Antifungal activities of *Bulbine frutescens* extracts against *Fusarium oxysporum*.

by

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DECLARATION

I, Zandile Maninjwa, declare that the contents of this thesis represent my own unaided work, and that the proposal 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.

Signed **Date**

DEDICATION

I dedicate this work to my mother Nosandla Phumza Maninjwa, my father Phumelele Hubert Maninjwa and my family for their love, prayers, and unconditional support throughout my academic career.

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I would like to thank God almighty for making a way in everything I do, giving me strength, courage, blessings and guidance throughout especially in times of discouragement. Not forgetting my ancestor's ooBhele kunye nooNdokose for their love and protection.

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LIST OF ACRONYMS

ANOVA	Analysis of Variance
B	Boron
Ca	Calcium
CPUT	Cape Peninsula University of Technology
Cu	Copper
Fe	Iron
HCl	Hydrochloric acid
Hr	Hour
HSD	Honest Significant Difference
INT	*p*-iodonitrotetrazolium chloride
K	Potassium
Mg	Magnesium
MIC	Minimum Inhibitory Concentration
Mn	Manganese
N	Nitrogen
Na	Sodium
Na_2CO_3	Sodium Carbonate
NH4+	Ammonium
NO3-	Nitrate
^oC	Degrees Celsius
P	Phosphorus

PDA	Potato Dextrose Agar
pH	Potential Hydrogen
RH	Relative Humidity
S	Sulphur
STD	Sexually Transmitted Disease
Zn	Zinc

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ABSTRACT

Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for production of new and safer plant fungicides. Due to implications caused by synthetic chemical fungicides there has been an increasing interest and demand for searching for a natural way that will be less toxic to human beings and environmental health. Medicinal plants are considered to be a natural source of antifungal agents. Thus leading to an increasing demand for medicinal plants and its cultivation strategies that will yield high amount of secondary metabolites and favours growth and development of these plants. The proper cultivation strategy including growing media can play a major role in increasing plant metabolites, growth and development. Therefore, *Bulbine frutescens* was cultivated under varied growth media combinations with the aim of evaluating the plants growth and physiological response as well as to determine the optimum growing media for increasing *B. frutescens* secondary metabolites production.

In chapters 1 and 2, the introduction and conceptual background and scientific realizations of the study are presented. In chapter 3, the research objective was to assess the effect of vermicompost, biochar and sand on plant growth and development in *B. frutescens.* Plant growth parameters such as number of leaves, leaf length, plant fresh weight, dry weight, root length, root weight and number of roots were recorded. Leaf samples were analysed for micro and macro nutrients. Results obtained showed a significant difference ($P < 0.05$) on the number of leaves, leaf length, plant fresh weight, dry weight, and number of roots among the treatments. When all the treatments were compared, T1 in the above mentioned parameters except for plant fresh and dry weight produced the best results among the treatments followed by T3, and T4, whilst T2 performed the least. Nutrient analysis results indicated that the treatments were significantly different ($P < 0.05$) on the uptake of N, P, Ca and Zn where T1 in N and Zn showed better uptake of nutrients in all the treatments. These results indicate that the different growing media have a better influence and also negative influence on growth of the plant depending on the ratio of the substrates incorporated. The nutrient uptake of *B. frutescens* was not influenced by the growth media. However, the treatment with high proportion of vermicompost indicated the best results among all other treatments.

In chapter 4, the study was conducted to (i) To evaluate the *in vitro* antifungal activity of extracts from *B. frutescens* cultivated on vermicompost, biochar and sand. (ii)To identify bioactive compounds in the extract which have shown promising antifungal activity. At 10 weeks post treatment, plants were harvested, washed and air dried before preparing them for bisassays. The acetone extracts of *B. frutescens* were then screened for antifungal activity against *F. oxysporum* using minimum inhibitory concentration (MIC) method. A preliminary study for anti *F. oxysporum* of normal soil grown *B. frutescens* leaves was also piloted. The MIC values of extracts of *B. frutescens* leaves following exposure to different media combinations were not statistically different ($P > 0.05$) among treatments. However the MIC values at 6 hr of plants grown in T2 (0.62 ± 0.12 mg/ml) showed high antifungal activity against *F. oxysporum* although it was not significantly different to other treatments and T1 (1.25 ± 0.25 mg/ml) values were lower compared to other treatments (T2, T3, T4) respectively. There was no significant difference ($P > 0.05$) in the MIC values among the treatments at 12 hr. Nonetheless, with T2 (0.50 ± 0.12 mg/ml) and T3 (0.50 ± 0.12 mg/ml) exhibited high anti *F. oxysporum* activity. The positive control at 6 and 12 hr showed activity while the negative control did not show any activity. Additionally, there was no significant difference ($P > 0.05$) between the extracts of *B. frutescens* grown in normal soil and the one exposed to different growing media. The leaf samples obtained from different treatments showed that *B. frutescens* have the following secondary metabolites: polyphenols, flavonols and alkaloids. The polyphenols (mg GAE/g) were significantly different ($P < 0.05$) and high in T1 (9.316 ± 0.17) while there was no significant difference ($P > 0.05$) in flavonol (mg QE/g) content among treatments. However, T1 produced higher flavonol content compared to other treatments. There was a significant difference ($P > 0.05$) in alkaloids content with T1 (6.29mg/g) recording the highest content of alkaloids.

These results indicate that *B. frutescens* has antifungal activities against *F. oxysporum.* This study also demonstrated that vermicompost, biochar and sand have a positive effect on antifungal activity of *B. frutescens* against *F. oxysporum* and the combination ration of 2:1:1 significantly favoured the accumulation of secondary metabolites.

CHAPTER ONE
General Introduction

1.1 General Introduction

Plant pathogenic fungi are heterotrophic organisms that produce a complex array of enzymes for the ingestion of plant host tissues during infection (Collins *et al*., 2013). They are known for being the utmost contagious agents in plants, initiating changes in the developmental stages of plants including post-harvest (Agrios, 2005b). There is an extensive kind of fungal genera in fruits and vegetables, that causes quality problems that are in relation to nutritional value, limited shelf life, and organoleptic characteristics (Agrios, 2005b). Additionally, in some cases these plant pathogenic fungi cause allergic or toxic disorders in consumers because of the production of mycotoxins or allergen by fungus such as *Fusarium oxysporum* (Dellavalle *et al.,* 2011).

Fusarium oxysporum f. sp*. lycopersici* is an ascomycetous fungus that is a widely known soil borne plant pathogen (O'Donnell *et al*., 1998; Ramanathan *et al*., 2010; Inami *et al*., 2014). It is one of the important plant pathogenic fungi that cause vascular wilt in many agricultural crops (Agrios, 2005a). The disease leads to severe losses in greenhouse and field crops as well as hydroponic cultures (Manzo *et al*., 2016). The severe losses occur particularly when the air and soil temperatures are high, mainly in a season with warm climates (Agrios, 2005a). For many years, the use of synthetic fungicides has been the most practiced method of protecting plants against fungal attack (Tapwal *et al.,* 2011; Yoon e*t al*., 2013; Jantasorn *et al.,* 2016). Unfortunately, the aftereffects resulting from the application of synthetic fungicides in plant production systems for control of crop diseases impact negatively on ecosystems (Castillo *et al.,* 2012). Tapwal *et al.* (2011); Castillo *et al.* (2012) and Ramaiah and Garampall (2015) agree that any synthetic chemical fungicides that are available in marketplaces are poisonous and have harmful effects on other organisms present in the environment. These chemical synthetic fungicides are not

biodegradable; can accumulate in the soil, plants and water, and consequently affect humans in the food chain (Tapwal *et al.,* 2011).

The high non-selective use and frequent application of synthetic fungicides leads to a loss in efficiency and increased resistance of the disease to the active ingredients (Castillo *et al.,* 2012) due to pathogen mutations (Fielding *et al*., 2015).

This increases chemical fungicide costs and environmental damage if the treatment is applied repetitively (Fielding *et al*., 2015). Thus, the development of pathogenic fungi resistance towards synthetic fungicides is a great concern (Tapwal *et al.,* 2011).

Even though synthetic chemical fungicides are very effective, due to the effects afore mentioned and in an attempt to decrease the use of these fungicides, intensive research has increasingly been focussing on the possible development of eco-friendly management strategies (Kim *et al*., 2005; Mandal *et al*., 2009; Tapwal *et al.,* 2011; Yoon e*t al*., 2013). These strategies include natural commercial botanical fungicides that are practically effective and safe for the environment and humans. To date, quite a few botanical fungicides have been registered and brought to the market for commercialisation e.g Amrut Fungistar. However, many scientists have reported successful management of plant diseases through isolation and characterization of a wide range of antifungal plant derivatives. Furthermore, they have been proven to be harmless and not phytotoxic, as opposed to chemical fungicides (Sharma and Kumar, 2009 and Yoon e*t al*., 2013).

The presence of antifungal compounds in plant species has long been regarded as a crucial factor in the control of plant diseases (Sales *et al*., 2016). Such compounds, being biodegradable and selective in their toxicity, are considered useful for controlling some plant diseases (Singh and Dwivedi, 1987). Except that natural fungicides are harmless and non-toxic to humans and the environment, it has been attested that plant extracts totally inhibit spore germination and viability of fungal spores.

Therefore, this research was undertaken to screen the antifungal potency of a medicinal plant (*Bulbine frutescens*) against the pathogen *Fusarium oxysporum.* It is

hoped that this plant may be used as an alternative source of antifungal agents in the horticultural and agricultural sector as a whole thereby increasing productivity.

1.2 Structure of this thesis

The current study is divided into five chapters as follows.

Chapter One: Introduction, and the structure of this thesis.

Chapter Two: Background of the research problem and literature review. It encompasses the background and validation of the study, as well as details pertaining to the problems which led to the undertaking of the study as well as the aims and specific objectives.

Chapter Three: Optimizing the cultivation strategy using varied growth media combinations for plant growth, development and increased production of secondary metabolites of *B. frutescens*. The main objective of this chapter is to evaluate growth parameters of *B. frutescens* cultivated in four different growing media combinations and the response of the micro and macronutrients in each treatment.

Chapter Four: In vitro antifungal activity and bioactive compounds of extracts from *B. frutescens* in response to varied growth medium combinations. The objectives of this chapter are to: (i) Evaluate changes *in vitro* of the antifungal activity of extracts from *B. frutescens* grown under varied growth medium combinations. The pilot study showed that *B. frutescens* possesses antifungal activity. The change would determine the significance of the pilot test results; and (ii) Identify bioactive compounds in the extract which showed good antifungal activity.

Chapter Five: General discussion, conclusions and recommendations.

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CHAPTER TWO

Background to the research problem and literature review

2.1 Background to the research problem

Fusarium wilt is one of the most common and damaging diseases caused by *Fusarium oxysporum*, causing infection that result in yield losses (Neela *et al.,* 2014). *Fusarium oxysporum* is an ascomycetous fungus that is a well-known soil borne plant pathogen (Inami *et al.,* 2014). It causes wilt disease in a wide range of crops all over the world (Roncero *et al.,* 2003; Ramaiah and Garampall 2015; Pérez Martín and Di Pietro, 2012). This disease leads to major losses in field crops, crops grown under controlled environments and hydroponic cultures (Manzo *et al.,* 2016). Chemical fungicides are used to control fusarium wilt in tomatoes (Ramaiah and Garampall 2015). Unfortunately, due to the negative effects they have on human health and the environment, their use is increasingly being limited (Dellavalle *et al.,* 2011). These synthetic fungicides are not biodegradable and appear to remain in the ecosystem for years, and this has led to certain fungi developing resistance to them (Ramaiah and Garampall 2015). However, it has been found that through mutations, plant pathogenic fungi can adapt and not respond to fungicide treatments leading to resistance and loss of fungicide efficacy (Hahn, 2014). This results in increases in the costs of chemical fungicides and ecological damage if frequent application is required (Fielding *et al.,* 2015).

Due to resistance and health hazards or implications of chemical synthetic fungicides, there is a high interest in searching for antimicrobial activities of plant origin, as well as isolating and identifying active compounds with potential use in integrated crop protection (Eksteen *et al.,* 2001; Tegegne *et al.,* 2008). Among others, Pretorius *et al*. (2002); Ramaiah and Garampall (2015) and Sharma and Kumar (2009) agree that the usage of natural products such as botanical additives or botanical extracts for the control of fungal diseases in plants is regarded a viable alternative for synthetic fungicides. This is because they possess fewer adverse impacts on humans and environmental wellbeing. It has been attested that plant extracts totally inhibit spore germination and viability of fungal spores. A number of higher plants and their constituents have been effective in the management of plant

diseases and unlike chemical fungicides, they have been proven to be harmless and not phytotoxic (Sharma and Kumar, 2009). The study of Mbambo *et al.* (2012) revealed that phytosterols extracted from *Bulbine natalensis* have an antifungal potential that can be used in the control of diseases caused by plant pathogenic fungal species. The majority of uses of the genus Bulbine are similar to those of Aloe (Coopoosamy, 2011). The study of Saks *et al.* (1995) and Sitara *et al.* (2011) indicated that the extract from *Aloe vera* leaves has an inhibitory effect on spore germination of plant pathogenetic fungi. This may be used in the formulation of new, safer and eco-friendly fungicides (Ramaiah and Garampall 2015).

2.2 Statement of the research problem

Fusarium oxysporum is a fungus which causes crops to wilt. Commercially, it is controlled by synthetic fungicides. These fungicides are not environmentally and human friendly and are prone to pathogenic fungal resistance. Therefore, the proposed study will assess the antimicrobial activity of *Bulbine frutescens* extracts against *Fusarium oxysporum* by growing the plant in varied combinations of growth media for better plant growth and development. This could impact positively on the sustainable use of the plant as a source of antifungal agents and hence a safer method of enhancing the management of pathogens such as *F. oxysporum*.

2.3 Literature review

2.3.1 *Bulbine frutescens*

Bulbine frutescens is a South African plant species widely cultivated for aesthetic purposes (Bringmann *et al*., 2008). It is known in Afrikaans as Rankkopieva, the Xhosa people from the Eastern cape call it ibhucu, while Zulu speaking people from Kwazulu Natal call it ithethe elimpofu (van Wyk *et al*., 2009) or stalked Bulbine in English (Coopoosamy, 2011). *B. frutescens* belongs to the family Asphodelaceae and subfamily Asphodeloideae (van Staden and Drewes, 1994). Members of the Asphodelaceae family are well known for their medicinal value (Joseph and Raj, 2010) and A*loe vera* is a renowned example that is now sold worldwide (Coopoosamy, 2011). *B. frutescens* is widely distributed in South Africa, where over

40 subspecies are known and it grows selectively on sandy soils (Coopoosamy, 2011). It is easily recognised by its branched woody stems (van Wyk *et al*., 2009). In several treatment modalities, fleshy leaves and roots of most species of this genus are used. In the treatment of wounds, burns, rashes, itches, broken lips and cracked skin, traditional healers and indigenous people predominantly use this plant's leaf sap (Coopoosamy, 2011).

2.3.1.1 Geographical distribution of *B. frutescens*

Bulbine frutescens is widespread in the provinces of Gauteng, Mpumalanga, Eastern Cape, Free State, KwaZulu-Natal, Limpopo, Northern Cape, North West and Western Cape of South Africa as shown in Fig 1 (South African National Biodiversity Institute, 2010). However, this plant also grows in other Southern African countries including Swaziland, Lesotho and Botswana (Brinckmann and Brendler, 2020; Coopoosamy, 2011; Mutanyatta *et al.,* 2005).

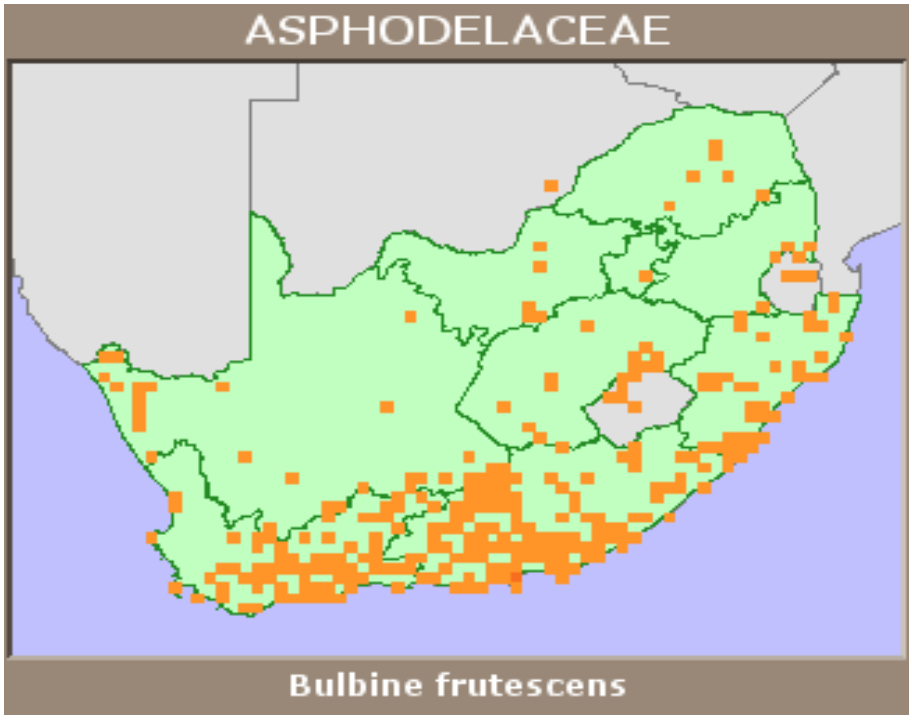

Figure 1: *Bulbine frutescens* distribution in South Africa

Source: http://redlist.sanbi.org/imgs/distribmaps/1/Plants-13708.png

2.3.1.2 Morphological characteristics of *B.frutescens*

B. frutescens has a rootstalk with several coarse roots; the leaves are 15 cm long and 4 to 8 mm thick, light green, fleshy and hairless. The plant bears yellow, orange or white flowers from April to August in thick, elongated clusters of flowers up to 30 cm long with hairy stamens (Colson, 2013).

2.3.1.3 Propagation and growth requirements of *B.frutescens*

The plant can be propagated through seed, cuttings or division of clumps. *B. frutescens* can be propagated from late winter to early spring till summer depending on the type of propagation employed. *B. frutescens* grows in full sun or semi shade position in well-drained media (sandy or sandy loam soil) and requires very little watering because overwatering can lead to rotting and onset of other fungal and bacterial infections (Harrison, 2006).

2.3.1.4 Compounds

The genus *Bulbine* also has *Aloe vera* properties and is used as a form of natural medicine in rural areas in much of Africa, where modern medicine is inaccessible and too expensive (Coopoosamy, 2011). The roots and stems of *Bulbine* species are reported to possess anthraquinones such as chrysophanol and knipholone (Van Staden & Drewes, 1994; Van Wyk, 1995). In the therapeutic process, which is most likely due to glycoproteins such as aloctin found in Aloe *arborescence* and *Aloe excelsa* leaf gels, these compounds may be of minor importance (Coopoosamy and Magwa, 2007; Coopoosamy, 2011). Rachuonyo *et al*. (2016a, 2016b) showed that *B. frutescens* has anti-bacterial and antifungal compounds against human pathogens causing ills/diseases. However, to the best of our knowledge either in ancient or modern times, fungicidal activity of this constituent on plant pathogens has never been recorded.

Research on the in vitro antifungal activity of *Aloe vera* pulp and liquid fraction against plant pathogenic fungi was performed by Jasso de Rodríguez *et al*. (2005). The results obtained indicate the presence of antifungal properties in *A. vera* pulp against *F. oxysporum.* These results are important in the current work since the genus *Bulbine* is known to contain the same properties as those of *A. vera*.

A study was also undertaken by Rachuonyo *et al*. (2016c) on *in vitro* antimicrobial activity of crude leaf extracts from *Bulbine frutescens* against *Salmonella typhi*.

Rachuonyo *et al*. (2016c) also reported on the in vitro antimicrobial activity of leaf extracts against *Salmonella typhi* from *Bulbine frutescens*. Phytochemical screening of the plant extracts revealed that all the methanolic extracts of leaves from the plant possessed the four types of secondary metabolites (saponins, tannins, flavonoids and alkaloids) under investigation. According to Castillo *et al*. (2012), the major groups with antifungal activity are terpenes, tannins, flavonoids, alkaloids, lecithin and polypeptides.

2.4 *Fusarium oxysporum*

2.4.1 Taxonomy

Fusarium oxyporum belongs to the Fungi kingdom and is classified under the Ascomycota and Sordariomycetes class divisions. It belongs to the order Hypocreales, the Nectriaceae family, the *Fusarium* genus and the *oxysporum* species (Michielse and Rep, 2009). *F. oxysporum* was described by O'Donnell *et al*. (1998) and Ramanathan *et al*. (2010) as a cosmopolitan soil-borne filamentous fungus. In a broad range of agricultural and ornamental host plant species, it is an anamorphic species that includes numerous plant pathogenic strains causing wilt diseases (Ramanathan *et al* 2010).

2.4.2 Ecology and dissemination of the pathogen

Fusarium wilt, most widespread on acidic and sandy soils, is a warm-weather disease (Bawa, 2016). The pathogen is soil-borne and survives for up to ten years in infested soils. For disease growth, soil and air temperatures of 28 ºC is optimal. If the soil temperature is ideal, but air temperatures are below optimal, the pathogen will spread to the lower sections of the stem, but external symptoms will not be displayed by the plants (Bawa, 2016). In warm climates and warm sandy soils, the disease is most damaging. The disease causes significant losses, particularly in vulnerable varieties, where soil and air temperatures during most of the season are very high (Agrios, 2005; Larkin and Fravel, 2002).

The pathogen is transmitted by water and infected farm machinery over short distances, and mostly in infected transplants or in the soil brought with them over long distances (Agrios, 2005). During the growth period, the pathogen is laterally

disseminated from plant to plant via root-to-root contact. The disease or infection can also be spread over soil infestation and subsequent root infection. Other means of dissemination of the disease can occur via chlamydospores that are present in the soil particles on contaminated shoes and plant stakes (Ozbay *et al*., 2004).

2.4.3 Fusarium wilt disease symptoms

Attack signs initially emerge as a mild clearing of the vein on the outer part of the young leaves, followed by older leaves exhibiting epinasty triggered by petiole drooping (Agrios, 2005). The wilting and dying of plants at seedling stage on occurrence of the first signs and symptoms is common. Furthermore, according to the same author, if the infection is serious and if the weather is conducive for the pathogen to grow and develop, older plants in the field can wilt and abruptly die. More often, vein clearing and leaf epinasty in older plants are accompanied by stunting of plants, yellowing of the lower leaves, infrequent development of adventitious roots, wilting of leaves and young stems, loss of leaves, marginal death of the remaining leaves and ultimately plant death (Agrios, 2005). The stem dies and the foliage is destroyed if these signs occur on one side of the stem and continue to advance upwards. Dark brown streaks become visible, running long ways across the stem if the main stem is cut. A brown ring is visible in the region of the vascular bundles in the cross segment near the base of the infected plant stem (Agrios, 2005). White, pink or orange fungal growth can be seen on the outside of affected stems, especially in wet conditions (Ajigbola and Babalola, 2013).

2.4.4 Morphology

2.4.4.1 Cultural characteristics

The mycelium of *Fusarium oxysporum* is initially colourless, but becomes pale pink cream-colored, pale yellow, or some-what purple as it ages (Agrios, 2005). The numerous special forms of *F. oxysporum* may have appearances that alter when they are in solid media culture, such as potato dextrose agar (PDA). The aerial mycelium first looks white, and then according to the strain of *F. oxysporum*, this may shift to a variety of colours ranging from violet to dark purple (Smith *et al*.,

1988). The culture may resemble a cream or orange colour if sporodochia is plentiful (Smith *et al*., 1988).

2.4.4.2 Morphological characteristics

Fusarium oxysporum is restricted by a set of morphological standards, mainly by the shape of the macroconidium, the form of the microconidiophore, and the development and nature of chlamydospores (Gordon and Martyn, 1997). Three types of asexual spores are produced by *F. oxysporum*: macroconidia, microconidia, and chlamydospores (Ohara and Tsuge, 2004). Microconidia are 1- or 2- celled and are the abundant and common types of spores produced under all conditions by the fungus. They are the most frequently developed forms of spores inside infected plant vessels (Agrios, 1988). Ebbole and Sachs (1990) reported that microconidia largely have a single nucleus and poorly germinate. Microconidia are thinly walled with a definite foot cell and a pointy apical cell, have gradually pointed and curved ends, and transpire on the surface of plants killed by the pathogen in sporodochia like groups (Nelson *et al* 1981). Chlamydospores are one or two thick-walled, round spores produced on older mycelium or in macroconidia within or terminally (Agrios, 2005). In fungal cultures and possibly in the soil, all three types of spores are produced, although only chlamydospores can survive for a long time in the soil (Agrios, 2005; Nelson *et al* 1981).

2.4.5 Disease cycle an epidemiology

When vigorous plants develop in uncontaminated soil, the germ tube of spores or the mycelium penetrates root tips directly (Agrios, 2005; Lyons *et al*., 2015; Olivain *et al*., 2003) or penetrate the roots through a wound or at the point of formation of lateral roots. The mycelium progresses through the root cortex, and when it reaches the xylem vessels, it passes in through the pits (Agrios, 2005). The mycelium then stays solely in the vessels and passes through them, often upwards, toward the plant's stem. The mycelium further branches and produces microconidia in the vessels, which are isolated and carried up in the sap stem (Agrios, 2005). At the point where their upward movement is stopped, microconidia germinate and the mycelium penetrates the vessel's upper wall, and the next vessel produces more microconidia. The mycelium also spreads through the pits into the adjacent vessels, penetrating

them (Agrios, 2005). This ultimately blocks the vascular system from transporting the rest of the host with water and nutrients (Ramaiah and Garampall 2015; Sharma and Kumar, 2009). When more water passes through the leaves than it gets from the roots, the stomata close and the leaves wilt and die, followed by the rest of the plant's death (Agrios, 2005).

2.5 The effects of substrates on plant growth, development and on production of secondary metabolites

A wide range of bioactive compounds and substances, such as flavonoids, phenolic acids, lignans, salicylates, stanols, sterols, and glucosinolates, are produced by plants (Hooper and Cassidy, 2006). The concentrations of various secondary plant metabolites are strongly dependent on the growing conditions and have an impact on their metabolic pathways responsible for the accumulation of the related natural products (Ramakrishna and Ravishankar, 2016). Organic fertilization imparts high organic matter in the soil, which influences higher production of plant phenols, flavonoids and stilbenes (Ibrahim *et al*., 2013). Plant metabolites like flavonoids, phenols, tannins, stilbenes and alkaloids are significantly influenced by the high nutrient content of the organic matter amendments applied to the soil or other growing media (Taie *et al*., 2008).

2.5.1 Compost

Compost can be obtained from a range of feedstock materials, including woody (trees, shrubs) and herbaceous (turf grass and small flowering plants) residues of green waste crops, bio solids (sewage sludge), animal manures, by-products of wood, bio-degradable packaging and construction materials and food scraps (Cayuela *et al* 2009; De Araujo *et al*., 2010). Composts differ according to the starting material and the particular operating parameters maintained throughout the process of composting (Sukesan and Watwood, 1998). This allows the difference in particle size and chemical decomposition to be affected (Cayuela *et al*., 2009; De De Araujo *et al*., 2010). A nutritional contribution is the most beneficial result of the use of compost in a growth medium. A large amount of N can be immobilized by non-

mature composts, but once stabilized, composts act as slow-release fertilizers to a great extent (Raviv *et al*., 2002).

2.5.2 Vermicompost

Vermicomposting is a straightforward composting technique in which some earthworm species are used to boost the waste conversion process and create a better end product. In some respects, vermicomposting varies from composting (Gandhi *et al*., 1997). Compared to the base or underlying soil and natural compost, it has a higher and more soluble amount of essential nutrients such as nitrogen, phosphorus, potassium, calcium and magnesium. The nutrients locked in organic waste are converted during the process into simpler and more readily accessible and absorbable forms in the worm's intestine such as nitrate or ammonium nitrogen, exchangeable phosphorus and soluble potassium, calcium and magnesium. Vermicompost is also treated as a fertilizer substitute and steadily releases major and minor nutrients with a substantial decrease in the C/N ratio, synchronizing with plant requirements (Pattnaik and Reddy, 2010).

An investigation into *Asparagus racemosus* roots grown under organic manures such as cow dung, compost and vermicompost without mineral or chemical fertilizer revealed that the total phenol and total flavonoid content was highest in the vermicompost treated soil plants. In the plants from compost treated soils, the antioxidant activity was highest (Saikia and Upadhyaya, 2011).

2.5.3 Biochar

Biochar is a carbon product obtained when raw materials such as compost from forest animals and residues from plants are heated without air in a closed storage area. Biochar is formed by seeming thermal decomposition of organic substances below incomplete oxygen supply (O^2) and at relatively low temperatures in many scientific and simpler standards (Shareef and Zhao, 2017).

Biochar soil application affects different physicochemical properties of the soil (Jien and Wang, 2013). Nutrient retention and nutrient availability were reportedly enhanced after biochar application because of the higher exchange potential, relevant surface area of biochar and direct nutrient additions (Glaser *et al*., 2002).

Biochar has a generally low nutrient content, so it is important to supply plant nutrients externally (Mann, 2000; Glaser and Birk, 2011). Only C and N can be generated in situ by photosynthetic organisms and biological N fixation respectively, with respect to potential nutrient sources. All other elements, such as P, K, Ca and Mg must be incorporated for nutrient accumulation in the environment (Glaser, 2007).

2.5.4 Sand

In order to improve drainage properties, several grades of sand are available and can be used as a growing medium or as a part of different substrate mixtures (Raviv *et al*., 2002). Anything with particle size ranging from 0.25 to 2.5 mm may be described as sand and particles may be angular or rounded (Harris, 1982). Sand contains almost no mineral nutrients, has no buffering capacity and is used mostly in combination with organic materials (Hartmann and Kester, 1983). Soils with low cation exchange capability, particularly sandy soils, benefit from the addition of organic soil matter that creates better conditions for plant nutrient retention and absorption. Compost contains humus, which binds nutrients loosely and builds up the soil's capacity to store and release plant growth nutrients (Masarirambi *et al*., 2012).

2.6 The effects of abiotic environmental factors on plant growth, development and production of secondary metabolites

The production of plant secondary metabolites is influenced by several abiotic environmental factors like temperature, nutrient supply, humidity, light intensity, minerals, CO_2 influence, water stress, waterlogging, frost, and pollution (Lambers *et al.,* 2008; Ramakrishna and Ravishankar, 2016). Secondary metabolites are related to primary metabolism by the rate of redirection of substrates from primary pathways to secondary biosynthetic pathways. Secondary metabolism is also influenced by numerous environmental elements affecting development, photosynthesis and other parts of primary metabolism (Ibrahim *et al.,* 2011).

2.6.1 Nutrient supply

Secondary metabolite production increases when plants are stressed because growth is often more inhibited than photosynthesis, and the carbon fixed is mainly allocated to secondary metabolites (Ramakrishna and Ravishankar, 2016). Nutrient stress also has a major impact on the level of phenolics in plant tissues, for example, phenolic compounds show a sensitive response to nutrient stress. Nitrogen (N) can either be taken up by plants as inorganic ions (NH4+ or NO3-), or as organic N. There is a lack of data on the impact of N type on production of secondary plant metabolites in plants. According to the Carbon/Nitrogen nutrient balance hypothesis, when N availability is reduced, metabolism shifts more to compounds containing carbon such as starch, cellulose, and non-N-containing secondary metabolites such as phenolics and terpenoids (Ibrahim *et al.,* 2013). Deficit in potassium, sulphur and magnesium are also reported to increase phenolic concentrations. Low iron levels can trigger increased release of phenolic acids from the roots (Ramakrishna and Ravishankar, 2016).

2.7 Hypothesis

a) Optimised cultivation strategy has an influence on plant growth and development and increases the production of secondary metabolites.
b) The type of solvent used in extracting compounds has an effect on antifungal activity.
c) *B. frutescens* possess antifungal activity against *F. oxysporum.*

2.8 The overall aim of the study

To evaluate the efficacy of antifungal activities of *Bulbine frutescens* extracts cultivated on various growth media against plant pathogenic fungi (*Fusarium oxysporum*); as well as to determine the influence of the growth media on plant growth and development.

2.9 Specific objectives of the research

a) To assess the effect of vermicompost, biochar and sand on plant growth and development in *B. frutescens.*
b) To evaluate the *in vitro* antifungal activity of extracts from *B. frutescens* cultivated on vermicompost, biochar and sand
c) To identify bioactive compounds in the extract which have shown promising antifungal activity.

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CHAPTER THREE

Effect of different growing media combinations on plant growth, development and nutrient uptake of *Bulbine frutescens.*

3.1 Introduction

The medicinal potential, including the anti-cancer (Kushwaha *et al*., 2019), antidiabetic and cyto-toxicity (Van Huyssteen *et al*., 2011; Odeyemi and Bradley, 2018), anti-malarial (Bringmann *et al*., 2008), antibacterial and chemical synopsis (Chunderika, 2000) as well the anti-HIV and antioxidant properties (Shikalepo *et al*., 2018) of *Bulbine frutescens* (burn jelly plant, yellow garlic, copaiva, snake flower, stalked bulbine) have been widely reported. The plant is geographically widespread in the Eastern, Western and Northern Cape of South Africa as well as in Lesotho. In the Eastern Cape where its commonly found, the folkloric uses of *B. frutescens* in the treatment of cracked lips, ringworms, STDs, dysentery, burns, itches, urinary tract infections and rashes have been reported (Otang-Mbeng *et al*., 2017). But the Zulu speaking people of the KwaZulu Natal Province use the roots to treat people who are going mad due to witchcraft (Chunderika, 2000). In Lesotho, the plant is used in the treatment of viral respiratory illnesses (Cock and Van Vuuren, 2020). There are also some indications that the leaves are boiled, and the infusion used in the treatment of colds, coughs, and arthritis (Unknown, 2015). According to the same author, the leaves are also consumed as a wild leafy vegetable. The vast pharmacological applications of this African plant indicate its importance in local traditions and culture and its nutraceutical potential. Be that as it may be, the plant is not yet cultivated. The domestication of plants is essential to both the environment and the pharmaceutical industry. Mass cultivation of any species has the potential to ameliorate the negative effects of overharvesting plants from the wild while large amounts are produced for pharmaceutical purposes. However, the cultivation of plants that were previously wild brings its own challenges. For example, the phytochemical properties may change due to failure to replicate the exact climatic and environmental conditions of the plant's original habitat. Plant growth and development are influenced by physical, chemical, and biological factors in the plant's environment. Some of the factors affecting plant growth and development are light, temperature, water, and nutrients (Gailliard, 2018). Plant growth and

development largely depend on the combination and concentration of nutrients that are available in the soil (Morgan, 2013). A lack of macro and micronutrients in the soil leads to the impairment of plant growth and reduced vigour and may result in plant death (Brukhin and Morozova, 2011). The impact of organic matter on soil physical, chemical, and biological properties has great effects on plant growth and yield (Hossain *et al*., 2017). As a direct source of slow-release nutrients, soil organic matter leads to improved chelation of microelements, helps to buffer soil pH, and increases soil cation and anion exchange capacity which improves plant nutrient availability and decreases leaching potential (Weil and Magdoff, 2004). The use of compost and manures not only influences the quantity of soil organic matter but contributes reactive humus-like substances that influence nutrient chelation, supply, and storage to plants (Rivero *et al*., 2004). Soil factors include texture, physical and chemical properties, water holding capacity and nutrients among others (Chen *et al*., 2015). Therefore, in the present trial, *B. frutescens* was subjected to varied organic and inorganic growth media to evaluate the plant's chemical as well as growth and physiological response. The compositions of this media could have an important impact not only on the nutritional and physiological; but also, the phytochemical and antioxidant properties which are essential in the plant's pharmacology.

3.2 Materials and methods

3.2.1 Plant Material

A total of one hundred (8 weeks old) *B. frutescens* plants that were grown in coir (60%): bark (30%): Sand (10%) and fertilized using Multicote were obtained from Shadowlands wholesale nursery Pty. Ltd. in Kuils River, Western Cape Province, South Africa. These plants were transported to the greenhouse at Cape Peninsula University of Technology, Bellville Campus, South Africa. The plants were washed to remove soil debris before being transplanted into 1.5 litre (15 cm x 13cm) pots filled with media components according to the treatments.

3.2.2 Growth medium preparation

This study comprised of four treatments of organic and inorganic media, with the same components but in different proportions as shown in Table 3.1. The components used were vermicompost (obtained from Soilsouls, Lynnwood, Pretoria), biochar (from NewCarbon, Sedgefield, Western Cape) and sand from the Department of Horticultural Sciences, Cape Peninsula University of Technology. The quantity of media blend in each pot was approximately 250 g. The media components were thoroughly mixed before filling into the pots.

Table 3 1: Composition of different media used in this study

Treatments	Composition
T1	Vermicompost + Biochar + Sand (2:1:1)
T2	Vermicompost + Biochar + Sand (1:2:1)
T3	Vermicompost + Biochar + Sand (1:1:2)
T4(Control)	Vermicompost + Biochar + Sand (1:1:1)

3.2.3 Experimental design/ Greenhouse experiment

The experiment was conducted in an environmentally controlled greenhouse of the Horticultural Sciences Department, Cape Peninsula University of Technology, Bellville Campus, Cape Town, South Africa and located at 33°55'47.1"S 18°38'35.5"E. The experiment was carried out under the following conditions: average day temperature of 28.9-36.9 ºC and average relative humidity of 51-59 % between December 2017 and February 2018.

Seedling roots were washed thoroughly to remove soil debris before being transplanted to 15 cm thermo-formed pots, consisting of media components (treatments). All plants were measured to obtain baseline data prior to their use in the experiment. Steel tables measuring 2.5 × 1 m were used as a flat surface for the pots used for the treatments. Plants were randomly allocated to four treatment groups with 25 plants each. The plants in all the treatments were hand watered with 350 ml of distilled water once a week. Nutrifeed® fertilizer supplied by Starke Ayres

Pty. Ltd., Cape Town was used as a source of nutrients to the plants. Nutrifeed 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 mg/kg Zn. Fertilizer group 1 Reg No: K2025 (Act 36/ 1947). The nutrient solution was prepared by dissolving 10 g of fertilizer in 5 L of distilled water, and 100 ml of this solution was supplied to the plants once in two weeks as a soil drench.

Figure 3.1: The experiment setup with treatments

3.2.4 Plant growth parameters

In response to media with the same components but different proportions, the following plant growth parameters were recorded on each plant: Number of leaves and roots, leaf length (cm), plant fresh weight (g), dry weight (g), root length (cm), and root weight (g). Plant growth parameters were evaluated prior to the greenhouse experiment to obtain baseline data and after the experiment.

3.2.5 Tissue analysis

Tissue analysis was conducted after the experiment of 10 weeks. Three leaf samples were per treatment were analysed for macro- and microelements by a certified commercial laboratory (Bemlab (Pty) Ltd in Somerset West, South Africa). Leaves were washed with Teepol solution, rinsed with de-ionised water and dried at 70°C overnight in an oven. The dried leaves were then milled and ashed at 480°C shaken up in a 50:50 HCl (50%) solution for extraction through filter paper (Campell & Plank, 1998; Miller, 1998). The Potassium (K), Phosphorus (P), Calcium (Ca),

Magnesium (Mg), Sodium (Na), Manganese (Mn), Iron (Fe), Copper (Cu), Zinc (Z) and Boron (B) contents of the extracts were analysed using the ash method (Campbell & Plank, 1998 and Miller, 1998). Total Nitrogen (N) content of the leaves was determined through total combustion in a Leco N-analyser. The amounts of N, P, K, Ca and Mg were converted from percentage (%) to mg/kg by a conversion factor of 10 000 (Xego *et al.,* 2017).

3.2.6 Statistical analysis

The experimental data collected were analysed using one-way analysis of variance (ANOVA) and Tukey HSD test was used to separate the means and were treated as significantly different at $P < 0.05$. These computations were performed using STATISTICA software (TIBCO 1984-2018).

3.3 Results

3.3.1 Growth Parameters

3.3.1.1 Number of leaves

There was a significant difference ($df = 3.36$; $F = 8.03$; $P < 0.05$) in *B. frutescens* number of leaves at ten weeks post treatment as shown in Table 3.2. When the means were compared, T1 produced the highest number of leaves (84) in comparison to other treatments which decreased in the order: T4 (64) >T3 (62) >T2 (42). There was no significant difference ($P > 0.05$) between T1, T3, and control.

3.3.1.2 Leaf length

At 10 weeks post treatment, there was a marked difference in leaf length among the treatments ($df = 3.36$; $F = 6.86$; $P < 0.05$). It was observed (Table 3.2) that T1 recorded the highest leaf length (18.36 cm) and this was significantly ($P < 0.05$) higher than other treatments; which decreased in the order T3 (15.54 cm) > T4 (14.52 cm) > T2 (14.41 cm).

3.3.1.3 Plant fresh weight

Treatments had a significant effect ($df = 3.76$; $F = 4.42$; $P < 0.05$) on *B. frutescens* plant weight as shown in Table 3.2 T4 produced the highest plant fresh weight

(102.20 g), followed by T1 (100.80 g) and T3 (90.00 g). The lowest plant fresh weight was obtained in T2 (66.45 g).

3.3.1.4 Plant dry weight

There was a marked difference (df = 3.76; F = 3, 37; P < 0.05) among the treatments at ten weeks post treatment. When the means were compared (Table 3.2), T4 produced the highest plant dry weight (7.00 g) which was significantly (P < 0.05) higher than T3 (5.35 g), T1 (5.22 g) and T2 (3.73 g) respectively.

3.3.1.5 Root length

There was no significant (p > 0.05) difference in root length among the treatments at 10 weeks post treatment (df = 3.76; F = 1, 29; P = 0,282) (Table 3.2). However, T1 produced the highest root length (35.03 cm) followed by T3 (32.78 cm), T4 (31.05 cm), and T2 (29.93 cm).

3.3.1.6 Number of roots

Treatments had a significant effect on the number of roots of *B. frutescens* among treatments (DF= 3, 36; F= 5.91; P < 0.05. By comparison of the leaf length means (Table 3.2), it was observed that the highest leaf length was obtained in T1 (7) which was significantly different (P < 0.05), followed by T3 (6 cm), T2 (6) and T4 (4).

Table 3 2: The effect of media combinations on *B. frutescens* growth parameters

Treatments	No of leaves	Leaf length (Long) cm	Number of roots	Plant wet weight g	Plant dry weight (g)	Root length (cm)
Treatment 1	84±6.27^{b}	18.36±0.50^{b}	7±0.67^{b}	100.80±9.54^{b}	5.22±0.39^{ab}	35.03±2.63^{a}
Treatment 2	42.4±4.43^{a}	14.41±0.99^{a}	5.7±0.33^{ab}	66.45±4.77^{a}	3.73±0.28^{a}	29.93±1.57^{a}
Treatment 3	62±5.73^{ab}	15.54±0.83^{a}	6±0.55^{b}	90.00±7.98^{ab}	5.35±0.32^{ab}	32.78±1.79^{a}
Control	63±7.27^{ab}	14.52±0.21^{a}	4±0.43^{a}	102.20±8.35^{b}	7.00±1.34^{b}	31.05±1.63^{a}

Values shown are mean ± S.E.

Means followed by same lowercase letters in the same column are not significantly different ($P < 0.05$) following comparison using Tukey test.

3.3.2 Nutrient analysis

3.3.2.1 Macronutrients

The levels of K and Mg in the leaves of *Bulbine frutescens* did not differ significantly (df = 3, 8; P > 0.05) among the treatments for all the macro nutrients assessed (Table 3.3). However, the uptake of N, P, and Ca varied significantly (P < 0.05). The uptake of N increased significantly in T1 (26300.00 mg/kg) and control as these were not significantly different, while P levels were higher in the control (3566.67 mg/kg) and Ca increased significantly in T3 (16800.00 mg/kg) and not significantly different to T1 and T2.

3.3.2.2 Micronutrients

As shown in Table 3.4, exposing *B. frutescens* to different growth media did not significantly (p > 0.05) affect the uptake of Na, Mn, Fe, Cu, and B (df = 3.8; P > 0.05). However, the uptake of Zn varied significantly (P < 0.05). Zinc (mg/ kg) decreased in the order T1 (53.67) > T4 (52.33) > T2 (41.67) > T3 (32) although T1 and T4 were not significantly different. Sodium (mg/ kg) was higher in T3 (11418.70) in comparison to T2 (9134.33), T1 (8088.67) and T4 (7251.67). Manganese (mg/kg) was high in T1 (30.67) and low in T2 (16.67). Although Fe (mg/kg) was high in T1 (82.33), this value was not significantly different from that reported in T3 (80.67). But, the lowest Fe (mg/kg) value was reported in T4 (48.67). Copper was high in T1 (9 mg/kg) and low in T4 (3 mg/kg) although T4 was not significantly different from T3 (3.33 mg/kg).

Table 3 3: The effect of media combinations on *B. frutescens* macronutrient uptake (mg/kg)

Treatments	N	P	K	Ca	Mg
Treatment 1	26300.00±1379.61[a]	3366.67±145.296[b]	60266.67±4308.65	14866.67±504.425[ab]	7933.33±260.34
Treatment 2	21966.67±145.29[b]	2666.67±296.27[ab]	57700.00±1081.67	13133.33±924.36[ab]	7733.33±405.52
Treatment 3	17833.33±656.59[c]	2400.00± 208.167[a]	49066.67±491.031	16800.00±1484.36[b]	7466.67±536.45
Control	25066.67±656.59[ab]	3566.67±120.19[b]	55866.67±2251.913	11600.00±600.000[a]	7966.67±120.19

Values shown are mean ± S.E.

Means followed by same lowercase letters in the same column are not significantly different ($P < 0.05$) following comparison using Tukey test.

Table 3 4: The effect of media combinations on *B. frutescens* micronutrient uptake (mg/kg)

Treatments	Na	Mn	Fe	Cu	Zn	B
Treatment 1	8088.67±181.26	30.67±6.3	82.33±2.03	9.00±3.00	53.67±5.17^{b}	25.67±1.45
Treatment 2	9134.33±603.41	16.67±2.03	70.33±19.40	4.33±0.88	41.67±3.67^{ab}	26.67±0.33
Treatment 3	11418.70±1941.52	21.67±1.45	80.67±4.10	3.33±0.33	32.00±1.15^{a}	25.33±3.18
Control	7251.67±495.68	20.00±7.00	48.67±9.02	3.00±1.15	52.33±4.06^{b}	23.67±1.20

Values shown are mean ± S.E.

Means followed by same lowercase letters in the same column are not significantly different ($P < 0.05$) following comparison using Tukey test.

3.4 Discussion

3.4.1 Growth parameters

These results indicate that the different growing media used led to some significant changes in growth and development of *Bulbine frutescens*.

The number of leaves, leaf length, number of roots, plant fresh weight and dry weight showed significant difference among the treatments. The combination of vermicompost: biochar: sand (2:1:1) in the above mentioned parameters except for plant fresh weight and dry weight produced the best results in comparison with other treatments. This was presumably due to the high composition of vermicompost. The different responses of plants to different doses of vermicompost may be a result of the release of variable amounts of available nutrients and other growth promoting substances in this type of compost (Tomati *et al.,* 1990; Grappelli *et al.,* 1989). However, vermicompost is an ideal organic manure for better plant growth (Joshi *et al*., 2015). The present results agree with those of Rekha *et al*. (2018) and Mistry (2015) who reported plant growth increase in plants treated with vermicompost. Meanwhile other authors including Olle (2016), Kashem *et al*. (2015) as well as Joshi *et al*. (2015) reported that vermicompost based growth substrate promoted plant growth parameters such as leaves, roots, shoots and root dry weight, stem diameter, plant fresh weight and fruits. The root length was not statically different among the treatments but with vermicompost: biochar: sand (2:1:1) producing the best results.

In comparison to other treatments, vermicompost + biochar + sand in the ratio 1:2:1 was the least to produce the best results in the number of leaves, leaf length, plant fresh weight, plant dry weight, and root length. It has been shown that application of biochar in the soil has a positive to neutral and even negative impact on crop production (Ding *et al.,* 2016). In line with these results, Spokas *et al.* (2012); Lentz and Ippolito (2012) and Ding *et al*. (2016) state that biochar's function is related to application rates, pyrolysis temperature, soil characteristics and plant species. Vermicompost + biochar + sand in the ratio 1:1:2 performed better in leaf length, number of roots, and root length when compared to the control. Therefore, the negative effects on plant growth may have occurred due to the application rate of biochar, as the treatment vermicompost + biochar + sand ratio 1:2:1 had high biochar content. A combination of biochar and compost is a powerful factor to

develop positive effects on plants by the application of such amendments (Bonanomi *et al.,* 2017). However, because of limited and fragmented knowledge, reliable guidelines about the amount of compost that should be mixed with biochar to enhance plant growth are lacking (Bonanomi *et al.,* 2017). Liu *et al.* (2018) reported that biochar application has the potential to improve soil fertility, nutrient retention and increase microbial activity by reducing soil density and increasing water holding capacity and soil temperature. According to Mabengwa (2013), plants grow well in media that holds water evenly and provide sufficient nutrient holding capacity. Meanwhile, vermicompost also provides optimum temperature; moisture and a balance between organic and inorganic nutrients needed in increasing plant growth (Singh *et al*., 2011). Liu *et al.* (2012) proved that the combined application of compost and biochar has a positive collaborative effect on water holding capacity and soil nutrient content. Furthermore, the combination of compost with biochar has been proven to be beneficial in terms of improving soil quality as well as improving and retaining nutrients, therefore enhancing plant growth (Agegnehu *et al.,* 2015; and Bonanomi *et al.,* 2017).

A combination of biochar and vermicompost offer a good approach to plant growth promotion and offer great environmental advantages while reducing environmental risk (Álvarez et al., 2017; Di *et al*., 2019).

3.4.2 Macro and Micronutrients

The present results indicate that the treatments did have a significant effect on the uptake of N, P, Ca, and Zn. The treatment that had the highest uptake of N, and Zn was vermicompost: biochar: sand in the ratio 2:1:1. Vermicompost has been shown to possess high levels of total and available nitrogen, phosphorous, potassium and some micronutrients (Parthasarathi *et al.,* 2008). Reddy and Reddy (1999) reported an increase in micronutrients in field soils after vermicompost applications. In other studies, the amount of soil nitrogen increased significantly after incorporating vermicompost into soils (Sreenivas *et al.,* 2000; Kale *et al.,* 1992) and the amount of available P and K also increased (Venkatesh *et al.,* 1998).

The present results show that the treatments did not have any significant effect on the uptake of K, Mg, Na, Mn, Fe, and Cu. Fageria (2007) reported that the presence of sufficient total quantities of nutrients in the soil does not guarantee that the nutrients will be available to growing plants because of factors such as soil-moisture content, soil temperature, pH, soil physical conditions and presence of toxic elements and/or salts (Etienne *et al*., 2018). However, vermicompost: biochar: sand (2:1:1) showed better uptake of nutrients in all the treatments.

3.5 Conclusion

The different growing media significantly influenced growth parameters of *B. frutescens*. This study demonstrated that vermicompost + biochar + sand have a positive and also negative effect on the growth of the plants depending at the ratio of the substrates incorporated. Vermicompost + biochar + sand (2:1:1) medium combination significantly favoured the growth followed by 1:1:2, whilst 1:2:1 with high incorporated biochar performed the least. This study also revealed that most nutrient composition was not influenced by the growth media used. Nevertheless, vermicompost + biochar + sand (2:1:1) proved to be effective by showing a better uptake in all the treatments. In conclusion this study indicated that vermicompost + biochar + sand (2:1:1) can be used to enhance the growth and development of *B.frutescens*.

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CHAPTER FOUR

Effect of varied growth medium combinations on in vitro antifungal activity and bioactive compounds of extracts from *Bulbine frutescens*.

4.1 Introduction

Secondary metabolites are compounds that are not necessary for a cell (organism) to live, but play a role in the interaction of the cell (organism) with its environment (Demain and Fang, 2000; Pagare *et al.,* 2015). The main classes of secondary metabolites include terpenoids and steroids, fatty acid-derived substances and polyketides, alkaloids, nonribosomal polypeptides, and enzyme cofactors (McMurry, 2015). Plants are major sources of secondary metabolites and about 80% of them in nature are produced by plants (Croteau *et al.,* 2000; Berdy, 2005).These secondary metabolites are classified according to their chemical structures into several classes (Hussein and El-Anssary, 2018; Thirumurugan *et al.,* 2018), which include phenolics, tannins, terpenoids, alkaloids, and flavonoids. Many of these secondary metabolites have been found to possess *in vitro* antimicrobial properties (Bourgaud *et al.,* 2001; Nguyen *et al.,* 2013; Hussein and El-Anssary, 2018). The bioactivities of these compounds provide the scientific basis for therapeutic investigations of plants and the validation of their use in the traditional medicine in many communities (Bourgaud *et al.,* 2001). Medicinal properties of plants include antiviral, antibacterial, antifungal and antiparasitic (Hussein and El-Anssary, 2018). Some of these compounds can protect plants against biotic (pathogens and herbivores) or abiotic (draught) stress (Pagare *et al.,* 2015). Evidence exists that a number of extrinsic factors do influence the production of secondary metabolites. One of the most limiting factors is nutrient availability (Waterman and Mole, 1989; Lambers *et al*., 2008). For example, the high nutrient content of the organic matter amendments applied to the soil or other growing media have been shown to significantly influence the production of secondary metabolites (Taie *et al*., 2008).

Over the last 50 years, studies on plant secondary metabolites have been increasing (Bourgaud *et al*., 2001). The conventional method of secondary metabolite production relies on extraction of metabolites from plant tissues by different phytochemical procedures, such as extraction using solvents (Thirumurugan *et al.,*

2018). Researchers from a variety of scientific disciplines are confronted with the challenge of extracting plant material with solvents of different polarities, often as a first step towards isolating and identifying the specific compounds responsible for biological activities associated with a plant or a plant extract (Pagare *et al.,* 2015). The type of solvent used in the extraction procedure determines the success of isolating compounds from plant material (Masoko *et al.,* 2008). Therefore, to extract all compounds it is important to extract using different solvents of varying polarity to cover the polarity range (Masoko *et al.,* 2008).

There is a growing recognition of the benefits of studying indigenous knowledge, validating traditional uses of plants and enhancing cultivation of medicinal plants. One of the species that needs to be studied further is *Bulbine frutescens*. It is a medicinal plant that belongs to the Asphodolaceae family and is widely cultivated for its aesthetic purposes (Bringmann *et al*., 2008). In previous studies, *B. frutescens* has been shown to possess antimicrobial activity against *Candida albicans*, *Candida tropicali, Aspergillus glaucus, Briotis cinerea, and Trichophyton mentagrophytes* among others (Ghuman and Coopoosamy, 2011; Fielding *et al.,* 2015). Rachuonyo *et al*. (2016a) and Rachuonyo *et al*. (2016b) showed that *B. frutescens* has anti-bacterial and antifungal compounds against pathogens causing ills/diseases in humans. However, to the best of our knowledge, fungicidal activity of this constituent on plant pathogens has never been reported either in ancient or modern times. Thus, the objectives of this chapter were (i) to evaluate the *in vitro* antifungal activity of extracts from *B. frutescens* cultivated on vermicompost, biochar and sand, (ii) to identify compounds in the extracts.

4.2 Materials and method

4.2.1 Plant material

A total of one hundred (8 weeks old) *B. frutescens* plants that were grown in coir (60%): bark (30%): Sand (10%) and fertilized using Multicote were obtained from Shadowlands wholesale nursery Pty. Ltd. in Kuils River, Western Cape Province, South Africa. These plants were transported to the greenhouse at Cape Peninsula University of Technology, Bellville Campus, South Africa. The plants were washed to remove soil debris before being transplanted into 1.5 litre (15 cm x 13cm) pots filled with media components according to the treatments.

4.2.2 Growth medium preparation

This study comprised of four treatments of organic and inorganic media, with the same components but in different proportions as shown in Table 3.1. The components used were vermicompost (obtained from Soilsouls, Lynnwood, Pretoria), biochar (from NewCarbon, Sedgefield, Western Cape) and sand from the Department of Horticultural Sciences, Cape Peninsula University of Technology. The quantity of media blend in each pot was approximately 250 g. The media components were thoroughly mixed before filling into the pots.

Table 4. 1: Composition of different media used in this study

Treatments	Composition
T1	Vermicompost + Biochar + Sand (2:1:1)
T2	Vermicompost + Biochar + Sand (1:2:1)
T3	Vermicompost + Biochar + Sand (1:1:2)
T4(Control)	Vermicompost + Biochar + Sand (1:1:1)

4.2.3 Experimental design/Greenhouse experiment

The experiment was conducted in an environmentally controlled greenhouse of the Horticultural Sciences Department, Cape Peninsula University of Technology, Bellville Campus, Cape Town, South Africa and located at 33°55'47.1"S

18°38'35.5"E. The experiment was carried out under the following conditions: an average day temperature of 28.9-36.9 ºC, night and average relative humidity of 51-59 % between December 2017 and February 2018.

Seedling roots were washed thoroughly to remove soil debris before being transplanted to 15 cm thermo-formed pots, consisting of media components (treatments). All plants were measured to obtain baseline data prior to their use in the experiment. Steel tables measuring 2.5 × 1 m were used as a flat surface for the pots used for the treatments. Plants were randomly allocated to four treatment groups with 25 plants each. The plants in all the treatments were hand watered with 350 ml of distilled water once a week. Nutrifeed® fertilizer supplied by Starke Ayres Pty. Ltd., Cape Town was used as a source of nutrients to the plants. Nutrifeed 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 mg/kg Zn. Fertilizer group 1 Reg No: K2025 (Act 36/ 1947). The nutrient solution was prepared by dissolving 10 g of fertilizer in 5 L of distilled water, and 100 ml of this solution was supplied to the plants once in two weeks as a soil drench.

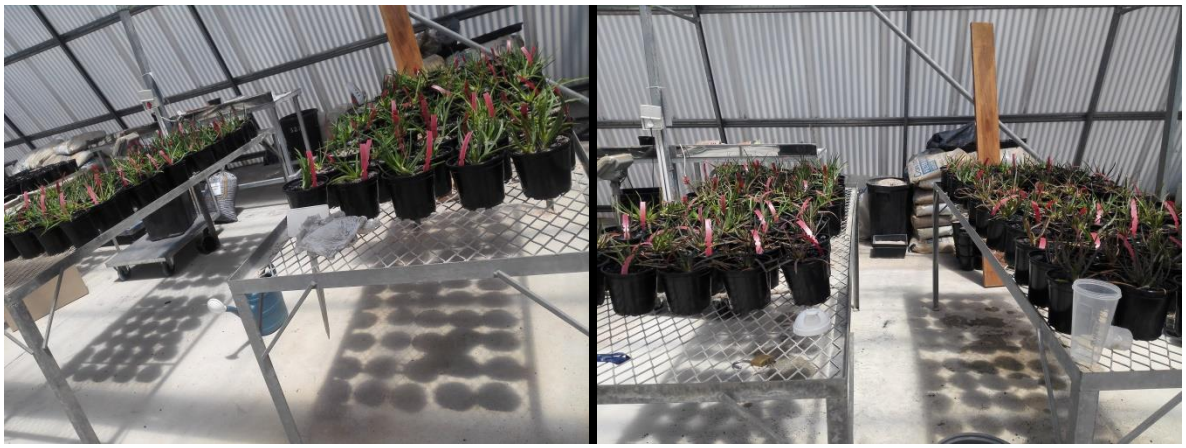

Figure 4.1: The experiment setup with different treatments

4.2.4 Solvent extraction of plant material

Fresh leaves were harvested at 10 weeks post treatment and were washed in running water to remove debris and dust particles and then rinsed using distilled water. The leaves from the different treatments were excised into smaller pieces and air dried at 29 ºC. The dried leaves were ground using a Jankel and Kunkel Model A 10 mill into fine powder. The powdered leaf material (5 g) was extracted with 100 ml of acetone in a glass beaker using a vortex mixer for 15 minutes and the supernatant filtered through Whatman No.1 filter paper. The plant extracts were left to air dry overnight in a fume cabinet at room temperature (22 ± 2 ºC).

4.2.5 Antifungal activities of extracts (MIC)

The microdilution method described by Eloff (1998) and Nchu *et al*. (2010) was adopted with minor modifications in determination of the minimum inhibitory concentration (MIC) for the extracts. One hundred microliters of starting concentration (6 mg/ml) of extracts for each treatment were serially diluted 50 % with water in 96-well microplates (two-fold serial dilution). *Fusarium oxysporum* sp. glycines strain (UPFC no. 21) obtained through the courtesy of the Phytomedicine Programme, University of Pretoria, was used as the pathogenic agent. C. Cronje originally isolated the fungus strain from roots of a maize plant in Delmas, Gauteng. *F. oxysporum* was sub-cultured from stock agar plates and grown into Nutrient Broth (Merck, South Africa) for four hours. The fungal concentration (100 µl) was added to each well of the 96-well microplates (10^5 cells/ml). A final fungal concentration (10^5 cells/ml) used for this study was determined by the aid of a haemocytometer. Mancozeb (160 µg/mL) was prepared as stock solution in autoclaved distilled water and served as a positive control and acetone used as a negative control. Forty micro litres (40 µl) of 0.2 mg/ml of *p*-iodonitrotetrazolium chloride (INT) (Sigma) dissolved in sterile distilled water was added to each microplate well, sealed in a plastic bag and incubated at 37 ºC and 100% RH. The MIC values were recorded after 6 and 12 hr. The antifungal bioassay (MIC) consisted of three replicates per treatment and per solvent.

4.2.6 Preliminary analysis

Antifungal activity of *Bulbine frutescens* that was originally grown in soil was analysed as described in 4.2.4 and 4.2.5 to compare the values with the one that was grown in different growing media used in the present study.

4.2.7 Bioactive compounds

4.2.7.1 Sample preparation

B. frutescens plants were harvested at 10 weeks post treatment and the leaves were even dried 35 ºC. The leaves of each plant were grounded separately using ceramic mortar and pestle for 2 minutes. The sample preparation technique was followed for all assays and all analyses were performed in triplicate.

4.2.7.2 Total polyphenol, flavonol and alkaloid content

A spectroscopic method described by Fadhil and Reza (2007) was used to determine total alkaloids in the plant. Briefly, dried *B. frutescens* leaves were powdered then extracted with 10 mL of 60 % ethanol and 40% of water for 2 hr, centrifuged (4000× g for 10 min), and the supernatant was used in the assay. Extract supernatant (2 ml) and atropine standard solutions 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 vigorously mixing using a vortex mixer. The absorbance at 417 nm was determined, and the concentration of the sample (mg/g) using a standard curve of atropine was calculated.

The total polyphenol content of the aqueous ethanol extracts of dried leaves was determined by the Folin–Ciocalteu method described by Daniels *et al.* (2011). Using a 96-well microplate, 25 µL of the sample was mixed with 125 µL Folin–Ciocalteu reagent (Merck, Cape Town, South Africa), diluted 1:10 with distilled water. After 5 min, 100 µL (7.5%) aqueous sodium carbonate (Na_2CO_3) (Sigma-Aldrich, Johannesburg, South Africa) was added to each well. The method described by Daniels *et al.,* (2011) was used to obtain the absorbance reading at 765 nm of the solution in the microplates using a Multiskan plate reader (Thermo Electron Corporation, USA), The standard curve was prepared using 0, 20, 50, 100, 250 and

500 mg/L gallic acid in 10% ethanol and the results were expressed as mg gallic acid equivalents per g dry weight (mg GAE/g DW).

The flavonol content of the aqueous ethanol extracts of dried leaves was determined using quercetin 0, 5, 10, 20, 40, and 80 mg/L in 95% ethanol (Sigma-Aldrich, South Africa) as standard, and the protocol was based on the method described by Daniels *et al.* (2011). In the sample wells, 12.5 µL of the crude sample extracts was mixed with 12.5 µL 0.1% hydrochloric acid (HCl) (Merck, Cape Town, South Africa) in 95% ethanol and incubated for 30 min at room temperature. The absorbance was read at 360 nm, at a temperature of 25 ºC The results were expressed as mg quercetin equivalent per g dry weight (mg QE/g DW).

4.2.8 Statistical analysis

The experimental data collected were analyzed using one-way analysis of variance (ANOVA) and Tukey HSD test was used to separate the means and were treated as significantly different at $P < 0.05$. These computations were performed using STATISTICA software (TIBCO 1984-2018).

4.3 Results

4.3.1 Acetone extracts on minimum inhibitory concentration (MIC)

There was no significant difference in MIC results among the treatments at 6 hr (DF: 8.0 F: 2.18 $P > 0.05$) and 12 hr (DF: 8.0 F: 1.88 $P > 0.05$) following ANOVA analysis (Table 4.2). It was observed that at 6 hr, T2 showed high antifungal activity against *F. oxysporum*, but it was not significantly different from the other treatments. Similarly, there was no significant difference in MIC results among the treatments at 12 hr. However, T2 and T3 exhibited the strongest anti-fungal activity against *F. oxysporum*. The positive control showed antifungal activity in both 6 (1.5 mg/l) and 12 hr (0.75 mg/ml) whilst negative control showed no activity. Additionally, the MIC value of *B. frutescens* normally grown in normal soil was not significantly different at both 6 and 12 hr (Table 4,3) compared to plants grown in different media combinations. The antifungal activity of the extracts showed better anti *F. fusarium* compared to positive control, especially at 12 hr. Generally, *B. frutescens* have

antifungal activities against *F. oxysporum* although there was no significant difference in treatments.

Table 4. 2: Results on (MIC) obtained from acetone extracts of *B. frutescens* leaves following exposure to different media combinations.

Treatment	MIC± SE(mg/ml)	
	6 hr	12 hr
T1	1.25±0.25A	1.12±0.37a
T2	0.62±0.12A	0.50±0.12a
T3	0.75±0.00A	0.50±0.12a
T4	1.0±0.25A	0.62±0.12a
Mancozeb	1.50±0A	No effect
Negative control	0.75±0.00	No effect

- Means followed by the same uppercase letters in the same row are not significantly different (P > 0.05), between 6 and 12 hr post treatment incubation in MIC, following comparison using Tukey test. Grey and white colours are used to differentiate treatments
- Means followed by the same lowercase letters in the same column are not significantly different (P > 0.05) among treatments following comparison using Tukey test.

Table 4. 3: Preliminary results on (MIC) obtained from *B. frutescens* grown in normal soil.

Hours	MIC± SE(mg/ml)
6 hr	2.0±0.5A
12hr	1.0±0.25a

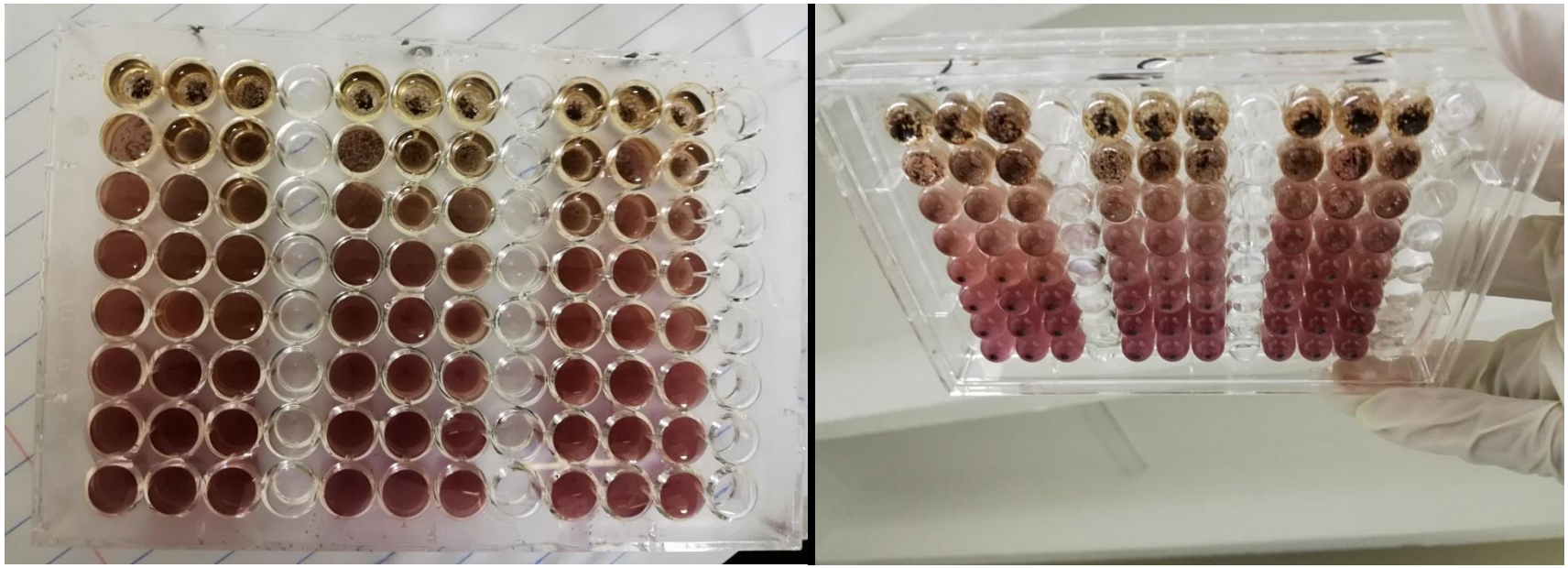

Figure 4.2: (Preliminary study) (Preliminary study) anti *F. oxysporum* activity of acetone extracts from *B. frutescens*

4.3.2 Secondary metabolites

The total polyphenols (mg GAE/g) were found to be significantly different (DF: 8.00 F: 9.05 P < 0.05) in the treatments. The total polyphenols were significantly high in T1 (9.316 ± 0.17) mg GAE/g and low in T4 (7.08±0.53), although T4, T3 (7.47±0.26) and T2 (7.90±0.17) were not significantly different from each other.

There was no significant difference (DF: 8.00 F: 2.21 P > 0.05) in flavonols content among treatments. However, T1 produced higher flavonols than the other treatments (Table 4.4) although it was not significantly different from T2, T3 and T4. There was a significant difference (DF: 8.00 F: 63.5 P < 0.05) in alkaloid content among the treatments, once more T1 (6.29mg/g) recorded the highest alkaloids. Broadly, *B. frutescens* contains alkaloids, polyphenols and flavonols and the substrate mix T1 (Vermicompost + Biochar + Sand (2:1:1) favoured higher accumulation of polyphenols, flavonols and alkaloids.

Table 4. 4: Mean ± SE of total polyphenol (mg GAE/g dry weight), flavonol (mg QE/g dry weight) and alkaloids (mg/g dry weight) content of the leaves of *B. frutescens*.

Treatments	Polyphenol (mg GAE/g)	Flavonols (mg QE/g)	Alkaloids (mg/g)
Treatment 1	9.31±0.17b	3.10±0.29a	6.29±0.08c
Treatment 2	7.90±0.17ab	2.39±0.14a	4.14±0.19b
Treatment 3	7.47±0.26a	2.65±0.26a	4.79±0.04a
Treatment 4(control)	7.08±0.53a	2.35±0.18a	4.67±0.09ab

Means in the same column with different letters are significantly different at P < 0.05.

4.4 Discussion

4.4.1 Minimum inhibitory concentration (MIC)

The results obtained from this study indicate that all the evaluated media combinations showed no significant difference in the mean of MIC values at both 6 and 12 hr on extracts of *B. frutescens* against *F. oxysporum*. However, extracts of *B. frutescens* plants that were grown in T2 (Table 4.2) exhibited the most antifungal effects bioactive against F. oxysporum at 6 hr compared to T1, T3 and T4. Similarly, Khalifa and Thabet (2015) observed that plants grown in amended substrate showed remarkable higher resistance to *F. oxysporum*. Biochar soil amendment does not only affect the crop yield and promoting the plant growth, it also has a suppressive effect against several soil borne fungal diseases (Kloss *et al*., 2014; Graber et al., 2014; Jaiswal *et al*., 2015). Graber *et al*. (2014) and Jaiswal *et al*. (2015) argued that biochar can potentially enhance plant resistance against various soil-borne pathogens and disease severity is biochar dose-dependent.

These results are in agreement with Akhter *et al.* (2015) who stated that soil amendments like compost and biochar are not known only to affect soil properties and plant growth but can also affect soil-borne plant pathogens. Szczech (1999), Szczech *et al.,* (1993); Borrero *et al*. (2004): observed that growth media formulated with composted organic waste such as vermicompost can suppress *F. oxysporum* and many studies document a suppressive effect of organic amendments like compost and organic wastes against *F.oxysporum* and other soil borne pathogens (Borrero *et al*., 2004; Bonanomi *et al*., 2007).

In previous studies *B. frutescens* has been shown to possess antimicrobial activity against *Candida albicans*, *Candida tropicali, Aspergillus glaucus, Briotis cinerea,* and *Trichophyton mentagrophytes* (Ghuman and Coopoosamy, 2011; Fielding *et al.,* 2015). However little or no information is available on *B. frutescens* and plant pathogenic fungi, therefore the results of this study show that *B. frutescens* can potentially be used to control *f. oxysporum.*

4.4.2 Secondary metabolite content

In the phytochemical analysis of this study, polyphenols, flavonols and alkaloids were detected in the extracts of *B. frutescens* leaves. Similarly, Shikalepo *et al.,* (2018) phytochemical screening revealed the presence of phenols, alkaloids and flavonoids in *B. frutescens*. Additionally, Castillo *et al*. (2012) reported that the principal groups with antifungal activity are terpenes, tannins, flavonoids, alkaloids, lecithin and polypeptides and studies suggest that the majority of secondary metabolites possess antifungal characteristics (Koga *et al.,* 1995). Relatively no information is available on these secondary metabolites in *B. frutescens* against plant pathogens. However, Rongai *et al.* (2015) and Salhi *et al.* (2017) found these types of secondary metabolites to possess an antifungal activity against *F. oxysporum* and the antifungal activity of these plant extracts may be due to the presence of secondary metabolites.

There was a significant difference ($P< 0.05$) (Table 4.4) in total polyphenol content and alkaloid content among treatments. This means that the different media combinations had an effect on the total polyphenol content and alkaloid content found in the leaves of *B. frutescens*. There was no marked difference in flavonol content. When the means were compared, T1 produced the highest level of flavonol found in the leaves of *B. frutescens* compared to other treatments. Treatment one had a high level of vermicompost compared to other treatments that imparts high organic matter in the soil, which influences higher production of plant phenols and flavonoids (Ibrahim *et al*., 2013). These results prove that the production of secondary metabolites is affected by abiotic environmental factors such as nutrient supply (Lambers *et al*., 2008; Ramakrishna and Ravishankar, 2016). Plant metabolites like flavonoids, phenols and alkaloids are significantly influenced by the high nutrient content of the organic matter amendments applied to the soil or other growing media (Taie *et al*., 2008). Ibrahim *et al.* (2011) found that nitrogen levels had a significant impact on the production of total phenolics and flavonoids. Vermicompost has been shown to have high levels of total and available nitrogen, phosphorous, potassium (NPK) and micro nutrients (Parthasarathi *et al.,* 2008).

Although the differences in secondary metabolites' levels was not reflected in antifungal activity of the different treatment, many factors may influence the antifungal activity of extract including the presence, quality and relative proportion of

the individual bioactive compounds in an extracts. Therefore, important future studies isolates and quantify the bioactive metabolites in *B. frutescens* metabolomics can shed light on the effect of substrates on *B. frutescens*.

4.5 Conclusion

In conclusion, while the different media combinations did not significantly influence the antifungal activity of *F. oxysporum*, this study demonstrated *B. frutescens* extracts are bioactive against the plant pathogen *F. oxysporum* and Vermicompost, biochar and sand incorporated media have a positive effect on anti *f. oxysporum* activity. The study also revealed that Vermicompost + Biochar + Sand (2:1:1) medium combination significantly favoured the accumulation of secondary metabolites in *B. frutescens*. Future studies in the effect of growth media on quantity and quality of specific bioactivity compounds are warranted.

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Chapter Five
General discussion, conclusion and recommendations

5.1 General discussion

Antifungal activities in higher plants have long been recognized, and an intensive search has increasingly been focusing on plants as natural strategies for eco-friendly management of plant pathogens. There is a growing recognition of the benefits of studying indigenous knowledge and enhancing the cultivation of medicinal plants. *B. frutescens* is one of the most important medicinal plant species that is wildly cultivated for its aesthetic purposes. However, there is a scarcity of studies on the pharmacological activities of this plant against *F. oxysporum.*

Results of the present study (Chapter 3) revealed that *B. frutescens* growth parameters: Number of leaves, leaf length, plant fresh weight, plant dry weight, root length and number of roots were significantly affected by the different growing media combinations. Plants cultivated in media with a high composition of vermicompost (vermicompost + biochar + sand – 2:1:1) in number of leaves and roots, leaf length and root length showed the best growth results when compared with other treatments. However, growth media with a low vermicompost ratio and high sand ratio (vermicompost + biochar + sand - 1:1:2) slowed down *B. frutescens* growth. Tomati et al. (1990) and Grappelli et al. (1989) reported that the different response of plants to different doses of vermicompost may be a result of favourable and optimum temperature, moisture and the release of variable amounts of available nutrients and other growth promoting substances. The control (1:1:1) performed better when compared with plants that had a ratio of 1:2:1 and 1:1:2. Furthermore it was found that there was a significant difference in micro and macronutrients (N, P, Ca, Zn) and no significant difference in Na, Mn, Fe, Cu, and B. However, T1 (2:1:1) performed better in the uptake of most nutrients, which potentially explains the exceptional increase in plant growth parameters. A higher composition of vermicompost improved the uptake on nutrients; therefore, growth improvement was presumably related to enhancement of nutrient uptake.

Acetone extracts of *B. frutescens* leaves subjected to different media combinations were found to be bioactive against *F. oxysporum*. Acetone extracts of *B. frutescens*

leaves that were cultivated in T2 (1:2:1) were the most bioactive against *F. oxysporum*. Additionally, the phytochemical analysis of this study revealed that *B. frutescens* possesses polypenols, flavonols and alkaloids and these are some important pharmacological secondary metabolites. The different media combinations significantly influenced the content of polyphenols and alkaloids among the treatments. In comparison with other treatments including the control, T1 (2:1:1) produced the highest contents of metabolites. Furthermore, flavonol contents were not significantly influenced by the growth media combinations; however, Treatment 1 produced more flavonol contents. Secondary metabolites found in the current study are reported to possess antifungal activity against *F. oxysporum.*

5.2 Conclusion

In conclusion, the cultivation of *B. frutescens* using different growing media combinations has the potential to optimize its growth and some secondary metabolites. While the different media combinations did not significantly influence antifungal activity against *F. oxysporum*, this study demonstrated that *B. frutescens* extracts are bioactive against *F. oxysporum*, and vermicompost, biochar as well as sand incorporated media possess anti *F. oxysporum* activity. The study also revealed that combining Vermicompost + Biochar + Sand in the ratio 2:1:1 significantly favoured the accumulation of secondary metabolites in *B. frutescens*. Although the differences in secondary metabolite compositions was not reflected in antifungal activity of the different treatments, many factors may influence the antifungal activity of the extract including the presence, quality and relative proportion of the individual bioactive compounds.

5.3 Recommendations

Based on the above conclusions, it is therefore recommended that future studies should investigate the effect of growth media on quantity and quality of bioactive metabolites in *B. frutescens*.

Appendices

Appendix 1: Data obtained from leaf nutrient analysis of *B. frutescens* used in chapter 3.

Species	**Orchard**	**Lab.**	**N**	**P**	**K**	**Ca**	**Mg**	**Na**	**Mn**	**Fe**	**Cu**	**Zn**	**B**
		Nr.	**%**	**%**	**%**	**%**	**%**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**
B. frutescens	Treatment 1	30895	2,42	0,31	5,8	1,56	0,75	8035	43	86	15	49	26
B. frutescens	Treatment 1	30896	2,58	0,34	5,42	1,39	0,79	8426	27	79	6	48	23
B. frutescens	Treatment 1	30897	2,89	0,36	6,86	1,51	0,84	7805	22	82	6	64	28
B. frutescens	Treatment 2	30898	2,2	0,21	5,56	1,32	0,78	9092	20	63	4	38	27
B. frutescens	Treatment 2	30899	2,22	0,31	5,83	1,47	0,84	8111	17	107	6	49	27
B. frutescens	Treatment 2	30900	2,17	0,28	5,92	1,15	0,7	10200	13	41	3	38	26
B. frutescens	Treatment 3	30901	1,91	0,27	4,81	1,39	0,81	9222	24	87	3	34	28
B. frutescens	Treatment 3	30902	1,75	0,25	4,94	1,88	0,79	9744	19	82	4	32	29
B. frutescens	Treatment 3	30903	1,69	0,2	4,97	1,77	0,64	15290	22	73	3	30	19
B. frutescens	Treatment 4	30904	2,38	0,38	5,86	1,22	0,82	8115	13	51	3	53	26
B. frutescens	Treatment 4	30905	2,6	0,35	5,14	1,04	0,79	6398	13	32	1	45	22
B. frutescens	Treatment 4	30906	2,54	0,34	5,76	1,22	0,78	7242	34	63	5	59	23

Appendix 2: Secondary metabolites of *B. frutescens* found in this study and the effect of medium combinations in metabolites content.

	Polyphenols				**Flavonols**				**Alkaloids**		
Sample	**mg GAE/g**				**mg QE/g**				**mg/g**		
#	**Repeat 1**	**Repeat 2**	**Repeat 3**		**Repeat 1**	**Repeat 2**	**Repeat 3**		**Repeat 1**	**Repeat 2**	**Repeat 3**
1	**8,98**	**9,55**	**9,42**		**2,51**	**3,46**	**3,33**		**6,15**	**6,43**	**6,29**
2	**7,88**	**8,21**	**7,61**		**2,57**	**2,50**	**2,11**		**3,81**	**4,47**	**4,14**
3	**7,06**	**7,96**	**7,39**		**2,13**	**2,84**	**2,98**		**4,72**	**4,86**	**4,79**
4	**6,49**	**6,60**	**8,16**		**2,00**	**2,64**	**2,42**		**4,51**	**4,84**	**4,67**
	GAE = Gallic acid equivalents										
	QE = Quercetin equivalents										
	CE = Catechin equivalents										