VARIATIONS IN GROWTH, YIELD AND METABOLITES OF AFRICAN GINGER (*SIPHONOCHILUS AETHIOPICUS*) IN RESPONSE TO IRRIGATION REGIMES AND NITROGEN LEVELS

by

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DECLARATION

I declare that the research reported in this thesis is based on my original work and it has not been submitted to this or any other institution of higher education. This research is being submitted for the degree Philosophy Doctor in Agriculture at the University of KwaZulu-Natal. I further declare that all sources cited or quoted are indicated and acknowledged by a list of references.

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LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of variance
ARC-VOP	Agricultural Research Council Vegetable and Ornamental Plant
ADL	Allowable depletion level
$AlCl_3$	Aluminium Chloride
BD	Bulk density
CV	Coefficient of variation
CO_2	Carbon Dioxide
DAP	Days after planting
DMRT	Duncan multiple range test
DPPH	2, 2'-diphenyl-1-picryl hydrazyl
ERD	Effective Rooting Depth
ET_0	Evapotranspiration
FC	Field capacity
FRAP	Ferric reducing antioxidant power
$FeCl_3$	Iron Chloride
GAE	Gallic Acid Equivalent
GC-MS	Gas chromatography mass spectrometry
HPLC-RID	High performance liquid chromatography-refractive index detector
KPa	Kilopascal
LAN	Limestone Ammonium Nitrate
LAI	Leaf Area Index
LA	Leaf area
LSD	Least significant difference
MASL	Meter above sea level
$NaNO_2$	Sodium nitrite
NM	Nanometre
NPK	Nitrogen, Phosphorus and Potassium
PWP	Permanent wilting point
PDP	Postgraduate Development Programme
PAW	Plant Available Water
RH	Relative humidity
RHn	Minimum relative humidity
RHx	Maximum relative humidity

SC	Stomatal Conductance
RSA	Radical scavenging activity
SANBI	South Africa National Biodiversity Institute
TLC	Thin Layer Chromatography
Tn	Minimum temperature
Tx	Maximum temperature
NRF	National Research Foundation
UV	Ultraviolet detector
WHO	World Health Organization
MAD	Maximum allowable depletion
QE	Quercetin
UV-Vis	Ultra violet visible spectrophotometry
USA	United State of America

VARIATIONS IN GROWTH, YIELD AND METABOLITES OF AFRICAN GINGER (*SIPHONOCHILUS AETHIOPICUS*) IN RESPONSE TO IRRIGATION REGIMES AND NITROGEN LEVELS

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ABSTRACT

Medicinal plants are valuable natural resources used as traditional medicine and have economic significance. African ginger (*S. aethiopicus)* (Schweinf.) B.L. Burtt is one of the most important rhizomatous plants, highly-valued for its medicinal properties and wide distribution in many regions of southern Africa. The plant is currently listed on the Red List of South African endangered species due to overharvesting. The increased demand for plant material has led to extinction in other areas of South Africa. The loss of wild populations harvested will destroy the natural habitats and genetic diversity in the long term. The demand for *S. aethiopicus* plant parts, particularly the rhizome is associated with the medicinal remedies possessed by the plant. The rhizomes have been traditionally used for the treatment of coughs, colds, asthma, headaches, pain, inflammation and malaria. Currently, there is limited scientific evidence on the cultivation and response of secondary metabolites of *S. aethiopicus* to agronomic practices. Cultivation of medicinal plants is a good approach to conserve species biodiversity and meet current demands for plant based products. This study investigated the variations in growth, yield and metabolites of *S. aethiopicus* in response to cultivation practices for commercial production and further development of medicinal products. In this study, total phenolic content, flavonoid content and antioxidant activity of *S. aethiopicus* leaf, rhizome and root from varying areas (Mpumalanga, KwaZulu-Natal, Limpopo and North West) were

evaluated. Total phenolic and flavonoid contents were investigated by Folin-Ciocalteu and aluminium chloride ($AlCl_3$) colorimetric methods, respectively. Antioxidant activity in different parts of *S. aethiopicus* was evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and ferric reducing power (FRAP). Furthermore, the study determined the variations in soluble sugars in the leaf, rhizome and root as influenced by varying growing areas. The results showed high concentration of sucrose, glucose and fructose in the leaf and root as influenced by different growing areas. A higher content of both total phenolics and flavonoids were found in the root from Mpumalanga (54.5±2.0 mg GAE/g and 14.83±0.06 µg QE/g, respectively) compared to the leaf and rhizome from other growing areas. KwaZulu-Natal also exhibited high flavonoids in the leaf (12.72±1.18 µg QE/g), rhizome (14.21±1.98 µg QE/g) and root (12.88±0.57 µg QE/g) compared to other growing areas. In both methods, the leaf exhibited higher antioxidant activity than the root and rhizome. The high antioxidant activities exhibited in the leaf from Mpumalanga suggest its adaptive capabilities to different environments. *S. aethiopicus* parts could be used as a potential source for antioxidant properties and encourage cultivation under different growing areas to conserve its biodiversity and increase species populations.

The effect of nitrogen levels and irrigation regimes on biomass yield, stomatal conductance, chlorophyll content and leaf area index was investigated under the rainshelter for two growing seasons. The results of this study conclusively reveal that the plant height and number of leaves per plant were significantly higher towards maturity. Plants grown with 50 and 100 kg N/ha had greater plant height, number of leaves per plant, LAI, SPAD values and biomass yield that eventually resulted in higher dry matter production. Stomatal conductance was higher throughout the growing period and decline in response water stressed treatment. The high amount of water utilized from well watered treatment (30% ADL) compared to moderate (50% ADL) and severe (70% ADL) treatments could be attributed to improved water availability and superior plant canopies. Further experiments should be conducted to evaluate different

combinations of agronomic practices to fully exploit the growth of *S. aethiopicus* under different conditions.

The high amount of water utilized from the well watered treatment (30% ADL) compared to moderate (50% ADL) and severe (70% ADL) treatments could be attributed to improved water availability and superior plant canopies. The well watered treatment (30% ADL) had a significantly higher total biomass, fresh and dry rhizome yield compared to other water stressed treatments. The response of water stress and nitrogen levels showed significant accumulation of plant flavonoids and phenolics in leaf, rhizome and root. In plant carbohydrates, root had high sucrose content with the application of low N under severely stressed (70% ADL) treatment.

The investigation of volatile components of leaf, rhizome and root in response to irrigation regimes and nitrogen levels were analysed by GC-MS. The results showed that the highest volatile components in the root and rhizome were terpenes, as compared to the increased components of aliphatic acids, benzenoids and aliphatic aldehydes in the leaf. In all treatments and parts, the odorant sesquiterpene (1E)-5-Methyl-1-(2, 6, 6-trimethyl-2, 4-cyclohexadien-1-yl)-1, 4-hexadien-3-one was the most abundant volatile compound. The 4-Hydroxy-4-methyl-2-pentanone was detected under severely stressed (70% ADL) treatment with the application of 100 kg N/ha. Severely stressed (70% ADL) treatment with minimal application of N induced the terpenes components in all plant parts. The study showed that volatile components of *S. aethiopicus* vary with plant sources, water stress and mineral nutrient deficiency. Knowledge on the impact of *S. aethiopicus* parts will provide a useful guide for selection towards identifying profiles of volatile compounds and explore the additional bioactive compounds for therapeutic use. Taken together, this study represents the importance of cultivation methods as an alternative approach to wild harvesting, conserving *S. aethiopicus* for commercial production and exposure to water stress conditions for high secondary metabolites.

Keywords: Nitrogen, water stress, medicinal plant, cultivation, part

CHAPTER ONE

GENERAL INTRODUCTION

The recent global increases in food and medicine prices have added pressure on the agricultural production and health care system. As a result, there is a growing interest in derivatives of plant origin for food, fuel and medicine. Plants containing inherent active ingredients used to cure or relieve pain are regarded as medicinal plants (Lucy and Edgar, 1999). These are valuable natural resources used as traditional medicine based on the indigenous knowledge, beliefs, and theoretical practices of different cultures. Approximately 80% of the world's inhabitants depend on traditional medicine for primary health care due to the extraordinary healing benefits, popularity as safe, cost effectiveness and easy accessibility (Owolabi *et al.* 2007).

There is a growing interest in traditional medicine due to their relevance as an alternative health care system in developed and developing countries. Modern therapeutic medicine based on indigenous therapies and ethno-pharmacological uses has become an important tool to identify new sources of pharmaceuticals (Cragg and Newman, 2013). The identified medicinal properties of plants contain chemical compounds exhibiting properties which are responsible for curative action of plants (Lambert *et al.* 1997). However, the information on the identification of bioactive compounds through isolation, purification and characterization of active ingredients in crude extracts using various analytical methods is still limited in many parts of Africa. Research and development for identification of many plants for medicinal benefits still have to be explored.

Due to the growing demand of plant-based drugs and advancement of research in medicine, heavy pressure is created on some medicinal species which results in over-exploitation. The global market for medicinal plants and pharmaceuticals, spices/herbs and cosmetics indicates the rapid growing demand expanding by 20% annually and projected to increase to USD 8

billion (ZAR 112 billion), USD 3 billion (ZAR 42 billion) and USD 1.5 billion (ZAR 2 billion), respectively (Mafimisebi *et al*. 2013). The high demand is due to the extensive use of traditional medicine. The unprecedented plea for herbal products, cosmetics, pharmaceuticals and food supplements posing a threat to biodiversity and genetic conservation of medicinal populations (Street and Prinsloo, 2012). Most medicinal plants are prone to extinction due to slow growing rates; reduced population densities and narrowed geographic ranges (Nautiyal *et al*. 2002). To maintain the endangered medicinal populations, it is important to cultivate many plant species at large scale. This may contribute to growing the economy and financial viability of medicinal plant species (Street and Prinsloo, 2012).

A number of medicinal plants still need to be studied with an intention to discover valuable phytochemicals. This is because their over-harvesting from the wild has led to their extinction and scarcity (Anon, 1998). Most traditional practitioners have the spiritual believes that medicinal plants harvested from the wild contain healing medicinal value than cultivated ones (Ngarivhume *et al*. 2015). The same finding was also reported in Kenya (Bussmann, 2006), Uganda (Okello and Ssegawa, 2007), Cameroon (Simbo, 2010) and Sudan (Musa *et al*. 2011). Evaluation and cultivation of these plants is necessary, both to substantiate the use of these plants by inhabitants, conserve the species biodiversity and also for possible lead in drug discovery from medicinal plant. This study has investigated the antioxidant properties of African ginger (*S. aethiopicus)* from different agro-ecological regions and the response of different parts to irrigation regimes and nitrogen levels under experimental trial. The study will provide valuable information about the cultivation practices as an effort to eliminate limitations of over-exploitation and drawbacks of herbal medicine.

1.1 Aims

The aim of the study was to investigate antioxidant properties of African ginger (*S. aethiopicus)* in response to cultivation practices for commercial production for further development of medicinal products.

1.2 Objectives of the study

The specific objectives were to:

- Evaluate the changes in phenolic composition, antioxidant activity and soluble sugars of *S. aethiopicus* parts grown in different regions of South Africa.

- Investigate the physio-morphological responses and plant growth of *S. aethiopicus* to irrigation regimes and fertilizer application.

- Determine the antioxidant activity and soluble sugars of *S. aethiopicus* in response to irrigation regimes and nitrogen levels.

- Investigate volatile profiling in different tissues of *S. aethiopicus* in response to irrigation regimes and nitrogen levels.

1.3 Rationale and justification for the study

In this study agronomic practices of African ginger (*S. aethiopicus)*, including the response of irrigation regimes and nitrogen levels on the morphological and physiological parameters; and assessment of biochemical analysis and characterisation of bioactive compounds using HPLC-RID and GC-MS analyses were investigated. The significance and focus of various aspects of this study included the medicinal potential of *S. aethiopicus*, which depends entirely on the successful cultivation using agronomic practices. The thesis presents an overview of the variations in phenolic composition, antioxidant activity and soluble sugars of *S. aethiopicus*

parts grown from different growing regions of South Africa. The section describes the variation in phytochemical profiling and biochemical analysis of *S. aethiopicus* parts from varying growing regions. This section also attempts to identify the agro-ecological location suitable for cultivation of *S. aethiopicus*. The information acquired in this study is important and will contribute to knowledge as few studies have reported on the phytochemical and biochemical screening of medicinal plants from varying locations in Africa.

The second section describes the influence of irrigation regimes and nitrogen application rates on the morphological (i.e., plant height, leaf area index, number of leaves per plant) and physiological (i.e., crop growth rate and stomatal conductance) parameters of *S. aethiopicus* grown under rain shelter. This section focused on the cultivation practices of *S. aethiopicus*, using different irrigation regimes and nitrogen application rates. The plant species was selected based on its medicinal value according to traditional practitioners from varying locations in South Africa, who harvested the rhizome from the wild and utilized it for medicinal purposes (van Wyk *et al.* 1997). Furthermore, *S. aethiopicus* is also listed in the African Herbal Pharmacopea, among the 51 most important medicinal plants in sub-Saharan Africa (Brendler *et al.* 2010). Increasingly, *S. aethiopicus* have received scientific and commercial attention which also increased the demand and pressure on the wild populations. Over-exploitation and indiscriminate collection has placed *S. aethiopicus* at risk of total extinction, hence the plant was listed on the Red List of South African endangered plants species. Populations remaining in the wild are critically low in many widespread areas of South Africa (The Red list of South African plants version 2015.1). Monitoring of subpopulations has indicated that unsustainable harvesting resulted in 84% decline in Mpumalanga Province in a period of only four years (The Red list of South African plants version 2015.1). The lack of programmes, legislation and policy for conservation and protection of *S. aethiopicus* will result in complete extinction of the species in the wild. It is important to incorporate wild species into the cultivation systems to reduce the loss of medicinal species biodiversity and over-exploitation (Lambert *et al.* 1997).

The approach of cultivating wild species is also advocated as a means to meet current and future demands and to reduce pressure of harvesting wild populations. Furthermore, cultivation of medicinal plants can reduce degradation and loss of genetic diversity (Cunningham, 1991). Analysed results clearly indicated that different N application rates and irrigation regimes has a significant effect on the growth and composition of *S. aethiopicus* plant. Application of nitrogen fertilizers and varying irrigation regimes increased biomass production and yield; however increased rates could compromise the accumulation of secondary metabolites and other elements which significantly contribute to medicinal properties.

Scientific investigation and validation of medicinal plants as a source of medicine is imperative for their safety, efficacy, quality and the appropriate dose of the plant material. This section explains the effect of irrigation regimes and nitrogen application levels on the antioxidant activities and soluble sugars of *S. aethiopicus*. The study attempts to investigate the antioxidants and soluble sugars produced in the leaf, rhizome and root of the plant. This information is important as few studies have investigated phytochemical profiling and soluble sugars secreted by different parts of medicinal plants from Africa and particularly South Africa. The results will contribute positively to the current information on the antioxidant capabilities and identified sugars to enhance utilization of the plant species.

The characterization of volatile compounds in most medicinal plants has resulted to the possible synthesis of potent drugs. This section investigated the bioactive agent from different plant parts (leaf, rhizome and root) to ascertain the volatile compounds. This research is an effective method to understand the ongoing research on phytochemistry of numerous medicinal plants, and demonstrate noteworthy of compounds. The information may also provide insight for potential future compound synthesis directives. Additionally, the data can also provide reliable botanical identification as a biotechnology tool to develop plant materials with agronomically and commercially desirable traits.

While scientific investigation has discovered phytochemistry of several medicinal plants, it is still vital to study in-detail the scientific evaluation of plants to identify suitable production practices, demonstrate the significant compounds and medicinal potential of *S. aethiopicus*. Cultivation of medicinal plants can assist to meet the current demand and contribute to the conservation of biodiversity of wild populations and endangered species.

1.4 Structure of the thesis

This thesis is divided into seven chapters as follows;

- Chapter One: This chapter covers the general introduction and background on the importance of medicinal plants. Focus is given to the medicinal plant industry and the importance of cultivation practices of medicinal plants. The rationale for the study, aim and objectives are also included in this chapter.
- Chapter Two: A review of literature regarding the origin, taxonomy, distribution, biodiversity conservation applications as well as cultivation practices importance of *S. aethiopicus* is discussed.
- Chapter Three: This chapter deals with the phenolic composition, antioxidant activity and soluble sugars of *S. aethiopicus* parts grown from different agro-ecological regions of South Africa.
- Chapter Four: This chapter describes influence of irrigation regimes and varying levels of nitrogen on the morphological (i.e., plant height, leaf area index and number leaves per plant) and physiological (i.e., crop growth rate and stomatal conductance) parameters.
- Chapter Five: This section illustrates the antioxidant activity and soluble sugars of *S. aethiopicus* parts in response to irrigation regimes and nitrogen levels.

- Chapter Six: This chapter deals with volatile profiling using GC-MS in different parts of *S. aethiopicus* in response to irrigation regimes and nitrogen levels.

- Chapter Seven: General discussion, conclusion and recommendations on the interconnectivity of all the results and findings are interpreted and discussed; suggestions for further research are provided.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Medicines derived from plants are widely prominent due to their low costs, easy availability and perceived safety (Wachtel-Galor and Benzie, 2011). Medicinal value lies in bioactive phytochemical constituents from different plant species and most are derived from the leaves, roots, rhizomes, seeds and flowers of plants (Kumar *et al*. 2010). Medicinal plants are considered a rich source of ingredients used in the drug development. They also play a crucial role in the development of human cultures, important source of nutrition and recommended for their therapeutic values (Ekor, 2014).

The Zingiberaceae is an important family, well-known for its medicinal properties, distributed widely throughout the world due to its important natural resources, which provide many useful products for food, spices and medicines. The family consist of 53 genera and 1200 species which makes it the largest family of the plant kingdom (Kress, 1990). Zingiberaceae are pantropical perennial terrestrial, rarely epiphytic, aromatic rhizomatous herbs with simple deciduous leaves. African ginger (*S. aethiopicus)* (Schweinf.) B.L. Burtt is a perennial rhizomatous herb and is classified as a member of the Zingiberaceae family due to its morphological characteristics (Makhuvha *et al*. 1997). Although, the plant is indigenous to South Africa (Makhuvha *et al*. 1997), it is regarded as an important medicinal plant in many regions of southern Africa. The plant is widely distributed in other regions of Africa (Figure 2.1), occurring from Senegal, Nigeria and Ethiopia to Zimbabwe, Malawi and Zambia (Makhuvha *et al*. 1997). The plant is reported to be extinct in many regions of southern Africa (Figure 2.1), specifically in South Africa (Jackson, 1990). The distribution patterns of *S. aethiopicus* in South Africa are restricted in areas of Mpumalanga, KwaZulu-Natal and Limpopo Province (van Wyk *et al.* 1997). As more plants are harvested and over-exploited for

financial gain, the need for cultivation to increase production levels of *S. aethiopicus* is imperative to conserve the biodiversity of its wild populations. Cultivation of medicinal plants through agronomic practices with the inclusion of fertilizers rates and irrigation regimes is advocated as a means of meeting current and future demands for large quantities of herbal drugs and relieve the pressure of harvesting wild populations.

Figure 2.1: Geographical distribution of African ginger (*S. aethiopicus*) in regions of South Africa and across southern Africa regions. Source: SANBI distribution data (2013).

2.2 Phylogeny and classification of Zingiberaceae species

Some members of Zingiberaceae have been utilized and cultivated for their attractive flowers, as spices, food, medicines, perfumes, dyes and aesthetics to man. Zingiberaceae is a family of flowering plants with many species of aromatic perennial herbs with tuberous rhizomes (Kress, 1990). Classifications of the family is mostly based on morphological traits such as height and

size. Although the family is still poorly known taxonomically with many species, current distribution of genus Renealmia are found in the Neotropics, while three genus Aframomum, Aulotandra and Siphonochilus are widely distributed and recognized in Africa (Kress *et al.* 2002). The distribution of Zingiberaceae from different zones across the world is distinguished by their various characteristics which make them adaptable to several climatic, geographic and topographic conditions.

The phylogenetic studies of Zingiberaceae have proceeded slowly from many genera and remain to be fully explored. Four sub-families namely Siphonochiloideae, Tamijioideae, Alpinioideae and Zingiberoideae and four tribes Siphonochileae, Tamijieae, Alpinieae and Zingibereae have been reported and recognized under the Zingiberaceae (Kress *et al.* 2002). African ginger (*S. aethiopicus)* (Schweinf.) B.L. Burtt forms part of Siphonochilus genera widely recognized and distributed in Africa (Kress *et al.* 2002). Taxonomy and classification of the Zingiberaceae will determine detailed understanding of species relationships within the family. Also, future studies to investigate and increase taxonomic sampling within the Siphonochilus subgenera will characterize patterns in the evolution within the subgenera.

2.3 *Siphonochilus aethiopicus* origins, description and current distribution levels

The genus *Siphonochilus* is the major group of angiosperms in the Zingiberaceae family (Makhuvha *et al.* 1997). The generic name *Siphonochilus* is derived from the Greek *siphono* meaning tube, and *chilus* referring to the edge shape of the flower. The specific name *aethiopicus* means the plant originated from southern Africa (Hutchings, 1996). Current distribution of African ginger in South Africa is restricted to Mpumalanga, Limpopo and KwaZulu-Natal (Figure 2.1). The plant is also considered important in other regions of Africa, including Zimbabwe, Swaziland, Malawi, and Zambia (van Wyk *et al.* 1997).

S. aethiopicus produces deciduous and sprout leaves that develop in spring from the underground rhizome. The leaves are lanced shaped and may grow to a height of up to 40 mm (Figure 2.2 B). The plant also produces purplish and yellowish attractive flowers, which are borne at ground level; they emerge before the leaves in spring and are very short-lived (Figure 2.2 A). *S. aethiopicus* has a coned-shaped rhizome which is borne to the ground (Figure 2.2 C) and the roots have been determined to reach up to 400 mm (Figure 2.2 D).

Some members of Zingiberaceae such as *Hedychium* and *Alpinia* species have become naturalized in certain areas (Smith, 1998). The increasing demand of herbal medicine worldwide has led to indiscriminate harvesting and as a result many plant species are becoming extinct and endangered (Diederichs *et al.* 2002). *S. aethiopicus* is also enlisted as a critically endangered species in South Africa and in other parts of Swaziland (The Red list of South African plants version 2015.1). As a result of the species extinction, the Venda tribe in Limpopo province are starting to travel to Zimbabwe to harvest the plant from the wild (Masevhe, 2004). The numbers remaining in the wild are critically low with 60% of the subpopulations consisting of fewer than 100 mature individuals (The Red list of South African plants version 2015.1). The medicinal value of the plant might be affected due to the geographical differences in different production locations throughout southern Africa (Ngarivhume *et al.* 2015). The primary ecological factors affecting the active ingredient contents could include temperatures, precipitation, radiation, soil pH and water levels.

Conservation of endangered medicinal plants using techniques such as plant part harvest substitution, agronomic practices and biotechnological interventions such as micro propagation, is imperative and will aid in future conservation of the species.

2.4 Utilization and contribution of *Siphonochilus aethiopicus* in the economy

Many members of the Zingiberaceae family are widely used as spices, flavouring agents, and medicines due to their unique flavour and medicinal properties (Crouch *et al.* 2000). As a species of Zingiberaceae, the ethno-botanical use of *S. aethiopicus* against a wide variety of ailments prompted our investigation of the rhizome of this plant with a view to isolating the compounds responsible for its medicinal value. In the *S. aethiopicus* producing areas of Africa, the rhizomatous part of the plant is mostly used. The rhizomes possess great medicinal benefits due to the presence of a medicinally significant compound siphonochilone (Gericke and van Wyk, 1997).

The herb has been traditionally used for the treatment of coughs, colds, asthma, headaches, pain, inflammation and malaria (van Wyk, 2008). The rhizome extracts contain anti-bacterial (Light *et al*. 2002), anti-inflammatory (Gericke, 2001), antimalarial (Lategan *et al*. 2009), and anti-candida properties (Verotta and Rogers, 1997). According to Fouché *et al.* (2008), a novel herbal extract from *S. aethiopicus* developed is BP4 which has potential for the treatment of asthma and allergies.

S. aethiopicus rhizomes and roots serve as a good source of spice, treat diarrhoea and stomach infections in East Africa (Burkill, 2000). In Nigeria the rhizome of *S. aethiopicus* serve as spice and flavour to enhance yam (Igoli *et al.* 2012). Traditionally, the mixture of rhizomes and roots has been reported to be used to treat hysteria and relieve dysmenorrhoea (Igoli *et al.* 2012). According to Lock (1999), the dried root of *Siphonochilus decorus* (Druten) can also be burned as incense and insect repellent. In Benin, the aqueous decoction of the roots and rhizomes of *S. aethiopicus* serve as the traditional medicine for treating female infertility and endometriosis (Noudogbessi *et al.* 2012).

S. aethiopicus production and processing is a valuable and lucrative business in South Africa. The plant is sold either as pieces or as dry powder and earns a decent income for traditional practitioners and shop owners. *S. aethiopicus* has been reported to have commercial production and financial viability potential (Street and Prinsloo, 2012).

It is also regarded as the second most frequently traded medicinal plant at muthi shops and by street vendors in different regions of South Africa. According to Mander (1998), it is estimated that between R140/kg and R450/kg (USD 10.08 and 32.40, respectively) could be obtained when selling *S. aethiopicus*. As a result of increased extinction and scarcity of plant species, in the Limpopo Province up to ZAR 800.00/kg (USD 59.59) could be obtained from the muthi shops and street traders (Moeng and Potgieter, 2011).

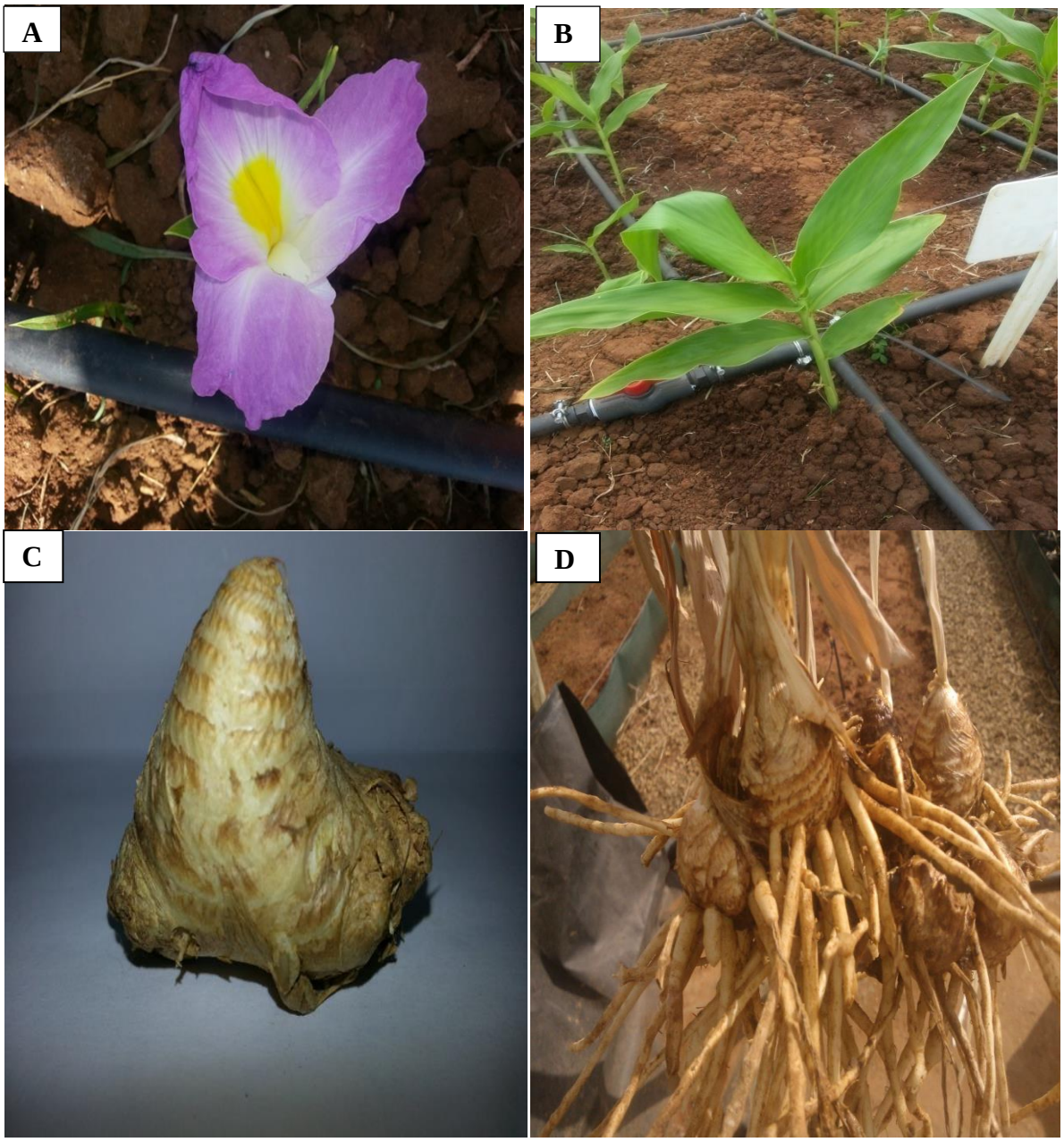

Figure 2.2: African ginger (*Siphonochilus aethiopicus*) flowers (A), deciduous leaves (B), cone-shaped rhizomes (C) and roots (D). (*Photo by Mokgehle S.N., 2016*).

2.5 Biodiversity conservation applications of *Siphonochilus aethiopicus*

Current trend of population growth, deforestation, overharvesting and bush fires have resulted in biodiversity loss for many medicinal plant species. Awareness and attempts on the importance of medicinal plant biodiversity has been considerably raised in the last decade both in developed and developing countries in Africa (Kokwara, 1993). However, the current biodiversity loss is as a result of no action. *S. aethiopicus* received the most attention in terms

of commercialization due to the rapid demand and short supply (Cunningham, 1993). In the search for cultivation and commercialization of the plant species, it is crucial that conservation efforts be adopted to preserve the natural habitats of endangered species. Fay (1992), suggest that effective conservation methods such as vitro techniques (plant propagation, seed germination, micro propagation and tissue culture) can play an essential role in the conservation of vulnerable plants.

A clear understanding of the plant propagation methods suitable for optimum response of medicinal plants will generate knowledge that will form the basis for genetic transformation research in plant improvement programmes. Suitable cultivation methods for conservation purposes (e.g. propagation, nutrient and irrigation managements) of *S. aethiopicus* are highly important.

2.5.1 Propagation and multiplication of medicinal plants

Several factors such as extinction, indemnity, restricted distribution and biodiversity loss have made it necessary to take steps to conserve medicinal plant species. Vegetative or asexual propagation is one of the methods of reproducing progeny of plants identical in genotype to a single source plant (Bedir *et al*. 2003). The method is recognized as a conservation strategy for plant species which are economically important and difficult to grow through seed multiplication process. Vegetative propagation is the most preferred and effective method of propagating *S. aethiopicus* due to the fact that the rhizome develops and mature underground. Cultivation and propagation of *S. aethiopicus* from rhizomes is happening in the warm areas of South Africa under small-scale (van Wyk, 2008). Efforts have been developed for propagation by using rhizome cuttings to assist in increasing cultivation of valuable medicinal plants. The results on vegetative propagation of *Paris polyphylla* through rhizome segments suggest increased opportunities for large scale production and reduced biodiversity loss (Danu *et al.* 2015).

To satisfy growing market demands for *S. aethiopicus* and other plant species, studies suggest that finding an alternative propagation protocol to produce large number of planting material for better yield and quality is essential. Micro propagation using tissue culture technique offers a rapid means of producing large numbers of clonal plants which can be used for restoration and conservation of endangered species (Chan and Thong, 2004). The method is considered a good approach because it ensures mass production and healthy seedlings with desirable characters. The method is not without hindrances. It is a complex technique involving multiple stages, precise formulations of nutrient media, careful surface sterilization and specific environmental growing conditions (Zheng *et al.* 2008). The type and age of plant material, time of year collected and treatment after collection are critical. Morphological characteristics can be visualized by looking at the plant. However, other plant characteristics such as shape, size, fruits and flower colour can only be assessed by transplanting and growing the plants under field conditions until flowering stage. The success of micro propagation for true ginger (*Z. officinale* Roscoe) from vegetative buds has been reported (Sharma and Singh, 1997). Plant regeneration of *Hedychium coronarium* through callus mediated somatic embryogenesis from leaves (Huang and Tsai, 2002); rhizome (Verma and Bansal, 2012) and axenic shoot tip (Bisht *et al.* 2012) have been reported.

Field assessment of tissue cultured plants indicated that two crop seasons are required in order to develop rhizomes that can be used for commercial cultivation. Although there are reports on micro propagation of true ginger (*Z. Officinale*) that has been established and utilized mostly for commercial production of high-quality plant-based medicines, to date the method is not widely used and incorporated on large scale for production. Micro propagation of *Hedychium coronarium* using rhizome buds, showed shoot proliferation with simultaneous rooting of the shoots (Huang and Tsai, 2002). This rapid and cost-effective micro propagation protocol

of *Hedychium coronarium* can reduce degradation of biodiversity species and be utilized in pharmaceutical industries (Mohanty *et al.* 2013).

2.5.2 Cultivation of medicinal plants

The increased demand for utilization and harvesting of medicinal plants from the wild is reducing the number of natural populations and leading to local extinction of many species and degradation of their habitats. Additionally, inadequate cultivation fields, the increased growth of medicinal trade, and lack of regulation of harvesting and overharvesting have led to reduced biodiversity of natural resources (Lambert *et al.* 1997). Medicinal plant production through cultivation can be an effective strategy for preservation of the plant populations and elevating the level of knowledge for sustainable utilization of these plants as traditional medicine. To date there is limited information on the cultivation and guidelines of medicinal plants in response to water levels and fertilizer requirements of most species.

Cultivation recommendations in most plant species are based on the plants with similar growth habits (Boyle and Craker, 1991). Cultivation of medicinal plant species under experimental conditions is one of the approaches that can clearly determine the fertilizer requirements (i.e. type of nutrient, rate of application and formulation) and soil water levels. Nutrients and moisture uptake from the soil influence growth development and phyto-nutritional status of the plant (Lichtenthaler, 1996). It is essential that recommended nutrients rates are applied to the soil to avoid yield reduction and impact on the biochemical and genetic functioning of the plant. Fertilizers such as nitrogen (N), phosphorus (P) and potassium (K) are of greatest significance for the balanced nutrition in the plant species production. They are categorized as organic and inorganic fertilizers, with the differences attributed to the source (Naguib, 2011). Nitrogen forms an integral component of many essential plant compounds. Although nitrogen is regarded as a deficient mineral nutrient limiting the productivity of different plant species in most African soils (Fricke, 1997), P and K are also essential for root development and promote

plant growth. Addition of nutrients, particularly K, can increase chlorophyll content. Photosynthetic organs are also protected to complete their role and increase the photosynthesis to avoid crop yield reduction under dry conditions. Nitrogen is an essential component of chlorophyll, proteins, carbohydrates and enzymes within a plant species. Application of fertilizers has been reported to enhance the yield, increase biosynthesis of carbon based secondary metabolites and benefit the primary functioning of plants (Marschner, 1995). Some investigations on the growth of thyme (*Thymus vulgaris*) found that N fertilizer increased herb yield, but essential oil content was not significant (Baranauskienne *et al.* 2003). The application of N fertilizer on Davana (*Artemisia pallens*) increased in the oil content, plant height, number of lateral shoots, fresh and dry weight (Kumar *et al.* 2009). Furthermore, significant increase in vegetative growth and oil yield content of dill (*Anethum graveolens*) in response to N fertilization was observed (Hellal *et al.* 2011).

A study on the cultivation of true ginger (*Z. officinale*) with nitrogen addition revealed markedly improved productivity in oil content and yield by enhancing the amount of biomass yields and photosynthetic rate (Sangwan *et al.* 2001). The role of nitrogen on vegetative growth and yield attributes is well documented by different studies. According to Akbarinia *et al.* (2006), the application of up to 90 kg N/ha significantly increased seed yield, essential oil content and fatty acids of coriander (*Coriandrum sativum*). The concentration of N, P and K (%) in different plant parts of true ginger (*Z. officinale*) showed significant yield by up to 32.8% and restricted rhizome rot disease occurrence (Sadanandan and Hamza, 1996).

Significant fraction can be saved by subjecting crops to periods of moisture stress with minimal effects on yields (Vandoorne *et al.* 2012). The technique requires knowledge of the soil characteristics, plant species rooting depth and water requirement (Sadras *et al.* 2005). The disproportion levels may be attributed to underdeveloped root system, low soil moisture availability and salts present in the irrigation water. Consequences of water stress may occur at

any stage of plant species development and growth. Furthermore, water stress may cause difference in morphological, physiological and biochemical changes of the plant. Reduced evapo-transpiration could result in stomatal closure, leaf senescence and canopy. Furthermore, reduced assimilation of carbon and cell development has decreased biomass production (Vandoorne *et al.* 2012). Some investigations on water stress reported increased yield of Chamomile (*Matricaria Chamomilla*) under 100% of field capacity and no significant variation on the yield of plants irrigated with 55% of field capacity (Pirzad *et al.* 2006). Effect of drought stress on yield and morphological parameters of Dracocephalum decreased shoot yield from 4126 to 2477 kg/ha. There was also an increase in water stress levels of 100% to 40% of field capacity (Safikhani *et al.* 2007). The two *Curcuma* species subjected to different irrigation levels showed that *C. Aromatic* recorded maximum values of the leaf, fresh and dry weight of rhizome when compared to *C. domestica* (Mohamed *et al.* 2014). A study that investigated the three irrigation treatments (100%, 80% and 60% of field capacity) of *Rosmarinus officinalis* (L.) reported a gradual increase in the chlorophyll content with increasing irrigation frequency (Hassan and Bazaid, 2013).

The investigations of medicinal species have discovered biological-active components such as morphine, cocaine, aspirin, codeine and guanine which contribute to traits of drug discovery today (Anon, 1996). Several secondary metabolites such as alkaloids, phenolic compounds, flavonoids, acids, aldehydes, alcohols, monoterpenes and sesquiterpenes, have been reported in different plants (Wink, 2015). The response of fertilizer applications and water regimes has been reported to produce variations in the secondary metabolites of different plant species during their development. Few studies have revealed the impact of environmental stress on the production of several secondary metabolites by plants (Jaleel *et al.* 2007). The accumulation of secondary metabolites has also been reported to be induced with exposure to nutrient deficiency (Stewart *et al.* 2001). In contrast, excess nitrogen application can result in excessive vegetative growth, increased susceptibility to diseases and delayed maturity (Gustfson, 2010). The

rhizome of *S. aethiopicus* possesses great medicinal benefits due to the presence of a medicinally significant compound siphonochilone (Gericke and van Wyk, 1997). Extracts of *S. aethiopicus* rhizome contains anti-bacterial, anti-inflammatory, antimalarial and anti-candida properties (Light *et al.* 2002; Lategan *et al.* 2009).

Soluble sugars, including monosaccharide and oligosaccharides play a major role in regulating metabolism, growth and development of plants. Plants have developed various adaptive strategies for their survival in nature and to avoid environmental stresses. However, the decrease in the efficiency of photosynthesis in source tissues may reduce the supply of soluble sugars to sink tissues due to variations in environmental factors such as light, water stress and temperature (Rosa *et al.* 2009). Plants are subjected to a combination of adaptive mechanisms such physiological and biochemical processes, hence plant's response to different stresses are unique. High concentration of soluble sugars in different tissues promote growth and carbohydrate storage, while, reduced soluble sugar status enhances photosynthesis and reserve mobilization (Rosa *et al.* 2004). The high levels of soluble sugar contents suggest a good regulated metabolic status of the plant, with low amounts of sugars indicating a potential metabolic deregulation. Soluble sugars increase during the winter season when temperatures are low and decrease in spring when plants are subjected to the gradual change in the environment. A study on the various parts of *C. Intybus* exhibited high total sugars and non-reducing sugar content compared to the seeds (Al-Snafi, 2016). The concentration of sucrose, glucose, and fructose for two varieties of ginger (*Z. officinale*) was significantly influenced by CO_2 concentration (Ghasemzadeh *et al.* 2014).

The characterization and proportion composition of volatile organic compounds (VOCs) provide an essential parameter for the classification and identification of the plant species. Characterization of VOCs is important to reveal the active ingredients which will show various useful compounds essential as fragrance and pharmacologically agents. The quality of VOCs

composition of plants can be influenced by agronomic practices, climatic conditions, cultivar, harvest time, storage, processing methods and agro ecological region (Asensio *et al.* 2007). Furthermore, the composition of different chemical constituents varies from one species to another, age and tissue type. Factors such as water stress, mineral nutrient deficiency, aluminium tolerance and plant microbe interactions have been reported to influence organic compounds in the root of varying plant species. Terpenoids, commonly monoterpenes and sesquiterpenes are some of the most important constituents identified in different plant species (Figure 2.3). The essential oil compositions of true ginger (*Z. officinale*) from different agro-ecological areas have been identified and reported (Wohlmuth *et al.* 2006). Investigation of chemical constituents in essential oils of true ginger exhibited the presence of zingiberene, α-curcumene, β-sesquiphellandrene camphene, from varying agro-ecologies (Singh *et al.* 2008). A study on *Polygonum minus* detected most volatiles as sesquiterpenes in the leaf and stem, followed by aliphatic and monoterpenes compounds (Ahmad *et al.* 2014). The most abundant sesquiterpenes in the leaf stem and root was β-caryophyllene, at 5.78%, 34.71% and 22.92%, respectively. Monoterpenes, such as eucalyptol, linalool, camphor, α-pinene, β-pinene, α-terpineol, borneol and many others, are the principal components of plant volatile oils (Figure 2.3). Production of most terpenes constituents in aerial parts increased with an increase in temperature regimes. The results obtained on *P. hydropiper* emitted more decanal and dodecanal constituents in the stems as compared to the presence of monoterpenoids emitted in the extracts of the rhizome contributed to the bioactivity of the plant species (Gabriel and Kesselmeier, 1999).

2.6 Quality analysis of medicinal plants

Various methods have been established for qualitatively and quantitatively, identification, separation and purification of several compounds and essential oils in different medicinal plant species. The choice of a particular analytical technique depends on the nature of the compounds involved and the plant species in question. The actual amounts of chemical constituents in

medicinal plants are known to be affected by the harvest season, plant parts collected, environmental factors (soil type, water availability, and temperature), drying method, plant origins and extraction procedure. One of the most important reasons for variation in estimated amounts of identified bioactive compounds is, however, the method employed (Siddiqui *et al.* 2013).

A spectrophotometric assay is a direct method which requires small sample volume of the plant materials. It allows the assay to be determined in a fast turn and could be applied to a single or several treatment samples at a time (Siddiqui *et al.* 2013). Antioxidant activities such as 2, 2'-diphenyl-1-picryl hydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) can be determined using spectrophotometric assays for plant materials. Assays are measured spectrophotometerically and amounts expressed using the recommended standard at the applicable wavelength.

Thin layer chromatography (TLC) has been designed for the analysis and identification of herbal products and quality control of medicinal plants. The technique is responsible for rapid characterization of natural products in extracts for an efficient and selective isolation procedure. The technique provides a multiple detection of compounds on the same plate and can also handle a high number of samples in a short analysis time (Wink, 2015). Hyphenated techniques such as High performance liquid chromatography (HPLC) and gas chromatography (GC) can provide useful structural information compounds prior to isolation. Several studies have described the use of HPLC for characterization and quantification of secondary metabolites in plant extracts, mostly phenol compounds, flavonoids and alkaloids (Khoddami *et al.* 2013). HPLC technique is regarded as the best due to its simplicity, versatility, and its ability to handle compounds of a diverse polarity and molecular mass (Wink, 2015). Gas chromatography (GC) is also useful in the discovery of novel compounds, metabolomics and synthesis of pathways. The technique is characterized by a high-sensitivity and better resolution on the identification

of specific compounds. The method remains the most powerful discovery tool for defining compounds and is considered imperative for isolation of volatile oil compounds analysis, but cannot be performed in high performance liquid chromatography (Pongsuwan *et al.* 2007).

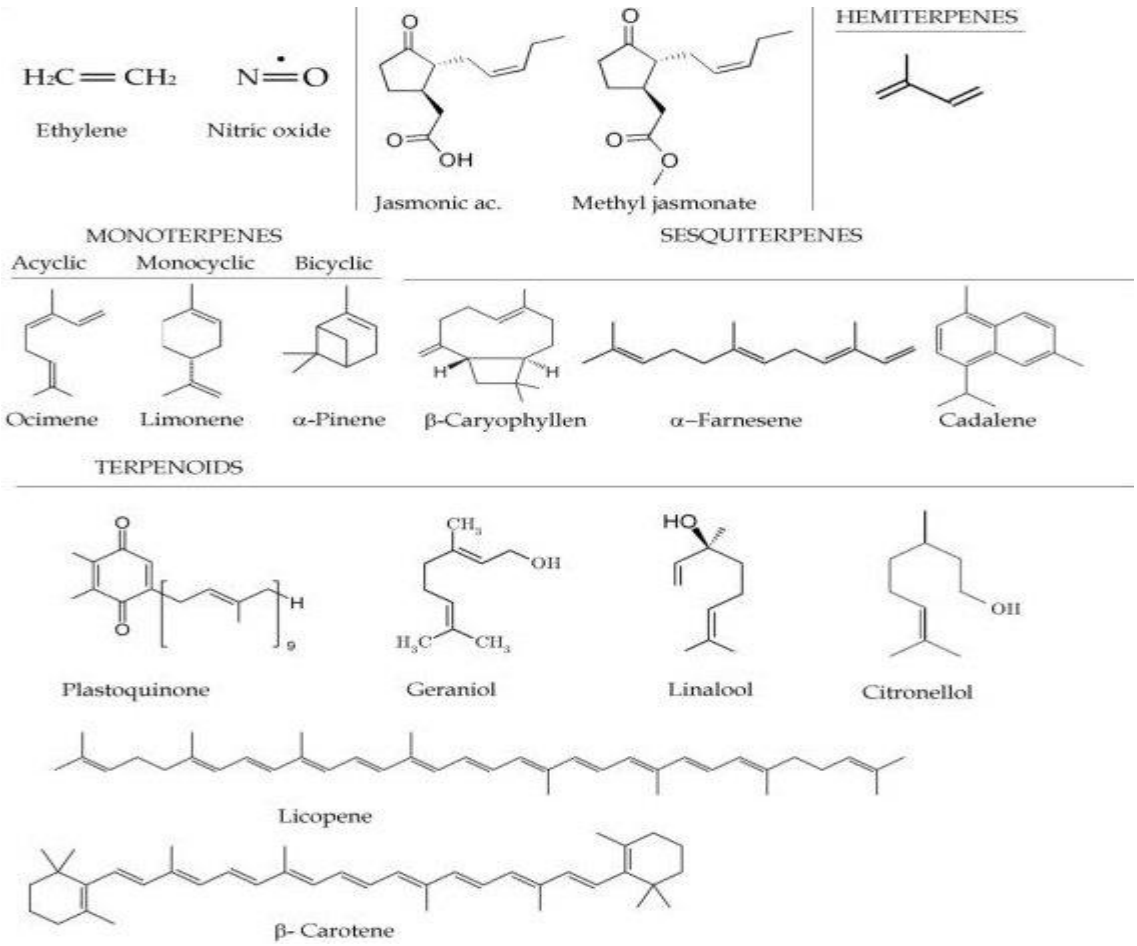

Figure 2.3: Chemical diversity of the different volatile organic compounds (VOCs) and related compounds present in the plant.

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CHAPTER THREE

PHENOLIC COMPOSITION AND ANTIOXIDANT ACTIVITY OF AFRICAN GINGER (*SIPHONOCHILUS AETHIOPICUS)* FROM DIFFERENT AREAS OF SOUTH AFRICA

ABSTRACT

African ginger (*S. aethiopicus*) is used as a medicinal plant containing bioactive constituents which provide health benefits. However, there is limited information describing its antioxidant properties from different parts and growing areas of South Africa. In this study, total phenolic content, flavonoid content and antioxidant activity of *S. aethiopicus* leaf, rhizome and root from varying areas (Mpumalanga, KwaZulu-Natal, Limpopo and North West) were evaluated. Total phenolic and flavonoid contents were investigated by Folin-Ciocalteu and aluminium chloride ($AlCl_3$) colorimetric methods, respectively. Antioxidant activity in different parts of *S. aethiopicus* was evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and ferric reducing power (FRAP). Furthermore, the study determined the variations in soluble sugars in the leaf, rhizome and root as influenced by varying growing areas. The results showed high concentration of sucrose, glucose and fructose in the leaf and root as influenced by different growing areas. A higher content of both total phenolics and flavonoids were found in the root from Mpumalanga (54.5±2.0 mg GAE/g and 14.83±0.06 µg QE/g, respectively) compared to the leaf and rhizome from other growing areas. KwaZulu-Natal also exhibited high flavonoids in the leaf (12.72±1.18 µg QE/g), rhizome (14.21±1.98 µg QE/g) and root (12.88±0.57 µg QE/g) compared to other growing areas. In both methods, the leaf exhibited higher antioxidant activity than the root and rhizome. The high antioxidant activities exhibited in the leaf from Mpumalanga suggest its adaptive capabilities to different environments. *S. aethiopicus* parts could be used as a potential source for antioxidant properties and encourage cultivation under different growing areas to conserve its biodiversity and increase species populations.

Keywords: Phytochemical screening, Phenolic, Medicinal plants, Free radicals

3.1 Introduction

The recent global increases in food and medicine prices have added pressure on the agricultural production and health care system. As a result, medicinal plants are screened for their phyto-constituents that can be used as drugs and various pharmaceutical products. Naturally occurring phyto-constituents such as tocopherols, carotenoids, polyphenolics and terpenoids from medicinal plants are effective in preventing various diseases caused by oxidative stress which generate free radicals (Saeed *et al.* 2012).

Medicinal species possess antioxidant properties with potential to prevent and treat diseases and maintain human health (Sofowora *et al.* 2013). Amongst them, *S. aethiopicus* is one of the mostly sought-after medicinal used for a variety of respiratory ailments. The plant is considered important and most popular of most traditional medicinal plants (Gericke *et al.* 2001). Chemical compounds isolated from the plant include sesquiterpenoids of the furanoid type (Holzapfel *et al.* 2002). The plant contains up to 0.2% of dry weight of the Siphonochilus siphonochilone (Viljoen *et al.* 2002).

S. aethiopicus has high popularity due to its medicinal properties and has been identified as one of the South African species with commercialization potential as a result of high demand and reduced supply. The plant is reported to contain health-promoting compounds and functional properties which prevent and treat several health conditions. Scientific studies have shown that various extracts of *S. aethiopicus* possess a wide range of pharmacological properties such as antimicrobial (Verotta and Rogers, 1997), anti-inflammatory (Gericke *et al.* 2001), and anti-candida properties (Light *et al.* 2002). Furthermore, some health benefits of *S. aethiopicus* rhizome and root include coughs, colds, asthma, headache, candida and malaria (van Wyk and Gericke, 2000).

The composition and concentration of soluble sugars have been of interest due to significant components in medicinal species and as food crops contributing to their nutritive value. Soluble sugars play a significant role as primary messengers and regulate the growth and metabolism in both sugar source and sink tissues (Rolland *et al.* 2006). Accumulations and variations in the levels of soluble sugars in different plant parts depend on a number of factors, such as environmental conditions in growing areas and the degree of maturity at harvest. Variations in soluble sugar concentrations exist not only among variant species, but also within the different plant parts of the same species. Plants employ several adaptive strategies in response to environmental stresses associated with metabolic adjustments which results in the accumulation of phenols and sugars (Tesfay *et al.* 2011).

The response of plant parts and soluble sugar concentration from varying growing regions will provide information fundamental data on physiological and biochemical mechanisms. Consequently, it is imperative to understand response of growing areas in relation to the plant secondary metabolites. The objective of this study was to investigate phenolic and flavonoid content, antioxidants activity and soluble sugars of different parts of *S. aethiopicus* from different growing areas in South Africa.

3.2 Materials and methods

3.2.1 Geographical description of study sites

S. aethiopicus plant materials were collected in April/May 2014 season from different growing areas in South Africa (namely; Mpumalanga, Limpopo, KwaZulu-Natal and North West). About 10-15 plants were randomly sampled from each site by walking in a zigzag manner across the length of the field starting from one corner. The areas have a unimodal rainy season that starts in October each year and ends in April the following year. Total rainfall received during the corresponding site was obtained from nearby automatic weather stations (Table 3.1)

Table 3.1: Site description study areas for *S. aethiopicus.*

	Limpopo	Kwazulu-Natal	North West	Mpumalanga
Geographical location (latitude and longitude)	23° 49' 59"S 30° 9' 48"E	28° 84' 52"S 31° 09' 99"E	25° 63' 44"S 27° 78' 11"S	25° 26' 25.3"S 30° 58' 55.9"E
Annual rainfall	600 mm	871 mm	540 mm	800 mm
Average Temperature	19–30 °C	16–26 °C	16–27 °C	15–28 °C
Frost occurrence (*during data collection)	None	Moderate	None	Moderate
Soil texture classes	Sand	Clay	Clay	Sandy loam

3.2.2 Harvesting and sample preparation

Fresh plant samples were harvested from the different growing areas and separated into leaf, rhizome and root. Plant materials for determination of soluble sugars were freeze-dried (Model FM25, St. Louis, Missouri) until analysis. The plant parts used for determination of antioxidant activity were oven-dried separately at 50 °C for 48 h, ground into fine powder and extracted with 50% aqueous methanol in an ultrasonic bath for 1 h. The infusions were filtered under vacuum through Whatman number 1 filter paper. The extracts were evaporated to dryness under vacuum at 30 °C using a rotary evaporator (Model RE300, Staffordshire, ST15 OSA,

UK) and entirely dried under a stream of air. Fresh extracts of 50% aqueous methanol were used in the antioxidant assays.

3.2.3 Determination of total phenolics and flavonoids

The concentration of total phenolics in plant parts was determined using the Folin-Ciocalteu colorimetric assay according to method by Li *et al.* (2008). The total phenolic content was expressed using Gallic acid as the standard (mg GAE/g dry weight basis). Flavonoids were quantified using aluminium chloride ($AlCl_3$) colorimetric according to method by Ordon *et al.* (2006). Quercetin was used as a standard. The absorbance was measured in triplicates at 765 and 510 nm using a spectrophotometer, for phenolics and flavonoids, respectively.

3.2.4 Determination of total antioxidant activities using DPPH assay

The antioxidant activity of *S. aethiopicus* leaf, rhizome and root was determined by the (DPPH 2, 2'-diphenyl-1-picryl hydrazyl) radical scavenging method according to Ndhlala *et al.* (2014). The reaction mixture consisted of 15 µL for each plant extract and diluted with 735 µL absolute methanol at different concentrations 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50 mg/mL. In addition, the freshly prepared DPPH solution (750 µL; 50 µM in methanol) was added to give a final volume of 1.5 mL in the mixture. The assay was run in triplicate and repeated twice with concentrations varying as follow; 0.065, 0.26, 0.52, 1.04, 6.25, 12.5, 25 and 50 mg/mL. The assay was performed under dimmed light and plates containing the reaction solution were incubated in the dark at room temperature for 30 minutes to complete the reaction mixture. Thereafter, the absorbance of the solution was measured at 517 nm using a UV/VIS spectrophotometer (Varian Cary 50, Varian Australia Pvt LTD, Sydney, Australia) with methanol as a blank. A standard antioxidant, ascorbic acid was used as positive control at different concentrations. DPPH scavenging activity was calculated as percentage (%) inhibition of DPPH.

Where; Abs_{517nm} sample is the absorbance of the reaction mixture, which contains the resuspended extract or positive control and Abs_{517nm} Neg control is the absorbance of the negative control. RSA (%) = 1- (Abs_{517nm} sample/ Abs_{517nm} Neg Control) ×100……. [Eq.1]

2.3.5 Determination of total antioxidant activity using FRAP assay

The ferric reducing antioxidant power assay (FRAP) of each plant part was measured according to Ndhlala *et al.* (2014). Each 96 well microtiter plate contained 30 µL of methanol and resuspended plant extract of 30 µL at a concentration of 50 mg/mL, a positive control (BHT dissolved in methanol) was added also and two-fold serial dilution was used in triplicate. About 40 µL potassium phosphate buffer (0.2 M, pH 7.2) and 40 µL potassium ferricyanide (1% in phosphate buffer, w/v) were added in each well. The plate was covered with aluminium foil and incubated at 50 °C for 20 min. After incubation 40 µL trichloro-acetic acid (10% in phosphate buffer, w/v), 150 µL distilled water and 30 µL $FeCl_3$ (0.1% in phosphate buffer, w/v) were added. Then again the plate was re-covered with foil and incubated at room temperature for 30 min to complete the reaction. The assay was repeated twice with absorbance read at 630 nm using a microtiter plate reader (Opsys MRTM, Dynex Technologies Inc., Palm City, FL, USA). The reducing antioxidant power of the extract and ascorbic acid were expressed by graphically plotting absorbance against concentration.

3.2.6 Determination of soluble sugar concentration

Freeze-dried, plant powder (0.05 to 0.10 g in dry weight basis) was mixed with 10 mL of the extraction solvent ethanol 80% (v/v). The solution was homogenized using an Ultra-Turrax (Model T25D, IKA, Germany) for 60 s, incubated in an 80 °C water bath for 60 min and kept at 4 °C overnight. Thereafter, tubes were centrifuged (12, 000 g, 4 °C), for 15 min and the supernatant was filtered through glass wool and dried in a Savant Vacuum Concentrator (SpeedVac, Savant, NY, USA). Dried samples were resuspended with 2 mL ultra-pure water,

filtered through 0.45 μm nylon filters and analysed using an HPLC-RID system (LC-20AT, Shimadzu Corporation, Kyoto, Japan) equipped with a refractive index detector (RID-10A, Shimadzu Corporation, Kyoto, Japan) and a Rezex RCM-Monosaccharide column (300 mm x 7.8 mm) (8-micron pore size; Phenomenex®, Torrance, CA, USA). The concentration of individual sugars was determined by comparison with authentic sugar standards (Tesfay *et al.* 2011).

3.2.7 Data analysis

All data was subjected to analysis of variance using GenStat software 17.1 editions (VSN International, Hemel Hempstead, UK). GraphPad Prism version 5.00 for Windows (GraphPad Software Inc., San Diego, CA) was used construction of graphs and calculation of IC_{50}/EC_{50} values. The treatment means were separated using $(p < 0.05)$ Duncan's multiple range test (DMRT). The results from different growing areas are presented as means ± standard error of six replicates.

3.3 Results and discussion

3.3.1 Total phenolic and flavonoid content

The total phenolic and flavonoid content of *S. aethiopicus* extracts are compiled and presented in Table 3.2. The result of the phytochemical screening carried out on the methanolic extracts of the leaf, rhizome and root revealed the presence of various levels of phenolic content from different growing areas. As indicated in Table 3.2, the total phenolic content of *S. aethiopicus* ranged from 10.50±2.4 to 56.69±6.9 mg GAE/g for rhizome and root from Limpopo province, respectively. Generally, high phenolic contents were observed in the root and lowest in the leaf and rhizome (Table 3.2). Among the plant parts sampled and analyzed, the root from Mpumalanga and Limpopo showed the highest phenolic content (56.6±6.9 mg GAE/g and 54.5±2.0 mg GAE/g), respectively. Significant amounts on total phenolic content were also obtained in other plant parts from Limpopo and Mpumalanga.

There was no significant effect on total phenolic content for the leaf, rhizome and root from North West province. The results showed the highest total flavonoids for the leaf, rhizome and root from KwaZulu-Natal province (Table 3.2). The highly significant amount of total phenolic content recorded in this study, suggest a strong influence of the environment from different varying areas on the biological performance of *S. aethiopicus* root (Table 3.2). These results indicate that the total phenolic content in this study had noticeable differences according to each agro-ecological location. This is due to environmental variations such as e.g. rainfall and temperature (Table 3.1) that may exert stress on the plant parts and exhibit expression of high phenolic content. Recent report indicated the expression of more plant secondary metabolites in response to environmental stressed conditions (Sampaio *et al.* 2016). The present results are comparable to total phenolic content reported on varieties of ginger (*Z. officinale*) varieties (Ghasemzadeh and Jaafar, 2011). The findings strongly suggest that phenolics are important components of medicinal plants and their pharmacological effects could be attributed to the presence of valuable constituents. The phenolic content exhibited in the leaf and rhizome of *S. aethiopicus* was within the same range as those of commonly consumed *Z. officinale* (Ghasemzadeh *et al.* 2010).

Accumulations of total flavonoids in different parts of *S. aethiopicus* from different areas are summarized in Table 3.2. The significant variations of flavonoid content (for the root) ranged from 4.73±0.86 µg QE/g for North West to 14.83±0.06 µg QE/g for Mpumalanga province. The flavonoid content followed an increasing trend for the leaf, followed by the rhizome and root from KwaZulu-Natal (Table 3.2). The high flavonoid content observed for the leaf from North West (13.34±1.18 µg QE/g), KwaZulu-Natal (12.72±1.18 µg QE/g) and Mpumalanga (10.41±1.41 µg QE/g) could be associated to rich flavones, which have been reported to play an essential role in plant leaf extracts (Mohamed *et al.* 2013). KwaZulu-Natal showed high flavonoid content in the leaf (12.72±1.18 µg QE/g), rhizome (14.21±1.98 µg QE/g) and root (12.88±0.57 µg QE/g) as compared to other sites (Table 3.2). The high total flavonoid content

for North West and KwaZulu-Natal are similar with reported content ginger (*Z. officinale*) leaf than the rhizome (Ghasemzadeh *et al.* 2010). This could be associated with flavonoids as agents for antioxidant activity which act as constituents involved biological activities such as anti-inflammatory processes (Rahman, 2007). Despite the lower rainfall amounts in North West and Limpopo (Table 3.1), highest phenolic and flavonoid content were reported for the root and leaf. The increase of flavonoid content could also be associated with plant mechanism to adapt to various environmental conditions. Furthermore, Jaleel *et al.* (2007) reported the high production of secondary metabolites in plants when subjected stressed environmental conditions. Several environmental conditions including the exposure to nutrient deficiency could offer a good strategy to induce and improve flavonoid content in different plant parts (Stewart *et al.* 2001).

Table 3.2: Total phenolic and flavonoid content of *S. aethiopicus* leaf, rhizome and root from different growing areas in South Africa.

Location	Plant part	Total phenolics (mg GAE/g)	Total flavonoids (µg QE/g)
Mpumalanga	Leaf	28.8±5.0^{b}	10.4±1.4^{ab}
	Rhizome	27.0±7.9^{b}	9.5±1.8^{bc}
	Root	54.5±2.0^{a}	14.8±0.06^{a}
Limpopo	Leaf	22.1±6.7^{bc}	9.9 ± 0.24^{bc}
	Rhizome	10.5±2.4^{c}	10.7±1.4^{ab}
	Root	56.6±6.9^{a}	8.9 ± 1.06^{bc}
KwaZulu-Natal	Leaf	14.4±2.4^{c}	12.7±1.1^{a}
	Rhizome	32.5±4.2^{b}	14.2±1.9^{a}
	Root	32.7±7.0^{b}	12.8±0.57^{a}
North West	Leaf	31.0±3.2^{b}	13.3±1.1^{a}
	Rhizome	27.6±9.1^{b}	4.8±1.3^{c}
	Root	24.5±1.7^{bc}	4.7±0.86^{c}
F-statistics		4.29**	3.15**

Values are Mean±SE (n=6) with dissimilar letters in a column are significantly different at $p < 0.05$ (**). Gallic acid (GAE) and Quercetin (QE).

3.3.2 DPPH 2, 2'-diphenyl-1-picryl hydrazyl radical scavenging activity

The EC_{50} values for the DPPH radical scavenging ability of *S. aethiopicus* leaf, rhizome and root are presented in Table 3.3. All plant parts showed a propensity to quench the free radicals, as indicated by low EC_{50} values. A low EC_{50} value is indicative of the stronger inhibitor of the biological process. The leaf and root extracts from Mpumalanga showed the lowest EC_{50} values, depicting its good antioxidant potential. Antioxidant activity of plants has been partly ascribed to phenolic compounds (Robards *et al.* 1999). However, high yield of phenolic compounds does not necessarily accompany high antioxidant, as the bioactivity of extracts can be influenced by structure and interactions between extracted phenolic compounds. For example extracts of the leaf from Mpumalanga exhibited the lowest EC_{50} values indicating high antioxidant activity (Table 3.3) with low phenolic content (Table 3.2) as compared to other plant parts. In contrary, the most active DPPH radical scavenger, the root from Mpumalanga exhibited the highest total phenolic and flavonoid content resulting in prominent antioxidant activity (Table 3.2 and 3.3). The presence of high total phenolic compounds in the root could be associated with high antioxidant properties (Table 3.2 and 3.3). According to Shad *et al.* (2013), the leaf of *Cichorium intybus* possessed good free radical scavenging capacity due to higher DPPH radical inhibition and lower EC_{50} values.

Table 3.3: Antioxidant activity of *S. aethiopicus* leaf, rhizome and root different growing areas in South Africa.

	Mpumalanga	Limpopo	KwaZulu-Natal	North West
Plant part	EC_{50} (µg/mL)			
Leaf	10.56 ± 0.15^{a}	33.52 ± 0.09^{c}	28.52 ± 0.10^{ab}	24.94 ± 0.14^{a}
Rhizome	20.91 ± 0.15^{a}	27.64 ± 0.14^{b}	20.77 ± 0.10^{a}	20.51 ± 0.14^{a}
Root	16.62 ± 0.11^{a}	30.19 ± 0.12^{b}	17.26 ± 0.13^{a}	17.22 ± 0.12^{a}
Ascorbic acid	78.41±0.22 µg/mL			

S. aethiopicus extracts with EC_{50} values (<78.41 µg/mL) are a measure of potent DPPH radical scavengers. The lower the EC_{50}, is indicative of the stronger inhibitor.

3.3.3 Total antioxidant activity by FRAP

The FRAP assay was used to evaluate the antioxidant activity in the leaf, rhizome and root from four areas in South Africa (Figure 3.1, 3.2 and 3.3). Generally, the greater antioxidant depicts the reduction of Fe^{3+} to various shades of blue ferrous form and higher absorbance values after the assay (Gülçin *et al.* 2007). The difference between the antioxidant activity of *S. aethiopicus* parts and growing areas differed significantly ($p < 0.05$). The FRAP values were higher in the leaf from Mpumalanga (0.53±0.03 mg/mL) and North West (0.46±0.06 mg/mL), however they did not differ significantly from each other. The leaf displayed a stronger antioxidant activity at all concentrations than the rhizome and root, irrespective of the where it was collected. As expected, reducing activity increased with the increase in the concentration of all plant parts. The investigation of antioxidant activity in the leaf grown from KwaZulu-Natal was significantly lower at all the concentrations (Figure 3.1).

The antioxidant activity decreased significantly irrespective of the growing area for the root. Therefore, the differences obtained in this study clearly indicate that FRAP assay has the potential for high precision measurement of antioxidant activity in different parts of medicinal plants. The reducing capacity of a plant is much related to the presence of biologically active compounds with potent donating abilities (Kasote *et al.* 2015). The increase in secondary metabolites and antioxidant activity from one growing area to another might be due to the variability of environmental conditions. The antioxidant potency observed in this study indicates the adaptation mechanism to varying growing areas by different parts of *S. aethiopicus* (leaf, rhizome and root). Their adaptation makes them ideally suitable to contribute to agronomic practices, which can potentially be cultivated and utilized in the future.

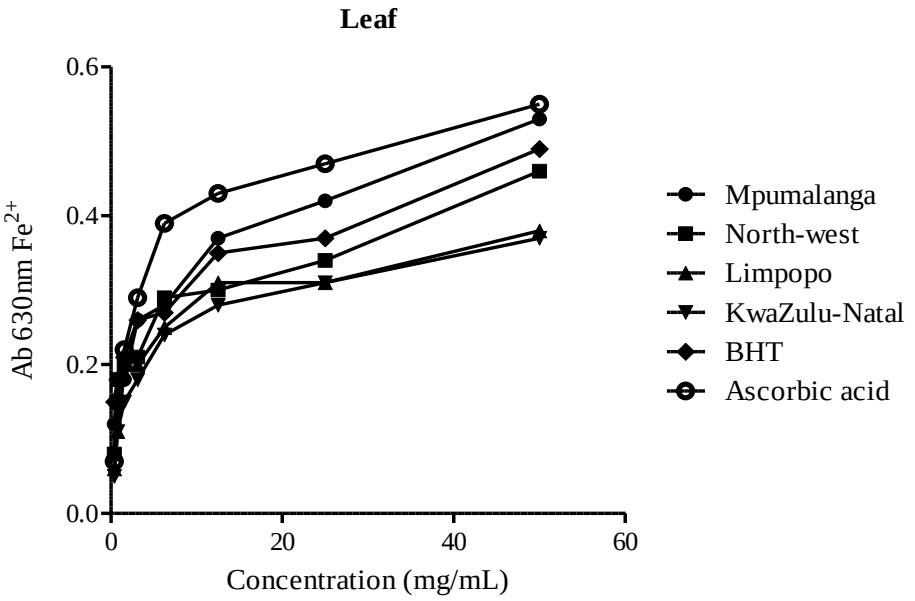

Figure 3.1: Ferric reducing power of leaf from Mpumalanga, North West, Limpopo and KwaZulu-Natal in South Africa. Positive controls (BHT and Ascorbic acid).

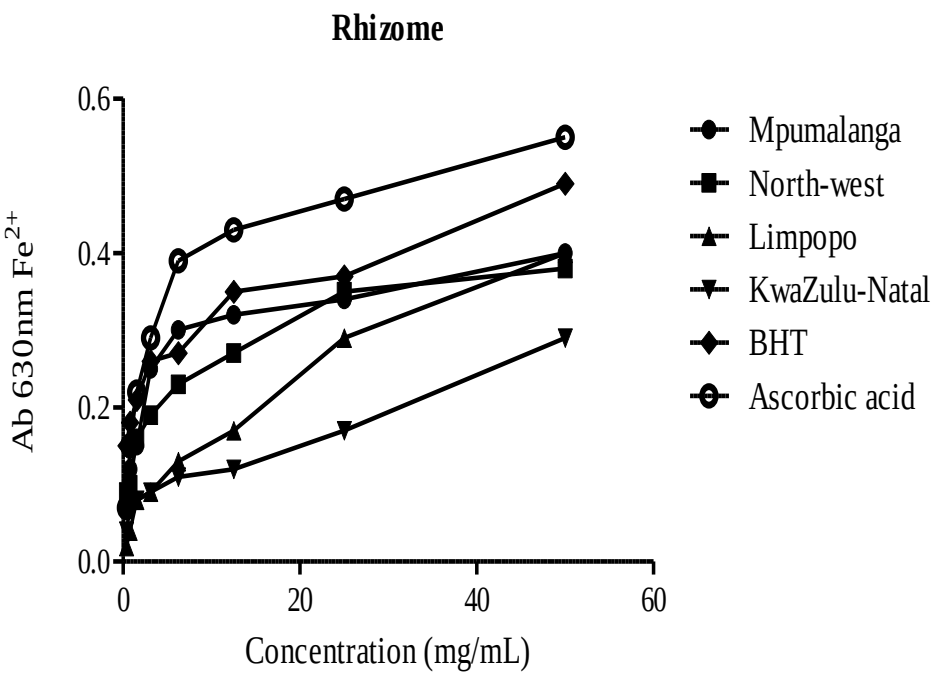

Figure 3.2: Ferric reducing power of rhizome from Mpumalanga, North West, Limpopo and KwaZulu-Natal in South Africa. Positive controls (BHT and Ascorbic acid).

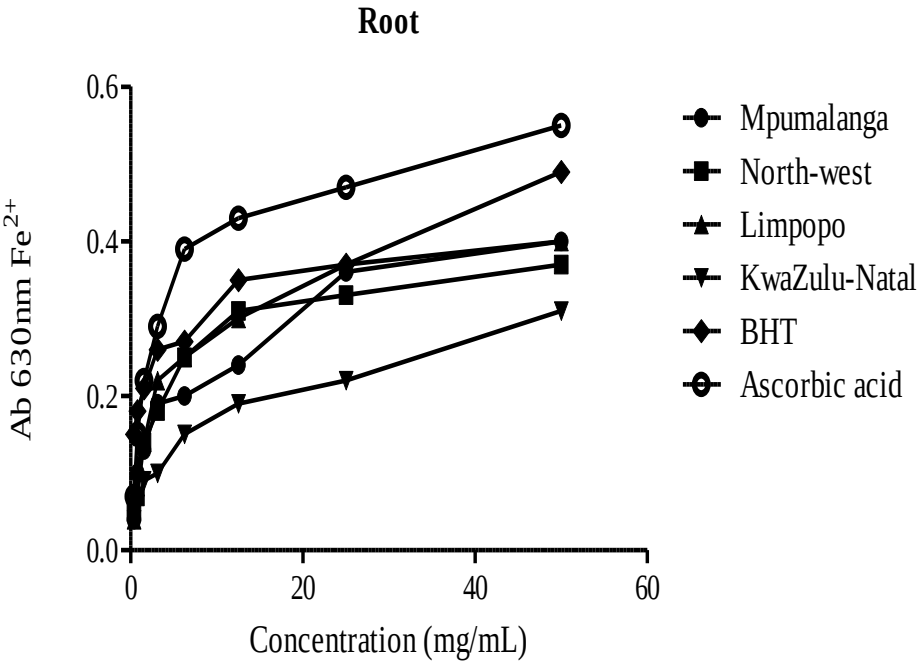

Figure 3.3: Ferric reducing power of root from Mpumalanga, North West, Limpopo and KwaZulu-Natal in South Africa. Positive controls (BHT and Ascorbic acid).

3.3.4 Effect of growing areas and soluble sugars content on plant parts

The soluble sugar (sucrose, glucose and fructose) were detected in the leaf, rhizome and root from different sites and the sugar composition varied with accession reflecting the variations among the plant parts (Table 3.4). Fructose was detected in all plant parts and from all areas, while sucrose and glucose were below the limit of detection. Sucrose content was high in the rhizome (7.48±0.46 mg/g DW) from North West and present in lower concentration in the rhizome from KwaZulu-Natal (Table 3.4). Soluble sugar concentrations in the rhizome from North West were three times higher than in the rhizome from KwaZulu-Natal and Mpumalanga (Table 3.4). The sugar composition reported in this study is similar to other storage plant parts investigated by other studies. For example, the rhizomes of bulrush (*Schoenoplectus lascustris* L.) contained high sucrose as the major soluble sugar. Furthermore; Cyr *et al.* (1990), reported sucrose as the main soluble sugar in the root of perennial plants chicory (*Cichorium intybus* L.) and dandelion (*Taraxacum officinale* L.). Glucose content was not detected in the rhizome and root from KwaZulu-Natal, Mpumalanga and Limpopo (Table 3.4).

Accumulation of glucose content in the leaf, rhizome and root of *S. aethiopicus* from North West followed the descending trend leaf > rhizome > root (Table 3.4). Ghasemzadeh *et al.* (2014) reported high sucrose content in the leaf and rhizome followed by glucose and fructose. Glucose content declined in the root from different sites but sucrose content was still high (Table 3.4). The variations in the leaf, rhizome and root could be attributed by the modifications of soluble sugars (sucrose, glucose and fructose) levels which do not follow the same patterns, signalling and developmental impact in a number of cases (Weber *et al.* 2005). As shown in Table 3.4, the increase in fructose content was greater in leaf, rhizome and root from KwaZulu-Natal and Mpumalanga, respectively. *S. aethiopicus* recorded highest fructose content in the leaf as compared to the rhizome and root (Table 3.4). Among the studied sugars, fructose content was higher compared to sucrose and glucose, although there were no significant variations between the study areas (Table 3.4). A number of studies suggest that soluble sugars could be part of groups useful to the plant as defenses and signalling agents (Brouquisse *et al.* 1991).

Although, fructose concentration was highest in all the growing areas and plant parts, the preferential effects of sucrose may be related to qualitative and quantitative importance of sucrose in higher plants as a major carbon structure and a form of transport throughout the plant. Understanding the mechanisms involved in soluble sugars concentrations can be used as a marker for providing insights on the ability and response of different plant parts and environmental conditions.

Table 3.4: Sucrose, glucose and fructose content in different tissues of *S. aethiopicus* from varying geographical locations.

Locations	Tissue	Sucrose	Glucose	Fructose
		mg/g DW		
Kwazulu-Natal	Leaf	1.45 ± 0.40^{cd}	0.54 ± 0.02^{bcd}	16.94 ± 1.49^{a}
	Rhizome	ND	ND	13.77 ± 1.97^{ab}
	Root	1.28 ± 0.28^{d}	ND	10.00 ± 4.39^{cd}
Mpumalanga	Leaf	1.72 ± 0.14^{cd}	1.00 ± 0.21^{b}	11.34 ± 1.05^{bcd}
	Rhizome	1.39 ± 0.13^{d}	ND	12.75 ± 3.75^{ab}
	Root	1.25 ± 0.28^{d}	ND	11.00 ± 2.49^{bcd}
North West	Leaf	ND	4.46 ± 0.34^{a}	1.00 ± 0.06^{d}
	Rhizome	7.48 ± 0.46^{a}	0.54 ± 0.04^{bcd}	0.93 ± 0.60^{d}
	Root	4.66 ± 0.28^{ab}	0.32 ± 0.02^{bcd}	11.24 ± 0.22^{bcd}
Limpopo	Leaf	1.65 ± 0.62^{cd}	0.36 ± 0.06^{bcd}	11.00 ± 1.71^{bcd}
	Rhizome	2.97 ± 0.15^{bcd}	ND	12.95 ± 1.14^{ab}
	Root	3.09 ± 0.23^{bcd}	ND	0.37 ± 0.01^{e}
	F Prob. (5%)	< 0.0001***	< 0.0001***	0.22^{ns}
	F-statistics	4.78	18.48	1.43

Notes: Data are means of triplicate measurements ± standard deviation; Means not sharing a common single letter for each measurement were significantly different at $p < 0.05$; Unit of all measurement are mg/g DW.

3.4 Conclusion

In the present study, different assays were used to evaluate the antioxidant potential of *S. aethiopicus* leaf, rhizome and root from different areas. The high antioxidant activities in the leaf suggest the effectiveness of antioxidants activities as an important therapeutic agents. The root from Mpumalanga showed the high amounts of total phenolic and flavonoid content and antioxidant activity. The high antioxidant activities observed in the leaf from Mpumalanga suggest its adaptive capabilities to different environments.

Soluble sugar content from different areas under stressed conditions is associated with adaptation of plants to various stresses. The antioxidant activity in the leaf could provide an alternative harvestable plant part and reduce over-harvesting. Further investigations are needed to improve knowledge on the effect of different environmental stresses on the secondary metabolites in different plant parts.

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CHAPTER FOUR

PHYSIO-MORPHOLOGICAL AND YIELD RESPONSE OF AFRICAN GINGER (*SIPHONOCHILUS AETHIOPICUS)* TO IRRIGATION REGIMES AND NITROGEN LEVELS

ABSTRACT

Plant growth is adversely affected by various forms of biotic and abiotic stress factors. Water deficit is one of the major abiotic stresses which limits agricultural productivity and causes losses in crop yield. African ginger (*S. aethiopicus)* is an important medicinal plant with great potential for treatment of many ailments. Although its cultivation is regarded as a good approach to reduce pressure on species populations and meet the high demands, there is limited information on the cultivation practices. The study evaluated the physiological and morphological response of *S. aethiopicus* growth to irrigation regimes and nitrogen application rates. This research was conducted under a rainshelter and exposed to three irrigation regimes (30, 50 and 70% allowable depletion level, ADL) and five nitrogen levels (0, 50, 100, 150 and 200 kg N/ha) for two cropping seasons. The data showed no significant interactions between the two seasons, however, the pooled data subjected to statistical analysis was significant *($p < 0.05$).* A significant interactive effect between irrigation regimes and nitrogen level was observed for parameters investigated, excluding stomatal conductance and biomass yield. The chlorophyll content, plant height, number of leaves per plant and leaf area index increased with an increase in N applied at 50 and 100 kg/ha. The high utilization of water from well watered treatment (30% ADL) compared to moderate (50% ADL) and severe (70% ADL) treatments could be attributed to improved water availability and superior plant canopies. The well watered treatment had a significantly higher total biomass, fresh and dry rhizome yield compared to other water stressed treatments. Addition of N fertilizer improved the rhizome yield. Taken together, the findings show that, adequate yields can be contributed by irrigation and nitrogen management strategies in cultivation of *S. aethiopicus.*

Keywords: medicinal plant, planting seasons, irrigation, allowable depletion level

4.1 Introduction

African ginger (*S. aethiopicus*) is one of the most important medicinal plants required in large quantities for the treatment of various respiratory ailments in humans, financial viability and commercial production potential. The large demand in *S. aethiopicus* is associated with treating aliments such as asthma, headaches, pain, inflammation and malaria (van Wyk, 2008). The use of *S. aethiopicus* has become a great trend as a household remedy for coughs and colds. The plant possesses antioxidant properties, exerts anti-inflammatory, antimalarial effects and is an effective anti-bacterial and anti-candida agent (Lategan *et al.* 2009).

Although, *S. aethiopicus* is regarded as an important medicinal plant with commercial potential and financial feasibility, the growing demand owing to its traditional use and local trade is putting a heavy pressure on the species populations. *S. aethiopicus* is listed on the Red List of South African endangered plants species due to over-exploitation and indiscriminate collection. Current populations in the wild are projected to be completely extinct if there are no approaches to conserve the species (The Red list of South African plants version 2015.1). Cultivation is an alternative and viable way to reduce the adverse situation on *S. aethiopicus* populations. This would enhance the limited information on the cultivation practices of medicinal plants, conserve biodiversity and ensure steady supply in the market.

Suitable cultivation practices are associated with water and nutrient management as major detrimental factors for plant growth, chemical composition and metabolic processes in plant species (Farooq *et al.* 2009). Application of mineral nutrients enhances plant productivity, improves plant physiological activities and is a tool to ameliorate the quality of medicinal species. One essential plant nutrient is nitrogen, which is commonly deficient in the soil and often contributing to reduced plant growth and decreased crop yields (Fricke, 1997). It plays several important roles in the improvement of leaf area index (LAI), metabolic and regulatory processes in plants (Hirel *et al.* 2007). Nitrogen shortage leads to loss of green pigmentation in

leaves, decrease leaf area and photosynthetic production in most plant species (Mofokeng *et al.* 2015). This is due to high percentage of total leaf N allocated to the photosynthetic activity (Makino and Osmond, 1991).

Inadequate supply of nitrogen may lead to environmental pollution matters, delay in crop maturation and encourage excessive vegetative growth of plants. Water deficit reduces plant growth and development, cause plant disorder such as reduction in photosynthesis and transpiration resulting in substantial yield reductions (Fereres and Soriano, 2006). Plant dry matter (DM) is associated with leaf area (LA) and leaf photosynthetic rates. Water stress limits leaf area production and the rate of transpiration. Reduced transpiration may delay plant survival by extending the period of available soil water in the root zone (Taiz and Zeiger, 2006). The stomatal closure is due to the reduced photosynthesis in water stressed leaves. Higher stomatal conductance increases CO_2 diffusion into the leaf and results in higher photosynthetic rates which may lead to higher biomass and higher yields (Ashraf and Harris, 2013).

Water supply and nutrient efficiency are closely related, balanced application and determination of their type, amounts and methods should be based not only on the nutrient-supplying capacity, but also on water status of soil (Farooq *et al.* 2009). Nitrogen deficiency and water may result in variations in the morphological, physiological and biochemical changes of the plant species (Anjum *et al.* 2011). A few studies have shown the potential that medicinal plants can reveal under farmers' fields or experimental plots (Mofokeng *et al.* 2015). However, the actual response of plant species to cultivation method can vary due to several biotic and abiotic factors (Gouinguene and Turlings, 2002).

While a number of studies have evaluated the suitable agronomic practices for medicinal plants, the data on physiological and morphological approaches to improving productivity of *S.*

aethiopicus is limiting. With the increased demand for *S. aethiopicus* material, there is a need to identify agronomic practices suitable for *S. aethiopicus* to increase production levels. The cultivation approach will provide information on agronomic parameters and also conserve the species biodiversity. The aim of this study was to evaluate morphological and physiological and parameters of *S. aethiopicus* in response of irrigation regimes and nitrogen application rates.

4.2 Materials and methods

4.2.1 Site description

The trial was conducted in a rainshelter at the Agricultural Research Council – Roodeplaat Vegetable and Ornamental Plant (ARC-VOP), Pretoria, South Africa (25°59′S; 28°35′E and 1 200 m.a.s.l.). The rainshelter (12 m × 24 m ground area and ridge height of 5.5 m) is designed to automatically open when there is no rain and close during a rainfall event, thus excluding rainfall factor from the experiment. Soil samples were collected from the experimental site at three depths of 0–20, 20–40 and 40–60 cm for physical and chemical properties analysis. The soil classification was sandy clay loam comprising of 56.6% sand, 9.5% silt and 21.0% clay (Soil Classification Working Group, 1991). Soil nutrient analysis (Total N, Mg and K) was determined using the ammonium acetate extraction (1 N NH40Ac) method (Araya *et al.* 2006). Analysis for Al, Ca, Fe and Mn was determined by atomic absorption spectroscopy (Araya *et al*. 2006). The physical and chemical properties of the soil are presented in Table 4.1 and 4.2. The weather data recorded by a weather station (Campbell Scientific, USA) at the experimental site during the experiment period is presented in Table 4.3.

Table 4.1: Physical properties of the soil at the experimental site.

Soil depth cm	Sand	Silt	Clay	Texture	PWP	FC	BD (mg/m^3)
	%						
0–20	58.5	9.9	19.6	Sandy loam	10.3	19.9	1.59
20–40	57.3	9.6	19.0	Sandy clay loam	12.9	25.5	1.56
40–60	54.1	9.1	24.6	Sandy clay loam	15.2	25.3	1.45

PWP-Permanent wilting point; FC-Field capacity and BD-Bulk density (BD was determined based on the calibration curves of soil profile)

Table 4.2: Chemical characteristics of sandy clay loam soil collected from the experimental site. The data are average of duplicate analysis of soil samples collected.

Soil depth (cm)	Fe	Mn	Ca	Mg	K	Total N	pH H_20
	mg/kg					%	
0–20	30.9	85.6	1007	349	275	0.028	7.13
20–40	42.8	99.3	1066	355	174	0.027	7.16
40–60	29.3	71.9	1314	481	132	0.024	7.26

Table 4.3: Summary of weather data collected during the experiment period.

Months	Temp (°C)		Relative humidity (%)		VPD (kPa)	Rainfall mm	ET0 (mm)
	Max	Min	Max	Min			
September	28.1	11.0	79.4	23.3	1.61	29.9	165.7
October	29.7	11.9	79.0	19.6	1.08	94.7	131.3
November	27.8	14.3	86.2	34.7	0.99	175.2	147.2
December	28.7	16.3	89.4	39.7	1.13	136.4	161.3
January	30.1	16.5	89.2	35.2	1.35	32.5	151.6
February	31.8	16.9	88.2	27.0	1.20	71.6	135.4
March	30.1	14.7	88.1	29.2	0.94	43.6	101.4
April	27.4	10.8	90.7	30.5	1.15	0.00	94.8
May	27.5	6.1	83.9	18.5	0.77	1.02	71.0
June	21.2	2.9	84.5	24.9	0.85	0.00	69.1
July	22.2	3.1	83.0	22.7	1.35	0.00	89.5
August	27.2	5.9	76.5	15.1	1.43	56.6	121.7

*Rainfall and ET_0 were not averaged but totalled. Max: maximum, min: minimum, VPD: vapour pressure deficit.

4.2.2 Experimental design and plant material

The mother material used for this study was obtained from Mpumalanga province (23° 49' 59"S and 30° 9' 48"E). The rhizomes were preserved for further use at ARC-VOP Gene bank (ARC-M2008/027) in Pretoria, South Africa. Rhizomes of *S. aethiopicus* were transplanted into the rainshelter in September 2014 and were established for four months, to ensure a good stand, before application of the treatments. The experiment was established in two seasons (1st season: 2014/2015 and 2nd season: 2015/2016) in a randomised complete block design with 15 treatments. Each treatment was replicated three times. An experimental plot with 3.9 m^2 size, had a spacing of 25 cm between plants and 60 cm between rows, which gave a total of 66 667 plants per ha. Each experimental plot per treatment was divided into five rows, the three centre rows were used for data collection, while the two rows on either side acted as border rows. The treatments were set up in factorial combinations of five nitrogen application rates (0, 50, 100, 150 and 200 kg N/ha) and three irrigation regimes (30, 50 and 70% allowable depletion level, ADL).

4.2.3 Irrigation and fertilizer application

Soil moisture was monitored on a weekly basis using a neutron probe (Waterman, Probe Version 1.6, 2005, Geotech, USA) based on volumetric basis and calibrated against different soil water contents to a depth of 0.4 m and at intervals of 0.2 m. The three irrigation treatments were 30, 50 and 70% allowable depletion level (ADL) of plant available water (PAW). The experimental plots were irrigated when the respective allowable depletion level (ADL) threshold values for each treatment were reached. The concept requires that a certain percentage be depleted from the effective rooting depth (ERD) before refilling the soil profile back to field capacity. The study observations determined effective rooting depth (ERD) of *S. aethiopicus* as 400 mm. The 30% ADL treatment was referred to as the well-watered control, while the 50% and 70% ADL were the moderately and severely stressed water treatments, respectively (Mofokeng *et al.* 2015). A controlled pressure-compensated drip irrigation system, with water

discharge rate of 2400 ml per hour at pressure range of 150–200 kPa was installed (Netafim, Cape Town, South Africa). The allowable soil water deficits for the three water levels were calculated based on the field capacity (FC), permanent wilting point (PWP) and bulk density (BD) as shown on Table 4.1.

$PAW_{(0-200\ mm)} = ((FC - PWP) \times BD) \times 200\ mm$

$= ((0.199 - 0.103) \times 1.59) \times 200\ mm$

$= \underline{30.5\ mm}$

$PAW_{(200-400\ mm)} = ((FC - PWP) \times BD) \times 200\ mm$

$= ((0.255 - 0.129) \times 1.56) \times 200\ mm$

$= \underline{39.3\ mm}$

Total PAW for $ERD_{(40mm)} = 30.5\ mm + 39.3\ mm$

$= \underline{69.8\ mm}$

30% ADL of PAW = 69.8 × 30%

$= \underline{20.94\ mm}$

30% ADL of PAW = 69.8 × 50%

$= \underline{34.9\ mm}$

30% ADL of PAW = 69.8 × 70%

$= \underline{48.86\ mm}$

The nitrogen (N) treatments levels were applied at the following rates; control (0), 50, 100, 150 and 200 kg/ha. The five nitrogen fertilizer rates were applied per plant (0, 0.75, 1.5, 2.25 and 3.0 g N/plant) as split application (25% before planting, 25% at planting and 50% at emergence). The source used was limestone ammonium nitrate (LAN, 28%N). The experiment received potassium chloride (50% K) at 50 kg/ha and single superphosphate (11% P) at a rate of 50 kg/ha at planting using the basal application method according to the soil nutrient status and estimated nutrient requirements of Turmeric (Haque *et al.* 2007). Weed control performed manually with hand hoes and continued when necessary.

4.2.4 Data collection

4.2.4.1 Plant growth

Plant growth and physiology data collected in this study included plant height, number of leaves per plant, leaf area index, stomatal conductance, chlorophyll content, total biomass yield, fresh and dry rhizome biomass yield. These growth parameters were recorded for the duration of the experiment during both cropping season.

4.2.4.2 Plant height, fresh and dry mass

Plant height (cm) was recorded using a tape measure and number of leaves was counted manually. The growth parameters were taken on a monthly basis after the treatment implementation. To determine yield parameters at harvesting, total biomass and rhizome yield were weighed for the whole nine data plant first and then rhizomes weighed separately on a field scale (Platform digital scale, W113, Richter scale). The rhizomes were then oven dried (Economy oven, 620 digital, Labotec) at 50 °C until constant mass to obtain rhizome yield.

4.2.4.3 Leaf area index

Leaf Area Index (LAI) was measured using LAI2200 plant canopy analyser (Li-Cor Bioscience, USA). The instrument has two cross calibrated sensors one devoted to above canopy measurements and the other moving below the canopy to calculate the light interception at five zenith angles. The 270° view cap was used to take one above canopy reading and four below readings replicated twice for all the treatments. LAI measurements were taken on a monthly basis after treatment implementation.

4.2.4.4 Stomatal conductance

Leaf stomatal conductance, was measured with a diffusion porometer (SC-1 Leaf porometer (Decagon Devices, USA) on a monthly basis. Stomatal conductance estimates the rate of passage of carbon dioxide or water vapour loss through the stomata of a leaf. Fully expanded

mature leaves for each treatment were measured on the abaxial (bottom) surface during midday when environmental conditions were at their peak. Without troubleshooting, each measurement took about 3 min, which was the minimum time allowed for the reading to stabilize before they were recorded.

4.2.4.5 Leaf chlorophyll content

Leaf chlorophyll content was measured on a fully matured leaf, with a chlorophyll content meter (SPAD 502 plus, Konica Minolta, Japan) at harvesting. Nine data plants per plot from the three replicates were used for this measurement.

4.2.5. Data analysis

All data was subjected to analysis of variance using GenStat software 17.1 edition (VSN International, Hemel Hempstead, UK). The treatment means were separated using $(p < 0.05)$ Duncan's multiple range test (DMRT).

4.3 Results and Discussion

Interactions between 2014/2015 (n=15) and 2015/2016 (n=15) cropping season for irrigation regimes and nitrogen levels application were not significantly different ($p < 0.05$) and data were therefore pooled (n=30) and subjected to statistical analysis.

4.3.1 Irrigation applied and soil water depletion patterns

The soil water deficits allowed per treatment before the amount of irrigation was initiated for two growing seasons of the experiment is presented in Figure 4.1, 4.2 and 4.3. The average amount of daily deficits and amount of water applied per nitrogen level for well watered treatment (30% ADL), moderately stressed (50% ADL) and severely stressed treatment (70% ADL) are presented in Table 4.4. The total relative evapotranspiration demand (ET_0) for the treatment period was 1440.48 mm (Table 4.3). In this study, well watered treatment (30% ADL) recorded the highest

water usage per nitrogen rate applied, followed by moderate (50% ADL) and least in severely stressed treatment (70% ADL).The recorded amounts of water supplied for each nitrogen level during the growth period is within the range reported by Darwish *et al*. (2006).

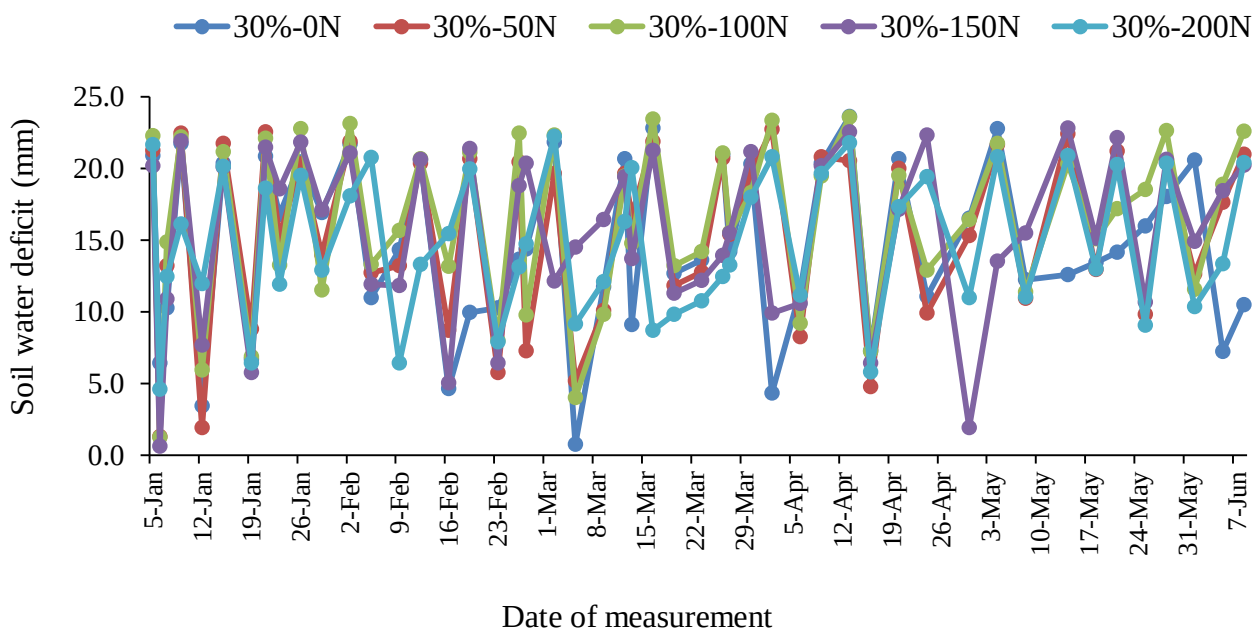

Figure4.1: Changes in soil water deficits (top 400 mm root zone) for the well watered (30% ADL) and five nitrogen levels (0, 50,100,150 and 200 kg/ha).

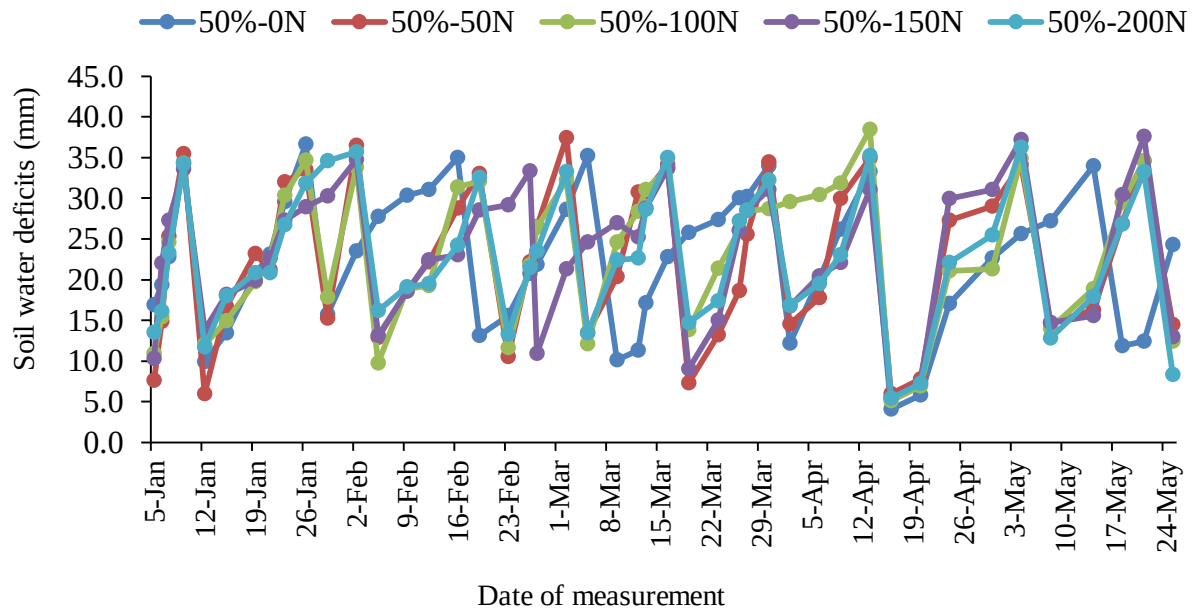

Figure 4.2: Changes in soil water deficits (top 400 mm root zone) for the moderately stressed (50% ADL) and five nitrogen levels (0, 50,100,150 and 200 kg/ha).

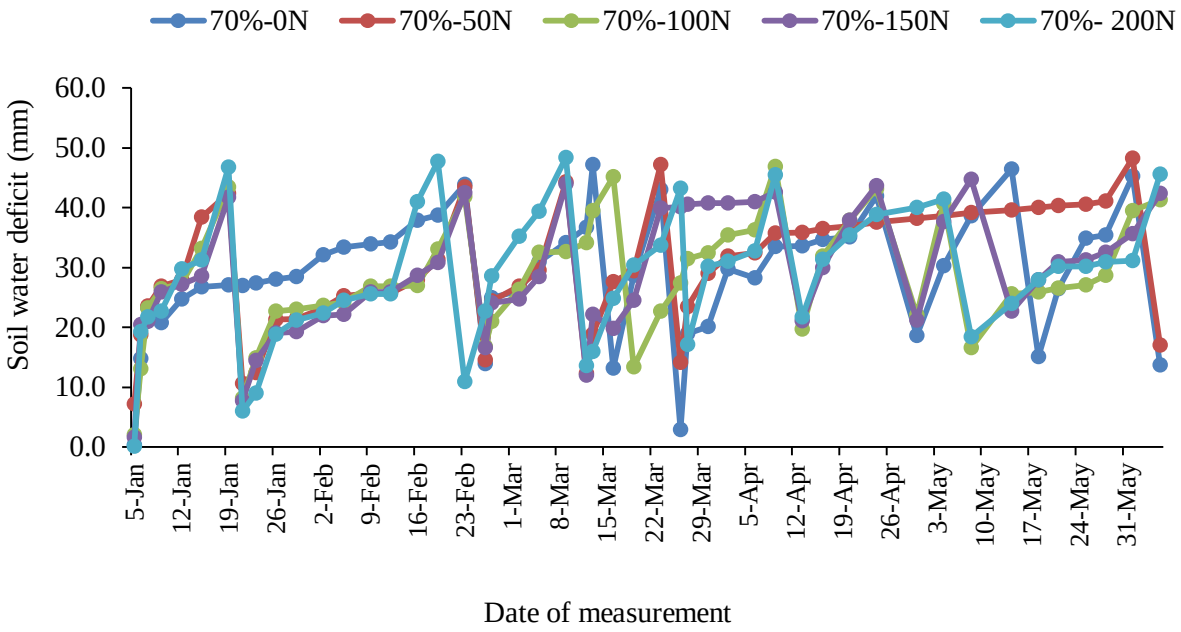

Figure 4.3: Changes in soil water deficits (top 400 mm root zone) for the severely stressed (70% ADL) and five nitrogen levels (0, 50,100,150 and 200 kg/ha).

Table 4.4: Mean variation in soil water deficits (top 400 mm root zone) during cropping season for the well-watered (30% ADL), moderately stressed (50% ADL) and severely stressed (70% ADL) treatments.

Allowable depletion (ADL)	Nitrogen levels	Average deficits (per day)	Total irrigation amount (per season)
%	N (kg/ha)	mm	mm
30	0	21.29	340
	50	21.05	484
	100	21.78	457
	150	21.14	401
	200	20.55	328
50	0	31.81	286
	50	34.82	348
	100	34.33	377
	150	33.91	305
	200	34.19	410
70	0	44.60	267
	50	45.10	225
	100	43.20	302
	150	43.08	301
	200	45.51	318

4.3.2 Plant growth parameters

4.3.2.1 Plant height

Plant height ranged from 15.06 cm in January to 41.91 cm in May. The results indicate significant differences for plant height in response to the growing period (Table 4.5). There were significant differences between some nitrogen application rates and irrigation regimes. The results indicate a gradual increase in plant height throughout the growing period (Table 4.5). Plant height was at its highest peak at 41 cm in May compared to other months after planting (Table 4.5). The morphological changes in the growth of *S. aethiopicus* can be considered as a morphological adaptation to species maturity. The maximum observed plant height (41 cm) is in agreement with the 40 cm reported for *S. aethiopicus* (Manzini, 2005).

The comparison of plant height in response to irrigation regimes and nitrogen application rates did not differ significantly (Table 4.6). Plant height indicated an increased with the application rate of 100 kg N/ha for well watered treatment (30% ADL), followed by moderately stressed treatment (Figure 4.4). Reduced plant height under severely stressed treatment (70% ADL) can be attributed to deficit irrigation which altered the morphology of the species reducing plant height and shoot growth. The increase in water stress levels of *Thymus daenensis* resulted in a decrease of plant height (Bahreininejad *et al.* 2012). Similar results were reported by Alishah *et al.* (2006) and Hedayati *et al.* (2013) for basil and Jatropha curcas, respectively. Growth reduction as a result of water deficit has been widely reported (Bettaieb *et al.* 2009; Ekren *et al.* 2012).

Our findings did not show significant differences between the nitrogen application rates, however, other studies revealed high plant height of sweet pepper in response to application of 100 and 150 kg/ha (Aminifard *et al*. 2012). Growth development of a plant species depend on the amount of inputs applied. Therefore, application of N coupled with irrigation management

in this study is significant for the development of production systems and profitability of the plant species.

4.3.1.2 Number of leaves per plant

This study examined the extent to which number of leaves is produced over a growing period. Number of leaves per plant increased significantly from March and reached the highest peak in May (Table 4.5). The results showed the same trend depicted for the plant height.

The results showed no significant differences in response to irrigation regimes, but there was an interaction effect between nitrogen application rates and water stress levels regimes (Table 4.6 and Figure 4.5). Well watered treatment (30% ADL) showed high number of leaves per plant with the application rate of 100 kg N/ha (Figure 4.5). Interpretation of number of leaves produced per plant can be associated with important physiological functions of individual leaves along environmental gradients including moisture and temperature. Moderately stressed (50% ADL) and severely stressed (70% ADL) treatment also showed high number of leaves per plant, though the variation was minimal (Figure 4.5). The reduced number of leaves for parsley and basil plants has been reported in response to water stressed levels (Petropoulos *et al*. 2008; Alishah *et al*. 2006). Hussain *et al*. (2006) reported the maximum number of branches per plant with the application of 90 kg N/ha on asparagus. Furthermore, Law and Egharevba (2009) indicated that increased application nitrogen rates can increased the yield and number of fruits per plant.

4.3.1.3 Leaf area index (LAI)

The mean leaf area index ranged between 1.17 and 1.64 (m^2 leaf area·m^2 ground area) for May and April, respectively. There was no significant variation for LAI over the growing period (Table 4.5). The plant recorded LAI in May, this is during the dormant stage that *S. aethiopicus.*

LAI of *S. aethiopicus* with different nitrogen application rates and irrigation regimes showed no substantial differences (Table 4.5). LAI increased reaching a peak of 1.80 (m^2 leaf area·m^2 ground area) and then decrease in response 0 kg N/ha irrespective of the water stress levels (Figure 4.7). Shafiq (2002) reported the highest LAI for irrigated plants than water stressed plants. Furthermore, Eiasu *et al*. (2009) reported a significant decline in LAI between the well watered control and the water stressed treatments of rose-scented geranium.

4.3.1.4 SPAD measurements

The average results on the response of SPAD measurements over the growing period are presented in Table 4.5. SPAD measurements were not significantly different in all months after planting (growth period). A significant interactive effect of N application rates and irrigation regimes was observed for SPAD measurements (Table 4.6). Maximum SPAD values were observed at 150 kg N/ha and declined progressively reaching the lowest with the application rate of 200 kg N/ha for severely stressed 70% ADL (Figure 4.8). The higher SPAD values observed in our study, corroborate findings of Zhao *et al.* (2005). The lower SPAD values at 200 kg N/ha might have been due to remobilization of N from leaves to reproductive organs as the rhizome mature and the effect of water stress levels. The reduction in SPAD values and chlorophyll content under severely stressed treatment has been reported in sunflower (*Helianthus annuus*) plants (Kiani *et al.* 2008). A low concentration of chlorophyll content is known to limit photosynthetic potential and directly decrease biomass production in plants (Mohammadian *et al*. 2005).

4.3.1.5 Stomatal conductance

Stomatal conductance of *S. aethiopicus* was evaluated in response to nitrogen application rates and irrigation regimes for better understanding and recommendation. The results showed significant variations in response to nitrogen application rates (Table 4.6). *S. aethiopicus* showed great tolerance to irrigation regimes, avoiding desiccation by decreasing stomatal

conductance as water became limiting. The results showed high stomatal conductance for well watered control (30% ADL) in February followed by moderately water stressed treatment (50% ADL). Decreases in stomatal conductance and assimilation values have been reported as some indicators of water stress in fruit trees (Machado *et al.* 1999).

Water stress levels have been reported to reduce both stomatal conductance and biomass production of plant species (Mofokeng *et al.* 2015). Plants have mechanisms for preventing turgor loss through stomata closure and osmotic adjustment. Eiasu *et al*. (2012) reported lower stomatal conductance in response to water stress treatments of *Pelargonium capitatum.*

The increase in stomatal conductance of *S. aethiopicus* reported in February irrespective of the water treatment is associated with vapor pressure deficits (VPD) during the growth period (Table 4.3). Conversely, the decrease in from March to May could be attributed to the dormant stage of the plant species. This period coincided with low demand for water by the plant as few leaves were transpiring. The higher stomatal conductance observed for the well watered control (30% ADL) can also be associated with the opening of stomata. Plants exposed to dormant conditions have been reported to partially close their stomata until lower shoot water potential is reached (Pembebleton *et al.* 2014). The lower stomatal conductance observed for severely stressed treatment is associated with the closing of stomata which limits water loss through transpiration and reduces intracellular carbon dioxide (CO_2) availability (Zhao *et al.* 2014). The lack of significant effect of nitrogen application rates and water stress on stomatal conductance was reported by Green and Mitchell (1992).

4.3.1.6 Total fresh and dry biomass yield

The results showed no interactive effect of N application rates and water stress treatment on total fresh and dry biomass yield. Irrigation regimes, however showed significant variations for total fresh and dry biomass (Figure 4.9 and 4.10). Well watered treatment (30% ADL) showed

significantly higher total fresh and dry biomass compared to moderate (50% ADL) and severe stressed (70% ADL) treatments. Total fresh and dry biomass of plants decreased due to exposure to severe water stress (Figures 4.9 and 4.10). Bahreininejad *et al*. (2013) reported tremendously decreased in fresh yield of *Thymus daenensis* for moderate water stress (at 50% MAD) and severe water stress (at 80% MAD). The non-stressed control (20% MAD) recorded high fresh biomass production. The decrease could be associated with the lower leaves senescence (Graça, *et al.* 2010). The reduced yield can also be effected by water which stimulate and regulate the photosynthetic enzymes of plants. Abdul-Hamid *et al.* (1990) reported that the reduction in chlorophyll content and photosynthesis efficiency could also lead to reduced biomass yield. Fresh and dry biomass yield of *Ocimum basilicum* (L.) exposed to water stress level was significantly decreased as plant water deficit increased (Simon *et al.* 1992). Furthermore, Baher *et al.* (2002) reported similar results on *Satureja hortensis* (L.) (Savory) plants whereby the fresh and dry weights significantly decreased under water stress conditions. This finding indicates that water stress is not a favorable component of increasing biomass production in most plant species.

The results showed significant increase in total fresh biomass in response to nitrogen application rates at 50 to 100 kg N/ha (Figure 4.11). The biomass production was largely a function of photosynthetic surface, which was favorably influenced by nitrogen application rates. The lowest biomass yield observed in the control (0 kg N/ha) can be associated with depletion of nutrients in the soil.

Plants with deficient nutrients exhibited stunted growth and were pale in colour when compared to greener plants. The increase in biomass production of *S. aethiopicus* due to nitrogen application rates was also reported by Magdatena (2003). The study increased leaf dry matter with an increase in nitrogen application rates. As a result of application rate of 90 kg N/ha, asparagus showed increased plant weight (Hussain *et al*. 2006). The increase in biomass

production can also be associated to an increase in number of branches and plant height, which can effectively enhance photosynthetic activity (Hussain *et al*. 2006). Mofokeng *et al.* (2015) reported increased total biomass with an increase of nitrogen application rates from 0 to 50 kg/ha for *Pelargonium sidoides.*

4.3.1.7 Interrelationships among morphological and physiological characters of *S. aethiopicus*

Plant characteristics are important because they reflect morphological and physiological functions of a species. They have the potential to summarize plant strategies in terms of water use efficiency, growth pattern and nutrient use. The results of this study show that functional growth traits (plant height and number of leaves per plant) are related to other physiological characteristics. The interrelationship is observed by the progressive increase of plant height, number of leaves per plant and leaf area index (LAI) over the growing period (Table 4.5). Nitrogen application rates also serve as determinant factor for photosynthetic ability of the leaves (Hussain *et al*. 2006). Nitrogen application is a functional factor in the increase of dry matter production. Dry matter production of *S. aethiopicus* increased with the application of 50 and 100 kg/ha (Figure 4.11).

4.4. Conclusion

The results of this study conclusively reveal that the plant height and number of leaves per plant were significantly higher towards maturity. Plants grown with 50 and 100 kg N/ha had greater plant height, number of leaves per plant, LAI, SPAD values and biomass yield that eventually resulted in higher dry matter production. Stomatal conductance was higher throughout the growing period and decline in response water stressed treatment. The high amount of water utilized from well watered treatment (30% ADL) compared to moderate (50% ADL) and severe (70% ADL) treatments could be attributed to improved water availability and superior plant canopies. Further experiments should be conducted to evaluate different combinations of agronomic practices to fully exploit the growth of *S. aethiopicus* under different conditions.

Table 4.5: Averages of leaf area index, plant height and number of leaves of *S. aethiopicus* over the growth period.

Growth period (*MAP)	Plant height	Number of leaves/plant	LAI (m^2 leaf area·m^2 ground area)	SPAD values
January	15.06^{c}	16.5^{c}	1.51^{b}	25.39^{b}
February	22.93^{bc}	21.5^{b}	1.47^{b}	27.91^{ab}
March	29.29^{b}	25.5^{b}	1.63^{ab}	37.55^{a}
April	35.88^{ab}	30.0^{ab}	1.64^{ab}	26.14^{b}
May	41.91^{a}	34.0^{a}	1.17^{a}	26.35^{b}
F-statistics	1.83**	6.81***	6.15**	1.54*

MAP=month after planting. Values with dissimilar letters in a column are significantly different at $p < 0.05$ using Duncan Multiple Range test.

Table 4.6: Comparison of plant height, number of leaves per plant, chlorophyll content, LAI and stomatal conductance of *S. aethiopicus* in response to irrigation regimes and nitrogen levels.

	Plant height (cm)	Number of leaves per plant	SPAD values	LAI (m^2 leaf area·m^2 ground area)	Stomatal conductance (mmol m^{-2} s^{-1})
Nitrogen (N)	61.4***	19.5***	1.3^{ns}	2.15^{ns}	1.3^{ns}
Irrigation	28.95***	1.84^{ns}	5.2^{ns}	2.55^{ns}	5.3**
Nitrogen × Irrigation	61.7**	23.7***	2.75*	2.8*	1.2^{ns}

p< 0.001 (***), p < 0.01 (**), p < 0.05 (*), ns=not significant.

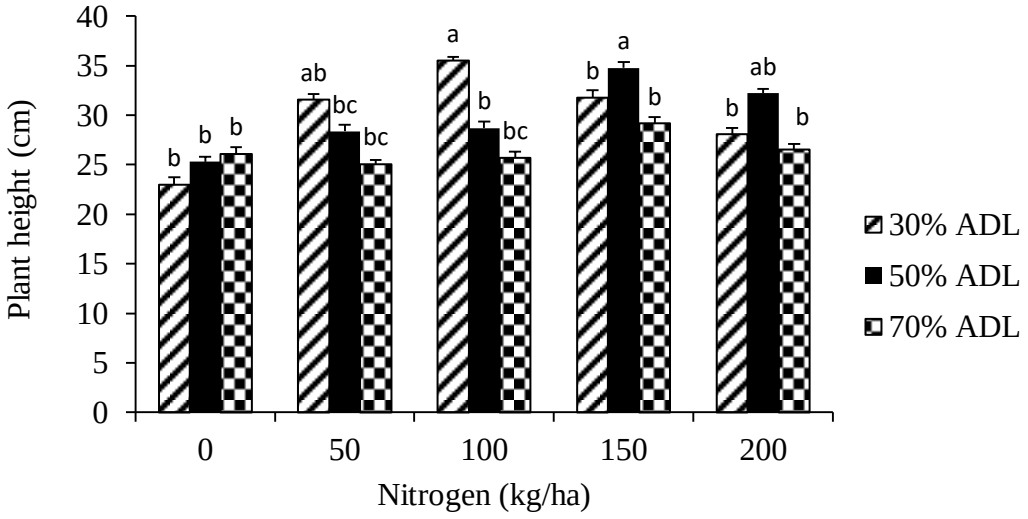

Figure 4.4: Interactive effect of nitrogen application rates × irrigation regimes on plant height of *S. aethiopicus.* Bars followed by dissimilar letters are significantly different at $p < 0.05$ and vertical lines on bars represent standard error (S.E).

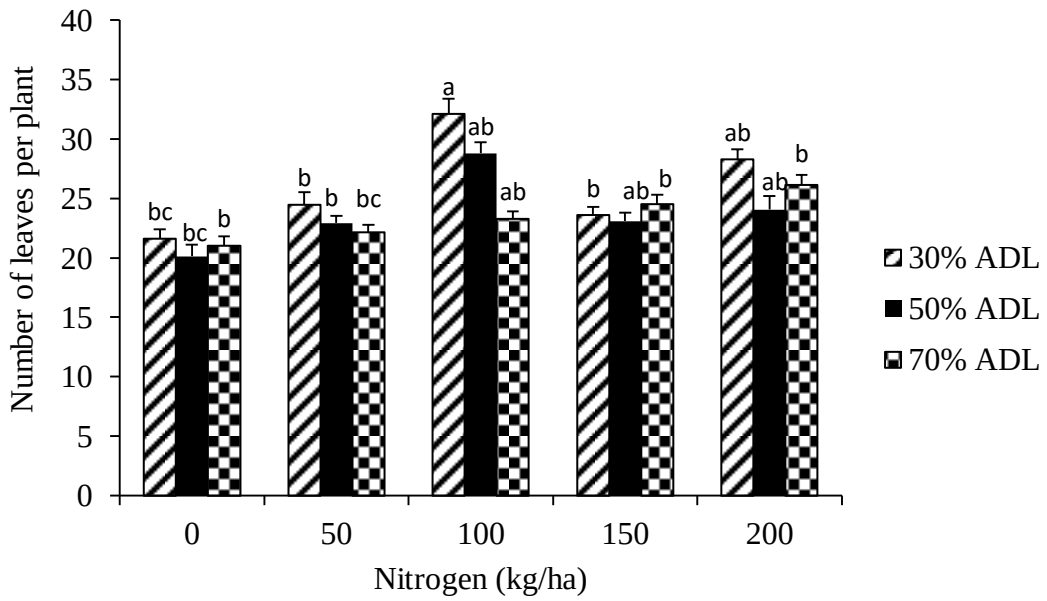

Figure 4.5: Interactive effect of nitrogen application rates × irrigation regimes on number of leaves per of *S. aethiopicus.* Bars followed by dissimilar letters are significantly different at $p < 0.05$ and vertical lines on bars represent standard error (S.E).

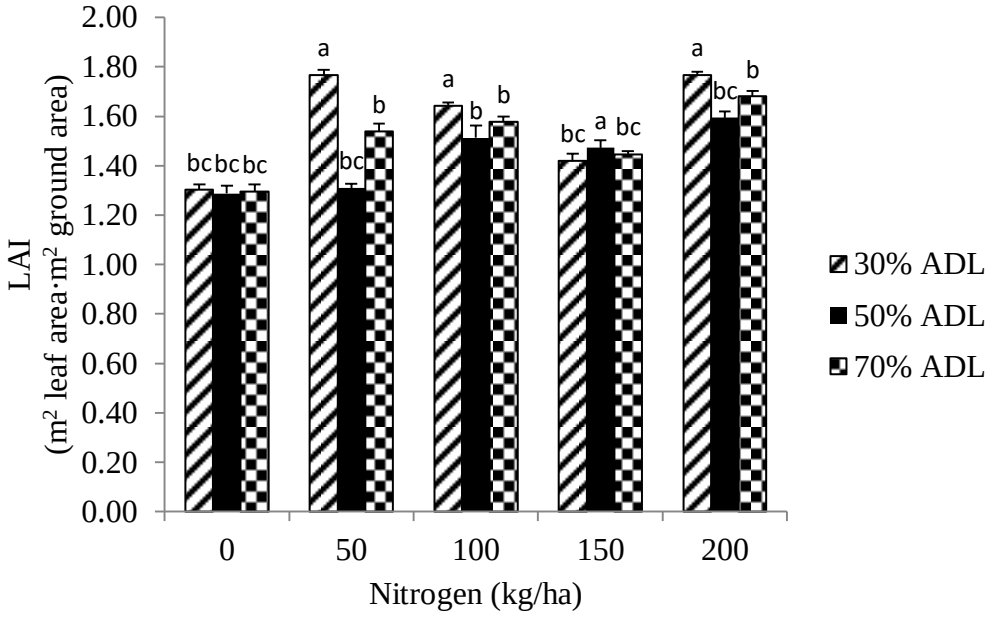

Figure 4.6: Interactive effect of nitrogen application rates × irrigation regimes on leaf area index (LAI) of *S. aethiopicus.* Bars followed by dissimilar letters are significantly different at $p < 0.05$ and vertical lines on bars represent standard error (S.E).

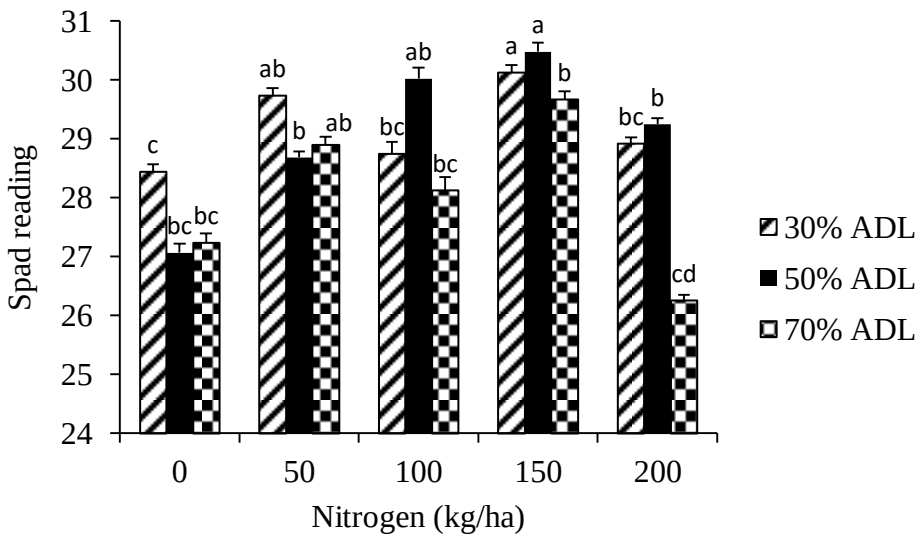

Figure 4.7: Interactive effect of nitrogen application rates × irrigation regimes on SPAD measurements of *S. aethiopicus.* Bars followed by dissimilar letters are significantly different at $p < 0.05$ and vertical lines on bars represent standard error (S.E).

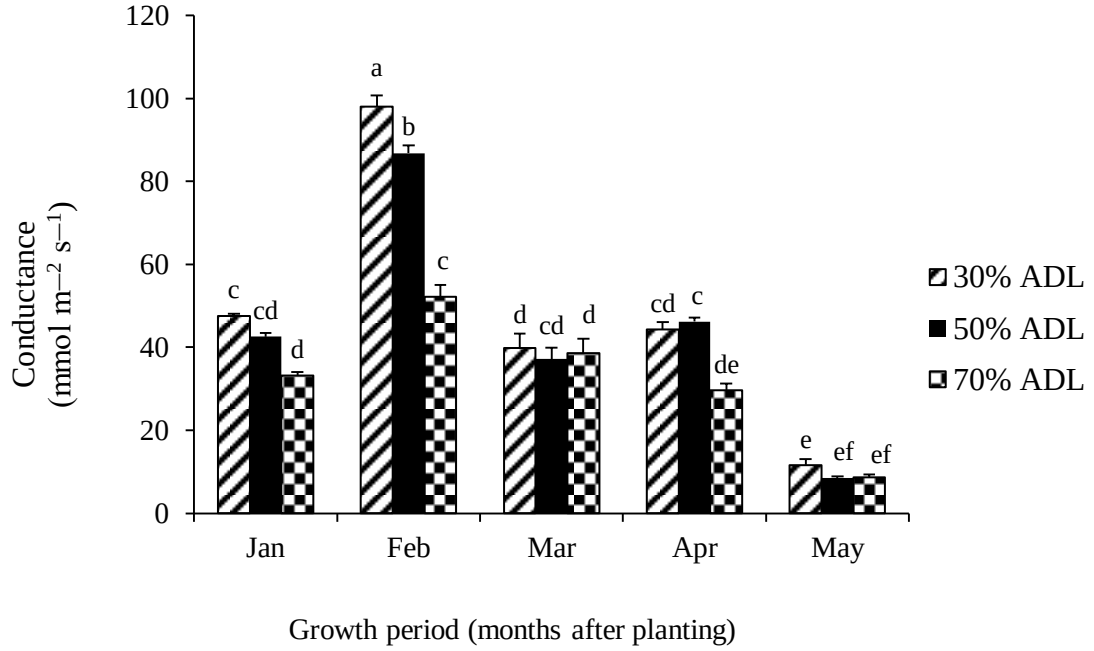

Figure 4.8: Average stomatal conductance of *S. aethiopicus* over the growing period in response to irrigation regimes. Bars followed by dissimilar letters are significantly different at $p < 0.05$ and vertical lines on bars represent standard error (S.E).

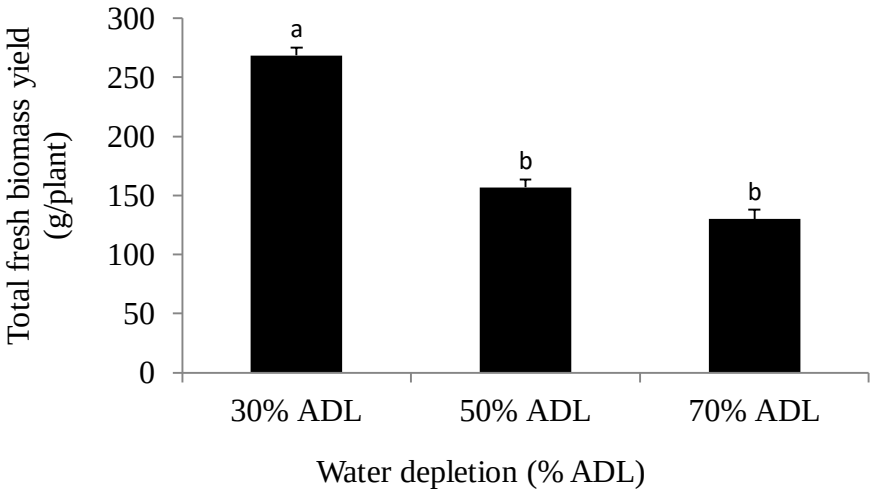

Figure 4.9: The effect of irrigation regimes on total fresh biomass yield of *S. aethiopicus.* Bars followed by dissimilar letters are significantly different at $p < 0.05$ and vertical lines on bars represent standard error (S.E).

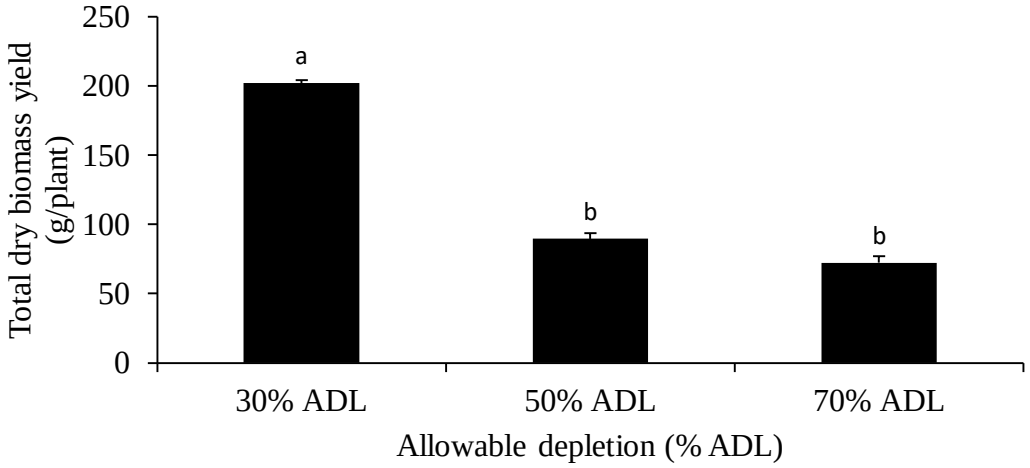

Figure 4.10: The effect of irrigation regimes on total dry biomass yield of *S. aethiopicus.* Bars followed by dissimilar letters are significantly different at $p < 0.05$ and vertical lines on bars represent standard error (S.E).

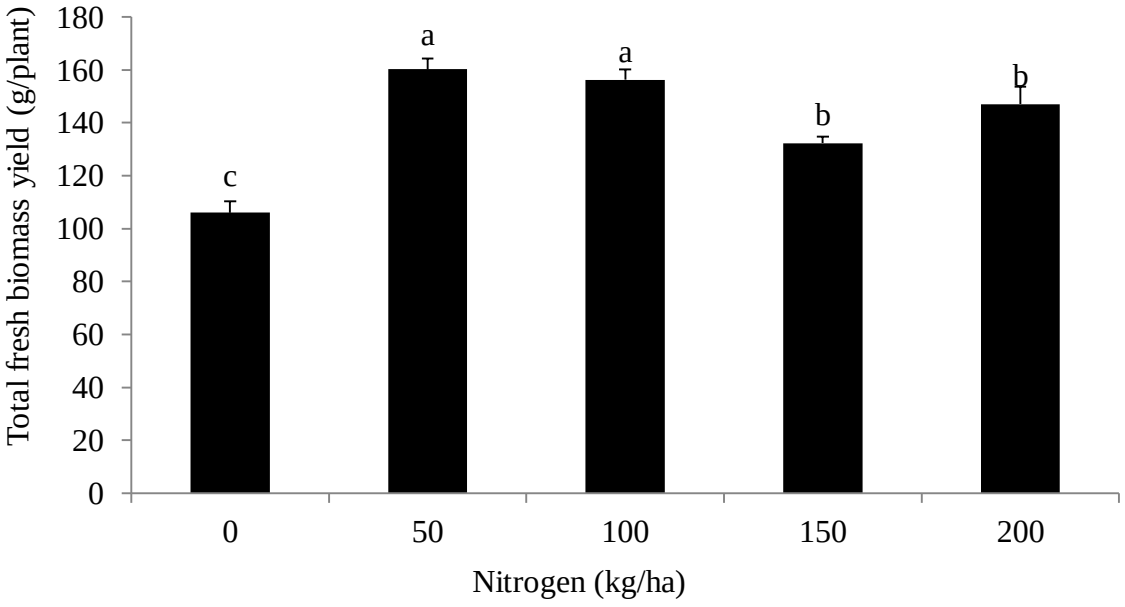

Figure 4.11: The effect of nitrogen fertilizer on total dry biomass yield of *S. aethiopicus.* Bars followed by dissimilar letters are significantly different at $p < 0.05$ and vertical lines on bars represent standard error (S.E).

Table 4.7: Effect of irrigation regime on the fresh and dry rhizome weights of *S. aethiopicus*

Water depletion (% ADL)	Fresh rhizome weight g/plant	Dry rhizome weight g/plant
30	45.26[a]	28.12[a]
50	39.01[ab]	23.30[ab]
70	37.72[ab]	15.20[b]
F-statistics	3.19**	2.01**

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CHAPTER FIVE

Antioxidant Activity and Soluble Sugars of African ginger (*Siphonochilus aethiopicus*) in response to Irrigation Regimen and Nitrogen levels.

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CHAPTER FIVE

ANTIOXIDANT ACTIVITY AND SOLUBLE SUGARS OF AFRICAN GINGER (*SIPHONOCHILUS AETHIOPICUS*) IN RESPONSE TO IRRIGATION REGIMEN AND NITROGEN LEVELS.

ABSTRACT

African ginger (*S. aethiopicus*), as a medicinal plant, is known for its medicinal properties, which contains various antioxidant compounds and carbohydrates. Rhizome yield is improved by water regimens and fertilizers applied at plant phenological stages. However, the rhizomatous herb, which is traditionally used for the treatment of asthma, inflammation and malaria, has limited information on water and nitrogen requirements for its production. This study assessed the effect of irrigation regimens (30, 50 and 70% allowable depletion level (ADL) and nitrogen levels (0, 50, 100, 150 and 200 kg/ha) on antioxidant activity and carbohydrates on plant leaf, root and rhizome. The interaction treatment effect of severely stressed (70% ADL) with the application of 100 kg N/ha had significant effect on leaf phenolic concentration (87.02±2.51 mg/g Gallic Acid Equivalent (GAE) compared to other water stress treatments (30% and 50% ADL) and Nitrogen (N) application rates (0, 50,150 and 200 kg N/ha). Interaction effect of moderately stressed (50% ADL) and severely stressed (70% ADL) treatment with application rate of 0 kg N/ha had significant effect on plant flavonoids and phenolics accumulated in leaf, rhizome and root parts. In plant carbohydrates, root had high sucrose content (47.68±9.0 mg/g DW, Dry Weight) with the application of low N (0 kg N/ha) grown under severely stressed (70% ADL) treatment. In conclusion, this implies that different *S. aethiopicus* parts can produce substantial amount of antioxidants and carbohydrates, as exhibited under low N and reduced water supply applied during the phenological cycle.

Keywords: Fertilizer effects, plant secondary metabolites, water deficits, soil moisture

5.1 Introduction

Medicinal plants play an essential role in the development of human culture, provide first-line and basic health to cure many ailments. The majority of the world's population in developing countries still rely on herbal medicines for health and healing needs (Ekor, 2014). While a significant portion of the South African population depend on synthetic drugs for medicine, utilization of herbs and medicinal plants remain the most affordable and easily accessible source of treatment for the primary health care system of resource poor communities (Hosseinzadeh *et al.* 2015).

S. aethiopicus (Schweinf.) B.L. Burtt, commonly known as African ginger, a rhizomatous herb, belonging to the family Zingiberaceae. The plant is indigenous to South Africa (van Wyk *et al.* 2009), and widely distributed in other regions of Africa, occurring from Senegal and Ethiopia to Zimbabwe, Malawi and Zambia (van Wyk, 2008). Some members of Zingiberaceae such as Hedychium and Alpinia species are naturalized in certain areas (Kress *et al.* 2002). While *S. aethiopicus* showed commercial economic value, it is becoming scarce in the wild due to overharvesting (Diederichs *et al.* 2002).

S. aethiopicus rhizomes possess great medicinal benefits due to the presence of a medicinally significant compound such as siphonochilone (van Wyk, 2008). The herb is used traditionally for the treatment of coughs, colds, asthma, headaches, pain, inflammation and malaria (Stafford *et al.* 2005). Furthermore, anti-bacterial (Coopoosamy *et al.* 2010), anti-inflammatory (Gericke, 2001), antimalarial (Lategan *et al.* 2009) and anti-candida properties (van Wyk, 2008) have been investigated in the rhizome extracts. Moreover, several bioactive compounds have been isolated from the rhizome of *S. aethiopicus* including two

furanoterpenoids, which represents 20% of the oil composition of the extract (Holzapfel *et al.* 2002).

Bioactive compound synthesis is enhanced by numerous biotic and abiotic stress factors such as water availability, limited nutrients, temperature and radiation (Reddy *et al.* 2004). Among these factors, water and nutrient supply can influence growth, biomass yield, and phyto-nutritional status of the plant (Mofokeng *et al.* 2015). In order to explore opportunities to improve the productivity and understand plant responses to varying factors, irrigation and nitrogen application has to be studied to achieve optimum production that is commercially viable. Application of fertilizers exhibit high yield and increase carbon based secondary metabolites syntheses, which are beneficial for the primary functioning in plants (Lemoine *et al.* 2013). Despite this finding, investigation of secondary metabolites from medicinal plants in response to fertilizer levels and water stress is limited. Few studies have revealed that under stressed conditions, plants exhibit high production levels of several secondary metabolites (Jaleel *et al.* 2007). The accumulation of secondary metabolites has also been reported to be induced with exposure to nutrient deficiency (Stewart *et al.* 2001), and high temperature (Couceiro *et al.* 2006).

It is essential to understand the antioxidant system and carbohydrate mobilization in response to water requirement and nitrogen application in different plant parts. Thus, the current study is aimed at evaluating the antioxidant activity and soluble sugars content of different parts of *S. aethiopicus* in response to irrigation regimens and nitrogen application rates.

5.2 Materials and methods

For details on site description, material source, experimental design, treatment application, harvesting and sample preparation please refer to Chapter Four.

5.2.1. Determination of total phenolic and flavonoid content

Total phenolic and flavonoid content of a crude extract was determined using the Folin-Ciocalteu and aluminium chloride ($AlCl_3$) colorimetric, respectively (Li *et al.* 2008; Ordon *et al.* 2006). The absorbance was measured in triplicates at 765 nm using a spectrophotometer and amounts were expressed using Gallic acid as the standard (mg GAE/g dry weight basis). The absorbance for total flavonoid content was measured at 510 nm in triplicates and a standard curve was plotted using Quercetin (mg QE/g dry weight basis).

5.2.2. Total antioxidant activities using FRAP and DPPH assay

The antioxidant activity of plant part (leaf, rhizome and root) was determined using the ferric reducing power assay and 2, 2'-diphenyl-1-picryl hydrazyl (Ndhlala *et al.* 2014). Butylated hydroxytoluene (BHT) and Ascorbic acid dissolved in methanol and distilled water, was used as standards for ferric reducing power assay. Samples for the assay was prepared in triplicate and repeated twice. The absorbance was read at 517 nm and 630 nm for ferric reducing power assay and 2, 2'-diphenyl-1-picryl hydrazyl using a microtiter plate reader (Enzyme-linked immunosorbent assay (ELISA), Microplate Reader, California, USA). The reducing antioxidant power of the extract, Butylated hydroxytoluene (BHT) and ascorbic acid were expressed by graphically plotting absorbance against concentration. The reaction mixture was incubated in the dark at room temperature for 30 minutes.

Percentage of DPPH scavenging activity was calculated as percentage (%) inhibition of DPPH [Eq. 1].

Where; Abs_{517nm} sample is the absorbance of the reaction mixture, which contains the resuspended extract or positive control and Abs_{517nm} Neg control is the absorbance of the negative control. The free radical scavenging activity (RSA) was calculated as follows;

$$RSA\ (\%) = 1 - (Abs_{517nm}\ sample / Abs_{517nm}\ Neg\ Control) \times 100 \quad \text{[Eq.1]}$$

5.2.3. Determination of soluble sugar concentration

Plant soluble sugars concentration was determined according to Tesfay *et al.* (2011), with slight modifications. Briefly, freeze-dried, plant powder (0.05 to 0.10 g in dry weight basis) was mixed with 10 mL 80 % (v/v) ethanol and homogenized using an Ultra-Turrax (Model T25D, Germany) for 60 s. The mixture was incubated in an 80 °C water bath for 60 min and kept at 4 °C overnight. After tubes were centrifuged (12, 000 g for 15 min, 4 °C), the supernatant was filtered through glass wool and dried in a Savant Vacuum Concentrator (SpeedVac, Savant, New York, USA). Dried samples were resuspended with 2 mL ultra-pure water, filtered through 0.45 μm nylon filters and analysed using an HPLC-RID (High performance liquid chromatography-refractive index detector) system (Liquid chromatography (LC-20AT), Shimadzu Corporation, Kyoto, Japan) equipped with a refractive index detector (Refractive index detector (RID-10A), Shimadzu Corporation, Kyoto, Japan) and a Rezex Monosaccharide column (300 mm x 7.8 mm, millimetre) (8-micron pore size; Phenomenex®, Torrance, California, USA). The concentration of individual sugars was determined by comparison with authentic sugar standards.

5.2.4 Statistical analysis

All data was subjected to analysis of variance using GenStat software 17.1 edition (VSN International, Hemel Hempstead, United Kingdom). GraphPad Prism version 5.00 for Windows (GraphPad Software., San Diego, California) was used construction of graphs and calculation of IC_{50}/EC_{50} (effective concentration) values. The treatment means were separated using ($p < 0.05$) Duncan's multiple range test (DMRT).

5.3 Results and discussion

An interaction between 2014/2015 (n=15) and 2015/2016 (n=15) cropping season for irrigation regimes and nitrogen levels application were not significantly different ($p < 0.05$) and data were therefore pooled (n=30) and subjected to statistical analysis.

The analysed data showed that various nitrogen levels and irrigation regimens (Table 5.4) influenced accumulation of total phenolics and flavonoids in different parts of *S. aethiopicus*. Generally, total phenolic content was highest in the leaf followed by root and rhizome. The results showed that leaf (87.02±2.51 mg/g GAE) had the highest total phenolic content, followed by rhizome (26.83±0.66 mg/g GAE) and root (23.06±0.60 mg/g GAE) with nitrogen and irrigation levels (Table 5.4). Total phenolic content showed an increasing trend for leaf (81.03±0.9 mg/g GAE), root (26.83±0.66 mg/g GAE) and rhizome (23.06±0.60 mg/g GAE) under severely stressed treatment (70% ADL). The current findings were in agreement with Ghasemzadeh *et al.* (2010) and Ibrahim *et al.* (2012), whereby high phenolic and flavonoid content was observed in the in the leaf, followed by rhizome and root extracts of *Labisia pumile.* As the plant received minimal nitrogen (N) levels (0 > 100 kg N/ha) the production of total phenolics and flavonoids was enhanced. It was apparent that optimum fertilization at 100 kg N/ha, increased high values in total phenolics and flavonoids

(87.02±2.51 mg/g GAE and 2.05 ±0.23 mg/g QE) of the leaf and root, respectively. Total flavonoids content followed the same trend as total phenolics where the highest content was observed in the root under moderately stressed (50% ADL; 377 mm per season) and severely stressed (70% ADL; 302 mm per season) water regimes (Table 5.2 and 5.4).

The increase in the production of total phenolics and flavonoids under low-N conditions in this study might be due to an over flow mechanism for carbon not utilized for plant growth due to limited nitrogen (Coviella *et al.* 2002). The reduced application of water indirectly enhanced the biosynthesis of total phenolics and flavonoids of *S. aethiopicus* treated with minimal nitrogen fertilizer. Water stress has been reported to limit protein synthesis and could therefore regulate total phenolic and flavonoid content (Kumar and Pandey, 2013). This implies that reduced amounts of nitrogen and water supply can enhance production of plant secondary metabolites in different parts of *S. aethiopicus.*

Antioxidant capacity of *S. aethiopicus* parts was significantly different, when plants were grown under various levels of nitrogen and irrigation regimens. Increasing the supply of nitrogen fertilization rates during the phenological cycle of the plant had significant effect on FRAP activity of *S. aethiopicus* parts. The FRAP values for the leaf, rhizome and root grown under two different nitrogen rates (150 and 200 kg/ha) were significantly lower than those under low-N levels (Figure 5.1A, B and C; Figure 5.2A, B and C; Figure 5.3A, B and C). Under low N-application (0 kg/ha) with moderately stressed (50% ADL; 286 mm per season) and severely stressed (70% ADL; 267 mm per season), leaf showed high reducing ability (Figure 5.1A, B and Table 5.2). Kovacik and Backor (2007) also reported similar results demonstrating that *Matricaria chamomilla* plants cultivated under nitrogen-poor condition contained more secondary metabolite compounds than under nitrogen-rich environment. The antioxidant activity of leaf and rhizome were higher than the root at 0 kg N/ha for severely

stressed (70% ADL) treatment (Figure 5.1A and 5.2A). The present study unambiguously demonstrates that, across three different irrigation regimens, high antioxidant activity was observed under moderately stressed (50% ADL) and severely stressed (70% ADL) for the leaf, rhizome and root. Water stress induced the accumulation of phenolic compounds in cultivars of Greek oil (*Olea europaea* L.), suggesting their role as antioxidants (Petridis *et al.* 2012). It can be observed that reduced application of nitrogen levels and water significantly enhanced antioxidant activity in different parts of *S. aethiopicus.* The current study indicated that supplying *S. aethiopicus* with nitrogen fertilizer improves the secondary metabolites production and antioxidant activity of this plant especially under low N (<100 kg N/ha).

Antioxidant capacity of *S. aethiopicus* parts was significantly different, when plants were grown under various nitrogen levels and irrigation regimens. In this study, the antioxidant activity measured by DPPH radical scavenging activity specifies that a decrease in absorbance of the plant extract indicate high antioxidant activity due to hydrogen atom-donating ability (Moyo *et al.* 2010). The EC_{50} (Effective concentration) values showed no significant variation for the antioxidant activity of rhizome (2.00±0.77 µg/mL) leaf (2.02±0.68 µg/mL) and root (2.02±0.62 µg/mL) under severely stressed treatment (70% ADL). A reduction in level of nitrogen from 100 to 0 kg/ha, EC_{50} increased. Irrigation regimens (70% ADL and 50% ADL) significantly increased DPPH antioxidant activity (Table 5.3). Relatively low amounts of water applied improved the bioavailability of secondary metabolites and the activities of antioxidant enzymes were increased during abiotic stress conditions (Tuteja *et al.* 2008). The EC_{50} values showed leaf (24.85±0.39 µg/mL) and root (8.30±0.47 µg/mL) had lower antioxidant activity under moderately stressed (50% ADL) treatment (Table 5.3). The free radical activities started to accumulate at 0 kg N/ha for the leaf, rhizome and root, and decreased gradually at 150 to 200 kg N/ha fertilization. Li *et al.*

(2008) showed that high nitrogen supply significantly reduced the DPPH radical scavenging activity of leaf Mustard genotypes. The high antioxidant activity recorded in the leaf, rhizome and root extracts across all irrigation regimens with low-N application indicate its rich source of antioxidants. Overall, the rhizomes displayed an increasing trend with the application of N between 0-100 kg/ha under all irrigation regimens (Table 5.3).

Sucrose, glucose and fructose levels were influenced significantly ($p < 0.05$) by nitrogen levels and irrigation regimens. *S. aethiopicus* grown under low-N fertilization showed the highest root soluble sugars when compared with rhizome and leaf. Sucrose levels were highest in the root (47.68±9.0 mg/g DW) and rhizome (5.54±0.70 mg/g DW) under 0 N application followed by the leaf (Table 5.5). Ibrahim *et al.* (2013) observed and reported on this phenomenon. Sucrose accumulation under low nitrogen fertilization might be due to reduction in sink strength of the plant when nitrogen is limiting, hence the reduction in translocation of sugars to the other plant parts (Meyer *et al.* 2006). This could be associated with plants consisting of high sink strength to accumulate high starch and soluble sugar in their plant parts (Lemoine *et al.* 2013).

S. aethiopicus sucrose content followed an ascending order of leaf (5.12±0.13 mg/g DW), rhizome (5.54±0.70 mg/g DW) and root (47.68±9.00 mg/g DW). Tognetti *et al.* (2013) reported that biosynthesis of carbon based metabolites played a significant role in root sucrose accumulation. It was predominantly found that the decrease in glucose content could be associated with glucose being directed towards synthesis of other major carbohydrates (Obendorf *et al.* 2008). The comparison of plant parts grown under 200 kg N/ha showed that the leaf (9.01±0.89 mg/g DW) and rhizome (2.27±0.33 mg/g DW) had higher glucose content than the root (Table 5.5). Under 100 kg N/ha, the root represents higher content of fructose

(24.24±6.45 mg/g DW) compared to the rhizome and leaf (Table 5.5). The high level of fructose concentration is an essential substrate responsible for lignin and phenolic compounds synthesis (Hilal *et al.* 2004).

The investigated irrigation regimes significantly affected soluble sugar accumulation in different parts of *S. aethiopicus* ($p < 0.05$; Figure 5.4A, B and C). Less soluble sugars (sucrose and fructose) were observed for the leaf (4.15±0.64 mg/g DW) and rhizome (3.2±0.12 mg/g DW) under well-watered control (Figure 5.4A and C). The same trend was observed for the moderately stressed (50% ADL) and severe stressed treatments (70% ADL). In Alfalfa (*Medicago sativa* L.), leaf soluble sugar determinations highlighted that fructose content was reduced under low water availability conditions (Aranjuelo *et al.* 2011). Contrary to our result, the elevated sugar content in leaf of the plant could increase under drought conditions (Irigoyen *et al.* 1992). Among the studied sugars, percentage enhancement of glucose content was higher in the leaf (5.38±0.56 mg/g DW), root (5.29±0.61 mg/g DW) and rhizome (1.49±0.26 mg/g DW) under well-watered (30% ADL) treatment (Figure 5.4B). Generally, sucrose content was higher in the root as compared to the leaf and rhizome. The highest accumulation of sucrose concentration was recorded in the root (42.85±4.58 mg/g DW) under severely stressed water condition (70% ADL) compared to the leaf and rhizome (Figure 5.4A). The accumulation of high sucrose content could be associated with hexose phosphate known as progenitor for sucrose synthesis, whereby an increase in hexose phosphate concentration concurrently enhances the synthesis sucrose (Paul and Foyer, 2001). In addition, soluble sugar accumulation under low water availability condition helps to maintain and protect the stability of the membranes, and keep protein functional (Lipiec *et al.* 2013). The current result implies that low-N and low water availability to different parts of *S. aethiopicus* plays an imperative role in the accumulation of soluble sugars. However,

variations in sugar concentrations do not follow a static model and vary with the plant part, variety and the stress factor (Castonguay *et al.* 1995).

Table 5.1: Chemical characteristics of sandy clay loam soil collected from the experimental site. The data are average of duplicate analysis of soil samples collected.

	Soil depth (cm)		
Soil nutrient	0–20	20–40	40–60
Ca	1007	1066	1314
Fe	30.9	42.8	29.3
K	275	174	132
Mg	349	355	481
Total N	0.028	0.027	0.024
pH	7.13	7.16	7.26

Table 5.2: Variation in soil water deficits (top 400mm root zone) for the well-watered (30% allowable depletion level, ADL), moderately stressed (50% ADL) and severely stressed (70% ADL) treatments.

Allowable depletion (ADL)	Nitrogen levels	Average deficits (per day)	Total irrigation amount (per season)
%	N (kg/ha)	mm	mm
30	0	21.29	340
	50	21.05	484
	100	21.78	457
	150	21.14	401
	200	20.55	328
50	0	31.81	286
	50	34.82	348
	100	34.33	377
	150	33.91	305
	200	34.19	410
70	0	44.60	267
	50	45.10	225
	100	43.20	302
	150	43.08	301
	200	45.51	318

Table 5.3: Antioxidant activity of African ginger tissues (leaf, rhizome and root) in response to three irrigation regimens and nitrogen levels determined by the (DPPH 2, 2'-diphenyl-1-picryl hydrazyl) scavenging activity.

ADL	Nitrogen levels	Antioxidant activity		
		Leaf	Rhizome	Root
%	N (kg/ha)	EC_{50} (µg/mL)		
30	0	3.57 ± 0.64^{ab}	2.39 ± 0.67^{ab}	11.22 ± 0.46^{e}
	50	7.27 ± 0.46^{bcd}	2.57 ± 0.45^{ab}	2.66 ± 0.63^{ab}
	100	7.88 ± 0.50^{bcd}	3.00 ± 0.57^{bc}	3.92 ± 0.38^{bc}
	150	10.44 ± 0.45^{de}	2.58 ± 0.63^{ab}	5.89 ± 0.54^{cde}
	200	8.91 ± 0.49^{bcd}	3.75 ± 0.63^{bc}	5.17 ± 0.37^{cde}
50	0	3.36 ± 0.57^{ab}	2.83 ± 0.71^{ab}	8.30 ± 0.47^{de}
	50	24.85 ± 0.39^{e}	2.58 ± 0.68^{ab}	2.81 ± 0.38^{ab}
	100	3.22 ± 0.56^{ab}	4.12 ± 0.62^{cde}	4.38 ± 0.55^{cde}
	150	11.18 ± 0.34^{de}	4.16 ± 0.58^{cde}	3.07 ± 0.61^{bc}
	200	7.64 ± 0.53^{bcd}	3.50 ± 0.63^{bc}	4.34 ± 0.49^{cde}
70	0	2.02 ± 0.68^{a}	2.00 ± 0.77^{a}	2.02 ± 0.62^{a}
	50	10.76 ± 0.41^{de}	2.14 ± 0.50^{a}	2.64 ± 0.70^{ab}
	100	4.81 ± 0.55^{b}	4.55 ± 0.55^{cde}	2.67 ± 0.63^{ab}
	150	9.61 ± 0.48^{de}	4.62 ± 0.51^{cde}	7.61 ± 0.53^{de}
	200	10.28 ± 0.48^{de}	3.44 ± 0.68^{bc}	3.36 ± 0.59^{bc}
Ascorbic acid		34.36 ± 0.33^{f}		

Plant parts with EC_{50} (Effective concentration) values (<34.36 µg/mL) are considered potent DPPH (2, 2'-diphenyl-1-picryl hydrazyl) radical scavengers. The lower the EC_{50}, the more rapidly the colour of DPPH radical was bleached and hence the more potent the antioxidant. ^{F} represents the antioxidant standard Ascorbic acid. Mean values (standard error-SE) in column with different letters are significantly different ($p < 0.05$).

Table 5.4: Total phenolic and flavonoid content of leaf, rhizome and root of African ginger in response to irrigation regimens and levels of nitrogen sampled from experimental field.

ADL	Nitrogen levels	Total Phenolic Content			Total Flavonoid content		
%	N (kg/ha)	mg/g GAE			mg/g QE		
		Leaf	Rhizome	Root	Leaf	Rhizome	Root
30	0	34.79±0.23^{de}	3.83±0.22^{cd}	1.71±0.24^{d}	1.07±0.12^{a}	0.32±0.06^{c}	0.68±0.20^{bcd}
	50	46.98±2.09^{d}	7.17±0.80^{b}	3.17±0.50^{bcd}	0.57±0.09^{bcd}	1.57±0.01^{a}	0.63±0.15^{bcd}
	100	47.04±1.36^{d}	2.23±0.26^{cd}	2.49±0.78^{bcd}	0.33±0.05^{bcd}	0.59±0.13^{bcd}	0.91±0.38^{bcd}
	150	62.58±0.74^{bcd}	2.39±2.29^{cd}	2.91±0.02^{bcd}	0.67±0.09^{ab}	0.87±0.10^{bcd}	1.73±0.30^{ab}
	200	65.43±0.32^{bcd}	1.47±0.61^{d}	2.73±0.07^{bcd}	0.31±0.01^{c}	0.60±0.07^{bcd}	1.22±0.13^{ab}
50	0	75.89±1.61^{ab}	3.83±0.27^{cd}	1.30±1.52^{d}	1.36±0.11^{a}	1.86±0.01^{a}	2.35±0.26^{a}
	50	76.56±0.52^{ab}	0.94±0.32^{d}	2.00±0.13^{bcd}	0.42±0.06^{bcd}	0.59±0.08^{bcd}	1.82±0.39^{ab}
	100	31.51±1.76^{de}	2.07±1.74^{cd}	8.12±0.27^{b}	0.64±0.10^{ab}	0.60±0.16^{bcd}	2.21±0.70^{a}
	150	50.65±1.39^{bcd}	4.90±1.96^{bcd}	7.26±0.50^{b}	0.26±0.07^{c}	1.25±0.28^{ab}	0.54±0.10^{bcd}
	200	62.17±2.06^{bcd}	0.21±0.50^{e}	1.67±0.06^{d}	0.66±0.19^{ab}	0.64±0.04^{bcd}	1.18±0.18^{ab}
70	0	64.20±2.68^{bcd}	5.32±2.65^{bcd}	6.45±1.41^{b}	0.61±0.19^{ab}	1.00±0.05^{ab}	0.83±0.02^{bcd}
	50	81.03±0.92^{a}	26.83±0.66^{a}	23.06±0.60^{a}	0.87±0.07^{ab}	0.51±0.15^{bcd}	0.58±0.06^{bcd}
	100	87.02±2.51^{a}	4.32±0.46^{bcd}	1.95±0.33^{d}	0.77±0.42^{ab}	1.22±0.23^{ab}	2.05±0.23^{a}
	150	60.14±2.20^{bcd}	1.99±0.24^{d}	2.79±0.35^{bcd}	0.78±0.07^{ab}	1.71±0.06^{a}	1.16±0.02^{ab}
	200	15.24±1.20^{e}	1.97±2.40^{d}	6.58±0.51^{b}	0.73±0.07^{ab}	0.54±0.03^{bcd}	1.60±0.54^{ab}
F-statistics		6.65**	20.00**	0.12**	3.20**	24.46**	15.89**

Values (Mean ± SE) with dissimilar letters in a column are significantly different at $p < 0.05$ using Duncan Multiple Range test. All analyses are the mean of triplicate measurements ± Standard error (SE); Gallic Acid Equivalent (GAE), Quercetin (QE).

Table 5.5: Amount of sucrose, glucose and fructose of *S. aethiopicus* tissues grown in the field in response to nitrogen application levels.

Nitrogen levels (kg N/ha)	Sucrose content (mg/g DW)		
	Root	Leaf	Rhizome
0	47.68±9.0^{a}	3.36±0.34^{ab}	5.54±0.70^{a}
50	36.64±3.2^{ab}	5.12±0.13^{a}	2.80±0.43^{ab}
100	44.68±0.35^{a}	4.80±0.35^{a}	1.07±0.54^{bc}
150	26.85±0.43^{ab}	3.05±0.43^{ab}	1.93±0.44^{bc}
200	18.24±0.20^{c}	4.85±0.20^{a}	0.83±0.21^{c}
	Glucose content (mg/g DW)		
	Root	Leaf	Rhizome
0	ND	1.17±0.16^{b}	0.29±0.11^{ab}
50	3.9±0.94^{ab}	0.40±0.20^{c}	1.03±0.32^{ab}
100	0.98±0.98^{c}	0.95±0.17^{c}	0.49±0.13^{ab}
150	7.80±1.26^{a}	1.07±0.10^{b}	0.72±0.25^{ab}
200	4.85±0.85^{ab}	9.01±0.89^{a}	2.27±0.33^{a}
	Fructose content mg/g DW		
	Root	Leaf	Rhizome
0	18.31±2.06^{ab}	2.97±0.70^{a}	0.96±0.34^{ab}
50	19.81±1.52^{ab}	2.23±0.80^{ab}	1.36±0.39^{ab}
100	24.24±6.45^{a}	1.29±0.13^{ab}	1.00±0.18^{ab}
150	21.82±1.83^{a}	1.89±0.27^{ab}	1.72±0.67^{ab}
200	14.53±1.82^{ab}	3.01±0.42^{a}	2.00±0.33^{a}

Values (Mean ± SE) with dissimilar letters in a column are significantly different at $p < 0.05$ using Duncan Multiple Range Test (DMRT). ND represents the non-detected; DW represents Dry weight.

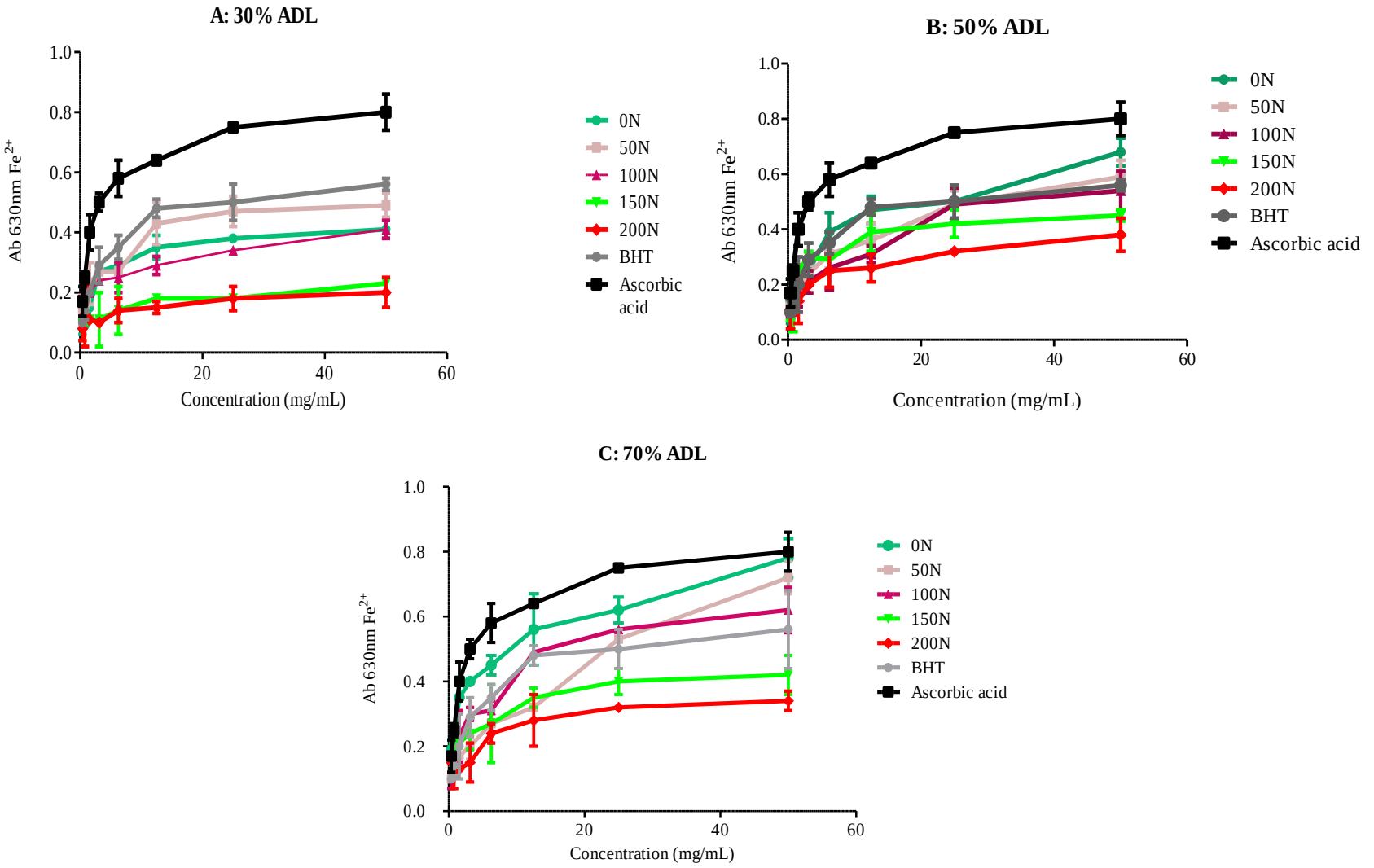

Figure 5.1: Ferric reducing power of leaf in response to irrigation regimes (A) well-watered 30% ADL, (B) moderately stressed 50% ADL, (C) severely stressed 70% ADL, different levels of nitrogen fertilizers and positive controls (BHT and Ascorbic acid).

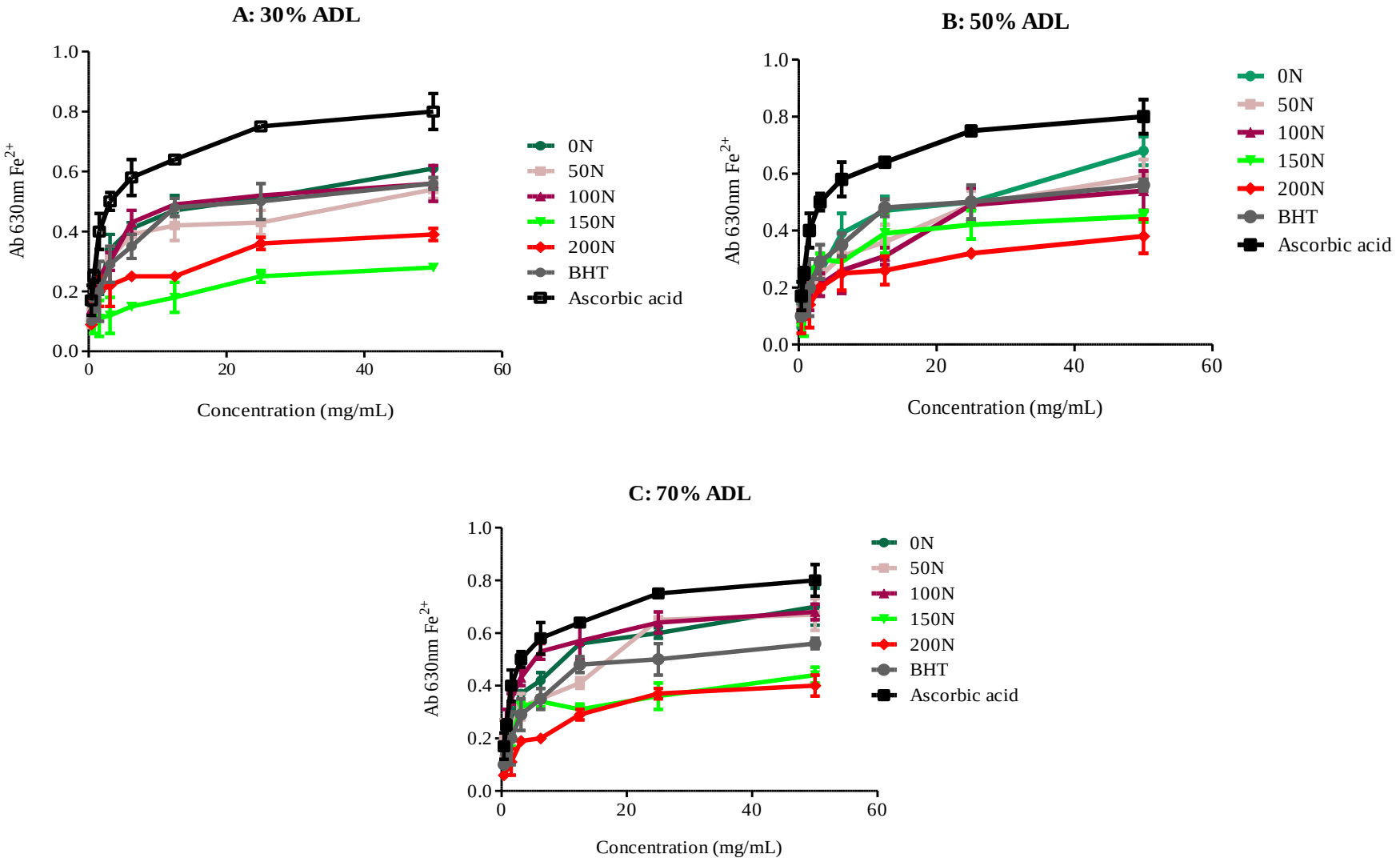

Figure 5.2: Ferric reducing power of rhizome in response to irrigation regimes (A) well-watered 30% ADL, (B) moderately stressed 50% ADL, (C) severely stressed 70% ADL, different levels of nitrogen fertilizers and positive controls (BHT and Ascorbic acid).

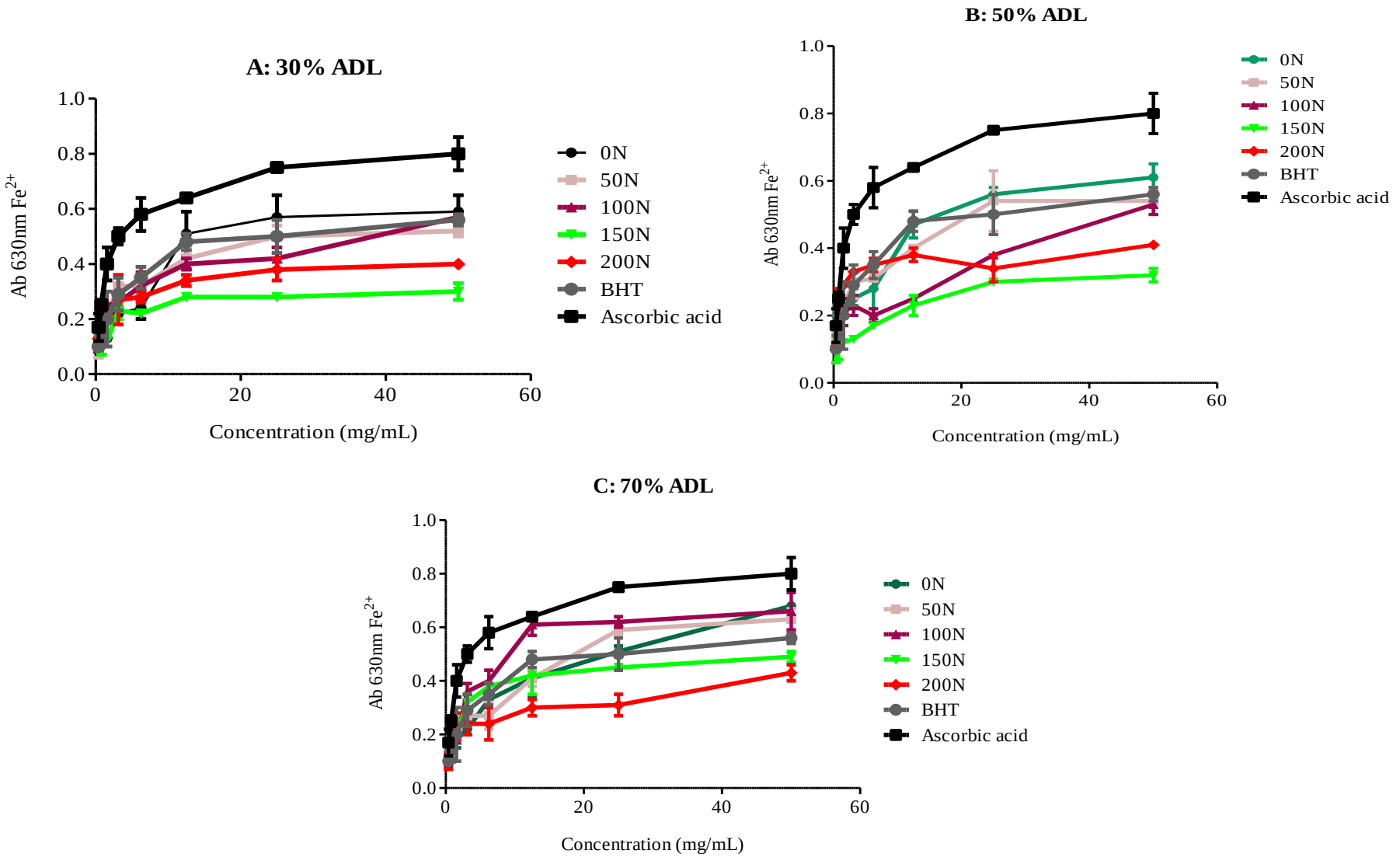

Figure 5.3: Ferric reducing power of rhizome in response to irrigation regimes (A) well-watered 30% ADL, (B) moderately stressed 50% ADL, (C) severely stressed 70% ADL, different levels of nitrogen fertilizers and positive controls (BHT and Ascorbic acid)

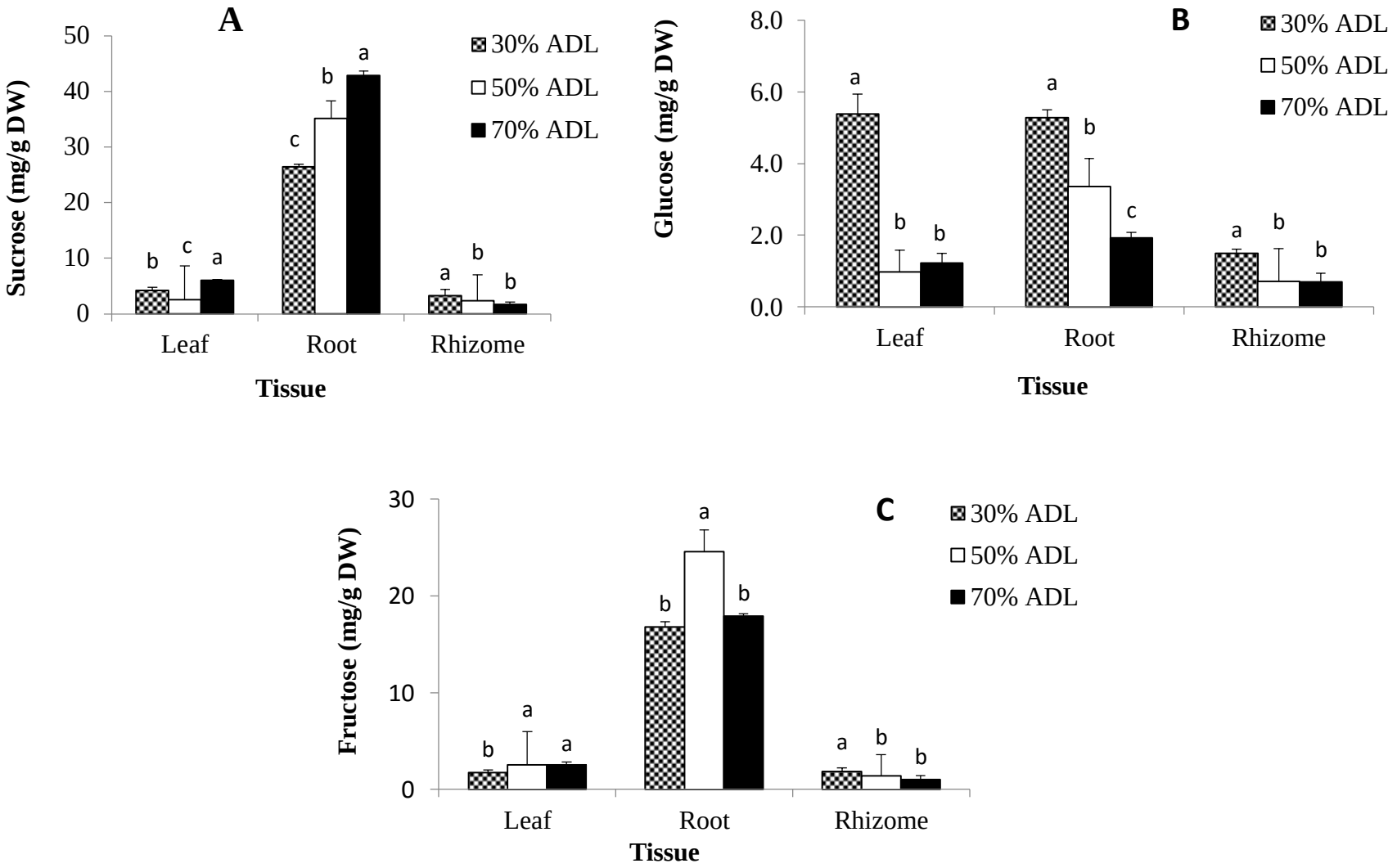

Figure 5.4: Amount of (A) sucrose, (B) glucose and (C) fructose of *S. aethiopicus* grown in the field in response to irrigation levels. Bars followed by dissimilar letters are significantly different at $p < 0.05$. Vertical lines on bars represent S.E. (n = 27).

5.4 Conclusion

Our results indicate that the manipulation of fertilizer, especially nitrogen, can possibly be an effective method to increase the expression of secondary metabolites in African ginger. Higher flavonoids, phenolics and antioxidant (by FRAP and DPPH) were demonstrated in rhizome and leaf fertilized with low N (<100 kg/ha) under severely stressed (70% ADL) treatment. The high antioxidant properties expressed in different tissues are important as an additional value for the medicine and flavour. Cultivation of African ginger at lower N fertilizer rate with moderately stressed (50% ADL) and severely stressed (70% ADL) irrigation regimens have enhanced soluble sugar concentration of the root. These results clearly demonstrate the potential of exploring secondary metabolites and bioactive medicinal components in different parts (especially in the leaf and rhizome).

5.5 References

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CHAPTER SIX

VOLATILE PROFILING OF AFRICAN GINGER (*SIPHONOCHILUS AETHIOPICUS*) PARTS IN RESPONSE TO IRRIGATION REGIMES AND NITROGEN LEVELS

ABSTRACT

This study investigated the volatile organic compounds of different parts of African ginger (*Siphonochilus aethiopicus*) as affected by irrigation regimes (30; 50 and 70 Allowable depletion level) and nitrogen levels (0, 50,100,150 and 200 kg/ha). Gas chromatography/mass spectrometry was used for volatile analysis. A maximum number of thirty-five (35) volatile organic compounds were detected in the rhizome, followed by thirty-three (33) in the leaf and least in the root (28). The volatile compounds detected were characterized according to eight chemical classes. The most abundant volatile components in the root and rhizome were terpenes, as compared to the increased components of aliphatic acids, benzenoids and aliphatic aldehydes in the leaf. The odorant sesquiterpene (1E)-5-Methyl-1-(2, 6, 6-trimethyl-2, 4-cyclohexadien-1-yl)-1, 4-hexadien-3-one was the most abundant across all treatments. Although 4-Hydroxy-4-methyl-2-pentanone (9.49%) was detected in all plant parts the concentration increased under severely stressed (70% ADL) with the application of 100 kg N/ha for the root. Low components of linalool was obtained from the rhizome (9.09%) and root (8.29%) grown without N application under severely stressed (70% ADL). Severely stressed (70% ADL) treatment with minimal application of N induced the terpenes concentration in all plant tissues. Knowledge on the impact of water stress and mineral nutrient deficiency of volatile components of African ginger parts provides a useful guide for selection towards improving phytochemical profiles of volatile compounds and explore the additional value of pharmacological properties.

Keywords: β-caryophyllene, volatile compound, fertilizer, plant tissues, irrigation regimes, gas chromatography

6.1 Introduction

Plants employ different strategies to accumulate a diverse group of natural products, which are significantly involved in interactions with the environment. Plant chemical products are important tools used in communications with microbes, animals, and other living species by emitting a blend of volatile organic compounds (Loreto and Schnitzler, 2010). The chemical composition and ratios of compounds in the blend constitute the plant scent induced by different stresses, both biotic and abiotic (Bruce *et al.* 2005). Volatile organic compounds (VOCs) are regarded as attractive or repellent to arthropods foraging for food. Plants emit VOCs which act as a signal to vascular in systemic responses (Niinemets *et al.* 2013).

African ginger (*S. aethiopicus*) is one of the most important rhizomatous medicinal plants associated with treatment of asthma, hysteria, cold, flu, coughs, pain relief, dysmenorrhea, influenza and hysteria (van Wyk, 2008). The rhizome extracts possess anti-inflammatory, antimalarial, antimutagenic, antibacterial activities and have high antioxidant activity (Coopoosamy *et al.* 2010). The plant is listed as one of the endangered species and it is becoming extinct in the wild due to overharvesting, hence conservation strategies are important.

Environmental stress such as physical damage, nutrient deficiency, salinity and drought alter the emission of volatile organic compounds (Jaleel *et al.* 2007). Variations in plant secondary metabolites and their composition were observed with water stress and nitrogen levels (Mirsa and Strivastava, 2000). Simon *et al*. (1992) reported reduced total fresh and dry weights for *Ocimum basilicum* (L.) as the plant water deficit increased. Essential oil content of *Satureja hortensis* (L.) was enhanced under severe water stress than moderate water stress treatment (Baher *et al.* 2002). The presence of volatile organic constituents of *Origanum dictamnus* (L.), were reported to be altered by nutrient levels (Economakis, 2005).

Several studies have been conducted on the composition of the volatile components of *S. aethiopicus*. The main components of the rhizome and root were mostly the sesquiterpenes (70%) and monoterpenes (3.5%) including 1, 8-cineole, (*E*)-β-ocimene and *cis*-alloocimene (Viljoen *et al.* 2002). The essential oil of *Curcuma sichuanensis* contained major constituents of terpenes of up to 87% (Zhou *et al.* 2007). Furthermore, the two furanoterpenoids representing 20% of the oil composition from the rhizome extracts were reported (Holzapfel *et al.* 2002). The antioxidant activities of *Zingiber officinale* and *Labisia pumila* cultivated under varying environmental conditions (Ghasemzadeh *et al.* 2011; Ibrahim *et al.* 2014).

Volatile and phytochemical profiling of medicinal species have been reported (Viljoen *et al.* 2002). However, information on the response of water deficits and nitrogen application rates of *S. aethiopicus* is limited. The objective of this study was to investigate the volatile components *S. aethiopicus* parts in response to nitrogen application and irrigation regimes. This study was designed to address this knowledge gap and gain a more integrated understanding of how volatile organic compounds production varies across nitrogen levels and water stress treatments.

6.2 Materials and methods

For details on site description, material source, experimental design, treatment application, harvesting and sample preparation please refer to Chapter 4.

6.2.1 Sample preparation

Plant materials of the same treatments were collected in paper bags from the experimental plots. Plants were separated into leaves, roots and rhizomes. The leaf, rhizome and root samples were freeze-dried and stored at -20°C. Approximately 100-150 g of leaf, rhizome and roots samples were ground and pulverized into powdered form using a mortar and the fractions were freeze-dried at -20°C until further analysis. Finally the leaf, rhizome and root (0.5g) were diluted in 5 mL of pentane before analysed by gas chromatography/ mass spectrometry (GC/MS).

6.2.2 Gas chromatography/ mass spectrometry (GC/MS) conditions

Volatile compounds were analyzed using coupled Varian 3800 gas chromatography (Varian Palo Alto, California, USA) and Varian 1200 mass spectrometry (GC-MS). The GC was equipped with an Alltech EC-WAX column of 30 m x 0.25 mm internal diameter x 0.25 μm film thickness (Alltech Associates Inc., Deerfield, Illinois, USA). Helium was used as the carrier gas at a flow rate of 1 mL/min. From each sample, 2 μL was injected into a chromatoprobe trap prepared by cutting glass tubes equaling the size of chromatoprobe quartz microvials (length: 15 mm; inner diameter: 2 mm) and filled with 2 mg of a 50:50 mixture of Tenax TA (Alltech Associates, USA) and graphitized carbon (Carbotrap™, Supelco, USA) and closed on both ends with glass wool. The chromatoprobe traps were placed in a Varian 1079 injector by means of a chromatoprobe fitting and thermally desorbed. The temperature of the injector was 40 °C, and was held for 2 minutes with a 20:1 split ratio and then increased to 200 °C, and then held at 200 °C min-1 in splitless mode for thermal desorption. Compound detection was delayed for 6 minutes. After a 3 minute hold at 40 °C, the GC oven was ramped up to 240 °C at 10 °C/min and held there for 12 minutes. Compound identification was carried

out using the NIST05 mass spectral library and comparisons with retention times of chemical standards, as well as comparisons between calculated Kovats retention indices and those published in the literature. Clean chromatoprobe traps were run in GC-MS as controls to identify background contamination. Compounds present at higher or similar percentages in the blanks were considered as contaminants and excluded from the analysis.

6.2.3 Data analysis

All data was subjected to analysis of variance using GenStat software 17.1 edition (VSN International, Hemel Hempstead, UK) to compare the volatile profiles in the leaf, root and rhizome. The treatment means were separated using ($p < 0.05$) Duncan's multiple range test (DMRT).

6.3 Results and discussion

6.3.1 Comparison of volatile components *S. aethiopicus* of plant parts

The relative abundances (%) of volatile organic compounds of *S. aethiopicus* leaf, rhizome and root of were identified by GC-MS (Tables 6.1). The volatile profiles of different parts are shown in Table 6.1 with clear separation and variation noticed concerning the isolated compounds, their amounts and the class of the components. In total, 107 volatile compounds were separated by GC and the relative peak values of only 96 were identified by mass spectrometry. Table 6.1 indicate a broad spectrum of main components found in different parts of *S. aethiopicus.*

In this study, a maximum number of thirty-five (35) volatile organic compounds were detected in the rhizome, followed by thirty-three (33) in the leaf and least in the root (28). The volatile compounds detected were characterised according to the chemical classes of aldehydes, alcohols, ketones, acids, benzenoids, terpenes, sulphur and N-containing compounds (Tables

6.1), while others were unidentified components and impurities. The results indicate that the large group of volatile compounds detected in rhizome and root were from sesquiterpenes and monoterpenes (Table 6.1). Major volatile components belonging to the chemical class of aldehydes, alcohols, ketones, acids, and benzenoids were detected in the leaf (Table 6.1). Volatile phytochemical composition and composition is Zingiberene ginger rhizome has been reported (Bhuiyan *et al.* 2008). Riyazi *at al.* (2007) also documented abundant volatile compounds such as beta-pinene, terpinolene and alpha-phellandrene in the rhizome of ginger (*Zingiber officinale*). The main components found two aldehydes compounds including decanal (1.74%) and nonanal (0.82%) were emitted in relatively small amounts in the leaf (Table 6.1). As can be observed, alcohols were in relatively higher abundance, representing 0.13-8.32% of the VOCs in the root (Table 6.1). Decanal was reported as the dominant volatile compound in the leaf part (11.63%) of *Polygonum minus* compared to the stem and root (Ahmad *et al.* 2014). Wu and Yong (1994) reported abundant volatile compounds of ginger rhizome in response to irradiation.

Table 6.1: Volatile profiling of leaf, rhizome and root of *S. aethiopicus* by using gas chromatography mass spectrometry

No	Compound	KRI	Percentage		
			Leaf	Rhizome	Root
	Alcohols				
1	1-butanol	1125	-	-	0.13
2	2-methyl-1-butanol	1179	-	-	0.72
3	2-hexanol	1189	-	-	0.65
4	1-Pentanol	1221	-	-	2.42
5	4-Hydroxy-4-methyl-2-pentanone	1349	2.90	0.44	0.46
6	2,3-Butanediol	1568	-	-	8.32
7	1-Octanol	1552	0.19	-	-
	Aldehydes		-	-	-
8	Nonanal	1381	0.82	-	-
9	Decanal	1498	1.74	-	-
	Acids		-	-	-
10	Acetic acid	1450	6.67	0.40	3.32
11	Nonanoic acid	2129	0.13	-	-
12	Decanoic acid	2214	0.21	-	-
13	Dodecanoic acid	2373	0.41	-	-
14	Tridecanoic acid	2450	0.23	-	-
15	Tetradecanoic acid	2532	3.38	-	-
	Ketones				
16	Crypton	1691	5.64	-	-
17	1-(1H-pyrrol-2-yl)-ethanone	1963	-	-	0.44
	Benzenoids				
18	Benzladehyde	1536	1.67	-	1.94
19	Methyl benzoate	1637	0.29	-	-
20	Benzene acetaldehyde	1655	-	-	0.45
21	*p*-Isopropylbenzaldehyde	1800	1.72	-	-
22	2,5-dimethylbenzaldehyde	1828	0.27	-	-
23	Butylated Hydroxytoluene	1911	1.11	-	-
24	Phenol	1993	0.97	-	0.05

Table 6.1: *Cont*

No	Compound	KRI	Percentage		
			Leaf	Rhizome	Root
	Terpenes (monoterpenes)				
25	*β*-Thujene	1062	-	-	5.73
26	α-Phellandrene	1072	1.78	0.64	-
27	3-Carene	1102	-	-	0.72
28	Sabinene	1117	-	-	1.05
29	Eucalyptol	1167	-	3.42	24.50
30	(*Z*)-3,7-dimethyl-1,3,6-Octatriene	1213	-	-	9.80
31	Cymene	1239	0.62	-	-
32	*trans*-*β*-Ocimene	1213	-	2.12	-
33	(*E,Z*)-2,6-dimethyl-2,4,6-Octatriene	1352	-	4.24	1.81
34	2,6-dimethyl-2,4,6-octatriene	1378	-	0.32	0.05
35	Linalool	1540	-	0.22	0.31
36	*cis*-*β*-terpineol	1544	-	0.12	0.21
37	*trans*-*β*-Terpineol	1549	0.40	0.05	-
38	Terpinen-4-ol	1606	-	0.81	2.57
39	α-terpineol	1708	1.03	1.10	1.61
40	*p*-Cymen-8-ol	1851	0.44	-	-
41	2-Hydroxycineol	1860	-	-	0.39
	Terpenes(Sesquiterpenes)				
42	*E,E*-Cosmene	1441	-	0.02	-
43	*β*-Elemene	1600	3.44	1.10	2.78
44	*β*-Caryophyllene	1611	7.68	-	-
45	Elixene	1644	-	0.49	0.36
46	(*E*)-*β*-Farnesene	1672	9.24	0.05	-
47	Germacrene D	1720	1.05	0.28	0.03
48	Epiglobulol	1783	2.57	1.91	3.18
49	Dihydro-*β*-agarofuran	1800	-	1.13	-
50	γ-Elemene	1842	3.62	3.05	1.02

Table 6.1: *Cont*

No	Compound[a]	KRI	Percentage		
			Leaf	Rhizome	Root
	Terpenes (Sesquiterpenes)				
51	Curzerene	1876	-	0.32	0.04
52	*β*-Vatirenene	1885	-	0.09	0.34
53	Caryophyllene oxide	1986	6.66	0.13	0.30
54	*E*-nerolidol	2032	-	0.03	-
55	Humulene-1,2-epoxide	2036	0.12	-	-
56	Elemol	2054	-	1.27	0.58
57	*γ*-Eudesmol	2078	-	1.04	-
58	Guaiol	2086	-	4.09	-
59	Cubenol	2115	-	0.27	-
60	*β*-Eudesmol	2128	-	0.69	-
61	*α*-Eudesmol	2138	-	0.29	-
62	*cis*-*Z*-*α*-Bisabolene epoxide	2238	-	0.69	0.36
63	Spathulenol	2293	-	0.75	0.09
64	Corymbolone	2312	-	0.60	-
65	Acorenone 1	2327	0.97	-	-
66	(1*E*)-5-Methyl-1-(2,6,6-trimethyl-2,4-cyclohexadien-1-yl)-1,4-hexadien-3-one	2544	21.35	64.50	13.73
67	Rhizoxin	2762	-	0.96	-
	Sulphur-containing compounds				
68	Dimethyl Sulfoxide	1586	8.35	-	-
	Nitrogen-containing compounds				
69	Tetramethyl-pyrazine	1470	-	-	0.20
70	Methoxy-phenyl oxime	1748	-	-	0.09
71	*N*,*N*-Dimethylacetamide	1754	-	-	0.26
72	*N*-Ethylacetamide	1756	-	-	0.15

6.3.2 Effect of nitrogen levels and irrigation regimes on volatile compounds

The effect of nitrogen levels and irrigation regimes of *S. aethiopicus* leaf, rhizome and root are presented in Tables 6.2, 6.3 and 6.4. Most of the volatile components were identified below 1% for the leaf and rhizome across all the nitrogen application rates and irrigation regimes. The highest components of aliphatic acids detected for the leaf was the components of nonanal with 50% ADL with 50 N kg/ha (Table 6.2). The distinct distribution of compounds between leaf, rhizome and root in this study suggest that different plant parts has the ability to detect varying chemical constituents (Aharoni *et al.* 2003).

The irrigation regimes and N levels had a significant effect on aliphatic ketones and benzenoids volatile components detected from leaf and root (Tables 6.2 and 6.4). Volatile components in other studies increased under moderate and severe water stress (Jaleel *et al.* 2007). The presence of Crypton ranged from 1.27% to 9.55% for *S. aethiopicus* leaf at 150 and 0 kg N/ha, respectively. The percentage of the components increased under well-watered control (30% ADL) compared to severely stressed (70% ADL) treatment (Table 6.2). The leaf also emitted high components of benzenoids such as Benzladehyde, *p*-Isopropylbenzaldehyde, butylated hydroxytoluene and phenol (Table 6.2). The subsequent decrease of 0.05% of phenol in the root was observed, which varied significantly from the leaf (0. 97%). High phenol components protect plant parts against damage and contain antioxidant properties in various medicinal plant species (Sytar *et al.* 2016).

Most of the volatiles components detected in the rhizome and root were sesquiterpenes and monoterpenes as shown in Tables 6.1 and 6.2. Volatile terpenes are mostly synthesized and accumulated in the root and rhizome of various plant species (Bos *et al.* 2002; Kovacevic *et al.* 2002). (1*E*)-5-Methyl-1-(2, 6, 6-trimethyl-2, 4-cyclohexadien-1-yl)-1, 4-hexadien-3-one was the dominant compounds in the leaf, root and rhizome (Tables 6.1, 6.2, 6.3 and 6.4). The significant effect was observed at 70% ADL for the leaf (9.6) and root (7.4%) at the application

rate of 0-50 kg N/ha (Tables 6.2 and 6.4). The odorant (1E)-5-Methyl-1-(2, 6, 6-trimethyl-2, 4-cyclohexadien-1-yl)-1, 4-hexadien-3-oneidentified in this study, is a sesquiterpenes previously isolated in honey (Blank, 1989) and in varying food and stimulants (Maarse and Vischer, 1989). Among volatile components of sesquiterpenes emitted in the leaf, (E)-β-Farnesene (9.24%), β-Caryophyllene (7.68%) and Caryophyllene oxide (6.66%) were the dominant compounds (Table 6.2). The high concentration of β-Caryophyllene detected in the leaf relates to the concentration determined for *S. salignus* species (Sánchez-Muñoz *et al.* 2012). β-Caryophyllene is well known for its potential as anti-inflammatory, insecticidal and fungicidal properties (Bayala *et al.* 2014). The high β-Caryophyllene in the leaf compared to the rhizome and root can be associated with exposure to oxidative stress and photosynthesis functioning.

Most terpenes are produced in all photosynthetically active plant parts and stored in the sub-epidermal compartments (Opitz *et al.* 2008). The differences in the volatile composition in different *S. aethiopicus* parts could be attributed to the method of extraction, geographic origin, irrigation and nitrogen levels. The results showed the presence of potent sesquiterpene volatile compound such as (1E)-5-Methyl-1-(2, 6, 6-trimethyl-2, 4-cyclohexadien-1-yl)-1, 4-hexadien-3-one, Caryophyllene oxide, γ-Elemene, β-Elemene and α-terpineol in all plant parts.

The presence of a high percentage of 4-Hydroxy-4-methyl-2-pentanone was emitted under severely stressed (70% ADL) for the root with the application of 100 kg N/ha (Table 6.4). Monoterpenes compounds abundant in the root and rhizome were eucalyptol, linalool, (*E*, *Z*)-2, 6-dimethyl-2, 4, 6-Octatriene and Terpinen-4-ol (Tables 6.1, 6.3 and 6.4). The presence of a high percentage of eucalyptol was observed at 9.63% and 9.69% for the rhizome and root under severely stressed (70% ADL) treatment with N application of 100 kg/ha, respectively (Tables 6.3 and 6.4). Nitrogen levels might have regulated the eucalyptol content due to the increasing trend in eucalyptol and N levels. According to Bayala *et al.* (2014) eucalyptol contributed about 35% of the volatile compounds in the herbal plant hence its significance. The eucalyptol

components in Chinese ginger showed a decreasing trend as compared to the increased components of 1, 3-cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl (Bayala *et al.*2014).

The low linalool was emitted in the rhizome (9.09%) and root (8.29%) grown without N application under severely stressed (70% ADL) treatment (Tables 6.3 and 6.5). In contrast, Hymete *et al.* (2008) and Eyob *et al.* (2007) reported higher percentage of linalool in seed oil content for *Aframomum giganteum* (44.3%) and *Aframomum corrorima* (39.3%). The lowest components of monoterpenes and sesquiterpenes compounds such as linalool and eucalyptol in plant parts could be attributed to the N levels applied. These results are in accordance studies on true ginger (*Z. officinale***)** and turmeric (*Curcuma longa*), whereby linalool was emitted in low amounts in the leaf and rhizome (Koo and Gang, 2012). The constituents obtained from *Ocimum sp* at 125% of field water capacity treatment increased the constituents of linalool (Khalid, 2006). Linalool is reported as a good phytochemical marker due to its anti-inflammatory, antiseptic and glutamatergic neurons activities (Sytar *et al.* 2016). The constituents of linalool (9.09%), germacre (9.87%) and cubenol (9.03%) were high in the rhizome without N application under severely stressed (70% ADL) treatment (Table 6.3).

In agreement with our findings, Kainulainen *et al.* (1996) reported the presence of a high concentration of monoterpenes and sesquiterpenes under limited nitrogen and mineral nutrients. The major constituents of terpenes including 1, 8-cineole, (*E*)-β-ocimene and *cis*-alloocimene were identified in the root and rhizome of *S. aethiopicus* (Viljoen *et al.* 2002). The characteristic of pleasant aroma in Chinese ginger analysed and identified using extracts dilution emitted the presence of odorant for volatile compounds such as linalool (Nishimura, 1995). The two major constituents of monoterpenes recorded high percentage of (Z)-3, 7-dimethyl-1, 3, 6-Octatriene (9.80%) and β-Thujene (5.73%). The highest components of sabinene were present in the root compared to the leaf and rhizome. The high percentage of sabinene was observed at 50% ADL (9.15%) and 70% ADL (7.87%) with low N application. Turtola *et al.* (2003) reported a decline in components of sabinene volatile oil for *Cassumunar*

ginger in response to days of water deficits. The high α-phellandrene content detected in the leaf (1.78%) was comparable to the α-phellandrene content that was abundant in the leaf of *Schinus terebinthifolius* and *Schinus molle species* (Ennigrou *et al.* 2011). The remaining chemical class of S-containing compound was only detected in the leaf (8.35%) compared to other plant parts. Some of the N-containing compounds identified in the root increased tetramethyl-pyrazine, methoxy-phenyl oxime, N, N-Dimethylacetamide and N-Ethylacetamide detected at different irrigation regimes and nitrogen level (Table 6.4).

There were more terpenes in the rhizome and root as compared to the increased components of aliphatic acids, benzenoids and aliphatic aldehydes in the leaf. Severely stressed 70% ADL treatment with minimal application of N also induced the terpenes components in plant parts. These can be associated with the composition and variation upon species type, age of the plant and tissue type, geographical conditions and environmental factors.Although it is common to find diverse profiles of volatile compounds obtained from different plant parts, a comparison of *S. aethiopicus* parts revealed significant variations in response to water stress and N levels. Further analysis of volatile organic compounds of different plant parts in response to other environmental factors and using analytical tools should be carried out to substantiate these findings.

6.4 Conclusion

Volatile profiling of different parts showed that terpenes were the major compounds in the rhizome and root, and many of its proven pharmacological properties are attributed by varying environmental factors. The most abundant volatile compounds components were influenced by water stress level and some components increased significantly upon stress such as (1*E*)-5-Methyl-1-(2, 6, 6-trimethyl-2, 4-cyclohexadien-1-yl)-1, 4-hexadien-3-one, γ-Elemene, *β*-Elemene and α-terpineol geraniol, carvacrol, and diisobutyl phthalate. The desired pharmacological components of *S. aethiopicus* might be oriented by manipulating agronomy factors.

Table 6.2: Relative concentrations of several classes of compound in *S. aethiopicus* leaf in response to irrigation regimes and nitrogen levels.

		Nitrogen levels (kg/ha)														
		0			50			100			150			200		
		Percentage														
No	Compound[a]	30	50	70	30	50	70	30	50	70	30	50	70	30	50	70
1	4-Hydroxy-4-methyl-2-pentanone	-	2.3	2.2	9.0	-	3.1	-	1.0	1.5	1.6	-	-	1.1	1.0	6.9
2	Decanal	6.4	-	-	2.6	-	-	4.4	-	-	4.1	-	-	-	-	6.7
3	Nonanal	-	-	-	-	6.4	-	-	-	-	-	-	4.3	-	-	3.7
4	Acetic acid	2.2	-	-	7.2	5.2	-	7.8	-	1.0	2,2	-	1.8	-	8.9	1.4
5	Crypton	9.5	1.6	1.8	1.1	2.1	7.7	2.1	2.2	2.6	1.6	2.4	1.2	1.4	5.5	2.6
6	Benzladehyde	5.7	-	1.8	-	-	-	-	-	-	-	-	4.5	-	-	4.2
7	p-Isopropylbenzaldehyde	3.7	3.4	4.9	-	7.9	7.1	9.9	5.7	1.0	1.9	1.0	9.7	2.7	5.4	5.5
8	2,5-dimethylbenzaldehyde	-	-	-	6.0	-	-	-	-	-	-	-	9.4	-	2.2	-
9	Butylated Hydroxytoluene	-	-	-	-	-	-	-	-	4.2	-	-	-	2.8	-	-
10	Phenol	-	-	-	-	-	-	2.2	-	-	-	-	1.6	-	1.5	-
11	Phellandrene	-	-	-	-	2.1	-	-	-	3.6	-	3.5	-	-	-	-
12	trans-β-Terpineol	-	5.3	3.1	-	2.5	5.3	-	-	3.8	-	3.6	-	-	-	-
13	α-terpineol	2.2	7.3	2.2	1.0	2.5	6.1	4.6	3.2	2.5	1.0	5.2	2.0	1.9	-	9.5
14	p-Cymen-8-ol	-	-	-	-	4.4	-	-	2.6	4.1	-	3.6	1.0	-	-	-
15	β-elemene	8.2	1.8	1.2	8.2	1.0	3.4	1.4	1.2	9.6	1.3	1.6	6.0	9.4	4.0	1.2
16	beta-Caryophyllene	1.3	2.5	1.6	1.4	1.5	4.2	2.1	2.1	1.6	1.8	2.4	1.2	1.6	9.1	2.8
17	(*E*)-*β*-Farnesene	2.2	4.4	2.7	2.7	2.1	9.9	4.1	4.1	2.1	3.2	3.6	2.9	3.1	-	5.8
18	Germacrene D	2.5	6.4	2.7	-	3.4	1.0	6.5	5.3	3.1	-	5.4	5.9	3.1	-	-
19	Epiglobulol	4.5	1.9	1.0	-	1.1	3.1	1.4	5.8	7.7	1.2	1.4	7.1	6.2	-	8.3
20	γ-Elemene	6.5	2.0	1.3	6.6	1.2	4.2	1.8	1.1	1.1	1.2	1.7	8.2	1.0	2.8	1.4
21	Caryophyllene oxide	1.3	2.9	2.1	9.8	1.8	7.6	3.2	2.4	2.3	4.0	3.0	9.9	1.7	6.5	4.1
22	(1E)-5-Methyl-1-(2,6,6-trimethyl-2,4-cyclohexadien-1-yl)- 1,4-hexadien-3-one	9.7	1.6	3.2	7.2	3.1	4.0	1.9	2.3	9.6	2.3	5.1	5.1	4.0	2.0	1.3
23	Dimethyl Sulfoxide	1.7	-	-	6.2	1.3	-	2.1	1.7	3.6	1.6	2.6	7.3	2.9	-	4.3

Table 6.3: Relative concentrations of several classes of compound in *S. aethiopicus* rhizome in response to irrigation regimes and nitrogen levels.

		Nitrogen levels (kg/ha)														
		0			50			100			150			200		
		Percentage														
No	Compound	30	50	70	30	50	70	30	50	70	30	50	70	30	50	70
1	4-Hydroxy-4-methyl-2-pentanone	1.2	1.7	1.3	1.4	3.1	7.2	8.2	2.3	1.0	1.9	8.0	2.1	1.0	3.8	1.5
2	Acetic acid	7.6	2.0	-	7.0	-	-	3.5	-	9.7	1.1	-	-	1.1	-	2.2
3	Eucalyptol	7.3	1.9	1.1	4.7	1.0	1.4	1.1	1.8	9.6	8.8	7.2	2.1	1.2	4.0	3.9
4	trans-β-Ocimene	3.9	1.1	8.5	2.3	1.0	7.0	1.7	1.2	9.1	5.7	2.3	1.7	5.8	2.8	3.3
5	2,4,6-Octatriene, 2,6-dimethyl-, (E,Z)-	9.1	2.2	2.0	4.7	2.0	1.7	4.2	3.3	2.0	1.5	3.4	3.6	1.1	4.8	5.0
6	Linalool	8.4	1.4	9.0	2.5	1.0	7.9	1.8	1.5	8.2	6.8	2.7	1.3	7.2	2.4	1.9
7	α-terpineol	6.6	6.8	4.2	1.7	4.4	4.1	1.8	8.0	4.7	3.4	1.0	6.2	4.0	9.3	9.1
8	β-elemene	6.4	6.3	4.3	1.7	4.9	4.2	1.6	8.4	3.9	3.9	7.6	7.9	4.1	1.1	9.1
9	Elixene	3.0	2.5	1.8	6.6	2.1	2.0	9.4	2.9	2.5	1.3	3.9	3.3	1.9	5.2	3.4
10	Germacrene D	1.9	1.5	9.8	3.1	1.0	1.2	5.9	2.8	1.0	9.0	1.5	1.9	1.0	2.7	2.5
11	Dihydro-β-agarofuran	7.8	6.2	4.2	1.6	4.3	4.4	2.0	9.1	4.6	4.7	6.6	6.8	4.8	1.1	8.1
12	γ-Elemene	2.1	1.5	1.2	3.8	1.2	1.1	6.1	2.4	1.3	1.1	1.9	2.0	1.0	3.0	2.5
13	β-Vatirenene	9.4	5.8	2.9	1.9	3.2	4.4	1.1	1.0	2.8	3.5	-	4.9	2.8	-	8.5
14	Caryophyllene oxide	1.2	9.7	4.5	-	7.8	7.5	1.0	1.8	1.1	5.7	-	8.1	7.4	-	1.2
15	Elemol	8.3	5.7	3.6	2.4	5.1	4.5	2.3	1.1	4.0	8.1	6.3	1.0	5.0	9.5	1.1
16	γ-Eudesmol	7.1	5.0	3.3	1.8	3.9	4.3	2.4	7.8	4.5	5.8	4.4	7.3	4.1	7.4	8.0
17	Cubenol	1.8	1.4	9.0	4.2	1.1	1.1	5.6	1.8	1.1	1.5	1.3	1.8	1.1	1.8	2.1
18	β-Eudesmol	4.8	3.2	1.8	1.0	5.1	2.1	1.5	8.5	2.6	3.2	2.8	3.3	2.6	3.3	4.7
19	*cis-Z-α*-Bisabolene epoxide	5.5	3.6	2.4	1.2	3.0	2.7	1.3	5.3	2.8	2.9	4.0	4.1	2.7	4.4	5.1
20	Spathulenol	7.5	3.3	2.7	1.5	3.4	2.8	1.6	5.8	3.0	3.6	4.1	4.3	2.6	4.2	4.9
21	(1E)-5-Methyl-1-(2,6,6-trimethyl-2,4-cyclohexadien-1-yl)-1,4-hexadien-3-one	4.1	3.2	2.1	1.5	2.7	2.9	1.3	4.0	2.5	3.2	3.1	4.1	2.4	4.9	5.8

Table 6.4: Relative concentrations of several classes of compound in *S. aethiopicus* root in response to irrigation regimes and nitrogen levels.

		Nitrogen levels (kg/ha)														
		0			50			100			150			200		
		Percentage														
No	Compound	30	50	70	30	50	70	30	50	70	30	50	70	30	50	70
1	4-Hydroxy-4-methyl-2-pentanone	5.6	2.3	1.9	7.1	5.1	3.2	2.1	1.8	9.4	-	1.6	5.0	1.3	1.3	-
2	2,3-Butanediol	-	5.7	1.1	-	-	3.2	-	2.3	4.3	-	5.1	3.4	3.4	6.5	4.0
3	Acetic acid	3.0	9.3	1.0	1.7	1.2	3.1	2.5	9.9	4.6	3.3	1.9	4.4	1.3	6.4	1.2
4	β-Thujene	8.7	1.5	1.4	1.0	2.9	1.4	3.2	1.1	9.6	2.5	1.2	1.2	7.8	9.5	2.6
5	Sabinene	-	2.2	1.6	-	9.1	2.1	-	4.2	7.8	2.9	2.6	1.9	1.5	2.1	-
6	Eucalyptol	5.3	1.0	8.0	1.4	1.2	8.2	3.6	8.3	1.2	6.3	5.5	9.6	1.7	2.2	1.0
7	1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	1.7	4.4	3.5	7.1	6.2	2.3	1.2	3.5	5.5	2.3	2.7	3.9	5.3	1.0	3.6
8	2,4,6-Octatriene, 2,6-dimethyl-, (E,Z)-	2.5	6.6	1.0	1.4	1.0	3.3	-	2.5	1.3	-	7.1	1.1	7.0	1.4	-
9	Linalool	8.2	2.1	4.1	6.7	-	-	7.7	-	-	1.0	2.5	-	-	-	-
10	*cis*-β-terpineol	6.2	1.8	1.9	3.1	-	-	9.4	-	-	8.4	1.2	-	-	-	-
11	Terpinen-4-ol	3.9	1.0	1.0	3.6	1.1	9.6	3.5	1.1	1.5	4.7	8.5	1.2	1.0	2.0	2.3
12	α-terpineol	2.6	8.2	6.4	3.4	5.5	5.8	3.7	7.6	7.2	3.4	4.0	5.9	7.3	-	6.7
13	2-Hydroxycineol	8.1	1.4	1.5	2.3	1.4	1.5	1.7	2.4	1.0	8.0	7.9	7.9	1.5	1.3	6.7
14	β-elemene	3.1	1.0	1.2	8.6	1.3	1.2	4.2	7.8	2.2	4.0	8.4	1.4	1.1	3.3	-
15	Elixene	2.1	1.5	1.9	2.6	1.2	-	-	1.4	3.5	-	1.3	2.6	2.0	2.2	-
16	Epiglobulol	4.0	1.9	1.5	1.4	1.1	1.1	7.2	1.4	1.6	1.1	1.2	9.9	1.2	3.2	2.5
17	γ-Elemene	1.0	3.0	5.2	1.6	3.4	1.3	9.9	3.5	6.2	-	3.4	4.3	2.4	2.1	7.2
18	β-Vatirenene	2.3	-	-	-	-	-	7.3	-	-	5.2	-	-	-	5.2	3.7
19	Caryophyllene oxide	1.8	1.5	1.2	1.4	1.0	1.6	6.6	1.7	1.3	5.9	1.3	8.4	8.0	-	-
20	Elemol	6.2	1.8	1.8	4.5	1.8	2.5	1.4	2.4	3.1	1.1	2.1	1.6	1.7	2.0	4.7
21	(1E)-5-Methyl-1-(2,6,6-trimethyl-2,4-cyclohexadien-1-yl)-1,4-hexadien-3-one	2.3	4.0	4.0	2.6	3.8	-	1.2	2.8	7.4	1.6	5.6	7.0	1.7	3.3	2.
22	N,N-Dimethylacetamide	-	1.6	1.6	-	1.0	2.0	1.9	2.4	3.0	8.8	-	-	1.1	2.2	-
23	N-Ethylacetamide	2.2	7.2	7.2	-	8.6	6.5	6.0	2.4	-	-	4.6	-	1.1	1.5	5.1

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CHAPTER SEVEN

GENERAL CONCLUSIONS AND RECOMMENDATIONS

Africa is renowned for its high plant biodiversity and valuable natural resources used as traditional medicine for primary health care and nutrition. The long history of traditional healing, indigenous knowledge, beliefs, and theoretical practices of different cultures are still important aspects in administering medications for various kinds of infectious diseases that are common in rural areas. In the past harvesting of medicinal plants was restricted to traditional health practitioners, who understood the conservation practices of wild plants. The rate at which human population is increasing, the growing demand on plant-based drugs for therapeutic purposes will exert pressure on the plant natural habitats and genetic diversity. Also, over-harvesting and exploitation is regarded as the main driver of medicinal plant depletion because the majority of unemployed South Africans rely on collection of plant species for trading and profit to combat economic circumstances. African ginger (*S. aethiopicus)* (Schweinf.) B.L. Burtt is one of the most important rhizomatous plants highly-valued due to its medicinal properties and widely used for respiratory ailments in many regions of the southern Africa. The current research on medicinal value of *S. aethiopicus* grown from different growing regions of South Africa was investigated, as well as the significance of cultivation practices through irrigation and nitrogen management and the assessment of plant metabolite content.

A study was initiated with an effort to contribute valuable knowledge to the medicinal database information relating to the potential medicinal value and strategies to conserve its populations. The specific objectives of the study were to investigate medicinal potential of different plant parts from varying regions in South Africa; evaluate the crop water and nitrogen requirements suitable for cultivation of *S. aethiopicus.* Investigate medicinal potential

through metabolite content determination and profiling volatile compounds in response to cultivation management practices.

In this study, total phenolic content, antioxidant activity and soluble sugars of *Siphonochilus aethiopicus* leaf, rhizome and root from varying growing areas (Mpumalanga, KwaZulu-Natal, Limpopo and North West) were evaluated. The high antioxidant activities observed in the leaf from Mpumalanga suggest its adaptive capabilities to different environments. The results from this study indicate that *Siphonochilus aethiopicus* parts could be used as a potential source for antioxidant properties and encourage cultivation under different growing areas to conserve biodiversity and increase species populations. Cultivation of this plant species should be extrapolated to other regions of South Africa to clearly understand the potential of other plant parts (root and leaf).

The importance of cultivation practices for commercial production and improvement of species biodiversity has made it necessary to investigate the relationship between water and nutrient required by the medicinal plants. The need for a better understanding of the irrigation and nutrient management, especially nitrogen, is important as they indirectly affect each other. Results from the two season data conducted under the rainshelter have shown a significant interactive effect between irrigation regimes and nitrogen levels. The high amount of water utilized from well watered treatment (30% ADL) than from the moderate (50% ADL) and severe (70% ADL) treatments could be attributed to improved water availability and superior plant canopies. Due to the increased temperatures and reduced rainfall as a result of climate change, understanding the correct shortage or excess of water to the plant is appropriate for irrigation management and saving water. The chlorophyll content, plant height, number of leaves per plant and leaf area index increased with increased N supply at 50 and 100 kg/ha.

The well watered treatment had a significantly higher total biomass, fresh and dry rhizome yield compared to other water stressed treatments. Addition of N fertilizer improved the rhizome yield. Taken together, the findings of this study show that, adequate yields can be contributed by irrigation and nitrogen management strategies in cultivation of *S. aethiopicus.*

The present study sought to establish the response of irrigation regime and nitrogen levels to metabolite content. The interaction treatment effect of severely stressed (70% ADL) with the application of 100 kg N/ha had significant effect on leaf phenolic concentration compared to other water stress treatments and N application rates. The results indicate that when different parts of *S. aethiopicus* were stressed, their antioxidant activity increased. This is due to the plant secondary metabolites produced under environmental stress and dominant non-enzymatic plant antioxidants. The high antioxidant activity is a desirable trait to humans due to the ability to scavenge for free radicals attributed to mechanisms of antimalarial, antimicrobial properties and detoxification of enzymes in the human body. In plant carbohydrates, root had high sucrose content with the application of low N grown under severely stressed (70% ADL) treatment. The findings of the study that different *S. aethiopicus* parts can produce substantial amount of antioxidants and carbohydrates, as exhibited under low N and reduced water supply applied during the phenological cycle. It is recommended that the sugar content investigated in this study should be corroborated with amino acids, starch and proline content for further understanding of *S.s aethiopicus* biochemical analysis.

The results showed that the highest volatile components in the root and rhizome were terpenes, as compared to the increased concentrations of aliphatic acids, benzenoids and aliphatic aldehydes in the leaf. In all treatments and samples, the odorant sesquiterpene (1E)-5-Methyl-1-(2, 6, 6-trimethyl-2, 4-cyclohexadien-1-yl)-1, 4-hexadien-3-one was the most

abundant volatile compound. The 4-Hydroxy-4-methyl-2-pentanone was detected under severely stressed (70% ADL) treatment with the application of 100 kg/ha. Severely stressed (70% ADL) treatment with minimal application of N induced the terpenes components in all plant parts. The study showed several bioactive volatile components present in different parts of *S. aethiopicus* which could be isolated and used for the therapeutic purpose. The study showed that volatile components of *S. aethiopicus* vary with plant sources, water stress and mineral nutrient deficiency. It is recommended that essential oil of *S. aethiopicus* be investigated their bioactive compounds.

FUTURE RESEARCH

- ❖ Evaluate emission of volatile organic compounds in response to abiotic stress
- ❖ Isolation and characterisation of bioactive compounds
- ❖ Phylogenetic diversity of medicinal plant species
- ❖ Evaluate photosynthetic carbon accumulation and dry matter in response to plant age

RESEARCH OUTPUTS

Conference proceedings and published paper

- Mokgehle S. N., Tesfay, S. Z. Araya H. T. & duPlooy C.P. (2017). Antioxidant activity and soluble sugars of African ginger (*Siphonochilus aethiopicus*) in response to Irrigation Regimen and Nitrogen levels. Acta Agriculturae Scandinavica, Section B — Soil & Plant Science, 2017. http://dx.doi.org/10.1080/09064710.2017.1293723.

- Mokgehle S. N., Tesfay, S. Z. Araya H. T. & duPlooy C.P. Phenolic content of African ginger (*Siphonochilus aethiopicus)* cultivated under different growth conditions. Combined Congress, George, Cape Town, South Africa, 21 – 25 January, 2015. (Oral presentation).

- Mokgehle S. N., Tesfay, S. Z. Araya H. T. & duPlooy C.P. (2017). Antioxidant activity and soluble sugars of African ginger (*Siphonochilus aethiopicus*) in response to Irrigation Regimen and Nitrogen levels. International conference in Agriculture and Horticulture, Cape Town, South Africa 25-28 June 2015 (Oral).

APPENDIX 1

Randomisation.gen
Randomised block design
3 sets (blocks or reps)
========== of 15 random numbers for 3 irrigation x 5 nitrogen levels)

TMTCOMB	IRRIGATION	NITROGEN
1	30%	0
2	30%	50
3	30%	75
4	30%	100
5	30%	125
6	50%	0
7	50%	50
8	50%	75
9	50%	100
10	50%	125
11	70%	0
12	70%	50
13	70%	75
14	70%	100
15	70%	125

===== BLOCK 1 =====

POSITION	TMTCOMB
1	2
2	4
3	3
4	1
5	15
6	8
7	10
8	5
9	9
10	14
11	7
12	13
13	11
14	6
15	12

===== BLOCK 2 =====

POSITION	TMTCOMB
1	4
2	11
3	13
4	9
5	12
6	14
7	3
8	8
9	6
10	7

11	2
12	1
13	5
14	10
15	15

===== BLOCK 3 =====

POSITION	TMTCOMB
1	10
2	4
3	12
4	5
5	13
6	14
7	15
8	7
9	1
10	3
11	8
12	9
13	11
14	2
15	6

End of Ngoakoana Mokgehle - ARC-VOPI - Project no PDP034. Current data space: 1 block, peak usage 1% at line 15.

GenStat 64-bit Release 15.1 (PC/Windows 7) 24 March 2014 08:17:41

Registered to: ARC