The effects of water deficiency and the fungus *Beauveria bassiana* on the bioactivity of *Allium cepa* (onion) extracts against grapevine mealybug (*Planococcus ficus*).

By

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DECLARATION

I, Lizeka Pretty Gana, declare that the contents of this dissertation/thesis represent my own unaided work, and it 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.

14/10/2022

Signed **Date**

DEDICATION

I dedicate this thesis:

- Firstly, to my parents, Noluviwe Gana-Mdludlu and Morris M. Mdluldu: I appreciate your constant encouragement to never give up despite all impediments and always instilling the value of education in us.
- Secondly, to my sister Nandipha Gana: Thank you for always holding my hand in both good and bad times and always celebrating my wins. You are truly heaven-sent.
- Thirdly, to my lovely niece Sinako Gana: May this serve as a reminder that you can achieve anything you set your mind to.
- Lastly, to my grandmother Nozameka Ngwangwaza: Ndiyabulela ngemithandazo yakho nangeemfundiso zakho Manqukhwe.

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LIST OF ACRONYMS

ANOVA	Analysis of Variance
B	Boron
C	Carbon
Ca	Calcium
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

For many years chemical insecticides have been utilized extensively in the agricultural sector to reduce the harm that insect pests inflict on plants. However, these substances are hazardous to humans and the environment. Studies that search for “green” alternatives that could replace synthetic insecticides are gaining significant attention. Biological control agents like fungi hold great promise as worthy alternative insecticides and plant growth stimulants. They are environmentally friendly and have proven efficacy against insect pest populations in greenhouse and field environments as well as their ability to enhance secondary metabolite production in plants. Furthermore, plants can develop symbiotic associations with some endophytic entomopathogenic fungus species, increasing the plants' resilience to abiotic stressors, such as water stress. Water deficiency is one of the most important abiotic factors limiting plant growth. However, despite the negative effects of water stress on plants, it can induce plants to produce more secondary metabolites. This study focuses on the singular and interactive effects of *Beauveria bassiana* inoculum and water stress on plant growth, secondary metabolite contents, antioxidant and repellent activities of *Allium cepa* extracts. The specific objectives were: (i) to determine the effects of water deficiency and the inoculation of a growth medium with *B. bassiana* conidia on the growth parameters (number of leaves, plant height, fresh and dry weights), (ii) to determine whether water deficiency and the fungus *B. bassiana* influence the nutrient contents of *A. cepa* tissues, (iii) to determine the variations in the profile of bioactive chemical constituents of *A. cepa* in response to water deficiency and the inoculation of growth medium with *B. bassiana* spores and (iv) to determine the anti-mealybug activities of *A. cepa* in response to water deficiency and inoculation of growth medium with *B. bassiana* spores.

The first experimental part of the study, presented in chapter three, evaluated the interactions between water deficiency and the inoculation of a growth medium with *B. bassiana* on plant growth, tissue nutrient contents, secondary metabolites, and antioxidant capacity of *Allium cepa*. *Allium cepa* seedlings were also exposed to *B. bassiana* or a no-fungus treatment and to one of three watering regime treatments (3-day, 5-day, or 7-day watering intervals). Plants treated with *B. bassiana* performed better than those in the no-fungus treatment, despite the longest watering interval causing reduced plant growth. The interaction between the watering schedule and the fungus had a significant impact on the contents of P, K, and Fe. Interestingly, the *B. bassiana*-treated plants had a considerably higher polyphenol (64.0 mg GAE/L) at the 7-day watering

interval than the plants that had not been exposed to fungus. Total flavonol is among the fungus-treated plants was significantly impacted by the watering interval. The quantity of flavonoids present in the onion bulbs and the antioxidant capacity in the FRAP bioassay were both substantially affected by the interaction between the watering interval and *B. bassiana* inoculation.

In the second part of the study, presented in chapter four, the effects of water stress and *B. bassiana* on the volatile organic compounds and the repellent activity of extracts of *Allium cepa* (onion) against grapevine mealybug were evaluated. Simultaneously, fungus and no-fungus treatment *A. cepa* seedlings were applied to one of three watering regimes (3-day, 5-day, or 7-day watering intervals). After ten weeks, *A. cepa* bulbs were collected, crushed, and then extracted with dichloromethane (DCM) for 24 hours at room temperature (25 °C). The DCM extract was filtered and dried, and then reformulated in DCM to obtain the following concentrations: 20% w/v, 10% w/v, and 5% w/v. These concentrations were then tested in a disc-repellent bioassay. The disc-repellent bioassay showed that repellencies differed significantly across the treatments (fungus, no-fungus, positive and negative control), regardless of the watering interval. When extracts were evaluated at 20% w/v, *B. bassiana* inoculation of *A. cepa* plants considerably increased the repellent ability against grapevine mealybugs. Plant extracts from the fungal treatments demonstrated the strongest insect repellent efficacy. The Gas chromatography–mass spectrometry analysis of the DCM extracts revealed that the *B. bassiana*-inoculated plants' bulbs produced considerably more volatile organic chemicals than the plants without the fungus.

The current study advances existing knowledge on the relationship of plants, fungi, and water deficiency in the contexts of plant response to stress and pest control.

1. CHAPTER ONE

Statement of research problem, main objectives, specific objectives and hypotheses

1.1 Introduction

Infestations of grapevine mealybugs cause substantial economic losses in both wine and table grapes. The honeydew produced by vine mealybugs can coat vine plants and cause sooty mold to form on the leaves and bunches (Daane et al., 2012). Mealybugs are phloem feeders that suck out plant juices with long, slender mouthparts (Wilson and Daane, 2017; Daane et al., 2018). They belong to the Pseudococcidae family, which contains some of the most important vineyard pests (Hardy et al., 2008). Mealybugs can feed on all vine sections, including the roots, making them of economic significance (Daane et al., 2006). Furthermore, they are carriers of grapevine leafroll-associated viruses, and they impair the vigor of the vine through yearly infestations. Managing mealybugs is extremely difficult, and currently, synthetic insecticides are widely used for controlling them (Daane et al., 2018).

Chemical insecticides and pesticides have been used in agricultural industries for many years to limit the damage caused by insects and pests (Özkara et al., 2015). Nevertheless, these chemicals are toxic to the environment and humans (Lichtfouse et al., 2009). Concerns about the harm caused by these agrochemicals have prompted researchers to look for alternatives that might be used to replace agrochemicals while avoiding the same adverse outcomes. The employment of biological control agents such as fungi and plants as a replacement for synthetic agrochemicals seems promising as they do not have the same negative consequences and have good prospects in managing insect pest populations under field and greenhouse conditions (Lahlali et al., 2022).

Many plant species, including *Allium cepa* L., can produce physiologically active secondary metabolites with insecticidal capabilities, and researchers are working to improve the cultivation of these anti-pest plants (Goławska et al., 2014). Many studies have shown that environmental stresses, such as drought and reduced light, causes plants to produce more bioactive constituents (Kessler and Kalske, 2018).

Drought is one of the major abiotic stress factors known to produce changes in the biochemical characteristics of plants and to increase the quantity of secondary metabolite production in diverse plants as a defensive strategy (Ren et al., 2007; Wang et al., 2007). Drought also slows plant growth by changing their cellular metabolism to adapt to severe environmental conditions (Zobayed et al., 2007).

Microorganisms, such as fungi and bacteria, aid in the production of bioactive chemicals in plants (Vey et al., 2001). Endophytic entomopathogenic fungi have a symbiotic connection with plants, colonizing plant tissues without infecting them and reducing insect infestation levels in plants (Bacon and White, 2000). It has also been shown that some endophytic fungi can help plants to cope with the detrimental impacts of external conditions such as drought (Schardl et al., 2004). As a result, exposing plants rich in anti-insecticidal chemicals to endophytic fungal inocula and water stress at the same time could boost the activity of their extracts, improve plant performance under stressful conditions, and increase secondary metabolites yield (Pang, 2004). Understanding the plants with insecticidal properties and endophytic fungi could lead to higher yields and higher quality of anti-insecticidal plant extracts, potentially reducing the usage of synthetic pesticides (Regnault-Roger et al., 2003). Additionally, it has been reported that extracts of *Allium* plants have compounds and secondary metabolites that are toxic to insects (Slimestad et al., 2007).

The aim of this study was to investigate the effect of water deficiency and the soil entomopathogenic fungi *Beauveria bassiana* on the growth of the common onion *A. cepa*, secondary metabolite contents and antimicrobial activity against the grapevine mealybugs. The dichloromethane extracts of *A. cepa* have been reported to have an acaricidal effect against ticks (Nchu, 2005); this raises the question of whether *A. cepa* extracts will also have insecticidal effects. While most studies on the insecticidal activities of plants in the *Allium* family have focused more on *Allium sativum* L. (garlic), this study focuses on the assessment of the interactive effects between *B. bassiana* and the abiotic factor (water deficiency) on the anti-insecticidal properties of *A. cepa* against grapevine mealybugs. The findings of this study could contribute to a better understanding of the link between plants, fungi, and water deficiency in terms of pest control, therefore, adding to the current body of knowledge. Furthermore, the results could also contribute to developing new techniques of controlling mealybugs using

biological agents such as entomopathogenic fungi, which could subsequently lead to the advancement of ecologically friendly methods of insect pest management.

1.2 Hypotheses of the study

B. bassiana conidia will mitigate the negative effect of water deficiency on *A. cepa*.

B. bassiana inoculum will enhance the growth of *A. cepa* plants.

B. bassiana inoculation will influence tissue nutrient contents of *A. cepa*.

The interaction between water stress and *B. bassiana* will induce *A. cepa* plants to increase the production of secondary metabolites.

B. bassiana inoculation and water deficiency will have enhanced anti-mealybug activities of *A. cepa* extracts.

1.3 Overall aim of the study:

The purpose of this study was to evaluate the bioactivities of *A. cepa* in response to water deficiency and endophytic entomopathogenic fungus *B. bassiana* to explore new ways of bio-prospecting the anti-mealybug activities of *A. cepa* plant extracts for the control of grapevine mealybugs.

1.4 Specific objectives of the study:

The specific objectives of this study were:

- To determine the effects of water deficiency and the inoculation of a growth medium with *B. bassiana* conidia on the growth parameters (number of leaves, plant height, fresh and dry weights).
- To determine whether water deficiency and the fungus *B. bassiana* influence the tissue nutrient contents of *A. cepa*.
- To determine the variations in the profile of bioactive chemical constituents of *A. cepa* in response to water deficiency and the inoculation of growth medium with *B. bassiana* spores.
- To determine the anti-mealybug activities of *A. cepa* in response to water deficiency and inoculation of growth medium with *B. bassiana* spores.

1.5 Structure of thesis

Chapter One: This chapter comprises an introduction and the background to the research problem.

Chapter Two: This chapter comprises the literature review.

Chapter Three: Presents with the interactive effects of water deficiency and endophytic *B. bassiana* on plant growth, nutrient uptake, secondary metabolite contents, and antioxidant activity of *A. cepa* L.

Chapter Four: covers water deficiency and endophytic *Beauveria bassiana* enhance repellent activity of *Allium cepa* L. (onion) extracts against grapevine mealybug.

Chapter five: provides a general discussion of the results, conclusion and recommendations.

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2. CHAPTER TWO

Literature Review

2.1 Grapevine mealybugs

2.1.1 Mealybug

Planococcus ficus (Signoret) is the scientific name for the vine mealybug, which belongs to Pseudococcidae family and the order Hemiptera (Millar, 2002; Ben-Dov and Miller, 2012). It is one of the insects that cause significant losses in the agricultural sector (Daane et al., 2006). Adult mealybugs have soft, segmented bodies that are covered in white powdered wax (Mani and Shivaraju, 2016; Chiotta et al., 2010). They are around 5 mm long and have unique filaments around the margins of their bodies. Adult females, nymphs, and eggs reside in clusters (Sharon et al., 2016). Female mealybugs have no wings and become more sessile when they are mature. Males go through a prepupal stage before becoming winged adult males. *Pseudococcus* species are 0.5 cm long, flat, oval-shaped, and contain wax filaments protruding from the body's perimeter (Figure 2.1).

Figure 2.1 Female *Planococcus ficus* (Grapevine mealybug)

Photo sourced from: www.sun.ac.za.

2.1.2 Lifecycle

Temperature is one of the most important factors in mealybug development (Satar et al., 2013). They thrive in warm weather with high humidity and temperatures around 25 °C (Braybrook, 2012). Winter generations survive under the bark of trees and in cracks in trellis supports, and the overwintering generation emerge from the vines as the buds open in the spring (Daane et al., 2003; Walton, 2003). Fruits and vegetation are the primary food sources for the first-generation crawler (Daane et al., 2003). Males have four larval instars, whereas females have three (Wakgari and Giliomee, 2003). This stage is known as the dispersal stage because the first instar moves swiftly in order to find a feeding location. The first instar nymphs, which are about 0.6 mm long, spend several days sheltering under the female before moving out of feed. In the second instar, third instar, and immature adult, there are three sequential molts that demonstrate an increase in size as well as the amount of wax secretion produced (Hardy et al., 2008). Majority of the females lay their overwintering eggs in old wood, but some oviposit in fruit clusters (Walton and Pringle, 2005; Waterworth et al., 2011).

2.1.3 Damages caused by mealybugs

Mealybugs are phloem feeders that feed on the roots, trunks, canes, leaves, and berry clusters of vines (Wilson and Daane, 2017; Daane et al., 2018). As they feed on the vines, they minimize the amount of carbohydrate-rich honeydew that accumulates on the leaves and in the grape clusters (Daane, et al., 2011). Honeydew acts as a breeding ground for sooty mold fungus, which can cause additional vine harm (Addison and Samways, 2000). When the mealybug population is large enough, honeydew can build up and form a hard, wax-like film that covers the afflicted plant (Figure 2.2). Furthermore, the honeydew can cause defoliation and limit photosynthesis (Reineke and Thiéry, 2016). Mealybug infestations can cause leaves to fall prematurely, affecting a canopy's capacity to develop a crop and store carbohydrate before dormancy (Braybrook, 2012). Defoliation can occur as a result of feeding damae, while vine mortality can happen after repeated yearly infestations (Walton and Pringle, 2017). They are also recognized as vectors for the spread of grapevine leafroll-associated viruses, which cause vine vegetative growth, yield, and fruit quality to suffer, as well as graft incompatibilities (Braybrook, 2012).

Figure 2.2 Grape infested with grapevine mealybugs and covered with honeydew.

Photo sourced from: www.wineland.co.za.

2.1.4 Control and management of mealybugs

A variety of pest management measures have been deployed to prevent and mitigate large economic losses caused by severe infestations on grapevines by vine mealybugs (Mansour et al., 2010). Monitoring mealybugs is one of the strategies used to avoid agricultural losses. Mealybug monitoring depend on population density, the number of samples required for a correct count is typically considerable because most mealybugs have a clumped distribution pattern, and with only a small percentage of vines being infested (Lentini et al., 2008). The right sampling depends on a variety of factors, such as the season, because the mealybug population changes with the seasons (Millar, 2002). Some mealybugs hide under the bark during the winter. Mealybug species differ in terms of annual generations and preferred feeding locations on grapevines.

Pheromone traps are also used as a method to control mealybugs. Yellow delta type traps, baited with the artificial sex pheromone of the female *P. ficus,* are commercially available and used to monitor the number of mealybug males in a vineyard. Sticky traps can be used to track the existence of mealybugs and catch the winged ones (Mansour et al., 2010).

The accumulation of ants is one of the signs of an infested grapevine (Daane et al., 2007). Because of the mutualistic relationship between ants and mealybugs, controlling ants is essential to control mealybugs. Chemicals should be applied to vine stems to create a barrier that prevents ants from entering the vine canopy (Braybrook, 2012).

During the growth season, both systemic and contact pesticides can be used to control mealybugs. Systemic pesticides are applied in various ways, including soil drenches, stem treatments, and full-cover sprays (Daane et al., 2004). Broad spectrum contact insecticides having highly adverse impacts on natural enemies and non-target species are generally authorized for full cover sprays throughout the growing season. To tackle overwintering mealybugs, contact insecticides are typically sprayed during dormancy, when the impact on non-target organisms is minimal (Daane et al., 2007).

2.2 Endophytic entomopathogenic fungus

Endophytic fungi are microorganisms that reside and thrive inside plant tissues above or below ground without creating disease symptoms (Bacon and White, 2000; Zabalgogeazcoa, 2008; Kusari et al., 2012). Both species are in a symbiotic relationship which means that they are not harmed during the relationship, but they do gain from one another (Millet et al., 2010; Zuccaro et al., 2011). Fungi and bacteria comprise most of the documented endophytes, at least one endophyte associated with every plant species (Arnold, 2007). Plants colonized by endophytic fungi produce several noxious alkaloids that can benefit the host by enhancing their tolerance to biotic and abiotic stresses (Schardl et al., 2004). Additionally, they may increase plant productivity, improve soil health, and improve plant nutrient uptake (Mei and Flinn, 2010).

Endophytic fungi can produce secondary metabolites that are used as biocontrol agents, immunosuppressive agents, and for other applications (Tan and Zou, 2001). Furthermore, endophytes fungi produce a variety of substances that can be employed in industries such as medicine, agriculture, and biotechnology (Strobel and Daisy, 2003). The interaction between fungi and plants may have a positive impact on plant productivity (Lugtenberg et al., 2002; Kamilova et al., 2005; Yadav et al., 2011).

Metarhizium anisopliae (Metschn.) Sorokn, *B. bassiana* (Bals.-criv.) Vuill, and other entomopathogenic fungal species are commonly utilized in the biological management of

agricultural pests (Azevedo et al., 2000). *B. bassiana* is an entomopathogenic fungus that infects a variety of insects and has the ability to colonize plants endophytically, making it a potential insect pest control agent (González-Mas et al., 2021). It infects the host insect regularly by passing through the cuticle (Fernanderz et al, 2001). The fungi invade the root systems of plants, increasing water and nutrient intake while the plant provides carbohydrates produced by photosynthesis to the fungus (Cook et al., 2016). Conidia then firmly adhere to the host cuticles, presumably by non-specific adhesion mechanisms assisted by the hydrophobic nature of the conidial cell wall (Boucias and Latgé, 1998). The propagule's final germination occurs after it makes contact with the host cuticle and germination structures give rise to penetration hypha. Several factors influence fungal penetration, including moisture levels and the presence of inhibitory substances within the cuticle (Inglis et al., 2001). Beauvercin, bassianin, bassianolide, beauverolides, oosporein, and tenellin are among the toxic compounds produced by *B. bassiana* and these compounds play a crucial role in the control of insects (Vey et al., 2001).

2.3 Water deficiency

One of the most important abiotic factors that affect plant development, photosynthesis, and biochemical characteristics is water deficiency (Flexas et al., 2004; Zobayed et al., 2007). Furthermore, water deficiency has been identified as one of the variables that causes the production of secondary metabolites in plants as a strategy to boost their resistance (de Matos Nunes, 2014; Niinemets, 2015). According to Winkel-Shirley (2001), water stress generates oxidative stress, which increases the number of phenolics and flavonoids that are subsequently used to protect plants throughout the period of water stress.

Plant growth, yield, and height are reduced/affected by water deficiency, which is caused by a reduction in soil moisture, affecting the availability of nutrients in the soil (Razmjoo et al., 2008). Water deficiency can cause physiological problems such as reduced photosynthesis and transpiration; plants adapt to these problems to limit water loss through transpiration (Ashraf and Iram, 2005; Azhar et al., 2011). Interestingly, because plants infected with endophytic fungi enhance production of alkaloids with potential beneficial effects in relation to plant resilience to biotic and abiotic stress factors, simultaneous exposure of seedling of *A. cepa* to *B. bassiana* spores and water stress could have an additive or antagonistic effects and a synergistic effect on secondary metabolite contents, and consequently, improve anti-insect activities of *A. cepa*.

2.4 Insecticidal activities of *Allium cepa* (Onion)

Allium cepa biennial or perennial belongs to Amaryllidaceae family (WHO, 1999). Allium plants such as onions, garlic and leeks are well-known for their medicinal properties, and they have exhibited potent pharmacological and nutritional activities (Yan et al., 2022). Furthermore, they are rich in organosulfur including allicin and phenolic chemicals such as gallic acid, quercetin, coumaric acid, and ferulic acid (Kucekova et al., 2011).

Synthetic insecticides have been used to control insects for many years because of their efficiency, but they are not environmentally friendly and pose serious hazards to humans (Uphoff and Dazzo, 2016). Hence, plants, which are thought to be a rich source of bioactive compounds, may provide viable alternatives for currently utilized insect pest control agents (Damalas, 2011). Plant extracts have also been reported to have minor negative effects on non-targeted organisms (Shaalan et al., 2005). Secondary metabolites from plants have been shown to influence insect growth and behavior by acting as anti-feedants, poisons, and insect growth regulators (Morimoto et al., 2006). A range of phytochemicals have been found in the bulbs of *A. cepa*, the bulk of which are hydrocarbons and their derivatives (Dini et al., 2008). Among them are allicin, diethyl sulfide (which has insecticidal properties), dimethyl disulphide (used as a gas odorant and in chemical synthesis), dipropyl disulphide, and other phytochemicals.

Combination of abiotic and biotic factors in influencing secondary metabolites has not been sufficiently studied. Previous studies have investigated the effects of abiotic and biotic factors on plants separately; however, this is the first study that focuses on combined effects of abiotic and biotic factors on plant secondary metabolites. This study is important because the results obtained will initiate interest in exploring new ways to enhance production of secondary metabolites on plants using abiotic and biotic factors simultaneously.

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3. CHAPTER THREE

Interactive effects of water deficiency and endophytic *Beauveria bassiana* on plant growth, nutrient uptake, secondary metabolite contents, and antioxidant activity of *Allium cepa* L.

Abstract

The main aim of this research study was to assess the interactive effects of water deficiency and the inoculation of a growth medium with *Beauveria bassiana* on plant growth, nutrient uptake, secondary metabolite contents, and antioxidant capacity of *Allium cepa*. *Allium cepa* seedlings were simultaneously exposed to one of three watering regime treatments (3-day, 5-day, and 7-day watering intervals) and *B. bassiana* or no-fungus treatment. While the longest watering interval induced reduced plant growth, plants inoculated with *B. bassiana* had better results than those in the no-fungus treatment. Significant interactive effects ($DF = 2.0$; $p < 0.05$) between fungus and the watering regime on P, K, and Fe contents were observed. Remarkably, at the 7-day watering interval, the polyphenol content (64.0 mg GAE/L) was significantly higher in the plants treated with *B. bassiana* than in the no-fungus-treated plants. The watering interval significantly affected ($DF = 2, 6$; $F = 7.4$; $p < 0.05$) total flavonol contents among the fungus-treated plants. The interaction of the watering interval and *B. bassiana* inoculation ($DF = 2.0$; $F = 3.8$; $p < 0.05$) significantly influenced the flavonol content in the onion bulbs and the antioxidant activities of onion bulbs in the FRAP assay ($DF = 2.0$; $F = 4.1$; $p < 0.05$).

Keywords: antioxidant activities; *Allium cepa*; *B. bassiana*; water deficit; endophytic fungus; polyphenol and flavonol.

3.1 Introduction

The rapidly growing demand for medicinal and nutraceutical plants is driving the search for more efficient strategies to cultivate medicinal and nutraceutical plants. Onion is one of the most consumed vegetables in the world (Pandey et al., 2011). However, because of increasing drought episodes worldwide and the high-water demand for onions (Gedam et al., 2021), it is necessary to develop efficient cultivation strategies that minimize water usage and optimize the plant's

nutraceutical qualities. According to Khokhar (2017), onions are more sensitive to water stress than other agronomic crops. Soil cultivation is still the most popular approach for cultivating onions. However, soil cultivation has many challenges, including exposure to pests, high pesticides, and fertilizers (Chen et al., 2016). Consequently, researchers are looking for other alternative cultivation methods that are more environmentally friendly and cost-effective. Hydroponics is a feasible, alternative approach for cultivating crops (Giurgiu et al., 2014). The biotic and abiotic factors can be manipulated in hydroponics to optimize plants' secondary metabolite production and bioactivities (Vu et al., 2006). Plants' secondary metabolites not only play vital ecological roles in plants' defense, protection, and signaling mechanisms (Griesser et al., 2015), but are also exploited for their pharmaceutical attributes. To achieve the optimum cultivation of plants, it is essential to understand how plants respond to abiotic and biotic environmental changes for the optimal biosynthesis of medicinal and nutraceutical bioactive compounds.

Water stress is one of the most prominent abiotic environmental factors influencing plant growth and secondary metabolite contents (Xu et al., 2010; Roos and Nchu 2021). It is a well-known limiting factor that affects various elements in plant growth and development (Sourour et al., 2017). Water stress can cause plant dehydration, stomatal closure, reduced gas exchanges, impairment of photosynthesis, and, ultimately, plant death (Verslues et al., 2006). However, plant survival varies with the plant species, growth stage, and the duration and severity of water deficiency (Zschocke et al., 2000). Water shortage also decreases the total soil nutrient accessibility and root nutrient distribution (Kheradmand et al., 2014). However, water stress can also influence secondary metabolite contents in plants (Isah, 2019). Indeed, many studies have revealed that water stress can induce higher concentrations of bioactive compounds in plants (Toscano et al., 2019; Roos and Nchu, 2021). Results from a study on *Chrysanthemum morifolium* L. cultivars by Hodaei et al. (2018) showed that the contents of six phenolic compounds such as chlorogenic acid, rutin, ferulic acid, quercetin, apigenin, and luteolin increased with increasing water stress and cultivar and flavonoid gene–environment interactions influenced these responses.

The diverse roles of fungal entomopathogens, ubiquitous within the soil and susceptible insects, in host plant growth promotion and pest and pathogen management have sparked a great deal of interest in the application of fungal endophytes in crop cultivation (Grabka et al., 2022). The

endophytic fungus–plant symbiotic relationship benefits the host plant and fungus (Heviefo et al., 2017; Ávila-Hernández et al., 2020). Some entomopathogenic fungi, such as *B. bassiana* and *Clonostachys rosea* (Hypocreales), can infect insects and colonize plant tissues as symptomless endophytes (Dannon et al., 2020). These fungi can live within plant tissues and proliferate under stressful environmental conditions, including drought (WHO, 1999). Entomopathogenic fungi have been shown to produce bioactive compounds that promote plant resistance to pests and diseases and increase the rate of nutrient uptake, tolerance to abiotic and biotic stresses, and overall plant growth (Heviefo et al., 2017). For example, in a recent study, entomopathogenic fungus *B. bassiana* optimized the yield and secondary metabolite contents of *A. cepa* plants in a hydroponic system (Ávila-Hernández et al. 2020). Many studies have demonstrated that *B. bassiana* strains can potentially be employed to optimize the commercial production of crops (Dannon et al., 2020). The current study focuses on the combined effects of water stress and endophytic fungus colonization on the plant growth, secondary metabolite production, and antioxidant activities of *A. cepa*, a member of the Allioideae subfamily and Amaryllidaceae family (WHO, 1999). While many studies have separately investigated the effects of water, light or nutrient stress and fungal endophytes on plant growth parameters and secondary metabolites, few studies have investigated the combined effects of abiotic and biotic factors in optimizing the medicinal or nutraceutical properties of plants. The Allium genus comprises over 850 species, making it one of the largest monocot genera worldwide (Peruzzi et al., 2017). *Allium* species, such as onions, garlic, and leeks, are known for their medicinal and nutraceutical properties and have demonstrated substantial pharmaceutical and nutritional activities (Sharifi-Rad et al., 2016). *Allium* spp. extracts contain several bioactive constituents, including phenolic compounds, organosulfur compounds, non-structural and soluble carbohydrates, organic acids, and various amino acids (Slimestad et al., 2007). Phenolic compounds, including gallic acid, quercetin, coumaric acid, and ferulic acid, are found in *Allium* spp. (Kucekova et al., 2011; Zeng et al., 2013). Onions are rich sources of flavonoids and organosulfur compounds (allicin), both of which are potent antioxidants (Nuutila, 2003). Hence, this investigation primarily aimed to determine the effects of combined water deficiency and the inoculation of growth medium with *B. bassiana* on plant growth, nutrient uptake, and secondary metabolite contents, as well as the antioxidant capacity of *A. cepa*.

3. 2. Materials and Methods

3.2.1. Plant Material

A total of 180 onion seedlings (Red Creole cultivar) sourced from a local retail nursery, Hart Nursery (PTY) Ltd. Ottery, Western Cape Province, South Africa, were selected for this experiment (Figure 3.1).

Figure 3.1: *Allium cepa* seedlings.

3.2.2. Preparation of fungus (*B. bassiana*)

The fungal cultures of a *B. bassiana* strain (SM 3), which is currently being maintained at the Horticultural Department, Cape Peninsula University of Technology, were used in this study. The isolate was initially isolated from a local vineyard and was identified molecularly by Moloinyane and Nchu (2019). The fungus was cultured on a medium containing half strength Potato Dextrose Agar (PDA) (19.5 g/1000 mL of water), 0.04 g streptomycin, and 0.02 g ampicillin sodium salt (Sigma-Aldrich (PTY) Ltd., Johannesburg, South Africa). The prepared medium was then

transferred into Petri dishes with 9 and 14 cm diameters. Fungal cultures were incubated in the dark for three weeks at 25 °C using a sterile spatula; the mature conidia of *B. bassiana* (Figure 3.2) were scraped and transferred into a 50 mL centrifuge tube containing 30 mL of sterile distilled water (0.05% of Tween 80) and glass beads. The conidial suspension was vigorously shaken for three minutes, and after that, it was vortexed for another three minutes again using a vortex mixer at 3000 rpm to homogenize the conidial suspension. The homogeneous conidial suspension was transferred into 1000 mL bottles. The required conidia concentration of 1×10^6 conidia mL^{-1} was determined using a hemocytometer. Sterile distilled water or conidial suspension was added when necessary to achieve the desired conidial concentration. A volume of 500 mL of conidial suspension was prepared in each 1000 mL bottle.

Figure 3.2 *B. bassiana* cultured on PDA.

3.2.3. Experimental Setup

The experiment was conducted in the greenhouse of the Department of Horticultural Sciences, Cape Peninsula University of Technology, Bellville Campus. The experiment ran for ten weeks

from spring to early summer (October–December) at 28.9–36.9 ℃ and 51–59% RH. A completely randomized design was used to test the main effects of two factors (*B. bassiana* inoculation and watering regime) consisting of two *B. bassiana* treatments and three watering regimes on *A. cepa* plants. The effects of the interactions of the two factors on the plant growth, tissue nutrients and secondary metabolite contents, and antioxidant activity of the bulb extracts of onions were also assessed.

An inert substrate mix containing different substrates (silica sand, vermiculite, perlite, and coco peat) was mixed in a 1:1:1:1 ratio. Two-week-old seedlings were transplanted into the potted substrate mix individually in 15 cm diameter pots after rinsing under running tap water to remove all the potting soil around the roots. The seedlings were divided into two groups. One group was inoculated with *B. bassiana*; the second was not. Plants in both groups received water stress treatments, i.e., no watering for 3, 5, or 7 days. Thirty potted plants were randomly allocated and treated to either *B. bassiana* or no *B. bassiana* inoculation and one of three watering regimes (3-day, 5-day, and 7-day watering intervals) (Figure 3.3). The watering interval was not extended beyond seven days because a pre-experimental investigation revealed that the plants wilted after seven days. In the fungus treatment, plant roots were inoculated with *B. bassiana* inoculum by drenching the substrate with 100 mL conidial suspension (1×10^6 conidia mL^{-1}) immediately after transplanting. *A. cepa* plants from each treatment were fed using hydroponics Nutrifeed fertilizer (Starke Ayres (PTY) Ltd., Cape Town, South Africa) consisting of 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 deionized water at a dosage of 10 g/5 L. Each plant received 100 mL of the nutritional solution fortnightly.

Figure 3.3 The experimental setup with treatments.

3.2.4. Plant Growth Parameters

After ten weeks post-treatment, i.e., ten weeks after the commencement of the experiment, the leaf length from the base of the leaf to the highest point was recorded using a 30 cm ruler for each replicate in the control and test treatments. *A. cepa* plants were uprooted, the bulb circumference was measured using a string (cm), and the bulbs' fresh weights were recorded. Subsamples of 15 randomly selected *A. cepa* bulbs from the different treatments were then chopped, transferred into a paper bag, and oven-dried at 35 ℃ for two weeks. After that, the dried materials from each plant were weighed to obtain the bulb dry weight (g).

3.2.5. Colonization of Tissues by Fungus

Four weeks after exposure of the experimental plants to the various treatments (watering regimes and *B. bassiana*), four leaf sections (1 × 1 cm^2), carefully excised from four randomly selected plants in each treatment, were rinsed with sterile distilled water. They were then sterilized with 70% ethanol for 10 s and again rinsed with sterilized distilled water for 60s. After that, they were placed on a Potato Dextrose Agar plate (PDA) containing half-strength Potato Dextrose Agar (PDA) (19.5 g/1000 mL), 0.04 g streptomycin, and 0.02 g ampicillin sodium salt. They were then incubated for 14 days in the dark at 25 ℃ (Figure 3.4).

Figure 3.4 Re-isolated *B. bassiana* from *A. cepa* leaves.

3.2.6. Plant Tissue Nutrient Analyses

Bulbs of randomly selected plants from each treatment were analyzed for macro-element and micro-element contents using an inductively coupled mass spectrometry (ICP-MS) and a LECO-nitrogen analyzer with appropriate standards (Bemlab (PTY) Ltd., Somerset West, South Africa). Each treatment had three replicates. Briefly, the bulbs were cleaned in a Teepol solution, rinsed with deionized water, and dried in an oven at 70 ℃ overnight. Thereafter, the dried bulbs were pulverized, ashed at 480 ℃, and agitated in a 50:50 HCl (50%) solution for extraction through filter paper (Campbell et al. 1998), and then used to determine the potassium (K), phosphorus (P), calcium (Ca), magnesium (Mg), sodium (Na), manganese (Mn), iron (Fe), copper (Cu), zinc (Z), and boron (B) content of the extracts. Total combustion in a Leco N-analyser was used to assess the bulbs' total nitrogen (N) contents. N, P, K, Ca, and Mg values were converted from percentages to mg/kg using a conversion factor of 10,000 (Xego et al., 2017).

3.2.7. Phytochemical Screening

After ten weeks in the glasshouse, three bulbs from each of the six treatments were randomly chosen and oven-dried for two weeks at 32 ℃. A Jankel and Kunkel Model A 10 mill was used

to grind each dry sample into a fine powder. The powdered material was labeled and packaged in sealable plastic bags.

3.2.7.1. Total Alkaloid Contents

The total alkaloids in plant extracts were determined using the method given by Fadhil and Reza (2007). A quantity of 0.1 g of powdered bulb plant material was mixed with 20 mL of 60% ethanol and 40% distilled water in a centrifuge and left in the dark for 24 h (Figure 3.5). The sample's absorbance at 417 nm was measured, and the concentration of mg atropine equivalent per g dry weight (mg AE/g DW) was estimated using an atropine 45 standard curve.

3.2.7.2. Total Flavonoid Contents

The total flavonoid contents were determined using quercetin as a reference for 0, 5, 10, 20, 40, and 80 mg/L in 95% ethanol (Sigma-Aldrich (PTY) Ltd., Johannesburg, South Africa), as described by Daniels et al. (2011). The crude extract solution was made by combining 12.5 L of 0.1% Hydrochloric acid (HCl) (Merck (PTY) Ltd., Cape Town, South Africa) in 95% ethanol in the sample wells, then incubating at room temperature for 30 min. In the ethanol extracts, total flavonoid concentrations were reported as mg quercetin equivalent per gram dry weight (mg QE/g DW).

3.2.7.3. Total Phenolic Contents

The total phenolic contents were determined using the Folin–Ciocalteau assay, as described by Singleton et al. (1999). Twenty-five grams of the material was mixed with 125 L of Folin–Ciocalteau reagent (1:10 dilution with distilled water) in a 96-well microplate (Merck (PTY) Ltd., Cape Town, South Africa). After 5 min, 100 L of the 7.5% Na_2CO_3 solution was added to each well's mixture. The total phenolic contents of *A. cepa* dry bulb were measured in milligrams of gallic acid equivalents (GAE) per 100 g dry mass (mg GAE/100 g DW). All analyses were carried out in duplicates.

3.2.8. Antioxidant Assays

3.2.8.1. Ferric Reducing Antioxidant Power (FRAP) Assay

Benzie and Strain (1996) describe a Ferric Reducing Antioxidant Power test comparable to the one employed here. The ferric tripyridyltriazine complex is reduced to its ferrous state in the presence of antioxidants in this test. The compounds used were as follows: 2.5 mL of a 10 mmol/L TPTZ (2, 4, 6-tripyridyl-s-triazine, Sigma) solution in 40 mmol/L HCl + 2.5 mL of 20

mmol/L $FeCl_3$ and 25 mL of 0.3 mol/L acetate buffer, kept at pH 3.6 and heated at 37 ℃. Forty microliters of the sample supernatant were divided into aliquots and mixed with 0.2 mL distilled water and 1.8 mL FRAP reagent. After a 10 min incubation period at 37 ℃, the absorbance of the reaction mixture was measured at 593 nm using a spectrophotometric technique. The standard solution was 1 mmol/L $FeSO_4$, and the result was represented as the concentration of antioxidants with a ferric reducing capacity of 1 mmol/L $FeSO_4$.

3.2.8.2. Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The antioxidant content in onion was measured using the TEAC method described by Miller et al. (1993). The antioxidant's ability to scavenge the blue green coloured ABTS•+ radical cation was compared to the water-soluble antioxidants' ability to scavenge the blue green coloured ABTS•+ radical cation.

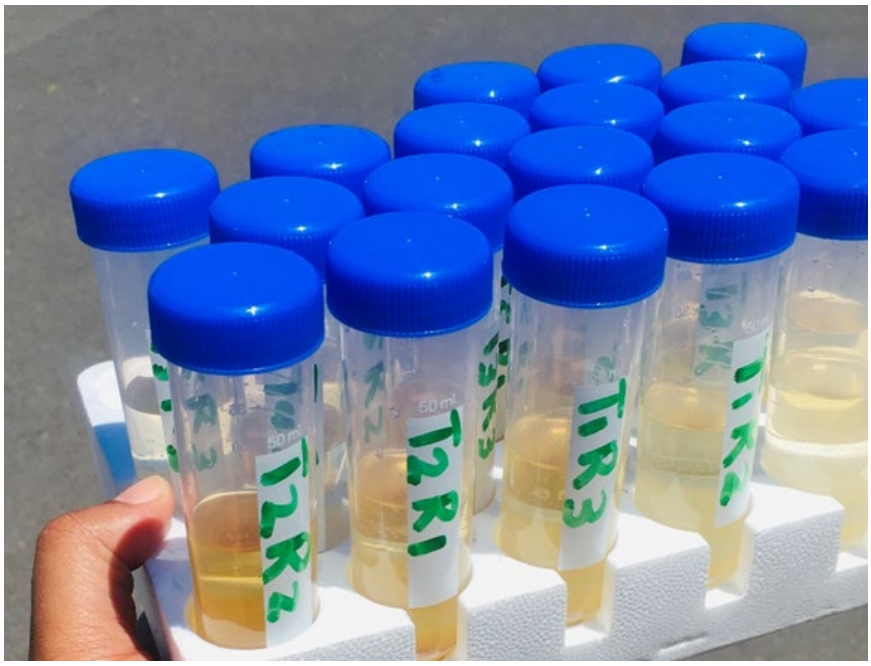

Figure 3.5 Centrifuge tubes containing mixture of powdered bulb plant material and 20 mL of solvent made up of 60% ethanol and 40% distilled water.

3.2.9. Data Analysis

The experimental data (leaf height, fresh bulb weight, bulb circumference, dry bulb weight, and tissue nutrient contents) are presented as mean ± SE in tables. The data were analyzed using one-way and two-way analyses of variance (ANOVA,) and the post-hoc Tukey HSD test was used to

separate the means at a level of significance, $p < 0.05$. These computations were performed using STATISTICA software (TIBCO 1984–2018).

3.3 Results

3.3.1. Colonization of Leaf Tissue

B. bassiana was successfully re-isolated from the leaves of the *A. cepa* plants. All the fungus-treated plants recorded mycelial outgrowth on their leaf sections, representing 100% fungal colonization. No fungal outgrowth occurred in the no-fungus plants.

3.3.2. Plant Growth Parameters

3.3.2.1. Leaf Length

At ten weeks post-treatment, fungus-treated plants had significantly ($DF = 1, 58$; $p < 0.01$) longer leaves than the no-fungus plants; this was the same for both plants exposed to the 3-day and 5-day watering intervals. However, there was no significant difference ($DF = 1, 58$; $F = 1.5$; $p > 0.05$) in the leaf length of *A. cepa* plants exposed to the 7-day watering regime between the fungus-treated plants and the no-fungus plants. Generally, longer leaves were associated with a shorter watering interval and *B. bassiana* inoculation. The interaction between the fungus and watering regime on leaf height of *A. cepa* was significant ($DF = 2$; $F = 22.3$; $p < 0.05$).

3.3.2.2. Bulb Circumference

There was no significant difference ($DF = 1, 30$; $F = 2.2$; $p = 0.1$) in the bulb circumference of the fungus and no-fungus plants exposed to low water stress (3-day watering interval) ten weeks post-treatment. However, the fungus treatment produced larger bulbs (8.8 ± 0.2 cm) than those with no fungus (8.2 ± 0.4 cm). Although the bulb circumferences varied significantly ($DF = 1, 30$; $p < 0.05$) between the fungus and the no-fungus treatments at the 5-day and 7-day watering intervals, there were no significant interactive effects ($DF = 2.0$; $F = 2.6$; $p > 0.05$) on fungus and watering regime.

3.3.2.3. Bulb Wet Weight

The wet weights of the onion bulbs varied significantly ($DF = 1, 30$; $p < 0.05$) between fungus and no-fungus treatments at the different watering regime treatments. Fungus treated plants watered at 3-day intervals had the highest mean (13.4 ± 0.7 g) bulb wet weight. There was a significant interactive effect ($DF = 2.0$; $F = 22.3$; $p < 0.05$) between fungus and watering regimes.

3.3.2.4. Bulb Dry Weight

At ten weeks post-treatment, there was no marked significant difference (DF = 1, 28; F = 3.4, $p > 0.05$) in the bulb dry weights of *A. cepa* plants exposed to the 3-day watering regime in the fungus and no-fungus treatments, ten weeks post-treatment (Table 3.1). There was a significant difference (DF = 1, 28; $p < 0.05$) in the bulb dry weight of *A. cepa* plants exposed to 5-day and 7-day watering regimes at ten weeks post-treatment (Table 1), with the fungus-treated plants having heavier bulbs than the no-fungus plants. Based on a two-way ANOVA, the interactive effect of watering regime and fungus (*B. bassiana* inoculum) on bulb dry weight was not significant (DF = 2.0; F = 0.9; $p > 0.05$).

Table 3.1 Effects of the watering regimes and endophytic *B. bassiana* on plant growth parameters (Mean±SE) of *A. cepa*.

Watering interval	Leaf length (cm)		Bulb circumference (cm)		Bulb wet weight (g)		Bulb dry weight (g)	
	Fungus	No fungus	Fungus	No fungus	Fungus	No fungus	Fungus	No fungus
3-day	60.75±0.36a(A)	44.41±0.96b(A)	8.83±0.24a(A)	8.17±0.36a(A)	13.39±0.68a(A)	10.38±0.76b(A)	2.27 ± 0.12a(A)	1.87±0.18a(A)
5-day	48.29±0.83a(B)	41.81±1.77b(AB)	6.81±0.23a(B)	5.00±0.17b(B)	7.47±0.37a(B)	3.70±0.23b(B)	1.21 ± 0.07a(B)	0.68± 0.05b(B)
7-day	41.16±1.02a(C)	39.24±1.18a(B)	6.46±0.20a(B)	5.34±0.21b(B)	6.88±0.36a(B)	4.41±0.37b(B)	1.15 ± 0.05a(B)	0.82±0.08b(B)
Two-way ANOVA	DF= 2.00; F= 22.34; p <0.05		DF=2.00; F=2.61; p >0.05		DF=2.00; F= 22.34; p < 0.05		DF=2.00; F=0.18; p> 0.05	

*Means followed by the same lowercase letters in the same row are not significantly different following Tukey's test (P > 0.05). Means followed by the same uppercase letters in the same column are not significantly different following Tukey's test (P > 0.05). DF: degree of freedom; F: F value.

3.3.3. Bulb Tissue Analysis

3.3.3.1. Macronutrients

There was no significant difference in the *A. cepa* plant tissue levels of C, N, P, K, Ca, and Mg on plants exposed to the 3-day watering regime ($DF = 1, 4$; $p > 0.05$), as shown in Table 3.2. This was the same for the 7-day watering regime, except for the mean Ca level in the fungus-treated plants, which was significantly higher compared with the no-fungus treatment ($DF = 1, 4$; $F = 11.0$; $p < 0.05$). Interestingly, there was a significant difference ($DF = 1, 4$; $p < 0.05$) in the bulb tissue levels of C, P, K, Ca, and Mg ($DF = 1, 4$; $p < 0.05$) in plants exposed to 5-day watering regimes, and higher concentrations of C, P, Ca, and Mg occurred in the fungus-treated plants. However, the N levels in the bulb tissue were not significantly different ($DF = 1, 4$; $F = 0.0$; $p > 0.05$) between plants in the fungus and no-fungus treatment at the different watering regimes. Significant interactive effects ($DF = 2.0$; $p < 0.05$) between fungus and watering regimes on P and K were observed.

3.3.3.2. Micronutrients

After ten weeks post-treatment, there was no significant difference between the fungus treated and no-fungus plants in Na, Fe, and Cu levels at the 3-day watering regime ($DF = 1, 4$; $p > 0.05$); however, the plant tissue levels of Mn and Zn in the bulbs varied significantly ($DF = 1, 6$; $p < 0.05$) with watering regimes among the fungus and no-fungus plants (Table 3.3). On the 3-day and 5-day watering regimes, the tissue nutrient uptake between the fungus-treated and no-fungus plants was significantly different only in the Zn levels ($DF = 1, 4$; $F = 8.3$, $p < 0.05$). The 7-day watering regime did not significantly differ in the tissues Na, Mn, Fe, Cu, and Zn ($DF = 1, 4$; $p > 0.05$) (Table 3). The interactive effect between fungus and watering regime on tissue Fe level was significant ($DF = 2.0$; $p < 0.05$).

Table 3.2 Effects of water deficiency and the inoculation of growth medium with *B. bassiana* on the macronutrient content in bulbs of *A. cepa*.

	C (mg/kg)		N (mg/kg)		P (mg/kg)		K (mg/kg)		Ca (mg/kg)		Mg (mg/kg)	
Watering interval	Fungus	No fungus	Fungus	No fungus	Fungus	No fungus	Fungus	No fungus	Fungus	No fungus	Fungus	No fungus
3-day	473350±5686.90a(A)	382416.67±7782.68a(A)	28900±866.02a(A)	31100±288.67a(A)	450±28.87a(B)	400±0.00a(B)	13450±28.87a(A)	9350±1587.71a(B)	11800±288.67a(B)	9000±1154.70a(A)	4300±173.20a(A)	3750±721.69a(A)
5-day	461350±2280.53a(A)	453950±663.95b(A)	27250±433.01a(A)	27450±1010.36a(B)	700±0.00a(A)	400±0.00b(B)	14250±490.75a(A)	10850±606.22b(AB)	14200±519.61a(A)	11100±288.67b(A)	5550±86.60a(A)	4150±144.34b(A)
7-day	459050±1760.92a(A)	472850±17176.17a(A)	26750±1587.71a(A)	26050±202.07a(B)	550±28.87a(B)	516.67±44.09a(A)	13100±115.47a(A)	16000±1674.31a(A)	11800±519.61a(B)	10050±86.60b(A)	4700±519.61a(A)	4150±86.60a(A)
Two-way ANOVA	DF=2.00; F=1.44; p > 0.05		DF=2.00; F=1.35; p>0.05		DF=2.00; F=37.50; p< 0.05		DF=2.00; F=7.50; p<0.05		DF=2.00; F=0.74; p>0.05		DF=2.00; F=0.84; p>0.05	

*Means followed by the same lowercase letters in the same row are not significantly different following Tukey's test (P > 0.05). Means followed by the same uppercase letters in the same column are not significantly different following Tukey's test (P > 0.05).

Table 3.3 . Effects of water deficiency and the inoculation of growth medium with *B. bassiana* on micronutrient content in leaves of *A. cepa*.

	Na (mg/kg)		Mn (mg/kg)		Fe (mg/kg)		Cu (mg/kg)		Zn (mg/kg)	
Watering interval	Fungus	No fungus	Fungus	No fungus	Fungus	No fungus	Fungus	No fungus	Fungus	No fungus
3-day	688. 5± 32.04a(A)	665.5 ± 101.32a(A)	32.75 ± 0.26a(A)	25.8 ± 1.20b(A)	251.5±21.65a(A)	352±101.61a(A)	3.0 ±0.23a(A)	3.5±0.40a(A)	6.3±0.63b(A)	8.13 ±0.09a(B)
5-day	1031±120.67a(A)	1286.67±330.97a(A)	45.8±1.62a(A)	43.5±3.41a(B)	321±74.48a(A)	122.05±35.19a(A)	3.0 ± 0.11a(A)	2.4±0.35a(A)	10.2±1.62b(A)	19.9±2.94a(A)
7-day	941.5±103.06a(A)	1076±112.00a(A)	38.85±8.40a(A)	43.3±0.52a(B)	407.5±114.03a(A)	124 ±6.93a(A)	2.75±0.144a(A)	2.93±0.32a(A)	9.3±2.14a(A)	5.13±1.18a(AB)
Two-way ANOVA	DF=2.00; F= 0.37; p> 0.05		DF=2.00; F=1.14; p>0.055		DF=2.00; F=3.98; p<0.05		DF=2.00; F=2.04; p>0.05		DF=2.00; F=2.63; p>0.05	

*Means followed by the same lowercase letters in the same row are not significantly different following Tukey's test (P > 0.05). Means followed by the same uppercase letters in the same column are not significantly different following Tukey's test (P > 0.05).

3.3.4. Phytochemical Contents

Alkaloids were not detected in this experiment in all the watering regimes and fungus treatments. There was no significant difference in the total polyphenol contents of plants at all the watering intervals (DF = 1, 4; $p > 0.05$) when the fungus and no-fungus treatments were compared (Table 3.4). Remarkably, at the 7-day watering interval, the polyphenol content (64.0 mg GAE/L) was significantly higher in plants treated with *B. bassiana* than in the no-fungus plants. Overall, however, the interaction of watering interval and fungus did not significantly (DF = 2.0; F = 3.4; $p > 0.05$) influence polyphenol contents in the onion bulbs based on a two-way ANOVA.

Generally, there was no significant difference in flavonol contents (DF = 1, 4; $p > 0.05$) between the plants inoculated with the fungus and those with no fungus at all three watering regimes (3-day, 5-day, and 7-day). However, the watering interval significantly affected (DF = 2, 6; F = 7.4; $p < 0.05$) total flavonol contents among the fungus-treated plants; the flavonol contents increased with the length of the watering interval in the fungus-treated plants. Noticeably, *B. bassiana* inoculation favoured a significantly higher accumulation of polyphenols in the onion bulbs at the 7-day watering interval compared to the no-fungus treatment (DF = 1, 4; $p < 0.05$); this is a reversal of the results obtained in the 3-day and 5-day treatments. The interaction of watering interval and fungus (DF = 2.0; F = 3.8; $p < 0.05$) significantly influenced flavonol contents in the onion bulbs, based on a two-way analysis of variance.

Table 3.4 The effects of *B. bassiana* inoculation and watering intervals on the secondary metabolite contents of *A. cepa*.

	Secondary metabolites			
	Total Polyphenol (mg GAE/L)		Total Flavonol (mg QE/L)	
Watering interval	Fungus	No fungus	Fungus	No fungus
3-day	27.45±6.34a(A)	30.05±3.19a(A)	7.38±0.79a(B)	10.67±1.35a(A)
5-day	42.20±12.67a(A)	45.96±2.34a(A)	11.39±3.87a(AB)	13.78±1.99a(A)
7-day	63.96±6.41a(A)	35.72±5.65b(A)	25.27±4.49a(A)	14.61±2.58a(A)
Two-way ANOVA	DF = 2.00; F=3.41; p>0.05		DF=2.00; F= 3.78; p< 0.05	

*Means followed by the same lowercase letters in the same row are not significantly different following Tukey's test (P > 0.05). Means followed by the same uppercase letters in the same column are not significantly different following Tukey's test (P > 0.05).

3.3.5. Antioxidant Activity

Interestingly, there were significant differences among plants exposed to the 7-day watering regime (most extended watering interval) (DF = 1, 4; F = 11.8; $p < 0.05$) in the FRAP and TEAC bioassays (Table 3.5), with the *B. bassiana*-treated plants yielding consistently higher antioxidant activity. Based on a two-way ANOVA, the interaction between water intervals and inoculation with *B. bassiana* significantly (DF = 2.0; $p < 0.05$) influenced the antioxidant activities of onion bulbs in the FRAP and TEAC bioassays.

Table 3.5 The effects of *B. bassiana* inoculation and watering intervals on the antioxidant capacity of *A. cepa.*

	FRAP (umol AAE/L)		TEAC (umol TE/L)	
Watering interval	Fungus	No fungus	Fungus	No Fungus
3-day	283.05±30.40a(A)	291.73±13.77a(A)	142.88±15.38a(B)	170.46±7.78a(B)
5-day	343.88±71.09a(A)	333.44±17.44a(A)	195.82±29.86a(AB)	220.76±14.21a(A)
7-day	492.17±54.51a(A)	289.81±22.04b(A)	273.47±14.95a(A)	171.63±8.83b(B)
Two-way ANOVA	DF = 2.00; F=4.11; p<0.05		DF = 2.00; F=9.70; p< 0.05	

*Means followed by the same lowercase letters in the same row indicate no significant difference between fungus and no fungus treatment following Tukey's test (P > 0.05). Means followed by the same uppercase letters in the same column are not significantly different following Tukey's test (P > 0.05).

3.4 Discussion

Water is essential for plant growth and survival. Depleting and disappearing freshwater resources, driven mainly by over-exploitation, pollution, and land-use changes, have led to a drastic scarcity of agricultural water. The fresh-water shortage, therefore, constitutes a danger to agriculture's long-term viability (Razmjoo et al., 2008; Xu et al., 2010; Ings et al., 2013). Water deficiency stress is an abiotic factor that impacts plant growth and is one of the most growth-limiting factors (Tátrai et al., 2016). In the current study, the results showed that exposing onions to a short watering interval and *B. bassiana* inoculation enhanced plant growth, whereas increasing the watering intervals from 3-days to 7-days consistently improved secondary metabolite contents and antioxidant activities among plants exposed to *B. bassiana* inoculum. However, the interactive effects of endophytic *B. bassiana* and watering regime on growth parameters, secondary metabolite contents, and antioxidant activities varied.

Water in a plant growth medium dissolves critical minerals and nutrients that are absorbed through the roots of plants. Hence, the higher growth obtained among plants in the 3-day watering interval than in the 5-day and 7-day intervals is expected. Previously, McDowell et al. (2011) and Breda et al. (2006) reported that water stress is a hindrance to plant growth and development because it restricts access to the resources needed for photosynthesis owing to stomatal closure and reduced internal water. Interestingly, although the growth results showed reductions in all growth parameters assessed, the reductions in growth were much more pronounced among plants that were not inoculated with *B. bassiana*. Prior studies reported that fungi that live as endophytes in host plants have plant growth-promoting properties (Lopez and Sword, 2015; Jaber and Enkerli, 2016). Numerous studies have shown that when used as an endophyte in agricultural plants, entomopathogenic fungi can enhance plant height, weight, and other growth indices (Sasan and Bidochka, 2012; Jaber and Enkerli, 2017). For example, endophytic *B. bassiana* and *Purpureocillium lilacinum* enhanced the growth and dry biomass of cotton plants (Jaber and Araj, 2018). Another mechanism through which endophytic fungi alleviate environmental stress on their hosts is by increasing mineral uptake. For example, García-Latorre et al. (2021) demonstrated that Sporormiella intermedia increased the mineral uptake of Ca, Cu, Mn, Pb, Tl, and Zn in *Trifolium subterraneum* (subclover); and *Mucor hiemalis* increased the uptake of K and Sr in *Poa pratensis* (Kentucky bluegrass). In the current study, phosphorus levels in the bulbs were significantly higher in the fungus-treated plants than in the no-fungus plants at the 5-day watering interval. Moreover, *B. bassiana* inoculation and watering

intervals had a significant interactive effect on P and K levels. Although the K level in the fungus-treated plants was lower than in plants in the no-fungus treatment, at the most extended watering interval (7 days), Ca, Mg, P, and N levels were more elevated in the fungus-treated plants, corroborating the argument that endophytic fungi alleviate the negative impacts of water stress by enhancing the uptake of essential macronutrients. These nutrients are essential for disease resistance, photosynthesis, cell membrane integrity, protein synthesis, resistance to abiotic and biotic stresses, pollen production, and enhancement of antioxidant enzymes and chlorophyll levels in plant tissues (Sarkar et al., 2021).

The mechanisms by which fungal endophytes induce water and nutrient uptake by plant hosts include increased production of phytohormones that increase root biomass; improved fungal colonization of roots and hyphal interception of nutrients; enhanced secretion of hydrolytic enzymes that enhance nutrient solubilization; increased activity of plasma membrane-associated proton-pumps, and elevated expression of phosphate transporters; and enhanced siderophore binding sites that chelate Fe^{3+} ions and bind to Cu^{2+}, $Zn^{2+,}$ and Mn^{3+} (Verma et al., 2021; García-Latorre et al., 2021). Interestingly, a significant interactive effect of watering interval and *B. bassiana* inoculation on tissue Fe content was observed in this study, with the fungus-treated plants having higher Fe content at 5-day and 7-day watering intervals. The Mn and Zn levels in plants varied significantly between the fungal treatments, with the fungus-treated plants recording higher Mn than the no-fungus plants at the 3-day watering interval, while Zn content was higher in the no-fungus plants than fungus-treated plants at the 5-day watering interval. When the watering interval was increased from 3 days to 7 days, the secondary metabolite contents also increased. Remarkably, the highest total polyphenol (64.0 ± 6.4 mg GAE/L) and flavonol (25.3 ± 4.5 mg QE/L) contents were obtained in plants exposed to both a 7-day watering regime and *B. bassiana* treatments. The results showed that the flavonol level in onion bulbs was enhanced when the watering interval was extended to 7 days, and *B. bassiana* inoculation and a long watering interval also enhanced the total polyphenol contents. Subjecting plants to reduced water can increase carbon-based secondary metabolites (Mundim and Pringle, 2018).

Plants employ adaptive physiological responses to cope with the environmental stress elicitors; for example, plants may produce chemotypes such as lipoic and ascorbic acid, flavonoids (quercetin), carotenoids, arylamines, aliphatic and unsaturated fatty acids as part of their chemical defense response to stress (Isah, 2019). However, water stress can have varied effects on plants'

production of secondary metabolites; for example, artemisinin, arteannuin-B, artemisinic acid, and dihydroartemisinic acid, major constituents of *Artemisia annua* L., were negatively influenced by water deficit stress, while sesquiterpenes and other low molecular weight volatiles were positively impacted by water deficits (Yadav et al., 2014). Roos and Nchu (2021) reported that subjecting *Salvia* species to different water stress levels influenced the accumulation of secondary metabolites.

Endophytic fungi are a well-known source of bioactive secondary metabolites (Patil et al., 2016). Their presence in plants could also induce plants to produce more secondary metabolites, conferring protection against biotic and abiotic stress factors. During the symbiotic interaction between a host plant and an endophyte, both organisms could engage in horizontal gene transfer and the synthesis, accumulation, and transfer of common metabolites to their corresponding symbiotic systems (Alam et al., 2021). Endophytes, including fungal endophytes, can produce siderophores, which chelate the ferric iron from a plant growth medium and make it available for microbial and plant cells (Singh et al., 2018). The current results showed higher levels of Fe (407.5 ± 114.0 mg/kg) in the fungus-treated plants. Antioxidant activities increased most in bulbs exposed to fungus and the most extended watering interval. This finding suggests that *B. bassiana* inoculation enhances antioxidant activities, especially at the long watering interval. These results also correlate with the level of total phenolic contents. Flavonols and phenolic compounds have antioxidant activities (López-Amorós et al., 2006). In addition, the antioxidant activities were associated with the higher P, N, Mg, Ca, and Fe content in this study. These elements are essentials in plant physiological functions and the synthesis of primary metabolites. Some primary metabolites are transformed into intermediary compounds that act as precursors for synthesizing carbon-based secondary metabolites by plants (Pott et al., 2019).

3.5 Conclusion

The key findings are that endophytic *B. bassiana* inoculation not only alleviates the adverse effects caused by water deficits on plant growth but also synergistically interacts with the most extended watering interval (7-day) to enhance total polyphenol and flavonol contents as well as the antioxidant activities of onion bulbs. The benefit of *B. bassiana* inoculation in improving the synthesis of secondary metabolites and antioxidant activities becomes evident under a high-water deficit condition. Furthermore, the results show that the action of the *B. bassiana* fungus on growth and secondary metabolites is potentially mediated by the enhanced acquisition of

essential nutrients such as the P, Ca, Mg, and Fe levels. However, it is necessary to study the role of phytohormones in mediating the growth-promoting effects of the fungus under water stress conditions. In conclusion, incorporating endophytic *B. bassiana* could improve the nutraceutical value of onions and reduce yield losses in water-scarce areas. Furthermore, given that extending the watering interval and *B. bassiana* inoculation induced higher levels in onion plants, it is possible that the extracts of plants exposed to these treatments will also increase bioactivities. In the next chapter, we studied the effect exposing onion to *B. bassiana* inoculum and watering interval on volatile constituents and insect repellency activity of bulb extracts.

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4. CHAPTER FOUR

Water deficiency and endophytic *Beauveria bassiana* enhance repellent activity of *Allium cepa* L. (onion) extracts against grapevine mealybug (*Planococcus ficus*).

Abstract

This study assessed the effects of water deficiency and *Beauveria bassiana* inoculum on the volatile organic compounds and the bioactivity of *Allium cepa* (onion) extracts against grapevine mealybug. *Allium cepa* seedlings were concurrently exposed to *B. bassiana* or a no-fungus treatment and one of three watering regime treatments (3-day, 5-day, or 7-day watering intervals). After ten weeks, *A. cepa* bulbs were harvested, crushed with a pestle and mortar for 2-3 minutes, and extracted with dichloromethane (DCM) at room temperature (25 ℃) for 24 hours. The DCM extract was filtered and evaporated to dryness, and then reformulated in DCM to achieve the following concentrations: 20% w/v, 10% w/v and 5% w/v, which were subsequently evaluated in a disc-repellent bioassay. The disc-repellent bioassay revealed that repellencies varied significantly ($DF = 3$, $p < 0.05$) among the treatments (fungus, no-fungus, positive and negative control) irrespective of the watering interval. *B. bassiana* inoculation of *A. cepa* plants significantly enhanced the repellent activity against grapevine mealybugs when extracts were tested at 20% w/v. The best insect repellent activity was recorded on plants extracts from the fungus treatments. The GC-MS analysis of the DCM extracts showed that bulbs of plants inoculated with *B. bassiana* produced significantly more volatile organic compounds than the no-fungus plants ($DF = 1$, $\chi2 = 5.7$, $p < 0\ .05$). In conclusion, endophytic *B. bassiana* inoculum could be used to optimize volatile organic constituents and the repellent activity of onion extracts against mealybugs.

4.1 Introduction

Using synthetic pesticides in agriculture poses severe threats to human lives and ecosystem services and goods (Uphoff and Dazzo, 2016). Concerns about the harmful effects of synthetic chemical pesticides used in the agricultural industry to control pests have prompted researchers to focus on developing environmentally friendly pest management strategies (Jacquet et al., 2022). Fungal entomopathogens are increasingly used as pest biocontrol agents (Chandi et al., 2018). Interestingly, some entomopathogenic fungal species can form symbiotic relationships with plants (Vega et al., 2008; Berendsen et al., 2012) and enhance their tolerance to biotic and abiotic

stresses. *Beauveria bassiana*, *Clonostachys rosea*, *Isaria farinosa*, and *Acremonium* sp. are among the few endophytic entomophatogenic fungi commonly used commercially.

Endophytes are microscopic organisms that live in plant tissue and do not harm them (Bacon and White, 2000). Once in plants, these fungi may have an impact on a variety of plant activities, including the generation of primary and secondary metabolites (Ownley et al., 2010). They also help plants to cope with stress (Fuentes et al., 2020). Endophytic fungi promote plant growth and help plants adapt to biotic and abiotic environmental stressors (Lugtenberg et al., 2016; Chand et al., 2020). Endophyte-associated plants have been shown to utilize less water and produce more biomass than the ones not associated with endophytes (Malinowski and Beleskey, 2000). This water stress tolerance could be explained by increased solute buildup in tissues of endophyte-infected plants relative to non-inoculated plants, decreased leaf conductivity, slower transpiration stream, or thicker cuticle formation (Malinowski and Beleskey, 2000). At least two other mechanisms are involved in symbiotically attained abiotic stress tolerance, the first one is the activation of host stress response systems shortly upon stress exposure, enabling the plants to avoid or minimize the effects of stressors, and the second one is endophyte biosynthesis of anti-stress biochemicals (Schulz et al., 2002). Some endophytic fungi function as bio-stimulants, assisting in distributing macronutrients and micronutrients (Saia et al., 2019). Endophytic fungi can synthesize secondary metabolites that protect plants from diseases and pests or help cope with water scarcity (Vinale et al., 2017).

The ability of plants to respond to abiotic stress is linked to their plasticity and the adaptability to changing water accessibility situations (Chaves et al., 2011). Water deficiency is one of the most researched of the limiting abiotic factors, as it is likely the most constraining factor in crop yield in arid and semi-arid places worldwide (Kabiri et al., 2014). Water deficiency causes plants to make a morpho-anatomical, physiological, and biochemical changes to compensate for the lack of water and maintain their hydric condition (Chaves et al., 2011). Plants create a variety of secondary metabolites in response to diverse environmental pressures, which aid in the plant's adaptation to those conditions (Hirt and Shinozaki, 2003; Berini et al., 2018). Plants can be induced to produce secondary metabolites by altering the biotic and abiotic conditions in which they are grown (Caretto et al., 2015). Terpenoids, nitrogen-containing alkaloids, sulphur-containing chemicals, flavonoids and related phenolic and polyphenolic compounds are the principal categories of plants' secondary metabolites. These secondary metabolites are defense

chemicals that protect plants from bacteria, viruses, and other competitive plants (Pusztahelyi et al., 2015). When cultivating specific, high-value medicinal plant species, the link between endophytic fungi and plants can be investigated to enhance the amount and quality of secondary metabolites in these plants, thereby improving their medicinal characteristics (Espinoza et al., 2019).

The simultaneous exposure of plants to endophytic fungus and water stress can favor enhanced production of anti-insect compounds while reducing the negative effect of water stress on plants (Coleman-Derr D and Tringe, 2014). A few studies have revealed that endophytic fungi can ameliorate the negative impact of water stress and effect on volatiles (Rodriguez et al., 2008; Lau and Lennon, 2012). The main aim of this chapter was to assess the effects of water deficiency and the fungus *B. bassiana* on the volatile contents as well as the bioactivity of *Allium cepa* (onion) extracts against grapevine mealybug (*Planococcus ficus).*

4.2 Materials and methods

4.2.1 Plant material

Thirty-six onion seedlings (Red Creole cultivar) sourced from a local retail nursery, Hart Nursery (PTY) Ltd. Ottery, Western Cape Province, South Africa, were used for this experiment.

4.2.2 Preparation of fungus (*B. bassiana*)

The fungal cultures of a *B. bassiana* strain (SM 3), which is currently being maintained at the Horticultural Department, Cape Peninsula University of Technology, were used in this study. The isolate was initially isolated from a local vineyard and was identified molecularly by Moloinyane and Nchu (2019). The fungus was cultured on a medium containing half strength Potato Dextrose Agar (PDA) (19.5 g/1000 mL of water), 0.04 g streptomycin, and 0.02 g ampicillin sodium salt (Sigma-Aldrich (PTY) Ltd., Johannesburg, South Africa). The prepared medium was transferred into Petri dishes with 9 and 14-cm diameters. Fungal cultures were incubated in the dark for three weeks at 25 ℃ using a sterile spatula; the mature conidia of *B. bassiana* were scraped and transferred into a 50 mL centrifuge tube containing 30 mL of sterile distilled water (0.05% of Tween 80) and glass beads. The conidial suspension was vigorously shaken for three minutes, and after that, it was vortexed for another three minutes again using a vortex mixer (MI0101002D Vortex Mixer) at 3000 rpm to homogenize the conidial suspension. The homogeneous conidial suspension was transferred into 1000 mL bottles. The required conidia concentration of 1×10^6

conidia mL^{-1} was determined using a hemocytometer. Sterile distilled water or conidial suspension was added when necessary to achieve the desired conidial concentration. A volume of 500 mL of conidial suspension was prepared in each 1000 mL bottle.

4.2.3 Experimental setup

The experiment was conducted in the greenhouse of the Department of Horticultural Sciences, Cape Peninsula University of Technology, Bellville Campus. The experiment ran for ten weeks from spring to early summer (October–December) at 28.9–36.9 ℃ and 51–59 % RH. A completely randomized design was used to test the main effects of two factors (*B. bassiana* inoculation and watering regime) consisting of two *B. bassiana* treatments and three watering regimes on *A. cepa* plants.

An inert substrate mix containing (silica sand, vermiculite, perlite, and coco peat) was mixed in a 1:1:1:1 ratio. Two-week-old seedlings were transplanted into the potted substrate mix individually in 15 cm diameter pots after rinsing under running tap water to remove all the potting soil around the roots.

A completely randomized design with two by three factors was used. The seedlings were divided into two groups. One group was inoculated with *B. bassiana*; the second (control group) was not. Plants in both groups received water stress treatments, i.e., no watering for 3, 5, or 7 days. Six potted plants were randomly allocated and treated to either *B. bassiana* or no *B. bassiana* inoculation and one of three watering regimes (3-day, 5-day, and 7-day watering intervals). The watering interval was not extended beyond seven days because a pre-experimental investigation revealed that the plants wilted after seven days. In the fungus treatment, plant roots were inoculated with *B. bassiana* inoculum by drenching the substrate with 100 mL of conidial suspension (1×10^6 conidia mL^{-1}) immediately after transplanting. *Allium cepa* plants from each treatment were fed using hydroponics Nutrifeed fertilizer (Starke Ayres (PTY) Ltd., Cape Town, South Africa) consisting of 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 deionized water at a dosage of 10 g/5 L. Each plant received 100 mL of the nutritional solution fortnightly.

4.2.4 Colonization of Tissues by Fungus

Four weeks after exposure of the experimental plants to the various treatments (watering regimes and *B. bassiana*), four leaf sections (1 × 1 cm^2), carefully excised from four randomly selected plants in each treatment, were rinsed with sterile distilled water. They were then sterilized with 70% ethanol for 10s and again rinsed with sterilized distilled water for 60 s. After that, they were placed on a Potato Dextrose Agar plate (PDA) containing half-strength Potato Dextrose Agar (PDA) (19.5 g/1000 mL), 0.04 g streptomycin, and 0.02 g ampicillin sodium salt. They were then incubated for 14 days in the dark at 25 ℃.

4.2.5 (GC-MS)

4.2.5.1 Sample Preparation

A GC-MS method described by Moloinyane and Nchu (2019) was used in this research. Fresh bulbs of *A. cepa* from each treatment at ten weeks post-treatment were freeze-dried at -80 ℃ overnight. The bulbs were pulverized using liquid nitrogen, and 1 g was weighed into an SPME vial. After that, each vial received 2 ml of 12 percent soaking alcohol solution (v/v) at pH 3.5, followed by 3 ml of 20 percent saturated NaCl solution. The headspace of the material was investigated using Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fibers after the vials were vortexed (gray). Eighteen plants, i.e., 3 plants per treatment were used for GC-MS analysis and another 18 plants were used for the repellency bioassay.

4.2.5.2 Chromatographic separation

A gas chromatograph (6890N, Agilent Technologies Network) was used to separate the volatile chemicals, which was connected to an inert XL EI/CI Mass Selective Detector (model 5975B, Agilent Technologies Inc., Palo Alto, CA). A CTC Analytics PAL autosampler was used in conjunction with the GC-MS system. A polar ZBWAX (30 m, 0.25 mm ID, 0.25 m film thickness) Zebron 7HGG007-11 capillary column was used to separate volatiles in the samples. A continuous flow rate of 1 mL/min of helium gas was used as the carrier. The split ratio was set to 5:1 and the injector temperature was set to 250 ℃. At 70-eV ionization energy, the Mass Selective Detector and mass spectrometer were used to scan from 35 to 500 m/z in full scan mode and electron impact mode, respectively. The volatile chemicals were tentatively identified by a retention time and mass spectrum that matched internal standards and a reference library by 70%.

4.2.6 Preparation of material extracts: Insect bioassay

After 10 weeks of cultivation with spore suspension, *A. cepa* bulbs were crushed using a pestle and mortar for a period of 2-3 minutes (Three bulbs per each treatment) (Figure 4.1). The crushed material was then used for extraction with dichloromethane (DCM); ten grams of crushed fresh *A. cepa* bulbs was extracted with 20 mL of DCM for 5 hours. The mixture of the plant material and the solvent was then filtered using Whatman no.1 filter paper. DCM was then evaporated from the mixture using a fan overnight to determine the dried extract yield. DCM was selected as the preferred solvent based on previous earlier reports (Nchu, 2004; Nchu et al., 2016). Eighteen plants, i.e., three plants per treatment were extracted.

4.2.7 Insect culture

Female *Planococcus ficus* grapevine mealybugs were obtained from the ARC Infruitec-Nietvoorbij (Agricultural Research Council) Stellenbosch, South Africa courtesy of Dr K.A. Achiano. The mealybugs were reared on butternut heads in a darkroom at 25 ℃ and 60 % RH (Figure 4.1). Mealybugs used for this research were five weeks old female mealybugs.

Figure 4.1 Butternut heads with mealybugs.

4.2.8 Repellency bioassay

A choice repellency bioassay was used to determine the effect of the extract at varying concentrations. A filter paper (Whatman no. 1) was divided into eight equal sections by drawing diametric lines through its center. A small circle (2-cm diameter) in the middle served as a neutral insect release zone. Each of the eight sections represented a test treatment or control treatment. The crude DCM extract mixtures (200 µL) were assessed. The control section was treated with pure DCM sixteen 21-day-old females of *Planococcus ficus* were released in the neutral section of the treated filter papers using a fine painter's brush (no. 3). Each of the eight sections was allocated to one of the treatments: TW1 (3-day fungus extract), TW2 (5-day fungus extract), TW3 (7-day fungus extract). CW1 (3-day no fungus extracts), CW2 (5-day no fungus extract), CW3 (7-day no fungus extract), PC (positive control: DEET insect repellency) and NC (negative control: Dichloromethane) (Figure 4.2 A). The positions of insects on each section were recorded 8-10 min after their release (Figure 4.2 B). Sections with the lowest number of insects were considered to be relatively more repellent. The repellency bioassay was replicated six times.

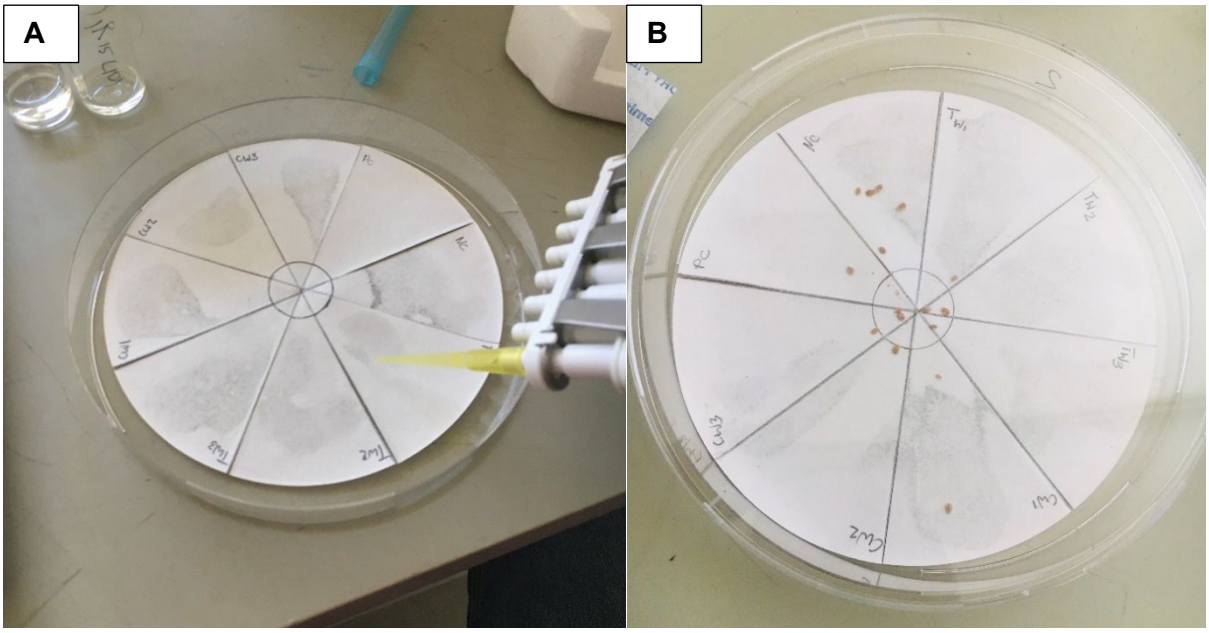

Figure 4.2 Adding plant extracts on repellency bioassay disk divided into eight sections (A) and (B) repellency bioassay disk with female mealybugs after 6 mins. TW1 (3-day fungus extract), TW2 (5-day fungus extract), TW3 (7-day fungus extract). CW1 (3-day no fungus extracts), CW2 (5-day no fungus extract), CW3 (7-day no fungus extract), PC (positive control: DEET insect repellency) and NC (negative control: Dichloromethane)

4.2.9 Statistical analyses

The experimental data (number of volatile compounds and insect repellency) are presented as Mean±SE in tables or graphs. The GC-MS data (number of compounds) was analyzed using Pearson Chi-square. The repellency data was analyzed using the non-parametric Kruskal-Wallis test followed by the post-hoc Mann-Whitney test. These computations were performed using STATISTICA software (TIBCO 1984-2018) and the PASTA version 3.21 (Hammer et al., 2018).

4.3 Results

4.3.1 Fungal re-isolation

B. bassiana was successfully re-isolated from the leaves of the *A. cepa* plants. All the fungus-treated plants recorded mycelial outgrowth on their leaf sections, representing 100% fungal colonization. No fungal outgrowth occurred in the no-fungus plants.

4.3.2 GC-MS

Generally, fungus-treated plants had more compounds (54) than the no-fungus plants (40) at the 7-day watering interval treatment, (DF = 1, $\chi 2$ = 5.7, p < 0 .05) (Table 4.1). However, no significant variations in the number of volatile compounds were observed between fungus and the no-fungus treatments at the shorter watering intervals (3-day and 5-day watering intervals).

Table 4.1 Volatile compounds with a match quality (reference library) from 70% upwards present in fungus and no-fungus treatments at all watering intervals of *A. cepa* plants.

	Compounds	Retention Time	Treatments Peak Area in the Chromatogram (Mean ± SE)					
			3-day		5-day		7-day	
			Fungus	No fungus	Fungus	No fungus	Fungus	No fungus
1.	Decane	5.47	0.26 ± 0.05a	0.16 ± 0.01b	0.25 ± 0.03b	0.40 ± 0.00a	0.26 ± 0.02b	0.31 ± 0.00a
2.	Cyclotetrasiloxane	5.93	0.82±0.01a	1.42±0.43b	0.61±0.06b	1.13±0.09a	0.62±0.04b	0.82±0.05a
3.	Spiro[2.4]hepta-2,6-diene	6.22	-	0.05±0.02a	-	-	-	-
4.	N-hexanal	7.68	-	0.06±0.03a	-	0.07±0.04a	-	-
5.	Dimethyl-disulfide	7.23	0.03 ± 0.01a	0.01 ± 0.00b	0.04 ± 0.00a	0.01 ± 0.00b	0.03 ± 0.01a	0.01 ± 0.00b
6.	Undecane	8.34	0.10 ± 0.01a	0.07 ± 0.00b	0.08 ± 0.00b	0.15 ± 0.00a	0.10 ± 0.00b	0.15 ± 0.00a
7.	p-Xylene	9.26	0.03 ± 0.00a	0.02 ± 0.00a	0.02 ± 0.00b	0.04 ± 0.00a	0.02 ± 0.00a	0.03 ± 0.00a
8.	Benzene, 1,3-dimethyl-	9.29	-	-	0.05±0.03b	0.18±0.00a	-	-
9.	Benzene, 1,2-dimethyl	9.30	-	-	0.04±0.02a	0.00±0.00b	-	-
10.	2,5-dimethyl_thiophene	9.33	0.05 ± 0.02a	0.01 ± 0.01a	0.04 ± 0.001a	_	0.02 ± 0.01a	0.00 ± 0.00a

11.	2-Methyl-2-pentenal	10.44	0.03 ± 0.01a	0.02 ± 0.01a	0.06 ± 0.02a	0.00 ± 0.00a	0.03 ± 0.00a	0.01 ± 0.00b
12.	2,3,4-Trithiapentane	11.11	1.05 ±0.53a	-	0.27 ± 0.04a	1.09 ± 0.54a	1.28 ± 0.54a	1.05 ± 0.52a
13.	o-Xylene	11.22	0.03 ± 0.01a	0.03 ± 0.00a	0.03 ± 0.00a	0.04 ± 0.00a	0.04 ± 0.00a	0.03 ± 0.00a
14.	Benzene, 1,4-dimethyl-	11.24	-	-	0.06±0.03b	0.11±0.06a	-	0.07±0.04a
15.	2,4-Dimethylthiophene	11.64	0.08 ± 0.01a	0.03 ± 0.01b	0.07 ± 0.02a	0.01 ± 0.00b	0.05 ± 0.02a	0.02 ± 0.00b
16.	Isocumene	12.51	0.04 ±0.01a	0.01 ±0.00b	0.01 ± 0.00b	0.02 ± 0.00a	0.02 ± 0.00a	0.02 ± 0.00a
17.	Cyclopentasiloxane	12.91	1.82 ±0.47b	5.10 ±1.86a	1.33 ±0.53b	3.30 ±0.93a	1.8 ±0.03a	1.42 ±0.29b
18.	Benzene, (1-methylethyl)-	12.95	-	-	-	0.03 ±0.02a	-	-
19.	o-Ethyltoluene	13.26	0.07 ± 0.02a	0.04 ± 0.00b	0.05 ±0.00b	0.07± 0.00a	0.07 ± 0.01b	0.22 ±0.16a
20.	Benzene, 1-ethyl-3-methyl-	13.29	0.13 ±0.08b	0.16 ±0.09a	0.24 ±0.01b	0.32 ±0.19a	0.02 ±0.01a	-
21.	Methyl_propyl_disulfide	13.57	0.22 ± 0.07a	0.12 ± 0.05b	0.31 ± 0.00a	0.02 ± 0.00b	0.17 ± 0.07a	0.05 ±0.00b

22.	3,4-dimethylthiophene	14.48	0.40 ± 0.05a	0.19 ± 0.06b	0.39 ± 0.05a	0.07 ±0.01b	0.33 ±0.09a	0.21 ± 0.02b
23.	Cumene	14.82	-	0.12±0.07a	-	-	-	-
24.	Styrene	14.83	0.12 ±0.01a	0.08 ± 0.01b	0.13 ± 0.00a	0.12 ± 0.00b	0.13 ± 0.01a	0.05 ±0.03b
25.	Methyl-trans-propenyl-disulfide	14.89	0.10 ± 0.03a	0.05 ± 0.03b	0.20 ± 0.00a	0.05 ± 0.00b	0.14 ± 0.07a	0.01 ±0.00b
26.	C3-Benzene	15.56	0.20±0.11a	-	0.22±0.13b	0.46±0.27a	0.33±0.00a	0.18±0.10b
27.	Benzene, 1,3,5-trimethyl-	15.57	-	-	-	-	-	0.04 ±0.02 a
28.	1,2,4-Trimethylbenzene	15.58	0.16 ± 0.03a	0.12 ± 0.01b	0.13 ± 0.00b	0.17 ± 0.00a	0.16 ± 0.01a	0.13 ± 0.00b
29.	Benzene, 1,2,3-trimethyl-	15.61	-	0.28±0.16a	-	0.27±0.16a	-	-
30.	Cyclohexanone	15.98	0.12 ± 0.01a	±0.00b	0.20 ± 0.01a	0.05 ± 0.00b	0.07 ± 0.00a	0.02 ±0.00b
31.	2-Heptenal, (E)-	17.44	-	-	-	0.08±0.07a	-	-
32.	Benzene, 1-ethyl-4-methyl-	17.56	0.10±0.06a	-	0.11±0.06a	-	0.14±0.08b	0.24±0.14a
33.	Benzene, 1-ethyl-2-methyl-	17.60	-	-	0.02±0.01a	-	-	-

34.	Ethyl n-heptanoate	18.03	-	-	-	-	0.40 ±0.23a	-
35.	Trisulfide, dimethyl	18.89	0.41 ±0.24a	0.30 ±0.17b	1.21 ±0.13a	-	0.65 ±0.38a	-
36.	Dipropyl_disulfide	19.18	2.71 ± 0.51b	4.28 ± 0.17a	4.12 ± 0.41a	1.20 ± 0.36b	2.66 ± 0.14a	2.02 ± 0.19b
37.	Disulfide, 1-methylethyl propyl	19.31	-	1.09 ±0.01a	-	-	-	-
38.	Propane, 2,2'-sulfonylbis-	19.32	-	-	0.57±0.33a	-	0.23±0.13a	-
39.	Disulfide, bis(1-methylethyl)	19.33	0.26±0.15b	0.55±0.32a	-	-	-	0.39±0.23a
40.	2-Nonanone	19.74	-	-	-	-	0.09±0.05a	-
41.	Nonanal	19.84	0.05 ± 0.02a	0.02 ± 0.00b	0.05 ± 0.01a	0.02 ± 0.00b	0.04 ±0.01a	-
42.	3-Octanol	20.20	1.60 ±0.93b	3.88 ±0.15a	3.96 ±0.03b	4.85 ±0.12a	2.81 ±0.40b	4.23 ±0.01a
43.	Allyl_propyl_disulfide	20.75	0.13 ± 0.06b	0.21 ± 0.12a	0.01 ± 0.00b	0.05 ± 0.02b	0.17 ± 0.09a	0.00± 0.00b
44.	trans-Propenyl_propyl_disulfide	20.9	0.70 ± 0.26a	0.75 ± 0.16a	0.90 ± 0.06a	0.12 ± 0.00b	0.61 ± 0.14a	0.38 ± 0.04b
45.	1H-Purine-8-carboxaldehyde	20.99	-	-	0.17 ±0.10a	-	-	-
46.	Octanoic acid, ethyl ester	21.27	-	-	-	-	0.11±0.06a	-

47.	1 Octen 3 ol	21.74	-	-	-	0.10 ±0.06a	-	-
48.	Tetradecamethylcycloheptasiloxane	23.78	0.33±0.02b	0.90±0.27a	0.51±0.13b	0.70±0.21a	0.56±0.10a	-
49.	Ethyl pelargonate	24.04	-	-	-	-	0.26±0.15a	-
50.	1-Octanol	24.61	0.01±0.01a	0.00 ± 0.00a	0.02 ± 0.01a	0.01 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.00a
51.	3-methyl-1,2,4-trithiane	24.98	0.02±0.01a	0.02 ± 0.01a	0.03±0.01a	0.00 ± 0.00b	0.04±0.01a	0.00 ± 0.00b
52.	2-Undecanone	25.59	-	-	-	-	3.08±1.78a	-
53.	Ethyl_isothiocyanate	26.59	0.01 ± 0.00a	0.02 ± 0.00a	0.04 ± 0.01a	0.01 ± 0.00b	0.02 ± 0.01a	0.00± 0.00a
54.	Diisoproply_trisulfide	27.37	1.21 ± 0.21a	1.42 ± 0.11a	1.24 ± 0.09a	0.52 ± 0.09b	1.12 ± 0.19a	1.30 ± 0.11a
55.	2-Nonen-4-one	28.39	-	-	-	-	0.13±0.07a	-
56.	Tetracosamethylcyclododecasiloxane	28.73	-	0.02±0.01	-	-	-	-
57.	3,6-Diethyl-2,4,5,7-tetrathiaoctane	28.79	0.02±0.00b	0.05 ± 0.01a	0.02 ± 0.00a	0.04 ± 0.01a	0.03± 0.02a	0.02 ± 0.01a
58.	2-Undecanol	29.44	-	-	-	-	0.59±0.35a	-
59.	3,5-diethyl-1,2,4-trithiane	30.16	0.02±0.00b	0.05 ± 0.01a	0.09 ± 0.02a	0.01 ± 0.00b	0.05 ± 0.01a	0.04 ± 0.00a

60.	2-Tridecanone	31.28	-	-	-	-	0.49±0.28a	-
61.	Cyclotrisiloxane, hexamethyl-	31.45	-	-	-	-	0.18±0.10a	-
62.	Arsenous acid, tris(trimethylsilyl) ester	31.46	-	0.04±0.03a	-	-	-	-
63.	Methanethioamide, N, N-dimethyl-	31.81	-	-	0.14±0.05a	-	0.06±0.03a	-
64.	Thiazolidine	31.82	0.08 ±0.04a	-	-	-	-	-
65.	Cyclohexasiloxane, dodecamethyl-	32.17	0.34±0.20a	-	-	3.65±2.07a	0.02±0.02b	1.05±0.61a
66.	Butyl_butyrate	32.25	0.02 ± 0.00a	0.02 ± 0.00a	0.03 ± 0.00a	0.02 ± 0.00a	0.03 ± 0.01a	0.03 ± 0.01a
67.	2,2-Dimethyl-1,3-propanediol isobutanoate	32.26	0.06 ±0.03a	-	-	-	-	-
68.	2-Methyl-5-(4'-methylphenyl)sulfonyl-4-nitroimidazole	32.46	-	0.06±0.04a	-	-	-	-
69.	Butylated_hydroxytoluene	32.91	0.04±0.01a	0.03 ± 0.01a	0.03 ± 0.00a	0.02 ± 0.00b	0.03 ± 0.00a	0.04 ± 0.01a
70.	2,3-Butandiol,o-(trimethylsilyl)-, monoacetat	33.49	-	-	0.07±0.04a	-	-	-
71.	3,4-Dimethyl-2,5-dihydrothiophene-2-one	33.62	-	-	0.02±0.01a	-	-	-

72.	5-Methyl-2-octyl-(2h)-furan-3-one	34.10	-	-	-	0.20 ±0.11a	1.56 ±0.90a	-
73.	Octanoic acid	34.83		-	-	-	0.05±0.03a	-
74.	Cepanone	36.61	-	-	-	-	0.05 ±0.03a	0.05 ±0.03a
75.	2-Ethylformanilide	37.11	0.12±0.07a	-	-	-	-	-
76.	2,4-Di-tert-butylphenol	37.43	0.23 ±0.03a	0.14 ± 0.01b	0.14 ± 0.00b	0.23 ± 0.01a	0.22 ± 0.01a	0.15 ± 0.00b
	TOTAL Number of COMPOUNDS		42	44	47	45	53*	39

*Means with the same lowercase in the same column are not significantly different (P > 0.05).

4.3.3 Insect repellency bioassay

The repellent bioassay showed that there was a significant difference (DF = 3, P < 0.05) in insect repellency between fungus and no fungus treatments at all watering intervals when extracts were assayed at 20 w/v %, with the fungus treatment producing the lowest number of insects (higher repellent activity) in the 3-day, 5-day, 7-day watering interval treatments as shown in Table 4. There was a significant difference (DF = 2; $\chi 2$ = 23.9; P < 0.05) among the fungus treatments with DCM extract of plants from the most extended watering interval (7-day) having the lowest number of insects or best insect repellency result (0.17 ± 0.17), while 3-day producing the highest mean number of insects (1.00) on the disc sections (Table 4.2). When no fungus treatments were compared, there was a significant difference; the 7-day watering interval treatment had the lowest mean number of insects or best insect repellency (1.67 ± 0.17), and the 3-day watering interval produced the highest mean (2.83±0.13).

Table 4.2 20 w/v % Repellent effects (mean number of unrepelled insects per disc section ± SE) of bulb extracts of *A. cepa* against grapevine mealybug the disc repellency bioassay.

Replicates	Treatment				
	Fungus	No fungus	PC	NC	
3-day	1.00±0.13aB	2.83±0.13cB	2.00±0.13b	2.67±0.13b	DF = 3; $\chi 2$ = 16.36; P < 0.05
5-day	0.67±0.18aAB	2.33±0.18bAB	2.00±0.18b	2.67±0.18b	DF = 3; $\chi 2$= 14.91; P < 0.05
7-day	0.17±0.17aA	1.67±0.17bA	2.00±0.17bc	2.67±0.17c	DF = 3; $\chi 2$= 16.36; P < 0.05
	DF = 2; $\chi 2$ = 23.9; P < 0.05	DF = 2; $\chi 2$ =11.52; P<0.05			

*Means with the same lowercase in the same row are not significantly different (P > 0.05). Means with the same uppercase in the same column are not significantly different (P > 0.05). The non-parametric Kruskal-Wallis test was used to compare the number of insects repelled between the fungus and no fungus plant extracts for the different solvents and extracts concentrations on different watering regimes, and it was followed by the post-hoc Mann-Whitney test to separate the means at a level of significance, P< 0.05.

The repellency bioassay showed that there was a significant difference (DF = 3, P < 0.05) in insect repellency on all watering intervals when extracts were assayed at 10 w/v % of DCM extracts, with fungus treatment producing the lowest mean number of insects or best insect repellency in all watering intervals (3-day, 5-day, 7-day) (Table 4.3). When fungal treatments were compared, there was a significant difference (DF = 2; $\chi 2$ = 21.22; P < 0.05), with 7 days providing the lowest mean number of insects (0.50±0.41) and 3 days producing the highest mean number of insects (1.50±0.35) on the demarcated disc sections. However, when the different watering interval treatments with no-fungus were compared, there was no significant difference (DF = 2; $\chi 2$ = 6.10; P > 0.05). Nevertheless, 7-day watering interval treatment recorded the lowest mean number of insects (1.83 ± 0.41) and 3-day produced the highest mean number of insects (2.83 ± 0.13) (Table 4.3).

Table 4.3 10 w/v % Repellent effects (mean number of unrepelled insects per disc section ± SE) of bulb extracts of *A. cepa* against grapevine mealybug in the disc repellency bioassay.

Replicates	Treatment				
	Fungus	No fungus	PC	NC	
3-day	1.50±0.35aB	2.83±0.13cB	3.17±0.38b	2.83±0.38b	DF = 3; $\chi 2$ = 9.24; P < 0.05
5-day	0.67±0.38aA	2.17±0.38bAB	3.17±0.38b	2.83±0.38b	DF = 3; $\chi 2$ = 12.61; P < 0.05
7-day	0.50±0.41aA	1.83±0.41aA	3.17±0.41b	2.83±0.41b	DF = 3; $\chi 2$ = 13.99; P < 0.05
	DF = 2; $\chi 2$ = 21.22; P < 0.05	DF = 2; $\chi 2$ = 6.10; P > 0.05			

* Means with the same lowercase in the same row are not significantly different (P > 0.05). Means with the same uppercase in the same column are not significantly different (P > 0.05). The non-parametric Kruskal-Wallis test was used to compare the number of insects repelled between the fungus and no fungus plant extracts for the different solvents and extracts concentrations on different watering regimes, and it was followed by the post-hoc Mann-Whitney test to separate the means at a level of significance, P< 0.05.

Overall, there was a significant difference (DF = 3; $P < 0.05$) in all watering interval treatments at 5 w/v % concentration of plant extracts, with the fungus treatment producing the lowest mean number of insects in all the watering intervals (3-day, 5-day, 7-day) (Table 4.3). When only the fungal treatments, i.e., the different watering interval treatments with fungus were compared, there was no significant difference (DF = 2; $\chi 2 = 13.68$; $P < 0.05$) with 7-day recording the lowest mean number of insects (0.50 ± 0.32) on the disc, followed by 3-day and 5-day (0.83±0.29). In the no-fungus treatments, there was a significant difference (DF = 2; $\chi 2 = 8.22$; $P < 0.05$) among the watering interval; 5-day and 7-day recorded a lower number of insects (1.83 ± 0.29) than the 3-day interval (2.33 ± 0.29) (Table 4.3).

Table 4.4 5 w/v % Repellent effects (mean number of unrepelled insects per disc section ± SE) of bulb extracts of *A. cepa* against grapevine mealybug in the disc repellency bioassay.

Replicates	Treatment				
	Fungus	No fungus	PC	NC	
3-day	0.83±0.29aA	2.33±0.29bB	2.50±0.29b	3.17±0.29b	DF = 3; $\chi 2 = 13.37$; $P < 0.05$
5-day	0.83±0.29aA	1.83±0.29abA	2.50±0.29bc	3.17±0.29c	DF = 3; $\chi 2 = 14.02$; $P < 0.05$
7-day	0.50±0.32aA	1.83±0.32abA	2.50±0.32b	3.17±0.32b	DF = 3; $\chi 2 = 14.06$; $P < 0.05$
	DF = 2; $\chi 2 = 13.68$; $P < 0.05$	DF = 2; $\chi 2 = 8.22$; $P < 0.05$			

* Means with the same lowercase in the same row are not significantly different ($P > 0.05$). Means with the same uppercase in the same column are not significantly different ($P > 0.05$). The non-parametric Kruskal-Wallis test was used to compare the number of insects repelled between the fungus and no fungus plant extracts for the different solvents and extracts concentrations on different watering regimes, and it was followed by the post-hoc Mann-Whitney test to separate the means at a level of significance, $P < 0.05$.

4.4 Discussion

The number of known anti-insect volatile compounds varied significantly (DF = 1, $\chi 2 = 5.7$, $p < 0.05$) among the six treatments of fungus and watering intervalshigher number of compounds occurred in plants inoculated with *B. bassiana.* These results were consistent in all watering intervals (3-day, 5-day and 7-day) *B. bassiana* inoculum enhancing repellency against mealybug. *Allium* species have been reported to contain bioactive agents such as organosulfur compounds, polyphenols, flavonoids, alkaloids and saponins (Slimestad et al., 2007; Radovanović et al., 2015). Similar results on repellency were found by Gao et al. (2019) who reported that essential oils from *Allium tuberosum* exhibited repellant and toxicity properties against *Plurtella xylostella* larvae. Additionally, in a study by Nchu et al. (2016), ticks were repelled by *Allium sativum* at low concentrations. Some endophytic fungi have been discovered to produce secondary metabolites that influence the reduction of insect infestations on their host plants (Jaber and Ownley, 2018).

Interestingly, the higher number of compounds observed among plants that were simultaneously exposed to the extended watering interval and fungus treatments in this study may suggest that the abiotic and abiotic factors could be contributing to the increased synthesis of bioactive secondary metabolites. An earlier study by Bennett (1994) showed that exposing plants to water stress resulted in increased synthesis of compounds. Plants exposed to water stress tend to produce more compounds (Gaspar et al., 2002). Endophytic fungi occurring in plants are abundant sources of new, bioactive, and structurally varied secondary metabolites and other natural products (Rustamova et al., 2020). Synthesis of bioactive substances, particularly those unique to their host plants, by fungal endophytes is significant from a biochemical and molecular aspect or ecologically perspective.

It is remarkable to observe the correlation between insect repellent activities and the number of compounds in the current study – the best results were obtained from plants that were exposed both to the fungus and extended watering interval. A number of important compounds which have been reported to have repellent activities and insecticidal properties were detected, for examples, 1-octen-3-ol, 3-octanone, and 1-octene (Khoja et al., 2019).

It is also known that endophytic fungi produce a number of significant secondary metabolites, such as anti-cancer, anti-fungal, anti-diabetic, and immunosuppressive substances (Schulz and Boyle, 2005). Endophytic fungi can act as an alternate source of significant plant secondary

metabolites because these compounds are often identical to those made by the corresponding host plants (Gunatilaka, 2006). According to Strobel and Daisy (2003) reported many researchers are interested in studying the endophytic fungus-produced secondary metabolites in plants. Numerous *Beauveria* species release anti-insect compounds that decrease insect viability or prevent pest reproduction (Gurulingappa et al., 2010).

Manipulating secondary metabolite contents using endophytic fungi and water stress is a potentially viable strategy to enhance the bioactivity as well as the medicinal, nutraceutical and pharmaceutical value of plants. It can also enhance return on investments and reduce harvesting of wild plants as well as reduce environmental degradation associated with conventional agriculture. The key findings of the study are that *B. bassiana* inoculation enhanced *A. cepa* volatile contents and repellency against grapevine mealybugs.

4.5 Conclusion

Manipulating secondary metabolite contents using endophytic fungi and water stress is a potentially viable strategy to enhance the medicinal, nutraceutical and pharmaceutical value of plants. It can also enhance return on investments and reduce harvesting of wild plants as well as reduce environmental degradation associated with conventional agriculture. The key findings of the study are that *B. bassiana* inoculation enhanced *A. cepa* volatile contents and repellency against grapevine mealybugs.

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CHAPTER FIVE

General Discussion, Conclusion and Recommendations

5.1.1 General Discussion and conclusion

One of the main limiting factors for plant growth is water deficit stress, an abiotic condition that affects plant growth. Overuse, pollution, and changes in land use are contributing to the deteriorating and disappearing freshwater supplies and massive shortage of agricultural water. The association of plants with endophytic entomopathogenic fungi can help them overcome debilitating stresses. This study has established that the enhancement of secondary metabolite production is a key mechanism by which endophytic fungi protect plants from disease and pests or assist them in mitigating the negative effects of water stress. This knowledge could be exploited for the enhanced production of anti-insect extracts and elucidates the relationship among fungi, plants, and water stress in terms of pest control. The key findings of this study are:

- *B. bassiana* enhanced plant growth, influenced tissue nutrient contents, induced the production of secondary metabolite and antioxidant activity of *Allium cepa* L. and insect repellent activities. These results are consistent with previous studies of Espinoza et al. (2019), Staffa et al. (2020) and Macuphe et al. (2021).
- Water stress enhanced plant growth, influenced tissue nutrient contents, induced the production of secondary metabolites and antioxidant activity of *Allium cepa* L and insect repellent activities. These results are consistent with the published works of Roos and Nchu (2021) and Azhar et al. (2011).
- Water stress and endophytic *B. bassiana* enhanced secondary metabolites, volatiles, plant growth and mitigates the negative impacts of water stress. This is a pioneer study.
- Furthermore, the study revealed another mechanism that may be involved in the reduction mitigation of the negative effects of water deficit and the enhanced bioactivities and secondary metabolites. It demonstrated the potential involvement of nutrients in influencing growth. It also demonstrates the synergistic effect of the two factors (water deficit and endophytic *B. bassiana*) on secondary metabolites, bioactivities and volatiles.
- Manipulating the cultivation of plants could greatly improve the production of high quality medicinal *A. cepa* plants with high commercial value. This study provides protocol for optimizing the cultivation of *A. cepa* under greenhouse and hydroponically.

5.1.2 Recommendations

- The current study was only limited to one plant species and insect pest. Future studies should consider including other different plant cultivars and species and insect species for a more comprehensive study.
- It is important to carry out large scale studies and cost benefit analyses
- It is interesting to characterize mycotoxin contents in *B. bassiana* inoculated *A. cepa.*

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