The search for a biological control agent to control invasive *Polistes dominula* wasps in the Western Cape region, South Africa

By

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Declaration

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Summary

Polistes dominula (Christ) (Hymenoptera: Vespidae: Polistinae), also known as the European paper wasp is globally notorious as a highly invasive wasp, and invaded and established itself in five continents, with the exception of the Antarctica. *Polistes dominula* had a negative impact in invaded regions, where it has been reported to displace native wasp species by exclusive competition. The first record of *P. dominula* in South Africa was in 2008 in the Western Cape Province. Since then, it has expanded its range to more areas in the Western Cape. However, there have been no reports of this invader in other provinces in South Africa.

Globally, there have been many methods used in the attempt to control invasive wasps. These methods include mechanical control, which involves the physical removal and destruction of nests, chemical control, which is the use of chemical pesticides, and biological control, which involves the use of a living organism to control the population of another. It has been found that mechanical control is labour intensive and chemical control is not environmentally friendly. This leaves biological control to be the best potential control strategy for the wasp.

Research has been carried out to find natural pathogens of invasive wasps and there are many potential control agents that could be used. These include entomopathogenic nematodes, entomopathogenic fungi, viruses, bacteria and parasitoids. These biocontrol agents tend to be host specific, and there has been no biological control agent developed against *P. dominula*.

In this study the fungi associated with *P. dominula* were isolated from the nests of wasps and identified. Most of the fungi isolated from the nest material were saprophytic fungi that are commonly found in soils and on plant material, with a few known to be pathogenic to plants and insects. The pathogenicity of selected fungi was tested against *P. dominula* second instar larvae and it was confirmed that, among the isolated fungi, *B. bassiana* was more virulent under laboratory conditions. This fungal isolate was able to kill 80% of the larval population within three days after exposure to the fungal treatment.

Furthermore, a field trial was conducted to determine the pathogenicity of the *B. bassiana* isolate that was successful in infecting *P. dominula* larvae under laboratory conditions. This fungal isolate was, however, not able to establish under the unpredictable field conditions, as there was no visible proof of infection to the larvae that were treated with the fungal isolate.

There are a number of biotic and abiotic factors that may hinder a fungal biological effect. In this study, temperature was one of the abiotic factors that played a role in hindering the fungal isolate from establishing in the field. Laboratory experiments showed that *B. bassiana* could only grow optimally at temperatures between 25 ˚C and 30 ˚C and at 40 ˚C fungal spores were not able to survive. The temperature in the field during the experiment, regularly reach above 30 ˚C.

Biotic factors, including other living organisms, also have an effect on the success or failure of a biological control agent. In this study, a *Bacillus* species were isolated from the nest material and were found to inhibit the growth of *B. bassiana*.

This study has provided valuable information by showing that the larvae are susceptible to the entomopathogenic fungi *B. bassiana* under laboratory conditions. Therefore, more research needs to be done to improve the performance of this pathogen in under field conditions.

Opsomming

Polistes dominula (Christ) (Hymenoptera: Vespidae: Polistinae), ook bekend as die Europese papierwespe, is wêreldwyd bekend as ‘n indringerspesie. Hierdie wespe het al vyf kontinente ingedring en gevestig, met uitsondering van die Arktiese gebied. *Polistes dominula* het ‘n negatiewe impak op die areas waar hy indring deur die inheemse wespe te verplaas deur middel van kompetisie. Die eerste keer dat *P. dominula* in Suid-Afrika aangemeld was, was in die Wes-Kaap in 2008. Sedertdien het dit in meer gebiede van die Wes-Kaap gevestig, maar daar is nog geen teken van hierdie indringer in ander provinsies voorkom nie.

Wêreldwyd is daar al verskeie metodes aangewend om indringerwespes te beheer. Dit sluit in meganiese beheers wat die fisiese verwydering en vernietiging van neste behels, chemiese beheer waar die gebruik van chemiese plaagdoders aangewend word; en biologiese beheer wat die gebruik van ‘n lewendige organisme vir die beheer van ‘n ander behels. Meganiese beheer kan arbeidsintensief wees en chemiese beheer is nie omgewingsvriendelik nie. Daarom is die beste potensiële strategie om wespes te beheer, dus die biologiese beheer.

Verskeie navorsings is al uitgevoer om ‘n natuurlike patogeen vir indringerwespes te identifiseer. Daar is verskeie potensiële beheermiddels wat gebruik kan word. Hierdie sluit in entomopatogeniese nematodes, entomopatogeniese swamme, virusse, bakterieë en parasitoïede. Hierdie beheermiddels is geneig om gasheer spesifiek te wees en daar was tot dusver nog geen beheermiddel ontwikkel teen *P. dominula* nie.

In hierdie studie is verskillende swamme wat met *P. dominula* assosieer, geïsoleer vanaf die neste en geïdentifiseer. Die meerderheid van die geïsoleerde swamme is saprofitiese swamme wat algemeen voorkom in grond en plantmateriaal. Slegs ‘n paar is bekend as patogene teen plante en insekte. Verskeie swam-isolate se patogenisiteit is teen *P. dominula* larwes getoets. Vanuit al die geïsoleerder swamme, het *B. bassiana* onder laboratoriumtoestande die hoogste virulensie

getoon. Binne drie dae na die blootstelling aan hierdie swambehandeling, het 80% van die larwes doodgegaan.

Verder is ‘n veldproef uitgevoer om die patogenisiteit van die *B. bassiana*-isolaat, wat suksesvol was onder laboratorium kondisies, te toets. Die onvoorspelbare veldkondisies het egter daartoe gelei dat die swam nie in die veldproef suksesvol was nie. Daar was geen sigbare bewys dat die larwes geïnfekteer is met die swam-isolaat nie.

Daar is verskeie biotiese en abiotiese faktore wat die biologiese effek van swamme kan beïnvloed. In hierdie studie het temperatuur veroorsaak dat die swam nie in die veld suksesvol nie. Eksperimentele werk in die laboratorium het getoon dat *B. bassiana* optimaal groei by temperature tussen 25 ˚C en 30 ˚C. Die swamspore het nie by temperature bo 40 ˚C oorleef nie. Tydens die veldproef het temperature gereeld bo 30 ˚C gestyg.

Biotiese faktore, soos ander lewendige organismes, het ook ‘n effek op die sukses of mislukking van ‘n biologiese beheersmiddel. In hierdie studie is ‘n *Bacillus*-spesie geïsoleer vanaf die nesmateriaal. Hierdie isolaat het die groei van *B. bassiana* effektief geïnhibeer.

Hierdie studie verskaf waardevolle inligting deur te bewys dat die larwes vatbaar is vir die entomopatogeniese fungi, *B. bassiana*, onder laboratorium kondisies. Daarom word meer navorsing benodig om die effek van hierdie patogeen in die veld te verbeter.

Dedication

To my dear fiancé, Toyosi Craig

Thank you for your encouragement, love, and patience – I wouldn't have come this far without your support

...Ololufe mi, modupe o

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Abbreviations and Acronyms

AIS	Alien invasive species
EPF	Entomopathogenic fungi
spp.	Species (plural)
sp.	Species (singular)
IPM	Integrated pest management

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Chapter 1

1. Invasive *Polistes dominula* in the Western Cape region of South Africa and their control.

1.1 Invasive wasps: *Polistes dominula* a global view

Alien invasive species (AIS), are plants, animals or microorganisms that have been, accidentally or purposefully introduced to areas beyond its native range, where they rapidly spread from the point of introduction to become abundant (Manfredini *et al.*, 2013; Kolar *et al.*, 2001). These alien species are notorious for having adverse impacts on the economy, the environment and human health (Lester *et al.*, 2014; Lymbery *et al.*, 2014). Invasive species are globally considered to be the leading threat to biodiversity (Gardner-gee & Beggs, 2013; Beggs *et al.*, 2011; Giliomee, 2011).

The risk of IAS invading new areas has increased with the rapid expansion of global trade in recent decades (Beggs *et al.*, 2011; Lester *et al.*, 2013). Most insect AIS have a close association with human transport and have a relatively low probability to be detected with standard border inspection protocols (Kenis *et al.*, 2007; Work *et al.*, 2005). One major reason which attributes to the success of invasive species is the phenomenon known as the enemy release hypothesis, which is based on the concept that the invader has escaped its natural enemies. This is because in its native range, an invader has a natural enemy that regulates its population, however, in the invaded range, the natural enemy is absent, leading to the increase in population size of the invader in the new location (Colautti *et al.*, 2004; Lester, 2014; Lester *et al.*, 2015; Liu & Stiling, 2006).

Social insects from the Order Hymenoptera, which includes bees, ants and wasps, have been the most successful invaders in the past 100 years (Moller, 1996). Possible reasons for the invasive success of social hymenoptera is that they are more adapted to disperse through human activities, especially transportation, because they can hide away in human goods and only a single fertilised queen is needed to start a new population. In addition, they have great dispersal abilities, high reproductive rates, generalist diets, a broad host range and their social lifestyle provides them with colony level responses (Beggs *et al.*, 2011; Moller, 1996).

Vespidae is a globally distributed, diverse wasp family with nearly 5000 species, which includes almost all species of eusocial wasps and also many solitary species (Beggs *et al*., 2011). *Polistes dominula* (Christ, 1791), also known as the European paper wasp, is one of the most abundant and wide spread *Polistes* species having successfully invaded and established in five continents namely, North America, South America, Asia, Africa and Australia (Cervo *et al*., 2000). In the USA, the first *P. dominula* wasps were observed in the Boston, Massachusetts (Eastern USA) area during the late 1970's. Since then more sightings were reported from surrounding states. In 1995, *P. dominula* were reported from the Sacramento area (Western USA) and within 25 years, *P. dominula* had spread across the USA and became the dominant species, displacing the native wasp species *P. fuscatus* (Cervo *et al*., 2000).

Similar to *P. dominula, Vespula germanica* invaded most continents of the world and both species exhibit the same flexible foraging behaviour, in that they both feed their larvae on protein based sources such as larvae of other soft bodied insects like Lepidoptera larvae or aphids, while the adults feed on crop liquids, such as nectar. Both species have economic, ecological, and social impacts in their invaded regions (Cervo *et al*., 2000; Buteler *et al*., 2016). Corley (2013) reported that *V. germanica* has become established in Argentina and currently is the most visibly abundant insect. In the 1940's, New Zealand was invaded by *V. germanica* and it has become a significant pest, but *Polistes dominula* has only recently made its way to New Zealand and is considered to have been in New Zealand since 2011 (Beggs *et al*., 2011).

1.2 *Polistes dominula*: South African context

The Cape Floristic region in the Western Cape of South Africa is a biodiversity hotspot (Cowling *et al*., 1999). Armstrong (1995) stated that in the Western Cape alone 80% of the listed endangered species are at risk due to invasion by alien invasive plants and animals. Eardley *et al*. (2009) was the first to record the presence of *P. dominula* in South Africa, where it was first sighted in 2008, in the suburb, Kuilsriver, and since then it has rapidly spread to many towns and cities including Stellenbosch, Elsenburg, Wellington, Jonkershoek, Franschoek, Paarl, Somerset West and Paarl (Benade, 2015; Benade *et al*., 2014). Not much is known about how the wasps spread, but literature revealed that their dispersal to different countries is mainly through anthropogenic activities (Corley, 2013). Fertilized queens find a hiding place in ships that carry goods, and due to low detection standards at the borders they can pass the borders. Benade *et al*. (2014) evaluated the spread of *P. dominula* in South Africa,

and observed that this species prefers humid areas, as no *P. dominula* specimens have yet been collected from drier regions. *P. dominula* is considered to be localized only in the Western Cape region, as there have not been any record of its presence anywhere else in South Africa to date (Eardley *et al*., 2009). The Western Cape region has weather conditions similar to the species' native region with wet winters and dry summers (Eardley *et al*., 2009). Hocherl *et al.* (2015), looked at the nesting behaviour of *P. dominula* and observed that this wasp species shows a very flexible and adaptive behaviour in response to climatic conditions. There is thus a concern that *P. dominula* could cross the boarders of the Western Cape and spread to other parts of South Africa (Benade *et al.*, 2014).

1.3 Biology

Polistes dominula, also known as the European paper wasp, has a bright yellow coat with black stripes across the abdomen (Fig.1A). The length of the body is on average 15-20 mm. The queen, also known as the foundress, is slightly bigger than the workers (Van Zyl 2016). At the beginning of the founding season, which is usually spring, the foundress emerges from over-wintering and looks for a suitable site where she can establish a colony. The foundress builds an aerial nest from chewed-up wood and turns it into a papier-mâché-like form, hence the term "paper" wasp. The nest cells are hexagon-shaped and face downwards. After a few cells have been built the queen then lays a single egg per cell and three days later the larvae hatch. The larvae moult and go through five larval instars in about five weeks before they pupate. The larvae are capped in the cells and later emerge as adults from the cocoon (Fig. 2) (van Zyl 2016). After the first workers emerged, the queen puts all her focus on laying eggs, while the workers are in charge of expanding the nest, feeding the larvae and protecting the nest from predators in general (Edwards 1980; van Zyl *et al*., 2016). The larvae of *P. dominula* have a generalist diet, and are fed chewed up soft bodied insects like aphids, caterpillars, bee and other wasp larvae (Cervo *et al*., 2000; Allsopp pers.comm., 2017; Benade, 2016).

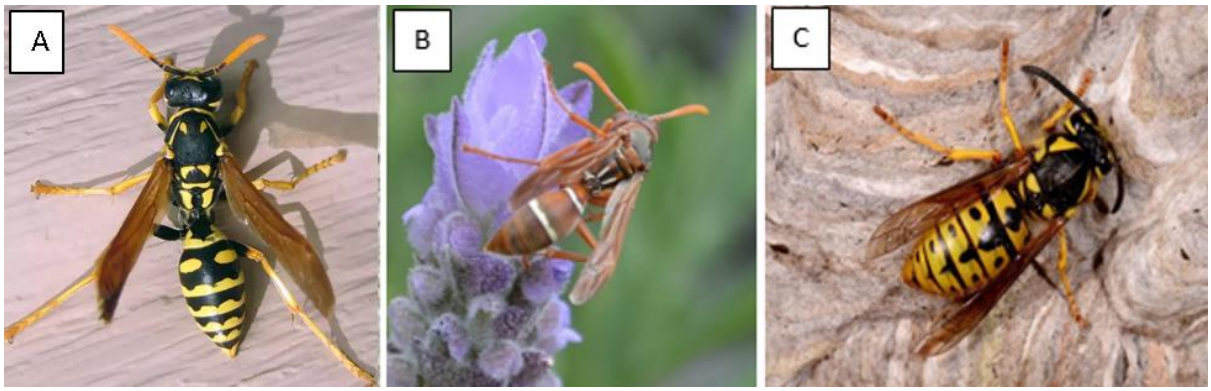

Figure 1. Social wasps A) *Polistes dominula* (invasive wasp species) Photo by John R. Maxwell (2008), B) *Polistes marginalis* (native wasp species) photo by Charles Griffiths and C) *Vespula germanica* (invasive wasp species) photo by Dr S van Noort

Figure 2. Aerial nest of *P. dominula*, the central cells are capped, these cells have cocoons that house the pupae. Photo by Carolien van Zyl (2016)

1.4 Impacts

1.4.1 Ecological impact

Polistes dominula adults feed mainly on carbohydrates or protein-based foods (variety of soft bodied insects for the larvae and nectar for the adults). This feeding behaviour is similar to that of *V. germanica* and to three native South African genera of Polistine paper wasps, *Belonogaster*, *Polistes* and *Ropalidia* (Scholtz & Holm 1985, Benade, 2016). Therefore, *P. dominula* pose an ecological threat to the native wasp species due to niche overlap. There is

an increased possibility that there is direct competition for food and shelter and also predation, since *P. dominula* feed their larvae soft bodied insects including the larvae of native wasps (Benade *et al*., 2014). Benade *et al*. (2014), in his field studies observed that *P. dominula* has become the dominant species potentially displacing the native species, *P. marginalis.*

Another factor that makes *P. dominula* a successful invader is their ability to withstand and adapt to broader weather conditions, which has allowed then to spread and persist in almost all continents with the exception of Antarctica (Beggs *et al*., 2011; Moller, 1996; Manfredini *et al*., 2013).

1.4.2 Economic impacts

In South Africa and in other countries, the viticulture industry experiences economic losses from this pest, as *P. dominula* readily build their nests inside the hollow part of metal grape vine trellises, the structures that holds the grape vines up. The wasps are aggressive and often prevent farm workers from harvesting the grapes, therefore, time is wasted trying to remove the nests. This often times lead to extra expenses in buying insecticides used to control wasp population in vineyards, which is also accompanied by extended harvest periods and more labour hours (Kenis *et al*., 2009; Beggs *et al*., 2011, Giliomee, 2011; Benade *et al*., 2014). For example, some states in Australia lose approximately 50% of the entire grape crop because farm workers cannot collect the grapes in time (Hendrichs *et al*., 1994). The fruit industry in the USA has also suffered losses due to *P. dominula*, as the wasps tend to damage fruits such as nectarines, raspberries and grapes. In 2005, some growers did not harvest anything due to damaged late-ripening grapes (Cranshaw *et al*., 2011).

In New Zealand approximately 10 000 bee hives are adversely affected each year by *V. germanica*, an equally invasive wasp, with similar dietary traits (Haupt, 2015), and with the continuous spread of *P. dominula* in South Africa, similar economic impacts as those of *V. germanica* can be expected. In South African apiculture, beekeepers have reported that *P. dominula* frequent bee hives but there is no available literature that has determined the economic loss due to predation of *P. dominula* on local bees (Moller, 1996; Allsopp, pers. comm. 2017).

1.4.3 Social impacts

P. dominula have been observed to construct their nests in close proximity to humans, under roof eaves, in between wall cracks, wooden planks of timber houses and on door frames. These wasps are a social nuisance at parks where people have outdoor activities such as picnics or at restaurants at wine farms, as these wasps are attracted to grapes (Cranshaw *et al*., 2011). *P. dominula* wasps are aggressive when humans approach their nests, and they have a painful sting which in some people who are allergic to the sting, could be fatal (Severino *et al*., 2006).

1.5 Management strategies

The eradication of invasive pests is globally considered to be vital in the protection of native biodiversity. Therefore, there is an increased need to find methods that will be effective yet not harm the native insects. Immediately after introduction of an invasive species , there is a very small window of opportunity for eradication, and once that opportunity is missed, the next focus would be to control and keep the wasp populations under the ecological damage threshold (Lester *et al*., 2013). In South Africa, the eradication window for both *P. dominula* and *V. germanica* (the other invasive wasp species that is problematic in the Western Cape Province of South Africa), were missed and the focus are now on ways to keep invasive wasp populations under the damage threshold.

According to literature, there are three major management approaches that exist, namely: mechanical control, chemical control and biological control (Beggs *et al*., 2011; Kenis *et al*., 2009; Lacey *et al*., 2015; Roberts, 1989; Shahid *et al*., 2012). These three management strategies are discussed below with respect to how they have been used in attempt to control invasive wasps.

1.5.1 Mechanical control

Mechanical control includes the manual removal and destruction of wasp nests. The advantage of this control method is that it is easy to achieve because it involves locating and destroying nests, and it targets only the intended insects. Beggs *et al.* (2011) stated that this method was difficult for the control of *V. germanica* as their nests are not easily located and the risk of being stung makes the nests dangerous to approach.

Mechanical control would not be effective in controlling *P. dominula* because their nests are not always easily accessible, as the nests are usually on eaves and roofs. Manual removal of nests is only effective if the nests are distributed over a small area, but, if the nests are distributed over a large area, such as a vineyard, it would be difficult and time consuming to individually remove each nest. This control method, is therefore, not the best option for controlling *P. dominula* because it is labour intensive (Beggs *et al*., 2008; Beggs *et al*., 2011; Van Zyl, 2016).

1.5.2 Chemical control

Chemical control includes the use of chemical insecticides that can either be sprayed directly onto the nests or insects. For the control of *V. germanica* which constructs its nests underground, the insecticide cannot be sprayed directly onto the nests and require the use of a baiting method (Beggs *et al.*, 2011). In the bait method the insecticide is mixed with a meat-based bait to attract insects, the target insect then carries the bait back to the nest to feed the brood and this aids the transmission of the poison to every nest mate. Only protein-based baits are used since sugary baits could also attract non-target insects such as bees (Rose *et al*., 1999).

In New Zealand, poison-baiting trials were carried out in 1986-1990 to manage *V. germanica*, where Compound 1080 (sodium monofluoroacetate) was mixed with canned sardines. The results were very positive and compound 1080 was able to reduce wasp foraging activity (where no wasps were leaving or entering the nest) by 100% within six hours. Though this compound was effective it was also found to be highly toxic to vertebrates (Spurr, 1991).

Chemical insecticides have a limited effectiveness, and at best they can achieve temporary localised control (Beggs *et al*., 1998). The disadvantages of this type of control are that chemical insecticides are expensive, labour intensive, potentially harmful to non-target organisms including humans, and the chemical residues that are left behind are not environmentally friendly (Rose *et al*., 1999).

Manual removal and chemical control are thus not ideal and are considered not feasible or practical in large areas such as farmlands because the nests are too numerous to attend to individually, as is in the case of vineyards in Stellenbosch, South Africa (Rose *et al*., 1999). Because of these drawbacks, the necessity for the search of alternative control methods cannot be overemphasized. An attractive alternative to these methods is biological control.

1.5.3 Biological control

Biological control is based on the idea that populations of living organisms are restrained by other naturally occurring biotic factors, such as other living organisms (Huffaker *et al.*, 1976; Roberts, 1989; Pal *et al*., 2006; Heydari & Pessaraki, 2010; Lacey *et al.*, 2015; Ruberson *et al*., 1999). Researchers from different disciplines have been drawn to biological control as they seek to find an alternative to chemical control (Burges & Hussey, 1971; Perkins, 1982; Graves *et al*., 1999). Table 1 illustrates the different strategies of biological control.

Entomopathogenic fungi (EPF), bacteria, viruses, protozoa and nematodes are pathogens of insects. These characteristic makes it possible to use them as biological control to restrict productivity and growth of insects. A number of biopesticides have been developed from these pathogenic organisms (Sáenz-de-Cabezón & López-Olguín, 2010).

A successful wasp pathogen is one that would have the ability to overcome the defence mechanisms of the wasp colony and be able to spread within the nest to kill the inhabitants of the nest (Rose *et al*., 1999; Inglis, & Goettel, 2007). Therefore, for a pathogen to be a successful long-term inoculative biological control agent, it would have to be able to survive throughout the winter and spread to new nests in spring when the conditions to establish a new nest are favourable.

Bacteria

Entomopathogenic bacteria, are those bacteria that have the ability to infect insect hosts (Harris *et al.*, 2000; Rose *et al*., 1999). Over the past three decades, most research focused on toxin-producing bacteria belonging to the genus *Bacillus*, and very little has been done on non-spore-forming bacteria. The reason for this could be that bacteria that do not undergo sporulation cannot tolerate unfavourable conditions such as the insect's internal environment and other abiotic factors (Lacey *et al*., 2015; Tohidi *et al*., 2013). There are a number of formulated bacterial products that have been developed and commercialised as biocontrol agents for several insect orders including the orders, Coleoptera (beetles), Diptera (flies) and Lepidoptera (moths and butterflies) (Gill *et al.*, 1992).

Serratia marcescens, S. entomophila, Lysinibacillus sphaericus, Paenibacillus spp. and a number of *B. thuringiensis* sub-species are bacterial species that have been confirmed through bioassays as pathogens of the order Hymenoptera (Rose *et al.*, 1999). *B. thuringiensis* is the

most commonly used commercial biological control agent of insects. It is a rod-shaped, gram-positive spore-forming bacterium, and during sporulation produces a variety of toxic crystal proteins, called endotoxins, that are insecticidal (Gill *et al.*, 1992; Knowles, 1994; Laridon *et al.*, 2006; Tohidi *et al.*, 2013). The production of the endotoxin is controlled by the CRY gene of the bacterium and the CRY genes are divided into classes *cryI, cryII, cryIII,*and *cryIV* (Tohidi *et al.*, 2013). Each class is specific for a different insect order, for example the CryI is lepidopteran-specific, CryII is lepidopteran and dipteran-specific, CryIII is coleopteran-specific and CryIV is dipteran specific (Laridon *et al.*, 2006; Tohidi *et al.*, 2013).

For the endotoxin of *B. thuringiensis* to induce its insecticidal affect, it needs to first be ingested by the insect host. Figure 3 illustrates the mechanism of action of *B. thuringiensis*. The ingested bacterium containing the spore and the endotoxin reach the mid-gut of the insect, and because of the high pH, the spores cannot germinate. However, the high pH dissolves the outer layer of the bacterium, releasing the protoxin, which is then activated by proteolysis in the insect gut. Upon activation, the protoxin attaches itself to the epithelium of the insect gut, causing holes in the gut lining. Blood and the haemocoel gets released in the gut, lowering the pH and providing the spores with enough nutrients to germinate. Bacteria proliferate within the insect, leading to the death of the insect (Gill *et al*., 1992; Laridon *et al*., 2006). Insects that have been infected by bacteria, exhibit symptoms such as sluggishness, loss of appetite, discharge from oral and anal cavities, discoloration, liquefaction and putrefaction of body tissues (Kiliç, & Lal, 2014).

The major disadvantage of this bacterium as a biological control agent is that it must be ingested by the target insect, because the toxin (protoxin) is only activated in the mid-gut of the insect, therefore, it would need to be incorporated into a bait that can be eaten. Another disadvantage of *B. thuringiensis* is that the CRY genes, which are responsible of producing the toxin, are highly specific and are not virulent to all insect orders. This is a disadvantage in the control of wasps because there is no Cry gene that has been found to code for a toxin that is effective against the order Hymenoptera (Tohidi *et al.*, 2013).

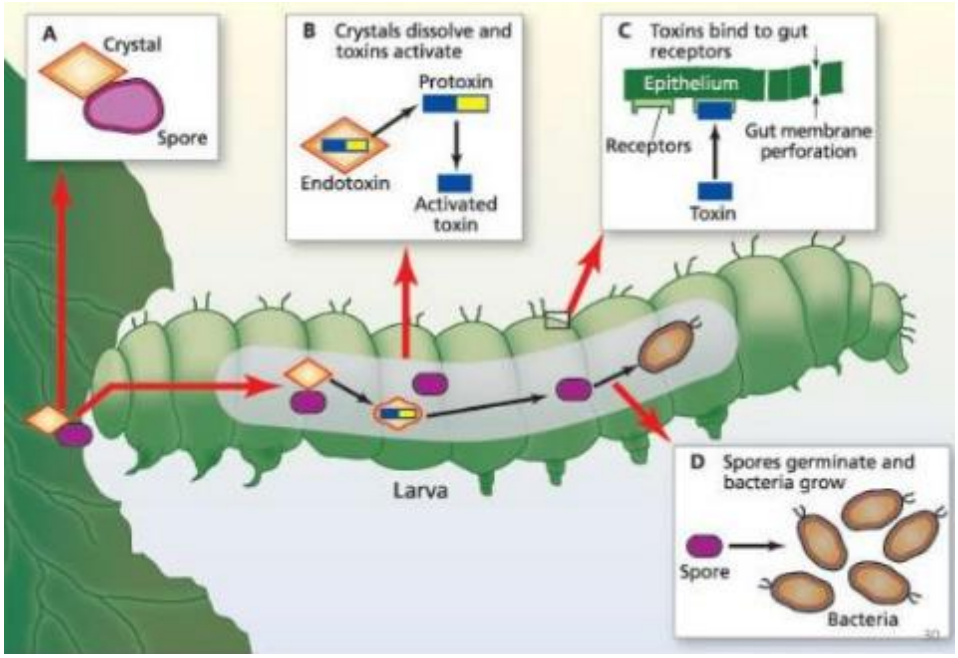

Figure 3. A simplified illustration of the mechanism of action of *B. thuringiensis* (Prabhu, 2015)

Viruses

There is a large number of viruses that have the potential to be biological control agents, with the most common entomopathogenic viruses from the Baculoviridae family (granuloviruses [GV] and nucleopolyhedroviruses [NPV]) (Lacey *et al.*, 2001; Rincón-castro, *et al.*, 2011). The larval stages of insects from the orders Lepidoptera and Hymenoptera have been known to be hosts of baculoviruses. One example of the use of baculovirus was its involvement in the control of *Anticarsia gemmatalis*, a pest moth of soybeans in America. This virus was isolated from the pest and mass produced and since been used to control soybean pests (Carter, 1984). Another example of entomopathogenic viruses include picorna-like RNA viruses such as the sacbrood virus, black queen cell virus and the deformed wing virus (Singh *et al.*, 2010).

Though there are a number of commercially available viral control agents for insects, there is very little information on other viruses as control agents that are commonly associated with the death of vespidae (Rose *et al.*, 1999). Viruses may be acquired either *per os* (orally), or through parasitism by vectors such as mites (Granados, 1980). The mode of action for Baculoviruses have been studied, and the most common entry route into the insect host is *per os* (Rincón-castro *et al.*, 2011). The virus travels to the mid-gut, which is lined with epithelial cells that allow virus attachment. Once inside the cells, the virus targets the nucleus and uses host cell

mechanisms to produce more viral particles and the virus-containing vesicles are then transported into the haemocoel leading to the death of the larvae. In sacbrood disease, infected larvae change colour from cream-white, to yellow and later to dark brown. The virus accumulates inside the dead larvae forming a sac-like structure filled with virus-containing fluid ready to infect any adult wasp that would come into contact with the dead larvae.

The disadvantage of using hymenopteran viral pathogens as a biological control agent is that they are not species specific. This is a disadvantage because if wasps are infected with the viral agent, they may easily transmit it to bees because wasps and bees might visit the same foraging sites (Chen *et al*., 2006; Levitt *et al*., 2013; Singh *et al*., 2010). Therefore, the low specificity of viral pathogens and the fact that to be activated they need to be ingested, makes viral pathogens an unfavourable option for the control of wasps.

Nematodes

Among other biological pesticides, entomopathogenic nematodes are potential biological agents for the control of various insects (Grewal *et al*., 2005; Somasekhar *et al*., 2002; Wilson & Grewal, 2005). Nematodes belonging to the genera *Steinernema* and *Heterorhabditis* spp. are obligate entomopathogens and have been tested to be virulent in a variety of insect orders (Harris *et al*., 2000; Shapiro-Ilan *et al*., 2012; Yadav & Lalramliana, 2012). These two genera have the attributes of an ideal control agent, because they are easy to mass produce, have a broad host range, high virulence and they are not harmful to the environment (Barberchek & Kaya, 1990; Choo *et al*., 2002).

Nematodes from the family Mermithidae are also pathogenic to many insect orders and ticks (İNCİ *et al*., 2014). Mermithid species have been used as an inundative control agent to supress mosquito larvae in rice-fields and ground pools (World Health Organization, 1982). Koehler *et al*. (1992) showed that *Steinernema carpocapsae* are pathogenic to cockroaches that inhabit moist habitats.

Most entomopathogenic nematodes belonging to *Steinernema* and *Heterorhabditis spp.* house pathogenic bacteria *Photorhabdus spp.* and *Xenorhabdus spp.* in their stomach (İNCİ *et al.*, 2014). These nematodes gain entry into the haemocoel of the insect host via natural openings such as spiracles, mouth, and anal cavity and in between the segments. The bacteria housed in the nematode intestines are then released and they are the primary cause of death of the insect

within 24-48 hours after exposure (Georgis, Mullens, & Meyer, 1987; Shapiro-Ilan *et al*., 2012). Though nematodes are effective control agents, they are intolerant to polluted organically enriched environments, and high temperatures, they are only capable of establishing in moist environments (Lacey *et al*., 2001).

There are a few studies that reported nematode species that are pathogenic to social wasps (Rose *et al*., 1999; Harris *et al.,* 2000; Lacey *et al.,* 2015). Van Zyl *et al*. (2016) conducted a study using South African indigenous species of entomopathogenic nematodes, *Heterorhabditis bacteriophora, H. noenieputensis* and *Steinernema yirgalemense* against *V. germanica* and *P. dominula* wasps. Both wasp species were infected and dead four days after exposure to pathogens. These findings suggest that the indigenous nematodes have the potential to be effective biological control agents and that they can be used in an integrated pest management programme to control invasive wasps in South Africa.

Parasitoids

A parasitoid is an organism that spends a significant part of its life attached to or inside a host organism , this association is only beneficial to the parasitoid because it ultimately leads to the death of the host organism (Bonet, 2009; Miller, Donnelly, & Gamboa, 2013; Severino *et al*., 2006).

The use of parasitoids as biological control agents has been attempted to control invasive wasp populations, but with limited success (Beggs *et al*., 2008). In New Zealand, the parasitoid *Sphecophaga vesparum* (Hymenoptera: Ichneumonidae) was introduced from its native range in Northern Hemisphere and has established in some areas (Donovan *et al*., 1989). It was predicted that the parasitoid will reduce the wasp population by 10% at most and this was not enough to solve the problem.

Three different parasitoid taxa have been isolated from both the American native *P. fuscatus* and the invasive *P. dominula.* These included *Dibrachys cavus* (Hymenoptera: Pteromalidae), *Chalcoela iphitalis* (Lepidoptera: Pyralidae) and *Sacophaga* sp. (Diptera: Sarcophagidae) (Beggs *et al*., 2008; Miller *et al*., 2013). *P. dominula* were more susceptible to being parasitized by *D. cavus* and *C. iphitalis*, whereas these parasotoids did not establish well in *P. fuscatus* (Miller *et al*., 2013).

One of the natural enemies of *P. dominula* in its native range is *Xenos vesparum*, a strepsipteran parasite (Beani, 2006; Manfredini *et al*., 2013; Miller *et al*., 2013). The first instar larvae of *Xenos vesparum* gains entry into immature stages of *P. dominula* through the soft parts of the cuticle. Infection by this parasitoid results in a behavioural change on the wasp, where infected workers are inactive and abandon the nest (Hughes *et al.*, 2004). An attempt was made in the United States, to use *Xenos vesparum* as a classical biological control agent, however, it was not successful, because, the rate of infection was too low to cause a colony wide response (Beani, 2006).

Benade *et al.* (2014) investigated parasitoids of both *P. dominula* and the indigenous *P. marginalis* in South Africa. He found that there were three parasitoids which were collected from the *Polistes* nests, two Hymenopteran parasitic wasps from the families Eurytomidae and Eupelmidae. The third parasitoid was a parasitic fly from the Tachinidae family. This fly is the most dominant parasitoid that parasitizes *P. dominula* nests. Parasitoids are not a desirable method of control, because the parasites are not just specific to *P. dominula* but can also infect and affect native *Polistes* spp.

Entomopathogenic fungi

Entomopathogenic fungi from the order Hypocreales have been known to regulate insect populations in soil environments (Roberts, 1989; Lacey *et al*., 2009; Shahid *et al*., 2012). A number of biopesticides have been commercially developed to control economically important agricultural pests (Acharya *et al.*, 2015; Shahid *et al*., 2012; Tamuli & Gurusubramanium, 2011). These commercialized products include among others, *Beauveria bassiana* which has been used against a wide variety of pests worldwide. *Beauveria brongniartii* which has been used against a wide variety of insect orders including, coleoptera, hemiptera, diptera and lepidoptera. *Metarhizium anisopliae* products have been developed commercially against termites, sugarcane spittle bugs and there is also a product available to control locusts and grasshoppers (Acharya *et al*, 2015).

Fungal agents are better candidates to control insects compared to bacterial or viral agents, which first need to be ingested by the insect before they start germinating internally to cause any notable damage (Laridon *et al*., 2006; Shahid *et al*., 2012). What makes fungi better biological control agents is that they are the only group with the ability to penetrate the insect exoskeleton (Lacey *et al.*, 2009; Wraight *et al*., 2007). The success or survival of

entomopathogenic fungi relies on a delicate interaction between the host insect, the fungus and the environment (Inglis *et al.*, 2012; Wraight *et al.*, 2007). The life cycle of entomopathogenic fungi is illustrated in Figure 4.

Harris *et al*. (1999) conducted a study to search for possible fungal pathogens associated with invasive wasps. Four fungal species were isolated namely *Aspergillus flavus*, *Beauveria sp*, *Cordyceps* sp. and *Metarhizium anisopliae*. *Beauveria bassiana*, *Metarhizium anisopliae* and *Cordyceps* are commonly found in soils and have been proven to reduce populations of agricultural pests such as diamondback moth, mosquitoes, aphids and potato beetles (Latifian, 2014; Lazzarini *et al*., 2006; Pal *et al*., 2006; Posadas *et al*., 2012; Tamuli & Gurusubramanium, 2011; Wang & Feng, 2014). However, none of these have been able to kill *P. dominula* wasps (Beggs *et al*., 2011; Rose *et al*., 1999).

Van Zyl (2016) did the first study in South Africa that examined the biological control of *Polistes dominula*, and compared the pathogenicity of entomopathogenic nematodes (EPN) (*H. bacteriophoras*), EPF (*Beauveria bassiana*) and a combination of EPF and EPN. The results showed that treatment with EPN alone gave 10% infected larvae, while treatment with EPF alone resulted in 20% infected larvae. However, when larvae were treated with a combination of EPN and EPF 40% of larvae were infected. These results further support the concept of integrated pest management.

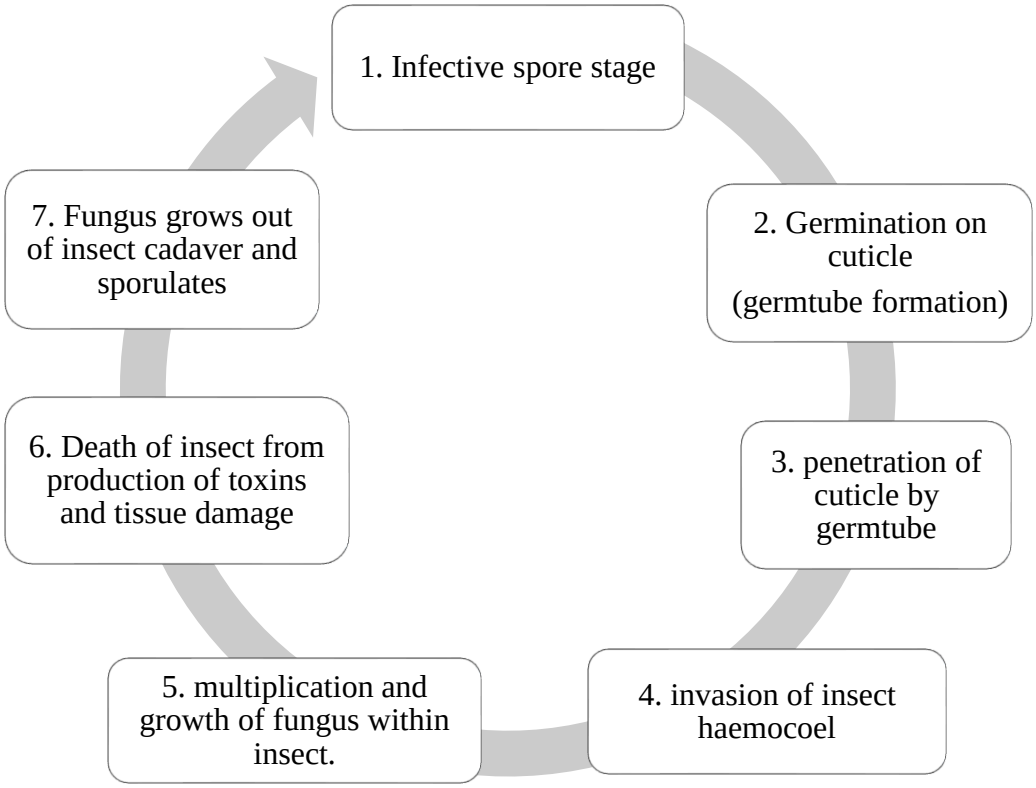

Figure 4. The life cycle of entomopathogenic fungi (EPF) (Wraight *et al*., 2007; Tamuli & Gurusubramanium, 2011).

Table 1. The definition of different approaches to biological control.

Strategy	Approach
Classical	The use of biological control agents against an introduced pest, which has established itself without its natural enemies. Research is done at its country of origin to seek the natural enemy of the pest. The natural enemy is then released into the introduced region to control the pest. This approach is extremely risky because another non-native species is introduced intentionally to control the invasive alien pest, this new biological control agent might not be specific to the pest and might switch hosts and start attacking non-target native organisms (Shah and Pell, 2003).
Augmentation	This strategy implies the need for mass production of the native biological control agent, and two approaches are proposed for agent application: 1) Inoculation approach requires that the biological control agent (e.g. entomopathogenic fungi used in plant protection) be applied early with the expectation that the agent will establish epizootics in the pest population and spread over time regulating pest populations below threatening limits (Radcliffe *et al*., 2009). 2) Inundative approach involves application of the mass produced biological control agent in large amounts for rapid short-term control with no expectation of epizootics (Lacey *et al.*, 2001; Pal and Mc Spadden Gardener, 2006)
Conservation	Conservation biological control presents various means to modify or manipulate the environment to enhance the activities and the persistence of native natural enemies of pests (Barbosa, 1998).
Autodissemination	Involves manipulating the pest population to facilitate the dispersal of the biological agent. For example, in the case of fungi, it allows the spread of fungal spores through horizontal transmission to the wider population and in the case of plant protection, the environmental conditions are modified to favour the persistence of the natural enemy of the pest being controlled. (Furlong and Pell, 2001; Dimbi *et al*., 2003; Shah and Pell, 2003).

1.6 Biotic and abiotic factors affecting biological control agents

Entomopathogenic fungi (EPF) are exposed to a number of biotic and abiotic factors which influence their ability to survive, infect, propagate and kill the target host (Wraight *et al*., 2007; Shahid *et al*., 2012). Temperature, humidity, soil moisture and solar ultraviolet radiation are some of the important environmental factors affecting survival and efficacy of entomopathogenic fungi (Benz 1987; Moller, 1996; Inglis *et al*., 2001).

Temperature is a major biotic factor that affects growth and survival of EPF in field conditions (Ahmad *et al*., 2016; Davidson *et al*., 2003; Nussenbaum *et al*., 2013). Ekesi *et al*. (1999) reported that *B. bassiana* and *Metarhizium anisopliae* had low germination, low radial growth and low pathogenic activity at 15 ℃. The optimal temperature for growth, germination and pathogenicity ranged between 25 – 30 ℃. These finding were also similar to those by Bugeme *et al*. (2009).

Fargues and Luz (2000) stated that humidity was also a crucial abiotic constraint for the EPF, *B. bassiana*. It was reported that, for the production of conidia at least 97% relative humidity is required, however, at humidity levels lower that 90% there was no conidia production (Milner & Lutton, 1986; Mini, 1973).

The efficacy of EPF is also affected by biotic factors such as, other naturally occurring microorganisms that may have beneficial associations with the target insect host (Shahid *et al*., 2012). The most important biotic constraint is the target insect. Insects have exoskeletons, with a cuticle that produces hydrocarbons that have antimicrobial activities (Boucias & Pendland, 1991). Some target hosts have relationships with other microbes that hinder the EPF to germinate. For successful germination, the EPF spore needs to be able to utilize or tolerate the cuticular compounds that may be toxic buy releasing toxin degrading enzymes (Boucias & Pendland, 1991; Ortiz-Urquiza & Keyhani, 2013; Shahid *et al*., 2012; Toledo *et al.*, 2015)

There are currently no entomopathogenic fungi developed to reduce the populations of invasive *P. dominula*, and with the continuous spread of the wasp in Western Cape region of South Africa, there is a need to search for potential native fungal pathogen. This study therefore focus on using standard microbiological methods and molecular techniques to isolate and identify fungi from soil and *P. dominula* nest material in search of a potential fungal biological control agent.

1.7 Problem statement

The negative impact of *P. dominula* has been identified by Beggs *et al* (1999) and their presence in South Africa has been confirmed by Benade (2015). Recent research conducted by Benade (2014) show that this wasp is expanding rapidly and may potentially have impact in the agriculture, viticulture and apiculture industries. Several control strategies, such as manual removal and destruction of nests, the use of chemical pesticides, and biological control has been identified to be desirable (Pal & Mc Spadden Gardener, 2006; Rose *et al.*, 1999; Usall, Torres, & Teixidó, 2016). Limited studies are available in literature on *P. dominula* in South Africa and only one study has been conducted on the biological control of these wasps (Van Zyl, 2016). Therefore, the role of microbial agents in the control of *P. dominula* populations is not well understood. This study seeks to identify a potential biological agent that can be used to reduce *P. dominula* populations in South Africa.

1.8 Research Questions

To understand the role of microbial agents in the control of invasive wasp populations, this study will answer the following questions:

1. Which fungi are associated with nests of *P. dominula?*
2. Can any of the associated fungi be used as a biological control agents of *P. dominula?*

Hypothesis: It is hypothesized that *P. dominula* nests will be associated with a variety of fungi including pathogenic fungi with the potential of being biological control agents.

1.9 Aim

The aim of this study is to deepen the knowledge about fungal associations of *P. dominula* and its susceptibility to entomopathogenic fungi

1.10 Objectives

1) Isolate and identify fungi from soil and wasp nests to determine which fungi are associated with *P. dominula*

2) To do a laboratory bioassay to test which of the isolates has the potential of being a biological control agent of *P. dominula*.

3) To carry out field trials to see if the selected fungal agent will be able to reduce *P. dominula* populations in the field.

4) To compare the pathogenicity of the selected field-isolated fungal agent to a commercial fungal strain.

References

Acharya, N., Seliga, R. A., Rajotte, E. G., Jenkins, N. E., & Thomas, M. B. (2015). Persistence and efficacy of a *Beauveria bassiana* biopesticide against the house fly, *Musca domestica*, on typical structural substrates of poultry houses. *Biocontrol Science and Technology*, 25(6), 697–715.

Ahmad, M. A., Ghazal, I., & Rajab, L. H. (2016). Laboratory evaluation of the effect of temperature and several media on the radial growth, conidia production and germination of the fungus *Beauveria bassiana* (Bals .) Vuil, *SSRG International Journal of Agriculture & Environmental Science*, 3(6), 39–46.

Allsopp, M. (2017). *Personal communication*.Nietvoorbij, Stellenbosch: ARC Infruitec.

Barberchek, M. E., & Kaya, H. K. (1990). Interactions between *Beauveria bassiana* and the entomogenous nematodes, *Steinernema feltiae* and *Heterorhabditis heliothidis*. *Journal of Invertebrate Pathology*, 55(2), 225–234.

Barbosa, P. (1998). Agroecosystems and conservation biological control. *Conservation biological control*. 39–54.

Beani, L. (2006). Crazy wasps: when parasites manipulate the *Polistes* phenotype. *Annales Zoologici Fennici*, 43(6), 564–574.

Beggs, J. R., Brockerhoff, E. G., Corley, J. C., Kenis, M., Masciocchi, M., Muller, F., Villemant, C. (2011). Ecological effects and management of invasive alien Vespidae. *BioControl*, *56*(4), 505–526.

Beggs, J. R., Rees, J. S., Toft, R. J., Dennis, T. E., & Barlow, N. D. (2008). Evaluating the impact of a biological control parasitoid on invasive Vespula wasps in a natural forest ecosystem. *Biological Control*, 44(3), 399–407.

Benade, P. C. (2015). Invaded range and competitive ability of the newly invasive *Polistes dominula* compared to that of its native congener species in the Western Cape , South Africa. Published thesis. South Africa: Stellenbosch University.

Benade, P. C., Veldtman, R., Samways, M. J., & Roets, F. (2014). Rapid range expansion of the invasive wasp *Polistes dominula* (Hymenoptera: Vespidae: Polistinae) and first record of parasitoids on this species and the native Polistes marginalis in the Western Cape

Province of South Africa. *African Entomology*, *22*(1), 220–225.

Bonet, A. (2009). Parasitoid Wasps, Natural Enemies of Insect. *Tropical Biology and Conservation Management*, 185–208.

Boucias D.G., Pendland J.C., Latge J.P. (1991) Nonspecific factors involved in attachment of entomopathogenic Deuteromycetes to host insect cuticle. *Applied Environmental Microbiology*. 54, 1795-1805.

Bugeme, D. M., Maniania, N. K., Knapp, M., & Boga, H. I. (2009). Effect of temperature on virulence of *Beauveria bassiana* and *Metarhizium anisopliae* isolates to *Tetranychus evansi*. *Diseases of mites and ticks*, 275–285.

Buteler, M., Lozada, M., Adamo, P. D., Buteler, M., & Kuperman, M. N. (2016). Social Learning in *Vespula Germanica* Wasps : Do they use collective foraging strategies ? *PLoS one* 11(3).

Carter, J. B. (1984). Viruses as pest-control agents. *Biotechnology and Genetic Engineering Reviews*, 1(1), 375–419.

Cervo, R., Zacchi, F., & Turillazzi, S. (2000). *Polistes dominulus* (Hymenoptera, Vespidae) invading North America: some hypotheses for its rapid spread. *Insectes Sociaux*, 47(2), 155–157.

Chen, Y., Evans, J., & Feldlaufer, M. (2006). Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. *Journal of Invertebrate Pathology*, 92(3), 152–159.

Choo, H. Y., Kaya, H. K., Huh, J., Lee, D. W., Kim, H. H., Lee, S. M., & Choo, Y. M. (2002). Entomopathogenic nematodes (*Steinernema* spp. and *Heterorhabditis bacteriophora*) and a fungus *Beauveria brongniartii* for biological control of the white grubs, *Ectinohoplia rufipes* and *Exomala orientalis*, in Korean golf courses. *BioControl*, 47(2), 177–192.

Colautti, R. I., Ricciardi, A., Grigorovich, I. A., & MacIsaac, H. J. (2004). Is invasion success explained by the enemy release hypothesis? *Ecology Letters*, 7(8), 721–733.

Corley, J. (2013). Distribution , dispersal and spread of the invasive social wasp *Vespula germanica* in Argentina, 162–168.

Cranshaw, W. S., Larsen, H. J., & Zimmerman, R. J. (2011). Notes on fruit damage by the European paper wasp, *Polistes dominula* (Christ) (Hymenoptera: Vespidae).

Southwestern Entomologist, 36(1), 103–105.

Davidson, G., Phelps, K., Sunderland, K. D., Pell, J. K., Ball, B. V, Shaw, K. E., & Chandler, D. (2003). Study of temperature – growth interactions of entomopathogenic fungi with potential for control of *Varroa destructor* (Acari : Mesostigmata) using a nonlinear model of poikilotherm development. *Journal of Applied Microbiology*, *94*, 816–825.

Eardley, C., Koch, F., & Wood, A. R. (2009). *Polistes dominulus* (Christ, 1791) (Hymenoptera: Polistinae: Vespidae) Newly Recorded from South Africa. *African Entomology*, *17*(2), 226–227.

Ekesi, S., Maniania, N. K., & Ampong-Nyarko, K. (1999). Effect of Temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti*. *Biocontrol Science and Technology*, *9*(2), 177–185.

Fargues, J., & Luz, C. (2000). Effects of fluctuating moisture and temperature regimes on the infection potential of B*eauveria bassiana* for *Rhodnius prolixus*. *Journal of Invertebrate Pathology*, *75*(3), 202–211.

Gardner-gee, R., & Beggs, J. (2013). Invasive wasps , not birds , dominate in a temperate honeydew system, *Austral Ecology*, 38, 346–354.

Georgis, R., Mullens, B. a, & Meyer, J. a. (1987). Survival and movement of insect parasitic nematodes in poultry manure and their infectivity against *Musca domestica*. *Journal of Nematology*, 19(3), 292–5.

Giliomee, J. H. (2011). Recent establishment of many alien insects in South Africa - a cause for concern. *African Entomology*, 19(1), 151–155.

Gill, S. S., Cowles, E. A., & Patricia, V. (1992). *Bacillus thuringiensis* endotoxins. *Annual Review in Entomology*, *37*, 615–636.

Granados, R. R. (1980). Infectivity and mode of action of Baculoviruses. *Biotechnology and Bioengineering*, *22*(7), 1377–1405.

Grewal, P. S., Ehlers, R. U., & Shapiro-Ilan, D. I. (2005). Nematodes as biocontrol agents. Nematodes as biocontrol agents. *Sustainable Agriculture* Reviews, 4. 45-50.

Harris, R. J., Harcourt, S. J., Glare, T. R., Rose, E. A. F., & Nelson, T. J. (2000). Susceptibility of *Vespula vulgaris* (Hymenoptera: Vespidae) to Generalist Entomopathogenic Fungi and

Their Potential for Wasp Control. *Journal of Invertebrate Pathology*, *75*(4), 251–258.

Haupt, K. (2015). Assessment of the invasive German wasp , Vespula germanica , in South Africa. Published thesis. South Africa: Stellenbosch University.

Heydari, A. & Pessaraki, M. (2010). A Review on Biological Control of Fungal Plant Pathogens Using Microbial Antagonists. *Journal of Biological Sciences*. 10(4), 273-290.

Huffaker, C. B., Messenger, P. S., & Adkisson, P. L. (1976). *Theory and practice of biological control*. Academic Press.

Hughes, D. P., Kathirithamby, J., Turillazzi, S., & Beani, L. (2004). Social wasps desert the colony and aggregate outside if parasitized: Parasite manipulation? *Behavioral Ecology*, 15(6), 1037–1043.

Inci, A., Kiliç, E., & Canhilal, R. (2014). Entomopathogens in control of urban pests. *Ankara Üniv Vet Fak Derg*, 61, 155–160.

Inglis, G. D., Enkerli, J., & Goettel, M. S. (2012). *Laboratory techniques used for entomopathogenic fungi. Hypocreales. Manual of Techniques in Invertebrate Pathology* (Second Edi). Elsevier Ltd.

Kenis, M., Auger-Rozenberg, M.-A., Roques, A., Timms, L., Péré, C., Cock, M. J. W., … Settele, J. (2009). Ecological effects of invasive alien insects. *Biol Invasions*, 11, 21–45.

Knowles, B. H. (1994). lnsecticidal endotoxins. *Advances in Insect Physiology*, *24*, 275–308.

Koehler, P. G., Patterson, R. S., & Martin, W. R. (1992). Susceptibility of cockroaches (Dictyoptera: Blattellidae, Blattidae) to infection by Steinernema carpocapsae. *Journal of Economic Entomology*, 85(4), 1184–1187.

Kolar, C. S., Lodge, D. M., & Lodge, D. M. (2001). Progress in invasion biology : predicting invaders, *Trends in ecology evolution*.16(4), 199–204.

Lacey, L. A., de la Rosa, F., & Horton, D. R. (2009). Insecticidal activity of entomopathogenic fungi (Hypocreales) for potato psyllid, Bactericera cockerelli (Hemiptera: Triozidae) *Biocontrol Science and Technology*, *19*(9), 957–970.

Lacey, L. A., Frutos, R., Kaya, H. K., & Vail, P. (2001). Insect Pathogens as Biological Control Agents : Do They Have a Future ? *Biological Control*, 248, 230–248.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., & Goettel, M. S.

(2015). Insect pathogens as biological control agents: Back to the future. *Journal of Invertebrate Pathology*, *132*, 1–41.

Laridon, N., Laridon, N., Mollett, J., & Gray, V. (2006). Isolation of a *Bacillus thuringiensis* strain from South African soils and the characterization of its cry gene sequence. Published thesis. South Africa: University of Witwatersrand.

Latifian, M. (2014). Study the pathogenecity of fungus *Beauveria bassiana* , *Beauveria brongniartii* and *Metarhizium anisopliae* on date horned beetle *Oryctes elegans* larvae based on different bioassay methods. *Scholars journal of agriculture and veterinary sciences 2*, 31–37.

Lazzarini, G. M. J., Rocha, L. F. N., & Luz, C. (2006). Impact of moisture on in vitro germination of *Metarhizium anisopliae* and *Beauveria bassiana* and their activity on *Triatoma infestans*. *Mycological Research*, 110(4), 485–492.

Lester, P. J. (2014). Determining the origin of invasions and demonstrating a lack of enemy release from.. *Diversity and distributions*. 20(8), 964–974.

Lester, P. J., Beggs, J., & Brown, R. L. (2013). The outlook for control of New Zealand ' s most abundant , widespread and damaging invertebrate pests : social wasps.

Lester, P. J., Corley, J. C., Lester, P. J., Bosch, P. J., Gruber, M. A. M., & Kapp, E. A. (2015). No evidence of enemy release in Pathogen and microbial communities of common wasps *Vespula vulgaris* in their native and introduced no evidence of enemy release in pathogen and microbial communities of common wasps *Vespula vulgaris* in their native. *PLoS one*. 10(3), 1-18.

Levitt, A. L., Singh, R., Cox-Foster, D. L., Rajotte, E., Hoover, K., Ostiguy, N., & Holmes, E. C. (2013). Cross-species transmission of honey bee viruses in associated arthropods. *Virus Research*, 176(1–2), 232–240.

Liu, H., & Stiling, P. (2006). Testing the enemy release hypothesis: A review and meta-analysis. *Biological Invasions*, 8(7), 1535–1545.

Lymbery, A. J., Morine, M., Kanani, H. G., Beatty, S. J., & Morgan, D. L. (2014). Co-invaders: The effects of alien parasites on native hosts. *International Journal for Parasitology: Parasites and Wildlife*, 3(2), 171–177.

Manfredini, F., Grozinger, C. M., & Beani, L. (2013). Examining the "evolution of increased

competitive ability" hypothesis in response to parasites and pathogens in the invasive paper wasp Polistes dominula. *Naturwissenschaften*, 100(3), 219–228.

Miller, G. L., Donnelly, C. R., & Gamboa, G. J. (2013). A ten-year comparative study of the population dynamics and parasitoidism in the native paper wasp Polistes fuscatus and the invasive P. dominulus. *Insectes Sociaux*, 60(1), 49–56.

Milner, R. J., & Lutton, G. G. (1986). Dependence of Verticillium lecanii (Fungi: Hyphomycetes) on high humidities for infection and sporulation using Myzus persicae (Homoptera: Aphididae). *Environ. Entomol.*, 15, 380–382.

Mini, L., 1973. Influence of relative humidity on the development of fungal infection caused by *Beauveria bassiana* [fungi imperfecti, moniliales] in imagines of *Acanthoscelides obtectus* [col.: bruchidae]., 22(4), 393–394.

Moller, H. (1996). Lessons for invasion theory from social insects. *Biological Conservation*, 78(1–2), 125–142. -

Nussenbaum, A. L., Lewylle, M. A., & Lecuona, R. E. (2013). Germination, radial growth and virulence to boll weevil of entomopathogenic fungi at different temperatures. *World Applied Sciences Journal*, 25(8), 1134–1140.

Ortiz-Urquiza, A., & Keyhani, N. O. (2013). Action on the surface: Entomopathogenic fungi versus the insect cuticle. *Insects*, 4(3), 357–374.

Pal, K. K., & Mc Spadden Gardener, B. (2006). Biological Control of Plant Pathogens. *The Plant Health Instructor*, 1–25.

Posadas, J. B., Comerio, R. M., Mini, J. I., Nussenbaum, A. L., & Lecuona, R. E. (2012). A novel dodine-free selective medium based on the use of cetyl trimethyl ammonium bromide (CTAB) to isolate Beauveria bassiana, Metarhizium anisopliae sensu lato and Paecilomyces lilacinus from soil. *Mycologia*, 104(4), 974–980.

Prabhu, G. (2015). Microbes in biological control,fermentation and enzyme technology. Retrieved May 3, 2017, from https://www.slideshare.net/gowrivprabhu/microbes-in-biological-controlfermentation-and-enzyme-technology

Radcliffe, E. B., Hutchison, W. D., & Cancelado, R. E. (2009). Integrated pest management: concepts, tactics, strategies and case studies. *Choice Reviews*, 46(11), 11-524.

Rincón-castro, M. C. Del, Ibarra, J. E., & Irapuato, I. C. P. (2011). Entomopathogenic Viruses. *Biological Control of Insect Pests*, (July), 29–64.

Roberts, D. W. (1989). World picture of biological control of insects by fungi. *Memórias Do Instituto Oswaldo Cruz*, 84(3), 89-100.

Rose, E. A. F., & Harris, R. J. (1999). Possible pathogens of social wasps (Hymenoptera: Vespidae) and their potential as biological control agents. *New Zealand Journal of Zoology*, 26, 179–190.

Sáenz-de-Cabezón, F. J., Zalom, F. G., & López-Olguín, J. F. (2010). A review of recent patents on macroorganisms as biological control agents. *Recent Patents on Biotechnology*, 4, 48–64.

Severino, M. G., Campi, P., MacChia, D., Manfredi, M., Turillazzi, S., Spadolini, I., … Bonifazi, F. (2006). European Polistes venom allergy. *Allergy: European Journal of Allergy and Clinical Immunology*, 61(7), 860–863.

Shahid, A. A., Rao, A. Q., Bakhsh, A., & Husnain, T. (2012). Entomopathogenic fungi as biological controllers: New insights into their virulence and pathogenicity. *Archives of Biological Sciences*, 64(1), 21–42.

Shapiro-Ilan, D. I., Han, R., & Dolinksi, C. (2012). Entomopathogenic nematode production and application technology. *Journal of Nematology*, 44(2), 206–17.

Singh, R., Levitt, A. L., Rajotte, E. G., Holmes, E. C., Ostiguy, N., Vanengelsdorp, D., … Cox-Foster, D. L. (2010). RNA viruses in hymenopteran pollinators: Evidence of inter-taxa virus transmission via pollen and potential impact on non-Apis hymenopteran species. *PLoS one*, 5(12).

Somasekhar, N., Grewal, P. S., De Nardo, E. A. B., & Stinner, B. R. (2002). Non-target effects of entomopathogenic nematodes on the soil nematode community. *Journal of Applied Ecology*, 39, 735–744.

Spurr, E. B. (1991). Reduction of wasp (Hymenoptera: Vespidae) populations by poison-baiting; experimental use of sodium monofluoroacetate (1080) in canned sardine. *New Zealand Journal of Zoology*, 18, 215–222.

Tamuli, A. K., & Gurusubramanium, G. (2011). Entomopathogenicity of white Muscardine Fungus *Beauveria bassiana* (Balls .) Vuill . (Deuteromyotina : Hyphomycetes) (BBFF-

135) Against Odontotermes (Rambur) (Isoptera : Termitidae)., (1986), 118–125.

Tohidi, F., Toulami, S., Tolami, H. F., Tameshkel, F. S., Nourani, A., Seyedinasab, S. R., … Jafarpour, M. (2013). Isolation and molecular identification of cry gene in Bacillus thuringiensis isolated from soils by semi-conserve PCR, 3(2), 452–456.

Toledo, A., López, S., Aulicino, M., de Remes Lenicov, A. M., & Balatti, P. (2015). Antagonism of entomopathogenic fungi by *Bacillus* spp. Associated with the integument of cicadellids and delphacids. *International Microbiology*, 18(2), 91–97.

Usall, J., Torres, R., & Teixidó, N. (2016). Biological control of postharvest diseases on fruit: a suitable alternative? *Current Opinion in Food Science*, 11, 51–55.

van Zyl, C. (2016). Status of the invasive wasp species, *Vespula germanic*a and *Polistes dominula* in South Africa, and the feasibility of various management strategies. Published thesis. South Africa: Stellenbosch University.

Chapter 2

2. Isolation of fungi associated with soils and nests of *P. dominula* in search of a biological control agent to control invasive wasps in the Western Cape Province, South Africa.

Abstract

Paper wasp nests are a previously untapped reservoir for fungi and other important microorganisms. In the past five years, a new *Mucor sp*. and a novel strain of Brewer's yeast were isolated from *P. dominula* nests. However, there is still not much known as to which fungal species are associated with *P. dominula* and their nests. This study aims to isolate and identify entomopathogenic fungi that are associated with the nests of *P. dominula* and the possible use of the isolates as biocontrol agents. Nest material was collected from Stellenbosch and surroundings. Fungi were isolated on selective media to select for entomopathogenic fungi. Selected strains were characterized using morphological and molecular characteristics. *Penicillium*, *Trichoderma*, *Aspergillus*, and *Fusarium* were the most frequent genera isolated. *Beauveria bassiana*, which is a well-studied entomopathogen, was also isolated from *P. dominula* nests. This study provides the first step in search of an effective biological control agent that may be used to regulate the population of invasive *P. dominula* wasp species in South Africa.

Key words: *P. dominula*, entomopathogenic fungi, nest material, fungal identification

2.1 Introduction

Polistes dominula has established itself across the globe and can be found in almost all the continents excluding Antarctica. The invasion success of these wasps is due to their high reproductive rate compared to the native wasps. Another reason why they are successful and abundant in the invaded region is the phenomenon known as the enemy release hypothesis (ERH), where the absence of their natural enemies in the new location leads to an increase in wasp populations (Lester *et al*., 2015; Liu & Stiling, 2006; Mlynarek, 2015).

Microorganisms, chiefly fungi and bacteria, play a major role in insects by forming symbiotic associations; where the microorganism may receive nutritional benefits from the insect host, while the insect may receive protection from pathogens Insects are usually influenced by the microorganisms that they harbour, and form symbiotic associations (Dillon *et al*., 2004; Jayaprakash *et al*., 2010; Ohkuma, 2003). For example, there are some termite species that cannot survive without their gut microbial symbionts, which help them to break lignocellulose down (Ohkuma, 2003). The microorganisms harboured by the insects are not all beneficial, but some of them might be pathogenic towards the insects and this is how they regulate insect populations (Coombes *et al*., 2013; Harris *et al*., 2000; Lacey *et al*., 2009; Zimmermann, 2007).

Fouillau & Morel (1995) stated that wasp nests may serve as a reservoir for fungi and yeasts. They recovered 31 fungal genera and identified approximately 52 species. *Mucor nidicola* sp. nov. was isolated from the nest of *P. dominula*, by Madden *et al*. (2012). Madden *et al*. (2013) conducted the first study which successfully isolated and characterized antimicrobial producing bacteria associated with *P. dominula* nests. The actinomycetes found included species from the genera *Actinoplanes, Micromonospora* and *Streptomyces.* Of these, thirty isolates showed antimicrobial activity against at least one challenge bacteria, namely *Escherichia coli, Serratia marcescens, Pseudomonas aeruginosa, Bacillus subtilis*, and *Staphylococcus aureus*.

There are relatively few reports of fungi that infect wasps directly, however, there are a number of records of fungi in the nest material, old food scraps in the nests and bits of dead wasps (Jayaprakash & Ebenezer, 2010; Rose *et al*., 1999). Usually, the fungi found on nest material occur and persist as saprotrophs associated with the decaying nest material or infecting dead wasps. The aim of this study was to determine the fungal species that are associated with *P. dominula* in South Africa, and to discover if there are any potential entomopathogenic fungi

that are associated with the nest material and the soil under or around the nest sites, as some of these soil-dwelling microorganisms could be potential biological control agents of wasps.

2.2 Materials and methods

2.2.1 Soil and nest sample collection

The reason for sampling the soil was that there are many soil-dwelling microorganisms that are involved in decomposing dead and decaying material, and some of that material could be larvae that fall off from the wasp nest or even dead wasps. Soil samples were collected from Welgevallen Experimental farm at Stellenbosch University (GPS coordinates: 33°56'46.2"S 18°52'00.5"E) and from Elsenburg farm (GPS coordinates: 33°51'03.0"S 18°49'31.5"E). These areas are densely populated with *P. dominula* nests. Twenty-five nests and six soil samples were collected during April to September 2015 for *P. dominula* in the Stellenbosch area. The soil was collected directly underneath the aerial nests, with the assumption that dead wasps or wasp material from the nest will fall on to the ground directly underneath the nest.

A metal hand trowel, sterilized with 70% ethanol, was used to dig up soil from 10-15 cm deep and approximately 1 kg of soil was placed in a plastic zip-lock bag and taken to the laboratory and stored at 4 °C. Eighteen active and seven inactive *P. dominula* nests were removed aseptically from roof crevasses and placed into zip-lock bags, labelled and taken back to the laboratory where they were stored at 4 °C till used. Nests were considered active if adult worker wasps were actively frequenting the nests and by the presence of live wasp larvae, and eggs. Nests were considered inactive if the nests were abandoned and empty with only capped cells (this indicated arrested development, pupae that never emerged) and no larvae.

2.2.2 Sample processing

In the laboratory, all adult wasps, larvae, and eggs were removed from the cells. The nests were visually examined, using a stereo microscope (Nikon Type 104), to determine if there was visible fungal growth on the nest material or on the larvae. The nest material was broken into smaller pieces using sterile forceps and one gram of the torn nest material pieces (including frass, nest paper, food material and honey for nests with honey) was placed into a test tube containing 10 ml of sterilized distilled water with 0.9% NaCl and vortexed using a Vortex Genie 2, at maximum speed for 5 minutes. The soil samples were mixed in the plastic bags and

one gram was placed in a test tube containing 10 ml of sterile distilled water with 0.9% NaCl and vortexed. 1 ml of the homogenate (from nest and soil suspensions) was diluted in a ten-fold serial dilution (10^{-1} – 10^{-5}) and fungal populations were plated by standard spread-plate dilution method described by Inglis *et al*. (2012), in triplicates per dilution for all samples (nest and soil).

2.2.3 Fungal isolation

For spread plates, 1000 µl of the soil and nest material dilutions were spread plated onto 95 mm petri dish plates with non-selective Oat Meal Agar (OA) with 15 g agar, 30 g oats and 1 L distilled water, and selective OA supplemented with 0.6 g Cetyltrimethylammonium bromide (CTAB), which is selective for entomopathogenic fungi (Mustafa *et al*., 2015). Both, the selective and non-selective media were supplemented with antibiotics, chloramphenicol (100 mg/ml) and ampicillin (100 mg/ml), to inhibit bacterial growth. Spread plates were done in triplicates per dilution and all the plates were incubated at 26°C for 7 days. One of the dead nests had fungal growth on the nest material and on the wasps found inside the nest cells, so a small piece of the nest material was broken from the nest and plated directly onto a fresh plate of OA and incubated at 26 °C for 7 days. After one-week incubation, colonies were counted per plate, per dilution and CFU/ml were determined (CFU is number of viable propagules per ml= number of colonies x suspension dilution factor x dilution series). After the CFUs were counted the colonies were examined under a light microscope. The fungal isolates were selected based on their morphological differences and transferred to fresh media to obtain pure cultures. This was done by transferring the spores of sporulating fungi and the mycelia of non-sporulating fungi to fresh PDA (Potato dextrose agar) to obtain pure fungal cultures, (fast-growing fungal plates were processed after 4 days to prevent plate overgrowth). These plates were incubated at 26°C for 7 days and then used for morphological identification.

2.2.4 Fungal identification

Morphology based identification

The phenotypic approach was used to group the different isolates into taxonomically recognizable units. In this approach units are defined based on their observable morphological characteristics (Guarro *et al*., 1999; Oliveira *et al*., 2013). A total of 229 morphologically different fungal isolates were obtained and identified at genus level based their macroscopic

and microscopic phenotypic appearance (Oliveira *et al*., 2013). Macroscopic features such as the texture, the colony colour, and exudates produced were examined using a Nikon Type 104 stereo microscope and recorded. In addition, an Olympus CX-31 compound light microscope was used to view wet mounts of the isolates, to examine their microscopic features such as hyphal, mycelial and spore shapes. Fungi were initially identified to genera level according to the identification key described by Samson *et al*. (2004).

From these 45 isolates which had distinct phenotypic features were selected for further analyses, with the aim of further identification at species level using molecular biology techniques.

Molecular identification

DNA was extracted from hyphae obtained from 1-week-old plates using the Zymo Research Fungal/Bacterial DNA MiniPrep ™ extraction kit (Zymo Research USA) according to the manufacturers' instructions. A 1 % agarose gel stained with 1 µl ethidium bromide was prepared and visualized under UV light to confirm the presence of DNA. The ITS region was amplified with PCR using the ITS1 and ITS4 (White *et al*., 1990; Martin & Rygiewicz, 2005). The ITS region was targeted because it is a conserved region in most fungi and it is also the commonly targeted locus for sequence-based identification of fungal isolates (White *et al*. 1990; Skouboe *et al*., 1999; Schoch *et al*., 2012).

In many genera of fungi, the ITS region is not variable enough for distinguishing closely related species, therefore, a secondary identification marker is often needed for identifying isolates to species level (Visagie *et al*., 2014). Two other gene segments were also targeted as secondary identification markers, namely, the partial elongation factor 1-alpha (EF1-α) and the beta-tubulin (BenA) region with primer set Bt2a/ Bt2b (Glass & Donaldson 1995). The ITS region was amplified for all isolates, but the secondary primers were done on selected isolates as indicated on Table 2. The reaction mixes for the ITS region had a final volume of 10 µl, consisting of 5 µl Taq polymerase Ready mix, 3.6 µl sterile MilliQ water, 1 µl of tempplate DNA and 0.2 µl of each primer (reverse and forward primer). The thermocycling conditions were programmed for 5 minutes denaturation at 94 °C, followed by 25 cycles of 30 seconds denaturation at 94 °C, annealing was at 55 °C for 30 seconds, and extension for 1 minute at 72 °C, and a final elongation for 7 minutes at 72 °C (White *et al*., 1990).

The PCR protocol for the BenA gene adapted from Visagie *et al.* (2014) was followed, with each reaction having a final volume of 25 µl consisting of 12.5 µl Ready mix, 10.5 sterile MilliQ water, 1µl template DNA and 0.5 µl of each primer. The thermocycling conditions were as follows, 5 minutes denaturation at 94 °C, followed by 36 cycles of 45 seconds denaturation at 94 °C, primer annealing at 56 °C for 45 seconds, and extension for 60 seconds at 72 °C, and a final 10 minutes elongation step at 72 °C. The same protocol and thermocycling conditions were followed for amplifying the EF1-α region.

The resulting amplicons were run on a 1% agarose gel and visualized under UV light, amplification was considered successful if there was a band with the correct fragment size on the agarose gel. A NanoDrop™ spectrophotometer (Thermo Scientific) was used to quantify the DNA concentration for sequencing.

The PCR fragments were sequenced directly in one direction using the respective forward primers. Each sequencing reaction mixture had a total reaction volume of 10 µl, containing 1 µl template DNA, 5 µl sterile MilliQ water, 1 µl of the forward primer, 1. 5 µl BigDye reagent and 1. 5 µl buffer. The thermocycling conditions for sequencing were set at 10 seconds denaturation at 94 °C, followed by 35 cycles of 20 seconds denaturation at 94 °C, primer annealing at 48 °C for 20 seconds, and extension for 4 minutes at 60 °C (Dunning *et al*., 2010). The sequence reactions were then taken to the Central Analytical Facility (CAF) at Stellenbosch University for further processing. Sequence chromatograms obtained from CAF were viewed Chromas Lite and trimmed to 500 base pairs which is the length of the sequenced region, then the trimmed sequences were exported to MEGA 7 for editing, this was done by replacing ambiguous base pairs (represented by letter other than the four base pairs ACGT) with the correct base pairs after analysing the correct peaks on the chromatograph. Sequence editing was done using Chromas Lite and MEGA 7. The sequences were then compared with reference sequences available in GeneBank databases using the Basic Local Alignment Search Tool comparison of nucleotide sequences (BLASTn) in the National Centre for Biotechnology Information (NCBI). Sequences retrieved were aligned with the most similar-type strains and Neighbour-joining analysis of trees were performed using Mega 7. Where there were conflicting results between the ITS and the secondary genes, the morphological identification data and the phylogenetic analysis results were taken into consideration to decide on the identity of an isolate.

2.3 Results

Total fungal counts

All the culture plates from 10^{-1} dilution had too many colonies and it was difficult to obtain an accurate count as the colonies were confluent and had to distinguish one colony from the other. The 10^{-5} dilution plates ranged from 3- 10 colonies per plate (for both soil and nest samples). The mean total fungal counts of each of the soil samples ranged from 6.9 x 10^5 cfu/ml -1.3 x 10^7 cfu/ml. From the nest material, the mean total fungal counts ranged from 3.14 x 10^5 – 2.7 x 10^7.

Diversity of fungi from soil and nest material

The phenotypic study showed an overview of the genera found in *P. dominula* nest material and soil analysed. In this study, 229 isolates were classified to genus level and 12 fungal genera were detected, including *Penicillium, Trichoderma, Aspergillus* and *Fusarium* being the most common genera found in the different wasp nests and soil sampled. Among the most represented genera identified in the nests and soils sampled, all of them have been reported to occur in soil environments (Schippers *et al*., 1982). More than 50% of the species identified belong to the genera *Penicillium* (30%), *Trichoderma* (26%), *Aspergillus* (14%) and *Fusarium* (12%). The frequencies of appearance of the different genera in all the occupied nests examined are illustrated in Fig. 5. These genera were present in 90% of the nests.

45 isolates were classified to species level. Among the 45 species (Table 2), 8 were recovered from the soil, 23 from wasp nest and 14 species isolated from both nest material and soil sampled. *Penicillium* spp. and *Aspergillus niger* were found in the unoccupied/abandoned nest, and the live/active nests had more fungal diversity when compared to unoccupied nests.

Molecular identification

Table 2 shows a list of the 29 different species (from the 45 that were sequenced, as there were some isolates that had the same sequence although they differed slightly in morphology) found to occur in wasp nest material and in soil environment.

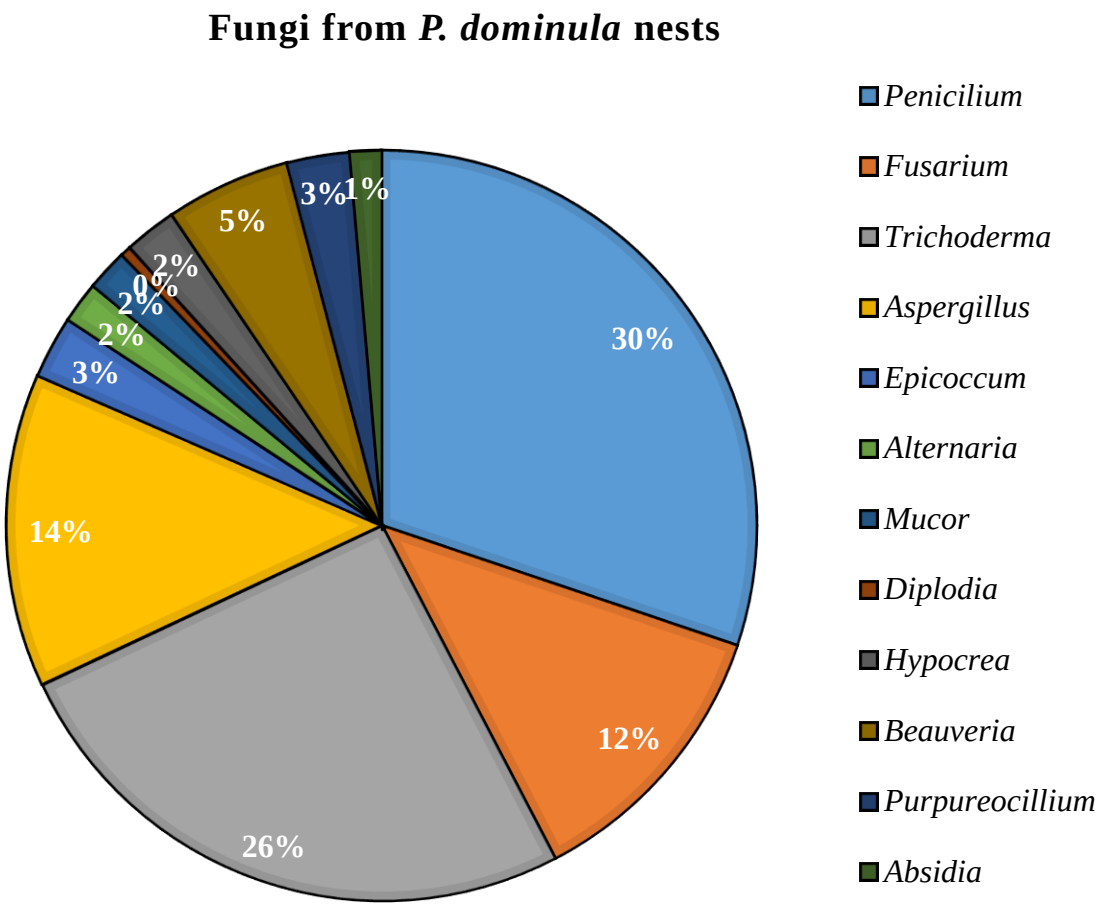

Figure 5. Pie chart, illustrating the frequencies of each genus isolated from *P. dominula* nests. The frequencies were calculated based on the number of total isolates analysed

	Beta TUBULIN			ITS1		ELONGATION FACTOR 1-alpha	
Sample ID	**Identification**	**Accession number**	**closest match**	**Accession no.**	**Closest match**	**Accession no.**	**Closest match**
T2	*Penicillium brevicompactum*	MF351756.1	*Penicillium brevicompactum*	MG647838.1	*Penicillium brevicompactum*	-	-
T3	*Penicillium rubens*	LT558974.1	*Penicillium rubens*	MG589559.1	*Penicillium rubens*	-	-
T14	*Fusarium equiseti.*	-	-	HQ384389.1	*Fusarium incarnatum*	KR108318.1	*Fusarium equiseti*
T16	*Trichoderma atroviride*	Z15055.1	*Trichoderma viride*	HM037928.1	*Trichoderma viride*	HG931224.1	*Trichoderma atroviride*
T18	*Epicoccum nigrum*	GU563380.1	*Epicoccum nigrum*	KX869965.1	*Epicoccum nigrum*	-	-
T19	*Aspergillus niger*	KR064523.1	*Aspergillus tubingensis*	KR912299.1	*Aspergillus niger*	AM270408.1	*Aspergillus niger*

T20	*Trichoderma atroviride*	XM_014090918.1	*Trichoderma atroviride*	HM037928.1	*Trichoderma viride*	HG931224.1	*Trichoderma atroviride*
T23	Trichoderma *atroviride*	-	-	FJ426394.1	*Trichoderma atroviride*	-	-
T24	*Penicillium sp.*	LT558974.1	*Penicillium rubens*	HM573339.1	*Penicillium sp.*	-	-
T25	*Alternaria sp*	HQ413316.1	*Alternaria alternata*	FJ949086.1	*Alternaria tenuissima*		
T26	*Fusarium cf. oxysporum*	KY553166.1	*Fusarium cf. oxysporum*	KT211527.1	*Fusarium oxysporum*	KU985430.1	*Fusarium oxysporum*
T27	*Trichoderma atroviride*	LT707601.1	*Trichoderma harzianum*	KY484994.1	*Trichoderma atroviride*	KJ871107.1	*Trichoderma atroviride*

		BTUBULIN		ITS1		ELONGATION FACTOR	
Sample ID	**Identification**	**Accession number**	**closest match**	**Accession no.**	**Closest match**	**Accession no.**	**Closest match**
T30	*Penicillium chrysogenum*	KX788178.1	*Penicillium chrysogenum*	JX139710.1	*Penicillium chrysogenum*	AM920437.1	*Penicillium chrysogenum*
T31	*Penicillium crustosum*	FJ004401.1	*Penicillium crustosum*	JN585931.1	*Penicillium crustosum*	-	-
T32	*Mucor racemosus*	-	-	KJ744354.1	*Mucor racemosus*	-	-
T33	*Epicoccum nigrum*	GU563380.1	*Epicoccum nigrum*	KX869965.1	*Epicoccum nigrum*	-	-
T34	*Diplodia seriata*	KX259170.1	*Diplodia seriate*	KT004551.1	*Diplodia seriata*	-	-

T36	*Aspergillus alliaceus*	FN185734.1	*Aspergillus alliaceus*	NR_121331.1	*Aspergillus alliaceus*		
T37	*Fusarium cf. incarnatum*	KT374271.1	*Fusarium cf. incarnatum*	FR822790.1	*Fusarium sp.*	JX118992.1	*Fusarium sp.*
T38	*Trichoderma sp.*	Z15055.1	*Trichoderma viride*	JF304975.1	*Trichoderma sp.*	AB558914.1	*Trichoderma atroviride*
M2	*Hypocrea lixii*	-	-	JN228901.1	*Hypocrea lixii*		
M3	*Purpureocillium lilacinum*	KU738451.1	*Purpureocillium lilacinum*	LC317747.1	*Purpureocillium lilacinum*	-	-
M4	*Penicillium sp.*	LT559017.1	*Penicillium pancosmium*	KP329822.1	*Penicillium sp.*	-	-
M8	*Absidia*	-	-	JN942685.1	*Absidia*	-	-

		BTUBULIN		ITS1		ELONGATION FACTOR	
Sample ID	**Identification**	**Accession number**	**closest match**	**Accession no.**	**Closest match**	**Accession no.**	**Closest match**
M11	*Aspergillus oryzae*	-	-	HQ832962.1	*Aspergillus sp.*	AB007770.1	*Aspergillus oryzae*
Bb4818	*Beauveria bassiana*	AB830334.1	*Beauveria bassiana*	KY640637.1	*Beauveria bassiana*	-	-
E6	*Fusarium solani*	-	-	KF494111.1	*Fusarium solani*	-	-
Bb4850	*Beauveria brongniartii*	EU604109.1	*Beauveria brongniartii*	JF947191.1	*Beauveria brongniartii*	-	-

Table 2. List of fungi isolated from nests of *P. dominula*, showing the three reference identification markers that were used to identify to species level. Only the matches with the highest similarity (>99% identity) were chosen.

2.4 Discussion

This study documented the first assessment to determine the diversity of fungi that are associated *with P. dominula* nests in South Africa. The results obtained from the total fungal counts from soil samples ranged from 10^5 – 10^7 cfu/ml, which fell within the range reported in other work done on fungal isolation from soil (Okoh *et al*., 1999; Ogunmwonyi *et al*., 2008).

From the 29 fungal species identified in this study, the most frequent genera observed were *Penicillium, Trichoderma, Aspergillus,* and *Fusarium,* comprising 30%, 26%, 14% and 12% of the isolated strains, respectively. Jayaprakash & Ebenezer, (2010), who conducted a fungal assessment on *Polistes* paper wasp nest material, also found that similar genera were common in nest material. These genera mainly consist of species that are associated with soil environments, as they are all typical saprotrophs (Boer *et al*., 2003; Fouillaud & Morel, 1995; Wraight *et al*., 2007).

Aspergillus niger was the most dominant of all the *Aspergillus* spp., it is no surprise that *Aspergillus niger* was dominant because it has been associated with black mould disease in grapes (Abrunhosa *et al*., 2001). The wasps in the Stellenbosch region spend most of their time foraging in vineyards and most of them build their nest in vineyards, so it is expected that the nest material will be associated with this fungus (Giliomee, 2011; Jayaprakash & Ebenezer, 2010; Tjamos *et al*., 2004). *Fusarium solani* was the most frequently isolated of all the *Fusarium* spp. It is a common soil fungus and a plant pathogen that is responsible for causing root rot on economically important crops such as passion fruit, peppers, avocado, peas and potatoes, and grapes (Pal *et al*., 2006; Barros *et al*., 2014; Madden *et al*, 2017). The presence of this species is also not unexpected as the wasps do come into contact with grapes and they may pick up the fungus back to the nest, during foraging or during collection of nest building material (Perez *et al*., 2011; Mikušová *et al*., 2013).

The other fungal species that are less dominant in the wasp nest material represent some opportunistic plant pathogens such as *Alternaria alternata* which causes leaf spots. It was, however, no surprise to isolate *A. alternata* in the nest material, because it is also an airborne species that is prevalent in outdoor environments (Salo *et al*., 2005). It is worth noting that since the nests were not surface sterilized for this experiment, therefore the presence of most airborne fungi found in *P. dominula* nests could be because of contaminants from the air. In

similar studies, Gambino & Thomas (1988) it was stated that the origin of the fungal content may be direct contamination by a variety of biotic and abiotic factors. This was further investigated by Fouillaud *et al.* (1995), who found that the source of fungi in *Polistes* nests is the gut content of larvae and the food material brought back to the nest by workers. This information is important because, most of the fungi isolated in this current study and previous studies are soil fungi previously reported from numerous soil and plant sources, which gives us information on how the fungi might have gotten into the nest material.

Many of these fungal species have been isolated from Hymenoptera nests previously, as the study by Jayaprakash and Ebenezer (2010) on paper nests of *Ropalidia marginata* showed. Most of these species identified in this study are common saprophytic fungi, but there are a few that are also considered entomopathogenic, such as *B.bassiana*, *Alternaria alternate* and *Purpureocillium lilacinum*. Of these entomopathogens, *Beauveria bassiana* constituted 5% of the fungi isolated in the current study which was the most frequently isolated in most nests of all the entomopathogenic species. The results of this study are consistent with those found by Jayaprakash and Ebenezer (2010), in their assessment of fungi in nests of the paper wasp, *Ropalidia marginata*, as they also found the similar genera to be common in paper wasps. Fouillaud *et al.* (1995) also found the same genera to be present in *Polistes* nest material.

The soil is known to consist of a variety of microorganisms that play a vital role in increasing soil fertility and promoting plant growth (Abed *et al*., 2017; Ullah *et al*., 2017). The soil has been extensively explored as one of the primary reservoirs for microorganisms other than plant and animal hosts. Madden *et al.* (2012) stated that *P. dominula* nest material is an untapped reservoir for fungal species and this study supports that statement because all the nest material samples had fungi isolated from them, whether the nest were active or unoccupied.

In a study conducted by Madden *et al.* (2012), a few *Mucor* spp. were isolated and they found a previously uncharacterized strain related to *Mucor heimalis* and *M. irregularis*, this novel strain was named *Mucor nidicola* sp. nov. However, in this study, *Mucor* spp. were not that prevalent, the only sp. identified was *M. racemosus* and it was one of the least abundant fungi, accounting for only 3% of the total isolates. This result is similar to that of Jayaprakash and Ebenezer (2010) who also found that *M. racemosus* was the only *Mucor* species represented in his study and it only accounted only 4 % of the total isolates in their study. It was not a surprise that *Mucor* spp. would be present in wasp nest material, as it is a common saprophyte in

decomposing plant matter, since wasps used decomposing plant matter, leaves and cardboard to create their nests (Ribes *et al*., 2000).

According to extensive literature search and according to our knowledge, the 29-fungal spp. identified in this study have not been reported in *P. dominula* nests, therefore, this study provides the first assessment of fungi associated with nests of *P. dominula* in South Africa.

Most of the saprotrophs isolated and identified in this study are opportunistic pathogens of plants, with a few insect pathogens. *Aspergillus* and *Beauveria* are the two genera isolated that have been used to control social insects such as ants, wasps, and termites with successful population reduction (Harris *et al*., 2000; Rose *et al*., 1999). While little is known about *P. dominula* pathogens, the EPFs isolated from their nests provide an insight as to which fungus could potentially be explored as a biological control agent for *P. dominula* wasps.

It is important to note that this study only isolated culturable fast-growing fungi that grew on culture media within the 7-day incubation period. Therefore, further studies would have to used advance molecular techniques to identify unculturable fungi to get the full assessment of the whole fungal diversity in *P. dominula* nests.

The aims of this chapter which was to isolate fungi from *P. dominula* nests and soil. However, further studies are important to determine which of these isolates have the potential to be pathogenic towards *P. dominula*. The findings of this study are crucial because they provide the first step to understanding the fungal diversity associated with these wasps and how they interact with their nests. This could lead to further studies on the impacts of microbes on wasp fitness. Further studies focusing on the full assessment of all the microbes associated with nest material need to be carried out. This will help increase our knowledge as to what the ecological value these microbes bring in wasp biology and how they can be exploited as biological control agents.

References

Abed, N. E., Salem, I. B., Khedher, M. B, Hamdi, M. M., & Hamdi, N. B. M. (2017). Isolation and identification of fungal communities in organic and conventional soils, *Int.J.Curr.Microbiol.App.Sci*, 6(4), 1111–1123.

Abrunhosa, L., Paterson, R. R. M., Kozakiewicz, Z., Lima, N., & Venâncio, A. (2001). Mycotoxin production from fungi isolated from grapes. *Letters in Applied Microbiology*, 32(4), 240–242.

Boer, W. De, Verheggen, P., Klein, P. J. A, Kowalchuk, G. A, Van, J. A, Gunnewiek, P. J. A K., & Veen, J. a Van. (2003). Microbial community composition affects soil fungistasis microbial community composition affects soil fungistasis. *Applied and Environmental Microbiology*, 69(2), 835–844.

Coombes, C. A., Hill, M. P., & Moore, S. D. (2013). Biocontrol science and technology persistence and virulence of promising entomopathogenic fungal isolates for use in citrus orchards in South Africa. *Biocontrol Science and Technology*, 23(9), 37–41.

Fouillau, M., & Morel, G. (1995). Fungi associated with nests of the paper wasp Polistes hebraeus (Hymenoptera: Vespidae) on La Réunion Island. *Environmental Entomology*, 24(2), 298–305.

Giliomee, J. H. (2011). Recent establishment of many alien insects in South Africa - a cause for concern. *African Entomology*, 19(1), 151–155.

Harris, R. J., Harcourt, S. J., Glare, T. R., Rose, E. A. F., & Nelson, T. J. (2000). Susceptibility of Vespula vulgaris (Hymenoptera: Vespidae) to generalist entomopathogenic fungi and their potential for wasp control. *J Inver. Journal of Invertebrate Pathology*, 75(4), 251–258.

Jayaprakash, A., & Ebenezer, P. (2010). A new report on mycobiota associated with Ropalidia marginata paper nests. *Indian Journal of Science and Technology*, 3(1), 6–8.

Lacey, L. A., de la Rosa, F., & Horton, D. R. (2009). Insecticidal activity of entomopathogenic fungi (Hypocreales) for potato psyllid, *Bactericera cockerelli* (Hemiptera: Triozidae) *Biocontrol Science and Technology*, *19*(9), 957–970.

Lester, P. J., Corley, J. C., Lester, P. J., Bosch, P. J., Gruber, M. A. M., & Kapp, E. A. (2015).

No evidence of enemy release in Pathogen and microbial communities of common wasps *Vespula vulgaris* in their native and introduced no evidence of enemy release in pathogen and microbial communities of common wasps *Vespula vulgaris* in their native , (April 2016). *PLoS one*. 10(3), 1-18.

Liu, H., & Stiling, P. (2006). Testing the enemy release hypothesis: A review and meta-analysis. *Biological Invasions*, 8(7), 1535–1545.

Lymbery, A. J., Morine, M., Kanani, H. G., Beatty, S. J., & Morgan, D. L. (2014). Co-invaders: The effects of alien parasites on native hosts. *International Journal for Parasitology: Parasites and Wildlife*, 3(2), 171–177.

Madden, A., Boyden, S., Soriano, J., Corey, T., Leff, W., Fierer, N. & Starkss, P. (2017). The emerging contribution of social wasps to grape rot disease ecology. *PeerJ* 5:e3223.

Madden, A. A., Stchigel, A. M., Guarro, J., Sutton, D., & Starks, P. T. (2012). Mucor nidicola sp. nov., a fungal species isolated from an invasive paper wasp nest. *International Journal of Systematic and Evolutionary Microbiology*, 62(7), 1710–1714.

Madden, A. a, Grassetti, A., Soriano, J. N., & Starks, P. T. (2013). Actinomycetes with antimicrobial activity isolated from paper wasp (Hymenoptera : Vespidae : Polistinae) nests. *Environmental Entomology*, 42(4), 703–710.

Martin, K. J., & Rygiewicz, P. T. (2005). Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiology*, 5, 28.

Mikušová, P., Šrobárová, A., Sulyok, M., & Santini, A. (2013). Fusarium fungi and associated metabolites presence on grapes from Slovakia. *Mycotoxin Research*, 29(2), 97–102.

Mlynarek, J. J. (2015). Testing the enemy release hypothesis in a native insect species with an expanding range. *PeerJ*, 3, e1415.

Mustafa, R. A, Abdullah, S. K, & Assaf, L. (2015). Isolation of Entomopathogenic and Opportunistic Fungi from Soil in Duhok Province, Kurdistan soil in Duhok Province, Kurdistan Region of Iraq. *Journal of Biology, Agriculture and Healthacare*, 5(4), 73-79.

Ohkuma, M. (2003a). Termite symbiotic systems: efficient bio-recycling of lignocellulose. *Applied Microbiology and Biotechnology*, 61(1), 1–9.

Oliveira, B. R., Barreto Crespo, M. T., San Romão, M. V., Benoliel, M. J., Samson, R. A., &

Pereira, V. J. (2013). New insights concerning the occurrence of fungi in water sources and their potential pathogenicity. *Water Research*, 47(16), 6338–6347.

Rose, E. A. F., & Harris, R. J. (1999). Possible pathogens of social wasps (Hymenoptera: Vespidae) and their potential as biological control agents. *New Zealand Journal of Zoology*, 26, 179–190.

Tjamos, S. E., Antoniou, P. P., Kazantzidou, A., Antonopoulos, D. F., Papageorgiou, I., & Tjamos, E. C. (2004). Aspergillus niger and Aspergillus carbonarius in Corinth raisin and wine-producing vineyards in Greece: Population composition, Ochratoxin A production and chemical control. *Journal of Phytopathology*, 152(4), 250–255.

Ullah, I., Khan, N. A., Jadoon, M. A., Ur, H., Khan, H., Rehman, M. U., Ali, S. (2017). Isolation and identification of different Rhizospheres fungi of Mansehra region , Pakistan, *Journal of Entomology and Zoology Studies*, 5(2), 437–442.

White, T. J., T. Bruns, S. Lee, and J. W. T. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications*, 315–322.

Wraight, S. P., Inglis, G. D., & Goettel, M. S. (2007). Fungi. In L. A. Lacey & H. K. Kaya (Eds.), *Field Manual of Techniques in Invertebrate Pathology*. Springer, 223–248.

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Science and Technology*, 17(6), 553–596.

Chapter 3

3. The virulence of entomopathogenic fungi on 2nd instar larvae of *P. dominula* in laboratory conditions

Abstract

Entomopathogenic fungi are commonly found in soil environments where they are involved in regulating the population of soil-dwelling insects. Over the past decades, there have been many entomopathogenic fungi that have been commercially produced to be used as biological agents. These include *Beauveria bassiana*, *Metarhizium anisopliae*, and *Entomophthora muscae* among many others. To date, there has not been any commercial biological agent developed against the invasive wasp, *Polistes dominula*. A variety of control strategies have been put in place, for example, chemical control and manual removal of this pest. Both methods have been proved to be ineffective as chemical pesticides are dangerous to the environment and mechanical removal is labour intensive, hence the need to find a biological control agent. Entomopathogenic fungi are the most promising biological control agents that can be used to control these wasps because of their ability to adhere to the wasps' cuticle and spread through horizontal transfer within the wasp nest infecting the whole colony. The present study aims to isolate and identify entomopathogenic fungal isolates from the wasp nests and develop these isolates into biological control agents. Wasp nests were collected from different locations in the Western Cape province, the nest material was then processed and inoculated onto selective media. Entomopathogenic fungal isolates from the genus *Beauveria* were the most prevalent species isolated from the nests. *B. bassiana, B. brongniartii and Penicillium lilacinium* strains were prepared as spore suspensions and applied to wasp larvae under laboratory conditions. *B. bassiana* isolates from local nests showed 100% mortality within four days. This study is the first step in providing a potential safe solution to the control of invasive wasp population in the Western Cape Province.

Key words: *Polistes dominula*, *Beauveria bassiana*, entomopathogenic fungi, biological control

3.1 Introduction

Polistes dominula has a negative impact on society, the environment, and economy. Social impacts include being a nuisance to humans because they build their nests in human settlements (Beggs *et al*., 2011; Benade *et al*., 2014; van Zyl, 2016). They are aggressive towards humans and they have a painful sting, that some people develop allergies to (Severino *et al*., 2006). Ecologically, *P. dominula* is a threat to biodiversity as they can replace indigenous wasp species such as the native *P. marginalis* by competing for food and shelter (Beggs *et al*., 2011). There are also records of economic impacts, as they have been reported to be a pest in some vineyards in Stellenbosch. Removing *P. dominula* nests from vineyards is expensive, time-consuming and labour intensive (Giliomee, 2011). All these negative impacts have increased the need to find a control method that will work fast and effectively to reduce *P. dominula* populations. Unfortunately, getting rid of these wasps has proved to be an arduous task (Gaertner, *et al*., 2015).

There have been different control strategies put in place to eradicate the rapidly expanding invasive wasp populations. Some of the control strategies include manual removal where wasp nests are physically removed and destroyed. Manual removal of nests is the control method used in South Africa to date (Benade, *et al*., 2014). This method of control is labour intensive and therefore not effective for large areas such as vineyards (Toft & Harris, 2004).

Chemical control is another method that is used, where chemical insecticides are sprayed onto the nests (Beggs, 2011). Although chemical control might be effective, it is not a desirable route because of the chemical residues that might remain in the environment contaminating the soil, air, and water after the chemical has been sprayed. Chemical insecticides also pose a risk to non-target insect species (Beggs, 2011) and they increase resistance, which may lead to the accumulation of pesticide resistant pests (Singh, *et al.*, 2015).

Since manual and chemical control methods are not effective, biological control is an attractive option as it could have a better chance because it utilizes natural pathogens which leave no chemical residues in the environment (Bidochka *et al.*, 1998). In the USA classical biological control using parasitoid *Xenos vesparum*, a parasitoid that is a natural enemy of *P. dominula*, was attempted and the trials were successful, as it was reported that infestation by this parasitoid resulted in a significant decline in the *P. dominula* population (Hughes *et al*., 2004; Manfredini *et al*., 2013; Miller *et al*., 2013). *X. vesparum* and other indigenous parasitoids

were able to affect *P. dominula* at the colony level. The disadvantage of these parasitoids was that they also affected the native *P. fuscatus*.

Entomopathogenic fungi from the order Hypocreales have been known to regulate insect populations and there has been several biopesticides developed to control economically important pests (Lacey *et al*. 2011; Zimmermann 2007; Inglis *et al*., 2001; Wraight *et al.* 2007; Shahid *et al*. 2012). Fungal agents are better candidates to control insects when compared to bacterial or viral agents, which first need to be ingested by the insect before they start germinating internally to cause any notable damage (Guetsky *et al*., 2002; Ruiu, 2015). Entomopathogenic fungal spores have the ability to recognize the host and attach to the insect cuticle, after which the spore germinates and forms a germ tube by which the hyphae elongates. Using enzymes, such as proteases and chitinases, and mechanical force, the fungus penetrates the insect cuticle through to the body cavity (haemocoel) where it develops and reproduces inside the insect, progressively digesting the insect from inside (Guetsky *et al*., 2002). After two to four days the hypha starts growing till it reaches the outside of the insect cuticle. By this time the insect is usually dead. Once the fungal spores are exposed, they can then be transmitted to other insects through horizontal transfer, usually body contact (Inci, *et al*., 2014). This makes EP fungi the best candidates to reduce invasive wasp populations.

Common examples of fungal pathogens that are used in biological control include but are not limited to *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* (Coombes *et al*., 2015; Lacey *et al*., 1999; Posadas *et al*., 2012). These fungal pathogens are commonly found in soils and have been proven to reduce populations of commercially important pests such as diamondback moth, mosquitoes, aphids and potato beetles (Castillo *et al*., 2014; Coombes *et al*., 2015; Sedehi, & Modarres, 2014; World Health Organization, 1982). However, there is no available literature showing that these EPFs could reduce *P. dominula* wasp populations.

The lack of a fungal pathogen known to reduce invasive *P. dominula* populations has necessitated the need to search for a potential fungal pathogen. In the previous chapter, we found that *P. dominula* nests are associated with a variety of fungi including pathogenic fungi with the potential of being biological control agents. Therefore, the aim of this study is to further investigate which isolate *P. dominula* is more susceptible to.

3.2 Methods and material

3.2.1 Insect larvae preparation

Polistes dominula nests with larvae were collected from the Welgevallen experimental farm in Stellenbosch in September 2016. Nests containing second instar larvae were carefully placed (Fig. 6A) in plastic containers and transported to the laboratory for further processing. In the laboratory, larvae were removed from the cells (Fig. 6B) using fine sterile forceps. Larvae were placed in 65 mm plastic petri dishes lined with a moist paper towel to maintain 45±5 % relative humidity (measured using a humidity and temperature recorder) and kept at room temperature for a maximum of one hour till treatments were applied. *P. dominula* larvae feed on a protein diet and this was not easy to simulate in the laboratory, therefore, for the duration of this experiment larvae were not fed. According to (Siekmann, *et al*., 2016), larvae can survive 5r to 5 days without feeding.

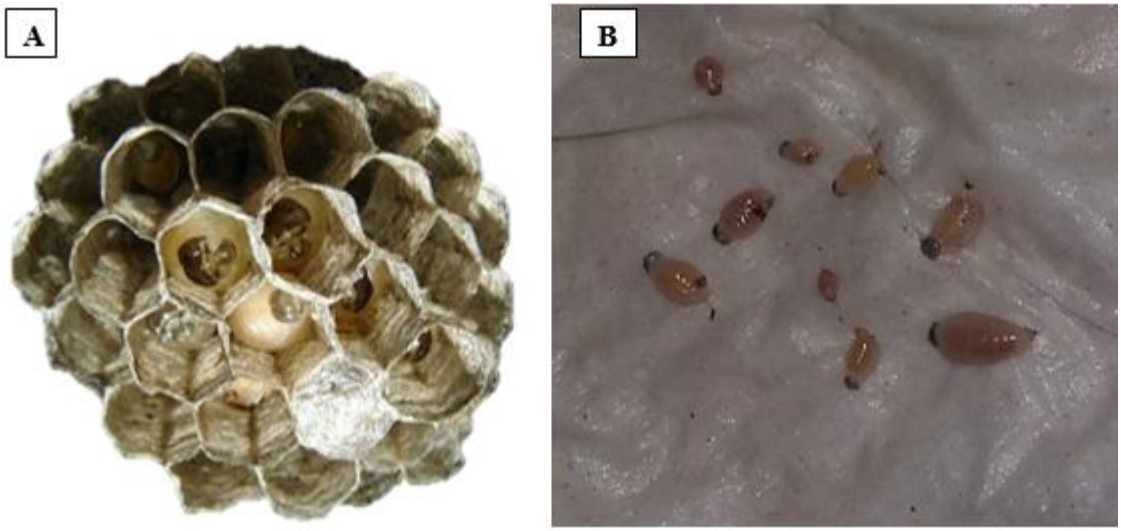

Figure 6. *P. dominula* nest containing: Second instar larvae (A); untreated 1st and 2nd instar larvae (B).

3.2.2 Fungal cultures

Fungal isolates were cultured on Sabouraud Dextrose Yeast Agar (SDYA), which was supplemented with chloramphenicol (100 mg/ml) and ampicillin (100 mg/ml) to prevent bacterial growth, in 95 mm plastic petri dishes and incubated in the dark at 30°C for two weeks to allow for optimal sporulation. This growth media was adapted from Uztan *et al*. (2016).

When cultured on solid media, the *B. bassiana* isolates were morphologically different (Fig. 7)., even though based on their sequences, they were all identified as *B. bassiana* (Chapter 1).

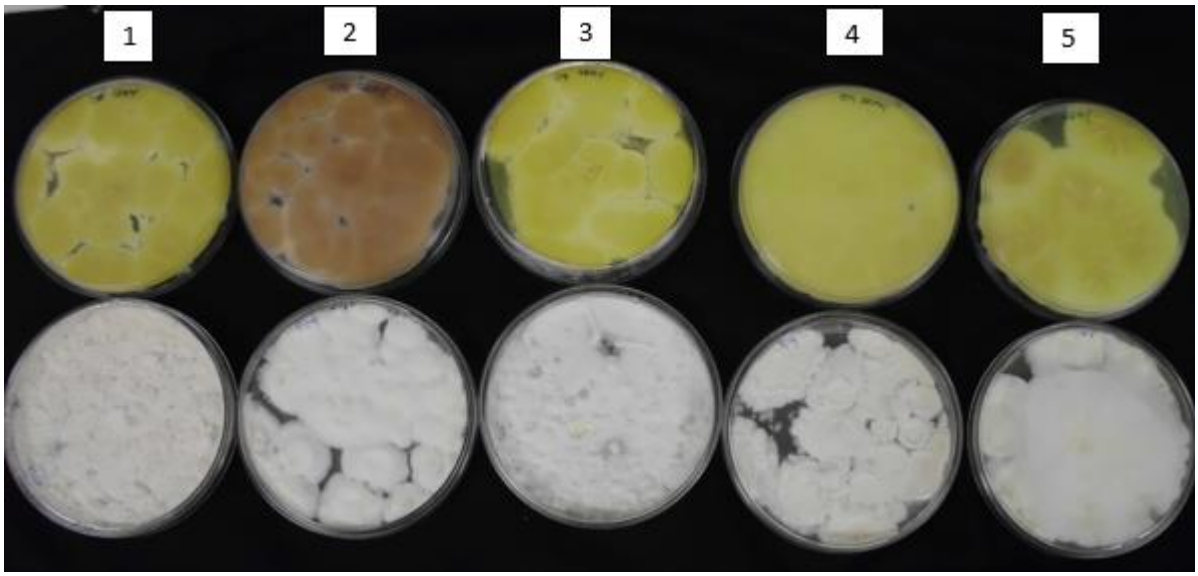

Figure 7. Two-week old *B. bassiana* grown on SDYA, illustrating the conidia and the reverse of the plate, to show the difference between the strains. 1) Bb4849 *B. bassiana*, 2) Bb4859 *B. brongniartii*, turns media pink/purple, 3) Bb4847 *B. bassiana*, 4) Bb4822 *B. bassiana* and 5) Bb4818 *B. bassiana*. Though all the isolates are *Beauveria spp*., they look different morphologically, with different textures and different distinctive growth patterns and colours on the reverse of the media plate.

Phylogenetic analysis of B. bassiana isolates

To further investigate the relatedness of the *B. bassiana* isolates, a phylogenetic analysis was conducted on the sequences (ITS1 region) of the respective isolates. MEGA 7 software was used for this analysis. *B. bassiana* sequences were downloaded from the NCBI databases including a type strain. These sequences were aligned with the 5 field-isolated *B. bassiana* sequences using the MUSCLE algorithm on MEGA. and a neighbour-joining method was used to construct the phylogenetic tree (Fig. 8). The bootstrap method with 1000 replicates was used to estimate the reliability of the tree.

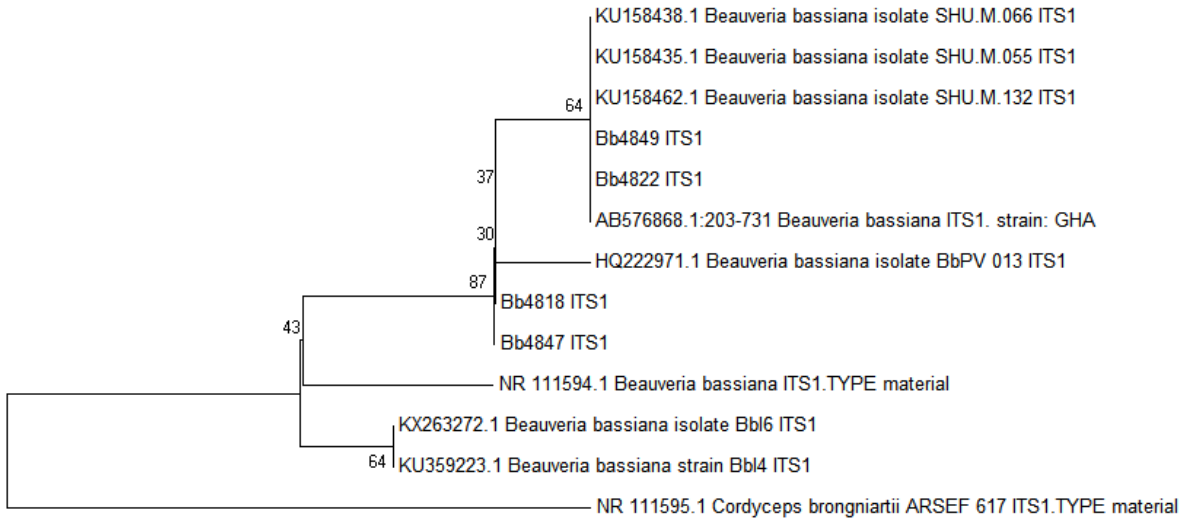

Figure 8. A rooted phylogenetic tree for *B. bassiana* and its related species based on ITS1 sequences constructed using the neighbour-joining method. *Cordyceps brongniartii* was used as an outgroup to root the tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree was constructed on MEGA v7 (Kumar *et al.*, 2015).

3.2.3 Conidial suspensions

After the two-week incubation period, fungal plates were flooded with 20 ml sterilized distilled water with 0.1 % Tween-80 and using a sterile glass rod, the spores were gently dislodged. The suspensions were then filtered using sterile cheesecloth into clean sterile 100 ml Schott bottles each containing spore suspensions from the respective isolates (Castillo *et al*., 2014). A Neubauer haemocytometer was used to determine conidia concentrations in the original suspensions and all the suspensions were adjusted to 2 $x10^9$ spores/ml, which is the recommended concentration for the commercial *B. bassiana* strain. The conidia suspensions were used within two hours after counting and preparation. The viability of conidia was examined by adjusting the concentration to 2 x 10^6 as described by Wraight, *et al*., (2007). The viability of conidia was determined by spread plating 0.1 ml of the conidial suspension with a concentration of 1 x 10^6 conidia/ml on SDYA media plates and a sterile microscope cover slip was placed at the centre of each plate. The plates were replicated four times per isolate. Plates were incubated at 26 ^{o}C for a 16-hour incubation period. After the incubation period, plates were examined under a light microscope at x40 magnification. Two hundred spores were

counted by counting number of germinated spores. Then the percentage germination was determined (no. of germinated/200). Spores were considered germinated and viable if the germination tube was double the size of the spore (Inglis *et al*., 2012).

3.2.4 Bioassay

Fungal cultures

The virulance of six fungal isolates (Table 3), which were isolated in the previous chapter, namely; three *Beauveria bassiana* isolates, one *Beauveria brongniartii* and one *Purpureocillium lilacinum*, were tested against the 2nd instar larvae of *P. dominula*. A commercial strain of *B. bassiana*, Eco-Bb® (Plant Health Products), which was provided by the Entomology Department, Stellenbosch University was also used in the bioassay as a positive control.

Table 3. Native entomopathogenic fungal isolates used against 2nd instar larvae in this study

Isolate Code	ID	Origin	Location	GPS coordinates
Eco-Bb®	*B. bassiana*	commercial strain	Entomology Department	
Bb4850	*B. brongniartii*	soil	Welgevallen Experimental Farm	33°56'33"S 18°51'56"E
Bb4818	*B. bassiana*	soil	Welgevallen Experimental Farm	33°56'33"S 18°51'56"E
Bb4849	*B. bassiana*	Nest material	Welgevallen Experimental Farm	33°56'33"S 18°51'56"E
Bb4847	*B. bassiana*	soil	Welgevallen Experimental Farm	33°56'33"S 18°51'56"E
Bb4822	*B. bassiana*	Nest material	Nooitgedacht Village	33°53'32"S 18°31'10"E

E14	*P. lilacinum*	soil	Welgevallen Farm	Experimental	33°56'33"S	18°51'56"E

Fungal infection of *P. dominula* larvae

Healthy *P. dominula* second instar larvae that were carefully removed from their nest (Fig. 6A), and placed on moist paper towels, were ovular with translucent glossy cuticle (Fig. 6B). To determine the virulence of the 7 isolates of entomopathogenic fungi against live second instar *P. dominula* larvae, 20 µl (2 x 10^9 spores/ ml) of the prepared conidial suspension was applied on the mouth and along the cuticle of the larvae using a micropipette. For each treatment (isolate) six 2nd instar *P. dominula* larvae were placed in each 95 mm plastic petri dish lined with filter paper which was moistened with sterile distilled water. Each treatment was done replicated 3 times, which means there were 3 petri dishes per treatment, each one containing six 2nd instar *P. dominula* larvae, therefore there were 18 larvae per treatment. A control group was also included, this group was only inoculated with 20 µl of distilled water with 0.1 % Tween-80 without spores as a negative control. Plates containing larvae treated with the same treatment were kept together in a 3 L rectangular transparent plastic container at room temperature with 65 ± 5% RH. Daily, plates were sprayed with 30 µl of sterile distilled water to prevent the paper towels used to line the plates from desiccating. Cumulative proportional mortality of the treated larvae was recorded and compared to that of the control group larvae. It should be noted that larvae were considered dead if they were not moving when probed with a pipette tip.

Mortality assessment

To confirm that death was due to fungal infection, dead larvae were surface sterilized by dipping the insect in 70 % ethanol for a few seconds and placed on a clean damp filter paper in airtight petri dishes and incubated at 26°C for a week to check if any fungal growth was visible. Fungal isolates from the mycosed larvae were re-isolated on fresh PDA media to confirm if it was the same taxon that was used to inoculate the respective larvae examined.

3.3 Statistical analysis

The aim of this section is to determine whether there are statistically significant differences between the means of dead larvae. A common method to achieve this objective in statistics is the use of Analysis of Variance (ANOVA), which is a collection of statistical models to analyse the differences among group means. There are several types of ANOVA (One-way ANOVA, One-way repeated ANOVA, Factorial ANOVA, Mixed ANOVA, ANCOVA, MANOVA), but the one-way ANOVA model was used in this study because we were testing for a significant difference among means of more than two groups.

One-way ANOVA was used to compare the percentage mortality of *P. dominula* larvae, 7 days after application of the respective treatments. The results from one-way ANOVA only shows that at least two groups are statistically different, it however, does not present which specific groups thereof, and to determine which groups differ from each other, a post-hoc test was performed using a t-test (α= 0.05) to compare virulence of each fungal isolate to the control and to each other.

One-way ANOVA analysis was performed on the data in Excel at a significance level of 0.05 to determine if the differences in the mean larvae mortality were significant. The output from this analysis is presented in Table 4. To determine if the ANOVA analysis perform was valid, a follow up test for equal variances was performed. This variance analysis assumption was done by finding the ratio of the largest to smallest variance.

3.4 Results

ANOVA

According to the test result, The P value= 0.79, F = 0.58, and with a critical value of 0.05, the F- critical = 2.115. Therefore, since the F is smaller than the F-critical value, this result shows that none of the treatments were significantly different, which means that the mean mortality caused by the treatments and the control were not significantly different. To determine if the ANOVA analysis perform was valid, a follow up test for equal variances was performed. This variance analysis assumption was done by finding the ratio of the largest to smallest variance.

which resulted to the value of 1.839 and because this value was less than/ close to 2, the one-way ANOVA was proven to be viable.

Table 4. ANOVA analysis for the assessment of measurement of error for larval mortality

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	15,59083	8	1,948854	0,584862	0,78589	2,115223
Within Groups	179,9365	54	3,332158			
Total	195,5273	62				

The results from the paired t-test showed a significant difference between Bb4822 and the control (P=0.01). When each treatment was compared to another, it was found that there was no significant difference.

Phylogenetic analysis

The field-isolated *B. bassiana* used in this study had nucleotide variation, however, all the isolates were clustered together (87 % bootstrap) into a monophyletic group though they formed a polyphyletic group to the *B. bassiana* type strain. The sequences show that there as minor differences in the sequences of these isolates as they are not all in one group, Bb4818 and 4847 clustered together while at a later node Bb4849 and Bb4822 clustered together.

Viability

The viability test revealed that all the isolates were viable with the germination rate ranging between 86- 95 %. Within two days after treatment, a colour change was observed in the infected larvae (Fig. 9a). By the third and fourth day, mycelial growth was observed from the infected larvae (Fig. 9b). Within 1 week after exposure all treated larvae had fungal growth,

except for the control treated with tween water, which resulted in some of the larvae liquefying without any fungal outgrowth (Fig. 9c).

Bioassay

The results of the mean proportional mortality of second instar *P. dominula* larvae treated with selected fungal isolates at 2 x 10^9 conidia/ml are presented in Figure 10 (A-F). Figure 10 showed the graphs comparing all the treatments to the control. Fungal species varied in their ability to infect *P. dominula*. Two days after application, treatment with *B. bassiana* isolates 4818, 4847 and 4849 showed a 40% mortality, while treatments with E14 (*P. lilacinium*) showed 30% which was similar to the commercial strain. Isolate 4822, showed the highest mortality rate of 80% 2 days after application. On the third day, isolates 4818 and 4849 had a mortality ratee of 80% while both 4847 and 4822 had 100% mortality. The commercial strain had a 40% mortality 3 days after application. On the 4th day all the treatments had reached 100% mortality, except for E14 with 60 % mortality, both the control and commercial strain with 80% mortality. Larvae treated with treated with *B. bassiana* changed to pinkish colour 2 days after treatment (Fig. 9A) and on the 4th day the whole larva was covered in mycelia (Fig. 9B). Some of the larvae from the control group, liquified after 2 days (Fig. 9C).

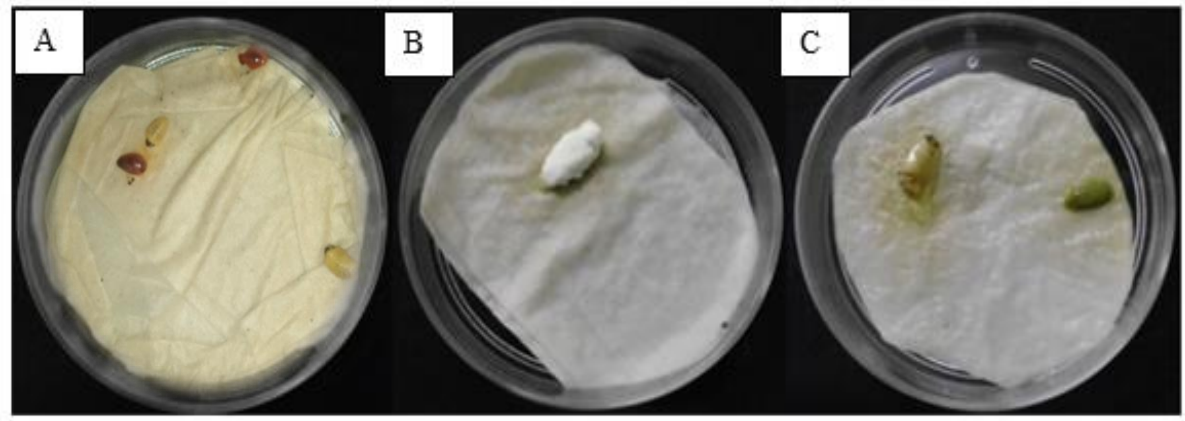

Figure 9. *P. dominula* larvae placed on moist paper towels in petri dishes. (A) Colour change on infected larva two days after exposure. (B) Larva 4 days after exposure. (C) Liquefied larva

The untreated control group had no fungal growth, the dead larvae just liquefied, this could due to bacterial or viral contamination. On the 4th day, larvae treated with the commercial strain

and E14 had 80% mortality and 60% mortality, respectively, while all the other isolates have 100 %.

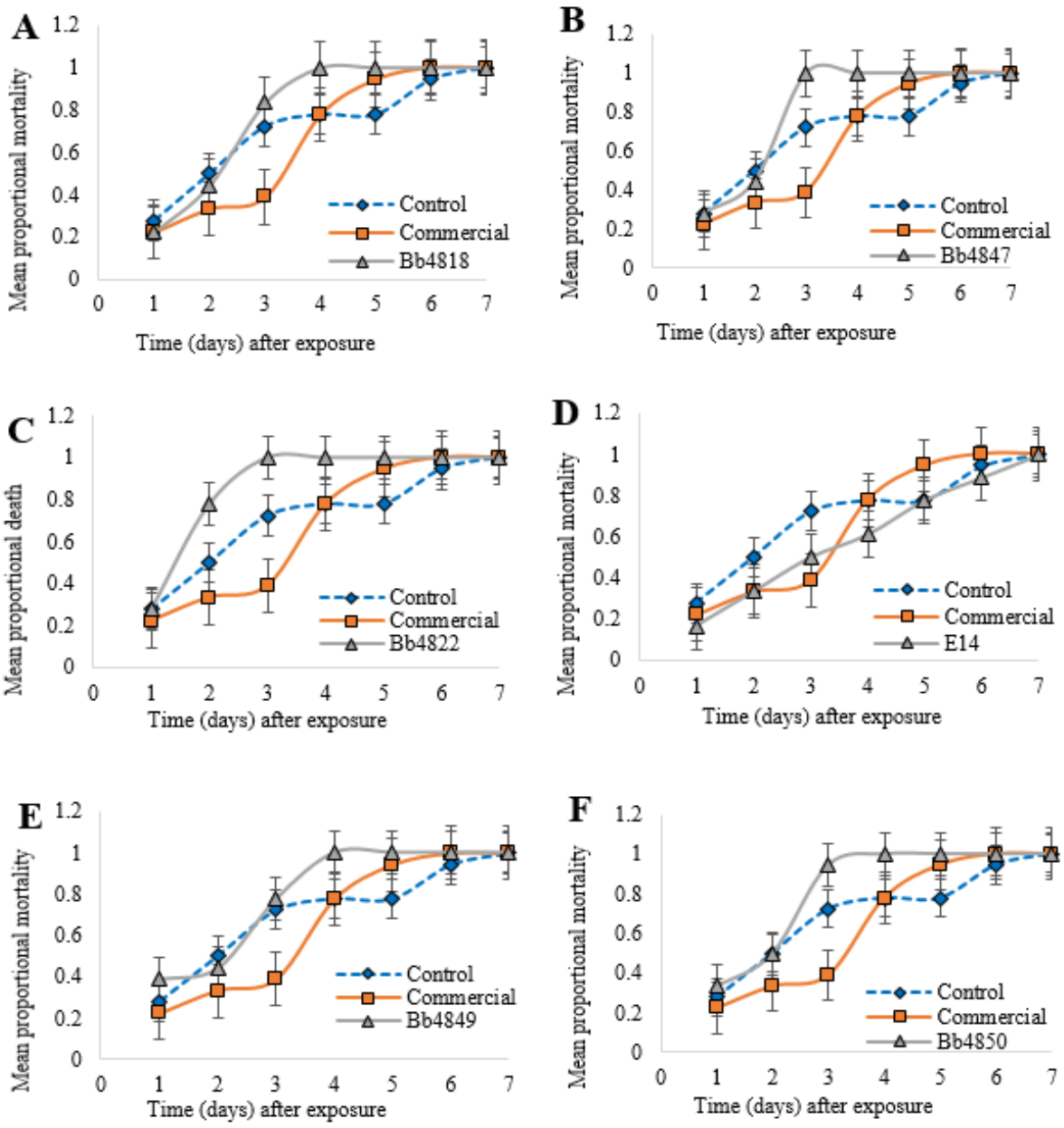

Figure 10. (A-F) Mean proportional cumulative mortality of second instar *P. dominula* larvae exposed to EP fungi formulation at 2 x 10^9 spores/ml with 0.1% Tween. Bars represent standard error.

3.5 Discussion

Fungal biological control agents have not been previously used to control *P. dominula* in South Africa. However, there have been some studies that used *B. bassiana* and *Metarhizium* spp. to control *Vespula germanica*, another invasive wasp in South Africa, they found that workers and larvae were susceptible to the fungal pathogens (Beggs, *et al*., 2011). In this study, *B. bassiana*, *B. brongniartii*, and *P. lilacinum* were the only entomopathogenic fungi isolated from the nest material and from soil. All the other isolates were saprophytic or opportunistic plant pathogens like *Alternaria*, *Aspergillus niger* and *Penicillium chrysogenum*. Therefore, only the entomopathogenic fungi were used in the bioassay. *P. lilacinum* was isolated from a soil sample that was collected directly underneath an abandoned nest, this fungus is a known pathogen of many nematode and tick species (Angelo, *et al*., 2012).

In this study, when the effects of *P. lilacinum* were compared to the effects of *B. bassiana* on *P. dominula* larvae, it was found that there was no statistical significant difference in mortality rates between them. *P. lilacinum* showed no significant difference to the untreated control group from day 1 to day 4 after application. These findings correspond to a study by (Lopez, *et al*., 2014), where the effects of *P. lilacinum* were compared to those of *B. bassiana* on cotton aphid and the *B. bassiana* resulted in aphid number reduction while *P. lilacinum* yielded results similar to the control group. When mean proportional mortality of the control group was compared to that of *P. lilacinum*, there was no significant difference observed, with the control resulting in an 80% mortality rate and *P. lilacinum* resulting in a 60% mortality rate on the fourth day. Singh *et al*. (2013) reported that *P. lilacinum* is also effective against the cotton root-knot nematode *Meloidogyne incognita*, where it inhibits nematode eggs from hatching. Although this fungus has been reported to be pathogenic to a wide range of insects and nematodes, the results from this study suggests that *P. dominula* larvae were less susceptible to it as no visible negative effect was detected (Oliveira *et al*., 2013; Lopez *et al*., 2014; Goffré & Folgarait, 2015; Lan *et al*., 2017). According to our knowledge this study also provided the first virulence test of *P. lilacinium* against *P. dominula* larvae.

Beauveria brongniartii is another common soil entomopathogenic fungus. Like *B. bassiana*, this fungus has been developed as a biological control agent for some insects. However, unlike *B. bassiana*, *B brongniartii* has a narrow host range infecting mostly coleopteran species (Strasser *et al*., 1994; Traugott, *et al*., 2005; Goble *et al*., 2015). In this study, *B. brongniartii*

was able to reach 94% mortality rate three days after exposure, and this performance was significantly better than that of the commercial strain which was only able to reach a 38 % mortality rate, three days after exposure a Student t-test gave P = 0.04. There was no statistical significance between the performance of *B. brongniartii* and *B. bassiana* isolates Bb4822 and Bb4847.

Isolates Bb4822 and Bb4847 were the most pathogenic against *P. dominula* larvae, and were then compared to each other, to see which isolate was the most virulent. Isolate Bb4822 reached 70% mortality two days after exposure, while Bb4847 was only able to reach 40 %. However, when the mean proportional mortality of both isolates was subjected to a Student's t-test, there was no statistically significant difference between the virulence of the two isolates (P=0.3).

Fungal agents isolated from the soil are more pathogenic than those isolated from insect cadavers (Sánchez-Peña, *et al*., 2007); most of the commercially available fungal biological control agents were originally isolated from infected insect hosts and mass produced for pest control (Sánchez-Peña, *et al*., 2007). This could explain why the commercial strain performed poorly compared to the other *B. bassiana* strains; 3 days after exposure the commercial strain had a mortality of 40% while the other B. bassiana spp. Had between 80%- 100% mortality. Sánchez-Peña, *et al*. (2007) recommended the use of soil derived strains in local biological control projects. Soil isolates are easier to obtain than locating infected insects and there are no legal restrictions because no exotic strain is being introduced to the environment when the agents are released.

The aim of this study was to determine which fungal isolate was pathogenic to *P. dominula* larvae and the results of this chapter show that under laboratory conditions, *P. dominula* is susceptible to all the entomopathogenic fungi tested in this study. Though there was no significant difference between the treatments, isolate Bb4822 was the only isolate that reached 70% mortality rate two days after application. This concludes that *P. dominula* is more susceptible to Bb4822. More studies are required to test its efficacy against larvae under field conditions.

References

Beggs, J. R., Brockerhoff, E. G., Corley, J. C., Kenis, M., Masciocchi, M., Muller, F., Villemant, C. (2011). Ecological effects and management of invasive alien Vespidae. *BioControl*, *56*(4), 505–526.

Beggs, J. R., Rees, J. S., Toft, R. J., Dennis, T. E., & Barlow, N. D. (2008). Evaluating the impact of a biological control parasitoid on invasive Vespula wasps in a natural forest ecosystem. *Biological Control*, *44*(3), 399–407.

Benade, P. C., Veldtman, R., Samways, M. J., & Roets, F. (2014). Rapid range expansion of the invasive wasp *Polistes dominula* (Hymenoptera: Vespidae: Polistinae) and first record of parasitoids on this species and the native Polistes marginalis in the Western Cape Province of South Africa. *African Entomology*, *22*(1), 220–225.

Castillo Lopez, D., Zhu-Salzman, K., Ek-Ramos, M. J. & Sword, G. A. (2014). The entomopathogenic fungal endophytes *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) and *Beauveria bassiana* negatively affect cotton aphid reproduction under both greenhouse and field conditions. *PloS one*, 9(8), e103891.

Coombes, C. A., Hill, M. P., Moore, S. D., Dames, J. F., & Fullard, T. (2015). *Beauveria* and *Metarhizium* against false codling moth (Lepidoptera : Tortricidae): A step towards selecting isolates for potential development of a mycoinsecticide, *African Entomology* 23(1), 239–242.

Enkerli, J., Widmer, F., & Keller, S. (2004). Long-term field persistence of Beauveria brongniartii strains applied as biocontrol agents against European cockchafer larvae in Switzerland. *Biological Control*, 29(1), 115–123.

Giliomee, J. H. (2011). Recent establishment of many alien insects in South Africa - a cause for concern. *African Entomology*, *19*(1), 151–155.

Gill, S. S., Cowles, E. A., & Patricia, V. (1992). *Bacillus thuringiensis* endotoxins. *Annual Review in Entomology*, *37*, 615–636.

Goble, T. A., Conlong, D. E., & Hill, M. P. (2015). Virulence of Beauveria brongniartii and *B. bassiana* against *Schizonycha affinis* white grubs and adults (Coleoptera: Scarabaeidae). *Journal of Applied Entomology*, 139(1–2), 134–145.

Goffré, D., & Folgarait, P. J. (2015). Purpureocillium lilacinum, potential agent for biological control of the leaf-cutting ant *Acromyrmex lundii*. *Journal of Invertebrate Pathology*, 130, 107–115.

Guetsky, R., Shtienberg, D., Elad, Y., Fischer, E., & Dinoor, A. (2002). Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Phytopathology*, 92(9), 976–985.

Harris, R. J., Harcourt, S. J., Glare, T. R., Rose, E. A. F., & Nelson, T. J. (2000). Susceptibility of Vespula vulgaris (Hymenoptera: Vespidae) to Generalist Entomopathogenic Fungi and Their Potential for Wasp Control. *Journal of Invertebrate Pathology*, *75*(4), 251–258.

Haupt, K. (2015). Assessment of the invasive German wasp , Vespula germanica , in South Africa. Published thesis. South Africa: Stellenbosch University.

Hughes, D. P., Kathirithamby, J., Turillazzi, S., & Beani, L. (2004). Social wasps desert the colony and aggregate outside if parasitized: Parasite manipulation? *Behavioral Ecology*, *15*(6), 1037–1043.

Inglis, G. D., Enkerli, J., & Goettel, M. S. (2012). Laboratory techniques used for entomopathogenic fungi. *Hypocreales. Manual of Techniques in Invertebrate Pathology* (Second Edi). Elsevier Ltd.

Lacey, L. A., Kirk, A. A., Millar, L., Mercadier, G., & Vidal, C. (1999). Ovicidal and larvicidal activity of conidia and blastospores of Paecilomyces fumosoroseus (*Deuteromycotina: Hyphomycetes*) against *Bemisia argentifolii* (Homoptera: Aleyrodidae) with a description of a bioassay system allowing prolonged survival of control. B*iocontrol Science and Technology*, 9, 9–18.

Lacey, L. A., Liu, T. X., Buchman, J. L., Munyaneza, J. E., Goolsby, J. A., & Horton, D. R. (2011). Entomopathogenic fungi (Hypocreales) for control of potato psyllid, *Bactericera cockerelli* (Šulc) (Hemiptera: Triozidae) in an area endemic for zebra chip disease of potato. *Biological Control*, 56(3), 271–278.

Liu, H., & Stiling, P. (2006). Testing the enemy release hypothesis: A review and meta-analysis. *Biological Invasions*, *8*(7), 1535–1545.

Manfredini, F., Grozinger, C. M., & Beani, L. (2013). Examining the “evolution of increased competitive ability” hypothesis in response to parasites and pathogens in the invasive

paper wasp *Polistes dominula*. Naturwissenschaften, 100(3), 219–228.

Miller, G. L., Donnelly, C. R., & Gamboa, G. J. (2013). A ten-year comparative study of the population dynamics and parasitoidism in the native paper wasp *Polistes fuscatus* and the invasive *P. dominulus*. *Insectes Sociaux*, 60(1), 49–56.

Oliveira, B. R., Barreto Crespo, M. T., San Romão, M. V., Benoliel, M. J., Samson, R. A., & Pereira, V. J. (2013). New insights concerning the occurrence of fungi in water sources and their potential pathogenicity. *Water Research*, 47(16), 6338–6347.

Posadas, J. B., Comerio, R. M., Mini, J. I., Nussenbaum, A. L., & Lecuona, R. E. (2012). A novel dodine-free selective medium based on the use of cetyl trimethyl ammonium bromide (CTAB) to isolate *Beauveria bassiana*, *Metarhizium anisopliae sensu lato* and *Paecilomyces lilacinus* from soil. *Mycologia*, 104(4), 974–980.

Rose, E. A. F., & Harris, R. J. (1999). Possible pathogens of social wasps (Hymenoptera: Vespidae) and their potential as biological control agents. *New Zealand Journal of Zoology*, 26, 179–190.

Ruiu, L. (2015). Insect pathogenic bacteria in integrated pest management. *Insects*, 6(2), 352–367.

Sedehi, A., Sedaghatfar, E., & Modarres Najafabadi, S. S. (2014). Studies on Effect of the Beauveria bassiana on eggs and larvae of Plodia interpunctella. *Canadian Journal of Basic and Applied Sciences*, 2(2), 40–45.

Severino, M. G., Campi, P., MacChia, D., Manfredi, M., Turillazzi, S., Spadolini, I., … Bonifazi, F. (2006). European Polistes venom allergy. *European Journal of Allergy and Clinical Immunology*, 61(7), 860–863.

Shahid, A. A., Rao, A. Q., Bakhsh, A., & Husnain, T. (2012). Entomopathogenic fungi as biological controllers: New insights into their virulence and pathogenicity. *Archives of Biological Sciences*, 64(1), 21–42.

Siekmann, G., Tenhumberg, B., & Keller, M. A. (2016). Nordic society Oikos feeding and survival in parasitic wasps : Sugar concentration and timing matter. *Wiley on behalf of Nordic Society Oikos Stable*. 95(3), 425–430.

Singh, R., Levitt, A. L., Rajotte, E. G., Holmes, E. C., Ostiguy, N., Vanengelsdorp, D., … Cox-Foster, D. L. (2010). RNA viruses in hymenopteran pollinators: Evidence of inter-taxa

virus transmission via pollen and potential impact on non-Apis hymenopteran species. *PLoS one*, *5*(12).

Strasser, H; Längle, T; Pernfuß, B. (1994). Beauveria brongniartii (Saccardo) Petch is a safe insecticide to control the European cockchafer. *Nucleic Acids Research*, 27, 38–45.

Uztan, A. H., Gunyar1, O. A., Yoltas, A., & Keskin, N. (2016). Isolation and identification of entomopathogenic fungi beauveria. *Fresenius Environmental Bulletin*, 26(12), 5180–5185.

Van Zyl, C. (2016). Status of the invasive wasp species, Vespula germanica and Polistes dominula in South Africa, and the feasibility of various management strategies. Published thesis. South Africa: Stellenbosch University

World Health Organization. (1982). Manual on environmental management for mosquito control with special emphasis on malaria vectors. *WHO Offset Publication*, (66), 1–283.

Wraight, S. P., Inglis, G. D., & Goettel, M. S. (2007). Fungi. In L. A. Lacey & H. K. Kaya (Eds.), *Field Manual of Techniques in Invertebrate Pathology*. Springer, 223–248.

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Science and Technology*, 17(6), 553–596.

Chapter 4

4. *In situ* pathogenicity of *B. bassiana*, isolated from *P. dominula* nests against *P. dominula* larvae

Abstract

P. dominula is an invasive wasp which has recently been introduced in the Western Cape Province of South Africa. It has established its population around the region and the possibilities of its negative ecological impacts have been identified. There are currently no long-term control methods for these wasps and biological control may be a possible solution.

In this study, field-isolated *B. bassiana* Bb4822 and commercially produced strain Eco- Bb® were tested in laboratory and field conditions to determine which strain was more virulent against *P. dominula* larvae. The effects of temperature and other microbial organisms, found on wasp material of *P. dominula*, were investigated to determine whether they affected the growth of *B. bassiana*

In the laboratory trial there was no notable difference between the pathogenicity of *B. bassiana* Bb 4822 and Eco-Bb®, as both strains resulted in mycosis. The results from these tests showed that *B. bassiana* was pathogenic against *P. dominula* larvae under laboratory conditions, while no infection of adult wasps or larvae was observed under field conditions. However, during the field trials, the parasitic flies that were found in the wasp nests were infected with *B. bassiana* which suggested that while *P. dominula* appears not to be susceptible, the parasitic fly found in the nests, were susceptible to *B. bassiana*.

In this study, the optimal temperature for radial growth and for sporulation of Bb4822 was found to be 26 ˚C and 30 ˚C respectively. The microbial organisms associated with the nest material of *P.dominula* were found to be bacterial species which were identified as: *Bacillus cereus*, *Bacillus subtilis*, *Bacillus velezensis*, *Bacillus pumilus*, *Paenibacillus polymyxa* and *Enterobacter cloacae*.

Key words: Entomopathogenic fungi, *P. dominula*, nest material, *B. bassiana*, field trials

4.1 Introduction

The European paper wasp, *P. dominula* is native to Mediterranean Europe and today found throughout the eastern and western hemisphere (Benade, 2015; Van Zyl, 2016; Eardley *et al.*, 2009; Haupt, 2015). Eardley *et al*. (2009) published the first record of *P. dominula* in South Africa, which was sighted in Kuilsriver in February of 2008. *P. dominula* has been recently recognized and recorded as an alien invasive species in South Africa (Benade, 2015; Benade *et al.*, 2014; Giliomee, 2011). Following its first record in 2008, *P. dominula* has rapidly increased in abundance reaching pest levels. However, its presence is considered to be restricted to the Western Cape Province, although there is a high potential for it to spread to other parts of South Africa (Eardley *et al*. 2009b). The restriction of *P. dominula* to the Western Cape Province increases the possibility of successful control and even gives the possibility to eradicate *P. dominula* populations if feasible (Van Zyl, 2016).

Many control methods have been developed globally to decrease and slow down the expansion of invasive wasp species, mostly *Vespula germanica* and *Vespula vulgaris* (Beggs *et al*., 2011; Harris *et al*., 2000). The primary control measure that has been used is chemical pesticides, however, chemical pesticides have shown to only achieve temporary suppression in a localized area (Beggs *et al*., 2008; Rose *et al.*, 1999). Though chemical control has been effective, there are many disadvantages that are associated with it, for example, chemical pesticides are not environmentally safe, they are labour intensive, costly and they are also not specific, which means that they may target beneficial insects (Fallis, 2013; Pal & Mc Spadden, 2006; Beggs *et al*., 2011; Shahid *et al*., 2012).

In South Africa, a method of control that is commonly used in conjunction with chemical control is mechanical control, where nests are physically removed by hand. This method seems to be well accepted by the public because it is not environmentally harmful (Van Zyl, 2016). This method is, however, not feasible for large areas with many nests, as this is time-consuming especially because the nests are not always easy to locate and access (Edwards *et al*., 2017).

Biological control is another method of controlling invasive species, however, it has not been extensively used against invasive alien Vespidae (Beggs *et al*., 2011). Parasitoids, predators, and pathogens such as parasitic wasps, beetles, entomopathogenic nematodes, mites, bacteria, entomopathogenic fungi and viruses have been recorded from wasp nest material and from wasps (Beggs *et al*., 2011; Fallis, 2013; Glare *et al*., 1993; Harris *et al*., 2000). None of the

microorganisms isolated from wasp nests have been confirmed to be pathogenic to *P. dominula* by the Koch's postulates (Harris *et al*., 2000). Entomopathogenic fungi such as *Aspergillus flavus*, a range of *Beauveria bassiana* and *Metarhizium anisopliae* strains have shown potential against social species, namely, *Vespula vulgaris*, *V. germanica* and *Polistes hebraeus* (Glare *et al*., 1993; Harris *et al*., 2000; Lacey *et al*., 2001).

Unlike chemical pesticides, entomopathogenic fungi (EPF) are greatly influenced by abiotic factors, for example, temperature, humidity, and sunlight or UV irradiation. Other factors that influence EPFs are biotic factors, for example, ecology (host-pathogen interaction), physiology and host associations (Wraight *et al*., 2007). These factors have an effect on the pathogen's survival and its ability to infect the host, but they also have an effect on the host's susceptibility and on the development of the infection inside the host (Nussenbaum *et al*., 2013). Despite all the research efforts to control social wasps globally, there is still no long-term remedy, to date (Beggs *et al*., 2011). Hence, the dire need to find a biological control agent to regulate the increasing invasive wasp population. The focus of this thesis is to find an alternative to the labour intensive mechanical control and chemical pesticides which are not environmentally friendly and also not target-specific. Though biological control is the best option, it is still understudied, especially when it comes to controlling *P. dominula* or wasps in general (Van Zyl, 2016).

To further understand the effects of the identified biological control (*B. bassiana* - Bb4822) on *P. dominula*, the study presented in this chapter was done to:

- determine the lethal concentration of the field-isolated *B. bassiana* Bb4822 and to test its efficacy under field conditions.
- assess for the effect of biotic and abiotic factors on the effectiveness of *B. bassiana* Bb4822
- to compare the pathogenicity of Bb4822 to Eco-Bb®,
- present a general discussion on the project

B. bassiana Bb4822 was selected to be used in the field trial because under laboratory conditions in Chapter 3, it was the most pathogenic against *P. dominula* larvae. Eco-Bb®, a commercial fungal biological control product from Plant Health Products (Pty) Ltd was used in field trials to compare the results of using Bb4822 to existing chemical control. The

commercial strain is not registered to control Hymenopteran insects, hence its inclusion in this field trial was to test if it will be able to infect the larvae under field conditions.

4.2 Methods and material

4.2.1 Fungal cultures

The *B. bassiana* Bb4822 that was used for this study was isolated from *P. dominula* nest material using selective media, isolation method is outlined in Chapter 2. The stock-culture of B. bassiana Bb4822 is maintained in the Microbiology Department at Stellenbosch University, Stellenbosch, South Africa. The commercial Eco-Bb®, used in this study, was obtained from the Conservation Ecology and Entomology Department, Stellenbosch University, South Africa. This strain of *B. bassiana* is commercially produced and is available in a wettable-powder formulation.

4.2.2 Virulence revival for fungi

Continuous sub-culturing of fungi on artificial media can lead to genetic changes that may affect the phenotype due to heterokaryosis (Inglis *et al*., 2012). This can cause the fungus to lose its virulence. According to Inglis *et al*. (2012), a single passage through an insect host can restore virulence. Therefore, in this study, to revive the virulence of Bb4822, meel worms obtained from the Entomology Department, Stellenbosch University, were inoculated with the fungus Bb4822. Meel worms were inoculated using the immersion method, where the insects were dipped into a prepared suspension of Bb4822 with 2 x 10^9 conidia/mL for 3 seconds and placed in a 95 mm petri dish lined with damp filter paper for moisture, the petri dish with the treated meel worms was then incubated at 26 ˚C. After a two-week incubation period the fungus was re-isolated from the meel worm cadaver and cultured on Sabouraud dextrose agar containing 1% yeast extract (SDYA) in 95 mm diameter plastic Petri dishes and incubated at 30 ˚C (optimal temperature for sporulation) in the dark for two weeks before being used in the trial (Arooni-Hesari *et al*., 2015). After the 12-day incubation period, the fungal isolate had sporulated (Fig. 11).

4.2.3 Conidia harvesting

B. bassiana conidia have hydrophobic cell walls and this makes them difficult to suspend in water, hence a surfactant was added to the water to solve this challenge. Spores of BB4822 were harvested by flooding the plate with 20 ml sterilized distilled water with 0.01 % Tween-80. Using a sterile glass rod the spores were gently dislodged. The suspension was then filtered using sterilized cheesecloth into a sterile 100 ml Schott bottle (Lopez *et al*., 2014). The suspension for the commercial strain was prepared according to the product label. A Neubauer haemocytometer was used to determine conidia concentrations in the original suspension of Bb4822. From the original suspension a series of aqueous suspensions with increasing concentrations of infective propagules were prepared. The concentrations were ranging from 1 x 10^5 to 1 x 10^9 conidia/mL (Inglis *et al.*, 2012).

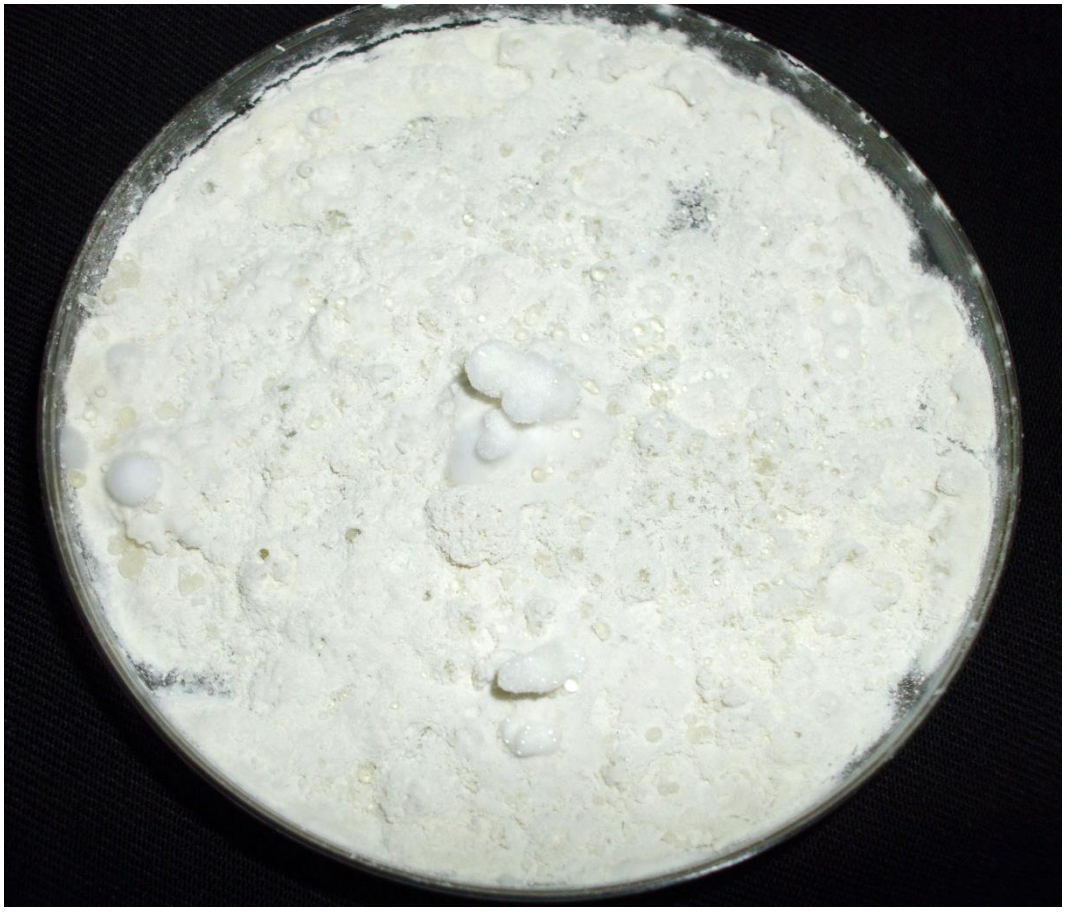

Figure 11. Two-week-old sporulating fungal isolate, grown on SDYA.

4.2.4 Effect of fungal concentration on larval mortality

This experiment was conducted to determine the most effective concentration that can be used for the field trial. Second instar larvae of *P. dominula* were used in this experiment. The larval stage was targeted because larvae have a very small chance of physically escaping the fungal treatment, unlike the adults that could fly away from the treated nests. Also, the cuticle of larvae has a lower chitin content compared to the cuticle of adults; which makes larvae more vulnerable to fungal penetration (Kaya *et al*., 2016).

In the field, *P. dominula* nests were first confirmed to contain at least 15 larvae, this was done by visually counting the cells that contained larvae. *Polistes dominula* nests containing larvae were collected from the Welgevallen experimental farm, in Stellenbosch. Nests were placed in a sterile plastic container and transported to the laboratory. In the laboratory, the adults that had just emerged from the enclosed cells were removed carefully and discarded because this study focused on determining the lethal concentration (LC50) of Bb4822 against 2nd instar larvae. To remove the larvae from their individual cells, sterile forceps were used to hold the nest material in place, while a sterile needle was used the tear the paper (nest material). Once the larva was exposed, it was gently removed from the nest, using sterilised forceps, and placed in 95 mm plastic petri dish. Three 2nd instar *P. dominula* larvae were placed in each 95 mm plastic petri dish lined with filter paper which was moistened with sterile distilled water.

To determine the lethal concentration of Bb4822, five concentrations were tested. For each of the 5 treatments (concentrations 1 x 10^5, 1 x 10^6, 1 x 10^7, 1 x 10^8 and 1 x 10^9 conidia/ml), there were 6 petri dishes, with each petri dish containing three 2nd instar *P. dominula* larvae, the experiment was replicated 3 times, therefore, there were 54 larvae used for each concentration. To prevent cross contamination, all larvae in a petri dish got the same treatment and plates with the same treatment were placed in their separate plastic container.

Most entomopathogenic fungi generally do not infect their host *per os* (orally), due to the high pH in the insect mid gut which could hinder fungal growth, rather they enter the host directly by adhering to the cuticle and penetrating the body (Inglis *et al*., 2012). In this study, a direct application was done by applying 10 µl of each treatment, using a micropipette on the surface of the insect cuticle. The inoculated insects were then incubated at room temperature in a 12:12 light to dark ratio, for 5 days. Mortality was recorded daily. Larvae were considered dead if they did not move after being probed with a sterile pipette tip and the number of dead larvae

was recorded daily. After the 5-day duration of the experiment, the LC_{50} (the concentration of spores that kill 50 % of the population) was calculated. The total daily death percentage was calculated and modified by Abbott equation and the LC_{50} values were calculated using Probit analysis, the steps followed to calculate the LC_{50} values are stated by Abbott (1925). Percentage larval mortality data for the laboratory trial were corrected for control mortality, which takes natural death into consideration, and the data was then normalized and transformed with the natural log, and subjected to statistical analysis on Excel (Abbott, 1925).

The concentration with the lowest LC_{50} value was selected to be used in the field trial.

4.2.5 Field trial

Experimental site

The field trial was carried out at the stables in the Welgevallen Experimental Farm at Stellenbosch University (33°56'45.8"S 18°52'01.9"E) and at the Agricultural Research Council Plant Protection Research Institute (ARC-PPRI) (33º 56’ 47” S, 18º 50’28” E), in Stellenbosch, Western Cape province of South Africa, because both sites had an abundance of *P. dominula* nests. The trail was conducted in March 2017, which is late summer. At the sites, experimental nests were chosen at random, and all the treatments (Table 5) had the same number of nests.

Table 5. Treatments used in the field trials of this study

Treatment	Contents
Bb4822	Field- isolated *Beauveria bassiana*
Eco-Bb®	Commercial strain
Control	Water without conidia

Spore suspension and viability test

Each treatment (Table 5) was prepared in a sterile 1L plastic spray bottle. The formulations were used within three hours of being prepared. Two and a half (2.5) ml of the suspension was sprayed onto a clear glass vial to see if the conidia could pass through the spray nozzle and wet

mounts were made from this suspension and viewed under the microscope to confirm the presence of conidia. The viability of conidia was determined by spread plating 0.1 ml of the conidial suspension with a concentration of 1 x 10^6 conidia/ml on SDYA media plates and a sterile microscope cover slip was placed at the centre of each plate, the experiment was replicated 4 times. Plates were incubated at 26 ^{o} C for a 16-hour incubation period. After the incubation period, plates were examined under a light microscope at x40 magnification. The percentage germination was determined from 200 spores for each plate (Spores were considered germinated and viable if the germination tube was double the size of the spore.

Field application

The day before the field application of the treatments, nests were marked with different colours, to identify which nest in a location will be getting which treatments. Most of the nests were parasitized by a parasitic fly from the genus *Anacamptomyia* (Tachinidae) and this was shown by the brown pupae (Figure 9) that occupy some of the cells in the wasp nests. This parasitic fly was also observed by Benade (2014) and Van Zyl (2016).

On the day of the field application, the nests were treated with the respective treatments, namely, the control with just water plus 0.1% Tween80, the BB4822 formulation and the commercial strain Eco-Bb®. Nests of *P. dominula* were sprayed until all cells were saturated with the suspension, this was done in the morning while the workers were still on the nests and inactive. Medium-sized nests were used for this treatment and they had an average ranging from 150 -300 cells. Only nests which were confirmed to have at least 15 larvae were used. After application, the nests were left in the field for three days before any of the nests were taken down for analysis.

Each treatment had twelve nests for the both sites combined (Table 5). Four nests per treatment were harvested on each of the following days: day 3, 6 and 9. Site effects were not taken into consideration because *P. dominula* search for micro-climatically perfect sites for nesting, therefore, it was assumed that nest conditions will be similar in both sites (Höcherl and Tautz, 2015). After the treated nests were harvested from the sites; to standardize number of individuals for analysis, the total number of mycosed or dead larvae would be compared to the total number of live larvae. After the trial period (9 days) was over, the nests, including nests

with fly pupae were incubated at room temperature for 5 extra days to observe if there would be visual mycosis.

4.3 Statistical analysis

Statistical analysis is about decision making based on a given data set, and they are often used to analyse the information given, to determine the trends, to suggest predictions and to draw conclusions (Bang *et al*., 2010).

ANOVA

The aim of this section was to determine whether there were statistically significant differences between the means of dead larvae from the 5 concentrations of the laboratory data. A common method to achieve this objective in statistics is the use of Analysis of Variance (ANOVA), which is a collection of statistical models to analyse the differences among group means.

There are several types of ANOVA, but the one-way ANOVA model was used in this study because we were testing for a significant difference among means of more than two groups.

A one-way ANOVA was used to compare the mean percentage mortality of *P. dominula* larvae, 3 days after application of the respective concentrations, a paired sample t-test was performed as a post-hoc test to compare the means between two treatments that were compared to each other.

The mean radial growth rate of the fungal isolate after exposure to different temperatures were compared by a single factor ANOVA. A paired t-test was performed to compare the mean error between two temperatures. A correlation analysis was performed in Microsoft Excel to determine if there was a correlation between temperature and fungal growth.

4.4 Assessment of factors that could affect fungi in the field

Entomopathogenic fungi (EPFs), in particular *B. bassiana*, have been extensively studied and have been developed as alternatives to chemical pesticides for the control of a wide variety of insects, since they have demonstrated to have a wide range of hosts (Acharya *et al*., 2015; Hill *et al*., 2015; Lacey *et al*., 2011; Latifian, 2014; Luz & Fargues, 1998; Sedehi *et al.*, 2014; Wraight *et al*., 2000). Though EPFs are potential alternatives to chemical pesticides, there are

a number of biotic and abiotic factors that may affect the efficiency of these EPFs. These factors have an impact on the survival of the pathogen and on its ability to infect its target host (Nussenbaum *et al*., 2013). The following section will examine the different factors that may affect the survival of the control agent.

4.4.1 Effects of temperature on fungal growth

It has been established in literature that abiotic factors play a significant role in the success of fungal agents (Ahmad *et al.,* 2016). Among several abiotic factors including: humidity, temperature and UV radiation, Ahmad *et al.* (2016) identified temperature as the most important environmental factor that affects the efficacy of EPFs. In this study, we examined the effect of temperature on the growth of the fungal isolate (Bb4822), which was isolated from a *P. dominula* nest. The effect of temperature on the vegetative growth of the fungi was assessed by inoculating the centre of 95 mm petri dishes, containing modified Sabouraud dextrose agar supplemented with 1 % yeast extract (SDYA) media (Inglis *et al*., 2012), with a 6 mm diameter mycelial agar plug taken from the margin of a week-old actively growing non-sporulating culture. Cultures were incubated at 4 ˚C, 20 ˚C, 26 ˚C, 30 ˚C, 37 ˚C and 40 ˚C. Three replicates were prepared for each temperature and incubated for a week to obtain maximum growth. Colony growth was measured daily (mm/day). The colony radial growth was calculated by measuring two perpendicular diameters of each plate and initial diameter of 6 mm from the inoculation plug was subtracted.

A preliminary test was done by centrally inoculating plates with the fungal culture and incubating at temperatures 4 ˚C, 20 ˚C, 30 ˚C, 37 ˚C and 40 ˚C. After 1 day of incubation, at the respective temperatures, all the plates were moved to 26 ˚C, to determine if the fungal spores were able to germinate after previously being exposed to temperatures that are not optimal for growth.

4.4.2 Effects of other microorganisms on fungal growth

The presence of other microorganisms may also influence the efficacy of the entomopathogen. Some hosts release chemicals that could potentially protect them from infections, while other hosts form symbiotic associations with microorganisms which can help them fight off infections or prevent infections (Madden *et al*., 2012). In this study, the *P. dominula* nest

material was tested to determine if it contained any microbes that may have inhibitory effects on the growth of Bb4822.

A 1 ml suspension of Bb4822 was spread plated on PDA media and after the surface of the plate had dried, holes with a diameter of 6mm were made in the media by using a cork borer. A homogenate of nest material was prepared by breaking 1 g of nest material into small pieces with sterile forceps in a test tube with 10 ml sterile distilled water, the mix was vortexed and then inoculated on to the plates by pipetting 1ml of homogenate in the plug holes. Dead wasp material, nest material containing honey were inoculated on the media containing Bb4822, each plate was replicated three times. 1 ml of sterile distilled water with 0.1% Tween 80 was used as a control. All the plates were then incubated at 26 ˚C in the dark for 7 days and observed to determine if there were any inhibition zones.

After incubation period was over, the plates with inhibition zones were viewed under the microscope and there was bacterial growth observed on the edge of the holes. And where ever bacterial growth was observed, the fungi was inhibited, this was signified by a clearing zone.

5 mm of the clearing zones were cut out and inoculated on fresh media to determine whether the inhibitors are fungistatic, or fungicidal, which means that they suppress the growth of fungi or fungicidal, which means that they kill the fungus. This was done by inoculating fresh MEA plates with the 5 mm agar inoculum, cut from the inhibition zone. Plates were incubated at 26 ˚C for 2 days and plates were examined for growth.

Each inhibition plate had a number of different bacterial colonies growing together, therefore, plates were purified by using the streak plate method, where each distinct bacterial colony was transferred on to fresh MEA media to obtain pure cultures, the plates were then incubated overnight at 26 ˚C. On the following day, DNA was extracted from the pure colony plates using the Bacterial/Fungal DNA extraction kit by ZymoResearch and a similar molecular identification procedure as detailed in chapter 3 of this study was followed to amplify the 16S rRNA gene, which was used to identify bacterial species.

4.5 Results

Effect of concentration on larval mortality under laboratory conditions

Analysing different concentrations of *B. bassiana* shows that there is a considerable difference in its effect on *P. dominula* larvae (Fig.12).

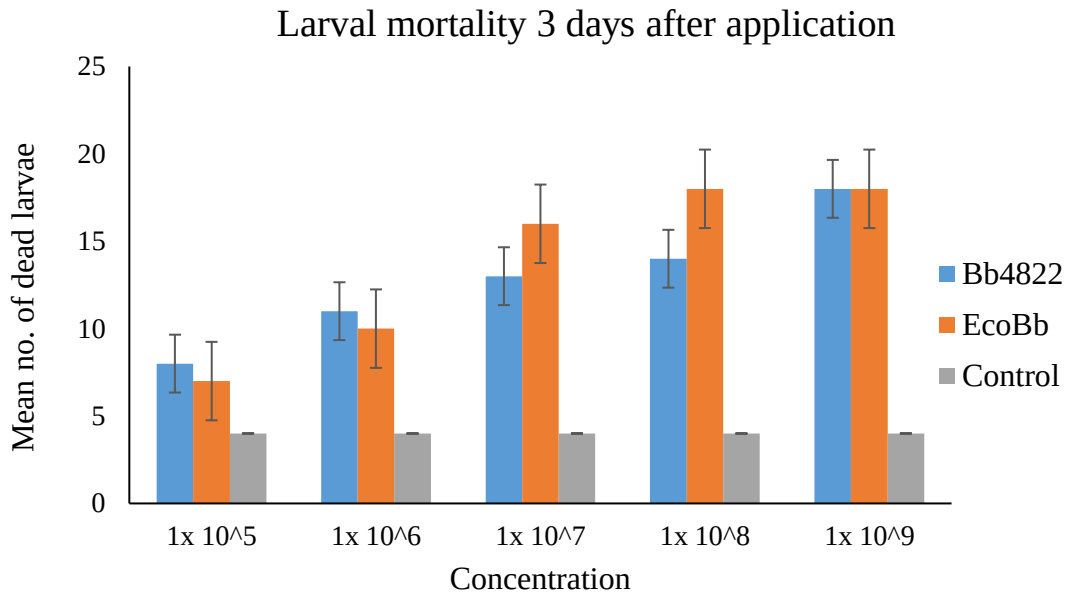

Figure 12. Concentration plotted against mean of dead larvae 3 days after exposure to EcoBb and Bb4822. A control group treated with water was added. Bars represent standard error.

ANOVA

According to the test result, The P value= 0.002, F = 11.213, and with a critical value of 0.05, the F- critical = 3.885. Therefore, since the F is greater than the F-critical value, this result shows that at least one of the treatments was significantly different from the others, which means that the mean number of dead larvae were significantly different between concentration. To determine if the ANOVA analysis perform was valid, a follow up test for equal variances was performed. This variance analysis assumption was done by finding the ratio of the largest

(25.2) to smallest (13.7) variance, which resulted to the value of 1.839 and because this value was less than/ close to 2, the one-way ANOVA was proven to be viable.

A t-test was performed to compare each of the treatments to one another and the values were p=0.39, p= 0.006, and p= 0.01 for Bb4822/EcoBb, Bb4822/control and EcoBb/control, respectively.

Table 6. ANOVA analysis for the assessment of different fungal spore concentrations on larval mortality.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	290.8	2	145.4	11.21337	0.001794	3.885294
Within Groups	155.6	12	12.96667			
Total	446.4	14				

ANOVA results from the two treatments, namely Bb4822 and Eco-Bb ®, were not significantly different from each other, p =0.729, $F_{(1, 8)} = 0.129$. There was a significant correlation between the concentration of fungi and mortality of larvae treated with Bb4822 (r = 0.98, p = 0. 00052), and larvae treated with Eco-Bb® (r =0. 99, p = 0.00012). The mean total of dead larvae increased as concentration increased. The LC_{50} value of treatment Bb4822 was calculated as 1 x 10^6 conidia/ml, which is similar to that of the commercial Eco-Bb®. The mean larval mortality in the control group was 33 %, with the lowest concentration (1 x10^5conidia/ml) larvae mortality caused by Bb4822 and Eco-Bb® 44 % and 38 %, respectively. In contrast, at the highest concentration (1 x 10^9 conidia/ml), mortality reached 100 % for both Bb4822 and Eco-Bb®.

Field trial

No larvae or adult from the field treated nests was observed to have fungal growth, the number of queens and works in the treated nests were observed to be the same throughout the field trial The field trials results showed that there were no dead or mycosed larvae from any of the treated nests that were collected from the field. In addition, the activity (number of adults) in the nests was observed to be the same throughout the trial. The nests were parasitized by fly pupae (Figure 13A), which was also recorded by (Benade, 2015; Benade *et al*., 2014; Van Zyl, 2016)The fly was identified as *Anacamptomyia* sp. from the family Tachinidae, which also parasitizes nests of native *Polistes marginalis* (Benade *et al*., 2014).

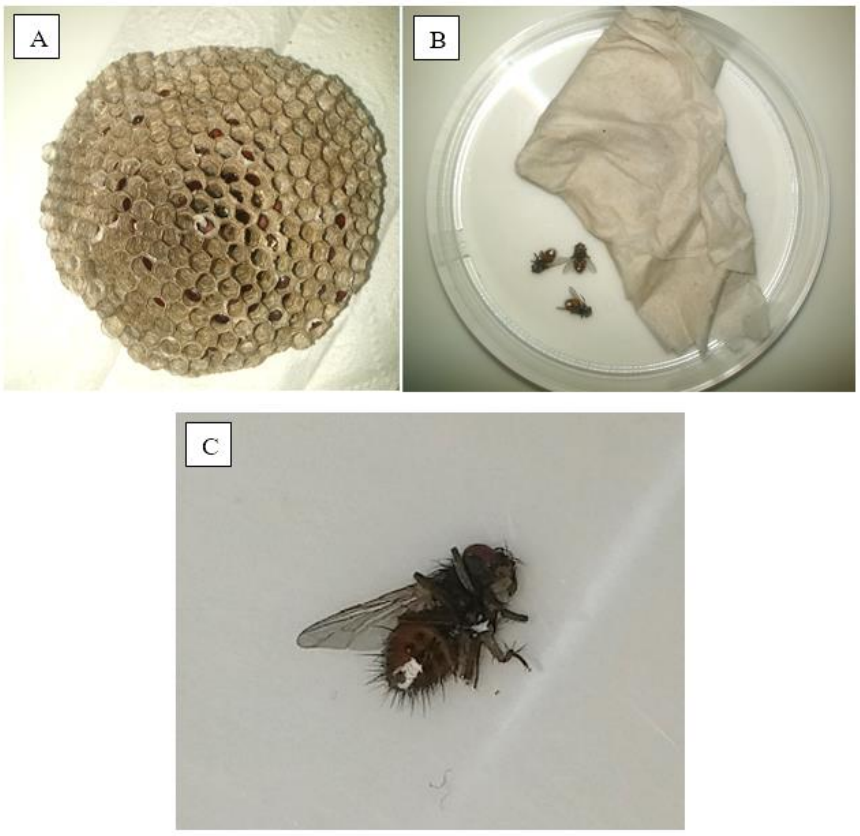

Figure 13. Field trial photos A) Wasp nest collected 7 days after application, nest is heavily parasitised with fly pupae, B) Flies that emerged in the lab after 11 days of exposure, C) A close up on the mycosed fly.

After the extra 5 days, adult flies emerged from the pupae in the nests and the wasp larvae still showed no sign of infection from the fungus. Conversely, it was interesting to note that the emerged adult flies had white spots on them and after a close inspection under a stereo microscope, the white spots on the flies were found to be *B. bassiana* spores (Figure 13 B and C).

4.5.1 Effect of temperature on fungal growth

The influence of temperature was examined to determine the optimal growth temperature. Fungal isolate Bb4822 was incubated at different temperatures for one week (Fig. 15). All replicate plates were able to grow at the temperatures ranging from 20 ˚C - 30 ˚C. The optimum temperature for mycelia growth was observed at 26 ˚C, with a mean diameter of 3.05 mm/day and the slowest growth was observed at 20 ˚C with 1.14 mm/day. At 4˚C, 37 ˚C and 40 ˚C, none of the replicates were able to grow. Radial growth rate (mm/day) for the isolate Bb4822 was calculated under experimental temperatures as means of the three replicates (Fig 14). It was also noted that all cultures that were incubated at 30 ˚C sporulated profusely.

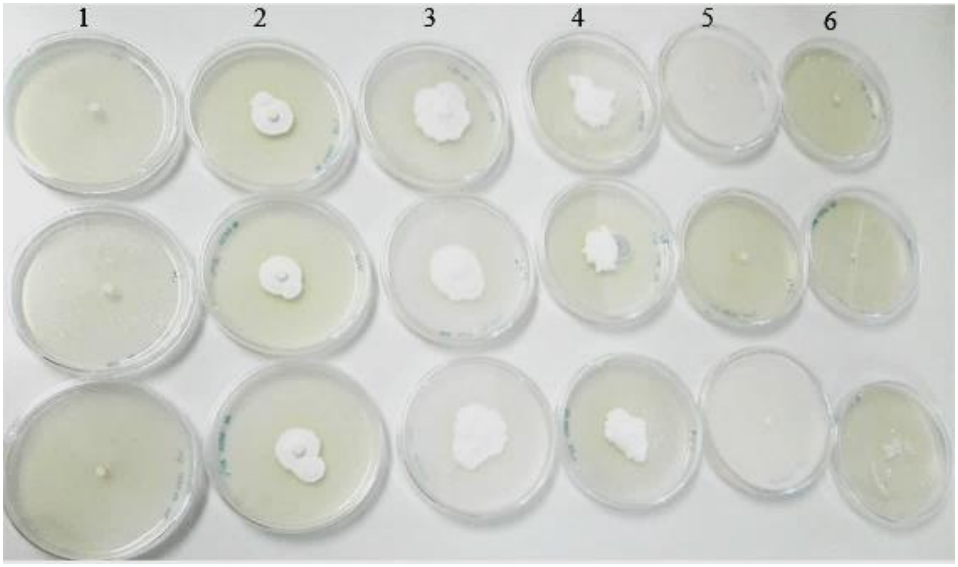

Figure 14. Fungal plates of Bb4822 incubated at six different temperatures. 1) 4˚C, 2) 30˚C, 3) 26˚C, 4) 20˚C, 5) 37˚C and 6) 40˚C

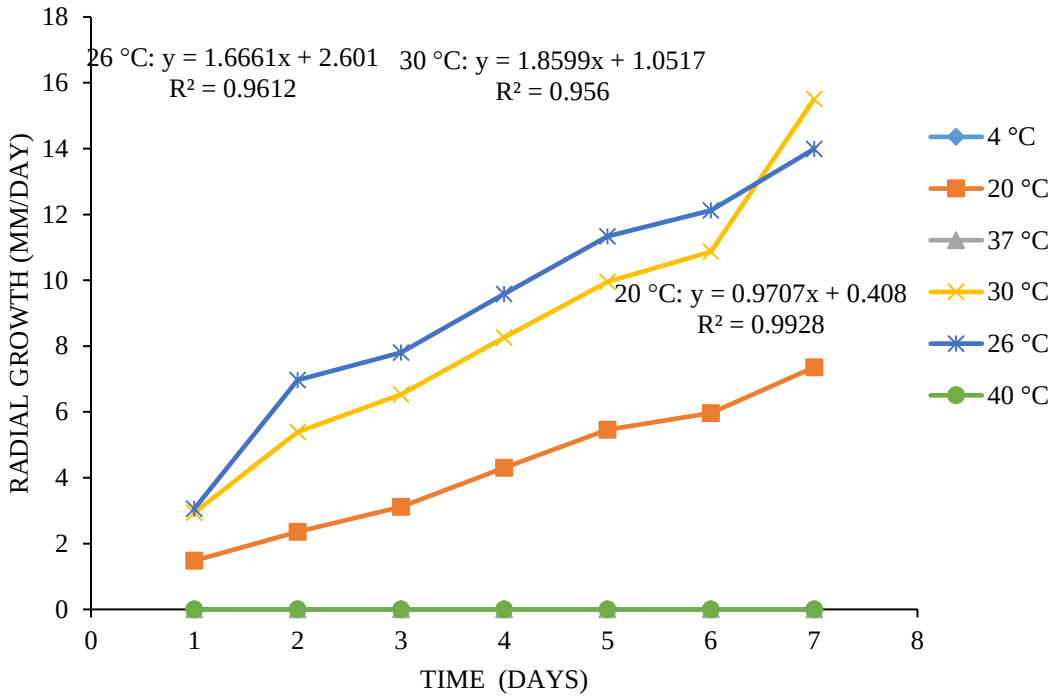

Figure 15. Mean radial growth rates (mm/day) of fungal isolate during 7 days incubation at temperatures 4, 20, 26,30, 37 and 40 °C.

Plates were also incubated at the respective temperatures for 1 day, removed and incubated at the optimal temperature (26 ˚C) to determine if they will recover. Plates that were incubated at 20 ˚C and 30 ˚C for one day were able to grow faster at optimal temperature, however, plates that were previously incubated at 4 ˚C, 37 ˚C and 40 ˚C, then taken to 26 ˚C after one day, were not able to recover, and no growth was observed.

Fungal isolate Bb4822 was incubated at different temperatures for one week (Fig. 14). Growth occurred at only three of the six temperatures 20 ˚C - 30 ˚C. The fastest growth rate was observed at 26 ˚C, with a mean diameter of 4.3 mm/day and the slowest growth was observed at 20 ˚C. At 4˚C, 37 ˚C and 40 ˚C, no growth was observed (Fig 15). ANOVA analysis showed that there was at least one group that was significantly different from each other (P-value =2.65 x 10 $^{-10}$, F= 23.01, F critical =2.47) (Table. 7). It was also noted that all cultures that were incubated at 30 ˚C sporulated profusely. Plates were also incubated at the respective

temperatures for 1 day, removed and incubated at the optimal temperature (26 ˚C) to determine if they will recover. Plates that were incubated at 20 ˚C and 30 ˚C for one day were able to grow faster at optimal temperature, however, plates that were previously incubated at 4 ˚C, 37 ˚C and 40 ˚C, then taken to 26 ˚C after one day, were not able to recover, and no growth was observed. The results from the correlation test resulted with a correlation value of -0.05, this showed that there was no statistical correlation between temperature and fungal growth.

Table 7. ANOVA analysis for the assessment of measurement of error for the mean radial growth

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	667.4186	5	133.4837	23.01998	2.65×10^{-10}	2.477169
Within Groups	208.7497	36	5.798603			
Total	876.1683	41				

4.5.2 Effect of other microorganisms on fungal growth

Fungal inhibition was observed, indicated by a clear zone. It was noted that plates with clear zones were plates with nest material (Fig. 16 A-D). Whereas, plates with wasp material did not demonstrate any inhibition as the wasp body was completely covered in fungi (Fig.16E), this is also seen in Figure 16B, where one side is nest material and the other is wasp material. The part with nest material had a small inhibition zone while wasp material was completely covered. The plates with the control were completely covered as no inhibition was observed in all the replicates. The inhibition zones were cut out and plated on clean agar, after incubation, no growth was observed (Figure 16F).

The organisms responsible for the inhibition were isolated and sub-cultured using the streak plate method with a sterile inoculation loop on malt extract agar (MEA) and incubated at 26 degrees Celsius in the dark for 24-48 hours. After the incubation period, plates were re-cultured

again to obtain pure cultures. Once the pure culture was obtain, wet mount slides were made and examined under a light microscope for identification. Based on morphology, isolates were characteristic of bacteria. Seven bacterial species were isolated and identified using morphological identification and sequencing to confirm the identifications. Table 8 shows that the exact species that were isolated and identified from the inhibition zones. Species from the genus *Bacillus* were the most common in this study.

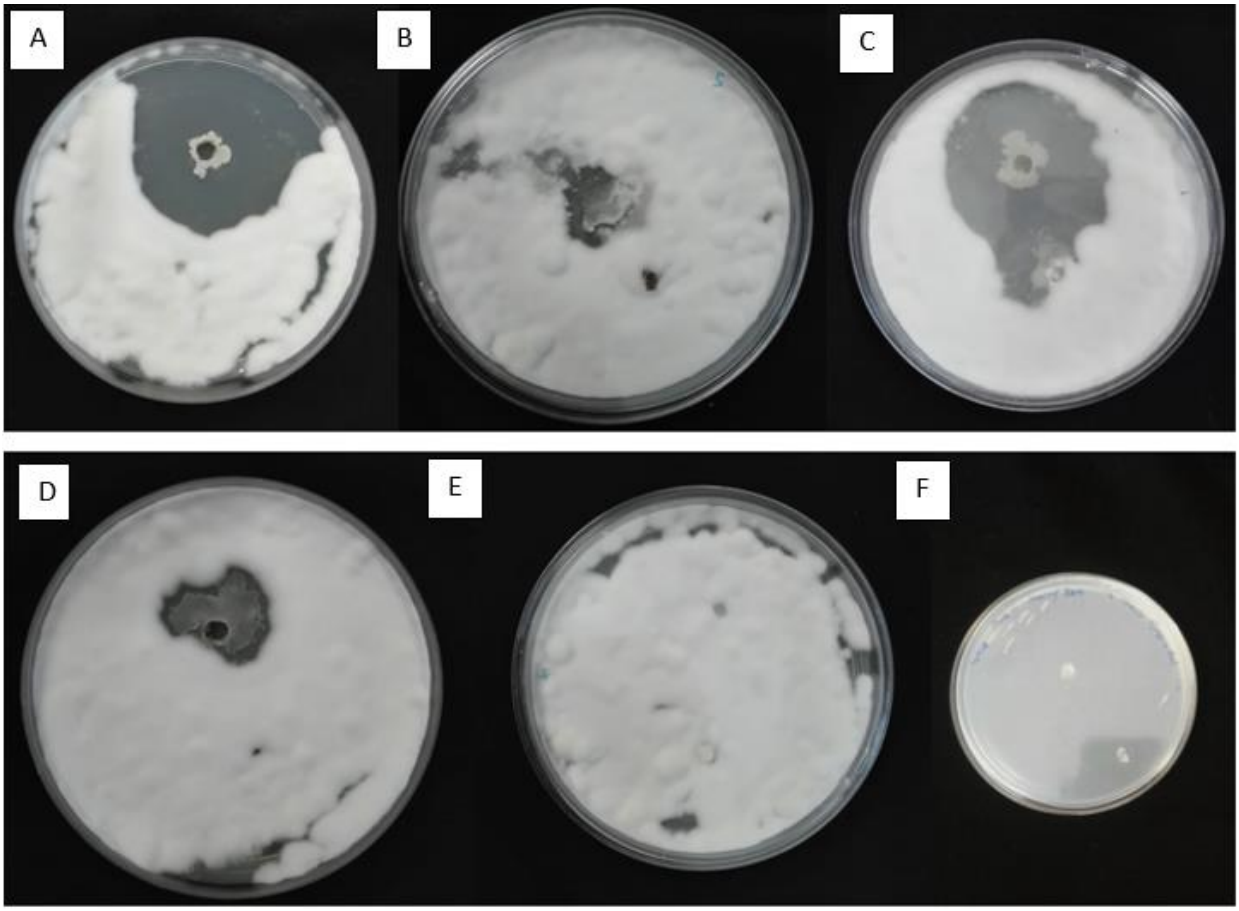

Figure 16. Spread plates of Bb4822 inoculated with A) nest material, B) wasp and nest material, C) nest material, D) nest material with honey substance, E) wasp material only and F) fungicidal inhibition zone

Table 8: A list of bacterial species isolated from inhibition plates

***Bacteria isolated from nest showing inhibitory effect**
Bacillus subtilis
Bacillus velezensis
Paenibacillus polymyxa
Bacillus pumilus
Bacillus cereus
Enterobacter cloacae

* The 16S rRNA gene was used to identify the bacterial species.

4.6 Discussion

The field isolated Bb4822 was formulated for field trials, the lethal concentration (LC50) was determined by testing different concentrations of the agent on 2nd instar larvae under laboratory conditions. The results showed that LC50 values of Bb4822 and the commercial strain Eco-Bb® were both 1 x 10^6 conidia/ml. This value is in accordance with similar studies, which found that the LC50 values of *B. bassiana* ranged from 1.98 x 10^5 conidia/ml to 2.16 x 10^6 conidia/ml (Coombes *et al*., 2015; Goble *et al*., 2011; Latifian, 2014). It means that of the 5 concentrations tested, 1 x 10^6 conidia per ml was able to kill 50 % of the population 3 days after application. A one-way ANOVA was performed to compare the mean number of larvae which died at each treatment, 3 days after exposure. The results from the ANOVA gave a p=0.002, a t-test showed that there was no significant difference between Bb4822 and EcoBb (p=0.39), however there was a significant difference when each treatment was compared to the control group as shown in the results section. For both isolates, virulence was directly proportional to concentration, which showed that the higher the concentration, the more virulence observed.

From the field trial results, there was no observable infection of larvae. These results are slightly different from the results obtained by a similar study by Van Zyl (2016), there was

very low infection rate, where the commercial strain EcoBb only caused 12% -14% mortality. Social insects are capable of hygienic behaviour, where that remove any infected individual from the nest to avoid the spread of the contaminant to the rest of the nest mates (Manfredini *et al*., 2013). A possible reason why Van Zyl could have observed infection was that, nests were only left in the field for 24 hours, this period is too short for the fungus to cause visible infection. However, in this current study, nests were left in the field for a minimum of three days, which give the fungus enough time to cause notable infection, as the laboratory trials in chapter 3 showed that 3 days after application, some of the larvae were covered with fungal mycelia. Therefore, because the nests were in the field longer, wasps could have removed infected individuals. In the Van Zyl (2016) study, while the treated nests were in the field, they were constantly kept moist by spraying water every two hours, to make the conditions more favourable for germination. In this study, nests were left in the field for the duration of the trial and the natural field conditions were not altered.

The nests that were in the field the longest, also did not show evidence of fungal infection. However, an interesting observation was made in a nest parasitized by fly pupae. When the treated nest material containing fly pupae were incubated at room temperature in moist conditions, the adult flies emerged and though some were alive, they were not as active. On close inspection under a microscope, the adult flies were examined and most of them were infected by the fungus which had started sporulating from the least sclerotized parts of their bodies. This finding disagrees with the results of Van Zyl, (2016), who stated that the emerging flies from *P. dominula* nest were not affected by the biocontrol agents applied to the nests. However, more research is needed to understand this disparity, as the pathogenicity of Bb4822 would need to be tested against this species of fly to understand the findings of this study. Van Zyl's study was testing the commercially produce strain of *B. bassiana,* and in this study, it was found that both the field isolated strain and the commercial strain, did not affect *P. dominula* larvae under field conditions.

Temperature is one of the most important abiotic factors that affect fungal growth among others, such as humidity and sunlight (UV irradiation) (Bugeme *et al*., 2009; Marin *et al.*, 1995; Wraight *et al*., 2007). In this study, it was found that the field isolated *B. bassiana* Bb4822 has

an optimal temperature of 26 ˚C with an upper limit of 30 ˚C. These results are supported by similar studies that found the optimal temperature of *B. bassiana* to be between 26 ˚C and 30 ˚C (Ahmad *et al*., 2016; Mwamburi *et al*., 2015). Walstad *et al*. (1970) reported that *B. bassiana* grew well at temperatures between 15 ˚C – 35 ˚C, while Hallsworth and Magan (1999) showed that the temperature required for the growth of *B. bassiana* was between 5˚C – 30 ˚C with an optimum temperature of 25 ˚C. Other studies had demonstrated that temperatures above 27 ˚C -30˚C inhibited fungal growth and killed the spores (Kessler *et al*., 2003; Cheong, 2015). Many studies that have investigated the effect of temperature on the growth of *B. bassiana* have shown varying results, this could be due to the vast genetic diversity in *B. bassiana* populations (Meyling & Eilenberg, 2007). This variation in growth temperature could also be due to the environment where the fungi was isolated from, as Inglis *et al*. (2001) stated that fungi isolated from warm areas may be able to tolerate higher temperatures, or conversely if it comes from cold areas, it may perform better at colder temperatures. Therefore, the results of this study show the optimum growth temperature ranges of the isolate Bb4822, this information is valuable because the thermotolerance of an entomopathogenic fungi is regarded as the major factor to their industrialization because they are used in different environments.

It was found that fungi were not capable of growing at 4 ˚C and at temperatures above 37 ˚C. The correlation analysis showed a value of -0.05, because the value was negative it was concluded that there is no correlation between temperature and growth of fungi. ANOVA analysis revealed that there was a significant difference between mean radial growth at different temperatures ($P= 2.65 \times 10^{-10}$). The t-test comparisons showed that there was no significant difference between the 26 and 30 ˚C (P= 0.115), while there was a significant difference between 20 and 26 and also between 20 and 30 ˚C, P=0.0002 and P= 0.001, respectively.

Rose, *et al*. (1999) conducted a study in search of possible pathogens of social wasps and found *Bacillus* sp. from the gut of *Vespula* larvae. In this study, bacterial species were isolated from the nest material because the wasps spend all their time as larvae in the nest, releasing the content of their gut onto the nest material which may contain bacteria. *Bacillus cereus, B. subtilis, B.velezensis, B. pumilus, Paenibacillus polymyxa* and *Enterobacter cloacae* were isolated from the nest of *P. dominula*. *Bacillus* species are often insect pathogens, however, in

this study when tested against Bb4822, these bacterial strains were found to have inhibitory effects on the growth of the fungus. Though the bacteria were acting as fungal antagonists, this could be beneficial to the wasp, as it prevents the fungal pathogen from germinating, this however, does not change the fact that *Bacillus* spp. could still be pathogenic to the wasps. Therefore, further studies testing the relationship between these bacterial species and the wasps are necessary.

Two of the bacteria isolated from nest material in this study, namely *B. pumilus* and *B. subtilis* had been previously used by Sicuia *et al*. (2014) in in integrated pest management (IPM) programs to control plant fungal pathogens. Which suggests that the bacterial species identified in this study could have reduced the effectiveness of the fungi in the field. In their study Sicuia *et al*. (2014) found that *B. pumilus* and *B. subtilis* inhibit the growth *B. bassiana*. Which explained by Bb4822 was not able to establish in the field in this study. This confirms why *B. pumilus* and *B. subtilis* inhibited the growth of Bb4822.These findings are important in IPM programs where more than one biocontrol agent is used. Therefore, it is pertinent to test the compatibility between beneficial microorganisms, such as testing for compatibility between entomopathogenic bacteria and entomopathogenic fungi, in order to prevent either of them from inhibiting the growth of the other.

Mechanical factors including the spraying method and the spraying distance could have limited the possibility of the fungi from establishing in the field as Inglis *et al*. (2012) had identified that mechanical shearing forces of the spraying tool could affect the viability and virulence of the fungal pathogen. Other factors such as weather conditions including ambient temperature, wind, and humidity; as well as the physical factors like *P.dominula* hygienic behaviour could also limit the establishment of the fungi in the field.

The nest paper material is hydrophobic, due to the proteins found in wasp saliva which is used during nest construction (Espelie & Himmelsbach, 1990). This hydrophobicity could have resulted in poor adhesion of the water-based fungal agent to nests. To increase adhesion, to limit desiccation and to lower solar radiation additives such as oil and UV protectants could be incorporated in the biological control formulation (Batta, 2016).

In this study, there was no infection observed from *P. dominula* larvae treated with the indigenous *B. bassiana* isolate and the commercial *B. bassiana* product under field conditions.

The aim of this study was to test the efficacy of isolate Bb4822 under field conditions and to compare its performance to the commercially produced *B. bassiana* strain. Though no infection by both treatments was observed in the field, it can be concluded that though under controlled laboratory conditions, the fungal pathogen performed well, in the field there are several biotic and abiotic factors that need to be considered when applying a biological control. Therefore, more research needs to focus on optimising field applications of fungal biological control on wasp nests, and more research is required to determine how the larvae and adult *P. dominula* protect themselves from pathogens in the field.

References

Abbott, W. S. (1925). The Value of the Dry Substitutes for Liquid Lime. *Journal of Economic Entomology*, 18, 265–267.

Acharya, N., Seliga, R. A., Rajotte, E. G., Jenkins, N. E., & Thomas, M. B. (2015). Persistence and efficacy of a *Beauveria bassiana* biopesticide against the house fly, *Musca domestica*, on typical structural substrates of poultry houses. *Biocontrol Science and Technology*, 25(6), 697–715.

Ahmad, M. A., Ghazal, I., & Rajab, L. H. (2016). Laboratory evaluation of the effect of temperature and several media on the radial growth, conidia production and germination of the fungus *Beauveria bassiana* (Bals .) Vuil, 3(6), 39–46.

Batta, Y. A. 2016. "Invert Emulsion: Method of preparation and application as proper formulation of entomopathogenic fungi." *MethodsX* 3: 119–27.

Beggs, J., Beggs, J. R., Brockerhoff, G., Corley, J. C., Masciocchi, M., Muller, F., Villemant, C. (2011). Ecological effects and management of invasive ecological effects and management of invasive alien Vespidae. Published thesis. South Africa: Stellenbosch University.

Beggs, J. R., Brockerhoff, E. G., Corley, J. C., Kenis, M., Masciocchi, M., Muller, F., … Villemant, C. (2011). Ecological effects and management of invasive alien Vespidae. *BioControl*, 56(4), 505–526.

Beggs, J. R., Rees, J. S., Toft, R. J., Dennis, T. E., & Barlow, N. D. (2008). Evaluating the impact of a biological control parasitoid on invasive Vespula wasps in a natural forest ecosystem. *Biological Control*, 44(3), 399–407.

Benade, P. C. (2015). Invaded range and competitive ability of the newly invasive *Polistes dominula* compared to that of its native congener species in the Western Cape, South Africa. Published thesis. South Africa: Stellenbosch University.

Benade, P. C., Veldtman, R., Samways, M. J., & Roets, F. (2014). Rapid range expansion of the invasive wasp *Polistes dominula* (Hymenoptera: Vespidae: Polistinae) and first record of parasitoids on this species and the native Polistes marginalis in the Western Cape

Province of South Africa. African Entomology, 22(1), 220–225.

Bugeme, D. M., Maniania, N. K., Knapp, M., & Boga, H. I. (2009). Effect of temperature on virulence of *Beauveria bassiana* and *Metarhizium anisopliae* isolates to *Tetranychus evansi*. *Diseases of Mites and Ticks*, 275–285.

Butt, T.M., Jackson, N. & Magan, C. 2001. "Fungi as biocontrol agents, progress, problems and potential." CABI publishing, UK, pp. 390.

Cheong, P. (2015). Bioactive metabolites of an isolate of entomopathogenic fungus *Beauveria bassiana.* Published thesis. Lincoln University: NewZealand.

Coombes, C. A., Hill, M. P., Moore, S. D., Dames, J. F., & Fullard, T. (2015). *Beauveria* and *Metarhizium* against false codling moth (Lepidoptera : Tortricidae): A step towards selecting isolates for potential development of a mycoinsecticide, 23(1), 239–242.

Eardley, C., Koch, F., & Wood, A. R. (2009). *Polistes dominulus* (Christ, 1791) (Hymenoptera: Polistinae: Vespidae) newly recorded from South Africa. *African entomology*, 17(2), 226–227.

Edwards, E., Toft, R., Joice, N., & Westbrooke, I. (2017). The efficacy of Vespex® wasp bait to control Vespula species (Hymenoptera: Vespidae) in New Zealand. International Journal of Pest Management, 63(3), 266–272.

Espelie, K. E., and Himmelsbach, D. S. 1990. Characterization of pedicel, paper, and larval silk from nest of *Polistes annularis* (L.). *Journal of Chemical Ecology* 16(12): 3467–77.

Fallis, A. . (2013). Options for biological control of vespula wasps in New Zealand. *Journal of Chemical Information and Modeling*, 53(9), 1689–1699.

Giliomee, J. H. (2011). Recent establishment of many alien insects in South Africa - a cause for concern. *African Entomology*, 19(1), 151–155.

Glare, T. R., O'Callaghan, M., & Wigley, P. J. (1993). Checklist of naturally occurring entomopathogenic microbes and nematodes in New Zealand. *New Zealand Journal of Zoology*, 20(2), 95–120.

Goble, T. A., Dames, J. F., Hill, M. P., & Moore, S. D. (2011). Investigation of native isolates of entomopathogenic fungi for the biological control of three citrus pests. *Biocontrol Science and Technology*, 21(10), 1193–1211.

Goettel, M. S., Inglis, G. D. & Wraight, S. P. (2000) "Fungi. field manual of techniques in invertebrate pathology. L. A. Lacey and H. K. Kaya", Eds. Kluwer Academic Publishers, Netherlands. pp. 255-282.

Hallsworth, J. E. & Magan, N. (1999). Water and temperature relations of growth of the entomopathogenic fungi *Beauvaria bassiana*, *Meterhizium anisopliae* and *Paecilomyces farinosus*. *J. Invertebrate Pathology*, 74: 261-266.

Harris, R. J., Harcourt, S. J., Glare, T. R., Rose, E. A. F., & Nelson, T. J. (2000). Susceptibility of *Vespula vulgaris* (Hymenoptera: Vespidae) to generalist entomopathogenic fungi and their potential for wasp control. *Journal of Invertebrate Pathology*, 75(4), 251–258.

Haupt, K. (2015). Assessment of the invasive German wasp, *Vespula germanica*, in South Africa. Published thesis. South Africa: Stellenbosch University.

Höcherl, N., Kennedy, S., & Tautz, J. (2016). Nest thermoregulation of the paper wasp *Polistes dominula*. *Journal of Thermal Biology*, 60, 171–179.

Höcherl, N., Tautz, J., (2015) Nesting behavior of the paper wasp *Polistes dominula* in Central Europe – a flexible system for expanding into new areas. *Ecosphere* 6, 262.

Inglis, G. D., Enkerli, J., & Goettel, M. S. (2012). *Laboratory techniques used for entomopathogenic fungi. Hypocreales. Manual of Techniques in Invertebrate Pathology* (Second Edi). Elsevier Ltd.

Kessler, P., Matzke, H. & Keller, S. (2003) "The effect of application time and soil factors on the occurrence of *Beauveria brongniartii* applied as a biological control agent in soil". *Journal of Invertebrate Pathology*. 84, 15–23.

Lacey, L. A., Frutos, R., Kaya, H. K., & Vail, P. (2001). Insect pathogens as biological control Agents : Do They Have a Future ? Biological Control, 248, 230–248.

Lacey, L. A., Liu, T. X., Buchman, J. L., Munyaneza, J. E., Goolsby, J. A., & Horton, D. R. (2011). Entomopathogenic fungi (Hypocreales) for control of potato psyllid, *Bactericera cockerelli* (Šulc) (Hemiptera: Triozidae) in an area endemic for zebra chip disease of potato. *Biological Control*, 56(3), 271–278.

Latifian, M. (2014). Study the pathogenecity of fungus *Beauveria bassiana*, *Beauveria brongniartii* and *Metarhizium anisopliae* on date horned beetle Oryctes elegans Prell larvae based on different bioassay methods, 2, 31–37.

Luz, C., & Fargues, J. (1998). Factors affecting conidial production of *Beauveria bassiana* from fungus-killed cadavers of *Rhodnius prolixus*. *Journal of invertebrate pathology*, 72(72), 97–103.

Marin, S., Sanchis, V., & Magan, N. (1995). Water activity, temperature, and pH effects on growth of *Fusarium moniliforme* and *Fusarium proliferatum* isolates from maize. *Canadian Journal of Microbiology*, 41(12), 1063–1070.

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

Mwamburi, L. A., Laing, M. D., & Miller, R. M. (2015). Effect of surfactants and temperature on germination and vegetative growth of *Beauveria bassiana*. *Brazilian Journal of Microbiology*, 46(1), 67–74.

Nussenbaum, A. L., Lewylle, M. A., & Lecuona, R. E. (2013). Germination, radial growth and virulence to boll weevil of entomopathogenic fungi at different temperatures. *World Applied Sciences Journal*, 25(8), 1134–1140.

Pal, K. K., & Mc Spadden Gardener, B. (2006). Biological Control of Plant Pathogens. *The Plant Health Instructor*, 1–25.

Rose, E. A. F., Harris, R. J., & Glare, T. R. (1999). Possible pathogens of social wasps (Hymenoptera: Vespidae) and their potential as biological control agents. New Zealand Journal of Zoology, 26(May 2015), 179–190.

Sedehi, A., Sedaghatfar, E., & Modarres Najafabadi, S. S. (2014). Studies on effect of the Beauveria bassiana on eggs and larvae of *Plodia interpunctella*. *Canadian Journal of Basic and Applied Sciences* ©PEARL Publication CJBAS, 2(2), 40–45.

Shahid, A. A., Rao, A. Q., Bakhsh, A., & Husnain, T. (2012). Entomopathogenic fungi as biological controllers: New insights into their virulence and pathogenicity. *Archives of Biological Sciences*, 64(1), 21–42.

Sicuia, O. A., Dinu, S., Dinu, M., Fătu, C., Valimareanu, D., Mincea, C., & Constantinescu, F. (2014). Pests and diseases management using compatible biocontrol bacteria and entomopathogenic fungal strains. *Scientific Bulletin*, 18, 66–72.

Van Zyl, C. (2016). Status of the invasive wasp species, *Vespula germanica* and *Polistes*

dominula in South Africa, and the feasibility of various management strategies. Published thesis. South Africa: Stellenbosch University.

Walstad, J.D., Anderson, R.F. & Stambaugh, W.J. (1970). Effect of environmental conditions on two species of muscardin fungi (*Beauveria bassiana* and *Meterhizium anisopliae*). *Journal of invertebrate pathology*, 2: 221- 226

Wraight, S. P., Carruthers, R. I., Jaronski, S. T., Bradley, C. A., & Garza, C. J. (2000). Evaluation of the entomopathogenic fungi *Beauveria bassiana* and *Paecilomyces fumosoroseus* for microbial control of the silverleaf, *Archives of Biological Sciences*, 217, 203–217.

Wraight, S. P., Inglis, G. D., & Goettel, M. S. (2007). Fungi. In L. A. Lacey & H. K. Kaya (Eds.), *Field Manual of Techniques in Invertebrate Pathology*. Springer. (pp. 223–248).

5. Conclusions and recommendations

The aim of this study was to isolate fungi from wasp nests and soil to test their virulence against *P. dominula* larvae. The results from this study illustrate that there are several fungi associated with *P. dominula* wasp nests and that some of those were entomopathogenic and were virulent towards *P. dominula* larvae under laboratory conditions. Overall, *P. dominula* larvae were more susceptible to infection by *B. bassiana* while *P. lilacinum* seemed to be the least pathogenic.

Under laboratory conditions, a 100 % mortality of *P. dominula* larvae was recorded four days after treatment with *B. bassiana* isolate Bb4822, as it was significantly different from the control. However, in the field trial, there was no visible mortality recorded and this could have been due to the unpredictable field conditions, like temperature, wind, and humidity or mechanical factors such as the shearing forces of the spraying tool which may have affected the viability, virulence of the fungal pathogen and the hygienic behaviour that social wasps demonstrate by removing infected larvae from the nest (Wraight *et al*., 2007).

An interesting result from the study was that, although the treatment did not infect the larvae from the field trials, the adult flies that parasitized *P. dominula* which emerged from the nests treated with fungi were found to be infected with *B. bassiana* spores. This indicated that the fungal isolate was able to establish itself in the nests with parasitic flies, however, the effect on the wasp larvae could not be observed.

This study also showed that the wasp nest material harboured not only fungi but also several *Bacillus* species, which when tested under laboratory conditions, were found to suppress the growth of *B. bassiana*. These bacteria could have affected the success of the fungal pathogen from establishing in the field. It is hereby recommended that future research should focus on determining other factors that could affect the efficacy of *B. bassiana* against *P. dominula* larvae under field conditions. The other recommendation is to optimize the application method to minimize the shear force damage to the fungal spores which may be caused by the application spray.

There is currently no biological control agent that is known to reduce populations of *P. dominula* in South Africa, and the results from this study contribute to the knowledge about *P. dominula*, the fungi associated with it and its susceptibility to entomopathogenic fungi

The results from this study slightly disagree with the findings of Van Zyl, who had earlier reported a 15% infection rate caused by a commercial strain of *B. bassiana* (EcoBb). This deviation could have been as a result of the difference in the number of days the nests were left in the field.

As a final point in this study, it is important to state that many fungi that perform well in the laboratory are less effective in the field (Butt *et al*., 2001), and results obtained in a laboratory may not necessarily reflect the true activity of the fungus. The results from this study confirm that such controlled experiments may also fail to take into consideration the effect of insect behaviour on the efficacy of the fungus and while this current study contributed immensely to the body knowledge, further studies are required.

Future research

Further studies on the formulation of the fungal pathogens are needed to determine a suitable carrier other than the water-based formulation that was used in this study. A suitable carrier will aid fungal spores to adhere to the wasp cuticle, provide the spores with a moist environment long enough for it to start germinating and protect spores from solar radiation.

Furthermore, there is still a need to identify other microorganisms, such as viruses and bacteria that are associated with *P. dominula* in South Africa, as this could identify other possible pathogens besides fungi. A full assessment of other microbial species will also aid in choosing a suitable biological control agent, one that will not be inhibited by other microorganisms that are found in the nest, but an agent that can co-exist with the existing microorganisms.

It would also be interesting for future studies to investigate what protects the larvae from fungal infections in the field as it was noted in this study that the parasitic flies were susceptible to the *B. bassiana* in the field while the wasp larvae were not infected.