Sampling and evaluation of entomopathogenic fungi for control of *Bathycoelia distincta* (Hemiptera: Pentatomidae) in South Africa

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DECLARATION BY THE CANDIDATE

I, LC. Linda, declare that the work presented in this MSc. thesis is my own work, that it is not been submitted for any degree or examination at any other University and that all the sources I have used or cited have been acknowledged by the complete reference.

Signature  Date: 10 December 2020

DECLARATION AND APPROVAL BY SUPERVISORS

We declare that the work presented in this thesis was carried out by the candidate under our supervision and we approve this submission.

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Abstract

The two-spotted stink bug, *Bathycoelia distincta* (Distant) (Hemiptera: Pentatomidae), is the most abundant stink bug species in macadamia orchards in South Africa. This pest is associated with serve macadamia nut damage and is mainly controlled with insecticides, placing selection pressure on the pest, which can result in resistance development. Chemical control also poses potential health and environmental risks, and alternative pest management tactics are therefore required. The use of effective entomopathogenic fungi (EPF), which are naturally occurring and environmentally friendly biological control agents with insecticidal action, may reduce these negative effects. The first aim of this study was to isolate and identify indigenous EPF isolates from *B. distincta* bugs in macadamia orchards. A survey was conducted in an unsprayed macadamia orchard, as well as on research and commercial farms in the Mpumalanga province in South Africa. *Beauveria bassiana* and *Purpureocillium lilacinum* were the only EPF isolated from naturally occurring stink bugs. Other insect-associated fungi genera isolated, were *Aspergillus, Chaetomium, Lasiodiplodia, Penicillium, Rhizopus, Trichoderma, Myriodontium, Fusarium, Epioccum, Talaromyces, Bionectria, Gelasinospora, Nigrospora* and *Pestalotiopsis.* The second aim, to assess the effect of temperature on growth of 11 of these *B. bassiana* isolates obtained, showed the optimal temperature for radial growth to be between 25-30 °C. Seven of these isolates did not differ significantly in terms of radial growth. To select the most virulent isolate for further evaluation under semi-field conditions, five *B. bassiana* isolates (PPRI 26695, 26696, 2667, 26700 and 26704) were selected and tested under laboratory conditions for their ability to infect and kill *B. distincta* adults. All five of these isolates were pathogenic towards *B. distincta*. The level of pathogenicity varied between the isolates and the mortalities caused by *B. bassiana* increased with time after application. *Beauveria bassiana* isolate (PPRI 26695) was selected for further assessment, based on 100 ± 8.61% efficacy of *B. distincta* control, mycosis of 86.67%, a LT_{50} of 6.99 and LT_{90} of 12.85 days. In the semi-field trial, the commercial *B. bassiana* product (Eco-Bb®) (not registered for control of stink bugs in South Africa), the experimental *B. bassiana* isolate, PPRI 26695 and insecticide, cypermethrin were compared for control of *B. distincta* adults. All three of these products provided effective control, with PPRI 26695, Eco-Bb® and cypermethrin that caused mortalities of 100, 90 and 80%, respectively, 18 days after treatment. The potential of *B. bassiana* for control of *B. distincta* was demonstrated in this study and further research in this regard is justified.

Keywords: *Bathycoelia distincta, Beauveria bassiana,* entomopathogenic fungi, laboratory bioassay, temperature, virulence

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Chapter 1

Introduction and literature review

1.1 Background

Macadamia F. Muell. (Proteaceae), are dicotyledonous, medium sized (20 m tall and 13 m wide) nut trees (Augstburger *et al.,* 2002; Gitonga *et al.,* 2008). The genus comprises of nine species with nuts of only two of these, *viz*. *M. integrifolia* Maiden & Betche and *M. tetraphylla* L.A.S Johnson, being edible (Abubaker *et al.,* 2018). The leaves of *M. integrifolia* have entire margins and very few spines, three leaves occur at a node and the nuts have a smooth shell (Alam *et al.*, 2018). The leaves of *M. tetraphylla* are spinier compared to *M. integrifolia,* four nearly sessile leaves occur in a whorl and the nutshell is rough (Alam *et al.*, 2018).

Macadamia is indigenous to Coastal-East Queensland and Northern New South Wales in Australia (Srichamnong, 2012). It served as an important source of food for the native inhabitants of the area (Augstburger *et al*., 2002, Carr, 2013; Alam *et al.*, 2018). Macadamia was introduced into Hawaii in the United States of America (USA) in the 1880's, but commercialisation was only established between 1930-1940 (Shigeura and Ooka, 1983; Barrueto *et al.,* 2018).

New macadamia varieties have been developed through the selection of superior germplasm, which supports sustainable crop production (Hardner *et al*., 2019). These varieties have improved agronomical traits and/or improved disease and pest resistance and some can tolerate a variety of environmental conditions (Langdon *et al.,* 2020). Macadamia is cultivated commercially in South Africa, USA (Hawaii), Guatemala, Costa Rica, Brazil, Kenya, Malawi, Zambia, Zimbabwe, Uganda, Thailand, Vietnam, Indonesia and China (Srichamnong, 2012). Due to the increase in popularity of macadamia, the demand continuously exceeds the supply (Augstburger *et al*., 2002). Macadamia nut quality is based on its physical appearance and oil content. Palmitoleic oil is a major component of macadamia nuts and its associated health benefits are used to gain leverage in various markets (Phatanayindee *et al*., 2012).

1.2. Macadamia production practices

Macadamia are best suited to altitudes lower than 600 m a.s.l. Trees planted at high altitude (above 600 m a.s.l.) grow slower, which results in yield reduction and these trees often yield nuts with a thick shell (Wiid-Hobson, 2003). Macadamia grows in a variety of soil types, but the trees flourish in deep, well-drained soil with high organic matter (Kuperus and Abercrombie, 2003). A loose, deep loam soil is preferred, with a pH between 5.0 - 6.5 (optimum 5.0 - 5.5) (Augstburger *et al*., 2002). Macadamia cultivars grow either upright or they spread (Wiid-Hobson, 2003). The plant spacing recommendation for upright growers is 2 - 2.5 m in-row spacing and 4.5 - 5 m work-row spacing, and for spreaders, 3 - 3.5 m in-row spacing and 6 - 7 m work-row spacing (Snijder, 2003).

Macadamia trees develop multiple leaders and therefore need to be trained, from 6 months after planting, to a central leader that supports wide-angled whorls. Trees require minimal pruning, but the 2 m alley between rows has to be maintained, as well as a manageable tree height (O'Hare *et al.,* 2004; McFadyen *et al.,* 2016). For optimal production, suitable fertilizer is required to maintain nutrient concentrations at the recommended level (Galanti and Cho, 2019). Lime is recommended to correct soil acidity since it contains calcium and magnesium, while gypsum is a preferred source of calcium and sulphur (Manson and Sheard, 2007). Application of fertilizers to macadamia can be through broadcasting, it can be placed in bands, or applied as sprays onto the foliage or through the irrigation system (Thomas, 2017). The pest and disease levels in orchards are reduced by means of an Integrated Pest Management (IPM) approach which includes cultural, biological and chemical control (Bright, 2018).

1.3 Nutritional value of macadamia

In the past decade, global macadamia production has continuously increased, faster than any other tree nut crop (Quiroz *et al.,* 2019; Langdon *et al.,* 2020). Currently, macadamia accounts for ±3% of global nut trade (Langdon *et al.,* 2020). The macadamia kernel has a high content of fatty acids, predominantly with oleic acid (±60%) and palmitoleic acids (±20%) which are both beneficial for human health (Aquino-Bolañosa *et al.,* 2016). Macadamia nuts can boost vascular health by efficiently reducing total cholesterol and low-density lipoproteins (Aquino-Bolañosa *et*

al., 2016). Macadamia vegetable oil is a potential source for biodiesel production and the nut shell could be a source of tar oil (Azad *et al*., 2017).

1.4 Worldwide macadamia production

The macadamia nut industry continues to grow rapidly worldwide. The global market size of the industry is estimated at US$ 822 million (Botha, 2018). The major macadamia producing countries are South Africa, Australia, Kenya, China, USA, Guatemala, Malawi, Brazil, Vietnam and Colombia (Table 1.1). These countries account for 80% of the world macadamia production. South Africa is the largest macadamia producer followed by Australia, Kenya and China (Botha, 2018; Quiroz *et al.,* 2019). The global trend of eating healthy food, contributed to the increase in global demand for these luxurious nuts and to increase in macadamia production (Abubaker *et al.,* 2018; Venter, 2019). The demand for macadamia are expected to remain high (Venter, 2019). Consequently, the worldwide production of macadamia is projected to surpass 700 000 MT with South Africa producing approximately 330 300 MT by 2025 (SAMAC, 2019).

Table 1.1: Macadamia nut production figures (MT), in shell as well as kernels during 2018 and 2019 (From SAMAC, 2019).

	2018		**2019**	
	In shell	**Kernel**	**In shell**	**Kernel**
South Africa	56 550	16 965	58 500	17 550
Australia	49 300	14 800	51 700	15 500
Kenya	38 500	7 750	32 000	8 389
China	21 400	6 000	29 962	6 400
USA	16 957	4 239	18 000	4 000
Guatemala	11 500	2 150	12 250	2 600
Malawi	6 980	1 619	7 678	1 781
Brazil	6 200	1 550	7 000	1 750
Vietnam	1 200	360	1 450	435
Colombia	1 350	338	1 000	250
Others	13 600	3 536	15 350	3 991
World total	**223 537**	**59 307**	**234 890**	**62 646**

1.4.1 South African macadamia industry

1.4.1.1 Cultivation areas and production

Macadamia was commercialised for the first time in South Africa during the 1960's when 60 000 *M. tetraphylla* seedlings were planted in the eastern seaboard area. Since then the industry has grown rapidly (Allan, 1996). Currently, approximately 44 776 hectares of macadamia have been established locally (SAMAC, 2020). Macadamia is mainly grown in three provinces, *viz*. Mpumalanga, the main macadamia nut producing province, followed by the Limpopo and KwaZulu-Natal provinces (South and North coast) (Botha, 2018). In 1991, 1 211 MT macadamia was produced and 58 500 MT in 2019 (SAMAC, 2019). Approximately 98% of annual production is exported to countries in Asia, Europe and North America with a total revenue of ZAR 4.8 billion during 2019 growing season (SAMAC, 2020).

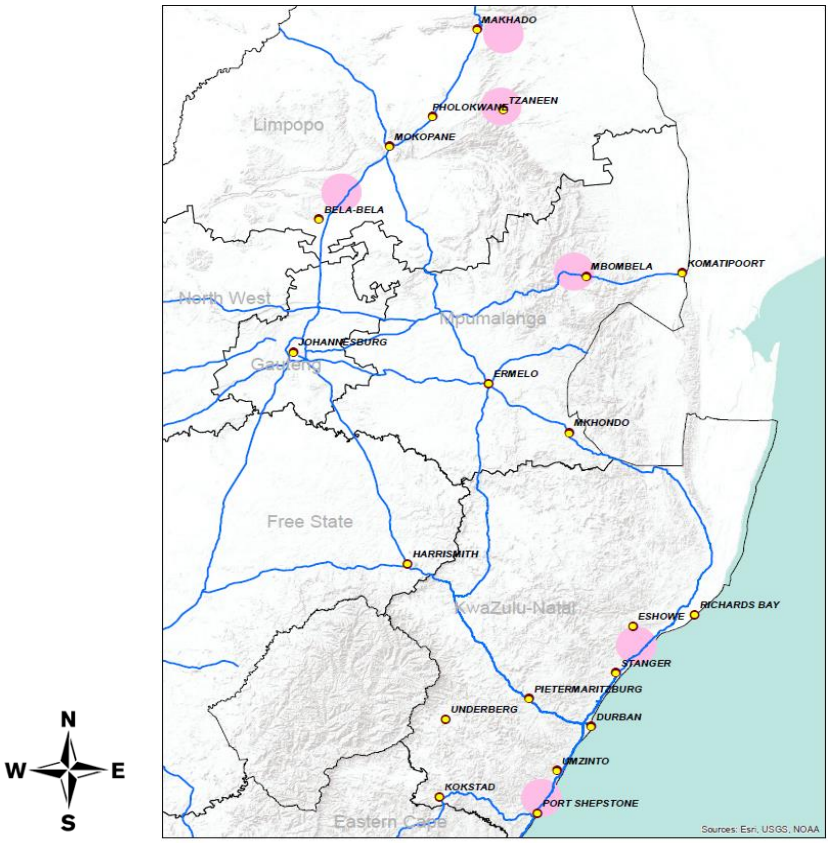


Figure 1.1: Macadamia producing areas in the main production region in South Africa, which is situated in the Mpumalanga, Limpopo and KwaZulu-Natal provinces (From Sourcebi, 2020).

1.4.1.2 Threats and challenges facing macadamia production in South Africa

With the increase in hectares planted to macadamia, the severity of diseases and pests associated with the crop, also increased (Schoeman, 2013). Although increases in crop production are achieved by varietal enhancement of genetic material, it is often restrained by diseases (viruses, bacteria and fungi) and pests, in particular insects, that can reduce nut quality and quantity (de Villiers *et al*., 2003; Akinsanmi, 2016; Wrona *et al*., 2020). Researchers, producers and related industries are, however, addressing these challenges to improve and ensure sustainable macadamia production (Schoeman, 2011; Azad *et al.,* 2017; Dardagan, 2018). For instance, fertilisers and pesticides are applied to improve tree and nut health (Thomas, 2017).

1.5 Insect pests and diseases of macadamia

Insect pests are considered as an economically important, limiting factor in macadamia crop production worldwide (van den Berg *et al.,* 1999; O'Hare, 2004; Schoeman, 2014b). In South Africa, more than 60 insect pests or potential pests are associated with macadamia crops, of which approximately 40 are heteropterans (Schoeman 2014b; Schoeman and Millar, 2018). Six species *viz*. the Afrotropical macadamia nut borer, *Thaumatotibia batrachopa* (Meyrick) (Lepidoptera: Tortricidae), false codling moth, *T*. *leucotreta* (Meyrick) (Lepidoptera: Tortricidae) (Timm *et al*., 2006; van der Merwe *et al*., 2017), coconut bug, *Pseudotheraptus wayi* (Brown) (Hemiptera; Coreidae) (Schoeman, 2013), macadamia felted coccid, *Eriococcus ironsidei* (Williams) (Hemiptera: Eriococcidae) (Zarders and Wright, 2016, Schoeman and Millar, 2018), citrus thrips, *Scirtothrips aurantii* Faure (Thysanoptera: Thripidae) (Thackeray *et al.,* 2015) as well as the two-spotted stink bug, *Bathycoelia distincta* (Distant) (Hemiptera: Pentatomidae) (Schoeman, 2013) are considered as major pests of macadamia in the country. Five of these species are endemic to Africa, with the exception of *E. ironsidei*, which is indigenous to Australia and was unintentionally introduced into South Africa (Schoeman and Millar, 2018).

The most common disease of macadamia caused by fungal infection include Botrytis blight (*Botrytis cinerea*), macadamia husk spot (*Pseudocerospora macadamiae),* Phytophthora blight (*Phytophthora capsici*), steam canker (*Phytophthora*

cinnamomi), rat tail disease (*Cladosporium*), blossom blight (*Botrytis cinerea*) and anthracnose (*Colletotrichum gloeosporiodes*) (De Villiers and Joubert, 2003).

1.6. The two-spotted stink bug

1.6.1. History, description and distribution in South Africa

The two-spotted stink bug, *B. distincta* (formerly known as *B. natalicola*), is indigenous to South Africa, and was reported for the first time in the country on macadamia in Levubu (Limpopo province) in 1984 (Bruwer, 1992; Schoeman, 2014a). *Bathycoelia distincta* and the yellow-spotted stink bug (*B. rodhaini* Schouteden) (Hemiptera: Pentatomidae) are morphologically similar and are therefore often confused (van den Berg *et al.,* 1999). However, the adults of the two-spotted stink bug are pale green in colour with a distinct white dot at each of the two top corners of the scutellum (Figure 1.2a). The yellow-spotted bug is pale, with yellow dots at the top corners of the scutellum (Figure 1.2b) (van den Berg *et al.,* 1999). *Bathycoelia rodhaini* is a sporadic pest that seldom occurs in unmanaged orchards and requires minimal management. *Bathycoelia distincta,* on the other hand, is a migratory pest with a wide host range which requires annual management (Schoeman, 2009; 2013; 2014a).

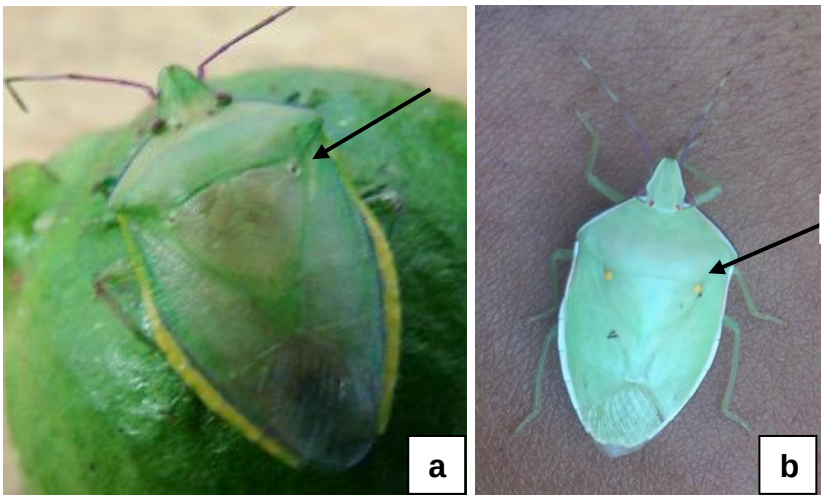


Figure 1.2: The morphological features of (a) the two-spotted stink bug and (b) the yellow-spotted stink bug. The arrows indicate the distinct spots to distinguish the two species (Photo: P.S. Schoeman, ARC-TSC).

Bruwer (1992) found *B. distincta* during a comprehensive study of heteropteran pests of macadamia in the Limpopo province. It was also reported in a follow-up

study by van den Berg *et al.* (1999) in Nelspruit, in an unsprayed orchard and at two commercial farms in Glenwood and Schagen in the Mpumalanga province. Since then, occurrence of *B. distincta* has also been documented in other major macadamia producing areas such as White River (Mpumalanga), Barberton (Mpumalanga), Kiepersol (Mpumalanga), Hazyview (Mpumalanga), Levubu (Limpopo), Letaba (Limpopo), Tzaneen (Limpopo) and surrounding areas, and in KwaZulu-Natal (PS Schoeman, pers. comm.). Little is known on the occurrence of *B. distincta* in the Eastern Cape province and other macadamia producing areas. According to Jones (2002*), B. distincta* is also common in countries such as in Malawi, Kenya (Muthoka *et al.,* 2008), Zimbabwe, Mozambique and Tanzania (PS Schoeman, pers. comm.).

1.6.2 Life stages

1.6.2.1 Eggs

Eggs of pentatomids are easily recognized by their barrel-shape (Matesco *et al.,* 2009). Eggs are always deposited in clusters and an operculum, which is surrounded by a ring of aero-micropylar processes, lifts during hatching (Matesco *et al.,* 2009). Females of *B. distincta* oviposit eggs in clusters which consist of 14 eggs firmly glued together in rows to a substrate (Figure 1.3a and b). *Bathycoelia distincta* females lay eggs throughout their adult stage. The eggs are small but visible to the naked eye, measuring ±2.5 mm in diameter (Bruwer, 1992). Newly laid eggs are light green in colour, but become cream to pink with development, and two red spots are prominent before hatching (Bruwer, 1992) (Figure 1.3b). Under optimal conditions, eggs hatch within 6-7 days. The eggs are laid on the main stem, leaves, branches, fruit and stalks of a host plant (Bruwer, 1992).

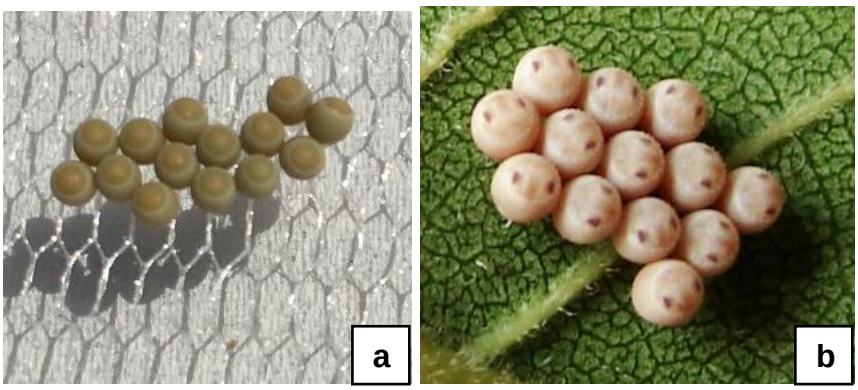


Figure 1.3: (a) Newly laid eggs of *Bathycoelia distincta*, (b) eggs firmly attached to a leaf with the two red spots visible prior to hatching (Photo: P.S. Schoeman, ARC-TSC).

1.6.2.2 Nymphs

After hatching, the two-spotted stink bug passes through five nymphal stages (Figure 1.4a-e). The nymphs are oval in shape, the first three nymphal instars are predominately black with a distinctive yellow and red pattern (Figures 1.4a-c). The fourth and fifth instar nymphs are lime coloured (Figures 1.5d-e). The neonate nymphs do not feed, but they aggregate on or around empty eggshells to acquire beneficial microbial symbionts (Bruwer, 1992; Oishi *et al.,* 2019). These symbionts provide amino acids and vitamins, which are essential for the development of nymphs, facilitate in resistance to natural enemies and degrade toxic chemicals (Taylor *et al.,* 2014, Oishi *et al.,* 2019). However, if the first instar nymphs are prevented from acquiring these microbial symbionts, the normal development is arrested and high levels of mortality occur (Bruwer, 1992; Taylor *et al.,* 2014). After 4-5 days, the first instar nymphs move away from the eggshells, but remain gregarious (Bruwer, 1992). According to Panizzi and Slansky (1985) and Bruwer (1992), this gregarious behaviour of the nymphs can be attributed to mutual and physical defence, that speeds up development and reduces mortality. At the fourth instar, all gregarious behaviour maintained during the first three instars disappears completely (Panizzi and Slansky, 1985).

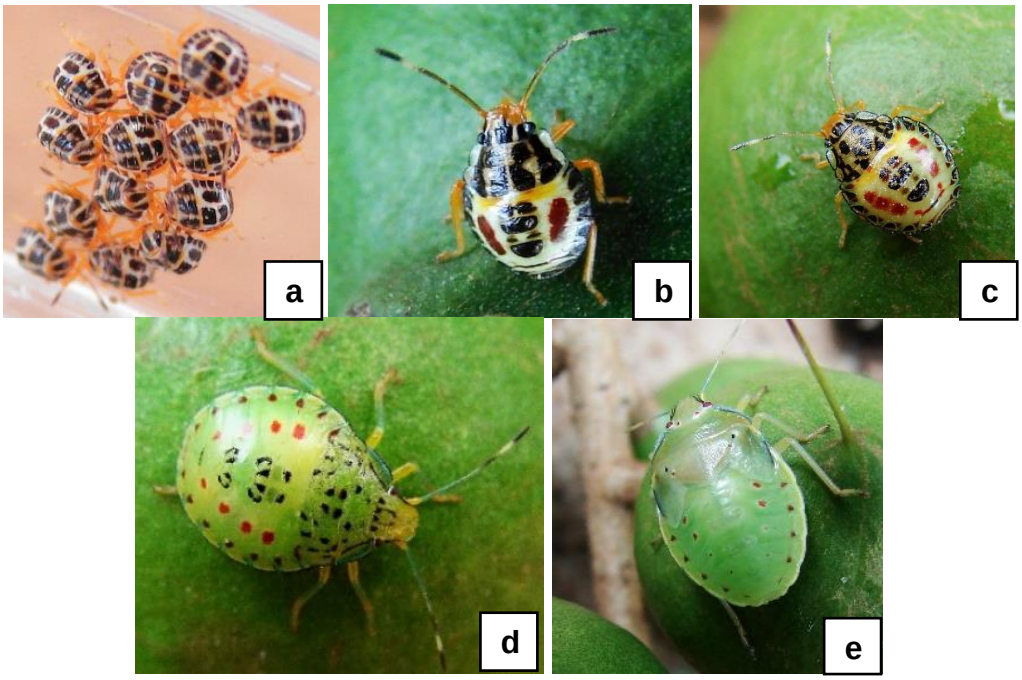


Figure 1.4: The different nymphal stages of *Bathycoelia distincta*; (a) gregarious first instar, (b) second instar, (c) third instar, (d) fourth instar and (e) fifth instar (Photo: P.S. Schoeman, ARC-TSC).

1.6.2.3 Adults

The growth rate of the two-spotted stink bug is highly dependent on temperature and the quality of the food source (Bruwer, 1992). A total development time, from egg to adult, of 43.58 days for *B. distincta* reared on green bean pods, at a constant temperature of 25 ± 2 °C and 75 ± 5% humidity (Bruwer,1992). Males (11 – 14 mm) are smaller than females, which are approximately 18 mm long (Bruwer, 1992) (Figure 1.5).

Figure 1.5: *Bathycoelia distincta* adults copulating (Photo: L. Linda, ARC-TSC).

Male and female stink bugs are distinguished by examining the ventral region near the tip of the abdomen. Males have claspers on the last abdominal segment that gives it a forked appearance (Jones, 2002) (Figure 1.6).

Figure 1.6: a) Ventral side of the abdomen of a stink bug female without claspers, and b) male, with claspers on the last abdominal segment. (From V.P. Jones, University of Hawaii).

1.6.3. Economic importance

Bathycoelia distincta is the most important pest of macadamia in South Africa (Schoeman, 2013; Schoeman, 2014a). From the second instar nymphs as well as adults of the two-spotted stink bug, attack macadamia. They extract fluids from the nuts with their piercing-sucking mouthparts (Daane *et al.,* 2014). This feeding damage may also introduce mould and fungi into the kernel, which causes crop loss as a result of premature nut abortion as well as lesions on mature nuts (van den Berg *et al*., 1999; Jones, 2002) (Figure 1.7a). The injuries inflicted by stink bugs to the crop, therefore not only result in premature nut drop, but also deformed and poor quality nuts, rendering the fruit not suitable for the market (Golden *et al.,* 2006; Schoeman, 2009) (Figure 1.6b). In South Africa, the two-spotted stink bug is associated with annual macadamia losses estimated at ZAR180 million (SAMAC, 2020). As a result, this major revenue loss incurred by the macadamia industry, necessitates the development of strategies to protect crops against this pest.

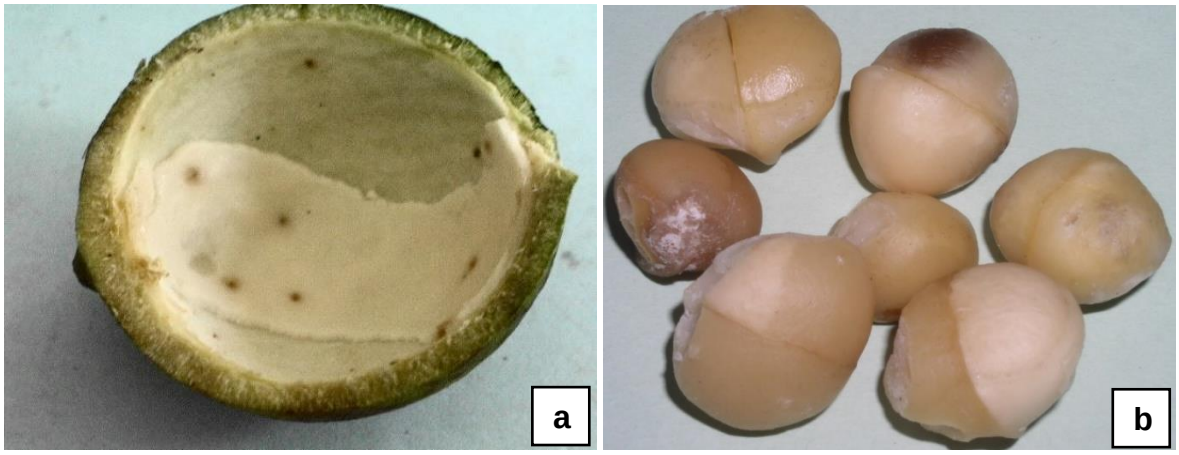


Figure 1.7: *Bathycoelia distincta* damage to (a) the husk, and (b) kernel of macadamia. (Photo: P.S. Schoeman, ARC-TSC, South Africa).

1.7 Control of the two-spotted stink bug

Monitoring and management of stink bugs in macadamia orchards are a challenge, since these pests are highly mobile (Krupke *et al.,* 2001; Daane *et al.,* 2014). The main strategies to reduce stink bug populations in the macadamia orchards include the use of insecticides, trap crops and biological control (Schoeman, 2014b).

1.7.1 Monitoring and scouting

It is very difficult to manage and monitor stink bugs, due to their high mobility and the lack of reliable monitoring techniques (Krupke *et al., 2001*). In addition, the goal of monitoring is often not well defined, and economic injury levels for different kinds of damage symptoms are not known. The counts of bugs obtained through monitoring is therefore difficult to put into a context of practical significance (Daane *et al.,* 2014). The primary goal of any monitoring program is not simply to measure pest abundance, but the ability to use the sampling method frequently, and to accurately detect small numbers of bugs soon after their arrival (Daane *et al.,* 2014). The following monitoring techniques were suggested by Schoeman (2011) for hemipteran pests in macadamia orchards:

i) Branch shaking: Randomly selected trees are shaken early in the morning and bugs that drop to the ground are collected, identified and recorded on scouting sheets.

ii) Knock-down: Chemical knock-down of stink bugs using dichlorvos.

iii) Egg scouting: Inspection of the main tree trunk for stink bug egg clusters.

iv) Nut dissection: Dissection of aborted nuts and inspection of husks and kernels for stink bug puncture marks.

These techniques are, however, not currently widely used by industry (Pers. obs.).

1.7.2 Chemical control

Chemical control is regarded as the most efficient and widely used strategy to control or prevent the outbreak of agricultural pests (Cardoso and Alves, 2012; Saeed *et al*., 2019). A limited number of insecticide active ingredients, belonging to only five Insecticide Resistance Action Committee (IRAC) groups, are currently registered for control of stink bugs on macadamia in South Africa (Table 1.2). Targeted insect pests are, however, known to evolve resistance to some insecticides due to the continuous use of these products (Wilson and Tisdell, 2001). Furthermore, there is continuous withdrawal of synthetic insecticides due to their high levels of toxicity towards beneficial, non-targeted insects, effects on other insects as well as to the terrestrial and aquatic ecosystems (Hepburn, 2015; Fair Trade USA, 2017). Due to poor spraying coverage in the apical regions of macadamia trees, it is speculated

that *B. distincta* has developed resistance against synthetic pyrethroids (Schoeman, 2014b; Schoeman, 2018). Various stink bug populations became less susceptible to acephate, endosulfan, organophosphate metamidophos, thiamethoxam and imidacloprid in Brazil (Sosa-Gomez *et al*., 2020).

Table 1.2: Chemical class, active ingredients and Insecticide Resistance Action Committee group for insecticides currently registered for the control of stink bugs on macadamia in South Africa (Agri-Intel, 2020).

Chemical class	**Group code**	**Active ingredient**
Diamides + pyrethroid	28 + 3A	Chlorantraniliprole + lambda-cyhalothrin
Microbial	11A	*Beauveria bassiana*
Neonicotinoid	4A	Clothianidin
		Thiamethoxan
Organophosphate	1B	Acephate
		Chlorpyrifos
	1B + 3A	Chlorpyrifos + cypermethrin
Pyrethroid	3A	Alpha-cypermethrin
		Beta-cyfluthrin
		Beta-cypermethrin
		Cypermethrin
		Esfenvalerate
		Gamma-cyhalothrin
		Lambda-cyhalothrin
		Tau-fluvalinate
Pyridine azomethine derivatives	9B	Zeta-cypermethrin
		Pymetrozine

1.7.3 Cultural control

Cultural control is aimed at reducing pest populations below economic injury levels by disrupting reproduction, dispersal and survival or allowing natural enemies to take effect (El-Shafie, 2018). In this regard, mechanical control and trap crops are often employed to control the two-spotted stink bug (Steyn, 2012). Stink bugs prefer to feed in the apical portions when trees become taller (Schoeman, 2014b). Therefore, reducing tree height and density by moderate pruning significantly reduce stink bug populations in macadamia orchards (Schoeman, 2014b). It is also important that nuts are harvested as soon as they mature, since *B. distincta* prefers fully developed nuts after the shell hardens. The longer the nuts remain on a tree, the higher the risk for damage (Nortjé and Schoeman, 2016).

1.7.3.1 Trap crops

The use of trap crops has the potential to reduce stink bug population levels in macadamia orchards (Steyn, 2012). Steyn (2012) and Radzilani *et al*. (2013) assessed various crops as potential trap crops to attract stink bugs from macadamia and avocado orchards. The trap crops assessed included mustard (*Brassica hirta* and *Brassica juncea*), buckwheat (*Fagopyrum esculentum*), okra (*Abelmoschus esculentus*), sunnhemp (*Crotolaria juncea*), red clover (*Trifolium pratense)*, cowpea (*Vigna unguiculata*)*,* rattle pod (*Crotalaria capensis*) and sunflower (*Helianthus annuus*)*.* Sunnhemp, cowpea and sunflower showed potential for trapping *Nezara viridula* (Hemiptera: Pentatomidae), *Chinavia pallidoconspera* (Hemiptera: Pentatomidae) and *B. distincta* in macadamia and avocado orchards in Levubu (Steyn, 2012; Radzilani *et al.,* 2013). Trap crops also increased the levels of functional biodiversity and beneficial arthropods, which has potential to reduce infestation levels of pest insects (Radzilani *et al*., 2013). However, this management strategy is most effective when used in conjunction with other pest management strategies such as chemical and biological control (Omondi, 2015).

1.7.4 Biological control

Biological control is an important component of Integrated Pest Management (IPM). Control can be achieved by using natural or living organisms to supress the population density of a specific pest resulting in reduced damage inflicted by the pest (Augustyniuk-Kram and Kram, 2012; Zhi-zhi *et al.,* 2019). Natural enemies that are

used to manage insect pests may include wasps and flies (Schoeman, 2009), nematomorphs (horsehair worms), bats (Weier *et al.,* 2019), viruses (Williamson and von Wechmar, 1994) and entomopathogenic fungi (EPF) (de Faria and Wraight, 2007).

1.7.4.1 Bats

Insectivorous bats are important bio-indicators (Jones et al., 2009; Taylor et al., 2013a) and provide biological control services by consuming arthropods, which are considered as major agricultural pests (Noer et al., 2011; Maine and Boyles 2015, Kolkert et al., 2019). In South African macadamia orchards, bats, particularly the Molossidae, prey on stink bugs and are economically beneficial to farmers (Taylor et al., 2013b; Weier et al., 2019). Predation of major insect pests by bats has also been documented in sugar plantations in Swaziland (Noer et al., 2011), as well as in maize fields and pecan orchards in the USA (Brown et al., 2015; Maine and Boyles, 2015).

1.7.4.2 Parasitoids

Parasitoids are organisms living in close association with their host, at the host's expense and eventually causing death of the host (Thierry *et al.,* 2019). Parasitoids may live inside a host as an endoparasitoid, where it grows and then emerges as an adult, while others live as ectoparasites, which paralyse and live on the outside, on the host (Hiroyoshi *et al.,* 2017). In South Africa, the two-spotted stink bug has been reported as a host of about six insect parasitoids *viz*. *Trissolcus* sp. A (Hymenoptera: Scelionidae), *Trissolcus* sp. B, *Pediobius* sp. (Hymenoptera: Eulophidae), *Pachyneuron* sp. (Hymenoptera: Pteromalidae), *Bogosia bequaerti* (Villeneuve) (Diptera: Tachinidae), *Cylindromyia eronis* (Curran) (Diptera: Tachinidae) and *Trichopoda giacomellii* (Blanchard) (Diptera: Tachinidae) (Bruwer, 1992; Schoeman, 2009). Although not scientifically recorded, horsehair worms were observed to parasitise the two-spotted stink bug (PS. Schoeman, Pers. comm.) (Figure 1.7).

Figure 1.8: A parasitic horsehair worm that infected *Bathycoelia distincta*. (Photo: P.S. Schoeman, ARC-TSC).

1.7.4.3 Viruses

Viruses are very rare in pentatomids, but extensive research has been conducted globally on insect pathogenic viruses (Nouri *et al*., 2018; dos Santos *et al*., 2019; Öhlund *et al*., 2019). In South Africa, the first and only viruses reported to infect stink bugs were isolated from *N. viridula,* and named NVV-1 and NVV-2 (Williamson and von Wechmar, 1992). NVV-1 is a single stranded RNA small picorna-like virus with physical properties similar to insect *picornaviruses,* while (NVV-2) is a double stranded RNA virus with properties similar to those of the *Totriviridae* (Williamson and von Wechmar, 1994). Typical infection symptoms include a dehydrated appearance, fluid retention in the wings, deformation of the wings, an abnormally large thorax relative to the abdomen, delayed development, the infected insects appear physically sick, and in some cases, the nymph does not develop past the fourth instar (Williamson and von Wechmar, 1992). These viruses are very contagious and can be transmitted horizontally in stink bug populations through surface contamination or vertically through the eggs (transovarially) (Williamson and von Wechmar, 1992). The yellow-edged and two-spotted stink bugs are hosts for both viruses. These viruses therefore have the potential to be used as biological

control agents to control stink bug populations (Williamson and von Wechmar, 1992; 1994).

1.7.4.4 Entomopathogenic fungi, with a focus on *Beauveria* spp.

Entomopathogenic fungi (EPF) occur naturally in terrestrial ecosystems and there are approximately 1 000 species that occur worldwide (Shrestha *et al.,* 2016; Mora *et al.,* 2017). These microorganisms parasitise the insect host and part of their life cycle is completed inside the host before killing it (Delgado and Murcia, 2011; Mora *et al.,* 2017). The majority of EPF are hyphomycetous, including *Aspergillus, Beauveria, Culicinomyces, Hirsutella, Metarhizium, Nomuraea, Paecilomyces, Tolypocladium* and *Verticillium* (Ingis *et al.,* 2001; Mweke *et al.,* 2016).

Beauveria bassiana (Balsamo) Vuillemin (Ascomycota: Hypocreales), was first discovered in Europe as a disease of silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) in the 18th century and was referred to as the white muscardine disease (Erler and Ates, 2015). The genus *Beauveria* contains about 26 species of which 23 species have been described (Bustamante *et al.,* 2019; Khonsanit *et al.,* 2020). Consequently, only six species, *B. bassiana*, *B. bassiana* cf. clade C, *B. brongniartii*, *B. caledonica*, *B. vermiconia*, and *B. amorpha,* are known and recognised as potential commercial pathogens to control insect pests in agro-ecosystems (Glare, 2004; Wang and Zheng, 2012; Saranraj and Jayaprakash, 2017). *Beauveria bassiana* is the preferred *Beauveria* spp. for biological control in agriculture in temperate regions (Glare, 2004; Wang and Zeng, 2012). Due to the effective control provided by *B. bassiana*, this fungal pathogen has gained interest and has been evaluated for control of various agricultural pests (Allegrucci *et al*., 2017; Davis *et al*., 2018; Nazir *et al*., 2018).

1.8 *Beauveria bassiana*

Beauveria bassiana is an ubiquitous entomogenous fungus (Fig. 1.9b) with a wide host range and geographical distribution (Khonsanit *et al.,* 2020). Using selective media, *B. bassiana* grows as white, later yellowish or occasionally reddish colonies that produces powdery conidia (Zimmermann, 2007) (Fig. 1.8b). Based on worldwide data, *B. bassiana* was reported to infect 707 insect species, comprising of 521 genera and 149 families of 15 orders. It also affects 13 host species of Acarina distributed in 7 genera and 6 families (Imoulan *et al.,* 2017). The 15 insect orders

susceptible to *B. bassiana* are Lepidoptera, Coleoptera, Hymenoptera, Homoptera, Diptera, Hemiptera, Orthoptera, Siphonaptera, Isoptera, Thysanoptera, Mantodea, Neuroptera, Dermaptera, Blattaria and Embioptera (Imoulan *et al.,* 2017).

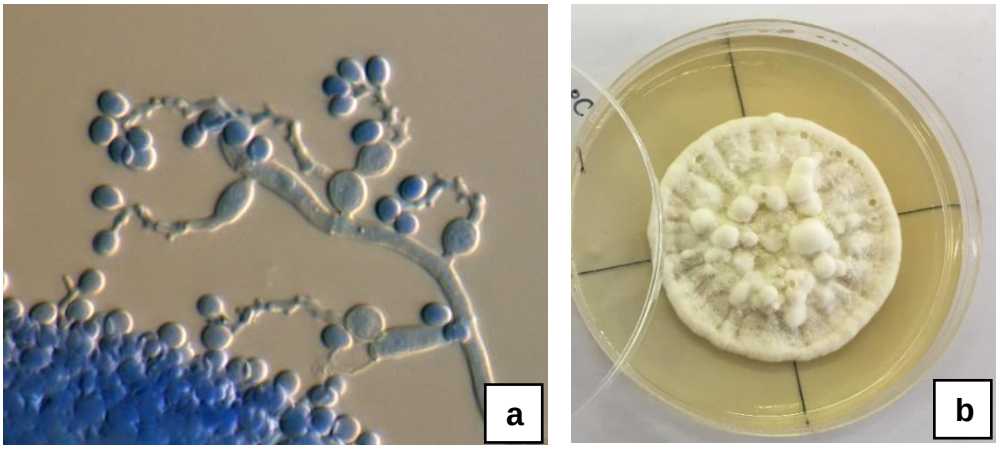


Figure 1.9: (a) A micrograph of *Beauveria bassiana* conidia and conidiogenous cells. (Photo: D. O'Brien, USDA Agricultural Researh Service, Brazil) (b) Fungal growth of *Beauveria bassiana* cultured on Sabouraud Dextrose Agar (SDA) medium.

Table 1.3: Summary of records of pentatomid pests controlled by the entomopathogenic fungus *Beauveria bassiana* and their host plants.

Host Insect	**Host crop**	**Reference**
Rice stink bug, *Oebalus pugnax* (F.)	Rice	Patel *et al.* (2005)
Redbanded stink bug, *Piezodorus guildnii* (Westwood)	Soybean	Parys and Portilla (2020)
Chinavia ubica (Rolston) *Euschistus heros* (Fabricius) *Dichelops melacanthus* (Dallas)	Soybean	Lopes *et al.* (2015); Oliveira *et al.* (2016)
Nezara viridula (Linnaeus)	Soybean, maize, green beans	Lopes *et al.* (2015); Raafat *et al.* (2015)
Bagrada hilaris (Burmeister)	Brassicaceae	Barrera-López *et al.* (2020)
Aelia rostrate (Boh.)	Wheat	Muştu *et al.* (2011)
Plautia stali (Scotto)	Fruit trees	Ihara *et al.* (2001)
Plomena prasina (L.)	Hazelnut fruit	Saruhan *et al.* (2016)

1.8.1 *Beauveria bassiana* mode of action

Beauveria bassiana can be isolated from soil, insect cadavers (Beilharz *et al*., 2002; Saranraj and Jayaprakash, 2017), the surfaces and the interior of plants (Zimmermann, 2007). Ingestion is a common route of infection for pathogens such as protozoa, bacteria and viruses (Schabel, 1976; Mannino *et al*., 2019). However, EPF are the only insect pathogens that are able to invade their hosts by direct penetration of the host cuticle (Mannino *et al*., 2019). Insects have highly heterogeneous cuticle structures that can vary in composition (Ortiz-Urquiza and Kehani, 2013). The cuticle has two layers, of which the outermost layer or epicuticle provides a hydrophobic barrier that is rich in lipids, followed by the procuticle that contains chitin and sclerotised protein (Ortiz-Urquiza and Keyhani, 2013). The entry of fungi into the host involves enzymatic degradation and mechanical pressure through both layers (Sexton and Howlett, 2006). For insect fungal pathogens to be successfully transmitted, a massive number of spores are required to be released into the environment (Mora *et al*., 2017). The infection pathway of *B. bassiana* is illustrated in Figure 1.10.

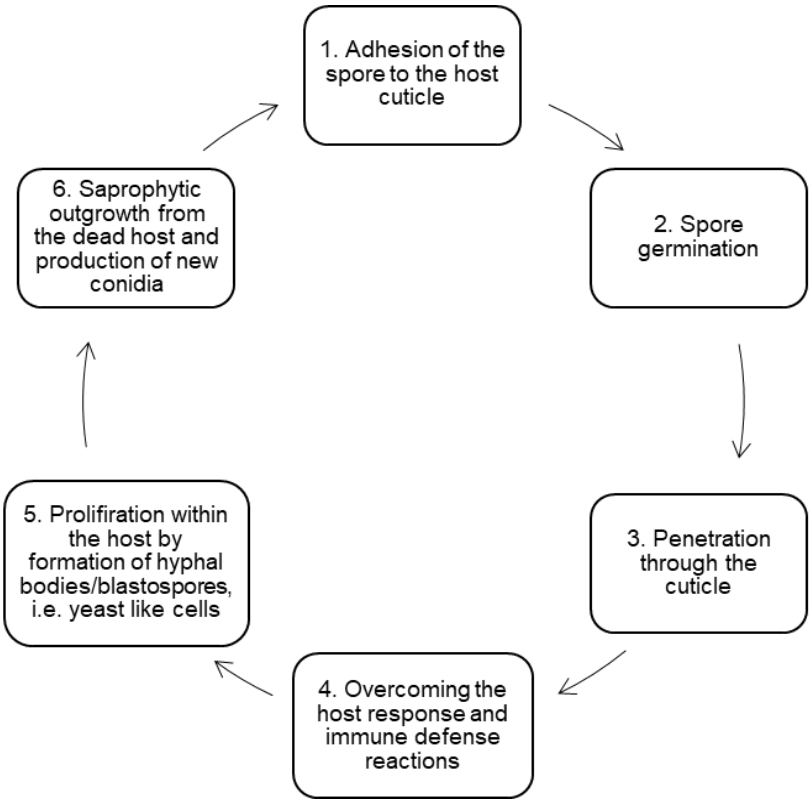


Figure 1.10: Infection process of entomopathogenic fungi. (From Zimmermann, 2007).

The first step in the development of mycosis, is adhesion to the cuticle. First a non-specific passive adsorption of fungal cells must occur, which is then followed by consolidation of the attachment (Boucias *et al*., 1988; Ortiz-Urquiza and Keyhani, 2013). Strong binding forces mediate the attachment of spores. After the pathogen reaches and adheres to the host surface, rapid germination occurs. Enzymes secreted by *B. bassiana* during germination, include protease, chitinase and lipases, and these, together with other factors, promote germination and break down the host cuticle (Ortiz-Urquiza and Keyhani, 2013; Sanjaya *et al.,* 2015). Spore growth is highly influenced by the availability of nutrients and oxygen, as well as by pH and temperature. Inside the haemocoel, the fungal pathogen enters the next phase. It develops yeast-like cells which invade muscle tissues and fatty bodies (Maina *et al.,* 2018). The fungus secretes secondary metabolites such as beauvericin (BEA), bassianolide and oosporein, which contribute to their survival (Cytryńska *et al.,* 2016). Oosporein is a red dibenzoquinone pigment known to have insecticidal activity (Fan *et al.,* 2017). Beauvericin is a natural bioactive compound reported to have numerous biological effects including antimicrobial, insecticidal, antiviral, antiplatelet aggregation, ionophoric, anti-flammatory, antimelanogenesis and antitumor effects (Lim *et al.,* 2020). Bassianolide is a cyclooctadepsipeptide, which acts as an ionophore and it is toxic towards insects (Sinha *et al.,* 2016). *Beauveria bassiana* colonizes insect body fat as well as the main organs producing defence molecules, and proliferates in the host. Insects infected by entomopathogenic fungi eventually die within 3-14 days due to the toxic action of intruder toxins (Maina *et al.,* 2018).

1.9 Mycoinsecticides

Since the 1960s, a large number of mycoinsecticides and mycoacaricides have been developed worldwide (de Faria and Wraight, 2007). The popularity of mycoinsecticides has increased, there are over 170 mycoinsecticide products registered for commmercial use worldwide (de Faria and Wraight 2007; Rice *et al*., 2019). The EPF genera *Metarhizium, Beauveria, Lecanillium, Isaria, Sporothix, Hirsutella, Aschersonia, Paecilomyces, Tolypocladium and Nimuraea* are the active ingredients in these products (de Faria and Wraight, 2007). Of these, *B. bassiana* (33.3%), *Metarhizium anisopliae* (33.9%), *Isaria fumosorosea* (5.8%) and *Beauveria bronginartii* (4.1%), are predominately used as active ingredients.

The negative impact of chemical insecticides continues to draw attention towards the use of naturally occurring microbial insect pathogens (Ortiz-Urquiza and Keyhani, 2014). Some of the advantages of mycoinsecticides are:

i. a wide hosts range and capability of infecting more than 100 crop pests, with a high degree of host specificity (Sandhu *et al.,* 2012).
ii. compatibility with predators, beneficial insects and non-target organisms (Sandhu *et al.,* 2012).
iii. low mammal toxicity (Zimmermann, 2007).
iv. lack of insect resistance development (Sandhu *et al*., 2012).
v. no pollution of the environment and long-term pest suppressive effects (Sandhu *et al*., 2012).

Sandhu *et al.* (2012) also outlined some of the constraints regarding the use of EPF:

i. Speed of kill of mycoinsecticides is very slow (14-21 days) whereas chemical insecticides may need only 2-3 hours.
ii. High relative humidity, low UVA radiation and favourable temperature are needed for application.
iii. Additional control agents may be needed for other pests occurring simultaneously, due to the host specificity of mycoinsecticides.
iv. Production is relatively expensive and spores have a short shelf life.
v. The persistence and efficacy of entomopathogenic fungi in the host population vary between different insect species.
vi. They pose a potential risk to immune-suppressive people.

1.10 Problem statement and justification of study

South Africa is currently the largest producer and exporter of macadamia nuts globally with more than 40 000 hectares under cultivation producing over 58 500 tonnes in the 2019 season (SAMAC, 2020). In South Africa, stink bugs are responsible for severe nut damage with annual crop losses estimated at approximately R 200 million (US $15.3 million) (Taylor *et al*., 2018). Due to their cryptic behaviour, stink bugs are difficult to monitor and control. Since the discovery of synthetic insecticides, the commercial farming sector has relied heavily on the use

of these products for crop protection (Rother *et al.,* 2008). In the near future, the availability of conventional pesticides is likely to decline due to new legislation, food safety concerns, environmental awareness amongst consumers and the evolution of resistance in pest populations (WHO, 2008). Strategies to produce food in an economical, sustainable and environmentally friendly manner has become main priorities globally. Therefore, alternative pest management tactics are needed.

1.11 General objective

To isolate and select highly virulent strains of entomopathogenic fungi to be incorporated in sustainable IPM strategies for control of stink bugs in macadamia, in South Africa.

Specific objectives

i. to sample, isolate and identify indigenous entomopathogenic fungi strains from macadamia orchards in Mpumalanga province.
ii. to determine the role of temperature of candidate fungal isolates (strain selection).
iii. to select the most virulent isolate against *B. distincta* adults under laboratory conditions.
iv. to conduct a semi-field assessment of the most promising isolate for control of *B. distincta* adults.

References

Abubaker, M., El Hawary, S.S., Mahrous, E.A. and Abd El-Kader, E.M. 2018. Botanical study and DNA fingerprinting of *Macadamia integrifolia* Maiden and Betche cultivated in Egypt. *Journal of Pharmacy Research* 12(3):298-304.

Akinsanmi, F. 2016. Branch dieback: a growing threat. *Australian Macadamia Society LTD* 58-59.

Alam, M.N., Neal, J., Howell., Russell, D. and Topp, B.L. 2018. 'MPM1: a macadamia hybrid showing breakdown of biological rhythm in morphogenesis. *International Symposia on Tropical and Temperate Horticulture* 631-636 DOI 10.17660/ActaHortic.2018.1205.78.

Allan, P. 1996. Quality of macadamia cultivars and selections in subtropical Natal. *Proceedings of the third Australian Society of Horticultural Science and the First Australian Macadamia Society Research Conference*, Australia 18-22 August 1996 216-220.

Allegrucci, N., Velazquez, M.S., Russo, M.L., Perez, E. and Scorsetti, A.C. 2017. Endophytic colonisation of tomato by the entomopathogenic fungus *Beauveria bassiana*: the use of different inoculation techniques and their effects on the tomato leafminer *Tuta absoluta* (Lepidoptera: Gelechiidae). *Journal of Plant Protection Research* 57(4):205-211.

Aquino-Bolañosa, E.N., Mapel-Velazco, L., Martín-del-Campo, S.T., Chávez-Servia, J.L., Martínez, A.J. and Verdalet-Guzmán, I. 2016. Fatty acids profile of oil from nine varieties of Macadamia nut. *International Journal of Food Properties* 20(6):1262-1269.

Augstburger, F., Berger, J., Censkowsky, U., Heid, P., Milz, J. and Streit, C. 2002. Organic farming in the tropics and subtropics. Special section: Organic Cultivation of Macadamia Nuts. Naturland e.V. 1st edition, Gräfelfing, Germany, pp. 1-17.

Augustyniuk-Kram, A. and Kram, K.J. 2012. Entomopathogenic fungi as an important natural regulator of insect outbreaks in Forests (Review), Forest Ecosystems- More than just trees, Dr Juan A. Blanco (Ed). ISBN: 978-953-51-0202-1.

Azad, A.K., Rasul, M., Khan, M.M. and Sharma, S. 2017. Macadamia biodiesel as a sustainable and alternative transport fuel in Australia. *Energy Procedia* 110:543-548.

Barrera-López, A.A., Guzmán-Franco, A.W., Santillán-Galicia, M.T., Tamayo-Mejía, F., Bujanos-Muñiz, R., García-Ávila, C.J. and López-Buenfil, J.A .2020. Differential susceptibility of *Bagrada hilaris* (Hemiptera: Pentatomidae) to different species of fungal pathogens. *Journal of Economic Entomology* 113(1):50-54.

Barrueto, A.K., Merz, J., Hodel, E. and Eckert, S. 2018. The suitability of *Macadamia* and *Juglans* for cultivation in Nepal: as assessment based on spatial probability modelling using climate scenarios and in situ data. *Regional Environmental Change* 18:859-871.

Beilharz, V.C., Parberry, D.G. and Swart, H.J. 2002. Dodine: A selective agent for certain soil fungi. *Transactions of the British Mycological Society* 79:507-511.

Botha, L. 2018. Macadamias: a sunrise industry poised for massive growth. *Macadamia SA* pp. 5.

Boucias, D.G., Pendlan, J.C. and Latge, J.P. 1988. Nonspecific factors involved in attachment of entomopathogenic Deuteromycetes to host insect cuticle. *Applied and Environmental Microbiology* 54(7):1795-1805.

Bright, J. 2018. Macadamia plant protection guide. Macadamia protection guide 2018-2019. pp. 40.

Brown, V.A., de Torrez, E.B. and McCracken, G.F. 2015. Crop pests eaten by bats in organic pecan orchards. *Crop Protection* 67:66-71.

Bruwer I.J. 1992. The influence of various hemipteran species on macadamia and some factors which can limit nut damage. *Unpublished PhD thesis*, University of Stellenbosch.

Bustamante, D.E., Oliva, M., Leiva, S., Mendoza, J.E., Bobadilla, L., Angulo, G. and Calderon, M.S. 2019. Phylogeny and species delimitations in the entomopathogenic genus *Beauveria* (Hypocreales, Ascomycota), including the description of *B. peruviensis* sp. nov. *MycoKeys* 58:47-68.

Cardoso, E.J.B.N. and Alves, P.R.L. 2012. Soil Ecotoxicology. 2012. Soil Ecotoxicology, Ecotoxicology, Begum, G (Ed.), ISBN: 978-953-51-0027-0, InTech, Available from: http://www.intechopen.com/books/ecotoxicology/soil-ecotoxicology

Carr, M.K.V. 2013. The water relations and irrigation requirements of macadamia (*Macadamia* spp.): a review. *Experimental Agriculture* 49(1):74-90.

Cytryńska, M., Wojda, I., Jakubowicz, T. 2016. How insects combat infections. In: Ballarin L, Cammarata M (Eds), Lessons in immunity: from single-cell organisms to mammals, Elsevier Academic Press, pp 117-128.

Daane, K.M., Millar, J.G., Rice, R.E., da Silva, P.G., Bentley, W.J., Beede, R.H. and Weinberger, G. 2014. Stink bugs and leaf footed bugs. Unidentified publication, University of California, pp. 186-196.

Dardagan, C. 2018. Expert advice from mayo macs. *Macadamia SA* pp. 35.

Davis, T.S., Mann, A.J., Malesky, D., Jankowski, E. and Bradley, C. 2018. Laboratory and field evaluation of the entomopathogenic fungus *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) for population management of spruce beetle, *Dendroctonus rufipennis* (Coleoptera: Scolytinae) in felled trees and factors limiting pathogen success. *Environmental Entomology* 47(3):594-602.

De Faria, M.R. and Wraight, S.P. 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. *Biological Control* 43:237-256.

Delgado, P.A.M. and Murcia, O.P. 2011. Entomopathogenic fungi as an alternative for the biological control of pests. *Journal of Applied Sciences* 6:77-90.

De Villiers, E.A., Joubert, P.H and van den Berg, M.A. 2003. Major pests. In: de Villiers, E.A and Joubert, P.H (Eds) *The cultivation of macadamia*. ARC-Institute for Tropical and Subtropical Crops, Nelspruit, South Africa 124-137.

Dos Santos, E.R., Trentin, L.B., Ecker, A., Silva, L.A., Borges, M., Mowery, J.D., Ribeiro, B.M., Harrison, R.L. and Ardisson-Araújo, D.M.P. 2019. An inflavirus found in stink bugs (Hemiptera: Pentatomidae) of four different species. *Virology* 534:72-79.

El-Shafie, H.A.F. 2018. Integrated insect pest management. *Pest Control and Acarology* DOI.org/10.5772/intechopen.81827

Erler, F. and Ates, A.O. 2015. Potential of two entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* (Coleoptera: Scarabaeidae), as biological control agents against the June beetle. *Journal of Insect Science* 15(1):44 DOI:10.1093/jisesa/iev029.

Fair Trade USA. 2017. Prohibited and restricted pesticide list (PRPL) pp. 1-13.

Fan, Y., Liu, X., Keyhani, N.O., Tang, G., Pei, Y., Zhang, W. and Tong, S. 2017. Regulatory cascade and biological activity of *Beauveria bassiana* oosporein that limits bacterial growth after host death. *Proceedings of the National Academy of Sciences of the United States of America* 114(9): E1578-E1586. DOI:10.1073/pnas.1616543114.

Galanti, R. and Cho, A. 2019. Soil amendments and soil profiling impact on macadamia growth and yield performance. *HortScience* 54(3):519-527.

Gitonga, L.N., Muigai, A.W.T., Kahangi, E.M., Ngamau, K. and Gichuki, S.T. 2008. Status of macadamia production in Kenya and the potential of biotechnology in enhancing its genetic improvement. *Journal of Plant Breeding and Crop Science* 1(3):49-59.

Glare, T.R. 2004. Molecular characterization in the entomopathogenic fungal genus *Beauveria*. *Laimburg Journal* 1:286-298.

Golden, M., Follett, P.A. and Wright, M.G. 2006. Assessing *Nezara viridula* (Hemiptera: Pentatomidae) feeding damage in macadamia nuts by using biological strain. *Horticultural Entomology* 99(3):822-827.

Hardner, C., e Silva, J.C., Williams, E., Meyers, N. and McConchie, C. 2019. Breeding new cultivars for the Australian macadamia industry. *HortScience* 54(4):621-628.

Hepburn, C. 2015. The phenologies of Macadamia (Proteacea) and thrips (Insecta: Thysanoptera) communities in Mpumalanga province, South Africa. *PhD thesis*, Rhodes University, South Africa.

Hiroyoshi, S., Harvey, J.A., Nakamatsu, Y., Nemoto, H., Mitsuhashi, J., Mitsunaga, T. and Tanaka, T. 2017. Potential host range of the larval endoparasitoid *Cotesia vestalis* (=*plutellae*) (Hymenoptera: Braconidae). *International Journal of Insect Science* 9:1-12.

Ihara, F., Yaginuma, K., Kobayashi, N., Mishiro, K. and Sato, T. 2001. Screening of entomopathogenic fungi against the brown-winged green bug, *Plautia stali* (Hemiptera: Pentatomidae). *Applied Entomology and Zoology* 36(4): 495-500.

Imoulan, A., Hussain, M., Kirk, P.M., Meziane, A.E and Yao, Y. 2017. Entomopathogenic fungus *Beauveria*: host specificity, ecology and significance of morpho-molecular characterization in accurate taxonomic classification. *Journal of Asia-Pacific Entomology* 20:1204-1212.

Ingis, G.D., Goettel, M.S., Butt, T.M. and Strasser, H. 2001. Use of Hyphomycetous fungi for managing insect pests. In: Butt, T.M., Jackson, C.W., Magan, N (Eds),

Fungi as biocontrol agents: progress, problems and potential. CABI International/AAGG, Wallingford, United Kingdom, pp. 23-69.

Jones, V.P. 2002. Macadamia integrated pest management: IPM of insects and mites attacking macadamia nuts in Hawaii. Honolulu (HI) University of Hawaii.

Jones, G., Jacobs, D.S., Kunz, T.H., Willig, M.R. and Racey, P.A. 2009. Carpe noctem: the importance of bats as bioindicators. *Endangered Species Research* 8:93-115.

Khonsanit, A., Luangsa-ard, J.J., Thanakitpipattana, D., Noisripoom, W., Chaitika, T. and Kobmoo, N. 2020. Cryptic diversity of the genus *Beauveria* with a new species from Thailand. *Mycological Progress* 19:291-315.

Kolkert, H., Andrew, R., Smith, R., Rader, R. and Reid, N. 2019. Insectivorous bats selectively source moths and eat mostly pest insects on dryland and irrigated cotton farms. *Ecology and Evolution* 10(1):371-388.

Krupke, C.H., Brunner, J.F., Doerr, M.D. and Kahn, A.D. 2001. Field attraction of the stink bug *Euschistus conspersus* (Hemiptera: Pentatomidae) to synthetic pheromone-baited host plants. *Horticultural Entomology* 94(6):1505-2001.

Kuperus, K.H. and Abercrombie, R.A. 2003. Soil requirements. In: de Villiers, E.A and Joubert, P.H (Eds) *The cultivation of macadamia*. ARC-Institute for Tropical and Subtropical Crops, Nelspruit, South Africa 14-19.

Langdon, K.S., King, G.J., Baten, A., Mauleon, R., Bundock, P.C., Topp, B.L. and Nock, C.J. 2020. Maximising recombination across macadamia populations to generate linkage maps for genome anchoring. *Scientific Reports* 10:5048 DOI:10.1038/s41598-020-61708-6.

Lim, H.N., Jang, J., Shin, H.J., Jang, J., Ahn, J.S. and Jung, H.J. 2020. Cytotoxic activities and molecular mechanisms of the beauvericin and beauvericin G_1 microbial products against melanoma cells. *Molecules* 25(1974):1-12.

Lopes, R.B., Laumann, R.A., Blassioli-Moraes, M.C., Borges, M. and Faria, M. 2015. The fungistatic and fungicidal effects of volatiles from metathoracic glands of soybean attacking stink bugs (Heteroptera: Pentatomidae) on the entomopathogen *Beauveria bassiana*. *Journal of Invertebrate Pathology* 132:77-85.

Maina, U.M., Galadima, I.B., Gambo, F.M. and Zakaria, D. 2018. A review on the use of entomopathogentic fungi in the management of insect pests of field crops. *Journal of Entomology and Zoology Studies* 6(1):27-32.

Maine, J.J. and Boyles, J.G. 2015. Bats initiate vital agroecological interactions in corn. *Proceedings of the National Academy of Sciences* 112(40):12438-12443.

Mannino, M.C., Huarte-Bonnet, C., Davyt-Colo, B. and Pedrini, N. 2019. Is the insect cuticle the only entry gate for fungal infection? Insight into alternative mode of action of entomopathogenic fungi. *Journal of Fungi* 5(33):1-9.

Manson, A. and Sheard, A. 2007. Macadamia fertilization in KwaZulu-Natal. *AgriReport* pp. 1-11.

Matesco, V.C., Fürstenau, B.B.R.J., Bernardes, J.L.C., Schwertner, C.F. and Grazia, J. 2009. Morphological features of the eggs of Pentatomidae (Hemiptera: Heteroptera). *Zootaxa* 1984:1-30.

McFadyen, L., Robertson, D., Morris, S. and Olesen, T. 2016. Effects of early tree training on macadamia production. *HortTechnology* 26(6):707-712. DOI:10.21273/HORTTECH03479-16

Mora, M.A.E., Castilho, A.M.C. and Fraga, M.E. 2017. Classification and infection mechanism of entomopathogenic fungi. *Arquivos do Instituto Biológico* 84:1-10.

Muştu, M., Demirci, F. and Koçak, E. 2011. Mortality of *Isaria farinose* (Holm.) and *Beauveria bassiana* (Balsamo) Vuillemin (Sordariomytes: Hypocreales) on *Aelia rostrate* Boh. (Hemiptera: Pentatomidae). *Turkish Journal of Entomology* 35(4):559-568.

Muthoka, N.M., Kiuru, P.D.N., Mbaka, J., Nyaga, A.N., Muriuki, S.J.N. and Waturu, C.N. 2008. Macadamia nut production and research in Kenya. *The African Journal of Plant Science and Biotechnology* 2(2):46-48.

Mweke, A., Ulrichs, C., Nguya, M.K. and Ekesi, S. 2016. Integration of entomopathogenic fungi as biopesticides for the management of cowpea aphid (*Aphis craccivora* Koch). *African Journal of Horticultural Science* 9:14-31.

Nazir, T., Basit, A., Hanan, A., Majeed, M.Z. and Qiu, D. 2018. In vitro pathogenicity of some entomopathogenic fungal strains against green peach aphid *Myzus persicae* (Homoptera: Aphidae). *Agronomy* 9(7) DOI:10.3390/agronomy9010007.

Noer, C.L., Dabelsteen, T., Bohmann, K. and Monadjem, A .2011. Molossid bats in an African agro-ecosystem select sugarcane fields as foraging habitat. *African Zoology* 47(1):1-11.

Nortjé, G.P. and Schoeman, P.S. 2016. Biology and management of stink bugs in Southern African macadamia orchards – current knowledge and recommendations. *SAMAC Stink bug Factsheet 1,* 8 August 2016. DOI: 10.13140/RG.2.2.36497.12644

Nouri, S., Matsumura, E.E., Kuo, Y. and Falk, B.W. 2018. Insect-specific viruses: from discovery to potential translational applications. *Current Opinion in Virology* 33:33-41.

O'Hare, P., Quinlan, K., Stephenson, R. and Vock, N. 2004. Growing guide: Macadamia grower's handbook. Queensland Dept. Primary Ind. Brisbane Australia.

Öhlund, P., Lundén, H. and Blomström, A. 2019. Insect-specific virus evolution and potential effects on vector competence. *Virus Genes* 55:127-137.

Oishi, S., Moriyama, M., Koga, R. and Fukatsu, T. 2019. Morphogenesis and development of midgut symbiotic organ of the stinkbug *Plautia stali* (Hemiptera: Pentatomidae). *Zoological Letters* 5:16. DOI: org/10.1186/s40851-019-0134-2

Oliveira, D.G.P., Dudczak, A.C., Alves, L.F.A. and Sosa-Gomez, D.R. 2016. Biological parameters of *Euschistus heros* (F.) (Heteroptera: Pentatomidae) and its susceptibility to entomopathogenic fungi when fed on different diets. *Brazilian Archives of Biology and Technology* 59:1-12.

Omondi, J.V. 2015. Effects of insecticide treated nets in the management of tomato pests and their impact on natural enemies and yield in Nairobi and Murang'a countries. *MSc. Thesis*, Kenyatta University, Kenya.

Ortiz-Urquiza, A. and Keyhani, N.O. 2013. Action on the surface: entomopathogenic fungi versus the insect cuticle. *Insects* 4:357-374.

Ortiz-Urquiza, A. and Keyhani, N.O. 2014. Improving mycoinsecticides for insect biological control. *Applied Microbiology and Biotechnology* DOI 10.1007/s00253-014-6270-x

Panizzi, A.R. and Slansky, F. 1985. Review of phytophagous Pentatomids (Hemiptera: Pentatomidae) associated with soybean in the Americas. *Florida Entomologist* 68(1):184- 214.

Parys, K.A. and Portilla, M. 2020. Effectiveness of *Beauveria bassiana* against *Piezodorus guildinii* (Hemiptera: Pentatomidae), a key pest of soybeans in the neotropics. *Biocontrol Science and Technology* 30(5):451-461.

Patel, D.T., Fuxa, J.R. and Stout, M.J. 2005. Evaluation of *Beauveria bassiana* for control of *Oebalus pugnax* (Hemiptera: Pentatomidae) in rice. *Journal of Entomological Science* 41(2):126-146.

Phatanayindee, S., Borompichaichartkul, C., Srzednicki, G., Craske, J. and Wootton, M. 2012. Changes of chemical and physical quality attributes of macadamia nuts during hybrid drying and processing. *Drying Technology* 30:1870-1880.

Quiroz, D., Kuepper, B., Wachira, J. and Emmott, A. 2019. Value chain analysis of macadamia nuts in Kenya. *Technical Report.* pp. 5

Raafat, I., Meshrif, W.S., Husseiny, E.M.E., El-Hariry, M. and Seif, A.I. 2015. *Nezara viridula* (Hemiptera: Pentatomidae) cuticle as a barrier for *Beauveria bassiana* and *Paecilomyces sp*. infection. *African Entomology* 23(1):75-87.

Radzilani, T., Schoeman, P.S., Botha, B. and de Lange, H.C. 2013. Trap crop management strategies to control stink bug in avocado orchards with emphasis on *Pseudotheraptus wayi* brown (Hemiptera: Coreidae). *South African Avocado Growers' Association Yearbook* 36:48-51.

Rice, S.J., Baker, D.K. and Leemon, D.M. 2019. Development of mycoinsecticide formulations with *Beauveria bassiana* and *Metarhizium anisopliae* for the control of lesser mealworm, *Alphitobius diaperinus*, in chicken broiler houses. *BioControl* 64:489-500.

Rother, H., Hall, R.H. and London, L. 2008. Pesticide use among emerging farmers in South Africa: contributing factors and stakeholder perspectives. *Development Southern Africa* 25(4):399-424.

Saeed, Q., Ahmad, F., Iqbal, N. and Zaka, S.M. 2019. Chemical control of polyphagous pests on their auxiliary hosts can minimize insecticide resistance: A case study of *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) in cotton agroecosystem. *Ecotoxicology and Environmental Safety* 171:721-727.

Sandhu, S.S., Sharma, A.K., Beniwal, V., Goel, G., Batra, P., Kumar, A., Jaglan, S., Sharma, A.K. and Malhotra, S. 2012. Myco-biocontrol of insect pests: factors involved mechanism, and regulation. *Journal of Pathogens* 2012:1-11. DOI:10.1155/2012/126819.

Sanjaya, Y., Ocampo, V.R. and Caoili, B.L. 2015. Infection process of entomopathogenic fungi *Beauveria bassiana* in the *Tetrancyhus kanzawai* (Kishida) (Tetranychidae: Acarina). *Arthropods* 4(3):90-97.

Saranraj, P. and Jayaprakash, A. 2017. Agrobeneficial entomopathogenic fungi-*Beauveria bassiana*: a review. *Indo-Asian Journal of Multidisciplinary Research* 3(2):1051-1087.

Saruhan, E.İ.İ., Akca, İ., Aksoy, H.M. and Tuncer, C. 2016. Evaluation of some entomopathogenic fungi for controlling the Green shield bug, *Palomena prasina* L. (Heteroptera: Pentatomidae). *Egyptian Journal of Biological Pest Control* 26(3):573 578.

Schabel, S. 1976. Oral infection of *Hylobius pales* by *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* 383:377-383.

Schoeman, P.S. 2009. Key biotic components of the indigenous Tortricidae and Heteroptera complexes occurring on macadamia in South Africa. *PhD thesis*, North-West University, South Africa.

Schoeman, P.S. 2011. Integrated management of macadamia pests with emphasis on alternative pesticides and more effective monitoring. *South African Macadamia Growers' Association Yearbook* 19:26-32.

Schoeman, P.S. 2013. Phytophagous stink bugs (Hemiptera: Pentatomidae; Coreidae) associated with macadamia in South Africa. *Open Journal of Animal Sciences* 3(3):179-183.

Schoeman, P.S. 2014a. Monitoring and damage of stink bugs on avocados. *South African Avocado Growers' Association Yearbook* 37:48-54.

Schoeman, P.S. 2014b. Stink bug IPM on macadamia in South Africa: current status and the road ahead. *Trends in Entomology* 10:87-95.

Schoeman, P.S. 2018. Relative occurrence of economically significant heteropterans (Pentatomidae and Coreidae) on macadamia in South Africa: implications for management. *African Entomology* 26(2):543-549.

Schoeman, P.S. and Millar, I.M. 2018. First report of *Eriococcus ironsidei* Williams (Hemiptera: Coccomorpha: Eriococcidae) on macadamia (*Macadamia integrifolia* Maiden & Betche and *Macadamia tetraphylla* Johnson: Proteaceae) in South Africa. *African Entomology* 26(1):247-249.

Sexton, A.C. and Howlett, B.J. 2006. Parallels in fungal pathogenesis on plant and animal hosts. *Eukaryotic Cell* 5(12):1941-1949.

Shigeura, G.T. and Ooka, H. 1983. Macadamia nuts in Hawaii: history and production Research Extension Series 39, Hawaii Institute of Tropical Agriculture and Human Resources, University of Hawaii, Honolulu, pp. 19.

Shrestha, B., Tanaka, E., Hyun, M.W., Han, J., Kim, C.S., Jo, J.W., Han, S., Oh, J. and Sung, G. 2016. Coleopteran and lepidopteran hosts of the entomopathogenic genus *Cordyceps* sensu lato. *Journal of Mycology* 1-14 DOI: 10.1155/2016/7648219

Sinha, K.K., Choudhary, A.K. and Kumari, P. 2016. Entomopathogenic fungi. In: Omkar, O (Ed), Ecofriendly pest management for food security. London Academic Press pp. 475-505.

Snijder, B. 2003. Plants and orchard establishment. In: de Villiers, E.A and Joubert, P.H (Eds) *The cultivation of macadamia*. ARC-Institute for Tropical and Subtropical Crops, Nelspruit, South Africa pp. 70-73.

South African Macadamia Growers' Association (SAMAC). 2019. SAMAC macadamia South Africa NPC journal pp. 1-72.

South African Macadamia Growers' Association (SAMAC). 2020. Statistics on the Southern African Macadamia industry, April 2020, viewed 9 May 2020, from https://www.samac.org.za/industry-statistics/

Sourcebi. 2020. Agriculture data analytics https://app.powerbi.com/view?r=eyJrIjoiMGYxMzExODQtZTJiMC00ODQyLWEwZWItZGU4ODc4NWQxNTliIiwidCI6IjAxZDg3MmQ1LThjZTQtNGUxMC1hNDgyLTczYzNhNjI1MDI2YyIsImMiOjh9

Sosa-Gómez, D.R., Corrêa-Ferreira, B.S., Kraemer, B., Pasini, A., Husch, P.E., Vieira, C.E.D., Martinez, C.B.R. and Lopes, I.O.N. 2020. Prevalence, damage, management and insecticide resistance of stink bug populations (Hemiptera: Pentatomidae). *Agricultural and Forest Entomology* 22:99-118.

Srichamnong, W. 2012. Effects of postharvest treatments on macadamia nut quality. *PhD Thesis,* The University of New South Wales, Australia.

Steyn, J.N. 2012. The potential use of trap crops and habitat manipulation for stink bug pest control in macadamia orchards. *South African Macadamia Growers Association Yearbook* 20:39-44.

Taylor, P.J., Monadjem, A. and Steyn, J.N. 2013a. Seasonal patterns of habitat use by insectivorous bats in a subtropical African agro-ecosystem dominated by macadamia orchards. *African Journal of Ecology* 51:552-561.

Taylor, P.J., Bohman, K., Steyn, J.N., Schoeman, M.C., Matamba, E., Zepeda-Mendoza, M.L., Nangammbi, T. and Gilbert, M.T.P. 2013b. Bats eat green vegetable stink bugs (*Nezara viridula):* diet analyses for seven insectivorous species of bats roosting and foraging in macadamia orchards. *South African Macadamia Growers Association Yearbook* 21:45-52.

Taylor, C.M., Coffey, P.L., DeLay, B.D. and Dively, G.P. 2014. The importance of the gut symbionts in the development of the brown marmorated stink bug, *Halyomorpha halys* (Stål). *PLoS One* 9(3):e90312. DOI:10.1371/journal.pone.0090312

Taylor, P.J., Grass, I., Alberts, A.J., Joubert, E. and Tscharntke, T. 2018. Economic value of bat predation services - A review and new estimates from macadamia orchards. *Ecosystem Services* 30:372-381.

Thackeray, S.R., Moore, S.D., Parkinson, M. and Hill, M. 2015. Citrus thrips, *Scirtothrips aurantii* (Thysanoptera: Thripidae), damage and infestation in the presence of molasses. *Crop Protection* 78:72-77.

Thierry, M., Hrček, J. and Lewis, O.T. 2019. Mechanisms structuring host-parasitoid networks in a global warming context: a review. *Ecological Entomology* 44:581-592.

Thomas, M.B. 2017. Myanmar macadamia industry guide to orchard best management practices. *Technical Report* 1-32. DOI:10.13140/RG.2.2.25420.31360

Timm, A.E., Geertsema, H. and Warnich, L. 2006. Analysis of population genetic structure of two closely related torticid species of economic importance on macadamias and litchis in South Africa. *Agricultural and Forest Entomology* 8:113-119.

Uniyal, V.P. and Shrivastava, A. 2012. Arthropods and their conservation in India (Insects and spiders), ENVIS bulletin: wildlife and protected areas. Vol.14, 2011. Printed in 2013; wildlife institute of India, Dehradun-248001, India.

Van den Berg, M.A., Steyn, W.P. and Greenland, J. 1999. Hemiptera occurring on macadamia in the Mpumalanga Lowveld of South Africa. *African Plant Protection* 5(2):89-92.

Van der Merwe, M., Jukes, M.D., Rabalski, L., Knox, C., Opoku-Debrah, J.K., Moore, S.D., Krejmer-Rabalska, M., Szewczyk, B. and Hill, M.P. 2017. Genome analysis and genetic stability of *Cryptophlebia leucotreta* granulovirus (CrleGV-SA) after 15 years of commercial use as a biopesticide. *International Journal of Molecular Sciences* 18(2327):1-14.

Venter, C. 2019. Local nut production continues to grow. *FarmBiz* 5(9):30-31.

Wang, J. and Zheng, C. 2012. Characterization of newly discovered *Beauveria bassiana* isolate to *Frankliniella occidentalis* Perganda, a non-native invasive species in China. *Microbial Research* 167:116-120.

Weier, S.M., Moodley, Y., Fraser, M.F., Linden, V.M.G., Grass, I., Tscharntke, T. and Taylor, P.J. 2019. Insect pest consumption by bats in macadamia orchards established by molecular diet analyses. *Global Ecology and Conservation* 18:e00626. DOI:org/10.1016/j.gecco.2019.e00626

Wiid-Hobson, E.M. 2003. Climatic requirements. In: de Villiers, E.A and Joubert, P.H (Eds) *The cultivation of macadamia*. ARC-Institute for Tropical and Subtropical Crops, Nelspruit, South Africa pp. 20-23.

Williamson, C. and von Wechmar, M.B. 1992. Two novel viruses associated with serve disease symptoms of the green stinkbug *Nezara viridula*. *Journal of General Virology* 73:2467- 2471.

Williamson, C. and von Wechmar, M.B. 1994. The effect of two viruses on the metamorphosis, fecundity and longevity of the green stinkbug, *Nezara viridula*. *Journal of Invertebrate Pathology* 65:174-178.

Wilson, C. and Tisdell, C. 2001. Why farmers continue to use pesticides despite environmental, health and sustainability costs. *Ecological Economics* 39:449-462.

World Health Organization (WHO). 2008. Pesticides Children's Health and the Environment WHO Training Package for the Health Sector.

Wrona, C.J., Mohankumar, V., Schoeman, M.H., Tan, Y.P., Shivas, R.G., Jeff-Ego, O.S and Akinsanmi, O.A. 2020. Phomopsis husk rot of macadamia in Australia and South Africa caused by novel *Diaporthe* species. *Plant Pathology* 69:911-921.

Zarders, D.R. and Wright, N.G. 2016. Macadamia felted coccid, *Eriococcus ironsidei*: biology and life cycle in Hawaii. *Proceedings of the Hawaiian Entomological Society* 48:51-55.

Zhi-zhi, W., Yin-quan, L., Min, S., Jian-hua, H. and Xue-xin, C. 2019. Parasitoid wasps as effective biological control agents. *Journal of Integrative Agriculture* 18(4):705-715.

Zimmermann, G. 2007. Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongiartii*. *Biocontrol Science and Technology* 17:553-596.

Chapter 2

Entomopathogenic fungi associated with stink bugs sampled in Mpumalanga province, South Africa

2.1 Abstract

Stink bugs, *Bathycoelia distincta* (Distant) (Hemiptera: Pentatomidae), *Nezara viridula* (Linnaeus) (Hemiptera: Pentatomidae), *Chinavia pallidoconspera* (Stål) (Hemiptera: Pentatomidae) are important insect pests of macadamia in South Africa. This study investigated the species diversity and occurrence of insect-associated fungi recovered from four pentatomid species. The pentatomids were sampled from an unsprayed macadamia orchard between January 2019 and October 2020 as well as from four macadamia farms. A total of 631 stink bugs were sampled in the unsprayed orchard, and 1015 stink bugs from the four macadamia farms. The most abundant stink bug species was *B. distincta.* Seventeen insect-associated fungi species, known to be entomopathogenic, opportunistic and secondary colonizers, were recovered. Only two species of EPF were isolated, *viz*. *Beauveria bassiana* (Balsamo) Vuillemin and *Purpureocillium lilacinum* (Thom) Samson.

Keywords: *Bathycoelia distincta, Chinavia pallidoconspera,* entomopathogenic fungi*,* macadamia, *Nezara viridula, Purpureocillium lilacinum.*

2.2 Introduction

Macadamia, with the two edible nut species, *Macadamia integrifolia* Maiden & Betche and *Macadamia tetraphylla* L.A.S Johnson (Proteaceae), is an important nut crop, native to Australia (Abubaker *et al.,* 2017). South Africa is the largest macadamia producer in the world and the industry continues to grow rapidly (SAMAC, 2019; Schoeman, 2019). There are currently an estimated 44 775 hectares of macadamia trees established in South Africa, with the largest growing regions in the Mpumalanga province, followed by the Limpopo and KwaZulu-Natal provinces (Botha, 2018). In 2019, the global macadamia crop was in excess of 200 000 tons with the expected global uptake estimated to reach 700 000 tons by 2025 (SAMAC, 2019). The South African macadamia industry produced approximately 58 500 tons nut in shell (NIS) and 17 550 tons of nut kernels during the 2019 harvesting season, with an estimated annual value of R 4.2 billion (SAMAC, 2020).

Insect pests affect macadamia nut production, resulting in significant losses (de Matos *et al.,* 2019). In South Africa, macadamia nuts are primarily attacked by indigenous pests belonging to the Pentatomidae and Coreidae (Bruwer, 1992; Schoeman, 2013). Pentatomidae is one of the largest and well-known families of the hemipteran order (Panizzi, 1997). There are an estimated 100 428 known hemipteran species, with the Pentatomidae family ranked the third largest, consisting of approximately 4 722 species from 896 genera (Uniyal and Shrivastava, 2012; Sharif *et al*., 2020). There are approximately 40 heteropteran species associated with macadamia in South Africa (Schoeman, 2014). Pentatomid stink bugs have become the most serious insect pests of macadamia, and these include the two-spotted stink bug, *Bathycoelia distincta* (Distant), green vegetable bug, *Nezara viridula* (Linnaeus) and yellow-edged stink bug *Chinavia pallidoconspera* (Stål) (Linden *et al.,* 2019).

The economic importance of stink bugs is associated with the magnitude of damage it causes to agricultural crops worldwide (Kamminga *et al*., 2012; Koch *et al*., 2017). For example, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) was responsible for economic losses close to US$37 million in the USA apple industry, in 2010 (Govindan and Hutchison, 2020). The South African macadamia industry is no exception. In 2019, SAMAC reported an economic loss estimated at R 96 million due to early-season stink bug damage and R 84 million due to late-season stink bug damage.

The control and management of stink bugs are difficult due to the lack of effective monitoring techniques (McBrien and Millar, 1999; Krupke *et al*., 2001). Chemical control application remains the main management tactic for most of the insect pests. Insecticides such as synthetic pyrethroids, neonicotinoids and organophosphates are generally relied upon for control of stink bugs (Nora *et al*., 2020). However, South African farmers have to practice precision farming and adhere to quality control practices to meet the stringent import control regulations of the European Union countries (Botha, 2018). Alternative control methods must therefore be investigated. One such a strategy is biological control, where living organisms, including invertebrates and a wide variety of microbial pathogens such as fungi, bacteria and viruses, are used as pest control agents to maintain low pest population densities (Ruberson *et al*., 1999).

Stink bugs are, however, capable of producing organic volatile substances that can suppress germination and growth of entomopathogenic fungi (Lecuona *et al.,* 1997; Nora *et al.,* 2020). Only one bioinsecticide is currently registered for the control of stink bugs on macadamia in South Africa. This study therefore aimed to investigate the occurrence of entomopathogenic fungi associated with stink bug species collected from macadamia orchards in the Mpumalanga province of South Africa, for use as possible biocontrol agents of stink bugs.

2.3 Material and methods

2.3.1 Stink bug collection from four commercial orchards

To determine the occurrence of indigenous EPF, surveys were conducted in commercial orchards at four farms during the summer, from October 2018 to February 2019. Collection was done at two Agricultural Research Council - Tropical and Subtropical Crops (ARC-TSC) research farms, *viz*. Burgershall (25°07’01S 31°05’04E), and Friedenheim (25°27’004S 30°59’29E), as well as from two commercial farms, Kudu and Esperia (25°38’45S 31°17’48E) and Langbult (25°04’19.4”S 31°02’00.2”E), in the Mpumalanga province of South Arica (Figure 2.1). At the commercial farms, stink bugs were collected during routine commercial scouting using the knockdown method described by (Schoeman, 2011). A dichlorvos spray mixture was applied with a high-pressure mist blower. A white sheet was placed under the drip zone of every sprayed tree. After an hour, stink bugs were manually collected, samples collected from Kudu and Esperia were stored in 70 % ethanol and transferred to the laboratory for processing.

Figure 2.1: Geographic locations of the four pentatomid stink bug sampling sites at ARC-TSC Burgershall (25°07'01S 31°05'04E), ARC-TSC Friedenheim (25°27'004S 30°59'29E), Kudu and Esperia Farm (25°38'45S 31°17'48E) and Langbult Farm (25°04'19.4"S 31°02'00.2"E).

2.3.2 Stink bug collection from an unsprayed macadamia orchard

Pentatomids (dead and alive) were collected fortnightly, over a 22-month period (January 2019 – October 2020) in an unsprayed, mixed cultivar, macadamia orchard at the ARC-TSC (25º27'18S 30º58'09E) in Nelspruit, South Africa (Figure 2.2). The orchard consisted of 60, 13-year-old trees which were planted at a spacing of 6 x 6 m (±1 ha). Stink bugs were collected in the morning (before 10:00 am) by shaking branches from the bottom 2 - 2.5 m of the trees as described by Schoeman (2013). All stink bugs that dropped were collected, counted and transferred to the laboratory. The stink bugs were maintained in the laboratory at room temperature (25 ± 1 ºC) in plastic containers (24.5 × 11 × 20.5 cm) closed with mesh infused lids to allow ventilation. Fresh macadamia leaves and nuts were provided as food, and moist cotton wool served as a source of water. The insects were maintained until death.

Figure 2.2: Macadamia orchard at the Agricultural Research Council (25º27’18S 30º58’09E) in Nelspruit, Mpumalanga province, where pentatomids were sampled from January 2019 until October 2020.

2.3.2. Isolation and identification of fungi

Sabouraud Dextrose Agar (SDA) (Oxoid), a selective media for the cultivation of fungi, was prepared, autoclaved for 20 min at 100 kPa (121 ºC), and allowed to cool down to 25 ºC. The agar was poured into 90 mm sterile Petri dish plates and left for 6 hours in a laminar flow hood to solidify. SDA plates were kept at 25 ± 1°C in an incubator (Memmert).

Stink bug cadavers were rinsed with distilled water for 2 min, surface-sterilized with 70% ethanol for 2 min and allowed to air dry on paper towels under a laminar flow hood. Four cadavers were placed per Petri dish (90 mm diameter). The bottom of each Petri dish was lined with filter paper (Whatman no. 1), and closed with a lid. The filter paper was kept moist with distilled water and incubated at 25 ± 1 ºC in complete darkness for 30 days or until fungal growth was visible. Visible fungal growth was scraped from the surface of the cadavers using a sterile needle and placed on the SDA in Petri dish plates for identification and preservation. All cadavers which showed no fungal infection, were counted and discarded.

Fungal colonies were aseptically removed from the SDA plates and mounted on microscope slides with distilled water and covered with a coverslip. Fungal samples

were submitted to the Agricultural Research Council - Plant Health and Protection (ARC-PHP) and Forestry and Agricultural Biotechnology Institute (FABI), Pretoria for molecular identification. All fungal pathogens recovered from this study were preserved in sterile 2 mL Eppendorf tubes with 15 % glycerol solution in a -20 ºC freezer at ARC-TSC, Nelspruit.

2.5 Data Analysis

The Frequency Procedure (PROC FREQ) of SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC, USA) were performed to analyse the data from four stink bug species, collected from four farms and also three stink bugs species collected over 22 months. All fungal species recovered were considered for statistical analyses. Chi-squared (χ^2) tests were used to compare the occurrence of stink bugs species from the four farms (research and commercial farms), and between stink bug sampled in 2019 and 2020 from unsprayed orchards. This test was also used to compare the recovery of fungal species from four farms and between samples obtained in 2019 and 2020.

2.6 Results

2.6.1 Stink bug and associated fungi collection from four commercial orchards

A total of 1015 stink bugs were recovered from four farms in Mpumalanga. The bark stink bug, *Coenomorpha nervosa* (Dallas) (Hemiptera: Pentatomidae), were collected from the bark of litchi (*Litchi chinensis*), while *Chinavia pallidoconspersa, B. distincta* and *N. viridula*, were sampled from macadamia orchards.

Bathycoelia distincta was the most abundant species from all four farms (χ^2=2009.77; df=3; p<0.0001) (Table 2.1). *Bathycoelia distincta* represented 93.79% at Burgershall research farm, 58.37% at Friedenheim research farm, 88.31% at Kudu and Esperia Farm and 100% at Langbult farm.

Table 2.1: Number of adults and nymphs of the four most dominant stink bug species sampled at four farms in Mpumalanga province.

Location	Occurrence	*Bathycoelia distincta*		*Chinavia pallidoconespera*	*Nezara viridula*	*Coenomorpha nervosa*	Total number of stink bugs collected
		Adults	Nymphs	Adults	Adults	Adults	
Burgershall	n	223	19	7	9	0	258
	%	86.43	7.36	2.71	3.49	0.00	
Friedenheim	n	114	22	9	5	83	233
	%	48.93	9.44	3.86	2.15	35.62	
Kudu and	n	134	100	6	25	0	265
Esperia	%	50.57	37.74	2.26	9.43	0.00	
Langbult	n	165	94	0	0	0	259
	%	63.71	36.29	0.00	0.00	0.00	
							1015

Thirteen insect-associated fungi species were isolated from pentatomids that were sampled from the orchards. The frequency of insect-associated fungi sampled from stink bug cadavers are shown in Table 2.2. Fungal species that were recovered were *Aspergillus* (*A. parasitIcus, A. niger, A. sclerotiorum* and *A. fisheri), B. bassiana, Chaetomium* (*C. subaffine, C. globosum*); *Lasiodiplodia (L. theobromae* and *L. pseudotheobromae), Penicillium* spp., *Rhizopus* spp., *Trichoderma* spp., *Myriodontium keratinophilum, Fusarium* (*F. gibbosum, F. equiseti*), *Epiococcum sorghinum, Bionectria* spp. *Talaromyces pionphilus* and *Purpureocillium lilacinum.*

Overall, the most frequently isolated fungi from all four farms were *Penicillium* spp. (8.57%), *Aspergillus* spp. (6.21%) and *Chaetomium* spp. (3.94%). Fungal species diversity differed significantly between the four farms (χ^2=522.53; df=13; p<0.0001). *Beauveria bassiana* and *P. lilacinum* were the only EPF recovered. *Beauveria bassiana* was isolated from Burgershall and Friedenheim research farms, with a frequency of 0.10% and 0.20%, respectively. *Purpureocillium lilacinum* was isolated from Burgershall at a frequency of 0.30%.

2.6.2 Stink bug collection from an unsprayed macadamia orchard

The number of *B. distincta, N. viridula* and *C. pallidoconspera* fluctuated over the 22 months of sampling at the ARC-TSC Nelspruit site (Figure 2.3). The highest numbers of stink bugs were sampled during November - March. Low pentatomid numbers were found in June - October (Figure 2.3). A total of 631 stink bugs were recovered between over the 2019/2020 season from the unsprayed orchard. Stink bug species rerecorded in 2019 (n=463) were *B. distincta* 94.6% (adult, n=187; nymphs, n=251), *C. pallidoconspera* 2.59% (adult, n=12), and *N. viridula* (2.81%) (adults, n=11; nymphs, n=2) and in 2020 (n=168) *B. distincta* 93.45% (adults n=90; nymphs, n=67), *C. pallidoconspera* 3.57% (adult, n=6) and *N. viridula* 1.82% (adult, n=5). *Bathycoelia distincta* was the most abundant species for both years and its numbers were significantly higher than those of other species (χ^2=1049.29; df=2; p<0.0001).

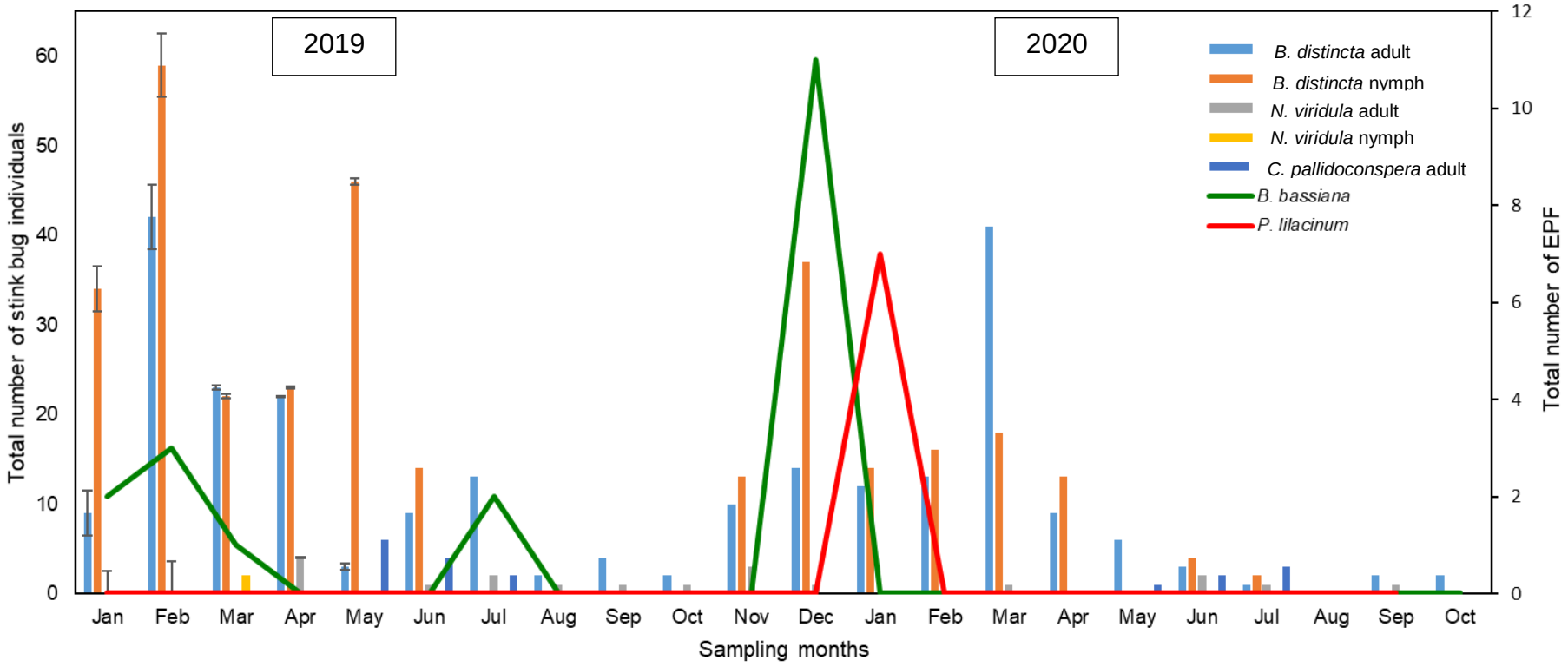


Figure 2.3: Number of *Bathycoelia distincta*, *Nezara viridula and Chinavia pallidoconspera* sampled per month in a macadamia orchard at ARC-TSC, Nelspruit from January 2019 until October 2020. (Total number of EPF isolated from infected stink bug = *Beauveria bassiana* and *Purpureocillium lilacinum).*

Thirteen insect-associated fungi genera were isolated from pentatomids that were sampled from the orchards. The percentage frequency of insect-associated fungi sampled from stink bug cadavers are shown in Table 2.3. Fungal species obtained were *Aspergillus* (*A. parasiticus, A. niger, A. sclerotiorum* and *A. fisheri), B. bassiana, Chaetomium* (*C. subaffine, C. globosum*); *Gelasinospora tetrasperma*, *Lasiodiplodia pseudotheobromae, Nigrospora sphaerica, Penicillium* spp*., Rhizopus* spp., *Trichoderma longibrachiatum, Fusarium (F. proliferatum F. equiseti* and *F. solani), Sordaria tomento-alba, Purpureocillium lilacinum* and *Pestalotiopsis dichaeta*. Photographs of selected fungi are provided in Figure 2.4.

Table 2.2: Frequency (% positive samples) of insect-associated fungi isolated from pentatomid cadavers sampled from four farms, in the Mpumalanga province, South Africa.

Fungal species *	**Research farm (% F)**		**Commercial farm (% F)**		***All farms (% F)**
	Burgershall n=258	Friedenheim n=233	Langbult n=259	Kudu Farm n=265	
All species	10.26	6.69	4.72	3.26	24.97
Aspergillus	2.86	1.67	1.18	0.49	6.20
Beauveria	0.10	0.20	0.00	0.00	0.30
Chaetomium	0.49	1.28	0.49	1.67	3.94
Lasiodiplodia	0.00	0.00	0.00	0.10	0.10
Non-sporulated mycelium	0.69	0.00	0.59	0.10	1.38
Penicillium	5.62	1.18	1.58	0.20	8.57
Rhizopus	0.10	0.00	0.39	0.00	0.49
Trichoderma	0.00	2.36	0.00	0.00	2.36
Myriodontium	0.00	0.00	0.10	0.00	0.10
Fusarium	0.00	0.00	0.39	0.30	0.69
Epioccum	0.00	0.00	0.00	0.30	0.30
Talaromyces	0.00	0.00	0.00	0.10	0.10
Bionectria	0.10	0.00	0.00	0.00	0.10
Purpureocillium	0.30	0.00	0.00	0.00	0.30

Frequency (%) of fungal species isolated per farm
*All species non-infected stink bugs (75.03%) - not included in table.

Overall, the most frequently isolated fungi were *Penicillium* spp. (17.20%), *Rhizopus* spp. (14.40%) and *Chaetomium* spp. (13.18%). Fungal species diversity differed significantly between 2019 and 2020 (χ^2=269.66; df=14; p<0.0001). *Beauveria bassiana* (4.32%) and *P. lilacinum* were the only EPF recovered. *Beauveria bassiana* was isolated only in 2019, with the majority of isolates recovered in December 2019. Seven *P. lilacinum* isolates were obtained in January 2020 (Figure 2.3). Pure cultures of *B. bassiana* and *P. lilacinum* isolated in this study, were assigned an accession number and deposited in the Mycology National Collection of Fungi at the Agricultural Research Council - Plant Health and Protection (ARC-PHP), Pretoria, South Africa. The code used is “PPRI”, the origin of the fungal isolates, their host source and year of isolation are provided in Appendix 2.1.

Table 2.3: Frequency (% positive samples) of fungi isolated from stink bug cadavers sampled from ARC- TSC Nelspruit, unsprayed macadamia orchard.

	2019		**2020**	
Fungal species	n	**% Frequency**	n	**% Frequency ***
All species		35.64		54.48
Aspergillus	32	6.91	3	1.80
Beauveria	20	4.32	0	0.00
Chaetomium	25	5.40	13	7.78
Gelasinospora	1	0.22	0	0.00
Lasiodiplodia	0	0.00	8	4.79
Nigrospora	0	0.00	32	9.58
Non-sporolated mycelium	10	2.16	6	3.59
Penicillium	63	13.61	6	3.59
Rhizopus	14	3.02	19	11.38
Trichoderma	0	0.00	1	0.60
Fusarium	0	0.00	3	1.79
Unknown	0	0.00	1	2.40
Sordaria	0	0.00	1	0.60
Purpureocillium	0	0.00	7	4.19
Pestalotiopsis	0	0.00	7	4.19

n = number of infected stink bugs
% Frequency = percentage frequency of fungi found in 2019 and 2020.

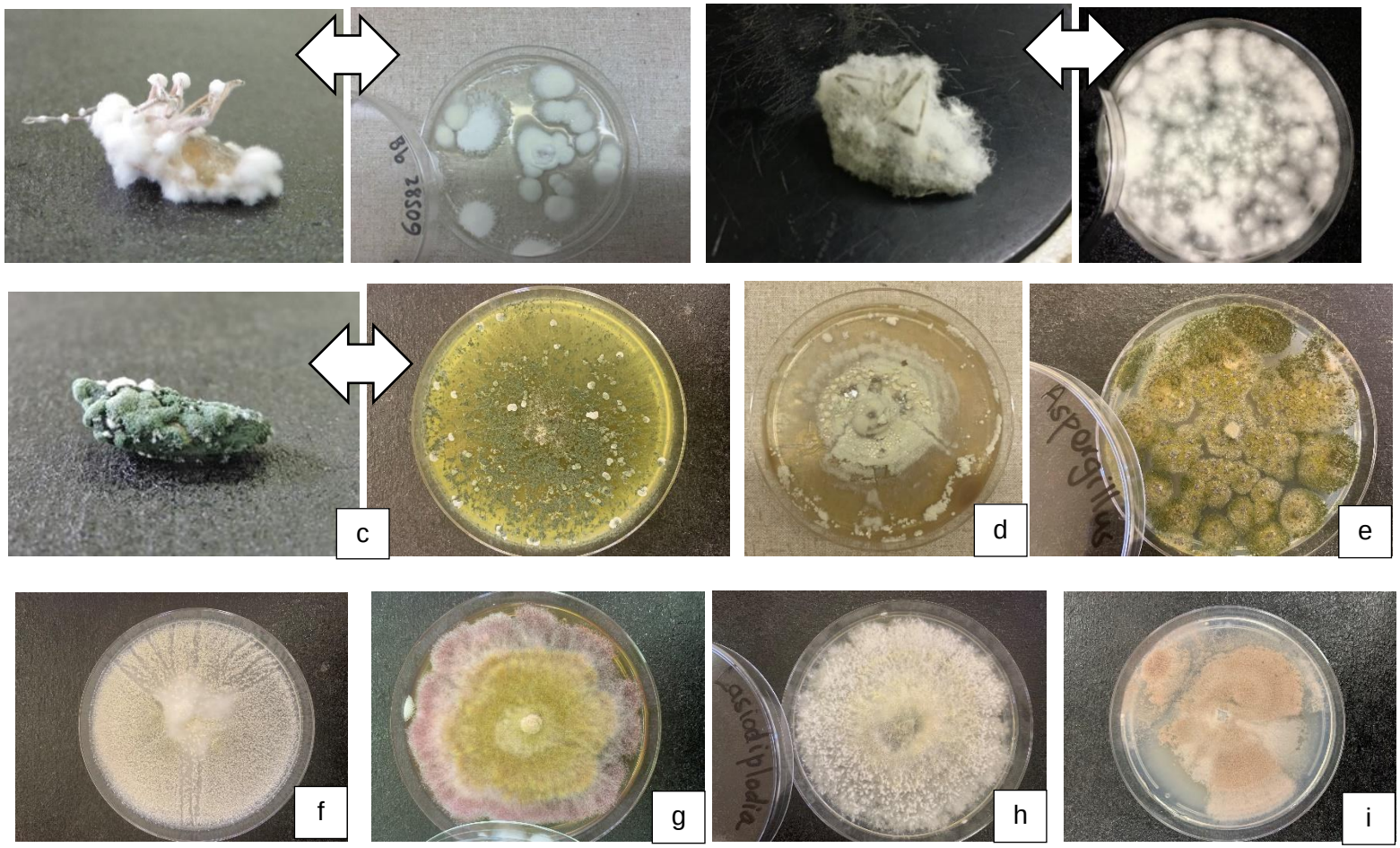


Figure 2.4: Fungi isolated from stink bugs. (a) Infection of an adult stink bug and colony development of *Beauveria bassiana,* (b) Infection of an adult stink bug and colony development of *Chaetomium globosum,* (c) adults infected by *Trichoderma longibrachiatum,* (d) *Myriodontium keratinophilum,* (e) *Aspergillus parasitucus,* (f) *Aspergillus fisheri,* (g) *Fusarium chlamydosporum,* (h) *Lasiodiplodia pseudotheobromae, and* (i) *Purpureocillium lilacinum.*

2.7 Discussion

Bathycoelia distincta was the most abundant stink bug species in this study. These findings were in accordance with Schoeman (2013), who reported that more than 90% of stink bugs sampled, was *B. distincta.* Schoeman (2018) reported *B. distincta* to breed successfully when macadamia nuts are available. During winter, when no nuts are available, other species occur in low numbers but they do not breed in macadamia orchards. *Bathycoelia distincta* adults migrate into macadamia orchards during flowering of trees to feed on newly formed nuts (Schoeman, 2018).

This study is the first to report on the insect-associated fungi isolated from pentatomid cadavers in Mpumalanga, South Africa. Fungal species recovered included four *Aspergillus* spp. (*A. parasitucus, A. niger, A. sclerotiorum* and *A. fisheri).* These were recovered in all orchards sampled. *Aspergillus* spp., *viz*. *A. tubingensis, A. nomius, A. leporis, A. sulphureus* and *A. ocharaceus* are known to produce metabolites that exhibit insecticidal activity (Tanada and Kaya, 1993; Cartagena *et al*., 2014; Kaur *et al*., 2016*). Aspergillus* spp. are generally known as opportunistic pathogens (Raper and Fennell, 1965; Leger *et al*., 2000). *Aspergillus parasiticus* is an endophytic entomopathogen capable of colonizing cotton, bean, corn, tomato and pumpkin plants (Gurulingappa *et al*., 2010). *Aspergillus* species has previously been isolated from cadavers of *Troglophilus neglectus* (Krauss) (Orthoptera: Rhaphidophoridae) (Gunde-Cimerman *et al.,* 1998) and *Culex pipiens* (Linnaeus) (Diptera: Culicidae) (Sur *et al.,* 1999).

Myriodontium keratinophilum was isolated once from a *B. distincta* specimen from Langbult farm. This fungus was recently described as a new EPF, which causes mycosis and mortality in *Gryllotalpa gryllotalpa* (Linnaeus) (Orthoptera: Gryllotalpidae) and *Agelastic alni* (Linnaeus) Coleoptera: Chrysomelidae), previously isolated from mole cricket nymphs (Sönmez *et al*., 2017). The natural source of *M. keratinophilum* is soil and different parts of mammals and keratinous substrates (Samon and Polonelli, 1978; Sönmez *et al*., 2016).

Two species of *Chaetomium* species, *viz*. *Chaetomium globosum* and *C. subaffine* were isolated from *C. pallodoconespera*, *N. viridula* and *C. nervosa. Chaetomium* spp. were recorded from all four farms including the unsprayed orchard. *Chaetomium subaffine* and *C. globosum* occur on a wide variety of substrates and

produce bioactive metabolites, which provide host resistance to numerous diseases caused by plant pathogens (Sekita *et al*., 1981; Zhou *et al*., 2016). *Chaetomium globosum* has been reported as a beneficial endophytic fungus (Zhai *et al.,* 2018). However, *C. subaffine* has been reported as an air contaminant causing human fungal infections (Wang *et al*., 2016).

Penicillium spp. were also found in all the orchards. These fungi represent a well-known cosmopolitan genus, which is primarily isolated from the soil, with decomposition as its main function (Visagie, 2008). *Trichoderma* sp. was mainly recovered from *C. nervosa* and *B. distincta* from the ARC research farm, Friedenheim. *Trichoderma* is a genus present in most forest and agricultural soils. It is a secondary opportunistic invasive fungi and a virulent plant symbiont (Vinale *et al*., 2008). *Trichoderma* spp. are, however, also fungal biological control agents used in agricultural fields, due to their antagonistic capacities. It is commercially sold as a biopesticide, biofertilizer, soil amendment and/or enhancer for plant growth (Gorai *et al.,* 2020). *Trichoderma longibrachiatum* was the species most frequently recovered. It has been reported to be pathogenic towards *Leucinodes orbonalis* (Guenee) (Lepidoptera: Crambidae) (Ghosh and Pal, 2015).

Fusarium spp. were obtained from the two commercial farms (Langbult and Kudu & Esperia farm) and also the unsprayed orchard in 2020. *Fusarium* spp. are saprotrophic and more than 13 *Fusarium* spp. have been reported to be pathogenic towards insects from the orders Coleoptera, Diptera, Hemiptera, Hymenoptera and Lepidoptera (Teetor-Barsch and Roberst, 1983; Humber, 1992; Pelizza *et al.,* 2011). *Fusarium equiseti,* isolated in this study, has previously been isolated from *Cephus cinctus* (Norton) (Hymenoptera: Cephidae), *Brahmina coriacea* (Hope) (Coleoptera: Scarabaeidae) *Aphis gossypii* (Glover) (Hemiptera: Aphididae), and *Bemisia tabaci* (Gennadius) (Hempitera: Aleyrodidae) (Sun and Liu, 2008; Wenda-Piesik *et al*., 2009; Sharma *et al.*, 2012; Anwar *et al.,* 2017). It was reported to be highly pathogenic towards *B. tabaci* nymphs (Anwar *et al.,* 2017, Santos *et al.,* 2020)

Epicoccum sorghinum was isolated from the farm, Kudu and Esperia, in this study. *Epicoccum sorghinum* (also known as *Phoma sorghina*), is leaf spot pathogen of sorghum, pokeweed and maize (do Amaral *et al*., 2004). This fungus occurs in a variety of plants, soil and substrates (Pažoutovă, 2009). It is regarded as an

important fungal contaminant of sorghum grain that causes economic losses by reducing crop yield (Oliveira *et al*., 2017). *Lasiodiplodia theobromae* and *Lasiodiplodia pseudotheobromae* are important plant pathogen that causes dieback, cancer, gummosis, leaf blight and root rot (Pitt and Hocking, 2009; Picos-Muñoz *et al*., 2014). Kamil *et al*. (2018) reported *L. theobromae* as a causal agent for dieback in mango in United Arab Emirates (UAE).

Results from this study indicate that stink bugs are susceptible to a wide range of soil-borne fungi which are opportunistic, secondary colonizers, plant pathogens or EPF. *Metarhizium anisopliae* (Metsch.) and *B. bassiana* are widely distributed and are the most studied entomopathogenic fungi worldwide (McGuire and Northfield, 2020). In this study, *Metarhizium* was not isolated from stink bug cadavers. *Beauveria bassiana* was, however, most commonly isolated from *B. distincta* cadavers, with the majority of the *B. bassiana* isolates recovered from the unsprayed macadamia orchard. Another isolated EPF was *P. lilacinum*. Both *B. bassiana* and *P. lilacinum* are biological control agents that already used commercially. However, *P. lilacinum* is mainly considered as a biological agent of *A. gossypii,* several phytoparasitic nematode species (Lopez *et al*., 2014), *Helicoverpa gelopoeon* (Dyar) (Lepidoptera: Noctuidae), *Diabrotica speciosa* (Germar) (Coleoptera: Chrysomelidae) (Vianna *et al*., 2020) and *Diaphorina citri* (Kuwayama (Hemiptera: Liviidae) (Ausique *et al.*, 2017).

Results from this study are in agreement with Chandler *et al.* (1997), who reported EPF to be common inhabitants of soils, but that the diversity of species is relatively low with only a few species occurring frequently. Pentatomids are well known to produce a blend of compounds, which include antifungal compounds that can reduce or prevent fungal infections (Borges *et al*., 1993; Quintela *et al.,* 2013). Da Silva *et al.* (2015) reported that the aldehydes, *(E)* -2-hexenal, *(E)*-2-octenal and *(E)*-2-decenal are contained in the defensive compounds of adult stink bugs, *Tibraca limbativentris* (Stål.) (Hemiptera: Pentatomidae). These contain compounds which strongly inhibit the growth of EPF. A fungistatic effect by *(E)*-2-decenal on the germination of *M. anisopliae* spores in *N. viridula,* was reported by Sosa-Gómez *et al*. (1997) and Lopes *et al*. (2015), implicating a low natural infection. Germination of *B. bassiana* was, however, not affected (Sosa-Gómez *et al*., 1997; Lopes *et al*., 2015).

From studies on the occurrence and diversity of EPF isolated from soil in agricultural fields, the most commonly isolated EPF were *Metarhizium, Beauveria* and *Paecilomyces (=Purpureocillium*) (Ali-Shtayeh *et al.*, 2002; Tuininga *et al*., 2009; Keyser *et al.*, 2015)*. Bathycoelia distincta*, *N. viridula* and *C. pallidoconspera* were found to be susceptible to *B. bassiana and P. lilacinum*, and to be colonized by the opportunistic fungi, *Aspergillus* spp*., Fusarium* spp, and *Penicillium* spp*.,* as well as to the secondary colonizers, *Chaetomium* and *Rhizopus*. In addition, infection of stink bugs by the secondary colonizer *M. keratinophilum,* is reported for the first time in South Africa, in this study. Plant pathogens such as *Epicoccum sorghinum* and *Lasiodiplodia theobromae* were, however, also isolated from the stink bugs.

References

Abubaker, M., El Hawary, S.S., Mahrous, E.A. and El-Kader, E.M.A. 2017. Study of nutritional contents of *Macadamia integrifolia* Maiden & Betche leaves, kernel and pericarp cultivated in Egypt. *International Journal of Pharmacognosy and Phytochemical Research* 9(12):1442-1445.

Ali-Shtayeh, M.S., Mara'i., A.B.M. and Jamous, R.M. 2002. Distribution, occurrence of entomopathogenic fungi in agricultural soil in the Palestinian area. *Mycopathologia* 156:235-244.

Anwar, W., Haider, M.S., Shahid, A.A., Mushtaq, H., Hameed, U., Rehman, M.Z.U. and Iqbal, M.J. 2017. Genetic diversity of *Fusarium* isolated from members of Sternorrhyncha (Hemiptera): entomopathogens against *Bemisia tabaci*. *Pakistan Journal Zoology* 49(2):639-645.

Ausique, J.J.S., D'Alessandro, C.P., Conceschi, M.R., Mascarin, G.M. and Junior I.D. 2017. Efficacy of entomopathogenic fungi against adult *Diaphorina citri* from laboratory to field applications. *Journal of Pest Science* 90:947-960. DOI 10.1007/s10340-017-0846-z

Borges, M., Leal, S.C., Tigano, M.S., Valadares, M.C.C., 1993. Efeito do feromônio de alarme do percevejo verde, *Nezara viridula* (L.) (Hemiptera: Pentatomidae), sobre fungo entomopatogênico *Metarhizium anisopliae* (Metsch.) Sorok. *Sociedade Entomológica do Brasil* 22 (3): 505-512.

Botha, L. 2018. Macadamias: a sunrise industry poised for massive growth. Macadamia South Africa. pp. 5.

Bruwer I.J .1992. The influence of various hemipteran species on macadamia and some factors which can limit nut damage. *PhD thesis*, University of Stellenbosch.

Cartagena, E., Marcinkevicius, K., Luciardi, C., Rodriguez, G., Bardon, A., Arena, M.E. 2014. Activity of a novel compound produced by *Aspergillus parasiticus* in the presence of red flour beetle *Tribolium castaneum* against *Pseudomonas aeruginosa* and coleopteran insects. *Journal of Pest Science* 87:521-530.

Chandler, D., Hay, D. and Reid, A.P. 1997. Sampling and occurrence of entomopathogenic fungi and nematodes in UK soils. *Applied Soil Ecology* 5:133-141.

Da Silva, R.A., Quintela, E.D., Mascarin, G.M., Pedrini, N., Liao, L.M. and Ferri, P.H. 2015. Unveiling chemical defense in the rice stalk stink bug against the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* 127:93-100.

De Matos, S.T.S., dos Santos-Cividanes, T.M., Cividanes, F.J., dos Santos, L.C., Suguino, E. and Ribeiro, A.A. 2019. First record of lepidopteran borers in macadamia nuts in Brazil. *Florida Entomologist* 102(3):649-650.

Do Amaral, A.L., de Carli, M.L., Neto, J.F.B. and Soglio, F.K.D. 2004. *Phoma sorghina*, a new pathogen associated with phaeosphaeria leaf spot on maize in Brazil. *Plant Pathology* 53:259.

Ghosh, S.K. and Pal, S. 2015. Entomopathogenic potential of *Trichoderma longibrachiatum* and its comparative evaluation with malathion against the insect pest *Leucinodes orbonalis. Environmental Monitoring and Assessment* 188:5-7.

Gorai, P.S., Barman, S., Gond, S.K. and Mandal, N.C. 2020. *Trichoderma*. Beneficial *Microbes in* Agro-Ecology pp. 571-591. DOI.org/10.1016/B978-0-12-823414-3.00028-9

Govindan, B.N. and Hutchison, W.D. 2020. Influence of temperature on age-stage, two-sex life tables for a Minnesota-acclimated population of the brown marmorated stink bug (*Halyomorpha halys*). *Insects* 11(108). DOI:10.3390/insects11020108

Gunde-cimerman, N Zalr, P. and Jeram, S. 1998. Mycoflora of cave cricket *Troglophilus neglectus* cadavers. *Mycopathologia* 141:111-114.

Gurulingappa, P., Sword, G.A., Murdoch, G. and McGee, P.A. 2010. Colonization of crop plants by fungal entomopathogens and their effects on two insect pests *when in planta. Biological Control* 55:34-41.

Humber, R.A. 1992. Collection of entomopathogenic fungal cultures: Catalog of strains, U.S. Department of Agriculture. Agricultural Research Service. Bulletin ARS-110.

Kamil, F.H., Saeed, E, El-Tarabily, K. and AbuQamar, S.F. 2018. Biological control of mango dieback disease caused by *Lasiodiplodia theobromae* using Streptomycete and non-streptomycete actionobacteria in the United Arab Emirates. *Frontiers in Microbiology* 9:829 DOI:10.3389/fmicb.2018.00829

Kamminga, K.L., Koppel, A.L., Herbert, D.A. and Kuhar, T.P. 2012. Biology and management of the green stink bug. *Journal of Integrated Pest Management* 3(3). DOI.org/10.1603/IPM12006

Kaur, T., Kaur, J., Kaur, A. and Kaur, S. 2016. Larvicidal and growth inhibitory effects of endophytic *Aspergillus niger* on a polyphagous pest, *Spodoptera litura*. *Phytoparasitica* 44:465-476.

Keyser, C.A., De Fine Licht, H.H., Steinwender, B.M. and Meyling, N.V. 2015. Diversity within the entomopathogenic fungal species *Metarhizium flavoviride* associated with agricultural crops in Denmark. *BMC Microbiology* 15-249. DOI 10.1186/s12866-015-0589-z

Koch, R., Pezzini, D.T., Michel, A.P. and Hunt, T. 2017. Identification, biology, impacts, and management of stink bugs (Hemiptera: Heteroptera: Pentatomidae) of soybean and corn in the Midwestern United States. *Journal of Integrated Pest Management* 8(1):1-14. DOI: 10.1093/jipm/pmx004

Krupke, C., Brunner, J.F., Doerr, M. and Kahn, A.D .2001. Field attraction of the stink bug *Euschistus conspersus* (Hemiptera: Pentatomidae) to synthetic pheromone-baited host plants. *Horticultural Entomology* 94(6):1500-1505.

Lecuona, R., Clement, J.L., Riba, G., Joulie, C. and Juárez, P. 1997. Spore germination and hyphal growth of *Beauveria* sp. on insect lipids. *Journal of Economic Entomology* 90(1):119-123.

Leger, R.J.S., Screen, S.E. and Shams-Pirzadeh, B. 2000. Lack of host specialization in *Aspergillus flavus*. *Applied and Environmental Microbiology* 66(1): 320-324.

Linden, V.M., Grass, I., Joubert, E., Tscharntke, T., Weier, S.M. and Taylor, P.J. 2019. Ecosystem services and disservices by birds, bats and monkeys change with macadamia landscape heterogeneity. *Journal of Applied Ecology* DOI: 10.1111/1365-2664.13424

Lopes, R.B., Laumann, R.A., Blassioli-Moraes, B., Borges, M. and Faria, M. 2015. The fungistatic and fungicidal effects of volatiles from metathoracic glands of soybean-attacking stink bugs (Heteroptera: Pentatomidae) on the entomopathogen *Beauveria bassiana*. *Journal of Invertebrate Pathology* 132:77-85.

Lopez, D.C., Zhu-Salzman, K., Ek-Ramos, M.J. and Sword, G. 2014. The entomopathogenic fungal endophytes *Purpureocillium lilacinum* (Formerly *Paecilomyces lilacinus*) and *Beauveria bassiana* negatively affect cotton aphid reproduction under both greenhouse and field conditions. *PLoS ONE* 9(8): e103891.

McBrien, H.L., and Millar, J.G. 1999. Phytophagous bugs. In J. Hardie and A. K. Minks (Eds), Pheromones of non-lepidopteran insects associated with agricultural plants pp. 277-304. CAB, Wallingford, England.

McGuire, A.V. and Northfield, T.D. 2020. Tropical occurrence and agricultural importance of *Beauveria bassiana* and *Metarhizium anisopliae*. *Frontiers in Sustainable Food Systems* 4(6). DOI: 10.3389/fsufs.2020.00006

Nora, D.D., Piovesan, B.C., Bellé, C., Stacke, R.S., Balardin, R.R., Guedes, J.V.C., Michaud, J.P. and Jacques, R.J.S. 2020. Isolation and evaluation of entomopathogenic fungi against the neotropical brown stink bug *Euschistus heros* (F.) (Hemiptera: Pentatomidae) under laboratory conditions. *Biocontrol Science and Technology* DOI:org/10.1080/09583157.2020.1826904

Oliveira, R.C., Davenport, K.W., Hovde, B., Silva, D., Chain, P.S.G., Correa, B. and Rodrigues, D. 2017. Draft genome sequence of sorghum grain mold fungus *Epicoccum sorghinum*, a producer of tenuazonic acid. *Genome Announcements* 5:e01495-16. DOI.org/10.1128/genomeA.01495-16.

Panizzi, A.R. 1997. Wild hosts of Pentatomids: ecological significance and role in their pest status on crops. *Annual Review of Entomology* 42:99-122.

Pažoutová, S. 2009. Genetic variation of *Phoma sorghina* isolates from Southern Africa and Texas. *Folia Microbiologica* 54(3):217-229.

Pelizza, S.A., Stenglein, S.A., Cabello, M.N., Dinolfo, M.L. and Lange. C.E. 2011. First record of *Fusarium verticillioides* as an entomopathogenic fungus of grasshoppers. *Journal of Insect Science* 70(11):1-8.

Picos-Muñoz, P.A., García-Estrada, R.S., León-Félix, J. and Allende-Molar, A.S.B.R. 2014. *Lasiodiplodia theobromae* in agricultural crops in Mexico: Taxonomy, host, diversity and control. *Revista Mexicana de Fitopatologia* 33(1):54-74.

Pitt, J. and Hocking, A. 2009. Fungi and Food Spoilage. 3 ed. Springer. pp 125-127.

Quintela, E.D., Mascarin, G.M., da Silva, R.A., Barrigossi, J.A.F. and Martins, J.F.S. 2013. Enhanced susceptibility of *Tibraca limbativentris* (Heteroptera: Pentatomidae) to *Metarhizum anisopliae* with sublethal doses of chemical insecticides. *Biological Control* 66:56-64.

Raper, K.K. and Fennell, D.I. 1965. The genus *Aspergillus*. Williams & Wilkins, Baltimore, Md, USA.

Ruberson, J.R., Nechols, J.R. and Tuaber, M.J. 1999. Biological control of arthropod pests. pp. 417-448. In: Handbook of pest management. (Ed. J.R. Ruberson). Marcel Dekker, Inc: New York.

Samson, R.A. and Polonelli, L. 1978. *Myridontium keratinophilum* Genet. Spnov. *Persoonia* 9:505-509.

Santos, A.C.S., Diniz, A.G., Tiago, P.V. and Oliveira, N.T. 2020. Entomopathogenic *Fusarium* species: a review of their potential for the biological control of insects, implications and prospects. *Fungal Biology Reviews* 34:41-57.

Schoeman, P.S. 2011. Integrated management of macadamia pests with emphasis on alternative pesticides and more effective monitoring. *Southern African Growers' Association Yearbook* 19:26-32.

Schoeman, P.S. 2013. Phytophagous stink bugs (Hemiptera: Pentatomidae; Coreidae) associated with macadamia in South Africa. *Open Journal of Animal Sciences* 3(3):179-183.

Schoeman, P.S. 2014. Stink bug IPM on macadamia in South Africa: current status and the road ahead. *Trends in Entomology* 10:87-95.

Schoeman, P.S. 2018. Relative seasonal occurrence of economically significant Heteropterans (Pentatomidae and Coreidae) on macadamia in South Africa: implications for management. *African Entomology* 26(2):543-549.

Schoeman, P.S. 2019. Damage potential of indigenous Heteroptera species occurring on Macadamia nuts (*Macadamia integrifolia* Maiden & Betche & *Macadamia tetraphylla* L. Johnson) in South Africa during the early and late season. *International Journal of Tropical Insect Science* 40:217-219.

Sekita S, Yoshihira K., Natori, S., Udagawa, Y., Muroi, T., Sugiyama, Y., Kurata, H. and Umeda, M.1981. Mycotoxin production by *Chaetomium* spp. and related fungi. *Canadian Journal of Microbiology* 27:766-72.

Sharif, T., Waheed, I., Bashir, A., Saleem, A., Aftab, M. and Ahmed, S. 2020. Taxonomic studies of family Pentatomidae (Hemiptera) four genera from district Faisalabad Punjab Pakistan with taxonomic keys. *Journal of Entomology and Zoology Studies* 8(1):1338-1344.

Sharma, M., Babu, K.T., Gaur, P.M., Ghosh, R., Rameshwar, T., Chaudhary, R.G., Upadhyay, J.P., Gupta, O., Saxena, D.R., Kaur, L., Dubey, S.C., Anandani, V.P., Harer, P.N., Rathore, A. and Pande, S. 2012. Identification and multi-environment validation of resistance to *Fusarium oxysporum* f. sp. *ciceris* in chickpea. *Field Crops Research* 135:82-88.

Sönmez, E., Sevim, A., Demirbag. and Demir, I. 2016. Isolation, characterization and virulence of entomopathogenic fungi from *Gryllotalpa gryllotalpa* (Orthoptera: Gryllotalpidae). *Applied Entomology and Zoology* 51:213-223.

Sönmez, E., Kocacewik, S., Sevim, A., Demirbag, Z. and Demir, I. 2017. Efficacy of entomopathogenic fungi against the alder leaf beetle *Agelastica alni* (L.) (Coleoptera: Chrysomelidae). *Applied Zoology* 69(4): 575-581.

Sosa-Gómez, D. R. and Moscardi, F .1997. Laboratory and field studies on the infection of stink bugs, *Nezara viridula*, *Piezodorus guildinii*, and *Euschistus heros* (Hemiptera: Pentatomidae) with *Metarhizium anisopliae* and *Beauveria bassiana*. *Journal of Invertebrate Pathology* 71(2):115–120.

South African Macadamia Growers' Association (SAMAC). 2019. SAMAC macadamia South Africa, NPC journal. pp. 1-72.

South African Macadamia Growers' Association (SAMAC). 2020. Statistics on the Southern African Macadamia industry, April 2020, viewed 10 October 2020, from https://www.samac.org.za/industry-statistics/

Sun, B. and Liu, X. 2008. Occurrence and diversity of insect-associated fungi in natural soils in China. *Applied Soil Ecology* 13:100-108.

Sur, B., Bihari, V., Sharma, A. and Basu, K. 1999. Survey of termite-inhabited soil and mosquito breeding sites in Lucknow, India for potential mycopathogens of *Anopheles stephensi*. *Mycopathologia* 144:77-80.

Tanada, Y and Kaya H.K.1993. Insect pathology. Academic Press, San Diego

Teetor-Barsch, G.H, and Roberts W.D. 1983. *Fusarium* species pathogens of insects. Review. *Mycopathologia* 84:3-16.

Tuininga, A.R., Miller, J.L., Morath, S.U., Daniels, T.J., Falco, R.C., Marchese, M., Sahabi, S., Rosa, D. and Stafford, K.C. 2009. Isolation of entomopathogenic fungi from soil and *Ixodes scapularis* (Acari: Ixodidae) Ticks: prevalence and methods. *Journal of Medical Entomology* 46(3):557-565.

Uniyal, V.P and Shrivastava, A. 2012. Arthropods and their conservation in India (Insects and spiders), ENVIS bulletin: wildlife and protected areas. Vol.14, 2011. Pritined in 2013; wildlife institute of India, Dehradun-248001, India.

Vianna, M.F., Pelizza, S., Russo, M.L., Toledo, A., Mourelos, C. and Scorsetti, C. 2020. ISSR markers to explore entomopathogenic fungi genetic diversity:

implications for biological control of tobacco pests. *Journal of Biosciences* 45(136). DOI: 10.1007/s12038-020-00108-4

Vinale, F., Sivasithamparam, K., Ghisalberti, E.L., Marra, R., Woo, S.L. and Lorito, M. 2008. *Trichoderma*-plant-pathogen interactions. *Soil Biology and Biochemistry* 40:1-10.

Visagie, C.M. 2008. Biodiversity in the genus *Penicillium* from coastal fynbos soil. *MSc* thesis, University of Stellenbosch.

Wang, X.W., Lombard, L., Groenewald, J.Z. and Videira, S.I.R. 2016. Phylogenetic reassessment of the *Chaetomium globosum* species complex. *Persoonia* 83-133. DOI.org/10.3767/003158516X689657

Wende-Piesik, A., Sun, Z., Grey, W.E., Weaver, D.K. and Morrill, W. 2009. Mycoses of wheat stem sawfly (Hymenoptera: Cephidae) larvae by *Fusarium* spp. isolates. *Environmental Entomology* 38(2):387-394.

Zhai, X., Luo, D., Li, X., Han, T, Jia, M., Kong, Z., Ji, J., Rahman, K., Qin, L. and Zheng, C. 2018. Endophyte *Chaetomium globosum* D38 promotes bioactive constituents accumulation and root production in *Salvia miltiorrhiza*. *Frontiers in Microbiology* 8:2694. DOI: 10.3389/fmicb.2017.02694

Zhou, W., Starr, J.L., Krumm, J.L. and Sword, G.A. 2016. The fungal endophyte *Chaetonium globosum* negatively affects both above- and belowground herbivores in cotton. *FEMS Microbiology Ecology* 92(10):1-15. DOI: 10.1093/femsec/fiw158

Appendix 2.1

The location of *Beauveria bassiana* and *Purpureocillium lilacinum* collected from macadamia orchards isolated from stink bug cadavers in the Mpumalanga province, South Africa.

Accession no.	Location	Coordinates	Host	Fungal species	Date of isolation
PPRI 26694	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	January 2019
PPRI 26695	Friedenheim, ARC-TSC	25°27’04S 30°59’29E	*Bathycoelia distincta*	*Beauveria bassiana*	September 2018
PPRI 26696	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	February 2019
PPRI 26697	Burgershall, ARC-TSC	25°07’01S 31°05’04E	*Nezara viridula*	*Beauveria bassiana*	July 2018
PPRI 26699	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	July 2018
PPRI 26700	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	January 2019
PPRI 26701	Friedenheim, ARC-TSC	25°27’004S 30°59’29E	*Bathycoelia distincta*	*Beauveria bassiana*	July 2018
PPRI 26702	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	February 2019
PPRI 26703	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	March 2019
PPRI 26704	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	January 2019
PPRI 28513	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	December 2019
PPRI 28505	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	July 2019
PPRI 28512	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	December 2019
PPRI 28511	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	December 2019
PPRI 28510	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	December 2019
PPRI 28508	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	December 2019

PPRI 28509	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	December 2019
PPRI 28503	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	February 2019
PPRI 28507	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	December 2019
PPRI 28506	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	July 2019
PPRI 28731	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Beauveria bassiana*	February 2019
PPRI 28964	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Purpureocillium lilacinum*	January 2020
PPRI 29096	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Purpureocillium lilacinum*	January 2020
PPRI 28963	Burgershall, ARC-TSC	25°07’01S 31°05’04E	*Bathycoelia distincta*	*Purpureocillium lilacinum*	July 2018
PPRI 28965	Nelspruit, ARC-TSC	25°27’18S 30°58’09E	*Bathycoelia distincta*	*Purpureocillium lilacinum*	January 2020
PPRI 28966	Burgershall, ARC-TSC	25°07’01S 31°05’04E	*Bathycoelia distincta*	*Purpureocillium lilacinum*	June 2018
PPRI 28962	Burgershall, ARC-TSC	25°07’01S 31°05’04E	*Bathycoelia distincta*	*Purpureocillium lilacinum*	July 2018

Chapter 3

Effect of temperature on radial growth of *Beauveria bassiana* isolates

3.1. Abstract

Beauveria bassiana (Balsamo) Vuillemin (Ascomycota: Hypocreales) isolates were isolated from the stink bugs, *Bathycoelia distincta* (Distant) and *Nezara viridula* (Linnaeus) (Hemiptera: Pentatomidae) sampled in orchards at Nelspruit, Mpumalanga. The effect of temperature on the radial growth of 11 of these *B. bassiana* isolates was assessed under constant temperature conditions in laboratory experiments. The radial growth of the different isolates was differentially, and significantly influenced by temperature. Radial growth of all isolates was significantly inhibited at 35 °C, but optimal temperature for growth was 25-30 °C. Seven of these isolates did not differ significantly in terms of radial growth after 21 days, *viz*. PPRI isolates 26701, 26703, 28505, 28503, 26695, 26696, and 26697.

Key words: *Bathycoelia distincta*, *Beauveria bassiana*, *Nezara viridula*, radial growth, temperature.

3.2. Introduction

Beauveria bassiana (Balsamo) Vuillemin (Ascomycota: Hypocreales) is a well-known and important filamentous fungus species with a wide distribution (Niemczyk *et al.,* 2019; Vertporokh *et al.,* 2019; McGuire and Northfield, 2020). This fungus is well adapted to terrestrial agro-ecosystems and well-studied because of its pathogenic action on numerous agricultural pests (Rodrigues *et al.,* 2016; Javed *et al.,* 2019). *Beauveria bassiana* occurs naturally in the soil, but it is generally isolated from the exterior surface of arthropod cadavers as well as from the surfaces and interior of plants (Zimmermann, 2007). This fungus occurs in a wide range of climatic regions including tropical (McGuire and Northfield, 2020), temperate (Shapiro-Ilan *et al.,* 2017) and artic regions (Meyling *et al.,* 2012).

Ultraviolet radiation, humidity and temperature are the most important abiotic factors affecting germination, growth, survival and pathogenicity of entomopathogenic fungi (EPF) (Ugine, 2011; Latifian *et al.,* 2018). *Beauveria bassiana* can grow at temperatures between 6-35 °C (Seid *et al*., 2019). Fargues *et al.* (1997) reported the

optimal temperature for growth, to be between 20-30 °C, while McGuire and Northfield (2020), refined it to be between 25-30 °C. Different strains may differ in their thermal optima (McGuire and Northfield, 2020), but temperatures below or above these ranges may have a negative impact on germination and viability of conidia (Athanassiou *et al.,* 2017). For example, *B. bassiana* is able to survive for one year at 8 °C, but under extreme heat conditions (>50 °C), spore viability is reduced and the fungi eventually die (Rodrigues *et al.,* 2016; Latifian *et al.,* 2018). The aim of this study was to assess the effect of temperature on radial growth of 11 *B. bassiana* isolates.

3.3. Material and methods

3.3.1 Fungal cultures

Ten *B. bassiana* isolates were isolated from infected *Bathycoelia distincta* (Distant) (Hemiptera: Pentatomidae), and one isolate from *Nezara viridula* (Linnaeus) (Hemiptera: Pentatomidae), collected from the Agricultural Research Council (ARC) - Tropical and Subtropical Crops (TSC) research farms in Nelspruit, South Africa. Detailed information on the sampling of these isolates are provided in Table 3.1. The isolates were collected from July 2018 – July 2019 (Table 3.1). Isolates were deposited and are currently maintained in the Mycology National Collection of Fungi at the ARC - Plant Health and Protection (ARC-PHP), Pretoria, South Africa. Fungal isolates used in this study were from stock cultures, preserved in a 15% glycerol solution and stored at -20 ºC at the ARC- TSC in Nelspruit, South Africa.

Table 3.1: *Beauveria bassiana* isolates used in laboratory trials to determine the effects of temperature on radial growth.

Isolate	Host species	Location	Date of isolation
PPRI 26694	*Bathycoelia distincta*	ARC-TSC Nelspruit (25°27'18S 30°58'09E)	Jan-19
PPRI 26695	*Bathycoelia distincta*	ARC-TSC Friedenheim (25°27'004S 30°59'29E)	Sep-18
PPRI 26696	*Bathycoelia distincta*	ARC-TSC Burgershall (25°27'18S 30°58'09E)	Feb-19
PPRI 26697	*Nezara viridula*	ARC-TSC Kiepersol (25°07'01S 31°05'04E)	Jul-18
PPRI 26699	*Bathycoelia distincta*	ARC-TSC Nelspruit (25°27'18S 30°58'09E)	Jul-18
PPRI 26700	*Bathycoelia distincta*	ARC- TSC Nelspruit (25°27'18S 30°58'09E)	Jan-19
PPRI 26701	*Bathycoelia distincta*	ARC-TSC Friedenheim (25°27'004S 30°59'29E)	Jul-18
PPRI 26703	*Bathycoelia distincta*	ARC-TSC Nelspruit (25°27'18S 30°58'09E)	Mar-19
PPRI 26704	*Bathycoelia distincta*	ARC-TSC Nelspruit (25°27'18S 30°58'09E)	Jan-19
PPRI 28504	*Bathycoelia distincta*	ARC-TSC Friedenheim (25°27'004S 30°59'29E)	Jul-19
PPRI 28503	*Bathycoelia distincta*	ARC-TSC Nelspruit (25°27'18S 30°58'09E)	Feb-19

3.3.2 Effect of temperature on radial growth

The 11 fungal isolates were cultured on Sabouraud agar (SDA) and maintained at 25±1 °C, in full darkness, for conidial production. Conidia were harvested after two weeks from sporulating cultures and suspended in sterile distilled water, with Tween 20 (Minema chemicals T9640) added as a surfactant to reduce clumping of conidia. The suspensions were vortexed (Vortex genie) for 5 min at 700 rpm in sterile 5 ml Eppendorf tubes, to obtain a homogenous suspension. Fifth-instar mealworm larvae, *Tenebrio molitor* (Linnaeus) (Coleoptera: Tenebrionidae), were dipped into these Eppendorf tubes for 3 sec and transferred to polystyrene Petri dishes (90 mm diameter), lined with moist filter paper. The larvae were kept without food in the Petri dishes at 25±1 °C in darkness for 14 days. After 14 days, conidia were harvested from the surface of the larval cadavers with a sterile scalpel blade, transferred onto SDA media and incubated at 25 ºC for 14 days in darkness. After 14 days, fungal conidia were harvested by flooding the Petri dishes with sterile distilled water containing 0.01% Tween 20. The prepared suspensions were then filtered through sterilized cheese cloth to remove hyphae and unsuspended conidia. The conidia

suspensions were diluted to obtain a concentration of 1 × 10^7 conidia/ml, of which 100-ul was spread evenly over the SDA media in Petri dishes. The Petri dishes were sealed with parafilm, and incubated in incubation chambers in darkness at 25±1 ºC for 72 h to obtain mycelial mats.

Unsporulated mycelia were cut into 5 mm (diameter), round plugs using a flame-sterilized cork-borer. These agar plugs were individually placed, upside down on the centre of newly prepared SDA medium, in Petri dishes (90 mm diameter). Plates were sealed with parafilm and incubated at 20, 25, 30 and 35 ± 1 ºC in darkness. Surface radial growth was measured using cardinal diameters, through two orthogonal axes drawn on the bottom of each petri dish to serve as a reference (Figure 3.1). Radial measurements were recorded at 6-day intervals, from three days after inoculation for a period of 21 days. The position of all plates were completely random to mitigate any possible bias and each isolate was replicated three times for each temperature treatment.

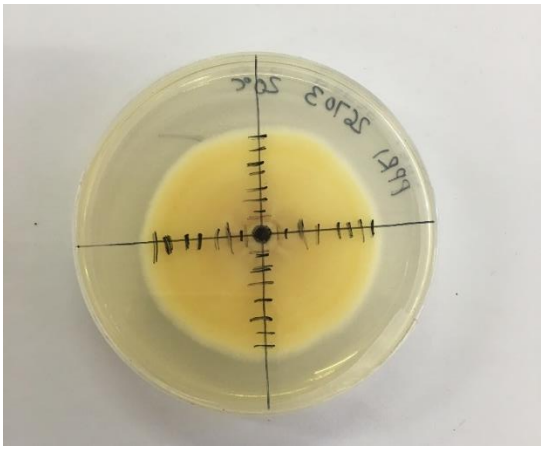


Figure 3.1: Surface radial growth was measured using cardinal diameters, through two orthogonal axes drawn on the bottom of each petri dish to serve as a reference

3.4. Data analysis

Repeated-measures ANOVA was used to analyse mean radial growth over time. Bonferroni correction was used to adjust for multiple mean comparisons. The data were analysed using TIBCO® Statistica™ software version 13.5 (TIBCO software Inc., 2018).

3.5. Results

3.5.1: Effect of temperature on radial growth

Mean radial growth of the respective isolates over a period of 21 days at four different temperatures are provided in Table 3.2. Radial growth of the different isolates was, however, differentially influenced by temperature ($F_{30,88}$ = 4.02; P < 0.0001) (Figure 3.2). (See also Appendix 3.1)

Table 3.2: Effect of temperature on radial growth (mm/day) of 11 *Beauveria bassiana* isolates at three-day intervals for a period of 21 days.

Temperature (°C)	Isolate	Days after inoculation			
		3	9	15	21
20	26694	10.83 ± 0.17	15.50 ± 0.76	32.67 ± 0.73	43.83 ± 1.83
	26695	12.17 ± 0.17	19.67 ± 0.17	40.17± 1.92	52.50 ± 3.25
	26696	14.33 ± 0.33	23.17 ± 0.17	42.00 ± 0.50	49.33 ± 0.67
	26697	11.00 ± 0.29	17.67 ± 0.60	43.83 ± 0.44	59.50 ± 1.50
	26699	12.00 ± 0.00	18.83 ± 0.44	37.33 ± 2.33	48.00 ± 3.06
	26700	12.00 ± 0.00	19.83 ± 0.17	34.83 ± 1.01	41.33 ± 2.52
	26701	12.83 ± 1.17	20.67 ± 1.86	38.50 ± 3.88	52.83 ± 2.89
	26703	10.33 ± 0.17	17.50 ± 0.29	38.83 ± 1.09	50.00 ± 0.76
	26704	11.67 ± 0.17	18.33 ± 0.44	35.00 ± 0.50	43.00 ± 1.00
	28505	10.67 ± 0.58	17.67 ± 0.44	38.17 ± 2.60	50.50 ± 4.37
	28503	11.83 ± 0.44	19.17 ± 1.59	40.00 ± 4.48	52.17 ± 5.45
25	26694	14.17 ± 0.60	21.33 ± 0.73	43.67 ± 1.33	56.50 ± 1.26
	26695	14.00 ± 0.00	23.67± 0.44	47.00 ± 1.44	57.17 ± 3.77
	26696	13.17 ± 0.17	21.67 ± 0.17	44.67 ± 0.44	52.33 ± 3.66
	26697	12.83 ± 0.44	22.00 ± 0.29	44.00 ± 3.55	54.50 ± 5.53
	26699	13.50 ± 0.50	22.17 ± 0.17	49.17 ± 1.01	57.50 ± 2.02
	26700	13.33 ± 0.88	22.33 ± 0.93	38.50 ± 3.28	44.83 ± 7.10
	26701	12.00 ± 0.50	20.33 ± 0.67	38.17 ± 3.17	49.33 ± 6.22
	26703	12.67 ± 0.17	19.83 ± 0.17	40.50 ± 0.76	45.67 ± 1.74
	26704	14.00 ± 0.00	21.67 ± 0.33	37.83 ± 3.00	42.50 ± 4.58
	28505	13.50 ± 0.29	22.00 ± 0.58	42.50 ± 2.02	50.83 ± 4.10
	28503	14.67 ± 0.33	23.50 ± 0.29	48.00 ± 0.76	55.67 ± 4.04
30	26694	15.50 ± 0.29	22.17 ± 1.17	44.00 ± 4.31	50.17 ± 4.00
	26695	13.50 ± 0.29	22.67 ± 1.09	48.17 ± 0.44	61.17 ± 2.96
	26696	15.00 ± 0.58	24.00 ± 0.58	45.83 ± 0.83	61.33 ± 0.60
	26697	12.33 ± 0.33	19.50 ± 0.50	37.17 ± 2.80	47.50 ± 5.35
	26699	11.67 ± 0.33	18.83 ± 0.73	35.67 ± 1.09	45.00 ± 1.15
	26700	14.67 ± 0.33	20.00 ± 0.76	37.67 ± 1.42	48.83 ± 3.90
	26701	11.83 ± 0.60	19.67 ± 0.33	36.50 ± 1.00	46.00 ± 2.75
	26703	13.50 ± 0.29	21.00 ± 0.76	46.83 ± 1.64	62.83 ± 1.30
	26704	14.50 ± 0.29	21.33 ± 0.73	37.33 ± 0.17	47.17 ± 1.83
	28505	13.50 ± 0.76	21.33 ± 0.60	39.67 ± 0.44	49.33 ± 0.67
	28503	14.83 ± 0.44	20.50 ± 0.76	34.83 ± 1.86	44.33 ± 2.03
35	26694	7.33 ± 0.17	8.00 ± 0.58	8.00 ± 0.58	8.00 ± 0.58
	26695	7.50 ± 0.29	7.83 ± 0.33	7.83 ± 0.33	7.83 ± 0.33
	26696	7.17 ± 0.17	7.17 ± 0.17	7.17 ± 0.17	7.17 ± 0.17
	26697	8.33 ± 0.17	8.33 ± 0.17	8.33 ± 0.17	8.33 ± 0.17
	26699	8.17 ± 0.17	8.17 ± 0.17	8.17 ± 0.17	8.17 ± 0.17
	26700	7.17 ± 0.17	7.17 ± 0.17	7.17 ± 0.17	7.17 ± 0.17
	26701	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00
	26703	7.00 ± 0.00	7.17 ± 0.17	7.50 ± 0.29	7.50 ± 0.29
	26704	7.50 ± 0.29	8.33 ± 0.60	8.33 ± 0.60	8.33 ± 0.60
	28505	8.50 ± 0.29	8.50 ± 0.29	8.50 ± 0.29	8.50 ± 0.29
	28503	7.17 ± 0.17	7.50 ± 0.29	7.50 ± 0.29	7.50 ± 0.29

Table 3.3: Repeated measures analysis of variance results of the effect of isolate, temperature and time on the mean radial growth of 11 *Beauveria bassiana* isolates.

Effects	Df	F-value	P-value
Isolate	10, 88	3.29	0.01
Temperature	3, 88	1010.51	0.0001
Time	38, 264	3399.43	0.0001
Isolate x Temperature	30, 88	4.02	0.0001
Time x Isolate	30, 264	3.18	0.0001
Time xTemperature	9, 264	375.11	0.0001
Time x Isolate x Temperature	90, 264	2.66	0.0001

The radial growth differed significantly between isolates (Table 3.3). Radial growth of isolates 26699 (23.5 mm) and 26704 (23.74 mm) was significantly slower compared to isolate 26703 (26.99 mm), but did not differ from all the other isolates (Figure 3.2). There was also no significant difference in radial growth of isolate 26703 and the other isolates (Figure 3.2).

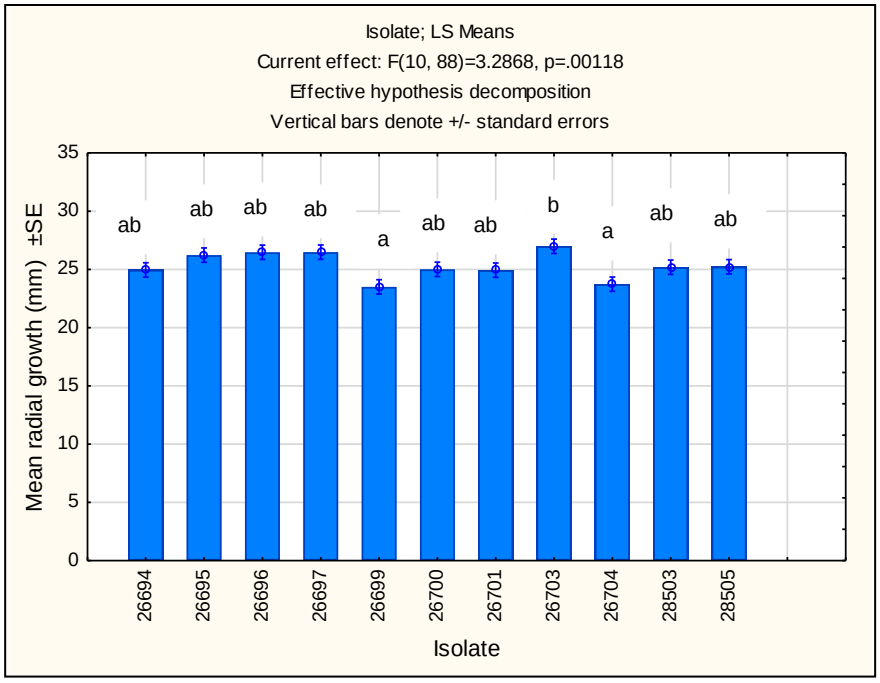


Figure 3.2: The mean radial growth (mm) ±SE of 11 *Beauveria bassiana* isolates.

Temperature had a significant effect on radial growth of the *B. bassiana* isolates (Table 3.3). Radial growth of all isolates was significantly inhibited at 35 °C

compared to growth at 20, 25 and 30 °C. Mean radial growth at 20 °C was also significantly slower than at 25 and 30 °C, but it did not differ significantly at the latter two temperatures (Figure 3.3).

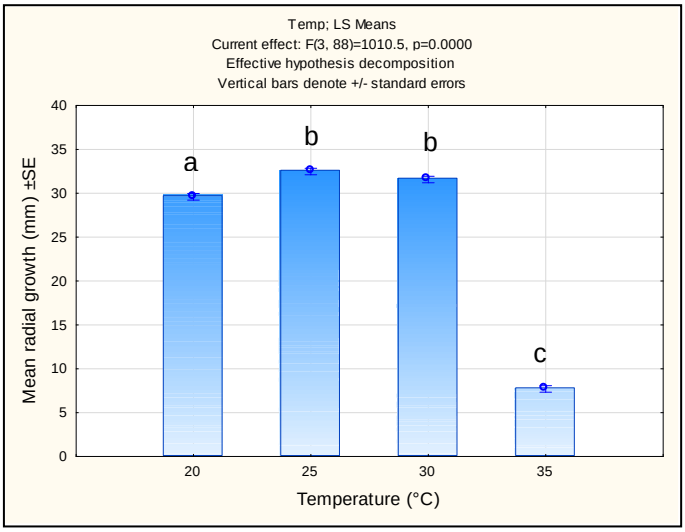


Figure 3.3: The effect of temperature on mean radial growth (±SE) of 11 *Beauveria bassiana* isolates.

Mean radial growth of the respective isolates was significantly affected by the interaction between the respective isolates and temperature (Table 3.3). All isolates did, however, grew significantly slower at 35 °C (Figure 3.4), (See also appendix 3.1a).

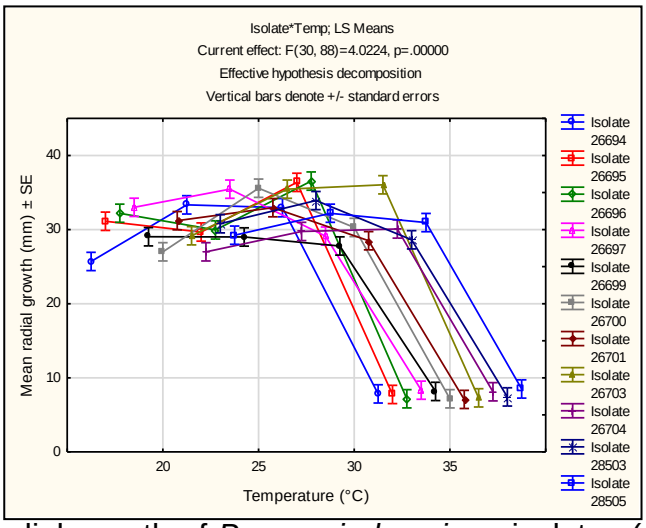


Figure 3.4: Mean radial growth of *Beauveria bassiana* isolates (±SE) as affected by the interaction between isolates and temperature.

Mean radial growth of the respective isolates increased differentially over time, as indicated by the significant interaction between time after inoculation and the respective isolates ($F_{30,264} = 3.18$; $P < 0.0001$) (Figure 3.5). (See also Appendix 3.1b)

However, at 21 days after inoculation, isolate 26703 had the longest radial growth, but it did not differ significantly from that of isolates 26701, 28505, 28503, 26696, 26695 and 26697 (Appendix 3.1b).

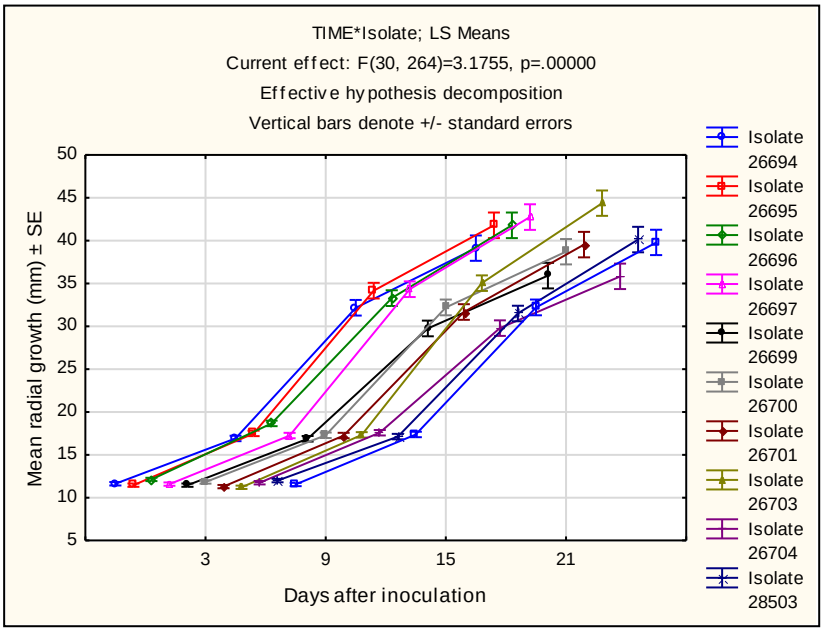


Figure 3.5: Mean radial growth of the *Beauveria bassiana* isolates (±SE) as affected by the interaction of the respective isolates with the time after inoculation.

The mean radial growth of the 11 *B. bassiana* isolates was also significantly affected by the time x isolate x temperature interaction ($F_{90,264} = 2.66$; $P < 0.0001$) (Table 3.3) (see also Appendix 3.2).

3.6 Discussion

All 11 *B. bassiana* isolates grew well over time, with their mean radial growth after 21 days being between 23.5 and 26.99 mm. Radial growth was, however, significantly affected by temperature. All *B. bassiana* isolates were able to grow at temperatures ranging between 20-30 °C, with optimal radial growth between 25 °C and 30 °C, and inhibited growth of all isolates at 35 °C. These findings were in agreement with that of Aker and Tuncer (2016) and Fargues *et al.* (1997) who reported that EPF belonging to the Hypomycetes are mesophilic with a temperature range between 15 - 35 °C, and optimum germination and growth between 25 and 30 °C. Similar findings were reported on the hyphomycetous fungi, *B. bassiana* and *Metarhizium anisopliae* (flavoviride) var. *acridum* (Ekesi *et al.,* 1999; Fargues *et al.,* 1997; Tefera

and Pringle 2003; Dimbi *et al.,* 2004). According to Yeo *et al.* (2003), the rate of growth was faster between 20 and 25 °C than at 10 °C and 15 °C. *Beauveria bassiana* isolate F-263 also grows between 10-33 °C, with optimal growth at 30 °C and no growth at 35 °C (Shimazu, 2004).

The physiology of fungal species may differ depending on their climatic region (Robinson, 2001). For example, most fungal isolates that occur in the Arctic and Antarctic are psychrotropic, they are tolerant to low temperatures and they have temperature growth optima at 20 °C or above (Robinson, 2001). Similar responses were reported in subarctic climate of the Yukon where entomopathogens showed favourable growth at 4-20 °C (Dobrotka *et al.,* 2019), while isolates from the Artic had favourable growth rates between 8-12 °C (Seid *et al.,* 2019). On the contrary, fungal isolates originating from temperate regions are tolerate to moderate to high temperatures (Tefera and Pringle 2003; Santoro *et al.,* 2015). The temperature response of *B. bassiana* isolates in this study, may therefore also be explained by the moderate climatic conditions of the area where these isolates were sampled. Nelspruit, in the Mpumalanga province, is in the temperate zone, with temperatures ranging between 14-30 °C. Temperature is therefore an important factor that can strongly affect the growth, pathogenicity, sporulation and distribution of filamentous fungi (Dimbi *et al.,* 2004; Aker and Tuncer 2016; Latifian *et al.,* 2018).

Seven of these isolates, *viz*. 26701, 26703, 28505, 28503, 26695, 26696, and 26697 did not differ in terms of radial growth 21 days after inoculation. These isolates could have potential for use in management of *B. distincta and N. viridula* and should be further evaluated.

References

Aker, O. and Tuncer, C. 2016. Efficacy of some entomopathogenic fungi in controlling filbert aphid, *Myzocallis coryli* Goetze (Hemiptera: Aphididae). *International Journal of Entomology Research* 1(5):49-53.

Athanassiou, C.G., Kavallieratos, N.G., Rumbos, C.I. and Kontodimas, D.C. 2017. Influence of temperature and relative humidity on the insecticidal efficacy of *Metarhizium anisopliae* against larvae of *Ephestia kuehniella* (Lepidoptera: Pyralidae) on wheat. *Journal of Insect Science* 17(1):22: 1-7.DOI: 10.1093/jisesa/iew107

Dimbi, S., Maniania, N.K., Lux, S.A. and Mueke, J.M. 2004. Effect of constant temperatures on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African tephritid fruit flies. *BioControl* 49:83-94.

Dobrotka, C.J., Krieger, M., Oakley, M., Trotter, J., Rodell, B.M., Stein, K.L., Klever, L.A., Fisher, K.A. and Yoder, J.A. 2019. Entomopathogenic fungi isolated from Yukon soil against unfed larvae of the winter tick, *Dermacentor albipictus*. *Studies in Fungi* 4(1):123-134.

Ekesi, S., Maniania, N.K. and Nyarko, A. 1999. Effect of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti*. *Biocontrol Science and Technology* 9(2):177-185. DOI: 10.1080/09583159929767

Fargues, J., Goettel, M.S., Smits, N., Ouedraogo, A. and Rougier, M. 1997. Effect of temperature on vegetative growth of *Beauveria bassiana* isolates from different origins. *Mycologia* 89(3):383-392.

Javed, K., Javed, H., Mukhatar, T. and Qiu, D. 2019. Efficacy of *Beauveria bassiana* and *Verticillium lecanii* for the management of whitefly and aphid. *Pakistan Journal of Agricultural Sciences* 56(3):669-674. DOI: 10.21162/PAKJAS/19.8396

Latifian, M., Ghazavi, M. and Soleimannejadian, E. 2018. The role of temperate on the pathogenicity of *Beauveria bassiana* in populations of sawtoothed grain beetle, *Oryzaephilus surinamensis* (Coleoptera: Silvanidae) fed on stored date fruits. *Journal of Crop Protection* 7(4):395-402.

McGuire, A.V. and Northfield, T.D. 2020. Tropical occurrence and agricultural importance of *Beauveria bassiana* and *Metarhizium anisopliae*. *Frontiers in Sustainable Food Systems* 4(6) DOI: 10.3389/fsufs.2020.00006

Meyling, N.V., Schmidt, N.M. and Eilenberg, J. 2012. Occurrence and diversity of fungal entomopathogens in soils of low and high Artic Greenland. *Polar Biology* 35:1439-1445. DOI: 10.1007/s00300-012-1183-6

Niemczyk, M., Sierpińska, A., Tereba, A., Sokołowski, K. and Przybylski, P. 2019. Natural occurrence of *Beauveria* spp. In outbreak areas of cockchafers (*Melolontha*

spp.) in forest soils from Poland. *BioControl* 64:159-172 DOI: org/10.1007/s10526-019-09927-3

Robinson, C.H. 2001. Cold adaptation in Arctic and Antarctic fungi. *New Phytologist* 151:341-353.

Rodrigues, I.M.W., Forim, M.R., da Silva, M.F.G.F., Fernandes, J.B. and Filho, A.B. 2016. Effect of ultraviolet radiation on fungi *Beauveria bassiana* and *Metarhizium anisopliae*, pure and encapsulated, and bio-insecticide action on *Diatrae saccharalis*. *Advances in Entomology* 4:151-162. DOI: 10.4236/ae.2016.43016

Santoro, P.H., Zorzetti, J., Constanski, K. and Neves, M.O.J. 2015. Quality of *Beauveria bassiana* conidia after successive passages through *Alphitobius diaperinus* (Coleoptera: Tenebrionidae). *Revista Colombiana de Entomologia* 41(1):87-94.

Seid, A.M., Fredensborg, B.L., Steinwender, B.M. and Meyling, N.V. 2019. Temperature-dependent germination, growth and co-infection of *Beauveria* spp. Isolates from different climatic regions. *Biocontrol Science and Technology* 29(5):411-426.

Shapiro-llan, D., Arthurs, S.P. and Lacey, L.A. 2017. Microbial control of arthropod pests of orchards in temperate climates. N: Lacey, L. A. (Ed.), Microbial Agents for Control of Insect Pests: from discovery to commercial development and use. Academic Press, pp. 253-267.

Shimazu, M. 2004. Effects of temperature on growth of *Beauveria bassiana* F-263, a strain highly virulent to the Japanese pine sawyer, *Monochamus alternatus*, especially tolerance to high temperatures. *Applied Entomology and Zoology* 39(3):469-475.

Tefera, T. and Pringle, K. 2003. Germination, radial growth, and sporulation of *Beauveria bassiana* and *Metarhizium anisopliae* isolates and their virulence to *Chilo partellus* (Lepidoptera: Pyralidae) at different temperatures. *Biocontrol Science and Technology* 13(7):699-704.

Ugine, T.A. 2011. The effect of temperature and exposure to *Beauveria bassiana* on tarnish plant bug *Lygus lineolaris* (Heteroptera: Miridae) population dynamics, and

the broader implications of treating insects with entomopathogenic fungi over a range of temperatures. *Biological Control* 59:373-383.

Vertyporokh, L., Hułas-Stasiak, M. and Wojda, I. 2019. Host-pathogen interaction after infection of *Galleria mellonella* with the filamentous fungus *Beauveria bassiana*. *Insect Science* 1-11 DOI: 10.1111/1744-7917.12706.

Yeo,H., Pell, J.K., Alderson, P.G., Clark, S.J. and Pye, B.J. 2003. Laboratory evaluation of temperature effects on the germination and growth of entomopathogenic fungi and on their pathogenicity to two aphid species. *Pest Management Science* 59:156-165. DOI: 10.1002/ps.622

Zimmermann, G. 2007. Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Science and Technology* 17(6):553-596 DOI: 10.1080/09583150701309006

Appendix 3.1

(a) Isolate*Temperature

Bonferroni test; variable DV_1 (Lungile for stats (B2:EC133))
Homogenous Groups, alpha = .05000 (Non-Exhaustive Search)
Error: Between MS = 18.229, df = 88.000

Cell No.	Isolate	Temp	DV_1 Mean	1	2	3	4	5	6
28	26701	35	7.08333	****					
12	26696	35	7.16667	****					
24	26700	35	7.16667	****					
32	26703	35	7.29167	****					
40	28503	35	7.41667	****					
8	26695	35	7.75000	****					
4	26694	35	7.83333	****					
36	26704	35	8.12500	****					
20	26699	35	8.16667	****					
16	26697	35	8.33333	****					
44	28505	35	8.50000	****					
1	26694	20	25.70833		****				
33	26704	20	27.00000		****	****			
21	26700	20	27.00000		****	****			
19	26699	30	27.79167		****	****			
27	26701	30	28.50000		****	****	****		
39	28503	30	28.62500		****	****	****		
18	26699	25	29.00000		****	****	****	****	
17	26699	20	29.04167		****	****	****	****	
15	26697	30	29.12500		****	****	****	****	
29	26703	20	29.16667		****	****	****	****	****
41	28505	20	29.25000		****	****	****	****	****
6	26695	25	29.66667		****	****	****	****	****
34	26704	25	29.75000		****	****	****	****	****
10	26696	25	29.95833		****	****	****	****	****
35	26704	30	30.08333		****	****	****	****	****
23	26700	30	30.29167		****	****	****	****	****
37	28503	20	30.79167		****	****	****	****	****
43	28505	30	30.95833		****	****	****	****	****
5	26695	20	31.12500		****	****	****	****	****
25	26701	20	31.20833		****	****	****	****	****
42	28505	25	32.20833		****	****	****	****	****
9	26696	20	32.20833		****	****	****	****	****
3	26694	30	32.95833		****	****	****	****	****
26	26701	25	32.95833		****	****	****	****	****
13	26697	20	33.00000		****	****	****	****	****
2	26694	25	33.33333			****	****	****	****
38	28503	25	33.91667			****	****	****	****
30	26703	25	35.45833				****	****	****
14	26697	25	35.45833				****	****	****
22	26700	25	35.58333				****	****	****
31	26703	30	36.04167					****	****
7	26695	30	36.37500					****	****
11	26696	30	36.54167						****

(b) Isolate*Time

Bonferroni test; variable DV_1 (Lungile for stats (B2:EC133))
Homogenous Groups, alpha = .05000 (Non-Exhaustive Search)
Error: Between; Within; Pooled MS = 9.5587, df = 276.25

Cell No.	Isolate	TIME	DV_1 Mean	1	2	3	4	5	6	7	8	9	10
29	26703	3	11.20833	****									
25	26701	3	11.29167	****									
17	26699	3	11.45833	****									
5	26695	3	11.45833	****									
41	28505	3	11.54167	****									
13	26697	3	11.58333	****									
1	26694	3	11.62500	****									
33	26704	3	11.75000	****	****								
21	26700	3	11.83333	****	****								
37	28503	3	12.00000	****	****								
9	26696	3	12.12500	****	****	****							
18	26699	9	16.87500		****	****	****						
2	26694	9	16.91667		****	****	****						
38	28503	9	17.12500			****	****						
26	26701	9	17.25000			****	****						
14	26697	9	17.25000			****	****						
22	26700	9	17.29167			****	****						
30	26703	9	17.33333				****						
42	28505	9	17.37500				****						
6	26695	9	17.50000				****						
34	26704	9	17.58333				****						
10	26696	9	18.66667				****						
19	26699	15	29.75000					****					
35	26704	15	29.79167					****					
39	28503	15	31.50000					****	****				
27	26701	15	31.66667					****	****				
3	26694	15	32.16667					****	****				
43	28505	15	32.20833					****	****				
23	26700	15	32.20833					****	****				
11	26696	15	33.29167					****	****				
7	26695	15	34.16667					****	****	****			
15	26697	15	34.33333					****	****	****			
31	26703	15	35.04167						****	****	****		
36	26704	21	35.83333						****	****	****		
20	26699	21	35.91667						****	****	****		
24	26700	21	38.70833							****	****	****	
4	26694	21	39.12500							****	****	****	
28	26701	21	39.54167								****	****	****
44	28505	21	39.79167								****	****	****
40	28503	21	40.12500								****	****	****
12	26696	21	41.79167									****	****
8	26695	21	41.79167									****	****
16	26697	21	42.75000									****	****
32	26703	21	44.37500										****

Appendix 3.2: Time*Isolate*Temperature

Scheffe test, variable DV_1 (Lungile for stats (32 EC133))
Homogeneous Groups, alpha = .05000 (Non-Exhaustive Search)
Error: Between, Within, Pooled MS = 9.5587, df = 276.25

Cell No.	Isolate	Temp	TIME	DV_1 Mean	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
111	26701	35	15	7.00000	****																																						
112	26701	35	21	7.00000	****																																						
110	26701	35	9	7.00000	****																																						
125	26702	35	3	7.00000	****																																						
93	26703	35	3	7.16666	****																																						
94	26703	35	9	7.16666	****																																						
48	26858	35	21	7.16666	****																																						
96	26703	35	21	7.16666	****																																						
157	26502	35	3	7.16666	****																																						
45	26858	35	3	7.16666	****																																						
47	26858	35	15	7.16666	****																																						
46	26858	35	9	7.16666	****																																						
95	26703	35	15	7.16666	****																																						
126	26702	35	9	7.16666	****																																						
13	26894	35	3	7.33333	****	***																																					
109	26701	35	3	7.33333	****	***																																					
128	26702	35	21	7.50000	****	***																																					
127	26702	35	15	7.50000	****	***																																					
158	26502	35	9	7.50000	****	***																																					
141	26704	35	3	7.50000	****	***																																					
159	26502	35	15	7.50000	****	***																																					
29	26858	35	3	7.50000	****	***																																					
160	26502	35	21	7.50000	****	***																																					
30	26858	35	9	7.83333	****	***	***																																				
32	26858	35	21	7.83333	****	***	***																																				
31	26858	35	15	7.83333	****	***	***																																				
16	26894	35	21	8.00000	****	***	***	**																																			
14	26894	35	9	8.00000	****	***	***	**																																			
15	26894	35	15	8.00000	****	***	***	**																																			
77	26855	35	3	8.16666	****	***	***	**	***																																		
79	26855	35	15	8.16666	****	***	***	**	***																																		
80	26855	35	21	8.16666	****	***	***	**	***																																		
78	26855	35	9	8.16666	****	***	***	**	***																																		
63	26859	35	15	8.33333	****	***	***	**	***																																		
142	26704	35	9	8.33333	****	***	***	**	***																																		
143	26704	35	15	8.33333	****	***	***	**	***																																		
62	26859	35	9	8.33333	****	***	***	**	***																																		
61	26859	35	3	8.33333	****	***	***	**	***																																		
144	26704	35	21	8.33333	****	***	***	**	***																																		
64	26859	35	21	8.33333	****	***	***	**	***																																		
175	26502	35	15	8.50000	****	***	***	**	***	***																																	
174	26502	35	9	8.50000	****	***	***	**	***	***																																	
173	26502	35	3	8.50000	****	***	***	**	***	***																																	
176	26502	35	21	8.50000	****	***	***	**	***	***																																	
113	26702	20	3	10.33333	****	***	***	**	***	***	***																																
161	26502	20	3	10.66666	****	***	***	**	***	***	***	***																															
1	26894	20	3	10.83333	****	***	***	**	***	***	***	***	***																														
49	26859	20	3	11.00000	****	***	***	**	***	***	***	***	***																														
129	26704	20	3	11.66666	****	***	***	**	***	***	***	***	***	***																													
73	26855	30	3	11.66666	****	***	***	**	***	***	***	***	***	***																													
105	26701	30	3	11.83333	****	***	***	**	***	***	***	***	***	***	***																												
145	26502	20	3	11.83333	****	***	***	**	***	***	***	***	***	***	***																												
65	26855	20	3	12.00000	****	***	***	**	***	***	***	***	***	***	***																												
81	26703	20	3	12.00000	****	***	***	**	***	***	***	***	***	***	***																												
37	26858	25	3	12.00000	****	***	***	**	***	***	***	***	***	***	***																												
17	26892	20	3	12.16666	****	***	***	**	***	***	***	***	***	***	***	**																												
57	26859	30	3	12.33333	****	***	***	**	***	***	***	***	***	***	***	**																												
21	26892	25	3	12.66666	****	***	***	**	***	***	***	***	***	***	***	**																												
5	26894	25	3	12.83333	****	***	***	**	***	***	***	***	***	***	***	**																												
97	26701	20	3	12.83333	****	***	***	**	***	***	***	***	***	***	***	**																												
101	26701	25	3	13.16666	****	***	***	**	***	***	***	***	***	***	***	**																												
133	26704	25	3	13.33333	****	***	***	**	***	***	***	***	***	***	***	**																												
25	26892	30	3	13.50000	****	***	***	**	***	***	***	***	***	***	***	**																												
165	26502	25	3	13.50000	****	***	***	**	***	***	***	***	***	***	***	**																												
121	26702	30	3	13.50000	****	***	***	**	***	***	***	***	***	***	***	**																												
169	26502	30	3	13.50000	****	***	***	**	***	***	***	***	***	***	***	**																												
85	26703	25	3	13.50000	****	***	***	**	***	***	***	***	***	***	***	**																												
69	26855	25	3	14.00000	****	***	***	**	***	***	***	***	***	***	***	**																												
117	26702	25	3	14.00000	****	***	***	**	***	***	***	***	***	***	***	**																												
149	26502	25	3	14.16666	****	***	***	**	***	***	***	***	***	***	***	**																												
33	26858	20	3	14.33333	****	***	***	**	***	***	***	***	***	***	***	**																												
137	26704	30	3	14.50000	****	***	***	**	***	***	***	***	***	***	***	**																												
53	26859	25	3	14.66666	****	***	***	**	***	***	***	***	***	***	***	**																												
89	26703	30	3	14.66666	****	***	***	**	***	***	***	***	***	***	***	**																												
153	26502	30	3	14.83333	****	***	***	**	***	***	***	***	***	***	***	**																												
41	26858	30	3	15.00000	****	***	***	**	***	***	***	***	***	***	***	**																												
9	26894	30	3	15.50000	****	***	***	**	***	***	***	***	***	***	***	**																												
2	26894	20	9	15.50000	****	***	***	**	***	***	***	***	***	***	***	**																												
114	26702	20	9	17.50000	****	***	***	**	***	***	***	***	***	***	***	**																												
50	26859	20	9	17.66666	****	***	***	**	***	***	***	***	***	***	***	**																												
162	26502	20	9	17.66666	****	***	***	**	***	***	***	***	***	***	***	**																												
130	26704	20	9	18.33333	****	***	***	**	***	***	***	***	***	***	***	**																												
74	26855	30	9	18.83333	****	***	***	**	***	***	***	***	***	***	***	**																												

Chapter 4

Control of *Bathycoelia distincta* (Hemiptera: Pentatomidae) with indigenous *Beauveria bassiana* isolates, under laboratory and semi-field conditions

4.1 Abstract

The damage by stink bugs to macadamia in South Africa, is currently estimated at R 180 million. The most dominant stink bug in terms of damage caused, is the indigenous two-spotted stink bug, *Bathycoelia distincta* (Distant) (Hemiptera: Pentatomidae). The aim of this study was to evaluate control of *B. distincta* with indigenous *Beauveria bassiana* isolates under laboratory and semi-field conditions. *Beauveria bassiana* was isolated from *B. distincta* and *Nezara viridula* (L.) (Hemiptera: Pentatomidae) adults. The pathogenicity of five of these isolates (PPRI 26695, 26696, 2667, 26700 and 26704), was tested against *B. distincta* under laboratory conditions. Adult stink bugs were individually inoculated with conidia suspensions from the respective isolates, and kept at room temperature under natural light. Stink bug mortality caused by *B. bassiana*, was confirmed with fungal growth from cadavers, 18 days after treatment. Based on the shortest lethal time, (LT_{50} of 6.99 and $LT_{90\ of}$ 12.85 d) as well as a high infection rate of 86.6%, isolate PPRI 26695 was selected for further assessment under semi-field conditions. The efficacy of control of *B. distincta* adults with the non-formulated, experimental *B. bassiana* isolate PPRI 26695, was compared to that of the formulated, commercially available product, Eco-Bb® and an insecticide, cypermethrin. All three these products provided effective control, with PPRI 26695, Eco-Bb® and cypermethrin that caused mortalities of 100, 90 and 80%, respectively, 18 days after treatment.

Keywords: *Bathycoelia distincta*, *Beauveria bassiana,* cypermethrin, Eco-Bb®, laboratory, pathogenicity, semi-field.

4.2 Introduction

The indigenous two-spotted stink bug, *Bathycoelia distincta* (Distant) (Hemiptera: Pentatomidae), is the dominant stink bug in macadamia orchards in South Africa where it causes severe nut damage (Schoeman, 2014a; 2018). Since its first report as a pest of macadamia in 1984 in Levubu, Limpopo province (Bruwer, 1992), it also

became problematic in other macadamia producing regions of South Africa (Schoeman, 2018).

Stink bugs feed by inserting their piercing mouthparts into the food source and extracting plant sap from leaves, stems and seed pods (Panizzi, 2000). The plant tissues are damaged due to the secretion of toxic saliva, which cause tissue necrosis (Panizzi, 2000). Feeding by the *B. distincta* nymphs and adults is detrimental to developing and mature macadamia nuts (Schoeman, 2009). When feeding occurs during early nut development, damage is visible as necrotic lesions in kernels. Damage inflicted by this pest in later nut development stages, results in oily, glazed, almost semi-transparent nuts. Nuts are also lost due to early nut drop (Schoeman, 2013). Uncontrolled populations can lead to yield losses of 80% (Schoeman, 2014a). The South African macadamia industry lost R 50 million due to damage caused by heteropterans in 2009. The current figure estimated for losses incurred through stink bug damage, is R 180 million (Taylor *et al.,* 2018; SAMAC, 2020). In the last decades, Bruwer (1992), van den Berg *et al.* (1999) and Schoeman (2013) monitored seasonal variations of stink bug populations on macadamia in South Africa. Changes in the population inside macadamia orchards are associated with immigration, seasonal variation in insect numbers as well as the quality and quantity of available food sources.

The South African macadamia industry has grown exponentially resulting in the establishment of large monocultures in close proximity to each other (Schoeman, 2018). Due to the lack of host plant diversity in monoculture orchards, a decline in predators/parasitoids occurred. This leads to increases in pest insect populations (Andow, 1983). The most common insect management practice in modern agriculture, effective against insect pests, is the application of insecticides (Cardoso and Alves, 2012; Saeed *et al.,* 2019). The use of insecticides aims to prevent economic damage with the lowest environmental risk (Karaağaç, 2012). Due to the regular and excessive use of insecticides, concerns have been raised regarding the sustainability of environmental stability, particularly aspects associated with health hazards of human, animal and non-target organisms (Mahmood *et al.,* 2016). The use of insecticides has resulted in resistance to insecticides, and to an increase in secondary pests (Schoeman, 2014b; Mohammed, 2020). Stink bug populations which have been heavily exposed to insecticides for several years, may develop

resistant phenotypes. For example, after exposure to organophosphates and pyrethroids for more than 45 years, the Neotropical stink bug, *Euschistus heros* (Hemiptera: Pentatomidae) was reported to be less susceptible to organophosphates and pyrethroids in Brazil (Sosa-Gomez *et al*., 2020). The effective application of insecticides for control of pests in macadamia trees in South Africa, is hampered by the trees being dense and tall (Schoeman, 2014b). Stink bugs mainly occur in the tree-tops, resulting in these insects being exposed to sub-lethal doses due to ineffective application of insecticides. This resulted in the evolution of resistance by *B. distincta* to synthetic pyrethroids (Schoeman, 2014b), and environmentally friendly alternatives are therefore necessary.

Entomopathogenic fungi (EPF) are bioinsecticides, which occur naturally in the soil. They are parasitic to arthropods and are therefore mainly isolated from arthropod cadavers (Behie and Bidochka, 2014; Litwin *et al.,* 2020). Entomopathogenic fungi have the ability to infect and kill arthropods and have been widely used as biological control agents for several insect pests or as supplements to chemical insecticides (Litwin *et al.,* 2020). Entomopathogens infect their host through penetration of the cuticle (Altinok *et al.,* 2019; Litwin *et al.,* 2020). Numerous studies have investigated the entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin for control of agricultural pests (Erler and Ates 2015; Sheng-yong *et al.,* 2015; Castrillo *et al.,* 2017). EPF have been proven to show great potential against various agricultural pests (Sánchez-Peña *et al*., 2011; Hwi-Geon *et al*., 2017; Chandra Teja and Rahman, 2020).

The objective of this study was to investigate the effects of entomopathogenic fungi on *B. distincta* adults in South Africa. In chapter 3, the radial growth of 11 *B. bassiana* isolates were reported and three promising *B. bassiana* isolates with the highest/vigorous radial growth, as well as two isolates with the lowest growth rate, were selected for use in this study.

The aims of this study were to select the most virulent isolate against *B. distincta* adults under laboratory conditions and to conduct a semi-field assessment of the most promising isolate for control of *B. distincta* adults.

4.3 Material and methods

Bathycoelia distincta stock colony

The *B. distincta* stock colony was established from adults and nymphs collected from an unsprayed, mixed cultivar macadamia orchard at the Burgershall research farm of the Agricultural Research Council - Tropical and Subtropical Crops (ARC-TSC), at Kiepersol (25°07'01S 31°05'04E). Sampling was also done at the ARC-TSC Friedenheim farm, Nelspruit (25°27'004S 30°59'29E), in mature (± 10 years old) macadamia orchards (cv. Beaumont). Stink bugs were collected early in the morning (before 10:00 am) by shaking of the branches from the bottom 2 - 2.5 m of randomly selected trees as described by Schoeman (2011). The stink bugs were maintained in translucent, plastic containers (24.5 × 11 × 20.5 cm), closed with mesh infused lids to allow ventilation. The containers were kept at room temperature (25 ± 1 ºC) and under the prevailing photoperiod. Stink bugs were provided with fresh macadamia leaves and nuts, and water was supplied by means of moistened cotton wool. The colony was provided with fresh food and cages were cleaned and the eggs collected, twice a week. The eggs were placed in 90 mm (diameter) plastic Petri dishes lined with moist filter paper (Whatman no. 1). Nymphs that emerged from these eggs were kept in the Petri dishes until they reached the 2nd instar. They were then transferred to plastic containers (24.5 × 11 × 20.5 cm) and provided with fresh macadamia leaves, nuts and moistened cotton wool, and reared until adults. Newly emerged adults were used in bioassays.

4.3.1 Screening of field collected *Beauveria bassiana* isolates against *Bathycoelia distincta*

Fungal cultures

The five *B. bassiana* fungal isolates screened in this study were selected on the basis of radial growth (see Chapter 3). These were isolates PPRI 26695, 26696, 26697 which exhibited rapid growth and isolates 26700 and 26704, with slow growth. These isolates were initially isolated from *B. distincta* and *Nezara viridula* (Linnaeus) (Hemiptera: Pentatomidae) (see Chapter 3), and maintained in stock cultures preserved in a 15% glycerol solution at -20 °C. The five isolates were cultured in Sabouraud dextrose agar (SDA) and kept for two weeks at 25 ± 1 °C in an incubator (Memmert), for conidial production. Conidia were suspended in sterile distilled water containing 0.01 % Tween 20 (Minema chemicals T9640) as a surfactant to reduce

clumping of conidia. The suspensions were vortexed (Vortex genie) for 5 min at 700 rpm in sterile 5 ml Eppendorf tubes to form homogenous mixtures. Each isolate was passed through mealworm, *Tenebrio molitor* (Linnaeus) (Coleoptera: Tenebrionidae) larvae to renew the pathogenicity of the isolates. Fifth-instar larvae were submerged in Eppendorf tubes containing the respective fungal conidia suspensions, for 3 seconds. The mealworms were maintained without food in 90 mm (diameter) plastic Petri dishes lined with wet filter paper. The Petri dishes were kept at 25 ± 1 °C in an incubator in complete darkness. After 14 days, conidia were harvested from the surface of the cadavers with a sterile scalpel blade and transferred to SDA and incubated at 25 ºC in complete darkness.

After 14 days, fungal conidia were harvested from SDA plates by flooding the Petri dishes with sterile distilled water containing 0.01% Tween 20. The conidia were gently scraped using a sterile blade into 20 mL Schott bottles. Tween 20 was used as a surfactant promoting fungal germination, and which allowed spore attachment on surfaces such as insect cuticles and leaves. Aqueous suspensions were filtered using a sterile cheese cloth to separate conidia from mycelial mats into sterile 50 mL centrifuge tubes containing 3 mm glass beads. The centrifuge tubes were sealed and vortexed for 5 min at approximately 700 rpm to break conidia clumps until homogenous conidial suspensions were formed. The concentrations of the suspensions were determined using a Neubauer haemocytometer (0.1 mm depth) (Boekel Scientific) under a compound microscope followed by serial dilutions in sterile water. The final concentrations for all five fungal isolates were adjusted to the standard concentration of 1×10^7 conidia/ml. Conidial suspensions were used within 6 hours of preparation.

Conidial viability

To evaluate the conidial viability, 100 µl from the prepared conidial suspension of each isolate, containing approximately 1×10^7 conidia/ml, was spread plated using 3 mm sterilize glass beads on SDA media plates (three replicates per isolate). A sterile cover slip (2 × 2 cm) was placed onto the agar in each plate, and the plates were incubated at 25 ± 1 ºC in complete darkness. Percentage germination was assessed after 24 hours under a compound microscope (Nikon eclipse Ni DS-Fi2) at 100-x magnification, by counting 100 spores and recording the number of spores with visible germ tubes (germinated) per Petri dish.

4.3.1.1 Laboratory screening

A conidial suspension (1 × 10^7 conidia/ml) was prepared for each fungal isolate as described above (see section 4.3.1). *Bathycoelia distincta* adults were placed individually on 90 mm Petri dishes and allowed to settle to facilitate application of the respective products on stationary specimens. Ten microliters (µl) of the conidial suspension, containing 0.01% Tween 20, was applied, with an auto-pipette, onto the thorax of the stink bug. Adults in the control treatment were inoculated with 10 µl sterilized, distilled water containing 0.01% Tween 20. Each treatment was replicated three times and each replicate consisted of five adults. Five treated stink bugs were transferred into 3 L translucent, plastic containers (24.5 × 11 × 20.5 cm), with mesh infused lids. Fresh macadamia leaves, green nuts as well as moist cotton wool were provided as food and water, respectively. All plant material was replaced once a week. The containers were kept, completely randomized in a laboratory at 25 ± 1 °C and ambient photoperiod.

Mortality assessments of stink bugs were done at 3-day intervals for a period of 18 days, post application. Stink bugs were counted as dead when they did not respond when they were gently tipped with a sterile paint brush. Dead stink bugs were removed from the 3 L containers to minimize cross contamination. Stink bugs that survived after day 18 were kept under observation until they reached the end of their natural life cycles. Treated and non-treated stink bugs were further examined. The dead stink bugs were surface sterilized under a laminar flow hood by dipping them into a 250 ml conical flask containing 70% ethanol for 2 minutes. All surface disinfected stink bugs were allowed to air dry under the laminar flow hood. Stink bug cadavers were then placed in Petri dishes (90 mm diameter) lined with moist filter paper, sealed with parafilm, and incubated at 25 ± 1 ºC under complete darkness to allow development of mycosis. Mortality as a result of fungal infection was confirmed once mycosis became visible.

4.3.1.2 Semi-field screening

A semi-field trial was conducted in a macadamia orchard at the ARC-TSC, Nelspruit, South Africa (25°27’18S 30°58’09E) to investigate the efficacy of control of the *B. bassiana* isolates PPRI 26695 (experimental) and commercially available Eco-Bb® (2 × 10^9 conidia/mL) (strain R444 - Plant Health Protection), as well as an insecticide registered for control of stink bugs on macadamia, cypermethrin (200g/L EC) (Villa

Crop Protection (Pty) Ltd). The experimental isolate, PPRI 26695, was included as a treatment in the semi-field experiment based on its efficacy of control of *B. distincta* in the laboratory experiment (see 4.2.1). Conidial suspension concentrations for PPRI 26695 and Eco-Bb® and were determined with a haemocytometer and the final concentration was adjusted to 2×10^9 conidia/ml.

Eco-Bb®, formulated as dry aerial conidia, is not registered for control of stink bugs, but it is registered for control of false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), on macadamia at a rate of 600 – 1000 g/ha and at an application rate of 1g/L water. The registered dosage rate of cypermethrin is 20 mL/100 L water to be applied as a high volume spray. Cypermethrin was included as the positive control treatment and water as the negative control treatment. Tween 20 (0.01%) was added to all treatments. The experiment was conducted from February to March 2020 under natural conditions. Weather data was obtained from Agroclimatology, Agricultural Research Council – Soil, Climate and Water (ARC – SCW), Stellenbosch, South Africa.

Isolate PPRI 26695 was recovered from a stock-culture preserved in a 15% glycerol solution, stored at -20 ºC and cultured as described above (see section 4.3.1). Conidia of PPRI 26695 were mass-produced on rice in three Erlenmeyer flasks (250 ml) (Figure 4.1). Each flask contained 75 g white rice grains (Aunt Caroline long grain Parboiled Rice Tiger Brands Limited) in 100 ml sterile water. The flasks were closed with cotton wool, covered with foil and autoclaved at 121 °C for 15 minutes. Once cooled down, each flask was inoculated with the *B. bassiana* PPRI 26695 isolate. A mycelial square (1 × 1 cm) excised from the starter culture was inserted into the rice in a laminar flow.

Figure 4.1: Rice overgrown with *Beauveria bassiana*, PPRI 26695

Rice grains were thoroughly mixed with the inoculated mycelial square using autoclaved laboratory spoons to evenly distribute fungal conidia throughout the rice in the flask. The flasks were incubated at 25 ± 1 °C in an incubator. After 14 days of incubation, the *B. bassiana* rice-grown cultures were placed on aluminium foil and oven dried at 55 °C for 24 hours. It was then grounded using an autoclaved mortar and pestle. Spores were harvested by dispensing it in 500 mL of distilled water containing 0.01% Tween 20. The suspension was sieved through a sterile cheese cloth, and transferred to a 2 L Scott bottle.

Spore viability and effective application of the fungi with a knap sack sprayer (Agpro) were confirmed, prior to application of the treatments in the semi-field trial. The respective treatments were applied to macadamia (cv. Beaumont) seedlings (2 years old). Prior to spraying, the trees were placed in plastic bags, covering the root system to prevent stink bugs from accidental contact with the soil and cross contamination (Figure 4.2a). Trees were placed individually in foldable insect rearing cages (34 × 34 × 61 cm) (Figure 4.2b), each assigned to a treatment and labelled accordingly. Cages were arranged in a completely randomized design in the orchard, with the four treatments, replicated four times. The cages were placed underneath the canopy of macadamia trees to limit the possible negative impact that sunlight exposure may have on the efficacy of the applied fungi. The cages were therefore

placed on the south-facing side of the trees, approximately 1 m away from irrigation sprinklers.

Figure 4.2: (a) A macadamia (cv. Beaumont) seedling with the base wrapped in plastic. (b) Seedlings were placed individually in foldable insect rearing cages placed underneath trees in an orchard.

Treatments were applied to the trees as full cover sprays at a spray volume of 125 ml per tree with a knap sack sprayer. To avoid cross contamination separate pressure sprayers were used for each treatment.

Immediately after spraying, five newly emerged *B. distincta* adults were placed into each cage (Figure 4.2b). Mortality assessments of stink bugs were done at 3-day intervals for a period of 18 days post application. Dead stink bugs were removed and the cadavers were surface sterilized with 70% ethanol under a laminar flow hood. The stink bugs were individually placed in a sterile Petri dish (9 mm diameter), lined with moist filter paper and stored in complete darkness until the development of mycosis. Mycosis from the cadavers sampled from the fungal treated trees, was used to confirm death as a result of the treatments that were applied.

4.4 Statistical Analysis

All data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) prior to analyses. Mortality data for the laboratory bioassay and semi-field screening of *B. distincta* expressed as a percentage, were Ln transformed and subjected to Repeated measures ANOVAs. Bonferroni correction was used to adjust for multiple mean comparisons. Fungal mycosis was subjected to one-way analysis of variance using General Linear Models Procedure (PROC GLM), SAS software (Version 9.4; SAS Institute Inc, Cary, USA). The treatment means were compared by means of Tukey's HSD post-hoc test at $p < 0.05$. To determine the number of days to 50% (LT_{50}) or 90% (LT_{90}) mortality, mortality data over time, was subjected to Probit analysis using Genstat software (Genstat for Windows 20th Edition, VSN International, Hemel Hempstead, UK).

4.5 Results

4.5.1 Screening of field collected *Beauveria bassiana* isolates against *Bathycoelia distincta*

4.5.1.1 Laboratory experiment

Before use in the experiment, spore viability of the respective isolates was determined, and exceeded 90%. The mean percentage mortalities of *B. distincta* adults caused by the respective *B. bassiana* isolates over time are provided in table 4.1

Table 4.1 Mean percentage mortalities (±SE) of *Bathycoelia distincta* adults caused by different *Beauveria bassiana* isolates under laboratory conditions over time.

Treatment	Mean mortality (%) (± SE)					
	Days after inoculation (DAI)					
	3	6	9	12	15	18
Control	0.00 ± 3.85	0.00 ± 6.09	6.67 ± 10.54	6.67 ± 11.86	13.33 ± 11.86	20.00 ± 8.61
PPRI 26695	6.67 ± 3.85	26.67 ± 6.09	73.33 ± 10.54	100.00 ± 11.86	100.00 ± 11.86	100.00 ± 8.61
PPRI 26696	0.00 ± 3.85	13.33 ± 6.09	73.33 ± 10.54	86.67 ± 11.86	86.67 ± 11.86	93.33 ± 8.61
PPRI 26997	6.67 ± 3.85	26.67 ± 6.09	73.33 ± 10.54	73.33 ± 11.86	86.67 ± 11.86	93.33 ± 8.61
PPRI 26700	0.00 ± 3.85	6.67 ± 6.09	46.67 ± 10.54	60.00 ± 11.86	60.00 ± 11.86	86.67 ± 8.61
PPRI 26704	0.00 ± 3.85	13.33 ± 6.09	53.33 ± 10.54	53.33 ± 11.86	66.67 ± 11.86	93.33 ± 8.61

Significant differences in the Isolate*Time (DAI) interaction are indicated in Appendix 4.1.

Table 4.2 Repeated measures analysis of variance results of the effect of time and isolate on pathogenicity of five *Beauveria bassiana* isolates in a laboratory experiment.

Effects	Df	F-value	P-value
Isolate	5, 12	9.52	0.001
Time	5, 60	131.40	0.0001
Time*Isolate	25, 60	4.30	0.0001

The results of the Repeated measures ANOVA showed that there was a significant main effect for Isolate ($F_{5,12}$ = 9.52; P < 0.001), with mortalities caused by all the isolates (Table 4.2). Isolate PPRI 26700 did not differ significantly from the percentage mycosis in the control treatment. There was, however, also no significant between the respective *B. bassiana* isolates in terms of the percentage mycosis caused by them.

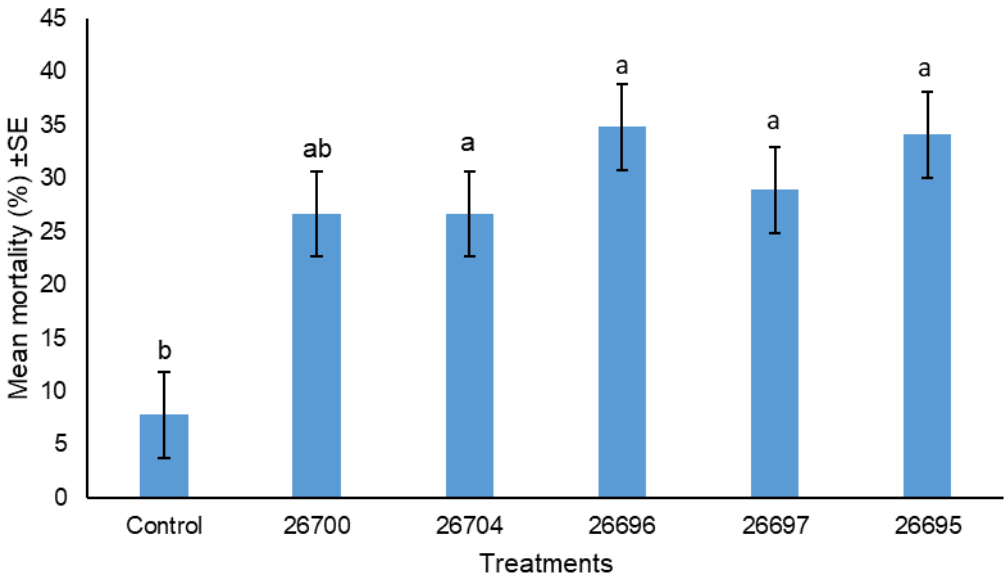


Figure 4.3: Mean percentage mortality of *Bathycoelia distincta* adults with five different *Beauveria bassiana* isolates under laboratory conditions.

The effect of Time (days after inoculation) on pathogenicity of the isolates, was also significant ($F_{5,60}$ = 131.40, P < 0.0001). Pathogenicity increased significantly over time, with mycosis 18 days after inoculation being significantly higher compared to earlier assessments (Figure 4.4).

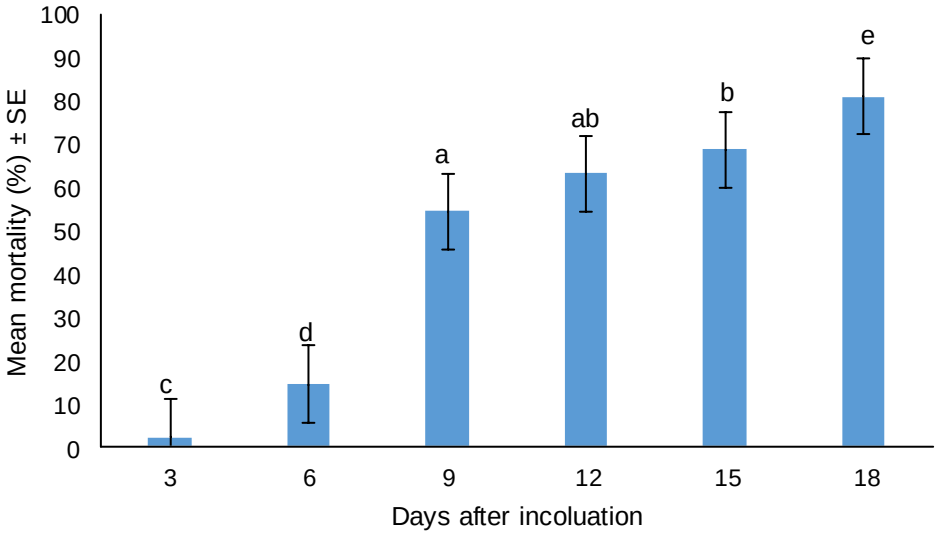


Figure 4.4: Mean percentage *Bathycoelia distincta* adult mortality over time after inoculation.

The interaction between Isolate and Time was also significant ($F_{25,60}$ = 4.30; P < 0.0001) (Figure 4.5). All five *B. bassiana* isolates were found to be pathogenic towards *B. distincta* adults, but the level of pathogenicity varied between the isolates over time resulting in a significant time x isolate interaction ($F_{25,60}$ = 4.30; p < 0.001) (Figure 4.5).

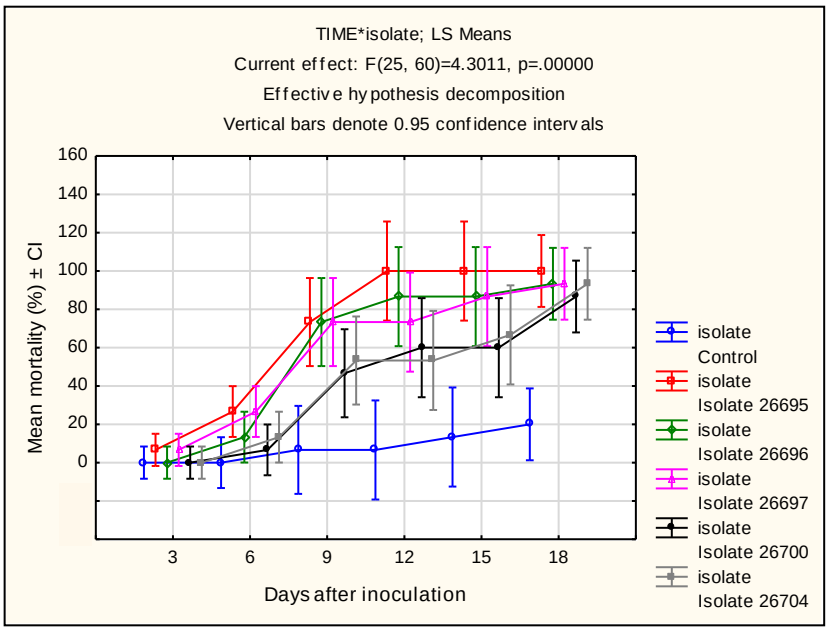


Figure 4.5: Mean percentage mortality of *Bathycoelia distincta* adults caused by different treatment, *viz*. *Beauveria bassiana* isolates and a control treatment, under laboratory conditions over time (Time x Isolate interaction).

According to the estimated LT_{50} and LT_{90} for the respective *B. bassiana* isolates, *B. distincta* adults were the most susceptible to isolate PPRI 26695, with 50% mortality at 6.99 days and 90% mortality estimated at 12.85 days. The isolate with the longest estimated LT_{50}, was PPRI 26700 at 8.80 days compared to the LT_{50} of the control, of 20.54 days (Table 4.3). There was, however, no significant difference in mycosis between the isolates ($F_{4,10} = 0.91$; $P = 0.49$), ranged between 66.67- 86.67%.

Table 4.3: Susceptibility of *Bathycoelia distincta* adults to different *Beauveria bassiana* isolates expressed as lethal time, for 50% and 90% mortality in a laboratory bioassay.

Treatment	LT_{50}	*95 % CI	LT_{90}	*95 % CI
Control	20.54	16.35 – 35.32	26.39	22.02 – 32.58
PPRI 26695	6.99	3.76 – 10.00	12.85	9.85 – 16.84
PPRI 26696	9.02	5.96 – 11.71	14.87	12.16 – 18.44
PPRI 26997	8.80	5.92 – 11.37	14.65	12.05 – 18.16
PPRI 26700	11.91	9.14 – 14.55	17.77	15.09 – 21.53
PPRI 26704	11.24	8.47 – 13.87	17.10	14.43 – 20.84

*CI=Confidence interval

4.5.1.2 Semi-field trial

The average maximum and minimum temperatures recorded during the semi-field trial period, were 30.57 and 17.13 °C, respectively. The average relative humidity was 68.31%. Before use in the experiment, spore viability of Eco-Bb® and PPRI 26695 was determined to be greater than 90%. The mean percentage mortalities of *B. distincta* adults caused by the different treatments over time are provided in table 4.4.

Table 4.4 Mean percentage mortality (% ± SE) of *Bathycoelia distincta* adults caused by different *Beauveria bassiana* isolates under semi-field conditions over time.

Treatment	Days after inoculation (DAI)					
	3	**6**	**9**	**12**	**15**	**18**
Control	0.00 ± 5.77	0.00 ± 7.91	5.00 ± 9.35	5.00 ± 6.12	10.00 ± 4.79	10.00 ± 5.77
Eco-Bb®	0.00 ± 5.77	0.00 ± 7.9	45.00 ± 9.35	60.00 ± 6.12	75.00 ± 4.79	80.00 ± 5.77
Cypermethrin	70.00 ± 5.77	85.00 ± 7.91	90.00 ± 9.35	90.00 ± 6.12	90.00 ± 4.79	90.00 ± 5.77
PPRI 26695	10.00 ± 5.77	15.00 ± 7.91	80.00 ± 9.35	95.00 ± 6.12	100.00 ± 4.79	100.00 ± 5.77

Significant differences in the Isolate*Time (DAI) interaction are indicated in Appendix 4.2.

Table 4.5 Repeated measures analysis of variance results of the effect of time and treatment on control *Bathycoelia distincta* adults in a semi-field trial.

Effects	Df	F-value	P-value
Isolate	3, 12	59.59	0.001
Time	5, 60	131.40	0.0001
Time*Isolate	25, 60	4.30	0.0001

The results of the Repeated measures ANOVA showed that there was a significant main effect for Treatment ($F_{3,12} = 59.59$; $P < 0.001$) (Table 4.3), with significantly better control of *B. distincta* adults obtained with Isolate 26695 and cypermethrin, compared to the Eco-Bb and control treatments (Figure 4.6). Percentage mortality of *B. distincta* adults did also differ significantly over time ($F_{5,60} = 131.40$; $P < 0.001$) (Figure 4.6).

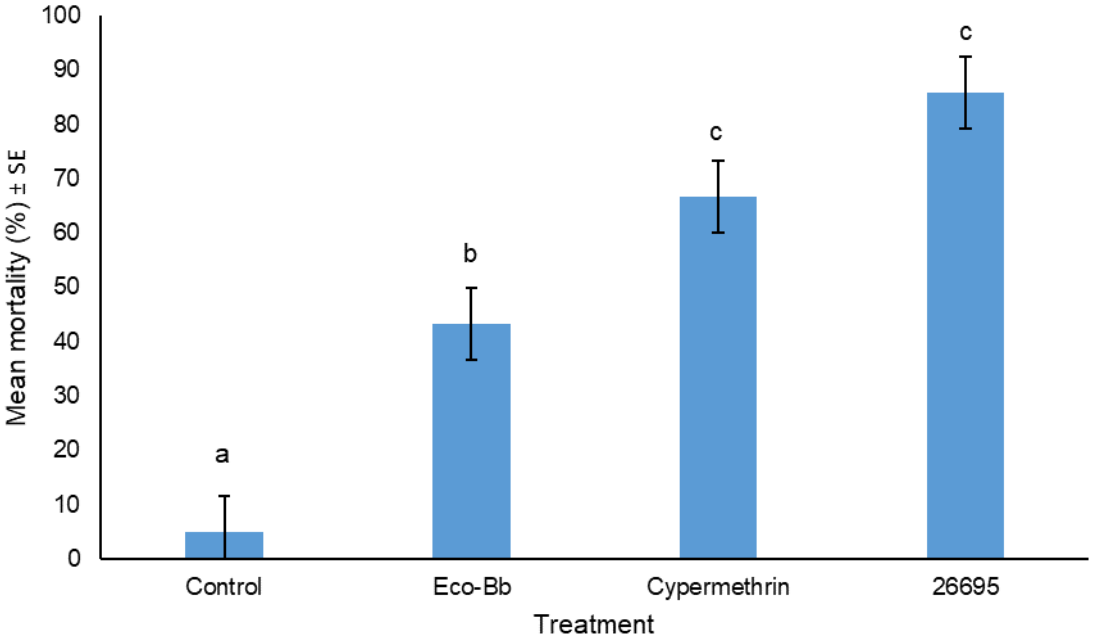


Figure 4.6: Mean percentage mortality of *Bathycoelia distincta* adults treated with two *Beauveria bassiana* isolates and a cypermethrin application application on macadamia seedlings under semi-field conditions.

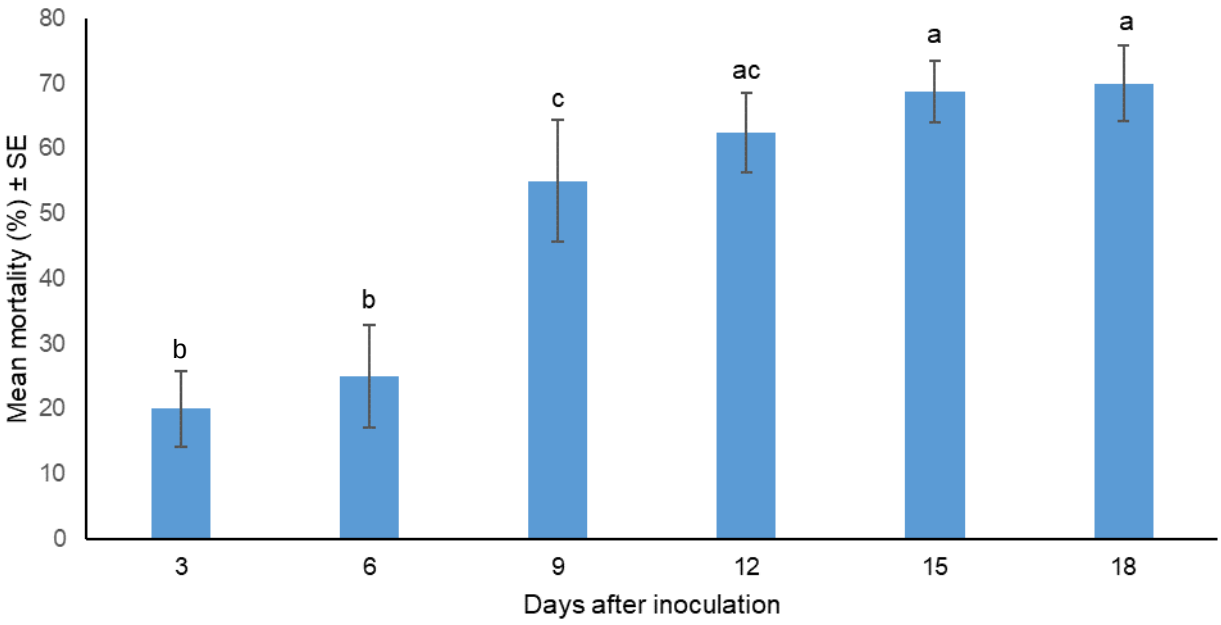


Figure 4.7: Mean percentage *Bathycoelia distincta* adult mortality over time after application of treatments.

The pathogenicity of the respective isolates varied over time, resulting in a significant time x isolate interaction ($F_{15,60}$ = 13.00; $p < 0.001$) (Table 4.2). A significantly higher percentage *B. distincta* mortality resulted from the application of cypermethrin, three and six days after treatment, compared to the two *B. bassiana* isolates and the control treatment (Appendix 4.2). From nine days after treatment, significantly higher control efficacy was recorded with all the treatments, compared to the control treatment. There was, however, no significant difference in mortality of *B. distincta* adults from 12 days after application, between the two *B. bassiana* isolates and cypermethrin. Very high efficacy was achieved from 15 days after treatment. At 18 days after treatment, mortality ranged between 80 - 100 % (Table 4.4), with no significant difference in efficacy of control between Eco-Bb®, PPRI 26695 and cypermethrin. The control achieved with the experimental PPRI 26695, was 100% (Appendix 4.2).

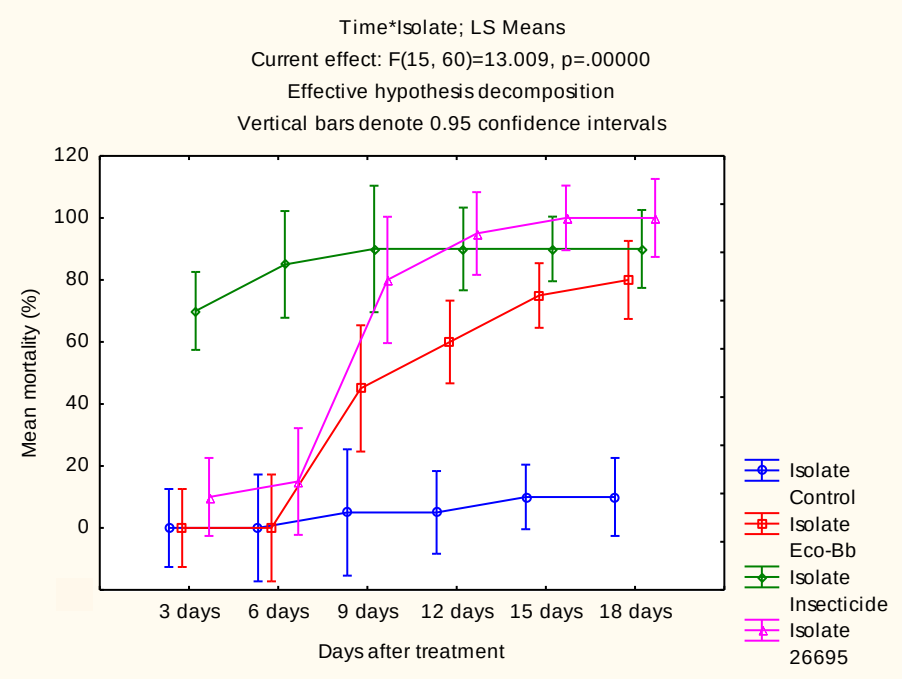


Figure 4.8: Mean percentage mortality (±SE) of *Bathycoelia distincta* adults caused by two *Beauveria bassiana* isolates under semi-field conditions over time.

Control of *B. distincta* adults was more rapid with cypermethrin (LT_{50}=2.57; LT_{90}=10.22 d), compared to PPRI 26695 (LT_{50}=6.81; LT_{90}=14.46 d), as well as to the Eco-Bb and control treatments (Table 4.6). There was no significant difference in mycosis between Eco-Bb® and 26695 ($F_{1,6}$ = 5.40; P = 0.06), mycosis percentage for Eco-Bb® and PPRI 26695 were 60% and 90%, respectively.

Table 4.6: Susceptibility of *Bathycoelia distincta* adults to different *Beauveria bassiana* isolates expressed as lethal time, for 50% and 90% mortality in a semi-field trial.

Treatment	LT_{50} (d)	*95 % CI	LT_{90} (d)	*95 % CI
Control	23.09	21.80 – 24.51	30.74	29.02 -32.72
Eco-Bb®	12.02	11.34 – 12.72	19.67	18.69 -20.80
Cypermethrin	2.57	1.60 – 3.47	10.22	9.37 -11.13
PPRI 26695	6.81	6.06 – 7.53	14.46	13.62 -15.39

*CI = confidence interval

4.6 Discussion

This is the first study to report effective control of *B. distincta* with *B. bassiana* under laboratory as well as semi-field conditions. Pathogenicity of five *B. bassiana* isolates, initially isolated from orchard-collected *B. distincta* and *N. viridula*, *viz*. PPRI 26695, 26696, 26697, 26700 and 26704, varied between 87 and 100%. Fungal mycosis, ranging between 67-87%, confirmed the susceptibility of *B*. *distincta* to these fungal isolates. Similar results were also reported with two *B. bassiana* isolates, causing 87 and 88% adult mortality of the redbanded stink bug, *Piezodorus guildinii* (Westwood) (Hemiptera: Pantatomidae) (Parys and Portilla, 2020). *Beauvaria bassiana* also controlled 67-100% brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) in a study by Gouli *et al.* (2012). Although *B. distincta* is currently controlled with insecticides, the apparent resistance problem of pyrethriods suggests that the current insect control practices are not sustainable (Schoeman, 2014c).

Entomopathogenic fungi (EPF) can be mass-produced (Roberts and Hajek, 1992), and some products are already available for commercial use (Maina *et al.,* 2018). The EPF most widely used for biological control of agricultural pests, are *Metarhizium anisopliae* and *B. bassiana* (de Faria and Wraight, 2007). *Beauvaria bassiana* is effective against a number of agricultural as well as glasshouse pests (Maina *et al.,* 2018; Fite *et al.,* 2020). The present study examined the pathogenicity of five new *B. bassiana* isolates against *B. distincta* under laboratory conditions. Further studies were also conducted to evaluate the pathogenicity of the unformulated isolate PPRI 26695, formulated isolate Eco-Bb® and the pyrethriod insecticide, cypermethrin, against *B. distincta* under semi-field conditions.

Beauveria bassiana has been reported as an effective control method, alternative to insecticide applications, against insect pests with piercing sucking mouthparts, which include stink bugs (Hatting *et al.,* 2004; Parys and Portilla, 2020). Fungal isolates can potentially kill target pests within 3-7 days after treatment (Baysal *et al*., 2018). The rate or speed of kill is highly dependent on the size of the target host. Approximately 7 days were reported to be necessary to obtain 100% mortality of smaller pest species with EPF. These included *Culicoides* biting midges (Diptera: Ceratopogonidae) (Ansari *et al*., 2011), onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) (Wu *et al*., 2013) and greenhouse aphid species (Hemiptera: Aphididae) (Mohammed *et al*., 2018). A longer incubation period of EPF (12-21 days) to obtain 100% mortality of larger insects, were reported for stink bugs (Muştu *et al*., 2011), grasshoppers (Jeffs *et al*., 1997) and beetles

(Clifton *et al*., 2020). Mortality caused by *B. bassiana* increased with time from the pilot study, where it took approximately 12-18 days to obtain maximum mortality. Similar responses were also reported for the green vegetable bug, *N. viridula* where it took approximately 10-12 days to obtain maximum mortality with conidial concentrations ranging between $3 \times 10^3 – 3 \times 10^5$ conidia/ml (Abdel-Raheem *et al.,* 2011).

The rate/speed of kill of EPF, therefore, not only varies between species, but virulence can also be affected by a number of other factors such as the time it takes for spores to germinate and penetrate the cuticle (Rafaat *et al*., 2015). For example, an increased conidial concentration, showed a positive correlation with percentage mortality of *N. viridula* (Abdel-Raheem *et al.,* 2011). In addition, a number of insects exhibit sexual dimorphism whereby one sex, usually the female, is larger or heavier than male (Teder and Tammaru, 2005). This has implications for pest management, which is rarely taken into consideration. Pentatomidae females are known to be significantly heavier than the males (Krupke *et al.*, 2008). Future studies should therefore also investigate differential susceptibility of males and females.

Studies by Lopes *et al.* (2015) suggested that the Pentatomidae family, specifically the green vegetable bug, has metahoracic glands, which secrete products that are fungistatic (inhibiting) and fungicidal. Responses to the *B. bassiana* isolates evaluated in this study, wwere evident from six days after treatment. It may be an indication of the time needed for conidial germination, which suggests a fungistatic effect. Most conidia do not germinate within 18 hours, but conidial germination is possible after 48 hours (Lopes *et al.,* 2015). The fungicidial properties of the metathoracic gland secretions of *N. viridula* confirmed limited attachment of EPF conidia of less than 2% (Rafaat *et al.,* 2015).

Rice overgrown isolates of PPRI 26695 resulted in an exceptionally high mortality of 100%. The area and host from which EPF isolates originate, has a major impact on the pathogenicity level (Dlamini *et al*., 2019). Isolate PPRI 26695 was originally isolated from *B. distincta* in the Mpumalanga province of South Africa (See chapter 3). Fungal pathogens isolated from host insects tend to be highly virulent towards the original host or closely related species (Dlamini *et al*., 2019). It explains the high pathogenicity of this isolate to *B. distincta*. Eco-Bb® with the active ingredient *B. bassiana* strain R444, is indigenous to the Western Cape, South Africa where it was originally isolated from the soil (Hatting *et al*., 2019). The pathogenicity of Eco-Bb® has been investigated to various

agricultural pests including the banded fruit weevil, *Phyctinus callosus* (Schonherr) (Coleoptera: Curculionidae), Sweet potato weevils, *Cylas puncticollis* (Boheman) and *Cylas formicarius* (Fabricius) (Coleoptera: Curculionidae) as well as the woolly apple aphid, *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae), with varying results (Dlamini *et al.*, 2019; Hlerema *et al*., 2017; Stokwe, 2016). Relatively high mortality of *B. distincta* was also recorded with Eco-Bb®. Mortality of *B. distincta* adults was, however, delayed in both the Eco-Bb® and non-formulated fungal isolates. The delayed mortality response might be due to more time required for conidial germination on -, and penetration of the stink bug cuticle, but also to the size of the stink bugs. The efficacy of the commercially available Eco-Bb®, formulated as a wettable powder, cannot be enhanced by the addition of wetters and adjuvants. The label indicated that living organisms are not compatible with adjuvants. This was confirmed by Melo *et al*. (2020), who reported the viability of *B. bassiana* formulated as a wettable powder, to be inhibited by surfactants (Melo *et al*., 2020).

Entomopathogenic fungi are morphologically, phylogenetically and ecologically diverse (Altinok *et al.,* 2019). The ecology of indigenous EPF should be understood to interpret their contributions to pest control and to predict the impact of agricultural practices and effects of environmental manipulations (Meyling and Elenberg, 2007). Knowledge about the ability of EPF to persist under environmental conditions is also important. Generally, the use of EPF remains a challenge, since they are negatively affected by high UV radiation or direct sunlight, since it may inactivate conidia (Kaiser *et al*., 2018). It may also delay germination, which may result in reduced pathogenicity of infective propagules (Fernandes *et al.,* 2015). However, a macadamia tree coverage provides protection from sunlight and radiation, which will protect conidia from these extreme conditions.

Insecticides play an important role in managing pests of various crops (Deep *et al*., 2018). The use of insecticides has increased drastically in commercial macadamia orchards due to the damage inflicted by *B. distincta* (Schoeman, 2018). There are concerns about the impact of contact insecticides on *B. distincta* and also on the general orchard ecology. In this study, cypermethrin, provided control of *B. distincta* adults of 70 ± 5.77% within 3 days, and 90 ± 5.77%, after 18 days. Some of the treated stink bugs did, however, recover after treatment. Recovery of ±10% after insecticide exposure has also been documented for the brown-marmorated stink bug (Leskey *et al*., 2012). With the rapid knock down effect of the contact insecticide, more rapid control was achieved compared to the *B.*

bassiana isolates, of which control only become evident from six days after treatment. A combination of EPF plus sublethal dose of chemical insecticide has been found to be a promising alternative (Pelizza *et al.,* 2018).

The potential of *B. bassiana* to be a viable alternative to chemical control of *B. distincta*, was indicated in this study, based on the high infection and excellent mortality rates of adults achieved with this EPF. Confirmation of the efficacy of the *B. bassiana* isolates against *B. distincta* nymphs, could enhance control of the two-spotted stink bug even more.

Both the laboratory and semi-field trials conducted in this study, confirmed the potential of *B. bassiana* in controlling *B. distincta* adults. However, fungal isolates should be tested under field conditions exposed to the harsh African climatic conditions also. Resulst obtained with cypermethrin indicated that *B. distincta* is effectively controlled by this insecticide on macadamia. Although it provides and effective chemical control option to macadamia producers, the application of cypermethrin may negatively affect beneficial arthropods that come into contact during foliar sprays. *Beauvaria bassiana* will be a valuable addition to an integrated pest management program for the two-spotted stink bug on macadamia, once its efficacy under field conditions is confirmed.

References

Abdel-Raheem, M.A., Ragab, Z.A. and Abdel-Rhman, I.E. 2011. Effect of entomopathogenic fungi on the green stink bug, *Nezara viridula* L. in sugar-beet in Egypt. *Bulletin of the National Research Centre* 36(2):145-152.

Altinok, H.H., Altinok, M.A. and Koca, A.S. 2019. Modes of action of entomopathogenic fungi. *Current Trends in Natural Sciences* 8(16):117-124.

Andow, D.1983.The extent of monoculture and its effects on insect pest populations with particular reference to wheat and cotton. *Agriculture, Ecosystems and Environment* 9:25-35.

Ansari, M.A., Pope, E.C., Carpenter, S., Scholte, E. and Butt, T.M. 2011. Entomopathogenic fungus as a biological control for an important vector of livestock disease: the *Culicoides* biting midge. *PLoS One* 6(1):e16108. DOI:10.1371/journal.pone.0016108

Baysal, E., Atay, T. and Yanar, Y. 2018. Efficacy of some local isolates of the fungus *Beauveria bassiana* (Balsamo) Vuillemin on the alfalfa weevil *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae) larvae under laboratory conditions. *Egyptian Journal of Biological Pest Control* 28:65. DOI.org/10.1186/s41938-018-0067-7

Behie, S.W. and Bidochka, M.J. 2014. Ubiquity of insect-derived nitrogen transfer to plants by endophytic insect-pathogenic fungi: an additional branch of the soil nitrogen cycle. *Applied Environmental Microbiology* 80:1553-1560.

Bruwer, S. 1992. The influence of various hemipteran species on macadamia and some factors which can limit nut damage. *PhD thesis*, University of Stellenbosch.

Cardoso, E.J.B.N. and Alves, P.R.L. 2012. Soil Ecotoxicology. 2012. Soil Ecotoxicology, Ecotoxicology, Begum, G (Ed.), ISBN: 978-953-51-0027-0, InTech, Available from: http://www.intechopen.com/books/ecotoxicology/soil-ecotoxicology

Castrillo, L.A., Mayfield, A.E., Griggs, M.H., Camp, R., Mudder, B., Taylor, A. and Vandenberg, J.D. 2017. Mortality and reduced brood production in walnut twig beetles*, Pityophthorus juglandis* (Coleoptera: Gurculionidae), following exposure to commercial strains of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium brunneum*. *Biological Control* 114:79-86.

Chandra Teja, K.N.P. and Rahman, S.J. 2020. Virulence of local isolates of entomopathogenic fungi on the common agricultural pest *Spodoptera litura* (Fabricius) Lepidoptera: Noctuidae. *International Journal of Current Microbiology and Applied Sciences* 9(2):2768-2778.

Clifton, E.H., Jaronski, S.T. and Hajek, A.E. 2020. Virulence of commercialized fungal entomopathogens against Asian Longhorned Beetle (Coleoptera: Cerambycidae). *Journal of Insect Science* 20(2):1-6.

De Faria, M.R. and Wraight, S.P. 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. *Biological Control* 43: 237-256.

Deep, G., Patil, J., Yadav, S., Kumer, B. and Nath, R. 2018. Novel insecticides: a potential tool for the management of insect pest. *Journal of Entomology and Zoology Studies* 6(5):277-281.

Dlamini, B.E., Malan, A.P. and Addison, P. 2019. Control of the banded fruit weevil, *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae), using entomopathogenic fungi. *African Entomology* 28(1):106-114.

Erler, F. and Ates, A.O. 2015. Potential of two entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* (Coleoptera: Scarabaeidae), as biological control agents against the June beetle. *Journal of Insect Science* 15(1):1-6.

Fernandes, E.K.K., Rangel, D.E.N., Braga, G.U.L. and Roberts, D.W. 2015. Tolerance of entomopathogenic fungi to ultraviolet radiation: a review on screening of strains and their formulation. *Current Genetics* 61:427-440.

Fite, T., Tefera, T., Negeri, M., Damte, T. and Sori, W .2020. Evaluation of *Beauveria bassiana*, *Metarhizium anisopliae*, and *Bacillus thuringiensis* for the management of *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) under laboratory and field conditions. *Biocontrol Science and Technology* 30(3):278-295.

Gouli, V., Gouli, S., Skinner, M., Hamilton, G., Kim, J.S. and Parker, B.L. 2012. Virulence of select entomopathogenic fungi to the brown marmorated stink bug, *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae). *Pest Management Science* 68:155-157.

Hatting, J.L., Wraight, S.P. and Miller, R.M. 2004. Efficacy of *Beauveria bassiana* (Hyphomycetes) for control of Russian wheat aphid (Homoptera: Aphididae) on resistant wheat under field conditions. *Biocontrol Science and Technology* 14(5):459-473.

Hatting, J.L., Moore, S.D. and Malan, A.P. 2019. Microbial control of phytophagous invertebrate pests in South Africa: Current status and future prospects. *Journal of Invertebrate Pathology* 165:54-66.

Hlerema, I., Laurie, S. and Eiasu, B. 2017. Preliminary observations on use of *Beauveria bassiana* for control of the sweet potato weevil (*Cylas* sp.) in South Africa. *Open Agriculture* 2(1):595-599.

Hwi-Geon, Y., Dong-Jun, K., Won-Seok, G., Tae-Young, S. and Soo-Dong, W. 2017. Entomopathogenic fungi as dual control agents against both the pest *Myzus persicae* and phytopathogen *Botrytis cinerea*. *Mycobiology* 45(3):192-198.

Jeffs, L.B., Feng, M., Falkowsky, J.E. and Khachatourians, G.G. 1997. Infection of the migratory grasshopper (Orthoptera: Acrididae) by ingestion of the entomopathogenic fungus *Beauveria bassiana*. *Biological and Microbial Control* 90(2):383-390.

Kaiser, D., Bacher, S., Mène-Saffrané, L. and Grabenweger, G. 2018. Efficiency of natural substances to protect *Beauveria bassiana* conidia from UV radiation. *Pest Management Science* 75:556-563.

Karaağaç, S.U. 2012. Insecticide Resistance, Insecticides - Advances in Integrated Pest Management, Dr. Farzana Perveen (Ed.), ISBN: 978-953-307-780-2.

Krupke, C.H., Brunner, J.F. and Jones, V.P. 2008. Factors influencing mate choice in *Euchitus conspersus* Uhler (Heteroptera: Pentatomidae). *Environmental Entomology* 37(1):192-197.

Leskey, T., Lee, D., Short, B.D. and Wright, S.E. 2012. Impact of insecticides on the invasive *Halyomorpha halys* (Hemiptera: Pentatomidae): analysis of insecticide lethality. *Journal of Economic Entomology* 105(5):1726-1735.

Litwin, A., Nowak, M. and Różalska, S. 2020. Entomopathogenic fungi: unconventional applications *Reviews in Environmental Sciences and Biotechnology* DOI.org/10.1007/s11157-020-09525-1

Lopes, R.B., Laumann, R.A., Blassioli-Moraes, M.C., Borges, M. and Faria, M. 2015. The fungistatic and fungicidal effects of volatiles from metathoracic glands of soybean-attacking stink bugs (Heteroptera: Pentatomidae) on the entomopathogen *Beauveria bassiana*. *Journal of Invertebrate Pathology* 132:77-85.

Mahmood, I., Imadi, S.R., Shazadi, K., Gul, A. and Hakeem, K.R. 2016. Effects of pesticides on environment. In: Hakeem K., Akhtar, M. and Abdullah, S. (Eds) *Plant Soil and Microbes.* Springer, Cham DOI.org/10.1007/978-3-319-27455-3_13

Maina, U.M., Galadima, I.B., Gambo, F.M. and Zakaria, D. 2018. A review on the use of entomopathogenic fungi in the management of insect pests of field crops. *Journal* of *Entomology and Zoology Studies* 6(1):27-32.

Melo, M.R., Zotarelli, M.F., Santos, L.D. and Silveira, E. 2020. Selection of surfactants for enhancing the wettability of powder formulations of *Beauveria bassiana* (bals.-criv.) Vuill.

(1912) (Ascomycota: Cordycipitaceae). *Biological Control* 151:104391. DOI.org/10.1016/j.biocontrol.2020.104391

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

Mohammed, A.A., Kadhim, J.H. and Kamaluddin, Z.N.A. 2018. Selection of highly virulent entomopathogenic fungal isolates to control the greenhouse aphid species in Iraq. *Egyptian Journal of Biological Pest Control* 28(71): 1-7.

Mohammed, A.A. 2020. Insecticidal potential of commercial neem-based insecticide, nimbecidine EC against the red flour beetle (*Tribolium castaneum*) in the laboratory. *Plant Archives* 20(1): 2618-2622.

Muştu, M., Demirci, F. and Koçak, E. 2011. Mortality effects of *Isaria farinose* (Holm.) and *Beauveria bassiana* (Balsamo) Vuillemin (Sordariomycetes: Hypocreales) on *Aelia rostrate* Boh. (Hemipetra: Pentatomidae). *Turkish Journal of Entomology* 35(4):559-568.

Panizzi, A.R. 2000. Suboptimal nutrition and feeding behaviour of Hemipterans on less preferred plant food sources. *Anais da Sociedade Entomológica do Brasil* 29(1):1-12.

Parys, K.A. and Portilla, M. 2020. Effectiveness of *Beauveria bassiana* against *Piezodorus guildinii* (Hemiptera: Pentatomidae), a key pest of soybeans in the neotropics. *Biocontrol Science and Technology* 30(5):451-461.

Pelizza, S.A., Schalamuk, S., Simon, M.R., Stenglein, S.A., Pacheco-Marino, S.G. and Scorsetti, A.C. 2018. Compatibility of chemical insecticides and entomopathogenic fungi for control of soybean defoliating pest. *Rachiplusia nu*. *Revista Argentina De Microbiologia* 50(2):189-201.

Raafat, I., Meshrif, W.S., El Husseiny, E.M., El-Hariry, M. and Seif, A.I. 2015. *Nezara viridula* (Hemiptera: Pentatomidae) cuticle as a barrier for *Beauveria bassiana* and *Paecilomyces* sp. Infection. *African Entomology* 23(1):75-87.

Roberts, D.W. and Hajek, A.E. (1992). Entomopathogenic fungi as bioinsecticides In*:* Leatham, C. (ed.) Frontiers in industrial Mycology. Chapman & Hall, New York: Springer. 144-159.

Saeed, Q., Ahmad, F., Iqbal, N. and Zaka, S.M. 2019. Chemical control of polyphagous pests on their auxiliary hosts can minimize insecticide resistance: A case study of *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) in cotton agroecosystem. *Ecotoxicology and Environmental Safety* 171:721-727.

Sánchez-Peña, S.R., Lara, J.S. and Medina, R.F. 2011. Occurrence of entomopathogenic fungi from agricultural and natural ecosystems in Saltillo, Mexico, and their virulence towards thrips and whiteflies. *Journal of Insect Science* 11(1). DOI:10.1673/031.011.0101

Schoeman, P.S. 2009. Key biotic components of the indigenous Tortricidae and Heteroptera complexes occurring on macadamia in South Africa. *PhD*, *thesis*, North West University, Potchefstroom, South Africa.

Schoeman, P. S. 2011. Integrated management of macadamia pests with emphasis on alternative pesticides and more effective monitoring. *Southern African Macadamia Growers' Association Yearbook* 19:26-32.

Schoeman, P.S. 2013. Phytophagous stink bugs (Hemiptera: Pentatomidae; Coreidae) associated with macadamia in South Africa. *Open Journal of Animal Sciences* 3(3):179-183.

Schoeman, P.S. 2014a. Aspects affecting distribution and dispersal of the indigenous Heteroptera complex (Heteroptera: Pentatomidae & Coreidae) in South African macadamia orchards*. African Entomology* 22(1): 191–196.

Schoeman P.S .2014b. Stink bug management in macadamia orchards: updates and latest recommendation. *South African Macadamia Growers Association Yearbook* 22, 2014.

Schoeman, P.S. 2014c. Stink bug IPM on macadamias in South Africa: current status and the road ahead. *Trends in Entomology*10:87-95.

Schoeman, P.S. 2018. Relative occurrence of economically significant heteropterans (Pentatomidae and Coreidae) on macadamias in South Africa: implications for management. *African Entomology* 26(2): 543-549.

Sheng-yong, W., Yu-lin, G., Xue-nong, X., Goettel, M.S. and Zhong-ren, L. 2015. Compatibility of *Beauveria bassiana* with *Neoseiulus barkeri* for control of *Frankliniella occidentalis*. *Journal of Integrative Agriculture* 14(1):98-105.

Sosa-Gómez, D.R., Corrêa-Ferreira, B.S., Kraemer, B., Pasini, A., Husch, P.E., Vieira, C.E.D., Martinez, C.B.R. and Lopes, I.O.N. 2020. Prevalence, damage, management and insecticide resistance of stink bug populations (Hemiptera: Pentatomidae). *Agricultural and Forest Entomology* 22:99-118.

South African Macadamia Growers' Association (SAMAC). 2020. Statistics on the Southern African Macadamia industry, April 2020, viewed 9 May 2020, from https://www.samac.org.za/industry-statistics/

Stokwe, N.F. 2016. Efficacy of entomopathogenic nematodes and fungi as biological agents of woolly apple aphid, *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae) under South African conditions. *PhD thesis*, University of Stellenbosch, South Africa.

Taylor, P.J., Grass, I., Alberts, A.J., Joubert, E. and Tscharntke, T. 2018. Economic value of bat predation services - A review and new estimates from macadamia orchards. *Ecosystem Services* 30:372-381.

Teder, T. and Tammaru, T. 2005. Sexual size dimorphism within species increases with body size in insects. *OIKOS* 108:321-334.

Van den Berg, M.A., Steyn, W.P. and Greenland, J. 1999. Hemiptera occurring on macadamia in the Mpumalanga Lowveld of South Africa. *African Plant Protection* 5(2):89-92.

Wu, S., Gao, Y., Xu, X., Zhang, Y., Wang, J., Lei, Z. and Smagghe, G. 2013. Laboratory and greenhouse evaluation of a new entomopathogenic strain of *Beauveria bassiana* for control of the onion thrips *Thrips tabaci*. *Biocontrol Science and Technology* 23(7):794-802.

Appendix 4.1

Significant differences between isolates over time (time*isolate interaction): laboratory experiment.

Cell No.	Bonferroni test; variable DV_1 (Lungile lab trial over time) Homogenous Groups, alpha = .05000 (Non-Exhaustive Search) Error: Between; Within; Pooled MS = .87433, df = 51.502						
	isolate	R1	DV_1 Mean	1	2	3	4
1	Control	ln_3 days	0.000000	****			
2	Control	ln_6 days	0.000000	****			
25	Isolate 26700	ln_3 days	0.000000	****			
13	Isolate 26696	ln_3 days	0.000000	****			
31	Isolate 26704	ln_3 days	0.000000	****			
7	Isolate 26695	ln_3 days	1.014841	****	****	****	
3	Control	ln_9 days	1.014841	****	****	****	
19	Isolate 26697	ln_3 days	1.014841	****	****	****	
26	Isolate 26700	ln_6 days	1.014841	****		****	
4	Control	ln_12 days	1.014841	****	****	****	
32	Isolate 26704	ln_6 days	2.029682	****	****	****	****
5	Control	ln_15 days	2.029682	****	****	****	****
14	Isolate 26696	ln_6 days	2.029682	****	****	****	****
6	Control	ln_18 days	2.252698	****	****	****	****
20	Isolate 26697	ln_6 days	3.267539	****	****	****	****
8	Isolate 26695	ln_6 days	3.267539	****	****	****	****
27	Isolate 26700	ln_9 days	3.755423		****		****
34	Isolate 26704	ln_12 days	3.940531		****	****	****
33	Isolate 26704	ln_9 days	3.940531		****	****	****
28	Isolate 26700	ln_12 days	3.944474		****		****
29	Isolate 26700	ln_15 days	3.944474		****		****
35	Isolate 26704	ln_15 days	4.167490		****	****	****
15	Isolate 26696	ln_9 days	4.278956		****	****	****
22	Isolate 26697	ln_12 days	4.299924				****
9	Isolate 26695	ln_9 days	4.299924				****
21	Isolate 26697	ln_9 days	4.299924				****
30	Isolate 26700	ln_18 days	4.447038				****
17	Isolate 26696	ln_15 days	4.447038				****
16	Isolate 26696	ln_12 days	4.447038				****
23	Isolate 26697	ln_15 days	4.468006				****
18	Isolate 26696	ln_18 days	4.541563				****
24	Isolate 26697	ln_18 days	4.541563				****
36	Isolate 26704	ln_18 days	4.541563				****
12	Isolate 26695	ln_18 days	4.615121				****
11	Isolate 26695	ln_15 days	4.615121				****
10	Isolate 26695	ln_12 days	4.615121				****

Appendix 4.2

Significant differences between treatments over time, (time*isolate interaction): semi-field trial.

Cell No.	Bonferroni test; variable DV_1 (mort semi field rep ANOVA in semi-field trial Lungile Suria) Homogenous Groups, alpha = .05000 (Non-Exhaustive Search) Error: Between; Within; Pooled MS = 184.72, df = 46.834							
	Isolate	R1	DV_1 Mean	1	2	3	4	5
1	Control	3 days	0.0000	****				
2	Control	6 days	0.0000	****				
8	Eco-Bb	6 days	0.0000	****				
7	Eco-Bb	3 days	0.0000	****				
4	Control	12 days	5.0000	****				
3	Control	9 days	5.0000	****				
6	Control	18 days	10.0000	****	****			
19	26695	3 days	10.0000	****	****			
5	Control	15 days	10.0000	****	****			
20	26695	6 days	15.0000	****	****			
9	Eco-Bb	9 days	45.0000		****	****		
10	Eco-Bb	12 days	60.0000			****	****	
13	Insecticide	3 days	70.0000			****	****	****
11	Eco-Bb	15 days	75.0000			****	****	****
21	26695	9 days	80.0000			****	****	****
12	Eco-Bb	18 days	80.0000				****	****
14	Insecticide	6 days	85.0000				****	****
17	Insecticide	15 days	90.0000				****	****
16	Insecticide	12 days	90.0000				****	****
15	Insecticide	9 days	90.0000				****	****
18	Insecticide	18 days	90.0000				****	****
22	26695	12 days	95.0000				****	****
24	26695	18 days	100.0000					****
23	26695	15 days	100.0000					****

Chapter 5

Conclusion and recommendation

Over the next 20 years crop production will need to increase significantly, in order to meet the demands of an increasing human population (FAO, 2017). Macadamia is an important tree crop, with an estimated global market size of US$ 822 million (Botha, 2018). South Africa is the largest macadamia producer in the world, followed by Australia, Kenya and China (Botha, 2018; Quiroz *et al.,* 2019). Insect pests are a major, economically important and limiting factors in macadamia production worldwide (van den Berg *et al.,* 1999; O'Hare, 2004; Schoeman, 2014b). The two-spotted stink bug, *Bathycoelia distincta* Distant (Hemiptera: Pentatomidae), is the most important pest of macadamia in South Africa (Schoeman, 2013; Schoeman, 2014a).

Management of pests such as *B. distincta* is important for food security. In this regard, the commercial farming sector rely heavily on the use of crop protection products (Chandler *et al.,* 2011). The public have also expressed concerns about the potential health and environmental impacts of chemical insecticides (Oluwole and Cheke, 2009). Therefore, environmentally friendly pest management methods are required.

Mycoinsecticides can potentially be a useful tool in sustainable agriculture, if it is included in an integrated pest management (IPM) strategy. It can also reduce the current reliance on chemical pesticides (Sani *et al*., 2020). The entomopathogenic fungi (EPF), *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* have been widely studied and found to be effective biological control agents of various agricultural pests, if applied under favourable conditions (Robles-Acosta *et al*., 2019). These entomopathogens have shown no negative effect towards non-target insects, they are also easy to produce, target specific, safe for the user and do not leave undesirable residues, which renders them safe to be applied near harvest (Damos *et al*., 2015).

This study is the first to report on the occurrence of insect-associated fungi for control of the two-spotted stink bug, *B. distincta* in South Africa. The first aim of this study (Chapter 2), was to investigate the occurrence of EPF associated with stink bug species, collected from macadamia orchards in the Mpumalanga province of South Africa, for use as possible biocontrol agents of these pests. Stink bugs in macadamia orchards were associated with a variety of fungal species. These included entomopathogenic fungi (EPF), potential pathogens, secondary colonizer and opportunistic fungi that pose no threat to

macadamia. The species recovered, included the following genera: *Aspergillus*, *Beauveria*, *Chaetonium, Gelasinospora*, *Lasiodiplodia, Nigrospora, Penicillum, Rhizopus, Trichoderma, Myriodontium, Fusarium, Talaromyces, Epioccum and Sordaria.* The EPF, *B. bassiana* was mainly isolated from an unsprayed macadamia orchard in Nelspruit. This study was also the first to report the presence of *Myriodontium keratinophilum* in South Africa. This fungus was classified as a new EPF that has been identified to be pathogenic to *Gryllotalpa gryllotalpa* (Linnaeus) (Orthoptera: Gryllotalpidae) and *Agelastic alni* (Linnaeus) (Coleoptera: Chrysomelidae).

The second aim of the study (Chapter 3), was to assess the effect of temperature on radial growth of eleven *B. bassiana* isolates, from the Nelspruit area, from which isolates for further pathogenicity tests could be selected. The radial growth of the different isolates was differentially, and significantly influenced by temperature. Radial growth of all isolates was significantly inhibited at 35 °C, but optimal temperature for growth was 25-30 °C. Seven of these isolates did not differ significantly in terms of radial growth. From these, selection of the most virulent isolate against *B. distincta* adults under laboratory conditions was done, and used in a semi-field assessment for control of *B. distincta* adults (Chapter 4). Five *B. bassiana* isolates (PPRI 26695, 26696, 2667, 26700 and 26704) tested under laboratory conditions were pathogenic towards *B. distincta*. The level of pathogenicity varied between the isolates and the mortalities caused by *B. bassiana* increased with time after application. *Beauveria bassiana* isolate (PPRI 26695) was selected for further assessment, based on 100 ± 8.61% efficacy of *B. distincta* control, mycosis of 86.67%, a LT_{50} of 6.99 and $LT_{90\ of}$ 12.85 days. Under semi-field conditions, the trial included the *B. bassiana* isolates PPRI 26695 (experimental) and commercially available Eco-Bb® (2×10^9 conidia/mL) (strain R444 - Plant Health Protection), as well as an insecticide registered for control of stink bugs on macadamia, cypermethrin (200g/L EC) (Villa Crop Protection (Pty) Ltd) and a control treatment. Good control was obtained with the experimental *B. bassiana* isolate, PPRI 26695 of 100 ± 5.77% mortality, 90% mycosis and a LT_{50} of 6.81 and LT_{90} of 14.46 days. Infected individuals were also easy to identify visually, as they developed a pinkish colour.

Beauveria bassiana has been previously reported to effectively control pests with piercing sucking mouthparts, which included stink bugs (Hatting *et al.,* 2004; Parys and Portilla, 2020). The rate or speed of kill of EPF is dependent on a number of factors, including the size of the target host (Jeffs *et al*., 1997; Muştu *et al*., 2011: Clifton *et al*., 2020). The

impact of females being bigger or heavier than males in a number of insect species, such as pentatomids (Krupke *et al.*, 2008), should therefore be taken into consideration when EPF are included in IPM strategies for stink bugs. The differential susceptibility of males and females with possible effects on the timing of application may therefore be of interest.

Insecticides have a faster knock down of insects compared to biocontrol agents such as EPF. Virulence of EPF is affected by the time it takes for spores to penetrate the cuticle (Raafat *et al*., 2015) as well as by increased conidial concentration (Abdel-Raheem *et al.,* 2011). Mortality of *B. distincta* adults was delayed after application of both the *B. bassiana* applications, *viz*. the commercially formulated, Eco-Bb® and non-formulated fungal isolates. The delayed mortality response might therefore have been due to a longer period required for conidial germination on, and penetration into the stink bug cuticle, and also to the size of the stink bugs.

One of the major factors that influence the field efficacy of EPF is the negative effect of high UV radiation or direct sunlight that inactivate conidia (Kaiser *et al*., 2018). This problem may be mitigated for when used for control of stink bugs in macadamia orchards, by the tree canopy coverage that will provide protection to conidia from sunlight and radiation. The potential of *B. bassiana* to be a viable alternative to chemical control of *B. distincta* provides an opportunity for future research in this regard.

Recommendations

The bioassays conducted in this study, must be repeated with application of the EPF done with a Burgejon spray tower instead of a micro-pipette. Only 11 *B. bassiana* isolates was investigated in this study. The remainder of the 21 *B. bassiana* isolates recovered in this study should also be explored further for control of stink bugs. Survival of the *B. bassiana* isolates exposed to UV radiation and re-isolation of mutant isolates for field trials is important. Although *Metarhizium* was not isolated from stink bugs in this study, laboratory bioassays should also be conducted on the virulence and pathogenicity of *Metarhizium*, *Myriodontium keratinophilum*, *Chaetomium* and *Fusarium* sp. to *B. distincta*. Positive outcomes of compatibility tests of candidate EPF isolates with insecticides registered for stink bug control on macadamia, as well as with fungicides, could improve an IPM strategy. Horizontal transmission of candidate isolates between *B. distincta* adults can increase the effectiveness of the EPF under field conditions and is an important area for future research. Surveys for candidate EPF should be continuous and screening of these

isolates for tolerance to harsh environmental conditions, for example temperatures exceeding 35 °C, will contribute tremendously to stink bug control in orchards.

References

Abdel-Raheem, M.A., Ragab, Z.A. and Abdel-Rhman, I.E. 2011. Effect of entomopathogenic fungi on the green stink bug, *Nezara viridula* L. in sugar-beet in Egypt. *Bulletin of the National Research Centre* 36(2):145-152.

Botha, L. 2018. Macadamias: a sunrise industry poised for massive growth. *Macadamia South Africa.* pp. 5.

Chandler, D., Bailey, A.S., Tatchell, G.M., Davidson, G., Greaves, J. and Grant, W.P. 2011. The development, regulation and use of biopesticide for integrated pest management. *Philosophical Transactions of the Royal Society B* 1573(366):1987-1998.

Clifton, E.H., Jaronski, S.T. and Hajek, A.E. 2020. Virulence of commercialized fungal entomopathogens against Asian Longhorned Beetle (Coleoptera: Cerambycidae). *Journal of Insect Science* 20(2):1-6.

Damos, P., Colmar, L.A. and Loriatti, C. 2015. Integrated fruit production and pest management in Europe: the apple case study and how far we are from the original concept? *Insects* 6(3):626-657.

Food and Agriculture Organization of the United Nations (FAO). 2017. The future of food and agriculture – Trends and challenges. Rome.

Hatting, J.L., Wraight, S.P. and Miller, R.M. 2004. Efficacy of *Beauveria bassiana* (Hyphomycetes) for control of Russian wheat aphid (Homoptera: Aphididae) on resistant wheat under field conditions. *Biocontrol Science and Technology* 14(5):459-473.

Jeffs, L.B., Feng, M., Falkowsky, J.E. and Khachatourians, G.G. 1997. Infection of the migratory grasshopper (Orthoptera: Acrididae) by ingestion of the entomopathogenic fungus *Beauveria bassiana*. *Biological and Microbial Control* 90(2):383-390.

Kaiser, D., Bacher, S., Mène-Saffrané, L. and Grabenweger, G. 2018. Efficiency of natural substances to protect *Beauveria bassiana* conidia from UV radiation. *Pest Management Science* 75:556-563.

Krupke, C.H., Brunner, J.F. and Jones, V.P. 2008. Factors influencing mate choice in *Euchitus conspersus* Uhler (Heteroptera: Pentatomidae). *Environmental Entomology* 37(1):192-197.

Muştu, M., Demirci, F. and Koçak, E. 2011. Mortality of *Isaria farinose* (Holm.) and *Beauveria bassiana* (Balsamo) Vuillemin (Sordariomytes: Hypocreales) on *Aelia rostrate* Boh. (Hemiptera: Pentatomidae). *Turkish Journal of Entomology* 35(4):559-568.

O'Hare, P., Quinlan, K., Stephenson, R. and Vock, N. 2004. Growing guide: Macadamia grower's handbook. Queensland Dept. Primary Ind. Brisbane Australia.

Oluwole, O. and Cheke, R.A. 2009. Health and environmental impacts of pesticide use practices: a case study of farmers in Ekiti State, Nigeria. *International Journal of Agricultural Sustainability* 7(3):153-163.

Parys, K.A. and Portilla, M. 2020. Effectiveness of *Beauveria bassiana* against *Piezodorus guildinii* (Hemiptera: Pentatomidae), a key pest of soybeans in the neotropics. *Biocontrol Science and Technology* 30(5):451-461.

Quiroz, D., Kuepper, B., Wachira, J. and Emmott, A. 2019. Value chain analysis of macadamia nuts in Kenya. *Technical Report.* pp.5

Raafat, I., Meshrif, W.S., El Husseiny, E.M., El-Hariry, M. and Seif, A.I. 2015. *Nezara viridula* (Hemiptera: Pentatomidae) cuticle as a barrier for *Beauveria bassiana* and *Paecilomyces* sp. Infection. *African Entomology* 23(1):75-87.

Robles-Acosta, I., Chacón-Hernández, J.C., Torres-Acosta, R.I., Landeros-Flores. J., Vanoye-Eligio, V. and Arredondo-Valdés, R. 2019. Entomopathogenic fungi as biological control agents of Phyllocoptruta oleivora (Prostigmata: Eriophyidae) under greenhouse conditions. *Florida Entomologist* 102(2):303-308. DOI.org/10.1653/024.102.0203

Sani, I., Ismail, S.I., Abdullah, S., Jalinas, J., Jamian, S. and Saad, N. 2020. A review of the biology and control of whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae), with special reference to biological control using entomopathogenic fungi. *Insects* 11:319 DOI:10.3390/insects11090619.

Schoeman, P.S. 2013. Phytophagous stink bugs (Hemiptera: Pentatomidae; Coreidae) associated with macadamia in South Africa. *Open Journal of Animal Sciences* 3(3):179-183.

Schoeman, P.S. 2014a. Monitoring and damage of stink bugs on avocados. *South African Avocado Growers' Association Yearbook* 37:48-54.

Schoeman, P.S. 2014b. Stink bug IPM on macadamia in South Africa: current status and the road ahead. *Trends in Entomology* 10:87-95.

Van den Berg, M.A., Steyn, W.P. and Greenland, J. 1999. Hemiptera occurring on macadamia in the Mpumalanga Lowveld of South Africa. *African Plant Protection* 5(2):89-92.

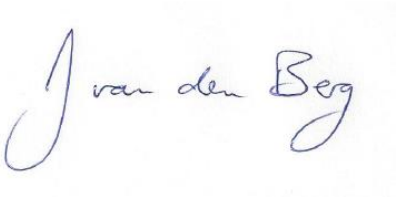
Appendix A

Declaration of language editing

Language editing statement

To whom this may concern,

I, Prof. J van den Berg, hereby declare that the thesis titled “Sampling and evaluation of entomopathogenic fungi for control of *Bathycoelia distincta* (Hemiptera: Pentatomidae) in South Africa” by LC. Linda has been edited for language correctness and spelling by the supervisors. No changes were made to the academic content or structure of this work.



Date : 10 December 2020

Appendix B

Ethics approval

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ETHICS APPROVAL LETTER OF STUDY

Based on the review by the **Faculty of Natural and Agricultural Sciences Ethics Committee (FNASREC)**, the Committee hereby clears your study as no ethical risk. This implies that the FNASREC grants permission that, provided the general conditions specified below are met, the study may be initiated, using the ethics number below.

Study title: Sampling and evaluation of entomopathogenic fungi for control of Chinavia pallidoconspera (Hemiptera: Pentatomidae)
Study Leader/Supervisor: Prof MJ du Plessis
Student: LC Linda

Ethics number: | N W U - 0 1 4 0 1 - | 2 | 0 | - | A | 9 |
Institution Study Number Year Status

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application type: Single **Risk Category:** **No Risk**

Commencement date: 01/02/2020
Expiry date: 01/04/2021

General conditions:
The following general terms and conditions apply:

- *The commencement date indicates the date when the study may be started.*
- *In the interest of ethical responsibility, the NWU-SCRE and FNASREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the FNASREC or that information has been false or misrepresented;*
    - *submission of the annual (or otherwise stipulated) monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and / or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *FNASREC can be contacted for further information or any report templates via Roelof.Burger@nwu.ac.za 018 299 4269*

The FNASREC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the FNASREC or the NWU-SCRE for any further enquiries or requests for assistance.

Yours sincerely,

Prof Roelof Burger
Chairperson Faculty of Natural and Agricultural Sciences Ethics Committee (FNASREC)

File reference: 9.1.5.4.2

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