Towards the development of a mycoinsecticide to control white grubs (Coleoptera: Scarabaeidae) in South African sugarcane

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ABSTRACT

In the KwaZulu-Natal (KZN) Midlands North region of South Africa, the importance and increased prevalence of endemic scarabaeids, particularly *Hypopholis sommeri* Burmeister and *Schizonycha affinis* Boheman (Coleoptera: Melolonthinae), as soil pests of sugarcane, and a need for their control was established. The development of a mycoinsecticide offers an environmentally friendly alternative to chemical insecticides. The identification of a diversity of white grub species, in two Scarabaeidae subfamilies, representing seven genera were collected in sugarcane as a pest complex. *Hypopholis sommeri* and *S. affinis* were the most prevalent species. The increased seasonal abundances, diversity and highly aggregated nature of these scarabaeid species in summer months, suggested that targeting and control strategies for these pests should be considered in this season. Increased rainfall, relative humidity and soil temperatures were linked to the increased occurrence of scarab adults and neonate grubs.

Beauveria brongniartii (Saccardo) Petch epizootics were recorded at two sites in the KZN Midlands North on *H. sommeri*. Seventeen different fluorescently-labelled microsatellite PCR primers were used to target 78 isolates of *Beauveria* sp. DNA. Microsatellite data resolved two distinct clusters of *Beauveria* isolates which represented the *Beauveria bassiana senso stricto* (Balsamo) Vuillemin and *B. brongniartii* species groups. These groupings were supported by two gene regions, the nuclear ribosomal Internal Transcribed Spacer (ITS) and the nuclear B locus (Bloc) gene of which 23 exemplar *Beauveria* isolates were represented and sequenced. When microsatellite data were analysed, 26 haplotypes among 58 isolates of *B. brongniartii* were distinguished. Relatively low levels of genetic diversity were detected in *B. brongniartii* and isolates were shown to be closely related. There was no genetic differentiation between the two sites, Harden Heights and Canema in the KZN Midlands North. High gene flow from swarming *H. sommeri* beetles is the proposed mechanism for this lack of genetic differentiation between populations. Microsatellite analyses also showed that *B. brongniartii* conidia were being cycled from arboreal to subterranean habitats in the environment by *H. sommeri* beetles. This was the first record of this species of fungus causing epizootics on the larvae and adults of *H. sommeri* in South Africa.

The virulence of 21 isolates of *Beauveria brongniartii* and two isolates of *B. bassiana* were evaluated against the adults and larvae of *S. affinis* and the adults of *H. sommeri* and *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae). Despite being closely-related, *B. brongniartii*

isolates varied significantly in their virulence towards different hosts and highlighted the host specific nature of *B. brongniartii* towards *S. affinis* when compared to *B. bassiana*. Adults of *S. affinis* were significantly more susceptible to *B. brongniartii* isolates than the second (L2) or third instar (L3) grubs. The median lethal time (LT_{50}) of the most virulent *B. brongniartii* isolate (C13) against *S. affinis* adults was 7.8 days and probit analysis estimated a median lethal concentration (LC_{50}) of 4.4×10^{7} conidia/ml^{-1}. When L2 grubs were treated with a concentration of 1.0×10^{8} conidia/ml^{-1}, *B. brongniartii* isolates HHWG1, HHB39A and C17 caused mortality in L2 grubs within 18.4-19.8 days (LT_{50}). *Beauveria brongniartii* isolate HHWG1 was tested against the L3 grubs of *S. affinis* at four different concentrations. At the lowest concentration (1×10^{6} conidia/ml^{-1}), the LT_{50} was 25.8 days, and at the highest concentration (1×10^{9} conidia/ml^{-1}) the LT_{50} dropped to 15.1 days.

The persistence of *B. bassiana* isolate 4222 formulated on rice and wheat bran and buried at eight field sites in the KZN Midlands North was evaluated by plating out a suspension of treated soil onto a selective medium. All eight field sites showed a significant decline in *B. bassiana* CFUs per gram of soil over time, with few conidia still present in the samples after a year. Greater declines in CFUs were observed at some sites but there were no significant differences observed in the persistence of conidia formulated on rice or wheat bran as carriers. Overall, poor persistence of *B. bassiana* isolate 4222 was attributed to suboptimum temperatures, rainfall, which rapidly degraded the nutritive carriers, attenuated fungal genotype and the action of antagonistic soil microbes.

Growers' perceptions of white grubs as pests and the feasibility of a mycoinsecticide market were evaluated by means of a semi-structured questionnaire. The study showed that the reduced feasibility of application, general lack of potential demand for a product, high cost factors and most importantly, the lack of pest perception, were factors which would negatively affect the adoption of a granular mycoinsecticide. Growers however exhibited a positive attitude towards mycoinsecticides, and showed all the relevant attributes for successful technology adoption. It is recommended that because *B. brongniartii* epizootics were recorded on target pests which indicated good host specificity, dispersal ability and persistence of the fungus in the intended environment of application; that a mycoinsecticide based on this fungal species be developed. What will likely increase adoption and success of a mycoinsecticide is collaboration between various industries partners to increases market potential in other crops such as *Acacia mearnsii* De Wild (Fabales: Fabaceae).

To my parents,

Clive and Jen

Thank you for your absolute love of nature

and your absolute nature of love.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PREFACE

Work from this thesis was presented at the following local and international conferences:

Entomological Society of Southern Africa (Bloemfontein) 3-6 July 2011:

Oral: Developing a *Beauveria brongniartii* based mycoinsecticide for the control of white grubs (Scarabaeidae) in KwaZulu-Natal Midlands sugarcane. Goble TA; Conlong DE; Rutherford RS; Hill MP.

45th Meeting of the Society of Invertebrate Pathology (Buenos Aires, Argentina) 5-9 August 2012:

Oral: Determining *Beauveria brongniartii* epizootics on white grubs attacking sugarcane in South Africa using molecular techniques. Goble TA; Costet L; Robene I; Nibouche S; Rutherford RS; Conlong DE; Hill MP.

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Oral: Fungal epizootics on white grub pests in Midlands North sugarcane. Goble TA; Costet L; Robene I; Nibouche S; Rutherford RS; Conlong DE; Hill M.

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Goble TA; Conlong DE; Hill MP. 2012. Susceptibility of *Schizonycha affinis* beetles to native strains of *Beauveria brongniartii* in South Africa. *Proceedings of the South African Sugar Technologists' Association* 85: 129-133.

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Goble TA. 2012. Controlling white grubs using indigenous fungi. *The Link*: *published by the South African Sugarcane Research Institute* 21(2): 10-11

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DECLARATION

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

_____ Date: _____

CHAPTER 1- General Introduction

Understanding of the interactions between the biology and ecology of potential insect pests and their fungal pathogens is critical to the successful development and implementation of mycoinsecticides. Entomopathogenic fungi are effective, natural regulators of insect populations, but their activity is dependent upon environmental conditions which may limit their effective control of target pests. Further variation within and among fungal species is still not well understood making effective strain selection difficult. Elucidating these key environmental and genetic variables can greatly enhance biological control efficacy and the subsequent strategies employed against target pests. Inundative biological control is such a strategy, which employs the intentional mass release of endemic fungal strains for long-term control of endemic pests and usually involves the development of mycoinsecticides (Eilenberg *et al.* 2001a). This thesis describes the occurrence of natural fungal epizootics which have been observed on white grubs (Coleoptera: Scarabaeidae) in the KwaZulu-Natal Midlands North sugarcane producing area of South Africa. Complete natural biological control of these pests has not been achieved nor has it limited their pest status, thus the development of a mycoinsecticide is required for their inundative control. The advances that have been made in the understanding of target pest biology and the environmental factors which govern their occurrence, as well as fungal pathogen genetics, ecology, biology, virulence, host range and persistence, all which contribute towards the successful development of a mycoinsecticide are discussed within this thesis.

1.1 *White grubs as pests of sugarcane*

Sugarcane is grown on over 20 million hectares in over 110 countries which ensures the livelihood of millions of growers in many rural areas (Goebel and Sallam, 2011). Insect pests are among the many constraints to sugarcane production around the world. There are more than 1500 recognised insects, including insect predators and parasites, associated with the cultivated sugarcane plant, *Saccharum officinarum* Linnaeus (Poaceae: Andropogoneae) worldwide (Wade, 1951; Box, 1953; Carnegie and Conlong, 1994). Very few of these insects have cosmopolitan distributions and 75% were listed from sugarcane in only one country or island (Pemberton and Williams, 1969), suggesting that every sugarcane system has its own

unique suite of associated insect pests, their predators and parasites. Commercially cultivated sugarcane originated from an ancestral host, *Saccharum robustum* Linnaeus (Poaceae: Andropogoneae) in New Guinea but many important insect pests of the world's sugarcane industries, as well as minor pests did not (Pemberton and Williams, 1969). 'The regional character of sugarcane pests, their host-plant interactions, the presumed origins of cultivated sugarcane and the close botanical affinity of cultivated sugarcane with wild canes and with grasses of other genera suggest that local insects have adopted sugarcane as a host simply as a consequence of its cultivation' (Pemberton and Williams, 1969, pg 3). There are no insect specialists associated with sugarcane and insects attacking the plant are generalist feeders or are feeders restricted to grasses, *Saccharum* species or their relatives (Pemberton and Williams, 1969).

Insect pests of sugarcane can generally be divided into three ecological categories
sap suckers and leaf feeders;
stalk borers and;
soil pests (Carnegie and Conlong, 1994).

Of the soil pests associated with sugarcane worldwide, the family Scarabaeidae is the dominant group both in terms of damage and distribution (Wilson, 1969; Allsopp *et al.* 1993; Carnegie and Conlong, 1994). Worldwide the Scarabaeidae are known pests of barley (Crocker *et al.* 1990), beans (Bellotti *et al.* 1997), cereals (Crocker *et al.* 1990), grape vines (Hammons *et al.* 2009), groundnuts (Wightman and Wightman, 1994), fruit trees (Prins, 1965), forestry (Govender, 2007), maize (du Toit, 1997), oats (Crocker *et al.* 1990), palms (Beaudoin-Ollivier *et al.* 2000), pastures (Townsend and Jackson, 1997), pineapples (Petty, 1976), potatoes (Bellotti *et al.* 1997), rice (Dale, 1994), sorghum (Teetes and Wiseman, 1979), soybeans (Teetes and Wiseman, 1979), sugarcane (Wilson, 1969) and turf grass (Potter, 1997).

The most important scarab subfamilies reported as pests of sugarcane are the Dynastinae (65 species), Melolonthinae (80 species) and the Rutelinae (40 species) (Box, 1953; Wilson, 1969). The impact these scarab subfamilies have on sugarcane vary in importance, with some inhabiting sugarcane but causing minor or sporadic damage while others are severe pests and can cause huge economic losses (Wilson, 1969). Most countries have a suite of endemic scarab species which attack sugarcane. However there have been some reports of accidental introduction of several species which have subsequently become major pests in their country

of introduction (Jackson and Klein, 2006). The accidental introduction of *Hoplochelus marginalis* Fairmaire (Coleoptera: Melolonthinae) from Madagascar into Reunion where it has become a major pest in sugarcane is an example (Vercambre *et al.* 1994). The Melolonthinae in particular have been problematic in sugarcane in Australia (Allsopp, *et al.* 1993), Florida (Cherry, 1991), Mauritius (Moutia, 1936), India (Saylor, 1940), Philippines (Quimio *et al.* 2001), Puerto Rico (Wilson, 1969), Reunion (Vercambre *et al.* 1994), South Africa (Carnegie, 1974; Carnegie, 1988) and Tanzania (Rajabalee, 1994).

In contrast to sugarcane producing areas such as Australia, Mauritius and other islands, Africa has, relatively speaking, been spared severe white grub infestations. Where infestations have occurred they have generally been localised and/or only of short duration (Conlong and Mugalula, 2003). Some exceptions are Swaziland and Zimbabwe which have both experienced heavy infestations of *Heteronychus licas* Klug (Coleoptera: Dynastinae) for many years in irrigated sugarcane (Sweeney, 1967; Cackett, 1992). Damage by this pest has also been recorded in Mozambique, Nigeria and South Africa (Taylor, 1966; Carnegie, 1974). In South Africa, *H. licas* has been recorded in sugarcane in KwaZulu-Natal, Pongola and Mpumalanga regions (Carnegie, 1988). *Adoretus fusculus* Fåhraeus (Coleoptera: Rutelinae) has been associated with noticeable but transient damage to ratoon sugarcane on several farms near Gingindlovu in KwaZulu-Natal (Carnegie, 1988). Serious outbreaks of the melolonthid, *Asthenopholis subfasciata* Blanch (Coleoptera: Melolonthinae) have also been recorded in ratoon sugarcane at Emoyeni in KwaZulu-Natal (Carnegie, 1988). Further, Jepson (1956) recorded serious damage to sugarcane by the melolonthid, *Cochliotis melolonthoides* Gerstaecker (Coleoptera: Melolonthinae) in Tanzania. More recently in Uganda at the Kinyara Sugar Works Ltd, huge areas of sugarcane were dying due to heavy infestations of a newly recorded sugarcane white grub species, *Idaecamenta eugeniae* Arrow (Coleoptera: Melolonthinae) (Conlong and Mugalula, 2003; Mugalula *et al.* 2006).

1.2 *White grub damage in sugarcane*

White grubs (the C-shaped, white larvae of polyphagous scarab beetles) feed on sugarcane roots and the underground stools that produce new shoots. This feeding severely reduces plant vigor, crop yield and sucrose content in the stalk (Allsopp *et al.* 1991). Excessive root feeding by white grubs in sugarcane can cause the leaves to turn yellow and the inner spindle of plants to wilt, creating a symptom known as ‘dead hearts’. Later, the leaves die and there is

consequent deterioration of the maturing stalks (Wilson, 1969) which leads to reduced sucrose storage. When the whole stool is stripped of its roots, the entire plant can topple under its own weight (Wilson, 1969). In Australia stools may be removed inadvertently during mechanical harvesting, further reducing subsequent yield potential and adding undesirable levels of soil to milling (Allsopp, 2010). In cases where root loss is severe, the ability of plants to regenerate and produce subsequent ratoon crops is weakened (Sosa, 1984). Damage to sugarcane is greatest when the third-instar grub is abundant in the soil, predominantly because this larger-bodied life stage feeds voraciously on sugarcane root hairs and roots, while younger instars tend to feed on organic matter and plant rootlets in the soil and consume less plant biomass as a consequence of their smaller body sizes (Wilson, 1969). Infestation of fields is irregular and usually starts from the field edges and spreads slowly throughout the field (Sosa, 1984). According to this author heavily infested fields are often not worth harvesting because sugarcane quality is compromised. There is also a report of some scarab adults feeding on sugarcane (Cackett, 1990). Damage to shoots and tillers in young plant and ratoon sugarcane is caused by *H. licas* beetles chewing into the sides of tillers just below the soil surface, creating a typical 'dead heart' which is visible in younger sugarcane (Cackett, 1990). Most scarabs however do not feed on sugarcane as adults; there is a preference for adults to feed on alternative host plants, often tree species, or they do not feed at all which is a consequence of their being sustained by large nutritional reserves acquired during the larval phase (Carnegie *et al.* 1974; Chinappen *et al.* 2003).

Damage caused by white grubs depends on various factors such as the size and abundance of grubs as well as the age and condition of the sugarcane crop being attacked. Mungomery (1949) suggested that just one grub of *Dermolepida albohirtum* Waterhouse (Coleoptera: Melolonthinae) per stool was enough to inflict serious damage to sugarcane in dry, red soils in Queensland, Australia. However, in rich alluvial soils it required 3-4 grubs to have the same effect (Mungomery, 1949). Historical white grub damage estimates in sugarcane in Puerto Rico, recorded yield reductions of 15-20 tonnes/acre (1 acre= 0.4 ha) when infested by *Phyllophaga portoricensis* Blanchard (Coleoptera: Melolonthinae) (Wilson, 1969). In the Philippines, as many as 24 *Leucopholis irrorata* Chevrolat (Coleoptera: Melolonthinae) grubs have been found infesting a single sugarcane stool (Lopez, 1931). Similarly, in Mauritius, 25-30 small-bodied white grubs of the species *Phyllophaga smithi* Arrow (Coleoptera: Melolonthinae) per stool were reported and overall crop losses of 10-15% per acre were recorded (Wilson, 1969). As many as 4-22 *Holotrichia consanguinea* Blanchard (Coleoptera:

Melolonthinae) grubs were found infesting a single sugarcane stool in India (Prasad and Thakur, 1961). In Tanzania the melolonthid, *C. melolonthoides* had the potential to reduce sugarcane yields by 45-80 tonnes/acre in plant cane and 30 tonnes/acre in ratoon cane (Jepson, 1956). Yield reductions in sugarcane in Florida, caused by the dynastid, *Ligyrus subtropicus* Blatchley (Coleoptera: Dynastinae) were 28% in tonnes of sugarcane per ha and 39% in tonnes of sugar per ha (Sosa, 1984). In Swaziland approximately 20 000 tonnes of sugarcane was reported lost due to *H. licas* infestations in 1967 (Sweeney, 1967).

More recent annual crop loss estimates associated with *D. albohirtum* in Australian sugarcane amount to AUS $10 million with periodic outbreaks where losses may reach AUS $40 million in damage and management expenses (Sallam, 2011). Chelvi *et al.* (2011) report a potential of 80-100% damage to sugarcane by white grubs in India. Estimated crop losses associated with white grubs in South African sugarcane are limited to one study. McArthur and Leslie (2004) estimated a 23-55% average reduction in sugarcane yield (tonnes sugarcane/ha) by various scarab species in the KwaZulu-Natal Midlands North region depending on sugarcane variety and season. There is a substantial need for additional studies on the economic impact of white grubs to be undertaken in South African sugarcane.

1.3 *The typical white grub life cycle*

The life history of the Scarabaeidae follows a typical pattern as depicted in Figure 1.1. Adult beetles occur from September to March in the southern hemisphere each year with mass emergences usually taking place in October-November (Prins, 1965, Carnegie, 1974). Beetles usually emerge from soil at dusk following favourable increases in temperature and rainfall (Wilson, 1969). Rainfall is thought to stimulate emergence, probably because of its softening of the soil and increasing moisture levels in the soil, which is thought to favour the survival and development of eggs and younger instars (Hawley, 1949). Mating occurs either on the soil or host plants, with or without an associated or subsequent feeding period on foliage. After mating, gravid females, sometimes followed by the males, return to the ground for oviposition (Wilson, 1969).

Most eggs are found at a depth of 8-15 cm, although in the South African large wattle chafer, *Hypopholis sommeri* Burmeister (Coleoptera: Melolonthinae) eggs can be found as deep as 23 cm in hard soil (Prins, 1965). The number of eggs a female can lay depends on the

species; in the case of *H. sommeri*, females can deposit up to 40-48 eggs in the soil (Prins, 1965). Eggs are oval, smooth and dirty white when they are first laid but increase dramatically in size when they undergo a moisture absorption phase and become spherical and duller (Figure 1.1). Average egg length ranges from 1.1 mm up to 2.7 mm just before they hatch depending on the species (Prins, 1965). Egg incubation periods also vary according to species but in *H. sommeri* ranged from 10-18 days, the longest incubation period recorded was 30 days (Prins, 1965).

After egg hatching, the first instars are pale white with light brown head capsules. Newly hatched first-instar grubs feed on organic matter in the soil and on young, tender roots of host plants and remain as first-instars for up to 95 days in some species. In *H. sommeri* the first instar period varies from 30-75 days (Prins, 1965). The insects then molt into second instars which are very similar to the first instar grub except for size, a darker colouration and the occurrence of secondary setae. The second instar phase can last for up to 165 days in some species but in *H. sommeri* the second instar phase lasts 37-142 days (average 85 days) (Prins, 1965). The third and final instar phase is the most damaging to sugarcane. They feed on roots and underground parts of sugarcane for up to 219 days in the case of *H. sommeri* (Figure 1.1). At the end of the third instar phase, the fully-grown grub burrows down into the soil to make an earthen cell in which pupation occurs. Metamorphosis takes place inside the cell and the pupae is at first pale yellow but turns reddish-brown towards the end of the pupal phase (Figure 1.1). Prins (1965) noted that the pupal phase in *H. sommeri* lasted for 20-30 days. Adult eclosion then takes place again when conditions become favourable.

A large majority of melolonthid white grubs have a one-year life cycle, for example: *D. albohirtum*, *P. portoricensis*, *L. irrorata*, *P. smithi* and *H. consanguinea* (Wilson, 1969). There are also examples of white grubs that have two-year life cycles such as *Lepidiota frenchi* Blackburn (Coleoptera: Melolonthinae) in Australia (Wilson, 1969). In South Africa, two melolonthid species: *H. sommeri* and *Schizonycha affinis* Boheman (Coleoptera: Melolonthinae) are thought to have two-year life cycles (Prins, 1965; Carnegie, 1974). In the maize belt in Springbok Flats, Potchefstroom, South Africa, the Rutelinae white grub species *Anomala ustulata* Arrow (Coleoptera: Rutelinae) has a recorded three-year life cycle (du Toit, 1996). European cockchafer, *Melolontha melolontha* Linnaeus (Coleoptera: Melolonthinae) is a serious pest of many crops in Europe but in Denmark where it attacks Christmas trees, it has a four-year-life cycle (Eilenberg *et al.* 2001b).

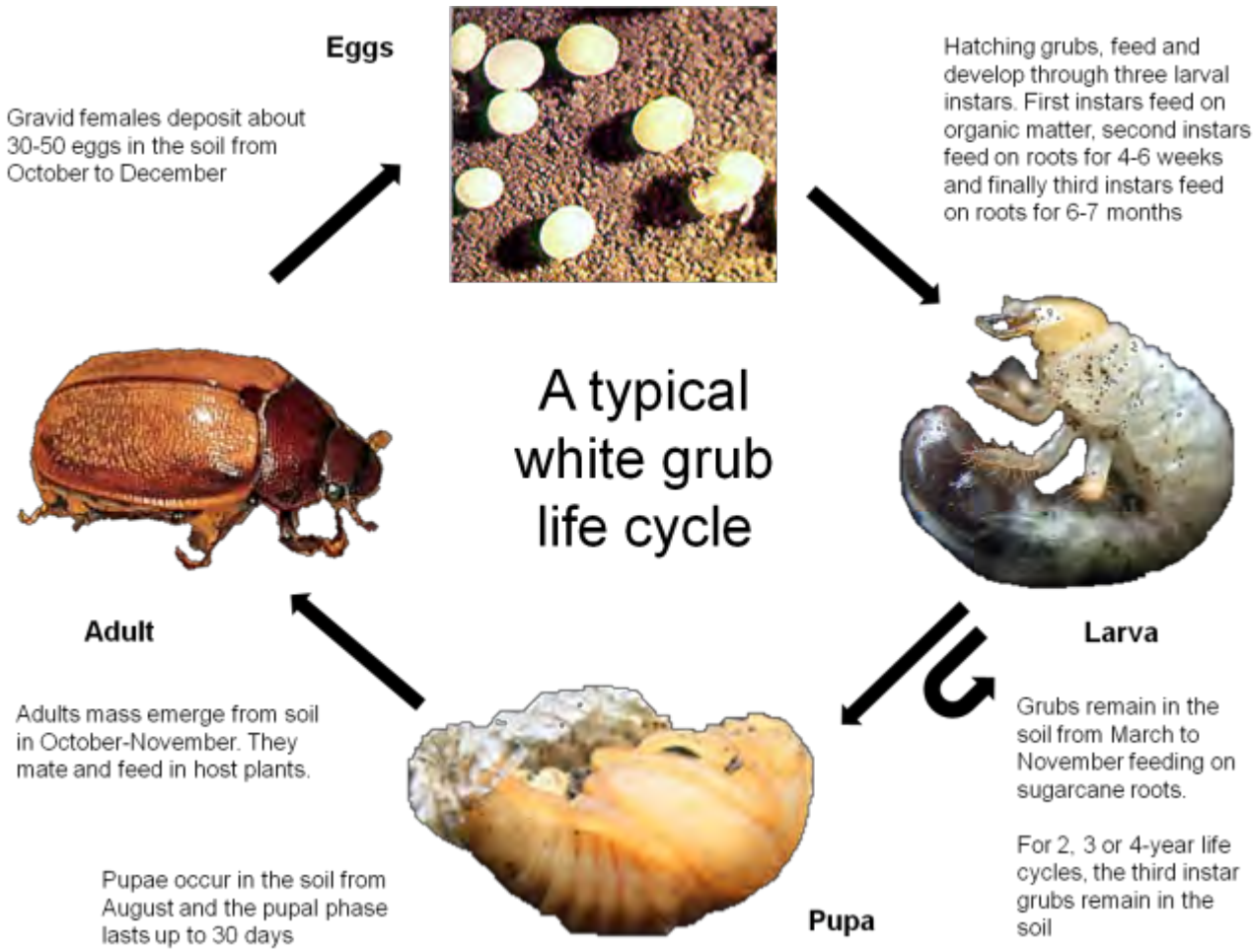

Figure 1.1: A typical scarab life cycle in South African sugarcane (photo credits: M. Way)

1.4 *White grub control in sugarcane*

In Australia chemical control forms the basis of the integrated control programme against white grubs while in other countries such as Mauritius, Hawaii and Barbados chemical control is discouraged and there is a stronger reliance on cultural and biological control (Fewkes and Greathead, 1978). Successful biological control in island countries such as Mauritius predates the introduction of chemicals and the success of this method obviates the need for chemical intervention (Fewkes and Greathead, 1978). Further, in countries with high human populations such as India, labour is relatively inexpensive and the need to employ people overrides the use of chemical insecticides. However, in Australia where labour is

expensive, chemical insecticides are cheaper and easier to use than labour (Fewkes and Greathead, 1978).

1.4.1 *Chemical control*

The history of chemical control against white grubs is best described by Australian researchers because these insects have been the primary pests of sugarcane on this continent since the introduction of the crop (Robertson *et al.* 1995). Thus most of the information provided here derives from Australian research. Chemical control is usually aimed at ovipositing females or at the first and second instar grubs by placing insecticides close to the sugarcane stools (Fewkes and Greathead, 1978). Between 1947 and 1987, the organochlorine insecticide benzene hexachloride (BHC) was used in Queensland against white grubs and provided cheap and effective control (Robertson *et al.* 1995). Other organochlorine insecticides used against white grubs were heptachlor, dieldrin and aldrin. Following the withdrawal of organochlorines in 1987 when residues were found in foodstuffs, new approaches were needed to ensure that the sugarcane industry did not rely solely on one control tactic as it had with residual insecticides (Robertson *et al.* 1995). Later, three organophosphate insecticides were registered: suSCon® Blue (140 g/kg chlorpyrifos in a controlled-release, granular formulation), Mocap® (100 g/kg ethoprophos) and Rugby® (100 g/kg cadusafos) in conventional clay-based granules. More than 85% of sugarcane producing areas in Australia used suSCon® Blue in 1995. Recently, a newer controlled-release formulation of systemic imidacloprid, suSCon® Maxi, was developed and it is currently being used (Allsopp, 2010). Chemical groups such as pyrethroids and carbamates do not work effectively against white grubs (Robertson *et al.* 1995). Liquid formulations of imidacloprid provided an alternative to controlled-release granules and were effective at controlling white grubs (Allsopp, 2010). They also served as a new class of insecticide which could be rotated with organophosphates to reduce insect resistance (Allsopp, 2010). The difficulties in using chemical control are numerous: targeted applications are difficult in ratoon crops; application failures due to accelerated loss of active ingredient often occur, the inappropriate placement of granules limits targeted application; the inconspicuous nature of white grubs in the soil and development of insect resistance against chlorpyrifos all have compromised chemical control (Robertson *et al.* 1995; Allsopp, 2010). In South Africa alone, there are over twenty chemical compounds registered for use against white grubs in forestry, green beans, soybeans, groundnuts, maize, ornamentals, flowers, turf grass (lawns), pineapples, potatoes, sorghum,

sunflowers and wheat (South African Department of Agriculture, 2007). There are however no registered chemical insecticides for the control of white grubs in sugarcane in South Africa (South African Department of Agriculture, 2007).

1.4.2 *Cultural control*

1.4.2.1 *Trap crops*

Sugarcane height appears to be of major importance in attracting ovipositing scarab females. Horsfield *et al.* (2002) discovered that late-planted or late-harvested fields were less damaged than fields which had been planted or harvested earlier. Beetles are clumsy flyers and tend to fly into and hit taller sugarcane and drop into the soil next to taller fields to oviposit. Trap crops are thus sections of sugarcane which have been deliberately planted earlier in the year or are those cut and ratooned earlier in the year so that at the time of beetle activity those taller sugarcane sections (trap crops) are available for beetles. A faster growing alternative crop such as sorghum may also be used as a trap crop. The idea is to attract ovipositing females to the taller sections of sugarcane and then treat those sections with an insecticide, thus targeting the pest better, reducing cost and increasing efficacy (Horsfield *et al.* 2002). In 1999-2001 harvest seasons in the Burdekin district in Australia, trap crops created by early-planting or early-harvesting reduced white grub numbers by 50-100% (Horsfield *et al.* 2002).

1.4.2.2 *Green trash blanket retention*

Another cultural control method is trash blanket retention (green trashing). Allsopp (2010) reported that fewer white grub larvae were found under sugarcane trash retained as mulch compared to areas where soil was left bare. Larvae were also found to die more readily or develop more slowly under green trash blankets presumably because of a higher incidence of disease (Allsopp, 2010). This method however may not have much relevance in South African sugarcane systems because almost all growers burn sugarcane trash owing to labour demands and the steep topography of the land under cultivation, which limits mechanical harvesting.

1.4.3 *Developing white grub-resistant sugarcane varieties*

Research in Australia has shown than sugarcane cultivars vary in resistance to white grubs (Allsopp, 2010). Pot and field trials showed a range of tolerance and antibiosis mechanisms in over 400 sugarcane clones and related species, illustrating that current commercial cultivars generally had poor tolerance and little antibiosis (Allsopp *et al.* 1995). Tolerances of some varieties are thought to be the result of differences in root architecture, tillering ability and ability to produce new roots after white grub feeding. The aim of this method is to select insecticidal genes or antimetabolites (lectins or proteinase inhibitors) which can be genetically engineered into sugarcane to increase resistance to white grubs in cultivated varieties (Allsopp *et al.* 1995). Positive attributes for breeding resistant sugarcane varieties are: season-long protection; insects are always treated at the most sensitive stage; protection is independent of climatic constraints; no application costs; only crop-eating insects are exposed; the material is confined to the plant tissues expressing it; does not leach into the environment and any residual material is likely to be removed during the milling process (Allsopp *et al.* 1995). Allsopp (2010) reported that this method has not been adopted by the sugarcane industry in Australia.

1.4.4 *Mechanical control*

One of the most effective ways to control white grubs is through the use of ploughing. White grubs which are present in the upper soil profile are either damaged by the metal blade as it moves through the soil or are crushed between harder pieces of soil. White grubs may also be lifted out of the soil by the blade which then exposes them at the soil surface to increased temperatures, leading to desiccation, or they are eaten by birds or other predators on the soil surface [1](Conlong, pers. comm.). Conlong and Mugalula (2003) showed that extensive ploughing reduced levels of *I. eugeniae* in heavily infested sugarcane fields from more than 20 grubs per pit in 2001 to fewer than 2 grubs per pit in late 2002 in Uganda at the Kinyara Sugar Works Ltd. It is unclear whether ripping or discing would have the same degree of efficacy as ploughing but the former methods usually involved shallower blade actions which may not be as effective in lifting grubs out of the soil or injuring them, particularly in dry months when grubs are deeper in the soil. Although effective, this method may be applied too

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infrequently for effective white grub control in the KwaZulu-Natal Midlands North region of South Africa because sugarcane in this area is harvested every 18-24 months, and growers will ratoon sugarcane up to six times before ploughing out their fields. This suggests that ploughing will occur only every decade at best.

1.4.5 *Biological control using natural enemies*

1.4.5.1 *Predators*

Predators can have a large impact on white grub larvae in the soil and can manage pest levels effectively. Ants (Formicidae), ground beetles (Carabidae) and rove beetles (Staphylinidae) have been shown to reduce various populations of white grubs (Jackson and Klein, 2006). The predacious elaterid, *Pyrophorus luminosus* Illiger (Coleoptera: Elateridae) was introduced into Barbados for the control of *P. smithi* (Wilson, 1969) and the Elateridae are also recognised predators of white grubs in Australia (Robertson *et al.* 1995). Robber fly larvae (Asilidae) and horse flies (Tabanidae) are known predators of white grubs in South Africa and elsewhere (Prins, 1965; Wilson, 1969; Robertson *et al.* 1995). Vertebrate predators include insectivorous lizards, frogs and small mammals such as rats, shrews and moles (Prins, 1965; Wilson, 1969). Of the birds that eat white grubs, owls, cuckoos, crows, various hawks and the hadedah ibis are reported from South Africa (Prins, 1965).

1.4.5.2 *Parasitoids*

1.4.5.2.1 *Dipteran parasitoids*

There are two Dipteran subfamilies of the Tachinidae that are known to parasitize the third instar larvae and adults of various scarab species. The Dexiinae has two recorded species: *Rutilia inornata* Guérin-Méneville which is a known parasitoid of the third instar grubs of *L. frenchi* and *Dasygnathus* sp. and a second unknown fly parasite species *Rutilia* sp. which attacks the third instar grubs of *Anoplognathus porosus* Dalman (Coleoptera: Melolonthinae) (Logan, 1999). Dexiinae are also known to parasitize the third-instar larvae of *H. sommeri* in South Africa (Prins, 1965) (Figure 1.2A and 1.2B). Some members of the Tachinidae are known parasitoids of scarab adults. These fly parasitoids have large maggots which develop inside the host's body cavity (Jackson and Klein, 2006). The Tachinid fly species *Palpostoma* sp. parasitizes *Antitrogus parvulus* (Coleoptera: Melolonthinae) Britton, *Lepidiota crinite*

Brenske (Coleoptera: Melolonthinae) and *Lepidiota noxia* Britton (Coleoptera: Melolonthinae) in Australia (Logan, 1999). The Tachinid, *Istocheta aldrichi* Mesnil, lays its eggs on the thorax of adult Japanese beetle, *Popillia japonica* Newman (Coleoptera: Melolonthinae). In New Zealand, the Tachinid *Prociscio cana* Hutton lays its eggs on the larvae of *Costelytra zealandica* White (Coleoptera: Melolonthinae) (Jackson and Klein, 2006). Jepson (1956) recommended that the Tachinid species, *Pexopsis pyrrhaspis* Villeneuve, which was an effective parasitoid of the white grub *H. sommeri*, could be a potential control agent for the Tanzanian chafer *C. melolonthoides*. Prins (1965) reported a Pyrgotidae fly, *Adapsilia latipennis* (=*Eupyrgota*) Walker as an effective parasitoid of adult *H. sommeri* in the KwaZulu-Natal province of South Africa. Another fly family, Bombyliidae has also been observed on chafers in South Africa (Prins, 1965). While these species can be effective at controlling white grub populations locally, parasitism levels are generally quite low (2-18%) (Logan, 1999).

1.4.5.2.2 *Hymenopteran parasitoids*

Various Hymenoptera also parasitize the larvae of scarabs. The wasp family Scoliidae comprises species such as *Campsomeris tasmaniensis* Saussure which attack the third instar grubs of *A. parvulus*, *Rhopaea* sp., *A. porosus*, *L. noxia* and *Lepidiota negatoria* Blackburn (Coleoptera: Melolonthinae) (Logan, 1999). Prins (1965) recorded the wasp families, Scoliidae and Tiphiidae parasitizing *H. sommeri* in KwaZulu-Natal (Figure 1.2F). *Tiphia* wasps are solitary ectoparasitoids of the larvae of numerous scarab beetles and find their hosts by following kairomones from the grub's frass (Jackson and Klein, 2006). The wasp species, *Tiphia vernalis* Rohwer is the most widespread parasitoid of the Japanese beetle, *P. japonica. Tiphia pygidialia* Allen is often found attacking the white grub, *Cyclocephala* sp. (Coleoptera: Rutelinae) (Jackson and Klein, 2006).

1.4.5.3 *Pathogens*

Scarabs share a close association with the soil and for this reason they are often infected by soil-borne pathogens. This is reflected in the number of diseases recorded from scarab pests worldwide. Further, the disease complex of scarabs is similar around the world, with various protozoan, viral, bacterial, nematodes and fungal pathogens recorded (Milner, 1997).

1.4.5.3.1 *Protozoa*

In Australia and New Zealand, microsporidians (*Nosema* sp. and *Vavraia* sp.), coccids (*Adelina* sp.) and neogregarines (*Mattesia* sp.) have been observed causing epizootics in white grub populations (Jackson and Klein, 2006). In Australia specifically, local white grub population extinctions have been observed due to a frequently encountered disease caused by a protozoan, *Adelina* sp. (Allsopp, 2010). This single-celled microorganism invades the haemocoel and fat bodies of a wide range of scarab beetle hosts and other arthropods, reducing glycogen accumulation and ultimately destroying the cells (Sallam *et al.* 2003). In north Queensland, a species of *Adelina* is frequently recovered from larvae of greyback canegrub, *D. albohirtum*. Infection by this protozoan is density-dependent and requires a living insect host to complete its life cycle, so mass production of the pathogen is difficult from a biological control perspective (Sallam *et al.* 2003). The microsporidian, *Ovavesicula popilliae* has been reported infecting the malpighian tubules of *P. japonica* (Jackson and Klein, 2006).

1.4.5.3.2 *Viruses*

Viruses among the Coleoptera are rare (Hajek and St Leger, 1994) however there are a few well-known examples of viruses which attack white grubs. The best known example is the non-occluded *Oryctes* virus which infects the adults of the rhinoceros beetle, *Oryctes rhinoceros* Linnaeus (Coleoptera: Dynastinae) (Huger, 2006). The virus infects the insect's gut cells and will reduce feeding and fecundity. It is particularly prevalent and transmitted during the aggregation behaviour of adult beetles. Scarabs are also infected by iridescent viruses (Iridoviridae) which are icosahedral in shape and replicate in the cytoplasm of infected cells. The larvae of the New Zealand endemic pasture pest *C. zealandica* have been observed carrying iridescent virus infection which characteristically turns the hindgut of the host a brilliant-blue colour (Moore *et al.* 1974). Scarabs are also known to carry entomopox viruses but these are very rarely encountered. An example is the recent observation of *Adoretus versutus* Harold (Coleoptera: Rutelinae) carrying entomopox virus infection in Fiji (Beaudoin *et al.* 1994; Dall *et al.* 1995). Viral infection also requires a living insect host for life cycle completion, so mass production of viral pathogens for biological control can be difficult.

1.4.5.3.3 *Bacteria*

Milky disease caused by the soil bacterium *Paenibacillus popilliae* Dusky is the most common and distinctive disease found only in scarab larvae (Jackson and Klein, 2006). The bacterium colonizes the host's haemolymph producing refractile spores that give the blood system a milky appearance and from the outside, grubs appear white in colour (Figure 1.2I). This well-known bacterium is the active ingredient of many commercial formulations and was first used in the United States to control *P. japonica* in the 1940 to the 1960s (Jackson and Klein, 2006). *Bacillus thuringiensis* Berliner surprisingly has had little or no effect against scarabs but recent research has shown that one isolate of the bacterium, the Buibui strain, was effective against Rutelinae scarab pests (Jackson and Klein, 2006). During an extensive survey for entomopathogens in KwaZulu-Natal, South Africa, the bacterium *Bacillus laevolacticus* was isolated from *H. sommeri* (Figure 1.2H) and three species of the non-spore forming bacteria from the genus, *Serratia* which included: *S. marcescens*, *S. liquefaciens* and *S. grimesii* were obtained from white grubs in South Africa (Hatting, 2008). In New Zealand, an unknown *Serratia* sp. is known to cause seasonal amber disease in C. zealandica (Figure 1.2J) (Jackson and Klein, 2006).

1.4.5.3.4 *Entomopathogenic nematodes*

Two families of nematodes, Steinernematidae and Heterorhabditidae have been used to control scarab pests (Figure 1.2C and 1.2G). Both nematode families carry symbiotic bacteria of the genus *Xenorhabdus* or *Photorhabdus* respectively and it is the symbiotic relationship between the nematode and its bacteria which kills the host by inducing septicaemia (Jackson and Klein, 2006). The most effective nematodes used thus far against white grubs are the larger-bodied species: *Steinernema glaseri* Steiner, *S. scarabaei* Stock and Koppenhofer, *Heterorhabditis megidis* Poiner, *H. bacteriophora* Poiner and *H. zealandica* Poiner (Jackson and Klein, 2006). Production and storage requirements are limitations in the use of entomopathogenic nematodes as biological control agents. Further, the effectiveness of nematodes is often limited by cooler soil temperatures where scarab larvae are still active (Jackson and Klein, 2006). Efficacy however may be improved when combined with low rates of insecticides, for example Koppenhofer *et al.* (2000) showed synergisms of two nematodes species, *S. glaseri* and *H. bacteriophora* with the chloronicotinyl insecticide imidacloprid against the Japanese beetle, *P. japonica*. The imidacloprid caused a disruption

in the normal nerve function of the third instar grubs resulting in drastically reduced activity by the insects; this then increased host attachment by juvenile nematodes (Koppenhofer *et al.* 2000).

1.4.5.3.5 *Entomopathogenic fungi*

Entomopathogenic fungi are unique in that they are able to infect insect hosts via the cuticle. In anamorphic species, asexual spores called conidia adhere to the insect cuticle via non-specific hydrophobic bonds (Inglis *et al.* 2001). Under the right environmental conditions the conidia germinate and a germ tube is produced. The appressorium (germ tube) penetrates the host cuticle through a combination of enzymatic and mechanical pressure. The cuticle is the host's first line of defense and has a central role in determining fungal specificity (Inglis *et al.* 2001). Within the host haemocoel, the fungus grows vegetatively and produces species-specific metabolites which aid in overcoming the host's immune system and later kill the host (Strasser *et al.* 2000). Upon insect death, fungal hyphae penetrate the less sclerotised areas of the cuticle (Inglis *et al.* 2001). If the fungus penetrates the cuticle, successful infection can only occur if the fungus can overcome the immune responses of the insect (Hoffman *et al.* 1999).

Some interesting fungal pathogens of the genus *Ophiocordyceps* (teleomorphic {sexual} fungi) have been recovered from scarabs in South Africa, Tanzania and other tropical areas. Evans *et al.* (1999) reported that there is a complex of *Cordyceps* species associated with white grub pests in general throughout the tropics. According to this author, over the past decade, most of the material received concerns infected sugarcane white grub pests from the Palaeotropics which have all been loosely assigned to the species *Cordyceps barnesii* Thwaiters. Collections include third instar scarab larvae (*Brachylepis* sp.) in Somalia where this pest is a serious constraint to sugarcane production (Figure 1.3C), from Indonesia, where white grubs belonging to the genera *Lepidiota* and *Dermolepida* are major pests and finally from Tanzania where many *C. melolonthoides* larvae, infected with *C. barnesii*, were collected from the TPC sugarcane plantations (Figure 1.3E) (Evans *et al.* 1999). In South Africa, an unknown *Ophiocordyceps* species was recorded [2](Way pers. comm.). The South African Sugarcane Research Institute collected a specimen from an infected third instar

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Anomala sp. larva at Eshowe in the KwaZulu-Natal Province (Figure 1.3B). While species of the *Ophiocordyceps* are interesting pathogens and considered useful in controlling white grubs, they cannot be grown on artificial media with ease thus their inundative biological control potential may therefore be limited.

Figure 1.2: Natural enemies of white grub larvae. Two Dexiinae parasitoid pupae found within the body cavity of a *Hypopholis sommeri* third instar larva (M. Way) (A and B); Nematodes of the families Steinernematidae and Heterorhabditidae are well-known parasites of scarabs [3](CSIRO, Australia) (C and G); *Beauveria* sp. (BT96) the active ingredient of Betel® used in Reunion against *Hoplochelus marginalis* [4](CIRAD, Réunion) (D); *Metarhizium anisopliae* isolate FI-1045, the active ingredient of the product BioCane® used in Australia against *Dermolepida albohirtum* [5](BSES, Bio-Care Technology Pty Ltd) (E); An unknown wasp larva of the family Scoliidae attached to the thorax of a scarab grub (M. Way) (F); The bacterium, *Bacillus laevolacticus* isolated from *H. sommeri* in South Africa (J. Hatting) (H); Milky disease caused by *Bacillus popilliae* (T. Jackson) (I); Grubs infected with *Serratia marcescens* (T. Jackson) (J).

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Figure 1.3: *Cordyceps* pathogens of white grub larvae: *Cordyceps melolonthae* var. *rickii* on melolonthid larvae in forest soil in Amazonian Ecuador (Evans *et al.* 1999) (A); Unknown *Ophiocordyceps* infecting a third instar *Anomala* sp. from Eshowe, KwaZulu-Natal, South Africa (M. Way) (B); Immature stroma on third instar larva of *Brachylepis* sp. excavated from sugarcane soil in Somalia (Evans *et al.* 1999) (C); *Ophiocordyceps* infecting the third instar larvae of *Cochliotus melolonthoides* from Tanzania plated on an egg-based agar (J. Hatting) (D); *Cordyceps barnesii*, young stages of stromata development, on *Cochliotus melolonthoides* larvae in Tanzania (Evans *et al.* 1999) (E).

Soil-borne entomopathogenic fungi of the genera *Beauveria* (Figure 1.2D) and *Metarhizium* (Figure 1.2E) are the most well-known fungal pathogens of the Scarabaeidae. Jackson and Klein (2006) concluded that consistent infection in scarabs was limited to specific strains mainly within the species *Beauveria brongniartii* (Saccardo) Petch (Ascomycota: Clavicipitaceae) and the large-spore variety *Metarhizium anisopliae* var. *majus* Metschnikoff (Ascomycota: Clavicipitaceae). Both species of fungus share a long history of scarab biological control. For example, *B. brongniartii* had already been recognised as a pathogen that could effectively control European cockchafer, *M. melolontha* more than 100 years ago (*Le Mould, 1893* cited by Kessler *et al.* 2004). Similarly, *M. anisopliae* was used by

Metchnikoff in Russia in 1878 for the control of cereal cockchafer, *Anisoplia austriaca* Herbst (Coleoptera: Rutelinae) (Glare, 2004; Lord, 2005). For this reason the present review will only focus on these two fungal species and the advances which have been made using these species to control scarab pests.

1.4.5.3.5.1 *Beauveria brongniartii*

Beauveria brongniartii is one of 12 recognised species within the monophyletic *Beauveria* genus. The genus comprises species which are soil-borne, have cosmopolitan distributions, reproduce asexually (anamorphic), are facultatively necrotrophic and are pathogens of insects and other arthropods (Rehner *et al.* 2011) (Figure 1.4E). Rehner *et al.* (2011) gives a full account of the taxonomy and various morphological characteristics of each species within the genus (Figure 1.4A, C, D). Within the genus, *B. brongniartii* is closely related to *Beauveria bassiana* (Balsamo) Vuillemin and they form a group clade. Sister to *B. brongniartii* are the species, *B. asiatica* Rehner and Humber and *B. australis* Rehner and Humber (Rehner *et al.* 2011). A teleomorph (sexual stage), *Cordyceps brongniartii* Shimazu was described and isolated from the scarab *Anomala cuprea* Hope (Coleoptera: Rutelinae) in Japan (Shimazu *et al.* 1988), but this anamorph-teleomorph link has not yet been confirmed with either molecular or cultural data (Rehner, 2005).

Beauveria brongniartii is commonly affiliated with Coleoptera and has a long history of scarab biological control. In Europe, *B. brongniartii* is considered a host-specific fungus with a limited host range and is known to infect all developmental stages of *M. melolontha* and *Melolontha hippocastani* Fabricius (Coleoptera: Melolonthinae) (Keller *et al.* 1997; Keller *et al.* 1999; Strasser and Pernfuss, 2005; Koller *et al.* 2005; Laengle *et al.* 2005). The fungus was tested against other scarabs *Amphimallon* sp. and *Phyllopertha horticola* Linnaeus (Coleoptera: Rutelinae) however limited infectivity was observed [6](Strasser pers. comm.). Further, the fungus was tested against important non-target predators such as the carabid, *Poecilus versicolor* Sturm (Coleoptera: Carabidae) and no significant infectivity was observed (Traugott *et al.* 2005). This highlighted the specific nature of *B. brongniartii* strains that have been formulated as the active ingredients of registered mycoinsecticides.

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There are currently seven registered products based on *B. brongniartii* which make up 4.1% of the total number of 171 registered mycoinsecticide and mycoacaricide products worldwide (Faria and Wraight, 2007). Three products based on *B. brongniartii* have been registered for control of scarabs in Switzerland since 1991. They are Beauveria-Schweizer® (Eric Schweizer Seeds Ltd.), Myzel® (LBBZ Arenenberg) and Engerlingspilz® (Andermatt Biocontrol) (Faria and Wraight, 2007). The fungus was also registered as the product Melocont®-Pilzgerste in Austria in 2000 (Kwizda Agro GmbH). No other EU countries have at present registered *B. brongniartii* as an active ingredient of any product. However, it is registered in non-European countries (Faria and Wraight, 2007). The product Betel® (Betel Reunion S.A) is available for scarab control in Reunion. In Japan the product Biolisa-Kamikiri® (Nitto Denko) has been registered against important forestry pests, the long horn beetles of the family Cerambycidae, and in Colombia the product Beauveria 50® (Ago Biocontrol) is available for scarab control, as well as for certain Hemiptera, Lepidoptera and Diptera (Faria and Wraight, 2007). South Africa has no registered products based on *B. brongniartii*; all the products currently listed in the country include either *B. bassiana* or *M. anisopliae* var. *acridum* as active ingredients. Faria and Wraight (2007) provide a comprehensive review of all globally registered mycoinsecticides and mycoacaricides.

Beauveria brongniartii produces a range of secondary metabolites: bassianin, beauvericin, tenellin and oosporein that are considered important pathogenicity determinants in the host-pathogen relationship (Abendstein *et al.* 2000). Strasser *et al.* (2000) however found that oosporein was the only major secondary metabolite produced by three commercial strains of *B. brongniartii* in submerged cultures and on sterilized barley kernels. Oosporein is a red-coloured C2 symmetrical 2, 5-dihydroxybenzoquinone which inhibits erythrocyte membrane ATPase activity in a dose-depended manner (Strasser *et al.* 2000) (Figure 1.4B). Oosporein has potent antibiotic (towards gram-positive bacteria only) and antiviral (inhibiting Herpes simplex virus-I DNA-polymerase) effects, and is believed to play a major role in the colonisation of infected insects by *B. brongniartii* (Strasser *et al.* 2000). When enough fungal biomass is present within a host insect, the fungus produces oosporein which suppresses the gut bacterial flora to allow for rapid colonisation and proliferation of *B. brongniartii* (Amin *et al.* 2010). Studies on the environmental build-up of oosporein in the soil, in plant material and infected insects following mass-release of the fungus concluded that water-soluble oosporein does not pose any risks and cannot be transferred to plant material (Strasser *et al.* 2000).

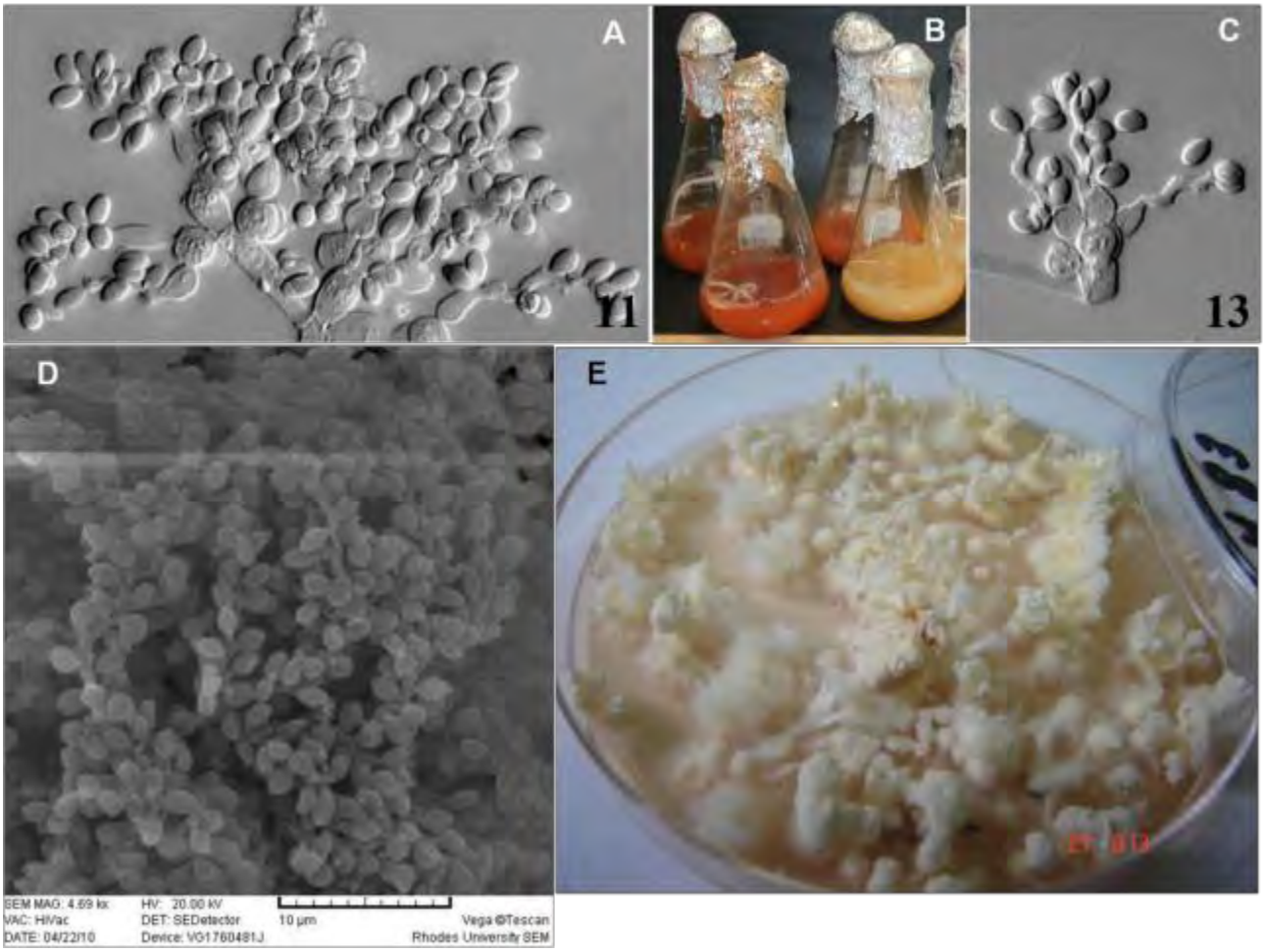

Figure 1.4: *Beauveria brongniartii* morphology: Conidiogenous cells and conidia of *Beauveria brongniartii* (ARSEF 617) (Rehner *et al.* 2011) (A and C); *B. brongniartii* produces the red, secondary metabolite, oosporein when enough fungal biomass is present (T. Goble) (B); Micrograph of the conidia of *B. brongniartii* SASRI 124 (T. Goble) (D); *B. brongniartii* isolate HH83 found in this study, grown on SDA agar. (T. Goble) (E).

Beauveria brongniartii is considered a Group 1 risk organism: biological control agents that are unlikely to cause human disease (Strasser *et al.* 2000). It also scores a low 16 points on the new Risk Index (RI) system proposed by Laengle and Strasser (2010) compared to other soil-applied fungi: *B. bassiana* (risk score: 96) and *M. anisopliae* (risk score: 96). Five basic concepts are proposed for the calculation of the overall environmental risk score for both chemical insecticides and biological control agents: persistence of the active ingredient, dispersal potential, range of non-target effects and direct and indirect effects on the ecosystem (Laengle and Strasser, 2010). Ongoing research for over 100 years using *B. brongniartii* has recorded no side-effects in humans or mammals. The fungus is unable to

grow above 33°C which means that pathogenicity towards warm-blooded animals may be excluded. Under natural field conditions, the fungus is a poor competitor and is suppressed naturally by other microorganisms in the soil; it has also never been recorded as an endophyte (able to colonise plant tissues) (Strasser *et al.* 2000). The ability of *B. brongniartii* to persist in the soil for up to 40 years is reported (Keller *et al.* 2003). In another field study, soil inoculation of different *B. brongniartii* strains showed that all strains were detected at all test sites for up to 14 years after the application (Enkerli *et al.* 2004). The ability to mass-produce *B. brongniartii*, long-term persistence of the fungus in the soil, its narrow host range and low risk index are desirable traits which make this fungus a good biological control agent.

1.4.5.3.5.2 *Metarhizium anisopliae*

Worldwide, *M. anisopliae* has been used far more extensively for scarab control than *B. brongniartii*. Products based on *M. anisopliae* comprise 33.9% of the total number of mycoinsecticides registered worldwide (Faria and Wraight, 2007). These authors list 18 products based on this fungus or its metabolites which have been registered specifically for scarab control in Austria, Italy, Switzerland, India, Australia, Mexico, USA, Colombia and Venezuela. No registered products based on *M. anisopliae* are available for scarabs in South Africa. However, Biological Control Products SA (now Becker Underwood Inc) produces a product based on *M. anisopliae* var. *acridum* for control of locusts and grasshoppers (Faria and Wraight, 2007).

The taxonomy of the *Metarhizium* genus has been complicated owing to poorly described morphological characteristics, which were defined on the basis of the arrangement of the phialides which bear chains of green, ovoid conidia (Bidochka and Small 2005; Zimmermann 2007). Poor molecular resolution due to the use of uninformative genes such as the nuclear ribosomal internal transcribed spacer (ITS) in earlier studies has added value to the taxonomy but not resolved it (Driver *et al.* 2000). Before a study by Bischoff *et al.* (2009), *M. anisopliae* was recognised as a species complex which had four varieties. Since the publication by Bischoff *et al.* (2009) the monophyly of nine terminal taxa in the *M. anisopliae* complex have been recognised as individual species: *M. anisopliae*, *M. guizhouense*, *M. pingshaense*, *M. acridum*, *M. lepidiotae* and *M. majus*. In addition they described three new species, *M. globosum*, *M. robertsii* and, *M. brunneum*, and showed that

M. taii is a later synonym of *M. guizhouense*. Bischoff *et al.* (2009) reported that the anamorphic *Metarhizium* had been linked developmentally and supported by subsequent molecular phylogenetic studies to the teleomorphic genus *Metacordyceps*.

Metarhizium species also produce a range of secondary metabolites and their function has been speculated to be inhibition of the cellular immune system response during fungal infection (Strasser *et al.* 2000). However, whether these secondary metabolites are important pathogenicity determinants for specific strains in the host-pathogen relationship is still unknown (Strasser *et al.* 2000). The first systematic studies of toxin production in *M. anisopliae* led to the discovery of two novel insecticidal substances, destruxin A and destruxin B (Strasser *et al.* 2000). Of the secondary metabolites 28 structurally-related types of destruxins are now known. The basic structure of these molecules consists of five amino acids and an α-hydroxy acid and may exist in many forms (Strasser *et al.* 2000). Insects vary in their susceptibility to destruxins (there are reports of *Lepidoptera* sp. being highly susceptible) and the symptoms of toxicosis also differ between insect species (Strasser *et al.* 2000). Some researchers report that destruxins (depending on the type and host species) have no contact toxicity while others report contact toxicity when applied to the insect cuticle (Strasser *et al.* 2000). These toxins are known to block multicellular defense reactions, disturb macromolecular synthesis and have antiviral properties. They also have a powerful inhibitory effect on the synthesis of DNA, RNA and proteins, even at low doses (Strasser *et al.* 2000).

The more extensive use of *M. anisopliae* worldwide for biological control of scarabs may be due to its broader host range when compared to the limited host range of *B. brongniartii*. However, host range is much more restricted when individual and specific fungal isolates/strains are considered (Vestergaard *et al.* 2003). The Scarabaeidae are a diverse group of insects and the economically important subfamilies form pest complexes in various cropping systems, particularly in sugarcane (Wilson, 1969; Allsopp, 2010). Thus, there is a need to control a variety of scarabs in these cropping systems and not just one particular subfamily, as has been the case using *B. brongniartii* with its specificity towards the Melolonthinae in Europe (Traugott *et al.* 2005; Strasser *et al.* 2000). Various authors report good pathogenicity of *M. anisopliae* against all three economically important scarab subfamilies, Melolonthinae (Milner *et al.* 2002; Guzman-Franco *et al.* 2011), Rutelinae (Lacey *et al.* 1994; Ansari *et al.* 2004) and Dynastinae (Raid and Cherry, 1992; Rath and

Worledge, 1995; Beron and Diaz, 2005; Makaka, 2008). The persistence of *M. anisopliae* is not as long-lasting as *B. brongniartii* and it is known to persist for at least three years post application (Vänninen *et al.* 2000; Milner *et al.* 2003). Other than a broader host range, what makes *M. anisopliae* more desirable is its tolerance against fungicides and insecticides which often accompany the application of the fungus in integrated pest management (IPM) approaches in various cropping systems (Samson *et al.* 2005; Quesada-Moraga *et al.* 2007). Samson *et al.* (2005) showed that only one out of eight fungicides and no insecticides had negative effects on spore viability of *M. anisopliae*, formulated as granules, when they were sprayed at very high rates in a sugarcane field trial. There are however known fungicides and insecticides which do inhibit or kill the fungus (Majchrowicz and Poprawski, 1993). A final consideration is the broader temperature tolerance of strains of *Metarhizium* compared to *Beauveria*. Under field conditions temperatures can vary significantly between regions and *Metarhizium* isolates are able to be infectious at temperatures as low as 5°C (Rath, 1992) and as high as 35°C (Milner, 1997).

1.5 *The history of black wattle, sugarcane and white grubs in South Africa*

The history of black wattle, *Acacia mearnsii* De Wild (Fabales: Fabaceae), in South Africa has interesting connections to present scarab pest prevalence and distribution, particularly for *H. sommeri* and *S. affinis*. Black wattle was first grown in KwaZulu-Natal (KZN) in 1864 by Charles Vanderplank who arrived from Australia with seeds and planted them on a farm in Camperdown (Sherry, 1973) (Figure 1.5). According to this author, seeds were also given to Mr Forbes who owned a farm near Noodsberg; some three years later, trees were taken to another farm near Wartburg and grown there (Sherry, 1973). Mr H.A. Carbutt was another black wattle pioneer who had established plantations at Harden Heights and Fawnleas (Seven Oaks district) by 1891 (Sherry, 1973). The commercial potential of black wattle was recognised in 1884 when the tree species became much sought after because of the royalties it gained in London from shipping the bark which was used in tanning. This profitable export business stimulated interest in wattle growing throughout the KwaZulu-Natal (KZN) Midlands (Sherry, 1973) (Figure 1.5).

Figure 1.5: The South African sugarcane-producing regions. Mill names are indicated by lower black font and city names are indicated in lower white font. The Midlands North region is indicated by a white, broken circle while the Midlands South region is indicated by a yellow broken circle (Source: South African Industry Directory 2004/2005).

At the end of the Second World War the demand for black wattle bark reached the highest level recorded and a large amount of afforestation took place in KZN at this time to meet the demand. KZN produced 60% of the total exportable bark which amounted to a value of ZAR 7 million at that time (Sherry, 1973). By 1958, just when substantial quantities of bark were flooding the market, the world demand for tanning materials decreased rapidly and a quota system was then imposed on all growers. This left many growers with too much cultivated and unused black wattle, so the industry advised growers either to sell the wood or convert the balance of their acreages to other crops (Sherry, 1973). Many growers in KZN took this advice and those areas which were climatically and topographically compatible were converted to sugarcane (Sherry, 1973).

Prins (1965) published the first comprehensive study on wattle chafers in South Africa. He studied two melolonthid species, *Monochelus calcaratus* Burmeister (Coleoptera: Melolonthinae), and *H. sommeri* and a ruteline, *Adoretus ictericus* Burmeister (Coleoptera: Rutelinae). He reported that the melolonthids were especially damaging to black wattle and that adults could completely defoliate these trees and fruit trees in the vicinity of black wattle plantations. He recorded a heavy outbreak of *H. sommeri* in litchi and plum trees on the South Coast near Hibberdene, KZN in 1957. He also reported that the worst affected areas suffering *H. sommeri* attacks fell within the KZN Midlands regions (Figure 1.5), in areas incorporating Greytown, Kranskop, Seven Oaks, New Hanover, Ixopo, Richmond, Harding, Wartburg and Hilton. Prins (1965) listed these areas as the worst affected by the large wattle chafer, *H. sommeri* and these areas were some of the first areas to cultivate black wattle on a commercial scale and were also the first areas to convert large areas of land to sugarcane. Later, Carnegie (1974) reported that in the KZN Midlands, where sugarcane had been grown on land that was previously under black wattle cultivation, that the species: *H. sommeri* and *S. affinis* had become damaging to sugarcane. Harrison (2013) provides an exceptional overview of the taxonomy revision, biology and ecology of the *Hypopholis* genus and Pope (1960) discussed the complicated taxonomic history of *S. affinis*.

Damage to sugarcane was severe enough for studies to be undertaken on their biology and on the use of various insecticides to try and control them (Carnegie, 1974). He concluded that the insecticides he had tested were unable to control white grubs. This author completed insecticide trials in the Seven Oaks district (Midlands North) and in Mid-Illovo (Midlands South) (Figure 1.5), because these areas were particularly hard hit by the pests. This again

highlighted the epicenters of wattle cultivation and subsequent cultivation of and damage to sugarcane. Almost a decade after this initial observation, Carnegie (1988) again reported that *H. sommeri* and *S. affinis* continued to damage sugarcane particularly when in the same vicinity as black wattle plantations (Figure 1.6). He commented that the range of white grub damage appeared to have expanded and that there were new reports received of damage in areas which had previously been free of these pests. He stated that although damage had not been geographically extensive, it had been locally very serious and that there were reports of damage by white grub species, particularly *A. fusculus* which had not been recognised before as crop pests in those areas (Carnegie, 1988).

After a long gap in white grub research, largely due to the extensive investigations which were being undertaken on the stem borer, *Eldana saccharina* Walker (Lepidoptera: Pyralidae), Way (1995, 1996, 1997) undertook studies on the abundance and diversity of scarabs in South African sugarcane to assess the severity and extent of white grub damage in the industry. The results of these surveys revealed that white grubs were present in a wide variety of geographic areas with different climatic conditions and soil types and that the number and diversity of white grubs varied from site to site (Way, 1995). *Schizonycha affinis* was the most widespread and abundant species and was particularly abundant in the KZN Midlands (Way, 1995). He recorded *H. sommeri* and *A. subfasciata* as the next most abundant species (Way, 1995). He further recorded how prevalent white grubs had become by listing 21 previously unknown scarab taxa in sugarcane and reported that white grubs were recovered from all 14 surveyed regions within the industry (Way, 1996). His final comments were that the prevalence of white grubs was highest in the Midlands regions, confirming previous studies (Carnegie, 1974), but that larval abundances were low with the highest recorded levels averaging only two larvae per pit (Way, 1997). Finally, white grub larval abundances and diversity were monitored for nine years (2001-2010; no monitoring occurred in 2002) in the KZN Midlands by Way *et al.* (2011). He again confirmed previous studies by reporting that the worst affected areas in the Midlands were those which lay close to Wartburg and the Seven Oaks district (the epicenters of historical wattle cultivation). He also stated that infestation levels were patchy but warranted control action.

Figure 1.6: A photograph taken from the Harden Heights farm in the Seven Oaks district of the Midlands North region, KwaZulu-Natal, South Africa. Notice the agricultural crop mosaic of black wattle, Acacia mearnsii grown in close proximity to sugarcane (PHOTO: T. Goble).

1.6 *The history of fungal pathogens in South Africa*

Prins (1965) listed the first records of the occurrence of two fungal species, *M. anisopliae* and an unknown *Cordyceps* species (Ascomycota: Cordycipitaceae) infecting wattle chafers, *M. calcaratus*, *H. sommeri* and *A. ictericus* in the KZN Midlands, and stated that these fungal species were responsible for the deaths of large numbers of white grubs in the soil, including adults. Carnegie (1974) also recorded natural fungal epizootics in the Seven Oaks district of the KZN Midlands North region in 1971 when he found that many larvae were dead or dying from 'an infection', which he thought had a progressive controlling effect (Carnegie 1974). It appears that natural biological control of white grubs in the KZN Midlands North has been continuous. However, these pathogens are only capable of suppressing a portion of the scarab pest populations, and have not controlled them sufficiently to limit their pest status. By

utilizing an inundative biological control approach with a virulent fungal isolate, sufficient control may be achieved (Eilenberg *et al.* 2001a).

In 2005, numerous fungal infections of epizootic proportion were observed on white grubs at Sunnyside farm in the Seven Oaks district of the KZN Midlands North region (Hatting, 2008). This author recorded a 20-30% natural prevalence of the fungal species causing the epizootic and through morphological techniques the causal agent was identified as *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Clavicipitaceae). Two fungal isolates were obtained directly from white grubs (SASRI 4222 and SASRI 4223) and tested for biological control potential in the laboratory against the larvae of *H. sommeri* and *S. affinis*. The study revealed that *B. bassiana* isolate SASRI 4222 could kill 50-60% of *S. affinis* larvae within 7 days and the isolate resulted in mycosis of 50% on both *H. sommeri* and *S. affinis*. There was however very high natural mortality recorded in the controls (Hatting, 2008). The isolate however had shown biological control potential at the field level when it was suspected to have caused the epizootic and laboratory bioassays had confirmed its virulence. So the isolate was passed to [7] Plant Health Products Pty Ltd., Nottingham Road in South Africa for mass production and formulation using a granular approach, as had been achieved with BioCane® in Australia (Milner *et al.* 2002). Experimental formulations on rice and on wheat bran were undertaken using *B. bassiana* isolate 4222.

1.7 *History and aims of the current study*

During field site surveys in 2010, two more fungal epizootics were observed at Harden Heights and Canema farms in the Seven Oaks district of the KZN Midlands North, approximately 15 km from the Sunnyside site discovered in 2005. Questions about the ecology of the fungal species and its occurrence on white grub hosts in this region were raised. Further, questions about which white grub species were hosts and which species could become infected by the fungus were considered. Furthermore, white grub species occurring at the sites were unknown, thus whether they were all economically important species or a pest complex in sugarcane had to be determined. It also raised concerns about whether or not *B. bassiana* isolate 4222 was the causal agent of the latter epizootics observed at Harden Heights and Canema farms, as had been assumed at Sunnyside in 2005. An important

[7] ***PLANT HEALTH PRODUCTS (PTY) LTD:*** *P.O. Box 207, Nottingham Road 3280, KwaZulu-Natal, South Africa*

consideration was the possibility that isolates with greater virulence might be obtained. It thus seemed prudent to study the latter epizootics in more detail to elucidate some of these important ecological aspects before undertaking small-scale field trials with *B. bassiana* isolate 4222. With all these considerations in mind and the specific idea of developing a mycoinsecticide, the objectives and aims of the present study are outlined below and in Figure 1.7.

1.8 *Objectives*

1.) *To identify the white grub species diversity occurring at sites where epizootics had occurred, and whether these were all economically important species or a pest complex in sugarcane.* Further aims were to assess spatial distributions of white grub species life stages within the field, and to determine which weather and soil parameters were important determinants in the distribution and occurrence of white grub species at the field site. These aims are important because successful application of any control method requires extensive knowledge of the pests' biology and ecology. The results of these studies are reported in Chapter 2.

2.) *To determine the causal agent of the 2010 epizootics*. This discovery would determine which fungal species/strain would form the active ingredient of the proposed mycoinsecticide. Further, to determine the identity of the causal agent of the epizootics, many fungal isolates obtained from infected white grub cadavers were analyzed using two molecular techniques: multi-locus gene sequencing and microsatellite markers (Chapter 3).

3.) *To determine the biological control potential of the identified fungal species/strains in the laboratory using bioassays against target white grub species.* This was essential because there are many species of white grub associated with sugarcane and it was important to know the host range of the proposed fungal agent and its virulence against various white grub species. It was also important to determine which white grub life stages were most susceptible to fungal infection as this would influence anticipated application strategies (Chapter 4).

4.) *To determine fungal persistence of B. bassiana isolate 4222 on rice and wheat bran carrier formulations to ascertain which carrier enhanced fungal persistence in the soil and could be used in field trials.* Prior to the discovery of the latter two epizootics reported in this study, isolate 4222 had been formulated on rice and wheat bran as carriers. Knowledge was needed on the persistence of the proposed granular product under different environmental conditions and a range of soil types to determine the feasibility of area-wide application of this fungal formulation in the KZN Midlands North region (Chapter 5).

5.) *To understand growers' perceptions and knowledge of white grubs as pests.* From a commercial viability perspective it was important to establish how sugarcane growers felt about the potential release of a granular mycoinsecticide because this would aid in the determination of a market for the proposed product. A semi-structured questionnaire was developed and distributed amongst growers from the two KZN Midlands regions. The results were analyzed to determine the viability of developing, selling and using a granular mycoinsecticide in these regions (Chapter 6).

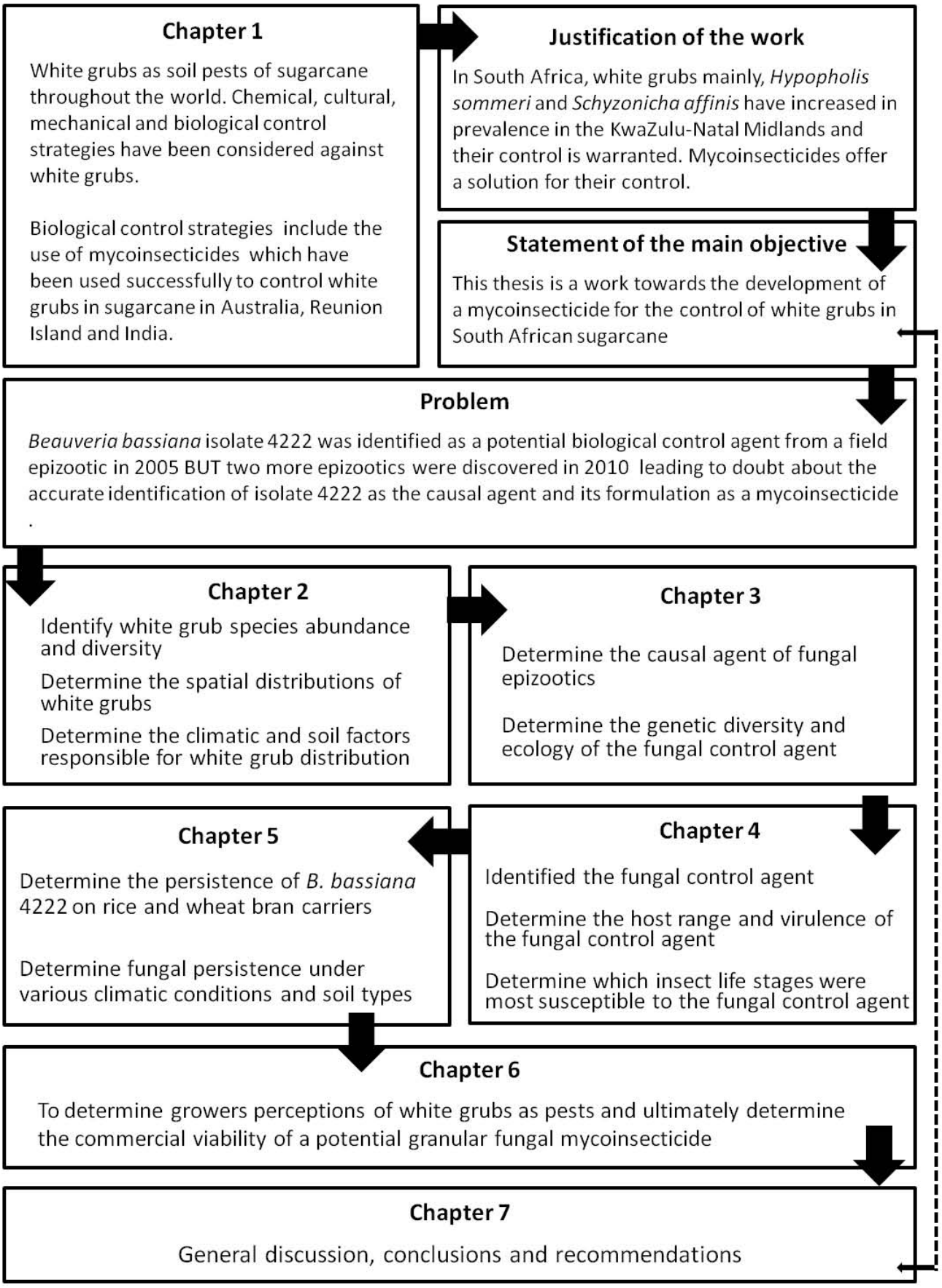

Figure 1.7: A summary of the main objectives and aims of the current study.

CHAPTER 2- White grub population dynamics and seasonality at the Harden Heights site in the Kwa-Zulu-Natal Midlands North

2.1 INTRODUCTION

Effective pest targeting in the application of mycoinsecticides requires accurate identification and extensive knowledge of the target species (Allsopp, 2010). This become particularly important when there is a suite of co-occurring white grub species which have different biology's and ecologies and vary in their susceptibility to various control strategies. It is also important in securing mycoinsecticide registrations, as regulators require these to be based on specific species (Allsopp, 2010) and importantly in understanding the possible non-target effects on non-pest scarab species. Allsopp (2010) further suggested that a good understanding of the systematics of white grubs could help in the understanding of the patterns in their ecology and biology. In Appendix 2.1, the importance of systematic in delineating white grub species in this study is shown. White grub ecology and their occurrence in the KwaZulu-Natal (KZN) Midlands North sugarcane region have been limited to a few short-term studies (Carnegie, 1974; Carnegie *et al.* 1974; Carnegie, 1988; Way, 1997). Carnegie (1974) provided an important overview of the occurrence and life history of two important white grub species, *H. sommeri* and *S. affinis* at two sites in the KZN Midlands regions. However, little biological, ecological and taxonomic literature exists on the lesser known species of white grubs which co-occur in sugarcane with important species such as the wattle chafers, *H. sommeri* and *S. affinis* and in most cases little is known about their effect on sugarcane production (McArthur and Leslie, 2004). Traditionally, within the South African sugar industry, the term 'white grub' has been used loosely to describe a group of root-feeding, soil insects with little regard for the delineation of species, their different biology's and the ecological niches they occupy.

The ecology of a species is closely linked to its distribution patterns and its exploitation of space and resources (Dalthorpe *et al.* 2000a). Spatial heterogeneity or the interspecific differences in the utilization of space by white grubs is a possible reason why many species can co-occur in sugarcane systems where competition exists that could exclude such dynamics (Dalthorpe *et al.* 2000a). The differential utilization of space is closely linked to the biology of a species, which highlights further the importance of understanding differences in white grub biology. 'The foundation of spatial ecology is the quantification of the patchiness of an

organism in space' (Dalthorpe *et al.* 2000b). It is important to understand the nature of patchiness in pest populations within agricultural systems, because some patches may be intense enough to damage a crop while other parts of the field remain undamaged (Dalthorpe *et al.* 2000a). White grubs are such pests which cause patchy damage. By applying a spatial ecology approach, the quantification, occurrence and distribution patterns of such patches can be better understood. Better understanding of the patchiness or spatial ecology of white grubs within sugarcane fields can aid in restricting management tactics to parts of the field where pest populations are high and can significantly enhance the efficacy of mycoinsecticides, limit chemical insecticide use and resistance and conserve natural enemies (Dalthorpe *et al.* 2000a).

The nature of white grub patches is driven by a number of factors and environmental variables, but these are often difficult to predict. For example, the density, distribution and dispersal of populations of Japanese beetle, *P. japonica* have been shown to be strongly affected by the distribution of rainfall, host plants, soil types, soil moisture and natural enemies (Hawley, 1949; Fleming, 1972; Regniere *et al.* 1983). In terms of host plants, it is known that the co-occurrence of black wattle, *A. mearnsii* and sugarcane in the KZN Midlands North and their close proximity to one another is a major contributing factor to the persistence of white grubs in this area, and will continue to aggravate the pest problem as long as they remain juxtaposed (Carnegie, 1974; Carnegie, 1988). Wattle chafers, *H. sommeri* and *S. affinis* utilize both crop species as host plants, with the adults feeding on the leaves of *A. mearnsii* and larvae feeding on the roots of the sugarcane plant (Carnegie *et al* 1974). What is less well-know are the environmental factors which drive white grub population dynamic in this region.

Way *et al.* (2011) collected data on *H. sommeri* and *S. affinis* larval abundance and distribution from 2001 to 2010 (Way *et al.* 2011). It was reported that average annual infestation levels were 2.11 larvae per pit (Way *et al.* 2011). The lowest infestations were observed in 2008 (drought year) when only 0.66 larvae/pit were found, while in 2009 (wet year) the highest record was found with 6.19 larvae/pit. Drought has historically been associated with large decreases in *P. japonica* grub populations and these populations also swell with the increased occurrence of rainfall and subsequent increases in soil moisture (Hawley, 1949; Dalthorpe *et al.* 2000b). Way *et al.* (2011) also found that larval distributions across the KZN Midlands North were inconsistent and that different eco-zones (areas of similar climate and similar potential for sugarcane production) had high, moderate or low infestation levels. These results clarified the patchy occurrence of white grubs in the region and alluded to the fact that climatic variables

may have an effect on white grub abundances. Literature also suggested that climatic variables may strongly determine white grub abundances, occurrence and distribution (Hawley, 1949; Fleming 1972; Regniere *et al.* 1983; Dalthorpe *et al.* 2000b) and recommended the importance of studying these factors in an effort to better understand white grub population dynamics in the KZN Midlands North. Further, understanding of white grub ecology can elucidate factors which cause population outbreaks so that area-wide management tactics maybe undertaken to prevent the spread of these pests (Dalthorpe *et al.* 2000a; Allsopp, 2010).

Therefore, the aims of this study were: 1.) To determine which species of white grubs occurred at a sugarcane field site in the KZN Midlands North; 2.) To determine their population dynamics, occurrences and abundances at the field site; 3.) To assess the spatial distributions of white grub species found at the site; 4.) To determine which weather and soil parameters were important for the distribution and occurrence of white grub species at the field site.

2.2 METHODS AND MATERIALS

2.2.1 *Population dynamics, spatial distributions and climatic associations of white grubs*

2.2.1.1 *Study site*

The study took place on the Harden Heights farm (field number 16) (29°14’54”S; 30°37’52’’E) in the Seven Oaks district of the KZN Midlands North region. This field was one of the locations of observed fungal infections on white grubs. Harden Heights farm is in eco-zone 7, which lies between 901-1400 meters above sea level and is prone to frost in winter (Webster *et al.* 2005). The total size of the field was 14.1 ha however, the panel sampled monthly was approximately 1.14 ha (Figure 2.1). The field was flanked on the eastern and western sides by *A. mearnsii* stands. The sugarcane variety planted was N12 and it was in a 3rd ratoon. Ratooning is a method of harvesting sugarcane which leaves the roots and underground parts of the crop uncut and undisturbed in the soil so that sprouting can occur. The average crop cycle for the field site was 24 months. The average yield for the field was 90 tons of sugarcane per ha.

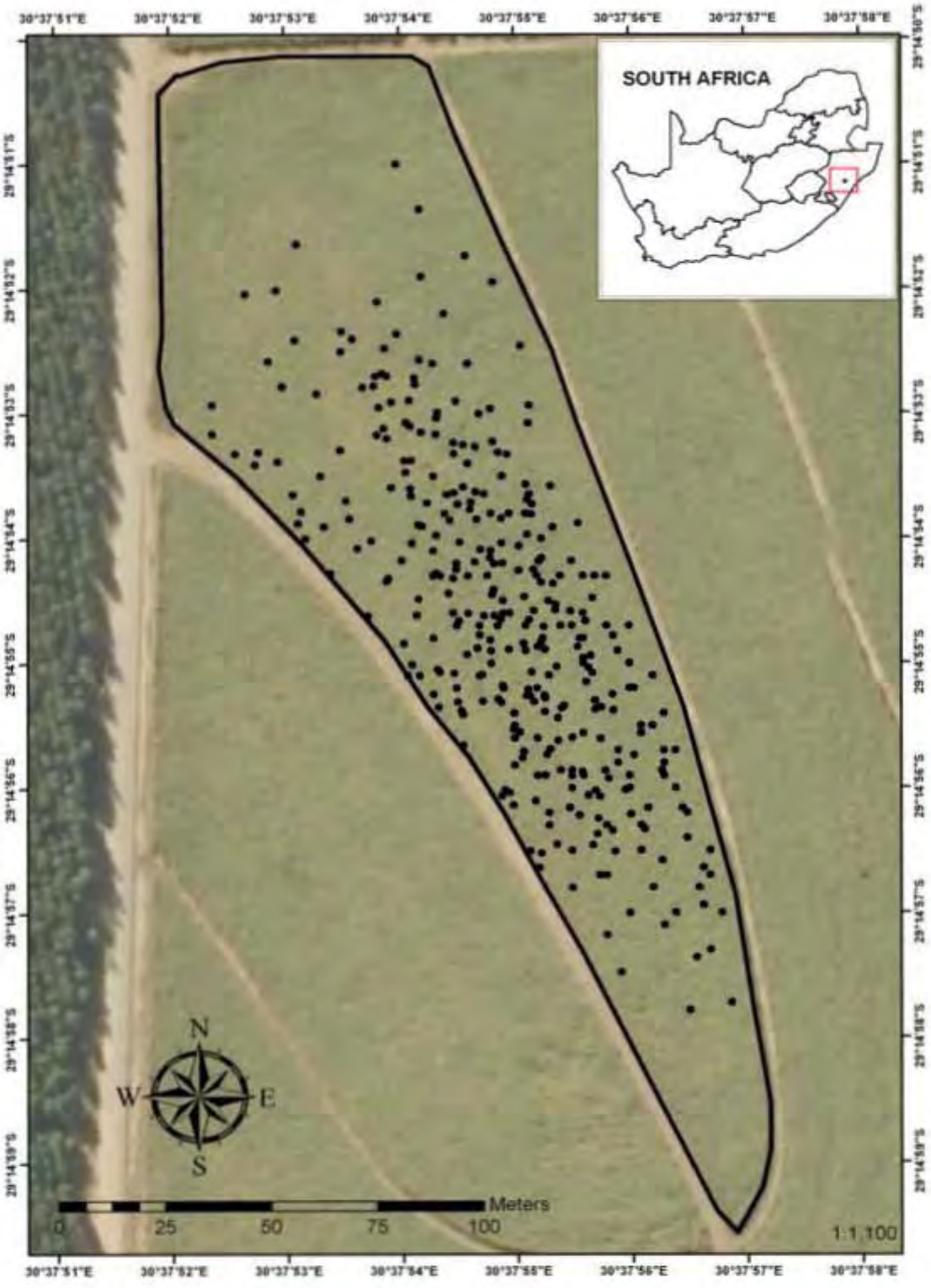

Figure 2.1: The study site: field number 16 (panel size 1.14 ha) of the Harden Heights farm in the Seven Oaks district of the Midlands North region in the KwaZulu-Natal Province. Circles represent sampling pits.

The field was fertilized with a ratio of Nitrogen (5): Phosphorus (1): Potassium (5) at a rate of 700 kg/ha in August 2010 before the study was undertaken and no other applications of fertilizer or gypsum (lime) was undertaken during the study. Sugarcane harvesting at the site took place in November 2012. Soils at this field site were determined as Inanda Form which is comprised of a humic A (thick dark brown powdery humic sandy clay loam to clay) and Red Apedal B (dark red porous friable sandy clay loam to non-structured clay) which overlies parent material (Natal Group Sandstone, Vryheid sediments, Dwyka tillite (SASA, 1999). Topsoil texture was clay loam. Features to note about this soil form are: 1.) rich in organic matter; 2.) physical properties: good and usually very deep; 3.) lime and gypsum are commonly required to correct aluminium toxicity and deficiencies of calcium and magnesium; 4.) phosphorus and zinc are commonly required to correct deficiencies; 5.) these soils mineralise considerable amounts of nitrogen and 6.) potassium requirements maybe higher than average (SASA, 1999).

2.2.1.2 *Sampling method*

White grub sampling took place every month from December 2010 to December 2011. At each sampling month, 40 pits ($30cm^3$) were dug in the sugarcane row incorporating sugarcane stools. The industry-based method for sampling white grubs (Way *et al.* 2011) was applied but modified as follows. The sampling strategy involved entering the field on the western side (Figure 2.1) and then digging and sampling pits in alternating transects, 100 m along the row (~8 pits) and 5 rows apart, until roughly 5 transects had been followed. Information on the row, pit and sample numbers was recorded, as well as the global positioning system (GPS) co-ordinates of each pit. Excavated soils from pits were carefully searched for white grub larvae and other life stages, as well as for fungal mycosed cadavers and parasitoids. All white grub life stages, fungal mycosed cadavers and parasitoid life stages found were collected in 30 ml plastic vials filled with autoclaved peat, sealed with a screw top, containing a gauze covered hole, to allow air to enter the vial, and brought back to the laboratory for identification. White grub larval identification and life stage determination involved analysing larval raster patterns on the ventral surface of the last abdominal segment, as well as recording the head capsule size which is used in the determination of the larval instar age (Wilson, 1969). A dissecting microscope with 16× magnification was used in conjunction with published taxonomic keys (Sweeney 1967; Dittrich-Schröder *et al.* 2009). Digital graphic images of white grub adults and larval stages were taken using a Nikon microscope SMZ1500 with a SLR camera Nikon D3100

(Appendix 2.2). White grub adult genus identification was undertaken by [8]Dr James Harrison at the University of the Witwatersrand, South Africa and [9]Dr Dirk Ahrens at the Zoologisches Staatssammlung München in Munich, Germany. The dipteran parasitoids (Appendix 2.2, plate A2.2.13) were identified by [10]Dr. David Barraclough of the University of KwaZulu-Natal, South Africa. Fungal mycosed cadavers were kept and analyzed using molecular methods which will be discussed in the next chapter (Appendix 2.2, plate A2.2.14). Sixteen unknown white grub larvae found at the site, which could not be identified using standard morphology from taxonomic keys were issued SASRI accession numbers, preserved in 90% ethanol and subjected to molecular analyses (Dittrich-Schröder *et al.* 2009), the results of this analysis can be viewed in Appendix 2.1.

2.2.1.3 *Soil sampling and analyses*

Nineteen soil samples were collected along five transects (see 3.2.1.2 *sampling method*) with four soil samples collected along each transect at a distance of 15 meters apart (transect A-D) and three soil samples collected from the last transect (transect E) within the field site sampling panel. Soil samples were collected in the sugarcane row at a depth of 10-20 cm with a beta auger and placed individually into labelled plastic bags which were transferred in a cooler box, filled with ice packs, directly to the laboratory at the Fertilizer Advisory Service (FAS) of the South African Sugarcane Research Institute (SASRI). All soil sampled sites were collected in March 2011 and recorded with global positioning system (GPS) co-ordinates. This was the only month soil samples were collected in because the cost of analysing many samples was beyond the scope of this study. The soil samples were analysed by FAS for pH which is measured in calcium chloride (CaCl), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), aluminium (Al) (all in ppm), and the percentages of organic matter (%), sand (%), clay (%) and ammonia (NH_3) were also determined according to the procedures of the FAS (www.sasa.org.za).

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2.2.1.4 *Weather data*

Daily weather data: rainfall (mm), soil water content (60 mm), mean/max/min temperature (°C), mean relative humidity of the air (%) and solar radiation (MJ/m^2/d) from December 2010-December 2011 were downloaded from the closest weather station (Noodsberg-Jaagbaan, station 23) (29°21'32"S; 30°41'15"E) on the [11]SASRI weather website. This weather station lay approximately 16 km from the Harden Heights field site. Soil water content was an important climatic parameter for this study and is calculated for rain fed conditions for a soil with total available moisture (TAM) of 60 mm and with a full canopy sugarcane crop growing on it. This weather station did not measure soil temperature which was more biologically relevant to this study than mean air temperature. Thus, soil temperature was calculated by plotting the mean soil temperatures over mean air temperatures (which are closely related) at Mount Edgecombe and using the obtained regression equation (y=1.2161x-1.9572) (R^2=0.8957) to solve for soil temperature (y) by substituting the mean air temperatures (x) obtained from Noodsberg-Jaagbaan station 23 in the KZN Midlands North into the equation. Some weather data namely, rainfall, relative humidity of the air, soil water content and minimum and maximum temperatures, were unusual compared to the long term mean (LTM) in 2011 and these variables were thus plotted against the LTM in Figure 2.2. Weather variables which did not deviate from the LTM were solar radiation and mean temperature and they were not plotted.

2.2.1.5 *Statistical analyses*

The spatial distributions of four commonly occurring white grub species (*H. sommeri*, *S. affinis*, *Maladera* sp11 and *Anomala* sp.) and fungal mycosed cadavers, as determined by sampling, were analysed within the field (see results), as the exact position of each sampling point in the field was known (GPS). Spatial analysis was also restricted to these species as other white grub species occurred too infrequently for a reliable analysis. This was done using the software programme Spatial Analysis of Distance Indices (SADIE) (Perry *et al.* 1999; Perry and Dixon, 2002). The method used information of the positions of the samples in two dimensional space as well as the count values of the samples. The SADIE programme compared the observed dataset with a large number of permutated randomisations of similar values.

[11] ***SASRI WEATHER WEB- http://portal.sasa.org.za/weatherweb***

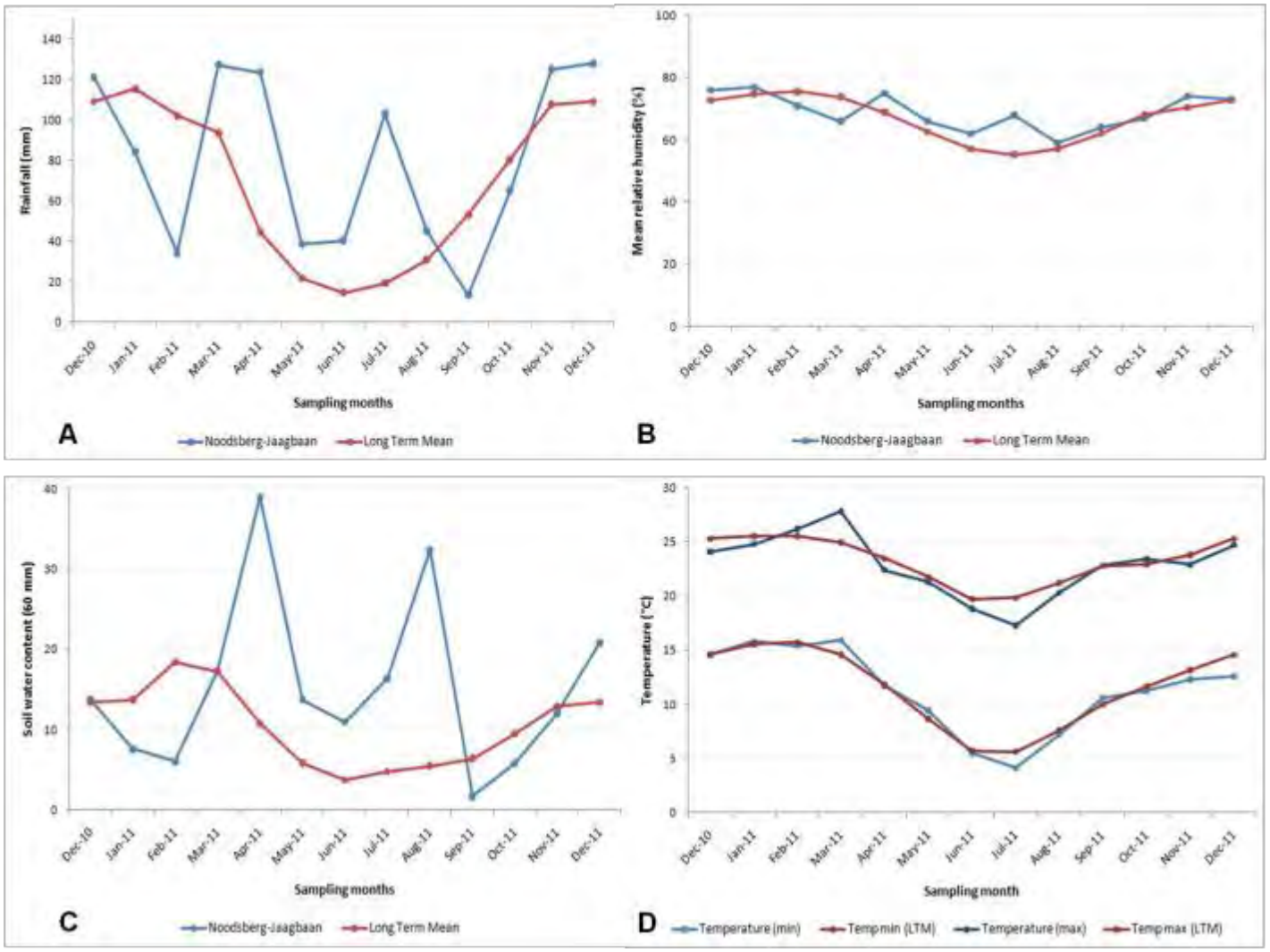

Figure 2.2: Mean rainfall (mm) which occurred over the sampling months plotted against the long term mean (LTM) which was calculated from a decade of recorded data (A); Mean relative humidity of the air (%) plotted against the LTM which was also calculated from a decade of recorded data (B); Soil water content (60 mm) plotted against the LTM which was calculated from a decade of recorded data (C); The maximum and minimum temperatures experienced over the sampling months plotted against their respective LTM's which were calculated from a decade of recorded data (D). All weather data was obtained from Noodsberg-Jaagbaan weather station number 23.

For each sample unit a dimensionless clustering index was identified based on the actual data and outcome of the randomisations. For each unit with count larger than average ('patch' unit) an outflow index, *vi*, was calculated and for each unit with count smaller than average ('gap' unit) an inflow index, *vj*, was found. A test for overall clustering was performed for

each data set by calculating an average index of *vi* and *vj*, respectively. This was then compared to the values of the randomisations. Thus, tests for both patches and gaps were made independently (Perry *et al.* 1999). By convention, clustering indices >1.5 indicated that the sampling units were members of a patch while clustering indices < -1.5 were interpreted as belonging to a gap area. The spatial locations of these indices identified patches (aggregations of units with large clustering indices) and gaps (aggregations of units with small clustering indices) (Perry *et al.* 1999). Maps were drawn using contours based on clustering indices which were interpolated between sample units by the package SURFER™ version 10 (Golden Software).

Different white grub life stages, fungal mycosed cadavers and parasitoids found per month, as well as weather data and soil characteristics, were analysed using principal component analysis (PCA) (Hotelling, 1933; ADE4 software, Thioulouse, 1997) which indicated the likelihood of relationships between components. Factorial maps produced from PCA showed the relationships between different months (treatments) and white grub life stages found. PCA factorial maps also showed the relationships between common white grub life stages, fungal mycosed cadavers, parasitoids, weather and soil data. Using co-inertia analysis, it was also possible to compare factorial maps of the above mentioned components against weather data to find relationships (Dray, 2003; Thioulouse, 2004). Co-inertia analysis is a two-table coupling method. The principle of co-inertia analysis is aimed at searching for axes that maximize the covariance between the row co-ordinates of the two tables (Dray, 2003; Thioulouse, 2004; Berry *et al.* 2011). The permutation test was done by changing the position of the rows of one of the tables and recalculating the total inertia of the co-inertia analysis. One thousand random permutations were performed and the frequency of simulations above and below the observed inertia value of the original row order calculated. If the number of simulations where $X \geq$ observed is greater than 50 then the two tables were not significantly related ($P \geq 0.05$). If number of simulations when $X \geq$ observed is less than 50 then the two tables were significantly ($P \leq 0.05$) related to each other.

All data were checked for normality and corrected if necessary using a square-root transformation. In some instances data were combined (*S. affinis* 1st and 2nd instars and *Anomala* sp. 1st and 2nd instars) because of low sampling numbers. Model building was undertaken by performing multiple regressions (generalized linear model) using the best subset model in Statistica 10 (StatsSoft Inc) (Appendix 2.3). Multicollinearity between

weather variables was checked by comparing Pearson correlation coefficients. Initially, six predictor variables were considered: rainfall, mean relative humidity, soil water content, solar radiation, mean temperature and soil temperature in the co-inertia analysis. There was however significant collinearity observed between some variables, particularly mean temperature and soil temperature (R^2=0.98, P≤0.001). Freckleton (2011) suggested that the most straight forward way to deal with collinear variables was to use a data reduction method such as PCA, which was undertaken as described above. Soil temperature was a more biologically relevant parameter for this study and was thus included in model building but mean temperature was excluded. The Akaike Information Criterion (AIC) (Akaike, 1983; Burnham and Anderson, 2002) was used to select the best models. Where the best models had multiple parameters, an impact factor (1 being the highest) was calculated by summing the Akaike weights (w_t) of those parameters in every valid model they appeared in. Further, parameter coefficients were checked for significance as another model variable selection method in Statistica 10 (StatsSoft Inc). Finally, simple or multiple regression analyses were used to validate the best models selected, which predicted the distributions or occurrences of each white grub species and their relevant life stages as well as fungal mycosed cadavers and parasitoids.

2.3 RESULTS

2.3.1 *Population dynamics of white grubs*

In total nine different larval species were determined from pit sampling using taxonomic keys and molecular analysis which represented six white grub genera (Appendix 2.1; Appendix 2.2, Plates A2.2.1-A2.2.5). Since molecular phylogenic analysis was able to determine some of the unknown SASRI larvae to genus level (Appendix 2.1), the larvae are referred to by genus name. These included: SASRI 30 (=*Adoretus* sp.), SASRI sp X (=*Congella* sp.), *H. sommeri*, SASRI 11A (=*Maladera* sp11), SASRI 11B (=*Maladera* sp11), SASRI 31(=*Maladera* sp1), SASRI 26 (not determined) and *S. affinis*. These larval sequences are currently stored on a database reserved by Mike Way and will form part of a larger molecular taxonomic study of white grubs which is ongoing at SASRI. *Anomala* sp. are referred to tentatively because larvae were identified using taxonomic keys only. There is subjectivity associated with taxonomic keys and further no *Anomala* sp. adults were collected at the site, bringing this species identification into question. Due to the difficulty in separating the

morphospecies SASRI 11A and 11B, they are referred to as one species for the remainder of this chapter, i.e: *Maladera* sp11.

In addition, six different Melolonthinae adult species were identified, these included the Melolonthini: *S. affinis* and *H. sommeri*, the Hopliini: *Congella* sp. and Sericini: *Trochalus* sp. and two *Maladera* sp. In addition, adult Rutelinae included only the Adoretini: *Adoretus* sp. Pinned adult beetles are currently housed in the SASRI collection maintained by Mike Way (Appendix 2.2, Plates A2.2.6-A2.2.12). These adult species identifications confirmed the results of phylogenic analysis (Appendix 2.1) and the presence of the above mentioned larvae at the Harden Heights site.

Parasitoids were found infecting *H. sommeri* 3rd instar larvae in August at the site. Usually, two fly larvae were found within one infested *H. sommeri* grub. They were identified as belonging to the Tachinidae: Dexiinae, known parasitoids of white grub larvae and especially *H. sommeri* (Harrison, 2013). Pinned adult flies are currently housed in the SASRI collection maintained by Mike Way (Appendix 2.2, Plate A2.2.13). The fungus that was found infecting white grubs (mycosed cadavers) was identified as belonging to the *Beauveria* genus using various molecular techniques which will be discussed in the next chapter (Chapter 3). For the remainder of this chapter we know refer to fungal mycosed cadavers as *Beauveria*-infected cadavers (Appendix 2.2, Plate A2.2.14). Fungal isolates are currently housed in the South African National Collection of Fungi at the Plant Protection Research Institute (Agricultural Research Council) in Pretoria, South Africa.

Distinct seasonal occurrences, fluctuations in abundance and different white grub species were observed during the 13 months of sampling at the Harden Height field site (Figure 2.3). Seasonal fluctuations in populations were observed with greater abundances of white grubs in summer months and a gradual decline in abundance in winter months. Only *H. sommeri* and *S. affinis* persisted at the site throughout the year (Figure 2.3) whilst other species seemed more abundant in either summer (*Anomala* sp. and *Adoretus* sp.) or winter (*Maladera* sp 11 and *Congella* sp.). Two white grub species, SASRI 31 (=*Maladera* sp.) and SASRI 26 were found once in summer but, were then not sampled again (Figure 2.3). A total of 121 *Beauveria*-infected cadavers were collected from monthly sampling at the Harden Heights and the occurrence of cadavers was different throughout the year with the greatest occurrences observed in April (14 cadavers) and September (22 cadavers) (Figure 2.3).

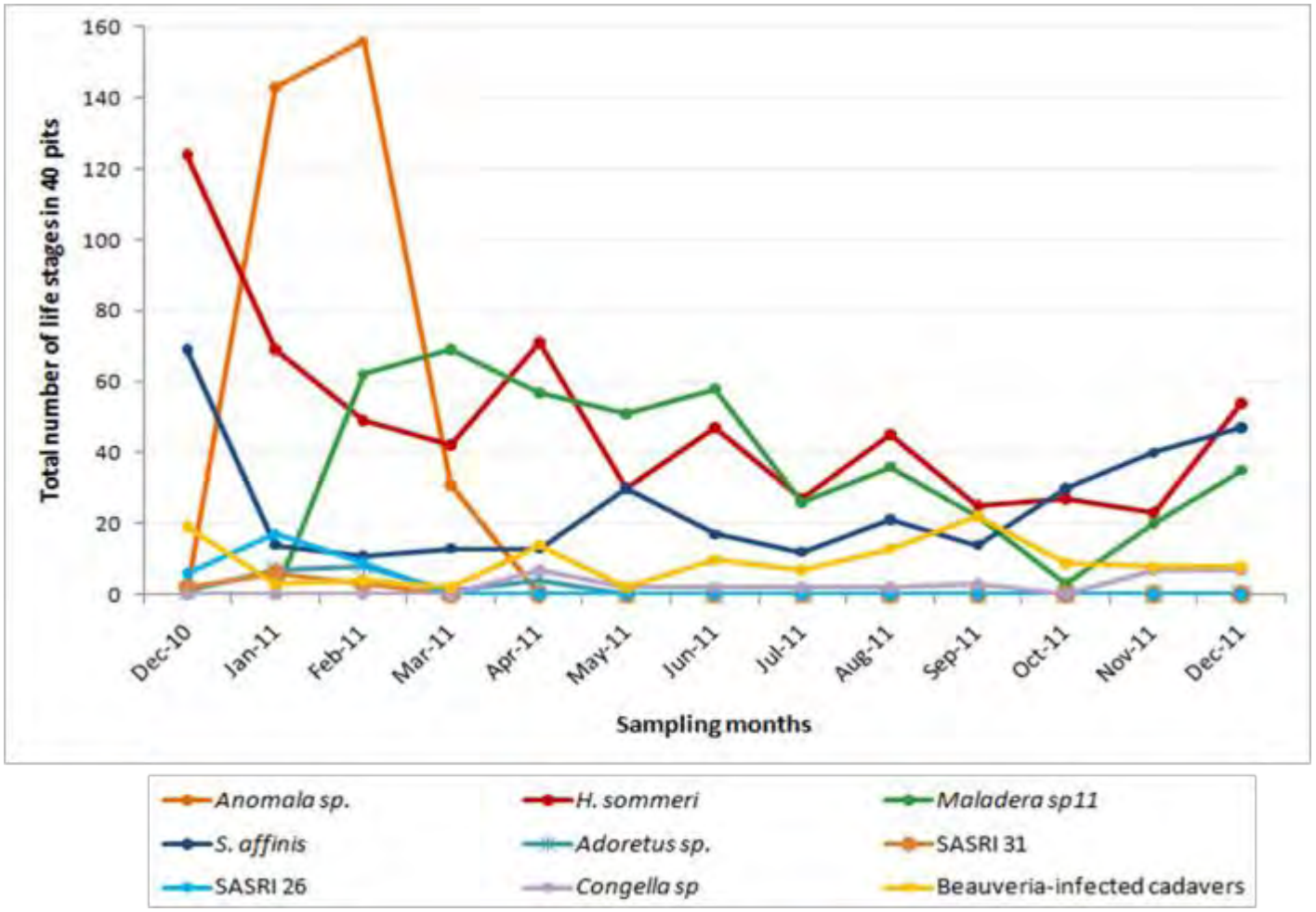

Figure 2.3: The total monthly abundance of white grub species and *Beauveria*-infected cadavers sampled at Harden Heights. The y-axis represents the total number of life stages that were sampled from 40 pits per month.

A total of 328 *S. affinis* life stages were obtained at the field site over the sampling period (Figure 2.4). This totalled 228 larvae (1^{st} instars=36; 2^{nd} instars=12; 3^{rd} instars=180), and 100 adults were found. Pupae were excluded from this analysis due to the difficulty in accurately determining *S. affinis* pupae because all species pupal sizes were very similar at Harden Heights and no pupae could be reared through to adulthood. *Schizonycha affinis* 1^{st} and 2^{nd} instar grubs occurred in high numbers in December months (Figure 2.4). Third instars occurred in the great abundance in December 2010 and then persisted throughout the year at stable numbers of approximately 10-18 larvae obtained in 40 pits per month. The adult occurrence was different between months with increases in adult numbers in May, August and November (Figure 2.4).

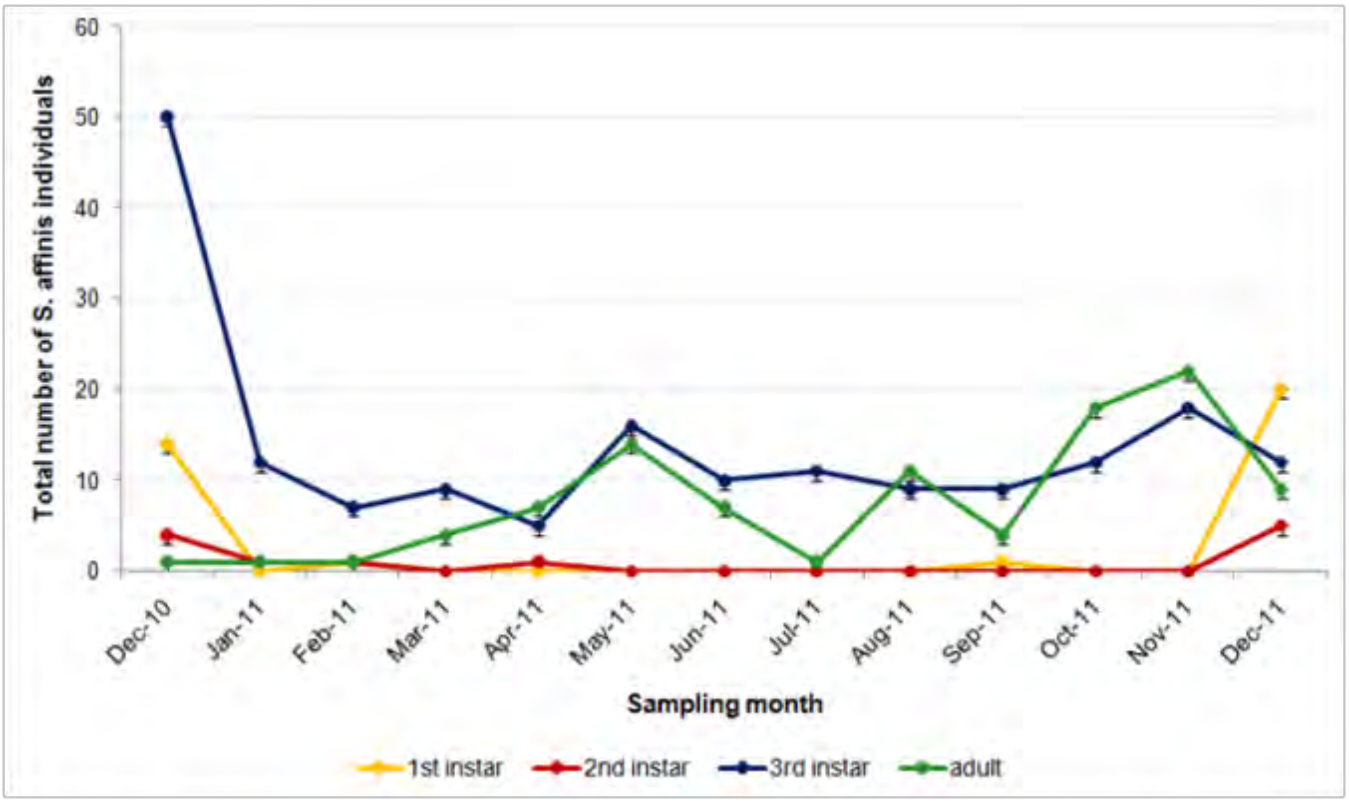

Figure 2.4: The seasonal abundance of the different life stages of *Schizonycha affinis* sampled at Harden Heights. The y-axis represents the total number of individuals that were sampled from 40 pits per month.

A total of 633 *H. sommeri* life stages which included 598 larvae (1st instars=85; 2nd instars=57; 3rd instars=456), 24 adults and 11 pupae were obtained over the sampling period (Appendix 2.2, plate 2.2.1, 2.2.9). *Hypopholis sommeri* 1st instars occurred with a defined peak in December and a 2nd instar peak occurred in January. Third instars persisted throughout the year but at fluctuating levels (Figure 2.5). A limited number of pupae were collected, except from September through to November (Figure 2.5). Pupae were included in this analysis because they were reared through to adulthood to confirm identity. In addition, *H. sommeri* pupae are larger than pupae of other white grub species occurring at Harden Heights. A few adults were collected from November through to January (Figure 2.5).

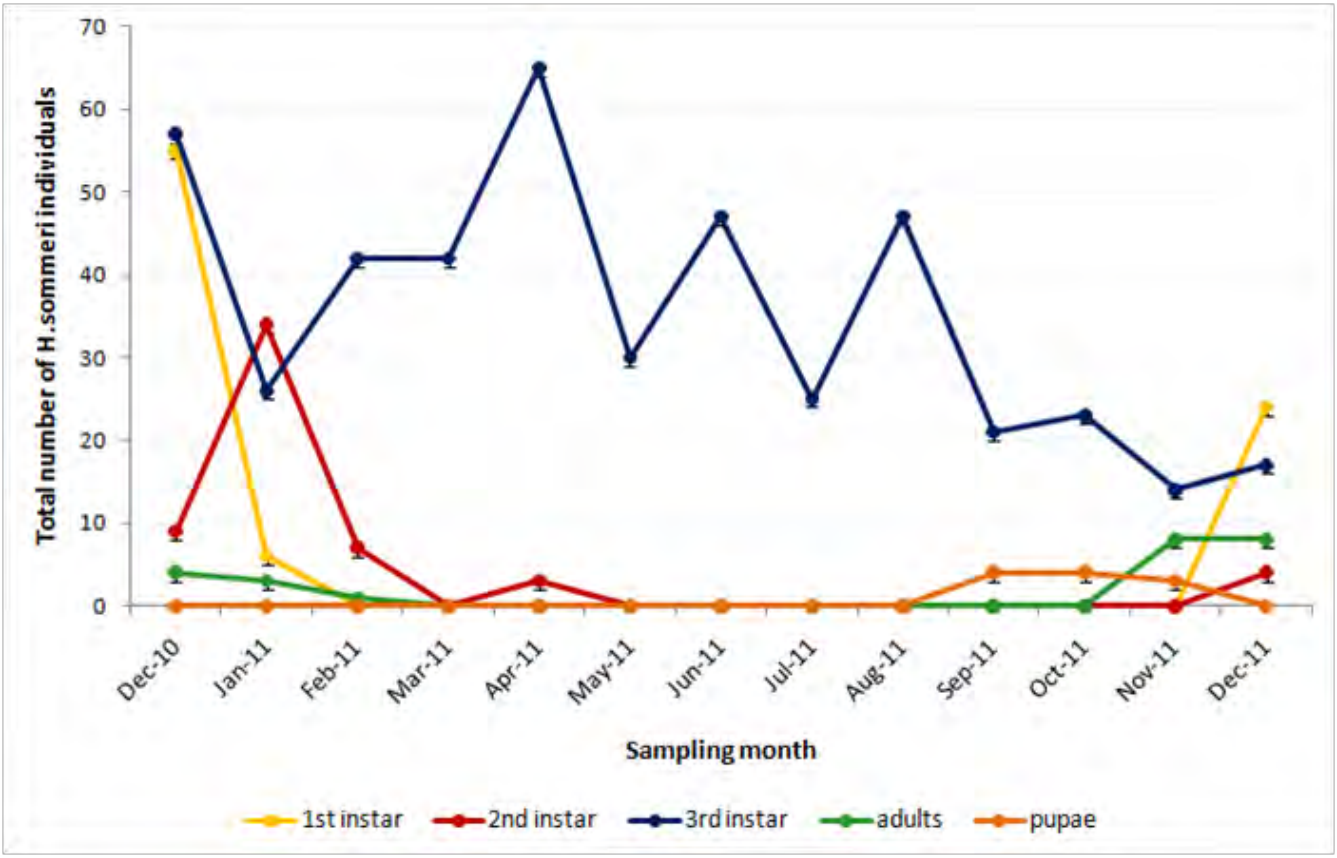

Figure 2.5: The seasonal abundance of all life stages of *Hypopholis sommeri* sampled at Harden Heights. The y-axis represents the total number of individuals that were sampled from 40 pits per month.

The ruteline white grub, *Anomala* sp. occurred in high numbers in the pits sampled (up to 120 individuals in February) from January to March but was not found again for the rest of the year (Figure 2.6). A total of 327 *Anomala* sp. larvae were obtained over the three months (1st instars=10; 2nd instars=59; 3rd instars=258), no adults or pupae were found. The 1st instar grubs were found in February only, while 2nd instars were found in January and February (Figure 2.6). The 3rd instar *Anomala* sp. grubs occurred from January to March in higher numbers compared to younger larval stages (Figure 2.6). In total, only 10 *Adoretus* sp adults (Appendix 2.2, plate A2.2.11) were collected and their occurrence was limited to January through to April. Three *Adoretus* sp 2nd instar larvae (included in the phylogenetic analysis) were collected in December 2010 and not found again (data not shown).

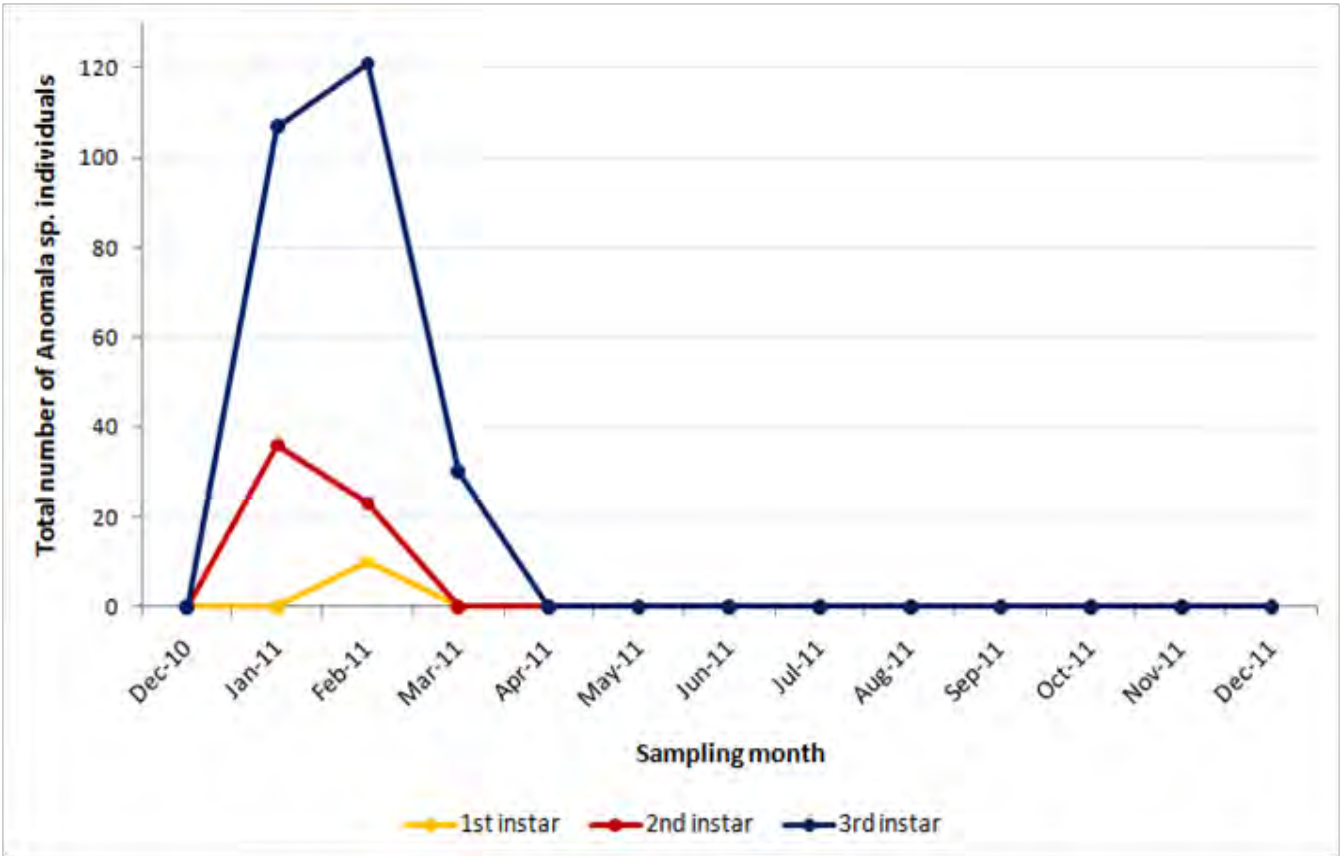

Figure 2.6: The seasonal abundance of all life stages of *Anomala* sp. sampled at Harden Heights. The y-axis represents the total number of individuals that were sampled from 40 pits per month.

Another commonly occurring white grub, *Maladera* sp11, appeared to have a life cycle that was different from other collected white grub species (Figure 2.7). A total of 439 *Maladera* sp11 larvae were obtained over the sampling period (1st instars=61; 2nd instars=109; 3rd instars=269), no confirmed pupae were found (Figure 2.7). Despite *Maladera* sp. adults being identified at the site it was not possible to link the adult occurrences with the larvae collected because phylogenetic comparison was not possible due to poor adult sequence chromatograms (unpublished data). *Maladera* sp11 1st and 2nd instar occurrences fluctuated from February to June while 3rd instars were found from March to October (Figure 2.7). Two different *Maladera* morphospecies (11A and 11B) were observed (Appendix 2.2, plate A2.2.2) during the sampling period but due to the difficulty in separating the two species in the laboratory, they were considered one species in these analyses (Appendix 2.1).

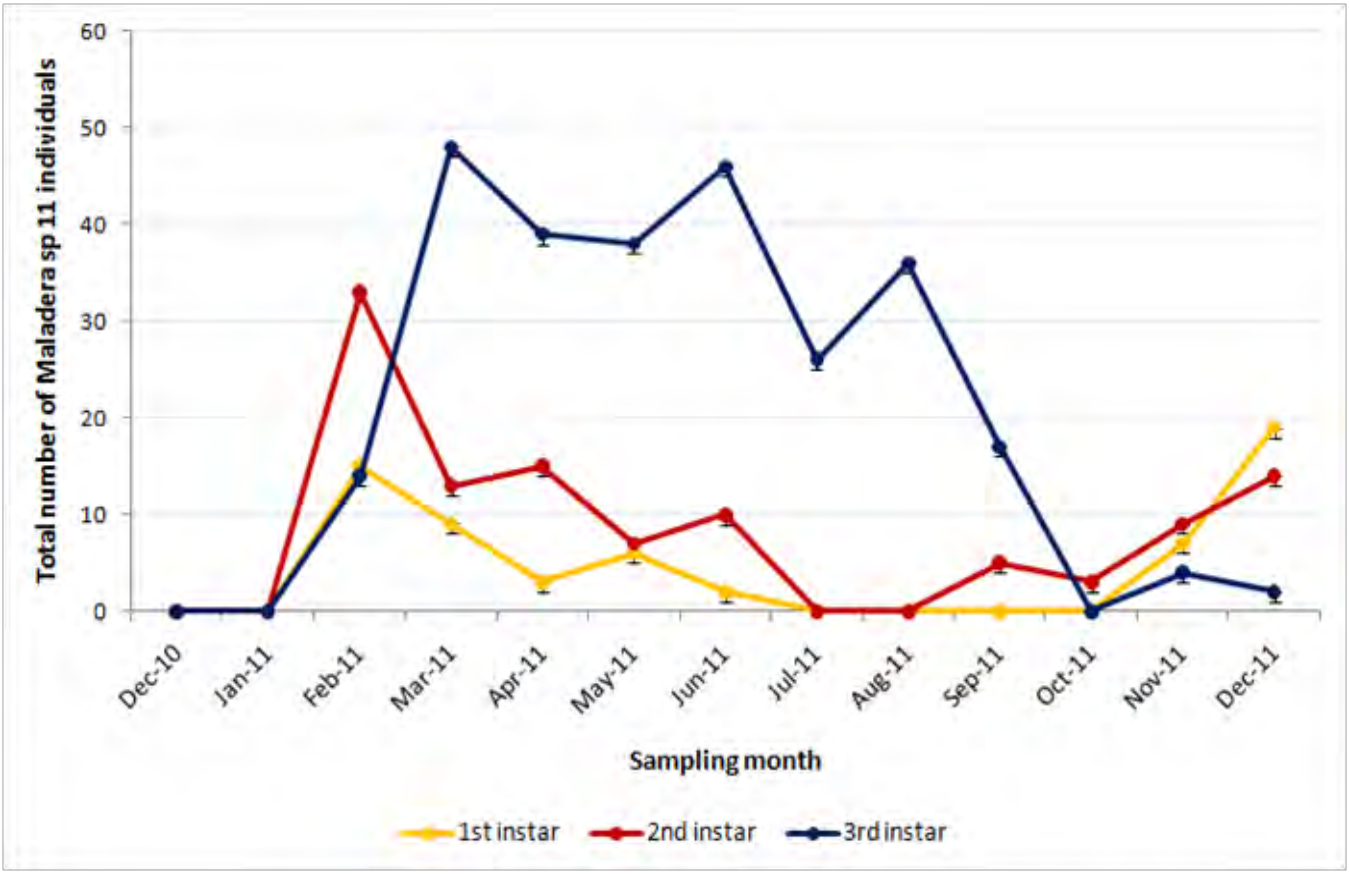

Figure 2.7: The seasonal abundance of all life stages of *Maladera* sp11 sampled at Harden Heights. The y-axis represents the total number of individuals that were sampled from 40 pits per month.

There were a number of white grub species which occurred in smaller abundances at Harden Heights. *Congella* sp. 1st, 2nd and a few 3rd instar larvae (Appendix 2.2, plate A2.2.3) were collected in April for the first time but not collected again until November. From April onwards, 3rd instars persisted throughout the year at relatively low numbers. *Congella* sp. adults (Appendix 2.2, plate A2.2.10) appeared in June and July. In November, *Congella* sp. 1st instars were collected again. A total of 37 larvae of the undetermined white grub species (SASRI 26) occurred from December to February. First and 2nd SASRI 26 instars (Appendix 2.2, plate A2.2.1) occurred together in January but only 2nd instars were collected in February. There was a limited occurrence (five individuals) of adult *Trochalus* sp. in November and December. Likewise five adults of the white grub *Maladera* sp1 were found in pits in November and December and three individual adult *Maladera* sp2 (Appendix 2.2, Plates A2.2.6-A2.2.8) were found only in pits in December. As the occurrence of these

uncommon white grub species were so limited, no further discussion or reference will be made to them again in this chapter.

2.3.2 *Spatial distributions of white grubs*

The factorial map of the PCA (describing 62% of the variability) shows more white grub species were collected in summer months compared to winter and greater numbers of individual species were collected in summer months as indicated by the long branches radiating from the circles (gravity centre's) in the plot (Figure 2.8). The gravity centre's of all summer months were located in the negative (left) section of the horizontal (F1) axis and winter months were in the opposite section of the plot and showed less variability, as indicated by the shorter lines radiating from the gravity centre's (Figure 2.8).

Summer (Dec 2010, Jan 2011, Feb, March, Oct, Nov and Dec 2011) and winter (April, May, June, July, Aug, Sep) months were determined by where they lay on the factorial map of the PCA and will be referred to as such for the remainder of this chapter. The spatial analysis (undertaken in SADIE) of the distribution of all *H. sommeri* larvae (1^{st}, 2^{nd} and 3^{rd} instars) in summer months showed significant clustering with respect to patches (average vi = 1.905; $P \leq$ 0.01) and gaps (average vj = -2.139; $P \leq 0.001$) (Figure 2.9 A). In winter however, no clustering within *H. sommeri* was observed as higher numbers of 3^{rd} instar larvae were shown to have a random distribution (Figure 2.9 B). When *H. sommeri* adults were considered over the whole year they occurred in patches (average vi = 1.521; $P \leq$ 0.01) but no significant gaps (average vj = -1.288; $P \leq 0.07$) were observed. All larval life stages of *S. affinis* in summer months showed significant clustering with regard to patches (average vi = 1.517; $P \leq 0.001$) and gaps (average vj = -1.583; $P \leq 0.01$) (Figure 2.9 C). However, 3^{rd} instars dominated in winter months and had a random spatial distribution with regard to patches (average vi = 0.877; $P \leq 0.73$) and gaps (average vj = -0.972; $P \leq 0.46$) (Figure 2.9 D). *Schizonycha affinis* adults were significantly clustered with regard to patches (average vi = 1.676; $P \leq 0.001$) and gaps (average vj = -1.843; $P \leq 0.001$) when they occurred in the field.

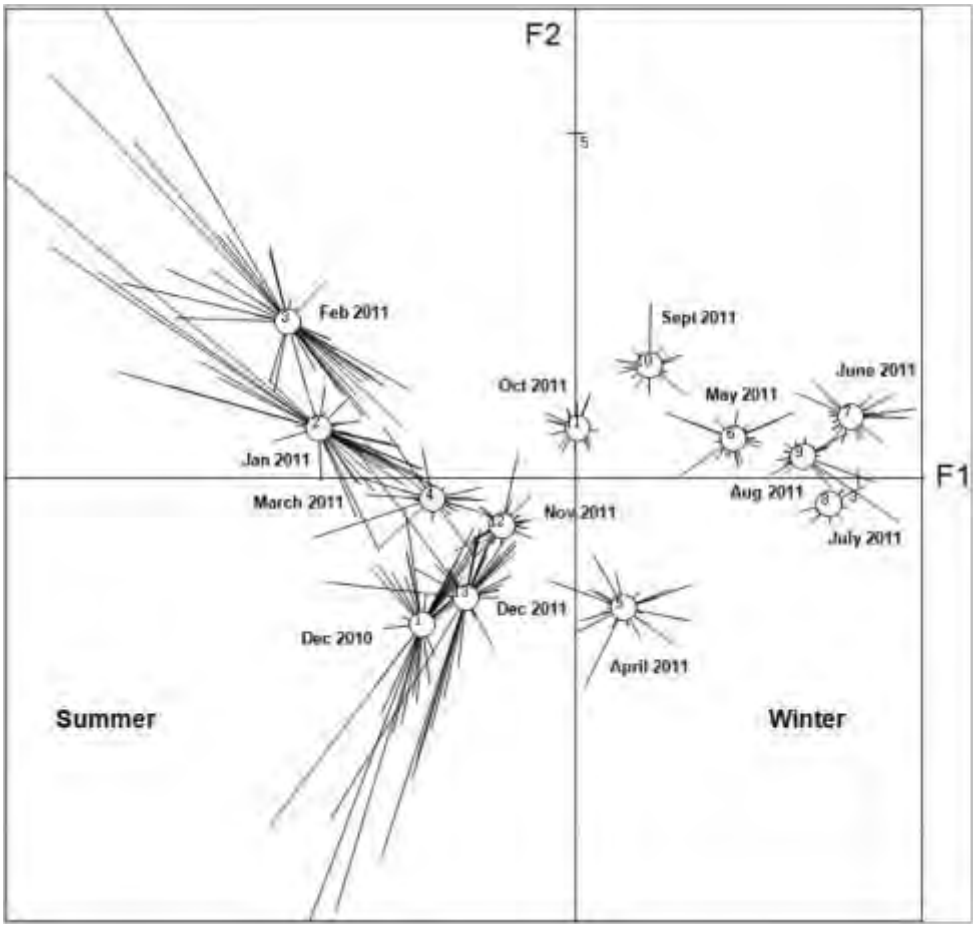

Figure 2.8: PCA factorial map of all white grub life stages collected per month, showing the relationship between summer and winter sampling months at Harden Heights. The circles (gravity centres) represent the various sampling months and each branch radiating from the circle represents the abundance and the variability of all the white grub life stages sampled per month.

All *Anomala* sp. larvae demonstrated significant clustering in summer (which was the only season this species was sampled) with regard to patches (average *vi* = 2.211; $P \leq 0.001$) and gaps (average *vj* = -2.029; $P \leq 0.001$) (data not shown). The spatial distributions of *Maladera* sp11 within the field was similar to the above mentioned white grub species. In summer larvae formed significant patches (average *vi* = 1.745; $P \leq 0.001$) but there were no significant gaps observed in larval distribution (average *vj* = -1.373; $P \leq 0.06$) (data not shown). In winter *Maladera* sp11 showed a random distribution pattern in terms of patches (average *vi* = 1.011; $P \leq 0.50$) and gaps (average *vj* = -0.965; $P \leq 0.51$) (data not shown).

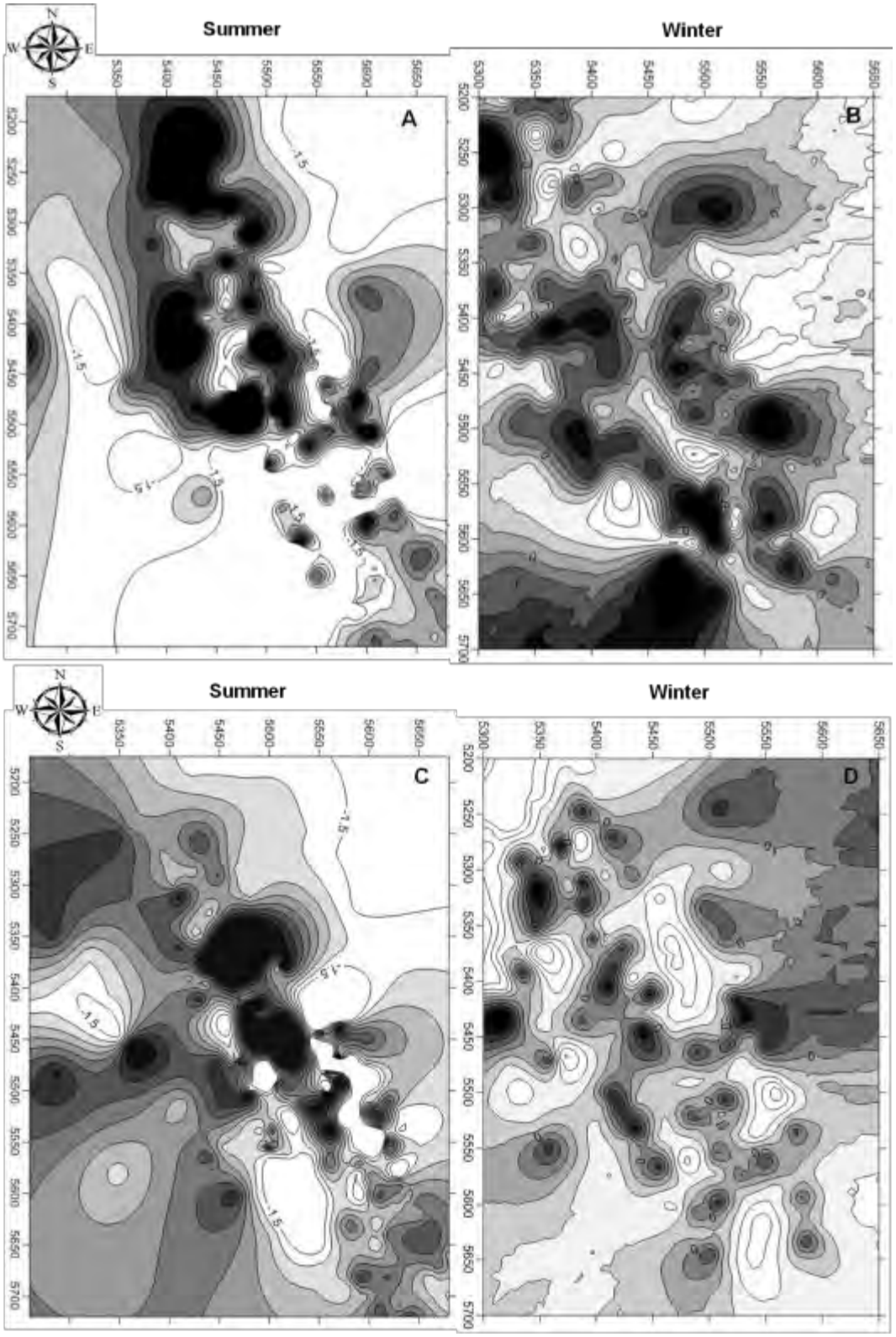

Figure 2.9: The spatial distribution of all *H. sommeri* larvae in summer (A); and winter (B); at the Harden Heights study site, and the spatial distribution of *S. affinis* larvae in summer (C); and winter (D); at the same site. Maps of clustering in larvae were calculated by the program, SADIE. Clustering indices equal to -1, 0 and 1 are represented in white. Grey shaded areas represent 'gaps' i.e: areas where clustering indices are below -1.5. Black areas are 'patches' were clustering indices are above 1.5. The scale of this figure is in seconds. The map was drawn in SURFER™.

Some of the observed summer clustering was associated with the co-occurrences of various white grub larval stages and adults of various species which co-occurred in the same sampling pits. There was a significant spatial association between *H. sommeri* 1st (Figure 2.10 A) and 3rd instar grubs (χ^2=0.104; df=1; P≤0.02) (Figure 2.10 C) and they occurred together in some summer sampling pits, particularly in December (data not shown). No significant spatial associations were observed when *H. sommeri* 2nd instars were compared to 1st and 3rd instar patches (Figure 2.10 B). *H. sommeri* adults were significantly associated with 1st instars (χ^2=0.35; df=1; P≤0.008) but not with 2nd instars (χ^2=0.07; df=1; P≤0.20).

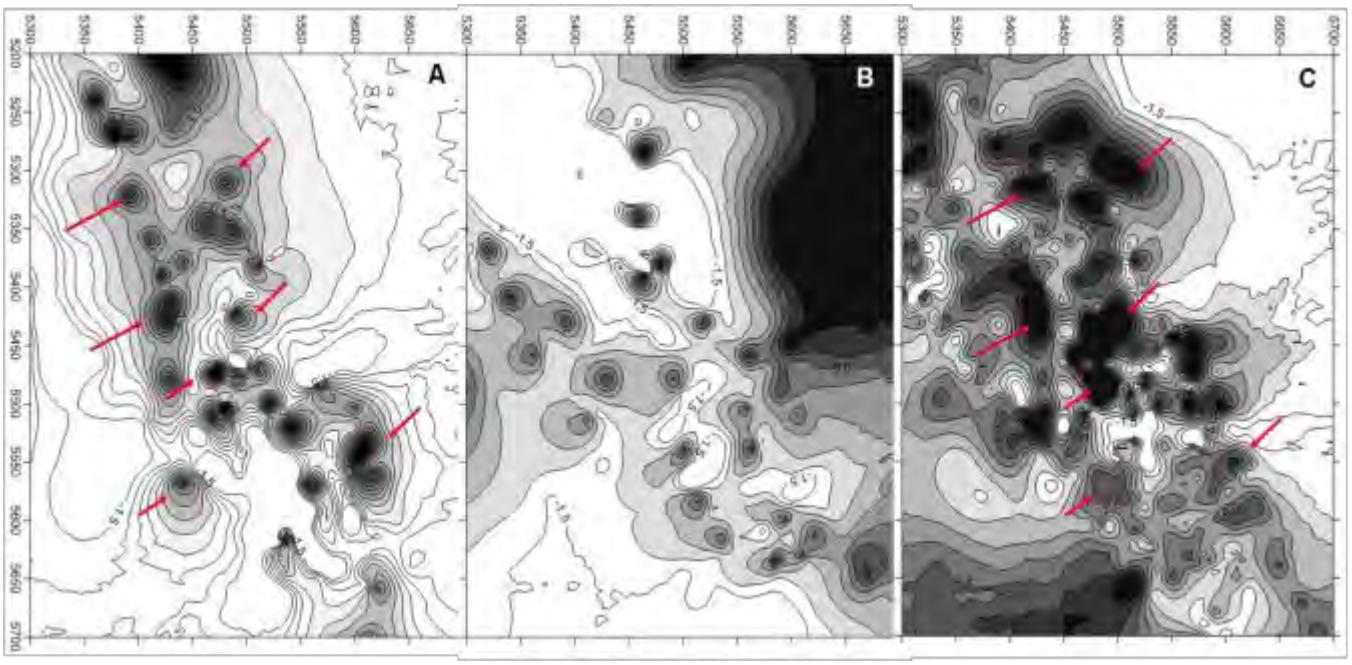

Figure 2.10: The spatial distributions of H. sommeri 1st instar larvae (A); second instar larvae (B); and third instar larvae (C); at the Harden Heights study site. Clustering indices equal to -1, 0 and 1 are represented in white. Grey shaded areas represent 'gaps' i.e: areas where clustering indices are below -1.5. Black areas are 'patches' were clustering indices are above 1.5. The scale of this figure is in seconds. Arrows indicate areas where aggregations of 1st and 3rd instars were similar.

A significant association was measured between the spatial distributions of *S. affinis* 1st and 2nd instar larvae (χ^2=0.407; df=1; P≤0.001) and they were frequently found together in the same sampling pits (data not shown). However, no significant associations were observed in the distribution of *S. affinis* 1st and 3rd instars or *S. affinis* 2nd and 3rd instars. Further, adult *S.*

affinis were not spatially associated with any larval instars (data not shown). There was a highly significant association between the spatial distributions of all *Anomala* sp larval stages (1st and 2nd: χ^2=0.54; df=1; P≤0.001) (2nd and 3rd: χ^2=0.52; df=1; P≤0.001) (3rd and 1st: χ^2=0.53; df=1; P≤0.001) which occurred together in sampling pits (data not shown). Finally, *Maladera* sp11 showed a significant spatial association between 1st (Figure 2.11 A) and 2nd instar larvae (χ^2=0.45; df=1; P≤0.001) (Figure 2.11 B) and a weaker association between 2nd and 3rd instars (Figure 2.11 C) which occurred in the same pits (χ^2=0.104; df=1; P≤0.01). The co-occurrence of *Maladera* sp11 1st and 3rd instars was not significant.

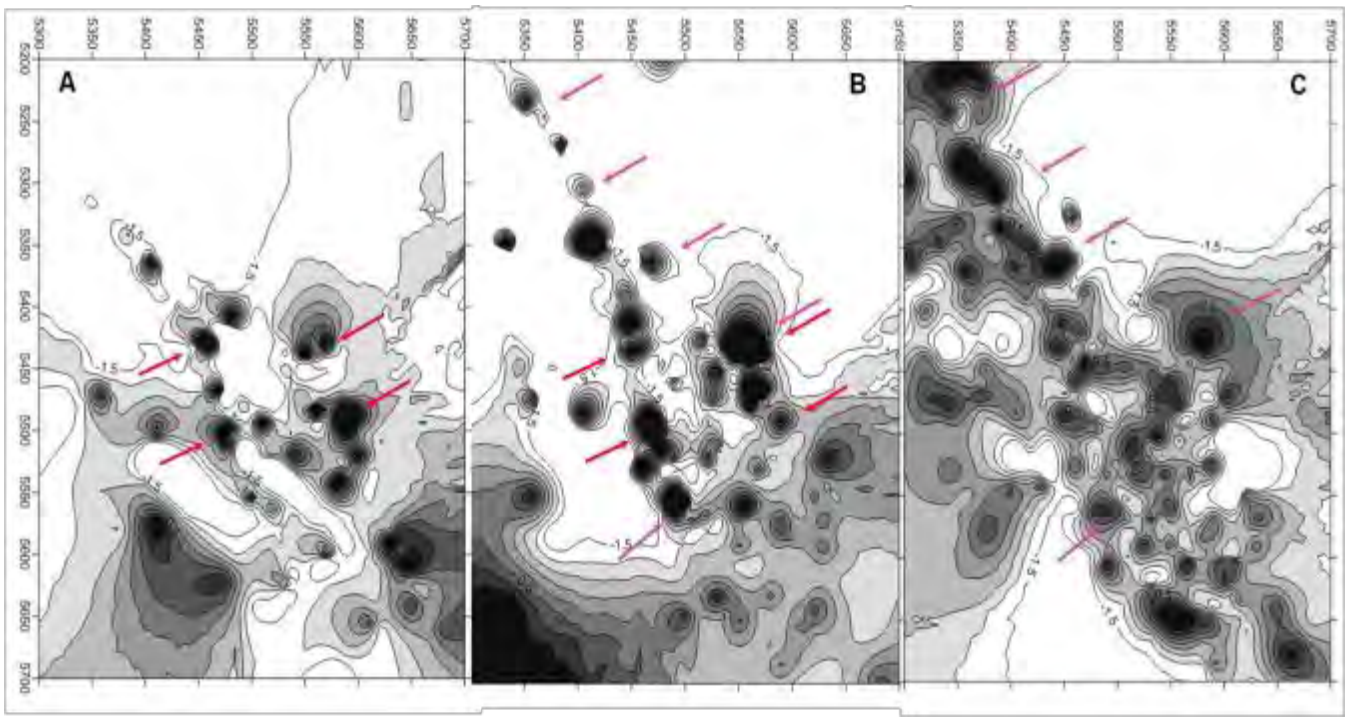

Figure 2.11: The spatial distribution of *Maladera* sp11 1st instar larvae (A); second instar larvae (B); and 3rd instar larvae (C); at the Harden Heights study site. Clustering indices equal to -1, 0 and 1 are represented in white. Grey shaded areas represent 'gaps' i.e: areas where clustering indices are below -1.5. Black areas are 'patches' were clustering indices are above 1.5. The scale of this figure is in seconds. Arrows indicate areas where aggregations of 1st and 2nd instars and 2nd and 3rd instars were similar.

The analysis of the spatial distribution of *Beauveria*-infected cadavers showed no significant clustering with respect to patches (average vi = 1.292; P≤0.192) or gaps (average vj = -1.287; P≤0.205) (Figure 2.12). The distribution pattern of *Beauveria*-infected cadavers over the

whole field surface could therefore not be distinguished from that of a random distribution (Figure 12). Further, there was no significant clustering when both summer (*vi* = 0.954; *P*≤ 0.474) (*vj* = -1.008; *P*≤0.346) or winter (*vi* = 0.988; *P*≤0.525) (*vj* = -0.974; *P*≤0.564) months were considered (Figure 2.12). During monthly sampling there were a number of *Beauveria*-infected cadavers which occurred in the same pits as *H. sommeri* 3rd instar larvae, and many of the infected cadavers were those of *H. sommeri* 3rd instar larvae. The spatial distribution of *Beauveria*-infected cadavers however did not match those of *H. sommeri* 3rd instar larvae (χ^2=0.041; df=1; *P*≤0.21) (data not shown).

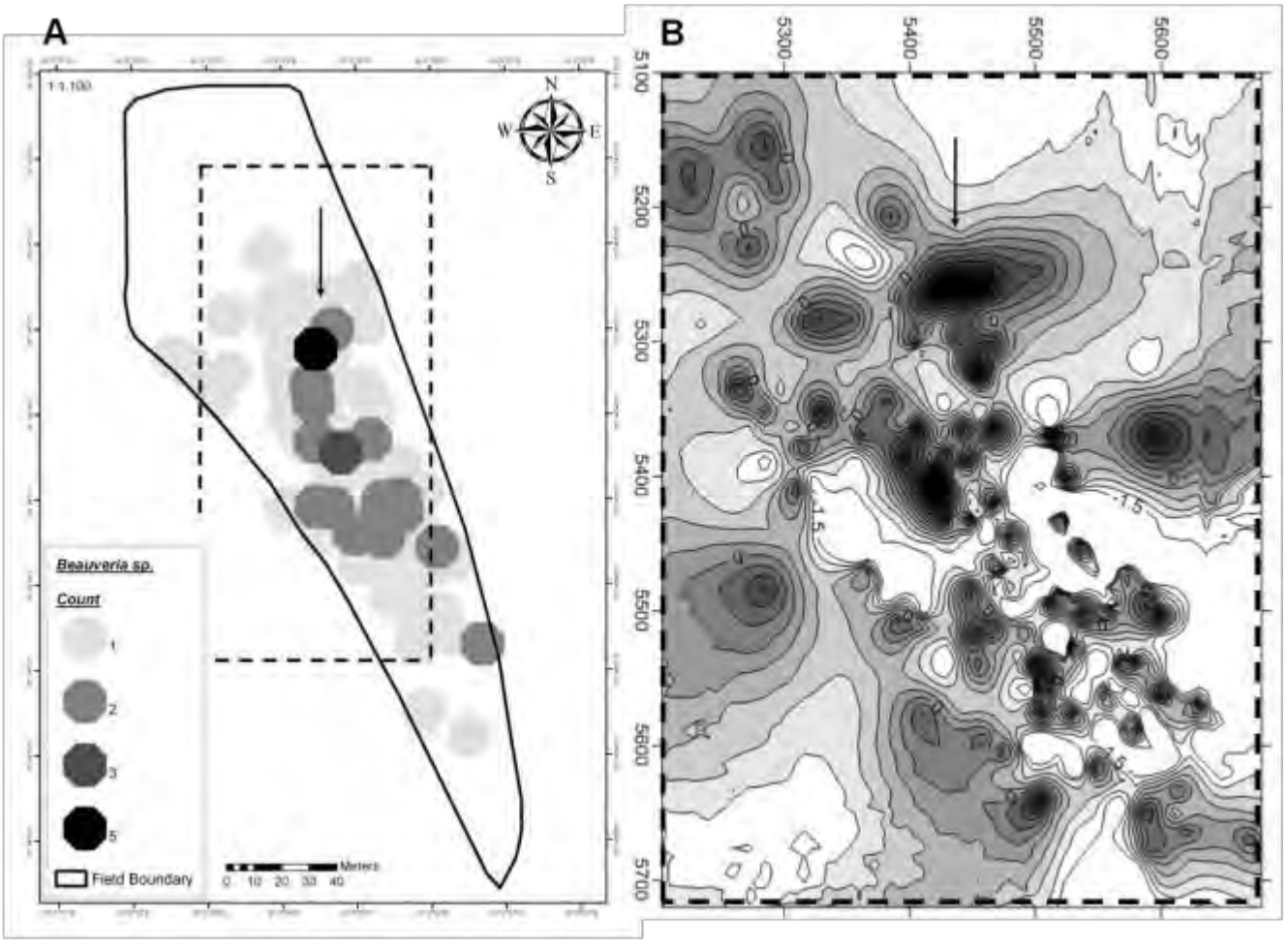

Figure 2.12: The spatial distribution and occurrence of all *Beauveria*-infected cadavers at the Harden Heights study site (A); The scale of this figure is in minute, degree, seconds and corresponds to the coordinates in the map of clustering in *Beauveria*-infected cadavers calculated by the program, SADIE (B). Clustering indices equal to -1, 0 and 1 are represented in white. Grey shaded areas represent 'gaps' i.e: areas where clustering indices are below -1.5 (lesser occurrences of *Beauveria* sp than should be expected from a random distribution). Black areas are 'patches' were clustering indices are above 1.5 (higher occurrences of *Beauveria* sp than what should be expected from a random distribution). The scale of this figure is in seconds. The map was drawn in SURFER™.

2.3.3 *Climatic associations of white grubs*

Co-inertia analysis showed that white grub life stages and weather data were significantly (P≤0.05) related to each other (Figure 2.14). All common white grub 1st and 2nd instar grubs were associated with the negative (left) part of the PCA factorial map which described 42% of the variability (Figure 2.13). The association of these components with the left part of the factorial map match those of summer months which also corresponded to this part of the factorial map in Figure 2.8. In the summer months, rainfall (χ^2=19.16, df=1, P≤0.001) and relative humidity of the air (χ^2=14.26, df=1, P≤0.001) were the best predictors of the occurrence and distribution of *S. affinis* 1st and 2nd instars (Appendix 2.3, Table A2.3.1). Rainfall had the highest correlation coefficient for 1st instars of this species while relative humidity of the air had the highest correlation coefficient for 2nd instars in the co-inertia analysis (Figure 2.14) and both were considered equal impact factors with a value of 1.

Rainfall and relative humidity were the best predictors of the occurrence and distribution of *H. sommeri* 1st instars and both factors were significant (P≤0.001) (Appendix 2.3, Table A2.3.1). So were both their respective correlation coefficients and both parameters also had equal impact factors of 1 (Figure 2.14). In addition, *H. sommeri* 2nd instars were linked to relative humidity and soil temperature which were both significant (P≤0.001). Relative humidity was considered to have the greatest impact factor on the distribution of *H. sommeri* 2nd instars (Appendix 2.3, Table A2.3.1). Relative humidity and solar radiation were selected as the best model to describe the distributions of both *Anomala* sp 1st and 2nd instars (Appendix 2.3, Table A2.3.1) and both parameters were significant (P≤0.001). Solar radiation had the highest correlation coefficient in the co-inertia and this was followed by soil temperature (Figure 2.14) but soil temperature is closely linked to solar radiation. However, relative humidity had the highest impact factor (0.80).

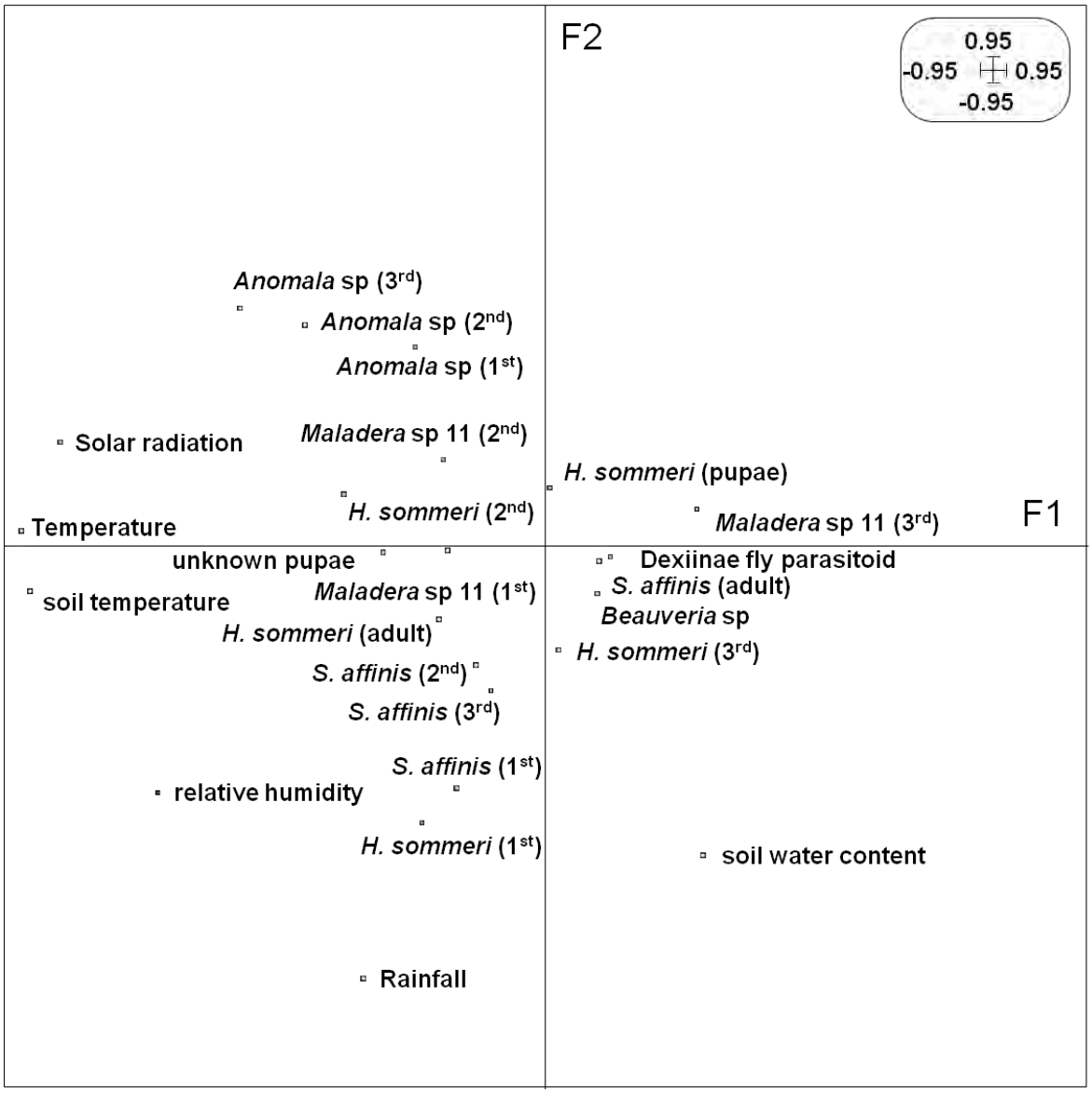

Figure 2.13: PCA factorial map showing the relationships between all common white grub species and life stages, *Beauveria*-infected cadavers and Dexiinae fly parasitoids collected per month, in relation to weather data at Harden Heights.

Due to the slightly later seasonal occurrences of *Maladera* sp11 1st instars, the best predictor model selected was soil water content ($P \leq 0.003$) and solar radiation ($P \leq 0.001$) and both parameters were significant (Appendix 2.3, Table A.2.3.2). Soil temperature again had a high correlation coefficient in the co-inertia analysis but this was an artefact of collinearity with solar radiation (Figure 2.14). Soil water content, rainfall and soil temperature had the greatest influence on the distribution of *Maladera* sp11 2nd instars and all parameters were significant ($P \leq 0.001$) (Appendix 2.3, Table A2.3.2). Despite solar radiation having a significant

correlation coefficient it did not have any significantly influence when a previous model, containing this parameter, was validated (Figure 2.14).

Hypopholis sommeri and *Maladera* sp 11 3rd instars were associated with the positive part of the factorial map (Figure 2.13) which corresponds to greater occurrences of 3rd instars of these two species in winter months in Figure 2.8. The best selected model to predict the occurrence of *Maladera* sp11 was soil water content × relative humidity × solar radiation × soil temperature and all parameters were significant ($P \leq 0.001$) (Appendix 2.3, Table A2.3.3). In the co-inertia analysis however, only soil water content was positively correlated with the species while all three other parameters were negatively correlated (Figure 2.14). All of the negative parameters had impact factors of 1 while soil water content had a lower impact factor of 0.98. *Hypopholis sommeri* 3rd instar larvae were significantly correlated to soil water content ($\chi^2=24.86$, df=1, $P \leq 0.001$), which was also the only weather variable with a significant correlation coefficient (Figures 2.13 and 2.14) and an impact factor of 1 (Appendix 2.3, Table A2.3.2).

The best model that described *S. affinis* 3rd instar distribution was rainfall and soil water content which were both significant ($P \leq 0.001$) (Appendix 2.3, Table A2.3.2). Many of the *S. affinis* 3rd instars were collected in December 2010, and then persisted at lower levels throughout the year. In addition, *Anomala* sp. 3rd instars were linked to soil water content ($P \leq 0.001$), soil temperature ($P \leq 0.001$) and solar radiation ($P \leq 0.07$). Despite trying to validate all the models listed, the above mentioned was the best model (Appendix 2.3, Table A2.3.2). Soil water content was negatively correlated to *Anomala* sp. 3rd instars while soil temperature was positively correlated.

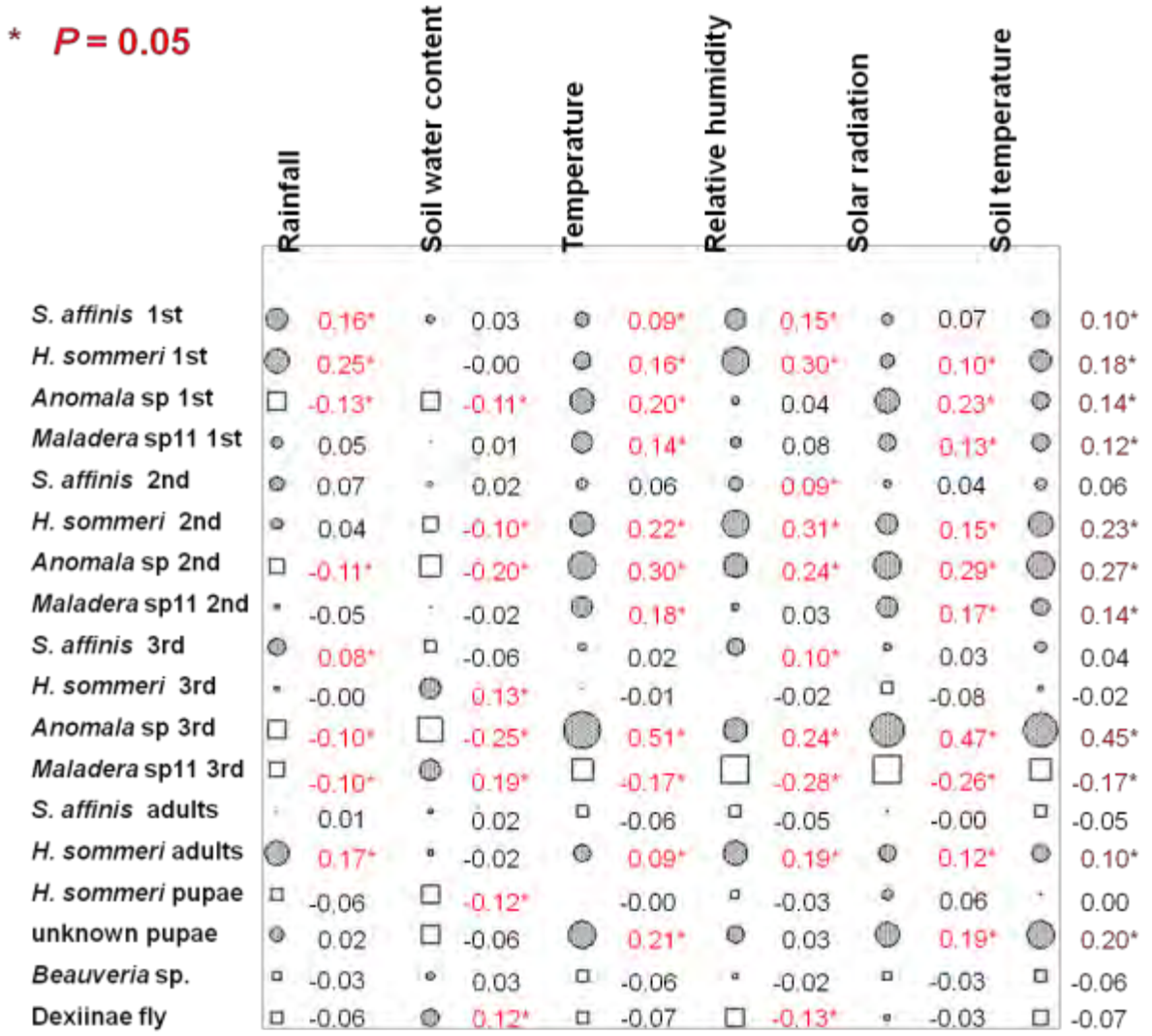

Figure 2.14: Co-inertia analysis of four common white grub species and their life stages, the occurrence of *Beauveria*-infected cadavers, Dexiinae fly parasitoids and their relationship with weather data. A square represents an anti-correlation (negative) relationship and a circle represents a positive correlation. *represents Pearson correlation coefficients with statistical significance levels of $P \leq 0.05$ while no asterisk represents no correlation. The larger the square or circle the greater the impact of the climatic variable; the smaller the size of the square or circle the smaller the impact of the climatic variable.

The best model that described *H. sommeri* adult distribution was rainfall, relative humidity and solar radiation which were all significant ($P \leq 0.001$) (Appendix 2.3, Table A2.3.3). Both parameters had equal impact factors of 1 and the highest correlation coefficients (Figure 2.14). The best predictor model of *H. sommeri* pupae was rainfall, soil water content and soil temperature which were all significant (Appendix 2.3, Table A2.3.3). Rainfall and soil water

content had the highest impact factors and correlation coefficients for *H. sommeri* pupae (Figure 2.14). The best predictor model of the occurrence of *S. affinis* adults was rainfall, solar radiation and soil temperature however none of the models were significant (Appendix 2.3, Table A2.3.3). Further, soil temperature had the highest impact factor (0.69) in this model.

Soil temperature alone was the best predictor of the occurrence of unknown pupae (χ^2=8.23, df=1, P≤0.004) (Appendix 2.3, Table A2.3.3) and *Beauveria*-infected cadavers but the latter association was not significant (Appendix 2.3, Table A2.3.4). Finally, the association of the Dexiinae fly parasitoids with the positive part of the factorial map highlights its occurrence in winter months as they were collected in August and were often found infesting *H. sommeri* 3rd instar larvae which were also associated with the F1 axis (Figure 2.8 and Figure 2.13). Relative humidity was negatively correlated to the occurrence of the parasitoids however this was not significant (Appendix 2.3, Table A2.3.4).

There was a relationship between the occurrence of *H. sommeri* 3rd instar larvae, *Beauveria*-infected cadavers and soil water content (Figure 2.15), and they were all located in the positive part of the F1 axis as shown in the factorial map (Figure 2.13). Pearson correlation coefficients showed that *H. sommeri* 3rd instars were significantly (P≤0.001) correlated to soil water content but *Beauveria*-infected cadavers were not (P≤0.43) (data not shown). However there was a significant correlation between *H. sommeri* 3rd instar larvae and *Beauveria*-infected cadavers (P≤0.01) as they often occurred in the same sampling pits. Figure 2.15 highlights increases in *H. sommeri* 3rd instar numbers as soil water content increases, likewise the incidence of *Beauveria*-infected cadavers' increases as *H. sommeri* 3rd instars increase.

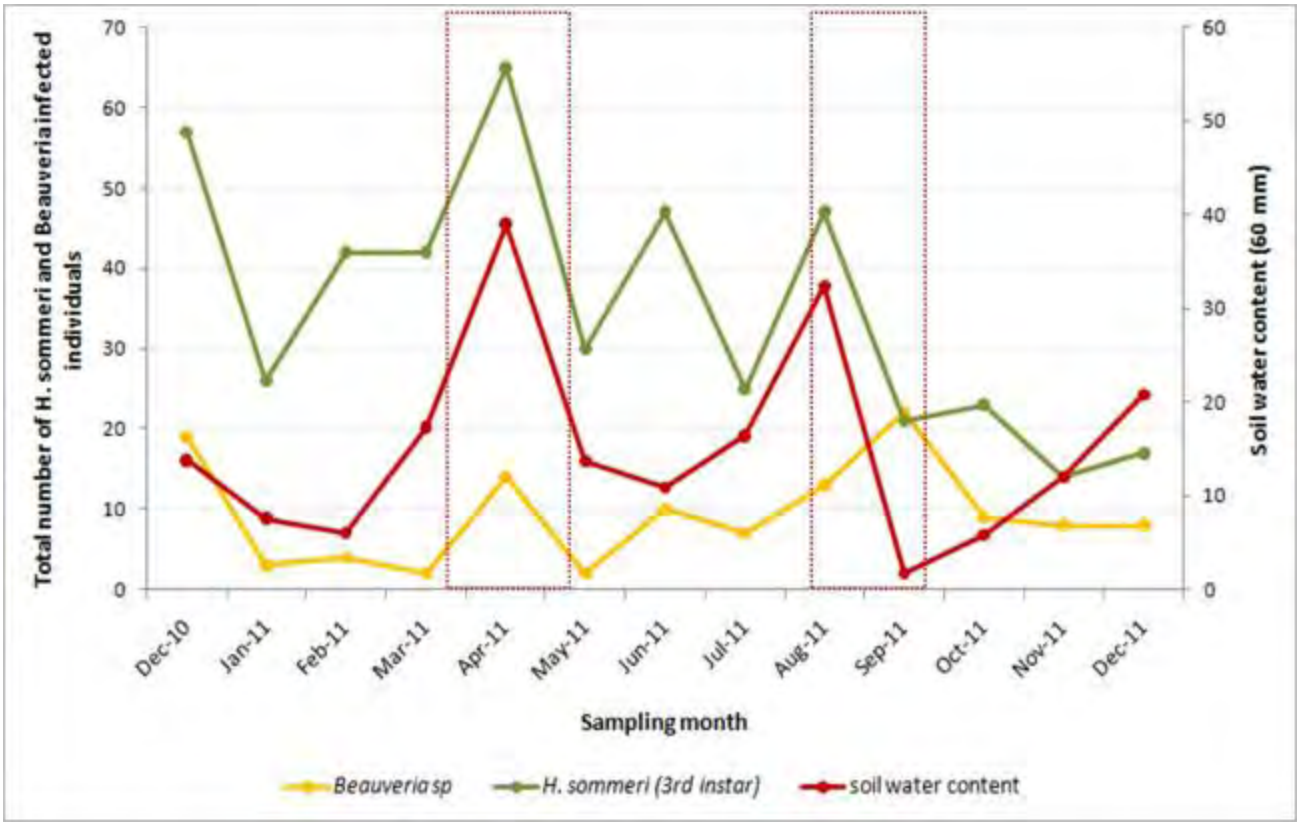

Figure 2.15: The relationship between *Hypopholis sommeri* and *Beauveria*-infected cadaver populations, and soil water content at the Harden Heights study site.

The PCA factorial map (describing 85% of the variability) showed relationships between some common white grub life stages, *Beauveria*-infected cadavers and soil characteristics in March 2011 (Figure 2.16). *Anomala* sp. 3rd instars and unknown pupae were correlated with the right (positive) part of the horizontal (F1) axis and were associated with high sand (%), aluminum (ppm) and the aluminum saturation index (%) but these correlations were not significant (Figure 2.16). The remaining white grub life stages and *Beauveria*-infected cadavers were correlated with the left (negative) part of the F1 axis. They were associated with higher percentages of potassium, clay, sodium, magnesium, organic matter, pH which was measured in calcium chloride (CaCl), calcium, phosphorus and NH_3 (ammonia). There was a significant correlation between *H. sommeri* 3rd instars, high organic matter (5.64%) (χ^2= 4.77, df= 1, P≤0.02) and high phosphorus (χ^2= 4.18, df= 1, P≤0.04). *Schizonycha affinis* 3rd instars were not significantly correlated with any soil characteristics. *Maladera* sp11 1st instar grubs were significantly correlated to high NH_3 (χ^2= 5.20, df= 1, P≤0.02). *Maladera* sp11 3rd instar grubs were significantly correlated to high potassium (χ^2= 6.16, df= 1, P≤0.01)

and high sand (%) (χ^2= 4.42, df= 1, P≤0.03). Likewise, *Beauveria*-infected cadavers were also significantly correlated with high potassium (χ^2= 7.78, df= 1, P≤0.001) and high sand (%) (χ^2= 4.82, df= 1, P≤0.02) (Figure 2.16).

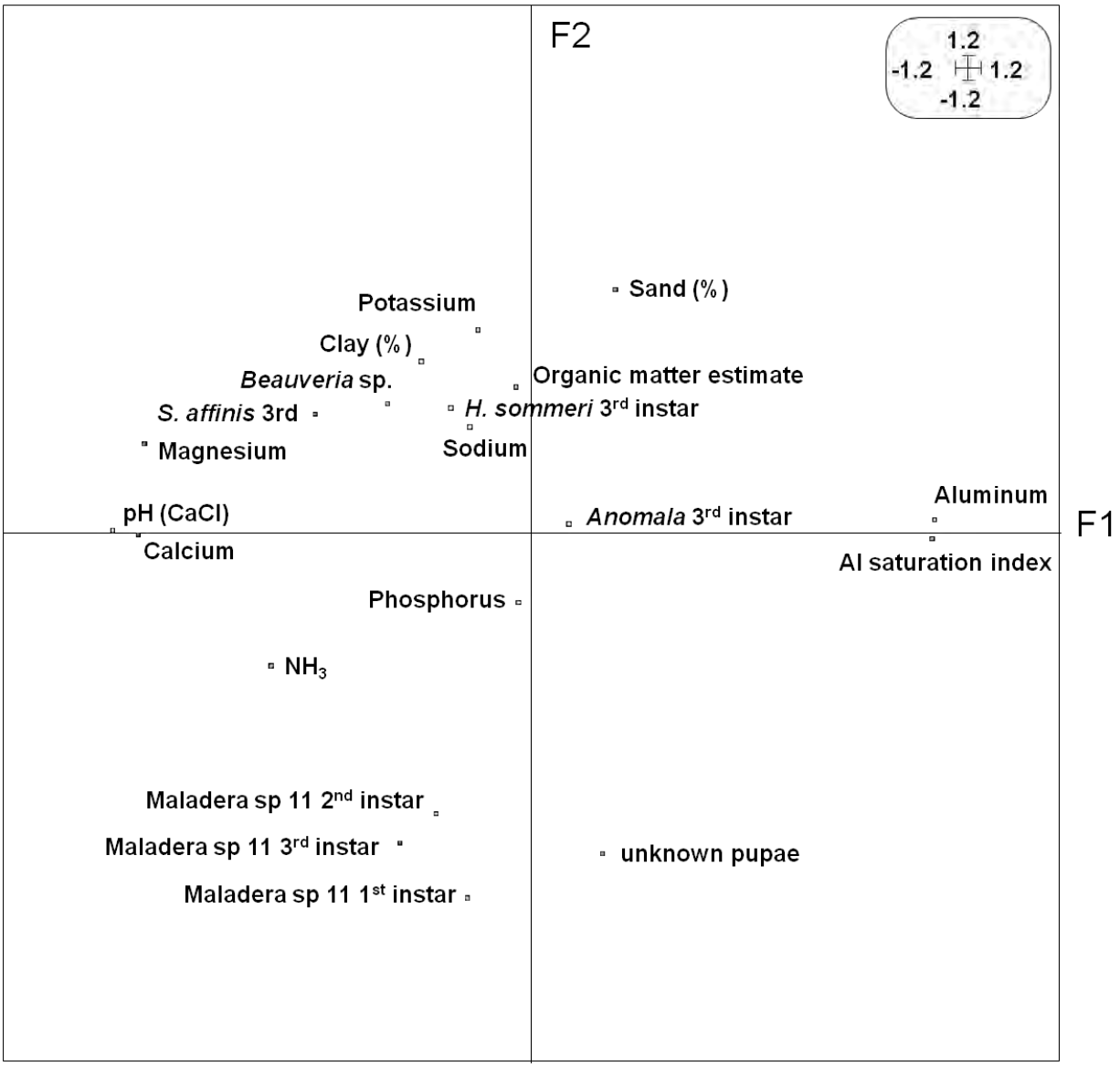

Figure 2.16: PCA factorial map showing the relationship between soil characteristics and the presence of white grub (*S. affinis*, *H. sommeri*, *Anomala* sp and *Maladera* sp11) life stages and *Beauveria*-infected cadavers in March 2011 at Harden Heights.

2.4 DISCUSSION

Two Scarabaeidae subfamilies, representing seven white grub genera: Rutelinae (*Anomala* and *Adoretus*) and Melolonthinae (*Congella, Hypopholis, Maladera*, *Schizonycha* and *Trochalus*) occurred within a relatively small area (~1.14 ha) in a sugarcane field at Harden Heights. Some species occurred in high numbers (*Anomala* sp., *H. sommeri*, *Maladera* sp 11 and *S. affinis*) within the field site, while other species (*Congella* sp., *Adoretus* sp., *Trochalus* sp., SASRI 26) occurred in lower, almost negligible abundances. Three of the four white grub genera which persisted at high levels throughout the year at Harden Heights were those of the subfamily Melolonthinae. This subfamily is the most diverse of the Scarabaeidae (Harrison, 2004) and has been particularly problematic in sugarcane in Australia (Allsopp *et al.* 1993). Carnegie (1988) already confirmed damage to sugarcane by the melolonthids, *H. sommeri* and *S. affinis* in the KZN Midlands North region and central inland Zululand. During an industry-wide survey undertaken by Way (1997) it was established that five of the white grub genera listed in the present study occurred throughout the South African sugar industry. However the occurrences of *Maladera* and *Congella* were not reported previously from sugarcane in the KZN Midlands North. Like other members of the Melolonthinae, they also feed on the leaves of angiosperm plants as adults and on roots in the larval stages (Ahrens, 2003; Ahrens and Vogler, 2008). Two *Maladera* species, *M. castanea* Arrow and *M. insanabilis* Brenske in particular are known agricultural pests, and *M. affinis* is a closely related species with disjunctive distributions in Madagascar, Reunion and India (Ahrens, 2003). *Maladera* are inconspicuous and morphologically uniform and their homogeneity has resulted in taxonomic difficulties and has limited studies on their distribution, ecology, and morphology (Ahrens and Vogler, 2008). While little is known about the occurrences of *Adoretus* sp. and *Anomala* sp. in the KZN Midlands North, they have been associated with transient crop losses in other parts of the South African sugar industry, such as Gingindlovu (Carnegie, 1988).

The distribution and population dynamics of *H. sommeri* and *S. affinis* in the KZN Midlands regions were evaluated by Carnegie (1974). He reported that a relative scarcity of *H. sommeri* grubs occurred between September and January. The results of this study indicated the persistence of 3rd instar grubs all year round with high occurrences in mid-summer (December) suggesting that populations of *H. sommeri* have increased in prevalence in KZN Midlands North since Carnegie's (1974) study. The high co-occurrence of 3rd instar grubs

with adults and 1st instar grubs, particularly in December, suggested that *H. sommeri* may have a 2-year life cycle. This hypothesis is further supported by Prins (1965) who found that times between hatching and pupation in *H. sommeri* could vary from 257 to over 600 days, making a 2-year life cycle possible. Further, Jackson and Klein (2006) reported that tachinid fly parasitoids can only parasitize two-year life cycle populations of white grubs because the third instar grubs have to coincide with summer fly emergences. Logan (1999) further reported that the Dexiinae fly parasitoid, *Rutilia inornata,* Guérin-Méneville (Diptera: Tachinidae) was found infesting the two-year white grub, *Lepidiota frenchi* Blackburn in spring which confirms that these parasitoids infect white grubs species with two-year life cycles. The Dexiinae fly parasitoid reported in the present study occurred as maggots, living within the 3rd instar grub of *H. sommeri*, which were collected in August. Parasitoid-infected *H. sommeri* hosts were brought back to the laboratory and two weeks later the adult flies emerged in September (spring). This confirmed that these parasitoids emerge as adults in spring and lay eggs on or near 3rd instar *H. sommeri* larvae (as these grubs are large enough to support the two growing internal maggots). It is important that 3rd instar *H. sommeri* grubs coincide with fly emergence to ensure the survival of the parasitoids. If *H. sommeri* had a one-year life cycle, very few 3rd instar larvae would have been collected because pupation would have taken place in August. The lack of *H. sommeri* pupae and an increased number of third instar larvae collected in August further confirmed a two-year life cycle for this species. Carnegie (1974) suspected that *S. affinis* may also have a 2-year life cycle. The present study established high 3rd instar occurrences in December which presumably were laid the year before, also suggesting the possibility of a 2-year life cycle. Further, temperatures in the KZN Midlands North are on average much cooler than the rest of the KwaZulu-Natal Province (Atkinson *et al.* 1981) which could also have had an effect on the biology of some white grubs in the area by reducing the rate of the life cycles.

Maladera sp11 had a different biology with 1st, 2nd and 3rd instar grubs occurring in winter months (April, May, June, July, Aug, Sep). The first occurrence of neonate (1st and 2nd instar) grubs at the study site was in February when soil temperatures (23°C) were high enough to induce successful embryonic development because according to Fleming (1972), who studied the biology of the Japanese beetle, *P. japonica*, no eggs hatched below 15°C. In the present study, 1st and 2nd instars persisted in the soil until June when mean soil temperatures reached 12°C. No *Maladera* sp11 1st or 2nd instars were sampled in July or August however,

presumably because grubs had migrated into deeper, warmer layers of the soil profile as suggested by Villani and Wright (1988). However, in early spring (September) only second instars were sampled. This may mean that development of 1st instars into 2nd instars could have continued at a slower rate during winter. Apparently, 1st instar *P. japonica* grubs which have fed sufficiently to accumulate a food reserved and have not entered into the early stages of ecdysis are able to overwinter (Fleming, 1972). This could explain the observed lack of 1st instars in early spring and the subsequent occurrence of 2nd instars only in the present study. Finally, because there was little overlap among developmental stages in spring (3rd instars were relatively scarce from October) (Carnegie, 1974), a univoltine life cycle is proposed for this species.

The importance of studying the biology of various white grub species now becomes apparent as *H. sommeri* and *S. affinis* are proposed to have two-year life cycles and *Maladera* sp11 is proposed to have a one-year life cycle. Allsopp (2010) suggested that any control application strategies which are undertaken on sugarcane fields that accommodate white grubs with different biology's would be difficult. This is because sugarcane crop height generally restricts application, except for the first few months after planting or after harvesting. This period coincides with the presence of young 3rd instar larvae (the damaging stage) of species with a two-year life cycle, but is about six months out of phase with the same stage of a one-year species (Allsopp, 2010). Hence, this author reported that the classical integrated pest management (IPM) approach of monitoring white grubs and then deciding to apply control tactics based on an action threshold did not work with one-year species and may be even more difficult to implement against two-year species. What is worthwhile however is a risk assessment strategy that advises whether pre-emptive control treatments are suitable (Allsopp, 2010).

In the present study, a strong seasonal pattern in white grub occurrence was seen at the Harden Heights study site, with a higher diversity and greater abundance of white grubs species sampled in summer months (Dec 2010, Jan 2011, Feb, March, Oct, Nov and Dec 2011). However, in winter months (April, May, June, July, Aug, Sep) a lower diversity of species were sampled with lower abundances. These observations in seasonality were most likely the result of the increased occurrence of neonate grubs which were encountered more frequently in the upper layers of the soil profile in summer months compared to winter. It has long been recognized that most white grub species are found nearer the surface of the soil in

summer months (McColloch and Hayes, 1923) and in some instances, crops under no tillage regimes (as was the case with our site which was in a 3rd sugarcane ratoon) have white grub larvae encountered more frequently at a shallower depth of 10 cm (*Santos 1992* reported by Oliveira *et al.* 2009). Strong seasonal distributions of the white grub, *Phyllophaga cuyabana* Moser (Coleoptera: Scarabaeidae) within the soil profile have been reported previously (Oliveira *et al*. 2009). These authors reported that a positive relationship existed between the percentage distribution of grubs in the soil profile and soil temperature. At a greater depth (up to 40 cm) the number of insects in the soil shared a positive relationship with air temperature and evapotranspiration (Oliveira *et al*. 2009). Likewise in the present study, increases in solar radiation (which increases soil temperature) and mean relative humidity of the air were considered important climatic variable parameters in models for the occurrence of all species of neonate grubs. High relative humidity is well-known to stimulate activity of above ground soil dwelling insects (Oliveira *et al*. 2009) and has been implicated as an inducer of hempiteran flight activity (Riis and Esbjerg, 1998). Likewise, relative humidity and rainfall were also found to be significant determiners in the occurrence of *H. sommeri* beetles in the present study. Soil temperatures at the Harden Heights study site were higher in summer months (when most of the neonate grubs were found) as evidenced by the climatic data provided in this study and increased soil temperature is known to increase the rate of insect development (McColloch and Hayes, 1923). During summer months at the site, soil temperatures were never above 25°C and neonate grubs could migrate to the upper surface of the soil to feed without exposing themselves to extreme temperatures. Fleming (1972) showed that *P. japonica* 1st and 2nd instar grubs could survive temperatures of up to 30°C which is well-above those experienced at Harden Heights. These would explain why so many 1st and 2nd instar grubs were found in pits dug to 30 cm during the summer season.

Well-defined seasonal patterns of vertical movement apparently associated with soil temperature have been recorded in some species of white grub (McColloch and Hayes, 1923; Villani and Wright, 1988). The occurrence of *Anomala* sp. in summer at Harden Heights was patchy, with all life stages occurring from January to March and then not sampled again. Their apparent lack of occurrence from April onwards is obscure. Villani and Wright (1988) radiographed soil blocks in the laboratory to study the temperature responses of three scarab grub species, which included: *P. japonica, Rhizotrugus majalis* Razoumowsky and *Anomala orientalis* Waterhouse (Coleoptera: Scarabaeidae). Temperature fluctuations had very little

impact on the position of *R. majalis* grubs in the soil (Villani and Wright, 1988). In contrast, the other two grub species responded rapidly to shifting temperatures by moving down in the soil profile at the onset of cooler temperatures. An increase in temperature caused *A. orientalis* grubs to move back up to the upper root zone (Villani and Wright, 1988). In the present study, *Anomala* sp. occurring at the Harden Heights study site were negatively linked to soil water content and positively correlated to soil temperature and solar radiation. This may mean that a combination of drier soil conditions in late summer and gradual decreases in temperature from April onwards, could have caused this white grub species to rapidly respond and move further down into the soil profile, which is possibly why it was not detected again in sampling pits. Unfortunately because we were unable to identify this white grub to species level it is difficult to make any conclusions about the life cycle. Some literature on *A. ustulata* suggests this white grub species has a three-year life cycle in the maize belt of the Springbok Flats in South Africa and that this species migrates down in winter and upward migration begins in early April (du Toit, 1996). Perhaps because of the above mentioned climatic conditions, this migration did not occur or was not apparent.

Not only are increases in soil temperature and relative humidity responsible for the increased occurrences of white grubs in summer months but Hawley (1949) further suggested that moisture was an important climatic variable for the occurrence of neonate grubs because dry soil conditions were unfavorable for newly hatched larvae because they were only lightly chitinized and not adapted to conserve water. Further, rainfall and the subsequent increases in soil water content are crucial factors in the survival of white grub eggs (Laughlin, 1957; Oliveira *et al.* 2009). *Hypopholis sommeri* and *S. affinis* adults and neonate grubs as well as *Maladera* sp11 neonate grubs were strongly linked to rainfall or soil water content in the present study and this suggested that the occurrence of these life stages were partially governed by moisture. Some authors suggest that localized populations of white grubs shrink and swell annually because of variations in rainfall and soil moisture (Hawley, 1949; Dalthorp *et al.* 2000b) highlighting the importance of moisture in the occurrence and distribution of white grubs.

Spatial analysis in summer months showed clumped distributions or aggregations of white grubs; an observation reported by Oliveira *et al.* (2009). It appears to be common for 1st and 2nd instar grubs to be aggregated together in the soil because of the nature of female oviposition for sites which are higher in organic matter, and it is the reproductive physiology of adult females

which play a part in population distribution (Brandhorst-Hubbard *et al.* 2001; Dimock, 2004; Dalthorp *et al.* 2000b). This was evidenced in the significant spatial associations observed between 1st and 2nd instars of *S. affinis*, *Maladera* sp11 and *Anomala* sp in the present study. White grub females lay egg clutches underneath sugarcane stools; a few centimeters below the soil surface (Carnegie, 1974). When neonate grubs hatch they feed on organic matter in the soil and later on sugarcane rootlets (Carnegie, 1974). Neonate grubs may stay within the females' chosen oviposition site where food sources are available but movement away from aggregations may be lethal if soil temperatures are high and soil is dry (Hawley, 1949). This author reported that soft-bodied young *P. japonica* grubs were unable to dig over long distances through the soil to find food because they were unable to replenish sufficient body moisture and may become desiccated. Due to lower soil water content, which was well-below the long term mean (LTM), experienced in summer months at the Harden Heights site, it is possible that neonate grubs may not have moved significantly far from oviposition sites to risk possible desiccation leading to the observed aggregations. Further, a significant spatial association between *H. sommeri* adults and 1st instars was found in summer which suggested that emerging 1st instar grubs did not migrate from oviposition sites either because of the difficulty of moving through the soil (Hawley, 1949) or because enough food was available in the form of organic matter to limit dispersal by 1st instar grubs. Logan and Kettle (2002) found that there was no evidence for density-dependant neonate survival or larval combat between 1st instars of *D. albohirtum* and that their survival and development were more related to food type and supply.

In the present study, the occurrence of *Maladera* sp11 1st instars in areas with higher organic matter was likely and hence there was no reason for them to redistribute. Female oviposition preference of the green June beetle, *Cotinis nitida* Linnaeus (Scarabaeidae: Coleoptera) for sites higher in organic matter have been reported (Brandhorst-Hubbard *et al.* 2001). These authors reported higher densities of *C. nitida* eggs in different organic fertilizers compared to an untreated control (loam soil). They suggested that strong odours in the organic fertilizers such as the presence of ammonia (NH_3) were attractive to ovipositing scarab females. Ammonia is a volatile compound that may be detected at great distances by both adult *C. nitida* beetles and grubs (Brandhorst-Hubbard *et al.* 2001). In the present study *Maladera* sp11 1st instar grubs were significantly correlated to NH_3 and these molecules were significantly correlated to organic matter (data not shown) because positive NH_3 molecules are attracted to negative sites on organic matter particles due to the dissociation of H^+ from weak acids

(Mengel *et al.* 2001). Suggesting the 1st instar grubs may move to areas within the soil profile that present better habitat if they are not already orientated so by female oviposition preference. This suggests that the distribution of *Maladera* sp11 1st instars may not only be determined by rainfall, soil water content and solar radiation but also by the presence of organic matter in the soil.

Another significant spatial association existed between *H. sommeri* 1st and 3rd instar grubs in summer. The intraspecific feeding niches of these two life stages are different with 3rd instars feeding on roots and 1st instars feeding on organic matter in the soil (Carnegie, 1974) therefore competition for resources is excluded. *Hypopholis sommeri* 3rd instar larvae were significantly correlated to organic matter and phosphorus in the present study and positive phosphorus cations are known to fix to organic matter (Mengel *et al.* 2001) which is probably why *H. sommeri* was correlated to both. Therefore, it is hypothesized that 3rd instars may have been attracted to sites where organic matter was high to either feed on roots which may grow better in the presence of organic matter (USDA, 2001) or to escape increasing soil temperature gradients in other parts of the field. It was drier in summer months at the site, soil water content was low from December to April and this may have influenced grub behaviour forcing them to seek out sites were soil moisture was suitably increased, particularly since this study showed a strong correlation between *H. sommeri* 3rd instar larvae and soil water content. Organic matter binds soil particles together into stable aggregations and improves porosity, infiltration, root penetration and improves the soils ability to hold water (USDA, 2001). Besides the obvious cooling effect of soil organic matter as a result of water retention which would attract 3rd instars in drier conditions, improved porosity and food resources would be favourable for 1st instars whose mobility is restricted to existing pores in the soil (Villani and Wright, 1990), leading to the observed aggregations of *H. sommeri* 1st and 3rd instar grubs.

Beauveria-infected cadavers were randomly distributed throughout the field site with higher occurrences of infection in April and September. There was a significant relationship between the occurrence of *H. sommeri* 3rd instar larvae and *Beauveria*-infected cadavers despite there being no significant spatial association detected. Dalthorp *et al.* (2000b) suggested that temperature extremes and subsequent stresses experienced by overwintering white grubs may result in increased spring mortalities by pathogens. In the current study two peaks in the occurrence of *Beauveria*-infected cadavers were seen, one peak following a relatively dry summer, which may have stressed *H. sommeri* 3rd instar grubs leading to increases infection

rates in April. A second infection peak occurred in September following a winter in which soil water content was particularly high, this might have increased the amount of *H. sommeri* 3rd instar grubs in the upper layers of the soil profile which might have increased the chance incident of uninfected larvae coming into contact with sporulating cadavers or fungal conidia.

In winter months, spatial patterns within the soil profile changed significantly from aggregated clumps to a random distribution of grubs. A greater number of 3rd instars persisted in these months because neonate larvae had grown into 3rd instars and dispersed from aggregations probably due to crowding by larger bodied grubs and competition for sugarcane roots. Larval combat is density-dependent and known to occur in a variety of 3rd instar grub species which may inflict a degree of mortality and leads to active grub dispersal (Regniere *et al.* 1981; Logan and Kettle, 2002; Allsopp, 2010). Further, movement down into the soil in winter protects larvae from unseasonable cold spells, which do not reduce soil temperatures in the deeper layers and could increase the randomness of grub distributions in the present study (Villani and Wright, 1988; Villani and Wright, 1990). However, heavy rainfall in both April and August at the site increased the soil water content considerably. This is probably why soil water content had the greatest impact factor on the distributions of *H. sommeri*, *S. affinis* and *Maladera* sp 3rd instar larvae as shown by models. Further, more *H. sommeri* 3rd instar larvae were sampled in these months, as grubs moved closer to the soil surface, presumably to escape high soil moisture gradients and seek out drier areas. Due to increased soil water content, 3rd instars would probably have moved horizontally within the upper layers of the soil rather than migrating down. Species-specific responses to changes in environmental temperature gradients have been observed (Villani and Wright, 1988) with some species remaining in the upper zones well into winter. This could also have attributed to the random distributions observed.

2.5 CONCLUSIONS

The increased seasonal abundances, diversity and highly aggregated nature of white grub species in summer months suggests that pest targeting and control strategies should be considered in this season. That way a greater diversity and abundance of species maybe effectively targeted and controlled. What will likely complicate successful control applications are the different biology's of white grubs which co-occur in sugarcane cropping

systems in the KZN Midlands North. Thus the development of an appropriate risk assessment strategy that advises whether pre-emptive control treatments are likely to be worthwhile should be developed. It is now know that increased rainfall, relative humidity and soil temperatures are linked to the increased occurrence of scarab adults and neonate grubs at the Harden Heights site. It is also suggested that localized populations of white grubs shrink and swell annually because of variations in rainfall and soil moisture thus climatic variables may play a big role in predicting the occurrence and abundance of some white grub species and may improve the development of a risk assessment strategy.

APPENDIX 2.1

Phylogenetic analyses of unknown white grubs

A.2.1.1 INTRODUCTION

Historically, identification of white grub species has relied on the use of morphological characteristics (Miller *et al.*, 1999; Allsopp and Miller, 2000; Allsopp and Lambkin, 2006; Sallam *et al.* 2007; Dittrich-Schröder *et al.* 2009). However, due to phenotypic variation within a single species it is not always possible to base identifications solely on morphology (Miller and Allsopp, 2000). This was the case in the present study when 16 unknown white grub larvae were found during collection studies to investigate which species of white grubs occurred at the Harden Heights field site in the KZN Midlands North. Literature suggested that advances in species identifications were made using molecular techniques based on the diversity of the mitochondrial *COII* or *COI* gene regions which then aided in the development of PCR-RFLP protocols for identifying closely related species and determining gene flow patterns (Miller *et al.* 1999; Miller and Allsopp, 2005; Ahrens *et al.* 2007; Dittrich *et al.* 2006; Dittrich-Schröder *et al.* 2009). These taxonomic advances are a start to the improvement of knowledge of the species composition of scarabaeid larvae in sugarcane fields in South Africa. Because accurate identification and extensive knowledge of the target species can aid in effective pest targeting with various control options (Allsopp, 2010). Thus it was of interest to identify the unknown larvae to genus level to better understand the occurrence of various species of white grub in this region.

A.2.1.2 METHODS AND MATERIALS

Sixteen unknown white grub larvae found at the Harden Heights study site, which could not be identified using standard morphology from taxonomic keys were issued SASRI accession numbers, preserved in 90% ethanol and subjected to molecular analyses (Table A2.1.1). In addition, twenty published GenBank white grub *cytochrome oxidase* subunit I gene (*CO1*) sequences were also included in the molecular analyses and their accession numbers appear in Figure A2.1.1

Table A2.1.1: The location, host plant information and collection date of 16 unknown SASRI larvae samples from which DNA was extracted and analysed using the *CO1* gene region.

SASRI code	Genus	Co-ordinates	Host plant	Collection date	Cane variety	Ratoon	Altitude (frost prone)
SASRI SCH sp1	*Schizonycha*	29° 14' 54.5" S 30° 37' 54.9" E	sugarcane (*Saccharum* , Poaceae)	6/12/2010	N12	3 rd	901-1400 m
SASRI SCH sp2	*Schizonycha*	29° 14' 54.7" S 30° 37' 55.4" E	sugarcane (*Saccharum* , Poaceae)	6/12/2010	N12	3 rd	901-1400 m
SASRI SCH sp3	*Schizonycha*	29° 14' 56.7" S 30° 37' 56.7" E	sugarcane (*Saccharum* , Poaceae)	8/03/2011	N12	3 rd	901-1400 m
SASRI SCH sp4	*Schizonycha*	29° 14' 56.0" S 30° 37' 56.5" E	sugarcane (*Saccharum* , Poaceae)	8/03/2011	N12	3 rd	901-1400 m
SASRI HYP 1	*Hypopholis*	29° 14' 54.9" S 30° 37' 55.1" E	sugarcane (*Saccharum* , Poaceae)	6/12/2010	N12	3 rd	901-1400 m
SASRI HYP 2	*Hypopholis*	29° 14' 55.7" S 30° 37' 55.4" E	sugarcane (*Saccharum* , Poaceae)	8/03/2011	N12	3 rd	901-1400 m
SASRI HYP 3	*Hypopholis*	29° 14' 56.0" S 30° 37' 56.0" E	sugarcane (*Saccharum* , Poaceae)	8/03/2011	N12	3 rd	901-1400 m
SASRI 11	morpho A	29° 14' 56.3" S 30° 37' 55.3" E	sugarcane (*Saccharum* , Poaceae)	10/05/2011	N12	3 rd	901-1400 m
SASRI 11	morpho A1	29° 14' 54.5" S 30° 37' 54.9" E	sugarcane (*Saccharum* , Poaceae)	8/03/2011	N12	3 rd	901-1400 m
SASRI 11	morpho B	29° 14' 55.2" S 30° 37' 55.2" E	sugarcane (*Saccharum* , Poaceae)	10/05/2011	N12	3 rd	901-1400 m
SASRI 11	morpho B1	29° 14' 54.5" S 30° 37' 55.3" E	sugarcane (*Saccharum* , Poaceae)	8/03/2011	N12	3 rd	901-1400 m
SASRI 30 A	unknown	29° 14' 56.5" S 30° 37' 56.1" E	sugarcane (*Saccharum* , Poaceae)	20/01/2011	N12	3 rd	901-1400 m
SASRI 30 B	unknown	29° 14' 54.7" S 30° 37' 55.4" E	sugarcane (*Saccharum* , Poaceae)	8/02/2011	N12	3 rd	901-1400 m
SASRI 30 C	unknown	29° 14' 54.3" S 30° 37' 55.8" E	sugarcane (*Saccharum* , Poaceae)	8/02/2011	N12	3 rd	901-1400 m
SASRI 31A	unknown	29° 14' 54.9" S 30° 37' 55.1" E	sugarcane (*Saccharum* , Poaceae)	8/02/2011	N12	3 rd	901-1400 m
SASRI Sp X	unknown	29° 14' 54.9" S 30° 37' 55.1" E	sugarcane (*Saccharum* , Poaceae)	4/04/2011	N12	3 rd	901-1400 m

Locality of all samples= Harden Heights farm, Seven Oaks district, KwaZulu/Natal Midlands North

DNA was isolated from unknown SASRI white grub larvae using a Kapa Xpress Xtract kit™ (Kapa Biosystems, Cape Town) according to the manufacturer's instructions. A portion of the mitochondrial *CO1* gene was amplified using the primers *CO1*F 5'-AAT TGG GGG GTT TGG AAA TTG-3'and *CO1*R 5'-GCT CGT GTA TCA ACG TCT AT TCC-3'. The PCR products were purified using the Wizard SV™ quick purification kit (Promega Corp, Johannesburg). Sequencing of both forward and reverse strands of the gene was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit™ (Applied Biosystems, Johannesburg). Sequence clean up was undertaken using BigDye X Terminator Purification Kit™ (Applied Biosystems) according to the manufacturer's instructions. Sequencing took place using an ABI 3500 Genetic Analyzer.

Unrefined sequence chromatograms were assembled and edited with Geneious™ 5.4 Pro (Drummond *et al.* 2011). Multiple sequence alignments and editing of sequences were undertaken with CLUSTAL*X* 2.0.11 (Larkin *et al.* 2007) and Bioedit™ 7.0.9 (Hall, 1999). The edited sequence data, which comprised 730 base pairs, were then imported into MEGA 4 (Tamura *et al.* 2007). The evolutionary history of the 36 taxa was inferred using the neighbour-

joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (30000 replicates) are shown next to phylogenic tree branches. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969). Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Larval species identification is part of an ongoing SASRI molecular project in which the *CO1* gene is used to infer species delineation and was not the focus of this study. Therefore, Bayesian Inference, Maximum Parsimony and Maximum Likelihood analyses were not undertaken.

A.2.1.3 RESULTS

The partial *CO1* gene data set comprised 730 aligned base pair (bp) positions of 36 white grub sequences (16 SASRI and 20 GenBank sequences) belonging to two scarab subfamilies, Rutelinae and Melolonthinae. Phylogenetic analysis resolved five well-supported clades (A-E) but the backbone of the tree was not supported due to poor taxon inclusion, the diversity of subfamilies and the lack of an out group which was not included in the analysis (Figure A2.1.1). Clade A represented the genus *Anomala* (subfamily: Rutelinae) but unfortunately no SASRI sequences were provided for this clade (Figure A2.1.1). Clade B represented the genus *Adoretus* (subfamily: Rutelinae) and three SASRI sequences (30A, 30B, 30C) fell within this clade with good bootstrap support (94%) but formed a group on their own. Clade C represented the first genus, *Maladera,* of the subfamily Melolonthinae. Five unknown SASRI sequences (11A, 11A1, 11B, 11B1 and 31A) fell within this clade with bootstrap support of 98% (Figure A2.1.1). An unknown SASRI larva (Sp X) fell within the *Congella* genus (clade D) with 100% bootstrap support. Finally, three SASRI *Hypopholis sommeri* Burmeister (1, 2 and 3) larvae grouped together and were sister to four SASRI *Schizonycha affinis* Boheman larvae (sp1, sp2, sp3 and sp4), which grouped together with 100% bootstrap support. These groupings made up the final clade E which represented the Melolonthinae subfamily (Figure A2.1.1).

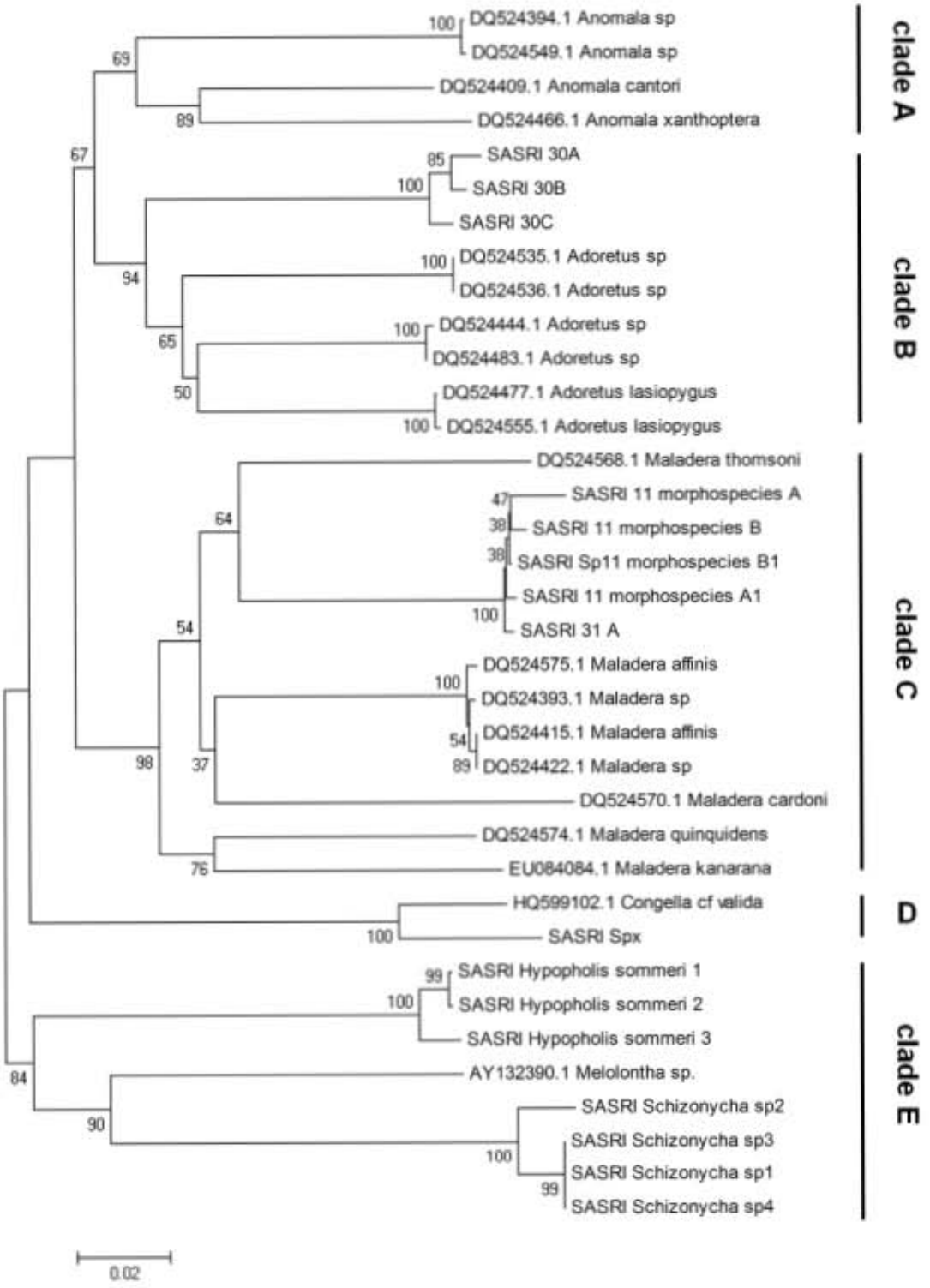

Figure A2.1.1: Neighbour-Joining (NJ) phylogeny for partial sequences of the *CO1* gene of 36 white grub sequences of the subfamilies: Melolonthinae and Rutelinae. Five clades were resolved highlighting six major genera: *Anomala* (A); *Adoretus* (B); belonging to the Rutelinae and *Maladera* (C); *Congella* (D); *Hypopholis* and *Schizonycha* (E) (all Melolonthinae). Statistical support generated in the NJ analyses for each node is shown.

A.2.1.4 DISCUSSION

This study showed that phylogenetic analysis was a useful tool in the delineation of collected, unknown larvae to genus level. This brief analysis was comparable in part with other studies on scarabaeid phylogeny (Ahrens and Vogler, 2008; Ahrens *et al.* 2011). Ahrens and Vogler (2008) undertook phylogenetic analyses which were based on partial 28S rRNA, cytochrome oxidase I (*cox1*) and 16S rRNA (*rrnL*) for 183 species of Scarabaeidae, representing all traditionally recognized subfamilies, with particular focus on Sericini (the tribe to which *Maladera* belongs). Ahrens and Vogler (2008) confirmed that the Anomalini were sister to the Adoretini; this arrangement is alike in the present study with clade A sister to clade B. Results of phylogenetic analyses concluded that the Sericini shared a close relationship with the Melolonthinae clade (Ahrens and Vogler, 2008). In the present study this relationship was not obvious as the Sericini (clade C) were more closely related to the Adoretini/Anomalini group, and the Melolonthinae were more distantly related to the Sericini probably because of poor taxon inclusion and limited phylogenetic information which restricted backbone support. This was not surprising however as the relationships of major Melolonthinae lineages differed substantially under various tree searching techniques according to Ahrens and Vogler, 2008. These authors found that Melolonthinae was paraphyletic in parsimony analysis and polyphyletic in Bayesian analysis, and that low branch support also affected its identification as the sister group to Sericini (Ahrens and Vogler, 2008). The arrangement of the taxa, particularly the *Maladera* Mulsant, 1842 (=*Autoserica* auct., non Brenske, 1897), within the Sericini in the present study highlighted the paraphyly of clade C which was already suggested by Ahrens and Vogler (2008). They showed that the generic level relationships were not consistent with the existing taxonomy as large genera such as the *Maladera* split into several distantly related branches as was seen in the present study. *Maladera* represents a collective, non-monophyletic group with several hundred species described from the Oriental and Afrotropical regions (Ahrens, 2003). The placement of clade D which represented the Hopliini tribe as a basal clade which is more closely related to the Sericini than the Melolonthinae was evident despite there being no backbone support in the present study; this placement is supported by the work of Ahrens and Vogler (2008). Finally, the close relationship of *H. sommeri* and *S. affinis* with *Melolontha* sp. is also synonymous with the work by Ahrens and Vogler (2008). This brief study is part of ongoing taxonomic research at the South African Sugarcane Research Institute and will add to the knowledge of how unknown larvae may be identified from sugarcane fields during ecological studies.

APPENDIX 2.2

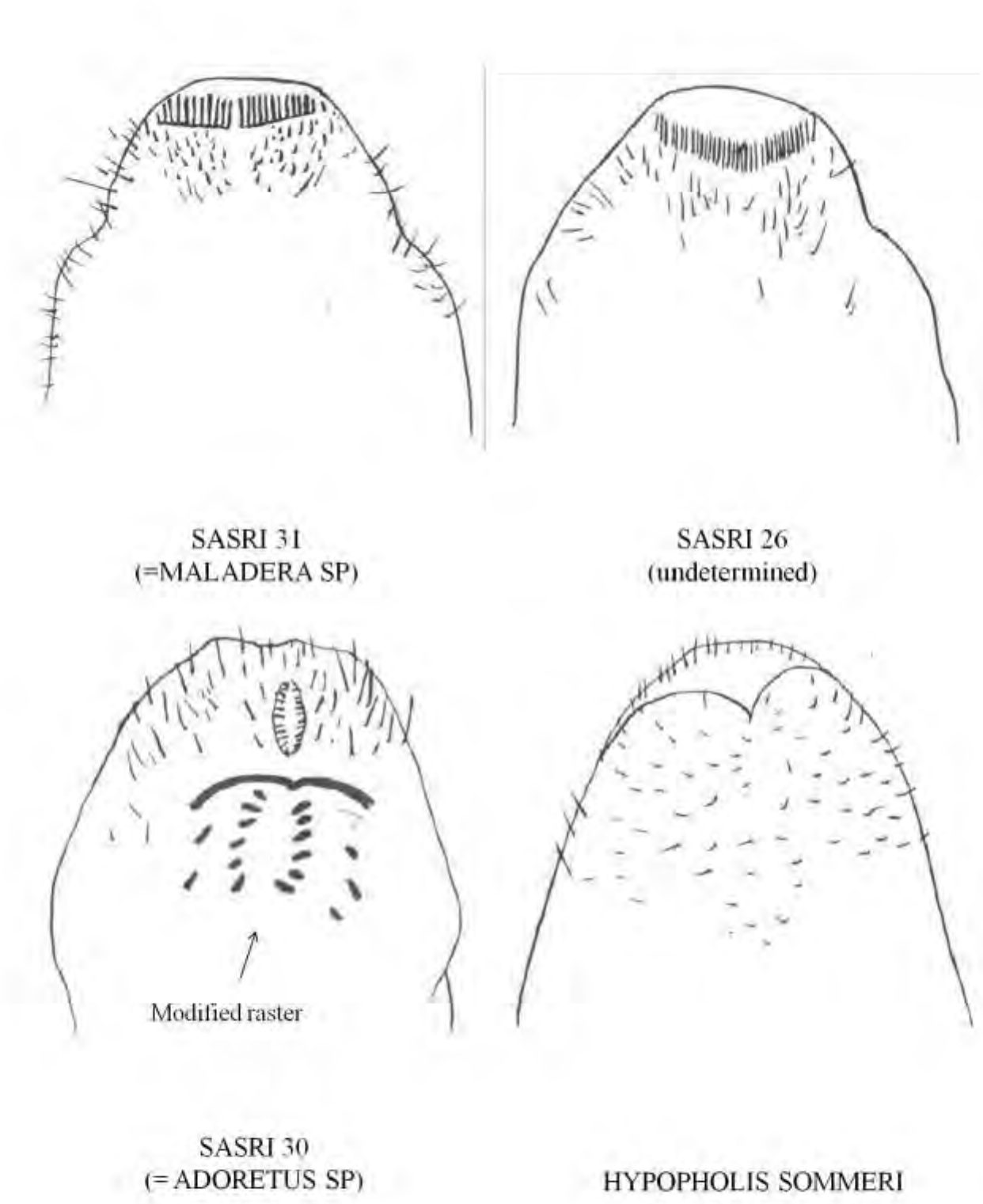

Plate A2.2.1: Schematic diagrams showing the raster patterns on the ventral surface of the last abdominal segment of four larval species found at the Harden Heights study site during monthly pit sampling.

Plate A2.2.2: Lateral view of a larva representing morphospecies SASRI 11A (=*Maladera* sp11) (A); the associated raster pattern on the ventral surface of the last abdominal segment of larval morphospecies 11A (B); lateral view of a larva representing morphospecies SASRI 11B (=*Maladera* sp) (C); the associated raster pattern on the ventral surface of the last abdominal segment of larval morphospecies 11B found at Harden Heights during monthly pit sampling (D). PHOTOS: M. Way

SASRI SP X (=CONGELLA SP)

SASRI WG 164
RSA. Midlands North
Harden Heights
April 2011

Plate A2.2.3: Lateral view of a larva representing SASRI Sp X (=*Congella* sp) (A); the associated raster pattern on the ventral surface of the last abdominal segment of larva SASRI Sp X found at Harden Heights during monthly pit sampling (B). PHOTOS: M. Way

ANOMALA SP.

SASRI WG 157
RSA. Midlands North
Harden Heights
March 2011

Plate A2.2.4: Lateral view of a larva representing *Anomala* sp (A); the associated raster pattern on the ventral surface of the last abdominal segment of *Anomala* sp. found at Harden Heights during monthly pit sampling (B). PHOTOS: M. Way A schematic representation of the *Anomala* sp. larval raster pattern in Sweeney, 1967 (C).

Plate A2.2.5: A *Schizonycha affinis* larva (A) PHOTO: T. Goble. Ventral view of a *S. affinis* larva showing the associated raster pattern (B) PHOTO: M. Way. *Schizonycha affinis* raster patter on the ventral surface of the last abdominal segment of a larva found at Harden Heights during monthly pit sampling (C). PHOTO: M. Way

Plate A2.2.6: Dorsal and lateral view of an adult *Maladera* sp1 found at Harden Heights in December during sampling. The scale is in mm. PHOTOS: M. Way

Plate A2.2.7: Dorsal and lateral view of an adult *Maladera* sp2 found at Harden Heights in December during sampling. The scale is in mm. PHOTOS: M. Way

Plate A2.2.8: Dorsal and lateral view of an adult *Trochalus* sp found at Harden Heights in December during sampling. The scale is in mm. PHOTOS: M. Way

Plate A2.2.9: Dorsal and lateral view of an adult *Hypopholis sommeri* found at Harden Heights in October during sampling. The scale is in mm. PHOTOS: M. Way

Plate A2.2.10: Dorsal and lateral view of an adult *Congella* sp found at Harden Heights in June during sampling. The scale is in mm. PHOTOS: M. Way

Plate A2.2.11: Dorsal and lateral view of an adult *Adoretus* sp found at Harden Heights in March during sampling. The scale is in mm. PHOTOS: M. Way

Plate A2.2.12: Dorsal and lateral view of an adult *Schizonycha affinis* found at Harden Heights in March during sampling. The scale is in mm. PHOTOS: M. Way

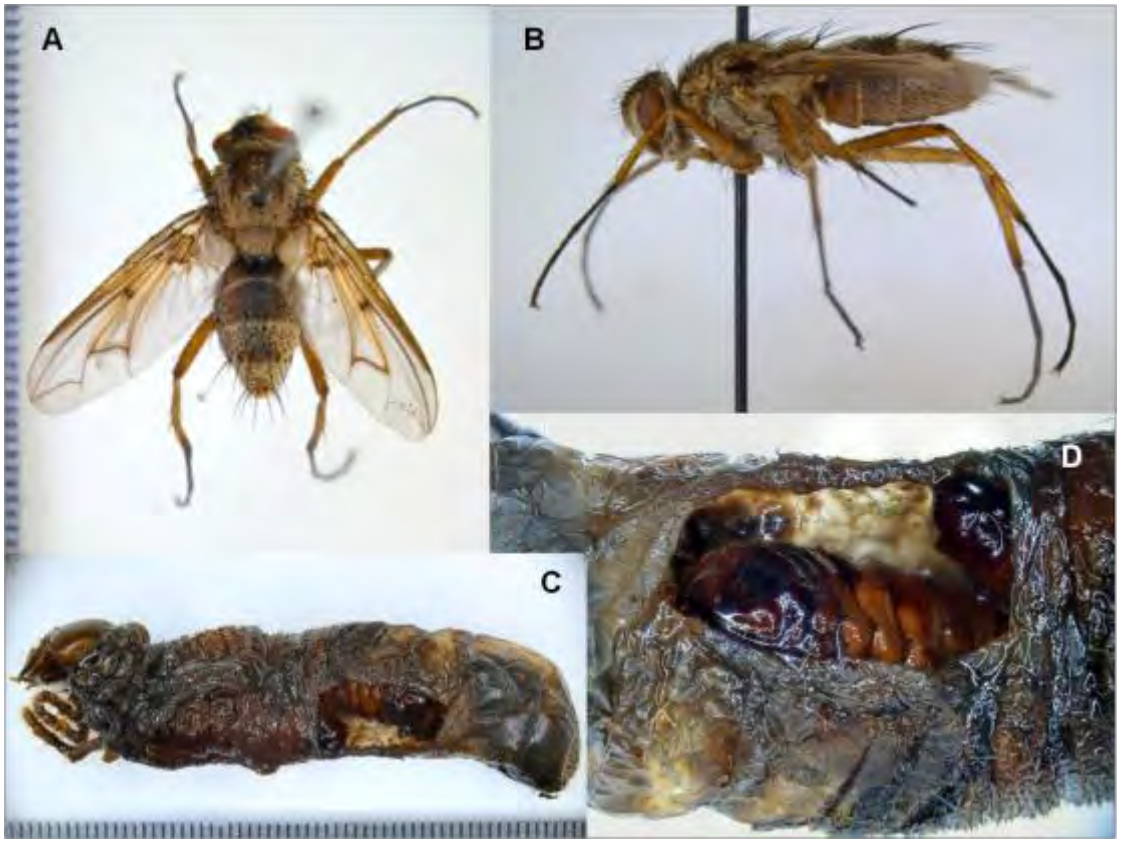

Plate A2.2.13: Dorsal view of a Dexiinae fly parasitoid (A); lateral view of the fly parasitoid showing long, clawed legs (B); two endoparasitic fly maggots are often found infesting *Hypopholis sommeri* 3rd instar grubs; one fly pupae can be seen inside the gut of the grub (C); a close up view of the fly parasitoid pupa inside the dead grub gut (D). The scale is in mm. PHOTOS: M. Way

Plate A2.2.14: *Hypopholis sommeri* 3rd instar grubs infected with the white muscadine fungal genus *Beauveria* found at Harden Heights in June 2011 (A); a unknown *Beauveria*-infected grub (B); unknown adult beetles infected with *Beauveria* sp found stuck to a black wattle *Acacia mearnsii*, tree trunk (C); close-up of the infected beetle (D); *Beauveria*-infected cadavers found in the soil at Harden Heights, showing heavy sporulation (E). The scale is in mm. PHOTOS: T. Goble

APPENDIX 2.3

Table A2.3.1: The best models for the white grubs: *S. affinis*, *Anomala* sp. and *H. sommeri* juvenile life stages selected from multiple logistic regression using information theoretic approach (Akaike 1983, Burnham and Anderson 2002). Bold font represents the best selected models and (*X*) represents the impact factor climatic variables.

			Variables included in the models										
species	**life stage**	**model no.**	**rainfall**	**soil water content**	**relative humidity**	**solar radiation**	**soil temperature**	**df**	**AIC**	**ΔAIC**	**w_i**	**w_t**	***P***
S. affinis	1st and 2nd instars	1	x	x	x	x	x	5	313.04	0.00	1.00	0.30	0.0000
S. affinis	**1st and 2nd instars**	**2**	**(x)**		**(x)**	**x**		**3**	**313.94**	**0.91**	**0.64**	**0.19**	**0.0000**
S. affinis	1st and 2nd instars	3	x		x	x	x	4	314.28	1.24	0.54	0.16	0.0000
S. affinis	1st and 2nd instars	4	x	x	x	x		4	314.66	1.62	0.44	0.13	0.0000
S. affinis	1st and 2nd instars	5	x		x		x	3	314.92	1.88	0.39	0.12	0.0000
S. affinis	1st and 2nd instars	6	x	x	x		x	4	316.42	3.38	0.18	0.06	0.0000
***Anomala* sp**	**1st and 2nd instars**	**1**			**(x)**	**x**		**2**	**231.15**	**0.00**	**1.00**	**0.17**	**0.0000**
Anomala sp	1st and 2nd instars	2	x		x			2	231.15	0.00	1.00	0.17	0.0000
Anomala sp	1st and 2nd instars	3		x	x		x	3	233.14	2.00	0.37	0.06	0.0000
Anomala sp	1st and 2nd instars	4	x	x			x	3	233.14	2.00	0.37	0.06	0.0000
Anomala sp	1st and 2nd instars	5		x	x	x		3	233.15	2.00	0.37	0.06	0.0000
Anomala sp	1st and 2nd instars	6	x	x	x			3	233.15	2.00	0.37	0.06	0.0000
Anomala sp	1st and 2nd instars	7	x		x	x		3	233.15	2.00	0.37	0.06	0.0000
Anomala sp	1st and 2nd instars	8	x		x		x	3	233.15	2.00	0.37	0.06	0.0000
Anomala sp	1st and 2nd instars	9		x		x	x	3	233.15	2.00	0.37	0.06	0.0000
H. sommeri	**1st instar**	**1**	**(x)**		**(x)**		**x**	**3**	**111.89**	**0.00**	**1.00**	**0.68**	**0.0000**
H. sommeri	1st instar	2	x		x	x	x	4	113.52	1.63	0.44	0.30	0.0000
H. sommeri	1st instar	3	x		x	x		3	119.25	7.36	0.03	0.02	0.0000
H. sommeri	**2nd instar**	**1**			**(x)**		**x**	**2**	**95.67**	**0.00**	**1.00**	**0.31**	**0.0000**
H. sommeri	2nd instar	2			x	x		2	97.39	1.71	0.42	0.13	0.0000
H. sommeri	2nd instar	3		x	x		x	3	97.54	1.87	0.39	0.12	0.0000
H. sommeri	2nd instar	4			x	x	x	3	97.58	1.90	0.39	0.12	0.0000
H. sommeri	2nd instar	5		x	x	x		3	97.63	1.95	0.38	0.12	0.0000

Table A2.3.2: The best models for the white grubs: *Maladera* sp11, *S. affinis*, *Anomala* sp and *H. sommeri* life stages selected from multiple logistic regression using information theoretic approach (Akaike 1983, Burnham and Anderson 2002). Bold font represents the best selected models and (*X*) represents the impact factor climatic variables.

			Variables included in the models									
species	**life stage**	**model no.**	**rainfall**	**soil water content**	**relative humidity**	**solar radiation**	**soil temperature**	**df**	**AIC**	**ΔAIC**	w_i	w_t	***P***
Maladera sp	1st instar	1	x	x		x		3	409.58	0.00	1.00	0.18	0.0000
Maladera sp	1st instar	2		x		x	x	3	409.96	0.38	0.83	0.15	0.0000
***Maladera* sp**	**1st instar**	**3**		**x**		**(x)**		**2**	**409.96**	**0.39**	**0.82**	**0.15**	**0.0000**
Maladera sp	1st instar	4	x	x		x	x	4	411.13	1.55	0.46	0.08	0.0000
Maladera sp	1st instar	5	x	x	x	x		4	411.50	1.93	0.38	0.07	0.0000
Maladera sp	2nd instar	1	x	x		x	x	4	614.54	0.00	1.00	0.30	0.0000
***Maladera* sp**	**2nd instar**	**2**	**x**	**(x)**			**x**	**3**	**616.04**	**1.49**	**0.47**	**0.14**	**0.0000**
Maladera sp	2nd instar	3	x	x		x		3	616.21	1.67	0.43	0.13	0.0000
Maladera sp	2nd instar	4	x	x	x	x	x	5	616.28	1.74	0.42	0.13	0.0000
S. affinis	3rd instar	1	x	x	x			3	825.79	0.00	1.00	0.32	0.0000
S. affinis	**3rd instar**	**2**	**x**	**(x)**				**2**	**826.68**	**0.89**	**0.64**	**0.21**	**0.0000**
S. affinis	3rd instar	3	x	x	x		x	4	826.68	0.89	0.64	0.21	0.0000
S. affinis	3rd instar	4	x	x			x	3	828.53	2.73	0.25	0.08	0.0000
H. sommeri	**3rd instar**	**1**		**(x)**				**1**	**1 683.42**	**0.00**	**1.00**	**0.13**	**0.0000**
H. sommeri	3rd instar	2	x	x		x	x	4	1 683.62	0.21	0.90	0.12	0.0002
H. sommeri	3rd instar	3	x	x			x	3	1 683.76	0.34	0.84	0.11	0.0001
H. sommeri	3rd instar	4		x			x	2	1 684.34	0.92	0.63	0.08	0.0001
H. sommeri	3rd instar	5		x		x	x	3	1 684.48	1.06	0.59	0.08	0.0002
H. sommeri	3rd instar	6	x	x				2	1 684.70	1.28	0.53	0.07	0.0002
H. sommeri	3rd instar	7	x	x	x			3	1 685.00	1.58	0.45	0.06	0.0002
H. sommeri	3rd instar	8	x	x	x		x	4	1 685.28	1.86	0.39	0.05	0.0003
H. sommeri	3rd instar	9		x	x			2	1 685.40	1.98	0.37	0.05	0.0002
***Anomala* sp**	**3rd instar**	**1**		**x**		**x**	**(x)**	**3**	**524.42**	**0.00**	**1.00**	**0.13**	**0.0000**
Anomala sp	3rd instar	2	x	x			x	3	524.42	0.00	1.00	0.13	0.0000
Anomala sp	3rd instar	3		x	x		x	3	524.43	0.00	1.00	0.13	0.0000
Anomala sp	3rd instar	4	x		x		x	3	524.43	0.00	1.00	0.13	0.0000
Anomala sp	3rd instar	5			x	x	x	3	524.43	0.01	1.00	0.13	0.0000
Anomala sp	3rd instar	6	x			x	x	3	524.45	0.02	0.99	0.13	0.0000

Table A2.3.3: The best models for the white grubs: *Maladera* sp11, *S. affinis* and *H. sommeri* life stages and unknown pupae selected from multiple logistic regression using information theoretic approach (Akaike 1983, Burnham and Anderson 2002). Bold font represents the best selected models and (*X*) represents the impact factor climatic variables.

			Variables included in the models									
species	**life stage**	**model no.**	**rainfall**	**soil water content**	**relative humidity**	**solar radiation**	**soil temperature**	**df**	**AIC**	**ΔAIC**	w_i	w_t	***P***
Maladera sp	3rd instar	1	x	x	x	x	x	5	998.33	0.00	1.00	0.49	0.0000
***Maladera* sp**	**3rd instar**	**2**		**x**	**(x)**	**x**	**(x)**	**4**	**998.38**	**0.05**	**0.98**	**0.48**	**0.0000**
Maladera sp	3rd instar	3	x		x	x	x	4	1003.97	5.64	0.06	0.03	0.0000
S. affinis	adults	1	x		x	x	x	4	556.14	0.00	1.00	0.10	0.0606
S. affinis	adults	2				x	x	2	556.33	0.20	0.91	0.09	0.0897
S. affinis	**adults**	**3**	**x**			**x**	**(x)**	**3**	**556.67**	**0.53**	**0.77**	**0.08**	**0.0902**
S. affinis	adults	4		x		x	x	3	556.99	0.85	0.65	0.07	0.1038
S. affinis	adults	5	x		x			2	557.32	1.19	0.55	0.06	0.1472
S. affinis	adults	6					x	1	557.53	1.39	0.50	0.05	0.2024
S. affinis	adults	7			x			1	557.58	1.45	0.48	0.05	0.2101
H. sommeri	**adults**	**1**	**(x)**		**(x)**	**x**		**3**	**69.73**	**0.00**	**1.00**	**0.19**	**0.0024**
H. sommeri	adults	2	x	x	x	x		4	71.32	1.60	0.45	0.09	0.0050
H. sommeri	adults	3	x		x	x	x	4	71.72	1.99	0.37	0.07	0.0060
H. sommeri	adults	4	x	x	x			3	72.05	2.33	0.31	0.06	0.0070
H. sommeri	**pupae**	**1**	**x**	**(x)**			**x**	**3**	**86.94**	**0.00**	**1.00**	**0.17**	**0.0000**
H. sommeri	pupae	2	x	x		x	x	4	86.95	0.01	0.99	0.17	0.0000
H. sommeri	pupae	3	x	x	x		x	4	86.95	0.01	0.99	0.17	0.0000
H. sommeri	pupae	4	x	x	x	x		4	86.96	0.01	0.99	0.17	0.0000
H. sommeri	pupae	5		x	x	x	x	4	86.96	0.02	0.99	0.17	0.0000
H. sommeri	pupae	6	x	x	x	x	x	5	88.96	2.01	0.37	0.06	0.0000
unknown	**pupae**	**1**					**(x)**	**1**	**178.72**	**0.00**	**1.00**	**0.17**	**0.0041**
unknown	pupae	2			x		x	2	179.90	1.18	0.55	0.09	0.0108
unknown	pupae	3				x		1	180.13	1.41	0.49	0.08	0.0090
unknown	pupae	4	x				x	2	180.31	1.59	0.45	0.08	0.0133
unknown	pupae	5		x		x	x	2	180.34	1.62	0.45	0.08	0.0134

Table A2.3.4: The best models for *Beauveria*-infected cadavers and Tachinid fly parasitoids selected from multiple logistic regression using information theoretic approach (Akaike 1983, Burnham and Anderson 2002). Bold font represents the best selected models and (*X*) represents the impact factor climatic variables.

			Variables included in the models									
species	**life stage**	**model no.**	**rainfall**	**soil water content**	**relative humidity**	**solar radiation**	**soil temperature**	**df**	**AIC**	**ΔAIC**	w_i	w_t	***P***
Beauveria	**cadavers**	**1**					**(x)**	**1**	**256.92**	**0.00**	**1.00**	**0.11**	**0.4583**
Beauveria	cadavers	2		x				1	257.30	0.37	0.83	0.09	0.6753
Beauveria	cadavers	3				x		1	257.32	0.40	0.82	0.09	0.6963
Beauveria	cadavers	4	x					1	257.34	0.41	0.81	0.09	0.7130
Beauveria	cadavers	5			x			1	257.41	0.49	0.78	0.08	0.8049
Tachinidae	**adult**	**1**			**(x)**			**1**	**13.96**	**0.00**	**1.00**	**0.18**	**0.0186**
Tachinidae	adult	2	x	x				2	15.96	2.00	0.37	0.07	0.0628
Tachinidae	adult	3		x	x			2	15.96	2.00	0.37	0.07	0.0628
Tachinidae	adult	4		x			x	2	15.96	2.00	0.37	0.07	0.0628
Tachinidae	adult	5			x	x		2	15.96	2.00	0.37	0.07	0.0628

CHAPTER 3 - Determining the taxonomy of fungal epizootics on white grubs in the KZN Midlands North using molecular techniques[12]

3.1 INTRODUCTION

In the previous chapter, the seasonal occurrence, diversity and distribution of various white grub species which occurred at the Harden Heights study site in the KZN Midlands North were described. This study site was also the location of an apparent fungal epizootic and during monthly sampling undertaken in the previous chapter many fungal mycosed white grub cadavers were collected. During the study period mentioned above, a second sugarcane cultivated field site, Canema, which is 5.5 km away from the Harden Heights study site, was discovered which also appeared to harboring many fungal mycosed white grub cadavers in the soil. The discovery of these infected insects at the Harden Heights and Canema sites prompted this study into the identity and ecology of the fungal species. In 2005, the first quantifiable incident of entomopathogenic fungal infection of white grubs in this region was observed on Sunnyside Farm (29°06'55.5"S, 30°46'59.1"E; altitude: 900-1400 meters) which is 15 km away from the Harden Heights and Canema sites, when many mycosed cadavers were dug up from sugarcane fields (Hatting, 2008). Using morphological identification, it was concluded that *B. bassiana* was responsible for the death of white grubs in this region (Hatting, 2008). *Beauveria bassiana* isolate 4222 was obtained directly from an infected white grub pupa at Sunnyside Farm in 2005, and showed good biological control potential in the laboratory against two white grub hosts, *H. sommeri* and *S. affinis* (Hatting, 2008).

The problem with the identification of the fungal species (*B. bassiana* isolate 4222) was that only morphology was considered, which is often highly subjective because fungal colony morphology can vary depending on the artificial media they are sub-cultured on (Brownbridge *et al.* 2001; Rehner, 2005). Further, morphological characteristics are not informative enough to delineate species in anamorphic (asexual) fungi and additional taxonomic characters need to be examined (Rehner, 2005). The power of molecular genetic techniques to address strain characterization and ecological research questions has opened a distinct interdisciplinary research area known as molecular ecology (Enkerli and Widmer,

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2010). Molecular ecology combines various aspects of population and evolutionary genetics, biodiversity or species-habitat interactions by employing molecular techniques which detect specific DNA sequence characteristics that are used as genetic markers for the delineation of species or to discriminate between taxonomic groups (Enkerli and Widmer, 2010). There are a number of studies which highlight the relevance and important of molecular techniques to study the ecology of fungal entomopathogens (Hesketh *et al.* 2010; Meyling and Hajek, 2010; Meyling *et al.* 2009).

Milner (1992) was the first to show how important molecular investigations were when evaluating fungal strains which occurred during epizootics. *Metarhizium anisopliae* is one of several species of *Metarhizium* which occur naturally in Australian soils (Samson *et al.* 2002). During the selection and characterization of *M. anisopliae* strains for the development of the mycoinsecticide, BioCane®, Milner (1992) reported that *M. anisopliae* was known to be highly genetically variable and often the most virulent isolates were obtained from natural infections in a particular host. Subsequent molecular studies using RAPD patterns, with a range of strains obtained from greyback grubs, *D. albohirtum*, showed that subsequent strains were genetically distinct from the original isolate obtained and formulated as BioCane® and that most were substantially less virulent than the original strain (Milner *et al.* 2002). Neuvéglise *et al.* (1997) also highlighted the importance of molecular studies in the determination of strains and their specificity for mycoinsecticide development. These authors showed that there was a correlation between the occurrence of characteristic group1-introns and virulent *B. brongniartii* isolates collected from the sugarcane pest *H. marginalis* in Reunion (Neuvéglise *et al.* 1997).

The most recent advances in the genetic diversity and molecular ecology of the *Beauveria* genus were described by Rehner *et al.* (2011). A multilocus phylogeny of *Beauveria* based on partial sequences of the *RPB1, RPB2, TEF* and *Bloc* gene regions was used to assess diversity within the genus and to evaluate species concepts and their taxonomic status (Rehner *et al.* 2011). The results concluded that *B. bassiana* and *B. brongniartii* represented species complexes and that the genus comprised 12 defined species. Primers which allow the amplification of various gene regions such as the elongation factor 1 alpha (*EF1α*) and *Bloc* (a nuclear intergenic region) are continually being developed for the *Beauveria* genus and these gene regions are now increasingly being used for the delineation of species in this fungal group (Rehner and Buckley, 2005; Meyling *et al.* 2009; Rehner *et al.* 2011).

Currently, microsatellite markers represent the most popular genetic markers used in the inference of fungal population structure, genetic variation and relatedness (Enkerli and Widmer, 2010). Microsatellites are simple sequences repeats (SSR) which consist of tandem repeats of 1-6 nucleotides and they are dispersed throughout the genome of most organisms (Enkerli and Widmer, 2010). Alleles of a given locus may vary in the number of repeat sequences resulting in polymorphism of the SSR alleles; it is ultimately this variation which allows for the specific detection and characterization of various fungal strains (Enkerli and Widmer, 2010). Many microsatellite markers have been developed for various entomopathogenic fungi, including the species, *B. bassiana* (Rehner and Buckley, 2003) and *B. brongniartii* (Enkerli *et al.* 2001). These genetic markers have been used to study insect-host associations (Leland *et al.* 2005), to identify and characterize strains with potential use as bio-insecticides (McGuire *et al.* 2005) and to monitor the fate and persistence of mass-released fungal isolates in the environment (Enkerli *et al.* 2004).

One of the most important aspects of the present study was to determine whether another fungal species was responsible for the death of white grubs in the KZN Midlands North region or to confirm the previously identified *B. bassiana* isolate 4222 as the causal fungal agent of the observed epizootics. Literature suggested that another fungal species, *B. brongniartii* may be responsible for the observed epizootics because this species is a well-known entomopathogen of Coleoptera, being quite specific to the Melolonthinae and known to cause epizootics elsewhere (Hurpin and Robert, 1972; Keller, 1986; Neuvéglise *et al.* 1997, Keller *et al.* 1999; Enkerli *et al.* 2001). Thus based on the literature which exists on various molecular markers and their relevance in delineating fungal species and their ability to discriminate fungal population structure, genetic variation and relatedness the aims of the present study were thus:

1.) To employed two molecular techniques: multilocus gene sequencing and microsatellite markers to ascertain which fungal species was causing the infections observed on white grubs in the KZN Midlands North region; 2.) To use microsatellite markers to investigate the genetic diversity and ecology of the most commonly occurring fungal species found in this region.

3.2 METHODS AND MATERIALS

3.2.1 *Collection of isolates*

A total of 78 *Beauveria* isolates (Appendix 3.1, Table A3.1.1) were considered in this study. Sixty-eight of these isolates were collected from two sites (Canema and Harden Heights) in the KZN Midlands North area of South Africa (Figure 3.1). The remaining ten *Beauveria* isolates included in this study came from other sites in South Africa, as well as an isolate from Reunion (Appendix 3.1, Table A3.1.1). The KZN Midlands North area is characterized by large tracks of land which are a mosaic of intensively cultivated sugarcane and black wattle plantations (Figure 3.1). At the two field sites, fungal isolates were collected from infected scarabaeid larvae, pupae and adults dug up from sugarcane fields. A standard industry-based method for sampling white grubs was applied (Way *et al.* 2011). Where infected larvae not showing signs of overt mycosis were found, raster patterns on the anus were investigated to confirm species identity (Sweeney, 1967). In addition, dead *H. sommeri* beetles attached to vegetation (mainly black wattle) at field edges were collected. Thirdly, soil samples collected randomly from both sites were baited with the greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae) to obtain *Beauveria* isolates (Zimmermann, 1986). These bait-soil samples did not overlap with collected insects or dug pits at either site and were evenly spaced throughout fields. Lastly, random and evenly spaced sampling of various plant surfaces, particularly the leaves of black wattle at the field edge was undertaken to isolate *Beauveria* spp. A leaf press was made onto an agar plate containing full-strength Sabouraud dextrose agar (SDA) (Strasser *et al.* 1996) supplemented with 50mg/l chloramphenicol, 25 mg/l cycloheximide and 50mg/l rifampicin. When white fungal colonies were observed they were carefully removed and sub-cultured on fresh SDA plates. All *Beauveria* isolates were maintained on full-strength SDA supplemented with the above mentioned antibiotics and kept at 23°C. Subsequently, single conidial preparations were made. Fungal isolates are currently housed in the South African National Collection of Fungi at the Plant Protection Research Institute (Agricultural Research Council) in Pretoria, South Africa.

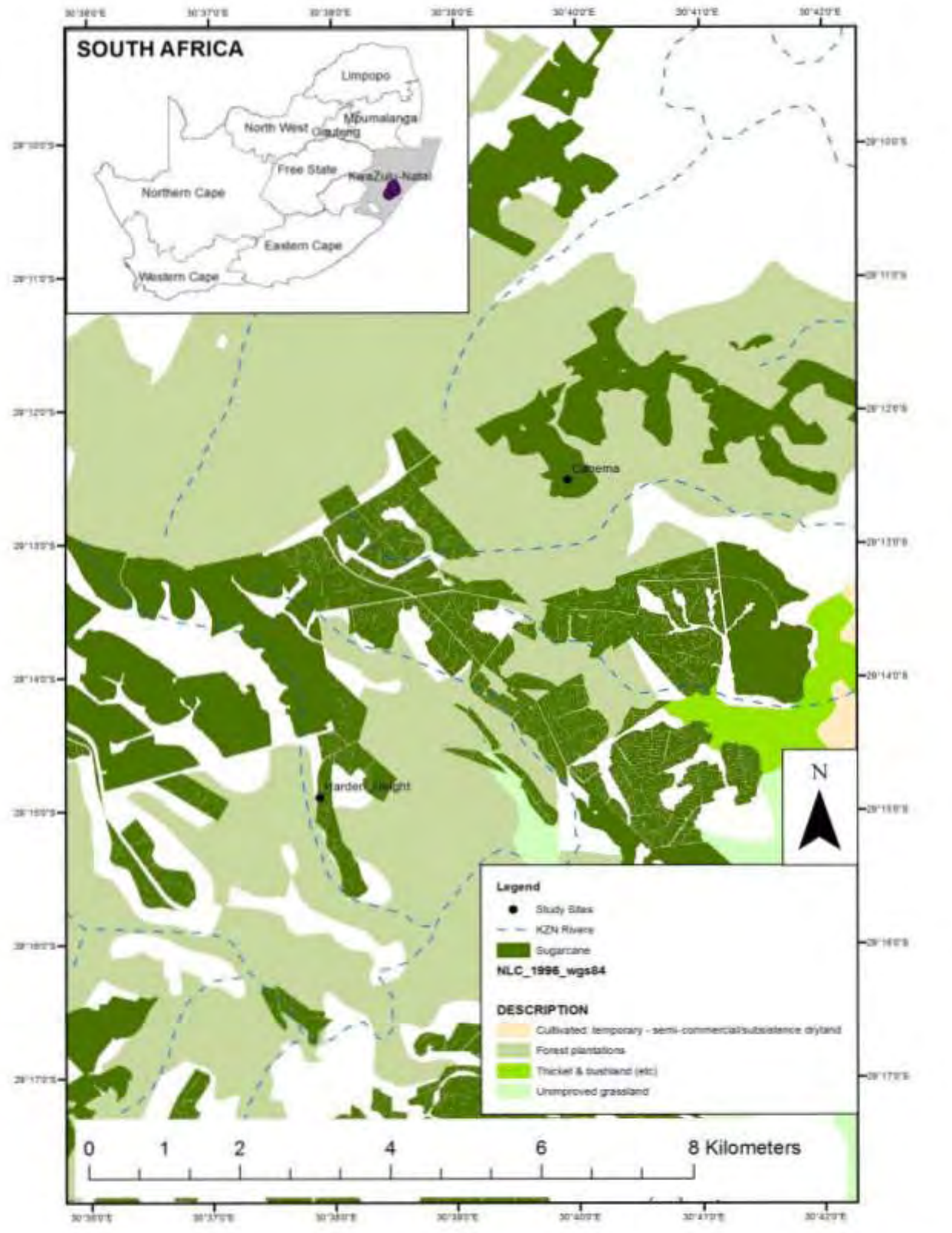

Figure 3.1: The two sites, Harden Heights and Canema where *Beauveria* isolates were collected in the KwaZulu-Natal Midlands North sugarcane producing region of South Africa. Dark green areas represent areas under sugarcane cultivation while those areas in lighter green/grey represent black wattle plantations.

3.2.2 *DNA extraction*

Conidia from fungal isolates were inoculated into flasks containing a sterilized liquid growth medium (3% sucrose, 2% peptone and 0.2% yeast extract) and placed on a rotary shaker at 180 rpm for 72 hours. The resulting mycelial mats were recovered by filtration and stored at -80°C for DNA extraction. Mycelia were ground with a mortar and pestle in liquid nitrogen until a fine powder remained. Ground mycelia (1.5 g) were re-suspended in extraction buffer (12.5 ml) consisting of 200 mM Tris-HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA and 0.5% SDS, after which phenol (pH 7.9) (8.75 ml) preheated to 60°C was added followed by the addition of chloroform/isoamylalcohol [24:1 (v/v)] (3.75 ml). After centrifugation (18900 x g) in a Beckman J2-21 centrifuge for 60 minutes at 4°C the top liquid phase containing the DNA was removed. RNA was removed from the liquid phase through the addition of 500 ul (5 mg/ml) RNase H and incubation for 20 minutes at 37°C. One part phenol was added to the mixture after incubation with the RNase and the mixture was again centrifuged (18900 x g for 20 minutes) at 4°C. The liquid phase was removed and the DNA was precipitated with 1:1 volumes of isopropanol followed by centrifugation. The resulting pellet was washed with 70% (v/v) ethanol, recentrifuged, after which the ethanol was aspirated and the pellet was dried. The pellet containing the isolated DNA was then dissolved in 1 ml TE buffer [10 mM Tris (pH 7.8) and 1 mM EDTA] and stored at -20°C for further manipulation.

3.2.3 *PCR amplification and sequencing*

The complete data set for phylogenetic analyses totalled 37 isolates of *Beauveria* and one out-group, *Isaria* sp. (Ascomycota: Cordycipitaceae) Twenty-three *Beauveria* isolates found in KZN, South Africa (eight *B. bassiana s.s* isolates and 15 *B. brongniartii* isolates) and the remaining isolates included 15 published, Bloc and ITS sequences, respectively from type-strain *Beauveria* species (Rehner *et al.* 2011). For the 23 South African isolates, two gene regions were partially amplified and sequenced: Bloc, an intergenic region often used as a phylogenetic marker for the genus *Beauveria* (Rehner *et al.* 2006) and the ribosomal internal transcribed spacer (ITS). The Bloc gene region was amplified with primers (B5.1F, B3.1R and B22U, B822L) obtained from Rehner *et al.* (2006). PCR amplifications were performed in a total volume of 20 µl, which included 10 µl of 2 × AmpliTaq Gold PCR master mix (DNA polymerase 250 U (0.05 U/µl) in buffer (30 mM Tris/HCl (pH 8), 100 mM KCl; dNTPs 400 µM each; $MgCl_2$ 5 mM) (Applied Biosystems, Johannesburg); 2 µl Q-solution

(Qiagen, Johannesburg); 1 µl of (10 µM) forward primer; 1 µl of (10 µM) reverse primer and 1 µl genomic DNA (30 ng) the rest was made up with water. PCR was performed using the following thermocycling regime: denaturation at 95°C for 5 min; 33 × cycles (94°C for 30 secs, 58°C for 90 secs, 72°C for 90 secs) and a final elongation step of 72°C for 30 min. The ITS gene was amplified and sequenced with primers ITS5 and ITS4 according to the specifications given in Rehner and Buckley (2005). Sequencing of both forward and reverse strands of both genes was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using 0.5 µl BigDye, 2 µl of 5× reaction buffer, 2 µl primer, 30 ng of DNA template and 4.5µl of water in a total volume of 10 µl. Sequence clean up was undertaken using BigDye X Terminator Purification Kit (Applied Biosystems) according to the manufacturer's instructions. Sequencing took place using an ABI 3500 Genetic Analyser. Genbank accession numbers for these sequences are provided in (Appendix 3.1, Table A3.1.1).

3.2.4 *Sequence editing and phylogenetic analyses*

Unrefined sequence chromatograms were assembled and edited with Geneious™ 5.4 Pro (Drummond *et al.* 2011). Multiple sequence alignments of edited sequences were created with MAFFT (Katoh *et al.* 2005; Katoh and Toh, 2008) using the FFT-NS-i alignment option. Maximum parsimony (MP) analyses were performed on individual and concatenated datasets. MP and nonparametric MP bootstrapping analyses were undertaken with PAUP*4.0b10 (Swofford, 2003) with all uninformative sites and gaps excluded, under equal character weighing, using a heuristic search option with TBR branch swapping, and executing 1000 random-addition replicate analyses. Heuristic MP bootstrap analyses were performed to assist clade support with 1000 bootstrap replicates. To determine whether different data partitions could be joined and analyzed together, a partition homogeneity test (PHT) was performed. Data were found to be congruent (>0.05) and the two genes (Bloc and ITS) were analyzed together. To determine the selection of nucleotide substitution model, implementing the Akaike information criterion (AIC) for Maximum likelihood (ML) and Bayesian (BI) analyses, jModelTest 0.1.1 (Posada, 2008) was used. The two-gene data set was partitioned, the substitution models for each partition were as follows: Bloc (TVM+G) and ITS (TIM2+G) and the combined data set (GTR+G). ML searches and 1000 ML bootstrap analyses were conducted in PAUP*4.0b10. Partitioned Bayesian analyses were carried out in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) and started from a random

tree using the program's default settings for the posterior probabilities which consisted of four chains, one cold and three hot and ran for 3×10^6 generations. A single tree was sampled ever 100^{th} generation. Plots of likelihood scores, tree length and averaged SD of split frequencies against number of generations showed that stationarity was reached within the first 10% of trees generated. Thus, the first 10% of trees generated were discarded using the burn-in function ensuring only trees generated at the stationary phase were included to calculate the posterior probabilities.

3.2.5 *Microsatellite DNA amplification and fragment analysis*

Initially, 17 fluorescently-labeled microsatellite PCR primers obtained from Enkerli *et al.* (2001) (Bb1F4, Bb2A3, Bb8D6, Bb10D4); Rehner and Buckley (2003) (Ba13); Meyling *et al.* (2009) (Ba06, Ba08, Ba12, Ba15, Ba17, Ba18, Ba21, Ba22, Ba25, Ba27, Ba28, Ba29) were used to target 78 isolates of *Beauveria* spp. DNA (Appendix 3.1, Table A3.1.1). It was unclear whether the epizootics were caused by *B. bassiana* or *B. brongniartii* so primers that would be able to discriminate both species were selected. A neighbour-joining analysis using DARwin™ 5.0 (Perrier and Jacquemoud-Collet, 2006) was undertaken.

Secondly, the genetic diversity of 58 *B. brongniartii* isolates obtained from two sites/populations: Harden Heights (35 isolates) and Canema (23 isolates), using the 17 microsatellite loci described above was undertaken. Two *B. brongniartii* isolates were not included due to poor quality of the chromatograms for some loci. PCR amplification reactions were performed in 15 µl reaction volumes with 1 µl (25 ng) of genomic DNA. 7.5 µl of 2×Qiagen multiplexing PCR master mix (Hotstart® DNA Polymerase, buffer and dNTP mix), 1.5 µl Q-solution and 1.5 µl of primer mixtures with fluorescent labeling either: VIC, PET, NED, 6-FAM were also added to the reaction (Applied Biosystems). PCR thermocycling parameters were as follows: 95°C for 15 min, 33 × cycles (94°C for 30 secs, 58°C for 90 secs, 72°C for 90 secs) and a final elongation step of 72°C for 30 min. After amplification 1 µl of PCR product was diluted 1/150 using distilled water. 1 µl of diluted PCR product was then added to 10.7 µl of formamide and 0.3 µl of molecular weight ladder GS 500 LIZ (Applied Biosystems). Mixtures were denatured at 95°C for 2 min and placed directly onto ice. Samples were then loaded into an ABI 3500 Genetic Analyser. Resulting fragment analysis was undertaken using GeneMapper™ 3.1 software (Applied Biosystems).

3.2.6 *Microsatellite population genetic analyses*

Population genetic diversity in terms of allelic frequencies (Na), the number of effective alleles (Ne), mean gene diversity (I) and unbiased diversity (h) by population was undertaken on microsatellite data with GenAlEx™6.3 (Peakall and Smouse, 2006). The number of effective alleles (Ne) measures the number of equally frequent alleles it would take to achieve a given level of gene diversity and allows us to compare populations where the number and distributions of alleles are drastically different. The information index (I) quantifies the expected value of information at loci. The program also identifies and estimates the number of microsatellite haplotypes and their frequencies (no. of observations) within populations. Genetic discontinuities between samples from different hosts (*G. mellonella*, scarabaeid larvae and *H. sommeri* adults) or sites/populations (Canema, Harden Heights) were quantified through analysis of molecular variance (AMOVA) using GenAlEx™6.3. Non-random associations of alleles among pairs of loci (linkage disequilibrium) were tested using two methods: gametic disequilibrium among pairs of loci was tested within each population using Fisher's exact test implemented in Genepop™4.0 and an index of multilocus linkage disequilibrium (r_d) was estimated within each population using Multilocus™2.2 (Agapow and Burt, 2001). Genic differentiation (exact G test) among populations and estimated spatial structure (F-statistic) for all populations was determined using Genepop™4.0 (Raymond and Rousset, 1995). The within locus G test were combined in a global χ^2-test, using the Fisher method (Fisher, 1938), carried out with Genepop. Considering that the existence of linkage disequilibrium among pair of loci could violate the independence assumption of the Fisher method, we also compared haplotype frequencies between both populations using a Fisher's exact test carried out with SAS proc Freq (SAS Institute, 2008).

3.3 RESULTS

An unweighted neighbor-joining tree (30 000 bootstraps replications) based on polymorphic microsatellite loci only and rooted on a *Beauveria* isolate BB1319 (BT126) from Reunion, revealed two distinct species groupings, supported by high bootstrap values (Figure 3.2). Of the 77 *Beauveria* isolates included as the ingroup, 17 were found to group within the smaller microsatellite (SSR) group 2 (22%), which was later confirmed as the *B. bassiana s.s* clade A; and the remaining 60 isolates (78%) fell within the larger SSR group 1 which represented a closely-related *B. brongniartii* clade C (Figure 3.2). It was the distinct separation of the data

into two groups which prompted sequencing to determine the taxonomy which is discussed below.

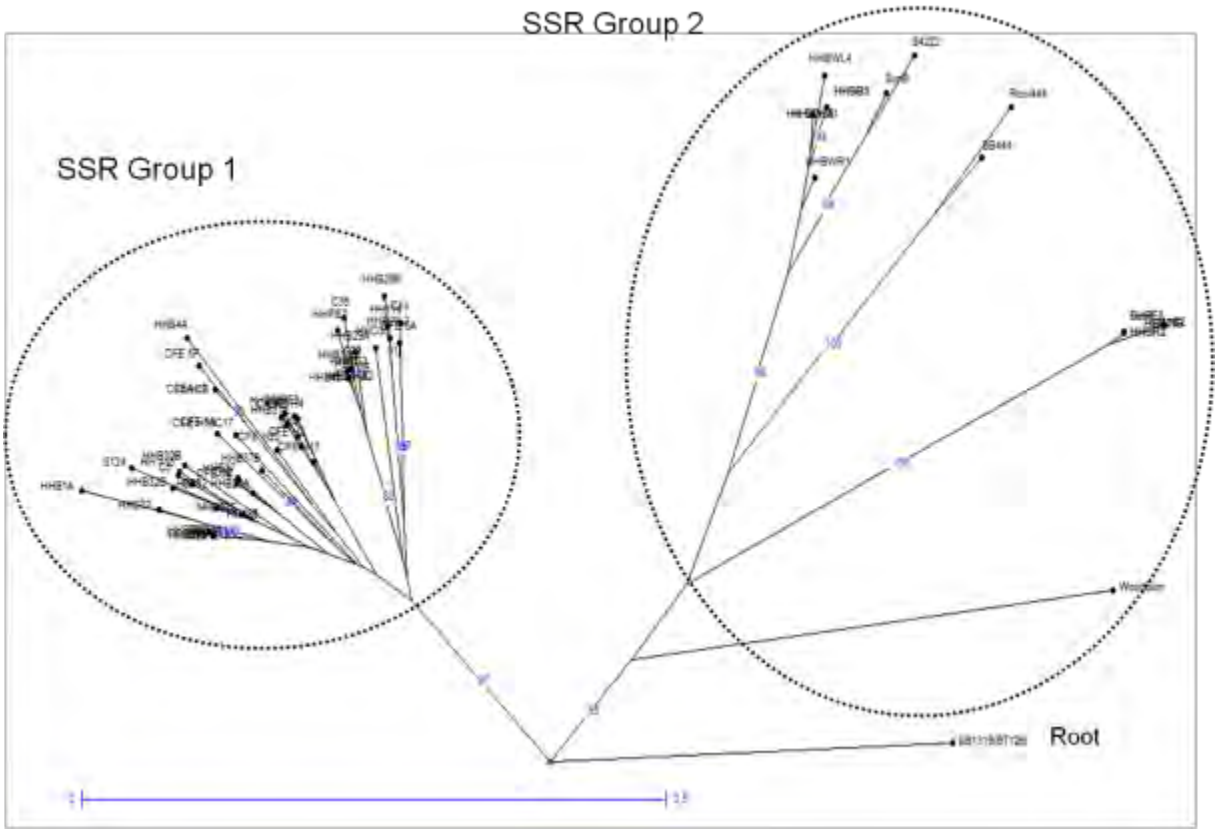

Figure 3.2: Unweighted, neighbour-joining tree in axial view, rooted on an isolate obtained from Reunion (BB1319-BT126), showing two distinct groupings of *Beauveria* isolates obtained from two sites in the KwaZulu-Natal Midlands North. Bootstraps > 80% are shown.

3.3.1 *Molecular sequencing*

The complete data set for phylogenetic analyses totalled 37 isolates of *Beauveria.* The two loci: Bloc and ITS consisted of 1308 and 532 aligned positions, respectively. When MP was considered, gapped or uninformative sites were excluded, rendering a final data set with 303 and 40 parsimony informative characters for Bloc and ITS respectively. The results of the analysis produced one most parsimonious tree with a length of 934 steps (CI: 0.525, RI: 0.779). ML analysis, under the GTR+G model, rendered a log-likelihood score of 9836.74 for the best tree. Finally Bayesian analyses converged on a stationary phase that yielded topologically identical 50% majority rule consensus trees. A phylogeny of *Beauveria* illustrating species relationships inferred from joint Bayesian Inference (BI) analysis of Bloc

and ITS gene sequences is shown in Figure 3.3. Bootstrap values from MP and ML analyses, as well as the posterior probabilities from Bayesian analyses, are provided (Figure 3.3).

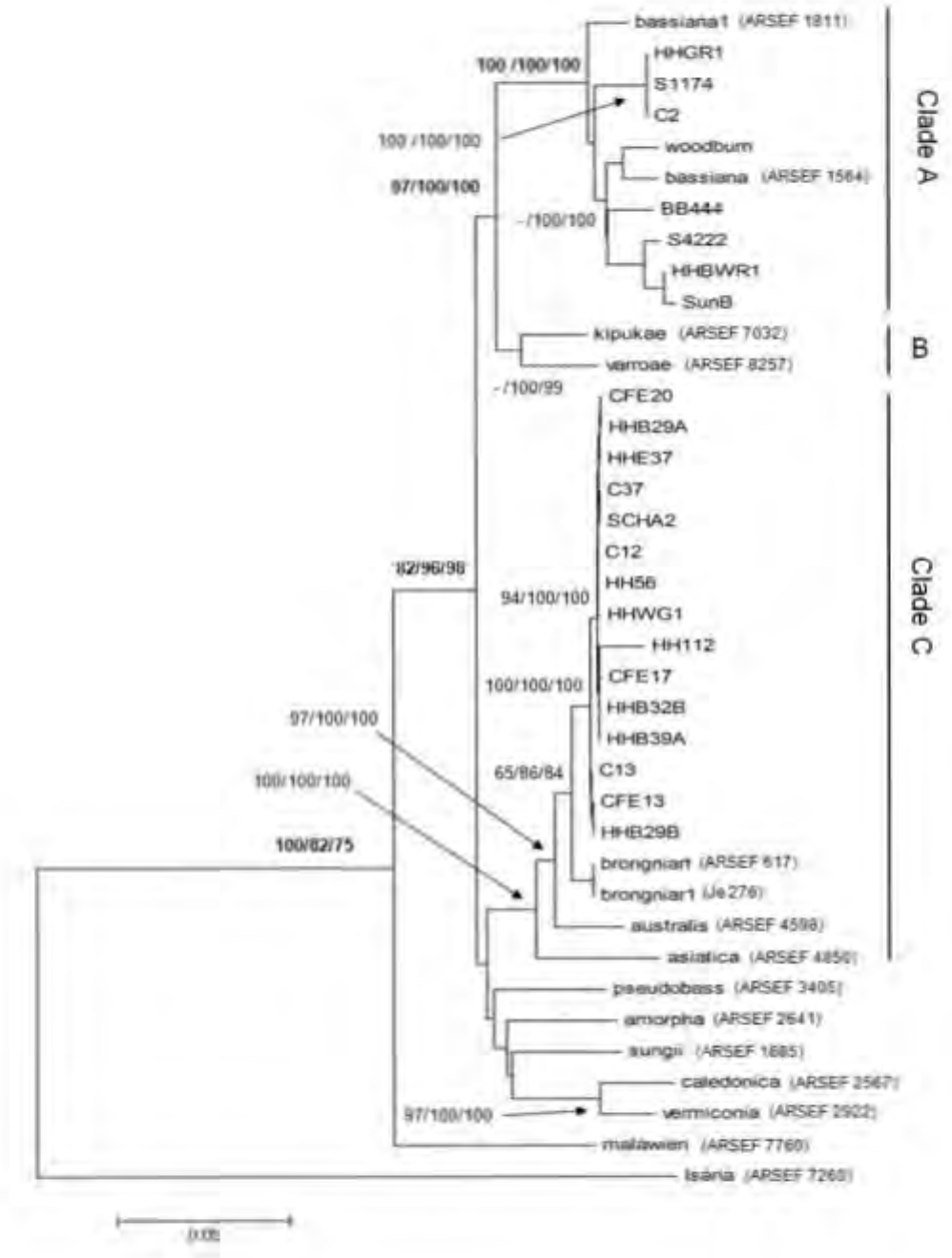

Figure 3.3: Phylogeny of *Beauveria* showing species relationships of South African isolates within the global *Beauveria* framework inferred from joint Bayesian Inference analysis of Bloc and ITS gene sequences. The bootstrap values above internal branches correspond to Maximum Parsimony (> 65), Maximum Likelihood (> 65) and Bayesian Inference posterior probabilities (> 95) respectively.

All three analyses (MP, ML and BI) yielded trees with similar tree topologies that each resolved three well-supported clades, A-C (Figure 3.3). Poor replication and inclusion of the basal taxa left the remaining clades indistinguishable and unsupported. Eight of the South African isolates (those not labelled with ARSEF numbers), which initially group into the smaller SSR group 2 (Figure 3.2), fell within the common *B. bassiana s.s* clade A in the phylogeny (Figure 3.3). Clade A was relatively diverse as seen by the deep internal branching and grouping of isolates, despite sampling localities being close together for some isolates. *Beauveria kipukae* and *B. varroae* formed a biphyletic clade B that was sister to *B. bassiana* in this study. Fifteen of the South African isolates (no ARSEF number), which grouped into the larger SSR group 1 (Figure 3.2) fell into the *B. brongniartii* clade C and were closely related as observed by very short branch lengths (Figure 3.3). *Beauveria australis* and *B. asiatica* were basal to *B. brongniartii* in this study with good bootstrap and posterior probability support. *Beauveria caledonica* and *B. vermiconia* grouped together with well-supported bootstrap and posterior probability support. Sister to this grouping was *B. sungii* with no support. Unfortunately, due to poor sample inclusion, an accurate placement of the remaining basal *Beauveria* taxa: *B. pseudobassiana*; *B. amorpha* and *B. malawiensis* were not observed (Figure 3.3).

3.3.1 *Microsatellite genotyping*

Beauveria brongniartii isolates showed relatively low levels of genetic diversity in this study (Table 3.1). Twelve of the 17 microsatellite loci used were polymorphic. Loci (Ba06, Ba08, Ba12, Ba18 and Ba21) revealed no genetic variation among isolates. In contrast, microsatellite markers Ba13, Ba22, Ba25 and especially Ba29 showed high levels of polymorphism, despite private alleles being thoroughly rechecked and despite them originally being cloned from *B. bassiana*. The percentage of polymorphic loci from Canema (47.06%) was lower than Harden Heights (70.59%) when rare alleles were considered (Table 3.1). Low levels of polymorphism were seen in the average number of alleles (Na) per locus which varied from 2.29 to 2.24 per site/population, respectively and a low mean expected heterozygosity was observed between Canema (0.25) and Harden Heights (0.24) (Table 3.1). Further, there was little genetic difference between the sites when effective alleles were considered: Canema (1.71) and Harden Heights (1.63).

Table 3.1: The number of alleles and their frequencies, as well as the diversity of 17 microsatellite loci from two South African populations of *B. brongniartii*

Population	Locus	N [a]	Na [b]	Ne [c]	I [d]	h [e]
Canema	BA18	23	1	1.00	0.00	0.00
Canema	BA15	23	1	1.00	0.00	0.00
Canema	BA25	23	2	1.63	0.57	0.39
Canema	BA17	23	1	1.00	0.00	0.00
Canema	BA22	23	3	2.63	1.02	0.62
Canema	BA21	23	1	1.00	0.00	0.00
Canema	BA29	23	3 (1)	2.99	1.21	0.67
Canema	BA27	23	1	1.00	0.00	0.00
Canema	BA08	23	1	1.00	0.00	0.00
Canema	BA06	23	1	1.00	0.00	0.00
Canema	BA12	23	1	1.00	0.00	0.00
Canema	BA28	23	1	1.00	0.00	0.00
Canema	BA13	23	3 (1)	2.46	1.08	0.59
Canema	Bb8D6	23	2	1.63	0.57	0.39
Canema	Bb1F4	23	5 (3)	5.24	1.83	0.81
Canema	Bb2A3	23	2 (3)	2.37	1.14	0.58
Canema	Bb10D4	23	1 (1)	1.19	0.30	0.16
Mean values			**2.29 ± 0.48**	**1.71 ± 0.28**	**0.45 ± 0.14**	**0.25±0.07**
Harden Heights	BA18	35	1	1.00	0.00	0.00
Harden Heights	BA15	35	1 (1)	1.06	0.13	0.06
Harden Heights	BA25	35	2	1.82	0.64	0.45
Harden Heights	BA17	35	1 (1)	1.06	0.13	0.06
Harden Heights	BA22	35	3	1.95	0.76	0.49
Harden Heights	BA21	35	1	1.00	0.00	0.00
Harden Heights	BA29	35	2 (1)	2.09	0.80	0.52
Harden Heights	BA27	35	1 (1)	1.12	0.22	0.11
Harden Heights	BA08	35	1	1.00	0.00	0.00
Harden Heights	BA06	35	1	1.00	0.00	0.00
Harden Heights	BA12	35	1	1.00	0.00	0.00
Harden Heights	BA28	35	1 (1)	1.06	0.13	0.06
Harden Heights	BA13	35	3 (2)	4.15	1.49	0.76
Harden Heights	Bb8D6	35	2	1.69	0.60	0.41
Harden Heights	Bb1F4	35	5	4.21	1.47	0.76
Harden Heights	Bb2A3	35	2 (1)	1.42	0.57	0.30
Harden Heights	Bb10D4	35	1 (1)	1.06	0.13	0.06
Mean values			**2.24 ± 0.30**	**1.63 ± 0.25**	**0.42 ± 0.12**	**0.24±0.07**

[a] Number of single-conidial strains analysed

[b] Number of alleles per locus (incl. private alleles) $p<0.05$

[c] Number of effective alleles- it measures the number of alleles at a locus and the equality of the allele frequencies at that locus

[d] Information index /same as Shannon-Weaver Index of ecology-measure of diversy

[e] Haploid genetic diversity-provides an indication that two individuals will be different (Nei, 1987)

AMOVA analyses showed that the total genetic differentiation among populations was 0.014 (PhiPT), and was not significant ($P \leq 0.180$) when based on permutation across the full data set, of which 99% (df=56; sum of squares=1115.77; variance=19.925) of the genetic variation is contained within each population, while only 1% (df=1; sum of squares=27.89; variance=0.287) occurs among populations (table not shown). The global fixation index was F_{ST}=0.03 indicating low genetic differentiation between the two sites/populations. However, when exact tests (G test) for population differentiation were performed looking at the distribution of alleles for all populations there was a highly significant difference in allele frequencies between the populations (χ^2=53.26; df= 24; $P \leq 0.001$).

Linkage disequilibrium was significant (non-random) for numerous pairs of loci within sites/ populations: for Canema 14 out of 28 possible tests were significant. There were fewer pairs of loci showing significant linkage disequilibrium at Harden Heights where 17 out of 66 possible tests were significant. The multilocus linkage disequilibrium index was significantly different from zero in both populations. It was highest at Canema (r_d = 0.196) and lower at Harden Heights (r_d = 0.141). These results indicate that both Canema and Harden Heights have non-recombining population structures. The comparison of haplotype frequencies between both populations (Fisher's exact test) concluded that there was no significant difference ($P \leq 0.09$), which is consistent with the AMOVA and low F_{ST} observed. These results suggest that Canema and Harden Heights represent the same, structured population of *B. brongniartii* but the population has been fragmented over time and space.

The highest number of alleles and private alleles came from *H. sommeri* adult beetles (Na=0.43 (private alleles=0.22) while both *G. mellonella* (Na=0.28(0.09) and scarabaeid larvae (Na=0.27 (0.09) had much lower allele numbers and private alleles. In the AMOVA analyses, the total differentiation among populations was 0.099 (PhiPT), and was significant ($P \leq 0.017$) when based on permutation across the full data set, of which 90% (df=52; sum of squares=976.42; variance=18.777) of the genetic variation is contained within each population, while only 10% (df=2; sum of squares=110.84; variance=2.067) occurs between populations (table not shown). The result indicates no genetic structure amongst the populations, and suggests there are significant differences in allele frequencies between the source types with low levels of gene flow.

The study revealed 26 haplotypes among 58 isolates of *B. brongniartii*. In the Harden Heights population 12 unique haplotypes were detected, some of them represented by only one strain. Nine unique haplotypes were observed at Canema, some of them also with only one strain. More microsatellite haplotypes were observed at Harden Heights (17) than at Canema (14) (Figure 3.4). Five haplotypes (H23, H5, H10, H7 and H3) were common to both populations however the frequency of occurrence of a particular haplotype was different in both populations (Figure 3.4). The most common haplotype, H23 was observed ten times and occurred in the same proportion in both populations (Figure 3.4 and Appendix 3.1, Table A3.1.2) when sample size was averaged. In the Harden Heights population the second most common haplotype was H5 which was observed six times, this haplotype occurred with less frequency at Canema where it was observed only twice (Figure 3.4 and Appendix 3.1, Table A3.1.2).

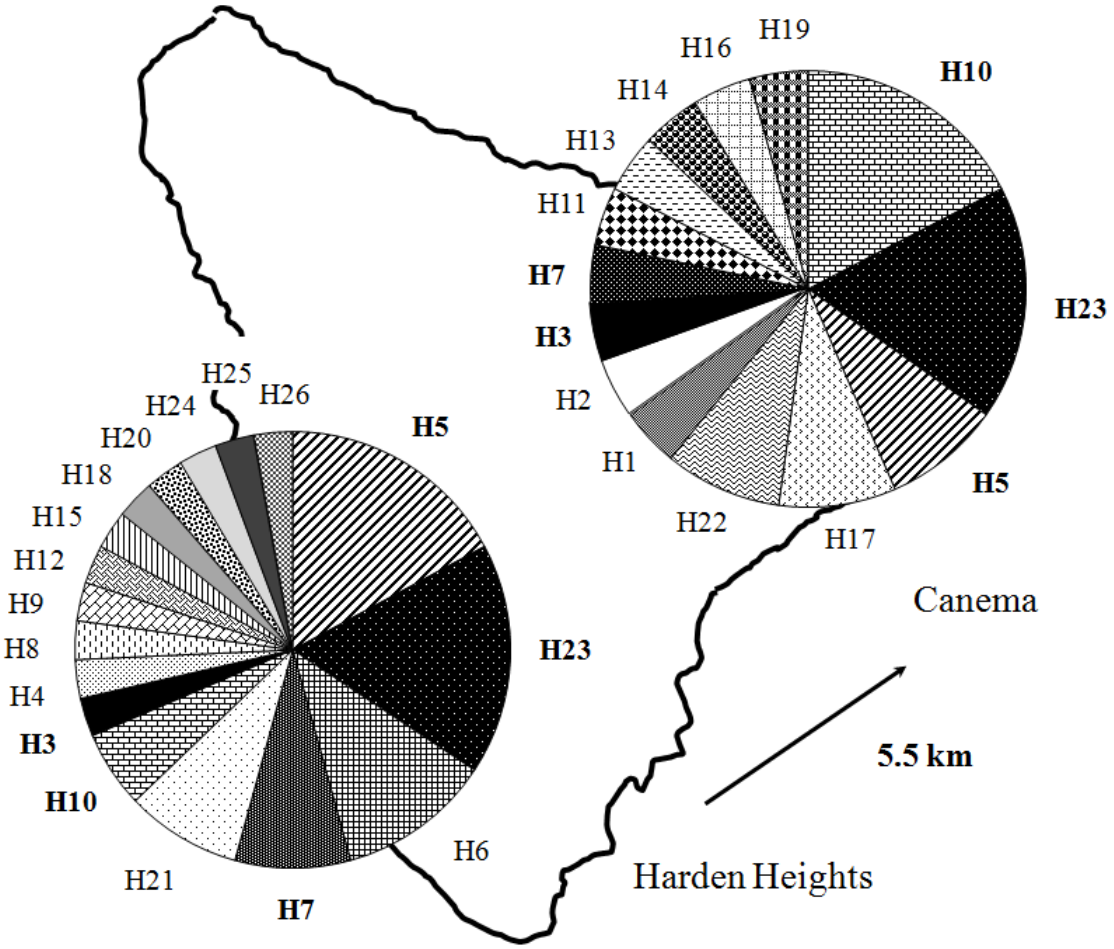

Figure 3.4: The geographic location and distribution of microsatellite haplotypes of two KwaZulu-Natal populations of *B. brongniartii* analysed in this study.

Some grouped haplotypes shared a common *H. sommeri* adult host (Figure 3.5; Appendix 3.1, Table A3.1.2). Besides these host groupings, no other structural patterns could be observed, even when sequence data, ecology or taxonomy was considered. A *B. brongniartii* haplotype (H6), shared three genetically identical strains/isolates (HH56; HHFE3 and HHBWL1 (HH219 was not considered) (Figure 3.5; Appendix 3.1, Table A3.1.2). HHFE3 was isolated from a *H. sommeri* adult found hanging off a black wattle leaf in a stand adjacent to the Harden Heights sugarcane field, the same strain was found on a black wattle leaf surface (HHBWL1) and finally the same strain was found within the sugarcane field from an infected *H. sommeri* L3 larva (HH56) (Figure 3.5). These results indicated the *B. brongniartii* was cycled from arboreal forest habitat to the subterranean sugarcane environment by the species, *H. sommeri.*

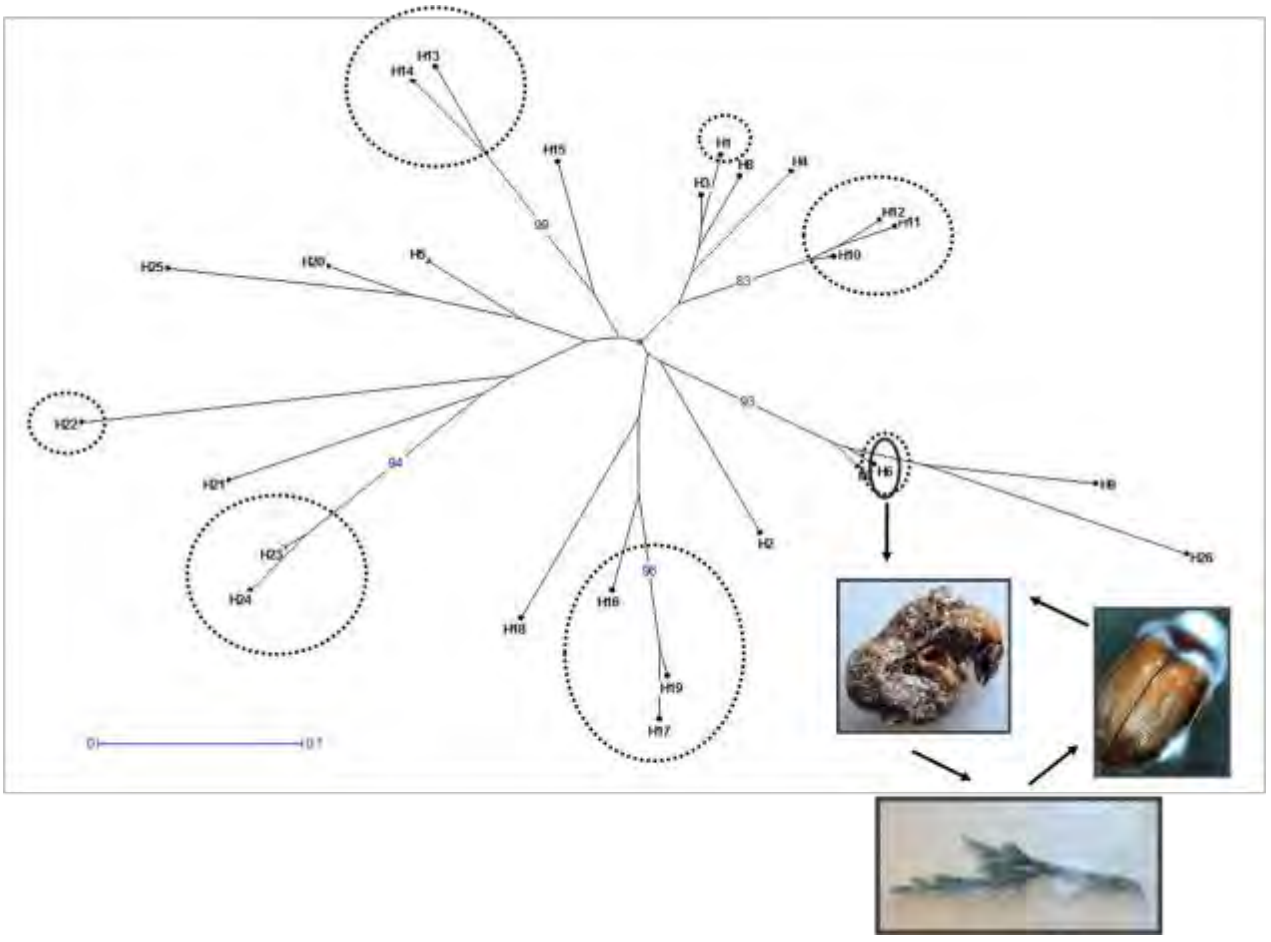

Figure 3.5: A Neighbour-Joining tree of *B. brongniartii* microsatellite haplotypes obtained from two populations in the KwaZulu-Natal Midlands North using a genetically related dice distance (Kosman and Leonard, 2005). A dotted circle represents those haplotypes which were collected from a *Hypopholis sommeri* adult host. A solid circle represents a *B. brongniartii* haplotype 6 which was being cycled in the environment.

3.4 DISCUSSION

Microsatellite analyses and multilocus gene sequence data revealed that the incidence of fungal entomopathogen infection occurring in white grubs and specifically, *H. sommeri* at two sites in the KZN Midlands North region of South Africa is as a result of *B. brongniartii* and not *B. bassiana* as previously thought. Of the 78 isolates obtained during this study, 60 isolates were confirmed *B. brongniartii* (78%) despite rigorous efforts to limit sampling bias by employing four different sampling strategies. Further, *B. brongniartii* is one of the most well-known, virulent and widespread pathogens of the Scarabaeidae, particularly within the subfamily Melolonthinae. Many countries in Europe for example Switzerland, Poland, German and Italy have observed natural infections of *B. brongniartii* which have lead to epizootics in the European cockchafer host, *M. melolontha* which have been known for over 100 years (Keller, 1986; Cravanzola *et al*. 1997; Piatti *et al.* 1998; Vestergaard *et al.* 2002; Keller *et al*. 2003). Similarly, *B. brongniartii* is known to cause epizootics with prevalence reaching up to 30% in grass grub, *C. zealandica* larvae sampled from soil in Waikato, New Zealand (Townsend *et al.* 1995). In Reunion, *H. marginalis* was introduced accidentally from Madagascar were it is endemic. Isolates of *B. brongniartii* were sourced in Madagascar and introduced into Reunion to control this pest in sugarcane, with has resulted in widespread control of *H. marginalis* (Vercambre *et al.* 1994; Callot *et al.* 1996; Neuvéglise *et al*. 1997). In India, the fungus has been collected from *Holotrichia* sp. which are serious soil pests in tropical upland rice and sugarcane (Rombach *et al.* 1994). Indian researchers confirmed pathogenicity of *B. brongniartii* towards the white grub *Holotrichia serrata* Fabricius (Coleoptera: Melolonthinae) in the laboratory, pot-culture and field experiments where it showed considerable field persistence (Easwaramoorthy *et al.* 2004; Srikanth *et al.* 2010; Srikanth *et al.* 2011). Our results complete an African link to the association of *B. brongniartii* on the melolonthid species, *H. sommeri*.

In the present study fungal isolates were shown to be closely related to each other however genetic diversity was relatively low compared to other studies (Enkerli *et al.* 2001). *Beauveria brongniartii* found at the two sites: Canema and Harden Heights can be considered one, structured, clonal population fragmented over a spatial scale of 5.5 km. Perhaps the geographical scale this study was undertaken on was too small and a greater genetic diversity may have been observed over a greater distance. Despite low overall genetic differentiation however, within sites many unique haplotypes were observed represented by only one strain.

As Enkerli *et al.* (2001) suggested 'the number of identical strains per haplotype were low, indicating that the search for existing haplotypes in the populations was not saturated'. This means the biodiversity of *B. brongniartii* in the KZN Midlands North region may be higher than this study indicates. Further, if more *B. brongniartii*-specific microsatellite markers were used, a better discriminating power (possibility a greater genetic diversity) could have been observed than what was seen in this study.

It may be possible that these small, statistically insignificant genetic differences between the two populations may be as a result of the inflow of private alleles carried by adult *H. sommeri* from other sources/areas, as observed by the significant differences in allele frequencies when different host sources were considered. Current gene flow appears to be very low between the sites according to the AMOVA analysis (by host), but historically high levels of gene flow must have occurred between Canema and Harden Heights to result in such low overall genetic differentiation. Gene flow, as a result of swarming *H. sommeri* adults, which have been observed many times in the KZN Midlands North region (personal observation), provides a mechanism for the observed occurrence of common alleles, as Enkerli *et al.* (2001) suggested. These authors suggested two possible mechanisms which could explain the occurrence of common alleles. They either co-evolved independently from each other through mutation or they are identical by descent and distributed through gene flow, as *B. brongniartii* can efficiently be disseminated by swarming cockchafers (Enkerli *et al.* 2001). The latter is supported by this study, as strong evidence for gene flow is provided when we consider that some *B. brongniartii* strains, which were isolated from adult *H. sommeri,* also belong to the two most common haplotypes observed in both populations. Significant linkage disequilibrium indicated that both Canema and Harden Heights have non-recombining population structures. The clonality of *B. brongniartii* was strengthened when a partition homogeneity test (PHT) was performed (Xu, 2006) and data was found to be congruent when Bloc and ITS gene sequences were considered. This suggests a slower in-situ mutation mechanism (because random recombination and ultimately the sharing of genetic material do not take place) and strengthens the idea that the spread of genetic variation is through gene flow via *H. sommeri* adults.

The study showed that *B. brongniartii* is also being cycled in the environment as evidenced by the common haplotype (H6) which was found as three genetically identical strains obtained from a black wattle leaf surface, within a *H. sommeri* adult and on a mycosed *H.*

sommeri larva. Meyling & Eilenberg (2007), in discussing the life cycle of *B. bassiana* (which was inferred from collected knowledge on the ecology of these fungi in temperate environments); suggest that fungal inoculum is cycled from arboreal habitats to subterranean environments via insect hosts. Swarming *H. sommeri* adults come into contact with *B. brongniartii* conidia on the surfaces of black wattle leaves when they are roosting, feeding or mating in trees. These adults become infected with the fungus and then fly to sugarcane fields were they lay their eggs. When the adults die in the soil due to fungal infection, they disseminate the fungal strain into the field where conidia are then able to infect larvae living in the soil and feeding on roots. Furthermore, adults emerging from pupation may come into contact with *B. brongniartii* conidia in the soil as they move through to the soil surface, and become infected. As death due to infection may take a few days, adults fly into adjacent black wattle stands to feed and mate; they then die on the plant surfaces and disseminate the conidia onto the leaf surfaces. We propose this process is cyclic and works in both directions.

Lastly, sequencing was also able to confirm a common mistake which is made while evaluating the *G. mellonella*-bait method which is often employed in the isolation of entomopathogenic fungi from subterranean environments (Zimmermann, 1986). One of the perceived limitations of the *G. mellonella*-bait method is the inability to distinguish between morphologically identical fungal isolates in the same sample plot, which has implications for diversity studies (Goble *et al.* 2010). This study highlighted that more than one genetically distinct isolate can occur in the same sample plot. Sequencing and microsatellite data showed that *B. brongniartii* isolates HHB29A and HHB29B, which were morphologically indistinguishable and found in the same sample plot, where in fact genetically quite dissimilar and did not share a haplotype (Figure 3.3). However, other *B. brongniartii* isolates (HHB32B, HHB32E and HHB32F) which were all found in the same sample plot did share the same haplotype (Appendix 3.1; Table A3.1.2). This information highlights the importance of using molecular techniques to accurately identify *Beauveria* species for future diversity studies, because morphology is subjective and simply not precise enough.

3.5 CONCLUSION

This study has broadened our knowledge of the occurrence, host range, genetic structure and ecology of *B. brongniartii* in the KZN Midlands North region of South Africa. To our best

knowledge this is the first record of *B. brongniartii* attacking the larvae, pupae and adults of *H. sommeri* or any other scarab crop pest in South Africa. There was a relatively low level of genetic diversity detected in *B. brongniartii* and isolates were shown to be closely related. No genetic differentiation was observed between the Harden Heights and Canema sites and the two populations may be considered one, structured population over a distance of 5.5 km. Historically, high levels of gene flow from swarming *H. sommeri* beetles is the proposed mechanism for this observed lack of genetic differentiation between populations. However, beetles may also be responsible for the inflow of private alleles from other sources/areas which over time may dominate and increase the genetic differentiation between Harden Heights and Canema. *Beauveria brongniartii* is being cycled from arboreal habitats to the subterranean environment by *H. sommeri*. One of the limitations to the present study was that it was not able to establish if the epizootic which occurred on white grubs at Sunnyside Farm in 2005 was caused by *B. brongniartii* or *B. bassiana*. Based on the confirmed occurrence of *B. brongniartii* at two sites in this region, as well as the environmental persistence of the fungus when it was shown to cycle in the environment and given the dispersal mechanism via swarming *H. sommeri* beetles, and the literature which states the fungus is highly host specific, it is likely that the epizootic observed at the Sunnyside site was also caused by *B. brongniartii.*

APPENDIX 3.1

Table A3.1.1: The locality, substrates and host plants of *B. bassiana* and *B. brongniartii* used for sequencing and microsatellite analyses

Strain name	Organism	Bloc Genbank	ITS Genbank	Locality	Co-ordinates	Host species/substrate	Host plant	Date isolated
BB1319 (BT 126)	*Beauveria spp.*	NS	NS	Reunion Island	no data	no data	no data	no data
BB444	*Beauveria bassiana*	JX110345	JX110368	Clanwilliam, Western Cape	no data	soil - *Galleria mellonella*	*Aspalathus linearis*	no data
C2	*Beauveria bassiana*	JX110348	JX110371	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 32.8" S 30° 39' 55.5" E	*Hypopholis sommeri* L3	sugarcane (*Saccharum* , Poaceae)	06/07/2010
C5	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 33.3" S 30° 39' 52.1" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	28/07/2010
C12	*Beauveria brongniartii*	JX110346	JX110369	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 34.3" S 30° 39' 46.4" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	28/07/2010
C13	*Beauveria brongniartii*	JX110347	JX110370	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 34.3" S 30° 39' 46.4" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	28/07/2010
C17	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 33.5" S 30° 39' 50.5" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	28/07/2010
C34	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 33.7" S 30° 39' 48.5" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	28/07/2010
C36	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 35.5" S 30° 39' 48.0" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	28/07/2010
C37	*Beauveria brongniartii*	JX110349	JX110372	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 35.5" S 30° 39' 48.0" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	28/07/2010
C38	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 33.7" S 30° 39' 47.6" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	28/07/2010
C39	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 33.5" S 30° 39' 48.6" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	28/07/2010
C40	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 33.6" S 30° 39' 49.4" E	soil - *Galleria mellonella*	sugarcane (Saccharum, Poaceae)	28/07/2010
C40A	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 33.6" S 30° 39' 49.4" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	28/07/2010
C FE3B	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 09.4" S 30° 38' 30.5" E	*Hypopholis sommeri* beetle	*Acacia mearnsii* (Fabaceae)	08/04/2011
C FE5A	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 08.7" S 30° 38' 31.2" E	*Hypopholis sommeri* beetle	*Acacia mearnsii* (Fabaceae)	08/04/2011
C FE5E	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 09.7" S 30° 38' 30.2" E	*Hypopholis sommeri* beetle	*Acacia mearnsii* (Fabaceae)	08/04/2011
C FE5F	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 08.7" S 30° 38' 31.2" E	*Hypopholis sommeri* beetle	*Acacia mearnsii* (Fabaceae)	08/04/2011
C 5ALB	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 34.1" S 30° 38' 00.2" E	unknown beetle	*Acacia mearnsii* (Fabaceae)	08/04/2011
C FE6A	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 09.2" S 30° 38' 30.8" E	*Hypopholis sommeri* beetle	*Acacia mearnsii* (Fabaceae)	08/04/2011
C FE3	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 38.2" S 30° 38' 11.4" E	*Hypopholis sommeri* beetle	*Acacia mearnsii* (Fabaceae)	08/04/2011
C FE4	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 38.2" S 30° 38' 11.4" E	*Hypopholis sommeri* beetle	*Acacia mearnsii* (Fabaceae)	08/04/2011
C FE13	*Beauveria brongniartii*	JX110350	JX110373	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 38.2" S 30° 38' 11.4" E	*Hypopholis sommeri* beetle	*Acacia mearnsii* (Fabaceae)	08/04/2011
C FE17	*Beauveria brongniartii*	JX110351	JX110374	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 38.2" S 30° 38' 11.4" E	*Hypopholis sommeri* beetle	*Acacia mearnsii* (Fabaceae)	08/04/2011
C FE18	*Beauveria brongniartii*	NS	NS	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 38.2" S 30° 38' 11.4" E	*Hypopholis sommeri* beetle	*Acacia mearnsii* (Fabaceae)	08/04/2011
C FE20	*Beauveria brongniartii*	JX110352	JX110375	Canema Farm, Seven Oaks, KZN Midlands North	29° 12' 38.2" S 30° 38' 11.4" E	*Hypopholis sommeri* beetle	*Acacia mearnsii* (Fabaceae)	08/04/2011
HH 2	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.2" S 30° 37' 54.5" E	unidentified larva	sugarcane (*Saccharum* , Poaceae)	21/12/2010
HH 110	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 54.9" S 30° 37' 55.1" E	*Hypopholis sommeri* larva	sugarcane (*Saccharum* , Poaceae)	08/02/2011
HH 112	*Beauveria brongniartii*	JX110354	JX110377	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.1" S 30° 37' 54.7" E	unidentified larva	sugarcane (*Saccharum* , Poaceae)	06/12/2010
HH 114	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 54.1" S 30° 37' 53.5" E	unidentified larva	sugarcane (*Saccharum* , Poaceae)	06/12/2010
HH 115	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 53.7" S 30° 37' 53.4" E	*Hypopholis sommeri* larva	sugarcane (*Saccharum* , Poaceae)	30/07/2010
HH 121	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.4" S 30° 37' 55.0" E	unidentified larva	sugarcane (*Saccharum* , Poaceae)	06/12/2010
HH 186	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 52.0" S 30° 37' 52.6" E	unidentified larva	sugarcane (*Saccharum* , Poaceae)	30/07/2010
HH 219	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 51.3" S 30° 37' 52.6" E	unidentified larva	sugarcane (*Saccharum* , Poaceae)	30/07/2010
HH 343	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.7" S 30° 37' 56.3" E	unidentified larva	sugarcane (*Saccharum* , Poaceae)	08/02/2011
HH 56	*Beauveria brongniartii*	JX110355	JX110378	Harden Heights, Dalton, KZN Midlands North	29° 14' 54.1" S 30° 37' 53.5" E	*Hypopholis sommeri* larva	sugarcane (*Saccharum* , Poaceae)	30/07/2010
HH D30	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 54.7" S 30° 37' 55.1" E	*Hypopholis sommeri* larva	sugarcane (*Saccharum* , Poaceae)	21/12/2010
HH E37	*Beauveria brongniartii*	JX110360	JX110383	Harden Heights, Dalton, KZN Midlands North	29° 14' 53.0" S 30° 37' 54.7" E	unidentified larva	sugarcane (*Saccharum* , Poaceae)	21/12/2010
HH E32	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.0" S 30° 37' 55.6" E	unidentified larva	sugarcane (*Saccharum* , Poaceae)	21/12/2010

Table A3.1.1 (continued): The locality and host plants of *B. bassiana* and *B. brongniartii* used for sequencing and microsatellite analyses

Strain name	Organism	Bloc Genbank	ITS Genbank	Locality	Co-ordinates	Host species/substrate	Host plant	Date isolated
HH WG1	*Beauveria brongniartii*	JX110362	JX110385	Harden Heights, Dalton, KZN Midlands North	29° 14' 54.2" S 30° 37' 52.6" E	*Hypopholis sommeri* larva	sugarcane (*Saccharum* , Poaceae)	30/07/2010
HH WG3	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 53.8" S 30° 37' 53.0" E	*Hypopholis sommeri* larva	sugarcane (*Saccharum* , Poaceae)	30/07/2010
HHWT 1	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 59.0" S 30° 37' 59.9" E	soil - *Galleria mellonella*	*Acacia mearnsii* (Fabaceae)	07/03/2011
HH B1A	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 56.8" S 30° 37' 55.5" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	22/02/2011
HH B4A	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.6" S 30° 37' 55.0" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	22/02/2011
HH B13D	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.7" S 30° 37' 55.4" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	22/02/2011
HH B14B	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.9" S 30° 37' 55.5" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	22/02/2011
HH B16B	*Beauveria bassiana*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 56.9" S 30° 37' 55.8" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	22/02/2011
HH B28A	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.5" S 30° 37' 56.2" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	22/02/2011
HH B29A	*Beauveria brongniartii*	JX110356	JX110379	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.8" S 30° 37' 56.3" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	22/02/2011
HH B29B	*Beauveria brongniartii*	JX110357	JX110380	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.8" S 30° 37' 56.3" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	22/02/2011
HH B30B	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 56.4" S 30° 37' 56.5" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	22/02/2011
HH B31E	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 56.8" S 30° 37' 56.6" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	22/02/2011
HH B32B	*Beauveria brongniartii*	JX110358	JX110381	Harden Heights, Dalton, KZN Midlands North	29° 14' 57.1" S 30° 37' 56.7" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	22/02/2011
HH B32E	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 57.1" S 30° 37' 56.7" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	22/02/2011
HH B32F	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 57.1" S 30° 37' 56.7" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	22/02/2011
HH B37B	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.5" S 30° 37' 56.2" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	22/02/2011
HH B39A	*Beauveria brongniartii*	JX110359	JX110382	Harden Heights, Dalton, KZN Midlands North	29° 14' 54.7" S 30° 37' 56.0" E	soil - *Galleria mellonella*	sugarcane (*Saccharum* , Poaceae)	22/02/2011
HH BWL1	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.3" S 30° 37' 59.1" E	Black wattle leaf print	*Acacia mearnsii* (Fabaceae)	08/02/2011
HH BWL2	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.3" S 30° 37' 59.1" E	Black wattle leaf print	*Acacia mearnsii* (Fabaceae)	08/03/2011
HH BWL4	*Beauveria bassiana*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 15' 04.6" S 30° 38' 01.1" E	Black wattle leaf print	*Acacia mearnsii* (Fabaceae)	08/03/2011
HH BWL5	*Beauveria bassiana*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 52.8" S 30° 37' 59.1" E	Black wattle leaf press	*Acacia mearnsii* (Fabaceae)	08/02/2011
HH BWR1	*Beauveria bassiana*	JX110353	JX110376	Harden Heights, Dalton, KZN Midlands North	29° 14' 54.2" S 30° 37' 58.9" E	Black wattle root press	*Acacia mearnsii* (Fabaceae)	08/02/2011
HH GR1	*Beauveria bassiana*	JX110361	JX110384	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.3" S 30° 37' 59.1" E	Grass root print	Grass sp. (Poaceae)	08/02/2011
HH GR2	*Beauveria bassiana*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.3" S 30° 37' 59.1" E	Grass root print	Grass sp. (Poaceae)	08/03/2011
HH GR3	*Beauveria bassiana*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 15' 04.6" S 30° 38' 01.1" E	Grass leaf print	Grass sp. (Poaceae)	08/03/2011
HH SC1	*Beauveria bassiana*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 15' 04.6" S 30° 38' 01.1" E	Sugarcane leaf press	sugarcane (*Saccharum* , Poaceae)	08/03/2011
HH FE3	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 55.3" S 30° 37' 59.1" E	*Hypopholis sommeri* beetle	Grass sp. (Poaceae)	08/03/2011
HH FE6	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 56.6" S 30° 37' 59.1" E	*Hypopholis sommeri* beetle	Grass sp. (Poaceae)	08/03/2011
HH FE7	*Beauveria brongniartii*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 14' 59.5" S 30° 38' 00.2" E	*Hypopholis sommeri* beetle	*Acacia mearnsii* (Fabaceae)	08/03/2011
HH FE8	*Beauveria bassiana*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 15' 00.9" S 30° 38' 00.5" E	*Hypopholis sommeri* beetle	*Acacia mearnsii* (Fabaceae)	08/02/2011
SCHA1	*Beauveria bassiana*	NS	NS	Harden Heights, Dalton, KZN Midlands North	29° 17' 00.5" S 30° 39' 24.0" E	*Schizonycha affinis* beetle	not applicable (light trap)	04/11/2010
SCHA2	*Beauveria brongniartii*	JX110365	JX110388	Harden Heights, Dalton, KZN Midlands North	29° 17' 00.5" S 30° 39' 24.0" E	*Schizonycha affinis* beetle	not applicable (light trap)	04/11/2010
S4222	*Beauveria bassiana*	JX110364	JX110387	Sunnyside Farm, Seven Oaks, KZN Midlands North	no data	*Hypopholis sommeri* L3	sugarcane (*Saccharum* , Poaceae)	28/05/2007
S1174	*Beauveria bassiana*	JX110363	JX110386	Sunnyside Farm, Seven Oaks, KZN Midlands North	no data	*Eldana saccharina* cadaver	sugarcane (*Saccharum* , Poaceae)	24/10/2003
S124	*Beauveria brongniartii*	NS	NS	Sunnyside Farm, Seven Oaks, KZN Midlands North	29° 06' 25.2" S 30° 46' 52.8" E	*Hypopholis sommeri* pupa	sugarcane (*Saccharum* , Poaceae)	04/02/2010
Sun A	*Beauveria bassiana*	NS	NS	Sunnyside Farm, Seven Oaks, KZN Midlands North	29° 06' 55.5" S 30° 46' 59.1" E	*Hypopholis sommeri* pupa	sugarcane (*Saccharum* , Poaceae)	26/02/2010
Sun B	*Beauveria bassiana*	JX110366	JX110389	Sunnyside Farm, Seven Oaks, KZN Midlands North	29° 06' 54.2" S 30° 46' 58.9" E	*Hypopholis sommeri* pupa	sugarcane (*Saccharum* , Poaceae)	26/02/2010
Woodburn	*Beauveria bassiana*	JX110367	JX110390	Ixopo, KZN South Coast	no data	scale insects (Diaspididae)	citrus (Rutaceae)	02/02/2011

NS: Not sequenced

KZN as an acronym for KwaZulu-Natal

Table A.3.1.2: The number of observed haplotypes, the isolates representing a particular haplotype and the host insect for each isolate obtained from two *B. brongniartii* populations.

Population	Haplotypes	N_{obs} [a]	Isolate [b]	Host
Canema	1	1	C FE5E [d]	*H. sommeri* adult
Canema	2	1	C17	*G. mellonella*
Canema and Harden Heights	3	2	C12 and HHB28A	*G. mellonella* only
Harden Heights	4	1	HHB30B	*G. mellonella*
Canema and Harden Heights	5	8	C5; C38; HH186 [e]; HH121 [e]; HH115 [e]; HHB32B; HHB32E; HHB32F	*G. mellonella,* scarab larvae
Harden Heights	6	4	HH 56 [c]; HH219 [e]; HHFE3 [c]; HHBWL1 [c]	*H. sommeri* adult, scarab larvae, leaf press
Canema and Harden Heights	7	4	C40; HHWT1; HHWG1 [e]; HH110 [e]	*G. mellonella,* scarab larvae
Harden Heights	8	1	HHE37 [e]	scarab larvae
Harden Heights	9	1	HHE32 [e]	scarab larvae
Canema and Harden Heights	10	6	C34; C FEH3 [d]; C FEH17 [d]; C40A; HHB31E; HHB39A	*G. mellonella, H. sommeri* adult
Canema	11	1	CFE H4 [d]	*H. sommeri* adult
Harden Heights	12	1	HHFE6 [d]	*H. sommeri* adult
Canema	13	1	C FEH18 [d]	*H. sommeri* adult
Canema	14	1	C FE5A [d]	*H. sommeri* adult
Harden Heights	15	1	HHB37B	*G. mellonella*
Canema	16	1	C FEH20 [d]	*H. sommeri* adult
Canema	17	2	C 5ALB [d]; C FEH13 [d]	*H. sommeri* adult
Harden Heights	18	1	HHB4A	*G. mellonella*
Canema	19	1	C FE5F [d]	*H. sommeri* adult
Harden Heights	20	1	HH 112 [e]	scarab larvae
Harden Heights	21	3	HH114 [e]; HHBWL2; HHB29B	scarab larvae, leaf press, *G. mellonella*
Canema	22	2	C13; C FE6A [d]	*G. mellonella, H. sommeri* adult
Canema and Harden Heights	23	10	C36; C37; C39; CFE3B [d]; HHWG3 [e]; HH2 [e]; HH343 [e]; HHB13D; HHB14B; HHB29A	*G. mellonella, H. sommeri* adult, scarab larvae
Harden Heights	24	1	HHFE7 [d]	*H. sommeri* adult
Harden Heights	25	1	HHD30 [e]	scarab larvae
Harden Heights	26	1	HHB1A	*G. mellonella*

[a] Number of times when a haplotype was observed in a population

[b] *B. brongniartii* isolates with a particular haplotype

[C] *B. brongniartii* isolates which imply fungi are cycled in the environment

[d] Isolate obtained from a *Hypopholis sommeri* adult

[e] Isolate obtained from scarab larvae

CHAPTER 4 - Determining the susceptibility of *Schizonycha affinis*, *Hypopholis sommeri* and *Tenebrio molitor* life stages to strains of *Beauveria brongniartii* and *B. bassiana*

4.1 INTRODUCTION

In the previous chapter, molecular analysis of fungal DNA revealed that the incidence of fungal infection occurring in white grubs, specifically *H. sommeri* at two sites in the KZN Midlands North region was the result of *B. brongniartii.* This was an important discovery because the causal agent of infection was confirmed which meant that this fungal species could be considered for further mycoinsecticide development. Milner (1992) stated that isolates obtained from field epizootics on target pests were often the best candidates for mycoinsecticide development because their behaviour under field conditions was already stable. He also warned that genetically closely related isolates may vary in their pathogenicity, even if they were obtained from the same field epizootics (Milner, 1992). *Beauveria brongniartii* isolates described in the previous chapter were shown to be closely related to one another, as low levels of genetic diversity were detected between isolates. In the previous chapter, 26 haplotypes among 58 isolates of *B. brongniartii* were identified. A haplotype is a group of genes or collection of short tandem repeat allele mutations within a genetic segment which are inherited together from a single parent (Kosman and Leonard, 2005). Because microsatellites are simple sequences repeats (SSR), consisting of tandem repeats which are distributed throughout the genome of most organisms (Enkerli and Widmer, 2010), it is possible that the genes responsible for virulence (or mutations in those genes) may be inherited similarly by organisms which share a haplotype. It is thus hypothesized that isolates of *B. brongniartii* which share a haplotype may have similar virulence towards scarab pests, provided the amplified microsatellite repeat sequences were regions within those virulence genes.

Milner, (1997) stated that accurate confirmation of fungal isolate performance or virulence against the target pest(s) through the employment of bioassays had to be undertaken and was an important next step in the development of mycoinsecticides. A 'biological assay' is defined as *"the measure of the potency of a stimulus (chemical, physical or biological) by means of the reactions that it produces in living matter"* and is employed to test the physiological host range of a particular fungal isolate against insect hosts under controlled conditions (Finney 1979;

Hatting and Wraight, 2007). Most commonly the median lethal concentration (LC_{50}) required to kill 50% of the test insect population is employed (Hatting and Wraight, 2007). Median lethal time (LT_{50}) is used to quantify the activity of virulent fungi and is important in expressing the time until insect death (which is important to farmers) (Hatting and Wraight, 2007). Scarabs are notoriously difficult to rear in the laboratory because of their long life cycles and are laborious to collect in the field (Carnegie, 1974; Milner, 1992) thus, mealworm *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae) were concurrently tested in bioassays as alternative hosts to determine fungal pathogenicity and to evaluate virulence in closely related *B. brongniartii* isolates.

Host specificity is considered an important trait of any potential biological control agent (BCA) because it limits non-target effects on other organisms which share the environment with the target pest(s). For example, in Chapter 2, Dexiinae fly parasitoids were found infecting *H. sommeri* 3rd instar larvae which are important antagonists of white grubs, sharing the same habitat, and may also be susceptible to *B. brongniartii*. Thus host specificity becomes an important consideration in the selection of a potential mycoinsecticide strain. In Europe, *B. brongniartii* is considered a host-specific fungus with a limited host range and is known to infect all developmental stages of *M. melolontha* and *M. hippocastani* (Keller *et al*. 1997; Keller *et al.* 1999; Strasser and Pernfuss, 2005; Koller *et al*. 2005; Laengle *et al.* 2005). However, the fungus was tested against other closely related scarabs *Amphimallon* sp. and *P. horticola* and limited infectivity was observed (Strasser pers. comm.). Further, the fungus was tested against an important non-target predator, the carabid, *P. versicolor* and no significant infectivity was observed (Traugott *et al.* 2005). These findings highlighted the host specificity of the fungal species and have been important in the registration of these mycoinsecticides in Europe because non-target effects are greatly reduced (Glare, 2004; Laengle and Strasser, 2010).

Host range should also not be so narrow that the practicability of a mycoinsecticide is compromised [13](Morris, pers. comm.). A broader spectrum of activity is sometimes desired when farmers are confronted with pest complexes, as is the case in Australia where nineteen

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endemic melolothine scarabs are key pests in sugarcane (Allsopp, 2010). Although the *M. anisopliae* based mycoinsecticide, BioCane® is registered for use against *D. albohirtum* in Australia the product is also able to control other closely related scarab pests, such as *L. negatoria* (Samson *et al.* 1999; Milner *et al.* 2002). In Chapter 2, it was shown that nine different white grub species, with different biology's, occurred within a relatively small area in sugarcane. In particular, the melolonthid pest species, *S. affinis* co-occurred with *H. sommeri* in the KZN Midlands North and has historically been equally troublesome in sugarcane and *A. mearnsii* in the region (Carnegie, 1974; Carnegie *et al.* 1974; Carnegie, 1988). It was shown in the previous chapter (3) that *B. brongniartii* isolates could infect the larvae, pupae and adults of *H. sommeri* under field conditions and that a mycoinsecticide based on this fungus would be able to infect and control *H. sommeri*. Thus it was prudent to test isolates of *B. brongniartii* against the pest species, *S. affinis*. If the host range of *B. brongniartii* isolates included this closely related scarab host species it would greatly improve the feasibility of a potential mycoinsecticide by being able to control two important sugarcane pests in the KZN Midlands North region. Another important consideration, which later becomes apparent for application efficacy, is in understanding which scarab life stages are most susceptible to *B. brongniartii* because effective pest targeting requires knowledge of the susceptibility of the life stages (Keller *et al.* 1997; Allsopp, 2010).

Thus the aims of the present study were: 1.) To determine whether genetically closely related *B. brongniartii* strains varied in their susceptibility towards *Tenebrio molitor* adults which were used as a screening host; 2.) To determine the pathogenicity of various strains of *B. brongniartii* and *B. bassiana* against the melolonthid pest, *S. affinis* by establishing the lethal concentration (LC_{50}) and lethal time (LT_{50}) values; 3.) To determine which life stages of *S. affinis* were most susceptible to strains of *B. brongniartii* and *B. bassiana.*

4.2 METHODS AND MATERIALS

4.2.1 *Test insects*

Tenebrio molitor were obtained from a laboratory culture held at the South African Sugarcane Research Institute (SASRI), Durban in South Africa, and maintained on a bran diet, supplemented with carrots and dog pellets (Bobtail™). *Schizonycha affinis* and *H. sommeri* adults were caught using wall-mounted light traps, consisting of a three meter PVC gutter,

leading into a funnel placed inside the neck of a 25 L plastic drum (Plate 4.2 A). Traps were erected just below security lights at workshops at three farm sites in the Dalton area (29°19'49''S, 30°42'22''E; 29°16'93''S, 30°39'53''E; 29°17'28''S, 30°39'77''E) of the KZN Midlands North in October 2011. Annual mass emergences of *S. affinis* and *H. sommeri* occur from late October until the end of November, and hundreds of beetles may be caught in a single light trap in one night. Traps, which consisted of a 25 L plastic drum, containing beetles, were emptied carefully into numerous 10 L plastic containers with perforated lids (to allow air flow) filled with black wattle leaves (to feed the adults). Traps were emptied every second day in the mornings. Care was taken not to place too many beetles in the same 10L container as this would crowd and stress the insects. These containers were then placed into large plastic cooler boxes filled with ice packs and transported back to the laboratory. Test beetles were sorted into species in the laboratory according to morphological descriptions (Pope, 1960; Harrison, 2013), and pinned specimens obtained in the previous chapter (which were identified by Dr James Harrison) were used as a reference library. Adults which were highly mobile (indicative of vigor) were selected and used immediately for bioassays.

White grub larvae (mainly *S. affinis*) were dug-up from soft, humic soil which had been tilled, at a field site near Wartburg (29°25'99'S; 30°39'46''E). The field site had previously been under sugarcane cultivation but was in the process of been prepared for a green manure crop, which meant the soil was easily accessible. Once grubs had been dug up they were individually placed individually into 30 ml plastic vials filled with autoclaved peat kept inside using perforated lids. Vials were collected and placed inside large plastic cooler boxes filled with ice packs and transported to the laboratory. In the laboratory, grubs were sorted into species by looking at the raster patterns on the ventral side of the last abdominal segment (Sweeney, 1967). Grubs were also sorted into instar sizes (second or third instar grubs) according to the size of the head capsule and general body size (Sweeney 1967; Wilson, 1969; Dittrich-Schröder *et al.* 2009). After identification and instar classing, grubs were placed individually into 90 ml clear plastic containers filled with autoclaved, moistened peat and sealed with perforated lids. These containers were placed onto large trays and were maintained at 23°C in a constant environment room for 10 days to allow the development of potential mycosis or other field-obtained diseases. After this screening period, surviving grubs were used for bioassays.

4.2.2 *Fungal isolates and preparation*

In total, 21 isolates of *Beauveria brongniartii* and two isolates of *B. bassiana* (Table 4.1) were evaluated in this study. Fungal isolates used in bioassays were grown in petri dishes on Sabouraud dextrose agar (SDA) supplemented with 50 mg/l^{-1} chloramphenicol, 25 mg/l^{-1} cyclohexamide and 50 mg/l^{-1} rifampicin and maintained at 23°C. Fungal isolates were in repeated subculture since their original isolation date and on one occasion they were passed through an insect host (*G. mellonella* or *T. molitor*) in an attempt to rejuvenate the strains. This was done because potential loss of virulence is highly relevant to the maintenance of strains in general and in the production of strains for comparative bioassays (Brownbridge *et al.* 2001). Strasser and Pernfuss (2005) however observed no loss in virulence of isolates of *B. brongniartii* stored in liquid nitrogen for sixteen months and then maintained on a chitin peptone agar. In the present study however chitin peptone was not used thus it was thought prudent to rejuvenate the isolates. All fungal isolates are currently housed in the South African National Collection of Fungi at the Plant Protection Research Institute (PPRI-Agricultural Research Council) in Pretoria, South Africa.

Fungal conidia were harvested from 3-week-old cultures by flooding the petri dishes with sterile distilled water containing 0.05% Triton X-100 and then tipping the conidial solution into sterile 20 ml glass bottles containing ⅕ glass beads (3 mm). Bottles were sealed with a lid and vortex mixed for 30 seconds to produce a homogenous conidial suspension. Conidial concentrations were determined using an improved Neubauer haemocytometer (0.1 mm depth) followed by dilution or serial dilution, depending on the desired conidial concentrations (Table 4.2), in sterile distilled water containing 0.05% Triton X-100 (Lacey, 1997). Serial dilution is the stepwise dilution of conidial solutions and is discussed in great detail by Lacey (1997). Conidial suspensions were used within 3 hours of enumeration. The percentage of viable conidia was determined prior to the bioassays by spread plating 0.1 ml of conidial suspension (titrated to 1×10^7 conidia per ml^{-1}) onto three SDA plates. A sterile cover slip was placed on each plate and incubated in complete darkness at ambient temperature. Percentage germination was examined after 24 h from 100-spore counts on each plate (Lacey, 1997).

Table 4.1: The haplotype, locality and collection substrate of *Beauveria bassiana* and *B. brongniartii* used for bioassays in this study

Isolate	Haplotype	Organism	Locality	Host species/substrate	Host plant	Date isolated
BB444	27	*B. bassiana*	Clanwilliam, Western Cape	soil - *Galleria mellonella*	*Aspalathus linearis*	no data
C5	5	*B. brongniartii*	Canema Farm, Seven Oaks, KZN	soil - *Galleria mellonella*	sugarcane	28/07/2010
C12	3	*B. brongniartii*	Canema Farm, Seven Oaks, KZN	soil - *Galleria mellonella*	sugarcane	28/07/2010
C13 *	22	*B. brongniartii*	Canema Farm, Seven Oaks, KZN	soil - *Galleria mellonella*	sugarcane	28/07/2010
C17 *	2	*B. brongniartii*	Canema Farm, Seven Oaks, KZN	soil - *Galleria mellonella*	sugarcane	28/07/2010
C FE5F *	19	*B. brongniartii*	Canema Farm, Seven Oaks, KZN	*Hypopholis sommeri* beetle	*Acacia mearnsii*	08/04/2011
C FE3 *	10	*B. brongniartii*	Canema Farm, Seven Oaks, KZN	*Hypopholis sommeri* beetle	*Acacia mearnsii*	08/04/2011
C FE13	17	*B. brongniartii*	Canema Farm, Seven Oaks, KZN	*Hypopholis sommeri* beetle	*Acacia mearnsii*	08/04/2011
C FE20	16	*B. brongniartii*	Canema Farm, Seven Oaks, KZN	*Hypopholis sommeri* beetle	*Acacia mearnsii*	08/04/2011
HH 112	20	*B. brongniartii*	Harden Heights, Dalton, KZN	unidentified larva	sugarcane	06/12/2010
HH 114 *	21	*B. brongniartii*	Harden Heights, Dalton, KZN	unidentified larva	sugarcane	06/12/2010
HH 115	5	*B. brongniartii*	Harden Heights, Dalton, KZN	*Hypopholis sommeri* larva	sugarcane	30/07/2010
HH 56 *	6	*B. brongniartii*	Harden Heights, Dalton, KZN	*Hypopholis sommeri* larva	sugarcane	30/07/2010
HH E37 *	8	*B. brongniartii*	Harden Heights, Dalton, KZN	unidentified larva	sugarcane	21/12/2010
HH E32	9	*B. brongniartii*	Harden Heights, Dalton, KZN	unidentified larva	sugarcane	21/12/2010
HH WG1 *	7	*B. brongniartii*	Harden Heights, Dalton, KZN	*Hypopholis sommeri* larva	sugarcane	30/07/2010
HH WT 1	7	*B. brongniartii*	Harden Heights, Dalton, KZN	soil - *Galleria mellonella*	*Acacia mearnsii*	07/03/2011
HH B1A	26	*B. brongniartii*	Harden Heights, Dalton, KZN	soil - *Galleria mellonella*	sugarcane	22/02/2011
HH B37B	15	*B. brongniartii*	Harden Heights, Dalton, KZN	soil - *Galleria mellonella*	sugarcane	22/02/2011
HH B39A *	10	*B. brongniartii*	Harden Heights, Dalton, KZN	soil - *Galleria mellonella*	sugarcane	22/02/2011
HH BWL2	21	*B. brongniartii*	Harden Heights, Dalton, KZN	Black wattle leaf print	*Acacia mearnsii*	08/03/2011
HH FE7	24	*B. brongniartii*	Harden Heights, Dalton, KZN	*Hypopholis sommeri* beetle	*Acacia mearnsii*	08/03/2011
S4222 *	28	*B. bassiana*	Sunnyside, Seven Oaks, KZN	*Hypopholis sommeri* L3	sugarcane	28/05/2007

KZN as an acronym for KwaZulu-Natal

***** Fungal isolates used against white grubs in bioassays

In the selection of fungal treatment concentrations published studies which have evaluated the virulence of entomopathogenic fungi against various scarab pests, conducted bioassays at an initial screen concentration of 1×10^{8} conidia/ml^{-1} (Beron and Diaz, 2005; Guzman-Franco *et al.* 2011; Srikanth *et al*. 2011) while other studies of this nature selected an initial screen concentration of 1-5$\times10^{7}$ conidia/ml^{-1} (Yaginuma *et al*. 2006; Traugott *et al.* 2005; Townsend *et al.* 2010). One study used a range of fungal concentrations, 1.4×10^{4}-1.4×10^{8} conidia/ml^{-1} against scarab pests (Makaka, 2008). Based on all the existing literature it was thought prudent to use 1×10^{8} conidia/ml^{-1} as the upper limit for initial screening against scarab hosts and also develop a concentration range to establish a more detailed comparison and evaluation of

isolates (Table 4.2). *Tenebrio molitor* hosts were tested at a lower concentration of 1×10^7 conidia/ml^{-1} (Table 4.2) because this was the upper limit of a study undertaken by Milner *et al*. (2002) against this laboratory insect.

4.2.3 *Bioassay overview*

Seven, replicated bioassays were conducted against mealworm, *T. molitor* (adults) and the melolonthid species: *H. sommeri* (adults) and *S. affinis* (adults and grubs) (Table 4.2 A-G). Twenty-one isolates of *Beauveria brongniartii* and one isolate of *B. bassiana* (BB444) (Table 4.1) were tested against the adults of *T. molitor* as a screening host (Table 4.2). *Tenebrio molitor* adults were considered a more appropriate life stage than the larvae because numerous larval instars make it difficult to accurately determine insect age and adds to variation in bioassays. *Schizonycha affinis* was tested in six, replicated bioassays (Table 4.2: B-G) using representative *B. brongniartii* strains against the adults (B-C) and grubs (D-G). Further, in one bioassay (C) the adults of *H. sommeri* were tested using a representative *B. brongniartii* strain.

Table 4.2: Bioassays performed using *Beauveria brongniartii* and *B. bassiana* against mealworm, *Tenebrio molitor* and the white grub species, *Schizonycha affinis* and *Hypopholis sommeri.*

Bioassay	Number of isolates	Treatment	Number of treatments	Insect species	Insect life stage	No. of insects per treatment
A	22	1 x 10^7 conidia/ml^{-1}	1	*Tenebrio molitor*	adults	120
B	5	1 x 10^3-1 x 10^8 conidia/ml^{-1}	6	*Schizonycha affinis*	adults	120
C	1	1 x 10^3-1 x 10^8 conidia/ml^{-1}	6	*S. affinis & H. sommeri*	adults	120
D	5	1 x 10^8 conidia/ml^{-1}	1	*Schizonycha affinis*	L2 grubs	60
E	5	1 x 10^8 conidia/ml^{-1}	1	*Schizonycha affinis*	L3 grubs	60
F	1	1 x 10^6-1 x 10^9 conidia/ml^{-1}	4	*Schizonycha affinis*	L3 grubs	60
G	3	1 x 10^8 conidia/ml^{-1}	1	*Schizonycha affinis*	L3 grubs	60

4.2.4 *Screening bioassays*

Bioassay A: *Tenebrio molitor*

A conidial suspension concentration 1×10^7 conidia/ml^{-1} of each of the 22 fungal isolates was prepared as described above. Forty adult beetles were used per replicate and three replicates (120 adults) were used per fungal isolate (Table 4.1). Four adults at a time were placed in a petri dish lid and rolled onto their dorsal surface using an autopipette to immobilize them. Once they were on their backs, they were inoculated topically with 10 µl of conidial suspension onto the ventral side of the abdomen using a calibrated autopipette. The four adults were allowed to dry for 15 minutes (with the petri dish now sealed with the bottom piece) before they were transferred to fresh 90 mm diameter petri dishes filled with autoclaved bran and dog pellets (Plate 4.1 A). This process was repeated until all the adults had been inoculated. All petri dishes containing the fungal inoculated insects were kept in a constant environment room at 23°C for seven days. On the 8th day, the bioassays were terminated and all living adults were counted and then discarded. All dead adults were surface sterilized by immersing them in 70% ethanol for 2 minutes (Plate 4.1 C) and then plating the whole insect onto fresh SDA agar plates. The criterion for scoring mycoses was: death of adults accompanied by fungal sporulation on the body (Plate 4.1 B, D).

4.2.5 *White grub adult bioassays*

Bioassay B: *Schizonycha affinis* adults

Four isolates of *B. brongniartii* (C13, CFe3, HH56 and HHE37) and one isolate of *B. bassiana* (S4222) were used to inoculate the adults of *S. affinis*. A conidial suspension concentration range of 1×10^3 to 1×10^8 conidia/ml^{-1} was prepared per fungal isolate. Thirty adults were used per replicate and four replicates (120 adults) were used per concentration (six concentrations) per isolate (5 isolates) (Table 4.2). Four adults at a time were placed in a petri dish lid and rolled onto their dorsal surface using an autopipette to immobilize them (Plate 4.2 D, E). Once they were on their backs, they were topically inoculated with 10 µl of conidial suspension onto the ventral side of the abdomen using a calibrated autopipette. This process was repeated until all the adults had been inoculated. Adults were allowed a drying period of 15 minutes (with the petri dish now sealed with the bottom piece) before they were transferred into cages (30cm×40cm×40cm). The cages consisted of a plastic, tray bottom which was lined with

autoclaved peat. An iron frame was then fixed onto the plastic tray bottom and a net mesh cover, especially designed to fit the iron frame, was draped over it and fixed to the bottom of the tray with an elastic band (Plate 4.2 H, I). Each cage contained ten branches of *A. mearnsii* leaves for the adults to feed on. All 120 inoculated adults from each concentration were kept together in a cage. The cages were maintained at 23°C in a constant environment room for seven days. On the 8th day, the bioassays were terminated and all living adults were counted and kept for observation for 5 days. All dead adults were surface sterilized by immersing them in 70% ethanol for 2 minutes and then plating the whole insect onto fresh SDA agar plates. The criterion for scoring mycoses was: death of adults accompanied with fungal sporulation on the insect body (Plate 4.2 K, L, M, N).

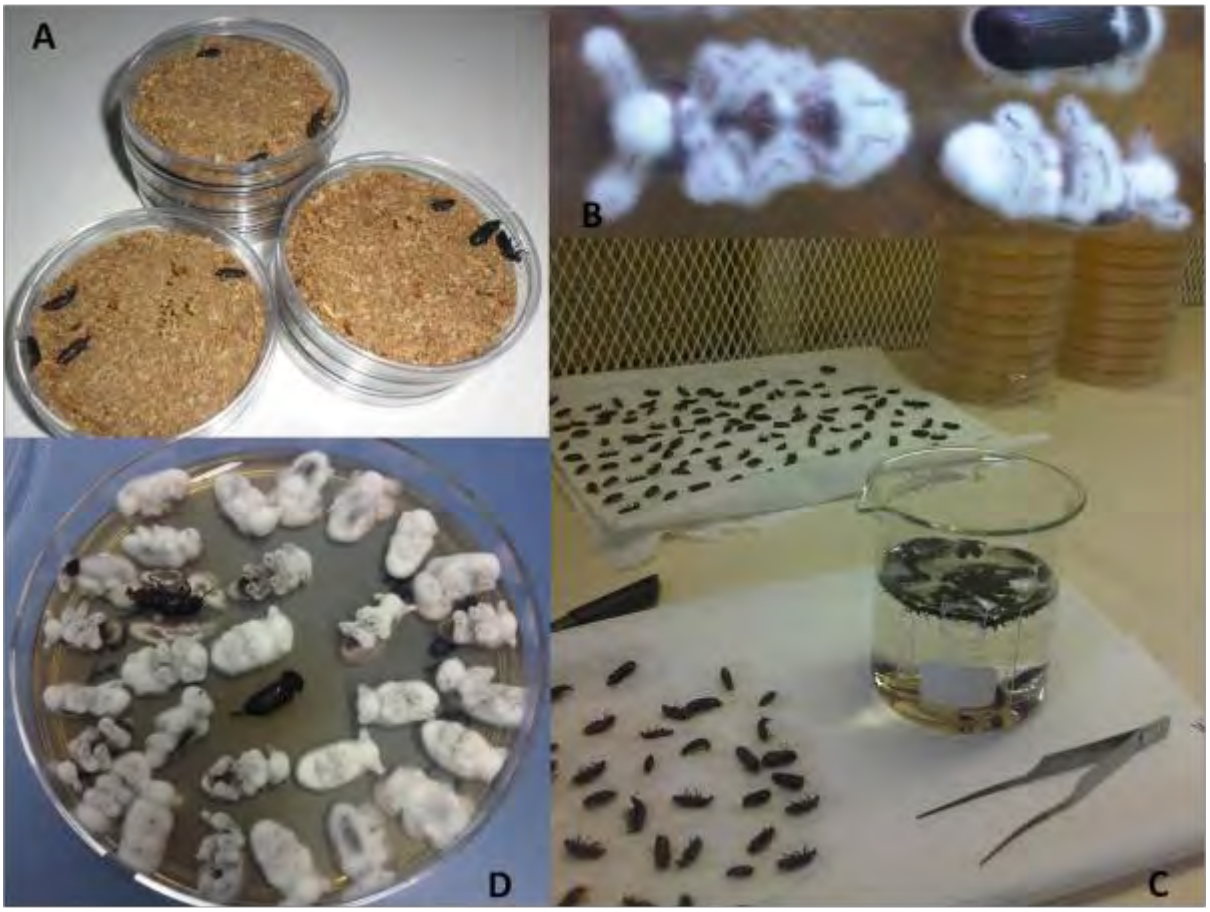

Plate 4.1: *Tenebrio molitor* were kept in Petri dishes (4 adults per dish) filled with autoclaved bran after treatment with fungi (A); overt mycosis observed in adults (B); surface sterilizing dead adults in 70% ethanol after treatment with fungi (C); surface sterilized adults were plated onto SDA plates (D).

Bioassay C: *Schizonycha affinis* and *H. sommeri* adults

An isolate of *B. brongniartii* (C13) was used to inoculate the adults of *S. affinis* and *H. sommeri*. A conidial-suspension concentration range of 1×10^{3} to 1×10^{8} conidia/ml^{-1} of the isolate was used (Table 4.2). Thirty *S. affinis* adults were used per replicate and four replicates (120 insects) were used per concentration (six concentrations) for isolate C13. Similarly, 30 *H. sommeri* adults were used per replicate and four replicates (120 adults) were used per concentration (six concentrations) for isolate C13. The adults of both *S. affinis* and *H. sommeri* were inoculated topically with 10 µl of conidial suspension onto the ventral side of the abdomen using a calibrated autopipette in the same manner as described above (Plate 4.3 B, C). Adults were allowed to dry for 15 minutes before they were transferred into cages (30cm×40cm×40cm) which have been described above (Plate 4.3 D, E). Each cage contained ten branches of *A. mearnsii* leaves for the adults to feed on. All 120 inoculated *S. affinis* adults from each concentration were kept together in a cage. Similarly, all 120 inoculated *H. sommeri* adults from each concentration were kept together in a cage. The cages were maintained as described above and all dead adults were surface sterilized and evaluated for mycosis as described above.

4.2.6 *White grub larval bioassays*

Bioassay D-E: *Schizonycha affinis* second and third instar grubs

Five isolates of *B. brongniartii* (C13, C17, HHE37, HHB39A and HHWG1) were used in these two bioassays against the second instar (L2) grubs and third instar (L3) grubs of *S. affinis*. A conidial concentration of 1×10^{8} conidia/ml^{-1} was prepared for each isolate (5 isolates) as described above. Surviving grubs which had undergone a screening period were used for bioassays. Sixty (60) second instar (L2) grubs (3 replicates of 20 grubs) were used per fungal isolate (5 isolates). Similarly, 60 third instar (L3) grubs were used per fungal isolate and also inoculated with the same five enumerated conidial suspensions (5 isolates) (Table 4.2). Grubs were placed individually into petri dishes and allowed to settle for a minute. Inoculations were made topically with 10 µl of conidial suspension by placing the fungal suspension onto the area just behind the head on the dorsal side (thorax) of the insect with an autopipette (Plate 4.4 B, C, D). It was easy to inoculate the grubs while they were moving because their posture allowed easier accesses to the thorax.

Plate 4.2: Wall-mounted light trap used to catch scarab beetles for bioassays (A); beetles were sorted in the laboratory (B, C); beetles were inoculated topically with fungus (D, E, F); after treatment beetles were kept in cages substituted with *A. mearnsii* leaves (G, H, I); surface sterilizing dead adults with 70% ethanol (J); infected *S. affinis* adults (K, L, M, N).

Plate 4.3: *Hypopholis sommeri* beetles were sorted in the laboratory (A); beetles were inoculated topically with fungus (B, C); after treatment beetles were kept in cages substituted with *A. mearnsii* leaves (D, E); surface sterilizing dead adults with 70% ethanol (F) infected *H. sommeri* adults (G, H, I).

After inoculation, grubs were allowed a 'drying period' of 15 minutes before they were transferred individually into 90 ml clear plastic containers filled with autoclaved, moistened peat and then sealed with a perforated lid (Plate 4.4 E, F). All 90 ml plastic containers (holding grubs) were grouped according to fungal isolate and placed on large trays which were kept on shelves at 23°C in a constant environment room for 37 days. Grubs were evaluated weekly (7, 14, 21, 28 and 37 days) for death by tipping out the contents (grub + peat) of the 90 ml container onto a sterile tray. After evaluation, the contents were then tipped back into the 90 ml container, and the grub either removed if it was dead or placed back if it was still alive, taking care not to cross-contaminate the containers or touch the grubs. All dead grubs were collected and surface sterilized with 70% ethanol by immersing them for 2 minutes. Surface sterilized grubs were then placed into petri dishes lined with moist filter paper to allow the development of mycosis. The criterion for scoring mycoses was: death of grubs accompanied with fungal

sporulation on the insect body. If grubs were already sporulating in the 90 ml containers, mycosis was recorded as such (Plate 4.4 G, H, I, J, K).

Bioassay F: *Schizonycha affinis* third instar grubs

One isolate of *B. brongniartii* (HHWG1) was used to inoculate the third instar (L3) grubs of *S. affinis*. A conidial-suspension concentration range of 1×10^{6} to 1×10^{9} conidia/ml^{-1} of the isolate was used. Surviving grubs which had undergone a screening period were used for bioassays. 60 third instar (L3) grubs (3 replicates of 20 grubs) were used per concentration (4 concentrations) (Table 4.2). Grubs were topically inoculated as described above (Plate 4.4 B, C, D) and then allowed to dry for 15 minutes before they were transferred individually into 90 ml clear plastic containers filled with autoclaved, moistened peat and then sealed with a perforated lid. All 90 ml plastic containers were placed on large trays which were kept on shelves at 23°C in a constant environment room for 30 days. Grubs evaluation was different in these bioassays as grubs were checked every three days up to 30 days for death by tipping out the contents (grub + peat) of the 90 ml container onto a sterile tray. It was decided to make the observation period more frequent so that a better evaluation of the time it took to kill the grubs could be established. After evaluation, the contents were then tipped back into the container, and the grub either removed if it was dead or placed back if it was still alive, taking care not to cross contaminate the containers or touch the grubs. All dead grubs were collected and surface sterilized with 70% ethanol by immersing them for 2 minutes. Surface sterilized grubs were then placed into petri dishes lined with moist filter paper to allow the development of mycosis. The criterion for scoring mycoses was: death of adults accompanied with fungal sporulation on the insect body. If grubs were already sporulating in the 90 ml containers, mycosis was recorded as such.

Bioassay G: *Schizonycha affinis* L3 grubs

In a final bioassay, three different isolates of *B. brongniartii* (CFe5F, HH114 and HHE37) calibrated to a concentration of 1×10^{8} conidia/ml^{-1} were prepared as described above and used to inoculate the third instar (L3) grubs of *S. affinis*. 60 third instar (L3) grubs (3 replicates of 20 grubs) were used per fungal isolate (Table 4.2). Grubs were inoculated, maintained, evaluated and scored for mycosis as described above in bioassay F.

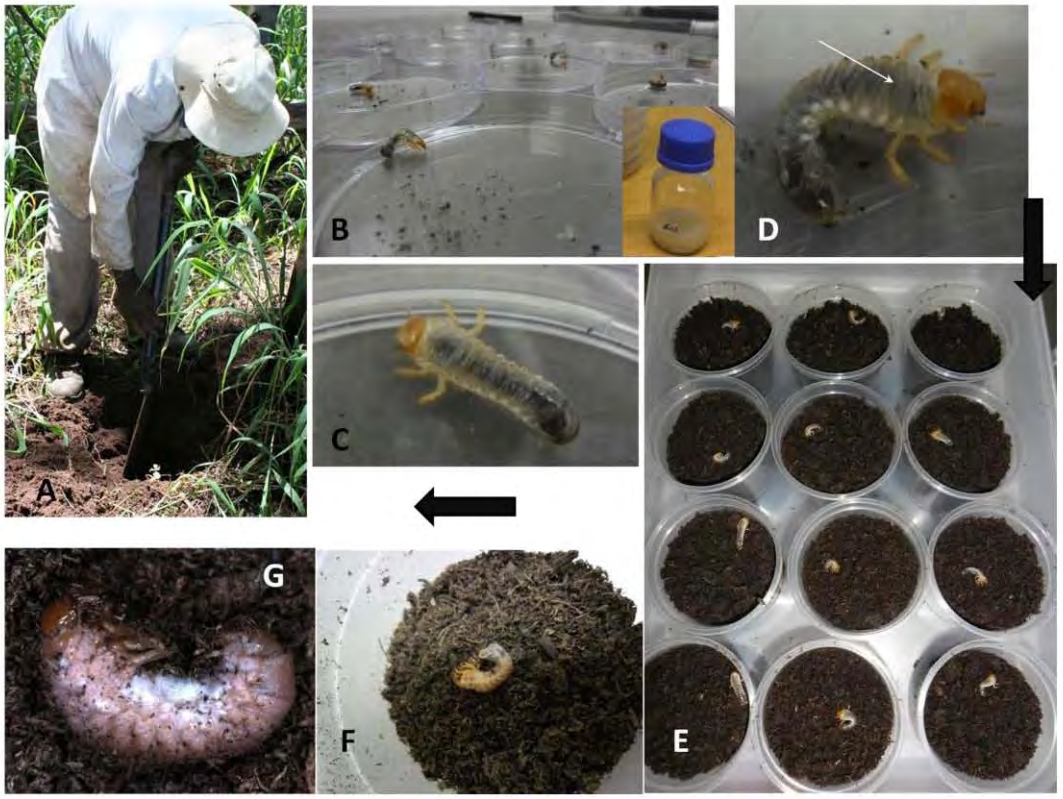

Plate 4.4: *Schizonycha affinis* grubs were dug up from soil (A); grubs were inoculated topically with fungi just behind the head (B, C, D); after treatment with fungi, grubs were placed into 90 ml cups filled with autoclaved peat (E); grubs were checked at various observation times for death (F, G, H); fungal infected grubs show typical signs of *B. brongniartii* infect (I, J, K,); oosporein, produced by *B. brongniartii*, turns the body pink (L).

4.2.7 *Statistical analyses*

Considering the screening bioassays (A) against *T. molitor* and bioassays (B and C) against *S. affinis* and *H. sommeri* adults, the percentage mortality data were corrected for control mortality (Abbott, 1925) and the data were normalized through arcsine transformation and then subjected to analysis of variance (ANOVA) followed by mean separation by the Tukey post hoc test ($P \leq 0.05$) using Statistica 10 (StatSoft Inc, 2011). A probit analysis was used to determine the functional relationship between log concentration of inoculum (five concentrations: 1×10^4 to 1×10^8 conidia/ml^{-1}) and probit of mortality for *S. affinis* adults (bioassay B). Probit analysis was also used to determine the relationship between log concentration of inoculum (six concentrations: 1×10^3 to 1×10^8 conidia/ml^{-1}) and probit of mortality for *S. affinis* and *H. sommeri* adults (bioassay C). The software program PROBAN (Van Ark, 1995) was used to calculate the lethal concentration (LC_{50} and LC_{90}) values of the fungal isolates used in these bioassays.

For bioassays D-G, standard probit analysis to determine the lethal times (LT_{50}) was not considered an appropriate test for comparing the susceptibility of insects and the virulence of isolates, since independent groups of individuals were not assessed at each time period but rather the same group of insects were monitored for up to 30 or 37 days (bioassay dependent) (Robertson and Preisler, 1992). Thus, regression analysis was undertaken using a complementary log-log (CLL) link function in Genstat (VSN International Ltd, 2011). This link function fitted the data best as there were no systematic deficiencies experienced compared to other link functions tested. However, some overdispersion ($\Phi > 1$) was noted. Overdispersion occurs because the data have larger variance than expected under the assumption of a binomial distribution. Nevertheless, one can still use the estimation algorithm for generalized linear models to find good parameter estimates and the data are still considered biologically relevant (McCullagn & Nelder, 1989). The complementary log-log link function was also specified to model the proportion of insects dead due to fungal infection (corrected for control mortality per day) over time for bioassays D, E and G. It was further specified to model the functional relationship between concentration and complementary log-log mortality (corrected for control mortality per day) in some bioassays (bioassay B, C and F). The LT_{50} (x) could be calculated by back transforming and solving the equation $y = a + bx$. Finally, regression lines within bioassays were compared using methods described by Armitage (1980).

4.3 RESULTS

4.3.1 *Screening bioassays*

Bioassay A: *Tenebrio molitor*

In all cases over 85% germination was observed for all fungal isolates in this study. Control mortality varied from 10-15%. In a one-way ANOVA fungal isolates were significantly different in their virulence against mealworm, *T. molitor* adults (F=15.41; df=22; $P \leq 0.001$) which included the untreated control (Figure 4.1). The only *B. bassiana* isolate (BB444) used against *T. molitor* in screening bioassays was the most virulent, causing 81% mortality. Only five *B. brongniartii* isolates caused over 70% mortality in *T. molitor* adults and the isolates were not significantly different from each other. Eleven *B. brongniartii* isolates screened against *T. molitor* (percentage mycosis ranged from 55-65%) did not differ significantly. Three *B. brongniartii* isolates (CFe13, CFe20 and HHB37B) performed relatively poorly against *T. molitor* adults, as less than 50% mortality was induced (Figure 4.1). Only 39% mortality was observed when isolate C12 was tested against *T. molitor* and did not differ from the untreated control.

Isolate HHWG1, which shared a common haplotype (7) with HHWT1 (Table 4.1) did not differ significantly in virulence when tested against *T. molitor*. Similarly, isolates HHB39A and CFe3, which also shared a haplotype (10), did not differ significantly from each other when used against this insect species. The same was true for isolates C5 and HH115 (sharing haplotype 5) and isolates HHBWL2 and HH114 (sharing haplotype 21). However, there were significant differences observed in pathogenicity between haplotypes (Figure 4.1).

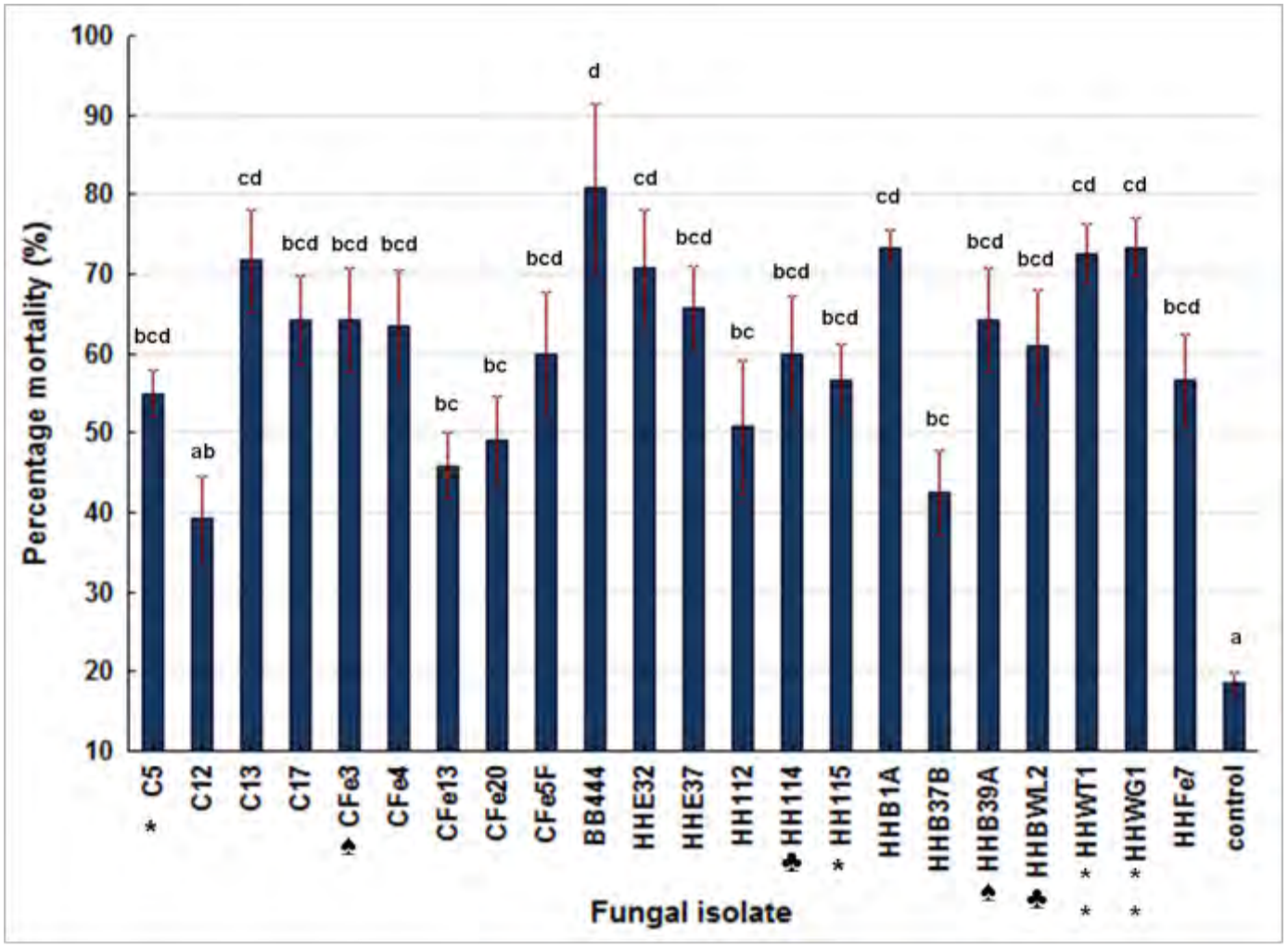

Figure 4.1: Pathogenicity of isolates of *Beauveria bassiana* (BB444) and *B. brongniartii* towards mealworm, *Tenebrio molitor* adults: Mean (±SE) of adult mycosis after treatment with a concentration of $1x10^7$ conidia/ ml^{-1}. Letters denote significant differences between isolates using a Tukey post hoc test. Shared haplotypes are denoted by symbols (*) haplotype 5 (♠) haplotype 10 (♣) haplotype 21 (**) haplotype 7. No symbol represents a unique haplotype.

4.3.2 *White grub adult bioassays*

Bioassay B: *Schizonycha affinis* adults

In a factorial ANOVA, four isolates of *B. brongniartii* and one *B. bassiana* (S4222) isolate varied significantly in their virulence towards the adults of *S. affinis* (F=37.87; df=4; P≤0.001). Adult mortality increased with an increase in concentration, with higher concentrations of conidia killing more adults (F=280.53; df=5; P≤0.001) (Figure 4.2). Control mortality was 12%. The interaction between fungal isolate and concentration was also significant (F=2.51;

df=20; $P \leq 0.001$). *Beauveria brongniartii* isolate C13 performed best against the adults of *S. affinis* as mycosis was observed in 79% of the test insects at the highest concentration (Figure 4.2). Regression analysis under the model (χ^2=24.66; df=9; error df=20; $P \leq 0.001$) showed that the relationship between concentration and isolate was significant however the effect of concentration on mortality was greater than the effect of isolate on mortality (Figure 4.3). One isolate (C13) had a significant effect on insect mortality and the estimated LT_{50} was 7.8 days (Table 4.4). Using probit analysis the estimated LC_{50} values for the C13 isolate against *S. affinis* was 4.4×10^7 conidia/ml^{-1} (Table 4.3).

Isolates HHE37 and HH56 did not differ significantly from C13 in their virulence against *S. affinis* and caused 58% and 57% mycosis respectively at the highest concentration (1×10^8 conidia/ml^{-1}) (Figure 4.2). The estimated LT_{50} values for these isolates were 8.6 and 8.7 days respectively (Table 4.4). It was further estimated that the LC_{50} values for these isolates were 7.5×10^7 conidia/ml^{-1} and 8.4×10^7 conidia/ml^{-1} respectively (Table 4.3). The isolate CFe3 and the only *B. bassiana* isolate S4222 differed significantly from isolates C13 and HHE37 and were considered less virulent against *S. affinis* adults (Figure 4.3). *Beauveria brongniartii* isolate CFe3 had an estimated LT_{50} value of 9 days while S4222 took an estimated 9.7 days to kill 50% of the test insects (Table 4.4). Their respective LC_{50} values for the abovementioned isolates were 9.7×10^7 conidia/ml^{-1} and 1.4×10^8 conidia/ml^{-1} (Table 4.3).

Table 4.3: The probit predicted LC_{50} and LC_{90} values for four *Beauveria brongniartii* and one *B. bassiana* (S4222) isolate tested against the adults of *Schizonycha affinis* and *Hypopholis sommeri*

Bioassay	Insect spp.	Life stage	Isolate	Coefficient intercept	Fit of the line			Lethal concentration			
					$\chi2$	df	*P*	LC_{50}	SE	LC_{90}	SE
B	*S. affinis*	adults	HH56	3.870	35.39	3	<0.0001	8.4×10^7	± 2.48	1.7×10^8	± 5.55
B	*S. affinis*	adults	CFe3	3.767	25.37	3	<0.0001	9.7×10^7	± 2.55	1.9×10^8	± 5.55
B	*S. affinis*	adults	HHE37	4.208	29.97	3	<0.0001	7.5×10^7	± 2.67	1.9×10^8	± 7.32
B	*S. affinis*	adults	C13	4.297	37.74	3	<0.0001	4.4×10^7	± 1.51	1.2×10^8	± 3.79
B	*S. affinis*	adults	S4222	3.754	22.09	3	<0.0001	1.4×10^8	± 5.32	2.8×10^8	± 1.16
C	*H. sommeri*	adults	C13	4.060	16.60	4	<0.002	6.1×10^7	± 2.05	1.4×10^8	± 4.76
C	*S. affinis*	adults	C13	4.156	15.24	4	<0.004	4.8×10^7	± 1.54	1.2×10^8	± 3.59

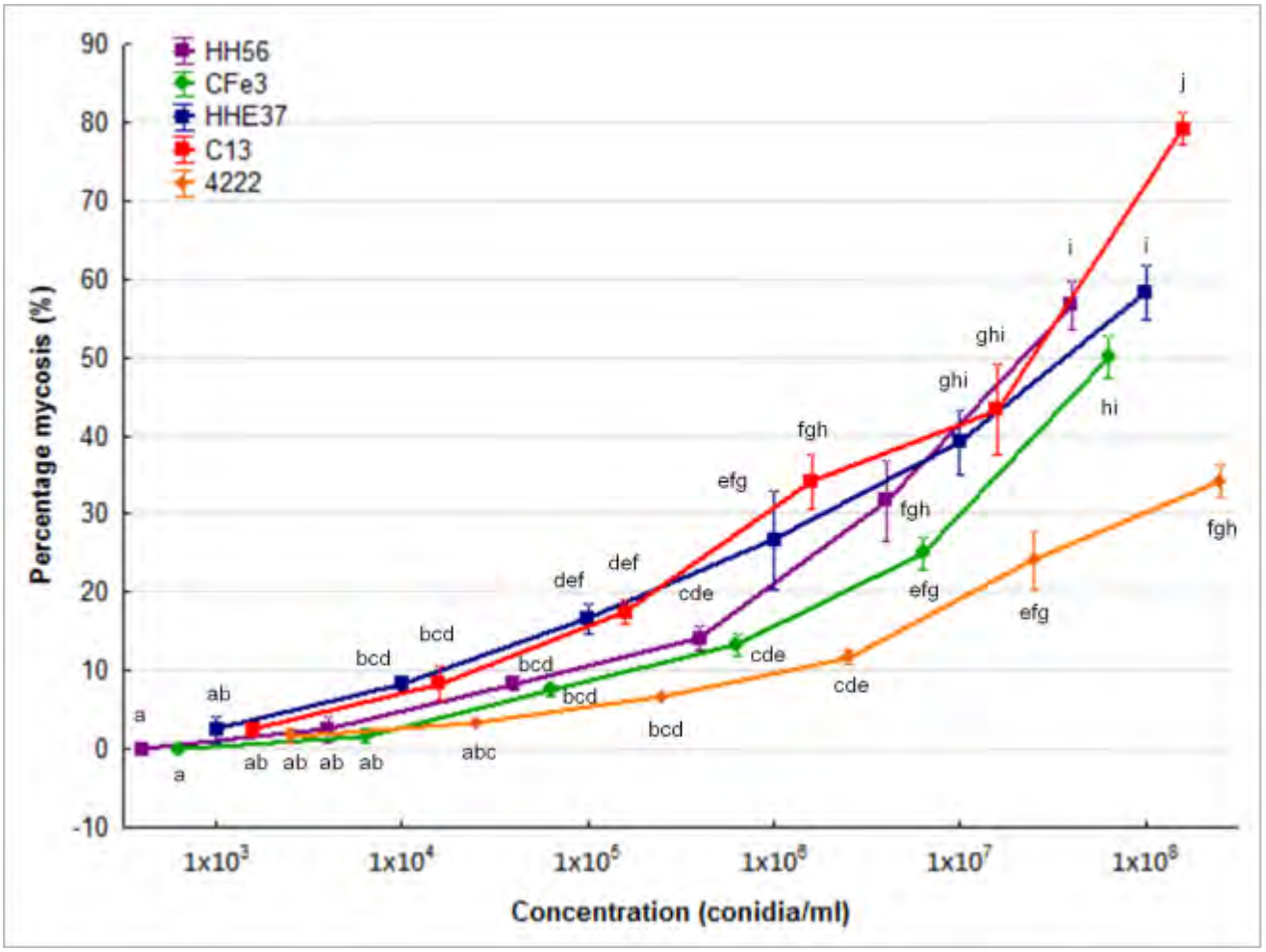

Figure 4.2: The mean (±SE) percentage mortality of the proportion of *Schizonycha affinis* adults killed over six concentrations of *Beauveria brongniartii* and *B. bassiana* (S4222) isolates. Data points are offset for clarity. Letters denote significant differences between treatments using a Tukey post hoc test.

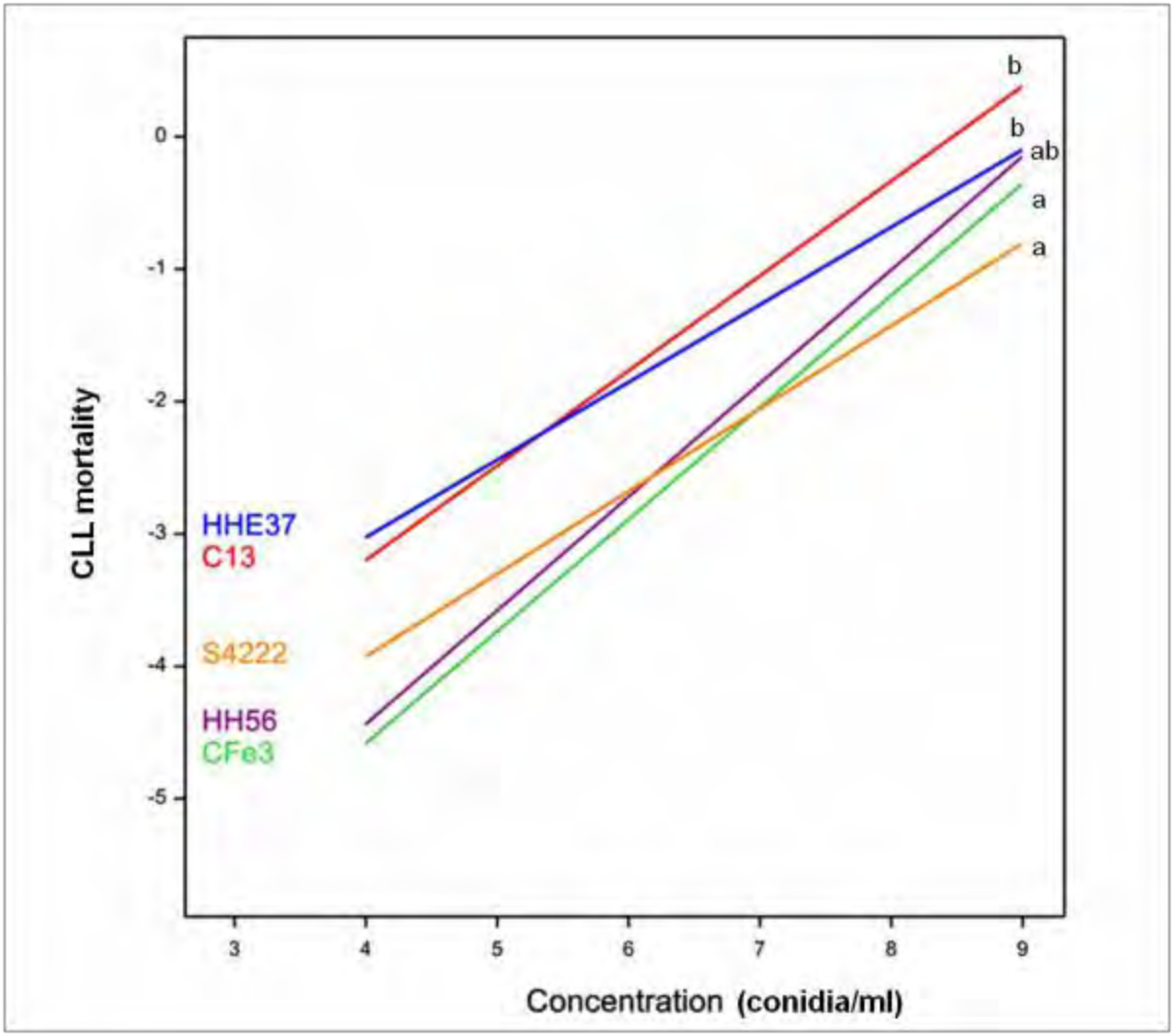

Figure 4.3: The predicted complementary log-log (CLL) transformations of the proportion of *Schizonycha affinis* adults killed over six concentrations of *Beauveria brongniartii* and *B. bassiana* (S4222) isolates. Letters denote significant differences between treatments.

Bioassay C: *Schizonycha affinis* and *H. sommeri* adults

In a factorial ANOVA, concentration had a significant effect on the adults of *S. affinis* and *H. sommeri* (F= 166.22; df=5; $P \leq 0.001$) with a higher concentration of C13 conidia killing more adults than at the lower concentrations (Figure 4.4). Control mortality was 12%. The adults of *S. affinis* were significantly more susceptible to *B. brongniartii* isolate C13 than *H. sommeri* (F= 8.76; df=1; $P \leq 0.005$) (Figure 4.4). The interaction between insect and concentration was not significant (F=0.76; df=5; $P \leq 0.57$). Regression analysis under the model (χ^2=38.04; df=3; error df=8; $P \leq 0.001$) of the relationship between concentration and insect species was significant

however, concentration had the greater effect on insect mortality than insect species had on mortality (figure not shown). The estimated LT_{50} value of isolate C13 against *S. affinis* adults was 7 days while it took longer to kill the adults of *H. sommeri* (LT_{50}=8.3 days) (Table 4.4). Probit analysis estimated that it required 4.8×10^{7} conidia/ml^{-1} of isolate C13 to kill 50% of the *S. affinis* test insects (Table 4.3). The estimated LC_{50} of the same isolate against *H. sommeri* adults was estimated at 6.1×10^{7} conidia/ml^{-1}.

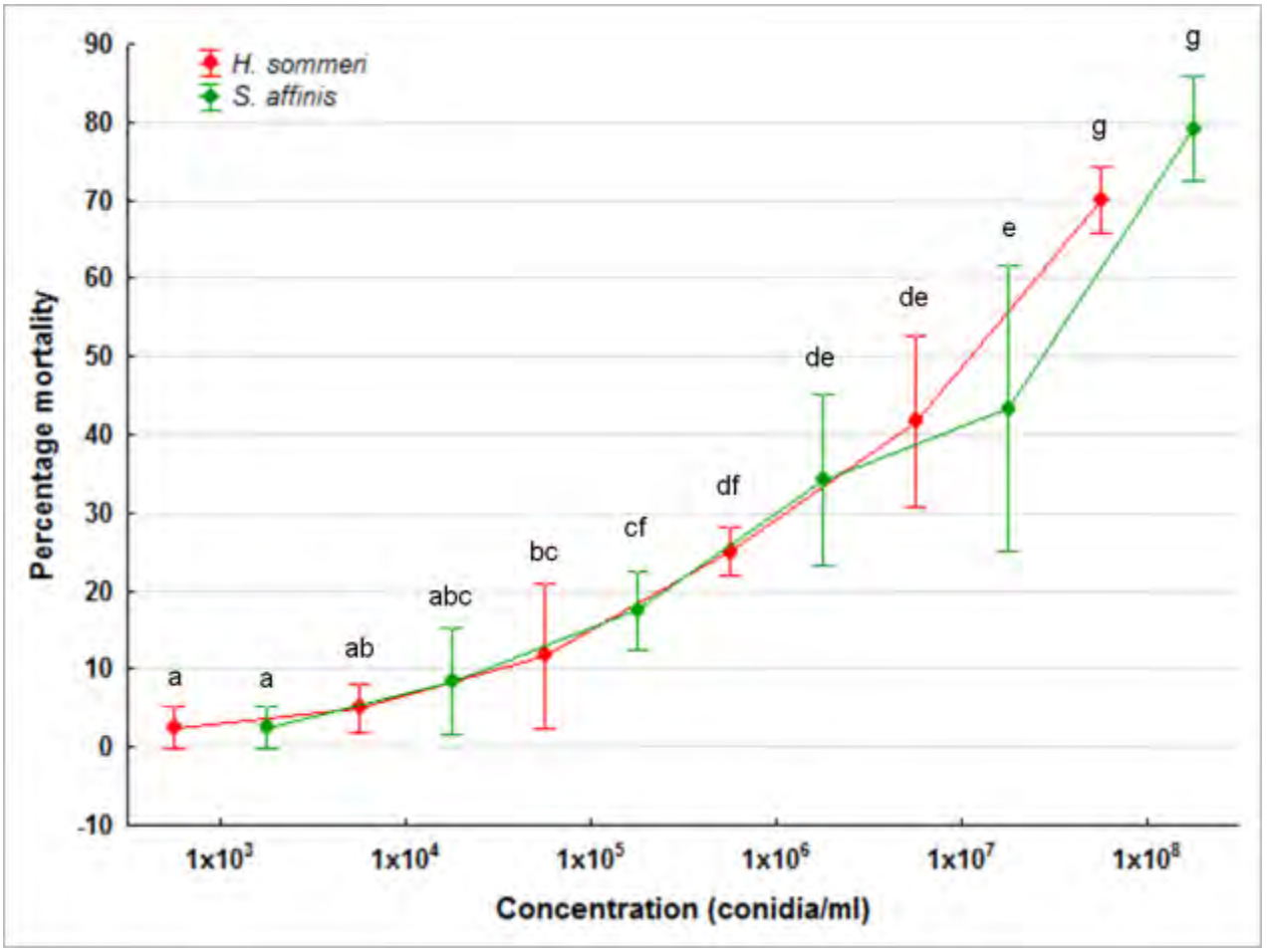

Figure 4.4: The mean (±SE) percentage mortality of the proportion of *Schizonycha affinis* and *Hypopholis sommeri* adults killed over six concentrations of *Beauveria brongniartii* isolate C13.

4.3.3 *White grub larval bioassays*

Bioassays D-E: *Schizonycha affinis* second and third instar grubs

In bioassay D, five *B. brongniartii* isolates tested against the second instar grubs (L2) of *S. affinis* were able to induce mortality in 72.2-88.9% of test insects after 37 days (Figure 4.5). Control mortality was 30%. Regression analysis under the model (χ^2=46.04; df=9; error df=15; $P \leq 0.001$) of the relationship between days after fungal treatment and isolate was significant. However, the number of days after treatment had a greater effect on insect mortality than isolate did, with an increase in insect mortality observed over time. The pathogenicity of some fungal isolates were also significantly different (Figure 4.9 A). Isolates HHWG1, HHB39A and C17 did not differ in virulence when L2 grub mortality was considered and were able to kill 50% (LT_{50}) of the L2 grubs within 18.4-19.8 days (Table 4.4). Isolates HHE37 and C13 were less effective and had LT_{50} values of 25 and 27.1 days respectively (Table 4.4).

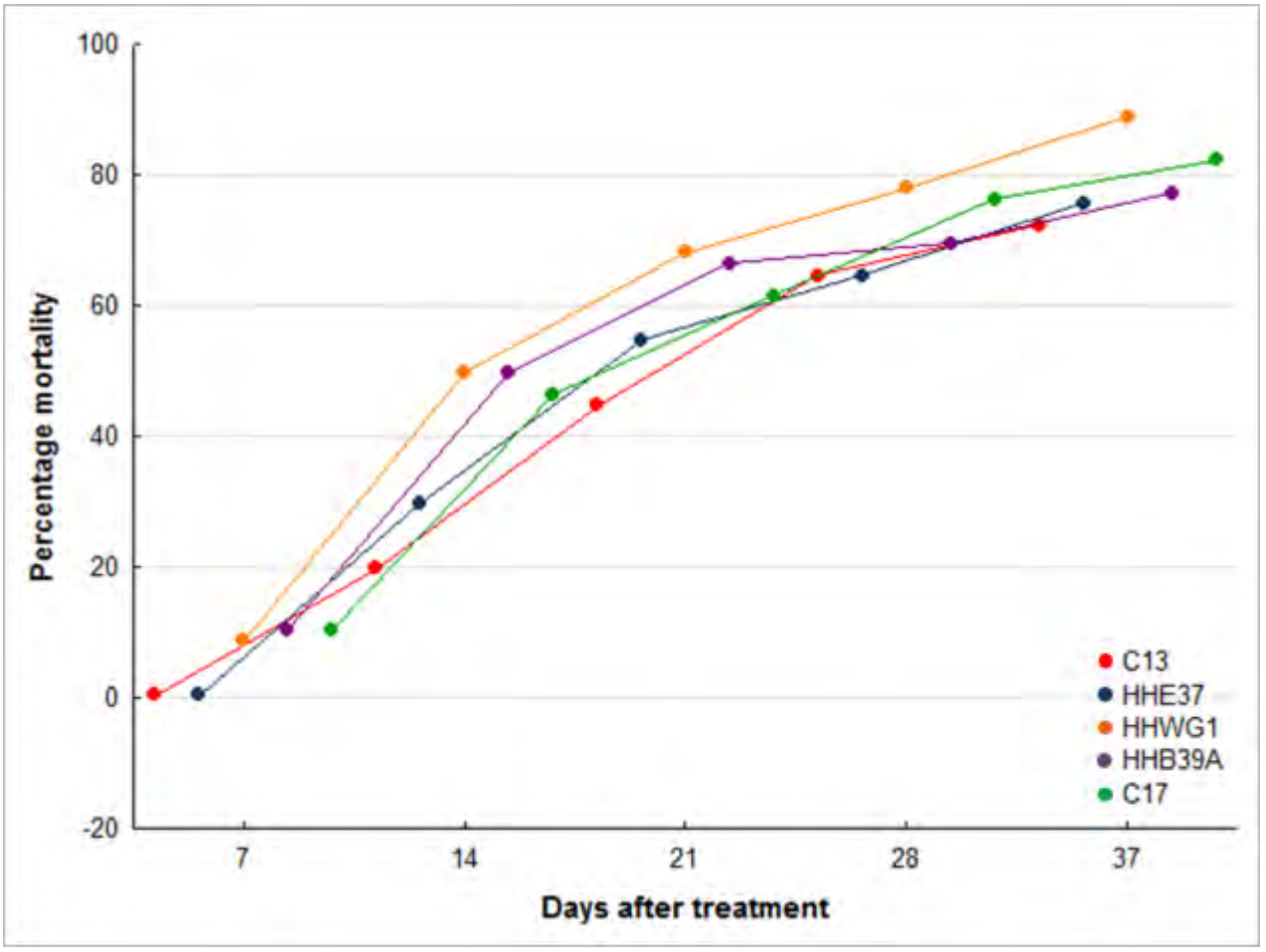

Figure 4.5: The percentage accumulated mortality of the proportion of *Schizonycha affinis* second instar grubs (L2) killed over time (days) by five isolates of *Beauveria brongniartii.*

In bioassay E, it took longer to kill L3 grubs than it did to kill L2 grubs when the same isolates were tested at the same concentration (1×10^8 conidia/ml^{-1}). Control mortality was 30%. The same three *B. brongniartii* isolates (HHWG1, HHB39A and C17) were able to induce mortality in 72-87.5% of the *S. affinis* L3 grubs after 37 days (Figure 4.6). Regression analysis under the model (χ^2=47.40; df=9; error df=15; $P\leq0.001$) of the relationship between days after fungal treatment and isolate was significant. The number of days after treatment had the greater effect on insect mortality than isolate. The pathogenicity of some fungal isolates were significantly different (Figure 4.9 B). Isolate HHWG1 was able to kill 50% of L3 grubs within 21.8 days (Table 4.4). Isolates C17 and HHB39B did not differ in significance when L3 grub mortality was considered and had LT_{50} values 22.4-26.9 days respectively (Table 4.4). Weaker isolates, C13 and HHE37 did not differ significantly in virulence and had estimated LT_{50} values of 28.7 days and 30.1 days respectively (Table 4.4).

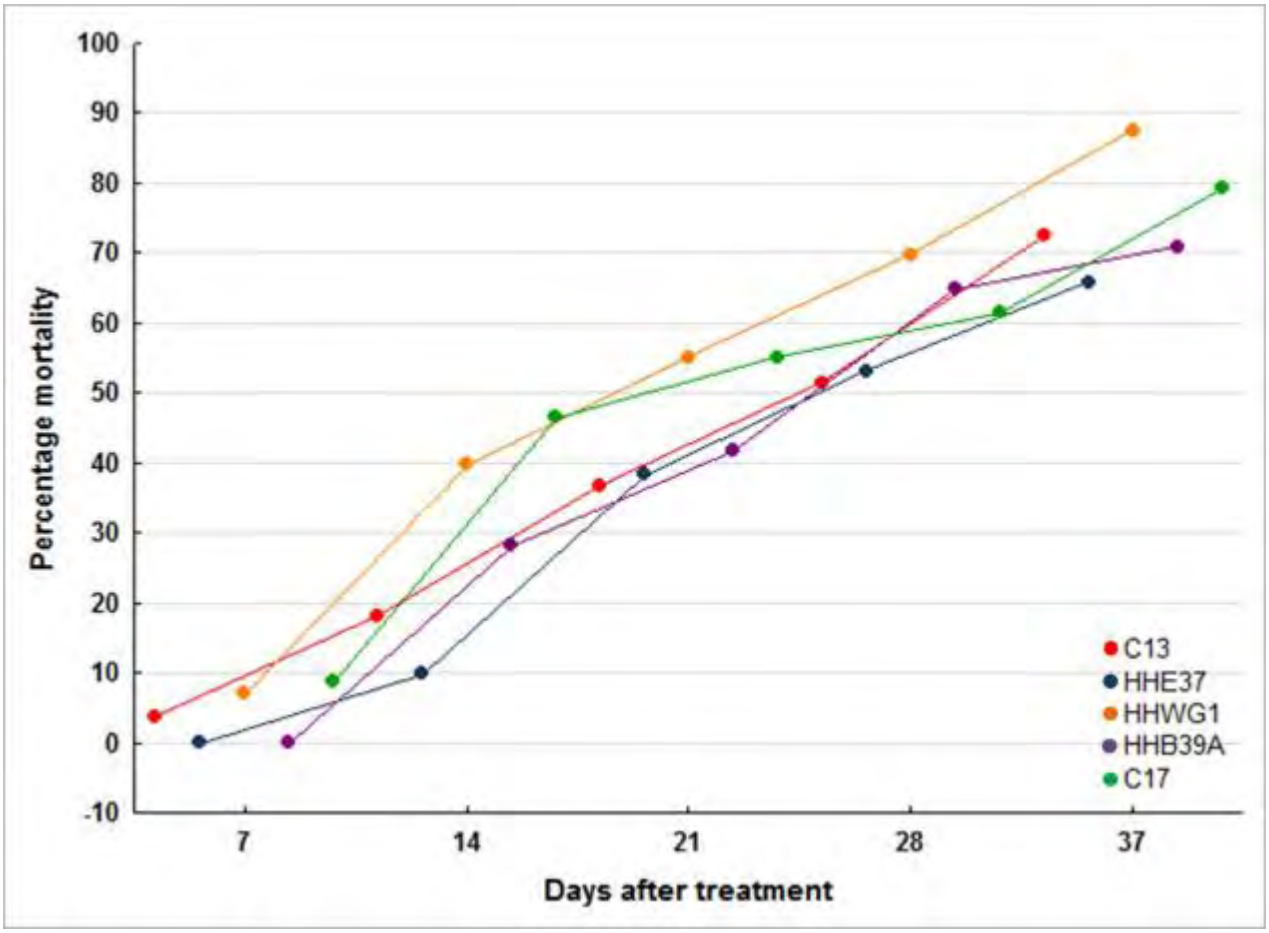

Figure 4.6: The percentage accumulated mortality of the proportion of *Schizonycha affinis* third instar grubs (L3) killed over time (days) by five isolates of *Beauveria brongniartii.*

Bioassay F: *Schizonycha affinis* third instar grubs

Only isolate HHWG1 was tested against the L3 grubs at four different concentrations. Mortality at the lowest concentration (1×10^{6} conidia/ml^{-1}) was 63.4% while at the highest concentration (1×10^{9} conidia/ml^{-1}), 95% mortality was observed after 30 days (Figure 4.7). Control mortality was 25%. Regression analysis under the model (χ^2=148.48; df=7; error df=32; $P\leq0.001$) was significant and number of days after treatment had a greater effect on insect mortality than concentration but there were significant differences observed between the different concentrations (Figure 4.9 C). At the lowest concentration, it took 25.8 days to kill 50% of the *S. affinis* L3 grubs (Table 4.4). At a concentration of 1×10^{7} conidia/ml^{-1} the LT_{50} was 22 days (Figure 4.7). At a 10-fold concentration increase (1×10^{8} conidia/ml^{-1}) the LT_{50} dropped to 19.2 days and finally at the highest concentration the LT_{50} dropped again to 15.1 days (Table 4.4).

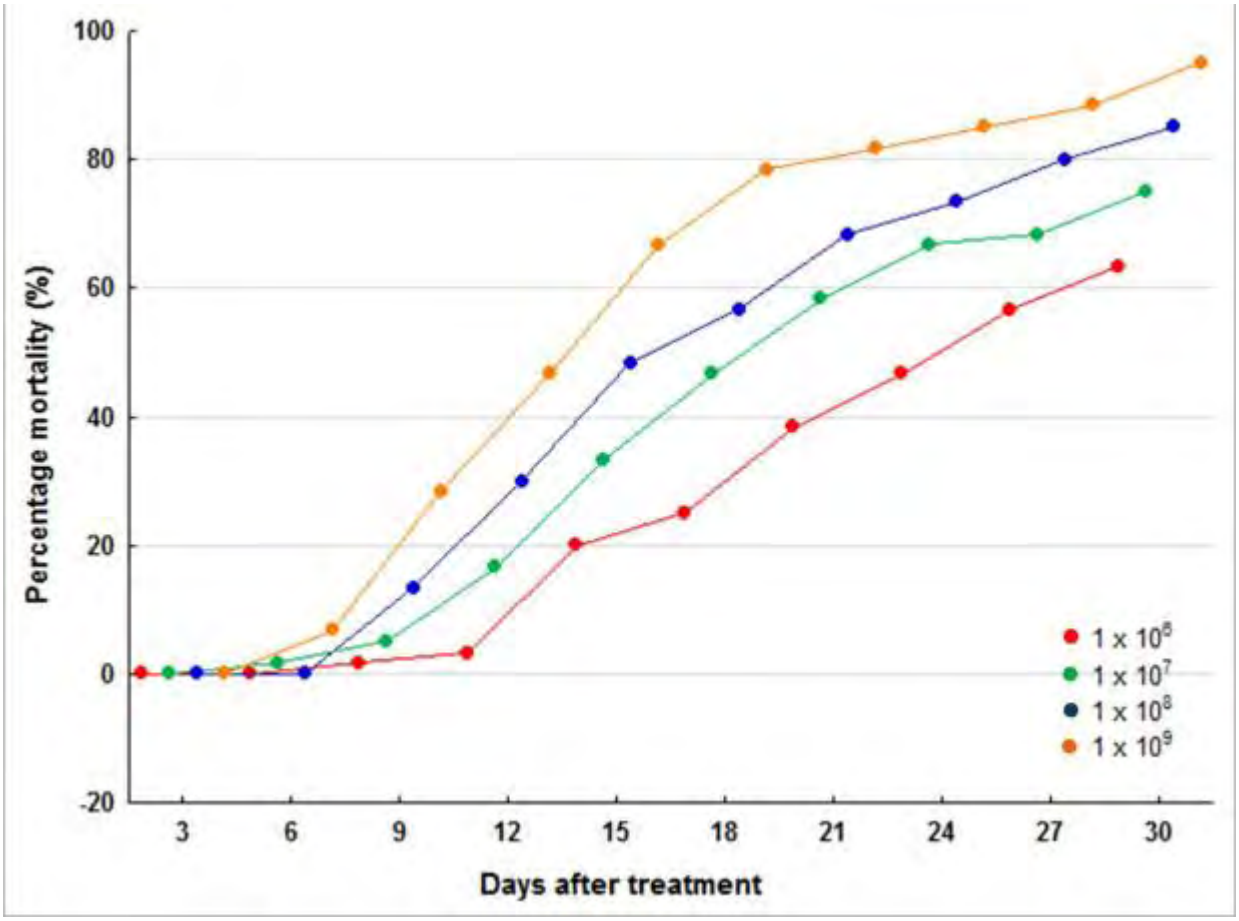

Figure 4.7: The percentage accumulated mortality of the proportion of *Schizonycha affinis* third instar grubs (L3) killed over time (days) by four concentrations of the *Beauveria brongniartii* isolate HHWG1.

Bioassay G: *Schizonycha affinis* L3 grubs

The percentage mortality for the most effective isolate (HH114) was 88.4% while isolate CFe5F caused 83.4% mortality and the weaker isolate (HHE37) caused 61.7% mortality in grubs (Figure 4.8). Control mortality was 28%. Regression analysis under the model (χ^2=156.55; df=5; error df=24; P≤0.001) was significant with the number of days after treatment having the greater effect on insect mortality than isolate (Figure 4.9 D). There were no significant differences in virulence when HH114 or CFe5F were considered and these isolates had estimated LT_{50} values of 15.8 days and 19.8 days respectively (Table 4.4). HHE37 took longer to kill L3 grubs and had an estimated LT_{50} value of 25.0 days (Table 4.4).

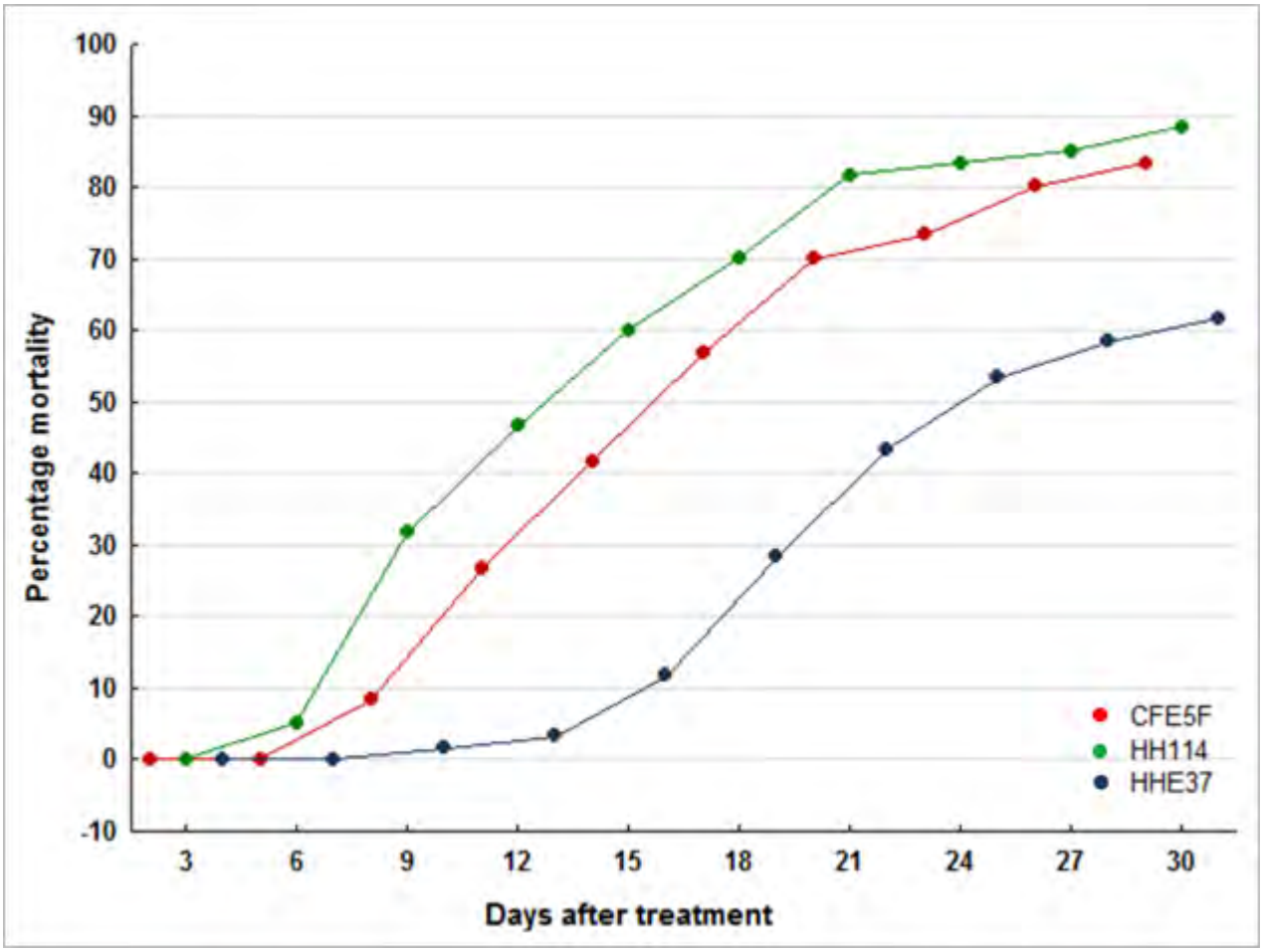

Figure 4.8: The percentage accumulated mortality of the proportion of *Schizonycha affinis* third instar grubs (L3) killed over time by three isolates of *Beauveria brongniartii.*

A

B

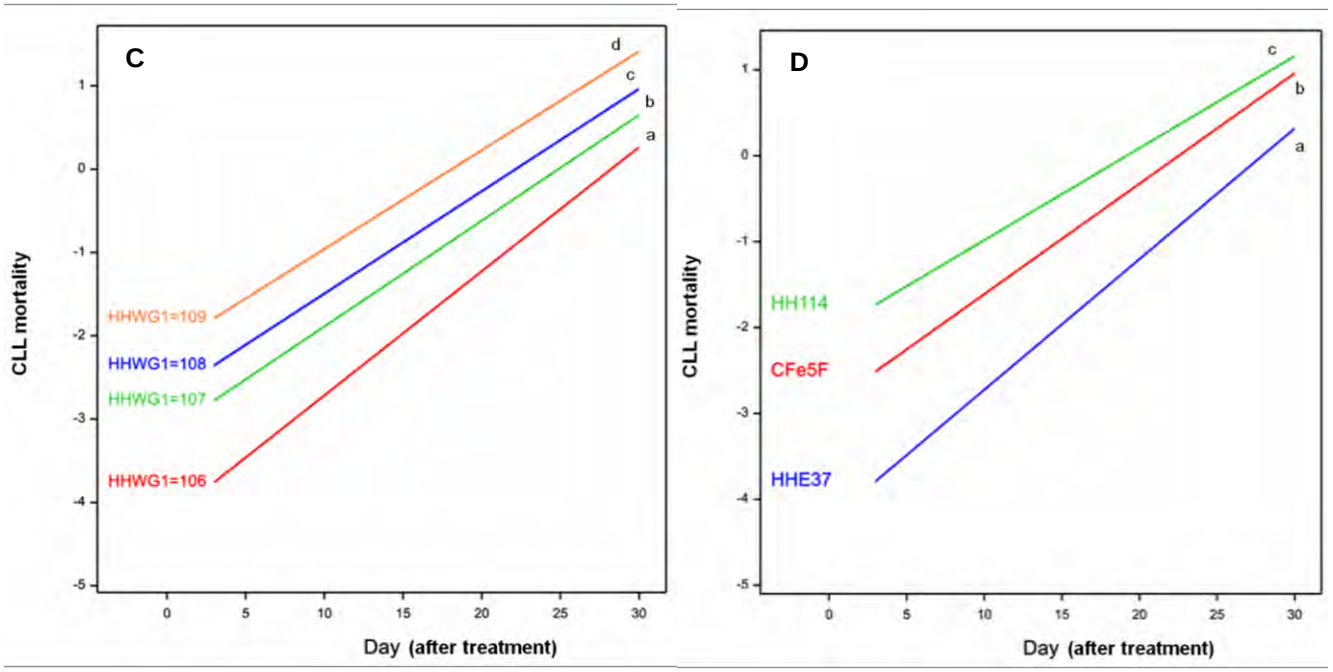

Figure 4.9: The predicted complementary log-log (CLL) transformations of the proportion of *Schizonycha affinis* second instar grubs (L2) (A); and third instar grubs (L3) (B); killed over time (days) by five isolates of *Beauveria brongniartii.* The predicted CLL transformations of *S. affinis* third instar grubs (L3) killed over time (days) by four concentrations of the *B. brongniartii* isolate HHWG1 (C); the predicted CLL transformations of *S. affinis* third instar grubs (L3) killed over time by three isolates of *B. brongniartii* (D)

Table 4.4: Regression analysis of time-mortality response in various life stages of *Schizonycha affinis* and *Hypopholis sommeri* following treatment with *Beauveria brongniartii* and *B. bassiana* (S4222).

Bioassay	Isolate name	Insect species	Life stage	Corrected mortality (%)	LT_{50} (days)	Lower LT_{10} (days)	Upper LT_{90} (days)	CLL regression equation	SE
B	C13	*S. affinis*	adults	79.2 (*)	7.8 ± 1.48	3.7 ± 2.48	13.6 ± 2.78	-6.065 + 0.716*x*	± 0.85
B	CFe3	*S. affinis*	adults	50.1 (*)	9.0 ± 1.54	1.1 ± 2.55	15.2 ± 3.01	-7.965 + 0.846*x*	± 1.63
B	HH56	*S. affinis*	adults	56.6 (*)	8.7 ± 1.22	1.2 ± 2.67	15.0 ± 1.22	-7.865 + 0.858*x*	± 1.54
B	HHE37	*S. affinis*	adults	58.3 (*)	8.6 ± 1.31	0.2 ± 3.21	14.4 ± 2.12	-5.375 + 0.586*x*	± 1.21
B	S4222	*S. affinis*	adults	34.2 (*)	9.7 ± 1.78	0.4 ± 5.32	15.3 ± 2.22	-6.425 + 0.624*x*	± 1.51
C	C13	*H. sommeri*	adults	70.1 (*)	8.3 ± 2.78	3.2 ± 2.48	14.4 ± 2.26	-5.903 + 0.761*x*	± 0.86
C	C13	*S. affinis*	adults	79.2 (*)	7.0 ± 2.18	2.1 ± 3.32	13.6 ± 2.18	-5.353 + 0.716*x*	± 1.14
D	C13	*S. affinis*	L2 grubs	72.2	27.1 ± 0.85	5.2 ± 2.17	40.8 ± 1.52	-2.707 + 0.087*x*	± 0.22
D	C17	*S. affinis*	L2 grubs	82.2	19.7 ± 1.08	0.2 ± 3.45	37.8 ± 1.67	-1.672 + 0.066*x*	± 0.17
D	HHE37	*S. affinis*	L2 grubs	75.6	25.0 ± 0.91	0.8 ± 2.56	40.3 ± 1.61	-2.320 + 0.078*x*	± 0.20
D	HHB39A	*S. affinis*	L2 grubs	77.2	19.8 ± 1.25	0.1 ± 4.46	40.9 ± 2.21	-1.476 + 0.056*x*	± 0.16
D	HHWG1	*S. affinis*	L2 grubs	88.9	18.4 ± 1.03	0.2 ± 3.06	34.9 ± 1.37	-1.717 + 0.074*x*	± 0.17
E	C13	*S. affinis*	L3 grubs	72.5	28.7 ± 0.89	7.0 ± 2.14	42.5 ± 1.68	-2.888 + 0.088*x*	± 0.24
E	C17	*S. affinis*	L3 grubs	79.2	22.4 ± 1.11	0.1 ± 3.76	41.8 ± 2.14	-1.761 + -0.063*x*	± 0.17
E	HHE37	*S. affinis*	L3 grubs	65.8	30.1 ± 0.87	9.9 ± 1.94	42.9 ± 1.64	-3.197 + 0.094*x*	± 0.26
E	HHB39A	*S. affinis*	L3 grubs	70.8	26.9 ± 0.92	3.05 ± 2.47	41.9 ± 1.74	-2.509 + 0.079*x*	± 0.21
E	HHWG1	*S. affinis*	L3 grubs	87.5	21.8 ± 0.90	0.1 ± 2.56	36.7 ± 1.34	-2.107 + 0.080*x*	± 0.19
F	HHWG1 (10^6)	*S. affinis*	L3 grubs	63.4	25.8 ± 0.45	13.1 ± 0.85	33.8 ± 0.82	-4.206 + 0.149*x*	± 0.25
F	HHWG1 (10^7)	*S. affinis*	L3 grubs	75.0	22.0 ± 0.43	7.1 ± 1.02	31.5 ± 0.73	-3.148 + 0.127*x*	± 0.18
F	HHWG1 (10^8)	*S. affinis*	L3 grubs	85.0	19.2 ± 0.42	3.9 ± 1.07	28.9 ± 0.63	-2.733 + 0.123*x*	± 0.15
F	HHWG1 (10^9)	*S. affinis*	L3 grubs	95.0	15.1 ± 0.46	0.1 ± 1.2	25.1 ± 0.56	-2.136 + 0.118*x*	± 0.13
G	CFe5F	*S. affinis*	L3 grubs	83.4	19.8 ± 0.41	4.9 ± 1.02	29.0 ± 0.61	-2.897 + 0.127*x*	± 0.16
G	HH114	*S. affinis*	L3 grubs	88.4	15.8 ± 0.49	0.1 ± 1.35	27.0 ± 0.65	-2.055 + 0.107*x*	± 0.13
G	HHE37	*S. affinis*	L3 grubs	61.7	25.0 ± 0.44	13.1 ± 0.83	33.4 ± 0.78	-4.246 + 0.152*x*	± 0.25

(*) only overt mycosis was recorded

4.4 DISCUSSION

Genetically closely related isolates of *B. brongniartii* did vary in their susceptibility towards *T. molitor* adults and *S. affinis* adults and grubs in this study as there were significant differences observed in virulence between haplotypes. These results are in accordance with findings by other researchers (Neuvéglise *et al.* 1997; Milner *et al.* 2002). Cravanzola *et al.* (1997) showed that genetic polymorphisms existed in strains of *B. brongniartii* collected at different European sites using RAPD-PCR. The study showed that the analysed strains were closely related but their ability to infect *M. melolontha* varied between 20% and 100%

mortality after 45 days in the host. Cruz *et al.* (2006) reported that cluster analysis produced three genetically distinct groups of *B. bassiana* and confirmed a low but significant intraspecific genetic diversity present among strains. This translated into significantly different strain virulence which ranged from 57.5% to 90% mortality against coffee borer, *Hypothenemus hampei* Ferrari (Curculionidae: Scolytinae) in Columbia. Neuvéglise *et al.* (1997) showed that the nuclear ribosomal DNA of *B. brongniartii* was polymorphic in terms of restriction site and length. Variation in the 28s rDNA group-1 introns appeared to be correlated with pathogenicity towards the white grub, *H. marginalis* while other genetic variations in this region translated into strains which were pathogenic towards the European cockchafer, *M. melolontha*.

Milner *et al.* (2002) evaluated the pathogenicity of various isolates of *M. anisopliae* again *T. molitor* and the scarab larvae of *D. albohirtum*. It was found that isolates differed significantly in virulence when tested against *T. molitor*. Specifically, *M. anisopliae* isolate FI-1045 which was highly virulent against *D. albohirtum* and was less virulent against *T. molitor* (Milner *et al.* 2002). These authors concluded that *T. molitor* was a good alternative screening host for various *M. anisopliae* isolates. The present study disagrees with this statement as some of the more virulent isolates against *S. affinis* were not as effective against *T. molitor* (114, CFe5F, C17). Isolates of *M. anisopliae* and *B. brongniartii* from scarabaeids are often highly host specific and are not able even to infect closely related scarab species (Milner *et al.* 2002; Traugott *et al.* 2005; Strasser pers. comm.). Therefore by screening such isolates against *T. molitor* a false-negative result may be obtained as Milner *et al.* (2002) alluded to when isolate FI-1045 (highly virulent against *D. albohirtum*) was less virulent against *T. molitor*. Rapid screening of isolates for mycoinsecticide development using alternative hosts may undervalue highly virulent and host specific isolates because these isolates may not perform as well on alternative hosts. Further, common laboratory-reared insects, such as *T. molitor*, are potentially affected by inbreeding which reduces adult condition and potentially makes them more susceptible to fungal infection (Rantala *et al.* 2011). These authors concluded that inbreeding reduced the realized immune response of *T. molitor* and its resistance to the fungus *B. bassiana*. Based on this, it may have been possible that some mortality results in the present study may have been overinflated as *T. molitor* may have succumbed to fungal infection sooner. It is therefore suggested that isolate screening against the intended target scarab species should be carried out without the use of alternative hosts for mycoinsecticide development.

In general, the isolates of *B. brongniartii* used in the present study were not shown to be highly virulent against *S. affinis* life stages compared with other studies on Scarabaeidae using other entomopathogenic fungi (Milner *et al.* 2002; Strasser and Pernfuss, 2005) but the results of the present study were in accordance with other research (Yaginuma *et al.* 2006; Townsend *et al.* 2010; Srikanth *et al*. 2011) and the results of the present study were better than other studies (Keller *et al*. 1999; Beron and Diaz; 2005; Traugott *et al.* 2005; Makana, 2008; Guzman-Franco *et al.* 2011).

The adults of *S. affinis* were more susceptible to isolates of *B. brongniartii* than the L2 or L3 grubs. These results are in accordance with Yaginuma *et al.* (2006) who found the adults of yellowish elongate chafer, *Heptophylla picea* Motschulsky (Coleoptera: Scarabaeidae) had average survival rates of 7-8.4 days while it took 15.9 days to kill the grubs at a concentration of 1×10^7 conidia/ml^{-1}. Some reasons for the observed anomaly may be due to the short-lived nature of the adults which reduces their exposure to the soil compared to the larvae. Reduced exposure to the soil limits exposure to entomopathogenic fungi, for which the soil is a natural reservoir; this may reduce the development of resistance against fungi. It is also hypothesized that adults may be more susceptible to fungi because activation of the immune response is costly and may be traded off against other energy-demanding aspects of the life history, such as reproduction which has been proposed by Rantala *et al.* (2011). Other studies undertaken on invertebrates have reported that mating results in trade-offs, with a down regulation of immune response (McKean and Nunney, 2001; Fedorka *et al.* 2004). So it is possible that white grub adults in particular (with their swarming flights) invest vast amounts of energy in reproduction and possibly reduce their realized immune response leading to higher fungal infection rates compared to the grubs.

When evaluating *S. affinis* (bioassay B) and *H. sommeri* (bioassay C) adults bioassays on the eighth day, only overt mycosis (fungus seen growing externally) was recorded in all dead *S. affinis* and *H. sommeri* adults. Further, percentage mortality levels were relatively low and the LC_{50} values appeared quite high in the present study. However, a lag in adult infection was observed after the termination of the bioassay (data not presented) when many recorded living adults died later due to fungal infection. Lacey *et al*. (1994) reported a delay in adult mortality of the Japanese beetle, *P. japonica* treated with *B. bassiana* and *M. anisopliae* until nine days after treatment and Milner *et al*. (2002) reported a marked increase in mortality in *T. molitor* seven days after treatment. It is therefore possible that because *S. affinis* and *H.*

sommeri adult bioassays were terminated a week after treatment that adult mortality data were under-estimated. According to Lacey *et al.* (1994), the observed delay in insect mortality following fungal treatment indicates good potential for dispersal of *B. brongniartii* within both *S. affinis* and *H. sommeri* population in the field.

Significant variation occurred during bioassays in the present study as was noted by overdispersion in the results when linear regressions were undertaken. Overdispersion occurs when heterogeneous residual variances are present and the predicted model cannot account for all the observed variation in the data. There are two possible reasons for the observed variation, strain attenuation and wild caught insects. Some strains (C13 and HHE37) did not perform as well in some bioassays (D, E and G) as they did against the adults of *T. molitor* and *S. affinis* (A and B). This discrepancy in virulence may have been due to intra-strain genetic variation which can lead to attenuation (Butt *et al.* 2006). Fungi are notorious for losing virulence or changing morphology when continuously sub-cultured on artificial media and strains can differ in the rate at which they decline in virulence (Butt *et al.* 2006). This author compared the different stages of the fungal invasion process (adhesion, germination, penetration and colonisation) of attenuated and virulent strains of entomopathogens. The study revealed only slight differences between the two types of strains overall, but the sum of the subtle differences in the fungal infection process, explained why attenuated strains had higher LT_{50} values compared with virulent strains (Butt *et al.* 2006). Relative loss in pathogenicity of isolates HHE37 and C13 in this case, may have been attributed to intra-strain genetic variation. Germination was always above 85% for these strains however any phase of the invasion and fungal development process may affect virulence, not just germination. Strains which are genetically and therefore morphologically less stable should not be considered as biological control agents.

In this study wild-caught insects were used due to the difficulty in rearing white grubs in the laboratory. There is no certainty that insects used were all exactly the same age. Insect age differences can add to variation in bioassays (Hatting and Wraight, 2007). Further, insect condition may have also influenced bioassay precision. As much care as possible was taken to ensure insects were fit enough for bioassays but because adults were caught in light traps, and grubs dug up from soils, one is not entirely sure what condition they are in prior to testing. This is the case with laboratory insects for that matter as inbreeding effects cause deterioration in insect condition, too (Rantala *et al.* 2011).

4.5 CONCLUSION

Despite close genetic relatedness of *B. brongniartii* isolates and their occurrences in the same field epizootics, pathogenicity varied significantly against *T. molitor* and white grubs in this study. This suggests that care should be taken when selecting a virulent strain for mass formulation as mycoinsecticides, particularly in light of strain attenuation. It also suggests that strains with greater virulence may be obtained if further bioassay work is undertaken. *Tenebrio molitor* adults are not considered a good screening host for isolates of *B. brongniartii* because the host specific nature of the fungal species may give false negative results and virulent strain selection may be compromised. The host range of *B. brongniartii* includes the melolonthid host, *S. affinis* as the grubs and adults were shown to be susceptible to various strains of the fungal species. It further suggests that inundative biological control efforts in the KZN Midlands North will encompass both *S. affinis* and *H. sommeri* species. *Schizonycha affinis* adults are the most susceptible life stage compared to second and third instar grubs and this will have consequences for biological control strategies and the timing of these technologies.

CHAPTER 5 - The persistence of *Beauveria bassiana* formulated on rice and bran in soils of eight sugarcane fields in the Midlands North

5.1 INTRODUCTION

In the previous chapter, strains of *B. brongniartii* were shown to vary in virulence despite being genetically closely related and different life stages of *S. affinis* varied in susceptibility to fungi which will have consequences for the timing of biological control strategies. In this chapter, biological control strategies, which include the use of granular formulations of fungi and their persistence under different environmental conditions, are discussed. The inundative use of fungi against soil dwelling pests such as white grubs in the soil environment represents a situation where all dose transfer to the insect is indirect; the insects have to come into contact with fungal conidia. Jaronski (2010) suggested that the idea was to create an infectious minefield through which insects have to pass when they are moving through soil pores to feed on plant roots and organic matter. Fungi are rarely applied as unformulated conidia to the soil (Jaronski, 2007).

Commercial formulations include wettable powders, aqueous suspensions, emulsifiable vegetable or mineral oils, clay coated conidia and granules (Jaronski, 2007; 2010). Soil drenches in the form of aqueous suspensions, oil formulations and as wettable dusts have been attempted (Vänninen *et al.* 2000; Ekesi *et al.* 2005; Garrido-Jurado *et al.* 2011) but generally these approaches do not carry conidia further than the top few centimeters of the soil. These formulations deliver 'naked' conidia which have a heterogeneous distribution in the soil even when soils are thoroughly drenched (Jaronski, 2010). As insects move through the soil environment they must accumulate sufficient conidia, through contact, for the disease to be elicited. This means that the application of many liters of suspension is required to inundate the area in which biological control is required (Jaronski, 2007). Application using fungi formulated on granules changes the quantity and distribution of conidia in the soil (Jaronski, 2010). With a granular formulation, fungal sporulation occurs on the granule carrier and as an insect moves through the soil may contact one granule (foci of greater than $1x10^7$ conidia) and obtain a lethal dose (Jaronski, 2007). The previous chapter showed that 50% of white grub larvae (L3) and adults could succumb to *B. brongniartii* infection within 22 days and 8 days respectively simply by contacting one of these potential granules.

Nutritive or non-nutritive granules coated with fungal conidia, granules colonized by fungus or granules composed of dry mycelium are examples of commercial formulations which have been used against soil pests (Jackson & Jaronski 2009). There are many examples of research which have successfully employed granules in the inundative biological control of several soil dwelling pests (Samson and Milner, 1999; Milner *et al*. 2003; Kessler *et al*. 2003; Kessler *et al.* 2004; Samson *et al.* 2006).

The earliest known example of a granular formulation is Betel®, which has been in existence since 1993 and is still manufactured in Reunion [14](Pastou, pers. comm.). Betel® is based on a strain of *B. brongniartii* which was obtained from native Madagascar to control an introduced scarab pest, *H. marginalis* in Reunion (Vercambre *et al*. 1991; Vercambre *et al.* 1994). The fungal isolate is highly virulent and is produced using a solid substrate fermentation process based on nutrient impregnated clay granules (Milner, 1997; Neuvéglise *et al.* 1997). The granules are recommended to be applied in furrow at sugarcane planting at a rate of 50 kg/ha together with a reduced rate of suSCon Blue® (chlorpyrifos in a controlled-release, granular formulation) (Milner, 1997). Betel® alone had a 90% efficacy rate and reduced the pest significantly within 3 years of its inundative release (Pastou, pers. comm.). Similarly, Australian researchers developed a successful granular biological control product called BioCane™ (Samson and Milner, 1999; Milner *et al*. 2002; Milner *et al*. 2003). The active ingredient is the viable conidia of *M. anisopliae* var. *anisopliae* strain FI-1045. The product is manufactured by Bio-Care Technology Pty Ltd and consists of grains of broken, parboiled rice on which the fungus has grown and sporulated. BioCane™ contains a minimum of 2×10^9 conidia/g^{-1} and is applied to plant sugarcane in furrow at the rate of 33 kg ha^{-1} for 1.5 m spacing. It was developed to control greyback canegrub, *D. albohirtum*. Large scale field trials consistently showed that when the fungus was applied correctly, it provided 50-60% control of the pest in the season of application with persistence effective in ratoon crops as well (Samson *et al*. 1999).

Survival of entomopathogenic fungi in the soil which ultimately leads to persistence in the soil environment has ranged from a few days or months to longer than three years (Vänninen *et al.* 2000; Milner *et al.* 2003; Ekesi *et al.* 2005) and in another case, up to 14 years (Enkerli *et al.* 2004). Several abiotic and biotic factors are known to affect fungal persistence in the

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soil. Climatic variables such as soil temperature, moisture and relative humidity are thought to have an effect on fungal persistence (Lingg and Donaldson, 1981). So are soil factors such as texture (the relative proportions of sand, silt and clay), structure (the arrangement of primary and secondary soil particles) and pH which have an impact on the accessibility of organic matter, nutrients, water and oxygen for soil microorganisms (Vänninen *et al.* 2000; Quesada-Moraga *et al.* 2007). Antagonistic organisms which co-occur in the soil environment can also affect fungal persistence (Lingg and Donaldson, 1981; Groden and Lockwood, 1991). Fungal formulation, fungal species and genotype affect persistence (Vänninen *et al.* 2000; Milner *et al.* 2003; Ekesi *et al*. 2005; Dolci *et al*. 2006). The most important factor however is the presence of insect hosts, which allow fungal inoculum levels to increase in the soil (Kessler *et al*. 2003; Kessler *et al.* 2004). Persistence studies, in the absence of insect hosts, have shown that only 50% of the applied conidia persisted for 12 months (Milner *et al.* 2002) while other authors have shown a 90% reduction in conidia in 16 months (Kessler *et al.* 2004).

After the success of Betel® and BioCane™, the development and implementation of a granular mycoinsecticide to control soil dwelling white grubs in South African sugarcane seemed prudent and feasible. However, prior to the discovery of the latter two *B. brongniartii* epizootics discussed in Chapter 3, a strain of *B. bassiana* (SASRI 4222) was initially isolated directly from a mycosed *H. sommeri* cadaver and assumed to be a good potential candidate for biological control of white grubs in the Midlands North (Hatting, 2008). *Beauveria bassiana* isolate 4222 was passed on to the company Plant Health Products (PHP) for formulation on wheat bran and rice which were to be field-tested as potential commercial formulations. However, the discovery that *B. brongniartii* was the causal agent of white grub epizootics occurred after the formulation of *B. bassiana* isolate 4222 or the implementation of this study (chapter). Hence, time constraints did not allow for the persistence of obtained and subsequently formulated *B. brongniartii* isolates to be investigated. But, knowledge on the persistence of these commercial formulations was still required.

Thus the aims of the present study were: 1.) To test the persistence of *B. bassiana* isolate 4222 formulated on wheat bran and rice as two nutritive carriers under field conditions, to know which carrier was better for fungal persistence; 2.) To test the persistence of *B. bassiana* isolate 4222 under different environmental conditions which included various field sites with a range of climates and soil types to determine the feasibility of area-wide application in the Midlands North region.

5.2 MATERIALS AND METHODS

5.2.1 *Formulations and net bag preparations*

Beauveria bassiana isolate (SASRI 4222) obtained from Sunnyside Farm (29°06'55.5"S, 30°46'59.1"E; altitude: 900-1400 meters) in the Seven Oaks district of the KZN Midlands North was grown on two nutritive carriers, wheat bran and rice. The first formulation consisted of dried, broken rice grains and the second, wheat bran that were inoculated with *B. bassiana* conidia and then incubated at 25°C for 3 weeks to produce conidia overgrown granules (Figure 5.1A). These potential commercial formulations were prepared and supplied by the company PHP. In a similar study by Milner *et al.* (2003), they mixed 0.5 g of four different formulations with soil taken from field sites to give a final concentration of 10^7 conidia g^{-1} in 100 g of soil, which is why 0.5 g of formulated rice and bran was used in this study. Thus, conidial counts were undertaken on both formulations (rice and wheat bran) after they were received from PHP with an improved Neubauer haemocytometer (0.1 mm depth), to ascertain the mean amount of conidia on the granules.

Precisely, 0.5 g of fungal-overgrown rice was suspended in 10 ml of triple distilled water containing the surfactant, 0.05% Triton-X100, in a plastic 20 ml tube and thoroughly vortex mixed for 2 minutes. Three, replicate rice suspensions were made and three separate conidial counts were made, the mean of which was 4.0×10^7 conidia/ml^{-1}. Separately, 0.5 grams of fungal-overgrown wheat bran was suspended in 10 ml of triple distilled water containing 0.05% Triton-X100 in a plastic 20 ml tube and thoroughly vortex mixed for 2 minutes. Three, replicate wheat bran suspensions were made and three separate conidial counts were made, the mean of which was 6.6×10^7 conidia/ml^{-1}. Then, 0.5 g of each formulated product (bran and rice) was mixed with 100 g of finely-sieved (2 mm mesh size), unsterilized soil taken from eight field sites in the KZN Midlands North (Figure 5.1B). These soils were taken within pits that were dug to 17 cm and described in 5.2.2 *Field sites.* The mixtures (0.5 g rice or bran + 100 g soil) were placed inside nylon net bags (15 cm×10 cm) with a pore diameter of 0.20 mm (Figure 5.1C). The net bags were sealed with plastic, cable ties and wrap around tree tag labels marked in water proof ink with the following information: The field site, the formulation type, the sampling month date. The net bags were placed inside a plastic container and transported to the eight selected field sites (Figure 5.1D).

Figure 5.1: *Beauveria bassiana* isolate 4222 grown on wheat bran (left) and rice (right) (A); 50 g of formulated bran and rice mixed with 100g of soil obtained from field sites (B); mixed soil and product were placed into net bags (C); bags were buried at field sites, note the white PVC pipe which marked each site (D).

5.2.2 *Field sites*

The eight sites chosen were commercial sugarcane fields that had a range of climates, soil types and were in areas where white grubs were known to be prevalent (Table 5.1). The presence of white grubs was ascertained from surveys which were undertaken before the initiation of this study (Goble, unpublished data). The eight field sites (where net bags were buried) included two farms from eco-zone 1: Cloud Hill and Ekukhanyeni. In eco-zone 4, three farms were selected: Strytegewoon 1, Strytegewoon 2 and Uitkyk and 3 farms in eco-zone 7: Harden Heights, Canema and Weltevreden (Figure 5.2). Eco-zones are areas of similar climate and similar potential for sugarcane production and are derived from the South African Department of Agriculture's Bio-Resource Units (BRU) and are marked in Figure 5.2 (Maher and Schulz, 2003; Webster *et al.* 2005; 2009). At each field site, a large flat (150 cm×80 cm) pit was dug approximately 17 cm below the surface of the soil in which the net

bags were placed (Figure 5.1.D). This depth is that recommended for the placement of BioCane™ granules when used commercially (Milner *et al.* 2002; Milner *et al.* 2003). The net bags were placed randomly in replicate rows within the pit at each site and covered immediately with soil. The bags were destructively sampled at time 0 and then every 3 months for up to a year. There were five sampling dates (0, 3, 6, 9 and 12 months) from September 2010 (spring) until the following spring 2011. Time zero counts were determined from net bags which were kept in the laboratory and not buried and counted the day after burial. A total of 30 net bags were buried at each of the eight field sites (three rice bag replicates per month; three bran bag replicates per month) and thus 240 bags were installed at all sites. All sites were maintained according to standard agronomic practices and no disturbance of the net bags, i.e. no ripping, discing or ploughing took place while the bags were installed. Each field site was marked with a 4 meter white PVC pole and the GPS co-ordinates of each site were also obtained.

Table 5.1: The field information, location, altitude and soil characteristics of eight field sites where net bags were buried for a year.

Farm name	Cloud Hill	Ekukhanyeni	Strytegewoon 1	Strytegewoon 2	Uitkyk	Canema	Harden Heights	Weltevreden
Farm owner	J. Hackland	E. Thole	R. Fortmann	W. Fortmann	R. Schroder	Mondi	A. Kotze	D. Schreuder
Eco-zone	1	1	4	4	4	7	7	7
Field number	C 8.1	11	C 08 B	71	708	DS 06	16	60
Field size (ha)	0.40	3.20	1.50	0.30	1.20	12.70	6.00	5.40
Sugarcane variety	N12	N12	N12	N12	N37	N12	N12	N37
Ratoon	second	plant cane	first	second	first	third	third	second
Latitude	29° 35' 40.8" S	29° 34' 26.4"S	29° 25' 21.4"S	29° 24' 56.4"S	29° 25' 08.8"S	29° 12' 31.4"S	29° 13' 54.1"S	29° 14' 03.5"S
Longitude	30° 27' 49.5" E	30° 29' 03.4" E	30° 41' 28.5" E	30° 41' 40.0" E	30° 43' 14.6" E	30° 39' 55.4" E	30° 39' 13.4" E	30° 39' 51.2" E
Altitude (meters)	451-900	451-900	901-1400	901-1400	901-1400	901-1400 (frost)	901-1400 (frost)	901-1400 (frost)
Clay (%)	57	17	27	31	45	43	27	33
Silt (%)	20	22	10	10	14	10	8	8
Sand (%)	23	61	63	59	41	47	65	59
Bulk density (kg/dm^3)	1.9	1.3	1.6	1.6	1.4	1.4	1.5	1.4
pH (water)	5.3	4.6	4.7	5.4	6.3	5.6	5.5	4.7

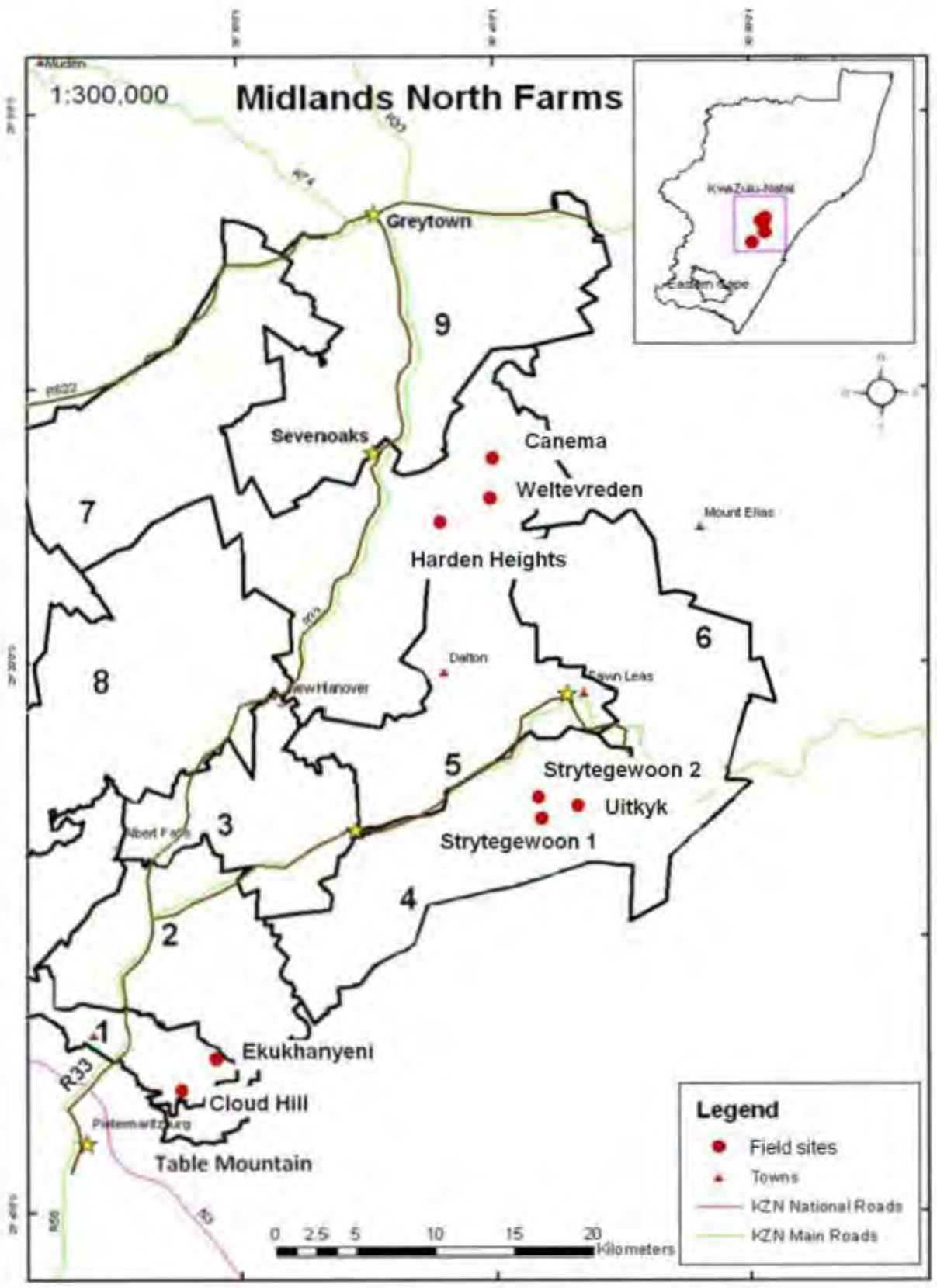

Figure 5.2: The geographic locations of the eight sites where net bags were buried for up to a year in the KZN Midlands North region. The numbers in the map represent the various eco-zones.

5.2.3 *Laboratory assessment of conidia viability using colony forming units (CFUs)*

The number of viable conidia remaining after burial was assessed by plating out a suspension of treated soil onto a selective medium (60 g Sabouraud Dextrose Agar (SDA) supplemented with 50 mg/l^{-1} chloramphenicol, 25 mg/l^{-1} cyclohexamide and 50 mg/l^{-1} rifampicin) in seven cm petri dishes and quantifying the number of colony forming units (CFUs) per gram of soil (Figure 5.3 D, E). This was done in the laboratory when 50 grams of soil in each field-collected, net bag was suspended in 300 ml of triple distilled water supplemented with 0.05% Triton-X100 in autoclaved bottles (500 ml) (Figure 5.3 A, B). The bottles were then placed on a rotor shaker (at a speed of 170 revolutions per minute) at room temperature for 30 minutes to homogenize the soil mixtures. Milner *et al.* (2003) sonicated their soil suspensions to ensure an even conidial distribution, but when this was attempted in the present study, no conidial germination was observed, suggesting that the sonication method may have prevented germination. Two milliliters (2 ml) of homogenized soil suspension was diluted further in 33.3 ml aqueous Triton-X100 solution. Finally, 10 µl of the diluted soil suspension was spread out onto three replicate SDA Petri-dishes using sterilized 3 mm beads. A second set of 10 µl samples was plated onto another three, replicate SDA plates. The petri dishes were incubated at 23°C for 1 week and the colonies of *B. bassiana* counted using a light box and magnifying glass (1.75× magnification) (Figure 5.3 D). The counts from all six replicate petri dishes per formulation (rice and bran) were averaged.

Figure 5.3: 50 g of soil from field-collected net bags was (A, C); diluted in 300 ml of water with a surfactant in 500 ml glass bottles (B); *Beauveria bassiana* colony forming units (CFU) seen growing on a selective SDA plate (D); *Beauveria bassiana* colonies can be distinguished more easily by their growth pattern seen from the underneath side of the plates (E

5.2.4 *Soil sample analyses*

Soil samples taken from the eight field sites in September 2010 were collected within the dug pit at a depth of 17 cm with a spade and placed individually into labelled bags which were transferred in a cooler box, filled with ice packs, directly to the laboratories of the [15]Soils Department and [16]Fertilizer Advisory Service of the South African Sugarcane Research Institute (SASRI). This was the only month soil samples were collect in because the cost of

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analyzing many samples was beyond the scope of this study. The soil samples were analyzed for the following: *Analytical Chemistry:* total organic nitrogen (ppm), total carbon (%), organic matter (%), NH_4 (ppm), NO_3 (ppm), macro-nutrients associated with salinity tests: magnesium (Mg), calcium (Ca), phosphorus (P) (all in molar equivalent/L), pH (water), electrical conductivity (molar equivalent/L), cation exchange capacity (CEC) (molar equivalent/L), sodium adsorption ratio (SAR) also known as EST. *Physical soil characteristics:* bulk density (kg/dm^3), clay (%), silt (%), sand (%), dry weight of soil (equivalent to 5g). *Soil Biology:* microbial biomass (µg carbon/g soil), basal respiration (µg of CO_2 carbon /g/ day), hot water extractable carbon (HWEC) (mg/kg), hot water extractable nitrogen (HWEN) (mg/kg).

5.2.5 *Weather data*

Daily weather data: rainfall (mm), plant available soil water content, mean temperature (°C), mean relative humidity of the air (%) and solar radiation (MJ/m^2/d) from September 2010-September 2011 were downloaded from three separate weather stations on the SASRI weatherweb website. Climatic data for eco-zone 1 was obtained from Pietermartizburg-Faulklands weather station 483 (29°33'0"S; 30°31'0"E) which is 7 km from field sites in this area. Climatic data for eco-zone 4 was obtained from Wartburg-Bruyns Hill weather station 455 (29°25'0"S; 30°41'0"E) which is 5 km from these field sites, and climatic data for eco-zone 7 was obtained from Noodsberg-Jaagbaan station 23 (29°21'32"S; 30°41'15"E), 16 km from field sites. Plant available soil water content was calculated for rain fed conditions for a soil with total available moisture (TAM) of 60 mm and with a full canopy of sugarcane. Soil temperature was calculated by plotting the mean soil temperatures over mean air temperatures (which are closely related) at Mount Edgecombe and using the obtained regression equation (y=1.2161x-1.9572) (R^2=0.8957) to solve for soil temperature (y) by substituting the mean air temperatures (x) obtained from the relevant weather station in the Midlands North into the equation. Rainfall, soil water content, mean temperature, soil temperature, relative humidity and solar radiation for all three weather stations were plotted against the long term mean (LTM) of each and are shown in figures 5.7-5.9.

5.2.6 *Statistical analyses*

Colony forming units (CFUs) count data were converted by adding +1 and then log transforming (Osborne, 2002; Milner *et al.* 2003) to accommodate zero counts and also correct for skewness. Generalized Linear Models (general custom design) were used to analyze LOG CFU data (dependent variable), two categorical predictors (field site/farm), carrier (rice and bran) and a continuous predictor (time/months) in Statistica 10 (StatSoft Inc, 2011). To determine which climatic variables were the best predictors of CFU decline, model building was also undertaken by performing multiple regressions (generalized linear model) using the best subset model in Statistica 10 (StatsSoft Inc). Multicollinearity between weather variables was checked by comparing Pearson correlation coefficients. Initially, six predictor variables were considered: rainfall, mean relative humidity, soil water content, solar radiation, mean temperature and soil temperature. There was however significant collinearity observed between some variables, particularly mean temperature and soil temperature (R^2=0.98, P=0.001). Soil temperature was a more biologically relevant parameter for this study and was thus included in model building but mean temperature was excluded. The Akaike Information Criterion (AIC) (Akaike, 1983; Burnham and Anderson, 2002) was used to select the best model. Finally, multiple regression analyses were used to validate the best model selected that predicted the decline of viable conidia at the eight field sites. Principal component analysis (PCA) (Hotelling, 1933; ADE4 software, Thioulouse, 1997) which indicated the likelihood of relationships between field sites, decline of colony forming units and soil characteristics was undertaken using ADE4 software.

5.3 RESULTS

All eight field sites showed a significant decline in *B. bassiana* CFUs per gram of soil over time with few conidia still present in the samples after a year (χ^2=823.09; df=1; P≤0.001) (Figures 5.4 and 5.5). There was a significant effect of field site on the decline of CFUs (χ^2=16.75; df=7; P≤0.01). Greater declines in CFUs were observed at Cloud Hill (eco-zone 1) for both rice and bran formulations while at Ekukhanyeni (eco-zone 1) the decline of CFUs of both formulations was significantly less (Figure 5.4). The other five field sites did not differ significantly from the above mentioned field sites (Figure 5.5). In the first six months there was a rapid decline in the number of *B. bassiana* CFUs remaining in the soil bags. CFUs gradually declined thereafter.

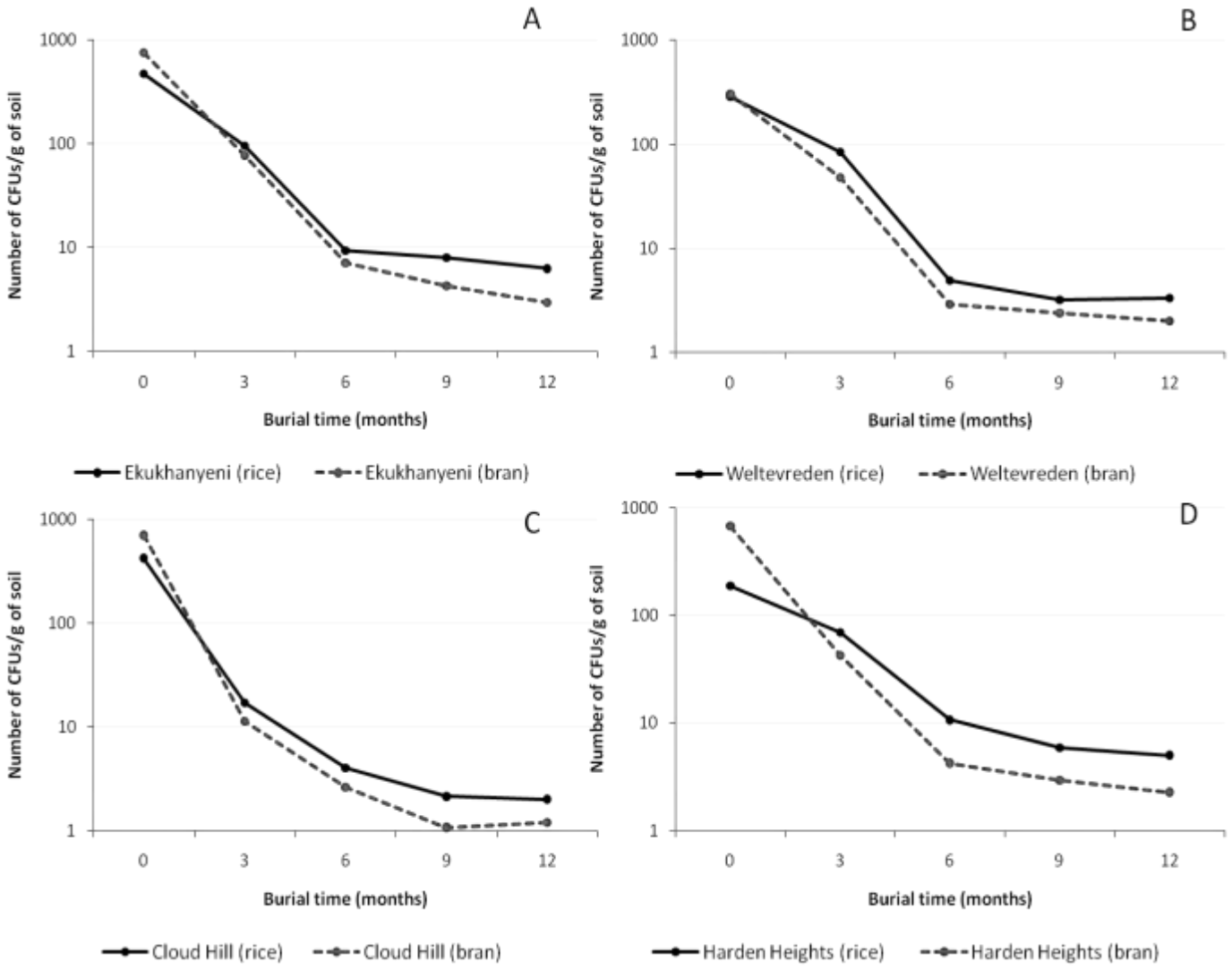

Figure 5.4: The effect of time and formulation (rice the solid line, bran the dotted line) on the number of colony forming units (CFUs) of *B. bassiana* isolate 4222 recovered from four field sites: Ekukhanyeni (A); Weltevreden (B); Cloud Hill (C) and Harden Heights (H). The data are represented on a log scale (y- axis).

At burying (time zero), bran supported higher or equal numbers of *B. bassiana* conidia compared to rice (Figures 5.4 and 5.5). In the field however, the rice formulation supported higher numbers of conidia for longer periods of time compared to the bran formulation. The effect of formulation on CFU decline however was not significant (χ^2=5.22; df=5, P≤0.38). When the interaction of field site and formulation were considered there were also no significant differences observed (χ^2=9.80; df=35; P≤0.99).

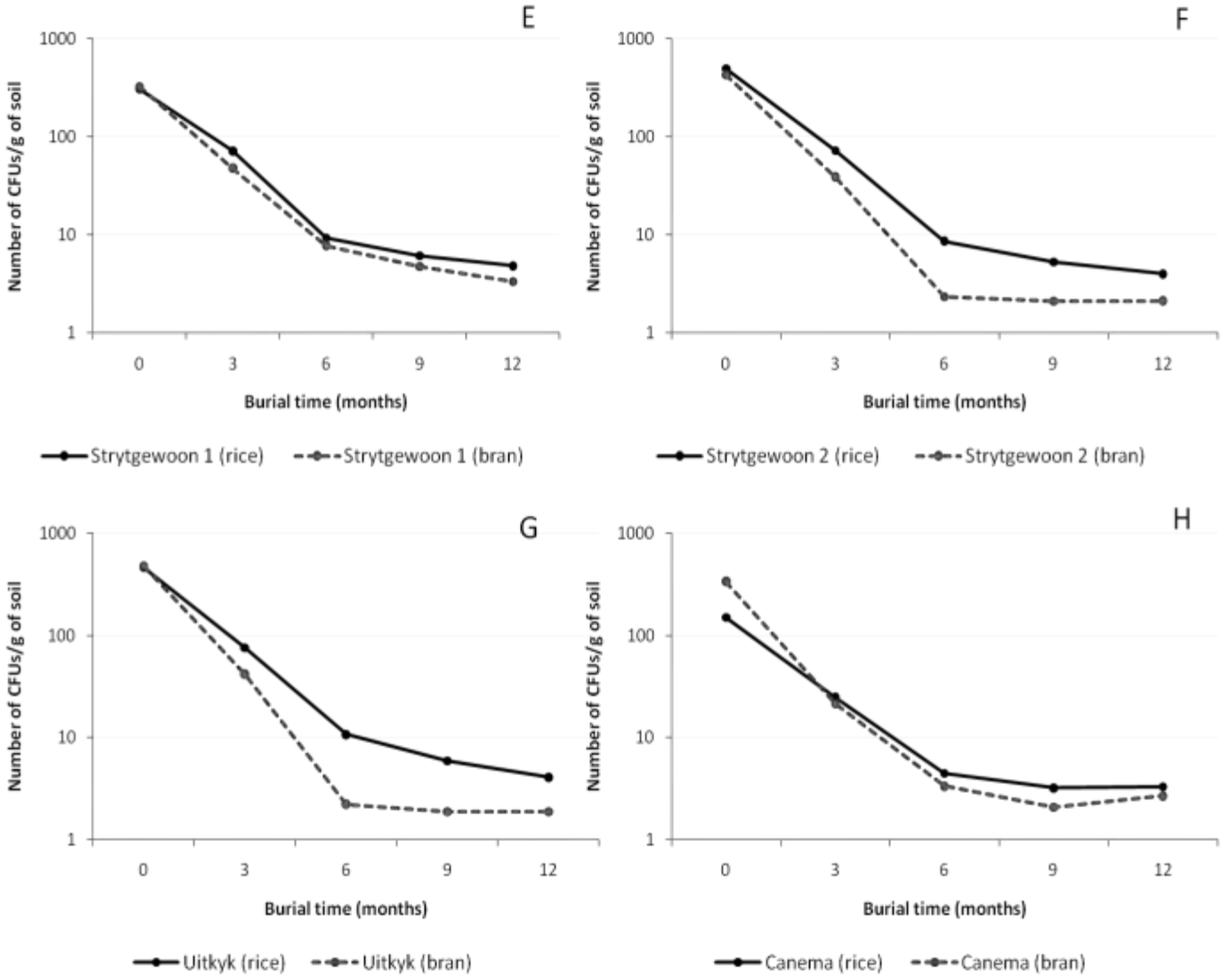

Figure 5.5: The effect of time and formulation (rice the solid line, bran the dotted line) on the number of colony forming units (CFUs) of *B. bassiana* isolate 4222 recovered from four field sites: Strytegewoon 1 (E); Strytegewoon 2 (F); Uitkyk (G) and Canema (H). The data are represented on a log scale (y- axis).

The effect of various climatic variables on the decline of CFUs at the eight field sites was significant. The best model predicted mean rainfall (χ^2=121.46; df=1; P≤0.001), mean soil water content (χ^2=142.63; df=1; P≤0.001), mean solar radiation (χ^2=15.72; df=1; P≤0.001) and mean soil temperature (χ^2=171.55; df=1; P≤0.001) to have an effect on conidia at the field sites (Table 5.2). When mean solar radiation was dropped as a parameter term in the model it was observed that the two best predictor variables were mean soil temperature (χ^2=22.42; df=1; P≤0.001) and mean soil water content (χ^2=264.64; df=1; P≤0.001) which both had a significant effect on *B. bassiana* CFUs at the field sites (Table 5.3; Figure 5.6).

Table 5.2: The best models selected from multiple logistic regression using information theoretic approach to describe the decrease in colony forming units (CFUs) of *B. bassiana* formulated on two carriers and buried at eight sites for a year

Model no.	Variables included in the models	df	AIC	ΔAIC	w_i	*P*
1	RAIN × SWC × SRAD × ST	4	2846.21	0.00	1.00	< 0.001
2	RAIN × SWC × RH × SRAD × ST	5	2847.20	0.99	0.61	< 0.001
3	SWC × SRAD × ST	3	2856.02	9.80	0.01	< 0.001
4	SWC × RH × SRAD × ST	4	2857.18	10.96	0.00	< 0.001

RAIN = Rainfall (mm)
SWC = Soil water content (60 cm)
SRAD = Solar radiation (MJ/m^2/day)
ST = Soil temperature (°C)
RH = Mean relative humidity (%)
df = degrees of freedom
AIC = Akaike information criterion
ΔAIC is the change in model AIC relative to the best fit model.
w_i: Akaike weight, the probability of the model being correct (see Burnham & Anderson 2001).

Table 5.3: The best models selected from multiple logistic regression after dropping mean solar radiation as a parameter using information theoretic approach to describe the decrease in colony forming units (CFUs) of *B. bassiana* formulated on two carriers and buried at eight sites for a year

Model no.	Variables included in the models	df	AIC	ΔAIC	w_i	*P*
1	SWC × ST	2	2871.07	0.00	1.00	< 0.001
2	SWC × RH × ST	3	2872.83	1.77	0.41	< 0.001
3	RAIN × SWC × ST	3	2873.06	2.00	0.37	< 0.001
4	RAIN × SWC × RH × ST	4	2874.72	3.66	0.16	< 0.001

RAIN = Rainfall (mm)
SWC = Soil water content (60 cm)
ST = Soil temperature (°C)
RH = Mean relative humidity (%)
df = degrees of freedom
AIC = Akaike information criterion
ΔAIC is the change in model AIC relative to the best fit model.
w_i: Akaike weight, the probability of the model being correct (see Burnham & Anderson 2001).

At low mean soil temperatures there were less CFUs observed. However, as soil temperature increased to an optimum 25°C there was an increase in the number of CFUs. Mean soil water content shared an inverse relationship with CFUs with higher mean soil water content decreasing the number of CFUs (Figure 5.6). At lower mean soil water content values, over a range of soil temperatures (12°C-28°C), the number of CFUs increased but as soil water content values increased the range of soil temperatures which could support higher CFUs decreased. Finally at the highest soil water content values, higher temperatures were required to increase conidia in the soil probably because of the cooling effect of moisture laden soils on temperature (Figure 5.6).

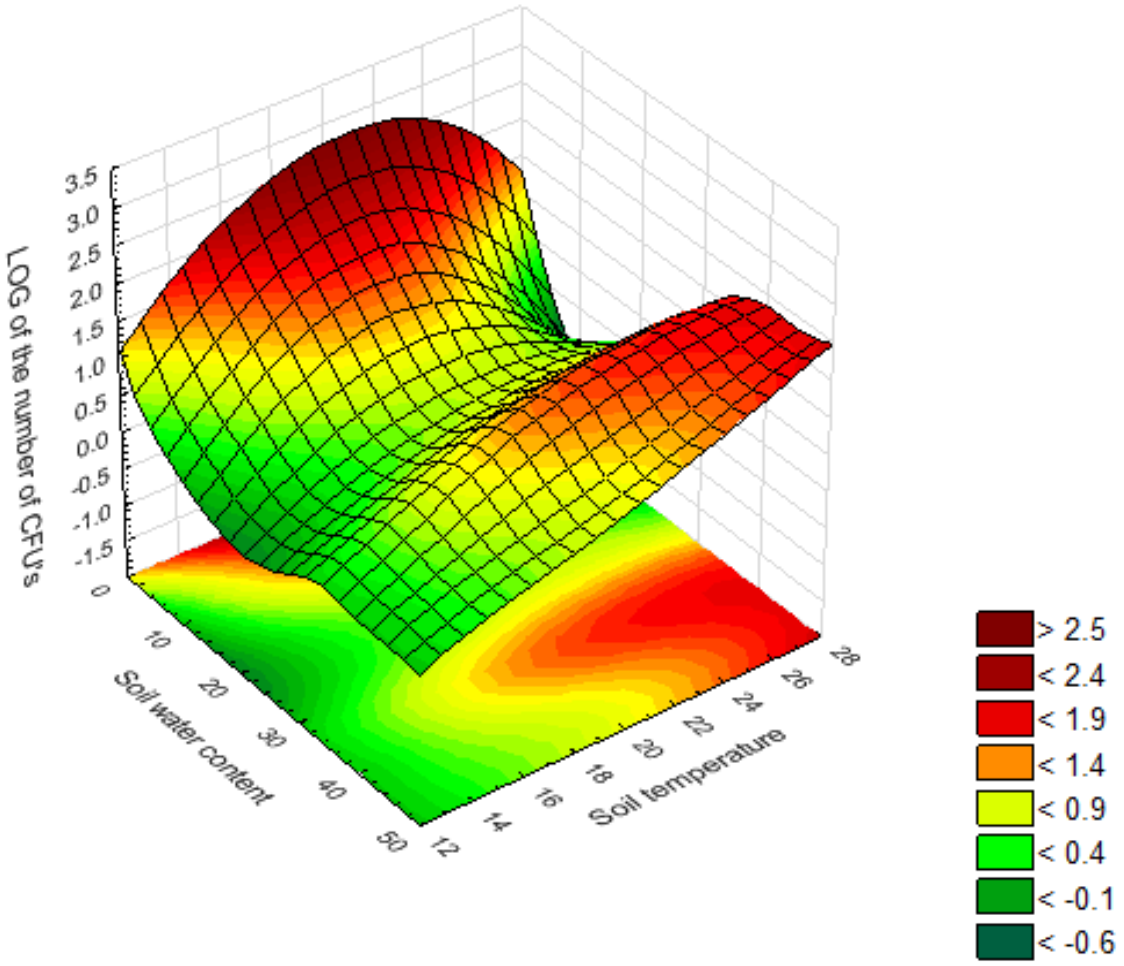

Figure 5.6: Three dimensional surface plot of the LOG number of colony forming units (CFUs) against soil temperature and soil water content. Colour squares represent the LOG number of CFUs.

Climatic variability between eco-zones was prominent as observed by Figures 5.7-5.9. Eco-zone 4 had the highest mean rainfall, soil water content and relative humidity and the lowest mean temperatures and soil temperatures (Figures 5.7- 5.9). Eco-zone 1 had the second highest recorded rainfall, soil water content and relative humidity but the highest mean temperatures and soil temperatures. Eco-zone 7 had the second highest mean temperatures and soil temperatures (Figure 5.8) but the lowest mean rainfall, soil water content and relative humidity (Figure 5.7 and Figure 5.9). Despite the observed climatic differences between eco-zones there were no significant differences observed in CFU decline between sites, except for the difference between Ekukhanyeni (eco-zone 1) and Cloud Hill (eco-zone 1). This suggested that *in situ* soil parameters and the effect of soil characteristics played a bigger role in the decline of conidia than climate did (Table 5.4).

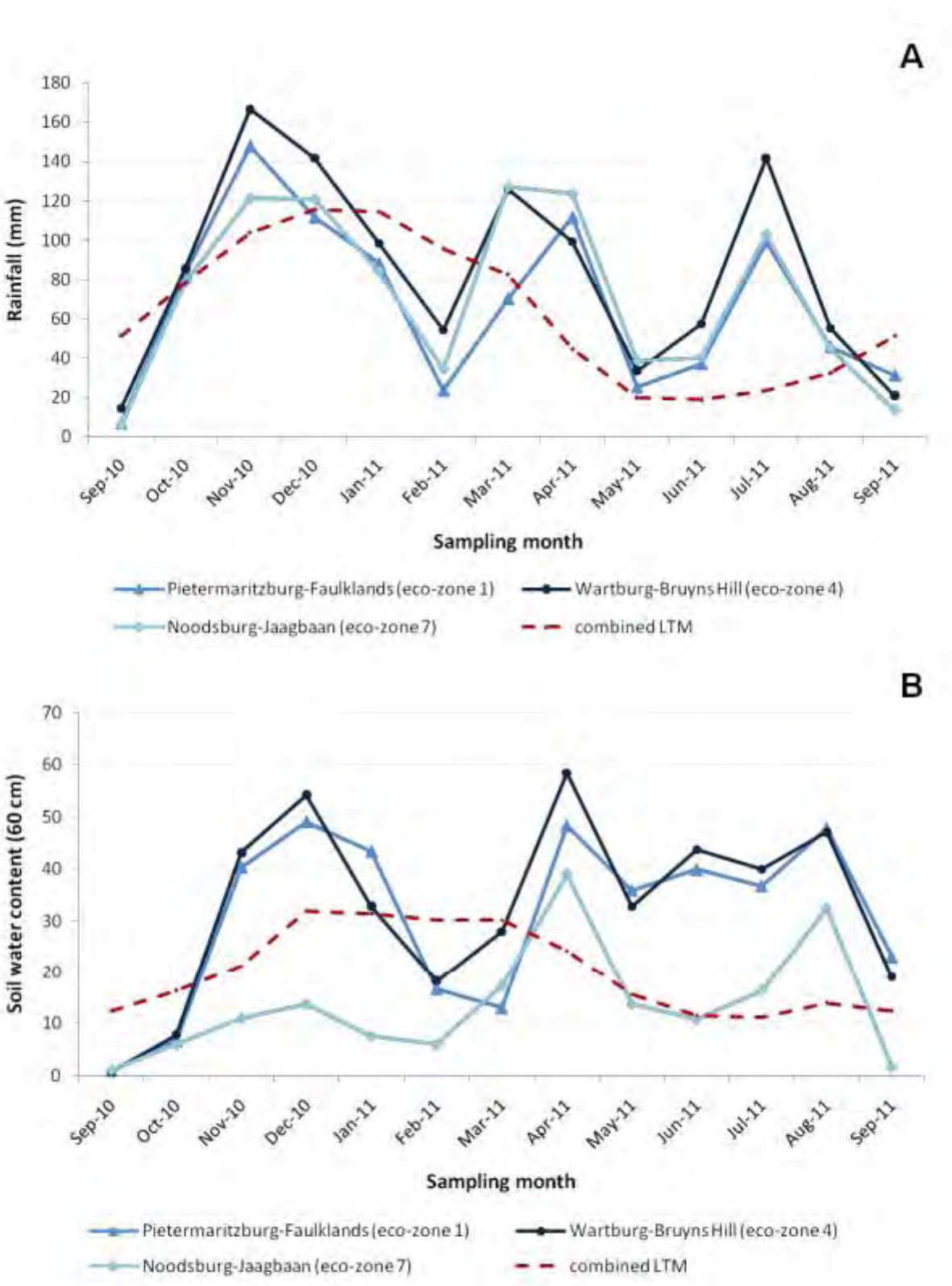

Figure 5.7: Rainfall (mm) which occurred over the sampling months plotted against the long term mean (LTM) which was calculated from a decade of recorded data, from three weather stations (A); soil water content (60 cm) which occurred over the sampling months from three weather stations plotted against the LTM which was calculated from a decade of recorded data (B).

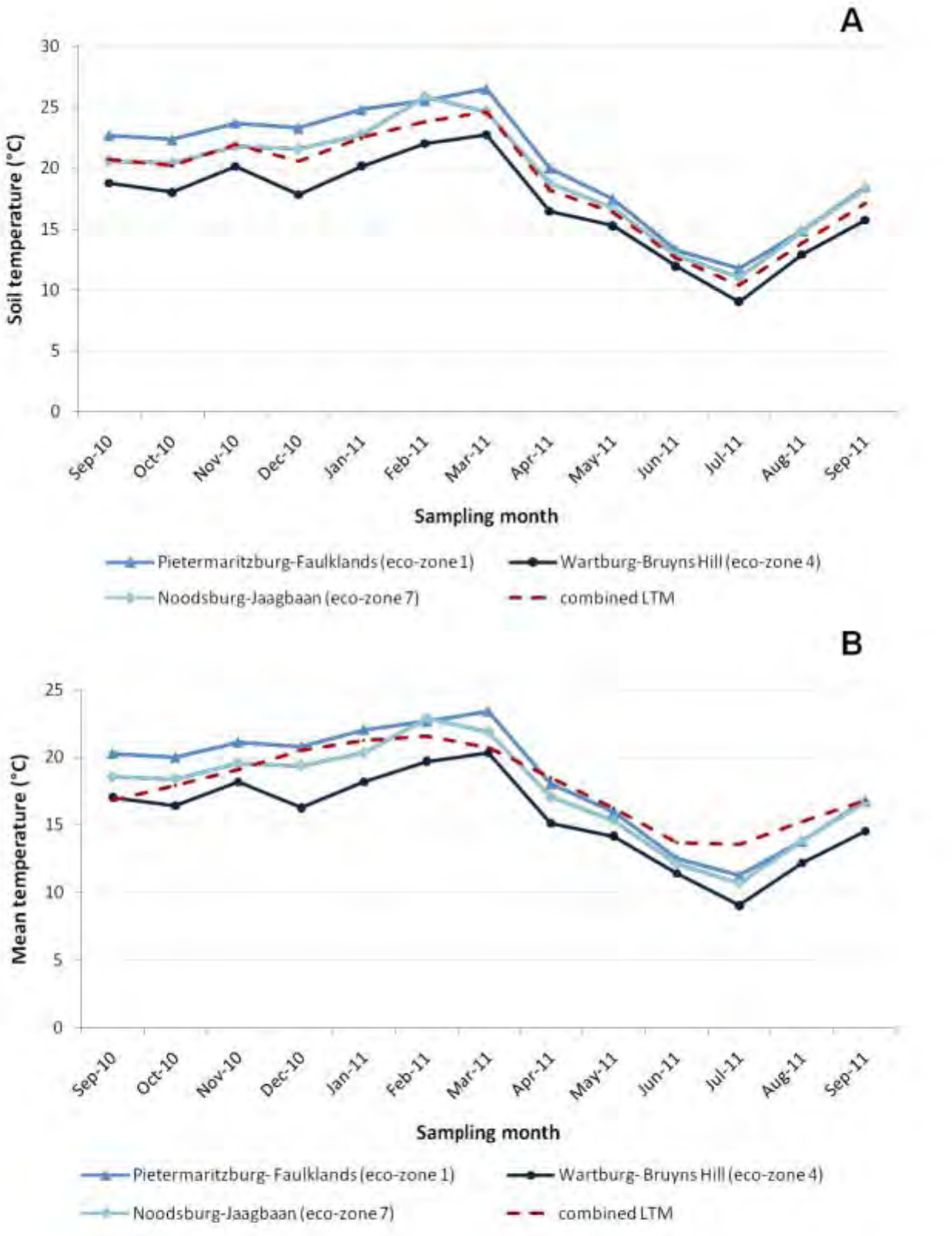

Figure 5.8: Soil temperature (°C) from three weather stations plotted against time (sampling months) (A); mean temperature (°C) which occurred over the sampling months from three weather stations plotted against the LTM which was calculated from a decade of recorded data (B).

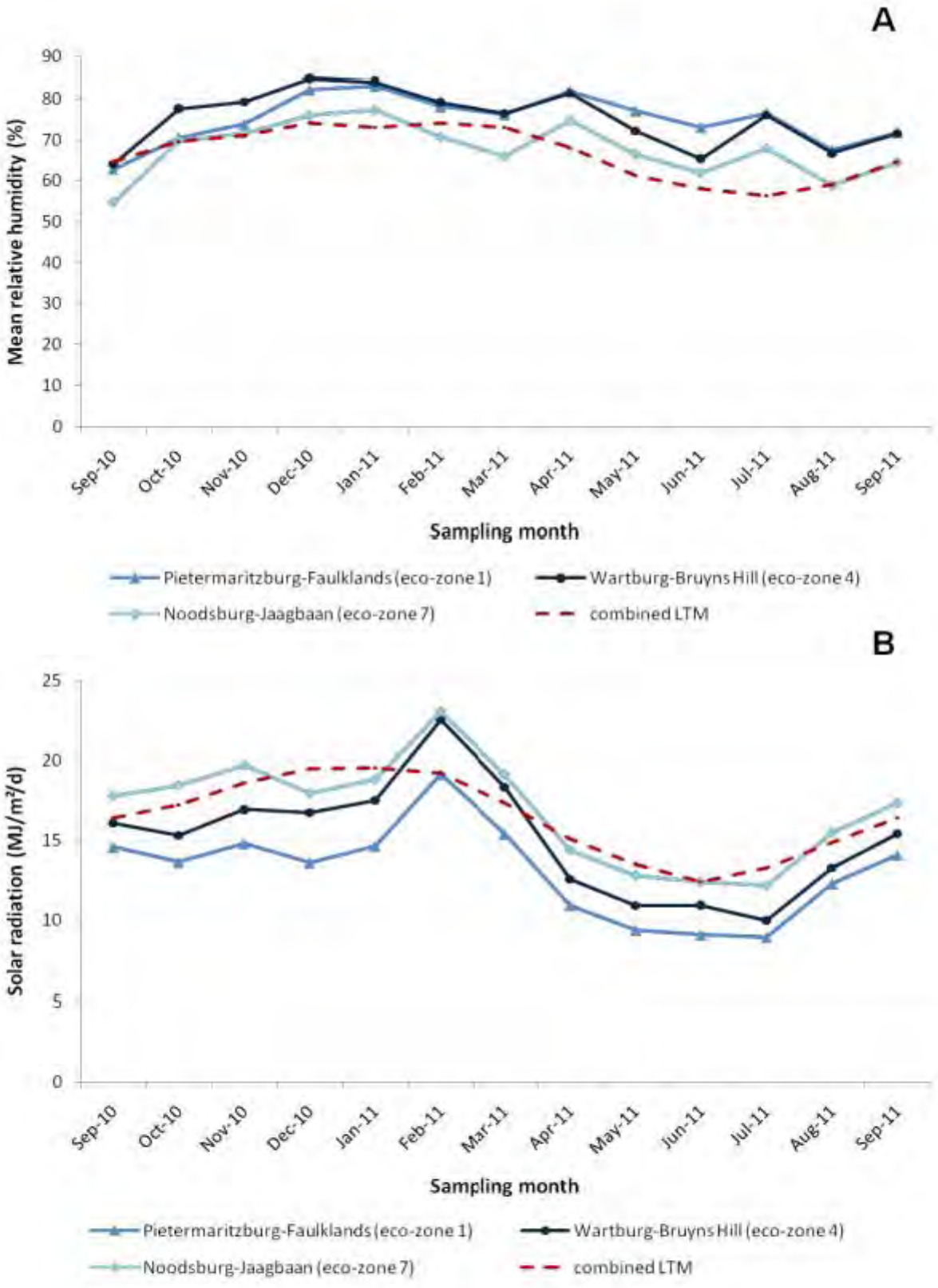

Figure 5.9: Mean relative humidity of the air (%) from three weather stations plotted against the LTM which was calculated from a decade of recorded data (A); solar radiation (MJ/m^2/d) which occurred over the sampling months from three weather stations plotted against the LTM which was calculated from a decade of recorded data (B).

Table 5.4: The soil characteristics of eight field sites where net bags were buried for a year.

Farm name	Cloud Hill	Ekukhanyeni	Strytegewoon 1	Strytegewoon 2	Uitkyk	Canema	Harden Heights	Weltevreden
Total Carbon (%)	1.2	2.9	1.7	1.5	2.3	2.5	2.6	2.3
Organic matter (%)	2.1	5.1	2.9	2.6	4.0	4.2	4.5	4.0
Total organic Nitrogen (ppm)	600.0	900.0	300.0	500.0	800.0	800.0	800.0	1000.0
NH_4 (ppm)	5.8	9.3	8.6	8.1	7.5	10.2	8.1	13.1
NO_3 (ppm)	0.9	18.6	7.2	11.0	10.3	27.5	13.5	17.3
Weight of the soil	94.7	84.1	94.7	93.7	90.3	89.7	87.7	91.0
Dry weight (equal to 5g)	4.7	4.2	4.7	4.7	4.5	4.5	4.4	4.6
Microbial biomass (ugC/g soil)	69.6	148.8	73.0	91.4	62.0	118.2	55.0	28.3
Respiration (ugCO2-C/g/day)	3.2	4.1	3.6	4.1	1.9	5.1	5.3	4.4
HWE Carbon (mg/kg)	501.6	656.4	510.0	543.0	615.2	710.1	949.1	655.0
HWE Nitrogen (mg/kg)	175.3	445.0	222.9	247.2	254.8	384.4	458.6	228.5
Clay (%)	57	17	27	31	45	43	27	33
Silt (%)	20	22	10	10	14	10	8	8
Sand (%)	23	61	63	59	41	47	65	59
Bulk density (kg/dm3)	1.9	1.3	1.6	1.6	1.4	1.4	1.5	1.4
pH (water)	5.3	4.6	4.7	5.4	6.3	5.6	5.5	4.7
Ca (meq/L)	0.4	1.5	1.8	1.0	0.9	1.5	1.1	0.7
Mg (meq/L)	0.5	1.2	1.1	0.7	0.9	1.3	0.8	0.6
Na (meq/L)	0.6	0.5	3.2	2.6	0.5	2.6	2.7	2.7
Electrical conductivity (meq/L)	28	57	71	38	41	55	45	59
Saturation (%)	42	127	51	39	65	76	50	87
Sodium acid ratio	1.0	0.4	2.6	2.9	0.5	2.2	2.8	3.3
Cation exchange capacity *	4.1	3.4	2.0	3.7	10.3	4.5	4.6	1.8

HWEC Hot water extractable Carbon
HWEN Hot water extractable Nitrogen
* meq/L (molar equivalent/L)

However, from the correlation circle of the principal component analysis (describing 75% of the variability) of field sites, CFU decline over time and soil characteristics, it was clear that there were also few differences between field sites. With all field sites strongly correlated to the F1 axis (Figure 5.10). There was however an association of field sites along the F1 axis, particularly in terms of some soil characteristics. Field sites in eco-zone 7 (Harden Heights, Canema and Weltevreden) shared higher total carbon (>2.34), higher organic matter (>4.02), higher total organic nitrogen (>800), higher hot water extractable carbon (HWEC) (>615) and lower bulk density (<1.48) and were closely grouped to Uitkyk and Ekukhanyeni which also shared these soil characteristics (Figure 5.10; Table 5.4). Where Cloud Hill and the two

Strytegewoon field sites differed from the former were in lower total carbon, lower organic matter, lower organic nitrogen, lower HWEC and higher bulk densities which leads to smaller pore spaces in the soil (Table 5.4, Figure 5.10).

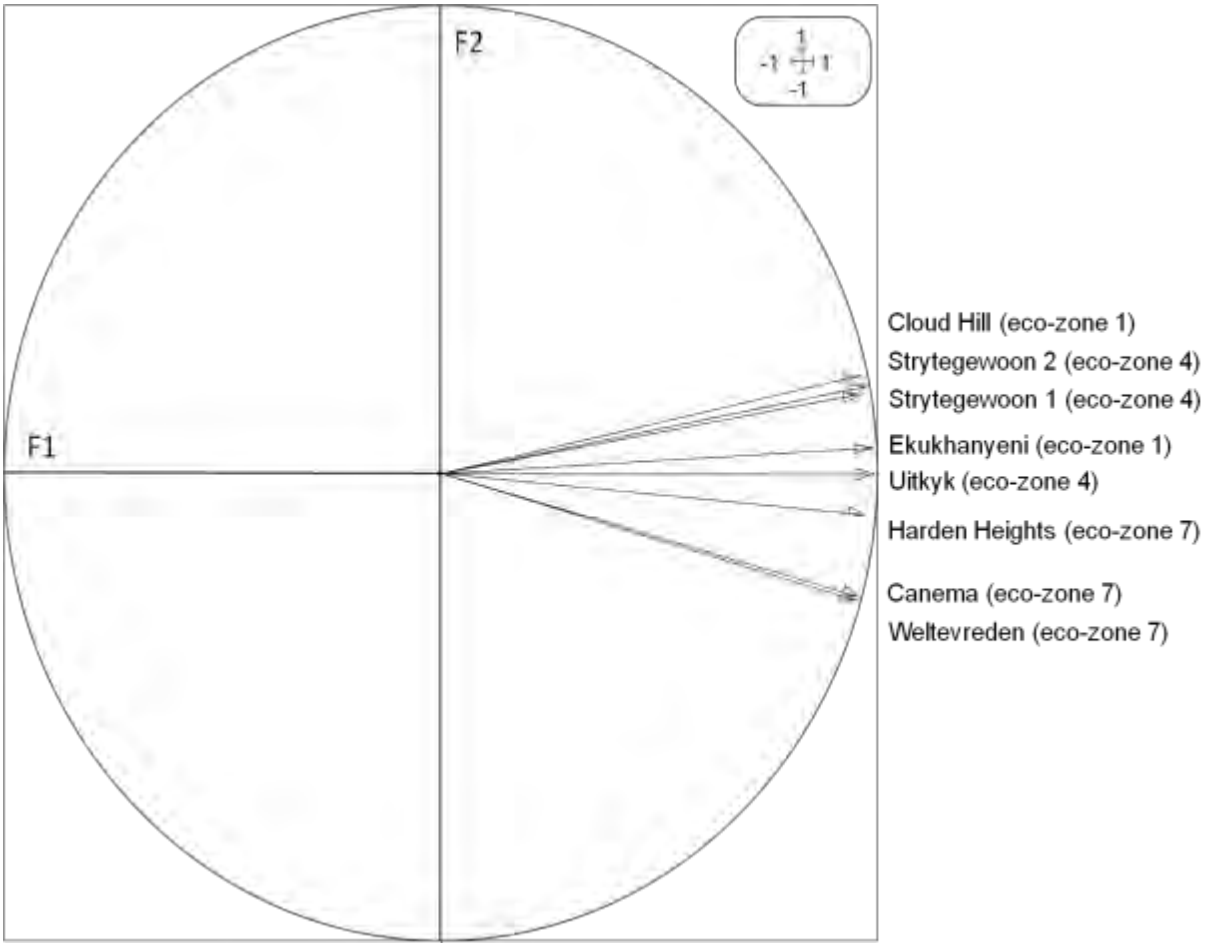

Figure 5.10: Principal component analysis correlation circle of the relationship between the eight field sites, their respective colony forming units over all sampling dates and various soil characteristics listed in Table 5.4.

5.4 DISCUSSION

Fungal persistence in soil is affected by many abiotic and biotic variables. These include soil temperature, soil moisture, soil texture, agricultural inputs, other microorganisms or macro-organisms, formulation, fungal species and genotype. Furthermore, conidia will persist in soils if they are not triggered to germinate by sufficient stimuli (Kessler *et al*. 2003; Jaronski,

2007). Therefore factors that would shorten persistence of conidia, because of conidial germination are optimum soil temperatures, moist conditions driving soil processes, aerobic conditions, lack of fungistasis (microbes reducing the germination potential of fungi) and lack of insect hosts (Milner *et al.* 2003). The rapid decline in *B. bassiana* CFUs in the present study at all field sites may have been the result of all of these factors acting together.

5.4.1 *The effect of soil temperature and moisture*

The effect of temperature on fungal persistence in soil is often complicated by moisture effects as well as biotic influences, and an inverse relationship exists between fungal persistence and soil temperature (Jaronski, 2007). Low soil temperatures and high soil water content values were shown to have a negative effect on the number of *B. bassiana* CFUs in the present study. On Sabouraud-dextrose-agar, *B. bassiana* isolate 4222 is able to grow successfully between 5°C and 33°C, with an optimum vegetative growth temperature of 27°C (Goble, unpublished data). In the present study, conidia were buried for the first time in spring (September) when mean soil temperatures at all field sites were 21°C. This was sufficiently warm enough to induce germination of conidia but was below the optimum vegetative growth temperature of isolate 4222 (27°C) and that reported for most entomopathogenic fungi (25-28°C) (Jaronski, 2007). Thus, temperature would have had a direct influence on the growth of *B. bassiana,* but because this growth was slow, it may have also reduced the sporulation potential or competitive ability of the fungus against other antagonistic microorganisms. Kessler *et al.* (2003) reported that suboptimum soil temperature was a major factor affecting the success or failure in the production and establishment of *B. brongniartii* inoculum for control of *M. melolontha* in Switzerland. Sub-optimum temperature effects may have been further compounded by higher-ranging soil water content values which were recorded at the sites within the first three months of burial.

Increased soil water content due to increased rainfall is known to potentially water-log soils, which reduces the amount of available oxygen in pore spaces in a heavy soil (Kessler *et al.* 2003). In the present study, annual spring/summer rains were experienced at all sites however, it is unlikely that water logging would have occurred because the high organic matter content of soils in the KZN Midlands North would have improved penetration and water holding capacity. The only site which may have experienced any water logging effects would have been Cloud Hill (the site where persistence was worst) because of the high clay

content and lowered amounts of organic matter (Jaronski, 2007). However, water logged soils would have a positive effect on persistence because a lack of oxygen would reduce the ability of conidia to germinate (Jaronski, 2007). Increased persistence however was not observed at Cloud Hill. Kessler *et al*. (2003) reported that soil water content and rainfall did not have a major influence in the growth and germination of *B. brongniartii* conidia. However, the best predicted model in the present study demonstrated that increased rainfall, soil water content, soil temperature and solar radiation were significant variables in the decline of *B. bassiana* CFUs. Further, when solar radiation was removed as a parameter variable in the second generated model, soil temperature and soil water content were important predictors of the decline in CFUs in the present study. This is consistent with literature, which shows that increased soil temperature and soil moisture are important determinants in the growth and sporulation of fungi which would decrease the number of CFUs (Jaronski, 2007). Further, the rate of organic matter decomposition is increased by these climatic variables, particularly soil moisture, which makes more labile fractions of soil organic matter available to microbes, and drives microbial community composition and diversity in the soil (Curiel Yuste *et al.* 2007; Ryan *et al*. 2009). Increased amounts of labile fractions of organic matter in the soil therefore increases competition between microbes and it is known that *B. bassiana* is a poor competitor for organic resources compared to opportunistic saprophytic fungi that are ubiquitous in soils (Keller and Zimmerman, 1989; Hajek, 1997). Therefore, climatic conditions would have allowed for the germination of *B. bassiana* conidia. However sub-optimum growth temperatures and the poor competitive ability of *B. bassiana* in the soil would have limited persistence of conidia and may explain the rapid decline in CFUs observed in this study.

5.4.2 *Agricultural inputs and organic matter*

All field sites in the present study had relatively high amounts of organic matter (> 2%) and microbial biomass (Hofman *et al.* 2004; Ryan *et al.* 2009). Organic amendments such a chicken litter, are favoured by sugarcane growers in the KZN Midlands North, and inorganic amendments such as fertilizers are applied annually. The fact that almost all field sites were either plant sugarcane or in an early ratoon meant that organic and/or inorganic amendments would have been particularly high, as it is common practice for growers to apply such

amendments to younger sugarcane fields [17](Rhodes, pers. comm.). The effects of these amendments were seen at Ekukhanyeni where *B. bassiana* persistence was greatest. This site had the highest organic nitrogen, highest plant and microorganism available NO_3 and highest NH_4. These cations are known to bind to organic matter in the soil (Mengel *et al*. 2001) which was also very high at this site (5.06%). Most of the other field sites were also relatively high in these cations but Cloud Hill was the lowest (worst persistence). Some authors report that increased amounts of organic matter can increase the occurrence of other antagonistic microorganisms in the soil which in turn can suppress entomopathogenic fungi which leads to greater persistence because conidia cannot germinate, or if they do germinate, sufficient sporulation may never be achieved (Lingg and Donaldson, 1981; Studdert and Kaya, 1990; Fargues and Robert, 1985; Keller and Zimmerman, 1989). For example, Lingg and Donaldson (1981) found that ammonium tartrate added to sterilized soils increased the numbers of *B. bassiana* but in amended non-sterile soils no *B. bassiana* was recovered apparently because of antagonistic effects from the fungus, *Penicillium urticae* (Ascomycota: Trichocomaceae). It is likely that the Ekukhanyeni site had the best conidial persistence, as reflected by CFUs, because it was highest in organic matter and microbial biomass and thus fungistasis would have suppressed germination of conidia. At Cloud Hill, organic matter and microbial biomass were low therefore persistence would have been worse because conidia were able to germinate.

Quesada-Moraga *et al.* (2007) however found that higher occurrences of *B. bassiana* and *M. anisopliae* were associated with soils with high organic matter content which was attributed to higher cation exchange capacities, with greater organic matter enhancing adsorption of fungal conidia. Further, many authors have reported greater occurrences of *B. bassiana* in organically managed soils than conventionally managed soils (Klingen *et al*. 2002; Goble *et al*. 2010), or *B. bassiana* to occur more frequently in natural habitats, which were higher in organic matter (Mietkiewski *et al*. 1997; Meyling *et al.* 2006). The most important consideration of all these studies however, was that the presence of insect hosts was discussed. *Beauveria bassiana* was thought to occur more frequently in areas with higher organic matter or in organically managed soils because amendments such as compost teas, manure or organic fertilizers, increased carbon loads in these soils which were favourable for soil inhabiting insects, which were potential hosts for *B. bassiana* (Keller and Zimmermann,

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1989). Furthermore, the diversity of natural habitats meant a greater diversity of insect hosts which should be considered the primary source of carbon for entomopathogenic fungi (Meyling *et al.* 2006). In the present study, the decline in isolate 4222 CFUs may have also been related to limited contact with insect hosts. Surveys at four of the field sites showed the presence of insect hosts, particularly scarabaeid life stages (Goble, unpublished data). Scarabaeid life stages however, in the first three months after burial were either eggs or first and second instar larvae which feed close to the soil surface on organic matter or sugarcane rootlets (see Chapter 2). The net bags which enclosed the soil samples and formulated *B. bassiana* conidia would have greatly reduced insect contact because insects may not have been able to penetrate the net bags. Further, *B. bassiana* isolate 4222 is not a host-specific fungus like strains of *B. brongniartii* (Kessler *et al*. 2003) which are particularly host-specific towards scarabaeids. So even if some insect contact had been made with formulated *B. bassiana* conidia, the fungal genotype may not have been specific enough to kill the insect hosts. These considerations bring into question the importance of fungal species and genotype in the persistence of entomopathogenic fungi.

5.4.3 *Fungal species and genotype*

The persistence of various species of entomopathogenic fungi can vary considerably in the soil (Klingen and Haukeland, 2001). Milner *et al.* (2003) for example, found that a small proportion of formulated *M. anisopliae* conidia persisted for 3.5 years at various sites in Australia. Similarly, Vänninen *et al*. (2000) found that three years post application there were still enough infectious *M. anisopliae* conidia in a clay soil, peat and two types of sandy soil to induce infections in 80% of the meal worm, *T. molitor* used as soil bait insects in Finland. However, Li and Holdom (1993) investigated the persistence of *M. anisopliae* by mixing conidia into air-dried soil and then incubating the soil at 25°C at various moistures in clay and sandy soil. In all cases, the conidia showed a rapid decline with only 10-40% surviving to the end of the experiment at 160 days (~5 months).

Vänninen *et al*. (2000) also evaluated the persistence of unformulated *B. bassiana* conidia in a clay soil, peat and two types of sandy soil and found that most of the augmented conidia disappeared during the first winter after application in the clay and one sandy soil. However, some *B. bassiana* conidia persistence was evident in the peat and other sandy soil one-year post application (Vänninen *et al*. 2000). Poorer persistence of *B. bassiana* in different soil

types, particularly agricultural soils, compared to *M. anisopliae* is known (Vänninen *et al*. 2000; Quesada-Moraga *et al.* 2007). It has been suggested that this is because *M. anisopliae* conidia can persist longer without repeated infection of hosts (Fargues and Robert, 1985; Vänninen, 1996) and is more pesticide tolerant (Quesada-Moraga *et al.* 2007). Further, it has been suggested that *B. bassiana* has a lower competitive ability compared to *M. anisopliae* (Bidochka *et al.* 1998) but several studies confirm that *B. bassiana* can tolerate a wider range of climatic conditions while *M. anisopliae* is more thermophilic (Klingen and Haukeland, 2001). The rapid decline of *B. bassiana* CFUs in the present study therefore is not surprising when we consider the above mentioned studies and knowledge of the persistence of various fungal species. Future studies on fungal formulation and longevity should consider that depending on isolate, *B. bassiana* may be less persistent and alternative fungal species should be considered.

Long-term persistence of *B. brongniartii* for up to 40 years is reported in the literature (Keller *et al.* 2003). In another field study, soil inoculation of different *B. brongniartii* strains showed that all strains were detected at all test sites for up to 14 years after the application (Enkerli *et al.* 2004). These studies suggest that a mycoinsecticide based on *B. brongniartii* may be more persistent than one based on *B. bassiana*. However, because of the host specific nature of *B. brongniartii*, under natural field conditions the fungus is a poor competitor and is suppressed naturally by other microorganisms in the soil (Strasser *et al.* 2000). In the absence of insect hosts, *B. brongniartii* is known to decline rapidly (Kessler *et al.* 2004). The survival of *B. brongniartii* as a biological control agent against *M. melolontha* was evaluated 16 months after application of 40-50 kg per ha of product which was tilled into different soil types in Switzerland. In the absence of the host the number of CFUs declined by as much as 90% but where the host was present the conidia survived significantly longer (Kessler *et al*. 2004). Thus, persistence studies based on this fungal species should be undertaken to evaluate survival under various soil conditions for further mycoinsecticide development in South Africa. Particularly, in light of the acquisition and host specificity of *B. brongniartii* strains which have been discussed in Chapters 3 and 4.

The rapid decline of *B. bassiana* isolate 4222 CFUs at all sites also brings into question the importance of fungal genotype. *Beauveria bassiana* isolate 4222 was isolated from a mycosed scarabaeid pupa during a field epizootic in the KZN Midlands North in 2005 (Hatting, 2008). It was assumed that this isolate was able to infect scarabaeids and was

considered cold tolerant as it was isolated from an area which has average cooler temperatures compared to the rest of the KwaZulu-Natal Province and could grow successfully at 5°C (Goble, unpublished data). During exploratory growth studies it was found that isolate 4222 had a good vegetative growth rate but sporulation was sub-optimum (Goble, unpublished data). Since its isolation seven years ago it has remained in repeated sub-culture only being rejuvenated by passing through an insect host on a few occasions. *Beauveria bassiana* is known to have a clonal population structure which can create a wide variety of phenotypic characteristics (Devi, 2006). For example, host specificity, morphology, growth rate, temperature optimum, sporulation potential, UV resistance and responses to abiotic and biotic soil factors could have changed over time in sub-culture for *B. bassiana* isolate 4222. Thus when it was formulated on rice and bran in 2010 it may have been genetically weaker and unable to with stand the environmentally competitive nature of the soil.

5.4.4 *Formulations*

The persistence of *B. bassiana* isolate 4222 formulated on rice and bran, as nutritive carriers were not significantly different and exponential decline in viable CFUs was observed with both carriers at all sites. This was similar to Milner's *et al.* (2003) study, which showed that formulation did not significantly affect the persistence of *M. anisopliae* formulated as rice granules (BioCane™), as a wettable powder derived from conidia screened from rice granules, as conidia off rice suspended in water and as conidia produced on agar plates, dried, and then mixed with water for adding to soil and buried in Australian commercial sugarcane fields. Previous studies have suggested that conidia formulated on rice are less persistent than conidia formulated as a clay powder (Milner *et al.* 2003). According to Jaronski (2007), there seems to be a consensus regarding entomopathogenic fungi formulated on granules in that soil fungistasis does not seem to operate on these carriers. This is because granules provide nutrients for entomopathogenic fungi which display rapid growth and sporulation. Subsequently, fungi produce antimicrobial compounds to keep soil saprophytes at bay (Jaronski, 2007). However, what have not been considered is the rapid breakdown of nutritive carriers like rice and bran under extremely moist conditions and the longevity of suppressed soil fungistasis under such circumstances. In the present study, nutritive carriers did initially supported rapid increases in conidial titers due to fungi growing and utilizing carbon on the rice or bran, as observed by time zero CFU counts. However, summer rainfall and increased

soil water content which occurred at all sites (which is also known to accelerate organic matter decomposition) (Ryan *et al.* 2009) would almost certainly affected rice or bran granules in the same way, rapidly degrading them too. An example of this was discussed by Ekesi *et al.* (2005) who studied the persistence and infectivity of aqueous, oil/aqueous (50:50) and granular formulations of *M. anisopliae* against pupating larvae of three fruit fly species (Diptera: Tephritidae) under field conditions in Kenya. The number of CFUs were determined by soil dilution and plating onto selective medium (Ekesi *et al.* 2005). There were no differences in soil persistence between the formulations for one year, at which point conidial levels for the two liquid formulations decreased about three orders of magnitude while conidial levels for the granular formulation decreased by 90%. This drop in CFUs coincided with a one-month period of rainfall (Ekesi *et al.* 2005).

Further, the combination of *B. bassiana* biomass increase and thus rapid utilization of more carbon from a nutritive granule would also lead to quicker decay of the granule. Nutritive carriers may also attract macro-organisms such as earthworms, mites or collembolans (Jaronski, 2007). Some field collected net bags had small holes in them and appeared to have been chewed through when they were destructively sampled in the laboratory indicating that some macro-organism activity had taken place at some sites. However, literature suggests that the effect of carrier and formulation does not greatly affect fungal conidial persistence but only the sporulation potential and distribution of conidia in the soil (Milner *et al*. 2003; Ekesi *et al.* 2005; Jaronski, 2007). *Metarhizium anisopliae* and *B. bassiana* sporulate better when the substrate is relatively poor in nutrient content. In a study by Nelson *et al.* (2006), when grains were supplemented with sugars and yeast additives, less conidia per gram of substrate was obtained than with grains alone. In Brazil, using rice bran/rice husk substrate mixtures, *M. anisopliae* has been found to produce conidial yields of 5–15 times higher than yields usually obtained for rice grains alone (Dorta *et al.* 1990). Thus, it is suggested that either rice/ bran mixtures be used as carriers for field trails but perhaps it would be prudent to select the carrier which is more cost effective in South Africa.

5.5 CONCLUSIONS

The conidia decline rates in the present study suggest that the persistence of a *B. bassiana* isolate 4222 granular mycoinsecticide is poor and would have to be applied regularly to be

effective. While this study has tried to elucidate some of the critical factors responsible for conidia decline at the eight field sites it remains difficult to point out any one particular driving element. Rainfall can play a critical role in degradation of nutritive carriers which provide *B. bassiana* with a competitive advantage over other soil microbes for a limited time by providing the initial carbon source for growth and sporulation. Seasonality, the subsequent increases in organic matter decay rates and microbial biomass which ultimately leads to competition and fungistasis in the soil have a great effect of *B. bassiana* conidial persistence. It appears that sites higher in organic matter, like those in the KZN Midlands North may be suppressive to the germination of *B. bassiana* which increases persistence. Insect hosts remain an important source of carbon for the buildup of conidia in the soil and their specialization in being able to infect insects remains their ecological niche. Fungal genotype will also determine the extent to which *B. bassiana* is able to infect a host and overcome environmental obstacles such as growth, sporulation and competition in the presence of other microorganisms. Because isolates of *B. brongniartii* have subsequently been discovered, which are more virulent towards white grubs than *B. bassiana* isolate 4222, it is suggested that these be investigated for persistence using the lessons learnt from this study.

CHAPTER 6 - Growers' perceptions of white grubs and the implementation of a mycoinsecticide in the KZN Midlands regions

6.1 INTRODUCTION

'Integrated Pest Management (IPM) is considered a farmers' best combination of control practices that are biologically, environmentally, economically, socially and culturally suited for that specific agro-ecosystem' (Litsinger *et al.* 2010 pg 119). The development of IPM was initiated by scientists, but the implementation of IPM is often carried out by farmers. The classical mode of 'top-down' recommendations for pest control, transferred from researchers via extension officers, has frequently failed at the farm level because researchers have been insufficiently aware of farmers' real problems and perceptions (Williamson, 1998). Furthermore, scientists and extension officers tend to use scientific terminology and information-laden approaches with farmers, and fail to recognise that experimental learning is the best way to change attitudes and farming practices (Williamson, 1998). This author reports that by involving the end-user in the planning, design and testing of the technology it often results in a much higher chance that farmers, as end-users, will buy or adopt the new technology.

Specifically, in biological control agent (BCA) research and development, researchers are mainly focused on the efficacy of these organisms against target pests with very little regard for the socio-economic environment in which they are applied and marketed (Moser *et al.* 2008). It is suggested that a market-orientated approach is an important prerequisite for the design of any new product and technology because it allows researchers the opportunity to understand the consumer, their expectations and behaviours, and identify problems or needs (Moser *et al.* 2008). This is critical because the degree of adoption of any new IPM strategy is based on farmers' knowledge and perception of pests and how those pests affect yield, their familiarity with the presented control techniques, and the interactions and cost-benefit of the various control tactics (Litsinger *et al.* 2010). Moser *et al.* (2008), when evaluating farmers' perceptions of BCAs in various strawberry production systems in Europe and Israel, found that growers were more aware of the positive aspects of BCAs than the negative ones because of extensive media coverage and word-of-mouth. Furthermore, hands on experience and suggestions made by growers associations were the next most mentioned factors influencing

growers' confidence in BCAs. Consumer-orientated production, the presence of grower associations, well-planned pest control programmes and an organised network that publicizes results of experimental trials are factors which may increase growers' knowledge of BCAs and their inclusion into IPM programmes (Bailey *et al.* 2010; Leng *et al.* 2011). Differences in growers' attitudes are related to the agricultural systems in which they operate. High value crops which are exported are more likely to have higher production input costs and the use of biological control agents (BCAs) is more likely (Moser *et al*. 2008).

There is a rising demand for bio-pesticides in agriculture which is in part due to increased application of IPM and organic farming practices (Bailey *et al*. 2010). North America has the largest percentage (44%) of the bio-pesticide market, followed by the European Union and Oceania (20% each), South American countries (10%), Asia (6%) and India (6%) (Bailey *et al*. 2010). In Africa, there is a long history of microbial control but these control approaches have rarely been developed into commercial products (Langewald *et al.* 2003). There are three features which differentiate bio-pesticides from conventional pesticides: 1.) Bio-pesticides can be used to manage insect resistance because many have different modes of action which reduce the chance of resistance occurring; 2.) bio-pesticides have low or no post harvest restriction entry levels; 3.) bio-pesticides have no maximum residue levels because they are considered safe, which has large implications for export markets, especially to the European Union (Faria *et al*. 2010). Despite these attributed features, the current rate of bio-pesticide adoption is low (Bailey *et al*. 2010). In the United States 30% of fruit farms, 20% of cotton farms, 10% of vegetable farms and less than 5% of grain crops and potatoes applied bio-pesticides in 2005 (Faria *et al*. 2010). In Canada, less than 2% of growers use bio-pesticides (Faria *et al*. 2010). The level of adoption may be a reflection of product availability because a recent survey indicated that biological control of weeds in field crops ranked 4 of 5 in importance by growers and needed more research (Faria *et al*. 2010). Bio-pesticides for field crops ranked 3.5 on the same scale indicating an interest in biological technologies by growers, but a lack of available products for specific pests (Faria *et al*. 2010). Bio-pesticide growth is projected at 10% annually in the United States but it is unpredictable in different areas and constrained by factors such as regulators, public and political attitudes, and limitations for market expansion (Bailey *et al*. 2010). It is this last sentiment which has captured the interest of this study because adoption of a mycoinsecticide in South African sugarcane will depend greatly on growers' attitudes towards white grub pests and their perceived need for a product to control such pests. Litsinger *et al.* (2010) importantly

suggested that farmers' perception surveys needed to precede applied scientific research to ensure that this research does not become another academic exercise.

Therefore the aims of this chapter were: 1.) To understand growers' general knowledge of white grubs, their biology and ecology; 2.) To understand growers' knowledge of white grub control and determine which control strategies were already in place; 3.) To determine growers' knowledge of mycoinsecticides and the feasibility of their implementation in sugarcane cropping systems.

6.2 METHODS AND MATERIALS

6.2.1 *Dissemination of the survey questionnaire*

Over 100 sugarcane growers from the Midlands North and Midlands South regions (Figure 1.5 in Chapter 1) of the KwaZulu-Natal sugarcane-growing belt were sent electronic survey questionnaires. Copies of the survey questionnaire were sent to extension managers at the Local Pest, Disease and Variety Control Committee (LPD&VCC) in Eston (Midlands South) and Wartburg (Midlands North) for dissemination by email. Growers in both regions were asked to complete the questionnaire and either scan and email them back or they were able to drop the questionnaire off at the LPD&VCC offices for our collection at a later date.

6.2.2 *Survey method and statistical analysis*

The survey was conducted by means of a self-administered, semi-structured questionnaire during March-April 2010. This is a quantitative survey method and is similar to the mail survey method described by Peshin *et al.* (2009) for evaluating IPM programmes and farmers' knowledge and perceptions. The main advantage of a mail survey is that it is less expensive and more time-efficient than a face-to-face interview (Peshin *et al.* 2009). The questionnaire planning and design followed guidelines for survey quality and ethical research from Fink (2009) and Babbie (2010), and assured farmers of the privacy of their information. The questionnaire included open-ended and closed questions and likert-type scale questions (i.e. do not agree, agree, strongly agree) (Fink, 2009) and was structured into five sections.

The first section provided personal information on farm size, farming area, age of the farmer, number of years farming and highest level of education. The second section examined growers' knowledge of white grubs, especially in terms of the occurrence and estimation of

damage caused by the pest. The third section dealt with growers' knowledge of white grub control and included knowledge about insecticides. Section four, determined growers' knowledge of mycoinsecticides, their application, and how much they would expect to pay for a product. The final section (section 5) dealt with growers perceptions on the feasibility of mycoinsecticide implementation. Thoughts about the feasibility of applying a fungal based mycoinsecticide with fertilizers on farms generated questions such as how often did growers apply fertilizers and how often did they ratoon their sugarcane fields.

For purposes of data analysis, responses to closed-questions were coded prior to data analysis. A content analysis was completed on open-ended questions to identify recurrent themes which could be quantified to determine farmers' perceptions (Fink, 2009). All categorical data were analyzed by means of contingency tables to show the effect of different groups or characteristics on farmers' responses. Chi-square tests were used to determine whether grouping effects identified using contingency tables were statistically significant (Babbie, 2010). Descriptive statistics such as frequencies and percentages were used for visual analysis and presentation of data in the form of tables and bar graphs.

6.3 RESULTS

6.3.1 *Personal and farm characteristics*

A total of 51 respondents from both regions completed the questionnaire. There were 17 respondents from the Midlands South region, made up 8 respondents from Eston, Mid-Illovo (3 respondents), Richmond (3), Thornville (2) and Ixopo (1) (Figure 6.1).

In the Midlands North region there were 34 respondents from across eco-zones which included: Bruins Hill (1 respondent), Cramond (3), Dalton (7), Fawn Leas (2), Glenside (2), Greytown (1), Harburg (3), Mount Ellis (1), New Hanover (2), Ottis Hill (1), Pietermaritzburg (1), Seven Oaks (3), Table Mountain (1) and Wartburg (6) (Figure 6.2). The Midlands North region is divided into eleven eco-zones (Figure 6.2). The eco-zone divisions are based on an environmental management system (Maher and Schulz, 2003). Eco-zones are areas of similar climate and similar sugarcane production potential and are derived from the Department of Agriculture's Bio-Resource Units (BRU) (Webster *et al.* 2005, 2009).

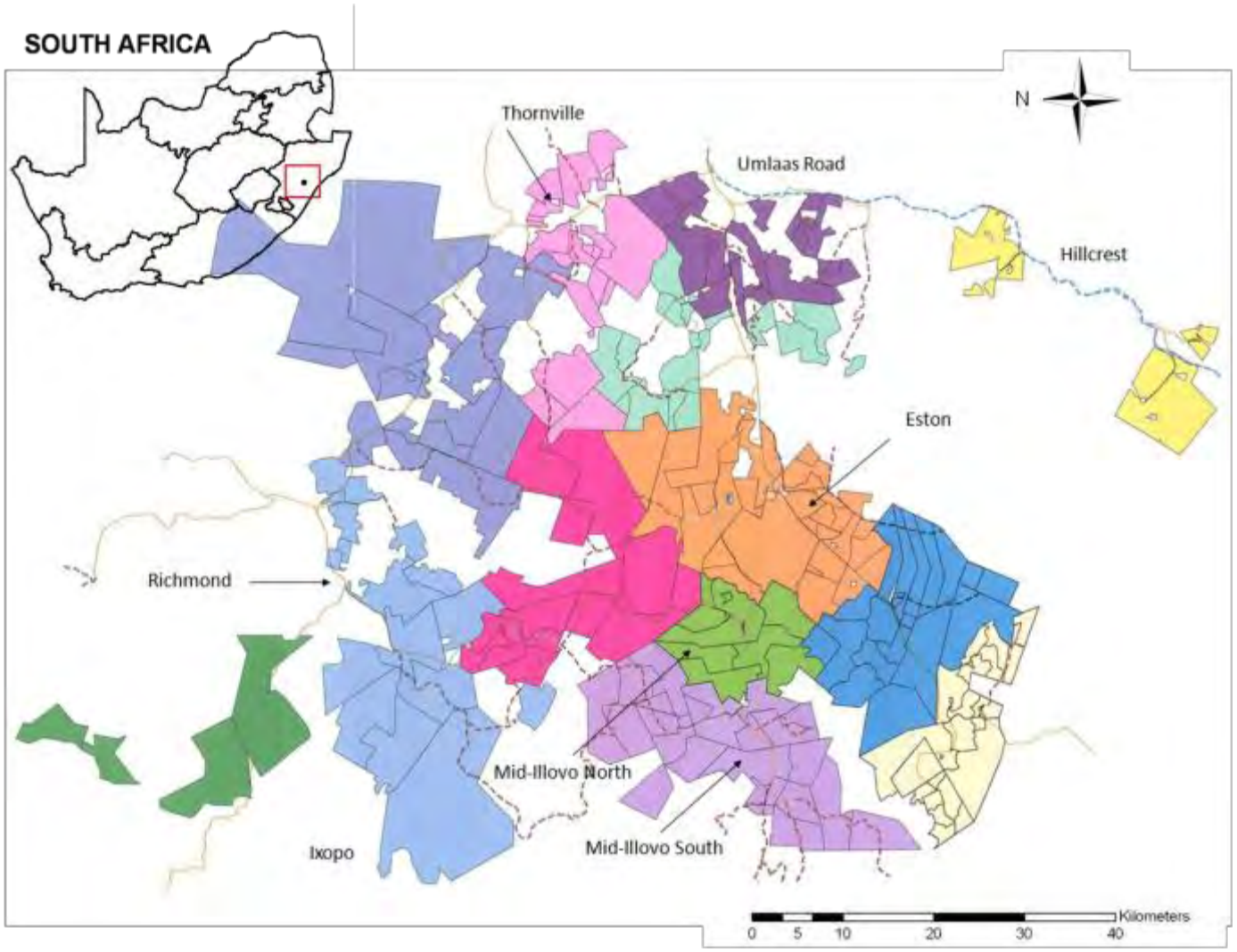

Figure 6.1: The Midlands South sugarcane producing region, showing the different growing areas shaded in different colours.

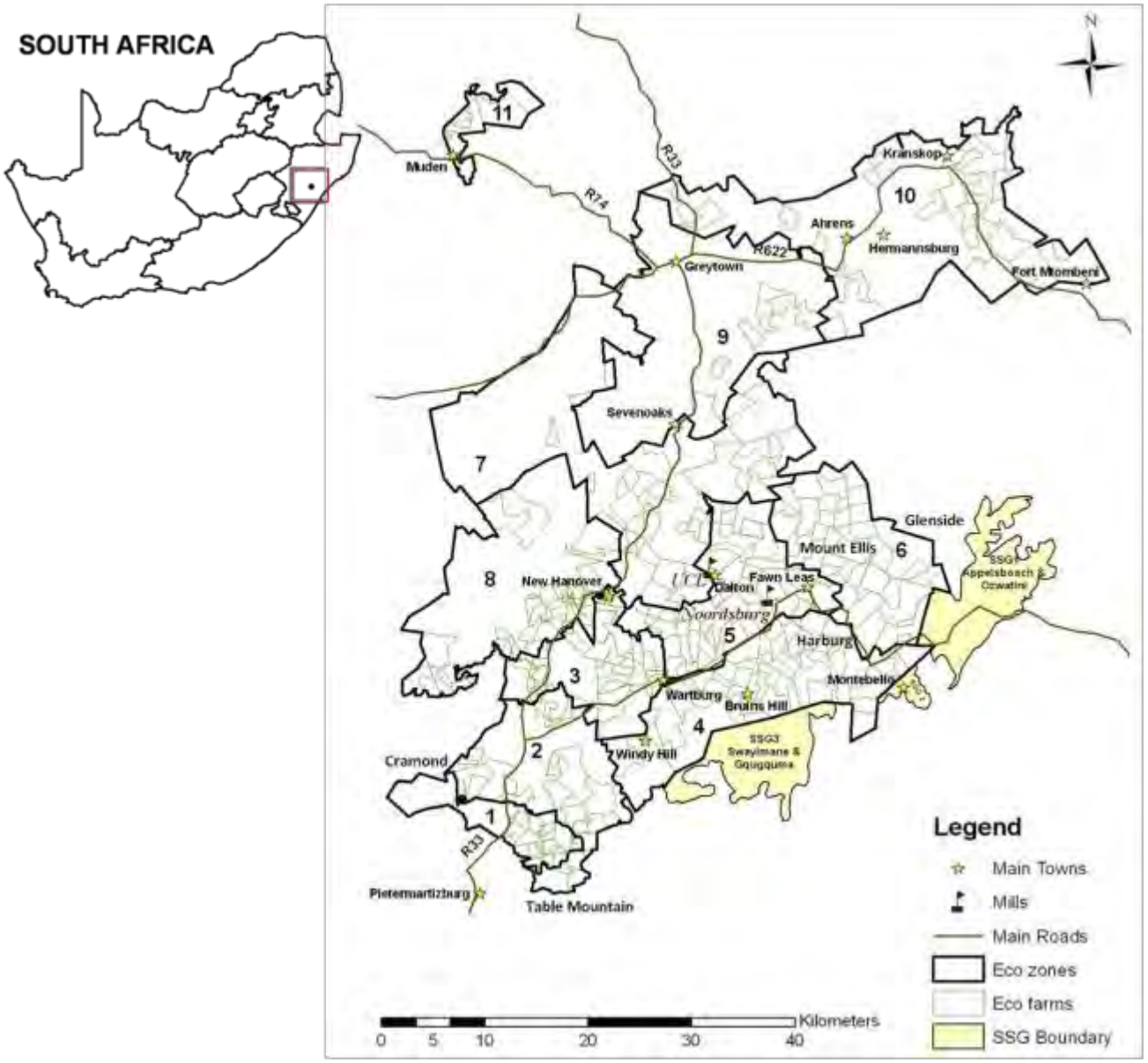

Figure 6.2: The Midlands North sugarcane producing region, showing the different eco-zone boundaries.

All 51 respondents were male (100%). Furthermore, most sugarcane growers (88.2% in the Midlands South and 55.9% in the Midlands North) owned farms that were 500 ha or smaller in size. A smaller percentage of growers in both regions owned farms larger than 500 ha (Table 6.1). In the Midlands North region, the largest proportion (36.4%) of growers were between 51-60 years old while in the Midlands South region the largest proportion (31.2%) were younger and ranged from 41-50 years old (Table 6.1). In both regions the largest proportion of growers had between 21-30 years farming experience (Table 6.1).

In terms of growers' education, both regions had a large proportion (68.8% in Midlands South and 76.5% in Midlands North) of respondents with tertiary education (Table 6.1).

Table 6.1: Personal information and farm characteristics of sugarcane growers in the Midlands North and South regions of KwaZulu-Natal.

	Midlands South	**Midlands North**
farm size (ha)	(% values)	(% values)
1-500	88.2	55.9
501-1000	5.9	29.4
1001-2000	5.9	0.0
2001-4000	0.0	14.7
age of growers (years)		
20-30	6.3	15.2
31-40	12.5	12.1
41-50	31.2	30.3
51-60	25.0	36.4
61+	25.0	6.0
number of years farming		
1-10	5.9	26.5
11-20	29.4	26.5
21-30	35.2	29.4
31+	29.5	17.6
education level		
none	0.0	0.0
primary	0.0	0.0
secondary	31.3	8.8
tertiary	68.8	76.5
other	0.0	14.7

6.3.2 *Knowledge of white grubs*

Just over half of the growers (56%) in the Midlands North had a history of white grub on their farms, while 35% indicated no history. Nine percent said they did not know if there was a history. Twenty-nine percent of growers in the Midlands North thought that white grub numbers were exacerbated by timber particularly black wattle, *A. mearnsii* in the region

(Figure 6.3). Conversely, 53% of growers in the Midlands South indicated no history of white grub on their farms. Of those Midlands South growers with a history of white grubs (24%), only 6% thought this was exacerbated by timber grown close by (Figure 6.3). Most growers in both regions: Midlands North (53%) and South (65%) however did not know if timber exacerbated the white grub problem (Figure 6.3). There was a significant response difference from Midlands North and South growers when they were asked to comment on white grub history and whether timber exacerbated this (χ^2=42.2; df=5; $P\leq$0.001), with more Midlands North growers experiencing a history of white grub damage and understanding that timber exacerbated the problem.

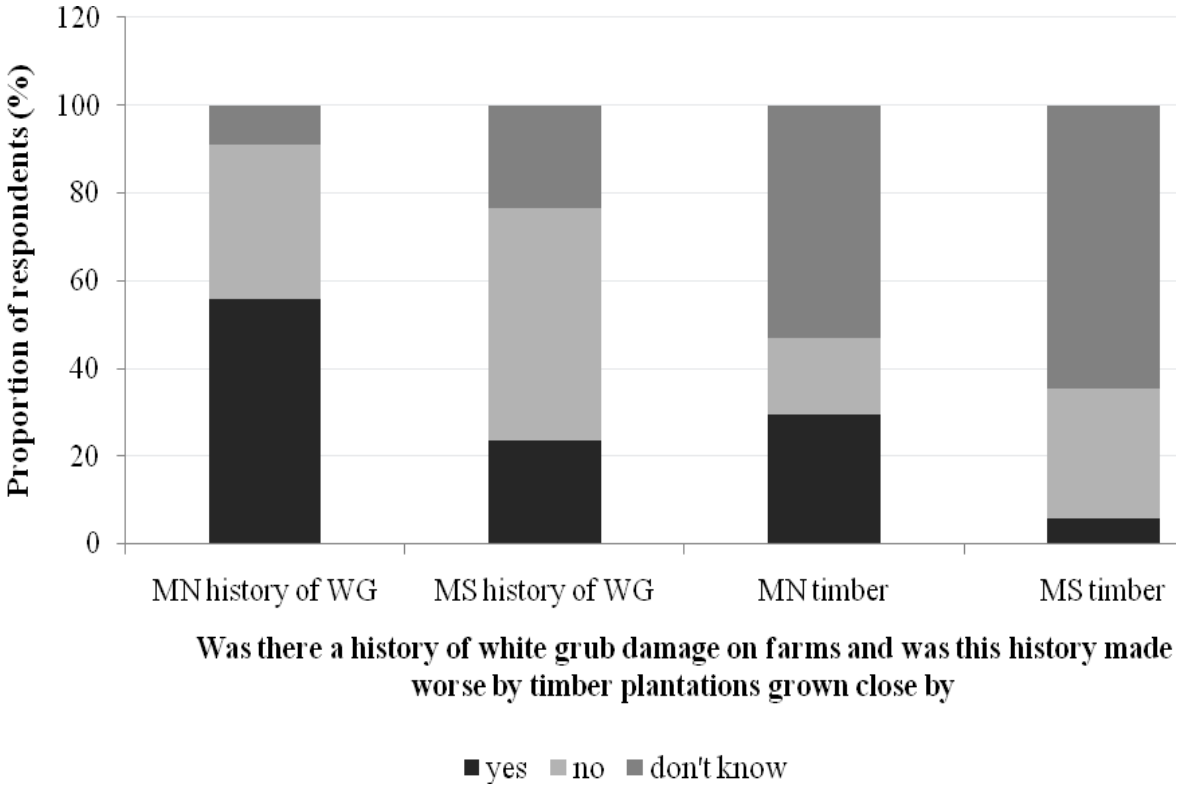

Figure 6.3: The proportion of respondents from the Midlands North (MN) and Midlands South (MS) who thought they had a white grub (WG) history on their farms and whether this was made worse by the fact that *A. mearnsii* (timber) was grown close by.

In the Midlands North, most growers (74%) recognised white grub damage in sugarcane, while in the Midlands South only 35% of growers could recognise white grub damage (Figure 6.4). Just over half (56%) of the growers in the Midlands North indicated that white grubs affected their final yields, while only 12% of Midlands South growers commented that white grubs affected their final yields (Figure 6.4). These difference between the two regions in terms of whether growers could recognise white grub damage and whether white grubs affected their final yields, were significant (χ^2=29.2; df=6; P≤0.001). Damage caused by white grubs was better recognised by Midlands North growers (Figure 6.4).

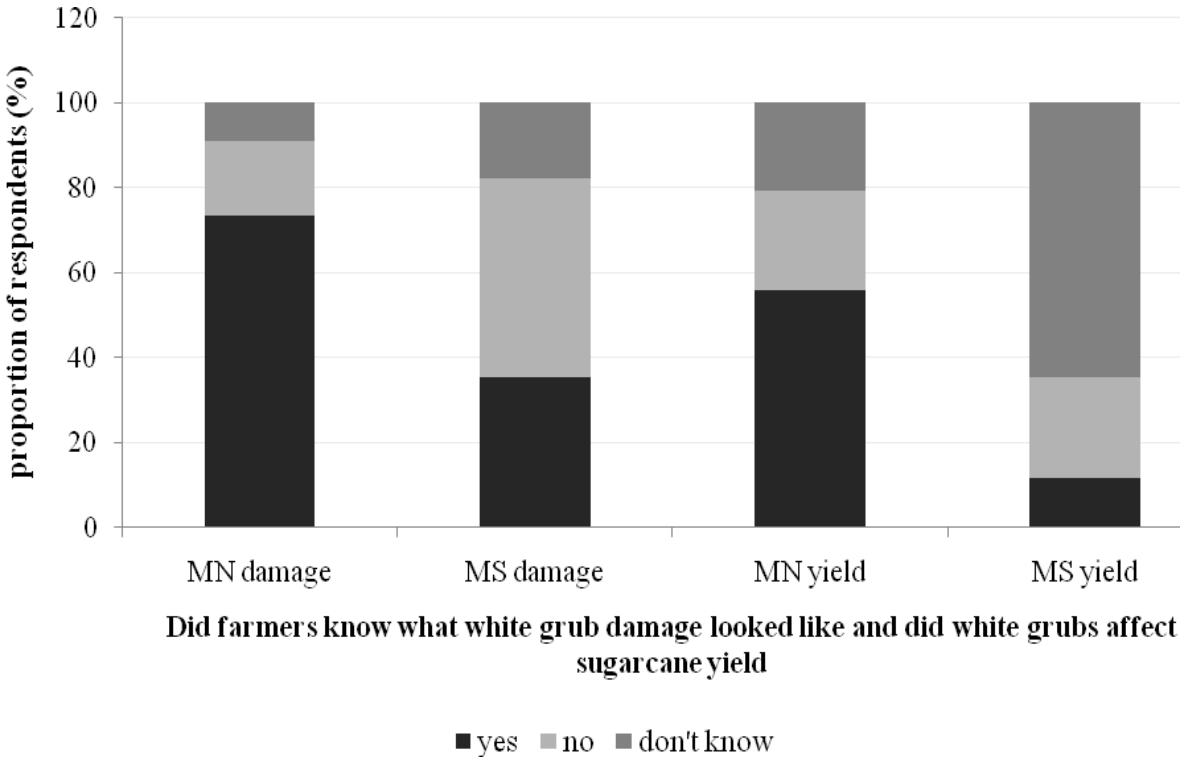

Figure 6.4: The proportion of respondents from the Midlands North (MN) and Midlands South (MS) who knew what white grub damage looked like in sugarcane and whether white grubs affected their final yields.

However, when growers were asked to estimate the percent damage caused by white grubs on their farms, 53% of the Midlands North growers said only 1-10% damage was incurred and 35.2% did not know (Figure 6.5). In contrast, 76.4% of Midlands South growers did not know what percentage of their final yield was affected by white grubs, while 24% of them

estimated 1-10% damage was incurred (Figure 6.5). When respondents were grouped according to number of years farming (experience), most relatively inexperienced growers gave a range of estimated white grub damage levels. While the most experienced growers either indicated very little damage (1-10%) was incurred or they did not know (Figure 6.5). There was no significant correlation between growers experience and the estimated white grub damage caused to sugarcane (χ^2=21.2; df=15; P≤0.130).

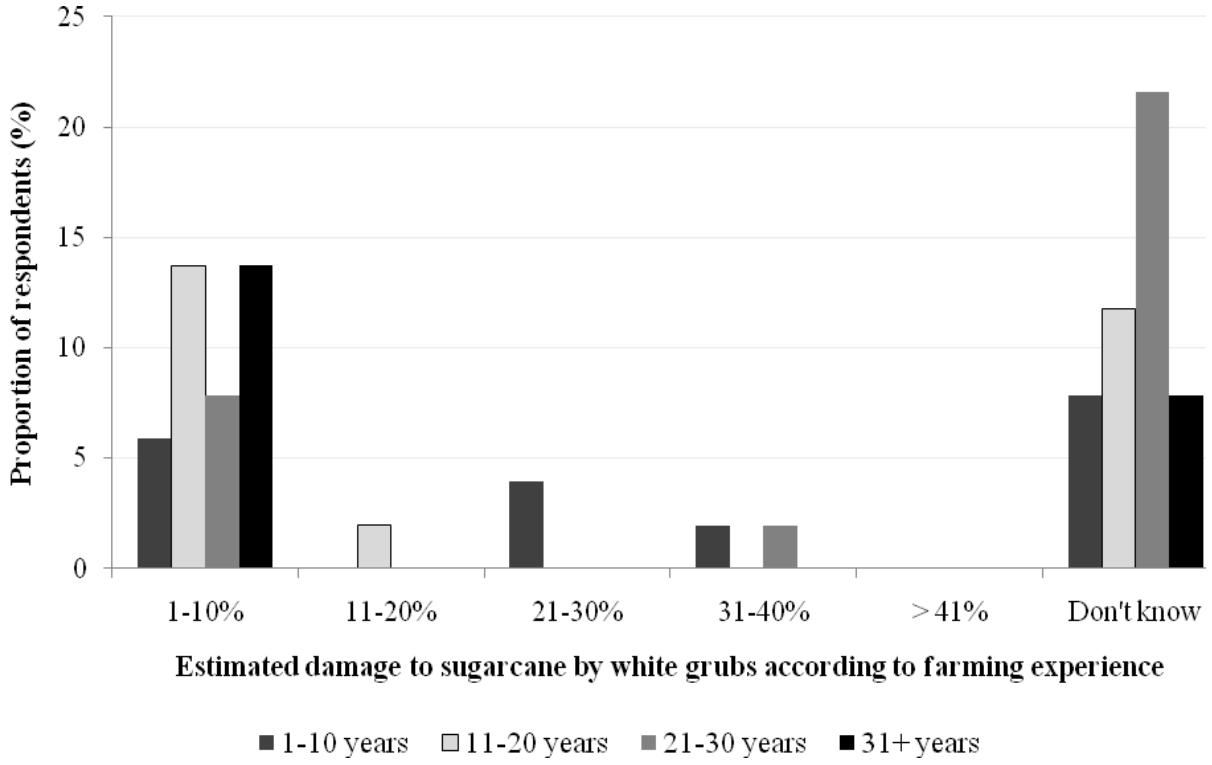

Figure 6.5: The proportion of respondents, grouped according to growers experience (years of farming), who estimated the percentage damage caused by white grubs.

6.3.3 *Knowledge of white grub control*

Eighty two percent (82%) of all respondents, from both regions, did not attempt to control white grubs using cultural control methods, while 18% said they did (data not shown). Of those that answered *yes*; cultural control methods included ploughing, ripping, and discing, leaving fields fallow for 6-9 months, and the use of a rotation crop, such as soybeans or black oats. All of the respondents that answered *yes* to cultural control were growers from the

Midlands North region (data not shown). This was significantly different to the Midlands South region (χ^2=5.46; df=1; $P\leq$0.01) which did not practice any cultural control methods.

When all respondents were asked if they knew which insecticides were available for white grub control, 80% did not know (data not shown). Twenty percent knew chemicals such as Lindane, Temik®, Pyrinex®250 CS, Avant and Deltamethrin were available for white grub control. However, these chemicals were used mainly to control white grubs in *A. mearnsii* plantations which were grown adjacent to sugarcane by some growers. There was no significant difference between the two regions when respondents were asked if they knew which insecticides were available for white grub control (χ^2=0.99; df=1; $P\leq$0.318). Ninety eight percent of all respondents did not use any insecticides to control white grubs on their farms (data not shown). Only 2% of respondents did, and these were Midlands North growers. When all respondents were asked to comment on how effective they thought chemical insecticides were, 61% did not know, 15% did not answer the question, 12% thought they were moderately effective, 8% thought they were not effective and 4% indicated that insecticides were very effective (data not shown).

6.3.4 *Knowledge of mycoinsecticides*

Despite the fact that most respondents from both regions did not use chemical or cultural methods to control white grubs, 72% of all respondents would use a mycoinsecticide, if one was available (data not shown). Eight percent did not respond to the question. The 20% of respondents that would not use a mycoinsecticide, answered so because they thought they did not have a big enough white grub pest problem. Some thought ploughing was sufficient for white grub control. Others did not understand the concept of mycoinsecticides and thought a mycoinsecticide product was as harmful to the environment as chemical insecticides. One respondent was concerned about the cost factor. When respondents were classed according to farming experience (number of years farming) and asked if they would use a mycoinsecticide if one was available. More experienced growers indicated *yes* to the use of a mycoinsecticide, while most relatively inexperienced growers said *no.* This however was not significant (χ^2=2.71; df=3; $P\leq$0.438) (data not shown).

When respondents were asked to comment on how often they would apply a mycoinsecticide, 26% of Midlands North and 53% of Midlands South growers did not answer the question

(Figure 6.6). There was a poor response to this question as most respondents indicated that they required more information to answer the question effectively or didn't understand the mode of action sufficiently to answer the question. Thirty-eight percent of Midlands North growers and 18% of Midlands South growers expected to apply it once. There was a similar response (21% Midlands North; 24% Midlands South) from both regions to apply the mycoinsecticide once a year (Figure 6.6). Small proportions of respondents thought it feasible to apply a mycoinsecticide every six months, every crop cycle (which would be 18-24 months) or as required (Figure 6.6). There was no significant difference observed in response to the question when the different regions were considered (χ^2=5.33; df=5; P≤0.377).

Further, there was no significant correlation between growers who had a history of white grub on their farm, and their need to apply mycoinsecticides more often (χ^2=6.74; df=5; P≤0.241) (data not shown).

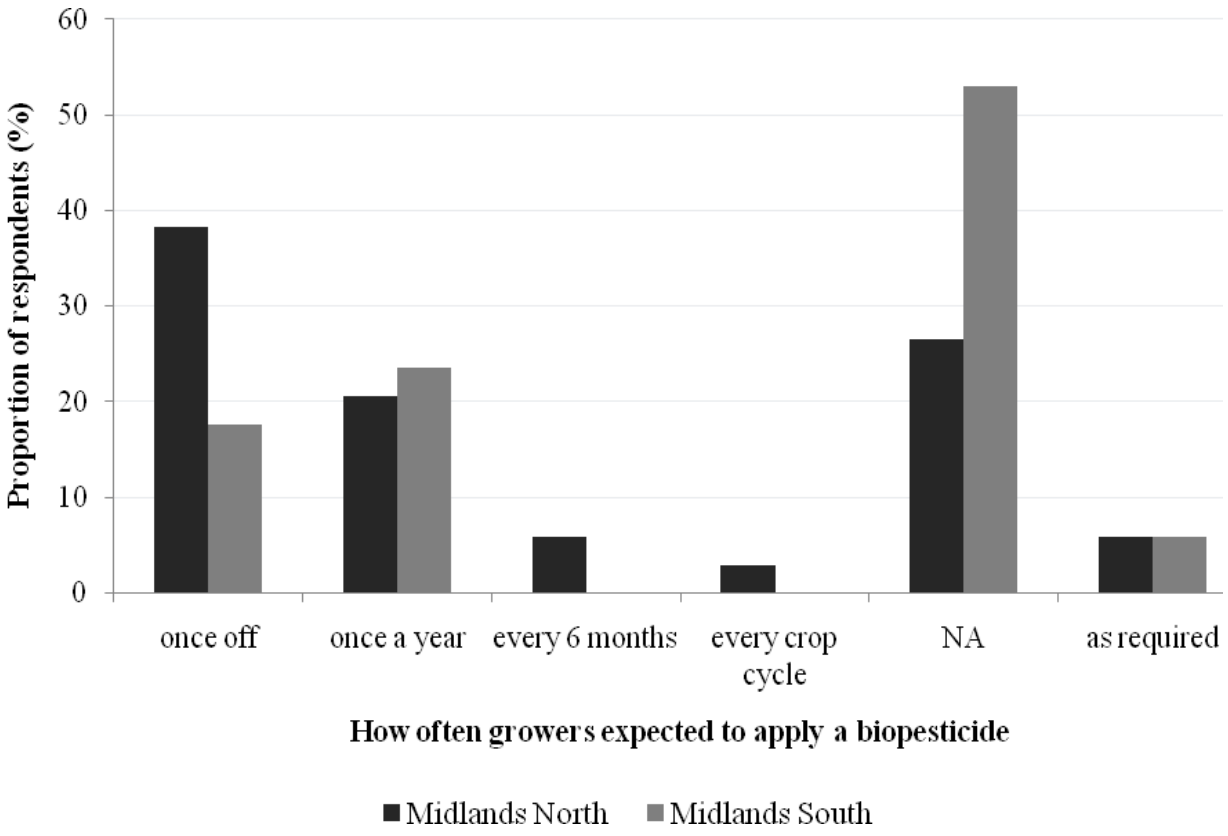

Figure 6.6: The proportion of respondents who were asked to comment on how often they would expect to apply a fungal-based mycoinsecticide per region. NA means not applicable and represents the proportion of respondents who did not answer the question.

Respondents from both regions were asked how much they would expect to pay for a 10 kg bag of mycoinsecticide. Forty-one percent from the Midlands North and 18% from the Midlands South expected to pay between R100-R500 per 10 kg bag (data not shown). Twenty-four percent of Midlands North and 18% of Midlands South growers expected to pay more for the mycoinsecticide (between R500-R1000). More Midlands South growers expected to pay in excess of R2000 for a 10 kg bag of mycoinsecticide (data not shown). However, there was no significant difference in response to this question by growers per region (χ^2=12.1; df=6; $P\leq$0.06). In addition, respondents were classed according to age and how much they would expect to pay for a 10 kg bag of fungal mycoinsecticide. Younger growers expected to pay less while older growers expected to pay more for the mycoinsecticide (Figure 6.7). This was not significantly correlated (χ^2=13.2; df=16; $P\leq$0.662).

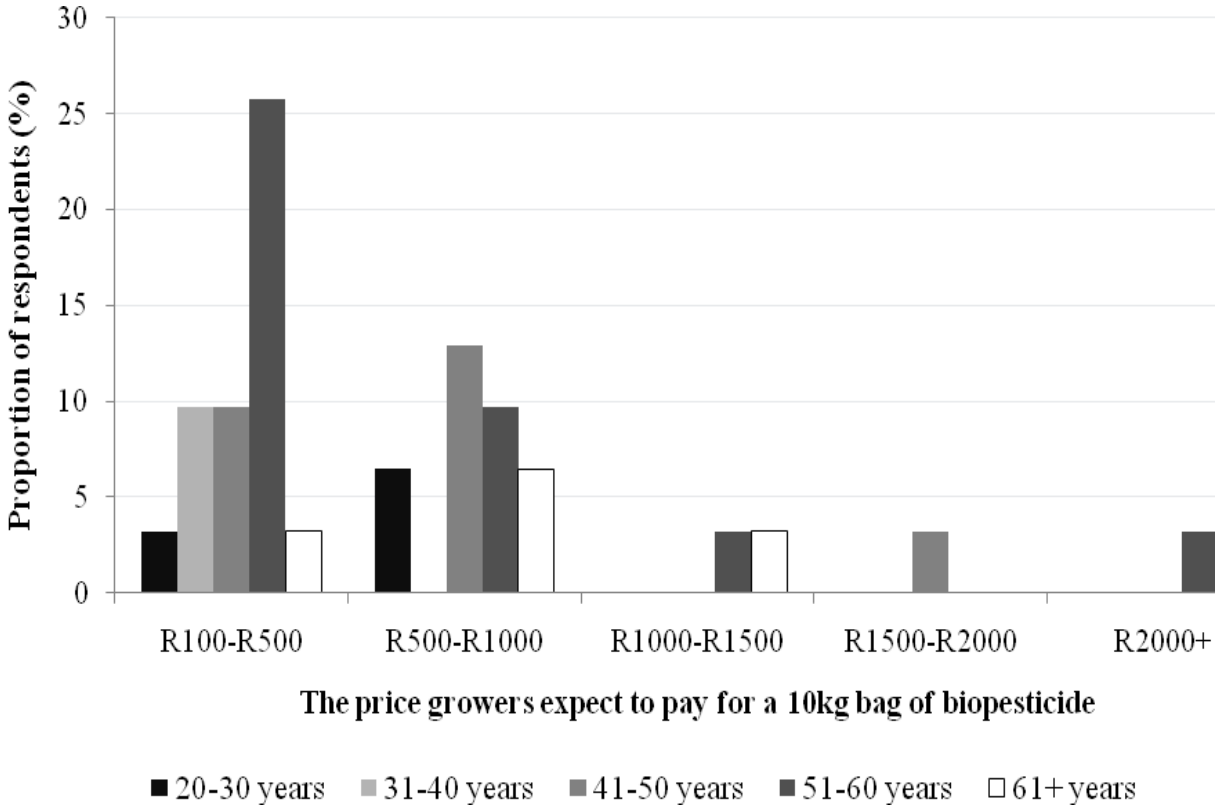

Figure 6.7: The proportion of respondents from both regions, grouped according to age, who were asked to comment on how much they would expect to pay for a 10 kg bag of fungal mycoinsecticide.

6.3.5 *Knowledge of mycoinsecticide feasibility*

Eighty percent of all respondents applied fertilizers annually, while 8% applied fertilizers every six months and a further 8% applied every crop cycle (18-24 months depending on the region). Only 4% of respondents did not answer the question (data not shown).

Ninety four percent of respondents owned a fertilizer applicator or could access one while 6% did not have access to a fertilizer applicator. There was a general trend for growers in both regions to plough out their sugarcane crop after the 5th, 6th and 7th rations (data not shown). Only growers in the Midlands North ploughed out their crop after a 4th ratoon. The annual sugarcane percentage replanting for all regions, grouped according to growers' experience, is shown in Figure 6.8. Most growers tended to replant smaller areas of their farms annually (1-20%), with only a small percentage of more experienced growers replanting larger areas per annum (Figure 6.8)

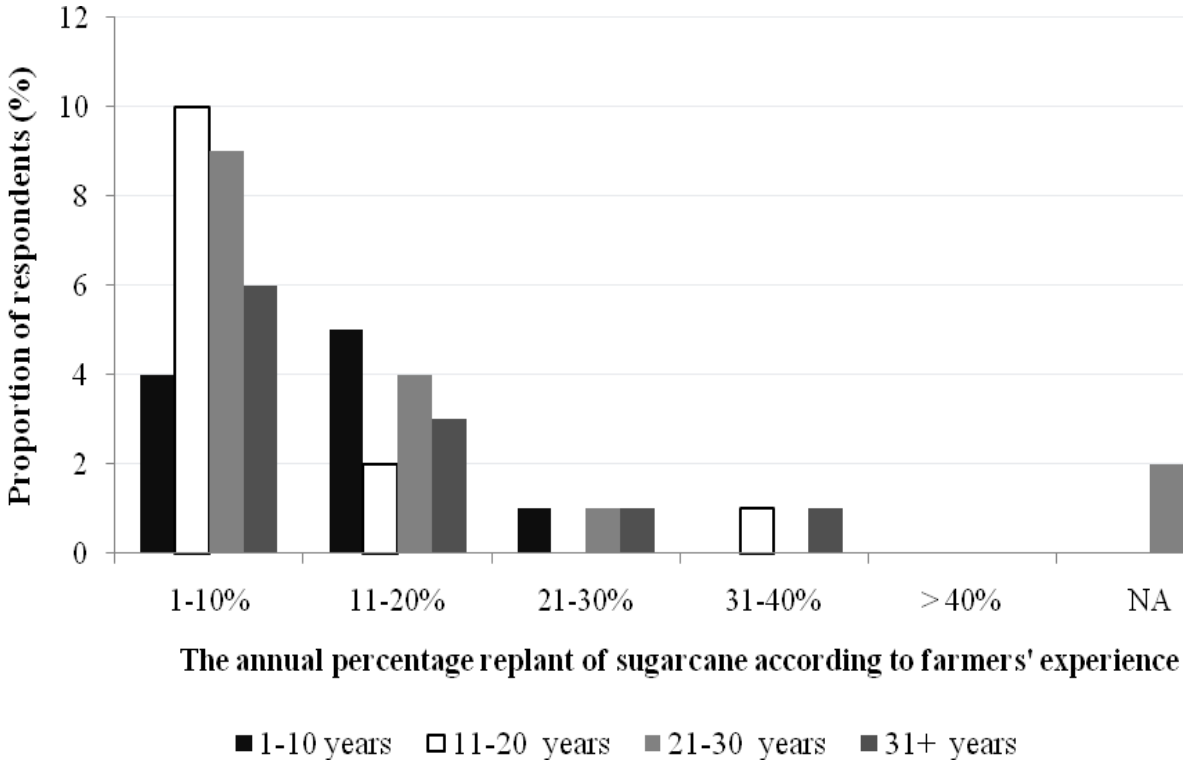

Figure 6.8: The proportion of respondents from both regions, grouped according to experience, who commented on what percentages of their farms were replanted anually. NA means not applicable and represents the proportion of respondents who did not answer the question.

6.4 DISCUSSION

6.4.1 *Personal and farm characteristics*

The Midlands North region is an area characterised by educated and experienced, older growers (51-60 years old) with large areas of self-owned land, sometimes in excess of 2000 ha in size. The Midlands South region is characterised by experienced, educated self-owned land proprietors who are relatively younger (41-50 years old) and own farms 500 ha in size. In terms of the demographic of the Midlands growers, Shennan *et al.* (2001) found a strong, positive relationship between grower age, education and IPM use. These factors were much more important determinants of IPM use than years of farming experience, in farmers, across five different crops in California, USA (Shennan *et al.* 2001). The age and education level of growers in both Midlands regions suggests that they may be open to mycoinsecticide adoption provided a significant perceived pest problem exists (Litsinger *et al*. 2010). The increased education level of these growers suggests that they have been exposed to information and literature, or attended courses to make them sufficiently aware of the existence and benefits of mycoinsecticides (Shennan *et al.* 2001). Hammond *et al*. (2006) further suggested that Wisconsin corn farmers who owned larger farms (882 ha) were more likely to adopt IPM practices than smaller farm owners (170 ha). He attributed this to the likelihood that larger farm owners relied more heavily on income generated on the farm, making economic optimization such as pest and pathogen control, labour management, crop rotation, crop price and weed resistance management (rotating herbicide families) more important to them. Larger fields require more consistent control to achieve the same absolute level of economic risk and farmers who own more land tend to do more pest management themselves (Feder *et al.* 1985; Hammond *et al*. 2006). Similarly, growers in the Midlands North and South regions, who own large farms, may be more likely to adopt or experiment with new technologies because they rely heavily on income generated on the farm. Shennan *et al.* (2001) confirmed that California growers on either very small farms or very large farms were the most intensive users of IPM and this overrode other characteristics such as land ownership, reliance upon farming for income, or the type of farm enterprise.

It has been shown that Midlands North growers are more willing to consider and adopt environmentally sustainable practices (Maher and Schulz, 2003; Webster *et al*. 2005; Webster *et al*. 2009; Cockburn *et al*. 2012). This is largely due to the Midlands North Local Pest, Disease and Variety Control Committee (MNLPD&VCC) which has been very active in

their dissemination of pest information and particularly in the development of an IPM system to prevent *E. saccharina* spread in the region (Webster *et al*. 2005; Webster *et al*. 2009; Cockburn *et al.* 2012). Furthermore, growers in the Midlands North have been well-aware of environmental issues and their adoption due to the development of SuSFarMS™ (Sustainable Sugarcane Farm Management System) – 'a management system that includes an audit of farming practices designed to help growers subscribing to it to manage their farms in an economically, socially and environmentally sustainable manner' (Webster *et al*. 2005). More recently, Cockburn *et al.* (2012) showed that the adoption of push-pull technology was well-received by large-scale growers and the likelihood of its implementation in the Midlands North is probable. Central to all of these studies in the region, were that growers recognised the value in adopting these environmentally sustainable strategies because the perceived risk of *E. saccharina* spread was so great (Webster *et al*. 2005; 2009). The environmental awareness of growers in the Midlands North region comes from the guidance of the MNLPD&VCC and the various projects that have been initiated in the region. It is thus likely that growers in the Midlands North would be open to the adoption of a granular mycoinsecticide, should there be a perceived risk.

In contrast, little is known about growers in the Midlands South or their attitudes towards IPM adoption or mycoinsecticides. What is known is that growers in this region are strongly influenced by representatives from agrochemical companies, who provide growers with information on chemicals, herbicides and fertilizers [18](Woodburn, pers. comm.). The influence of agrochemical companies is often a perceived barrier to IPM adoption (Shennan *et al*. 2001; Pretty, 2005). Moser *et al.* (2008) reported that agrochemical companies do not wish to see their sales figures decrease and therefore develop strong arguments and spend substantial amounts of money on advertising to convince farmers that chemicals are easy, effective and cheap to use, and are constantly being improved to ensure safety. McDonald and Glynn (1994) reported that apple growers in New York who trusted chemical company representatives were less likely to scout and monitor pests in their orchards. Furthermore, Shennan *et al*. (2001) found that a higher intensity of IPM was used by growers whose pest control advisors were not affiliated with agricultural chemical companies than for those who were. Literature suggests that growers in the Midlands South may thus be less likely to adopt a granular mycoinsecticide.

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6.4.2 *Knowledge of white grubs*

Most of the growers in the Midlands North and Midlands South perceive white grubs as minor pests and commented that while they can recognise white grub damage in the field, and white grubs do affect their final yields, only a small percent yield loss is perceived. Litsinger *et al*. (2010) suggested that it was important to know farmers' perceptions of yield loss because such understanding shapes farmers' attitudes and pest control decision making. If growers do not perceive their yield losses to be great, they are less likely to take a decision to control white grubs. This may limit growers' willingness to use a mycoinsecticide. Growers in both Midlands regions did not understand white grub biology or the pests' ecological utilization of host plants. Litsinger *et al*. (2010) suggested that the adoption of any IPM strategy is based on growers' knowledge and perception of pests and the ecological principles that govern pest populations. Of concern, was that a significant amount of Midlands South growers did not know that *A. mearnsii* exacerbated white grubs in the area because the pest utilizes both host plants as part of its life cycle (Prins, 1965; Carnegie, 1974; Carnegie, 1988). This suggests that knowledge dissemination from the Midlands South Local Pest, Disease and Variety Control Committee has been limited which may further restrict the adoption of a mycoinsecticide in this region.

Further lack of perception of white grubs as major pests came from insufficient understanding of their biology, their cryptic nature (Wightman and Wightman, 1994), confusion about damage and other, greater perceived threats such as the spread of the stem borer, *E. saccharina* into the Midlands regions. Apart from white grub adults that are most notable for two months of the year starting from mid-October, the most damaging life stage to sugarcane, the third instar grub, is cryptic in the soil (Carnegie, 1974). Some growers do not recognise the life cycle link between adults flying around workshop lights at night and grubs feeding on adjacent sugarcane roots in the soil (Woodburn, pers. comm.). Wightman and Wightman (1994) suggested that soil pests are the most difficult to manage because they cannot be detected without considerable effort. This means that farmers have to be convinced that investment in their control tactics would create sufficient return to cover the costs of any benefits (Wightman and Wightman, 1994). The cryptic nature of white grubs thus limits growers' perceptions of the pest problem and therefore may limit adoption of a mycoinsecticide. However, on the positive side, helping farmers to learn more about insect biology can result in the development of novel pest management techniques as farmers combine their own knowledge and experience with new information (Williamson, 1998).

Some growers confused white grub damage with macronutrient deficiencies in sugarcane which further reduces their pest status (Woodburn, pers. comm.). For example, white grub infestations may cause sugarcane to turn yellow in patches across a field (Wilson, 1969; Sosa, 1984). Similarly, a nitrogen deficiency in some patches in the soil will also cause sugarcane to appear yellow and patchy (SASRI, 2000). Thus, while growers say they can recognise white grub damage, unless they dig underneath these yellow patches and confirm the presence of white grubs in the soil, it may be difficult to confirm the damage as that of white grubs. Even when growers have large infestations of white grubs in their fields, they may still achieve high yields as a result of fertile soils (Chapter 2). This tends to cloud growers' perceptions of the pest problem because the yield is perceived not to be affected; a perceived limitation to IPM adoption (Litsinger *et al*. 2010). Finally, the threat of other pests is a far greater perceived problem for growers in these areas. For example, the spread of the stem borer, *E. saccharina* from coastal areas in to the Midlands North and Midlands South could potentially cripple sugarcane production in these areas (Webster *et al*. 2005; Webster *et al*. 2009). It is imperative that future studies be undertaken in both the Midlands North and South regions to determine accurately the extent of white grub damage in these areas, so that growers can make educated decisions about future control technologies.

6.4.3 *Knowledge of white grub control*

Further lack of pest perception was evidenced by growers' limited use of cultural control practices and insecticide applications, even in the Midlands North where yields were being affected by white grubs. In the present study however, a few Midlands North growers did apply chemical insecticides (Lindane, Pyrinex®250 CS, Avant and Deltamethrin) but did so mainly to control white grubs in alternative crops such as *A. mearnsii*, as these insecticides are registered for use in forestry and not sugarcane (South African Department of Agriculture, 2007). The fact that 92% of growers did not use chemical insecticides may increase the likelihood of adoption of a mycoinsecticide by growers, should a sufficient pest problem arise because the influence of agrochemical companies is limited (Shennan *et al*. 2001; Pretty, 2005). However, a small proportion of Midlands North growers, who were older, employed a higher rate of cultural control such as ploughing, than Midlands South growers, who were relatively younger and did not employ any cultural control methods for white grub control. Thus, our results are not in accordance with other researchers who found a negative association between grower age and the use of scouting, cultural control and

biologically based practices (McDonald and Glynn, 1994; Shennan *et al*. 2001). These authors found that younger farmers were more likely to practice IPM and many of them ran organic enterprises whilst older farmers (50 years +) showed decrease emphasis on IPM practices. This was not the case in the present study.

6.4.4 *Knowledge of mycoinsecticides*

The lack of pest perception was further mirrored by the low cost values and once-off application of a mycoinsecticide expected by growers in both Midlands regions. A large proportion of growers from both regions expected to pay very little for the product (<R500). Moser *et al.* (2008) demonstrated that Italian and German strawberry growers themselves behaved like consumers during a survey study and were not willing to pay more for strawberries just because they had been grown with a biological control agent. These growers were also among the few who also had less pest constrainsts (Moser *et al.* 2008). While Moser's *et al.* (2008) study cannot explain Midlands growers' expectation to pay less for a mycoinsecticide, it suggests that growers perceptions of a pest will govern their willingness to pay for such products. Most growers from both Midlands regions expected that the product need only be applied once off. Most of the marketing surrounding the use of bio-pesticides has encouraged their use as once-off biological versions of conventional pesticides (Williamson, 1998). This gives growers the impression that bio-pesticides are as effective as chemical when applied once off, and may thus explain the expectation of a once-off application in the Midlands regions. Moser *et al.* (2008) found that if a grower has confidence in a biological control agent, then they were more willing to experiment with the product or adopt an IPM program. Conversely, if a farmer did not trust these biological control agents, they would be less prone to experiment, as this would imply more effort (Moser *et al.* 2008). However, these under-estimations of the product value and its application may simply have been a lack of knowlegde of the nature of biological control agents and their actual costs (Williamson, 1998; Moser *et al.* 2008).

There were comments about the environmental safety of a granular mycoinsecticide, as some growers were worried that they may contaminate soils, highlighting a lack of understanding of mycoinsecticides. Most respondents did not answer a question related to mycoinsecticide application. This may have been because growers felt they did not have enough information to answer the question sufficiently. Or growers did not answer the question because they did

not understand the mode of action of the proposed product. Williamson (1998) for example, commented that farmers' lack of understanding of the mode of action of many microbial control agents, and even the fact that they are living organisms, is not limited to less educated farmers. An example of this concept was observed in the UK glasshouse industry, where growers were using a new product, Vertalec® based on the entomopathogenic fungus, *Verticillium lecanii* Zimmermann to control aphids. Despite warning labels, growers did not understand that they could not mix the fungal biological control agent in the product with the fungicide, benomyl. This resulted in complete failure of the product and its withdrawal from the market (Williamson, 1998). Moser *et al*. (2008) also found that educated strawberry growers in various countries did not understand the basic mode of action of biological control agents but understood that they had ''less environmental impact", entail ''higher costs of monitoring the crop", and are ''more sensitive to climatic factors", and that they ''have a slower and weaker effect" than chemical pesticides. These studies highlighted that despite a higher level of education and limited basic knowledge of the mode of action of biological control agents, that growers generally exhibited a positive attitude and were more aware of the positive aspects of biological control agents than the negative ones (Williamson, 1998; Moser *et al*. 2008).

Seventy-two percent of growers from both Midlands regions demonstrated a positive attitude towards a granular mycoinsecticide. Although this was particularly true of Midlands North growers, as more respondents responded positively to a mycoinsecticide and were prepared to apply it more often. This was unsurprising given the willingness of Midlands North growers to consider and adopt environmentally sustainable practices (Maher and Schulz, 2003; Webster *et al*. 2005; Webster *et al*. 2009; Cockburn *et al*. 2012). However, growers are more likely to respond favourably to questions which are specific to their interest and have some practical application (Shennan *et al*. 2001). Thus, it is expected that most growers answered with a positive attitude even though they perceived a minor pest problem. Perhaps growers recognised the future possibility of a perceived risk that could have further influenced their response to the possible use of a mycoinsecticide This information suggests that educated growers in the Midlands North and Midlands South could be open to adoption and understand the technology of mycoinsecticides and thus utilize them correctly, if extension services or free training were offered (Moser *et al.* 2008). Moser *et al.* (2008) commented that strawberry production managers in Italy believed that the most important strategy in promoting the expansion of biological control agents would be subsidies to cover additional

costs associated with the use of biological control agents followed by free technical support. This makes technology transfer and implementation much easier and more successful (Moser *et al*. 2008).

6.4.5 *Knowledge of mycoinsecticide feasibility*

What will likely further influence the adoption of a granular mycoinsecticide in sugarcane is integrating the new product into growers' existing application methods and lower pricing of the end product (Bailey *et al*. 2010). This was the idea behind the last set of questions in the survey when we asked growers how often they applied fertilizers and how often they ratoon sugarcane. It was thought feasible to apply a granular mycoinsecticide with fertilizer applications, that way ease-of-product-use could be achieved. The feasibility of applying a mycoinsecticide to sugarcane soils more than once a year may not be likely if adoption of this product was undertaken, because almost all growers in both regions apply fertilizers annually only. This fertilizer application regime is also strongly influenced by agrochemical representatives, the Fertilizer Advisory Service of the South African Sugarcane Research Institute and historical practices (SASRI, 2000). It is unlikely that farmers would want to apply a mycoinsecticide more than once a year when the perceived need is less than that of fertilizers and they are only applied once a year.

Applications of the Australian fungal-based product, BioCane™, are recommended to be made at sugarcane planting because BioCane™ was not rigorously tested for application in ratoon crops (Samson *et al.* 1999; Samson *et al.* 2006). Since applying BioCane™ to ratoon crops damaged sugarcane stools and efficacy was thought to vary, the best results of BioCane™ application were achieved in plant sugarcane (Samson *et al.* 2006). It was of interest to learn how often a granular mycoinsecticide could be applied in Midlands regions if using the product at plant was best. Growers from both regions plough out their sugarcane crop from the 5th, 6th or 7th ratoon. When we consider that crop cycles in these regions are 18-24 months long, it may be a decade before some fields are replanted. Furthermore, only small percentages of land are replanted annually in both Midlands regions so demand for a possible mycoinsecticide product in sugarcane may not be great enough to maintain economic viability for some microbial control companies. Also, BioCane™ is recommended to be applied at a rate of 33 kg per hectare (Milner *et al.* 2002). To apply this rate in South Africa using a granular mycoinsecticide would also not be commercially viable for microbial control

companies (Morris, pers. comm.). Even at a significantly reduced application rate (20 kg per ha), the cost to grower is in the region of about R2000 or US $233 per ha (Morris, pers. comm.). What may be important to consider is the use and marketing of a mycoinsecticide in alternative crops such as *A. mearnsii* because since the recent banning of the chemical insecticide, Deltamethrin, there exists a need to control these pests in this crop, [19](Norris, pers. comm.).

6.5 CONCLUSION

The adoption of any IPM strategy is based on growers' decisions, which are based on their knowledge and perception of pests, the ecological principles that govern pest populations, their relationships to yield and the cost-benefit of the control strategies. While this study is limited in its nature, it is likely that a granular mycoinsecticide would not be adopted by growers in the Midlands North or Midlands South regions. This is mainly because growers in these regions only perceived white grubs as minor pests. The limited use of cultural and chemical control strategies in sugarcane (even when yields were being affected) as well as the low cost value assigned to a possible mycoinsecticide further confirmed the reduced pest status of white grubs. This comes from insufficient understanding of white grub ecology, biology, their cryptic nature, confusion about damage and other, greater perceived threats such as, *E. saccharina.* Growers in the Midlands North in particular exhibited a positive attitude towards mycoinsecticides, and showed all the relevant attributes for successful technology adoption. However, the reduced feasibility of application, general lack of potential demand for a product, high cost factors and most importantly the lack of pest perception would negatively affect growers' attitudes towards a mycoinsecticide. This study highlighted key areas of research and knowledge transfer that are most certainly required for growers in these regions. Information on the actual crop losses associated with white grubs in sugarcane is required in both regions. Grower days are often the best way for practical learning and perhaps these could lead to better understanding of the pest's biology, ecology and management.

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CHAPTER 7-General Discussion

7.1 INTRODUCTION

In South Africa, the importance and increased prevalence of endemic scarabs, particularly *H. sommeri* and *S. affinis* as soil pests of sugarcane in the KZN Midlands North region, and a need for their control was established (Carnegie, 1974; Carnegie, 1988; Way, 1996; Way, 1997; Way *et al.* 2011). Previous research on the development, feasibility and success of fungal mycoinsecticides as alternative white grub control strategies within a greater integrated pest management (IPM) approach in sugarcane has been demonstrated (Vercambre *et al.* 1991; Vercambre *et al.* 1994; Milner, 1997; Neuvéglise *et al.* 1997; Samson *et al.* 1999; Samson *et al.* 2001; Milner *et al.* 2002; Milner *et al.* 2003). Thus, the development of a mycoinsecticide to control white grubs in South African sugarcane is important and has relevance; may serve as an environmentally friendly alternative to chemical insecticides; and would serve to support growers in controlling white grubs should they reach economically significant levels. Particularly, since historical records of the occurrence of entomopathogenic fungi in the KZN Midlands North suggest that natural fungal biological control of white grubs has been in progress in this region (Prins, 1965; Carnegie, 1974; Hatting, 2008). Mycoinsecticide development is further justified by the discovery of two new sites where *B. brongniartii* was found to occur in this study, and it is likely that more sites may harbour this fungal species, should field site surveys be undertaken in the region and also in the KZN Midlands South region. There are a number of steps involved in the development of a successful mycoinsecticide. These include: 1.) the identification of a pest problem; 2.) the identification of a suitable pathogen; 3.) pathogen strain selection; 4.) formulation and application; 5.) socio-economic and market analysis; 6.) financial support and collaboration; 7.) scale-up production; 8.) large-scale field trials; 9.) product registration; 10.) marketing, distributing and selling (Dent, 1998). This thesis has investigated the first five steps to the success development of a mycoinsecticide for scarabaeid control in South African sugarcane. There were however, some steps which were not entirely completed and thus recommendations for future research are discussed.

7.2 THE IDENTIFICATION OF A PEST PROBLEM

Chapter 1 highlighted the global importance of the Scarabaeidae, as the dominant group of soil pests, both in terms of distribution and damage to a large number of agricultural crops, including sugarcane (Prins, 1965; Wilson, 1969; Petty, 1976; Teetes and Wiseman, 1979; Crocker *et al.* 1990; Wightman and Wightman, 1994; Bellotti *et al.* 1997; du Toit, 1997; Potter, 1997; Townsend and Jackson, 1997; Beaudoin-Ollivier *et al.* 2000; Govender, 2007; Hammons *et al.* 2009). In other sugarcane producing countries such as Australia and India, these pests cause huge economic losses in damage and management expenses (Chelvi *et al.* 2011; Sallam, 2011). While scarabaeids may not be perceived by growers as major pests of sugarcane in the KZN Midlands regions (Chapter 6), their global distribution and damage levels highlight potential for their increased pest status in South Africa.

What will likely continue to aggravate the problem is the close cropping of *A. mearnsii* and sugarcane in the KZN Midlands North region. This agricultural crop mosaic creates the ideal environment for the proliferation of *H. sommeri* and *S. affinis*, as both pests feed on sugarcane as larvae and *A. mearnsii* as adults (Carnegie, 1974). Further, the KZN Midlands North region has particularly rich, humic soils which contain large amount of organic matter and are ideal for the development of neonate grub instars (Carnegie *et al.* 1974; Logan and Kettle, 2002; SASA, 1999). Sugarcane in this region is harvested every 18-24 months and growers can ratoon their fields seven times before sugarcane is ploughed out (Chapter 6). This means that sugarcane stools remain undistributed in the soil for up to 14 years in some fields. The application of lime or gypsum requires some ripping which disturbs the soil, but it is not known what damage, the shallower blade action of this method, would cause to white grubs. The general lack of soil disturbance increases white grub survival particularly in the presence of high quality sugarcane roots which are present all year round. It is likely that this lack of soil disturbance has also increased the development of fungal diseases, particularly *B. brongniartii*, in the KZN Midlands North (Allsopp, 2010). Similarly, white grubs in Australia became problematic in sugarcane because of habitat transformation. Endemic Australian melolonthids initially feed on tussock grasses in rangelands where soil contained little organic matter and was undisturbed (Allsopp, 2010). The quantity and quality of this food source probably limited white grub numbers, and the heterogeneity of these natural environments meant an abundance of parasitoids and pathogens (Allsopp, 2010). Later, these natural environments were replaced with extensive areas of sugarcane, with high quality

roots. Loss of trees limited parasitoids and disturbance of the soil meant decreases in pathogens, which all accelerated white grub pest status in Australian sugarcane (Allsopp, 2010). Agriculture is full of examples of the important concept that natural habitat loss, leads to altered host utilization by insects, which involves a monoculture crop, which then leads to increased prevalence of pests. Conlong (1994) showed that the stem borer, *E. saccharina,* preferentially fed on its indigenous hosts, which are mainly sedges rather than on recent hosts, the graminaceous crop plants. He also reported that this pest subsequently became problematic in sugarcane because of a loss of wetlands, its natural habitat (Conlong, 1994).

While ecological understanding of both the natural and altered habitats of insect pests can increase the efficacy of various control tactics, literature highlights that accurate identification of the target pests and their respective life histories are also necessary steps in the implementation of efficient control tactics (Allsopp, 2010). The results of Chapter 2 facilitated the accurate identification of a diversity of white grub species which co-occurred in sugarcane as a pest complex. It confirmed that *H. sommeri* and *S. affinis* were prevalent species in the KZN Midlands region, both of which have two-year life cycles. This study also highlighted the prevalence of other melolonthid species in sugarcane in this region. In Australia, the key pests of sugarcane are 19 different endemic melolonthids (Allsopp, 2010). Similarly, countries such as Madagascar, Mauritius, India, Philippines, Reunion and Tanzania report melolonthids as primary scarabaeid pests in sugarcane (Moutia, 1936; Saylor, 1940; Rajabalee, 1994; Vercambre *et al.* 1994; Quimio *et al.* 2001).

7.3 THE IDENTIFICATION OF A SUITABLE PATHOGEN

There are three important considerations when identifying a suitable pathogen for biological control. The first, involves identifying a naturally occurring fungal species on the target hosts, in this case are the melolonthids *H. sommeri* and *S. affinis*, or within the intended environment of use (Milner, 1992). The second involves accurately determining the identity of the pathogen (Milner *et al*. 2002) and finally, confirmation of the occurrence or efficacy of the pathogen on the target pest from the literature is required. Chapter 3 facilitated the accurate molecular identification of *B. brongniartii* as the causal agent of at least two observed epizootics in the KZN Midlands North in 2010 on the melolonthid, *H. sommeri*. It subsequently discredited *B. bassiana* isolate 4222 as the causal agent of these epizootics and therefore as the active ingredient of the mycoinsecticide for scarab control in South Africa

which saved considerable time and expense. Milner (1992) stated that isolates which were obtained from field epizootics against target pests were often the best to formulate and use as mycoinsecticides because their behaviour under field conditions was already stable. Natural biological control of scarabaeids was already occurring in the KZN Midlands North and thus suggested that *B. brongniartii* strains from these epizootics needed to be investigated for mycoinsecticide development. Chapter 3 also highlighted the importance of using molecular techniques over simple morphological examination to accurately identify genetically distinct strains and to subsequently evaluate strains thoroughly for mycoinsecticide development which had already been addressed in the literature (Milner, 1992; Cravanzola *et al*. 1997; Neuvéglise *et al.* 1997; Piatti *et al*. 1998; Samson *et al.* 1999; Samson *et al.* 2001; Samson *et al.* 2002; Milner *et al.* 2002.

It was initially concluded that isolate 4222 was the causal agent of epizootics observed at Sunnyside in 2005 because morphological identification was undertaken on two isolates from the site, confirming the species, *B. bassiana* (Hatting, 2008). Further, preliminary bioassay results had suggested that isolate 4222 was virulent against scarab hosts (Hatting, 2008). It is not unusual for *B. bassiana* to cause epizootics in scarab hosts. For example, *B. bassiana* caused an epizootic with prevalence reaching up to 99% in *C. zealandica* larvae sampled from soil in New Zealand (Townsend *et al.* 1995). However, these observations in scarabs are rare and *B. bassiana* epizootics are more common in other orders of insects such as the Lepidoptera (Bing and Lewis, 1993). *Beauveria bassiana* caused up to 84% mortality in overwintering larvae of *Ostrinia nubilalis* (Lepidoptera: Pyralidae) in corn residues that were laying on the soil surface (Bing and Lewis, 1993). While these examples allude to the broader host range of *B. bassiana*, consistent infection in scarabs has been limited to specific strains mainly within the species *B. brongniartii* and *M. anisopliae* (Jackson and Klein, 2006). Thus it is not unlikely that *B. bassiana* does infect scarabs in the KZN Midlands North but due to the more extensive research on the occurrence of *B. brongniartii* in this area, particularly as the confirmed causal agent of at least two epizootics, it shows that *B. brongniartii* is the better choice of pathogen for mycoinsecticide development.

Chapter 2 highlighted the predominance of melolonthid species in the KZN Midlands North. *Beauveria brongniartii* is one of the most virulent and widespread pathogens of the Scarabaeidae, and shares a particularly close association with the subfamily Melolonthinae in counties such as Austria, Belgium, Czech Republic, France, Germany, India, Italy,

Madagascar, New Zealand, Poland, Reunion and Switzerland (Keller, 1986; Rombach *et al.* 1994; Vercambre *et al*. 1994; Townsend *et al.* 1995; Callot *et al.* 1996; Cravanzola *et al*. 1997; Neuvéglise *et al*. 1997; Piatti *et al*. 1998; Vestergaard *et al.* 2002; Keller *et al.* 2003; Easwaramoorthy *et al.* 2004; Srikanth *et al.* 2010; Srikanth *et al*. 2011). The results of Chapters 3 and 4 completed an African link to the association of *B. brongniartii* on the melolonthid species, *H. sommeri* and *S. affinis*. With the widespread occurrence of this fungal species on melolonthids worldwide and the particular success of the product, Betel® within sugarcane systems (Vercambre *et al*. 1994), it is further recommended that strains of *B. brongniartii* be considered as the active ingredient of future mycoinsecticide products against melolonthid scarab pests in South Africa.

Advances which have been made using molecular techniques in Chapter 3, particularly the use of microsatellites include: ecological knowledge on the life cycle of *B. brongniartii*, its natural occurrence in the KZN Midlands North; dispersal mechanisms; host associations and fungal population characteristics. All which could not have been achieved without the use of microsatellites. Thus, it is suggested that any further research undertaken on the ecology of both scarabaeid hosts and their fungal pathogens should strongly consider the use of microsatellites. The twenty-six *B. brongniartii* strains which were discovered between the Canema and Harden Heights sites were the first ever record of this species of fungus infecting the melolonthid species, *H. sommeri* in South Africa and highlighted the natural occurrence of this fungal species in the country, which has implications for product registration. The study also showed that *B. brongniartii* was cycled from arboreal habitats to subterranean environments via *H. sommeri* adults. This highlighted an adaption to the host's biology and demonstrated the life cycle of the fungus. This aspect of the life cycle further reflected the pathogens ability to persist and self perpetuate in the environment, an advantage to the use of biological control agents over chemicals (Inglis *et al.* 2001; Lacey *et al.* 2001). Milner (1997) and Meyling and Eilenberg (2007) suggested that pathogens are able to cycle in the environment and thus, provide control for a number of years. Further, the ability of *B. brongniartii* to disperse via swarming *H. sommeri* beetles was shown when molecular techniques identified that a population of *B. brongniartii* spanned an area of 5.5 km between the two sites, Canema and Harden Heights. The low genetic diversity detected in *B. brongniartii* isolates indicated that high gene flow had occurred between the two sites. This phenomenon was also reported by Enkerli *et al*. (2001) when *B. brongniartii* isolates were obtained from two Swiss populations of *M. melolontha* and were shown to be genetically

closely related via gene flow. These authors suggested that since *M. melolontha* populations fluctuated in size and distribution, that it was possible that separated populations had been interacting at some point in the past (Enkerli *et al*. 2001). This aspect was important because it also suggested that beetle movement and population dynamics of *H. sommeri* within the KZN Midlands North are governed by the agricultural crop mosaic of intensively cultivated sugarcane and *A. mearnsii*. The establishment of the area-wide occurrence of *B. brongniartii* in the KZN Midlands North is prudent because this would allow insight into the occurrences of white grub hosts and the environmental parameters which may exclude the fungus, which would subsequently have significance for inundative application failures in the future for example.

It was shown that *B. brongniartii* occurs naturally in *H. sommeri* populations in the region and that there is an old association (Aldrich, 1995) between this melolonthid scarab species and the fungus. This hypothesis was based on the molecular results and historical records of the occurrence of fungi in the KZN Midlands North (Prins, 1965; Carnegie, 1974). Roy *et al.* (2006) reported that host-specific pathogens engage in a tight process of co-evolution, whereas generalist pathogens interact more diffusely. However, Pimentel (1963) reported that co-evolution may lead to physiological equilibrium that could prevent long-associated pathogens from being effective biological control agents. The fact that epizootics have been observed in *H. sommeri* populations suggests that equilibrium may not yet have been reached in this case. Further, during microsatellite analysis (Chapter 3) the numbers of identical *B. brongniartii* strains per haplotype were low indicating that the search for existing haplotypes in the fungal population was not yet saturated, which confirms the work of Enkerli *et al.* (2001). *Schizonycha affinis* may also share an old association with *B. brongniartii* because many fungal infected beetles and larvae were observed in the field and laboratory (personal observation). Further, molecular results confirmed *B. brongniartii* infections in *S. affinis* however, some beetles were also found to harbour *B. bassiana* infections. Although some strains of *B. brongniartii* were virulent against *S. affinis* during bioassays (Chapter 4), there were other strains which were not particularly virulent, suggesting possible co-evolution. The fact that *S. affinis* is also a melolonthid species and literature suggests that melolonthids are particularly susceptible to strains of *B. brongniartii* (Vercambre *et al.* 1994; Kessler *et al*. 2004; Strasser and Pernfuss, 2005; Traugott *et al.* 2005), and given the long-term occurrence of this species in the KZN Midlands North (Carnegie, 1974) it is suggested that *S. affinis* also shares an old association with *B. brongniartii*.

7.4 PATHOGEN STRAIN SELECTION

Beauveria brongniartii was shown to be a suitable pathogen against melolonthids and host specificity is an important trait because it limits non-target effects. Literature also suggested that host range and virulence were important considerations for the efficacy and practicability of mycoinsecticides (Lacey *et al.* 2001; Milner *et al.* 2002; Ansari *et al*. 2004; Strasser and Pernfuss, 2005; Hatting and Wraight, 2007). Further understanding of insect life stage susceptibility to fungal infection can aid in effective pest targeting (Keller *et al.* 1999; Traugott *et al.* 2005; Yaginuma *et al*. 2006; Townsend *et al.* 2010; Srikanth *et al*. 2011). Chapter 4 highlighted that strains of *B. brongniartii* were good candidates for formulation as mycoinsecticides for the control of *S. affinis* and *H. sommeri.* Pathogenicity of *B. brongniartii* isolates were equivalent to those reported as having good biological control potential in the literature (Yaginuma *et al.* 2006; Townsend *et al*. 2010; Srikanth *et al.* 2011). Even the weakest strains of *B. brongniartii* were superior biological control agents compared to *B. bassiana* 4222, Further the specificity of *B. brongniartii* towards the host, *S. affinis* was shown, especially when compared to the reduced infectivity observed on *T. molitor.* Strains of *B. brongniartii* produced oosporein when they were grown in shaker flasks for DNA isolation in Chapter 3. The production of secondary metabolites such as oosporein aid more aggressive strains of entomopathogenic fungi in penetrating the host cuticle, overcoming the hosts immune system, colonising the host by excluding competing microbes and are thought to be involved in the determination of host specificity (Strasser *et al*. 2000; Glare, 2004; Amin *et al.* 2010).

In Europe, it is well known that *B. brongniartii* is a host specific fungus with a limited host range (Keller *et al.* 1997; Keller *et al.* 1999; Strasser and Pernfuss, 2005; Koller *et al.* 2005; Laengle *et al.* 2005). Chapter 4 was limited in that only a few genetically distinct *B. brongniartii* isolates were screened and tested against *S. affinis.* The host range of the fungus was not investigated against other melolonthid pest species. It is important to determine accurately the host range of the obtained native strains of *B. brongniartii* because mycoinsecticide viability will rely on strains which are active against a range of scarabaeid species (Samson *et al.* 1999). It would be prudent to investigate *B. brongniartii* strain pathogenicity against a number of known melolonthid pest species in South African sugarcane such as: *A. subfasciata*, *Congella* sp., *Maladera* sp., *Trochalus aerugineus* Burmeister, *Apogonia ovata* Fahraeus, *Autoserica* sp., and other *Schizonycha* sp. (Way,

1997). In Europe for example, commercial strains of *B. brongniartii* are pathogenic towards species within the *Melolontha* but are not effective against other melolonthid species (Strasser pers. comm.). Thus, it is unclear whether the host range of native *B. brongniartii* strains could control other melolonthids or other scarabaeid subfamilies such as the Rutelinae and Dynastinae. Host range is an important consideration particularly as two ruteline pest genera, *Anomala* and *Adoretus* were also found in the KZN Midlands North (Chapter 2). According to Way (1997) there are more ruteline pest species present throughout the sugar industry and it would be opportune if a mycoinsecticide could include the control of such species. Further, the dynastid, *H. licas* is an important species to control because damage to sugarcane by this pest is often extensive due to heavy infestations, particularly in irrigated sugarcane (Sweeney, 1967; Carnegie, 1988; Cackett, 1990; Cackett, 1992). *Heteronychus licas* is also a well-known pest of several other crops in South Africa, thus there may be vast scope for the implementation and profitability of a mycoinsecticide based on *B. brongniartii* in other crops.

Literature however suggests that isolates of *M. anisopliae* are most effective against the Rutelinae and Dynastinae (Rath and Worledge, 1995; Ansari *et al.* 2004; Beron and Diaz, 2005; Makaka, 2008). There are also reports in the literature of the Melolonthinae being controlled effectively with *M. anisopliae* (Milner, 1997; Samson *et al.* 1999; Milner *et al.* 2002; Guzman-Franco *et al*. 2011). In Australia, *M. anisopliae* is used successfully to control an endemic melolonthid pest complex in sugarcane (Allsopp, 2010) but this is because *B. brongniartii* is not known to occur there (Milner, 1997). There are also reports of *B. brongniartii* infecting species within the Dynastinae (Theunis and Aloali'I, 1998) and Rutelinae (Ansari *et al.* 2004) however *B. brongniartii* isolates appear to be less virulent towards these two subfamilies compared to *M. anisopliae* isolates. Milner (1997) suggested that the relative importance of various pathogen species in controlling white grubs varies with the host and environmental conditions. For example, in New Zealand, protozoan pathogens are major scarab population regulators in the North Island while in the South Island, the bacterial genus, *Serratia* dominates as a major scarab population control agent (Milner, 1997). The same appears true of fungal pathogens. In Australia, *M. anisopliae* is the dominant pathogen of scarabs and *B. brongniartii* is the major fungal species that occurs on scarabs in Europe (Keller, 1986; Vestergaard *et al*. 2002; Keller *et al.* 2003). It appears that the natural occurrence of either *M. anisopliae* or *B. brongniartii* in various countries determines the use of these fungal species against scarabaeid hosts (Milner, 1997). This

becomes particularly important when epizootics are observed (Milner, 1992). Thus, whether *M. anisopliae* or *B. brongniartii* is used against such hosts will depend on which fungal species is most prevalent in a respective country. Certainly, in the KZN Midlands North region *B. brongniartii* is the dominant fungal species but it would be of interest to determine if this is true of all areas of the sugar industry in South Africa. Future study should consider the host range of native strains of *B. brongniartii* to establish if this fungal species can infect and effectively control other melolonthids, dynastids and rutelines.

The advantages of using a pathogen such as *B. brongniartii* which shares an old association with its hosts are numerous, and have been encouraged by many biological control practitioners (Aldrich, 1995). One of the most important advantages is non-target effects through evolved host specificity. Harrison (2013) cautions that biological control efforts implemented against *H. sommeri* may potentially threaten closely-related, non-pest scarab species such as *Hypopholis pondoensis* Arrow (Coleoptera: Melolonthinae), which are rare and endemic to forest habitats. Also, *Hypopholis vittata* Fåhraeus in Boheman (Coleoptera: Melolonthinae) which is more widespread in southern Africa (Harrison, 2013). While we know *B. brongniartii* is capable of causing epizootics in *H. sommeri,* it is assumed that the fungus would also be able to infect closely related species such as *H. pondoensis* and *H. vittata.* European researchers found that commercially available strains of *B. brongniartii* were highly specific to species within the genus *Melolontha,* and were also capable of infecting the forest cockchafer, *M. hippocastani* (Koller *et al.* 2005; Laengle *et al.* 2005). Further, these two *Melolontha* species are sympatric in some areas in Europe which renders biological control very useful (Reinecke *et al.* 2006b). It is thought that because an indigenous fungal isolate will be considered for mycoinsecticide development in South Africa, species such as *H. pondoensis* would have been exposed to the fungal species at some point during their evolution in South Africa. This suggests that some resistance to the pathogen may already exist in these rare species populations, especially with respect to the more restricted Karkloof population of *H. pondoensis* which lie in close proximity to the KZN Midlands North *H. sommeri* populations (Harrison, 2013). One could also argue that because *H. pondoensis* is confined to forest habitats, and the intention to use a fungal mycoinsecticide in sugarcane and possibly *A. mearnsii* would probably limit the dispersal of the fungus into these natural forested areas. Harrison (2013) reported that IPM approaches using *B. brongniartii* should be restricted to agricultural systems to reduce the potential risk of infections in *H. pondoensis.* However, if an exotic strain of *B. brongniartii* was released in

South Africa, the potential threat of non-target effects against these rare scarabs would almost certainly come into question, particularly because all three *Hypopholis* species occur in KZN (Harrison, 2013). It would be of interest to survey these rare *H. pondoensis* populations, to note if *B. brongniartii* is naturally present.

Chapter 4 showed that genetically closely-related *B. brongniartii* strains varied significantly in virulence towards the host, *S. affinis* and the laboratory-reared, *T. molitor* despite them being obtained from the same field epizootics and belonging to the same fungal population. Similar findings on genetically closely-related *B. brongniartii* and *M. anisopliae* isolates, and virulence of individual strains against various scarab hosts, are not uncommon in the literature (Cravanzola *et al.* 1997; Neuvéglise *et al.* 1997; Piatti *et al.* 1998; Milner *et al.* 2002). The existence of genetically distinct and virulent strains within a field epizootic brings into consideration the possibility of co-formulation of fungal strains to increase biological control efficacy. Co-formulation of strains and the potential synergisms which may arise from having two or more fungal strains formulated into one product has been considered by some researchers (Inglis *et al.* 1997; Leal-Bertioli *et al*. 2000; Wang *et al*. 2002). One of the perceived limitations of fungal biological control agents is that they may only show efficacy under a narrow set of environmental conditions (Wang *et al*. 2002). Inglis *et al.* (1997) for example, investigated the combined use of *M. anisopliae* and *B. bassiana* for control of locusts and grasshoppers, to overcome the temperature limitations of both fungal species. Most growers have to deal with pest complexes, which is why they often prefer to use broad-spectrum insecticides (Wang *et al*. 2002). If co-formulation of different fungal strains could enhance pathogenicity, host range, environmental competency and reduce the development of resistance, it would improve the status of mycoinsecticide pest control (Wang *et al*. 2002). Unfortunately such studies reported no synergistic effects in virulence between genetically distinct fungal strains when co-formulated and assayed against insects hosts (Leal-Bertioli *et al*. 2000; Wang *et al*. 2002). In fact one aggressive strain always dominated and could displace a less competitive strain and time to insect death was extended because of dilution of the more virulent strain (Leal-Bertioli *et al*. 2000). These authors cautioned that parasexual recombination was possible with indigenous strains which may result in heterokaryons (cells that contain multiple genetically different nuclei) with altered virulence and host specificity, and may in turn impact hugely on non-target insect populations and biodiversity (Leal-Bertioli *et al*. 2000).

Bioassay results in Chapter 4 showed that the adults of *S. affinis* were more susceptible to *B. brongniartii* and could be killed within a shorter time period than the second or third instar grubs. These aspects of life stage susceptibility suggest that adults and neonate grubs should be considered as primary targets in pest control using mycoinsecticides, and suggests that late spring applications (late September) are the correct time to target these susceptible life stages. The increased susceptibility of adults compared to grubs was attributed to them being short-lived, which limited exposure to *B. brongniartii* in the soil and resulted in delayed resistance development. Further, it was thought that activation and use of the immune response was costly and thus was traded off against other energy-demanding aspects of the life history, such as reproduction leading to higher susceptibility to pathogens (McKean and Nunney, 2001; Fedorka *et al.* 2004; Rantala *et al.* 2011). Other authors have also confirmed the increased susceptibility of adults compared with grubs to fungi, and have suggested that adults are appropriate targets for mycoinsecticide application (Lacey *et al.* 1994; Yaginuma *et al.* 2006). A delay in beetle infection was noted in Chapter 4. This observed delay in insect mortality following fungal treatment indicates good potential for dispersal of *B. brongniartii* within both *S. affinis* and *H. sommeri* populations in the field (Lacey *et al.* 1994), and compliments the results of the molecular study (Chapter 3) which showed the dispersal ability of beetles and *B. brongniartii*. These have implications for biological control strategies using adults as fungal vectors to contaminate breeding sites as has been done in Switzerland (Keller *et al.* 1999) and considered in New Zealand (Glare *et al.* 2002).

7.5 PERSISTENCE, APPLICATION AND FORMULATION

Strain selection should not only be based on host range or virulence towards the target pest alone, but should also consider aspects such as persistence, easy of production and strain consistency (Dent, 1998; Brownbridge *et al.* 2001). The literature suggests that the long-term persistence of mycoinsecticides in the environment is an important prerequisite for long-term control of pests, their widespread use and ultimate adoption by growers. Doubts about the efficacy of fungal formulations can lead to disuse and product removal among growers and policy makers (Vänninen *et al*. 2000; Kessler *et al.* 2003; Milner *et al.* 2003; Kessler *et al.* 2004; Laengle *et al.* 2005; Sallam *et al.* 2007). Chapter 5 highlighted the poorer persistence of *B. bassiana* 4222 formulated on rice and wheat bran compared to other studies using fungal species such as *M. anisopliae* and *B. brongniartii* (Milner *et al.* 2003; Enkerli *et al.*

2004). Overall, poor persistence was mainly attributed to suboptimum temperatures, rainfall, which rapidly degraded the nutritive carriers, attenuated fungal genotype and the action of antagonistic soil microbes. Thus, based on the requirements of long-term persistence and strain consistency for successful mycoinsecticide practicality, *B. bassiana* 4222 can be excluded. This further confirms the rejection of the isolate as the active ingredient of a mycoinsecticide. Chapter 5 was limited in that *B. brongniartii* was not field tested to investigate the persistence of various strains, especially since this pathogen's efficacy against melolonthids was confirmed by literature and bioassays in Chapter 4. Thus it would be of interest to know if strains of *B. brongniartii* could persist for long periods of time in South African soils, which would almost certainly increase the attractiveness and adoption of a potential mycoinsecticide. For this reason a discussion around the persistence, application and formulation of a *B. brongniartii* mycoinsecticide will ensue rather than discussing these features for *B. bassiana*.

Environmental stability and persistence is an important consideration for the inundative release of mycoinsecticide based on *B. brongniartii.* Literature suggests that *B. brongniartii* can persist in the environment for considerable periods of time, sometimes for up to 40 years (Keller *et al.* 2003). The reason inundative or augmentative biological control approaches are used is because the effects on the target pest species are normally limited by the pathogens ability to proliferate and disperse (Eilenberg *et al.* 2001a; Glare, 2004). However we have shown that proliferation and persistence are good with respect to *B. brongniartii* which is why epizootics occur in the KZN Midlands North. However, while *B. brongniartii* exists to suppress these target scarab pests, total suppression is never achieved usually as a consequence of co-evolution (Roy *et al*. 2006). Further, the constraints of abiotic environmental factors such as temperature and moisture or, biotic factors such as competition from other microorganisms, and scarabaeid host resistance, may also limit persistence and proliferation of the fungus (Strasser *et al.* 2000; Kessler *et al.* 2003). *Beauveria brongniartii* requires insect hosts to build-up enough infectious material to create infections in the soil (Kessler *et al.* 2004). This close host-pathogen relationship leads to a mutual control, expressed in fluctuations in the host population (Klingen and Haukeland, 2001; Kessler *et al.* 2004). As the host decreases, so too does the number of infectious propagules of the pathogen in the environment (Eilenberg *et al*. 2001a). Kessler *et al.* (2004) showed that in the absence of the host, *M. melolontha* the number of *B. brongniartii* CFUs declined by as much as 90% but where the host was present the conidia survived significantly longer. Therefore, the

success and/or failure of biological control of scarabs with *B. brongniartii* will strongly depend on insect hosts and the subsequent production of infectious material in the soil to elicit an epizootic (Kessler *et al*. 2004). The environmental stability of a native fungal pathogen is another aspect that will likely enhance biological control efficacy. Such strains have evolved naturally under abiotic and biotic environmental conditions and are thus better suited for biological control in terms of field efficacy against the target pest, compared to exotic strains (Milner, 1997).

With the knowledge that *B. brongniartii* requires insect hosts to persist, and with the knowledge that the most susceptible life stages occur in late spring and subsequent summer months (October-March) (Chapters 4), it is recommended that pest targeting using mycoinsecticides should be considered in late September (Chapter 2). In these months a greater diversity of white grubs may be found which exhibit a highly aggregated distribution in the soil, which also increases application efficacy. Chapter 2 also showed that there were higher occurrences of mycosed cadavers in September (spring). The higher occurrences of cadavers in spring were attributed to increased soil water content which forced stressed scarab hosts to the upper levels of the soil profile which increased the contact of host and pathogen and therefore the chance incident of infection. Other authors have commented that depending on the severity of winter temperatures white grub fungal pathogens may result in naturally higher spring mortalities because pests are stressed (Dalthorp *et al*. 2000). Likewise, dry summer months or drought can have a significant stress effect on white grubs and can lead to increased incidences of fungal mortality (Carnegie, 1974). An autumn peak in the occurrence of mycosed cadavers was also observed, which followed a relatively dry summer. These natural increases in fungal infection give insight into the timing of application strategies and suggest that spring or autumn application times may better suit the growth and development of *B. brongniartii*.

Spring applications (late September) of formulated *B. brongniartii* will coincide with beetle emergence and the subsequent neonate life stages which follow. Chemical control for example, is usually aimed at ovipositing females or at the first and second instar grubs by placing insecticides close to the sugarcane setts (Fewkes and Greathead, 1978). When beetles emerge from soil they can become infected with fungus which has a two-fold effect. The adults themselves may die from fungal infection as they emerge from soil or, the subsequent swarming adults may horizontally transfer the infection to healthy adults during mating or

when feeding in trees. This would further enhance dissemination as well as augmenting natural infection levels (Lacey *et al.* 1994). Following potential infection of the adults, those surviving, ovipositing females may become infected with fungus as they bury into the soil to lay their eggs. Subsequently, the first and second instars may then become infected with residual fungal material from sporulating adults or from residual material positioned during spring applications.

In Europe for example, two methods are used to apply *B. brongniartii.* Firstly, swarming *M. melolontha* adults are sprayed with an aqueous suspension of fungal blastospores with the aim of using the returning females as vectors of the fungus to contaminate breeding sites (Keller *et al.* 1999). Second, the fungus is grown on sterilized barley kernels and applied with a conventional tractor-powered seed drill in areas infested with *M. melolontha* larvae and emerging adults (Keller *et al.* 1999). In Switzerland, the timing of the latter method for mycoinsecticide applications against *M. melolontha* were made in spring, summer and autumn to evaluate the most appropriate season for the proliferation of *B. brongniartii* (Kessler *et al*. 2003). Spring and summer applications resulted in proliferation of the fungus however, the autumn applications failed to yield any colony forming units (CFUs) (Kessler *et al*. 2003). These authors attributed the failed applications to a number of environmental variables, the most important being suboptimum temperatures (Kessler *et al.* 2003). These results suggested that seasonality was an important consideration and highlighted spring or summer as potential application times.

The timing of mycoinsecticide application in the soil is complicated by the harvesting method of sugarcane and by the life cycle of a particular pest species (Samson *et al.* 1999). In Australia, *D. albohirtum* has a one year life cycle and damages sugarcane in late summer (February) and autumn (April) (Samson *et al.* 1999). The *M. anisopliae* based mycoinsecticide, BioCane™ is usually applied as a prophylactic treatment at plant (Samson *et al.* 1999), the timing of which may vary with region in Australia but attempts are made at coinciding applications with scarabaeid host occurrences in spring (Allsopp, 2010). *Lepidiota negatoria* occurs in central Queensland and has a two-year life cycle, and damages sugarcane in early-spring and summer (Samson *et al*. 1999). BioCane™ applications against this grub species has mostly targeted ratoon sugarcane because of the long life cycle (Samson *et al.* 1999). However, additional chemical control is usually required because of the difficulty in pest targeting in ratoon sugarcane (Samson *et al.* 1999). Similarly, in South Africa the

different life histories of white grubs which co-occur in sugarcane cropping systems in the KZN Midlands North will likely complicate successful control applications. As *H. sommeri* and *S. affinis* have two-year life cycles and *Maladera* sp11 have a one-year life cycle (Chapter 2). Allsopp (2010) suggested that any control application strategy which is undertaken on sugarcane fields that accommodate white grubs with different life histories would be difficult. Thus the development of an appropriate risk assessment strategy that advises whether pre-emptive control treatments are likely to be worthwhile should be developed (Allsopp, 2010). It is also suggested that localized populations of white grubs shrink and swell annually because of variations in rainfall and soil moisture (Hawley, 1949; Dalthorpe *et al.* 2000b) thus climatic variables may play a big role in predicting the occurrence and abundance of some white grub species and may improve the development of a risk assessment strategy.

Chapter 5 showed that environmental variables such as soil temperature, moisture and other microbes will play the biggest roles in the persistence of mycoinsecticides in spring and subsequent summer months in South Africa. Temperature is known to be one of the most important factors influencing the development and persistence of *Beauveria* species (Lingg and Donaldson, 1981; Kessler *et al.* 2003; Jaronski, 2007). Chapter 3 was limited in that strains of *B. brongniartii* were only obtained from the KZN Midlands North, an area characterised by average cooler temperatures compared to the rest of the province. Therefore it is anticipated, based on limited, unpublished data that the optimum temperature range of *B. brongniartii* strains may lie closer to 20°C than 25°C, the latter being the optimum temperature generally reported for entomopathogenic fungi (Jaronski, 2007). The temperature profile of *B. brongniartii* strains in South Africa (Goble, unpublished data), in Europe (Kessler *et al.* 2003) and Reunion (Vercambre *et al.* 1994) suggests that the fungus cannot survive field temperatures above 33°C and sporulation is halted at 28°C (Kessler *et al.* 2003). This may limit mycoinsecticide application in terms of persistence and efficacy significantly in summer months in many parts of the South African sugar industry. In summer months, particularly December, January, February and March soil temperatures above 33°C are often recorded within the top 10 cm of the soil profile in various areas within the sugar industry [20](Sithole, pers. comm.). Soil temperatures above the critical threshold of *B. brongniartii*

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conidia survival have also been recorded in the KZN Midlands North but these are recorded less frequently and usually only in January and February compared to other regions within the sugar industry (Sithole, pers. comm.).

However, the temperature profiles of various entomopathogenic fungi vary substantially between different strains (Jaronski, 2007). This author examined the temperature tolerances of 34 isolates of *B. bassiana* from sugar beet fields and three *M. anisopliae* isolates from other sources. Twenty-three isolates grew slowly at 10°C; very few grew at 7°C; only a few isolates grew at 35°C and none of the isolates grew at 37°C. This suggests that future studies should consider the temperature tolerances of various strains of *B. brongniartii* to ensure the selected strain is temperature resilient. Jaronski, (2007) reported that it was more relevant to consider the effects of microhabitats rather than regional soil temperatures at the time of inundative use. For example, in treating pupating foliar pests this microhabitat may be within the first 1-2 cm of the soil surface. However, in the case of our intended use, this microhabitat will be deeper, at least 15-20 cm, if a granular formulation is used which will buffer temperature extremes (Jaronski, 2007).

Further, extreme temperature effects may be buffered by moisture effects (Jaronski, 2007) particularly since the KZN Midlands regions receive summer rainfall (Chapter 5). However, summer rainfall and the subsequent increases in soil water content could also negatively affect mycoinsecticide persistence. The rapid degradation of *B. bassiana* formulated on rice and bran carriers in part was attributed to heavy rainfall (Chapter 5) and confirms the work of Ekesi *et al.* (2005). Further, soil types which are higher in organic matter will also affect persistence of a mycoinsecticide in summer (Chapter 5). Increased rainfall causes accelerated decomposition of organic matter and increases soil microfauna which feed on organic matter (Curiel Yuste *et al.* 2007; Ryan *et al.* 2009). Catalase activity is also highly correlated to organic content of the soil and can negatively affect *B. brongniartii* (Kessler *et al.* 2003; Quesada-Moraga *et al*. 2007). Other microorganisms in the soil are higher in summer and may thus either outcompete *B. brongniartii* for carbon in the absence of insect hosts or have a suppressive effect on the growth of the fungus *in situ.* It is anticipated that the antagonistic effects of other soil microorganisms however will be less pronounced if there is an abundance of insect hosts on which the fungus can proliferate. Kessler *et al.* (2003) found that in most cases there was a significant increase in the number of colony forming units (CFU) of *B.*

brongniartii in the soil following application of the mycoinsecticide, Beauveria-Schweizer® (granular formulation with fungus grown on barley kernels) in spring and summer.

In contrast, these authors found that applications in autumn did not increase *B. brongniartii* density in the soil and that applications had failed to control *M. melolontha* (Kessler *et al.* 2003). Similar to the situation in Europe, autumn applications of a mycoinsecticide in South Africa may also show poorer persistence than spring applications because although soil temperatures, rainfall and other microorganisms are reduced so too are the number of susceptible hosts (Chapter 2). Insect pest life stage targeting is narrowed because only the third instar larvae are present and usually at deep levels within the soil, suggesting that pest targeting may be greatly reduced (Chapter 2). Further, the third instars larvae are the least susceptible to the effects of *B. brongniartii* (Chapter 4). Kessler *et al*. (2003) attributed failed autumn applications to that fact that optimum growth temperatures of the fungus were never achieved. Information from weather stations in Chapter 5 suggest that if autumn (April) applications were made in the KZN Midlands North, soil temperatures would average 15°C or lower which is about 5°C lower than the anticipated optimum temperature for growth of *B. brongniartii* in South Africa. Even if fungal growth was only slowed during autumn application, sporulation may never be achieved and thus increases in the level of fungal inoculum in the soil may be too low to elicit host infections (Kessler *et al.* 2003). Further, weather information showed that uncharacteristically high winter rainfall is possible in the KZN Midlands North which would rapidly decrease formulated mycoinsecticides in the soil and limit persistence of a product (Chapter 5). Thus autumn applications are considered less effective and in the light of grower's perceptions (Chapter 6), only one application per year would be acceptable to them, thus a late spring application is suggested.

Biological control using mycoinsecticides will always be considered a prophylactic treatment because initial control is usually too slow to prevent extensive damage by white grubs if numbers are high (Milner, 1997). Thus, is it suggested that areas which experience high white grub densities should consider using chemical control in conjunction with mycoinsecticides. For example, in Australia and Reunion, where large infestations of white grubs are known to occur, integrated pest management (IPM) in sugarcane usually relies on a suite of different tactics which include chemical and mycoinsecticide control (Milner, 1997; Allsopp, 2010). In both countries, a controlled-release, granular formulation of chlorpyrifos (suSCon® Blue) or imidacloprid (suSCon® Maxi) is applied in furrow at planting which provides initial

knockdown kill of white grubs. Simultaneously, granules of the mycoinsecticides, BioCane® (Australia) or Betel® (Reunion) are also applied in furrow for long term control (Milner, 1997; Allsopp, 2010). In areas where white grubs exhibit low-to-moderate larval populations, the mycoinsecticide may be applied alone in furrow at plant or to subsequent ratoon crops as is done in Australia (Allsopp, 2010).

A key factor in the successful development of a mycoinsecticide and its adoption is the type of formulation that is used, which should be effective, cheap and presumably use existing equipment (Milner, 1997). To date, there are eleven recognised formulation types, with technical concentrates (fungus-colonized substrates) (26.3%), wettable powders (20.5%) and oil dispersions being the most common (Faria and Wraight, 2007). The high cost factors associated with mycoinsecticides tend to discourage growers (Bailey *et al*. 2010). If a granular formulation of *B. brongniartii* was developed and implemented in soil to control white grub larvae at similar rates to those abroad the cost would amount to approximately R2000 or US $233 per ha in South Africa, which is simply not viable for microbial control companies or growers (Morris, pers. comm.). Further, the lack of perception of white grubs as major pests in sugarcane will limit the viability of a granular mycoinsecticide (Chapter 6). Thus other formulation types using *B. brongniartii* as the active ingredient should be addressed and tested for efficacy in the field. Substantial coverage using oil or aqueous formulations of the fungus for example, may be achieved in sugarcane which would amount to approximately R500 or US $58 per ha (Morris, pers. comm.). While substantial coverage may be achieved however, the persistence and efficacy of oil or aqueous formulations still need to be assessed, as these formulations generally do not carry conidia further than the top few centimeters of the soil (Jaronski, 2010). Based on the results of other studies it is likely that persistence of aqueous formulations will generally be lower than oil formulations (Ekesi *et al*. 2005; Jaronski, 2007). Oil formulations in particular have been very successful. During the LUBILOSA project the importance of fungal formulation was realised when the *M. acridum* based mycoinsecticide, Green Muscle™ was formulated as an oil-based product (Lomer *et al.* 1999; Langewald *et al*. 2003). Formulation was important because it allowed the persistence of Green Muscle™ and efficacy of the product under hot, dry desert conditions (Lomer *et al.* 1999; Langewald *et al*. 2003). It is thus suggested that oil formulations of *B. brongniartii* be investigated for inundative soil control, particularly in light of summer temperatures which may limit the persistence of a mycoinsecticide in some hotter parts of the sugar industry.

7.6 SOCIO-ECONOMIC AND MARKET ANALYSIS

Chapter 6 suggested that sugarcane growers in the KZN Midlands regions were not likely to adopt a fungal mycoinsecticide. Growers in these regions generally exhibited a positive attitude towards mycoinsecticides, and showed all the relevant attributes for successful technology adoption. However, the reduced feasibility of application, general lack of potential demand for a product, high cost factors, and most importantly the lack of pest perception would negatively affect growers' attitudes towards a mycoinsecticide. It is not uncommon for bio-pesticides to be superfluous at this stage of the development of a product (Bailey *et al.* 2010; Leng *et al.* 2011). Researchers often pursue academic exercises and interests in bio-pesticides but may not be able to overcome the challenges in product development, registration, commercialization or market (Bailey *et al.* 2010). Bailey *et al.* (2010) suggested that 'the key to successful product adoption lies with the development of a production system which allows for scale-up of a quality product that someone wants to buy.' An example of a product which failed at the market level is the mycoherbicide, BioMal® which was registered to control the prolific weed, *Malva rotundifolia*, in field crops (Boyetchko *et al*. 2007). The product however, was not successfully adopted in the market place because of issues with scale-up and market size (Boyetchko *et al*. 2007). In contrast, in Canada, a bio-pesticide product Chontrol® based on the fungus, *Chondrostereum purpureum*, is registered for use in forestry for the inhibition of stump resprouting in Sitka and red alder trees (Bailey *et al.* 2010). This product was quickly adopted by the market because the forestry sector was under increasing pressure to move away from conventional herbicides, leaving few alternatives for vegetation management (Bailey *et al.* 2010). Importantly, this product had similar pricing to alternative control tactics and for that reason has also become a successful technology (Bailey *et al.* 2010).

What will likely increase the adoption of a *B. brongniartii* based mycoinsecticide is a broader-spectrum activity against a range of scarab pests and the use of the potential product in many different types of crops, as there is currently no anticipated market in sugarcane. Both *H. sommeri* and *S. affinis* are not only pests in sugarcane (Carnegie 1974; Carnegie 1988) but are also serious pests of *A. mearnsii* and *Eucalyptus* sp. (Prins, 1965; Govender, 2007; Harrison, 2013) and other crops (Petty, 1976; du Toit, 1997). Further, many growers in the KZN Midlands regions cultivate both sugarcane and *A. mearnsii*, and commented that they undertake chemical control in *A. mearnsii* plantations particularly when seedlings are

establishing (Chapter 6). However, with the recent banning of Deltamethrin in the forestry industry, many growers have been left with no alternative control strategies against white grub pests (Norris, pers. comm.). Similarly, there are reports of white grubs causing serious damage to soybeans in the Bergville, 28° 44'S; 29° 22'E (1073 m), South Africa, and growers are also in need of alternative control strategies (Morris, pers. comm.). Thus a mycoinsecticide would offer growers in forestry and soybeans an alternative form of control, and likely increase the demand for the product albeit in alternative crops first. If there are no doubts about the efficacy of the formulation and growers establish that the product works, area-wide adoption or adoption in sugarcane is more likely.

Although there is a growing demand for bio-pesticides worldwide (Bailey *et al.* 2010; Leng *et al.* 2011), there is insufficient awareness of these emerging technologies because the modes of action of many bio-pesticides are so different compared to conventional insecticides (Bailey *et al*. 2010). For bio-pesticide adoption to be successful and embraced by farmers, there are a number of aspects which must be considered. It is necessary to inform farmers about the availability of new products and educated them about the way a specific product is used (Bailey *et al.* 2010). A considerable challenge has been informing farmers that bio-pesticides are products that actually do work (Moser *et al.* 2008). It appears that simple, stand-alone technologies are much easier to understand and more readily adopted by farmers than technologies which form part of a large pest management package (Williamson, 1998). Furthermore, experimental learning through demonstration trials is the best way to change farmers' attitudes and control practises; this may be done by stewarding growers' organisations and provincial experts to set up trial plots for field-site demonstration training (Williamson, 1998; Cockburn *et al.* 2012). Fact sheets describing new technologies or discoveries can be handed out at growers' workshops or demonstration trials (Bailey *et al.* 2010). This in particular was achieved during the current thesis when an article describing the occurrence of *B. brongniartii* in the Midlands regions and its effect of the pest, *H. sommeri* was handed out at various workshops and grower days (See published articles). By involving the end-user in the planning, design and testing of the technology it often results in a much higher chance that farmers, as end-users, will buy or adopt the new technology (Williamson, 1998). Further adoption of mycoinsecticides is likely if there are (1) development of better products, (2) development and implementation of truly integrated pest management strategies in which biological options are emphasized (Lomer *et al*. 1999; Lacey *et al.* 2001), (3) the ability of bio-pesticide manufacturers/retailers to maintain marketing and product support

teams, (4) cultural changes (acceptance by farmers of slow-acting, narrow-host-range products), and (5) sound, knowledge- based recommendations for product use (Bailey *et al.* 2010; Leng *et al.* 2011).

7.7 FINANCIAL COLLABORATION AND REGISTRATIONS

What the socio-economic study (Chapter 6) showed was that a consortium of partners is required for the successful development of a mycoinsecticide. In many respects this thesis was a collaboration between three industry partners. The South African Sugarcane Research Industry identified a pest problem; the Agricultural Research Council-Small Grains Unit initially screened fungal pathogens for white grub control in 2005; and the microbial control company Plant Health Products was available to produce various formulations (Hatting, 2008). What is now required is a consortium of partners which should include the forestry industry (NCT Forestry Cooperative Limited) and other field crops (The South African Soybean Value Chain) because the main challenges in the commercialization process are still ahead. These will include (1) developing a fermentation strategy for product quantity and shelf life, (2) controlling the product identity and efficacy, (3) controlling product purity and safety, (4) keeping down the costs for growth media and formulation components, (5) integrating the new product into existing application methods of the end user, and (6) pricing of the product for the end user (Bailey *et al.* 2010). This requires cooperation between companies to reduce production costs and enhance competitiveness (Leng *et al*. 2011). Merging, reorganising and association of strong enterprises are one of the trends in the development of safe and efficient pesticides and has seen these strategies applied to large companies such as Bayer, Syngenta, BASF, DuPont, Dow AgroSciences, and Monsanto (Leng *et al*. 2011). Further, cooperation with foreign enterprises will introduce new products, make novel technologies and optimize existing ones, enhance product quality, saves resources, reduces costs, enhances the market competitiveness and expand exports (Leng *et al.* 2011). By strengthening relationships with foreign enterprises, the encouragement and support of bio-pesticides is enhanced (Leng *et al.* 2011).

Before the discovery of *B. brongniartii* in Chapter 3 the occurrence of this fungal species on scarabaeid species in South Africa was not previously known [21](Truter, pers. comm.). This meant that the importation of exotic *B. brongniartii* strains would have been stopped until evidence of the fungal species existence in the country was shown or an application following rigorous research on non-target and toxicology effects was produced. For small mycoinsecticide companies aiming to development a range of niche products, the cost of toxicity testing is at least US $150,000 and could represent a serious constraint to registering new mycoinsecticide product (Leng *et al.* 2011). Therefore, cheaper product registration costs are an advantage to the use of native fungal strains. It is possible to register native fungal strains under agricultural remedies in terms of Act. No 36 of 1947 in South Africa (Morris, pers. comm.). Following strain registration, successful field data over a two year time period are required for the registration to be completed. However, if the fungal strain is exotic, an import permit and permission to release document must be obtained from the Quarantine Division of the Department of Agriculture, Fisheries and Forestry in South Africa (Morris pers. comm.). This aspect is managed by the Biodiversity Act which is administered by the Department of Environmental Affairs in South Africa and can be very complicated, time consuming and expensive (Morris pers. comm.). Therefore this thesis has also contributed towards the cost effective registration of a potential mycoinsecticide.

7.8 RECOMMENDATIONS

The study was limited in various aspects of *B. brongniartii's* host range, physiological information on strains, such as temperature profiles and growth rates and other application technologies such as attractive traps. Further, ecological and molecular studies alluded to the possibility of predicting pest population dynamics using variations in crop height at the time of beetle emergence. Lastly, growers' perceptions highlighted a key knowledge gap in the limited knowledge of the economic impact associated with white grubs in the sugar industry. Therefore, the following recommendations for future research are made:

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7.8.1 *Physiological fungal strain exploration studies*

It is well-known that different fungal strains vary considerably in their temperature profiles which ultimately govern their vegetative growth and sporulation potential in the field (Callot *et al.* 1996; Jaronski, 2007). The present study was limited in that *B. brongniartii* isolates were only obtained from the KZN Midlands North, an area characterized by average cooler temperatures. This suggests that strains obtained from this region may be adapted to this cooler environment and thus is unclear whether the temperature profiles of these obtained isolates would prevail under more extreme temperatures in other areas of sugar industry. *Aregger-Zavadil, 1992* reported by Kessler *et al.* (2003) found that European strains of *B. brongniartii* grown on Sabouraud-dextrose-agar and on barley kernels grew vegetatively at temperatures between 2 and 27°C, sporulated between 5 and 28°C and the optimum growth temperature lay between 20 and 25°C. However, researchers in Reunion Island found that *B. brongniartii* isolate Bt96 grew optimally at higher temperatures of 25-28°C (Vercambre *et al.* 1994; Callot *et al.* 1996). It is thus recommended that candidate strains be physiologically evaluated to ascertain their temperature profiles so that if future applications succeed or fail this aspect of fungal physiology could explain such occurrences.

7.8.2 *Host range against other scarab species*

The literature suggests that strains of *B. brongniartii* are effective against melolonthid hosts (Keller, 1986; Vercambre *et al.* 1994; Townsend *et al.* 1995; Strasser and Pernfuss, 2005; Srikanth *et al.* 2010). *Beauveria brongniartii* is able to infect a number of known melolonthid pest species in South African sugarcane but it is unclear at this stage whether the host range of native *B. brongniartii* strains could include other melolonthids or other subfamilies such as the Rutelinae and Dynastinae. It is crucial that these *B. brongniartii* strains are tested against key sugarcane white grub pests such as *H. licas*, *Adoretus* species, *A. ustulata*, *A. subfasciata* and other species of *Schizonycha* sp. Further, this improved information on host range will likely improve the feasibility and adoption of a mycoinsecticide in sugarcane and other crops such as *A. mearnsii*.

7.8.3 *Adult trap technology*

Based on the finding that *B. brongniartii* is being cycled from arboreal habitats to subterranean soil environments by *H. sommeri* and given the increased susceptibility of *S.*

affinis adults to some strains of the fungus compared to grubs, future work should investigate the use of attractant traps formulated with *B. brongniartii*. Such an approach has been investigated for *M. anisopliae* against *P. japonica* beetles in the Azores (Klein and Lacey 1999). Infecting adult beetles with the fungus in attractant traps and then allowing them to disseminate spores could potentially augment natural fungal infections in crop soils as infected adult beetles make their way back to these soils to lay eggs. When the adults die in the soil they become an infective focal point for white grub larvae in the soil and the fungal disease can progress. This field-based technology (traps) offers farmers a cheap, effective and area-wide method for controlling white grubs in sugarcane and *A. mearnsii*. Research in the biological control of forest cockchafer, *M. hippocastani* in Germany showed that male beetles could act as vectors when they were contaminated by *B. brongniartii* spores after passage through an attractant trap (Koller *et al*. 2005). They showed that adult male beetles were highly attracted to the combination of green leave volatiles and the sex pheromone 1, 4-benzoquinone. The use of chemical lures makes the traps very specific to the species of white grub we want to target and reduces non-target effects (Ruther *et al.* 2000; Ruther and Hilker, 2003; Reinecke *et al.* 2006 a; Reinecke *et al.* 2006 b). Research showed that traps could catch up to 260 males in a couple of hours and that the height of the trap in the tree was also significant (Koller *et al*. 2005). The number of captured males increased with the trap height within a tree. Male infection rates were significantly increased from transfer of spores from the inoculation traps. The transfer of fungal inoculum from males to females was also shown (Koller *et al*. 2005).

7.8.4 *Studies on the economic impact of white grubs*

The adoption of a mycoinsecticide in sugarcane in South Africa will rely on studies and reports on the economic losses associated with white grubs in the KZN Midlands regions and elsewhere in the sugar industry. Growers perceived very little damage to sugarcane and are thus unable to make educated decisions about future control technologies. Apart from a few studies undertaken in South Africa (McArthur and Leslie, 2004) and Swaziland (Sweeney, 1967) there are no accurate figures of yield losses associated with white grubs. It is thus highly recommended that such studies be undertaken on an industry-wide base but if this is impractical then it is suggested that focus takes place in eco-zones 3, 4 and 7 in the KZN Midlands North, areas which were identified by Way *et al.* (2011) as the worse affected by white grubs.

7.8.5 *Predicting white grub occurrences and subsequent control action*

This study was limited in that the population dynamics of *H. sommeri*, *S. affinis* and some other scarab pests were restricted to one site. It was also limited in that only white grubs and adults in the soil were considered and not the dispersing adults which drive populations dynamics. Molecular investigations (Chapter 3) showed that *H. sommeri* beetle movement occurred frequently between sites in the KZN Midlands North. Damage by *H. sommeri* and *S. affinis* may be predicted by the occurrence of tall *A. mearnsii* stands at the time of beetle emergence and aggregation. Dalthorpe *et al.* (2000b) discovered that large contiguous areas of woodland, could present barriers to white grub beetle migration. *Acacia mearnsii* stands are harvested approximately every seven years and sugarcane approximately every 18-24 months. The transient nature of tall *A. mearnsii* stands can act as barriers to beetle movement and dispersal in some years. However, when these stands (barriers) are harvested, passages for beetle movement and dispersion may open up distributing the adults further within the region. Horsfield *et al.* (2002) for example, discovered that late-planted or late-harvested fields were less damaged than fields which had been planted or harvested earlier because beetles were clumsy flyers and tended to fly into and hit taller sugarcane and drop into the soil next to taller fields to oviposit.

Therefore, emerging and dispersing adults in the Midlands regions may be similarly attracted to taller *A. mearnsii* stands where they feed and mate. Subsequently, ovipositing females may then fly back into sugarcane fields which lie adjacent to these trees. This suggests that sugarcane fields which lie adjacent to such *A. mearnsii* stands would have higher occurrences of white grubs in the sugarcane soils. Most KZN Midlands North growers have land use plans which determine which crops (sugarcane and *A. mearnsii*) are planted where and when these fields will be harvested. Thus the assimilation of all or even some of these land use plans in this region would highlight when tall *A. mearnsii* stands would be available at the time of beetle emergence and a predictive model could be generated. Once this predictive model had been established, investigatory studies on white grub abundances in sugarcane fields that lie adjacent to such predicted *A. mearnsii* stand could be undertaken on some of these farms. Based on historical records, the Seven Oaks district may be a good starting point to generate information about the population dynamics of *H. sommeri* and the Mid-Illovo district of the Midlands South for *S. affinis* population dynamics to be undertaken (Carnegie *et al.* 1974). Such a predictive model on the population dynamics of white grubs and subsequent damage estimates would greatly enhance our ability to target applications cheaply and effectively.

7.9 FINAL CONCLUSIONS

It was the intention of this thesis to provide initial research and insight into the development of a mycoinsecticide to control important scarabaeid pests in sugarcane in the KZN Midlands regions. It is recommended that because *B. brongniartii* epizootics were recorded on one of the target pests, *H. sommeri*, which indicated good host specificity, dispersal ability and persistence of the fungus in the intended environment of application; that a future mycoinsecticide should include an aggressive and environmentally stable strain of *B. brongniartii* as the active ingredient. This is further supported by the fact that *B. brongniartii* strains exhibited good pathogenicity towards the grubs and adults of *S. affinis* and literature has shown that *B. brongniartii* is a successful pathogen of melolonthid scarabaeid pests in sugarcane. It is recommended that prophylactic spring applications of a mycoinsecticide be made in areas with lower white grub abundances, and that areas which experience high white grub densities should consider using chemical control in the form of controlled-release granular formulations of chlorpyrifos (suSCon® Blue) or imidacloprid (suSCon® Maxi) applied in furrow at planting which will provide initial knockdown kill of white grubs, in conjunction with mycoinsecticides. Spring applications will ensure that the most susceptible scarabaeid life stages are targeted. Environmental variables which will likely affect mycoinsecticide persistence are extremes in temperatures and moisture and soils which are high in organic matter (<3%). The presence of insect hosts will greatly enhance persistence of a mycoinsecticide. The general lack of pest perception by growers' would likely limit adoption of a mycoinsecticide in sugarcane, but there is scope for collaboration between various industries partners to increases market potential in other crops such as *A. mearnsii* and soybeans.

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