Investigation of entomopathogenic fungi for control of false codling moth, *Thaumatotibia leucotreta*, Mediterranean fruit fly, *Ceratitis capitata* and Natal fruit fly, *C. rosa* in South African Citrus.

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Abstract

The biology of key citrus pests *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Tortricidae), *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) and *Ceratitis rosa* Karsch (Diptera: Tephritidae) includes their dropping from host plants to pupate in the soil below citrus trees. Since most EP fungi are soil-borne microorganisms, the development and formulation of alternative control strategies using these fungi as subterranean control agents, targeted at larvae and pupae in the soil, can potentially benefit existing IPM management of citrus in South Africa. Thus, a survey of occurrence of entomopathogenic fungi was undertaken on soils from citrus orchards and natural vegetation (refugia) on conventionally and organically managed farms in the Eastern Cape Province in South Africa. A method for baiting soil samples with citrus pest *T. leucotreta* and *C. capitata* larvae, as well as with the standard bait insect, *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae), was implemented. Sixty-two potentially useful entomopathogenic fungal isolates belonging to four genera were collected from 288 soil samples, an occurrence frequency of 21.53%. The most frequently isolated entomopathogenic fungal species was *Beauveria bassiana* (Balsamo) Vuillemin (15.63%), followed by *Metarhizium anisopliae* var. *anisopliae* (Metschnikoff) Sorokin (3.82%). *Galleria mellonella* was the most effective insect used to isolate fungal species (χ^2=40.13, df=2, $P\leq$ 0.005), with a total of 45 isolates obtained, followed by *C. capitata* with 11 isolates, and *T. leucotreta* with six isolates recovered. There was a significantly (χ^2=11.65, df=1, $P\leq$ 0.005) higher occurrence of entomopathogenic fungi in soil samples taken from refugia compared to cultivated orchards of both organically and conventionally managed farms. No significant differences were observed in the recovery of fungal isolates when soil samples from both farming systems were compared.

The physiological effects and host range of 21 indigenous fungal isolates obtained in the Eastern Cape were investigated in the laboratory to establish whether these isolates could be effectively used as biological control agents against the subterranean life stages of *C. rosa*, *C. capitata* and *T. leucotreta*. When these pests were treated with a fungal concentration of 1 x 10^7 conidia ml^{-1}, the percentage of *T. leucotreta* adults which emerged in fungal treated sand ranged from 5 to 60% (F=33.295; df=21; *P*=0.0001) depending on fungal isolate and the percentage of pupae with visible signs of mycosis ranged from 21 to 93% (F= 96.436; df=21; *P*=0.0001). Based on fungal isolates, the percentage adult survival in *C. rosa* and *C. capitata* ranged from 30 to 90% and 55 to 86% respectively. The percentage of *C. rosa* and *C. capitata* puparia with visible signs of

mycosis ranged from 1 to 14% and 1 to 11% respectively. Deferred mortality due to mycosis in *C. rosa* and *C. capitata* adult flies ranged from 1 to 58% and 1 to 33% respectively, depending on fungal isolate. Entomopathogenic fungal isolates had a significantly greater effect on the adults of *C. rosa* and *C. capitata* than they did on the puparia of these two fruit fly species. Further, *C. rosa* and *C. capitata* did not differ significantly in their response to entomopathogenic fungi when adult survival or adult and pupal mycosis were considered.

The relative potency of the four most virulent *Beauveria* isolates as well as the commercially available *Beauveria bassiana* product, Bb Plus® (Biological Control Products, South Africa), were compared against one another as log-probit regressions of mortality against *C. rosa*, *C. capitata* and *T. leucotreta* which all exhibited a dose-dependent response. Against fruit flies the estimated LC_{50} values of all five *Beauveria* isolates ranged from 5.5 x 10^{11} to 2.8 x 10^{12} conidia/ml^{-1}. There were no significant differences between the relative potencies of these five fungal isolates. When *T. leucotreta* was considered, isolates: *G Moss R10* and *G 14 2 B5* and Bb Plus® were significantly more pathogenic than *G B Ar 23 B3* and *FCM 10 13 L1*. The estimated LC_{50} values of the three most pathogenic isolates ranged from 6.8 x 10^5 to 2.1 x 10^6 conidia/ml^{-1}, while those of the least pathogenic ranged from 1.6 x 10^7 to 3.7 x 10^7 conidia/ml^{-1}. *Thaumatotibia leucotreta* final instar larvae were exposed to two conidial concentrations, at four different exposure times (12, 48, 72 and 96 hrs) and showed an exposure time-dependant relationship ($F=5.43$; $df=3$; $P=0.001$). At 1 x 10^7conidia/ml^{-1} two *Beauveria* isolates: *G Moss R10* and *G 14 2 B5* were able to elicit a response in 50% of test insects at 72 hrs (3 days) exposure.

Although a limited amount of mycosis was observed in the puparia of both fruit fly species, deferred adult mortality due to mycosis was high. The increased incidence of adult mortality suggests that post emergence mycosis in adult fruit flies may play a more significant role in field suppression than the control of fruit flies at the pupal stage. The increased incidence of pupal mortality, as well as the relatively low concentrations of conidia required to elicit meaningful responses in *T. leucotreta* pupae may suggest that pre-emergent control of false codling moth will play a more significant role in field suppression than the control of adult life stages using indigenous isolates of entomopathogenic fungi. Various entomopathogenic fungal application techniques targeted at key insect pests within integrated pest management (IPM) systems of citrus are discussed.

To my parents,

Clive and Jen

Thank you both for your incredible love of nature

and your incredible nature of love

Declaration

The following thesis has not been submitted to any university other than Rhodes University, Grahamstown, South Africa. The work presented here is that of the author.

_____ Date: _____

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List of Abbreviations

χ^2	chi-squared
µl	micro litre
%	percent
ºC	degrees Celsius
BCA	biological control agent
CGA	Citrus Growers Association
cc	centimetre cubed
cm	centimetre
CRI	Citrus Research International
EP	entomopathogenic
EU	European Union
FCM	false codling moth
F	frequency
g	grams
hrs	hours
ha	hectare
IGR	insect growth regulator
IPM	integrated pest management
ITS	internal transcribed spacer
kg	kilograms
L	litre
LC_{50}	medial lethal concentration
LD_{50}	medial lethal dose
LT_{50}	medial lethal exposure time
m	metre
mg/L	milligrams per litre
min	minute
ml	milliliter
mm	millimeter
MPa	mega Pascal
n	number of replicates
nm	nano metre
pH	potential of hydrogen ions
SIT	sterile insect technique
s.s.	*sensu stricto*
UK	United Kingdom
USA	United States of America
US$	United States Dollar
UV	ultraviolet
ZAR	South African Rand

I

GENERAL INTRODUCTION

1.1 CITRUS IN SOUTH AFRICA

1.1.1 The genus Citrus

Citrus (Rutaceae) Linnaeus is a genus of flowering, fruit-bearing trees which originated in the tropical and subtropical areas of Southeast Asia (McCoy *et al.* 2007) and then spread to other continents. The genus has had a long history in South Africa with some of the first orange and lemon orchards planted over three centuries ago, in the 1650s when seedling trees, originating from St. Helena Island, were planted in the gardens of the Dutch East India Company in Cape Town, over 100 years before citrus orchards were planted in California (Mather & Rowcroft 2004). The precise number of natural species is unknown and genetic evidence suggests that some wild, true-breeding species are of hybrid origin thus leaving the taxonomy and systematics of the genus complicated (Nicolosi *et al*. 2000). Historically studies on the relationships between citrus genera and species have been largely based on morphological characteristics. However, recent molecular studies reveal that citrus can be divided into two subgenera, citrus and *Papeda* (non-edible species) (Nicolosi *et al*. 2000). Almost all cultivated citrus belongs to the sub-genus citrus, and the numerous cultivars are derived from three true breeding species based on genetic studies where citron, pummelo and mandarin were placed into three distinct groups (Nicolosi *et al*. 2000).

1.1.2 Citrus production areas

Citrus production occurs mainly in the Western and Eastern Cape, Mpumalanga, KwaZulu-Natal, and Limpopo provinces (Bedford 1998) (Fig. 1.1). There are important differences between production regions in South Africa based on climate and farm structure. The Western Cape and Eastern Cape are considered to be ‘cooler’ citrus growing areas and production is

focused on navel oranges and lemons (Mather 2003). The cooler climate has allowed farmers to respond to consumer demand for easy peelers (soft citrus) such as Clementines and Satsuma mandarins, and most of the country's easy peelers are produced in these two regions (Fig. 1.1). The farm sizes in the Western and Eastern Cape Provinces are small, and most citrus is packed by privatised cooperatives within facilities that are amongst the largest in the world (Mather 2003). In Mpumalanga, Limpopo and KwaZulu-Natal the climate is warmer and better suited to the cultivation of grapefruit and Valencia oranges (Mather 2003) (Fig. 1.1). Navel orange trees, however, tend to set smaller crops in the warmer parts of the citrus areas (Agricultural Research Council 2003). Farm sizes in these warmer regions are larger and most farmers pack in smaller, privately owned facilities. In terms of area under citrus cultivation (Ha), the Limpopo and Western Cape Provinces have the largest total amounts of land under cultivation, followed by the Eastern Cape and Mpumalanga (Fig. 1.1).

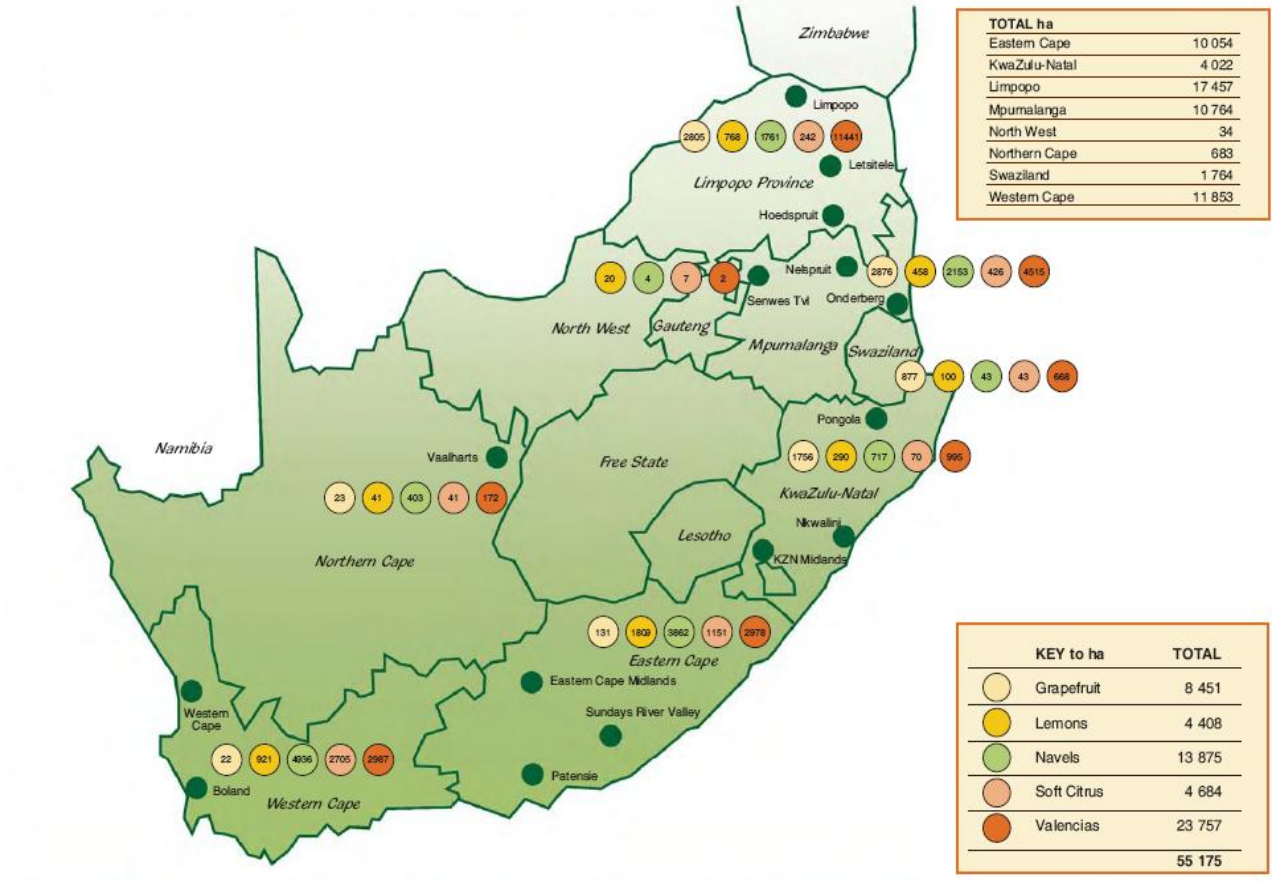

Figure 1.1 The citrus producing regions of South Africa and Swaziland showing the total citrus production per province and the total area planted to various citrus cultivars per province (CGA Annual Report 2008/9).

1.1.3 Climatic requirements

Climatic conditions vary considerably among the different citrus producing regions and even within regions, and the effects of temperature can have significant impacts on production (Bedford 1998). Production in South Africa is confined to areas with mild and almost frost-free winters where temperatures rarely drop below -2°C (Agricultural Research Council 2003). Citrus does well in most areas where the temperatures are between 10 to 30°C (the optimum temperature between 20 to 28°C). However, temperature limits growth above 37.7°C and prolonged temperatures above 30°C can cause fruit drop (Wellington 1960; Veldman 2001). Night temperatures of between 10 to 14°C in June and July induce more intense blossom, and mean minimum temperatures below 13°C in autumn (early March to early June), induce external fruit colour (Veldman 2001). Most cultivars start to bear during the third year, although in hotter climes it can be earlier and in colder areas as much as a year later. Lemons start bearing fruit a year earlier because of their vigorous growth habit (Veldman 2001). Most citrus cultivar production tends to peak after 8-12 years (Veldman 2001). Citrus (excluding lemons) require shorter days and cooler temperatures in winter for a normal production rhythm. In the more tropical areas the flowering pattern is much less clearly defined and main-season crops tend to be considerably smaller (Agricultural Research Council 2003).

1.1.4 Soil requirements

Citrus can be grown in a wide range of soil types provided they are well-drained; usually sandy loams of colluvial or alluvial origin with the most productive localities situated on the floors or slopes of river valleys (Wellington 1960). Fertile, well-aerated soils with a pH of between 6 and 6.5 are ideal (Agricultural Research Council 2003). In the drier parts of the citrus growing regions in South Africa, and in particular the Sundays River Valley in the Eastern Cape, soils of a loam or clay nature may become alkaline (pH 9) due to lower rainfall, although careful drainage and the addition of compost can counteract these effects (Wellington 1960). An unusual condition in South African citrus soils is the low phosphorus content. The high acid-sugar ratio of citrus fruit is thought to be as a result of this soil condition (Wellington 1960). An ideal citrus soil with respect to optimum water holding capacity would be red, yellow-brown or brown, have a clay content of between 10 to 40% and no clay or structural layers within one meter of the soil surface (Agricultural Research Council 2003).

1.1.5 Rootstocks

All citrus produced in South Africa are budded onto rootstocks, and the majority of these are certified trees (Veldman 2001). Farmers should have certification of their plantings which would include the type of cultivar and the rootstock used, as well as the nursery information from where they obtained their trees. This certification names the budwood, which is used to produce the trees, and it is a requirement of Eurogap that the trees are true to type and are cleared of any foreign pathogens (Lee 2009). Certification of trees and rootstocks means that they have various degrees of resistance to soil- borne diseases and viruses (Lee 2009). Rootstocks influence tree and fruit characteristics and are used to regulate tree height and improve tree age. They can further improve some physiological problems associated with some cultivars, such as creasing, cold or drought resistance and some stocks can sustain flooding situations (Veldman 2001). The commercial rootstocks available in South Africa include the most widely used rootstock, Swingle citrumelo; Rough lemon is often used on virgin soils and areas that induce high internal fruit quality, it is also widely used on lemons and is adaptable to high pH soils (Veldman 2001). Troyer/Carrizo citrange can be used on a wide range of soil types and cultivars; the rootstock X639 is semi-dwarfed and used for high pH soils. The following rootstocks are used to a lesser extent in South Africa: C35 citrange, Minneola x Trifoliate, Yuma citrange and Benton citrange (Veldman 2001). For further reading on the importance of certification of citrus in integrated pest management (IPM) it is suggested that Lee (2009) be consulted.

1.1.6 Irrigation systems

Citrus production requires vast amounts of water, especially when the canopy volume (tree age) increases (Veldman 2001). Water is particularly important during the blossoming and fruit-setting periods, which occur from August to November in South Africa. Citrus will grow where there is available water equivalent to a rainfall of 760-860 mm a year (Wellington 1960). The mean annual rainfall in South African citrus areas varies from as much as 743 mm at Letaba and 949 mm at Richmond in the summer rainfall areas to 237 mm at Clanwilliam in the winter rainfall area (Bedford 1998). Summer rainfall can be extremely unpredictable in the Eastern Cape over these important phenological periods, so irrigation is particularly important. Wellington (1960) however reported that a slight drying out of the soil from February to June results in better quality fruits.

The most commonly used systems are: (1) overhead irrigation, where stationary sprayers are situated approximately 15 m apart and wet the overall area and (2) under foliage irrigation which involves three techniques; open hydroponic systems (OHS), micro irrigation and drip irrigation. OHS irrigation is very technical and the whole system is computerized. Usually the irrigation scheduling is pulsed with trees receiving water and nutrients at intervals each day as per the requirement for any given day, but this is an extremely expensive method (Veldman 2001). With micro irrigation, small cone sprays which are used to concentrate the wetting area are placed under each tree. Wider sprayers are used at a later stage to enlarge the wetting area. However, wider sprayers increase the likelihood of evaporation and use a vast amount of water (Veldman 2001). This factor motivated a move away from this system to drip irrigation, especially in water scarce areas. Drip irrigation consists of 1-4 inline drippers that can also be used for fertigation (applying liquid fertilizers to trees) which makes the system cost effective and also ensures a better nutrient uptake (Veldman 2001). Throughout the world, farmers are increasingly adopting the use of drip irrigation. As the quality and automation of these irrigation systems improves, the feasibility of using them to apply insecticides, growth regulators and nutrients also increases (Grout & Stephen 2005).

Understanding the growth and environmental parameters of a particular cropping system is crucial for the successful implementation and use of microbial biological control agents. Tritrophic interactions between crop plants, their pests and microbial control agents applied to control pests cannot be ignored and must be considered together. A greater understanding of the biology and ecology of crops will enhance our understanding of the environments in which microbial control agents are required and better adapted strains or isolates of these control agents may be applied.

1.1.7 The South African citrus export industry

The South African citrus industry was established in the early 1800s. However, it was not until 1926, with the founding of the single-desk exporter, the South Africa Co-operative Citrus Exchange, that the industry started realising its enormous potential in the export market (Mather & Greenberg 2003; Mather & Rowcroft 2004). Exports of citrus to the United Kingdom (UK) started at the turn of the century; by the 1960s, South Africa was exporting over half of all southern hemisphere fresh citrus produce and was ranked amongst the top five fresh citrus

exporters in the world (Mather 2003). In 1995, the citrus industry exported 43 million cartons of citrus to 60 countries with the gross value of ZAR 1.6 billion (FABI 1998). New market legislation was passed in 1996 which broke the export monopoly and allowed growers to choose independent export agents, which inspired a strong private sector response (Mather 1999; Mather & Greenberg 2003).

The South African citrus industry is now the second largest exporter of citrus in the world after Spain (FAO: citrus Fruit Annual Statistics 2006), with Europe, Japan, China and the USA being the largest export markets (CGA Annual Report 2008/9). In 2008, up to 89 million cartons (68% of total citrus production in South Africa) of citrus fruit were exported, which generated an annual income of approximately ZAR 5.1 billion for South Africa (Edmonds, pers. comm.). Citrus varieties such as Valencia and Navel oranges are the most popular on the overseas markets with approximately 41 million cartons and 20 million cartons of 15 kg each exported respectively. These are followed by grapefruit (13 million cartons), lemons (8 million cartons) and soft citrus (7 million cartons) (CGA Annual Report 2007/8).

1.1.8 Citrus pests

Citrus is host to as many as 875 insects and mites, however only 144 are well known major pest species worldwide (Smith & Peña 2002). South Africa is known to harbor over 100 citrus pest species of economic importance (Bedford 1998). Approximately 30-60% of known citrus pests constitute the Hemiptera, such as aphids, scale insects, mealybugs, leafhoppers and whiteflies. These are followed by the Lepidoptera (fruit boring moths, piercing moths, leaf rollers and leaf miners), Acari, Coleoptera, Homoptera, Diptera and Thysanoptera (Smith & Peña 2002) (Table 1.1). As mentioned climatic conditions vary among production areas which restrict the distribution of some citrus pests. Some pests are more sensitive to extremes in temperature and humidity, thus each citrus producing region has its own complex of major and minor pests (Bedford 1998). Citrus pests are generally more abundant and more difficult to control in the hotter, low-lying areas of the Northern Province and Mpumalanga. In other areas such as Citrusdal in the Western Cape, where temperature extremes vary considerably during winter and summer, very few pests require regular control strategies (Bedford 1998).

Table 1.1 Important insect and mite pests of citrus in South Africa

Citrus pests	Common name	Scientific name
Armoured scale	Red scale*	*Aonidiella aurantii**
	Circular purple scale ▲	*Chrysomphalus aonidium* ▲
	Citrus mussel scale	*Cornuaspis becki*
Soft scale	Soft brown scale	*Coccus hesperidum*
	Soft green scale	*Pulvinaria aethiopica*
Wax scale	Citrus wax scale	*Gascardia brevicauda*
	White wax scale	*Gascardia destructor*
Cottony cushion scale	Cottony cushion scale ▲	*Icerya purchasi* ▲
Mealybugs	Citrus mealybug ▲	*Planococcus citri* ▲
	Karoo thorn mealybug	*Nipaecoccus vastator*
	Oleander mealybug	*Paracoccus burnerae*
	Spherical mealybug ▲	*Nipaeoccus viridis* ▲
Aphids	Black Citrus aphid ▲	*Toxoptera citricidus* ▲
	Cotton aphid	*Aphis gossypii*
Leafhoppers	Citrus leafhopper ▲	*Penthimeola bella* ▲
	Green Citrus leafhopper	*Empoasca* citrus*a*
Blackflies	Citrus blackfly	*Aleurocanthus woglumi*
	Spiny blackfly ▲	*Aleurocanthus spiniferus* ▲
Psyllids	Citrus psylla*	*Trioza erytreae**
Thrips	Citrus thrips*	*Scirtothrips aurantii**
Caterpillars	Citrus swallowtail	*Papilio demodocus*
	False codling moth*	*Thaumatotibia leucotreta**
	Apple leaf roller	*Tortrix capensana*
	Citrus leaf roller	*Archips occidentalis*
	Citrus looper	*Ascotis selenaria reciprocaria*
	African bollworm	*Helicoverpa armigera*
Fruit flies	Mediterranean fruit fly *	*Ceratitis capitata **
	Natal fruit fly *	*Ceratitis rosa **
	Marula fruit fly	*Ceratitis cosyra*
Mites	Citrus bud mite ▲	*Aceria sheldoni* ▲
	Citrus grey mite	*Calacarus citrifolii*
	Citrus rust mite ▲	*Phyllocoptruta oleivora* ▲
	Citrus red mite ▲	*Panonychus citri* ▲
	Red spider mite	*Tetranychus cinnabarinus*
	Citrus flat mite	*Brevipalpus californicus*
	Citrus silver mite ▲	*Polyphagotarsonemus latus* ▲
	Oriental spider mite ▲	*Eutetranychus orientalis* ▲

* represents key pests of citrus, ▲ represents major or occasional important pests of citrus, all other pests are minor or sporadic pests of citrus (Annecke & Moran 1982; Bedford 1998; Smith & Peña 2002).

1.1.9 Control of citrus pests - towards an integrated pest management approach

Pesticides used in the early days of citriculture in South Africa had very short residual action and included: resinwash, lime sulphur, sulphur dust and nicotine sulphate (Bedford 1998). Until 1948, citrus pest control relied substantially on annual fumigation of trees which were enclosed under tarpaulins, using the highly toxic gas, hydrogen cyanide (HCN) (Annecke & Moran 1982). From 1948 onwards, fumigation and sulphur dust were replaced by parathion, an organophosphate insecticide, which was much quicker to apply to trees and was used to combat Californian red scale, *Aonidiella aurantii* and also controlled other important citrus pests (Bedford 1998). It was noted however that resurgence of secondary pests (soft brown scale, *Coccus hesperidum* and citrus red mite, *Panonychus citri*) occurred mainly as a result of the disappearance of natural enemies; this was due to the toxic effect of the organophosphate sprays (Annecke & Moran 1982). As a result of the resurgence of insect pests at this time, the cycle of injudicious spraying of chemical insecticides continued which eventually lead to insecticide resistance. This prompted the use of new insecticides (chlorpyrifos, mercaptothion and methidathion), with newer active ingredients and the combining of chemical pesticides to kill insects (Bedford 1998). As a result of this 'pesticide treadmill' in an effort to control red scale, citrus growers were forced to move toward an integrated pest management (IPM) approach and the use of narrow range petroleum oils replaced organophosphates (Bedford 1998). Synthetic pyrethroids were registered in 1988 to control citrus thrips, *Scirtothrips aurantii*, and were highly successful to a point but were also found to disrupt populations of natural enemies and biological control agents released to control scale insects. At this time insect growth regulators (IGR's) were applied to citrus in an attempt to amalgamate control tactics as part of an IPM strategy to control resurgences of red scale, following failed oil sprays (Bedford 1998). Tartar emetic and bait sprays (methiocarb and formetanate) were introduced at this time as part of an IPM strategy against thrips in citrus orchards. Further application of new products such as Confidor® (imidacloprid) (Bayer CropScience, Germany), a stem applied systemic which was considered more effective and safer to natural enemies than foliar sprays, already showed potential disruption of the biological control of *Icerya purchasi* (Bedford 1998).

IPM had became an integral part of citrus production in the 1980s but later in the 1990s there was a dramatic increase in the use of thripicides in citrus which disrupted the biological control of red scale and mealybugs. The increased use of thripicides together with the reductions in organophosphate usage led to drastic increases in mealybug populations during the 1990s (Bedford 1998).

The control of citrus in South Africa has relied almost exclusively on chemical control in the last 100 years and biological control had been largely ignored or unstudied which is ironic because as early as 1892, before the extensive use of chemicals in citrus, the Vedalia ladybird beetle *Rodolia cardinalis*, was introduced to control *I. purchasi* and was highly successful (Bedford 1998). Bedford (1998) suggested that "*the only way to avoid pesticide-induced outbreaks of pests is to strive towards integrated control*". The term integrated control refers to the combined usage of the advantageous aspects of both chemical and biological control, thus killing insect pests with minimal disruption to natural enemies and maintaining a greater permanence of pest suppression (Orr 2003). The South African citrus industry has now developed standardised testing procedures, in collaboration with the Agricultural Research Council, to screen new plant protection agents to ensure that they comply with citrus IPM programmes (Bedford 1998). Further, numerous studies on the implementation of predators and parasitoids in citrus orchard systems have been undertaken, reiterating the need for a reduction in pesticides which are highly potent and have persistent residual action (Bedford 1998).

1.2 TARGET PESTS

Tephritid fruit flies, *Ceratitis capitata* (Wiedemann) and *C. rosa* (Karsch) constitute major pests of citrus in South Africa and are also considered economically important fruit pests worldwide (Ekesi *et al.* 2002; Smith & Peña 2002; Dimbi *et al.* 2003). Similarly, false codling moth, *Thaumatotibia leucotreta* (Meyrick), is considered a major problem in most cultivated fruits, vegetables and nuts in South Africa and other countries (Kirkman & Moore 2007; Stibick 2008). The highly phytophagous nature of all three of these insect pests, their relative phytosanitary importance and the economic importance of fruit loss makes it vital to control these pests in citrus.

1.2.1 False codling moth, Thaumatotibia leucotreta

The false codling moth, *Cryptophlebia* (=*Thaumatotibia*) *leucotreta* (Lepidoptera: Tortricidae), revised by Komai (1999), is regarded as a major insect pest on citrus in South Africa (Newton 1998; Van den Berg 2001). This moth was first described as a pest on citrus in KwaZulu-Natal by Fuller in 1901, who called it the Natal codling moth; it was later found in the northern areas

of South Africa and named the orange codling moth (Newton 1998). False codling moth (*T. leucotreta*) is found throughout sub- Saharan Africa (where it originates) as well as the neighboring islands of the Indian and Atlantic Oceans (Stibick 2008) (Fig. 1.2). The species has a broad host range, being recorded from over 80 different species of trees and shrubs, which includes important economic crops such as avocados, citrus, cotton, litchis, macadamias, pome and stone fruit (Van den Berg 2001; Venette *et al.* 2003; Kirkman 2007; Kirkman & Moore 2007; Stibick 2008). In citrus, navels appears to be the variety most heavily damaged by *T. leucotreta*. Grapefruits and naartjies are less susceptible to attack by *T. leucotreta*, and larval development is rarely, if ever, completed in lemons and limes, possibly due to the acidity content in this cultivar (Newton 1998). *Thaumatotibia leucotreta* larvae are capable of developing in hard, green fruit before control measures can be implemented and once the fruit is damaged, it becomes susceptible to fungal infection and scavengers and drops to the ground (Annecke & Moran 1982).

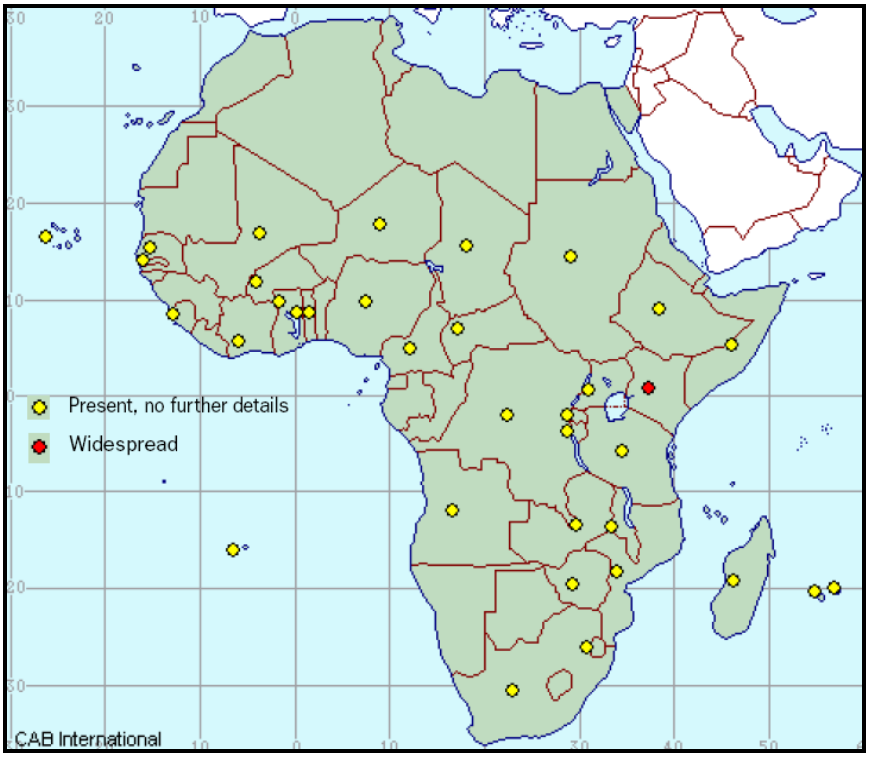

Figure 1.2 The geographical distribution of false codling moth, *Thaumatotibia leucotreta* in Africa and neighbouring islands (Stibick 2008).

1.2.1.1 Life cycle of false codling moth

The life stages of *T. leucotreta* are described in detail by Stibick (2008). Female moths lay pearl-white, oval eggs (which turn slightly reddish with a black spot shortly before neonate larvae hatch) singly on the rind of fruit. At higher insect densities, more than one egg is laid on a single fruit (Newton 1998). The eggs (Fig. 1.3 A) are flattened with a reticulate surface and measure approximately 0.77 mm in length and 0.60 mm in width (Van den Berg 2001); egg incubation varies according to temperature but generally lasts for up to 12 days in winter months (Kirkman 2007). There are five recognized larval instars which are determined by the width of the head capsule (Kirkman 2007). Larval development varies according to temperature but averages 35 days in summer and 67 days in winter (Kirkman 2007). Neonate larvae are cream-white with a dark brown head and measure approximately 1.5 mm in length and generally bore into fruit within 24 hrs of hatching (Wysoki *et al.* 2002). The entire larval stage progresses inside the fruit until the final instar larval stage, which is pinkish-red (Fig. 1.3 B) and measures 12-15 mm in length, exits the fruit to pupate in the soil (Van den Berg 2001). Final instar larvae drop to the ground below host plants and begin spinning cocoons with silk and soil particles (Wysoki *et al.* 2002; Stibick 2008). The pupal stage (Fig. 1.3 C), like both the egg and larval stages, is also temperature dependent and can last from 21 to 80 days; there is no diapause stage (Stibick 2008). Adult moths (Fig. 1.3 D) emerge with mottled dark grey forewings and pale, fringed hind wings and generally live for 2 to 3 weeks in the field. The sex ratio of field populations is generally 1:1 (Newton 1998). Males are usually smaller than females and have a grayish anal tuft of scales and a scent organ near the anal angle of each hind wing (Van den Berg 2001). A female moth can lay up to 450 eggs during her lifetime, and as many as 5 or 6 generations a year may occur, however this is influenced by several factors, including temperature, food availability and quality, photoperiod, humidity, latitude and the effect of predators and diseases (Stibick 2008).

1.2.1.2 Economic damage

T. leucotreta causes annual losses in excess of ZAR 100 million to the South African citrus industry, mainly as a result of pre-harvest fruit loss caused by cryptic, internal feeding of the larvae, and as a result of post-harvest decay of fruit (Kirkman & Moore 2007). Larval feeding and development can affect fruit development at any stage, causing premature ripening and infested fruit may drop as early as November when they are no more than 15-20 cm in diameter (Newton 1998). Estimates of long term crop losses in citrus in Nelspruit during 1971 were recorded as 24-45 fruit per tree (Newton 1998). Foreign market rejection of fruit due to pest

presence is considered to be the most important factor pertaining to this recognized phytosanitary pest and contributes to further loss of revenue for the industry (Moore 2002). False codling moth has as yet not established in the United States of America despite numerous interceptions at port entries. It does represent a significant import threat to the USA particularly because large areas in the southern and south western United States are under agricultural production and these areas have similar climate parameters to South Africa; establishment of the pest in the USA would result in US$ billions in economic losses (Stibick 2008). Large export markets such as China will only accept fruit from guaranteed *T. leucotreta*-free orchards and sensitive markets such as the USA will reject large consignments of fruit due to the presence of *T. leucotreta*, which constitutes further losses in revenue for the export industry (Kirkman 2007).

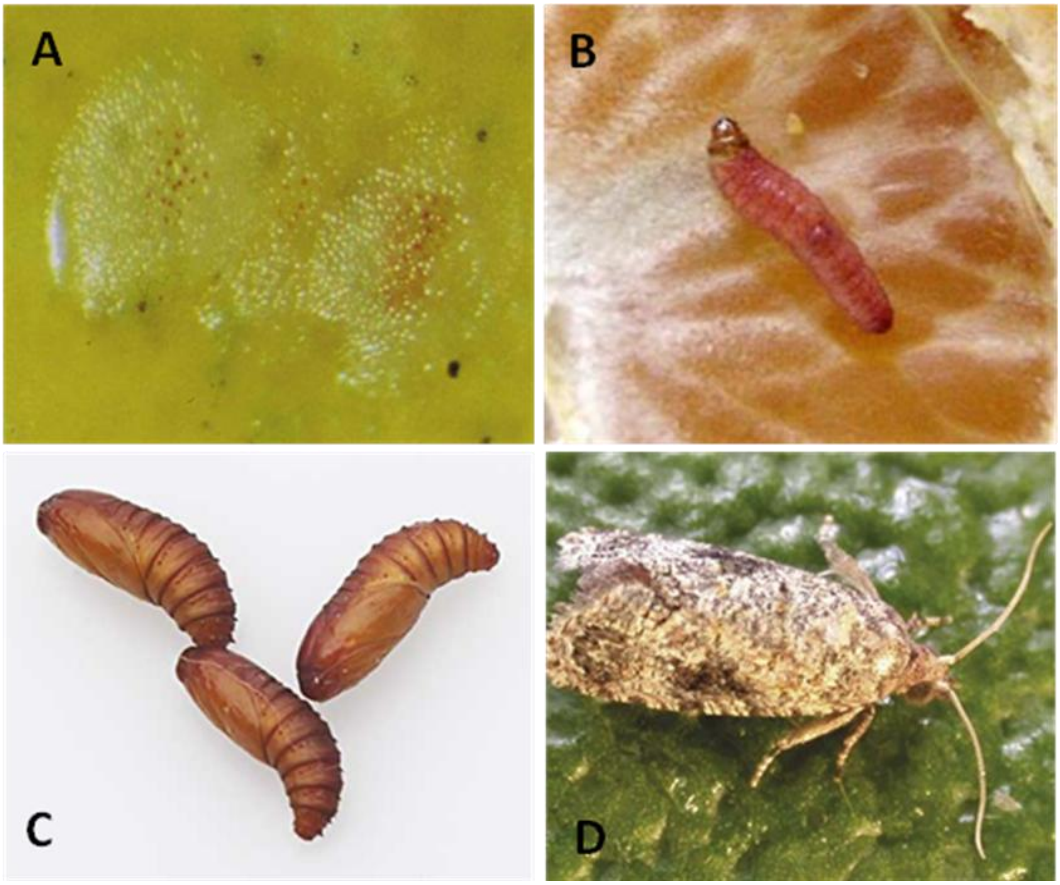

Figure 1.3 *Thaumatotibia leucotreta* **(A)** egg laid singly on the rind of fruit (**B)** fifth instar larva with characteristic red colouring **(C)** sclerotised brown pupae **(D)** mottled grey adult moth. Photo Credits: (A, C, D) River Bioscience (B) A.M. Varela, ICIPE

1.2.1.3 Control of false codling moth

Control of *T. leucotreta* in citrus in South Africa has included:

1.) *Pre-harvest monitoring:* The Lorelei trap which contains a female-based pheromone dispenser and an adhesive is used to ascertain the numbers of moths present in or near orchards; the threshold value of trap catches is 10 males per trap per week (Moore *et al*. 2008).

2.) *Chemical control:* Registered chemicals used include, two chitin synthesis inhibitors, Alsystin® (Bayer CropScience, Germany) and Nomolt® (Cyanamid, South Africa). These chemicals however are known to cause sterilization in predatory beetles and result in population explosions of citrus red mite and oriental mite. Further, these chemicals may not be used on fruit destined for export to the USA (Kirkman 2007). An organophosphate insecticide called Penncap-M® (Elf Atochem, Netherlands) which kills larvae on contact and two pyrethroids, Meothrin (Sanachem, South Africa) and cypermethrin (Agropharm, South Africa) are also registered for use on *T. leucotreta* in citrus (Kirkman 2007).

3.) *Cultural control:* Practices such as orchard sanitation, can contribute largely to the control of *T. leucotreta*; as much as 75% of fruit picked up weekly from orchard floors can still be infested with *T. leucotreta* larvae (Moore & Kirkman 2009).

4.) *Behavioural control*: Mating disruption using synthetic female pheromones to confuse or disorientate males finding females for mating can subsequently lead to a reduction of fertile eggs in the field (Moore 2002). The attract and kill technique is another mating disruption method which attracts male moths to a synthetic female pheromone. When the male moths make contact with an incorporated insecticide they are killed (Kirkman 2007).

5.) *Genetic control*: Sterile insect technique (SIT) is a control measure that employs gamma irradiation to induce sterility in male moths. This is followed by mass releases of sterilized male moths into citrus areas where mating with females of wild populations results in infertile eggs (Kirkman 2007).

6.) *Post harvest control:* includes cold sterilization of exported fruit which maintains fruit sterility on route to lucrative markets such as China, Japan and the USA (Kirkman 2007).

7.) *Biological control*: The use of natural enemies against *T. leucotreta* includes a suite of insects (mainly Hymenoptera) and microorganisms which vary in their respective levels of control. Moore (2002) lists 14 hymenopteran egg larval and pupal parasitoids, of which *Trichogrammatoidea cryptophlebiae*, *Agathis bishopi* and *Apophua leucotreta* are considered to be common and elicit effective suppression of *T. leucotreta*. Three Tachinid fly species are known to parasitize the larvae and pupae of *T. leucotreta* (Moore 2002). Further, three

hemipterous predators (two *Orius* sp. and *Rhynocoris albopunctatus*) and a mite (*Pediculoides* sp.) exert limited natural control on *T. leucotreta* populations in the field (Newton 1998). The use of microorganisms such as viruses and fungi has also contributed to the control of *T. leucotreta*. Viruses in particular have been very successful, the *Cryptophlebia leucotreta* granulovirus (CrleGV), is highly pathogenic to *T. leucotreta* and can cause extensive epizootics in nature (Moore 2002). Likewise the entomopathogenic fungus *Beauveria bassiana* (Balsamo) has been recorded on *T. leucotreta* pupae amongst the leaf litter in Zebediela Estate, although attempts to culture and apply this fungal pathogen have been met with little success to date (Newton 1998).

1.2.2 Fruit flies

Mediterranean fruit fly, *Ceratitis capitata*

Ceratitis capitata (Diptera: Tephritidae) is one of the world's most damaging fruit pest species and originates from the tropical zones of Central Africa (Van den Berg 2001). This species is widespread throughout the world and occurs in all South African provinces (Du Toit 1998). *Ceratitis capitata* is a highly polyphagous insect species, recorded from over 260 different plant species including some important economic crops such as avocado, coffee, citrus, custard apple, granadilla, guavas, litchi, mangos and persimmons (Thomas *et al.* 2001; Van den Berg 2001). Adult Mediterranean fruit fly are approximately 4 mm long with a black scutellum and defined ivory markings which do not enclose three black areas, as in the Natal fruit fly (Fig. 1.4 B). The males are easily recognised by the two spatulate setae on the head (Du Toit 1998).

Natal Fruit Fly, *Ceratitis rosa*

Ceratitis rosa (Diptera: Tephritidae) is an economically important fruit fly species with a widespread distribution throughout Africa and the islands of Mauritius and Réunion (Botha *et al.* 2004). Its distribution in South Africa is more humidity dependent than *Ceratitis capitata* and is confined to the subtropical regions of KwaZulu-Natal (where it was first found), the Northern Province, Mpumalanga and along the coastal regions of the Western and Eastern Cape Provinces (Ware 2002; de Meyer *et al.* 2008). *Ceratitis rosa* is a pest of over 100 different plant species, which include important agricultural species such as apple, apricot, avocado, blackberry, citrus, coffee, custard apple, fig, guava, Kei-apple or umkokolo, loquat, mango, papaya, peach, pear,

persimmon, plum, quince, roseapple, soursop, and *Chrysophyllum natalense*, *Eugenia cordata*, *Garcinia livingstonei*, *Opuntia* sp., *Rawsonia lucida*, *Solanum auriculatum*, and *Solanum giganteum* (Weems 2002; Botha *et al.* 2004). The appearance of this fruit fly looks very similar to that of the Mediterranean fruit fly; however it has a larger body size (length of the fly 4 to 5 mm). Females can be separated from other fruit fly species by their thoraxes which bear ivory bands enclosing three black areas on the scutellum (Fig. 1.4 C). Males have characteristic dark bristles on the tibia of the mid-pair of legs (Weems 2002).

1.2.2.1 Life cycle of fruit flies

The life cycles of both Mediterranean and Natal fruit flies are similar (Du Toit 1998) and are discussed together. *Ceratitis* females puncture the rind of the fruit and oviposit clusters of about 10-20 small, white, sausage-shaped eggs in the rind tissue. Larvae hatch and burrow through the flesh (Smith & Peña 2002) (Fig. 1.4 A). The life cycle is temperature dependant and is shorter in warmer climates and takes longer in cooler environments. Fruit flies lay eggs on mature green and ripening fruit with some species laying eggs in unripe fruit (Botha *et al.* 2004). Two to four days after oviposition the eggs hatch. The larvae emerge as white maggots and pass through three larval instars, which usually take 5 to 11 days (Thomas *et al*. 2001) (Fig. 1.4 A). After 4 to 17 days the third instar maggots leave the fruit, making holes in the skin, and drop to pupate in the soil; this pupal stage may last for 10 to 20 days (Botha *et al.* 2004). The pupae are white, brown or black and approximately 4 to 6 mm long (Du Toit 1998). They are found in the soil 2 to 5 cm beneath the host plant (Thomas *et al*. 2001). Adult flies emerge from the pupae 10 to 15 days after pupation (Du Toit 1998). Oviposition may take place as early as four to five days after emergence in warm temperatures but can take several days after emergence at 20°C; and adult longevity is 2 to 3 months depending on the season (Thomas *et al*. 2001). Some *Ceratitis* species populations can produce up to 10 generations per annum depending on the availability of host plants and suitable climatic conditions (Thomas *et al*. 2001; Weems 2002).

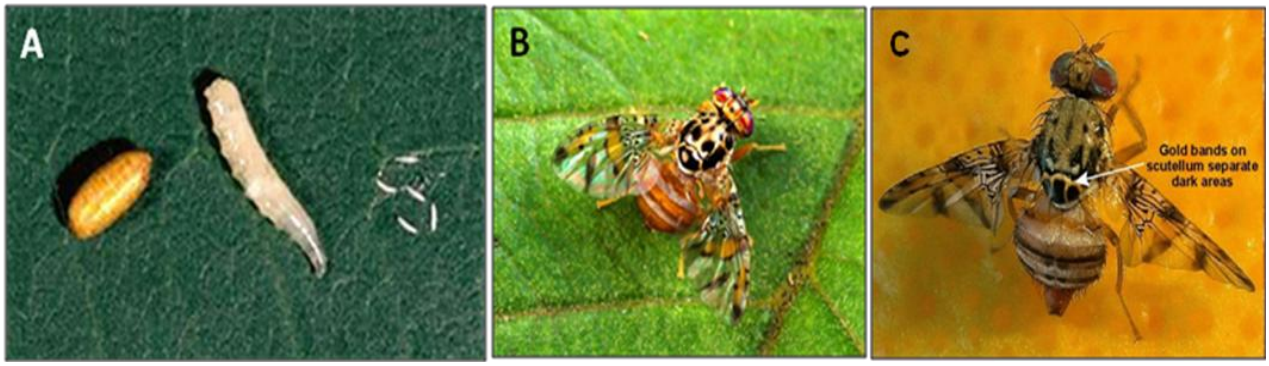

Figure 1.4 (A) Pupa, larva and eggs of *Ceratitis capitata* (**B)** Adult *C. capitata* fruit fly **(C)** Adult female *C. rosa* fruit fly. Photo Credits: (A) California Department of Food and Agriculture (B) Scott Bauer, USDA (C) Citrus Research International.

1.2.2.2 Economic damage

The economic importance of fruit flies has been well described by Du Toit (1998); Ekesi *et al.* (2002) and Dimbi *et al.* (2003, 2004). Fruit flies affect the production of quality fruits by infestation which causes both direct and indirect loss of product. Direct loss occurs through fruit flies feeding on citrus which causes fruit drop and renders the fruit inedible because of fungal infections which invade the fruit after puncture marks are created by ovipositing females (Du Toit 1998). Indirect loss arises from phytosanitary restrictions imposed by importing countries to prevent entry and establishment of fruit flies (Ekesi *et al.* 2007).

1.2.2.3 Control of fruit flies

Control methods against fruit flies in South African citrus include:

1.) *Pre-harvest monitoring*: Includes the use of Sensus® traps which contain synthetic attractants to ascertain the number of fruit flies present in or near orchards (Smith & Peña 2002). Suitable economic thresholds have not been determined for the control of fruit fly in all citrus areas but a recommendation for producers to try and reduce fly numbers to an average of one female fly per trap per week when Questlure® (Quest Developments CC, Brits) is used as an attractant is suggested by Moore *et al.* 2008. Treatment thresholds for use with the Sensus trap are four fruit flies per trap per week when Capilure® is used. If Ceratitislure® is used the thresh-hold is either zero female fruit flies or four male fruit flies per trap per week (Moore *et al*. 2008).

2.) *Cultural control*: Orchard sanitation involves the collection and destruction of all senesced citrus fruit in orchards (Ekesi *et al.* 2007). Fruit flies are notorious for infesting decaying fruit

and piles of fallen fruit can cause unnecessary build up of fruit fly numbers in orchards (Moore & Kirkman 2009).

3.) *Behavioural control*: The most common method used to control fruit flies in South Africa is the attract and kill technique, whereby a protein attractant is mixed with a toxicant in a bait station (Ware *et al.* 2003). The male annihilation technique involves the arrangement of high density trapping stations in which a female-based attractant is combined with an insecticide; the aim is to reduce the male population so that low level mating occurs (Ekesi *et al.* 2007).

4.) *Genetic control*: Sterile insect technique (SIT) includes the genetic sterilization of male fruit flies but does not affect their ability to mate; the aim is to introduce a genetic mutation in the male sperm which induces sterility in the sperm but does not affect sperm function, males then mate but no eggs can be fertilized (Ekesi *et al.* 2007).

5.) *Chemical control:* In citrus orchards chemicals are usually applied as weekly applications of toxic bait in the form of large droplets over half the canopy of every alternate tree. Baits consist of mixtures of mercaptothion or trichlorfon and protein hydrolysate (Du Toit 1998). The method targets adult flies, mainly females (because they require a protein meal before oviposition) and aims to attract and poison them before they cause damage to fruit

6.) *Biological control:* The use of natural enemies such as predators or parasitoids has always been appealing because they are safe and self perpetuating. There are three fruit fly parasitoids which occur in South Africa, *Opius concolor* and *O. humilis* (Braconidae) which both attack the larvae, and a pupal parasitoid, *Trichopria capesis* (Diapriidae). However, these natural enemies are unable to control fruit fly in citrus (Du Toit 1998). The role of entomopathogenic fungi as it is known from these target insect pests species is such that epizootics of fungal disease of fruit fly in nature is uncommon and natural infection rates are low (Ekesi *et al.* 2007). There have only been a few reports of natural infection of *Ceratitis capitata* adults by the entomopathogenic fungus *Entomophthora muscae* and *E. schizophorae* in Israel (Ekesi *et al.* 2007). Ekesi *et al.* (2007) reported that *Isaria fumosorosea* and *Beauveria bassiana* also caused natural infections in *C. capitata* populations in Spain.

1.2.3 Justification for alternative control tactics

The production of citrus in South Africa plays a major role in providing food security for the country; it further provides employment and opportunity for foreign export and trade. As it currently stands, the South African citrus industry is the second largest exporter of citrus in the world after Spain (FAO: Citrus Fruit Annual Statistics 2006) and generates an annual income of

approximately ZAR R5.1 billion in currency for South Africa (Edmonds, pers. comm.). Present control of insect pests on citrus still relies on insecticide use because the cosmetic integrity of this crop is vital for export. Citrus exports to the European Union (EU) however have now been restricted by the recent lowering of maximum residue limits of certain insecticides, such as dimethoate (Grout & Stephen 2005). This is due to the EU's drive to reduce unnecessary and harmful chemical pesticide use (Mather 1999) and, to a lesser extent, the recent consumer interest in organically produced foods, which prohibits the use of pesticides (Zehnder *et al.* 2007). Further large export markets will continue to impose strict phytosanitary restrictions on key citrus pests such as Natal fruit fly and false codling moth because these pests have not as yet become established in Europe, USA, China or Japan (Botha *et al.* 2004; Stibick 2008), which makes their control in citrus orchards crucial. South African citrus growers exporting fruit to these overseas markets are obliged to reconsider their pest control practices, and in particular the use of some chemical pesticides. As a consequence, sustainable agriculture may rely increasingly on alternative control strategies that are environmentally friendly and reduce the amount of human contact with pesticides; further the use of these alternative control strategies within IPM programmes must be considered. To maintain a stronghold in overseas export markets, it has become important for the South African citrus industry to investigate other facets of IPM, which includes the study and use of microbial control agents, such as entomopathogenic fungi (hereafter referred to as EP fungi). The biology of the key citrus pests, *T. leucotreta*; *C. capitata* and *C. rosa*, includes their dropping from their host plants to pupate in the soil or leaf litter below citrus trees. Since most EP fungi are soil-borne microorganisms, the development and formulation of alternative control strategies using these fungi as subterranean control agents, targeted at larvae and pupae in the soil, can potentially benefit existing IPM management of citrus in South Africa.

1.3 ENTOMOPATHOGENIC FUNGI

1.3.1 General taxonomy

Fungi are a diverse group of unicellular (yeasts) or hyphal (filamentous fungi), eukaryotic microorganisms that are all heterotrophic, meaning that they have absorptive nutrition and reproduce either sexually or asexually by means of spores (Madigan *et al.* 2005). The most common characteristic used to assign fungi to their four divisions, based on genetic relationships, relies on sexual fruiting structures (Inglis *et al.* 2001). Until recently, it was thought that

Hyphomycete EP fungi had lost the ability to produce sexual spores. These fungi were traditionally placed in the division, *Deuteromycota* within the superficial class Hyphomycete. They are characterized by mycelia forms that produce asexual spores called conidia which are borne on specialized conidiogenous cells (Inglis *et al.* 2001). However, with the advent of molecular technologies, some EP taxa have now been connected with a sexual state and most of these taxa exhibit ascomycetous affinities which include characteristics such as septate hyphae with lamellate hyphal walls with a thin electron-dense outer layer and a thicker electron-transparent inner layer (Inglis *et al.* 2001; Rehner & Buckley 2005). When a mitotic fungus has been connected with a sexual state it is called the 'anamorph'. The sexual or perfect state is called the 'teleomorphic state'. For example, *Beauveria bassiana* (the anamorphic state) has been linked to the teleomorphic species *Cordyceps bassiana* (Rehner & Buckley 2005). Most EP fungi now fall under the order Hypocreales which belongs to the division *Ascomycota* and the most common EP hyphomycetous genera include: *Aspergillus* (Micheli), *Beauveria* (Balsamo), *Culicinomyces*, *Hirsutella* (Patouillard), *Metarhizium* (Metschnikoff), *Nomuraea* (Yasuda), *Isaria* (=*Paecilomyces*) (Samson), *Tolypocladium* and *Lecanicillium* (=*Verticillium*) (Gams and Zare) (Inglis *et al.* 2001).

1.3.2 Fungal infection process and environmental cycling

All EP fungi have the same basic mode of action when infecting insect hosts and are unique in that they can infect insect hosts through the cuticle (Goettel 1995). The conidia, or asexual spores, come into direct contact with the host and adhere to the cuticle via nonspecific hydrophobic mechanisms (Inglis *et al.* 2001). Under specific environmental conditions the conidia germinate and a germ tube, or appressorium (penetration-peg structure), are produced (Inglis *et al.* 2001) (Fig. 1.5 A). The cuticle is penetrated by a combination of mechanical pressure from the appressorium and the action of cuticle-degrading enzymes, such as trypsin, metalloproteases and aminopeptidases (Bidochka & Small 2005). The fungus grows by vegetative growth in the host haemocoel and external conidia are produced upon death of the host when fungal hyphae exit through the less sclerotised areas of the cuticle (Inglis *et al.* 2001) (Fig. 1.5 B). Some EP fungal species, such as *Metarhizium* and *Beauveria*, produce powerful insecticidal cyclic peptides called destruxins and a toxic metabolite called oosporein (Goettel *et al.* 1989; Inglis *et al.* 2001). Comprehensive reviews on the function and mode of action of destruxins and oosporeins are reported in the literature (Zimmermann 2007a, b). Fungal strains which produce these toxins are sought after for commercialisation because although fungi grow

sparsely in the insect haemocoel, they kill the insect relatively quickly. Strains which do not produce these toxins grow profusely in the insect haemocoel but take much longer to kill their hosts (Bidochka & Small 2005). The host cuticle is the first line of defense against fungal infection and has a central role in determining fungal specificity. If the fungus breaches the cuticle, successful infection can only occur if the fungus can overcome the innate immune response of the insect (Hoffman *et al.* 1999). Insects respond in both a cellular and humoral manner to fungal infection, with immune activation occurring as early as the point of cuticle degradation during the penetration step (Gillespie *et al.* 2000). Fungi overcome host defense responses by developing cryptic growth forms that are effectively masked from the insect defense responses, or by the production of immune-modulating substances that suppress the host defense system as mentioned (Bidochka & Small 2005) (Fig. 1.5 A).

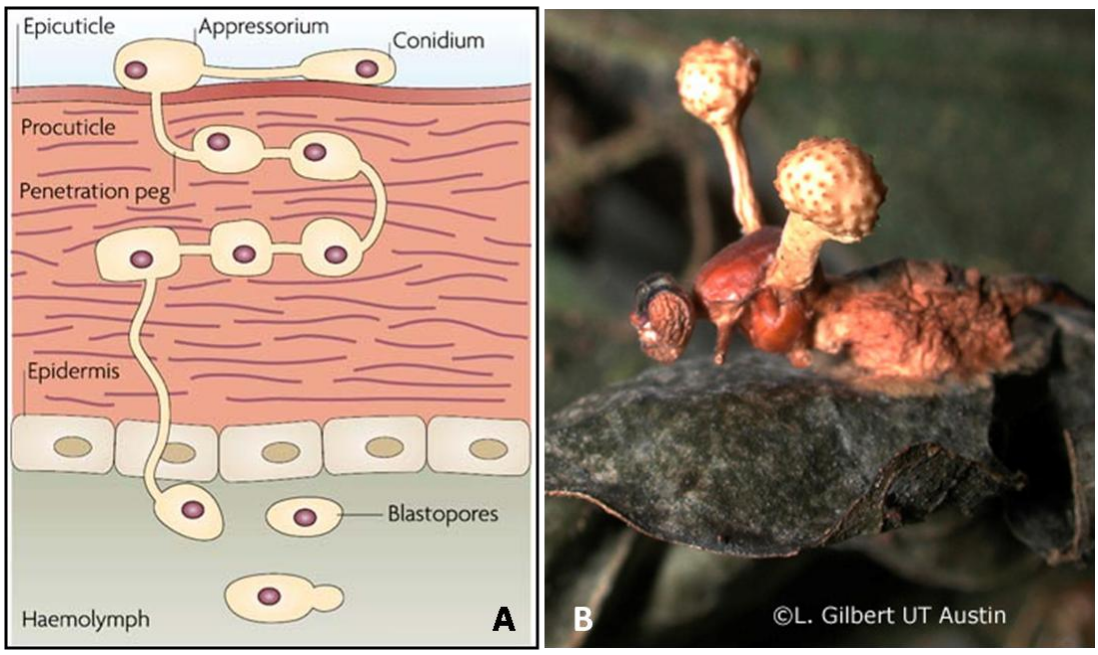

Figure 1.5 (A) Schematic diagram of an EP fungus invading the host insect cuticle **(B)** *Cordyceps* sp. fungus growing out of the thorax of an unidentified fly species. Photo Credits: (A) Thomas & Read 2007 (B) Lawrence Gilbert, University of Texas Austin

1.3.3 Fungi as biological control agents

Soil-inhabiting EP fungi are an important and widespread component of most terrestrial ecosystems and play a key role in regulating some soil-dwelling insect populations (Meyling & Eilenberg 2007; Quesada-Moraga *et al.* 2007). The majority of fungi that naturally regulate insect and mite populations in the field belong to the orders Hypocreales and Entomophthorales.

However, the latter has not been possible to mass produce and commercialisation or inundative application of this fungal order has not occurred, unlike the Hypocreales which are amenable to mass production and commercialisation (Dolinski & Lacey 2007).

The use of microbial control agents specifically EP fungi, have been investigated for the control of a wide range of orchard pests (Cross *et al.* 1999; Puterka 1999; Lacey & Shapiro-Ilan 2003; Alves *et al.* 2005; Castrillo *et al.* 2005; Dolinski & Lacey 2007; Lacey & Shapiro-Ilan 2008). Further the use of EP fungi against various citrus pests has been successful (Table 1.2). Numerous entomopathogens (fungi, viruses, nematodes and microsporidia) impose a high level of natural control on citrus pests throughout much of the humid, warmer climates of the world. EP fungi appear to be the most prevalent pathogens attacking citrus pests; to date over 24 mycopathogens have been recorded (McCoy *et al.* 2007).

The use of EP fungi in the form of mycoinsecticides has many desirable traits, EP fungi leave no toxic residues on crops, and are generally harmless to beneficial and other non target insects (Goettel 1995; Zimmermann 2007a, b). They also pose minimal risks to humans and animals and there is no damage to the environment because ground water and riparian habitats are not contaminated by EP fungi, as is the case with pesticides (Zimmerman 2007a). Their host specific nature enhances their potential role in IPM, as it preserves natural enemies which then make a greater contribution to the overall regulation of pests (Inglis *et al.* 2001). The main impact of EP fungi on pest populations however may only occur once economic thresholds have been surpassed. Because chemical control can be really effective at immediately suppressing a growing pest population it is unlikely that EP fungi will ever supersede chemical control and it is highly unlikely that the disuse of chemicals will occur (Inglis *et al.* 2001). Thus, it becomes more important to select fungal strains which are pesticide tolerant and can survive in the cropping systems for which they are required. The use of EP fungi in cropping systems will require augmentation of isolates, most often applied as a prophylactic treatment rather than a once off application (Ekesi *et al.* 2007). Some of the most versatile and widely used fungal biological control agents are from the order Hypocreales and some important genera are discussed further below.

Table 1.2 EP fungi used in the control of citrus pests worldwide (Dolinski & Lacey 2007)

Common name	Pest species	Fungal control	References
Citrus rust mite	*Phyllocoptruta oleivora*	*Beauveria bassiana* *Hirsutella thompsonii*	Alves *et al.* (2005) McCoy (1996)
Citrus red mite	*Panonychus citri*	*B. bassiana*	Shi & Feng (2006)
Citrus broad mite	*Polyphagotarsonemus latus*	*H. thompsonii*, *B. bassiana*, *Isaria fumosoroseus*	Peña *et al.* (1996)
False spider mite	*Brevipalpus phoenicis*	*H. thompsonii*	Rossi-Zalaf & Alves (2006)
Whiteflies	*Dialeurodes citri*	*Aschersonia spp.*	Dolinski & Lacey (2007)
Citrus black fly	*Aleurocanthus woglumi*	*Aschersonia aleyrodis*	Elizondo & Quezada (1990)
Scales	*Parlatoria ziziphi*	*A. aleyrodis* *A. goldiana*	El-Choubassi *et al.* (2001)
	Selenaspidus articulates	*A. aleyrodis*	Gravena *et al.* (1988
	Chrysomphalus aonidum *Lepidosaphes beckii*	*Podonectria coccicol* *Pseudomicrocera henningsii*, *Sphaerostilbe aurantiicola*	Yen & Tsai (1969)
Aphids	*Toxoptera citricida*	*B. bassiana*	Poprawski *et al.* (1999)
Diaprepes root weevil	*Diaprepes abbreviatus*	*B. bassiana*	McCoy *et al.* (2007)
Fruit flies	*Ceratitis capitata*	*B. bassiana* *M. anisopliae*	Ekesi *et al.* (2002; 2003; 2005) Dimbi *et al.* (2003a; 2003b) Castillo *et al.* (2000)
		I. fumosoroseus *B. brongniartii*	Konstantopoulou & Mazomenos (2005)

1.3.4 Genus Beauveria

Beauveria (Balsamo) Vuillemin (Ascomycota: Hypocreales) is a cosmopolitan genus of soil borne EP moulds, first discovered as a devastating disease which affected European silkworms in the 18th and 19th centuries, known as the white muscardine disease (Rehner 2005). Although the initial investigation of *Beauveria* was instigated from a need to protect domesticated insects such as silkworms and honey bees, it is also an important natural pathogen of insects, capable of infecting more than 700 species of arthropods (Lord 2005). Its hosts include many economically important pests and its wide variation in virulence towards different insect hosts makes it one of the more versatile candidates of EP fungi for the biological control of insect pests (Rehner 2005).

Other aspects which have contributed to the large amount of research focused on this fungal genus are firstly, its broad distribution in nature which makes *Beauveria* easy to recognise and has lead to it being noted as the most frequently encountered of all EP fungi (Rehner 2005). Secondly, *Beauveria* is an extremely traceable organism as it can be easily isolated from insect cadavers or from the soil using simple media, antibiotics and selective agents (Rehner 2005). Thirdly, the genus can be easily cultured in the laboratory on simple media and can be conserved by storing conidia in glycerol solutions at ultra-low temperatures or by freeze-drying (Rehner 2005). Furthermore, *Beauveria* is amenable to large-scale production and formulation as a mycoinsecticide. Despite two centuries of research and several comprehensive taxonomic studies, there are still significant problems in the identification, taxonomy and nomenclature of species in this genus and progress in elucidating the genetics and evolution of *Beauveria* is just beginning (Rehner 2005; Rehner & Buckley 2005).

1.3.4.1 Taxonomy

Vuillemin (1912) formally described the genus *Beauveria*, assigning *Botrytis bassiana* as the type species, this later became *Beauveria bassiana* in recognition of J. Beauverie in 1914, after his invaluable contribution to the study of white muscardine disease (Rehner 2005). The genus is easy to distinguish morphologically; its most distinctive features are the sympodial to whorled clusters of short-globose to flask-shaped conidiogenous cells that produce a succession of one-celled, sessile and hyaline conidia on an elongating 'zigzag' rachis (Domsch *et al.* 2007) (Fig. 1.6 A).

In culture, *Beauveria* species typically produce white coloured mycelium and conidia (Fig. 1.6 B), although some isolates may produce yellow pigment in the older, central parts of the colony (Domsch *et al.* 2007). Colony growth tends to be rapid, and the texture of the mycelium is typically lanate to woolly and synnemalike projections can occasionally be observed (Rehner 2005). *Beauveria* conidial production can often be profuse, frequently creating a chalky, mealy or powdery appearance on the colony surface (Rehner 2005). Many isolates can excrete a red pigment into the culture medium, although this does not occur under all culture conditions (Rehner 2005).

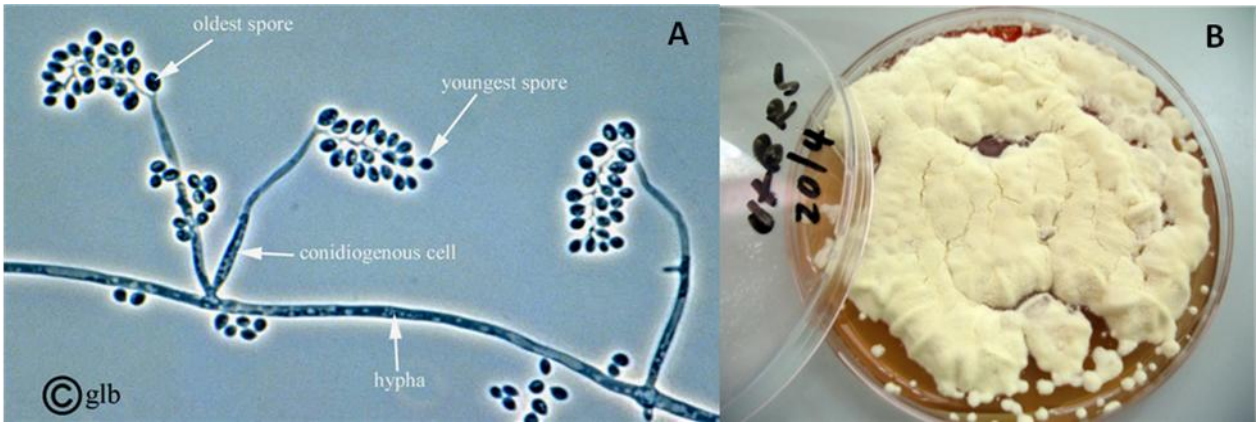

Figure 1.6 (A) A photomicrograph showing three conidiogenous cells on a hypha of *Beauveria bassiana*. The asexual conidia are produced in acropetal succession (youngest at the tip) **(B)** *B. bassiana* grown on Sauboraud dextrose agar (SDA) at 25 °C. Photo Credits: (A) George Barron, University of Guelph, Canada (B) T. Goble, Rhodes University, South Africa.

The conidial form is the only morphological feature of the genus that has proven helpful for species delineation, as conidia vary from globose, ellipsoid, cylindrical and vermiform and range in sizes from 1.8 to 6.0 µm in their greatest dimensions (Rehner 2005; Domsch *et al.* 2007). In total 49 species have been classified in the genus of which 22 are currently considered valued (Rehner, 2005). Overlapping variation in the size of conidia among many species has raised questions of the validity of routine species identifications. Further, cultural features of *Beauveria* are influenced by culture conditions and are thus unreliable as taxonomic features (Rehner 2005). Six morphological species can be discerned in the genus *Beauveria*: *B. amorpha, B. bassiana, B. brongniartii, B. caledonica, B. velata* and *B. vermiconia* (Fig. 1.7). There is however much confusion with regard to the species *B. bassiana* for which many morphologically similar species have been described; as it stands seven species are synonymous with *B. bassiana* (Rehner 2005).

Current molecular techniques based on the comparison of available internal transcribed spacer (ITS) sequences and other molecular markers among species, has shown great promise in elucidating the genetic taxonomy of this genus (Rehner & Buckley 2005; Meyling *et al.* 2009). An important inference obtained using molecular analyses was the discovery of the close relatedness between the asexual or anamorphic genus *Beauveria*, and the sexual or teleomorphic genus *Cordyceps. Beauveria bassiana s.s.* reproduces asexually by mitosporic conidia and for some time it has been presumed to be exclusively clonal (Meyling *et al.* 2009). Phylogenetic and developmental studies have linked *B. bassiana s.s.* to the Asian sexual species *Cordyceps bassiana* which provides evidence that *B. bassiana* is in fact facultatively sexual (Meyling *et al.* 2009).

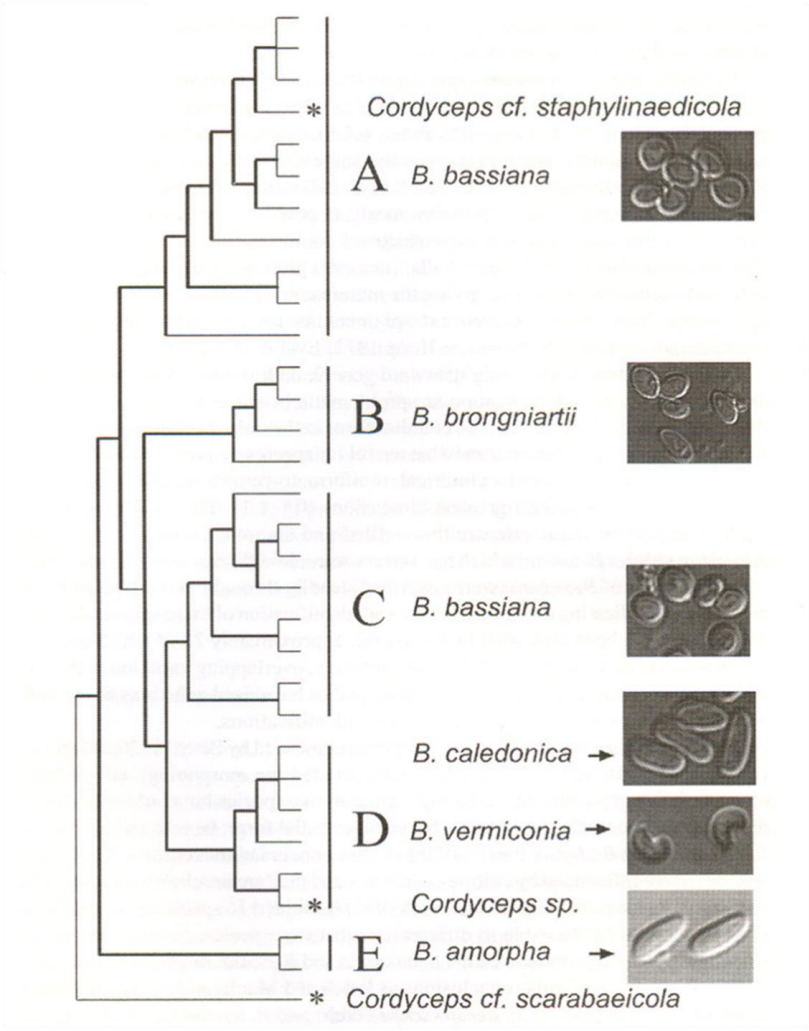

Figure 1.7 The phylogenetic relationships of the genus *Beauveria* based on several independent Bayesian and parsimony analyses. Clades A-E are labelled according to their morphological species identification and micrographs of representative conidia (Rehner 2005).

1.3.5 Genus Metarhizium

Metarhizium (Metschnikoff) (Hypocreales: Ascomycota) is a haploid cosmopolitan genus of soil borne EP moulds. Elie Metchnikoff, a Russian microbiologist, was the first person to isolate and name the green muscardine fungus, *Entomophthora anisopliae* in the 1880s (now known as *Metarhizium anisopliae*) which was found infecting the grain beetles he worked on (Lord 2005). *Metarhizium* species have a wide range of virulence and are known to infect more than 200 different insect species, many of which are major agricultural pests, such as sugarcane stem-borers, scarab grubs and grasshoppers (Bidochka & Small 2005). Further *Metarhizium* is capable of infecting insects from over 14 different orders including some non target orders such as Malacostrata (Amphipods), Acari, Ephemeroptera and Dermaptera (Zimmermann 2007a).

1.3.5.1 Taxonomy

As with *Beauveria*, the earlier classification of this genus was based on morphological characteristics, but because these characteristics are fairly limited it has complicated the taxonomy (Bidochka & Small 2005). The genus is morphologically defined on the basis of the arrangement of the phialides which bear chains and columns of green, dry and slightly ovoid conidia (Zimmermann 2007a) (Fig. 1.8 A,B). Tulloch used the morphological characteristics of the conidia to distinguish between the two forms of *M. anisopliae;* the short-spore (5-8 µm) form called *M. anisopliae* var. *anisopliae* and the long-conidial form (10-16 µm) called *M. anisopliae* var. *majus* (Zimmermann 2007a). Since then nine additional taxa have been described, although each may not be formally recognised as a separate *Metarhizium* species (Bidochka & Small 2005). *Metarhizium flavoviride* (Gams and Rozsypal) (Fig. 1.8 C, D) differs from *M. anisopliae* by having wider, greyish to yellow conidia (Zimmermann 2007a).

A population genetic analysis by St Leger *et al.* in 1992, using allozyme markers however discounted any evidence for a tight association between morphological characteristics such as conidial shape and colour (Zimmermann 2007a). DNA and other molecular markers have recognised areas of consensus concerning the taxonomy of *Metarhizium.* (1) *Metarhizium anisopliae* is recognised as a monophyletic group but that the designation of varieties or cryptic species within the group is still unclear. (2) *Metarhizium anisopliae* is related to *M. flavoviride* but can be distinguished using molecular markers. There seems to be genetically distinguishable clades or cryptic species within the *M. anisopliae* and *M. flavoviride.* (3) It is estimated that 10-15 different cryptic species comprise the genus *Metarhizium* and *M. anisopliae*; *M. flavoviride*

and *M. album* are recognised as separate species. (4) An association of *M. anisopliae* genotypes occurs with habitat type in temperate and polar regions and (5) Southeast Asia is the probable origin of the evolution and diversity of *M. anisopliae* (Bidochka & Small 2005; Zimmermann 2007a). Until 1991, there was no teleomorph of the genus *Metarhizium*, until *Cordyceps taii* was discovered which had an anamorphic state called *Metarhizium taii*, later *Cordyceps brittlebankisoides* was isolated which proved the teleomorph of *M. anisopliae* var. *majus* (Zimmermann 2007a). A taxonomic revision of *Metarhizium* is greatly needed however this requires larger data sets as well as informative nucleotide characteristics which are not available at this time (Bidochka & Small 2005).

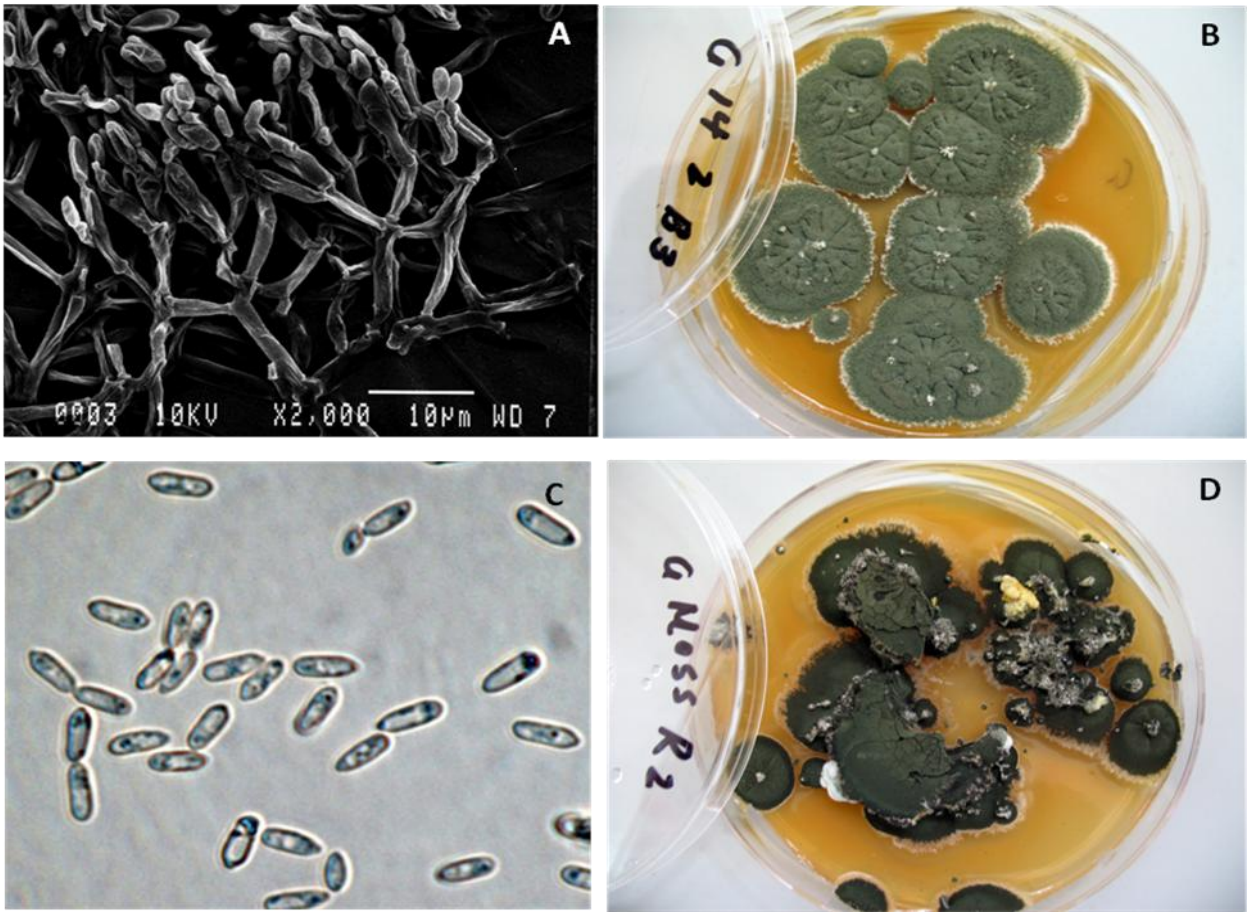

Figure 1.8 (A) A transmission electron micrograph showing conidiogenous cells on the hyphae of *Metarhizium anisopliae* **(B)** *M. anisopliae* grown on Sauboraud dextrose agar (SDA) at 25 °C **(C)** Conidia of *M. flavoviride* at 1000X under oil immersion (Zeiss Imager A1 DIC microscope with Canon Powershot **(D)** *M. flavoviride* grown on Sauboraud dextrose agar (SDA) at 25°C. Photo Credits: (A) Fumio Ihara, National Institute of Fruit Tree Science, Japan (B, D) T. Goble, Rhodes University, South Africa (C) Dan Johnson, University of Lethbridge, Canada

Several commercial endeavours have registered strains of *Metarhizium* as the active ingredient of various products for insect pest management namely: *Metarhizium* 50® (AgoBiocontrol) for garden pest control in Columbia; Biogreen® and Green Guard® in Australia for red-headed cockchafer, *Adoryphouse couloni* and locusts respectively, BIO 1020® in Germany for vine weevils, *Otiorhynchus sulcatus* and Green Muscle® (Biological Control Products, South Africa) against locusts, *Locustana pardalina* and grasshoppers (Bidochka & Small 2005). Many other registered *Metarhizium* products are currently available and a comprehensive list can be found in Faria & Wraight (2007). There are many criteria which become important in selecting a strain of *Metarhizium* for commercialisation: a high level of virulence towards the target pest, the utilisation of indigenous strains is preferable because exotic strains could be politically and ecologically problematic. Finally, the ability to distinguish and track the formulated strain against background populations of EP fungi, either through genotyping or genetic tagging becomes crucial to ascertaining the effectiveness of the applied strain (Bidochka & Small 2005).

1.3.6 Genus Conidiobolus

Conidiobolus is the only genus of the entomophthorales which is commonly isolated from the soil environment (Domsch *et al.* 2007). The vegetative mycelium consists of short hyphal bodies. Colonies grow rapidly and are flat, cream in colour becoming folded and covered by a fine powdery surface mycelium and conidiophores (Fig. 1.9 B). The colony colour becomes tan with age. The conidia are forcibly discharged by the conidiophores which are simple, forming solitary, terminal conidia which are spherical (10-25 µm in diameter), single celled and have prominent papilla (Domsch *et al.* 2007) (Fig. 1.9 A). The conidia may also produce hair-like structures called villae, and when conidia germinate they produce either (1) single or multiple hyphal tubes that may also become conidiophores or (2) produce multiple short conidiophores, each bearing a small secondary conidium (Domsch *et al.* 2007).

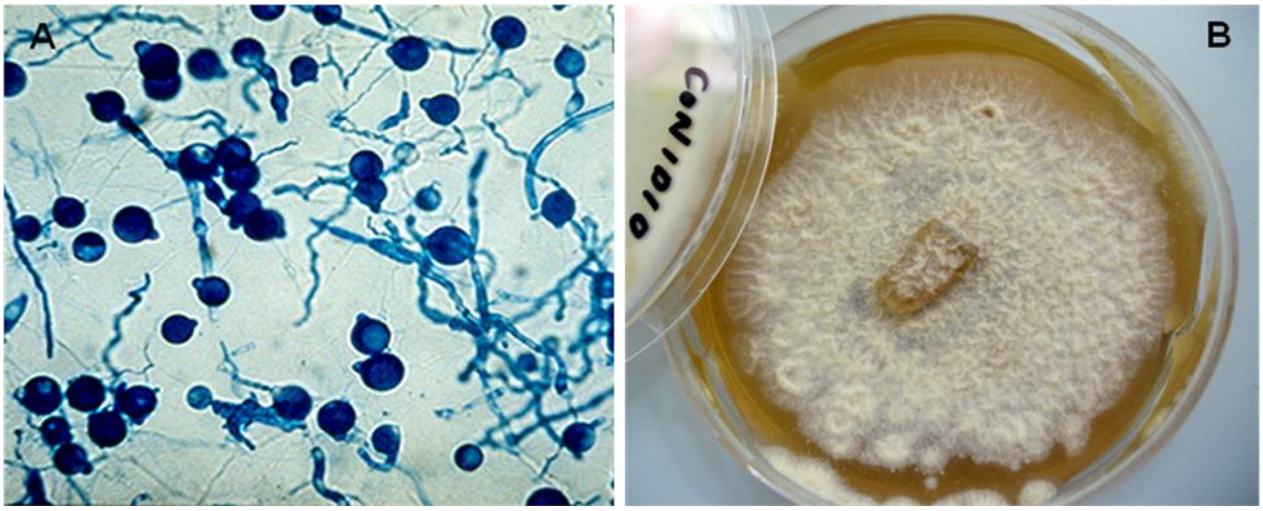

Figure 1.9 (A) A photomicrograph of *Conidiobolus coronatus* showing characteristic conidia with prominent papilla **(B)** *C. coronatus* grown on Sauboraud dextrose agar (SDA) at 25°C (photo credits: (A) Dr. K. Salfelder, Universidad de Los Andes, Venezuela (B) T. Goble, Rhodes University, South Africa

1.3.7 Factors influencing the efficacy of EP fungi

A variety of factors may influence fungal host range and the specificity of fungal pathogens, for example: the fungal strain, the host's physiological state, nutrition, defense, host mechanisms and the insect cuticle (Inglis *et al.* 2001). Further, the development of epizootics is controlled by a variety of environmental factors and is influenced by the distribution of both the host and pathogen populations (Goettel 1995). Fungal genera with broad host ranges, such as *Beauveria* and *Metarhizium* are generally facultative pathogens which are ubiquitous in the soil and are prime candidates for inundative biological control of insect pests (Goettel 1995).

1.3.7.1 The insect host

There is a complex array of morphological and physiological factors which influence the susceptibility of insect pests to EP fungi. One of the most important concepts however is that 'stressed insects', either through overcrowding, poor nutrition, exposure to chemicals or harsh environmental factors, are more susceptible to fungi than non-stressed insects (Inglis *et al.* 2001). Inadequate insect nutrition from feeding on plants which produce volatiles or alkaloids and stress the insect, can greatly enhance the efficacy of EP fungi (Inglis *et al.* 2001); conversely insects may feed on plants such as cow-pea and obtain plant-derived fungistatic compounds which greatly reduces fungal efficacy (Ekesi *et al.* 2000). Another important factor is the developmental stage of an insect pest as not all stages in an insect's life cycle are equally susceptible to fungal infection. In some cases, as with the susceptibility of immature stages of the

legume flower thrips, *Megalurothrips sjostedti*, to the EP fungus *Metarhizium anisopliae*, the adult stage was found to be more susceptible to infection than the larval and pupal stages (Ekesi & Maniania 2000). In other cases the adults of the western flower thrips, *Frankliniella occidentalis* were more susceptible to *L. lecanii* than the nymphs (Vestergaard *et al*. 1995). Differential susceptibility in insect host stage is likely further related to loss of infection through ecdysis, as the insect molts during instar development, so it may loose fungal inocula as the cuticle is removed. Insect density is also very important for the development of epizootics, as insect density increases there is a higher probability of insects coming into contact with infected individuals. The behaviour of insects can also greatly affect the persistence of fungal disease, for example sun-basking (to increase body temperature) and crowding behaviour (to elicit a behavioural fever to raise the body temperature) in locusts has been shown to slow the infection rate of *Metarhizium anisopliae* var. *acridum* (Wilson *et al.* 2002). Grooming behaviour in termites can spread the conidia of EP fungi to other individuals (Inglis *et al.* 2001), but this sanitation behaviour can also remove inoculum and limit the spread of disease and instill resistance in some social insect groups (Yanagawa *et al.* 2008, 2009).

1.3.7.2 The environment

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

Solar radiation

The effect of irradiance, particularly in epigeal habitats, is one of the most important environmental parameters for the persistence of EP fungal spores; exposure to sunlight for even a few hours, particularly the UV-B portion of the spectrum (285 - 315 nm) can completely inactivate the infectious conidia of *Metarhizium* (Braga *et al.* 2001). There are significant differences in susceptibility to irradiation among fungal strains and species, and mechanisms for resistance against irradiation should be considered when selecting candidate EP fungi for product formulation (Inglis *et al.* 2001). Microhabitats play an important role in enhancing the persistence of fungal conidia by providing shade under tree canopies for example (Inglis *et al.* 2001). The rapid inactivation of propagules by sunlight is a major hindrance to the successful commercialisation of entomopathogens and thus much research has focused on enhancing conidial persistence in the field by using solar blockers or UV-B absorbing substances

(sunscreens such as clay) and optical brighteners, such as Tinopal™ (Ciba Chemical Company, Switerland) or stilbene (Ibargutxi *et al.* 2008). Unlike the canopy layer, the soil environment is less prone to UV-B irradiance penetration and there are less dramatic fluctuations in temperature (Ansari *et al.* 2007), making this habitat the seeming ideal place for the persistence of conidia when considering commercial product application (Ekesi *et al.* 2003). However, temperature conditions in the top few centimeters of the soil layer may be well over 40°C in some instances, therefore incorporation of conidia into soil to enhance persistence will depend on climatic factors as well as shading or depth of conidia in the soil (Inglis *et al.* 2001).

Temperature

Ambient temperature influences the rate of insect infection and may restrict the use of EP fungi in biological control. Most EP fungi are mesophilic (they can grow at a range of temperatures) but grow optimally at temperatures between 25 and 35°C. Species such as *Beauveria bassiana* can grow at a wide range of temperatures (8 to 35°C) with a maximum threshold of 37°C (Fernandes *et al.* 2008). Selecting EP fungal strains which can withstand environmental temperature fluctuations is an important consideration in the development of a commercial product and often strains that come from hotter areas perform better at higher temperatures and likewise those that come from cold climates perform better at lower temperatures (Inglis *et al.* 2001). Recent evidence suggests that even when *Beauveria* strains were found in the same geographic location their variability to thermotolerance was great, indicating that microhabitat adaption is just as important as the macro-environment factor (Fernandes *et al.* 2008). However, *M. anisopliae* isolates found in agricultural soils were found to be better adapted to heat and UV-B exposure than isolates which were found in cold, forested habitats of similar latitude (Bidochka *et al.* 2001, 2002; Fernandes *et al.* 2008). There is general agreement that fungal isolates from higher latitudes are more cold-tolerant than isolates from nearer the equator (Rangel *et al.* 2005). Behaviour such as basking and fevers which increase host temperature may also influence the rate and severity of fungal infection and thus selecting commercial fungal strains which elicit better infection at higher temperatures should be considered.

Relative humidity

Environmental moisture is a very important parameter for the persistence and germination of conidia in nature. Conidiogenesis on insect surfaces requires high moisture (Inglis *et al.* 2001), if

this factor is low, conidia production slows and the horizontal transmission of spores is reduced, slowing the transfer of infection. A number of studies have indicated that dry conditions immediately after the application of EP fungi are less injurious than previously thought as *Beauveria* and *Metarhizium* strains were able to germinate and effectively infect insects at low ambient humidity, provided that there was sufficient moisture within the microhabitats (Inglis *et al.* 2001). The requirement for high environmental moisture was lessened with the breakthrough technology of formulating fungal spores in oil (Bateman & Alves 2000). The conidia remain active inside the oil droplets and research now concludes that oil enhances the insect infectivity at low humidity levels (Inglis *et al.* 2001).

Rainfall

Precipitation can play a vital role in the dispersal of conidia from substrates, but there is strong evidence to suggest that significant amounts of fungal conidia may be 'washed off' the insect cuticle during rainfall despite the lipophilic mechanisms of conidial adhesion (Inglis *et al.* 2000). Despite these evidences, confounding factors like canopy density and architecture as well as insect behaviour on the persistence of fungal conidia in these environments, remains to be resolved (Inglis *et al.* 2001). Research on the use of stickers in the persistence of fungal conidia has been limited but their use is cautioned because they may retard the transfer of conidia from plant leaves to insects in a natural environment (Inglis *et al.* 2000).

Soil factors

Many EP fungi are soil-borne microorganisms and this environment represents a complex set of biotic and abiotic factors which can affect the efficacy and persistence of fungi. These abiotic factors include: soil type (texture, cation exchange capacities, organic matter content, pH etc), moisture and the occurrence of other soil microflora (Ali-Shtayeh *et al.* 2002; Ekesi *et al.* 2003; Jaronski 2007; Quesada-Moraga *et al.* 2007). Soil moisture has been shown to adversely affect the persistence of *B. bassiana*, *M. anisopliae* and *L. lecanii* because vertical movement of conidia in the soil is affected and loss of inoculum occurs (Inglis *et al.* 2001). Soil texture and organic matter appear to be the most important factors governing the vertical movement of fungi with sandy-textured soils low in organic matter retaining fewer propagules than clay-textured and organic soils (Jaronski 2007). Mietkiewski *et al.* (1997) observed that the isolation of *B. bassiana* from arable soils in the UK was usually associated with natural undisturbed habitats

which were higher in organic matter and Quesada-Moraga *et al.* (2007) suggested that soils with greater organic matter content have higher cation exchange capacities with greater organic matter enhancing conidia absorption. Quesada-Moraga *et al.* (2007) found that *M. anisopliae* predominated in soils with a pH lower than 7 and was better adapted to slightly more acidic soils than *B. bassiana*. This said however, the effects of pH seem to be minimal (Inglis *et al.* 2001).

Management practices such as tillage may also affect the presence of EP fungi substantially, because during these processes conidia may be moved to the surface of the soil, where they become exposed to UV-B radiation and higher temperatures (Jaronski 2007; Meyling & Eilenberg 2007). The effects of biotic factors, such as other soil microorganisms, may have an effect on the persistence of EP fungal species. Meyling & Eilenberg (2006b) found that organic matter content and subsequent biological activity in the soil adversely affected the persistence of this fungal species due to antagonistic effects. Mainly because both *Beauveria* and *Metarhizium* are poor competitors for organic resources compared to opportunistic saprophytic fungi that are ubiquitous in the soil (Meyling & Eilenberg 2006b). Understanding the ecology of EP fungi in the environment, particularly the soil, is an important prerequisite to applying these fungi correctly in the field. Meyling & Eilenberg (2007) provide a schematic diagram which looks at the ecology of both *Beauveria* and *Metarhizium* in the soil and how these species are dispersed and cycle in the environment. (Fig. 1.10).

1.3.8 Compatibility of fungi with agrochemicals

It is important to realize that EP fungi will not displace chemical pesticides in all commercial crop production systems, often because livelihoods may be at stake and chemical control may be needed to hurriedly suppress a rapidly expanding pest population or simply because EP fungi may not target all insect pests (Inglis *et al.* 2001). Much of the research on EP fungi has focused on the influence and compatibility of agrochemicals and the germination of EP fungi *in vitro* and their inhibitory properties which vary within and among chemical classes. Agrochemical insecticides, herbicides and fungicides are often applied to conventional agricultural systems and can either have a significantly deleterious effect (Poprawski & Majchrowicz 1995) on the occurrence of EP fungi or work synergistically with fungal biological control agents (Purwar & Sachan 2006).

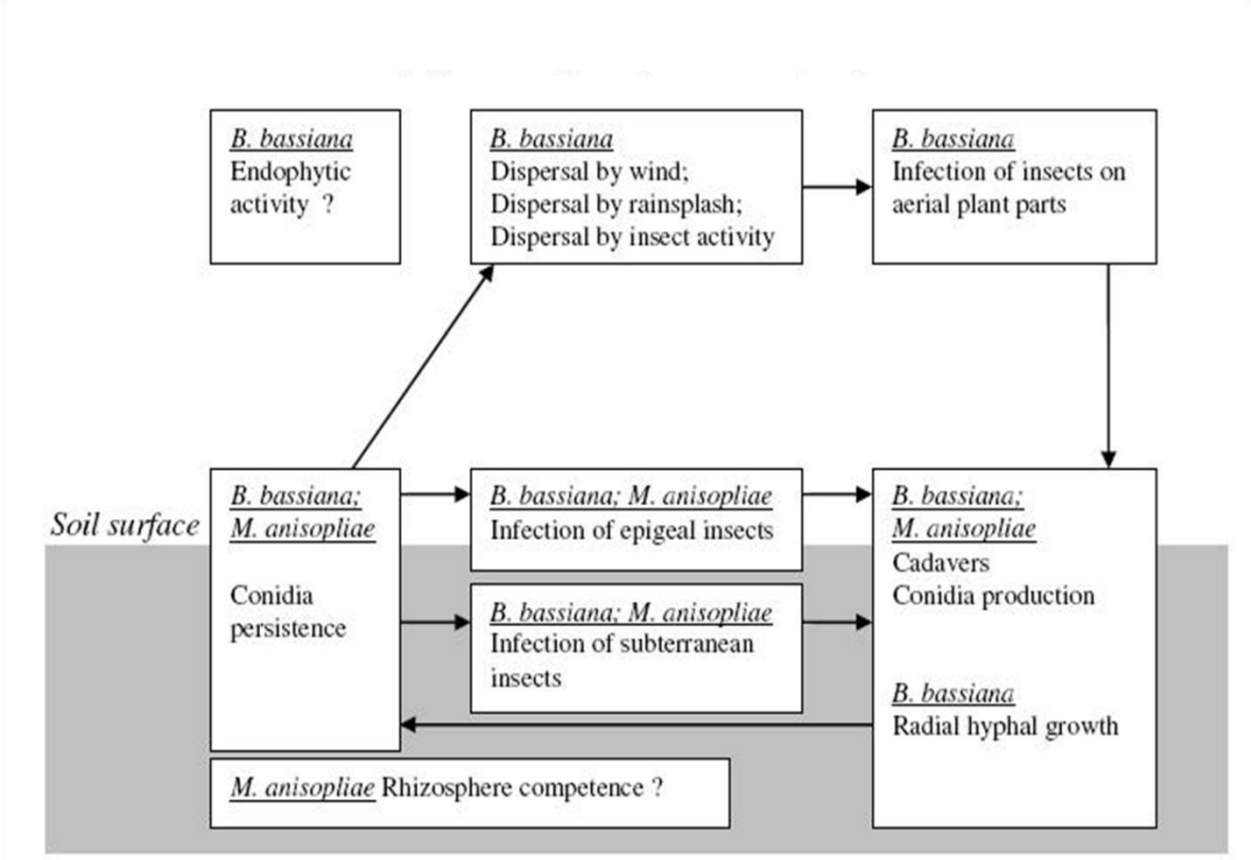

Figure 1.10 A schematic diagram summarizing the compartments in temperate terrestrial ecosystems in which *B. bassiana* and *M. anisopliae* occur naturally. Compartments are connected with lines to indicate dispersal and infection pathways. Plant associations are included as potential compartments for the natural occurrence of *B. bassiana* and *M. anisopliae* (Meyling & Eilenberg 2007).

EP fungi can be adversely affected by chemical pesticides in several ways. In addition to direct toxic effects on the fungi themselves, chemicals may have an indirect effect of killing the potential fungal hosts (Jaros-Su *et al.* 1999). In general, it appears that germinating EP fungal spores are more sensitive to pesticides than the fungal mycelia (Sterk *et al.* 2002). Agrochemical pesticides can inhibit vegetative growth, conidiogenesis, fungal sporulation and cause genetic mutations which can result in low conidial viability and virulence towards insect pests (Mochi *et al.* 2006).

Imidacloprid, the active ingredient of Confidor® (Bayer CropScience, Germany), is a neonicotinoid (neuro-active insecticide modeled after nicotine) which is often used in citrus to control whiteflies and scale insects. James & Elzen (2001) investigated the dual use of this insecticide with *B. bassiana* and found antagonism in that *B. bassiana* inhibited the effectiveness

of imidacloprid. The authors hypothesize that *B. bassiana* caused a behavioural response that reduced insect feeding and uptake of imidacloprid. *Beauveria bassiana* spore germination and colony formation were not inhibited by this chemical but *B. bassiana* did not adsorb or degrade imidacloprid in a tank mix either. However, McCoy *et al.* (2007) found that *B. bassiana*, the active ingredient of the commercial product, Mycotrol® (BioWorks, New York), formulated in oil or a combination of fungus and a sub-lethal dose of imidacloprid were efficacious against the neonate larvae and adults of root feeding weevils, *Diaprepes* species, when applied as a soil barrier in citrus.

Insecticide treatments against citrus rust mite, *Phyllocoptruta oleivora* (Ashmead), on navel oranges have also been shown to significantly reduce the incidence of *Hirsutella thompsonii*, while methidathion and chlorobenzilate were previously shown to be moderately toxic to other EP fungi (Puterka 1999). The fungicide, azoxystrobin, is commonly used to control citrus black spot in orchards; it possesses the broadest spectrum of activity of all presently known antifungals (Anonymous 2001). Sterk *et al.* (2002) looked at the toxicity of this compound against four beneficial fungal species namely: *I. fumosoroseus, L. lecanii, B. bassiana* and *T. harzianum* and found azoxystrobin to be less toxic than expected. Azoxystrobin also seemed to be compatible with these beneficial fungi when there were gaps of a few days between applications of EP fungi and fungicides (Sterk *et al.* 2002). Majchrowicz & Poprawski (1993) examined the *in vitro* effects of fungicides on EP fungi and concluded that the susceptibility of fungi to fungicides varied widely depending on the chemicals and species of fungus. They found that dithiocarbamate derivatives zineb and copper oxychloride together or mancozeb alone completely inhibited germination of the fungi tested, including *B. bassiana.* The inhibitory effects were directly proportional to the dose of the active ingredient. Mochi *et al.* (2006) investigated the effects of various pesticides in the soil which could affect the pathogenicity of the EP fungus *M. anisopliae* which was used against *Ceratitis capitata* in orchard systems. Two fungicides based on the chemicals: chlorothalonyl and tebuconazol, an acaricide based on abamectin, an insecticide based on trichlorfon and a herbicide based on ametrin were incorporated into soil at the manufacturers recommended doses. The pesticides had negligible influences on the action of *M. anisopliae* but the fungicides effected fungal conidia during the pupal stage and decreased the pathogenicity of the fungus against *C. capitata* (Mochi *et al.* 2006). In South African citrus orchards, the use of the insecticide chlorpyrifos is often employed to control African bollworm (*Helicoverpa armigera*), however the fungicidal effects of this insecticide have been noted by de Oliveira *et al.* (2003), who found that *B. bassiana* germination

was 100% inhibited by the insecticide when compatibility tests were carried out in Brazilian coffee plantations.

1.3.9 Compatibility with natural enemies and other biological control agents

The contribution that predators, parasitoids and indigenous pathogens provide in cropping systems is crucial for the suppression of many insect pest populations and is an important aspect to consider in IPM strategies. Reports on the natural prevalence of EP fungi and particularly the hyphomycetes have shown that these fungi occur widely in soils and on insects in the aerial environment illustrating the long evolutionary history of coexistence which EP fungi share with other microorganisms (Zimmermann 2007b). Some hyphomycetes have antagonistic properties against other microorganisms and similarly other microorganisms may have a suppressive effect on EP fungi which may affect their efficacy in the field (Inglis *et al.* 2001). The main concern in terms of inundatively applying mycoinsecticides to systems is their potential to displace natural, non-target populations of microorganisms and similarly these biological control agents have to persist in the soil themselves and should not be affected by other microbes. *Metarhizium anisopliae* and *B. bassiana* have been shown to suppress phytopathogenic fungi such as *Ophistoma ulmi*, the causal agent of Dutch Elm disease (Zimmermann 2007a). Further *B. bassiana* has been shown to have antagonistic effects and reduces the disease incidence of *Phoma betae*, a common soil fungus which attacks weak plants and is the causal agent of blackleg of beet. *Beauveria bassiana* was also able to reduce *Rhizotonia solani* (damping-off of tomato) in greenhouses and protect cotton against a seedling disease complex (Zimmermann 2007b). The compatibility of *M. anisopliae* and other EP fungi with the mycoparasites, *Gliocladium* (now *Clonostachys*), *Trichoderma harzianum* and *Lecanicillium lecanii*, have recently been investigated and it was shown that *M. anisopliae* and *B. bassiana* were highly susceptible (suppressed growth of these EP fungi) to all mycoparasites tested, but that co-application of mycoparasites and EP fungi did not affect their biological control efficacy *in vivo* (Zimmermann 2007a). The inundative application of strains of *B. bassiana* against the masson pine moth, *Dendrolimus punctatus*, in China showed that during the course of a year, indigenous and exotic strains were reisolated, and it was found that indigenous strains continued to persist and were not displaced by the exotic *B. bassiana* strains (Zimmermann 2007b). In studying the vegetative compatibility of indigenous and mass-released strains of *B. bassiana* in North America, it was suggested that the likelihood of genetic exchange between these strain types was

very low due to a large number of vegetative compatibility groups which had self/non-self recognition systems (Castrillo *et al.* 2004).

It is surprising that very little attention has been given to the co-application of EP fungi and other entomopathogens such as viruses, nematodes and bacteria, as the perceived enhancement of biological control efficacy seems promising, particularly because these microbial organisms have different modes of action, which would reduce inter-species antagonisms. In a study by Rao *et al.* (2006) the co-application of two EP fungi, *B. bassiana* and *N. rileyi*, were assessed against the second instar larvae of *Spodoptera litura* and no synergistic effects on insect mortality were found. In other studies synergistic effects were observed when *M. anisopliae* and *Serratia entomophila* (an *Enterobacteriaceae* agent which causes amber disease in grass grubs) were co-applied against the early instar grass grub, *Costelytra zealandica* (Inglis *et al.* 2001). Inglis *et al.* (1997) demonstrated the effective use of 'fungal cocktails' when they tested the hypothesis that co-applied *B. bassiana* and *M. flavoviride* could be used to increase the temperature ranges over which the fungi on their own would elicit an infection. In the hot temperature environment, less mortality was observed for *B. bassiana* (3%) than for *M. flavoviride* (52%). Conversely, in the cool temperature environment, less mortality was observed for *M. flavoviride* (46%) than for *B. bassiana* (100%). Co-application induced greater mortality than if *M. flavoviride* was applied alone in a simulated hot-temperature environment or *B. bassiana* alone in a simulated cool-temperature.

The safety of EP fungi towards non-target arthropods, earthworms and pollinators is also a very important consideration for the use of mycoinsecticides because of the broad host range of some members, such as *B. bassiana*, which is capable of infecting over 700 different insect species across 14 insect and amphipod orders (Rehner 2005; Zimmermann 2007b). At this stage it is important to note that there are differences in the physiological and ecological host ranges where pathogens are concerned. The physiological host range demonstrates which organisms can be infected in the laboratory, which is often much broader because insects may become stressed and larger concentrations of conidia are used (Zimmermann 2007a). While the ecological host range demonstrates the organisms which may be infected in nature, which is often narrower because pathogens may not occupy the same ecological niches as some organisms or infect all organisms in the environment (Zimmermann 2007a). The capability of *B. bassiana* to infect non-target organisms such as collembolans, carabid beetle predators and coccinellids has been demonstrated under stress conditions however in these studies, earthworms and honeybees were not affected

(Zimmermann 2007b). *Metarhizium anisopliae* has been shown to infect a wide range of insects in laboratory experiments and some non-target effects have been observed in the field, however lower non-target effects were seen in the field (Zimmermann 2007a). This EP fungus showed pathogenicity towards two natural enemies in laboratory experiments: the green lace wing, *Chrysoperla carnea* and a mirid predator, *Dicyphus tamaninii*. The mirid predator showed greater susceptibility than *C. carnea* with corrected mortalities of 10 and 4%, respectively (Thungrabeab & Tongma 2007). The literature concludes that EP fungi have minimal impact on non-target organisms, especially when isolate selection and spacio-temporal factors are considered, and are generally safe and compatible with a number of beneficial insects (Inglis *et al.* 2001; Hajek & Goettel 2007; Thungrabeab & Tongma 2007; Zimmermann 2007a, b).

1.3.10 Safety of EP fungi towards vertebrates

The registration requirements of a new mycoinsecticide product are such that detailed vertebrate safety tests have to be included; all safety tests using EP fungi such as *Beauveria, Metarhizium* and *Paecilomyces* carried out against fish, amphibians, reptiles, birds and small mammals revealed that no serious detrimental effects were observed in these groups (Zimmermann 2007a, b). Allergies in humans are rare but have been recorded and may be as a result of extended exposure to EP fungi, particularly in production facilities (Zimmermann 2007b). Some orders in the class Zygomycete, which includes the EP fungi in the order Entomophthorales, are pathogenic to humans. Pathogens such as *Conidiobolus coronatus* cause various forms of zygomycosis, which causes serious nasal infection and is considered a possible cause of rhino facial swelling in immigrants from equatorial Africa, Asia, and America (Fischer 2008).

1.4 AIMS OF THE STUDY

Based on the literature there appears to be great interest in the development of mycopathogens for IPM in cropping systems because the natural incidence of these microorganisms in agricultural environments has been shown to significantly suppress pest populations. By inundatively applying conidia of EP fungi to the soil in agricultural systems for the control of pests with subterranean life stages, citrus growers and other IPM cropping systems are offered another tool for insect control which is environmentally friendly and safe. A greater understanding of the ecology of EP fungi in nature and in cropping systems will enhance our

knowledge of environmental persistence and aid in strain selection which would lead to the enhancement of biological control efficacy. Thus, the aims of this study were (1) to recover and identify indigenous isolates of EP fungi from soil samples taken from organically and conventionally managed citrus orchards and (2) to compare the recovery of fungal isolates and species from the bait insects, *Galleria mellonella* (Lepidoptera: Pyralidae), *T. leucotreta* and *C. capitata.* This study also served to (3) compare both the distribution and abundance of EP fungi in conventionally versus organically farmed citrus soils, and (4) to compare cultivated orchards and orchard margin (refugia) areas in the Sunday's River Valley citrus growing region in the Eastern Cape Province in South Africa.

Further aims were (5) to compare the pathogenicity of 21 obtained indigenous isolates of EP fungi against final instar larvae of *T. leucotreta, C. capitata* and *C. rosa* using sand-conidial suspension incorporation bioassays. Screening trials were undertaken to determine the most virulent fungal isolates, which had broader host ranges and were capable of infecting all three insect test species. Further aims were (6) to test the four most virulent isolates with a commercially produced *Beauveria* product, Bb Plus® (Biological Control Products, South Africa), against the three citrus pests using various concentrations of fungal conidia incorporated into sand to ascertain the LC_{50} and LC_{90} values of each isolate and (7) to test the three most virulent fungal isolates which were highly pathogenic to *T. leucotreta* in concentration-response bioassays against the final instar larvae of *T. leucotreta* using four different exposure times, 12, 48, 72 and 96 hrs and two conidial concentrations.

II

SURVEYING FOR INDIGENOUS ISOLATES OF ENTOMOPATHOGENIC FUNGI

2.1 INTRODUCTION

Soil-inhabiting entomopathogenic (EP) fungi are among the many natural enemies of arthropod pests in natural and agricultural systems; numerous fungal genera have cosmopolitan distributions and have been recognised as playing a key role in pest regulation and suppression (Ali-Shtayeh *et al.* 2002; Meyling & Eilenberg 2007). Studies on the natural occurrence and distribution of EP fungi have been conducted in Canada (Bidochka *et al.* 1998), China (Bing-Da & Xing-Zhong 2008), Denmark (Meyling & Eilenberg 2006b), Mauritius (Sookar *et al.* 2008), Norway (Klingen *et al.* 2002), Pacific Northwest of the USA (Bruck 2004), Palestine (Ali-Shtayeh *et al.* 2002), Spain (Asensio *et al.* 2003; Quesada-Moraga *et al.* 2007), Switzerland (Keller *et al.* 2003; Rodrigues *et al.* 2005) and the United Kingdom (Chandler *et al.* 1997).

An enhanced understanding of the ecology of indigenous populations of EP fungi within cropping systems and their surrounds is an important prerequisite for the evaluation of their contributions to pest control (Meyling & Eilenberg 2007). It is also important to understand the impact of agricultural practices and management strategies on naturally occurring EP fungal populations. Many economically important ascomycete fungi, such as *Beauveria bassiana* and *Metarhizium anisopliae* are facultative organisms capable of both saprophytic growth and survival in the soil environment, and show pathogenicity towards insect hosts which they colonise (Bidochka *et al.* 2001; Meyling & Eilenberg 2007). Understanding the nature of these interactions could facilitate more effective exploitation of fungi for biological pest control strategies (Vega *et al.* 2009).

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Studies on *M. anisopliae* revealed that this fungal species had rhizosphere competence (the ability of a micro-organism to colonize and become established in the narrow zone of soil which is subject to the influence of living plant roots) (St Leger 2008). *Beauveria bassiana* has been included in the spectrum of fungi with endophytic activity; studies suggest that the presence of this fungus in vascular plant tissues is an adaptive protection against herbivorous insects and its natural occurrence has been found within corn, cocoa, poppy, coffee and tomato (Meyling & Eilenberg 2007). This facultative ability has enormous potential for biological control; if insect hosts become scarce in a particular agricultural habitat, these EP fungi can adapt and persist in the environment until hosts become available again. Furthermore, EP fungi play additional roles in nature including being antagonists of plant pathogens (Goettel *et al.* 2008) and plant growth promoters (Vega *et al.* 2009).

There is a present paradigm that assumes that the genotype of an EP fungus coevolves with a particular insect host taxon which it infects (Bidochka *et al.* 2002). Both *Beauveria bassiana* and *Metarhizium anisopliae* are capable of infecting hundreds of different insect species (Bidochka & Small 2005; Rehner 2005) and both, are considered facultative pathogens (Meyling & Eilenberg 2007). A recent study in Canada analysed various genetic populations of *B. bassiana* taken from forested, agricultural and Canadian Arctic habitats. Three distinct genetic groups of *B. bassiana* were observed which were distinctly correlated with these habitats. Further, within each group, recombining population structures as well as clonally reproducing lineages were noted (Bidochka *et al.* 2002). *Beauveria bassiana* genotypes isolated from forested and Arctic habitats exhibited cold tolerances and were able to grow at low temperatures (8°C), while those genotypes isolated from agricultural habitats were able to grow at 37°C and exhibited UV tolerances (Bidochka *et al.* 2002). Interestingly, there were no clear associations between genetic groups of *B. bassiana* and the ability to infect different lepidopteran and coleopteran insect hosts (Bidochka *et al.* 2002). This was further observed in a population genetic study with the EP fungal species, *M. anisopliae* sampled from forested (showed cold-active growth) and agricultural habitats (grew at higher temperatures and had UV tolerance) (Bidochka *et al.* 2001). Two distinct, reproductively isolated, genetic groups were observed which were strongly associated with different habitats yet no associations with insect hosts were found (Bidochka *et al.* 2001). These genetic studies suggest that *M. anisopliae* may be an assemblage of cryptic species (reproductively isolated species with similar/identical morphology) which may each be adapted to various environmental conditions. The elucidation of fungal genetics will continue to

increase our understanding of the taxonomy of these microbes as well as the important ecological functions they perform.

One method of determining EP fungal population densities and occurrences includes isolating fungi on selective media and quantifying the number of colony forming units (CFU) per gram of soil by diluting the soil sample (Keller *et al.* 2003). Most EP fungi and a range of bacteria can grow on artificial media *in vitro*, and specific media have been developed to select for certain groups of microorganisms (Veen & Ferron 1966). The development of selective media for the isolation of EP fungi includes the inhibition of bacteria by the application of broad-spectrum antibiotics such as chloramphenicol, tetracycline or streptomycin. Hypocrealean fungi grow relatively slowly in comparison to the ubiquitous opportunistic saprophytic fungi found in the soil environment. Thus, the contents of the media need to include substances that prevent these fungi from out-competing and overgrowing the species of interest (Meyling 2007). The disadvantages of using soil suspensions on selective media include overgrowth of opportunistic soil fungi on media plates, and often small soil samples are used (1 g), therefore the risk of not sampling the fungus is increased because EP fungi have a clumped distribution in the soil (Meyling 2007). Furthermore, the use of the soil dilution plating method is convoluted because of the preparation of soil subsamples, the determination of water content, dilution and plating of suspensions, as well as the initial production of media (Jaronski 2007). Often lower numbers of samples are included which limits the sample area and the true reflection of occurrence of fungi.

Presently most studies of EP fungal biodiversity in natural and agricultural systems are conducted with the use of standard bait insects, such as *Galleria mellonella* (Lepidoptera) or *Tenebrio molitor* (Coleoptera) (Klingen *et al.* 2002; Meyling *et al.* 2009). The method was originally developed by Zimmermann (1986) to isolate EP nematodes from the soil, but fungi were also isolated. Insect baiting was suggested to be a more sensitive method than traditional soil suspensions plated on selective media and could be employed as a standard technique for obtaining EP fungi from soil environments (Meyling 2007). Standard bait insects are easily obtained and cultured on artificial diet, and are also susceptible to fungal infection (Chandler *et al.* 1997). Often a large number of fungal isolates are obtained using these bait insects, but fungal species diversity is usually low (Klingen *et al.* 2002), and it has been suggested that lepidopteran or colepteran-specific isolates may be obtained whilst employing the standard bait insects (Chandler *et al.* 1997). Some perceived disadvantages to employing this technique are: a) that some insect bait species may select for specific fungal pathogens and thus may not entirely be

representative of all the potential fungal species present in the soil environment; b) baiting may be regarded as a semi-quantitative measure at best (Jaronski 2007); c) moist soils may enhance the infection of nematodes which can also utilise the bait insects; and d) it may be difficult to quantify inoculum levels (Meyling 2007).

If soils baited with *G. mellonella* are incubated at specific temperatures, it may enhance or decrease selection of specific fungal species present in the sample pot, for example Jaronski (2007) showed that when forest soil samples were incubated at 18°C, *B. bassiana, Isaria farinosus* and *I. fumosoroseus* were observed. However, when baiting was done at 25°C, *M. anisopliae* predominated while *I. farinosus* was greatly reduced. Baiting soil samples with larvae of *G. mellonella* is a widely applied screening tool for the determination of the distribution and occurrence of indigenous species of EP fungi (Ali-Shtayeh *et al.* 2002; Klingen *et al.* 2002; Asensio *et al.* 2003; Keller *et al.* 2003; Rodrigues *et al.* 2005; Sookar *et al.* 2008; Meyling *et al.* 2009). It is unlikely however that any single protocol will be an adequate method of estimating the total diversity of EP fungi in soil environment because isolation techniques are selective in one way or another (Klingen *et al.* 2002). Therefore Klingen *et al.* (2002) suggested that if a more representative range of EP fungi is to be sampled, it may be necessary to use a selection of insect species or bait methods with complimentary susceptibilities to fungal pathogens.

Biodiversity is usually evaluated by the assessment of species diversity, however the assessment of EP fungal populations and communities has been difficult because fungal taxonomy has been limited and fungal taxa usually comprise cryptic species (Meyling & Eilenberg 2007). Meyling *et al.* (2009) inferred species diversity and reproductive potential using molecular techniques on *Beauveria* within a single arable field (~ 28 ha in size) and bordering hedgerow in Denmark. Seven phylogenetic species were discovered including five phylogenetic species within *B. bassiana senso stricto.* Mating-type polymerase chain reaction (PCR) assays showed that all five *B. bassiana s.s.* species possessed bipolar out-crossing mating systems. These analyses demonstrated the genetic diversity of *B. bassiana* within a relatively limited geographic area (Meyling *et al.* 2009) and the greatest genetic diversity was observed in the semi-natural habitat of the hedgerow (~ 0.35 ha in size) compared to the arable field. The simultaneous occurrence of phylogenetically similar members of *B. bassiana* from populations below and above ground suggests that EP fungi may cycle between the soil environment and the phylloplanes of vegetation and insect hosts in natural habitats (Meyling & Eilenberg 2007; Meyling *et al.* 2009). These authors concluded that natural habitats offered structural diversity to groups of organisms

(fungi and their insect hosts) and were important reservoirs for fungal species diversity compared to the cultivated fields. Evidence suggests that maintaining landscape heterogeneity in agricultural habitats is crucial for the maintenance of fungal species diversity which allows fungal environmental adaptation, which in turn contributes to pest management in sustainable agriculture (Meyling & Eilenberg 2007).

The effects of agricultural practices can have an important impact on the distribution and occurrence of EP fungi. Annually cropped vegetable systems for example are highly disturbed environments due to tillage regimes and the fungal communities in arable fields are different to those communities of less disturbed habitats (Meyling & Eilenberg 2007). Meyling & Eilenberg (2007) showed that systems with no-till or strip-till versus those that were ploughed or disked had higher *B. bassiana* and *M. anisopliae* densities. Tillage practices turn the soil over and expose EP fungal conidia to UV-B and temperature increases, both of which inactivate fungal germination (Braga *et al.* 2001). Temperature, moisture and UV radiation appear to be the most important factors concerning EP fungal survival in the soil (Meyling & Eilenberg 2007) (cf Chapter I). In citrus orchards these environmental factors are considerably reduced depending on tree age and canopy size which provide shade and reduces temperature and UV-B radiation compared to annual vegetable cropping systems (McCoy *et al.* 2007). Furthermore, citrus orchards are perennial habitats and may support larger, more stable microbial communities than annual vegetable cropping systems. The use of irrigation systems and the need to frequently water citrus, offers continuous soil moisture for soil inhabiting EP fungi (Ali-Shtayeh *et al.* 2002). Reducing areas of bare ground through the use of mulches may reduce the sizes of pest populations because these areas then become favourable for ground-dwelling predators (Meyling & Eilenberg 2007). Mulching however may be unfavourable for EP fungi because increased amounts of organic matter in the soil have been shown to increase the antagonistic effects of opportunistic saprophytic fungi and other microbes against EP fungi, which have a slower grow-rate and are poorer competitors for organic resources (Meyling & Eilenberg 2007).

The development of virulent EP fungal biopesticides against insect pests has been the sole focus of much of the literature, which has reduced our ability to improve biological control efficacy and persistence in the field (Wang *et al.* 2005), but these microbial agents have the potential to play multiple roles in crop protection against plant pathogens and insects (Vega *et al.* 2009). These findings indicate that the ecological role of EP fungi in the environment is not yet fully understood and limits our ability to employ them successfully for pest management. Thus, the

continuation of exploratory collection studies allows information about the natural history of these microbes to be explored, particularly in a context outside of their insect hosts. Ecological data can aid in isolate selection and the development of application strategies for the implementation of EP fungi (Castrillo *et al.* 2005). It further ensures that microbial control efforts are not limited to currently available mycoinsecticides and their inundative releases. Meyling & Eilenberg (2007) discuss the concept of conservation biological control (CBC): 'the manipulation of farming management practices to enhance the living conditions of specific natural enemies of a pest, with the primary focus being suppression of that pest species.' This concept serves to conserve any natural enemies/microbial populations already present, rather than inundating an area with maladapted, non-indigenous strains or species, reiterating the need for ecological collection studies of EP fungi.

Habitat influences the distribution and abundance of insect species and it also drives the distribution of EP fungi; any indication of insect/host related population structure within EP fungi should be seen principally as the result of the habitat in which the insects and fungi co-occur (Bidochka *et al.* 2001) and not as a result of the occurrence of insect hosts alone. Increasing evidence suggests that habitat selection, and not insect host selection, drives the population structure of EP fungi and that adaption to a specific habitat type is a more important selection criterion for identifying potential biological control candidates (Bidochka *et al.* 2002). While it may be important to obtain highly infective and virulent strains of EP fungi to control pests, it is even more important to look for isolates which are also capable of surviving and persisting in the environments and habitats for which they are required. For the abovementioned reasons it was thought pertinent to investigate the distribution and occurrence of indigenous isolates of EP fungi from *citrus* growing regions and their surrounds, for the subsequent control of *citrus* pests.

Thus, the aims of this chapter were (1) to recover and identify indigenous isolates of EP fungi from soil samples, so as to assess the diversity and occurrence of fungi from citrus orchards and natural habitats; and (2) to compare the recovery of fungal isolates and species from the bait insects, *Galleria mellonella* (Lepidoptera: Pyralidae), *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) and *Ceratitis capitata* (Diptera: Tephritidae). The purpose being that by baiting with these key citrus pests, one would obtain fungal isolates which may be more virulent towards *T. leucotreta* and *C. capitata.* This study also served to (3) compare both the distribution and abundance of EP fungi in conventionally versus organically farmed citrus soils; and (4) compare

cultivated orchards versus natural (refugia) areas in the Sunday's River Valley citrus growing region and surrounding areas in the Eastern Cape Province in South Africa.

2.2 METHODS & MATERIALS

2.2.1 Insect Cultures

Thaumatotibia leucotreta larvae were obtained as final instar larvae from a continuous laboratory culture held at River Bioscience, Addo, South Africa. *Ceratitis capitata* larvae were obtained from a continuous laboratory culture held at Citrus Research International (CRI), Nelspruit, South Africa, and maintained on artificial diet until final instar larvae were needed. Larvae of the greater wax moth, *Galleria mellonella*, are often used as a standard bait insect for the isolation of EP fungi, because the larvae are susceptible to infection by these fungi (Chandler *et al.* 1997; Meyling 2007). *Galleria mellonella* larvae were obtained from absconded bee hives held at Grahamstown (33°23′54″S; 26°25′41″E), cultured on honeybee wax (Meyling 2007) and maintained in constant darkness at 24°C.

2.2.2 Soil sampling

A total of 288 soil samples were collected from three conventional citrus farms and three organic citrus farms (as defined by the EU Council Regulation 2092/91) in the Eastern Cape Province, South Africa (Fig. 2.2.1). The locations of the sampled soils were recorded using global positioning system (GPS) equipment (Garmin: E-Trex). Conventional citrus sites included, Arundel Farm near Addo (33°30′57″S; 25°39′11″E), Mosslands Farm near Grahamstown (33°23′54″S; 26°25′41″E) and J & B citrus near Cookhouse (32°45′56″S; 25°45′46″E). The organic sites included: Rosedale Farm near Addo (33°32′21″S; 25°41′39″E); Hippo Pools Farm near Kirkwood (33°24′42″S; 25°24′34″E) and Olifantskop near Addo (33°37′14″S; 25°40′49″E) (Fig. 2.2.1).

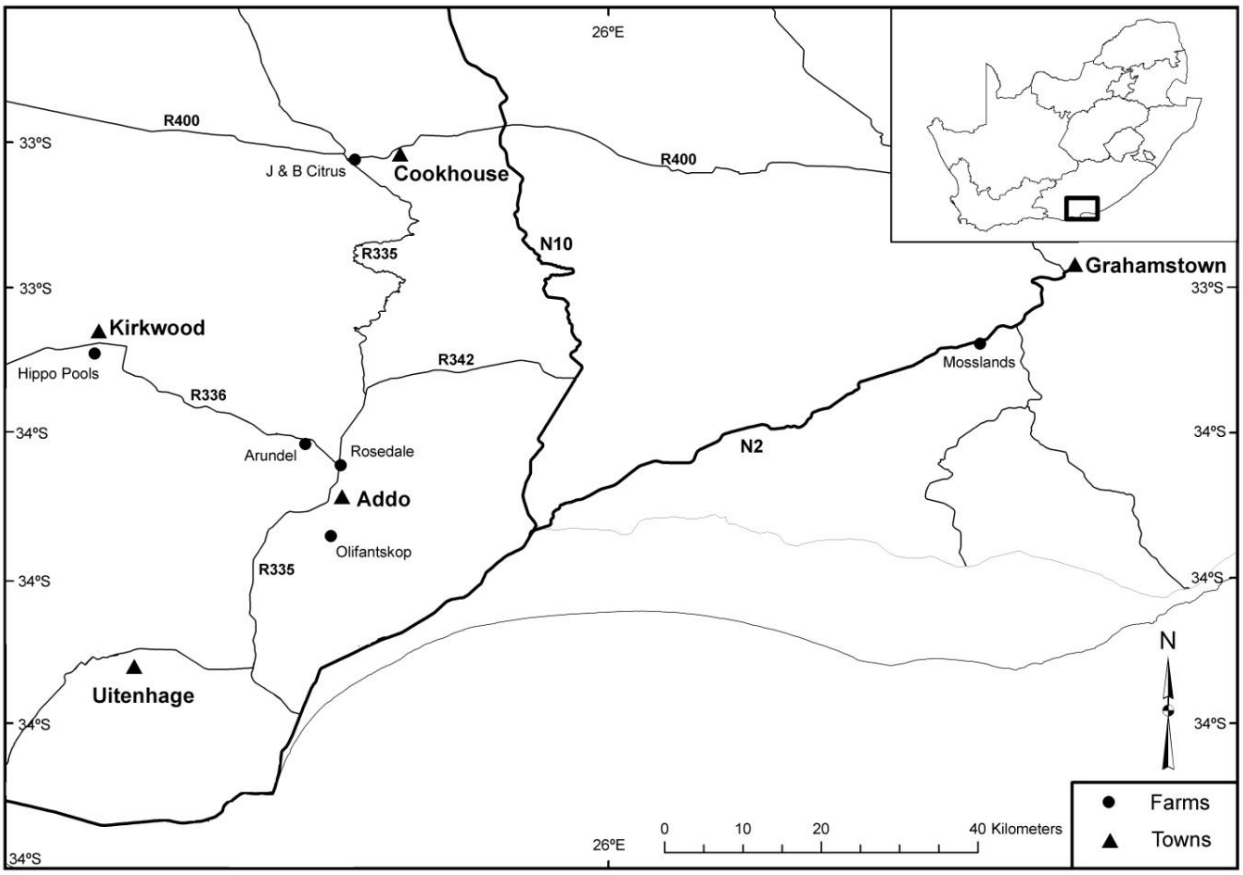

Figure 2.2.1 Map of the Sunday's River Valley citrus growing region and surrounding areas in the Eastern Cape Province, South Africa, with the locations of the six farms, marked with a bullet (•), where soils were sampled in this study.

At each of the six farms, three orchards were selected. A total of 12 soil samples were collected per orchard, along two intersecting transects, until the end of the orchard was reached (Fig. 2.2.2). Additionally, indigenous vegetation within 1-2 km of orchard sites was randomly sampled at 12 points, which were at least 18 m apart; hereafter referred to as refugia (Fig. 2.2.2). Soil samples were collected with the use of a cylindrical soil auger (7 cm x 14 cm) volume 538 cm^3 to a depth of 15 cm. Surface litter was removed and samples were placed in clear plastic bags (31 × 20 cm) and labelled. Orchard soil samples were collected from under the tree canopies at the beginning of autumn (March) 2008, stored at 4°C and individually baited within two months of the sampling period. All orchards had trees of similar sizes and the height of the tree canopies was consistent. Agricultural soil type data and other agronomic information were obtained from farm owners (Table 2.2.1). The classification of South African soils is based on

the recognition of diagnostic topsoils and soil horizons, and soil forms are defined in terms of these sequences (Agricultural Research Council 2009).

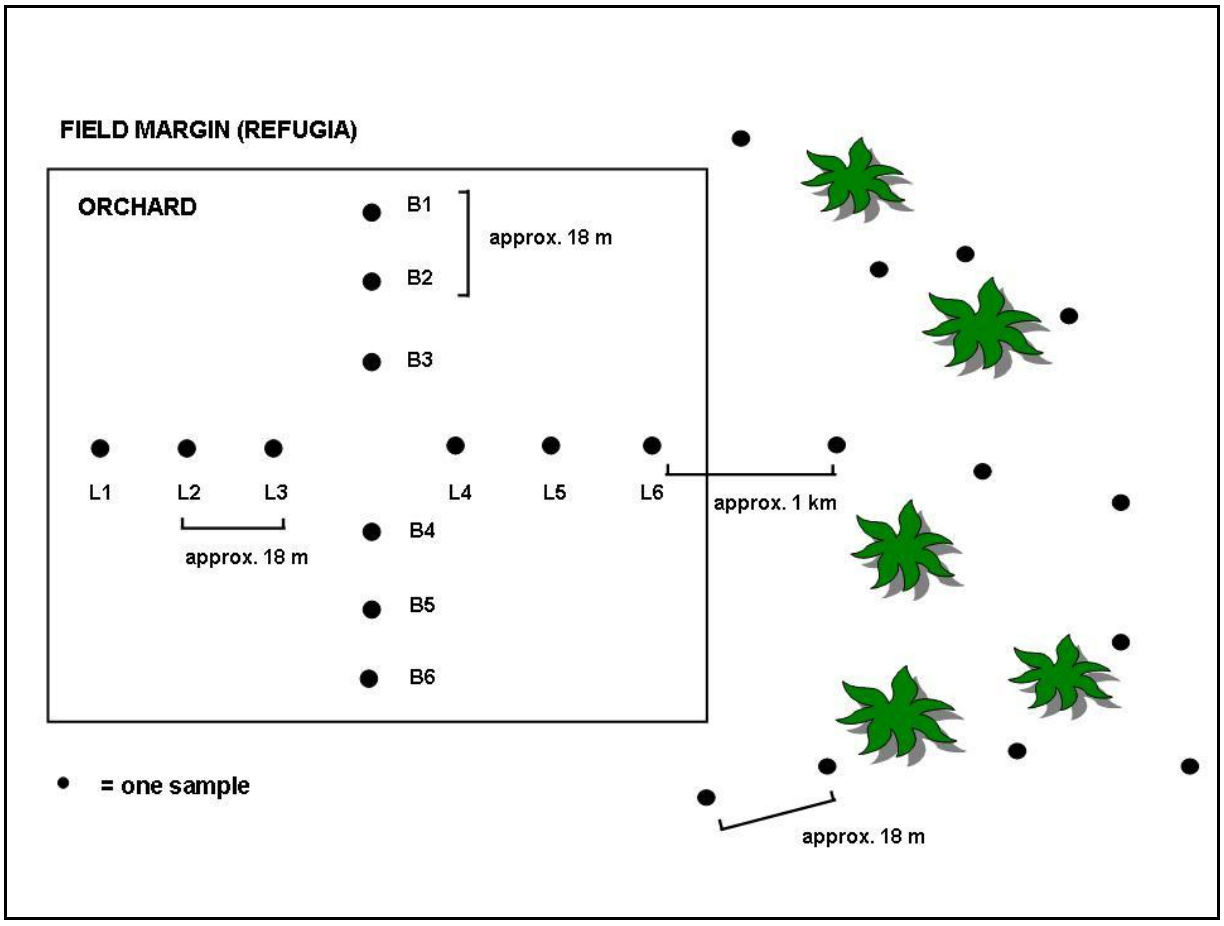

Figure 2.2.2 Sampling points in each orchard and the adjacent field margin (refugia).

2.2.3 Baiting procedures

In the laboratory, each soil sample was thoroughly mixed and then sieved through a metal sieve with a mesh size of 4 mm. Soil samples were divided into three 200 ml portions and transferred to 400 ml transparent plastic pots which were then sealed with perforated lids. If soil samples were too dry they were moistened with distilled water to maintain humidity during baiting. EP fungi were isolated from soil samples using variations of the '*Galleria* bait method' (Zimmermann 1986; Chandler *et al.* 1997; Ali-Shtayeh *et al.* 2002; Klingen *et al.* 2002; Keller *et al.* 2003; Meyling & Eilenberg 2006b). Three separate experiments were run to test *T. leucotreta*, *C. capitata* and *G. mellonella* larvae individually as bait insects. The *Galleria* bait method was conducted using 10 final instar larvae which were placed on the surface of each soil sample container. *Galleria mellonella* larvae were heat-treated according to Meyling (2007) to

prevent excessive webbing in the soil. The same protocol was undertaken using 10 *T. leucotreta* and 10 *C. capitata* larvae per soil sample; however there was no heat-treatment applied to these species because excessive webbing in the soil was not experienced with these insects. All soil samples were incubated at 22°C in the dark. Containers were inverted daily for the first week to make bait insects penetrate the soil as much as possible. After initial baiting, samples were checked for the presence of dead larvae every 3-4 days for three weeks. All dead larvae were surface sterilised with 70% ethanol prior to incubation in a moisture chamber (Petri dish with moistened filter paper) to prevent opportunistic external saprophytic fungi from growing on the cadavers. Sporulating larvae and/or pupae were placed on appropriate media to isolate fungal cultures.

2.2.4 Isolation and identification of fungi

Fungal isolations were made using selective media adapted from Meyling (2007) with the following composition and preparation: 60 g SDA (Sabouraud Dextrose Agar, Merck) supplemented with 1 ml Dodine, 50 mg/L Chloramphenicol, 50 mg/L Ampicillin or 50 mg/L Rifampicin. Plates were incubated at 22°C in the dark. All potential EP fungi were identified microscopically using tape mounts, according to morphological characteristics described in taxonomic keys and other relevant literature (Barnett 1960; Domsch *et al.* 2007). Selected strains were sent to the Mycology Unit at the Plant Protection Research Institute (PPRI) in Pretoria, South Africa, for morphological identification. Once identified each isolate was allocated an accession number and stored in the South African National Collection of Fungi held at PPRI. The PPRI identification and accession numbers for these cultures were in turn used as a reference catalogue. All saprophytic fungi arising from the baiting procedures were discarded.

Table 2.2.1 Agronomic data of orchards of the six farms in the Eastern Cape from which soils were sampled and used in insect baiting.

Farm Name	Orchard No.	Soil Type	Citrus Cultivar	Size (Ha)	Rootstock	Fungicides and Other Additives
Arundel	1	Loam	Navel Oranges	1.00	Troyer citrange	Abamectin, Tartox, Cryptogran
	17	Loam	Navel Oranges	0.65	Carizzo citrange	Abamectin, Tartox, Cryptogran
	23	Loam	Navel Oranges	0.50	Swingle citrumelo	Imidacloprid, Cryptogran
Mosslands	10	Oakleaf	Navel Oranges	0.82	Rough lemon	Abamectin
	11	Oakleaf	Navel Oranges	0.25	Rough lemon	Abamectin
	14	Glenrosa	Navel Oranges	0.98	Rough lemon	Copper oxychloride, Abamectin, Chlorphyripos, Mercaptothion
J&B Citrus	14	Oakleaf	Cara caras	2.17	Carizzo citrange	Imidacloprid, Methidathion, Mancozeb, Abamectin, Azoxystrobin
	20	Oakleaf	Cara caras	1.90	Carizzo citrange	Imidacloprid, Methidathion, Mancozeb, Abamectin, Azoxystrobin
	22	Sterkspruit	Late Navels	1.63	Carizzo citrange	Imidacloprid, Methidathion, Mancozeb, Abamectin, Azoxystrobin
Rosedale	3	Oakleaf	Navel Oranges	0.90	Carizzo citrange	Copper spray, Neem & Pyrethrum, Cryptogran, Broadband, compost extract
	15	Oakleaf	Clementine's	0.90	Carizzo citrange	Neem & pyrethrum, Cryptogran, Copper spray, Broadband, compost extract
	20	Oakleaf	Lane Late Navels	1.10	Carizzo citrange	Neem & Pyrethrum, Cryptogran, compost extract
Hippo Pools	14	Loam	Palmer Navels	0.40	Rough Lemon	Neem & Pyrethrum, copper spary, fish emulsion, mineral oil, compost tea
	21	Loam	Palmer Navels	0.45	Swingle citrumelo	Neem & Pyrethrum, copper spary, fish emulsion, mineral oil, compost tea
	24	Loam	Navel Oranges	0.50	Carizzo citrange	Neem & Pyrethrum, copper spary, fish emulsion, mineral oil, compost tea
Olifantskop	13	Loam	Palmer Navels	0.80	Rough lemon	Neem & Pyrethrum, copper spray, fish emulsion, mineral oil, compost tea
	15	Loam	Palmer Navels	0.80	Rough lemon	Neem & Pyrethrum, copper spray, fish emulsion, mineral oil, compost tea
	19	Loam	Palmer Navels	0.50	Rough lemon	Neem & Pyrethrum, copper spray, fish emulsion, mineral oil, compost tea

2.2.5 Quick screening method to detect entomopathogenic fungi

To separate EP fungi from opportunistic saprophytic fungal species, a rapid screening method was adapted from Ali-Shtayeh *et al.* (2002). Isolated fungal cultures were first grown on SDA media for 10-12 days until sporulation was reached. Fungal suspensions were prepared by incising small pieces of hyphae and conidia from sporulating cultures and placing them into sterilized 1.5 µl microcentrifuge tubes, containing 1ml of sterilized distilled water and 0.05% Triton X, then vortex mixed. Five final instar *G. mellonella* larvae were dipped into each fungal suspension for two seconds. The larvae were then placed into moisture chambers and incubated in the dark at 22°C. Petri-dishes were checked daily for the presence of dead larvae. Dead larvae were placed onto SDA agar until sporulation was again detected. Koch's postulates (Madigan *et*

al. 2005) were employed and *Galleria* larvae were re-infected with spores from these cultures (Fig. 2.2.3). Upon insect mortality, larvae were again placed onto SDA agar until sporulation was detected. Sporulating EP fungal cultures were labelled and sent to the Mycology Unit at PPRI in Pretoria, for morphological identification. Cultures that were not sent to PPRI were morphologically identified using the methods described above.

Figure 2.2.3 *Galleria mellonella* larvae infected with conidia from various EP fungal species during the employed quick-screening method (all images were taken with a Leica EZ 4D dissecting microscope at ~ X16) (Photo Credits: T. Goble, Rhodes University).

2.2.6 Data analysis

Only EP fungal species were considered for all statistical analyses. Chi-squared (χ^2) tests were used to compare the recovery of EP isolates from the three bait insects used and specifically the recovery of *Beauveria bassiana* from these insects. Chi-squared tests were also used to compare the occurrence of EP fungi in soils sampled from organically versus conventionally farmed soil, and between soils sampled in cultivated orchards versus refugia. This test was also used to compare the recovery of *B. bassiana* from organic versus conventionally managed soils. Infections were registered qualitatively per sample pot. This meant that whether one or several larvae infected with the same EP fungal species were observed in the same sample pot, this was

registered as one infection. All analyses are based on 288 soil samples from six localities (farms); 108 from organically farmed soil; 108 from conventionally farmed soil and 72 from refugia. In all analyses these ratios were adjusted accordingly.

2.3 RESULTS

2.3.1 Bait insects and fungal species

The fungal species and the number of fungal isolates obtained during this study differed significantly according to the three insects used as bait (Fig. 2.3.1). When the standard bait insect, *G. mellonella,* was used, a total of 45 EP fungal isolates were obtained, this was significantly higher (χ^2=40.13, df=2, $P \leq 0.005$) than those recovered from either *C. capitata* (11 isolates) or *T. leucotreta* (6 isolates) (Fig. 2.3.1). *Beauveria bassiana* was isolated significantly more frequently (χ^2=43.72, df=2, $P \leq 0.005$) than any other fungal species from all bait insects (Fig. 2.3.1). More *Metarhizium anisopliae* var. *anisopliae* isolates were obtained using *G. mellonella* than any other bait insect and the only isolate of *Metarhizium flavoviride* Gams and Rozsypal was also obtained from this bait insect species. *Ceratitis capitata* was the only bait insect to isolate the fungal species, *Conidiobolus coronatus* (Constantin) Batko and *Lecanicillium psalliotae* Treschew. *Lecanicillium psalliotae* is recognised as a nematophagous fungal species (Gan *et al.* 2007), but was included as an EP fungal species because of its capability of infecting arthropods (Pirali-Kheirabadi *et al.* 2006) and because of its close relatedness to *Lecanicillium lecanii* (a well-known EP fungus) (Zare & Gams 2008). The genus *Metarhizium* was not isolated from *C. capitata* (Fig. 2.3.1).

2.3.2 Abundance and geographic distribution of entomopathogenic fungi

The distribution and frequency (% positive samples) of EP fungi in soil sampled from cultivated orchards and refugia on six citrus farms in the Eastern Cape Province are shown in Table 2.3.1. In total, 62 EP fungal isolates belonging to 4 genera were recovered from 288 soil samples baited with each of the three insect bait species at 22°C, an occurrence frequency of 21.53% (Table 2.3.1). The most frequently isolated EP fungal species was *B. bassiana,* which was recovered from 15.63% of all soil samples baited. This was followed by *M. anisopliae* var. *anisopliae,* which was found to occur in fewer soil samples at a lower frequency of 3.82%. *Lecanicillium psalliotae* was isolated with a frequency of 1.39%, while other species such as *M. flavoviride*

(0.35%) and *C. coronatus* (0.35%) were only isolated once from soil samples (Table 2.3.1). Only 1.38% (4 out of 288) of the soil samples collected yielded two EP fungal species in the same sample.

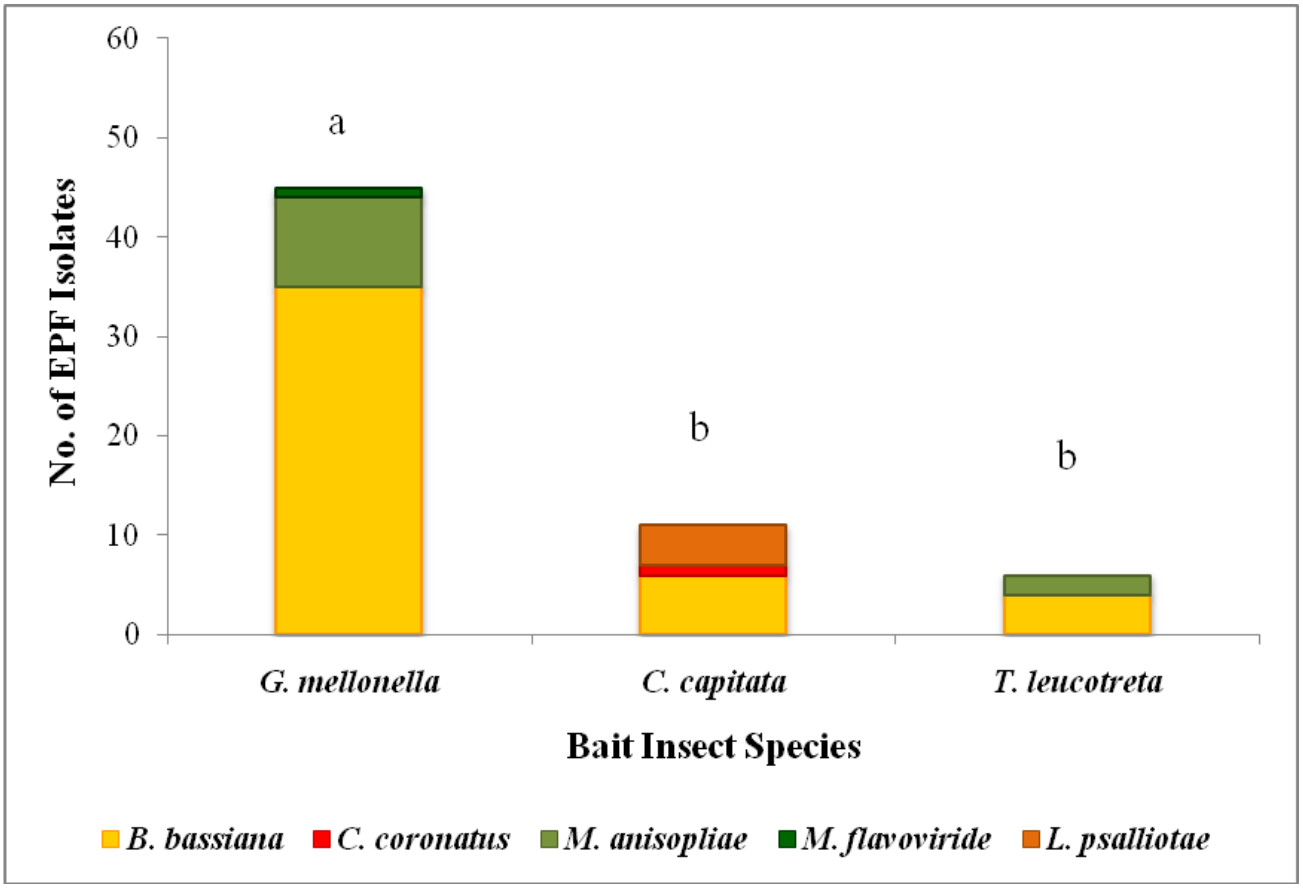

Figure 2.3.1 The total number of EP fungal isolates and species recovered from the three different bait insects used. Letters above a column denote significant differences using χ^2 test, $P \leq 0.005$.

Table 2.3.1 Distribution and occurrence frequency (% positive samples) of EP fungi in 288 soil samples, from orchards and refugia on three organic and three conventionally managed citrus farms, in the Eastern Cape Province, South Africa.

EPF species*	Conventional farms (% F)						Organic farms (% F)						% F **
	Mosslands		Arundel		J&B Citrus		Rosedale		Hippo Pools		Olifantskop		
	N= 36 orchards	N=12 refugia	N= 36 orchards	N=12 refugia	N= 36 orchards	N=12 refugia	N= 36 orchards	N=12 refugia	N= 36 orchards	N=12 refugia	N= 36 orchards	N=12 refugia	
All species	**27.7**	**66.5**	**8.2**	**8.3**	**5.5**	**24.9**	**22.2**	**33.3**	**5.5**	**16.6**	**33.1**	**58.2**	**21.5**
B. bassiana	11.1	41.6	5.5	0.0	5.5	16.6	22.2	33.3	5.5	8.3	27.7	41.6	**15.6**
C. coronatus	0.0	0.0	0.0	0.0	0.0	8.3	0.0	0.0	0.0	0.0	0.0	0.0	**0.3**
M. anisopliae	16.6	8.3	2.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.7	16.6	**3.8**
M. flavoviride	0.0	8.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**0.3**
L. psalliotae	0.0	8.3	0.0	8.3	0.0	0.0	0.0	0.0	0.0	8.3	2.7	0.0	**1.4**

***Percentage frequency of isolates found per farm**

**** Percentage frequency % F based on the total number of isolates found on all farms/288 soil samples**

The greatest diversity of EP fungal species was recorded from the conventional citrus farm, Mosslands. Eighteen isolates belonging to four fungal species (three genera) were obtained (Table 2.3.1). The only isolate of *M. flavoviride* was obtained from one soil sample taken from refugia at this farm. At Arundel, four isolates belonging to three species of EP fungi were recovered. Five isolates belonging to two EP fungal species were retrieved from J & B citrus and the only isolate of *C. coronatus* found in this study was retrieved from a soil sample taken from refugia at this location. The farm, Rosedale, yielded the lowest diversity of EP fungal species. Only *B. bassiana* was retrieved from soil samples at this farm. However, a large number of isolates were obtained (12 isolates). At Hippo Pools, four fungal isolates belonging to two species were recovered. At Olifantskop, the largest number of EP fungal isolates was recovered (19 isolates) but the diversity of fungal species (three species) was lower than was observed at Mosslands. The sixty two isolates of EP fungi which were collected from cultivated orchards and refugia, from the six farms in the Eastern Cape are listed in Tables 2.3.2 and 2.3.3.

2.3.3 Farming systems and field margins

More EP fungal isolates were recovered from organic (35 isolates) than conventionally (25 isolates) farmed soil samples although this was not significant (Fig. 2.3.2). *Beauveria bassiana* was isolated significantly more frequently in samples from organically farmed soils (30 isolates) than from samples of conventionally farmed soils (15 isolates) (χ^2=5.00, df=1, $P \leq 0.05$). In contrast, *M. anisopliae* var. *anisopliae* was isolated more frequently in samples from conventionally cultivated soils (8 isolates) than organically farmed soil samples (3 isolates) but this result was not significant. *Lecanicillium psalliotae* occurred equally in both organically and conventionally farmed soil samples. The single occurrences of *M. flavoviride* and *C. coronatus* were not considered further. When comparing cultivated lands and refugia, significantly more EP fungi were found in refugial habitats than cultivated land habitats (χ^2=11.65, df=1, $P \leq 0.005$) (Fig. 2.3.2). However, when comparing cultivated lands and refugia separately, there were no significant differences observed in the occurrence of EP fungi from the soil samples when both farming systems were compared (Fig. 2.3.2).

Table 2.3.2 The location and ecological data of *Beauvaria bassiana* collected from citrus orchards and refugia soils in the Eastern Cape Province, South Africa.

Farm	Type	Habitat	Area (Ha)	Soil Type	Rootstock	Bait Type	Accession no.
Arundel	conventional	orchard	0.50	Loam	carizzo citrange	*Galleria*	RHO Ar 17 L6
Arundel	conventional	orchard	0.50	Loam	carizzo citrange	*Galleria*	PPRI 9679
Mosslands	conventional	orchard	0.98	Oakleaf Richley	rough lemon	*Galleria*	PPRI 9555
Mosslands	conventional	refugia	2.00	Unknown	NA	*Galleria*	PPRI 9774
Mosslands	conventional	refugia	2.00	Unknown	NA	*Galleria*	PPRI 9773
Mosslands	conventional	refugia	2.00	Red sandy	NA	*Galleria*	PPRI 9554
Mosslands	conventional	refugia	2.00	Red sandy	NA	*Galleria*	PPRI 9557
Mosslands	conventional	refugia	2.00	Red sandy	NA	*Galleria*	PPRI 9593
Mosslands	conventional	orchard	0.82	Oakleaf Caledon	rough lemon	FCM	PPRI 9680
Mosslands	conventional	orchard	0.25	Oakleaf Caledon	rough lemon	FCM	PPRI 9592
Mosslands	conventional	orchard	0.25	Oakleaf Caledon	rough lemon	FCM	PPRI 9594
J&B Citrus	conventional	orchard	0.74	Oakleaf Caledon	rough lemon	*Galleria*	PPRI 9678
J&B Citrus	conventional	orchard	1.11	Sterkspruit	rough lemon	FF	PPRI 9693
J&B Citrus	conventional	refugia	2.00	Unknown	NA	FF	PPRI 9556
J&B Citrus	conventional	refugia	2.00	Unknown	NA	FF	PPRI 9553
Olifantskop	organic	orchard	0.80	Loam	rough lemon	*Galleria*	RHO OL 13 L1
Olifantskop	organic	orchard	0.80	Loam	rough lemon	*Galleria*	PPRI 9688
Olifantskop	organic	orchard	0.80	Loam	rough lemon	*Galleria*	RHO OL 15 L5
Olifantskop	organic	orchard	0.80	Loam	rough lemon	*Galleria*	RHO OL 15 B3
Olifantskop	organic	orchard	0.50	Loam	rough lemon	*Galleria*	PPRI 9689
Olifantskop	organic	orchard	0.50	Loam	rough lemon	*Galleria*	RHO OL 19 B1
Olifantskop	organic	orchard	0.50	Loam	rough lemon	*Galleria*	RHO OL 19 B2
Olifantskop	organic	orchard	0.50	Loam	rough lemon	*Galleria*	RHO OL 19 B3
Olifantskop	organic	orchard	0.50	Loam	rough lemon	*Galleria*	RHO OL 19 B5
Olifantskop	organic	orchard	0.50	Loam	rough lemon	*Galleria*	RHO OL 19 B6
Olifantskop	organic	refugia	2.00	Unknown	NA	*Galleria*	PPRI 9772
Olifantskop	organic	refugia	2.00	Unknown	NA	*Galleria*	PPRI 9771
Olifantskop	organic	refugia	2.00	Unknown	NA	*Galleria*	PPRI 9692
Olifantskop	organic	refugia	2.00	Unknown	NA	*Galleria*	PPRI 9691
Olifantskop	organic	refugia	2.00	Unknown	NA	*Galleria*	PPRI 9690
Rosedale	organic	orchard	0.90	Oakleaf Letaba	troyer citrange	*Galleria*	RHO Rose. 3 L2
Rosedale	organic	orchard	0.90	Oakleaf Letaba	troyer citrange	*Galleria*	PPRI 9681
Rosedale	organic	orchard	0.90	Oakleaf Letaba	troyer citrange	*Galleria*	RHO Rose. 3 B2
Rosedale	organic	orchard	0.90	Oakleaf Letaba	carizzo citrange	*Galleria*	PPRI 9682
Rosedale	organic	orchard	1.10	Oakleaf Limpopo	carizzo citrange	*Galleria*	RHO Rose.23 L1
Rosedale	organic	orchard	1.10	Oakleaf Limpopo	carizzo citrange	*Galleria*	PPRI 9687
Rosedale	organic	orchard	1.10	Oakleaf Limpopo	carizzo citrange	*Galleria*	RHO Rose 23 L4
Rosedale	organic	orchard	1.10	Oakleaf Limpopo	carizzo citrange	*Galleria*	RHO Rose 23 L5
Rosedale	organic	refugia	1.20	Unknown	NA	*Galleria*	PPRI 9683
Rosedale	organic	refugia	1.20	Unknown	NA	*Galleria*	PPRI 9686
Rosedale	organic	refugia	1.20	Unknown	NA	*Galleria*	PPRI 9684
Rosedale	organic	refugia	1.20	Unknown	NA	FCM	PPRI 9685
Hippo Pools	organic	orchard	0.40	Loam	rough lemon	FF	RHO HP 14 L5
Hippo Pools	organic	orchard	0.40	Loam	rough lemon	FF	RHO HP 14 B5
Hippo Pools	organic	refugia	1.00	Unknown	NA	FF	PPRI RHO R1

NA = not apllicable, **RHO** = Rhodes University accession numbers

FCM = *Thaumatotibia leucotreta*, **FF** = *Ceratitis capitata*, **PPRI** = Plant Protection Research Institute accession numbers

Table 2.3.3 The location and ecological data of *Conidiobolus coronatus, Lecanicillium psalliotae, Metarhizium anisopliae* and *M. flavoviride* collected from citrus orchards and refugia soils in the Eastern Cape Province, South Africa.

Farm	Type	Habitat	Area (Ha)	Soil Type	Rootstock	Bait Type	Fungal genus or species	Accession No.
J&B Citrus	conventional	refugia	2.00	Unknown	NA	FF	*Conidiobolus coronatus*	PPRI 9695
Arundel	conventional	refugia	1.00	Unknown	NA	FF	*Lecanicillium psalliotae*	PPRI 9768
Mosslands	conventional	refugia	2.00	Red sandy	NA	FF	*Lecanicillium psalliotae*	PPRI 9767
Olifantskop	organic	orchard	0.80	Loam	rough lemon	FF	*Lecanicillium psalliotae*	PPRI 9766
Hippo Pools	organic	refugia	1.00	Unknown	NA	FF	*Lecanicillium psalliotae*	RHO 14 B4
Arundel	conventional	orchard	0.50	Loam	swingle citrange	FCM	*M. anisopliae group*	PPRI 9561
Mosslands	conventional	orchard	0.98	Oakleaf Richley	rough lemon	FCM	*M. anisopliae var. anisopliae*	RHO F 14 2 B5
Mosslands	conventional	orchard	0.98	Oakleaf Richley	rough lemon	*Galleria*	*M. anisopliae var. anisopliae*	PPRI 9558
Mosslands	conventional	orchard	0.98	Oakleaf Richley	rough lemon	*Galleria*	*M. anisopliae var. anisopliae*	RHO 14 2 L6
Mosslands	conventional	orchard	0.98	Oakleaf Richley	rough lemon	*Galleria*	*M. anisopliae var. anisopliae*	PPRI 9562
Mosslands	conventional	orchard	0.98	Oakleaf Richley	rough lemon	*Galleria*	*M. anisopliae var. anisopliae*	RHO G 14 2 B5
Mosslands	conventional	orchard	0.25	Oakleaf Caledon	rough lemon	*Galleria*	*M. anisopliae var. anisopliae*	PPRI 9803
Mosslands	conventional	refugia	2.00	Unknown	NA	*Galleria*	*M. anisopliae var. anisopliae*	PPRI 9559
Olifantskop	organic	orchard	0.50	Loam	rough lemon	*Galleria*	*M. anisopliae var. anisopliae*	PPRI 9802
Olifantskop	organic	refugia	2.00	Unknown	NA	*Galleria*	*M. anisopliae var. anisopliae*	PPRI 9800
Olifantskop	organic	refugia	2.00	Unknown	NA	*Galleria*	*M. anisopliae var. anisopliae*	PPRI 9801
Mosslands	conventional	refugia	2.00	Unknown	NA	*Galleria*	*Metarhizium flavoviridae*	PPRI 9560

NA = not apllicable, **RHO** = Rhodes University accession numbers

FCM = *Thaumatotibia leucotreta*, **FF** = *Ceratitis capitata*, **PPRI** = Plant Protection Research Institute accession numbers

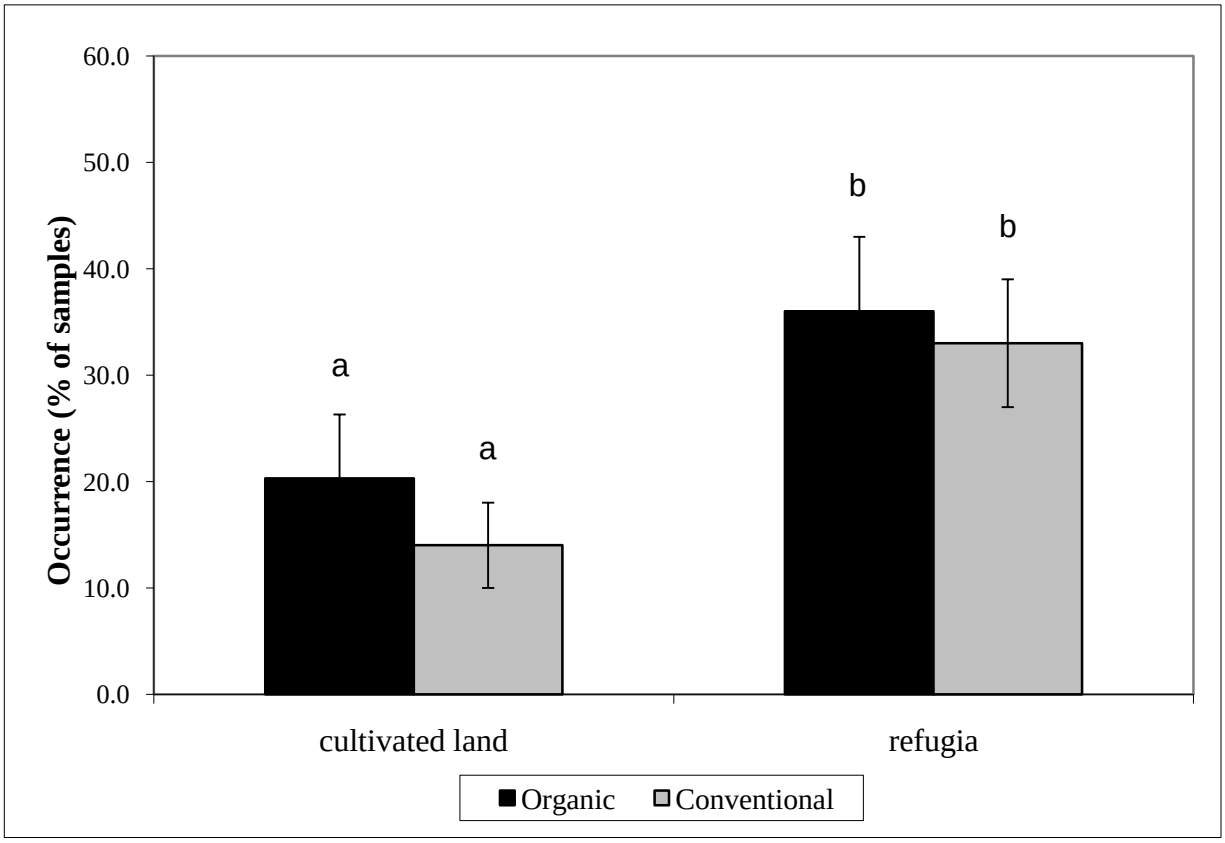

Figure 2.3.2 Mean (% ± SE) of the frequency (% of samples) of EP fungi occurring in soil samples from orchards and refugia of organic and conventionally managed farms. Letters above a column denote significant differences using χ^2 test, $P \leq 0.005$.

2.4 DISCUSSION

2.4.1 Bait insects and fungal species

This study confirmed the sensitivity of the *Galleria* bait method, as significantly more EP fungal isolates were obtained using this insect species than either *T. leucotreta* or *C. capitata*. The observed differences in isolation of EP fungal species and isolate numbers in this study may in part be attributed to the biology of the bait insect species used (Smith 1937; Thomas *et al.* 2001; Smith & Peña 2002; Stibick 2008), as well as their relative susceptibilities to fungal infection (Vilcinskas *et al.* 1997; Konstantopoulou & Mazomenos 2005; Begemann 2008). *Beauveria bassiana* was isolated significantly more often from *G. mellonella* than was any other EP fungal species from any other bait insect. This fungal species was first described from South African citrus orchards at Zebediela in the Northern Province by Schoeman in 1960, who found it

growing on pupae of the citrus measuring worm, *Ascotis selenaria receprocaria* (Begemann 2008). In an extensive survey undertaken by Hatting *et al.* (2004), *B. bassiana* was the most commonly isolated EP fungus (87%) from South African soils baited with *G. mellonella.* Various other authors have also reported *B. bassiana* to be the most commonly recovered EP fungal species, using the bait insect *G. mellonella*, from soil samples (Chandler *et al.* 1997; Meyling & Eilenberg 2006b; Quesada-Moraga *et al.* 2007). Klingen *et al.* (2002) investigated and compared the use of *G. mellonella* and *Delia floralis* as bait insects. No significant differences were observed in the number of isolates of EP fungi recovered from both insect species but the fungal species, *Tolypocladium cylindrosporum*, was isolated more frequently from *D. floralis* (Anthomyiidae) than *G. mellonella*. When *C. capitata* was used as a bait insect in the present study, it was the only insect to isolate the fungal species, *L. psalliotae.* The larvae of *G. mellonella* are known to be highly susceptible to insect pathogens (Chandler *et al.* 1997; Vilcinskas *et al.* 1997; Meyling 2007) and this may explain the large number of EP fungal isolates which were recovered from this insect bait species when compared to the other bait insects used. The soil environment is an integral habitat for the completion of the lifecycles of both *T. leucotreta* and *C. capitata,* therefore these bait species may be better adapted to the adversities of this environment, which may include lower susceptibility to fungal infections, compared to *G. mellonella,* to which the soil is not a natural habitat. One feature of the biology of *T. leucotreta* is their ability to spin a cocoon made from silk and sand particles prior to pupation (Stibick 2008). This cocoon may minimize the possibility of the spores of EP fungi coming into direct contact with the cuticle of the insect. The spores of EP fungi may adhere to the cocoon but because of the lack of direct contact with the insect cuticle may fail to germinate. Perhaps future baiting procedures using this insect species should consider heat-treating the larvae, as is done with *G. mellonella*, but this warrants further investigation.

2.4.2 Fungal ecology

The 21.53% occurrence of five species of EP fungi in soils from the Sunday's River Valley citrus producing area and surrounding areas in Eastern Cape Province, South Africa, is lower than the expected range when compared to studies in various other countries. Ali-Shtayeh *et al.* (2002) found insect-pathogenic fungi to occur in 33.6% of the soil samples studied in irrigated vegetable fields and citrus orchards in Palestine. Other typical recovery rates include: 38.6% in Mauritius (Sookar *et al.* 2008), 52% in the Pacific North West, USA (Bruck 2004), 55.5% in China (Bing-Da & Xing-Zhong 2008), 71.7% in Spain (Quesada-Moraga *et al.* 2007), 91% in

Ontario, Canada (Bidochka *et al.* 1998), and 96% in Switzerland (Keller *et al.* 2003). Chandler *et al.* (1997) reported that EP fungi were common inhabitants of the soil biota, but the diversity of species was low, usually with one or two species occurring frequently; this observation proved true for the present study and has been noted by other authors (Keller *et al.* 2003; Quesada-Moraga *et al.* 2007).

The occurrence of *L. psalliotae* was low and was only recovered from *C. capitata* larvae. Literature is scarce on the distribution and occurrence of this nematophagous fungal species (Gan *et al.* 2007) which can also be EP and capable of infecting other arthropod hosts (Evans & Whitehead 2005; Pirali-Kheirabadi *et al.* 2006). *Metarhizium flavoviride* was only isolated once from a soil sample in this study however its' isolation has been recorded in other studies and this fungal species is considered to have a wide geographic distribution (Braga *et al.* 2001). Meyling & Eilenberg (2006b) for example found *M. flavoviride* to occur commonly (21.9%) in soils of a Danish research farm in Copenhagen. *Conidiobolus coronatus* was also isolated once in this study. Hatting *et al.* (1999) conducted a survey on the fungal pathogens of aphids from South Africa and revealed that *C. coronatus* was not isolated from field-collected cereal aphids in South Africa; in fact the only record of this fungal species was obtained from a mycosed aphid cadaver in an aphid-rearing facility in Bethlehem, South Africa. The study however did not include soil samples and thus the limited occurrence of *C. coronatus* must be carefully considered. However, Ali-Shtayeh *et al.* (2002) found *C. coronatus* to be the most frequently isolated EP fungus (31.4%) in citrus orchard soils in Palestine. The limited number of fungal isolates obtained from the species *M. flavoviride, C. coronatus* and *L. psalliotae* may mean that these fungal species are locally rare or simply that they may not have been detected by the bait method protocol for various reasons which are discussed further below. However, based on the studies mentioned, these species of fungi do occur with more prevalence worldwide.

The low incidence of soil samples which yielded more than one EP fungal species in the same sample (1.38%) may be an artifact or limitation of the method of qualitative quantification of infection constantly used in the *Galleria* baiting technique. As long as there were more than one morphologically identifiable fungal species within a sample pot, detection of multiple EP fungal species was easy. However, genetically different *Beauveria* species within the same pot may have been missed due to the extreme difficulty in determining genetically distinct *Beauveria* species based on morphology alone. Meyling *et al.* (2009) inferred species diversity and reproductive potential using molecular techniques on *Beauveria* within a single arable field and

bordering hedgerow in Denmark. Seven phylogenetic species were discovered including five phylogenetic species within *B. bassiana sensu stricto.* These analyses demonstrate the genetic diversity of *B. bassiana* within a relatively limited geographical area with the greatest genetic diversity observed in the semi-natural habitat of the hedgerow compared to the arable field. Furthermore, it was found that *Beauveria* phylogenetic species also occupied overlapping niches within the hedgerow habitat. Based on these results it may be possible that some genetically distinct *Beauveria* isolates could have been overlooked in a particular sample during the fungal recovery process using the insect bait technique.

This study showed a greater likelihood of finding EP fungi in refugia than in cultivated habitats, but no differences were observed in the recovery of fungi when organic and conventional farming systems were considered. Klingen *et al.* (2002) however, found more EP fungi in cultivated fields of organically farmed soils and, no significant differences in the occurrence of EP fungi were found between the refugia of the two cropping systems (Klingen *et al*. 2002). There was a common result in that a positive relationship existed between occurrence of fungi and organically farmed soils in the present study. *Beauveria bassiana* and *M. anisopliae* var. *anisopliae* were the most commonly isolated EP fungal species in soils from the Eastern Cape Province. On five of the six farms, Arundel Farm being the only exception, *B. bassiana* was recovered more often from soil samples taken from refugia than from cultivated lands. Furthermore, there was a significant difference in the recovery of *B. bassiana* from organic compared to conventionally farmed soils.

Mietkiewski *et al.* (1997) and Chandler *et al.* (1997) in the United Kingdom both showed that the frequency of occurrence of EP fungi in intensively cultivated soils was lower than in marginal habitats. Meyling *et al.* (2009) attributed greater *Beauveria* diversity in seminatural habitats of hedgerows in Denmark to increased humidity, reduced ultra-violet light, long-term environmental stability and a greater abundance and diversity of insect hosts. Natural habitats are expected to host more diverse and stable insect communities compared to cultivated lands, due to the absence of various pest control tactics, and the structurally more complex, shaded and undisturbed composition of these habitats. Meyling & Eilenberg (2007) suggested that insect hosts should still be considered the primary source of organic matter for fungal population build up. An increase in organic matter content (from surrounding vegetation) in the soils of natural habitats may also favour the prevalence of EP fungi. Quesada-Moraga *et al.* (2007) suggested that soils with greater organic matter content have higher cation exchange capacities with greater

organic matter enhancing conidia adsorption. Meyling & Eilenberg (2006b) however found that organic matter content and subsequent increase in the biological activity in the soil adversely affected the persistence of EP fungal species due to antagonistic effects. Mainly because both *Beauveria* and *Metarhizium* are poor competitors for organic resources compared to opportunistic saprophytic fungi that are ubiquitous in the soil (Meyling & Eilenberg 2007).

The natural occurrence of EP fungi in the soil is influenced by a complicated set of abiotic and biotic factors such as soil structure (Jaronski 2007), organic matter in the soil (Quesada-Moraga *et al.* 2007), ultra-violet light (UV-B) (Braga *et al.* 2001), cultural practices: pesticide applications, tillage regimes, crop cover and rotations, fertilizers (Filho *et al.* 2001; Saenz-de-Cabez Irigaray *et al.* 2003; Jaronski 2007; Meyling & Eilenberg 2007) and insect hosts. In light of some of the abovementioned factors, it appears that organically farmed soil may be a more suitable habitat for insect-pathogenic fungi compared to cultivated, conventionally farmed soils (Klingen *et al.* 2002; Meyling & Eilenberg 2006b), in which the presence of insecticides and especially fungicides may have a direct killing effect on EP fungi (Khalil *et al.* 1985; Klingen *et al.* 2002; Meyling & Eilenberg 2007). One of the conventional farms in this study, J&B Citrus, regularly applied the fungicide mancozeb to sample orchards for the control of citrus Black Spot, *Guignardia citricarpa*. Lagnaoui and Radcliffe (1998) however, found mancozeb to be strongly inhibitory of germination of EP fungal conidia. Majchrowicz and Poprawski (1993) examined the *in vitro* effects of fungicides on fungi and found that dithiocarbamate derivatives zineb and copper oxychloride applied together or mancozeb applied alone, completely inhibited the germination of six fungi tested, including *B. bassiana*. Further the uses of the broad-spectrum fungicide azoxystrobin, and the organophosphate insecticide methidathion, within the orchards at J&B Citrus may also have contributed, in part, to lower incidences of fungal isolates from this farm because both compounds have been shown to negatively affect EP fungi *in vitro* (Puterka 1999; Jaronski 2007).

Cultivated citrus orchards are perennial habitats and may support more diverse and stable microbial communities compared to annual cropping systems (Ali-Shtayeh *et al.* 2002); tree canopies provide shade and maintain humidity levels in the soil, which in turn reduce harmful UVB rays from penetrating the soil and desiccating fungal spores (McCoy *et al.* 2007). The use of compost teas (and manure) or organic fertilizers may have a positive effect on the occurrence of EP fungal species because an increased carbon load in the soil is favourable to soil inhabiting insects, which themselves, are potential hosts for fungi (Ali-Shtayeh *et al.* 2002; Klingen *et al.*

2002). This together with the fact that conventional farming practices in the Eastern Cape are considered 'soft' with emphasis placed on IPM strategies rather than intensive pesticide applications, may be one reason for the limited differences between farming systems when comparing cultivated orchards. This was shown by the large recovery of EP fungal isolates (18 isolates) from Mosslands Farm. Further, the conventional farm Arundel, showed proportionally more diversity of EP fungi in the soils. Very few agrochemicals and other additives were applied in citrus orchards at this location. The soils at Arundel Farm are predominantly sandy loam to which supplements of humic acid and compost were made periodically. The locality of this farm is such that the Sundays River flows through it, thus the soils could be considered younger in age and more underdeveloped (less clay content) compared to those further away from the river, such as at Rosedale (Finnemore, pers. comm.). Loamy soils are composed of sand, silt, and clay in a relatively even concentration (about 40-40-20% respectively), and generally contain more nutrients and humus than sandy soils and have better infiltration and drainage than silt soils (Soil Classification Working Group 1991). In contrast, the organic farm Hippo Pools yielded a low incidence of fungal isolates; the owner reported that the farm was initially managed as a conventional citrus farm with an intense pesticide regime prior to his acquisition of the property. It had been managed as an organic citrus farm for the past six years. Thus, it is also important to consider the history of a sampling area during collection studies. Similarly, at the organic farm Olifantskop, where a large number of fungal isolates were obtained (19 isolates), the farmer reported that his orchards were all first time plantings on virgin soils, which essentially were natural habitats, and this may explain the high occurrence of fungi at this farm.

The direct application of EP fungi, in the form of microbial products, in organic and some conventional farming practices may also have a significant influence on the occurrence and recovery of fungi in these systems. The organic citrus farm, Rosedale for example, made use of a *Beauveria bassiana* formulated product called Bb Plus® (Biological Control Products (BCP), South Africa) which was applied to two of the orchards from which soil samples were collected, six months prior to the sampling period. Interestingly enough only four *Beauveria* isolates were recovered from the two orchards augmented with the Bb Plus® product. The rest of the isolates were recovered from the only orchard to which the product had not been applied, and from the natural areas. Thus, the incidence of occurrence of *B. bassiana* isolates from the cultivated orchard sites 3, and 15 at Rosedale may or may not be attributed to residual spores from the direct application of Bb Plus®. The only way of determining the presence of residual Bb Plus® conidia would be to genetically analyze the isolates. However, this was not the focus of this

study and there were only four isolates obtained from the areas where the product had been applied. Vänninen *et al.* (2000) in discussing the persistence of augmented *M. anisopliae* and *B. bassiana* in Finnish agricultural soils, found lower abundances (a 44% reduction) of fungal conidia in peat soil sites compared to both sandy (a 37% reduction) and clay (a 14% reduction) soils, just one year after application. *Beauveria bassiana* conidia were generally less persistent in all soil types compared to *M. anisopliae* (Vänninen *et al.* 2000). The agricultural soils at Rosedale were all classified as Oakleaf form, which comprise 20% clay content and are calcareous (Finnemore, pers. comm.). These soils drain well but are relatively depauperate of nutrients and organic matter; soils are annually supplemented with compost and compost teas are applied every two months (Finnemore, pers. comm). This practice however could stimulate the growth of saprophytic fungi which could subsequently antagonise EP fungal persistence in the soil. The conidia of the applied Bb Plus® product may still have been present in the soil of orchards 3 and 15, however determination of conidial persistence is complex (Vänninen *et al.* 2000; Jaronski 2007).

Increasing evidence suggests that habitat selection and not insect host selection drives the population structures of deuteromycete EP fungi (Bidochka *et al.* 2001; Bidochka *et al.* 2002) particularly in light of recent studies which advocate that some EP fungal genera are facultative pathogens, capable of multiple ecological roles (Goettel *et al.* 2008; Vega *et al.* 2009) and that adaption to a specific habitat type is a more important selection criterion for identifying potential biological control candidates (Bidochka *et al.* 2002). While it may be important to obtain highly infective and virulent strains of fungi to control insect pests, it is even more important to look for isolates which are also capable of surviving and persisting in the environments and habitats for which they are required.

2.4.3 Potential for biological control

Twenty-one of the 62 collected indigenous fungal isolates were selected for testing against the subterranean life stages of *T. leucotreta*, *C. capitata* and *C. rosa* in conidial-sand incorporated bioassays to evaluate their potential as biological control agents (cf Chapter III). Indigenous isolates were selected so as to represent an even geographic spread from the six farms in the Eastern Cape. The EP fungal isolates selected for conidial-sand incorporation bioassays are shown in Figs 2.4.1-2.4.6. Fungal isolates were grown on SDA supplemented with 1 ml/L Dodine, 50 mg/L Chloramphenicol, 50 mg/L Ampicillin or 50 mg/L Rifampicin in Petri dishes

and maintained at an ambient temperature of 25°C. Fungal isolates were labelled according to the bait insect species they were originally isolated from, followed by the farm name and then the orchard or refugia sample number.

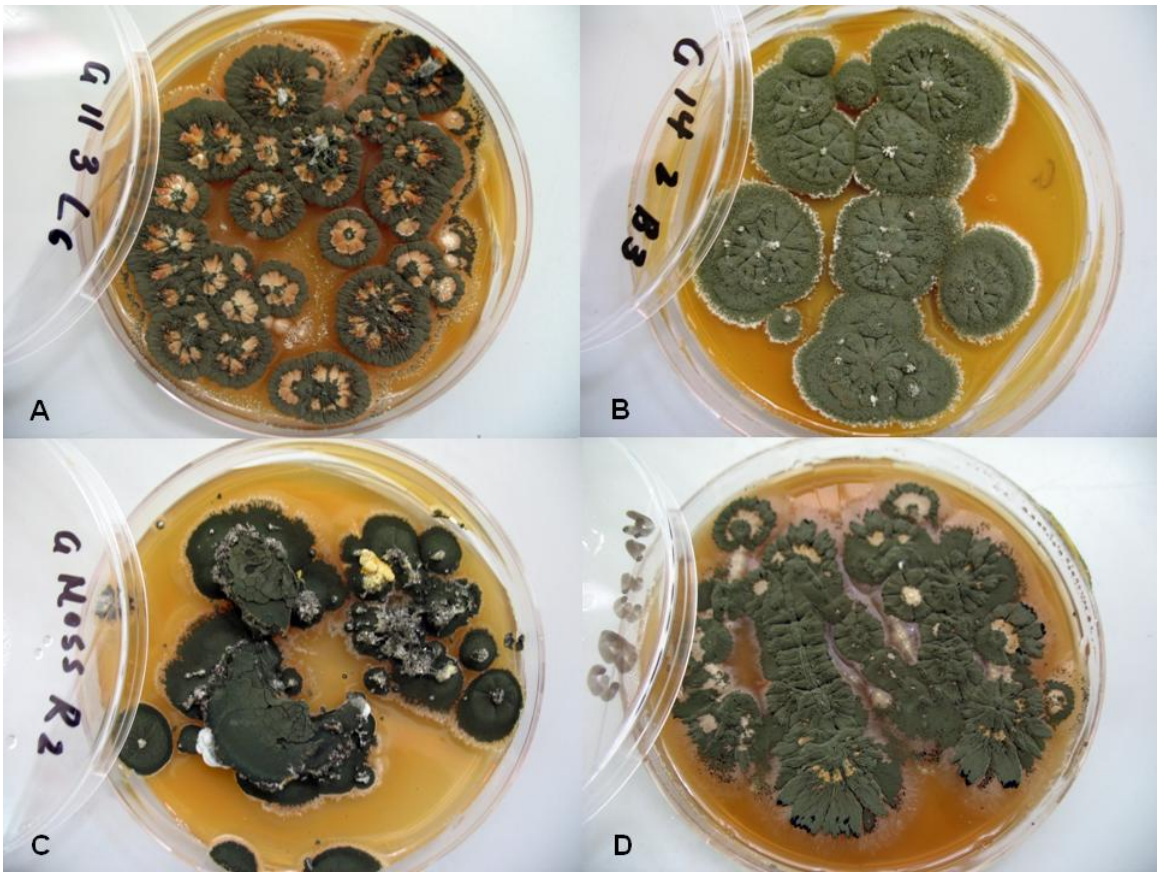

Figure 2.4.1 **A, B, D**- *Metarhizium anisopliae* var. *anisopliae* **C**- *Metarhizium flavoviride*

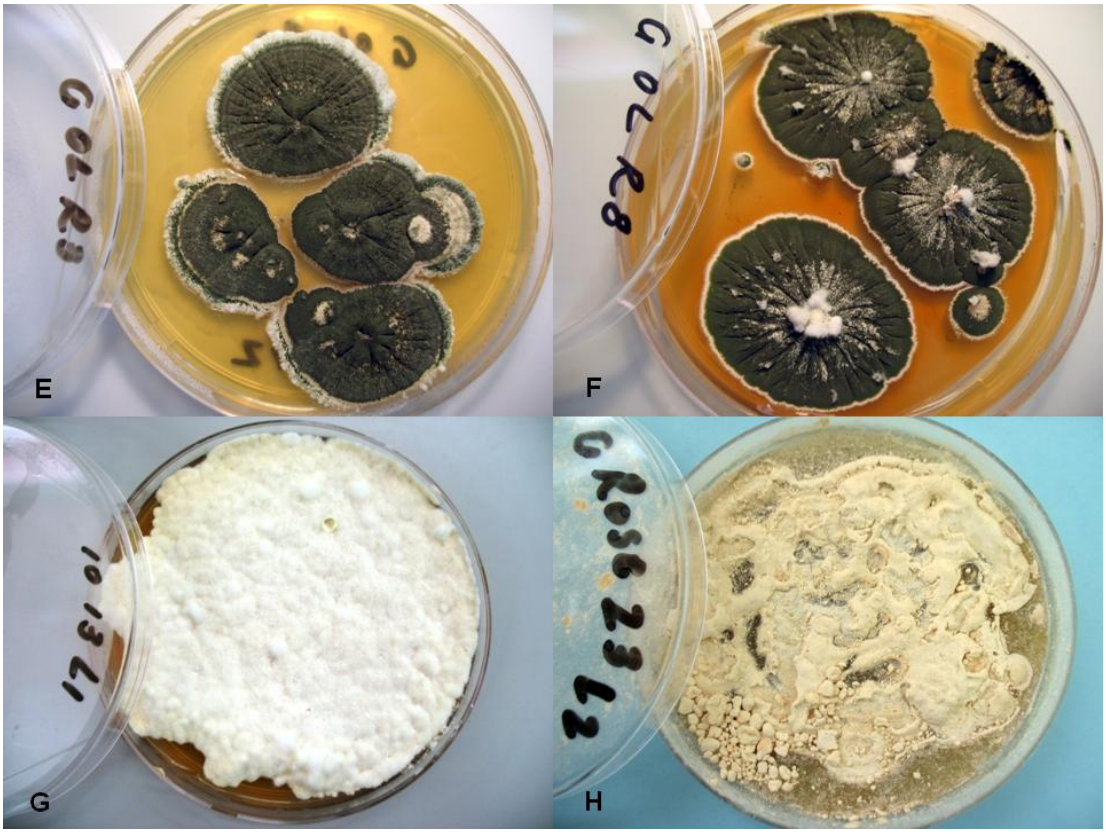

Figure 2.4.2 **E, F**- *Metarhizium anisopliae* **G, H**- *Beauveria bassiana*

Figure 2.4.3 **I, J, K, L**- *Beauveria bassiana*

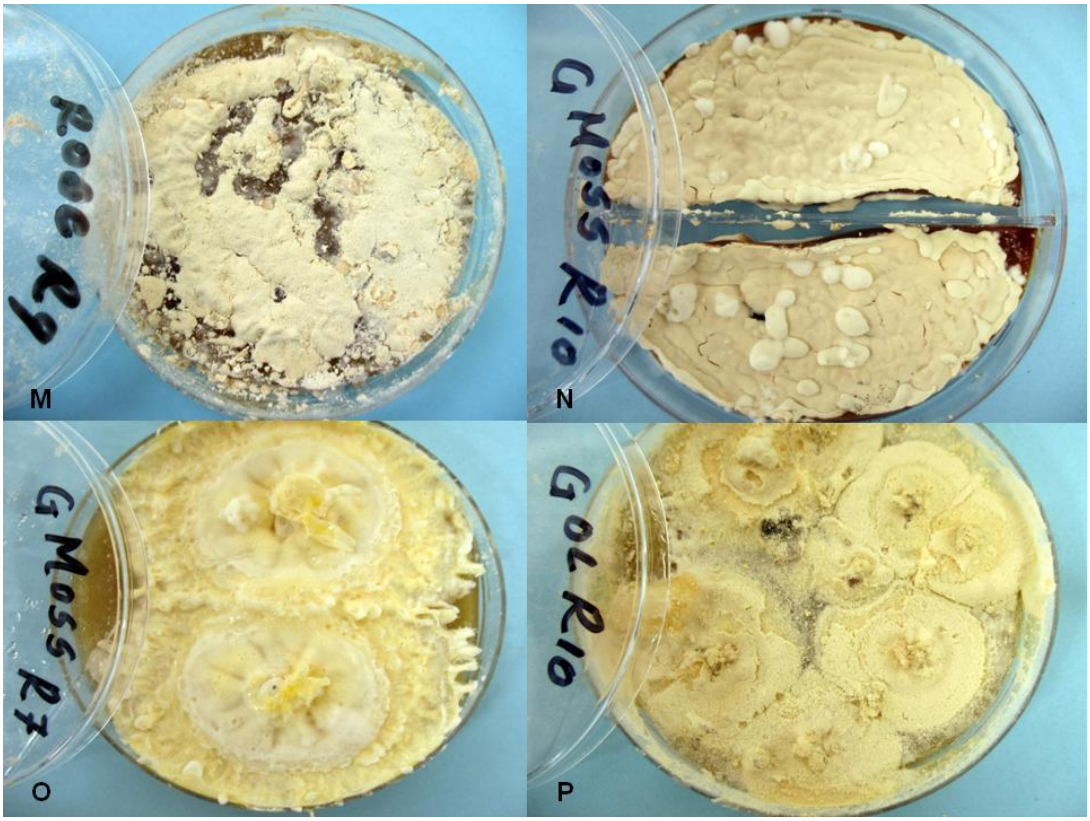

Figure 2.4.4 M, N, O, P- *Beauveria bassiana*

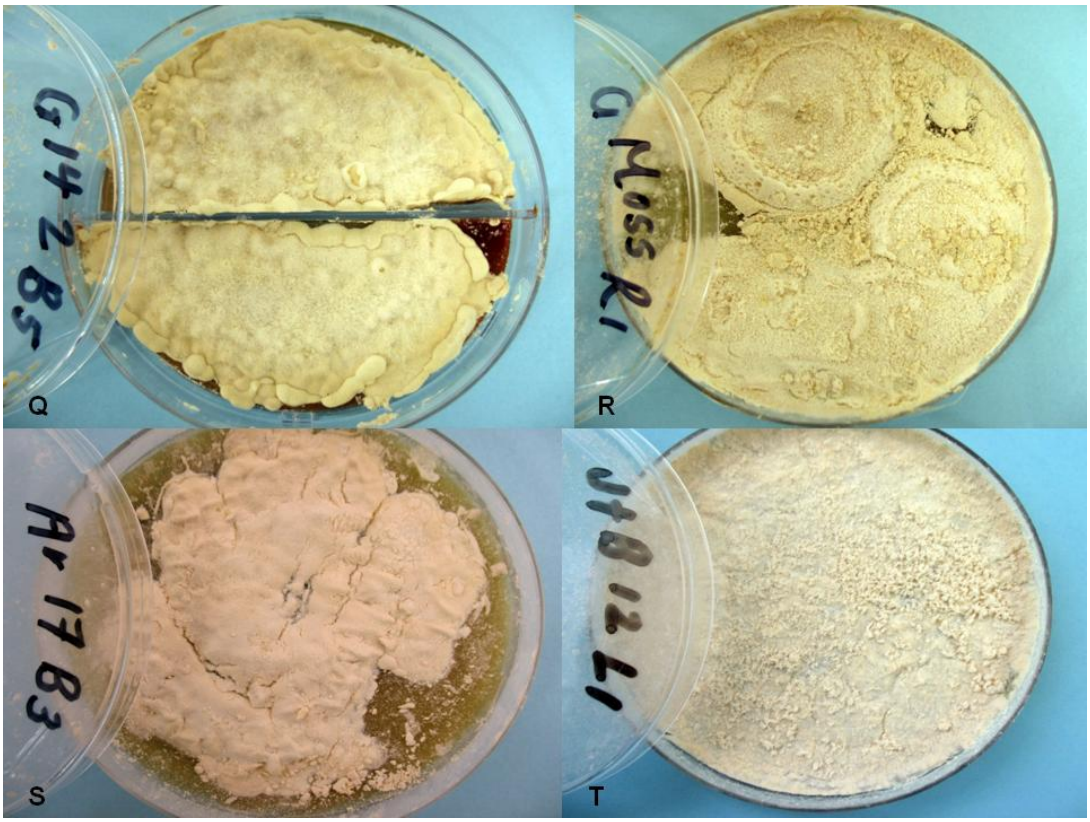

Figure 2.4.5 Q, R, S, T- *Beauveria bassiana*

Figure 2.4.6 **U**- *Beauveria bassiana* (Photo Credits, T. Goble, Rhodes University)

III

ASSESSING THE VIRULENCE OF ENTOMOPATHOGENIC FUNGI AGAINST CITRUS PESTS

3.1 INTRODUCTION

The ecological distribution and occurrence of indigenous isolates of EP fungi from citrus orchards and refugia was examined in Chapter II and their potential use as biological control agents (BCA's) against fruit flies and false codling moth was recognized. This chapter will investigate the physiological effects and host specificity of various indigenous fungal isolates obtained in the Eastern Cape, to establish whether these isolates can be effectively used as control agents against the subterranean life stages of *C. capitata*, *C. rosa* and *T. leucotreta*. The biology of these key citrus pest species includes their dropping directly from fruit trees to pupate in the soil or leaf litter below (Thomas *et al.* 2001; Ekesi *et al.* 2007; Begemann 2008, Stibick 2008). Since most EP fungi are soil-borne microorganisms, the investigation of a management strategy option in citrus, targeted at the pre-pupating larvae and pupae of these insect pest species in the soil, needs to be investigated. For this reason, it was decided to explore soil as an inoculation medium for citrus pest control. Unlike arborial habitats, the soil environment is less prone to dramatic environmental fluctuations in temperature and humidity, which can have negative effects on the recycling and persistence of EP fungi (Ansari *et al.* 2007). Van den Berg (2001) reported that natural infection by *Beauveria bassiana* was often recorded in *T. leucotreta* pupae found amongst the leaf litter in Zebediela Citrus Estate, Northern Province, South Africa. Fungi with broad host ranges, such as *B. bassiana*, are frequently even more specific under field conditions because of complex abiotic and biotic factors, thus reports from Van den Berg (2001) that natural infections have already been observed in *T. leucotreta* illustrates a potential for control strategies in these orchard environments. Begemann (2008) studied the potential use of *B. bassiana* against the subterranean life stages of *T. leucotreta* in South Africa and concluded

that, under natural conditions, the final instar migrating larvae become exposed to the fungus when leaving the fruit to pupate in the soil. Despite limited reports of epizootics of fungal disease of fruit flies in nature (Ekesi *et al.* 2007) numerous studies have demonstrated the potential success of soil treatments with EP fungi for control management strategies against fruit flies (Ekesi *et al.* 2002; Ekesi *et al.* 2005; Mochi *et al.* 2006; Ekesi *et al.* 2007).

Accurate confirmation of fungal performance through the employment of bioassays is an important step in the development of a mycoinsecticide. A 'biological assay' is defined as *"the measure of the potency of a stimulus (chemical, physical or biological) by means of the reactions that it produces in living matter"* (Hatting & Wraight 2007) and is employed to test the physiological host range of a particular fungal isolate against insect hosts under controlled conditions (Finney 1979). When using EP fungi as the *stimulus* against insect pests, the most common infectious units employed are the conidia or the yeast-like hyphal bodies (blastospores) and the intensity of response among the treated insects is used as the measure of the effect of this stimulus or "*the disease power of the microorganism*" (Hatting & Wraight 2007). The median lethal dose (LD_{50}) is the most precise expression of virulence. However, this is very difficult to determine and often fungal virulence is expressed as a concentration (Hatting & Wraight 2007) and most commonly the median lethal concentration (LC_{50}) required to kill 50% of the test insect population is employed. Median lethal time (LT_{50}) is used to quantify the activity of virulent fungi and is important in expressing the time until insect death (which is important to farmers). Hatting and Wraight (2007) report that lethal time is proposed as *"the period of exposure to a pathogenic stimulus which will produce death in half the test subjects. The length of exposure is a direct measure of the dosage, and an increase in the period of exposure results in an increased uptake and true dose in the same ratio..."* Further understanding of bioassay procedure and the practice of statistical inference can be obtained in Finney (1979).

Experimental accuracy is absolutely crucial in bioassay procedures and the effects of what may go wrong are discussed in detail by Hatting & Wraight (2007). Not only is the accuracy of the bioassay important, but there are essential factors which need to be considered in conjunction with these assays. These factors include issues such as attenuation of fungi due to repeated sub-culturing in the laboratory. Wang *et al.* (2005) reported spontaneous phenotypic degeneration which resulted in sterile fungal culture sectors when sub-culturing *Metarhizium anisopliae* on artificial media *in vitro* (Fig. 3.1). Fungal strains which are continually grown on artificial media can show culture degradation which results in either a loss or reduction in sporulation and

virulence, and cultures are often observed with a fluffy-mycelium type growth (Wang *et al.* 2005). Unfortunately, fungal culture degradation is irreversible and inheritable and can result in extensive commercial losses (Wang *et al.* 2005). These authors attempted to grow sterile fungal cultures (those indicative of colony sectorization) on host-related media. However, this did not result in the recovery of wild-type characteristics nor was rejuvenation experienced. They concluded that perhaps these data may alleviate the long standing dispute as to whether passage through insect hosts can or cannot restore the virulence of attenuated EP fungal strains. Hatting & Wraight (2007) however, reported that numerous studies have demonstrated successful restoration of virulence in *M. anisopliae, B. bassiana* and *Isaria farinosa*, following passage through an insect host. Furthermore, Dimbi *et al.* (2004) reported that the virulence of *Metarhizium* strains was maintained by regular passage through insect hosts during sub-culturing and maintenance of fungal strains. However, in the laboratory, isolates of fungi with broad host ranges are generally considered most virulent to the host insect from which they were first isolated (Goettel 1995). So the question of whether passage through any insect host would maintain this virulence needs to be considered. Brownbridge *et al.* (2001) reported that the *B. bassiana* GHA strain, which is the active ingredient of the commercial products, BotaniGard® and Mycotrol®, after being passed through three different insect host species, maintained its virulence and stability *in vitro*. Further, Aemprapa (2007) noted that no fungal isolates of dipteran origin were used in the screening of fungi against *Bactrocera dorsalis* in Thailand, and yet all fungi were highly pathogenic towards this fly species. This suggests that fungi from any insect origin would have the potential to elicit a high level of pathogenicity against test insects, provided they are susceptible.

Standardization of the growth substrate is important to reduce variability among fungal isolates during bioassay procedure (Hatting & Wraight 2007), and it is important that EP fungal species receive the best nutrition *in vitro* at temperatures optimal for growth, germination and sporulation. The use of Sabouraud Dextrose Agar (SDA) which contains peptone (10 g), dextrose (40 g) and agar (10 g), is often used in the isolation and maintenance of most hyphomycete fungal strains, and can be further supplemented with 0.1% yeast extract and glucose (10 g) (Brownbridge *et al.* 2001; Meyling 2007).

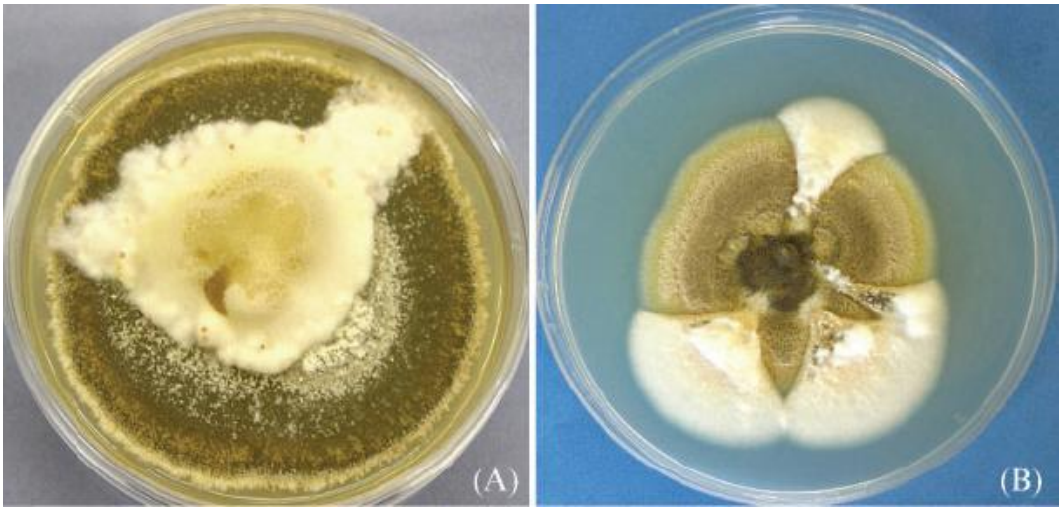

Figure 3.1 A, B Different types of culture colonies of *Metarhizium anisopliae* indicating sterile sectorization and the restoration of conidiation in some areas (Wang *et al.* 2005).

The ratio of antibiotics and fungicides in the media, which are used to reduce the amount of bacteria and saprophytic fungal species growth when culturing EP fungi *in vitro,* are also essential. Hu & St Leger (2002) found that by using Veen's semi-selective media minus the fungicide cyclohexamide, more species of EP fungi were observed from collected soil samples than when the fungicide was included in the medium. The discovery that dodecylguanidine monoacetate (dodine) can be used to selectively inhibit non-EP fungi has simplified isolation of EP fungi and enhanced their maintenance on artificial media (Jaronski 2007). The addition of strong concentrations (greater than 50 mg/L) of the antibiotic, Rifampicin, can reduce mycelia growth of indigenous isolates of EP fungi (personal observation). For more information regarding the isolation and culturing of EP fungi *in vitro* and the effects various media have on fungal growth factors, it is suggested that more classic works be referred to (Chase *et al.* 1986; Li & Holdom 1995; Ibrahim *et al.* 2002; Meyling 2007; Safavi *et al.* 2007).

Insect hosts factors such as development stage, age, sex and activity may influence insect response to EP fungi. Test insects must be selected carefully to limit the effects of these factors so as not to confound the effects of the conidial concentrations applied during bioassay procedures (Van Ark 1995). In a study by Begemann (2008), various concentrations of *B. bassiana* in suspension and as dry preparations were tested against the final instar larvae of *T. leucotreta*. The author found that non-migrating larvae illustrated a dose-dependant response with LC_{50} and LC_{90} values in the range of 5.2×10^4 to 1.0×10^6 and were more susceptible to the

fungus. There was however no dose-dependant response observed for migrating *T. leucotreta* larvae and this was attributed to lowered susceptibility by migrating larvae, possibly due to the moulting process already taking place.

When considering fruit flies as tests insects during conidial-sand incorporation bioassays, it is crucial to use third instar larvae which are 'jumping'. This indicates fitness of the test insect and ensures that the correct life stage has been utilised. Hatting & Wraight (2007) further suggest that homogenisation (thorough mixing) of stock suspensions of conidia or blastospores, and the investigation of the viability of conidia (generally measured in terms of percentage germination), are also important deliberations during bioassay preparation. Consideration of all the abovementioned factors may reduce the amount of variation within and among fungal isolates tested in bioassays.

As a group, fungi have the widest host ranges among insect pathogens, but host ranges vary considerably depending on fungal species (Inglis *et al.* 2001). An attribute of good fungal BCA's includes that their host ranges are not too specific, as is case with some of the entomophthoralean species of fungi which infect only one genus of insect, as in *Formica* ants (Boer, 2008) or the fungus *Nomuraea rileyi* which infects Lepidoptera in the Noctuidae (Inglis *et al.* 2001). Fungal genera such as *Beauveria* and *Metarhizium* are known for their broad host ranges which is desirable in being able to knock down more than one species of insect (Goettel 1995). Concurrently, insect pests should exhibit susceptibility to fungal pathogens and occupy similar niches in the environment so as to increase the advantage of fungal BCA's and their use (Ekesi *et al.* 2002).

Other important considerations are virulence (which is the quantitative amount of disease a pathogen can stimulate in insect hosts) and subsequent speed of kill. Fungal strains which are more virulent will require less inoculum in the environment and production of these strains will therefore be cheaper (Inglis *et al.* 2001). The use of genetic engineering by the directional addition of one or more genes coding for pathogenicity is the most common approach to shifting the physiology of EP fungi to enhance virulence and has been described in greater detail by St Leger & Screen (2001). A high level of sporulation of fungal cultures, either on host cadavers or on artificial media, is also considered a desirable trait because each pupa dying in the soil constitutes an infection site. The more prolific the conidial growth on the dying host, the more easily the increased inoculum is dispersed (Ekesi *et al.* 2002) and the more desirable the fungal

isolate becomes when commercialisation is considered (Brownbridge *et al.* 2001). Increasing evidence suggests that while it may be important to obtain highly infective and virulent strains of fungi to control insect pests, it is even more important to look for isolates which are also capable of surviving and persisting in the environments and habitats for which they are required (Bidochka *et al.* 2001; Bidochka *et al.* 2002). A comprehensive list of environmental considerations of fungal BCA's is listed in Chapter I.

Soil moisture is considered an important environmental factor in determining efficacy of fungal isolates when controlling subterranean life stages. At 20-30°C, mortality in pupae was highest at a water potential of -0.1 and -0.01 mega Pascal (MPa) and lowest at a water potential of -0.0055 and -0.0035 MPa (Ekesi *et al.* 2003). This is important in the use of EP fungi as an integrated pest control tactic because of the large amounts of water required in citrus production (Wellington 1960). Some studies have shown that soil moisture content in excess of 30% moisture holding capacity is detrimental to *B. bassiana* longevity in the soil. This could be attributed to reduced oxygen concentrations in wet soil as well as higher bacterial decomposition of organic matter, which is known to occur in soils with water potential approaching zero (Ekesi *et al.* 2003). Jaronski (2007) observed that the infectivity of four *B. bassiana* isolates against the Southern Corn Rootworm, *Diabrotica howardii*, was inversely proportional to soil moisture above 25% field capacity, but that the controls were unaffected, implying that soil moisture affected the fungal infection process. Thus, it is crucial during bioassay procedure that appropriate soil moisture levels are maintained, preferably using moisture levels which would be indicative of a field scenario.

Consideration of all the abovementioned points will ensure, to a large degree that the correct assumptions are made about environmental factors which affect fungal performance and subsequent variation which may occur among replicates during bioassay procedures. It further ensures that the physiological effects and host range of various indigenous fungal isolates are correctly quantified for the control of subterranean life stages of citrus insect pests.

The aims of this chapter were 1.) To compare the pathogenicity of 21 indigenous isolates of EP fungi against *T. leucotreta, C. capitata* and *C. rosa* final instar larvae using sand-conidial suspension incorporation bioassays. These tests were essentially screening trials to determine the most virulent fungal isolates, which had broad host ranges and were capable of infecting all three insect test species; and 2.) Test the four most virulent isolates, and a commercially available

product Bb Plus® (BCP, South Africa) (which has *Beauveria bassiana* as the active ingredient) against the three citrus pests using various concentrations of fungal conidia incorporated into sand to ascertain the LC_{50} and LC_{90} values of each isolate; and 3.) Test the three most virulent fungal isolates which were highly pathogenic to *T. leucotreta* in concentration-response trials against final instar *T. leucotreta* larvae using four different exposure times, 12, 48, 72 and 96 hrs.

3.2 METHODS & MATERIALS

3.2.1 Fungal cultures

The pathogenicity of 15 *Beauveria bassiana*, five *Metarhizium anisopliae* and one *Metarhizium flavoviride* isolates, obtained using the insect-bait method (Table 3.2.1; cf Chapter II: Figs 2.4.1-2.4.6), were tested against *T. leucotreta*, *C. capitata* and *C. rosa* final instar larvae. All isolates, except one (RHO *G B Ar 23 B3*), are currently housed in the South African National Collection of Fungi at the Plant Protection Research Institute (Agricultural Research Council) in Pretoria, South Africa. Fungal isolates used in this study were grown on Sabouraud Dextrose Agar (SDA) supplemented with 1 ml Dodine, 50 mg/L Chloramphenicol, 50 mg/L Ampicillin or 50 mg/L Rifampicin in Petri dishes and maintained at an ambient temperature of 25°C. Fungal isolates are labelled according to the bait insect species they were originally isolated from, followed by the name of the farm and then the orchard or refugia soil sample number.

Table 3.2.1 Twenty one indigenous EP fungal isolates tested against *Thaumatotibia leucotreta, Ceratitis capitata* and *C. rosa* final instar larvae

Fungal Species	Rhodes Code	Isolate No.	Host Species	Locality	Soil Type	Co-ordinates	Date of Isolation
B. bassiana	FCM 11 3 B2	PPRI 9592	*T. leucotreta*	Mosslands	Oakleaf Caledon	33°23′54″S; 26°25′41″E	April 2008
	G 14 2 B5	PPRI 9555	*Galleria*	Mosslands	Oakleaf Richie	33°23′54″S; 26°25′41″E	May 2008
	G Moss R12	PPRI 9593	*Galleria*	Mosslands	Sandy red soil	33°23′54″S; 26°25′41″E	May 2008
	FCM 10 13 L1	PPRI 9680	*T. leucotreta*	Mosslands	Oakleaf Caledon	33°23′54″S; 26°25′41″E	April 2008
	G Moss R1	PPRI 9774	*Galleria*	Mosslands	Unknown	33°23′54″S; 26°25′41″E	June 2008
	G Moss R10	PPRI 9557	*Galleria*	Mosslands	Sandy red soil	33°23′54″S; 26°25′41″E	June 2008
	G Moss R7	PPRI 9554	*Galleria*	Mosslands	Loam	33°23′54″S; 26°25′41″E	May 2008
	G Ar 17 B3	PPRI 9679	*Galleria*	Arundel	Loam	33°30′57″S; 25°39′11″E	May 2008
	G B Ar 23 B3	RHO Ar 23 B3	*Galleria*	Arundel	Loam	33°30′57″S; 25°39′11″E	May 2008
	FF J&B R5	PPRI 9556	*C. capitata*	J&B Citrus	Unknown	32°45′56″S; 25°45′46″E	April 2008
	G J&B 14 L2	PPRI 9678	*Galleria*	J&B Citrus	Unknown	32°45′56″S; 25°45′46″E	June 2008
	G OL R10	PPRI 9691	*Galleria*	Olifantskop	Unknown	33°37′14″S; 25°40′49″E	July 2008
	G OL R11	PPRI 9690	*Galleria*	Olifantskop	Unknown	33°37′14″S; 25°40′49″E	July 2008
	G Rose 20 L2	PPRI 9687	*Galleria*	Rosedale	Oakleaf Limpopo	33°32′21″S; 25°41′39″E	August 2008
	FCM Rose R9	PPRI 9685	*T. leucotreta*	Rosedale	Unknown	33°32′21″S; 25°41′39″E	August 2008
M. anisopliae	G 14 2 B3	PPRI 9562	*Galleria*	Mosslands	Oakleaf Richie	33°23′54″S; 26°25′41″E	March 2008
	G 11 3 L6	PPRI 9803	*Galleria*	Mosslands	Oakleaf Caledon	33°23′54″S; 26°25′41″E	April 2008
	FCM AR 23 B3	PPRI 9561	*T. leucotreta*	Arundel	Loam	33°30′57″S; 25°39′11″E	April 2008
	G OL R3	PPRI 9800	*Galleria*	Olifantskop	Unknown	33°37′14″S; 25°40′49″E	July 2008
	G OL R8	PPRI 9801	*Galleria*	Olifantskop	Unknown	33°37′14″S; 25°40′49″E	July 2008
M. flavoviride	G Moss R2	PPRI 9560	*Galleria*	Mosslands	Unknown	33°23′54″S; 26°25′41″E	June 2008

PPRI = Plant Protection Research Institute Fungal Collection accession no **RHO** = Rhodes University accession no.
FCM = False codling moth, *Thaumatotibia leucotreta* **FF** = Fruit fly, *Ceratitis capitata* ***Galleria*** - *G. mellonella*

3.2.2 Preparation of conidial suspensions

Fungal conidia were harvested from 2 to 3 week old surface cultures by scraping with a glass rod. Spores were suspended in 20 ml sterile distilled water supplemented with 0.05% Triton X-100 in sterile glass bottles containing 3 mm glass beads. Bottles were sealed and vortex mixed for 2 minutes to produce a homogenous conidial suspension. Conidial concentrations were determined using a Neubauer haemocytometer following serial dilution in sterile distilled water and conidial suspensions were used within 3 hrs of enumeration. Viability of conidia was determined by spread plating 0.1 ml of conidial suspension (titrated to 1×10^7 conidia per ml^{-1}) on three SDA plates. A sterile cover slip was placed on each plate and incubated in complete darkness at ambient temperature of 25°C. Percentage germination was examined after 24 hrs from 100-spore counts on each plate (Ekesi *et al.* 2002).

3.2.3 Inoculation of insects

Sterile, dried river sand was sieved through a 16-mesh screen and transferred into 90 mm diameter Petri dishes. A standard concentration of 1 x 10^7 conidia ml^{-1} (5ml) was used to inoculate 50 g of sand, which was then vigorously mixed with a spatula. Controls were treated with sterilized distilled water containing 0.05% Triton X-100. Bioassays were also conducted at a higher dose concentration of 1 x 10^8 conidia ml^{-1} and were initiated using four fungal isolates which performed well at the lower concentration (1 x 10^7 conidia ml^{-1}). Twenty *T. leucotreta*, *C. capitata* and *C. rosa* final instar larvae, which would be ready to pupate within the next 24 hrs, were then introduced seperately into each Petri dish. The dishes were incubated at ambient temperature (25°C) under a photoperiod of L12: D12. Seven days after the introduction of larvae, pupae were removed from Petri dishes of treated sand and transferred into new Petri dishes containing sterilized, untreated sand. Sterilized plastic bottles were cut 15 cm below the neck of the bottle and used as individual emergence chambers. The Petri dishes were placed in trays filled with autoclaved Pearlite and covered with cut plastic bottles (Fig. 3.2.3A). For fruit flies, a cotton bud soaked in water and a small lid (3 x 3 cm) containing a 4:1 mixture of sugar and yeast hydrolysate, were introduced into the neck of the bottle and the emergence chamber respectively to provide food. For *T. leucotreta* adults, an 8% sugar solution was provided. Test insects were maintained at the same conditions as described above. The number of adult flies and moths that emerged from treated and control sand were recorded every second day until 10 days

after the first emergence. Records were also kept of the number of pupae that failed to emerge. Adult flies and moths that died during this period and pupae or larvae that failed to emerge were surface sterilized in 70% ethanol and transferred onto SDA (Fig. 3.2.3B). The criteria for scoring mycoses were (1) failure of pupae to emerge followed by fungal sporulation and (2) death of adults followed by fungal sporulation. Each treatment was replicated four times with 20 insects per replicate.

3.2.4 Concentration-response of citrus pests to EP fungi

In a similar set up as described above (Section 3.2.3), sand-conidial incorporation bioassays using four fungal isolates that were highly virulent to all three test insect species in previous bioassays were selected to assess the concentration-mortality relationship and to determine the lethal concentration LC_{50} and LC_{90} values for each fungal isolate. The registered microbial control product, Bb Plus® (BCP, South Africa) was also included as an additional isolate in the concentration-mortality trials. This product was stored and used according to the manufacturer's instructions. Conidial concentrations of the five isolates were determined using a Neubauer haemocytometer following five-fold serial dilution in sterile distilled water supplemented with 0.05% Triton X-100. Based on previous bioassays, four concentrations of fungal inocula were calibrated for bioassays with *T. leucotreta* with the following values: 1×10^4, 1×10^5, 1×10^6, 1×10^7 conidia ml^{-1}. For *C. capitata* and *C. rosa* the following concentration inocula were used 1×10^7, 1×10^8, 1×10^9, 1×10^{10} conidia ml^{-1}. It was determined that concentrations of 1×10^{11} and $1x10^{12}$ conidia ml^{-1} would most likely be needed for the fruit fly inoculums. However, due to the difficulty in obtaining enough conidial suspension volume, only the concentrations stipulated were used. Viability of conidia was determined by spread plating (as described in Section 3.2.2), and percentage germination was examined after 24 hrs from 100-spore counts on each plate. The criteria for scoring mycosis were undertaken as described in Section 3.2.3. Each treatment was replicated four times with 20 insects per replicate.

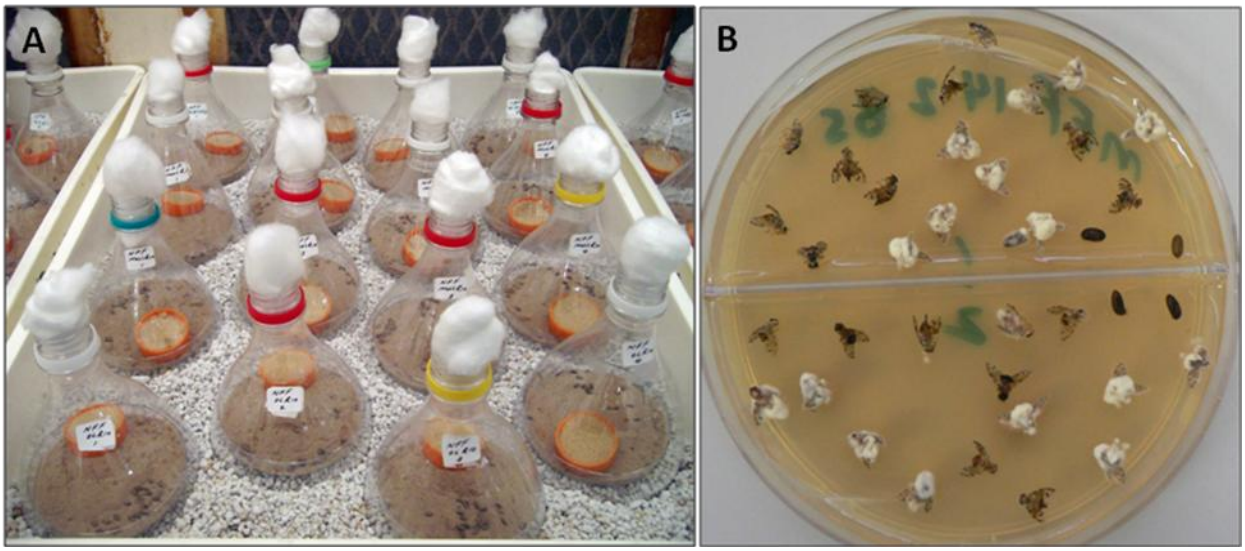

Figure 3.2.3 (A) Cut plastic bottles used as emergence chambers placed on Pearlite during bioassay experiments. Note the cotton wool bungs used to introduce water into the chambers. Orange lids were filled with 1:4 yeast hydrolysate and sugar mixture to feed flies **(B)** During the scoring of mycosis dead insects were placed onto SDA agar. (Photo Credits: T. Goble, Rhodes University).

3.2.5 Exposure time-response trial of Thaumatotibia leucotreta *to EP fungi*

In a similar experimental set up as described in Section 3.2.3, the exposure time-mortality relationship of three isolates which had the lowest LC_{50} values against *T. leucotreta* in the concentration-mortality trials (Section 3.2.4), were selected to be tested against *T. leucotreta* final instar larvae. Based on previous bioassays it did not seem meaningful to ascertain lethal exposure time (LT) values for fruit flies because concentration values were already far too high to be viable. *Thaumatotibia leucotreta* larvae were subjected to EP fungal isolates at four different time periods: 12, 48, 72 and 96 hrs after insect inoculation, to evaluate the effect of exposure time on the incidence of mycosis (mortality) using two concentrations of fungal conidia, 1×10^4 and 1×10^7 conidia ml^{-1}. Viability of conidia was determined by spread plating, as described in Section 3.2.2 and percentage germination was examined after 24 hrs from 100-spore counts on each plate. The criteria for scoring mycosis were undertaken as described in Section 3.2.3. Each treatment was replicated four times with 20 insects per replicate.

3.2.6 Statistical analyses

Percentage mortality data (Section 3.2.3) were corrected for control mortality (Abbott 1925) and the data were normalized through Arcsine transformation and then subjected to analysis of

variance (ANOVA) followed by mean separation by the Student-Newman-Keul's test (P=0.05). To investigate whether fungal isolates from orchards or natural habitats showed grouping in terms of pathogenicity, this mortality data was normalized through Arcsine transformation and subjected to ANOVA. Regression analysis was used (Section 3.2.4) to determine the functional relationship between log concentration of inoculum and probit of mortality (pupae and adults mycosis combined) using PROBAN (Van Ark 1995). PROBAN utilises a chi-squared (χ^2) test to determine the fit of a probit line and ascertains whether deviations from this line are homogenous. The program also calculates a G-value which is used to calculate the amount of response variation during bioassay procedures and in the calculation of the fiducial limits Percentage mortality data from preliminary exposure time-response tests (Section 3.2.5) were normalized through Arcsine transformation and then subjected to ANOVA.

3.3 RESULTS

In fungal conidial viability tests, germination for all isolates ranged from 88 to 95%. *Thaumatotibia leucotreta* final instar larvae treated with EP fungi did not always pupate normally because larvae were sometimes found mycosed (Fig. 3.3.1 A). For the purpose of analyses, those mycosed larvae were grouped with mycosed pupae and considered as one grouped life stage. Few *T. leucotreta* adults were found mycosed (Fig. 3.3.1 B) and for this reason only adult emergence and not adult mycosis was recorded. In both fruit fly species, *C. capitata* and *C. rosa*, all final instar larvae treated with fungi pupated normally but infection established in puparia and emerging adults. Total adult survival was recorded for fruit flies; these were recorded as total adult emergence minus mycosed adults.

3.3.1 The effects of EP fungi on **Thaumatotibia leucotreta** *adult emergence and pupal mycosis*

Thirteen EP fungal isolates had a significant effect in reducing *T. leucotreta* adult emergence to below 20%. Percentage adult emergence in the control treatment was 100%, and varied from 5 to 60% in fungal treated sand depending on fungal isolate (F=33.295; df=21; P=0.0001), with five *Beauveria* isolates showing a marked reduction in adult emergence (Fig. 3.3.2).

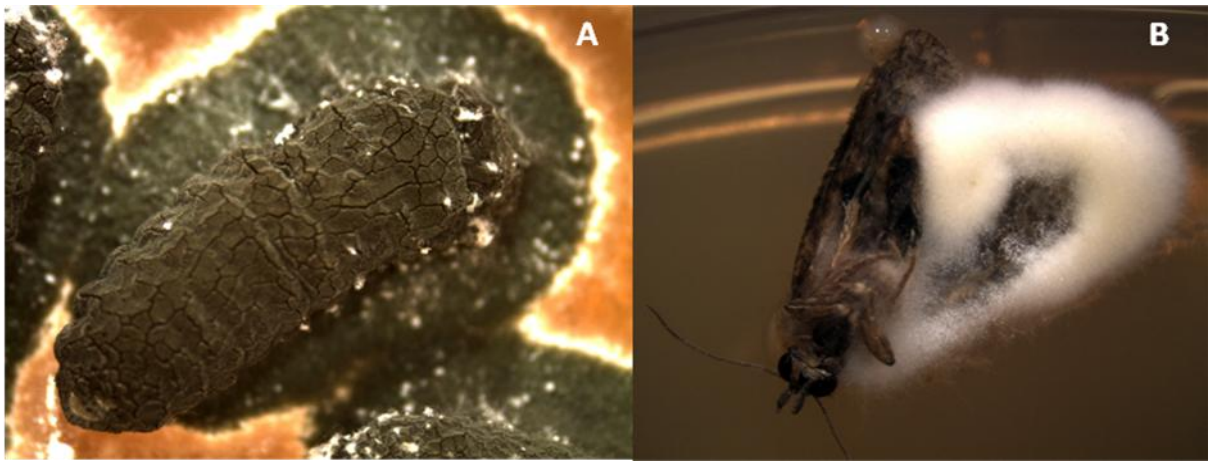

Figure 3.3.1 (A) A *Thaumatotibia leucotreta* wandering larva infected with *Metarhzium anisopliae* var. *anisopliae* **(B)** An adult *T. leucotreta* moth infected with *Beauveria bassiana* (all images were taken with a Leica EZ 4D dissecting microscope at ~ X16) (Photo Credits, T. Goble, Rhodes University).

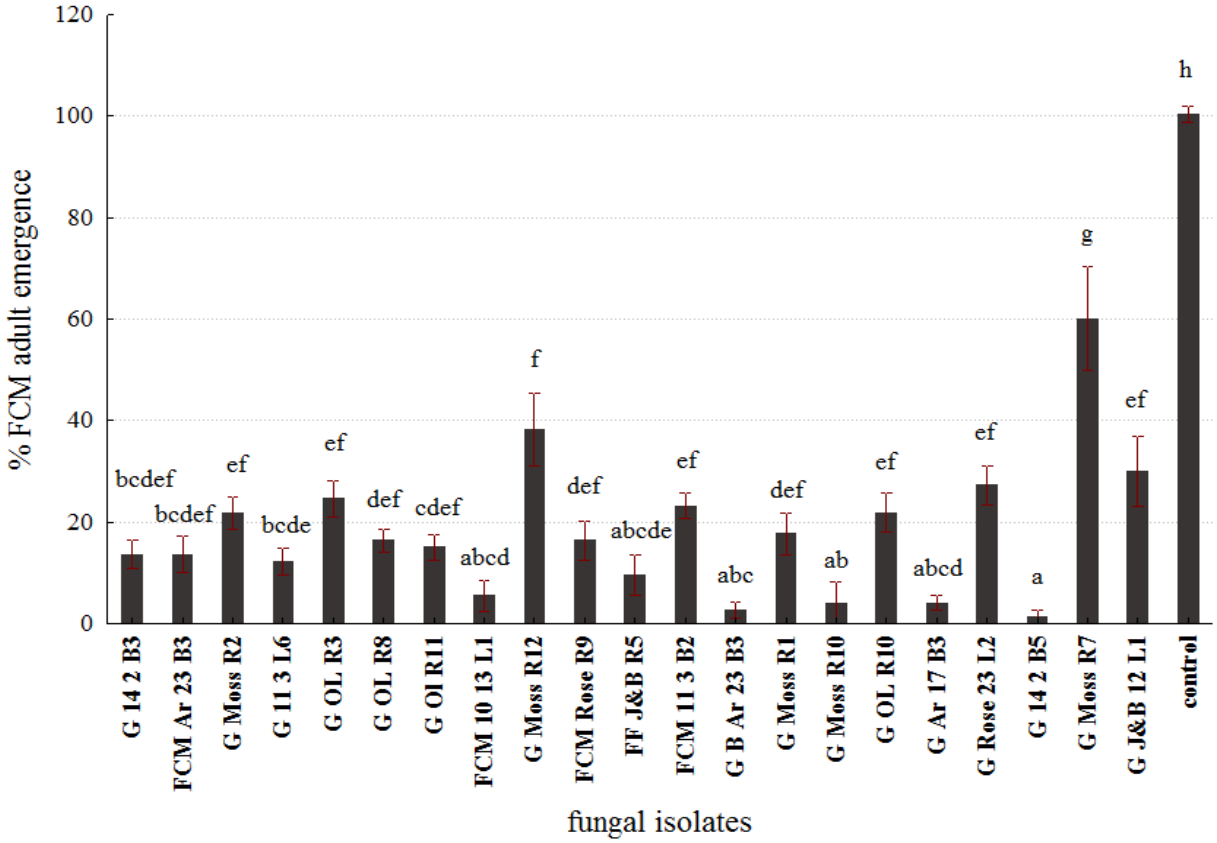

Figure 3.3.2 Pathogenicity of isolates of *Beauveria bassiana* and *Metarhizium anisopliae* to *Thaumatotibia leucotreta*: Mean (% ± SE) of adult emergence after treatment with a concentration of 1 x 10^7 conidia ml^{-1}. Bars with the same letter do not differ significantly by SNK test (P= 0.05).

The percentage of pupae with visible signs of mycosis ranged from 21 to 93% depending on the fungal isolate (F=96.436; df=21; *P*=0.0001) (Fig. 3.3.3). Four *Beauveria* isolates, *FCM 10 13 L1*, *G B Ar 23 B3*, *G Moss R10* and *G 14 2 B5* showed the greatest pathogenicity towards *T. leucotreta* as both a decrease in adult emergence and an increase in percentage pupal mycosis was observed (Fig. 3.3.3). The effect of fungal isolate habitat (either orchards or refugia) on the incidence of mycosis was investigated to determine if isolates obtained from natural habitats were more virulent towards *T. leucotreta*. Most orchard isolates seemed to show a greater incidence of mycosis. However, there were no significant differences observed in the incidence of mycosis by various fungal isolates grouped according to habitat ($F_{(1,19)}$=1.63; *P*=0.21).

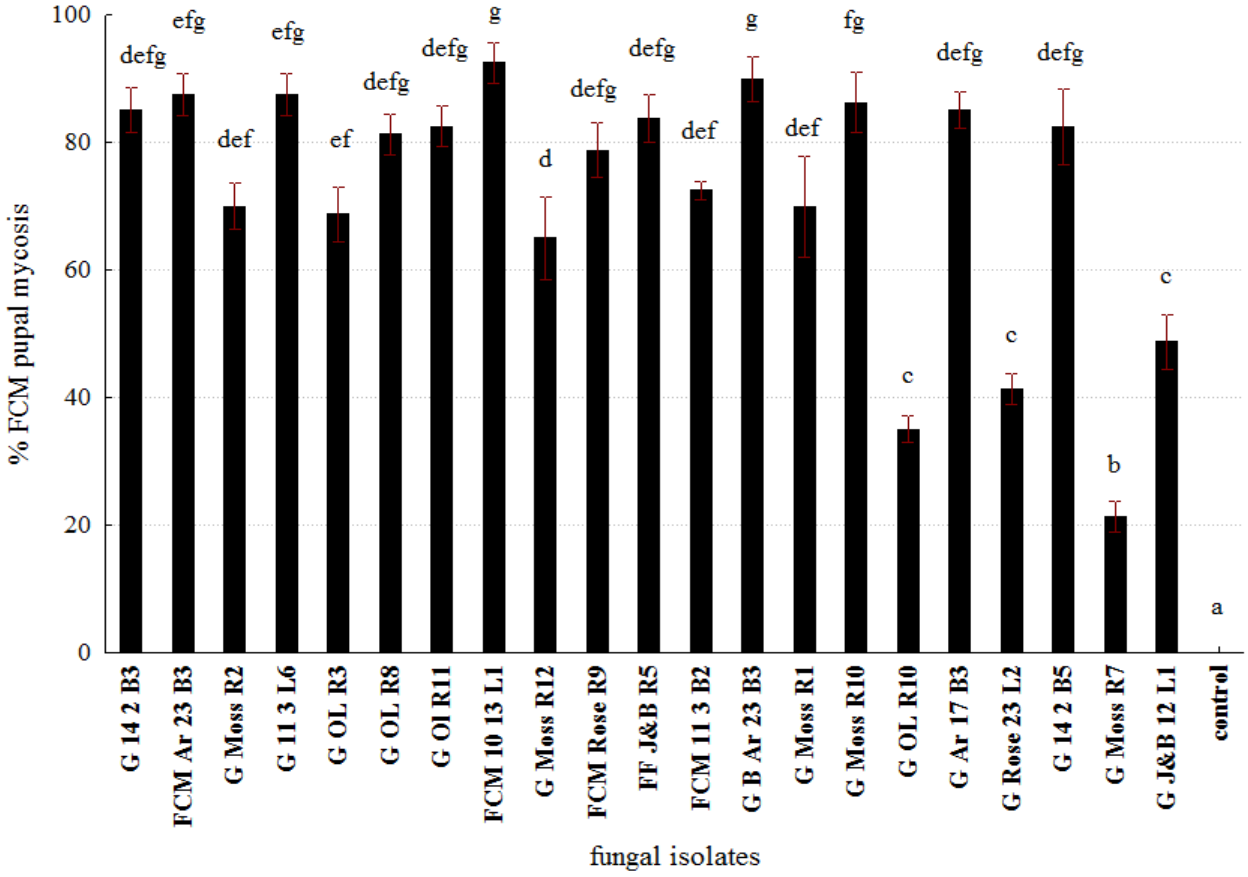

Figure 3.3.3 Pathogenicity of *Beauveria bassiana* and *Metarhizium anisopliae* to *Thaumatotibia leucotreta*: Mean (% ± SE) of pupal mycosis after treatment with a concentration of 1 x 10^7 conidia ml^{-1}. Bars with the same letter do not differ significantly by SNK test (*P*= 0.05).

3.3.2 The effects of EP fungi on Ceratitis rosa *and* C. capitata *adult survival*

When treating *C. rosa* and *C. capitata* final instar larvae to a fungal concentration of $1x10^7$ conidia ml^{-1}, percentage adult emergence in the control treatments were 88% and 86% respectively. Percentage adult survival in *C. rosa* ranged from 30 to 90% depending on the fungal isolate, while the percentage of *C. capitata* adults which survived various fungal treatments at this concentration ranged from 55 to 86% (Fig. 3.3.4). Two *Beauveria* isolates: *G OL R11* and *FCM 10 13 L1* had a significant effect on adult fruit fly survival compared to other isolates (F=10.70; df=12; *P*=0.0001). However, there were no significant differences observed in fruit fly species (*C. rosa* versus *C. capitata*) response to various fungal isolates (F=0.18; df=1; *P*=0.66) (Fig. 3.3.4).

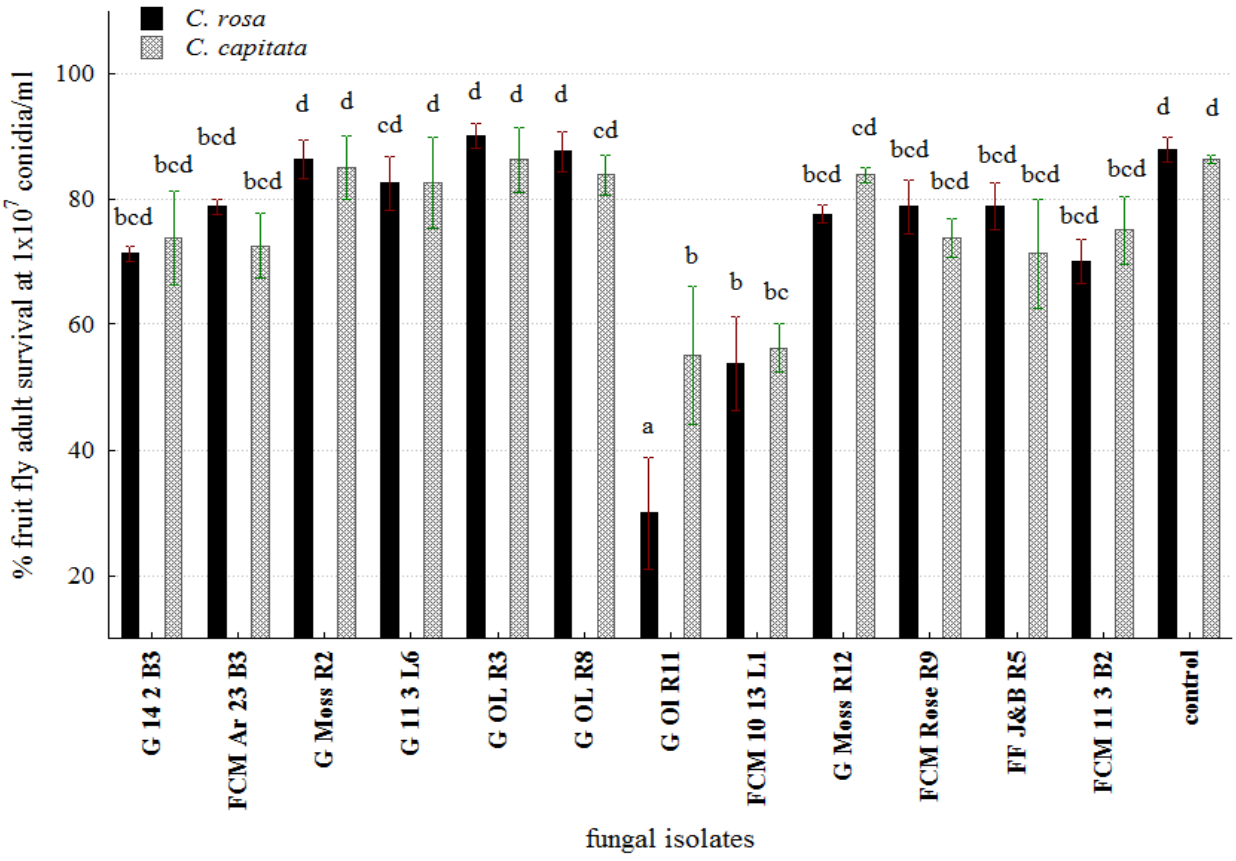

Figure 3.3.4 Pathogenicity of indigenous fungal isolates of *Beauveria bassiana* and *Metarhizium anisopliae* to *Ceratitis rosa* (black bars) and *C. capitata* (grey bars): Mean (% ± SE) of fruit fly adult survival after treatment with a concentration of 1 x 10^7 conidia ml^{-1}. Bars with the same letter do not differ significantly by SNK test (*P*= 0.05).

Compared to *T. leucotreta*, there was little effect on the reduction of fruit fly survival by fungal isolates in the previous trial, thus bioassays at a higher dose concentration of 1 x 10^8 conidia ml^{-1}, using fungal isolates (four isolates) which performed well at the lower concentration (1 x 10^7 conidia ml^{-1}), and by the addition of new fungal isolates (seven isolates) were initiated. Percentage adult emergence in the control treatments were 87 and 89% for *C. rosa* and *C. capitata* respectively (Fig. 3.3.5). Percentage adult survival in *C. rosa* differed significantly and ranged from 47 to 85% depending on the fungal isolate (F=6.46; df=11; *P*=0.0001), while the percentage of *C. capitata* adults which survived various fungal treatments also differed significantly (F=7.74; df=11; *P*=0.0001) and ranged from 34 to 84% depending on the fungal isolate (Fig. 3.3.5). Four *Beauveria* isolates: *G 14 2 B5, G Moss R10, G B Ar 23 B3* and *FCM 10 13 L1* had a significant effect on adult fruit fly survival compared to other isolates (F=14.03; df=11; *P*=0.0001). However, there were no significant differences observed in fruit fly species (*C. rosa* versus *C. capitata*) response to various fungal isolates (F=0.17; df=1; *P*=0.67) as both fruit fly species showed similar susceptibilities to the fungal isolates (Fig. 3.3.5).

In Fig. 3.3.5, there appeared to be a greater reduction in adult fruit fly survival at a higher conidial concentration. However, with the addition of new fungal isolates, these observed reductions were not due to the increase in concentration, but rather from the addition of more virulent fungal isolates. This was confirmed through the four fungal isolates (*G14 2 B3*, *FCM Ar23 B3*, *G OL R11* and *FCM 10 13 L1*), which were used in both bioassay trials at dose concentrations of 1 x 10^7 and 1 x 10^8 conidia ml^{-1} being analysed for significant interactions between concentration, insect species and fungal isolate. There was a weakly significant difference observed between the two fungal concentrations (F=5.30; df=1; *P*=0.02), with the weaker conidial concentration showing a greater reduction in fruit fly survival. However, there was again no significant difference observed in the adult survival response to fungal isolates by both fruit fly species (F=0.004; df=1; *P*=0.94), illustrating that both fruit fly species, *C. rosa* and *C. capitata*, responded similarly to the effects of EP fungal isolates. The fungal isolates had a significant effect on adult fruit fly survival with both *Beauveria* isolates, *G OL R11* and *FCM 10 13 L1*, showing a marked decrease in adult fruit fly survival compared to the two *Metarhizium* isolates *G14 2 B3* and *FCM Ar23 B3* (F=17.41; df=4; *P*=0.0001) (Fig. 3.3.5).

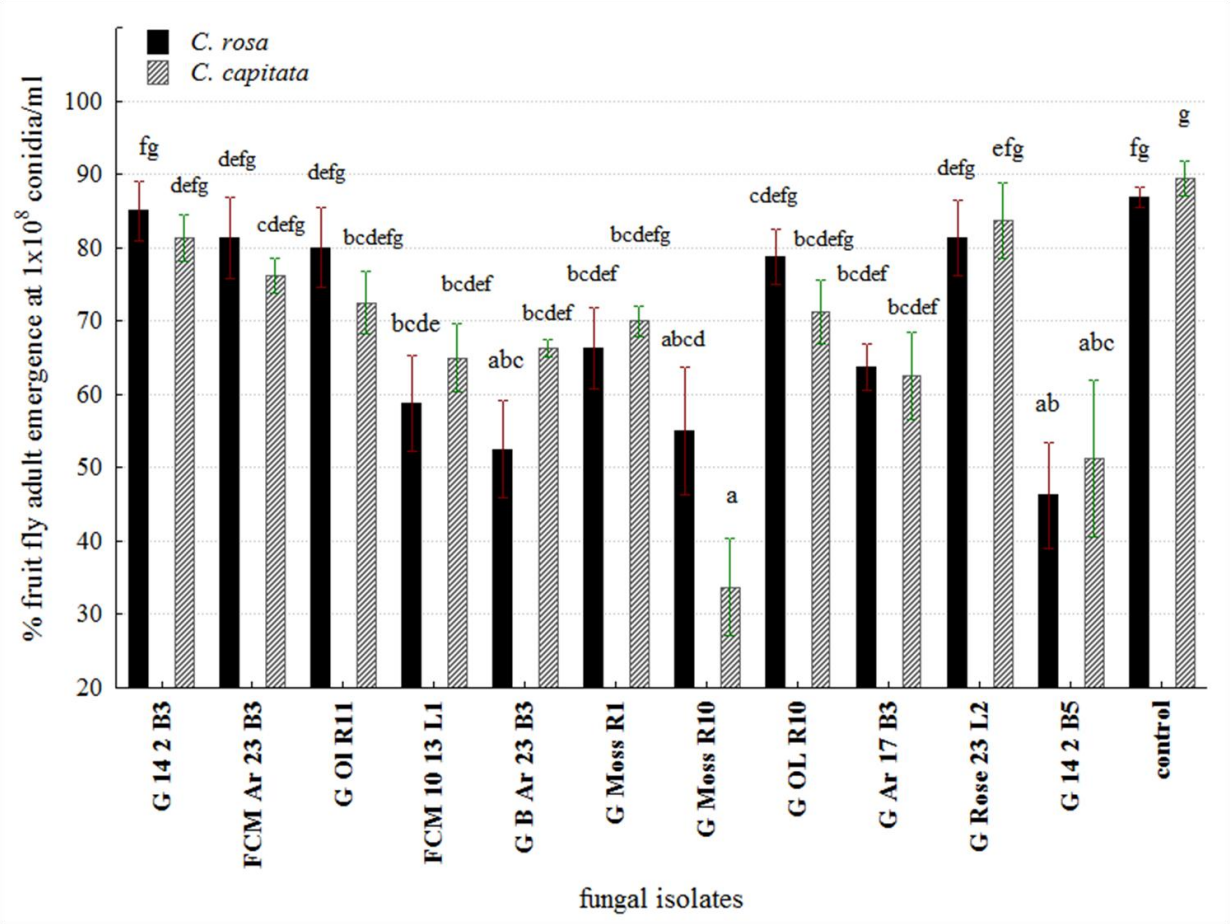

Figure 3.3.5 Pathogenicity of indigenous fungal isolates of *Beauveria bassiana* and *Metarhizium anisopliae* to *Ceratitis rosa* (black bars) and *C. capitata* (grey bars): Mean (% ± SE) of fruit fly adult survival after treatment with a concentration of 1 x 10^8 conidia ml^{-1}. Bars with the same letter do not differ significantly by SNK test (*P*= 0.05).

3.3.3 The effects of EP fungi on Ceratitis rosa *and* C. capitata *adult and pupal mycosis*

When treating *C. rosa* final instar larvae with a fungal concentration of 1 x 10^7 conidia ml^{-1}, the percentage of puparia with visible signs of mycosis ranged from 1 to 14% depending on the fungal isolate (F=4.37; df=12; *P*=0.0001) (Fig. 3.3.6 and Fig. 3.3.10). The *Metarhizium* isolate, *FCM Ar 23 B3*, was the most pathogenic towards *C. rosa* puparia at this concentration. Deferred mortality due to mycosis in *C. rosa* adult flies ranged from 1 to 58% depending on the fungal isolate (F=27.01; df=12; *P*=0.0001) (Fig. 3.3.6), with two of the *Beauveria* isolates, *G OL R11* and *FCM 10 13 L1*, showing the greatest pathogenicity towards *C. rosa* adults. Natural mortality in the untreated controls never exceeded 10%. EP fungal isolates had a significantly greater effect on the adults of *C. rosa* than they did on the puparia ($F_{(1,12)}$=13.11; *P*=0.0001) (Fig. 3.3.6). *Beauveria* isolates, *G OL R11*, *FCM 10 13 L1*, *G Moss R12*, *FCM Rose R9*, *FF J&B R5* and

FCM 11 3 B2 showed a noticeable increase in deferred adult mortality compared to the *Metarhizium* isolates, *FCM Ar 23 B3*, *G Moss R2*, *G 11 3 L6*, *G OL R3* and *G OL R8*.

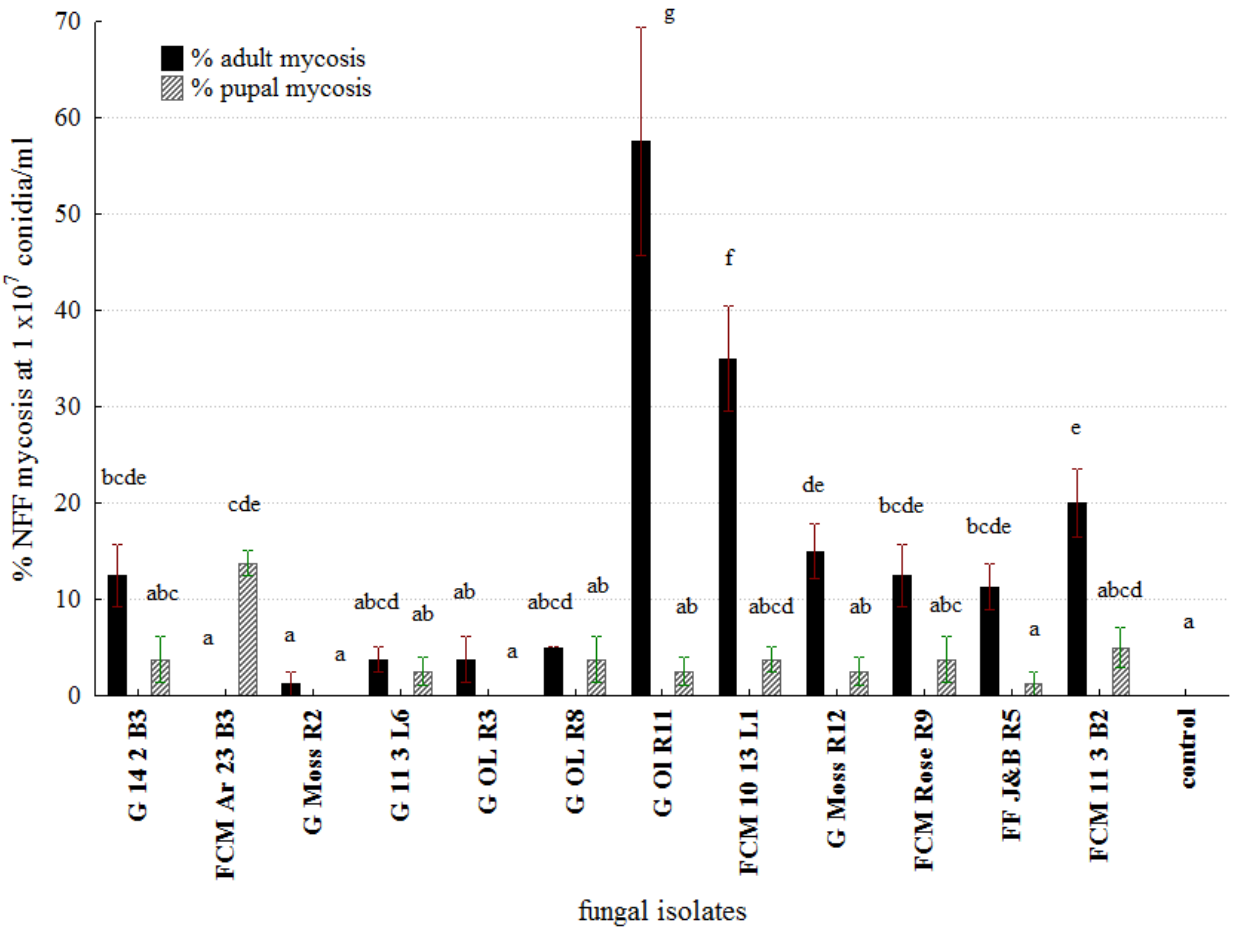

Figure 3.3.6 Pathogenicity of indigenous fungal isolates of *Beauveria bassiana* and *Metarhizium anisopliae* to *Ceratitis rosa:* Mean (% ± SE) of *Ceratitis rosa* adult (black bars) and pupal (grey bars) mycosis after treatment with a concentration of 1 x 10^7 conidia ml^{-1}. Bars with the same letter do not differ significantly by SNK test (*P*= 0.05). Note: Y-axis goes to 70%.

A subsequent bioassay, at a higher dose concentration of 1 x 10^8 conidia ml^{-1}, was initiated using four fungal isolates which performed well at the lower concentration (1 x 10^7 conidia ml^{-1}) and by the addition of seven new fungal isolates. The percentage of *C. rosa* puparia with visible signs of mycosis ranged from 1 to 25% (F=11.04; df=11; *P*=0.0001) (Fig. 3.3.7). One *Beauveria* isolate, *G 14 2 B5,* showed the greatest pathogenicity towards *C. rosa* puparia. Deferred mortality in *C. rosa* adult flies ranged from 3 to 32% depending on the fungal isolate (F=13.53;

df=11; *P*=0.0001) (Fig. 3.3.7), with two *Beauveria* isolates, *G B Ar 23 B23* and *FCM 10 13 L1*, showing the greatest pathogenicity towards *C. rosa* adults. Natural mortality in the controls never exceeded 10%. Fungal isolate had a significant effect on pupal and adult fruit fly mycosis ($F_{(1,11)}$=12.55; *P*=0.0001) with a greater percentage mycosis occurring in adults than puparia (Fig. 3.3.7). Four *Beauveria* isolates, *G14 2 B5*, *G B Ar23 B3*, *FCM 10 13 L1* and *G Moss R10*, showed the greatest potential as possible biological control agents as a significantly marked increase in adult and pupal mycosis was observed with these isolates.

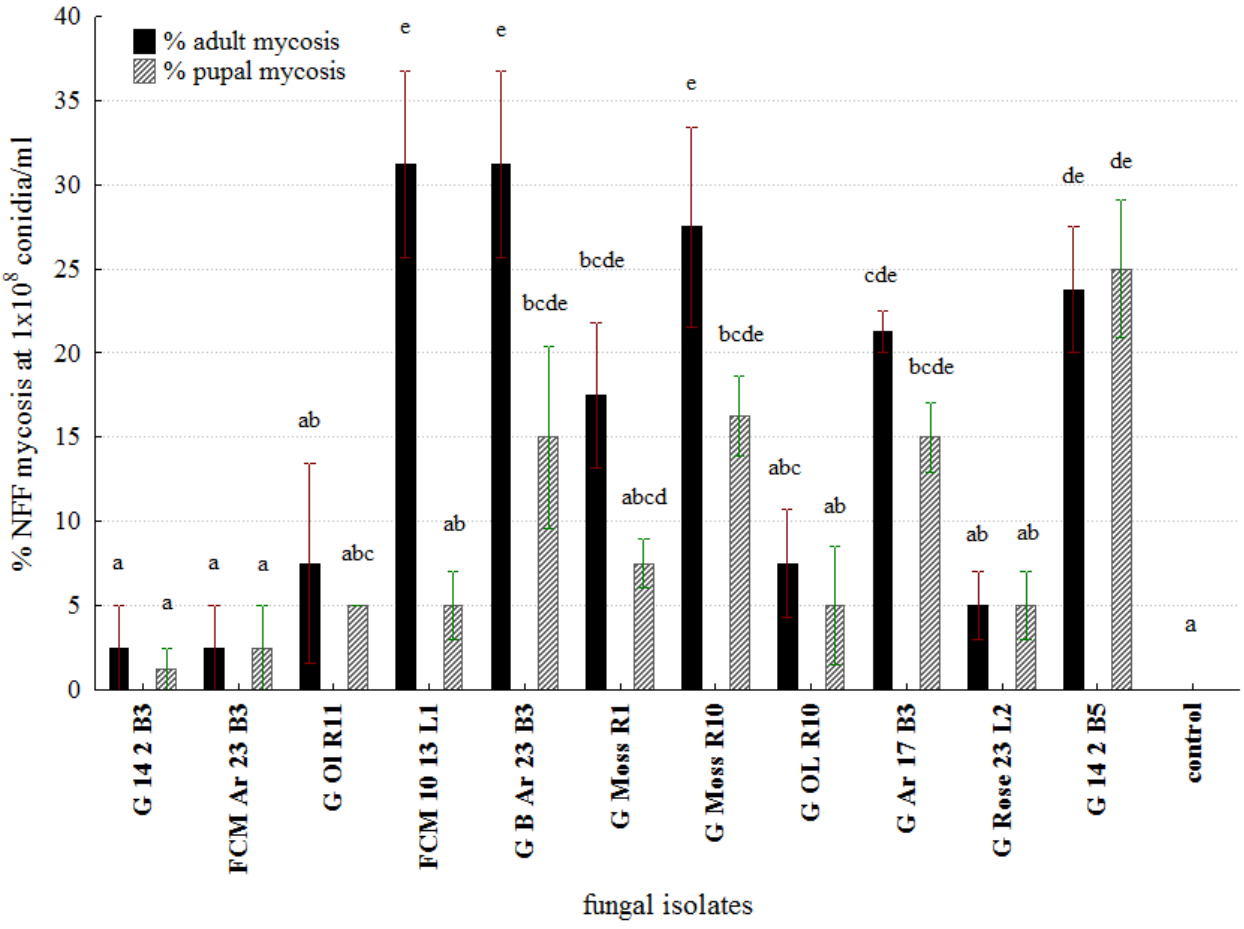

Figure 3.3.7 Pathogenicity of indigenous fungal isolates of *Beauveria bassiana* and *Metarhizium anisopliae* to *Ceratitis rosa:* Mean (% ± SE) of *Ceratitis rosa* adult (black bars) and pupal (grey bars) mycosis after treatment with a concentration of 1 x 10^8 conidia ml^{-1}. Bars with the same letter do not differ significantly by SNK test (*P*= 0.05). Note: Y-axis goes to 40%.

When treating *C. capitata* final instar larvae with a fungal concentration of 1 x 10^7 conidia ml^{-1}, the percentage of puparia with visible signs of mycosis ranged from 1 to 11% depending on fungal isolate (F=3.76; df=12; *P*=0.0005) (Fig. 3.3.8). The *Metarhizium* isolate, *FCM Ar23 B3*, and the *Beauveria* isolate, *G OL R11*, were the most pathogenic to the puparia of *C. capitata.* Deferred mortality in *C. capitata* adult flies ranged from 1 to 33% depending on fungal isolate (F=10.95; df=12; *P*=0.0001) (Fig. 3.3.8), with the same two *Beauveria* isolates, *G OL R11* and *FCM 10 13 L1*, exhibiting the greatest pathogenicity towards *C. capitata* adults, as was seen with *C. rosa* adults at the same concentration. Natural mortality in the untreated controls never exceeded 10%. EP fungal isolates had a significantly greater effect on *C. capitata* adults than on the puparia ($F_{(1,12)}$=3.17; *P*=0.0005) (Fig. 3.3.8).

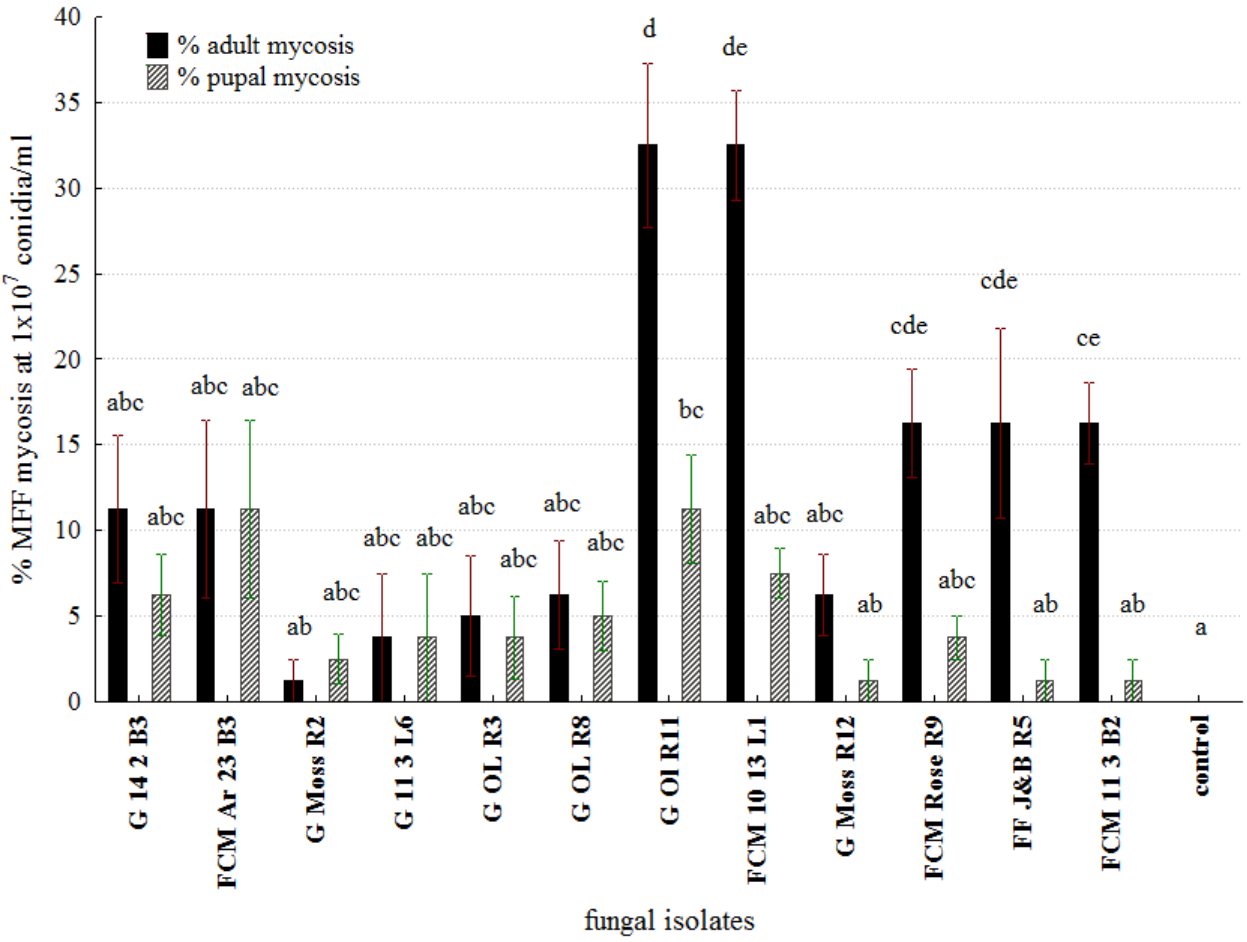

Figure 3.3.8 Pathogenicity of indigenous fungal isolates of *Beauveria bassiana* and *Metarhizium anisopliae* to *Ceratitis capitata:* Mean (% ± SE) of *Ceratitis capitata* adult (black bars) and pupal (grey bars) mycosis after treatment with a concentration of 1 x 10^7 conidia ml^{-1}. Bars with the same letter do not differ significantly by SNK test (*P*= 0.05).

In a subsequent bioassay using a higher concentration of 1 x 10^8 conidia ml^{-1}, four fungal isolates which performed well at the lower concentration (1 x 10^7 conidia ml^{-1}) were included and seven new fungal isolates were also incorporated. The percentage of *C. capitata* puparia with visible signs of mycosis ranged from 1 to 10% (F=2.68; df=11; *P*=0.01) (Fig. 3.3.9). One *Beauveria* isolate, *G Moss R10*, showed the greatest pathogenicity to *C. capitata* puparia in this bioassay. Deferred mortality in *C. capitata* adults ranged from 4 to 41% (F=8.31; df=11; *P*=0.0001) depending on the fungal isolate (Fig. 3.3.9), with two *Beauveria* isolates, *G Moss R10* and *G 14 2 B5*, showing the greatest pathogenicity towards *C. capitata* adults. Natural mortality in the untreated controls never exceeded 10%. When the interaction of life stage (pupal and adult mortality) and isolate were analysed, there was a significant difference observed with more adults than puparia exhibiting mycosis ($F_{(1,11)}$=4.19; *P*=0.0008) (Fig. 3.3.9).

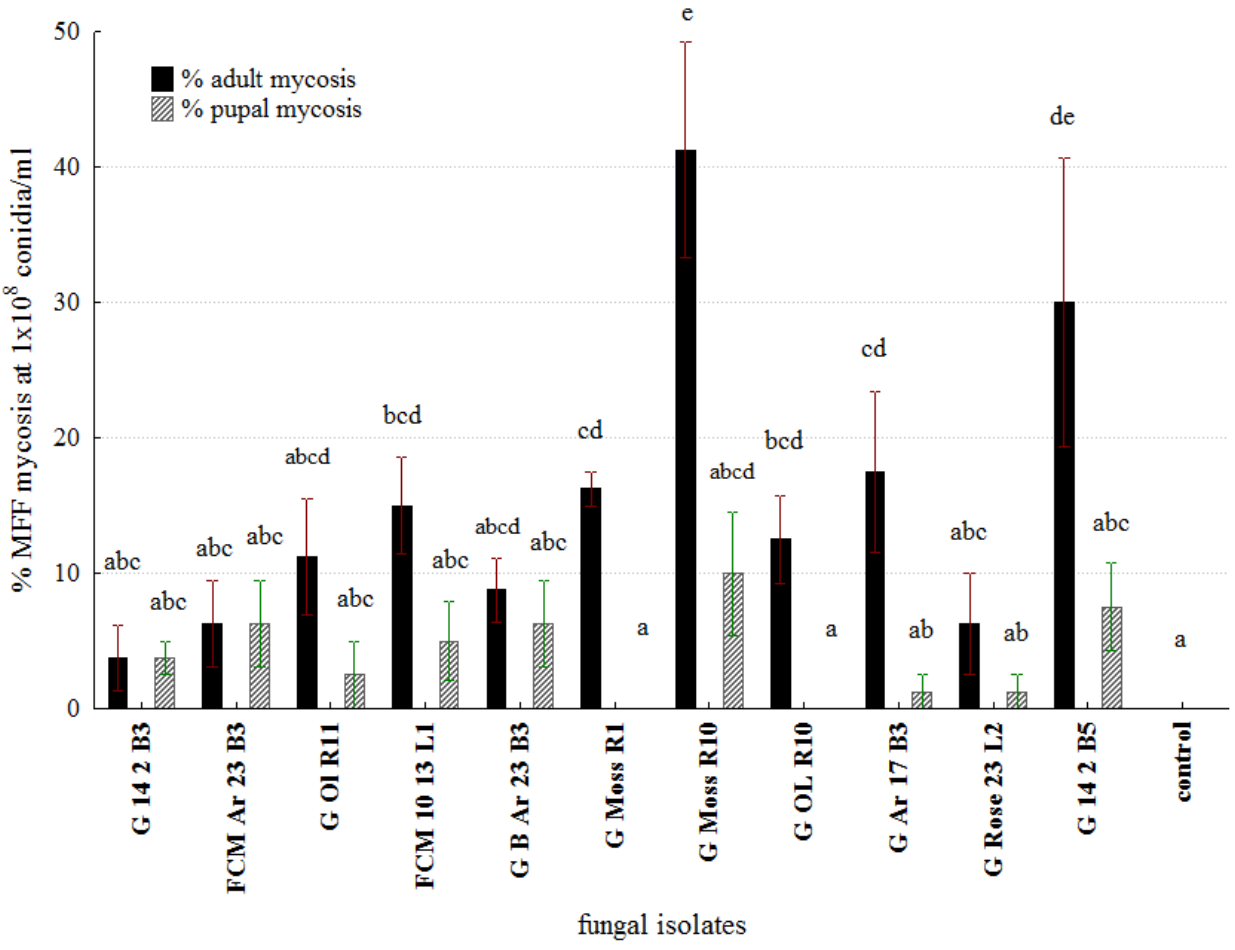

Figure 3.3.9 Pathogenicity of indigenous fungal isolates of *Beauveria bassiana* and *Metarhizium anisopliae* to *Ceratitis capitata:* Mean (% ± SE) of *Ceratitis capitata* adult (black bars) and pupal (grey bars) mycosis after treatment with a concentration of 1 x 10^8 conidia ml^{-1}. Bars with the same letter do not differ significantly by SNK test (*P*= 0.05).

The four fungal isolates which were used in both pupal and adult mycosis bioassay, against both fruit fly species, at dose concentrations of 1 x 10^7 and 1 x 10^8 conidia ml^{-1} were analysed to see if there were any significant interactions between dose concentrations, insect species and fungal isolates. A weakly significant difference was observed between the two conidial concentrations used (F=4.03; df=1; *P*=0.04), with the lower concentration showing a greater increase in adult and pupal mycosis. The interaction of concentration, insect species and stage was not significant (F=0.27; df=3; *P*=0.59) despite the fact that higher deferred adult mortality was observed than pupal mortality. There were no significant differences observed between *C. rosa* and *C. capitata* (F=0.003; df=1; *P*=0.95), illustrating again that both fruit fly species responded similarly to the effects of EP fungi.

The effect of isolate habitat (either orchards or refugia) on the incidence of mycosis (both adult and pupal) was investigated to determine if isolates obtained from natural habitats maybe more pathogenic towards fruit flies. At a conidial concentration of 1 x 10^7 conidia ml^{-1}, there were no significant differences observed in the incidence of mycosis by various fungal isolates grouped according to habitat ($F_{(1, 20)}$=0.02; *P*=0.87). At a conidial concentration of 1 x 10^8 conidia ml^{-1}, there were also no significant differences observed in the incidence of mycosis by various fungal isolates grouped according to habitat ($F_{(1, 18)}$=0.07; *P*=0.78).

Figure 3.3.10 (A) *Ceratitis capitata* pupa infected with *Metarhizium flavoviride* **(B)** *Ceratitis rosa* adult infected with *Beauveria bassiana* **(C)** *C. capitata* infected with *M. anisopliae* (all images were taken with a Leica EZ 4D dissecting microscope at ~ X16) (Photos Credits: T. Goble, Rhodes University).

3.3.4 The effect of fungal concentration on fruit fly mortality

In fungal viability tests, germination of conidia for *Beauveria* isolates, *G 14 2 B5*, *G Moss R10*, *G B Ar 23 B3*, *FCM 10 13 L1* and Bb Plus® ranged from 80 to 92%. The relative potencies of the five isolates were compared against one another as log-probit regressions of mortality against *C. rosa* and *C. capitata* (Fig. 3.3.11). For *C. rosa*, when the five fungal regression lines were compared against one another a positive relationship between increased conidial concentration and fruit fly mortality was observed (Fig. 3.3.11). When log-probit regression lines of mortality were compared, Bartlett's test found residual variances to be homogenous (χ^2=0.38; df=4; *P*=0.98) and the slopes of the lines were therefore comparable. The slopes of the lines were parallel (χ^2=0.25; df=4; *P*=0.99) and their elevations were significantly different ($F_{(4,14)}$=5.23; *P*=0.009), but when multiple comparisons of the elevations and slopes were undertaken using Bonferroni's method (*P*=0.005), no significant differences were observed between the five fungal isolates (Fig. 3.3.11).

The information regarding each fungal isolate probit line and the estimated LC_{50} and LC_{90} values for each fungal isolate tested against *C. rosa* are shown in Table 3.3.1. The estimated LC_{50} values of isolates *G B Ar 23 B3* and *G 14 2 B5* were lower than isolates *G Moss R10*; Bb Plus® and *FCM 10 13 L1*, indicating greater potency of the prior isolates. However, the slopes of the latter three isolates were steeper than both the former isolates indicating that target insects may be more vulnerable over a given time period to increasing concentrations of conidia.

With *C. capitata*, residual variances of the five fungal regression lines (Fig. 3.3.11) were homogenous (χ^2=0.13; df=4; *P*=0.99) and the slopes of the lines were compared. Slopes were parallel (χ^2=0.85; df=4; P=0.93) and the elevations were compared. The elevations of the lines were significantly different ($F_{(4,14)}$=5.72; *P*=0.006). However, when multiple comparisons of the elevations and slopes of the lines were compared (*P*=0.005), no significant differences were observed between the five isolates (Fig. 3.3.11). Despite there being no significant differences between fungal isolates, there were differences observed in the LC_{50} and LC_{90} values among the five isolates (Table 3.3.1) with *G B Ar 23 B3* and *G 14 2 B5* showing lower estimated LC_{50} values than those of Bb Plus®; *G Moss R10* and *FCM 10 13 L1*. Bb Plus® and *G B Ar 23 B3* had steeper slopes than the other isolates, indicating stronger concentration responses by the test insects.

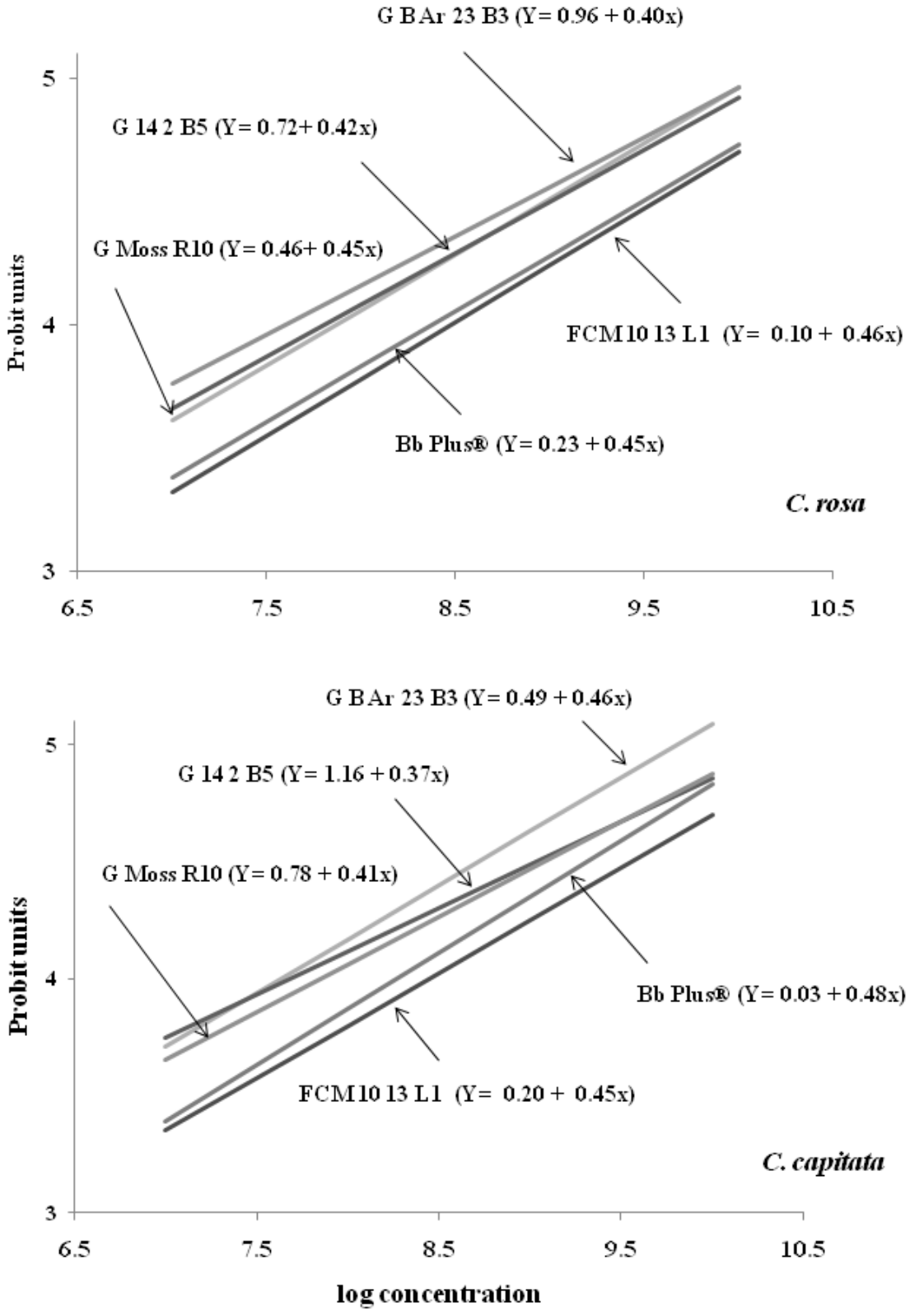

Figure 3.3.11 The log-probit regressions of mortality (pupal and adult combined) caused by five isolates of *Beauveria bassiana* against *Ceratitis rosa* and *C. capitata.*

Table 3.3.1 The calculated probit data of each of the five fungal isolates tested against *Ceratitis rosa, C. capitata* and *Thaumatotibia leucotreta*. Highlighted areas indicate isolates which were considered the most pathogenic by relative potency comparison tests using PROBAN.

Insect *spp*	Isolate	Fit of the Line		Intercept	R- Coefficient	Fiducial	Lethal Concentration			
		$\chi 2$	*P*	a	b	G	LC_{50}	LC_{50} (SE)	LC_{90}	LC_{90} (SE)
C. rosa	G B Ar 23 B3	1.65	0.44	0.96	0.40	0.17	7.3×10^{11}	5.2 ±	1.0×10^{15}	2.1 ±
	G 14 2 B5	1.24	0.53	0.72	0.42	0.17	8.9×10^{11}	6.3 ±	8.6×10^{14}	1.7 ±
	G Moss R10	0.25	0.87	0.46	0.45	0.17	1.0×10^{12}	7.2 ±	7.0×10^{14}	1.3 ±
	Bb Plus®	1.57	0.45	0.23	0.45	0.21	2.6×10^{12}	2.4 ±	1.7×10^{15}	4.0 ±
	FCM 10 13 L1	0.66	0.72	0.10	0.46	0.22	2.6×10^{12}	2.3 ±	1.5×10^{15}	3.2 ±
C. capitata	G B Ar 23 B3	0.38	0.82	0.49	0.46	0.14	5.5×10^{11}	3.3 ±	3.3×10^{14}	5.6 ±
	G 14 2 B5	0.40	0.81	1.16	0.37	0.21	1.3×10^{12}	1.1 ±	3.2×10^{15}	8.4 ±
	G Moss R10	0.50	0.78	0.78	0.41	0.20	1.5×10^{12}	1.2 ±	1.9×10^{15}	4.4 ±
	Bb Plus®	1.01	0.60	0.03	0.48	0.18	1.3×10^{12}	9.9 ±	5.7×10^{14}	1.0 ±
	FCM 10 13 L1	0.70	0.70	0.20	0.45	0.22	2.8×10^{12}	2.6 ±	1.7×10^{15}	4.2 ±
T. leucotreta	G B Ar 23 B3	0.73	0.69	3.26	0.33	0.18	1.6×10^{7}	9.4 ±	1.1×10^{11}	2.2 ±
	G 14 2 B5	1.86	0.39	3.17	0.46	0.11	8.8×10^{5}	5.9 ±	5.2×10^{8}	3.8 ±
	G Moss R10	2.44	0.29	3.19	0.47	0.12	6.8×10^{5}	4.8 ±	3.6×10^{8}	2.5 ±
	Bb Plus®	0.26	0.87	3.50	0.34	0.17	2.1×10^{6}	1.6 ±	1.1×10^{10}	1.5 ±
	FCM 10 13 L1	0.64	0.73	2.93	0.37	0.15	3.7×10^{7}	1.8 ±	1.0×10^{11}	1.8 ±

Both the LC_{50} and LC_{90} values of all five fungal isolates tested against *C. rosa* and *C. capitata* however fall outside of the tested concentration range (which was 1 x 10^7 to 1 x 10^{10} conidia/ml^{-1}), because the number of exposed insects at the highest conidial concentration only just exceeded 50% of the total response by all insects. Furthermore, there were large G-values observed for each isolate, indicating a wide variation in response by test insects (Table 3.3.1). Bonferroni's test showed no significant differences among the five isolates tested against fruit flies, indicating poor resolution between the relative potencies of these fungal isolates. LC_{50} values were higher than the highest concentration used in this study and the further use of increased conidial concentrations is impractical and unaffordable when translated into field required inoculums levels.

3.3.5 The effect of fungal concentration on **Thaumatotibia leucotreta** *mortality*

The concentration-mortality response of *T. leucotreta* to the five isolates was far greater than that observed for fruit flies (indicating a more susceptible insect species to the effects of these indigenous isolates of fungi). There was a positive relationship between increased conidial concentration and *T. leucotreta* mortality. Fig. 3.3.12 illustrates the plots of the probit regression estimates against concentration for the five fungal isolates tested against *T. leucotreta*. When the regression lines were compared, residual variances were homogenous (χ^2=0.568; df=4; *P*=0.96) and the slopes of the lines were compared. The slopes of the lines were found to be parallel (χ^2=2.821; df=4; P=0.59) and the elevations were compared. The elevations of the lines were significantly different ($F_{(4,14)}$=28.11; *P*=0.0001) and when multiple comparisons of the elevation and the slopes of the lines was undertaken using Bonferroni's test, mean separation (*P*=0.005) showed that the isolates *G Moss R10, G 14 2 B5* and Bb Plus® were significantly different from *G B Ar 23 B3* and *FCM 10 13 L1*. These differences were observed in the relative LC_{50} and LC_{90} values of each isolate are shown in Table 3.3.1. The slopes of the regression lines for isolates: *G Moss R10* and *G 14 2 B5* were steep, indicating a stronger concentration response to these isolates, than to Bb Plus®. A weak concentration response was observed against isolates: *G B Ar 23 B3* and *FCM 10 13 L1* (Fig. 3.3.12). The LC_{50} values of all isolates fell within the tested concentration range (which was 1 x 10^4 to 1 x 10^7 conidia/ml^{-1}) and the accuracy of these values remains intact. Only the LC_{90} values fell outside of the observed concentration range.

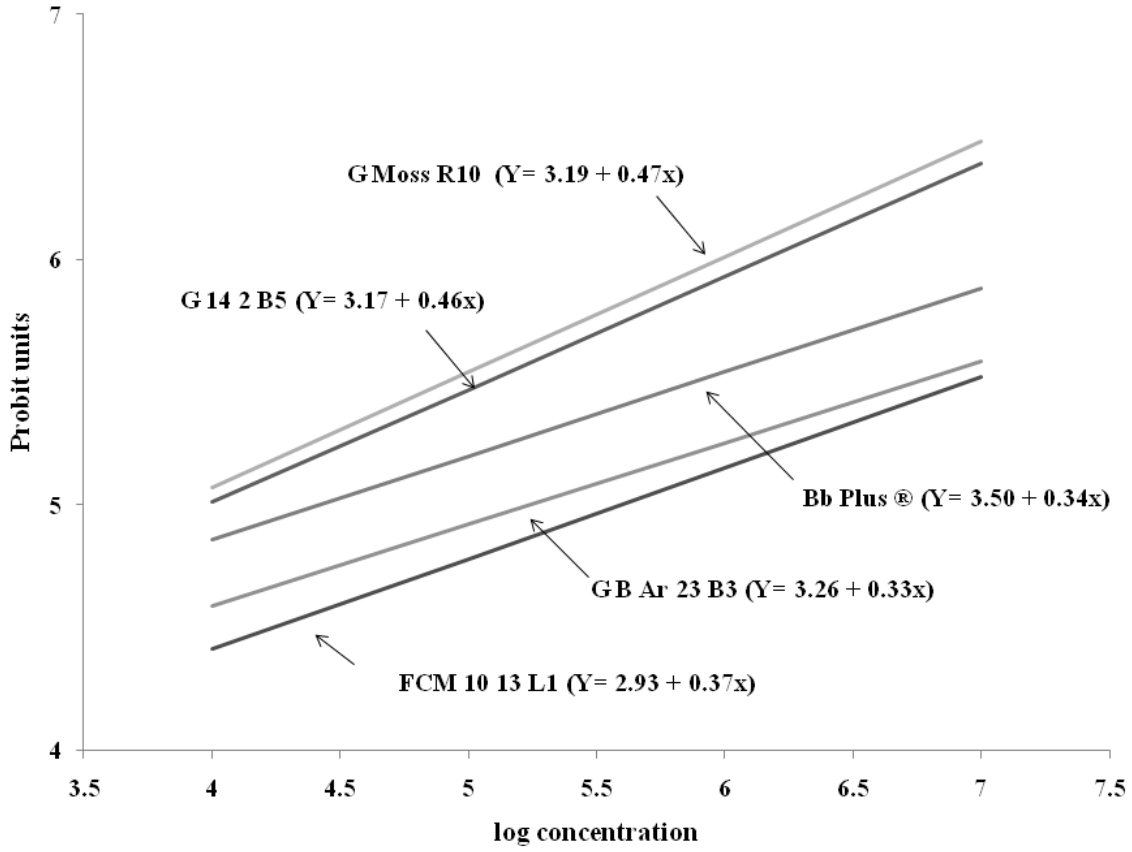

Figure 3.3.12 The log-probit regressions of mortality (pupal and adult combined) caused by five isolates of *Beauveria bassiana* against *Thaumatotibia leucotreta.*

3.3.6 Exposure time-mortality relationship study between EP fungi and Thaumatotibia leucotreta

In fungal viability tests, germination of conidia for three *Beauveria* isolates, Bb Plus®, *G Moss R10* and *G 14 2 B5* ranged from 82, 90 and 92% respectively. Exposure time-response trials of *T. leucotreta* final instar larvae, exposed to two conidial concentrations, at four different exposure times, showed a positive relationship. An increase in total percentage mycosis was observed with an increase in exposure time (F=5.43; df=3; *P*=0.001) (Fig. 3.3.13). At a higher concentration (1 x 10^7conidia/ml^{-1}) and shorter exposure time (12 hrs), percentage mycosis in *T. leucotreta* ranged from 22.7 to 46.2%, while at the longest exposure time (96 hrs), mortality due to mycosis ranged from 46 to 56.16% depending on the fungal isolate. At a lower concentration (1 x 10^4 conidia/ml^{-1}) across all time exposures, percentage mycosis ranged from 12.53 to 27% (Fig. 3.3.13). There was a significant difference observed in percentage mycosis between the two conidial concentrations, with the higher concentration resulting in a greater percentage mycosis (F=74.13;

df=1; *P*=0.0001). A significant difference was observed between the isolates at a higher concentration (F=3.51; df=2; *P*=0.03), with *G Moss R10* and *G 14 2 B5* showing greater percentage mycosis of pupae over time compared to Bb Plus®. At the higher concentration, isolates *G Moss R10* and *G 14 2 B5* elicited a response in 50% of test insects at 72 hrs (3 days) exposure (Fig. 3.3.13). No significant differences were observed between isolates when they were compared against one another at a lower concentration (F=1.33; df=2; P=0.27).

An outlier was observed in that a large percentage of pupae exposed to isolate *G Moss R10* were mycosed at 12 hrs. The interaction of fungal isolate over time was not significant because of the positive trend observed in mycosis with increased exposure time for all isolates. A significant increase in adult moth emergence was observed at 1 x 10^4 conidia/ml^{-1}compared to the higher concentration (F=49.38; df=1; *P*=0.0001), for which many more pupae were noted. These results are considered preliminary and could be used as a guideline for future work regarding exposure time-response in *T. leucotreta* using these fungal isolates.

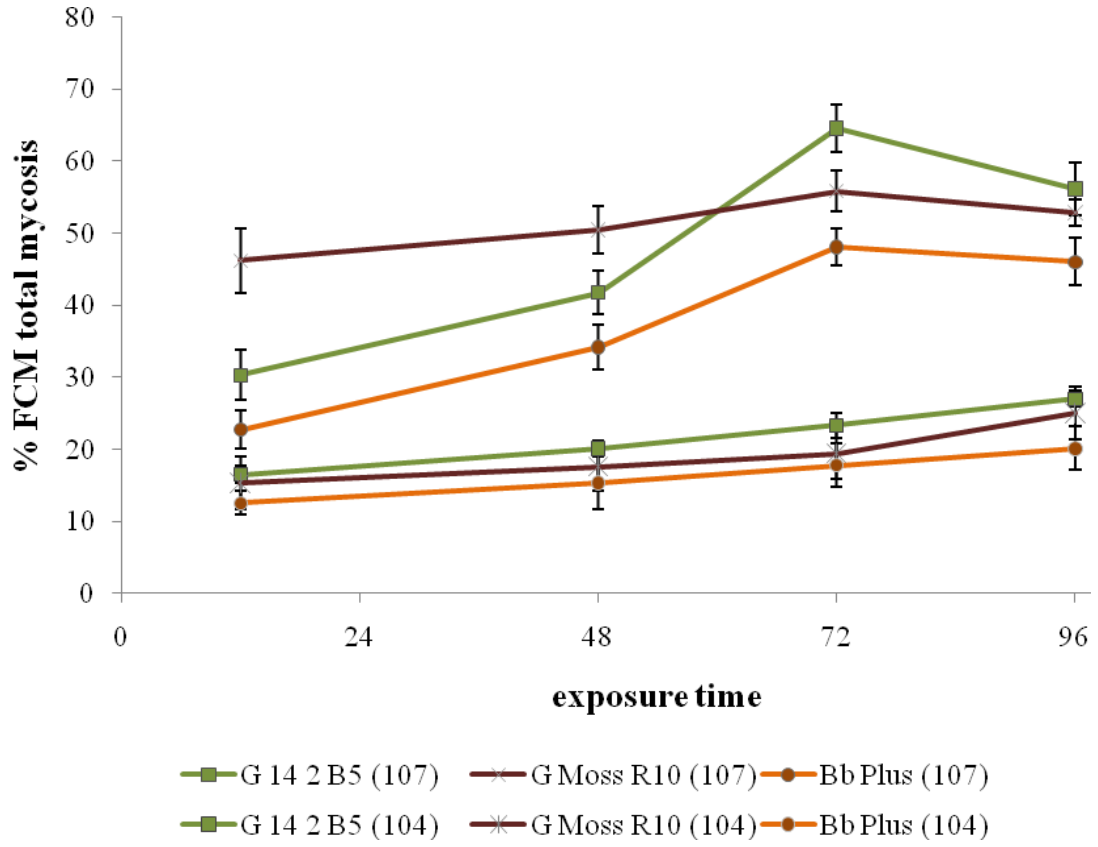

Figure 3.3.13 *Thaumatotibia leucotreta* total adult and pupal mycosis after final instar larvae were exposed to three fungal isolates at two concentrations, for 12, 48, 72 and 96 hrs time periods.

3.4 DISCUSSION

3.4.1 The effects of EP fungi on fruit flies, Ceratitis rosa *and* C. capitata

Twenty-one indigenous isolates of EP fungi were screened at conidial concentrations of 1 x 10^7 and 1 x 10^8 conidia/ml^{-1}. Five *Beauveria bassiana* isolates, *G Moss R10, GB Ar 23 B3, FCM 10 13 L1, G14 2 B5* and *G OL R11,* were able to reduce *C. capitata* and *C. rosa* adult survival to below 60%, however adult survival was generally high for most other EP isolates (greater than 80%). De la Rosa *et al.* (2002) also showed high adult emergence when Mexican fruit fly, *Anastrepha ludens* larvae were dipped into *B. bassiana* conidial suspensions of 1.6 x 10^8 conidia/ml^{-1} and then allowed to pupae on sand, from which between 92 to 98% adult emergence occurred. Similarly, the effects of *Metarhizium anisopliae* against the western cherry fruit fly, *Rhagoletis indifferens*, were determined when adults were exposed to fungal spores mixed into soil at concentrations ranging from 7.6 x 10^5 to 1.6 x 10^6 conidia/gram. The results revealed that adult emergence was not reduced (Yee & Lacey 2005). During fruit fly adult survival trials using 1 x 10^8 conidia/ml^{-1} in the present study, there appeared to be a greater reduction in the rate of survival. This was the result of the additional screening of more virulent isolates. The isolates, *G OL R11* and *FCM 10 13 L1,* which showed a marked decrease in adult fruit fly survival at 1 x 10^7 conidia/ml^{-1}, did not show the same resilience during bioassays conducted at a higher concentration (1 x 10^8 conidia/ml^{-1}). This was confirmed when analysing the four fungal isolates, *G14 2 B3, FCM Ar23 B3, G OL R11* and *FCM 10 13 L1,* which were used in both bioassay trials at the abovementioned conidial concentrations. A weakly significant difference was observed between the two fungal concentrations, with the weaker conidial concentration showing a greater reduction of adult fruit fly survival. This trend was further observed during pupal and adult mycosis trials against *C. capitata* and *C. rosa,* when *G OL R11* and *FCM 10 13 L1* elicited a greater amount of mycosis during bioassays at lower concentrations than subsequent assays at higher conidial concentrations. These variations were as a result of the marked increase in deferred adult mortality which subsequently affected the survival rate and percentage adult and pupal mycosis rate of fruit flies against these particular fungal isolates. A higher incidence of deferred adult mortality at lower concentrations may have been as a result of any of the three fungal inoculum transfer modes which are discussed in more detail below.

Indigenous isolates were not able to induce high mortality in *C. capitata* and *C. rosa,* puparia when they were exposed to sand treated with conidia suspensions relative to other studies (Castillo *et al.* 2000; Ekesi *et al.* 2002). During the initial fungal virulence screening trials, pupal

mycosis of both fruit fly species never exceeded 25% by any given fungal isolate, even at concentrations of 1 x 10^8 conidia/ml^{-1}. Toledo *et al.* (2006), in an investigation of various *Beauveria bassiana* and *Metarhizium anisopliae* isolates and formulated products against *Anastrepha ludens* and *C. capitata,* showed that EP fungi had no effect on the larvae or pupae of these fruit fly species but that all isolates were highly virulent to the adults. Similarly, De la Rosa *et al.* (2002) reported that the mortality caused by eight EP fungal isolates at a concentration of 1.6 x 10^8 conidia/ml^{-1} applied by dipping the immature stages of Mexican fruit fly, *A. ludens*, was low, 2 to 8% in larvae and 0% in pupae, however very high levels of adult mortality were noted against three *Beauveria* isolates causing 98 to 100% mycosis. In the evaluation of susceptibility of *Delia antique* (Anthomyiidae) to eleven isolates of hyphomycetes, pupae were generally found not to be susceptible to mycosis (Poprawski *et al.* 1985).

The low incidence of mycosis observed on fruit fly puparia may be attributed in part to the introduction of wet conidial suspensions which caused clumping of the sand and may have reduced the even distribution of conidia in the soil. Thus, as third instar larvae burrow into the soil they could have missed the fungal inoculum. Jaronski (2007) suggested that soil pore structure is an important consideration during the application of EP fungi to soils because as soils are hydrated, increasingly larger pores become filled with water. Insects burrowing down into the soil will follow the path of least resistance and thus favour the larger pore spaces, whereas fungal conidia are more commonly contained within the smaller pore sizes, thus migrating insects may not have come into contact with enough conidia (Jaronski 2007). The fact that river sand was used, which is finer in texture, limits these larger soil pore spaces. However, it does not negate conidia from becoming buried within soil clumps. Some authors have reported that if soils are too moist, this may negatively affect conidial efficacy by reducing sporulation because of lower oxygen concentrations (Ekesi *et al.* 2003). However, because no measurement of soil moisture was recorded these suggestions should be considered lightly. Further, because the number of mycosed puparia in the soil was low and infected puparia represent a site of infection, the ability to infect other surrounding puparia in the soil, by increasing inoculum levels, may have been reduced. Limited mycosis in puparia was only noted with fruit flies and was not apparent with *T. leucotreta* pre-pupating larvae; these observations could possibly be due to the relatively small sizes of fruit fly larvae (7 to 9 mm in length) (Thomas *et al.* 2001), compared to *T. leucotreta* larvae (12 to 15 mm in length) (Stibick 2008), which limits body contact with conidia as insect larvae burrow into the sand. It could also mean that the immature stages of fruit

flies are simply less susceptible to EP fungi than the adults (Poprawski *et al.* 1985; De la Rosa *et al.* 2002; Toledo *et al.* 2006).

Deferred adult mortality due to mycosis on the other hand, reached 58% during one screening trial. Deferred adult mortality occurs when immature insect stages are not affected by EP fungi but as they emerge as adults so the cuticle is soft and they become susceptible to fungal infection. It was observed consistently that mycosis due to deferred adult mortality was significantly higher for most fungal isolates than mycosis of fruit fly puparia. During the evaluation of SDA plates at the end of the 10 day bioassay trial period, more adults were observed with fungal infection than puparia. Yee & Lacey (2005) reported that during the evaluation of *M. anisopliae* against *R. indifferens*, adult emergence was not reduced however 14.9 to 68% of emerging adults were infected at death. Poprawski *et al.* (1985) noted that deferred mortality was observed in *D. antique* adults which emerged after treatment with various EP fungal species. Ekesi *et al.* (2002) reported that 11 of their 15 *Metarhizium* and *Beauveria* isolates showed greater deferred adult mortality in *C. capitata* (due to mycosis) than pupal mycosis although pupal mycosis was also very high (10 of the isolates were able to induce over 60% pupal mycosis). A couple of reasons could explain this observation. Firstly, when third instar fruit fly larvae enter the fungal treated sand they pupate within less than 12 hrs of entering (personal observation) and the puparia may become sclerotised before the fungal conidia can germinate and penetrate the pupal case. Ekesi *et al.* (2002) showed that mortality of puparia decreased with an increase in pupal age, as it was found that older pupae (96 hrs old) had a lower level of fungal infection due to the sclerotization of the pupal case. Thus, the spores remain on the sclerotised fruit fly pupae until emergence begins. Upon emergence, adult fruit flies break open the pupal case and adult flies contort their bodies to rid themselves of the exuviae. At this time the cuticle of the newly emerged adults may come into contact with the fungal conidia, which then germinate and cause insect death (Ekesi *et al.* 2002).

Secondly, when adult fruit flies were infected with fungi as a result of deferred adult mortality, then died in the emergence chambers early on in the 10 day bioassay trial period, they became a source of infection and caused healthy adults to become infected simply by contact. In a study by Dimbi *et al.* (2003a), the age of the host, in this case, *C. capitata*, had a significant effect on mortality, with younger flies (0 to 7 days old) succumbing to infection by EP fungi earlier than older flies (7 to 14 days). If the chance incidence of young fruit flies with deferred fungal infection is experienced early on in the bioassay trial period, the incidence of mycosis in adult

flies could become higher because fungal inocula levels are increased in the emergence chambers. This may explain to some degree why adult mycosis was high for some *Beauveria* isolates during the investigation of puparia and adult mycosis of *C. capitata* and *C. rosa* at lower conidial concentrations, which showed a slight significant difference from higher conidial concentrations. Perhaps future research should evaluate the use of plastic bottles as emergence chambers to reduce confounding factors, such as limited space, which may have increased mycosis scores during bioassay procedures. In the evaluation of EP fungi against fruit flies, Ekesi *et al.* (2002) placed Petri dishes containing untreated sand and puparia in Plexi-glass cages which were 15 x 15 x 20 cm in size and therefore not much bigger than the sterilised plastic bottles which were used in this study. Despite this, it is still suggested that this aspect be investigated to optimise bioassay procedures and reduce variation.

Another possible reason for the observed increase in adult mortality due to mycosis compared to pupal mortality could be horizontal transmission. During the 10 day bioassay trial period, adult fruit flies of both species were observed mating and eggs were also found on the cotton wool plugs which were used to introduce water into the emergence chambers. Horizontal transmission of pathogens within the same target species is called autodissemination (Quesada-Moraga *et al.* 2008) and occurs when fungal infected individual flies mate with healthy flies. Whilst evaluating the SDA plates after the trials had terminated, it was found that both male and female *C. rosa* and *C. capitata* adults had EP fungi growing on the tip of the abdomen and on the thoracic sclerites (Fig. 3.4.1). Quesada-Moraga *et al.* (2008) reported that when uninfected *C. capitata* females were paired with *Metarhizium* inoculated males for 24 hrs, these females showed a higher density of conidia in the dorsal thoracic scelrites. Conidia were also observed on the sclerites and inter-segmental regions of the dorsal, lateral and ventral areas of the abdomen. Inoculated males exhibited conidia on the aedeagi and females had conidia on the ovipositor. Quesada-Moraga *et al.* (2008) reported that in Spain, wild *C. capitata* females will first oviposit four to seven days after emergence. Thomas *et al.* (2001) reported that *C. capitata* females were able to oviposit as early as four to five days after emergence. This was also observed in this study for both fruit fly species. Horizontal transmission is more effective when infected male flies mate with healthy females because female *C. capitata* fruit flies are monogamous or oligogamous (Quesada-Moraga *et al.* 2008). In addition to the possible transmission of EP fungi between male and female fruit flies, calling male flies also show the tendency of numerous attempts at homosexual mating (Ekesi *et al.* 2007). These male to male mounting attempts as well as heterosexual fruit fly mating could almost certainly have increased horizontal

transmission of EP fungi in adults in the emergence chambers during bioassay procedures. Horizontal transmission of EP fungi in the laboratory has also been noted in diamondback moth, *Plutella xylostella* (Furlong & Pell 2001) and in the Malaria vector, *Anopheles gambiae* (Scholte *et al.* 2004). This was not the focus of this study however, but would be worthwhile investigating in greater detail in future research, particularly in light of recent literature which lists horizontal transmission as an important mechanism for EP fungal inoculum transfer in nature (Ekesi *et al.* 2007; Dimbi *et al.* 2009).

Figure 3.4.1 (A) Female adult *C. rosa* fruit fly showing *B. bassiana* growing on the abdominal segments **(B)** Female fly showing *B. bassiana* growth on the dorsal and lateral thoracic scelrites **(C)** Male adult *C. rosa* fruit fly showing *B. bassiana* growing on the abdominal segments (all images were taken with a Leica EZ 4D dissecting microscope at ~ X16) (Photo Credits: T. Goble, Rhodes University).

A high level of sporulation was observed for some fungal isolates, particularly some of the *Beauveria* isolates (personal observation) and sporulation levels increase the amount of conidia which are produced from cadavers. This increases the chances of other individuals becoming infected due to the higher level of inoculum present in the emergence chambers. The level of sporulation is considered an important function for selected biological control agents. As contaminated pupae or adults represent a concentrated source of infection and ensures that inoculum levels and contact chances between conidia and healthy individuals are increased which subsequently ensures disease transmission (Ekesi *et al.* 2002). Apart from two *Beauveria* isolates, *FCM 10 13 L1* and *G OL R11*, which induced 58% and 41% mycosis respectively in adult fruit flies, other virulent isolates showing a greater increase in adult mycosis were *G Moss R10*, *G 14 2 B5* and *G Ar 17 B3*. It is concluded that although a limited amount of mycosis was observed in puparia of both fruit fly species, deferred adult mortality due to mycosis was sufficiently high enough to warrant further investigation, particularly of the abovementioned

isolates. The increased incidence of deferred adult mortality suggests that post emergence mycosis in adult fruit flies may play a more significant role in field suppression than the control of fruit flies at the pupal stage.

During the concentration-response regression analyses of fruit flies, there was very little delineation among fungal isolates in terms of virulence, and isolates did not vary significantly in their effect against *C. capitata* and *C. rosa*. The results of the regression analyses however suggest that these indigenous fungal isolates, which were considered some of the most virulent, require a large amount of inoculum (LC_{50} values range from 1 x 10^{11} to 1 x 10^{15}conidia/ml $^{-1}$) to elicit a meaningful response in fruit flies. Both the LC_{50} and LC_{90} values of all five fungal isolates tested against *C. rosa* and *C. capitata* fell outside of the tested concentration range because the number of exposed insects at the highest conidial concentration only just exceeded 50% of the total response by all insects. This unfortunately was due to the difficulty in obtaining large enough volumes of conidial suspension to use concentrations of 1 x 10^{11} and 1 x 10^{12} conidia/ml^{-1} in the laboratory. LC_{50} values were higher than the highest concentration used in this study and the further use of increased conidial concentrations is impractical and unaffordable when translated in field required inoculum levels. Ekesi *et al.* (2002) reported high mortality in *C. capitata* puparia and a significant reduction in adult emergence using conidial sand incorporation bioassays, and also found that the fungal isolates used to control fruit fly puparia had median lethal concentration (LC_{50}) values of between: 1.7 x 10^5 to 7.7 x 10^6 conidia/ml, depending on which fruit fly species the isolates were tested against. Ekesi *et al.* (2007) also reported that the pathogenicity of several isolates of EP fungi was demonstrated against *Bactrocera invadens* final instar larvae when exposed to soil treated with 1 x 10^9 conidia/ml^{-1}, reducing adult emergence to below 8 and 10%. Other typical LC_{50} values of the most virulent fungal isolates used against adult fruit flies include 4.9 x 10^5 to 2.0 x 10^6 conidia/ml^{-1}. However, these authors sprayed fruit flies with various conidial suspensions instead of applying the fungal inoculum to the soil (Quesada-Moraga *et al.* 2006). These studies reiterate the poor pathogenicity observed in the effects of our indigenous isolates of EP fungi against the immature life stages fruit flies, *C. capitata* and *C. rosa*.

Both *C. capitata* and *C. rosa* responded in a similar way to the effects of indigenous isolates of fungi as there were no significant differences observed between fruit fly species response when adult survival, or adult and pupal mycosis were compared. There were also limited observed differences in fruit fly response to various concentrations of selected isolates during regression

analyses. This is of great importance when considering a biological control agent against these fruit fly species because they occupy similar ecological niches in the warmer areas of southern Africa (de Meyer *et al.* 2008). Ekesi *et al.* (2002) suggested that an isolate with a broader range of activity would be a more desirable trait than an isolate which was species-specific. This was the concept which prompted the selection of fungal isolates for further analyses which may not necessarily have been the most pathogenic towards *T. leucotreta* alone but were most virulent towards all three insect species.

Indigenous EP fungal isolates were collected from orchard and natural habitats and it was of interest to ascertain whether grouping isolates from these habitats would reveal a pattern in pathogenicity. For example, isolates from natural habitats may prove to be more pathogenic towards citrus pests because there may be a greater diversity and abundance of insect hosts present in these natural habitats (Meyling *et al.* 2009). Isolates originating from these natural habitats may therefore have broader host ranges and greater virulence. Meyling *et al.* (2009) concluded that natural habitats offered structural diversity to groups of organisms (fungi and their insect hosts) and were important reservoirs for fungal species diversity compared to cultivated fields. Thus, an increase in species diversity may translate into a greater probability of encountering virulent fungal isolates. Conversely, attributes of fungal isolates obtained from orchard habitats may include pesticide and UV-B tolerances or exhibit resilience towards agronomic practices. However, no significant differences were observed in the incidence of mycosis by various fungal isolates grouped according to habitat. This result is valid since increasing evidence suggests that habitat selection and not insect host selection drives the population structures of EP fungi (Bidochka *et al.* 2001; Bidochka *et al.* 2002) (cf Chapter II) and virulence is linked to insect host susceptibility. Fungal species with broad host ranges are generally facultative pathogens and relatively less pathogenic compared to highly specific, generally obligatory EP fungi (Goettel 1995).

Konstantopoulou & Mazomenos (2005) demonstrated that both *Bactrocera oleae* and *Ceratitis capitata* adult fruit flies showed different degrees of susceptibility to two EP fungal species, *Beauveria bassiana* and *B. brongniartii.* Dimbi *et al.* (2003b) reported considerable variation in virulence among fungal isolates tested against *C. capitata*, *C. rosa* var. *fasciventris* and *C. cosyra*. If it is possible for fruit flies species to vary so much in susceptibility to EP fungi and fungi to exhibit variations in virulence, then it would stand to reason that a moth species, such as *T. leucotreta,* may also vary in its susceptibility to specific fungal isolates. Despite fruit flies

exhibiting greater resistance to the effects of indigenous isolates of EP fungi, these isolates were found to be highly pathogenic towards *T. leucotreta*.

3.4.2 The effects of EP fungi on false codling moth, Thaumatotibia leucotreta

Most of the indigenous isolates of EP fungi which were tested in this study were able to induce high mortality in *T. leucotreta* final instar larvae and pupae when exposed to sand treated with conidia suspension. The overall effect of this subterranean treatment led to a significant reduction in adult moth emergence (less than 20% emergence was achieved with most EP fungal isolates). Eleven of the initial twenty-one fungal isolates tested showed marked pathogenicity towards the pupae (mycosis greater than 80%) of this insect species. Loc & Chi (2007) reported that *B. bassiana*, *M. anisopliae* and two commercially available biopesticides (Crymax® 35 WP and Atabron® 5EC) applied at concentrations of 1 x 10^7 conidia/ml^{-1} were able to induce 70 to 87.3% mortality in diamondback moth, *Plutella xylostella,* larvae seven days after exposure when applied directly as sprays. Tefera & Pringle (2003) reported high mortality (98 to 100%) in the stem borer, *Chilo partellus*, after exposing second instar larvae to *Beauveria bassiana* at concentrations of 1.25 x 10^7 conidia/ml. The authors concluded that the development of *B. bassiana* on cadavers of *C. partellus* was influenced by temperature, conidial concentrations and exposure methods.

Concentration-response trials against *T. leucotreta* revealed a dose-dependant relationship and two selected isolates, *G 14 2 B5* and *G Moss R10* could induce mycosis in 50% of the test population (LC_{50}) at concentrations of 8.8 x 10^5 and 6.8 x 10^5 conidia/ml^{-1} respectively. The steeper slopes of these two isolates also indicated that target insects will be more vulnerable over a given time period to increasing concentrations of conidia. Anand *et al.* (2009) reported that EP fungi applied to *Spodoptera litura* pupae in the soil, as a drench (conidial suspensions at a titre of 10^8 conidia/g of soil) reduced adult emergence by 81.3% and 72.5% depending on the fungal isolate. However, when premixing the conidial suspensions into the soil, fewer pupae were killed by the fungi; this was attributed to the accumulation of conidia up to 10 cm from the soil surface during drenching. The authors reported LC_{50} and LC_{90} values of *M. anisopliae* (the most virulent isolate) as 1.2 x 10^7 and 4.3 x 10^8 conidia/g respectively. In the investigation of EP fungi against *Thaumetopoea pityocampa* (Thaumatopoeidae) at concentrations of 1 x 10^5 conidia/ml^{-1}, all tested isolates resulted in various levels of mortality (16 to 100%) and when dose-mortality tests

were undertaken by spraying five conidial concentrations on larvae, the estimated LC_{50} value was 3.4 x 10^6 conidia/ml^{-1} (Er *et al.* 2007).

The registered microbial control product, Bb Plus®, elicited a weaker concentration response in *T. leucotreta* which was subsequently noted in the preliminary time-response trial when this product competed poorly with two indigenous isolates. Further, *G 14 2 B5* and *G Moss R10* were significantly more pathogenic towards *T. leucotreta* than *G B Ar 23 B3* and *FCM 10 13 L1*. This was interesting because *G B Ar 23 B3* was the most pathogenic isolate towards fruit flies, indicating variation in virulence of fungal isolates towards various insect hosts.

The preliminary time-response trials did not compliment the results of the concentration-response trial for *T. leucotreta* because a concentration of 1 x 10^5conidia/ml^{-1} (LC_{50} values of *G 14 2 B5* and *G Moss R10*) was not used. The nearest concentration to that was 1 x 10^4 conidia/ml^{-1}, which only induced 12.53 to 27% mycosis in *T. leucotreta* pupae at the longest exposure time (96 hrs). At the higher concentration (1 x 10^7conidia/ml^{-1}), isolates *G Moss R10* and *G 14 2 B5*, elicited a response in 50% of test insects after 72 hrs (3 days) exposure. Based on earlier trials, when pupae were left in fungal treated sand for up to seven days, over 80% mycosis was observed for isolates *G 14 2 B5* and *G Moss R10*; this suggests that *T. leucotreta* requires a longer exposure time period than 72 hrs to elicit mortality in the region of 80%, at a concentration of 1 x 10^7conidia/ml^{-1}. This is valid since more pupae become infected over time with an increase in time as fungal inoculum levels in the soil increase; subsequently more pupae or emerging adults become infected. A time-response extrapolation at the lowest concentration (1 x 10^4 conidia/ml^{-1}) suggests that it requires a longer time period than 96 hrs to elicit mycosis greater than 27% in the test insects. Unfortunately the exposure time-response results are inconclusive and can only be considered as guideline information regarding future research with these fungal isolates against *T. leucotreta*.

During the time-response trials an outlier was observed in that a large percentage of pupae exposed to isolate *G Moss R10* were mycosed at 12 hrs. This observation was as a result of extensive bunching of pupae (larvae spin pupal cases adjacent to one another) leading to the higher mycosis of pupae observed during the trial. *Thaumatotibia leucotreta* pupae are sometimes grouped in the soil, possibly due to the confines of the Petri-dish, (personal observation) and this may increase the incidence of mycosis because if infected pupae are positioned next to healthy pupae fungal hyphae may grow onto the adjacent pupae (Fig. 3.4.2).

Krueger & Roberts (1997) noted that dry mycelial particles, as a form of inoculum, function in the same way as fungal-killed insects. When water in the soil hydrates the fungal mycelia, new mycelial growth is experienced and infectious conidia are produced on the new mycelial growth. During fungal preparations, cultures were scraped with a glass rod; upon enumeration of fungal concentrations, bits of hyphae were often observed. Shah & Pell (2003) suggested that infection by *Beauveria* spp. in soil environments was improved by the radiation of modified hyphal strands away from cadavers. Thus, it is valid that fungal mycelia can grow while bioassay procedures are carried out, and further investigation is warranted.

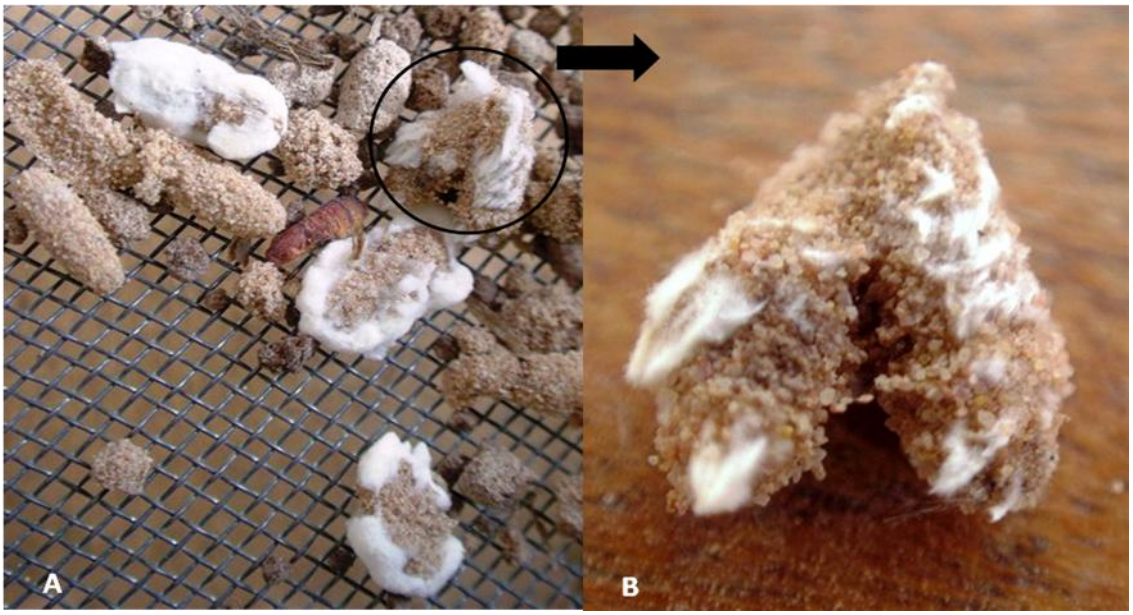

Figure 3.4.2 (A) *Thaumatotibia leucotreta* larvae spin cocoons around themselves and may become attached to one another in Petri-dishes. **(B)** *Beauveria bassiana* fungal mycelia seen growing from two adjacent *T. leucotreta* pupae (Photo Credits: T. Goble, Rhodes University).

Some variation during bioassay procedures occurred in this study in that attenuation of the isolate *FCM 10 13 L1* was observed. This isolate exhibited a change in mycelia growth form (Fig. 3.4.3) as described by Wang *et al.* (2005). In subsequently conducted germination tests, a lower percentage of germination occurred prior to its use in concentration and time-response trials. During initial fungal screening trials against *C. capitata*, *C. rosa* and *T. leucotreta*, this isolate was particularly pathogenic and a significant effect was seen in its ability to reduce adult emergence and elicit mycosis in all three test insect species. Subsequent trials revealed a less virulent *FCM 10 13 L1* isolate. It is empirically unknown why this isolate exhibited morphologically altered growth in culture. Perhaps the isolate is inherently unstable, as

Brownbridge *et al.* (2000) suggested that the effects of repeated *in vitro* sub-culturing and subsequent morphological characteristics vary considerably among isolates and fungal species. A loss of pathogenicity has been noted in isolates of *B. bassiana*; whereas other isolates retain their virulence after repeated sub-culturing under the same conditions (Brownbridge *et al.* 2000). Furthermore, an isolate which is inherently unstable is undesirable as a biological control agent because large commercial losses may be experienced if isolates lose their virulence (Wang *et al.* 2005).

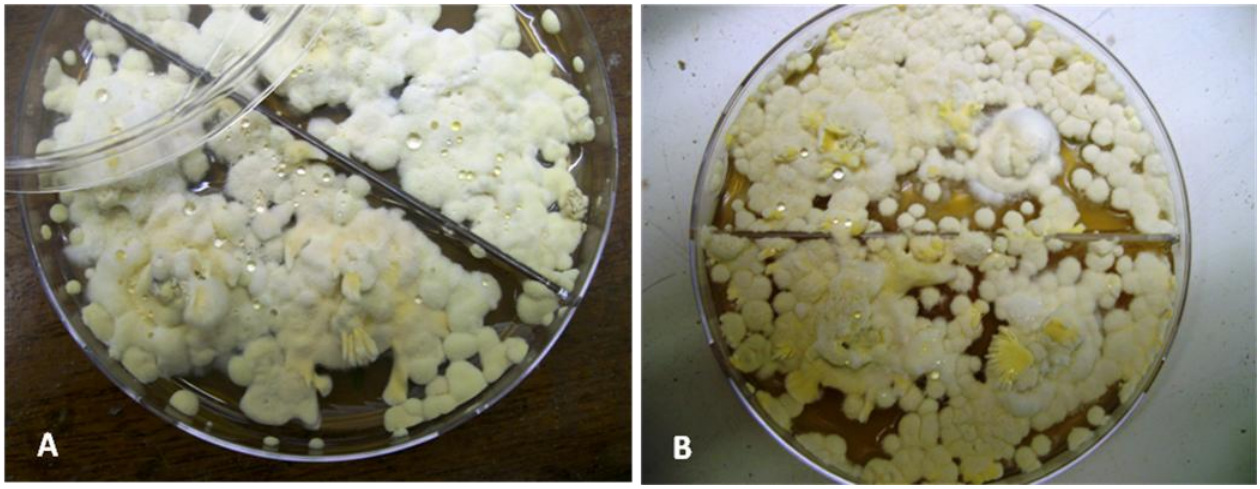

Figure 3.4.3 (A & B) *Beauveria bassiana* isolate *FCM 10 13 L1* showing *in vitro* culture degeneration (fluffy-mycelia growth) (Photo Credits: T. Goble, Rhodes University).

As mentioned, selected isolates were chosen on the basis that they were able to induce mortality in all three insect test species, thus there is wide scope for future research on *T. leucotreta* with isolates which were not focused on in this study. Isolates of noteworthy mention are the three *Metarhizium* isolates, *G 14 2 B3*, *FCM Ar 23 B3* and *G 11 3 L6*, and four *Beauveria* isolates, *G Ar 17 B3*, *G OL R11*, *G Rose R9* and *FF J&B R5*. The increased incidence of pupal mortality, as well as the relatively low concentrations of conidia required to elicit meaningful responses in *T. leucotreta* pupae may suggest that pre-emergent or prophylactic control of *T. leucotreta* will play a more significant role in field suppression than the control of adult life stages using indigenous isolates of EP fungi.

IV

GENERAL DISCUSSION

4.1 Integrated Pest Management (IPM) strategies for the use of EP fungi in citrus

Citrus in southern Africa has moved towards an IPM approach in the last three decades and the viability of IPM in citrus in southern Africa has been pioneered (Terblanche 1998). IPM has now become an established management strategy, particularly in light of the large export earnings of the citrus industry (Terblanche 1998). There is no universally agreed upon definition for IPM and its initial conception was to rationalise the use of pesticides to delay the resurgences of pest populations that had become resistant to chemicals and to protect natural enemies (Orr 2003). Presently, there is much concern over the injudicious use of pesticides and IPM may be defined as: *"A flexible and holistic approach which views the agroecosystem as an interrelated whole and utilises a variety of biological, cultural, genetic, physical and chemical techniques to hold pests below economically damaging levels with a minimum amount of disruption to the cropping ecosystem and the surrounding environment"* (Orr 2003). Further, the establishment of an International IPM Facility in 1995 with the support of the World Bank illustrated a global desire to reassess plant protection procedures to enhance IPM and restrict the use of chemicals to those which were absolutely necessary (Terblanche 1998).

Citrus has had a long history of cultivation in southern Africa and the literature on citrus pests and their control is already extensive, particularly with work done on the biology of pests and their control with chemicals (Bedford 1998). Citrus IPM strategies against *Thaumatotibia leucotreta*, *Ceratitis capitata* and *C. rosa* include: pre-harvest monitoring, orchard sanitation, sterile insect technique (SIT), attract and kill, mating disruption, male annihilation technique and biological control using predators, parasitoids and microbes (viruses) (cf Chapter I). In contrast the use of microbial biological control agents, such as EP fungi within citrus IPM in southern Africa is still in its infancy. However, with the growing demands of overseas markets to limit chemical pesticides, and reduce phytosanitary risk as well as the recent consumer interest in

organically produced foods, microbial biological control using EP fungi as an additional IPM management tool holds promise.

Based on the biodiversity studies using bait insects presented in this study, it appears that some EP fungal genera are a widespread component of the soil environments of both cultivated citrus orchards and the natural areas surrounding orchards in the Eastern Cape Province in South Africa. Fungal occurrence in natural areas is significantly higher than in cultivated citrus orchards, due to the structurally complex nature of natural habitats which are free from pest control practices and include diverse abundances of insect hosts (Meyling *et al.* 2009). The present study also revealed that there were no significant differences observed in the occurrence of EP fungi from conventional and organic citrus farms when cultivated orchards and refugia were considered separately. This illustrates that both farming strategies will be amenable to EP fungal application because the use of fungicides and insecticides did not exclude the occurrence of these microbes in conventional citrus cropping systems. Despite these observations, it appears that very few EP fungal genera are present in citrus soils and natural areas surrounding orchards. This has been noted in other countries and different environments by Chandler *et al.* (1997), Klingen *et al.* (2002) and Quesada-Moraga *et al.* (2007). Furthermore, EP fungal inocula levels in citrus soils are too low to elicit any meaningful level of pest control by these microorganisms.

The application of EP fungi as biological control agents in citrus cropping systems will require inundative application, as has been observed with most microbial control strategies using entomopathogens to date (McCoy 2007). This strategy may be a better application tactic than either classical biological control of insect pests with exotic fungal isolates or conservation biological control which serves to preserve and promote exisiting EPF populations within southern African citrus. The application of existing microbial control products seems useful because the costly and time consuming research and testing of commercial products has already been undertaken. However, their application is limited by existing formulations which may not necessarily be practical or compatible in citrus cropping systems. The intentional introduction of exotic pathogens into South Africa for classical biological control has generally been difficult because of regulatory restrictions imposed by the National Environmental Management Biodiversity Act (DEAT 2004). The potential for maladapted exotic fungal isolates is also high.

Beauveria bassiana and *Metarhizium anisopliae* are two facultative EP fungal species which have been studied in great detail for the control of citrus pests and their formulation and use as

microbial control products in these cropping systems has been extensive (Lacey *et al.* 2001; Lacey & Shapiro-Ilan 2003; Dolinski & Lacey 2007). In the present study, both *B. bassiana* and *M. anisopliae* were the most commonly isolated EP fungi in citrus soils and soils from natural areas. Their broad host ranges (Bidochka & Small 2005; Rehner 2005) and large genetic variation within species (Bidochka *et al.* 2001; Meyling *et al.* 2009) suggests that microbial control efforts should not be limited to the currently available mycoinsecticides and their inundative releases. Rather, indigenous fungal isolates, which may be more virulent and better adapted to local citrus cropping systems, should be utilised. The present study identified a number of virulent EP fungal isolates which showed potential as biological control agents in the laboratory, particularly against *T. leucotreta*. Further investigations in environmental competence and virulence may greatly increase our understanding of the persistence and efficacy of microbial control products, which are based on indigenous fungal isolates, in the field. When EP fungi are capable of becoming established in a particular cropping system they are then able to confer long-term regulation of pest populations, which is the ultimate goal of biological control (Dolinski & Lacey 2007). Thus, the presence of both *B. bassiana* and *M. anisopliae* in citrus cropping systems in the Eastern Cape illustrates their establishment and ability to persist in these soil environments albeit at low densities. It is however unclear as to what extent these indigenous isolates exert natural control pressures on citrus pests in the field, an area of research which requires attention in southern African citrus.

The results of the present study revealed that indigenous fungal isolates were not able to induce high mortality in *C. capitata* and *C. rosa* puparia when exposed to sand treated with conidia suspensions. This illustrated the lack of susceptibility of the immature life stages of fruit flies to the effects of EP fungi which has been observed by Poprawski *et al.* (1985), De la Rosa *et al.* (2002) and Toledo *et al.* (2006). Deferred adult mortality, which resulted in a greater incidence of adult fruit flies mycosed by EP fungi after treatment of soil, was sufficiently high to consider the use of EP fungi as a subterranean treatment against emerging fruit fly adults. The observation that increased incidences of deferred adult mortality due to mycosis, as a subsequent result of subterranean treatment of immature life stages of fruit flies, has also been reported by Poprawski *et al.* (1985), Ekesi *et al.* (2002) and Yee & Lacey (2005). Thus, the initial aim of the study, which set out to investigate the potential application of EP fungi against the larvae and pupae of fruit flies, did not show potential control of these immature life stages but did show potential control of emerging adults at a subterranean level. Most of the indigenous isolates of EP fungi tested in this study were able to induce high mortality in *T. leucotreta* final instar larvae and

pupae when exposed to sand treated with conidial suspension. The overall effect of this subterranean treatment led to a significant reduction in adult moth emergence and eleven of the initial twenty-one fungal isolates tested showed marked pathogenicity towards the pupae of this insect species. Based on the results of the present study, it appears that effective control of *T. leucotreta* immature life stages may occur if EP fungi are applied as a subterranean soil barrier below citrus trees. During bioassays in the laboratory, low levels of fungal inocula were required to elicit meaningful control of *T. leucotreta*; reiterating the potency of these indigenous fungal isolates against this insect pest species and the practicality of utilising these isolates in the field. Likewise, this subterranean control method may reduce the incidence of fruit fly adult survival because adults become infected with fungi upon emergence. However, much higher levels of fungal inocula were required to elicit meaningful control of fruit flies in the laboratory and these levels may translate into uneconomical or impractical inocula levels in the field. Thus, the utilisation of alternative EP fungal control strategies may need to be employed which would lower the required fungal inocula levels by enhancing the efficacy of the dissemination of fungal conidia, as has been observed with the use of autoinoculation devices, discussed below.

Augmentation as a biological control strategy, using identified indigenous EP fungal isolates in citrus, has a relatively low implementation cost and could increase presently low fungal density levels in the soil (McCoy & Tigano-Milani 1992). There are two approaches which may be undertaken, inoculation and inundation. The inoculation approach using fungal isolates involves applying small amounts of inocula early on in the crop cycle, with the premise that fungi will cycle and establish epizootics in pest populations and spread over a period of time, maintaining pest populations below economic thresholds (Shah & Pell 2003). The main impact of EP fungi on pest populations however, may only occur once economic thresholds have been surpassed (Inglis *et al*. 2001). The utilisation of this approach alone may be difficult to undertake against fruit flies and *T. leucotreta* because the economic thresholds of these pests are usually very low (Moore *et al*. 2008). Furthermore, the effects of EP fungi are density dependant and will require increased pest population numbers to elicit epizootics in orchards to maintain fungal recycling (Inglis *et al*. 2001). This may be difficult to maintain because various IPM strategies targeting and controlling these insect pests may reduce pest populations to levels which are unable to sustain fungal recycling.

The most common method of employing EP fungi for pest control is through inundation using mycoinsecticides for short-term, rapid control of insect pests, much like the use of chemical

insecticides (Shah & Pell 2003). Fungi may be administered via herbicide applicators or other existing equipment which makes this approach appealing (McCoy *et al*. 2007) and there is no expectation that fungi will cause secondary infection or cycle in the environment (Shah & Pell 2003). If inundation is to be considered as a biological control option using indigenous EP fungal isolates, which are applied as a soil barrier below citrus trees for the control of *T. leucotreta* and emerging fruit fly adults, formulation of bare conidia will need to be undertaken to enhance the persistence and infectivity of fungi in the soil. This is an important prerequisite for the successful biological control of fruit flies and *T. leucotreta* pupae which are often sedentary or less susceptible in immature life stages (cf Chapter III). Efficacy of application will be determined by the larvae of these soil-dwelling pests contacting a sufficient number of conidia to become infected (Jaronski *et al.* 2005). The infectivity of unformulated conidia for example, is greatly affected by soil texture and moisture which is thought to be a result of pore structure (Jaronski *et al.* 2007). As the soil hydrates, increasingly larger pore spaces become filled with water. Insects moving in the soil tend to take the path of least resistance and thus move through these larger pore spaces. Unformulated conidia tend to remain in the smaller soil pores and the result of this is the ineffective or sublethal contact of insects and conidia (Jaronski *et al.* 2007). The effect of soil texture on formulated granules however is far less and discussed further below.

Vänninen *et al.* (2000) investigated the persistence and penetration of unformulated fungal conidia in various soil types over three years in Finland. The fungal species, *M. anisopliae*, generally persisted better than *B. bassiana* in all soil types although persistence decreased with soil depth and time. *Beauveria bassiana,* when applied as an aqueous soil suspension, showed an 85 to 95% reduction of viable conidia at 12 days post-application. However, three years post-application there were still enough infectious *M. anisopliae* conidia in soils at all sites to infect over 80% of *Tenebrio molitor* insects employed as baits (Vänninen *et al.* 2000). The authors attributed the decrease in *Beauveria* inoculum levels in field soils to three processes: biodegradation (considered the most important factor), physical weathering and percolation into deeper soil layers. However, percolation into deeper soil layers is thought to be the least important factor because most conidia are retained in the top 5 cm regardless of soil texture (Ekesi *et al.* 2007). Pupating insects, such as *Ceratitis* fruit flies and *T. leucotreta,* never exceed a depth of 7 cm in the soil, thus infection by EP fungi is maximised in this upper region of the soil (Ekesi *et al,* 2007; Stibick, 2008). In terms of soil type, clay soils are considered to have the highest carrying capacity of fungal conidia; clay can also function as a protectant against biodeterioration, resulting in an increase in stability and longevity of conidia (Vänninen *et al.*

2000). Sandy soils allow for a faster percolation of fungal conidia (depending on the size of the particles) and are generally considered warmer soils during the summer months because of the increased abundances of quartz (a good conductor of heat), compared to clay.

Commercial interest in using EP fungi to control soil dwelling pests have resulted in formulations which are based on liquid culture-produced mycelial pellets, encapsulated fungal biomass or solid substrate-produced conidia, either in aqueous or oil suspensions or on granular carriers (Jackson & Jaronski 2009). Recently, the discovery of microslerotia (compact hyphal aggregates which are often melanised and highly resistant to desiccation), have shown to be effective fungal application inocula (Jackson & Jaronski 2009). The ingredients selected for formulation should not interfere with the fungal infection process in anyway and at best should enhance viability, disease transmission and field persistence of EP fungi.

Nutritive or non-nutritive granules coated with EP fungal conidia, granules colonised by fungus or granules composed of dry mycelium are examples of commercial formulations which have been used against soil pests (Jackson & Jaronski 2009). The principal behind granular formulations is that EP fungi will grow out onto the granular carrier and resporulate in the presence of water (water activity greater than 0.98 or water potential of -3 MPa), much like an insect cadaver (Jaronski 2007). Granules seem to show more resilience in the soil because fungal sporulation occurs on the granules. However, increases in fungal titres (sporulation) in the soil are rarely observed when application by aqueous suspensions or dry powders are made. Krueger *et al*. (1992) cited by Jaronksi (2007) showed that *M. anisopliae* mycelial-based granules (0.25-0.5 mm diameter) applied to the soil for the control of corn rootworm, *Diabrotica undecimpunctata,* at a rate of 120 granules/cm^3 increased conidial titres in the soil by 1.1 x 10^4 colony forming units per granule. This author concluded that granules are readily able to support EP fungal growth and that the inability of bare conidia to germinate in soil is due to the absence of sufficient stimuli. Based on the above considerations, if application of indigenous EP fungal isolates as a soil barrier is to be considered then granular formulation may be the most effective way to formulate fungal conidia to enhance persistence and efficacy.

Jaronski *et al.* (2007) suggested that the use of EP fungal granules should be compatible with farming equipment. The size and bulk density of the granules should not exceed 0.5-1.5 mm in diameter and an upper practical limit of 22 kg granules per hectare, if they are to be properly applied by existing equipment. Most conventional insecticide granules are applied at 11-17 kg/ha

(Jaronski *et al.* 2007). These authors mentioned that a critical concentration of granules is required for high efficacy. In bioassays against third instar larvae of the sugarbeet root maggot, *Tetanops myopaeformis* (Ulidid fly), in clay soil at optimal temperature and moisture for *M. anisopliae*, four or more granules per gram (corn grit granules 0.5-1 mm diameter coated with *M. anisopliae* and having a bulk density of 1400 granules per gram) were needed for greater than 90% efficacy. At anything less than that critical density, sharp decreases in infection rates were observed in sugarbeet root maggot larvae (Jaronski 2007). At four granules per centimetre cubed (cc) of soil, 158 kg granules per hectare would be required to apply a 15 cm band over a row of sugarbeet incorporated to a depth of 2.5 cm. These application rates were uneconomical and impractical. Jaronski *et al.* (2007) concluded that critical concentration (4 granules/cc of soil) could be achieved at 11 kg/ha when granules were applied using in-furrow applications in sugarbeet. Granule sizes of 0.5-1 mm in diameter create foci of greater than 1 x 10^7 conidia, depending on the sporulation potential of the particular EP fungal isolate (Jaronski 2007). However, if unformulated conidia are present in the soil, an insect would have to encounter many unformulated conidia to obtain a lethal dose. With granular formulation, sporulation occurs on the granule carrier and thus an insect coming into contact with just one granule (foci of greater than 1x10^7 conidia) may obtain a lethal dose (Jaronski 2007). Granular applications are most effective where insects

4.2 How can soil application of EP fungi work in citrus?

Soil application of EP fungi within citrus IPM is an exciting prospect and the timing of application will depend on the type of citrus cultivar and the geographic region in which citrus is grown. Unlike viruses, which are only pathogenic towards the larvae of *T. leucotreta* (Moore 2009), EP fungi are pathogenic towards *T. leucotreta* adults, larvae and pupae. Thus application would not be limited to the control of only pupae in the soil, but can include the additional control of adults (which is discussed below). Soil application of EP fungi against fruit flies in the present study concluded that the immature life stages were less susceptible to EP fungi, but that adults were more susceptible and adults emerging from the soil could become infected with EP fungi. This deferred adult mortality due to fungi is recognised as an important mechanism for the recycling of fungal inocula. Recycling occurs from the soil environment where pupae and emerging adults reside to epigeal habitats where adults mate and live out their lifecycles (Ekesi *et al.* 2007) and which may greatly enhance IPM using soil treatments.

Soil application of EP fungi, particularly as granules, offers the most effective formulation for the application of EP fungi to the soil. This application method can serve as a 'bi-catch' strategy within citrus IPM and could possibly reduce the frequency of orchard sanitation, and at best improve the control of *T. leucotreta* and fruit flies. Having EP fungi in citrus soils offers growers a 'safety blanket' for fruit which drop throughout the year as a result of the staggered maturation of various citrus cultivars (Lee 2009). Fungi can be effective during the hotter months of the citrus growing season, just prior to harvest, and as a post harvest control measure. The application of EP fungi can further promote the use of parasitoids and be available to 'catch' escaping insects when orchard sanitation cannot, as when stung fruit remains on trees and is not spotted by orchard cleanup crews. The use of granular formuations is normally associated with seed treatments in row crops, protecting plant roots where insects are expected to congregate to feed (Jaronski *et al.* 2005). Thus, one limitation to granular application below citrus trees may be the inadequate coverage for pests dropping from trees to pupate in the soil, in this case aqueous suspensions may be a more economical approach to control citrus pests.

Orchard sanitation is usually recommended from as early as November in some areas because small fruit (15-20 mm in diameter) can become infested with *T. leucotreta* or fruit flies and drop off trees (Newton 1998). Raking up abscised, small fruit has been suggested to remove infestation potential. However, this is very labour intensive and usually only about 30% of these fruit are infested with *T. leucotreta* (Moore & Kirkman 2009). If EP fungi are present in the soils at these times, the need to remove such fruit is reduced because the small percentage of escaping larvae entering the soil to pupate can become infected with EP fungi, this in turn can reduce labour and time costs. Soil treatment can be particularly useful in summer, as higher numbers of *T. leucotreta* pupae have been recovered from citrus orchards from October to mid-December than at any other time in the season (Moore & Kirkman 2009). This illustrates that *T. leucotreta* activity and population numbers are highest during these months, an observation which is also true of fruit flies (Du Toit 1998). Higher numbers of pest populations are required to elicit epizootics as transmission is increased. The increased incidence of humidity during the summer months can also greatly accelerate the fungal infection process (Inglis *et al.* 2001).

Orchard sanitation reduces citrus pest larval populations in orchards. However, it reduces the number of larval parasitoids which themselves are employed as biological control agents, particularly against *T. leucotreta* (Moore & Kirkman 2009). A reduction in orchard sanitation has been suggested to promote the activity of *T. leucotreta* larval parasitoids (Keeton 2007).

Thus, the benefits of soil application of EP fungi will include promoting the use of existing *T. leucotreta* parasitoids, *Agathis bishopi* and *Apophua leucotreta,* which require larvae to complete their lifecycles. Fruit fly parasitoids are not mentioned here because their presence in citrus orchards has a negligible effect on fruit fly control (Du Toit 1998). Competition between fungi and parasitoids is reduced because fungi are applied to the soil. Thus, parasitoids will host-seek wandering larvae from fruit on the ground or on trees and those that are missed by these natural enemies will subsequently be colonised by fungi in the soil.

The requirement of humidity for fungi to germinate suggests that they may be more effective at controlling pests in the northern, hotter and more humid citrus growing regions of South Africa such as KwaZulu-Natal, Mpumalanga and the Northern provinces. Further, natural infection by *Beauveria bassiana* has often been recorded in pupae of *T. leucotreta* amongst the leaf litter in Zebediela Citrus Estate in the Northern Province (Van den Berg 2001). The developmental rates of citrus pests are higher in these regions and the percentage of dropped fruit still infested with *T. leucotreta* and fruit flies is lower at these times due to faster insect development rates (Moore & Kirkman 2009). In the hotter months of the citrus growing season, the percentage of fruit which was still infested with *T. leucotreta* when collected weekly, dropped from 75% to between 60 and 70% (Moore & Kirkman 2009). Larval movement out of fruit is much faster, due to increased temperatures and subsequent developmental rates and the employment of orchard sanitation is not as effective because larvae have already exited fruit to pupate in the ground (Moore & Kirkman 2009). Current control measures employ the use of labour to remove dropped fruit twice a week, a practice that is expensive and requires time. Thus, the presence of EP fungi in the soils offers additional protection as escaping larvae, which then pupate in the soil, could subsequently become infected with fungi. This could reduce the need for twice weekly orchard sanitation to a weekly practice even during summer months. Fruit fly larvae rarely leave the fruit before it has fallen off citrus trees (Moore & Kirkman 2009), thus orchard sanitation still remains an important control tactic.

The argument that fungi require hosts in order to build up populations in these systems and elicit epizootics is valid. The fact that every IPM strategy in citrus, in various ways, targets the same insect pests which are hosts to EP fungi, suggests that not enough hosts will be available for fungi to recycle. However, the concept of the inundation approach is that EP fungi are often applied in large amounts (usually as mycoinsecticides), for short-term, rapid control of insect pests and there is often no expectation that fungi will cause secondary infection or cycle in the

environment (Shah & Pell 2003). Further, by applying the conservation biological control strategy, an ecological approach, which usually requires the modification of the physical environment or the management of other crop production practices (McCoy & Tigano-Milani 1992). The persistence of EP fungi in the soil may be increased which may subsequently reduce follow-up applications as fungi may then survive for longer.

There are many citrus crop production and protection practices which could be considered to enhance the persistence of EP fungi. Irrigation strategies can increase available moisture, which is an important aspect of fungal germination (McCoy *et al.* 2007). The timing of pesticides and especially fungicide used in citrus orchards will affect the survival of fungal conidia (Shah & Pell 2003). Mulching can increase the presence of EP fungi because an increased carbon load in the soil is favourable for soil dwelling pests, which themselves are potential hosts for EP fungi (Klingen *et al.* 2002). Citrus orchards are perennial habitats and management practices which increase disturbance, such as tilling or ploughing, are thus negligible in these systems. Furthermore, tree canopies provide continuous shade for microbes in the soil and reduce the amount of UV-B irradiation which is detrimental to EP fungal germination (Braga *et al.* 2001). These factors support the implementation of conservation biological control in citrus cropping systems and the abovementioned considerations can greatly increase the presence of EP fungi in these soils which then subsequently increase IPM potential. Inundation and conservation biological control strategies will benefit greatly from being used together or in conjunction with other cultural methods used in citrus IPM (Shah & Pell 2003). The use of mycoinsecticides, for example, are applied as products and their use is for short-term pest control however, the persistence of fungal inocula in cropping systems may be greatly increased by applying the conservation biological control strategy in citrus cropping systems, particularly because of the perennial nature of these systems.

The importance of removing infested fruit from citrus trees, as well as out-of-season fruit once harvest has finished, is crucial for the control of subsequent *T. leucotreta* population build-up and for the control of various post-harvest fungal diseases such as sour rot, blue/green moulds (*Penicillium* spp.) and *Alternaria.* It can however be difficult to detect all fruit fly stung fruit on trees and the removal of such fruit is labour intensive. Thus, fruit that is missed by orchard cleanups, due to the difficulty in detecting all stung fruit, may subsequently be protected by the presence of EP fungi in the soils. Further, in areas such as Mpumalanga, where the coffee bean weevil (*Araecerus coffeae*) infests mummified fruits (Moore & Kirkman 2009), EP fungi may

also serve as a control measure against these secondary pests, although further study is required. The presence of EP fungi as a post-harvest control tactic is also valid and fungi will continue to self-replicate in these systems provided the correct environmental conditions persist and the appropriate conservation control tactics are applied (i.e. timing of fungicides should not coincide with the application of EP fungi).

The timing of EP fungal application should consider the biology of insect pests and climatic conditions of a particular citrus area which dictate the number of generations per year. Both *T. leucotreta* and fruit flies are generally present throughout the year but at various levels depending on the time of year, different growing regions and various cultivars. Successful soil application of EP fungi will coincide with periods when the highest percentage of the *T. leucotreta* population are wandering larvae and pupae, which can be as early as October in some regions (Moore & Kirkman 2009). Emerging fruit fly adults is the recommended life stage to target these pests in citrus using soil applications. *Ceratitis* fruit flies become highly active around the fruit colouring period, which may be as early as January in early cultivar varieties such as, Satsuma mandarins and some early navel oranges (Lee 2009). Du Toit (1998) reported that in the Sundays River Valley in the Eastern Cape, *C. capitata* populations reached their peak as early as December over five consecutive years. Monitoring of pest populations will reflect the best times at which to apply fungi and the targeted life stage of each pest must be considered. For best results it is recommended that fungi be applied before insects numbers are too high, as a prophylactic treatment so as to give fungal inoculum levels a chance to increase in the soil, particularly if granular applications are made. October would be a good month to initiate EP fungal application in the soil against *T. leucotreta* and December would be the recommended month to initiate application against fruit flies in some areas. It might however be impractical to stagger these applications by just two months. It is recommended that perhaps just one initial application be made against both pests in November, provided that both pests occur and are targeted in that area.

The use of granules may greatly increase the efficacy and persistence of EP fungi in soils compared to liquid formulations. High levels of EP fungal conidia (4.9 to 9.5 x 10^4 cfu/g) were still recovered from soil treated with a granular formulation 668 days post-treatment in a study by Ekesi *et al.* (2005). These persistence levels suggest that perhaps only one application to the soil per growing season may be required when granular formulation is considered. In the case of aqueous suspensions, when unformulated conidia are used, no sporulation is observed in the soil

because of a lack of stimuli (Jaronski 2007). However, Ekesi *et al.* (2005) observed that the infectivity of aqueous and oil/aqueous (50:50) formulations of *Metarhizium anisopliae* persisted in the soil until one year post-application, at which point conidial levels of these two liquid formulations decreased significantly. So it is possible that only one application per growing season may be required in the case of aqueous suspensions too. McCoy *et al.* (2007) for example, reported that EP fungi were successfully applied beneath citrus trees as aqueous suspensions for the control of *Diaprepes* weevil using herbicide applicators and injection via low volume irrigation systems. Using a tractor-mounted herbicide sprayer (boom size 1.5-2.5 m) a fungal treated band was uniformly applied to the soil beneath citrus trees at the point between the trunk and the canopy edge (McCoy *et al.* 2007). McCoy *et al.* (2007) further suggested that the application of EP fungi via microsprinkler irrigation could be a more cost effective method for applying these microbes beneath citrus trees and that sprinklers be positioned about 30 cm from the tree trunk and be suitable to cover about 80% of the root area. However, because of the competitive nature of soil and the complicated abiotic factors associated with this environment, persistence is not straight forward and future studies will be needed to determine appropriate application rates. Furthermore, the distribution of conidia in the soil may be greatly increased if repeated application is made.

Timing may be predicted with existing pre-harvest monitoring traps, such as the Delta or PVC pipe traps fitted with a Lorelei dispenser for *T. leucotreta* (Moore 2009). *Thaumatotibia leucotreta* flight peaks usually occur as early as October, with a main flight peak occurring at the end of November depending on the region; in the cooler climes of the Western Cape these peaks are less clearly defined (Moore & Kirkman 2009). Subsequent moth peaks will be smaller from January to March and then tail off as winter approaches. For fruit flies, Sensus® traps fitted with a Questlure® or Capilure® attractant are often employed to monitor fruit fly populations (Ware 2002; Ware *et al.* 2003). Timing of EP fungal application against fruit flies will be difficult because of the low economic threshold of this pest and traps are not there to indicate when timing of an application programme should be initiated but rather when a fixed control programme is inadequate (Moore *et al.* 2008). Trap surveys should be initiated during November until harvest (Moore *et al.* 2008) and application of EP fungi should be made when trap catches exceed their thresholds.

4.3 Autoinoculation devices for the use of EP fungi in citrus IPM

Strategy options, in terms of the implementation of EP fungi in the field as part of an IPM programme against fruit flies, have been summarised by Ekesi *et al.* (2007). Soil inoculation has been discussed and due to the low mortality rates in the immature life stages of fruit flies as well as the large inoculum rates required to suppress fruit flies in the soil, the application of alternative techniques is required. Horizontal transmission was a listed factor which may have increased the rate of adult mortality during bioassay procedure in the present study, and was sufficiently high to further investigate the potential use of control strategies which are based on this factor, such as the use of autoinoculation devices.

The assisted autodissemination control option using devices in which fungal conidia are spread by adult insects, has recently been proposed for insect pests which respond to visual and olfactory cues (Ekesi *et al.* 2007). The strategy is to attract insect pests using visual, chemical and food lures to the focal point of EP fungi. Insects then inadvertently pick up the fungal inoculum and disseminate the infective conidia via horizontal transmission to other healthy individuals of the pest population (Dimbi *et al.* 2003a). Autoinoculation devices have been widely used for a number of insect and fungal species; some notable studies include their use against fruit flies with *M. anisopliae* (Dimbi *et al.* 2003a; Ekesi *et al.* 2007) and diamond back moth, *Plutella xylostella* with the fungus, *Zoopthora radicans* (Pell *et al*. 1993; Furlong *et al.* 1995; Furlong & Pell 2001). The technique has many advantages over the mass application of fungi: (1) the strategy is highly efficient, particularly when a food bait or pheromone lure is incorporated into the device; (2) it may be species-specific; (3) it is cost effective because the required fungal inoculum levels are low; (4) the autoinoculation devices are easy to build and maintain and (5) infected insects will return and transmit EP fungi to the pest population (Baverstock *et al.* 2009). Autoinoculation devices could contain an insecticide, which is what the 'attract and kill' method of pest control is based on, for both fruit flies and *T. leucotreta* in citrus (Ware *et al.* 2003; Kirkman 2007). However, by incorporating EP fungi, which themselves are self-replicating, it is ensured that vectoring and infection is then spread throughout a pest population and persistence is enhanced (Baverstock *et al.* 2009).

Autoinoculation devices are based on 750 ml plastic bottles that hold both the autoinoculators and the food/pheromone attractants. Autoinoculators which have been investigated thus far consist of a maize cob (Ekesi *et al.* 2007), cheesecloth (Dimbi *et al.* 2003a), non-woven fabric

(Baverstock *et al.* 2009) or a Petri dish (Ekesi *et al.* 2007). The plastic bottle is usually divided into three compartments: (A) the upper compartment is used to introduce the autoinoculators; (B) the middle compartment where the autoinoculators are located and where flies and moths become contaminated with EP fungi and (C) the lower compartment which is separated from with a mesh gauze (20 mm diameter) to prevent insects from drowning. The lower compartment contains a food bait or pheromone to lure insects to the device. Entrance and exit holes are made to allow free movement of insects from the device (Dimbi *et al.* 2003a).

Laboratory and field caged experiments using autoinoculation devices have shown that the number of conidia picked up by individual flies is in the range of 4.2×10^5 and 1.0×10^6 conidia per fly (Dimbi *et al.* 2003a). Castillo *et al.* (2000) reported that adult *C. capitata* fruit flies, when inoculated with various EP fungal isolates on the abdomen, required from 1×10^2 to 1×10^6 conidia per fly to elicit mortality greater than 50% at 10 days post inoculation. During the investigation of *B. bassiana* as a pathogen in the autoinoculation device used to infect *P. xylostella*, male moths acquired a lethal dose of *B. bassiana* within 1 second of exposure to all concentrations tested: 0.24 to 6.2 µg conidia/mm^2. This suggested that the use of these devices to inoculate moths provided an efficient method for transferring inoculum to the rest of the population (Furlong & Pell 2001). These authors showed the overall rate of mortality was not influenced by concentration of *B. bassiana* conidia on the surface of the inoculator but time until insect death was affected by both exposure time and conidia concentration. As moths spent more time walking around the inoculation chamber, they acquired increased levels of fungal inoculums. Thus, by designing devices which maximise time spent by moths in these chambers, one ensures that lethal doses are then transferred to the susceptible target population (Furlong & Pell 2001).

Population suppression by autodissemination is a function of horizontal transmission between healthy insects and fungal contaminated insects (Ekesi *et al.* 2007) and is greatly increased during insect mating. Mating competitiveness in fruit flies is a function of predisposition and compatibility (Ekesi *et al.* 2007). The ability of insects to find a receptive mate and successfully copulate will be crucial to the functioning of horizontal transmission of EP fungi in the field. Dimbi *et al.* (2009) showed that healthy male fruit flies belonging to three species (*C. capitata, C. cosyra* and *C. fasciventris*) showed no mating preference for healthy female flies over fungus infected females. It was concluded that fungus treated male fruit flies competed equally for virgin females on days 0 to 2 post inoculation. However, at day three, untreated males had a

significantly higher percentage of pairing with females compared to fungal treated males (Dimbi *et al.* 2009). These authors further reported a significant delay among fungus infected male flies at the onset of calling and mating due to grooming efforts to remove fungal inoculum. Grooming behaviour further resulted in the spread of fungal inoculum to areas of the body that were not contaminated, particularly the mouthparts (Dimbi *et al.* 2009). Feeding flies, which are also known to regurgitate food and feed one another, may thus also spread EP fungal inoculum to fruit or other individuals where conidia may be picked up by healthy flies.

Female fruit flies are oligogamous (Quesada-Moraga *et al.* 2008) and choose mates based on male pheromone; if male mating behaviour is altered and females preferentially chose healthy males over fungus infected males the effectiveness of horizontal transmission may be lost (Ekesi *et al.* 2007). However, when considering an attractant which is more female specific, as in the Questlure® attractant, more females are attracted to the autoinoculation devices. Female-bias mating will thus not affect horizontal transmission because female flies are then also contaminated with EP fungi. Fruit flies form leks (which are groups of calling males) and this aspect of their biology may be a useful point for the autodissemination of fungal inoculum among healthy individuals because of the close proximity of flies at this time which increases contact chances and pathogen transmission (Ekesi *et al.* 2007). In addition, calling male flies also show the tendency of numerous attempts at homosexual mating (Ekesi *et al.* 2007). These male to male mounting attempts as well as heterosexual fruit fly mating will almost certainly increase horizontal transmission of EP fungi in the field.

In small scale field plot experiments undertaken in Kenya using *Metarhizium anisopliae* in autoinoculation devices against mango fruit fly, *Ceratitis cosyra*, Ekesi *et al.* (2007) demonstrated the efficacy of using these novel devices to significantly reduce fruit flies in this crop. Application of autoinoculation devices has considerable advantage over mass application using soil treatments because relatively low amounts of fungal inoculum levels are required for field application. The case study revealed that dry conidia applied to the devices remained viable for five weeks (68% germination) but then dropped to 27% after six weeks. Longer persistence of fungal conidia was attributed to the nature of the device and placement (under tree canopies) which significantly reduced UV radiation. Currently, the cost of production of *M. anisopliae* used in the biopesticide, Green Muscle® (BCP, South Africa) for locust control in Africa is US$ 200/kg of dry spores which makes application rates unaffordable in developing countries (Ekesi

et al. 2007). However, by using these autoinoculation devices about 0.8-1.0 g of conidia are required per device, which translates into a weekly application of 80-100 g of conidia to treat one hectare of a crop (100 trees/ha). However, fungal inoculum could persist for one month, therefore weekly application may not have been necessary, and this could have further reduced cost. Further, the manufacturing of the devices is inexpensive and maintenance simple (Ekesi *et al.* 2007).

The implementation of autoinoculation devices in citrus IPM as an additional tool for the control of fruit flies and *T. leucotreta* seems promising, particularly because the ratio of fungal inoculum to hosts is greatly reduced compared to the mass application of fungi in soils. Further horizontal transmission appears to be an important aspect of fungal dissemination within insect populations. Based on the study by Ekesi *et al.* (2007) it appears that the implementation of autoinoculation devices within citrus must coincide with insect flight peaks, because peaks determine when mating is at its highest. Data from monitoring trap systems within specific citrus areas will dictate population peaks and the deployment of autoinoculation devices. The biology and occurrence of fruit flies within specific geographic regions may require greater understanding because trap monitoring does not dictate when to apply a strategy but rather when a particular strategy is not working (Moore *et al.* 2008). The ultimate aim of citrus pest management is to reduce oviposition by gravid females (Ekesi *et al.* 2007). Apart from obvious death elicited by EP fungi, Yee & Lacey (2005) reported that the western cherry fruit fly, *Rhagoletis indifferens*, females infected with *M. anisopliae* laid fewer eggs between three and seven days post-infection. Castillo *et al.* (2000) reported that a reduction in *C. capitata* fecundity was evident four days post infection. In fruit flies, young gravid females are more susceptible to fungal infection than older flies, and autoinoculation must target this age group which will reduce the level of oviposition in field populations and contribute to overall insect management. Based on bioassay experiments in the present study, it appears that the adults of *T. leucotreta* are also susceptible to fungal infection, but it is unclear as yet as to whether younger moths will be more susceptible than older moths and warrants further investigation if autoinoculation is to be considered against *T. leucotreta*.

Fruit fly population numbers may peak in some citrus growing areas as early as December, weather dependant (Du Toit 1998). *Thaumatotibia leucotreta* population peaks may be as early as October in some areas (Moore & Kirkman 2009). Autoinoculation devices can be employed in early October or December (depending on insect species) just before insect populations reach

their peak. Further, if devices are baited with pheromones or parapheromones just prior to insect peaks, the device efficacy may be increased because insects seeking mates will be attracted to the devices and become infected with EP fungi. Upon habituation of the pheromone within devices, fungal infected insects will then seek out mates again and copulate. Mating increases horizontal transmission of fungi and thus inoculum may be spread through mating insect populations faster, thus ultimately decreasing egg laying. In the current control of *T. leucotreta* using traps, only synthetic female pheromones have been discovered which attract males (Newton 1998), thus the incidence of female fungal infection may be low. However, *T. leucotreta* exhibits multiple mating in both males and females (Newton 1998), sometimes moths can mate up to several times per day (Stibick 2008), and this aspect of their biology would greatly increase horizontal transmission.

The replacement of devices, according to the persistence of EP fungal conidia, may need to be done every month from October or December onwards, until pest population numbers decrease. A one hectare citrus orchard with tree spacing of approximately 2 m may contain over 1000 trees in the orchard (Knott, pers. comm.). However, the most common modern citrus planting distance is 6 m x 3 m, which gives 555 trees/ha (Moore, pers. comm.). Based on Ekesi *et al.* (2007), it is suggested that a citrus orchard of approximately one hectare may require a weekly application of 800-1000 g (1kg/ha) of conidia (1000 trees/ha), which is still well within the application rate of most conventional insecticides which are applied at 11-17 kg/ha (Jaronski *et al.* 2007). However, based on the study by Ekesi *et al.* (2007), mango trees were widely spaced (7 m x7 m) and perhaps the suggested rates above are excessive. Further study within citrus orchards will estimate these values more precisely. Depending on fungal persistence (germination percentage), the weekly application rate may be reduced to a monthly application treatment, as Ekesi *et al.* (2007) showed that *M. anisopliae* conidia could last for up to five weeks. Furthermore, most fruit fly monitoring trap lures are replaced after 4 to 6 weeks anyway because the lure attractant fades (Grout, pers. comm.). Thus, the compatibility of fungal conidia and attractants is valid and they appear to have similar periods of residual efficacies. This reiterates the practicality of using these devices within citrus orchards to disseminate fungal conidia effectively for the control of adult fruit flies and *T. leucotreta*.

Fruit flies are present throughout the year but overwinter in the adult stage and food, water, and shelter are important overwintering factors (Weems 2002). *T. leucotreta* is also present throughout the year at all life stages however, over winter developmental rates are slowed and

larvae will either spend a substantial amount of time in fruit or pupate and spend up to several weeks (29 to 40 days) in the soil (Newton 1998). The possibility of erecting autoinoculation devices, baited with food attractants in winter to try and reduce overwintering pest populations was considered, however in the case of fruit flies, they are poorly attracted to traps during winter months (Botha *et al.* 2004). Thus, the dissemination of fungal inoculum in winter may be slow and negligible but worth investigating in future studies as these pests are present all year round.

4.4 Conclusion

No single microbial control agent will provide sustainable control of an insect pest or complex of pests. However, the prospect of applying EP fungi in citrus as an additional insect management strategy holds promise, particularly as applications via existing equipment, irrigation and monitoring systems are already available. Research will continue to identify the limitations on efficacy and field persistence of EP fungi to improve their use within cropping systems, particularly because there is much concern over the injudicous use of pesticides which greatly affects the lucrative southern African citrus export industry. Integration of EP fungi within IPM systems will require detailed compatibility studies and the development of effective guidelines for their use (Inglis *et al.* 2001). EP fungal integration within citrus IPM will require further monitoring to optimise the levels of control achieved with each application and the strategy used. One of the major limitations in using EP fungi is the inconsistency of efficacy when useful isolates reach the stage of large scale field testing (Whipps & Lumsden 2001). Fungal isolates must survive application and then persist in the environments for which they are required. Thus, it is crucial that selection, screening and further development processes adopt an ecological approach, which takes into account the features of the required environment (Whipps & Lumsden 2001). Subsequent steps in the development of EP fungi as mycoinsecticides are the inoculum production, formulation and application technologies which need to be cost effective and easy to implement (Butt *et al.* 2001). Further, cost/benefit analyses of the EP fungal commercialisation process have often slowed development because of small niche markets which require EP fungi as biological control agents (Whipps & Lumsden 2001). The cost of producing microbial control products must be judged in terms of the value of the crop protected by using the control agent and in comparison to the cost of chemical control options (Dolinski & Lacey 2007). In Florida, for example, the use of EP nematodes is an integral part of citrus IPM and this indicates that the cost/benefit relationship is positive (Dolinski & Lacey 2007).

Technology transfer is also crucial to the successful implementation of EP fungi within cropping systems and a greater understanding of the ecological requirements of these biological control agents must be met by growers and microbial product representatives. If all the above considerations are adhered to then the utilisation of EP fungi within citrus cropping systems may have a bright future.

4.5 Future work

The acquisition of EP fungal isolates from citrus soils and soils of natural areas surrounding orchards in the Eastern Cape Province has offered some insight into their biodiversity and occurrence. Implemented bioassays against target citrus pests have offered further insight into the relative potencies (as shown by the LC_{50} and LC_{90} values) of these obtained indigenous isolates against *T. leucotreta* and fruit flies in the laboratory. However, bioassays need to be repeated, particularly because the LC_{50} and LC_{90} values against fruit flies were higher than the tested concentration range. Future work should include the repetition of bioassays against *T. leucotreta* with the existing concentration range: 1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8 conidia/ml^{-1}. Repeated bioassays against fruit flies should be initiated at higher concentrations. It is suggested that a starting concentration range against fruit flies will include: 1×10^8, 1×10^9, 1×10^{10}, 1×10^{11}, 1×10^{12}, 1×10^{13} conidia/ml^{-1}, to accurately determine the LC values against these insect pests.

Furthermore, time-response trials against fruit flies and *T. leucotreta* should be undertaken to ascertain LT_{50} and LT_{90} values for the most virulent isolates. It is suggested that *Beauveria* isolates, *G Moss R10, G 14 2 B5* and *G B Ar 23 B3* be considered primarily in repeated bioassays. However, the selection of isolates was based on their ability to induce mortality in all three insect test species, thus there is more scope for future work on *T. leucotreta* with isolates which were not focused on in this study. Isolates of noteworthy mention are: three *Metarhizium* isolates, *G 14 2 B3, T. leucotreta Ar 23 B3* and *G 11 3 L6* and four *Beauveria* isolates *G Ar 17 B3, G OL R11, G Rose R9* and *FF J&B R5.*

A much needed step following laboratory bioassays should include the characterisation of the ecological constraints of the candidate isolates. Environmental competence is an important prerequisite particularly as isolates need to persist to be effective against soil pests.

1.) Environmental competence tests will include temperature or thermo-tolerances (Ouedraogo *et al.* 1997; Ekesi *et al.* 2003; Devi *et al.* 2005; Rangel *et al.* 2005a; Fernandes *et al.* 2008), UV-B tolerance and range (Braga *et al.* 2001; Rangel *et al.* 2005b; Fernandes *et al.* 2007) and moisture or drought tolerance (Ekesi *et al.* 2003; Devi *et al.* 2005) of isolates in the soil. The effect of these factors on fungal virulence must also be considered. It is suggested that these environmental competence tests be carried out in conjunction with known environmental parameters in various citrus growing regions.

2.) Other environmental competence factors which should be investigated to ensure environmental compatibility in citrus cropping systems include:

- The effects of various soil types (clay, loam, sandy) and the carrying capacity and persistence of conidia (Vänninen *et al.* 2000; Jaronski 2007) present in the soil.

- Citrus pesticide and fungicide compatibility tests using candidate isolates may offer some insight into application techniques. For example, the viral biopesticide, Cryptogran® (River Bioscience, South Africa) has been shown to be compatible in tank mixes with the following chemicals mancozeb, benomyl, strobilurins, abamectin, methomyl, methidathion and pyriproxyfen which has greatly increased the ease of application and acceptance for its use in citrus (Moore 2009). Some noteworthy studies have been undertaken to evaluate the effects of pesticides and fungicides on EP fungi by Filho *et al.* (2001), James & Elzen (2001), Shapiro-Ilan *et al.* (2002), Sterk *et al.* (2002), de Oliveira *et al.* (2003), Saenz-de-Cabez Irigaray *et al.* (2003), de Oliveira & Neves (2004), Mochi *et al.* (2005, 2006), Purwar & Sachan (2006) and Thompson & Brandenburg (2006).

3.) The effects of various growth media on the sporulation, growth and germination of candidate isolates need to be investigated, particularly if mass production of fungal conidia is to be considered (Campbell *et al.* 1978; Li & Holdom 1995; Ibrahim *et al.* 2002; Safavi *et al.* 2007). Fungal radial growth, sporulation potential and germination speed have been shown to change not only with fungal species and isolates, but also with the carbon/nitrogen ratios of the media used for production. It is important to note that formation of distinct morphological variants or sectors is often observed in fungal cultures maintained on artificial media, thus the stability of isolates needs to be further investigated

in conjunction with the effects of nutrients if mass production is considered (Brownbridge *et al.* 2001; Shah & Butt 2005; Wang *et al.* 2005).

4.) Genetic characterisation studies of candidate EP fungal isolates are also an important prerequisite for the identification and tracking of strains in nature and the determination of genetic diversity. Molecular techniques used to ascertain genetic diversity in EP fungi include restriction fragment length polymorphism (RFLP), the use of the ribosomal internal transcribed spacer region (ITS) and the nuclear gene, elongation factor- 1á (EF-1á) (Rehner & Buckley 2005). Further, multilocus microsatellite genotyping mapping (Meyling *et al.* 2009) and universally primed (UP) PCR fingerprinting methods (Meyling & Eilenberg 2006a) are additional genetic methods employed.

5.) If autoinoculation devices are to be considered in citrus IPM, then the following biological and physiological aspects need to be evaluated:

- The horizontal transmission potential of candidate isolates against citrus pest adults (Furlong & Pell 2001; Quesada-Moraga *et al.* 2008).
- The investigation of the effects of candidate isolates on mating behaviour of citrus pests (Dimbi *et al.* 2009).
- The effects of candidate isolates on the fecundity and egg fertility of citrus pests (Ekesi & Maniania 2000).

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