Cape Peninsula University of Technology

# ANTI-TICK ACTIVITIES OF EXTRACTS OF *TULBAGHIA VIOLACEA* (ALLIACEAE) CULTIVATED IN HYDROPONIC MEDIA AMENDED WITH ENTOMOPATHOGENIC FUNGI (HYPOCREALES)

By

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## DECLARATION

I, Pumla Staffa, 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.

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**Signed** **Date**

## DEDICATION

To God almighty

To My family

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First and foremost, I would like to thank God almighty for having made everything possible by giving me strength and courage to do this work. If it was not for the Lord, I would have never could have made it, the enemy could have swallowed me.

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## LIST OF ACRONYMS

| Acronym | Meaning |
|---|---|
| ANOVA | Analysis of Variance |
| CPUT | Cape Peninsula University of Technology |
| EC | Electrical conductivity |
| N | Nitrogen |
| Na | Sodium |
| P | Phosphorus |
| K | Potassium |
| Zn | Zinc |
| Mn | Manganese |
| Ca | Calcium |
| B | Boron |
| WHO | World Health Organization |
| EPF | Entomopathogenic Fungi |
| DMDS | Dimethyl disulfide |
| NaCl | Sodium chloride |
| EFC | East Coast fever |

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## ABSTRACT

Ticks and tick-borne diseases are important limiting factors to the attainment of sustainable animal and human health, affecting livelihood of resource poor farming communities in developing countries. *Rhipicephalus appendiculatus* and *Amblyomma variegatum* (Ixodidae) are among the most troubling tick species in Africa. While ticks can be controlled by applying chemical acaricides, these chemicals are quite expensive, especially, for small-scale famers in developing countries. Hence, the quest for alternative tick control over the years have revealed that entomopathogenic fungi (EPF) and plant extracts have huge prospects as sustainable alternatives for tick control. *Beauveria bassiana* (Hypocreales) is a fungal entomopathogen with the ability to colonize plants endophytically and induce secondary metabolite production in plants, and it has been found to be a potential biological control agent against a wide range of arthropods. Several plant species including plant species belonging to the family Alliaceae possess anti-tick activities (repellent and toxic); therefore, integrating the two strategies by inoculating *Tulbaghia violacea* with *B. bassiana* could enhance secondary metabolite contents in extracts obtained from the plant and increase medicinal materials.

The primary purpose of the present study was to evaluate the effect of indigenous endophytic entomopathogens inoculation of *T. violacea* on the plant growth, tissue nutrient contents and secondary metabolites, and anti-tick activities of extracts of *T. violacea*. The specific objectives were: (i) to carry out a study on the effects of inoculating *T. violacea* with fungal strains of *B. bassiana* and *Clonostachys rosea* on plant growth and tissue nutrient contents in extracts of *T. violacea* with the view of selecting one of the two fungi for further investigation (ii) to compare secondary metabolite profiles of extracts obtained from plants exposed to fungus (EPF) inoculum and control treatment during cultivation, and (iii) to determine whether exposure of *T. violacea* to an endophytic fungus (*B. bassiana*) during cultivation affects the repellent and acaricidal activities of extracts of *T. violacea* on *A. variegatum* and *R. appendiculatus.*

The study had two experiments, presented in chapters two and three. In the first experiment, eight weeks old potted seedlings of *T. violacea* were inoculated separately with *B. bassiana* (strain SM3) and *C. rosea* (strain SM8) conidia suspended at concentrations of 1 x $10^6$ conidia $mL^{-1}$. Plant growth parameters, such as number of leaves, plant height (aerial part), fresh weights of aerial parts, and tissue nutrient contents were assessed. Results indicated that *B. bassiana* induced higher growth of plants than *C. rosea*. Inoculation with *B. bassiana* did not significantly ($P > 0.05$)

influence most of the growth parameters, number of leaves, fresht weight of roots and of fresh weight aerial parts (leaves) of *T. violacea* assessed in the current study. However, mean plant heights and root lengths were significantly ($P < 0.05$) higher in favour of *B. bassiana* treated plants compared to those in *C. rosea*. Fe contents in the roots (1416.3 ± 305.10 mg/kg) were found to be positively influenced ($P < 0.05$) by the fungal inoculation. There was a significant difference in roots ($P < 0.05$) on the uptake of Mn in *C. rosea* treated plants (243 ± 19 mg/kg) compared to the control group (169 ± 16.37 mg/kg) and *B. bassiana* treated roots (161.3 ± 14.44 mg/kg). Macro nutrients up take did not differ significantly among treatments on both leaves and roots ($P > 0.05$). On the basis of the plant growth results, *B. bassiana* was therefore, selected for further evaluation in the proceeding chapter (chapter three).

The indigenous *B. bassiana* strain (SM8) was used in the proceeding experiment, which investigated the effect of fungal inoculation on colonization and and anti-tick activities of the *T. violacea*. Eight weeks old seedlings of *T. violacea* plants were cultivated in growth medium in the presence of *B. bassiana* inoculum, while the control medium had no fungal inoculum. The growth medium consisted of a mixture of inert materials (peat, silica sand, perlite and vermiculite) supplemented with hydroponic nutrient solution and water. The experiment carried on for twelve weeks. The following parameters were recorded and assessed: tissue colonization by conidia of *B. bassiana*, anti-tick bioactivities (repellency and toxicity) and secondary metabolite content. Five grams of fresh plant materials (roots and leaves) were crushed separately and extracted with acetone (25 ml). The extracted materials were used to test repellency of the leaf and root extracts of *T. violacea* against *R. appendiculatus* larvae in a repellency bioassay. For the toxicity bioassay, the juice of crushed leaves and roots of fungus-exposed and unexposed plants were used. The acaricidal activities of the juice extracts of *T. violacea* against adults of the hard ticks *R. appendiculatus* and *A. variegatum* were carried out in a contact tick toxicity bioassay. In order to determine secondary metabolite contents, plant materials were analyzed for total polyphenols and total alkaloids. The chemical profile of the volatiles was also determined using Headspace Gas Chromatography/Mass Spectrometry method.

Successfully tissue colonization (75% of leaves and 91.6% of roots) by *B. bassiana* conidia among fungus inoculated plants were achieved. The fungus did not have any significant effect on the polyphenol content ($P > 0.05$). In the GC-MS analysis, besides 4-Terpineol and 9,12-Octadecadienoic acid- ethyl that were significantly different ($P < 0.05$) in area ratios between fungus and control treated plants, the analysis revealed that broadly the fungus did not affect the profile of most the volatile chemical constituents. The repellent effects on *R. appendiculatus* larvae

showed that when extracts of plants were exposed to fungal inoculation were assessed at a high concentration (20 w/v%), it produced the least repellent effects. Generally, the results of this study revealed that juice extracts of *T. violacea* were highly toxic to both tick species, with both fungus and control treatments recording high tick mortality (> 90%); no significant effect of fungus was detected in this study.

In conclusion, while the fungal inoculation, generally, did not significantly influence most of the tissue macro- and micro-nutrients, rare observations of significant effects on the uptake of Fe, and Mn were detected and are worth noting. The experimental fungal inoculation of plants (*T. violacea*) produced variable effects on the plant growth and tick reppellency of extracts of *T. violacea*. The study further showed that extracts of *T. violacea* are repellent against *R. appendiculatus larvae* and juice extract from aerial parts possess potent acaricidal activity against adults of *R. appendiculatus* and *A. variegatum*.

# CHAPTER ONE

## General introduction and literature review

### 1.1: Introduction

Ticks are medically and veterinary important parasites that affect both domestic and wildlife animals. They cause direct harm to animals and also transmit important pathogens (Rajput et al., 2006). Invasion of ticks can result in huge losses in livestock farming (Eyo et al., 2014). Ticks and tick-borne diseases (TBDs) are the most widespread of all the major livestock problems in Africa (Dipeolu et al., 1992; Walker et al., 2003). There have been many reports of tick parasitizing humans (Horak et al., 2002; Louly et al., 2008). For examples, *Hyalomma rufipes* and *Amblyomma variegatum* (Acari) have been reported to feed on humans in South Africa (Parola et al., 2001. Horak et al., 2002). Ixodid ticks from the genus *Hyalomma, sub-genus Boophilus, Rhipicephalus* and *Amblyomma* are among the most economically important parasites of livestock in the tropical and sub-tropical parts of the world, including South Africa (Spickett et al., 2011).

Conventional control of ticks has relied on synthetic chemicals; however, with the increasing recognition of the risks associated with these synthetic acaricides and that they become unaffordable to most livestock farmers (Njoroge & Bussmann, 2006), more consumers are seeking alternative tick control agents such as botanicals. Plant extracts from many species, including *Tagetes minuta, Lippia javanica* and *Azadirachta indica* etc., are known to be bioactive against ticks (Borges et al., 2003; Isman, 2006; Ribeiro et al., 2007). Recently, few commercial botanical products against ticks have been registered (Habeeb, 2010), for example, Tre-san®, MiteStop®, Wash Away Louse® and Picksan LouseStop®, all based on neem seed extracts (Schmahl et el., 2010). Some species of the genus *Allium* have been demonstrated to have acaricidal and tick repellent properties, for example *Allium cepa* and *Allium sativum* (Jarial, 2001; Nchu et al., 2005). These plants have insecticidal chemical constituents such as allicin. The genus, *Tulbaghia* is said to be closely related to the genus *Allium* (Lyantagaye, 2011) and also has chemical constituents such as allicin. Allicin (diallyl thiosulfinate) is a defense molecule from garlic (*Allium sativum*) with a broad range of biological activities (Borlinghaus et al., 2014). As the herbal product industry develops, the demand for highly efficacious and standardized efficacy of plant extracts also increases. Therefore, cultivation methods play an important role in the efficacy of plant extracts. Results from recent studies suggest that the manipulation of plant nutrient concentration can influence secondary metabolites production (Ncube et al., 2012).

Plants grown hydroponically have an advantage over field grown plants in that production is all year round and chemical constituents in the plants can be manipulated by controlling growing conditions (Treftz & Omaye, 2016). The level at which human use medicinal plants is very high (Patel et al., 2012). Primary economy is highly dependent on medicinal and aromatic plants because of the nonstop and increased demand for their products at local, national and international markets (Kumari & Prasad, 2013). Medicinal plants are largely used for their contents of bioactive compounds and are increasingly cultivated on a commercial scale to satisfy the large demand for natural remedies (Maggini et al., 2012). The way wild plant resources are used and managed will significantly influence the sustainability of people's livelihood and the conservation plant's biodiversity (Amujoyegbe et al., 2012). Therefore, cultivation of medicinal plants is vital. It gives an opportunity to increase yields and good quality plants (Amujoyegbe et al., 2012). Medicinal plant production through the cultivation can reduce the amount to which wild populations are harvested, but it also may lead to environmental degradation and loss of genetic diversity, as well as loss of incentives to conserve wild populations (Schippmann et al., 2002). According to Zabalgogeazcoa (2008) plant species are associated with fungal endophytes.

Endophytic fungi or endophytes are vital constituents of plant micro-ecosystems which commonly occur inside the healthy tissues of living plants (Jia et al., 2016). It has been shown that endophytic fungi played a very essential role in affecting the quality and quantity of crude drugs (Faeth & Fagan, 2002). Through a particular fungus-host interaction, these fungi demonstrate that interactions between endophytic fungi and medicinal plants are required for promoting crude drug production (Faeth & Fagan, 2002). Fungal endophytes produce some bioactive compounds often refer to as secondary metabolites (Zhang et al., 2006; Rodriguez et al., 2009). Numerous plants have been used to isolate endophytic fungal strains, for example fodders, vegetables, fruits and other crops (Rosenblueth & Romero, 2006). However, there are scarce reports on the efficacy of *T. violacea* against ticks. It is hypothesized that amending growth medium with endophytic fungal spores will induce higher anti-tick activities of extracts of hydroponically-cultivated *T. violacea*.

In this study, a plant growth substrate mix was amended with conidia of the entomopathogenic fungi (EPF), *B. bassiana*. EPF promote plant growth, act as antagonists of plant pathogens and may influence secondary metabolite constituents of plants (Lopez et al., 2014). The findings

from this research project may benefit small-scale livestock farmers, who are struggling to control ticks and the diseases they transmit.

### 1.2: Structure of the dissertation

This thesis consists of three chapters, which are briefly described.

**Chapter one:** Introduction, literature review/background problem statement: this chapter presents the theoretical framework of the research, provides scientific justification of the study and the aims and specific objectives of the study.

**Chapter two:** This chapter focuses on the assessment of the effects of *Beauveria bassiana* and *Clonostachys rosea* inoculation on growth parameters of *Tulbaghia violacea*.

**Chapter three:** This chapter presents findings of a detailed assessment of the effects of *Beauveria bassiana* inoculation on tissue colonization by fungal conidia, secondary metabolites and anti-tick activities (repellency and toxicity) of *Tulbaghia violacea* (alliaceae).

### 1.3: Literature review

#### 1.3.1: Introduction to the literature review

The use of ethnoveterinary medicine for treatment of animal diseases, including wounds, has been in practice for a long time in the Eastern Cape Province (Masika, et al., 1997; Van Wyk, 1997) especially for resource-poor farmers. Herbal products have played an important role in the drug discovery and development processes (Eloff, 1998; Newman & Gragg, 2007). Traditional remedies had been the main source of livestock ailment treatments in regions of poor resources of South Africa (Magwede et al., 2014). The genus *Allium* is well-recognized for their anti-tick activities as well as their bioactive secondary metabolites (Jarial, 2001; Nchu et al., 2005). Bioactive compounds are thought to occur in most members of this family e.g. *A. sativum* and *A. cepa*. Many studies have revealed that the quantity and quality of secondary metabolites are influenced by environmental conditions, which include biotic and abiotic factors (Akula & Ravishankar, 2011). Secondary metabolites play a vital role in the adaptation of plants to the altering environments (Ncube et al., 2012). Inoculation of plants with fungus enhanced secondary metabolite in species (Ding et al., 2018; Adolfsson et al., 2017).

Some species of EPF such as *B. bassiana* and *Metarhizium anisopliae* (hypocreales) are currently used against a wide range of arthropods, mainly insect pests (Strasser et al., 2000). EPF are reported to be major pathogens of ticks, and are more promising than other potential biological control agents (Kaaya, 1992; Samish & Rehacek, 1999). Incorporation of these fungi in plant growth media could result in many advantages; plant growth and secondary metabolite production could be improved, thereby increasing the efficacy of extracts. Therefore, development of effective cultivation strategies that incorporate endophytic EPF is important. Apart from being good in controlling arthropods in animals, fungi are increasingly being used as soil bio-amendments. Some of these fungi are endophytic and are able to enhance plant defenses against parasites (Gawande et al., 2013). However, integration of EPF into plant growth media could enhance plant secondary metabolite production and increase bio-efficacy of plant extracts against ticks.

Ticks are a major problem for livestock, especially in developing countries, such as those in Africa (Minjauw & McLeod, 2003; Nyahangare et al., 2015, Nyangiwe et al., 2017) and TBDs have adverse impact on animal and human health leading to major negative impacts on the livelihoods of resource-poor farming communities in developing countries (Jongejan, 2004; Jabbar et al., 2015). The hard-bodied ticks consist of the most important group of vectors that transmit pathogens of veterinary importance (Hoogstraal, 1985; Jongejan & Uilberg, 2004). There are six main genera of ixodid ticks (hard bodied ticks) including the subgenus *Boophilus*, and these are *Amblyomma*, *Dermacentor, Haemaphysalis, Hyalomma, Ixodes* and *Rhipicephalus* (Jongejan & Uilberg, 2004). This study focuses on two tick species under two genera, namely, *R. appendiculatus*, and *A. variegatum*.

### 1.3.2: *Tulbaghia violacea*

*Tulbaghia* is a small plant genus in the Alliaceae family and it has about 30 species, which are mostly indigenous to the Southern Africa region (Van Wyk & Gericke, 2000; Lyantagaye, 2011; Vosa 2000, Vosa & Condy, 2006). It occurs in Southern Tanzania, Malawi, Botswana, Zimbabwe, Mozambique, South Africa, Lesotho and Swaziland (Van Wyk & Gericke, 2000; Vosa & Condy, 2001). The genus *Tulbaghia* is quite closely related to genus *Allium* and produces the ‘garlic-like odour’ (alliaceous odour). The odour is associated with cysteine-derived sulphur compounds released from wounded or decaying tissues and organs, such as the leaves and rhizomes (Kubec et al., 2013). For centuries, its nutritive, ornamental and

medicinal values, and consequently its economic importance have been recognized and highlighted (Reinten and Coetzee, 2011; Van Wyk, 2011a; Van Wyk, 2011b). Among the species in this genus, *T. violacea* remains the most popular, widely utilized and it's a highly investigated species. *Tulbaghia* is generally known as "wild garlic", "sweet garlic" or "pink agapanthus" due to its similarity to the genus *Agapanthus* (Kubec et al., 2013).

*T. violacea* is a highly utilized medicinal plant species in the Eastern Cape and KwaZulu-Natal regions (Burton, 1990; Van Wyk et al., 2000). Like many medicinal plants, there are many chemical constituents that have been identified in the Alliaceae family. Such compounds are responsible for the many biological activities of medicinal plants (Aremu et al., 2012). Based on reports from various studies (Van Wyk & Gericke, 2000; Raji et al., 2012), *Tulbaghia* may possess biological activities similar to garlic (*A. sativum*) since both belong to the family Alliaceae. For example, the bioactive components of garlic are mainly responsible for its healing properties (Kallel et al., 2014).

#### 1.3.2.1: Taxonomic classification and general characteristics of the species *T. violacea.*

*T. violacea* is a small perennial bulbous herb with corm-like rhizomes and narrowly linear, evergreen aromatic leaves, with hairless leaves with a white, fleshy stalk (Van Wyk et al., 1997; Kubec et al., 2013) (Figure 1.1). It has mauve or pale purple flowers, occurring in groups of about ten at the tip of the slender stalk. *T. violacea* prefers partial shade or partial sun to full sun; and dry to moist soils. Mature height of *T. violacea* ranges from 30 cm to 120 cm depending on the environmental conditions. *T. violacea* is commonly known as wild garlic, "wilde knoffel" (Afrikaans), "isihaqa" (Zulu) or "itswele lomlambo" (Xhosa) and is indigenous to the Eastern Cape Province (Van Wyk et al., 1997). To our knowledge, there is no scientific evidence that support *T. violacea's* use in ethnoveterinary medicine, especially in tick control.

**Figure 1.1:** *Tulbaghia violacea* showing pinkish-mauve tubular flowers (source: http://plantinfo.co.za/plant/tulbaghia-violacea/).

### 1.3.2.2: Medicinal and other uses of *T. violacea*

*T. violacea* is frequently used in the southern African region for combating fever, asthma, constipation, oesophageal cancer and hypertension (Van Wyk & Wink, 2004). Different plant parts of *T. violacea* are used in the treatment of a variety of conditions (Ngunge et al., 2011). Bulbs and leaves of *T. violacea* are conventionally used in the southern African region for the treatment of gastrointestinal ailments, asthma, (Ncube et al., 2011; Van Wyk & Wink, 2004). Bulbs are also used as an aphrodisiac medicine and a salvnake repellent (Van Wyk et al., 1997). Crushed bulbs repel insects and are also used for coughs and colds (Lyantagaye, 2011). The Zulu tribe eats the leaves, flowers and uses the leaves for seasoning meat and potatoes (Hutchings et al., 1996). Roots, bulbs and leaves of *T. violacea* are used in South Africa to make tea (Roberts, 1990). Apart from the medicinal and nutritive value, the plant is also used extensively as an ornamental in South Africa (Bryan, 2002). Horticultural potential of *T. violacea*, as a bedding plant, street landscapes and parks due to its attractive foliage as well as its pretty flowers has been reported (Kubitzki, 1998; Bryan, 2002). In some cultures, the leaves of *T. violacea* are used as a substitute for chives and garlic (Kubec, 2002).

#### 1.3.2.3: Propagation methods that are used

*T. violacea* can be propagated through seeds and cuttings (Aremu & Van Staden, 2013). Interestingly, micropropagation protocols have been developed for *T. violacea* (Hunter et al., 2006).

### 1.4: Cultivation of medicinal plants

Many plant species are not only used as a source of making food, but they are used as sources of non-food industrial products as well. Oils, carbohydrates, and fibers are obtained from various crops and such crops are normally cultivated on a large scale (Lubbea & Verpoorte, 2011). Medicinal plants are considered a good source of livelihoods in remote areas and play a huge role in the healthcare of livestock, therefore, their cultivation is essential (Kepe, 2007). Local communities have a strong history and beliefs of primary healthcare that is constructed on medicinal plants (Caniago & Siebert, 1998; Tanzin et al., 2010). Louw (2016) conducted a study on cultivation and gathering of medicinal plants and reported that cultivation and gathering of these medicinal plants are very important because they offer income to a large number of inhabitants. Even with the increase in synthetic drugs; however, people still rely on medicinal plants specifically in rural communities (WHO, 2004; Rao et al., 2004). In most countries, people gather medicinal plants from wild vegetation; therefore, as the demand of medicinal plants is escalating globally, these plants will have to be cultivated so that they can be supplied on a regular basis and conserved (Rao et al., 2004). A number of challenges that affect the conventional cultivation of medicinal plants. These include land availability, climate, season, water availability, diseases and pests, and slow growth of plants (Arikat et al., 2004). Hence, application of technologies that minimized the impact of these constraints in the cultivation of medicinal plants may reduce excessive harvesting of wild medicinal plants of endangered species and fast production of pharmacological compounds irrespective of seasonal and climatic conditions (Arikat et al., 2004). The success of this strategy, however, depends on the validation of micropropagated plants through pharmacological screening on their suitability for use in traditional medicine. Currently, medical plants are mostly grown under field conditions; however, it is quite hard to control ecological and climatic factors and to maintain high yield (Chen et al., 2016). Therefore, cultivation creates a chance to attain consistency on the yield of medicinal plant.

### 1.4.1: Optimizing cultivation of medicinal plants using hydroponic technology.

There is a great demand for natural remedies, as a result medicinal plants are gradually being cultivated on a commercial scale to fulfill these demands. A substitute to conventional crop production of medicinal species is hydroponic cultivation (Montanari et al., 2008). Hydroponics produces quality plant material throughout the year in consideration of the possibility to control growing conditions and to encourage secondary metabolism by correct manipulation of mineral nutrition (Maggini et al., 2012). Hydroponics can simply be defined as the growing of plants in a water and fertilizer solution holding essential nutrients for plant growth (Jensen, 1997). It can also be defined as soilless agriculture or growing plants in soilless medium (Sheikh, 2006). The advantage in hydroponics is that, plant growth is quicker and yield is higher than in the soil, and contamination by pollutants and microorganisms is lesser (Letchamo et al., 2002). Also, when growing plants in hydroponics, growing environment can be controlled to produce consistent plant material for the industrial extraction of bioactive compounds (Zheng et al., 2006).

Hydroponic growing systems are very useful in horticultural plant production and are the subject of intensive research activities (Furtner, 2006). Economic and environmental factors in particular have led to the growth of hydroponic plant production in recent decades (Nelson, 1998). The benefits of growing plants hydroponically includes high-density crop cultivation, maximum crop yield, crop production can be carried out in areas where good soil for production is not available and plants can be grown any time. Hydroponics is used mainly as a controlled system for the production of out of season crops, for growing crops in areas where the soil is not suitable for cultivation, or where water supply is limited (Putra & Yuliando, 2015). Maggini et al. (2012) conducted study on assessing the production of *Echinacea* and Basil in hydropics (floating raft system), these crops are among the commonly active medicinal plants that contain numerous bioactive molecules. They indicated that yield of *Echina*cea was higher than that of typical field crops and it grew quicker, and the basil total biomass production was about three times higher.

### 1.4.2: Exploitation of medicinal plants in South Africa

Medicinal plants have been used since time immemorial, and many cultures still rely on indigenous medicinal plants for their primary health care needs (Veilleux & King, 1996; Tripathi & Tripathi, 2003). South Africa, a country with a strong history of traditional healing, hosts around 30,000 flowering plant species (Louw et al., 2002). Medicinal plants are now commonly recognized as the basis for a number of critical human health, social and economic support

systems and benefits (Mander et al., 2006). Many cultures depend on indigenous medicinal plants for their primary health care needs (Veilleux & King, 1996; Dery, 1999). Harvesting and selling of medicinal plants provide support to many households as source of income. About 80% of the population in South Africa use traditional medicines to meet their primary health care needs; but, for financial gain, only a few indigenous medicinal plants have been exploited (Street & Prinsloo, 2012). However, Wiersum et al. (2006) reported severe harvesting of wild medicinal plants due to the increasing use has resulted in overexploitation and is a serious threat to biodiversity in the region. This severe harvesting has led to local plant extinction (Mander, 1998). The only option for many species is cultivation at a large scale so that the wild species are maintained (Cunningham, 1993).

### 1.4.3: Secondary metabolites that are found in *T. violacea*.

Bungu et al. (2002) and Aremu et al. (2013) postulated that *T. violacea* has related secondary metabolites to *A. sativum,* and they have the specific sulphur odor associated with garlic. Sulphur compounds of garlic and *T. violacea* are ascribed with the medicinal properties of garlic (Kubec et al., 2002; Kubec et al., 2013). *T. violacea* oil is rich in sulfur-containing compounds that are similar to those found in *A. sativa* (El-meleigy et al., 2010; Martinez-Velazquez et al., 2011). During crush of garlic bulb sulphur-containing compounds are formed when the enzyme allinase reacts with allicin. (Kubec et al., 2002). Bioflavonoids such as quercetin (Hutchings et al., 1996) have also been isolated from extracts of *T. violacea*.

## 1.5: Ticks

### 1.5.1: Tick classification

Ticks are ecto-parasites, which belong to phylum Arthropoda, class (Arachnida) and an order Acari (Latif & Walker, 2004). They are divided into two main families, Argasidae and Ixodidae. Argasids have soft leather scutum and are known as soft ticks while ixodid ticks possess hard plate on their scutum and is hence known as hard ticks (Walker et al., 2003). Ticks feed on their hosts, and feeding of hard ticks is slow; it takes a number of days while soft ticks feed more rapidly, for up to several hours (Walker et al., 2003; Latif & Walker, 2004). Questing of ticks parasitizes suitable hosts putting them at risk of pathogen infection (Gilbert et al., 2014). *Rhipicephalus* and *Haemaphysalis* ticks have the hunting behavior, they trail their potential host and creep through the skin to find an appropriate place to attach and feed, whereas

*Amblyomma* and *Hyalomma* run across the ground to seek host that are nearby(Walker et al., 2003). This study focused on two tick genera of the family Ixodidae; *Amblyomma* and *Rhipicephalus*.

### 1.5.2: Damages

Ticks are vectors of disease-causing pathogens to humans and livestock globally (Jernigan et al., 2000; Horak et al. 2002). They are the most important economically ectoparasite of cattle, they cause extensive losses, and tick infestations often results to the death of their hosts (Eyo et al., 2014). Damages caused by *Rhipicephalus* (Boophilus) microplus infestation in Australia during 1974, were estimated at USD 62 million, and in Brazil, loses around USD 2 billion per year were recorded (Rajput et al., 2006). Ocaido et al. (2009) reported significant damages caused by ticks in three districts of Uganda. In Tanzania a study by Kivaria (2006) showed an estimated annual loss of US$364 million with estimated mortalities of 1.3 million cattle due to tick-borne diseases. Moreover, tick infestations are responsible for severe losses through blood loss, damage to hides and udders, the transmission of toxins, or through morbidity or mortality caused by the diseases they transmit (FAO 1984). Ticks transmit diseases, produce paralysis or toxicosis, and cause physical damage to livestock (Rajput et al., 2006). Huge numbers of ticks feeding on a host can cause a decrease in weight and anemia (FAO, 1998). Tick feeding spread diseases and allows entry sites for secondary infections and myiasis while causing decreased live weight gains, milk yields and skin quality. *R. appendiculatus, Amblyomma hebraeum* Koch and *A. variegatum* are among the major economic important ticks that cause production losses in Africa. The use of chemical acaricides is currently the main defense for controlling ticks (Rajput et al., 2006; Nyangiwe et al., 2018).

## 1.5.3: Tick Control

### 1.5.3.1: Chemical control

Even though several potential alternatives for control of ticks have been documented, people still rely on chemical acaricides. Control of tick infestations through the use of acaricides is one of the methods that can be used to reduce tick-borne diseases (Spickett & Fivaz, 1992), but recently various studies have shown that ticks have developed resistance against a range of acaricides (Rajput et al., 2006; Moyo & Masika, 2009; Vudriko, et al., 2016; Nyangiwe et al., 2018). Acaricides, including arsenical, chlorinated hydrocarbons, organophosphates,

carbamates, formamidines, and synthetic pyrethroids are used for controlling ticks on livestock (Rajput al., 2006). Of these acaricides, several studies have reported the resistance of ticks to organophosphate, synthetic pyrethroids, and formamidines (amitraz) in regions where the cattle tick, *R*. (*Boophilus*) *microplus* is found (Ntondini et al., 2008; Vudriko, et al., 2016; Nyangiwe et al., 2018). Dipping, spraying, ear tagging and pour on, have been used to apply chemicals to protect livestock against ticks. Nevertheless, the use of acaricides is commonly accompanied by serious drawbacks, including chemical residues in food (meat and milk products) and environmental contamination (Rajput et al., 2006; Vudriko, et al., 2016).

**1.5.3.2: Entomopathogenic fungi (EPF)**

Various studies have aimed at developing EPF as alternative biological control agents on insects, mites and ticks (Butt et al., 2001; Goettel et al., 2005). Studies by Samish and Rehacek (1999) reported EPF as the main pathogen of ticks and it has shown most promising results among potential biological control of ticks. *B. bassiana* and *M. anisopliae* are amongst the most important fungi which are presently used against an array of arthropods, especially insect and ticks (Butt et al., 2001; Zimmerman 2007a, b). According to Fernandes et al. (2008) and Kaaya et al. (1996) some isolates of *M. anisopliae* and *B. bassiana* are capable of causing diseases to all stages of tick species, suggesting that they have high prospects as biocontrol agents of ticks. According to Benjamin et al. (2002) several strains of *M. anisopliae* are pathogenic to *I. scapularis* and can be of importance in tick management and control. A study by Campos et al. (2010) demonstrated that laboratory assays are useful to determine whether they are virulent and are selectable for field testing; they can be used to test mortality, inhibitory effects on egg production, egg hatchability and repellency. However, field trials often showed low mortality levels than those observed under laboratory conditions (Ginsberg et al., 2002; Fernandes et al., 2008).

According to Samish et al. (2004), penetration and germination of conidia under field condition is not effective enough to penetrate the cuticle or may show less lethal effects, and this may be caused by various factors, such as temperature and humidity variations, solar radiation, and host microclimatic factors (Polar et al., 2005c; Zimmerman, 2007b). It has been shown that fungal spores penetrate ticks via cuticle (Alveb, 1998). Fungal spores that successfully penetrate the cuticle attack the internal organs, produce toxic substances, and kill the host

(Kaaya et al., 1991). Studies by Roberts (1981) and Alves (1998) showed that fungal spores of *B. bassiana* and *M. anisopliae* emit toxic metabolites that cause development of disease. Kaaya et al. (1996) reported that increased number of conidia on the ticks' cuticle lead to increased mortality rate.

#### 1.5.3.2.1: *Beauveria bassiana*

*B. bassiana* is an EPF species, which is able to colonize plants endophytically. As an endophyte, *B. bassiana* may play a role in protecting plants from herbivory and disease (Parsa et al., 2013). *B. bassiana* has been reported as an endophyte in a variety of plants, including maize (Bing & Lewis 1991, 1992a, b; Wagner & Lewis, 2000) and *Theobroma gileri* (Evans et al., 2003). Research has revealed that endophytic fungi could play an essential role in affecting the quality and quantity of the crude drugs through fungus-plant host interaction (Faeth & Fagan, 2002). Also, Jia et al. (2016) mentioned that even though endophytic fungi are among the vital constituents in plant micro-ecosystems that should have significant influences on the growth and development of host plants, the knowledge about the precise connection between endophytic fungi and their host plants is still very partial. As yet, no reports are available on the potential of inoculating *B. bassiana* in *T. violacea*, an important medical plant of Africa.

#### 1.5.3.2.2: *Clonostachys rosea*

According to Sutton et al. (1997), *C. rosea* is a soil fungus that occurs in a wide range of environments. Also, it has been reported to endophytically colonize the roots of plants, such as cucumber and tomato and to induce the expression of defense related genes in wheat and canola (Alvarez Nordstrom, 2014). Interestingly, *C. rosea* can act as an antagonist to numerous plant pathogens. For example, there is evidence that *C. rosea* decreases symptoms of root disease in canola roots and chinese cabbage (Lahlali & Peng, 2013; Moller et al., 2003). Disappoint

### 1.5.3.3: Anti-tick plants and plant extracts

The use of medicinal plants to treat various diseases has been part of human culture since early times (Mewrwe et al., 2011). In Africa, farmers have used indigenous methods to treat livestock diseases, such as crude extracts of medicinal plants, manipulative techniques and herd

management (Gabalebats et al., 2013). The potential of some local plants and plant-based products to repel ticks has been demonstrated previously (Webb & David, 2002). The use of crude plant extracts in ethno-veterinary medicine represents a cheaper and easily accessible method for tick control. Most rural farmers turn to it when financial conditions do not allow them to purchase modern western medicines. This approach offers sustainable strategies directed toward developing appropriate animal health care systems suitable to rural communities for improved livestock performance and production (Wanzala et al*.,* 2014).

Extracts of tropical and subtropical plants have acaricidal effects on *R. (B.) microplus* and *R. appendiculatus* (Abdel-Shafy & Zayed, 2002). Some ingredients obtained from plant extracts, seeds, leaves and barks of trees, tubers and roots of various trees are considered valuable in animal health (Alawa et al., 2002). Such ingredients are mainly secondary metabolites which include thousands of alkaloids, terpenoids, phenolics and minor secondary chemicals (Arnason et al., 1988). The most common secondary metabolites with protective action against arthropods include non-protein amino acids, steroids, phenols, flavonoids, glycosides, alkaloids, glucosinolates, quinines, tannins and terpenoids (Pino et al., 2013). A wide variety of medicinal plants have been reported to have repellent and acaricidal effects. For example, water extracts of *Lippia javanica* (Verbenaceae) are acaricidal against *R. appendiculatus (*Nyahangare et al., 2012). Also, *Azadirachta indica (Meliaceae) have repellent and acaridiidal effects on ticks (Abdisa, 2017). Garlic extracts have high acaricidal and repellent properties (Nchu et al., 2016). Essential oil of Tagetes minuta (Asteraceae) repelled ticks and delayed hatching of ticks in vitro experiments (Nchu et al., 2012).*

**1.6: Research problem**

Ticks spread many diseases which are important to humans and livestock. The use of chemical acaricides to control ticks is currently popular. However, their uncontrolled use has led to environmental toxicity and rapid development of tick resistance to acaricide, and they are quite costly, especially for resource-poor farmers in rural areas. This has favoured the use of plant-based insecticides as suitable alternatives to synthetic insecticides. Hence, optimizing anti-ticks activities of cultivated medicinal plants, such as *T. violacea* in hydroponic medium amended with endophytic EPF (Hypocreales) may favor the production of anti-tick metabolites by plants and improve the efficacy of plants extracts against ticks.

### 1.7: Aim of the study

To evaluate the effects of amending plant growth medium with endophytic EPF (*B. bassiana*) on anti-tick activities of *T. violacea* cultivated hydroponically.

### 1.8: Specific objectives

- To carry out a trial on the effects of inoculating *T. violacea* with fungal strains of *B. bassiana* and *C. rosea* on plant growth (plant height, number of leaves, fresh weight) and tissue nutrient contents in extracts of *T. violacea* with the view of selecting the one with higher positive effects on growth parameters for a more detailed assessment.

- To compare secondary metabolite profiles of extracts obtained from plants exposed to fungus (EPF) inoculum or control treatments during cultivation.

- To determine whether exposure of *T. violacea* during cultivation affects the repellent and acaricidal activities of extracts of *T. violacea* on *A. variegatum* and *R. appendiculatus.*

### 1.9: Hypothesis

- Addition of fungal spores in hydroponic growth medium will induce production of greater quantities of anti-tick secondary metabolites by *T. violacea* compared to the corresponding growth medium with no fungal spores.

- Addition of fungal spores in hydroponic growth medium will induce higher anti-tick activities of extracts of *T. violacea* compared to the corresponding growth medium with no fungal spores.

# CHAPTER TWO

## The effects of *Beauveria bassiana* and *Clonostachys rosea* inoculation on growth parameters of *Tulbaghia violacea*

### 2.1: Introduction

Endophytic fungi form a symbiotic relationship with plants (Zhai et al., 2017). They colonize plant tissues and can improve growth and medicinal properties by increasing secondary metabolite production in plants (Muvea et al., 2014; Akello et al., 2007; Jia et al., 2016). However, recent studies have shown that they have variable physiological effects on plants depending on species and isolates of the fungi (Parsa et al., 2013). Dara et al. (2017) indicated that *B. bassiana* showed a positive influence on the survival, growth, length, and dry weight of cabbage. Kello et al. (2007) found that all plant growth parameters (plant height, leaf length and width, fresh and dry shoot weight, and the number of leaves) were significantly higher for plants dipped in a conidial suspension than for those injected with a conidial suspension of *B. bassiana.* On the other hand, Jaber and Enkerli (2017) stated that there is absence of consistency in the plant growth promotion obtained by inoculation with entomopathogenic fungi. It is plausible to think that the effect of endophytic fungi on plants is mediated through the uptake of essential plant growth nutrients. Previous studies have revealed that EPF increases the bioavailability of certain heavy metals, such as Cd, Cu, Pb and Zn (Raya-Díaz et al., 2017). Jia et al. (2016) mentioned that even though endophytic fungi are amongst the vital constituents in plant micro-ecosystems that should have significant influences on the growth and development of host plants, the knowledge about the precise connection between endophytic fungi and their host plants is still very partial.

The high demand for ethnoveterinary plants and the subsequent excessive harvesting and decrease of these plants from the wild as well as the high demand for consistency and quality medicinal materials has favored the search for new cultivation approaches. Many studies have revealed that the quantity and quality of secondary metabolites are influenced by environmental conditions, which include biotic and abiotic factors (Morison & Lawlor, 1999; Bapela et al., 2007). *Tulbaghia violacea* is among the most well-known medicinal plant species in the genus, especially in the Eastern Cape and KwaZulu-Natal regions (Burton, 1990; Van Wyk et al., 2000). This species occurs is spread accross South Africa due to cultivation in gardens and in

the commercial medicinal plant farms (Van Wyk et al., 2000). The objective of this chapter was to assess the effects of inoculating *T. violacea* with fungal strains of *B. bassiana* and *Clonostachys rosea* on plant growth and tissue nutrient contents in extracts of *T. violacea* with the view of selecting one of the two fungi for further investigation in chapter three.

## 2.2: Materials and methods

### 2.2.1: Fungi

Indigenous strains of *B. bassiana* (SM3) and *C. rosea* (SM8) were selected for the present experiment. Cultures of fungal strains were obtained from Cape Peninsula University of Technology (CPUT), Horticultural Sciences laboratory. Prior to their use, a conidial germination test to determine conidial viability was carried out according to the method described by Inglis et al. (2012) with modifications. The viability of conidia was determined by spread-plating 0.1 ml of conidia suspension, titrated at 1 x $10^6$ conidia $ml^{-1}$ on PDA plates. Two replicated sterile microscope cover slips were placed on each plate and incubated at 26 ± 2 °C. Plates were then examined after 24 h and percentage germination determined from 100-spore counts under each cover slip. The germination percentage was over 90%.

The fungi were cultured on half-strength PDA (Potato dextrose agar) amended with 0.02 g/L of ampicillin (Sigma-Aldrich), and 0.04 g/L streptomycin (Sigma-Aldrich). Clean fungal sub-cultures on agar were prepared in 9 cm diameters Petri dishes and incubated at 25 °C. *C. rosea* and *B. bassiana* conidia were harvested by scrapping 3-4 weeks old cultures using sterile spatula and suspended into 100 ml bottles of sterile distilled water containing sterile 0.01% Tween 80. The suspension was mix using a vortex shaker for 5 min to ensure separation of spores. Conidial concentrations were determined using an improved Neubauer haemocytometer and the suspensions were adjusted to 1 × $10^6$ conidia $mL^{-1}$ in sterile distilled water.

### 2.2.2: Greenhouse experiment

The experiment was conducted in a greenhouse within the nursery of the Department of Horticultural Sciences, Cape Peninsula University of Technology (CPUT), Western Cape Province, South Africa. Seedlings trays of *T. violacea* were obtained from a wholesale nursery, Western Cape Seedlings, Cape Town. Six weeks old seedlings were transplanted separately into 14 cm pots containing a substrate mix of peat, silica sand, perlite and vermiculite (1:1:1:1 in volume). There were three treatments: plants that were inoculated with *B. bassiana*, plants that

were inoculated with *C. rosea* and those that were not exposed to a fungus (control group). Plants were supplied Nutrifeed fertilizer (Stodels Garden Centre, Cape Town) containing the following ingredients: 65 g/kg N, 27 g/kg P, 130 g/kg K; 70 mg/kg Ca, 20 mg/kg Cu, 1500 mg/kg Fe, 10 mg/kg Mo, 22 mg/kg Mg, 240 mg/kg Mn, 75 mg/kg S, 240 mg/kg B and 240 mg/kg Zn. Nutrient solutions were prepared by dissolving 5 g of fertilizer in tap water in a 10-L watering can. The treatments were arranged in a randomized block design with a total of 150 plants, 50 replicates per treatment. Steel table (2.5 × 1 m) was used as a flat surface. Each treatment consisted of fifty replicates. Fifty millilitres of fungal conidial suspension ($1 \times 10^6$ conidia/ $mL^{-1}$.) belonging to *C. rosea* and *B. bassiana* was added separately in the test treatments by soil drenching. Control treatment was not exposed to fungal spores, only 50 ml of sterile 0.01% Tween 80 was added to each control replicate. After three weeks, this treatment procedure was repeated. Plants were irrigated once a week, each plant was watered with 100 mL reverse osmosis water.

The greenhouse had the following experimental conditions; average temperature; morning (18-27 °C), day (26-49 °C) and evening (18-35 °C), relative humidity; morning (64-92%), day (82-96%) and evening (81-92%). The experiment was arranged in a randomized block design and the experiment continued for 12 weeks.

### 2.2.3: Plant growth parameters

Growth measurements were taken before (baseline) and at the end of the experiment. Observations on the following parameters were recorded: Plant height (aerial part), root length, number of leaves (cm) and plant total fresh weight (g). Plant height cm (aerial part) was taken by setting a ruler from the centre of growing medium level to the tip of the long leaf of the plant, leaves were counted for each plant and new shoots were also counted per plant.

### 2.2.4: Plant tissue nutrient analyses

Plant foliage from subsamples of plants from each treatment was sent to Bemlab (Pty) Ltd (Somerset West, South Africa) for analysis. Leaves and roots were washed with Teepool solution, rinsed with deionised water and dried at 70 $^0$C overnight in an oven. Total N content of the leaves was determined through total combustion in a Leco N-analyser. The methods described by Campbell & Plank (1998) and Miller (1998) was used to determine total P, K, Ca, Mg and micro-nutrient analyses in plant tissue. 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 were analysed using Ash method.

## 2.3: Results

### 2.3.1: Growth of *T. violacea*

Growth of the plants inoculated with *B. bassiana*, *C. rosea* and the control treatments showed significant differences ($P < 0.05$) at 12 weeks after inoculation. Root length of *T. violacea* varied significantly ($df = 2, 14$; $f = 6.71$; $P < 0.05$). When comparing T1 (plants inoculated with *B. bassiana*) and T2 (plants inoculated with *C. rosea*) the highest root length (25.3 ± 0.54 cm) was obtained in plants that were inoculated with *B. bassiana* (Table 2.1). Correspondingly, the highest root weight (24.2 ± 0.84 g) was also obtained in *B. bassiana* treated plants ($df = 2, 14$; $f = 10.11$; $P < 0.05$). Also, plant height (aerial part) was statistically different between fungi treated and the control plants ($df = 2. 14$; $F = 5.76$; $P < 0.05$). Nevertheless, highest measurement was observed in *B. bassiana* treated plants (34.1 ± 0.37 cm) compared to *C. rosea* exposed plants. As well, mean fresh weight of roots showed significant differences ($df = 2, 14$; $f = 10.68$; $P < 0.05$) with *B. bassiana* treatment showing highest weight (24.2 ± 0.84 g) (Table 2.1).

**Table 2.1:** The mean ±se growth obtained following inoculation of *Tulbaghia violacea* with conidia of *Beauveria bassiana* and *Clonostachys rosea* at 12 weeks post-treatment.

| | **Root length (cm)** | **Root fresh weight (g)** | **Plant Height (aerial part) (cm)** | **Plant fresh weight (aerial part) (cm)** | **Number of leaves** |
|---|---|---|---|---|---|
| **T1** | 25.3 ± 0.54 a | 24.2 ± 0.84 a | 34.1 ± 0.37 a | 17.2 ± 0.64 b | 7.8 ± 0.23 b |
| **T2** | 22.4 ± 0.52 b | 19.1 ± 0.74 b | 33.8 ± 0.37 a | 20.4 ± 0.49 a | 8.14 ± 0.19 b |
| **Control** | 23.5 ± 0.63 a | 23.1 ± 0.97a | 32.1 ± 0.56 b | 20.4 ± 0.54 a | 9.38 ± 0. 24 a |

Means followed by lowercase letters in the same column are significantly different ($P < 0.05$) following comparison using Tukey test. T1 and T2 represent plants treated with *B. bassiana* and with *C. rosea* inocula, respectively.

### 2.3.2: Tissue analysis

#### 2.3.2.1: Macronutrients

The levels of N, P, K and Ca on leaves of *T. violacea* did not differred significantly among the tests and control treatments (df = 2, 6; P > 0.05) for all of the macro-nutrients assessed (Table 2.2a). However, for most of the macro-nutrients, *B. bassiana* showed higher mean value compared to *C. rosea* (Table 2.2a). The uptake of nutrients in the roots of *T. violacea* showed no significant variations among the three threatments for all the macronutrients assessed (N, P, K, Mg, Ca and Na (df = 2, 6; P > 0.05). However, when the two fungi were compared, *C. rosea* exposed plants had significantly higher root P compared to *B. bassiana*-exposed plants. In contrast, the tissue content of K was higher in the roots of plants that were inoculated with *B. bassiana* conidia (Table 2.2 a).

#### 2.3.2.2: Micronutrients

As shown in Table (2.2b), uptake of Mn, Fe, Cu, and Zn varied significantly (df = 2, 6; P > 0.05) in leaves of *T. violacea* among the tests and control treatments. However, there was a significantly higher (P < 0.05) up take of B in the leaves of *C. rosea* exposed plants. Similarly, there were variations in the micro-nutrients contents in the root tissue among the different treatments with *C. rosea* plants recording the highest root tissue contents of Mn and Fe (Table 2.2 b).

**Table 2.2 a:** The mean ±se (mg/kg) tissue macronutrient contents following cultivation of *Tulbaghia violacea* in the presence and absence of either *Beauveria bassiana* or *Clonostachys rosea* inoculum.

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | **LEAVES** | | | |
| **Treatments** | **N** | **P** | **K** | **Ca** | **Mg** | **Na** |
| T 1 | 52333 3± 494.13 a | 6000 ± 208.16 a | 59166 6 ± 5548.07 a | 13100 ±808.2 a | 7666.6 ± 33.3 a | 1525± 45.23 a |
| T 2 | 55866.6 ± 4252.18 a | 5833.3 ± 33.33 a | 666.0 ± 15552 a | 10466 ±60911 a | 8000 ± 115.47 a | 1511 ± 58.96 a |
| Control | 60900 ± 3044.66 a | 6133 ± 88.19 a | 68433.3 ± 1451.81 a | 12200 ±895.28 a | 7933.3 ± 185.59 a | 1691.3 ± 54.46 a |
| | | | **ROOTS** | | | |
| T 1 | 47300± 404.14 a | 17866.6 ± 896.90 a | 94500 ± 3036.99 a | 13666.6 ± 330.08 a | 10100 ± 585.94 a | 3697.6 ± 239.18 a |
| T 2 | 43466 ± 1747.69 a | 20166.6 ± 688.79 a | 88033.3 ± 3546.98 a | 13900 ±1300 a | 9900 ± 702.37 a | 3536.3 ± 203.2 a |
| Control | 48400 ± 1081.66 a | 17333.3 ± 716.52 a | 90933.3 ± 5318.31 a | 11300 ±907.37 a | 98833.3 ± 817.17 a | 3250.3 ± 225.39 a |

Means followed by lowercase letters in the same column are significantly different (P< 0.05) following comparison using Tukey test. T1 represents plants treated with *B. bassiana*, T2 represents plants treated with *C. rosea*

**Table 2.2 b:** The mean ±se (mg/kg) tissue micronutrient contents following cultivation of *Tulbaghia violacea* in the presence and absence of either *Beauveria bassiana* or *Clonostachys rosea* inoculum.

| | | | | | |
|---|---|---|---|---|---|
| | | | **LEAVES** | | |
| **Treatments** | **Mn** | **Fe** | **Cu** | **Zn** | **B** |
| T 1 | 113.2 ± 4.04a | 197.6 ±15.49 a | 7.3 ± 1.45 a | 45.6 ± 1.76 a | 103.3 ± 3.33 b |
| T2 | 141.3 ± 10.58 a | 148.3 ± 44.94 a | 5 ± 0.57 a | 52.33 ± 2.84 a | 131 ± 8.66 a |
| Control | 125 ± 5.07 a | 134.6 ± 7.00 a | 5.3 ± 0.33 a | 53.6 ± 1.76 a | 115.6 ± 5.53 b |
| | | | **ROOTS** | | |
| T 1 | 161.3 ± 14.4 b | 802 ± 18.45 b | 10.6 ± 0.66 a | 44.6 ± 1.45 a | 31 ± 0.57 a |
| T 2 | 243 ± 19 a | 1416.3 ± 44.94 a | 13.3 ± 2.40 a | 48.6 ± 2.33 a | 33.6 ± 1.20 a |
| Control | 169 ± 16.37 b | 511.2 ± 51.71 b | 10.3 ± 0.33 a | 45 ± 0.57 a | 30 ± 0 a |

Means followed by lowercase letters in the same column are significantly different ($P < 0.05$) following comparison using Tukey test. Grey and white colours are used to differentiate columns with control treatments. T1 represents plants treated with *B. bassiana*; T2 represents plants treated with *C. rosea.*

## 2.4: Discussion

These results indicated that *B. bassiana* had a better influence on growth of plants following inoculation compared to *C. rosea*. This is not unusual given that there are many previous reports of successful inoculation, colonization and enhancement of plant with *B. bassiana* (Posada et al., 2007; Muvea et al., 2014; Pelizza et al., 2017). Our findings support a previous report by Tefera and Vidal (2009), which found that *B. bassiana* had a positive influence on the survival, growth, health, length, and dry weight of cabbage. It is worth noting that endophytism is influenced by fungal species, fungal strain and inoculation method (Akello et al., 2007; Brownbridge et al., 2012; Muvea et al., 2014). The control group (plants without fungi) in many instances, performed better when compared to *C. rosea,* but when compared with plants that were inoculated with *B. bassiana*, there was no significant difference. This study demonstrates that soil drench inoculation with conidia of entomopathogenic fungi, *B. bassiana* had variable effects on the different growth parameters: *B. bassiana* did not significantly influence the number of leaves, new shoots, and fresh weight of *T. violacea* when compared with the control plants, but did influence plant height and root length significantly when compared with *C. rosea*.

There are many factors that may affect the relationship between a plant and a fungal endophyte, and consequently the effect of endophyte on plant growth. These are fungal isolates, plant host, fungal species and environmental factors (Parsa et al., 2013). For example, the study by Tefera and Vidal (2009) showed that sterile soil had an impact on roots, stem and leaf colonization of fungi on sorghum plants. Inoculation method was found to influence plant growth in bread and durum wheat plants that were inoculated with *B. bassiana* (Sánchez-Rodríguez et al., 2018).

Fungi can influence uptake of nutrients by plants (Behl et al., 2012). Evidences suggest that fungi can also influence availablility of vital growth elements in growth medium (Rashid et al., 2016). In the current study, Fe and B significantly varied among the three treatments with *C. rosea* having the highest tissue Fe contents both in roots and in B in leaves. These results are in agreement with previous reports, which showed that EPF increases the bioavailability of certain heavy metals, such as Cd, Cu, Pb and Zn (Raya-Díaz et al., 2017). However, regarding macro-nutrients, there were no significant differences in N, P, K, Ca, Mg and Na content in leaf and root tissue among *T. violacea* exposed to *B. bassiana*, *C. rosea* and control treatments. Perhaps constant optimal supply of nutrient in the hydroponics medium might have minimized the influence of the fungi on the uptake and availability of the nutrients.

Based on these results, it was concluded that the specific strain of *B. bassiana* used induced better plant growth; hence, it was selected for more detailed assessments in chapter three.

### 2.5 Conclusion and recomandation

In conclusion, application of *B. bassiana* throught soil drench increased plant height (aerial part), roots and fresh weight of *T. violacea*. In addition, the fungi also influenced the uptake of some macro - and micronutirents. However, this study provides a gap for a further investigation to determine the factors that might have caused *C. rosea* to perform poorely in *T. violacea* when compared with *B. bassiana*.

# CHAPTER THREE

## Effects of *Beauveria bassiana* inoculation on growth, tissue nutrient content, secondary metabolites and anti-tick activities of *Tulbaghia violac*ea

### 3.1: Introduction

Ticks are associated with many diseases of humans, livestock and wildlife. Developing countries, especially those in Africa are heavily burdened by tick infestations and record huge annual economic losses (Nyahangare et al., 2015). Ticks and tick-borne diseases have adverse impacts on livestock and human health, placing a huge burden on the livelihoods of resource-poor farming communities in developing countries (Jongejan, 2004; Jabbar et al., 2015). In Africa, tick-borne diseases are among the most important zoonotic and emerging diseases (Minjauw and McLeod, 2003). The hard-bodied ticks are among the most important group of vectors of pathogens (Hoogstraal, 1985); they are capable of transmitting a wide range of pathogens including bacteria, viruses, and parasites. Two of the most important tick species in the African continent are *Rhipicephalus appendiculatus* and *Amblyomma variegatum*; they are vectors of important diseases, such as tick-borne rickettsioses (Parola et al., 2013; Yssouf et al., 2014), Heartwater, and East Coast Fever (ECF) (Mtambo et al., 2007; Madder et al., 2013).

Traditional remedies are still the main approach of controlling ticks and tick-borne diseases in Africa, especially in resource-poor regions (Magwede et al., 2014). The use of ethnoveterinary medicine for treatment of animal health problems, including wounds, tick infestations and tick-borne diseases is widely practiced in South Africa (Masika, et al., 1997; Van Wyk, 1997). Ethno medicinal knowledge has played a crucial role in the drug discovery and development processes (Eloff, 1998; Newman & Gragg, 2007). Extracts from several plant species have been used to suppress tick populations and some herbal-based products are available commercially (Maia & Moore, 2011). The genus *Allium* is well-recognized for their anti-tick activities and their bioactive secondary metabolites, and some species belonging to the Liliaceae family, such as *Allium cepa* and *Allium sativum* have been demonstrated to have acaricidal and tick repellent properties (Jarial, 2001; Nchu et al., 2005).

These plants have the bioactive chemical constituent allicin. The genus, *Tulbaghia*, which is closely related to the genus *Allium* (Lyantagaye, 2011) also, has the bioactive allicin, an important secondary metabolite. Allicin (diallyl thiosulfinate) is a defense compound with a broad range of biological activities (Borlinghaus et al., 2014). The genus, *Tulbaghia*, which is closely

related to the genus *Allium* (Lyantagaye, 2011). Currently, there are scarce reports on the efficacy of *T. violacea* on ticks.

Secondary metabolites contribute towards the adaptation of plants to the altering environments (Ncube et al., 2012); they protect plants against pathogens and protect plants from oxidative stress. Since secondary metabolites are responsible for the bioactivities and medicinal properties of plant species, many studies have investigated strategies to improve medicinal constituents in plants by manipulating biotic and abiotic factors (Coley, 1987: Ncube et al., 2012). Inoculation of plants with fungus is a biotic approach that enhances secondary metabolite in host plant species (Adolfsson et al., 2017; Ding et al., 2018). A well-known endophytic arthropod pathogenic fungus is *Beauveria bassiana* (Hypocreales). *B. bassiana* is an endophyte in a variety of plants, including maize (Bing & Lewis 1991, 1992a, b; Wagner & Lewis, 2000). A fungal endophyte may protect plants from herbivory and disease (Parsa et al., 2013) mediated through changes in volatile and alkaloid constituents of host plants (Parisi et al., 2014). There is substantial justification, therefore, to understand soundly the plant-fungus interaction within the context of improving the quality and quantity of medicinal properties of plants and the promotion of crude drug production. As far as it can be ascertained, there are no reports available on the potential of inoculating *B. bassiana* in *T. violacea*, an important medicinal plant in Africa.

The objective of this study was to evaluate the effects of amending plant growth medium with inoculum of an endophytic entomopathogenic fungus (*B. bassiana*) on growth, secondary metabolites and anti-tick (*R. appendiculatus and A. variegatum)* activities of *T. violacea* cultivated hydroponically.

### 3.2: Materials and methods

#### 3.2.1: Tick colonies

Nine days old adults of *R. appendiculatus,* and 14 days old larvae of *R. appendiculatus* and *A. variegatum* colonies used in this study were obtained from the Division of Livestock and Human Diseases Vector Control, Tropical Pesticides Research Institute, Arusha Tanzania. They were reared in a room that had relative humidity of 70% and temperature range of 26-28°C. Ticks were kept in small tubes with gauze stopper that were kept in small cylindrical containers half-filled with moist sand. Nymphs and larvae were fed on New Zealand White rabbit and adult ticks

were fed on sheep. The animals were handled humanely, in accordance with prescribed ethical guidelines of the Tropical Pesticide Research Institute. Distract

### 3.2.2 Fungus

An indigenous strain (SM3) of *B. bassiana* was used in the present study. Cultures of the fungal strain were obtained from the Department of Horticultural Sciences, Cape Peninsula University of Technology (CPUT). Prior to its use, a conidial germination test to determine conidial viability was carried out according to the method described by Inglis et al. (2012) with modifications. The viability of conidia was determined by spread-plating 0.1 ml of conidia suspension, titrated at 1 x $10^6$ conidia $ml^{-1}$ on PDA plates. Two sterile microscope cover slips were placed on each plate and incubated at 26 ± 2 °C. Plates were then examined after 24 h and percentage germination determined from 100-spore counts under each cover slip. The germination percentage was over 90%.

The fungus was cultured on half-strength PDA (Potato dextrose agar) amended with 0.02 g/L of ampicillin (Sigma-Aldrich, South Africa), and 0.04 g/L streptomycin (Sigma-Aldrich, South Africa). Clean fungal sub-cultures on agar were prepared in 9 cm diameter Petri dishes and incubated at 25 °C. *B. bassiana* conidia were harvested by scrapping 3-4 weeks old culture using a sterile spatula and suspended into 100 ml bottles of sterile distilled water containing sterile 0.01% Tween 80. The suspension was mixed using a vortex shaker for 5 minutes to ensure separation of spores. The conidial concentrations were determined using an improved Neubauer haemocytometer and the suspensions were adjusted to 1 × $10^6$ conidia $mL^{-1}$ in sterile distilled water.

### 3.2.3: Greenhouse experiment

The experiment was conducted in a greenhouse of the Department of Horticultural Sciences, Cape Peninsula University of Technology, Bellville campus, Western Cape, Province, South Africa. Seedlings trays of *T. violacea* were obtained from a wholesale nursery, Western Cape Seedlings, Cape Town. Eight weeks old seedlings were transplanted into 14 cm pots containing substrate mix made of peat, silica sand, perlite and vermiculite in equal volume, and were placed in a controlled greenhouse (Figure 3.1). There were two treatments: plants that were inoculated with the fungus *B. bassiana* (test group) and those that were not exposed to the fungus (control group). One hundred plants were randomly allocated to each block with 50

replicates per treatment. The potted plants were placed on flat surface steel tables (2.5 X 1 m). Plants were fed with Nutrifeed fertilizer obtained from Stodels PTY LTD., (Garden Centre, Cape Town), and the fertilizer contains the following ingredients: 65 g/kg N, 27 g/kg P, 130 g/kg K; 70 mg/kg Ca, 20 mg/kg Cu, 1500 mg/kg Fe, 10 mg/kg Mo, 22 mg/kg Mg, 240 mg/kg Mn, 75 mg/kg S, 240 mg/kg B and 240 mg/kg Zn. The nutrient solution was prepared by dissolving 60 g of the fertilizer in 60 L reservoir with tap water. Plants were irrigated once a week, and each plant was watered with 100 mL of the nutrient solution. Each treatment consisted of fifty replicates. Fungal conidial suspension (50 mL of 1 x $10^6$ conidia/ $mL^{-1}$) belonging to *B. bassiana* was added separately in the test treatments by soil drenching, after 3 weeks this procedure was repeated. Control treatment was not exposed to fungal spores, only 50 ml of sterile 0.01% Tween 80 was added to each control replicate. After three weeks, this treatment procedure was repeated. The greenhouse had the following experimental conditions: 25 ± 5 $^{O}$C and 65 ± 5 RH. The experiment was arranged in a randomized block design and the experiment continued for 12 weeks. Plant samples were analysed for secondary metabolites and extracts of plants exposed to the two treatments tested for anti-tick activities.

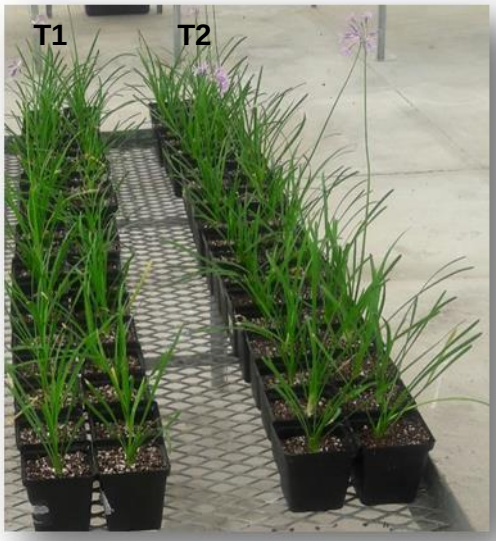


**Figure 3.1:** The experimental group (T1) and control group (T2), in different mixture of media substrates.

### 3.2.4: Re-isolation of fungus

As described by Muvea. (2014) re-isolation of *B. bassiana* in tissue of *T. violacea* was determined at three weeks after inoculation with *B. bassiana*. Randomly selected seedlings

were carefully removed from pots and the leaves separated from the roots. The leaves and roots were then softly washed with tap water, and then placed on sterile tissue paper in a laminar flow cabinet. From these, four leaf (1-2 $mm^2$) and root (1 cm long) sections were excised. These parts were sterilized with 70% ethanol for 1 min, 1% sodium hypochlorite for 1 min, rinsed twice with sterilized distilled water and placed separately on the surface of the selective medium; half strength Potato dextrose agar (19.5 g/L) amended with 0.02 g/L of ampicillin (Sigma-Aldrich), and 0.04 g/L streptomycin (Sigma-Aldrich). The presence and absence of *B. bassiana* growth on the pieces was recorded after ten days at 25 °C. A total of 60 plant species were examined in test and control treatments. The presence of *B. bassiana* in at least one of the leaf sections was considered as an indication of successful colonization of a plant. The data was expressed as percentage colonization ([number of plant replicates colonized/ number of plant replicates excised] × 100).

### 3.2.5: Plant material and extract preparation

Extract preparation was conducted as described by (Nchu et al., 2016). Extracts of *T. violacea* used in the repellency bioassay were prepared by manually crushing 10 g of fresh leaves and roots using porcelain mortar for 15 minutes followed by extraction with 25 ml of acetone. The extraction process lasted 5 hours, followed by filtration with Whatman no 1 filter paper into clean centrifuge tube. Afterwards, 5 ml of the filtrate yield was mixed with 5 ml of clean acetone in order to obtain the 20 w/v % (2 g/10 ml). 10 w/v % was obtained by taking out 5 ml from the 20 w/v % and mixed with 5 ml of clean acetone (1 g/ 10 ml), lastly another 5 ml was taken from10 w/v % and mixed with 5 ml of clean acetone to obtain the 5 w/v % (0.5 g/10 ml).

### 3.2.6: Headspace GC-MS analysis

#### 3.2.6.1: Sample Preparation

Sample preparation was conducted as described by (). Roots and leaves were excissed from fresh *T. violacea* plants and frozen at -80 ˚C (overnight). The leaves and roots samples were freeze-dried and liquid nitrogen ($N_2$) were added. The samples were immediately crushed and 1 g was weighed into a solid phase micro extraction (SPME) vial. Two milliliters of 12% alcohol solution (v/v) at pH 3.5 was added into the vial, followed by 3 ml of 20% NaCl solution. The samples were vortexed and analyzed by SPME-GC-MS (with a grey fiber (divinylbenzene/ carboxen/ polydimethylsiloxane (DVB/CAR/PDMS). About 5 ml of Milli-Q (ultra pure) water was

added to 5 ml of the sample into a solid phase micro extraction (SPME) vial, followed by addition of 3 ml of a 20% sodium chloride (NaCl) solution and vortexed. The headspace of the sample was analysed using a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber (gray).

#### 3.2.6.2: Chromatographic separation

Chromatographic separation was carried out on a gas chromatograph (6890N, Agilent technologies network) coupled to an Agilent technologies inert XL EI/CI Mass Selective Detector (MSD) (5975B, Agilent technologies Inc., Palo Alto, CA). The carrier gas was Helium, and it was used at a flow rate of 1 ml/min. The following conditions were maintained: injector temperature 250°C; the split ratio 5:1; the oven temperature was set to 35 °C for 6 min, at a rate of 3 °C/min to 70 °C for 5 min, then at 4 °C/min to 120°C for 1 min and finally increased to 240 °C at a rate of 20 °C/min for 2.9 min. The electron impact mode of the mass spectrometer maintained at an ionization energy of 70eV, scanning from 35 to 500 m/z. The identification of the volatile compounds was achieved through mass spectrum and retention time with 90% matching with internal standards and reference library.

### 3.2.7: Total polyphenol contents

The Folin-Ciocalteu method described in Daniels et al. (2011) was used to determine the total polyphenol content of the crude extracts of whole plant and the results are presented as mg gallic acid equivalents per g dry weight (mg GAE/g DW).

### 3.2.8: The total alkaloid contents

A spectroscopic method described earlier by Fadhil and Reza (2007) was used to determine total alkaloids in the plant extracts. Dried whole plant material of *T. violacea* was first powdered, and then extracted with 10 mL of 60% ethanol for 2 h. The mixture was centrifuged (4000 x g for 10 min) and the supernatant was used in the assay. Atropine standard solutions mixed with two milliliters of the extract supernatant were mixed with 5 mL sodium phosphate buffer and 12 mL bromocresol green solution. This was followed by the addition of 12 mL of chloroform to the solution and vigorous mixing. The absorbance reading was fixed at 417 nm. A standard curve of atropine to calculate the concentration of the sample.

### 3.2.9: Repellency bioassay

A disk bioassay was used in the repellency bioassay, as described by (Carrol et al., 2004). The 12.5 cm Whatman filter paper was divided into six sections of similar size by drawing diametric lines passing through the center of the filter paper with pencil, and a small circle in the middle, which served as a neutral tick release zone (Figure 3.2). The six sections represented treatments: TR (extracts of roots from plants treated with EPF), TL (extracts of leaves from plants treated with EPF), CR (extracts of roots from plants that were not treated), CL (extracts of leaves from plants that were not treated), Deet and Acetone. The ticks were distributed in the small circle at the centre of the filter paper (neutral). This test was made-up of 3 concentrations, 20 w/v %, 10 w/v % and 5 w/v %. Each concentration had 5 replicates. Fourteen-day old larvae of *R. appendiculatus* were removed from the rim of the specimen tube using a fine painter's brush (no. 3). One hundred larvae of *R. appendiculatus* were released separately on the neutral section of filter papers. Five replications were performed. The positions of ticks on each section were recorded three minutes after their release. Sections with the lowest number of ticks were considered to have repellent activity.

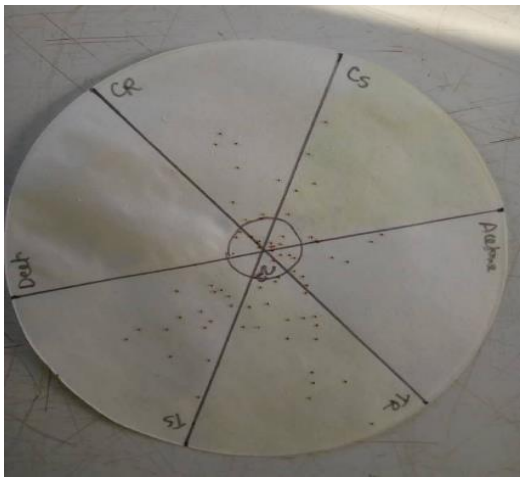


**Figure 3.2:** Disk repellency bioassay of *Tulbaghia violacea* on *Rhipicephalus appendiculatus* larvae. The Whatman No.1 filter paper divided into 6 sections, representing treatments (Test roots, Test leaves, Control roots, Control leaves, Deet and Acetone).

### 3.2.10: Toxicity bioassays

The toxicity bioassay used in this study was a modification of that described by Nchu et al. (2005). Extracts of *T. violacea* were prepared by manually crushing 10 g of fresh leaves and roots separately using porcelain mortar for 15 minutes. Then, 5 g of each crushed leaves and roots were immediately squeezed into respective 15 ml centrifuge tubes and labeled accordingly (TL, TR, CL and CR). Two other centrifuge tubes were included; one containing control (Pc) and the other negative control (Nc). A gluey tape (2 x 2 $cm^2$) was stuck on the bottom surface of a 12.5 cm diameter Petri dishes, each of which represented TR, TL, CR, CL, Pc or NC. The Petri dish labeled Pc had 0.0075% Cypermethrin dip wash prepared from Cybadip® 15 EC (Bajuta General Vetagro, Arusha, Tanzania). A total of 10 un-sexed adult ticks (5 *R. appendiculatus* and 5 *A. variegatum*) were distributed in each petri dish on gluey tape to prevent movement. NC contained distilled water. Thereafter, using micropipette, 2 µL was measured and dispensed on the dorsum of each tick as per respective designated treatment. Petri dish lids were used to cover the containers and kept under the condition of subsequent storage. This experiment was replicated 5 times. The percentage mortality was checked after 24h. Ticks that did not respond to human breath ($CO_2$) and tactile stimulus were considered dead.

**Figure 3.3:** Juice extracts of *Tulbaghia violacea* applied topically on adults of *Rhipicephalus appendiculatus* and *Amblyomma variegatum*.

### 3.2.11 Statistical analysis

The experimental data collected; mean difference on plant growth parameters, mean percentage mortality of toxicity bioassay, mean percentage of repellent bioassay, mean effect on tissue analysis and mean number of GC-MS analysis were analysed using one-way analysis of variance (ANOVA) and Tukey HSD test was used to separate the means at a level of significance, $P< 0.05$. These computations were performed using PAST software (Hammer et al., 2001).

## 3.3: Results and discussion

### 3.3.1: Re-isolation of fungus from leaf and root materials

The *B. bassiana* was successfully re-isolated from the leaf and root samples (Figure 3.4). The percentage colonization showed that the *B. bassiana* colonization occurred in 75% of leaf tissues, while root sections produced 91.6% *B. bassiana* colonization.

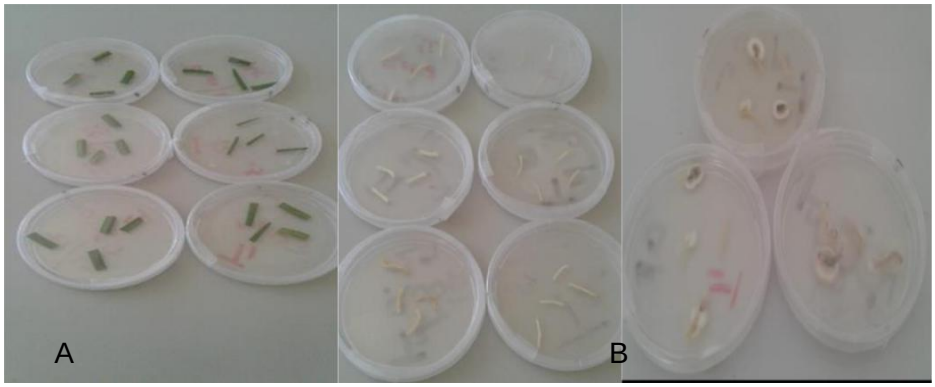


**Figure 3.4:** Representative results of inoculation treatments on endophytic colonization of *Tulbaghia violacea* roots by *Beauveria bassiana*. **A:** control plates with no growth, **B:** endophytic *Beauveria bassiana* conidia outgrowth on leaf and root sections as seen under a dissecting microscope at 100X.

### 3.3.2: Total polyphenol content

The fungus did not have any significant effect on the polyphenol content ($P < 0.05$) between control (T2 ) and fungus treatment ( T1) (Figure 3.5).

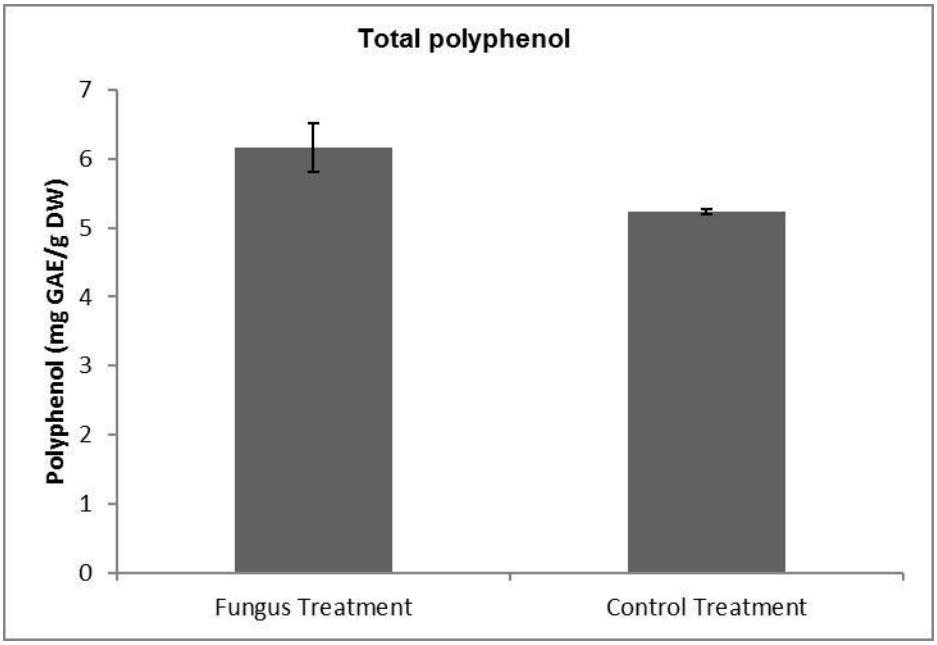


**Figure 3.5:** The effect of *Beauveria bassiana* inoculation on total polyphenol content of whole plant extracts of *Tulbaghia violacea.*

### 3.3.3: GC- MS analysis anticipation

GC-MS analysis was carried out on extract of aerial parts and root part of *T. violacea.*

In the present study, varieties of compounds were detected (Table 3.1). Some of the compounds included Dimethyl disulfide, Alpha pinene, Limonene, 4- Methyl-2- pentanol, Cumene, gamma-terpinene. Generally, the diversity of the compounds obtained in fungus exposed and unexposed plants were similar; hence, the number of volatile compounds produced by plants did not vary significantly ($P > 0.05$) between the fungus inoculated and the control plants for both leaves and roots of *T. violacea*. However, for significant variations ($df = 1,4$; $P < 0.05$) in the quantity of detected compounds (ratio of area of chromatogram of a compound in relation to its corresponding internal standard) between fungus inoculated and control *T. Violacea* with no clear pattern.

**Table 3.1:** Effect of *Beauveria bassiana* inoculation on the area ratio of volatile compound (mean ± se) of aerial (leaves) and root parts of *Tulbaghia violacea.*

| Volatile compounds | Leaves | | Roots | |
|---|---|---|---|---|
| | *B. Bassiana* treatment | Control Treatment | *B. bassiana* treatment | Control treatment |
| Dimethy disulfide | 0.5 ± 0.24 | 0.2 ± 0.09 | 1.5 ± 0.18 | 0.6 ± 0.30 |
| Alpha-pinene | 0.1± 0.02 | 0.1± 0.03 | 0.1 ± 0.02 | 0.0± 0.04 |
| Limonene | 2.4 ± 0.53 | 2.5 ± 0.65 | 1.9 ± 0.65 | 2.3 ± 0.53 |
| Cumene | 0.1 ± 0.00 | 0.1±0.0 | 0.1 ± 0.0 | 0.1 ± 0.15 |
| 4- Terpineol | 0.0 ± 0.6 | 0.0± 0.5 | 0.0 ± 0.00 | 0.00 ± 0.0 |
| Gamma-terpinene | 0.5 ± 0.02 | 0.4 ± 0.10 | 0.6 ±0.01 | 0.3 ± 0.11 |
| Styrene | 1.9 ± 0.33 b | 1.2 ± 0.37 b | 4.6 ± 0.58 a | 2.6 ± 0.77 b |
| Octenone | 0.1 ± 0.0 | 0.1 ± 0.00 | 0.4 ± 0.33 | 0.1 ± 0.00 |
| Dimethyl-trisulphide | 0.3 ± 0.07 | 0.4 ± 0.26 | 1.3 ±0.3 | 0.9 ± 0.63 |
| Nonanal | 0.5 ± 0.23 b | 3.7 ± 1.72 a | 1 ± 0 b | 4.0 ± 1.5 a |
| Sec-butyl sultany | 0.2 ± 0.00 a | 0.1 ± 0.03 b | 0.2 ± 0.01 a | 1 ± 0.04 a |
| Durene | 1.2 ± 0.15 a | 0.7 ± 0.20 | 1.3 ± 0.19 a | 0.9 ± 0.22 a |
| Iso-derene | 0.3 ± 0.17 a | 0.6 ± 0.39 | 0.2 ± 0.05 a | 0.3 ± 0.23 a |
| 1,1- demethyl propul ben | 0.08 ± 0.00 a | 0.08 ± 0.03 | 0.1 ± 0.02 a | 0.09 ± 0.01 a |
| Iso-durene (trans) | 1.2 ± 0.09 a | 0.8 ± 0.02 b | 0.2 ± 0.04 b | 0.8 ± 1.62 b |
| 4-methyl-indene | 0.3 ± 0.02 a | 0.2 ± 0.08 a | 0.5 ± 0.01 a | 0.3 ± 0.10 a |
| 2-2 Sulfanyl ethy | 7.5 ± 0.81 a | 5.2 ± 1.92 b | 6.7 ± 2.08 b | 10.1 ± 2.07 a |
| Alpha-cylocitral | 0.12 ± 0.03 | 0.04 ± 0.00 | 0.6 ± 0.32 | 1 ± 0 |
| Methyl (methyl thio) methyl | 0.1 ± 0.02 a | 0.2 ± 1.13 a | 25.1 ± 4.58 a | 9.9 ± 6.7 b |
| Trans(+) carveol | 0.0 ± 0.01 | 0.3 ± 0.32 | 4.0 ± 1.37 | 1.3 ± 0.70 |
| Naphthalene | 0.9± 0.09 a | 0.6± 0.1 b | 1.7 ± 0.2 a | 1.2 ± 0.4 a |

| | | | | |
|---|---|---|---|---|
| 1,2,4-trithiolane | 0.1 ± 0.02 | 0.2 ± 0.13 | 0.1 ± 0.06 | 0.1± 0.10 |
| Cis-genenaineol | 0.3 ± 0.01 a | 0.1 ± 0.03 a | 0.2 ± 0.00 a | 0.1 ± 0.01 a |
| Dimethy-trithiocarbonate | 0.1 ± 0.03 a | 0.1 ± 0.03 a | 0.0 ± 0.00 a | 0.1 ± 0.03 a |
| 4-Propyl benzamide | 0.2 ± 0.16a | 0.1 ± 0.03 a | 0.7 ± 0.13 a | 0.2 ± 0.05 a |
| Tris(methyl thio met) | 0.2 ± 0.05 a | 0.1 ± 0.03 a | 0.7 ± 0.01 a | 0.5 ± 0.01 a |
| 9,12-octadecadienoic-acid-ethyl-ester | 0.0 ± 0.04 a | 0.00 ± 0.00 a | 0.05 ±0.00 a | 0.00 ± 0.00 b |

Mean followed by same lowercase letters in the same row are not significantly different (P > 0.05) following comparison of fungus and control treatments using Tukey test; roots and leaves were separated.

### 3.3.4: Repellency bioassay

When comparing repellent activities between treated and control extracts of *T. violacea* at concentration of 20 w/v% dissolved in acetone, the difference in repellency was significant (P< 0.05) (Table 3.2). At the highest concentration more larval ticks were found in the sections treated with extracts from the plants exposed to the fungal inoculum. Also, at 10 w/v% and 5 w/v% results indicated significant repellent effects ( P < 0.05), at 5% (P < 0.05) and the root extracts of plants exposed to fungus performed better than the corresponding control extracts in the repellency bioassay (Table 3.2).

**Table 3.2:** The mean (±se) number of *R. appendiculatus* larvae that stayed on the fungus and control treated sections in the disk bioassay

| Treatments | Mean number of ticks present ± SE | | |
|---|---|---|---|
| | 20% concentration | 10% concentration | 5% concentration |
| Test leaves | 21.4 ± 20.1 a | 8.4 ± 2.78 b | 7.8 ± 2.10 a |
| Test roots | 20.4 ± 4.27 a | 5.6 ± 1.88 b | 6.8 ± 2.74 a |
| Control leaves | 5.0 ± 1.22 b | 9.0 ± 2.60 b | 8.2 ± 2.49 a |
| Control roots | 9.6 ± 1.96 b | 17.2 ± 4.11 a | 14.2 ± 14.27a |
| DEET | 6.0 ± 1.87 b | 2.8 ± 1.01 b | 3.8 ± 1.31 b |
| Acetone | 9.4 ± 2.08 b | 11.0 ± 2.02 a | 10.8 ± 2.88 a |

Mean followed with lowercase letters on the same column (20%, 10% and 5%) are not significantly different, following Tukey posthoc test at P > 0.05 level of significance.

### 3.3.5: Toxicity bioassay

Tick mortality resulting from extracts of *T. violacea* against adults of *A. variegatum* used in this bioassay was generally high in both control and fungus exposed plants, ranging from 4.2/5 to 4.8/5 mean mortality against adults of *A. variegatum* (Table 3.3a) and this was comparable to tick mortality caused by the commercial acaricide (Cybadip EC). Similar high mean mortality against Adults of *R. appediculatus* was induced by plant extracts from *T. violacea* plants from both control and fung us treatments; mean tick mortality ranged from 4.8/5-5/5 (Table 3.3b). Statistically, there was no significant difference ($P > 0.05$) between the two treatments at the high concentration used.

**Table 3.3a:** Mean percentage mortality ±se of adults of *Amblyomma variegatum* at 24 h following treatment with leaf and roots juice extracts of *Tulbaghia violacea* in contact toxicity bioassay (n = 5 ticks/exposure × 5 replications).

| Treatments | Plant part | Mean mortality ± SE |
|---|---|---|
| Test (TL) | leaves | 4.8 ±0.2 |
| Control (CL) | leaves | 4.2 ±0.37 |
| Test (TR) | Roots | 4.8 ±0.2 |
| Control (CR) | Roots | 4.8 ±0.2 |
| Pc (Cybadip EC ) | - | 4.8± 0.2 |
| Nc (distilled water) | - | 1 ± 0 |

**Table3.3b:** Mean percentage mortality ±se of adults of *Rhipicephalus appediculatus* at 24 h following treatment with leaf and roots juice extracts of *T. violacea* in contact toxicity bioassay (n = 5 ticks/exposure × 5 replications)

| Treatments | Plant part | Mean mortality ± SE |
|---|---|---|
| Test (TL) | Leaves | 5 ± 0 |
| Control (CL) | Leaves | 5 ± 0 |
| Test (TR) | Roots | 5 ± 0 |
| Control (CR) | Roots | 5 ± 0 |
| Pc (Cybadip EC ) | - | 4.8 ± 0.2 |
| Nc (distilled water) | - | 1 ± 0 |

## 3.4: Discussion

### 3.4.1 Tissue colonization by *B. basssiana* inoculum

The fungal strain of *B. bassiana* used in this study was successfully re-isolated from the leaf and root tissue suggesting that the fungus successfully colonized tissues of *T. vio*lacea. While endophytes in plants have been shown to occur in many plant species, especially plants of the gramineae family, fewer medicinal plants have been experimentally inoculated with fungal endophytes with the view to improve medicinal properties. Successful colonization of many plant species following inoculation with *B. bassiana* has been reported previously (Tefera & Vidal, 2009; Sánchez-Rodríguez et al., 2015; Waqas et al., 2015; Russo et al., 2018). This study also demonstrated that soil drench inoculation with conidia of entomopathogenic fungi, *B. bassiana* had variable effects on the different growth parameters: *B. bassiana* did not significantly influence the number of leaves, new shoots, dry and fresh weight of *T. violacea* when compared with the control plants, but did influence plant height and root length significantly.

### 3.4.2: Total polyphenol and Volatiles

In this study, phytochemical analysis revealed that the leaves and roots of *T. violacea* treated with *B. bassiana* and untreated plants contained polyphenols did not vary significantly. It was observed that there was no significant difference in the number of occurring in plants in control and fungus treatments. However, the quantity of essential oil in extracts of *T. violacea* varied between control and fungus treatments. The results also showed that in both treatments essential oils were rich in polyphenols based on GC-MS analysis. Chemical analysis carried out on fresh rhizomes of *T. violacea* detected dimethy disulfide, naphthalene, dimethy trisulfide, 2, 4-dithiapentane and (methylthio) acetic acid, and 2- (methylthio) ethanol, 3-(methylthio) propanenitrile (Olorunnisola et al., 2012). Some of these compounds were also contained in the leaves and roots extracts of *T. violacea* evaluated in this study. Interestingly, sulfur-based compounds, such as dimethyl disulfide (DMDS) are among compounds that were also detected in the current study even though it was not significantly different among treatments. These compounds are released by quite a number of plants in the atmosphere, and they are toxic to some insects; Dugravot et al. (2003) demonstrated that DMDS induced an uncommon complex neurotoxic activity. Also, DMDS was identified in leaves of garlic wood plant (*Gallesia integrifolia*); essential oil from its leaves was more efficient in inhibiting egg hatching of

*Rhipichephalus* (Boophilus) *microplus* (Raimundo et al., 2017). Geraniol is commonly used as an insecticide and it exhibits various biochemical and pharmacological properties (Chen & Viljoen, 2010). In addition, geraniol is used as a natural pest control agent and is thought to have low toxicity to untargeted species; research has shown geraniol to be an effective plant-based insect repellent (Barnard & Xue, 2004).

### 3.4.3: Toxicity bioassay

Even though EPF and plant extracts have been evaluated with promising results against tick species, this is the first study that evaluated the effects of inoculating plants with fungal inoculum, and the subsequent effects on anti-tick activities of plant extracts. The use of EPF is one of the approaches being considered as alternative to chemical acaricides (Maniania et al., 2007).

The results on the evaluation of toxic effects of *T. violacea* grown under growing medium amended with EPF, *B. bassiana* exhibited high mortality towards adult of *R. appendiculatus* and *A. variegatum*. Equally, squeezed juice of both leaves and roots from the control group showed very high tick mortality percentage. There was no significant statistical difference between the test and control treatments ($P>0.05$). Juice materials from ethnoveterinary plants parts are used in traditionally for control of ticks on livestock, for example, in Ethiopia, juice extracts of different plant parts were done on engorged female *Boophilus decoloratus* (Regassa, 2000). The toxic effects of numerous plant extracts have been reported. Previous study by Nyahangare (2015) showed that water extracts of *Lippia javanica* are acaricidal against *R. appendiculatus*. Nchu et al. (2005) reported that DCM extract of *A. sativum* was toxic against ticks and could be a potential source of novel acaricidal agents.

### 3.4.4: Repellent bioassay

In the present study, the acetone extracts of *T. violacea* amended with EPF were assessed against larvae of *R. appendiculatus*. There was a significant difference among extracts of *T. violacea* that were inoculated with fungi when compared with the control test. At 20 w/v %, concentration, extracts from fungus inoculated plants perfomed poorly, even showing net positive concentration. However, at 10 w/v % and 5 w/v % concentrations of extracts of *T. violacea* repellent activities on ticks was positive. Previously, Nchu et al. (2016) reported that

DCM extract of garlic showed positive repellent effects on ticks at a lower range of concentrations in repellency bioassays. In a review paper, Wanzala et al. (2014) presented repellent activities of eight plants essential oils, including *Tagetes minuta, Tithonia diversifolia, Juniperus procera, Solanecio mannii, Senna didymobotrya, Lantana camara, Securidaca longepedunculata,* and *Hoslundia opposita*), against *R*. *appendiculatus*.

### 3.4.5: Conclusion

We can conclude that *B. bassiana* isolate used in the present study was able to endophytically colonize *T. violacea* plant through soil drench method.The results show high percentage of endophytic colonization on leaves and roots of *T. violacea.*The exposure of entomopathogenic fungi did not improve polyphenol content of *T. violacea*.This study also revealed that extracts of *T. violacea* exposed to both fungus and control treatments equally repelled larvae of *R. appendiculatus* at lower concentrations, but, interestingly, at high concentration the extracts from fungus treated plants showed significantly negative repellency compared to the control treatment. Remarkably, juice extracts of *T. violacea* from both in fungus-treated and control group induced high mortality. Therefore, we can conclude that fungus had minimal effects on growth, and anti-tick activities of *T. violacea.* Further study is necessary to evaluate different formulations of the juice extracts against ticks and the possibility of using fungus inoculated *T. violacea* simultaneously as an attractant and acaricide.

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**Appendix 1**: Evidence of manuscript submission