

**Development of *Beauveria brongniartii* as a
Bio-Insecticide to Control White Grub
(Coleoptera: Scarabaeidae) Species
Attacking Sugarcane in South Africa**

by

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degree of Master of Science in the School of Life Sciences,
Discipline of Entomology, University of KwaZulu-Natal,
Pietermaritzburg, South Africa

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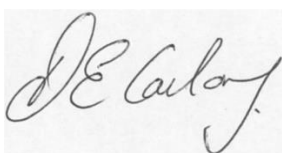
*To my mother
Celephi Ngcobo,
For enormous love and support.*

PREFACE

The research contained in this dissertation was completed by the candidate while based at the Crop Biology Resource Centre of the South African Sugarcane Research Institute, and registered with the School of Life Sciences of the College of Agriculture, Engineering and Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was financially supported by the South African Sugarcane Research Institute (SASRI).

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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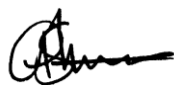
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I, Nozipho Kheswa, declare that:

(i) The research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) This dissertation has not been submitted in full or in part for any degree or examination to any other university;

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(v) Where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) This dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

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DECLARATION 2: PUBLICATIONS

This work was presented in symposia and a local congress orally, and as a peer reviewed paper in the proceedings of the congress. The * indicates corresponding author.

Symposiums

South African Sugarcane Research Institute Post-graduate symposium (03 December 2015)

Oral: Development of *Beauveria brongniartii* as a Bio-insecticide to Control White Grub (Coleoptera: Scarabaeidae) Species of Sugarcane in South Africa. Kheswa N*, Conlong D and Laing M

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ABSTRACT

White grubs (Coleoptera: Scarabaeidae) are serious pests of sugarcane. Their larvae are soil-dwellers, whilst adults are free flying. Larvae feed on sugarcane roots, thereby damaging the crop and this damage is associated with substantial loss of quality and yield. Different control measures, such as the use of chemical insecticides and cultural practices have been employed to control white grub infestations. However, none of these showed satisfactory results as larvae are cryptic, and patchy in distribution. Alternatively, entomopathogenic fungi such as *Beauveria bassiana* and *Beauveria brongniartii* show promise as biological control agents (BCA), as they occur in the soil and are proven control agents against many crop pests, including white grubs. In the South African sugarcane industry, indigenous isolates of *B. brongniartii* (C17 and HHWG1) were highly pathogenic against *Schizonycha affinis* and *Pegylis sommeri* in the Midlands North region of KwaZulu-Natal.

In order to assess *B. brongniartii*'s host range, it was therefore critical to know whether the C17 and HHWG1 isolates were pathogenic against other white grub species affecting sugarcane found in different regions of South Africa. Hence, the pathogenicity of these isolates were evaluated against larvae and adults of *Heteronychus licas*, *Asthenopholis minor*, *H. tristis* and *Temnorhynchus clypeatus*; and larvae of *Schizonycha neglecta*. Individuals of all species were inoculated with 1×10^9 conidia/ml concentration of both isolates in laboratory bioassays, and the effect of the isolates was evaluated over time. The results indicated that *S. neglecta*, *H. tristis* and *T. clypeatus* larvae were most susceptible to HHWG1 and had 80-90% mortality overall, compared to 5-60% mortality when treated with C17. In contrast, adults of *T. clypeatus*, *H. licas*, *A. minor* and *H. tristis* were highly susceptible to C17 (60-80% mortality) compared to HHWG1 (10-45% mortality). It was concluded that both C17 and HHWG1 isolates have potential as bio-insecticides against adults and larvae of white grubs, respectively, as they have a wider host range than just the species from which they were collected. It was further concluded that both isolates need to be tested in replicated field trials to confirm their suitability as potential bio-insecticides.

Although *B. brongniartii* isolates are thus pathogenic against a number of white grub species in South Africa, they have never been recorded or established as endophytes of plants for long term protection against pest insects, except for one record from coffee (*Frangula californica*) in Hawaii. In this study the potential of *B. brongniartii* isolates to be established as endophytes of sugarcane roots for protection against white grub species was examined by conducting a

glasshouse trial. N12 and N48 sugarcane variety setts were treated with C17 and HHWG1 inoculum at 1×10^7 conidia/ml concentrations using a dip inoculation method and were planted in sugarcane seedling trays using a randomised complete block design. To assess endophytic colonization of C17 and HHWG1 isolates in sugarcane roots, one month after dipping and growing, sugarcane seedlings had their roots disinfected with 10% Sodium hypochlorite, 70% ethanol and distilled water. Characters of fungal colony establishment, conidia and conidiophores were searched for in the roots of the inoculated sett material, using a microscope, and several representative micrographs were taken. These showed no *Beauveria* species characteristics. Other fungi (*Fusarium* spp. and *Penicillium* sp.) were detected as endophytes of sugarcane roots. Factors such as inoculation method and aggressive antagonistic species may have played a role in preventing *B. brongniartii* from becoming established as an endophyte. It was concluded that future research should focus on finding alternative control measures, such as known endophytic *B. bassiana* isolates, for long term protection against white grubs.

As a final step before consideration as a potential commercial bio-insecticide, the efficacy of *B. brongniartii* as a pathogen of white grubs in field trials had to be evaluated. Here the pathogenicity of C17 and HHWG1 on a species of white grub was evaluated in pot trials under field conditions, and the resultant impact on seedling growth determined. In these pot trials, C17 and HHWG1 inoculum was applied as a soil drench and root dip inoculation. Three small and three large white grub larvae of *Schizonycha affinis* each were introduced 5 cm deep into soil in their respective pots containing sugarcane seedlings, and the pots were inoculated with 100 ml of C17 and HHWG1 at 1×10^9 conidia/ml suspension, 100 ml insecticide (as a positive control) and distilled water with 0.05% Triton-X (as a negative control).

Beauveria brongniartii isolates and insecticide were effective against the larvae of *S. affinis*. However, insecticide was more effective in the early stages of the trial, and caused mortality within the first 3 days after treatment (DAT) as compared to the fungal isolates which took longer to cause mortality of the larvae. It was observed that insecticide was highly virulent against small larvae, causing 100% mortality within 6 DAT as compared to 60% mortality against large larvae. Although insecticide was highly virulent from the first few DAT, there was no significant difference ($P > 0.05$) in mortality of the larvae between the HHWG1 isolate and insecticide at 21-30 DAT. Both isolates of the fungus were pathogenic against the larvae, and there was no significant difference ($P > 0.05$) between mortality of the small and large larvae. Seedlings in the control (with larvae but no treatments) were heavily grazed, with 80-93%

reduction of root dry weight (DW) as compared to 0-10% reduction in the insecticide and 10-24% in the HHWG1 treated seedlings. Consequently, sugarcane seedling biomass recorded from the control in the presence of *S. affinis* larvae was lower than the seedling biomass recorded in the HHWG1 and insecticide treated pots. Hence, insecticide and HHWG1 exhibited some protection against the larvae of white grubs.

It was concluded that the locally discovered isolates HHWG1 and C17 of *B. brongniartii* remain potential bio-control agents for white grub species attacking sugarcane in South Africa. Even though they could not be established as endophytes, this study showed that they did cause significant mortality of different life stages of a number of local white grub species attacking sugarcane. In addition, in pot trials it was shown that these isolates, especially HHWG1, were as effective at killing as many larval stages of *S. affinis* as insecticides, but only after a period of time, and also afforded the treated sugarcane plants as much protection against root feeding, as did the insecticide treatments. The further development of these isolates, through registration and longevity trials, as a commercial bio-insecticide product is thus recommended.

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Lastly, my sincere gratitude goes to the South African Sugarcane Research Institute (SASRI) for opening their doors for me and for funding my research project.

“Ukubekwezela kuzala impumelelo”
“Patience is the mother of success”

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CHAPTER 1: INTRODUCTION

1.1 Problem statement

Pests contribute to 80% of sugarcane yield losses (McArthur and Leslie, 2004), and white grubs (Coleoptera: Scarabaeidae) are amongst the most destructive of these sugarcane insect pests (Avasthy, 1967). The larvae of white grubs feed on sugarcane roots and some white grub adults (e.g. *Heteronychus licas* Klug) feed on the young shoots of sugarcane plants, thus reducing vigour, yield, and sugar content, and increase chances of pathogen infections (Allsopp *et al.*, 1991; Chelvi *et al.*, 2011; Way *et al.*, 2011; Cock and Allard, 2013; Gyawaly *et al.*, 2016). The larvae have three immature life stages, viz. first, second and third instar. The final instar is the most damaging stage to sugarcane roots (Way, 1997). The number of larvae found underneath one sugarcane stool can range between 3-25 grubs in highly infested areas (Way *et al.*, 2013). More than 5 different species of white grub have been found attacking sugarcane in South Africa (Dittrich *et al.*, 2006; Dittrich-Schröder *et al.*, 2009).

White grubs cause an estimated loss between 23 and 55 tons/ha of sugarcane in South Africa (McArthur and Leslie, 2004). Cherry (2008) reported 39% reduction of sugarcane yield at harvest in heavily white grub infested fields in Australia. This is a serious constraint to the production of sugarcane in all sugarcane producing countries (Allsopp *et al.*, 1991; Goble, 2012) and immediate interventions for controlling white grub species are thus necessary across the world and in South Africa.

Pest control studies in sugarcane have mostly focused on the control of lepidopteran stem borers (Khan *et al.*, 2014; Vargas *et al.*, 2015). However, for the past few years white grubs have frequently been reported as serious pests of sugarcane in South Africa (Way, 1997; McArthur and Leslie, 2004). White grubs have a long complex life cycle spanning between 12 and 24 months and this makes them difficult to control (Sweeney, 1967; Wilson, 1969). Chemical insecticides such as organochlorides, aldrin, dieldrin, heptachlor (Niemczyk and Lawrence, 1973); DDT, malathion, endrin (Kaunsale *et al.*, 1978) and confidor (Conlong and Mugalula, 2003), have been either tested and/or used to alleviate white grub infestations. However, they were found to be generally ineffective since white grub larvae are protected in the soil and the insecticide does not reach the larvae below the soil (Niemczyk and Lawrence, 1973). Furthermore, high usage of chemical insecticides can lead to residues of harmful substances in the soil that pose a danger for non-target organisms and the environment (Lacey *et al.*, 2001;

Guzmán-Franco *et al.*, 2012; Bhandari, 2014). Consequently, chemical insecticide applications have been banned in most agricultural areas (Bhandari, 2014). Because of these constraints, insecticides should be used as a supportive control measure rather than as a principal solution with unrestricted use (Bhandari, 2014). Moreover, cultural control methods, such as crop rotation, weed control, raising cutting height and mid-season plowing have also provided little protection against white grubs (Potter *et al.*, 1996; FIELD CROPS IPM, 2009). With the goal to minimise the use of insecticides to control white grubs, attempts have been made to find alternative, environmentally friendly control measures, such as the use of biological control agents (BCAs) (Lacey *et al.*, 2001; Guzmán-Franco *et al.*, 2012). This is the usage of natural agents, including pathogens (such as entomopathogenic fungi) that have potential to control the population density of a particular pest (Alston, 2011).

Contrary to agrochemicals, entomopathogenic fungi (EPFs) are highly persistent, and they infect many insect species (Zimmermann, 2007). In addition, EPFs are environmentally friendly, cost effective, and non-hazardous to humans and animals (Samson *et al.*, 2006; Zimmermann, 2007; Fegrouch *et al.*, 2014). Fungal isolates of *Beauveria brongniartii* (Sacc.) Petch (Deuteromycotina: Hyphomycetes), serve as a potential biological control for white grubs (Keller *et al.*, 1999; Zimmermann, 2007; Mane and Mohite, 2015). *Beauveria brongniartii* isolates have been identified to cause mortality against white grub adults and larvae in countries such as Australia, India, Switzerland and South Africa (Keller *et al.*, 1999; Kessler *et al.*, 2004; Dolci *et al.*, 2006; Goble *et al.*, 2012). Strains of *B. brongniartii* have since been registered as commercial products to control white grub species in a number of countries (e.g. India, Reunion Island and Australia) (Enkerli *et al.*, 2004; Zimmermann, 2007; Goble *et al.*, 2016).

Furthermore, Goble *et al.* (2012) identified a number of *B. brongniartii* isolates affecting adults and larvae of *Pegylis sommeri* Burmeister (Coleoptera: Scarabaeidae) (previously known as *Hypopholis sommeri*) in sugarcane, for the first time in South Africa. She tested these isolates against adults and larvae of *Schizonycha affinis* Boheman (Coleoptera: Scarabaeidae) affecting sugarcane in the Midlands North region of KwaZulu-Natal, using laboratory bioassays (Goble *et al.*, 2015). Amongst these *B. brongniartii* isolates, C17 and HHWG1 showed great potential for control of these scarabs, due to their high virulence, causing 80 and 95% mortality of *S. affinis* adults and larvae, respectively (Goble *et al.*, 2015).

1.2 Aims and objectives

The general aim of this study was to build on and expand the work of Goble *et al.* (2012), to develop her two native and most virulent *B. brongniartii* isolates (C17 and HHWG1) as potential commercial bio-insecticides against immatures and adults of white grub species occurring in the South African sugarcane industry.

To attain the above aim, the specific objectives of the study were as follows:

- i. To test the infectivity of *B. brongniartii* isolates C17 and HHWG1 in laboratory bioassays against adults and larvae of other white grub species occurring in South African sugarcane growing areas, thereby assessing the host range of *B. brongniartii*;
- ii. To test C17 and HHWG1 isolates for their potential to become established as endophytes of sugarcane roots for long term protection against white grub larvae; and
- iii. To evaluate the efficacy of C17 and HHWG1 isolates to control *S. affinis* larvae in simulated field conditions in pot trials using different pathogen inoculation methods.

1.3 Thesis general overview

This thesis is made up of six chapters. The first chapter gives an overview of the current problem, explores what has been achieved so far, and outlines the aims and objectives of the current study. The following chapter (Chapter 2) is a literature review, highlighting the impact of white grubs globally and in South Africa, and the control measures that have been used to reduce their impacts. White grub collections comprising different species, from different South African sugarcane growing locations, were screened against two *B. brongniartii* isolates C17 and HHWG1 and this formed the basis of Chapter 3. Chapter 4 focuses on the potential of the *B. brongniartii* isolates to be established as endophytes of sugarcane roots and trials attempted to do this. Preliminary soil applications of *B. brongniartii* isolates and insecticide against white grub larvae were carried out in pot trials, and their impact on sugarcane growth evaluated. The results and discussion are presented in Chapter 5; and lastly general conclusions and future directions are given in Chapter 6.

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CHAPTER 2: LITERATURE REVIEW

2.1 White grubs as pests of sugarcane

Sugarcane is a vital cash crop cultivated mainly for its sucrose to produce sugar (Cherry, 2008; Goble, 2012; Singels *et al.*, 2015). There are over 100 countries which produce sugarcane for commercial use (FAOSTAT, 2013). However, sugarcane production is affected by plant pathogens, insect pests and other biotic and abiotic conditions. Worldwide, more than 200 pests cause serious problems for sugarcane production (Cherry, 2008; Chelvi *et al.*, 2011; Cock and Allard, 2013). The damaging pests of sugarcane found in South Africa are grouped into three ecological categories: (1) soil insects, (2) sap suckers and leaf feeders, and, (3) stem borers (Goble, 2012). Amongst the soil insects, white grub species (Coleoptera: Scarabaeidae) remain important pests in sugarcane (Goble *et al.*, 2012; Cock and Allard, 2013). The common name “white grubs” is used to identify or name the soil dwelling larvae of scarabaeid species feeding on plant roots (Allsopp *et al.*, 1991; Way *et al.*, 2013; Goble *et al.*, 2015). The most problematic white grub species belong to the subfamilies Dynastinae, Melolonthinae, and Rutelinae. These subfamilies have also been identified in Kenya (Harrison, 2009; Hajek *et al.*, 2005), Uganda (Harrison, 2009), Tanzania (Harrison, 2009), and in different South African regions (Dittrich *et al.*, 2006; Dittrich-Schröder *et al.*, 2009, Goble, 2012). The level of white grub infestations varies between different regions and countries (Keller, *et al.*, 1999).

In the KwaZulu-Natal (KZN) Midlands sugarcane growing region of South Africa, white grubs were originally associated with black wattle trees (*Acacia mearnsii* De Wild., Fabales: Fabaceae), before the land was used for sugarcane cultivation (Carnegie, 1974a). After that, sugarcane became a secondary host plant for white grubs in the region, which have now become seriously damaging pests of sugarcane (Carnegie, 1974a). White grub adult females deposit their eggs in the soil, next to the sugarcane stool thereby providing the emerging larvae with a food supply (Carnegie, 1974a). The damage caused by white grubs is easily detected by examining the roots, or by the yellowing of leaves and stem lodging where the stems no longer exhibit the typical erect, parallel habit of healthy sugarcane plants (Carnegie, 1974a).

White grub infestations have been reported from various sugarcane growing industries worldwide (Way, 1997). Several studies have reported yield reduction due to high white grub infestations in sugarcane (Wilson, 1969; Sosa, 1984; Carnegie, 1988; Allsopp *et al.*, 1991; Allsopp, 1995; McArthur and Leslie, 2004). McArthur and Leslie (2004) found an average of

23–55% reduction in sugarcane yield (tons cane/ha) in the KZN Midlands North area after taking into consideration three factors: (1) sugarcane variety, (2) season, and (3) white grub infestation level. In Florida, United States of America (USA), 39% sugarcane yield reduction was reported due to high white grub infestations (Raid and Cherry, 1992). In Australia, 80–100% sugarcane damage is associated with white grub infestations (Chelvi *et al.*, 2011). White grub infestations are thus a serious threat to sugarcane production and sustainable control measures are urgently needed to prevent reductions in sugarcane yield both locally and worldwide.

Biological control agents (BCAs) are critical regulators of various agricultural pests, including white grubs (Zimmermann, 2007). There are several advantages of using BCAs compared to the use of chemical insecticides. The BCAs, particularly entomopathogenic fungi (EPFs) are natural pest regulators, they multiply very rapidly within the host and are persistent within the environment in which they occur (Zimmermann, 2007). *Beauveria brongniartii* (Sacc.) Petch (Ascomycota: Clavicipitaceae) is an entomopathogenic fungus with virulent isolates causing significant mortality in a number of white grub species (Keller *et al.*, 1997; Strasser *et al.*, 2000; Zimmermann, 2007; Chelvi *et al.*, 2011; Goble *et al.*, 2012). Closer to home, native virulent isolates of *B. brongniartii* were found causing epizootics in two white grub species, *Pegylis sommeri* Burmeister and *Schizonycha affinis* Boheman (Coleoptera: Scarabaeidae: Melolonthinae) populations found in the KZN Midlands North sugarcane growing area (Goble *et al.*, 2012). Goble *et al.* (2015) tested *B. brongniartii* isolates against these white grub species and found that mortality of both immature and adult white grub species increased with the increasing conidia concentration of *B. brongniartii* isolates, and the virulent isolates at 1×10^9 conidia/ml induced at least 80% white grub mortality.

Understanding the biology of natural control agents of white grubs, and the white grubs themselves, is vital and serves as a step towards developing control methods to lessen the pest's detrimental effects on sugarcane (Conlong and Rutherford, 2009). At a later stage, the developed biological control strategy can be included in the integrated pest management (IPM) strategies against pests in sugarcane.

2.2 Biology of white grubs

The Scarabaeidae is the largest and most widespread family in the order Coleoptera (Mishra and Singh, 1999). It comprises some 35,000 known species belonging to seven subfamilies (Allsopp, 1995). Amongst several subfamilies in the Scarabaeidae, species attacking sugarcane

occur in at least three subfamilies (Table 2.1; Wilson, 1969; Way, 1997; Dittrich *et al.*, 2006; Cock and Allard, 2013). White grubs are known by their long life cycle which takes one year and sometimes two to three years to complete (Way, 1997; Cock and Allard, 2013). The adults have hard-shell bodies (Figure 2.1), commonly darker in colour, and the larvae, commonly called white grubs (Cock and Allard, 2013), have C- shaped, white-to-cream coloured bodies (Figure 2.1), commonly with a darkened posterior (Cherry, 2008).

White grubs are detrimental pests of many different fruit-bearing plants, vegetables, ornamental plants, crops, pastures, turf and meadow grasses, lawns, golf courses and forest trees in Africa and globally (Goble, 2012). They cause damage on roots and underground stems (Way *et al.*, 2011; Way *et al.*, 2013). White grub third instars are reported to be the most destructive stage to sugarcane roots (Goble, 2012). White grub adults in the Dynastinae subfamily are distinct from other adults in other subfamilies (Melolonthinae and Rutelinae) found in sugarcane, because they also feed on young sugarcane plants, and have the most damaging larval stage (Wilson, 1969; Cock and Allard, 2013). Two melolonthid species, *P. sommeri* and *S. affinis* are the most dominant white grub species in the KZN Midlands North areas (Way *et al.*, 2011).

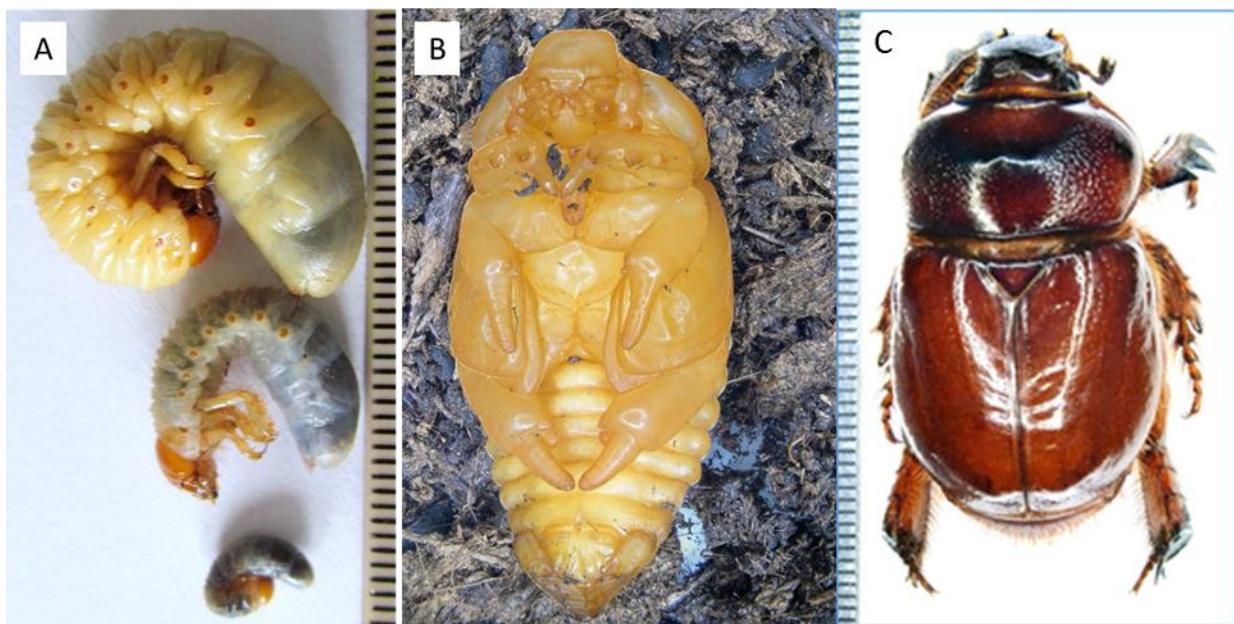


Figure 2.1: Life stages of *Temnorhynchus clypeatus* Klug in the subfamily Dynastinae, to demonstrate the typical white grub morphology; (A) Larval stages (first, second and third instar, from bottom to top); (B) Pupal stage; and (C) Adult stage. Lines on scale bars are 1mm apart. Photo credit: Mike Way.

In 1967, Sweeney used raster patterns on the last abdominal segment of a white grub larva, to identify and classify white grubs into different species (Figure 2.2). Similar methods have been adopted and several studies conducted in Africa have shown that white grub species larvae can be differentiated from each other by using details of their raster patterns (Dittrich *et al.*, 2006; Goble, 2012; Way *et al.*, 2013). Raster patterns are the distinct patterns of spines and hairs at the posterior end of the larval abdomen (Way *et al.*, 2013). Other features that have been used to identify white grub species include the mouthparts (Sweeney, 1967). The width of the larval head capsule has been used to determine the larval instar stage (Wilson, 1969; Sweeney, 1967; Dittrich *et al.*, 2006, and Dittrich-Schröder *et al.* 2009).

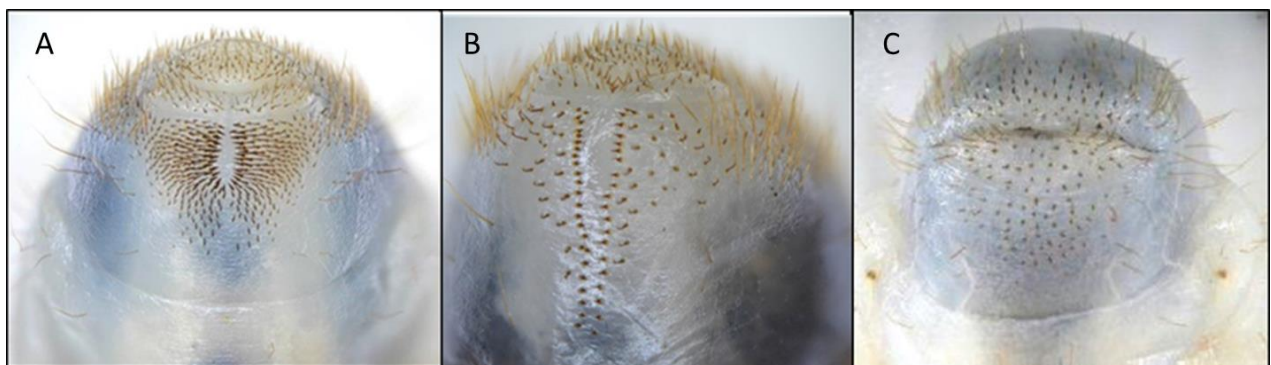


Figure 2.2: Different raster patterns on the ventral side of the abdomen of larval white grub species: (A) *Asthenopholis minor* Brenske (Coleoptera: Scarabaeidae: Melolonthinae); (B) *Schizonycha neglecta* Boheman (Coleoptera: Scarabaeidae: Melolonthinae); and (C) *Heteronycha licas* Klug (Coleoptera: Scarabaeidae: Dynastinae). Raster patterns are used to distinguish different white grub species. Photo credit: Mike Way.

2.2.1 Life cycle of white grubs

White grubs have a complete life cycle with four life stages: eggs, larval instars, pupae and adults (Cherry, 2008). The female adult lays eggs in the soil and all the immature life stages including the pupae remain in the soil (Cherry, 2008). Different white grub species have the same life cycle. However, the time of adult emergence, egg laying, larval period and time of pupation may differ between different species due to the fact that they may experience different climatic conditions (Carnegie, 1974a; Carnegie, 1974b). Most melolonthid white grubs have a one-year life cycle (Carnegie, 1974a; Carnegie, 1974b); however, there are other white grubs in the subfamily Melolonthinae that have life cycles that last for more than two years (e.g. *Lepidiota frenchi* Blackburn Coleoptera: Scarabaeidae: Melolonthinae; Goble, 2012). Adults of

the common white grub species in the sugar industry in South Africa, occur between September and March, but peak from October to November, when temperatures and rainfall are favourable (Goble, 2012).

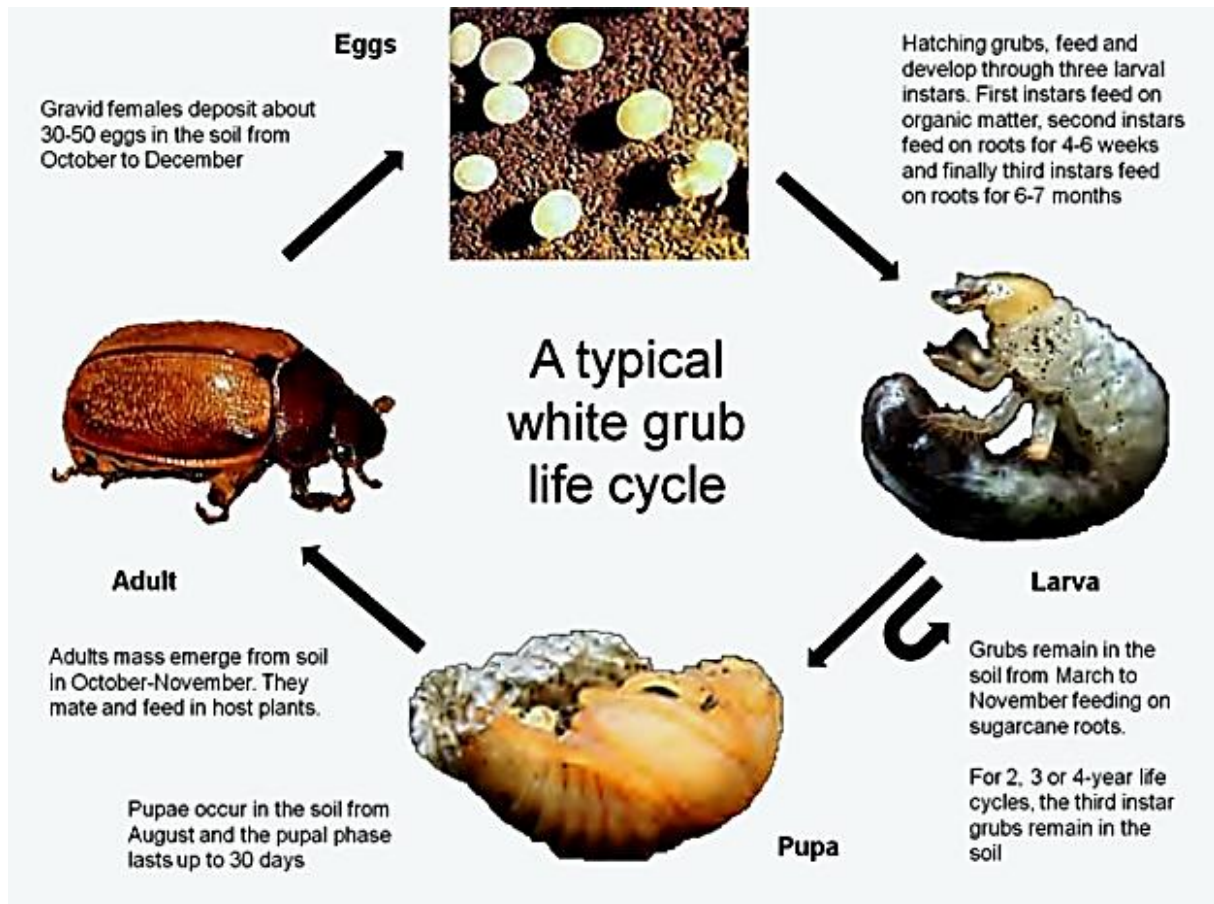


Figure 2.3: A typical life cycle of white grubs. Adults lay eggs in the soil and the emerging larvae remain in the soil and feed on organic matter and sugarcane roots (from Goble, 2012).

Adult females lay their eggs at soil depths of 8-15 cm (Goble, 2012). However, *P. sommeri* adults can lay their eggs as deep as 23 cm (Goble, 2012). *Pegylis sommeri* females can deposit a total of 48 eggs in the soil (Figure 2.3). The eggs are oval shaped, soft and whitish in their earliest stage. Eggs would be very small when they are first laid and increase in size as they imbibe water after being oviposited (Goble, 2012). The first instars, the pale neonates with light brown head capsules emerge, feed on organic matter in the soil and on younger small host plant roots. After 95 days first instars develop to second instars which will have a much darker brown head capsule and have developed secondary setae and they will persist in this stage for approximately 165 days (Goble, 2012). After this period, a third instar develops. This is the

most destructive stage on numerous plants and sugarcane. Third instar larvae feed on roots and underground sugarcane stools and they remain in the soil for 219 days; after which they move deeper down the soil profile, and make an earthen cell within which they will pupate (Goble, 2012). The resulting pupae are light yellow and will change to reddish brown in 20-30 days. Thereafter, when development conditions are met, adults ecdyse from the pupae, and emerge from the soil to mate and oviposit (Goble, 2012).

2.2.2 Distribution of different white grub species

The larvae of white grub species are mostly soil-dwellers and phytophagous (García *et al.*, 2011). Their population densities are highly influenced by several factors, including geographic area, host plants and climatic conditions, which play a role in white grub distribution and survival success (Hawley, 1949; Dalthorp *et al.*, 2000). Many white grub species occur in large numbers in arid or semi-arid regions and their distribution is generally localized (Dalthorp *et al.*, 2000). Their outbreaks are prevalent in areas with sandy or sandy loamy soils, however they can also be found in clay soils (Laznik *et al.*, 2012). For example, sites that are affected by the melolonthid cockchafer *Melolontha melolontha* Linnaeus (Coleoptera: Scarabaeidae: Melolonthinae) in Austria are mostly valleys and plains that are 300 to 600 m above sea level; and these sites have annual mean temperatures of not less than 7°C (Hann *et al.*, 2015). Female cockchafers oviposit in the upper layer of soil, close to vegetation in warmer areas (Hann *et al.*, 2015). They prefer warm, dry, slightly permeable soil with a high nutritional content (Hann *et al.*, 2015). These micro-habitat requirements vary between species, however, and other white grub species are known to prefer different densities of vegetation at oviposition sites. For example, adult *Phyllopertha horticola* Linnaeus (Coleoptera: Scarabaeidae: Rutelinae), a white grub occurring in British grasslands, prefers to oviposit in much denser vegetation compared to the oviposition sites of other female cockchafers (Bocksch, 2003).

White grub species also occur in several African countries, including Swaziland, Uganda, and South Africa. Carnegie (1988) reported heavy infestations of *Heteronychus licas* Klug (Coleoptera: Scarabaeidae: Dynastinae) in Swaziland. In Uganda, negative impacts of *Idaecamenta eugeniae* Arrow (Coleoptera: Scarabaeidae: Melolonthinae) on sugarcane growth and yield were observed (Conlong and Mugalula, 2003).

In South Africa, *Pegylis sommeri* and *Schizonycha* spp. are the most abundant species in the sugarcane growing industries (Allsopp, 1995; Way *et al.*, 2011; Harrison, 2014); followed by *Heteronychus* spp. and *Temnorhynchus* spp. (Coleoptera: Scarabaeidae: Dynastinae) (Carnegie

1988; Dittrich-Schröder *et al.*, 2009; Visser and Stals, 2012). The distribution of white grub species, particularly melolothid scarabs (i.e. *Schizonycha* spp. and *P. sommeri*) in the Midlands regions of KwaZulu-Natal, is strongly associated with the occurrence of *A. mearnsii* (Carnegie, 1974a; Carnegie, 1988). Way (1997) studied white grub species abundance, diversity and the amount of damage caused on sugarcane in KZN Midlands areas of South Africa. He recorded thirteen different white grub species (Table 2.1) and revealed that the Midlands and surrounding areas were highly affected by white grubs.

Pegylis sommeri occurs in the eastern regions and has also been recorded in the western parts of South Africa, with only two records in Mozambique (Harrison, 2014). Harrison (2014) suggested that *P. sommeri* recorded in the western region might have been introduced through transportation of turf from Northern Mpumalanga. Adults of *P. sommeri* feed on different plant species including *Saccharum* species (Poales: Poaceae), *Solanum tuberosum* Linnaeus (Solanales: Solanaceae), *Eucalyptus* species (Myrtales: Myrtaceae), *Erythrina* species (Fabales: Fabaceae) and some *Acacia* species (Harrison, 2014).

Schizonycha affinis is a widely distributed species in the KZN Midlands areas (Goble, 2012). The first instar larvae of *S. affinis* occur in December, however, all instars can be found throughout the year (Goble, 2012). The larvae of *S. affinis* feed on sugarcane roots and similar to the other melolothid species, *S. affinis* also feeds on other angiosperm plants (Goble, 2012). Other *Schizonycha* species such as *S. fimbriata* Brenske (Coleoptera: Scarabaeidae: Melolonthinae) have been recorded feeding on soya beans and *A. mearnsii* in KZN (Harrison and Wingfield, 2016).

Table 2.1: White grub species recovered from surveys conducted in South African sugarcane (from Way, 1997).

Subfamily	Species
Dynastinae	<i>Heteronychus licas</i> Klug
	<i>H. rusticus</i> Klug
Melolonthinae	<i>Apogonia ovata</i> Fahraeus
	<i>Asthenopholis subfasciata</i> Blanch
	<i>Autoserica</i> sp.
	<i>Pegylis sommeri</i> Burmeister
	<i>Schizonycha affinis</i> Boheman
	<i>Schizonycha</i> sp.
Rutelinae	<i>Trochalus aerugineus</i> Burmeister
	<i>Adoretus</i> sp.
	<i>Anomala ustulata</i> Arrow
	<i>A. caffra</i> Burmeister
	<i>A. prob. resplendens</i> Fahraeus
	<i>A. prob. zambesicola</i> Peringuey

In addition, *Temnorhynchus* spp. are found in most parts of the Afrotropical region. They have been recorded in all regional biomes and are highly abundant in the Savanna Biome (Visser and Stals, 2012). There are five *Temnorhynchus* species that have been reported in South Africa, viz. *T. coronatus*, *T. retusus* (Fabricius), *T. elongatus* Arrow, *T. clypeatus* Klug and *T. zambeziensis* Krell (Visser and Stals, 2012). The common species that is affecting sugarcane in

South Africa, *T. clypeatus*, is mainly found in the Northern provinces and along the KZN coast (Visser and Stals, 2012).

The adults of *Heteronychus* spp. commonly known as black beetles, are strongly associated with maize plants (*Zea mays* Linnaeus; Poales: Poaceae; Venter and Louw, 1978). However, they are also pests of other agricultural plants such as *S. tuberosum* (Venter and Louw, 1978) and sugarcane (Figure 2.4) (Carnegie, 1988; Dittrich-Schröder *et al.*, 2009). The common species that is affecting sugarcane in South Africa, *H. licas*, has been associated mainly with irrigated fields. This species has significantly damaged sugarcane in the Lowveld regions of Mpumalanga with sparse damage in Pongola and severe but localised damage in the Northern part of the KwaZulu-Natal, Umfolozi River flats (Carnegie, 1988). Other white grub species such as *Asthenopholis* species (Coleoptera: Scarabaeidae: Melolonthinae) have been recorded in South Africa. *Asthenopholis* species have been recorded damaging pineapple plants in cool, high altitude areas and wet or cool coastal areas, and they are typically associated with red sandy, loamy (Harrison, 2009) and heavy clay soils (Sweeney, 1967).

2.3 White grubs as pests of agricultural crops

Scarabaeid species remain the most serious soil pests of agricultural crops worldwide (Sapkota, 2006). White grubs attack many agricultural plants including sugarcane, maize, millet and sorghum (Sapkota, 2006; Rahama *et al.*, 2014). They feed on the roots and young shoots, therefore reducing yield (Sosa, 1984). Root damage weakens the plants and, in severe cases, can result in the death of the plants. Damage by white grubs can easily be observed on plant roots, which experience reduction of lateral roots and removal of root hairs. According to Sapkota, (2006), even the slightest white grub infestations can increase plant lodging and reduce plant yields. The physical damage caused by white grubs also increases the chances of pathogen infections (Ueckert, 1979).

A preliminary study conducted by Ueckert (1979) to quantify white grub damage on perennial grasses showed that grass roots were damaged up to 20-30 cm below the soil surface which later resulted in the death of the plants. More recently, Anitha *et al.* (2006) studied the distribution and abundance of *Schizonycha* species on groundnut plants in southern India. They found that plant damage was positively correlated with white grub density. White grubs have also been reported as pests of teak seedlings (*Tectona grandis* L.f.; Lamiales: Lamiaceae; Kulkarni *et al.*, (2009). *Cotinis nitida* Linnaeus (Coleoptera: Scarabaeidae: Cetoniinae) or June scarab adults are problematic in many other grown berries (Hammons *et al.*, 2009). The adults

damage grape berry by tearing the skin from the pedicel with their tarsal claws (Hammons *et al.*, 2009). According to Hammons *et al.*, (2009) June scarab adults can reduce harvestable grape clusters on untreated vines by 95% or more, causing substantial loss for vine farmers. Raid and Cherry (1992) reported sugarcane yield loss of up to 39% caused by June adult white grubs in USA. Kulkarni *et al.* (2009) reported wilting and death of *T. grandis* as a consequence of damage to the root systems by *Holotrichia mucida* Gyllenhal and *H. rustica* Burmeister (Coleoptera: Scarabaeidae: Melolonthinae).

It is clear that control methods for white grub pests are needed across various agricultural industries. Some of the most promising control techniques include the use of natural enemies such as parasites and entomopathogens as biocontrol agents (Dolci *et al.*, 2006). Examples which have produced positive results against many white grub species include larvae of *Promachus yesonicus* Bigot (Diptera: Asilidae; Wei *et al.*, 1995), entomopathogenic nematodes (EPNs) such as *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae; Koppenhöfer *et al.*, 2000, Koppenhöfer and Fuzy 2008), and EPFs such as *B. brongniartii* and *B. bassiana* (Balsamo-Crivelli) Vuillemin (Ascomycota: Hypocreales; Goble *et al.*, 2015).

2.4 The impact of white grubs in South African sugarcane

South Africa is one of several countries in which sugarcane is commercially grown to produce sugar (Cheavegatti-Gianotto *et al.*, 2011; Meyer and Clowes, 2011; Moore and Ming, 2011). The production of sugarcane in South Africa also plays a significant role in the livelihoods of many South Africans through creation of job opportunities (van den Berg and Singels, 2013). The provinces that mass cultivate sugarcane in South Africa include KwaZulu-Natal, Eastern Cape and Mpumalanga (Maloa, 2001; Goble *et al.*, 2012). An average of 19.9 million metric tons of sugar is produced each season in South Africa (Morokolo, 2011; SASA, 2015). Sugarcane production, however, is negatively affected by various abiotic and biotic factors. White grubs represent one the major biotic challenges affecting sugarcane production. McArthur and Leslie (2004) estimated that white grubs were responsible for 80% of the sugarcane yield losses in the years 1996-1999. In the Midlands areas of KwaZulu-Natal, damage by white grubs ranged between 23-55 % in 2004, with more than 10 grubs per pit being recovered in some areas (McArthur and Leslie, 2004). Control measures are therefore, increasingly being required to regulate white grubs and the potential for biocontrol measures have recently been highlighted (Goble *et al.*, 2012; Goble *et al.*, 2015). The use of natural

enemies such as entomopathogens have gained popularity in South Africa as they represent a way to minimize chemical usage against white grubs (Du Rand, 2009; Goble *et al.*, 2015).



Figure 2.4: Adult white grub of *Heteronychus tristis* Boheman (Coleoptera: Scarabaeidae: Dynastinae) observed damaging sugarcane. (Photo credit: Mike Way).

2.5 Biological control agents against white grub species

2.5.1 The use of entomopathogenic nematodes (EPNs) to control white grubs

Entomopathogenic nematodes and their symbiotic bacteria are found in many different soil types and use many different insects as their hosts (Pillay *et al.*, 2009). Once the host has been penetrated, the EPN feeds in the insect host, and releases bacteria, which cause death (Pillay *et al.*, 2009). Several EPN species have been used to reduce white grub populations. For example, Koppenhöfer *et al.*, (2000) used *H. bacteriophora* and *Steinernema glaseri* Steiner (Rhabditida: Steinernematidae) strains as control agents against *Popillia japonica* Newman (Coleoptera: Scarabaeidae: Rutelinae), and showed that 3rd instar white grub populations were killed at levels similar to that of an organophosphate insecticide.

Countries such as India and Brazil commercially mass-produce EPNs such as *H. indica* Poinar (Rhabditida: Heterorhabditidae), *S. braziliense* n. sp., *S. carpocapsae*, *S. glaseri*, and *S. thermophilum* (Alteromonadales: Shewanellaceae) for use as control agents (Goble *et al.*, 2016). These EPNs are, however, poorly used due to the fact that they are expensive to mass-produce, and also require mass production of their respective bacteria on different medium

ingredients (Goble *et al.*, 2016). Cost-effective production of EPNs for use as biocontrol agents is thus seldom possible.

2.5.2 The use of predators to control white grubs

Predators of white grubs have not been widely used. A study by Wei *et al.* (1995) investigated the potential of predatory robber flies, *Promachus fitchii* Osten Sacken and *P. vertebratus* Say (Diptera: Asilidae) against white grub species in the soil. They measured robber fly predation of white grubs and correlated this with the reduction of damage to wheat plants. They found that the flies had the potential to reduce the number of grubs by more than 95%, and wheat damage was significantly lower in plots artificially infested with robber flies than in the control plots. However, there is little information available about the behavior of predators of white grubs, making it difficult to identify potential predators for use as biocontrol agents. These limitations have resulted in the investigation of alternative biocontrol agents, such as EPFs (Keller *et al.*, 1999).

2.5.3 The use of entomopathogenic spore-forming bacteria and entomopathogenic fungi (EPFs) to control white grubs

The potential to use bacteria (for example *Bacillus popilliae* Dutky (Bacillales: Bacillaceae)) and fungi (for example *Beauveria bassiana* (Bals.-Criv.) Vuill. and *B. brongniartii*) as biological control agents has also been pursued (Paray *et al.*, 2012; Goble, 2012). *Bacillus popilliae* is known to cause “milky disease” in the larvae of *Tomarus subtropicus* Blatchley (Coleoptera: Scarabaeidae: Dynastinae; Paray *et al.*, 2012; Augustyniuk-Kram and Kram, 2012). A study conducted by Du Rand (2009) revealed that entomopathogenic spore-forming bacterial isolates of *Bacillus* spp. were able to cause a minimum of 40% mortality against larvae of *P. sommeri*. However, *Bacillus* spp. such as *Bacillus thuringiensis* (Bt) (Bacillales: Bacillaceae) are costly (Goble *et al.*, 2016). According to Goble *et al.* (2016), the relatively low use of Bt products to control scarabs in developing countries is strongly associated with costs of importing products from European countries. The effectiveness of an entomopathogen also depends on its ability to persist within the environment (Zimmermann, 2007), and the ability of the entomopathogens to persist in the soil remains vital for the development of bacterial control agents for white grub larvae (Keller *et al.*, 2003).

Goble *et al.* (2012) identified an entomopathogenic fungus (*B. brongniartii*) causing epizootics against adults and larvae of the same species, *P. sommeri*, evaluated by Du Rand (2009). Goble (2012) then showed that *B. brongniartii* isolates were highly virulent against adults and

immature stages of *S. affinis* and *P. sommeri* causing 95% mortalities. The future of using EPFs to control white grubs in sugarcane is promising. There are several advantages of using EPFs to control pests including: a) production of EPFs is cheaper compared to bacteria and nematodes; b) just one fungal spore is enough to infect insect pests; and, c) EPFs can cause infections against both immature and adult insect stages (Goble *et al.*, 2016).

Entomopathogenic fungi are very infectious and have been used to control many different pests (Zimmermann, 2007; Goble *et al.*, 2016). They are widely distributed and occur in most terrestrial ecosystems (Zimmermann, 2007; Goble *et al.*, 2016). Entomopathogenic fungi differ from chemical insecticides because they can be developed and maintained under laboratory conditions for a long time (Goble *et al.*, 2016). Most entomopathogenic fungi infections on pests are systemic (Zimmermann, 2007; Keller *et al.*, 1997), as the EPFs have spores that attach to the body of the host and then penetrate and colonize the pest (Holder, 2005). Once the fungus has established itself, it spreads throughout the insect's body resulting in mortality due to mechanical damage and toxins released by the EPF (Zimmermann, 2007; Keller *et al.*, 1997). *Beauveria brongniartii*, *B. bassiana* and *Metarhizium anisopliae* Metchinikoff (Ascomycota: Clavicipitaceae) are characterised by the ability to persist in the soil for long periods of time, and they are therefore considered to have a great potential as commercial bio-insecticides (Keller *et al.*, 2003). *Metarhizium anisopliae* has been used as a bio-insecticide of many different insect species, especially in the orders Lepidoptera and Coleoptera (Manisegaran *et al.*, 2011). Like any other EPF, *M. anisopliae* causes diseases when a single spore comes into contact with the insect cuticle (Manisegaran *et al.*, 2011).

The role of entomopathogenic fungi as endophytes

The term endophyte was first used by the German scientist Heinrich Anton De Bary in 1884, to describe the fungi or bacteria that establish inside plant parts without causing obvious plant pathogenic symptoms (Vega *et al.*, 2008). Fungi with this kind of biology have been isolated in at least a hundred plants, including wheat, bananas, soybeans and tomatoes (Breen, 1994; Elmi *et al.*, 2000; Larran *et al.*, 2001; Larran *et al.*, 2002a; Larran *et al.*, 2002b; Cao *et al.*, 2002; Dingle and McGee, 2003; Wicklow *et al.*, 2005; Vega *et al.*, 2008). Fungal endophytes are important, as they have an ability to infect herbivorous insects and thus provide protection for plants (Conlong, 1990; Conlong and Rutherford, 2009).

Beauveria bassiana and *M. anisopliae* have been isolated from numerous plants and this has drawn attention to their potential as endophytic entomopathogens. Vega *et al.* (2008) revealed

that *B. bassiana* was able to colonize coffee tissues, and was also re-isolated from the peduncle, epicarp, crown, and the seeds of berries. *Beauveria bassiana* endophytism studies conducted by Cherry *et al.* (1999) and Cherry *et al.* (2004) in Africa provided evidence of insect feeding reduction, because infected (or mycosed) larvae were lighter in weight than the larvae in the un-inoculated (control) plants. Maize leaves sprayed with a *B. bassiana* suspension, deterred infestation by maize stem borer (*Sesamia calamistis* Hampson; Lepidoptera: Noctuidae) (Cherry *et al.*, 2004). Furthermore, Vega *et al.* (2008) showed minimal tunneling by *S. calamistis*. Akello *et al.*, (2008) showed that *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae) eggs, larvae and adults were infected after feeding on banana plants that were inoculated with an endophytic *B. bassiana* strain. In another study, larvae of a leaf mining fly, *Liriomyza huidobrensis* Blanchard (Diptera: Agromyzidae) were found to be susceptible to *B. bassiana* endophytic inoculum, resulting in reduced pupation and reduced development of pupae into adults (Vidal and Jaber, 2015). Lewis *et al.* (1996) have suggested that *B. bassiana* strains may be able to reduce tunneling by *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae), although their study could not show any form of insect mycoses post inoculation. In a local study, Conlong, (1990) found that *Eldana saccharina* Walker (Lepidoptera: Pyralidae) was highly susceptible to *B. bassiana* present as an endophytic pathogen in *Cyperus papyrus* Linnaeus (Poales: Cyperaceae).

It has been shown that antagonistic behavior of other endophytic fungi such as *Fusarium* spp. (Hypocreales: Nectriaceae) can affect the performance of an endophytic EPF (Conlong and Rutherford, 2009). Conlong and Rutherford, (2009) for example, showed that *Fusarium* spp. prevented *B. bassiana* from colonizing sugarcane plants. Their results are supported by other studies, which reported antagonistic behaviour of *Fusarium* species towards *Beauveria* species (Geetha *et al.*, 2012; Mulaw *et al.*, 2013).

Beauveria bassiana appears to be relatively widespread as an endophyte. In contrast, *B. brongniartii* has only been recovered once from the crown of a coffee berry (*Frangula californica* (Eschsch.)) collected in Hawaii (Vega *et al.*, 2008). There is little information available on *B. brongniartii* as a potential endophytic fungus of sugarcane plants, for long term protection against damage from white grubs. Nonetheless, *B. brongniartii* has been applied in fields by contaminating the soil, and the results showed that application of *B. brongniartii* fungal isolates reduce white grub populations (Keller *et al.*, 1997; Keller *et al.*, 1999; Keller, 2000; Enkerli *et al.*, 2001; Keller *et al.*, 2003; Kessler *et al.*, 2004; Laengle *et al.*, 2005; Dolci

et al., 2006; Manisegaran *et al.*, 2011). Since the identification of native *B. brongniartii* isolates in South Africa (Goble *et al.*, 2012), *B. brongniartii* has also been considered a great potential bio-insecticide of white grubs affecting sugarcane in South Africa.

2.6 Biology of *Beauveria brongniartii*

2.6.1 *Beauveria brongniartii*

Beauveria brongniartii is a toxin producing fungus (Zimmermann, 2007; Paray *et al.*, 2012) which was first discovered in the early 19th century (Jones, 1994). It has since been used against *M. melolontha* for more than 100 years (Zimmermann, 2007). It is a soil borne saprophyte with a widespread distribution (Zimmermann, 2007; Keller *et al.*, 1997; Anitha *et al.*, 2006; Perez-Gonzalez *et al.*, 2014). Virulent isolates of *B. brongniartii* (Figure 2.5) have been studied extensively for protection against a number of pests including Blattodea, Coleoptera, Diptera, Hemiptera, Lepidoptera, and Orthoptera (Leatherdale, 1970; Jones, 1994; Keller *et al.*, 1997; 2003; Khan *et al.*, 2012).

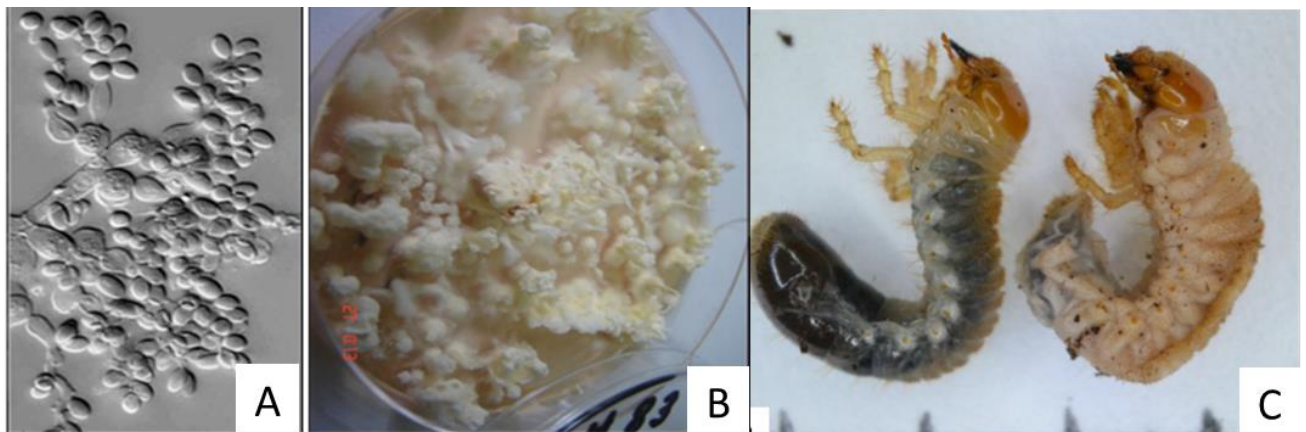


Figure 2.5: Morphology of *Beauveria brongniartii*. (A) Micrograph of *B. brongniartii* conidiogenous cells and conidia (Rehner *et al.*, 2011); (B) Fungal growth of *B. brongniartii* isolate culture on Sabouraud Dextrose Agar medium; and (C) Healthy (left) and infected (right) white grub larvae (from Goble, 2012).

Beauveria brongniartii is more favourable than the use of chemical insecticides due to the fact that it is persistent in the environment. Keller *et al.* (2003) reported persistence of *B. brongniartii* for more than 30 years. Enkerli *et al.* (2004) also reported persistence of *B. brongniartii* strains in the soil for at least 14 years after soil inoculations. These studies concluded that the ability of *B. brongniartii* to persist in the soil increases its pathogenicity, thus increasing its potential as a good biological control agent.

There are 171 registered mycoinsecticides and mycoacaricides worldwide, of which only seven incorporate *B. brongniartii*, making up 4.1 % of the total of the registered mycoinsecticides (Faria and Wraight, 2007; Goble, 2012). Of the seven registered *B. brongniartii* products, six are used to target scarabs and these are: Beauveria-Schweizer® (Eric Schweizer Seeds Ltd.), Myzel® (LBBZ Arenenberg) and Engerlingspilz® (Andermatt Biocontrol), produced in Switzerland; Beauveria 50® and Beauveria (Ago Biocontrol), produced in Colombia; and Betel® (Betel Reunion S.A), which is produced in the Reunion Island (Keller *et al.*, 2003; Faria and Wraight, 2007). The final registered *B. brongniartii* product (Biolisa-Kamikiri® (Nitto Denko)) is produced in Japan and is registered for use against forestry pests, especially long horn beetles in the family Cerambycidae. South Africa does not have any registered *B. brongniartii* based products as commercial bio-control agents to control scarab species. There are, however, products containing *B. bassiana* and *M. anisopliae* as active ingredients, which are both available commercially, and used to control a variety of agricultural pests (Goble, 2012). Therefore, intensive research effort is required in order for *B. brongniartii* to be commercialized and used as a biological control agent against scarab pests in South Africa.

2.6.2 Host insects of *Beauveria brongniartii*

Beauveria brongniartii causes the death of many soil-dwelling scarab pests (Goble *et al.*, 2012). According to Zimmermann (2007) *B. brongniartii* is a highly specific pathogenic fungus of scarabs. Furthermore, there are about 70 scarab species whose larvae are hosts for entomopathogenic fungi. Table 2.2 lists some of the host insects of *B. brongniartii*.

Table 2.2: Some major scarab hosts of *Beauveria brongniartii* and their geographic origin.

Host insects	Origin	References
<i>Melolontha melontha</i>	Stuttgart, Germany	Hadapad <i>et al.</i> (2006)
<i>M. hippocastani</i>	Freiburg, Germany	Hadapad <i>et al.</i> (2006)
<i>Holotrichia morosa</i>	China	Hadapad <i>et al.</i> (2006)
<i>H. parallela</i>	Cangzhou, China	Hadapad <i>et al.</i> (2006)
<i>Pachnaeus litus</i>	Florida, USA	Hadapad <i>et al.</i> (2006)
<i>Spodoptera litura</i>	Guntur, India	Hadapad <i>et al.</i> (2006)
<i>Melolontha melontha</i>	Valley of Aosta, northwest Italy	Dolci <i>et al.</i> (2006)
<i>Schizonycha affinis</i>	KZN, South Africa	Goble <i>et al.</i> (2012)
<i>Pegylis sommeri</i>	KZN, South Africa	Goble <i>et al.</i> (2012)

2.6.3 *Beauveria brongniartii* life cycle and mode of infection

Beauveria brongniartii is a spore bearing fungus (Shahid *et al.*, 2012). The fungus releases spores that survive well in the soil and they form an important source of inoculum (Shahid *et al.*, 2012). *Beauveria brongniartii* produces insecticidal toxins such as oxalic acid and oosporein that increase infection processes (Strasser *et al.*, 2000; Zimmermann, 2007). *Beauveria brongniartii* has both asexual and sexual reproduction (Zimmermann, 2007). In the asexual life cycle (Figure 2.6) without a host, *B. brongniartii* grows through spore germination and filamentous growth and the formation of conidia (Khan *et al.*, 2012). In the sexual life cycle on the insect host, the hyphae multiply over the cuticle until suitable access into the insect internal body is found (Holder, 2005). The hyphae then enter the insect haemocoel, paralysing and causing death of the insect and the hyphal growth then changes to a yeast like growth and blastospores multiply through the host (Holder, 2005). When the host dies, blastospores change into hyphae again, which grow and cover the insect cuticle (Figure 2.6) (Holder, 2005). The fungus is characterised by white and yellowish to pinkish or reddish colonies (Ownley *et al.*, 2008; Perez-Gonzalez *et al.*, 2014).

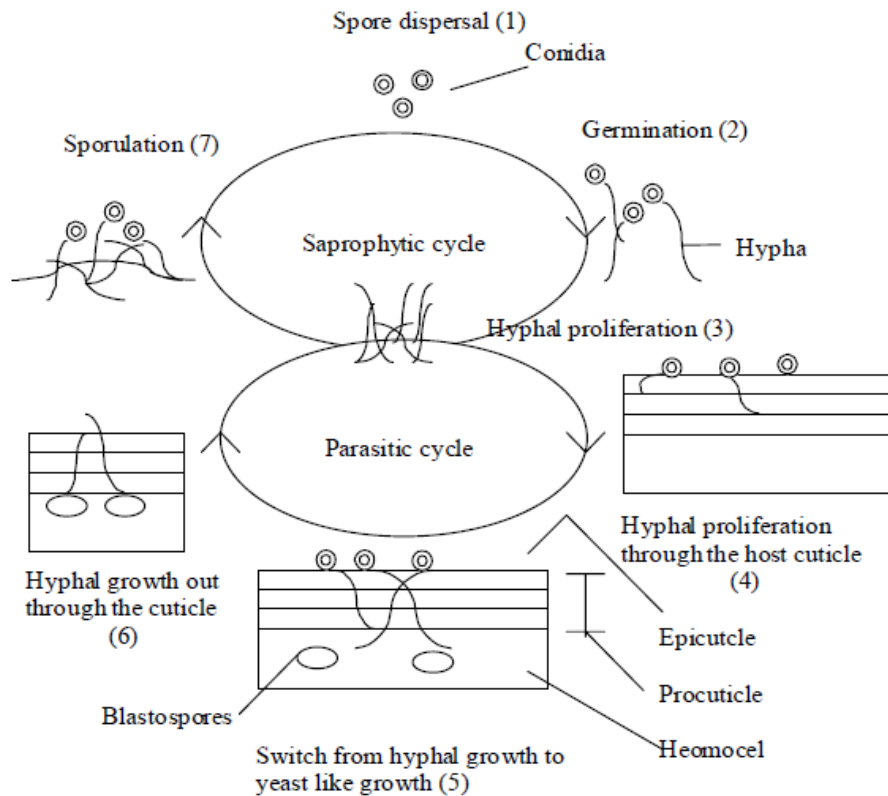


Figure 2.6: A typical life cycle of a hyphomycete entomopathogen (from Holder, 2005).

2.6.4 Taxonomy of *Beauveria brongniartii*

Beauveria brongniartii was discovered by Beauverie and has been given several names since then (Jones, 1994). The genus *Beauveria* was previously known as *Botrytis*, but was changed to *Beauveria* in 1911 in honor of Beauverie, its founder. There are several species in the genus *Beauveria* Vuill, including the well-known species *Beauveria alba* Limber, *B. brongniartii*, and *B. bassiana* (Holder, 2005). These are all well-known entomopathogenic fungi with a wide host range (Ownley *et al.*, 2008). Species in the genus *Beauveria* are characterised by globose to flask-shaped conidiogenous cells from which one-celled, terminal holoblastic conidia are produced in sympodial succession on an indeterminate and denticulate rachis (Figure 2.6) (Rehner *et al.*, 2011). They have distinct conidia that are smooth-walled, hyaline, usually 1.5–5.5 μm and globose to cylindrical or vermiform (Rehner *et al.*, 2011).

Beauveria brongniartii (Saccardo) Petch taxonomy was reported by Minnis *et al.*, (1989). According to Minnis *et al.* (1989), *B. brongniartii* (Saccardo) Petch, previously known as *Botrytis brongniartii* was first described by Saccardo (1892) from Algeria, in Africa. This fungus was reported to have been originally isolated from the desert locust (*Acridio peregrine*), now known as *Schistocerca gregaria* Forsskål (Orthoptera: Acrididae). However, Rehner *et al.*

(2011) stated that, in Europe, where *B. brongniartii* is commonly found; it is believed to have been originally isolated from *M. melolontha* and there is no confirmation that *B. brongniartii* infects Orthoptera. It is believed that Brongniart evaluated fungal cultures and recorded two isolates, one with oval conidia which was later named *Botrytis brongniartii* (Minnis *et al.*, 1989) and the second with globose conidia which was named *Botrytis delacroixii* Sacc. (= *Beauveria delacroixii* (Sacc.) Petch). The latter is now known as *Beauveria bassiana* (Bals. Criv.) Vuill. Both fungus names were suspected to have been used to honour a person who isolated the pathogenic fungus from the locusts. However, Minnis *et al.* (1989) argued that there was not enough and traceable evidence for the original isolates used by Brongniart thus, Petch changed both of Saccardo's pathogenic fungi from *Botrytis* species to *Beauveria*.

Table 2.3: Classification of *Beauveria brongniartii* (after Rehner *et al.*, 2011)

Kingdom	Fungi
Division	Ascomycota
Class	Ascomycetes
Order	Hypocreales
Family	Cordycipitaceae
Genus	<i>Beauveria</i>

Recent literature by Rehner *et al.* (2011) examined the phylogeny of anamorphic *Beauveria*. Rehner *et al.* (2011) cited a revision by de Hoog (1972), who listed three species that belong to the genus *Beauveria*: viz. *B. bassiana*, *B. brongniartii* and *B. alba* (Limber) Saccas (now known as *Engyodontium album*) (Limber) de Hoog. Since then, many other species in this same genus have been recorded. However, former research placed *B. bassiana* and *B. brongniartii* within

the Cordyceps Fr. teleomorphs (Shimazu *et al.*, 1988; Huang *et al.*, 2002) and molecular phylogenetic analyses proved that both these *Beauveria* species belong to the family Cordycipitaceae (Hypocreales; Sung *et al.* 2007). The recent classification of *B. brongniartii* is summarised in Table 2.3. After the revision by de Hoog (1972), *B. brongniartii* was then recognized to have a wide host range encompassing mostly Coleoptera (Rehner *et al.*, 2011). In South Africa, *B. brongniartii* was recorded for the first time (by Goble *et al.* 2012) infecting the scarab species *P. sommeri* and *S. affinis*, in sugarcane. Since then, a number of *B. brongniartii* isolates have been identified and tested against these scarab species affecting sugarcane in South Africa (Goble, 2012).

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CHAPTER 3: EVALUATION OF TWO *BEAUVERIA BRONGNIARTII* ISOLATES FOR PATHOGENICITY AGAINST DIFFERENT LIFE STAGES OF WHITE GRUB SPECIES (COLEOPTERA: SCARABAEIDAE), IN SOUTH AFRICAN SUGARCANE

Abstract

Two formulated isolates of *Beauveria brongniartii* (C17 and HHWG1) obtained from the white grub species *Pegylis sommeri* and *Schizonycha affinis* in the KwaZulu-Natal Midlands, were used to test their pathogenicity on larvae and adults of *Heteronychus licas*, *Asthenopholis minor* and *H. tristis*, and larvae of *Temnorhynchus clypeatus* and *Schizonycha neglecta*. Conidial suspensions of each isolate (10 µl) at a concentration of 1×10^9 conidia/ml were used to inoculate these white grub species' life stages. Larvae were kept at a temperature of 23°C for 35 days and the adults at 23-35°C for eight days after treatment. Larvae were checked for mortality every fifth day and adults were checked once on the eighth day. Dead specimens were disinfected with 70% ethanol and plated onto a Sabouraud dextrose agar medium to assess the cause of death. *Schizonycha neglecta*, *H. tristis* and *T. clypeatus* larvae inoculated with HHWG1 had 80-90% mortality, compared to 5-60% mortality when treated with C17. Adults of *T. clypeatus*, *H. licas*, *A. minor* and *H. tristis* were highly susceptible to C17 (60-80% mortality) compared to HHWG1 (10-45% mortality). It was concluded that both *B. brongniartii* isolates, C17 and HHWG1 have potential as bio-insecticides against adults and larvae, respectively, as they have a wider host range than just the species from which they were collected. However, both isolates still need to be tested in replicated field trials.

3.1 Introduction

Sugarcane is an important commercial crop in Africa and in tropical regions. Production is influenced by numerous biotic and abiotic factors such as insect pests and diseases. More than 200 pests cause serious yield loss in sugarcane worldwide (Chelvi *et al.*, 2011). White grub species (Coleoptera: Scarabaeidae) cause serious damage in some sugarcane industries (Cherry, 1998; Goble *et al.*, 2012; Cock and Allard, 2013). Infestations of white grubs in sugarcane have been reported from various regions in Africa (Way, 1997; Conlong and Mugalula, 2003). The most problematic species in South African sugarcane belong to the subfamilies Dynastinae, Melolonthinae and Rutelinae. These include *Pegylis* (formerly *Hypopholis*) *sommeri* Burmeister (Melolonthinae), *Schizonycha affinis* Boheman (Melolonthinae), *Asthenopholis minor* Brenske (Melolonthinae) and *Heteronychus licas* Klug (Dynastinae) (Way, 1997; Goble *et al.*, 2012). Recent infestations of *A. minor* and *H. licas* have been reported from sugarcane in Swaziland (Way *et al.*, 2013). Adult white grubs lay eggs in the soil of sugarcane fields and the emerging larvae feed on sugarcane roots and underground stools thus reducing vigour, yield and sugar content (Chelvi *et al.*, 2011; Way *et al.*, 2011; Cock and Allard, 2013; Way *et al.*, 2013).

Insecticides have been used to control pests in agriculture; however, these are costly, environmentally ‘unfriendly’ and represent health hazards to humans and animals (Elena *et al.*, 2011). Biological control is an alternative method, aimed at alleviating problems that are associated with the use of chemical insecticides (Zimmermann, 2007). *Beauveria brongniartii* (Sacc.) Petch (Ascomycota: Clavicipitaceae) is an entomopathogenic fungus with virulent isolates that have been isolated from various white grub life stages (Strasser *et al.*, 2000; Zimmermann, 2007; Goble *et al.*, 2012). It is thus a potential biological control agent against white grubs (Keller *et al.*, 1997; Chelvi *et al.*, 2011; Goble *et al.*, 2012). Preliminary sugarcane studies demonstrated the capacity of virulent isolates of *B. brongniartii* to cause mortality of white grub species (Keller *et al.*, 1997; Chelvi *et al.*, 2011). Goble *et al.*, (2015) demonstrated that *B. brongniartii* isolates were effective biocontrol agents against white grubs in South African sugarcane. Furthermore, they identified virulent isolates of *B. brongniartii* (HHWG1 and C17) from *P. sommeri*, a white grub pest of sugarcane in the KwaZulu-Natal Midlands of South Africa (Goble *et al.*, 2012). HHWG1 caused mortality of up to 95% in third instar *S. affinis* larvae, within a period of 15 days at a concentration of 1×10^9 conidia/ml. Moreover, both virulent isolates of this *B. brongniartii* were able to induce mortalities of up to 50% (LT₅₀) in

second instar *S. affinis* larvae within 18.4 to 19.8 days. Goble *et al.* (2015) showed that HHWG1 was highly virulent to larvae, whereas C17 was highly virulent to adults of these species.

However, it is not known how these entomopathogenic fungal isolates perform on other species of white grubs. This study thus investigated the host range of these two isolates of *B. brongniartii* against other white grub species that occur in South African sugarcane.

3.2 Material and methods

The study was conducted at the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, in South Africa.

3.2.1 Test insects

Three white grub species in the subfamily Dynastinae and two in the subfamily Melolonthinae were collected from various sugarcane sites in South Africa (Table 3.1), to be tested against the locally collected C17 and HHWG1 isolates of *B. brongniartii*. Species identity was confirmed according to morphological descriptions of Harrison (2014) and SASRI pinned specimens of adult white grubs were used as a reference library (previously identified by Dr James Harrison¹). Characters used in these identifications are shown in Figure 3.1.

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Table 3.1: Collection sites, their characteristics, and the species, life stage and number of white grubs collected from each site in South Africa for evaluation of *Beauveria brongniartii* isolates HHWG1 and C17.

Sites	Site characteristic	Cane variety	Species	Larvae	Adults	GPS coordinates
Tomahawk farm Mpumalanga	3 months old plants, clay soil	N49	<i>T. clypeatus</i>	500	-	25° 37' 25" S; 31° 34' 54" E
Mandalay Estate farm Mpumalanga	3 rd ratoon cane, clay soil	N49	<i>H. licas</i>	400	-	25° 30' 02" S; 31° 31' 23" E
Lower Tugela – PR Mathir's farm	3 rd ratoon cane, clay soil	N49	<i>H. licas</i>	-	200	29° 12' 17" S; 31° 27' 52" E
Amasundu farm KZN	Sandy soil	N39	<i>A. minor</i>	400	250	31° 40' 5" S; 28° 57' 29" E
Frost farm KZN	Sandy soil, 9 th ratoon sugarcane	N41	<i>H. tristis</i>	200	400	31° 40' 22" S; 28° 57' 27" E
	Sandy soil, 9 th ratoon sugarcane	N41	<i>S. neglecta</i>	100	-	31° 40' 22" S; 28° 57' 27" E
Tomahawk farm Mpumalanga	3 months old, 3 rd ratoon, clay soil	N39	<i>T. clypeatus</i>	908	90	25° 37' 25" S; 31° 34' 54" E

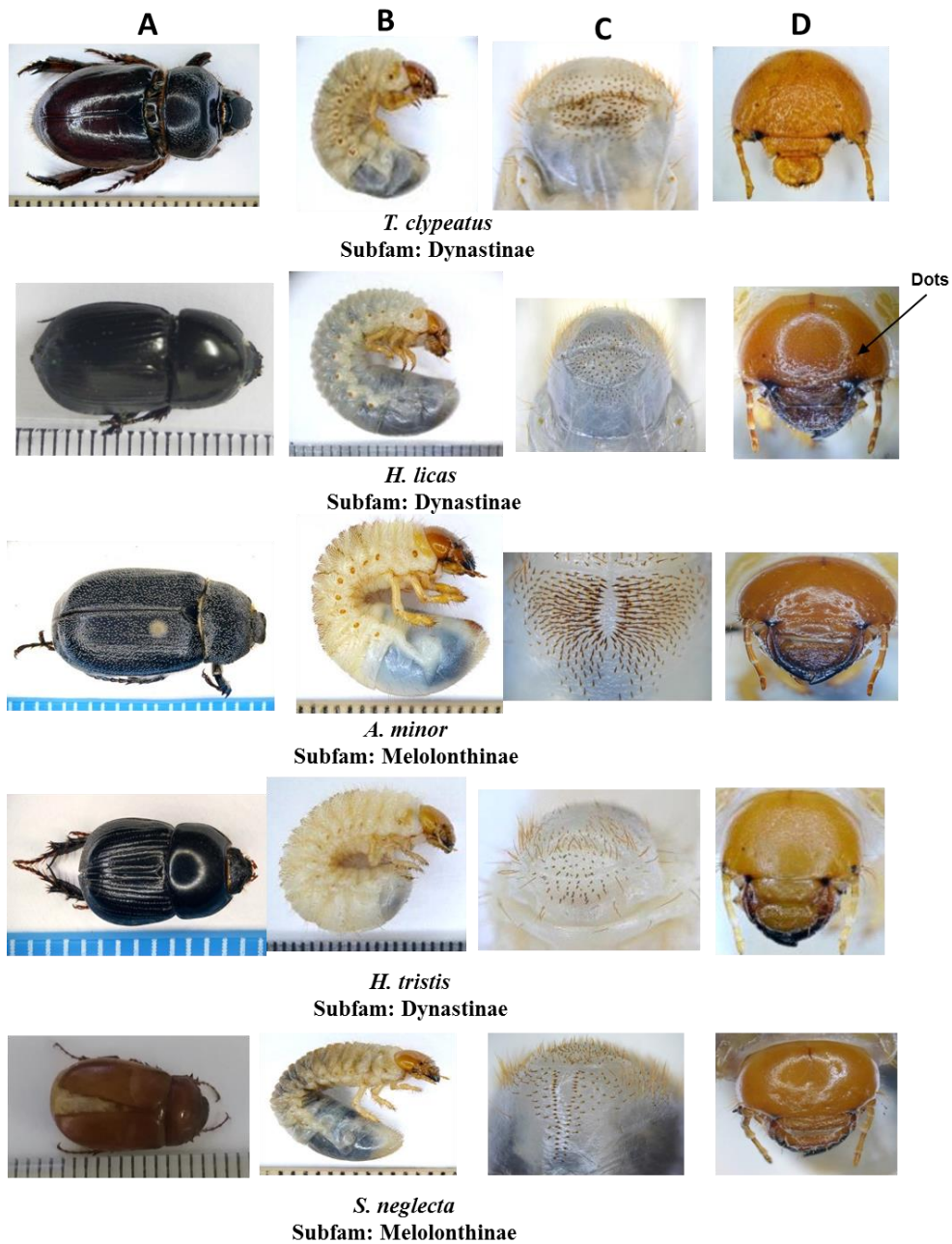


Figure 3.1: White grub larval and adult stages used to test pathogenicity of two virulent isolates of *Beauveria brongniartii* (C17 and HHWG1). Column A: adults (scale in mm given below each adult), Column B: larvae (scale given in mm below each larva), Column C: raster pattern on the last abdominal segment of the larvae shown in column B, Column D: head capsules and traits (e.g. black dots) used to distinguish between species of the larvae shown in column B, (Photo credit: Mike Way).

3.2.2 Larval collection

White grub larvae were collected by digging out sugarcane roots and soil in 0.30×0.30×0.30 m pits. Pits were positioned across the sugarcane stools. Larvae found were placed into plastic vials (30 ml) containing autoclaved peat moss produced by Grovida c.c.². Peat was autoclaved for 15 min at 121°C and cooled before use. Vials were sealed with perforated lids. To minimise mortality, vials were placed in large cooler boxes with ice packs during the collection period, and were transported from the field to the laboratory. In the laboratory, white grub larvae in their plastic vials were packed into plastic 4 litre (L) trays and were maintained at 23°C and 75% Relative humidity (RH) in the Insect Rearing Unit (IRU) Quarantine room at SASRI until they were used. All larvae were left for 10 days under these conditions to allow diseased larvae to die. Only larvae surviving the 10 days screening period were used in bioassays.

Prior to conducting bioassays, grubs were identified to species level by examining their raster patterns on the ventral surface of the last abdominal segment (Sweeney 1967; Dittrich *et al.*, 2006; See Figure 3.1). Specimens were grouped on the basis of head capsule width into small (2 mm) (second instar) and large (3-4 mm) larvae (third instar) by measuring head capsule width using a Digital Caliper³ (0-150 mm) (Sweeney, 1967; Wilson, 1969; Cock and Allard, 2013; Way *et al.*, 2013; Goble *et al.*, 2015).

3.2.3 Adult collection

3.2.3.1 *Heteronychus licas*

Adults were collected by digging out sugarcane roots and soil in 0.30x0.30x0.30 m pits. Infected plants were identified by the presence of a dead heart in the young sugarcane shoot (Conlong, 2015). Pits were positioned across the infected sugarcane stools. Each adult was placed into a plastic vial (30 ml) containing autoclaved peat. Vials were sealed with perforated lids. Plastic cooler boxes with ice packs were used for transportation. Adults were sexed according the morphology of the fore-legs. Information to identify *H. licas* adults and to distinguish between adult male and female is described in detail by Sweeney (1967), Wilson (1969), and Dittrich-Schröder *et al.* (2009).

²Peat Moss bale 275L, by Grovida c.c. Horticultural Products at 400 Sydney Road, PO Box 18163, Dalbridge, Durban 4014

³Digital Caliper by MARSHALTOOLS™

3.2.3.2 *Heteronychus tristis*

Adults of this species were found at the site described in Table 3.1, without digging up the sugarcane stool. They were hand collected from groups feeding at the base of young sugarcane stalks. Adults were packed, transported from the field, identified and sexed as described for *H. licas* above.

3.2.3.3 *Asthenopholis minor*

At the collection site (Table 3.1) on the sampling day, adult *A. minor* were observed flying about 0.5 m above the ground at about 9 am. The day was hot and clear. Adults were flying over a gravel road adjacent to a sugarcane field. Most were caught using a sweep net and transferred into 2 L plastic containers sealed with perforated lids. The adults were transported using a vehicle to the laboratory at SASRI. All adults were used within 12 hours of arrival at the laboratory because they have a short life span (Harrison, 2009), and were not sexed because there was no clear sexual dimorphism evident.

3.2.3.4 *Temnorhynchus clypeatus*

Adults and larvae were collected by digging out sugarcane roots and soil in 0.30x0.30x0.30 m pits. Pits were positioned across the infected sugarcane stools. Adults and larvae were placed individually into plastic vials (30 ml) containing autoclaved peat. Vials were sealed with perforated lids. Plastic cooler boxes with ice packs were used for transportation. For the first bioassay conducted, 90 adults were used on their arrival at the laboratory, because they have short life span (Harrison, 2009), and were not sexed because there was no clear sexual dimorphism evident. For the second bioassay, larvae were reared through to adults by placing them individually into plastic vials (30 ml) containing autoclaved peat which was changed twice per week. Larvae were fed pieces of carrot as food supplement during the rearing process.

3.2.4 Fungal isolates and preparations

Beauveria brongniartii isolates C17 and HHWG1 were mass-produced and formulated by Plant Health Products (PHP)⁴. Five grams of conidia were mixed thoroughly in a sterile 200 ml bottle containing 10 ml distilled water with 0.05% Triton X-100. Bottles were sealed with a lid and the conidial mixture was vortexed for one minute to produce a homogenous conidial suspension. Conidial spore counts were determined using a Neubauer haemocytometer (0.1 mm depth) and conidial concentrations were determined through serial dilutions to get 1×10^9

⁴Plant Health Products (Pty) Ltd in Strathdean farm, Gowrie Avenue, Pietermaritzburg, South Africa

conidial/ml concentration, as per Lacey (1997). This concentration was chosen as the most appropriate concentration to use based on the study of Goble (2012) which indicated that 1×10^9 conidial/ml of *B. brongniartii* isolate HHWG1 was highly infectious and caused 95% mortalities on large larvae of *S. affinis*, 30 days after treatment (DAT). Conidial suspensions were used within 3 hours of mixing and dilution to minimise reduction of isolate viability. Furthermore, to assess whether the formulated isolates were still viable, conidial viability was evaluated by plating out 0.1 ml of conidial suspension onto three Sabouraud Dextrose Agar (SDA) plates and then incubating them for 4 days at 23-25 °C. SDA is a selective solid media prepared by dissolving 60g of SDA 4% agar (Merck) in 1 L of distilled water and autoclaved for 15 min at 121°C and cooled to 55°C. The medium was also supplemented with 0.05g/ml rifampicin (Sigma-Aldrich; St. Louis), 0.05 g/ml cycloheximide (Calbiochem; Canada), 0.05g/ml chloramphenicol (Sigma-Aldrich; St. Louis) and 0.02g/ml dodine (Sigma-Aldrich; St. Louis) antibiotics to prevent bacterial and saprophytic fungal growth (Goble *et al.*, 2015). The mixture was decanted into and stored in 90mm plastic petri dishes which were placed in the laboratory at 25°C ambient temperature until used.

3.2.5 Bioassays against white grub larvae

A conidial suspension (1×10^9 conidia/ml) was prepared per fungal isolate as described in the above section. White grub larvae were grouped into small and large categories (as described in the “3.2.2 larval collection” section). The number of specimens used in the bioassays per species and per category are described in Table 3.2, “No. of specimens” column. Larvae were placed individually into petri dishes (90 mm diameter) and allowed to settle for a minute because it was easier to inoculate on stationary specimens. The Goble *et al.*, (2015) method was used to inoculate specimens whereby 10 microlitres (μ l) of the conidial suspension was pipetted using an auto-pipette onto the dorsal side of the thorax of the larvae. Ten μ l of distilled water with 0.05% Triton X-100 was inoculated in the same way onto control larvae. Specimens were allowed to dry for 15 minutes to ensure attachment of spores. Specimens were then put into vials with autoclaved moist peat. A small piece (10mm \times 10 mm \times 10mm) of carrot was placed in each vial for larvae to feed. All vials were labelled by isolate name and size of larvae, and sealed with a perforated lid. The vials with the larvae were then grouped according to the fungal isolates inoculated on them, and placed into 4 L trays at 23°C, with 72% RH and 12:12 h as an L:D cycle for 35 days. Laboratory temperature and RH were evaluated every day. Larvae were evaluated at 5 day intervals up to 35 days by carefully tipping them from their vials into a petri dish to determine mortality. All live grubs were replaced into their respective vials with fresh

peat, and peat was moistened when necessary. All dead grubs were examined, counted and recorded. Dead grubs with overt mycosis were counted and recorded. Dead grubs without clear mycosis and dead grubs in the controls were surface disinfected under laminar flow by dipping them into a 250 ml conical flask containing 70% ethanol for 2 minutes. All surface disinfected grubs were dried by placing each grub on a sterile paper towel for one minute. They were then plated out onto petri dishes (90 mm diameter) with SDA medium and incubated at 23°C to allow for the development of mycosis. Cause of death was assessed by scoring mycosis on the grubs' bodies at time of collection from the vials and after incubation.

Table 3.2: A summary of the bioassays used to evaluate the efficacy of two virulent isolates of *Beauveria brongniartii* (C17 and HHWG1) against white grubs collected in South African sugarcane.

Bioassay	No. of treatments	Species	Life stage	Sex	No. of specimens	Collection Date
A	3	<i>T. clypeatus</i>	Small ^a	-	21	15 Jul 2015
			Large ^b	-	21	
B	3	<i>H. licas</i>	Small ^a	-	60	13 Aug 2015
			Large ^b	-	60	
C	3	<i>A. minor</i>	Small ^a	-	15	07 Oct 2015
			Large ^b	-	15	
D	3	<i>H. tristis</i>	Large ^b	-	38	13 Jan 2016
E	3	<i>S. neglecta</i>	Large ^b	-	15	13 Jan 2016
F	3	<i>H. licas</i>	Adults	Males	20	21 Oct 2015
				Females	20	
G	3	<i>A. minor</i>	Adults	-	90	07 Oct 2015
H	3	<i>H. tristis</i>	Adults	Males	20	13 Jan 2016
				Females	20	
J	3	<i>T. clypeatus</i>	Adults	-	180	14 Jul 2016

^aSecond instar; and

^bThird instar, determined by measuring diameter of their head capsule.

3.2.6 Bioassays against white grub adults

Adult white grubs were identified to species and sexed (“3.2.3 adult collection”, section). Adults were placed into a 9 cm plastic petri dish and inoculated using the method of Goble *et al.* (2015). Ten µl of 1×10^9 conidial/ml concentration of C17 and HHWG1 using an auto-pipette was placed on the dorsal portion of the thorax just behind the head. In the controls, adults

were inoculated with 10 µl of 1 L distilled water with 0.05% Triton X-100 solution. This inoculation method was used for all adults and they were left to dry in a petri dish for 15 minutes before they were placed into cages. Cages (0.30 m × 0.40 m × 0.40 m) were prepared in advance before performing bioassays. Each cage used was made up of an iron frame fixed onto a 4 L plastic tray (Goble *et al.* 2015). The trays were half filled with sterile peat, and the cage was covered with a net mesh which was the same size as a cage (Goble *et al.* 2015). Each treatment consisted of two replicates of 10 female and 10 male adults for *H. licas* and *H. tristis*, respectively, and three replicates of 10 adults per treatment if adults were not sexed, as for *A. minor*. Bioassays for all other adults except for *A. minor* were conducted in a laboratory at a room temperature of 23°C, with 72% RH and L:D cycle of 12:12 h. Bioassays for *A. minor* were done at a room temperature of 30°C with 40% RH and 12:12 h as an L:D cycle, because *A. minor* were collected during daytime on hot days (30°C). Adults were fed young sugarcane plants by placing two young sugarcane plants in an Oasis Wet⁵ block (0.08 m × 0.08 m × 0.08 m), previously soaked with distilled water. The effect of *B. brongniartii* isolates was evaluated after eight days by counting dead adults with clear mycosis. The ones without overt mycosis were disinfected with 70% ethanol and plated onto SDA medium and incubated for 4 days at 23°C for further mycosis development.

3.3 Statistical analyses

Cumulative mortality percentages of the small and large larvae of white grub species were calculated and recorded using Abbott's formula (Abbott, 1925):

$$CM = \frac{\% \text{ mortality in treatment} - \% \text{ mortality in control}}{100 - \% \text{ mortality in control}} \times 100 \quad (3.1)$$

Percentage mortality used to describe results were corrected for any control mortality.

For bioassays of small and large white grub larvae, Simple Linear Regression with groups was used to determine whether proportional mortality varied between fungal isolate treatments, and the interaction effect between the isolates and days after treatment was assessed (Guzmán-Franco *et al.*, 2012). There were no systematic deficiencies experienced when using this

⁵Oasis Wet 20 per box, by Grovida c.c. Horticultural Products at 400 Sydney Road, PO Box 18163, Dalbridge, Durban 4014

analysis and the model was desirable for the data as compared to other regression link functions tested. The analysis was conducted using GenSTAT 18th edition (VSN International Ltd, 2016). Median survival times (ST_{50}) for treated larvae were determined using Kaplan–Meier tests, which were used to determine the time (i.e. days) at which death occurs in 50% of the tested insects after treatment with the fungal isolates (Lacey, 2012; Goble *et al.*, 2015). The analysis was conducted in GenSTAT 18th edition (VSN International Ltd, 2016).

The statistical differences between the adults (females and males) of *H. licas* and *H. tristis* and a control were analysed using a two-way Analysis of Variance. The interaction effect between treatment were compared using a Tukey’s multiple comparison test ($P=0.05$) (Crichton, 1999). The virulence of isolates toward adults of *A. minor* and *T. clypeatus* was determined using a one-way ANOVA ($P=0.05$). The analysis was conducted in GenSTAT 18th edition (VSN International Ltd, 2016).

3.4 Results

3.4.1 Bioassays against white grub larvae

Overt mycosis of *T. clypeatus* small larvae treated with C17 and HHWG1 isolates was recorded, while no mycosis was observed in larvae treated with distilled water and 0.05% Triton-X solution (control) (examples are shown in Figure 3.2).

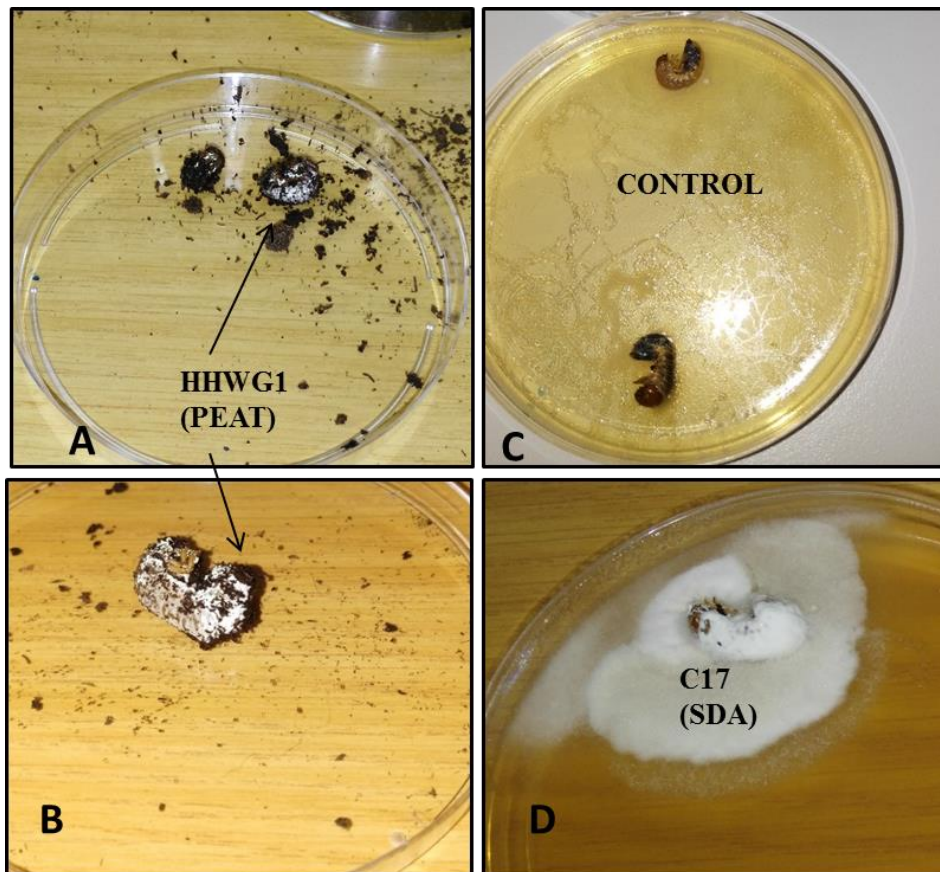


Figure 3.2: Mortality evaluation in larvae of white grubs after they were treated with *Beauveria brongniartii* isolates C17 and HHWG1, showing white powdery growth covering their body after treatment. (A and B) Dead larvae treated with HHWG1 inoculum, that were recovered from a peat vial during mortality evaluation ; (C) No fungal growth in dead larvae in the control ; and (D) dead larva treated with C17 inoculum, plated on SDA medium covered with mycosis, after 4 days of incubation at 23°C.

3.4.1.1 Small and large larvae of *Temnorhynchus clypeatus*

There was a significant difference ($\chi^2=105.03$; $df=11$; $P=0.017$) between small and large larvae at 35 DAT with both C17 and HHWG1, respectively (Figure 3.3). The HHWG1 isolate induced higher mortality (85%) compared to C17 (76%) (Figure 3.4A). Both isolates (C17 and HHWG1) caused low mortalities of large larvae (5 and 12%), respectively (Figure 3.4A). The median survival time (ST_{50}) was 15 days (95% CI: 15 days) for HHWG1 treated small larvae, whereas for C17 treated small larvae, ST_{50} was 20 days (95% CI: 20 days) (Figure 3.3B).

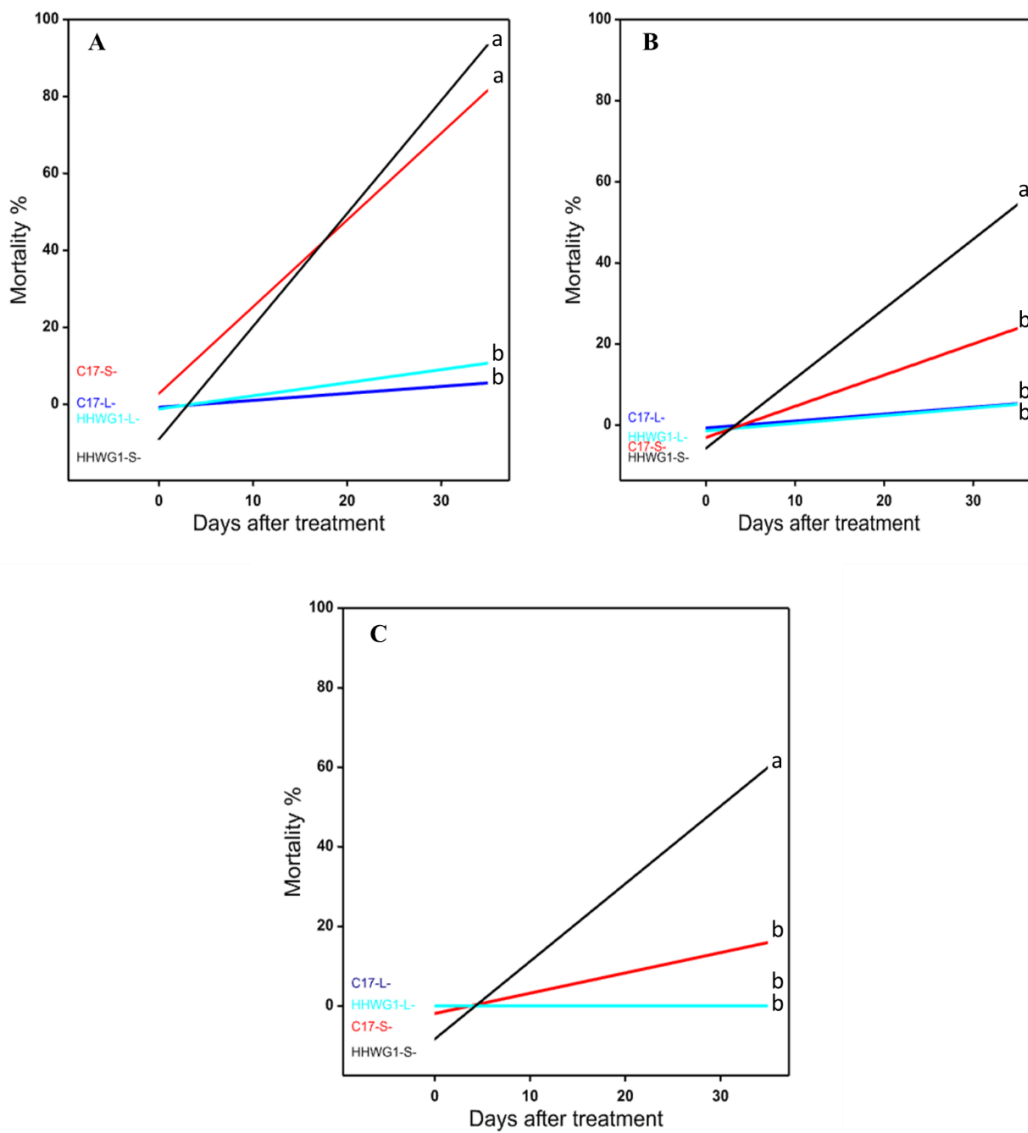


Figure 3.3: The predicted mortality percentages for small (S) and large (L) larvae of (A) *Temnorhynchus clypeatus*; (B) *Heteronychus licas*; and (C) *Asthenopholis minor* showing the efficacy of *Beauveria brongniartii* isolates (C17 and HHWG1), 35 days after treatment. Different letters indicate significant differences between the treatments.

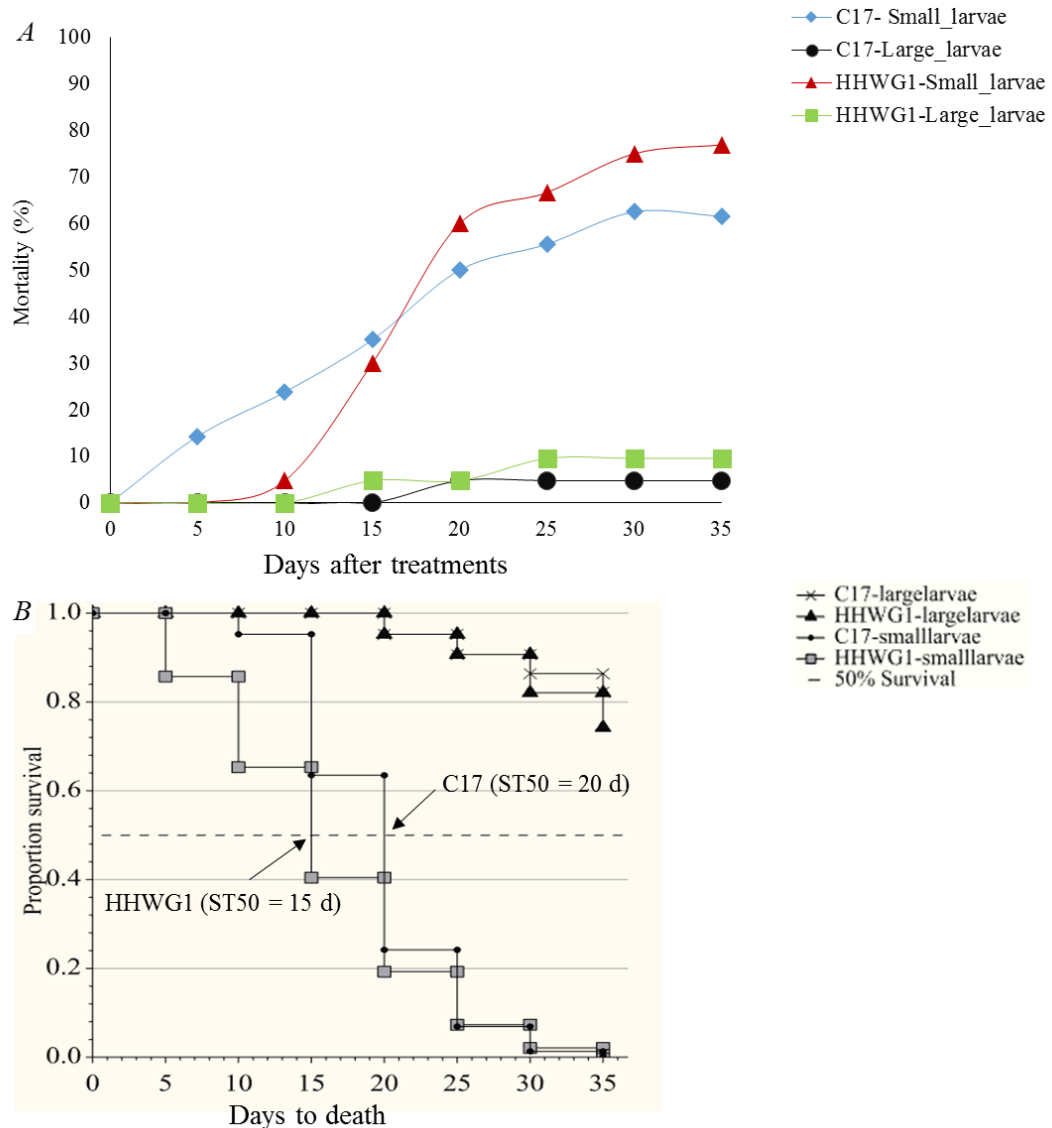


Figure 3.4: Cumulative mortality (A); and (B) estimates of survival (ST₅₀) of larvae of *Temnorhynchus clypeatus* 35 days after treatment showing the efficacy of two *Beauveria brongniartii* isolates (C17 and HHWG1) at a concentration of 1 x 10⁹ conidia/ml suspension maintained at 23°C, 12:12 h L:D.

3.4.1.2 Small and large larvae of *Heteronychus licas*

There was also a significant difference between the effect of C17 and HHWG1 against the small larvae 35 days after treatment ($\chi^2=109.31$; df =11; $P<0.001$) (Figure 3.3). As with *T. clypeatus*, although the isolates were not as virulent, small larvae of *H. licas* were more susceptible to both isolates of *B. brongniartii* than large larvae (Figure 3.5). HHWG1 caused higher mortalities (53%) than C17 (29%) in small larvae (Figure 3.5A). C17 and HHWG1 caused low mortalities of large larvae (5 and 7%), respectively, (Figure 3.5A). The effect of *B. brongniartii* isolates

(C17 and HHWG1) was observed 5 DAT in small larvae compared to 20 DAT in large larvae, and ST_{50} was 25 days (95% CI: 25 days for HHWG1 and 35 days for C17) in small larvae (Figure 3.5B).

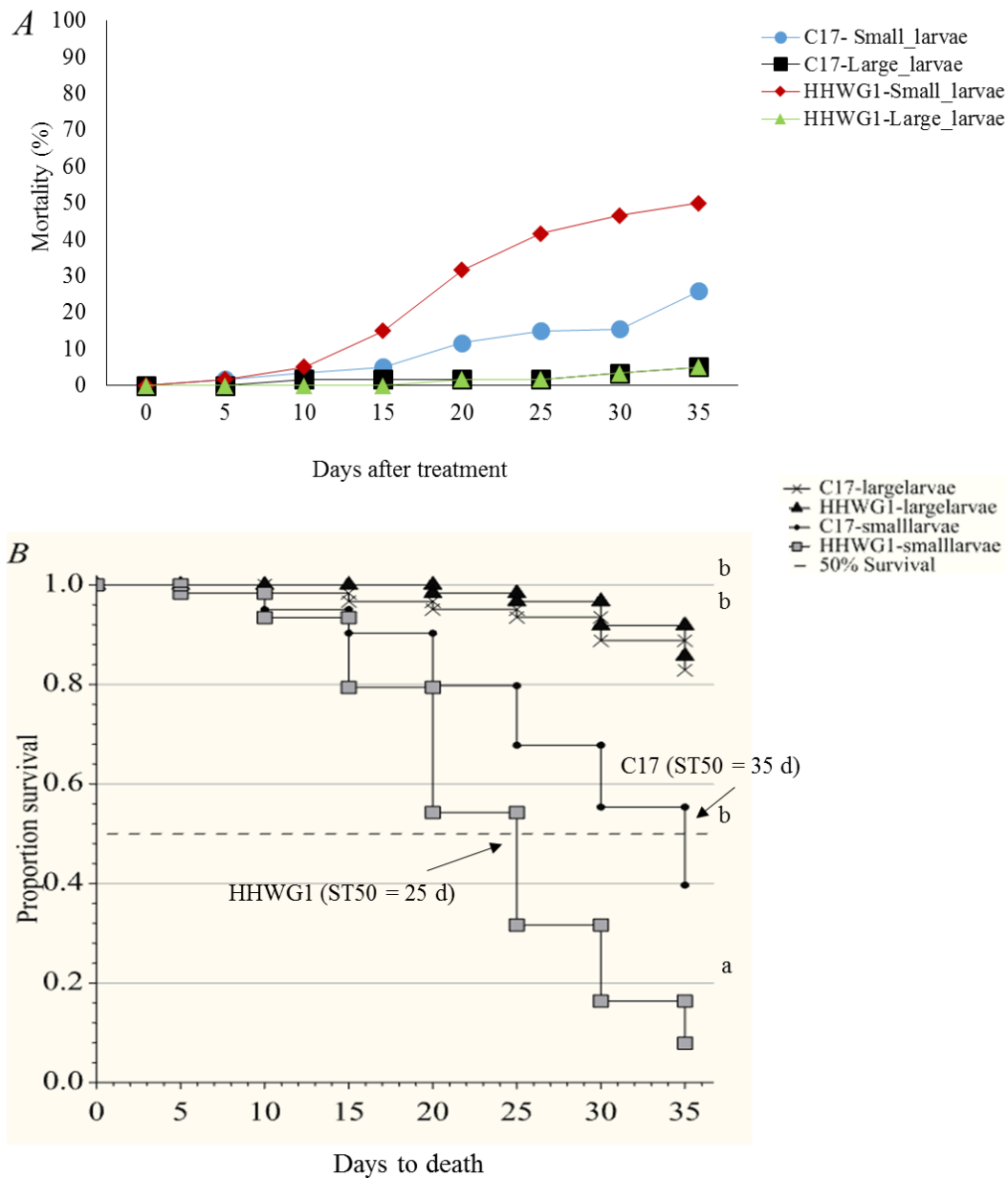


Figure 3.5: Cumulative mortality (A); and (B) estimates of survival (ST_{50}) of larvae of *Heteronychus licas*, 35 days after treatment with two *Beauveria brongniartii* isolates (C17 and HHWG1) at a concentration of 1×10^9 conidia/ml suspension, and maintained at 23°C, 12:12 h L:D.

3.4.1.3 Small and large larvae of *Asthenopholis minor*

Once again small larvae were most susceptible to the two isolates, and HHWG1 was more virulent than C17. There was a significant difference in mortalities caused by HHWG1 and C17 ($\chi^2=51.42$; $df=11$; $P<0.001$) (Figure 3.3), in small larvae. No mortality due to the two isolates was recorded in large larvae. HHWG1 caused significantly high mortality in small (52%) and large larvae (19%) (Figure 3.6A), 35 days post-inoculation; and there was a significant difference between small and large larvae treated with HHWG1 and C17 isolates, respectively. Both isolates took longer to cause death in small larvae and ST_{50} was 25 days (95% CI: 25 days) post inoculations (Figure 3.6B).

Small larvae of *T. clypeatus*, *H. licas* and *A. minor* were most susceptible to the two fungal entomopathogenic isolates (C17 and HHWG1) when compared to the large larvae, with large larvae being far more resistant to them. Small larvae of *T. clypeatus* were most susceptible to both isolates, with at least 20% more mortality compared to the two other species. The most virulent isolate for all small larvae was HHWG1.

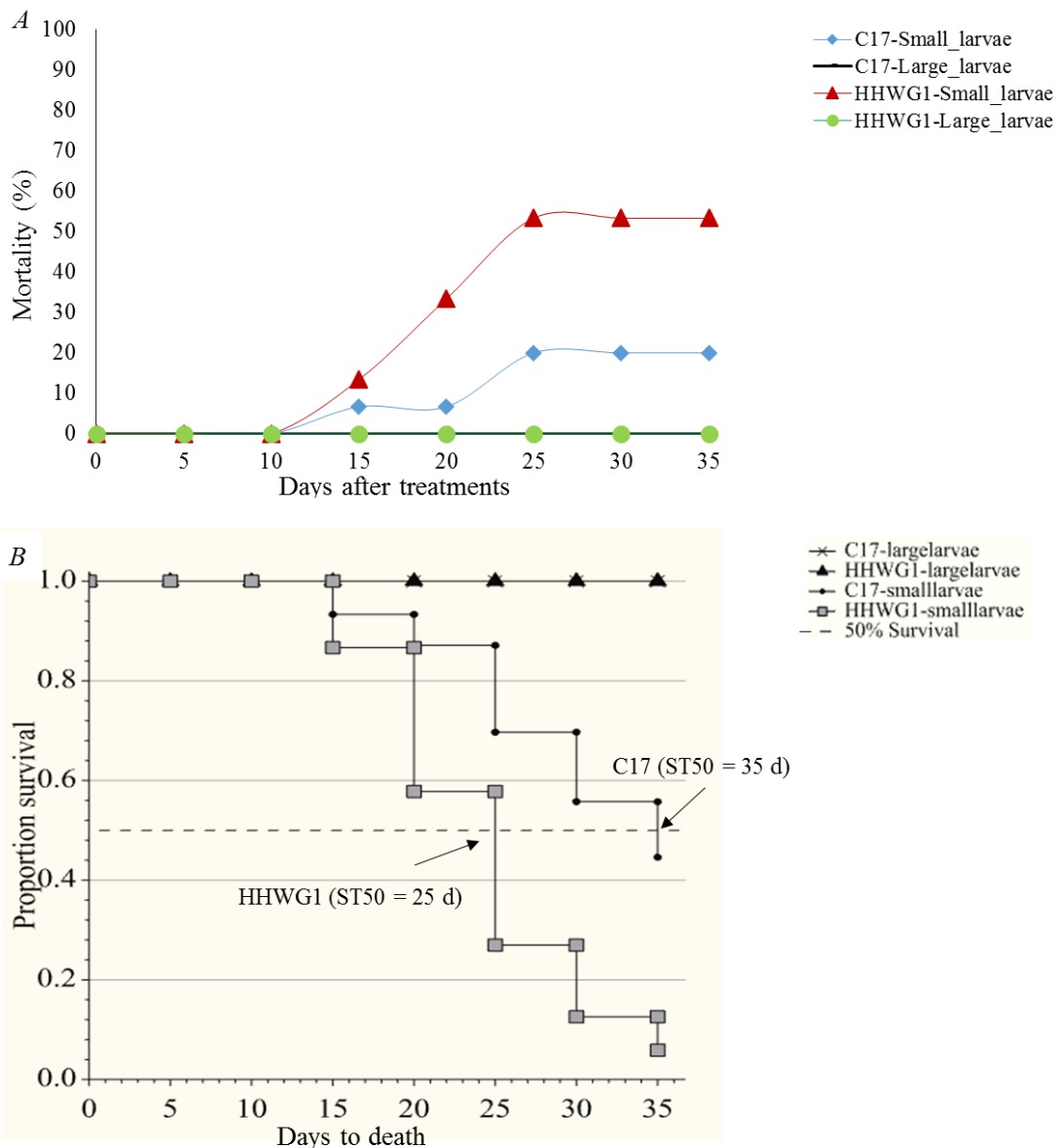


Figure 3.6: Cumulative mortality (A); and (B) estimates of survival (ST₅₀) of larvae of *Asthenopholis minor*, 35 days after treatment with two *Beauveria brongniartii* isolates (C17 and HHWG1) at a concentration of 1×10^9 conidia/ml suspension, and maintained at 23°C, 12:12 h L:D.

3.4.1.4 Large larvae of *Schizonycha neglecta* and *Heteronychus tristis*

There was no significant difference between the mortalities caused by the two isolates of *B. brongniartii* in large larvae of *S. neglecta* ($\chi^2 = 62.78$; $df = 5$; $P = 0.071$) (Figure 3.7). Large larvae of *S. neglecta* were however, highly susceptible to HHWG1 (80%) and less so to C17 (49%) (Figure 3.8A). HHWG1 caused mortality within 5 days post-inoculation in large larvae of *S. neglecta* and ST₅₀ was 10 days (95% CI: 10 days) (Figure 3.8B).

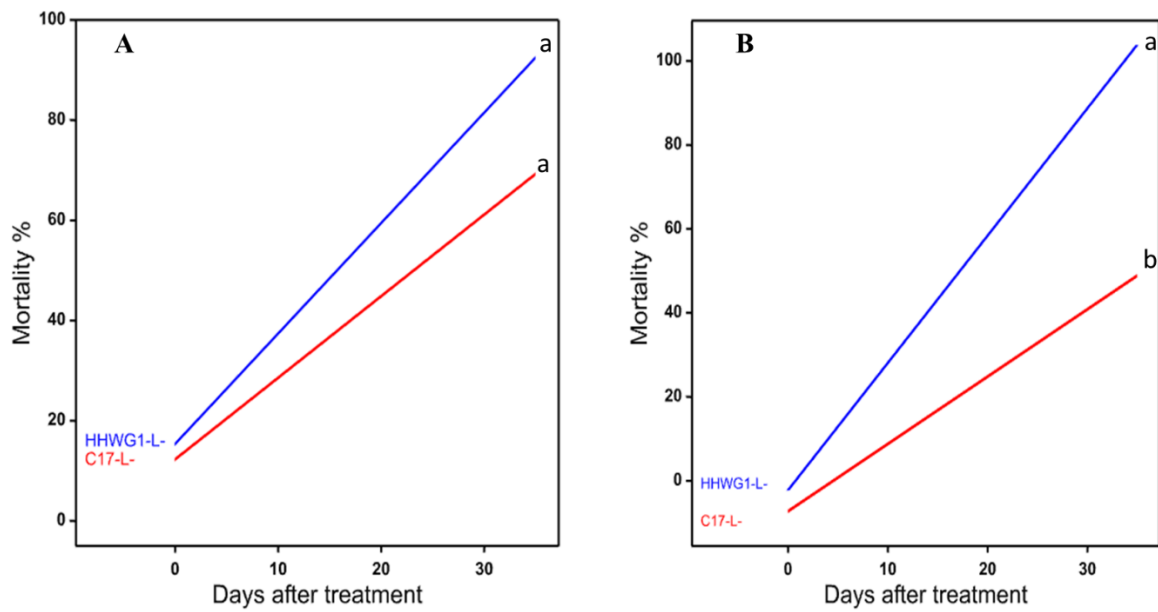


Figure 3.7: The predicted mortality percentages for large (L) larvae of (A) *Schizonycha neglecta*; and (B) *Heteronychus tristis* showing the efficacy of *Beauveria brongniartii* isolates (C17 and HHWG1), 35 days after treatment. Different letters indicate significant differences between the treatments.

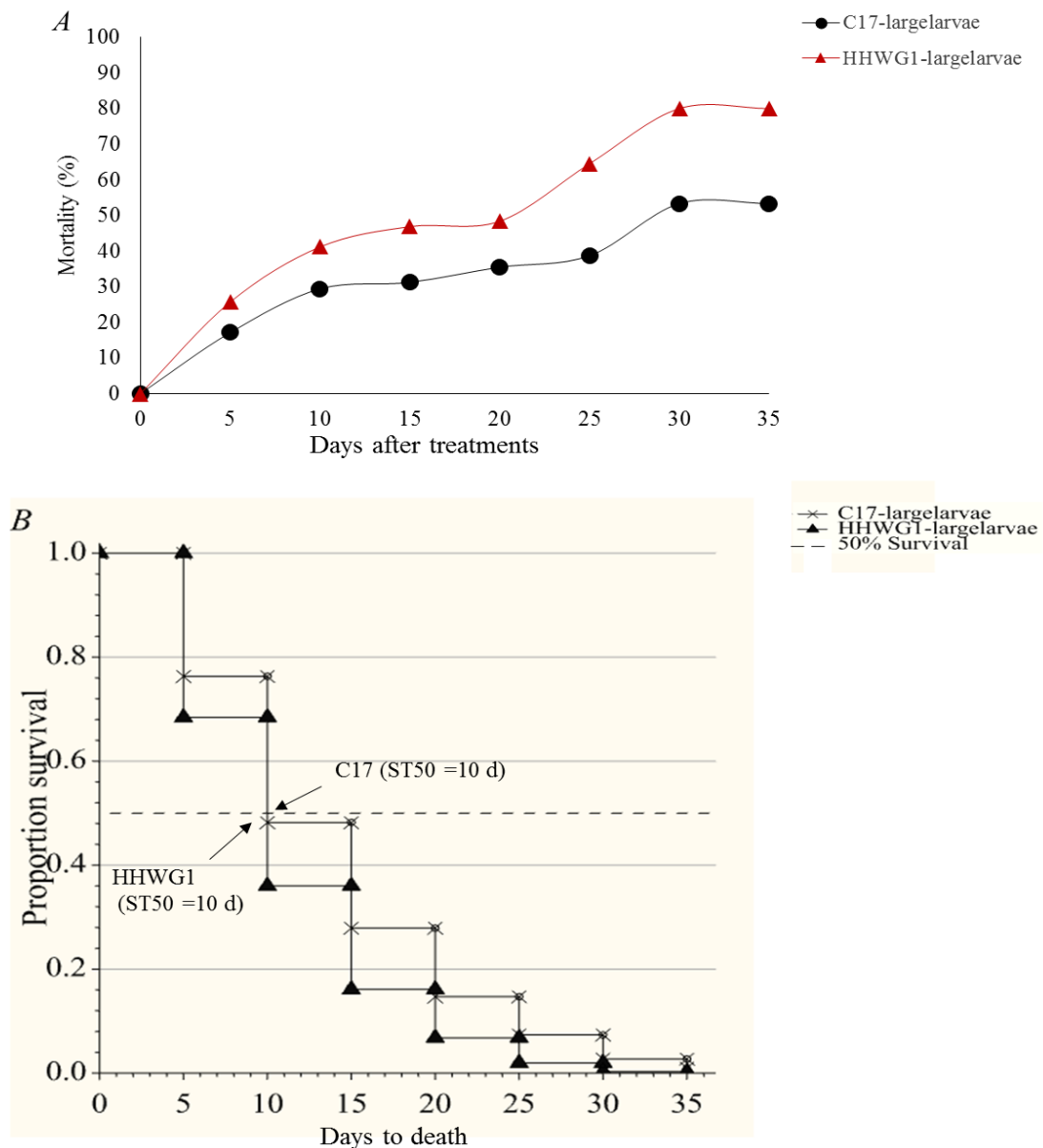


Figure 3.8: Cumulative mortality (A); and (B) estimates of survival (ST₅₀) of large larvae of *Schizonycha neglecta*, 35 days after treatment with two *Beauveria brongniartii* isolates (C17 and HHWG1) at a concentration of 1×10^9 conidia/ml suspension, and maintained at 23°C, 12:12 h L: D.

In contrast to *S. neglecta* larvae, there was a significant difference between the mortalities caused by the two isolates of *B. brongniartii* in large larvae of *H. tristis* ($\chi^2 = 72.52$; df = 5; $P < 0.001$) (Figure 3.7). Large larvae of *H. tristis* were highly susceptible to HHWG1 (93%) and less so to C17 (20%) (Figure 3.9A); and ST₅₀ was 15 days (95% CI: 15 days) after treatment (Figure 3.9B). Overall, large larvae of *T. clypeatus*, *H. licas* and *A. minor*, were much more susceptible to the two *B. brongniartii* isolates, with HHWG1 being particularly pathogenic.

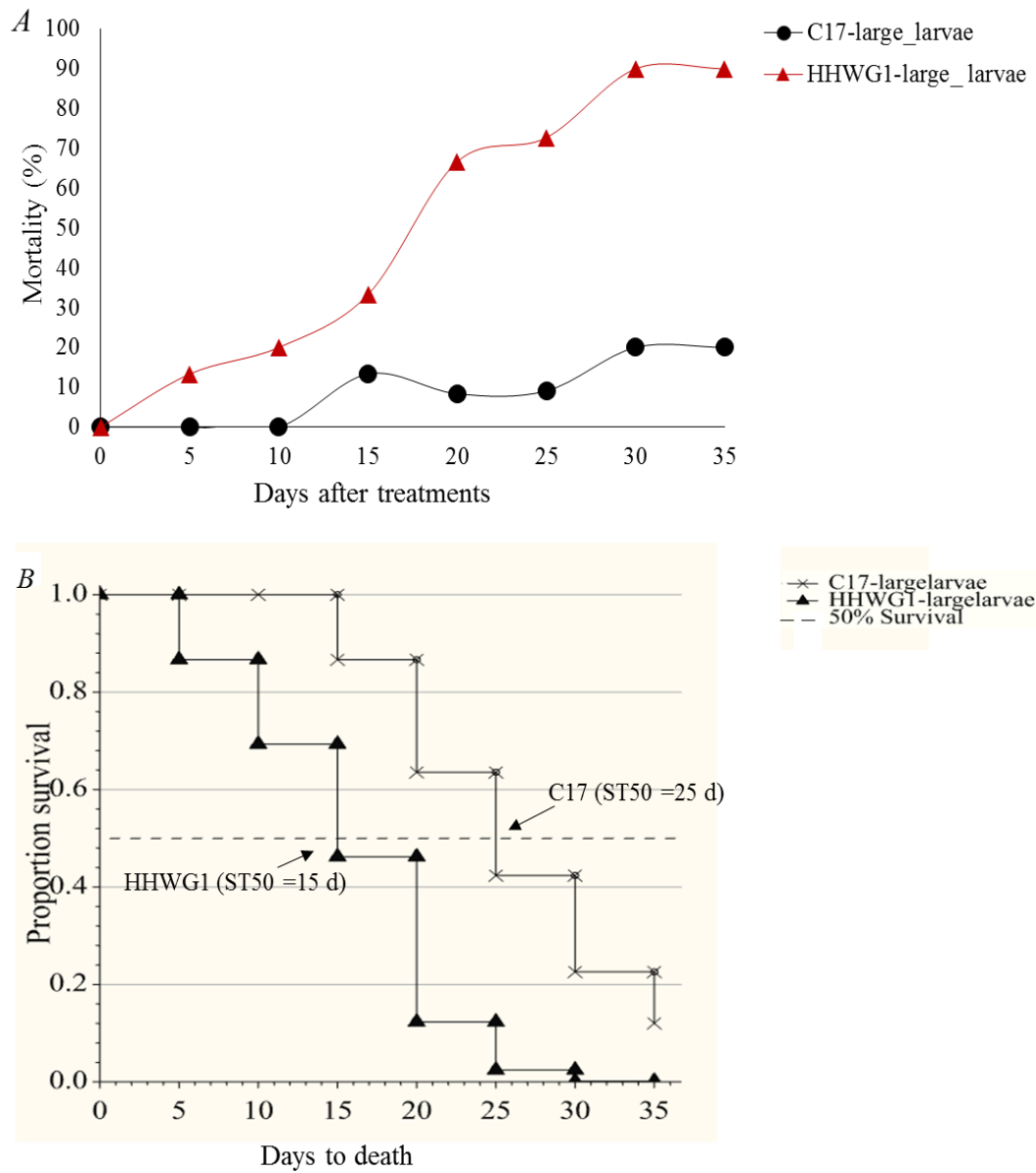


Figure 3.9: Cumulative mortality (A); and (B) estimates of survival time (ST₅₀) of large larvae of *Heteronychus tristis*, 35 days after treatment with two *Beauveria brongniartii* isolates (C17 and HHWG1) at a concentration of 1×10^9 conidia/ml suspension, and maintained at 23°C, 12:12 h L: D.

3.4.2 Bioassays against white grub adults

Heteronychus licas, *Heteronychus tristis* and *Asthenopholis minor*

No mycosis was observed after 4 days of incubation when mycosis development was evaluated in the control for the adult bioassays (Figure 3.10).

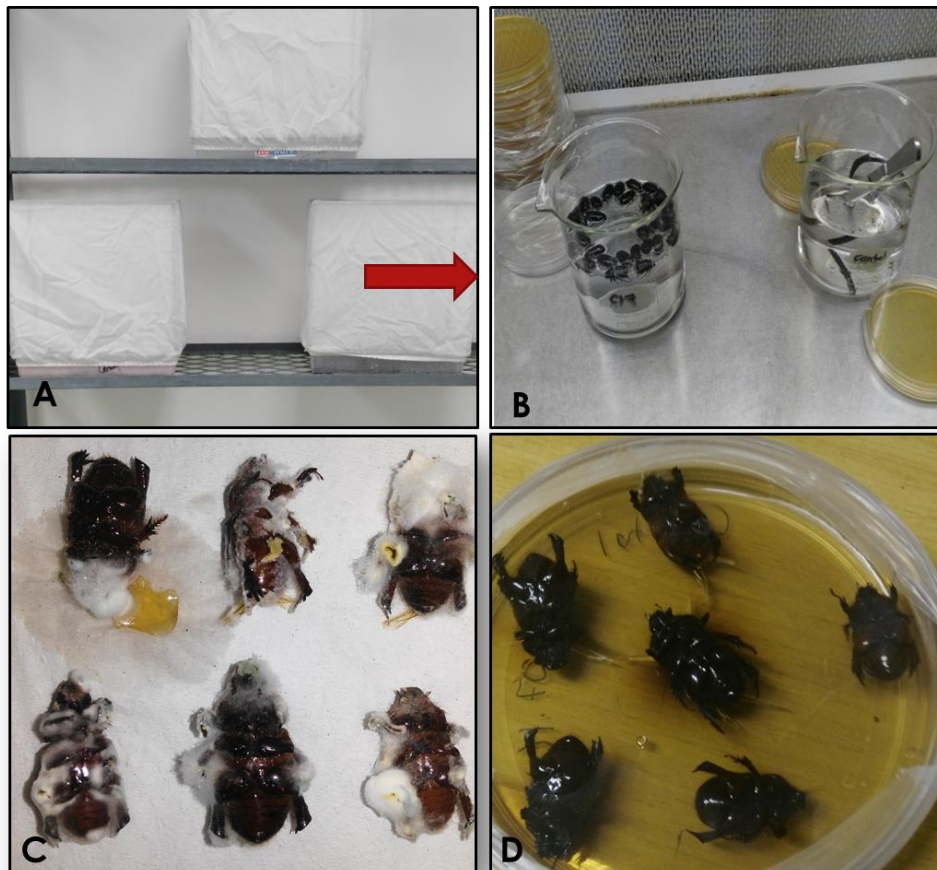


Figure 3.10: Development of fungal mycosis on adult white grubs after inoculating with *Beauveria brongniartii* isolates (C17 and HHWG1). (A) Cages used to hold adults; (B) Adult surface disinfected with 70% ethanol to evaluate further development of mycosis; (C) Mycosed adults; (D) Control adults with no overt mycosis development.

Adults of all four species (*H. licas*, *H. tristis*, *A. minor* and *T. clypeatus*) were more susceptible to *B. brongniartii* isolate C17 compared to isolate HHWG1 (Figure 3.11-3.14). The former caused around 50% more adult mortality than the latter in all species (Figure 3.11-3.14). Adult males of *H. licas* were more susceptible (85% mortality) to C17 than females (63% mortality)

(Figure 3.11); and C17 isolate caused significantly high mortality ($F_{(2, 4)}=100.33$; $P<0.001$) compared to HHWG1 and the Control on both female and male adults of *H. licas*.

The C17 isolate was effective against females and males of *H. tristis* with mortality of 69% and 76% compared to 10% and 12% mortality caused by HHWG1 isolate, respectively (Figure 3.12); and C17 isolate also caused significantly higher mortality ($F_{(2, 2)}=46.08$; $P<0.001$) than HHWG1 and the Control on both females and males (Figure 3.12).

Adults of *A. minor* were highly susceptible to C17 ($F_{(2, 2)}=19.50$; $P=0.002$); with C17 causing 85% mortality as compared to 45% mortality in HHWG1-treated adults (Figure 3.13). *Temnorhynchus clypeatus* adults were also highly susceptible to C17 ($F_{(2, 2)}=25.40$; $P=0.001$); with C17 causing 72% mortality compared to 46% mortality in HHWG1-treated adults (Figure 3.14).

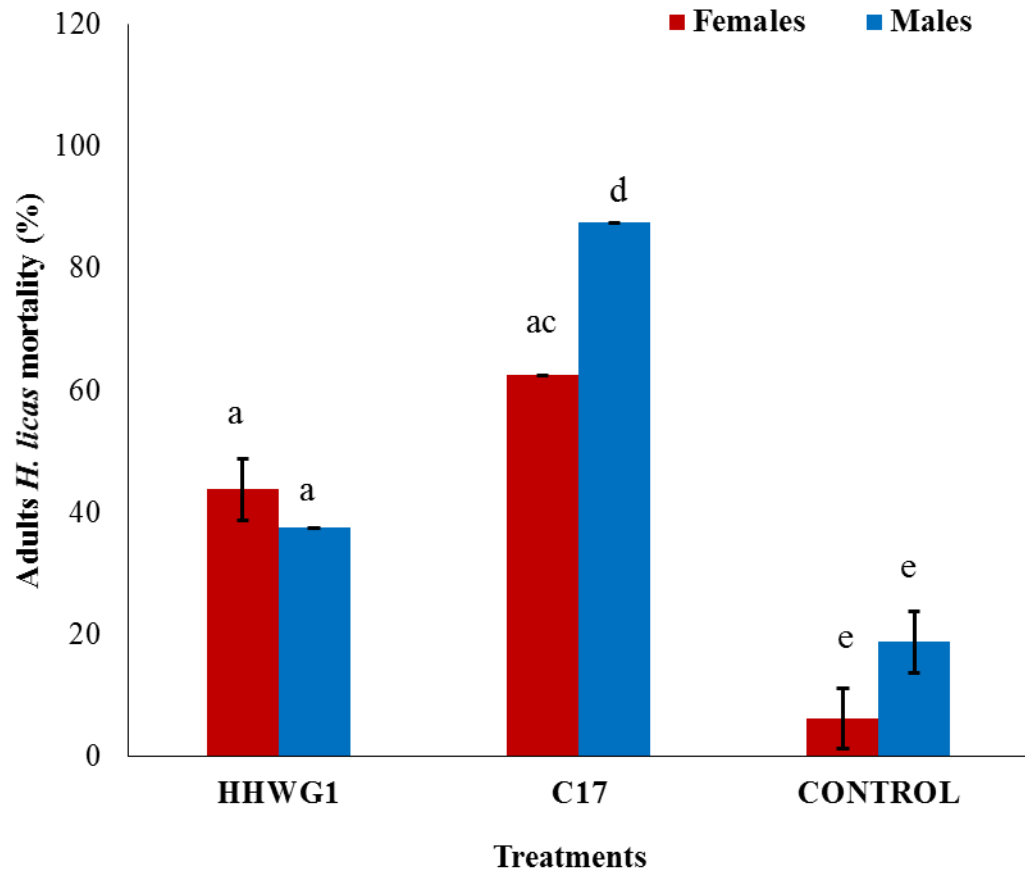


Figure 3.11: Mean mortality percentage (\pm SE) of *Heteronychus licas* adults eight days after treatment, showing the effect of two *Beauveria brongniartii* isolates (C17 and HHWG1) at a concentration of 1×10^9 conidia/ml and 1 L distilled water with 0.05% Triton X-100 solution for the control. Eight days after treatment, dead adult white grubs were surface disinfected with 70% ethanol to evaluate further development of mycosis. Different letters above the histograms indicate significant differences at $P \leq 0.05$ calculated using Tukey multi-comparisons test. SE: Standard error.

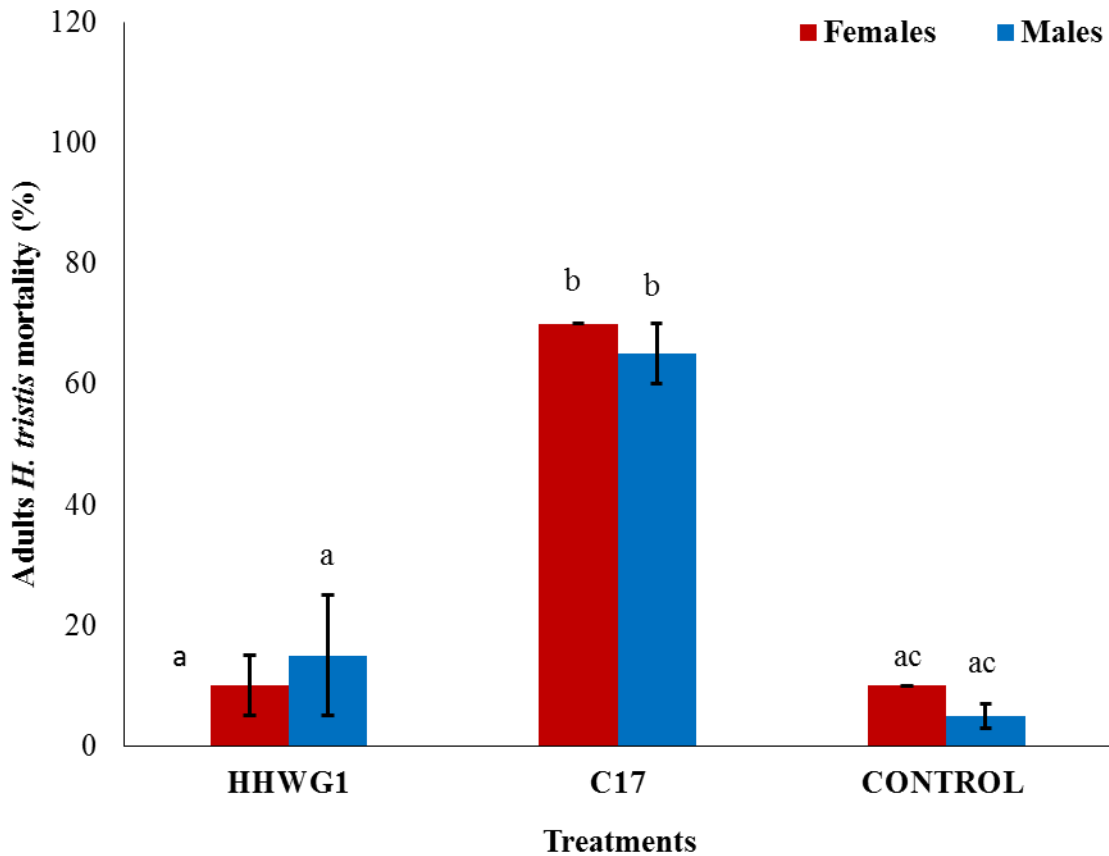


Figure 3.12: Mean mortality percentage (\pm SE) of *Heteronychus tristis* adults eight days after treatment, showing the effect of two *Beauveria brongniartii* isolates (C17 and HHWG1) at a concentration of 1×10^9 conidia/ml and 1 L distilled water with 0.05% Triton X-100 solution for the control. Eight days after treatment, dead adult white grubs were surface disinfected with 70% ethanol to evaluate further development of mycosis. Different letters above the histograms indicate significant differences at $P \leq 0.05$ calculated using Tukey multi-comparisons test. SE: Standard error.

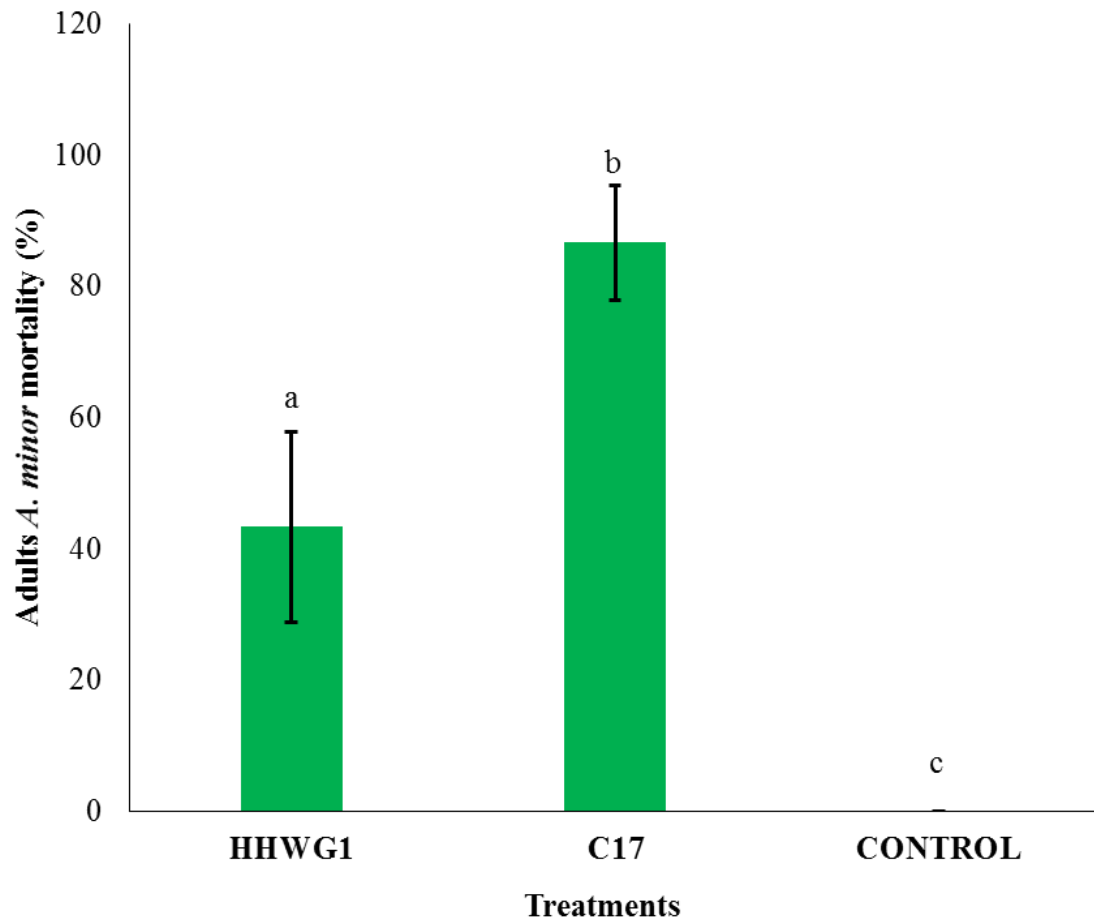


Figure 3.13: Mean mortality percentage (\pm SE) of *Asthenopholis minor* adults eight days after treatment, showing the effect of two *Beauveria brongniartii* isolates (C17 and HHWG1) at a concentration of 1×10^9 conidia/ml and 1 L distilled water with 0.05% Triton X-100 solution for the control. Eight days after treatment, dead adult white grubs were surface disinfected with 70% ethanol to evaluate further development of mycosis. Different letters above the histograms indicate significant differences at $P \leq 0.05$ calculated using Tukey multi-comparisons test. SE: Standard error.

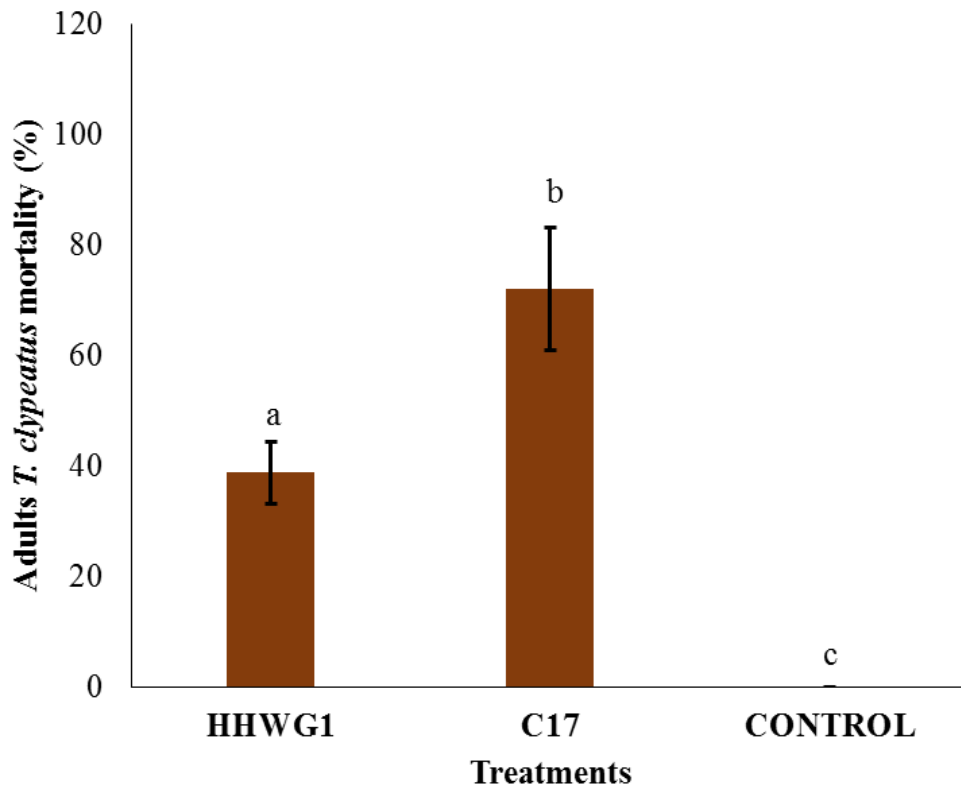


Figure 3.14: Mean mortality percentage (\pm SE) of *Temnorhynchus clypeatus* adults eight days after treatment, showing the effect of two *Beauveria brongniartii* isolates (C17 and HHWG1) at a concentration of 1×10^9 conidia/ml and 1 L distilled water with 0.05% Triton X-100 solution for the control. Eight days after treatment, dead adult white grubs were surface disinfected with 70% ethanol to evaluate further development of mycosis. Different letters above the histograms indicate significant differences at $P \leq 0.05$ calculated using Tukey multi-comparisons test. SE: Standard error.

3.5 Discussion

Beauveria bassiana Balsamo-Crivelli Vuillemin (Ascomycota: Hypocreales), *Metarhizium anisopliae* Metchinikoff (Ascomycota: Clavicipitaceae) and *B. brongniartii* are important biological control agents, isolates of which have been developed as bio-insecticides against a number of agricultural plant pests (Samson *et al.*, 2006; Goble *et al.*, 2015). Goble *et al.* (2015) found epizootics caused by *B. brongniartii* in two white grub species (*P. sommeri* and *S. affinis*), attacking sugarcane in the Midlands North area of KZN. She further identified two isolates of *B. brongniartii* (C17 and HHWG1) that were highly pathogenic to these two white grub species, causing 95% mortality under laboratory conditions. In order for a bio-insecticide to become a

commercial proposition, it should either be applied over a very large area against a pest of broad distribution, or it should have a wide host range.

This study aimed to evaluate the C17 and HHWG1 isolates of *B. brongniartii* found by Goble *et al.* (2015), against other white grub species affecting sugarcane in South Africa, in order to broaden knowledge of their host insect range. Life stages of five species that were common and causing damage to sugarcane were collected from sugarcane fields in Mpumalanga and the north coast of KZN, and were tested against these two isolates. This study revealed three very important concepts: 1) Different white grub species sharing similar subfamilies had different resistances/susceptibilities to the same pathogens; 2) Within a species, the life stages had different susceptibilities to the same pathogens; 3) Isolates of pathogens found to be most pathogenic to a certain life stage of its original insect host, generally were most pathogenic to the same life stage in other hosts of the same family/subfamily. Dissecting these concepts in the above order a little more:

1) Different white grub species sharing similar subfamilies had different resistances/susceptibilities to the same pathogens

Small larvae of white grubs were all susceptible to the two *B. brongniartii* isolates, but to varying degrees. Species such as *T. clypeatus*, *S. neglecta* and *H. tristis* were more susceptible to the *B. brongniartii* isolates, while other species such as *A. minor* and *H. licas* had low susceptibility to the isolates tested. Keller *et al.* (1999) reported similar results for two populations of Melolonthinae from Italy and Switzerland. Melolonthinae species from Italy were susceptible (72-94%), compared to Melolonthinae species from Switzerland which were significantly less prone (28-72%) to *B. brongniartii* isolates. Keller *et al.* (1999) argued that entomopathogenic fungal isolates might not show similar efficacy to other species within the same subfamily.

2) Within a species, the life stages had different susceptibilities to the same pathogens

In this study it was shown that different life stages of the same species differed in terms of susceptibility to the tested fungal isolates. This was also discovered for *P. sommeri* and *S. affinis* by Goble *et al.* (2015). Furthermore, Goble *et al.* (2015) demonstrated that younger larvae of *P. sommeri* and *S. affinis* were more susceptible to her *B. brongniartii* isolates compared to

large larvae. In our study, the younger larvae of *T. clypeatus* were highly susceptible to both *B. brongniartii* isolates, as was found on the species worked on by Goble *et al.* (2015). However, in this study it was observed that small larvae of *H. licas* and *A. minor* were less susceptible to the HHWG1 and C17 *B. brongniartii* isolates. In addition, it was found that large larvae of *H. tristis* and *S. neglecta* were highly susceptible to both *B. brongniartii* isolates. This study has demonstrated that the infectivity of *B. brongniartii* isolates on different insect life stages varies with the different species tested. Isolates were highly virulent on large larvae of *S. neglecta*, resulting in high mortality. Different susceptibilities in the life stages of white grub larvae to other entomopathogenic fungi have been reported (Kowalska, 2008; Berón and Diaz, 2005). Berón and Diaz (2005) demonstrated that a *B. bassiana* isolate caused higher mortality (70%) of third instar larvae than first instars at 40 days after treatment. Kowalska (2008) also indicated significant differences between the numbers of resistant white grub larvae at different life stages after inoculation with *B. brongniartii*.

3) Isolates of pathogens found to be most pathogenic to a certain life stage of its original insect host, generally were most pathogenic to the same life stage in other hosts of the same family/subfamily

The HHWG1 isolate was most virulent to small and in some cases large larvae, and C17 was highly virulent to adult white grubs. The observed differences of *B. brongniartii* isolate's effectiveness confirm the results of Goble *et al.* (2015). Goble *et al.* (2012) and Enkerli *et al.* (2001) argued that genetically distinct groups of *B. brongniartii* isolates that originated from specific insect life stages may have the same virulent potential against the same life stages that they originated from, regardless of the tested insect species. This applies to other species of fungal entomopathogens, for example *M. anisopliae*. Mazodze and Zvoutete (1999) found *M. anisopliae* isolates less effective on *H. licas* larvae compared to adults, and isolates were slow acting in the soil. Low mortality responses observed in this study, especially for *H. licas* larvae, corroborated Mazodze and Zvoutete's (1999) study. In the present study, *H. licas* larvae were less susceptible to both isolates of *B. brongniartii*, compared to *H. tristis*, with HHWG1 being the most virulent isolate to all larvae.

Adults of *H. licas* and *A. minor* were highly susceptible to C17 compared to small larvae of the same species. This was also indicated by Goble *et al.* (2015). Goble *et al.* (2015) found that adults of *S. affinis* were highly susceptible to *B. brongniartii* isolates compared to the larvae. This can be explained by the findings from other studies that moulting of larvae could remove

the inoculum from the insect cuticle, whereas this process does not occur in adults (Berón and Diaz, 2005; Ortiz-Urquiza and Keyhani, 2013; Goble *et al.*, 2015).

3.6 Conclusion

Building on a former study which demonstrated the potential of *B. brongniartii* isolates C17 and HHWG1 to cause death of two white grub pest species (*S. affinis* and *P. sommeri*) of sugarcane in South Africa, the current study showed these isolates to be effective against a further five species (*T. clypeatus*, *H. licas*, *A. minor*, *H. tristis* and *S. neglecta*), thus increasing their host range. This increased host range adds potential to the *B. brongniartii* isolates HHWG1 and C17 to be developed as potential bio-insecticides for these white grub species in South Africa. However, the isolates C17 and HHWG1 should be tested in replicated field trials against the most susceptible white grub species (*P. sommeri*, *S. affinis*, *T. clypeatus*, *H. trisis* and *S. neglecta*) to prove their efficacy in a field situation.

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CHAPTER 4: CAN *BEAUVERIA BRONGNIARTII* STRAINS BE ESTABLISHED AS ENDOPHYTES OF SUGARCANE ROOTS FOR LONG TERM PROTECTION AGAINST WHITE GRUB SPECIES (COLEOPTERA: SCARABAEIDAE)?

Abstract

Entomopathogenic fungi as endophytes of plants are important to protect plants against herbivorous insects. *Beauveria brongniartii* is pathogenic to white grub species (Coleoptera: Scarabaeidae) that are pests of sugarcane in South Africa. However, little is known about *B. brongniartii* as an endophyte of sugarcane plants. The aim of this study was to evaluate two virulent *B. brongniartii* isolates (C17 and HHWG1) for potential to be established as endophytes of sugarcane roots for possible long term protection against white grubs. Two sugarcane varieties (N12 and N48) were cut into setts and treated with C17 and HHWG1 inoculum at 1×10^7 conidia/ml concentrations. Twenty four replicates per treatment and for the control were used. Sugarcane setts were planted using a randomised complete block design in polystyrene trays and placed inside a controlled temperature glasshouse cubicle. At 30 days post-planting, sugarcane roots per treated sugarcane plant were disinfected with 10% Sodium hypochlorite (NaOCl), 70% ethanol and washed three times with distilled water, to assess endophytic colonization of C17 and HHWG1 isolates in sugarcane roots. Endophytic fungi in sugarcane roots were re-isolated by plating root sections onto Sabouraud Dextrose Agar (SDA) medium and by staining the root sections with lactophenol cotton blue. Endophytic fungal growth in SDA medium was examined visually and by using a bright field microscope using a wet mount. Characters of fungal colony, conidia and conidiophore were examined using a microscope and several representative micrographs were taken. These showed no *Beauveria* species characteristics. Other fungi (*Fusarium* spp. and *Penicillium* sp.) were detected as endophytes of sugarcane roots. Factors such as inoculation method and aggressive antagonistic species might have played a role in preventing *B. brongniartii* being established as endophytes. It was concluded that future research should focus on alternative potential controls such as virulent endophytic *B. bassiana* isolates for long term protection of sugarcane against white grubs.

4.1 Introduction

White grubs (Coleoptera: Scarabaeidae) are among the most damaging pests of sugarcane (*Saccharum sp.* L.) (Wei *et al.*, 1995, Goble, 2012). Feeding damage by white grubs can be easily detected by sugarcane stalk lodging and yellowing of leaves (Sapkota, 2006; Rahama *et al.*, 2014). This then results in high sugarcane yield losses (Goble, 2012). It is therefore of importance to find an effective control strategy in order to alleviate white grub damage in sugarcane plantations. Towards the development of an effective control strategy, *Beauveria brongniartii* (Sacc.) Petch (Ascomycota: Clavicipitaceae) is a potential entomopathogenic fungus (EPF) control agent that has been isolated from white grub species collected from the Midlands North area of KwaZulu-Natal (Goble, 2012). Studies have shown *B. brongniartii* to cause death of white grubs, in laboratory bioassays (Goble *et al.*, 2015) and in field trials (Keller *et al.*, 1999). Moreover, *B. brongniartii* has similar traits to those of *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Ascomycota: Hypocreales), as a potential EPF to control pests. For instance, *B. bassiana* is an important EPF and has been studied intensively because of its ability to endophytically colonize plants (Vega, 2008; Vega *et al.*, 2008; Vidal and Jaber, 2015). The ability of *B. bassiana* to colonize plants has been shown for many species including bananas (*Musa spp.* L.) (Akello *et al.*, 2007; Akello *et al.*, 2008), coffee plants (*Coffea arabica* L.) (Posada *et al.*, 2007; Vega *et al.*, 2010), strawberries (*Fragaria spp.*) (Dara, 2013), sugarcane (Memela, 2015), and cassava (*Manihot esculenta* Crantz) (Greenfield *et al.*, 2016).

Fungal endophytic colonization has been associated with improvements in plant growth, and herbivore and disease protection (Conlong and Rutherford, 2009; Vidal and Jaber, 2015). Studies also inferred that some fungal endophytes have a mutualistic relationship with some plants, and can protect their host plants against pathogens and insect herbivores (Vega *et al.*, 2010). However, the ability of an endophytic fungal entomopathogen to provide pest protection to the plant is hard to detect. This is due to no signs of mycosis development on insects' cuticle after they have fed on EPF inoculated plant tissues (Vega *et al.*, 2008). However, tested insect larvae have shown minimal development after being fed plants with endophytic EPFs, compared to larvae that were fed plants without endophytic EPFs (Cherry *et al.*, 1999, 2004; Vidal and Jaber, 2015).

In contrast to *B. bassiana*, *B. brongniartii* has only been recovered once as an endophyte from the crown of a coffee berry (*Frangula californica* (Eschsch.)) collected in Hawaii (Vega *et al.*, 2008). Goble (2012) isolated *B. brongniartii* isolates from cadavers of two important white grub larvae and adult pests, *Schizonycha affinis* (Boheman) and *Pegylis sommeri* Burmeister

(Coleoptera: Scarabaeidae), of sugarcane in the KwaZulu-Natal Midlands North area of South Africa. Goble *et al.* (2015) further showed 95% mortality caused by *B. brongniartii* isolates (C17 and HHWG1) against immature and adult stages of these white grub species in laboratory bioassays. However, little information is available on *B. brongniartii* as a potential endophytic fungus of sugarcane plants, for long term protection against damage from white grubs. It is also not known whether *B. brongniartii* can be established as an endophyte of plant roots. Therefore, the aim of this study was to determine whether these two isolates of *B. brongniartii* (C17 and HHWG1), found by Goble (2012), could be established as endophytes of sugarcane roots for their long term protection against white grub species, and to subsequently act as biocontrol agents against them.

4.2 Material and methods

4.2.1 Trial design: Study sites and Plant tests

The study was conducted in a glasshouse at the South African Sugarcane Research Institute (SASRI), in Mount Edgecombe, Durban, South Africa. Two commercially grown sugarcane varieties (N12 and N48) were used to test the potential of two *B. brongniartii* isolates (C17 and HHWG1) to be established as endophytes of sugarcane roots for long term protection of the plants against white grub larval damage. The varieties chosen had several good agronomic qualities such as being resistant to *Eldana saccharina* Walker (Lepidoptera: Pyralidae) a sugarcane stalk borer, tolerant to drought and most suited for growing in the Midlands region of KwaZulu-Natal (Mcintyre and Nuss, 1998; Zhou, 2010). The *B. brongniartii* isolates C17 and HHWG1 were formulated and mass-produced by Plant Health Products (Pty) Ltd, in South Africa⁶.

Sugarcane stalks used for inoculation were cut from SASRI fields and transported to the laboratory. Sugarcane stalks were cut into single budded setts (40 mm) which is the section of stalk containing the node with a bud. The internode at both ends of the node are cut off using secateurs, being careful not damage the bud (See Figure 4.1A). Prior to inoculation, the cut setts were sterilized using a hot water treatment (HWT) method, by placing them into separate sterile glass beakers (1000 ml) labelled with variety type, containing sterile distilled water. The glass beakers with cut setts were then placed in a water bath with half-filled with tap water at 55 °C and hot water treated for 30 min. After 30 minutes, the beakers were removed from the water

⁶ Plant Health Products (Pty) Ltd in Strathdean farm, Gowrie Avenue, Nottingham Road, South Africa

bath and the setts were placed onto autoclaved (121 °C for 15 min) sterile paper towels for 5 min to cool down. The cut setts were used on the same day for the experiments.

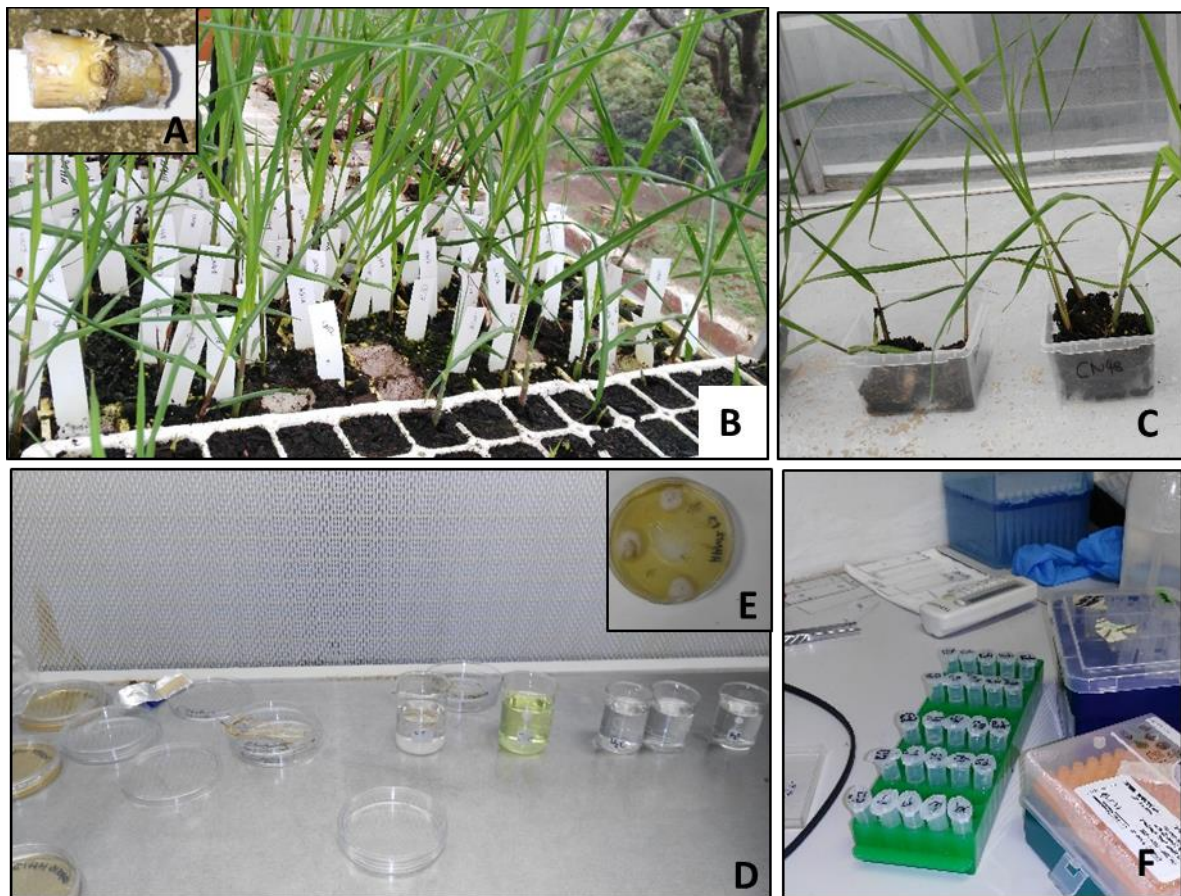


Figure 4.1: Two sugarcane varieties (N12 and N48) were artificially inoculated with two *Beauveria brongniartii* isolates (C17 and HHWG1). (A) Sugarcane setts treated with *B. brongniartii* isolates and sealed with molten wax to prevent opportunistic colonization of other microorganisms; (B) setts planted in polystyrene planting trays filled with composted pine bark; (C) Sugarcane plantlets per treatment uprooted, placed into Sterivent high containers; (D) Sugarcane seedling roots cut into 20 mm pieces and disinfected by soaking them into 10% Sodium hypochlorite for 5 min, 70% ethanol for 1 min and rinsed three times in sterile distilled water to evaluate colonization of endophytic *B. brongniartii*; (E) Roots pieces plated onto SDA medium; (F) Roots cut into small sections and stained using lactophenol solution; and fungal endophytic colonization examined under a bright field contrast microscope.

4.2.2 Preparation of inoculum and inoculation method

To inoculate the prepared setts, 12 g of each isolate (C17 and HHWG1) was mixed thoroughly with 100 ml distilled water containing 0.05% Triton X-100 in a sterile 250 ml glass bottle.

Bottles were sealed with a plastic lid and the conidial mixture was vortexed for 1 minute to produce a homogenous conidial suspension. A Neubauer haemocytometer (0.1 mm depth) was used to estimate the spore concentration and suspensions were adjusted to a final concentration of 1×10^7 conidia/ml for both isolates through serial dilutions, as per Lacey (1997). Thereafter, C17 and HHWG1 conidial suspensions were used to dip-inoculate sugarcane setts (Goodall *et al.*, 1998). The dip method was the most suitable method over the soil drenching method because setts had their cut ends inoculated with conidia suspensions and conidia spores were left to germinate on sett ends before they were sealed with molten wax and planted, to minimise setts being colonized by other opportunistic microorganisms. In a previous study by Memela (2015), minimal *B. bassiana* root colonization was observed when soil drenching was used as an inoculation method. The inoculation process was conducted in the laboratory in a laminar flow cabinet. A group of 20 setts was placed into three 500 ml sterile glass beakers. Homogenous conidial suspension (100 ml) in the treatments and sterile distilled water with 0.05% Triton X-100 (100 ml) in the control were poured into the respective beakers containing setts. Three replicates were used per treatment and control. In total, 48 N12 (16 setts per replicate) and 48 N48 (16 setts per replicate) setts were treated with C17 and HHWG1 conidial suspensions. Further, 48 N12 (16 setts per replicate) and 48 N48 (16 setts per replicate) setts were used in the control. The beakers were labelled with the names of treatments and variety to avoid cross-contamination between treatments. Thereafter, beakers were gently stirred manually for 5 min. After stirring the setts were removed individually from the beakers using autoclaved (121 °C for 15 min) tweezers. Setts were placed into Sterivent containers (107 x 94 x 96 mm) containing a damp paper towel to maintain high humidity. The containers were then placed in an incubator (IncoTherm[®]- Labotec) at 27 °C for four days to induce bud germination. On the fifth day, the setts were assessed for buds and conidial germination and setts without sprouting buds nor visible conidial germination were omitted from the trial.

4.2.3 Sugarcane plants grown to study *Beauveria brongniartii* as an endophyte

After the inoculation procedure, setts were sealed, to minimise colonization by other microorganisms, by dipping them on each of the cut ends in molten wax. A randomised complete block design (Tudu *et al.*, 2007) was used for growing the inoculated setts. The setts were grown with their buds facing upwards in a 96-cell (16 x 6 mm) polystyrene planting tray, and setts were covered with sterile composted pine bark (autoclaved twice at 121 °C for 15 min) (Figure 4.1B). Planting trays containing sugarcane setts were grown by placing the trays in a glasshouse cubicle at 30°C; with 85±5% relative humidity. The planting trays were irrigated

twice every day using automatic irrigating sprinklers for 5 min (600 ml/min) for the sugarcane growth duration time.

4.2.4 Evaluating endophytic colonization of *Beauveria brongniartii* isolates in sugarcane plants

Thirty days post inoculation, three sugarcane plants per treatment were randomly selected using sterile tweezers. Selected plants were removed from their respective cells in the planting trays. The removed plant roots were washed clean of composted pine bark using running tap water, and plants were transported to the laboratory ensuring that no cross-contamination occurred between collected sugarcane plants, by placing them into Sterivent high containers (Figure 4.1C) labelled with the treatment name and variety they contained.

4.2.5 Surface sterilization procedure to ensure that sterile root tissues are used to study endophytes of sugarcane roots

In preparation for re-isolation of possible endophytic *B. brongniartii*, sugarcane roots were cut into small sections (20 mm), using sterile scissors (autoclaved at 121 °C for 15 min), from their respective sugarcane settling plants. Root tissues were pressed onto Sabouraud Dextrose Agar (SDA) medium to examine fungal epiphytes; and were then surface sterilized by dipping them for 1 min in 96% ethanol (Figure 4.1D). The root tissues were removed from the ethanol and dipped in 10% sodium hypochlorite (NaOCl) for 5 min. Thereafter, the root pieces from the NaOCl solution were rinsed three times in three separate 200 ml glass beakers containing distilled water (Reay *et al.*, 2010). The sugarcane roots were then left to dry on sterilized paper towel for 1 min.

4.2.6 Method used to verify the surface sterilization process

To verify whether the above surface sterilization procedure was successful, sugarcane roots were cut into small pieces (20 mm) and were pressed onto SDA medium plates using sterilized tweezers (Figure 4.2B). Further, 100µl distilled water from the final rinse was plated onto SDA medium plates using a pipette. Four SDA medium plates were left open on the laminar flow cabinet during surface sterilization process, to examine whether other airborne fungi were present in the laboratory room during this period. All SDA medium plates used in the sterilization procedure were incubated in an incubator (IncoTherm^e- Labotec) at 23°C for four days to evaluate fungal development. Clear SDA plates without colony growth confirmed reliability of the surface sterilization procedure (Figure 4.2C).

4.2.7 The ability of Beauveria brongniartii to colonize sugarcane roots endophytically

4.2.7.1 Beauveria brongniartii re-isolation using SDA medium

After surface sterilization, roots were cut using sterile surgical blades (Sinorgmed-China) to remove dead root skin, then cut into cross-wise A sections to get ~1.1 cm pieces and to create growth space for an endophytic fungus (Reay *et al.*, 2010). Using sterilized tweezers, all root pieces were plated onto SDA medium petri dishes. Petri dishes were sealed with Parafilm® (Pechiney Plastic Packaging, Chicago) and incubated in an incubator at 25-27 °C for 8 days. After this period, colonies (Figure 4.2D) that showed *B. brongniartii* growth traits (a cottony to powdery white mycelia) were sub-cultured repeatedly onto clean SDA petri dish plates, and incubated at 23°C for 4 days. Fungal colonies which grew following incubation were then examined to find *B. brongniartii* conidia, using wet mounted slides. Wet mounted slides were prepared by scraping a small sample of the growing fungal colony from SDA plates and placing it on a clean glass slide using a tweezer, adding a small droplet of distilled water and covering with a cover slip. The fungal colonies on the slides were examined for conidial shape, size and presence of conidiophores, and hyphal septae under 400x magnification using a bright field contrast microscope (Nikon Eclipse 50i).

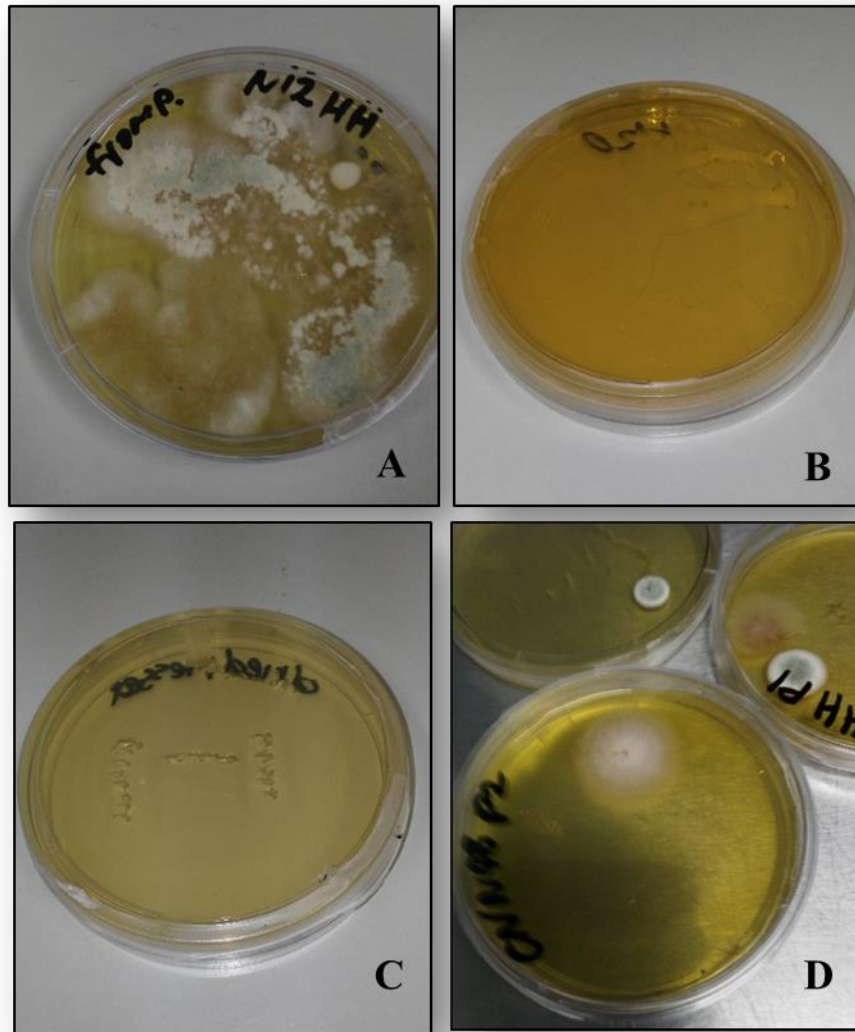


Figure 4.2: The Sabouraud Dextrose Agar (SDA) medium plates used to examine the efficacy of the plant roots surface disinfection procedure. (A) Fungal epiphytes after unsterile plant roots were pressed onto SDA medium and incubated; (B) Clear SDA plate without colony growth confirmed the sterility of the final water rinse (100 μ l) spread plated onto a clean SDA medium and incubated, all SDA plates were incubated at 23°C for four days; (C) Clear SDA plate without colony growth confirmed the sterility of root pieces after the disinfection procedure; and (D) Endophytic fungal growth of disinfected and pressed roots plated onto SDA medium.

4.2.7.2 Staining and microscopic analysis

Small portions of root pieces from the sugarcane plants (4.2.7.1 *B. brongniartii* re-isolation, section), were also used to examine for endophytic fungal conidia of two *B. brongniartii* isolates (C17 and HHWG1) using a staining method under a bright field contrast microscope (Nikon Eclipse 50i). Ten pieces of root pieces per sugarcane plant were cut longitudinally into two halves using a sterilized surgical blade (Sinorgmed-China). The staining process was conducted under a fume hood cabinet (Sprechert-Schuh IP65). Using tweezers, the halved root pieces were placed individually into 1.5 ml microfuge tubes containing Liquefied Phenol (Sigma, St. Louis, USA). All tubes were then incubated using a heating bar (TECHINE-Dri-block DB-2D, Labotec) set at 80 °C for 8 min. After 8 min, root pieces were removed from the microfuge tubes using tweezers and placed in clean microfuge tubes containing 70% (v/v) lactophenol cotton blue solution (Sigma, St. Louis, USA) (25% (v/v) lactic acid, 25% (v/v) phenol, 50% (v/v) glycerol) staining solution for 5 minutes. Wet mounts were used to examine for endophytic fungal growth in the roots and were prepared using the stained root sections. The stained root sections were placed onto glass slides. Cover slips were placed to cover root sections and the slides were examined for endophytic colonization in root sections using a bright field microscope (Nikon Eclipse 50i) at 400x magnification. Representative micrographs of endophytic fungal growth in root sections were taken using an attached camera (AxionCam Zeiss MZi2s).

4.2.7.3 Microbiological characterization

Wet mounts to examine endophytic fungal colonies that grew from sugarcane root sections were prepared. Small mycelia were picked from eight day old SDA medium cultures using a sterile tooth pick (autoclaved at 121 °C for 15 min) and placed onto a glass slide with a small drop of distilled water. Cover slips were placed to cover the mycelia on the slides and the slides were examined using a bright field microscope (Nikon Eclipse 50i) at 400x magnification, to identify and describe endophytic fungal colonies characteristics. The characteristics of the fungal colonies (colour and mycelia growth) under the microscope were used to describe the hyphae, conidiophores and conidia shapes on the slides. To identify fungal colonies of *Beauveria* species, fungal growth traits such as mealy and cottony/white powdery colonies (Terefe *et al.*, 2012), that grew onto SDA medium plates were inspected. In order to identify hyphae of *Beauveria* species, characteristics such as globose shaped conidiophores, a zig zag rachis and single celled globose shaped conidia were also contemplated under the bright field microscope (Terefe *et al.*, 2012). Representative micrographs of all the endophytic fungal growth colonies

and fungal growth traits were recorded and captured using an AxionCam Zeiss MZi2s camera, which was attached to the microscope. Further, fungal characterization and identifications from previous studies by Zhang *et al.* (2009); Barik *et al.* (2010); Xei *et al.* (2012) and Khan *et al.* (2013), were used as a library to confirm the identity of fungal colonies, conidia and conidiophore morphologies recovered in the present study.

4.3 Results

4.3.1 Beauveria brongniartii re-isolation using SDA medium

None of the fungal conidial growth from both sugarcane varieties (N12 and N48) inoculated could be identified as *Beauveria* species. A number of other endophytic fungal colonies were cultivated on SDA medium plates though and three of these were re-isolated from N12 sugarcane root sections. These were fluffy whitish, greenish to pinkish. An isolate, greenish at the centre with dense whitish edge of mycelia was observed (Figure 4.3A). This was identified as a *Penicillium* species (Eurotiales: Trichocomaceae), using characteristics described by Lugauskas *et al.*, (2011) (Figure 4.3B). The pink to whitish conidia isolate of endophytic fungi (Figure 4.4A) were identified as a *Fusarium* species (Hypocreales: Nectriaceae), using characteristics described by Zhang *et al.*, (2009) (Figure 4.4B). The third isolate, a whitish endophytic fungus colony (Figure 4.4C) was identified as another *Fusarium* species, from characteristics described by Xei *et al.*, (2012) (Figure 4.4D).

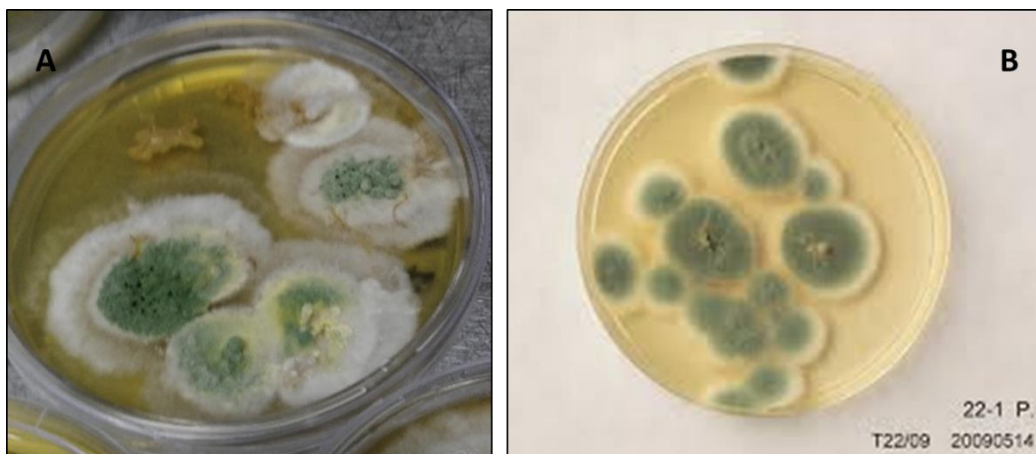


Figure 4.3: Colonies of endophytic fungi developing from sugarcane root sections, grown on SDA medium to evaluate endophytism of *Beauveria brongniartii* isolates C17 and HHWG1. (A) The common green saprophytic *Penicillium* sp. colonies 4 days after incubation in darkness; and (B) these colonies conform to that of *Penicillium* sp. identified by Lugauskas *et al.* (2011).

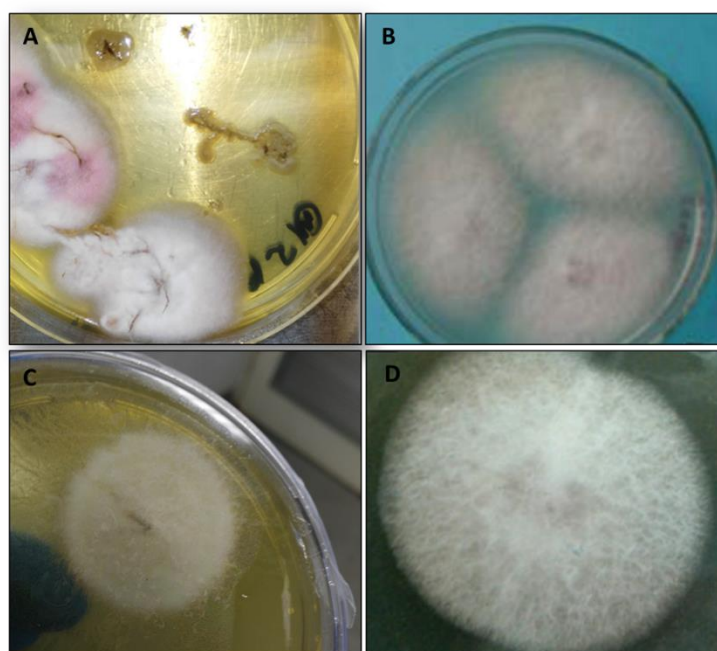


Figure 4.4: Colonies of endophytic fungi developing from N12 sugarcane root sections, grown on SDA medium to evaluate endophytism of *Beauveria brongniartii* isolates C17 and HHWG1. (A) Pinkish colonies of endophytic fungal isolate 4 days after incubation in darkness conform to that of *Fusarium* spp.; (B) identified by Zhang *et al.*, (2009); (C) A whitish colony 4 days after incubation in darkness conforms to that of another *Fusarium* sp.; (D) identified by Xie *et al.*, (2012).

4.3.2 Staining and microscopic analysis

Results presented in Figure 4.5 and 4.6 show differences in endophyte colonization of roots between the control, C17 and HHWG1 treated N12 and N48 sugarcane varieties. Root sections of N12 and N48 in the control, and root sections of N12 in the treatments showed evidence of endophytic fungal colonization (Figure 4.5 and 4.6). However, micrographs under bright field microscopy of N12 root sections after treatment with both isolates were not different from those of control N12 (Figure 4.6A and B). Some micrographs of variety N12 treated with C17 and HHWG1 had a distinct endophytic colonization (red arrows), whereas variety N48 root sections did not show endophytic fungal colonization (Figure 4.6C and D).

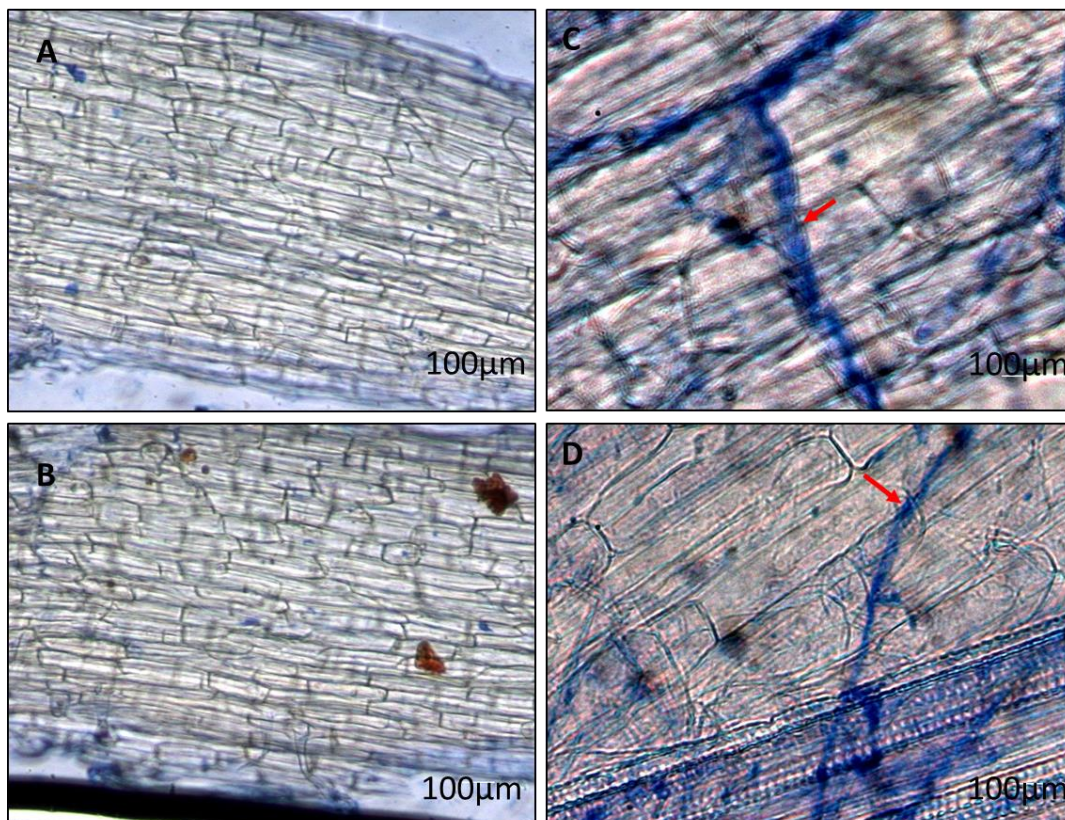


Figure 4.5: Light micrographs of root sections of sugarcane stained with lactophenol cotton blue, from the control, under bright field microscopy (10 x 100 magnification). (A&B) Root sections from control variety N12 and N48 respectively showing no endophytic colonization; relative to (C&D) images of the same varieties, also from the control, showing endophytic fungal growth (red arrows).

Root sections of N12 variety were further examined to identify the endophytic fungal growth under a bright field microscope at 400x magnification (Figure 4.7 & 4.8). Micrograph results presented in Figure 4.7 A and B showed an endophytic fungus with brownish round cells. The brownish cells observed in Figure 4.7 A and B are the characteristics (brownish yeast-like cells) of an endophytic *Penicillium* species (Figure 4.7 C and D). The endophytic fungi in stained micrographs were further examined to identify the endophytic fungi in root sections under a bright field microscope at 400x magnification. The traits of this endophytic fungus (Figure 4.8 A and B) are similar to the traits of *Fusarium* species (Figure 4.8 C and D).

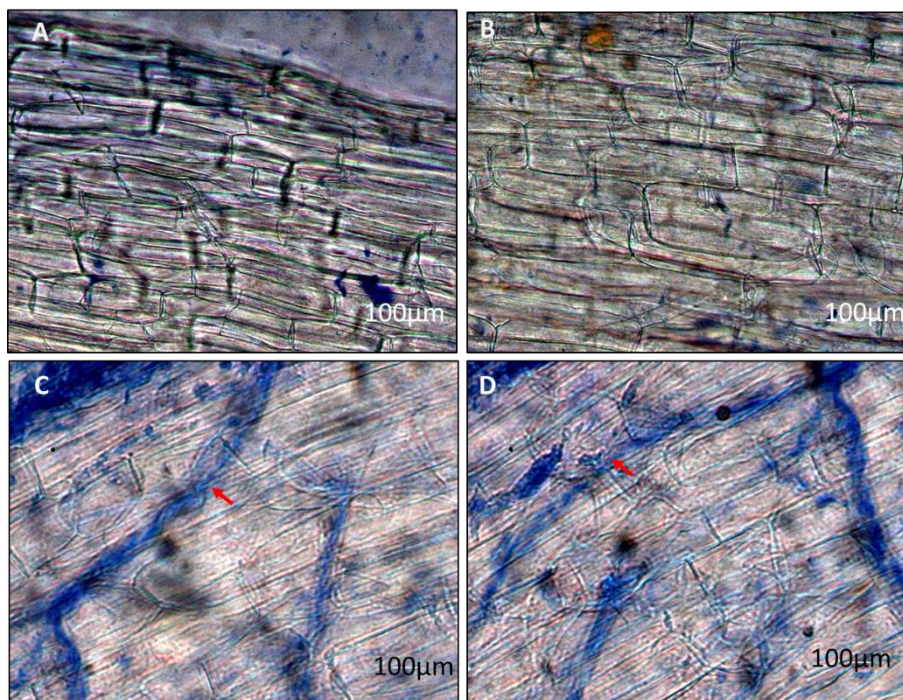


Figure 4.6: Light micrographs of root sections of sugarcane varieties N12 and N48, stained with lactophenol cotton blue under bright field microscopy (10 x 100 magnification). (A) Microscopic images of root sections from variety N48 treated with C17; and (B) HHWG1 show no endophytic fungal colonization; whereas, (C) microscopic images from the N12 variety treated with C17; and (D) HHWG1 show endophytic fungal colonization (straight mycelia growth- red arrows).

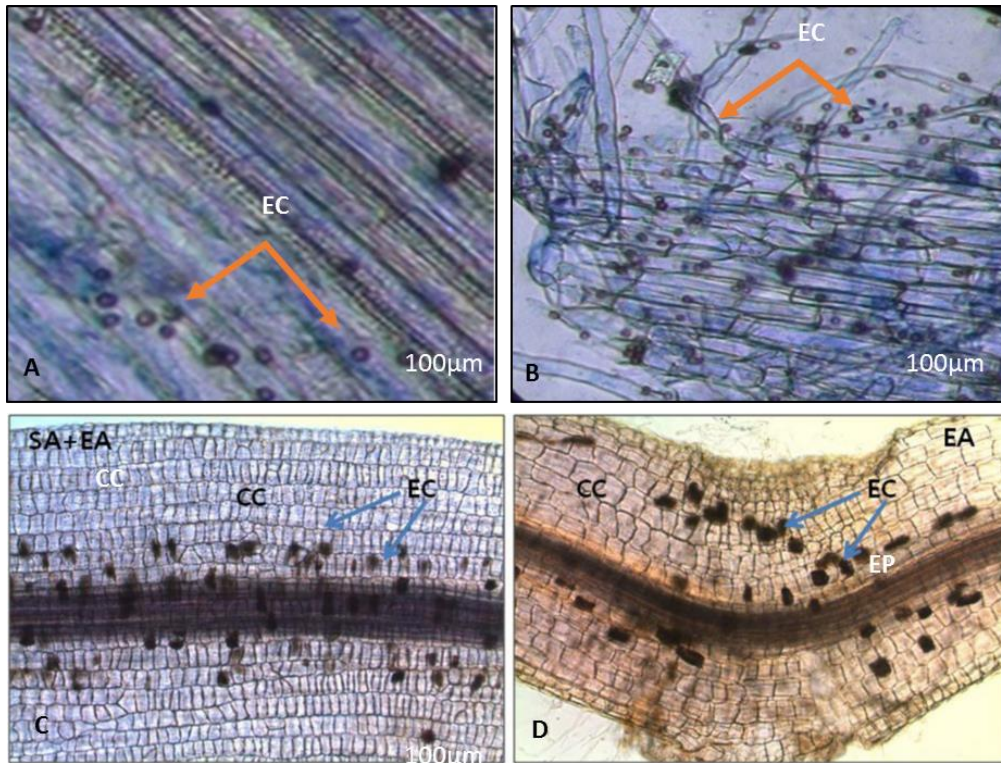


Figure 4.7: Light micrographs of N12 sugarcane root sections stained with lactophenol cotton blue under bright field microscopy (10 x 100 magnification) (A&B) showing endophytic fungal growth traits (EC) (brownish yeast-like cells), which are similar to that of endophytic *Penicillium resedanum* McLennan (C&D) identified by Khan *et al.*, (2013).

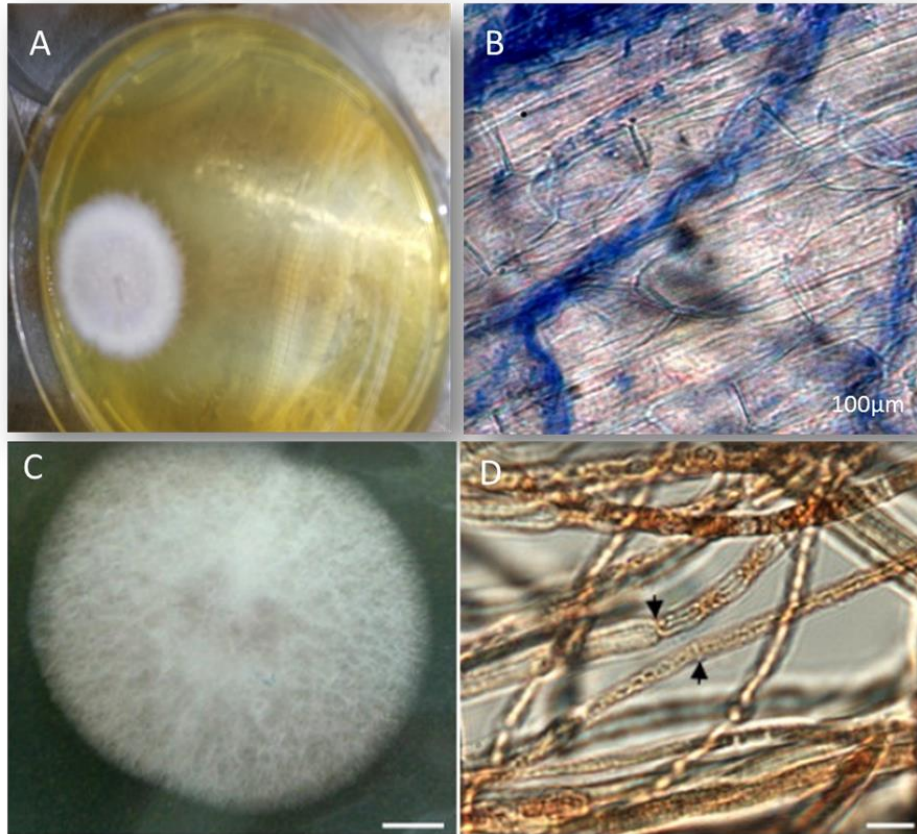


Figure 4.8: Morphology of endophytic fungus re-isolated from N12 sugarcane root sections treated with *Beauveria brongniartii* isolates (C17 and HHWG1), using re-isolation and staining method and incubated for 4 days in darkness at 23 °C. (A) Endophytic fungal growth from roots sections used in re-isolation; (B) A stained micrograph conform to the fungal growth habit of *Fusarium* sp. (C&D) identified by Xie *et al.*, (2012); Scale bars are 1 cm (C) and 5 µm (D).

4.3.3 Microbiological characterization

The visual characteristics of the endophytic fungal isolate on SDA medium was pinkish to dense white (Figure 4.9A). Micrographs of the pinkish fungal isolates were visualised as narrow, elongated and sausage shaped microconidia (Figure 4.9B). The characteristics of the pinkish endophytic fungi were identified as similar to those narrow, elongated and sausage shaped microconidia characteristics of *Fusarium* sp. (Figure 4.9C) identified by Zhang *et al.*, (2009) and Barik *et al.*, (2010).

Micrographs in Figure 4.10 (A and B) showed elongated conidiophores with globose conidial characteristics. The elongated conidiophore and globose conidia in the fungal growth in Figure 4.10 C is similar to the elongated conidiophore and globose conidia of an identified *Penicillium* sp. in Figure 4.10 (D and E).

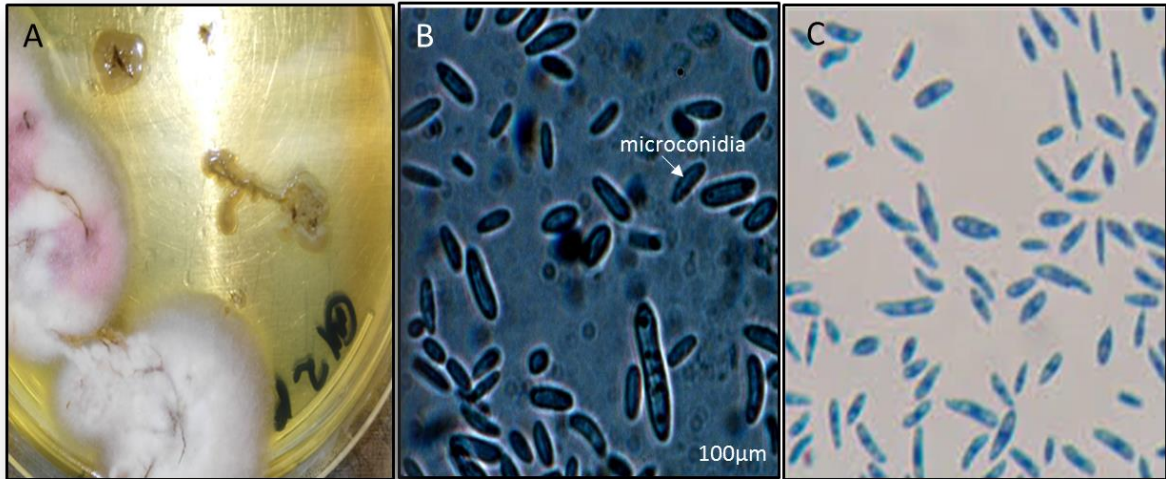


Figure 4.9: Endophytic *Fusarium* sp. conidia associated with sugarcane root sections under a bright field microscope (x100). (A) A pinkish fungal colony grew onto the SDA medium after sugarcane root sections were incubated for 4 days in darkness; (B) Morphology of endophytic colony (narrow and elongated sausage-shaped microconidia); which (C) confirm the morphology of the *Fusarium* sp. conidia identified by Zhang *et al.* (2009) and Barik *et al.* (2010).

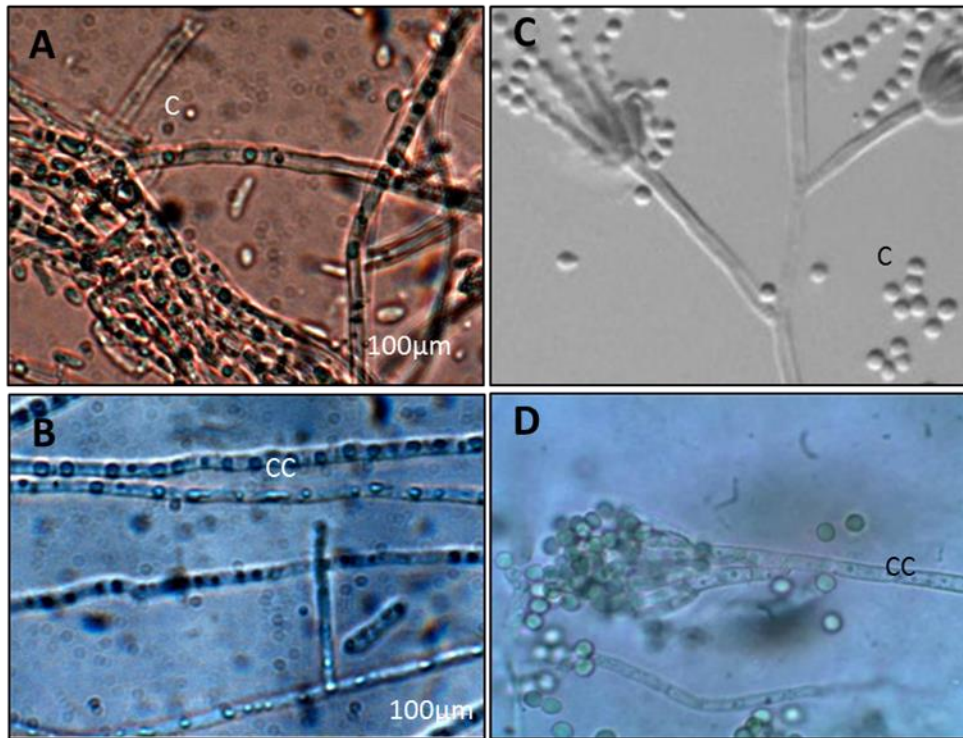


Figure 4.10: Morphology of endophytic *Penicillium* sp. conidia and conidiophore after N12 sugarcane root sections were plated onto SDA medium and incubated for 4 days in darkness. The conidia and conidiophore morphology was visualised under bright field microscope (x100). (A&B) Morphology of endophytic *Penicillium* sp. conidia (C) and conidiophore (CC), (100µm); and (C&D) morphology of *Penicillium* sp. conidia identified by Zhang *et al.* (2009) and El-Fadaly *et al.* (2015).

4.4 Discussion

Beauveria spp. have been reported as plant endophytes (Akello *et al.* 2007; Akello *et al.* 2008; Vega *et al.* 2008; Greenfield *et al.* 2016). These have all been confirmed as *B. bassiana* (Akello *et al.* 2007; Greenfield *et al.* 2016). However, there is only limited data available on *B. brongniartii* as an endophyte of plants. This study has shown that *B. brongniartii* did not efficiently colonize sugarcane roots. Fungi re-isolated as endophytes of sugarcane roots were identified as *Fusarium* sp. (Zhang *et al.* 2009; Barik *et al.* 2010; Xei *et al.* 2012) and *Penicillium* sp. (Lugauskas *et al.* 2011; Khan *et al.* 2013). These species are known to be effective colonizers of plant parts (Vega *et al.* 2008; Mulaw *et al.* 2013; Fouda *et al.* 2015). They are also known to be aggressive colonizers (Geetha *et al.* 2012; Mulaw *et al.* 2013), often out-competing less aggressive endophytes such as *B. bassiana* (Geetha *et al.* 2012; Mulaw *et al.* 2013). Furthermore, roots may not be a favoured endophytic colonization site of *Beauveria*, as Tefera

and Vidal (2009) found significant colonization of *Sorghum* sp. (Poales: Poaceae) leaves and stems, and less colonization in roots.

The inability of *Beauveria* to colonize sugarcane plant tissues, could be because of antagonistic behaviour of other species of fungi colonising the plant tissue. This has been reported previously (Vega *et al.* 2008; Conlong and Rutherford, 2009). The study by Geetha *et al.* (2012) on interactions of EPFs (*B. bassiana* and *B. brongniartii*) and other opportunistic fungi (*Fusarium* sp., *Penicillium* sp. and *Aspergillus* sp.) found that both the *Beauveria* EPFs were not competitive when occurring together with opportunistic fungi. They concluded that the presence of other fungi had an antagonistic effect on the behaviour of the *Beauveria* EPFs. Vega *et al.* (2008) reported that two common soil fungi, *Penicillium urticae* Bainier and *Aspergillus clavatus* Desm. (Eurotiales: Trichocomaceae) exhibited antagonism, preventing germination of *B. bassiana* conidia and root colonization. *Penicillium urticae* is known to produce water soluble inhibitors and *A. clavatus* produces metabolites that are fungicidal to *B. bassiana* (Vega *et al.*, 2008).

Even though *B. brongniartii* is not regarded as an efficient endophyte of plants (Vega *et al.*, 2008), there are many other possible reasons that could have prevented the establishment of *B. brongniartii* isolates in sugarcane roots. Firstly, the inoculation method used in this study may not have been the most suitable method to use. A dip inoculation method was used to inoculate sugarcane setts in the present study. Memela (2015) found that stem the direct injection method was the one causing the highest endophytic colonization of *B. bassiana* isolates, whereas soil drenching colonization was detected only on sugarcane stems. Foliar spray inoculation method was the better method for leaf colonization. Posada *et al.* (2007) showed highest colonization when a direct inoculation method was adopted. However, Akello *et al.* (2007) showed that the dipping method was efficient when introducing *B. bassiana* isolates into tissue cultured banana plants.

Tefera and Vidal (2009) introduced *B. bassiana* into sorghum plants using a dip inoculation method. They found that leaves and stems had greater endophytic colonization compared to roots. The reason for lower recovery of *B. bassiana* in the roots was unknown. However, Akello *et al.* (2007) reported higher endophytic colonization of *B. bassiana* in the roots and rhizomes than in the pseudostem bases of banana plants. Both studies by Tefera and Vidal (2009) and Akello *et al.* (2007) emphasized that *Beauveria* spp. prefer specific plant tissues and that certain plant tissue conditions are required for successful endophytic colonization.

Secondly, the disinfection method to ensure that clean plant parts were used may also have played a role in unsuccessful establishment of *B. brongniartii* isolates in sugarcane roots (Tefera and Vidal, 2009; Parsa *et al.*, 2013). In the current study, hot water treatment and waxing was adopted. However, Memela (2015) showed that hot water plus waxing of setts is not an efficient method in sterilizing sugarcane setts and concluded that the use of tissue cultured sugarcane plants provided better endophytic results compared to using hot water treated plus waxed sugarcane setts. All these factors were not critically evaluated in the current study and may have affected the establishment of *B. brongniartii* isolates as potential endophytic colonizers of sugarcane roots.

Finally, the effect of sugarcane age on endophytic colonization of an EPF was not evaluated. Agrios (2005) discussed plant age as another critical factor that affects colonization of plants by fungi. He explains that pathogens found in plants depend on plant age and that old plants tend to have more pathogens compared to young plants. The sugarcane used for this study was at the third ratooned stage and it is then possible that it had high levels of antagonistic fungal pathogens established in it that could have prevented *B. brongniartii* colonization.

Future research should include the use of tissue cultured sugarcane plants to ensure that sterile and clean sugarcane are used. Thereafter, sugarcane setts that show fungal growth of any other opportunistic fungi should be discarded and only clean setts should be used.

4.5 Conclusion

Beauveria brongniartii isolates C17 and HHWG1 were not endophytic colonizers of sugarcane roots. Rather, virulent *B. bassiana* isolates need to be tested against white grubs and because several studies have shown the ability of *B. bassiana* as endophytes of sugarcane plants, this will serve as a better strategy to reduce and repel white grubs in sugarcane plantations.

4.6 References

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CHAPTER 5: THE IMPACT OF *BEAUVERIA BRONGNIARTII* ISOLATES AGAINST LARVAE OF *SCHIZONYCHA AFFINIS* (COLEOPTERA: SCARABAEIDAE) IN SUGARCANE PLOT TRIALS, AND THE RESULTANT SUGARCANE PLANT GROWTH

Abstract

The aim of the study was to evaluate the effect of two *Beauveria brongniartii* isolates (C17 and HHWG1), insecticide as a positive control and distilled water with 0.05% Triton X-100 as a negative control, against larvae of *Schizonycha affinis*, using two inoculation methods (a soil drench inoculation and root dip inoculation), and the impact of *S. affinis* larvae on the sugarcane biomass under these treatments and controls. The impact of *S. affinis* larvae was evaluated at 10 and 20 days after treatments (DAT) by randomly selecting three seedlings per treatment. Sampled seedlings were measured for root dry weight (DW) and foliar cover (leaf dry weight, seedling height and the number of green and dead leaves). The effect of the treatments was evaluated every 3rd day for 30 DAT; and the results were compared between the treatments and the control.

Sugarcane seedlings were planted in plastic pots containing composted pine bark potting medium. Ten days post-planting, 3 larvae were introduced 5 cm deep in the soil per single pot. One day later, the pots were treated by drenching the soil with treatments near the sugarcane seedling stalk. Using a root dip inoculation method, sugarcane seedlings were also planted in pots containing composted pine bark potting medium. Five days post-planting, sugarcane seedlings were detached from the soil and were treated by dipping the plant roots into 100 ml of each treatment and control for 1 minute and replanted into corresponding pots. Three larvae were then introduced 5 cm deep per single pot with sugarcane seedling.

In the soil drench inoculation method, seedlings in the water only control were highly damaged with 80-93% reduction of root DW as compared to 0-10% reduction in the insecticide treated seedlings. Consequently leaf DW, height of seedlings and number of green leaves recorded from the control in the presence of *S. affinis* larvae were lower than the seedlings that were treated with HHWG1 and insecticide. Insecticide and HHWG1 showed some protection against the larvae at 20 DAT. As a result, the study showed that insecticide was more effective and caused larval mortality within the first 3 DAT as compared to HHWG1 and C17 isolates which started to cause infections at 9 DAT. Insecticide caused 100% mortality of small larvae within 6 DAT as compared to large larvae mortality (30-60%). Although insecticide was highly virulent from the first few DAT, at 24-30 DAT the pathogenicity of the HHWG1 isolate was

comparable to the effect of an insecticide treatment, hence, there was no significant difference ($P>0.05$) between HHWG1 isolate and insecticide. For the root dip inoculation method, no mortality of larvae at 3-9 DAT was recorded for both the insecticide and *B. brongniartii* isolates. However, larval mortality developed at 12 DAT in the insecticide treated pots as compared to the development of larval mortality at 18 DAT in the HHWG1 isolate treated seedlings. Overall larval mortality was 30% for HHWG1 isolate and 60% in the insecticide treated larvae.

This study shows that white grub larvae drastically affect sugarcane biomass, and that the HHWG1 *B. brongniartii* isolate and insecticide show potential to reduce white grub larvae impact on sugarcane plants. Furthermore, the soil drench inoculation method was more effective than the root dip inoculation method at infecting *S. affinis* larvae. However, further studies need to be conducted to evaluate the yield losses over a longer period (preferably up to harvest time) and with a larger sample size. Thus, further evaluation of *B. brongniartii* isolates against white grub larvae under replicated field conditions remain the priority.

5.1 Introduction

Soil dwelling pests are seen to have a critical impact on plant biomass (Coale and Cherry, 1989). They usually consume plant roots thus affecting water and nutrients uptake and plant stability (Allsopp *et al.*, 1991; Erb and Lu, 2013). The greatest concern is that it is difficult to control soil-dwelling pests, because they are difficult to access since they spend most of their life cycle deep in the soil (Wilson, 1969; Jackson *et al.*, 2000). This is one of the major constraints limiting the regulation of soil-dwelling insect pests.

White grub larvae (Coleoptera: Scarabaeidae) are soil-dwelling herbivores that feed on many agricultural plants including *Solanum tuberosum* Linnaeus (Solanales: Solanaceae), *Pennisetum glaucum* Linnaeus (Poales: Poaceae), *Saccharum* spp. Linnaeus (Poales: Poaceae) and many other graminaceous crops (Cherry, 1988). Larvae feed on sugarcane roots and cause substantial losses in yield and production (Cherry, 1991; McArthur and Leslie, 2004). White grub larval infestations in sugarcane can be severe, and the number of larvae found underneath one sugarcane stool can range between 3-25 grubs/stool/pit in highly infested areas (Way *et al.*, 2011); a “pit” is basically a hole (30×30×30 cm) dug to survey white grubs in the soil and it is routinely used by the South African sugar industry as a standard procedure to conduct surveys (Way *et al.*, 2011).

White grubs are increasingly destructive pests of sugarcane in South Africa (Carnegie, 1988; Way, 1997; Way *et al.*, 2011; Conlong, 2015; Goble *et al.*, 2015). To date, more than five different white grub species have been found occurring in abundance in South African sugarcane plantations (Dittrich *et al.*, 2006; Dittrich-Schröder *et al.*, 2009; Chapter 3 in this thesis). Their larvae feed on sugarcane roots and cause yield losses (Cherry, 1998; Goble, 2012; Cock and Allard, 2013) of around estimated 66 tons/ha in South Africa (Chelvi *et al.*, 2011). Cherry (1988) reported 39% reduction of sugarcane yield at harvest in heavily infested fields. White grub larvae have three instars and the final third instar is the most damaging stage to sugarcane roots (Way, 1997). White grubs generally have a long, complex and largely cryptic life cycle, which has contributed to poor management strategies against them (Sweeney, 1967; Wilson, 1969).

According to Ueckert (1979), heavy white grub larvae infestations result in poor rangeland vegetation, crops, pastures and lawns. Sometimes even low infestations can have great vegetation impacts like enhancing drought effects and increasing entry points for plant pathogen infections. Wightman *et al.* (1994) stated that the damage caused by white grubs does not only

affect nutrients uptake by plants and their drought intolerance, but grubs also reduce the plants mechanisms to control apical bud dominance, stomatal opening and leaf senescence. Ueckert, (1979) reported a huge reduction of live plant cover of *Bouteloua dactyloides* Nutt (Poales: Poaceae), *Aristida purpurea* Nutt (Poales: Poaceae), and *Erioneuron pilosum* Buckley (Poales: Poaceae) from 94% to 11% in white grub-infested areas. McArthur and Leslie (2004) studied the impact of white grub on sugarcane yields in the Midlands north region of South Africa, and reported yield reductions and heavy root damage in white grub infested (10 grubs/pit) sugarcane fields. The decline in sugarcane yields in these fields were between 40 and 50%.

To date, chemical insecticides are the most used pest management measure to control white grubs in sugarcane (Allsopp *et al.*, 1995). The use of insecticides to control pests, however, has been restricted due to the negative impacts these have on the environment, as well as to non-target flora and fauna (Zimmermann, 2007; Strasser *et al.*, 2000; Elena *et al.*, 2011; Chelvi *et al.*, 2011). A study conducted in Uganda showed minimal reduction of white grubs when using insecticidal control measures (Conlong and Magalula, 2003). Although efforts of controlling white grubs are in progress, one cannot ignore the fact that sugarcane yield losses due to white grub infestations have drawn attention to seek alternative control strategies, with the emphasis on those that are more environmentally friendly. This introduces the term biological control in agriculture. Biological control is a phenomenon whereby a natural enemy is used to control the population/density of a particular organism, either a plant or an animal (Alston, 2011). *Beauveria brongniartii* (Sacc.) Petch (Deuteromycota, Hyphomycetes) is an entomopathogenic fungus (EPF) that has been recognized as a promising biological control agent to reduce white grub populations in South Africa (Goble *et al.*, 2012; Kheswa *et al.*, 2016). Entomopathogenic fungi have in recent years been used as an alternative to insecticides (Reddy *et al.*, 2013; Rai *et al.* 2014).

Isolates of *B. brongniartii* were isolated from two white grub species, *Schizonycha affinis* (Boheman) and *Pegylis sommeri* (Burmeister) (Coleoptera: Scarabaeidae: Melolonthinae) found in sugarcane in the Midlands North region of KwaZulu-Natal (Goble *et al.*, 2012). These promising biological control agents were evaluated in laboratory bioassays and showed great potential as control agents under laboratory conditions, with 80-95% mortalities of *B. brongniartii* C17 and HHWG1 isolates against both larvae and adults of *S. affinis* and *P. sommeri* (Goble *et al.*, 2015). Similar results were obtained in a study to determine the host range of these isolates (Kheswa *et al.*, 2016).

Keller *et al.* (1999) showed that *B. brongniartii* was persistent and effective against white grubs in soil application studies. Dolci *et al.* (2006) in northwest Italy, found that *B. brongniartii* was also persistent and caused death to white grub larvae after soil inoculation trials. In Switzerland, soil application studies showed that *B. brongniartii* was host specific and its population was elevated in soil, in the presence of its host *Melolontha melolontha* Linnaeus (Coleoptera: Scarabaeidae: Melolonthinae) as compared to host free trials (Kessler *et al.*, 2004). The efficacy of *B. brongniartii* as a pathogen of white grubs has however, never been evaluated in field trials in South Africa. This study is the first step in the investigation of these indigenous *B. brongniartii* (C17 and HHWG1) isolates in field applications.

Furthermore, the resultant control impacts of the sugarcane seedlings in the pots was assessed. It is known that white grubs affect plant yield, but it has never been clearly quantified. Further, their impact of root feeding on leaf growth and senescence have also not been quantified. There is, however, limited literature (cited in this section already) explicitly demonstrating the intensity at which white grub larvae reduce sugarcane plant biomass. The present study measured the impact, on unprotected plants and those protected by insecticide and two *B. brongniartii* isolates, in replicated pot trials, with small and large larvae of *S. affinis* as the target.

5.2 Material and methods

5.2.1 Experimental study site

A trial study was conducted from the 2nd May-30th June 2016 at the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, in South Africa. The test trials were conducted outdoors at the sugarcane site (29° 42' 14.598"S; 31° 2' 39.908"E). The maximum temperature and rainfall data, was obtained from SASRI Weather Web, 2016⁷. The rainfall and temperature conditions at the site during the study are shown in Figure 5.1.

5.2.2 Test insects

Schizonycha affinis larvae to be tested against the locally collected C17 and HHWG1 isolates of *B. brongniartii* were collected from 3rd ratoon N12 sugarcane at Sultan farm, Wartburg (29° 25' 29.284"S; 30° 39' 30.902"E) in KwaZulu-Natal. Prior to being used in the soil trial, the field collected larvae were identified to species level by examining their raster patterns on the ventral surface of their last abdominal segment (Sweeney, 1967; Dittrich *et al.*, 2006). Further,

⁷ SASRI Weather Web, 2016. <http://portal.sasa.org.za/weatherweb/weatherweb>

specimens were grouped into 2 instar categories on the basis of their head capsule width; small (2 mm) (second instar) and large (3-4 mm) (third instar) (Figure 5.2A). Head capsule width was measured using a Digital Caliper⁸ (0-150 mm) (Sweeney, 1967; Wilson, 1969; Cock and Allard, 2013; Way *et al.*, 2013; Goble *et al.*, 2015).

5.2.3 White grub larval collection

Larvae were collected by digging out sugarcane roots and soil in 30×30×30 cm pits. Pits were positioned across infected sugarcane stools. Larvae found were placed into plastic vials (30 ml) containing autoclaved peat moss produced by Grovida c.c.⁹. Peat was autoclaved for 15 min at 121°C and cooled before use, to sterilize it and kill any pathogens it may harbor. Vials were sealed with perforated lids. To minimize mortality, vials were placed in large cooler boxes with ice packs during the collection period, and when transported from the field to the laboratory (Goble, 2012). In the laboratory, after species identification, white grub larvae in their plastic vials were packed into 4L plastic trays and were maintained at 23°C and 75% relative humidity (RH) in the Insect Rearing Unit (IRU) Quarantine room at SASRI until they were used. During the larval collection procedure, larvae may suffer from bruises and some may be collected diseased, therefore, all larvae were left for 10 days under these conditions to stabilize in their new environment, and only larvae surviving the 10 days screening period were used in experiments (Goble, 2012).

5.2.4 Sugarcane plants

Five month old sugarcane seedlings (N12 variety) were planted into the pots used for the soil application trial. Prior to the trial study, sugarcane seedlings were maintained in the trays they were supplied in at the SASRI sugarcane seedling nursery, for 10 days. Thereafter, one sugarcane seedling was planted into a 220 mm diameter plastic pot (Figure 5.2B). The pots were then filled with composted pine bark as a plant growth medium and placed in 1.7×0.47×0.2 m aluminum plant troughs.

⁸Digital Caliper by MARSHALTOOLS™

⁹Peat Moss bale 275L, by Grovida c.c. Horticultural Products at 400 Sydney Road, PO Box 18163, Dalbridge, Durban 4014

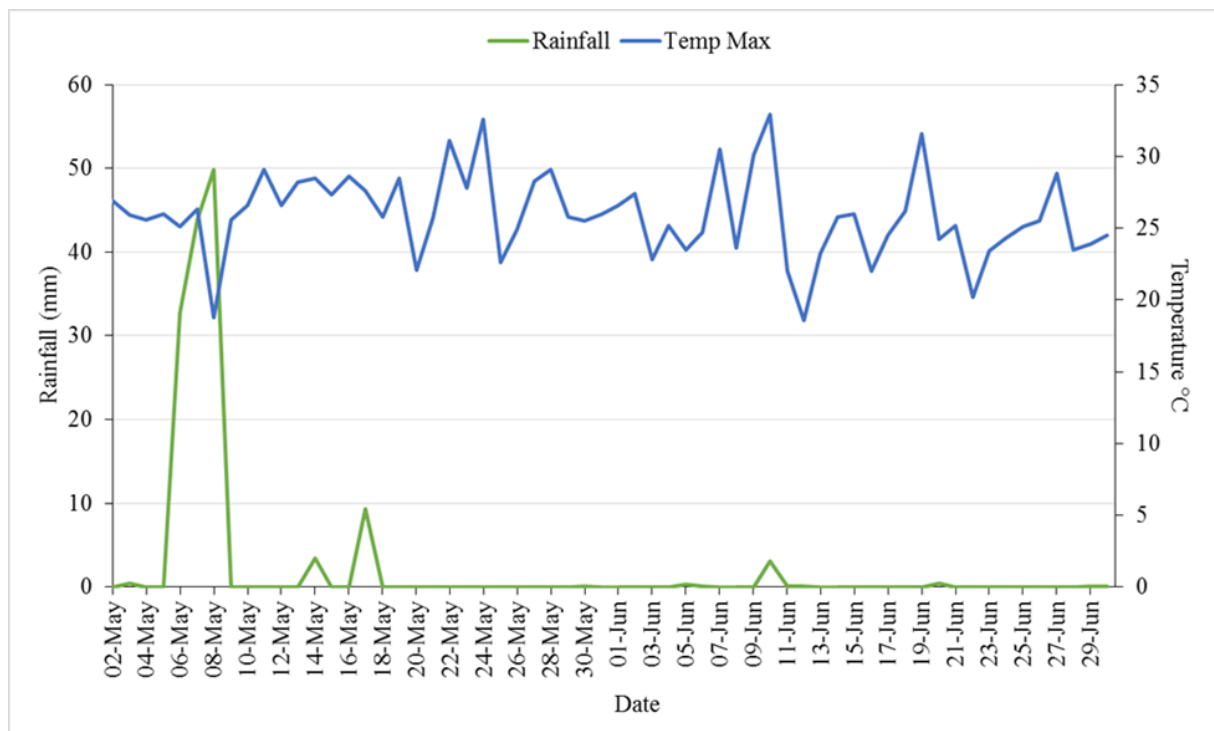


Figure 5.1: Rainfall (mm) and temperatures (°C) at Mount Edgecombe, KwaZulu-Natal during the trial study period (From SASRI Weather Web).

5.2.5 The efficacy of C17 and HHWG1 *Beauveria brongniartii* isolates and insecticide treatment against *Schizonycha affinis* larvae

5.2.5.1 *Beauveria brongniartii* isolates inoculum preparations

The *B. brongniartii* isolates C17 and HHWG1 were grown on Sabouraud Dextrose Agar (SDA) plates and then incubated at 23 °C until sporulation. SDA is a selective solid media prepared by dissolving 60g of 4% SDA agar (Merck) in 1 L of distilled water and autoclaved for 15 min at 121°C then cooled to 55°C. The medium was supplemented with 0.05g/ml rifampicin (Sigma-Aldrich; St. Louis), 0.05 g/ml cycloheximide (Calbiochem; Canada), 0.05g/ml chloramphenicol (Sigma-Aldrich; St. Louis) and 0.02g/ml dodine (Sigma-Aldrich; St. Louis) antibiotics to prevent bacterial and saprophytic fungal growth (Goble *et al.*, 2015). The mixture was transferred into and stored in 90 mm plastic petri dishes and then placed in the laboratory at 25°C ambient temperature until used. Fungal isolates were repeatedly sub-cultured since their original isolation date and periodically passed through a susceptible insect host (*Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae). The goal of this was to restore their virulence, as described by Mohammadbeigi (2013). This was necessary as it is known that EPF isolates lose virulence the more they are sub-cultured. However, virulence can be restored by passing

them through an insect host (Brownbridge *et al.*, 2001). Fungal inoculum for the trial was prepared from 4-week-old colonies, by flooding the petri dishes containing the isolates with sterile distilled water mixed with 0.05% Triton X-100, and then tipping the solution into sterile 1000 ml glass bottles containing 500 ml distilled water with 0.05% Triton X-100 and a stirrer bar. Bottles were sealed with a lid and the conidial mixture was vortexed for one minute to produce a homogenous conidial suspension. Conidial spore counts were determined using a Neubauer haemocytometer (0.1 mm depth) and conidial concentrations were determined through serial dilutions as described by Lacey (1997), to get 1×10^9 conidia/ml concentration. This concentration was chosen because a former study (Goble, 2012) showed that 1×10^9 conidia/ml of HHWG1 isolate was the most virulent concentration against white grub larvae of *S. affinis* and *P. sommeri* in laboratory bioassays. Conidial suspensions were used within 3 hours of mixing and dilution. Furthermore, to assess whether the formulated isolates were still viable, conidial viability was evaluated by plating out 0.1 ml of conidial suspension on SDA plates and then incubating for 4 days at 23 °C.

5.2.5.2 Insecticide (positive control) preparations

Decis-Forte (Bayer Crop Science[®]) insecticide was used as a positive control in the soil trial infested with *S. affinis* larvae. To prepare a positive control solution, 7.5 L of tap water was poured into a 25 L container. Water pH was adjusted to 5.5 with an all buffer solution and measured by using a waterproof pH Meter (Designer Water[®]) before making up the insecticide solution. Using a syringe, 2.2 ml of Decis-Forte as put into the 25 L container, and 7.5 ml of Surfactant, (BREAK-THRU[®], Technology for Agriculture) was poured into the container. The suspension was mixed thoroughly using a wooden stick. Prior to soil application, the insecticide solution was used to directly inoculate small (n=10) and large (n=10) *S. affinis* larvae by pipetting 10µl of the prepared insecticide, using an auto-pipette, onto the dorsal side of the thorax of the larvae; this was done to evaluate the efficacy of the insecticide. After the efficacy evaluation procedure proved positive larval mortality, the insecticide solution was used within 3 hours in soil application trials.

5.2.5.3 Experimental design

The study was conducted using two soil inoculum application methods in the pots, a soil drench and a sugarcane root dip method. Further, positive control (insecticide) and negative control (distilled water containing 0.05% Triton X-100) treatments were added to the design. Twenty

four replicates were used per treatment and each replicate contained 3 larvae per size category, respectively. A randomized complete block design was used for the trial.

5.2.5.4 Inoculation methods

Soil drenching method

Sugarcane seedlings were planted in pots on the 2nd May 2016. Ten days post-planting, 3 *S. affinis* larvae of the same larval stage were introduced into the soil with sugarcane seedlings, per pot. The larvae were introduced in pots by carefully removing the planted sugarcane seedling, and placing the larvae into the soil (approx. 5 cm deep), then the seedlings were carefully replaced into the pot. One day post-larval inoculation, the soil was drenched with 100 ml inoculum, near the base of the seedling stalk. In the inoculated controls, the soil was drenched with the Decis-Forte (100 ml), insecticide. In the untreated control, the soil was drenched with 100 ml distilled water with 0.05% Triton X-100 solution. The pots were manually irrigated with tap water twice every day, using a brass faced rose plastic watering can (10 L) for 1 min per pot (~250 ml/min), before inoculations. After inoculation, pots were irrigated every 3rd day thereafter, by half filling the aluminum plant troughs with 7.5 L of tap water and the level of water in the plant pot saucers was monitored every day for the duration of the trials (30 days). This irrigation procedure was used to prevent potting soil from receiving excessive water content; and it was essential to reduce runoff and prevent the treatments from getting washed off (esp. the conidial spores), since germination of *Beauveria* spp. conidia is strongly affected by moisture conditions (Luz and Fargues, 1997).

The effect of C17 and HHWG1 isolates, and insecticide against second and third instar larvae of white grubs were evaluated at three day intervals for 30 days, post soil inoculations. Three pots per treatment were randomly selected at each interval, and the effect of the treatments was assessed by searching for all three placed larvae per pot. Larvae in the soil were collected by gently tipping out soil, hand sieving the soil and detaching the sugarcane plants carefully from the pots (Figure 5.2C). All dead larvae were collected and recorded. Dead larvae recovered from the soil with mycosis were recorded as such. Collected dead larvae per pot were placed into petri dishes (90mm), labelled with treatment and larval size. All live larvae collected per pot were placed in plastic vials (30 ml) containing autoclaved peat and sealed with perforated lids. The vials were also labelled with treatment name and larval size to avoid cross-contamination between treatments. Collected dead larvae without clear mycosis during the evaluation period were put separately into labelled petri dishes and taken into the laboratory

where they were surface disinfected by dipping them into 250 ml conical flasks containing 70% ethanol for 2 minutes. All surface disinfected larvae were dried by placing each on a sterile paper towel for one minute. They were then plated onto petri dishes (90 mm diameter) with SDA medium and incubated at 23°C to allow for the development of mycosis. The cause of death was assessed by scoring mycosis on grubs' bodies at time of collection from the SDA plates and after incubation.

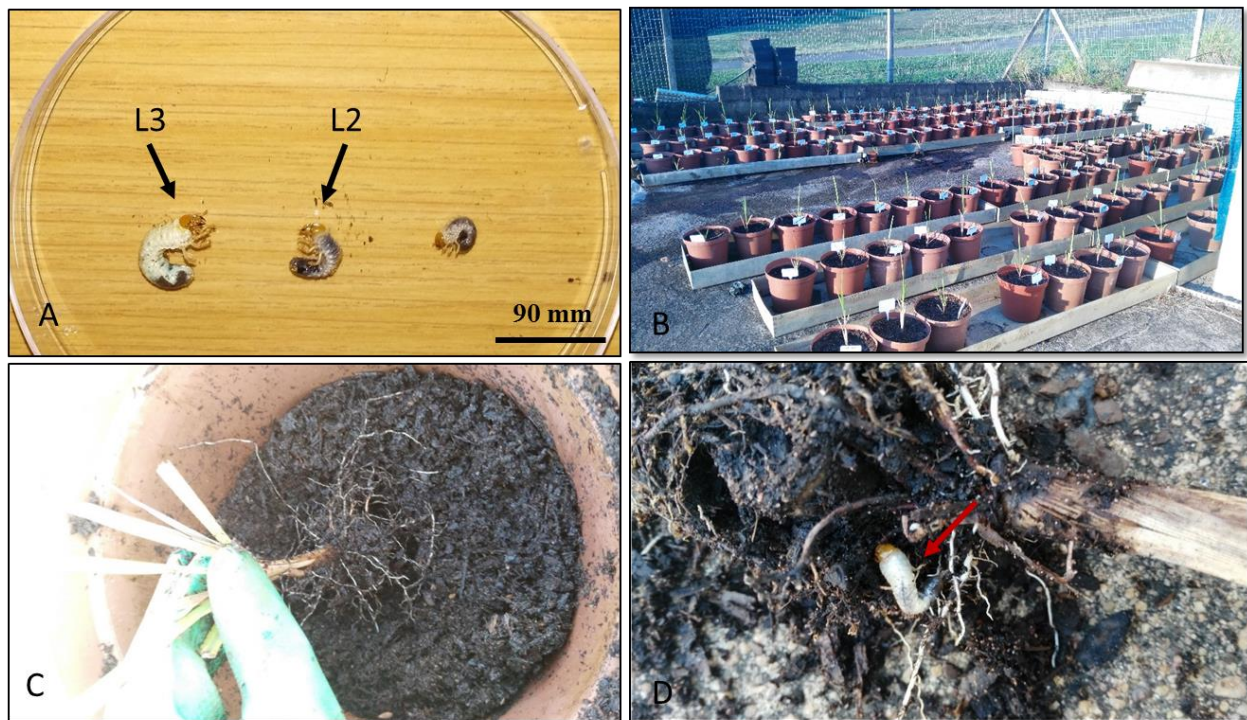


Figure 5.2: Experimental procedure to evaluate the effect of *Beauveria brongniartii* isolates (C17 and HHWG1) and insecticide against white grub larvae of *Schizonycha affinis* in pot trials. (A) *S. affinis* larval categories used (L2: small and L3: large instar); (B) Sugarcane seedlings planted into pots, 3 larvae were introduced per pot and inoculated with fungal isolates and insecticide; (C) Sugarcane seedlings removed from the pots during evaluation, the soil was tipped-out from the pots and sieved to identify the white grub larvae every 3rd day for 30 days after treatments; and (D) Red arrow indicates *S. affinis* larva found feeding on sugarcane roots.

Sugarcane root dip inoculation method

Sugarcane seedlings were planted in pots on the 2nd May 2016, as described above. On the 5th day, plants were carefully removed from the soil in the pots and the respective seedling sett

with intact roots, was dipped into 100 ml of C17 or HHWG1 inoculum, 100 ml of inoculated control (insecticide) and 100 ml of un-inoculated control (distilled water containing 0.05% Triton X-100) for 1 minute, respectively. After dipping the seedling roots, seedlings were carefully replanted into their soil pots. One day post-replanting, 3 larvae were placed per pot, by digging 3 separate small holes (approximately 5 cm deep and 2 finger sizes wide), near the sugarcane seedling stalk in the soil. Thereafter, one larva was placed per hole and holes were immediately covered with the potting soil. Pots containing seedlings and larvae were irrigated as described in the “5.2.5.4 Soil drenching method” section above. The effect of the treatments was evaluated every third day for 30 days, by randomly selecting 3 pots per treatment and searching for mycosed and/or healthy larvae. The effect of the treatments was assessed using the procedure mentioned in the “5.2.5.4 Soil drenching method” above.

5.2.6 Impact of *Schizonycha affinis* larvae on sugarcane seedling foliar and root biomass

A total of 108 pots were used to study the impact of *S. affinis* larvae on sugarcane plants, using two treatment methods, a soil drench and a root-dip. Fifty four pots per inoculation method were randomly selected to evaluate the impact of larvae on sugarcane seedling biomass at 10 and 20 days intervals post-inoculations. Three pots per treatment and per control containing sugarcane seedlings were randomly selected. Sugarcane plants were removed from the soil, roots were washed thoroughly but carefully with running tap water, to remove the composted pine bark and were then placed in brown paper bags (labelled with grub size and treatment name) (Figure 5.3A). Paper bags containing plants were then taken to the laboratory. In the laboratory, one plant at a time was cut into three parts (stem and shoots, sett roots and setts) (Figure 5.3B). The sugarcane seedling height was measured using a ruler (mm) from above the sett to the youngest emerging leaf apex (Figure 5.3C) and recorded. All three sugarcane seedling parts were then placed separately into paper bags. Paper bags were labelled with sugarcane seedling part, treatment name, inoculation method and a date; to ensure that correct measurements are recorded for the corresponding sugarcane plant. All samples were then oven-dried at 65 °C for 3 days using a sugarcane drying oven (Memmert, West Germany). On the third day, seedling parts were cut into small pieces and weighed (g) using a Model ML 54 (Mettler Toledo, Switzerland) weighing balance and the weight for each sample was recorded in a prepared data collection sheet.

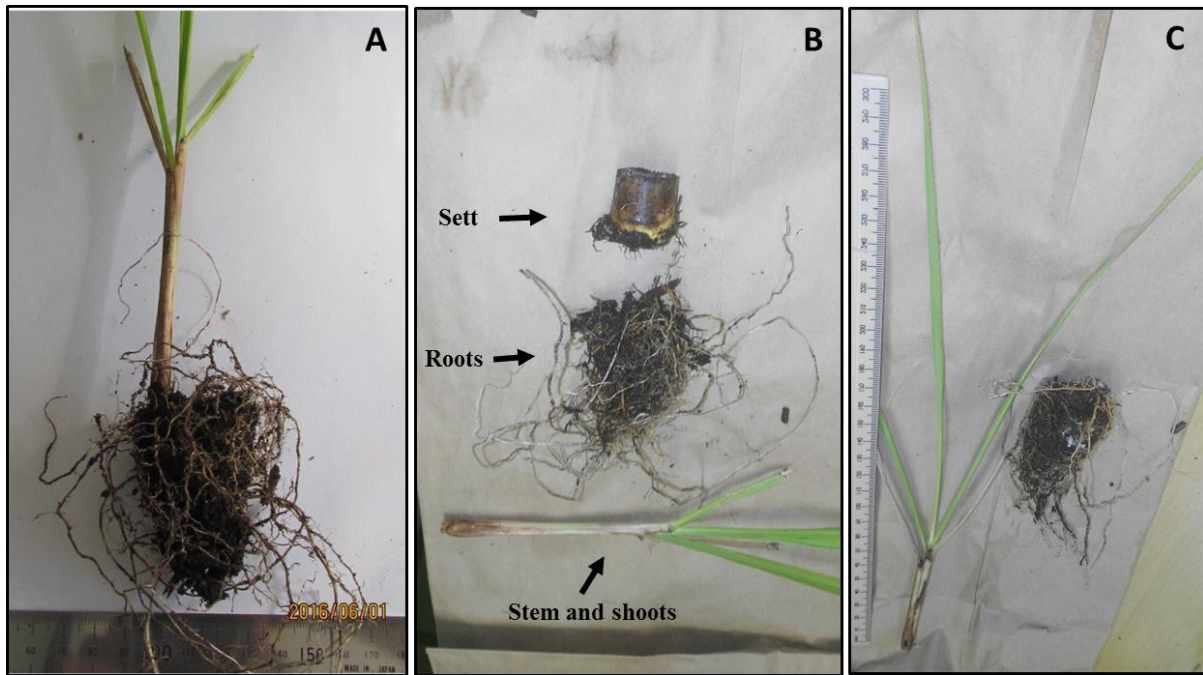


Figure 5.3: Evaluation of the impact of *Schizonycha affinis* larvae on sugarcane seedling biomass, 10 and 20 days after the experiment. (A) Sugarcane seedling detached from the pots; (B) Seedlings placed in brown paper bags, transported into the laboratory and divided into three parts (stem and shoots, setts and sett roots); and (C) Height (mm) of the seedlings were determined, oven dried at 65 °C and weighed. The impact of larvae was evaluated at 10 and 20 days post-experiment.

5.3 Statistical analyses

5.3.1 Impact of larvae on sugarcane biomass

Two-way analysis of variance (ANOVA) was used to analyse the data for differences between treatments and the control (no grub and no treatment). Tukey's multiple comparison test and probability (P) of 0.05 was used to determine significant differences between treatments using GenStat version 18.0 software (Crichton, 1999).

The mean ($N=3$) root dry weight percentages were estimated by measuring the amount of root dry weight that remained intact on the seedlings in the presence of grubs and treatment and in the absence of both, in the same aged seedlings (Shreve *et al.* 2006).

The dry weight percentage of root biomass was determined using this formula:

$$DW \% = \frac{UC-TC}{UC} \times 100 \quad (5.1)$$

Where: DW is dry weight percentage, UC is the root dry weight of untreated seedlings, (no grubs, and no treatment)

TC is the root dry weight of treated seedlings, (isolates or insecticide)

5.3.2 Efficacy of *Beauveria brongniartii* isolates, soil application

Mortality percentage was corrected for the control mortality using the formula:

$$CM \% = \left(\frac{T-C}{100-C} \right) \times 100 \quad (5.2)$$

Where: CM is corrected mortality, T is percent mortality of treated larvae and C is percent mortality of larvae in the control (Abbott, 1925).

Mortality data obtained from the study were subjected to a two-way Analysis of Variance (ANOVA) (Mane and Mohite, 2015). Tukey's multiple comparison test ($P= 0.05$) was used to determine significant differences between treatments using GenStat version 18.0 software (Crichton, 1999).

5.4 Results

5.4.1 Impact of *Schizonycha affinis* larvae on sugarcane seedling foliar and root biomass

Root damage was observed in sugarcane seedlings previously infested with larvae of *S. affinis*, at 10 days after treatment (DAT) (Figure 5.4). The seedlings in the control with larvae, but no protection, had fewer roots remaining compared to the seedlings in the no grub no treatment control (Figure 5.4A). Some seedlings had no roots remaining in the C17 treated and also untreated control with larvae at 10 DAT; and the results also showed that the seedlings treated with HHWG1 isolate and an insecticide, tended to have less root damage, compared to the seedlings in the control with grubs (Figure 5.4B). Moreover, seedlings in the untreated control exhibited leaf chlorosis (Figure 5.4C), compared to no larvae, no treatment seedlings which had many green/healthy leaves (Figure 5.4D).

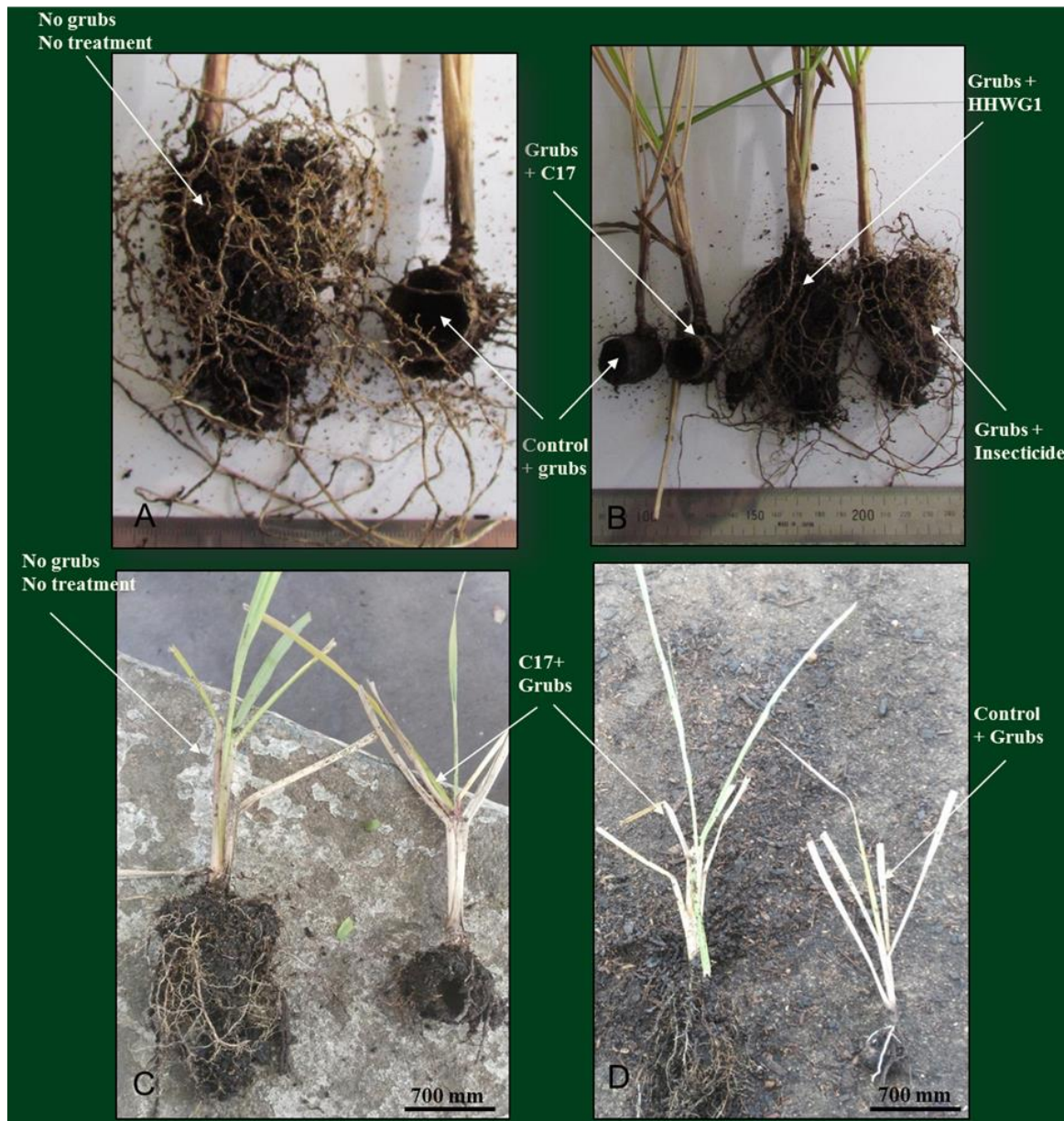


Figure 5.4: Impact of *Schizonycha affinis* larvae on root volume and aerial foliage of sugarcane seedlings. (A) 10 days post experiment, plant removed from untreated/ un-infested pots, and plant removed from pots infested with larvae and inoculated with distilled water with 0.05% Triton X-100 (control); and (B) Plants removed from pots treated with Decis (insecticide), distilled water with 0.05% Triton X-100 and *Beauveria brongniartii* isolates (C17 and HHWG1); (C) Sugarcane seedlings showing leaf chlorosis; and (D) Healthy leaves in a no larvae, no treatment seedling and root damaged seedling.

5.4.1.1 Root dry weight

In the no larva, no treatment control there was higher root biomass compared to the control with larvae. High root damage was observed in the control seedlings with larvae with the root dry

weight ranging from 0.1-0.2g as compared to 0.4-1.4g in the seedlings treated with HHWG1 isolate and insecticide, respectively at both 10 and 20 DAT, in the soil drenching inoculation (Figure 5.5). There was a significant difference ($P < 0.001$) in dry root biomass between the control with larvae and the control without larvae (Table 5.1 and 5.2).

The no larva, no treatment control had ± 1.6 g root dry weight at 10 and 20 days. Insecticide afforded some protection against large larvae at 10 DAT but less protection at 20 DAT (Figure 5.5A). The unprotected controls were heavily grazed (Figure 5.5A). It was also observed that insecticide was effective against small grubs, as was HHWG1 at 10 and 20 days (Figure 5.5A). In contrast, C17 gave the least protection to the sugarcane roots against small and large larvae. The insecticide treatments in the soil drench method were not as effective as in the root dip method against large larvae. Nevertheless, at 20 days it was effective against small larvae (Figure 5.5B). HHWG1 was effective against small larvae at 10 days but not at 20 days. All other treatments were more or less the same, but high protection at 20 days was recorded, especially with HHWG1 against large larvae (Figure 5.5B).

Table 5.1: Impact of *Schizonycha affinis* larvae ($N=3$) on sugarcane seedling above ground plant parts and mean root dry weight (DW) percentage (%). Plants were post inoculated with insecticide and *Beauveria brongniartii* isolates (C17 and HHWG1) using a soil drench inoculation method and results were recorded 10 and 20 days after treatment (DAT).

Treatments	10 DAT					20 DAT					Mean roots Dry weight (%)	
	No. of green leaves	No. of dead leaves	Roots DW (g)	Leaves DW (g)	Height (mm)	No. of green leaves	No. of dead leaves	Roots DW (g)	Leaves DW (g)	Height (mm)	10 days	20 days
Insecticide_Small	4.3cd	2.0ab	1.2ab	0.8ab	428.3bc	3.3bc	2.3ab	1.1b	0.8ab	373.3bc	81.11	79.23
Insecticide_Large	4.0cd	2.7b	1.4b	0.8ab	393.3bc	4.3cd	2.6bc	1.1b	0.8ab	400.0bc	100	78.5
HHWG1_Small	3.7c	2.7b	1.3b	0.9ab	371.7bc	3.7c	2.3ab	1.0b	0.7ab	382.7bc	90.32	75.85
HHWG1_Large	3.0b	2.7b	0.8ab	0.9ab	363.3bc	4.0cd	3.0bc	0.7ab	0.6a	341.7bc	55.76	48.31
dH2O (Control)_Small	2.7ab	3.0bc	0.2a	0.9ab	299.7b	2.0a	5.0d	0.1a	0.6a	216.7a	15.9	6.76
dH2O (Control)_Large	2.0ab	4.3cd	0.3a	0.8ab	285.0b	2.3a	3.7c	0.2a	0.9ab	366.7bc	17.51	15.22
C17_Small	2.7ab	2.7b	1.0ab	0.7ab	363.3bc	3.3bc	2.7b	0.7ab	0.8ab	340.0bc	65.67	49.52
C17_Large	3.3bc	3.3bc	0.8ab	1.0ab	380.0bc	3.7c	3.7c	0.5ab	0.6a	423.3bc	56.22	39.61
No grub no treat (Control)	4.0cd	2.0ab	1.4b	1.0ab	476.7c	5.7d	1.3a	1.4b	1.6b	616.7d	100	100
<i>P</i>	< 0.031	< 0.001	< 0.001	< 0.044	< 0.001	< 0.001	< 0.001	< 0.001	≤ 0.045	< 0.001		
All Treatments*Days								> 0.279	> 0.855	> 0.157		
All Treatments*Size	> 0.056											
All Treatments*Methods								> 0.895	> 0.924	> 0.334		

Values are means of 3 replicates. Different letter(s) next to the means within a column and the same variables tested indicate significant differences (Tukey comparison test, $P \leq 0.05$)

Table 5.2: Impact of *Schizonycha affinis* larvae ($N=3$) on sugarcane seedling above ground plant parts and mean root dry weight (DW) percentage (%). Plants were post inoculated with insecticide and *Beauveria brongniartii* isolates (C17 and HHWG1) using a root dip inoculation method and results were recorded 10 and 20 days after treatment (DAT).

Treatments	10 DAT					20 DAT					Mean roots Dry weight (%)		
	No. of green leaves	No. of dead leaves	Roots DW (g)	Leaves DW (g)	Height (mm)	No. of green leaves	No. of dead leaves	Roots DW (g)	Leaves DW (g)	Height (mm)	10 days	20 days	
Insecticide_Small	4.7cd	0.6a	0.5ab	0.8ab	366.7c	3.7cd	2.3ab	1.3b	0.8ab	376.7cd	34.56	92.75	
Insecticide_Large	4.7cd	1.7ab	1.2b	0.7ab	310.7ab	3.7cd	2.0ab	0.7ab	0.6ab	336.7bc	82.49	52.17	
HHWG1_Small	4.0cd	1.0a	1.2b	0.7ab	410.0cd	2.7cd	2.7b	0.4a	0.7ab	300.0bc	82.26	32.37	
HHWG1_Large	3.7cd	2.7b	1.0ab	0.7ab	391.7c	3.0cd	2.3ab	1.1b	0.6ab	353.3bc	68.43	78.5	
dH2O (Control)_Small	2.3ab	3.3bc	0.1a	0.8ab	346.7ab	1.0a	5.0d	0.2a	0.7ab	313.3bc	9.68	11.35	
dH2O (Control)_Large	1.3a	4.7cd	0.1a	0.5a	276.7a	2.7ab	4.0cd	0.3a	1.0b	396.7cd	9.49	24.4	
C17_Small	3.0c	3.0c	1.0ab	1.1b	380.0c	3.7cd	2.0ab	1.0ab	0.4a	365.0c	70.05	74.4	
C17_Large	3.7cd	1.7ab	1.1ab	0.8ab	343.3ab	3.3c	2.0ab	0.5ab	0.9ab	266.7b	75.35	37.92	
No grub no treat (Control)	4.0cd	2.0ab	1.4b	1.0b	476.7cd	5.7d	1.3a	1.4b	1.6bc	616.7d	100	100	
<i>P</i> < 0.001 < 0.001 < 0.001 > 0.100 < 0.001 < 0.001 < 0.001 < 0.001 > 0.1000 < 0.001													
All Treatments*Days									> 0.479 > 0.093 > 0.064				
All Treatments*Size 1.000													
All Treatments*Methods									> 0.895 > 0.924 > 0.334				

Values are means of 3 replicates. Different letter(s) next to the means within a column and the same variables tested indicate significant differences (Tukey comparison test, $P \leq 0.05$)

Furthermore, root dry weight was reduced severely in the presence of either small or large larvae, especially in the untreated control seedlings in both soil drench and root dip inoculation methods (Table 5.1 and 5.2). Hence, at 20 DAT, 90-100% root dry weight remained intact in the insecticide and HHWG1 treated seedlings in the presence of white grub larvae as compared to 6-15% root dry weight in the control (Table 5.1 and 5.2). In the presence of larvae, their impact reached a maximum of 75-93% root weight reduction in the control with larvae, compared to the control without larvae, at 10 and 20 DAT (Table 5.1 and 5.2).

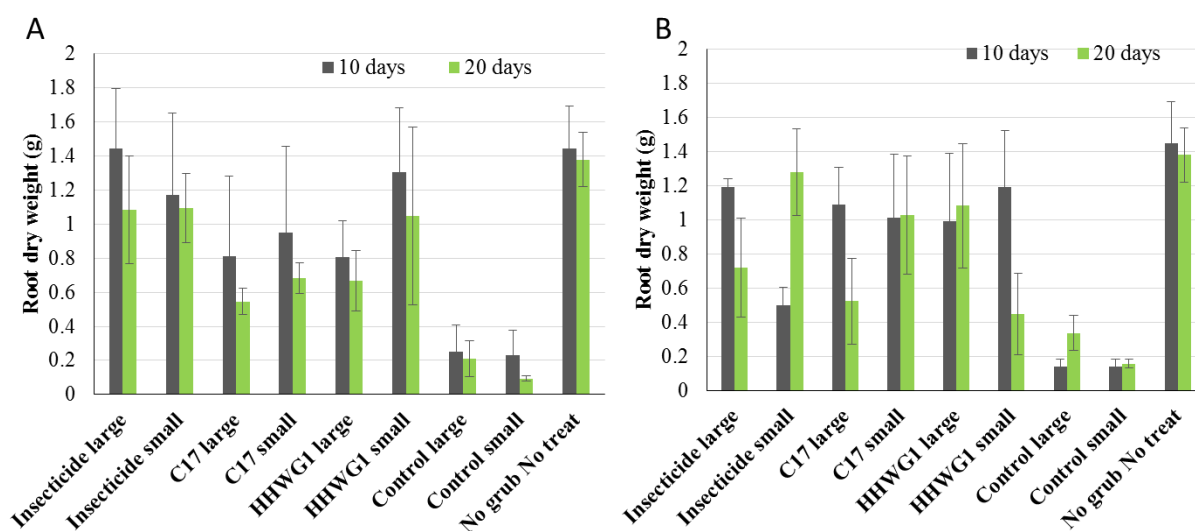


Figure 5.5: Mean (\pm S.E.) root dry weight (g) of sugarcane seedlings in the presence of small and large larvae of *Schizonycha affinis*, 10 and 20 days after treatment with fungal isolates (HHWG1 and C17) and insecticide using a soil drench (A) and root dip (B) inoculation methods. The sugarcane seedling root damage caused by *S. affinis* larvae was compared to the two controls (i.e. with larvae, but with no seedling protection and without larvae).

5.4.1.2 Leaf dry weight

Results showed that *S. affinis* larvae had a significant impact on leaf dry weight. The impact was not clear at 10 DAT as there was no significant difference ($P>0.05$) between leaf dry weight (DW) of the seedlings in the control without larvae and in all the treated seedlings as well as in the control with larvae (Table 5.1 and 5.2). However, the difference was significant ($P<0.05$) and highly noticeable, at 20 DAT. The treated seedlings after larval introduction had relatively low leaf DW of 0.6-1g as compared to 1.6g of seedlings in the control without larvae (Figure 5.6A and B). Most important and interesting to see is the strikingly high difference in leaf DW

between the controls without larvae and without treatment and the controls with larval infestations but without treatments (Figure 5.6). Both inoculation methods displayed a similar trend, the controls without larvae and without treatment had a significantly ($P<0.05$) higher leaf DW than the controls with larval infestations but without treatments at 20 days.

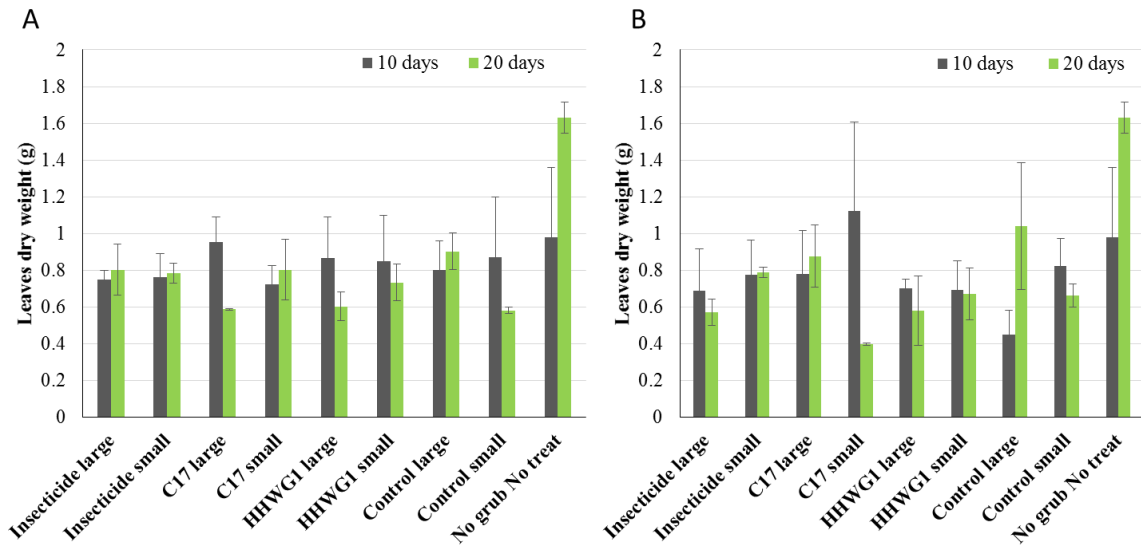


Figure 5.6: Mean (\pm S.E.) leaf dry weight (g) of sugarcane seedlings in the presence of small and large larvae of *Schizonycha affinis*, 10 and 20 days after treatment with fungal isolates (HHWG1 and C17) and insecticide using soil drench (A) and root dip (B) inoculation methods. The sugarcane seedling leaf dry weight was compared to the two controls (with larvae but without seedling protection, and without larvae).

5.4.1.3 Total number of leaves

There were many more green leaves (4-4.5) recorded for all treated seedlings, compared to the number of green leaves (2-3) of seedlings in the control with larvae (Figure 5.7A and B). The number of green leaves recorded for the no larva no treatment control seedlings were even more (4.5-6) than those in the treated seedlings, reflecting the type of growth that could be expected under the environmental conditions under which the trial was conducted, in the absence of *S. affinis* larvae.. The total number of dead leaves was also lower in the control seedlings without larvae than in all treatments, including the control with larvae (Figure 5.7A and B). However, the difference was only significant ($P<0.05$) between the control with larvae and the control without larvae.

There were also significant differences ($P<0.05$) in numbers of green and dead leaves between the seedlings in the control without larvae and the seedlings in some of the treatments, however there was no consistency (Table 5.1 and 5.2). The seedlings in the control with larvae were the only ones that had a higher number of dead leaves (3.5-5) as compared to green leaves (Figure 5.7A and B). Similar results were observed for both soil drench and root dip inoculation methods. Hence, the total number of dead leaves recorded at 20 DAT post infestations in the control was significantly lower ($P<0.05$) compared to the total number of dead leaves in the treated seedlings and in the control (no grub no treatment) seedlings (Table 5.1 and 5.2).

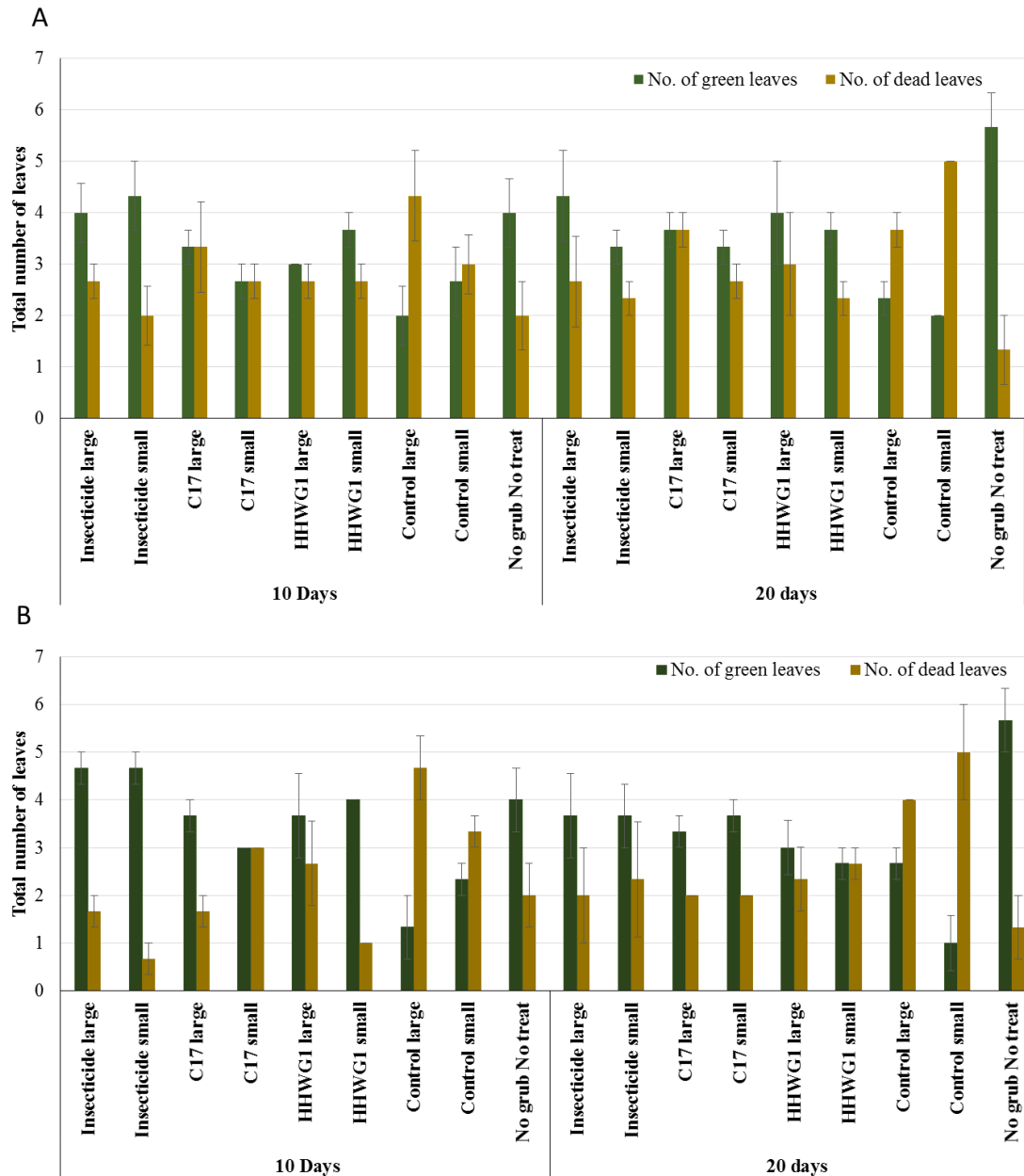


Figure 5.7: Mean (\pm S.E.) total number of green and dead leaves of sugarcane seedlings in the presence of small and large larvae of *Schizonycha affinis*, 10 and 20 days after treatment with fungal isolates (HHWG1 and C17) and insecticide using soil drench (A) and root dip (B) inoculation methods. The sugarcane leaves status was compared to the two controls (with larvae but without seedling protection, and without larvae).

5.4.1.4 Seedling height

Sugarcane seedling height was significantly higher ($P < 0.05$) in the no grub no treatment than in the control with larvae and all other treatments, this difference was visible at 20 DAT (Table 5.1 and 5.2). Hence, the results show that the presence of white grub larvae slow down the rate of plant growth in terms of height (Figure 5.8A and B). A similar outcome was shown for both inoculation methods that were used.

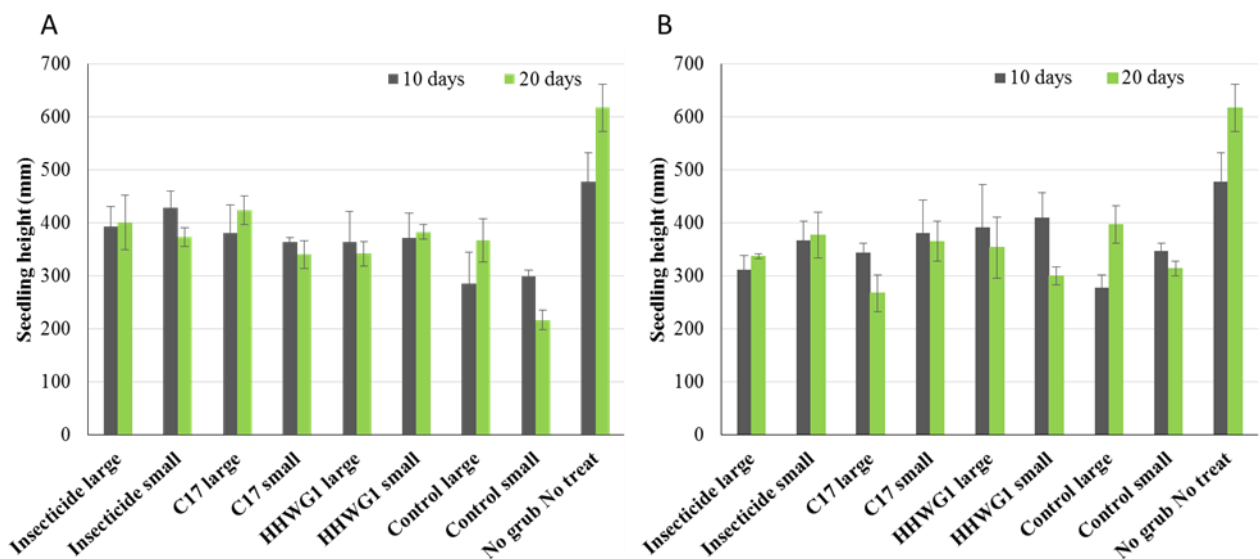


Figure 5.8: Mean (\pm S.E.) height (mm) of sugarcane seedlings in the presence of small and large larvae of *Schizonycha affinis*, 10 and 20 days after treatment with fungal isolates (HHWG1 and C17) and insecticide using soil drench (A) and root dip (B) inoculation methods. The sugarcane seedling height was compared to the two controls (with larvae but without seedling protection, and without larvae).

5.4.1.5 General results description

Although root DW was significantly different ($P < 0.001$) between the treatments, there was no significant interaction effect ($P > 0.05$) between treatments and the number of days elapsed post inoculations in root DW using soil drench and root dip methods. This was also observed in the case of leaf DW and height (Table 5.1 and 5.2). Furthermore, no significant interaction ($P > 0.05$) was observed between the inoculation methods used in the pot trial and there was also no significant larval impact ($P > 0.05$) observed between the small and large larvae (Table 5.1). Further, when looking at the larval damage in terms of the number of green leaves remaining

and number of dead leaves post-infestations, the effect of treatments, the interaction between treatments and condition of leaves were highly significant ($P < 0.001$) (Table 5.1 and 5.2).

5.4.2 The efficacy of C17 and HHWG1 *Beauveria brongniartii* isolates and insecticide treatment against *Schizonycha affinis* larvae

The numbers of *S. affinis* larvae recorded in the treated pots were notably lower than in the control pots, and most importantly no dead larvae were recovered in the control pots. However, *B. brongniartii* isolates (C17 and HHWG1) and insecticide were differentially effective against *S. affinis* white grub larvae, for both the soil drench and root dip application trials.

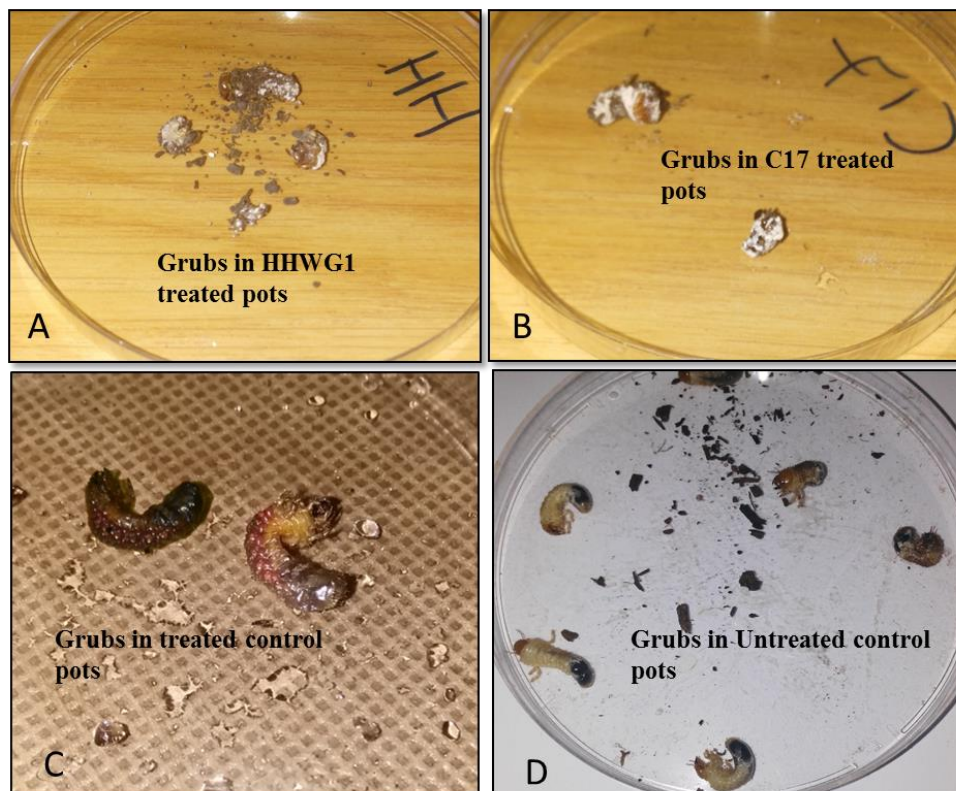


Figure 5.9: *Schizonycha affinis* larvae from pot trials treated with *Beauveria brongniartii* isolates (C17 and HHWG1), insecticide and the untreated controls. (A) Examples of mycosed larvae recovered from HHWG1 treated pots; (B) Examples of mycosed larvae recovered from C17 treated pots; (C) Examples of dead larvae recovered from insecticide treated pots; and (D) healthy larvae recovered from the control. Insecticide was used as a treated control and distilled water with 0.05 Triton X-100 was an untreated control. The effect of the treatments was evaluated every 3rd day for 30 days after treatment.

5.4.2.1 Soil drench inoculation method

Larval mycosis was observed in pots treated with C17 and HHWG1 isolates (Figure 5.9A and B). Reddish dead larvae (Figure 5.9C) were recovered from the insecticide treated pots from the 12th day after treatment (DAT). No mycosed larvae were recovered from the control pots, only healthy ones (Figure 5.9D).

Schizonycha affinis larvae surviving per pot during the experiment varied between the treatments. Survival rate of 100% of larvae was observed in the negative control (distilled water with 0.05% Triton X-100) pots for both soil drench and root dip inoculation methods. High larval mortality was recorded in the insecticide as well as in the HHWG1 fungal isolate treated pots. Insecticide caused significantly higher mortality of larvae. At 3 days after treatment (DAT), dead larvae were recovered from the insecticide treated pots and high mortality was recorded thereafter until 30 DAT (Figure 5.10). Insecticide was highly virulent to small larvae, more so than to large larvae. Mortality of 100% and 80% was recorded after application of the insecticide compared to 20% and 10% after application of fungal isolates at 6-12 DAT, on both small and large larvae, respectively (Figure 5.10). The best effect of fungal isolates against small and large larvae was recorded 21 DAT, where no significant difference ($F_{(2, 3)} = 1.00$; $P > 0.05$) in mortality was recorded between the insecticide and HHWG1 treatments of both the small and large larvae (Table 5.1).

Both *B. brongniartii* isolates caused relatively low mortality (10-40%) against small and large larvae at 9-18 DAT (Figure 5.10). At 21 DAT, however, small larvae were more susceptible to the HHWG1 isolate compared to C17. The HHWG1 isolate caused 55% mortality in small larvae and 40% mortality in large larvae at 21 DAT (Figure 5.10), whereas the C17 isolate caused 20% mortality in small larvae and 20% mortality against large larvae at 21 DAT. Although the treatments did not cause significant ($F_{(2, 39)} = 1.09$; $P > 0.05$; Appendix 1) mortality in small and large larvae; 66% mortality was observed at 27 and 30 DAT in HHWG1 treated small larvae. Further, the results also showed that there was a significant interaction ($F_{(2, 63)} = 1.04$; $P < 0.05$; Appendix 1) between treatment and days after treatment for both small and large larvae.

Overall results indicated that insecticide was the most virulent treatment against the larvae as compared to both *B. brongniartii* isolates, especially for the first few days after treatment application; however, after 21 days, the effect of the HHWG1 isolate escalated. Towards the end of the trial, thus, there was no significant difference ($P > 0.05$) between mortality caused by

the insecticide and HHWG1 isolate treatments at 21, 24, 27 and 30 DAT (Figure 5.10; Appendix 1).

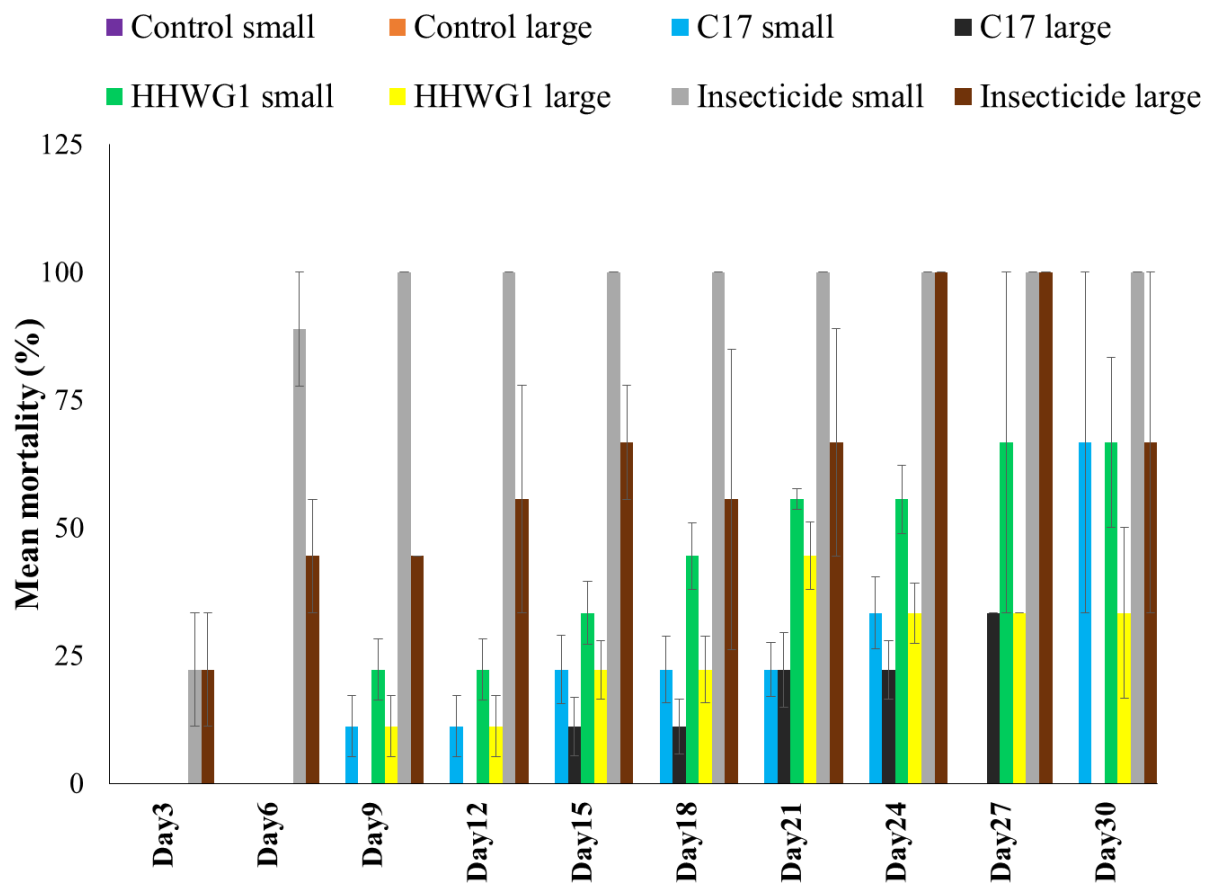


Figure 5.10: Mean mortality (%) of small and large larvae of *Schizonycha affinis* at three day intervals until 30 days after treatment with solutions of *Beauveria brongniarii* isolates (C17 and HHWG1), insecticide and water (control) in pot trials, using a soil drench method. Bars are standard errors of the mean.

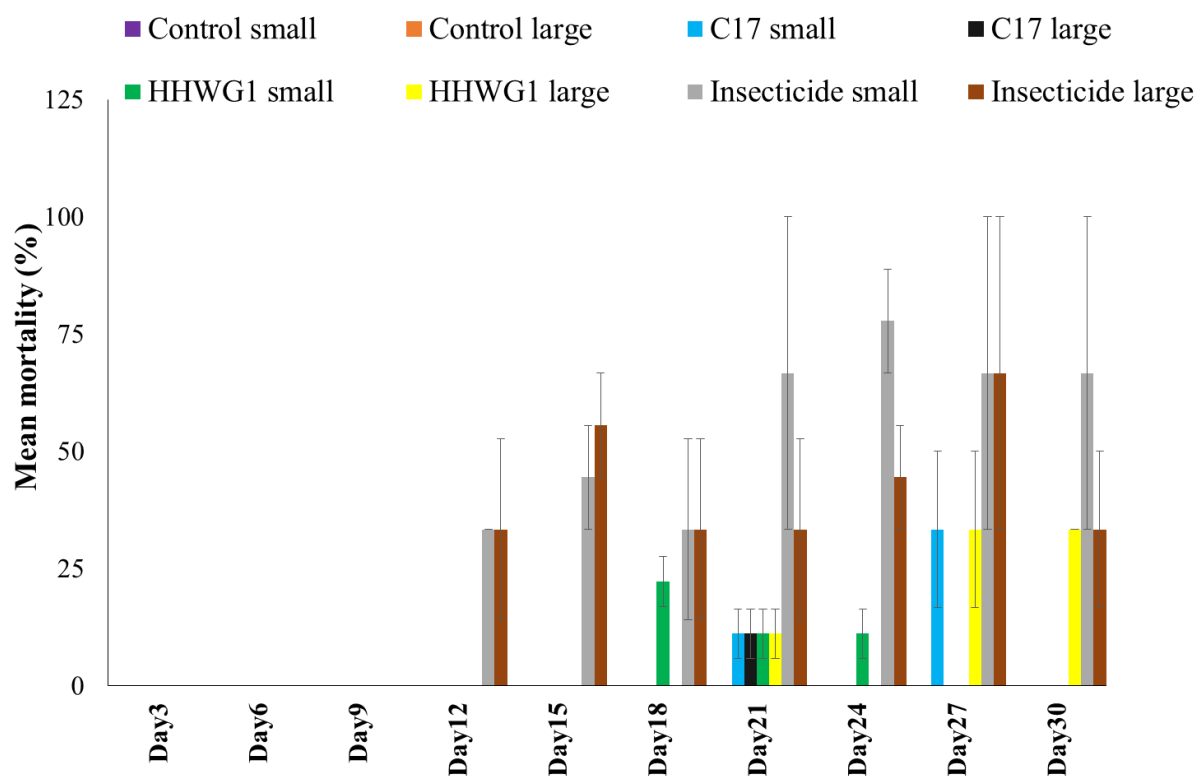


Figure 5.11: Mean mortality (%) of small and large larvae of *Schizonycha affinis* at three day intervals until 30 days after treatment with a suspension of *Beauveria brongniartii* isolates (C17 and HHWG1), insecticide and water (control), using a root dip inoculation method. Bars are standard errors of the mean.

5.4.2.2 Root dip inoculation method

There were no dead and mycosed white grub larvae recovered from the control pots throughout the trial (Figure 5.11). No dead larvae were recovered from any pots for the first 9 DAT (Figure 5.11). Insecticide caused 30% mortality in both small and large larvae at 12 DAT as compared to 0% mortality recorded in fungal treated pots at 12 DAT (Figure 5.11). The C17 and HHWG1 isolates took longer to cause mortality in the small and large larvae than insecticide. Hence, the insecticide caused the highest mortality, ranging from 70-80%, as compared to 10-30% mortality caused by C17 and HHWG1 (Figure 5.11). Treatments started to be effective as the number of days progressed, at 12 days after application. Hence, there was no significant difference ($F_{(2, 3)} = 2.00$; $P > 0.05$; Appendix 2) in mortality recorded at 12 and 27 days after treatments, between the small and large larvae. However, the number of days post inoculations were essential and yielded significant results; hence, there was a significant ($F_{(2, 63)} = 2.17$;

$P < 0.001$; Appendix 2) interaction between treatment and the number of days after treatment for both small and large larvae. The results showed that insecticide was highly effective, and yielded mortality that was significantly higher ($F_{(2, 3)} = 216.00$; $P < 0.001$; Appendix 2) than mortality in the control pots for both small and large larvae at the same DAT intervals.

The effect of *B. brongniartii* isolates on the larvae was observed at 18 DAT. At 18 DAT, 20% mortality was recorded after application of the HHWG1 isolate while there was 0% mortality recorded after application of C17 (Figure 5.11); thus, there was significant difference ($F_{(2, 3)} = 4.00$; $P < 0.001$; Appendix 2) between mortality recorded for both fungal isolates and that in the control, for both small and large larvae. Hence, the results indicated that insecticide was the most virulent treatment against both small and large larvae as compared to both *B. brongniartii* isolates (C17 and HHWG1) when using the root dip inoculation method.

5.4.2.3 Soil drench versus root dip inoculation methods

The effect of *B. brongniartii* isolates and insecticide on small and large larvae was dependent on the inoculation method used, and days after treatment. The soil drench inoculation method yielded strikingly good treatment effects against the larvae. It was thus found that the effect of treatments was significantly higher ($F_{(2, 79)} = 3.06$; $P < 0.001$ Appendix 1) in the soil drench, compared to the root dip inoculation method. Larval mortality was recorded during the first 3 days of application of an insecticide using a soil drench inoculation method, as compared to 12 DAT using the root dip inoculation method (Figure 5.10 and 5.11). For *B. brongniartii* isolates, mortality was recorded 9 DAT using a soil drench inoculation method, as compared to 18 days after application using the root dip inoculation method (Figure 5.10 and 5.11).

5.5 Discussion

5.5.1 Impact of Schizonycha affinis larvae on sugarcane seedling foliar and root biomass

The results demonstrated the impact of *S. affinis* larvae on sugarcane seedlings. Root damage to sugarcane seedlings found in the presence of larvae, in the control pots was up to 93%. Similar results were obtained by Coale and Cherry (1989), who studied the effect of white grub larvae (*Ligyris* sp. Ritcher; Coleoptera: Scarabaeidae: Dynastinae) infestations on sugarcane root: shoot relationships, and found that *Ligyris* larvae were responsible for 59% reduction of sugarcane root mass. They further demonstrated that heavy white grub larval infestations in their trial buckets caused severe losses in sugarcane root dry weight as well as in shoot dry weight. Comparing those results with that of current study, it is quite clear that root consumption by the white grub larvae attributes to the loss of the sugarcane plant aerial foliage because of

the high number of dead leaves in the larvae infested pots. This also explains the severe leaf chlorosis that was observed after sugarcane seedlings were infested with the *S. affinis* larvae. Thus, indicating that white grub larvae can have massive negative impacts on sugarcane biomass.

Allsopp *et al.* (1995) studied the effect of the insecticide suSCon Blue (active ingredients= 140g/kg chlorpyrifos) against larvae of *Lepidiota picticollis* Lea (Coleoptera: Scarabaeidae: Melolonthinae) in Australian sugarcane, and the impact of the larvae on sugarcane yields. They showed that the number of larvae were higher in the untreated plots as compared to the suSCon treated pots, which resulted in greater sugarcane yields in the treated plots than in the untreated plots. Comparing their results with the results acquired in the present study, it was found that white grub larvae surviving per pot during the current study varied between the treatments. High larval mortalities were recorded in the insecticide as well as in the HHWG1 fungal isolate treated pots, whereas, high survival of white grub larvae was observed in the control (distilled water) pots for both soil drench and root dip inoculation methods, as compared to relatively low larval survival in the insecticide and HHWG1 treated pots in soil the drench inoculation method. This is supporting information that explain the possible reasons for the results obtained. For example, the low root damage in insecticide treated seedlings can be linked to high larval mortalities, whereas high root damage in the control can be due to high larval survival resulting in continuous feeding on the roots. Thus indicating that insecticide and the HHWG1 isolate had a negative effect on the number of *S. affinis* larvae found under the sugarcane seedling stool.

The impact of white grub larvae on other agricultural crops has been researched and reported on. Rogers *et al.* (2005) studied the damage of two white grub species *Holotrichia serrata* Hope (Coleoptera: Scarabaeidae: Melolonthinae), a root feeding species and *Heteronyx piceus* Blanchard (Coleoptera: Scarabaeidae: Melolonthinae), a pod grazer on groundnut. They showed that *H. serrata* grubs caused drastic yield losses of 7.52 g/larva and *H. piceus* caused a yield loss of 4.20g/larva. Their study corroborates the data presented in the current study, by indicating that white grub larvae consume excessive amount of plant roots which therefore affect plant quality. Furthermore, reduced dry weight of the sugarcane seedlings in the control with *S. affinis* larvae in the present study, confirms the impact of this species of white grub in sugarcane. It was shown that insecticide afforded the sugarcane protection against *S. affinis* larvae. These results correspond to the findings of Kulkarni *et al.* (2007), who evaluated the incidence of white grub larvae, *Schizonycha ruficollis* Fabricius (Coleoptera: Scarabaeidae:

Melolonthinae) on teak (*Tectona grandis* L. f.; Lamiales: Lamiaceae), and found low biomass losses and low white grub larval survival in seedlings treated with insecticides (phorate and chlorpyrifos).

5.5.2 The efficacy of C17 and HHWG1 Beauveria brongniartii isolates and insecticide treatment against Schizonycha affinis larvae

The results showed that insecticide treatment was highly virulent (30-100%) against *S. affinis* larvae, compared to the *B. brongniartii* isolates. Present results are therefore consistent with results of a study by Carnegie (1988), which indicated that insecticide reduced white grub larvae of *S. affinis* and *Asthenopholis minor* Brenske (Coleoptera: Scarabaeidae: Melolonthinae) in field trials in South Africa. These results also confirm the results of Kowalska (2008), who evaluated the effect of *B. brongniartii* and insecticide against larvae of *Otiorhynchus sulcatus* Fabricius (Coleoptera: Curculionidae) species. He showed that insecticide was effective during the first few days after inoculation, compared to *B. brongniartii* which took longer to kill the target larvae.

Further collaborative results of control of white grubs using EPF's and insecticide have been reported by Benker and Leuprecht (2005), who evaluated the effect of *B. brongniartii* and two insecticides (Imidacloprod and Carbofuran) against *M. melolontha* in field replicated studies. Both insecticides were highly effective 8 DAT, compared to *B. brongniartii* which caused low mortality during the first week after application, but increased in efficacy after 10-15 days after treatments. This corresponds with the results obtained in the current study. Our study showed that subsequently at 30 days after treatment, the HHWG1 isolate was comparably as effective as the insecticide used. From 18 DAT, it was observed that HHWG1 isolate infection on *S. affinis* larvae increased as days after treatment were increasing, indicating that the EPF takes longer to cause death compared to insecticide, which caused larval death from 3 days after treatment. Although insecticides are effective short term control measures, Benker and Leuprecht (2005) stated that the use of EPF have the potential of producing long-term protection against white grub species, because if they are effective, they produce higher spore loads from infected cadavers, increasing their ability to infect more larvae of the target species. In the current study, increasing infections and persistence of *B. brongniartii* isolates days after treatment is supported by findings reported by Dolci *et al.* (2006), that *B. brongniartii* persists in the presence of the host species in the soil, post-inoculation.

Current results showed that the most virulent isolate of *B. brongniartii* (HHWG1) at 1×10^9 conidia/ml concentration, caused 60% mortality of *S. affinis* larvae at 30 DAT. Mane and Mohite, (2015) studied the pathogenicity of a range of entomopathogenic fungi against white grub larvae of *Leucopholis lepidophora* Blanchard (Coleoptera: Scarabaeidae: Melolonthinae) infesting sugarcane in a pot trial. They found that *B. brongniartii* at 2×10^8 conidia/ml was virulent against *L. lepidophora* larvae, causing 24% mortality at 30 DAT and 58.62% mortality at 45 DAT. This revealed that high conidial concentration yields better results, within a shorter period after treatment. This also suggests that the higher the concentration, the higher the mortality caused. Thus, the concentration of pathogenic fungi concentration plays a crucial role in the pathogenicity of EPFs against white grubs. When considering using the EPF as a commercial bio-insecticide though, using the prospective isolate at higher concentrations has budget cost implications, which may outweigh the benefits of the control obtained by using the commercial EPF.

However, the EPF concentration necessary to give the best results is still controversial. For instance, Malik *et al.* (2016) showed that high mortalities of *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) were observed at a lower concentration rate (1×10^6 conidia/ml) of *B. bassiana* when they evaluated the effect of imidacloprid and *B. bassiana* against the larvae of *R. ferrugineus*. They argued that low conidial dosage results in higher mycosis development and sporulation than high concentrations. This viewpoint was shared by Tefera and Pringle (2003), who argued that high doses of conidia were likely to result in the conidial self-inhibition scenario, which is defined as “*low conidia germination caused by amassing of spores*” (Lingappa and Lingappa, 1965). These findings are, however, contrary to those of Vandenberg (1992) and Goble (2012). Vandenberg (1992) recorded high sporulation and mycosis development at intermediate concentrations of *B. bassiana* on *Megachile rotundata* F. (Hymenoptera: Megachilidae). Goble (2012) indicated that at the lower concentration (1×10^6 conidia/ml), the time at which mortality of treated larvae of *P. sommeri* reached 50% (LT50) was 25 days, and at a higher concentration (1×10^9 conidia/ml) the LT50 decreased to 15 days.

These contrary results could be explained in part by differential susceptibilities of the host specimens in question, as has been shown in Chapter 3 of this thesis, and between host life stages as demonstrated by Goble (2012) and Kheswa *et al.* (2016). The degree of susceptibility of different host specimens and their life stages is influenced by a number of factors including the insect cuticle thickness, sclerotization and the specificity of the EPF (Vega *et al.*, 2008).

According to Fox (1961) the young larvae are highly susceptible to fungal infection compared to the older larvae with more sclerotized cuticles.

The fungal isolates application using a root dip inoculation method were less effective on small and large larvae of white grubs than observed in the soil drench application method. Paray and Rajabalee (1997) indicated that a soil drench inoculation method used to control white grub adults of *Phyllophaga smithi* Arrow (Coleoptera: Scarabaeidae: Melolonthinae) produced high mortalities, compared to dipping the white grub species into a conidial suspension of EPF. Consequently, applying fungal isolates in the soil using a root dipping method produced relatively low mortality of *S. affinis* larvae in the present study, possibly because fewer spores were attached to the roots of the sugarcane seedlings, which therefore resulted in less fungal conidia being transferred to the soil in the pots.

These results revealed that inoculum application method is an important factor to consider in field applications, which can enhance physical contact of fungal spores and larvae in the soil. Keller (2000) reported the persistence of *B. brongniartii* for over 5 years post soil applications with a granular formulation. Townsend *et al.* (2010) reported that as fungal dose increased, so did the mortality of manuka beetle larvae (*Pyronota* sp.; Coleoptera: Scarabaeidae: Melolonthinae) after inoculation with *B. brongniartii* strain F636. They also stated that field application of *B. brongniartii* in the form of granules may yield adequate results and enhance establishment of fungal pathogenicity in the target host population. There is therefore a need to further evaluate field application methods of *B. brongniartii* using high conidia dosage or alternatively using the spores in a form of conidial powder or granular formulation.

Biotic factors can also play a part in the successful use of EPF's. In the present study heavy rainfall occurred during the first 4 days after applying *B. brongniartii* isolates in the pots (see Figure 5.1), which could have contributed to the poor recovery of mycosed larvae from the treated pots for both the soil drenching and root dipping inoculation methods. According to Gupta *et al.* (2003) rainfall is the most crucial factor known to affect the efficacy of EPF's against white grubs.

5.6 Conclusion

Towards the development of *B. brongniartii* as a bio-insecticide for white grub species in South Africa, this was the first study to compare the efficacy of *B. brongniartii* (HHWG1 and C17) isolates and insecticide (Decis) to alleviate white grub infestation in pot trials under outside environmental conditions in South Africa, and to measure the impact of this protection on the

growth of sugarcane. The results indicated that the insecticide used was highly virulent to small and large larvae of *S. affinis* and the *B. brongniartii* HHWG1 isolate was most virulent against *S. affinis* larvae 18 days after treatment. Furthermore, protection of seedling growth against *S. affinis* larvae was afforded by the insecticide and HHWG1 treatments in particular, as the high larval mortality recorded in these treatments, was complemented by more root growth, which resulted in better sugarcane seedling growth. The results also highlighted the importance of inoculation method used, with the soil drenching inoculation method providing adequate results by reducing white grub larvae under the sugarcane stool, compared to the root dipping method. The *B. brongniartii* HHWG1 isolate thus remains a potential bio-insecticide that can be commercially produced to control white grub species in sugarcane and possibly other crops in South Africa. Further evaluation of this *B. brongniartii* isolate in replicated field trials is supported by this trial.

5.7 References

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Appendix 1

Mean mortality percentage of small and large white grub larvae of *Schizonycha affinis* ($N=3$) treated with two *Beauveria brongniartii* isolates (C17 and HHWG1), insecticide (treated control) and untreated control (distilled water (dH2O)) at three day intervals until 30 days after inoculation using a soil drench inoculation method.

Treatments	Larval size	Mean mortality percentage days after treatment									
		Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 24	Day 27	Day 30
dH2O	Small	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a
dH2O	Large	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a
Insecticide	Small	22.2±11.1ab	88.9±11.1bc	100±0.0c	100±0.0c	100±0.0c	100±0.0bc	100±33.3bc	100±0.0c	100.0±0.0c	100±0.0c
Insecticide	Large	22.2±11.1ab	44.4±11.1bc	44.4±0.0ab	55.6±22.2bc	66.7±11.1abc	100±29.4bc	66.7±19.2bc	100±0.0c	100.0±0.0c	66.7±33.3bc
C17	Small	0±0.0a	0±0.0a	11.11±6.0ab	11.1±6.0ab	22.2±6.7abc	22.2±6.2abc	22.2±2.0ab	33.3±7.0ab	0±0.0a	66.7±33.3bc
C17	Large	0±0.0a	0±0.0a	0±0.0a	0±0.0a	11.1±5.7ab	11.1±5.3ab	22.2±6.6ab	22.2±5.7ab	33.3±0.0ab	0±0.0a
HHWG1	Small	0±0.0a	0±0.0a	22.2±6.0ab	22.2±6.0ab	33.3±6.2ab	44.4±6.5bc	55.6±5.3bc	55.6±6.7bc	66.7±33.3bc	66.7±16.7bc
HHWG1	Large	0±0.0a	0±0.0a	11.1±6.0ab	11.1±6.0ab	22.2±5.7ab	22.2±6.5ab	33.3±7.3ab	33.3±5.9ab	33.3±0.0ab	33.3±16.7ab
	<i>P</i> -value	> 0.155	< 0.001	< 0.001	< 0.001	< 0.001	≥ 0.053	≥ 0.051	< 0.001	< 0.001	< 0.001
All Treatments*Size	> 0.344	-	-	-	-	-	-	-	-	-	-
All Treatments*Days	< 0.038	-	-	-	-	-	-	-	-	-	-
All Treatments*method	< 0.001	-	-	-	-	-	-	-	-	-	-

Values are means of 3 replicates per treatment. Mean ± S.E. values with different letters are significantly different (Tukey HSD test, $P \leq 0.05$)

Appendix 2

Mean mortality percentage of small and large white grub larvae of *Schizonycha affinis* ($N=3$) treated with *Beauveria brongniartii* isolates (C17 and HHWG1, insecticide (treated control) and untreated control (dH2O) at three day intervals until 30 days after inoculation using a root dip inoculation method.

Treatments	Larval size	Mean larval mortality percentage days after treatment									
		Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 24	Day 27	Day 30
dH2O	Small	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a
dH2O	Large	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a
Insecticide	Small	0±0.0a	0±0.0a	0±0.0a	33.3±0.0abc	44.4±11.1bcd	33.3±19.3bc	66.6±33.3cd	77.8±11.1d	66.7±33.3abcd	66.7±33.3cd
Insecticide	Large	0±0.0a	0±0.0a	0±0.0a	33.3±19.3abc	55.7±11.1bcd	33.3±19.3bc	33.3±19.3bc	44.4±11.1bcd	66.7±33.3abcd	33.3±16.7abc
C17	Small	0±0.0a	0±0.0a	0±0.0a	0±0.0a	0±0.0a	0±0.0a	11.1±5.3ab	0±0.0a	33.3±16.7abc	0±0.0a
C17	Large	0±0.0a	0±0.0a	0±0.0a	0±0.0a	0±0.0a	0±0.0a	11.1±5.3ab	0±0.0a	0±0.0a	0±0.0a
HHWG1	Small	0±0.0a	0±0.0a	0±0.0a	0±0.0a	0±0.0a	22.22±5.3abc	11.1±5.3ab	11.1±5.3ab	0±0.0a	0±0.0a
HHWG1	Large	0±0.0a	0±0.0a	0±0.0a	0±0.0a	0±0.0a	0±0.0a	11.1±5.3ab	0±0.0a	33.3±16.7abc	33.3±16.7abc
	<i>P</i> -value	-	-	-	> 0.155	< 0.001	< 0.001	< 0.001	< 0.001	> 0.323	< 0.014
All Treatments*Size	< 0.009	-	-	-	-	-	-	-	-	-	-
All Treatments*Days	< 0.001	-	-	-	-	-	-	-	-	-	-
All Treatments*Method	< 0.001	-	-	-	-	-	-	-	-	-	-

Values are means of 3 replicates per treatment. Mean ± S.E. values with different letters are significantly different (Tukey HSD test, $P \leq 0.05$)

CHAPTER 6: GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 Introduction

The impact of white grub species on South African sugarcane is well documented (Carnegie, 1974; Carnegie, 1988; Way, 1997; McArthur and Leslie, 2004; Way *et al.*, 2011). More recent research recognized the potential use of biological control strategies to reduce the impact of white grub species affecting sugarcane as an alternative control measure to the more commonly used chemical insecticides (Milner *et al.*, 2002; Milner *et al.*, 2003; Samson *et al.*, 2006; Goble *et al.*, 2012; Goble *et al.*, 2015, Kheswa *et al.*, 2016). Shifting towards the development of mycoinsecticides to control white grubs in South Africa, Goble *et al.* (2012) identified endemic *Beauveria brongniartii* causing epizootics on *Pegylis sommeri* and *Schizonycha affinis* adults and larvae in the Midlands North area of KwaZulu-Natal. In 2015, it was shown through laboratory bioassays, that amongst several identified *B. brongniartii* isolates, the strains C17 and HHWG1 were highly pathogenic against adults and larvae of *P. sommeri* and *S. affinis* (Goble *et al.*, 2015).

In developing an effective mycoinsecticide, there are at least 10 procedures that are followed (Goble, 2012). Goble (2012) in her study, completed the first 5 procedures, viz. a) recognizing the pest species; b) recognition of a potential entomopathogen; c) entomopathogen strain selection; d) isolate preparation and testing; and e) socio-economic and market analysis. Goble (2012) therefore, recommended that future research should further evaluate: a) *B. brongniartii*'s host range, due to the fact that it was unknown whether the host range of endemic *B. brongniartii* isolates could be infectious against other species in the Melolonthinae or other subfamilies such as the Rutelinae and Dynastinae in South Africa; b) the efficacy of formulated *B. brongniartii* isolates tested in soil application trials; and c) the economic impact of white grub in the sugar industry, since there is limited information available on this aspect.

Building from Goble's (2012) study, the current study broadened *B. brongniartii*'s host range; provided knowledge about the low potential to establish *B. brongniartii* as an endophyte of sugarcane roots; and showed the efficacy of *B. brongniartii* isolates against the larvae of *S. affinis* and their subsequent impact on sugarcane seedling growth in pot trials. To build on the findings of the study, the following future directions are recommended.

6.2 Host range of the *Beauveria brongniartii* isolates

In Chapter 3, the host range of two native *B. brongniartii* isolates (HHWG1 and C17) against adults and larvae of melolonthine and dynastine white grub species, which included

Asthenopholis minor, *Heteronychus licas*, *Temnorhynchus clypeatus*, *Heteronychus tristis* and larvae of *Schizonycha neglecta*, was evaluated. It was shown that these *B. brongniartii* isolates were variably effective against these species of white grubs. This wide host range of *B. brongniartii* has been found by other studies (Theunis and Aloali'i, 1998; Hadapad *et al.*, 2006; Srikanth *et al.*, 2010). This characteristic increases *B. brongniartii* practicability to be developed into a cost effective mycoinsecticide, as it broadens its host range (Goble, 2012).

However, in the current study it was found that different white grub species, even within the same subfamilies, had different susceptibilities to the same pathogens. This is in keeping with Keller *et al.* (1999) findings. The isolates were less effective against larvae of the dynastine species, *H. licas* and melolonthine species, *A. minor*. The highly virulent *B. brongniartii* isolate HHWG1 caused 50% mortality in second instar larvae of *H. licas* and *A. minor*; compared to 80-93% mortality in the third instar larvae of *H. tristis*, *S. neglecta* and second instar larvae of *T. clypeatus*. According to Thungrabeab *et al.* (2006) the virulence of entomopathogenic fungi (EPF) can be grouped into three pathogenic categories, based on the target insects' mortality: 1) highly pathogenic, 2) moderately pathogenic and 3) weakly pathogenic; where a highly pathogenic EPF causes >64.49% mortality, a moderate one from 30.99-64.49% mortality and a weakly pathogenic one <30.99% mortality. We may thus conclude that the effect of isolates against the least susceptible species in this current study was moderate, while that on the susceptible species and life stages was highly pathogenic. However, Terefe *et al.* (2012) stated that the efficacy of an EPF against *Sesamia calamistis* was highly pathogenic if mortality was 81-100%, moderate at 71-80% and weak if mortality ranged from 60-70%. For this reason low mortality of larvae of *H. licas* and *A. minor* obtained in Chapter 3 requires future studies, which should evaluate other alternative entomopathogenic agents against the least susceptible white grub life stages and species. For instance, the literature suggests that isolates of *Metarhizium anisopliae* are the ones that are highly pathogenic towards species within the Dynastinae (Rath and Worledge, 1995; Ansari *et al.*, 2004; Beron and Diaz, 2005; Makaka, 2008) and Melolonthinae subfamilies (Milner *et al.*, 2002; Guzmán-Franco *et al.*, 2012).

Moreover, it has been shown that the efficacy of entomopathogenic fungi (EPFs) against targeted insect pests may be affected by several factors. Keane and Kerr (1997) included the geographic region of origin and host species differing resistances as two factors. This is supported by Keller *et al.* (1999), who noted differences in susceptibility of similar white grub species found in different geographic regions towards the same pathogens. Keller *et al.* (2003) reported that *Metarhizium anisopliae* was the most effective pathogen of white grubs in

Australia and *B. brongniartii* was highly pathogenic against white grubs in Europe. Srikanth *et al.* (2016) showed that biocontrol agents of a given pest in different geographic regions can have different pathogenicity. Finding a biocontrol agent that can maintain its virulence across all geographic ranges can be advantageous. A biocontrol agent with a large host range assists in its implementation and introduction in different regions and in non-native pest areas (Srikanth *et al.*, 2016). However, care should be taken to ensure that they do not attack beneficial, non-pest species in the latter instance.

Consequently, efficient control of white grub species can be achieved by evaluating and testing the efficacy of *B. brongniartii* against all the life stages of other white grub species found in South African sugarcane fields. Hence, surveys for more effective strains of biocontrol agents should be a continuous activity across the whole range of occurrence of pest species.

6.3 Establishment of *Beauveria brongniartii* as endophytes of sugarcane roots

Unlike with *Beauveria bassiana*, there is very little literature reporting the establishment of *B. brongniartii* as an endophyte in plants. *Beauveria brongniartii* was isolated once from coffee berry plants (Vega, 2008). In Chapter 4, the potential of C17 and HHWG1 isolates to be established as endophytes of sugarcane roots for long term protection against white grub larvae was evaluated. Unfortunately, after four weeks post inoculation of setts of two sugarcane varieties (N12 and N48), no fungi with *Beauveria* spp. characteristics could be re-isolated from sugarcane roots. However, *Fusarium* spp. and *Penicillium* species were detected. Literature (Vega, 2008; Mulaw *et al.*, 2013; Fouda *et al.*, 2015) reported *Fusarium* spp. and *Penicillium* spp. as aggressive endophytes of plant parts. They outcompeted other fungal endophytes, including *B. bassiana*, in plant tissues (Geetha *et al.*, 2008; Mulaw *et al.*, 2013). Geetha *et al.* (2008) suggested that the presence of other opportunistic endophytic fungi may suppress the performance of *B. bassiana* and *B. brongniartii* (known as antagonism behaviour of opportunistic fungi towards entomopathogenic fungi). The inability of *Beauveria* spp. to colonize plants effectively has also been reported by Vega *et al.* (2008), and Conlong and Rutherford (2009).

It is possible that a number of other factors could have affected the establishment of *B. brongniartii* in sugarcane roots. Memela (2015) stated that sugarcane defence mechanisms could prevent colonization of fungal isolates other than sugarcane's own endophytic isolates. According to Ownley *et al.* (2010) plants induce defences such as induced systemic resistance (ISR) compounds which deter and repel herbivorous insects. However, this may be an

exception since *Fusarium* species, such as *F. verticillioides* (Sacc.) Nirenberg (Hypocreales: Nectriaceae) are capable of degrading the plant's defensive compounds (Hashimoto and Shudo, 1996; Richardson and Bacon, 1995; Memela, 2015).

Other factors may include inoculation methods used in the present study and inoculated sugarcane age. In the present study, the dip inoculation method used did not provide good results. A study by Posada *et al.* (2007) found that direct inoculation methods resulted in highest colonization of endophytic fungi in plants. Also, Memela (2015) showed that a direct injection method of *B. bassiana* yielded sufficient results in terms of establishment of *B. bassiana* as an endophyte of sugarcane plants. In 2009, Tefera and Vidal revealed that a dip inoculation method of *B. bassiana* into sorghum plants favoured leaf and stem colonization compared to root colonization; and further stated that the reason for low root colonization was still unclear. It is therefore assumed that *Beauveria* spp. favour specific plant tissues and may favour specific plant tissue conditions for efficient colonization (Tefera and Vidal, 2009; Akello *et al.*, 2007). Because there is currently no literature that has proven successful establishment of *B. brongniartii* as an endophyte of plants, it is suggested that future research should focus on evaluating virulent *B. bassiana* isolates, which establish more easily as endophytes of sugarcane, for long term protection of sugarcane roots against white grub species.

6.4 Impact of *Beauveria brongniartii* isolates against larvae of *Schizonycha affinis* in sugarcane pot trials, and the resultant sugarcane plant growth

White grubs continue to cause serious damage to sugarcane in the Midlands North of KwaZulu-Natal region. During white grub surveys conducted for this study, more than 5000 larvae were recovered under sugarcane stools in the surveyed areas. In South Africa, the potential of *B. brongniartii* to control white grubs has been established in the laboratory; but not in the field. There is thus a need to evaluate the performance of effective *B. brongniartii* isolates such as C17 and HHWG1 under open field conditions. Several studies (Keller *et al.*, 1999; Kessler *et al.*, 2004; Srikant and Santhalakshmi, 2004; Dolci *et al.*, 2006) have shown that soil application of *B. brongniartii* does reduce white grub populations in the soil. This study (Chapter 5) promisingly showed that the isolates C17 and HHWG1 were varyingly effective against *S. affinis* larvae in pot trials, under field conditions. The efficacy of these *B. brongniartii* isolates were however low during the first few days after application, compared to the insecticide treatment, which caused highest mortality of small and large larvae. Benker and Leuprecht (2005) and Kowalska (2008) showed that insecticides were highly effective within the first few

days post inoculations against white grub larvae, but *B. brongniartii* took longer to cause significant mortality. The HHWG1 isolate, in particular was the best treatment after the insecticide treatment. HHWG1 caused mortality of 60% of the second instar larvae of *S. affinis* in the soil drench inoculation method, compared to insecticide treatment which caused 100% mortality, 30 days after treatment. This moderate effectiveness of EPFs against pests has also been recorded in other studies. According to the literature *M. anisopliae* at 1×10^{13} spores/ha, was the best treatment after an insecticide (chlorpyrifos) in alleviating white grub numbers, but the application of this EPF increased sugarcane yield (Ramanujam *et al.*, 2014). The present study confirmed that *B. brongniartii* was less effective during the first few days of experimentation, but its efficacy improved over time, and caused significant mortality of larvae at 30 days after treatment. Hence, results revealed the likelihood of persistence of *B. brongniartii* for several days after treatment as was reported by Dolci *et al.* (2006); and also the results are consistent with findings of Easwaramoorthy *et al.* (2004) and Srikanth *et al.* (2010). Easwaramoorthy *et al.* (2004) showed that *B. brongniartii* at 1×10^{14} - 10^{16} spores/ha continuously affected white grub larvae of *Holotrichia serrata* for over 4 years under laboratory and pot-culture bioassays.

Chapter 5 also investigated the efficacy of inoculum application methods, namely a soil drench and root dip inoculation method, to control *S. affinis* larvae under pot trials. The results showed that a soil drench inoculation method was a better method compared to a root dip inoculation method. In the soil drench inoculation method, the effect of the isolates was recorded during the 9th day after treatment compared to the 18th day after treatment in the root dip inoculation method. HHWG1 caused 60% mortality against the second instar larvae of *S. affinis* in the soil drench inoculation method, compared to 40% mortality in the root dip inoculation method, 30 days after treatment. Monitoring *B. brongniartii* isolates over a longer period of time to investigate the persistence of the fungal isolates is critical. It is suggested that further evaluation of the soil application method needs to be completed to optimize the efficacy of the entomopathogenic fungus.

6.5 Studies on the impact of white grubs in sugarcane

Previous research by Goble (2012) emphasized the shortfall of information available on sugarcane yield losses caused by white grubs, and there is currently not much done to reduce their infestations. Goble (2012) stated that acceptance of *B. brongniartii* as a mycoinsecticide to control grubs in South Africa will depend on demonstrating cost benefits in its use. In the present study it was shown that a number of three larvae per pot were severely damaging to

roots of seedlings in the control, findings similar to that of Coale and Cherry (1989), working with *Ligyris* sp. on sugarcane. This indicates the impact that white grub larvae have on sugarcane and expected yield losses in white grub infested areas.

Beauveria brongniartii and insecticides have been used to reduce white grub infestations in sugarcane (Allsopp *et al.*, 1995; Benker and Leuprecht, 2005; Kowalska, 2008). The pot trials discussed in Chapter 5 demonstrated the potential of especially the HHWG1 isolate under open environmental conditions, and the impact of white grub larvae on sugarcane growth. The results showed the potential of *B. brongniartii* isolate HHWG1 and insecticide to reduce white grub infestations. It was observed that sugarcane seedling's biomass was heavily affected in the control pots compared to the HHWG1 and insecticide treated pots. This indicates that the HHWG1 and insecticides had, to some extent, provided protection against the larvae of *S. affinis*. Similar results were reported by Manisegaran *et al.* (2011) and Chelvi *et al.* (2011), where a known EPF, *M. anisopliae* at 4×10^9 conidia/ha, and 3×10^{12} conidia/ha protected the sugarcane against the larvae of white grub species, and as a result the sugarcane yield improved. Nonetheless, future research is needed to evaluate the impact of white grub larvae using replicated field trials and yield losses over a longer period (preferably up to harvest time). Evaluating the impact of white grub larvae over a long period will demonstrate the impact of white grubs on sucrose levels, which is the measure of sugarcane quality, and the findings will clearly demonstrate to the farmers the urgent need of finding a cost effective and practical control measure (Keller, 2000).

6.6 Conclusions

The results of this study highlighted important information about the host range of native *B. brongniartii* isolates in South Africa. Other white grub species were highly susceptible to the fungal isolates in the laboratory bioassays. In addition results showed the potential of fungal isolates to control white grub larvae under open environmental conditions. The general conclusions of this study are summarized below:

1. White grubs remain important pest species in sugarcane production in South Africa. Towards the development of a bio-insecticide to control white grub species, *B. brongniartii* isolates are promising agents of two damaging white grub pests of sugarcane, *P. sommeri* and *S. affinis* in South Africa. The indigenous *B. brongniartii* isolates, C17 and HHWG1, have been shown to have a wider host range. The isolates were as pathogenic against *T. clypeatus*, *H. tristis* and *S. neglecta* as they were against

the original host species, *P. sommeri* and *S. affinis*. This increased host range adds potential to the *B. brongniartii* isolates C17 and HHWG1 to be developed as potential bio-insecticides for white grub species in South Africa.

2. Attempts to establish *B. brongniartii* isolates C17 and HHWG1 as endophytes of sugarcane roots were made. However both isolates were not endophytic colonizers of sugarcane roots. Future research may however, investigate endophytic *B. bassiana* isolates for further long term protection of white grubs.
3. The pot application trial indicated the efficacy of *B. brongniartii* isolates against the larvae of *S. affinis*, under open environmental conditions, which is a promising first step in developing a bio-insecticide. It was shown that the HHWG1 isolate was comparable to the positive control (insecticide) at 21-30 days after treatment. These results increase the chances of pursuing the goal of developing the native *B. brongniartii* isolates as biological agents to control white grubs in South Africa.
4. White grub larvae cause severe damage to sugarcane which compromises the yield and quality of sugarcane. The impact of *S. affinis* larvae on sugarcane seedling dry weight was severe on the seedling sugarcane plants in the control, whereas HHWG1 and insecticide provided protection against the larvae of *S. affinis* in the pot trials. The results highlighted the importance of application methods, with the soil drenching inoculation method providing adequate results by reducing white grub larval numbers in the soil.

6.7 Concerns and specific future directions

Future research should investigate:

- i. Ways of preserving native *B. brongniartii* in the field to ensure that more formulated spores are available to conduct replicated field trials; and further field application methods of viable *B. brongniartii* isolates against white grubs should be investigated.
- ii. Ways of preserving native *B. brongniartii* as formulated products, and the factors that affect the product storage of formulated *B. brongniartii*
- iii. *Beauveria brongniartii* isolates were not tested in replicated field trials, at the known white grub hotspots in South Africa. The reason behind this was because formulated *B. brongniartii* isolates were losing their viability. Additional efforts must be done to produce formulated *B. brongniartii* isolates, which is a critical step for the successful

development of the fungal inoculum for field conditions (Easwaramoorthy *et al.*, 2002; Horaczek and Viernstein 2004; Tamizharasi *et al.*, 2005). It is therefore essential to establish methods for the preservation of *B. brongniartii*, so that it can be tested in the field trials.

- iv. The efficacy of *B. brongniartii* isolates yielded adequate results in the laboratory bioassays, and results convincingly showed that isolates have a larger host range. It is however, known that laboratory conditions generally enhance the effectiveness of EPFs, since the laboratory conditions such as temperature, moisture, and pest insects of interest are monitored in the laboratory. Although, a soil drench inoculation method that was used in this study showed some efficacy of regulating white grub larvae under the sugarcane seedling stool, which further shows the promising future of the development of *B. brongniartii* as a bio-insecticide for regulating white grubs, future studies are required to evaluate the efficacy and establishment of the isolates in soils, by looking at the short and long term impacts of regulating white grub population densities.

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