The effect of the fungus *Beauveria bassiana* (Hypocreales) on the growth of *Tulbaghia violacea* and its ability to synthesize nano-particle insecticidal to mealybug

By

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DECLARATION

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

DEDICATION

I dedicate this thesis:

To My Late Mom Lulama Elizabeth Ludwaba and my late grandmother Meriam Nolili Ludwaba.

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Contents

LIST OF ACRONYMS

ANOVA	**Analysis of Variance**
CA	**Correspondence Analysis**
CAF	**Central Analysis Facilities**
EPF	**Entomopathogenic fungi**
GC-MS	**Gas Chromatography – Mass Spectrometry**
ºC	**Degrees Celsius**
PDA	**Potato Dextrose Agar**
AuNPs	**Gold nanoparticles**
NPs	**Nanoparticles**
GDP	**Gross domestic product**
CPUT	**Cape Peninsula University of Technology**
WHO	**World Health Organization**
DMSO	**Dimethyl sulfoxide**

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ABSTRACT

Few studies have investigated the effects of biotic factors on the yield and plant production of secondary metabolites. This study aimed to evaluate the effect of the entomopathogenic fungus *Beauveria bassiana* (Hypocreales) on the growth and ability of *Tulbaghia violacea* (wild garlic) to synthesise nanoparticles. *T. violacea* Harv. plants were inoculated by drenching with different concentrations of *B. bassiana* conidia, concentrations (0 mL^{-1}, 1 x 10^6 mL^{-1}, 1 x 10^7, and 1 x 10^8 conidia mL^{-1}). A UV-VIS spectrometer (SPECTROstar nano) was used to determine the presence of nanoparticles. Conidial colonization of plant tissue in the different fungal treatments was evaluated. Gas chromatography-mass spectrometry was used to analyze volatile constituents in the plants. Each of the polyphenols contents was determined using Folin-Ciocalteu reagent with gallic acid as the standard to measure. Furthermore, antioxidant activities were assessed using (TEAC, DPPH, and FRAP). Up to 57% of plants were successfully colonized at the highest concentration (1 x 10^8 conidia mL^{-1}). Generally, the results showed that *B. bassiana* treatment had no significant effect ($P>0.05$) on the growth parameters (height, number of leaves, aerial part fresh weight, aerial part dry weight, root fresh weight, and root dry weight) after four weeks post treatment. The aqueous extracts of *T. violacea* from both control and *B. bassiana*-treated plants successfully synthesized gold nanoparticles. Furthermore, the aqueous extracts of plants and gold nanoparticle from both fungus and control treatments were equally toxic to mealybug and induced high mortality. The GC-MS results revealed that the number of compounds detected in plants subjected to the different concentrations of fungal inoculum and control did not vary significantly ($P>0.05$) in leaves and roots. The polyphenol contents in the aerial parts were not significantly different ($P>0.05$) among treatments. Similarly, the antioxidant activities were not significantly influenced ($P>0.05$) by fungal treatment on both leaves and roots.

In conclusion, although *B. bassiana*'s conidia endophytically colonized *T. violacea* plants following drenching, this had no significant influence on the plant growth. However, an interesting finding of this study is the ability of *T. violacea* extracts to reduce aqueous gold to gold nanoparticles in both control and fungus-treated plant.

CHAPTER 1

Introduction and the background of the research problem

1.1 General introduction

Synthetic pesticides are used worldwide to control grapevine pests (Sabatier *et al*., 2014). But many pests have evolved and developed resistance over time towards synthetic chemical control (Marrone, 2019). Over the years, pest resistance to well-known pesticides, including synthetic insecticides, has been increasing at an alarming rate (Auteri *et al*., 2018). Additionally, chemical pesticides are expensive and toxic to the environment (World Health Organization, 1990). Many consumers, weary of synthetic insecticides, prefer organically cultivated produce (Georghiou & Mellon, 1983:; Huang, 1996). Thus, the search for biological approaches to help slow down the resistance rate is intensifying.

Green syntheses of biological active insecticidal nanoparticles from plant extracts have been successfully achieved (Mubayi *et al*., 2012). Some nanoparticles exhibit anti-parasitic activities comparable to synthetic chemicals (Amhed *et al*., 2016). Green synthesis of nanoparticles has also been recorded in fungi and bacteria (Sundhu *et al*., 2017), especially entomopathogenic ones. Singh *et al.* (2016) reported that endophytic fungi have good prospects as nanoparticle synthesizers because they produce substantial amounts of enzymes and can be easily handled in the laboratory. Verma *et al*. (2010) argue that endophytic fungi are highly advantageous over chemical synthesis and provide a sustainable source of pesticides. Moreover, they can enhance plant growth and influence plant nutrient uptake (Rodriguez *et al*., 2009).

Endophytic fungi present an excellent opportunity to optimize the production of nanoparticles. They can enhance the biosynthesis of secondary metabolites, which could serve as a precursor for the synthesis of nanoparticles (Kaur *et al*., 2021). Furthermore, many fungi can synthesize nanoparticles and be manipulated to colonize plants systemically during cultivation. Recent studies suggest that manipulating plant nutrient concentration can influence secondary metabolite production (Ncube *et al*., 2012; Rustamova *et al*., 2019). Hence, it is expected that fungal treatment of plant growth medium could enhance the green synthesis of nanoparticles and the

bioactivities of plant extracts against insects. In the present study, the species *T. violacea,* known to have insecticidal properties was selected.

As the demand for highly efficacious and standardized plant extracts develop, so does the need to optimize the cultivation of medicinal plants. Hydroponics technology has a high uptake, quality, and yield, and all year-round production can be achieved using this technology. Growing conditions are controlled throughout the year, and the enhancement of secondary metabolism can be achieved by ensuring accurate manipulation of mineral nutrition (Treftz & Omaye, 2016b). The demand for medicinal and aromatic plants continues to increase in local, national and international markets (Lubbe & Verpoorte, 2011). Sustainable cultivation could help meet the large demand for natural remedies (Chemat *et al*., 2019). It can relieve the pressure on the wild plant populations. This study could contribute towards developing a novel alternative insect control strategy.

1.2 Statement of the research problem

Pest resistance to commonly used pesticides is a major limiting factor to agricultural productivity. Recent results have indicated that plant-synthesized nanoparticles have the potential to control insects had motivated the current study, which seeks to the optimise biosynthesis of insecticidal nanoparticles against grapevine mealybug (*Planococcus ficus*) by wild garlic (*T. violacea*). Plant growth medium was inoculated with conidia of the entomopathogenic endophyte *B. bassiana*, and its effects on plant growth, the antioxidant activity of extracts, biosynthesis of nanoparticles and insect toxicity were assessed.

1.3 Objectives of the study

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The main objectives of this study were to evaluate the endophytic effects of *B. bassiana* (Hypocreales) on the growth and synthesis of nanoparticle, anti-grapevine mealybug (*Planococcus ficus)* activities of *T. violacea*.

Specific objectives were to:

1. Assess the growth and effect of antioxidant activity of *T. violacea* following drenching with *B. bassiana* conidial suspensions ($1x10^6$ conidial/mL, $1x10^7$ conidial/mL and $1x10^8$ conidial/mL)

2. Determine the effect of inoculating plant growth media with an endophytic fungus (*B. bassiana*) on the green synthesis of nanoparticles by extracts of wild garlic.

3. Evaluate the effect of inoculating *T. violacea* with *B. bassiana*'s conidia on secondary metabolite contents (volatile and non-volatile constituents)

4. Evaluate the toxicity of the aqueous nanoparticle solution against grapevine mealybug.

1.4 Hypothesis

1. Inoculating plants with the fungus *B. bassiana* will positively influence growth parameters (height, crown size, root length, dry weight, fresh weight and antioxidant activity).
2. Inoculating *T. violacea* with endophytic *B bassiana* will enhance the biosynthesis of insecticidal nanoparticles.
3. Inoculating *T. violacea* plants with *B. bassiana* will positively influence the secondary metabolite content (anti-insect volatiles and non-volatiles).
4. Aqueous nanoparticles solution will be toxic against grapevine mealybug.

1.5 Literature review

1.5.1. Background

Recently, Staffa *et al*. (2020) studied the effect of *B. bassiana* inoculation on *T. violacea* growth and secondary metabolite production. The findings revealed that *B. bassiana* successfully colonised leaf and roots with the fungal hyphae occurring in 75% and 91.6% of the plant. Although, *B. bassiana* significantly influenced ($P<0.05$) root length and plant height. However, there were no notable effects on the weight and leaf number of *T. violacea*. Staffa *et al*. (2020) disclosed that the fungus did not significantly affect the overall number of compounds; however, significant variations in the quantity (area ratio) were observed in at least four detected compounds. Building upon that study, the current study examined the effects of varying concentrations on *B. bassiana* conidia on plant growth, antioxidant activities, green synthesis of the nanoparticle and the anti-insect activity by extracts of *T. violacea*.

1.5.2 History and geographical distribution of Mealybug

Mealybug was first described in the Western Cape Province of South Africa in 1943 (Walton & Pringle, 2017). Mealybug (Hemiptera: *Pseudococcidae*) is an invasive pest in many parts of the world affecting horticulture and agriculture worldwide (Figure 1.1) (Gutierrez *et al.,* 2008). Downie and Gullan (2004) describes mealybug as small plant-sucking insects that are the second-largest recorded family of scale insects (Coccoidea). Currently, in South Africa, about 50 genera of *Pseudococcidae* have been recorded (Le Vieux & Malan, 2013). However, mealybugs comprise a very diverse group, with 2291 species belonging to 274 genera described worldwide (Correa *et al*., 2012). These species are morphologically similar, which makes it hard to identify. Current methods for identifying mealybug species are based on key morphological characteristics of the adult female under the microscope (Correa *et al*., 2012). Figure 1.1 showing mealybug covered in a white-grey mealy wax.

Figure 1.1 Grapevine mealybug (adapted from http://www.ecoport.org)

1.5.2.1 Morphometric and life cycle

Mealybug adults are soft, oval, and segmented insects covered with white or grey mealy wax (Millar, 2002). They lay 300-600 eggs over two weeks, with the female dying shortly after all eggs are laid. Like other bugs, the vine mealybugs represent two distinct sexual forms, dimorphism and nymph. Mealybug females undergo incomplete metamorphosis; nymphs go through three instars, with a generation taking as little as a month, depending on temperature. The fully matured females are 4 mm long, 1.5 mm thick and 2 mm wide, and short-lived males are 3 mm long (Walton, 2003). Male nymphs go through five instars. The first two instars exist solely to fertilize the females. According to Faure (2015), they are very small, 1 mm body length, have no mouthparts, single pair of wings on the metathorax, and two long filamentous setae.

1.5.2.2 Mealybug (Family: *Pseudococcidae*) damage on plants

Mealybugs are one vital group of phytophagous insects that cause major damages on a range of horticultural crops across the world (Tanga, 2012). It is believed that at high numbers, they cause leaf yellowing, curling as the plant defoliates and loss of nutrients due to toxic saliva they inject while extracting plant sap (Walton & Pringle, 2017). Furthermore, they cause the build-up of honeydew and associated sooty mould

fungus, reducing fruit quality and lowering tree vitality through the loss of photosynthetic capacity (Kerns *et al*., 2004).

In Western Cape, the common mealybug is *Planococcus ficus* which causes damage to grapevine crops, one of the leading contributors to the gross domestic product (GDP) of South Africa. According to Lentini *et al.* (2017), *P. ficus* causes high yield loss in the wine and table grape vineyards. *P. ficus* feeds on the phloem of the plants and hide underneath the leaves and sucks sap from plant tissues, causing severe damage to plants (Sarwar, 2014).

1.5.2.3 Mealybug damage control

1.5.2.3.1 Monitoring

Early detection and isolation of infested plants are vital to prevent mealybug outbreak claims (Ripa & Rojas,1990). They can be seen on the underside of leaves, petiole, leaf junction, and base of the plant and below ground (Millar, 2002). An appropriate method of control to be used depends on the season, as mealybugs can move between hosts. Effective management requires integrated control methods, based on the combination of different control methods, such as chemical and biological control (Daane *et al*., 2012).

1.5.2.3.2 Chemical control

Mealybugs tend to hide in protected locations and form dense colonies, making controlling them difficult (Daane *et al*., 2008). Chemical control is considered the most common management strategy (Franco *et al.,* 2009). However, the mealybug waxy covering helps to protect them from chemical exposure (Daane *et al*., 2012). Synthetic insecticides are often released under high pressure to achieve control (Dusfour *et al*., 2019). Nevertheless, excessive, and repetitive applications of chemicals promote insecticide resistance (Sarwar, 2014). Moreover, only a few available insecticides on the market have shown positive results in reducing the outbreak of mealybug populations (Mansour *et al*., 2018).

1.5.2.3.3 Biological control

Different biological approaches have been used to control mealybugs in greenhouses and fields. Most predators that are used to control mealybug are generalists that also feed on non-targeted small soft-bodied arthropods (Mgocheki & Addisson, 2009). The most known predator of mealybug is the mealybug destroyer (*Cryptolaemus montrouzieri*) whose adults and larvae prey on mealybug (Tanwar *et al.*, 2010). The efficacy of predators is less against mealybugs than parasitoids. *Cryptolaemus mentrouzieri,* which is the predatory ladybird beetle known as the destroyer, may be

used to control mealybug populations (Tanwar *et al*., 2010). Researchers such as DeBach, (1949) reported ladybird beetles as another group of predators feeding on mealybugs. The challenge faced in using predators is that many predators have a low tolerance to winter temperatures (Bielza *et al*., 2020). Lacewings have also been shown to suppress mealybug populations by killing small mealybugs. However, lacewings have difficulty feeding on eggs because they are protected by wax secretions of the mealybug (Daane *et al*., 2008).

1.5.3 Nanoparticles

Sandhu *et al.* (2017) defined nanoparticles as materials at nano-scale levels with dimensions between 1 and 100 nm. Scientific researchers are conducting more studies on nanoparticles because they bridge the gap between bulk materials and atomic or molecular structures (Timoshenko *et al*., 2018; Vollath *et al*., 2018). According to Daraee *et al.* (2016), the branch involving novel strategies to design, manipulate, synthesis and apply particles with a dimension smaller than 100 nm is called nanotechnology.

Recently, the use of nanoparticles has grown, leading to high demands for their synthesis (Nune *et al.,* 2009; Mubayi *et al.,* 2012). Mubayi *et al*. (2012) claimed that in most cases, the success of nanoparticle synthesis is achieved from the extract of plant parts such as leaves. Green synthesis of nanoparticles can happen at low concentrations of leaf extract without using additional unsafe chemical/physical methods (Ahmed *et al.,* 2016). On the other hand, physical and chemical methods to synthesise nanoparticles result in harmful environmental by-products (Patra and Baek, 2014). Hence, green synthesis of nanoparticles is a safer and eco-friendly alternative for synthesising nanoparticle materials for pest management (Bhan *et al*., 2018). Evidences show that green synthesis of nanoparticles can be achieved from microorganisms, plants and viruses or their by-products (Raja *et al*., 2017).

Fungi are perfect contenders for the environmentally friendly synthesis of nanoparticles (Qamandar & Shafeeq, 2018). Atef & Kim (2018) claim that fungi are easy to manage in the laboratory and need simple nutrients. Furthermore, the

synthesis of nanoparticles using this approach is cost-effective because it can be easily scaled up to produce nanoeconomics for economic purposes using renewable natural materials (Biao *et al*., 2018).

1.5.3.1 Plant synthesis of nanoparticles (Mechanisms)

There are three methods for the synthesis of nanoparticles. These are physical, chemical, and biological syntheses of nanoparticles. They are briefly discussed here.

1.5.3.2 Physical synthesis

According to Singh *et al*. (2016), evaporation-condensation and laser ablation are the most important physical approaches. Physical synthesis is advantageous over chemical because of the absence of solvent contamination in preparation for nanoparticle distribution. The physical synthesis of nanoparticles using a tube furnace at atmospheric pressure has disadvantages; hence, a small ceramic heater with a local heating area is used to evaporate source materials (Li *et al*., 2020). Nanoparticles like silver could be synthesized by laser ablation of bulk materials in solution. An essential advantage of the laser ablation technique compared to other methods to produce metal colloids is the absence of chemical reagents in the solution (Dheyab *et al*., 2021).

1.5.3.3 Chemical synthesis (Chemical reduction)

The chemical synthesis of nanoparticles is a straightforward, common, and inexpensive approach (Yaqoob *et al*., 2020). Nanoparticles synthesised by chemical methods form colloids. For example, the reduction of $AgNo_3$ (silver nitrate) in an aqueous solution by a reducing agent in the presence of an appropriate stabiliser. The chemical method consists of two methods: the sol-gel processing and solution-based synthesis. The sol-gel process is a wet-chemical technique that uses either a chemical solution or colloidal particles to produce an integrated network (Gudikandula & Charya Maringanti, 2016). It consists of two processes: nucleation and growth (Phan & Nguyen, 2017). While according to Chandra *et al*. (2021) Solution-based chemical

synthetic strategies provide simple and powerful routes to nanocrystals. Furthermore, Solution-based synthesis is adaptable with existing nanofabrication processes, is scalable at low cost and has already been proclaimed to produce high quality MoS2 films using a sole source precursor such as ammonium tetrathiomolybdate (NH4)2MoS4 through thermal decomposition for electronic devices applications (Abbas *et al*., 2020).

1.5.3.4 Biological synthesis of nanoparticles

Plant extracts and microorganisms such as Bacteria, fungi and yeast reportedly possess high ability to synthesize nanoparticles. (Ahmed *et al*., 2015; Singh *et al*., 2016). A plausible explanation for such synthesis is detoxification. The mechanisms involve restricting the entry of the toxic ions into the cell, sequestering toxic species in or outside the cell, intracellular or extracellular sequestration, activating energy-dependent efflux pathways to eliminate the toxic species and enzyme-catalysed oxidation or reducing toxic species to less harmful form (Pasricha *et al*., 2021).

Several studies show that many factors affect the synthesis of nanoparticles, including pH of the solution, temperature, the concentration of the extracts used, the concentration of the raw materials used, size, and above all, the methods that are followed for the synthesis process (Patra & Baek, 2014; Ebrahiminezhad *et al*., 2018; Aboelfetoh *et al*., 2020). Researchers have discovered that the pH of a solution medium influences the size and texture of the synthesized nanoparticle. The physical method requires a high temperature (>350 ^{O}C), whereas chemical methods only require a temperature of less than 350 ^{O}C. In most cases, the synthesis of nanoparticles using green technology requires temperatures less than 100 ^{O}C or ambient temperature.

1.5.4 *Beauveria bassiana*

Beauveria bassiana is an entomopathogenic fungus, which belongs to the class Sordariomycetes, order: Hypocreales, and family: Clavicipitaceae, and they naturally occur worldwide (Ownley *et al.,* 2008). *B. bassiana* is known to be an endophytic

organism of various plant species. It protects plants against insect pests and pathogens (Ownley *et al*., 2008). Endophytic fungus is an endobiont that lives within a plant in a mutual beneficial relationship and is a source of biosynthesized nanoparticles (Rana *et al*., 2008). However, there are limited studies on the use of entomopathogenic fungi for the biosynthesis of nanoparticles. Researchers such as Govindappa *et al*. (2016) are among the few who reported nanoparticles synthesis using endophytic fungal species. Kamil *et al*. (2017) have reported good results on the green synthesis of nanoparticles using endophytic fungi.

1.5.5 Field and laboratory experiments of fungus *B. bassiana* as a control for insects

Field and laboratory studies on the pathogenicity of the fungus *B. bassiana* against insects has been reported before. In the laboratory tests against the adults *of C. tarsalis, C. pipiens, A. aegypti, A. sierrensis, A. nigromaculis*, and *A. albimanus*, conidia of *B. bassiana* induced 100% mortality within five days after exposure, while less than 50% occurred in corresponding controls (Clark *et al*., 1968). Ausique *et al*. (2017) reported the efficacy of entomopathogenic fungi against adult *Diaphorina citri* in laboratory and field experiments. In addition, Lecocq *et al*. (2021) evaluated susceptibility of adult *Hermetia illucens* infection by the fungus *B. bassiana*, and the findings showed that adult flies are susceptible to the biocontrol agent *B. bassiana*.

1.5.6 *Tulbaghia violacea*

T. violacea is a small plant species from the Alliaceae family, consisting of about 30 genera and approximately 600 species, mainly indigenous to the Southern African region (Ncise, 2018). *T. violacea* is commonly known as wild garlic, “wilde knoffel” (Afrikaans), “isihaqa” (Zulu) or “itswele lomlambo” (Xhosa), and it is indigenous to the Eastern Cape, South Africa (van Wyk *et al*., 1997). The genus is widely distributed in Southern Tanzania, Malawi, Botswana, Zimbabwe, Mozambique, South Africa, Lesotho and Swaziland (Van Wyk & Gericke, 2000; Vosa & Condy, 2001). The genus belongs to the Alliaceae family, which was once included in the monocot family Liliaceae, but is now recognised by many as a separate plant family (Van Wyk &

Gericke, 2000; Vosa, 2000, Vosa & Condy, 2006; Lyantagaye, 2011). *T. violacea* produces a 'garlic-like odour' (alliaceous odour), which is associated with cysteine derived sulphur compounds released from wounded or decaying tissues and organs, such as leaves and rhizomes (Kubec *et al*., 2013). Furthermore, the strong garlic smell has been ascribed mainly to allicin, a compound also found in garlic (van Wyk & Wink, 2004). For many more years, *T. violacea* has been highly recognized for its nutritive, ornamental, and medicinal values, also its economic importance (Reinten & Coetzee, 2011; van Wyk, 2011a; van Wyk, 2011b).

The study by Staffa *et al*. (2020) suggested that *T. violacea* contains some volatile compounds that are repellent or toxic to insects. Olorunnisola *et al*. (2012) reported some of the compounds of *T. violacea*. Compounds such as dimethyl disulfide (DMDS) and methylsulfonylmethane (MSM), which are sulphur-based, were among the compounds detected. These compounds are commonly released by several plants in their defence against insect pest (Bottger *et al*., 2018).

1.5.6.1 Propagation methods

T. violacea is commonly propagated using seeds and divisions (Ncube *et al*., 2011). However, recent studies showed that micropropagation strategies had been developed (Kulkarni *et al*., 2005; van Wyk *et al*., 2009).

1.5.6.2 Cultivation of medicinal plants

The World Health Organization estimates that more than 80% of the world's population depends on herbal medicine for primary healthcare needs (WHO, 2003). Thus, the demand for natural remedies is high, resulting in them being cultivated on a large commercial scale (Schippmann *et al*., 2002). The hydroponic system is the best technique for producing quality plant material throughout the year. It is possible to control growing conditions and enhance secondary metabolism by using accurate manipulation of mineral nutrition (Xego, 2017; Staffa, 2018). Chhetr *et al*. (2022) describes hydroponics as a technique for growing plants in nutrient solutions with or without the use of an artificial medium to supply mechanical support.

As several materials used in herbal medicine and vitamin supplements are taken from wild plants, the fast-growing demand for medicinal plants puts pressure on many species being over-exploited. Hence, the recommendation for governments and agencies is to bring wild medicinal plants into the cultivation system (WHO, 2004). According to Yuan *et al.* (2010), cultivation of medicinal plants is not only a way of meeting current and future demands for large-volume production of plant-based drugs and herbal remedies, but also a way of relieving harvest pressure on the wild population. Schippmann *et al*. (2002) argued that, in certain circumstances, such as traditional agriculture, cultivation could serve as an important reservoir of genetic variability.

1.5.6.3 Anti-insect activities and medicinal use of *Tulbaghia violacea*

T. violacea is used in southern Africa for the prevention of fever, asthma, oesophageal cancer and constipation and hypertension (van Wyk & Wink, 2004; Ncube *et al*., 2011). Various parts of *T. violacea* are used to treat various conditions such as fever, colds, asthma, cryptococcal meningitis, tuberculosis, oesophagus cancer and high blood pressure (Ngunge, 2011). According to Raji (2012), *T. violacea* can lower blood pressure and heart rate. Many traditional healers boil fresh bulbs and leaves in water and then soak them daily to treat fever, arthritis and rheumatism (Van Wyk *et al*., 1997; Charles & Bonareri, 2020). Crushed bulbs are also used to repel insects and for coughs and colds (Van Wyk *et al*., 1997). The Zulu tribe eats the leaves, flowers, and uses the leaves for seasoning meat and potatoes (Ncube *et al*., 2011). *T. violacea* in some cultures, is used as a substitute for chives and garlic (Kubec, 2002). Mfengwana, (2019) states that apart from the medicinal and nutritive value, *T. violacea* is highly used as an ornamental plant in Southern Africa.

1.5.7 Secondary metabolites that are found in *T. violacea*.

Bungu *et al*. (2008) and Krstin *et al*. (2018) postulated that *T. violacea* has related secondary metabolites to garlic (*A. sativum*), and it has the specific sulphur odour associated with garlic. The sulphur-containing compounds of garlic and *T. violacea* are ascribed to many medicinal properties (Kubec *et al*., 2002; El-meleigy *et al.*, 2010; Martinez-Velazquez *et al*., 2011; Kubec *et al*., 2013). *T. violacea* oil is rich in sulphur-containing compounds that are similar to those found in *A. sativa* (Staffa, 2018). During the crush of garlic bulbs, sulphur-containing compounds are formed when the enzyme alliinase reacts with alliin (Kubec *et al*., 2002). Alliin is present in plants belonging to the genus *Allium* (Kubec *et al*., 2002; Kubec *et al*., 2013). Flavonoids such as quercetin have also been isolated from extracts of *T. violacea* (Hutchings *et al*., 1996). The sulphur-based volatile compounds have been linked to the anti-arthropod properties and repellent activities on fleas, ticks, and mosquitoes of *T. violacea* and *A. sativum* (Nchu *et al*., 2016; Webb & David, 2002).

Secondary metabolites may be organic compounds that are not directly involved in essential plant functions like growth and development. They are crucial in certain stress conditions/environments of the plant (Zhang *et al*., 2022). Secondary metabolites may reduce long-term damage. Plant phenolics compounds such as flavonoids and lignin gather in response to various biotic and abiotic stresses (Bourgaud *et al*., 2001). Some secondary metabolites have anti-insect, anti-microbial and antioxidant properties (Hameed *et al*., 2018).

1.5.8 Antioxidants

Most environmental stresses affect the production of active oxygen species in plants, causing oxidative stress (Sharma, 2019). Environmental factors such as drought, metal toxicity, extreme temperature, ultraviolet-B (UV-B) radiation, pesticides, and pathogen infection lead to substantial oxidative stress in plants (Xie *et al*., 2019). Plants can produce antioxidants to mitigate oxidative stress. Antioxidants are substances that are widely distributed among plants and animals and can significantly prevent or inhibit the oxidative damage to cells (Mitra, 2020). Antioxidants are

scientifically interesting compounds with numerous benefits such anti-ageing and anti-inflammatory (Zehiroglu *et al*., 2019).

1.5.9 Scientific rationale

Many researchers have focused on secondary metabolites. Moloinyane and Nchu (2019) argued that the production of secondary metabolites could be influenced by fungal endophytes, hence, conferring protection plants. Espinonza *et al*. (2019) detected higher total alkaloid content in leaves of chives inoculated with *B. bassiana* compared to control-treated plants. Further evidence shows that the synthesis of secondary metabolites by fungi in the plant tissues may increase the yield of compounds (Alvin *et al*., 2014). Similar results were obtained with suspension cultures of *Taxus cuspidate* with the addition of endophytes, which led to the increased yield of paclitaxel (Anaya *et al*., 2013).

Secondary metabolite contents in a plant or a fungus have a bearing on the ability of the extracts to synthesise nanoparticles. The biomolecules, such as flavonoids, polyphenols, proteins, phenolic acids, and polysaccharides in the plants can reduce metal ions into nanoparticles and can act as a stabilising agent in the synthesis of nanoparticles (Yulizar *et al*., 2017). Since the endophytic fungus can colonise plant tissues without causing infection and enhance the secondary metabolite contents in plants, it makes sense to hypothesise that inoculating *T. violacea* with *B. bassiana* would enhance the secondary metabolite contents and the ability of extracts to synthesis nanoparticles.

CHAPTER 2

Effect of fungus *Beauveria bassiana* inoculation on growth and antioxidants activity of *Tulbaghia violacea*

2.1 Introduction

The high demand for medicinal plants leads to an excessive harvest of wild plants, threatening the existence of many indigenous species (Chen *et al*., 2016). Furthermore, the high demand for consistent and quality medicinal plants has motivated researchers to look for new cultivation methods (Badola & Aitken, 2003). One of the effective cultivation methods is the hydroponic system. Treftz and Omaye (2016a) mentioned that hydroponic plants have a considerable advantage over plants grown in other growing techniques. Several benefits of this technique include less growing time for crops than conventional plant cultivation — all-year-round production, minimal disease and pest incidence, and weeding (Sharma *et al*., 2018).

Plant growth-promoting rhizobacteria (PGPR) can directly activate plant genetic and molecular pathways, leading to increases in plant growth and induction of plant resistance and tolerance (Rosier *et al*., 2018). Vigneshwari *et al.* (2019) stated that the ecological importance, together with the continual metabolic interactions between the fungus and the plant seems to serve as a strong growing pressure for the endophytes to synthesize secondary metabolites. Additionally, synthesis of? these bioactive compounds can also benefit the host plant as they may play an important role in a plant's interaction with the environment for adaptation and defence (Narayanan & Glick, 2022).

Beauveria bassiana is an endophyte that can colonize the internal tissues of plants, and they can reside internally in plant tissues without causing any harm to their host (Khan *et al*., 2017). Many studies have demonstrated that they can enhance growth and control diseases of plants (Zinniel *et al*., 2002). Furthermore, Tuli *et al. (*2014) reported that an endophytic fungus is an endobiont that lives within a plant in a mutually beneficial relationship. Tefera and Vidal (2009) claim that fungal endophytes,

such as *B. bassiana,* are active against phytophagous insects. For these reasons, they are used in agriculture to improve crop performance (Atugala & Deshappriya, 2015).

Results from recent studies showed that plants inoculated with *B. bassiana* have variable physiological responses depending on the species and fungal strain (Parsa *et al*., 2013). Jaber and Enkerli (2017) indicated that there is an absence of consistency in the plant growth promotion obtained by inoculating plants with entomopathogenic fungi. Nevertheless, Dara *et al*. (2017) stated that *B. bassiana* had a positive impact on the survival, growth, length and dry weight of cabbage.

Tulbaghia violacea is one of the most well-known medicinal plant species in the genus, particularly in the Eastern Cape and KwaZulu-Natal regions (van Wyk & Gericke, 2000; van Wyk *et al*., 2000). This species occurs across South Africa due to cultivation in gardens and commercial medicinal plant farms (van Wyk *et al*., 2000). *T. violacea* leaves are used to repel ticks, mosquitoes and other pests when crushed (Bungu *et al*., 2006; Staffa *et al*., 2020).

The objective of this chapter was to assess the effect of fungus *B. bassiana* inoculation on the growth and antioxidants activity of *T. violacea*.

2.2 Materials and methods

2.2.1 Experimental design

Six weeks old *T. violacea* plants of the same cultivar *T. violacea* Harv. (Wild garlic), purchased from Stodels Garden Centre, Cape Town, were randomly allocated to four fungal treatments in a randomised complete block design. The treatments were based on four concentrations (0 mL^{-1}, 1 x 10^6 mL^{-1}, 1 x 10^7, and 1 x 10^8 conidia mL^{-1}) of *B. bassiana* conidial suspension.

2.2.2 Substrate preparation

A substrate mixes of peat, silica sand, perlite and vermiculite (1:1:1:1 in volume) was prepared in 14 cm pots. The silica sand was obtained from Consol in Cape Town, South Africa. While peat, vermiculite, and perlite were obtained from Grow-rite Pty Ltd in Cape Town. The sand and peat were soaked and washed in sterile distilled water. Peat was also soaked in water first to break the blocks and expand, making mixing easy.

2.2.3 Plant Material

Large clumps of *T. violacea* seedlings were divided and were gently washed under a running tap for 4- 5 minutes. Thereafter, they were transplanted into 15 cm pots with substrate mix as explained above. Each pot was then irrigated twice a week with 200 mL of deionized water containing nutrient solution (Nutrifeed from Stodels Pty Ltd.), which consist of the following ingredients: (65 mg kg^{-1}) N, (27 mg kg^{-1}) P, (130 mg kg^{-1}) K; (70 mg kg^{-1}) Ca, (20 mg kg^{-1}) Cu, (1500 mg kg^{-1}) Fe, (10 mg kg^{-1}) Mo, (22 mg kg^{-1}) Mg, (240 mg kg^{-1}) Mn, (75 mg kg^{-1}) S, (240 mg kg^{-1}) B, and (240 mg kg^{-1}) Zn.

2.2.4 Fungi

Entomopathogenic fungal culture of *B. bassiana* strain (SM3) obtained from existing cultures in the Department of Horticultural Sciences Research Laboratory, the Cape Peninsula University of Technology was used. The fungus was originally isolated from a soil sample collected in the Cape Winelands (Strain: SM3) by Moloinyane & Nchu (2019). The fungus was cultured on potato dextrose agar (half-strength) supplemented with 0.02 g L^{-1} of ampicillin (Sigma-Aldrich, Cape Town, South Africa), and 0.04 g L^{-1} streptomycin (Sigma-Aldrich, Cape Town, South Africa). Uncontaminated fungal sub-cultures on agar were prepared in 9 cm diameter Petri dishes and followed by incubation at 25 °C. Three to four weeks old cultures of *B. bassiana* conidia (Figure: 2.1) were scrapped using a sterile spatula and suspended into 500 mL bottles of sterile distilled water containing sterile 0.01% Tween 80 v/v. To ensure separation of spores, the suspension was vortexed for 5 minutes. The conidial concentrations were

determined using an improved Neubauer hemocytometer (Merck KGaA, Darmstadt, Germany) and the suspensions were adjusted to 1 × 10^6 conidia mL^{-1} in sterile distilled water.

Figure: 2.1 cultures of *B. bassiana* conidia

2.2.5 Green-house experiment

The experiment was conducted in a laboratory and an environmentally controlled research greenhouse at Cape Peninsula University of Technology (Bellville Campus), Symphony Way, Bellville, South Industrial, 7535 (S33 55.927; E18 38.379). Plant roots were washed thoroughly to remove soil debris before being transplanted to 15 cm pots, filled with the substrate mix (peat, perlite, vermiculite and silica sand). Plants were allocated randomly to one of four treatment groups (control and fungus treatments [1x10^6, 1x10^7 and 1x10^8 conidia/mL]). Each treatment was randomly allocated 25 plants, which makes 100 the number of total plants required for this research. Each plant was planted separately in a 15 cm-pot containing the following substrate mixes mentioned above. One hundred millilitres of fungal conidial suspension (1x10^6,1x10^7 and 1x10^8 conidia/mL) belonging to *B. bassiana* was added separately in the test treatments using soil drenching method. Plants in the control treatment were not exposed to fungal spores. Conidial inoculation of potted plants was repeated after three weeks. They were irrigated twice a week with 200 mL of deionized

water containing the Nutrifeed nutrient solution. The fungal suspension was added twice over a period of two weeks apart. To determine plant growth in response to combinations of substrates parameters such as the number of shoots, plant height were measured weekly. The experiment was allowed to run for four weeks, after which aerial and root fresh and dry weights were recorded. The following ambient conditions were maintained: temperature (25 ± 2 ^{o}C) and relative humidity (70%). The experiment was laid out in a randomized complete block design.

2.2.6 Assessment of colonization

The colonization of *B. bassiana* on leaves and roots of *T. violacea* was assessed after four weeks of inoculation following the method described by (Posada & Vega, 2006). One plant was used from each treatment: T1 (1×10^{6} conidial/mL), T2 (1×10^{7} conidial/mL), and T3 (1×10^{8} conidial/mL). Each plant was then washed gently with tap water, roots and leaves were separated. They were then transferred to the laboratory soaked in distilled water. Leaf sections of 1-2 mm^{2} were cut under a laminar flow hood. The sections were then surface-sterilised by dipping them in 0.5% sodium hypochlorite for 2 minutes, followed by 70% ethanol for 2 min and rinsed twice in sterile distilled water and placed on the selective medium (19.5 g Potato Dextrose Agar [PDA], 0.02 g/L of ampicillin (Sigma-Aldrich), and 0.04 g/L streptomycin [Sigma-Aldrich]) Petri dishes. They were incubated in the dark at 25 ^{o}C for daily observation, after which the presence of fungal growth was observed. Positive colonisation was scored by counting the number of plants showing outgrowth of mycelia from at least one leaf section (Figure 2.2). The data were expressed as percentage colonisation ([number of plant replicates colonised/ number of plants replicates excised] × 100). Only the plants in the fungus treatment that showed successful *B. bassiana* colonization were used.

Figure: 2.2 Mycelia outgrowth from leaf section of *T. violacea* indicating successful isolation.

2.2.7 Growth parameters

Growth was measured after the experiment. Monitoring and observation of the following parameters were recorded: Number of leaves, root length (cm), plant height (aerial part) and plant total fresh weight (g). Leaves were counted for each plant, and new shoots were also counted per plant. Plant height was measured by setting a ruler from the surface of the medium where the plant is coming out to the tip of the longest leaf of the plant.

2.2.8 Antioxidants

2.2.8.1 Sample material

At the end of the greenhouse experiment (six weeks post-treatment), plants were randomly selected according to their fungal inoculation. They were then oven-dried separately for a week at 35 °C in paper bags. Then, dried plant material was then ground, and the powder plant material was surrendered into plastic bags. Three samples from each treatment, signifying three replicates, were selected and weighed. A mass of 0.1 g of powdered plant material was transferred into centrifuge tubes. The samples were separately extracted d with 25 mL of 60% ethanol and placed inside the incubator for 24 hours. Only plants from the fungus treatments that were successfully

colonised and *B. bassiana* re-isolated from were used for this analysis. Control plants were also included in the antioxidant capacity bioassays.

2.2.8.2. FRAP

For Ferric Reducing Antioxidant Power (FRAP) assay, a method described by Benzie and Strain (1996), was followed. This assay is based on the reduction of ferric-tripyridyl triazine complex to its ferrous in the presence of antioxidants. Reagents were prepared as required by mixing 2.5 mL of a 10 mmol/L TPTZ (2,4,6- tripyridyl-s-triazine, Sigma-Aldrich, Cape Town, South Africa) solution in 40 mmol/L HCl plus 2.5 mL of 20 mmol/L $FeCl_3$ and 25 mL of 0.3 mol/L acetate buffer, and maintained at pH 3.6 prepared freshly and warmed at 37°C. Aliquots of 40 µL of the sample supernatant were mixed with 0.2 mL distilled water and 1.8 mL FRAP reagent. After incubation at 37 °C for 10 min, the spectrophotometric method was applied to read the absorbance of the reaction mixture at 593 nm. The standard solution was 1 mmol/L of $FeSO_4$, and the result was expressed as the concentration of antioxidants having a ferric-reducing ability equivalent to 1 mmol/L $FeSO_4$.

2.2.8.3 Trolox equivalent antioxidant capacity (TEAC)

The antioxidant activity of the *T. violacea* was measured following the TEAC method described by Miller *et al*. (1993). The TEAC values were measured on the antioxidants' ability to scavenge the blue-green coloured $ABTS^{\bullet+}$ radical cation relative to the $ABTS^{\bullet+}$ radical cation scavenging ability water-soluble.

2.2.8.4 DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate)

The DPPH radical scavenging abilities were determined according to the procedure described by Brand-Williams *et al*. (1995) with minor modification. Each sample with a fixed volume of 0.1 mL was mixed with 0.9 mL DPPH solution (0.15 mM in methanol) and stored in the dark at room temperature for 60 min (the time for reactions to reach a plateau). Then, its absorbance was measured at 517 nm using a microplate reader (Synergy H1, BioTek, CA, USA). The DPPH radical scavenging activity was expressed

as the percentage of inhibition of the DPPH according to the expression: $(A_0-A_t)/A_0 \times 100\%$, where A_0 is the initial absorbance and A_t is the absorbance at 60 min."

2.2.9 Statistical analysis

The data on plant fresh weight, roots fresh weight, plant dry weight, roots dry weight, FRAP (µmol AAE/g), TEAC (µmol TE/g), DPPH (µmol TE/L), plant height and root length were collected. The data were analysed using one-way ANOVA (TIBCO Statistica® 13.3.0 Dell Inc., USA). The post hoc Turkey HSD was applied for the separation of means.

2.3 Results

2.3.1 Re-isolation of fungus from *T. violacea*

After four weeks of inoculation, *B. bassiana* was successfully isolated from the three-fungal treatments. Mycelia outgrowth on *T. violacea* leaves was 58.82% in treatment 1 (1×10^6), 47.06% from treatment 2 (1×10^7), and 64.71% from treatment 3 (1×10^8). No fungal outgrowth occurred in control plants.

2.3.2 Growth of *T. violacea*

Results of the study showed that plants inoculated with *B. bassiana* and control treatment showed no significant difference ($P>0.05$) at four weeks post-treatment (Table 2.1). There was no significant difference in heights among different treatments ($DF = 3,76$; $F= 0.293$; $P = 0.830$). However, T2 (1×10^7) showed the highest height compared to other treatments. The number of leaves showed no significant difference between treatments ($DF = 3, 96$; $F = 2.014$; $P = 0.119$). Nevertheless, T2 (1×10^7) and control had more leaves than the other treatments. For the aerial part fresh weight (g), no significant difference ($DF = 3.60$; $F = 0.815$; $P = 0.491$) was observed among treatments, even though T1 (1×10^6) led to the highest weight and T2 (1×10^7) had the lowest weight (Table 2.1). Moreover, no significant difference was shown in the root fresh weights (g) ($DF = 3.60$; $F = 0.133$; $P = 0.939$). Also, no significant difference was recorded in the dry root weights (g) ($DF = 3.60$; $F = 0.593$; $P = 0.622$).

Table 2.1 The effects of *B. bassiana* inoculation with varying conidial suspensions on different growth parameters of *T. violacea* at four weeks post-treatment.

Treatments Conidia/ml	Aerial part height (cm)	Number of leaves	Aerial part fresh weight (g)	Aerial part dry weight (g)	Root length (cm)	Root fresh weight (g)	Root dry weight (g)
T1 (1×10^6)	5.6±0.7a	2.9±0.2a	11.2±1.3a	1.3±0.1a	26.8±0.6a	17.9±1.4a	3.1±0.3a
T2 (1×10^7)	6.1±05a	3.7±0.2a	9.5±0.5a	1.3±0.1a	27.7±0.5a	17.5±1.3a	2.6±0.3a
T3 (1×10^8)	5.8±0.8a	3.1±0.2a	10.0±0.4a	1.0±0.1a	27.8±0.6a	17.1±1.3a	2.9±0.2a
Control	5.3±0.6a	3.6±04a	10.8±0.9a	1.3±0.1a	25.8±0.8a	18.3±1.6a	2.8±0.3a

Means followed by the same lowercase letters in a column (Table 2.1) are not significantly different ($p > 0.05$) following the comparison of treatments using Tukey's test. T1 (1×10^6 conidial/mL), T2 (1×10^7 conidial/mL), and T3 (1×10^8 conidial/mL)

2.3.3 Antioxidants content

Generally, results showed significant differences amongst treatments for DPPH (DF = 3,8 F = 35.6; P>0.01) on the aerial part of the plants, while there was no significant difference (P>0.05) on the roots among treatments. Furthermore, no significant difference for (TEAC, DF = 3,8; $p > 0.05$) and (FRAP, DF = 3,8; P> 0.05) in both the aerial parts and roots among treatments (Table 2.2).

Table 2.2 Antioxidant capacity of the leaf and root samples of *Tulbaghia violacea* inoculated with different concentrations of conidia of *Beauveria bassiana* at four weeks post-treatment.

	DPPH (µmol TE/L)		FRAP (µmol AAE/g)		TEAC (µmol TE/g)	
Treatments	**Leaves**	**Roots**	**Leaves**	**Roots**	**Leaves**	**Roots**
Control	**151.6±2.8 a**	**84.0±1.6a**	**42.3 ± 5.1a**	**48.2 ± 4.9a**	**233.6 ± 14.7a**	**194.5 ± 13.6a**
T1 ($1x10^6$)	**101.7± 1.2b**	**71.5±0.9b**	**43.4 ± 4.2a**	**59.0 ± 6.7a**	**214.6 ± 8.1a**	**189.7 ± 2.7a**
T2 ($1x10^7$)	**120.2± 5.7c**	**82.6±1.9a**	**42.8 ± 3.4a**	**54.1 ± 8.0a**	**227.1 ± 10.9a**	**201.7 ± 20.3a**
T3 ($1x10^8$)	**135.6±3.0ac**	**87.3±2.5a**	**43.4 ± 3.6a**	**55.6 ± 4.9a**	**227.3 ± 6.0a**	**212.2 ± 5.3a**

Means with same lowercase letters in the same column, for roots or leaves are not significantly different (P> 0.05) following comparison using Tukey test. T1 (1×10^6 conidial/mL), T2 (1×10^7 conidial/mL), and T3 (1×10^8 conidial/mL)

2.4 Discussion

B. bassiana conidia successfully colonised 58-64% of leaf and root tissues of *T. violacea*. This was an indication that the fungus was moderately endophytic, and the inoculum was transferred from roots to the leaves. It is worth mentioning that successful (75%) colonisation of *B. bassiana* in *T. violacea* has been reported previously on leaves (Staffa *et al*., 2020). The success of colonisation of B. bassiana is influenced by inoculation method, biotic and abiotic factors (Tefera & Vidal, 2009).

Despite evidence of successful colonisation of *T. violacea*, entomopathogenic fungi *B. bassiana* had no significant effect (P> 0.05) on growth among treatments nor showed any significant difference in the number of leaves. Similar results were also obtained by Moloinyane & Nchu (2019) in a study when the same *B. bassiana* strain (SM3) was inoculated in *Vitis vinifera*. Tall and Meyling (2018) reported that some strains of entomopathogenic fungi induce growth in plants. On the other hand, Espinoza *et al*. (2019) reported the limited effect of *B. bassiania* on the growth of chives (*Allium schoenoprasum* L. [Amaryllidaceae]). Jaber & Enkerli (year) stated that there are

inconsistencies in the plant growth parameters of plants inoculated with entomopathogenic fungi. However, researchers such as Bamisile *et al*. (2018) and Dera *et al*. (2017) have indicated that *B. bassiana* positively influences growth, length, development, and health of chive and cabbage.

In the current study, chemical analysis showed that the leaves of T. violacea inoculated with B. bassiana and the control plants contained antioxidant capacity measured by FRAP, DPPH and TEAC assays. Results showed that there were significant differences ($P < 0.05$) between the different treatments in DPPH, TEAC and FRAP. However, there was no significant difference ($P>0.05$) on the roots among treatments. Interestingly, leaves from the control treatment had higher antioxidant capacity than leaves of the fugus-treated plants. Luximon-Ramma *et al*. (2002) reported that antioxidants capacity were strongly correlated with total phenols. Furthermore, the positive high correlation between antioxidant capacities and total phenolic content indicates that phenolic compounds are a major contributor to the antioxidant activity of these plants (Song *et al*., 2010). However, the antioxidant capacities of samples might be influenced by many factors such as test system and may not be fully described by a single method (Fu *et al*., 2011). Alcalde et al. (2019) claim that most natural antioxidants are multifunctional, and the behaviour of each compound varies in response to different methods. Therefore, in the current study, different antioxidant activity assessments could have yielded different results. Besides, the current results indicate that B. bassiana inoculation did not influence antioxidants of T. violacea.

2.5 Conclusion

The fungus successfully colonized the plants that were inoculated. However, there was no evidence that endophytic fungus positively influenced the plant growth and the antioxidant activity. This study contributes to the current body of knowledge on the interaction between endophytic fungus and plant *T. violacea.*

CHAPTER 3

Assessment of the effect of *Beauveria bassiana* inoculation on the toxicity of *Tulbaghia violacea* extracts and synthesized gold nanoparticles against grapevine mealybug

3.1 Introduction

Mealybugs (Hemiptera: Pseudococcidae) are classified as small soft-bodied plant sap-sucking insects that represents the largest family of scale insects (Hemiptera: Coccoidea), with more than 2000 described species and 290 genera (Franco *et al*., 2009). Sap-sucking insects such as mealybugs are a big concern in horticultural systems (Schulze-Sylvester *et al*., 2021). Vijay *et al*. (2020) claims that mealybugs have a wide distribution. Their infestation is quicker due to their polyphagous status which drives extreme outbreaks in many crops. They cause economic damage to many crops and losses are estimated to be in the range of 10-60% depending on the crop (Diwan *et al*., 2020).

Mealybugs are difficult to control with insecticides due to their protection from sprays by their cryptic lifestyle (Ji *et al*., 2020). Two chemical insecticides that are widely used to control mealybug are organophosphates and neonicotinoid (Daane *et al.* 2020). However, using chemical insecticides repeatedly to control mealybug could lead to insecticide resistance (Cocco *et al*., 2021). Hence, the use of insecticides with novel modes of action and long-lasting effectiveness combined with eco-friendly semiochemical based tools is a sustainable approach (Mansour *et al*., 2018).

Recently, there is increasing evidence proving that entomopathogenic fungi such as *Beauveria bassiana* are effective biological control agents against insect pests (Dannon *et al*., 2020). Some fungi can colonize plant tissues without harming the host plants (Agbessenou *et al*., 2020). Plants colonized by endophytic fungi may have enhanced production of volatile compounds (González-Mas *et al*., 2021). Some of the volatiles enhanced by the endophytic fungus *B*. *bassiana* have been reported to have insecticidal or insect repellent properties (Moloinyane and Nchu, 2019; González-Mas *et al*., 2021).

Synthesis of nanoparticles from *B. bassiana* has been reported before (Tyagi *et al*., 2019; Bhadani *et al*., 2022). Santos *et al.* (2021) reported biomass production and synthesis of silver nanoparticles using entomopathogenic fungi isolates. There have been reports on synthesis of nanoparticles using plants and different plant parts (Matussin *et al*., 2020). A previous study carried out by Ogunyemi *et al*. (2019) reported successful synthesis of zinc oxide nanoparticles (ZnONPs) using plant extract of chamomile flower (*Matricaria chamomilla* L.), olive leaves (*Olea europaea*) and red tomato fruit (*Lycopersicon esculentum* M). Hence, the hypothesis for this study is that fungal inoculation will possibly enhance nanoparticle synthesis because endophytic fungi can enhance the synthesis of secondary metabolites in plants.

The aim of this chapter was to assess the effect of *B. bassiana* inoculation on the synthesis of gold nanoparticle and toxicity against grapevine mealybug.

3.2 Materials and methods

3.2.1 Plant material

Six-week-old *T. violacea* Harv. plants were purchased from Stodels Garden Centre, Cape Town. Large clumps of *T. violacea* seedlings were divided and gently washed under a running tap for 4- 5 min. After that, they were transplanted into 15 cm pots with a substrate mix of peat, silica sand, perlite and vermiculite (1:1:1:1 in volume). Each pot was then irrigated twice a week with 200 mL of deionized water containing nutrient solution (Nutrifeed® from Stodels Pty Ltd), which consist 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}), Fe (1500 mg kg^{-1}), Mo (10 mg kg^{-1}), Mg (22 mg kg^{-1}), Mn (240 mg kg^{-1}), S (75 mg kg^{-1}), B (240 mg kg^{-1}), and Zn (240 mg kg^{-1}).

3.2.2 Insect rearing

Adult females of *Planococcus ficus* were obtained from the ARC 21 Infruitec-Nietvoorbij (Agricultural Research Council) Stellenbosch, South Africa courtesy of Dr K.A. Achiano. The mealybugs were reared on butternut squash in a darkroom at 25 ºC and 60% RH (Figure 3.1). Ten adult female mealybug was used for each treatment in this study.

Figure:3.1 Mealybugs rearing on butternuts

3.2.3 Fungal culture

Entomopathogenic fungal culture of *B. bassiana* strain (SM3) obtained from existing cultures in the Department of Horticultural Sciences Research Laboratory, Cape Peninsula University of Technology, was used for this study. The fungus was isolated from a soil sample collected in the Cape Winelands by Moloinyane & Nchu (2019). The fungus was cultured on Potato dextrose agar (half-strength) supplemented with 0.02 g/L of ampicillin (Sigma-Aldrich, Johannesburg, South Africa), and 0.04 g/L streptomycin (Sigma-Aldrich, South Africa). Uncontaminated fungal sub-cultures were grown on Potato Dextrose Agar (PDA) in 9 cm diameter Petri dishes and incubated at 25 °C for 21 days. Three to four weeks old cultures of *B. bassiana* conidia were scrapped using a sterile spatula and suspended into 500 mL bottles of sterile distilled water containing sterile 0.01% Tween 80 v/v. The suspension was vortexed for 5 min to ensure the separation of conidia. The conidial concentrations were determined

using an improved Neubauer haemocytometer (Merck KGaA, Darmstadt, Germany), and the suspensions were adjusted to 1 × 10^6 conidia mL^{-1} in sterile distilled water.

3.2.4 Colonization of *B. bassiana*

The colonization of *B. bassiana* on leaves and roots of *T. violacea* was assessed after four weeks of inoculation following the method described by (Posada & Vega, 2006). One plant was used from each treatment: T1 (1×10^6 conidial/mL), T2 (1×10^7 conidial/mL), and T3 (1×10^8 conidial/mL). Each plant was then washed gently with tap water, roots and leaves were separated. They were then transferred to the laboratory soaked in distilled water. Leaf sections of 1-2 mm^2 were cut under a laminar flow hood. The sections were then surface-sterilised by dipping them in 0.5% sodium hypochlorite for 2 minutes, followed by 70% ethanol for 2 min and rinsed twice in sterile distilled water and placed on the selective medium (19.5 g Potato Dextrose Agar [PDA], 0.02 g/L of ampicillin (Sigma-Aldrich), and 0.04 g/L streptomycin [Sigma-Aldrich]) Petri dishes. They were incubated in the dark at 25 ^oC for daily observation, after which the presence of fungal growth was observed. Positive colonisation was scored by counting the number of plants showing outgrowth of mycelia from at least one leaf section. The data were expressed as percentage colonisation ([number of plant replicates colonised/ number of plants replicates excised] × 100). Only the plants in the fungus treatment that showed successful *B. bassiana* colonization were used.

3.2.5 Greenhouse experiment

One hundred plants were gently washed under a running tap for 4- 5 min. Plants were allocated randomly to one of four treatment groups (control 0 and fungus treatments [1×10^6, 1×10^7 and 1×10^8 conidia/mL]). Each treatment was randomly allocated 25 plants, which makes a total of 100 plants. Each plant was planted in a 15 cm pot containing the following substrate mix as previously described. One hundred millilitres of fungal conidia suspension (1×10^6,1×10^7 and 1×10^8 spores/mL) belonging to *B. bassiana* was applied to the substrate mix in the test treatments by soil drenching. Plants in the control treatment were not exposed to conidia. Conidial inoculation of potted plants was repeated after three weeks. They were irrigated twice a week with 200 mL of deionized water containing nutrient solution (Nutrifeed® from Stodels Pty

Ltd, Bellville, Cape Town). Number of shoots and height were measured twice weekly under the temperature (25 ± 2^{o}C) and relative humidity (70%).

3.2.6 GC-MS Analysis (Head Space)

3.2.6.1 Sample Preparation

The fresh successful colonized fungus treated *T. violacea* leaves and roots (Figure 3.2) were frozen at -80 ˚C (overnight). The leaves and root samples were freeze-dried, and liquid nitrogen (N_2) was added. Only plants from the fungus treatments that were successfully colonised were used for this analysis and three replicates were used per treatment. The samples were then crushed, 1 g was weighed into a solid phase microextraction (SPME) vial and 2 mL of 12% alcohol solution (v/v) at pH 3.5 were added into the vial, followed by 3 mL of 20% NaCl solution. The samples were vortexed, and the headspace of the samples was analysed using a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre (gray).

Figure 3.2 Preparation of samples per treatment

3.2.6.2 Chromatographic identification of constituents

Separation of volatile compounds was performed with a gas chromatograph (6890N, Agilent Technologies Network) coupled to an inert XL EI/CI Mass Selective Detector (model 5975B, Agilent Technologies Inc., Palo Alto, CA). The GC-MS system was coupled to a CTC Analytics PAL autosampler, and the separation of volatiles present in the samples was achieved on a polar ZB-WAX (30 m, 0.25 mm ID, 0.25 µm film thickness) Zebron 7HGG007-11 capillary column. Helium gas was used as the carrier at a flow rate of 1 mL/min. The injector temperature was maintained at 250 °C, and the split ratio was set at 5:1. The oven temperature was programmed as follows: 35 °C for 6 min, at a rate of 3 °C/min to 70 °C for 5 mins, then at 4 °C/min to 120 C for 1 min and finally increased to 240 °C at a rate of 20 °C/min and held for 2.89 min. The mass selective detector was operated in a full scan mode and the source and quad temperatures were maintained at 230 °C and 150 °C, respectively. The transfer line temperature was maintained at 250 °C. The mass spectrometer was operated under electron impact mode at ionisation energy of 70 eV, scanning from 35 to 500 m/z. Relative ratios were calculated using the expression (peak area/IS peak area) × IS concentration (IS = internal standard), and hence, are only approximate values. Only the organic volatile compounds with a match quality of at least 90% were identified and reported.

3.2.7 Analysis of secondary metabolites in leaves of inoculated plants

For the analysis of secondary metabolites, only plants from the fungus treatments that were successfully colonised were used for this analysis. Control plants were also selected and in total three replicates per treatment were used. *T. violacea* materials were air-dried for 30 days (Figure 3.3?) and ground.

Figure:3.3 *T. violacea* material air-dried

Total alkaloids: The spectroscopic method was used to determine total alkaloids in the plant (Fadhil *et al*., 2007). Briefly, 0.1 g of powdered *T. violacea* leaves were extracted with 25 mL of 60% ethanol and 40% of sterile distilled water for 24 hours in total darkness, centrifuged (4000 x g for 10 min), and the supernatant was used in the assay. Subsequently, two mL of the extract supernatant and atropine standard solutions were mixed with 12 mL bromocresol green solution and 5 mL Concentration and pH of the buffer. Twelve mL of chloroform was added to the above-mentioned solution, and the solution was mixed using a vortex mixer. The spectrometric absorbance at 417 nm and a standard curve of atropine ranges between 1 and 20 µmol/L were used to determine the concentration of mg atropine equivalent per g dry weight (mg AE/g DW) in the sample.

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

Total flavonol: The determination of flavonol content was done using the protocol described by Daniels *et al*. (2011). Quercetin standard concentrations of 0, 5, 10, 20, 40, and 80 mg/L in 95% ethanol (Sigma-Aldrich, South Africa) were used. 12.5 µL of

the crude sample extracts were mixed with 12.5 µL 0.1% hydrochloric acid (HCl) (Merck, South Africa) in 95% ethanol in the sample wells, and then incubated for 30 min at room temperature. The results are expressed as mg quercetin equivalent per g dry weight (mg QE/g DW).

3.2.8 Biosynthesis of gold nanoparticles

The *T. violacea* materials were air-dried for 30 days and ground to powder using???. Ten grams of ground materials were used for extraction. One hundred millilitres (100 mL) boiled distilled water was mixed with the 10 g of ground plant material to reduce the chances of fermentation due to contamination. After cooling down, the gravimetric filtration was carried out in a centrifuge tube by centrifugation. The solution was then purified further using a filter syringe and placed in the freezer and lyophilized. During the lyophilization process, the first step involves condensation-freezing liquid product to solid at a minimum temperature of -40 ºC, followed by the sublimation process whereby moisture is extracted from the frozen product to gas (vapour), while still maintaining the materials crystalline structures. Thereafter, the vacuum removed the moisture and non-condensable vapour from the frozen product chamber or tray. This lyophilization process was carried out for 24 h.

Sixteen milligrams of the lyophilized *T. violacea* plant leaves and roots was dissolved in 2 mL of distilled water. After which, 8.5 mL of 0.01wt% of $NaAuCl_4 \cdot 2H_2O$ was measured and 1.7 mL of the plant extract was added in a 96- well cell culture plate dropwise. The solution was heated at 70 ℃ in a standard oven for 1 h. Thereafter, the solution was allowed to cool down at room temperature and diluted in a 1:1 ratio twice, resulting in two samples diluted from the solution sample treatment. This method was similar to that of Mohadeseh *et al*. (2021).

3.2.8.1 Extraction preparation

Extract of *T. violacea* were prepared by manually crushing of fresh leaves and roots from all treatments separately using Cemotec™ laboratory grinder. Then, 12 mg each crushed leaves and roots was poured into test tubes with 25 mL of 100% ethanol. The samples were mixed well using a vortex mixer, labelled accordingly, and then placed in a dark room for 24 hours. The supernatant was separated using Whatman filter

paper no 1. The filtrate from the extracts was transferred into new centrifuge tubes that were pre-weighed. The solvent was evaporated from the filtrate using a fan, and the yield of the extract was determined. Only plants from the fungus treatments that were successfully colonised were used for this analysis. Plants from the control treatment were also selected for extraction and insect bioassay. Three replicates per treatment were used.

3.2.9 Toxicity bioassay

The toxicity bioassay used in this study was a modification of that described by Nchu *et al*. (2005). After the extraction, the dried extracts from the four treatments: treatment 1 ($1x10^6$ conidia/mL), treatment 2($1x10^7$ conidia/mL), treatment 3($1x10^8$ conidia/mL) and control treatment were mixed with .DMSO. To achieve the final concentration of 0.005% w/v, 2 mg of ethanol extract in 22 mL of DMSO (dimethyl sulfoxide) was prepared for the anti-insect bioassay. Each of the four treatments had three replicates. The negative control had only DMSO. KEMPRIN 200 EC from ARYSTA LifeScience South Africa (Pty) Ltd. was used as the positive control at the recommended dose of 1.0 mL/10 L. Each Petri dish was labelled accordingly. A total of 10 adult female mealybugs (*P. ficus*) were treated with 2 µL of extract mixture dispensed topically on the dorsal side of 10 mealybugs in each petri dish. The percentage mortality was checked after 24h. Dimethyl sulfoxide (DMSO) may be toxic certain insects due to concentration. However, in this current study it was not found toxic.

3.2.10 Toxicity of extracts of AuNPs

Adult females of *Planococcus ficus* were obtained from the ARC 21 Infruitec-Nietvoorbij (Agricultural Research Council) Stellenbosch, South Africa courtesy of Dr K.A. Achiano. The mealybugs were reared on butternut squash in a darkroom at 25 ºC and 60% RH (Figure 3.4). An immersion bioassay was performed following a method described by Zaheer *et al*. (2021). For each aqueous nanoparticle solution,10 mealybugs were immersed in 1 mL of each solution of the replica for 1 min. Mealybugs were then transferred to Petri plates and kept in an incubator room at 25 ºC for a period of 4 days.

3.2.11 Statistical analysis

The mortality was analysed using one-way way ANOVA (TIBCO Statistica® 13.3.0 Dell Inc., USA). The post hoc Tukey HSD was applied for separation of means. To compare the number of volatiles produced by plants in fungus and control, Pearson Chi-square test was used. To analyse synthesis of nanoparticles UV-visible spectroscopy (UV-Vis) spectroscopy (BMG LABTECH-SPECTROstar-Nano) was used.

3.3 Results

3.3.1 Re-isolation of fungus from *T. violacea*

After four weeks of inoculation, *B. bassiana* was successfully isolated from the three-fungal treatments. Mycelia outgrowth on *T. violacea* leaves was 58.82% in treatment 1 (1×10^6), 47.06% from treatment 2 (1×10^7), and 64.71% from treatment 3 (1×10^8). No fungal outgrowth occurred in control plants.

3.3.2 GC-MS Analysis (Head Space)

Several volatile compounds were detected in the GC-MS analysis carried out on plant extracts (leaves and roots) of *T. violacea* (Table 3.1). Some of the detected compounds included well-known anti-insecticide volatiles such as Methionol, Dimethyldisulfide, N-hexanal, Undecane, Verbenene, Isoamyl_acetate, Alpha-Terpinene, Limonene, Eucalyptol, Trans-2-hexenal, P-cymene, 1-Octen-3-ol, Phenylethyl_alcohol, Tetradecanoic acid, ethyl_ester, Trans-Caryophyllene, Gamma-terpinene and 2-pentylfuran (Table 3.2). The number of compounds obtained in plants subjected to different concentrations of fungal inoculum and control did not vary significantly in both leaves (χ^2 = 2.57; P>0.05) and roots (χ^2 =0.92; P>0.05). There was no clear trend in the quantity (relative area ratio) of the detected compounds for both leaves and roots of *T. violacea* between plants exposed to different concentrations of fungal inoculum and control treatment (P>0.05). However, it was observed that roots had a higher number of compounds than leaves (Table 3.1).

Table: 3.1 Volatile organic compounds with a match quality of at least 90% present in fungal treated and control roots and leaves of *T. violacea*.

Roots				
Compounds	**Control**	**T1 ($1x10^6$)**	**T2 ($1x10^7$)**	**T3 ($1x10^8$)**
Methionol	+	+	+	+
Dimethyldisulfide	+	+	+	+
N-hexanal	+	+	+	+
Ndecane	+	+	+	+
Verbenene	+	+	+	+
Isoamyl acetate	-	-	+	-
Alpha-terpinene	+	+	+	+
Limonene	+	+	+	+
Eucalyptol	+	+	+	+
Trans-2-hexenal	+	+	+	+
2-pentylfuran	+	+	+	+
Ethyl hexanoate	+	+	+	+
P-cymene	+	+	+	+
Cis-3-Hexenyl formate	+	+	+	+
2,4-dithiapentane	+	+	+	+
Methyl heptanoate	+	+	+	+
2-Pentyn-1-ol	+	+	+	+
Ethyl_n-heptanoate	+	+	+	+
Dimethyl trisulfide	+	+	+	+
Verbenyl ethyl ether	+	+	+	+
2 octenal	+	+	+	+
Ethyl octanoate	+	+	+	+
Acetic acid	+	+	+	+
1-Octen-3-ol	+	+	+	+
2-sec-Butyl-3-methoxypyrazine	+	+	+	+
Ethyl nonanoate	+	+	+	+

L-linalool	+	+	+	+
1-octanol	+	+	+	+
Alpha-trans-beta-bergamotene	+	+	+	+
Methyl methylthiomethyl disulfide	+	+	+	+
Trans-pinocarveol	+	+	+	+
Cis-verbenol	+	+	+	+
1,4-dimethyltetrasulfide	+	+	+	+
1,2,4-trithiolane	+	+	+	+
6-camphenol	+	+	+	+
Methyl-N-Hydroxybenzene carboximidoate	+	+	+	+
1,1 bis(methylmercapto)methyl sul	+	+	+	+
Hexanoic acid	+	+	+	+
Ethyl laurate	+	+	+	+
Benzyl alcohol	+	+	+	+
Phenylethyl alcohol	+	+	+	+
Tetradecanoic acid ethyl ester	+	+	+	+
Methyl palmitate	+	+	+	+
Ethyl palmitate	+	+	+	+
Bis(2-sulfhydrylethyl)-disulfide	+	+	+	+
Ethyl (11E)-11-hexadecenoate	+	+	+	+
Benzyl benzoate	+	+	+	+
Total number of compounds	46	46	47	46

Leaves

1-propanethiol	+	+	+	+
Methionol	+	+	+	+
Dimethyldisulfide	+	+	+	+
Limonene	+	+	+	+
Trans-2-hexenal	+	+	+	+
2-pentylfuran	+	+	+	+
Gamma-terpinene	+	+	+	+
Ethyl hexanoate	+	+	+	+
P-cymene	+	+	+	+

Cis-3-Hexenyl_formate	+	+	+	+
2,4-dithiapentane	+	+	+	+
Cis-3-Hexenyl acetate	+	+	+	+
Ethyl n-heptanoate	+	+	+	+
Dimethyl_trisulfide	+	+	+	+
Verbenyl ethyl ether	+	+	+	+
Cis-3-hexenol	+	+	+	+
2_octenal	+	+	+	+
Ethyl octanoate	+	+	+	+
Acetic acid	+	+	+	+
1-Octen-3-ol	+	+	+	+
Cis-3-hexenyl 2-methylbutanoate	+	+	+	+
Trans,trans-2,4-Heptadienal	+	+	+	+
L-linalool	+	+	+	+
1-octanol	+	+	+	+
Trans-caryophyllene	+	+	+	+
4-terpineol	+	+	+	+
Beta-cyclocitral	+	+	+	+
Methyl methylthiomethyl disulfide	+	+	+	+
(Z)-3-hexenyl pentenoate	+	+	+	+
Benzyl formate	+	+	+	+
Linalyl propionate	+	+	+	+
1,4-dimethyltetrasulfide	+	+	+	+
Dimethyl trithiocarbonate	+	+	+	+
B-phenylethyl formate	+	+	+	+
Methyl-N-hydroxybenzenecarboximidoate	+	+	+	+
Nerol	+	+	+	+
1,1_bis(methylmercapto)methyl sul	+	+	+	+
Benzyl alcohol	+	+	+	+
Phenylethyl alcohol	+	+	+	+
Beta-ionone	+	+	+	+
(-)-Nerolidol	+	+	+	+
Tetradecanoic acid ethyl ester	+	+	+	+

Methyl palmitate	+	+	+	+
Ethyl palmitate	+	+	+	+
Ethyl (11E)-11-hexadecenoate	+	+	+	+
Dihydroactinidiolide	+	+	+	+
1,2,4,6-tetrathiepane	+	+	-	+
8,11-Octadecadienoic acid methyl ester	+	+	-	+
Ethyl linoleate	+	+	+	+
Linolenic acid methyl ester	+	+	+	+
Benzyl benzoate	+	+	+	+
Total number of compounds	51	51	49	51

(+) Indicates that the compound is detected in that particular treatment while (–) indicates that the compound is not detected in that particular treatment.

Table 3.2 Selected and well-known insecticidal volatiles detected in *T. violacea* and their relative area ratios following gas chromatography-linked mass spectrometry analysis of control and fungus treated plants.

Compound	Reference	Plant part	Mean ± T1 ($1x10^6$)	T2 ($1x10^7$)	T3 ($1x10^8$)	Control
	Pavela & Vrchotova (2012)	Roots	1.88±0.52a	2.00±0.21a	2.07±0.96a	0.40±0.03a
Methionol*		Leaves	1.07±1.07a	3.61±1.39a	4.31±0.22a	3.96±1.07a
Dimethyldisulfid	Diaz-mantano & Trumble (2012)	Roots	3.53±1.17a	2.85±0.41a	3.03±1.23a	1.28±0.14a
e*		Leaves	9.39±1.00a	6.75±1.04a	8.17±1.34a	6.22±0.48a
N-Hexanal	Prates et al. (1998)	Roots	22.87±6.37a	15.02±3.20a	13.50±2.24a	11.68±0.42a
		Leaves	-	-	-	-
Undecane	Ebandollahi & Taghinezhad (2020)	Roots	0.10±0.07a	0.06 ± 0.02a	0.05 ± 0.02a	0.31 ± 0.02b
		Leaves	-	-	-	-
Verbenene	Herrera et al. (2015)	Roots	0.26 ± 0.03a	0.28 ± 0.03 a	0.41 ± 0.02a	0.32 ± 0.09a
		Leaves	-	-	-	-
Isoamyl acetate	Velayutham & Ramanibai	Roots	0.01±0.01a	0.04 ± 0.03a	-	0.33 ± 0.33a
		Leaves	-	-	-	-
alpha-Terpinene	Pavela (2011) R	Roots	0.40±0.12a	0.37±0.30a	0.60±0.38a	0.34±0.15a
		Leaves	-	-	-	-
Limonene*	Hebeish et al. (2008)	Roots	1.03±0.06a	1.25±0.34a	1.11±0.07a	1.26±0.90a
		Leaves	0.42±0.16a	0.53±0.17a	0.72±0.21a	0.48±0.10a
Eucalyptol	Morreti et al. (2014) R	Roots	0.61±0.54a	1.41±0.18a	0.58±0.25a	0.24±0.03a

		Leaves	-	-	-	-
Trans-2-Hexenal*	Chen et al. (2015)	Roots	0.29±0.02a	0.30±0.06a	0.32±0.07a	0.17±0.02a
		Leaves	9.17±2.79a	7.30±2.23a	6.62±1.00a	7.68±1.76a
p-cymene*	Sener et al., (2009)	Roots	0.35±0.21a	0.250.08a	0.42±0.09a	0.210.07a
		Leaves	0.48±0.06a	0.32±0.23aa	0.76±0.20a	0.98±0.15a
1-Octen-3-ol	Herrera et al. (2015)	Roots	0.35±0.12a	0.33±0.11a	0.58±0.04a	0.20±0.07a
		Leaves	-	-	-	-
Phenylethyl_Alcohol*	Prates et al. (1998)	Roots	0.92±0.06a	1.11±0.40a	1.55±0.43a	1.12±0.11a
		Leaves	-	-	-	-
Tetradecanoic acid, ethyl_ester	Okonkwo & Onyeji (2018)	Roots	0.20±0.05a	0.92±0.44a	0.50±0.13a	0.93±0.41a
		Leaves	-	-	-	-
trans-Caryophyllene	Jung (2015)	Roots	-	-	-	-
		Leaves	0.54±0.21a	0.11±0.07a	0.31±0.16a	0.20±0.07a
gamma-Terpinene	Jung (2015)	Roots	.	-	-	-
		Leaves	1.41±1.30a	1.70±0.63a	1.82±0.58a	1.76±0.69a
2-Pentylfuran	Cha et al., (2020) R	Roots	0.80±0.12a	0.47±0.22a	0.50±0.08a	0.33±0.18a

	Leaves	0.22±0.03a	0.17±0.11a	0.27±0.08a	0.20±0.08a
Total number of compounds	Roots	15	15	14	15
	Leaves	8	8	8	8

Means for anti-insecticidal compounds with same lowercase letters in the same column, for roots and leaves are not significantly different ($p < 0.05$) following comparison using Tukey test. 8

3.3.3 Effect of fungus on secondary metabolites

The results showed that there was no significant difference between treatments (Table 3.3) in the total flavonol (mg QE/L) (DF = 3.8; F = 2.05; p =0.19) and total polyphenol (mg GAE/g) contents. Alkaloids (mg/L) were not detected on both leaves and roots.

Table:3.3 Effect of *B. bassiana* inoculation on secondary metabolites of *Tulbaghia violacea* on different treatments.

	Flavonols (mg QE/L)		Polyphenols (mg GAE/g)		Alkaloids (mg/L)	
Treatments	**Leaves**	**Roots**	**Leaves**	**Roots**	**Leaves**	**Roots**
Control	**8.5 ± 1.5a**	**N. D**	**9.7 ± 0.7a**	**7.4 ± 0.3a**	**N. D**	**N. D**
T1 ($1x10^6$) conidia mL-1	**6.4 ± 2.7a**	**N. D**	**9.5 ± 0.6a**	**8.5 ± 0.7a**	**N. D**	**N. D**
T2 ($1x10^7$) conidia mL-1	**5.0 ± 1.0a**	**N. D**	**10.0 ± 0.8a**	**8.2 ± 0.9a**	**N. D**	**N. D**
T3 ($1x10^8$) conidia mL-1	**4.9 ± 2.3a**	**N. D**	**10.0 ± 0.6a**	**7.7 ± 0.7a**	**N. D**	**N. D**

Means with same lowercase letters in the same column, for roots and leaves are not significantly different (P> 0.05) following comparison using Tukey's pairwise.

3.3.4 Biosynthesis of gold nanoparticles

3.3.4.1 AuNP Formation

The addition of *T. violacea* leaf extract solution (50 µL) from different treatments into gold metal (250 µL) resulted in the development of an array of colours after the reaction mixture at 70 °C, revealing the formation of gold nanoparticles (Figure 3.4). No significant change in the appearance of the product was observed over several hours of maintaining the solution, indicating that the synthesized Au NPs were stable.

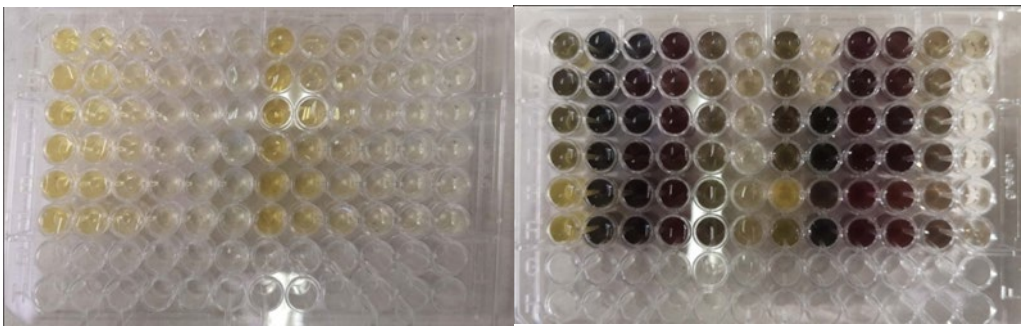

Figure:3.4 Showing (left) plate before the oven and showing (right) plate after the oven (Reaction of AuNPs).

Figure 3.5 presents the dynamic UV-visible absorption spectroscopy of the Au-NPs synthesized using aqueous *T. violacea* extract on three different treatments (treatment 1 (1×10^6 conidia/ml), treatment 2 (1×10^7 conidia/mL), treatment 3 (1×10^8 conidia/mL)) including control treatment. The UV-visible spectroscopy was only used to analyse the surface plasmon resonance (SPR) peak absorption of AuNPs of the treatments at different wavelength. However, size and morphology of AuNPs was not tested in this study.

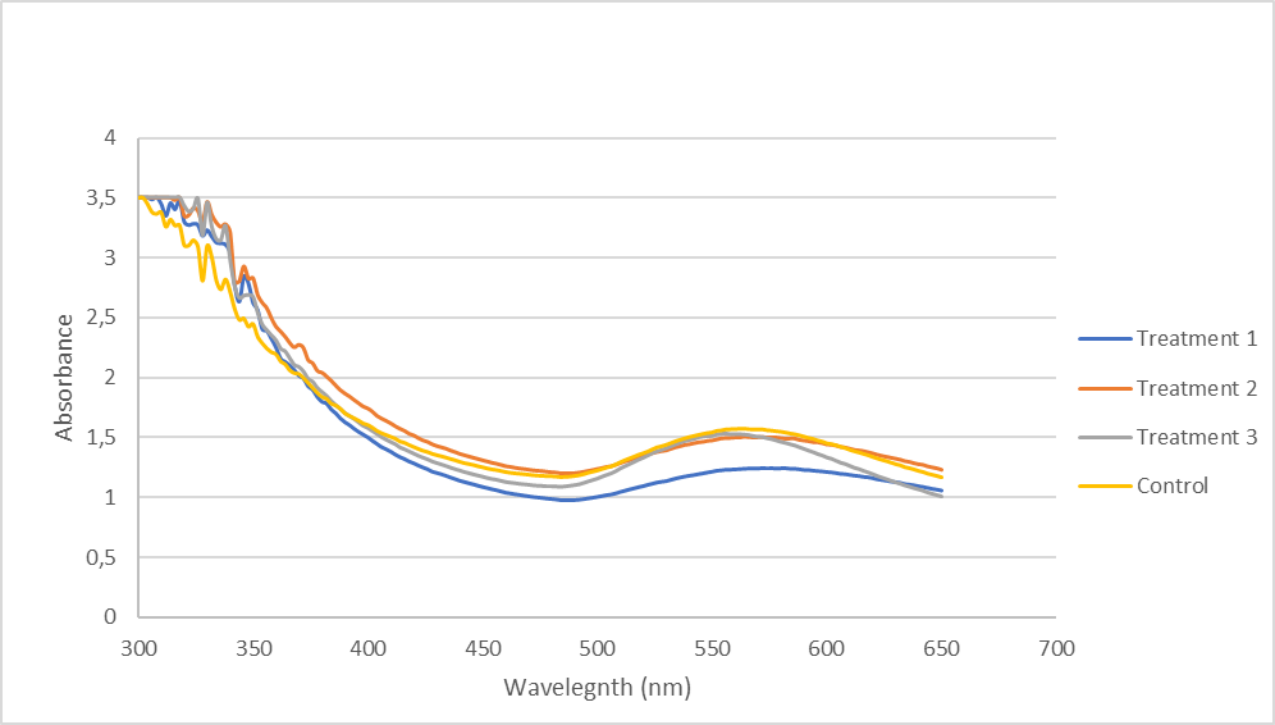

Figure:3.5 UV-Visible spectrums of synthesized AuNPs showing a peak at different wavelength per treatment. Different colours representing different treatments peaks and different wavelengths.

3.3.5 Toxicity bioassay

There was a significance difference (P<0.01) in insect mortality among the treatments on both leaves (DF = 5,12; F=2.99; P = 0.05; P = 0.01) and root extracts(DF = 5,12; F=5.39; P = 0.001) (Table: 3.4); leaves. Positive control had the highest mortality followed by extract from treatment 3 and negative control had the least mortality on leaves. The 2 controls one was a solvent and the other one a treatment control.

Table:3.4 Effect of *B. bassiana* inoculation on female *P. ficus* mortality of extracts of *T. violacea*

	Insect mortality	
Treatments	**Leaves**	**Roots**
	24h	**24h**
T 1	7,67±1,02ab	8±2ab
T2	7,33±1,45ab	10±3,33a
T 3	8±2ab	7,67±1,20ab
Control	7±0,58ab	8,67±0,88ab
Negative control	3,33±0,88b	3,33±0,88b
Positive control	10±3,33a	10±6,67a

Means with same lowercase letters in the same column, for roots and leaves are not significantly different (P> 0.05) following comparison using Tukey's pairwise. T1, 1×10^6 conidia mL^{-1}; T2, 1×10^7 conidia mL^{-1}; T3, 1×10^8 conidia mL^{-1}; Control, 0 conidia mL^{-1}

3.3.6 Toxicity of extracts of AuNPs

Broadly, results showed that the gold nanoparticle synthesized by extracts of *T. violacea* induced mealybug mortality. However, gold nanoparticle solution from the plants that were inoculated with fungus conidia mL^{-1} induced higher mortality compared with the control treatment (DF = 5.12; F = 17.3 P < 0.01). It is worth noting because the aqueous component of the gold solution was not evaporated and separated from the solid AuPs, the exact concentration of the gold nano-particle were not determined in this study. The two controls, one was a solvent and one a treatment control.

Table: 3.5 Percentage of mortality mean ± SE of female *P. ficus* exposed to Aqueous gold nanoparticles (AuNPs) solution of plants inoculated with *B. bassiana* at different treatments as well as a negative and positive control.

Treatments	**Mortality**
-VE control	0±0a
Control 0	1±0.33ab
Treatment 1	7±0.3bc
Treatment 2	5±0.33abc
Treatment 3	6±1.15bc
+VE control	10±0c

Means ± SE within columns followed by the same lower case is not significantly different (P>0.05) following comparison of Tukey HSD test. T1, 1×10^{6} conidia mL^{-1}; T2, 1×10^{7} conidia mL^{-1}; T3, 1×10^{8} conidia mL^{-1}; Control, 0 conidia mL^{-1}

3.4 Discussion

Green synthesis of gold nanoparticles by *T. violacea* inoculated with *B. bassiana* was successfully achieved in this study, and this was confirmed through a UV-Vis spectrometer. However, the nanoparticles were not characterized in this study. Future studies will characterize and test the exact concentration of synthesized gold nanoparticles. This was not the first time *T. violacea* was used for green synthesis of nanoparticles. Similar findings were found in the Mbenga *et al.* (2022) study, which looked at nanoarchitectonics of ZnO nanoparticles mediated by extract of *T. violacea* and their cytotoxicity evaluation. Furthermore, insect toxicity evaluation of aqueous solution of nanoparticles synthesised by extract of *T. violacea* was carried out for the different treatments. Although there was a significant difference ($DF = 3$; $P < 0.05$) in insect mortality, with aqueous AuPs solutions of *B. bassiana* treated plants having higher mortality, these results need to be interpreted with caution because the exact concentrations of the AuPs in the solutions was not determined. Benelli (2018) revealed that there is a rise on the nanotechnology and arthropod control science to manage pest population. Numerous researchers have reported on the toxicity of nanoparticles towards various arthropod species (Pavoni *et al*. 2019; Pilaquinga *et al*. 2019).

There was a significant difference ($P<0.01$) amongst treatments in insect mortality induced by the leaf and root extracts from different treatments including negative and positive treatments. There was a significant difference between negative control and positive control on leaves. Furthermore, roots showed there was significant difference amongst the treatments. Interestingly, treatment 2 and positive control were significantly higher (Table 3.4). The significant difference might be due to Allium species being toxic against insects. Jimenez *et al*. (2019) reported Allium species are toxic to some insects with sucking including aphids. Furthermore, Staffa *et al*. (2020) had positive findings on inoculation of *T. violacea* with an endophytic arthropod-pathogenic fungus against ticks. Interestingly, Nchu *et al*. (2016) reported that dichloromethane extract of garlic showed positive effects against ticks.

Secondary metabolite compounds protect plants against various biotic and abiotic stresses (Tian *et al*., 2020). In this study it was observed that the leaves and roots extracts of *T. violacea* produced several well-known anti-insect compounds such as Methionol, Dimethyldisulfide, N-hexanal, Undecane, Verbenene, Isoamyl_acetate, Alpha-Terpinene, Limonene, Eucalyptol, Trans-2-hexenal, P-cymene, 1-Octen-3-ol, Phenylethyl_alcohol, Tetradecanoic acid, ethyl_ester, Trans-Caryophyllene, Gamma-terpinene and 2-pentylfuran (Table 3.2). However, the number of compounds obtained in plants subjected to different concentrations of fungal inoculum and control did not vary significantly ($P > 0.05$) in both leaves and roots, which indicates that the fungal inoculum did not affect the number of compounds (Table 3.2). It is not surprising given that the secondary metabolite contents, both volatile and non-volatile constituents, did not vary significantly with fungal inoculation. Baron & Rigobelo (2022) states that endophytic fungi influence on secondary metabolites is influenced by the plant species and the portion of the plant colonized.

Manipulation of abiotic and biotic factors to improve secondary metabolites production in plants have yielded encouraging results. Factors such as light, water and nutrients can influence the production of secondary metabolites in plants (Bont *et al*., 2020). In recent years interests in the use of biotic agents, such as fungi and bacteria, to optimize secondary metabolites production in plants have been growing (Thakur *et al*., 2019). Some microbes are beneficial to plants. They perform the same role as chemical fertilizers and pesticides (Timmusk *et al*., 2017). *T. violacea* inoculated with *B. bassiana* conidia was assessed for potential influence on secondary metabolites. There was no significant difference ($P>0.05$) among treatments. Polyphenol contents were not influenced by the exposure of *B. bassiana* on leaves. Furthermore, flavonols also showed no significant influence on leaves. Alkaloids were not detected on both roots and leaves. Rocchetti *et al*. (2022) mentioned low detection of alkaloids on many allium species. Furthermore, Esienanwan *et al*. (2020) reported an absence of alkaloids in some of the allium species. Noticeably, flavonols were highly concentrated in the control treatment on leaves compared to the other three treatments. Previously, Espinoza *et al*. (2019) reported similar findings looking at the effect of the same fungal strain on the secondary metabolite content of chives.

3.5 Conclusion

Generally, in the current study, the key finding is that the extracts of leaves and roots of *T. violacea* in both fungal and control treatments were able to synthesis gold nanoparticles. However, *B. bassiana* did not have a significant influence on the number of compounds obtained from the leaves and roots. Also, the exposure of entomopathogenic fungi did not improve polyphenol and Flavonols content of *T. violacea*. Remarkably, the extracts of *T. violacea* exposed to both fungus and control treatments were equally toxic to mealybug induced high mortality. This study provides insights into the endophytic *B. bassiana*-*T. violacea*-mealybug relationship and further recommends studies on characteristics of nanoparticles induced by the fungal endophyte.

Chapter four

4.1 General discussion and recommendations

Insect pests are mainly controlled using chemical insecticides, which are environmentally unfriendly (Kaur *et al*., 2019; Mpumi *et al*., 2020). Also, pests tend to develop resistance towards synthetic chemicals over time (Kole *et al*., 2019). This has motivated researchers to investigate procedures to control insects (Deng *et al*., 2018). The use of entomopathogenic fungi has gained interest among many researchers (Islam *et al*., 2021; Santos *et al*., 2022). Endophytic fungi do not only act protect plants against pests but also influences plant growth positively (Mantzoukas & Eliopoulos, 2020).

Despite successful colonization, the results showed that plants inoculated with *B. bassiana* and control treatments had no significant influence ($P>0.05$) in heights, number of leaves, aerial part fresh weights, root lengths, root fresh weights and root dry weights among different treatments. Numerous researchers have reported on the inconsistency of entomopathogenic fungi (Jaber, 2018; Moloinyane & Nchu, 2019; Staffa *et al*., 2020). Interestingly, results showed that leaves of *T. violacea* inoculated with *B. bassiana* and the control plants exhibited antioxidant activities in FRAP, DPPH and TEAC assays. Furthermore, the was a significance differences among the treatments in DPPH, FRAP and TEAC assays.

Secondary metabolites were not affected by the *B. bassiana* inoculation. There were no significant differences amongst treatments on polyphenol and flavonols. Interestingly, alkaloids were not detected. Li *et al*. (2020b) mentioned that synthesis and accumulation of secondary metabolites are very complex, thus affected by many factors such as internal developmental genetic (enzyme, regulated gene) and by external environmental factors such as light, temperature, water etc. However, in the current study there were several volatile compounds detected in the fungus-treated plants and in control treated plants. It was also observed that the leaves and roots extracts of *T. violacea* produced several well-known anti-insect compounds such as Methionol, Dimethyldisulfide, N-

hexanal to name few. However, they did not vary significantly. Litwin *et al*. (2020) mentioned that plants emit several anti-insects compounds as a defence against insects. Moreover, several researchers reported endophytic fungi to produce anti-insecticide volatile compounds (González-Mas *et al*., 2019; Moloinyane & Nchu. 2019).

Green synthesis of nanoparticles has recently emerged as a promising approach due to its eco-friendliness (Soni *et al*., 2018). Synthesis of nanoparticles from B. *bassiana* has been reported before (Khooshe-bast *et al*., 2016). In the current study, green synthesis of gold nanoparticles by *T. violacea* was successfully achieve. Interestingly, for the formation of AuNPs the addition of *T. violacea* leaf extract solution (50 µL) from different treatments into gold metal (250 µL) resulted in the development of an array of colours after the reaction mixture at 70 °C, revealing the formation of gold nanoparticles (Figure:3.5). The UV-visible spectroscopy (UV-Vis) spectroscopy (BMG LABTECH-SPECTROstar-Nano) was applied for analysis (Figure 3.6). However, in this study, we could not differentiate exactly the quantity of nanoparticles produced by fungus -plant and nanoparticles produced by control. However, it is possible that one way of getting around this problem could be to look at the scanning electron microscope (SEM) and transmission electron microscope (TEM) analyses in the future to be able to characterise and see the differences in nanoparticle obtained from plants exposed to fungus and control treatments.

Toxicity evaluation of *T. violacea* inoculated with *B. bassiana* against mealybugs was carried out on different treatments. With mortality rate evaluated at 24 hrs, There was significant difference among the treatments including negative and positive control. Positive control performed better on leaves compare to other treatments. However, on roots both treatment 2 ($1x10^7$) and positive control performed better.

4.2 Conclusion

This study contributes to a deeper understanding of the endophytic fungus-plant-insect relationship, effect of volatile organic compounds in crop protection and mediation of insect. Filling up the knowledge gap in using green synthesis of nanoparticles and fungi as bio-control agents. However, more research and analysis needed in understanding the relationship between fungi and nanoparticle characteristics against insects.

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