Cape Peninsula
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THE EFFECT OF WATER DEFICITS ON THE GROWTH OF INDIGENOUS *SALVIA* SPECIES AND THE ANTI-FUNGAL ACTIVITY OF THEIR LEAF EXTRACTS AGAINST *FUSARIUM OXYSPORUM* SCHITDL. (HYPOCREALES), A CAUSAL AGENT OF SEEDLING BLIGHT IN MAIZE.

by

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DECLARATION

I, Shaheed Roos, 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.

December 2020

Signed **Date**

DEDICATION

In the name of God, the Most Gracious, the Most Merciful,

I would like to dedicate this thesis to my entire family from my late grandparents to my nieces and nephews and all in between including my esteemed parents, my siblings, my aunts and uncles and all my beloved first and second cousins and their children and grandchildren.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Synthetic chemicals come with negative implications on human health and also cause environmental degradation. There is a call for natural, environmentally friendly products to be used in the cultivation, storage, and processing of food crops. The laws and restrictions on some of the harmful synthetic chemicals are becoming stricter. The Republic of South Africa is rich in medicinal plants, of which its traditional healers use 3000 species to treat a plethora of illnesses and ailments. Most plants' healing properties are due or at least partly due to their phenolic compounds. One of the uses of secondary metabolites by the plants is for protection against pests and pathogens. Thus, humans can use these phytochemicals during crop production to manage diseases caused by phytopathogenic fungi and bacteria. Products utilising these natural compounds are the alternative to synthetic agricultural products. By producing these green products, South Africa can contribute to the global organic market worth multi-billions of US dollars, increases its GDP, and creates employment opportunities for citizens. South Africa is also a water-scarce country, which can be a limiting factor in crop production.

Studies have shown that cultivating medicinal plants under water deficits enhances the quality and quantity of the same compound intended to be extracted to produce these green products. It is, therefore, why this study was initiated. Six species of indigenous *Salvia* were screened for their anti-fungal activity against *Fusarium oxyproum*, a phytopathogenic pathogen that causes seedling blight in *Zea mays* (Maize). Maize is one of the most important food crops in many countries across the globe. Poor stands due to seedling blight are financially devastating for commercial farmers, but it could mean starvation for subsistence farmers and developing countries. Fungicides are recommended for the management of seedling blight. However, the indiscriminate use of these chemicals causes the degradation of human health and the world's environment.

In the first part of this study (chapter 2), the aim was to evaluate the anti-fungal activity of extracts against the phytopathogenic fungus *F. oxysporum*. The plant extracts were screened *in vitro* for their anti-fungal activity against *F. oxysporum* in a Minimal Inhibitory Concentration (MIC) assay. Extracts of all the six species inhibited the *F. oxysporum* at the 6, 12, 18, and 24 h post-treatment. The MIC values of the extracts ranged from 0.75 mg/ml and 12 mg/ml. However, *S. dolomitica*, *S. namaensis*, and *S. repens* with MIC values of 3.0, 4.2, and 9.6 mg/ml, respectively, continued to inhibit fungal growth beyond the 18th hour time period

suggesting extracts from these species have fungistatic activities. *S. chameleagnea* showed the weakest activity compared to the other species in all four of the tested time slots. In contrast, *S. dolomitica* and *S. namaensis* showed the best activity was subsequently selected for further study. In conclusion, the six *Salvia* spp. studied are potential sources of anti-fungal extracts and compounds.

In the second part of this study (Chapter 4), effects of different levels of water deficit treatments on the growth, concentration of polyphenols, and anti-*Fusarium oxysporum* activity of *S. dolomitica* and *S. namaensis* were assessed. Four-weeks old seedlings of the two species were subjected to 3-day, 6-day, 9-day, or 12-day water deficit regimes. Secondary metabolites such as polyphenols, alkaloids, and flavonols were assessed using spectroscopic methods, and the anti-fungal activities of crude extracts obtained from plants exposed to the various treatments were evaluated in a micro-dilution bioassay. In all treatments, the plant height, crown width, the number of stems and leaves, and fresh and dry weights reduced at increased intervals between irrigation. Acetone extracts from all treatments showed anti-fungal activity in MIC bioassay — the MIC values ranged from 0.75–6 mg/ml and 1.5–6 mg/ml for *S. dolomitica* and *S. namaensis*, respectively. However, extracts from the treatment with moderate water deficit (6-day watering interval) recorded significantly ($P<0.01$) better inhibition of *F. oxysporum* at the 18 h post-incubation than Mancozeb. This research has revealed that moderate water deficit level favours the accumulation of polyphenols, flavonols, and alkaloids in *S. dolomitica*. In contrast, severe water deficits treatment was associated with higher alkaloid content in *S. namaensis* extracts. Generally, total alkaloid content increased as the level of water deficits increased in both species. There is a possible correlation between this increase in plant secondary metabolites and the enhanced anti-fungal activity compared to the extracts of plants that received no water deficits.

The final part of the study (Chapter 5) aimed to observe the effects of the acetone extracts of *S. dolomitica* plants on the germination of maize seeds when used as a seed treatment in the presence and absence of thy phyto-pathogenic fungus, *F. oxysporum*. The extracts were derived from plants subjected to four different water treatments (none, mild, moderate, severe), and each extracts having three concentrations (10, 20, 30 mg/ml). The germination bio-assay was conducted in sterilized closed systems in a research laboratory maintained at 25 °C for 14 days. Most of the extracts tested had no significant effects ($P > 0.05$) on the germination of the maize seeds; however, the extracts were associated with shorter shoot lengths and shoot lengths of the

maize seedlings. The bioassays results indicated that the extracts of *S. dolomitica* might contain allopathic compounds that may be used to develop green alternatives to synthetic herbicides.

The present study's findings pave the way for developing bioactive natural products as a green alternative to synthetic fungicides and using less water to cultivate these medicinal plants, which benefit a water-scarce country like South Africa.

CHAPTER ONE

STATEMENT OF RESEARCH PROBLEM, MAIN OBJECTIVES, SPECIFIC OBJECTIVES AND HYPOTHESES

1.1 Statement of the Research Problem and Importance of the Study

Plant diseases negatively impact humans' well-being through agricultural and economic loss (Anderson *et al*., 2004). Economic impacts of plant diseases stem from losses in productivity and the cost of disease management (Chakraborty *et al*., 1999). *Fusarium oxysporum* Schitdl and *Fusarium verticiloides* (Saccardo) Nirenberg cause seedling blight of maize plants. The pathogenic fungus *F. oxysporum* is one of the most common pathogens associated with diseases in maize. While fungicides are often recommended for the control of seedling blights of maize (Stuckey *et al*., 1993), contamination due to the incorrect use and disposal of synthetic chemicals is common. It has become a primary environmental concern (Mobasser & Tavassoli, 2013). The increased use of synthetic pesticides, bactericides, and fungicides to combat plant diseases can be toxic to humans, animals, and ecosystems. (Anderson *et al*., 2004). Consumers prefer food that is nutritional, free of toxic residues, and is produced in an environmentally friendly manner (Messias *et al*., 2013). Consequently, there is also a worldwide trend towards environmentally safe methods, especially in sustainable agriculture, by reducing the use of synthetic fungicides and developing environmentally safe, long-lasting, and effective bio-control methods for the management of plant diseases (Nashwa & Abo-Elyousr, 2012). The plant kingdom has an extensive reservoir of natural anti-pathogenic microbial compounds and is a source of safe and effective alternatives to synthetic compounds (Wilson *et al*., 1996).

Various studies have shown that pathogenic fungi are becoming increasingly resistant to commercial fungicides. Therefore, the discovery and research on new natural-based fungicides must become our priority. Natural fungicides from plants are traditionally used for their medicinal properties, and it is believed that they, unlike synthetic compounds, are less toxic to humans and livestock. Generally, plant-derived materials are readily biodegradable and therefore, less likely to contaminate the environment than synthetic pesticides (Rani & Devanand, 2011).

Efforts to optimize the yield of medicinal plant materials through cultivation methods or techniques have been receiving much attention lately due to the rise of multidrug-resistant organisms (MDROs) (Chen *et al*., 2016; Isah, 2019). The success rate of developing a new drug from natural medicinal origins is higher than synthetic drugs, with the latter being very expensive and difficult to develop (Pan *et al*., 2013).

Several plant cultivation protocols to enhance bioactivities of plant-derived materials are being sought. Manipulation of the ambient environmental factors has been used to successfully enhance secondary metabolite production by plants (Ramakrishna & Ravishankar 2011). For example, many studies have revealed that medicinal plants grown under certain water deficit conditions resulted in significantly higher concentrations of relevant secondary metabolites than their unstressed counterparts (Kleinwächte & Selmar, 2014). Metabolic reactions triggered by drought stress are responsible for the higher natural product accumulation in plants grown in semi-arid regions (Al-Gabbiesh *et al*., 2014). In this context, it is plausible to think that exposing indigenous *Salvia* plants to water stressing during cultivation could enhance the production of secondary metabolites, thereby increasing the plant extracts' efficacy against *F. oxysporum*.

There is not much research on South African *Salvia* species in terms of their anti-fungal properties. However, studies on other non-indigenous members of the *Salvia* genus showed that plant extracts and essential oils of *Salvia officinalis*, *Salvia tomentosa*, and *Salvia cryptantha* were significantly effective at inhibiting *F. oxysporum* mycelium (Yılar & Kadıoğlu, 2016). Several species of the *Salvia* genus in the Lamiaceae family have been used in traditional folk medicine for their antiseptic, antibacterial, diuretic, spasmolytic, stomachic, and carminative agents. *Salvia* spp. Plants and their essential oils are economically important worldwide as agents in food flavouring products, perfumery, and cosmetics. They contain bioactive principles; most of the phenolic acids in *Salvia* species are exclusively those of caffeic acid derivatives. The *Salvia* species that will be used in this study are indigenous to South Africa and include: *Salvia africana-lutea* L. (Lamiaceae) and *Salvia chameleagnea* Berg. (Lamiaceae). These genus members are extensively used in South Africa to treat infections during healing rites (Kamatou *et al*., 2009).

This Master's research's main hypothesis is that applying water stress to selected indigenous South African plants will increase their anti-fungal secondary metabolites' profiles in aerial plant tissue extracts. Crude extracts of water-stressed *Salvia* species will inhibit seedling blight

in maize caused by *F. oxysporum* when used as a seed treatment compared to a control treatment. Research needs to be conducted on our indigenous medicinal plants to ascertain whether they are viable sources of bioactive extracts against phytopathogenic fungi, especially the economically significant fungal pathogens of staple food crops maize. This study will lead to the development of an optimum cultivation protocol for the cultivation of *Salvia* species to enhance the medicinal quality of its extracts.

1.2 The Main Objectives of the Research

The main objectives of the research were (i) to screen six indigenous *Salvia* species to obtain the top two performing species based on their anti-*F. oxysporum* activity in a micro dilution bioassay, and then (ii) to assess the effect of applying water stress on the two selected species during cultivation with the view of increasing the anti-fungal efficacy of their crude extracts for pathogenic fungal control of seedling blight in maize..

1.3 Specific Objectives of the Research

1. Screen aerial plant tissue extracts of six indigenous *Salvia* species cultivated under greenhouse conditions against *F. oxysporum* in a minimum inhibitory concentrations (MIC) bioassay to assess the anti-fungal activity and subsequently, select the top-performing two species for further study.

2. Determine the height, crown width, and biomass, two selected indigenous *Salvia* spp. were subjected to different water-deficit treatments to establish the effects of drought stress on the fore-mentioned plant growth parameters.

3. Assess the minimum inhibitory concentrations (MIC) of the aerial plant tissue extracts of the two selected indigenous *Salvia* species, subjected to different water-deficit treatments, against *F. oxysporum* to assess the anti-fungal activities from the differently treated plants against the fungal pathogen.

4. Measure the phenolic constituents of the extracts of the aerial parts of the two selected indigenous *Salvia* species subjected to different water-deficit treatments in order to assess the effect of water stress on secondary metabolites.

5. Determine the *in vivo* protective effect (symptomatic suppression) of aerial plant extracts of the more potent of the two indigenous *Salvia* species following exposure to different water-deficit treatments on *F. oxysporum* -contaminated maize seedlings in order to assess the efficacy of the extracts in inhibiting *F. oxysporum*, a causal agent of seedling blight disease in maize seedlings.

1.4 The Hypotheses of the Research

1. There are differences in the anti-fungal activity (based on MIC values) of the six *Salvia* spp.

2. The growth parameters, viz. height, crown width, and biomass in response to varying water-stress levels will vary significantly between the two selected indigenous *Salvia* spp.

3. The anti-fungal activity (MIC values) following exposure to varying water-stress levels will be significantly different between the two selected indigenous *Salvia* spp.

4. There will be a significant difference in the phenolic constituents of the extracts of the aerial parts of the two selected indigenous *Salvia* spp. following exposure to the different watering treatments.

5. There will be a significant difference in the symptomatic suppression of seedling blight disease in maize seedlings by the extracts of the aerial parts of the two selected indigenous *Salvia* spp. following exposure to different watering treatments.

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CHAPTER TWO

LITERATURE REVIEW

2.1.1 Seedling blight of Maize

There are two types of seedling blight namely pre-emergence and post-emergence seedling blight (Govender, 2008). Seeds may rot and die before they emerge and in some cases where the seeds do emerge, the roots may be discoloured with dark brown to black sunken lesions on it. Water and nutrient uptake of the seedlings is affected (Viviers 2014). Seedling blight can cause poor maize plant stands, it can cause uneven growth and some seedlings may also be chlorotic and stunted (Dodd, 1980). *F. oxysporum* is a plant pathogen with many *formae specialis* (f.sp.), which means that they have different specialized forms. These forms can infect a variety of plant species causing various diseases. Although some of the forms are limited in their degree of distribution, *F. oxysporum* is known to be cosmopolitan, which means it is widely distributed and occurs on many continents. Some of the disease symptoms in plants caused by *F. oxysporum* are vascular wilt, the rotting of corms, root rot, and damping-off. At the seedling stage, the plants infected by *F. oxysporum* may wilt and die soon after symptoms appear (Gonsalves & Ferreira). In a study conducted by Viviers (2014), a total of 101 maize fields were sampled in the South African commercial maize producing areas for two years. Root discolouration was found in 73% of the seedling samples. Seventy fungal genera were isolated from the roots, with the most common fungi groups being *Aspergillus* spp., *Clonostachys* spp., *Fusarium* spp., and *Penicillium* spp. Of the *Fusarium* group, the most common species isolated were *F. fujikuroi*, *F. solani*, and *F. oxysporum*.

2.1.2 *Fusarium oxysporum*

Kingdom Fungi; Phylum Ascomycota; Class Sordariomycetes; Order Hypocreales; Family Nectriaceae; genus *Fusarium*. More than 120 different *formae speciales* (f.sp.) have been identified. This identification was made based on the different f.sp. specific to host plant species belonging to a wide range of plant families (Michielse & Rep, 2009). The different f.sp of *F. oxysporum* may differ in appearance but in general, the aerial mycelium first appears white and then may change to various colours according to the strain or form of *F. oxysporum*. The

various colours range from violet to dark purple. Culture of *F. oxysporum* may appear cream or orange in colour if sporodochia are present in large quantities (Smith *et al*., 1988). *F. oxysporum* produces three types of asexual spores which are microconidia, macroconidia, and chlamydospores (Agrios, 1988). The one to two-celled microconidia is the type of spore that is most often produced within the vessels of plants infected by the fungus and the macroconidia] which are three to five celled, are the spores commonly found on the surface of plants killed by this pathogenic fungus (Agrios, 1988). Some specific forms of *F. oxysporum* are pathogenic to plants (Smith *et al*., 1988), while others are saprophytic. This saprophytic ability enables the fungus to survive in the soil between crop cycles. When the host plant is not available, the fungus can survive in the infected debris of the previous growth cycle of the plant present in the soil as mycelium or as any of its three different spore types (Agrios, 1988).

Most of the seedling rots caused by species within the *Fusarium* complex result from the infection of chlamydospores, mycelia, and conidia that has survived in the soil (Young and Kucharek, 1977). Susceptible plants can become infected by *F. oxysporum* if the soil they are growing is contaminated with the fungus, which can penetrate the root tips or at the formation point of the lateral roots or enter through wounds present on the roots of the host plants (Agrios, 1988). A study conducted by Bacon *et al*. (1994) screened maize hybrids for resistance to seedling blight disease and found that as the pericarp of the seed breaks and the roots start to develop, the seedling blight fungi present in the soil will grow towards the roots and infect the maize seedling.

The mycelium grows through the root cortex intracellulary, and when the mycelium reaches the xylem, it invades the vessels by entering via the xylem's pits. From this point, the mycelium remains in the vessels, where it usually grows upwards towards the plant's aerial parts. When the fungus produces microconidia inside the plant's tissues, these spores move in the plant's sap stream within the vessels. The fungus can move sideways by growing the mycelium into the adjacent xylem vessels through the xylem pits (Agrios, 1988).

This fungal growth within the plant's vascular tissue affects its water supply as less water becomes available to the plant, the lack of water induces the stomata of the leaves to close. The leaves wilt, and the plant eventually dies. Within the desiccated plant, the fungus invades the plant's parenchymatous tissue. It moves towards the surface of the dead tissue, where it sporulates profusely, and these spores can become the inoculum for the spread of the fungal disease (Agrios, 1988).

2.1.3 Lack of efficacy of synthetic fungicides in controlling fungal pathogens

Seed treatment is defined as a substance applied to the seed's surface to control pests or microorganisms and the diseases associated with them. Before seed treatments were applied to destroy the pathogens on the seed or to protect germinating seeds from soil-borne pathogens, but with the development of systemic fungicides, internal pathogens in the seed can be controlled (Evans, 1997). Some of the active ingredients used in the formulations of seed treatments include captab, fludioxonil/mefenoxam and thiram (Nel *et al.,* 2003). Fungicides such as carboxin with thiram, Kodiak HB, ipconazole, metaconazole fludioxonil. Mefenoxam, and trifloxystrobin are effective as seed treatments against root rot diseases caused by *Fusarium* species (Ruden, 2013). Fungicides such as Captan, Fludioxonil, Maneb, and Thiram control most types of soil-borne pathogens, except for the root rot organisms (Ruden, 2013). Products associated with seed surface protection have a short residual effect. Systemic fungicides that are absorbed into the seedling and inhibits or kill the fungus inside the plant tissue include, Carboxin, Azoxystrobin, Metalaxyl, Thiabendazole, Mefenoxam, and Trifloxystrobin (Ruden, 2013).

To reduce the risks of seedling blight and seedling borne diseases, maize seeds are treated with fungicides (Solorzano and Malvick, 2011). Captan was once widely used to control soil and seed-borne fungal diseases but has since been replaced with more efficient treatments (Solorzano and Malvick, 2011). The overuse of synthetic pesticides has caused an increase in the number of pathogens becoming resistant to these chemicals. In an experiment comparing plant extracts and a standard fungicide, Eksteen *et al*. (2001) recorded poor inhibition of *R. solani* by the broad-spectrum standard fungicide, indicating the level of inherent resistance of this organism against the broad spectrum fungicide used in the assays. Some studies have shown that maize seeds produced by plants grown organically had lower *Fusarium* infection than those produced from conventional plants.

2.1.4 Alternative control measures

Plants are an essential source of anti-fungal agents and can carry out combinational chemistry by mixing, matching and evolving the gene products required for secondary metabolite

biosynthetic pathways. This creates an unlimited pool of beneficial chemical compounds which mankind uses in both traditional and modern medicinal systems (Ncube *et al*., 2011).

One of the characteristic features of higher plants is their capacity to synthesize an extremely large variety of organic molecules known as secondary metabolites (Wink, 1987). These secondary metabolites play an important role in the resistance of plants against herbivores and pathogens and can protect plants against viruses, bacteria and fungi. These secondary metabolites can be extracted and used for the benefit of man not just medicinally but also for pest control mechanisms.

According to Mondali *et al*. (2009), many chemicals have been developed for the control of plant diseases. Nevertheless, due to ever growing awareness of these chemicals' hazardous side effects, in nature and agriculture, more and more emphasis is being given to the use of bio-control agents. A major challenge of plant pathologists is to introduce eco-friendly and safe alternative control strategies, which leads researchers to turn their attention to plants and microorganisms as sources of biocontrol agents. Plant-derived materials are considered readily biodegradable and, therefore, less likely to contaminate the environment than synthetic pesticides (Rani & Devanand, 2011). The antimicrobial activities of 50% methanolic extracts obtained from *S. africana* stored for 16 years at room temperature showed lower MIC values than those of the extracts made from fresh materials. These findings indicate that stored plant materials retain their biological activities over a long period of time and that its activities can be enhanced, probably due to more potent metabolites arising from the breakdown of some of their constituent chemicals (Amoo *et al*., 2012).

Plants can synthesise inhibitory proteins or enzymes that could degrade the cell walls of pathogenic microbes. Some plants synthesize peroxidase and phenoloxidase, which could help to inactivate phytotoxins given off by microbes (Wink, 1987). Results of an investigation by Mondali *et al*. (2009) show that the growth of the saprophytic fungi *Rhizopua* and *Aspergillus* was inhibited with the crude aqueous and alcoholic extracts of *Azadirachla indica* leaves. This is just one of a plethora of examples where plant extracts have proven its anti-pathogenic characteristics.

2.1.5 Effect of water stress on plant growth

The abiotic factor, water deficit stress, is one of the most common environmental factors that affect plant growth (Bohnert and Jensen, 1996; Xu *et al*., 2010), and is considered one of the most growth-limiting factor that decrease plant growth (Tátra *et al*. 2016). Drought stress occurs

when the available water in the soil is reduced to such critical levels. Drought stress tolerance is seen in all plants, yet the extent of tolerance varies from species to species. Exposure to drought causes many common reactions in plants, such as cellular dehydration, which causes osmotic stress and water removal from the cytoplasm to vacuoles (Ramakrishna & Ravishankar, 2011). A reduction in chlorophyll content may occur under drought stress (Massacci *et al*., 2008)

Water deficits can adversely impact many aspects of the physiology of plants, especially photosynthetic capacity and prolonged drought stress may severely diminish plant growth and productivity. (Osakabe *et al*., 2014). Water stress affects plants' photosynthesis by reducing the Carbon dioxide conductance in leaves due to the changes in the stomatal aperture with the decrease in the leaf water potential. In some plants, the translocation of photoassimilates from leaves to the stems assists in the maintenance of growth under water stress conditions (Ohashi *et al*., 2000). (LI *et al*., 2009)

Increased water deficits can have a profound effect on the structural changes in the piliferous zone of roots of some plants, causing a significant decrease in the diameter of newly formed adventitious roots by reducing the number of the cortical parenchyma, through the reduction of cell size and the diameter of vessels in the primary xylem (Labdelli *et al*., 2014). In some instances, mild water deficits may increase medicinal compounds' yield without negative effects on growth of roots.

2.1.6 Effect of water stress on plant secondary metabolites and anti-fungal activity

Different environmental stresses cause plants to produce various secondary metabolites that help the plant adapt to those stresses (Kroymann, 2011; Berini *et al*., 2018). The production of secondary metabolites by plants can be elicited by changing the biotic and abiotic factors under which they are cultivated. (Rejeb *et al*, 2014; Caretto et al., 2015; Narayani & Srivastava, 2018). Moreover, the presence of secondary metabolites that are affected under water shortage can impact the commercial medicinal value of plants (Tátra *et al*., 2016).

These secondary metabolites are preformed inhibitors produced in healthy plants (phytoanticipins) or may be synthesized *de novo* as phytoalexins in response to attack by pathogens or by various non-biological stress factors. The same compound maybe a preformed anti-fungal substance in one species or a phytoalexin in another (Pusztahelyi *et al*., 2015). Plant secondary metabolites can be divided into three major groups, namely terpenoids; nitrogen-

containing alkaloids and sulphur-containing compounds and flavonoids and allied phenolic and polyphenolic compounds.

These secondary metabolites act as defence molecules against microbes, viruses, or other competing plants (Wink, 2003). Antimicrobial compounds restrict pathogens' growth by activating specific signalling pathways leading to Ca2+ elevation and Reactive oxygen species (ROS) burst by binding to cell membranes followed by leakage of cell components of-pathogenic fungi such as *F. oxyporum* (Ito *et al*., 2007).

Drought often causes oxidative stress, thereby increasing the amounts of flavonoids and phenolic acids in plant tissues. (Larson, 1998). Water stress can also increase the tannin and saponin contents of plants (Umebese & Falana, 2013) which are reported to have anti-fungal properties (Arif *et al*, 2009). Under drought stress, the closing of the stomata results in a series of consequent physiological and/or biochemical adjustments aimed at balancing the photosynthetic process as well as at enhancing the plant defence barriers against the drought-promoted stress. These barriers include the stimulation of antioxidant systems, stimulation of aquaporin synthesis and the accumulation of osmolytes which some of the actions the plant takes to overcome the unfavourable period of water deficit (Kampoor *et al*., 2020).

2.1.7 Indigenous *Salvia* species screened in this study

2.1.7.1 *Salvia africana-lutea* L.

Family: Lamiaceae

Common names: golden sage, beach sage, dune sage, sand sage

Salvia africana-lutea is an evergreen (Figure 2.1), fast growing, and aromatic shrub. It can grow to a height of up to two meters. The leaves are greyish green in colour. The shrubs bloom in early spring. The new flowers start as bright yellow flowers that fade to rusty-orange and finally become reddish brown. The f calyxes are a dark rusty colour on the side that faces the sunlight and olive green on the side that faces away from the light. After the petals fall, the large campanulate calyxes becomes papery and remain on the plants. The distribution of *S. africana-lutea* extends from Namaqualand to the Cape Peninsula and eastwards to Port Alfred and forms part of the natural coastal vegetation on coastal dunes (Notten, 2015).

Figure 2.1: *Salvia africana-lutea*

Sourced from: www.operationwildflower.org.za

2.1.7.2 *Salvia chamelaeagnea* P.J.Bergius

Common names: Rough Blue Sage

Salvia chamelaeagnea is a heavily flowering shrub that can grow up to 2 meters high. Its upright stems are strong and plentiful (Figure 2.2). The bright green leaves are oppositely arranged and are slightly hairy with toothed edges. The leaves are dotted with glands that emit a very strong odour when touched. The flowers are formed in whorls with the more common colour being the dark blue top lip and white lower lip but can vary in colour from blue, mauve, pink to pure white. The flowers occur on the small side branches with the calyxes usually a reddish-purple colour. It is covered with small hairs and is also dotted with glands. *Salvia chamelaeagnea*, grows wild in the south Western Cape and is commonly found in sandy soil in open fields and streambeds along roadsides (Clebsch, 2003; van der Walt, 2001).

Figure 2.2: *Salvia chamelaeagnea*

Photo sourced from: www.wildflowernursery.co.za

2.1.7.3 *Salvia disermas* L.

Common names: wild giant sage, Transvaal sage

Salvia disermas is a hardy herbaceous perennial with a slightly woody rootstock, which occurs in a variety of colours and growth forms (Figure 2.3). It is a relatively fast-growing shrub that can grow up to 60 cm with multiple square stems growing from a slightly woody rootstock. The plants are covered in soft hairs and the long and narrow leaves are opposite, lobed and bluntly toothed with a rough texture and greyish-green in colour. When crushed, the leaves produce a sweet scent. Many inflorescences are born from each stem. The flower is a two-lipped corolla. The stigma and anthers are found on the upper lip, which is hooded, while the lower lip is cupped with the edges turned down, providing a platform for pollinators. Flower colours can range from being icy white, pink, blue, and in bicolored forms. Flowering occurs in spring and summer. *S. disermas* occurs throughout South Africa and in Namibia. It occurs at altitudes of 360 to 1555 metres in well-drained, rocky or stony soil (Clebsch, 2003; McQuillan, 2009).

Figure 2.3: *Salvia disermas*
Photo sourced from: www.triggplants.com.au

2.1.7.4 *Salvia dolomitica* Codd

Common names: dolomite sage, pilgrim's rest pink sage

Salvia dolomitica a fast-growing shrub that can grow up to 1.5 meters high (Figure 2.4). The stems is covered in short glandular hairs. The simple elliptic leaves are green when young and turn grey-green as the leaves mature. The leaves are shaped like flattened eggs, the tips are pointed, and the bases of the leaves is wedge-shaped. The leaves are densely covered with fine grey hairs. The flowers are shaped in the two-lipped corolla style, which is typical of the Sage family. The upper lip is narrow and bends downwards, while the lower lip is open and flattened. The flowers are multi coloured with the lilac and white corolla usually having yellow throat markings. The pea green oily calyxs remain after flowering and turns a deep pinkish-purple until the nutlets forming within it ripen and fall out, after which the calyx then dries and falls off. Each of the four dark brown nutlets, each containing a single seed. *S. dolomitica* is restricted to the provinces of Limpopo and Mpumalanga, where it grows on dolomite rock outcrops at altitudes of 1150-1900 m (Clebsch, 2003; McQuillan, 2013).

Figure 2.4: *Salvia dolomitica*

Photo sourced from: www.herbgarden.co.za

2.1.7.5 *Salvia namaensis* Schinz

Common names: Nama sage

Salvia namaensis is a fast growing semi-woody perennial shrub (Figure 2.5). It can grow from 0.3 to 1.2 metres high. The shrub has a light and airy appearance. The leaves which are wrinkly are split into more than three lobes. The terminal lobes are large and rounded and the lateral lobes are smaller which tends to get even smaller towards the bases of the leaves. The young stems and leaves are densely covered in fine, glandless and curled hairs as well as oil globules. The foliage is aromatic and the flowers are produced in whorls from two to six flowers borne along flowering stems. These floral leaves are long and end in sharp points. The flower has a two-lipped corolla with the upper lip hooded and the lower lip cupped with the edges turned downwards. The lower corolla is deeply cleft into two parts near the middle. The whitish to pale blue flowers are borne in spring and summer. The black or dark brown nutlets are visible at the base of the calyx and fall out when ripe. *S. namaensis* comes from Namaqualand, Namibia, or the Northern Cape, as indicated by the epithet *namaensis*. It grows at altitudes of 400 to 1700 metres on top of limestone and dolerite hills in well-drained, rocky or sandy soil (Clebsch, 2003; McQuillan, 2011).

Figure 2.5: *Salvia namaensis*

Photo sourced from: www.randomharvest.co.za

2.1.7.6 *Salvia repens* Burch. ex Benth.

Common names: kruipsalie

Salvia repens is a fast growing herbaceous perennial and has rhizomes that spread to form small clumps (Figure 2.6). The plants can grow up to 40 cm tall with soft, hairy stems that can be simple or branched. The leaves are oppositely arranged and are rough and hairy with toothed and irregular margins. The leaves are bigger and more crowded at the base of the stems. The flowers are formed in clusters close to the stem. The inflorescences can be simple or branched. The flowers can vary in colour from white to purple or a deep shade of blue. The oil glands on the calyx and leaves have a light herblike scent that it gives off when stroked. *Salvia repens* is widespread throughout the summer rainfall, the eastern part of South Africa, and most commonly found in the grasslands of the Highveld (Clebsch, 2003; van de Walt, 2003).

Figure 2.6: *Salvia repens*

Photo sourced from: www.garden.org

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CHAPTER THREE

ANTI-FUNGAL ACTIVITY OF LEAF EXTRACTS OF SIX SOUTH AFRICAN *SALVIA* SPECIES AGAINST *FUSARIUM OXYSPRORUM*, A CAUSAL AGENT OF SEEDLING BLIGHT IN MAIZE

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This study aimed to evaluate the anti-fungal activity of extracts of six indigenous *Salvia* species against the phyto-pathogenic fungus *Fusarium oxysporum*. The plant extracts were screened in vitro for their anti-fungal activity against *F. oxysporum* in a Minimal Inhibitory Concentration (MIC) assay. Extracts of all the six species inhibited the *F. oxysporum* at the 6, 12, 18 and 24 h post-treatment. The MIC values of the extracts ranged between 0.75 mg/ml and 12 mg/ml. Furthermore, *S. dolomitica*, *S. namaensis* and *S. repens* with MIC values of 3.0, 4.2, and 9.6 mg/ml, respectively, continued to inhibit fungal growth beyond the 18th hour time period suggesting extracts from these species have fungistatic activities. S. *chameleagnea* showed the weakest activity than the other species at all four of the tested time slots, while *S. dolomitica* and *S. namaensis* showed the best activity and were subsequently selected for further study. In conclusion, the six *Salvia* spp. studied could be considered as potential sources of anti-fungal extracts and compounds.

Key words: *Salvia* spp., Minimal inhibitory concentration, *Fusarium oxysporum*, Seedling blight

3.1 Introduction

Maize is one of the major crops cultivated worldwide, especially in developing countries (Messias *et al*., 2013). Seedling blight of maize significantly influences the stands and seedling vigour of field crops in South Africa and occurs in different areas of the country and over various seasons. The seedling blight of maize is not only a problem in South Africa but also worldwide, with severe cases resulting in financial losses to farmers (Viviers, 2014). In South Africa maize cultivation is continuously under threat from pests including pathogens, such as

F. oxysporum (Smit, 2000). *F. oxysporum* is an opportunistic human pathogen and a phytopathogen (Paiva *et al*., 2010). Conventionally, synthetic fungicide is often used to control fungal phytopathogens. Seeds are treated with fungicides to control soil- and seed-borne fungal diseases (Ruden, 2013). In South Africa, synthetic fungicides, used as seed treatments, is the primary method used to protect grain crops against *Fusarium* spp. and other soil-borne pathogens (Gerber, 2010). However, the indiscriminate use of synthetic chemicals leads to environmental degradation (Kookana *et al*., 1998, Komarek *et al*., 2010) and negatively affects human health (Schwarzman, 1996; Zaker, 2016). Compounds for the production of green fungicides can be sourced from medicinal plants (Chuang *et al*., 2007), and South Africa is a country rich in medical plants with 3000 known species being used by its traditional healers (Tewari, 2004).

Some of these medicinal plants being used are from the genus *Salvia*. The *Salvia* genus constitutes almost one-quarter of the Lamiaceae family (Ebani *et al*., 2018) as is widely distributed globally. Approximately 400 species of *Salvia* is used in traditional and modern medicine (Franz & Novak, 2010). Twenty species are indigenous to southern Africa, and most of those from South Africa predominantly occur in the Cape region (Kamatou *et al*., 2008; Paton, 1991). The essential oils produced by the leaves of sage plants are known to have beneficial uses in the food, cosmetics, perfumery, and pharmaceutical industries (Bassolino *et al*., 2015; Coisin *et al*., 2012; Kamatou *et al*., 2006; Russo *et al*., 2013) and chemical analyses of some species such as *S. albicaulis*, *S. muirii*, and *S. runcinata* and S. *namaensis* suggest that they may be considered as alternative commercial sources of natural rosmarinic acid and carnosol (Kamatou *et al*, 2010). The antimicrobial properties of these plants have been explored in pharmacology, pharmaceutical botany, and phytopathology as well as to medical and clinical microbiology fields (Zhiming *et al*., 2013). Indigenous species have been widely adopted in traditional medicine due to their various phytochemicals properties (Ahmed *et al*., 1994; Bassolino *et al*., 2014; Russo *et al*., 2013). The in-vitro system used in a research by Ramogola (2009) on the high potency of the *S. africana-lutea* extracts against pathogenic bacteria and fungi can be scaled-up to benefit both the pharmacology and the agricultural sectors and Caffeic acid.

There is limited research on South African *Salvia* species in terms of their anti-fungal properties, especially against *F. oxysporum*. Nevertheless, studies on other non-indigenous members of the *Salvia* genus showed that plant extracts and essential oils of *S. officinalis*,

S. tomentosa, and *S. cryptantha* were found to be significantly effective at inhibiting *F. oxysporum* mycelium (Yılar & Kadıoğlu, 2016).
The essential oils of *S. fruticosa* were slightly effective against *F. oxysporum* f. sp. *dianthi* and *Fusarium proliferatum*, and highly effective against *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Fusarium solani* f. sp. *cucurbitae* (Pitarokili *et al*., 2003)

This chapter's objective was to screen extracts of six cultivated *Salvia* spp. against *F. oxysorum* with the view of identifying the two most active species. Furthermore, we will conduct further research on the selected species to determine the effects of water-deficits on plants' anti-fungal activity of their extracts against *Fusarium oxysporum*.

3.2. Material and Methods

3.2.1 Experimental design

The experiment was conducted in a research greenhouse at the Cape Peninsula University of Technology, Bellville, Western Cape, South Africa S33° 54' 0, E18° 38'0 from September 2017 to January 2018.
Fifty rooted cuttings of each species was individually transplanted into 15 cm brown plastic pots filled with sterilized medium containing equal parts 1:1:1 silica sand, coir and perlite. The potted saplings were placed in a random block design on a cement floor inside the research greenhouse where the plants were exposed to natural sunlight that entered through the polycarbonate roof. The light intensity measured.
1 cm above the plants ranged from 25.31 Klux to 34.27 Klux. Temperatures inside the greenhouse were 24 – 26 °C during the day and 15 – 20 °C at night. The average relative humidity was 74% relative humidity (RH).

3.2.2 Plant material

Semi-hardwood cutting materials from indigenous *Salvia africana-lutea*, *S. chamelaeagnea*, *S. disermas*, *S. dolomitica*, *S. namaensis*, and *S. repens* were obtained from the Kirstenbosch National Botanical Garden courtesy of the curator at the time, the late Mr Philip le Roux. In August 2017, one hundred 10 cm cuttings of each species was propagated in 128 cell Styrofoam

trays filled containing equal volumes 1:1 rivers sand, and perlite. These substrate materials were pre-sterilized with Captab (4 g in 1 L) before use. The trays of cuttings were placed on hotbeds maintained at a temperature of 26.5 °C under intermittent mist in the production glasshouse (22°C; 100% RH) until the roots appeared through the drainage holes of the trays.

3.2.2.1 Irrigation and fertilization programme

The plants were hand irrigated every four days at midday for twelve weeks, with 250 ml reverse osmosis (RO) water (Figure 3.1). The water was poured slowly around the stem of each plant to give the media enough time to absorb most of the water, thus eliminating excess runoff through the drainage holes of the pots. Every 16 days Nutrifeed fertilizer (Starke Ayres, Cape Town) was added to the irrigation water (5 g per 5 L) containing the following ingredients: N (65 mg/kg), P (27 mg/kg), Ca (70 mg/kg), K (130 mg/kg), Cu (20 mg/kg), Fe (1500 mg/kg), Mg (22 mg/kg), Mo (10 mg/kg), Mn (240 mg/kg), S (75 mg/kg), Zn (240 mg/kg) and B (240 mg/kg).

Figure 3.1: 50 replicates of 6 species of *Salvia* placed in randomized block design on the greenhouse floor and irrigated by hand every four days with 250 ml RO water.

3.2.3. Preparing crude plant extracts

3.2.3.1 Drying and milling the plant material

Aerial plant materials (leaves and stems) from harvested plants of each of the six *Salvia* species were placed into individual labelled brown paper bags and dried in an oven at 40 °C for 14 days. The dried plant materials were removed from the oven. Each sample was milled into a fine powder using a Jankel and Kunkel Model A 10 mill. The powdered material was stored in sealable plastic bags (ABSA coin bags) and labelled accordingly.

3.2.3.2 Solvent extraction

Five grams of the milled material for each species was taken from five sample plants at random. The milled material was mixed with 50 ml of acetone, placed into a glass jar with a lid on, and extracted overnight. The supernatant formed after the precipitate has settled in the jars was filtered out, using Whatman no. 1 filter paper, into pre-weighed plastic test tubes. The filtrate was placed in a fume hood under a cooling fan until the acetone evaporated, after which the extracted plant material was weighed. The dried extracts were reconstituted to a stock concentration of 24 mg/ml. This stock solution was used in the MIC bioassay.

3.2.4 *In vitro* screening using Minimum inhibitory concentration (MIC) assay

The MIC, as described by Ntobela *et al*., 2020), was carried out on 96-microwell plates. The starting concentration was 6 mg/ml, which was further diluted in successive wells by two-fold serial dilution. The strain of *Fusarium oxysporum* sp.*glycines* strain (UPFC no. 21) that was used in this bioassay was obtained from existing cultures in the Horticulture Research Laboratory of Cape Peninsula University of Technology, Bellville campus. *F. oxysporum* was sub-cultured from stock agar plates and transferred into Nutrient Broth (Merck, South Africa) for four hours. The fungal cultures (100 µl) were added to each well of the 96-well microplates (105 cells/ ml). Mancozeb (Stodels Pty Ltd. Garden Centre, Cape Town, South Africa) served as the positive control (6 mg/ml). Forty microliter (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 °C and 100% RH. The MIC values were recorded at the 6^{th}, 12^{th}, 18^{th}, and 24^{th} hour of incubation of the microtitre plates and the MIC values was recorded after comparing the colour of dyed wells to known concentration markers for individual rows of the microtitre plates using visual observations (Figure 3.2). The anti-fungal bioassay (MIC) consisted of three replicates per treatment.

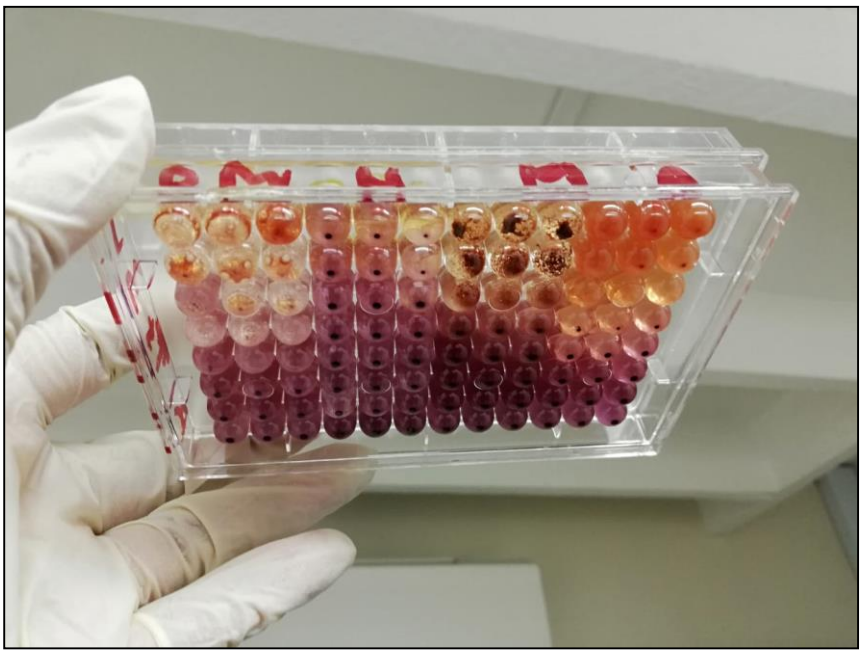

Figure 3.2: Covered microtitre plate being held up to the light for visual observations for the recording of MIC values.

3.2.5 Statistical analysis

The MIC data were recorded as mean ± standard error, and the one-way analysis of variance (ANOVA) was used to compare the means amongst the treatments.

All computations were completed with the software programs STATISTICA® (13.5.0.17) and the Paleontological Statistics package for education and data analysis (PAST 3.14). Post hoc analysis based on Tukey test was used to separate the means.

P values of <0.05 were regarded as significant, and P values of <0.01 as very significant.

3.3 Results

At six hours incubation, a significant difference (P<0.01) was found in the minimum inhibitory concentrations among the different species (df = 6, 28; F = 12.54; P = 7.80E-07). The MIC values of the leaf extracts of the six tested *Salvia* species against *F. oxysporum* ranged from 0.75 mg/ml to > 6.0 mg/ml. The results at 6 hours incubation of the microtitre plates at 26 ºC± 2 ºC indicates that *S. africana-lutea* and *S. disermis* had the best fungistatic results, both having a MIC value of 0.75 mg/ml (Table 3.1), which was comparable to the synthetic fungicide Mancozeb used as the positive control and *S. chameleagnea* had the worst results with an MIC value of 5.4 mg/ml.

At twelve hours after inoculation there was a significant difference (P<0.01) in the minimum inhibitory concentrations among the species (df = 6, 28; F = 4.267; P = 0.00356). The top two species with the best fungistatic results were *S. disermis* and *S. dolitica*. They had MIC values of 1.8 mg/m land 1.95 mg/m respectively, whereas *S. repens* and *S. chameleagnea* both showed the highest MIC concentration of 5.4 mg/ml.

Table 3.1: The MIC (Mean ± SE,) of acetone extracts of 6 *Salvia* species against *F. oxysporum.*

***Salvia* species**	**MIC value at 06 hours**	**MIC value at 12 hours**	**MIC value at 18 hours**	**MIC value at 24 hours**
Salvia africana-lutea	00.75±0.00^{a}	05.10±1.87^{a}	05.70±1.80^{a}	12.00±0.00 ^{a}
Salvia chameleagnea	05.40±0.60^{b}	05.40±0.60^{a}	06.60±1.46^{a}	12.00±0.00 ^{a}
Salvia disermis	00.75±0.00^{a}	01.80±1.05^{ab}	05.40±1.74^{a}	12.00±0.00 ^{a}
Salvia dolomitica	01.95±0.45^{ac}	01.95±0.45^{ab}	02.25±0.47^{a}	03.00±0.00^{b}
Salvia namaensis	02.40±0.36^{ac}	03.00±0.82^{ab}	03.00±0.82^{a}	04.20±0.73^{bc}
Salvia repens	04.20±1.10^{bc}	05.40±0.60^{a}	05.40±0.60^{a}	09.60±1.46^{a}
Control				
Mancozeb (6mg/ml)	00.75±0.00^{a}	00.75±0.00^{b}	01.50 ±.0.00^{a}	06.0±0.00^{c}

The values tabulated are the means ± SE.
Significant differences among the means are evaluated using the Tukey HSD test.
Values with the same letters in a column are not significantly different at the 0.05 probability level.

Eighteen hours after inoculation there was a significant difference (P<0.05) in the minimum inhibitory concentrations (df = 6, 28; F = 2.808; P = 0.02882) (Figure 3.3). The MIC bio-assay results at 18 hours exposure indicated that the two species with the best fungistatic results belonged to *S. dolomitica* and *S. namensis*, with MIC values of 2.25, and 3.0 mg/ml, respectively. *Salvia chameleagnea* yielded the least active results with an MIC value of 6.6 mg/ml.

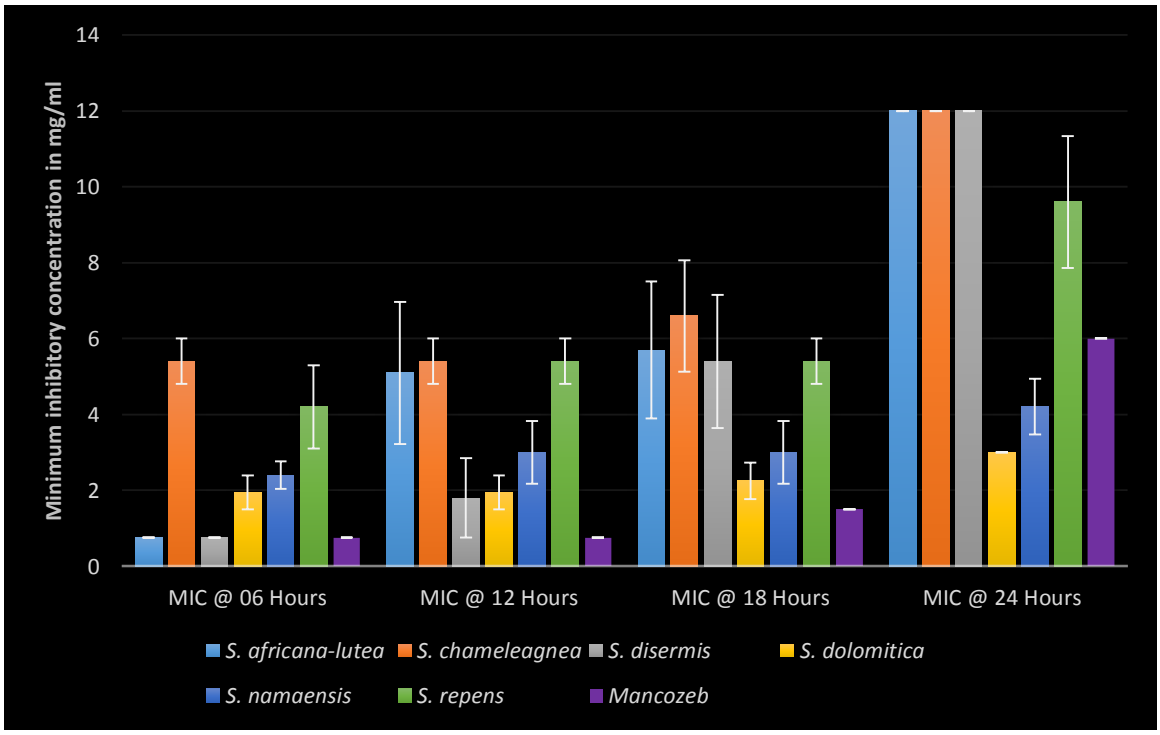

Figure 3.3: MIC values of six *Salvia* extracts exposed to *Fusarium oxysporum* (10^5 cells/ ml) at 6, 12, 18 and 24 hours of incubation.

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At 24 h post inoculation, there was a significant difference (P<0.01) in the minimum inhibitory concentrations among the extracts of the different species
(df = 6, 28; F = 44.06; P = 1.06E-12). When the microtitre plates were incubated for 24 hours, *S. africana-lutea*, *S. chameleagnea*, *S. disermis*, and *S. repens* showed MIC values of more than 6 mg/ml (Figure 3.3). The two species with the lowest MIC values at 24 h of incubation were *S. dolomitica* and *S. namaensis* with MIC values of 3.0 mg/ml and 4.2 mg/ml respectively. This was less that of the synthetic fungicide, Mancozeb, which had a MIC value of 6.0 mg/ml at 24 hours of exposure (Figure 3.3).

3.4 Discussion

In this study, the results of the MIC values recorded at the 6th hour of incubation of the microtitre plates indicated that the acetonic extracts of *S. africana-lutea* and S. *disermis* showed the highest anti-fungal activity of the six species screened and were comparable to the synthetic fungicide Mancozeb (control). The anti-*Fusarium* activity of the *S. africana- lutea* was previously demonstrated by Nkomo *et al*. (2014). In the study, the anti-fungal activities of *S. africana-lutea* were tested against Isolates of two *Fusarium* species namely *F. verticillioides* (MRC 826 and 8267) and *F. proliferatum* (MRC 7140 and 6908), and it was concluded that all the test plant samples showed good in vitro anti-fungal activity against these strains of Fusarium, with MIC values ranging between 0.031 mg/ml and 0.5 mg/ml. Also, Ramagola (2009) showed in his study that both the hairy root and *in vitro* plantlet extracts of *S. africana-lutea* were highly potent against the phyto- pathogenic fungi *F. verticilliodes* and *F. proliferatum* with the MIC values ranging from 0.02 to 0.64 mg/ml for *F. verticilliodes* and 0.08 to 0.64 mg/ml for *F. proliferatum*. Ncube *et al*. (2011) also recorded anti-fungal activities with dichloromethane: methanol (1:1 v/v) crude plant extracts of *S. africana-lutea* on *F. proliferatum* and F. verticillioides. In the same study, all test samples gathered from five different *S. africana-lutea* exhibited good MIC bioassay activity, having values ranging from 0.031 mg ml^{-1} to 0.5 mg ml^{-1}. In the current study, after the 6th h of incubation the extracts of *S. africana-lutea* became less active.

In contrast, the extracts of *S. disermis* and *S. dolomitica* recorded the highest activity by displaying the lowest MIC values at the 12th hour of incubation. In a microdilution assay conducted by Ebani *et al*. (2018), it was observed that, *Mucor* spp. and *Trichothecium roseum* did not show visible growth when subjected to 4.06 mg/ml (IC_{50} = 2.07 mg/ml) of the essential oil of *S. dolomitica*. *S. chameleagnea* showed the weakest activity overall but extracts from the same plant material obtained with solvents of different characteristics have distinct biological properties. The bio-composition of secondary metabolites in plants can differ in plants from the same species. Combining chemical components can show different responses concerning the potential for the inhibition of pathogens (Sales *et al*., 2018).

3.5 Conclusion

Salvia dolomtica and *S. namaensis* showed stronger activity than the synthetic fungicide at 24 h of incubation and outperformed most of the tested extracts from the 12th hour onwards, these two species has been selected for further study in the next chapter.

3.6 Acknowledgements

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CHAPTER FOUR

EFFECT OF WATER DEFICITS ON THE GROWTH AND PHENOLIC COMPOUNDS OF *SALVIA DOLOMITICA* CODD. AND *SALVIA NAMAENSIS* SCHINZ.

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Salvia dolomitica and *Salvia namaensis* are indigenous to southern Africa and are used as medicinal plants in folk medicine for the treatment of many diseases. These species are rich in secondary metabolites. Findings from recent studies suggest manipulation of abiotic factors such as water availability can enhance secondary metabolite and medicinal, pharmaceutical and commercial values indigenous medicinal plants. This study aimed to assess the effects of different levels of water deficit treatments on the growth, concentration of polyphenols, and anti-*Fusarium oxysporum* activity of *Salvia dolomitica* and *Salvia namaensis*. Analyses of plant material were carried out in the Department of Horticultural Sciences, Cape Peninsula University of Technology. Four weeks old seedlings of the two species were subjected to 3-day, 6-day, 9-day or 12-day water deficit regimes. Secondary metabolites such as polyphenols, alkaloids, and flavonols were assessed by the spectroscopic methods. The anti-fungal activities of crude extracts obtained from plants exposed to the various treatments were evaluated in a micro-dilution bioassay. In all treatments, the plant height, crown width, number of stems and leaves, and fresh and dry weights reduced as intervals between water irrigation increased. Acetone extracts from all treatments showed anti-fungal activity in the micro-dilution bioassay — the MIC values ranged from 0.75–6 mg/ml and 1.5–6 mg/ml for *S. dolomitica* and *S. namaensis*, respectively. However, extracts from the treatment with moderate water deficit (6-day watering interval) recorded significantly ($p<0.01$) better inhibition of *F. oxysporum* at the 18 h post incubation than the commercial Mancozeb. This research has revealed that mild to moderate water deficit level favours the accumulation of alkaloids in *S. dolomitica*. Meanwhile, mild to severe water deficit was associated with significantly lower flavonol content in *S. namaensis*, suggesting water stress negatively affected flavonol accumulation in the species. There was a possible correlation between this increase in plant secondary metabolites, specifically total alkaloid contents, water deficit and the enhanced anti-fungal activity of extracts of *S. dolomitica*. The present study's findings pave the way for optimised cultivation of medicinal plants and development of bioactive natural products as a green alternative to

synthetic fungicide. Moreover, it revealed that restricting water supply is suitable for cultivating these medicinal plants in South Africa, a water-scarce country.

Key words: Polyphenols, Alkaloids, Flavonols, *Fusarium oxysporum*, Water deficit, Salvia species

4.1 Introduction

South Africa is ranked as the 30th driest country globally (World Resources Institute, 2015). According to the World Economic Forum (2017), the third-highest risk for doing business in South Africa is its crisis with access to fresh water. According to the W.W.F. (2016), a two-degree increase globally means a four-degree increase for South Africa. As global temperatures are increasing, droughts are becoming more frequent and intense. Even though South Africa is already using too much water, it is forecasted that more water will be needed in the agricultural, industrial, and municipal sectors in the future (Donnefeld *et al*., 2018).

Even though South Africa is a water-scarce country (Muller *et al*., 2009; Goldin, 2010), it is home to 6% of the world's plant diversity (Raimondo, 2015) and has the richest temperate flora globally (Staden & Lotter, 2015). South Africa has many indigenous medical species that have prospects of becoming sources for natural pesticides. The global demand for natural products is increasing as the world becomes more conscious of synthetic products' negative impacts on human and environmental health (Saha *et al*., 2005).

In India, scientists in collaboration with the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and local Indian farming communities selected drought-resistant medicinal plants that the farmers can be intercropped with the food crops that are commonly cultivated. The intention was for farmers to generate more income. Moreover, farmers who had started growing medicinal plants had doubled or even tripled their incomes (Antonucci, 2016). In 2016, India's prime minister initiated a project called the "Aroma Mission," which promotes the cultivation of marketable medicinal and aromatic crops in unproductive areas, including those affected by water scarcity, drought, salinity, or floods (Srivastava, 2016). There are also several similar initiatives in South Africa, supported by provincial governments and municipalities (van Wyk, A. & Prinsloo, 2018). One of the South African government mandates, as stated by a previously presiding Minister of Environmental Affairs, is to create

jobs through research on the cultivation of medicinal plants on a commercial scale and promotion of indigenous knowledge sytems (Molewa, 2015).

Efforts to optimize the yield of medicinal plant materials through cultivation methods or techniques have been receiving much attention lately due to the rise of multidrug-resistant organisms (MDROs) (Chen *et al*., 2016; Isah, 2019). The success rate of developing a new drug from natural medicinal origins is higher compared to synthetic drugs, with the latter being very expensive and difficult to develop (Pan *et al*., 2013). Manipulation of the ambient environmental factors has been used to successfully enhance secondary metabolite production by plants (Ramakrishna & Ravishankar 2011). For example, many studies have revealed that medicinal plants grown under certain water deficit conditions resulted in significantly higher concentrations of relevant secondary metabolites than their unstressed counterparts (Kleinwächte & Selmar, 2014). Metabolic reactions triggered by drought stress are responsible for the higher natural product accumulation in plants grown in semi-arid regions (Al-Gabbiesh *et al*., 2014).

Different environmental stresses cause plants to produce various secondary metabolites that help the plant adapt to those stresses (Kroymann, 2011; Berini *et al*., 2018). The production of secondary metabolites by plants can be elicited by changing the biotic and abiotic factors under which they are cultivated (Rejeb *et al*, 2014; Caretto et al., 2015; Narayani & Srivastava, 2018). Moreover, the presence of secondary metabolites that are affected under water shortage can impact the commercial medicinal value of plants (Tátra *et al*., 2016). Plant secondary metabolites can be divided into three major groups, namely terpenoids; nitrogen-containing alkaloids and sulphur-containing compounds and flavonoids and allied phenolic and polyphenolic compounds. These secondary metabolites act as defence molecules against microbes, viruses, or other competing plants (Wink, 2003; Pusztahelyi *et al*., 2015). Antimicrobial compounds restrict pathogens' growth by activating specific signalling pathways leading to Ca2+ elevation and Reactive oxygen species (ROS) burst by binding to cell membranes followed by leakage of cell components of-pathogenic fungi such as *F. oxyporum* (Ito *et al*., 2007).

Drought often causes oxidative stress, thereby increasing the amounts of flavonoids and phenolic acids in plant tissues. (Larson, 1998). Water stress can also increase the tannin and

saponin contents of plants (Umebese & Falana, 2013) which are reported to have anti-fungal properties (Arif *et al*, 2009). Under drought stress, the closing of the stomata results in a series of consequent physiological and/or biochemical adjustments aimed at balancing the photosynthetic process as well as at enhancing the plant defence barriers against the drought-promoted stress. These barriers include the stimulation of antioxidant systems, stimulation of aquaporin synthesis and the accumulation of osmolytes which some of the actions the plant takes to overcome the unfavourable period of water deficit (Kampoor *et al*., 2020). In the current context, it is plausible to think that water stressing indigenous *Salvia* plants during cultivation could enhance the production of secondary metabolites, thereby increasing the plant extracts' efficacy against *F. oxysporum*.

Salvia dolomitica Codd, commonly known as dolomite sage or pilgrim's rest pink sage, is a fast growing shrub and is restricted to the provinces of Limpopo and Mpumalanga where it grows on dolomite rock outcrops at altitudes of 1150-1900 m (Clebsch, 2003; McQuillan, 2013). The major compounds in *S. dolomitica* are α-pinene (7.1%), δ-3 carene (7.5%), limonene (9.7%), 1.8-cineole (17.6%), β-caryophyllene (17.4%) and borneol (8.5%). The methanol:chloroform (1:1) extracts of *S. dolomitica* contain: Betulafolientriol oxide, Caffeic acid, Oleanolic/ursolic acid, and Rosmarinic acid (Kamatou *et al*., 2007).

Salvia namaensis Schinz, commonly known as Nama sage, is a well branched, semi-woody perennial shrub that is fast growing. The species comes from Namaqualand, Namibia or the Northern Cape as indicated by the epithet *namaensis*. It grows at 400-1700 m in well-drained, rocky, or sandy soil on limestone and dolerite hills (Clebsch, 2003; McQuillan, 2011). The methanol: chloroform (1:1) extracts of *S. namaensis* contain: 7-O-Methylepirosmanol, Betulafolientriol oxide, Caffeic acid, Carnosol, Carnosic acid, Oleanolic/ursolic acid, and Rosmarinic acid (Kamatou *et al*., 2007).

This investigation aimed to determine the effect of water deficits on the phenolic compounds of the aerial parts of two indigenous South African Salvia species, namely *Salvia dolomitica* and *Salvia namaensis*.

4.2 Materials and Methods

4.2.1 Propagation of *Salvia dolomitica* and *Salvia namaensis* cuttings

Semi-hardwood cutting materials from indigenous *S. dolomitica* and *S. namaensis* were obtained from the Kirstenbosch National Botanical Garden, Cape Town, courtesy of the curator at the time, the late Mr Philip le Roux. One hundred 10 cm cuttings of each species were propagated in 128 cell Styrofoam trays filled containing equal volumes rivers sand and perlite (Figure 4.1). These substrate materials were pre-sterilized with Captab (4 g in 1 L) before use. The trays of cuttings were placed on hotbeds maintained at a temperature of 26.5 ℃ under intermittent mist in the production glasshouse (22°C; 100% RH) until the roots appeared through the drainage holes of the trays.

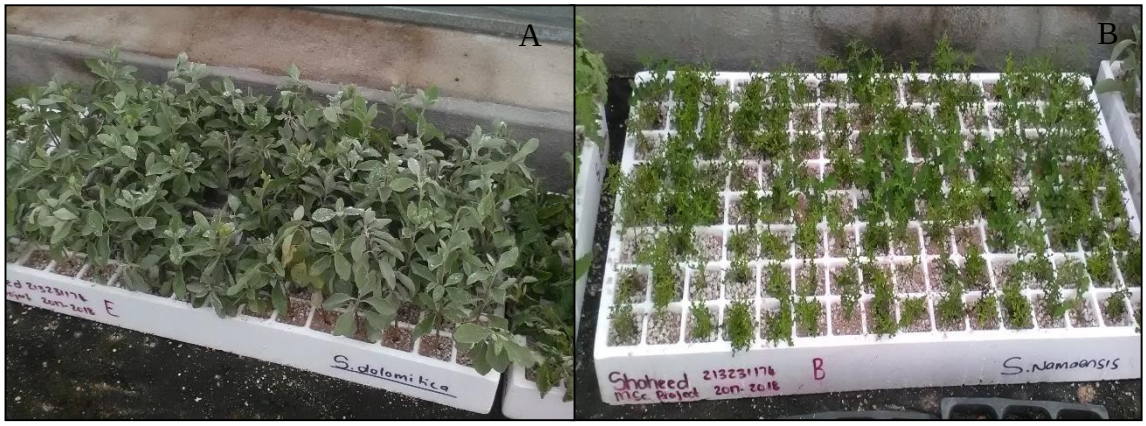

Figure 4.1: Semi-hardwood cuttings of *Salvia dolomitica* (A) and *Salvia namaensis* (B) rooting on hotbeds in production nursery at CPUT, Bellville Campus.

4.2.2. Greenhouse experiment

The experiment was conducted in a research greenhouse at the Cape Peninsula University of Technology, Bellville, Western Cape, South Africa S33° 54' 0, E18° 38'0 from December 2018 to March 2019. Four-week-old rooted cuttings of *S. dolomitica* and *S. namaensis* were individually transplanted into 15 cm brown plastic pots filled with sterilized medium containing equal parts of 1:1:1 silica sand, coir, and perlite. Sixty four, 4-week old potted cuttings of each of the two *Salvia* species was used in the water deficit experiment. The two groups of plants was divided into four treatments of 16 replicates each. Preliminary tests have shown that

watering the plants with 250 ml of RO water every three days was sufficient to prevent wilting, and thus, this watering regime was used as the treatment one (T1 – no water deficits). In treatment 2 (T2 – mild deficits), sixteen plants of each species were irrigated with 250 ml of water every six days. In treatment 3 (T3 - moderate), sixteen plants of each of the two species were watered every nine days, and plants in the fourth treatment (T4 - severe) were watered every 12 days. The experiment was conducted for eleven weeks and the plants were irrigated with reverse osmosis water according to the irrigation regimes per treatment. The potted plants were placed on the cement floor inside the research greenhouse, where the plants were exposed to natural sunlight that entered through the polycarbonate roof. The experiment followed a complete randomised block design. Temperatures inside were maintained between 24 – 26 °C during the day and 15 – 20 °C during night and 74% Relative humidity (RH). The plants' height was measured at the beginning and end of the experiment using a measuring tape from the surface of the medium inside the plant pot to the tip of the tallest shoot. The Crown width for each plant was measured from the tips of the two shoots that grew furthest from each other on a horizontal plane. The biomass for each of the replicates was calculated post-harvest. Individual plants were harvested at the end of the experiment by cutting the stems at the base level to the growth medium's surface. The plants were weighed, and their fresh weight was recorded. After being dried in a thermo-oven at 40 °C for 7-14 days in individual brown labelled paper bags, dry weights were recorded and subtracted from the wet weights to calculate the biomass.

4.2.3 *In vitro* screening using minimum inhibitory concentration (MIC) assay

As described by Ntobela et al. (2020), the micro-dilution method was employed to determine the minimum inhibitory concentration (MIC) for the extracts. 5 g of milled material of 5 replicates per treatment of *S. dolomitica* and *S. namensis* was extracted with 25 ml acetone and evaporated under a fan. The extracts were diluted into acetone to obtain a starting concentration of 6 mg/ml. The starting concentration was diluted two-fold in each successive serial dilution. The strain of *Fusarium oxysporum* sp.glycines strain (UPFC no. 21) that was used in this bioassay was obtained from existing cultures in the Horticulture Research Laboratory of Cape Peninsula University of Technology, Bellville campus. *F. oxysporum* (Figure 4.2) was sub-cultured from stock agar plates and transferred into Nutrient Broth (Merck, South Africa) for four hours. The fungal cultures (100 µl) were added to each well of the 96-well microplates

(105 cells/ ml). Mancozeb (Stodels Pty Ltd. Garden Centre, Cape Town, South Africa) served as the positive control. Forty microliter (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 °C and 100% RH. The MIC values were recorded at the 6th, 12th, 18th, and 24th hours of incubation of the microtitre plates. MIC values was recorded after comparing the colour of dyed wells to known concentration markers for individual rows of the microtitre plates using visual observations. The anti-fungal bioassay (MIC) consisted of three replicates per treatment.

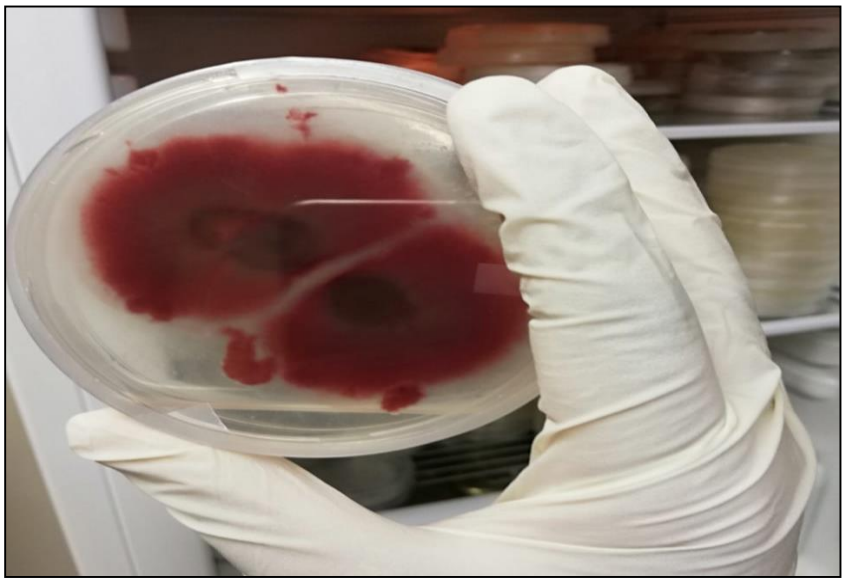

Figure 4.2: Two week old subculture of *F. oxysporum* showing dark pink mycelia that will eventually turn purple at about twelve weeks.

4.2.4 Determination of Polyphenol, Flavonol and Alkaloid contents

The method, described by Fadhil & Reza (2007), was used to determine total alkaloids in the plant extracts. 100 mg of the milled aerial (leaf and stem) materials was extracted separately with 10 ml of aqueous ethanol (a mixture of 60% ethanol and 40% water) for two hours after which it was centrifuged (4000 x g for 10 min) and the supernatant was used in the assay. Two millilitres of the extract supernatant and atropine standard solutions were mixed with 5 ml sodium phosphate buffer and 12 ml bromocresol green solution. Thereafter, 12 ml of chloroform was added to the solution, and the solution was mixed vigorously using a vortex mixer. The absorbance at 417 nm was determined, and the concentration of mg atropine equivalent per g dry weight (mg AE/g DW) in the sample using a standard curve of atropine

was calculated. The total polyphenol content of the aqueous ethanol extracts of dried *S. dolomitica* and *S. namaensis* materials were determined by the Folin-Ciocalteu method (Singleton *et al.*, 1999; Swain & Hills, 1959). The method of Swain and Hills (1959) was adapted for the plate reader. A 96-well microplate was used for the MIC. 25 µL of the sample was mixed with 125 µL Folin-Ciocalteu reagent (Merck, South Africa) and diluted 1:10 with distilled water. After 5 minutes, 100 µL (7.5%) aqueous Na_2CO_3 (Sigma-Aldrich, South Africa) was added to the well. The plates were incubated at room temperature for two hours before the absorbance was read at 765 nm 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 aerial materials of the *Salvia* plants were determined using quercetin 0, 5, 10, 20, 40, and 80 mg/L in 95% ethanol (Sigma-Aldrich, South Africa) as standard. In the sample wells, 12.5 µL of the crude aqueous extracts were mixed with 12.5 µL 0.1% HCl (Merck, South Africa) in 95% ethanol and 225 µL 2% HCl and incubated at room temperature for thirty minutes. The absorbance was read at 360 nm, at a temperature of 25°C (Mazza *et al.*, 1999). The results were expressed as mg quercetin equivalent per g dry weight (mg QE/g DW).

4.3. Statistical analysis

The plant growth, total phenolic, and MIC data are reported as Mean ± SE, and the one-way analysis of variance (ANOVA) was used to compare the means amongst the treatments. All computations were completed with the software programs STATISTICA® (13.5.0.17) and the Paleontological Statistics package for education and data analysis (PAST 3.14). Post hoc analysis based on the Tukey test was used to separate the means. P values of <0.05 were regarded as significant, and P values of <0.01 as very significant.

4.4 Results

4.4.1 Height and crown width.

Results of this study showed a significant difference in the height of the *Salvia dolomitica* plants among watering interval treatments (df = 3, 48; F = 18.95; P < 0.01). Treatment two (6-day watering interval) had the highest mean of 31.50 cm, followed by the shortest watering interval (3-day watering interval) with a mean of 30.45 cm. Treatments three (9-day watering interval) and four (12-day watering interval) recorded the shortest plants with 22.46 cm and 18.26 cm, respectively. There was also a significant difference P<0.01 in the heights of the *Salvia namaensis* plants between treatments (df = 3, 48; F = 5.345; P = 0.004875). Treatment one had the highest mean height of 19.80 cm, followed by treatment two with a mean height of 16.00 cm. The lowest means belonged to treatment three, 14.50 cm, and treatment four, with a mean of 13.25 cm.

A significant difference in the crown widths of the *Salvia dolomitica* plants (df = 3, 48; F = 22.24; P < 0.01) was observed with the largest widths belonging to treatment 1 (3-day watering interval) with a mean of 13.458 cm followed by treatment two and three with measurements of 119.41 mm and 97.50 mm, respectively. Treatment four had the lowest mean of 63.33 mm (Figure 4.3).

There was a significant difference in the crown width of the *S. namaensis* plants (df = 3, 48; F = 3.312; P = 0.03433). The widest crown size measurements belonged to treatment two with a mean of 21.33 cm, followed by treatment one 20.69 cm. Treatments three and four both had the lowest mean of 14.50 cm each.

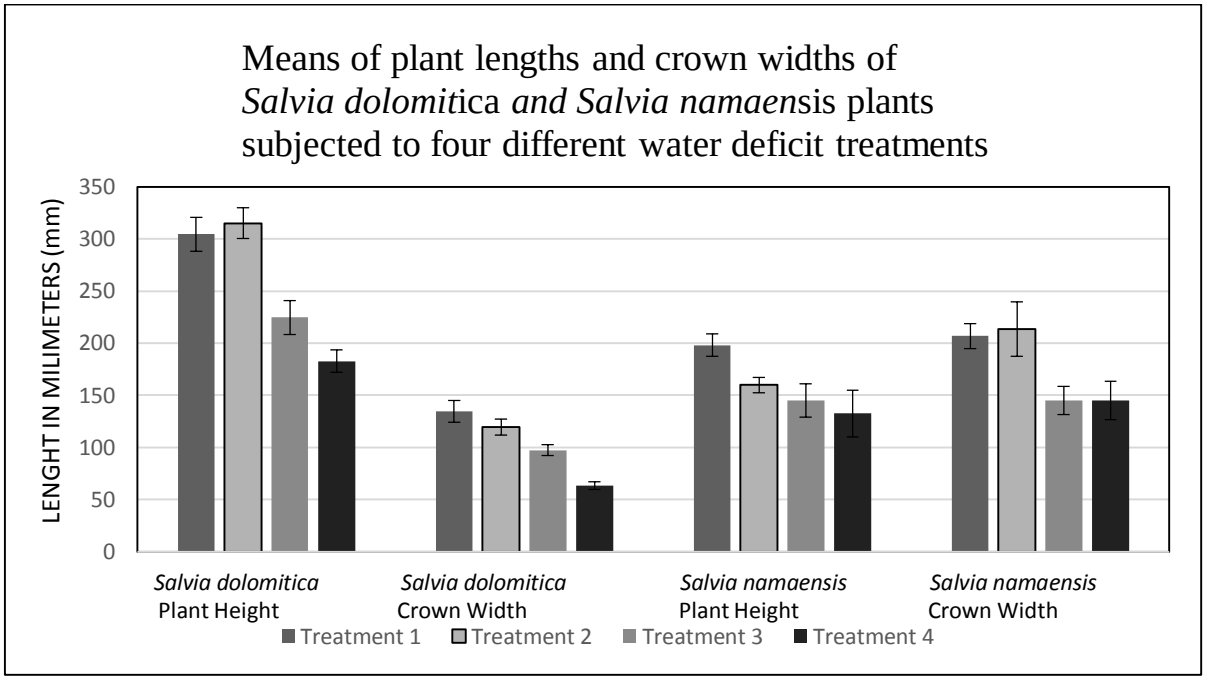

Figure 4.3: Means of plant lengths and crown widths of *Salvia dolomitica* and *Salvia namaensis* plants subjected to four different water deficit treatments.

4.4.2 Fresh and dry weights

There was a significant difference in the fresh weight of the *Salvia dolomitica* plants (df = 3, 48; F = 61.62; P < 0.01). The shortest watering interval had the highest mean of 11.25 g, followed by treatment two with fresh weights of 8.9 g and 4.13 g, respectively. Treatment four had the lowest mean fresh weight (1.52 g) (Table 4.1).
A significant difference was also found in the fresh weights of the *S. namaensis* plants (df = 3, 48; F = 58.56; P < 0.01). Treatment one had the highest mean of 8.52 g, followed by treatment two and three with 3.52 g and 1.46 g, respectively. Treatment four had the lowest mean fresh weight (1.15 g).

Table 4.1: The mean ± SE fresh and dry weights of *Salvia dolomitica* and *Salvia Namaensis* species subjected to four different water-deficit treatments.

***Salvia* species**	**Fresh Weight**	**Dry Weight**
Salvia dolomitica		
Treatment one	11.25±0.93 [a]	3.98±0.43 [a]
Treatment two	8.90±0.50 [b]	3.46±0.23 [a]
Treatment three	4.13±0.35 [c]	1.85±0.14 [b]
Treatment four	1.52±0.12 [d]	1.37±0.10 [b]
Salvia namaensis		
Treatment one	8.52±0.64 [a]	3.69±0.29 [a]
Treatment two	3.52±0.48 [b]	2.14±0.26 [b]
Treatment three	1.46±0.12 [c]	1.38±0.11 [bc]
Treatment four	1.15±0.08 [c]	1.01±0.07 [c]

The values tabulated are the means (± SE).
Significant differences among the means are evaluated using the Tukey HSD test.
Values with the same letters in a column are not significantly different at the 0.05 probability level.

There was a significant difference in the dry weight of the Salvia *dolomitica* plants (df = 3, 48; F = 22.72; P < 0.01). Treatment one had the highest mean dry weight (3.98 g), followed by treatment two and three with measurements of 3.46 g and 1.85 g, respectively. Treatment four had the lowest mean of 1.37 g.

Results also show a significant difference P < 0.01 in the dry weight of the *Salvia namaensis* plants (df = 3, 48; F = 31.6; p < 0.01). Treatment one had the highest mean dry weights (3.69 ± 0.29 g), followed by treatment two and three with measurements of 2.14 ± 0.26 g and 1.38 ± 0.26 g, respectively. Treatment four had the lowest mean of 1.01 g.

4.4.3 Number of stems and leaves

Due to the nature of the growth habit of *S. namaensis*, the number of stems and leaves for the different treatments have not been recorded as they are too numerous to count, and even mild to moderate water deficit treatments made the leaves too brittle to handle. The data for *S. dolomitica* is tabulated below. The number of stems and number of leaves decreased with increasing water stress. A significant difference was found among treatments for both the number of stems (df =3; F=3.259; P = 0.02944) and leaves (df =3, 48; F=15.71; P < 0.01) of the S. *dolomitica* plants subjected to different water-deficit

treatments (Table 4.2). Treatment one had the highest mean recorded for the stems, 3.07 ± 0.66 stems, while the lowest mean 1.38 ± 0.21 stems was recorded for treatment four. For the number of leaves, treatment one had the highest mean number of leaves (109.15 ± 9.71), while the lowest mean number of leaves (44.33 ±4.33) was recorded for treatment four (Table 4.2).

Table 4.2: The mean ± SE number of stems and leaves of *Salvia dolomitica* subjected to four different water-deficit treatments.

Species: ***Salvia dolomitica***	**Number of Stems**	**Number of leaves**
Treatment one	3.07±0.66 [a]	109.15±9.71 [a]
Treatment two	1.84±0.37 [ab]	90.46±6.22 [a]
Treatment three	1.53±0.31 [ab]	58.08±6.07 [b]
Treatment four	1.38±0.21 [b]	44.33±4.33 [b]

The values tabulated are the means (± SE).
Significant differences among the means are evaluated using the Tukey HSD test.
Values with the same letters in a column are not significantly different at the 0.05 probability level.

4.4.4 Secondary metabolites

Table 4.3: The Mean ± SE number of stems and leaves of *Salvia dolomitica* subjected to four different water-deficit treatments.

	Polyphenols in mg GAE/g	**Flavonols in mg QE/g**	**Alkaloids in mg AE/g**
Salvia dolomitica			
Treatment one	66.43±5.23 [a]	08.84±0.90 [a]	05.91±0.33 [ab]
Treatment two	64.51±2.96 [a]	08.53±0.48 [a]	04.57±0.26 [b]
Treatment three	73.67±4.91 [a]	10.36±0.76 [a]	06.67±0.54 [a]
Treatment four	64.39±3.40 [a]	08.09±0.11 [a]	04.75±0.53 [ab]
	Polyphenols in mg GAE/g	**Flavonols in mg QE/g**	**Alkaloids in mg AE/g**
Salvia namaensis			
Treatment one	61.22±1.79 [a]	08.24±0.30 [a]	02.10±0.20 [a]
Treatment two	55.33±2.31 [a]	07.45±0.44 [ab]	02.89±0.30 [a]
Treatment three	52.88±3.53 [a]	06.13±0.34 [b]	02.75±0.68 [a]
Treatment four	51.57±3.97 [a]	06.33±0.60 [ab]	04.05±0.96 [a]

The values tabulated are the means (± SE).
Significant differences among the means are evaluated using the Tukey HSD test.
Values with the same letters in a column are not significantly different at the 0.05 probability level.

4.4.4.1 Polyphenols mg GAE/g

There was no statistical difference amongst the treatments (df = 3, 8; F = 107; P = 0.4147); however, treatments three recorded the highest mean of 73.67±4.91 GAE/g and treatment four had the lowest amount of polyphenols tested, recording a mean of 64.39±3.40 GAE/g (see table 4.3). Similarly, no statistical difference in polyphenol was observed among the treatments for the *S. namaensis* (df = 3, 8; F = 1.986; P = 0.1947); however, treatment one had the highest content of polyphenols, 61.22±1.79 GAE/g, followed by treatment two, 55.33±2.31 GAE/g and treatment three, 52.88±3.53 GAE/g, and the lowest amount recorded was from treatment four with 51.57±3.97 GAE/g.

4.4.4.2 Flavonols in mg QE/g

There was no statistical difference in the total flavonol contents in the extracts of the differently treated *S. dolomitica* plant (df = 3, 8; F = 2.401; P = 0.1432). The values of mg QE/g ranged from 8.09±0.11 mg QE/g to 10.36±0.76 mg QE/g, with treatment four containing the lowest amount of flavonols and treatment three the highest (Table 4.3). Treatment one and two, and two contained 8.84±0.90 mg QE/g and 8.53±0.48 mg QE/g, respectively.

On the other hand, flavonol contents in *S. namaensis* plants varied significantly among the treatments (df = 3, 8; F = 5.171; P = 0.02811), with the shortest watering interval yielding the highest amount 8.24±0.30 mg QE/g, followed by treatment two 7.45±0.44 mg QE/g and treatment four with 06.33±0.60 mg QE/g Treatment three contained the lowest with a value of 6.13±0.34 mg QE/g.

4.4.4.3 Alkaloids mg AE/g

Extracts of *S. dolomitica* exposed to treatment three (6.67±0.54 mg AE/g) produced significantly higher alkaloid content compared to the other treatments (df = 3, 8; F = 5.211; P = 0.02758) (Table 4.3). No statistical difference in total alkaloid contents (P>0.05) was observed amongst the treatments (df = 3, 8; F = 1.716; P = 0.2405) of the *S. namaensis* extracts. The values of mg AE/g ranged from 2.10±0.20 mg AE/g to 4.05±0.96 mg AE/g, with the treatment one having the lowest and Treatment four the highest. Treatment two and three contained 2.89±0.30 mg AE/g and 2.75±0.68 mg AE/g, respectively.

4.4.5 Minimum inhibitory concentration

4.4.5.1 MIC of *Salvia dolomitica* extracts

There was a significant difference among treatments after the 6th and 12th hour of incubation (df = 4, 10; F = 7.51; P < 0.01). The mild, treatment two, and moderate, treatment three, water deficit treatments produced higher activity than the synthetic fungicide, Mancozeb (Figure 4.4). A significant difference was observed amongst the treatments at the eighteenth hour of incubation (df = 4, 10; P < 0.01). Treatments one, two, and three each had a MIC of 1.5 mg/ml, followed by treatment four with a recording of 3 mg/ml. All the treatments showed fungistatic activity higher than Mancozeb, which had a MIC value of 6 mg/ml.

After 24 h of incubation, there was a significant difference in the fungistatic activities of the acetone extracts of *S. dolomitica* (df = 4, 10; P < 0.01). All the treatments had higher activity than the synthetic fungicide, Mancozeb that had a MIC reading of 6 mg/ml at this period. Treatment one and treatment four recorded a MIC value of 3 mg/ml, and both treatment two and three recorded a better MIC value of 1.5 mg/ml.

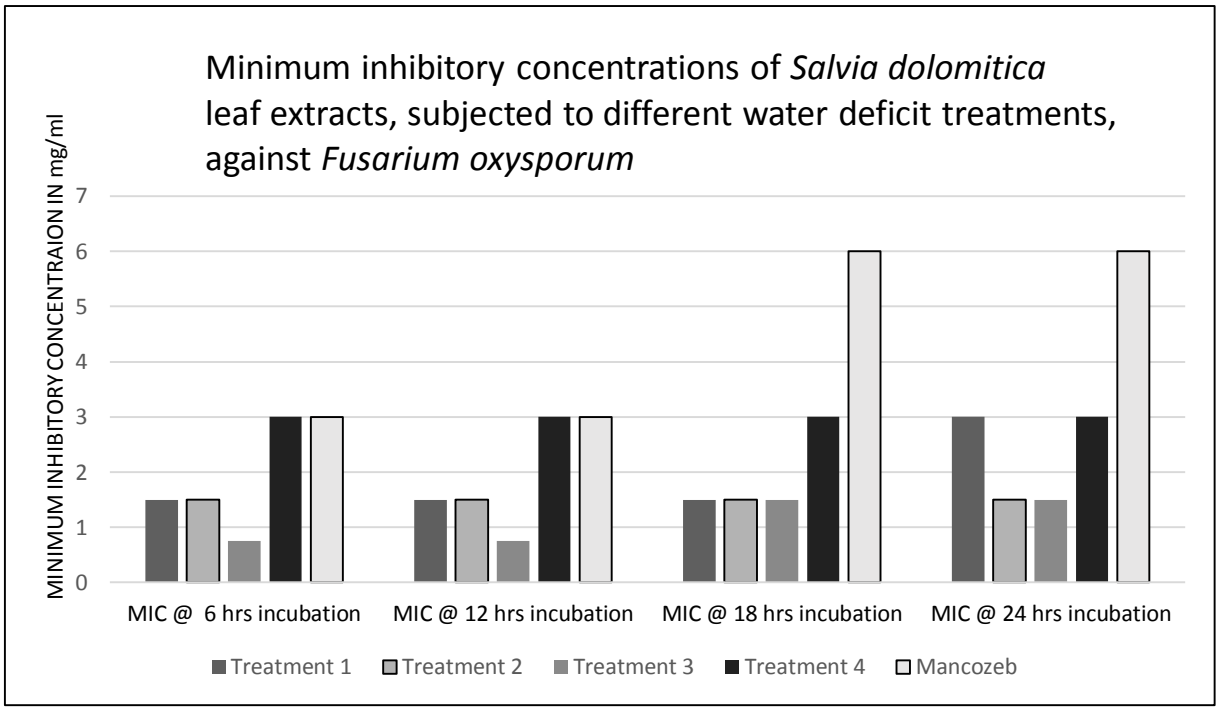

Figure 4.4: Minimum inhibitory concentration values (mg/ml) of *Salvia dolomitica* leaf extracts, subjected to different water deficit treatments, against *Fusarium oxysporum.*

4.4.5.2 MIC of *Salvia namaensis* extracts

There was a significant difference amongst treatments at the 6 and 12 h of incubation (df = 4, 10; F = 1.5; P < 0.01) (Figure 3). Treatment one and treatment four, both having a MIC of 6 mg/ml, showed weaker activity than that of Mancozeb, which had a reading of 3 mg/ml. Treatment three was comparable to that of Mancozeb with the same MIC value, and treatment two showed the highest activity of 1.5 mg/ml of the extracts tested (Figure 4.5).
Treatment two had significantly higher fungistatic activity than the other treatments (df = 4, 10; F = 5.4; P < 0.01) with a MIC of 3 mg/ml. A reading of 6 mg/ml was observed at the eighteenth hour of incubation for treatment one, treatment three, treatment four, and the synthetic fungicide, Mancozeb.
There was no change in the MIC values at 24 h. MICs for treatments one, three, and four were comparable to the fungistatic activity of Mancozeb. All had a MIC value of 6 mg/ml.

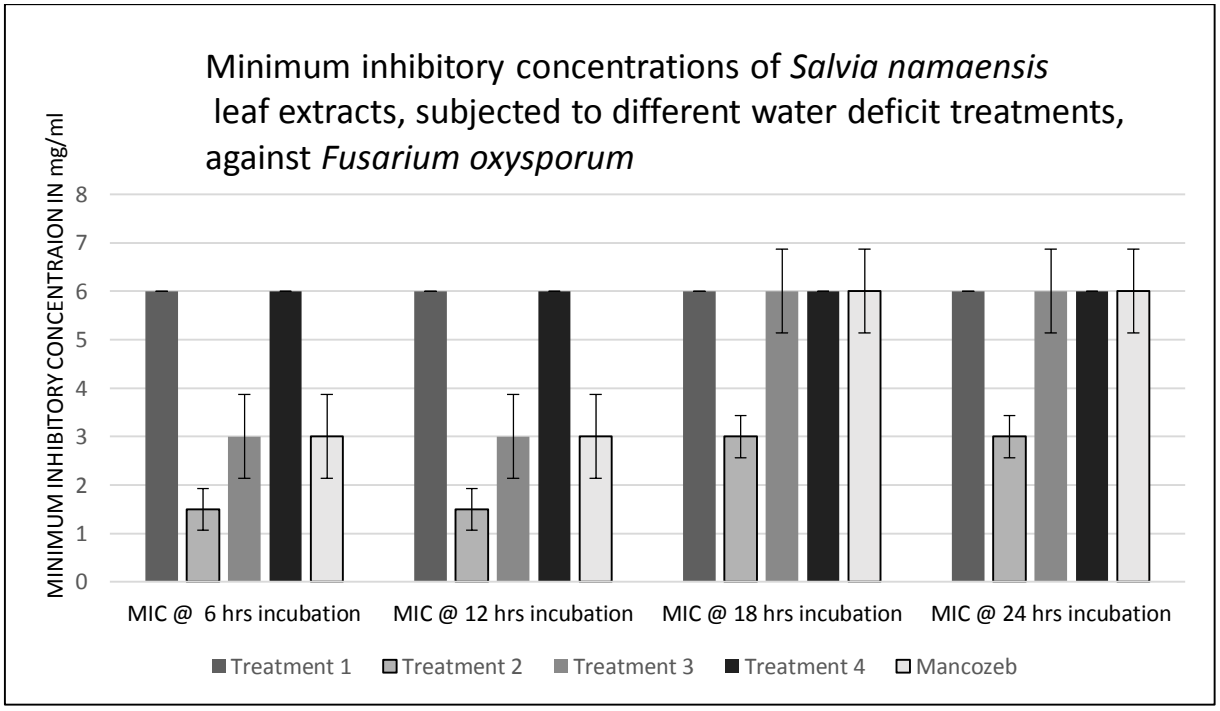

Figure 4.5: Minimum inhibitory concentration values mg/ml of *Salvia namaensis* leaf extracts, subjected to different water deficit treatments, against *Fusarium oxysporum.*

4.5 Discussion

4.5.1 Height and crown width

Water deficit is one of the most important factors affecting plant growth and poses a substantial threat to sustainable agriculture (Ings *et al*., 2013). This study's findings suggest that increasing water deficits lead to a decrease in plant height and crown width in *Salvia dolomitica* and *Salvia namaensis* plants overall. It is in agreement with the findings of Rahimi *et al*. (2017). They found that dehydration reduces overall plant growth due to the considerable reduction of photosynthesis, cell turgidity, cell growth, and increasing evapotranspiration. Moderate water deficit did increase the length of *S. dolomitica* and the crown width of *S. namaenis* plants in the current study. Similar findings were reported by Luvaha *et al*. (2008), who found that mild water deficits had a positive effect on the stem length of *Mangifera indica*. Guo *et al*. (2016) also showed that the height and crown width of *L. ruthenicum* increases under mild water deficit conditions.

4.5.2 Fresh and dry weight

The fresh and dry weights decreased as the water deficits increased for all the treatments of both species of Salvia tested in this study. However, the drop in fresh weight is more drastic than the decrease in dry weight. Ramos *et al*. (1999) showed that water deficit inhibits the accumulation of fresh plant mass significantly than dry biomass of common bean plant (*Phaseolus vulgaris* L.). Malejane *et al*. (2018) concluded based on their trial that overall, increasing water deficit impacted negatively on the fresh weight of both the green leafy lettuce cultivars tested. Water deficit also reduced the plant height, crown width, fresh and dry mass of *Melissa officinalis* L. (Radácsi *et al*., 2016). In this study, the number of leaves and stems of *S. dolomitica* significantly decreased as the water deficits' intensity increased. These findings can be corroborated by Gugliuzza *et al*. (2020) studies, in which drought reduced all growth in Loquat plants (*Eriobotrya japonica*). Radácsi *et al*. (2016) showed that water-deficit levels significantly affected the quantity and quality of lemon balm plants.

4.5.3 Number of stems and leaves

The abiotic factor, water deficit stress, is one of the most common environmental factors that affect plant growth (Bohnert and Jensen, 1996; Xu *et al*., 2010), and is considered one of the most growth-limiting factor that decrease plant growth (Tátra *et al*. 2016). Drought stress occurs when the available water in the soil is reduced to such critical levels. Drought stress tolerance is seen in all plants, yet the extent of tolerance varies from species to species. Exposure to drought causes many common reactions in plants, such as cellular dehydration, which causes osmotic stress and water removal from the cytoplasm to vacuoles (Ramakrishna & Ravishankar, 2011). A reduction in chlorophyll content may occur under drought stress (Massacci *et al*., 2008). Water deficits can adversely impact many aspects of the physiology of plants, especially photosynthetic capacity and prolonged drought stress may severely diminish plant growth and productivity. (Osakabe *et al*., 2014). Water stress affects plants' photosynthesis by reducing the carbon dioxide conductance in leaves due to the changes in the stomatal aperture with the decrease in the leaf water potential. In some plants, the translocation of photoassimilates from leaves to the stems assists in the maintenance of growth under water stress conditions (Ohashi *et al*., 2000; LI *et al*., 2009)

4.5.4 Total Phenolics

The effects of water-stress on phenolic content varied with plant species in this study, suggesting that water deficit can increase or decrease phytochemicals in plants (Umebese, C. & Falana, 2013). Specifically, moderate water deficit treatment increased the level of polyphenols in the extracts of *S. dolomitica*. Similar results were obtained by Marchese *et al*. (2010) when they examined the effect of water deficits on accumulating biomass and artemisinin in annual wormwood (*Artemisia annua* L.) and results showed that moderate water deficit not only induced artemisinin accumulation but also reduce time and costs in drying the crop. These, in combination, increase crop profit margins coupled with water-saving and can only lead to further mitigation of environmental degradation and sustainable business practices. According to McKiernan *et al*. (2017) juvenile *E. globulus* can tolerate periods of continuous yet moderate water availability with little impact on concentrations of secondary plant metabolites.

On the other hand, increasing water deficits resulted in a decrease in polyphenolic content in *S. namaensis*, which is in contrast to the results recorded by Lee & Oh (2017) in their study on the effects of water deficits on the contents of bioactive compounds in Dropwort; the total phenolic contents of all the water deficit treatments increased significantly compared to the controls. A study by Giamperi *et al*. (2012) compared the total content of polyphenolic compounds in eight *Salvia* spp. exudates and reported that the second highest concentration of polyphenols was detected in *S. namaensis* with 20 µg polyphenols recorded. It is interesting that the water deficit treatments in this study did not have a positive effect on the accumulation of polyphenols in *S. namaensis*. The levels of phenolic compounds in plants are genetically controlled and environmentally induced (Lattanzio, 2013). Further study on the effects of abiotic factors on the polyphenolic content of this species is recommended.

This study indicates that moderate water deficits increased the flavonol content of *S. dolomitica* extracts, and mild and severe water deficit treatments decreased the flavonols' levels. Umebese & Falana (2013) investigated the impact of water stress on the concentrations of phytochemicals of *Bryophyllum pinnatum* L. and found that flavonoids increased with increased water deficit intervals. Various biotic and abiotic stress affects the accumulation of flavonoids in plant vegetative tissues and organs (Braidot *et al.*, 2008). Water deficits affect the accumulation of flavonoids in plants (Hernandez *et al*., 2004). The results of a trial by Yuan *et al*. (2012) clearly showed that water deficit affected flavonoid accumulation by regulating hormone metabolism in *Scutellaria baicalensis*.
Water deficit influence the flavonol content negatively. All the water deficit treatments decreased the *S. namaensis* extracts' flavonol content compared to the controls. Radácsi *et al*. (2016) also found that under two of the stress treatments they subjected *Melissa officinalis* plants to, the accumulation of flavonoids was decreased by 14–22%.

As was the case in the Flavonol content of the extracts in this study, mild and sever water deficit treatments decreased the Alkaloid content in the *S. dolomitica* extracts but an increase in alkaloid content was observed in the extracts exposed to moderate water deficits, significantly more than the extracts treated to mild water deficits. With the *S. namaensis* extracts, the alkaloid content increased as the level of water deficits increased, the association was not significant.

Alkaloids are secondary metabolites synthesized and accumulated by many higher plant species (Umbese *et al*., 2012). Several alkaloids affect the biological function of fungi at low concentrations (Singh *et al*., 2007). The differences in accumulation of secondary metabolites of *Salvia* species at various degrees of water deficits may be due to the genetic makeup of the individual species (Isah, 2019; Matveeva et al., 2015) as well as changes in nutrient uptake which may cause changes in secondary metabolite production (Prescott et al., 2020)

4.5.7 Minimum inhibitory concentration

The extracts of *S. dolomitica* plants exposed to mild and moderate water deficits displayed significantly better fungistatic activity against *F. oxysporum* than the controls, the severe water deficit treated extracts, and the synthetic fungicide Mancozeb. With the *S. namaensis*, the mild water deficit treated extracts displayed more potent activity than all other treatments. Broadly, these results suggested that mild to moderate water stress enhances second antifungal activity of acetone extracts of both *Salvia spp*. studied. When el Bouzidia *et al*. (2012) examined the anti-fungal activities of *Achillea ageratum* against *Candida* species, and they claimed that the MIC values ranging from 5.83 to 8.42 mg/ml were quite remarkable. In this study, the MIC values ranged from 0.075 – 6 mg/ml for *S. dolomitica* extracts and 1.5-6 mg/ml for *S. namaensis* extracts. These MIC values can, therefore, also be considered as remarkable levels of anti-fungal activity. Orhan *et al*. (2010) also described the MIC levels of their tested compounds with MIC levels ranging from 32 to128 mg/ml as strong anti-fungal activity against isolated strains of the pathogen, and some of the compounds with MIC levels of 32 mg/ml possessed more potent anti-fungal activity than fluconazole (64 mg/ml). The anti-fungal activity of the tested extracts can be correlated to the changes in the phenolic content when subjected to water deficits. Similarly, results of the study on the antimicrobial properties of ethanol extracts of *Gymnema montanum* L. against *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Candida albicans* indicated that the presence of antimicrobial properties in the leaf extract of *G. montanum* correlated to its phenolic compound content (Devi *et al*.; 2017).

4.6 Conclusion and Recommendations

The present study results indicate that the cultivation of *S. dolomitica* under mild and moderate water deficits correlated with their alkaloid metabolites as well as the fungistatic activity of the acetone extracts against *F. oxysporum*. However, the data presented here reflect *in vitro* and greenhouse experiments, and we recommend that field experiments and further investigations need to be conducted to confirm this conclusion. The application of controlled water deficits during the cultivation of medicinal plants will save water, freeing up water for other uses or decreasing the water bill and enhancing profit margins of medicinal plant cultivators. Saving water is critical for a water-scarce country like South Africa.

Based on the results of this study it is possible to speculate that moderate water stress can be beneficial for secondary metabolite production *Salvia* spp. and this knowledge could be further exploited by pharmaceutical industries to develop new drugs and formulations.

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CHAPTER FIVE

THE EFFECTS OF EXTRACTS OF *SALVIA DOLOMITICA* CODD. ON THE GERMINATION OF MAIZE SEEDS WHEN USED AS A SEED TREATMENT.

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This trial aimed to assess the effects of the acetone extracts of *Salvia dolomitica* plants on the germination of maize seeds when used as a seed treatment against the phytopathogenic fungus, *Fusarium oxysporum*. The extracts were derived from plants subjected to four different water treatments (none, mild, moderate, severe), and the extracts were tested in three concentrations (10, 20, 30 mg/ml). The germination bio-assay was conducted in sterilized closed systems as described by Del Barrio-Duque *et al*., 2020) with slight modifications in a research laboratory with the room temperature maintained at 25 °C for 14 days. Most of the extracts tested caused no significant effects (P>0.05) on the germination of the maize seeds; however, the extracts were associated with shorter shoot lengths of the maize seedlings. The bioassays results indicated that the extracts of *S. dolomitica* may contain bioactive compounds that may be used for the development of green alternatives to synthetic herbicides.

Key words: fungicide, bio-assay, *Fusarium oxysporum*, germination, coleoptile

5.1 Introduction

Worldwide, the organic market is a multi-billion dollar industry (Willer & Lernoud, 2019) and is continually growing at a fast pace (Willer & lernoud, 2019).

Organic agriculture has seen tremendous economic growth in the last two decades as consumers globally become aware of the dangers of synthetic agricultural products have on their health and the environment. Farmers have come to the realisation that consumers are willing to pay more for products that have been cultivated in more natural and organic ways (Morgera *et al.*, 2012). The department of Agriculture, Forestry, and Fisheries of South Africa claims that

Labour intensive organic productions would create endless working opportunities and that Self-subsistence would be accompanied by self-employment, which, in turn, would contribute to a progressive transformation of the informal economy and rural sector into a more vibrant economy (DAFF, 2019)

Plant pathogens are developing, and some have already acquired resistance to widely used chemical products and many chemicals are not allowed in organic farming (Šernaitė, 2017). To conduct business in this market, farmers will have to cultivate their crops according to organic standards, promoting the use of natural substances instead of synthetic substances (McEvoy, 2008). Seed treatments from natural origins could benefit organic farmers by allowing earlier sowing times, which could also result in higher yields (Christain, 2007). The National Policy on Organic Seed Production recognises the need to promote organic seeds (African Centre for Biodiversity, 2016).

Globally there is need for alternative environmentally friendly products in the market because of increased opposition to the use of synthetic chemical antimicrobial agents, and these products are losing their potency (Dellavalle *et al*., 2010; Rautenbach *et al*., 2015; Tebbets *et al*., 2013). The ever increasing resistance of microorganisms against available antimicrobial agents is of significant concern not only amongst scientists but also clinicians all over the world (Orhan *et al*., 2010). The increasing resistance of pathogens to synthetic agricultural products is also justifying the search for novel active molecules and new strategies to control diseases (Dellavalle *et al*., 2010). A desirable alternative to synthetic plant protection in agriculture is to use natural products developed from the compounds of plants with naturally occurring anti-fungal properties (Pretorius & van der Watt, 2015). In 2018, the fungicide market was valued at 13.41 billion US dollars and is expected to reach a value of 15.74 billion dollars during the forecasted period of 2019 to 2024 (Wood, 2018). In recent years, bio-fungicides' popularity has increased among farmers, producers, and end consumers because of factors regarding food safety and environmental degradation. The development of bio-pesticides or eco-friendly crop protection products has become a priority, and profits in organic-based farming are influencing farmers to look toward the synthetic chemical alternatives to control plant diseases (Wood, 2018).

The correct application of production inputs that will sustain the environment and agricultural production is important for successful maize production (du Plessis, 2003). Significant

quantities of active substances are required when applying plant protection compounds as soil amendments or sprayed directly on the plants. However, an alternative approach is to apply the crop protection compounds directly to the seed before sowing. This requires minimal quantities of active substances and is a targeted method of controlling pests and diseases. Mancozeb (Meridian Agrochemical Company (Pty) Ltd) is a broad-spectrum, largely protectant-contact fungicide effective against many fungi that cause seed rot and seedling blight (Dastogee, 2013). The application of synthetic fungicides results in environmental contamination, the development of pathogen resistance to many available pesticides used currently as well as pesticide residues on fruit crops (Yang & Jiang, 2015). The use of biodegradable plant products, especially from medicinal plants, is gaining importance in plant disease control (Singh *et al*., 2007). Medicinal plants have been used as a safe form of medication, either in crude or isolated pure molecules, for thousands of years by ancient cultures throughout the world (Khan 2014), and the traditional practice of using plant preparations to combat fungal infections has gained attention. The focus is currently on detecting new anti-fungal components from plants that have no adverse effect on the environment or animal and human systems (Saha *et al*., 2005). The anti-fungal activity of plant extracts is partly due to bioactive polyphenol compounds, which interfere with fungi life process by binding their protein molecules, acting as chelating agents altering structural component synthesis, weakening or destroying the permeability barrier of the cell membranes. In a trial by Riaz *et al*. (2008), the plant extracts of *Tagetes erectus* L (marigold), *Helianthus annus* L (sunflower), and *Capsicum annum* L (chilies) were found to be highly effective against the phytopathogenic fungi, *Fusarium oxysporum,* where all the employed extract concentrations significantly reduced the fungal biomass by 54 to 79%, 33 to 85% and 45 to 57%, respectively.

South Africa is rich in medicinal plants (Tewari, 2004) whose extracts can be used to create natural plant-based anti-fungal products to be used in the organic food industry, such as maize production. Maize is the most important grain crop in South Africa. It is the leading feed grain and the staple food of the majority of the South African population (DAFF, 2016). Seedling diseases of maize, including seed rot, seedling blight, and root rot are caused by seed-and soil-borne fungi (Soonthornpoct *et al*., 2001). Soil-borne pathogens cause seedling blights of maize during the critical stage of emergence (Brien, 2017). Seed and seedling diseases of maize are generally caused by one or a complex of soil-inhabiting fungi such as *Fusarium* spp., *Pythium* spp., *Rhizoctonia* spp., *Stenocarpella* spp. and *Penicillium* sp. (Flett & Hugo, 2011).

Rodriguez-Brljevich (2008) found in a study on the interaction of fungicide seed treatments and the *Fusarium*-maize (*Zea mays* L.) pathosystem that *Fusarium* species were the most common seed-borne fungi in maize seed. Faloon (1982) stated that *F. oxysporum* along with *T. koningii* were the most common fungi isolated that is capable of stunting maize seedlings or killing them. By using broad spectrum fungicides or fungicide combinations with similar broad anti-fungal activities as seed treatments, the maize seedlings are protected from pre-emergence pathogenic fungi (Faloon, 1982). *Fusarium* spp. are environmental hyaline moulds that can be pathogenic to plants and opportunistic pathogens in humans (Lainhart *et al*., 2018). More than fifty species of *Fusarium* have been identified (Nucci & Anaissie, 2007). *Fusarium* species are also some of the most drug-resistant fungi (Dignani & Anaisie, 2004).
In the previous chapters we demonstrated that S. *dolomitica*'s crude was active against *Fusarium oxysporum* activities could be enhanced by growing plants under water stress conditions. Hence, in this chapter we investigated the effect of water-deficits on S. *dolomitica*'s extracts anti-*F. oxysporum* activities as seed treatments on maize seeds at three different concentrations (10, 20 and 30mg/ml).

5.2 Material and Methods

5.2.1 Laboratory experiment (evaluating the effects of seed treatment with plant extracts on germination)

The experiment was conducted in the Biology Research Laboratory at the Cape Peninsula University of Technology, Bellville, Western Cape, South Africa S33° 54' 0, E18° 38'0 from September 2019 to December 2019. Maize seeds were treated with three concentrations (10, 20 & 30 mg/ml) of acetone extracts obtained from aerial parts (leaves and stems) of *Salvia dolomitica* cultivated under four different water deficit regimes. The seeds were placed in sealed germination containers on a work bench in the laboratory in a completely randomized design. After 14 days, the germination percentage and shoot length of the seedlings were evaluated to determine the effects of these extracts on the germination of maize seeds in the absence and presence of experimental exposure to *F. oxysporum*.

5.2.1 Plant materials

Four weeks old rooted cuttings of *S. dolomitica* and was individually transplanted into 15 cm brown plastic pots filled with sterilized medium containing equal parts 1:1:1 silica sand, coir, and perlite and established for four weeks. Thereafter, sixty-four, 4-week old potted cuttings were divided into four treatments: 16 replicates each and subjected to four water-deficit treatments. Preliminary tests have shown that watering the plants with 250 ml of RO water every three days was sufficient to prevent wilting, and thus, this watering regime was used as treatment one (T1 – no water deficits). In treatment 2 (T2 – mild deficits), sixteen plants (corresponding to 16 replicates) were irrigated with 250 ml of water every six days. In treatment 3 (T3 - moderate), sixteen replicates were watered every nine days, and plants in the fourth treatment (T4 - severe) were watered every 12 days. The experiment was conducted for eleven weeks, and during this time fertilization was withheld, and the plants were irrigated with reverse osmosis water according to the irrigation regimes per treatment.

The potted plants were placed on cement floor inside the research greenhouse where the plants were exposed to natural sunlight that entered through the polycarbonate roof. The experiment followed a complete randomised block design. Temperatures inside was maintained between 24 – 26 °C during the day and 15 – 20 °C during night and 74% Relative humidity (RH).

5.2.2 Preparation of extracts

Dried milled aerial plant material of five replicates of *S. dolomitica* plants for each treatment, plants, was extracted with acetone for 24 hours in 3 separate glass jars per treatment contain 25 g of milled material and 25 ml acetone each. One glass jar of extract would later be reconstituted to 10g/ml, the second jar to 20 mg/ml, and the third jar to 30 mg/ml. After the extraction period, the extracts were filtered and decanted into weighed and labelled 50 ml plastic test tubes and allowed to dry under a fan in the fume hood. The dry weight of the extract was calculated, and then the extract was reconstituted with acetone so that each water deficit treatment had the extract concentrations of 10, 20, and 30 mg/ml. The test tubes were capped and stored in the dark at four °C until used.

5.2.4 Preparation of germination containers

Foil containers 15.5 cm x 8.2 cm x 4 cm were half-filled with equal part 1:1 coir and river sand moistened with 15 ml reverse osmosis water. Each container was covered with a layer of heavy aluminium foil and autoclaved for 15 minutes at 121°C. When the containers had cooled down, they were autoclaved again (Figure 5.1). The still covered containers were store under laminar flow hood until needed.

Figure 5.1: Foil containers were half filled with medium and covered with single layer of thick aluminum foil (A) and then autoclaved at 121°C for 15 minutes twice (B).

5.2.2 Germination bio-assay

The methodology described by Del Barrio *et al*. (2020) was used for the germination bio-assay with slight modifications. Maize seeds used in this study were rinsed three times with autoclaved water and placed on a sheet of absorbent paper under the laminar flow to dry. Forty-five seeds were placed in a small clear plastic bag (18cm x 9.5 cm) for each of the three acetone extracts (10, 20, and 30 mg/ml) of plants material subjected to no, mild, moderate, and severe water deficits. Each bag was labelled according to acetone concentration and water deficit treatment. For each of the tested concentrations, two millilitres of extracts were released inside the bags using a clean 5 ml plastic pipette. The seeds were gently mixed inside the bag for 10 seconds to make sure they are evenly coated. The coated seeds were removed from the plastic bag to dry under laminar flow before being placed in a dry labelled plastic bag and closed. The bags were stores under the laminar flow hood until used. The positive control was prepared by coating three bags of seeds with 10, 20, and 30 mg/ml concentrations of Mancozeb (Stodels Pty Ltd. Garden Centre, Cape Town, South Africa). Three bags coated with RO water only served as the negative controls.

The containers were placed into clear A4 plastic sheets, which were then placed in a tray. The trays were placed on a workbench in the research laboratory with 24 hours of illumination via overhead florescent lights on the ceiling of the laboratory. The room temperature was 25 °C for the duration of the experiment. The seeds subjected to 100% RH inside plastic bags.

The germinated seeds were counted after 14 days to calculate the germination percentage (GP) as the following:

$$\frac{\text{Number of germinated seeds}}{\text{Total number of seeds in container}} \text{ X } 100$$

For the Bio-assay in the presence of *F. oxysporum* contamination, the same methodology as described above was used in addition to a 5 mm plug of agar containing *F. oxysorum* mycelia being placed adjacent to each maize seed. Established cultures of *F. oxysporum* sp.*glycines* strain (UPFC no. 21) was used in the germination bioassay courtesy of the Phytomedicine Programme, University of Pretoria. The fungus was sub-cultured for 3-4 weeks onto stock agar plates, made up of half-strength potatoes dextrose agar.

During the germination bioassay, multiple 5 mm cores was depressed into the agar of each petri dish, and these plugs were transferred into the germination containers under laminar flow using sterilized tweezers as needed (Figure 5.2).

Figure 5.2: Each maize seed had a 5mm plug of agar containing *Fusarium oxysporum* mycelia placed adjacent to it.

5.2.3 Statistical analysis

The germination percentages and shoot lengths were recorded as means ± se. One-way analysis of variance (ANOVA) was used to compare the means amongst the treatments. All analyses were carried using the software programs STATISTICA® (13.5.0.17) and the Paleontological Statistics package for education and data analysis (PAST 3.14). Post hoc analysis based on Tukey test was used to separate the means. P values of <0.05 were regarded as significant, and p values of <0.01 as very significant.

5.3 Results

5.3.1 Effect of acetone extract (10, 20, and 30 mg/ml) of *S. dolomitica* on maize seed germination

There was no significant difference in the percentage of maize seeds that germinated after being coated with the acetone extracts at 10 mg/ml (df = 5, 12; F = 0.7358; P = 0.6107), 20 mg/ml (df = 5, 12; F = 1.603; P = 0.2327) and 30 mg/ml (df = 5, 12; F = 1.383; P = 0.2977) from the different water deficit treatments in the absence of experimental *F. oxysporum* exposure. In other words, coatings with the acetone extracts did not improve germination (Table 5.1).

Table 5.1: The values (Mean ± SE) of percentage of maize seeds, coated with a 10, 20 and 30 mg/ml concentrations of *Salvia dolomitica* plant extracts, which germinated in the absence of *Fusarium oxysporum* in the propagation media.

***Salvia dolomitica* Acetone extract concentration and treatments**	**% of Maze seeds Germinated with 10 mg/ml acetone extracts**	**% of Maze seeds Germinated with 20 mg/ml acetone extracts**	**% of Maze seeds Germinated with 30 mg/ml acetone extracts**
Water deficit treatment 2 (Mild water deficit)	93.33±06.66 [a]	91.00±02.00 [a]	93.33±03.75 [a]
Water deficit treatment 3 (Moderate water deficit)	91.00±05.85 [a]	97.66±02.33 [a]	97.66±02.33 [a]
Water deficit treatment 4 (Severe water deficit)	97.66±02.33 [a]	93.00±00.00 [a]	89.00±02.00 [a]
	% of Maze seeds Germinated with 10 mg/ml Mancozeb	**% of Maze seeds Germinated with 20 mg/ml Mancozeb**	**% of Maze seeds Germinated with 30 mg/ml Mancozeb**
Positive control Mancozeb	93.00±05.77 [a]	95.33±02.33 [a]	84.33±05.92 [a]
Negative control Non-coated	95.33±02.33 [a]	95.33±02.33 [a]	95.33±02.33 [a]

The values tabulated are the means ± SE.
Significant differences among the means are evaluated using the Tukey HSD test.
Values with the same letters in a column are not significantly different at the 0.05 probability level.

5.3.2 Effect of acetone extract (10, 20, and 30 mg/ml) of *S. dolomitica* on maize seed germination following experimental exposure to *F. oxysporum* inocula

For the coated maize seeds placed on sterilized medium in the presence of intentional *F. oxysporum* contamination, there was no significant difference in the percentage of maize seeds that germinated after being coated with the treatments at a concentration level of 10 mg/ml (df = 5, 12; F = 0.6479; P = 0.6686), 20 mg/ml (df = 5, 12; F = 1.224; P = 0.3563) and 30 mg/ml (df = 5, 12; F = 0.7896; P = 0.5769). None of the extracts improved germination (Table 5.2).

Table 5.2: The values (Mean ± SE) in the table below represents the mean percentage of maize seeds, coated with a 10, 20 and 30 mg/ml concentrations of *Salvia dolomitica* plant extracts, that germinated in the presence of *Fusarium oxysporum* in the propagation media.

***Salvia* dolomitica Acetonic extract concentration and treatments**	**% of Maze seeds Germinated with 10 mg/ml acetonic extracts**	**% of Maze seeds Germinated with 20 mg/ml acetonic extracts**	**% of Maze seeds Germinated with 30 mg/ml acetonic extracts**
Water deficit treatment 1 (No water deficit)	91.00±02.00 [a]	93.00±00.00 [a]	95.33±02.33 [a]
Water deficit treatment 2 (Mild water deficit)	91.00±02.00 [a]	86.66±03.75 [a]	91.00±05.85 [a]
Water deficit treatment 3 (Moderate water deficit)	88.66±04.33 [a]	84.66±02.33 [a]	86.66±03.75 [a]
Water deficit treatment 4 (Severe water deficit)	89.00±02.00 [a]	95.66±04.33 [a]	82.33±02.33 [a]
	% of Maze seeds Germinated with 10 mg/ml Mancozeb	**% of Maze seeds Germinated with 20 mg/ml Mancozeb**	**% of Maze seeds Germinated with 30 mg/ml Mancozeb**
Positive control Mancozeb	82.00±05.85 [a]	93.33±03.75 [a]	86.33±06.66 [a]
Negative control Non-coated	82.33±09.59 [a]	82.33±09.59 [a]	82.33±09.59 [a]

The values tabulated are the means (± SE).
Significant differences among the means are evaluated using the Tukey HSD test.
Values with the same letters in a column are not significantly different at the 0.05 probability level.

5.3.3 Length of shoots of germinating seeds treated with extracts

There were no significant differences (df = 5, 12; P>0.05) in the mean shoot lengths of the germinated maize seeds treated with coatings of extracts from plants subjected to various water deficit treatments, 10, 20, and 30 mg/ml extract concentration levels (Figure 5.3). However, the non-treated (control seeds) seeds recorded the longest mean length (136.00±0.00 mm) compared to the treated seeds for the different plant extracts concentrations.

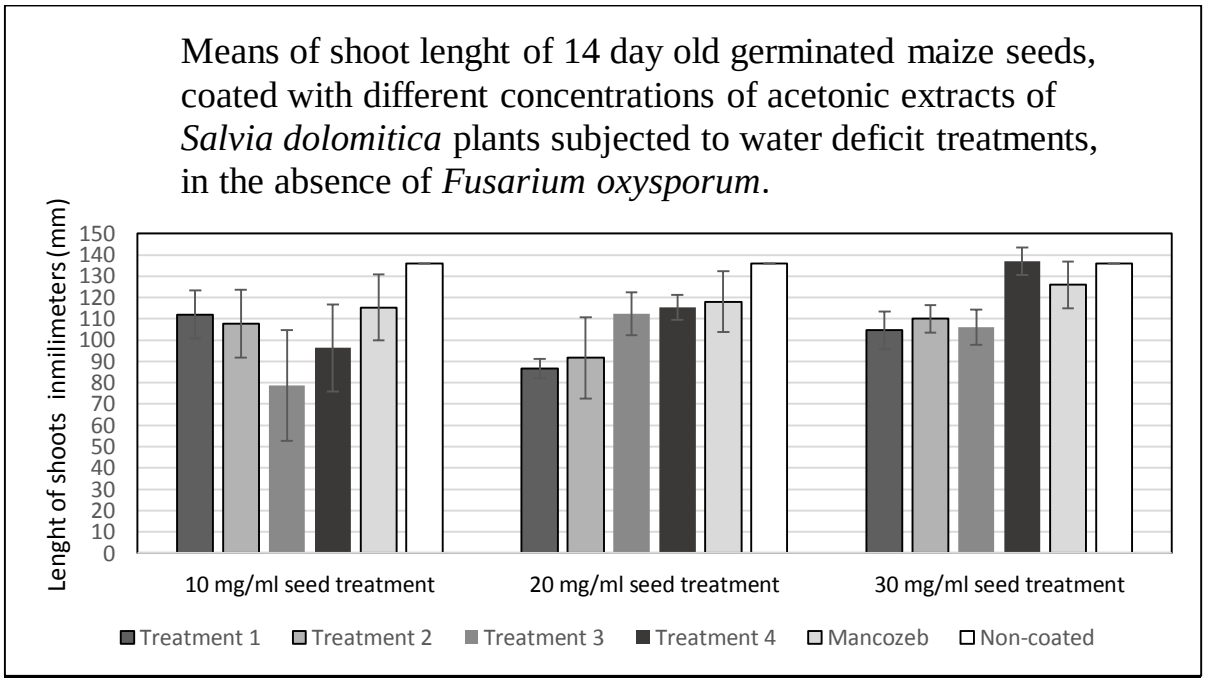

Figure 5.3: Means of shoot length of 14 day old treated, germinated maize seeds.

5.4 Discussion

5.4.1 Germination Percentage

Seed-borne pathogens cause seed abortion, seed necrosis, and reduction or elimination of germination capacity (Abo el-dahab *et al*., 2016). There is a high demand for organic agricultural products and a need for research for environmentally friendly products, such as fungicides, to be used as seed treatments (Masangwa *et al*., 2016). In this study, all the three tested concentrations of the differently treated acetone extracts of *S. dolomitica* in the absence of deliberate contamination with *F. oxysprorum*, showed no significant differences in the percentage of germination of the maize seeds amongst treatments. Some of the extracts did show higher percentages than the synthetic fungicide Mancozeb® and the non-treated seeds,

but it was not significant (P > 0.05). In the presence of deliberate contamination with *F. oxysprorum*, all three tested concentrations of the differently treated extracts reported no significant differences in the maize seeds' percentage of germination. These results corroborate with Rani & Devan and (2011), where maize seeds treated with *C. papaya, C. indica, M. charantia*, and *S. melongena* extracts at all the tested concentrations resulted in normal germination that is comparable with that of the uncoated seeds.

Furthermore, when Tagne *et al*. (2013) investigated the controlling of seed-borne *Fusarium moniliforme* on maize seeds coated with different concentrations of the essential oil from *O. gratissimum*, it was revealed that at the rate of 10%, *F. moniliforme* was eradicated. However, it also inhibited the seeds by increasing the number of dead seeds from 7.5% in the untreated seeds to 70%.

The changes in the number of secondary metabolites, such as alkaloids, in the extracts treated to different water deficits, did not seem to influence the germination percentages of the maize seeds, whether exposed to fungus or not. Further research needs to be conducted to ascertain at what levels, do concentrations of the extracts not show any phytotocicity at all to the germinating seeds.

5.4.2 Shoot length

In the absence of deliberate *F. oxysprorum* contamination the non-coated seeds and those treated with Macozeb had longer shoots lengths than those of the treated seeds at all three concentration levels, although not significant. Findings of the present study were corroborated with the inhibitory effect of leaf extracts of Citrus reticulate Blanco (Mandarin orange) leaf extracts on the shoot length of *Capsicum annum* L. (chilli), *Glycine max* (L.) Merr. (Soybean), *Zea mays* L. (maize), *Oryza sativa* L. (paddy) and *Abelmoschus esculentus* (L.) Moench (lady's finger) (Sahoo *et al.*, 2015). These findings are similar to those of a trial by Lawan *et al*. (2011) in which concentrations of the aqueous extracts of *Azadiracta indica* caused significant inhibitory effects on shoot elongation and that in two of the varieties of cowpea tested, the 40% and 50% extracts resulted in the shoot not forming at all. The results of their bio-assay indicated that the inhibitory effect was proportional to the concentrations of the extract. As concentration increased, the extent of inhibition also increased. The extracts of certain *Salvia* species were found to be alleopathic to maize seeds as indicated in trial conducted by Husna *et al*. (2016), which found that *Salvia plebeia* aqueous root extract at different concentrations

(5g, 10g, and 15g) affect the plumules of maize negatively. Rowshan & Karimi (2013) found that aqueous extracts of *Salvia macrosiphon* Boiss reduced the coleoptile sections elongation of germinating maize seeds.

When Kadio & Yanar (2014) tested the allopathic Effects of 22 plant extracts against seed germination of 9 different plants considered common weeds, they concluded that some of these extracts may be used herbicides to control the germination and growth of some other weeds. The result of this study may indicate that although the extracts of *S. dolomitica* may not be effective as a seed treatment for maize seeds, it could be considered to be a source of allopathic chemicals to be used for green alternatives to herbicides on other weeds and more research is warranted in this regard.

5.5 Conclusion

Broadly, this *in vivo* study showed that exposing *S. dolomitica* to water deficit stress did not improve anti-*F. oxysporum* activity. High seed germination was observed in all treatments. It is also likely that the fungus was not virulent. The effect on germination was minimal even in untreated plants (not exposed to *F. oxysporum*), and this might have obscured the effect of the plant extracts.

Further investigations regarding the shorter shoot length recorded in extract coated seeds are essential to characterize the *S. dolomitica*'s active compounds with allopathic characteristics.

5.6 Acknowledgment

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CHAPTER SIX
GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

6.1 General Discussion, conclusion and recommendations

The Republic of South Africa is water-scarce country. Nevertheless, it can still partake in the cultivation of medicinal plants for the production of green alternatives for synthetic fungicides. The global organic industry is worth billions of US dollars. Applying water deficits during the cultivation of medicinal plants has been shown to increase secondary anti-fungal metabolites in plants. South Africa is a country rich in medicinal plants that can be a source for environmentally friendly products to be used to cultivate food crops such as maize, which is the staple diet of the citizens of many countries. This study provides a simple protocol for cultivating indigenous *Salvia* species. The study further revealed that acetone extracts of *S. dolomtica* and *S. namaensis* showed stronger activity than the synthetic fungicide in MIC bioassay at 24 hours of incubation, and water stress favours secondary metabolite accumulation in extracts of *Salvia* spp.

Conclusion

While these results are in agreement with other studies, as mentioned in the preceding chapters, this is the first study that specifically focused on enhancing secondary metabolite contents in *Salvia* spp. An unexpected yet exciting observation in this study was the harmful effect of the acetone extracts of *S. dolomtica* shoot length of seedlings. This information can be exploited for control of seedlings of some weeds. A further investigation of this aspect is certainly warranted.

Recommendations

- By observations of the characteristics of the differently treated extracts of *S. dolomitica* in terms of its enhanced activity in the MIC bioassays, as well as the higher amounts of phenolic contents it possesses when compared to *S. namaensis*, it is possible to speculate that moderate water stress can be beneficial for secondary metabolite production in *S. dolomitica*.
- The *S. dolomitica* species is therefore a good candidate to produce highly valuable plant metabolites that could be further exploited by pharmaceutical industries to develop new drugs and formulations.

- The extracts were associated with lower shoot lengths of germinated seeds treated with the extracts and allopathically active extracts of *S. dolomitica* to be used to produce these green products thus leading to job creation and South Africa joining the lucrative organic market of the global economy.

APPENDIX A

POSTER PRESENTED AT THE POSTGRADUATE CONFERENCE 2019, HOSTED BY THE CENTRE FOR POSTGRADUATE STUDIES.

The poster was awarded 3rd place at the post graduate conference.

Cape Peninsula University of Technology

National Research Foundation

THE EFFECT OF WATER DEFICITS ON THE ANTI-FUNGAL ACTIVITY OF THE LEAF EXTRACTS OF INDIGENOUS *SALVIA* SPP. AGAINST *FUSARIUM OXYSPORUM* SCHITDL. (HYPOCREALES)

Shaheed Roos and Felix Nchu
Cape Peninsular University of Technology

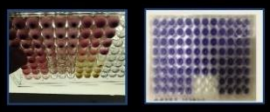
INTRODUCTION

There is a global demand for natural and environmentally safe agricultural products[1]. South Africa contains 6% of the world's flora and our indigenous medicinal plants may be a source of ingredients for these natural products. South Africa is also a water scarce country ravaged by droughts. New cultivation strategies can be used to optimise the yield of medicinal secondary metabolites in plants. The purpose of this study was to determine the effect of water deficits on the anti-fungal properties of indigenous Salvia species whose extracts may then be used to combat seedling blight of maize, a disease which lead to economic loss for farmers.

MATERIALS AND METHODS

- Six species of indigenous Sage were screened for their anti-fungal activities against *Fusarium oxysporum*, a fungus that causes seedling blight in maize.
- Ten week old cuttings of the top two performing species were subjected to different water deficit treatments.
- The leaf extracts of these differently treated plants was subjected to MIC and total phenolic assays in order to determine what effects the water deficits had on the secondary metabolites and anti-fungal activity.

The Following Water Deficit Treatments were used :
Controls was irrigated with 250 ml water every 3 days,
T1=every 6 days, T2=every 9 days and T3=every 12 Days



Plant extracts subjected to MIC and Total phenolic assays to test for anti-fungal activity and phenolic content.

RESULTS AND DISCUSSION

The plant extracts of Treatment 2 of *S. dolomitica* showed the highest mean value for Polyphenol ,Flavonol and Alkaloid content. The extracts of treatment 1 of *S. namaensis* showed the highest mean value for Alkaloids. The MIC bioassay showed that treatment 1 and 2 of the *Salvia dolomitica* extracts was significantly more active (P>0.01) than the synthetic fungicide Mancozeb.
The results are in accordance with the findings by Al-Gabbiesh *et al.*(2015) that deliberate water stress can enhance the concentration and quality of natural products such as isoprenoids, phenols or alkaloids[2].

There are 26 indigenous species of Sage in Southern Africa

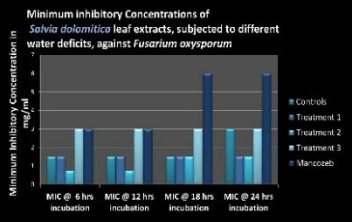


Table 1.1: Effects of different water deficit treatments on the total phenolic values of *Salvia dolomitica* leaf extracts.

	Polyphenols in mg GAE/g	Flavonols in mg QE/g	Alkaloids in mg AE/g
Salvia dolomitica			
Controls	66.43±5.23	8.84±0.90	5.91±0.33
Treatment 1	64.51±2.96	8.53±0.48	4.57±0.26
Treatment 2	73.67±4.91	10.36±0.76	6.67±0.54
Treatment 3	64.39±3.40	8.09±0.11	4.75±0.53

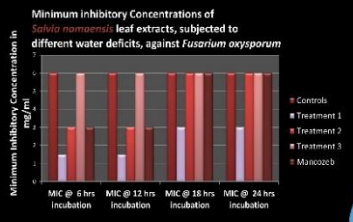


Table 1.2: Effects of different water deficit treatments on the total phenolic values of *Salvia namaensis* leaf extracts.

	Polyphenols in mg GAE/g	Flavonols in mg QE/g	Alkaloids in mg AE/g
Salvia namaensis			
Controls	61.22±1.79	8.24±0.30	2.10±0.20
Treatment 1	55.33±2.31	7.45±0.44	2.89±0.30
Treatment 2	52.88±3.53	6.13±0.34	2.75±0.68
Treatment 3	51.57±3.97	6.33±0.60	4.05±0.96

Plant phenolic compounds may contain anti-oxidant, anti-microbial & anti-cancer properties

CONCLUSION

The results of the MIC and Total Phenolic assays concluded that some levels of water deficits do favourably affect some of the secondary metabolites present in the plants which may be responsible for the anti-fungal activity against the pathogenic fungus, *Fusarium oxysporum* .

Statistical Analysis

The one-way analysis of variance (ANOVA) was used to compare means among treatments and Post hoc analysis based on the Tukey test was used to separate the means.

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