exposure. Similar results were seen when thrips are exposed to *M. anisopliae,* Ansari *et al.* (2007) found that it was more effective than chemical insecticides against the pupae of WFT, causing 70 % mortality.

The zone where the EP fungi would likely have the greatest effect would be the upper layers of the soil or leaf litter. EP fungi naturally occur with the soil (Quesada-Moraga *et al.*, 2007) and the prepupal

and pupal life stages of *S. aurantii* occur within the upper layers of soil or leaf litter (Gilbert and Bedford, 1998). Thus, this soil zone may be the perfect interface for intersection between pathogen and its host. Infection in the soil also has an added benefit in that the soil provides protection for the EP fungul conidia from harsh elements which could render them inert (Zahn and Morse, 2013). Even if not directly applied to the soil, conidia are often removed from the foliage due to rain or irrigation (Thompson *et al.*, 2006). Thus, the potential for these species of EP fungi to be used against citrus thrips in the field is very high, though future research is needed to test the EP fungi effectiveness on adult, larval and pupal life stages on a larger scale under laboratory conditions, followed by field trials.

Chapter 4

General discussion

4.1 Entomopathogenic fungi as biological control agents

From this study the *M. anisopliae* isolate FCM AR 23 B3 has potential to be produced as a myco or biopesticide for the control of citrus mealybug and perhaps citrus thrips. Biopesticides are mass-produced biological control agents formulated from living micro-organisms or natural products, then sold to control crop pests. There are currently at least 170 different mycopesticide products based on EP fungi registered for the use against approximately 5 different insect and arcarine orders, the majority of which are strains of *B. bassiana* or *M. anisopliae.* When implementing BCAs, many factors must be taken into consideration, some of which are considered advantages, while others are disadvantages from a marketing standpoint. These factors are environmental safety, speed and mode of action, persistence, natural epizootic potential, host age-dependent susceptibility, environmental sensitivity, host contact, host response to dose, host resistance, compatibility with other natural enemies, mass production, product registration and compatibility with agrochemicals (Wraight and Hajek, 2009).

One advantage of EP fungi as mycopesticides is that they environmentally safe in that they leave no toxic residues, specific strains have relatively narrow host ranges and both *M. anisopliae* and *B. bassiana* have been tested for pathogenic effects against humans, mammals and fish and are considered safe with minimal risks (Zimmermann, 2007 a, b). They are capable of persisting in different environments, in the soil and within the host population. Running costs for mass-production of the fungal propagules (see Section 2.4 for fermentation details) are significantly lower in comparison to chemical pesticide production costs (Wraight and hajek, 2009; Li *et al.*, 2010; Chandler *et al.*, 2011). This minimizes the high doses required for control from becoming a disadvantage. Resistance amongst hosts for BCA is usually low. Insect death occurs due to infection rather then toxinosis,

which makes resistance less likely. Their compatibility with other natural enemies within the agroecosystem is still being studied, but due to the specificity of host range per strain, the risk is minimal. Many different EP fungi are compatible with a number of pesticides, thus allowing them to be used in IPM programmes (Section 4.2). Synergistic effects have even been recorded when exposed to sublethal doses of specific pesticides (Wraight and Hajek, 2009; Chandler *et al.*, 2011). Otherwise they are often used in situations where chemical pesticides are banned or in the process of being phased out (Butt *et al.*, 2001). Another advantage of EP fungal biopesticides is that they can be applied using existing conventional spray equipment which the majority of farmers already posses (Chandler *et al.*, 2011).

Some disadvantages of EP fungal biopesticides are that it takes longer to observe the effects than commercial pesticides, since hosts do not die immediately after infection. Thus, they may continue to damage crops for several days or weeks. While not necessarily a disadvantage, many EP fungi are more virulent against the immature larval life stages of their host, as in the case of FCM AR 23 B3 against mealybug. Although, (Ekesi and Maniania, 2000) determined that the most susceptible life stage of legume flowering thrips (*Megalurothrips sjostedti*) to infection from *M. anisopliae* were the adults. While the isolates in this study were only tested against adult thrips, GAR 17 B3 proved to be highly effective. Hence, farmers must have knowledge of the pests life cycle to spray at times when the biopesticide would be the most effective (Wraight and Hajek, 2009; Chandler *et al.*, 2011).

Adverse abiotic conditions for example high temperatures, low moisture and exposure to UV can be extremely detrimental to EP fungi. Steps must be taken to circumvent these factors, usually in the formulation of the product, such as the addition of "sunscreens," or the cultivation of strains with increased UV resistance (Zimmermann, 2008). The biggest disadvantage to the production of biopesticides is the absence of universally accepted registration procedures. Most follow procedures originally were set up for the registration of chemical pesticides and have not been adapted. For many developed products their intended market is too small to justify the expensive and tedious registration process (Butt *et al.*, 2001; Wraight and Hajek, 2009; Menzler-Hokkan, 2006). Although the registration of indigenous isolates, such as FCM AR 23 B3, are slightly less rigorous (Wraight and Hajek, 2009).

EP fungi as mycopesticides have great potential for the control of many crop pests including citrus. Biopesticides are usually applied in one of three ways; classical biological control, augmentation or conservation. Classical biological control is the use of a natural enemies against what is usually an exotic pest and thus without local natural enemies (Shah and Pell, 2003). It is not often used because large amounts of research must be conducted to determine which natural enemies to use, since there must be no short or long-term detrimental effects on the environment or non-target organisms (Goettel and Hajek, 2001; Klingen and Haukeland, 2006; Wraight and Hajek, 2009). Classical control is considered successful when the introduced natural enemy permanently establishes in and controls

the host population (Goettel and Hajek, 2001; Klingen and Haukeland, 2006). When successful, they provide excellent long-term control (Shah and Pell, 2003; Wraight and Hajek, 2009). The best candidates for this kind of control along with parasites, such as *Leptomastix dactylopii*, are pathogens which have the ability to decrease the host population in its endemic area and have a narrow host range to prevent predation on other insects in the agrosystem (Goettel and Hajek, 2001). *Leptomastix dactylopii* is considered to be the primary parasitoid of *P. citri* and has proven to be highly efficient in reducing mealybug population sizes on their host plants (Mansour *et al.*, 2012).

Augmentation control occurs when the natural enemies are already present in the agrosystem but are too few to control the pest. In this case the amount of the natural enemy is augmented. Augmentation occurs in one of two ways; inoculation or inundation. Inoculation is when the application of the pathogen occurs often and in small amounts, usually early in the crop's growing season. This form is used when the fungus is expected to establish and spread within the population and provide long-term control of the pest (Shah and Pell, 2003). For example *M. anisopliae* was reisolated 3 years after application with 80% infection of the bait insects and *Beauveria brongniartii* was reisolated 40 years after application (Klingen and Haukeland, 2006). Inundation closely resembles the application of chemical pesticides, in that the fungus is applied often and in large amounts for rapid short-term control. This is the system mycopesticides are most often developed for (Goettel and Hajek, 2001). For these systems the EP fungi are usually applied in aqueous suspensions with additional components that improve their chances of survival, such as UV-protectors (discussed below). One example of a mycopesticide often used in inundative control is *B. bassiana* which has been developed into the registered product "Mycotrol" marketed as a replacement for synthetic insecticides. Thus, Mycotrol requires frequent application and timing similar to regular insecticides (Shah and Pell, 2003).

Conservation control differs from the above two systems in that EP fungi are not released. Rather, farming practices are adapted to enhance the activity and population sizes of the natural fungi (Shah and Pell, 2003; Klingen and Haukeland, 2006). It has two stages: (1) reduce pesticides which would result in EP fungi mortality, (2) manipulate the habitat in ways to enhance the survival of the fungi (Klingen and Haukeland, 2006; Wraight and Hajek, 2009). EP fungi survival can be enhanced by increasing the moisture in the field, adding overwintering sites for alternative hosts as well as maintaining soil carbon (Shah and Pell, 2003).

While a worthwhile system, conservation control would be difficult to implement for the control of citrus mealybug in citrus orchards. EP fungi rarely occur above soil, and mealybug have no soil-born stages, thus, the EP fungi would have to be applied to the canopy where they are found. Alternatively, thrips prepupa develop within the soil (Gilbert and Bedford, 1998). Thus, EP fungi could be applied directly to the soil for their control. Ansari *et al.* (2008) tested different strains and species of EP fungi against WFT soil born life stages and determined that two *M. anisopliae* isolates were the most effective resulting in 85-96% mortalities. Different methods of application to the soil were also tested

for their efficacy. No significant differences were found in the efficacy between the drench in the soil with the conidial solution nor premixed soil with dry conidia. Though the length of time for which they would remain viable was not tested. Viability and persistence of dry conidia premixed within soil of citrus orchards against FCM larvae of both isolated FCM AR 23 B3 and GAR 17 B3 were tested by Coombes *et al.* (2013) and it was determined that the conidia were able to remain viable for a minimum of 6 months.

4.2 EP fungi in integrated pest management (IPM) programmes

Since their introduction, synthetic chemical insecticides have been the preferred solution for pest control (Charnley and Collins, 2007). Some of the older chemicals used have been linked to severe health problems,usually due to improper control and application. Many of these have been subsequently withdrawn from markets. The rate at which newer and safer chemicals are becoming available is very slow due to a decrease in the rate of discovery of new active molecules and lengthy registration procedures and costs (Butt *et al.*, 2001; Chandler *et al.*, 2011). It is estimated that companies have to screen a minimum of 140 000 chemicals to find one new, commercially viable synthetic pesticide. It costs approximately $250 million US dollars and 10 years just to develop one new synthetic pesticide product in comparison with the roughly 3 years and $3-5 US million to produce a new biopesticide product (Glare *et al.*, 2012). Recently the rise in insecticide resistance, pest resurgences and public concern over the impact these chemicals are having on the environment and human health has demonstrated the need to search for alternative forms of biological based forms of pest control (Charnley and Collins, 2007).

The global biopesticide market is growing rapidly, but it still dominated by bacterium-based products. Overall fungi-based biopesticides are only the 4th most commonly sold biopesticide after *Bacillus thuringiensis* based products, other bacterial biopesticides, and virus-based products. Still, they are the second highest selling in Africa and the Middle East (Table 4.1) (Glare *et al.*, 2012). Worldwide over 500 species of pest have become resistant to one or more insecticides. This urgent need to search for alternative pest control is also related to the significant need for increase in crop production which is required to meet the needs of the increasing human population. Even so, pesticides will never fully be replaced or removed from the market (Chandler *et al.*, 2011). Ways must be found to work with them. One such solution is IPM, the combination of biological control and insecticide use.

Table 4.1: Estimated sales of microbial biopesticides by type, for 2010 ($US million) (Glare *et al.*, 2012)

Biopesticide	**Sale estimates ($)**					
	North America	**Europe**	**Asia and Australasia**	**Latin America**	**Africa and the Middle East**	**Total**
Total Bt[a]	72.0	27.57	74.75	30.19	6.28	210.79
Other bacteria	23.94	6.30	14.05	4.56	0.40	49.25
Viruses	5.57	7.47	23.90	3.80	0.48	41.22
Fungi	15.85	5.64	18.85	35.96	0.78	77.08
Nematodes and others	9.4	7.50	0.95	0.16	0.13	18.14
Total	**126.76**	**54.48**	**132.5**	**74.67**	**8.07**	**396.48**

[a] Products based on *Bacillus thuringiensis* serotypes

IPM is the "*Flexible and holistic approach which views the agrosystem as an interrelated whole and utilizes a variety of biological, cultural, genetic, physical and chemical techniques, as required to hold pests below economically damaging levels with minimal amount of disruption to the cropping ecosystem and, surrounding environment*" (Malena, 1994). These programmes are designed to keep plants healthy and economically productive while reducing environmental impact (Lee, 2009). In sum, it is an approach which combines many different crop protection systems; cultural, physical, mechanical, chemical and biological, along with the careful monitoring of pests and their natural enemies together, with a belief that the combined practices overcome the others pitfalls (Ambethgar, 2009; Chandler *et al.*, 2011; Mamun and Ahmed, 2011). Unlike pesticides, IPM programmes do not aim at completely eradicating the targeted pest but rather to reduce and manage them at levels determined as below economic importance. To determine these levels it is important to know the Economic Threshold Level (ETL) of the pest. The ETL is the pest population density at which control measures should be implemented to prevent an increasing pest population from reaching economic importance injury levels. Establishing ETL helps farmers to determine what kind and amount of pest control must be applied (Orr, 2003; Mamun and Ahmed, 2011). These levels are different for each pest and thus can be difficult for farmers to determine and implement (Orr, 2003).

IPM programmes are acknowledged as one of the most robust contribution to agricultural science during the second half of the 20th century (Mamun and Ahmed, 2011). Many of the projects have been shown to substantially reduce the application of pesticides (Chandler *et al.*, 2011). Every year the number of countries which have experienced success with biological products in IPM programmes increases (Waage, 2001). Within these programmes EP fungal products are important components. Biopesticides used in combination with chemical controls reduce the need for large amounts of insecticides, improve the efficiency of pest control, reduce environmental contamination risks and delay insecticide resistance emergence in pests. Thus, interest in mycopesticides is increasing every year (Ambethgar, 2009).

IPM programmes often combine biological and chemical control methods. It is therefore important to know the effect of pesticides on the EP fungi to be used. While EP fungi are compatible with some agrochemicals, some increase their pathogenicity (Purwar and Sachan, 2006), most reduce their efficiency and growth (Shapiro-Ilan *et al.*, 2002). The insecticide compatibility of each EP fungal product must be determined. Research in this area is essential. Kouassi *et al.* (2003) found that timing is also very important, the effect of the fungicides metalaxyl, mancozeb and copper oxide, differed depending on when they were applied in relation with a *B. bassiana* isolate. If the fungicides were applied 2-4 days after the EP fungus, the insecticidal effect was increased, but if the application order was switched the opposite occurred. This also occurred if they were applied at the same time. This suggests that essential fungal biopesticides must become established in order to remain effective. Mietkiewski *et al.* (1997) compared the effects of pesticides in arable soils under laboratory and field conditions and determined that under field conditions pesticides are unlikely to kill all the EP fungi, nor limit their recolonization. Sublethal doses of insecticides have been found to synergistically increase insect susceptibility when combined with EP fungi. Conidial germination is believed to be stimulated at sub-lethal insecticide concentration (Ambethgar, 2009). Insecticides are not the only pesticides used in agrosystems. Fungal plant pathogens must also be controlled. Shah *et al.* (2009) tested the effect of 15 different commercially available fungicides on fungal germination rate, mycelial growth and virulence of *M. anisopliae, B. bassiana, Isaria fumosorosea* and *Lecanillium longisporum*. Most of the fungicides retarded conidial germination rates and affected the mycelial growth rate of certain fungi, they had little affect on fungal virulence against their target pests. Thus they could be used to control mealybug. These examples indicate that pesticides are not necessarily deleterious to EP fungi. An IPM program combining EP fungal products and pesticides can improve pest control, but that their use in combination requires careful planning.

Biopesticides are not solely microorganisms. Some are natural products derived from microorganisms. The most common of these are the *Cry* proteins produced by the entomopathogenic bacterium *Bacillus thuringiensis.* These pathogenic proteins are produced when the organism is under nutrient stress. Each protein has a narrow host range and is selectively toxic to different species of invertebrate phyla (Sansinenea, 2012). *Bacillus thuringiensis* enters the system of its host via ingestion. The toxic proteins bind to the glycoreceptors on the epithelium midgut cells, which disrupt the cytoplasmic membrane and causes cell lysis. Formulations based on these toxins have been produced for many decades and, like other biopesticides, are considered environmentally friendly and harmless to humans and other invertebrates (Sansinenea, 2012; Mantzoukas *et al.,* 2013). The development of biological control products combining multiple BCAs such as EP fungi and bacteria is an emerging field in which sizable amounts of research are being conducted (Prabhukarthikeyan *et al.,* 2013). Insects infected by multiple pathogens have increased mortality rates (Mantzoukas *et al.,* 2013). Numerous studies on the combined application of EP fungi and specific *Cry* toxins have resulted in positive interactions between the pathogens, which increased mortality of the host insect under laboratory

conditions. Wraight and Ramos (2005) combined *B. bassiana* and *B. thuringiensis* based biopesticides against Colorado potato beetle larvae which resulted in increased mortalities when compared with either biopesticide alone. Gao et al (2012) also tested the effect of combined *B. bassiana* and *B. thuringiensis* against spotted asparagus beetle larvae. When the larvae were exposed to sub-lethal doses of *B. thuringiensis* and *B. bassiana* their mortality significantly increased. Mantzoukas *et al.* (2013) tested the combined effect of *M. robertsii* and *B. thuringiensis* against the multivoltine *Sesamia nonagrioides.* After 16 days larval mortality observed was between 56-100%. All these studies found that the combination of EP fungi and *B. thuringiensis* resulted in higher larval mortality then either BCA achieved alone. It is probable that this combination, as with the application of sub-lethal doses of pesticides and EP fungi, cause a reduction in fitness on the insects, which increased the EP fungal infectivity.

4.2.1 IPM in citrus

A good example of successful IPM programmes in citrus was presented as a case study by Zalucki *et al.* (2009), on the implementation and subsequent success of IPM in Queensland, Australia. IPM in Australia began in 1973 due to the failure of insecticides to control their many non-indigenous pests. The programs used against imported pests were classical biological control, and augmentation where needed against the dominant scale insects. In the absence of the disruptive insecticides, such as bait sprays, the BCAs were able to provide effective control of the major scale pests. It also increased the mortality of minor pests because the negative impacts on their natural enemies were removed. Since a major grower adopted this IPM program in 1978, the number of growers changing over to IPM increased to 40% in 1985 and 75% by 1991.

IPM programmes are also in place to help control graft-transmissible pathogens (GTP) which come from infected germplasm, referred to as certification programmes. These IPM programmes are designed to produce "pathogen-tested" citrus seedling for planting. Certification programmes consist of 3 phases; (1) a quarantine program, (2) a clean stock program and (3) a certification program. Quarantine programmes are straightforward. They consist of isolating new germplasm. During quarantine the new germplasm are tested for the presence of pathogens. Clean stock programmes are the recovery and subsequent cultivation of healthy plants recovered from cultivars infected with GTP. It consists of 6 different steps; (1) selection of mother trees from local cultivars, (2) indexing those trees, (3) therapy for those that are infected, (4) indexing those which received therapy to ensure the pathogens have been eliminated, (5) horticultural evaluation of the recovered, healthy plants and (6) the maintenance of healthy plants. Certification programmes make sure the seedlings in nurseries are tested for GTP and are of the highest quality possible (Lee, 2009).

South Africa's version of this program is known as the Citrus Improvement Program (CIP). The CIP monitors GTP by producing the propagating material in a central location, the Citrus Foundation Block, Uitenhage which produces trees certified to be disease free (Mather, 1999). In Africa foreign markets have driven the citrus industry to adopt IPM (Orr, 2003), as a result of stricter regulations for pesticides residues on exported fruit and other photo-sanitary issues. As with Australia, this led to a drive towards "bio-intensive" programmes (Mather, 1999). As a result of this drive EP fungal products are being developed and marketed. The isolate FCM AR 23 B3 could have potential to become one of these product, and, after further study GAR 17 B3.

4.3 Future work

This project was undertaken with the main aim of finding an EP fungal isolate which could be developed as a mycopesticide to control citrus mealybug, and as an alternative to chemical pesticides or for the use in IPM programmes. For FCM AR 23 B3 to be developed into such a product useful in IPM programmes, considerable work must be undertaken.

The bioassays conducted for this study provided insight into the potential of indigenous EP fungal strains against citrus pests. However, the dose-dependent bioassay against adult female mealybug with the isolate GB AR 23 13 3 needs to be repeated as 50% mortality was not achieved. The goal would be to confirm whether the isolate had merely lost viability, since it had 41% average percentage mortality against adults on the initial bioassay at a concentration of 1 x 10^7 ml^{-1}. Thus, an increased percentage mortality would be expected at increased concentrations.

Expanding the thrips bioassay should also be undertaken. Both larval and adult thrips should be tested against a wider range of fungal isolates, since it is probable that an isolate which was not tested against could be more virulent against thrips than mealybug. The number of repeats per isolate should be increased for a more robust statistical analysis, and tested against the soil-born life stages of thrips as well as those found in the citrus canopy. Dose-dependent bioassays of the most virulent of isolates against the thrips should be undertaken to determine LC_{50} and LC_{90}. This would be best achieved by establishing a *S. aurantii* culture at Rhodes University. While tedious and time-consuming, methods for thrips cultures which allow access to each life stage have been formulated (Laughling, 1971) and would be used if this area of study is undertaken.

Future research is also needed to define the ecological constraints of FCM AR 23 B3. If it is to be developed into a mycopesticide, much more information is required on the isolate's viability and in-

teraction with conditions in the field. Its persistence in the field must be determined by determining its reaction to important ecological factors. FCM AR 23 b3's ability to tolerate temperature, thermotolerance and cold-activity (Rangel *et al.*, 2005; Fernandes *et al.*, 2008; Nishi *et al.*, 2013), UV-B tolerance, as well as the affect of "sunscreens" would have on virulent (Inglis *et al.*, 1995 a; Rangel *et al.*, 2005 b; Fernandes *et al.*, 2007; Rangel *et al.*, 2008), and its ability to survive in habitats with low relative humidity (Bouamama *et al.*, 2010). The effect that all of these factors have on FCM AR 23 B3's virulence must be would need to be quantified. Along with its interact with other insects in citrus orchards, and effects on other beneficial natural enemies in the field must be established.

If FCM AR 23 B3 is to be marketed as an IPM product its compatibility to the more common insecticides and fungicides must be investigated (Mietkiewski *et al.*, 1997). Knowing its pesticide compatibility would provide insight into the ways into which it may be applied in the citrus orchards. Specifically as the likelihood of it being applied with same the equipment used to apply the pesticides is high. Its compatibility with other biopesticides and their combined effects on the targeted pests should also be investigated, since this would provide insight into how FCM AR 23 B3 should be incorporated into specific IPM programmes.

The best approach to mass producing of the isolate must also be investigated. The fermentation process would have to be optimized, be it liquid, solid-state or diphase fermentation (Ye *et al.*, 2006; Mar *et al.*, 2012). If the latter, which cereal grains, temperature and moisture combinations would produce the highest yields? From these results a mass production method to yield a concentration of 1 x $10^{9\text{-}10}g^{-1}$is necessary.

4.4 Conclusion

This study was designed to identify an EP fungal strain, which was virulent against citrus mealybug *P. citri* and citrus thrips, *S. aurantii.* The results showed that the *M. anisopliae* strain FCM AR 23 B3 was the most significantly virulent isolate tested against the mealybug. It also showed pathogenic activity against the citrus thrips. The *B. bassiana* isolate GAR 17 B3 was the most pathogenic against adults thrips. All of the other experimental aims of the study: establishing a successful *P. citri* culture, maintaining the fungal isolates, bioassays with both mealybug and thrips, morphological and molecular identification of the significantly virulent isolates, determining the LC_{50} and LC_{90} concentrations of those isolates and determining the mode of infection using SEM, were achieved to some degree. It was not possible to determine the mode of fungal infection via SEM due to the obstruction caused by the large number of wax fragments present of the mealybug's cuticle. Although, fungal growth out through the cuticle was confirmed after insect death. Future investigations should be focused on the

isolates FCM AR 23 B3 and GAR 17 B3 as they have great potential to become EP fungal products used in citrus orchards.

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Appendix

FCM AR 23 B3 aligned with *Metarhizium anisopliae* isolate CG626 accession number: KF056847.1

Score	Expect	Identities	Gaps	Strand
889 bits(481)	0.0	488/491(99%)	2/491(0%)	Plus/Minus

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Sbjct  491  GTTGGGGGGTTTTACGGCAGTGGACCGCGCCGGGCTCCTGTTGCGAGTGCTTTACTACTG  432

Query  61   CGCAGAGGAGGGCCACGGCGAGACCGCCAATTAATTTAAGGGACGGCTGTGCTGGAAAAC  120
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Sbjct  431  CGCAGAGGAGGGCCACGGCGAGACCGCCAATTAATTTAAGGGACGGCTGTGCTGGAAAAC  372

Query  121  CAGCCTCGCCGATCCCCAACACCAAGTCCCACAGGGGACTTGAGGGGCGTAATGACGCTC  180
            |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  371  CAGCCTCGCCGATCCCCAACACCAAGTCC-ACAGGGGACTTGAGGGGCGTAATGACGCTC  313

Query  181  GAACAGGCATGCCCGCCAGAATACTGACGGGCGCAATGTGCGTTCAAAGATTCGATGATT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  312  GAACAGGCATGCCCGCCAGAATACTGACGGGCGCAATGTGCGTTCAAAGATTCGATGATT  253

Query  241  CACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  252  CACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAG  193

Query  301  AACCAAGAGATCCGTTGTTGAAAGTTTTGATTCAtttttttttAACCACTCAGAAGATACT  360
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Query  361  TATTAAAAAAATTCAGAAGGTTTGGGTCCCCGGCGGGCGCGAAGTCCCGCCGAAGCAACAA  420
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Sbjct  132  TATTAAAAAATTCAGAAGGTTTGGGTCCCCGGCGGGCGCGAAGTCCCGCCGAAGCAACAA  73

Query  421  TTAAAGGTATAATTCACAGGGGTTGGGAGTTGGATAACTCGGTAATGATCCCTCCGCAGG  480
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  72   TTAAAGGTATGATTCACAGGGGTTGGGAGTTGGATAACTCGGTAATGATCCCTCCGCAGG  13

Query  481  TTCAACCTACG  491
            |||| ||||||
Sbjct  12   TTCA-CCTACG  3
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GAR 17 B3 aligned with *Beauveria bassiana* isolate 1572 accession number: JQ861946.1

Score	Expect	Identities	Gaps	Strand
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Sbjct  508  GAAGTTGGGTGTTTTACGGCGTGGCCGCGTCGGGGTTCCGGTGCGAGCTGTATTACTACG  449

Query  61   CAGAGGTCGCCGCGGACGGGCCGCCACTCCATTTCAGGGCCGGCGGTGTGCTGCCGGTCC  120
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Query  121  CCAACGCCGACCTCCCCCAGGGGAGGTCGAGGGTTGAAATGACGCTCGAACAGGCATGCC  180
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Query  181  CGCCAGAATGCTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGGATTCTGC  240
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Sbjct  328  CGCCAGAATGCTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGGATTCTGC  269

Query  241  AATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAGCCAAGAGATCC  300
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Sbjct  268  AATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAGCCAAGAGATCC  209

Query  301  GTTGTTGAAAGTTTTGATTCATTTGTTTTGCCTTGCGGCGTATTCAGAAGATGCTGGAAT  360
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Sbjct  208  GTTGTTGAAAGTTTTGATTCATTTGTTTTGCCTTGCGGCGTATTCAGAAGATGCTGGAAT  149

Query  361  ACAAGAGTTTGAGGTCCCCGGCGGGCCGCTGGTCCAGTCCGCGTCCGGGCTGGGGCGAGT  420
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Sbjct  148  ACAAGAGTTTGAGGTCCCCGGCGGGCCGCTGGTCCAGTCCGCGTCCGGGCTGGGGCGAGT  89

Query  421  CCGCCGAAGCAACGATAGGTAGGTTCACAGAAGGGTTAGGGAGTTGAAAACTCGGTAATG  480
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Sbjct  88   CCGCCGAAGCAACGATAGGTAGGTTCACAGAAGGGTTAGGGAGTTGAAAACTCGGTAATG  29

Query  481  ATCCCTCCGCAGGTTCA  497
            |||||||||||||||||
Sbjct  28   ATCCCTCCGCAGGTTCA  12
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GB AR 23 B3 aligned with *Beauveria bassiana* isolate A117 accession number: KC461113.1

Score	Expect	Identities	Gaps	Strand
917 bits(496)	0.0	498/499(99%)	0/499(0%)	Plus/Minus

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Sbjct  458  CGCAGAGGTCGCCGCGGACGGGCCGCCACTCCATTTCAGGGCCGGCGGTGTGCTGCCGGT  399

Query  121  CCCCAACGCCGACCTCCCCCAGGGGAGGTCGAGGGTTGAAATGACGCTCGAACAGGCATG  180
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Sbjct  398  CCCCAACGCCGACCTCCCCCAGGGGAGGTCGAGGGTTGAAATGACGCTCGAACAGGCATG  339

Query  181  CCCGCCAGAATGCTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGGATTCT  240
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Sbjct  338  CCCGCCAGAATGCTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGGATTCT  279

Query  241  GCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAGCCAAGAGAT  300
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Sbjct  278  GCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAGCCAAGAGAT  219

Query  301  CCGTTGTTGAAAGTTTTGATTCATTTGTTTTGCCTTGCGGCGTATTCAGAAGATGCTGGA  360
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Sbjct  218  CCGTTGTTGAAAGTTTTGATTCATTTGTTTTGCCTTGCGGCGTATTCAGAAGATGCTGGA  159

Query  361  ATACAAGAGTTTGAGGTCCCCGGCGGGCCGCTGGTCCAGTCCGCGTCCGGGCTGGGGCGA  420
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Sbjct  158  ATACAAGAGTTTGAGGTCCCCGGCGGGCCGCTGGTCCAGTCCGCGTCCGGGCTGGGGCGA  99

Query  421  GTCCGCCGAAGCAACGATAGGTAGGTTCACAGAAGGGTTAGGGAGTTGAAAACTCGGTAA  480
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Query  481  TGATCCCTCCGCAGGTTCA  499
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