The effect of the entomopathogenic fungus *Beauveria bassiana* on growth, physiological responses and control of aphid (*Myzus persicae*) infestation on *Lactuca sativa* L.

By

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Thesis submitted in fulfilment of the requirements for the degree

Master of Horticultural Science

in the Faculty of Applied Sciences

CAPE PENINSULA UNIVERSITY OF TECHNOLOGY

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Bellville

2020

DECLARATION

I, Neo Macuphe, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

2020

Signed **Date**

DEDICATION

I dedicate this thesis to:

- To my late grandmother Makelello Beauty Lehana-Macuphe for showing passion in education and encouraging me to study. May her soul rest in peace.
- Dr Mirriam Macuphe-Matandela for support through calls and asking about my progress, and always motivating me to do better.
- Kefuoe Macuphe for the passion and love you showed me during this period.

ACKNOWLEDGEMENTS

I would like to thank God and my ancestors for guiding me in difficult situations and giving me strength and courage to complete this project.

- I would like to express my gratitude to my supervisor, Professor Felix Nchu, for his continuous guidance, for his patience, encouragement, motivation, support and sacrifice throughout this project. His knowledge in this field made it possible for me to finish this study. Lastly, for showing love and transporting me when the department's vehicles were not available.
- My Co-supervisor Professor Oluwafemi Omoniyi Oguntibeju, for his encouragement, guidance, support, calmness and NRF-bursary he secured for me, all of which were keys for me to complete this study.
- I want to also express my gratitude to my family Kefuoe Macuphe, Thabile Macuphe, Dr Mirriam Macuphe-Matandela, Mpotseng Macuphe, Makereke Monica Vani Ayanda Vani, Tshepo Macope, my siblings Busiswa and Emihle and all my cousins, thanks for valuable guidance and support throughout my studies. Without your continued support, I would have stopped these studies a long time ago. You have been amazing.
- The departmental technicians Terrence Hlokane Mabela, Phumlani Roto Deborah Erasmus and Mvuyisi Mtshisazwe showed me love and welcomed me to C.P.U.T from the first day. You used your cars sometimes to transport me. You made it possible for me to finish my experiments. I am loss of words for your support during this project.
- Student Learning staff, Dr Vuyokazi Mntuyedwa, Dr Xena Cupido and Jean thanks for the support and love you gave me.
- I am grateful to my colleagues and friends for their assistance, Bandile Ludwaba, Bulelwa Ntobela, Zandile Maninjwa, Shaheed Roos, Siphokazi Moloinyane, Mvuyisi Qotyiwe, Odwa Mnyapa, Ata Kyemere, Yamkelani Pityoi, and Sinethemba Ngcongolo.
- The study was financially funded by NRF (National Research Foundation) and Cape Peninsula University of Technology.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

ANOVA	Analysis of Variance
B	Boron
C	Carbon
Ca	Calcium
EPF	Entomopathogenic fungi
GC-MS	Gas Chromatography – Mass Spectrometry
K	Potassium
Mg	Magnesium
Mn	Manganese
N	Nitrogen
ºC	Degrees Celsius
P	Phosphorus
PDA	Potato Dextrose Agar
S	Sulphur
Micronutrients	(Mn, Fe, Cu, Zn, and B)
Macronutrients	(C, N, P, K, Ca, Mg, and Na)

Abstract

Green peach aphid (*Myzus persicae*) is among the most destructive pests of agricultural and horticultural crops. This sap-sucking insect species reduces crop productivity and is a vector to many phytopathogens, and are often difficult to control with conventional synthetic insecticides. The quests for alternative environmentally-friendly insecticides for controlling aphids are intensifying. The use of entomopathogenic fungus presents an enticing opportunity to control sap-sucking insects such as *M. persicae*. Endophytic fungus such as *B. bassiana* is a natural parasite of insects and is a safer alternative to synthetic chemicals that are potentially toxic to human and environmental health.

The objectives of this study were to (i) assess the *Beauveria bassiana* colonization on *Lectuca sativa* plants, (ii) assess pathogenicity *of B. bassiana* against *M. persicae* in the laboratory, (iii) assess the effect of *B. bassiana* on antioxidant contents of *L. sativa*, (iv) assess the effect of *B. bassiana* on proximate components of *L. sativa,* (v) assess the effect of *Beauveria bassiana* inoculation on secondary metabolites contents of lettuce, and (vi) assess the effect of *B. bassiana* inoculation on *M. persicae* infestation level on lettuce plants in a greenhouse.

The *B. bassiana* strain (SM3) used in this study was first evaluated in an insect mortality bioassay to determine its pathogenicity and suitability for further study. Insects in each treatment group were exposed to one of four fungal conidial concentrations: 0, 1 x 10^6, 1 x 10^7 and 1 x 10^8 conidia mL^{-1}. The strain was pathogenic against *M. persicae*, with an LC_{50} of 1.1 to 1.6 x 10^6 conidia mL^{-1}.

Potted-lettuce plants were allocated to one of four conidial concentrations (0, 1x 10^6, 1 x $10^{7,}$ and 1 x 10^8 conidia mL^{-1}), following a complete randomized design, with a single-factor. The effects of fungal inoculation on plant growth, plant physiology, plant secondary metabolites, and aphid infestations were assessed.

After 21 days post-treatment, fresh leaves were picked off plants and taken to the laboratory to assess whether the fungus colonized the plant tissue. Leaf sections from the harvested leaves were surfaced sterilized. The leaves were then placed on selective solid potatoes dextrose agar (PDA) plates of half strength of 19.5 g/1000 ml, containing

0.04 g streptomycin, and 0.02 g ampicillin sodium salt. The plates were incubated at 25 ± 2 ^{o}C. Based on the mycelia outgrowth from the leaf sections, the fungus colonized up to 76% of plants in 1 x 10^{8} conidia mL^{-1} suggesting that tissue colonization by the conidia of *B. bassiana* was high.

The fungus did not significantly affect the growing parameters (P>0.05); however, there was a significant difference in crown size and plant height (P<0.001). The tissue contents of the micronutrients Mn, Fe, Cu, and B increased significantly (P<0.05) with the fungal treatment. On the other parameters assessed, antioxidant activities of the extracts and protein content of lettuce varied with conidial treatments. Interestingly, the values of antioxidant activities, carbon (C) content, and protein content in the control (no fungus) and highest conidial treatment were consistently higher than the moderate conidial treatments. The fatty acids and chlorophyll contents were not significantly (P>0.05) influenced by conidial inoculation.

Chapter three results showed that the fungus did not affect insect infestation levels (P>0.05). However, there was a statistical difference among the treatments on total polyphenol content (P<0.001). Flavonols were not significantly affected (P > 0.05) by the fungal inoculation. A wide range of volatile compounds was detected using GC-MS analysis. Some are well-known insect repellents and antifeedents, such as Limonene and 3-octanol. The 3-octanol and 2,4-Di-tert-butyl-phenol were significantly (P<0.01) more concentrated in the fungal treated plants than the control plants. Generally, while *B. bassiana* inoculation significantly affected total polyphenol and micronutrient (Mn, Fe, B, Cu) contents, it did not significantly affect flavonol level nor insect infestation levels.

In conclusion, the *B. bassiana* strain used in this study successfully colonized the lettuce plants. Overall, the fungus had minimal effects on plant growth and protection against aphid infestations. Exposure to the fungus did not significantly induce increased macronutrient contents in the plant tissue of nutrients and some volatile chemical constituents. This study provides insights into the effect of fungal inoculation on the growth, physiology, and aphid infestation of lettuce plants**.** The study also highlights the need for further studies to better understand the endophytic fungus-host plant-herbivorous insect relationship.

Key words: *Beauveria bassiana*, endophytic fungus, *Myzus persicae*, Lettuce and entomopathogenic

Chapter One

Introduction and Literature review

1.1 Introduction

Aphids have adapted to living off and feeding off plants. They are sap-sucking plant pests that feed by inserting their stylets into plants and sucking the phloem sap (Elzinga and Jander, 2013). Regrettably, this feeding behaviour facilitates the transmission of phytopathogens since aphids are efficient vectors of plants'plants' well-known infectious agents (Stavrinides e*t al*., 2010).

Myzus persicae (Sulzer) (Homoptera: Aphididae) is among the most damaging insect species of agricultural and horticultural crops (Vieira *et al*., 2016). This phytophagous insect feeds on more than 50 plant families worldwide. Aphids are known to reduce crop production and quality, leading to massive economic losses to large and emerging farmers (Dedryver *et al*., 2010). Consequently, farmers tend to use insecticides to mitigate the losses and retain high yield.

The control of these hemipterans is complicated: they can reproduce asexually in a short period (Augustinos *et al*., 2011; Ben-Issa *et al*., 2017), both male and female can produce alates (wings morph) that can disperse quickly and locate new host plants and increase their colony in a short period (Webster, 2012; Charaabi *et al*., 2018). Most of the aphids have developed a strategy of hiding on the leaves' underside, making it very challenging to control them (Webster, 2012).

Conventional control of aphids relies primarily on synthetic chemicals; however, these chemicals are not bio-rational and are toxic to the environment and human health (Laamari *et al*., 2008; Soffan and Aldawood, 2014). Because of the increased recognition of the dangers associated with synthetic acaricides, in the past few years, studies that focus on the development of efficient bio-rational control methods for insects are on the increase (Sadeghi *et al*., 2009; Sallam *et al*., 2009).

The use of entomopathogenic fungi presents an enticing opportunity/prospect for controlling insect pests because they are natural parasites to insects (Ahmed and Leather, 1994). Furthermore, some entomopathogenic fungi are endophytic (Vidal and Jaber, 2015). They can colonize plant tissues (Mejia *et al*., 2008; Vega *et al*., 2008; Rodriguez *et al*., 2009; Aly *et al*., 2011). Endophytic fungi cause physiological and defensive reactions in plants (Vilcinksas and Matha, 1997). Once they colonized plants, these fungi may affect varied processes in plants, such as the production of secondary and primary metabolites and nutrient uptake (Ownley *et al*., 2010; Rohlfs and Churchill, 2011), as well as insect herbivory.

However, entomopathogenic fungi are living organisms and are susceptible to environmental stresses (Ortiz-Urquiza and Keyhani, 2015). Under uncontrolled environmental conditions in field trials, many EPF have produced inconsistent efficacy, hindering the widespread uptake of EPF. Encouragingly, investigations to minimise this and other setbacks of EPF are ongoing. These fungi are currently applied as suspension (classical, augmentative, inundative or broadcast spray) (Jaronski, 2010), which is wasteful and may contaminate non-target species. On the other hand, a systemic application approach is perhaps a more feasible and efficient approaches, especially if the target insect is a sap-sucking insect with piercing mouthparts like aphids.

Besides assessing the effects of *B. bassiana* inoculation on growth and aphid infestation on *Lactuca sativa*, this study will provide insight into plants, fungi, and insects, thus filling a critical knowledge gap in the use of fungi as bio-control agents.

1.2 Literature review

1.2.1 *Beauveria bassiana* overview

Beauveria bassiana (Balsamo) Vuillemin is an entomopathogenic fungus that has a cosmopolitan distribution (Ownley *et al*., 2008). It occurs in diverse environmental habitats. This fungus belongs to the order Hypocreales (Brownbridge *et al*., 2012).

These hypocrealeans are soil-borne (Vanninen, 1995; Quesada-Moraga *et al*., 2007). However, some strains are both endophytic and pathogenic to insects (Khosravi *et al*., 2015). Endophytes occur in plant tissue without causing any harm to the plants (Bongiorno *et al*., 2016). Endophytes form a mutualistic relationship with plants by protecting plants against insects (Clay, 1988; Carroll, 1988; Saikkon *et al*., 1998; Ownley, 2010). For a fungus to successfully infect an insect, it must first come into contact with the cuticle of the insect, and its spores must germinate on the cuticle (Shah and Pell, 2003). Entomopathogenic fungi use a blend of enzymes and mechanical mechanisms to penetrate the cuticle and infect the insects (Inglis *et al*., 2001).

1.2.2 Lettuce

Lettuce (*Lactuca sativa* L.) is one of the most consumed leafy vegetables worldwide. It is a member of the Asteraceae family, which has around 23 000 - 30 000 species (Still, 2007:71; Noumedem *et al*., 2017). According to Noumedem *et al*. (2017), it is used worldwide for its nutritional content and medicinal properties. It is cultivated worldwide, with China being the leading cabbage producer, accounting for approximately 49.2% of the world total (Still, 2007:72).

1.2.2.1 Challenges facing lettuce cultivation

Production of lettuce is hindered by aphid infestations (Lu *et al*., 2011). Aphid infestations result in unmarketable lettuce and yield loss for growers (Fagan *et al*., 2010). Aphids infest all growth phases of the plants, from the young to mature plants (McCreight, 2008) and transmit the destructive lettuce mosaic disease between lettuce plants (Moreno *et al*., 2007).

1.2.3 The genus *Myzus*

The genus Myzus consists of approximately 50 species. Members of this genus are small-bodied insects that feed on host plants' phloem by sucking the juice with their pierce-sucking mouth-part (Panini *et al*., 2017). Aphid species are competent vectors, transmitting approximately 55% of the plants' viral diseases (Linz *et al*., 2015).

The aphid species *Myzus persicae* (Sulzer) (Homoptera: Aphidae) is one of the most destructive pests in the agricultural and horticultural sectors. It has a worldwide distribution and is capable of causing damage to more than 50 families of plants (Ghaedi and Andrew, 2016). *Myzus persicae* (green peach aphid) is well-known for transferring phytopathogenic viruses between plant families (Ghaedi and Andrew, 2016). This pest can transfer diseases, including the lettuce mosaic virus (Tiwari and Singh, 2018).

1.2.3.1 Historical and distribution of *Myzus persicae*

The *M. persicae* is a polyphagous pest insect with a worldwide distribution feeding on more than 1600 plant species across 60 plant families (Tiwari and Singh, 2018). In India, it affects approximately 300 plant species in families such as Solanaceae, Brassicaceae, Poaceae, Asteraceae, Rosaceae (Tiwari and Singh, 2018).

1.2.3.2 Morphology

The green peach aphids can be alates or apterea depending on environmental conditions. The aletes can grow up to 2.5 mm long and have dark green color. The alates can be identified by their head black head and thorax with the yellow-green abdomen (CAFE, 2013). Furthermore, the apterous aphid has different colors ranging from green, pale yellow-greenish, and pink or reddish (Goodarzifar *et al*., 2016). Nymphs of green peach aphid can reproduce asexually or sexually. Parthenogenetic reproduction is a common asexual reproductive strategy in aphids when they find a suitable host (Capinera, 2011).

1.2.3.3 Female aphids

Apterea green peach aphid females are easily identifiable, they resemble the yellow-green colour with three brownish longitudinal lines, one is located at mid-dorsum and the others are on the left and right of the dorsum (Smith, 2015). The female can grow up to 2.5 mm long with green pale siphunculi and a swollen middle (Smith, 2015). Females reproduce sexually and asexually through pathogenesis, giving birth to live young

aphids (Cooper, 2012:69). The nymph may be alate or apterea (Figure 1). The alates fly and locate the food (Cooper, 2012:69-70).

Figure 1: Adult apterea female *M. persicae*.

Source (http://entnemdept.ufl.edu/Creatures/veg/aphid/green_peach_aphid.htm).

1.2.3.4 Male aphids

The male aphids are holocyclic — they can undergo sexual reproduction with females to produce alates or apterous nymphs (Margaritopoulos *et al*., 2002). Alates male can disperse for long distances to finds mates and host plant. On the contrary, apterous males cannot fly, but they develop quickly to compete with alates on the host plant (Braendle *et al*., 2006).

1.2.3.5 Life cycle

Green peach aphids can reproduce sexually and asexually depending on the suitability of the environmental conditions. According to Margaritopoulos *et al*. (2002), aphids' life cycle has three categories: holocyclic, anholocyclic, and androcyclic. Holocyclic reproduces sexually; male mates with females and give birth to young ones. However, anholocyclic morphs are not able to mate. They produce females that are

parthenogenetically active and overwinter on host plants (Margaritopoulos *et al*., 2002). Androcyclic morphs reproduce parthenogenetically morphs that can mate and reproduce (Margaritopoulos *et al*., 2002).

1.2.3.6 Host plants

This polyphagous aphid feeds on more than a hundred plant species in more than 50 plant families (Shannag *et al*., 2014). It occurs worldwide, feeding on agricultural and horticultural crops (Shannag *et al*., 2014).

1.2.4 Control Methods

1.2.4.1 Chemical

Most farmers use systemic insecticides to control aphid infestations on plants (Obopile and Ositile, 2010; Mahmoud *et al*., 2017). Chlorinated hydrocarbons, carbamates and organophosphates are commonly used chemical pesticides to control aphids (Tewary *et al*., 2006). However, these systemic insecticides' continuous use has contributed to the rampant insecticide resistance in insects (Torkey *et al*., 2009; Sial *et al*., 2018). Furthermore, their use is correlated with harmful effects on the environment and human health (Tewary *et al*., 2006; Misra *et al*., 2020).

1.2.4.2 Biological control

The control of aphids using biological control methods is gaining favour among farmers and researchers. Thus far, two groups of organisms — parasitoids and entomopathogens – have produced promising results in controlling aphid infestations. Aphid parasitoids are natural enemies of aphids and can reduce aphid densities under field conditions (Kaser and Heimpel, 2018). According to Rakhshani *et al*. (2005), aphid is a natural host for numerous aphidiines. Based on the research performed by Cardinale *et al*. (2003), the introduction of *Nabis* sp. (Hemiptera: Nabidae) played a major role in the suppression of small soft body aphids. *Nabis* sp. uses their piercing-sucking mouthpart to attack the aphids (Cardinale *et al*., 2003).

Some strains of entomopathogenic fungal species, including *B. bassiana*, have proven efficacies against many insect pests (Rondot and Reineke, 2018; Javed *et al*., 2019). A few strains of these species are active ingredients in well-known commercial mycoinsecticides, such as Broadband and Real Metarhizium 69 OD (Jaronski and Mascarin, 2017), BotaniGard 22WP® (Kapongo *et al*., 2007). The use of entomopathogenic fungi has other advantages, for example, they can increase fitness and survival of the host plants (Latz *et al*., 2018). Furthermore, they colonize plant tissues without being pathogenic to the host plants (Latz *et al*., 2020). In addition, they have the ability to control insects over a long period of time and are compatible with systemic insecticide application method (Silva *et al*., 2020). *Beauveria bassiana* forms a mutual relationship with the plants and can colonize plant tissues (Ownley et al., 2010). According to Wagner and Lewis (2000), *B. bassiana* can be applied as foliar or injection to the plants.

1.2.4.2.1 Entomopathogenic fungus (*B. bassiana*)

1.2.4.2.1.1 Mode of action

Beauveria bassiana (Ascomycota: Hypocreales) conidia are able to germinate under humid conditions (Feng *et al*., 1994). *Beauveria bassiana* penetrates the insect host by developing the germ tube that invades the insect's hemocoel. Once the spore establishes contact with the cuticle of a suitable insect host, it secretes diverse hydrolytic enzymes that damage protein, chitins, and lipids (Feng *et al*., 1994). The steps of fungal insect colonization begin with adhesion to the insect cuticle, then penetration, followed by colonization and proliferation in the haemocoel. Eventually, *B. bassiana* eliminates their host by starvation and toxicity (Quesada-Moraga and Vey, 2003).

1.2.5 Potential benefits of entomopathogenic fungi in plant cultivation

Applying endophytes on seeds or growing medium can lead to endophytic fungal colonization of plants (Tefera and Vidal 2009). The fungus-colonized plants may be protected against insect pests. Endophytes live within plants without causing any

apparent damage to plants (Latch *et al*., 1985). According to Meyling and Eilenberg (2007), some entomopathogenic fungi, such as *Metarhizium anisopliae* can occur in the rhizophere and protect plants against pathogens. These are known as rhizopheric fungi and have many important ecological roles (Pattnaik and Busi, 2019). They maintain the soil health and plant health and breakdown organic matter (Dundas *et al*., 2018). Rhizospheric fungi also form a mutual relationship with plant roots and promote plant growth through nutrient absorption and enable a protective mechanism against pathogens and pests (Meena *et al*., 2017; Hassan *et al*., 2019; Fuentes *et al*., 2020). They also enhance plants' ability to tolerate environmental stresses (Fuentes *et al*., 2020). Some genera of rhizospheric fungi can be used for the management of parasitic nematodes. These genera (*Penicillium, Verticillium, Chaetomium, Fusarium*, and *Arthrobotrys*) can affect nematodes through parasitism, predation, and antagonism (Zhou *et al*., 2016).

1.2.6 Integrated pest management

The development of entomopathogenic fungus as bio-insecticides, especially against aphids, has received much interest among those interested in integrated pest management (Yeo *et al*., 2003; Edson *et al*., 2013). *Beauveria bassiana* is commonly used as bio-insecticides because it is pathogenic against many insects (Kanzok and Jacobs-Lorena, 2006; Kergunteuil *et al*., 2016). The combined use of an entomopathogenic fungus and safer pesticides could be very effective against aphid infestations (Alizadeh *et al*., 2007; Sayed *et al*., 2019). An entomopathogenic fungus was successfully used to control *Dociostaurus maroccanus*; the fungus secreted toxic secondary metabolites such as, cyclic peptides and insecticidal properties against the insect (Quesada-Moraga and Vey, 2004). *Beauveria bassiana* based insecticides have been developed for commercial use such as BotaniGard 22WP® (Wraight *et al*., 2000; Kapongo *et al*., 2007; Aak *et al*., 2018).

1.2.7 Mycotoxins

Endophytic fungi, including those in the order hypocreales, produce mycotoxins that are detrimental to herbivorous insects (Faeth, 2002). Moreover, these mycotoxins are being explored to control insects (Paszkiewicz *et al*., 2017). Endophytic fungi produce two main classes of mycotoxins - nonribosomal peptide (NRP) and polyketide (PK) synthase (Hu *et al*., 2016). The mycotoxins that have been detected in some of the hypocreales fungi are Fumonisin B1, Beauvericin, Enniatin A, Enniatin B, and Destruxin A (Paszkiewicz *et al*., 2017).

Endophytic fungi can also induce host plants to produce secondary metabolites (Ludwig-Müller, 2015; Kusari *et al*., 2012). Although researchers have made progress in the characterization of endophytic fungi mycotoxin in grasses and some woody plants, few studies have focused on the effects of endophytic fungal colonization on lettuce and other vegetables. This study examined the effect of endophytic fungal entomopathogens on the production of secondary metabolites by lettuce.

1.2.8 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are by-products of normal cellular metabolism in plants induced by a pathogen's stress or infection (Karuppanapandian *et al*., 2011). ROS plays a significant role in plants by regulating hormonal gene response against pathogen infection (Kreslavski *et al*., 2012). ROS induced redox systems in the plasmalemma and increased ROS generation in the apoplast are among the common plant cell responses to stress (Kreslavski *et al*., 2012). Endophytes have been found to increase antioxidants' production in host plants (White Jr and Torres, 2010), which can neutralize the ROS in plants (Hamayun *et al*., 2018).

1.2.9 Proximate analysis

A study by Hamayun *et al*. (2018) showed that plants that are inoculated with endophytes have increased sugar contents than untreated plants. White Jr and Torres (2010) suggested that plants with endophytes produce more glucose and fructose than non-endophytes. However, available literature focusing on endophytes and

carbohydrates interaction is still limited. Therefore, it is interesting to study the effect of endophytes on sugar content in lettuce — an important food crop — with the view of getting a broader understanding of the role of fungal endophytes on plant response to stress and insect herbivory. A few studies have demonstrated that inoculating plants with fungal endophytes leads to an initial increase in insect infestation level and a drop over time (Akello and Sikora, 2012).

1.3 Problem statement

Conventional control of *Myzus persicae* has relied on chemical insecticides; however, despite their efficacies, they have challenges in maintaining sustainable aphid control. Insect resistance to insecticides and toxicity of insecticides are some of the main challenges. The search for alternative and benign bio-control agents such as entomopathogenic fungal endophytes is justified. Endophytic fungi can colonize plant tissue and, therefore, be delivered systematically, targeting sucking insects like *M. persicae* through plant growth medium.

1.4 Aim of the study:

To evaluate the effect of the entomopathogenic fungus (*B. bassiana* [Hypocreales]) on growth, physiological responses and control of aphid (*M. persicae* [Hemiptera]) infestations on lettuce.

1.5 Specific Objectives

- To assess the pathogenicity *of B. bassiana* against *M. persicae* in the laboratory.
- To assess the colonization of lettuce by endophytic fungus (*Beauveria bassiana* SM3- strain).
- To assess the effect of endophytic fungus *B. bassiana* on growth parameters (height, crown size, roots length, dry weight, and fresh weight).
- To assess *B. bassiana* inoculation on macro – and micro–nutrients contents on the plants' aerial parts.

- To assess the effect of *B. bassiana* on physiology of plant (antioxidant contents, proximate components and chlorophyll content).
- To assess the effect of *B. bassiana* inoculation on secondary metabolites contents of lettuce, and to assess the effect of *B. bassiana* inoculation on *M. persicae* infestation level on lettuce plants in a greenhouse.

1.6 Hypothesis

- Higher concentration of *B. bassiana* will cause higher mortality of *M. persicae*
- Inoculation of plants with fungus will influence growth parameters (height, crown size, roots length, dry weight, and fresh weight).
- Inoculating lettuce plants with *B. bassiana* will positively influence the physiology (antioxidants, proximate components and chlorophyll content).
- Inoculating lettuce plants with *B. bassiana* will positively influence the secondary metabolites (polyphenol, alkaloid and flavonol) in plants.
- Lettuce plants will emit more insect-repelling *volatiles* than the control treated lettuce plants when exposed to *B. bassiana* inocula
- The number of aphids infesting lettuce plants will be comparatively lower on *B. bassiana*-treated plants than on control-treated plants.

1.7 Rationale of the study and significance

Vegetables and fruits play a massive role in supplying food and preventing degenerative disease (Serafini *et al*., 2002). Lettuce is one of the most consumed vegetables worldwide (Rouphael *et al*., 2017). However, its cultivation and yield is hampered by insects' infestations, including aphids, on lettuce plants (Fagan *et al*., 2010). To improve the yield of lettuce, effective pest management strategies would have to be developed.

The search for environmentally friendly insect control methods is ramping up. Many researchers are exploring the symbiotic relationship between entomopathogenic fungi and plants (Marquez *et al*., 2007). Entomopathogenic fungi are infective to insects (Mannino *et al*., 2019). Furthermore, some entomopathogenic fungi are also endophytic and rhizospheric (Rivas-Franco *et al*., 2020). Hence, the use of endophytic

entomopathogenic fungi could enhance protection of host plants against foraging by phytophagous insects. Results from many studies suggest the mutually beneficial relationship between endophytic fungi and plants may enhance plants' defence against herbivorous insects, thereby, reducing the rate of insects' infestations on plants (Clay, 1988; Raps and Vidal, 1998; Lehtonen *et al*., 2005). The presence of endophytic fungus in plant tissues can influence the quantity and quality of certain secondary and primary metabolites produced by plants and, consequently, reduces insect infestations (Strasser *et al*., 2000).

It is worth-noting that endophytic fungal conidia can be made available for uptake by plants during cultivation (Tefera and Vidal, 2009). The endophytic spores can be applied directly to the medium during plant cultivation (Kumar *et al*., 2011). Based on the above-mentioned arguments, incorporating endophytic conidia in plant growth is an interesting approach of plant cultivation. According to Inglis *et al.* (1993) application of *B. bassiana* is mostly done as a foliar application. But because the fungus is susceptible to sunlight, it tends to become ineffective; hence, systemic application of the fungus is a viable method. This study seeks to provide an insight into the interactions of plant, fungus and insects and, thus, filling an important knowledge gap in the use of fungi as bio-control agents.

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Chapter two

Evaluating the endophytic activities of *Beauveria bassiana* on the physiology, growth and antioxidant activities of extracts of lettuce (*Lactuca sativa* L.)

Abstract

Endophytic entomopathogens have growth promoting and anti-insects properties that could enhance the cultivation of lettuce, a vegetable crop that has high demand but highly susceptible to aphid infestations. This study's objective was to assess the colonization of the plant by conidia of *Beauveria bassiana* (SM3) (Hypocreales) as well as the effects of fungal inoculation on growth and physiology of the lettuce plants. Firstly, the pathogenicity of *B. bassiana* (SM3) was evaluated in an insect mortality bioassay, at 0, 1x 10^6, 1 x $10^{7,}$ and 1 x 10^8 conidia mL^{-1}, to determine whether it was suitable for the greenhouse study. For the greenhouse study, potted lettuce plants were allocated to one of four treatment groups in a complete randomized design. Plants in each treatment group were exposed to one of four fungal conidial concentrations: 0, 1x 10^6, 1 x $10^{7,}$ and 1 x 10^8 conidia mL^{-1}. The effects of fungal inoculation on tissue colonization, plant growth, plant physiology, and proximate composition were assessed. The strain was pathogenic against *M. persicae*, with a mean insect mortality of 8 per 10 insects recorded at the highest concentration (1 x 10^8 conidia mL^{-1}) and an LC_{50} of 1.1 to 1.6 x 10^6 conidia mL^{-1}. The *B. bassiana* endophytically colonized up to 76% of plants exposed to 1 x 10^8 conidia mL^{-1}. Except for crown size and plant height, there was no significant difference in all the other growth parameters (roots length, dry weight and fresh weight). Among the plant macronutrients, only carbon tissue content was significantly ($P<0.01$) affected by conidial treatments. However, most of the micronutrients, viz. Mn, Fe, Cu, and B were remarkably higher ($P < 0.05$) in the fungal treated plants than the control. Similarly, antioxidant activities (FRAP and TEAC) of plant extracts were also significantly ($P<0.001$) enhanced in higher conidial treatments. In conclusion, the *B. bassiana* strain was endophytic in lettuce, pathogenic against

the *M. persicae*, and induced increased micronutrient tissue contents and antioxidant activities.

2.1 Introduction

Lactuca (Asteraceae) is one of the most consumed salad vegetables in North America, South America, Europe, Australia, and New Zealand. Aphid infestations on this crop often lead to declines in lettuce yields and economic losses among commercial and small-scale farmers (Barriere *et al*., 2015). Aphids are sap-feeding insects capable of causing direct injury to plants and vectoring many damaging phytopathogens, including the lettuce mosaic virus (Barriere *et al*., 2015). Reducing aphid infestations is an efficient strategy to manage viruses and other vector-borne phytopathogens (Chandi *et al*., 2018). However, conventional aphid control still depends mostly on synthetic insecticides, which are often toxic to the environment (Koch *et al*., 2018; Ricupero *et al*., 2020). Furthermore, prolonged, excessive, and widespread use of these chemicals have been associated with insect resistance to insecticide and reduced soil fertility (Furlan *et al*., 2018). To achieve high quality and yield of lettuce, alternative, benign measures of aphid control needs to be developed.

Endophytic fungi can colonize plant tissues without causing damage or disease to host plants (Nair and Padmavathy, 2014). Fungal endophytes have been found in different agricultural crops (Vega *et al*., 2008). Fungal endophytes are vital in the agricultural and horticultural sectors because they provide protection against herbivorous insects and improve plant health (Vega *et al*., 2008). Endophytic fungi have many effects on host plants. The literature suggests that endophytic fungi does not only enhance plant growth directly or indirectly (Shah *et al*., 2018) but also favor plant adaptation to adverse conditions including, biotic and abiotic stresses (Lugtenberg *et al*., 2016; Tiwari and Lata, 2018; Omomowo and Babalola, 2019, Chand *et al*., 2020).

Researchers are interested in the mechanisms through which endophytic fungi protect plants. For example, *B. bassiana* can help plants adapt to different conditions and facilitate the transfer of nutrients from the soil to the roots (Afandhi *et al*., 2019). Some

endophytic fungi act as biostimulants that help disseminate macronutrients and micronutrients (Saia *et al*., 2019). Nutrient translocation and uptake can enhance plant growth by modifying phytohomornes (Ren *et al*., 2011; Lugtenberg *et al*., 2016). Endophytic fungi can produce or assist plants produce secondary metabolites that can defend the plant from pathogens and pests (White Jr and Torres, 2010; Vinale *et al*., 2017). White Jr and Torres (2010) reported that colonized plants by endophytes produce more glucose and fructose. Some studies suggest that endophytic fungi stimulate antioxidants production in plants, which is essential for neutralizing reactive oxygen species (Pan *et al*., 2017). Fungal endophytes can also solubilize phosphate (Otieno *et al*., 2015) and produce phytohormones such as cytokinins, indole acetic acid (IAA), gibberellin (GAs), and siderophore. Some fungi supply essential vitamins to the plant host (Waqas *et al*., 2012; Ismail *et al*., 2016; Jaber and Enkerli, 2017; Lata *et al*., 2018; Ikram *et al*., 2018; Omomowo and Babalola, 2019). A study by Hamayun *et al*. (2018) suggested that endophytic fungi enhanced proximate composition in plants.

Although many studies have examined the effects of endophytic fungi on plant growth and secondary metabolites, few studies have simultaneously and comprehensively investigated the effects on tissue colonization, plant growth, nutrient uptake, antioxidant content, and proximate components. This study intended to provide a deeper understanding of the physiological effects of endophytic fungus on *L. sativa* for improved cultivation

The objectives of this chapter were (i) to assess the pathogenicity *of B. bassiana* against *M. persicae* in the laboratory, (ii) to assess colonization of lettuce by endophytic fungus (*B. bassiana* SM3- strain), (iii) to assess effect of endophytic fungus *B. bassiana* on growth parameters (height, width, roots length, dry weight and wet weight), (iv) to assess the effect of *B. bassiana* on macronutrient and micronutrient contents on aerial parts of the plants, (v) to assess the effect of *B. bassiana* on antioxidant contents, (vi) to assess the effect of *B. bassiana* on proximate components of *L. sativa*, and (vii) to assess the effect of *B. bassiana* on chlorophyll content of lettuce.

2.2 Materials and methods

2.2.1 Research design

Laboratory and greenhouse experiments were carried out in this study. The selected *Beauveria bassiana* (SM3) virulence was evaluated in an insect mortality bioassay to determine its virulence and whether it could be considered for further study — the greenhouse study. In the greenhouse study, potted lettuce plants were allocated to one of four treatment groups in a complete randomized design, with a single factor. Plants in each treatment group were exposed to one of four fungal conidial concentrations: 0, 1x 10^6, 1 x $10^{7,}$ and 1 x 10^8 conidia mL^{-1} (Figure 2.1). The effects of fungal inoculation on plant growth, plant physiology, plant secondary metabolites, and insect infestations were assessed. All experimental plants were maintained under the same environmental conditions.

Figure 2. 1 Glasshouse experiment, the yellow tag is control , blue tag is 1x 10^6 conidial mL^{-1}, red tag 1x 10^7 conidial mL^{-1} and green tag 1x 10^8 conidial mL^{-1}.

2.2.2 Plants material

Lettuce (*L*. *sativa*) seedlings (cultivar: Green Oak) were sourced from Stodels Nurseries (Pty) Ltd in Bellville, Western Cape Province, South Africa. They were kept in the greenhouse of the Cape Peninsula University of Technology (CPUT), Bellville, South

Africa under the following conditions: 25 ± 2 ^{o}C, 60-80% RH, and 14/10 natural light/ dark regime. The individual plant was gently removed from the six-pack tray and transplanted into a 15 cm pot diameter containing a substrate mix: one-part silica sand, one-part perlite, one-part perlite, and one-part peat moss. Before its use, the substrate mix was sterilized with 1% sodium hypochlorite for 30 minutes and was rinsed with sterile distilled water three times. Plants were fed using recommended hydroponics Nutrifeed® hydroponic fertilizer (Starke Ayres Pty. Ltd., South Africa). The fertilizer was mixed with sterile distilled water at a concentration of 10g/ 5000 ml, and 200 ml was added in each plant once a week. Subsequently, each plant was watered with distilled water once a week for the six weeks.

2.2.3 Fungus preparation

An existing *B*. *bassiana* (SM3) strain that was previously isolated from a vineyard and identified molecularly by Moloinyane and Nchu (2019) was used in this study. The fungus was cultured on a selective medium: half-strength (19.5 g/ 1000 ml) of Potato Dextrose Agar (PDA) (Sigma-Aldrich PTY. LTD., South Africa), 0.04 g streptomycin, and 0.02 g ampicillin sodium salt. The PDA was prepared in 9 cm- and 14 cm-diameter Petri dishes. Fungal cultures were incubated for three weeks at 25 ± 2 ^{o}C in the darkness. The mature conidia of *B. bassiana* were harvested using a sterile spatula and transferred into a 50 ml centrifuge tube containing 30 ml sterile water. The tube was capped and shaken for 3 min and mixed vigorously for two minutes using a vortex mixer (MI0101002D Vortex Mixer) at 3000 rpm to homogenise the conidial suspension. The homogenous conidial suspension was transferred into 1000 ml bottles containing 500 ml sterile distilled water and 0.05% Tween 80 (Polysorbate, Sigma-Aldrich, South Africa). The conidia concentration was determined using a haemocytometer (Bright-Line, Sigma-Aldrich, South Africa) and observed with a light microscope at 400X magnification to determine the required concentration of (1x 10^{8}, 1x $10^{7,}$ and 1x 10^{6} conidia mL^{-1}). Germination percentage was assessed on 100 spore count at 40 x magnification (Latifian and Rad, 2012). Each plate was replicated four times, and over 90% germination was observed.

2.2.4 Pathogenicity test against *Myzus persicae*

The pathogenicity of the *B. bassiana* strain (SM3) on *Myzus persicae* was tested against the three different conidial suspensions of endophytic fungus *Beauveria bassiana* (SM3). The leaf dip method was adopted for pathogenicity bioassay. Three conidial concentrations and control were used to determine the virulence of the fungus against the aphid. The spores were adjusted into three concentrations (1x 10^8, 1x $10^{7,}$ and 1x 10^6 conidia mL^{-1}), and the control had only 0.05% Tween 80 and sterile water. A lettuce leaf section (with a diameter of 50 mm was cut for each treatment and immersed into 5 ml conidia suspension for 10 s for control 50 mm was immersed into sterile water with 0.05%Tween 80. Each treated leaf section was then placed on a Whatman No.1 sterile filter paper for 15 minutes to remove excessive conidia suspension (Nazir *et al*., 2018). Each treated leaf section was transferred into a Petri dish (90 mm diameter, 15 mm depth) lined with moistened Whatman No.1 sterile filter. After that, 10 adult apterous aphids were transferred onto each leaf section using a camel hairbrush and under a light microscope. Each treatment had six replicates, and each replicate had ten apterous adults aphid. The petri dishes were sealed with parafilm and incubated in the growing chamber at 25 °C and a photoperiod of 12:12 (L: D) h for seven days. The mortality was observed after five days. Insects were considered dead if they remained unresponsive after probing with camel hairbrush. Aphids that died were sterilised by dipping them into 70% ethanol for 10 s and rinsed with sterile distilled water for 1 minute. The cadavers were moved to Petri dishes lined with damp filter paper and were incubated at 25 ^{0}C in the dark, with 90% relative humidity to increase fungal growth and sporulation to confirm that the insects died from fungus.

2.2.5 Greenhouse study

This experiment took place at CPUT in the Department of Horticultural Sciences, Bellville Campus, South Africa. Greenhouse's mean temperature was 27 ± 3 ^{o}C, 70 ± 3% relative humidity, and the average light intensity was 31.77 kilo lux. Two weeks old lettuce seedlings were transferred into 15 cm pots containing a substrate mix of 25% silica sand 25% coco peat 25% perlite and 25% vermiculite. One hundred plants were

each planted into a 15 cm pot. This experiment had four treatments, and each treatment had twenty-five replicates. The first treatment was the control, which was drenched with 100 ml of sterile distilled water with 0.05% Tween 20. Treatment one was drenched with 100 ml conidial suspension of 1x 10^6 conidia mL^{-1}. Treatment two was drenched with 100 ml conidial suspension of 1x 10^7 conidia mL^{-1}. Treatment three was drenched with 100 ml conidial suspension of 1 x 1x 10^8 conidia mL^{-1}. Plants were fed using recommended hydroponics Nutrifeed fertilizer (Starke Ayres Pty. Ltd., South Africa) comprising the following ingredients: N (65 mg kg^{-1}), P (27 mg kg^{-1}), K (130 mg kg^{-1}), Ca (70 mg kg^{-1}), Cu (20 mg kg^{-1}), Mo (10 mg kg^{-1}), Fe (1500 mg kg^{-1}), Mg (22 mg kg^{-1}), S (75 mg kg^{-1}), B (240 mg kg^{-1}), Mn (240 mg kg^{-1}), and Zn (240 mg kg^{-1}). The fertilizer was mixed with sterile distilled water at a concentration of 10g/ 5000 ml, and 200 ml was added to each plant once a week. Each plant was watered with distilled water twice a week. The data was collected, plant height was measured from the soil surface to the top of the highest leaf, and crown size was measured from the widest of the plant to the widest leaf. After 21 days post-treatment, fresh leaves were pick-off plants and taken to the laboratory to assess fungal colonization. Leaf sections were surfaced sterilized in the following sequence: 0.5% of sodium hypochlorite for two minutes, 70% ethanol for two minutes, and then rinsed with sterile distilled water for 1 minute. The sterilized leaf sections were placed on a selective solid agar plates made up of potatoes dextrose agar (PDA) half strength of 19.5 g/1000 ml of sterile water containing 0.04 g streptomycin and 0.02 g ampicillin sodium salt and were incubated at 25 ± 2 ^oC. After six weeks post-inoculation, plants were uprooted from the pots, and roots height (cm $plant^{-1}$) and fresh weight (g $plant^{-1}$) of plants and roots were measured. Roots were separated from the aerial parts. Sub-samples of lettuce were oven-dried at 35 ^oC for 168 hours, after which the dried plants were weighed to record the weight (g $plant^{-1}$) of roots and plants. The experiment was repeated twice.

2.2.6 Antioxidants

2.2.6.1 Sample material

At the end of the glasshouse, experiment plants were randomly selected based on fungal colonization. Plants were oven-dried at 35 °C for 168 hours. The dried plant materials were ground, and the powered material transfered into plastic bags. Three samples representing three replicates were weighed for each treatment, and 0.1g of powdered plant material was transferred into centrifuge tubes. The samples were extracted with 25 ml of 60% ethanol and placed inside the incubator for 24 hours.

2.2.6.2. FRAP

The Ferric Reducing Antioxidant Power assay used is similar to the procedure discribed by Benzie and Strain (1996). This assay is based on the reduction of ferric-tripyridyltriazine complex to its ferrous in the presence of antioxidants. The following reagents were used: 2.5 ml of a 10 mmol/L TPTZ (2,4,6- tripyridyl-s-triazine, Sigma) solution in 40 mmol/L HCl plus 2.5 ml of 20 mmol/L $FeCl_3$ and 25 ml of 0.3 mol/L acetate buffer, and maintained at pH 3.6 was prepared freshly and warmed at 37°C. Aliquots of 40 µl of the sample supernatant were mixed with 0.2 ml distilled water and 1.8 ml FRAP reagent. After incubation at 37 °C for 10 min, we employed spectrophotometric method to read the absorbance of the reaction mixture at 593 nm. The standard soluation was 1 mmol/L of FeSO4, and the final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to 1 mmol/L FeSO4.

2.2.6.3 Trolox equivalent antioxidant capacity (TEAC)

The presence of antioxidants in lettuce was measured using the TEAC method described by Miller *et al*. (1993). The TEAC values were measured on the antioxidants' ability to scavenge the blue-green coloured $ABTS^{\bullet+}$ radical cation relative to the $ABTS^{\bullet+}$ radical cation scavenging ability of the water-soluble.

2.2.7 Tissue Nutrient Content Analyses

After six weeks post-inoculation, 12 plants that showed fungal colonization, three from each treatment, were taken for analysis of macronutrients and micronutrients at a commercial laboratory, Bemlab (Pty) Ltd (Gant's Sentrum, 16 Van Der Berg Cres, Strand, Cape Town, 7140, South Africa. Before the analyses, the lettuce leaves were washed with teepol solution, followed by rinsing with sterile distilled water, and then drying at 65 °C overnight in an oven. Briefly, 5g of dried leaves were milled and ashed at 480 °C, shaken up in a 50:50 HCl (50%) solution for extraction through filter paper (Xego *et al*., 2017). The phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), Sodium (Na), Iron (Fe), manganese (Mn), zinc (Z), Boron (B), copper (Cu), and carbon (C) content of the extracts were analyzed using Ash method. Total nitrogen (N) content of the leaves was assessed through total combustion in a Leco N-analyser. The unit of the macronutrients was g kg^{-1} while micronutrients were expressed as mg kg^{-1}.

2.2.8 Proximate analysis

2.2.8.1 Sample preparation

Briefly, after 30 minutes of harvest, lettuce plants were frozen at -20 °C until they became lyophilized. The damaged leaves were carefully removed during this preparation. Dried materials were grounded with an ultracentrifuge mill.

2.2.8.2 Protein analysis

The method used was adopted from Chikwanha *et al*. (2019); nitrogen content was analyzed using the method described by Duma of macro-Nitrogen analyzer (LECO® FP528, LECO Corporation, Miami, USA). Total protein content was determined by multiplying the N content by a factor of 6.25. The total protein percentage was converted into g kg^{-1}.

2.2.8.3 Fatty acid analysis

The method was adopted from Sukhija and Palmquist (1998) with minor adjustment. Fatty acids were analysed on Agilent 7890A Gas Chromatography– Flame Ionisation Detector System. The column used was HP88 (100 m x 250 µm, 0.250 µm); the temperature was set at 50 ^{o}C hold for 2 min, increase at 5 ^{o}C/min to 250 ^{o}C, and hold for 15 min. Carrier gas: Nitrogen with a flow rate set at 1.0 ml/min. Injection volume: 1 µl (split; 50:1). The fatty acids were detected by evaluation of their retention times with that of internal standard. The fatty acids that were detected were expressed as mg kg^{-1}.

2.2.9 Chlorophyll content Analysis

The chlorophyll estimation was adopted in the method of (Rajalakshmi and Banu, 2014). Briefly, one gram of freshly harvested plants was ground with 20-40ml of acetone. It was then centrifuged for 5 min at 5000 –10000rpm. After that, the supernatant was transferred, and the procedure was constant until residue become colorless. Then the absorbance of the solution was red at 645nm and 663nm against the solvent (acetone) blank.

2.2.9.1 Estimation of chlorophyll content

The concentrations of total chlorophyll, chlorophyll a, and chlorophyll b were calculated using the following equations.

Total Chlorophyll: $20.2(A_{645}) + 8.02(A_{663})$

Chlorophyll a: $12.7(A_{663}) – 2.69(A_{645})$

Chlorophyll b: $22.9(A_{645}) – 4.68(A_{663})$

2.2.10 Statistical analysis

The data collected were plant height, crown size, roots length, plant dry weight, roots dry weight, plant fresh weight, roots fresh weight, FRAP, TEAC, macronutrients, micronutrients, protein, and fatty acids, and chlorophyll. The growth parameters' data of

the first and second experiments were pooled since no significant differences were observed when the growth results were compared. The data were then analyzed using one-way ANOVA. Dosage mortality response was subjected to Finney's probit analysis method (1952)] to obtain the LC_{50}. The analyses were performed using the statistical software TIBCO Statistica® 13.3.0 Dell Inc., USA. Count mortality data was arcsine square root transformed and analysed using one-way analysis of variance (anova). The post hoc Turkey HSD was applied to separate means.

2.3 Results

2.3.1 Pathogenicity assessment

Generally, the results showed that *B. bassiana* (strain: SM3) was pathogenic against *M. persicae.* Insect mortality increased with conidial concentration. The highest concentration (1×10^8 conidia mL^{-1}) caused highest insect mortality (8) compared to other treatments ($DF=3, 20$; $F=21.57$; $P<0.01$) see table 2. The isolate had LC_{50} value of 1.1 to 1.6×10^6 conidia mL^{-1} (Table 2.1).

Table 2.1. LC_{50} values following leaf immersion of *Beauveria bassiana* against *Myzus persicae* in the laboratory.

Slope ± SE	LC_{50} (conidia ml^{-1}) (95%FL)	X^2 (DF)
0.411 ± 0.0582	$1.1 \times 10^6 – 1.6 \times 10^6$	0.995 (1)

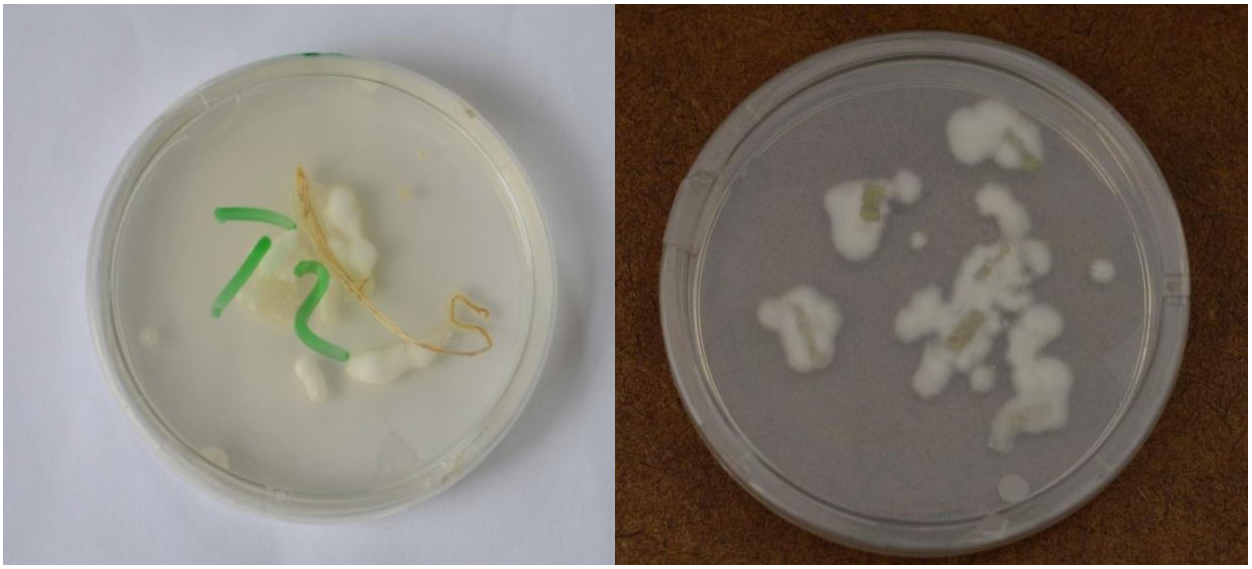
Table 2.1.1. The pathogenicity (mean ± SE number of dead insects and (Abbott-corrected percentage mortality), of *Beauveria bassiana* against *Myzus persicae* in the laboratory after five days following leaf immersion on fungal spores.

Treatments	Mean ± SE number of dead insects and (Abbott-corrected percentage mortality)
Control	2 ± 0.55a (0%)
1x 10^6 conidia mL^{-1}	6 ± 0.49b (48%)
1x 10^7 conidia mL^{-1}	7 ± 0.31b (66%)
1x 10^8 conidia mL^{-1}	8 ± 0.17b (78%)

Means with the same lowercase letters in the column indicates means ± SE are not significantly different using Tukey HSD test at P = 0.05 level of significance.

2.3.2 Colonization of tissues by fungus

Beauveria bassiana was successfully re-isolated from the leaves of the plants after three weeks. All the fungal treatments recorded fungal colonization of lettuce leaves and roots but at varying levels, with treatment three (1 x 10^8) showing the highest colonization percentage (76%), followed by treatments two (1 x 10^7) and one (1 x 10^6), 64% and 56%, respectively. Control did not show any fungal outgrowth in leaves. No fungal contamination was observed. *B. bassiana* was re-isolated from the roots and similar results were obtained with the leaves were also found in the roots; 1 x 10^8 conidia mL^{-1} showed the highest colonization percentage (76%), followed by treatments two (1 x 10^7) and one (1 x 10^6), 64% and 56% (Figure 2.3). No fungal outgrowth occurred in the control.



A B

Figure 2.3 Mycelia outgrowth from leaf sections demonstrating successful colonization of roots (a) and leaf (b) tissues by endophytic fungus *Beauveria bassiana.*

2.3.3 Effect of fungus on plant height, crown size and roots length.

Generally, the *B. bassiana* inoculation significantly increased plant height (DF=3, 196; F=3.61; P<0.01); the heights ranged from 15.52 to 16.04 ± 0.18 (Table 2.2). Similarly, there was a significantly difference among the treatments in terms of crown size of the plant (DF=3, 196; F= 14.52; P<0.001); generally, fungus treated plants had larger crown size (Table 2.2). Unlike plant height and crown size there was no significantly difference in roots lenght (DF=3, 116; F= 0.996; P=0.40).

2.3.3.1 Effect of fungus on fresh weight and dry weight of roots and the plant

The fungus inoculation did not significantly affect (DF = 3, 116; P> 0.05) both fresh and the dry weights of the roots and aerial parts (Table 2.2.1 respectively).

Table 2.2 The effect of endophytic fungus (*Beauveria bassiana*) on root length, plant height and crown size of the plants on different concentrations of spores and control.

Treatments	Roots Length (cm)	Plant height (cm)	Crown size (cm)
Control	19.50 ± 0.54a	15.52 ± 0.10a	25.94 ± 0.19a
1x 10^6 conidia mL^{-1}	19.33 ± 0.47a	16.02 ± 0.12b	27.50 ± 0.27bc
1x 10^7 conidia mL^{-1}	20.10 ± 0.51a	16.02 ± 0.12b	26.96 ± 0.29b
1x 10^8 conidia mL^{-1}	20.33 ± 0.38a	16.04 ± 0.18b	28.14 ± 0.22c

The same lowercase letters in the same column indicates means ± SE are not significantly different using Tukey HSD test at P = 0.05 level of significance.

Table 2.2.1 The effect of fungus (*Beauveria bassiana*) on Mean ± SE dry and fresh weights of plants and roots exposed to different conidial concentrations.

Treatments	Roots fresh Weight (g)	Plant fresh Weight (g)	Roots Dry Weight(g)	Plant Dry Weight (g)
Control	23.57 ± 0.62a	58.70 ± 1.60a	3.18 ± 0.12a	4.24 ± 0.11a
1x 10^6 conidia mL^{-1}	23.12 ± 1.21a	61.70 ± 1.53a	3.30 ± 0.06a	4.49 ± 0.13a
1x 10^7 conidia mL^{-1}	20. 48 ± 1.00a	54.17 ± 2.26a	3.39 ± 0.11a	4.61 ± 0.11a
1x 10^8 conidia mL^{-1}	21.28 ± 0.95a	57.65 ±2.43 a	3.32 ± 0.10a	4.49 ± 0.18a

The same lowercase letters in the same column indicates means ± SE are not significantly different using Tukey HSD test at P = 0.05 level of significance.

2.3.4 Effect of fungus (*Beauveria bassiana*) on antioxidant capacity

The treatment significantly influenced antioxidant capacity in plant extracts (DF=3, 8; F=6.067; P<0.001 FRAP $_{(umol\ AAE/g)}$; DF=3, 8; F=31.669; P<0.001 ABTS $_{(umol\ TE/g)}$), with higher levels occurring in the plants inoculated with the highest fungal conidial concentration (1 x 10^8 conidia mL^{-1}) and in control treatments (Table 2.3) compared to the lower conidial concentrations of 1x 10^6 conidia mL^{-1} and 1x 10^7 conidia mL^{-1}.

Table 2. 3 The effect of endophytic fungus concentration on antioxidant capacity of lettuce extracts following exposure of plants to *Beauveria bassiana* conidial during cultivation.

Treatments	**Frap (Umol AAE/g)**	**TEAC (Umol TE/g)**
	Mean ± SE	**Mean ± SE**
Control	86.13 ± 6.35a	88.92 ± 7.02a
1 x 10^6 conidia mL^{-1}	46.15 ± 3.61b	32.17 ± 6.43 b
1x 10^7 conidia mL^{-1}	55.81 ± 13.15b	43.14 ± 4.40b
1x 10^8 conidia mL^{-1}	89.43 ± 9.10a	97.97 ± 5.30a

The same lowercase letters in the same column indicates means ± SE are not significantly different using Tukey HSD test at P = 0.05 level of significance.

2.3.5 Effect of fungus (*Beauveria bassiana*) on tissue analysis

2.3.5.1 Macronutrients

Among all the macronutrients, only carbon (C) tissue content was significantly (DF=3, 8; F=34.67; P<0.01) influenced by fungal treatment. Carbon varied significantly with fungal treatments, with plants in the control and highest conidial concentration recording higher tissue carbon contents than the moderate conidial treatments (1 x 10^6 conidia mL^{-1} and 1x 10^7 conidia mL^{-1}). No significant differences in N, P, K, Ca, and Mg tissue contents were found among treatments (Table 2.4a).

2.3.5.2 Micronutrients

Unlike the macronutrients, most micronutrients were significantly affected by conidial concentration in this study. Apart from Zn, all the other tissue micronutrients (Mn, Fe, B, Cu) assessed varied significantly (DF=3, 8; $P < 0.05$) among treatments, with a discernible association of fungal treatments and higher plant tissue micro-nutrient contents. The highest values for Mn (81.03 ± 4.39), Cu (5.90 ± 0.26), and B (50.27 ± 1.01) were observed at 1x 10^7 conidia mL^{-1} (Table 2.4b). The tissue Fe (iron) content was highest at the highest conidial treatment and lowest in the control treatment, and the differences among treatments was statistically significant (DF=3, 8; F=7.956; P<0.01).

Table 2.4a Effects of inoculating lettuce plants with different conidial concentrations of *Beauveria bassiana* on tissue macronutrients contents (g kg-1).

Treatments	C	N	P	K	Ca	Mg	Na
Control	407.36 ± 0.41a	20.90± 0.60a	4.10 ± 0.60a	55.33 ± 0.33a	10.10± 0.45a	4.27 ± 0.15a	2.43 ± 0.14a
1x 10^6 conidia mL^{-1}	365.50 ± 3.36b	21.37± 0.75a	4.67 ± 0.38a	60.00 ± 4.16a	11.67± 0.88a	5.27 ± 0.56a	2.58 ± 0.19a
1x 10^7 conidia mL^{-1}	363.13 ± 1.48b	22.13 ± 0.47a	4.53 ± 0.18a	60.67 ± 0.67a	10.33 ± 0.33 a	5.20 ± 0.23a	2.42 ± 0.04a
1x 10^8 conidia mL^{-1}	396.33 ± 6.55a	21.20 ± 0.81a	4.60 ± 0.30a	58.33 ± 2.73a	10.03 ± 0.48a	4.63 ± 0.20a	2.44 ± 0.29a

The same lowercase letters in the same column indicates means ± SE are not significantly different using Tukey HSD test at P = 0.05 level of significance.

Table 2.4b Effects of inoculating lettuce plants with different conidial concentrations of *Beauveria bassiana* on tissue micronutrients contents (mg kg^{-1}).

Treatments	Mn	Fe	Cu	Zn	B
Control	54.73± 5.25a	286.00 ± 17.47a	2.87 ± 0.33a	47.27± 7.13a	38.70 ± 1.29a
1x 10^6 conidia mL^{-1}	70.63± 7.58ab	439.33± 41.91b	5.00± 0.15b	39.77± 3.48a	45.43± 2.57ab
1x 10^7 conidia mL^{-1}	81.03± 4.39b	427.33 ± 2.85b	5.90± 0.26b	38.33 ± 4.99a	50.27 ± 1.01b
1x 10^8 conidia mL^{-1}	72.60± 1.99ab	464.67 ± 34.36b	3.57± 0.35a	36.10± 2.21a	46.27± 3.44ab

The means ± SE followed by the same lowercase letters column indicates means ± SE are not significantly different using Tukey HSD test at P = 0.05 level of significance.

2.3.6 Proximate results

2.3.6.1 Protein

Generally, there was a significantly different in protein contents among treatments (DF=3, 8; F=5.18; P<0.05). Plants in the treatment 1x 10^6 conidia mL^{-1} had the lowest

protein content compared to other treatments (Table 2.5). Furthermore, there was no other significant difference among different treatments (Table 2.5).

Table 2.5 Effects of inoculating lettuce plants with different conidial concentrations *of Beauveria bassiana* on protein contents (g kg^{-1}).

Treatments	**Protein (g kg^{-1})**
	Mean ± SE
Control	27.87 ± 2.02ab
1x 10^6 conidia mL^{-1}	23.20 ± 1.19a
1x 10^7 conidia mL^{-1}	32.01 ± 2.25ab
1x 10^8 conidia mL^{-1}	34.06 ± 2.26b

The same lowercase letters in the same column column indicates means ± SE are not significantly different using Tukey HSD test at P = 0.05 level of significance.

2.3.6.2 Fatty acids

Fungal inoculation had no influence on palmitic acid (DF=3, 8; F=0.66 P>0.05), linoleic acid (DF=3, 8; F=4.00; P>0.05), linolenic acid (DF=3, 8; F=3.14; P>0.05) and total fatty acids (DF=3, 8; F=1.17; P>0.05). However, the total fatty acids were quite high in the control plants (1566.67 ± 233.33) than in those of fungal treatments (Table 2.6).

Table 2.6. Influence of endophytic fungus on proximate composition of *Lactuca sativa* (mg kg-1).

Treatments	Palmitic acid Mean ± SE	Stearic Acid Mean ± SE	Oleic acid Mean ± SE	Linoleic acid Mean ± SE	Linolenic acid Mean ± SE	Total Fatty Acids Mean ± SE
Control	366.67 ± 33.33a	ND	ND	266.67 ± 33.33a	933.33 ± 166.67a	1566.67 ± 233.33a
1x 10^6 conidia mL^{-1}	466.67 ± 166.67a	ND	ND	200.00 ± 0.00a	566.67 ± 33.33a	1233.33 ± 185.59a
1x 10^7 conidia mL^{-1}	300.00 ± 0.00a	ND	ND	200.00 ± 0.00a	733.33 ± 33.33a	1233.00 ± 33.33a
1x 10^8 conidia mL^{-1}	400.00 ± 0.00a	ND	ND	200.00 ± 0.00a	833.33 ± 33.33a	1433.33 ± 33.33a

The same lowercase letters in the same column indicates means ± SE are not significantly different using Tukey HSD test at P = 0.05 level of significance. n.d denotes not detected

2.3.7 Chlorophyll

Generally, the exposure to *B. bassiana* conidium did not statistically affect chlorophyll contents (DF=3, 8; F=2.45; P>0.05; total chlorophyll ug g^{-1}), (DF=3, 8; F=2.26; P=0.05; chlorophyll a ug g^{-1}) and (DF=3, 8; F=2.96; P=0.05; chlorophyll b ug g^{-1}), respectively. However, the chlorophyll contents were higher at 1 x 10^8 conidia mL^{-1} (Table 2.7).

Table 2.7. Effects of inoculating lettuce plants with different conidial concentrations of *Beauveria bassiana* on total chlorophyll and chlorophyll contents a and b (Mean ± SE µg g^{-1}).

Treatments	Total Chlorophyll	Chlorophyll a	Chlorophyll b
Control	503.13 ± 36.73a	368.51 ± 28.31a	134.75 ± 8.45a
1x 10^6 conidia mL^{-1}	488.93 ± 6.71a	355.16 ± 5.15a	133.89 ± 1.56a
1x 10^7 conidia mL^{-1}	520.84 ± 25.97a	381.12 ± 17.63a	139.85 ± 8.38a
1x 10^8 conidia mL^{-1}	586.51 ± 31.21a	425.39 ± 22.37a	161.26 ± 8.82a

Means with the same lowercase letters in the same column are not significantly different following comparison using the Tukey HSD at P = 0.05 level of significance.

2.4 Discussion

The *B. bassiana* used in this study induced aphid mean mortalities ranging 2 ± 0.55 - 8 ± 0.17 of 10 insects in the *in vitro* bioassay, increasing significantly (P<0.001) with concentrations and demonstrating that this fungus is pathogenic against aphids (Javed *et al*., 2019; Motholo *et al*., 2020; Cheong *et al*., 2020). *Beauveria bassiana* species have been known to be particularly virulent against sap-sucking homoteran because they can secrete proteases and chitinases that degrade insect cuticles (Fang *et al*, 2009). Also, this study demonstrated that, depending on the concentration, the *B. bassiana* SM3 strain colonized 56 to 76% of lettuce's leaf tissue after six weeks. Despite the evidence of successful tissue colonization by the fungus, minimal effects were

observed on plants' growth. However, interestingly, conidial inoculation significantly influenced tissue micronutrient contents, carbon tissue content, and antioxidant contents.

The conidial colonization observed in this study could be described as moderate to high. The colonization of the plant tissues by a fungus can be influenced by several factors, which include the concentration of fungal conidia, age of the plant (Biswas *et al*., 2012), and type of fungal strain selected (Jaber and Ownley, 2018; Moloinyane and Nchu, 2019). It is worth mentioning that some inoculation methods enhance colonization of the plant tissues by an EFP fungus (Muvea *et al.,* 2014). For example, seed inoculation produced higher colonization compared with the seedling inoculation in a study by Muvea *et al*. (2014) on the colonization of onion plants by fungal endophytes.

Plants colonized by endophytic fungus tend to perform better in terms of growth and tolerance to biotic and abiotic stresses (Abdelaziz *et al*., 2017). While the moderate to high fungal colonization was observed in this study, the influence of the *B. bassiana* isolate used in this study was observed on plant height and crown size of the plants. Moreover, plants that were inoculated with fungus showed increase in length and crown size compared to the control plants ($P<0.01$). This is in agreement with Dash *et al*. (2018) who reported that entomopathogenic fungi such as *B. bassiana*, *Isaria fumosorosea,* and *Lecanicillium lecanii* has potential to improve the growth of plants. Although the mechanism is not well document, EFP fungi produced siderophores and organic acids and can cause some nutrients to be available to the plants (Dash *et al*., 2018). However, the *B. bassiana* isolate used in this study had a minimal effect on frest and dry weight of the plant ($P>0.05$) despite evidence of tissue colonization. Broadly, while these results are contrary to the widely held expectations that fungal endophyte promotes plant growth, many recent studies have demonstrated that their influence on plant growth varies with host and isolates (Moloinyane and Nchu, 2019).

Despite the successful colonization of plant tissues, there was no statistical difference in tissue macronutrients for most macronutrients measured, i.e., N, P, K, Ca, and Mg. Moloinyane and Nchu (2019) reported similar results with the same fungal strain. Because N, P, K, Ca, and Mg are important for increased plant growth and biomass accumulation, it is therefore not surprising the results in the current showed that the fungus had little effect on the plant growth. However, the fungus affected C tissue content as it was higher in the control plants compared to plants in the other treatments (1×10^6 and 1×10^7), with the exception of the highest concentration of $1x\ 10^8$ conidial mL^{-1} (Table 2.4a). The high carbon content could be linked to higher amounts of structural carbon or carbon-based compounds (Gayler *et al*., 2008) and increased carbohydrate accumulation due to increased photosynthesis (Araya *et al*., 2010; Ainsworth and Bush, 2011). Carbon is an essential element for the photosynthesis process (Smith and Stitt, 2007). Carbon is an essential element for the photosynthesis process [39]. However, the reason for the lower tissue carbon in treatments 1×10^6 and 1×10^7 conidia mL^{-1} is not clear. It is worth-mentioning that carbon is used by fungus for hyphal growth (Sun and Liu, 2006). Similarly, nitrogen and carbon are used by an EFP fungus as sources for conidia germination and growth (Safavi *et al*., 2007).

The trend of the tissue micronutrient contents were quite obvious and interesting, as shown in the current results. There were statistical differences ($P < 0.05$) between fungal treated plants and control in three (Mn, Fe, and Cu) of the five micronutrients assessed, with control plants having lower levels of Mn, Fe, Cu, and B than fungus inoculated plants. Endophytic fungus can synthesize some of these micronutrients and improve the uptake of these nutrients (Vergara *et al*., 2019). While these elements are needed in small quantities, they have important physiological roles in plants: Fe is needed for the development of chlorophyll in plants (Rout and Sahoo, 2015); B is an essential microelement in the metabolism of nucleic acid, carbohydrates and protein (Uluisik *et al*., 2018); Cu plays critical roles in the physiological processes of plant such as photosynthesis, respiration, carbohydrate distribution, nitrogen fixation, metabolic of protein and antioxidant activity (Ruscitti *et al*., 2017) and Mn is significant for

metabolism and plant development (Schmidt and Husted, 2019). The deficiency or too much of these micronutrients can be detrimental to plants (Luciano *et al*., 2017; Singh *et al*., 2018). For example, Fe deficiency can cause yellowing and chlorosis on new leaves and reduces sugar metabolic enzymes (Das *et al*., 2017). Furthermore, the production of secondary metabolites is influenced by the concentration of micronutrients in plants, and these micronutrients are needed in small amounts (Luciano *et al*., 2017; Singh *et al*., 2018).

Generally, our results showed that inoculating plants with varying conidial concentration influenced antioxidant content of *L. sativa*. Antioxidants play an essential role as an inhibitor of reactive oxygen species when plants are under stress whether it is caused by abiotic or biotic factors (Hasanuzzaman *et al*., 2017). Remarkably, similar to C content, antioxidant content was lower among plants inoculated with conidial concentrations of 1×10^6 and 1×10^7 compared to control and 1×10^8 and corroborated with the results of the antioxidant capacity observed in the TEAC and FRAP assays. It is worth-noting that carbon-based secondary metabolites have antioxidant properties (Bidart-Bouzat and Imeh-Nathaniel, 2008). Nevertheless, the association and dip in both antioxidant capacity and tissue carbon in plants exposed to 1×10^6 and 1×10^7 needs further investigations. A proximate analysis may help clarify the nature of the carbon in the tissue. A study by Chatterjee *et al*. (2019) suggests that some endophytic fungus strains are a source of antioxidants. Future studies on metabolomics may also help elucidate the relationship between carbon contents and secondary metabolite production.

The endophytic fungi have capability of producing biochemical metabolites that can be exploited in agricultural (Ray *et al*., 2016; Shahabivand *et al*., 2017). Based on the proximate analysis protein was significantly different ($P<0.05$). Protein play a pivotal in growth of the plants and mediating the antioxidants (Rasheed *et al*., 2020). However, further investigations are needed to determine the types of protein that enhance the plant growth. The fatty acids' contents did not vary significantly ($P>0.05$) among conidial concentrations.

The chlorophyll content in plants has been studied as a response to abiotic stress. The endophytic fungus is well-known for increasing the plant's tolerance on biotic and abiotic factors (Idhan *et al*., 2018). In this study, the fungus did not influence chlorophyll content in plants despite the colonizing them. In the current study some of the key nutrients that are responsible for the synthesis chlorophyll were not affected by fungal treatment, Previously, Rozpądek *et al*. (2015) demonstrated that endophytic fungus improve chlorophyll content in plants.

2.5 Conclusion

The fungus successful colonized the plants that were inoculated. The laboratory results showed that the fungus is effective against the *M. persicae*. There was no evidence on that endophytic fungus influencing the fresh and dry weight except the plant height and crown size. The phytochemical showed that the fungal inoculation influences the C and micronutrients such as Mn, Fe, B, and Cu. Also, the endophytic fungus influenced the antioxidants. This study provides the knowledge on endophytic fungus and physiological aspect of the plants.

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Chapter Three

Evaluating the effect of *Beauveria bassiana* on secondary metabolite contents and green peach aphid (*Myzus persicae*) infestation level on lettuce (*Lactuca sativa* L.)

Abstract

Endophytic fungus could play a crucial role in the protection of food crops against phytophagous insects by endophytism and inducing the production of anti-insect secondary metabolites in plants. This study's objectives were to assess the effects of *Beauveria bassiana* (Hypocreales) inoculation on secondary metabolites, green peach aphid (*Myzus persicae*) infestation on lettuce plants, and plant volatile compounds, especially those with insect repellent properties. In this greenhouse study, two sets of potted lettuce plants were inoculated with one of four fungal conidial concentrations: 0, 1×10^6, 1×10^7, and 1×10^8 conidia mL^{-1}. The first set of plants was used to test the effect of *B. bassiana* inoculation on aphid infestation level on lettuce in meshed boxes. The second set of plants was used for assessing the effects of fungal inoculation on secondary metabolite contents (volatile and non-volatile compounds). The results showed that the fungus did not significantly ($P > 0.05$) affect insect infestation level. However, total polyphenol contents varied significantly with conidial concentrations. The flavanol content was not significantly ($P > 0.05$) affected by the fungal inoculation. GC-MS analysis detected a wide range of volatile compounds, including limonene and 3-octanol, which are well-known insect repellents and anti-feedents. The 3-octanol and 2,4-Di-tert-butyl-phenol were significantly ($P<0.01$) more concentrated in the fungal treated plants than the control plants. In conclusion, *B. bassiana* inoculation significantly affected polyphenol and the quantity of some volatile compounds; however, it did not significantly influence flavonol level nor reduce insect infestation levels.

3.1 Introduction

Green peach aphid (*Myzus persicae*) is among the most devastating pests of agriculture and horticulture crops (Davis *et al*., 2006; Kang *et al*., 2017; Tian *et al*., 2017). The financial loss caused by this pest is estimated at several billion US dollars per annum (Ren *et al*., 2015). They transmit some highly infectious viral agents to plants. These include potato virus y (PVY), mosaic virus, and beet western yellows virus (BWYV) (Lai *et al*., 2017; He *et al.*, 2018; Yoshida and Tamada, 2019). Furthermore, high sums of money are spent on their control (Sarwar, 2013), which has mostly relied on the use of synthetic insecticide.

Despite advances in synthetic insecticides, it is hard to achieve adequate control of green peach aphid. They can reproduce at a relatively fast rate (Madanat *et al*., 2016; Rix *et al*., 2016). Both females and males can develop wings and disperse to locate new host plants when food is scarce (Brisson, 2010). Furthermore, alate aphids are more alert to predators and parasitoids (Brisson, 2010). In the past century, the primary control method of aphids is chemical, espercially synthetic insecticides, such as organophosphates, carbamates, pyrethroids, and neonicotinoids (Foster *et al*., 2012; Silva *et al*., 2012). However, besides the efficacy challenges due to insecticide resistance, insecticides are toxic to the environment and human beings (Bass *et al*., 2011; Faraone *et al*., 2015).

Consequently, biorational control is gaining ground. Biological control agents, such as fungi, have good prospects in managing insect pest populations under field and greenhouse conditions, based on published results (Card *et al*., 2016). However, inconsistent stability and efficacy under adverse environmental conditions negatively affect their widespread use (Ortiz-Urquiza and Keyhani, 2015). Despite these setbacks, entomopathogenic fungi are quite versatile; they have specific characteristics that can be exploited to enhance their efficacy against some insects, such as aphids (Mascarin and Jaronski, 2016). Some entomopathogenic fungi are endophytic. They can live in the tissues of plants without causing visible symptoms nor damage to the host plants

(Kusari *et al*., 2012). *Beauveria bassiana* and *Clonostachys spp.* can successfully colonized plant tissues, offering protection against insects and pathogens, and enhancing growth (Gurulingappa *et al*., 2010; Guesmi-Jouini *et al*., 2014; Bamisile *et al*., 2018; Jaber and Ownley, 2018). Also, some entomopathogenic fungi can be easily mass-produced and formulated.

Chemical analysis of plant tissues colonized by endophytic fungi revealed that the fungi enhance secondary metabolite contents (Chandra, 2012; Kusari *et al*., 2012; Venugopalan and Srivastava, 2015). Ren and Dai (2012) demonstrated that inoculating plants with fungus enhanced Jasmonic acid (JA) and increased the production of volatile oil compounds in plant hosts. *Beauveria bassiana* and *M*etarhizium *robertsii* can produce volatile organic compounds (VOC) that are insecticidal or repellent (Lozano-Soria *et al*., 2020). Nitrosoamide, produced by *Muscodor* spp., is a classic example of a fungal volatile compound that is detrimental to insects (Lozano-Soria *et al*., 2020). Recently, Moloinyane and Nchu (2019) found higher volatile compounds on fungal treated grapevines than control, and interestingly, the fungus-treated plants released nephtalene, which has repellent and insecticidal properties. *Beauveria* and *Metarhizium* can produce volatile compounds that have repellent and pesticidal properties, and the most known volatile compounds produced by these endophytic fungi are 1-octen-3-ol, 3-octanone, and 1-octene (Khoja *et al*., 2019). The most known volatile compounds produced by these endophytic fungi are 1-octen-3-ol, 3-octanone, and 1-octene (Khoja *et al*., 2019). Some species of the genus *Beauveria* produce toxic metabolites that reduce insects' survival or delay pest reproduction (Gurulingappa et al., 2011). Hence, endophytic fungi are enticing biocontrol agents (Gange *et al*., 2019).

Our hypotheses for this study were: (i) inoculating lettuce plants with *B. bassiana* will positively influence secondary metabolite contents (polyphenol, alkaloid and flavonol) in lettuce plants, (ii) lettuce plants inoculated with *B. bassiana* will emit more volatile compounds that are potentially repelling to insects including *M. persicae* than the control treated plants, and (iii) the number of aphids infesting lettuce plants will be comparatively lower on *B. bassiana*-treated plants than on control-treated plants. This

chapter's objectives were to assess the effects of *B. bassiana* inoculation on secondary metabolites contents of lettuce and assess the effect of *B. bassiana* inoculation on *M. persicae* infestation level on lettuce plants in a greenhouse.

3.2 Materials and methods

3.2.1 Fungus preparation

An existing *B*. *bassiana* (SM3) strain that was previously isolated from a vineyard and identified molecularly by Moloinyane and Nchu (2019) was used in this study. The fungus was cultured on a selective medium: half-strength (19.5 g/ 1000 ml) of Potato Dextrose Agar (PDA) (Sigma-Aldrich PTY. LTD., South Africa), 0.04 g streptomycin, and 0.02 g ampicillin sodium salt. The PDA was prepared in 9 cm- and 14 cm-diameter Petri dishes. Fungal cultures were incubated for three weeks at 25 ± 2 ^{o}C in the darkness. The mature conidia of *B. bassiana* were harvested using a sterile spatula and transferred into a 50 ml centrifuge tube containing 30 ml sterile water. The tube was capped and shaken for 3 min and mixed vigorously for two minutes using a vortex mixer (MI0101002D Vortex Mixer) at 3000 rpm to homogenise the conidial suspension. The homogenous conidial suspension was transferred into 1000 ml bottles containing 500 ml sterile distilled water and 0.05% Tween 80 (Polysorbate, Sigma-Aldrich, South Africa). The conidia concentration was determined using a haemocytometer (Bright-Line, Sigma-Aldrich, South Africa) and observed with a light microscope at 400X magnification to determine the required concentration of (0, 1x 10^{6}, 1x $10^{7,}$ and 1x 10^{8} conidia mL^{-1}). Germination percentage was assessed on 100 spore count at 40 x magnification (Latifian and Rad, 2012)**.** Each plate was replicated four times, and over 90% germination was observed.

3.2.2 Plants

Two week-old lettuce (*Lactuca sativa* L.; cultivar Green Oak) purchased from Stodels Nurseries (Pty) Ltd in Bellville, Western Cape Province, South Africa were used in this study. Plants were maintained at the Cape Peninsula University of Technology's

greenhouse in Bellville, South Africa under the following conditions: at 25 ± 2 ^{o}C, 60-80% RH, and 14/10 natural light/ dark regime.

3.2.3 Aphid rearing

Green peach aphid *M. persicae* Sulzer (Homoptera: Aphididae) were reared on Lettuce (*Lactuca sativa* L.; cultivar Green Oak), Cape Peninsula University of Technology greenhouse. Aphids were reared on lettuce in a greenhouse with controlled conditions: 60– 65 % RH, 26 ± 2 ^{o}C and 12:12 light: dark (L: D) photoperiod.

3.2.4 Greenhouse study two

Research design/greenhouse experiment

For the greenhouse study, two sets of potted lettuce plants were allocated to one of four treatment groups in a randomized complete design. Plants in each treatment group were exposed to 1 of four fungal conidial concentrations: 0, 1 x 10^{6}, 1 x 10^{7} and 1 x 10^{8} conidia mL^{-1}. The first set of plants were used to study the effect of fungal inoculation on insect infestation. The plants were confined in meshed boxes (with a mesh size of 0.6 mm) to prevent insects from moving on different treatments. The second set of plants was not infested with insects, rather was used for assessing the effects of fungal inoculation on secondary metabolite contents. The greenhouse conditions were: temperature 27 ± 3 ^{o}C, 70 ± 3% relative humidity, and the average light intensity was 31.77 kilo lux. Two week-old lettuce seedlings were transferred into 15 cm pots containing a substrate mix of 25% silica sand, 25% coco peat, 25% perlite, and 25% vermiculite. Plants were fed using recommended hydroponics Nutrifeed fertilizer (Starke Ayres Pty. Ltd., South Africa) comprising the following ingredients: P (27 mg kg^{-1}), N (65 mg kg^{-1}), Ca (70 mg kg^{-1}), K (130 mg kg^{-1}), Cu (20 mg kg^{-1}), Mo (10 mg kg^{-1}), Fe (1500 mg kg^{-1}), Mg (22 mg kg^{-1}), S (75 mg kg^{-1}), B (240 mg kg^{-1}), Mn (240 mg kg^{-1}), and Zn (240 mg kg^{-1}). The fertilizer was mixed with sterile distilled water at a concentration of 10 g/ 5000 ml, and 200 ml was added to each plant once a week. Additionally, each plant was watered with distilled water twice a week.

Insect infestation

After 35 days, the plants were infested with 10 adult female aphids using a camel hairbrush, and the number of infested plants was counted on the fifth day using handheld magnifying lenses to check the number of adult and number of nymphs aphids per plant. In each meshed box with a size of 0.6 mm, there were five plants and 10 adults per plant of *M. persicae.* The effect of fungal inoculation on insect infestations was assessed.

3.2.5 Sample material

At the end of the greenhouse experiments, plants that showed successful fungal colonization were randomly selected for the analysis of secondary metabolite contents. The successful fungal colonization of the tissues was determined using the method decribed in Moloinayne and Nchu (2019). Briefly, after 21 days post-treatment, fresh leaves were pick-off plants and taken to the laboratory to assess fungal colonization. Leaf sections were surfaced sterilized in the following sequence: 0.5% of sodium hypochlorite for two minutes, 70% ethanol for two minutes, and then rinsed with sterile distilled water for 1 minute. The sterilized leaf sections were placed on a selective solid agar plates made up of potatoes dextrose agar (PDA) half strength of 19.5 g/1000 ml of sterile water containing 0.04 g streptomycin and 0.02 g ampicillin sodium salt and were incubated at 25 ± 2 ^{o}C. Plants were oven-dried at 35 °C for 168 hours and were ground into plastic bags. For each treatment, three replicates were prepared. 0.1 g of each of the powdered materials from each replicate was transferred into separate centrifuge tubes. The samples were extracted with 25 ml of 60% ethanol and placed inside the incubator for 24 hours.

3.2.5.1 Analysis of secondary metabolites on leaves of inoculated plants

Total alkaloids: The spectroscopic method was used to determine total alkaloids in the plant (Fadhil *et al*., 2007). Briefly, 0.1 g of powdered lettuce leaves were extracted with 25 mL of 60% ethanol and 40% of sterile distilled water for 24 hours in total darkness, centrifuged (4000 x g for 10 min), and the supernatant was used in the assay.

Subsequently, two millimetres of the extract supernatant and atropine standard solutions were mixed with 12 mL bromocresol green solution and 5 mL sodium phosphate buffer. We added 12 mL chloroform was added to the above-mentioned solution, and the solution was mixed using a vortex mixer. The spectrometrc absorbance at 417 nm and a standard curve of atropine were used to determine the concentration of mg atropine equivalent per g dry weight (mg AE/g DW) in the sample.

Total polyphenol: The Folin-Ciocalteu method was used to determine total polyphenol content of the crude extracts of leaves (Singleton *et al*., 1999; Swain & Hillis, 1959). 25 µL of the crude extract sample was mixed with 125 µL Folin-Ciocalteu reagent (diluted 1:10 with distilled water) (Merck, South Africa). 100 µL (7.5%) aqueous sodium carbonate (Na_2CO_3) (Sigma-Aldrich, South Africa) was added to each well after 5 min. This was followed by the absorbance reading of the solution in the microplates, and results are expressed as mg gallic acid equivalents per gram dry weight (mg GAE/g DW).

Total flavonol: The flavonol content was determined using the protocol described by Daniels *et al*. (2015). Quercetin standard concentrations of 0, 5, 10, 20, 40, and 80 mg/L in 95% ethanol (Sigma-Aldrich, South Africa) were used. 12.5 µL of the crude sample extracts were mixed with 12.5 µL 0.1% hydrochloric acid (HCl) (Merck, South Africa) in 95% ethanol in the sample wells, and then incubated for 30 min at room temperature. The results were expressed as mg quercetin equivalent per g dry weight (mg QE/g DW).

3.2.6 GC-MS analysis

3.2.6.1 Sample preparation

Twelve potted plants, three from each fungal treatment, were used for this analysis. Only plants that showed fungal colonization among the fungus treated plants were used for GC-MS analysis.

3.2.6.2 GC-MS Analysis (Headspace)

The GC-MS method described by Moloinyane and Nchu (2019) was adopted for this study. We removed whole leaves from the fresh lettuce plants and freeze-dried them at -80 ^{0}C (overnight). We crushed the leaves in liquid nitrogen, and transferred 1 g of the crushed leaves into a solid-phase microextraction (SPME) vial, and then transferred 2 ml of 12% ethanol solution (v/v) at pg 3.5 and 3 ml of 20% NaCl in the vial. The samples were mixed vigorously using a vortex mixer. Finally, we analysed the headspace of the samples using a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre (gray).

3.2.6.3 Chromatographic separation

We identified and separated the volatile compounds in the lettuce plants using a gas chromatograph (6890N, Agilent Technologies Network) coupled to an inert Xl EI/CI Mass selective detector (model 5975B, Agilent Technologies Inc., Palo Alto CA). The protocol used is described in Moloinyane and Nchu [28]. The GC-MS system used was combined to a CTC Analytics PAL autosampler. The volatiles were separated on a polar ZB-WAX (30 m, 0.25 mm ID, 0.25 µm film thickness) Zebron 7HG-G007-11 capillary column. We used helium as the carrier gas. The flow rate of the helium was maintained at 1 mL/min. The injector temperature was 250 °C, with a split ratio of 5:1 and oven temperature was timed at 35 °C for 6 min, at a rate of 3 °C/min to 70 °C for 5 min, then at 4 °C/min to 120 °C for 1 min, and finally increased to 240 °C at a rate of 20 °C/min and maintained for 2.89 min. We operated the mass selective detector in full scan mode while maintaining the source, quad, and transfer temperatures at 230 °C, 150 °C, and 250 °C, respectively. The electron impact mode at ionization energy of the mass spectrometer was run below 70 eV, scanning from 35 to 500 *m*/*z*. To estimate quantities, relative ratios were used and calculated using the expression (peak area/IS peak area) × IS concentration (IS = internal standard). A cut-off match quality of at least 90% for organic volatile compound identities were used.

3.2.7 Statistical analysis

The data collected were secondary metabolites, insect infestation, and volatile compounds. Count data for insect infestation was arcsin square root transformed, and then analysed using one-way ANOVA. The post hoc Turkey HSD was performed to separate the different means. The data were analyzed using Statistica (TIBCO Statistica® 13.3.0 Dell Inc., USA).

3.3 Results

3.3.1 Effect of fungus on secondary metabolites

Generally, there was a significant difference (DF=3, 8; F=15.518; P<0.001) among treatments for the total polyphenol contents in plants. The highest total polyphenol content was recorded among plants treated with the highest conidial concentration (1x 10^8 conidia mL^{-1})(Table 1). The fungus had no effect on total flavonols (mg/QE/g) at (DF=3, 8; F=3.68 P>0.05); however, 1x 10^8 conidia mL^{-1} showed the best result (7.46 ± 0.68 mg QE/g) and 1x 10^6 conidia mL^{-1} showed the lowest number (45 ± 0.59 mg QE/g) (Table 3.1.) Alkaloids were not detected in the lettuce plants in this study.

Table 3. 1 Effect of *Beauveria bassiana* inoculation on secondary metabolites of *Lactuca sativa* on different treatments.

Treatments	Polyphenols (*Mean ± SE mg GAE/g)	Flavonols (*Mean ± SE mg QE/g)	Total alkaloids
Control	65.93 ± 4.22a	7.11 ± 0.63a	ND
1x 10^6 conidia mL^{-1}	34.98 ± 0.27b	4.45 ± 0.59a	ND
1x 10^7 conidia mL^{-1}	43.21 ± 7.30 b	5.94 ± 0.89a	ND
1x 10^8 conidia mL^{-1}	71.54 ± 2.94a	7.46 ± 0.68a	ND

The same lowercase letters in the same column indicates means ± SE are not significantly different using the Tukey HSD test at P = 0.05 level of significance. ND denotes not detected.

3.3.2 Infestation level of aphid in the greenhouse

The inoculation of plant with *B. bassiana* did not significantly affect immature aphids infestation (DF=3, 16; F=1.86 P>0.05). Similar results were obtained for the immature and the adult aphids (DF=3, 16; F=2.14; P>0.05). But the highest fungal concentration (1x 10^8 conidia mL^{-1}) showed lower infestation by adult aphids, 29.40 ± 0.68 (Table 3.2).). Generally, the fungus-inoculated plants showed lower infestation by immature aphids compared to control (Table 3.2).

Table 3. 2 Effect of endophytic fungus (*Beauveria bassiana*) on the infestation level of aphids (*Myzus persicae*) (Mean ± Se number of insects per plant) in the greenhouse.

Treatments	Immature aphids	Adults aphids
Control	51.20 ± 1.71a	33.40 ± 1.21a
1x 10^6 conidia mL^{-1}	48.00 ± 1.10a	32.00 ± 1.30a
1x 10^7 conidia mL^{-1}	47.40 ± 1.25a	30.80 ± 1.36a
1x 10^8 conidia mL^{-1}	48.00 ± 0. 89a	29.40 ± 0.68a

The same lowercase letters in the same column indicates means ± SE are not significantly different using the Tukey HSD test at P = 0.05 level of significance.

3.3.3 Volatile compounds following GC-MS analysis

In the current study, diverse volatile compounds were identified following the GC-MS analyses in all treatments (Table 3.3). Some well-known insects' repellents and semiochemicals, such as limonene, dodacane, hexadene, benzaldehyde, hexadene, beta-cyclocitral, aromadendrene, and hexadecenal were detected in plants from all the treatments. Interestingly, the quantities of 3-Octanol (DF=3, 8; F=18.94; P<0.01) and 2,4-Di-tert-butyl-phenol (DF=3,8; F=27.53; P<0.01) were significantly higher in fungal treated plants.

Table 3. 3 The mean area ratio of volatile compound (mean ±SE) of *Lactuca sativa* after exposure in different fungal concentration.

Compound volatiles	control	1x 10^6 conidia mL^{-1}	1x 10^7 conidia mL^{-1}	1x 10^8 conidia mL^{-1}
Dodecane	0.076 ± 0. 012A	0.046 ± 0.022A	0.067 ± 0.014A	0.068 ± 0.009A
Limonene	0.018 ± 0.002A	0.015 ± 0.005A	0.015 ± 0.006A	0.018 ± 0.006A
Nonanal	0.238 ± 0.026A	0.177 ± 0.019A	0.188 ± 0.066A	0.136 ± 0.016A
1,3-Di-tert-butylbenzene	ND	ND	ND	0.1 ± 0.001
Pentacedene	0.032 ± 0.006A	0.039 ± 0.008A	0.037 ± 0.008A	0.034 ± 0.009A
Trans,trans_2,4_heptadeinal	0.021 ± 0.002A	0.021 ± 0.004A	0.021 ± 0.002A	0.020 ± 0.004A
1-Octanol	0.023 ±0.001A	0.020 ± 0.003A	0.024 ± 0.004A	0.017 ± 0.002A
Benzaldehyde	0.036 ± 0.012A	0.053 ± 0.008A	0.044 ± 0.009A	0.033 ± 0.008A
Hexadene	0.036 ± 0.013A	0.058 ± 0.009A	0.045 ± 0.010A	0.033 ± 0.008A

Table 3.3 continues

Undercanal	0.010 ± 0.003A	0.012 ± 0.004A	0.006 ± 0.001A	0.011 ± 0.002A
Ethyl-caprate	0.019 ± 0.011A	0.012 ± 0.004A	0.008 ± 0.003A	0.010 ± 0.002A
Beta-cyclocitral	0.029 ± 0.007A	0.057 ± 0.023A	0.040 ± 0.019A	0.045 ± 0.022A
Heptadecene	0.015 ± 0.006A	0.028 ± 0.006A	0.022 ± 0.006 A	0.017 ± 0.006A
1-Dodecanal	0.054 ± 0.013A	0.084 ± 0.035A	0.074 ± 0.035A	0.091 ± 0.034A
Aromadendrene	0.028 ± 0.004A	0.019 ± 0.006A	0.021 ± 0.008A	0.012 ± 0.004A
Delta-cadine	0.016 ± 0.010A	0.016 ± 0.008A	0.011 ± 0.004A	0.012 ± 0.002A
Ethyl-laurate	0.033 ± 0.012A	0.086 ± 0.054A	0.034 ± 0.013A	0.055 ± 0.012A
Pentadecanal-	0.033 ± 0.012A	0.086 ± 0.054A	0.034 ± 0.013A	0.055 ± 0.012A
2,6-Dimethylbenzaldehyde	0.135 ± 0.026A	0.224 ± 0.070A	0.224 ± 0.089A	0.180 ± 0.054A
Ethyl-linoleate	0.047 ± 0.018A	0.295 ± 0.183A	0.223 ± 0.117A	0.268 ± 0.106A

Table 3.3 continues

Beta-Ionone	0.148 ± 0.022A	0.202 ± 0.070A	0.244 ± 0.100A	0.244 ± 0.072A
trans-beta-ionone-5,6-epoxide	0.040 ± 0.010A	0.077 ± 0.026A	0.073 ± 0.035A	0.055 ± 0.016A
Ethyl-myristate	0.030 ± 0.008A	0.064 ± 0.022A	0.087 ± 0.042A	0.063 ± 0.019A
Ethyl-decanate	0.010 ± 0.003A	0.020 ± 0.007A	0.016 ± 0.006A	0.024 ± 0.010A
(z,z,z)-9,12,15-octadecatrienoic-acid,-methyl-e	0.185 ± 0.086A	1.013 ± 0.499A	0.600 ± 0.269A	0.666 ± 0.261A
Hexadecenal	0.018 ± 0.006A	0.045 ± 0.015A	0.039 ± 0.012A	0.036 ± 0.012A
Hexadecanol	0.198 ± 0.084A	1.060 ± 0.520A	0.631 ± 0.286A	0.087 ± 0.040A
Ethyl-stearate	0.020 ± 0.006A	0.053 ± 0.023A	0.037 ± 0.020A	0.038 ± 0.015A
Nonanoic-acid	0.003 ± 0.000A	0.004 ± 0.001A	0.004 ± 0.001A	0.003 ± 0.000A
6-Amyl-alpha-pyrone	0.898 ± 0.142A	1.331 ± 1.072A	0.756 ± 0.356A	2.633 ± 1.030A

Table 3.3 continues

Ethyl-palminate	0.896 ± 0.140A	2.003 ± 0.894A	2.631 ± 1.029A	2.217 ± 0.900A
Alpha-humelene	0.931 ± 0.164A	2.163 ± 0.861A	0.763 ± 0.345AB	4.088 ± 0.225B
Ethyl-9-hexadecenoate	0.055 ± 0.009A	0.288 ± 0.078A	0.255 ± 0.092A	0.363 ± 0.149A
2,4-Di-tert-butyl-phenol	0.062 ± 0.007A	2.462 ± 0.352B	2.801 ± 0.365B	3.773 ± 0.321B
3-Octanol	0.00A	2.775 ± 0.237B	2.801 ± 0.630B	3.744 ± 0.313B

The same uppercase letters in the same row indicates means ± SE are not significantly different following the Tukey HSD test at P = 0.05 level of significance. ND denotes that the volatile were not detected.

3.4 Discussion

Inoculation of lettuce with *B. bassiana* conidia had varied influences on secondary metabolites. The total polyphenol content was significantly influenced by *B. bassiana* exposure, while the total flavanol content was not affected. It is worth mentioning that the total polyphenol content was higher in the plants inoculated with the highest conidial concentration of *B. bassiana*. Similar findings were found in a study by Espinoza *et al*. (2019) that focused on the effect of the same fungal strain on the secondary metabolite content of chives. Previously, Song *et al*. (2017) reported that some endophytic fungal strains could increase the synthesis of secondary metabolites such as flavanoids in host plants. The role of flavonoids in plants has been studied extensively in plant resistance against phytophagous insects (Bentivenha *et al*., 2018; Hay *et al*., 2020).

In this study three volatile compounds were found in the fungus-treated plants at significantly higher levels ($P < 0.05$) following the GC-MS analysis. Among these volatile compounds is a well-known insects' repellent 3-Octanol (Mburu *et al*., 2013). The other volatile compound that was significantly correlated with fungal treatment was 2,4-Di-tert-butyl-phenol. Although the effect of the higher amounts of these two volatile compounds did not translate into a reduction of insect infestation level, volatile compounds can elicit repellent and insecticidal activities against some insects.

Despite the detected positive effect of fungus inoculation on the yield of some secondary metabolites, the number of adults and immature insects foraging on the lettuce plants were not affected by *B. bassiana* treatment. This result is different from that of Mahmood *et al*. (2019), which showed a reduction in aphid population and delayed fecundity on plants that were inoculated with endophytic fungus *B. bassiana*. Again, just as in plant growth, fungal strain, host plants, and insect species may influence endophytes' effects on insect herbivory. These findings suggest highlight the complex relationship between secondary metabolites on insect herbivory and foraging.

Studies that focus on the effect of fungal endophytism on lettuce are scarce. The results obtained in this study provide insights on the effects of *B. bassiana* inoculation on secondary metabolite production and aphid infestation on lettuce plants as well as the complex yet intriguing endophytic fungus-lettuce-aphid relationship. It would be interesting to study the longterm sublethal effects of the fungus on the foraging aphids in a future study.

3.5 Conclusion

Generally, while *B. bassiana* inoculation significantly affected total polyphenol content, and the quantity of some volatile compounds, but its influence on flavonol level or insect infestation levels was not significant. This study provides some insights into the endophytic *B. bassiana*-lettuce-aphid relationship and recommends further studies on the chemical characteristics of fungal strains, host plants, and insects.

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Chapter four

4.1 General discussion

Insect control is mostly still based on chemical insecticides that are not environmental friendly (Sarwar, 2016; Sparks *et al*., 2019; Karkanis and Athanassiou, 2020). The excessive use of insecticides has led many researchers to find alternative measures to control insects (Nardoni *et al*., 2018). The use of entomopathogenic fungus is gaining popularity amongst researchers (Mahmoudi *et al*., 2018; Lee and Kim, 2019). Endophytic fungi do not only protect plants from insects, it also improves plant growth (Espinoza *et al*., 2019; Staffa *et al*., 2020). These endophytic fungi can colonize the plant tissues without causing apparent symptoms to the host plant (Vergara *et al*., 2017; Jaber and Ownley, 2018; Yan *et al*., 2019). In this study, *B. bassiana* colonized up to 76% of the fungus inoculated plants.

Interestingly, the successful colonization of lettuce tissue by the fungus, in the current study, did not favourably influence the macronutrient contents nor fresh and dry weight of the plant. Nitrogen, phosphorus, and potassium are essential macronutrients required by plants for development (Shin *et al*., 2005). This insignificant effect of the fungal exposure on growth of lettuce could be explained by the minimal effect of the *B. bassiana* inoculation on the tissue macronutrients' contents.

Remarkably, *B. bassiana* induced higher micronutrients in the fungus-treated plants. Although these micronutrients are required in small quantities, they do, however, play a significant role in synthesis of secondary metabolite production in plants (Dordas, 2008). For example, B is a vital microelement that plays a role in the metabolism of nucleic acid, carbohydrates, and protein (Uluisik *et al*., 2018). There were noticeable variations in lettuce tissue C (carbon) content with treatments, and 1 x 10^8 conidia ml^{-1} recorded the highest tissue C content among the fungal treatments (Table 2.4a), with plants in the highest conidial concentration yielding the tissue C content. Carbohydrates are produced during photosynthesis (Hajihashemi *et al*., 2018). Moreover, photosynthesis

use light, absorb through the chlorophyll, and CO_2 to produce carbohydrates (Hajihashemi and Sofo, 2018). This study did not find any effect of fungus treatment on the chlorophyll contents nor fatty acids. However, these results need further interrogating to determine whether the C content is due to structural carbohydrates or secondary metabolites.

Although endophytic fungus may not be detrimental to the host plants, it can influence plants' physiology. In this study, antioxidant activities and secondary metabolites in lettuce plant extracts were affected by *B. bassiana* inoculation. Specifically, polyphenols and antioxidants activities (FRAP and TEAC) in plant extracts were higher in control, and the highest conidial concentration than in moderate conidial treatments. Few studies have investigated the effects of endophytes on antioxidant activities and secondary metabolites (Rahmawati *et al*., 2019). Some secondary metabolites triggered by endophytic fungus are antiviral, insecticidal, plant growth, and regulatory activities (Lee *et al*., 2017).

While the *B. bassiana* strain (SM3) used in this study was pathogenic against *M. persicae* in our laboratory study and could colonise the lettuces' tissue in the greenhouse study, it did not reduce aphid infestations. Many factors influence the fungus-plant-insect relationship: the ability to colonize tissue varies between strains and species (Garrido-Jurado *et al*., 2017), host plant characteristics in terms of secondary metabolites and defense mechanisms varies (Zaynab *et al*., 2018), and insect adaptations varies (Sharma *et al*., 2020). An initial increase followed by reduction insect infestation levels has been observed in previous studies (Akello and Sikora, 2012). Since plants normally provide nutrient to endophytes, colonization by fungus may lead to an initial drop in important nutrients required by plants, causing stress to plants and increasing insect infestation levels (Faeth, 2002).

In this study, two bioactive volatile compounds, 2,4-Di-tert-butyl-phenol and 3-Octanol, were significantly higher in fungal treated lettuce plants than control plants. Some endophytic fungi induce volatile compounds in plants (Herrera *et al*., 2015). This could

explain why plants inoculated with conidia showed higher volatile compounds than the control plants. However, although the fungus treatment induced a higher number of volatile repellent compounds, it did not translate to a reduction in insect infestations.

This study provides insight into plant, fungus, and insects' interactions, thus filling a critical knowledge gap in using fungi as bio-control agents. However, more physiological studies are recommended to improve our understanding of the mechanisms through which endophytic fungus can reduce insect infestation levels.

4.2 Recommendations

Based on the outcomes of this study, the following recommendations are suggested for future studies.

I. The reactive oxygen species (ROS) needs to be assessed to determine whether the fungus caused stress to the plant.
II. The mycotoxins that are produced by the fungus needs to be assessed to find out whether they can cause harm to human beings.
III. Metabolomic studies can help decipher the interactions endophytic entomopathogens and host plants
IV. The inoculation of seeds by endophytic fungus to assess whether the seeds will improve colonization when compared to seedlings inoculation.

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