Isolation of endophytic *Beauveria bassiana* and investigation of interactions between *Fusarium* spp., *Eldana saccharina* and the sugarcane plant for dual biological control

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PREFACE

The experimental work described in this dissertation was carried out in two departments: 1) the Plant Pathology Department of the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, Durban, SA, under the supervision of Rd. Stuart Rutherford (SASRI; Honorary Lecturer School of Life Sciences, University of KwaZulu-Natal) and Prof. rd. Stefan Schmidt (Discipline of Microbiology, University of KwaZulu-Natal). 2) The Agricultural Entomology Department at the University of Göttingen is under the supervision of Prof. Dr. Stefan Vidal (Chapters 4 and 5). The research was financially supported by the South African Sugarcane Research Institute (SASRI), the National Research Foundation (NRF), and INSPIRE: Erasmus Mundus (PhD mobility in Germany).

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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N.S. Memela
Date: December 2023

As the candidate's supervisor I have approved this dissertation for submission

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Supervisor: Professor S. Schmidt

Date: December 2023

……

Co-Supervisor: DR. R.S. Rutherford

Date: December 2023

DECLARATION 1 – PLAGIARISM

I, **Nongcebo Sandra Memela**, declare that:

1. The research reported in this thesis, except where otherwise indicated, is my own and has been generated by me as a result of my own original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other persons.

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5. Where other written sources have been quoted, then:

    a. Their words have been re-written, but the general information attributed to them has been referenced.

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N.S. Memela

December 2023

DECLARATION 2: PUBLICATIONS AND CONFERENCES

Short non-refereed paper: Memela, N.S., Rutherford, R.S., Schmidt, S. (2021). Plant-endophyte-pest interactions: Investigating the biological control of *E. saccharina* and stem rot disease in sugarcane. *Proc S Afr Sug Technol Ass*., **93:** 139-144.

Poster presentation: Memela, N.S.; Rutherford, R.S.; Schmidt, S.; Vidal, S., Conlong, D.E. Inoculation of *Beauveria bassiana* into sugarcane plants for endophytic control of *Eldana saccharina*. **Conference:** International Symposium, Microbe-assisted crop production – opportunities, challenges & needs; 4-7 December 2017; Vienna, Austria.

Poster presentation: Memela, N.S.; Rutherford, R.S.; Schmidt, S.; Conlong, D.E. In an effort to control the South African sugarcane stem borer *Eldana saccharina*. **Conference:** Deutsche Gesellschaft für allgemeine und angewandte Entomologie (DGaaE) Entomologentagung; 13-16 March 2017; Freising, Germany.

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NS Memela

December 2023

ABSTRACT

The South African Sugarcane Research Institute is exploring the use of the fungal entomopathogen *Beauveria bassiana* to control the borer pest *Eldana saccharina*, a major threat to the South African sugar industry. This study aimed to understand the natural occurrence of *B. bassiana* in various sugarcane genotypes and host plants of *E. saccharina*, and its interactions with the pest and a common plant co-occurring pathogen, *Fusarium* spp. which is beneficial to the pest. Therefore, this study aimed to survey the natural occurrence of *B. bassiana* as an endophyte in 28 sugarcane genotypes and *E. saccharina* host plants, to identify isolates that are virulent and can be used as endophytes in biological control programs to control the boring stages of *E. saccharina* and to explore the interaction of isolated *B. bassiana* isolates with *Fusarium* spp. Furthermore, this study investigated the persistence of *B. bassiana* as an endophyte in sugarcane plants and the ability of the endophytic association to reduce *E. saccharina* feeding. In this study, 128 fungal isolates morphologically characterized as *Beauveria* spp. were isolated as endophytes from internal plant tissues following the sampling of 326 plants collected from five locations in sugarcane-producing areas (KwaZulu-Natal, South Africa). Analysis of the nuclear ribosomal internal transcribed spacer (ITS) region confirmed the identity of 16 representative isolates as *B. bassiana* s.l., with >98% sequence similarity to reference strains. For the first time, 120 endophytic *Beauveria* spp. isolates were obtained from 22 sugarcane genotypes and eight from *E. saccharina* host plants. Sixteen *B. bassiana* isolates were repellent towards *E. saccharina* first instar larvae during larval choice assays, detrimental towards *E. saccharina* development when *B. bassiana* isolates were incorporated into *E. saccharina* artificial diets and virulent towards 3rd and 5th instar larval stages of *E. saccharina* during topical conidia spray bioassays. Interactions of two isolated *B. bassiana* with four *Fusarium* spp. strains revealed that both genera can reduce and limit the other's growth when grown with culture filtrates or in Petri dish co-culture assays. Artificial inoculated *B. bassiana* isolates endophytically colonized sugarcane plants. For the first time, this study revealed that endophytic colonization can persist from 1 to 6 months in sugarcane with colonies resembling *Beauveria* spp. morphology re-isolated using selective agar. Endophytic colonization persistence decreased with an increase in time post inoculation, was found to be variety dependent and influenced by the inoculation method, plant pre-treatment, and fungal strain used. Sugarcane plants inoculation with *B. bassiana* TL-leaf strain via conidia spray inoculation, weighed significantly more than control plants 3 months post inoculation, with no statistical significance in plant height when compared to control plants. When *E. saccharina* larvae were allowed to feed on plants 16 months post *B. bassiana* inoculation, no statistically significant differences were observed within control and *B. bassiana* plants in reducing boring length and larval weights.

Furthermore, no *B. bassiana* colonies were re-isolated 16 months post inoculation into sugarcane. The results of this study demonstrated the abundance of *B. bassiana* as an endophyte within plant species in the sugarcane ecosystem. It highlighted the importance of conducting bioassays when selecting biological control strains, as levels of repellence and virulence differ amongst *B. bassiana* strains. Additionally, the study revealed that using an appropriate *B. bassiana* biocontrol strain and artificial endophytic colonization of sugarcane is achievable in different sugarcane varieties. Moreover, results accentuate the importance of determining persistence of artificially inoculated *B. bassiana* strains as an endophyte in the plant ensuring *B. bassiana* is present as an endophyte in the plant when the target pest feeds.

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This research study could not have been accomplished if not for the unfailing mercies of the Most High God. So many people have contributed to the completion of this research project; they have shared my challenges and my celebrations and have imparted on me valuable knowledge and skills that I will use both in life and in my career. I would like to express my utmost gratitude to the following people:

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Lisakhanya ikusasa
"The future is still
bright".

TABLE OF CONTENT

This thesis represents a compilation of different manuscript styles using different reference styles for the intended journal publications, where each chapter is an individual entity, therefore, some repetition between chapters has been unavoidable.

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CHAPTER 1

Literature review

1.1 Sugarcane

Sugarcane (*Saccharum* Linnaeus spp. hybrids) (*Poaceae*) is a tropical and sub-tropical perennial grass crop (Raza *et al*., 2021; Thibane *et al*., 2023), grown in over 200 countries (Raza *et al*., 2021). It is mainly cultivated for the commercial production of cane sugar (sucrose) which accumulates in its matured stalks (Raza *et al*., 2021; Wani, *et al*., 2023). By products generated during sugar manufacturing are used by many industries. In addition, they have potential for energy and biofuel production such as, molasses-based ethanol production, cattle feed and supply of industrial enzymes (Budeguer *et al*., 2021; Kurmi *et al*., 2022; Singels *et al*., 2023). The *Saccharum* genus is comprised of two wild species of *S. spontaneum* L and *S. robustum* Brandes and Jeswiet and four domesticated species *S. edule, S. barberi*, *S. sinense* and *S. officinarum* (Bakker, 1999; Xiong *et al*., 2022). According to Bakker (1999) the latter was amongst the first form of "chewing cane" as it produced sweet soft tasting cane. *Saccharum officinarum* L. was thus initially grown as a domestic garden crop in the New Guinea area and associated islands of the Malayan archipelago, which are considered to be the center of diversity of these "noble" canes (Figure 1.1) (Blackburn, 1984; Rutherford, 1993; Mirajkar *et al*., 2019).

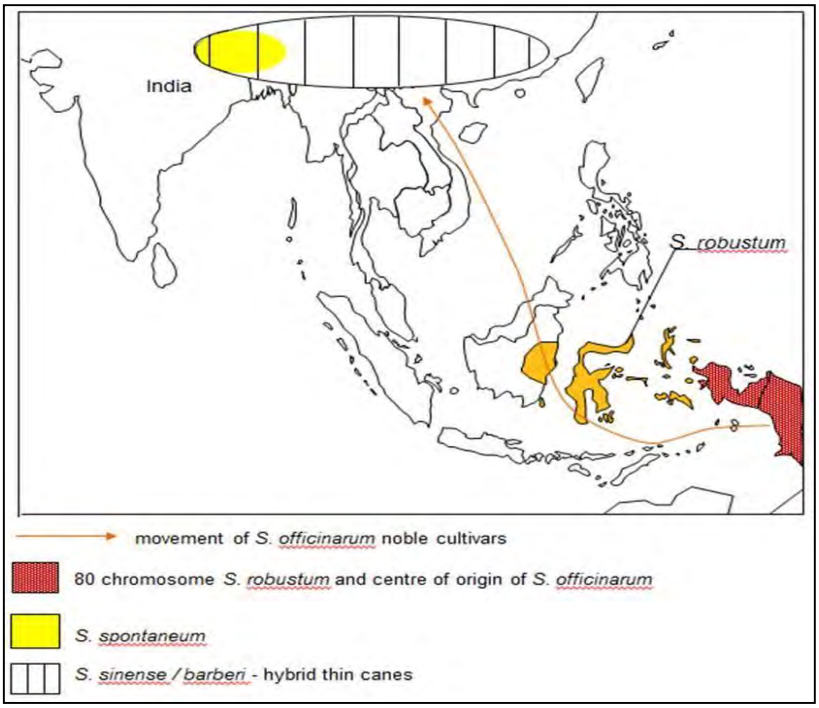

Figure 1.1: Centers of diversity for the sugarcanes, *Saccharum* spp. (Rutherford, 1993).

Genetically, *S. officinarum L.* is believed to have been derived from *S. robustum* Brandes and Jeswiet (ex Grassl) (2n=80) through chance mutations, while *S. robustum* has been suggested to have resulted from complex introgressions amongst *Saccharum spontaneum* L. and *Miscanthus floridulus* (Labill) (Grivet *et al*., 2004; Mirajkar *et al*., 2019).

Modern sugarcane varieties are highly genetically complex polyploid hybrids (Budeguer *et al*., 2021; Xiong *et al*., 2022**)** derived from the initial interspecific hybridization of *S. spontaneum* clones (Glagah (2n=112) with 'noble' sweet canes of *S. officinarum,* about 100 years ago in Indonesia (Java). Figure 1.2 shows the 'nobilization' of the clone Glagah. The production of hybrids is considered desirable since *S. spontaneum* clones have many adaptive traits, such as drought, flood, high salinity, insect pest and disease resistances, which are useful in breeding programs (Roach, 1986; Zhou *et al*., 2013; Yadav *et al*., 2020). *Saccharum* species hybrids derived from such crosses have since been distributed around the globe.

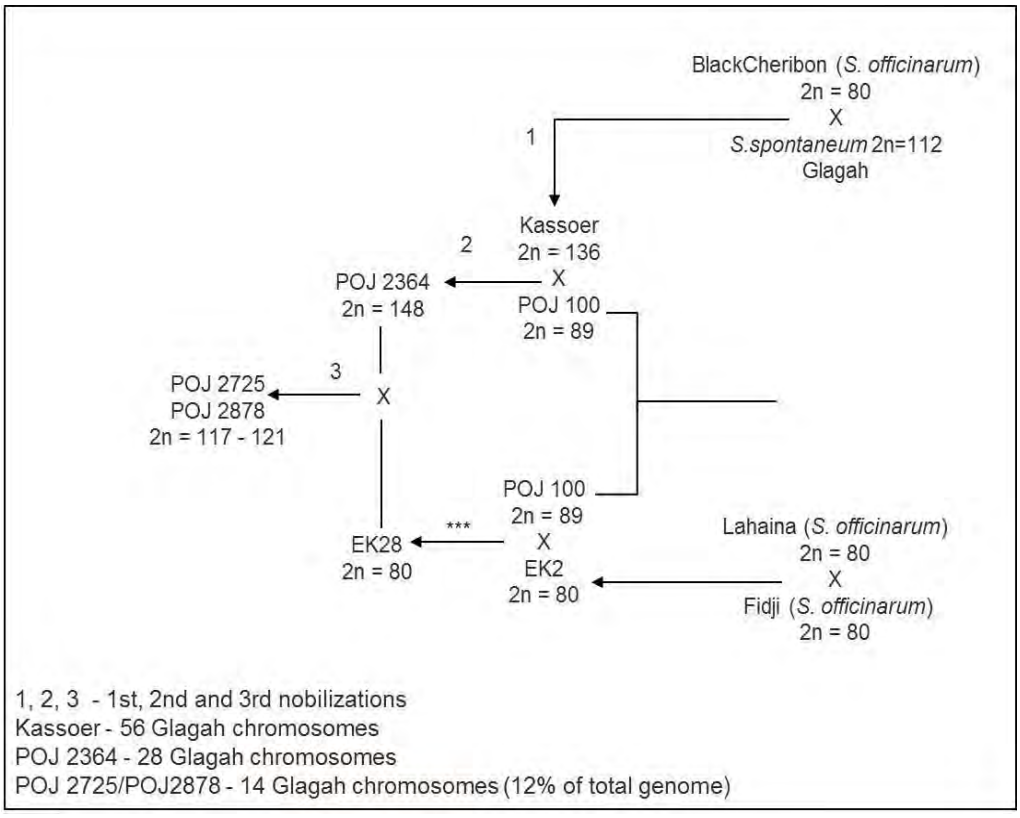

Figure 1.2: The Java nobilization of *S. spontaneum* clone Glagah (Roach, 1972).

In South Africa, the sugar industry generates an annual direct income of R18 billion per annum and contributes substantially to the national economy of the country, sustainable development, and the employment (SASA, 2023). Sugarcane cultivation began in 1847 in Durban after being imported from Mauritius (Lewis, 1990). Today, commercial sugarcane plantations extend from

Mpumalanga, KwaZulu-Natal (KZN) to the Eastern Cape provinces (covering approximately 362,824 hectares of land) (Zhou, 2022). The growth cycle of sugarcane is dependent on the geographic location it is cultivated, with the average harvest age currently 13 months on the coast and 16 months in the hinterland (Rutherford, 2015).

Sugarcane cultivation is, however, faced with several challenges which negatively affect its productivity, thus compromising, plant growth, sucrose yields, quality and profit gained from sugarcane in South Africa and worldwide (Rutherford, 2015; Yadav *et al*., 2020). Sugarcane health and maturity is influenced by factors such as climatic conditions, soil fertility, water supply and pest and disease occurrence (Blackburn, 1984; Yadav et al., 2020). Variety breeding programmes in South Africa have developed several commercially available sugarcane hybrids such as “NCo”, and the more recent “N” varieties, which display better adaptations to disease, pest and various agro- climatic environments (Zhou, 2022). However, many insect species belonging to Orders Coleoptera, Homoptera, Hemiptera, Isoptera, Lepidoptera and Orthoptera are still a problem in the South African sugar industry, they feed on sugarcane, and some cause significant damage either as leaf, stalk or root feeders (Leslie, 2004; Goble *et al*., 2017). Stalk borers including larvae of Lepidoptera and some Coleoptera are often the most damaging pests of sugarcane in most countries growing this crop (Muniappan *et al*., 2004; Goble *et al*., 2017). Amongst the many stalk borers *Eldana saccharina* Walker (Lepidoptera: Pyralidae), *Busseola fusca* Fuller (Lepidoptera: Noctuidae), *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae), *Chilo orichalcociliellus* Strand (Lepidoptera: Pyralidae) and *Chilo partellus* Swinhoe (Lepidoptera: Crambidae) have been reported as important stalk borers of graminaceous crops in Africa (Conlong, 2001; Haile and Hofsvang, 2001; Bancole *et al*., 2020; Péné *et al*., 2020). In South Africa, the stalk borer *E. saccharina* is currently causing economically significant crop losses in the production of sugarcane (Goebel and Sallam, 2011; Zhou, 2022).

1.2 The pest *Eldana saccharina*

1.2.1 History and distribution

Eldana saccharina is indigenous to Africa with its first description in Sierra Leone in 1865 from sugarcane (Rutherford, 2015). It has since been discovered in most of the tropical and subtropical continental regions of Africa, occurring throughout sub-Saharan Africa, East Africa (e.g.: Uganda), West Africa (e.g.: Ivory Coast) (Péné *et al*., 2020) Southeast Africa (e.g.: Mozambique) and in South Africa feeding on economically important crops such as maize, sorghum and sugarcane (Atkinson 1979; Conlong, 2001; Assefa *et al*., 2008; Péné *et al*., 2020).

Although known as “The sugarcane stalk borer” or the “African sugarcane stem borer”, *E. saccharina’*s natural host plants are the wetland sedges in the family Cyperaceae (*Cyperus papyrus* L., *Cyperus dives* L. and *Cyperus fastigiatus* L.) and wild grasses in the family Graminaceae (S*orghum bicolor* L., *Coix lacryma-jobi* L.*, Panicum maximum* L.), Typhaceae (*Typha latifolia* L.) and Juncaceae (Atkinson, 1979; Atkinson and Carnegie, 1989; Rutherford, 1993; Conlong, 2001; Leslie, 2004).

Eldana saccharina was first observed as a sugarcane pest in South Africa after two outbreaks, the first occurring in the Umfolozi area in 1939 and the second occurring in 1970 at the Hluhluwe area (Atkinson, 1980; van Vuuren *et al*., 2018). Thereafter, *E. saccharina* spread both north and south of Hluhluwe, becoming a pest in Mpumalanga, Pongola, Swaziland and in the lower altitudes of KZN (Figure 1.3) (Atkinson, 1979). The distribution of stem borers is reported to be dependent on altitude, temperature, rainfall, and availability of food (Haile and Hofsvang, 2001). The spread of *E. saccharina* was therefore, thought to be limited by the low winter temperatures in the southern areas of the sugarcane belt (Atkinson, 1979). However, in recent years, *E. saccharina* has rapidly spread being observed further south, towards the higher altitudes, and observed in colder climates (Midlands North and South) extending to the Limpopo province in the north and Mkambati Nature Reserve in the Southeastern Cape, with new limits of *E. saccharina* observed at the Boskop dam in the North-West province (Rutherford, 2015; Kleynhans *et al*., 2018).

It is hypothesized that the removal of *E. saccharina* natural host plants (sedges and grasses) and the subsequent planting of sugarcane as a monoculture crop in these wetland areas may have led to *E. saccharina* altering hosts, feeding on the available sugarcane plants (Conlong, 1994; Conlong, 2001; Rutherford, 2015). Several factors such as (i) the increased use of nitrogenous fertilizer during sugarcane cultivation, (ii) planting of susceptible sugarcane varieties, (iii) water stressed sugarcane, (iv) planting older sugarcane plants (13 months or older), and (v) the cryptic oviposition sites of *E. saccharina* further contributed to the establishment of *E. saccharina* as a sugarcane pest (Atkinson and Nuss, 1989; Rutherford, 1993; Leslie, 2004; Keeping *et al*., 2014; Rutherford, 2015).

Figure 1.3: The history of *Eldana saccharina* outbreaks in the sugarcane growing regions of South Africa (Rutherford, 2015).

1.2.2 Biology

Eldana saccharina is holometabolous, metamorphosing through egg, larval, pupal and adult moth developmental stages (Hossler, 2010). Time spent at each stage of the life cycle is variable and depends on food quality and temperature experienced during growth (Leslie, 2004). *Eldana saccharina* breeds continuously throughout the year, however seasonal moth peaks are typically observed in April and November. It takes approximately 1.5 to 2.5 months for *E. saccharina* to complete a life cycle (Figure 1.4) (Rutherford, 2015; Kleynhans *et al*., 2018).

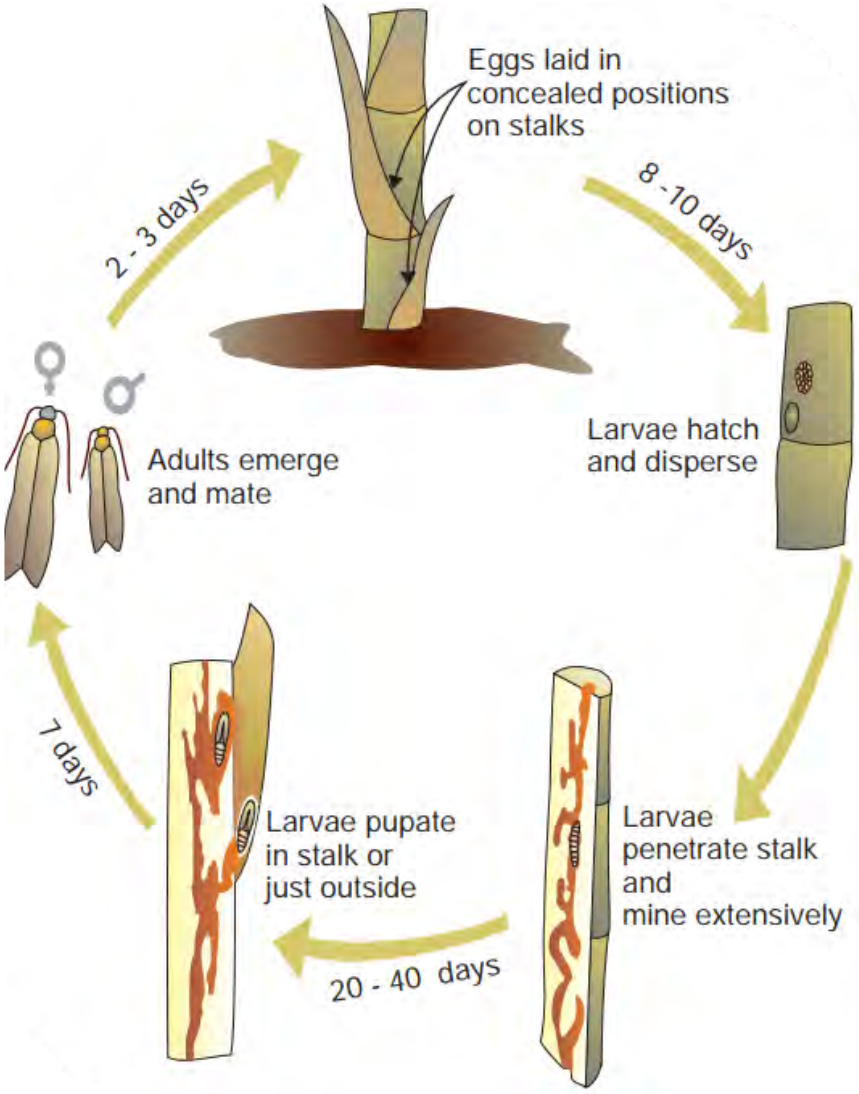

Figure 1.4: Schematic life cycle of *Eldana saccharina* in sugarcane (Rutherford, 2015).

The non-feeding adult stage (Figure 1.4 and Figure 1.5A) of *E. saccharina* is nocturnal, living on average seven days. Females are typically larger in size than males and mating occur predominately on the first and second night after males' emergence (Walton and Conlong, 2016). Walton and Conlong (2016) reported that females lay a maximum of 798 eggs. The females lay their eggs in different places in different crops. For instance, in sugarcane *E. saccharina* females lay their eggs in clumps on lower parts of the sugarcane plant on concealed dry/dead or senescent leaf sheaths (Leslie, 2004), while in maize *E. saccharina* females lay eggs on plant debris on soil or below the cobs in larger maize (Ndemah *et al*., 2000; Atachi *et al*., 2005). When laid, the eggs are white but become pink to orange as the neonates develop in them (Figure 1.5B), and then blackish before hatching of neonate larvae. Larvae take between eight to ten days to hatch depending on temperature conditions (Figure 1.4) (Rutherford, 2015).

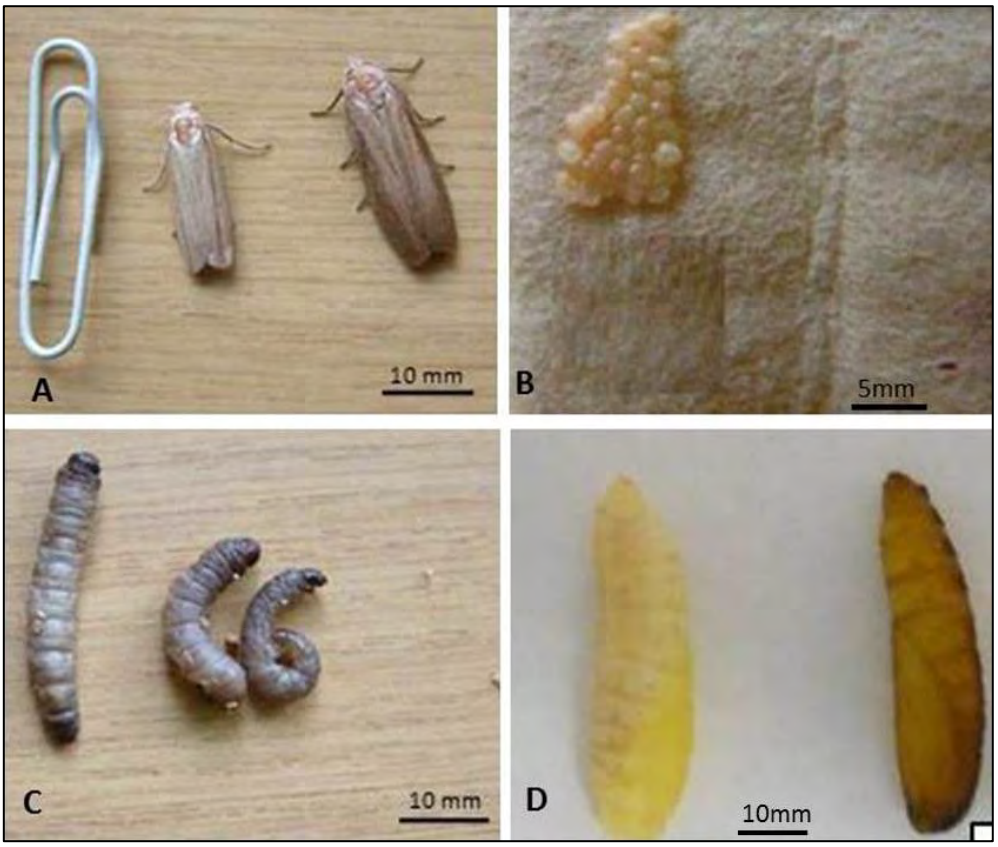

Figure 1.5: *Eldana saccharina* developmental stages: (A) Adult moth, (B) Yellow oval eggs typically laid in a clump, (C) larval stages, (D) Pupae stage, freshly pupated on the right.

Eldana saccharina larvae are brown/grey in colour (Figure 1.5C) and undergo six or seven moults before pupation occurs (Leslie, 2004). Different larval stages feed on different parts of the plants. In sedges (Cyperaceae) and grasses the smaller instars larvae feed on the soft green umbel rays (Conlong, 2001), the inflorescences, unsubmerged rhizome and on surfaces of the stalk (Atkinson, 1979) while, the larger larvae bore into the stem and growing regions on the rhizome and complete their development in this hidden environment (Conlong, 2001). In sugarcane the small instar larvae (1st instar) scavenge on surfaces of the middle and lower parts of mature stalks, feeding initially on decaying leaf matter and the softer parts of the stems (Conlong, 2001). At 2nd and 3rd instar stage, larvae begin to feed on the stem, boring and penetrating the lower parts of the stem via the bud, root band and cracks. Once inside the stem the larvae feed on the soft internal tissues of sugarcane taking 20-60 days to complete this developmental stage. Larvae may cross nodal plates of several internodes during development inside the sugarcane stem (Leslie, 2004). Thereafter the larvae create a moth exit hole on the stem, spin a protective silk cocoon and pupate. Pupation occurs behind leaf sheaths or just within the hollowed stem (Leslie, 2004). The pupa changes from yellow-brown hardening into a red-brown appearance (Figure 1.5D), from which adults emerge. Depending on temperature

and other environmental factors the non-feeding pupal stage can last two to three weeks before adult emergence occurs (Leslie, 2004; Kleynhans *et al.*, 2018). Upon adult emergence from pupae, the life cycle begins again.

1.2.3 Sugarcane damage and economic implications

Eldana saccharina larvae borers sugarcane of all ages, however, the most serious damage occurs typically in mature carry-over cane of 13 months or older (Rutherford, 2015). *Eldana saccharina* bores and tunnels mainly in the lower third of the sugarcane stem (bottom node and internode), however, can cross internodes, reducing sucrose quality and crop productivity (Leslie, 2004; Goble *et al.*, 2017). Up to 14 *E. saccharina* have been reordered per sugarcane internode (Rutherford, 2015). “Frass Spuren” (~feeding waste) can be observed on the outside of the stalk, which is used as an indicator of larval boring (Kasl, 2004) (Figure 1.6B). Internal feeding of *E. saccharina* causes secondary infections from *Fusarium* spp. and the soft rot fungus *Glomerella tucumanensis* (Speg.) (Arx & Müller.), which convert sucrose into glucose, turning the white sugarcane tissues (Figure 1.6C) into a red discoloration (Figure 1.6D). This discoloration reduces sucrose quality observed during sugarcane milling. It is estimated that for every one *E. saccharina* larvae per 100 stalks, there is a loss of 0.5-ton cane/hectare (Rutherford, 2015).

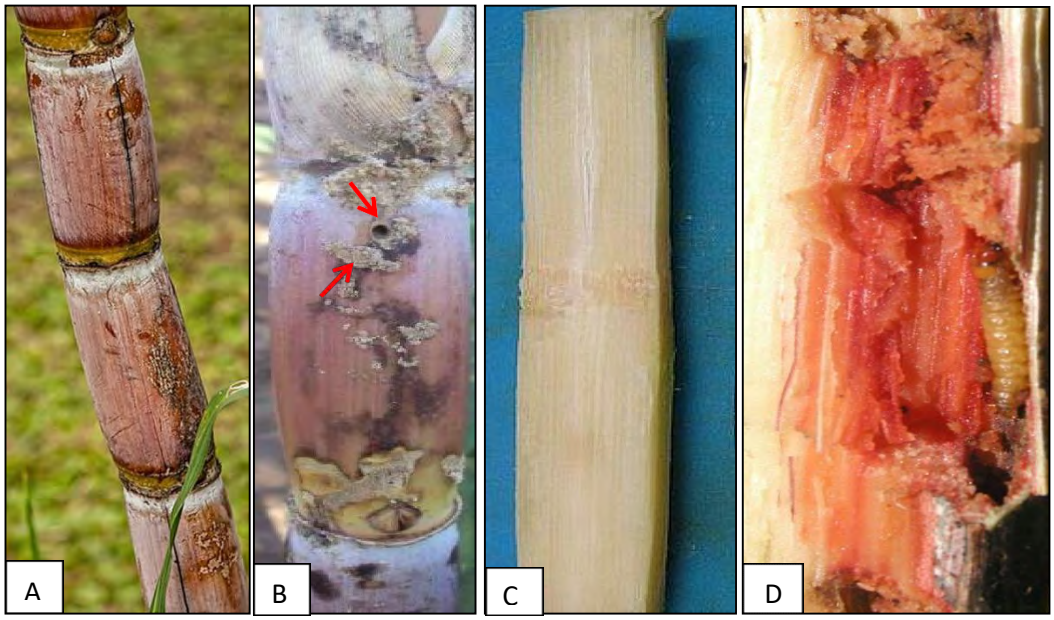

Figure 1.6: Illustration of healthy sugarcane plant tissues in comparison to damage symptoms resulting from *E. saccharina* boring on sugarcane: (A) healthy undamaged sugarcane stem; (B) *E. saccharina* boring holes and brown frass (red arrows) on the sugarcane stem; (C) cross section of undamaged stem; (D) cross section showing red discoloration of internal stem tissues resulting from co-infections by *Fusarium* spp. and *Glomerella tucumanensis.*

Revenue losses in sugar production due to *E. saccharina* have been increasing since its establishment as a sugarcane pest. The annual average losses in sugarcane due to *E. saccharina* damage during the drought years of 1980 to 1986 were estimated at R6 million (Rutherford, 1993). In 1995, Keeping reported annual losses estimated at R50 million per annum. In 2002, Butterfield reported sugarcane losses due to *E. saccharina* ranging between R97 and R150 million during the 2001/2002 milling season. Currently losses due to *E. saccharina* in South Africa are estimated at US$62 million per annum (Zhou, 2022). These data alone show the devastating impact that *E. saccharina* has had in the South African sugar industry. Due to these economic losses, extensive research has been conducted on *E. saccharina* by SASRI since 1974 to understand the ecology of the pest and establish management practices to control the pest (Assefa *et al*., 2006).

1.2.4 *Eldana saccharina- Fusarium* spp. association

The reddening of sugarcane tissues that proliferates losses due to *E. saccharina* borings is largely resultant from infections of the damaged tissue by various *Fusarium* species (McFarlane *et al*., 2009; Mahlanza, 2015; Rutherford *et al*., 2020). *Fusarium* is a genus of filamentous fungi belonging to the order Hypocreales and phylum *Ascomycota* (Pelizza *et al*., 2011; Nikitin *et al*., 2023). Species in this genus form associations with soil, plants, and insects. In soil, *Fusarium* is known to exist as a saprophyte and opportunistic insect-pathogen being isolated from dead insects (Sharma and Marques, 2018). In plants, *Fusarium* spp. form complex associations and are notorious as phytopathogens and recently also commonly isolated as symptomless endophytes (Rodriguez *et al*., 2012; Thio *et al*., 2021; Kisaakye *et al*., 2022). For instance, *Fusarium verticillioides* (Sacc.) Nirenberg (1976) (teleomorph *Gibberella moniliformis*) has been associated with “pokkah boeng” infections in sugarcane (McFarlane *et al*., 2009) and ear rot in maize plants (Gai *et al*., 2018) and is a known pathogen of rice (*Oryza sativa* L.) and sorghum (*Sorghum bicolor* L.). However, some *F. verticillioides* strains are reported to be able to colonize maize plants and exist as symptomless endophytes, causing no apparent infections (Bacon *et al*., 2008; Bennett *et al*., 2023), these strains have been shown to have the ability to cause limited cellular disruption due to their ability to catabolize plant secondary metabolites such as benzoxazinoids before these compounds are converted into their bioactive (i.e., aglycones) deferring forms (Hashimoto and Shudo, 1996; Saunders *et al*., 2010).

Moreover, *Fusarium* spp. have been shown to attract insects, benefit insect feeding, development and survival (Ako *et al*., 2003; McFarlane *et al*., 2009; Blacutt *et al*., 2018; Franco *et al*., 2021). The association of *Fusarium* spp. with insects is well documented (Bosque-Perez

and Mareck, 1991; Schulthess *et al*., 2002; Ako *et al*., 2003; Vernard and Vaillancourt, 2007; McFarlane *et al.*, 2009; Sharma and Marques, 2018; Rutherford *et al*., 2020) with plant feeding insects able to co-exist with *Fusarium* spp. and form mutualistic associations. The insects boring on plant tissues provides an entry point for the pathogen and in turn the microorganism provides ergosterols (Clayton,1964; McFarlane *et al*., 2009), essential for normal insect development and fecundity, as insects are unable to synthesis esterols. Sharma and Marques (2018) reported that *Fusarium* strains (*Fusarium solani* species complex), can act as insect mutualists supporting insect growth and fecundity. Vernard and Vaillancourt (2007) reported on the association of European corn-borer and *Colletotrichum graminicola* (Ces.) (the causal agent of maize anthracnose), that although the fungus can cause infection without insect wound damage, the infection is not as efficient as infection via wounds. Similarly, infections by the fungal phytopathogen *F. verticillioides* occur in association with *Diatraea saccharalis* (F.) caterpillars in sugarcane (Franco *et al*., 2021), demonstrating the complexity of microbial-plant and insect interactions.

Fungi-insect association has been shown to proliferate insect infestation. *Sesamia calamistis* (Lepidoptera: Pyralidae), *E. saccharina* and *Mussidia nigrivenella* Ragonot (Lepidoptera: Pyralidae) the maize ear borer were found to survive longer and had lower mortality on maize plants inoculated with *F*. *verticillioides* (Schulthess *et al*., 2002). A study by McFarlane *et al.* (2009) reported *Fusarium* isolates *F. proliferatum* and *F. pseudonygamai* (closely related to *F. verticillioides)* recovered from sugarcane to significantly improve development and survival of *E. saccharina* larvae during *in vitro* assays. While Ako *et al*. (2003) reported fourfold increases in the number of eggs laid by *E. saccharina* females when maize was infected by *F. verticillioides*. Bosque-Perez and Mareck (1991) reported that *E. saccharina* infestations significantly increased the severity of stalk rot on maize. Furthermore, insects in the family Nitidulidae have been shown to prefer feeding in plants infected with *Fusarium*, as *Fusarium* spp. produce a large array of volatile compounds such as namely, alcohols, esters and aldehydes, which are attractive to these insects (Bartelt and Wicklow, 1999; McFarlane *et al.*, 2009).

Although some *Fusarium* spp. form beneficial associations with *E. saccharina,* some strains have been reported as entomopathogens having the ability to infect insects and defer insect feeding (McFarlane *et al*., 2009; Pelizza *et al*., 2011; Sharma and Marques, 2018). Some strains of *F. sacchari* form antagonistic relationships with *E. saccharina* (McFarlane *et al*., 2009). In addition, *Fusarium* spp. are known to produce an array of mycotoxins and secondary metabolites, such as moniliformin, fumonisins, fusaric acid, beauvericin and fusaproliferin (Ferrigo *et al*., 2016), with the latter two known to have insecticidal properties. Although these

mycotoxins are produced in varying degrees by different species, *Fusarium* spp. lacking the cancerogenic fumonisins are seen as potential biocontrol agents (Bennett *et al*., 2023). The beneficial and antagonistic interactions between *E. saccharina* and microorganisms is thus important to consider when developing control strategies, especially in biological control.

1.2.5 Current *E. saccharina* control methods and their limitations

Similar to most lepidoptera pests *E. saccharina* control in sugarcane is difficult due to the pests' cryptic life cycle (Conlong, 1994; Péné *et al*., 2016; Goble *et al*., 2017). Research into controlling *E. saccharina* has thus been conducted around an Integrated Pest Management (IPM) approach described by Conlong and Rutherford (2009) as a "knowledge- based" farming approach that integrates different methods that can reduce pest levels in crops, requiring knowledge of the identity and biology of the pest. These strategies incorporate cultural, chemical, variety resistant and biological control methods collectively aimed at restricting *E. saccharina* damage to minimal levels such that profitability from sugarcane is maintained. Furthermore, reduction of risks to human health and the environment is also considered (Rutherford, 2015).

1.2.5.1 Cultural control

Cultural control is defined as a deliberate man-made alteration of crop production systems to reduce the prevalence of pest damage and pest numbers. It involves employing agronomic practices such as planting regulations, crop rotation and altering harvesting dates to control a pest (Bosque-Pérez, 1995; Leslie, 2004). Although the ultimate solution to combating *E. saccharina* has not been found, research into habitat, soil and crop management conducted at SASRI over the years has led to methods which have shown some degrees of efficiency in reducing *E. saccharina* infestation and damage (Zhou and Mokwele, 2015). Several agronomic practices have been put in place, such as reducing the use of nitrogen fertilizers (Rutherford, 2022), sugarcane pre-trashing to remove *E. saccharina* oviposition and early harvesting of sugarcane to reduce incidence of *E. saccharina* on older sugarcane plants (Keeping *et al*., 2014; Rutherford, 2015). Although early harvesting reduces *E. saccharina* incidence in older sugarcane, this practice reduces the potential sucrose production by sugarcane. Research has shown that the optimal physiological and economic harvest age of sugarcane is obtained at 15-22 months in coastal and inland regions (Ramburan, 2015). Thus, early harvesting contributes to the indirect economic losses in the South African sugar industry experienced from *E. saccharina* damage (Keeping *et al*., 2014; Rutherford, 2015).

1.2.5.2 Chemical control

Chemical control is defined as the use of chemical insecticides to kill and/or reduce pests or their activity (Bosque-Pérez, 1995). This control method has been used worldwide to control pests, however due to the negative environmental impact resulting from insecticides, their use has been limited (Pathak *et al*., 2022). Moreover, the use of chemical insecticides in ultimately controlling *E. saccharina* has been difficult because larvae are only exposed to the environment for short periods after hatching and before they bore into the stem (Leslie and Keeping, 1996). Insecticides containing alpha-cypermethrin as the active ingredient were effective in limiting severe incidence of *E. saccharina* in carry-over sugarcane (Rutherford, 2015). Recently, insecticides such as lamda-cyhalothrin (pyrethroid), chlorantaniliprole (diamide), cyantraniliprole (diamide) and indoxacarb (oxadiazine) have been registered against *E. saccharina* (Anon, 2023). The potential of timed insecticide application during moth peaks, which result in subsequent neonate larval peak, has been investigated as a control option for *E. saccharina* (Leslie, 2004). However, this would require repeated applications of insecticides for effective control, due to the multivoltine behavior of *E. saccharina.* Therefore, a control approach which is not dependent on the sole use of chemical insecticides but integrates their use with other strategies would offer a more environmentally friendly, sustainable control system.

1.2.5.3 Varietal resistance control

Varietal resistance control is based on the inherent resistance that a plant has towards insect pests. It enables the plant to avoid, minimize, tolerate or recover from injuries caused by insects (Bosque-Pérez, 1995). Several sugarcane varieties are commercially available and have been developed through breeding programmes, to better suit the agro-climatic requirements in the various sugarcane growing areas of South Africa. Furthermore, these varieties are bred for improved pest and disease resistance and the production of superior sucrose (Zhou, 2022). Resistant variety breeding programmes in SASRI are currently the most promising control method for *E. saccharina*. Breeding for *E. saccharina* resistance involves crossing parents originating from replicated testing stages, introgression breeding and genotypes imported from other countries and maintained as a germplasm collection. These parents have high genetic and breeding values, known to possess high levels of resistance to produce selection populations which are then tested (Zhou, 2015; Zhou, 2022). They are aimed at developing *E. saccharina* resistant sugarcane varieties (having a hard external stalk making it hard for larval boring) while also maintaining high sucrose yields, and adaptability of varieties to the major agro-climatic environments of the sugarcane producing areas (Keeping, 1995; Zhou, 2022).

All commonly grown sugarcane varieties in South Africa have some degree of susceptibility to *E. saccharina* attack, with newer varieties showing higher degrees of resistance such as N21, N33, N70, and intermediate resistant varieties such as N12, N41 have been released to replace the susceptible NCo376 variety initially colonized by *E. saccharina* (Ramburan, 2014, Zhou 2022). Resistant varieties are continuously being developed in the breeding screening programs (Zhou, 2022). These varieties show resistance and intermediate resistance to *E. saccharina* damage and pathogen infections such as smut. The development of improved varieties is however a lengthy process taking on average 10 to 15 years of testing, screening and selection before varieties are commercially released with up to three new sugarcane varieties (N varieties) released each year (SASRI, 2022). Therefore, this control approach is also faced with limitations, as the current pest status of *E. saccharina* requires prompt control.

1.2.5.4 Biological control

Biological control is defined as the use of living organisms to reduce plant diseases, weeds and pest populations, to reduce crop losses (Stenberg *et al*., 2021). Three strategies in particular are used in biological control; (i) Classical, (ii) augmentation (inoculation and inundation) and (iii) conservation biological control (Shah and Pell, 2003; Glare, 2004; Stenberg *et al*., 2021; Bellone *et al*., 2023). The use of biological control agents against *E. saccharina* is considered the better option of control in both sugarcane and other graminaceous crops in Africa (Conlong, 1997; 2001). Since the development of a biological control unit in Mount Edgecombe in 1981, research has been devoted into the search of biological predators, pathogens and parasitoids of *E. saccharina* in both sugarcane and its indigenous host plants (Conlong, 1990). Parasitoids (*Schembria eldanae* Barraclough), entomopathogenic nematodes (*Heterorhabditis* sp.) bacteria (*Bacillus thuringiensis*), and an entomopathogenic fungus, *Beauveria bassiana* a member of the fungal order Hypocreales were discovered in South Africa, in indigenous sedges in Ethiopia and South Africa (Assefa et al. 2006). These potential biological control agents have, however, not been developed and used in commercial sugarcane pest control strategies in South Africa.

Since the discovery of the fungal entomopathogen *B. bassiana* on *Cyperus papyrus* L. umbels in South Africa, infecting and killing all *E. saccharina* larval instars and pupae stage (Conlong, 1990; 2001), this microorganism is seen as a potential biological control agent which can be integrated into the newer area-wide integrated pest management (AW-IPM) approach for *E. saccharina* control (Conlong and Rutherford, 2009). This approach aims to combine the traditional control methods with ecological understanding of the pest population over a wide geographic range. It additionally involves chemical ecology, habitat management, sterile

insect techniques (SIT) and a distinct understanding of the environmental (Kleynhans *et al*., 2018) interactions between plant, pathogen, endophytes and insects. Therefore, in order to use this fungal entomopathogen it is important to understand its ecology, interactions with *E. saccharina*, sugarcane and co-occurring pathogen, *Fusarium* spp.

1.3 Endophytic entomopathogens in biological control

1.3.1 Plant endophytes

The term endophyte is broadly used to refer to any microorganism found living within plant tissues (Arnold and Lewis, 2005; Nair and Padmavathy, 2014). Mycorrhizal fungi colonize plant roots and penetrate intracellular and extracellular space of plant tissues are termed endophytes (Jumpponen, 2001; Andrade-Linares and Franken, 2013). Microorganisms that form pathogenic symbioses within plant hosts are also termed endophytes. In this chapter the term endophyte will refer to fungi which for all or part of their life cycle can live within and colonize tissues of living plants without causing any symptoms of disease or infection to the host plant (Arnold and Lewis, 2005; Vega, 2008; Rodriguez *et al*., 2009; Quesada-Moraga *et al*., 2022; Rajab *et al*., 2023).

Plants are believed to “allow” endophytic colonization by beneficial microorganisms as plants have well developed chemical systems to attract such microbes and with stand biotic and/or abiotic stressors (Ownley *et al*., 2010; Rutherford, 2014). For instance, when plants are infected with plant pathogenic microorganisms, attacked by insect or treated with chemical compounds, they can trigger a series of induced systemic resistance (ISR) responses. These defense responses result in the expression of defense related genes which can release the biosynthesis of phenolic compounds at the site of infection (hypersensitive response) or at a distance (signalled by methyl salicylate) (Heil and Bostock, 2002; Mandal *et al*., 2010; Beniušytė *et al*., 2023), thus limiting the spread of the pathogen and damage (Durrant and Dong, 2004). However, when the plant is infected by a beneficial endophyte, mild ISR responses are triggered, thereby allowing the establishment and colonization of the plant by the endophyte such as Rhizobacter attracted to enter root tissues (Mushtaq *et al*., 2023). These mild ISR responses are reported to be facilitated by the jasmonic acid and ethylene signaling pathways (Turner *et al*., 2002; Mandal *et al*., 2010; Ownley *et al*., 2010; Rutherford, 2014; Beniušytė *et al*., 2023).

Endophytes are an essential factor in the ecology and fitness of plant communities (Rodriguez *et al*., 2009). All plant species studied to date form symbiotic relationships with endophytes and a single plant species can occupy hundreds of endophytic species within its healthy

tissues (Tan and Zou, 2001; Arnold and Lewis, 2005; Rodriguez *et al*., 2009; Rajab *et al*., 2023). Fungal entomopathogens have likewise been reported to form endophytic relationships with plants (Rajab *et al*., 2023). Entomopathogenic fungal endophytes belonging to the family *Clavicipitaceae* have been seen to have beneficial functions in plants (Vega *et al*., 2008, Samal *et al*., 2023) and are known to infect 300 species of grasses and sedges belonging to families Poaceae, Juncaceae, and Cyperaceae (Vega *et al*., 2008). These endophytes are seen colonizing the plant rhizosphere, phyllosphere, vascular tissues and interior parts of plant reproductive structures (Arnold and Lewis, 2005; Maheshwari, 2006; Rodriguez *et al*., 2009; González-Mas *et al*., 2021), improving plant defense against pests and diseases (Francis *et al*., 2022; Bellone *et al*., 2023).

1.3.2 Evolution of entomopathogenic endophytes

Fungal endophytes are categories into two major groups, (1) clavicipitaceous and (2) non-clavicipitaceous fungal endophytes. Clavicipitaceous fungal endophytes are found associated mainly with grasses and are termed “true endophytes”. These endophytes are transmitted vertically through seeds from one plant to another and tend to have an obligate relationship with host plants (Kuldau and Bacon, 2008; Lopez *et al*., 2014). Endophytic entomopathogenic fungi are categorized as non-clavicipitaceous, are typically horizontally transmitted and are prevalent in vascular and non-vascular plant species (Bamisile *et al*., 2018). Horizontally transmitted endophytes are typically transferred via airborne spores colonizing plants (Bamisile *et al*., 2018). It is hypothesized that entomopathogenic fungi arose from plant endophytes developing an ability to infect insects and being able to switch genes allowing these endophytes to live multifunctional lifestyles (Bamisile *et al*., 2018; Quesada-Moraga, 2020; Stone and Bidochka, 2020; Vidhate *et al*., 2023). Based on phylogenetic, proteomic and genome-based evolutive models, entomopathogenic fungi (*Beauveria* spp. and *Metarhizium* spp.) are closely related to grass endophytes (Barelli *et al*., 2015). These fungi are believed to have evolved; with insect pathogenic genes being co-selected from those associated with endophytic colonization. This could explain Quesada-Moraga *et al*. (2014) findings, reporting the vertical transmission of *B. bassiana* in opium poppy plants through seeds following artificial seed inoculation. With regards to evolutionary lineage, Gonzalez-Mas *et al*. (2021) stipulated the lack of knowledge on the evolutionary pathway of endophytic entomopathogenic fungi. The authors suggested that endophytic entomopathogens are less evolved than strict endophytes with the former following a different evolutionary pathway. Furthermore, the report discusses on endophytic entomopathogens maintaining both insect pathogenicity and endophytic colonization genes, with virulence-related genes being silenced during plant colonization.

1.3.3 Endophytes in plant protection

Endophytes can benefit the plant host and promote plant growth by enhancing resistance to abiotic stressors, diseases and insects (Vega *et al*., 2008; Bamisile *et al*., 2018). Several different mechanisms have been reported to be involved in fungal endophyte-mediated plant defence (Ownley *et al*., 2010). These mechanisms include direct antagonism resulting from microbial action (i.e.: antibiosis, competition and mycoparasitism) and indirect mechanisms which trigger plant responses as a result of endophytic colonization (i.e.: induced systemic resistance) (Gao *et al*., 2010; Ownley *et al*., 2010; Constantin *et al*., 2019). Antibiosis is a mechanism involving the secretion of antibiotics, enzymes and bioactive volatile organic compounds (VOCs) that are lethal and deterrent to pathogens and insects thereby restricting insect feeding and pathogen growth in the plant (Gao *et al*., 2010; Vega *et al*., 2010). These secondary metabolites produced by endophytes can also induce the synthesis of plant growth hormones, thereby promoting plant growth (Zhenhua *et al*., 2012; Quesada-Moraga, 2020).

Endophytes also exert plant protection by competing against other endophytes (i.e.: pathogens) present in the plant for nutrition and space. The successful suppression of plant pathogens by an endophytic biological organism is said to be resultant by the ability of the biological organism to out compete the plant pathogen, utilizing the nutrients available in the plant first. For instance, plant growth promoting *Proteobacteria* (now *Pseudomonadota)* from genera such as *Pseudomonas* and *Burkholderia* can reduce plant diseases through the competition for nutrients and through the release of secondary metabolites (antibiosis) thereby suppressing soil borne plant pathogens (Rutherford *et al*., 2002).

Mycoparasitism is the parasitism of one fungus by another (Ownley *et al*., 2010). It occurs by direct contact of the biological control endophyte with the plant pathogen. For example, the biological control fungus *Trichoderma harzianum* has the ability to detect fungal hosts and thereafter secret an extracellular exochitinase which diffuses towards the host cell and catalyzes the hydrolysis of the fungal host cell walls Such *Trichoderma* spp. release fungitoxic endochitinases and other hydrolytic enzymes, which degrade the cell wall of their host fungus (i.e.: plant pathogen) (Ownley *et al*., 2010; Vega *et al*., 2010).

Indirect mechanisms are also suggested to play a part in plant protection as endophyte colonization can trigger ISR responses in the plant (Dubois *et al*., 2006; Gao *et al*., 2010; Francis *et al*., 2022). For instance, during endophytic colonization of plants by symptomless endophytes, the jasmonic acid signalling pathway is triggered and is reported to facilitate endophytic colonization of non-pathogenic organisms. This signaling pathway is seen to be

associated with increased jasmonate (JA) defence signalling, suggested to increase plant resistance to pathogenic fungi and insects (Navarro-Meléndez and Heil, 2014; Constantin *et al*., 2019). The same mechanism has been observed when plant growth promoting bacteria colonize plants. The colonization of plants by these microorganisms' triggers bacteria mediated ISR, which in turn increases plant resistance to pathogens and promotes plant growth (Oliveira *et al*., 2009). Furthermore, plants can trigger the release of bioactive secondary metabolites as defense in response to endophytic colonization by pathogenic endophytes or tissue damage caused by insects feeding. These metabolites include flavonoids, cyanogenic glycosides and glucosinolates, which cause limitation of colonization and defer insect feeding (Weston *et al*., 2013; Rutherford, 2014; Yactayo-Chang *et al*., 2020). The entomopathogen *B. bassiana* has been shown to protect plants against pathogens and insect damage and is believed to employ the above-mentioned mechanisms (Vega *et al*., 2008; Ownley *et al*., 2010; Samal *et al*., 2023).

1.4 *Beauveria bassiana*

1.4.1 Microbiology

The anamorphic species, *B. bassiana,* is morphologically characterized by the formation of white to yellowish pigmented mycelia and conidia forming overt mycoses on insect cadavers. In culture, *B. bassiana* colonies grow moderately slow, typically taking 2 weeks to sporulate at 25°C (Rehner *et al*., 2011). Conidia appear mealy, cottony to powdery on the surface of the colony. Under low magnification *B. bassiana* shows little white, powdery balls on the aerial hyphae. A key diagnostic feature of this species is the presence of globose to flask shaped conidiophores forming one-celled holoblastic conidia, which are produced in sympodial succession on an elongating zig-zag rachis that can be detected by light and electron microscopy. Hyphae appear hyaline, smooth, and thin walled with septate segments. Conidia size (1.5-5.5 µm) and shape (globose to cylindrical or vermiform) are used as primary identification of species within the genus *Beauveria* (Rehner *et al*., 2011). Morphological identification is however, limited in delineating anamorphic fungal species and additional taxonomic characterization is deemed necessary (Robène-Soustrade *et al*., 2015).

Improvements in molecular techniques based on the analysis of DNA by the polymerase chain reaction (PCR) have enabled the development of accurate placement and renaming of *Beauveria* species, with evidence that *B. bassiana* species form cryptic lineages, belonging to a *B. bassiana* complex, with these lineages bring adapted to specific hosts or ecologies, (Bich *et al*., 2021; Wang *et al*., 2022). Phylogenetically the asexual genus *Beauveria* is monophyletic within the teleomorphic family Cordycipitaceae (*Cordyceps bassiana*) (Rehner

et al., 2011; Wang *et al*., 2021). Furthermore, Wang *et al*. (2022) stipulated that the cosmopolitan group of soilborne necrotrophic, arthropod-pathogenic fungi is made up of five species, within the *B. bassiana* complex. *Beauveria bassiana* isolates have great potential in the management and control of various insect pests as they are easily isolated from infected insects and soil using routine culture media, easily stored and mass propagated.

1.4.2 Entomopathogenic activity of *B. bassiana*

In natural environments the life cycle of the entomopathogen *B. bassiana* is hemibiotrophic synchronized with life stages of host insects. It has saprotrophic and biotrophic phases with the former involving colonization of arthropod cadavers and the latter phase, parasitism involving infection of the host (Ferreira and Soares, 2023; Sharma *et al*., 2023). The fungal infection process is initiated by the passive dispersal of conidia/spores (infective units) by wind or water from infected cadavers or from soil environments. Insect host recognition is a vital step for the fungal infection process to commence with G protein–coupled receptors (GPCR) and transmembrane proteins facilitating host recognition (Vidhate *et al*., 2023). Conidia land on and attach to the cuticle surface of susceptible hosts, facilitated by hydrophobic rodlets which cover the conidia. However, at times more specific structures may be formed (Charnley, 1989; Clarkson and Charnley,1996; Boomsma *et al*., 2014).

Under favorable environmental conditions, conidia germinate forming penetrating structures termed appressoria or germ tubes on the insect cuticle (Wang *et al*., 2021). Entomopathogens with a wide host range such as *B. bassiana* produce conidia which germinate in response to non-specific carbon and nitrogen sources present on the host cuticle, while entomopathogens with narrow host range such as *Beauveria brongniartii* have more specific requirements for germination (Charnley, 1989; Clarkson and Charnley,1996). Conidia germination is also dependent on the ability of the fungal entomopathogen to tolerate toxic compounds such as antimicrobial lipids, proteins, and metabolites present on the surface of the host cuticle (Shahid *et al*., 2012; Ortiz-Urquiza and Keyhani, 2013). Upon germination appressoria penetrate through the epicuticular and procuticle layers of the cuticle by a combination of physical forces and enzymatic activity.

Appressoria penetration can occur through insect joints, between segments, segmented cuticles, and mouth parts of insects (Charnley, 1989; Clarkson and Charnley,1996). Upon successful penetration through the chitinous exoskeleton and entry of hyphae into the haemocoel of insects, the fungal pathogen releases a cascade of inducible extracellular hydrolytic enzymes namely proteases, chitinases and lipases (St Leger *et al*., 1986; Charnley, 1989; Ali *et al*., 2010; Shahid *et al*., 2012). Protease and chitinase are critical for the virulence

of the entomopathogen. Protease expose the chitin fibers present in the insect epicuticle, when the fungus is in the procuticle, it then offsets the secretion N-acetyl-glycosaminidases and chitinases, which facilitate complete hydrolysis of chitin (Ferreira and Soares, 2023). The penetrating fungus is later converted into yeast-like propagules (hyphal bodies) (Figure 1.7), which spread throughout the insect's haemocoel, producing species specific metabolites such as oosporein, beauvericin and tenellin which contribute to insect immune system impairment (Wang *et al*. 2021).

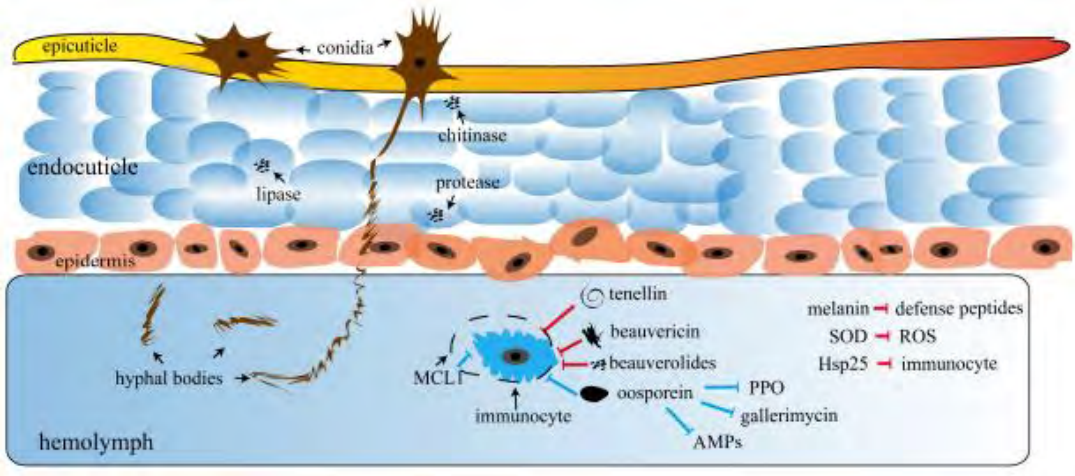

Figure 1.7: Schematic diagram of a fungal entomopathogen invading the insect host via the cuticle (Wang *et al*., 2021). AMPs: antimicrobial peptides; PPO: polyphenol oxidase; MCL1: myeloid cell leukemia sequence, an antiapoptotic protein; SOD: superoxide dismutase; ROS: reactive oxygen species; Hsp25: heat shock factor 25).

Insect death results from internal colonization of tissue, stress reactions and production of toxins released by the pathogen (Trizelia, 2010; Wang *et al*., 2021). After host death the fungus colonizes the cadaver in the form of white mold and mycelia continues to produce infectious spores, surviving on the mummified cadavers or as conidia in soil (saprophytic stage of fungi), or forms aerial hyphae which sporulate and are spreading conidia to new hosts. *Beauveria* species produce secondary metabolites such as oosporein, bassianin, bassiacridin, beauverolides, tenellin, cyclosporine A and oxalic acid, important in insect pathogenicity (Roberts *et al*., 1992; Abendstein *et al*., 2000; Amin *et al*., 2011; Rehner and Buckley, 2005; Rehner *et al*., 2011; Sahab, 2012; Deb *et al*., 2022). Oosporein has antibiotic, antiviral, antifungal, and insecticidal activities, which the fungus uses against gut bacteria the insect (Wang *et al*., 2021). *Beauveria bassiana* produces two major depsipeptide: beauvericin and the bassianolide, which have insecticidal and antibacterial activity. Due to the entomopathogenic activity of *B. bassiana,* its ability to produce insecticidal metabolites, and be mass produced; this microorganism has been explored in mycoinsecticide production

(Mascarin and Jaronski, 2016; Deb *et al*., 2022).

1.4.3 Application in biological control

1.4.3.1 Mycoinsecticides

Insect associations with microorganisms and diseases of insects have been recorded for more than a thousand years (Glare, 2004; Davidson, 2012; Wang *et al*., 2021). However, it was only in the 18th century when microorganisms were first used for insect control (Davidson, 2012). Bacterial toxin forming species, such as *Bacillus thuringiensis* (Bt), nematodes in the families *Steinernematidae* and *Heterorhabditidae*, Baculoviruses and entomopathogenic species within the fungal order *Hypocreales* have been successfully used in biological control of insects (El-Husseini, 2006; Dolinski and Lacey, 2007; Golijan-Pantović and Sečanski, 2022). However, entomopathogenic fungi pose distinct advantages when compared to other microbial control agents, in that entomopathogenic fungi can infect all developmental stages of susceptible insect pests (Anand *et al*., 2008; Dannon *et al*., 2020). Infections occur through direct contact with the insect cuticle rather than by ingestion; therefore, entomopathogenic fungi are capable of infecting non-feeding stages such as eggs, larvae, pupae, and adults (Hussain *et al*., 2012; Dannon *et al*., 2020). These fungal insect pathogens mainly belong to the orders *Hypocreales* and *Entomophthorales*.

The entomopathogenic *Hypocreales* have mostly been developed as commercial biological control products against forest and agricultural insect pests because they can be easily mass propagated (Dolinski & Lacey 2007; Jaronski, 2010; Mascarin and Jaronski, 2016; Sharma *et al*., 2023). These entomopathogens include species of the genera *Beauveria, Cordyceps*, *Akanthomyces* and *Metarhizium* are commercially available worldwide for insect control in many agricultural sectors (Baverstock *et al*., 2010; Jaronski, 2010; Kepler *et al*., 2017; Bamisile *et al*., 2021). The active ingredients of mycoinsecticides are the viable conidia of the entomopathogenic fungal strain and the efficacy of these products are therefore dependent on the conidial viability, their persistence and infectivity upon application, regeneration and cycling in the environment (Sharma *et al*., 2023).

De-Faria and Wraight (2007) and Maina *et al*. (2018) reported that 171 commercial products of entomopathogenic fungi have been developed. The use of biological pesticides in pest control accounted for 65% of the market share in 2019 and has been projected to increase in a compound annual growth rate (CAGR) of 14.7% based on the forecast period 2020-2025 (GlobeNewswire 2022). Mycoinsecticide product formulations range from liquid emulsions to powered products of fungi grown in different growth media (Glare, 2004; Mascarin and

Jaronski, 2016). Namely, products such as Bb weevil® and Bb Plus® formulated using *B. bassiana* strains are commercially available against Hemiptera (Aphididae), Acari (Tetranychidae) and Coleoptera (Curculionidae) (De-Faria and Wraight, 2007) insect pests in South Africa.

Although the use of mycoinsecticides is favourable in sustainable agriculture, challenges are associated with product efficacy in field application, when mycoinsecticides are exposed to abiotic factors (sunlight, rainfall, temperature, and humidity) affecting the integrity of conidia and hence product activity and performance (Glare, 2004; Fernandes *et al*., 2007; Yao *et al*., 2009; Bamisile *et al*., 2021; Ebani *et al*., 2021). The high production costs associated with production of large volumes of inoculum to achieve significant pest reduction may have also added to the slow commercial usage of mycoinsecticides (Vega, 2008; Azevedo *et al*., 2000; Jaronski, 2010). However, with the recent increased regulation of chemical insecticide, companies are investing in the production of entomopathogenic fungi and formulations that are stable under different environmental conditions.

Beauveria bassiana is readily isolated from internal plant tissues, naturally colonizing plants and insect host can been artificially inoculated into plants for biological control of plant pathogens and insect pests (Vega, 2008). The artificial establishment of an entomopathogen in plants is seen as a novel technique, which would potentially solve the constraints associated with field applications of mycoinsecticides (Azevedo *et al*., 2000; Bamisile *et al*., 2021). The entomopathogen would not be exposed to external abiotic factors that affect its activity as the entomopathogen would be inoculated into the plant and can develop inside the plant. The use of artificial inoculation and subsequent endophytic plant colonization by entomopathogens would drastically reduce application costs associated with inundative use of entomopathogens as mycoinsecticide formulations in insect pest as only little inoculum will be required to achieve control because the entomopathogen would be inoculated into the plant (Akello *et al*., 2007; Quesada-Moraga, 2020; Bamisile *et al*., 2021).

Posada and Vega (2005) reported that *B. bassiana* can re-cycle within the ecosystem after artificially inoculating cocoa seeds, with the fungus being isolated from surfaces of cocoa plants. Furthermore Quesada-Moraga *et al*. (2014) demonstrated the ability of *B. bassiana* strains to be vertically transmitted from endophytically colonized maternal plants. This ability of the fungus to translocate would further provide a continual natural inoculum for insect control. Artificial establishment of fungal entomopathogens into plants such as sugarcane would also ensure the continuous presence of the entomopathogen within the plant thereby providing continuous pest control throughout the crop cycle.

1.4.3.2 Artificial establishment *of Beauveria bassiana* as an endophyte

Artificial inoculations of entomopathogenic fungi have been achieved using various inoculation techniques such as conidia stem injection, conidia foliar spray or soil drench and plant dip applications (Quesada-Moraga, 2020; Deb *et al*., 2022). For instance, cocoa seedlings were endophytically colonized by *B. bassiana* after radicle inoculation with *B. bassiana* conidia (Posada and Vega, 2005). Maize leaves were endophytically colonized by *B. bassiana* after conidia were applied onto leaves using a hand brush (Wagner and Lewis, 2000). Sorghum leaves, stems and roots were also colonized by *B. bassiana* after seeds were immersed for 10 minutes in *B. bassiana* conidia. In addition, leaves were sprayed, and soil was drenched with a 3ml (1 x 10^8conidia ml^{-1}) *B. bassiana* conidial suspension (Tefera and Vidal, 2009). Brownbridge *et al.* (2012) colonized *B. bassiana* in pine seedlings after pine seeds were coated and pine roots were dipped into (2 x 10^6 and 1 x 10^7 conidia ml^{-1}) *B. bassiana* conidial suspensions. These inoculation techniques allowed recovery of *B. bassiana* from pine plants for up to 9-month post inoculation. Recently, Donga *et al*. (2018) demonstrated the successful inoculation and colonization of sugarcane plants by *B. bassiana* following stem injection, foliar spray or root drench methods when colonization was evaluated for 16 days post inoculation.

Endophytic colonization of plants by *B. bassiana* was shown by Wagner and Lewis (2000) to result from the conidia germinating on plant surfaces producing hyphae, which grow randomly across the plant surface and penetrate the plant using hydrolytic enzymes, forming small entrance holes. Hyphae were also seen to enter internal plant tissues randomly through stomatal openings on leaves. Once inside the plant, hyphae developed into branched mycelial networks which grew directly into neighbouring epidermal cells and palisade parenchyma and/ or enter intercellular spaces. Hyphae were also observed in xylem vessels, thus potentially moving throughout the plant via the interconnecting xylem tissues, thereby colonizing the entire plant (Wagner and Lewis, 2000; Gómez-Vidal *et al*., 2006).

Artificial inoculation of *B. bassiana* into agriculturally economically important crops has shown great potential for controlling tunnelling insect pests (Akello *et al*., 2007; Vega, 2008; Bamisile *et al.,* 2021). When artificially inoculated into maize plants, *B. bassiana* provided season-long control of the maize borer *O. nubilalis* (Wagner and Lewis, 2000) when compared to plants with no endophytic colonization by *B. bassiana*. A study by Cherry *et al*. (2004) also demonstrated that *B. bassiana* inoculated maize plants showed enhanced resistance against stem borer damage, and significantly reduced tunnelling by *S. calamistis*. Banana weevil (*C. sordidus*) damage was also reduced when tissue cultured banana plants were colonized by *B. bassiana* (Akello *et al*., 2007; Akello *et al*., 2008). Vidal and Tefera (2013) likewise reported

that an endophytic *B. bassiana* in *Vicia faba* L. (Fabales: Fabaceae) resulted in larval mortality and induced weight loss in H*. armigera Hübner* larvae feeding on plants carrying *B. bassiana* as an endophyte. Notably endophytic *B. bassiana* established in cotton seedlings reduced the disease resulting from the pathogen *Rhizoctonia solani* in cotton seedlings (Vega, 2008; Gurulingappa *et al*., 2010; Ownley *et al*., 2010). Furthermore, inoculation of entomopathogenic fungi into plants has shown enhancement of plant biomasss, with Donga *et al*. (2018) reporting enhancement the numbers of sett roots following spraying sugarcane leaves and drenching the soil with *B. bassiana*. Furthermore, a number of authors have reported on improved plant growth when plants were grown in the presence of entomopathogenic fungi (Lee *et al*., 1999; Maniania *et al*., 2003; Khan *et al*., 2012; Vega 2018; Quesada Moraga, 2020). Although these positive results have been obtained when establishing *B. bassiana* as an endophyte in plants, there are concerns regarding the presence of this microorganism in the environment and food chain as *B. bassiana* is known to produce potentially toxic secondary metabolites.

1.4.3.3 Safety in the food chain

Entomopathogens produce a range of bioactive secondary metabolites; some of these have been shown to have cytotoxic, neurotoxic and ionophoric properties when tested on mammalian cell lines during *in vitro* assays (Strasser *et al*., 2000; Zimmermann, 2007; Chen *et al*., 2020). For instance, some *Fusarium* species are notorious for their production and secretion of metabolites toxic to mammals, which upon ingestion of contaminated food led to health risks such as cancer of the oesophagus (Zain, 2011). Furthermore, these mycotoxins are seen to cause plant infections and diseases (Yazar and Omurtag, 2008). Like *Fusarium* species, *Beauveria* spp. produce a bioactive metabolite called beauvericin. Therefore, there are concerns that artificial inoculation of these endophytes into plants may exacerbate these health effects should they enter the food chain (Strasser *et al*., 2000; Vega, 2008; Chen *et al*., 2020).

The safety of a biological based control agent (i.e.: mycoinsecticides), to the environment and mammals has to be investigated intensively prior to registration, (i.e.: mycoinsecticides), organizations such as the European Food Safety Authority (EFSA) therefore conduct risk assessments to evaluate factors such as operator, worker, bystander, consumer risk, as well as risks to aquatic organisms, non-target organisms and possible contamination of ground water by secondary metabolites produced by mycoinsecticides(EFSA, 2020).

Unlike saprophytic plant pathogenic fungi, entomopathogenic fungi have not been shown to secrete metabolites in quantities that cause harm to plants and/or cause health risks (Strasser

et al., 2000). EFSA (2013) reported that *B. bassiana* strains ATCC-74040 and GHA are not pathogenic to humans. Additionally, 50 ppm of beauvericin and bassianolide did not display adverse effects on tested mammals. Nevertheless, Henke *et al*. (2002) and Tucker *et al*., (2004) reported that persons with Leukemia developed disseminated *B. bassiana* infections. Both studies stated the weakness of their findings due to the recovery of the fungus from only one infected site. In 2004, Glare reported that although secondary metabolites are produced by entomopathogenic fungi, their production *in vivo* is far lower than the levels produced in nutrient rich liquid cultures, and that entomopathogens do not produce all the metabolites at once when present. Research on the environmental impact and accumulation of *B. bassiana* metabolites in the environment showed that mycoinsecticides are safe and not expected to result in metabolite accumulating to harmful levels in the environment (Strasser *et al*., 2000). EFSA (2013) assessments on the persistence and mobility of mycoinsecticide in air and water, showed degradation of *B. bassiana* conidia within 2 days if suitable insect host are absent, as conidia will be degraded by sun-exposure. In addition, *B. bassiana* conidia are not efficiently transported in water due to their hydrophobic globose/ovoid conidial shape, while Zimmerman (2007) reported that no side effects or phytopathogenic activities caused by *B. bassiana* on plants are known. Additionally, Hu *et al*. (2016) reported that mycotoxins from mycoinsecticides have limited ways to enter environments. Likewise, entomopathogenic fungi are seen as important biological control agents, as they can reduce the use of toxic pesticides and reduce global environmental pollution from synthetic insecticides (Litwin *et al*., 2020).

Therefore, the use of the entomopathogen *B. bassiana* as an artificial endophyte in sugarcane could (1) Provide an environmentally friendly option to control *E. saccharina* when compared to toxic chemical insecticides, as *B. bassiana* would not accumulate in the environment. (2) The cryptic life cycle of *E. saccharina* in sugarcane has made it difficult to control this insect pest. The cryptic larval stage inside the stem is the most damaging stage. The artificial establishment of this entomopathogen into sugarcane would thus target this cryptic boring stage. (3) The artificial inoculation of sugarcane plants and the endophytically established entomopathogen *B. bassiana* would potentially provide prolonged pest control within the plant without need for repeated application. This would therefore reduce costs associated with repeated application of chemical control agents. (4) Concerns of secondary metabolites of the pathogen being released into the sugarcane plant and subsequently being passed into the food chain are negligible, due to the manufacturing process of sugar involving a series of high-pressure vacuum-boiling process to form sugar crystals. Therefore, if any metabolites present in the plant would be denatured.

Currently the entomopathogen *B. bassiana* is not used as a control agent of *E. saccharina* in

sugarcane in South Africa. Based on the literature available it appears that is will be beneficial to explore *B. bassiana* as an endophytic biological control agent in South African sugarcane. It is therefore imperative to survey the presence of entomopathogenic *Beauveria* species within the sugarcane agro-ecosystems, and to investigate the applicability of this inoculation technique in sugarcane plants.

The aims of this study were therefore to survey for the natural occurrence of entomopathogenic *Beauveria bassiana* from internal tissues (as apparent endophytes) from *E. saccharina* host plants and 28 sugarcane varieties found growing in the sugarcane agroecosystems. Thereafter to identify isolates using both microbiologically and molecular techniques. To determine the virulence of the characterized entomopathogenic endophytic isolates against *E. saccharina* larval instar stages. Followed by investigating interactions of *Fusarium* spp. with *B. bassiana* isolates. Finally, to artificially establish the most virulent endophytic entomopathogenic strains into sugarcane plantlets using different inoculation techniques, testing persistence and effect of endophyte on *E. saccharina* larvae 16 months post inoculation.

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CHAPTER 2

First time isolation of endophytic *Beauveria bassiana* from plant species in the vicinity of sugarcane fields in South Africa

Abstract

Eldana saccharina Walker is a serve sugarcane stalk borer pest in South Africa. Twenty-eight sugarcane (*Saccharum* L. spp.) genotypes and seven different species of host plants of *E. saccharina* were examined for the presence of endophytic *Beauveria bassiana*. Collectively, 326 plant samples (roots, stems, or leaves) were collected from five locations in sugarcane-producing areas (KwaZulu-Natal; South Africa). Following plant surface disinfection, 128 fungal colonies morphologically resembling *Beauveria* spp. were isolated from internal plant tissues. Sequence analysis of the internal transcribed spacer region (ITS) confirmed 13 representative isolates to be *B. bassiana.* Eight *B. bassiana* isolates were recovered from *E. saccharina* natural host plants, and 120 from different sugarcane stem parts (top node, top internodes, bottom node, and bottom internode). Endophytic *Beauveria bassiana* isolates were recovered from 22 of 28 sugarcane genotypes surveyed. The number of *B. bassiana* isolates obtained from different sugarcane parts differed significantly, with more *B. bassiana* isolates obtained from the top than the bottom internodes. Furthermore, sugarcane genotypes N31 and N41 yielded *B. bassiana* from all plant parts sampled, while 5 sugarcane genotypes harbored no *B. bassiana* isolates. This study demonstrates for the first time the natural occurrence of endophytic *B. bassiana* isolates in 22 sugarcane genotypes and *E. saccharina* host plants in South Africa.

2.1 Introduction

The South African sugar industry generates R18 billion per annum from sugarcane (*Saccharum* L. spp.) production, contributing substantially to employment, sustainable development, and the country's national economy (SASA 2023). Plantations of this tropical crop are predominately located in the province of KwaZulu-Natal, on the Eastern coast of South Africa (Thibane et al. 2023). The province has a subtropical climate, with sugarcane cultivation mostly taking place in locations matching the Köppen-Geiger classifications Cfa and Cfb, with warm and humid weather in summer and typically mild temperatures in winter, providing suitable conditions for sugarcane cultivation (Kottek et al. 2006; Thibane et al. 2023). Sugarcane growth and productivity are, however, constrained by many factors, such as insect pest infestation, with damage caused by root, leaf, or stem-feeding pests, compromising sucrose yields, and profit gained from the crop (Rutherford 2015; Goble et al. 2017; Hatting et al. 2019).

In South Africa, the stem feeder *Eldana saccharina* Walker (Lepidoptera: Pyralidae) is currently the most profit-limiting pest, causing losses estimated at US$62 million per annum (Zhou 2022). It is assumed that the initial cultivation of sugarcane within the natural habitat of *E. saccharina* and the subsequent removal of wetland sedges (Cyperaceae, Juncaceae, Typhaceae), which are identified as natural host plants of *E. saccharina* (Assefa et al. 2006), prompted the transition and host shift of *E. saccharina* to utilize sugarcane as its host (Conlong 2001; Rutherford 2015). The biological interaction of *E. saccharina* with sugarcane makes it difficult to control; its larvae feed on the buds and root bands of the lower third parts of the stem (bottom nodes), penetrating the soft tissues. Once inside, larvae feed on the soft internal tissues, boring the bottom internodes of the stem, spending 20-40 days before boring an exit hole, followed by pupating and emerging as adult moths (Conlong 2001; Rutherford 2015). *Eldana saccharina* is, therefore, protected inside the stem for up to 40 days from any applied pest control strategies. Controlling the internal larval feeding stage inside the sugarcane stem is imperative, and appropriate control strategies focusing on deferring *E. saccharina* feeding or causing larvae mortalities should be investigated.

Numerous biological control strategies to regulate lepidopteran stalk borers of sugarcane have been reported globally (Goble et al. 2017). The South African Sugarcane Research Institute (SASRI) has conducted surveys within the sugarcane ecosystem and indigenous host plants to identify natural control agents of *E. saccharina*. Parasitoids (*Schembria eldanae* Barraclough, *Goniozus natalensis* Gordh), entomopathogenic bacteria (*Bacillus thuringiensis*), and entomopathogenic fungi (*Beauveria bassiana, Entomophthora* spp.) were identified as

factors causing mortality in *E. saccharina* larvae in indigenous sedges in Ethiopia and South Africa (Assefa et al. 2006). Similarly, entomopathogenic nematodes (*Heterorhabditis* sp.) and lepidopteran viruses are known to infect *E. saccharina* larvae (Spaull 1992; Cherry et al. 1999). However, these control approaches have not been developed into commercial products or applications for South African sugarcane farmers.

The entomopathogenic fungal genus *Beauveria* has been surveyed intensively in the South African sugarcane ecosystem (Goble et al. 2012; Hatting et al. 2019) for biological control of sugarcane pests due to the ease of mass production, cosmopolitan distribution, limited impact on beneficial non-target organisms and their ability to form beneficial endophytic associations with plants (Mascarin and Jaronski 2016; Bamisile et al. 2021; Feng et al. 2023). Virulent *B. bassiana* strains used commercially as biological control agents (De Faria and Wraight 2007) can be isolated from naturally infected target insects (insect cadavers), soil, and plants (Sevim et al. 2010; Alfiky 2022; Quesada-Moraga et al. 2022). In plants, *B. bassiana* is isolated from plant surfaces as epiphytes and internal plant tissues as endophytes (Doberski and Tribe 1980; Meyling and Eilenberg 2006; Reay et al. 2010). This fungal species can naturally colonize plant tissues without causing disease or infection to the host plant (Gómez-Vidal et al. 2006; Arnold and Lewis 2005; Vega 2008; Quesada-Moraga et al. 2022). Such endophytic symbioses are reported to increase plant resistance to pathogen infection and insect damage (Meyling and Eilenberg 2006; Ownley et al. 2008) with the ability to suppress stem borer pests (Bing and Lewis 1993; Wagner and Lewis 2000; Akello et al. 2008; Feng et al. 2023). Studies conducted at SASRI (Hatting 2008; Goble et al. 2012) showed the prevalence of indigenous entomopathogenic fungi within the sugarcane ecosystem.

Nevertheless, no surveys of internal sugarcane and *E. saccharina* host plant tissues for endophytic entomopathogens have been conducted. Goble et al. (2012) found epiphytic *B. bassiana* isolates from one sugarcane leaf press, and only recently, sugarcane plants harboring *B. bassiana* in internal tissues were reported in Malawi (Donga et al. 2021). So far, sugarcane plants harboring fungal entomopathogens in internal tissues have not been reported in South Africa. Thus, *B. bassiana* could qualify as a natural in-field pest control candidate due to forming an endophytic symbiosis with sugarcane tissues, even though such a relationship has not been previously demonstrated in South Africa. Therefore, this study aimed to determine if *B. bassiana* is a prominent endophyte in different sugarcane varieties and representative *E. saccharina* natural host plants by surveying 28 sugarcane genotypes and seven *E. saccharina* host plant species found in the sugarcane agro-ecosystem in KwaZulu-Natal, South Africa.

2.2 Materials and Methods

2.2.1 Sample collection

Five representative sugarcane growing regions, four further than 80 km away from the research station, Mount Edgecombe, were randomly selected to improve the chances of isolating endophytic *B. bassiana* within the KwaZulu-Natal sugarcane production ecosystem. Sampling locations were (i) Mount Edgecombe (29°45' S, 31°51' E) (ii) Harden Heights (29°14' S, 30°37' E) (~116 km from Mount Edgecombe); (iii) Canema (29°12' S, 30°38' E) (~129 km from Mount Edgecombe); (iv) Eston (~89 km from Mount Edgecombe) and (v) Sezela (29°50' S, 30°30' E) (~99 km from Mount Edgecombe). The plant species collected were *Phragmites australis* L., *Saccharum* L. spp., *Sorghum bicolor* L., *Coix lacryma-jobi* L., *Panicum maximum* Jacq, *Cyperus papyrus* L., *Cyperus dives* L., *Cyperus sexangularis* L. and *Typha latifolia* L. Collectively 326 plant samples (leaves, stem and roots, collected from healthy, and in case of sugar cane, ten-month-old plants) were collected during the months of January to April, with average temperatures typically exceeding 25ºC from December to March and 20ºC from April to August (Ndlovu and Demlie 2020). Leaves and stem samples were collected by detaching them from the plant using disinfected (70% ethanol) secateurs, while root samples were dug out and separated from the soil. The individual plant samples were placed in clean (disinfected with 70% ethanol), sealable polyethylene bags and transported to the laboratory in polystyrene cooler boxes (22 L) lined with ice blocks, stored in a refrigerator at 4°C, and processed within 72 hours. Only sugarcane stem samples were collected to reflect the biology of the pest *E. saccharina,* which feeds on sugarcane stems. Two stalks from 28 sugarcane varieties were collected in Mount Edgecombe. One stalk from 18 varieties was collected at the Kearsney Research Farm due to the limited number of varieties and stalks available for sampling. Sugarcane genotype information has been reported previously (SASRI, 2022), and the breeding department at SASRI provided field layout information for sampling. Sugarcane plant samples were transported, stored, and processed as specified above.

2.2.2 Isolation of fungi from plant samples

Plant samples were surface disinfected by placing them in 100% ethanol for 1 minute, 10% sodium hypochlorite (NaOCl) for 5 minutes, and rinsing twice in autoclaved distilled water for 1-minute intervals each as described by Reay et al. (2010). For *Saccharum* L. spp. varieties, stem samples were cleaned (leaves removed), and three buds were counted from the upper natural breaking point. The 3rd bud (top node) and 3rd top internode from the breaking point were cut and used for *Beauveria* spp. isolation. Likewise, focusing isolations on the typical boring sites of *E. saccharina* larvae, the 3rd bottom node and bottom internode from the bottom part of the stem (e.g., from the roots) were also cut and surface disinfected as described above.

Plant samples were allowed to air dry in sterile Petri dishes in a laminar flow for 5 minutes. To confirm the efficiency of the surface disinfection process to remove possible *Beauveria* spp. present on plant surfaces, air-dried plant samples were aseptically pressed onto Sabouraud dextrose agar (SDA, Merck) supplemented with 50 mg/l chloramphenicol, 25 mg/l cycloheximide, 50 mg/l rifampicin (Goble et al. 2012) and 20mg/l dodine, as dodine selectively inhibits non-entomopathogenic fungi (Posadas et al. 2012). Following this, all edges of the plant sample were cut using a sterile surgical blade to remove dead plant tissue and create fungal emergence sites (Reay et al. 2010). Plant samples were thereafter cut transversely into thin, smaller sections (1.5 x 2 cm) and placed onto supplemented SDA plates. All plates were then sealed with Parafilm and incubated at ambient temperature (25-27°C) in the dark for 14 days. The absence of fungal growth on agar test-plates indicated the efficiency of the plant surface disinfection process. Only fungal colonies that grew from surface disinfected cut edges of plant samples were considered endophytes.

2.2.3 Morphological identification

Morphological characterization of 128 isolates to the genus *Beauveria* was performed according to the criteria specified by Humber (1997). White fungal colonies with cottony to powdery conidia that grew from plant segments were examined using a stereomicroscope (Leica MZ 125). Using phase-contrast microscopy (Zeiss Axio Scope A1, Germany, fitted with a Zeiss AxioCam ICc3), samples were examined for conidial shape, conidiophores, and hyphae septation. Fungal colonies that exhibited the typical morphology of *Beauveria* spp., as described by Rehner and Buckley (2005) and Rehner et al. (2011), were sub-cultured onto fresh SDA plates. *Beauveria* spp. isolates were thereafter stored and preserved using either 15% glycerol at -20°C or on supplemented SDA plates, which were sub-cultured every 2-3 weeks (Abdo et al. 2008).

Electron microscopy was used to support and confirm conidia size, formation pattern, and conidial and conidiophore shape, as noted with phase-contrast microscopy. An agar block (0.5 x 0.5 cm) was cut out using a sterile blade from the *Beauveria* spp. colonies grown for 2 weeks and treated using the Glutaraldehyde/Sodium-cacodylate buffer fixation method described by Wagner and Lewis (2000) with the following modifications. Samples were placed into small glass bottles fixed by soaking for 2 hours with 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2) and thereafter washed with fresh 50 mM sodium cacodylate buffer (pH 7.2) for 30 minutes. After fixation, samples were dehydrated in 30, 50, 70, 80, 90, and 100% ethanol each for 10-minute intervals. The samples were finally washed three times in 100% ethanol. Thereafter, samples were transferred into critical point drying (CPD) baskets, soaked in 100% ethanol, and dried (Hitachi HCP-2: critical point dryer) by exposure to liquid

CO_2 for 1 hour. The samples were then placed on carbon tape mounted on a specimen stub and sputter coated with gold-palladium using a Polaron E5100 sputter coater (Eiko IB-3, Japan) for 2 minutes. Samples were examined using a Philips XL 130 environmental scanning electron microscope (Philips, Netherlands) at optimally adjusted vacuum, temperature, and humidity levels.

2.2.4 Molecular characterization

DNA of 13 representative isolates was extracted using the Prep Man Ultra sample preparation reagent protocol (Applied Biosystems, California, USA). The purity and concentration of extracted DNA was thereafter, quantified using a spectrophotometer (NanoDrop ND-1000 Technologies, Delaware, USA). Isolated DNA samples were stored at -20°C until use. PCR amplification of the ITS1-5.8S rRNA gene-ITS2 region of the *Beauveria* spp. isolates was performed using universal primers (ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3')) (White et al. 1990) custom synthesized by Inqaba Biotech (Pretoria, South Africa). PCR amplification was performed for each of the extracted samples according to Sevim et al. (2010) with the following amendments. Using a KAPA Taq PCR Kit (KAPA Biosystems, Lasec, South Africa), a reaction tube with a final volume of 50µl contained 5µl 10x Taq-DNA polymerase reaction buffer, 1mM Mg^{2+}, 200µM dNTPs, 1 µl genomic DNA (25ng/µl), 0.4µM each of the opposing amplification primers and 0.5 units of Hot-start Taq DNA polymerase. A negative control containing PCR-grade water (Merck Sigma Aldrich, South Africa) served as a non-template control, and DNA from *B. bassiana* strain SASRI BB 444 (JX110368) was used as a positive control. PCR reactions were performed using a thermocycler (GeneAmp PCR System 9700, Applied Biosystems, California, USA) with the following cycling parameters: initial template denaturation at 95°C for 5 min, followed by 25 cycles at 95°C denaturation for 1 min, 55°C annealing for 55 s., 72°C extension for 2 min.; and a final extension at 72°C for 10min.

PCR amplicons (~600bp amplicon size) were separated and visualized under UV light (320nm) after loading 15µl mixed with 4 µl of 6X gel loading dye (Fermentas, USA) into 1.5% agarose (Whitehead Scientific, South Africa) gel wells alongside a molecular weight marker (O'GeneRuler 100bp Plus DNA Ladder, Fermentas, USA) and running gels for 45 min. The gel was prepared with 1x TBE buffer (45mM Tris, 45mM boric acid, 1mM EDTA; pH 8.0) and stained with ethidium bromide at a final concentration of 0.5µg/ml. The amplicons from the PCR reaction were purified using the Wizard SV gel PCR clean-up system (Promega, USA), according to the manufacturer. The purified PCR amplicons were quantified and sequenced using both forward and reverse primers (ITS5 and ITS4) in separate reaction tubes using an ABI3500 instrument, as previously described (Goble et al. 2012). The resulting DNA

sequences were checked for base-calling errors and edited to establish consensus sequences (Geneious Prime 2022.2.1). Consensus sequences were compared to ITS1-5.8S rRNA gene-ITS2 region sequences deposited in GenBank using the NCBI Basic Local Algorithm Search Tool (BLAST, http://www.ncbi.nlm.nih.gov), and matches were considered reliable if sequence similarities were greater than the established 98.5% SH threshold (Hoang et al. 2019; Nilsson et al. 2019) to fungal ITS reference sequences. Sequence alignments of sequences from isolates and reference sequences were created using MEGA 11 (Tamura et al. 2021). Of the 128 *Beauveria* spp. isolates, 13 isolates representing different locations and plant species were selected and used to construct a maximum likelihood phylogenetic tree using MEGA 11 (1000 bootstrap replications) based on the sequence alignment (Clustal W) of selected isolated *Beauveria* spp. and published ITS reference sequences for *Beauveria bassiana* isolates and type material of the genus *Beauveria* deposited in GenBank. The sequence from *Cordyceps succavus* (MFLU 18-1890-T) was used as an out-group. The tree topology was confirmed using the Neighbour-Joining algorithm. Sequences of the 13 representative isolates selected were assigned accession numbers and deposited in Genbank (Table 3).

2.2.5 Statistical analysis

The number of *B. bassiana* isolates obtained from the top internode, top node, bottom internode, and bottom nodes of sugarcane plants were recorded. Data were subjected to a normality test (Shapiro-Wilk test for normality) using GenStat (18th edition, VSN International, Hemel Hempstead, UK) and analysed via a one-way ANOVA and Sidak post hoc test to separate the means and determine significant differences. A significance threshold of 0.01 (probability level) was used.

2.3 Results

2.3.1 Isolation of endophytic *Beauveria bassiana* from plant samples

Colonies of isolates from surface disinfected plant tissues generally appeared white to cream-yellow in colour, and the conidia texture of isolates ranged from cottony and flaky to powdery (Figure 2.1).

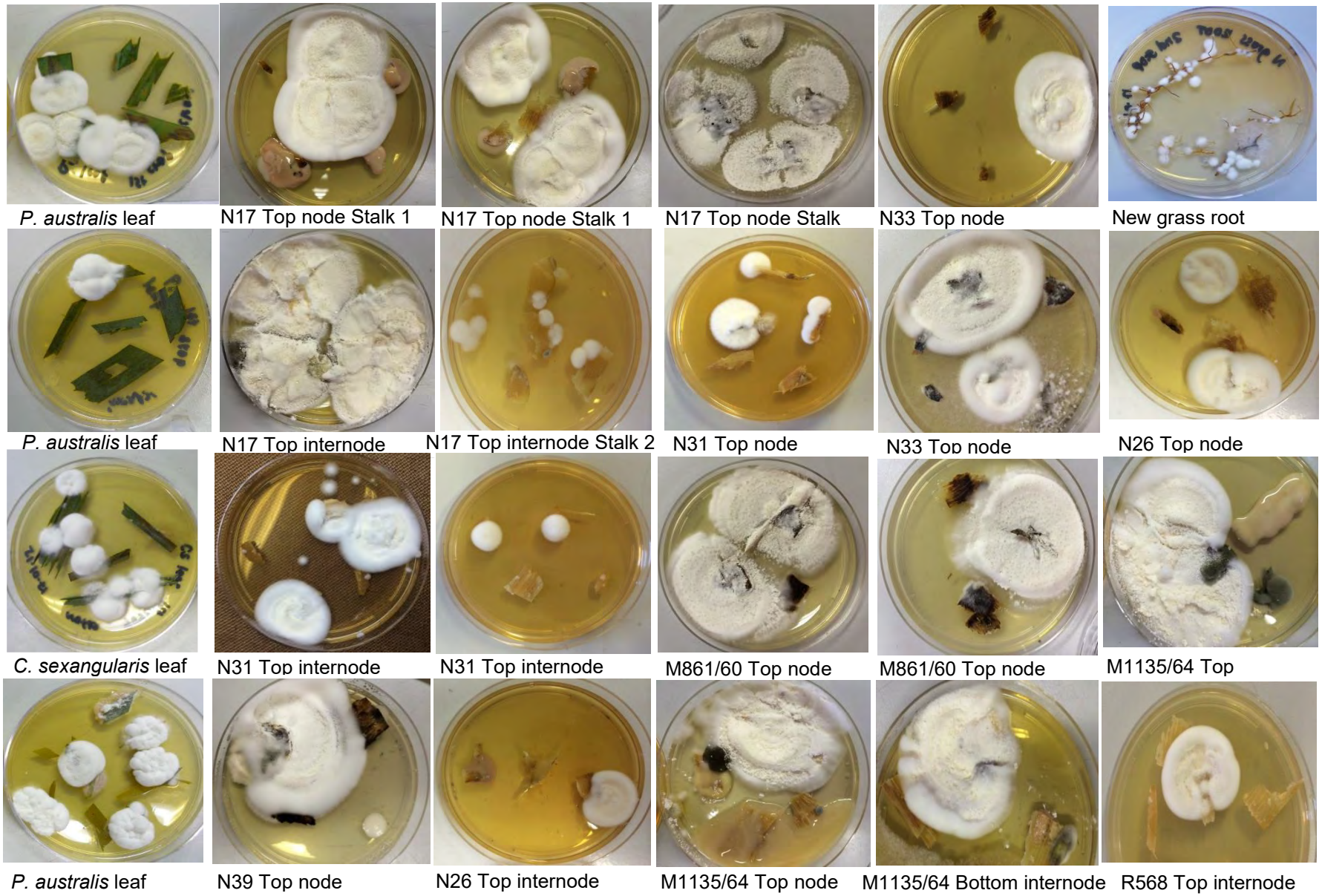

Figure 2.1: Colonies resembling *Beauveria* spp. isolated from internal tissues of plants growing within sugarcane fields.

Stereomicroscopy conducted on selected isolates revealed that mycelia were bearing snowball-like aerial conidia, while phase contrast microscopy revealed the presence of hyaline, septate hyphae, and flask-shaped conidiophores (Figure 2.2). In addition, clusters of sympodial globose conidiogenous cells producing spherical conidia (2.0 µm x 1.5 µm) on an elongating zig-zag rachis (Figure 2.2) were observed using scanning electron microscopy, all of which are diagnostic features of the genus *Beauveria* (Rehner et al. 2011).

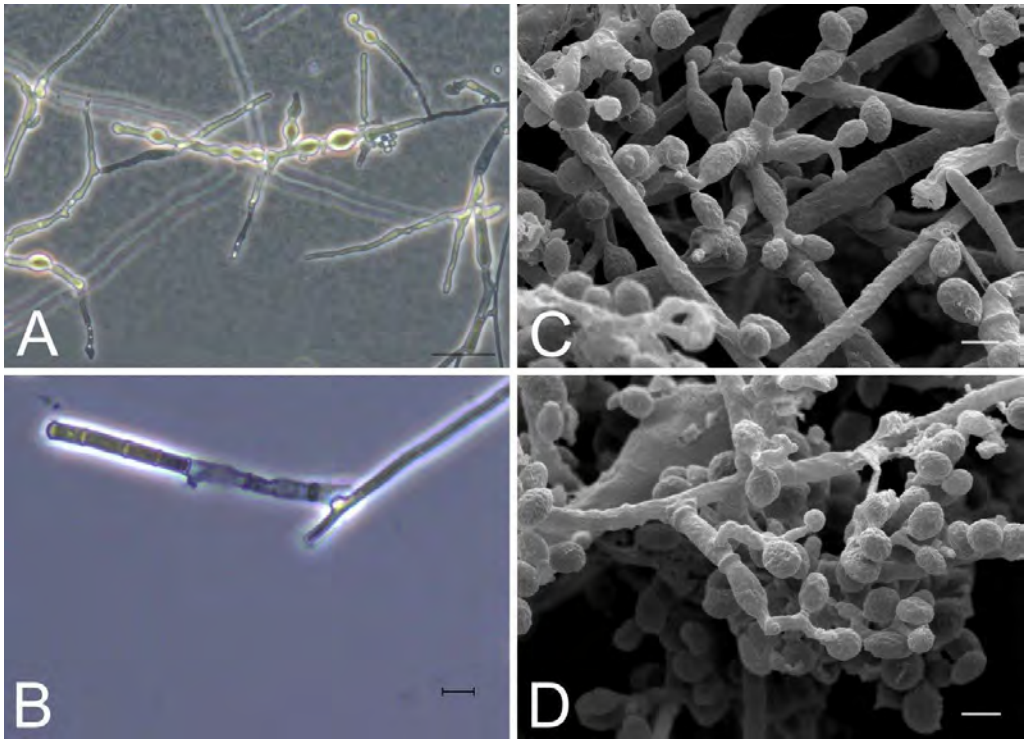

Figure 2.2: Phase contrast (A&B) and ESEM (C&D) micrographs of *Beauveria bassiana* isolates. (A) Formation of conidiophores from hyphae, Scale bar= 20 µm, (isolate: TL-leaf). (B) Hyaline, septate on *B. bassiana* hyphae (isolate: Site 55 roots), Scale bar= 20 µm. (C) Globose to flask-shaped conidiophores producing spherical conidia. Scale bar= 2µm, (isolate: S2root). (D) Zig-zag elongated rachis with septation (arrows), Scale bar= 2µm (isolate: S2root).

A total of 128 fungal isolates with colonies morphologically resembling *Beauveria* spp. were recovered, with 8 from plants of the families Cyperaceae, Typhaceae, and Poaceae and 120 from sugarcane varieties (Table 2.1, Table 2.2, and Table 2.3) and stored in glycerol at -80ºC and -20ºC at SASRI.

Table 2.1: Endophytic *Beauveria* spp. isolates recovered from sugarcane varieties in Mount Edgecombe.

Variety Number	Variety	*Beauveria* spp. isolates/variety	Sugarcane parts sampled[#]			
			Top internode	Top Node	Bottom internode	Bottom node
1	N31	8	2	2	2	2
2	N41	7	2	2	2	1
3	N32	6	2	2	1	1
4	M1135/64	6	2	2	1	1
5	R576	5	2	1	1	1
6	R568	5	2	2	1	0
7	N29	5	2	1	1	1
8	N28	5	2	1	1	1
9	N21	5	2	1	1	1
10	N26	4	1	1	1	1
11	N17	4	2	2	0	0
12	N12	4	2	1	1	0
13	N25	4	1	1	1	1
14	R572	3	1	0	1	1
15	N24	2	1	0	0	1
16	N22	2	1	0	0	1
17	N39	2	0	2	0	0
18	N33	2	1	1	0	0
19	Co6505	2	0	1	0	1
20	M861/60	2	0	1	1	0
21	N52/219	2	1	0	0	1
22	Co1287	1	0	0	1	0
23	NCo376	0	0	0	0	0
24	N11	0	0	0	0	0
25	N27	0	0	0	0	0
26	M1025/70	0	0	0	0	0
27	R570	0	0	0	0	0
28	R573	0	0	0	0	0
	Total *Beauveria* isolates	86	29	24	17	16

#: 2 sugarcane stalks sampled

Of the 120 isolates, 86 were from Mt. Edgecombe (isolated from 22 of the 28 sugarcane varieties), while 34 were obtained from Kearsney Research Farm from 15 of the 18 varieties. Variety N31 yielded the highest number of endophytic *Beauveria* isolates from all sugarcane stalk parts sampled, followed by N41 with seven isolates from both localities. In Mount

Edgecombe, the number of *Beauveria* spp. isolates obtained from the different sugarcane parts (top internode, top node, bottom internode, and bottom node) (Table 2.1) differed significantly ($F(3, 81) = 5.20$, $p = 0.002$). Significantly more ($F(1, 27) = 10.80$, $p = 0.003$) *Beauveria* spp. isolates were obtained from the top internodes (29 isolates) than the bottom internodes (17). However, significant differences were not detected ($F(1, 27) = 3.13$, $p = 0.088$) between the number of isolates found in the top nodes (24) versus bottom nodes (16) (Table 2.1).

Variety number	Variety	*Beauveria* spp. isolates/variety	Sugarcane parts sampled[#]			
			Top internode	Top Node	Bottom internode	Bottom node
1	N31	4	1	1	1	1
2	N41	4	1	1	1	1
3	N22	4	1	1	1	1
4	N25	3	1	1	0	1
5	N28	3	1	0	1	1
6	N27	3	1	0	1	1
7	N32	2	1	1	0	0
8	N24	2	1	0	0	1
9	N29	2	1	1	0	0
10	N39	2	0	1	1	0
11	N33	1	0	0	0	1
12	N52/219	1	0	0	1	0
13	N21	1	1	0	0	0
14	N17	1	0	0	1	0
15	N12	1	1	0	0	0
16	N11	0	0	0	0	0
17	NCo376	0	0	0	0	0
18	N26	0	0	0	0	0
	Total *Beauveria* isolates	34	11	7	8	8

Table 2.2: Endophytic *Beauveria* spp. isolates recovered from sugarcane varieties in Kearsney Farm

#: 1 sugarcane stalks sampled

In Kearsney Research Farm Station, significant differences ($F(3, 51) = 0.89$, $p = 0.45$) in the number of *Beauveria* spp. isolates obtained from the different parts of the sugarcane varieties sampled were not revealed. No colonies resembling *B. bassiana* morphology were obtained from sugarcane varieties Nco376 and N11 at both Mt. Edgecombe and Kearsney Research Station (Tables 2.1 and Table 2.2).

2.3.2 Molecular characterization

Sequence analysis of the ITS1-5.8S rRNA gene-ITS2 region confirmed the assignment to *B. bassiana*, as a comparison of the sequences obtained for the selected isolates to matching *B. bassiana* type strain ARSEF 1564 sequences in GenBank, showed that all 13 representative isolates had >98.5% sequence (Table 2.3; Table A2.1) similarity.

In addition, high similarity values (>99%) were obtained for matching sequences available for *B. bassiana* s.l. isolates deposited in GenBank (Table A2.1). The established phylogenetic tree showed that all 13 *B. bassiana* s.l. isolates representing different locations (e.g., Mount Edgecombe, Canema, Eston, Harden Heights) grouped closely with *B. bassiana* reference strain sequences (Figure 2.3).

Table 2.3: Endophytic *B. bassiana* s.l. isolates recovered from representative Poaceae, Cyperaceae and Typhaceae families within the sugarcane. ecosystem.

	Isolate name	Species identity	Percentage (%) identity to type strain ARSEF 1564	ITS Genbank	Isolation substrate/Vegetation	Collection area
					Poaceae, Cyperaceae and Typhaceae representatives	
1	P2 leaf	*Beauveria bassiana*	98,72	MF802502	*Phragmites australis* leaf	Eston
2	S2root	*Beauveria bassiana*	98,95	MF802501	Sorghum biocolor root	Mount Edgecombe
3	CS leaf	*Beauveria bassiana*	99,05	MF802495	*Cyperus sexangularis* leaf	Eston
4	TL-leaf	*Beauveria bassiana*	98,69	MF802503	*Typha latifolia* leaf	Mount Edgecombe
5	CC4 root	*Beauveria bassiana*	98,77	MF802494	*Panicum maximum* Jacq root	Canema
6	Site 55 roots	*Beauveria bassiana*	99,12	MF802497	*Panicum maximum* Jacq root	Harden Heights
7	JT leaf	*Beauveria bassiana*	99,08	MF802493	*Coix lacryma-jobi* leaf	Mount Edgecombe
8	NG root	*Beauveria bassiana*	99,24	MF802496	*Panicum maximum* Jacq root	Eston
					Sugarcane plants	
9	N41S1TI	*Beauveria bassiana*	99,1	MF802488	*Saccharum* spp. *N41 Top internode Stalk 1*	Mount Edgecombe
10	N41S2BN	*Beauveria bassiana*	98,5	MF802491	*Saccharum* spp. *N41 Bottom node Stalk 2*	Mount Edgecombe
11	N41S2TNa	*Beauveria bassiana*	99,1	MF802489	*Saccharum* spp. *N41a Top node Stalk 2*	Mount Edgecombe
12	N41S2TNb	*Beauveria bassiana*	98,77	MF802492	*Saccharum* spp. *N41b Top node Stalk 2*	Mount Edgecombe
13	R568S2TN	*Beauveria bassiana*	99,1	MF802490	*Saccharum* spp. *R568 Top node Stalk 2*	Mount Edgecombe

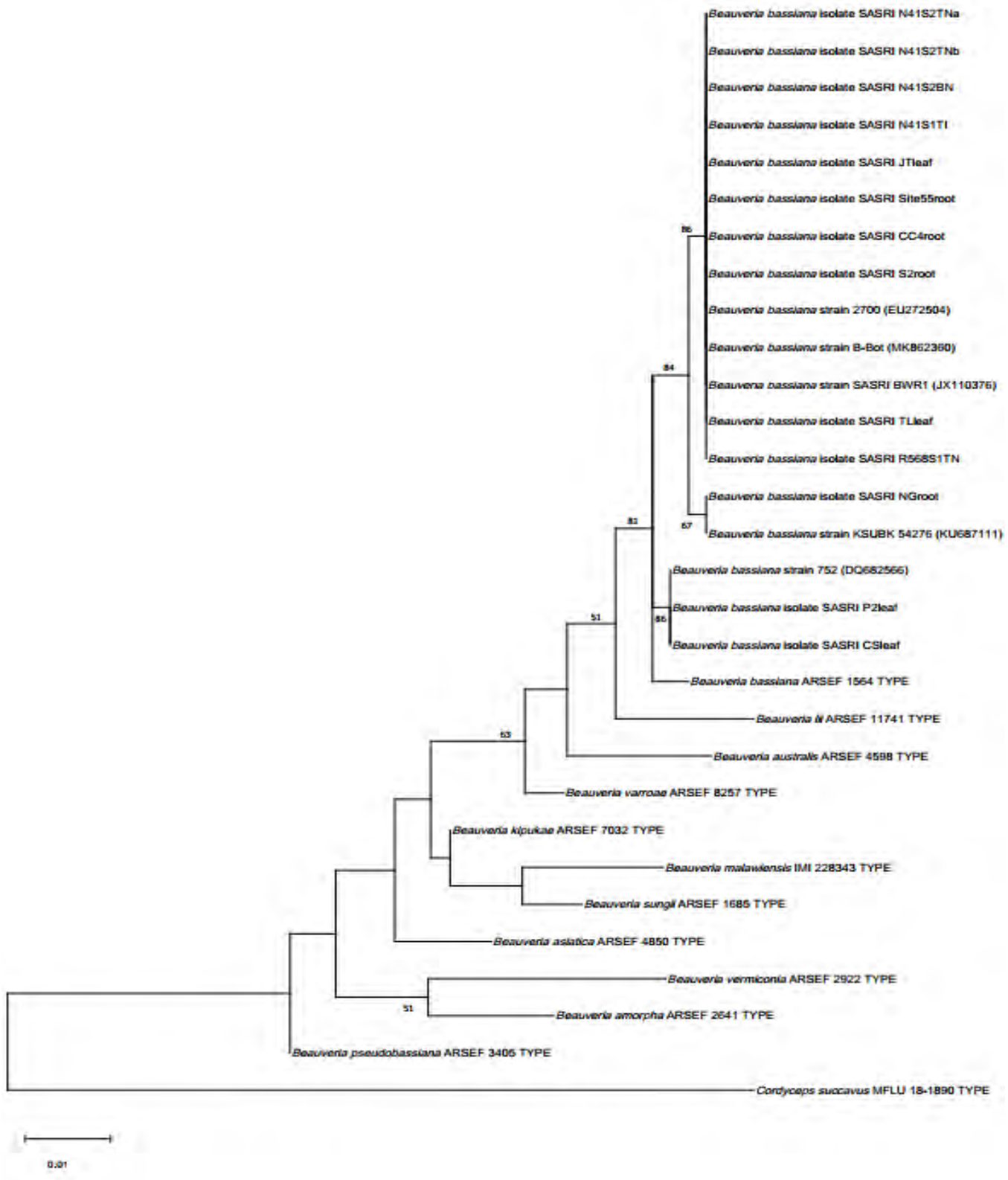

Figure 2.3: Maximum likelihood tree showing the phylogenetic affiliation of 13 *Beauveria bassiana* isolates obtained from plant tissues in comparison to reference strain sequences obtained from NCBI GenBank based on the ITS1-5.8S rRNA gene-ITS2 region. *Cordyceps cf. scarabaeicola* AY532058: Nepal, was used as an out-group. The scale bar indicates two estimated changes per 100 nucleotides. Bootstrap values are shown at topology nodes.

2.4 Discussion

Upon surveying 28 sugarcane genotypes from two sites in KwaZulu-Natal and *E. saccharina* natural host plants (Cyperaceae, Typhaceae) and grasses (Poaceae) belonging to seven different plant species in five different geographic locations, endophytic *B. bassiana* isolates were isolated from all plant species. A higher number of isolates were obtained from Mount Edgecombe due to sampling two stems from 28 varieties compared to one stem sampled from 18 varieties in Kearsney Research Farm, as there was a limited number of varieties and stems available in Kearsney Research Farm during sampling. All isolates shared key morphological and microscopic characteristics reported for the genus *Beauveria* by Rehner et al. (2011). Sequence analysis of the ITS1-5.8-S-ITS2 gene region identified representative isolates as *B. bassiana* as they shared similarities greater than 98.5% to *B. bassiana* ITS reference sequences, the benchmark for assignment to species level (Hoang et al. 2019; Nilsson et al. 2019). The *B. bassiana* isolates, representing different plant species and locations (e.g., Mount Edgecombe, Canema, Eston, Harden Heights) that were more than 80 km apart, shared a close phylogenetic relationship. Goble et al. (2012) reported similar results for *Beauveria brongniartii* isolates obtained from soil and insects at two matching locations in KwaZulu-Natal (Harden Heights, Canema). Interestingly, endophytic *B. bassiana* isolates CS leaf and P2 leaf, isolated in this study, grouped closely with an endophytic isolate obtained from coffee plants (*B. bassiana*, DQ682566) in the USA (Posada et al. 2007); while the endophytic *B. bassiana* isolate N41S2TNb matched closely with an endophytic *B. bassiana* isolate (EU272504) obtained from *Espeletia grandiflora* in Colombia (Miles et al. 2012). Nevertheless, using additional sequencing targets (e.g., the rpb genes) would enable a more detailed phylogenetic analysis of isolates.

As an insect pathogen, *B bassiana* has a multifunctional lifestyle (Ortiz-Urquiza, 2021) and a broad endophytic capability as it has been isolated from a large variety of plant hosts (Hu and Bidochka 2021). Arnold and Lewis (2005) reported endophytic *B. bassiana* from *Zea mays* L. (*Poaceae*), Vega (2008) from cotton, and Reay et al. (2010) isolated *B. bassiana* as an endophyte from needles, seed, and root material of *Pinus radiata* in New Zealand. *Beauveria bassiana* was also recently detected as an endophytic fungus in sugarcane tissues in Malawi (Donga et al. 2021). The isolation of 120 endophytic *B. bassiana* isolates from 22 sugarcane genotypes and 8 isolates from *E. saccharina* host plants in this study, evidently shows for the first time the natural occurrence of *B. bassiana* as an endophyte in different sugarcane varieties and *E. saccharina* host plants in South Africa.

Natural control of *E. saccharina* by endophytic *B. bassiana* in sugarcane plants has not been reported in South Africa, raising the question of whether endophytic *B. bassiana* can naturally control *E. saccharina* in the sugarcane plant. In this study, the number of *B. bassiana* isolated from the top internode was significantly higher than the bottom internode in Mount Edgecombe, with the same trend observed in isolates obtained from sugarcane stems collected in Kearsney Research Farm. The reason for a higher prevalence of endophytic *B. bassiana* in the top internodes compared to the bottom internodes is unclear. However, endophytic fungi are reported to display tissue specificity and are optimally adapted to conditions present in specific host plant parts (Akello et al. 2007a; 2007b; Tefera and Vidal 2009; Mantzoukas et al. 2021).

The apparently limited in-field control of *E. saccharina* larvae by endophytic *B. bassiana* may be due to the higher frequency of *B. bassiana* isolates in top internodes and top nodes compared to bottom internodes and bottom nodes. Based on the biological interaction of *E. saccharin*a with sugarcane, *E. saccharina* enters sugarcane by boring in the bottom nodes of the stem, followed by larval feeding on the bottom internodes (Conlong, 2001). Therefore, the natural incidence of *E. saccharina* encountering *B. bassiana* endophytes is low due to the higher frequency of endophytic colonization in the top parts of the plant. Nevertheless, insect pests can develop mycosis following feeding on *B. bassiana* colonized plants, as Powell et al. (2007) observed mycosis of *Helicoverpa zea* larvae following endophytic colonization of tomato plants with *B. bassiana,* and Akello et al. (2008) verified pest mycosis following feeding on *B. bassiana* inoculated banana plants. Bing and Lewis (1993) attributed the apparent lack of mycosis of *O. nubilalis*, fed on *B. bassiana* colonized corn, to low fungal abundance in the colonized plants, suggesting that levels of *B. bassiana* present in the plant tissues is crucial for insect pest control.

Furthermore, *E. saccharina* boring and feeding on internal sugarcane tissues is frequently coupled with the development of a secondary plant infection by *Fusarium* spp. *Fusarium* species have an endophytic and necrotic relationship with sugarcane, benefiting *E. saccharina* growth and development (McFarlane et al. 2009; Berasategui et al. 2023). However, the presence of *Fusarium* spp. may affect the entomopathogenic capacity of endophytic *B. bassiana* in sugarcane tissues. Indeed, *Fusarium* spp. can produce mycotoxins such as enniatin, inhibiting the growth of *B. bassiana* (Meca et al. 2010) and may additionally interfere via their secondary metabolites with plant defense systems (Rodriguez et al. 2009; Rutherford 2014). In this study, endophytic *B. bassiana* were isolated from varieties N41 and N31, categorized by the breeding department at SASRI to exhibit intermediate resistance to *E. saccharina.* However, no endophytic *B. bassiana* isolates were recovered from the susceptible

varieties NCo376 and N11 at both sampling locations (Mount Edgecombe and Kearsney). In South Africa, sugarcane variety resistance against *E. saccharina* has been attributed to morphological and physiological plant characteristics such as stalk surface wax chemistry, internode rind hardness, bud scale flavonoids, and chlorogenate profiles, which contribute to larval antixenosis and early-instar antibiosis (Keeping and Rutherford 2004; Kvedaras and Keeping 2007). However, so far, no studies are available attributing sugarcane resistance to the presence of endophytic *B. bassiana*. Therefore, vigorous sampling of more varieties from multiple localities should be conducted to investigate the occurrence of *B. bassiana*-mediated pest resistance.

Even though this study did not explore the mode of transmission of the *Beauveria* spp. endophytes in sugarcane, the presence of *B. bassiana* in sugarcane nodes is promising as nodes are the growing point when sugarcane is propagated. The stem node consists of the growth ring, root primordia, and bud, which germinate when planted, forming new tillers and generating stalks (Rae et al. 2013). The presence of *B. bassiana* as an endophyte in nodes would promote the transfer of *B. bassiana* endophytes into new plant tillers, as endophytes can be vertically transmitted from plant to seeds (Quesada-Moraga et al. 2014) or horizontally transmitted via asexual or sexual spores infecting the plant (Hu and Bidochka 2021). *Beauveria bassiana* may be horizontally transmitted as an endophyte as it is clonally propagated. Donga et al. (2021) suggested the possible transmission of the entomopathogen *B. bassiana* as an endophyte through fungal propagules via insect feeding on the plant, and Goble et al. (2012) described the cycling of the entomopathogen *Beauveria* spp. in South Africa as they found three genetically identical strains from the black wattle leaf surface, inside a *Hypopholis sommeri* Burmeister (Coleoptera: Scarabaeidae) adult and on a mycosed larva. Only recently, using an NGS approach, Watson et al. (2022) reported a highly diverse fungal symbiont community for the fall armyworm (*Spodoptera frugiperda*), with reads for *Beauveria* detected in insect but not soil or leaf samples.

Endophytic *B. bassiana* isolate were isolated from root, stem, and leaf samples of *E. saccharina* natural host plants. The isolation of endophytic *B. bassiana* from roots was not surprising as species within the genus *Beauveria* are reported to be ubiquitous in soil environments and were previously thought to be exclusive soil insect pathogens (Rehner and Buckley 2005), which could account for the recovery of *B. bassiana* from roots. In this study, however, endophytic isolates were also recovered from leaf samples. The occurrence of *B. bassiana* in leaf tissues could be explained by the ability of *B. bassiana* conidia to germinate, penetrate, and endophytically colonize leaves after being deposited on leaves via air currents (Meyling and Eilenberg 2006; Donga et al. 2021). Wagner and Lewis (2000) demonstrated

that *B. bassiana* endophytically colonized maize leaves after *in vivo* application of conidia onto maize leaf surfaces using a hand brush.

It would be expected that if *B. bassiana* forms stable endophytic associations with sugarcane plants, natural control of *E. saccharina* inside the stalk should be observed in the field. For instance, Cyperaceae species are natural host plants of *E. saccharina,* and *B. bassiana* isolates were indeed isolated from *Cyperus sexangularis* leaf in this study. This matches the reported presence of *B. bassiana* in natural host plants of *E. saccharina* (Assefa et al. 2006; Conlong and Way 2015), with *B. bassiana* infected *E. saccharina* larvae detected in *C. papyrus* umbels. Furthermore, McKinnon et al. (2017) reported that plants harboring endophytic *B. bassiana* defer and reduce insect feeding, while the biocontrol of plant-feeding insects by *B. bassiana* based products was shown for insect pests from various orders (Smagghe et al. 2023). However, no field-infected *E. saccharina* larvae were observed during sample collection in the present study. The lack of infected *E. saccharina* cadavers could be explained by the dynamics existing between insect and pathogen, given that Meyling and Eilenberg (2006) and Hesketh et al. (2010) reported that the density of insect hosts limits the abundance of insect pathogens.

In conclusion, this study confirmed the natural occurrence and association of *B. bassiana* with different plant species in South Africa. It demonstrated for the first time the occurrence of *B. bassiana* as an endophyte in 22 sugarcane genotypes in KwaZulu-Natal, South Africa. The isolation of 128 endophytic *B. bassiana* isolates from sugarcane and plant species belonging to the families Poaceae, Typhaceae, and Cyperaceae found growing in the sugarcane ecosystem shows the prevalence of indigenous endophytic *B. bassiana* in five sugarcane growing regions in the province of KwaZulu-Natal. Although it was not investigated if the presence of endophytic *B. bassiana* leads to in-field biocontrol, the presence of this entomopathogen in sugarcane is promising for an endophyte-based biological control of plant-feeding insects. Inoculation of *B. bassiana* into sugarcane plants could verify if native isolates are compatible as biocontrol agents for *E. saccharina* or against other plant-feeding pests of sugarcane. Research is currently ongoing to determine the virulence of *B. bassiana* isolates toward *E. saccharina* under laboratory conditions and to verify whether artificially inoculated sugarcane plants can exhibit control of *E. saccharina*.

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Appendix

Table A2.1: Highest sequence similarities of representative endophytic *Beauveria bassiana* isolates from KwaZulu-Natal with sequences from *B. bassiana* isolates deposited in Genbank.

Isolate	Accession number	Highest similarity match (accession number)	Geographic origin
B. bassiana P2 leaf	MF802502	99.29% (DQ682566)	Endophytic *B. bassiana* strain 752 (coffee plant), USA
B. bassiana S2root	MF802501	99.83% (MK862360)	*B. bassiana* strain B-Bot, Palestine
B. bassiana CS leaf	MF802495	99.63% (DQ682566)	Endophytic *B. bassiana* strain 752 (coffee plant), USA
B. bassiana TL-leaf	MF802503	99.82% (MK862360)	*B. bassiana* strain B-Bot, Palestine
B. bassiana CC4 root	MF802494	99.66% (MK862360)	*B. bassiana* strain B-Bot, Palestine
B. bassiana Site 55 roots	MF802497	100% (MK862360)	*B. bassiana* strain B-Bot, Palestine
B. bassiana JT leaf	MF802493	100% (MK862360)	*B. bassiana* strain B-Bot, Palestine
B. bassiana NG root	MF802496	99.45% (KU687111)	*B. bassiana* strain KSUBK 54276 (flour beetle), Turkey
B. bassiana N41S1TI	MF802488	100% (MK862360)	*B. bassiana* strain B-Bot, Palestine
B. bassiana N41S2BN	MF802491	99.83% (EU272504)	Endophytic *B. bassiana* strain 2700 (Espeletia grandiflora), Colombia
B. bassiana N41S2TNa	MF802489	100% (MK862360)	*B. bassiana* strain B-Bot, Palestine
B. bassiana N41S2TNb	MF802492	99.33% (JX110376)	*B. bassiana* strain SASRI BWR1, root press black wattle, South Africa
B. bassiana R568S2TN	MF802490	100% (JX110376)	*B. bassiana* strain SASRI BWR1, root press black wattle, South Africa

CHAPTER 3

Assessment of virulence for *Beauveria bassiana* isolates against *Eldana saccharina* larvae.

Abstract

Larval choice, diet inclusion, and topical spray bioassay experiments were conducted to investigate the repellency, larval development, and virulence of 16 endophytic *Beauveria bassiana* isolates toward *Eldana saccharina* larval stages. *Eldana saccharina* first instar larvae were repelled from all *B. bassiana* isolates during larval choice assays when larvae were placed in a choice arena with uninoculated and *B. bassiana* inoculated maize kernels, with larvae mainly found feeding on uninoculated maize kernels. Repellence levels, however differed among *B. bassiana* isolates ranging from 65-95%. Diet inclusion assays, incorporating a *B. bassiana* isolate, obtained from *Typha latifolia* L. leaf (TL-leaf) significantly reduced survival of *E. saccharina* larvae to 34%, when larvae were fed on this diet for 21 days, while control diets had 100% *E. saccharina* survival. Furthermore, the surviving larvae fed on *B. bassiana* N41S1TI isolate weighed significantly less (0.01 g ± 0) than larvae reared in control diets (0.20 g ± 0.02). Similarly, seven *B. bassiana* isolates reduced *E. saccharina* development during diet inclusion assays, with no pupae recorded after 21 days, whilst 80% pupation was observed using the control diets. Topical spray bioassays showed all sixteen *B. bassiana* isolates to be virulent towards *E. saccharina* 3rd and 5th instar larvae with 3rd instar being more susceptible than 5th instars. Median lethal doses (LD_{50}) of the most virulent *B. bassiana* isolates against 3rd instar larvae were 17±3 and 23±5 conidia/mm^2 based on the total counted conidia. *Beauveria bassiana* isolates that were highly repellent, resulted in the highest reduction of *E. saccharina* larval weight and reduced *E. saccharina* development and were highly virulent during topical spray bioassays. Likewise, *B. bassiana* isolates that had the lowest repellence (NG-root, and JT-leaf) similarly caused lowest *E. saccharina* mortalities during topical spray bioassays. The *B. bassiana* isolates tested can be separated into classes of highly virulent, intermediate, and weakly virulent to *E. saccharina* larvae. The endophytic *B. bassiana* isolates show potential as biological control agents against *E. saccharina*.

3.1 Introduction

The African sugarcane stem borer, *Eldana saccharina* Walker (Lepidoptera: Pyralidae), is a problematic pest in sugarcane fields in southern and eastern Africa (Kleynhans et al., 2018). Species in this order comprise of four distinct stages: egg, larva, pupa, and adult (moth) with larvae depending on plants for their growth and survival. They tunnel into the stems, fruits and seeds of plants causing defoliation, economic losses, and food insecurity (Hatting et al., 2019; Ouaba et al., 2022).

The life cycle of *E. saccharina* in sugarcane is cryptic. Second and third instar larvae bore and penetrate the lower parts (bottom nodes) of the sugarcane stalk via the bud, root band and cracks (Rutherford, 2015). Once inside, larvae feed (in bottom internodes), causing increasing tunneling in the stalk, developing into fourth, fifth and sixth instar stages; before pupating within the hollowed stem (Way, 1995; Leslie, 2004). This internal feeding reduces sucrose production in the crop (Leslie, 1993; Rutherford, 2015) causing economic losses estimated at around US$62 million per annum for the South African sugar industry (Zhou, 2022). This cryptic nature of *E. saccharina*, has made it difficult to control using traditional pest management methods (Harraca et al., 2012; Rutherford, 2015). The complete eradication of *E. saccharina* populations is impossible as it is an indigenous insect with many wild host plants. Thus, alternative methods that can be collectively applied should result in the reduction of *E. saccharina* damage (Rutherford, 2015; Kleynhans et al., 2018). The South African Sugarcane Research Institute (SASRI) has promoted the implementation of an area-wide integrated pest management (AW-IPM) system to achieve sustainable control of *E. saccharina* (Conlong and Rutherford, 2009; Kleynhans et al., 2018). The potential establishment of an entomopathogenic fungus *Beauveria bassiana*, as an endophyte into sugarcane to target the 3rd, 4th, 5th, and 6th instar larval stages of *E. saccharina*, and/or to repel the insect, is a possible alternative control method. However, effective implementation of this biological control approach within the AW-IPM system requires an understanding of the multitrophic interactions between plants, endophytes, and insects (Conlong and Rutherford, 2009; Kaur et al., 2010; Kleynhans et al., 2018; Quesada-Moraga et al., 2022).

Entomopathogenic fungi infect insects through direct contact of conidia with susceptible insect host(s). Infections occur after viable conidia germinate on insect epicuticle forming appressoria (germ tubes) which penetrate insect cuticle and epidermis layer through soft integuments between joints, segments and/or mouth parts of the insect (Trizelia, 2010; Mannino et al., 2019). During penetration, the entomopathogenic fungi release a cascade of

extracellular enzymes such as chitinase and protease, which degrade the insect cuticle (St Leger et al., 1986; Bali et al., 2022). Once penetrated, the entomopathogenic fungus multiples by blastospore formation and spreads throughout the insect hemocoel producing secondary toxic metabolites, which cause tissue destruction, paralysis and death. Upon insect death, hyphae penetrate outwards onto the insect surface forming conidia and overt mycosis on the insect cadaver (Trizelia, 2010; Augustyniuk-Kram and Kram, 2012; Bali et al., 2022; El-Maraghy et al., 2023).

Insects have, however, developed innate or adaptive defense mechanisms to avoid infections (Cooper and Eleftherianos, 2017; Ramirez et al., 2018, Bali et al., 2022). For instance, *Tribolium castaneum* (Herbst), the red flour beetle, has evolved resistance to entomopathogenic infection by embedding its cuticle with antifungal compounds inhibiting fungal germination on the cuticle (Davyt-Colo et al., 2022). Besides the epicuticle defense system, insects have chemoreception responses (Mburu et al., 2013; Ortiz-Urquiza and Keyhani, 2013), enabling them to detect volatile compounds produced by fungal pathogens and thus avoid contact with the pathogen (Meyling and Pell, 2006). Virulent entomopathogenic fungal isolates of *Metarhizium anisopliae* and *B. bassiana* have been shown to be repellent to insects such as termites (Mburu et al., 2013). Likewise, *E. saccharina* larvae are known to respond to different chemical signals, particularly those released by plants, with larvae either attracted to or repelled by these volatile cues (Harraca et al., 2011). *Eldana saccharina* moths can detect volatile cues that repel larvae and therefore avoid ovipositing on plants emitting certain volatiles (Harraca et al., 2011), it is thus, important to investigate *E. saccharina* larval responses towards endophytic *B. bassiana* isolates, as such responses may affect the efficacy of *B. bassiana.*

Furthermore, *B. bassiana* isolates may affect *E. saccharina* larval development and survival differently, as microorganisms have been reported to have variable interactions with insects. For instance, endophytic *Fusarium verticillioides* can increase *E. saccharina* survival and plant damage on maize plants (Ako et al., 2003). Additionally, *Fusarium* isolates within the same species (i.e.: *F. pseudonygamai)* affected *E. saccharina* larval development differently, with some isolates showing beneficial effects, while others were antagonistic (McFarlane et al., 2009). Hence, it is essential to assess interactions between endophytic *B. bassiana* isolates and *E. saccharina* when selecting an entomopathogen as a control option. Controlled laboratory biological assays (bioassays) have been widely used as primary criteria for assessment of virulence; defined as median lethal dose (LD_{50}) or median lethal concentration (LC_{50}) of infective conidia per unit area (cm^2 or mm^2) required to kill 50% of the insect test population (Hatting and Wraight, 2007; Hatting 2019).

Numerous *B. bassiana* strains have been tested in laboratory and field trials for virulence against various pests including stem borers (Legaspi et al., 2000; Rodríguez et al., 2009; Wraight et al., 2010; Gabarty et al., 2014; Ramirez et al., 2018; Bancole et al., 2022). *Beauveria bassiana* strains have been reported to have varying virulence towards insects (Trizelia, 2010) with different instar stages having different susceptibilities towards infections (Hastuli et al., 1999; Legaspi et al., 2000; Srikanth et al., 2011; Husseini, 2019). Other than an assessment of natural field pathogenicity of *B. bassiana* against *E. saccharina* larvae in *Cyperus papyrus* L. (Conlong, 1990), literature is scarce on *E. saccharina/ B. bassiana* interactions in sugarcane plants. Laboratory evaluations for virulent strains are the initial steps used to select for candidate biological control agents (Quesada-Moraga et al., 2006). To use indigenous endophytic *B. bassiana* strains in biological control programmes, their virulence towards the target pest (*E. saccharina*) must be tested, and the most virulent strain selected to target the most susceptible life stage of the pest.

Therefore, the aim of this study was to (1) Evaluate the response of *E. saccharina* first instar larvae to *B. bassiana* in choice bioassays. (2) To evaluate development and survival of *E. saccharina* larvae when fed on artificial diet containing attenuated *B. bassiana* fermented rice and (3) to select the most virulent *B. bassiana* isolates towards *E. saccharina* 3rd and 5th instar stages by quantifying LD_{50} and LC_{50} values.

3.2 Materials and Methods

Three laboratory assays were conducted to investigate the effect of 16 *B. bassiana* isolates on *E. saccharina* larvae (Table 3.1).

Mycelial squares of the fungal isolates which were stored at -20°C in 10% glycerol, were thawed and placed on Sabouraud dextrose agar (SDA) supplemented with antibiotics (50 mg/l chloramphenicol, 25 mg/l cycloheximide, 50 mg/l rifampicin and 20mg/l dodine) for 2 - 4 weeks until sporulation. The resultant colonies were used as starter cultures in each of the insect bioassays. These were: 1) Larval choice assay; 2) *Beauveria – Eldana saccharina* diet inclusion assay; and 3) Conidia topical spray bioassays.

Table 3.1: Sixteen *Beauveria* spp. isolates used in virulence experiments showing isolate names, isolation substrate and location obtained.

Isolate name	Isolation substrate/Vegetation	Collection area
P2 leaf	*Phragmites australis* leaf	Eston
S2root	Sorghum biocolor root	Mount Edgecombe
CS leaf	*Cyperus sexangularis* leaf	Eston
TL-leaf	*Typha latifolia* leaf	Mount Edgecombe
CC4 root	*Panicum maximum* Jacq root	Canema
Site 55 roots	*Panicum maximum* Jacq root	Harden Heights
CP2root	*Cyperus papyrus* root	Mount Edgecombe
SC-leaf	*Saccharum spp. leaf*	Eston
P2srem	*Phragmites australis leaf*	Eston
JT leaf	*Coix lacryma-jobi* leaf	Mount Edgecombe
NG root	*Panicum maximum* Jacq root	Eston
N41S1TI	*Saccharum* spp*. N41 Top internode Stalk 1*	Mount Edgecombe
N41S2BN	*Saccharum* spp. *N41 Bottom node Stalk 2*	Mount Edgecombe
N41S2TNa	*Saccharum* spp. *N41a Top node Stalk 2*	Mount edgecombe
N41S2TNb	*Saccharum* spp*. N41b Top node Stalk 2*	Mount Edgecombe
R568S2TN	*Saccharum* spp. *R568 Top node Stalk 2*	Mount Edgecombe

3.2.1 Larval choice assay

To evaluate the repellency of each of the *B. bassiana* isolates towards *E. saccharina* 1st instar larvae, larval choice assays were conducted according to a modified method of McFarlane et al. (2009). Sixteen McCartney bottles, each containing 5 ml distilled water and five dried maize kernels (Brennco, whole yellow maize, P.O. Box 13132, Hatfield, 0028, Pretoria, South Africa), were autoclaved at 121°C for 15 minutes. Thereafter, using aseptic techniques, the distilled water was decanted and each of the 16 McCartney bottles respectively inoculated with a sporulating square (5 x 5 mm) of the 16 *B. bassiana* isolates. The bottles were vortexed (Genie2) for 1 minute to mix the kernels with the sporulated square and were then incubated at 25°C (IncoTherm- Labotec), in the dark for 2 weeks. Control maize kernels were prepared similarly, excluding fungal culture inoculum.

The larval choice assays were carried out in 90 mm sterile plastic Petri dishes. Using forceps, two drawing pins were individually flame-sterilized (Fireboy Plus; IBS Integra Biosciences) and whilst hot, each pinpoint was punctured into opposite ends (10mm from edge of lid) of the Petri dish lid (i.e.: the pinpoints were punctured from the outer lid surface so to point inwards when the Petri dish was closed).

Following puncturing the drawing pins through the Petri dish lids; using pre-cooled flame sterilized forceps, sterile filter paper (no 1, Whatman) was pushed through the pinpoints (Figure 3.1A) to line the Petri dish lids. Thereafter, 1 ml sterile distilled water was pipetted onto the filter paper to create surface tension and minimize movement of 1st instar larvae across the lid during inoculation. Using pre-cooled flame sterilized forceps, two maize kernels: one inoculated with *B. bassiana* and one uninoculated control kernel, were aseptically mounted onto the drawing pins, by pushing the maize kernels through the pin so to touch the Petri dish lid (Figure 3.1A). Newly eclosed 1st instar *E. saccharina* larvae were obtained from the insect rearing unit (IRU) at SASRI (Mount Edgecombe, Durban). On a laminar flow bench, twenty larvae were carefully picked from their transparent plastic transportation bag using a paint brush (Cotman-Winsor & Newton, size: 0000) previously dipped in 70% ethanol and sterile distilled water. Larvae were placed in the center of each Petri dish lid.

The Petri dish base was placed on the lid and edges sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL). Petri dishes were placed in the cupboard in the dark at room temperature (± 23°C) for 24 hours. Following 24 hours inoculation, the Parafilm, and Petri dish base were removed, leaving the maize kernel mounted on the lids. The number of larvae feeding on maize kernels inoculated with *B. bassiana* isolates and on uninoculated control maize kernel was counted (Figure 3.1B and C). Maize kernels were individually lifted and removed from the pins using forceps so to count larvae on the underside of the kernel Furthermore, the kernels were dissected using a surgical blade to count larvae inside the kernel. The number of larvae that had not made a choice and were not feeding on any kernels were recorded. The experiment was replicated five times for each of the 16 *B. bassiana* isolates.

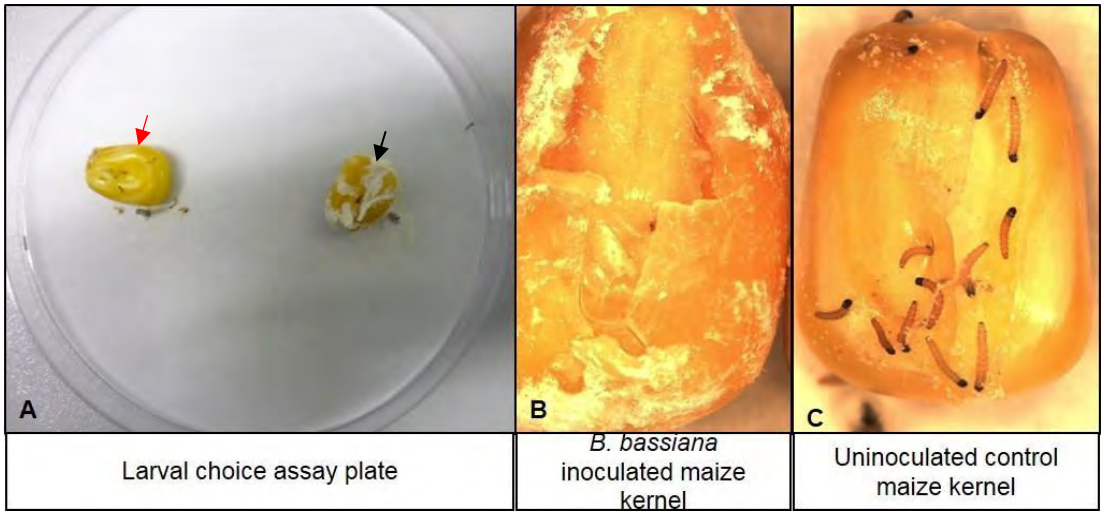

Figure 3.1: Illustration of the larval choice assay set up. (A) Uninoculated control maize kernel (red arrow) pinned adjacent to a *B. bassiana* inoculated maize kernel (black arrow). (B) *B. bassiana* inoculated maize kernel. (C) Larvae associated with uninoculated maize kernel following termination of the experiment at 24 hours.

3.2.2 *Beauveria bassiana- Eldana saccharina* diet inclusion assay

Diet inclusion assays were conducted to assess the toxicity of presence secondary metabolites of *B. bassiana* isolates to *E. saccharina* larval development, when larvae were fed on diets incorporating *B. bassiana* isolates. The assay was modified from a method described by McFarlane et al. (2009), in that *B. bassiana* isolates were individually grown on rice, followed by' incorporation into the regular *E. saccharina* diet described by Walton (2011).

3.2.2.1 *Beauveria* bassiana fermented on rice: Inoculation.

Sixteen Erlenmeyer flasks (250 ml) containing 75 g white rice grains (Aunt Caroline long grain Parboiled Rice; Tiger Brands Limited, 3010 William Nicol Drive, Bryanston) and 100 ml distilled water, were capped with cotton wool, covered with foil, and autoclaved at 121°C for 15 minutes. Once cooled (±25°C) each flask was individually inoculated with one *B. bassiana* strain in the laminar flow bench by placing a sporulating fungal square (1 x 1 cm) excised from starter cultures.

Rice was mixed with inoculated mycelial squares using autoclaved laboratory spoons to evenly distribute fungal conidia throughout the rice in the flask. Inoculated flasks were then incubated in a 25°C incubator (IncoTherm- Labotec) for 2 weeks in the dark. *B. bassiana* rice grown cultures and uninoculated control rice grains (autoclaved and cooled) were taken out of their respective Erlenmeyer flasks using autoclaved laboratory spoons and placed on sterile aluminum foil and oven dried (Scientific series 9000 oven Spectralabs) at 55°C for 24

hours. Thereafter the dried *B. bassiana* rice grown cultures and control rice were individually grounded to a fine powder using a Waring blender cleaned (wash with autoclaved distilled water, sprayed with 70% ethanol and a second wash with autoclave distilled water) between each *B. bassiana* rice culture and stored in clean, transparent zip-lock bags in the cupboard until further use.

3.2.2.2 Diet preparation

Eldana saccharina artificial diets incorporating each of the 16 *B. bassiana* isolates (previously grown on rice, dried and crashed into powder (3.2.2.1) were prepared according to Walton (2011), with the following amendments:

I. A 1L glass beaker containing 7.25 g Bacteriological agar (Merck) dissolved in 640 ml distilled water was placed on a hot stirring plate with stirrer bar until boiled. Ten milliliters of 1 M Potassium hydroxide (KOH) was then mixed into the boiling water, which, on cooling, produced a firmer, more brittle agar gel (Morton, 1979). This replaced the food grade agar normally used in the diet.

II. In a separate mixing bowl, 65 g whole wheat flour, 20 g milk powder and 25 g egg powder were mixed and added to the agar before it cooled. The whole content was mixed with a disinfected wooden spoon (cleaned with autoclave water and 70% ethanol between mixes) and the beaker then covered with tin foil and autoclaved (121°C for 15 minutes).

III. While still hot, 30 g of crushed and dried sugarcane powder (NCo376 stem crushed and dried at SASRI, Mount Edgecombe), 1 ml wheat germ oil and 65 g dried *B. bassiana* rice powder from each strain was added into each beaker and mixed with a wooden spoon, disinfected with 70% ethanol.

IV. Thereafter, a 100 ml solution (3 g yeast extract, 10 g sucrose, 1 g Wesson's salts mixture), 2.5 g ascorbic acid, 0.05 g tetracycline-HCl, and 3.5 ml mold inhibitor (4 ml phosphoric acid, 42 ml propionic acid, 54 ml distilled water) which did not have adverse effect on *B. bassiana* were added and mixed with a 70% ethanol-disinfected wooden spoon.

V. The final step in diet preparation was the mixing and addition of 1.5 g nipagin dissolved in 10 ml ethanol to inhibit *Aspergillus* spp.

VI. Boiling distilled water was added to make up 1 kg of diet.

VII. Diet representing each *B. bassiana* strain was then poured into 3 plastic multicell trays (32 activities /tray; 8 ml diet /cavity) in the laminar flow cabinet and allowed to cool.

3.2.2.3 Larval inoculation

Cooled diets incorporating 6.5% *B. bassiana* rice fermented powder was scarified with a flame-sterile laboratory forceps to allow neonate larvae to gain access into the solidified media. Newly hatched first instar *E. saccharina* larvae were collected from the insect rearing unit at SASRI and maintained at ± 25°C until inoculation. One 1st instar *E. saccharina* larvae was introduced per cavity of a multicell tray and inoculated using a fine paint brush disinfected by dipping in 70% ethanol and sterile distilled water. Each cell of the 32-cell tray was used as a pseudoreplicate, giving 32 replicates in each tray, and 3 trays, for each *B. bassiana* strain. Sago (from *Metroxylon* palms) mixed with Dithane M45 (0.2 g dithane in 500 g Sago) was placed on the surface of the diet to discourage larval escape from the cells. Dithane M45 is a fungicide and was added to prevent the growth of contaminating fungi (such as *Aspergillus* spp.) on diet surface during the 21-day rearing period. Trays were then covered with plastic cling wrap, punctured above each cell to allow air flow into and out, and incubated at the insect rearing unit larval growth rooms maintained at 28± 2 °C; 75±5% relative humidity and at 0:24 L:D photo-phase for 21 days. Thereafter, the multicell trays were removed from the insect rearing larval growth rooms. Larvae and pupae were harvested and individually weighed. Numbers of surviving larvae and pupae were similarly recorded for each diet composed from the 16 different *B. bassiana* isolates and control diets, which were not inoculated with fungal isolates.

3.2.3 *Eldana saccharina* bioassay: Conidial topical spray application

The topical spray bioassay investigated virulence of the 16 *B. bassiana* isolates towards *E. saccharina* 3rd and 5th instar larvae. These instar stages were selected based on the ecological biology of *E. saccharina* in sugarcane, with 3rd instar larvae initially boring into sugarcane and 5th instars larval stages boring and feeding inside sugarcane tissues. This experiment involved rearing *E. saccharina* larvae, preparation of fungal inoculum, spray inoculation and monitoring larvae post inoculation.

3.2.3.1 Rearing of *Eldana saccharina* larvae

To attain 3rd and 5th instar *E. saccharina* larvae, newly hatched first instar larvae were collected from the insect rearing unit at SASRI and maintained at ±25°C until use. Plastic multicell trays (32 cavity) containing 8 ml artificial diet per cell were reared according to Walton (2011) with the modification of omitting the fungicide Dithane M45 (to avoid fungicide contamination of larvae used for topical application of *B. bassiana* conidia), were inoculated with one *E. saccharina* first instar larva using a paint brush decontaminated by dipping in 70% ethanol and sterile distilled water. Thereafter, multicell trays were incubated at 28 ± 2°C; 75±5% relative humidity and at 0:24 L:D photo-phase in the IRU. To determine instar stage, every week, up to 5 weeks, larval head capsules were collected from the artificial diet surface and each well was viewed under a dissecting microscope. Using an insect mounting pin the head capsules were picked up and placed in clean labelled Petri dishes. Head capsules were sorted according to week obtained. The larval instar stages were determined by measuring the head capsule width (Figure 3.2) using a binocular light microscope as described by Way (1995). *E. saccharina* larvae were reared for 2.5 and 4.5 weeks in the insect rearing larval growth rooms to obtain 3rd and 5th instar larvae, respectively.

Figure 3. 2: Illustration of *Eldana saccharina* larval head capsules collected once a week from reared diet for 5 weeks (from left to right). Head capsule widths measured as depicted (red double arrow) to determine instar stage. The graduations at the bottom of the picture are 1mm apart.

3.2.3.2 Fungal inoculum preparations

Conidia were obtained from 3-week-old starter cultures by flooding plates with 15 ml sterile distilled water containing 0.1% Triton X-100 surfactant. The resultant conidial suspension was pipetted into sterile 50 ml plastic Corning tubes and mixed using a vortex mixer for 5 minutes to produce a homogenous conidial suspension. Actual concentrations of the conidial

suspensions (conidia/ml) were determined by using a Neubauer counting chamber and adjusted by serial dilution to 10^4-10^8 conidia/ml using sterile distilled water supplemented with 0.1% Triton-X100. Final concentrations were confirmed using the same counting chamber. The five conidial concentrations (10^4, 10^5, 10^6, 10^7, 10^8 conidia/ml) were tested within 5 hours of preparation. Conidia concentrations referred to in this chapter refer to counted conidia.

3.2.3.3 Larval spray preparations

a. Collection of 3rd and 5th instar *Eldana saccharina* larvae for inoculation

Larvae reared for 2.5 (3rd instar) and 4.5 weeks (5th instar) were removed from the larval growth rooms in the insect rearing unit and harvested by gently removing artificial diet from the larvae. Ten 3rd and 5th instar larvae were placed in separate plastic rectangular trays (21 x 32 cm diameter) with wire vented lids. To reduce mobility of larvae during inoculation, the rectangular plastic trays holding the larvae were placed in the refrigerator at 4°C for 1-2 minutes before assays were conducted.

b. Spray apparatus and inoculation platform

A Burgerjon topical spray tower was used to inoculate larvae, as described by Hatting and Wraight (2007). The spray tower was connected to a compressor by a pipe to provide a constant airflow pressure into the tower at a rate of 5 litre/min. On the top of the tower was another inoculum input pipe which drew conidial suspension into an air atomizing nozzle (Fluid Cap 2850 + Air Cap 67147-ENP) which administrated a fine mist of the conidial suspension at a constant rate into the tower. The base of the spray tower was fitted with a rotating turntable (30 rpm) driven by a 12 V battery where two 90 mm Petri dish lids with *E. saccharina* larvae were placed on opposite ends, and three agar plates (one SDA and two water agar) were placed during inoculation. Where SDA plates were used to confirm percentage germination and water agar to determine conidia dose (conidia/mm^2).

c. Inoculation of *Eldana saccharina* larvae

Third and fifth instar larvae were spray-inoculated separately. Prior to inoculation, two Petri dish lids (90 mm) were lined with filter paper using forceps. The filter paper was moistened by pipetting 100 µl sterile distilled water to absorb excess inoculum during inoculation, (i.e. inoculum that did not land on the larvae during spraying). Plastic rectangular holding trays containing *E. saccharina* larvae were removed from the refrigerator and 5 larvae were gently placed using forceps onto each of the two Petri dish lids lined with moist filter paper. These were placed diagonal to each other on the

turn table. Each larva was positioned using a paintbrush so not to shield other larvae from acquiring similar inoculum during spraying. Larvae were immediately sprayed with 15 ml aliquots of distilled water supplemented with 0.1% Triton X- 100 serving as a control. Thereafter, treatment larvae were sprayed with 15 ml conidial suspension with doses administered in ascending concentrations (5 doses ranging from 10^4 to 10^8 conidia/ml). Spray inoculations were administrated swiftly while larvae were slow moving (lethargic behavior) following cold treatment in refrigerator.

Experiments were independently replicated three times for each of the 16 *B. bassiana* isolates on different days. In each replicate, 10 larvae at each concentration were sprayed. In total, thirty 3rd instar and thirty 5th instar larvae were sprayed for each the isolates (16 x *B. bassiana* isolates x 5 concentrations x 10 larvae per concentration x 3 replicates x 2 instars = 4800 larvae). Between spraying of different isolates, the inoculum input pipe and spray nozzle were flooded with 100% ethanol for 30 seconds, followed by 10% sodium hypochlorite (NaOCl) (3.5 v/w) for 30 seconds, and sterile distilled water for 1 minute. Thereafter, SDA agar plates were placed on the turn table and sterile distilled water sprayed through the nozzle to confirm a clean inoculum inlet pipe and nozzle between spraying of the different isolates. Ten control 3rd and 5th instar larvae were sprayed with 0.1% Triton X- 100 at each replication (30 larvae at each instar).

During administration of each conidial concentration, two water agar Petri dish plates were placed adjacent to the two Petri dish lids (holding the 5 *E. saccharina* larvae) on the turntable. These water agar Petri dish plates were used to quantify conidial deposition into the spray tower during inoculum application. Conidia counts (dose count values) were expressed as the number of conidia per square millimetre (conidia/mm^2) deposited on the agar plate. Conidial dose counts were counted under the light microscope at 20x magnification for each concentration for all the 16 *B. bassiana* isolates tested. Conidia counts were on three randomly selected areas of 1 mm^2 on each water agar plate. These counts were used as estimates for conidia deposited onto *E. saccharina* per mm^2 surface area.

In addition to this, one SDA plate was placed in the center of the turntable to collect conidia deposited in the spray mist during inoculation. This was agar plate was used to calculate the percentage of viable conidia able to germinate for all the conidial suspensions used for each isolate. Percentage conidial germination was estimated by randomly inspecting 100 conidia on the SDA plate under light microscopy, after

incubation for 24 hours at 25°C (Posada and Vega, 2006). Conidia were considered germinated if the germ tube was longer than the conidial diameter (Sevim et al., 2010).

d. *Eldana saccharina* maintenance post inoculation and mortality monitoring

Following topical spray inoculations, larvae were transferred by gently moving them from the filter paper with a paint brush into rectangular plastic containers (22 x 34 cm diameter). Containers had been previously disinfected with 70% ethanol and placed under UV light for 10 minutes in a laminar flow. Containers were closed with metal gauze-ventilated lids to prevent larvae from escaping and left on the laminar flow bench to dry for 5 minutes.

Thereafter, using sterilized forceps, larvae were individually placed into plastic vials (5 x 2 cm diameter) containing artificial diet (Walton, 2011) without Dithane M45. The vials were closed with metal gauze ventilated lids and placed in the insect rearing room maintained at 25°C (75±5% relative humidity and at 0:24 L:D photo- phase). Mortality of the larval instars, exposed to the different concentrations of the *B. bassiana* isolates, and their overt mycosis was recorded every 2nd day for 4 weeks. Larvae that pupated during this period were placed in multicell trays (32 cavities) and cling-wrapped to prevent adult escape. Ventilation openings were made with a pin. Multicell trays were then incubated for 14 days in the IRU (27 ± 2 °C; 75 ± 5% RH; 8:16 L: D photophase), to record moth emergence.

Adults that failed to emerge from the puparium and larvae that died during this period were surface disinfected by immersing them in 70% ethanol for 2 minutes and air dried for 5 minutes on the laminar flow bench, placed onto SDA plates and incubated at 25°C for 7 days. SDA plates were checked daily, and overt mycosis of larvae and pupae was recorded. Insects that died of bacterial or *Aspergillus* spp. contamination were recorded. Mortality was corrected according to Abbott's formula (1925):

Abbott's corrected mortality:

= ((% mortality in treatment - % mortality in control)/ (100 - % mortality in control)) x 100

3.2.4 Statistical analyses

3.2.4.1 Larval choice assay

The number of larvae feeding on *B. bassiana* inoculated maize kernels, those found feeding on uninoculated (control) and those found around (no choice) were counted. Data were analyzed to determine the relative repellence of the individual isolates. Data was subjected to a normality test (Shapiro-Wilk test for normality) using GenStat (18th edition, VSN International, Hemel Hempstead, UK). The normal data was analysed via a one-way ANOVA (three runs) to compare larval behavior among (1) *B. bassiana* inoculated maize kernels, (2) uninoculated (control) and (3) those found around (no choice). Comparison for each *B. bassiana* strain was conducted to determine most repellent strain based on the number of larvae found in each parameter (*B. bassiana* inoculated, uninoculated maize and no choice) was likewise analyzed using a one-way ANOVA. The means were compared using Fisher's unprotected t-test (least significant difference – LSD) at the 5% level of significance. Furthermore, interactions between *B. bassiana*, uninoculated maize and no choice were investigated.

3.2.4.2 *Beauveria bassiana – Eldana saccharina* diet inclusion assay

The number of larvae and pupae surviving after rearing for 21 days in *B. bassiana* inclusion and control diets were counted. Additionally, larval, and pupal weights (g) were recorded. Data was subjected to a normality test (Shapiro-Wilk test for normality) using GenStat. Data not normality balanced was analyzed using Restricted Maximum Likelihood (REML) using the Wald statistic to determine the fixed degree of freedom (n.d.f) and Sidak post hoc test to separate the means and determine the influence of the *B. bassiana* isolates on *E. saccharina* larval survival, and development. A significance threshold of 0.05 (probability level) was used.

3.2.4.3 *Eldana saccharina* bioassay: Conidial topical spray application

The number of 3rd and 5th instar larvae that died and/or presented with overt mycosis at the different conidial concentrations for each *B. bassiana* isolates was recorded. Overt mycosis observed on dead larvae, larvae that died while moulting into pupae and larvae that pupated however failed to emerge, were recorded. The number of dead larvae that developed bacterial, *Aspergillus* spp. or yeast infections, was recorded. Abbott's formula was used to correct for mortality in the control treatments (Abbott, 1925). Percentage mortality values were calculated using Microsoft Excel. Data not normality distributed was analysed using Restricted Maximum Likelihood (REML) using the Wald statistic to determine the fixed degree of freedom (n.d.f) in GenStat, and the Sidak post hoc test was used to separate the means and determine significant differences between isolates. Probit analysis was used to

determine the relationship between log concentrations (five conidial concentrations: 10^4 to 10^8 conidia/ml) and larval response (mortality) when compared to number of larvae sprayed (30 larvae per concentration per *B. bassiana* isolate) and represented as Chi squared probability values (chi pr.). Probit was used to determine lethal concentration able to kill 50% (LC_{50}) and 90% (LC_{90}) of *E. saccharina* larvae tested for each of the 16 *B. bassiana* isolates, using GenStat Probit regression analysis.

3.3 Results

3.3.1 Larval choice assay

More *E. saccharina* larvae were found feeding on uninoculated control maize kernels than in *B. bassiana* inoculated kernels (Table 3.2). The number of *Eldana saccharina* larva found feeding on *B. bassiana* maize kernels was significantly different (P<.001; d.f.=15) across all isolates tested. Maize kernels inoculated with *B. bassiana* N41S2TNa had an average of one larva feeding on the maize, when compared to JT-leaf which had seven larvae. Repellency was 95% and 65% respectively for these isolates (Table 3.2).

A significant difference (P<.001; d.f.=2) was observed between strain and treatments (*B. bassiana* inoculated, uninoculated maize control and no choice) as fewer neonate larvae were found feeding on kernels inoculated with *B. bassiana* isolates and no choice when compared to uninoculated maize control. When comparing *B. bassiana* isolates individually, it was found that four isolates (CS-leaf and SC-leaf, NG-root, and JT-leaf) were least effective in repelling *E. saccharina* with the number of larvae found feeding on these isolates having no significant differences to control maize kernels (Figure 3.3: B and C, J and M) respectively.

Table 3. 2: Mean number of larvae found feeding on 16 *B. bassiana* maize inoculated kernels, uninoculated control and those with no choice during larval choice assays.

Isolates	Larvae feeding selection		
	Bb maize	**Maize control**	**No choice**
N41S2TNa	1^{a}	10^{abc}	4^{b}
N41 S1TI	2^{ab}	12^{abcdef}	3^{ab}
N41S2BN	2^{ab}	12^{bcdef}	4^{b}
S2root	2^{ab}	13^{cdef}	3^{ab}
TL-leaf	2^{ab}	14^{ef}	3^{ab}
R568S1TN	2^{ab}	15^{f}	1^{a}
CP2root	3^{abc}	10^{abcd}	2^{ab}
P2stem	3^{abcd}	12^{bcdef}	2^{ab}
P2leaf	4^{bcdef}	12^{abcdef}	3^{ab}
N41S2TNb	4^{abcde}	14^{def}	4^{ab}
Site 55-root	5^{cdef}	11^{abcde}	4^{b}
CC4-root	6^{def}	9^{ab}	4^{ab}
CS-leaf	6^{ef}	11^{abcde}	3^{ab}
NG-root	7^{ef}	9^{a}	3^{ab}
SC-leaf	7^{ef}	9^{abc}	2^{ab}
JT-leaf	7^{f}	9^{ab}	4^{b}

Different letters depict significant differences at the 5% test level for the given larval feeding selection within a column.

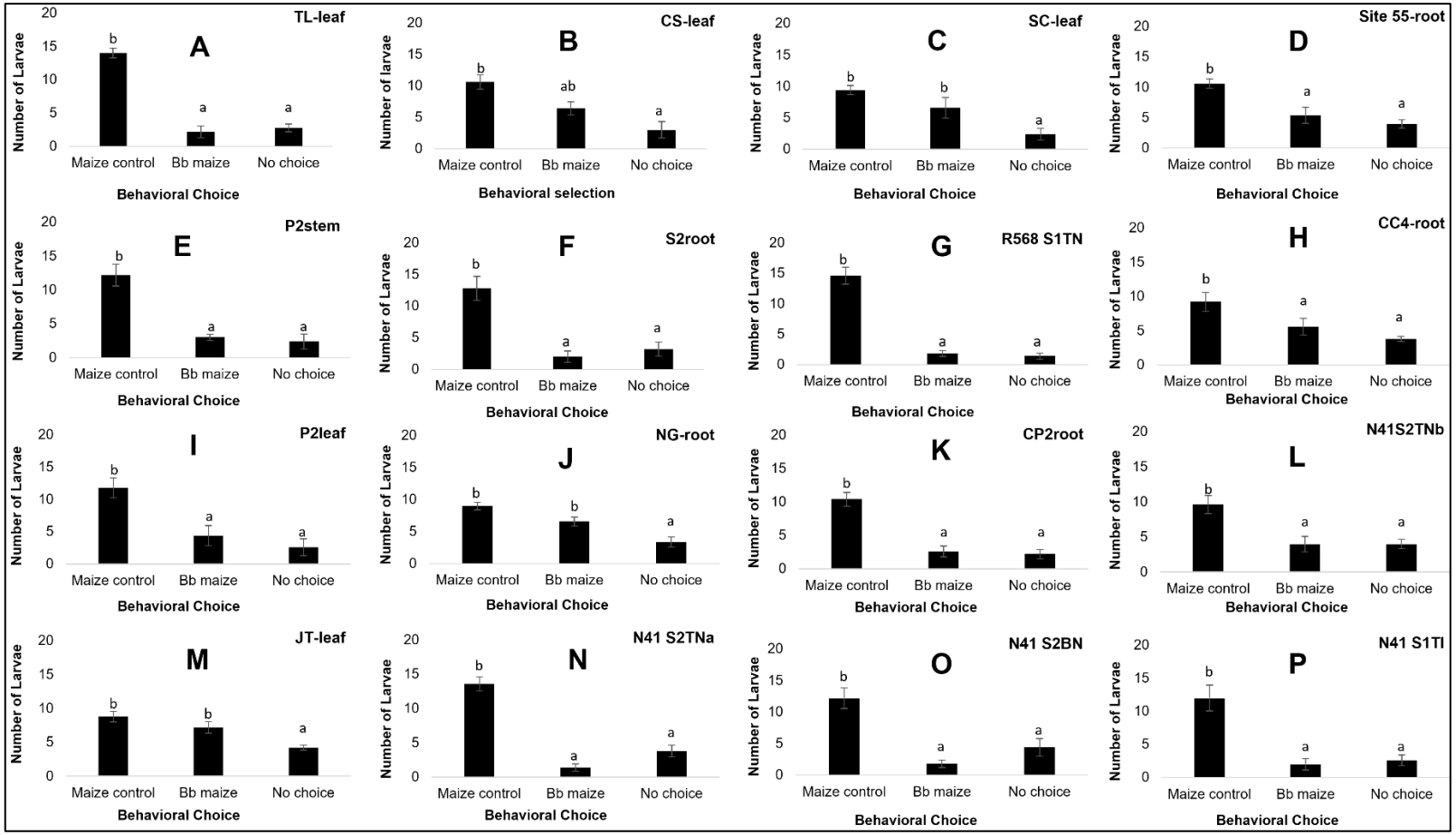

Figure 3.3: Mean number of *E. saccharina* first instar larvae feeding on *B. bassiana* inoculated, uninoculated control maize kernels and larvae that did not make a choice (No choice) during the larval choice assays when 20 larvae were placed in a choice arena. Within each graph, bars (mean ± SE of individual assays) with different letters indicate significant differences at the 5% test level.

3.3.2 *Beauveria bassiana- Eldana saccharina* diet inclusion assay

Following 21 days of rearing *E. saccharina* larvae on 16 artificial diets, each incorporating a different *B. bassiana* strain, *E. saccharina* larvae and pupae were recovered. A higher number of larvae were found in *B. bassiana* isolates (Figure 3.4A) when compared to the control which had more pupa at 21 days (Figure 3.4B). Seven isolates (TL-leaf, S2root, N41S1TI, N41S2BN, R568S1TN, N41S2TNb and N41S2TNa) had zero pupae, per tray at 21 days whilst the matching control had 25 pupae (Figure 3.4B). There was a significant difference (P<.001; n.d.f.=16) in the number of pupae found in each of the *B. bassiana* isolates tested when compared to the control. *B. bassiana* strain NG-root and CC4-root had higher numbers of pupae, (24 and 23 respectively), while some isolates (P2stem and P2leaf) had only 4 and 5 pupae respectively (Figure 3.4B).

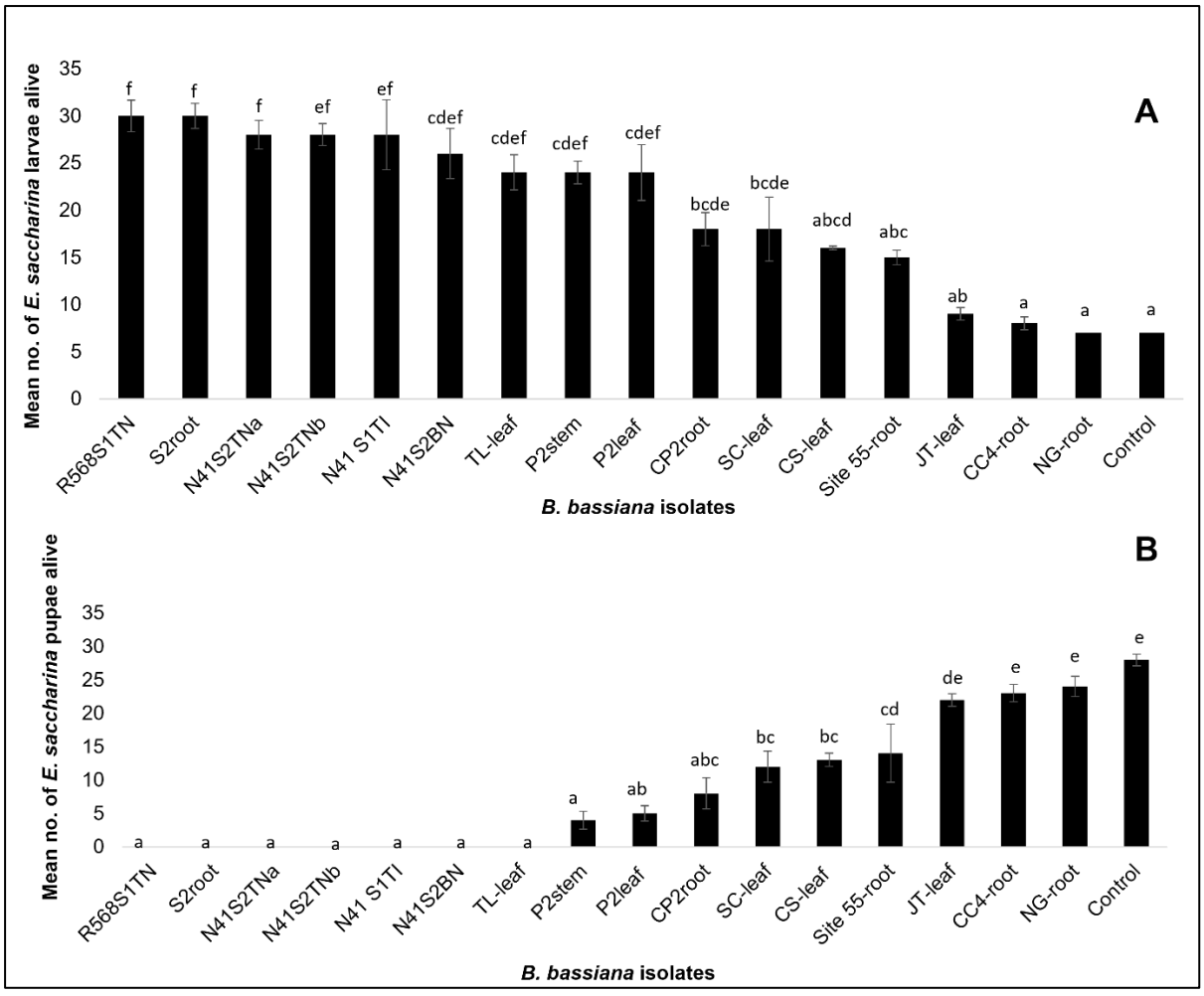

Figure 3.4: Mean number of *E. saccharina*: (A) larvae (B) and pupae after a 21-day feeding period on 16 diets incorporating different *B. bassiana* isolates. Bars (for either larvae or pupae) with different letters, differ significantly at the 5% test level. Error bars indicate the standard error of the mean.

Different larval and pupal weights were observed for *E. saccharina* reared on the different *B. bassiana* isolates (Figure 3.5). There were significant differences in the weight of larvae and pupae when fed on diets containing *B. bassiana* (P<.001; n.d.f. =16). *E. saccharina* larvae reared on uninoculated control diets, weighed on average: 0.20 g ± 0.02 (Figure 3.5A) and pupae averaged: 0.16 g ± 0.01 (Figure 3.5B). Larvae reared on diet incorporating *B. bassiana* isolates N41S1TI and TL-leaf, weighed significantly less (0.01 g ± 0.00 and, 0.04 g ± 0.01, respectively) than matching controls. Seven *B. bassiana* isolates showed antagonism to *E. saccharina* development with no pupae recovered after 21 days when reared in these diets when compared to the control diet (Figure 3.5B). These isolates resulted in low numbers and lower weights of *E. saccharina* larvae and pupae. Pupae developed in all the other nine *B. bassiana* isolates tested (Figure 3.5B).

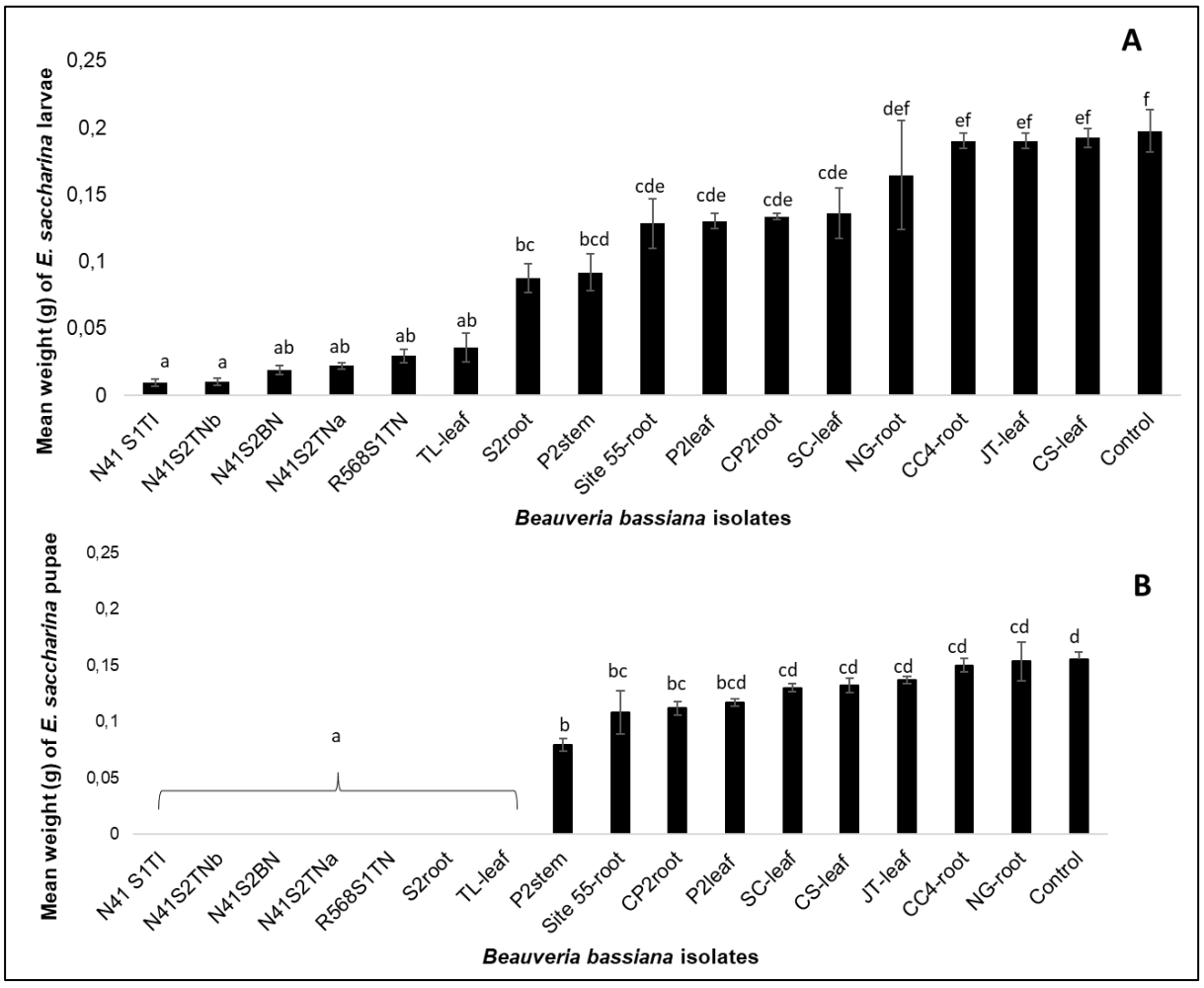

Figure 3.5: Mean individual weight (g) of *E. saccharina* larvae (A) and pupae (B) after rearing on diet incorporating 16 *B. bassiana* isolates for 21 days during dietary inclusion assays. Bars represent the standard error of the mean and different letters symbolize significant differences between isolates.

There was a significant difference (P = 0.003, n.d.f. = 16) in the number of *E. saccharina* larvae and pupae collectively alive after 21 days. Only 66% (21) larvae and pupae survived on *B. bassiana* isolates TL-leaf while control had 100% (32) survival. Survival for the remaining 15 *B. bassiana* treatments was above 80% (Figure 3.9).

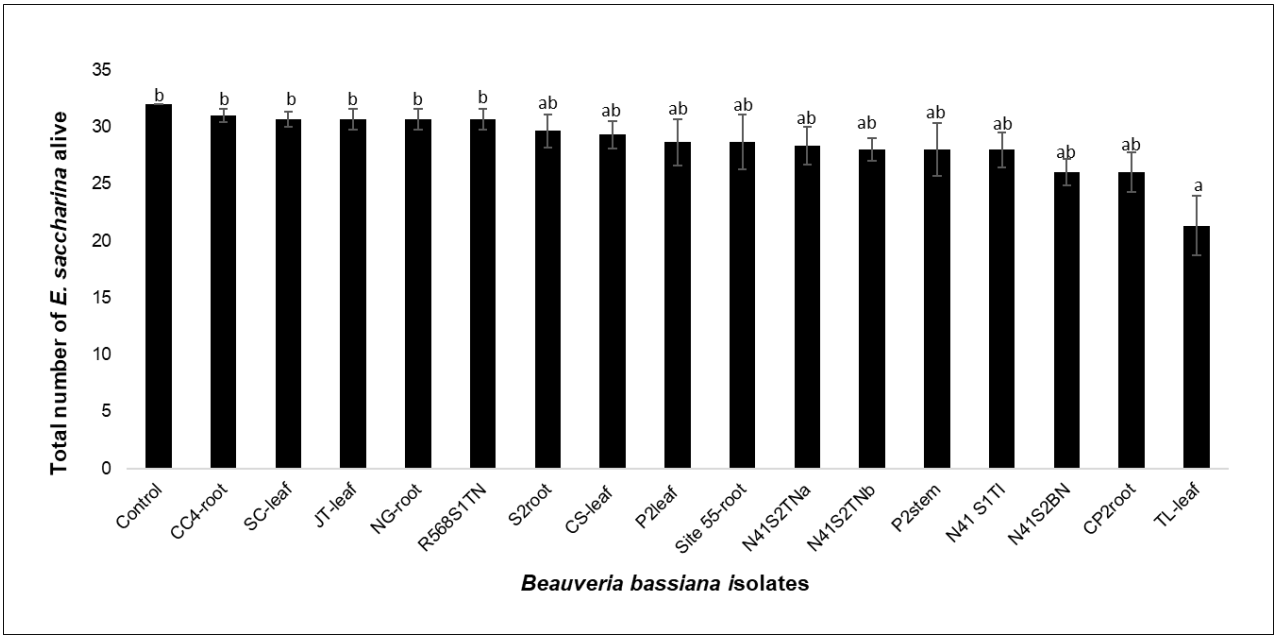

Figure 3.6: Mean number of surviving *E. saccharina* after a 21-day rearing period on diet incorporating *B. bassiana* isolates grown on rice when 32 larvae were initially inoculated. Error bars indicate the standard error of the mean and different letters above error bars symbolize significant differences between isolates.

3.3.3 *Eldana saccharina* bioassay: Conidial topical spray application

Conidial germination for all 16 *B. bassiana* isolates used in these topical spray bioassays ranged from 75-92%. Following surface disinfection of dead *E. saccharina* larvae and pupae and plating onto SDA, overt mycosis occurred on larvae (Figure 3.7) and pupae (Figure 3.8).

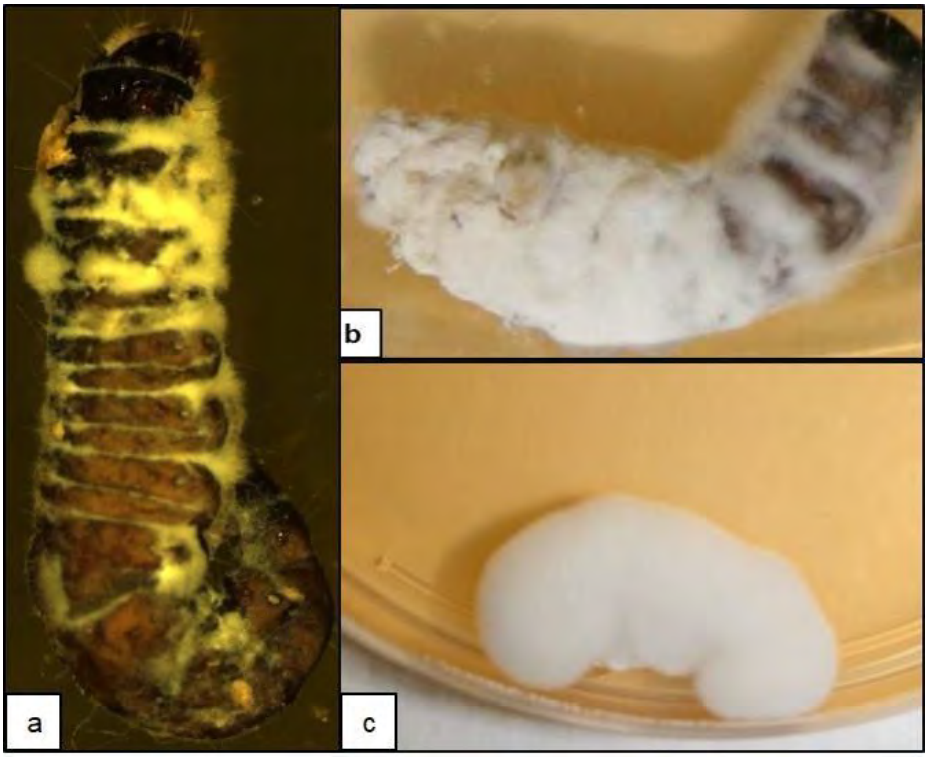

Figure 3.7: Dead overt mycosed *Eldana saccharina* 3rd and 5th instar larvae after being sprayed with *Beauveria bassiana* conidia. (a) 5th instar larvae; (b & c) 3rd instar larvae.

Eldana saccharina 3rd and 5th instar larvae that died after spraying with *B. bassiana* conidia became hard and developed mycosis (Figure 3.7 a-c). Similarly, mycosis developed on 5th instar larvae that pupated after conidial spray application, however failed to emerge into adults (moths) (Figure 3.8a-d) plus those that died and hardened whilst molting into pupae (Figure 3.8 e). Some dead *E. saccharina* did not present overt mycosis. However, these developed bacterial and fungal contamination. The mortality of control 3rd and 5th instar larvae was 27% and 20% respectively.

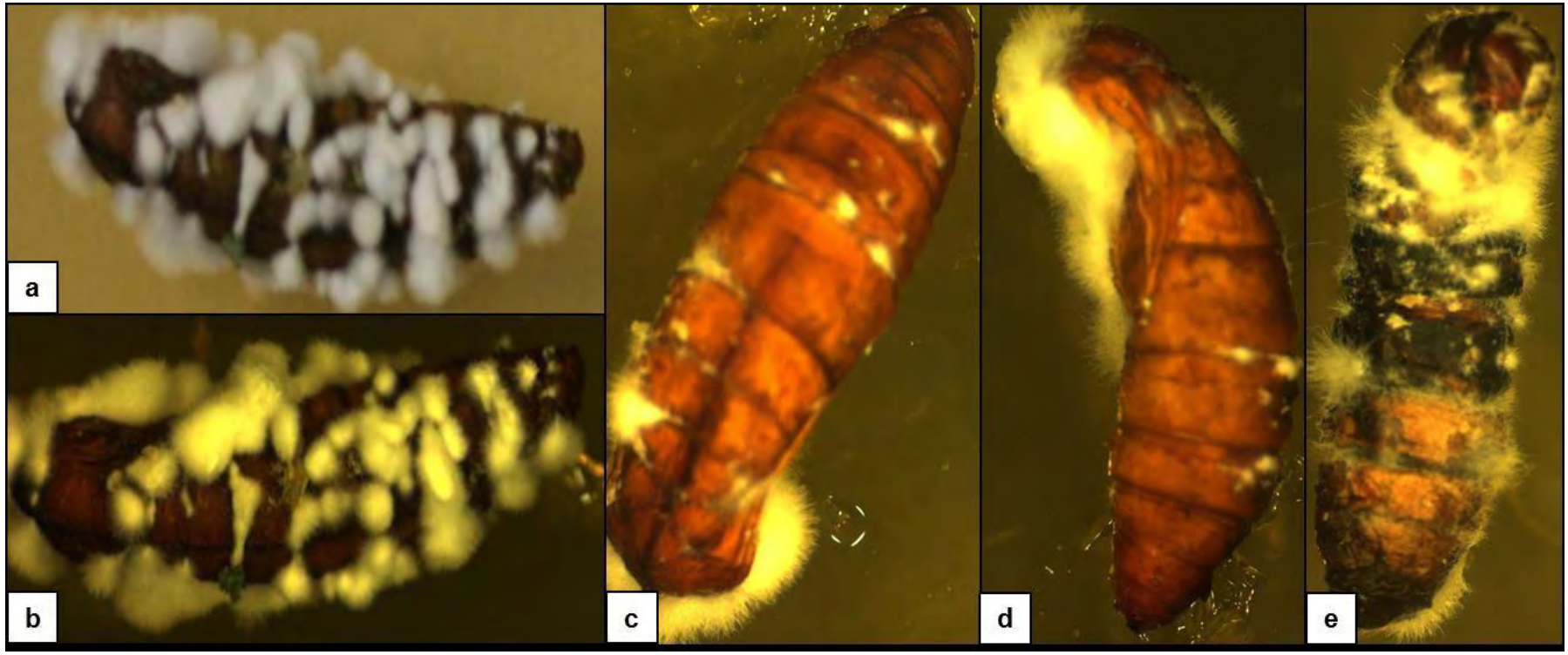

Figure 3.8: Overt mycosis of *Eldana saccharina* pupae infected with *Beauveria bassiana* after larvae were sprayed with conidia. (a & b): Larvae sprayed at 3rd instar (10^4 conidia/ml); (c & d): Larvae sprayed at 3rd instar (10^8 conidia/ml); (e) Larvae sprayed at 5th instar (10^8 conidia/ml).

a. Mortality of 3rd instar larvae

The mean percentage mortality of *E. saccharina* 3rd instar larvae at the five conidial concentrations tested is presented in Table 3.3. The 16 *B. bassiana* isolates differed significantly ($P < 0.001$; n.d.f. = 15) in their virulence against *E. saccharina* 3rd instar larvae. *B. bassiana* strain CC4-root was the least virulent, causing 59% mortality at the highest conidial concentration tested (10^8 conidia/ml) while nine *B. bassiana* isolates (S2root, SC-leaf, N41 S1TI, CS-leaf, R568S1TN, N41 S2TNa, P2leaf, P2stem and TL-leaf) caused over 70% mortality at 10^8 conidia/ml. Strain P2stem and TL-leaf caused the highest mortality above 80% at 10^8 conidia/ml. Strain TL-leaf was the most virulent, it resulted in 84±3.8% mortality (Table 3.3) when 1525±86 conidia/mm² ± SE were deposited (Tabe A1), while strain P2stem caused 82± 2.8% mortality when 1799±55 conidia/mm² ± SE were deposited in the spray tower (Table 3.3; Table A3.1). The *B. bassiana* strain JT-leaf resulted in a low mortality at 10^4 conidia/ml and decreased to zero when corrected for the mortality of controls at 27%.

Table 3.3: Mean Abbott-corrected mortality (percentages) of 3rd instar *Eldana saccharina* sprayed with 16 *Beauveria bassiana* isolates at different concentrations (conidia/ml ± SE).

	Conidia concentration (conidia/ml)					
Isolates	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**	**#P= <0.05 (Sidak)**
CC4-root	25±4.4	32±8.6	38±3.2	43±6.2	59± 6.1	a
NG-root	27±4.7	43±4.7	44±8.6	55±3.9	59± 4.3	a
JT-leaf	0	48±6.5	57±5.4	53±1.8	62±11.6	ab
Site55-root	23±8.3	38±6.9	59±4.8	63 ±6.1	67±2.4	abc
CP2root	38±4.8	50±4.2	52±2.6	57±1.2	67±9.9	abc
N41S2TNb	24±7.8	37±6.4	43±7.8	50±7.8	67±8.6	abc
N41S2BN	40±2.2	56±3.5	60±3.5	69±3.9	69±3.9	bcd
S2root	45±6.4	57±5.4	65±4.3	63±6.1	71±5.9	cdef
SC-leaf	40±2.6	58±3.0	62±4.6	69±3.40	73±4.4	def
N41 S1TI	43±5.4	57±2.9	61±3.9	69±3.9	73±3.9	def
CS-leaf	30±7.6	46±0.7	55±1.8	64±1.9	74±6.0	efg
R568S1TN	33±4.4	58±3.5	65±2.8	72±7.4	75±0.9	efg
N41S2TNa	41±6.8	59±2.6	61±3.9	73±3.9	75±0.9	efg
P2leaf	31±15.7	47±6.8	60±16.9	65±9.6	77±9.0	fg
P2stem	37±2.7	59±1.3	65±3.0	75±4.2	82±2.8	g
TL-leaf	43±2.9	52±8.4	66±5.01	79 ±3.8	84±3.8	g

#: Probability values equal or smaller than 0.05. Sidak pos hoc test separating means.

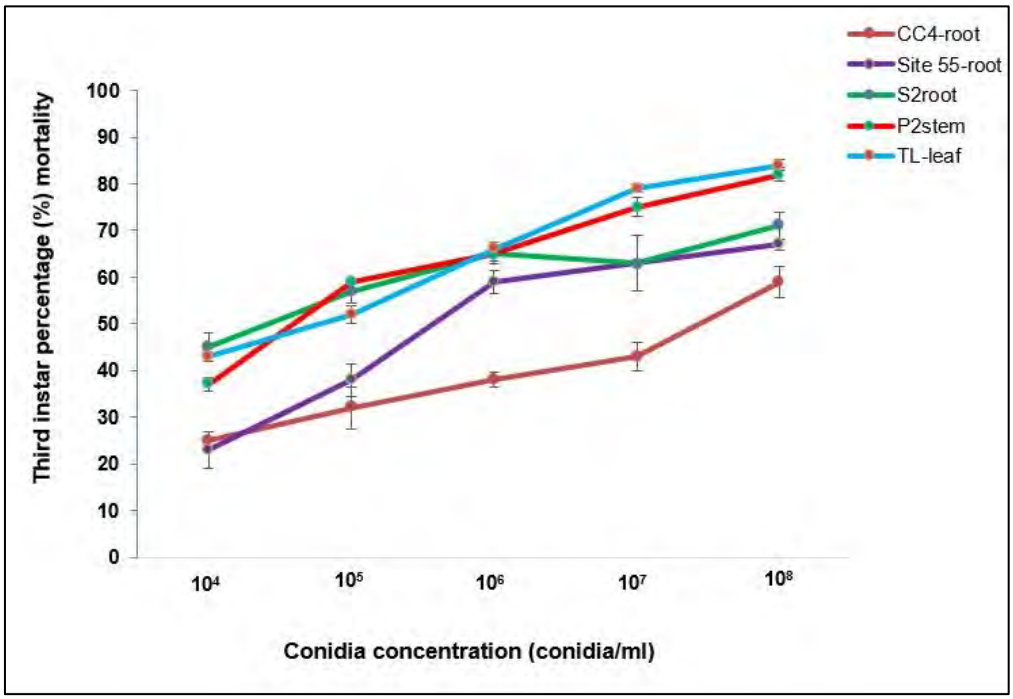

Figure 3.9: Mean percentage (%) mortalities of *Eldana saccharina* 3rd instar larvae killed at five conidial concentrations of five *B. bassiana* isolates. Bars represent mean percentage mortality ± standard errors.

Larval death and mycosis were dose dependent, higher conidial concentration resulted in increased larval mortality. There were no significant interactions between *B. bassiana* isolates and conidial concentration **(**P= 0.283; n.d.f. = 60) when tested against 3rd instar as all isolates caused increasing mycosis with increasing conidial concentration (Figure 3.9).

Probit regression analysis showed that the 16 *B. bassiana* isolates tested differed significantly (chi pr. < 0.001; d.f. 16) in their virulence towards 3rd instar larvae. No interactions (chi pr. = 0.693) were observed between *B. bassiana* strain and log concentrations, *E. saccharina* mortality increased with increase in concentration for all *B. bassiana* isolates tested. The regression analysis revealed a positive relationship between increased conidial concentrations and larval death. Using probit analysis LC_{50} and LC_{90} values of the 16 *B. bassiana* towards 3rd instar larvae obtained are presented in Table 3.4. *Beauveria bassiana* isolate TL-leaf showed the lowest LC_{50} of 1.9E+04 conidia/ml and only 23±5 conidia/mm^2 were needed to cause a mortality of 50% of the 3rd instar test population (Table 3.4). Calculated conidia deposited (conidia/mm^2) varied across isolates and dosed tested (Table A3.1).

Table 3.4: Probit predicted LC_{50} and LC_{90} values for *Beauveria bassiana* isolates tested against *Eldana saccharina* 3rd instar larvae with calculated conidia dose counts (conidia/mm^2 ± SE and (CV%)).

Isolate	Lethal concentrations				
	LC_{50} conidia/ml	S. E	#Calculated conidia/mm^2 at LC_{50}	LC_{90} conidia/ml	S. E
N41S2TNa	6.2E+05	± 3.0	20±4 (22%)	1.21E+10	± 5.2
N41S2TNb	3.0E+05	± 2.9	30±5 (23%)	5.86E+10	± 5.0
N41S2BN	9.3E+05	± 3.9	32±3 (25%)	1.82E+10	± 7.6
CC4-root	2.5E+07	± 2.9	221±10 (8%)	4.93E+12	± 4.9
CP2root	8.8E+05	± 2.8	25±2 (18%)	1.72E+11	± 4.0
CS-leaf	4.7E+05	± 2.8	49±3 (11%)	9.24E+10	± 3.9
SC-leaf	1.1E+05	± 2.9	32±4 (22%)	1.00E+10	± 3.6
JT-leaf	5.1E+06	± 2.8	127±20 (26%)	9.95E+11	± 4.4
NG-root	4.4E+07	± 2.8	175±26 (28%)	8.58E+12	± 4.4
P2stem	2.0E+04	± 3.0	17±3 (30%)	3.85E+09	± 3.5
P2leaf	6.6E+05	± 2.8	175±26 (28%)	1.29E+11	± 4.0
R568S1TN	1.5E+05	± 3.1	36±6 (29%)	3.02E+10	± 5.5
S2root	2.0E+05	± 2.8	31±3 (14%)	3.98E+10	± 3.8
Site 55-root	9.9E+05	± 2.8	31±4 (24%)	1.94E+11	± 4.1
N41 S1TI	4.9E+05	± 3.7	29±2 (18%)	9.58E+10	± 7.2
TL-leaf	1.9E+04	± 2.2	23±5 (34%)	3.73E+09	± 2.7

#: Calculated conidia/mm^2: calculated dose counts of conidia as represented in Table A3.1.

*: LC_{50} beyond tested concentration range, thus conidia/mm^2 could not be counted.

b. Mortality of 5th instar larvae

Similarly, *E. saccharina* 5th instar larvae were affected by the 16 *B. bassiana* isolates, causing death and mycosis over the five conidial concentrations tested (Table 3.5).

Table 3.5: Mean mortality percentages of 5th instar *Eldana saccharina* sprayed with sixteen *Beauveria bassiana* isolates at different concentrations (conidia/ml).

	Conidia concentration (conidia/ml)					
Isolates	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**	**#:P= <0.05 (Sidak)**
R568S1TN	9±0.0	17±4.0	20±7.0	24±3.8	36±3.8	a
N41S2TNb	9.±0.0	20±0.0	28±4.0	28±10.0	39±10.0	a
CC4-root	1±0.7	8±2.2	24±4.9	39±6.7	47±8.3	ab
S2root	25±6.5	38±6.2	49±8.7	52±10.4	55±11.9	c
JT-leaf	0	21±6.9	33±4.3	37±6.8	56±7.3	cbd
NG-root	15±3.8	38±6.8	49±4.0	51±3.8	57±11.6	cd
Site55-root	28±1.6	36±2.8	43±4.3	50±2.5	58±2.9	cd
CS-leaf	22±5.5	36±6.2	52±5.7	60±2.03	63±3.2	cde
N41S2BN	23±2.0	39±4.0	54±0.0	62±3.8	65±3.8	cde
CP2root	27±4.7	51±5.7	55±3.2	51±7.7	66±3.9	cde
P2stem	35±4.4	46±8.0	60±1.3	64±1.7	67±5.5	de
N41S2TNa	20±0.0	32±7.0	36±8.0	48±3.8	68±3.8	cde
SC-leaf	33±1.7	44±4.8	57±2.2	60±1.9	68±4.3	de
P2leaf	28±11.9	46±19.0	44±6.3	57±15.6	68±7.1	de
N41 S1TI	31±16.0	40±7.0	45±17.0	56±9.6	69±9.0	de
TL-leaf	48±4.3	62±1.3	63±6.1	71±2.9	78±5.4	e

#Probability values equal or smaller than 0.05. Sidak pos hoc test separating means.

Isolates were found to be significantly different ($P < 0.001$; n.d.f. = 15) in their virulence against *E. saccharina* 5th instar larvae, however varied significantly in their pathogenicity towards *E. saccharina* 5th instar larvae. *Beauveria bassiana* isolates (i.e.: TL-leaf, P2stem) caused higher mortality while CC4-root and NG-root resulted in low mortalities at the same conidial

concentration (10^8 conidia/ml) (Table 3.5). Percentage mortality of 5th instars by JT-leaf at 10^4 conidia/ml was reduced to zero when the percentage mortality data was corrected for the control mortality (20%). Conidial concentration (10^4-10^8 conidia/ml) was found to have a significant effect (P <0.001; d.f. = 4) on percentage mortality of 5th instar larvae, with higher concentrations killing more larvae than lower concentrations (Table 3.5 and Figure 3.10). Mortality of 5th instar larvae generally increased in a dose dependent manner, with a higher conidial concentration resulting in increased larval mortality (Table 3.5). There were no significant interactions between *B. bassiana* isolates and log concentration of conidia (P= 0.954; d.f. = 60) when tested against 5th instar larvae. Isolates generally caused increasing mortality with increase in conidial concentration (Table 3.5).

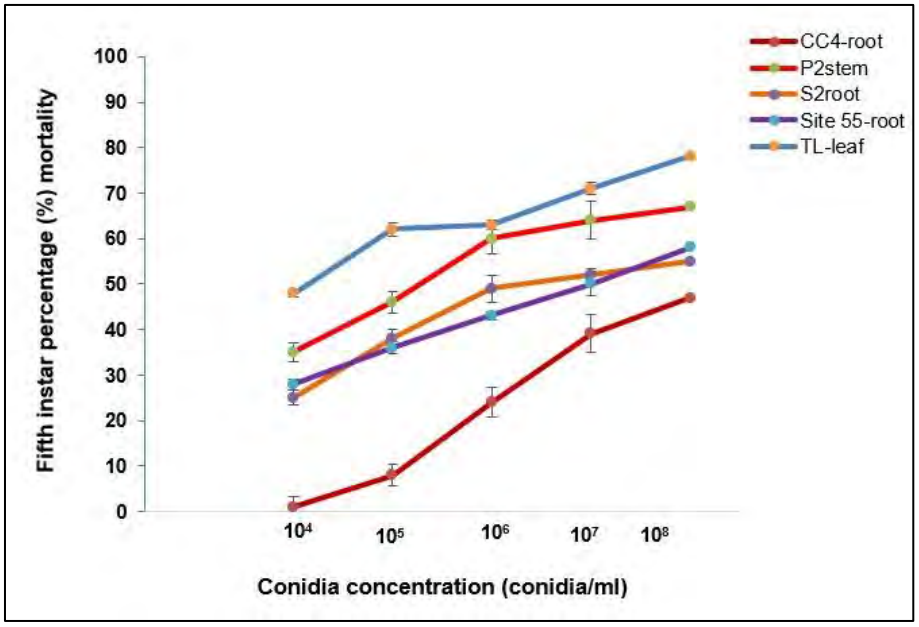

Figure 3.10: Mean percentage mortality of *Eldana saccharina* 5th instar larvae killed at five conidial concentrations of five *Beauveria bassiana* isolates. Bars represent percentage mortality standard errors.

Probit regression analysis showed significant differences (chi pr. < 0.001) for the virulence of the *B. bassiana* isolates towards 5th instar larvae. Percentage mortalities were different even when *B. bassiana* isolates were sprayed at the same conidial concentrations. No interactions (chi pr. = 0.229) were observed between strain and log concentrations as mortality of *E. saccharina* 5th instar larvae increased with increase in conidia concentration. The regression lines for isolates showed a positive relationship between increase conidial concentration and larval death. For 5th instar larvae, mortality was not always seen to increase with a 10-fold increase in concentration (ie: TL-leaf 10^5: 62±1.3%; 10^6 63±6.1%).

Using probit analysis LC_{50} and LC_{90} values of the 16 *B. bassiana* isolates towards 5th instar

larvae are presented in Table 3.6 Again the *B. bassiana* strain TL-leaf performed best as it had the lowest LC_{50} of 3.21 x 104 conidia/ml and only 23 ± 5 conidia/mm^2 needed to cause mortality of 50% of the 5th instar test population. *Beauveria bassiana* strain CC4-root and N41S2TNb showed the lowest percentage mortality of 36% and 39% respectively for 5th instar at the highest conidial concentration tested (10^8 conidia/ml). The predicted LC_{50} for these isolates was beyond the tested concentrations (Table 3.6).

Table 3.6: Probit predicted LC_{50} and LC_{90} values for *Beauveria bassiana* isolates tested against *Eldana saccharina* 5th instar larvae with calculated conidia dose counts (conidia/mm^2 ± SE and (CV%)).

Isolates	**Lethal concentrations**				
	LC_{50} conidia/ml	**S. E**	**#Calculated conidia/mm^2 at LC_{50}**	**LC_{90} conidia/ml**	**S. E**
N41S2TNa	1.6E+07	±1.09	232±11 (9%)	5.6E+12	±3.84
N41S2TNb	1.7E+09	±1.09	*	7.6E+14	±5.52
N41S2BN	3.3E+06	±1.09	32±3 (25%)	1.4E+11	±3.96
CC4-root	2.9E+09	±1.11	*	1.1E+14	±7.98
CP2root	2.2E+05	±1.08	26±4 (22%)	1.3E+11	±4.37
CS-leaf	5.4E+06	±1.08	106±6 (10%)	3.4E+11	±4.58
SC-leaf	2.0E+06	±1.09	56±5 (17%)	7.2E+09	±3.87
JT-leaf	1.1E+07	±1.10	127±20 (26%)	1.5E+12	±6.84
NG-root	2.4E+06	±1.08	61±6 (18%)	1.5E+12	±4.98
P2stem	4.9E+05	±1.08	25±4 (24%)	3.1E+11	±4.56
P2leaf	7.9E+05	±1.08	43±2 (10%)	5.0E+11	±4.68
R568S1TN	2.8E+09	±1.09	*	1.2E+14	±3.94
S2root	2.3E+06	±1.08	68±9 (25%)	3.3E+12	±5.23
Site 55-root	6.0E+06	±1.09	85±4 (9%)	1.4E+12	±4.96
N41 S1TI	2.1E+05	±1.09	29±2 (18%)	8.0E+11	±3.89
TL-leaf	2.1E+04	±1.06	23±5 (34%)	8.1E+09	±3.02

#: Calculated conidia/mm^2: calculated dose counts of conidia as represented in Table A3.1

*: LC_{50} beyond tested concentration range, thus conidia/mm^2 could not be counted.

Third instar larvae were more susceptible than 5th instars. In all the three laboratory assays conducted on the 16 *B. bassiana* isolates, strain TL-leaf, and P2stem were the best performing isolates while strain CC4-root was the least effective when compared to the other isolates tested.

3.4 Discussion

3.4.1 Larval choice assay

Tested *B. bassiana* isolates were repellent towards *E. saccharina* first instar larvae when uninoculated and *B. bassiana* inoculated maize kernels were placed in a choice arena; larvae avoided feeding on *B. bassiana* inoculated maize kernels and preferred feeding on the uninoculated kernels or did not feed at all (larvae with no choice). Work done by Meyling and Pell (2006) supports this repellent effect. When these authors placed the flower bug (*Anthocoris nemorum* L. (Heteroptera: Anthocoridae)) onto *B. bassiana* inoculated nettle (*Urtica dioica* L.) leaf surfaces, the flower bug strongly avoided the leaf surfaces. This repellent behavior was suggested to be due to insects avoiding pathogenic fungi because of chemoreception. Insects can detect volatile or contact compounds emitted by pathogenic fungi (Mburu et al., 2009; Mburu et al., 2013; Pedrini, 2022; Quesada-Moraga et al., 2022). Similarly, Zhang et al (2022) reported the entomopathogen *M. anisopliae* to produce the volatile compound phenylethyl alcohol (PEA) that caused behavioral avoidance in locusts. Repellence of insects by pathogenic fungi has similarly been reported by Mburu et al. (2009). They reported termites, *Macrotermes michaelseni* Sjölstedt (Isoptera: Macrotermitidae), to be able to detect and avoid direct physical contact with *M. anisopliae* and *B. bassiana* during laboratory olfactory assays.

Furthermore, when *E. saccharina* 1st instar larvae were exposed to uninoculated and *Fusarium sacchari* inoculated maize kernels, larvae tended to avoid feeding on *F. sacchari* inoculated kernels (McFarlane et al., 2009). These authors suggested larval repellence could be due to repellent metabolites released by *Fusarium* spp., as these fungi are known to produce volatile organic compounds (VOCs) during growth, nevertheless, contact chemoreception was not ruled out as a cause of avoidance/repellence. Neither the less, fungal species are widely reported to produce VOCs which can function as attractants or deterrents to insects (Crespo et al., 2008; Mora et al., 2017; Davyt-Colo et al., 2022). Like *Fusarium* spp., *B. bassiana* produce volatile organic compounds such as diisopropyl naphthalenes, ethanol and n-decane (Ownley et al., 2010). Recently Jalinas et al. (2022) showed that *B. bassiana* produced VOCs such as ethenyl benzene and benzothiazole repelling *Rhyncophorus ferrugineus* (Oliver) females. Therefore, it is possible that *E. saccharina* larvae avoided movement towards *B. bassiana* inoculated maize kernels due to the presence of such compounds, although the compounds

released by *B. bassiana* isolates tested in this study were not analyzed.

Different levels of repellence were observed between the sixteen *B. bassiana* isolates. *Beauveria bassiana* isolates N41S2TNa, TL-leaf and S2root showed superior repellent activity while isolates NG-root, SC-leaf and JT-leaf were the least repellent towards *E. saccharina* 1st instar larvae. The differences in repellency between the *B. bassiana* isolates in this study are similar to the results reported by Mburu et al. (2009). These authors reported that a correlation existed between repellency and virulence amongst different *B. bassiana* fungal strains. Likewise, Meyling and Pell (2006) reported that highest repellence of termites during an olfactory assay was observed with the most virulent *M. anisopliae* fungal strains. This was confirmed in this study as the most repellent strain TL-leaf was found to be again, the most virulent strain during laboratory topical spray bioassays towards *E. saccharina* 3rd instars.

3.4.2 Beauveria bassiana - Eldana saccharina diet inclusion assay

Eldana saccharina larvae reared on artificial diets incorporating dried, ground mycelia of *B. bassiana* isolates had delayed development and significantly lower body mass (g) when compared to those reared on an uninoculated control diet. For instance, seven *B. bassiana* isolates (R568S1TN, S2root, N41S2TNa, N41S2TNb, N41S1TI, N41S2BN, TI-L-leaf) significantly reduced *E. saccharina* development with no pupae recorded after 21 days while control assays had 78% pupal development. Leckie et al. (2008) reported similar deleterious effects on corn earworm, *Helicoverpa zea* (Lepidoptera: Noctuidae), larvae fed on dried, ground *B. bassiana* mycelia incorporated diets, with larvae having low weights and delayed development. They suggested delayed development to be attributed to the toxicity of mycelia and fungal metabolites which have antifeeding activities. Isolates of *B. bassiana* have been demonstrated to produce toxic metabolites such as beauvericin, bassianolide, and isarolide which have insecticidal activities (Pedrini, 2022), therefore deleterious effects could result when ingested by insects (Leckie et al., 2008).

Reduced body mass and development of *E. saccharina* larvae may have as well been attributed to starvation or feeding avoidance as a defensive response. Thomsen and Eilenberg (2000) and Leckie et al. (2008) reported that insects can avoid feeding on diet containing fungal mycelia. Insects can induce early diapause and delay development as a defense strategy in response to unfavourable conditions such as temperature, chemicals, food depletion and infection. They can trigger physiological changes to adapt to these stressors (Perić-Mataruga et al., 2006; Sau et al., 2022). Some Lepidoptera species have been reported to induce onsets of larval diapause in response of environmental stressors and it is hypothesized that stress-induced diapause results in insects switching on mechanisms of passive defense against

damage, by temporarily blocking growth (Perić- Mataruga et al., 2006). Therefore, it is possible that the presence of *B. bassiana* secondary metabolites in *E. saccharina* artificial diet resulted in larval antifeeding and reducing food consumption as a defensive mechanism. This therefore reduced overall larval weights and delayed pupal formation when larvae were fed on diet containing grounded *B bassiana* grown on rice.

Deleterious effects of the fungal diet on *E. saccharina* depended on the fungal isolate incorporated. *E. saccharina* fed on six isolates (N41S1TI, N41S2TNb, N41S2BN, N41S2TNa, R568S1TN and TL-leaf) had lower larval mass (g) and developed slower than those fed on CC4-root, JT-leaf, and CS-leaf. Leckie et al. (2008) explained the differences between fungal isolates to cause detrimental effects on insect to depend on the amounts of mycotoxins produced by each isolate. Several entomopathogenic fungi have been reported to produce variable toxic metabolites or volatile organic compounds (Strasser et al., 2000; Hummadi et al., 2022). These reports may explain variation amongst *B. bassiana* isolates used in this study.

3.4.3 Eldana saccharina bioassay: Conidial topical spray application

Topical spray bioassays showed all sixteen *B. bassiana* isolates to be virulent to *E. saccharina* 3rd and 5th instar larval stages. Faraji et al. (2013) reported *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) larvae to be highly susceptible to five *B. bassiana* isolates. After dipping *E. kuehniella* larvae in *B. bassiana* conidial suspensions (10^8 conidia/ml) for 5 seconds, they reported the *B. bassiana* isolates to cause more than 80% mortality of larvae. Additionally, Legaspi et al. (2000) tested the virulence of a *B. bassiana* (strain GHA) towards the Mexican rice borer, *Eoreuma loftini* (Dyar) (Lepidoptera: Pyralidae), an important pest of sugarcane in North and Central America, and the sugarcane borer, *D. saccharalis*. Both stalk borers were found to be susceptible to *B. bassiana*, with *B. bassiana* causing 1st, 2nd, and 3rd instar larval mortality. Furthermore, Wraight et al. (2010) reported susceptibility of eight lepidopteran species to forty-three *B. bassiana* isolates during topical spray laboratory bioassays. Likewise, *Chilo partellus* (Lepidoptera: Pyralidae) 2nd instar larvae were reported susceptible to *B. bassiana* isolates, with isolates causing over 90% mortality of larvae after conidial spray applications (Tefera and Pringle 2003a, 2003b). These studies, all demonstrated the ability of *B. bassiana* isolates to cause mortality to Pyralidae insect pests and match the mortality results obtained in this study.

In this study, *B. bassiana* isolates varied in virulence towards *E. saccharina* larvae, causing significantly different mortality levels in 3rd and 5th instar larvae and different LC_{50} values. Previous studies have likewise reported variation in virulence between entomopathogenic fungal isolates (Hicks et al., 2001; Safavi et al., 2010; Golzan, et al., 2023). Mburu et al. (2013)

reported that intra-specific differences in virulence between two *B. bassiana* isolates in their study were due to genotypic differences. Similarly, in this study *B. bassiana* isolate NG-root was part of a separate branch of *B. bassiana* isolates (the same node) during phylogenetic analysis caused low virulence against 3rd instar larvae and was closely related to *B. bassiana* CC4-root (Figure 2.3) which also had low virulence to *E. saccharina*. Differences in virulence between *B. bassiana* isolates has further been reported to be related to physiological characteristics such as differences in conidia surface proteins (Zhang et al., 2011), conidia germination (Trizelia, 2010; Mohammadbeigi, 2013), variation in the production of polymer hydrolyzing enzymes, toxic metabolites (Safavi et al., 2010 Apirajkamol et al., 2023) and differences in the susceptibility of the isolates to lose their virulence following successive *in vitro* sub-culturing (Hatting and Wraight, 2007; Mohammadbeigi, 2013). *Beauveria bassiana* isolates in this study had varying conidial germination percentages (75 - 92%) and conidial concentration counts which may have contributed to the isolate's variation in virulence towards *E. saccharina*.

Pooled values of estimated median lethal concentrations required to kill 50% of the tested *E. saccharina* population in this study were 48 conidia/mm^2 and 57 conidia/mm^2 for 3rd and 5th instar respectively. These results are similar to those obtained by Vandenberg et al. (1998) who reported LC_{50} of 52 and 61 conidia/mm^2 after spraying two *B. bassiana* isolates against the diamondback moth, *Lymantria dispar* (Lepidoptera: Plutellidae). Results obtained in this study were, however, slightly lower than those reported by Hatting and Wraight (2007). When spraying *B. bassiana* conidia against an aphid species (Hemiptera) they obtained an LC_{50} of 83 conidia/mm^2. This slightly higher LC_{50} value may be attributed to the rigid exoskeleton cuticle of aphids, when compared to soft bodied lepidopteran larvae. Therefore, aphids may be more resistant to *B. bassiana,* requiring higher conidial concentration to obtain mortality.

Median lethal concentrations (LC_{50}) reported by Wraight et al. (2010) were apparently even lower (LC_{50} of <5 conidia/mm^2). They sprayed 43 *B. bassiana* strains against eight lepidopteran pests of vegetable crops. The lowest LC_{50} values obtained in this study; 23 ± 5 conidia/mm^2 for *B. bassiana* strain TL-leaf and 20±4 conidia/mm^2 for N41S2TNa on 3rd instar respectively. However, these authors highlighted that their exceedingly low LC_{50} was due to technical artifacts of the assay system used, providing highly favourable conditions for inoculation and infection, albeit using a Burgerjon topical spray tower as used in this study. Therefore, these differences may be attributed to conidial solution sprayed into the chamber and the deposition rates of conidia. Souza et al. (1999) attributed differences in their LC_{50} values to the antomophysiological differences between insects and the ability of entomopathogenic isolates to adhere and germinate on insect cuticle after conidia application.

Third instar *E. saccharina* larvae were found to be more susceptible to *B. bassiana* infections than 5th instars. Similarly, Hastuli et al. (1999) observed 1st and 2nd instar larvae of *Paropsis charybdis* (Coleoptera: Chrysomelidae), a pest of eucalyptus, to be more susceptible to three *B. bassiana* isolates while older larvae and adults were more tolerant to infections. Similarly, Apirajkamol et al. (2023) attributed difference in susceptible of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) 3rd and 6th instars to *B. bassiana* isolates to be due to older instars having a more developed immune system and thicker skin. Some unexpected variations in percentage mortality for some *B. bassiana* isolates (i.e., CP2root), a smaller increase in percentage mortality with a 10-fold increase in spore counts (10^7 to 10^8) for some of the tested *B. bassiana* isolates in this study were observed. In some instances, higher conidial concentrations resulted in a drop in larval percentage mortality for 5th instar larvae. When *B. bassiana* isolate CP2root was applied to 5th instar larvae, a higher mortality was obtained at 10^6 conidial concentrations and a lower mortality at a 10^7 conidia/ml concentration. Furthermore, a three percent mortality increase was observed with a 10-fold increase in conidial concentration for *B. bassiana* isolate CS-leaf at concentrations of 10^7 and 10^8 conidia/ml against 5th instar larvae. These variations were not expected as a positive correlation is reported to exist between conidial concentration and percentage mortality of insects (Hastuli et al., 1999). Hatting and Wraight (2007) assumed that differences in insect age may attribute to variation in bioassay results. However, in this study all larval instar stages were tested and verified before the bioassay was conducted as *E. saccharina* head capsules were measured according to Way (1995) and insects used were all at the same instar stages (i.e.: 3rd or 5th). Nevertheless, it was difficult to assess when larvae had moulted into that instar stage. Therefore, some degree of variation may have resulted from spraying 3rd and 5th instar larvae having recently moulted into those stages, while some larvae may have been sprayed just before moulting from 5th to 6th instar stages. In the latter case, cuticles might be expected to have a greater degree of sclerotization, which could increase resistance to infection (Ekesi et al., 2002). In addition, moulting has been shown to remove fungal conidia when insects moult their skins. Larval moulting soon after fungal spray has been reported to interfere with fungal penetration as insect shed their exoskeletons, along with any applied conidia (Vandenberg et al., 1998; Hatting, 2012, Ortiz-Urquiza and Keyhani, 2013).

In this study, many 5th instar larvae were found to pupate soon after spraying with the *B. bassiana* isolates. This pupation subsequently resulted in lower mortality percentages for 5th instars. Similar to this study, Hatting (2012) reported acceleration of pupation by cotton bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) larvae, following topical applications of *B. bassiana* and *Metarhizium rileyi* conidial suspensions. This author attributed the acceleration of pupation to be linked to chitinase enzymes which break down the insect cuticle during the infection and penetration of entomopathogens. This penetration process acts

as a stress factor, which may induce earlier pupation. Early pupation of insects has been suggested to be a defense mechanism of insects to avoid external stress such as infections (Hatting, 2012). Therefore, an increase in conidial concentration may have resulted in increased stress and might have triggered early pupation of 5th instar larvae, before the fungus could become established (Vandenberg et al., 1998; Wraight et al., 2010). Moreover, Ekesi et al. (2002) and Apirajkamol et al. (2023) elaborated that onsetting cuticular sclerotization via melanisation of the exoskeleton speeds up wound healing as an evolved defence mechanisms for pupae. Thereafter, explaining that older instar stages have developed innate immune system thus increasing resistance to fungal penetration. This may explain the higher numbers of pupae in 5th instars which subsequently developed into moths thereby reducing percentage mortality results in the bioassays performed.

Control mortalities of 27% and 20% were obtained in 3rd and 5th instar bioassays respectively. Bacterial, yeast and *Aspergillus* spp. contamination developed in artificial diet during the bioassay experiments. Higher mortalities due to contamination were observed for 3rd instar than in 5th instar. *Eldana saccharina* artificial diet contains preservatives and antimicrobial constituents (i.e.: Dithane M45 and sodium propionate) that minimize microbial development during the rearing process. Dithane M45 (Mancozeb) is included in the *E. saccharina* artificial diet as a preservative and antifungal component. During this study Dithane M45 was omitted due to the antifungal activity of this compound on *B. bassiana* as this might in turn impact infectivity on sprayed larvae. This may have allowed the development of spoilage microorganisms in the diet affecting the results by causing high control mortality. Wraight et al. (2010) similarly reported development of *Aspergillus* spp. contamination in their artificial diets, in the absence of antimicrobial (fungicidal) ingredients in their larval diets during bioassay experiments.

In conclusion, *B. bassiana* isolates originating as endophytes from *E. saccharina* host and sugarcane plants varied in their virulence toward *E. saccharina* larvae. Therefore, these results highlight the importance of laboratory bioassay-based screening to select sufficiently pathogenic isolates. Third instar stages of *E. saccharina* were more susceptible than 5th instar stages. The results showed that the endophytic *B. bassiana* isolates were virulent to *E. saccharina* and are therefore potential biological control agents for *E. saccharina*. Whilst the *B. bassiana* strain TL-leaf appears the most pathogenic towards *E. saccharina*, the most suitable isolate for further development must not only be pathogenic, however, it must too be capable of limiting endophytic *Fusarium* spp. which associate with *E. saccharina* in sugarcane. Persistent of endophytic colonization of sugarcane plants to target the internal feeding of *E. saccharina* inside the stem must likewise be investigated.

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Appendix

Table A3.1: Mean dose counts of conidia (conidia/mm^2) with standard error (coefficient of variation) deposited in each concentration on sampling plates during spray tower assay for 3rd and 5th instar *E. saccharina* larvae.

Calculated conidia deposition (conidia/mm² ± SE) (CV) per isolate at each concentration					
Isolates	**104**	**105**	**106**	**107**	**108**
TL-leaf	23±5 (34%)	39±5 (25%)	85±6 (13%)	220±12 (9%)	1525±86 (10%)
P2stem	17±3 (30%)	25±4 (24%)	104±6 (12%)	548±31 (11%)	1799±55 (5%)
S2root	18±2 (16%)	31±3 (14%)	68±9 (25%)	214±18 (14%)	1536±130 (18%)
P2leaf	15±2 (18%)	43±2 (10%)	93±7 (24%)	181±4 (4%)	1510±30 (3%)
CC4-root	11±1 (19%)	26±4 (22%)	57±5 (17%)	221±10 (8%)	1375±60 (7%)
Site55-root	20±2 (20%)	31±4 (24%)	85±4 (9%)	309±13 (7%)	1733±68 (7%)
JT-leaf	14±3 (33%)	20±3 (22%)	34±5 (22%)	127±20 (26%)	1269±82 (11%)
CS-leaf	22±2 (18%)	49±3 (11%)	106±6 (10%)	209±17 (14%)	2186±27 (2%)
NG-root	11±2 (35%)	38±5 (22%)	61±6 (18%)	175±26 (28%)	1375±48 (6%)
SC-leaf	14±2 (28%)	32±4 (22%)	56±5 (17%)	136±8 (11%)	1262±98 (14%)
CP2root	8±1 (31%)	25±2 (18%)	36±6 (29%)	124±18 (22%)	1238±42 (6%)

CHAPTER 4

Fusarium spp. x *Beauveria bassiana* interaction

Abstract

Species of the genus *Fusarium* form complex relationships with sugarcane, existing as sugarcane endophytes, and plant pathogen, yet also forming beneficial or antagonistic relationships with the sugarcane stalk borer *Eldana saccharina* Walker. Control of *Fusarium* spp. infections is essential, as the subsequent red discoloration of sugarcane tissues following *E. saccharina* boring causes a decline in sugarcane sucrose quality. The potential of two *B. bassiana* (TL-leaf and N41S1TI) isolates as control agents for four *Fusarium* strains (*F. sacchari:* PNG40, MN57a and *F. pseudonygamai*: SC17, ZN7) was investigated *in vitro*. When *B. bassiana* s.l. isolates were grown in *Fusarium* spp. culture filtrate (3.6 w/v%) and vice versa, both genera produced significantly lower percentage biomass. *Beauveria bassiana* isolate N41S1TI and *Fusarium* SC17 stains achieved lower percentage biomass than *B. bassiana* TL-leaf and *Fusarium* PNG40 strains, indicating differences in the degree of susceptibility and resistance to secondary metabolites to be strain-dependent. The growth pigmentation in broth of *Fusarium* sp. strain PNG40 changed from purple to yellow when grown in 3.6 w/v% *B. bassiana* culture filtrate while control broths remained purple, highlighting the potential of *B. bassiana* to affect the metabolic processes of *Fusarium* spp. Plate co-culture assays revealed both genera to have the ability to limit each other's growth. *Fusarium* spp. strains grew significantly faster than *B. bassiana* when radial growth was measured every 3rd day for 12 days. A demarcation line was observed when *F. sacchari* strains (MN57 and PNG40) were grown in co-culture with *B. bassiana* isolates. Deferred inhibition was likewise observed when *B. bassiana* isolates were grown in co-culture with *F. pseudonygamai* strains (ZN7 and SC17), highlighting the potential of these strains to use different antagonism strategies. Results from this study show that antagonisms may exist between the tested *B. bassiana* and *Fusarium* spp. strains.

4.1 Introduction

Fungi form complex ecological interactions in agricultural systems, i.e., with soil, plants, insects, and other microorganisms (Gentry *et al*., 2014; Ishaq, 2017; Bahram and Netherway, 2022). In plants, they occur as endophytes (living asymptomatic inside plant tissues), and as epiphytes (on the surface of the plant), or as latent pathogens with the ability to form pathogenic associations (Rodriguez *et al*., 2009; Alam *et al*., 2021). Furthermore, fungi form convoluted interactions with insects, which can be beneficial, e.g., insect attraction, improved insect development, and improved survival. (Schulthess et *al*., 2002; Ako *et al*., 2003; McFarlane *et al*., 2009; Blacutt *et al*., 2018; Nicoletti and Becchimanzi, 2022). Moreover, fungi have been further reported as entomopathogens, able to infect and kill insects (Teetor-Barsch and Roberts, 1983; Kurtz et al., 2010; Pelizza *et al*., 2011; Sharma and Marques, 2018; Feng *et al*., 2023).

In sugarcane, the most profit-limiting stem borer pest is *Eldana saccharina* Walker, it forms ambiguous associations with *Fusarium* species within the plant, which propagate damages and economic losses resulting from the borer pest. *Fusarium* spp. have been reported to form dynamic interactions with sugarcane and maize plants, existing as both an asymptomatic endophyte and a pathogen (de Lamo and Takken, 2020; Thio *et al*., 2021). In sugarcane, *Fusarium* spp. such as *F. sacchari* (PNG40) form entomopathogenic associations, while *F. pseudonygamai* (SC17) forms beneficial associations with *E. saccharina* (McFarlane *et al*., 2009). *Eldana saccharina* is difficult to control using conventional control strategies; thus, the potential adaptation of an endophytic entomopathogenic fungus like *Beauveria bassiana* to control the internal feeding stage of *E. saccharina* within the plant is seen as a viable option. It is, however, essential to understand the interactions the potential biological control agent may have with the plant, pests, and plant pathogens (Kaur *et al*., 2010).

Beauveria bassiana can naturally occur as a non-pathogenic endophyte in plants (Arnold and Lewis, 2005; Rajab *et al*., 2023). In addition, *B. bassiana* has been artificially inoculated into several plants and has enhanced plant defences against insects (Wagner and Lewis, 2000; Vidal and Tefera, 2013; Bancole *et al*., 2022). The control of different insect pests using *B. bassiana* has been widely reported in various crops such as maize, sorghum, tomato, rice, and banana plants (Wagner and Lewis, 2000; Cherry *et al*., 2004; Vidal and Tefera, 2013; Mascarin and Jaronski, 2016; García-Estrada *et al*., 2016; Bancole *et al*., 2022). Moreover, antagonistic *in vitro* and *in vivo* interactions between *B. bassiana* and several plant pathogens such as *Botrytis cinerea* (Sui *et al*., 2023), *Fusarium oxysporum* (López-López *et al*., 2023), *Pythium* sp. (Bamisile *et al*., 2018), and *Rhizoctonia solani* (Ownley *et al*., 2004; 2008) have been reported. Further benefits that have been reported include enhanced plant growth and yield promotion (Raad *et al*., 2019; Dara, 2019; Quesada-Moraga *et al*., 2022).

Microorganisms interact with each other and produce an array of secondary metabolites during growth, which assist in competition and survival within their ecological niche (Akram *et al*., 2023). *Fusarium* spp. and *Beauveria* spp. have been reported to produce an array of secondary metabolites during growth, which play a pivotal role in their survival and ability to colonize their hosts (Demain and Fang, 2000). In addition, they both have been reported to occur naturally in sugarcane (McFarlane *et al*., 2009; Donga *et al*., 2021). Therefore, understanding secondary metabolite production and interactions between *Fusarium* spp. and *Beauveria* spp. is essential if *B. bassiana* is to be used as a biological control of *Fusarium* spp. in sugarcane.

The successful employment of *B. bassiana* as an endophyte for biological control is dependent on the ability of the biocontrol agent to compete with endophytes already in the plant (Akram *et al*., 2023). Moreover, the effectiveness of *B. bassiana* as a dual control agent against *E. saccharina* and plant pathogenic *Fusarium* spp. is dependent on the entomopathogenicity against *E. saccharina* and the antagonism towards *Fusarium* spp. According to Ownley *et al.* (2004), endophytes that outcompete and antagonize other microorganisms have a higher chance of establishing endophytic based control. Hence, the aim of this study was to investigate the interactions and competition that may exist between *B. bassiana* and *Fusarium* spp. strains *in vitro* using culture filtrate and co-culture assays.

4.2 Material and methods

To evaluate the interactions between *B. bassiana* (TL-leaf and N41S1TI) and *Fusarium* spp. strains *F. sacchari* (PNG40, MN57a) and *F. pseudonygamai* (SC17 and ZN7), two *in vitro* assays were conducted: 1) Culture filtrate; and 2) Co-culture assays. Mycelial squares (1 x 1 cm) of *B. bassiana* and *Fusarium* spp. strains (Table 4.1) stored at -20°C in 10% glycerol were thawed and placed on Sabouraud dextrose agar (SDA) (Merck, Sigma Aldrich, Johannesburg, South Africa) for 2-4 weeks at 25°C to grow *B. bassiana* and Potato Dextrose Agar (PDA) (Merck, Sigma Aldrich, Johannesburg, South Africa) for 1-2 weeks at 28°C to grow *Fusarium* spp. strains. The resultant colonies were used as starter cultures in each of the interaction assays.

Table 4.1: *Beauveria bassiana* and *Fusarium* spp. fungal isolates used during experiments.

Isolate code	Species	Cultivar	Isolation substrate	Location, ZA	*E. saccharina* interaction	Isolator
N41S1TI	*B. bassiana*	Sugarcane N41	Healthy sc* stem tissues	Mount Edgecombe	Entomopathogenic	NS Memela
TL-leaf	*B. bassiana*	*Typha latifolia*	Healthy *T. latifolia* leaf	Mount Edgecombe	Entomopathogenic	NS Memela
PNG40	*F. sacchari*	Sugarcane N30	*E. saccharina* damaged sc* tissue	Pongola	Antagonistic	McFarlane *et al*. (2009)
MN57a	*F. sacchari*	Sugarcane N16	Healthy sc* tissue	Midlands North	Antagonistic	McFarlane *et al*. (2009)
SC17	*F. pseudonygamai*	Sugarcane N41	Pokkah boeng infected sc* stem tissue	South Coast	Beneficial	McFarlane *et al*. (2009)
ZN7	*F. pseudonygamai*	Sugarcane N41	*E. saccharina* damaged sc* tissue	Zululand North	Beneficial	McFarlane *et al*. (2009)

sc*=sugarcane

4.2.1 *Fusarium* spp. and *Beauveria bassiana* culture filtrate

Two culture filtrate (CF) concentrations (1.8 w/v% or 3.6 w/v%) were prepared to evaluate the effect of *Fusarium* spp. CF on *B. bassiana* growth and *B. bassiana* CF on *Fusarium* spp. growth in terms of biomass production. Two *Fusarium* spp. strains (*F. pseudonygamai:* SC17 and *F. sacchari:* PNG40) and two *B. bassiana* (TL-leaf and N41S1TI) were used in these assays.

a. Culture filtrate preparation

Culture filtrates were prepared as described by Mahlanza *et al.* (2013). Agar-grown *Fusarium* spp. (F. *pseudonygamai*: SC17 and *F. sacchari*: PNG40) and *B. bassiana* (TL-leaf and N41S1TI) starter cultures were excised, using flame sterilized scalpel blades to cut a mycelial square from the leading edge of each colony. Mycelial squares (1 x 1 cm) were individually transferred into Erlenmeyer flasks containing 250 ml of autoclaved (121°C for 15 mins and cooled at 25°C) Potato dextrose broth (PDB) (Sigma Aldrich, Johannesburg, South Africa). The cultures were incubated for 7 days in a shaker (Lab Companion Incubator Tabletop) at 145 rpm at 25°C for *Fusarium* spp. and *B. bassiana* cultures.

Thereafter, each culture (Figure 4.1) was transferred into sterile 50-ml corning tubes and centrifuged (Thermo Scientific HeraeusTM MegafugeTM 40R) at 15616 g for 5 min. The

supernatant was transferred into sterile Schott bottles while the pellet was collected through a sterile cloth (Supa Wipes). The cloth for each fungal strain was wrapped with aluminium foil, and the fresh mass (g) of the pellet was recorded while accounting for the cloth weight. Thereafter, the pellet was dried at 80°C for 24 h, and the dry mass (g) was recorded. This was used to calculate the actual weight (g) and determine culture filtrate concentrations (m/v%).

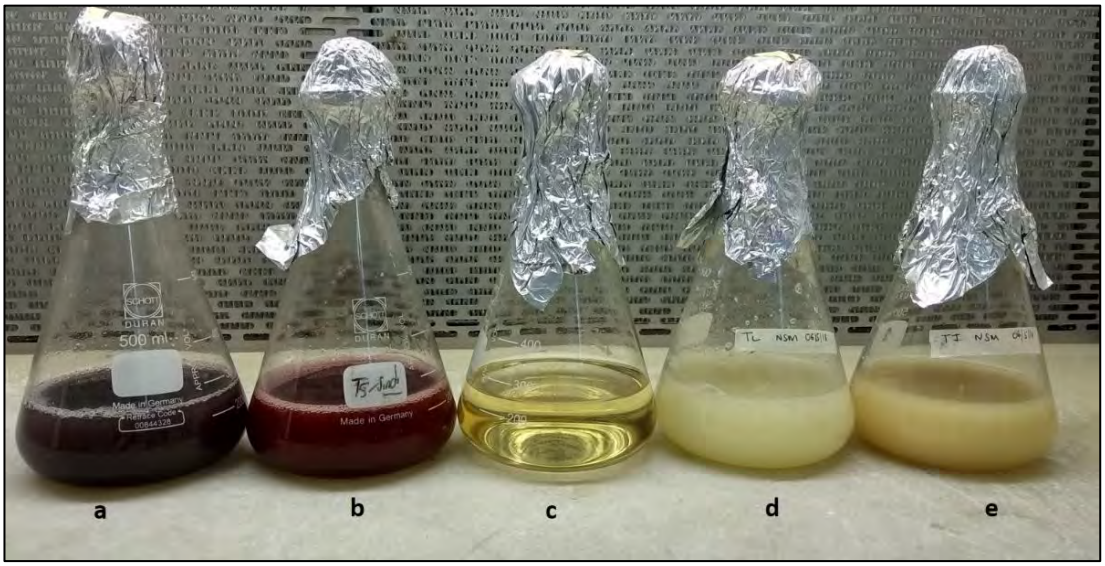

Figure 4.1: Appearance of (a) *Fusarium sacchari* strain PNG40, (b) *F. pseudonygamai* strain SC17 culture in PDB, (c) Control Potato Dextrose Broth (PDB), (d) *B. bassiana* strain TL-leaf, and (e) *B. bassiana* strain N41S1TI culture in PDB following incubation at 145 rpm at 25°C

To remove excess spores in the supernatant, the supernatant for each fungal strain was aseptically and sequentially filtered through 1) a Whatman No. 1 filter paper via Buchner filtration, 2) a 0.45 µm membrane filter (Millipore, Ireland) placed on a sintered glass filter (Millipore) attached to a vacuum pump, and 3) through a 0.2 µm syringe filter (Millipore) into sterile Schott bottles (Figure 4.2). The Schott bottles containing the culture filtrate were then kept at 4°C for 24 h. After the dry weight of the pellet was determined, the concentration of each fungal CF was calculated and expressed as the fungal dry mass/volume of PDB (250 ml) used in liquid culture.

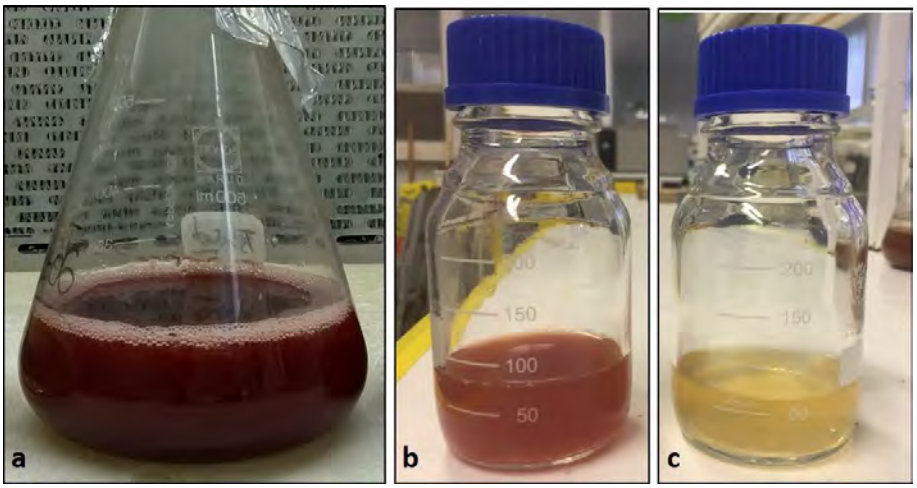

Figure 4.2: Image depicting culture filtrate preparation (a) *F. pseudonygamai* strain SC17 culture in PDB, (b) *F. pseudonygamai* strain SC17 culture filtrate after filtration through 0.45 µm and (c) 0.22 µm filters.

b. Culture filtrate assay

The experiment was initiated by aseptically inoculating *B. bassiana* TL-leaf or N41S1TI starter culture mycelial squares (5 x 5 mm) into Erlenmeyer flasks containing sterile PDB spiked with *F. pseudonygamai* SC17 or *F. sacchari* PNG40 CF at a concentration of 1.8 w/v% or 3.6 w/v% and vice versa (Table 4.2).

Table 4.2: Experimental setup showing concentrations (w/v%) of *F. pseudonygamai* (SC17) or *F. sacchari* (PNG40) and *B. bassiana* (TL-leaf or N41S1TI) CF combinations tested, and fungal mycelia (inoculum) inoculated into each CF concentration.

Fungal cultures	Culture filtrate concentration for each tested strain	Inoculum
F. pseudonygamai SC17	1.8 w/v%	*B. bassiana* TL-leaf
F. pseudonygamai SC17	3.6 w/v%	*B. bassiana* TL-leaf
F. pseudonygamai SC17	1.8 w/v%	*B. bassiana* N41S1TI
F. pseudonygamai SC17	3.6 w/v%	*B. bassiana* N41S1TI
F. sacchari PNG40	1.8 w/v%	*B. bassiana* TL-leaf
F. sacchari PNG40	3.6 w/v%	*B. bassiana* TL-leaf
F. sacchari PNG40	1.8 w/v%	*B. bassiana* N41S1TI
F. sacchari PNG40	3.6 w/v%	*B. bassiana* N41S1TI
B. bassiana TL-leaf	1.8 w/v%	*F. pseudonygamai* SC17
B. bassiana TL-leaf	3.6 w/v%	*F. pseudonygamai* SC17
B. bassiana TL-leaf	1.8 w/v%	*F. sacchari* PNG40
B. bassiana TL-leaf	3.6 w/v%	*F. sacchari* PNG40
B. bassiana N41S1TI	1.8 w/v%	*F. pseudonygamai* SC17
B. bassiana N41S1TI	3.6 w/v%	*F. pseudonygamai* SC17
B. bassiana N41S1TI	1.8 w/v%	*F. sacchari* PNG40
B. bassiana N41S1TI	3.6 w/v%	*F. sacchari* PNG40

The cultures were incubated for 7 days in a shaker (Lab Companion Incubator Tabletop) at 145 rpm at 25°C for *B. bassiana* mycelia-inoculated flasks. Following incubations, culture pigmentation was observed at each concentration. Thereafter, each culture was transferred into sterile corning tubes and centrifuged (Thermo Scientific Heraeus™ Megafuge™ 40R) at 15616 g for 5 min. The pellet was washed and harvested through a sterile cloth (Supa Wipes). The cloth for each fungal strain was wrapped with aluminium foil, and the fresh mass (g) of the pellet was recorded while accounting for the cloth weight. Thereafter, the pellet was dried at 80°C for 24 h, and the dry mass (g) was reordered. The difference in fresh and dry mass was used to calculate the actual biomass (g) of the test fungus following growth in *F. pseudonygamai* SC17 or *F. sacchari* PNG40 CF at different concentrations.

Vice versa, experiments were conducted with *F. pseudonygamai* SC17 or *F. sacchari* PNG40 mycelial squares being inoculated into *B. bassiana* TL-leaf or N41S1TI culture filtrates of 1.8 w/v% or 3.6 w/v% biomass concentrations (Table 4.2). The cultures were incubated for 7 days in a shaker (Lab Companion Incubator TableTop) at 145 rpm at 25°C. Thereafter, cultures were centrifuged, dried, and biomass recorded as described above. Controls were *Fusarium* spp. and *B. bassiana* isolates grown solely in PDB without culture filtrate. Three replicates for each combination were prepared.

4.2.2 *Beauveria bassiana* - *Fusarium* spp. co-culture

Co-culture assays were conducted to investigate interaction between two selected *B. bassiana* (TL-leaf and N41S1TI) and four *F. sacchari* (PNG40, MN57a) and *F. pseudonygamai* (SC17 and ZN7) strains. Co-culture experiments were conducted at the Agricultural Entomology department: Göttingen University.

a. Spore harvest

Following growing *Fusarium* spp. and *B. bassiana* for 3 weeks at 25°C on PDA, fungal starter culture spores were harvested by flooding plates with sterile water containing 0.1% Tween 20 in a laminar flow. The plates were gently swerved, and suspension was poured through an autoclave stainless steel woven wire mesh to remove mycelial biomass. Spore concentrations were determined by counting spores using a haemocytometer, and concentrations were adjusted via serial dilution for all fungal isolates to 10^6 conidia/ml.

b. Co-culture assay

Co-culture assays were performed on PDA in 90-mm Petri dishes as described in Rahman *et al*. (2009) and Thanh *et al*. (2014), with the following amendments: A line was drawn on the centre of the plate using a permanent marker, and two marks were drawn 2 cm from the

edge of the Petri dish on opposite sides. Using a pipette, 10µl of a 10^6 conidia/ml suspension of *B. bassiana* TL-leaf or N41S1TI was pipetted on the 2cm mark, with 10µl of a 10^6 conidia/ml suspension of the four *Fusarium* strains: *F. sacchari* (PNG40, MN57a) or *F. pseudonygamai* (SC17, ZN7) pipetted on the opposite side (Figure 4.3a) in separate assays. For controls, each fungal strain was placed 2cm from the edge of the Petri dish and allowed to grow alone (Figure 4.3b).

Eighteen replicates were conducted for each *B. bassiana* and *Fusarium* spp. co-culture combination and control plates. All plates were incubated at 25°C in the dark, and radial growth was measured every 3 days up until day 12, when growth seized. Percentage inhibition of radial growth (PIRG) was calculated using a formula developed by Skidmore and Dickinson (1976).

$$\text{PIRG}\ (\%) = \frac{R1 - R2}{R1} \times 100$$

Where, R1 represented radial growth of control plates colonies and R2 represents radial growth of the colony in the presence of the antagonist during co-culture assays.

At day 12, growth on co-cultured plates was observed and recorded in terms of overgrowth, deferred inhibition, formation of a demarcation line or ridge (hyphae met but did not overgrow one another) or indeterminate (colonies failed to meet or the interaction was unclear).

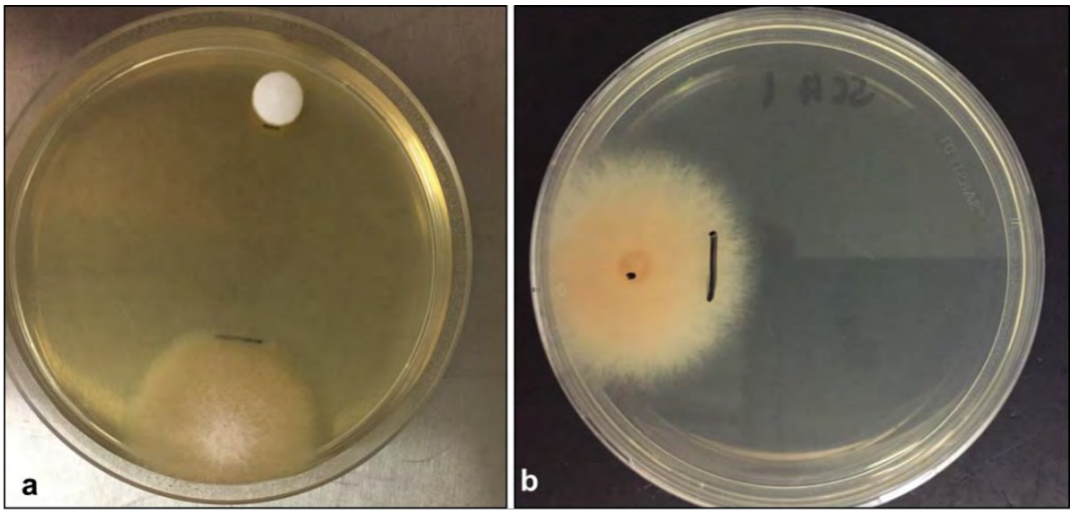

Figure 4.3: Illustration of the co-culture plate set up. (a) *Beauveria bassiana* TL-leaf strain (white colony) and *F. pseudonygamai* SC17 (orange colony) plated 2cm from the edge of a Petri dish containing PDA. (b) Control plate with *F. pseudonygamai* SC17 strain grown individually.

4.2.3 Statistical analysis

4.2.3.1 Culture filtrate

The actual biomass (g) of test fungi following growth in 1.8 w/v% or 3.6 w/v% culture filtrate concentrations was recorded. The actual biomass (g) of fungal isolates grown in the absence of CF (controls) was also recorded. The results were expressed as percentages of the control weight. Data was subjected to a normality test (Shapiro-Wilk test for normality) on GenStat (18th edition, VSN International, Hemel Hempstead, UK). The normal data was analysed using a two-way ANOVA. Interactions between CF concentrations (1.8 w/v% or 3.6 w/v%) and fungal isolates were run, and a Sidak post hoc test was used to separate the means and determine significant differences between CF concentrations and the percentage biomass of fungal isolates. Detected differences were significant at $P \leq 0.05$.

4.2.3.2 Co-culture

Regression analyses were conducted on radial growth measurements collected at 3, 6, 9, and 12 days for control assays to compare and determine the radial growth of *B. bassiana* and *Fusarium* spp. strains over time. Radial growth data was subjected to a normality test (Shapiro-Wilk test for normality) on GenStat (18th edition, VSN International, Hemel Hempstead, UK), and an ANOVA was conducted on normal data to determine if radial growth over time of *Fusarium* spp. or *B. bassiana* isolates was affected when isolates were grown in co-culture versus when grown alone. Data for each co-culture combination was analysed separately. A Sidak post hoc test was used to separate the means and determine significant differences (significant at $P \leq 0.05$) in radial growth over time (0, 3, 6, 9, and 12 days) for each of the co-culture combinations.

Radial growth measurements (cm) of fungal strains growing in the presence of the antagonist (Figure 4.3a) and when growing alone in a Petri dish (Figure 4.3b) were recorded every 3rd day for 12 days. Using this data, the percentage inhibition of radial growth (PIRG) at day 12 was calculated as reported by Thanh *et al.* (2014) and Skidmore and Dickinson (1976). The data was then analysed in GenStat (18th edition, VSN International, Hemel Hempstead, UK) and checked for normality (Shapiro-Wilk test for normality), and a two-sided T-test with a confidence limit of 95% was conducted to determine the significance of inhibition between *B. bassiana* and *Fusarium* spp. co-cultures at day 12.

4.3 Results

4.3.1 Effects of *Fusarium* spp. culture filtrate on *B. bassiana* growth

Fusarium sacchari (PNG40) and *F. pseudonygamai* (SC17) culture filtrate had a significant inhibitory effect (P< 0.043, d.f. = 1) on *B. bassiana* growth (biomass). Culture filtrates from 3.6 w/v% resulted in reduced percentage *B. bassiana* biomass when compared to 1.8 w/v% (Figure 4.4). Average control biomass of *F. sacchari* (PNG40) was found to be 4.23g, *F. pseudonygamai* (SC17): 6.41g, while control biomass for *B. bassiana* isolates TL-leaf was 5.65g and N41S1TI: 4.95g.

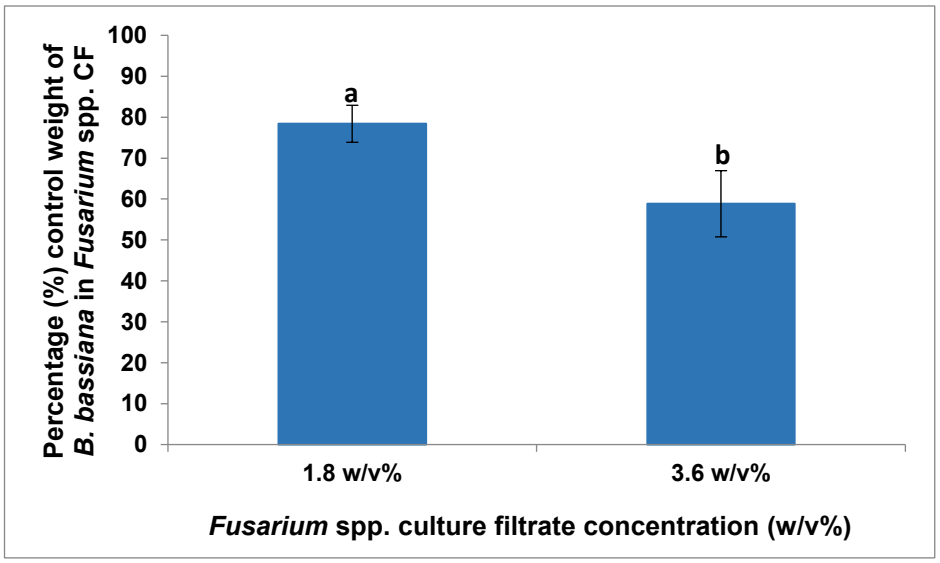

Figure 4.4: Collective mean percentage biomass yield of *B. bassiana* isolates (TL-leaf and N41S1TI) when grown in *Fusarium* spp. (*F. sacchari*: PNG40 or *F. pseudonygamai*: SC17) culture filtrates of 1.8 w/v% and 3.6 w/v%. Data expressed as percentage of control weight. Error bars represent the standard error of means and different letters above error bars symbolize significant differences (n=3).

No distinct changes in culture pigmentation were observed during visual observations when *B. bassiana* TL-leaf or N41S1TI were grown in either *F. sacchari* PNG40 or *F. pseudonygamai* SC17 culture filtrates.

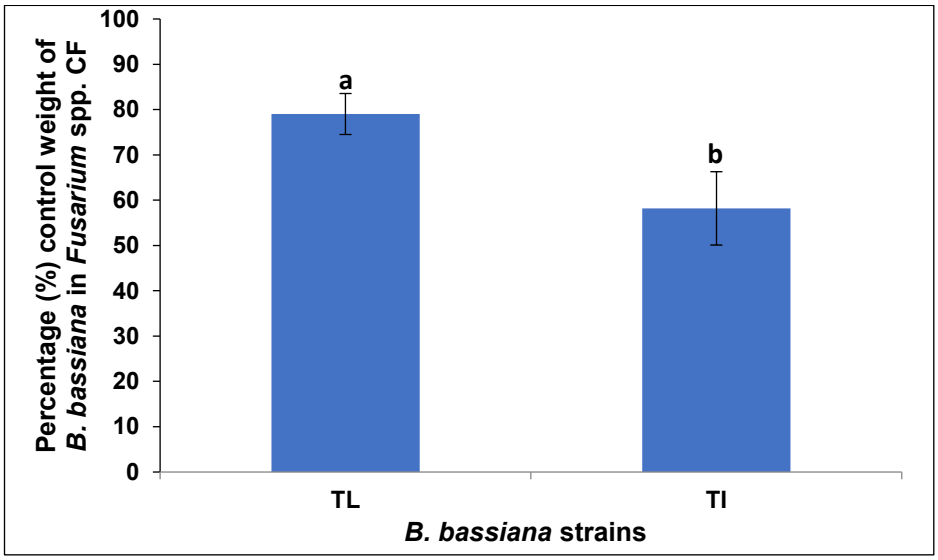

Figure 4.5: Collective mean percentage biomass yield of *Beauveria bassiana* isolates (TL=TL-leaf and TI=N41S1TI) when grown in *Fusarium* spp. (*F. sacchari*: PNG40 or *F. pseudonygamai*: SC17) culture filtrates. Data is expressed as percentage of control. Error bars indicate the standard error of means and different letters above error bars symbolize significant differences between isolates (n=3).

There were significant differences (P< 0.033, d.f. = 1) in biomass between the two *B. bassiana* isolates when grown in the presence of *Fusarium* spp. CF (Figure 4.5). Statistically, there were no significant interaction between *B. bassiana* isolates, concentration and *Fusarium* culture filtrate. *Beauveria bassiana* strain TL-leaf always weighed more than strain *B. bassiana* N41S1TI when grown in *F. sacchari* PNG40 or *F. pseudonygamai* SC17 culture filtrates of different concentrations (Figure 4.5).

4.3.2 Effects of *B. bassiana* culture filtrate on *Fusarium* spp. growth

Beauveria bassiana (TL-leaf and N41S1TI) culture filtrate resulted in a significant inhibitory effect (P< 0.031, d.f. = 1) on *F. sacchari* PNG40 and *F. pseudonygamai* SC17 percentage biomass. Both *F. sacchari* PNG40 and *F. pseudonygamai* SC17 biomass was reduced in the presence of increased *B. bassiana* CF concentrations, with CF concentrations of 3.6 w/v% resulting in lower biomass when compared to 1.8 w/v% (Figure 4.6). There were no significant interactions between *Fusarium* spp. strains and *B. bassiana* CF concentration. Higher *B. bassiana* CF concentrations resulted in reduced percentage weights of both *F. sacchari* PNG40 and *F. pseudonygamai* SC17 when grown in *B. bassiana* culture filtrate concentration of 3.6 w/v% as compared to 1.8 w/v% (Figure 4.6).

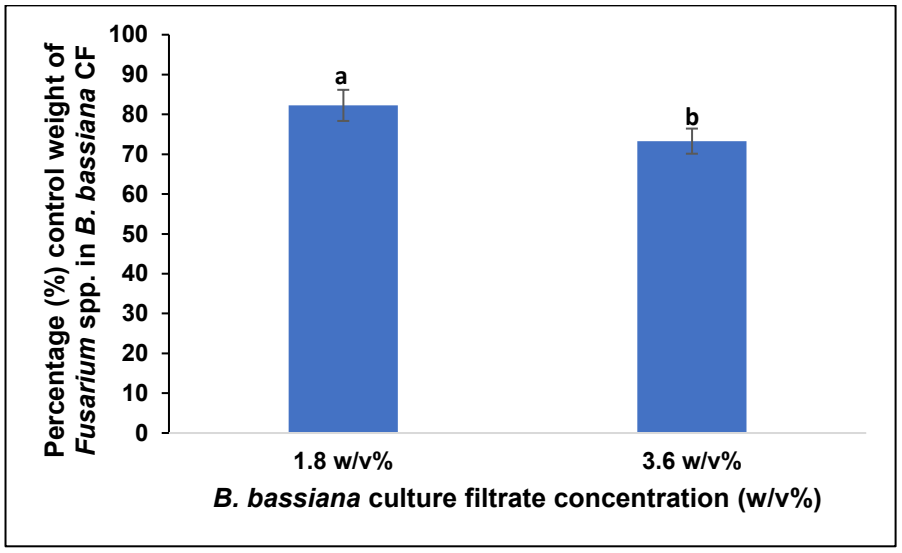

Figure 4.6: Collective mean percentage biomass yield of *F. sacchari* PNG40 and *F. pseudonygamai* SC17 when grown in *B. bassiana* (TL-leaf or N41S1TI) culture filtrates of 1.8 w/v% and 3.6 w/v% concentrations. Data expressed as percentage of control weight. Error bars indicate the standard error of means and different letters above error bars symbolize significant differences between isolates (n=3).

Distinct changes in culture pigmentation were observed during visual observations when *F. sacchari* PNG40 or *F. pseudonygamai* SC17 was grown in *B. bassiana* CF (Figure 4.7). *Fusarium pseudonygamai* SC17 control produced a red pigmentated culture when grown alone in PDB, however, when grown in *B. bassiana* CF broth pigmentation changed and became darker (Figure 4.7a). *Fusarium sacchari* PNG40 produced purple pigmented culture when grown alone (control) in PDB (control), however, turned yellow when grown in *B. bassiana* CF (Figure 4.7b).

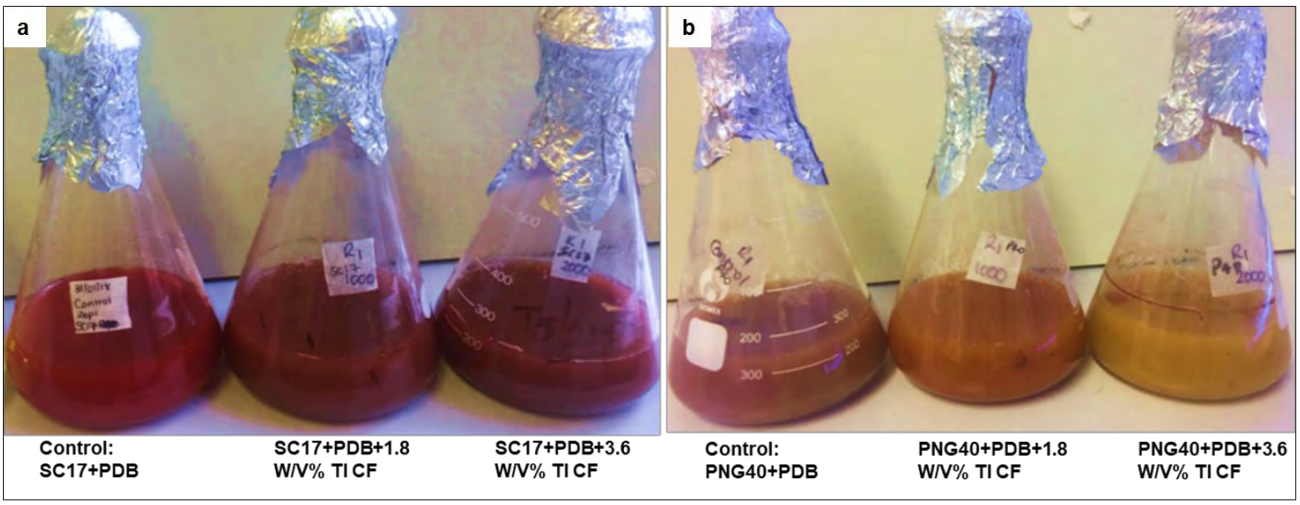

Figure 4.7: Representative *Fusarium* spp. cultures in PDB. (a) *F. pseudonygamai* SC17 in PDB (control), 1.8 w/v% and 3.6 w/v% *B. bassiana* N41S1TI CF. (b) *Fusarium sacchari* PNG40 in PDB (control), 1.8 w/v% and 3.6 w/v% *B. bassiana* N41S1TI CF.

There was a significant reduction (P<0.053, d.f.=1) of biomass formed by the two *Fusarium* strains. *Fusarium sacchari* PNG40 achieved a higher percentage of biomass control weight than *F. pseudonygamai* SC17 strain when exposed to *B. bassiana* CF (TL-leaf or N41S1TI) (Figure 4.8).

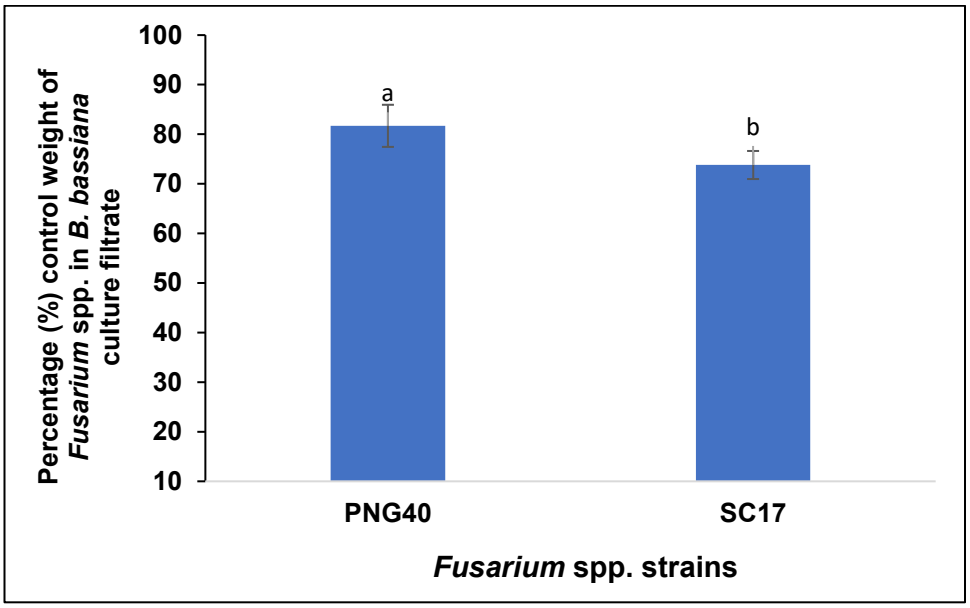

Figure 4.8: Collective mean percentage biomass yield of *F. sacchari* (PNG40) and *F. pseudonygamai* (SC17) when grown in *B. bassiana* (TL-leaf or N41S1TI) culture filtrates. Data is expressed as percentage of the control. Error bars indicate the standard error of the mean and different letters above error bars symbolize significant differences between isolates (n=3).

Significant biomass yield differences were observed (P<0.020; d.f.=1) between *Fusarium* spp. strains *F. sacchari* PNG40 and *F. pseudonygamai* SC17 and *B. bassiana* CF. No significant interactions occurred between *Fusarium* spp., *B. bassiana* CF concentration and *B. bassiana* isolates (TL-leaf or N41S1TI) used in the culture filtrate were observed. *Fusarium sacchari* PNG40 biomass percentage relevant to control was higher than *Fusarium pseudonygamai* SC17 when grown in *B. bassiana* culture filtrates of different concentrations (Figure 4.8).

4.3.3 *Beauveria* - *Fusarium* co-culture

Fusarium spp. strains grew faster and had larger radial growth (cm) when compared to the two tested *B. bassiana* isolates which had lower radial growth (cm) and grew slower (Figure 4.9). Regression analysis was conducted to compare radial growths of *Fusarium* spp. and *B. bassiana* isolates over time (days). Radial growth (cm) of *B. bassiana* TL-leaf and N41S1TI were significantly smaller ($p < 0.001$) to those of *Fusarium* spp. strains. *Beauveria bassiana* TL-leaf and N41S1TI had half (0.10031; 0.10019 respectively) radial growths (cm) than that of *F. sacchari* MN57 (0.24598) and PNG40 (0.24370) *F. pseudonygamai* SC17 (0.23174) and ZN7 (0.22148) (Figure 4.9). The radial growth of *Fusarium* spp. strains over 12 days was higher when compared to *B. bassiana* isolates (Figure 4.9).

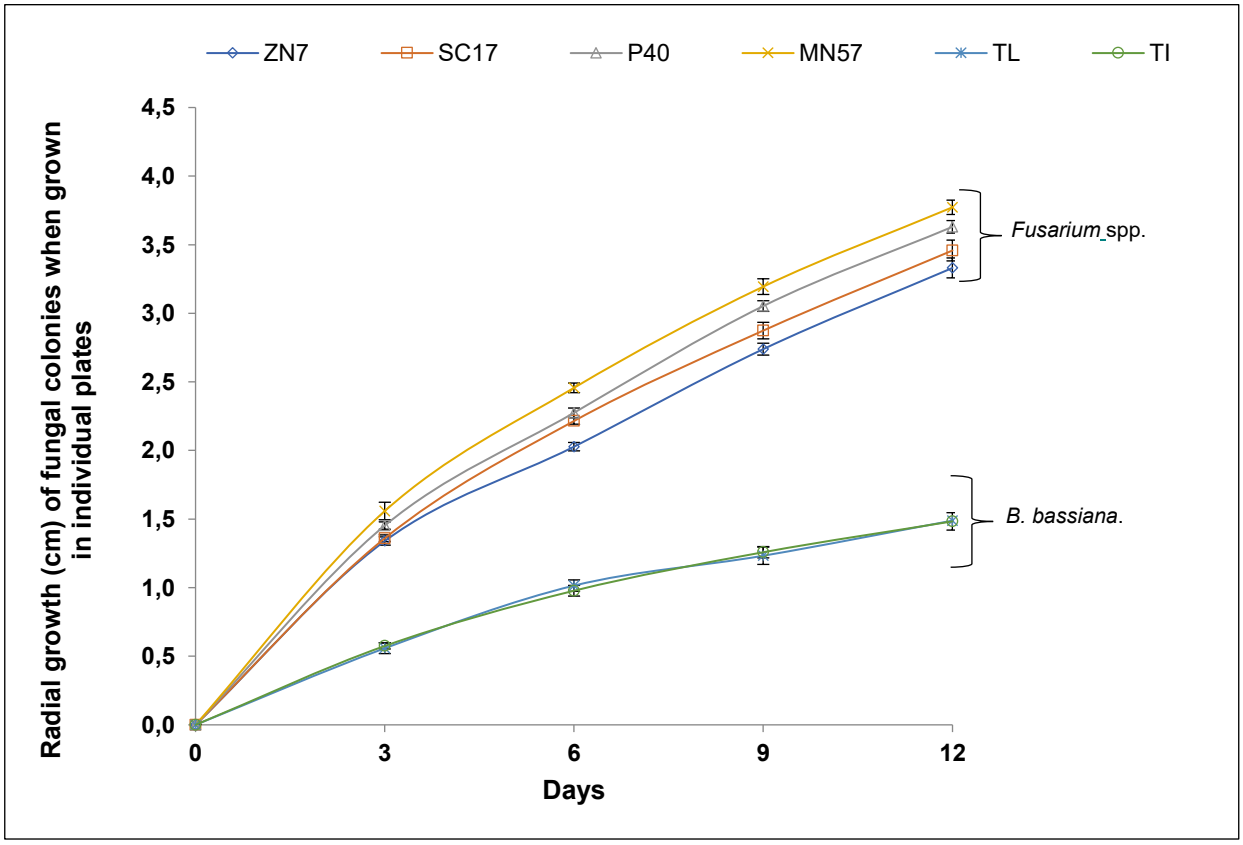

Figure 4.9: Colony radial growth (cm) measured at 3-day intervals for *F. sacchari* (MN57 and P40=PNG40), *F. pseudonygamai* (SC17 and ZN7) and *B. bassiana* (TL=TL-leaf, TI=N41S1TI) isolates when grown individually on PDA for 12 days at 25°C. Bars indicate the standard errors of means (n=18).

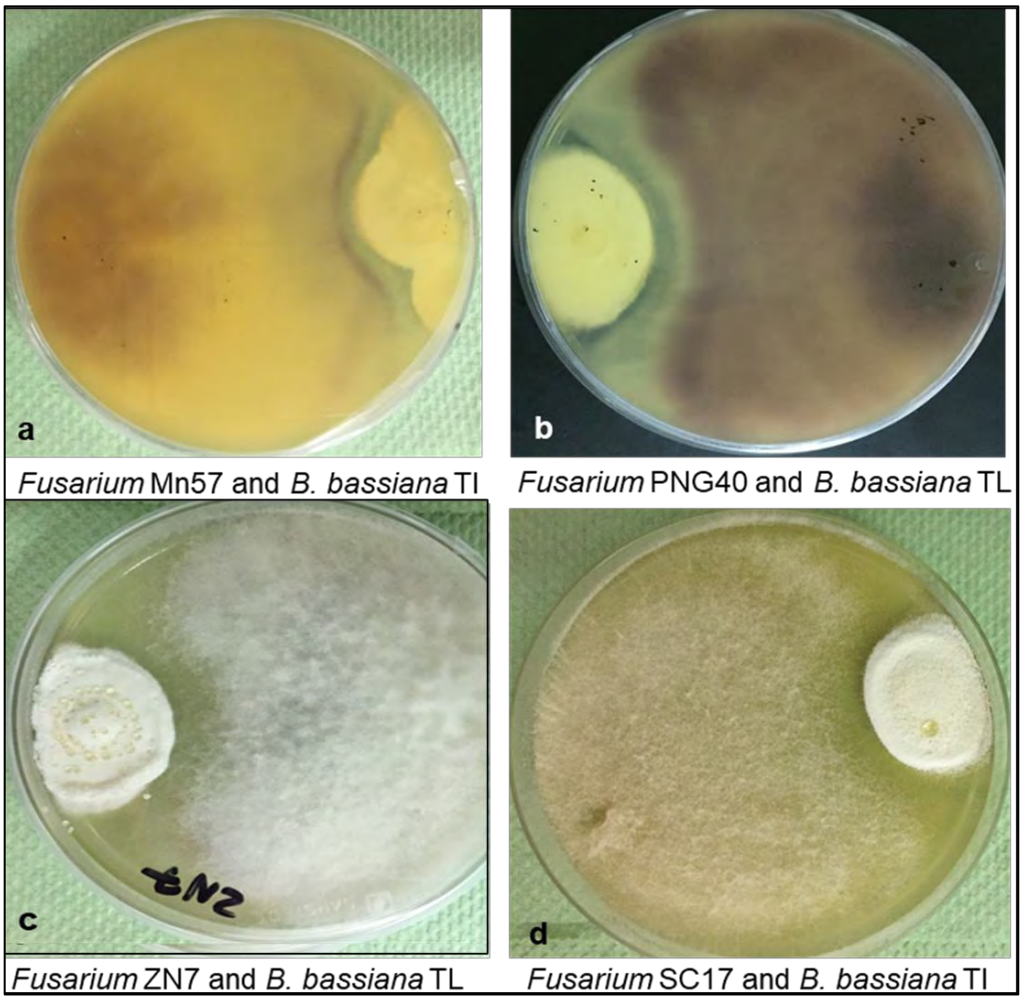

Figure 4.10: *Fusarium* spp. and *B. bassiana* isolates grown in co-culture at 25°C on day 12 (n=18).

A demarcation line was observed when *F. sacchari* MN57 was grown in co-culture with *B. bassiana* (TI=N41S1TI) (Figure 4.10a) and *F. sacchari* PNG40 with *B. bassiana* (TL=TL-leaf) (Figure 4.10b). When *B. bassiana* (TL=TL-leaf) strain was co-cultured with *F. pseudonygamai* ZN7 strain and *B. bassiana* (TI=N41S1TI) with *F. pseudonygamai* SC17 deferred growth was observed. *Beauveria bassiana* growth was localized on the assay plates, while *Fusarium* spp. strains occupied more space on the Petri dish (Figure 4.10).

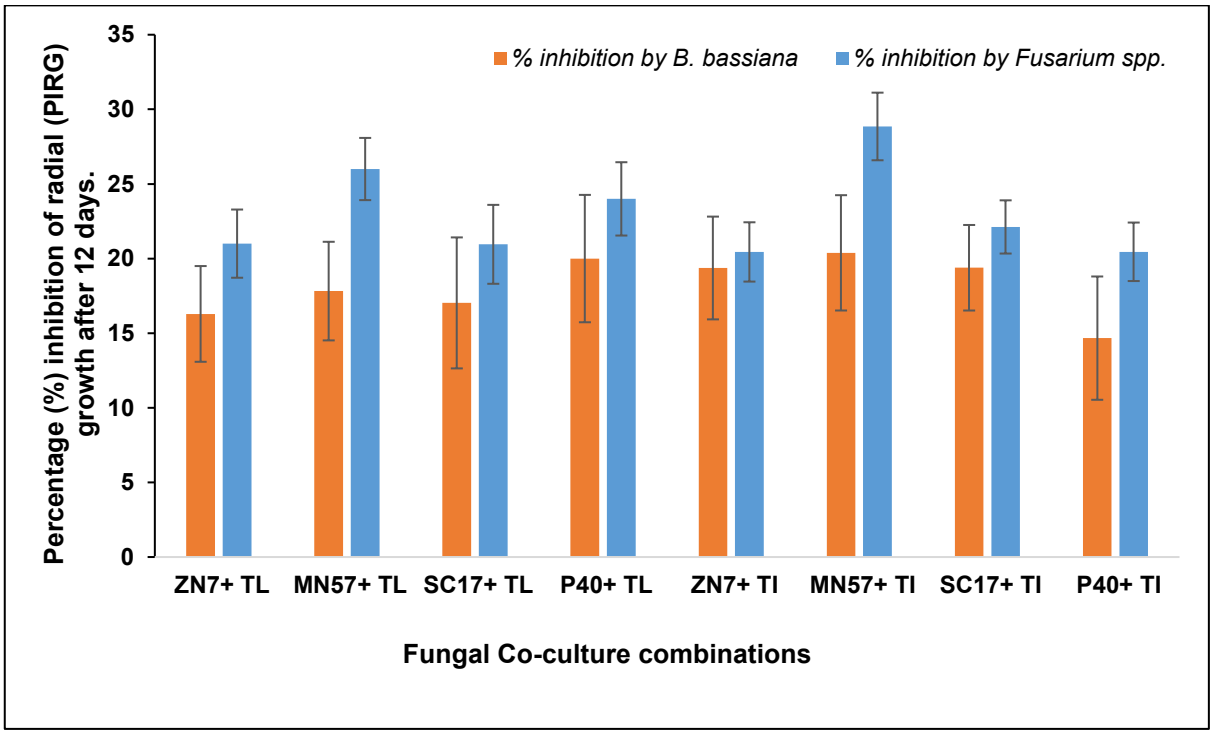

Figure 4.11: Percentage inhibition of radial growth (PIRG) at day 12 when *F. sacchari* (MN57 and PNG40) and *F. pseudonygamai* (SC17 and ZN7), and *B. bassiana* (TL=TL-leaf, TI=N41S1TI) isolates were grown alongside in co-culture (designated with + sign). Bars indicate standard errors of means (n=18).

When the four *Fusarium* spp. strains were sequentially grown in co-cultures with the two *B. bassiana* isolates, *Fusarium* spp. strains caused radial growth inhibitions ranging from 20-26% towards *B. bassiana* radial growth, while *B. bassiana* resulted in inhibitions of 15-20% on *Fusarium* spp. strains when the percentage inhibition of radial growth (PIRG) was calculated at day 12 (Figure 4.11). However, no significant differences were revealed between *Fusarium* spp. and *B. bassiana* percentage inhibitions and vice versa for all combinations tested at day 12 when T-tests were conducted for each combination.

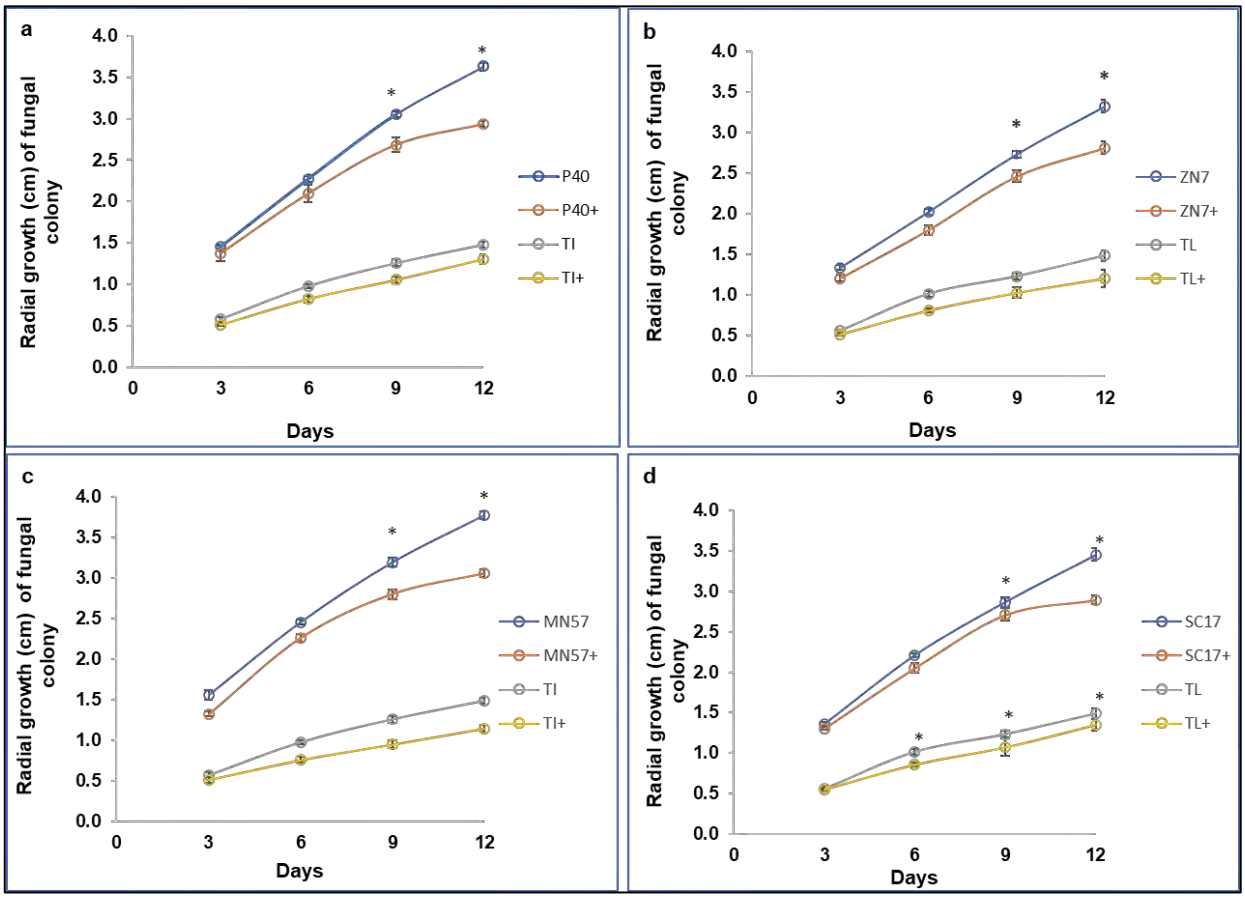

Figure 4.12: Radial growth (cm) of *Fusarium* spp. and *B. bassiana* isolates grown in co-culture plates (designated with + sign) in comparison to those grown individually over 12-day incubation at 25ºC. Bars show standard errors of means (n=18). * Indicates significant differences

Both *Fusarium* spp. and *B. bassiana* radial growth increased over time whether grown in co-culture or alone (Figure 4.12). However, there was a reduction in radial growth when both genera were grown in co-culture (designated with +; Figure 4.12). There was a significant interaction (P=0.010; d.f.=3) between days and *Fusarium* spp. (alone and in co-culture) for all the *Fusarium* spp. strains tested, *Fusarium* spp. did not always grow faster than *B. bassiana* isolates in co-culture, it depended on the days after inoculation. At day 9 *F. sacchari* PNG40 radial growth reduced in co-culture plates (designated with +) when compared to when grown alone (control) (Figure 4.12a).

4.4 Discussion

Fungi interact with each other in various ecological niches. The nature of microbe-microbe interactions can be mutual (beneficial to each other) or detrimental (Grabka *et al*., 2022). Subsequently, they compete for space and nutrients and produce secondary metabolites in the microenvironment, limiting and antagonizing the growth of other microorganisms (Ownley *et al*., 2010; Dara, 2019). Thus, to potentially use *B. bassiana* as a biological control of *Fusarium* spp. in the sugarcane niche, it is important to investigate the interaction that may exist between these species. During this study, *in vitro* assays were carried out to gain insight on the interactions that may exist between *Fusarium* spp. (a sugarcane endophyte and pathogen) and *B. bassiana* (a potential biological control agent).

4.4.1 Culture filtrates

The ability of microorganisms to produce secondary metabolites or mycotoxins and enzymes during growth is well recorded (Künzler, 2018; Venkatesh and Keller, 2019). These mycotoxins give microorganisms a competitive advantage and can antagonize microorganisms within the same niche (Nesic *et al*., 2014; Venkatesh and Keller, 2019). Culture filtrate assays were conducted on *Fusarium* spp.: *F. sacchari* PNG40, *F. pseudonygamai* SC17, and *B. bassiana* TL-leaf and N41S1TI isolates to investigate the effect of metabolites excreted during growth on each genus. *Fusarium* spp. culture filtrate concentrations of 1.8 w/v% and 3.6 w/v% reduced *B. bassiana* percentage biomass yield relative to control and vice versa in *Fusarium* spp. cultures. Both genera had significantly lower biomass when grown on higher (3.6 w/v%) culture filtrate concentrations of the respective genus. Likewise, when Yu *et al*. (2017) used *B. bassiana* culture filtrate of different concentrations on *Botrytis cinerea*, higher concentrations of filtrate showed significantly higher antifungal activities when compared to lower concentrations. Yu *et al*. (2017) attributed the repression of *B. cinerea* by *B. bassiana* strains to the presence of antifungal substances in the culture filtrates. Although these authors did not identify antifungal substances present in the culture filtrates, Ávila-Hernández *et al*. (2022) used HPLC-ESI-MS^2 to identify a metabolite produced by the *B. bassiana* PQ2 strain that inhibited the germination of *Gibberella moniliformis* (a teleomorph of *Fusarium verticillioides*) spores as oosporein. Although metabolites present in the broth media were not investigated in this study, it is well recorded that both *Fusarium* and *Beauveria* species produce extracellular secondary metabolites such as beauvericin (Venkatesh and Keller, 2019). *Beauveria bassiana* is known to produce metabolites in culture such as tenellin, bassianin, pyridovericin and pyridomacrolidin produced as a yellow pigmentation involved in pathogenicity (Ownley *et al*., 2004; Zimmermann, 2007; Yun *et al*., 2017; Ávila-Hernández *et al*., 2020; Deb and Dutta, 2021; Elkhateeb and Daba, 2023). *Fusarium* spp. are also notorious for producing pigmented secondary metabolites such as Naphthaquinones (purple) Bikaverin (red) with antibiotic activities, fusaric acid, and enniatin, to

name a few (Meca *et al*., 2010; Estrada *et al*., 2012; Nesic *et al*., 2014; Ekwomadu *et al*., 2021; Elkhateeb and Daba, 2023). These secondary metabolites can inhibit the biosynthesis of the fungal cell wall and have antibacterial and insecticidal activities. Moreover, beauvericin has been shown to block ABC transporters against *Candida albicans* (Tong *et al*., 2016; Caloni *et al*., 2020). Thus, although secondary metabolites produced by isolates tested were not characterized in this study, reduction in percentage biomass can be attributed to the production of metabolites by both *B. bassiana* and *Fusarium* spp. strains during growth as supported by literature.

Fusarium spp. are known to produce red and purple-pigmented naphthoquinones known to be antifungal and antibacterial (Duarte and Archer, 2003; Studt *et al*., 2012; Westphal *et al*., 2018). Duarte and Archer (2003) found that *F. solani* strains that produced red pigments in culture media produced more efficient biologically active filtrates than those that produced pink or clear culture filtrates, suggesting that pigmentation is associated with toxigenic activity. In this study, *F. pseudonygamai* SC17 generally produced a red-pigmented culture when grown in PDB, while *F. sacchari* PNG40 produced purple coloration. However, when the isolates were grown in *B. bassiana* culture filtrates, pigmentation varied and became darker for *F. pseudonygamai* SC17, while *F. sacchari* PNG40 cultures turned yellow. Sanivada and Challa, (2014) showed that the antifungal activity of entomopathogenic strains against *Colletotrichum falcatum* increased with the production of chitinolytic enzymes. Chitinase is known to cause cell wall degradation in susceptible fungi and inhibition of cell separation (Hartl *et al*., 2012; Le *et al*., 2019). Therefore, the reduction in biomass formation at 3.6 w/v% CF concentration and the change in culture pigmentation seen during this study may be due to metabolites or enzymes present in the culture filtrate obtained from both *Fusarium* spp. strains and *B. bassiana* isolates.

Furthermore, antagonistic growth effects were observed during culture filtrate assays. *Beauveria bassiana* TL-leaf isolated from *Typha latifolia* L. percentage weight was significantly higher when compared to *B. bassiana* N41S1TI, isolated from sugarcane internodes, when grown in the presence of *Fusarium* spp. culture filtrates. In the presence of *B. bassiana* culture filtrates, *F. sacchari* PNG40 percentage weight was significantly higher when compared to *F. pseudonygamai* SC17. These results show that the production of metabolites is both species- and strain-dependent (Duarte and Archer, 2003).

4.4.2 Co-culture

When *B. bassiana* isolates were inoculated and grown simultaneously in co-cultures with *Fusarium* spp. strains, both *Fusarium* spp. and *B. bassiana* isolates expressed reduced radial growth when compared to controls when isolates were grown alone. This indicated the potential

of both genera to reduce, limit, and antagonize each other's growth when grown in the same niche. An important factor that determines the efficiency of the antagonist to colonize its niche is growth rate (Culebro-Ricaldi *et al*., 2017). The results showed that during co-cultures, *B. bassiana* isolates always had smaller radial growths when compared to *Fusarium* spp. Culebro-Ricaldi *et al*. (2017) showed similar results, when *B. bassiana* and *Fusarium oxysporum f. sp. Lycopersici* strains were applied simultaneously on agar plates during co-culture assays, *B. bassiana* radial growth was slower than *F. oxysporum*. In sugarcane, some *Fusarium* spp. strains are natural endophytes prior to forming pathogenic infections (McFarlane *et al*., 2009). Radial growth of *F. oxysporum* was found to be lower than *B. bassiana* when *F. oxysporum* was plated onto agar plates two days after *B. bassiana* inoculation (Culebro-Ricaldi *et al*., 2017). They also reported that inoculating *B. bassiana* on agar plates prior to *F. oxysporum* allowed *B. bassiana* to produce diffusible hydrolytic enzymes (i.e., protease and lipase) effective in reducing *F. oxysporum* growth when *F. oxysporum* application or introduction was delayed.

Percentage inhibition of radial growth (PIRG) at day 12 was found to be low and not significant for both genera. *Fusarium* spp. resulted in PIRG towards *B. bassiana* ranging from 20–26%, while *B. bassiana* isolates resulted in 15-20% inhibitions of *Fusarium* spp., suggesting that *Fusarium* spp. is not detrimental to *B. bassiana* and both genera cause a degree of reduction in each other's radial growths. Culebro-Ricaldi *et al*. (2017) also found inefficient antagonism between the two genera when *F. oxysporum* and *B. bassiana* were applied simultaneously on agar plates. In this study, antagonism was different at different days for the *Fusarium* spp. strains, with greater antagonism experienced at day 12 as radial growth was significantly lower in co-cultured plates for the *Fusarium* spp. strains when compared to when grown alone. These results highlight the potential for better antagonism of the pathogen in the plant with delayed pathogen occurrence. This can be supported by findings by Ownley *et al*. (2008) when they treated tomato seeds with *B. bassiana* 11–98 and sowed the seeds in *Rhizoctonia solani* (damping agent)-infected soil, damping-off was significantly reduced and similar findings of reduced plant disease development upon pre-treatment with *B. bassiana* strains have been reported (Renwick *et al*., 1991; Bark *et al*., 1996; Reisenzein and Tiefenbrunner, 1997; Deb and Dutta, 2021). Since *B. bassiana* was found in this study to grow significantly slower than the four tested *Fusarium* spp. strains, for *B. bassiana* to be an effective antagonist to *Fusarium* spp. in sugarcane, the possibility of inoculating tissue culture plants with *B. bassiana* prior to planting in the field must be explored. Initially inoculating sugarcane tissue culture plants with *B. bassiana* would give *B. bassiana* a competitive advantage to colonize the plant first and potentially lower the growth of secondary colonizers such as *Fusarium* spp. if its incidence in sugarcane is delayed.

Additionally, a demarcation line and mycelial growth inhibition were apparent during co-culture assays; a line was seen for *F. sacchari* strains (MN57 and PNG40), while deferred inhibition was seen for *F. pseudonygamai* strains (ZN7 and SC17). This highlights the potential of microorganisms to use different strategies during antagonisms. Moreover, when *B. bassiana* was grown with *Fusarium* spp., *B. bassiana* radial growth was seen to be localized on the plate, with *Fusarium* spp. occupying a larger area on the agar Petri dish. This was due to the fast radial growth rate of *Fusarium* spp. Culebro-Ricaldi *et al*. (2017) found that *B. bassiana* produced inhibitory diffusible metabolites in media and reported that overgrowth of the pathogen (*F. oxysporum*) by the antagonist (*B. bassiana*) is not required to inhibit the fungal pathogen. This may be true for *B. bassiana* isolates TL-leaf and N41S1TI, as there were no significant differences in PIRG at day 12 for *B. bassiana* and *Fusarium* spp., even though *Fusarium* spp. occupied most of the plate. However, space limitation (Petri dish size) may have also played a role, as at day 12, most of the Petri dish was colonized by *Fusarium* spp.

In conclusion, this study revealed that both *B. bassiana* and *Fusarium* spp. can reduce each other's growth (% biomass formation and radial growth) when grown *in vitro,* in the presence of culture filtrate, or in co-culture plates. *Fusarium* spp. strains grew faster than *B. bassiana* isolates, however, no significant inhibitions were observed. Therefore, to apply *B. bassiana* as a dual control against *E. saccharina* and *Fusarium* spp., inoculation of tissue culture plants with *B. bassiana* followed by *Fusarium* spp. should be investigated to determine if *B. bassiana* isolates can colonize sugarcane plants first and restrict *Fusarium* spp. growth. Furthermore, the interaction of *Fusarium* spp. and *Beauveria* spp. may vary in a plant host, due to the interactions of these fungi with other endophytic microorganisms colonizing the plant, thus interaction must be investigated with plant inoculations.

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CHAPTER 5

Establishment and persistence of *Beauveria bassiana* in sugarcane plantlets (*Saccharum* spp.) and its virulence against *Eldana saccharina* (Lepidoptera: Pyralidae)

Abstract

Literature on the establishment of *B. bassiana* as an endophyte in sugarcane for the biological control of *E. saccharina* is scarce. The objective of this study was to establish *B. bassiana* in sugarcane and investigate plant and pest response to colonization. Four independent experiments were conducted. The first experiment, sugarcane plants were inoculated with three *B. bassiana* isolates using three inoculation and three plant pre-treatment methods to evaluate the best method to establish endophytic colonization. All three *B. bassiana* isolates colonized sugarcane stem and leaf tissues and were re-isolated from surface disinfected sugarcane plants for up to 4 months. Tissue cultured sugarcane plants were better colonized than plants grown from hot water treated setts (HWT), hot water treated and waxed setts (HWT+WAX), while soil drench, stem injection or foliar spray applications achieved different levels of stem and leaves colonization. The second experiment used dip and spray conidia inoculation methods, using two *B. bassiana* isolates TL-leaf and N41S1TI to determine the better plant colonizer and plant responses (plant height and weight) to colonization. Three months post inoculation, *B. bassiana* inoculated plants had increased above and below weights (g) when compared to control plants with DNA of *B. bassiana* N41S1TI strain detected in sprayed sugarcane plants in root and leaves (0,31±0,01; 0,41±0,018 DNA pg/100g respectively) using RT-PCR. The third experiment used 28 sugarcane varieties to inoculate *B. bassiana* TL-leaf strain and colonization persistence evaluated at 3- and 6-months. Results showed the degree of endophytic colonization to be variety dependent and decreased with increase in post inoculation time, with *Beauveria* spp. re-isolated from only seven of the 28 varieties at 6 months. The fourth experiment inoculated two *B. bassiana* strain conidia and control plants with 0.1% Triton X-100 into sugarcane var. N41 for 16 months. No statistically significant differences were observed within control and *B. bassiana* plants in reducing boring length and larval weights when *E. saccharina* larvae were fed on plants 16 months post *B. bassiana* inoculation, with no *B. bassiana* colonies re-isolated 16 months post inoculation. These results demonstrated endophytic establishment of *B. bassiana* isolates into sugarcane for 1 to 6 months. Endophytic colonization was influenced by inoculation method, plant pre-treatment, and fungal strain. Results highlight the importance of determining endophytic persistence and ensuring *B. bassiana* is present as an endophyte.

5.1 Introduction

Endophytic fungal entomopathogens can live and colonize healthy plant tissues without causing any symptoms of disease to the plant (Arnold and Lewis, 2005; Maheshwari, 2006). Their symbiosis with plants is reported to enhance plant defense against insect damage, causing antibiosis and feeding deterrence in insects (Vega, 2008; Rodriguez *et al*., 2009). Furthermore, the presence of these organisms in plant tissues has been demonstrated to enhance plant tolerance to plant pathogens (Ownley *et al*., 2008). Fungal entomopathogens *Beauveria bassiana, Akanthomyces lecanii* (formerly *Lecanicillium lecanii*) and Cordyceps *formerly Paecilomyces* spp. have been artificially inoculated into plants for potential insect pests biological control agents (Ownley *et al*., 2004; Vega, 2008; Lopez *et al*., 2014; Kepler *et al*., 2017).

Beauveria bassiana has been demonstrated to colonize root, stem and leaf plant tissues (Posada and Vega, 2006) with hyphae able to move and translocate within inter-cellular spaces after conidial application onto plants (Wagner and Leslie, 2000; Gómez-Vidal *et al*., 2006; Quesada-Moraga *et al*., 2014). Establishing this entomopathogen in plants as an endophyte has been shown to reduce stem borer tunnelling in plants. For instance, Cherry *et al*. (2004) inoculated *B. bassiana* conidia into maize plants and its endophytic presence significantly reduced the tunnelling length of the stalk borer *Sesamia calamistis* Hampson (Lepidoptera, Noctuidae) on maize plants. Banana weevil [Cosmopolites sordidus Germar (Coleoptera: Curculionidae)] survival and damage was also reduced when tissue culture banana plants were inoculated and endophytically colonized by *B. bassiana* (Akello *et al.,* 2008). Furthermore, when *B. bassiana* was established endophytically in broad bean plants, it resulted in larval mortality and weight loss when the cotton bollworm *Helicoverpa armigera* Hübner (Lepidoptera, Noctuidae) pest larvae were fed on colonized plants (Vidal and Tefera, 2013). Donga *et al*. (2018) documented the establishment of *B. bassiana* as an endophyte in sugarcane for 16 days in Malawi. In South Africa endophytic establishment of *B. bassiana* has been reported on rice cultivars and tested efficacy against rice stem borer, *Sesamia calamistis* (Bancole *et al*., 2020) and in sorghum against *Chilo partellus* Swinhoe (Bancole *et al*., 2022). However, the endophytic establishment of *B. bassiana* in sugarcane plants as part of an integrated pest management strategy for *E. saccharina* biocontrol has not been reported.

Eldana saccharina is a profit limiting pest in the South African Sugar Industry, its larvae bore into older carry-over sugarcane (10-15 months) and develop inside the sugarcane stem, feeding on the internal stem tissues (Leslie, 2004). This boring stage leads to the development of secondary infections by *Glomerella tucumanensi*s and *Fusarium* species, which convert sugarcane sucrose into glucose (Way and Goebel, 2003) reducing cane quality during milling.

Conventional control strategies against *E. saccharina* have shown limited effectiveness due to the cryptic life cycle of the insect (Way and Goebel, 2003; Meyer and Clowes, 2011). Hence, biological control involving the establishment of an entomopathogenic fungus as an endophyte in sugarcane is being investigated by the South African Sugarcane Research Institute (SASRI).

To implement endophyte-based control strategies, it is essential to investigate if *B. bassiana* can colonize sugarcane plants after inoculation and the extent to which the plant is colonized. Furthermore, colonization levels have been reported to vary amongst *B. bassiana* strains with some strains having higher infection capabilities (Posada *et al*., 2007). It will, therefore, be important in *B. bassiana*-mediated biological control strategies to establish the infectivity of *B. bassiana* strains to sugarcane plants prior to its application as a biological control agent of *E. saccharina*. Moreover, it is important to determine the best and most practical method of inoculating *B. bassiana* into sugarcane plants as the type of inoculation method used is reported to influence establishment of the fungus in different plant parts (Tefera and Vidal, 2009). Plant species and growth media have also been reported to influence degree of endophytic colonization (Tefera and Vidal, 2009). In addition to this, studies have reported *B. bassiana* colonization levels to decrease in plants with time post inoculation (Posada *et al*., 2007). Hence, it is imperative to ascertain persistence of *B. bassiana* isolates inoculated into sugarcane plants, as it is essential to know if the endophyte will be present for 10 months and beyond, as *E. saccharina* is primarily a pest of mature cane (Rutherford, 2015; Anon, 2023).

Control strategies directed at the larval feeding stage inside the sugarcane plant are likely to be more effective in managing the damage caused by *E. saccharina* during feeding and development inside the stem. *Beauveria bassiana* was found as a natural endophyte in *E. saccharina* natural host plants growing within sugarcane agro-ecosystems and in 22 sugarcane varieties. These endophytic isolates were further shown to be pathogenic towards *E. saccharina* 3rd and 5th instar larvae. Therefore, the aim of this study was to determine if endophytic *B. bassiana* isolates isolated from Poaceae and Typhaceae plant species can be inoculated and established as endophytes in sugarcane plants using different inoculation methods. In addition, the persistence of these endophytic strains in sugarcane plants and their virulence on feeding *E. saccharina* larvae was investigated.

5.2 Material and Methods

5.2.1 Fungal culture preparation

Four independently designed experiments were conducted. Fungal culture preparation for each *B. bassiana* strain (TL-leaf, S2roots, P2stem or N41S1TI) previously isolated as endophytes from surface disinfected plant tissues were retrieved from glycerol stock cultures stored at −20 °C and

sub-cultured onto full strength Sabouraud Dextrose Agar (SDA), supplemented with antimicrobial antibiotics (50 mg/l rifampicin (Sigma-Aldrich; St. Louis), 25 mg/l cycloheximide (Calbiochem; Canada), 50 mg/l chloramphenicol (Sigma-Aldrich; St. Louis) (Gobel *et al*., 2012) and 20 mg/l dodine (Sigma-Aldrich; St. Louis). Culture plates were then maintained at 25 °C for 2-3 weeks in the dark until sporulation and used as starter cultures for all four experiments. Spore suspensions were prepared in a sterile laminar flow bench (Thermo Fisher Scientific). Conidia were harvested by flooding *B. bassiana* culture plates with sterile distilled water supplemented with 0.1% Triton X-100 (Sigma-Aldrich) (Experiment 1,3 and 4) or 0.1% Tween 80 (Difco) (Experiment 2) surfactant solution. Conidia were gently scraped off using a sterile surgical blade. The resulting conidia suspension was sieved through a sterile cheese cloth or wire mash to remove any traces of mycelia and dispensed into sterile Schott bottles and mixed using a magnetic stirrer for 5 minutes to produce a homogenous conidial solution. The concentration of conidial suspensions (conidia/ml) was determined using a Neubauer counting chamber and brightfield microscopy at 400 x magnification and the final concentration was adjusted to 10^7 conidia/ml by serial dilution for each isolate. Conidial viability was determined by counting percentage germination for each isolate as described by Sevim *et al*. (2010) for all suspensions used in each experiment. Sugarcane plants were inoculated with conidial suspensions from one of the four *B. bassiana* isolates (TL-leaf, S2roots, P2stem or N41S1TI) and control treatments were inoculated with 0.1% surfactant using inoculation methods described in the following section.

5.2.2 Experiment 1

Experiments were conducted in a glasshouse, at the South African Sugarcane Research Institute (SASRI) in Mount Edgecombe. A commercially grown sugarcane variety (N41) with intermediate resistance to *E. saccharina* was used for artificial inoculation of *B. bassiana* conidia suspensions. The initial experiment investigated the potential endophytic colonization of sugarcane plants grown using three plant pre-treatment growth methods, three conidia inoculation methods and three *B. bassiana* isolates. The sugarcane plants were prepared in 3 different ways (i) Hot water treated (HWT), (ii) Hot water treated and waxed (HWT+Wax) and (iii) tissue cultured plants (TC) as described below under 5.2.2.1- 5.2.2.3 to determine the effect of plant growth method on degree of endophytic colonization. Three *B. bassiana* isolates (*Typha latifolia* L.; leaf, (TL-leaf); *Phragmites australis* L. stem (P2stem) and *Sorghum bicolor* L. root (S_2roots)), were artificially inoculated into sugarcane plants to determine whether differences exist amongst *B. bassiana* isolates in the ability to colonize sugarcane plants. *Beauveria bassiana* cultures for the three isolates were prepared as described above (5.2.1). The three inoculation methods tested were (i) soil drench, (ii) stem injection and (iii) spray inoculation as described below: 5.2.2.4-5.2.2.6 to evaluate the effect of inoculation method on

the degree of endophytic colonization. Concurrently, endophytic persistence was evaluated every month for up to four months (5.2.2.7). Sugarcane tissue staining was used to observe colonization microscopically (5.2.2.8).

5.2.2.1 Plant pre-treatment: *Sugarcane plants grown from hot water treated setts (HWT)*

Sugarcane stalks (N41 var.) were collected from SASRI fields (Mount Edgecombe, Durban, South Africa) and cut into single budded setts using secateurs. Setts were then hot water treated (HWT) at 50 °C for 30 minutes in a water bath. Thereafter, setts were placed in Sterivent high containers (107 x 94 x 96 mm) lined with damp paper towel and incubated (IncoTherm^{e}- Labotec) at 30°C for 24 hours to induce bud germination. Setts were then planted with buds facing upwards in 96-cell polystyrene seedling trays containing pre-cooled, autoclaved (121 °C for 15 minutes) potting mix and vermiculite (1:1; v/v) (Hygrotech, Pretoria, South Africa) previously autoclaved for 3 consecutive days. The polystyrene seedling trays with planted setts were placed in a glasshouse (21/30°C; day/night) and watered twice a day using automatic sprayers for 5 min (600 ml/min) for eight weeks.

5.2.2.2: Plant pre-treatment: *Sugarcane plants grown from hot water treated and waxed setts (HWT+WAX)*

Sugarcane stalks were collected from the field, cut into setts, hot water-treated and incubated to induce bud germination as described above (5.2.2.1). Prior to planting, the cut ends of the setts were sealed by dipping in molten wax to prevent colonization of setts by microorganisms which may grow in potting mix and vermiculite over time. The waxed setts were then planted and maintained as described above (5.2.2.1).

5.2.2.3. Plant pre-treatment: *Tissue cultured sugarcane plants (TC)*

Sugarcane plants were propagated *in vitro* using a shoot tip culture method for sugarcane multiplication (Ramgareeb *et al*., 2010). Briefly, shoots were produced from meristems, and multiplied by a series of sub-culturing in proliferation media. Shoots were rooted in root media for 3 weeks and after root development, plants were removed from the medium and washed with tap water to remove excess media. Rooted plants were transferred into 96-cell polystyrene seedling trays containing pre-cooled autoclaved (121 °C for 15 minutes) potting mix and vermiculite (1:1; v/v) (Hygrotech, Pretoria, South Africa) previously autoclaved for 3 consecutive days. The polystyrene seedling trays with tissue cultured plantlets were placed in a glasshouse, watered twice a day using automatic sprayers for 5 min (600 ml/min) and fertilized (0.2 g of N: P: K; 2:3:2) once a month. Plants were allowed to harden for eight weeks prior to inoculation experiments.

5.2.2.4 Plant inoculation method: *Soil drench inoculation*

Seedling plants were uprooted from soil (potting mix and vermiculite) and roots were washed with running tap water. Approximately 2 cm of the roots were trimmed using scissors previously disinfected with 70% ethanol. The stem base (close to the germinating bud point) of the plant was pricked with an autoclaved toothpick to create a wound. Plants were then re- planted into clean 96-cell polystyrene seedling trays containing pre-cooled autoclaved (121 °C for 15 minutes) potting mix and vermiculite (1:1; v/v) previously autoclaved for 3 consecutive days. The soil surface of each polystyrene cell containing the test plants was individually drenched with 10 ml conidial suspensions containing 10^7 conidia/ml of the *B. bassiana* isolates (*Typha latifolia* L.; leaf, (TL-leaf); *Phragmites australis* L. stem (P2stem) or *Sorghum bicolor* L. root (S2roots. The entire seedling tray with soil drenched inoculated plants was then covered with a black plastic bag for 24 hours to maintain high humidity (Posada *et al*., 2007).

5.2.2.5 Plant inoculation method: *Stem injection*

Four-week-old seedling plants were uprooted from soil and the outer leaf covering of the plant was removed to expose the stem. The stem was rinsed with tap water and a wound was made on the lower part of the stem using an autoclaved toothpick (Figure 5.1A). Using a micropipette, 3 µl of the 10^7 conidia/ml suspension was administrated into the stem. Thereafter conidia inoculated plants were re-planted into clean 96 polystyrene seedling trays containing pre-cooled autoclaved potting mix and vermiculite.

5.2.2.6 Plant inoculation method*: Foliar spray inoculation*

Seedling leaves were trimmed (approximately 2 cm) and 5 ml conidia suspension containing 10^7 conidia/ml was applied per plant using a hand sprayer as described by Posada *et al*. (2007). The conidial suspension was sprayed on leaves and the entire seedling tray with foliar inoculated plants was also covered with a black plastic bag for 24 hours to maintain high humidity. All inoculated plants were maintained in the glass house and watered twice a day using automatic sprayers for 5 min (600 ml/min).

5.2.2.7 Endophytic evaluation and persistence

Once a month for up to four months sugarcane plants were collected from the glass house, uprooted and individually rinsed with running tap water. The outer leaf covering of the plant was removed to expose the stem (Figure 5.1B). The stem and upper leaves were subsequently placed in sterile 50 ml Corning plastic tubes. Plants were then surface disinfected by placing them in 100% ethanol for 1 minute, 10% sodium hypochlorite (NaOCl) for 5 minutes and rinsing twice in autoclaved distilled water for 1-minute intervals each (Reay *et al*., 2010). After surface

disinfection, plant samples were allowed to air dry on sterile Petri dishes in the laminar flow cabinet for 1 minute. To confirm the efficiency of the surface disinfection in removing epiphytic fungi, stem and leaves were pressed (by placing the upper surface of the plant onto one half of the agar plate then turning plant material to expose the lower surface onto the other half of the agar plate) onto SDA plates using flame sterilized forceps.

Stems were cut longitudinally into two halves. One half of the stem was used for staining with lactophenol cotton blue (Sigma, St. Louis, USA) (5.2.5.2) while the other half was further cut into transverse pieces (0.5 cm x 0.5 cm) dividing the stem into bottom, middle and top sections. (Figure 5.1C) while leaves were then cut into 0.5 cm x 0.5 cm transverse sections (Figure 5.1 D) using a pre-cooled, flame sterilized surgical blade (Sinorgmed-China). The leaves and stem sections were placed onto clean SDA plates using pre-cooled flame sterilized forceps. Plates were sealed with Parafilm and incubated at 25 °C in the dark and monitored for up to 14 days. At 14 days, fungal colonies that emerged from plant pieces (Figure 5.1D) were examined on wet mounted slide preparations using phase contrast microscopy at 400 x magnification. Colonies that showed a morphology resembling *Beauveria* spp. were sub-cultured onto SDA plates to obtain pure cultures. Control plants were also evaluated for the presence of endophytic *Beauveria* spp.

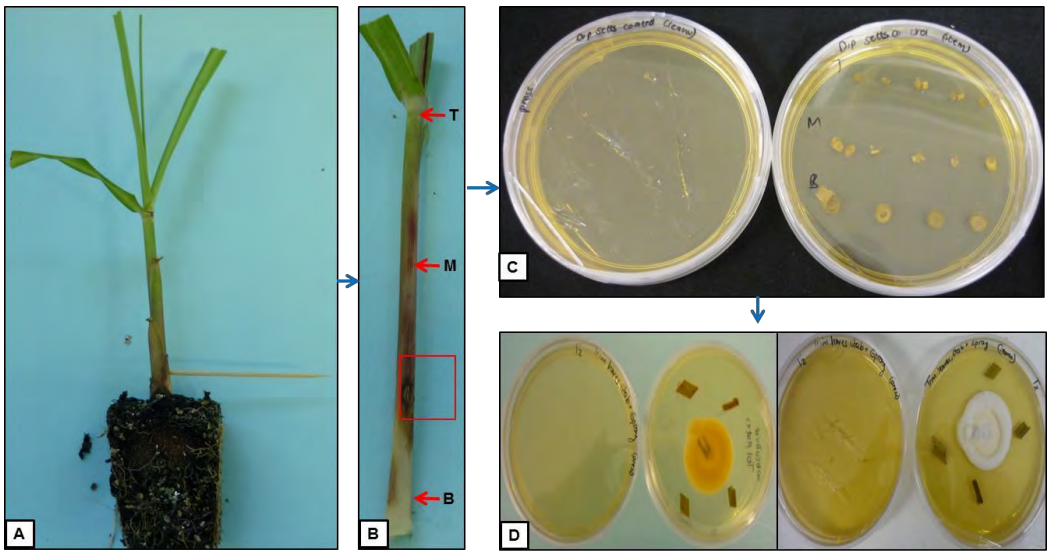

Figure 5.1: Schematic representation of re-isolation of *B. bassiana* from inoculated sugarcane plants. A) Demonstration of induced stem wound using autoclave toothpick, prior to stem injection conidia inoculation. B) Re-isolation was conducted from plant section. Red arrows indicating- B: bottom M: middle and T: top sections of the stem. C) Transverse stem pieces on SDA plate showing plant placement. D) shows leave placement on SDA and *Beauveria* sp.

5.2.2.8 Endophytic evaluation: *Stem tissue staining and microscopic analysis.*

The halved stem was further cut into thin longitudinal transparent pieces using a sterile surgical blade. Using forceps, the resulting pieces were placed into 1.5 ml microfuge tubes containing 1 ml of 5% (v/v) KOH. Tubes were then placed in a water bath at 65 °C for 30 minutes to leach out plant pigments. Solutions were decanted and stem pieces were rinsed with 1% (v/v) HCl and twice with distilled water. The microfuge tubes with stem pieces were then filled with 70% (v/v) lactophenol cotton blue solution (Sigma) diluted with lactophenol (25% (v/v) lactic acid, 25% (v/v) phenol, 50% (v/v) glycerol) and placed in a water bath at 65 °C for 30 minutes. The staining solution was decanted, and stem tissues were destained using lactophenol solution by incubating tubes at 65 °C for 30 minutes. If tissues were darkly stained, the destaining step was repeated. A wet mount of the stained stem tissues was then performed by placing tissue sections onto a glass slide with a drop of 98% lactic acid (Sigma) and glycerol (1:1) (v/v) solution. A cover slip was placed over the specimen and the samples were examined by brightfield microscopy at 400 x magnification to identify fungal hyphae colonizing stem tissues. Control stems were also stained as described above.

5.2.2.9 Statistical analysis

The number of plant pieces (stem or leaves) plated on SDA that developed colonies resembling *B. bassiana* were recorded. Plant pieces that had no *B. bassiana* colonies were also recorded. Endophytic colonization was expressed as a percentage of plated plant pieces developing colonies resembling *Beauveria* spp. to those that did not have colonies. The standard error of the mean was calculated for colonization percentages for each *B. bassiana* isolate. Stem and leaf colonization percentage data were subjected to a Shapiro–Wilk normality test (*W*-test) test using GenStat statistical package 18th edition (VSN International, Hemel Hempstead, UK). An analysis of variance (ANOVA) was then conducted on the normal data to determine the interactions of post time inoculation, fungal strain, inoculation method and plant growth method on endophytic colonization of sugarcane. A Sidak post hoc test separated the mean percentage colonization data and significant differences between fungal isolates, inoculation methods, plant growth method and time (months post inoculation). Significance was determined at $P < 0.05$.

5.2.3 Experiment 2

Experiments were conducted at the University of Göttingen, Agricultural entomology department (Germany). Two *B. bassiana* stains *Typha latifolia* L.; leaf, (TL-leaf) and N41S1TI (TI) were artificially inoculated into sugarcane plants to determine the better plant colonizer. Two field appropriate conidia inoculation methods (methods easily adopted in field) were tested to establish endophytic colonization of inoculated plants: (1) pre-treated setts dip inoculation

and (2) conidia spray inoculation (5.2.3.2-5.2.3.3). Endophytic colonization was evaluated at 3 months post inoculation using real time polymerase chain reaction (RT-PCR) analysis. Additionally, plant responses to colonization were determined by recording height and weight 3 months post inoculation (5.2.3.4 a&b).

5.2.3.1 Sugarcane plant material preparation

Sugarcane setts were transported from the South African sugarcane research institute (SASRI) to the University of Göttingen. Prior to transportation sugarcane stalks (N41 var.) were collected in the field in Mount Edgecombe, SASRI and brought to the laboratory for processing. Leaves were removed and stalk cut into single budded setts which were hot water treated at 50°C for 30 minutes. Thereafter, cut ends were individually sealed with wax to prevent tissue drying during transportation. Setts were subsequently placed in plastic bags lined with moist paper towel and sealed for moist retainment and germinated at 30°C for 2 days. Upon arrival at the University of Göttingen, setts were placed in a container lined with damp paper towel and incubated at 30 °C for 3 days to further induce bud germination and elongation, containers were covered to increase humidity and paper towel were moistened with sterile water if paper towel showed signs of drying out. Two inoculation methods (5.2.3.2-5.2.3.3) were investigated to establish endophytic colonization of *B. bassiana* on sugarcane.

5.2.3.2 Pre-treated setts: *dip inoculation*

Fungal spore suspension was prepared as described (5.2.1). Wax was peeled off the edges of 24 setts. Control treatments (8 setts) were dipped in 2L 0.1% Tween 80, 8 setts in 2L *B. bassiana* TL-leaf and 8 setts in *B. bassiana* N41S1TI 10^7 conidia/ml spore suspension for 12 hours (Figure 5.2) Starting with control, germinated setts with buds facing upwards in individual pots (± 3L) containing sterilized soil mixture (Fruhstorfer Erde Typ T, Hawita Gruppe GmbH, Vechta, Germany) and 0,3mm sand (3:1). Pots were placed in the greenhouse (25-28°C; day/night) and watered twice a day with 200ml sterile water. All plants were fertilized one month post inoculation NPK (5:1:5) and maintained for three months.

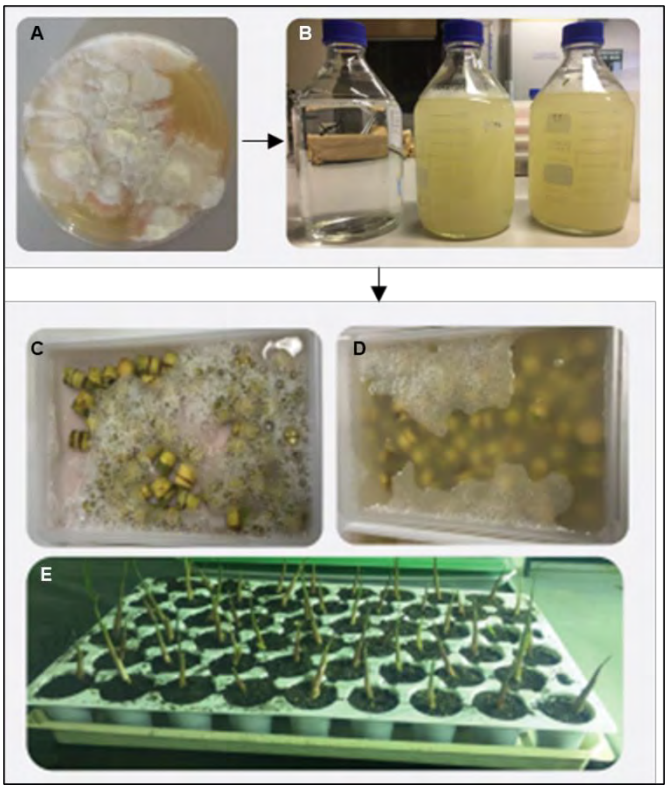

Figure 5.2: Diagrammatic representation of pre-treated sett dip inoculation method. A: Sporulated *B. bassiana* isolate in SDA plate. (B): Left: Control (2L 0.1% Tween 80) 2L *B. bassiana* TL-leaf and *B. bassiana* N41S1TI 10^7 conidia/ml spore suspension. (C &D): Sugarcane setts dipped in 0.1% Tween 80 and *B. bassiana* conidia suspension for 12 hours. (E) Treated germinated setts with buds facing upwards planted individual in seedling tray.

5.2.3.3 Conidia spray inoculation

Upon sett germination. Setts were planted as described above (5.2.3.2). Eight replicates were grown for each treatment (control, *B. bassiana* TL-leaf and N41S1TI). After four weeks of acclimation, seedlings were inoculated with10ml suspension of 10^7 conidia/ml via spraying onto 2cm trimmed leaves. Suspension was allowed to drizzle on the enter stem. Plants were maintained for three months in the green house and watered as described above (5.2.3.2).

5.2.3.4 Endophytic evaluation and plant response

a. *Re-isolation*

Three months post inoculation plants were harvested to determine colonization. Plants were harvested by gently uprooting from the pots, avoiding roots from breaking. Above and below ground measurements (plant height, and weight at harvest) were recorded for both inoculation methods (pre-treated setts dip and spray) and for each treatment (control, *B. bassiana* TL-leaf and N41S1TI). Plant height measurements (cm) were recorded from above the root to natural

breaking point, Root length (cm), stem and root weights (g) were likewise recorded. In the laboratory, stems were surface disinfected by soaking in 96% ethanol for 1 min, then in 10% sodium hypochlorite (NaOCl) for 5 min, rinsed twice in sterile distilled water for 1 min intervals each and finally once in sterile diethylpyrocarbonate (DEPC)-treated water. Roots were rinsed of excess soil in sterile water and leaves detached from stem, thereafter, soaked in 70% ethanol for 1 minute, 2% sodium hypochlorite for 30 seconds and 70% ethanol for 1 minute, followed by immersion in sterile distilled water for 30 seconds twice and once in sterile diethylpyrocarbonate (DEPC) as described in Jayanti, (2020). To confirm successful disinfection, 100µl DEPC water, used in the final rinse was spread plated onto SDA and incubated for 2-weeks at 25 °C in the dark. Lack of *Beauveria* spp. growth indicated successful surface disinfection.

b. *DNA extraction and RT-PCR quantification*

Surface disinfected leaves, stems and roots from spray inoculation were grounded to a fine powder using sterile mortar and pestle and stored at -20°C until DNA extraction. DNA extraction from plant samples was done using a peqGOLD Plant DNA Mini kit (Peqlab, Erlangen, Germany). The purity and concentration of extracted DNA was determined using a spectrophotometer (NanoDrop, USA). To confirm endophytic colonization of plant tissues, real time PCR was conducted using species specific primers Bsn1-2-forward "GCGTCAAGGTGCTCGAAGACAG" and Bsn1-2-reverse "TCTGGGCGGCATCCCTATTGT" (Zhang, 2014). Real time PCR amplification was performed for each of the extracted samples in a reaction tube with a final volume of 10µl containing 5µl 2× qPCRBIO SyGreen Master Mix (PCR Biosystems Ltd., England), 0.2µl, 10µM each of the opposing amplification primers, 1µl of sample DNA and 3.6µl sterile water with reactions performed using an iCycler System CFX384 Real time system (BioRad, Hercules, CA, USA) with the following cycling parameters: initial template denaturation at 95°C for 2 min followed by 39 cycles at 95°C denaturation for 10 s., 65°C annealing for 15 s., 72°C extension for 15 s., and a plate read at the end of each cycle. Subsequently a final extension at 72ºC for 1 min. The amplification of the melting curve analyses was performed at a range of 95°C to 55°C held for 30 s. Serial diluted standard curve concentrations 100 pg, 33.33 pg, 11.11 pg, 3.70 pg, 1.23 pg, 0.41 pg, 0.13 pg, 0.045 pg, 0.015 pg, and 0.0005 pg run in triplicates were obtained from stock solutions available at the Agriculture Entomology department and included together with a negative control.

5.2.3.5 Statistical analysis

Collected data for above (stem and leaves) and below ground (roots) measurements for plant height (cm), and weight (g) at harvest for both inoculation methods (pre-treated setts dip and spray) and for each treatment (control, *B. bassiana* TL-leaf and N41S1TI) were checked for

normality using the Shapiro-Wilk test for Normality in Genstat 18th edition. The normal data was subjected to a one-way ANOVA (analysis of variance) and Sidak post hoc test to separate the means and determine the effect of B*. bassiana* isolates on plant height and weight when compared to control treatments. A significance threshold of 0.05 (probability level) was used.

Data from RT-PCR (qPCR) quantification of DNA (pg) obtained from roots, stem or leaves inoculated with *B. bassiana* using spray inoculation were analysed. Control DNA samples were also included. Separated values of average number of gene copies expressed as pg DNA/100 g were analysed using one way ANOVA. The means within each treatment were compared for statistical significance differences using a Bonferroni test at a significance level for $p< 0.05$ in Genstat 18th edition.

5.2.4 Experiment 3

Experiments were conducted at SASRI. Twenty-eight sugarcane varieties collected in Chapter 2 used to isolate endophytic *B. bassiana* from sugarcane, were additionally used to artificially inoculate *B. bassiana* TL-leaf strain conidia via dipping setts, drenching soil and foliar spray (5.2.4.1). Endophytic colonization of inoculated plants by *B. bassiana* was evaluated at 3- and 6-months post inoculation (5.2.4.2) to determine persistence of colonization and define whether artificial colonization is variety dependent.

5.2.4.1 Sugarcane plant material

Sugarcane varieties were collected from the field and cut into single budded setts. Setts were thereafter hot water treated at 50°C for 20 minutes. Setts were either dipped into $x10^7$ conidia/ml suspension of *B. bassiana* isolate TL-leaf (treatment) or 0.1% Triton X-100 (control) for 2 minutes. Dipped setts were subsequently placed in clean steri-vent containers and covered with moist autoclaved vermiculite and incubated at 30°C until bud germination and root emergence (± 5 days). Once germinated, setts were placed into seedling trays and covered with autoclaved soil, which was then drenched with 10 ml of a conidial suspension (10^7 conidia/ml). Trays were then placed in the glass house to allow plants to grow for four weeks. Thereafter, leaves from emerged plants were trimmed and sprayed with 5ml of a 10^7 conidia/ml suspension of *B. bassiana* TL-leaf. Control setts and plants were treated similarly with 0.1% Triton X-100.

5.2.4.2 Endophytic evaluation and plant response

At 3- and 6-months post inoculation 2 stalks per variety were collected to determine colonization. Stalks were cut and divided into bottom, middle, top and leaf sections, and subsequently surface disinfected by soaking in 96% ethanol for 1 min, then in 10% sodium hypochlorite (NaOCl) for 5 min, then finally rinsed twice in sterile distilled water for 1 min intervals each (Reay *et al*., 2010).

Surface disinfected plants were placed in sterile Petri dishes and allowed to air dry in the laminar flow bench for ±5 minutes. Five pieces from the respective sections were cut from each section and placed onto SDA agar plates which contained antibiotics and used to re-isolate previously inoculated *B. bassiana* TL-leaf (40 plant pieces were plated per sugarcane variety). Two weeks following plate inoculation and incubation at 25°C in the dark, the number of plant pieces observed with *B. bassiana* was recorded. The degree of plant colonization was expressed as the sum of plant pieces observed with colonies resembling *Beauveria* species.

5.2.4.3 Statistical analysis

Data of the number of *B. bassiana* colonies re-isolated from 28 sugarcane varieties at 3- and 6-months post inoculation was subjected to a normality test (Shapiro-Wilk test for normality) using GenStat (18th edition, VSN International, Hemel Hempstead, UK). The normal data was analysed using one-way analysis of variances (ANOVA) in a randomized block design. The means within each variety were compared for statistical significance differences using a Sidak post hoc test at a significance level for $p< 0.05$. The number of colonies re-isolated from plant pieces at 6 months was analysed similarly.

5.2.5 Experiment 4:

Experiments were conducted at SASRI glasshouses. Sugarcane variety (N41) was inoculated with *B. bassiana* isolate TL-leaf and N41S1TI conidia prepared as described in 5.2.1. Control plants were inoculated with 0.1% Triton X-100. Conidia dip and spray inoculations were tested for each *B. bassiana* isolate and control plants (5.2.5.1-5.2.5.2). Thereafter, endophytic colonization was evaluated 16 months post inoculation (5.2.5.3). Furthermore, 16 months post inoculation, *E. saccharina* 2nd instar larvae were allowed to feed on inoculated sugarcane plants (5.2.5.4) as in the field, *E. saccharina* generally attacks old carry over sugarcane plants (10 month and older sugarcane). Effect of colonization on sugarcane growth and *E. saccharina* weights and boring was investigated (5.2.5.4).

5.2.5.1 Plant inoculation method: *Dip inoculation*

Sugarcane stalks (N41 var.) were collected from the field and cut into setts. Three hundred setts were hot water treated (50ºC for 30mins), 50 setts were dipped in 0.1% Triton X- 100 which served as controls; 50 into 10^7 conidia/ml suspension of TL-leaf and similarly, 50 into N41S1TI suspension. All setts were dipped for 12 hours in the respective inoculum and thereafter planted in 98 polystyrene seedling trays for 8 weeks in glasshouse. Forty germinated seedlings from each treatment were transferred into big 10L black pots (4 seedlings per pot) containing river sand (10 replicates per treatment).

5.2.5.2 Plant inoculation method: *Spray inoculation*

Following hot water treatment, 150 setts were planted in 98 polystyrene seedling trays for 8 weeks. Forty germinated seedlings from each treatment were transferred into big 10L black pots (4 seedlings per pot) containing river sand. Thereafter, leaves were trimmed and sprayed with either 10ml of 0.1% Triton x- 100 (control), or 10^7 conidia/ml suspension of *B. bassiana* TL-leaf or N41S1TI isolates. All plants were fertilized (0.2 g of N: P: K; 2:3:2) once a month and maintained in the green house for the duration of the experiment. Each treatment had ten 10L black pots.

5.2.5.3 Endophytic evaluation and plant response

Ten plants from each treatment (dip, spray, and control inoculation) were harvested at 16 months with plant height (cm) and stalk weight (g) measured during harvest. Root, stalk and leaf samples were collected from each stalk and surface sterilized to investigate colonization. For re-isolation, stalk samples were cleaned, and three buds were counted from the top breaking point. The 3rd bud (node) and 3rd internode from the breaking point was cut and used for *Beauveria* spp. re-isolation. Likewise, the 3rd node and internode from the bottom part of the stem (from the roots) were also cut and surface sterilized as described by Reay *et al*. (2010) and left to dry in laminar flow on sterile Petri dishes. Once dry, all edges of the plant samples were cut using a flame sterile surgical blade (Sinorgmed- China) to remove dead plant tissue and create fungal emergence sites (Reay *et al*. 2010). Plant samples were then cut transversely into smaller sections (1.5 cm x 2 cm) and placed onto SDA agar (supplemented with antibiotics) using flame sterilized forceps. All plates were then sealed with Parafilm (Pechiney Plastic Packaging, Chicago) and incubated at ambient temperature (25-27°C) in the dark for 14 days.

5.2.5.4 *Eldana saccharina* feeding response in *B. bassiana* inoculated sugarcane plants.

The remaining stalks in the pots were used for *E. saccharina* feeding experiments, where larvae were allowed to feed on 16 post *B. bassiana* inoculated sugarcane plants. A randomized design was used to re-organize the 10L pots with stalks in the glass house. Using a handheld drill, the stalks were drilled at two points: 3 nodes from breaking point (top) and 3 nodes above soil in the bottom of the stalk. Ten stalks from each treatment (60 stalks) were drilled: (10 stalks control, ten for *B. bassiana* TL-leaf and 10 for *B. bassiana* N41S1TI strain x 2 inoculation methods (Dip and Spray).

Eldana saccharina 2nd instar larvae were collected from the insect rearing unit (IRU) at SASRI and weighed in the laboratory to obtain initial larval weights before feeding. Larvae were individually placed in labelled 1ml Eppendorf tubes (Eppendorf bottom ends were previously cut

using a hot flamed surgical blade and a Prestik used to bud the cut end to prevent larvae from escaping). In the green house, weighed larvae in labelled Eppendorf tubes were mounted onto drilled points (top and bottom) on the stalk, where the cut Eppendorf ends were placed and attached to the stalk using the Prestik. A hundred and twenty larvae were inoculated in total for all treatments ((20 larvae (control), 20 (TL-leaf), 20 (N41S1TI) x 2 inoculation methods (Dip and Spray)). The larvae were allowed to feed on the stalk for 3 weeks before harvesting. Thereafter, larvae were collected from each stalk and weighed in the laboratory. In addition, boring length (cm) was recorded for each treatment. During harvest, sugarcane weights and stalk height were likewise recorded.

5.2.5.5 Statistical analysis

Measurements of sugarcane stalk height (cm) and weight (g) 16 months post inoculation with the two *B. bassiana* isolates and control, using two inoculation methods were recorded. The data was subjected to a normality test (Shapiro-Wilk test for normality) using GenStat statistical package 18th edition (VSN International, Hemel Hempstead, UK). The normality distributed data was analyzed using Restricted Maximum Likelihood (REML) (as larvae were not always recovered in all inoculated stalks, data was not balanced). The Wald statistic was used to determine the fixed degree of freedom (n.d.f) and Sidak post hoc test to separate the means and determine the influence of the *B. bassiana* isolates on sugarcane stalk height and weight when compared to control treatments. A significance threshold of 0.05 (probability level) was used. *Eldana saccharina* boring length (mm) and larval weight (g) data, following feeding on sugarcane inoculated plants was analysed similarly. The means within each treatment (isolates, inoculation method) were compared for statistical significance differences using a Sidak post hoc test at a significance level for $p< 0.05$ to determine differences in boring length and larval weights when compared to control treatments.

5.3 Results

5.3.1 Experiment 1

Conidial suspensions had conidial viability above 80% for all the three *B. bassiana* isolates used in this experiment. Following surface disinfection of sugarcane plants (three plant pre-treatment growth methods) individually inoculated with 3 *B. bassiana* isolates using 3 inoculation methods, no fungal growth was observed after pressing surface disinfected stem and leaves on SDA plates, indicating the efficiency of the surface disinfection method in removing epiphytic fungi. Furthermore, no *B. bassiana* was recovered from control plants. However, endophytic *Fusarium* spp. and bacteria was re- isolated from some plant pieces. Plants inoculated with *B. bassiana* showed no symptoms of damage that would suggest that

the fungus might be detrimental to the plant.

5.3.1.1 Effect of plant growth method on endophytic colonization of sugarcane by *Beauveria bassiana*

Beauveria bassiana isolates were successfully established endophytically in sugarcane stem and leaves of Hot water treated (HWT), hot water treated and waxed (HWT+WAX) and tissue cultured (TC) seedling plants, as fungal mycelia were observed emerging from plant pieces plated on SDA plates following surface disinfection (Figure 5.3).

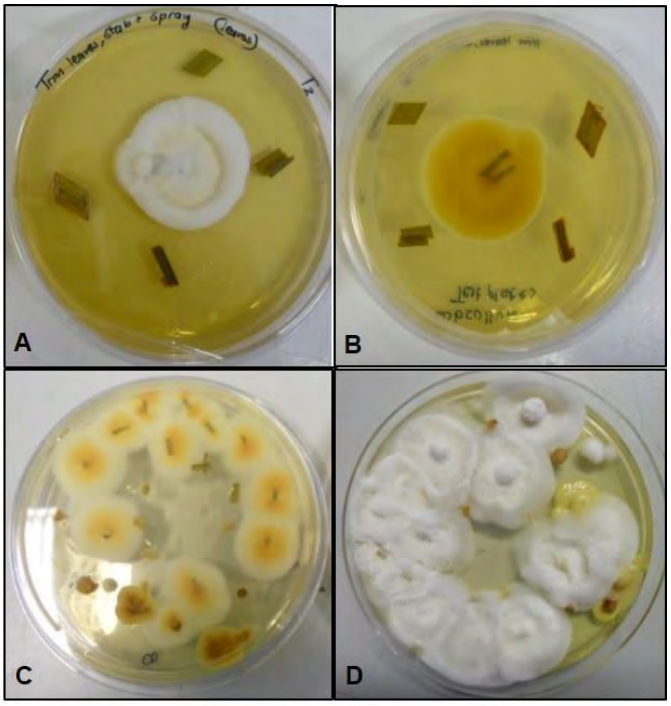

Figure 5.3: Fungi morphologically resembling *Beauveria* sp. re-isolated from inoculated tissue cultured (TC) sugarcane plants. A & B: colonies emerging from leaf after conidia foliar spray; C & D: colonies emerging from bottom, middle and top stem sections after stem injection with conidia.

Furthermore, microscopic analyses of the fungal mycelia found emerging from the plant pieces showed morphologies matching *Beauveria* sp., with globose conidiophores, single celled globose conidia, zig zag hyphal rachis and hyaline hyphae (Figure 5.4).

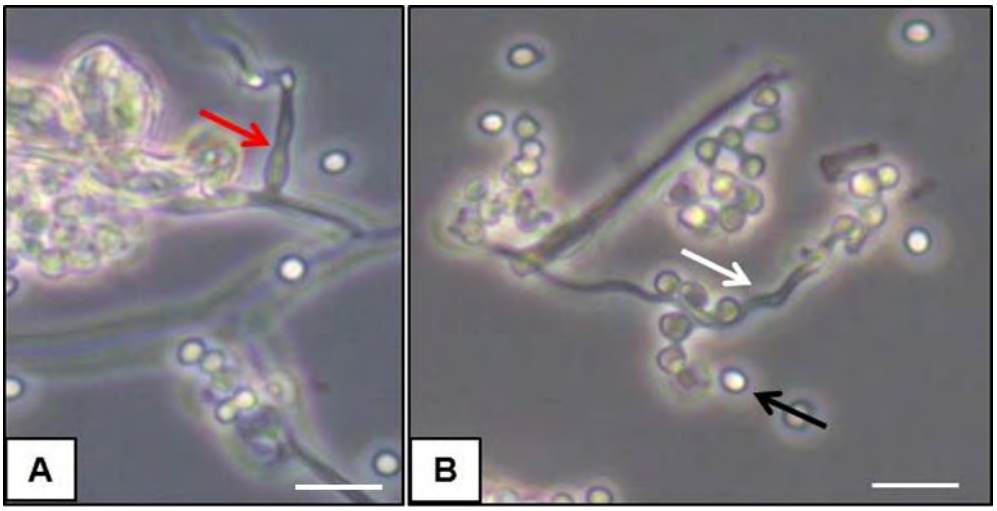

Figure 5.4: Wet mount samples from *Beauveria* sp. colonies re-isolated from plant pieces following artificial inoculation with *Beauveria bassiana* s.l. conidia into sugarcane plants. (A) Red arrow: globose shaped conidiophores. Scale bar=60µm. (B) White arrow: zig zag rachis; Black arrow: single celled globose shaped conidia. Scale bar= 50µm.

There was a significant difference (P<.001; d.f. = 2) in colonization of HWT, HWT+WAX and TC plants as percentage plant colonization varied amongst the plants. Overall, TC plants were better colonized by *B. bassiana* isolates as higher percentage stem (Figure 5.5) and leaf (Figure 5.6) colonization were observed over time. For instance, 80% stem colonization was obtained for TC plants, one month post inoculation when plants were inoculated with *B. bassiana* isolate TL-leaf using conidia stem injection method, while lower stem colonization were obtained in HWT+WAX (33.3%) and HWT (20%) (Figure 5.5).

Percentage colonization of sugarcane plants (HWT, HWT+WAX or TC) was influenced by inoculation method, *B. bassiana* isolate and time post inoculation, as a significant interaction (P<.001; d.f. = 24) between these main effects was obtained, percentage colonization varied with a variation of one factor.

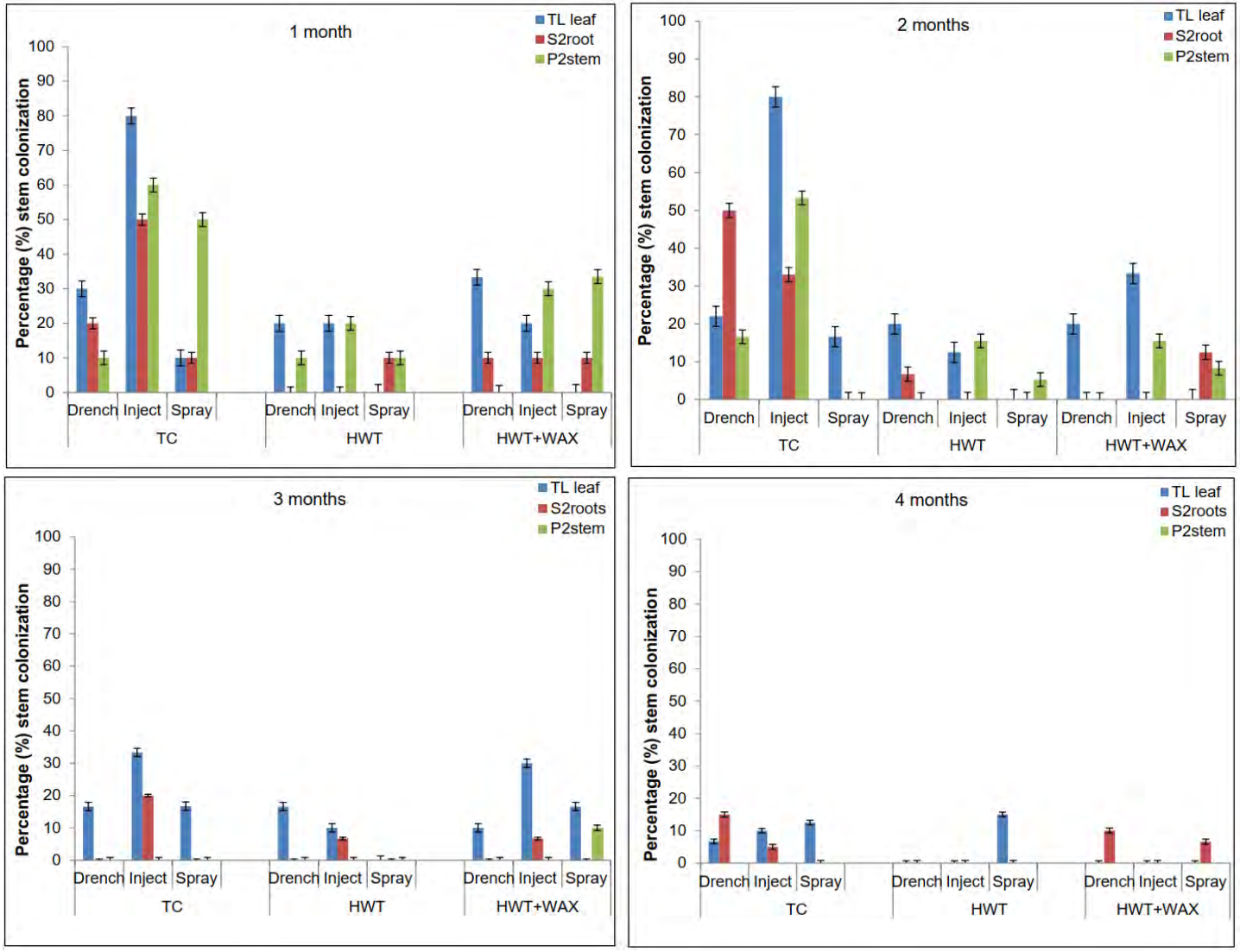

Figure 5.5: Percentage colonization of sugarcane (N41) stem tissue at 1 to 4 months post inoculation with *Beauveria bassiana* isolates TL-leaf, S2root or P2stem. Sugarcane plants were grown from 1) TC: tissue cultured plants, 2) HWT: hot water treated setts or 3) HWT+WAX: hot water treated and wax setts and inoculated with *B. bassiana* conidia using 1) Soil Drench 2) Inject stem; or 3) Spray inoculation. Error bars indicate standard error for percentage mean colonization.

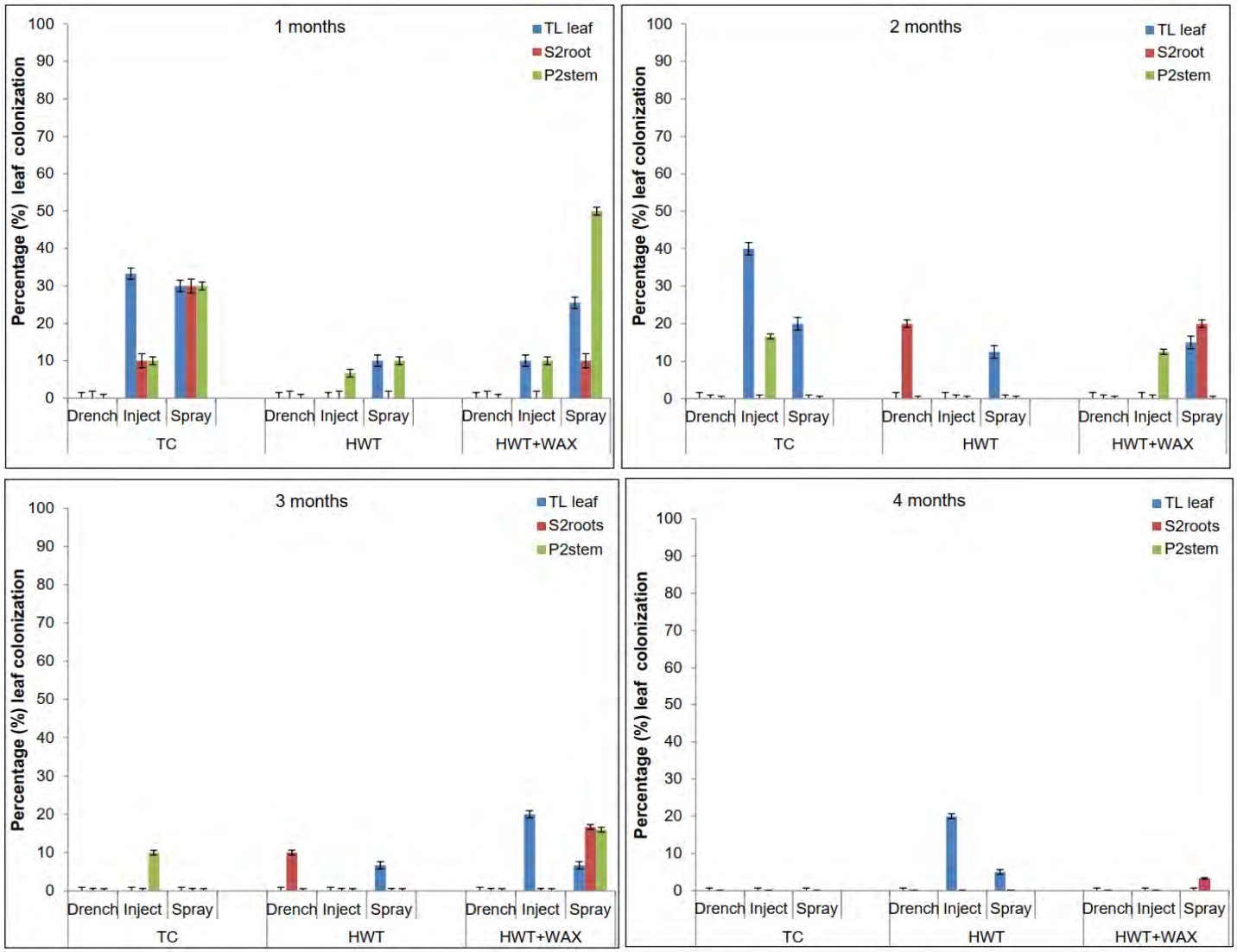

Figure 5.6: Percentage colonization of sugarcane (N41) leaf tissues at 1 to 4 months post inoculation with *Beauveria bassiana* isolates TL-leaf, S2root or P2stem. Sugarcane plants were grown from 1) TC: tissue cultured plants, 2) HWT: hot water treated setts or 3) HWT+WAX: hot water treated and wax setts and inoculated with *B. bassiana* conidia using 1) Drench: 2) stem inject or 3) Spray: inoculation. Error bars indicate standard error for percentage mean colonization.

Seedling plants grown from HWT setts and HWT+WAX setts displayed stem and leaf colonization percentages lower than 50% in all the re-isolation months (Figure 5.5- Figure 5.6). During re-isolation from HWT and HWT+WAX plants, endophytic fungi other than *B. bassiana* were also isolated. Endophytic *Fusarium* spp. and bacterial species were recovered from these plants (Figure 5.7).

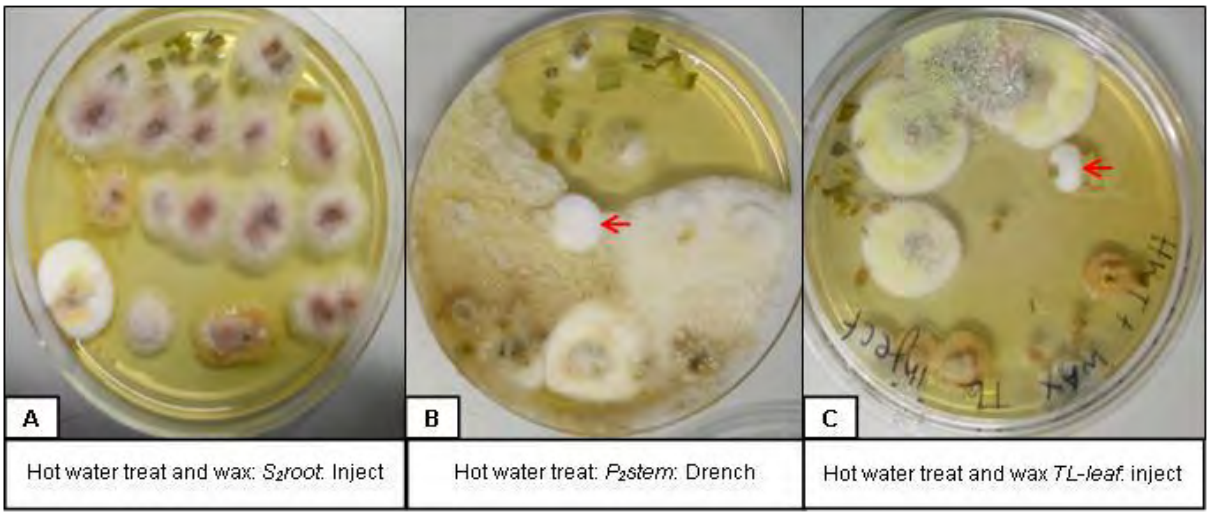

Figure 5.7: Colonies re-isolation from *B. bassiana* inoculated sugarcane plants. (A): *Fusarium* spp. emerging from seedlings grown from hot water treated and waxed setts (HWT+WAX) following injecting plants with conidia from *B. bassiana* isolate S2root. (B): Fungal colonies emerging from seedlings grown from hot water treated setts (HWT) following drenching plants with conidia from *B. bassiana* isolate P2stem. (C): Fungal colonies merging from seedlings grown from HWT+WAX setts after injecting plants with *B. bassiana* TL-leaf strain. Red arrows (B and C) indicate *B. bassiana* re-isolated from plant pieces.

5.3.1.2 Effect of *Beauveria bassiana* isolates on endophytic colonization of sugarcane plants.

There was a significant difference (P<.001; d.f. = 2) in the percentage colonization obtained for each of the fungal isolates and as percentage colonization varied amongst the fungal isolates over the four months and when the same plants (HWT, HWT+WAX or TC) and inoculation methods were used. For instance, at one month post inoculation, tissue cultured (TC) sugarcane plants inoculated with *B. bassiana* TL-leaf strain, using stem injection method exhibited 80% and 33% colonization of stems and leaves respectively. However, when TC plants were inoculated with conidia from *B. bassiana* P2stem, 60% and 10% stem and leaf colonization resulted one-month post inoculation respectively (Figure 5.5 and Figure 5.6), while *B. bassiana* S2root strain resulted in 50% and 10% stem and leaf colonization respectively, at the same month following

stem injection of conidia into TC plants. Overall *B. bassiana* TL-leaf strain resulted in higher colonization percentages of sugarcane stem and leaves across all tested factors (Figure 5.5 Figure 5.6). Likewise, percentage colonization of sugarcane plants by *B. bassiana* isolates was influenced by time, plant growth method and inoculation method used, as significant interactions (P<.001; d.f. = 24) and varying percentage colonization's were observed for each fungal strain across these tested effects.

5.3.1.3 Effect of inoculation method on endophytic colonization of sugarcane plants by *Beauveria bassiana* isolates

All inoculation methods were successful in artificially inoculating and establishing *B. bassiana* as an endophyte in sugarcane plants as *B. bassiana* colonies were re-isolated from plants up to four-month post inoculation (Figure 5.5 and Figure 5.6). There were significant differences (P = 0.002; d.f. = 2) in colonization of sugarcane plant parts when different inoculation methods were used, as percentage stem and leaf colonization varied with inoculation method. Inoculation methods were seen to influence the plant part colonized. For instance, higher stem colonization was obtained when conidia were inoculated by the stem injection method which injected conidia directly into the stem. Whilst leaves were colonized to a greater degree when the foliar spray inoculation method was used (Figure 5.6). When the soil drench method was used, no colonization of leaves was observed for TC and HWT+WAX plants in all the post inoculation months (Figure 5.5 and Figure 5.6). The drench inoculation method only achieved stem colonization for TC and HWT+WAX plants. Soil drench inoculation resulted in lower overall percentage colonization of leaves (Figure 5.6).

5.3.1.4 Evaluation of endophytic colonization and persistence of *Beauveria bassiana* in sugarcane plants

Endophytic *B. bassiana* colonization of sugarcane stems were verified when blue-stained fungal hyphae were detected microscopically in *B. bassiana* inoculated sugarcane stem tissues, following lactophenol blue staining (Figure 5.8B), while uninoculated control stems had no stained hyphae (Figure 5.8A).

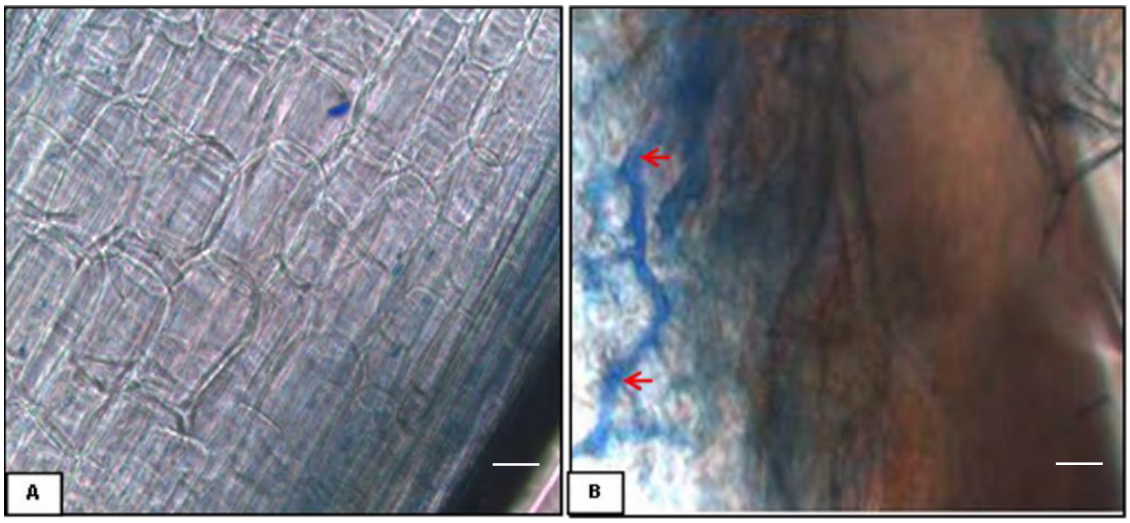

Figure 5.8: Lactophenol blue stained sugarcane stem tissues. (A): Control stem tissue inoculated with 0.1% Triton X-100, with no fungal stained hyphae visible. (B): *Beauveria bassiana* inoculated stem tissue with visible fungal stained hyphae present. Red arrows indicate blue-stained fungal hyphae present in an inoculated plant. Scale bar =10µm.

Endophytic colonization was significantly (P. <.001; d.f. = 3) influenced by time, with higher colonization at one month post inoculation, while lower colonization was observed at four months post inoculation (Figure 5.5 and Figure 5.6), for all isolates, inoculation methods, and sugarcane plants. At four months post inoculation only 20% leave colonization was obtained in HWT plants injected with *B. bassiana* TL-leaf conidia, whilst HWT+WAX plants and TC plants had no *B. bassiana* re-isolations from leaves four months post conidia injection with *B. bassiana* TL-leaf strain (Figure 5.6). Persistence was greatest in sugarcane stems when TC plants were inoculated with stem injection.

5.3.2 Experiment 2

5.3.2.1 Endophytic evaluation and plant response

During spray inoculation *B. bassiana* isolates TL-leaf (TL) and N41S1TI (TI) increased above and below weights (g) of sugarcane plants measured at three months post endophytic inoculation when compared to control plants (Figure 5.9 A&B; D&E). Although control plants were taller than *B. bassiana* inoculated plants using spray inoculation method, plant height was not significantly different. Spray inoculated plants had taller plant when compared to dip inoculated plants (Figure 5.9 C&F).

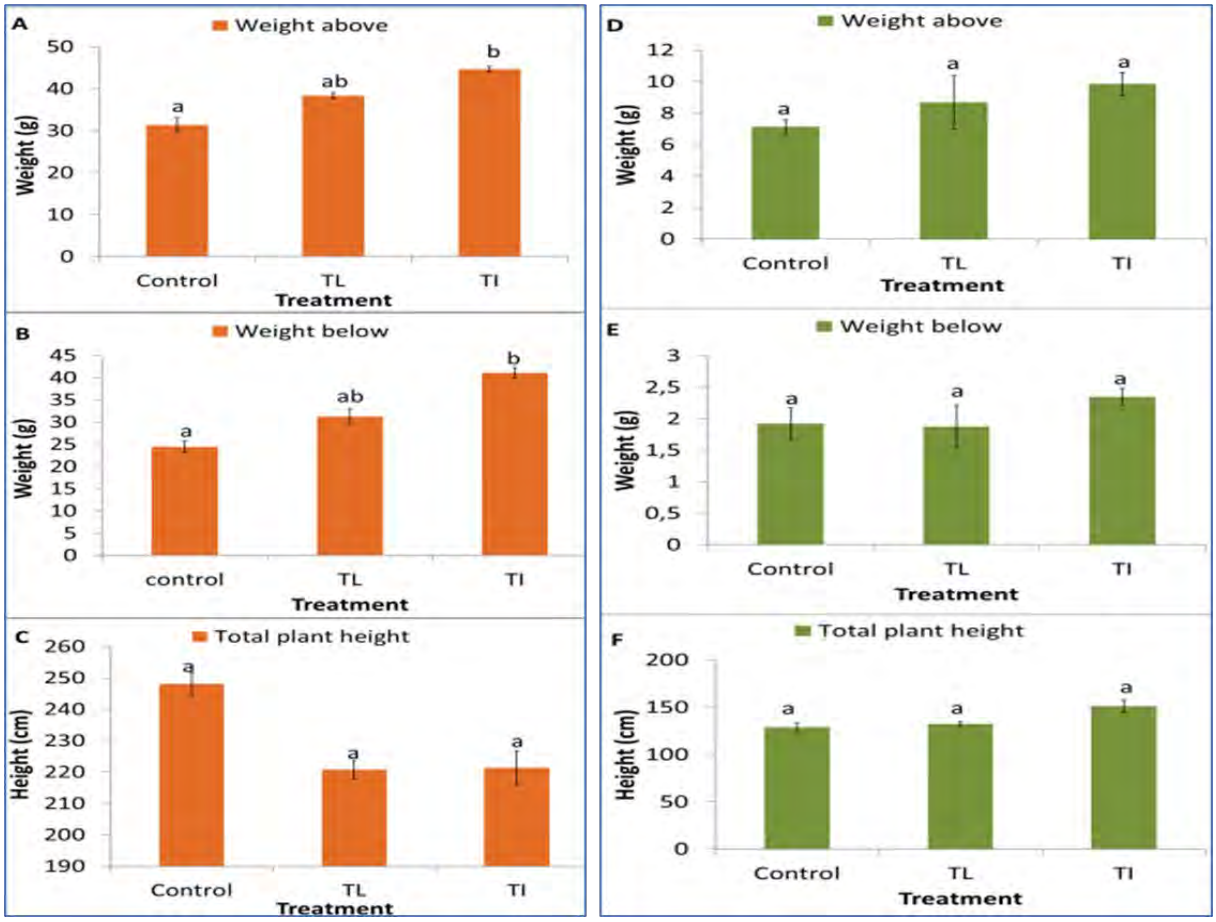

Figure 5.9: Growth parameters of sugarcane plants inoculated with *Beauveria bassiana* isolates TL=leaf (TL), N41S1TI (TI) and Control (0.1% Tween 80) using spray inoculation (A-C) (n=9) and (D-F) using Dip inoculation (n=6) 3 months post inoculation Bars represent standard error of means and different letters indicate significant differences between isolates.

Beauveria bassiana DNA was detected in sprayed sugarcane plants using qPCR. *Beauveria bassiana* strain N41S1TI (TI) was detected in root, stem, and leaves (0,31±0,01; 0,03±0,008; 0,41±0,018 DNA pg/100g) (Figure 5.10). Significant *B. bassiana* DNA was detected in roots inoculated with *B. bassiana* strain N41S1T (TI) (Root: F pr.= 0,017). Only N41S1T (TI) was detected in stem and leaves (Stem: F pr.= 0,182; Leaves: F pr.=0,007). Low levels of DNA were detected in roots of control and *B. bassiana* TL-leaf strain samples. In this experiment B. bassiana N41S1TI improved above and below sugarcane plant weight when compared to control for spray inoculations.

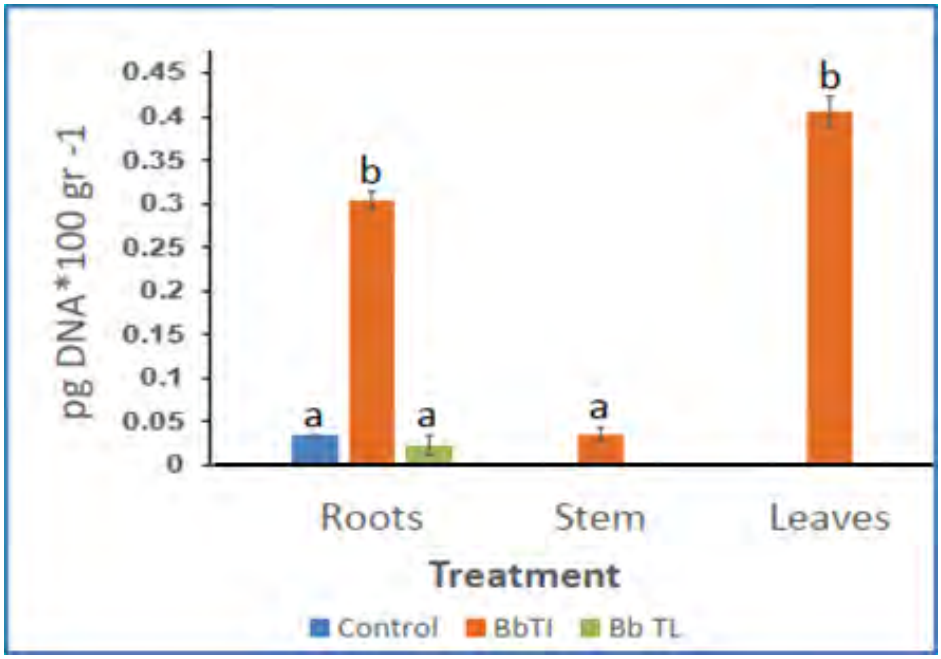

Figure 5.10: Amount of *Beauveria bassiana* TL=leaf (TL), N41S1TI (TI) DNA (pg DNA/100gr) detected in sugarcane (roots, stem and leaves) plants 3 months post spray inoculation (n=9). Bars represent standard error of means and different letters indicate significant differences between treatments.

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5.3.3 Experiment 3

5.3.3.1 Endophytic evaluation and plant response

Twenty-eight sugarcane varieties artificially inoculated with conidia of *B. bassiana* TL-leaf strain, conidia via dipping setts, drenching soil and foliar spray were evaluated at 3- and 6-months post inoculation, for persistence of endophytic colonization and to determine whether artificial colonization is variety dependent. At 3 months, there was a significant difference (F pr. 0.001; d.f. 27) in the number of colonies resembling *Beauveria* spp. re-isolated from the sugarcane varieties. Twenty-six of the 28 varieties were colonized by *B. bassiana* at different degrees, R573 and Nco376 had the highest number of *Beauveria* spp. re-isolated, with 17 and 13 plant pieces yielding colonies resembling *B. bassiana* morphology. Sugarcane varieties R570 and N26 had zero *Beauveria* spp. colonies re-isolated from bottom, middle, top and leaf sections at 3 months post *B. bassiana* inoculation (Figure 5.11).

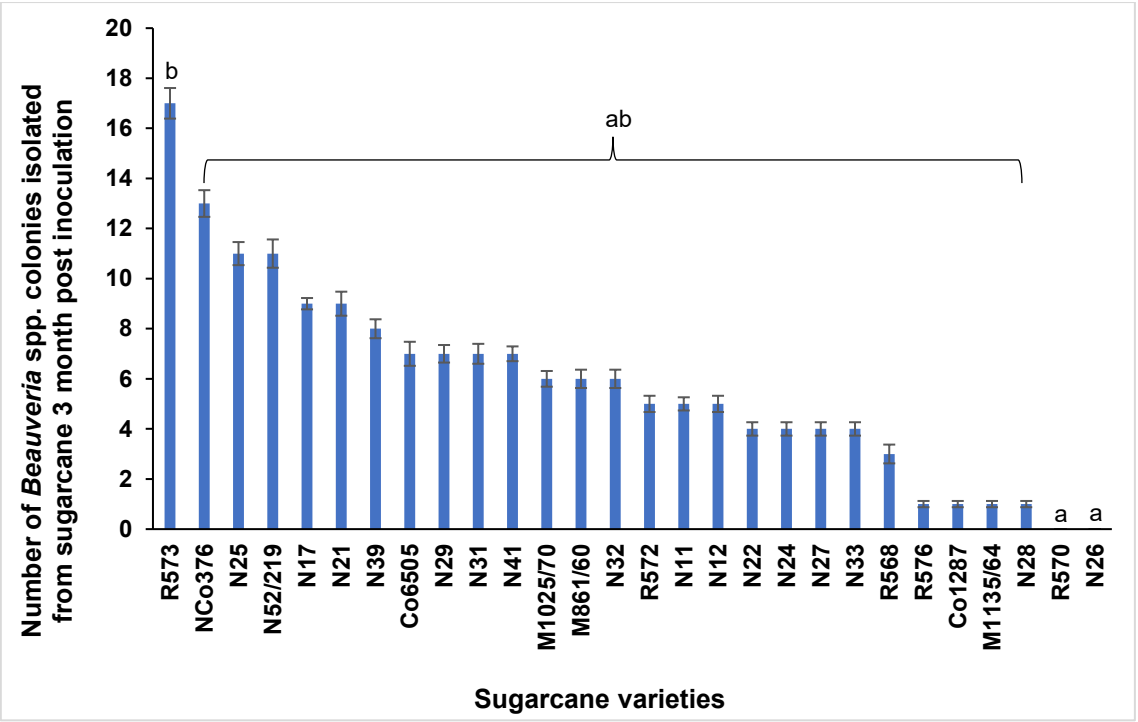

Figure 5.11: Collective number of colonies resembling *Beauveria* spp. isolated 3 months post *B. bassiana* TL-leaf inoculation following isolation from 2 sugarcane stalks from top, middle and bottom stem parts . Bars represent standard error of means and different letters indicate significant differences between varieties.

Beauveria bassiana colonization decreased with increase in post inoculation time from 3 to 6 months. The number of colonies re-isolated from sugarcane varieties differed significantly (F pr. 0.025; d.f. 27) with only 7 of the 28 varieties having colonies resembling *Beauveria* spp. re-isolated at 6 months post *B. bassiana* TL-leaf inoculation (Figure 5.12). Sugarcane variety R570 had 3 colonies resembling *Beauveria* spp. at 6 months, with on colonies re-isolated at 3 months post inoculation, while N26 had no colonization at both months evaluated (Figure 5.11 and Figure 5.12).

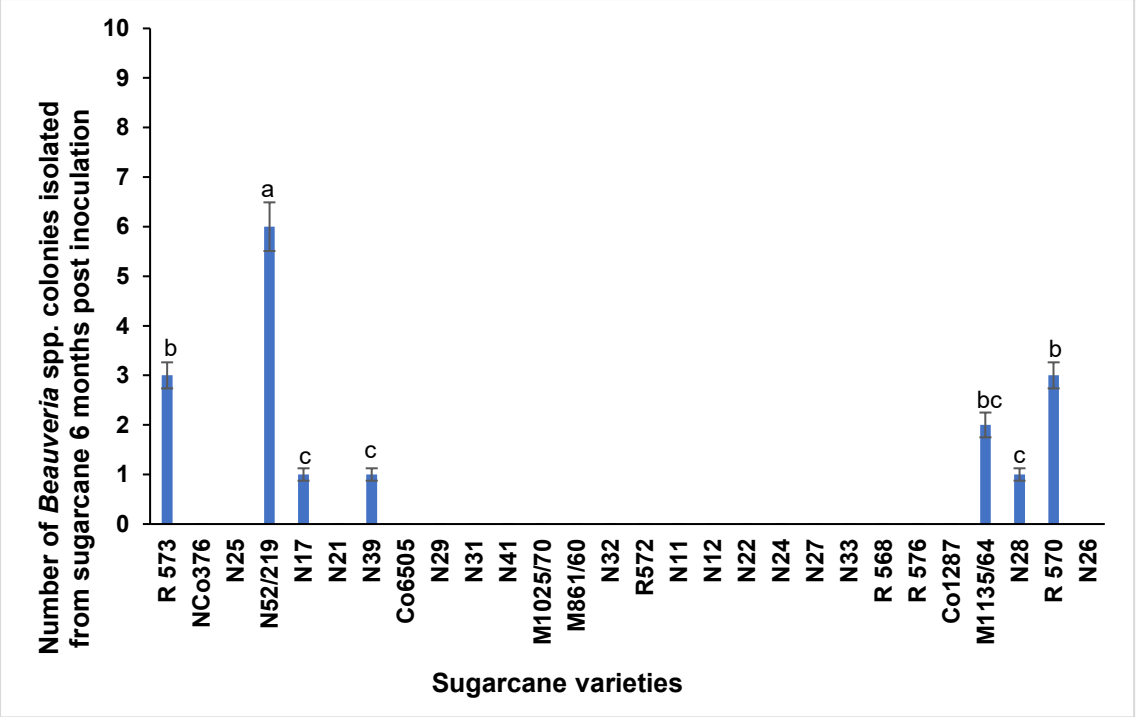

Figure 5.12: Collective number of colonies resembling *Beauveria* spp. isolated 6 months post *B. bassiana* TL-leaf inoculation following isolation from 2 sugarcane stalks from top, middle and bottom stem parts. Bars represent standard error of means and different letters indicate significant differences between varieties.

5.3.4 Experiment 4

5.3.4.1 Endophytic evaluation and plant response

Sugarcane variety (N41) inoculated with *B. bassiana* strain TL-leaf, N41S1TI and 0.1% Triton X-100 (control) were evaluated for persistence of endophytic colonization, plant, and pest response 16 months post inoculation. No colonies resembling *Beauveria* spp. morphology were re-isolated from sugarcane plant samples 16 months post inoculation with *B. bassiana* TL-leaf and N41S1TI isolates following dip and spray conidia inoculations.

There were no significant differences (F pr. 0.081; n.d.f.=2) in height between plants inoculated with *B. bassiana* to control plants inoculated with 0.1% Triton X-100 at 16 months post inoculation (Figure 5.13). Albeit there were significant differences (F pr <0.001; n.d.f.=5) between inoculation methods, with dip plants having taller stalks when compared to spray plants (Figure 5.13).

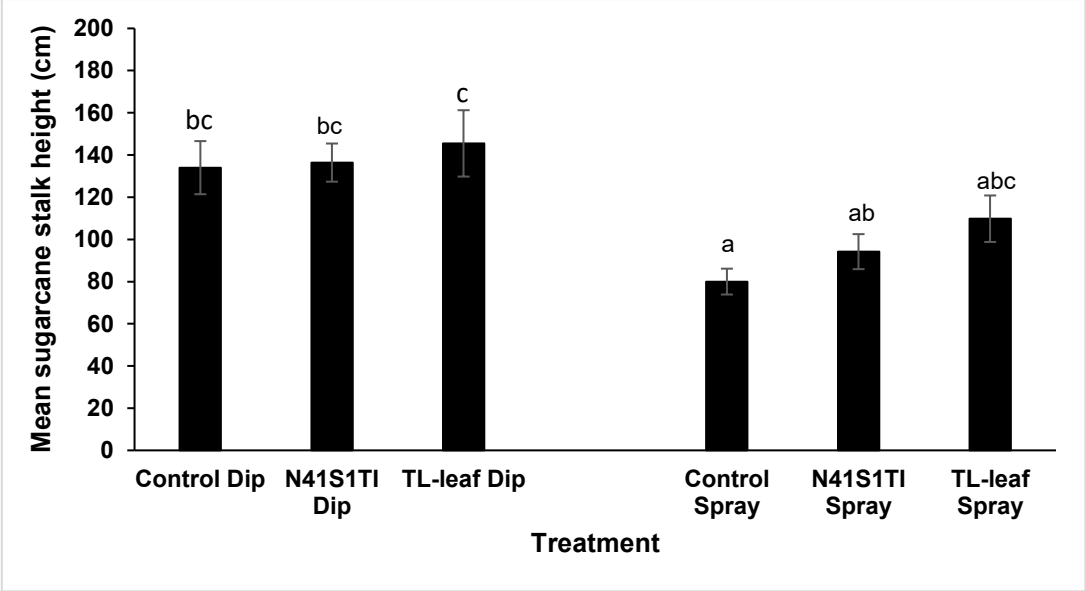

Figure 5.13: Mean sugarcane stalk height (cm) at 16 months following inoculation with *B. bassiana* N41S1TI and TL-leaf and 0.1% Triton X-100 (control) using Dip and Spray inoculation methods. Dip and Spray data were analyzed separately. Bars represent standard error of means and different letters indicate significant differences between treatments.

Furthermore, no statistically significant differences (F pr. 0.428; n.d.f. 2) in sugarcane stalk weights were observed for sugarcane plants inoculated with *B. bassiana* TL-leaf and N41S1TI conidia via dip and spray, 16 months post inoculation (Figure 5.14).

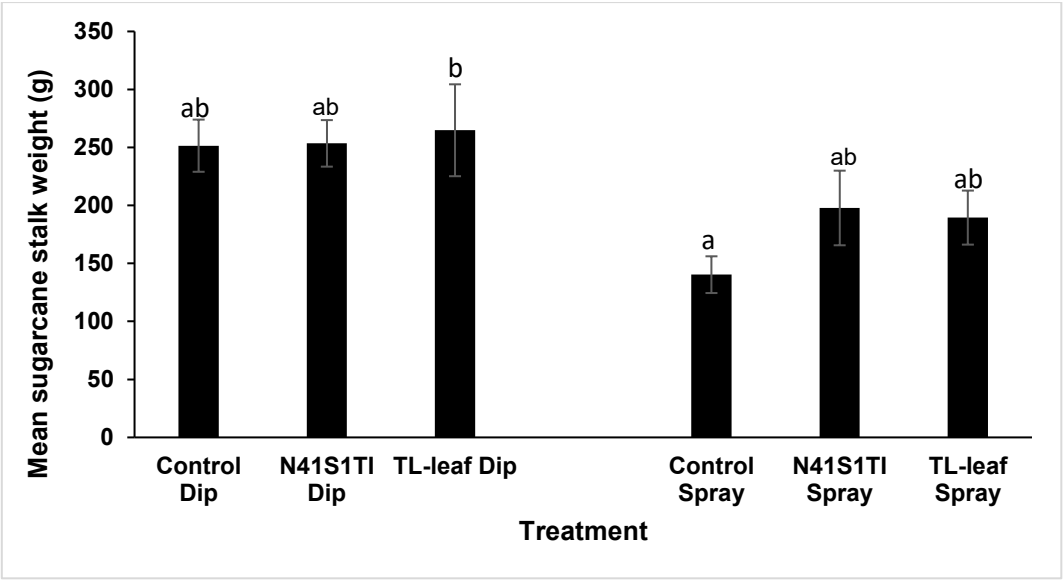

Figure 5.14: Mean sugarcane stalk weight (g) at 16 months following inoculation with *B. bassiana* N41S1TI and TL-leaf and 0.1% Triton X-100 (control) using conidial Dip and Spray inoculation methods. Dip and Spray data were analyzed separately. Bars represent standard error of means and different letters indicate significant differences between treatments.

Inoculation methods had a significant effect on stalk weight 16 months post inoculation (F. pr. <0.001; n.d.f.= 1), with dipped plants weighing more than spray inoculated plants (Figure 5.14).

5.3.4.2 *Eldana saccharina* feeding response in *B. bassiana* inoculated sugarcane plants.

No significant differences (F. pr. 0.039; n.d.f.=5) were observed in boring length of *E. saccharina* fed on control plants to plants inoculated with *B. bassiana* TL-leaf and N41S1TI conidia 16 months post inoculation. Conidia dip inoculated plants with *B. bassiana* N41S1TI isolates had larger boring lengths, however not significantly different to control treated stalks (Figure 5.15).

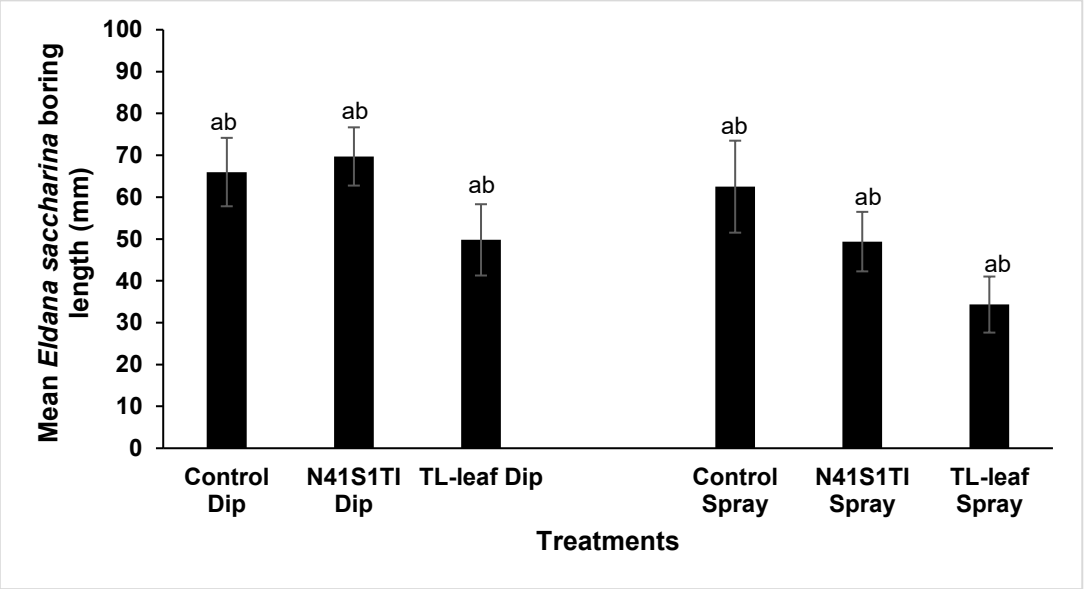

Figure 5.15: Mean *E. saccharina* boring length (mm) following feeding for 3 weeks on sugarcane stalks 16 months post inoculation with *B. bassiana* N41S1TI and TL-leaf and 0.1% Triton X-100 (Control) using conidial Dip and Spray inoculation methods. Dip and Spray data were analyzed separately. Bars represent standard error of means and different letters indicate significant differences between treatments.

Likewise, no statistically significant differences (F. pr. 0.181; n.d.f.= 5) were observed in *E. saccharina* larval weights in stalks inoculated with *B. bassiana* N41S1TI or TL-leaf or 01 % Triton X-100 (control) 16 months prior to *E. saccharina* feeding experiments (Figure 5.16).

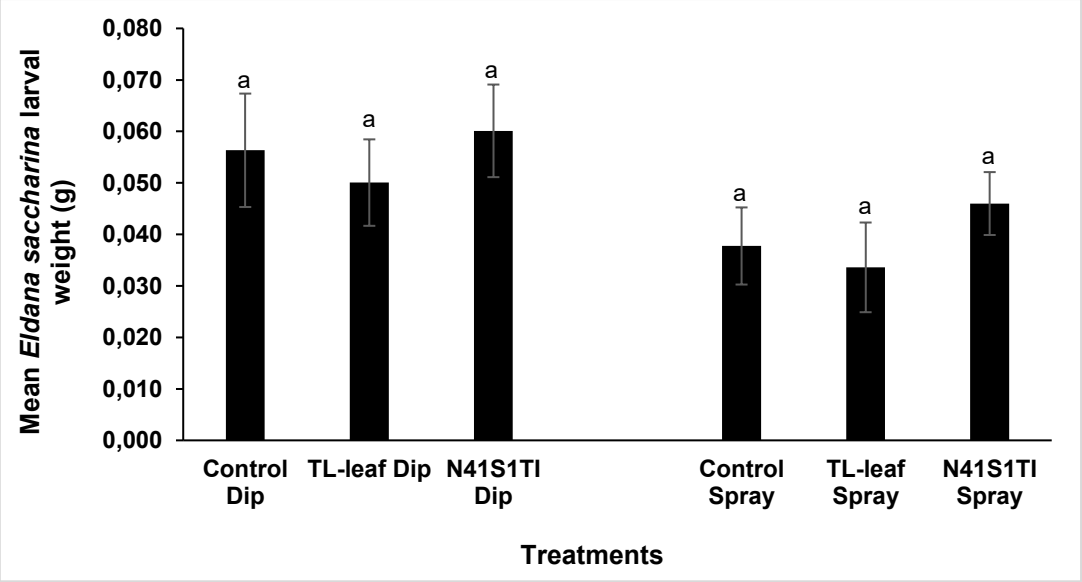

Figure 5.16: Mean *E. saccharina* weights (g) following feeding for 3 weeks on sugarcane stalks 16 months post inoculation with *B. bassiana* N41S1TI or TL-leaf or 0.1% Triton X-100 (control) using Dip and Spray inoculation methods. Dip and Spray data were analyzed separately. Bars represent standard error of means and different letters indicate significant differences between treatments.

5.4 Discussion

The present study investigated the establishment of four *B. bassiana* isolates (TL-leaf, S2roots, P2stem or N41S1TI) as endophytes in sugarcane roots, stems and leaves following conidia application to plants using foliar spray, stem injection, soil drench or sett dip inoculation methods. Persistence of colonization over time and plant-pest interactions were also investigated. Literature is available for the artificial inoculation of *B. bassiana* in plants; however, it is scarce for the control of sugarcane pest *E. saccharina*. Successful endophytic establishment of *B. bassiana* has been reported in several plant species for biological control strategies to control insect pest feeding in agricultural crops. Quesada-Moraga *et al*. (2006) inoculated *B. bassiana* in opium poppy plants after foliar spray applications of *B. bassiana* conidial suspensions for control of *Timaspis papaveris* (Kieffer) borer pest. Posada *et al*. (2007) established *B. bassiana* as an endophyte in coffee plants following injecting stems using 10^8 conidia/ml suspensions against coffee berry borer *Hypothenemus hampei* (Coleoptera: Curculionidae) pest while, Tefera and Vidal (2009) endophytically colonized *B. bassiana* in sorghum plants for potential control of stem borer *Chilo partellus* Swinhoe (Crambidae), *Busseola fusca* Fuller (Noctuidae), and *Sesamia calamistis* Hampson (Noctuidae). This study reports the successful establishment of *B. bassiana* into sugarcane plants for 3, 4- and 6-months post inoculation with colonies re-isolated from plant tissues, *B. bassiana* hyphae

observed in sugarcane stem tissue following staining with lactophenol blue and *B. bassiana* DNA detected in sugarcane plants. A number of factors such as fungal strain, time post inoculation, inoculation method, plant tissue type are reported to influence effectiveness of this control strategy. These factors were investigated in this study and compared with available literature to determine if they stand true for the sugarcane crop.

5.4.1 Effect of plant growth method and plant varieties on degree of endophytic colonization of sugarcane by B. bassiana

Endophytic colonization of sugarcane by the *B. bassiana* isolates was found to be dependent on the way in which the sugarcane seedlings were prepared prior to inoculation and on the sugarcane variety used. Similarly, Tefera and Vidal (2009) reported plant growth medium (sterile soil, non-sterile soil, or vermiculite) to influence levels of *B. bassiana* endophytic colonization of sorghum. Endophytic colonization of N41 plants grown from HWT and HWT+WAX setts were clearly lower when compared to N41 tissue cultured (TC) plants. Similarly, *V. vinifera* plants (var. Sideritis) grown from cuttings and those that were self-rooted showed different levels of endophytic colonization (Mantzoukas *et al*., 2021). Furthermore, the 28 sugarcane varieties used in this study, were colonized by *B. bassiana* TL-leaf strain at different degrees, with cdetain varieties allowing better endophytic colonization (R573; NCo376) while others (N26) showed no colonization at 3- and 6-months post inoculation. Comparably, Veloz-Badillo *et al*. (2019) reported endophytic colonization levels of barley (*Hordeum vulgare*) by *B. bassiana* strain Bb 882.5 to be variety dependent, following inoculating two barley varieties. Additionally, Toffa *et al*. (2021) found tomato varieties (var. Tounvi and Padma) to exhibit significantly different percentage colonization of stem and roots when B. *bassiana* strain Bb115 conidia were inoculated through seed coating. Differences in the degree of endophytic colonization occurring in plants may be explained by the varying degrees of microbial competition existing within plants. Ownley *et al*. (2010) reported that the successful establishment of an endophyte is based on the ability of the microorganism to out-compete other endophytes already present in the plant for nutrients and space and the initial colonizer will likely out-compete the secondary colonizer.

Competition levels in TC plants might be expected to be low as these plants were propagated *in vitro* from apical meristems, which would exclude endophytic microorganisms initially present in the plant (Snyman *et al*., 2008; Ramgareeb *et al*., 2010; Tiwari *et al*., 2011). Inoculated *B. bassiana* isolates were therefore the “initial colonizers” of the TC plants allowing higher colonization. In contrast, HWT and HWT+WAX plants may already have had endophytic colonization by other microorganisms as sugarcane plants are reported to naturally harbor a wide number of endophytic fungi and bacteria within their stem tissues (McFarlane

et al., 2009). For instance, McFarlane *et al*. (2009) isolated 71 endophytic *Fusarium* species from symptomless sugarcane stems. It is, therefore, possible that the establishment of *B. bassiana* within HWT and HWT+WAX sugarcane plants were limited by endophytes already present in the sugarcane plant. Even though hot water treatment (HWT) of sugarcane setts was performed to eliminate microorganisms present in the setts (Damayanti *et al*., 2010) it is possible that HWT may not have been completely effective in eliminating all endophytes in the setts. Thus, colonization competition between the inoculated *B. bassiana* and natural sugarcane endophytes may have caused the low percentage colonization of *B. bassiana* obtained for HWT plants. The presence of other endophytic microorganisms in sugarcane plants was evident during re-isolation of *B. bassiana*, as bacteria and fungi, other than *B. bassiana* were isolated from HWT and HWT+WAX plants. Additionally, competition for endophytic colonization may have arisen from soil microorganisms developing over time despite soil sterilization. Sugarcane plants grown from HWT+WAX setts were better colonized by *B. bassiana* isolates than plants grown from HWT setts. Waxing of sugarcane setts seals the cut edges which would prevent entry and further colonization of setts by soil microorganisms. Waxing of setts may have thus reduced competition allowing better colonization of plants by *B. bassiana*.

Low recovery of *B. bassiana* in leaves or stem or roots may have been a consequence of the method used to evaluate colonization. Although the SDA agar used for *B. bassiana* re-isolation was supplemented with antibiotics (e.g., rifampicin, cycloheximide, chloramphenicol and dodine), making the media selective to *B. bassiana*, other fungal species still grew from HWT and HWT+WAX plant pieces. The outgrowth and re-isolation of *B. bassiana* from plant pieces may have been limited by rapidly growing *Fusarium* and bacterial species which were often isolated from HWT and HWT+WAX plants. *Beauveria bassiana* is slow growing when compared to *Fusarium* spp. which spread across the plate and made it difficult to observe any re-isolated *B. bassiana*. When *Fusarium* spp. and *B. bassiana* strains were grown in co-culture, *Fusarium* spp. strains were seen to grow faster than *B. bassiana* isolates. During endophytic establishment, *B. bassiana* isolate S2root was not recovered from HWT+WAX stem 3 months post foliar spray inoculations with conidia, however, this strain was recovered at 4 months post inoculation. Furthermore, *B. bassiana* TL-leaf strain was not recovered in HWT stem 3 months post spray inoculation though was re-isolated at 4 months. Similar results were obtained when 28 sugarcane varieties were HWT and subsequently inoculated with *B. bassiana* isolate TL-leaf. At 3 months post inoculation no colonies resembling *Beauveria* spp. were re-isolated from sugarcane R570 variety, yet colonies were re-isolated at 6 months. It is apparent that *B. bassiana* is a slow colonizer in certain varieties and would eventually emerge from plant sections with minimal competition. Posada *et al*. (2007);

Quesada-Moraga *et al*. (2009); Brownbridge *et al*. (2012) and Parsa *et al*. (2013) also reported similar problems of endophytic fungal species natural found in plants, hampering the recovery *B. bassiana* from inoculated plants.

5.4.2 Effect of B. bassiana strain on endophytic colonization of sugarcane plants

Differences in levels of endophytic colonization by different *B. bassiana* strains have been previously reported (Posada *et al*., 2007; Qin et al., 2021; Wilberts et al., 2023). Levels of endophytic colonization of sugarcane by the four *B. bassiana* isolates (TL-leaf, S2roots, P2stem or N41S1TI) varied in this study. *Beauveria bassiana* TL-leaf and P2stem isolates resulted in greater colonization of sugarcane stems and leaves, when compared to *B. bassiana* isolate S2root, which was a poor stem and leaf colonizer. Endophytic fungi show degree of tissue specificity being adapted to conditions present in different organs (such as differences in microbial and physiological conditions present among plant parts (Akello *et al*., 2007b; Mengistu, 2020; Mantzoukas *et al*., 2021). Akello *et al*. (2007b) reported variation in plant tissue colonization by *B. bassiana* strains WA, G41 and S204, with the latter two strains colonizing tissue cultured banana plants to a higher degree then strain WA. They hypothesized the ability of a fungal strain to colonize plant parts to be related to the origin of the strain. They attributed low colonization of banana plants by *B. bassiana* strain WA to be due to its isolation from insect cadaver (banana weevil) attributing *B. bassiana* strain WA to be less adapted to a saprophytic lifestyle compared to better adapted strains G41 and S204 isolated from soil. Low sugarcane stem and leaf colonization percentages obtained from strain S2root may be attributed to the strain being isolated from roots, whereas isolates TL-leaf and P2stem were isolated from leaf and stem plant parts, respectively. When *B. bassiana* isolate N41S1TI isolated from sugarcane top internode stalks (var. N41) was inoculated alongside TL-leaf using spray conidia inoculation, *B. bassiana* N41S1TI was a better colonizer, and qPCR detected DNA of this strain in root, stem and leave 3 months post inoculation, while TL-leaf DNA was only detected in roots. Posada *et al*. (2007) reported colonization variation among their *B. bassiana* isolates (1480, CS16-1, 5486 and 2687) and attributed the variations to different innate characteristics, infectivity, and capabilities of the isolates to colonize coffee plant tissues.

5.4.3 Effect of inoculation method on endophytic colonization of sugarcane plants by B. bassiana isolates

Colonization percentage of *B. bassiana* has been previously reported to be dependent on the crop and inoculation method used (Veloz-Badillo *et al*., 2019; Mengistu, 2020; Altaf et al., 2023). Likewise, inoculation methods used to introduce *B. bassiana* conidia into sugarcane

plants determined the extent of *B. bassiana* endophytic establishment in the different plant parts. For instance, the highest stem colonization by *B. bassiana* isolates occurred on tissue culture plants when conidia suspension was delivered directly into the stem via stem injection. Furthermore, *B. bassiana* conidia application by foliar spray inoculation, resulted in greater colonization of sugarcane leaves by the *B. bassiana* isolates. Soil drench with *B. bassiana* conidia suspension was not an effective method for establishing *B. bassiana* as an endophyte in sugarcane leaves as 0-20% colonization percentages were obtained (experiment 1). Similarly, *B. bassiana* strains were unable to colonize coffee leaves when applied conidia via soil drench application (Posada *et al*., 2007). Many endophytic fungi have been shown to have tissue specificity being optimally adapted to conditions present in specific plant parts (Akello *et al*., 2007a; 2007b; Tefera and Vidal, 2009; Mantzoukas *et al*., 2021).

Although stem injection resulted in higher stem colonization, this method will be difficult to implement in field systems. As reported by Posada *et al*. (2007), foliar spray inoculation is the easiest and most practical method to accomplish in field settings. It was seen in this study that sugarcane stems were colonized following foliar spray applications. However, levels of stem colonization were low. Overall, sugarcane stems were better colonized following stem injection with conidia than leaves, as higher re-isolation percentages were obtained in stem pieces. A higher level of colonization in sugarcane stems is desirable if endophytic control of *E. saccharina* is to be implemented as endophytic colonization would target boring feeding of *E. saccharina* larvae in sugarcane stems. Nevertheless, *B. bassiana* endophytic colonization of different plant organs is a positive attribute, as endophytic establishment could potentially be used to target multiple sugarcane insect pest feeders. Literature is available on *B. bassiana* causing mortalities in root, leave and stem feeders (Goble *et al*., 2014; Motholo *et al*., 2023) The detection of higher colonization in roots could be used to control root feeders such as white grubs (order: Coleoptera). Goble *et al*. (2014) showed *Beauveria* spp. namely *B. bassiana* and *B. brongniartii* strains to effectively cause mortality in sugarcane root feeder pests *Schizonycha affinis* (Coleoptera: Scarabaeidae). Additionally, the recovery of *B. bassiana* DNA in leaves during this study, confirms colonization indicating that *B. bassiana* endophytic colonization could potentially be applied to target leave feeders, such as Aphids (order: Hemiptera). Motholo *et al*. (2023) reported the endophytic potential of *B. bassiana* strain PPRI 7598 for suppression of Russian Wheat Aphid*, Diuraphis noxia* and demonstrated that overall, *B. bassiana* endophytic colonization of three wheat varieties significantly reduced *D. noxia*.

5.4.4 Evaluation of B. bassiana endophytic colonization and persistence in sugarcane plants

Studies have shown that levels of endophytic colonization are dependent on time post inoculation, as endophytism reduces over time (Posada *et al*., 2007; Greenfield et al., 2016; Altaf et al., 2023). It is thus imperative to determine persistence of endophytism when aiming to employ this control strategy for pest control. When endophytic colonization was evaluated monthly for 4 months (Experiment 1), 3 and 6 months (Experiment 2 and 3) and 16 months (Experiments 4), re-isolation of endophytic colonization reduced over time.

When *B. bassiana* was evaluated for persistence at 3-, 4- and 6-months post inoculation the fungus was re-isolated from sugarcane tissues while, no endophytic colonization was observed at 16 months post inoculation. Endophytic colonization of sugarcane plants declined with increasing post inoculation time. Akello *et al*. (2007a), Posada *et al*. (2007) and Biswas *et al*. (2012) reported reduction in *B. bassiana* endophytic colonization with increasing post inoculation time. Biswas *et al*. (2012) attributed reduction in endophytic colonization in fibrous jute (*Corchorus olitorius*) plants to be due to fiber formation in the plants and secondary cell wall thickening, which resulted from phenolic compound deposition on xylem and phloem during plant growth. Likewise, sugarcane becomes fibrous with age and reduction in colonization of *B. bassiana* may have been due to the same factors reported by Biswas *et al*. (2012), however, this was not investigated here. Nevertheless, Posada *et al*. (2007) and Brownbridge *et al*. (2012) attributed decreases in recovery of *B. bassiana* over time in coffee and pine seedlings, to increases in microbial competition over time within the plant, leading to inhibition of *B. bassiana* endophytic growth. While reduction of *B. bassiana* colonization may have been a consequence of the plant defense system, which may have limited endophytic colonization by *B. bassiana* isolates as plants have well developed chemical systems and secondary plant metabolites such as benzoxazinoids, which upon colonization by a foreign entity, trigger induced systemic plant responses limiting persistence of colonization (Couture *et al*., 1971; Singh *et al*., 2003; Ownley *et al*., 2008).

At four months post inoculation, *B. bassiana* strain TL-leaf and S2root could still be re-isolated from both sugarcane stem and leaves, while isolate P2stem was not recovered. Likewise, Posada *et al*. (2007) reported *B. bassiana* isolates persistence to vary. Following inoculating coffee plants with four *B. bassiana* isolates (1480, CS16-1, 5486 and 2687), they reported that only one isolate (1480) was detected at four- and six-months post inoculation, while the remaining three isolates were re-isolated from plants only at two months post inoculation. Posada *et al*. (2007) emphasized the necessity to evaluate fungal strain suitability for endophytism. Results presented here corroborate this.

5.4.5 Eldana saccharina feeding response in B. bassiana inoculated sugarcane plants.

Plants inoculated with *B. bassiana* have been shown to reduce pest feeding (Motholo *et al*., 2023; Bancole *et al*., 2020; Russo *et al*., 2019; Lopez and Sword, 2015). Bancole *et al*. (2020) showed rice plants inoculated with *B. bassiana* to cause mortality on the African pink stem borer, Sesamia *calamistis*. Motholo *et al*. (2023) showed *B. bassiana* inoculated wheat to negatively affect development of the Russian Wheat Aphid, *Diuraphis noxia*. During this study sugarcane plants inoculated with *Beauveria* spp. were allowed to grow for 16 months prior to *E. saccharina* feeding assays. This was to assess whether the endophyte would persist *in planta* until the target plant age when *E. saccharina* attacks sugarcane infield, as *E. saccharina* is a generally a pest of carry over cane, older than 10 months (Rutherford, 2015).

No *Beauveria* spp. was re-isolated 16 months post inoculation of sugarcane plants in this study. Literature has reported of endophytic colonization for short assessment intervals, namely, at 144 hours in opium poppy, *Papaver somniferum* (Quesada-Moraga et al., 2006), 28 days post-inoculation in tobacco, corn, wheat and soybeans (Russo et al., 2015) 36 days after inoculation of strawberry plants (Dara et al., 2013) and 28 days on *Zea mays* L. (maize) (Altaf et al., 2023). Endophytic colonization of *B. bassiana* has been reported as an effective control during trails using plants with a short growth cycle and when target pest insects attack plant at an early plant growth stage. Maize has a short growth cycle when compared to sugarcane, with endophytic colonization used to target fall armyworm (*Spodoptera frugiperda*) which target early growth stages of maize (Idrees et al., 2022). Sugarcane has been inoculated with *B. bassiana* by Donga et al. (2018), however colonization was assessed 14 days post inoculation, yet sugarcane has a growth cycle of up 10-24 months depending on growth location (Rutherford, 2015). No significant results in reducing *E. saccharina* larval weight or boring lengths nor improving sugarcane height or weight were observed in this study at 16 months post *B. bassiana* inoculation. This may be attributed to the loss of endophytic colonization by 16 months. However, at 3 months post *B. bassiana* inoculation, significant improvement in above weights were seen, suggesting potential enhancement of plant growth at early plant growth stages.

In conclusion, this study has demonstrated the establishment of four *B. bassiana* isolates as endophytes in sugarcane roots, stem and leaves. Although TC plants, stem injection and *B. bassiana* strain TL-leaf resulted in higher percentage colonization; results showed that endophytic colonization of sugarcane plants was influenced by multiple factors such as plant growth method (HWT, HWT+WAX or TC), *B. bassiana* strain, inoculation method used,

sugarcane variety and time post inoculation. Persistence of endophytic colonization was visible till 6 months post inoculation and not at 16 months. Inoculated plants had improved above weights when compared to controls at 3 months with no significance at 16 months. Therefore, revealing importance of studying endophytism control at plant age when pest feeds on the plant if this control strategy is to be implemented.

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Chapter 6

Concluding remarks and future work

The stem borer *E. saccharina* is difficult to control, and the *E. saccharina* - *Fusarium* spp. interactions worsen damages resulting from this insect pest in sugarcane. Endophytic entomopathogenic *B. bassiana* strains have been shown to successfully control stem borers in other C4 plants, such as maize (Cherry *et al*., 2004; Feng *et al*., 2023) and sorghum (Reddy *et al*., 2009; Bancole *et al*., 2022). Therefore, in the present study, 128 endophytic fungal isolates morphologically resembling *Beauveria* spp. were isolated from internal (endophytic) tissue samples, leaves, stems, and roots using selective microbiological media. Eight *B. bassiana* isolates were recovered from typical *E. saccharina* natural host plants (*Cyperus sexangularis* L*., Phragmites australis L*, *Sorghum bicolor L.*, *Typha latifolia L.*, *Coix lacryma-jobi* and *Panicum maximum* Jacq.) and 120 from stems of 22 sugarcane varieties found within the sugarcane agroecosystem. Together with various reported studies (Doberski and Tribe, 1980; Meyling and Eilenberg, 2006; Ownley *et al*., 2008; Vega, 2008; Reay *et al*., 2010; Donga *et al*., 2021; Hu and Bidochka, 2021), this confirms that the entomopathogen *B. bassiana* has a wide host plant range and endophytic capability resulting in complex, multifunctional lifestyle ecological interactions. Bioassays provided insight into the virulence of 16 representative isolated *B. bassiana* s.l. isolates, taxonomically confirmed using ITS sequencing. Although *E. saccharina* larvae reduced feeding or moulting soon following conidia spray application, as a defensive response to the tested *B. bassiana* isolates, the 16 isolates affected *E. saccharina* larval behavior negatively, reduce larval development, and caused 3rd and 5th instar larval mortalities, with 3rd instar being more susceptible to infection than 5th instar. The degree of pathogenicity depended, however, on multiple factors such as fungal isolate, conidia concentration or larval instar stage. Previous studies supported the findings in this study with early larval stages of insects' being more susceptible to fungal entomopathogens together with the ability of insect to induce defense strategies such as feedings avoidance, repellency towards pathogenic microorganisms has been reported (Vandenberg *et al*., 1998; Leckie *et al*., 2008; Thomsen and Eilenberg, 2000; Perić-Mataruga *et al*., 2006; Hatting, 2012; Ortiz-Urquiza and Keyhani, 2013; Idrees, *et al*., 2022). Bioassay results provided in this study emphasize the necessity of isolate pathogenicity screening prior to use in biological control programs (Hicks *et al*., 2001; Safavi *et al*., 2010; Trizelia, 2010; Vidal and Tefera, 2013). The presence of *Fusarium* spp. as a natural endophyte in sugarcane and its association with *E. saccharina* raised the question of the impact this fungal genus may have on *B. bassiana* establishment and its effectiveness in controlling *E. saccharina* upon endophytic colonization. *Fusarium* spp. were commonly isolated on "Veens" (Veen and Ferron, 1966) selective medium

modified by the addition of dodine (Liu *et al*., 1993), which was used for *B. bassiana* isolation in this study. Infection by *Fusarium* spp. may deter other fungi (Wicklow *et al*., 1988; Rodriguez *et al*., 2009; Meca *et al.,* 2010; Estrada *et al.,* 2012). The investigation of the *Beauveria-Fusarium* spp. interaction *in vitro* revealed that tested *Fusarium* spp. strains grew faster than *B. bassiana* isolates selected for testing during dual co-cultures. However, when statistically analyzed, no significant differences were evident between the two fungal genera's ability to inhibit each other's growth. Thus, both genera can reduce and limit each other's growth. For the first time, this study revealed that endophytic colonization of sugarcane by *B. bassiana* can persist from 1 to 6 months for the tested *B. bassiana* isolates, with *B. bassiana* DNA detected in sugarcane tissues using qPCR techniques at 3 months post inoculation. Endophytic colonization persistence decreased with an increase in post inoculation time, with no *B. bassiana* colonies re-isolated 16 months after inoculation into sugarcane plants. Declined colonization has been reported (Akello *et al*., 2007; Posada *et al*., 2007), and endophytic colonization was sugarcane variety dependent and influenced by the inoculation method, plant pre-treatment, and fungal strain used. When *E. saccharina* larvae were allowed to feed on plants 16 months post *B. bassiana* inoculation, no statistically significant differences were observed between control and *B. bassiana* inoculated plants in reducing boring length and larval weights. This may be due to the loss of endophytic colonization by 16 months, as neither *B. bassiana* isolates were re-isolated from inoculated plants 16 months post inoculation. Donga *et al*. (2018) demonstrated for the 1st time that *B. bassiana* can endophytically colonize sugarcane plants, although persistence was only evaluated for 16 days. Commercially, sugarcane is grown and harvested after 13-16 months depending on location (Rutherford, 2015). Therefore, the results in the present study highlight the importance of evaluating the persistence of endophytic colonization and determining if the endophyte will be present when the targeted pest feeds on the plant. Furthermore, this study contributes information accentuating the different interactions existing between *B. bassiana* isolates, the insect pest (*E. saccharina*), a fungal plant pathogen (*Fusarium* spp.), and the plant (sugarcane), which are crucial when considering using *B. bassiana* strains for endophytic based control of *E. saccharina* in sugarcane.\

Limitations of the study

With the improvement in molecular identification of fungi by genome sequencing, the usage of ITS primers is increasingly improved and refined by targeting other taxonomically relevant genes such as the 1-alpha (EF1-apha) elongation factor primers and nuclear intergenic BLOC regions primers according to Rehner and Buckley, 2005. Furthermore, the use of gene sequencing targeting genes associated with pathogenicity, the production of secondary metabolites and identification of genomic lineages existing between *B. bassiana* strains

isolated from different sampled area should be considered (Solano-González *et al*., 2023). This was not implemented in the current study.

Investigating the *B. bassiana* virulence to all the metamorphosing stages of *E. saccharina* (eggs and moths) would have been valuable in assessing the biocontrol potential of *B. bassiana* isolates on these growth stages of the pest.

Although not investigated, identifying secondary metabolites produced by both *Fusarium* spp. and *B. bassiana* isolates during growth would have given insight into the metabolites responsible for reducing the growth of the respective genera. Dual plant inoculation of *B. bassiana* and *Fusarium* spp. to investigate endophytic competition *in vitro* using molecular techniques would have provided insight into *in planta* competition.

Future studies may be directed as follows:

Based on the limitations of this study, investigating additional genes should be considered to improve the taxonomic grouping and differentiation of isolates from different locations.

Higher *B. bassiana* colonies were isolated from top sugarcane internodes and nodes during this study. Furthermore, higher levels of DNA (pg DNA/100gr) were detected on leaves of inoculated plants at 3 months post *B. bassiana* inoculation. Based on these results, the biocontrol capacity of *B. bassiana* endophytically colonized plants towards insect pests that feed on young sugarcane plants, top borers, and leave feeders should be investigated. These sugarcane pests include the Yellow Sugarcane Aphid (YSA), *Chilo sacchariphagus* and sugarcane thrips (*Fulmekiola serrata* (*Kobus*)) are suggested.

Testing and characterization of metabolites produced during liquid culture would be provide knowledge as to which metabolites are extracted and used to inhibit Fusarium biomass formation during culture filtrate experiments. Furthermore, testing of crude metabolite cultures according to methods described in Lozano-Tovar et al. (2013) and Yousef et al. (2013) would give inside of which metabolites affected *E. saccharina* growth.

Finally, assessing a larger sample size of sugarcane genotypes characterized as susceptible and moderately susceptible to *E. saccharina* for the presence of endophytic *B. bassiana* would confirm if the natural presence of *B. bassiana* as an endophyte in sugarcane plays a role in resistance against insect pests. Furthermore, entomopathogens such as *Metarhizium, Lecanicillium* (= *Akanthomyces*) which are reported to form endophytic association with plants such also be considered.

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