BIOLOGICAL CONTROL OF SORGHUM AND RICE STEM BORERS, *CHILO PARTELLUS* AND *SESSAMIA CALAMISTIS* USING ENDOPHYTIC STRAINS OF *BEAUVERIA BASSIANA*

By

W. Bernice Armelle Bancole

MSc, Faculty of Sciences (Entomology)

University of Abomey-Calavi, Benin

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PREFACE

The experimental work presented in this thesis was conducted in the School of Applied Environmental Sciences, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Professor M. D. Laing and Dr K. S. Yobo.

These studies represented in this thesis, is my original work and have not otherwise been submitted in any form for any degree or examination at any other University.
Where use has been made of the work of others, it is duly acknowledged in the text

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W.B.A. Bancole (Candidate)

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Professor M. D. Laing (Supervisor)

……

Dr K. S. Yobo (Co-supervisor)

THESIS SUMMARY

Sorghum and rice are two of the major cereals grown across the world. Both of these crops are subjected to a range of abiotic and biotic constraints. Insect pests are important biotic stress factors, which affect both of the crops at all of their growth stages. Stem borers from the family of Lepidoptera, e.g. *Chilo partellus* (Lepidoptera: Pyralidae) and *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) are important pests that attack these cereals. Control of *C. partellus* and *S. calamistis* has largely been with pesticides. However, chemical pesticides are too expensive for most small-scale farmers in Africa, leaving their crops unprotected. Biological control is one of the measures that have been advocated for the management of stem borers. Various strains of *Beauveria bassiana* (Vuillemin) have been documented as being endophytes infecting a wide range of plants, as well as being pathogenic on numerous insect pests. Successes in biological control research have led to the development of various *B. bassiana* products, which are available commercially, but these are largely epiphytic strains. Biological control studies were therefore conducted with several endophytic strains of *B. bassiana* against sorghum stem borer, *C. partellus,* and the rice stem borer, *S. calamistis*. The fungi were tested by endophytic behaviour and the ability to control the 3rd larval instars of both stem borers, in the laboratory and greenhouse. The interactions of *B. bassiana* strains and a commercially available *Trichoderma* product, Eco-T®, were tested in sorghum and rice plants.

In vivo and i*n vitro* screening were initially undertaken to evaluate the endophytic behaviour of 20 *B. bassiana* strains, using two inoculation methods. Subsequently, the best endophytic *B. bassiana* strains and the best inoculation method were tested at 30 and 60 days after inoculation. The strains were screened *in vivo* using seed treatments and foliar sprays, under greenhouse conditions, for endophytic behaviour in sorghum and rice plants. There were highly significant differences between the *B. bassiana* strains ($P = 0.0001$). Depending upon the inoculation method, the *B. bassiana* strains that successfully colonized the sorghum and rice plants could be selected after 30 and 60 days. Five strains of *B. bassiana* strains (Bb3, Bb4, Bb10, Bb21 and Bb35) were found to be endophytic in both crops, and to provide biological control against the two borers.

The best five *B. bassiana* strains were tested for their pathogenicity on the 3rd instar larvae of *C. partellus* and *S. calamistis*. Out of the five endophytic strains of *B. bassiana*, two (Bb35 and Bb3) were the most pathogenic on *C. partellus*, with the greatest mortality of 80 % being achieved within 28 days after treatment. The *B. bassiana* strains Bb35 and Bb4 were the most effective strains against *S. calamistis*, killing 93.33 and 76.66% of the 3rd larval instar at 28 days, respectively. The cumulative mortality of the 3rd instar larvae of both stem borers increased over time at 21 days after inoculation for all five *B. bassiana* strains.

A field trial was conducted to evaluate the biocontrol efficacy of the five best endophytic strains of *B. bassiana* against *C. partellus*, compared to pyrethroid pesticide, Karate. Three of the endophytic strains of *B. bassiana* strains were as effective as Karate sprays when they were applied as seed treatments, reducing damage by *C. partellus* as much as Karate did.

In vitro and *in vivo* screening were carried out under laboratory and greenhouse conditions, using various inoculation methods, to assess the interaction between the five B. *bassiana* strains and a commercially available *Trichoderma harzianum* product, Eco-T®. In the *in vitro* dual culture bioassay, one of the five endophytic *B. bassiana* strains (Strain Bb35) was not inhibited by *T. harzianum* Strain Kd (TKD) at 15 days after inoculation at 30 days after *in vitro* inoculation. None of the five endophytic *B. bassiana* strains grew in the presence of TKD. Only the TKD grew all over the plates. In greenhouse trials, various interactions occurred between the two fungi, according to the inoculation methods. When a mixture of conidia of the two fungi was used at the same time as a seed treatment, there was a strong inhibitory effect by TKD toward the five *B. bassiana* strains. However, if sorghum plants were seed treated with the five *B. bassiana* strains, followed by drenching of the plant roots with a TKD suspension, then the *B. bassiana* strains appeared to be able to colonize the stems of the plants whilst the TKD colonized the roots. Sorghum roots were rapidly colonized by the TKDwhen it was used alone for the seed treatment.

The endophytic behaviour of some strains of *B. bassiana* in sorghum and rice plants can be used as powerful tool to enhance their biological control activity against stem borers of these crops. However, the tested TKD and *B. bassiana* strains were not compatible in the same space, such as the rhizosphere, but could be used sequentially to secure the benefits of insect control by the *B. bassiana* strains, as well as the biological control and plant growth stimulation activities provided by *T. harzianum* strains.

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DEDICATION

To my beloved Mother for her endless spiritual support

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Thesis Introduction

Background

Sorghum and rice are major cereal crops that are produced across the world (Rosenzweig and Parry, 1994; Taylor, 2003; Leff *et al*., 2004; Jordan *et al*., 2012; Kumari *et al*., 2016). Sorghum is a significant staple food, mainly in semi-arid tropical areas of Africa and Asia due to its resistance to severe weather conditions. It is also an important feed grain and fodder crop in the Americas and Australia (Rao *et al*., 2014; Ediage *et al*., 2015; Abdelhaleem *et al*., 2016). Estimated production shows that sorghum is grown throughout the world, and its uses are also on the rise (Arkin *et al*., 1976; Mullet *et al*., 2014; Cauvain, 2015). In Southern Africa, sorghum consumption is increasing. The production of sorghum in South Africa varies from 100.000 tons to 180.000 tons a year, compared to 3 million tons of wheat and 11.5 million tons of maize a year.

Rice is a staple food of most of the world's humans, including those in both developed and developing countries (Datta, 1981; Maclean, 2002; Muthayya *et al*., 2014). Africa is the only continent where the two domesticated rice species, *Oryza sativa L*. and *O. glaberrima* Steud. are being cultivated (Chang, 1976; Khush, 1997; Khush, 2005). *Oryza sativa* L., the most widely grown rice, is the staple food of an estimated 3.5 billion people worldwide (Muthayya *et al*., 2014). Apart from being the staple food of more than half of the world's population (Schalbroeck, 2001; Jena and MacKill, 2008), rice is also the main source of carbohydrate for calorific intake (Meharg and Rahman, 2003). Rice production worldwide is about 710 million tons of paddy rice (FAO, 2006). Rosegrant *et al*. (1995) estimated the actual sub-Saharan Africa's rice production (17.2 million tons annually) could reach 22.23 million tons by the year 2025, whereas a 75% increase is predicted for world demand by 2025 (Khush, 2005; Roetter and Van Keulen, 2007). In West Africa, rice consumption is increasing at an annual rate of 4.5% (AfricaRice Center, 2010). More than seventeen million tons of milled rice are consumed annually, while the production for the same period is 13.2 million tons for rice (AfricaRice Center, 2008). Nearly 40% of the rice consumed in Africa is imported (Lancon and Benz, 2007; Seck *et al*., 2010).

Both of these crops are subjected to many abiotic and biotic constraints, which results in low productivity. Insect pests are important biotic stress that affects sorghum and rice at all their growth stages. Stem borers from the family Lepidoptera, e.g., *Chilo partellus* (Swinhoe) and

Sesamia calamistis (Hampson), are the most important pests, causing significant yield losses in these regions. There are no available cultivars that are resistant to the stem borers. Several control measures have been advocated for the management of stem borers. Chemical control of stem borers is only partially effective, and is not affordable for small farmers. The use of biocontrol agents could be a useful tool to reduce crop losses caused by stem borers. In this study, novel strains of the entomopathogenic fungus *Beauveria bassiana* (Vuillemin) were used as biocontrol agents to develop a new approach to the control of sorghum and rice stem borers, *C. partellus* and *S. calamistis*.

Goal and objectives of the study

The overall goal of the study was to identify one or more strains of *B. bassiana* that combined both endophytic behaviour and entomopathogenic activity, to be developed as biocontrol agents against two stem borers, *C. partellus* and *S. calamistis,* of sorghum and rice. The following specific objectives were established to achieve that goal.

- Isolate numerous strains of *B. bassiana.*
- Identify the best endophytic strains of *B. bassiana* in the collection.
- Quantify any variation in the endophytic behaviour of the *B. bassiana* strains toward sorghum and rice cultivars.
- Identify the best inoculation method.
- Verify if the *B. bassiana* strains enhance the growth of sorghum and rice plants.
- Identify the best *B. bassiana* strain for the control of the 3rd instar larvae of *C. partellus* and *S. calamistis* in sorghum and rice, respectively.

Structure of the thesis

The Thesis Introduction outlines the importance of sorghum and rice, points out their major constraints including stem borer problem. It then establishes the primary goals of the thesis. Chapter 1 encompasses a review of the literature about the thesis topic, in order to develop a framework for studies on the control of stem borers. Chapter 2 presents a study on the endophytic competence of multiple strains of *Beauveria bassiana* (Balsamo) Vuillemin in sorghum cultivars. Chapter 3 covers studies on the establishment of selected *B. bassiana* strains as endophytes in rice cultivars. The susceptibility of *C. partellus* and *S. calamistis* third instar larvae to the selected *B. bassiana* strains was covered in Chapter 4. Chapter 5 covers the field evaluation of selected, systemic strains of *B. bassiana* against a sorghum stem borer, *Chilo partellus* Swinhoe. Chapter 6 reports on a study of the compatibility of *Trichoderma harzianum* Rifai Strain Kd (Eco-T®) with selected systemic strains of *B. bassiana*.

Chapter 7 provides an overview of the research undertaken, and the implications of the results. Furthermore, suggestions are made for future research that are inspired by these results.

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Chapter 1 Literature review

1.1 Sorghum

Crop History

Sorghum bicolor (L) is known under a variety of names: great millet and guinea corn in West Africa, kafir corn in South Africa, dura in Sudan, mtama in eastern Africa, jowar in India and kaoliang in China (Purseglove, 1972; Sadhana *et al*., 2016). The earliest known record of sorghum comes from an archaeological dig at Nabta Playa, near the Egyptian-Sudanese border, dated 8000 B.C. Sorghum was first domesticated in Africa (Doggett, 1988; Kimber, 2000; Wang *et al*., 2013). It is believed to have originated from Northern Africa. The distribution and spread of sorghum is attributed to the movement of various tribal groups in Africa (Sadhana *et al*., 2016). Documented sorghum races are bicolor, kafir, caudatum, durra and guinea (Wang *et al*., 2013; Westengen *et al*., 2014). It is now distributed all over the world (Smith and Frederiksen, 2000; Taylor, 2003; Morris *et al*., 2013; Luo *et al*., 2016). Sorghum is a source of food for over 500 million people in 98 countries (Pennisi, 2009; Magalhaes *et al*., 2016). It is cultivated widely in tropical and subtropical regions (Caniato *et al*., 2014).

There are two primary types of grain sorghum: bitter (high tannin) and low-tannin sorghum (Deenanath *et al*., 2012). There is also sweet stem sorghum, which is grown for its sugars that accumulate in the stem, much like sugar cane (Mangena et al, 2018). Sorghum adapts to a wide range of environments (Assar *et al*., 2016). It is highly resistant to drought (Steduto *et al*., 1997; Pennisi, 2009), salinity (Almodares *et al*., 2008), and water logging (Promkhambut *et al*., 2010).

Morphology, Growth Stages and Conditions

Grain sorghum is an annual grass similar in appearance to *Zea mays* L. (Vaillancourt and Hananu, 1992), although it has more tillers (stems) and more finely branched roots than maize (Carter *et al*., 1989). Many researchers have described the morphological features and growth stages of *S. bicolor* (Harlan and De Wet, 1972; Doggett, 1988; House, 1995). Sorghum leaves are wide, similar to maize leaves, but differ from maize by developing toothed margins on the leaves. They are 50-100 mm wide and 0.5-0.8 m long. These waxy leaves can roll into tubes when moisture stressed, an adaptation that helps the plant to be

more drought resistant than other grains (Carter *et al*., 1989). Like maize stems, sorghum stems are oval and have a pronounced groove or indentation (Harlan and De Wet, 1972). They are strong, hard and smooth, and are divided by nodes. The height and diameter of sorghum stems depends on the genetics of the cultivar. Sorghum root system include prolific lateral roots and root hairs that are able to absorb water and nutrients efficiently compare to other cereals. Besides subterranean root system, sorghum forms strong aerial roots permeating through the soil, to ensure stability. The inflorescence of sorghum is a characteristic part of the plant that takes a number of specific shapes and sizes, based on the race of the sorghum. Panicles may be erect, drooping or reflexed. Damage to sorghum panicles reduces grain production (Steduto *et al*., 2012; Knott *et al*., 2016). This may result from pest and disease attack (Harris-Shultz *et al*., 2015; Okrikata *et al*., 2015). On the panicle, the spikelet are in pairs and bear round, oval or heart-shaped grains. These may beand can occur in white, creamy, yellow, pink, brown or violet coloured. Dark brown or red grains are typically high in tannins (Carter *et al*., 1989). Spikelets contain one flower and are gathered in bunches of 2, 3 or 4 on second branches. Sorghum plants are autogamous, and they are normally self-fertilizing. However, they cross pollinate readily. Through breeding efforts, newer varieties may have 2-3 dwarf genes, resulting in a plant .6 to 1.2m tall, which makes then easier to combine harvest, and reduces lodging in windy conditions (Carter *et al*., 1989). Sorghum grows in a wide range of temperature. It has been reported to tolerate annual temperatures of 7.8 – 27.8°C, soil pH values of 4.3 – 8.7, and it is adapted to tropical and subtropical summer rainfall climates, with annual rainfall ranging from 250-1250 mm (Logsdon, 2007). Structural and morpho-physiological evolutionary features of sorghum play a role in its high levels of water use efficiency (Paterson *et al*., 2009). Moderately well drained soils are most suitable for sorghums. Sorghum prefers moderately acid soil (Rai et al., 2008). Small amounts of alkali in the soil reduce the performance of sorghum considerably. Tolerance to salinity is moderate (Almodares and Hadi, 2009; Bavei *et al*., 2011).

According to Vanderlip (1993) there are nine stages that can be used in understanding the growth and development of grain sorghum. These stages are from stage 0 (emergence) to stage 9 (physiological maturity) with each stage having some distinctive characteristics. Time required to reach each stage depends both on the hybrid and the environment in which it is growing. Other factors such as soil fertility, insect or disease damage, moisture stress, plant population, and weed competition may also affect both timing of the various stages of

development and condition of the plants at each stage of development (Figure 1-1). Following is an outline of the development stages in grain sorghum based on Vanderlip's description:

1. **Emergence**: When the plant first breaks through the soil surface (generally occurs 3 to 10 days after planting).
2. **Three-Leaf Stage**: Leaves may be counted when the collar of the leaf can be seen, without tearing the plant apart. The collar is the area where the leaf blade and leaf sheath attach.
3. **Five-Leaf Stage**: Approximately 3 weeks after it emerges, a sorghum plant has 5 leaves fully expanded; its root system develops rapidly; roots produced at the lower nodes affect the development of the leaf which may drop off from the plant. This usually does not affect the identification of the five-leaf stage because the lower leaf has a rounded tip.
4. **Growing Point Differentiation**: This happens about 30 days after sorghum emerges. Approximately one-third of the total leaf area has fully developed and the plant has 7-10 leaves, depending on maturity class. The lower 1 – 3 leaves may have been lost.
5. **Flag Leaf Stage**: Following growing point differentiation, rapid culm elongation and rapid leaf development occur simultaneously until the flag leaf (final leaf) is visible in the whorl. Most leaves are fully expanded except for the final 3-4 leaves.
6. **Boot Stage**: All leaves are now fully expanded, providing maximum leaf area and light interception. The head has now developed to nearly full size and is enclosed in the flag-leaf sheath.
7. **Half-bloom**: This is usually defined as when one-half of the number of plants in a field or area are in some stage of bloom. However, because an individual sorghum head flowers from the tip downward over 4-9 days, half-bloom of an individual plant is when the flowering has progressed half-way down the head. The period between planting to half-bloom depends on the days-to-maturity of the hybrid, and on environmental conditions.
8. **Soft-Dough Stage**: Between half-bloom and soft-dough, the grain fills rapidly; almost half of its dry weight is accumulated in this period. The stalk weight increases slightly following half-bloom; then, is forming rapidly, the stalk loses weight. The loss in stalk weight may account for as much as 10 percent of the grain weight.

9. **Hard-Dough Stage**: By hard-dough stage, about three-quarters of the grain dry weight has accumulated.
10. **Physiological Maturity**: Maximum total dry weight of the plant has occurred. The time from flowering to because grain physiological maturity varies with hybrid and environmental conditions. Grain moisture content at physiological maturity is usually between 25 and 35%, but this varies with each variety, and on growing conditions. If temperature and moisture conditions are favorable, branches may start to grow from several of the upper nodes (places where leaves attach).

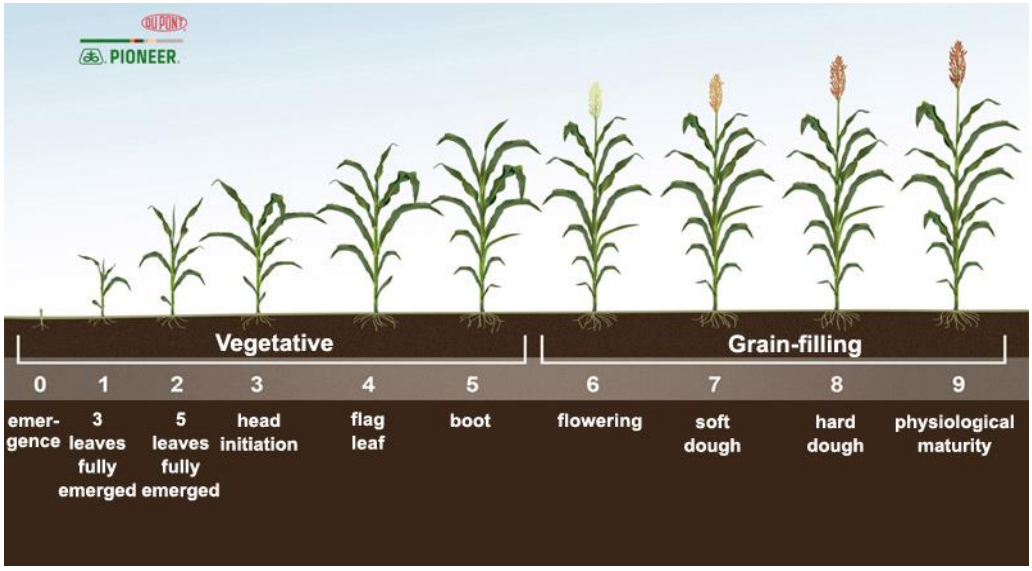


Figure 1-1 Stage of development of sorghum plant.

(Source: www.pioneer.com. Assessed on 21/05/2017)

Production and Use of Sorghum

Production

Sorghum is cultivated in many parts of the world today and is a staple food for over 500 million people in the tropical and subtropical regions (Ramatoulaye *et al*., 2016). World sorghum production in 2015 was estimated to be 63 million ton year^{-1} (Popescu and Condei, 2014), compared to 61 million tons in 2010 (Backoulou *et al*., 2014). The introduction of improved varieties, combined with improved management practices, has led to increased sorghum productivity (Edgerton, 2009; Mindaye et al., 2016). Africa produces about 26 million tonnes (FAOSTAT, 2015), making sorghum the second most important cereal grain in Africa after maize. The production of sorghum in South Africa was estimated at 175 million tons in 2016. Sorghum production in South Africa varies from province to province

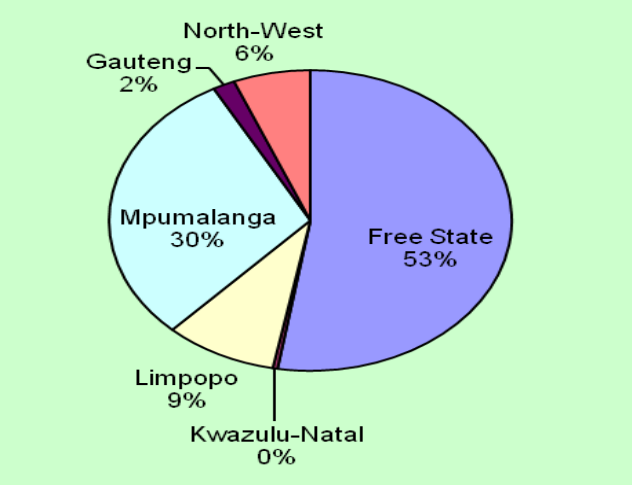


Figure 1-2 Grain sorghum production by province in South Africa in 2010/2011

(Source: Statistics and Economic Analysis
Department of Agriculture, Forestry and Fisheries. South Africa.)

Use of Sorghum

Sorghum is used for human consumption, livestock feed, and industrial production of bio-ethanol (Paterson *et al*., 2009; Dahlberg *et al*., 2011; Cao *et al*., 2012; Li *et al*., 2013). Estimated production shows that sorghum is grown throughout the world, and the diversity of its uses are increasing (Borrell *et al*., 2014). Sorghum often malted and then used as the main ingredient in brewing indigenous beers across Africa, often needed for cultural ceremonies. These are usually low alcohol, opaque beers (Holmes *et al*., 2016; Ndubisi *et al*., 2016). However, sorghum is not well adapted for Western beer making, especially lager and stout beers, due to the absence of a hull (Dufour *et al*., 1992). Traditional fermented food and beverages made from sorghum are common among the peoples of West Africa (Adebiyi *et al*., 2016; Muyonga *et al*., 2016)

Biotic constraints to sorghum production

One constraints affecting small scale sorghum farmers is insect pests. Nearly 150 species of insects have been documented to be pests on sorghum (Jotwani *et al*., 1980; Sharma, 1993). Among the 21 economically important lepidopteran stem borers in Africa, the indigenous *Busseola fusca* Füller (Noctuidae) and the invasive *Chilo partellus* Swinhoe (Crambidae) (Reddy and Walker, 1990; Maes, 1998) are the most damaging in sub-Saharan Africa (Kfir *et al*., 2002). Damage caused by the larval stages of the stem borers lead to yield losses, depending on the cultivar, developmental stage of the plants at infestation, infestation rate and prevailing environmental conditions, among other factors (Kfir *et al*., 2002). Some insect

attacks are specific to development phase of the sorghum plant (Ben-Hammouda *et al*., 1995). Losses to stem borer damage globally have been estimated to be over US$ 300 million annually (Sharma *et al*., 2003). In Kenya, De Groote *et al*. (2003) that stem borers cause annual losses of 13.5%, valued at US$ 80 million, in Kenya. Similarly, Ogeto *et al*. (2012) and Midingoyi *et al*. (2016) estimated the annual yield losses in sorghum in Kenya due to stem borers to be USD 90 million p.a.

The stem borer, *C. partellus,* is one of the economically most damaging pests in Asia and Africa, attacking all parts of the plant except the roots (Reddy *et al*., 2009; Sharma *et al*., 2010) causing yield losses of 18 to 53% in Kenya (Gethi *et al*., 2002). Other estimates were of field infestations of sorghum plants by stem borers ranging from 30-100%, and causing crop losses in sorghum of up to 88% (Reddy and Walker, 1990; Midingoyi *et al*., 2016). A number of genotypes with resistance to *C. partellus* have been identified, but the levels of resistance are low to moderate (Sharma *et al*., 2003). Most of the sources of resistance belong to durra group of sorghums of Indian origin followed by caudatum, conspicuum, caffrorum, dochna, roxburghii, cernuum, and nervosum kaoling. Some of these sources are also resistant to other stem borer species infesting sorghum in Africa and Latin America (Reddy, 1983). Sorghum varieties with good resistance to stem borers has not been widely reported in the literature (Sharma *et al*., 2007). The issue is that the resistance is polygenic, and is largely governed by additive genes (Sharma *et al*. 2007). This requires recurrent selection to accumulate these genes. However, it is hard to do recurrent selection in sorghum because it is strongly self-pollinating, and recurrent selection is based on repeated cross pollination of many plants and their progeny (Sharma *et al*. 2007; Marulasiddesha *et al*., 2007; Singh *et al*., 2011; Padmaja *et al*., 2012; Muturi, 2013).

Figures 1-3 Symptoms of stem borer damage in sorghum crops

Source: Ricaud et *al*., 2008

1.2 Sorghum stem borers in Africa and South Africa

1.2.1 Taxonomy

There are two primary species of stem borers of sorghum in Africa:

Order: Lepidoptera

Family: Pyralidae

Genus: *Chilo*

Species: *Chilo partellus*

Family: Noctuidae

Genus: *Busseola*

Species: *Busseola fusca*

1.2.2 Geographical Distribution and Host Range

The spatial and temporal distribution and abundance of stem borers vary among and within host plants due to variations in their suitability for oviposition and larval development.Hence, different varieties exhibit varying levels of susceptibility to borer attacks (Barry and Darrah, 1991). Microclimatic factors (temperature and precipitation) and biotic factors such as natural enemies and alternative host plants may influenced the distribution and abundance of the stem borers (Addo-Bediako and Thanguane, 2012). Soil nutrients have been found to play an

important role in determining the distribution of pests (Allsopp *et al*., 1993; Setamou *et al*., 1993; Addo-Bediako and Thanguane, 2012).

One of the many pests of sorghum, *Chilo partellus* Swinhoe, is not native to Africa, but was introduced from Asia. It is essentially a pest of hot lowland areas, and is seldom found above an altitude of 1500 m. Since its introduction on the African continent, it has continuously expanded its distribution into the warm, low-altitude regions of eastern and southern Africa (Figure 1-4) (Khan *et al*., 2000; Kfir *et al*., 2002; Hell and Mutegi, 2011), and has recently spread to the Mediterranean region (Yonow *et al*., 2017). This stem borer is dominant in India (Duale and Nwanze, 1999; Poveda *et al*., 2008; Divya *et al*., 2010; Sharma *et al*., 2010) and South East Asia (Paini *et al*., 2010). It is now the most economically important stem borer of maize and sorghum in many areas of Africa.

In contrast, *Busseola fusca* Fuller and *Sesamia* species are distributed all over sub-Saharan Africa. The presence of *B. fusca* depends on the altitude (Kfir *et al*., 2002). *Sesamia calamistis* Hampson is the most important borer amongst the *Sesamia* species. *Eldana saccharina* Walker is abundant in Africa. In West Africa it is a pest of *Oryza sativa* Steud, maize and sugar cane (*Saccharum officinarum* L.) but in South Africa it infests mostly sugarcane (Kfir *et al*., 2002). In South Africa, *C. partellus* and *B. fusca* are the most important borers that infest sorghum and maize. In West Africa *S. calamist*, *Chilo* species, *B. fusca* and *S. saccharina* are the most devastating pests of rice, sorghum and maize (Kfir *et al*., 2002).

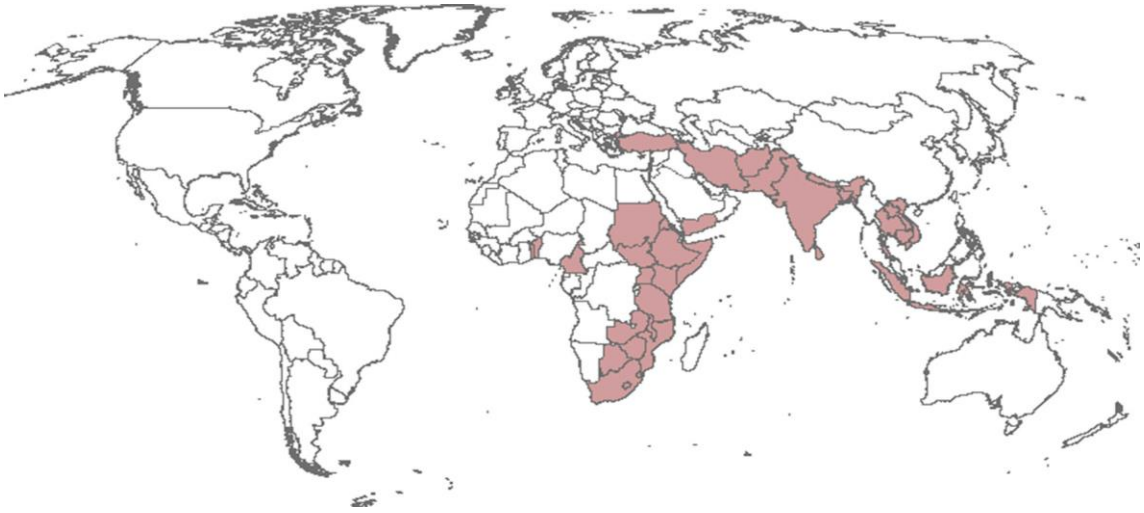


Figure 1-4 Map of the world indicating those countries in which *Chilo partellus* has been previously recorded (marked in brown)

Source: Yonow *et al*. (2017)

1.2.3 *Chilo partellus*: Life Cycle, Biology and Symptoms

1.2.3.1 Life Cycle and Biology

Adult moths are straw-coloured and small (approx. 15 mm long). Its hind wings are white, while its forewings are straw-coloured with darker scale patterns forming longitudinal stripes close to the wing margins. Adult moths rest with wings folded over the abdomen. The adult stage lasts from 2-12 days. Adult females can lay between 200 and 600 eggs during a lifetime, in separate batches of 10-80 eggs. *Chilo partellus* eggs are flattened, white, scale-like, ovoid and 1.5 mm wide (Visarada *et al*., 2014). Typically, they are deposited in overlapping clusters on the underside of leaves close to the midribs, with an incubation period of 4-5 days in the warm season. This species prefers smooth surface for oviposition and lays most of its eggs on the lower surfaces of the leaves and upper part of midribs. Depending on the environmental conditions, eggs take 5-7 days to hatch (Alghali, 1985; Sithole, 1990; Ben-Yakir *et al*., 2013). Immature larvae are yellowish, spotted (Figure 1-5), and 1-2 mm in length. Mature larvae are crystalline and spotted with black warts on each body segment (Figure 1-5). They have four purple/brown longitudinal stripes on the back of the body, 20-25 mm in length, and have a prominent reddish-brown head. Larvae stage last 14-28 days. Upon hatching, the first instar larvae of *S. calamistis* are unusual because they bore directly into the stem (Bosque-Perez and Schulthess, 1998; Polaszek et al., 2000). In contrast, the first stage larvae of most stem borer species initially feed on young leaf tissues, while older larvae tunnel into the stem tissues and feed internally (Bosque-Perez and Schulthess, 1998). The larval stage of stem borers may last 25-58 days and may have 6-8 stages. Pupation normally takes 5-14 days after which adult moths emerge (Harris, 1990; Maes, 1998). Pupae are light yellow/brown to dark red/brown. Pupae have belts of small spines on the dorsal anterior margins, from the fifth to seventh abdominal segments, with six dorsal spines, and two large flattened ventral spines on the last abdominal segment. Pupation occurs within the tunneled stem. Pupae are about 15 mm long (Figure 1-6).A thin, exterior wall window in the plant stem is left at the end of the tunnel to enable adults to emerge.

Figure 1-5 Larvae of *Chilo partellus* in two instars larvae

Source: Hutchison *et al*. (2008)

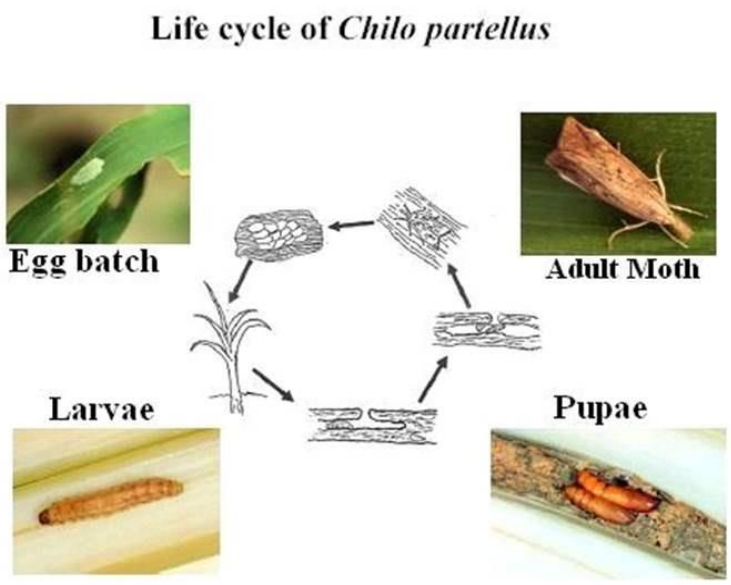


Figure 1-6 Life Cycle of *Chilo partellus*.

Source: Hutchison *et al*. (2008)

1.2.3.2 Mechanism of damage

Infestation starts approximately 2 weeks after seedling emergence. Young caterpillars of spotted stem borer feed on the tender leaves of the plants. The first symptom of damage is the presence of irregular scars, holes and windows caused by early instar larval feeding, and the infested plants exhibit a ragged appearance. Feeding and stem tunnelling by the stem borer

larvae result in crop losses as a consequence of destruction of the growing point, early leaf senescence, and interference with the translocation of metabolites and nutrients that result in peduncle breakage, and production of completely or partially chaffy panicles called "**white panicle**" (Figure 1-3) (Taneja and Leuschner, 1984). Similar symptoms are produced by other species of cereal stem borers (Soul-Kifouly et al., 2016). A longitudinal dissection of the stalks will reveal the larvae. In older plants, the upper part of the stem usually dies, due to the boring of the larvae in the stem. Feeding in the whorl and midribs of leaves can lead to the "**dead heart**" symptom, due to the death of central leaves (Figure 1-3), which can terminate plant growth. This may cause the development of many tillers that are barren. Damage is most critical if infestation results in dead heart or excessive crop lodging. Plants with dead heart are unlikely to produce ears, and lodging will decrease harvested grain (Tefera et al., 2016). The level of economic losses will depend on the level of infestation and its timing. Early attacked plants are stunted in growth and the ears are poorly developed. Losses are also incurred due to loss of photosynthetic leaf area caused by larval feeding.

1.2.4 Feeding Behaviour and Symptoms

Upon hatching, with the exception of *S. calamistis* that bore directly into the stem, the first stage larvae of most stem borer species initially feed on young leaf tissues, while older larvae tunnel into the stem tissues and feed internally (Bosque-Perez and Schulthess, 1998). Depending on the species, the larval stage may last 25-58 days and may have 6-8 stages. Pupation normally takes 5-14 days after which adult moths emerge (Harris, 1990; Maes, 1998; Kfir *et al*., 2002). The first indication of stem borer infestation is the appearance of small, elongated windows or round holes due to feeding by the young larvae (Visarada *et al*., 2014). These symptoms on the leaves appear in 15-25 days old plants. The third stage larvae migrate to the base of the plant, bore into the shoot, and damage the growing point, resulting in the production of a dead heart in 25-45 days old plants. Normally, at least two leaves dry up because of stem borer damage, while only one leaf dries up due to shoot fly damage. Stem borer larvae also feed inside the stem and cause extensive tunnelling, which is not apparent unless the stem is split opened. Heavy damage in the stems and peduncle result in peduncle breakage or partial seed set. Extended periods of drought and poor plant growth increases the levels of damage caused by the stem borers.

1.3 Management of insect pests

The spotted stem borer, *C. partellus*, and the African stem borer, *B. fusca*, are the most destructive pests of sorghum in southern Africa. Management of these insect pests is usually through cultural methods, chemical control or biological control.

1.3.1 Cultural method

Cultural methods include crop rotation, seedbed preparation and seed treatment. These methods minimize the build up of sorghum pests combined with selection of proper hybrids that are well adapted to the environment, have vigorous growth, and have good disease resistance (Khan *et al*., 2015). It has been reported that in west Kenya, early sowed sorghum developed less infestations by stem borers than late planted sorghum (Haile, 2015; Leonard, 2015). Several researchers have suggested that the destruction of crop residues, stubble, volunteer and alternate host plants can reduce borer infestations (Reddy, 1987; Kfir *et al*., 2002). These cultural approaches aim to decrease pest populations by focusing on disrupting the pest life cycle. Hence, a detailed knowledge of the life cycle of each specific stem borer is required to ensure the efficiency of the cultural control methods (Belay *et al*., 2009). Other reported cultural methods include shredding of stalks, post-harvest (Kumar *et al*., 2014). However, these methods are time consuming and costly, which is not feasible for small farmers and are difficult to accomplish for all farmers. Thus, the commercial farmers mostly rely on control methods based on insecticides or GMO resistant crop.

Push-Pull, another technique developed by the international centre of insect physiology and ecology (icipe) in collaboration with Rothamsted Research in United Kingdom, and national partners in East Africa aim of improving productivity and incomes of smallholder farmers through integrated management of stem borers. It was first conceived as a strategy for insect pest management by Pyke *et al*. (1987), later formalised and refined by Miller and Cowles (1990), involves use of behaviour-modifying stimuli (e.g. semiochemicals) to manipulate the distribution and abundance of pest and/or beneficial insects for the management of pest (Cook *et al*., 2007). The approach used in Push-Pull technique combined inter and trap cropping systems where stem borers are attracted and trapped in a perimeter trap plant, and are driving away from the cereal crop by antagonistic/repellent intercrops. Napier grass, *Pennisetum purpureum* Schumach, Sudan grass, *Sorghum sudanense* Stapf are the most attractive and antagonistic plant species for use as trap and repellent intercrops identified by icipe and partners. *P. purpureum* Schumach and *S. sudanense*, both folder plants were

preferred to maize for oviposition by gravid stem borers' moths, and were used as trap crop in field. Khan *et al*. (2000) reported highly significant reductions in stem borer infestation in maize with concomitant yied increases of 1-1.5 t ha^{-1} as a result of planting Napier gras as a border crop around plots of maize. Another fodder crop, *Melinis minutiflora* was found and after subsequent studies that confirmed its emitted repellent semiochemicals, was used as a repellent plant in the push-pull strategy (Khan *et al*., 1997). Both *M. minutiflora* and forage legumes in the genus Desmodium use as an intercrop with maize provided effective control of these pest (Khan *et al*. 2000). Over 80% in stem borer infestation were controlled after using *M. minutiflora* intercropped with maize (Khan *et al*. 2000). The Push-Pull approach herein is so far the choice for the majority of smallholder farmer in eastern Africa where this constraints affect cereal production (Cook et al., 2007).

1.3.2 Chemical control

Chemical methods used to manage stem borers are not completely effective, and are not affordable for resource poor farmers. It is essential to apply the insecticides before larvae enter the sorghum stalks to ensure effective control. When larvae have already entered the stalks, insecticides can be applied to reduce the damage. However, the use of insecticides to control borers without integration with other control methods has been relatively ineffective (Haile, 2015).

Insecticidal control measures against sorghum stem borers are based on the use of both contact and systemic insecticides, applied as sprays to the foliage (Sithole, 1986). The use of chemical pesticides, however, has resulted in numerous problems. Moreover, many of the commonly used insecticides are considered environmentally unsafe. For example, endosulfan and monocrotophos have been banned for their potential adverse health effects on animals and humans (Rameash *et al*., 2012; Gill and Garg, 2014). Furthermore, many insects develop resistance to regularly applied insecticides, making these compounds less effective (Shah and Pell, 2003). Foliar sprays with insecticides usually causes death of beneficial insects such as pollinators and natural predators of the target pest.

As a result of these problems with insecticides, there is great interest in the development of alternatives to insecticides, such as the use of entomopathogenic fungi to replace insecticides (Shah and Pell, 2003).

1.3.3 Biological Control

A promising alternative for pest control is the use of entomopathogenic fungi as biological insecticides. Use of fungal biocontrol agents could reduce the use of insecticides. Fungal entomopathogens have been widely investigated as biological control agents of pest insects in attempts to improve the sustainability of crop protection (Roy *et al*., 2010). Under suitable conditions, entomopathogenic fungi are capable of causing epizootics among the pests, which makes them attractive to use in Integrated Pest Management programs (IPM) (Mehra *et al*., 2014). More than 700 species of fungi, belonging to 90 genera, are pathogenic to insects (Ingelis *et al*., 2001), and are capable of infecting all developmental stages of a broad range of pests in storage sheds, greenhouses and under field conditions (Barbarin et al., 2012). Most research on entomopathogenic fungi has been aimed at developing them as inundative biological control agents of insects, mites and ticks. In some cases, they have been used classical biocontrol strategies (Vincent *et al*., 2007).

1.4 Rice

1.4.1 Crop origin, genetics and morphology

1.4.1.1 Origin and Genetics of Rice

Rice is a cereal belonging to the family Poaceae, in the genus *Oryza*. There are about twenty-five species, of which only two are found in Africa as cultivated crops: *Oryza sativa* Linne (Asian origin) and *Oryza glaberrima* Steud (African origin) (Carpenter, 1978). The centre of origin and centres of diversity of the two cultivated species, *O. sativa* and *O. glaberrima,* have been identified using genetic diversity, historical and archaeological evidence, and geographical distribution. It is generally agreed that the river valleys of the Yangtze and Mekong Rivers could be the primary center of origin of *O. sativa.* The delta of the Niger River in Africa is considered to be the primary center of origin of *O. glaberrima* (Porteres, 1956). The inner delta of Niger River and some areas around Guinean coast of Africa are considered to be secondary centres of diversity of *O. glaberrima* (Chang, 1976; Oka, 2012).

Oryza sativa is the species most cultivated in the world today, and has evolved a wide variety of colours, shapes, scents and cooking qualities. The progenitors of *O sativa* are widely considered to have evolved from the Asian AA genome of a diploid species *Oryza rufipogon* Griff. (Second, 1982). In this theory, the new species spread and diversified to form two ecological groups: *indica* and *japonica*. A contrasting theory is that the two groups were derived independently from the domestication of two different wilds rice in China, and India

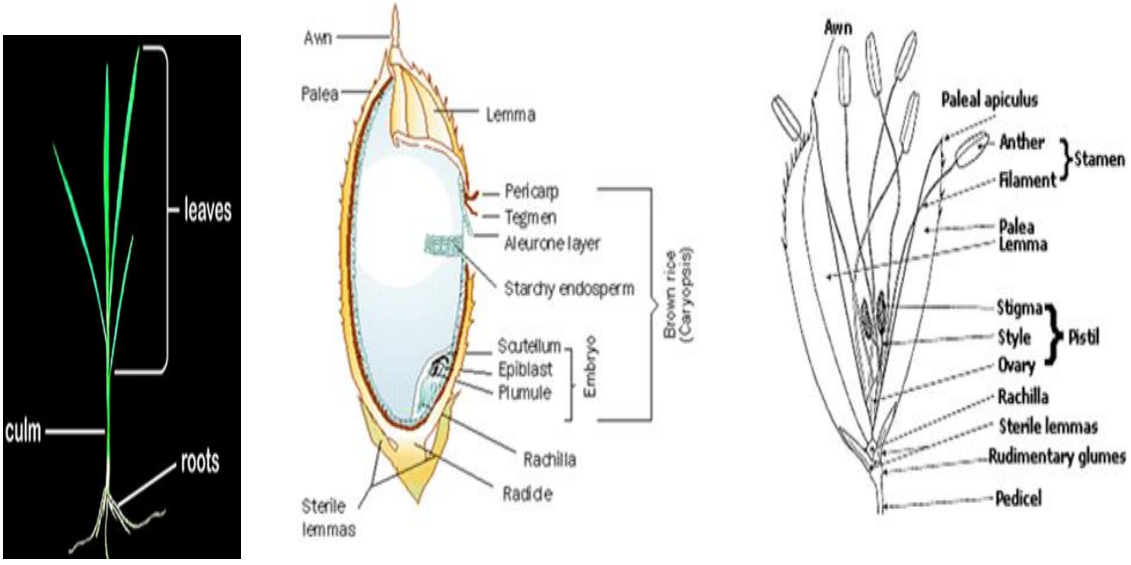
respectively (Second, 1982; Second, 1985). The *indica* group appear to have been developed in tropical Asia, and is characterized by its high tillering ability, and long and thin grains. The *japonica* group developed in the temperate and sub-tropical zones of Asia. Its tillering ability is medium, and it has short and round grains. *O. sativa* was introduced into Africa by the Portuguese in 1500 B.C. (Porteres, 1956). *Japonica* types are well adapted to the rainfed, upland conditions used for rice production in West Africa.

The progenitors of *O. glaberrima* are considered to be two African diploid species, *O. barthii* and *O. longistaminata* (Chang, 1976). *O. glaberrima*, with the genotype AgAg, has a center of origin in West Africa (Van Andel, 2010) and was domesticated in the central delta of the Niger River in Mali (Bonjean and Picard, 1990). Its cultivation spread from Cape Verde to Chad (Porteres, 1956), the Gambia, Casamance and the Sokoto Basin (Carpenter, 1978). It has been cultivated from at least 3500 years. *O. glaberrima* landraces are gradually being displaced by *O. sativa* which yields more grain per ha (Sie, 1991). However, it is still widely cultivated in West Africa because of its resistance to drought and waterlogging, and its ability to tolerate acidic soil, iron toxicity and heat. It also has superior resistance to rice blast, *Helminthosporium* leaf spot, rice yellow mottle virus (RMYV), insects and nematodes. With its high level of plant vigour, it has the ability to contain and tolerate weeds (Pham, 1992; Adeyemi and Vodouhe, 1996; Jones *et al*., 1997). Apart from their different geographic origins, *O. glaberrima* and *O. sativa* are morphologically different. The ligule is short, roundish and tough in *O. glaberrima* but is pointed, bifid and long in *O. sativa*. At maturity, *O. glaberrima* plants show an open panicle, whereas they are closed in *O. sativa*. In general, a higher number of spikelets are observed on the panicle of the Asian species (Porteres, 1956).

1.4.1.2 Morphology of rice

Rice is herbaceous annual, semi-aquatic, glabrous, erect or spreading stubble, varying in height from less than a meter to five meters for floating rice. It is prone to tillering, forming a cluster of stems and fibrous roots containing rootlets and root hairs (Figure 1-7). Uniform spikelets are grouped in panicles 20-30 cm, which may be erect or pendulous (Angledette, 1996). Roots, stem and leaves constitute the vegetative apparatus. The roots are typically fibrous and have rootlets and root hairs. The stem is fully enclosed within the sheath until heading. The main stem is divided into internodes separated by swollen nodes. The node carries a leaf and a bud. The height of the main stem is dependent on the number of

internodes, which may vary from 10-20, depending on the variety. The lower leaf axils of the main stem and the nodal axillary bud can give rise to the first order of tiller. The phenomenon is repeated on primary tillers, giving birth to second order tillers, developing in a plane perpendicular to that of the first order tillers. The phenomenon of tillering thus leading to the formation of a clump. This phenomenon depends on varietal factors and environmental conditions (Angladette, 1996). The leaves are unsheathed and originate along the tillers at the nodes. They consist mainly of two parts: the leaf sheath and the blade. On the sleeve joint/limb these are two small structures: the auricle and ligule (Angladette, 1996).



Parts of a rice plant | Morphology of a rice grain | Morphology of the rice flower

Figure 1-7 Morphology of a rice plant, grain and flower

Source: Bardenas and Chang (1965)

1.4.2 Growth and development stage

The rice development cycle follows grain germination, the vegetative stage, the reproductive stage and the maturity stage (Figure 1-9) Counce *et al*. (2000). Its duration is 3-6 months, depending on the variety and the environment in which it is grown (Second. 1985). Rice is not strictly annual or strictly perennial. Some rice species, such as *Oryza barthii* A. Chev and *O. meridionalis* Ng., are annual (Cheng et al., 2002), whereas others such as *O. longistaminata* A. Chev. and *O. nivara* Sharma and Shastry are perennial (Chang, 1984; Khush, 1997; Vaughan and Morishima, 2003). *Oryza rufipogon* Griff. and *O. glumae patula* Steud. vary in perenniality (Cheng *et al*., 2002). The variable life cycle is determined by the vegetative phase.

The vegetative phase (Figure 1-8) takes 60 days for varieties having a cycle of 120-130 days, and more than 60 days for late cycle varieties. This stage includes germination, emergence and tillering. Germination takes 5-20 days depending on the temperature (5 days in hot condition, and 20 days under low temperature). It has been estimated that germination is triggered when the grain has absorbed a quarter of its weight of water, and the mean temperatures are above 13°C (Dobelmann, 1976). The lifting stage is from emergence to the four-leaf stage and lasts 15-25 days depending on the temperature. Tillering starts from the five leaf stage, about 15 days after sowing (Dobelmann, 1976), and has a variable duration depending on temperature and variety. The duration of this phase can be differentiated into short, medium and long cycle varieties.

The reproductive phase (Figure 1-8) is dependent on various factors such as moisture, nutrition, and especially temperature and day length. This phase goes from panicle initiation to flowering, including panicle initiation, bolting, heading and flowering. The reproductive phase lasts from 19-20 days. During this phase, the plant is very sensitive to drought and low temperatures. Panicle initiation (PI) occurs at the start of the reproductive phase of rice development. It is when the actual panicle (terminal flowering head) begins to form in the base of the stems. The formation of the panicle marks the end of the tillering or vegetative phase and the beginning of the reproductive phase. PI is defined as when 3 out of 10 main stems have a panicle 1-3 mm long. At this stage, three leaves will still emerge before the panicle finally emerges. In short-duration varieties, the panicle becomes visible with 1.0-1.5 mm long. It first emerges from the main culm and then from tillers, where it emerges in an uneven pattern. It can be seen developing by dissecting the stem. As the panicle continues to develop, the spikelets become distinguishable. The young panicle increase in size and its upward extension inside the flag leaf sheath causes the leaf sheath to bulge, which is called booting. The booting is most likely to occur first in the main culm. At booting, senescence (aging and dying) of leaves and non-bearing tillers are noticeable at the base of the plant. Heading is marked by the emergence of the panicle tip from the flag leaf sheath. The panicle continues to emerge until it partially or completely protrudes from the sheath. Flowering occurs a day after heading and the process continues until most of the spikelets in the panicle are in bloom. It takes about 7 days for all spikelets in a panicle to open. At flowering, 3-5 leaves are still active.

The maturity phase (Figure 1-8) lasts 30-40 days. It begins from flowering to the ripening of the grain that goes through different stages. In the milk grain stage, the grain starts to fill with a white, milky liquid, which can be squeezed out by pressing the grain between the fingers.

The panicle looks green and starts to bend. Senescence at the base of the tillers is progressive. The flag leaves and the two lower leaves remain green. In the dough grain stage, the milky portion of the grain first turns into soft dough and later into hard dough. The grains in the panicle begin to change from green to yellow. Senescence of tillers and leaves are noticeable. The field starts to look yellowish. As the panicle turns yellow, the last two remaining leaves of each tiller begin to dry at the tips. In the mature grain stage, the individual grain is mature, fully developed, hard and turned to yellow. The upper leaves are now drying rapidly although the leaves of some varieties remain green. A considerable amount of dead leaves accumulate at the base of the plant.

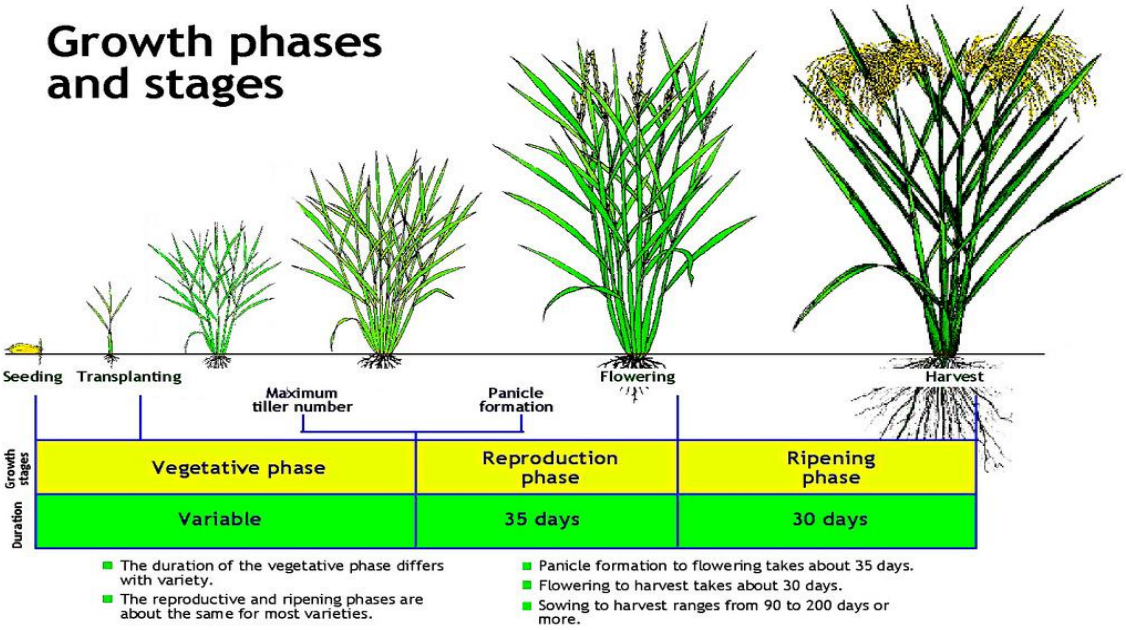


Figure 1-8 Growth stage of rice plant.

Source: https://www.flickr.com/photos/ricephotos/13596607373/ (Accesses on 21st June 2017)

Figure 3 : Cycle de croissance de la plante de riz : 1 Grain ; 2 Plantule issue de la germination ; 3 Plant végétatif ; 4 Début du tallage ; 5 Plant mature.

Figure 1-9 Life cycle of rice plant.

Source: Bert Maertens et Michiel de Vries (2014).

1.4.3 Taxonomy of rice

Rice belongs to the genus *Oryza* and the tribe Oryzeae of the family Gramineae (Poaceae). The genus *Oryza* contains 25 recognized species, of which 23 are wild species and two; *O. sativa* and *O. glaberrima*, are cultivated (Morishima, 1969; Vaughan, 1994). *O. sativa* is the most widely grown of the two cultivated species. *O. glaberrima* is only grown in some West African countries.

- **Scientific classification**

Kingdom: Plantae

Division: Magnoliophyta

Class: Liliopsida

Order: Poales

Family: Poaceae or Gramineae

Genus: *Oryza*

Species: *Oryza glaberrima*

Oryza sativa

1.4.4 Rice production constraints

Africa rice ecosystems are labelled on the basis of the source of water, include mangrove swamps, deep-water floating, irrigated lowland, Sahelian irrigated, rainfed lowland and rainfed upland, and are among the most diversified in the world (Buddenhagen, 1978; Andriesse and Fresco, 1991). The various ecosystems are subject to various constraints, some of which are specific to particular ecosystems, while others are general and cut across ecosystems and regions. The general constraints affecting rice production in Africa include the unreliability of rainfall in the transitional zones of West, Central and East Africa (Tanaka *et al*., 2013), regular destruction of rice fields by cyclones in some countries such as Madagascar (Abdur *et al*., 2013; Rakotobe *et al*., 2016), yield losses caused by low solar radiation owing to clouds (Schmitter *et al*., 2015), extremes of temperature which result in yield losses (Diagne et al., 2013), and the absence of suitable varieties to withstand these stresses (Rodenburg *et al*., 2014).

Biotic constraints also affect rice production across the world. Insect pests are possibly the most harmful of the biotic factors, causing annual losses of about 18% (Oerke, 1994; Seck *et al*., 2012; Todaka *et al*., 2015). Over 100 species of insects are considered to be pests of rice, but only few of them are of major economic importance (Diagne *et al*., 2013; Tanaka *et al*., 2013). The pests infest all parts of the rice plant at various stages of development. Breeding for resistance to these pests would be an ideal solution to the problem that they pose (Chaudhary *et al*., 2003). However, rice is difficult to breed for pest resistance, and secondly, the importance of each pest may vary considerably from one region to another, from one field to another, and from one year to another (Heinrichs, 2004). Variable incidence and severity is typical of the stem borers (Lepidoptera and Diptera) (Tanaka *et al*., 2013; Leonard, 2015).

1.4.5 Rice stems borers

These are especially Diptera (families of Diopsidaes and Cecidomyidae) and Lepidoptera (Crambidae, Pyralidae and Noctuidae), and they are considered to be the most damaging group affecting rice production (Breniere, 1983; Heinrichs, 2004). The larvae of these borers cause significant yield losses, by the formation of dead hearts and white panicles, during the vegetative stage and heading stage, respectively, which prevents the development of the panicle (Breniere, 1983; Nwilene *et al*., 2008). The major stem borers encountered in Africa that are lepidopteran include *Chilo zacconius* Bleszynski, *C. partellus* Swinhoe, *Sesamia calamistis* Hampson, *Maliarpha separatella* Rag and the *Scirpophaga spp.* The important

dipterans include the African rice gall midge, *Orseolia oryzivora* (Harris and Gagné), and *Diopsis* spp. (Umeh *et al*., 1993; Nwilene *et al*., 2006).

***Sesamia calamistis* Hampson (African pink stem borer)**

This species is found in sub-Saharan Africa and some of the islands in the Indian Ocean (Nwilene *et al*., 2013). It commonly occurs in wetter localities at altitudes from sea-level to 2400 m. The main crops affected are sorghum, rice, maize, pearl millet, wheat, and sugarcane. *S. calamistis* generally is not a major pest in Eastern and Southern Africa, whereas in West Africa this species is one of the most damaging to rice, sorghum and maize (Barhahahkana *et al*., 2014).

Life Cycle of *Sesamia calamistis*

African pink stem borer larvae (3-4 cm) usually pupate at the base of the stem or within a cocoon in an old leaf sheath. The adult form has light-brown forewings with dark stripes and silvery white hind wings. This moth, like the African maize stem borer, lay eggs between the lower leaf sheaths and the stem in batches of 10-40, arranged in 2-4 contiguous rows. Each female lays around 300 eggs in a period of five days. Egg may be laid on rice plants from the time the plants are two weeks old until they are flowering. As the time for hatching approaches, eggs become pinkish and then turn brown (Woy and Bowling, 2013). Larvae penetrate the stem shortly after they emerge from their eggs. Feeding might result in dead hearts. The tunnelling and girdling activity of the larvae often results in stalk breakage. Development of the larvae takes four to six weeks, after which they pupate within the stem or maize cobs (Tefera, 2013; Woy and Bowling, 2013).

1.4.6 Feeding Behaviour and Symptoms of Borers

The damage caused by stem borers depends on the development of the larvae in the stem. The damage depends on the species of Lepidopteran borer. The yield losses the larvae cause are higher when they infect young plants than when the damage occurs late in the plants' development, when they have completed their development (Breniere, 1983). The yield losses from stem borers are estimated at 25-30% of potential yields (Bux et al., 2013). Chaudhary *et al*., (2003) reported that dipterans of the genus *Diopsis* could cause yield losses of up to 38%. In Africa, the losses caused by "drillers" damage are estimated between 2-38% of the crop yield (Nwilene *et al*., 2013).

Lepidoptera are characterized during their larval life by two distinct periods. They cause two main types of damage to rice plants. An early attack occurs during the vegetative phase,

resulting in the destruction of the central bud, and drying of the central leaf. The larvae are phyllophagous, and consume the parenchyma of leaves and sheaths in seedlings at the beginning and during tillering. These attacks are characterized by the **dead heart** symptom (Figure 1-10). When the attack occurs during the reproductive stage (flowering), the young larvae (especially the 2nd generation) are found in the panicle, where they feed inside the scape, which dries to give a completely empty spikelet, which is a symptom of **white panicle** (Figure 1-10) (Breniere, 1983). Breakage of the stem may occur thereafter. The damage caused by the Asian gall midge, *Orseolia oryzivora* Wood-Mason, are due to the activity of the larvae that feeds inside young infested tillers. They eat the buds, causing elongation of the tissues and the formation of tubular structures called galls, turning the stem a silver colour, so they start to resemble onion leaves (hence the name **onion tubes**) (Harris and Gagne, 1982; Umeh *et al*., 1992). Therefore, the formation of galls always results in the loss of tillers. The attack of the stem borer is less damaging when it occurs at tillering, because the plant can compensate by producing other tillers (Polaszek *et al*., 2000).

Figure 1-10 Rice plants with dead heart and white panicle symptoms

Source: Togola (2006)

1.4.7 Strategy to Control Stem Borers of Rice

1.4.7.1 Detection methods

African pink stem borer (*S. calamistis*) infestations may be detected by scouting through young crops for characteristic feeding marks on funnel leaves, and looking for the presence of dead hearts and holes in tunnelled stems. Samples of affected stems can then be cut open to find larvae and pupae. It is best to rear them until they reach the adult stage to identify the species because it is difficult to identify the species from the larvae and pupae. African pink

stem borer may be detected in older crops and in crop residues by dissecting random samples of stems to find the larvae and pupae.

1.4.7.2 Cultural practices

Intercropping rice with non-hosts crops, such as *Manihot esculenta* Crantz or legumes like *Vigna unguiculata* (L.) Walp., can reduce spotted stem borer damage. Alternatively, cereal crops can be intercropped with a repellent plant such as silver leaf desmodium (*Desmodium uncinatum* Jacq.), or planted with a border of a trap crop such as Napier grass (*Pennisetum purpureum* Schumach.), or molasses grass (*Melinis minutiflora* Beauv.). The trap plant draws the adult female away from the crop. More eggs are laid on the trap plant than on the main crop but the larvae develop poorly and not at all on the trap plant. This practice is known as Push-Pull (Murage et al., 2015).The destruction of rice residues by burning to get rid of the larvae and pupae within the stems, and the removal of volunteer crop plants and/or alternate hosts, prevents carry-over populations (Mahajan and Chauhan, 2013). This helps in limiting the initial establishment of stem borers that would infest the next crop. Early slashing of rice stubble and laying it out on the ground, where the sun's heat destroys the larvae and pupae within the dead stems, can also be utilized.

1.4.7.3 Chemical control

Chemical control can be achieved by applications of insecticidal granules or dusts to the leaf whorl early in crop growth to kill early larval instars. This method has limited effectiveness once the larvae bore into the stem. Spray or granular applications of carbaryl, trichlorfon, carbofuran, diazinon or fenitrothion have been recommended in the past. Application of a 50:50 mixture of powdered neem kernels and sawdust to the leaf whorls of young plants is also reportedly effective. (Wam *et al*., 2014).

1.4.7.4 Biological Control

1.5 *Beauveria bassiana* as Biological Control Agent

Myco-insecticides based on the white muscardine fungus, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin, are being used against agricultural, veterinary and medical insect pests globally. *B. bassiana* is ubiquitous in distribution and is pathogenic to a wide spectrum of arthropods, with its host range spanning most orders of class Insecta (Butti and Goettelz,

2000; Lacey et al., 2001; Zimmermann, 2007) It has been isolated from over 700 insect species, and is also present in the soil as a saprophyte (Wraight et al., 2000) (Table 1-1).
It also has the ability to colonize plants as a symbiont or endophyte (Wagner and Lewis, 2000). It has been reported to be an endophyte of many plants, both monocots and dicots (Zimmermann, 2007). The endophytic properties of *B. bassiana* would enhance its potential to protect crops from pests because its presence inside plant tissues will be for longer than the conidia can persist on leaf surfaces, exposed to the elements (Table 1-1).

1.5.1 Taxonomy

Kingdom: Fungi
Phylum: Ascomycota
Class: Sordariomycetes
Order: Hypocreales
Family: Clavicipitaceae
Genus: *Beauveria*
Species: *B. bassiana*

1.5.2 Morphology and Life Cycle

B. bassiana is a polymorphic fungus whose life cycle (Figure 1-11) includes both single and multi-cellular stages (Tucker et al., 2004). It is a ubiquitous saprobe and can be found in soil or decaying plant material, where it grows as a multi-cellular mycelium by absorbing nutrients from decaying matter. Reproduction and dispersion of progeny is accomplished by the production of asexual spores called conidia (Westwood *et al*., 2005). Conidia are produced from conidiogenic cells, produced in a zigzag structure from hyphae (Kisla *et al*., 2000). The conidia remain in the environment in a dormant, state until appropriate conditions activate germination. Humidity is a major factor in activation of conidia, independent of a host (Tucker *et al*., 2004). On cultural media, the fungus grows as a white mould, producing an abundance of dry, powdery conidia in white spore balls (Ownley *et al*., 2008). In the absence of a host, the fungus follows an asexual vegetative cycle where germination, filamentous growth and the formation of conidia occurs. In the presence of a host, the fungus follows a sexual cycle where the conidia germinate on the cuticle of insects, followed by formation of hyphal tubes that penetrate the integument of the host directly. From there the fungus grows in a yeast-like phase that produces hyphal bodies that circulate in the

haemolymph and proliferate by budding. This results to the death of the host, after which fungal growth reverts to its original hyphal growth.

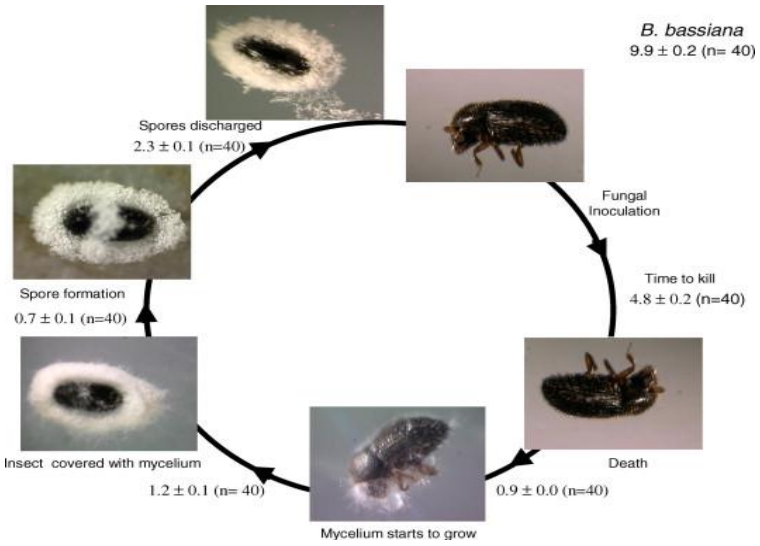


Figure 1-11 Life Cycle of *Beauveria bassiana*.

Source: Kisla *et al*. (2000)

1.5.3 Epidemiology

The growth of all fungi is affected by the environmental conditions (temperature, humidity or moisture and solar radiation). Optimum growth of *B. bassiana* requires a temperature range from 23-28°C. Minimal growth occurs at a minimum temperature at 5-10°C. This depends on the strain of *B. bassiana*. Spores lose their viability when exposed to temperatures of 50°C for 10 minutes or more (Zimmermann, 2007).

Humidity is a vital factor when it comes to the growth of *B. bassiana*. Successful infection of insect pests has been reported when RH ranges from 60-70%. However, successful infection has been accomplished, in the presence of oil, with low RH levels. The longevity of conidia decreases with increasing temperatures and RH (Zimmermann, 2007).

Conidia of a culture of *Beauveria bassiana* that is exposed to 99% solar radiation for a period of about 16 minutes loses its viability. However, some isolates may show different responses to sunlight exposure, though the general effect is detrimental to the germination and viability of conidia (Zimmermann, 2007).

1.5.4 Infection process

The life cycle of insect mycopathogens involve the dissemination of conidia, adhesion to the host, germination, penetration, invasion and colonization in the haemocoel, tissues, and organs, followed by sporulation. The timing of each of these stages is variable, according to

fungal species, the host and the environmental conditions. Conidiogenesis normally occurs after the external growth of the fungus from the dead host. During fungal growth, the production of toxins may occur in the insects' body. Moreover, the time from infection to insect death varies with the concentration of conidia applied, and the virulence of isolates. Unlike bacteria and viruses, which must be consumed, infection by entomopathogenic fungi most often occurs after contact of the fungal conidia with the host cuticle. The process of infection begins with the attachment and germination of the conidia in the cuticle. This stage is said to be random and is aided by wind or water. Hydrophobic rodlets on dry conidia are considered to be responsible for adhesion, which is due to hydrophobic forces exerted by the rodlets, which attracts the conidia to the waxy cuticle of the host insect. Under favourable conditions, rapid germination of the conidia is followed by penetration of the host through the cuticle. Penetration involves both mechanical and enzymatic actions (Zimmermann, 2007). A range of proteases, chitinases and lipases are produced to degrade the cuticle of a host. When the fungal hypha reaches the insect haemocoel, it may encounter cellular and humeral immune responses of the host. Successful attack and proliferation within a host depends on the ability of the fungus to overcome or avoid the host's immune responses to fungal infection (Gillespie *et al*., 1998). As part of its arsenal to defeat the insect resistance reaction, *B. bassiana* produces toxins in the body of its host. These toxins have various effects on the tissue of the host. If the host fails to defend itself, it will die due to tissue damage, toxicosis and a lack of nutrient resources.

1.5.5 Endophytic activity

There are several reports of endophytic colonization of plants by *B. bassiana*, either occurring naturally, or because of inoculation (Wagner and Lewis, 2000). The process of endophytic infection is similar to the infection process that all pathogenic fungi follow. However, the fungus does not develop appressoria on the host plant as it does on insect cuticles (Wagner and Lewis, 2000). Signalling must occur between the fungus and host to establish the symbiotic relationship, and to stop the host plant from killing the invading fungus. *B. bassiana* does not require precise orientation to invade the plant. Instead, hyphae grow randomly on the surface of the leaves, roots or stem, depending on mode of inoculation. The fungus penetrate sorghum, rice and other crops but does cause any disease in these crops as it exhibits a beneficial relationship with the host plant (Kisla *et al*., 2000).

Table 1-1: Summary of the Use of *Beauveria bassiana* as a Bioinsecticide: Target Insects, Crops and its Mode of Application

Fungus	Intended use	Target crop	Mode of application	Reference
Beauveria bassiana	*Ostrinia nubilalis* Hampson (Lepidoptera: Pyralidae)	Maize (*Zea mays* L.)	aqueous or granular formulation	Lewis and Cossentine, 1986; Lewis and Bing, 1991
	Sesamia calamistis H. (Lepidoptera : Noctuidae)		Treat maize seeds with dry conidia; by spraying conidial suspensions in the leaf axils; injecting a conidial suspension in the stem	Cherry *et al*., 1999, 2004 ; Lomer *et al*., 1997
	Sitophilus zeamais Motsch (Coleoptera : Curculionidae).		Dry formulation of conidia applied to maize seeds	Adane *et al.*, 1996
	Hypothenemus hampei Ferrari (Coleoptera: Curculionidae and Scolytidae)	Coffee (*Coffea arabica* L)	Aqueous suspension with Tween	De La Rosa *et al.*, 1997
	Cosmopolites sordidus Germar (Coleoptera: Curculionidae)	Banana (*Musa spp*),	Plantlets colonization. Root and rhizome dipping in a *B. bassiana* conidial suspension or plant injection with a *B. bassiana* conidial suspension	Griesbach, 2000; Paparu, 2004*;* Akello *et al.*, 2008
	Metamasius. spinolae Gyllenhal (Coleoptera: Curculionidae)	Cactus (*Opuntia ficus-indica*	applied *B. bassiana* conidial suspension in a spray tower	Tafoya *et al.*, 2004
	White Grub *Laniifera cyclades* Druce (Lepidoptera: Pyralidae)	Prickly pear cactus *(Opuntia spp.)*	Sprayed conidial suspension first to *Galleria mellonella* L. moth. In the field, introduction of a *G. mellonella* cadaver into the orifices made by *L. cyclades* larvae in the prickly pear plants. In the greenhouse, the orifices were made manually and larvae of *L. cyclades* were inserted into the orifices.	Lozano-Gutiérrez and España-Luna, 2008

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Chapter 2 Evaluation of the endophytic competence of multiple strains of Beauveria bassiana in sorghum cultivars

Abstract

Sorghum bicolor (L.) is a drought tolerant cereal crop. It is extensively cultivated in the semi-arid tropics of Africa and India, is used for both grain and cattle fodder. The stem borer, *Chilo partellus* Swinhoe (Lepidoptera: Pyralidae), is among the most economically important pests affecting this crop, routinely causing yield losses of 80–100%. The primary aim of this study was to determine the endophytic competencies of 20 *Beauveria bassiana* strains as endophytes in five sorghum cultivars, as potential biocontrol agent against *C. partellus*. Two inoculation methods (seed and foliar applications) were tested. Plants were grown from surface-sterilized seeds before being inoculated with a conidial suspension of one of the *B. bassiana* strains, or sterile distilled water as a control. Samples of leaves, stems and roots were harvested after 30 and 60 days, and were then assessed for the presence of *B. bassiana.* Some of the *B. bassiana* strains established themselves as endophytes in all plant parts sampled, regardless of the inoculation method, with varying degree of colonization and persistence. With the foliar spray, 45% of the strains colonized all of the plant parts sampled, while the seed treatment resulted in only 25% of the strains colonizing all three of the plant tissues sampled. However, the greatest persistence of colonization was observed with seed treatments. Only three strains (15%) colonized all the plant parts when used either as a seed treatment or as a foliar spray. Colonization by *B. bassiana* was not observed in the untreated control sorghum plants. Significant differences were recorded between the application methods (P = 0.0001), the strains (P = 0.0001) and the cultivars used (P = 0. 0001). With both application methods, significant interaction effects were also recorded between cultivars x strains (P = 0.0001), strains x time of application method (P = 0.0098 for seed treatment and P = 0.0189 for foliar spray), and strains x cultivars x time of application method (P = 0.05). The results showed that some *B. bassiana* strains are endophytic in sorghum, and that both seed treatments and foliar sprays with their conidia may result in colonization of sorghum plants.

Keywords: *Beauveria bassiana*, entomopathogenic fungus, *Chilo partellus* and inoculation methods.

2.1 Introduction

Beauveria bassiana (Balsamo) Vuillemin is a biological control agent of numerous insect pests of many orders (Ownley *et al*., 2008; Ownley *et al*., 2010). Many characteristics of *B. bassiana*, such as endophytic colonization, parasitism of pests and eases of production (Azevedo *et al*., 2000; Vega *et al*., 2009), contribute to its potential as a mycopesticide (Ferron *et al*., 1991; Feng *et al*., 1994; Wagner and Lewis, 2000; Charnley, 2003; Zhang *et al*., 2012). As an entomopathogen, *B. bassiana* is known to infect many insect species, belonging to most insect orders (Rai and Ingle, 2012). Several commercial products of *B. bassiana* have been developed (Butt *et al*., 2001).

Beauveria bassiana has also been shown to be an endophyte in several crops. Fungal endophytes may play an important role in protecting plants against insects and pathogens (Azevedo *et al*., 2000; Ownley and Windham, 2007). Evidence suggests that endophytic *B. bassiana* has the potential to protect plants not only from arthropod pests (Bing and Lewis, 1991; Bing and Lewis 1993; Gurulingappa *et al*., 2011), but also from some plant pathogens (Ownley *et al*., 2008).

Chilo partellus Swinhoe (Lepidoptera: Pyralidae), the spotted stem borer, is a highly destructive insect on sorghum [*Sorghum bicolor* (L)] in much of Africa. It is present in eastern and southern Africa, often becoming the most prevalent stem borer (Kfir, 1997a; Kfir, 1997b; Abate *et al*., 2000). *C. partellus* is also a major pest of sorghum in Asia (Khan *et al*., 1997; Girijashankar *et al*., 2005; Prasad *et al*., 2015). In South Africa, two lepidopteran stem borers, *C. partellus* and *Busseola fusca* Fuller (Lepidoptera: Noctuidae), are important stem borers of sorghum and maize [*Zea mays* (L)] (Kfir, 1998). Females lay eggs on the underside of the leaves and the first instar larvae feed on the leaf whorls on young leaves (Tefera and Pringle, 2003). The third instar larvae penetrate the stem tissue to feed internally, producing extensive tunnelling in stems. After excavating emergence windows to facilitate the exit of the adult moths, the fifth instar larvae pupate in the tunnels (Ownley *et al*., 2008). The estimated yield losses in South Africa, due to *C. partellus* in sorghum and maize often exceed 50% (Kfir, 1998). For many years, the control of *C. partellus* by farmers has been based on application of chemical insecticides such as phorate, cypermethrin, carbofuran, spinosad and chlorpyriphos. Pesticides are not very effective against *C. partellus* because of the cryptic feeding of this pest, as well as being costly and toxic to spray applicators and the environment. As an alternative, of the pest might be achieved with an endophytic biological control agent such as *B. bassiana*. Conidia of *B. bassiana* infect hosts through conidial

adhesion to the cuticle of the insect, germination, and penetration directly into the host insect (Ferron *et al*., 1991; Vega *et al*., 2009). However, a range of environmental conditions affect the infection cycle. In contrast, the environment does not affect endophytes living inside host plants, which are then eaten by pests, which they infect from the gut of the insects. The present study was undertaken to investigate the endophytic ability of 20 strains of *B. bassiana* applied to five sorghum cultivars in *in vitro* and *in vivo* tests to evaluate their potential for the biological control of *C. partellus*. Furthermore, the duration of their endophytic behaviour in the sorghum cultivars were assessed *in vitro,* to estimate the persistence of the fungi, given the issue of endophytic succession (Crous *et al*., 1995).

2.2 Materials and methods

2.2.1 Sorghum Cultivars

The experiments were conducted using five cultivars of sorghum (Sugargraze, AS 18, AS 71, AS 79 and PAN 8816) provided by Mr Ian Doidge from the University of KwaZulu-Natal experimental farm, Pietermaritzburg, South Africa. The characteristics of the sorghum cultivars are as follows:

Sugargraze: This is a forage sorghum, very susceptible to ergot (grain) and Northern corn leaf blight. It is a three way hybrid bred for cattle feed in Australia. It produces about 50 tons ha^{-1} of biomass.

AS 18: Originally bred in Mexico, this is a dual-purpose restorer line sweet sorghum and grain. It produces a grain yield of about 3.0 tons ha^{-1} and a biomass yield of 30 tons ha^{-1}. It has a good disease resistance.

AS 79: This is a sweet stem line with high brix. It produces a grain yield of about 3.0 tons ha^{-1} and a biomass yield of 50 tons ha^{-1}. It has good disease resistance, but is lightly susceptible to rust. It was originally bred in the USA.

AS 71: This is a dwarf grain sorghum breeding line with a high grain yield of 4-5 tons ha^{-1} and a low biomass yield of 25 tons ha^{-1}. It is also sold as a medium tannin sorghum variety called Dwarf Wonder, bred in Southern Africa.

PAN 8816: This is a Pannar grain hybrid designed for high tannin grain production. PAN 8816 is large-seeded grain sorghum with a high grain yield (4.5-6 tons ha^{-1} but low biomass), and good threshing ability. This cultivar is classified as a good malt (GM) quality, a uniform growth habit, good standability, good leaf disease resistance but is susceptible to stem borers.

It is widely grown in various agronomic regions of South Africa, where the grain is used for various purpose (human consumption, animal feed, and ethanol and beer production).

2.2.2 Beauveria bassiana strains

A collection of 20 *B. bassiana* strains, provided by the Department of Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg South Africa, were initially used for the study. They were previously isolated from a variety of soil, including soils collected from the rhizosphere of mangoes (*Mangifera indica* L.), rooibos tea (*Aspalathus linearis* Burm. F.) and wheat (*Triticum aestivum* L.). They were initially isolated and characterized by the Plant Protection Research Institute (PPRI), Pretoria, South Africa.

2.2.3 Production of sorghum plants for greenhouse studies

Seeds of sorghum cultivars were surface sterilized in 3% sodium hypochlorite for 3 minutes followed by 70% ethanol for 2 minutes. They were rinsed three times with distilled water, air dried and divided into two sets. The first set was used for seed inoculation and the second for foliar spray experiments. The second set of seeds used for the foliar spray application was sown in Speedling® 24 trays filled with composted pine bark (CPB) growing medium. The seedlings were watered with tap water and placed under greenhouse conditions held at a range of 20-28°C night and day. Two weeks after germination, the seedlings were transplanted into 30 cm diameter pots filled with CPB growing medium and placed under the same greenhouse conditions night/day. The plants were allowed to grow for seven days before the foliar spray inoculation took place. Plants were irrigated three times a day with irrigation water containing NPK fertilizer [3: 1: 3 (38)] (50%) together with calcium nitrate (50%) and trace elements (TE).

2.2.4 Production of a conidial suspensions of 20 *Beauveria bassiana* strains

The conidial suspensions used in the studies were prepared following the method of Parsa *et al*. (2013). The conidia were obtained from fungal cultures of the 20 *B. bassiana* strains. The fungal strains were cultured in 90 mm diameter plastic Petri dishes containing potato dextrose agar (PDA) plus antibiotics (100 mg L^{-1} of ampicillin and streptomycin) and incubated at 28°C. The cultures were allowed to grow for 14-18 days, after which the conidia were harvested. The conidia were harvested under sterile conditions by gently scraping the fungal growth from the surface of the agar with a sterile spatula, and rinsing with sterile distilled

water. The suspensions were filtering using sterile cheesecloth to remove mycelium and agar debris. Conidial density was determined using an improved Neubauer haemocytometer and adjusted to 2 x 10^6 conidia ml^{-1} with sterile distilled water containing Tween-80 (0.01%). The viability of each batch of conidia for all the experiments was evaluated by taking a 100 μL sample of each batch, spreading it onto PDA, incubating the conidia at 25°C and assessing their germination 24 hours later. The percentage germination of conidia was determined from 100 randomly selected conidia under a light microscope. Germination of conidia was assumed to be complete when hyphae were visible, or the germ tube was about twice the length of the conidium. For each strain the mean of three replicates were used to assess the viability of each strain. The inoculum was used for the seed treatment and foliar spray inoculations.

2.2.5 Initial screening of 20 *Beauveria bassiana* strains for endophytic colonization in sorghum

2.2.5.1 Seed Treatment Inoculation

Surface sterilized sorghum seeds (as described in Section 2.2.3) for each cultivar were separately dipped in the conidial suspension of each *B. bassiana* strains [5 ml of the prepared inoculum (2×10^6 conidia ml^{-1})] then air-dried. The seeds were then planted into Speedling® 24 trays filled with CPB growing medium. The control plants consisted of uninoculated seeds treated in a similar manner with distilled water. Two weeks after germination the seedlings were transplanted into 30 cm diameter pots filled with CPB growing medium and placed under greenhouse conditions at 20-28°C. Three plants were planted per pot, which were then were arranged in the greenhouse in a randomized complete blocks design with three replicates. Plants were irrigated as previously described under Section 2.2.3. The plants were allowed to grow for 30 days or 60 days before being harvested, and three tissues (roots, stems and leaves) were screened for endophytic colonization.

2.2.5.2 Foliar Spray Inoculation

Two weeks old seedlings of the five sorghum cultivars (Section 2.2.3) were transplanted into 30 cm diameter pots that contained CPB growing medium. After 15 days, a hand sprayer was used to inoculate the leaves of the plants with the conidial suspensions of the *B. bassiana* strains, as prepared above. 50 ml of the inoculum of each *B. bassiana* strains was used per plant. The top of the pots were covered with aluminum foil, folded around the base of the

plants, before the leaves were inoculated with the conidial suspension of *B. bassiana* strains, in order to prevent inoculum runoff onto the roots. Plastic bags were used to cover the leaves for 24 hours to increase humidity. For the control plants, sterile distilled water was applied in a similar manner as described for the *B. bassiana* treatments. All the treated plants (three plants per pot) were placed in a greenhouse (20-28°C night/day) using a randomized complete blocks design with three replicates. The plants were irrigated as previously described under Section 2.2.3.

2.2.6 Evaluation of endophytic colonization

From each sorghum cultivar x *B. bassiana* treatment combination, the leaves, the stem and roots were sampled at 30 and 60 days, and evaluated for endophytic colonization by the *B. bassiana* strains. The seedlings were carefully removed from the pots and gently washed with tap water to remove all debris. The plants were cut into leaves, stems, and roots. Each of those parts were surface-sterilized by immersing them in 0.3% sodium hypochlorite for 2 minutes, followed by 70% ethanol for 2 minutes, and rinsing three times with sterile distilled water for 15 seconds. The surface sterilized samples were placed on sterile tissue paper under a laminar flow cabinet for drying. After drying, six pieces of each of the samples (leaves, stem and roots), from each plant were randomly taken and plated separately onto a *B. bassiana* selective medium (Appendix 2) and incubated for 15 days at 25°C. To confirm that the surface sterilization was effective, 10ml of the sterile distilled water used to rinse the samples during the surface sterilization procedure was spread onto Petri dishes containing *B. bassiana* selective medium. The plates were incubated for 10-15 days at 25°C before counting the colonies that had formed. However, the sterilization process was effective, and resulted in clean plates. Therefore, B. *bassiana* growth from surface-sterilized tissues was assumed to have originated from within plant tissues as endophytes. The plates that contained plant samples were monitored every 2-3 days for emergence of fungal mycelia from the tissues. After 10 or 15 days, **t**he presence or absence of *B. bassiana* growth from the leaves, stem and root samples plated on the selective media for each treatment combination was recorded. The fungal colonies grown from the samples were confirmed to be *B. bassiana*, based on morphological characteristics.

2.2.7 Secondary screening of the best five *B. bassiana* strains for endophytic colonization of three sorghum cultivars

From the initial screening, three sorghum cultivars and the five best *B. bassiana* strains were selected for this study. The same procedures were followed for this study as previously described under Sections 2.2.3 - 2.2.6. This study was done to confirm the endophytic colonization ability of the five best *B. bassiana* strains x 3 best sorghum cultivars for further studies in *in vivo* greenhouse and field biocontrol experiments, using *C. partellus*.

2.2.8 Data analysis

The colonization of plant tissues resulting from the various treatment combinations were analyzed using SAS (Version 9.4). A general linear model (GLM) was used for the ANOVA. If the ANOVA F-test was significant, ($P < 0.05$), then treatment means were separated using the Duncan's Multiple Range Test (DMRT).

2.3 Results

2.3.1 Initial screening of 20 B. bassiana strains for endophytic colonization

2.3.1.1 Evaluation of endophytic colonization at 30 days

Following the foliar spray inoculation, all the three tissues (root, stem and leaves) of four Cultivars (C2, C3, C4 and C5) were fully colonized by *B. bassiana* strains (Table 2-1). However, after seed inoculation at 30 days, all the three tissues of Cultivar 3 (C3) were fully colonized by four *B. bassiana* strains. Only one *B. bassiana* strain were endophytic in all three tissues of Cultivar 4 (C4). The rest of the strains were endophytic either in one or two tissues (Table 2-3).

2.3.1.2 Evaluation of endophytic colonization at 60 days

Following the foliar spray inoculation, none of the *B. bassiana* strains was able to fully colonize the tissues of all five cultivars (Table 2-1). However, at the same date after seed inoculation, Cultivar 3 tissues was colonized fully by three *B. bassiana* strains (Table 2-3).

The number of *B. bassiana* strains that colonized the plant tissues at 30 and 60 days after either seed or foliar spray inoculation varied considerably (Table 2-2 and 2-4). The degree of colonization of the stems of three cultivars (C2, C3 and C5) by *B. bassiana* strains and their presence at both 30 and 60 days was consistent using both inoculation methods (Table 2-2 and 2-4).

Table 2-1: Numbers of *Beauveria bassiana* strains that were endophytic in various sorghum cultivars sections after foliar spray inoculation

Cultivars	30 Days After Inoculation				60 Days After Inoculation			
	All three tissues	Two tissues	One tissue	None	All three tissues	Two tissues	One tissue	None
C1	0	6	6	8	0	0	2	18
C2	5	8	3	4	0	0	5	15
C3	2	6	8	2	0	0	2	18
C4	1	5	9	5	0	0	6	14
C5	2	9	3	8	0	0	4	16

Table 2-2: Number of *B. bassiana* strains that were endophytic in specific sorghum tissues after foliar spray inoculation

Cultivars	30 Days After Inoculation				60 Days After Inoculation			
	Stems	Roots	Leaves	None	Stems	Roots	Leaves	None
C1	6	8	4	8	1	0	1	18
C2	1	11	10	4	3	1	1	15
C3	8	11	9	2	0	1	1	18
C4	8	10	4	5	1	4	1	14
C5	1	8	6	8	1	1	2	16

Table 2-3: Number of *B. bassiana* strains that were endophytic in specific sorghum tissues after seed inoculation

Cultivars	30 Days After Inoculation				60 Days After Inoculation			
	All three sections	Two sections	One section	None	All three sections	Two sections	One section	None
C1	0	4	12	4	0	0	2	18
C2	0	2	9	9	0	5	5	10
C3	4	3	8	5	3	2	4	11
C4	1	5	11	3	0	0	4	16
C5	0	8	8	4	0	2	5	13

Table 2-4: Number of *B. bassiana* strains that were endophytic in each tissues after seed inoculation

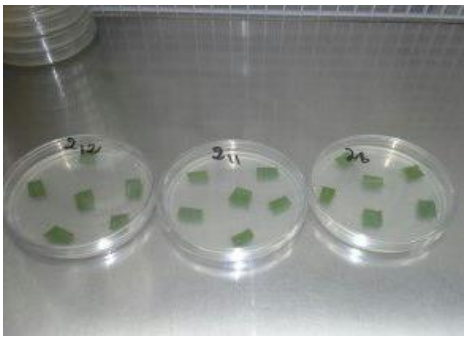
Cultivars	30 Days After Inoculation				60 Days After Inoculation			
	Stems	Roots	Leaves	None	Stems	Roots	Leaves	None
C1	4	14	2	3	0	0	2	18
C2	8	5	0	9	7	4	4	10
C3	10	4	11	5	9	4	3	11
C4	14	4	6	3	0	0	4	16
C5	8	5	11	4	5	0	4	13

Note: C1= Sugargraze, C2 = AS 18, C3 = AS 79, C4 = AS 71 and C5 = PAN 8816

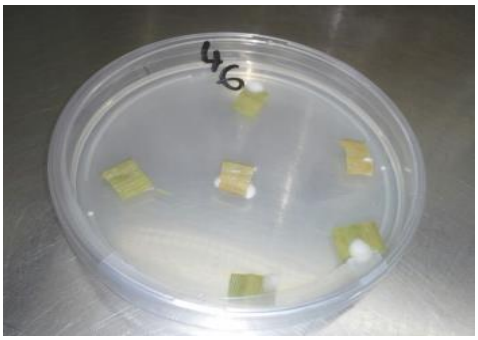
2.3.2 *In vivo* screening to confirm the five best *B. bassiana* strains interacting with three sorghum cultivars

Significant differences were observed between the strains, the inoculation methods, the sorghum cultivar, the plants tissues (roots, stem and leaves) and the time of inoculation with to *B. bassiana* strains (Table 2-5 and 2-6). All the sorghum plant parts were colonized by the *B. bassiana* strains (Figure 2-1), using both inoculation methods. However, the colonization of sorghum tissues was specific to the cultivar, *B. bassiana* strains and inoculation methods used. The roots and stem were more colonized using seed inoculation, while the leaves and the stem were more colonized using foliar spray inoculation. Colonization by *B. bassiana* strains of the sorghum tissues changed with the time and was dependent on the inoculation methods and *B. bassiana* strains. Some *B. bassiana* strains remained endophytic in the stem at both 30 and 60 days after inoculation. No endophytic colonization by *B. bassiana* was observed in the untreated sorghum plants parts (control plants).

Colonization of sorghum stem after the seed inoculation method revealed highly significant differences ($P = 0.0001$) between the *B. bassiana* strains at both 30 and 60 days (Table 2-5). The sorghum root colonization by *B. bassiana* strains was highly significant ($P = 0.0001$), and was dependent on the sorghum cultivars and days after inoculation (Table 2-5).



1. Plates of sorghum leaf samples



2. B. bassiana emerging from stem tissues after 7 days

3. Mature *B. bassiana* colonies grown from root tissues

4. Re-isolation of *B. bassiana* from sorghum plant tissues

Figure 2-1 Sorghum cultivar tissues (leaves, stems and roots), showing *B. bassiana* colonization after inoculation

(T = stem, F = Leaves, R = Roots)

Table 2-5: I*n vivo* seed treatment evaluation for endophytic behaviour of *B. bassiana* strains inside sorghum plants

***Beauveria bassiana* strains**	**Sorghum Cultivars**	**Time (Days)**	**No. of root sections colonised**	**No. of stem sections colonised**	**No. of leaves sections colonised**
Bb3	Pan 8886	30	4 c	5.33 abc	4 b
Bb3	AS 18	30	2 d	2.66 def	2 def
Bb3	AS 79	30	5.33 b	6a	6a
Bb4	Pan 8886	30	4 c	4 bcde	4 b
Bb4	AS 18	30	2 d	1.33 fgh	1.33 ghi
Bb4	AS 79	30	4 c	1.33 fgh	2.66 cde
Bb10	Pan 8886	30	5.66 ab	5.66 ab	5.66 a
Bb10	AS 18	30	6.0 a	6.0 a	6.0 a
Bb10	AS 79	30	2 d	1.33 fgh	0.33 hi
Bb21	Pan 8886	30	4 c	3.66 cde	3 bcd
Bb21	AS 18	30	2 d	1.66 fgh	1.66 efg
Bb21	AS 79	30	4 c	4 bcde	4 b
Bb35	Pan 8886	30	4 c	4 bcde	4 b
Bb35	AS 18	30	4 c	4 bcde	4 b
Bb35	AS 79	30	4 c	4 bcde	4 b
Bb3	Pan 8886	60	2.33 d	1.33 fgh	1 fghi
Bb3	AS 18	60	0.33 e	0.66 gh	0 i
Bb3	AS 79	60	6.0 a	6.0 a	6.0 a
Bb4	Pan 8886	60	4 c	4 bcde	4 b
Bb4	AS 18	60	1.66 d	1.33 fgh	0.66 ghi
Bb4	AS 79	60	0 e	0 h	0 i
Bb10	Pan 8886	60	4 c	3.66 cde	3.66 bc
Bb10	AS 18	60	4 c	4.33 abcd	4 b
Bb10	AS 79	60	0 e	0 h	0 i
Bb21	Pan 8886	60	2.33 d	2.33 efg	2 def
Bb21	AS 18	60	2 d	1.33 fgh	1.33 fgh
Bb21	AS 79	60	4 c	4 bcde	4 b
Bb35	Pan 8886	60	2 d	4 bcde	2 def
Bb35	AS 18	60	2 d	4 bcde	2 def
Bb35	AS 79	60	2 d	4 bcde	2 def
Control 1	Pan 8886	60	0 e	0 h	0 i
Control 2	AS 18	60	0 e	0 h	0 i
Control 3	AS 79	60	0 e	0 h	0 i

Effects	**F values**	**P values**	**F values**	**P values**	**F values**	**P values**
Strains	19.05	0.0001***	12.61	0.0001***	12.25	0.0001***
Cultivars	60.07	0.0001***	9.93	0.0002***	23.69	0.0001***
Time	310.04	0.0001***	21.78	0.0001***	96.13	0.0001***
Strain x cultivar	127.47	0.0001***	23.45	0.0001***	65.7	0.0001***
Strain x time	13.21	0.0001***	3.66	0.0098 **	4.47	0.0031 **
Cultivar x time	1.08	0.3455 ns	1.93	0.1546 ns	1.74	0.1836 ns
Strain x cultivar x time	21.39	0.0001***	1.68 ns	0.1223 ns	5.24	0.0001 ns
% CV	11.69		29.64		22.09	

Means with the same letter are not significantly different (P = 0.05) according to DMRT

*** = Highly Significant, * = Significant, ns = Not Significant, Bb = *Beauveria bassiana* strain.

After the foliar spray inoculation, the colonization of the stems was highly dependent (P = 0.0001) upon the *B. bassiana* strains, both at 30 and 60 days (Table 2-6). The leaf colonization by *B. bassiana* strains was not time dependent. A significant interactions was observed (P = 0.0001) between the sorghum cultivars and *B. bassiana* strains (Table 2-6).

The colonization of the root and leaf tissues by *B. bassiana* strains was dependent on the *B. bassiana* strains, sorghum cultivars and the time of inoculation when foliar spray inoculation was used (Table 2-6).

Table 2-6: I*n vivo* foliar spray evaluation for endophytic behaviour of *B. bassiana* Strains inside sorghum plants

***Beauveria bassiana* strains**	**Sorghum Cultivars**	**Time (Days)**	**No. of root sections colonised**	**No. of stem sections colonised**	**No. of leaves sections colonised**
Bb3	Pan 8886	30	6 a	6 a	6 a
Bb3	AS 18	30	3.33 c	3.33 d	4 b
Bb3	AS 79	30	2 d	6 a	6 a
Bb4	Pan 8886	30	6 a	6 a	6 a
Bb4	AS 18	30	2 d	6 a	6 a
Bb4	AS 79	30	0 e	0 e	2 c
Bb10	Pan 8886	30	6 a	5.66 a	6 a
Bb10	AS 18	30	2 d	5 b	6 a
Bb10	AS 79	30	0 e	4 c	4 b
Bb21	Pan 8886	30	0 a	6 a	6 a
Bb21	AS 18	30	0 e	0 e	4 b
Bb21	AS 79	30	0 e	0 e	4 b
Bb35	Pan 8886	30	6 a	6 a	6 a
Bb35	AS 18	30	2 d	6 a	6 a
Bb35	AS 79	30	2 d	6 a	4 b
Bb3	Pan 8886	60	6 a	6 a	6 a
Bb3	AS 18	60	0 e	6 a	6 a
Bb3	AS 79	60	6 a	6 a	6 a
Bb4	Pan 8886	60	0 e	6 a	6 a
Bb4	AS 18	60	0 e	0 e	2 c
Bb4	AS 79	60	6 a	6 a	6 a
Bb10	Pan 8886	60	2 d	6 a	6 a
Bb10	AS 18	60	0 e	4 c	4 b
Bb10	AS 79	60	6.a	6 a	6 a
Bb21	Pan 8886	60	0 e	0 e	4 b
Bb21	AS 18	60	0 e	0 e	4 b
Bb21	AS 79	60	6 a	6 a	6 a
Bb35	Pan 8886	60	2 d	6 a	6 a
Bb35	AS 18	60	2 d	6 a	4 b
Bb35	AS 79	60	0 e	0 e	0 d
Control 1	Pan 8886	60	0e	0 e	0 d
Control 2	AS 18	60	0 e	0 e	0 d
Control 3	AS 79	60	6 a	6 a	6 a

Effects	**F values**	**P values**	**F values**	**P values**	**F values**	**P values**
Strains	175	0.0001***	780.83	0.0001***	0.61	0.04532*
Cultivars	5474.31	0.0001***	1055.55	0.0001***	3.34	0.0387*
Time	25	0.0001***	7.2	0.0094**	3.46	0.0654 ns
Strain x cultivar	91.75	0.0001***	458.95	0.0001***	316	0.0027**
Strain x time	10	0.0001***	3.2	0.0189**	0.1	0.9823 ns
Cultivar x time	7	0.0019**	4.2	0.0196**	0.03	0.0087**
Strain x cultivar x time	25.75	0.0001***	1.7	0.1169 ns	0.21	0.0098**
% CV	7.58		5.27		64.53	

Means with the same letter are not significantly different (P = 0.05) according to DMRT.

*** = Highly Significant, * = Significant, ns = Not Significant, Bb = *Beauveria bassiana* strain

2.4 Discussion

Beauveria bassiana occurs naturally in soil and plant residues (Akello *et al*., 2007). *B. bassiana* has been isolated as a fungal endophyte from many plant tissues under natural conditions, as well as in plants inoculated using various methods (Wagner and Lewis, 2000; Posada *et al*., 2007; Ownley *et al*., 2008; Vega, 2008; Vega *et al*., 2008). The sorghum tissues colonized by *B. bassiana* strains were dependent upon cultivar, time (30 and 60 days) and application method (seed treatment and foliar spray). Of the 20 *B. bassiana* strains originally inoculated onto sorghum cultivars, five performed significantly better *in vivo* as endophytes on the sorghum cultivars, using either seed treatment or foliar spraying. These results are similar to those of Vega *et al*. (2008). Ownley *et al*. (2008) found that inoculated *B. bassiana* strains could be recovered from roots, stem and leaves sections of surface-sterilized sorghum plants with standard plating procedures on selective medium, as in our study. This gave a good indication of endophytic establishment of the five best *B. bassiana* strains. Our study also demonstrated that *B. bassiana* could be induced to form an artificial endophytic relationship in the sorghum cultivars through various inoculation method, as described in other studies (Bing and Lewis, 1991; Wagner and Lewis, 2000; Ownley *et al*., 2004). Five strains of *B. bassiana* from the 20 used in the study were endophytic in the sorghum cultivars when the seed or the seedlings were inoculated through seed treatment or a foliar spray. The recovery of *B. bassiana* strains from stems and leaves, in addition to the roots, also shows that *B. bassiana* can translocate throughout the plant tissues. The *B. bassiana* strains colonization duration (30-60 days) in some sorghum cultivars tissues indicated that they could be useful strains of endophytic *B. bassiana*, a result similar to the results obtained by Bing and Lewis (1991). The lack of any visual symptoms on the seedlings also showed that *B. bassiana* could colonize sorghum cultivars without negatively affecting its growth. No differences between the conidial morphology of the five *B. bassiana* strains where observed. This is similar to the result obtained by Thakur *et al*. (2005). The colonization of stems by some *B. bassiana* strains introduced the possibility that these strains are potential biocontrol agents for the control of sorghum stem borers, applied as a seed treatment or a foliar spray. As reported in various studies, plants such as wheat, opium poppy (*Papaver somniferum* L.), banana (*Musa spp*. L) and maize that were colonized by *B. bassiana* supported a significantly reduced reproduction of *Aphis gossypii* Glover (Hemiptera: Aphididae) and *Cosmopolites sordidus* Germa (Coleoptera: Curculionidae) (Akello *et al*., 2008), as well as causing a weight reduction in *Chortoicetes terminifera* Walker (Orthoptera: Acrididae) (Gurulingappa *et al*., 2010). The endophytic behaviour of *B. bassiana* strains has also been used as for the

biological control of *Iraella luteipes* Thompson (Hymenoptera: Cynipidae) (Quesada-Moraga *et al*., 2009), *C. sordidus* Germar (Akello *et al*., 2008), Ostrinia *nubilalis* Hubner (Lepidoptera: Crambidae) in maize (Bing and Lewis, 1991), and *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) (Cherry *et al*., 2004). Most of these studies have attributed the reduction in damage by insect pests to the accumulation of the entomopathogenic toxins in plant tissues by *B. bassiana*.

In this study, we were able to show that five of the *B. bassiana* strains out of the 20 strains could be tested as active endophytes with potential for the biological control of the sorghum borer, *C. partellus*.

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Chapter 3 Potential establishment of *Beauveria bassiana* strains as endophytes in rice cultivars

Abstract

Possible *in vivo* colonization of rice cultivars parts (leaves, stem and roots) by *Beauveria bassiana* strains as endophytes was investigated. The present study was conducted at the University of KwaZulu-Natal, Pietermaritzburg, South Africa. Five promising *B. bassiana* strains were evaluated in rice cultivars plants parts for endophytic colonization, as well as the effect of three inoculation methods. Strains of *B. bassiana* have been found as endophytes colonizing sorghum [*Sorghum bicolor* (L.)], maize [*Zea mays* (L)]. The plant tissues infected is often dependent upon the inoculation method (seed treatment, drench treatment and foliar spray). The levels of colonization of the rice tissues by five *B. bassiana* strains were assessed at 30 and 60 days after seed inoculation and foliar spray. Plants of all three cultivars were colonized by *B. bassiana* strains using both inoculation methods, but the colonization of the tissues was affected by the interactions of cultivars x *B. bassiana* strains x inoculation methods. The colonization of the cultivars plant tissues (root, stem and leaf) varied over time (30-60 days), and was affected by the inoculation methods. For drenching and foliar spray treatments, highly significant differences were observed in the roots and the leaves over the time (30 and 60 days) [*B. bassiana* strains x cultivar, *B. bassiana* strains x time, cultivars x time and *B. bassiana* strains x cultivars x time $P = 0.0001$]. However, with seed treatment, there was no difference in levels of colonization in stems by the *B. bassiana* strains x time ($P = 0.3213$).

Depending on the *B. bassiana* strains and the inoculation methods, *B. bassiana* can form an endophytic relationship with rice plants. Therefore, these strains have the potential to control *Sesamia calamistis,* one of the major rice pests.

Keywords: *Beauveria bassiana* strains, endophyte, inoculation methods, rice cultivars; *Sesamia calamistis*

3.1 Introduction

Rice (*Oryza sp* L.) is one of the world's most important crops, providing food for more than half of the world's population (Khush, 2005; Van Nguyen and Ferrero, 2006; Chapagain and Hoekstra, 2011). Rice and wheat (*Triticum sp* L.) together contribute about 21% of the total energy consumed by humans (Kikuchi *et al*., 2015). In West Africa it has become the main source of calories for low-income householders (AfricaRice Center, 2011). Rice cultivation is associated with Asia, but it is a commodity of strategic significance, and is the fastest-growing food source in Africa, where it has been cultivated for centuries (Rice, 2006). Two *Oryza* species are cultivated globally, Asian rice (*Oryza sativa* L.) and African rice (*Oryza glaberrima* S.), whose cultivation is limited to tropical West Africa (Bardenas and Chang, 1965). Rice is now grown and consumed in more than 40 countries in the African continent (Rice, 2006). Growth in the consumption of rice is increasing rapidly in Africa, making rice the second largest source of carbohydrates in Sub-Saharan Africa (Kikuchi *et al*., 2015). Imports of rice account for nearly 40 % of the total rice consumption of the region (Somado *et al*., 2008; Seck *et al*., 2010).

Biotic factors such as pests, diseases and weeds reduce yields of rice worldwide. According to FAO estimates, diseases, insects and weeds cause as much as 25% yield losses annually in cereal crops (Khush, 2005). The most serious pests of rice plant worldwide are rice stem borers, which belonged to three families (Noctuidae, Pyralidae and Diopsidae). These attack plants from the seedling stage to mature plants. Indigenous predators, parasites and entomopathogens are the most commonly used biological control agents in tropical Asia and Africa to control these stem borers (Pathak and Khan, 1994). Some strains of the entomopathogen *Beauveria bassiana* are endophytic in a range of crops. It is has been introduced into a several plant species [maize (*Zea mays* L), banana (*Musa sp*), tomatoes (*Solanum lycopersicum* L.*)*, sorghum (*Sorghum bicolor* L), coffee *(Coffea arabica* L.*)*, wheat and pumpkins (*Cucurbita sp* Duchesne) to control various insects (Bing and Lewis, 1992; Wagner and Lewis, 2000; Akello *et al*., 2007b; Posada *et al*., 2007). The fungus is commercially available as a mycoinsecticide in many countries globally, including South Africa. Various inoculation method have been used to establish *B. bassiana* as endophyte in cereals crops including seed treatments, soil drenches, foliar and flower sprays and stem injections. The logic of this study was that endophytic strains of *B. bassiana* in rice might

provide protection against *Sesamia calamistis* (Hampson), one of the rice stem borers prevalent in West Africa.

3.2 Materials and methods

3.2.1 Production of a conidial suspensions of five *Beauveria bassiana* strains

Five *B. bassiana* strains (Bb3, Bb4, Bb10, Bb21 and Bb35) were used for this study. These had been isolated from various soils, including soil collected from the rhizosphere of mangoes (*Mangifera indica* L.), rooibos tea (*Aspalathus linearis* Burm. f.) and wheat. They were characterized by the Plant Protection Research Institute (PPRI), Pretoria, South Africa. In prior research, they were selected for their endophytic abilities on sorghum plant parts (leave, stem and root). Conidial suspensions used for the study were prepared following the method of Parsa *et al*. (2013). The conidia were obtained from fungal cultures of the five *B. bassiana* strains. The *B. bassiana* strains were cultured in 90 mm diameter plastic Petri dishes containing potato dextrose agar (PDA) plus antibiotics (100 mg L^{-1} of ampicillin and streptomycin), and incubated at 28°C. The cultures were allowed to grow for 14-18 days, after which the conidia were harvested. The conidia were harvested under sterile conditions by gently scraping the fungal growth from the surface of the agar with a sterile spatula, and rinsing with sterile distilled water. The suspensions were filtering using sterile cheesecloth to remove mycelium and agar debris. Conidial density was determined using an improved Neubauer haemocytometer, and adjusted to 2 x 10^6 conidia ml^{-1} with sterile distilled water containing Tween-80 (1 ml^{-1}). The viability of the conidia for all the experiments was evaluated by taking a 100 µL sample of each strain, spreading it on PDA and incubating them at 25°C, and assessing the germination 24 hours later. The percentage germination of conidia was determined from 100 randomly selected conidia under a light microscope. The germination of conidia was assumed when the hyphae were visible or the germ tube was about twice the length of the conidium. For each strain, the mean of three replicates was used to assess the viability of the conidia of each strain. The inoculum was used for seed treatment and foliar spray experiments.

3.2.1 Production of rice plants for greenhouse studies

Three rice cultivars (NERICA1, NERICA8 and NERICA-L19) (Somado *et al.* 2008) were used as the host plants because there may be differential interactions between host plants and endophytic strains of fungi. Seeds of each cultivar were separately surface sterilized in 3%

sodium hypochlorite for 3 minutes followed by 70% ethanol for 2 minutes. They were rinsed three times with distilled water, air dried and divided into two sets. The first set was used for seed inoculation and the second for foliar spray experiments. The second set of seeds used for foliar spray experiment were sown in Speedling® 24 trays filled with Composted Pine Bask (CPB) growing medium. The seedlings were watered with tap water and placed under greenhouse conditions at 20-28°C night/day. Two weeks after germination, the seedlings were transplanted into 30 cm diameter pots filled with composted pine bark (CPB) growing medium and placed under greenhouse conditions at 20-28°C night/day. The plants were allowed to grow for seven days before being used in the foliar spray experiment. Plants were irrigated three times a day with irrigation water containing NPK fertilizer [3: 1: 3 (38)] Complete, plus calcium nitrate and trace elements (TE).

3.2.3 Inoculation of *B. bassiana* strains for endophytic colonization in rice cultivars

3.2.3.1 Seed Treatment

The seeds of the three rice cultivars were surface sterilized (as described in Section 3.2.2). After surface sterilization, the seeds of each cultivar were separately dipped into the conidial suspension of each *B. bassiana* strains [5 ml of the prepared inoculum (2×10^6conidia ml^{-1})], then air-dried. The seeds were then planted in Speedling® 24 trays filled with CPB growing medium. The control plants consisted of uninoculated seeds treated in a similar manner using distilled water. After two weeks, the emerging seedlings were transplanted into 30 cm diameter pots filled with CPB growing medium and placed under greenhouse conditions at 20-28°C night/day. Three plants per pot were arranged in the greenhouse in a randomized complete block design with three replicates. Plants were irrigated three times a day with irrigation water containing NPK fertilizer [3: 1: 3 (38)] Complete, plus calcium nitrate and TE. The plants were grown for 30 and 60 days before they were harvested, and the roots, stems and leaves were evaluated for evidence of endophytic colonization.

3.2.3.2 Foliar Spray

Plants of the three rice cultivars (Section 3.2.2) were sprayed 15 days after transplanting into pots. A hand spray was used to inoculate the rice plants leaves with the inocula of the *B. bassiana* strains. 50 ml of the inoculum of each *B. bassiana* strains was used per plant. Before the leaves were sprayed, the base of each pot was covered with aluminium foil, with holes to allow the plants out. This was to stop inoculum running off the leaves onto the roots,

and therefore to create a root drenching situation. Plastic bags were used to cover the entire plants for 24 hours to increase humidity. For the control plants, sterile distilled water was applied in a similar manner as described for the *B. bassiana* treatments. The treated plants (three plants per pot) were then placed in a greenhouse (20-28°C night/day) using a randomized complete block design with three replicates. Plants were irrigated as described in Section 3.2.3.1. The roots, stems and leaves of each plant were harvested after 30 days and 60 days, for evaluation for endophytic colonization.

3.2.4 Evaluation of endophytic colonization of the five *B. bassiana* strains

The colonization of rice plants parts by *B. bassiana* was determined 30 and 60 days after inoculation with each *B. bassiana* strain. From each rice cultivar x *B. bassiana* treatment combination, plants were carefully removed from their pots and dissected into leaves, stems and roots. The roots were gently washed with tap water to remove residues of CPB. The plant parts were surface-sterilized by immersing them in 0.3% sodium hypochlorite for 2 minutes, followed by 70% ethanol for 2 minutes. They were rinsed three times with sterile distilled water. The surface sterilized samples were placed on sterile tissue paper under a laminar flow cabinet for air-drying. After drying, six pieces of each of the samples (leaves, stem and roots) from each treated plant were randomly taken and plated separately onto a *B. bassiana* selective medium (Appendix 2) and incubated for 15 days at 25°C. To confirm that the surface sterilization was effective, 10 ml of the sterile distilled water used to rinse the samples during the surface sterilization procedure was spread onto Petri dishes containing the *B. bassiana* selective media. The plates were incubated for 10-15 days at 25°C to count the colony forming units. However, the sterilization resulted in clean plates. Therefore, any *B. bassiana* mycelium emerging from surface-sterilized plant tissues was assumed to have originated from within the plant tissues as an endophyte. The plates that contained plant samples (leave, stem and roots) were monitored every 2-3 days for the emergence of fungal mycelia. After 10 or 15 days, the presence or absence of *B. bassiana* colonies were recorded. The fungal colonies grown from the samples were confirmed to be *B. bassiana* based on morphological characteristics.

3.2.5 Data analysis

The colonization of plant tissues resulting from various treatment combinations were analysed using SAS (Version 9.4). A general linear model (GLM) was used for the ANOVA.

If the ANOVA F-test was significant, ($P < 0.05$), then treatment means were separated using the Duncan Multiple Range Test (DMRT).

3.3 Results

The rice plants were colonized by *B. bassiana* strains using both inoculation methods, but the colonization of each rice plants parts were cultivar, strain and inoculation method dependent. The colonization of the rice tissues (root, stem and leaf) varied also with time (30-60 days) and the inoculation methods used (Tables 3-1 and 3-2).

Following seed treatment, highly significant differences were observed in the colonization of plant tissues (roots, stem and leaves) x *B. bassiana* strains ($P = 0.0001$) and x rice cultivar ($P = 0.0001$). Highly significant differences were observed in the roots and the leaves, both at 30 and 60 days for all interactions: strains x cultivars, strains x time, cultivars x time and strains x cultivars x time ($P = 0.0001$). There was no interaction between strain x time ($P = 0.3213$).

After foliar sprays of inoculum, (Table 3-2), highly significant differences were observed in the levels of colonization of the roots between *strains*, rice cultivars, strains x cultivars, strains x time, cultivars x time and strains x cultivars x time ($P = 0.0001$). Highly significant differences was observed in the leaves between strains, cultivars, time (30-60 days), strains x cultivars, strains x time, cultivars x time and strains x cultivars x time ($P = 0.0001$). In the stem, significant differences were observed between strains ($P = 0.0054$), time ($P = 0.0235$) and cultivars x time ($P = 0.0018$). The interactions of strains x cultivars and *strains* x cultivars x time were highly significant ($P = 0.0001$ or 0.0002). No colonization by *B. bassiana* strains were observed in the tissues of the Control plants, as a result of both inoculation methods (Table 3-1 and 3-2)].

Table 3-1: Evaluation of endophytic behaviour of *B. bassiana* strains in rice plants following seed inoculation

Beauveria bassiana strain	Rice Cultivar	Time (Days)	No. of root sections colonized	No. of stem sections colonized	No. of leaf sections colonized
Bb3	NERICA1	30	6.0 a	6.0 a	4 b
Bb3	NERICA8	30	6.0 a	6.0 a	2 c
Bb3	NERICA.L.19	30	3.33 c	2 c	0 e
Bb4	NERICA1	30	4 b	4 b	2 c
Bb4	NERICA8	30	6.0 a	4 b	4 b
Bb4	NERICA.L.19	30	4 b	4 b	2 c
Bb10	NERICA1	30	6.0 a	6.0 a	2 c
Bb10	NERICA8	30	6.0 a	6.0 a	6.0 a
Bb10	NERICA.L.19	30	3 d	2 c	0.66 d
Bb21	NERICA1	30	6.0 a	6.0 a	0 e
Bb21	NERICA8	30	4 b	4 b	4 b
Bb21	NERICA.L.19	30	0 g	0 d	0 e
Bb35	NERICA1	30	6.0 a	6.0 a	6.0 a
Bb35	NERICA8	30	6.0 a	6.0 a	2 c
Bb35	NERICA.L.19	30	0 g	0 d	0 e
Bb3	NERICA1	60	6.0 a	6.0 a	2 c
Bb3	NERICA8	60	6.0 a	6.0 a	2 c
Bb3	NERICA.L.19	60	2 e	0 d	0 e
Bb4	NERICA1	60	6.0 a	6.0 a	2 c
Bb4	NERICA8	60	6.0 a	6.0 a	6.0 a
Bb4	NERICA.L.19	60	4 b	2 c	0 e
Bb10	NERICA1	60	2 e	2 c	0 e
Bb10	NERICA8	60	6.0 a	6.0 a	0 e
Bb10	NERICA.L.19	60	6.0 a	6.0 a	2 c
Bb21	NERICA1	60	6.0 a	6.0 a	2 c
Bb21	NERICA8	60	2 e	0 d	0 e
Bb21	NERICA.L.19	60	6.0 a	2 c	4 b
Bb35	NERICA1	60	6.0 a	6.0 a	2 c
Bb35	NERICA8	60	1 f	0 d	0 e
Bb35	NERICA.L.19	60	2 e	2 c	0 e
Control 1	NERICA1	60	0 g	0 d	0 e
Control 2	NERICA8	60	0 g	0 d	0 e
Control 3	NERICA.L.19	60	0 g	0 d	0 e

Effects	**F values**	**P values**	**F values**	**P values**	**F values**	**P values**
Strains	176.91	0.0001***	3.87	0.0054**	310.23	0.0001***
Cultivars	1050.54	0.0001***	9.95	0.0001**	2225.67	0.0001***
Time	1	0.3213 ns	5.26	0.0235*	1444.96	0.0001***
Strain x cultivar	245.02	0.0001***	9.55	0.0001***	1129.84	0.0001***
Strain x time	89.16	0.0001***	1.18	0.3213 ns	634.5	0.0001***
Cultivar x time	493.85	0.0001***	6.68	0.0018**	1264.83	0.0001***
Strain x cultivar x time	252.87	0.0001***	4.18	0.0002**	1039.79	0.0001***
% CV	4.74		46.41			

Means with the same letter are not significantly different (P = 0.05) according to DMRT.

*** = Highly Significant, * = Significant, ns = Not Significant, Bb = *Beauveria bassiana* strain.

Table 3-2: Evaluation of endophytic behaviour of *B. bassiana* strains in rice plants using foliar sprays inoculation

Beauveria bassiana strain	Rice Cultivar	Time (Days)	No. of root sections colonized	No. of stem sections colonized	No. of leaf sections colonized
Bb3	NERICA1	30	0 g	6.0 a	6.0 a
Bb3	NERICA8	30	6.0 a	6.0 a	6.0 a
Bb3	NERICA.L.19	30	2 f	2 c	6.0 a
Bb4	NERICA1	30	0 g	0 d	2 c
Bb4	NERICA8	30	0 g	4 b	4 b
Bb4	NERICA.L.19	30	4 dc	4 b	6.0 a
Bb10	NERICA1	30	4 dc	4 b	4 b
Bb10	NERICA8	30	4 dc	6.0 a	6.0 a
Bb10	NERICA.L.19	30	0 g	4 b	6.0 a
Bb21	NERICA1	30	4 dc	4 b	4 b
Bb21	NERICA8	30	0 g	0 d	0 e
Bb21	NERICA.L.19	30	0 g	4 b	4 b
Bb35	NERICA1	30	2 f	6.0 a	6.0 a
Bb35	NERICA8	30	0 g	0 d	0 e
Bb35	NERICA.L.19	30	4 dc	4 b	4 b
Bb3	NERICA1	60	0 g	4 b	4 b
Bb3	NERICA8	60	2 f	6.0 a	6.0 a
Bb3	NERICA.L.19	60	2 f	6.0 a	6.0 a
Bb4	NERICA1	60	6.0 a	6.0 a	6.0 a
Bb4	NERICA8	60	5.33 b	6.0 a	6.0 a
Bb4	NERICA.L.19	60	4 dc	4 b	6.0 a
Bb10	NERICA1	60	4 dc	6.0 a	4 b
Bb10	NERICA8	60	3.33 e	6.0 a	6.0 a
Bb10	NERICA.L.19	60	4.33 c	6.0 a	6.0 a
Bb21	NERICA1	60	6.0 a	6.0 a	6.0 a
Bb21	NERICA8	60	0 g	0 d	0 e
Bb21	NERICA.L.19	60	0 g	6.0 a	1.33 d
Bb35	NERICA1	60	6.0 a	6.0 a	6.0 a
Bb35	NERICA8	60	0 g	0 d	0 e
Bb35	NERICA.L.19	60	3.66 d	4 b	2 c
Control 1	NERICA1	60	0 g	0 d	0 e
Control 2	NERICA8	60	0 g	0 d	0 e
Control 3	NERICA.L.19	60	0 g	0 d	0 e

Effects	**F values**	**P values**	**F values**	**P values**	**F values**	**P values**
Strains	209	0.0001***	3.87	0.0054**	3302.81	0.0001***
Cultivars	232.79	0.0001***	9.95	0.0001**	1683.04	0.0001***
Time	600.4	0.3213 ns	5.26	0.0235*	16.01	0.0002**
Strain x cultivar	572.81	0.0001***	9.55	0.0001***	1998.31	0.0001***
Strain x time	336.57	0.0001***	1.18	0.3213 ns	495.88	0.0001***
Cultivar x time	232.83	0.0001***	6.68	0.0018**	555.78	0.0001***
Strain x cultivar x time	205.28	0.0001***	4.18	0.0002**	270.94	0.0001***
% CV	8.28		46.41		2.44	

Means with the same letter are not significantly different (P = 0.05) according to DMRT.

*** = Highly Significant, * = Significant, ns = Not Significant, Bb = *Beauveria bassiana* strain.

3.4 Discussions

Beauveria bassiana has been reported to colonize many plants as an endophyte (Wagner and Lewis, 2000; Vega *et al*., 2008; Quesada-Moraga *et al*., 2009; Tefera and Vidal, 2009), which correlates with the results of our study. Colonization of plants by *B. bassiana* depends on the inoculation method, fungal isolation and plant species. In coffee, the highest colonization rates have occurred after leaf injections, when banana roots were drenched with conidia (Akello *et al*., 2008), when tomato seeds were treated with a conidial spray, and when maize and opium poppy plants were treated with conidia as foliar sprays (Tefera and Vidal, 2009). Some strains of *B. bassiana* were able to invade maize plants via the epidermis, thereafter persisting in the plant though the entire growing season, and reducing tunnelling by *Ostrinia nubilalis* (Lepidoptera: Pyralidae) (Bing and Lewis, 1991). *B. bassiana* can become established as endophyte in rice when seeds or seedlings are inoculated, as revealed in several studies (Akello *et al*., 2007a; Ownley *et al*., 2008; Parsa *et al*., 2013). However, the levels of colonization of the various tissues differs according to the *B. bassiana* strains and the rice cultivars. Both of the inoculation methods used conferred good colonization of the rice stem by some of the *B. bassiana* strains. Our study confirmed that there are several possible pathways to inoculate and recovery *B. bassiana* from plant tissues (Gurulingappa *et al*., 2010). Both inoculation methods (seed treatment and foliar spray) resulted in high levels of leaf and root colonization. The inoculation method did not appear to favour a specific pattern of local colonization of the rice cultivars. This is opposite to the results of Posada *et al*. (2007) study. They found that foliar sprays favoured leaf colonization, whereas soil drenching favoured root colonization in coffee. The same result was found in common bean by Parsa *et al*. (2013).

The systemic spread of each *B. Bassiana* strain differed over the two times (30-60 day). A reduction in levels of colonization over time may have been caused by a host resistance response to the heterotrophic fungal, or because of competition from other endophytes in the rice tissues (Posada *et al*., 2007). The colonization of the rice cultivars by the *B. bassiana* strains did not cause any apparent negative effects on the growth of the rice plants, as was reported by Van Bael *et al*. (2005). This study revealed that rice plants could be colonized by *B. bassiana* strains. The five *B. bassiana* strains tested were all endophytic and could be used as biological control agents against the rice stem borer, *S. calamistis*.

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CHAPTER 4 Susceptibility of *Chilo partellus* (Swinhoe) and *Sesamia calamistis* Hampson third instar larvae to strains of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin

Abstract

Moth species belonging to the families Crambidae, Pyralidae and Noctuidae constitute important cereal pests in most of Africa. Complex control methods are often used to manage them. The use of biological control methods is now receiving attention since the biocontrol agents are non-polluting, ecologically sound and their use should be sustainable. The use of an entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin as a biological control agent of several insect has been reported worldwide. For this study, five endophytic strains of *B. bassiana* were tested for their pathogenicity against third instar larvae of *Chilo partellus* Swinhoe and *Sesamia calamistis* Hampson on sorghum and rice, respectively. The results of the pathogenicity tests revealed that most of the five *B. bassiana* strains were highly pathogenic on both sets of larvae, causing mortalities of more than 50% at 28 days after treatment. However, the virulence of the five *B. bassiana* strains varied. On sorghum, the three strains of *B. bassiana* (Bb3, Bb10 and Bb35) that colonized sorghum plants (Chapter 2) controlled the 3rd instar larvae of *C. partellus*, causing high mortality levels. On rice, the endophytic *B. bassiana* strains (Bb4, Bb10 and Bb35) controlled the 3rd instar larvae of *S. calamistis*, causing high mortality levels. Strains Bb3 and Bb35 were equally effective against 3rd instar larvae of *C. partellus*. Strains Bb 4 and Bb 35 were equally effective against 3rd instar larvae of *S. calamistis*. Strain Bb21 was not able control to both *C. partellus and S. calamistis*.

Keywords: Pathogenicity, *Beauveria bassiana*, entomopathogen, *Chilo partellus*, *Sesamia calamistis*, endophytic strains

4.1 Introduction

Chilo partellus Swinhoe (Lepidoptera: Pyralidae) is considered to be one of the most damaging pests of sorghum [*Sorghum bicolor* (L.)] crops throughout the world (Reddy, 1988), and is one of the most prevalent pests of sorghum in Southern Africa (Hoekstra and Kfir, 1997). It is an exotic stem borer of cereal crops in Africa that invaded from Asia earlier this century (Kfir, 1994; Overholt *et al*., 1997). Losses caused by this insect range between 20 and 100% (Setamou and Schulthess, 1995). *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) is a pest of grain crops including sorghum, rice (*Oryza s*p L.), maize (*Zea mays* L.), pearl millet (*Pennisetum sp* L.), wheat (*Triticum sp*), and sugarcane (*Saccharum officinarum* L.). It is one of the major pest that attacks rice and maize in West Africa. Control of these two borers by commercial farmers has mainly relied on the application of synthetic insecticides (Warui and Kuria, 1983; Van den Berg and Van Rensburg, 1996; Reddy, 1998). However, their control using chemicals is difficult because of a prolonged emergence pattern, multiple generations and their cryptic feeding behaviour (Kfir, 1992). A further issue is that these borers have developed resistance to chemicals. In addition to their high costs and their inefficacy against the borers, chemical insecticides can have adverse effects on non-target organisms. Their presence as residues in food and water can also be dangerous for humans being (Pimental *et al*., 1992). Chemical pesticides may also cause ecological problems, and are usually unaffordable for small-scale farmers (Pimentel *et al*., 1992; Deedat, 1994; Kfir, 1994; Goulson, 2013). The demand for the sustainable production of food has increased, coupled with an increased restriction in the use of chemical control agents. The need for alternative methods for the control of major pests has driven research to develop biological control products. Entomopathogenic fungi are important among biological control agents due to their broad host range, their diverse mechanisms of pathogenicity, and their environmental safeness (Maniania, 1992; Cherry *et al*., 1999; Qazi and Khachatourians, 2005). As an alternative to chemical control, *B. bassiana* may have a role in managing *C. partellus* and *S. calamistis*. Previous studies have demonstrated the potential of *B. bassiana* for the control of *C. partellus* (Odindo *et al*., 1989; Hoekstra and Kfir, 1997; Cherry et *al*., 1999). Nevertheless, empirical evidence for this approach is still scanty. This study was conducted to identify endophytic strains of *B. bassiana* that could be used as biological control agents against *C. partellus* and *S. calamistis*.

4.2 Material and Methods

4.2.1 Biological Agents

For the experiments, five strains of *B. bassiana* were used. These had been previous identified as endophytes in sorghum and rice plants (Chapter 2, Section 2.2.3 and 2.2.6)]. Third larval instars of *C. partellus* and *S. calamistis* were used as the test insects.

4.2.2 Production of conidial suspensions of the five *B. bassiana* strains

To prepare the fungal inoculum, the five strains of *B. bassiana* were cultured on potato dextrose agar (PDA) amended with 100 mg L^{-1} of ampicillin and streptomycin, and incubated at 28°C for 14-18 days. The conidia were then harvested under sterile conditions by gently scraping the fungal growth from the surface of the agar with a sterile spatula, and rinsing with sterile distilled water. The suspensions were filtering using sterile cheesecloth to remove mycelium and agar debris. Conidial density was determined using an improved Neubauer haemocytometer and adjusted to 2 x 10^6 conidia ml^{-1} with sterile distilled water containing Tween-80.

4.2.3 Production of endophytic stems of sorghum and rice plants

Seeds of a sorghum cultivar (PAN 8816) and a rice cultivar (NERICA 1) were each surface sterilized separately in 3% sodium hypochlorite for 2 minutes, followed by 70% ethanol for 2 minutes. They were rinsed three times with distilled water and air-dried. The surface sterilized sorghum and rice seeds were then dipped separately in a conidial suspension of each of the five *B. bassiana* strains [5 ml of the prepared inoculum (2×10^6 conidia ml^{-1})] before air-drying the seeds. Each crop (sorghum and rice) seeds were then planted in Speedling® 24 trays filled with a composted pine bark (CPB) growing medium. After two weeks, the seedlings were transplanted into 30 cm diameter pots filled with CPB growing medium and placed under controlled greenhouse conditions set at 20-28°C night/day. Three plants per pot were arranged in the greenhouse in three replicates per treatment, in an RCB design. Plants were irrigated three times a day with irrigation water containing NPK fertilizer [3: 1: 3 (38) Complete] plus calcium nitrate and trace elements (TE). The plants were allowed to grow for 30 days before one plant per treatment from each crop (sorghum and rice) were harvested and sampled (roots, stems and leaves) to confirm the endophytic colonization of the tissues by *B. bassiana* strains. Each of the sorghum and rice plants tissues were separately surface-sterilized by immersing them in 0.3% sodium hypochlorite for 2 minutes, followed

by 70% ethanol for 2 minutes. They were separately rinsed three times with sterile distilled water and placed on a sterile paper tower in a laminar flow cabinet for drying. After drying, six pieces of each of the samples (leaves, stems and roots) from each plant were randomly taken and plated separately onto *B. bassiana* selective medium (Appendix 2), and incubated for 15 days at 25°C. The plates were monitored every 2-3 days for the emergence of fungal mycelia. After the colonization of the stems by *B. bassiana* strains was confirmed, the remaining plants of each crop (sorghum and rice) was harvested, washed with tap water, and the stems were then used for pathogenicity testing on the two stem borers.

4.2.4 Mass rearing of *Chilo partellus* larvae

Larvae of *Chilo partellus* can be reared using casein, glucose, a salt mixture, yeast, choline chloride, cholesterol, cellulose, a leaf factor, agar agar, water and methyl paraben (Pant et al. 1960). Stem borer species can also be reared on wheat germ, casein, a salt mixture, cellulose, a vitamin mixture, ascorbic acid, aureomycin, methyl paraben, agar, potassium hydroxide (KOH), formaldehyde and water (Chatterji et al. 1968). A successful diet was reported 28 years ago for the rearing of *C. partellus*, based on kabuli gram (chickpea, *Cicer arietinum* L.) (Taneja and Nwanze 1990). The insect cultures of the *C. partellus* larvae used for this study was mass reared in the laboratory of Plant Pathology at the University of KwaZulu-Natal, Pietermaritzburg, South Africa. To start the rearing process, colonies of *C. partellus* eggs were obtained from SASRI (South Africa Sugarcane Research Institute, Mount Edgecombe, Durban, South Africa). The eggs colonies were kept in an incubator at 25°C until they hatched. The artificial diet that was used for the rearing is presented in Table 4-1 below. The distilled water from Fraction A was boiled and allowed to cool at 60-70°C before mixing in the weighted ingredients from Fraction A (except nipagin) for 3 minutes. The agar powder from Fraction B was added to distilled water provided in Fraction B, boiled and cooled at 60-70 °C before being mixed with the pre-prepared Fraction A solution, plus nipargin dissolved in Denol. Fraction C was added to the above suspension and mixed for 3 minutes. Under a laminar airflow, the whole mixture was poured into plastic containers for solidification. After 24 hours, it was used as an artificial diet to feed the first larval instars of *C. partellus*. A small hole was made in the artificial medium, and two 1st instar larvae of *C. partellus* that had already hatched from the eggs colonies were put into the hole. The containers that contained the 1st instar larvae were kept in an incubator at 25°C until they developed into 3rd instar larvae.

Table 4-1: Components of the artificial diet used for the rearing of *Chilo partellus*

	Design	**2 L**	**10 L**
Fraction A	Distilled Water	800 g	4000 mL
	Brewer's yeast	45.0 g	225 g
	Sorbic acid	1.3 g	6.5 g
	Nipargin	2.85 g	14.25 g
	Denol	100 mL	200 mL
	Ascorbic acid	5.0 g	25.0 g
	Vitamin E powder	4.2 g	21.0 g
	Crushed cane leaf powder	50 g	250 g
	Ground chickpea	175 g	875 g
	Sucrose	70 g	350 g
Fraction B	Agar powder	25.0 g	125 g
	Distilled water	800 mL	4000 mL
Fraction C	Formaldehyde	**4.2** L	33.25 L

4.2.5 Artificial rearing of *Sesamia calamistis*

A suitable number of *S. calamistis* pupae collected from a maize field were placed into cages that contained cotton soaked in sugar/honey water that served as food for the moths once they emerged from the pupae. Sheet of transparent paper were wound around wooden rods (40cm) to create a slots for the females to lay their eggs in. These eggs were harvested from the slots by scraping the wooden rods with a sterile spatula. The eggs were placed into plastic containers on a sterile paper towel. The containers were incubated at 26°C with an RH of 60% (+/-10%), and a photoperiod of 12 hours light and 12 hours dark (L:D 12:12). The containers were monitored daily until the larvae hatched, creating the stage called "black heads". These young larvae were transferred into other transparent plastic containers whose lids were perforated but covered with mosquito netting to ensure permanent ventilation, and were incubated at 26°C. Fresh maize stalks were harvested and put into the plastic containers to serve as food for the black head larvae. After 3 days, the maize stalks were dissected and the larvae were extracted and transferred into new containers that contained fresh corn stalks (Figure 4-1), and kept in an incubator at 26°C. This procedure was repeated until larvae of the desired 3rd instar larval stage developed. The number of days for each stage varied from one larval stage to the next. Development from the 2nd instar to the 3rd instar took five days, while development from the 3rd instar to the 4th instar took 8 -10 days.

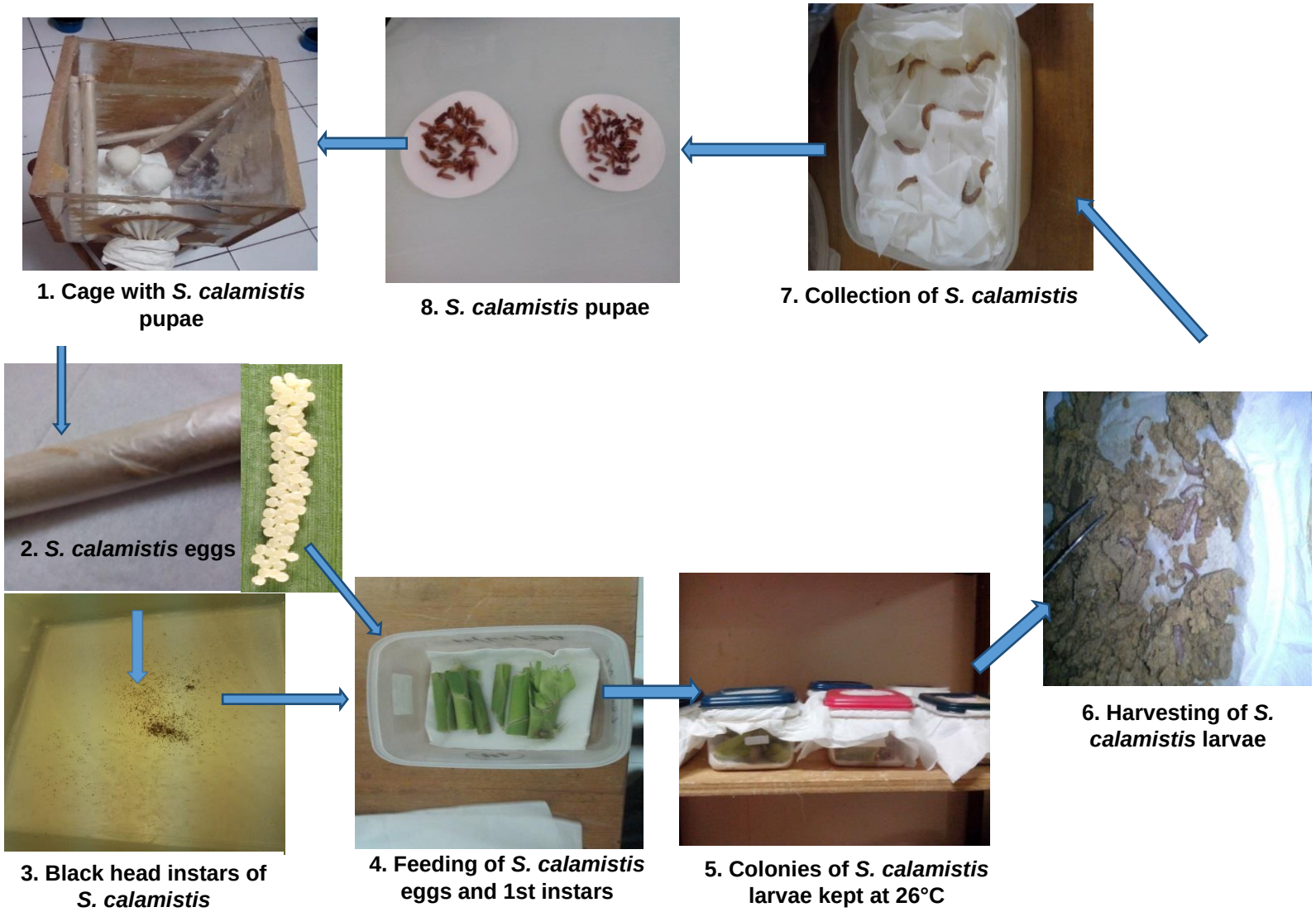


Figure 4-1 Steps in the artificial rearing of *Sesamia calamistis*

4.2.6 Efficacy of five endophytic *B. bassiana* strains against 3rd instar larvae of *Chilo partellus*

Third instar larvae of *C. partellus* and sorghum plants infected with endophytic *B. bassiana* in their stems were obtained as described in Section 4.2.3. Stems from the treated plants were harvested, and the stems were washed using sterile distilled water. Ten 3rd instar larvae of *C. partellus* were put into plastic containers that contained 10g of clean *B. bassiana* infected stems of sorghum, and incubated at 25°C. For a control, larvae were fed with uninoculated sorghum stems. The larvae in each container were monitored and the mortality data were recorded by counting the dead cadavers at 7, 14, 21 and 28 days. The dead larvae were removed and place in a plastic containers that contained Whatman filter paper wetted with sterile distilled water. Two to three days after being collected, the dead larvae were surface-sterilized in 0.3% sodium hypochlorite for 1 minute followed by 70% ethanol for 1 minute. They were rinsed three times with sterile distilled water for 15 seconds. The surface sterilized dead larvae were placed on sterile paper tower under a laminar flow cabinet for air-drying. The dried, dead larvae were plated onto Petri dishes that contained a *B. bassiana* selective media (Appendix 2) (Figure 4-2). The plates were kept in an incubator at 25°C and monitored every 2-3 days. The fungi that appeared on the surface sterilized dead larvae were harvested and sub-cultured onto fresh PDA plates for pure culture and identification. After 15 days, the colonies were evaluated and compared to the original endophytic *B. bassiana* strain that had been inoculated onto the sorghum stems. The experiment was repeated for three times to confirm the pathogenicity of the *B. bassiana* strains.

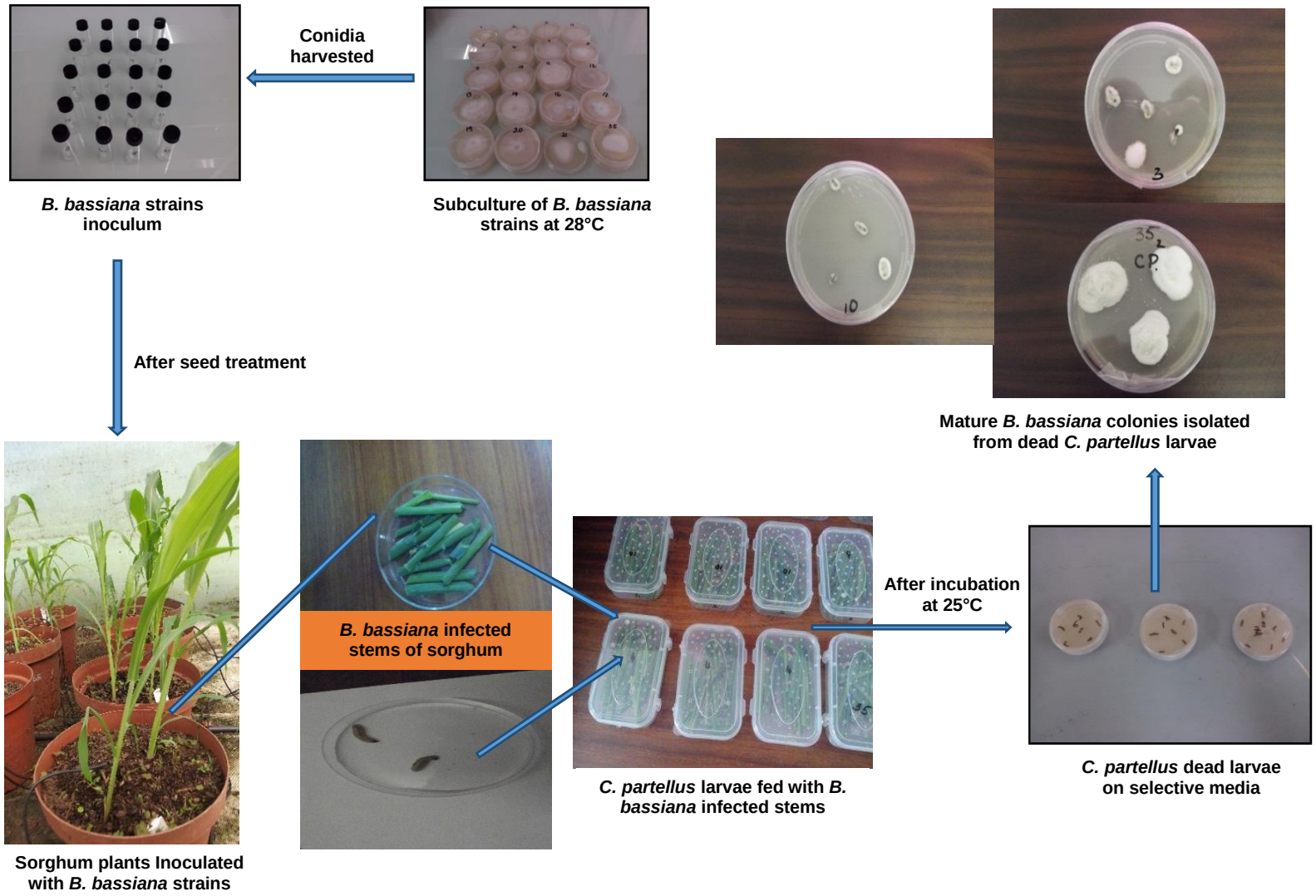


Figure 4-2 *In vivo* screening of endophytic strains of *Beauveria bassiana* for pathogenicity to *Chilo partellus*

4.2.7 Efficacy of *B. bassiana* strains against 3rd instar larvae of *Sesamia calamistis*

Rice seed was inoculated with *B. bassiana* strains, as described in Session 4.2.3. The inoculated seed was sown and one month later, the stems were tested for the endophytic presence of each of the five *B. bassiana* strains. The stems were then were harvested as described in Section 4.2.3, and washed with distilled water, before being fed to the 3rd instar larvae of *S. calamistis*. Ten 3rd instar larvae of *S. calamistis* were placed into plastic containers that contained 10g of *B. bassiana* infected stems of rice. The containers were placed into an incubator at 28°C. Mortality of the larvae was recorded after 7, 14, 21 and 28 days. For the control, larvae were fed with uninoculated rice stems. Dead larvae were collected at 7, 14, 21 and 28 days and were maintained in plastic containers that contained Whatman filter paper previously wetted with sterile distilled water. Two to three days after being collected, the dead larvae were surface-sterilized in 0.3% sodium hypochlorite for 1 minute followed by 70% ethanol for 1 minute. They were then rinsed three times with sterile distilled water for 15 seconds. The surface sterilized dead larvae were placed on sterile paper towels under a laminar flow cabinet for air-drying. The dried dead larvae were plated onto Petri dishes that contained a *B. bassiana* selective media (Appendix 2) (Figure 4-2). The plates were kept in an incubator at 26-28°C and monitored every 2-3 days. Fungi that appeared on the surface sterilized larvae of *S. calamistis* were harvested and sub-cultured onto a fresh PDA plates for pure culture and identification. After 15 days, the colonies were compared to the endophytic *B. bassiana* strains that were initially inoculated into the rice seed. The experiment was repeated three times to confirm the pathogenicity of the *B. bassiana* strains.

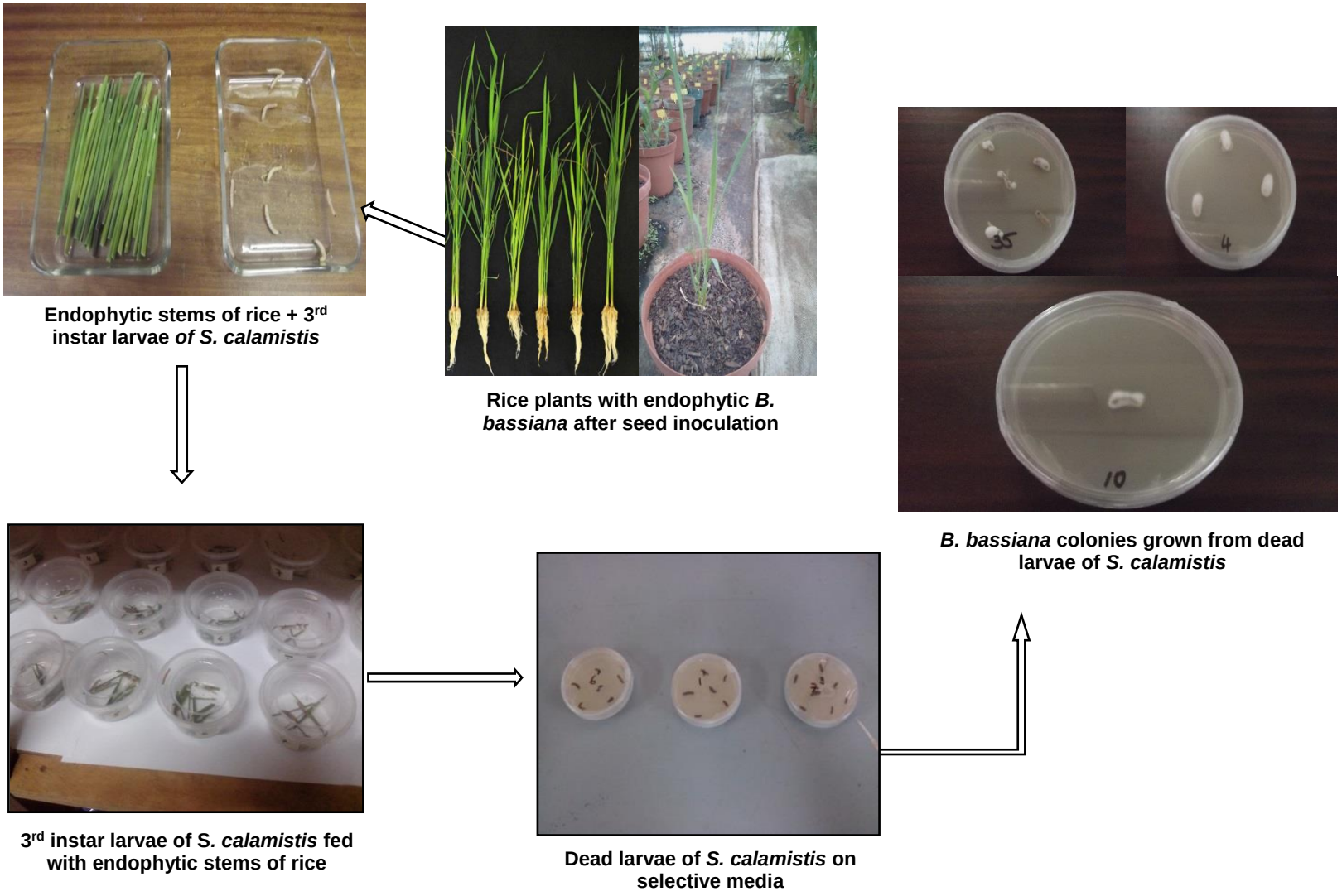


Figure 4-3 *In vivo* screening of endophytic strains of *Beauveria bassiana* for pathogenicity to *Sesamia calamistis*

4.2.8 Data Analysis

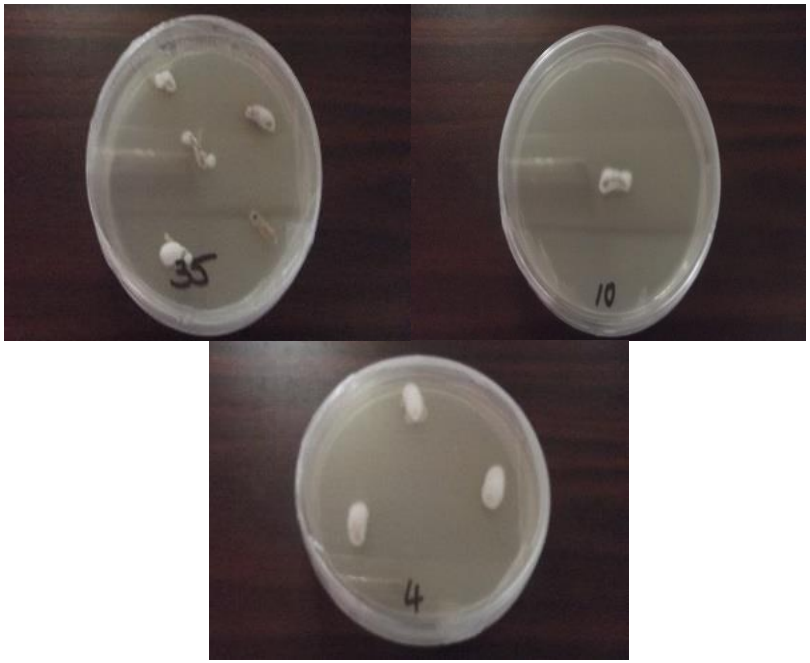
The cumulative percentage of mortality of *C. partellus* and *S. calamistis* was collected, and the Area Under the Mortality Progress Curve (AUMPC) figures were calculated. The data analysis was done using GenStat 18th Edition. A two factorial ANOVA was run where the *B. bassiana* strains and the time (days) of the death of the stem borers were the factors.

4.3 Results

4.3.1 Evaluation of the pathogenicity of the five *B. bassiana* strains on 3rd instar larvae of *C. partellus*

The five *B. bassiana* strains were pathogenic on the 3rd instar larvae of *C. partellus* and *S. calamistis*, causing various mortality levels (Figure 4-4). There was a highly significant difference between the pathogenic effect of the five *B. bassiana* strains (P = 0.001) when used against *C. partellus* (Table 4-2). The most effective strains of *B. bassiana* were Bb35 and Bb3 (P = 0.7606, Table 4-2). The highest mortality rate of 80 % was recorded at 28 days using both Bb35 and Bb3 (Figure 4-5). The cumulative mortality of the 3rd instar larvae of *C. partellus* increased over time as result of four (Bb3, Bb4, Bb21 and Bb35) out of the five *B. bassiana* strains. However, the cumulative mortality of the stem borer decreased after 21 days with the use of Bb10 (Figure 4-5). The AUMPC analysis revealed that two of the *B. bassiana* strains, Bb3 and Bb35, caused similar levels of pathogenicity, and that they were superior to the other strains, Bb 4, Bb10 and Bb 21 (Table 4-2).

Mature *B. bassiana* colonies grown from dead larvae of *C. partellus*



Mature *B. bassiana* colonies grown from dead larvae of *S. calamistis*

Figure 4-4 3rd instar larvae of *Chilo partellus* and *Sesamia calamistis* infected by *Beauveria bassiana*

Table 4-2: Evaluation of the pathogenicity of five *B. bassiana* strains against *C. partellus*

***Beauveria bassiana* strains**	**AUMPC mean after 28 days**	
Bb3	933 a	
Bb4	1657 c	
Bb10	1377 b	
Bb21	2065 d	
Bb35	910 a	
Effects	**P values**	**Significance**
Strains	0.001	xx
Bb35 vs Bb3	0.7606	NS
Bb35 vs Bb10	0.0001	xxx
Bb35 vs Bb4	0.0001	xxx
Bb35 vs Bb21	0.0001	xxx
Bb3 vs Bb10	0.0001	xxx
Bb3 vs Bb4	0.0001	xxx
Bb3 vs Bb21	0.0001	xxx
Bb10 vs Bb4	0.0037	xx
Bb10 vs Bb21	0.0001	xxx
Bb4 vs Bb21	0.0003	xxx
% CV	6.6	

AUMPC = area under the mortality progress curve

Means with the same letter are not significantly different (P = 0.05)

xxx = Highly significant, xx = Significant, NS = Not Significant

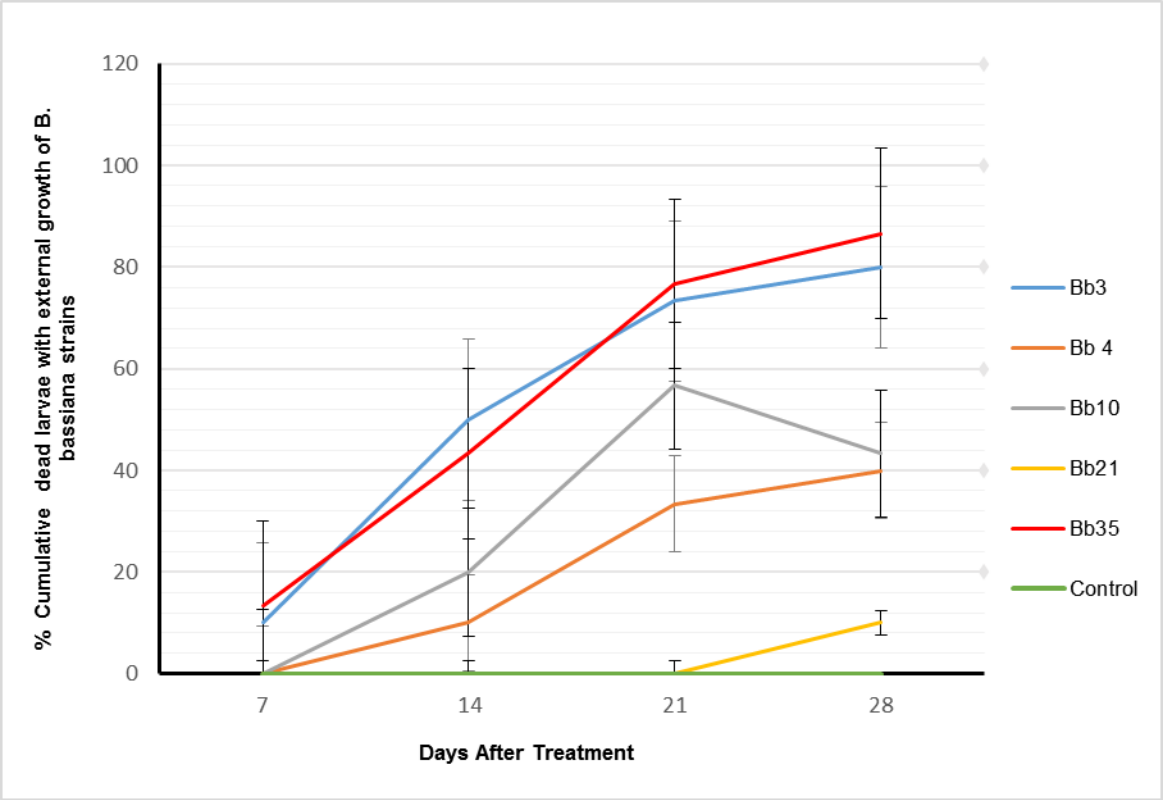


Figure 4-5 Cumulative mortality of 3rd instar larvae of *Chilo partellus* (%) caused by five endophytic *Beauveria bassiana* strains

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4.3.2 Cumulative mortality of 3rd instar larvae of *S. calamistis* caused by five *B. bassiana* strains

There were highly significant differences between the pathogenicity of the five *B. bassiana* strains that were used against the 3rd instar larvae of *S. calamistis*, P = 0.001 (Table 4-3). Strains Bb4 and Bb35 were the most effective strains, killing 93.33% and 76.66 of *S. calamistis* larvae at 28 days (Figure 4-6). The AUMPC data revealed that the *B. bassiana* strains, Bb4 vs Bb35, Bb35 vs Bb10 and Bb 10 x Bb 3 showed similar levels of pathogenicity (Table 4-3).

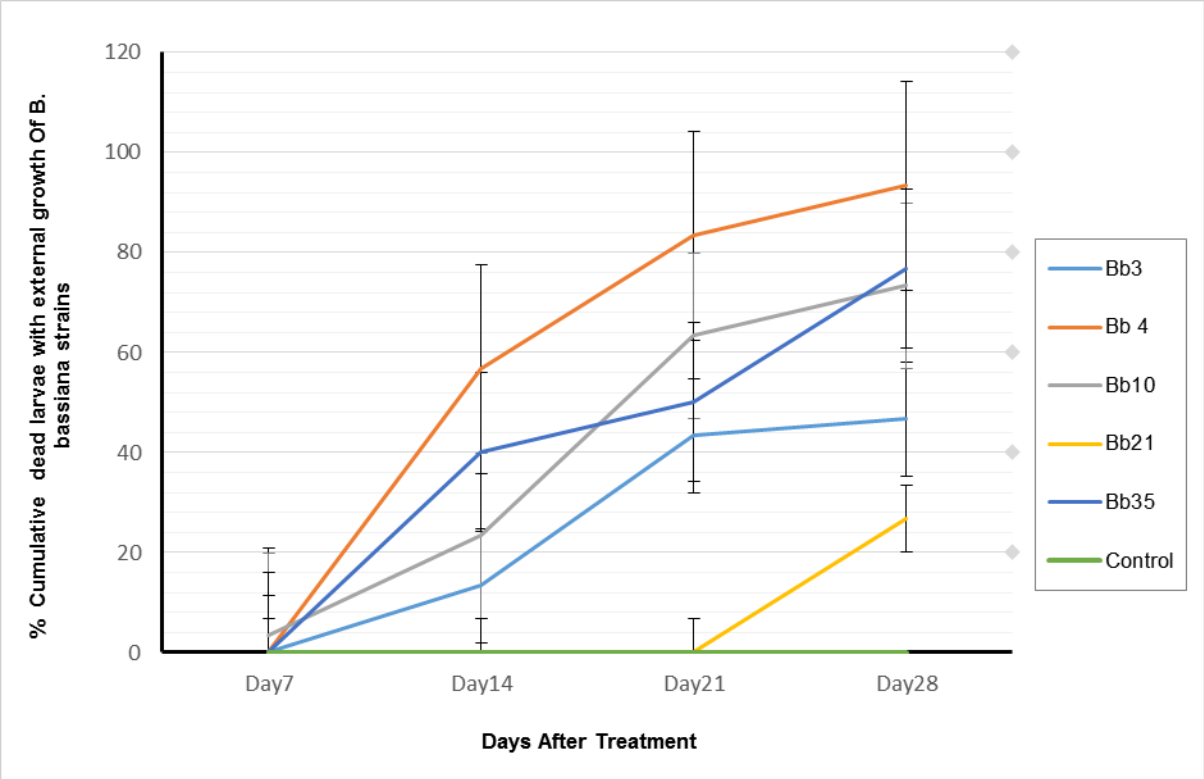

Figure 4-6 Cumulative mortality of 3rd instar larvae of *Sesamia calamistis* (%) caused by five endophytic *Beauveria bassiana* strains

Table 4-3: Evaluation of the pathogenicity of five *B. bassiana* strains against *S. calamistis*

***Beauveria bassiana* strains**	**AUMPC mean after 28 days**	
Bb3	1540 c	
Bb4	793 a	
Bb10	1225 bc	
Bb21	2007 d	
Bb35	1108 ab	
Effects	**P values**	**Significance**
Strains	0.001	xx
Bb4 vs Bb35	0.0599	NS
Bb4 vs Bb10	0.0156	xx
Bb4 vs Bb3	0.0005	xxx
Bb4 vs Bb21	0.0001	xxx
Bb35 vs Bb10	0.4503	NS
Bb35 vs Bb3	0.0156	xx
Bb35 vs Bb21	0.0001	xxx
Bb10 vs Bb3	0.0599	NS
Bb10 vs Bb21	0.0004	xxx
Bb3 vs Bb21	0.0105	xx
% CV	13.6	

AUMPC = area under the mortality progress curve

Means with the same letter are not significantly different (P = 0.05)

xxx = Highly significant, xx = Significant, NS = Not Significant

4.4 Discussion

The five *B. bassiana* strains showed differential pathogenicity against the 3rd instar larvae of *C. partellus* and *S. calamistis*. This outcome is similar to the result of Ignoffo *et al*. (1976) who found that fungal isolates from different insect hosts have varying degree of virulence to the different insects.

In this study, mortalities of 80 % and 93.33% were achieved with selected strains on 3rd instar larvae of *C. partellus* and *S. calamistis*. Similarly, *B. bassiana* strains caused high levels of mortality in larvae of diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae) (Valda *et al*., 2003; Godonou *et al*. (2009); Batta (2013), and in adult banana

weevils, *Cosmopolite sordidus* (Coleoptera: Curculionidae) in Uganda (Nankinga, 1994). Hemalatha et *al*. (2016) also found that *B. bassiana* had the potential to control *Myzus persicae* Sulzer (Homoptera: Aphididae) on chilli (*Capsicum spp* L.), as did Tesfaye *et al*. (2010), who isolated various strains of *B. bassiana* that caused mortalities of >75% of adults of *M. persicae*.

Insect pathogens are cheaper and more environmentally safe than other methods of pest management and they fit into the concept of integrated pest management (IPM) (Narayanan, 1997). Research have showed that *B. bassiana* can protect plants from herbivorous insects (Jallow *et al*., 2008) and some plant pathogens (Ownley *et al*., 2008). The results of this study indicate that the five tested *B. bassiana* strains hold potential as biological control agents for the IPM of cereal stem borers.

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CHAPTER 5 Field evaluation of systemic strains of Beauveria bassiana (Balsamo) Vuillemin activity against sorghum stem borer (Chilo partellus) damages

Abstract

Apart from their performance as artificial endophytes in several plant species, some commercial formulations of *Beauveria bassiana* Vuillemin have been reported to be effective for the control of insect pests of crops, including *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae), *Ostrinia nubilalis* Hubner (Lepidoptera: Crambidae) and *Sylepta derogate* Fabricus (Lepidoptera: Pyraustidae). Several systemic strains of *B. bassiana* were previously found to be effective for the control of 3rd instar larvae of *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) in laboratory bioassays. This study was therefore aimed at assessing the efficacy of endophytic *B. bassiana* strains and a pesticide, Karate (lambda cyhalothrin), for the control of sorghum stem borer in the field. Conidial suspensions of the five endophytic *B. bassiana* strains were applied as seed treatments to sorghum seed. Karate was applied to the positive control plants as a foliar spray, to see if the *B. bassiana* strains could match it for efficacy. The damage caused by the stem borers was estimated in each plot for dead heart and chaffy panicles. The extent of damage reduction in our study was positively correlated with the endophytic colonization of the stems by strains of *B. bassiana*. Sorghum plants treated with *B. bassiana* strains and Karate produced half-chaffy panicles. In the control plots, the panicles were completely chaffy. For the plant traits measured, such as the number of panicles and the panicle weight of the sorghum plants, those plants treated with *B. bassiana* or Karate were greater than the untreated sorghum plants. *B. bassiana* strains could be used for the control of sorghum borer, *C. partellus*, as an alternative to synthetic chemical pesticides such as Karate.

Keywords: Systemic *B. bassiana* strains, lambda cyhalothrin, Karate, dead heart, chaffy panicles.

5.1 Introduction

People in Africa continue to face food insecurity due to high population growth, and both biotic and abiotic constraints to crop production. The continent has the highest population growth in the world, with its human population more than tripling in the second half of the 20th century, from 230 to 811 million (FAO, 2011). Despite the surge in human population in the continent, levels of food production have stagnated (World Bank, 2008). A complex of stem borers are one of the major constraints affecting cereal production in Africa. Yield losses resulting from stem borer damage range from 10 to 80% (Kfir *et al*., 2002). Among the lepidopteran stem borers, *Chilo partellus* Swinhoe (Lepidoptera: Crambidae) is one of the species causing economic yield losses (Kfir *et al*., 2002). Control of stem borers is usually difficult due to the cryptic and nocturnal habits of adult moths (Ampofo *et al*., 1986). One of the main methods to control the stem borers recommended to smallholder farmers by public extension agents in most of the sub-Saharan Africa's region is the use of pesticides. However, the use of chemical control against stem borers is uneconomical, unaffordable, and impractical for most resource-poor farmers, and is not environmentally friendly (Van den Berg and Nur, 1998a; Gill and Garg, 2014). Moreover, stem borers may develop resistance to the pesticide (Shah and Pell, 2003), have adverse effects on non-target organisms and result in pesticide residues in food (van den Berg and Nur, 1998a; Varela *et al*., 2003). There is need for effective, affordable and safe alternatives to pesticides. Biological control is one option. Several potential biocontrol roles have been identified for fungal endophytes, including providing protection against herbivorous insects (Breen, 1994), plant pathogens (Dingle and McGee, 2003; Wicklow *et al*., 2005) and plant parasitic nematodes (Elmi *et al*., 2000). Some strains of *B. bassiana* has been demonstrated to be endophytic in several plant species such as banana (*Musa spp*.), maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L.) (Posada and Vega, 2005; Posada and Vega, 2006; Ownley *et al*., 2008) and these strains were pathogenic against *C. sordidus*, *Aphis gossypii* Glover (Homoptera: Aphididae) and *O. nubitalis* (Lewis and Cossentine, 1986; Lewis and Bing, 1991; Inglis *et al*., 2001; Akello *et al*., 2007; Herlinda, 2010). This study aimed at assessing the efficacy of five endophytic *B. bassiana* strains, compared with a pyrethroid insecticide, lambda-cyhalothrin (Karate), in controlling sorghum stem borer in the field.

5.2 Materials and methods

5.2.1 Experimental site

The experiment was conducted in the summer of 2017 at the Ukulinga Research Farm of the School of Agriculture, Earth and Environment, University of KwaZulu-Natal, Pietermaritzburg South Africa, latitude 29.67 S, longitude 30.41 E, situated at 809 meters above sea level.

5.2.2 Sorghum cultivars and *Beauveria bassiana* strains

A commercial sorghum cultivar, PAN 8816, which is grown in various agronomic regions of South Africa, was used for the field study. Five strains of *B. bassiana* were selected, based on prior research showing them to be endophytic in sorghum and pathogenic on *C. partellus.*

5.2.3 Experimental design and management

The experiment was laid out in a randomized complete block design (RCBD) with three replicates. The land was demarcated into three blocks. Each block measured 20.8 meters and had seven plots. Each plot was divided into three rows that contained 10 plants/row. The inter-row distance was 50 cm and the intra-row distance was 20 cm. The distance between the plots was 80 cm.

Before sowing, the sorghum seeds were surface sterilized in 3% sodium hypochlorite for 3 minutes, followed by 70% ethanol for 2 minutes. They were rinsed three times with distilled water and air-dried. They were then inoculated with each of the five endophytic *B. bassiana* strains. The treated seeds were planted in Speedling® 24 trays filled with composted pine bark (CPB) growing medium. After 15 days, the seedlings were transplanted in the field. Sterile distilled water was used to treat the seed of the negative control. Seed of the positive control were not treated with anything but were treated the same as the other seed. The positive control plant were treated every fortnight with a suspension of Karate at the recommended rate (0.6ml per 100m row). A hydraulic hand sprayer was used to apply the pesticide to the plants.

Infestation of the crop by *C. partellus* was based on adult moths arriving from neighbouring crops of maize and other cereals in the region. No inoculations were made. All normal agronomic practices such as weeding, fertilization and irrigation were carried out as per standard practices. The weed population was kept at minimum by regular hand weeding.

5.2.4 Data Collection

Estimates were made for the levels of stem borer symptoms, and a number plant traits were measured in each plot. The characters measured following the sorghum descriptor list from the International Board of Plant Genetic Resources, IBPGR/ICRISAT (1993).

5.2.4.1 Borer symptoms and plant performance

The number of plants showing the death of the central leaves was recorded, a symptom commonly called **dead heart (DH),** which is a symptom boring of the caterpillars into the stem. Ten (10) plants from the middle row were tagged and used for assessed for dead hearts at the vegetative stage.

% Dead hearts: This was based on plants showing dead heart symptoms:

$$\%\ \text{Deadherts} = \frac{\text{Numbers of plants with with deadhearts}}{\text{Number of plants tagged per plot (10 plants)}}\ \text{x } 100$$

The **white head (WH)** or chaffy panicle symptom is caused by the stem borer larvae tunnelling into the stem. It results in the destruction of the growing point, early leaf senescence, and interferes with the translocation of metabolites and nutrients to the panicle. The number of complete or partially chaffy panicles in 10 plants chosen randomly from the middle of the ridge of each plot was recorded at the flowering stage.

$$\%\ \text{Chaffy Panicles} = \frac{\text{Number of plants with chaffy panicles}}{\text{Number of plants tagged per plot (10 plants)}}\ \text{x } 100$$

Borer presence/absence inside the stem

Plants from the border row of each plot were harvested at 90 days after transplanting. The stems were dissected and the number of stem borers was recorded.

Plant height (PH) was measured in cm at 90 days after treatment. The measurements of 10 plants/plot that were randomly selected from the middle of the ridge were taken from the base of the plant to the tip of the head.

The **number of tillers (NT)** was counted at 90 days after planting on 10 plants randomly selected in the middle of the ridge of each plot.

The **grain weight (GW)** in gram of 100 seeds was assessed at physiological maturity when the seeds were at approximately at 12% moisture.

The **number of panicles (NP)** from each plot was recorded at physiological maturity of the crop.

The weight in gram of 10 panicles **(PW)** was obtained from 10 plants randomly selected from the middle of the ridge of each plot, and was recorded at the mature stage when the panicles were dry.

5.2.4.2 Presence of the biocontrol agent

Endophytic detection

Plants from the border row of each plot were harvested at 30, 60 and 90 days after treatment. The plants leaves, stems and roots were tested for the presence of *B. bassiana* strains. The presence or absence of each *B. bassiana* strain on the samples were accessed using the same methodology used in Chapter 2, Section 2.2.6.

5.2.5 Statistical analysis

Analysis of variance was carried out on the data collected using SAS (Version 9.4). A general linear model (GLM) was used for the ANOVA. Treatment means were compared using least significant difference (P = 0.05). The mean values for each character was used to calculate correlation coefficients.

5.3 Results

At the vegetative stage, all the treated sorghum plants showed symptom of damage caused by 1st instar larvae of *C. partellus* (Figure 5-1).

At 30 days after transplanting, the endophytic colonization of the stem of the plants treated with each *B. bassiana* strains was confirmed. The application of three *B. bassiana* strains

proved to be effective. The plants treated with the *B. bassiana* strains Bb35, Bb10 and Bb4 recovered from the 1st instar larvae attack. They produced partially chaffy panicles, similar to the plants treated with the pesticide Karate (Figure 5-2).

Only the control plants produced panicles that were completely chaffy, confirming stem borer (*C. partellus*) attack (Figure 5-3).

Scars and holes caused by the 1st instar larvae of *Chilo partellus*

Figure 5-1 Sorghum plant with 1st instar larvae symptoms

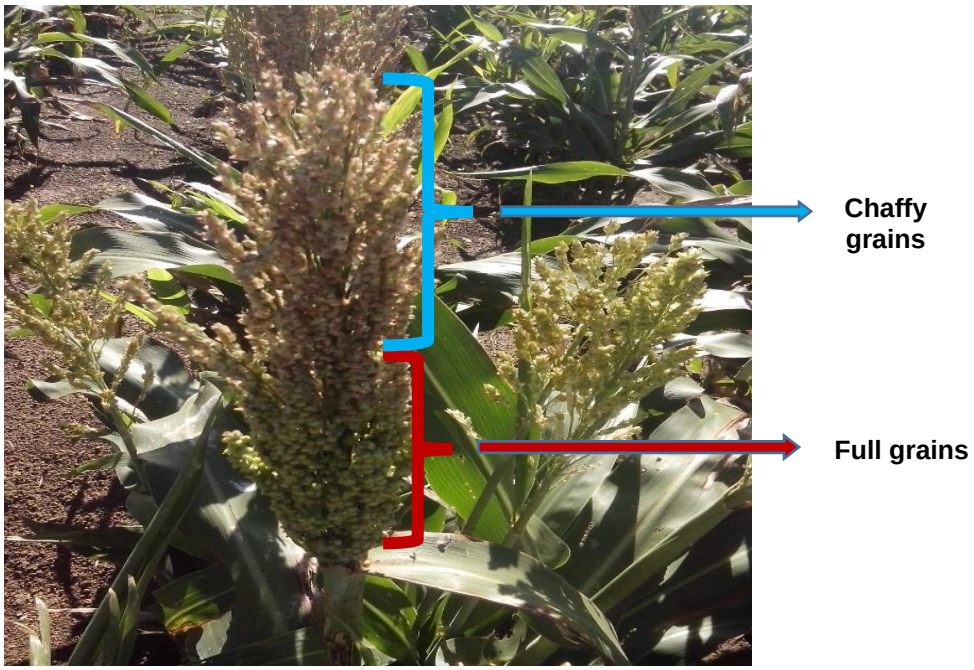

Figure 5-2 Sorghum plants with partially chaffy panicle

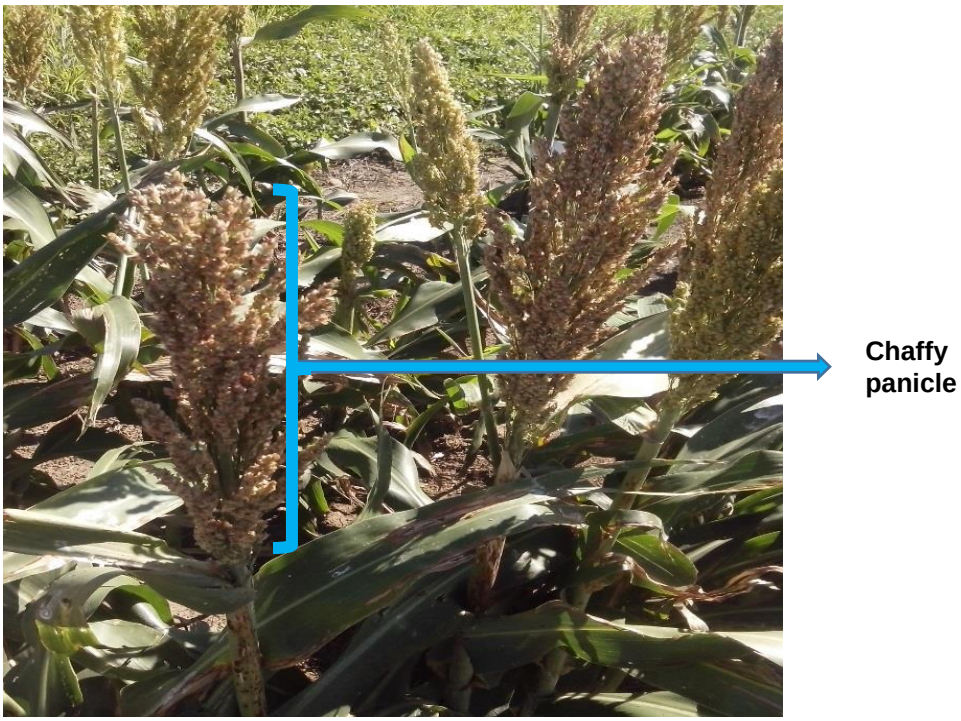

Figure 5-3 Sorghum plant with symptom of 3rd instar larval attack

The control plants had the highest percentage of "Dead Heart" (% DH). Most of the sorghum plants treated with the *B. bassiana* strains and Karate did not develop the symptom of DH. The highest level of "White Head" (WH) occurred on the control plants. The level of WH was 50% for the plants treated with *B. bassiana* strains Bb3 and Bb21, while those treated with *B. bassiana* strains Bb4, Bb10 and Bb35, and Karate were at around 30% (Figure 5-4).

All the plants treated with *B. bassiana* strains and Karate produced more panicles than the control plants. The number of panicles (NP) of the plants treated with *B. bassiana* strains Bb4, Bb10 and Bb35 was same as those treated with Karate but those treated with strains Bb3 and Bb21, and the negative control, had fewer panicles (Figure 5-4).

The weight of the panicle is related to the weight of 100 grains. The higher the weight of 100 grains, the higher the panicle weight and vice versa. The panicle weight (PW) of the plants treated with Bb4 and Bb10 were the highest compared to those treated with Karate and the other *B. bassiana* strains (Figure 5-4).

Table 5-1: Effect of *Beauveria bassiana* strains on sorghum plants compare to sorghum plants treated with Karate and untreated sorghum plants

Bb strains	% Dead Heart (DH)	% White Head (WH)	Number of Panicles (NP)	Grain Weight (GW)	Panicle Weight (PW)	Presence/Absence of Tunnel
Bb3	0	50b	10	1.3a	125.94a	+++
Bb4	20	36.66a	13	2.54b	494.46g	+
Bb10	0	33.33a	13	2.26b	438.72f	+
Bb21	0	53.33b	10	2.46b	175.82c	+++
Bb35	0	30a	14	2.45b	279.27d	++
Control 1-Karate	0	30a	14	2.31b	324.23e	++
Control 2-untreated	60	70c	7	2.57b	153.12b	+++
F value		20.22		10.62	12169.45	
P value		0.001		0.001	0.001	
%CV		13.4		10.4	0.8	

Means with the same letter are not significantly different (P = 0.05) according to least significant difference.

5.4 Discussion

Endophytic *B. bassiana* strains affect the development and survivorship of larvae of the sorghum borer, *C. partellus,* inside sorghum plants, and reduce the damage that they cause. In the current experiment, all the control plants were attacked by the larvae of *C. partellus*, while the plants treated with the five *B. bassiana* strains and Karate recovered from the attack of the borer. After the attack of the 1st instar larvae of the borer at the vegetative stage, most of the *B. bassiana* treated plants were able to eliminate the pest, and resume their growth, going on to produce panicles that were only partially chaffy. In contrast, the panicles of the untreated control plants were completely chaffy. Bing and Lewis (1991; 1993) reported similar findings with maize plants colonized by *B. bassiana* strains, where *O. nubilalis* damage was reduced by 14.6 - 41%. Cherry *et al*. (2004) reported that in maize, endophytic *B. bassiana* strains provided protection against *Sesamia calamistis* Hampson. Some commercial formulations of *B. bassiana* have been effective against cotton weevils but their endophytic nature is not clear (Wright and Chandler, 1992; Lacey and Goettel, 1995). In the current study, we did not determine the primary cause of damage reduction in *B. bassiana* treated plants. However, based on the reduction in the levels of levels of damage in plants inoculated with endophytic *B. bassiana*, we suggest that most of the endophytic *B. bassiana*

strains negatively affected larval feeding and development, resulting in reduced plant damage. Cessation of feeding by *C. partellus* occurs as early as 1-4 days after inoculation with *B. bassiana* (Tefera and Pringle, 2003), which we also found. In addition, Roy *et al*. (2006) found that a range of altered behaviors was exhibited by infected insects, including reduced feeding. Sorghum plants inoculated with three of the *B. bassiana strains* showed similar levels damage of as those treated with Karate. This result suggests that some strains of this fungus could be used as an alternative to the pesticide (Karate) for the control of the borer, *C. partellus,* in sorghum. Further field trials are needed to confirm this finding.

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CHAPTER 6 Compatibility of Trichoderma harzianum Rifai Strain Kd (Eco-T®) with selected systemic strains of Beauveria bassiana (Balsamo) Vuillemin

Abstract

The study was undertaken in order to evaluate the possible interaction effects between *Trichoderma harzianum* Rifai Strain Kd (sold as Eco-T®) (TKD) and selected *Beauveria bassiana* (Balsamo) Vuillemin strains that previously showed potential as biological control agents against stem borers of sorghum and rice. The strains of both fungi were inoculated *in vivo* and *in vitro* for their interactions with each other. *In vitro* bioassays indicated that TKD inhibited four of the five selected *B. bassiana* strains at 15 days after inoculation. Only the *B. bassiana* Strain Bb 35 grew in the presence of TKD. A total inhibition of the growth of the five selected *B. bassiana* strains by TKD was observed at 30 days after *in vitro* inoculation. Various interaction effects were observed between the fungi according to the inoculation methods. When the five selected *B. bassiana* strains were inoculated onto seed of sorghum and rice together with TKD, the inhibitory effect of TKD toward the *B. bassiana* strains was strongest. The inhibitory effect of TKD toward the *B. bassiana* strains was less when the five *B. bassiana* strains were inoculated as seed treatments, and the seed sown and grown for 7 days before suspension of TKD conidia was drenched onto the roots of the plants. The endophytic behaviour of each *B. bassiana* strains was observed in plant stems when the seeds were treated using only included the *B. bassiana* strains. The same result was obtained when the seeds were initially treated with each of the *B. bassiana* strains, and the seedlings were drenched 7 days later with TKD. The root colonization by TKD on rice and sorghum varied according to the inoculation methods. There was an effective colonization of the plant roots by TKD when its suspension was applied as a drench 7 days after the *B. bassiana* strains were inoculated onto the seed. Dual colonization was not possible TKD and the five *B. bassiana* strains.

Keywords: *T. harzianum* Rifai strain Kd (Eco-T®), *B. bassiana*, compatibility, inhibition, biocontrol, endophyte.

6.1 Introduction

Bio-pesticides are microbes that are naturally occurring organisms, which are able to control pests by a number of mechanisms. *Beauveria bassiana* (Vuillemin) is a fungus found globally that is a parasite of a wide range of insects (Wagner et *al*, 2000). It has been commercialized successfully in many parts of the world, including South Africa. Some strains are endophytic, symbiotic and entomopathogenic. Another symbiotic fungus is *Trichoderma harzianum* Rifai, which has been investigated as a biological control agent for over 70 years (Lumsden *et al*., 1993; Monte, 2001). Strains of *Trichoderma* species have become available commercially as biocontrol agents and growth stimulants (Lumsden *et al*., 1993; Monte, 2001). *T. harzianum* has multiple modes of action to provide host plants with protection from fungal pathogens, but also to enhance tolerance of abiotic stresses such as drought and waterlogging (Papavizas, 1985). Application of conidia of strains of *T. harzianum* to plants may result in improved seed germination, increased plant size, leaf area and dry weight (Inbar *et al*., 1994; Altomare *et al.,* 1999). *Trichoderma spp*. are potent agents for the biocontrol of plant pathogens (Sivan and Chet, 1992; Inbar *et al*., 1994). Some strains of *Trichoderma* have evolved mechanisms for both attacking other fungi and enhancing plant and root growth (Harman, 2000). Among their mechanisms, there is mycoparasitism with a concomitant production of enzymes that degrade fungal cell walls (Chet, 1987). The colonization of the root system by rhizosphere competent strains of *Trichoderma* results in increased development of root and/or aerial systems and crop yields (Harman and Björkman, 1998). The induction of plant systemic resistance and an antagonistic effect towards plant pathogenic nematodes (Sharon *et al*., 2001) have also been described.

B. bassiana and *T. harzianum* can both live endophytically inside plants, between plant cells, without causing negative effects on plant growth and development. Many of these endophytic strains enhance pest and disease resistance (Segarra et al., 2007; Shakeri and Foster, 2007; Shoresh and Herman, 2008; Gomez-Vidal et *al*., 2009). Both of the fungi produce a wide array of enzymes that can attack substrates as diverse as plant cell walls, insect cuticles, and oomycetous and fungal plant pathogens. Their also produce hydrophobins that have been suggested to play a role in the formation of protective layers and the structural components of cell walls, and which reduce surface tension to allow aerial growth and conidial thermotolerance (Ying and Feng, 2004; Viterbo and Chet, 2006; Linder, 2009). There is no information on their combined use to control soil-borne diseases, stem borers and abiotic stress. Even though the scenario of a combined systemic bio-pesticide and plant growth promoter has great market potential, there is a need to determine whether it is possible to

combine two systemic bio-pesticides for field control of both insects and diseases. The aims of this study were to first assess the efficacy of both *B. bassiana* and *T.* harzianum Kd (Eco-T®) (TKD) applied singly or in a combination to sorghum and rice plants. Secondly, the study was undertaking to provide information about the interactions between TKD and each of the five *B. bassiana* strains.

6.2 Materials and methods

6.2.1 Fungal strains

Five endophytic *B. bassiana* strains: Bb 3, Bb 4, Bb 10, Bb 21 and Bb 35, were selected, based on previous *in vitro* and *in vivo* screening trials (Chapters 2 and 3) for biological control activity against *C. partellus* and *S. calamistis*. One commercial strain of TKD was used in this study, which was provided by Plant Health Products (PHP, Nottingham Road, South Africa.

6.2.2 *In vitro* dual culture bioassay studies of TKD and *B. bassiana* strains interactions

Both of the fungal strains were initially subcultured in Petri dishes, which contained potato dextrose agar medium (PDA). The TKD plates were incubated at 25°C for 7 days. Each plate of *B. bassiana* was incubated at 28°C for 15 days. Under a laminar airflow, 4mm diameter plugs were cut from actively growing edges of both TKD and each *B. bassiana* strain. The mycelial plugs from each fungi were placed opposite each other (25mm apart) and on PDA in 90mm diameter Petri dishes. The plate were incubated for 30 days at 28°C. Each treatment was replicated three times. At 15 and 30 days post-inoculation, each plate was examined for interactions between the strains of both fungi.

6.2.3 *In vivo* dual culture bioassay of the interactions between TKD and five *B. bassiana* strains in the greenhouse

Initially, TKD was cultured on PDA for 7 days at 25°C. Each of the *B. bassiana* strains was cultured on PDA for 15 days at 28°C. Under a laminar airflow, the conidia of both fungi were harvested as described in Chapter 2, Section 2.2.4. Seed of sorghum and rice were surface sterilized in 3% sodium hypochlorite for 3 minutes, followed by 70% ethanol for 2 minutes. They were rinsed three times with distilled water, air dried and divided into three sets. Three inoculation methods were used for the treatments.

For the first set, seeds of each crop were separately dipped in the conidial suspension of each fungus strains [5 ml of the prepared inoculum (2×10^6 conidia ml^{-1})]. The seeds were then planted in Speedling® 24 trays filled with composted pine bark (CPB) growing medium. After 7 days, the seedlings were transplanted into 30 cm diameter pots filled with CPB growing medium and placed under controlled greenhouse conditions set at 20-28°C (night and day).

The seeds of each crop from the second set were separately dipped in a mixed suspension of the TKD and each *B. bassiana* strain. The seeds of each crop were then separately planted in Speedling® 24 trays previously filled with CPB growing medium. The seedlings were transplanted after 7 days into 30 cm diameter pots filled with CPB growing medium and placed under controlled greenhouse conditions set at 20-28°C.

For the third set, the seeds of each crop were initially dipped in the conidial suspension of each *B. bassiana* strains [5 ml of the prepared inoculum (2×10^6 conidia ml^{-1})]. The seeds of each crop were then planted separately in Speedling® 24 trays filled with CPB growing medium. The treated seedlings were transplanted into 30 cm diameter pots that contained CPB growing medium. After 7 days, a hand sprayer was used to drench the plants of each crop with a conidial suspension of the TKD. 50 ml of the inoculum of TKD was used per plant. Three plants per pot were arranged in the greenhouse in a randomized complete blocks design with three replicates.

Three different control were used for both crops. The first control plants consisted of uninoculated seeds. For the second set of control, after being surface sterilized, the seeds were directly inoculated with the conidial suspension of TKD only. For the third control set, the seedlings of each crop were drenched at 7 days after planting with the standard conidial suspension of TKD.

6.2.4 Evaluation of T. harzianum and B. bassiana colonization

At 15 and 30 days after inoculation of each crop, one plant per treatment from each crop was separately harvested and thoroughly washed with tap water. From each treatment combination (each crop x *B. bassiana*, or each crop x *B. bassiana* x TKD, or each crop x TKD), plants were sampled for their root and stem tissues. The roots were evaluated for colonization by both fungi. The stems were only tested for *B. bassiana* colonization. The stems and roots of each crop were separately surface sterilized in 0.3% sodium hypochlorite for 2 minutes, followed by 70% ethanol for 2 minutes. The samples were then rinsing three

times with sterile distilled water for 15 seconds. After being dried on sterile tissue paper under a laminar flow cabinet, six pieces of the surface sterilized stem from each treatment of the two crops were randomly taken and plated separately onto a *B. bassiana* selective medium (Appendix 2). The plates were incubated for 15 days at 25°C. After being dried in the same way, six pieces of roots from each treatment of the two crops were plated separately onto *Trichoderma* selective medium (TSM). The plates were kept in an incubator at 25°C for 7-15 days. To confirm the effectiveness of the surface sterilization, 10 ml of sterile distilled water used to rinse the samples (roots and stems) during the surface sterilization procedure was spread onto Petri dishes containing TSM and *B. bassiana* selective medium. The plates were incubated for 7-15 days at 25°C. However, the sterilization process followed resulted in clean plates. Thus, any growth of *B. bassiana* or *T. harzianum* from surface-sterilized tissues was assumed to have originated from within plant tissues as endophytes. All the plates that contained plant samples (roots and stems) were monitored every 2-3 days for emergence of mycelium growing. The presence or absence of *T. harzianum* and *B. bassiana* from root samples was recorded 7 or 15 days later. The *B. bassiana* growth from stem samples was recorded similarly. The fungal colonies grown from the samples were confirmed to be *T. harzianum* or *B. bassiana* strains, based on their morphological characteristics. At the maturity stage of each crop, one plant from each treatment combination was dried at 70°C for 2 days and the dry weight was recorded.

6.3 Results

6.3.1 *In vitro* dual culture bioassay studies of *T. harzianum* and *B. bassiana* interactions

Interaction effects between the TKD and each of the *B. bassiana* strains occurred four days after inoculation. At 7 days, the TKD was less inhibitory to *B. bassiana* strain Bb 35 than the other *B. bassiana* strains. However, after 15 days, the TKD also overgrew the Bb 35 colony (Figure 6-1). The TKD was highly inhibitory towards *B. bassiana* strains [Bb 3, Bb 4, Bb 10 and Bb 21, (Figure 6-2)], at 15 and 30 days post-inoculation. Full sporulation of the TKD occurred within 30 days of inoculation with the formation of spores on all sections of the plates. The greenish colouration spread over the plates as the *Trichoderma* strain grew over the *B. bassiana* strains.

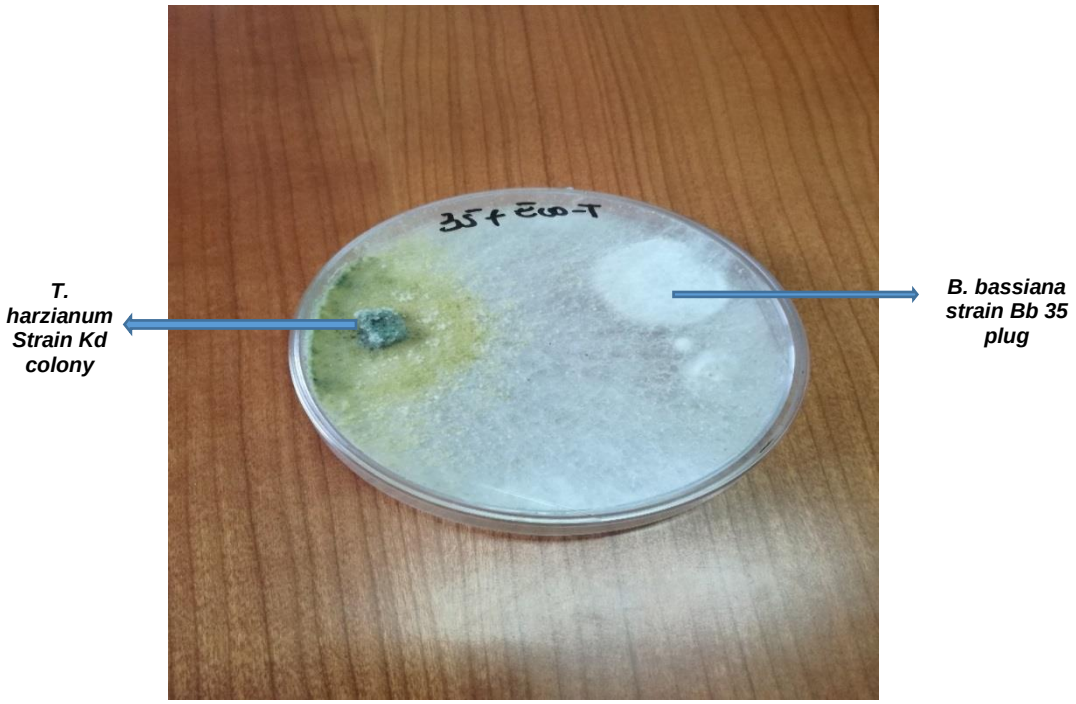


Figure 6-1 *In vitro* interaction between *Trichoderma harzianum* strain Kd and *Beauveria bassiana* Strain Bb35 at 15 days in dual culture

.

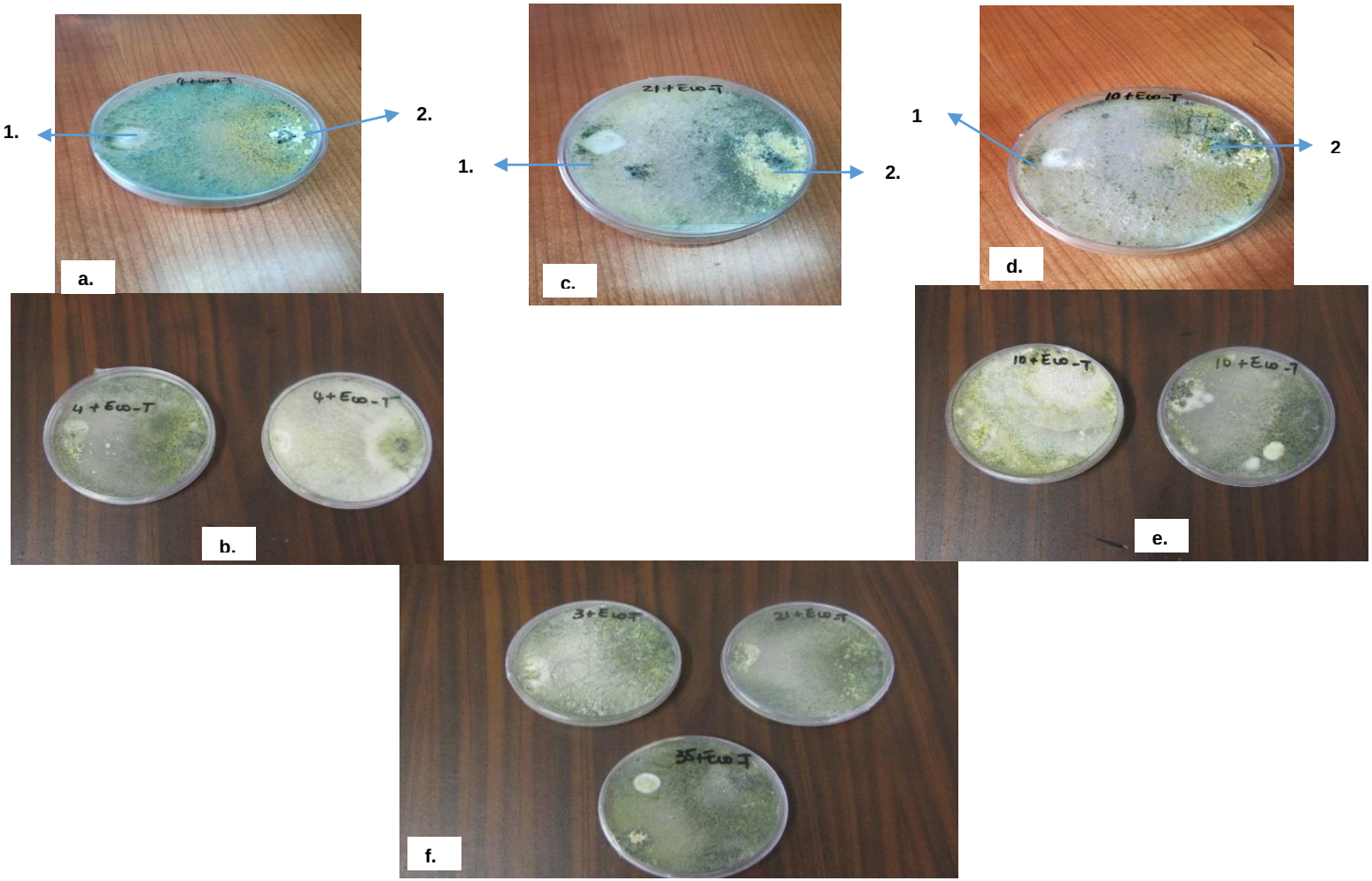


Figure 6-2 Inhibitory action of *Trichoderma harzianum* (Strain Kd) towards *Beauveria bassiana* strains

a = Bb4 + TKD dual culture after 15 days; b = Bb4 + TKD dual culture after 30 days; c = Bb21 + TKD dual culture after 15 days
d = Bb10 + TKD dual culture after 15 days, e = Bb10 + TKD dual culture after 30 days; f = Bb3, Bb21 and Bb35 + TKD dual culture after 30 days
1 = *B. bassiana*; 2 = *T. harzianum* (Strain Kd).

6.3.2 *In vivo* dual culture bioassay of TKD and *B. bassiana* strains interactions in the greenhouse

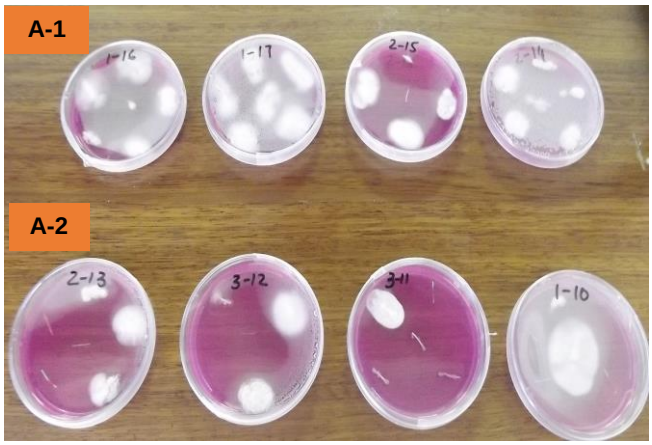
All of the *B. bassiana* strains colonized well the roots and the stem of both crops when they were inoculated alone without TKD as a seed treatment. The TKD and *B. bassiana* strains did not inhibit each other's growth with all the combinations of treatment (Table 6-1, Figure 6-3 and Figure 6-4). There was less inhibition of growth of both fungi in roots when the *B. bassiana* strains were used as a seed treatment and the TKD was applied as a drench 7 days later (Figure 6-3-D). The inhibition of the TKD by some of the *B. bassiana* strains occurred in the roots when a mixture of their conidial suspensions was used as a seed treatment at the same time [Table 6-1-6-6, Figure 6-3 (A1 and C) and Figure 6-4-A]. *B. bassiana* strain Bb35 colonized the sorghum and rice stems when it was used as a seed treatment combined with TKD, when the TKD was applied as a drench treatment [Table 6-2, Figure 6-3-B and Figure 6-4-C (3-17)]. The *B. bassiana* strain Bb3 colonized the rice stem after being used together with TKD as a combined seed treatment [Figure 6-4-C (1-8)].

Table 6-1: *In-vivo* sorghum tissue colonization by both fungi after 15 days using various inoculation methods

Inoculation methods	Sorghum tissues	Five Bb strains (Bb3, 4, 10, 21, 35)	Bb3 x TKD	Bb4 x TKD	Bb10 x TKD	Bb21 x TKD	Bb35 x TKD
Bb strains singly applied as seed treatment	Roots	Yes	-	-	-	-	-
Mixed inoculum of Bb strains and TKD	Roots	-	No	No	Yes	Yes	Yes
Bb strains applied as seed treatment + TKD applied as drench	Roots	-	Yes	No	No	No	Yes
Bb strains singly applied as seed treatment	Stems	Yes	-	-	-	-	-
Mixed inoculum of Bb strains and TKD	Stems	-	No	No	No	No	Yes
Bb strains applied as seed treatment + TKD applied as drench	Stems	-	No	Yes	Yes	No	Yes

Table 6-2: *In-vivo* colonization of rice tissue by both fungi after 15 days, using various inoculation methods

Inoculation methods	Sorghum tissues	Five Bb strains (Bb3, 4, 10, 21, 35)	Bb3 x Eco-T	Bb4 x Eco-T	Bb10 x Eco-T	Bb21 x Eco-T	Bb35 x Eco-T
Bb strains singly applied as seed treatment	Roots	Yes	-	-	-	-	-
Mixed inoculum of Bb strains and TKD	Roots	-	No	Yes	No	No	No
Bb strains applied as seed treatment + TKD applied as drench	Roots	-	Yes	No	No	No	Yes
Bb strains singly applied as seed treatment	Stems	Yes					
Mixed inoculum of Bb strains and TKD	Stems	-	Yes	No	No	No	No
Bb strains applied as seed treatment + TKD applied as a drench	Stems	-	No	No	No	No	Yes

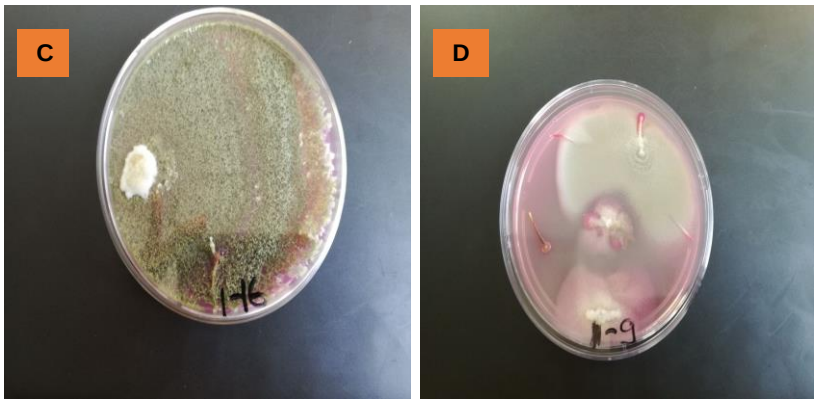


A- 1 (1-10; 3-11 and 3-12) = Sorghum roots colonized by Bb10, Bb21 and Bb35 at 15 days after using a mixed inoculum of the 3 *B. bassiana* strains and TKD as a seed treatment

A-2- (1-16; 1-17; 2-13; 2-14 and 2-15) = Sorghum roots colonized 15 days by the five *B. bassiana* strains after using *B. bassiana* strains inoculum as a seed treatment and TKD applied after 7 days as a drench treatment

B-
3-17 = Sorghum stem colonized by Bb35 at 15 days after using Bb35 inoculum as a seed treatment and TKD applied as a drench treatment after 7 days

1-7 = Sorghum stem colonized by Bb35 at 15 days after using only Bb35 inoculum as a seed treatment

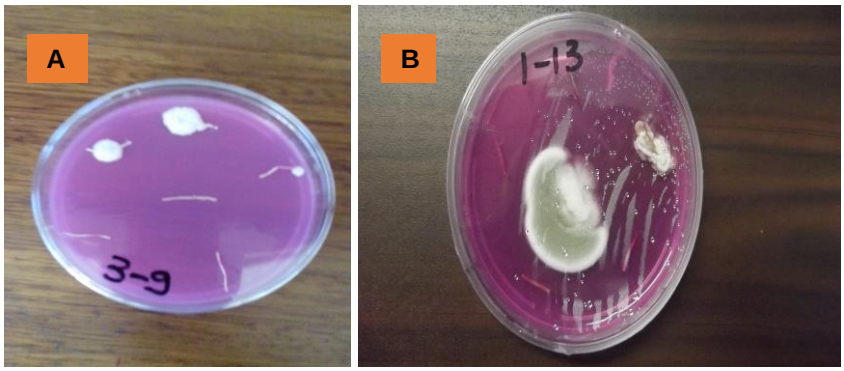


C- Interaction between Bb21 and TKD showing inhibitory response toward Bb21 and overgrowth of TKD at 15 days after using Bb21 inoculum as a seed treatment and TKD applied after 7 days as drench treatment

D- Sorghum roots colonized by TKD and Bb4 at 30 days after seed treatment using a mixed inoculum of both fungi

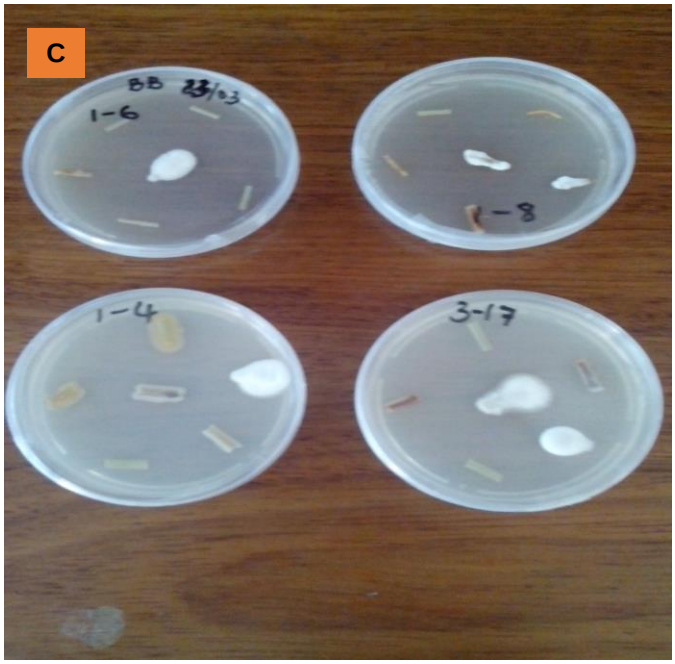
Figure 6-3 *In vitro* sorghum tissue colonization by both *Beauveria bassiana* strains and *Trichoderma* strain (Strain Kd) (TKD) using various inoculation methods. (A, B, C and D).

Bb = *Beauveria bassiana* strain



A- Rice roots colonized at 15 days by only Bb4 after seed treatment using a mixture inoculum of Bb4 and TKD

B- Rice roots colonized at 15 days using both Bb3 inoculum as a seed treatment and TKD applied as a drench treatment



C-
(1-4; 1-7) = Rice stem colonized by Bb4, Bb21 at 15 days after using both *B. bassiana* strains suspensions as a seed treatment

1-8 = Rice stem colonized at 15 days by Bb3 after using the mixture of Bb3 and TKD suspension as a seed treatment

3-17 = Rice stem colonized at 15 days by Bb35 after using its suspension as seed treatment and TKD applied as drench treatment

Figure 6-4 *In vitro* colonization of rice tissues by both *Beauveria bassiana* strains and *Trichoderma harzianum* strain (Strain Kd) (TKD), using various inoculation method (A, B and C).

Bb = *Beauveria bassiana* strain

6.4 Discussion

Understanding the interactions between various biological control agents (BCAs) in relation to their biological control mechanisms against plant pests or pathogens is vital for the optimization and implementation of integrated biological control products. Their antagonism or inhibitory effects, and their ability to increase plant growth are need to be known.

Fungi such as *B. bassiana* and *Lecanicillium spp*. are already well known for their entomopathogenic characteristics, but have recently been shown to engage in plant-fungus interactions (Vega, 2008; Vega et *al*., 2008). Entomopathogenic fungi are known to produce

metabolites (Robert, 1981; Gillespie and Claydon, 1989; Strasser *et al*., 2000). T. *harzianum* is traditionally used to control fungal pathogens. However, some strains may have antagonistic activities to insects. It is a powerful producer of antifungal metabolites, which could be responsible for the inhibitory response toward the *B. bassiana* strains revealed in our study (Ghisalberti and Sivasithamparam, 1991; Goettel *et al*., 2008). This is similar to results described by Shields et *al*. (1981), Wainwright et *al*. (1986) and Castlebury *et al*. (1999). Inhibition of other fungi through the production of enzymes by *T. harzianum* has been also reported in many studies. The chitinolytic enzymes from *T. harzianum* control a wide range of fungi (Lorito, 1993). In this regard, the inhibitory response of the *in-vivo* growth showed by TKD toward the *B. bassiana* strains could have been due to its chitinolytic enzymes production.

There is no prior information on the relationship between *B. bassiana* and *T. harzianum* after being inoculated together, using various application methods. Although much has been accomplished in the development of *B. bassiana* and *T. harzianum* as epiphytes and endophytes for the suppression of plant diseases and pests, more still need to be done to understand the relationship between *B. bassiana* and *T. harzianum*. The use of these two fungi in various inoculation methods may overcome some of the challenges faced in plant pest control. In conclusion, this study clearly showed that there was a powerful inhibitory action by TKD toward the endophytic *B. bassiana* strains in the *in vivo* bioassays that were performed. The inhibitory response of TKD toward the endophytic *B. bassiana* strains varied, depending on the inoculation methods used, and their timing. These results may constitute an important step in our understanding of the potential dual role, and the relationship between the two fungi. Further studies could be done to determine the metabolites and enzymes responsible for the inhibitory response observed between the fungi.

The use of two or several methods of control can be more effective for managing several pests and diseases of crops than using a single product. It was found in this study that strains of the biocontrol agent, *B. bassiana*, were endophytic inside the stems and roots of sorghum and rice plants when applied alone, or were applied 7 days before TKD was applied as a drench treatment. Most of *B. bassiana* strains were killed before they could colonize host plants, when they were inoculated concurrently with TKD. By managing their antagonism by sequential applications, both biocontrol agents can be used on a single crop to manage fungal root diseases and stem borers' attacks.

6.5 References

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THESIS OVERVIEW

Introduction

Sorghum and rice (*Sorghum bicolor* L. and *Oryza sp*.) are two of the major cereals grown across the world (Mendez, 2010; Ramatoulaye *et al.*, 2016), to produce grains for human consumption and animal feed (Rooney and Waniska, 2000; Schalbroek, 2001; Taylor, 2003; Dahlberg *et al*., 2011; Partnership, 2013). About 167,000 tons of sorghum are consumed each year in South Africa. In West Africa, rice consumption is increasing at an annual rate of 4.5% (AfricaRice, 2010). The current import costs of rice to African countries are at least $4 billion per year for approximately 10 million tons of rice (AfricaRice, 2010), indicating the economic importance of rice in the lives of many Africans. More than 17.0 Mt of milled rice are consumed annually in West Africa, while the production for the same period is 13.2 Mt (AfricaRice, 2008). Therefore, West African countries have to imports about 4 Mt p.a. to meet 40% of their rice demands (AfricaRice, 2010).

Both of these crops are subjected to abiotic and biotic constraints. The insect pests are the most important biotic stress that affects sorghum and rice at all of their growth stages. Stem borers from the family of Lepidoptera (e.g., *Chilo partellus* and *Sesamia calamistis*) are the most important pests, and significantly reduce the yields of both cereals. Several control measures have been advocated for the management of stem borers. Most control measures, including chemical control, are only partially effective. Furthermore, chemical control is not affordable for most smallholder farmers. Biocontrol agents may provide an alternative option for their control. This study was therefore initiated to investigate the use of strains of a fungal entomopathogen, *B. bassiana*, looking for endophytic colonization of sorghum and rice stems, a pathogenic effect against the two stem borers, *C. partellus* and *S. calamistis*. The study was undertaken also to evaluate the compatibility of a widely used biocontrol agent, *Trichoderma harzianum* with *B. bassiana* strains, to understand whether they could be used together to control root diseases and stem borers, respectively.

Findings from the study

The major findings of the research are presented and discussed within the context of their implications for future research and development. The first findings presented in this thesis resulted from the evaluation of the endophytic competence of multiple strains of *B. bassiana* in sorghum and rice cultivars. Two inoculation methods (seed and foliar applications) were used to establish whether 20 *B. bassiana* strains had the potential to establish themselves as

endophytes in sorghum and rice plants. Out of the 20 *B. bassiana* strains tested, five were endophytic in both crops and both stems and roots, regardless of the inoculation method, with varying degree of colonization and persistence. The greatest persistence of colonization resulted from seed treatments. Significant differences were recorded between the application methods, the strains and the cultivars (P = 0. 0001). With both application methods, significant interaction effects were also recorded between cultivars x strains, strains x time of application method and strains x cultivars x time of application. These findings confirmed that *B. bassiana* can form an endophytic relationship with sorghum and rice plants when their seeds or seedlings were inoculated, and that they caused no harmful effects, as revealed in many other studies (Wagner and Lewis, 2000; Akello *et al*., 2007b; Posada *et al*., 2007 and Ownley *et al*., 2008). Our study showed also that there are several possible pathways to inoculate and recover *B. bassiana* from plant tissues, as revealed by Gurulingappa *et al*. (2010).

The efficacy of the best five endophytic *B. bassiana* strains were investigated against *C. partellus* and *S. calamistis*. Most strains were highly pathogenic on 3rd instar larvae of both *C. partellus* and *S. calamistis*. However, their virulence varied: on sorghum, three of the five *B. bassiana* strains (Bb3, Bb10 and Bb35) controlled the 3rd instar larvae of *C. partellus* and caused high mortality levels. On rice, three *B. bassiana* strains (Bb4, Bb10 and Bb35) provided effective control of the 3rd instar larvae of *S. calamistis* by causing high mortality levels. These findings are similar to those documented in previous studies that demonstrated the potential of *B. bassiana* as biocontrol agent against *C. partellus* (Odindo *et al*., 1989; Hoekstra and Kfir, 1997; Cherry et *al*., 1999).

The efficacy of the endophytic *B. bassiana* strains and a pesticide, Karate (lambda cyhalothrin) were tested for the control of sorghum stem borer in the field. Damage (dead heart and chaffy panicles) caused by the stem borer, *Chilo partellus*, was reduced when the seeds were treated with the five *B. bassiana* strains, and the plants were sprayed with Karate fortnightly. The treated sorghum plants produced partially chaffy panicles, compared to completely chaffy panicles in the untreated control. Plant traits such as the number of panicles and panicle weight of the sorghum plants treated with *B. bassiana* strains and Karate were greater than those of the untreated control plants.

In order to assist farmers better, who might want to combine two successful biological control agents such as *Trichoderma harzianum* Rifai Strain Kd (TKD) and *B. bassiana* strains for root and stem protection, the compatibility of TKD with five systemic strains of *B. bassiana* was investigated. *In vitro* dual culture bioassays TKD and the five *B. bassiana*

strains showed that there was an interaction between the fungi strains 4 days after inoculation. At 7 days, post-inoculation only one *B.bassiana* strain (Bb35) growth was not inhibited by TKD. TKD strongly inhibited all the *B. bassiana* strains at 15 and 30 days post-inoculation.

In trials on plants concurrent inoculation of TKD and the five *B. bassiana* strains, TKD inhibited all of the *B. bassiana* strains when they were applied as seed treatments, using a mixed inoculum of the fungi. However, satisfactory colonization of the stems of treated plants by *B. bassiana* occurred when the strains were inoculated as seed treatments and the *T. harzianum* strain was inoculated only 7 days later using a drench treatment onto growing plants.

Conclusion and recommendations

The primary objective of this thesis was to isolate and screen strains of *B. bassiana* for the biological control of two stem borers of sorghum and rice (*C. partellus* and *S. calamistis*), respectively. The most effective inoculation method to ensure colonization of the stems of sorghum and rice plants was seed treatment. It also provided for the most control of the two stem borers. Further research is needed to determine the mechanism of infection of the borers. Large-scale field trials at multiple sites and in multiple seasons need to be done. Field trials will be conducted on both paddy rice and upland rice, using the same methods. Trials need to be conducted to evaluate fertilized versus unfertilized crops. The cost of applications need to be quantified versus the value of the increased yields achieved in practice. Evaluation of endophytic behaviour of the *B. bassiana* strains in other cereals (such as maize, millet, and sugar cane) and their efficacy against other borers, such as African Rice Gall Midge (AfRGM) on rice and *Busseola fusca* on sorghum need to be determined.

It would be important to work with industry partners to create the best possible formulations of *B. bassiana* as commercial products for use on agricultural crops. Obstacles that would need to be overcome include large-scale production, harvesting of conidia, shelflife issues, optimizing formulations, optimizing storage conditions, balancing cost with affordability and international registrations and logistics. For this to become a reality there is a need for the further development of appropriate selection criteria for the best *B. bassiana* strains, and for strategies leading to product formulation and implementation.

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APPENDIX 1

(*TRICHODERMA* SELECTIVE MEDIUM) (TSM) (Composition)

The basal medium used consisted of the following:

$MgSO_4.7H_2O$ = 0.2g

K_2HPO_4 = 0.9g

KCl = 0.15g

NH_4NO_3 = 1.0g

Glucose (anhydrous) = 3.0g

Rose Bengal = 0.15g

Agar = 20.0g

The constituents were added to 900ml of distilled water and autoclaved at 121°C for 15min.

The biocidal ingredients were:

Chloramphenicol (Crystallized) = 0.25g

Quintozene (PNCB wettable powder) = 0.2g

Captan (Kaptan wettable powder) = 0.2g

Propamocarb-hydrochloride (Previcur, solution concentrate) = 1.2ml

Mix the ingredients in 50ml of sterilized (autoclaved at 121°C for 15min) distilled water and added to the autoclaved basal medium.

APPENDIX 2

***Beauveria bassiana* selective medium**

A- Potato Dextrose Agar (PDA) = 39g

B- Yeast Extract = 2g

C- Dodine = 1.1g

D- Antibiotic ingredients (Streptomycin = 100mg/L and Ampicillin = 100mg/L)

The ingredients A and B are mixed together in 1L of sterilized distilled water and autoclaved at 121°C for 15min.

Mix the ingredient C in 1L separately and Autoclaved at 121°C for 15min.

The autoclaved constituents were mixed together with the antibiotics ingredients