EFFECT OF IRRIGATION RATE ON GROWTH PARAMETERS, YIELD AND TEA QUALITY OF *CYCLOPIA SUBTERNATA*

by

MARY-JANE SEJI MAHLARE

Thesis submitted in fulfilment of the requirements for the degree of Master of Agriculture (MAgric)

Faculty of Applied Sciences

Cape Peninsula University of Technology

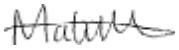
Supervisor: Prof F.B Lewu

Co-supervisor: Dr M.N Lewu

Wellington
2023

DECLARATION

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



June 2023

Signed **Date**

ABSTRACT

Cyclopia, generally known as honeybush, and belonging to the *Fabaceae* family, originates from the Cape Floristic Region of the Eastern and Western Cape provinces of South Africa. Honeybush (*Cyclopia* spp.) plants are a rich source of antioxidant properties and phenolic compounds, and the extracts are utilized as beverage and other aesthetic products. Currently, six honeybush species are commercially cultivated, but to date, there are limited trials attempting to study their agronomic water demand. Two pot trials and a field study were conducted at three different sites where *Cyclopia subternata* plants were cultivated on different soil types (Stellenbosch granite, Stellenbosch shale and Stellenbosch clovelly) and subjected to three different water deficit stress levels (well-watered, semi-stressed and stressed). Remarkably, irrigation treatments and soil types did not significantly affect the growth of the plants. However, the well-watered treatment consistently had higher yields compared to the other two treatments. Proline which generally accumulates in plants that undergo different stresses (biotic and abiotic) was studied in all three experiments using the colorimetric method. The water-stressed (semi-stressed and stressed) treatments had higher proline concentrations with lower relative water contents (RWC), which signify water stress. Stomatal conductance was also investigated only in one pot study and field experiment and generally, lower in stressed plants and highest in well-watered plants. The drop in stomatal conductance in the stressed plants was due to the induction of stomatal closure which is a coping mechanism to aid survival by reducing transpiration rate. The development, growth and yields of the plant can be limited by water availability. Thus, this study also investigated the changes in molecular functions, cellular components, and biological processes of *C. subternata* exposed to different water stress conditions (T1, T2, T3, T13, T17, and T19). The proteins found in *C. subternata* leaves were differentially identified and quantified with quantitative mass spectrometry. A total of 11 proteins were differentially expressed proteins (DEPs) were identified using Fisher's Exact Test ($p < 0.00100$). Only α-glucan phosphorylase was found to be statistically common between T17 and T19 ($p < 0.00100$). In T19, 5 DEPs were upregulated and 6 were downregulated. Based on gene ontology, the DEPs in the stressed plant were associated with cellular and metabolic processes, response to stimulus, binding, catalytic activity, and cellular anatomical entity. Majority of these proteins were involved in photosynthesis, phenylpropanoid biosynthesis, thiamine, and purine metabolism. This study revealed the presence of trans-cinnamate 4-monooxygenase, an intermediate for the biosynthesis of large number of substances, such as phenylpropanoids, coumarins, and flavonoids. As much as consumers prefer healthy food, they are mostly not eager to compromise on taste and other sensory properties. Studies have proven that water stress has various effect on the sensory quality of different plant food products. In terms of tea quality, a

descriptive sensory analysis was used for field grown *C. subternata* to compare the infusions prepared from three water deficit treatments. Severe water-deficit stress seems to boost the sensory profile of the infusions, in particular the ‘woody’, ‘fynbos-floral’, ‘rose perfume’, ‘fynbos-sweet’ and ‘sweet spice’ aromas as well as a sweet taste.

Key words: *Cyclopia subternata*, relative water content, proline, proteins, volatile organic compounds, water deficit stress**,** stomatal conductance, tea

ACKNOWLEDGMENTS

I would like to pass my heartfelt gratitude to the following individuals and institutions:

- My **Heavenly Father** for the gift of life, bravery to start this journey, strength to stay strong and for the physical and mental ability to reach the finishing line.
- Agricultural Research Council (ARC, Infruitec-Nietvoorbij) for allowing me to use their laboratories and all facilities.
- Department of Science and Technology for funding the project.
- Cape Peninsula University of Technology for funding my studies (M08/023354) / (UNOPS FUNDING-C/C E084).
- Department of Higher Education and Training for funding my studies through Nurturing Emerging Scholars Programme (NESP).
- My supervisors, **Dr Nike Lewu** and **Prof Francis Lewu** for their time, mentorship, and guidance from the conceptualisation of the project until completion of thesis writing.
- **Dr Cecilia Bester**, for funding acquisition and project management.
- **Dr Oluwafemi Caleb** (Stellenbosch University, Faculty of AgriSciences) - for his time, support, and guidance.
- **Dr Lizex Husselmann** and **Mrs Gadija Mohamed** (University of the Western Cape, Proteomic Unit) - for their time, patience, support and assistance with the proteomic analysis and result interpretation.
- **Dr Brigitte Du Preez** and **Dr Erika Moleich** of Sensory Research Facility, Department of Food Science, Stellenbosch University for sensory analysis of the *C. subternata* irrigation trial samples.
- **Prof Lizette Joubert** of Post-Harvest and Agro-Processing Technologies of ARC Infruitec-Nietvoorbij is thanked for her time and guidance on writing and editing the sensory analysis chapter.
- **Mr George Dico** of the Post-Harvest and Agro-Processing Technologies of ARC Infruitec-Nietvoorbij is thanked for the herbal tea processing of the plant material samples of the *C. subternata* irrigation trial.
- **Dr Marieta van der Rijst**, for her assistance with statistical analysis throughout the study.
- Staff of Soil and Water Science division at the ARC Infruitec-Nietvoorbij, **Mr Ncedo Ndololwana**, **Mr Francois Baron**, **Mr Trevor Harris**, **Mr Jeffrey Adams**, **Mr Lester Sassman, Dr Carolyn Howell** and **Mrs Isabella van Huyssteen** for their time, respective contributions, and technical support. I wouldn't have done it without their assistance.
- My mother (**Machoshane Mahlare**), father (**Mohlotsoane Mahlare**), brothers (**Mantake** and **Tseke Mahlare**) and aunt (**Maselepe Mphahlele**) for their daily prayers, encouragement, love and support.

- My cheerleader, **Tshepho Sekwati** for his love, support, encouragement, and believing so much in me even during gloomiest and stormy days.
- **Dr Lutendo Nethononda** for his valuable advice, prayers and constantly reminding me of my capabilities.

Opinions expressed in this thesis and the conclusions arrived at, are those of the author, and are not necessarily to be attributed to the Department of Higher Education and Training.

DEDICATION

To my sweet little darling, **Thato Mahlare**, you may not understand what I am doing right now but it will all make sense one day.

To those who came before me, I hope you are beaming with pride wherever you are resting.

RESEARCH OUTPUTS

Published articles:

Mary-Jane Seji Mahlare[1], Muinat Nike Lewu[2], Francis Bayo Lewu[1*] and Cecilia Bester[2]**.** 2023. *Cyclopia subternata* growth, yield, proline and relative water content in response to water deficit stress. DOI**:** https://doi.org/10.17159/wsa/2023.v49.i1.3988

Mary-Jane S. Mahlare[1, 2], Lizex Husselmann[3], Muinat N. Lewu[1*], Cecilia Bester[1], Francis B. Lewu[2], Oluwafemi James Caleb[4,5*]. 2023. Analysis of the differentially expressed proteins and metabolic pathways of honeybush (*Cyclopia subternata*) in response to water deficit stress. *Plants.* DOI: 10.3390/plants12112181

Mary-Jane Seji Mahlare[1], Muinat Nike Lewu[2], Francis Bayo Lewu[1*] and Cecilia Bester[2]. 2022. Response of *Cyclopia Subternata* to Watering Frequency: Stomatal Conductance, Proline, and Relative Water Content. DOI: 10.17758/IICBE4.C1122203

Conference presentations:

Mary-Jane Seji Mahlare[1], Muinat Nike Lewu[2], Francis Bayo Lewu[1*] and Cecelia Bester[2]. 2022. Response of *Cyclopia Subternata* to Watering Frequency: Stomatal Conductance, Proline, and Relative Water Content – Oral presentation. JOHANNESBURG International Conference on "Chemical, Biological and Environmental Engineering" (ICCBEE-22) scheduled on Nov. 28-29, 2022, Johannesburg (South Africa). Presented on the 29th of November 2022.

Mary-Jane Seji Mahlare[1], Muinat Nike Lewu[2], Francis Bayo Lewu[1*] and Cecelia Bester[2]. 2021. Estimation of water stress on growth, proline and relative water content of honeybush cultivated on different soil types – Oral presentation. The Environment, Climate Change and Sustainability, CPUT Postgraduate conference, Western Cape, South Africa. Presented on the 30th of November 2021.

Mary-Jane Seji Mahlare[1], Muinat Nike Lewu[2], Francis Bayo Lewu[1*] and Cecelia Bester[2]. 2021. Estimation of drought stress on growth, proline and relative water content of honeybush cultivated on different soil types – Oral presentation. Indigenous Plant Use Forum (University of Johannesburg, IPUF 2021). Presented on the 5th of July 2021.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

Abbreviations	Descriptions
ANOVA	Analysis of variance
ARC	Agricultural Research Council
CAF	Central Analytical Facilities
CRF	Cape Floristic Region
DEP	Differentially expressed proteins
EC	Enzyme code
KEGG	Kyoto Encyclopedia of Genes and Genomes
IG	Infruitec greenhouse
NF	Nietvoorbij field
NG	Nietvoorbij greenhouse
PC	Pot capacity
PAW	Plant available water
RBD	Randomised block design
RPM	Revolutions per minute
RWC	Relative water content
SAHTA	South African Honeybush Tea Association
VOC	Volatile organic compound
%	Percentage
mL	Millilitre
°C	Degree Celsius
g	Gram
L	Litre
nm	Namo meter

CHAPTER ONE
GENERAL INTRODUCTION

1.1 Introduction

A country's economy can be greatly impacted by the marketing of indigenous plants and animals (Rampedi and Olivier, 2008). Several studies have demonstrated that indigenous teas of South Africa provide essential nutrient supplements and have economic potential (Rampedi and Olivier 2005; McGaw *et al*., 2007). Despite challenges associated with cultivating honeybush tea plants, the South African Honeybush Tea Association (SAHTA, 2016) is striving to unite the industry. In recent years, honeybush (*Cyclopia* species) tea has grown in popularity due to its many health benefits, and it has become necessary to commercialize the industry (McGregor, 2017). Tea is the second most widely consumed beverage after water. Various types of tea, including oolong, green, dark, and herbal teas are produced depending on the post-harvest treatment (Soni *et al*., 2015).

South Africa is home to rooibos (*Aspalathus linearis*), bush (*Athrixia phylicoides*) and honeybush (*Cyclopia* species) teas. Even though the commercialisation of bush tea is still in the infancy stage, honeybush has picked up recognition while rooibos is the most known and well-established in the industry (Van Wyk and Gericke, 2000; Joubert *et al*., 2008; Joubert *et al*., 2011). Before the year 2005, a fraction of these tea plants (particularly honeybush) was not wholly developed as commercial products. People in urban areas had to depend on supplies from rural community members who live in regions where these tea plants grow naturally. However, various studies have documented that these indigenous tea species contain essential nutrients that can promote health and provide economic benefits (Rampedi and Olivier 2005; McGaw *et al*., 2007). These findings may have spurred consumer interest further in the consumption of indigenous teas.

Honeybush tea is sold as either black or green which is fermented or unfermented respectively (Horn, 2019). It contains flavonoid properties, which have shown cancer prevention agent impacts, due to its ability to scavenge free radicals and act as antioxidants. The phenolic compounds are perceived as credibly useful to wellbeing. The polyphenols as active segments associated with honeybush tea have beneficial effects on human wellbeing (Soni *et al*., 2015).

The demand for indigenous tea has prompted concerns of over-exploitation of natural populations of the *Cyclopia* species. A significant factor contributing to the growing market of the overall honeybush industry is the consumers being aware of its potential for wellbeing due

to its antioxidants (Joubert *et al*., 2011). Previously, harvesting practices contributed to the decrease and even disappearance of populations of the species (Du Toit *et al*., 1998). To ensure sustainable production, commercial honeybush plantations were established (Joubert *et al*., 2011).

Even though more than 20 honeybush species grow naturally in the wild, only a few of them are commercially used to produce tea. Up to this point, *C. intermedia, C. genistoides* and *C. subternata* are exported, as the demand surpasses supply. Others, such as *C. maculata, C. longifolia* and *C. sessiliflora* were incorporated to cater for the bigger demand (Joubert *et al*., 2011).

1.1 Statement of the research problem

Honeybush (*Cyclopia* species) is an endemic fynbos shrub growing naturally in the Western and Eastern Cape Provinces. Despite its potential as herbal tea, honeybush tea remained a cottage industry until the mid-1990s when its commercial potential was realised, and a few producers established plantations. Through research, the species has been labelled a “healthy beverage”, bringing this unique niche crop under the attention of national and international tea lovers. The healthy beverage status of the *Cyclopia species* is a key driver of consumption.

South Africa's honeybush industry is still very young, producing an average of over 200 tons per annum of processed tea (Joubert *et al*., 2011; McGrevor, 2017). Even though some species of *Cyclopia* are commercially cultivated, about 85% of the country's production still comes from harvesting wild-growing honeybush plants. The industry is currently unable to cope with the enormous international demand that outweighs local supply. The increased rate of wild harvesting diminishes the natural population, thus, making the exploitation of *Cyclopia* species unsustainable, hence, threatening the population of the species in their natural habitat.

Commercial production, therefore, is becoming increasingly important to save the natural habitat from a decline in the genetic diversity of wild populations and the gene pool of the species. Other factors threatening the growth of the honeybush industry include drought and veld fires. Although, some species of honeybush plants are commercially cultivated, to date, very limited field studies have attempted to investigate the water deficit stress of the species. However, the water needs of the honeybush have not been investigated.

Even though farmers do irrigate honeybush whenever they feel there is a need for it, the amount of water applied has never been quantified and the water use of these species is still

unknown (Mahlare *et al*., 2023). Incidents of climate change now make it more important to irrigate honeybush tea plants to ensure the sustainability of the honeybush tea industry. Science based studies of this nature are deemed important since some of the cultivated and naturally growing honeybush species were lost due to the heat wave and drought conditions of the recent past in the Western Cape, because of climate change.

Therefore, a field study to determine the irrigation water requirement of *C. subternata* honeybush species was conducted. *C. subternata* was selected because it is among the cultivated honeybush species currently advocated for commercialisation on a large scale and thus, of commercial interest. Since the *Cyclopia* species is a niche tea produce of South African origin, irrigation guidelines for this crop are a priority.

1.2 Aims and objectives

Broad Aim: this experiment aims to evaluate the effects of three different irrigation levels on growth parameters, yield, and tea quality of *C. subternata*.

Specific Objectives:

- To determine the effect of irrigation at three different levels of plant available water (PAW) depletion from transplanting to harvesting on the shoot growth of a cultivated *C. subternata* honeybush species.
- To document the effect of irrigation at three different levels on the yield of *C. subternata*.
- To determine the tea quality of *C. subternata* through evaluation of the sensory analysis.
- To evaluate the accumulation of proteomes on *C. subternata* subjected to three different levels of PAW depletion during plant growth.
- To study the effect of irrigation at three different levels for water use efficiency of *C. subternata*.

1.3 Hypotheses and research questions

1.4.1 Hypotheses:

1.4.1.1 Variation in irrigation levels will have minimum impact on the quality of the *C. subternata* tea.

1.4.1.2 Different irrigation rates will result in significant differences in the growth parameters and yield of *C. subternata*.

1.4.1.3 The different irrigation levels will influence the formation and availability of volatile organic compounds in *C. subternata*.

1.4.2 Research questions:

1.4.2.1 What will the effects of three different irrigation levels be on the quality of *C. subternata* tea?

1.4.2.2 How will different irrigation levels affect the growth parameters of *C. subternata*?

1.4.2.3 What will the effects of irrigation at three different levels be on the yield of *C. subternata*?

1.4.3 Delineation of the research

Other South African indigenous tea other than *Cyclopia* species.

REFERENCES

Du Toit, J., Joubert, E. and Britz, T.J., 1998. Honeybush tea–a rediscovered indigenous South African herbal tea. Journal of Sustainable Agriculture, 12(2-3), pp.67-84.

Horn, A., 2019. Honey Bush Tea - Small Industry, big potential. 36 - 38.

Joubert, E., Gelderblom, W.C.A., Louw, A. and De Beer, D., 2008. South African herbal teas: Aspalathus linearis, *Cyclopia* spp. and Athrixia phylicoides—A review. *Journal of ethnopharmacology*, 119(3), pp.376-412.

Joubert, E., Joubert, M.E., Bester, C., De Beer, D. and De Lange, J.H., 2011. Honeybush (*Cyclopia* spp.): From local cottage industry to global markets—The catalytic and supporting role of research. *South African Journal of Botany*, 77(4), pp.887-907.

Joubert, E.D.B.D. and de Beer, D., 2011. Rooibos (*Aspalathus linearis*) beyond the farm gate: From herbal tea to potential phytopharmaceutical. *South African Journal of Botany*, 77(4), pp.869-886.

Mahlare, M.S., Lewu, M.N., Lewu, F.B., & Bester, B. (2023). *Cyclopia subternata* growth, yield, proline and relative water content in response to water deficit stress. *Water SA*, 49(1), 64–72. https://doi.org/10.17159/wsa/2023.v49.i1.3988

McGaw, L.J., Steenkamp, V. & Eloff, J.N., 2007. Evaluation of *Athrixia* bush tea for cytotoxicity, antioxidant activity, caffeine content and presence of pyrrolizidine alkaloids. *Journal of Ethnopharmacology*, 110(1), pp.16.

McGregor, G., 2017. An overview of the honeybush industry. Department of Environmental Affairs and Development Planning, Cape Town. pg, 2(1), p.3.

Rampedi, I. & Olivier, J., 2005. The use and potential commercial development of *Athrixia phylicoides*. *Acta Academica*, 37(3), pp.165-183.

Rampedi, I. & Olivier, J., 2008. The development path of rooibos tea–a review of patterns and lessons learnt for the commercialisation of other indigenous teas in South Africa. *International Journal of African Renaissance Studies*, *3*(2), pp.5-20.

Soni, R.P. Katoch, M., Kumar, A., Ladohiya, R. & Verma, P., 2015. Tea: Production, Composition, Consumption and its Potential as an Antioxidant and Antimicrobial Agent. *International Journal of Food and Fermentation Technology*, 5(2), pp.95-106.

South African Honeybush Tea Association Business Plan, 2012-2016, For: Western Cape Department of Agriculture.

Van Wyk, B.E., & Gericke, N., 2000. People's Plants. A Guide to Useful Plants of South Africa. First Ed. Briza Publication, Pretoria, South Africa, p. 102.

CHAPTER TWO
LITERATURE REVIEW

2.1 Introduction

Honeybush is the general term used to classify all the known types of Cyclopia species, a genus in the leguminous family (Schutte and Van Wyk, 1996). It is native to the fynbos biome in the Western and Eastern Cape Provinces of South Africa and its tea has a sweet, honey-like taste (Du Toit and Joubert, 1998). *Cyclopia* species are among the few wild plants that are turned into commercial commodities in South Africa. Although 23 species grow in the wild, only a few, that is, *C. subternata, C. intermedia, C. genistoides, C. maculata, C. longifolia* and *C. sessiliflora* are currently used to make tea (Mabizela, 2020; Koen *et al*., 2021). The popularity of honeybush cannot only be attributed to its unique taste, but also to its low tannin content, the absence of caffeine, and the presence of antioxidants. The tea is usually enjoyed in its fermented state, although the unfermented (green) one is also marketed (Le Roux *et al*., 2012).

Increasing awareness of the health benefits of *Cyclopia* species is one of the factors contributing to the market's growth (Joubert *et al*., 2011). However, at a point, the absence of standardisation of good quality tea was recognized as a significant weakness in the effective commercialisation and headway of the honeybush business (Du Toit *et al*., 1998; SAHTA, 2014). Hence, Theron *et al*. (2014) built up a non-exclusive honeybush sensory wheel and lexicon dependent on the tangible compounds of six *Cyclopia* species. With consumers gradually being more wellbeing conscious, there is now a bigger appreciation for herbal goods including organic teas (Payne *et al*., 2014). The resultant effect is the rapid expansion of the honeybush tea market in the recent years, with demand surpassing supply (Joubert *et al*., 2011). Not so long ago, markets comprised primarily of *C. intermedia, C. genistoides* and *C. subternata*, but as the demand continues to surpass supply, other *Cyclopia* species were incorporated to cater for the higher demand (Joubert *et al*., 2011).

The high demands for these indigenous teas have raised concerns of harvesting *Cyclopia* populations excessively beyond the sustainable limit. Previously, harvesting led to the deterioration and decline of the species populations (Du Toit *et al*., 1998). Later, commercial plantations were set up to guarantee effective and continuous production of honeybush (Joubert *et al*., 2011). The honeybush industry intends to formalize production practices that will comprise of emerging as well as small scale farmers. The aim is to keep the industry competitive and contribute to the critical economic growth of rural communities of the Eastern and Western Cape provinces (Den Hartigh, 2011). The natural occurrence of *Cyclopia* species in specific areas is helping with the identification and mapping of suitable areas for commercial

cultivation of honeybush plants; which is, however, dependent on effective propagation and cultivation practices (Jacobs, 2007; Jacobs, 2008).

Currently, more than 200 ha of mostly *C. genistoides* and *C. subternata* are under cultivation to cater for demand. However, *C. intermedia* is not favoured for cultivation as it can only be harvested every second or third year as frequent harvesting results in die-back due to insufficient build-up of energy reserves in the rootstock, deeming it uneconomical for cultivation (Joubert *et al*., 2011). Soil type and precipitation are the most significant factors in deciding areas that are well-suited for honeybush development (Jacobs, 2008). Wild harvesting, particularly of *C. intermedia*, is a significant supporter of the annual production (Bester, 2013).

2.2 Botanical description of honeybush plants

Fully developed honeybush shrubs are long-term perennials that differ in their development environment from little, multi-stemmed shrubs to tall, single-stemmed trees (Schutte and Van Wyk, 1996). The life expectancy of the shrub is around 15 years and afterward it ought to be supplanted. The flowers have single-bloomed inflorescences and nicely scented with brilliant yellow colour. Leaf shape and size vary among the species; however, most have slim, needle-like to long leaves (DAFF, 2016).

Bond and Goldblatt (1984) reported that most honeybush plants may grow up to 1.5 meters tall, while others may be 3 meters high (Figure 2.1A). The leaves are trifoliated with difference in structure, from narrow to flat leaves (Mbangcolo, 2018). Honeybush plants can be effectively distinguished in the field during the blooming season as they have distinctive yellow flowers with nectar-like aroma (Figure 2.1B). They normally start flowering in spring (September through October), apart from *C. sessiliflora* that blossoms late, early autumn or early winter (May and June) (Schutte, 1995). DAFF (2016) additionally reported that they have woody stems and hard-shelled seeds which are framed in tiny pods that turn brown and split open as the seeds mature. The seeds are dicotyledonous and share numerous anatomical appearances with other vegetable species (Ma *et al*., 2004)

Figure 2.1: Matured *C. subternata* plant at Napier (Soetmuisberg) farm (A) and *C. subternata* during flowering stage at Nietvoorbij (Stellenbosch) field (B)

2.3 Geographic distribution of *Cyclopia* species

The *Cyclopia* species are endemic to the Cape Floristic Region (CFR) and can grow under a variety of conditions. Generally, they prefer cooler, shady southern slopes, except for *C. genistoides*, which inhabits sandy and flat grounds. (Turpie *et al*., 2003; Mabizela, 2020). The shrubs can be produced efficiently on acidic soils with good drainage, low phosphorus concentrations and minimum nematode infestation. Therefore, they can grow successfully on soils that are not fit for the cultivation of most crops. SAHTA (2011) stated that *Cyclopia* is ideal for rural community projects as it is cost-effective. *Cyclopia* is harvested from the wild on an estimated 200+ hectares (approx. 75%) (Figure 2.2), as well as under cultivation (SAHTA, 2011; Bester, 2013).

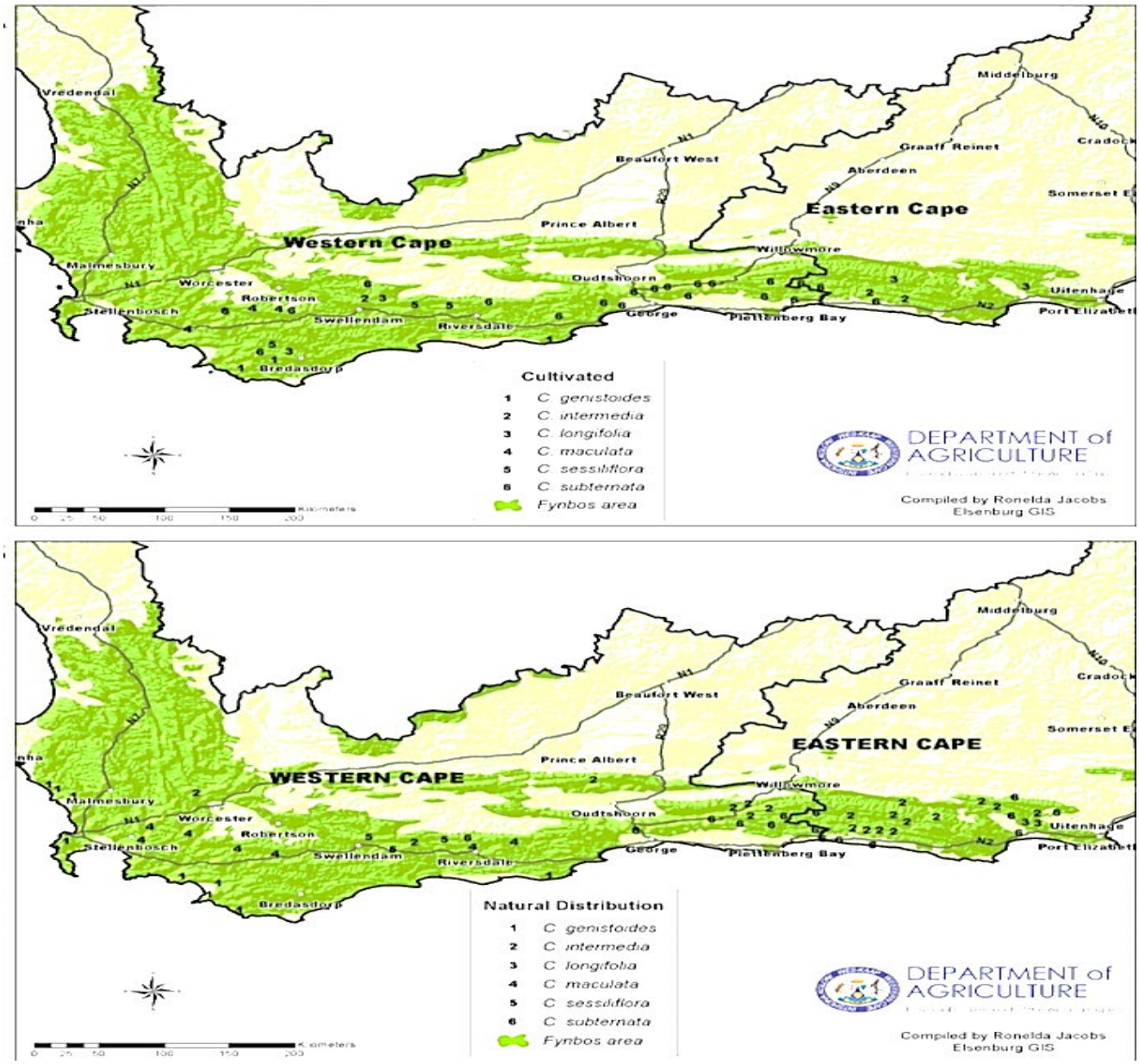


Figure 2.2: Areas where different *Cyclopia* species are cultivated (top) and occur naturally (bottom) (Joubert et al., 2011).

The CFR covers a region of 85 240 km^2, through which all wild honeybush grows naturally. *C. subternata* and *C. genistoides* are the most cultivated species. Their development is confined from Overberg region to the Langkloof, with around 100 ha under cultivation (SAHTA, 2011). *C. subternata* grows mostly on sandy loam soil in valleys of Langkloof, Waboomskraal close to George and Riversdale territory (SAHTA, 2012). The required soil pH for optimum establishment of honeybush ranges from 4.0 – 5.0 (Joubert *et al*., 2007; Spriggs and Dakora, 2007; Kokotkiewicz *et al*., 2012). The rising demand of honeybush tea can bring about the extinction of the wild populations, which may be brought about by the uncontrolled harvesting techniques (Mbangcolo, 2008). As indicated by SAHTA (2011), the cultivation of *Cyclopia*

species has diminished from 230 ha before (1993) to 200 ha (2011), signifying the decline in *Cyclopia* plantations. Currently, *Cyclopia* contributes 16% to the commercialised teas in the country compared to the 30% that was reported previously because of the diminishing in *Cyclopia* plantations.

Therefore, the cultivation of *Cyclopia* species is encouraged to secure a consistent and reliable supply while alleviating the strain on the wild population as well as meeting high market demands (Hobson and Joubert, 2011; Joubert *et al*., 2011). A market profile of honeybush indicates that it declined to 120 ha in 2013, and then inclined to 200 ha in 2015 (Joubert *et al*., 2011, Hobson and Joubert, 2011; Erasmus, 2012; DAFF, 2011; 2013; 2015). The Agricultural Research Council (ARC) then proceeded to domesticate the wild plant species for commercial production. Utilizing improved seeds, ARC showed the capacity to harvest as much as 10 tons of *C. subternata* per hectare (Bester, 2016).

2.4 Health benefits of honeybush tea

Tea is the most consumed healthy drink as demonstrated by numerous scientific investigations in the world. It is one of the most investigated plant-based remedies (Soni *et al*., 2015). Indigenous teas like red bush and honeybush are progressively common in the medical care diet due to their caffeine-free properties (Joubert *et al*., 2008). Extracts of these teas are also utilized in food and other aesthetic products (Joubert and De Beer, 2011; Joubert *et al*., 2011). These herbal teas are famous for their rich organic antioxidant properties and phenolic compounds which are helpful in treating colon, throat and lung ailments, heartburn, ulcers, nausea, and urinary tract infections (Soni *et al*., 2015; Joubert *et al*., 2019). Polyphenols in the unfermented honeybush tea have been broadly perceived as having anti-cancerous properties (Jankun *et al*., 1997; Kanwar *et al*. 2012). Honeybush tea contains the following nutritional components as depicted in Table 2.1:

Table 2.1: Nutritional composition of honeybush tea (Bates *et al*., 2000).

Nutrients	Function in Body
Calcium (Ca)	Strengthens teeth and bones
Magnesium (Mg)	Maintains healthy nervous systems
Sodium (Na)	Maintains fluid and acid-base balance
Copper (Cu)	Essential to the various metabolic processes
Zinc (Zn)	Ensures healthy skin growth and development
Potassium (K)	An essential component of metabolic processes
Manganese (Mn)	Metabolic functions, bone growth, and development
Iron (Fe)	Essential for the transportation of oxygen in the body

2.5 The market for processed honeybush tea

The exported yield of honeybush has expanded with an increasing demand during the most recent 15 years. It is estimated that 90 percent of the honeybush tea crop is exported to the Netherlands (± 44%), Germany (± 30%), United Kingdom (± 8%), and the United States (± 7%), while the remaining percentage is packaged for local consumption in South Africa (McGregor, 2017; Karsen *et al*., 2022). Certain honeybush combinations have 10 times the higher retail value in the international markets than in local markets (Horn, 2019). Joubert *et al*. (2011) provided the export data for 1999–2009, whereas Perishable Produce Export Certification Agency annual reports provided statistics for 2010–2019 (PPECB, 2020) as shown in Figure 2.3. However, it is difficult to estimate the total amount of tea plant material produced annually since the local market does not record the percentage of tea sold (Karsen *et al*., 2022). Secondary products are created by utilizing high-value components in tea and turning them into makeup, which offers a higher financial incentive than the actual tea (Horn, 2019). The export of honeybush has steadily increased over the past 19 years from 50 t in 1999 to a peak of 632 t in 2011. Thereafter, it decreased to an all-time low of 195 tons in 2017, before increasing again to 584 tons in 2019, indicating a positive outlook (Karsen *et al*., 2022).

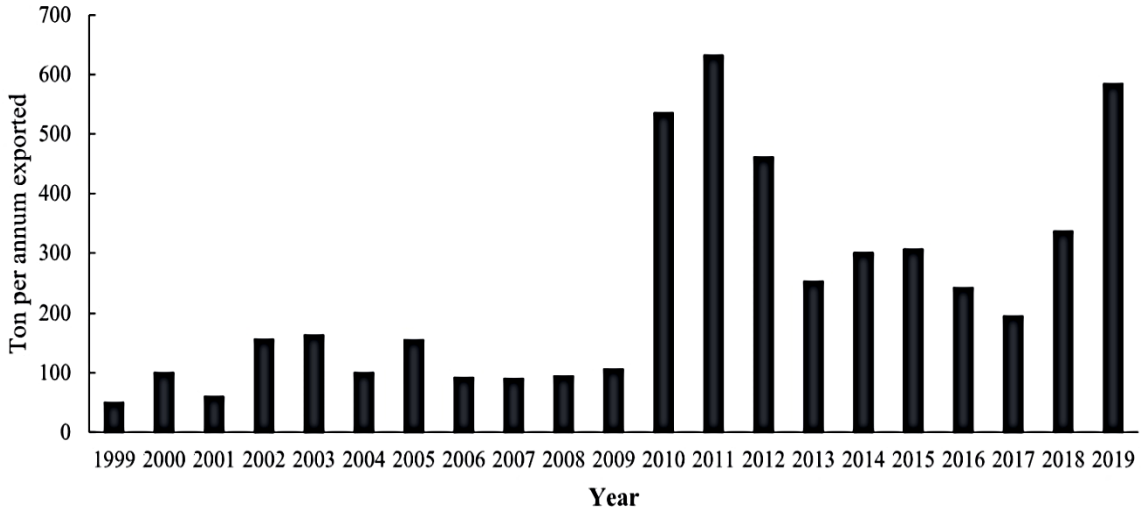


Figure 2.3: The quantity of processed honeybush tea exported from South Africa in tons over time (Joubert *et al*., 2011; PPECB, 2020).

2.6 Harvesting and processing of honeybush tea

Sprouters like *C. intermedia, C. genistoides* and *C. sessiliflora* can be harvested 2 to 3 years after planting (Viljoen, 2001). They are cut at the soil level to encourage the development of new shoots from the rootstock. The non-sprouter like *C. subternata* generally grows quicker and can be harvested annually by cutting the shoots in the range of 30 and 50 cm over the ground level. (Joubert *et al*., 2011). Motorized feed cutters are utilized to cut the plant material into tiny pieces (2-3 mm long) to quicken the fermentation process. (Du Toit *et al*., 1998). Cut

plant material is pre-treated with water to bring about more consistent earthy, brown-coloured tea leaves with enhanced attributes (Du Toit and Joubert, 1998). The end-product is passed through an electrically operated tube-shaped sifter with a 6.5 mm gap screen, to eliminate all the thicker pieces (Viljoen, 1994). The quality norms, regarding taste and aroma, for the market price of honeybush tea are characterized by the Agricultural Product Standards (Act no. 119 of 1990).

2.7 Quality attributes of honeybush tea

2.7.1 Flavour and taste

Flavour is an important part of the tea and is due to a combination of aroma, taste and usually one or more of other factors such as taste sensation, texture, and temperature. Among these contributors, taste sensation, aroma and basic taste are greatly dependent on chemicals and their structural variations (Kulka, 1967). The bitter taste is notably in the unfermented teas. Consumers relate honeybush tea made from various *Cyclopia* species with a sweet taste, so the bitter taste is viewed as inconvenient to product approval. The bitter taste is often the result of the presence of phenolic compounds (Vermeulen, 2015). Phytonutrients like polyphenolic corrosive derivates, flavonoids, isoflavones, terpenes and glucosinolates are generally identified as bitter (Drewnowski and Gomez-Carneros, 2000). Bitter compounds arise in numerous varieties; the most grounded and significant agents are alkaloids, terpenoids and flavonoids (Ley, 2008).

2.7.2 Aroma

Tea quality is determined by colour, freshness, strength, and aroma. The phenolic compounds in tea are responsible for its colour and taste, while the volatile compounds contribute to its odour and aroma (Yang *et al*., 2013). Honeybush and sweet aroma could be delegated as non-exclusive aroma, since this attribute is common to all honeybush species. However, the other four scents, namely, Earl Gray, lemon, plant-like and rooibos can be delegated as species-explicit aromas. It was similarly recommended that further study is required to profile the sensory characteristics of every *Cyclopia* species to build up a substantial flavour lexicon for honeybush tea (Cronje, 2010). Aroma plays an important role in determining the sensory characteristics and economic value of tea (Guo, 2021).

2.7.3 Active compounds responsible for aroma

A significant responsibility in the chemistry of flavour is to recognize the aroma active compounds and the less smelly or scentless compounds present. It is known that honeybush tea, like many other natural products, contains many volatile organic compounds (VOCs) that

have diverse properties both physiologically and chemically. However, the perception of aroma is not due to all these volatile compounds (Ntlhokwe, 2016). VOCs have distinctive fragrance actions that can be credited to three significant properties of a compound namely the total edge, the strength of the compound as an element of its dilution and its quality (Delahunty *et al*., 2006).

A couple of compound mixes in a composite aroma blend adds to the general scent because of their various properties. Furthermore, aromas of various qualities can mix or smother each other to create a new aroma. Mixes with comparable characteristics can mix and produce another aroma (Delahunty *et al*., 2006). Black tea has been found to contain hundreds of volatiles, while oolong and green tea have fewer due to the lesser degree of fermentation. Black tea can contain more than 600 volatiles, and 41 of these contribute significantly to its aroma (Schuh and Schieberle, 2006). Certain compounds present in concentrations beneath their aroma perimeter, or which has no aroma movement when surveyed exclusively, can add to the aroma when they are in a blend (Delahunty *et al*., 2006). Gas-chromatography-olfactometry (GC-O) is utilized to decide the scent group of unsteady natural compounds in samples and to appoint a relative significance to every one of these mixes (Delahunty *et al*., 2006).

2.7.4 Aroma analysis

Sensory analysis can be defined as a “scientific discipline used to evoke, measure, analyse and interpret reactions to characteristics of foods and beverages as they are perceived by the senses” (Stone and Sidel, 1993). Sensory evaluation is viewed as a definitive technique to determine aroma as concoction and instrumental strategies do not have the intensity of the human sensation and the capacity to coordinate recognitions (Aparicio *et al*., 1996). Nonetheless, sensory analysis with a human board can be mistaken, labour intensive and time consuming because of exhaustion, contamination and perspective (Dutta *et al*., 2003). A sensory wheel is basically a lot of terms used to describe the scent, flavour, taste as well as mouthfeel of a particular product (Drake and Civille, 2002).

To create a trustworthy sensory lexicon, a few points ought to be considered: trait intensities must be secured reliably; terms must be accurate, clear, and correctly defined; reference guidelines given; and the terms must be selective, straight-forward, related and not more than is needed (Meilgaard *et al*., 1999; Drake and Civille, 2002). The one created for tea is restricted to canned teas (Cho *et al*., 2005), green tea (Lee and Chambers, 2007; Lee *et al*., 2008) and currently for rooibos tea (*Aspalathus linearis*) (Koch, 2011).

2.8 Significance of water in agriculture

The Earth's surface is covered by 70% water, which plays an important role in all living organisms' biochemical and metabolic processes as well as transportation of nutrients (Wood, 2007; Harb *et al*., 2010; Long and Pijanowski, 2017). Presently, the shortage of water has massively increased because of an ever-increasing population, industrialisation, water pollution and poor management, climate change and others, which destroys the water resource quality and quantities from local to global scales (WWAP, 2012; Connor, 2015; Long and Pijanowski, 2017). About 60% of the available hydric resources is utilised for irrigation purposes in developed countries while developing countries can use up to 90% (Adeyemi *et al*., 2017; Velasco-Muñoz *et al*., 2018).

Water has always been of utmost importance for almost all food contributing about 85% to 95% of the mass of most plants. Insufficiency of water inhibits essential processes such as photosynthesis, respiration and transpiration, thus, restraining growth and development of plants (Bartels and Souer, 2003; Henry and Krutz, 2016; Farooqi *et al*., 2020). In simple terms, plant growth cannot be efficient without water, more especially those that are grown for commercial purposes (Nielsen *et at*., 2009; Henry and Krutz, 2016). Although irrigation studies are not new, the focus of the previous research were mostly limited to plant performance in response to irrigation (Jermár 1989; Pereira, 2017). A study conducted by Carruthers *et al*. (1997) reported that it is crucial to investigate "the links between water scarcity, food production, food security, and environmental sustainability".

It is evident that good fertilizer application and enhanced farming systems increase yields and performance; however, water and irrigation are playing a vital role in achieving and maintaining continuous food security to cater for an ever-increasing population (Pereira, 2017). Although, 275 million hectares of cultivated land are in existence, with yearly increase of 1.3%, only 23% is devoted to irrigated farming. Irrigated farmlands contribute a total of 45% to food production, which ought to increase by 70% to meet food demand by 2050 (IWMI, 2007; Hedley *et al*., 2014; Singh, 2014; Gago *et al*., 2015; Wu and Ma, 2015; Velasco-Muñoz *et al*., 2018).

2.8.1 An overview of irrigation and water management

The typical way of sustaining crop production in agriculture during dry periods is through irrigation (Dessalegn, 1999, Worku, 2011). It is a crucial customary way of operation in agriculture to produce food, pasture, and fibre in dry regions (Koech and Langat, 2018). Schedules for irrigation are either derived from actual measurements of soil water status (SWS) or derived from physiological measurements such as plant water status (PWS). The soil-based measurements are quick and easy to automate, but the response of a plant to a

particular level of soil moisture depends on a complex interaction between evaporative demand and soil moisture (van Zyl, 2014). The water that is present in the soil is not always available to plants to support growth and development. Therefore, the accessible water to plants for absorption is known as plant-available water which is the difference between field capacity and the wilting point (Easton and Bock, 2016).

According to Gruber and Schultz (2005), soil variability is mainly due to texture, structure, organic content, and gravel content, which affect the plant's capacity for retaining water. Amongst important processes responsible for growth is the exchange of water for carbon dioxide through the stomata. Stomatal openings on the plant leaves open for carbon dioxide intake and in the process, water is lost through transpiration, and this can promote wilting. Water use efficiency of the plant influences this gas exchange (Medlyn *et al*., 2017). Koech and Langat (2018) defined water use efficiency as “the proportion of the water supplied through irrigation that is productively or beneficially used by the plant”.

2.8.1.1 The correlation between soil and water

Soil is part of the Earth’s surface consisting of humus, disintegrated rocks and pores that permits water mobility to allow plant’s growth. Easton and Bock (2016) defined SWS as “the amount of water a given soil can store, primarily influenced by the soil texture and the soil organic matter content. It can be conveyed as volumetric or gravimetric water content”. They further stated that gravimetric water content is the quantity of water per unit quantity of parched soil. The pores are spaces between particles that are filled by either air or water and their size depends entirely on the texture of the soil, bulk density, and structure (Scherer *et al*., 2017).

2.8.1.2 Factors that influence soil water dynamics

a) Soil texture

Soil texture can be referred to as an assortment of particle sizes comprising of coarse sand, loam, and clay (Fernandez-illescas *et al*., 2001; van Schalkwyk, 2018). According to Adugna and Abegaz (2015), the fraction of sand in the soil has a strong effect on the increment and decrement of soil depth. The size and positioning of soil particles regulate the porosity of the soil that is why the texture plays an important role in the mobility of the water (Hacke *et al*., 2000; Hultine *et al*., 2005; van Schalkwyk, 2018).

b) Soil bulk density and porosity

Soil bulk density expresses the ratio of the mass of dry soil to the total volume occupied in the soil. The total volume includes both the solids (mineral and organic) and the pore spaces

(Easton and Bock, 2016). However, soil porosity can be defined as the percentage of pores in a soil (Easton and Bock, 2016). Therefore, soil bulk density aids in giving signal on the fraction of pores within the soil. The bulk density is usually greater in sandy soils compared to clayey soil (USDA, 1998; Neves *et al*., 2003; Chaudhari *et al*., 2013; van Schalkwyk, 2018). The porosity of the soil is mostly determined by texture, aggregation, penetration of the roots and tunnelling by various organisms (Easton and Bock, 2016).

c) Soil structure

Soil structure can be defined as an arrangement and embodiment of soil fragments into assemblages of aggregation (Easton and Bock, 2016; Williams, 2019). Structure determines the texture of the soil which can alleviate root penetration as well as the ability of water and air to move freely within the soil. The movement of water is quite faster in single-grained sandy soils but very slow in massive thick soils such as clays (Easton and Bock, 2016).

2.8.2 Stress associated with water in plants

Heat and water stress have the potential to compromise sustainable crop production on a global scale (Mittler, 2006; Zhou *et al*., 2017). A plant can be affected by water stress in two different ways: drought (too little water) or waterlogging (too much water), which reduces oxygen in the soil and impairs nutrient uptake (Abid *et al*., 2018; Wojtyla *et al*., 2020). Changing climatic conditions, high salinity and drought are components of the water deficit, which occurs when plant transpiration exceeds absorption (Abdul Qados, 2011; Osakabe *et al*., 2014; Hatfield and Prueger, 2015; Farooqi *et al*., 2020). Lack of water can alter plant chemistry and negatively influence their growth by disrupting metabolism and pigments, thereby decreasing photosynthesis, and thus, preventing food production (Foyer and Noctor 2000, Fu and Huang, 2001; Ahmad *et al*., 2018; Hussain *et al*., 2018)

It is difficult to have a universal definition of drought due to its multiple categories, physical processes, and degrees of severity; but it can be described as a period in which there is no considerable rainfall (Lloyd-Hughes, 2014; Mukherjee *et al*., 2018). An agricultural water deficit is the absence of sufficient water in the soil to support normal plant growth (Jaleel *et al*., 2009; Tanveer *et al*., 2019; Taha *et al*., 2020; Mabizela, 2020). Aridity occurs naturally at a slow pace with different types including meteorological (a lack of rain), agricultural (a lack of soil moisture), socioeconomic (lack of water for daily use by the public) and hydrological (a lack of water in rivers and lakes) droughts (Wilhite, 2000; Mishra and Singh, 2010; Mukherjee, 2018).

Honeybush grows in South Africa, a country prone to drought. The species grows naturally in climates that are hot and dry in summer, and cool and wet in winter. Summers are frequently characterized by drought conditions (Gibberd *et al*., 1996; Lionello *et al*., 2006; Joubert *et al*., 2011). Mediterranean climate plants are expected to experience extreme stresses, but the severity and response to water stress depends on the type of plant, its stage of development, how long the stress lasts, and how harsh it is (Tekle and Alemu, 2016; Mabizela, 2020).

2.8.3 Defence mechanisms and adaptation of plants to water stress

Plants that grow in their natural habitats can adapt to water stress through a variety of mechanisms. The mechanisms range from short-term responses to low soil moisture to major survival mechanisms such as early flowering, which enables plants to escape drought stress (Basu *et al*., 2016). There is a great diversity of plant species growing in climatic regions that experience extreme dry conditions. This suggests that plants have evolved morphological, physiological, and biochemical adaptations to cope with drought stress (Bohnert *et al*., 1995). A plant with drought tolerance can resist dehydration through various physiological mechanisms, such as osmotic adjustment induced by osmoprotectants (Luo, 2010). A drought escape occurs when a plant grows quickly so that it can reproduce before its life cycle ends (Mabizela, 2020). In drought avoidance, a plant completes its life cycle ahead of drought's impact (McCann and Huang, 2008). The ability of a plant to recover from extreme drought stress is known as drought recovery (Manavalan *et al*., 2009).

a) Stomatal conductance

The regulation of stomatal conductance plays a significant role in a plant's response to water stress. The first response to water shortage is stomatal closure (Flexas *et al*., 2014). Leaf stomata regulate CO_2 assimilation, water loss, and evaporative cooling by permitting gaseous diffusion between the leaf and the external atmosphere (Faralli *et al*., 2019). In agricultural areas where sufficient water is available, a large stomatal conductance correlates with improved crop yields, while CO_2 downregulation may result in suboptimal yields (Engineer *et al*., 2016).

There is a greater inhibition of cell enlargement by water stress than there is on cell division. As a result, it reduces plant growth by inhibiting photosynthetic processes, respiration, translocation, ion uptake, carbohydrate and nutrient metabolism, and growth promotion (Farooq *et al*., 2009). Drought promotes reduction in leaf expansion, acceleration of leaf senescence, as well as reduction in leaf tissue density. Therefore, the measurement of transpiration rate provides an excellent way to determine a crop's drought tolerance (De Souza *et al*., 1997; Alves and Setter, 2004; Chowdhury *et al*., 2016).

b) Proline

Responses of plants to water deficit stress include changes in the levels of metabolites and the activity of metabolic pathways (Sharma *et al*., 2011). Different environmental stresses lead to the accumulation of proline in a wide range of plants as an adaptive mechanism (Mattioli *et al*., 2009; Dar *et al*., 2016). There are some authors who dispute the link between proline accumulation and stress adaptation, but it is generally accepted that plant cells benefit from a rise in proline content following stress injury (Mattioli *et al*., 2009). Proline accumulation depends on the level of stress and can also vary among species (Hayat *et al*., 2012).

Plants that have been subjected to heat stress produce larger amounts of proline within their cells, acting as osmo-protectants (Kumar *et al*., 2012). When plants are stressed, proline accumulates within their tissue and functions as stabilisers of proteins and enzymes, and scavengers of reactive oxygen species (Anaytullah and Bose, 2012; Hameed *et al*., 2014). According to existing literature, proline may have a dual role in flowering and development as a small molecule and as a metabolite. Research on the proline production of honeybush plants under drought stress could help determine which plants are more drought-tolerant for the best planting choices in low moisture areas (Mattioli *et al*. 2009; Mabizela, 2020).

c) Relative water content (RWC)

A plant's ability to keep turgor during water deficit periods is key to ensuring a smooth metabolic process (Čereković *et al*., 2013). Plant relative water content (RWC) is helpful in determining plant water status under drought and heat stress to assess the physiological consequences of cellular water deficit (Surendar *et al*., 2013; Soltys-Kalina *et al*., 2016). Therefore, based on cell turgor and osmotic potential, RWC reflects the relative change in cell volume, and does not directly determine cell volume, since it depends on cell wall rigidity and solute concentration (Bolat *et al*., 2014). A study of the impact of water uses and stress on plant growth is therefore critical for production sustainability in agriculture (Harb, *et al*., 2010).

d) Proteomics

Plants are continually exposed to biotic stress in natural and agricultural environments, which can threaten their survival and growth (Zipfel *et al*., 2017). There have been numerous molecular programs in plants that have evolved in response to these changes of circumstances to quickly detect and adapt to environmental changes. Over the recent decade, the use of proteomics for crop plant analysis has amplified drastically (Komatsu *et al*., 2013).

The scarcity of genomic information has hampered crop proteomics applications. However, with the successful development of "next generation" sequencing technology, the identification and annotation of proteins and their isoforms in a specific crop species is becoming considerably easier (Komatsu *et al*., 2013). Proteomics analysis aims to provide an inclusive profile of various proteins found in a specified organism in response to different biotic or abiotic stresses (Jorrin-Novo *et al*., 2019; Mabizela, 2020). Plant proteins play a crucial role in both biotic and abiotic stress response because they regulate physiological characteristics directly to adapt to changes in the environment. They are also crucial executors of cellular mechanisms and key players in cellular homeostasis (Liu *et al*., 2019). Studies of metabolic changes in response to various stress conditions can be conducted using proteomics. The analysis of plant proteomes is becoming more and more important as a means of determining a plant's functional characteristics (Chandrasekhar *et al*., 2014). Researchers are now able to study plants in greater detail using mass spectrometry-based proteomics. Plant resistance mechanisms have been discovered by using proteomics as a discovery tool in several studies (Liu *et al*., 2019).

CHAPTER THREE

CYCLOPIA SUBTERNATA GROWTH, YIELD, PROLINE AND RELATIVE WATER CONTENT IN RESPONSE TO WATER DEFICIT STRESS

(This chapter has been published in *Water SA* 49(1) 64–72 / Jan 2023. DOI: https://doi.org/10.17159/wsa/2023.v49.i1.3988 . Please refer to appendix 1).

ABSTRACT

Cyclopia, generally known as honeybush, and belonging to the *Fabaceae* family, originates from the Cape Floristic Region of the Eastern and Western Cape provinces of South Africa. Currently, six honeybush species are commercially cultivated, but to date, there are limited trials attempting to study their agronomic water demand. A pot trial was conducted where *Cyclopia subternata* plants were cultivated on different soil types (Stellenbosch granite, Stellenbosch shale and Stellenbosch clovelly) and subjected to three different water deficit stress levels (well-watered, semi-stressed and stressed). Remarkably, irrigation treatments and soil types did not significantly affect the growth of the plants. However, the well-watered treatment consistently had higher yields compared to the other two treatments. The water-stressed (semi-stressed and stressed) treatments had lower relative water contents (RWC) with higher concentrations of proline, which signify water stress, compared to the control treatment. Higher proline and lower RWC contents in this study are indications of water stress.

3.1 Introduction

South Africa, a drought-prone country, is home to rooibos (*Aspalathus linearis*), bush (*Athrixia phylicoides*) and honeybush (*Cyclopia* species) teas (Joubert *et al*., 2011). The teas are sold as either black or green (fermented or unfermented, respectively) (Horn, 2019). Even though the commercialization of some of these remedial teas is still in its infancy stage, honeybush has gained recognition, while rooibos is the most well-known and well established in the industry (Van Wyk and Gericke, 2000; Joubert *et al*., 2008; Joubert *et al*., 2011).

Studies state that these South African indigenous tea species have essential nutrients (iron, calcium, magnesium, copper, and potassium) that can improve wellbeing and/or prevent diseases and have economic potential (Rampedi and Olivier 2005; McGaw *et al*., 2007). These herbal teas are famous for their rich caffeine-free and organic antioxidant properties, which are helpful in colon, throat and lung illnesses, prevention of urinary stone and tooth caries and other medical problems (Soni *et al*., 2015).

The demand for honeybush tea has prompted concerns of over-exploitation of natural populations of the *Cyclopia* species. The increased rate of wild harvesting diminishes the natural population, thus making the exploitation of *Cyclopia* species unsustainable. Harvesting practices have contributed to the decrease and even disappearance of populations of the wild *Cyclopia* species (Du Toit *et al*., 1998). Other factors threatening the growth of the honeybush industry include drought and veld fires. To ensure sustainable production, commercial honeybush plantations have been established (Joubert *et al*., 2011).

Commercial production is therefore becoming increasingly important to save the natural populations from decline while ensuring consistent supply. Cultivation of *Cyclopia* species will not only contribute to sustainability and conservation of the species but will also improve the livelihoods of rural harvester communities. Although cultivated honeybush plants receive water through irrigation in addition to rainfall, irrigation volume is at the discretion of farmers, without any understanding of the water requirements of the species. Presently, the shortage of water has massively increased in some parts of the world, including some regions in South Africa, due to a variety of reasons such as an ever-increasing population, industrialization, water pollution and poor management, climate change and others (WWAP, 2012; Connor, 2015; Long and Pijanowski, 2017).

In addition, the South African Department of Water and Sanitation (DWS) has reduced agricultural allocations significantly, and irrigation volume for the agricultural sector is unlikely to increase anytime soon. For example, in 2015, DWS restricted an irrigation water allocation in KwaZulu-Natal by 40–100% due to a water shortage caused by insufficient rain (RSA, 2015). Also, agriculture in the Western Cape has had to cut its water use by 60% since 2017 (WWF, 2018). As a result, research that focuses on the sustainability of water-use in agriculture is gaining huge interest (Velasco-Muñoz *et al*., 2018). Environmental factors, including water stress, tend to interfere with crucial physiological processes and biochemical mechanisms; resulting in yield loss (Per *et al*., 2017).

Therefore, research on water-use and the effects of stress on plant growth is crucial for production sustainability in agriculture (Harb *et al*., 2010). Plants have proven to use protective mechanisms such as proline and carbohydrate accumulation to cope with water-deficit situations (Mabizela, 2020). Proline is a water-soluble amino acid and beneficial solute that accumulates in plants under different kinds of stresses, such as drought, cold, heat, heavy metal, nutrient, and salt stress (Siddique and Dubey, 2017).

Relative water content (RWC) is a useful measure of plant water status in terms of the physiological consequences of cellular water deficit and may indicate the degree of water stress expressed under drought and heat stress (Surendar *et al*., 2013; Soltys-Kalina *et al*., 2016). It combines leaf water potential and the effect of osmotic regulation to quantify plant

water status (Lugojan and Ciulcas, 2011; Kardile *et al*., 2018). Insufficient water in plants due to stress results in low RWC (Chakhchar *et al*., 2015). A plant's ability to retain turgor during water-deficit periods guarantees smooth metabolic processes for growth (Čereković *et al*., 2013). Several studies have stated that RWC determination is an efficient method of assessing drought tolerance and plant water status (Slabbert and Krüger, 2004; Li-Ping *et al*., 2006; Jones, 2007; Obidiegwu *et al*., 2015). To date, limited studies have been conducted to investigate the water needs of *Cyclopia* species. Therefore, the aim of this study was to evaluate the effects of 3 different irrigation treatments on growth, yield, proline and relative water content of *Cyclopia subternata* species of honeybush.

3.2 Methods and Materials

3.2.1 Experimental site and layout

A greenhouse pot trial was conducted at the Agricultural Research Council (ARC), Infruitec-Nietvoorbij (latitude −33.914395° and longitude 18.861390°) in Stellenbosch, South Africa, to determine the effect of water stress on *C. subternata*. The experiment was conducted for 140 days (from end-July to mid-December 2020). The experimental design was a randomised block design (RBD) with 9 treatment combinations (irrigation x soil type) replicated at random in each of 4 block replicates. The treatment structure was a 3 x 3 factorial with 3 irrigation levels (well-watered, semi-stressed and stressed) and 3 soil types (Stellenbosch granite, Stellenbosch shale and Stellenbosch clovelly).

3.2.2 Soil collection, preparation, and planting

Soil collection was carried out from three different sites at the ARC Nietvoorbij research farm. For each site, soil samples were collected from the 0–30 cm soil depth, sieved with a 3 mm sieve to remove large fragments, followed by baseline physicochemical analysis of the composited samples at a commercial laboratory (Bemlab, Strand). 14 kg of soil was weighed into a 30 cm plastic pot, using a digital scale. The soil in each pot was irrigated to pot capacity (PC) before planting. Nine-month-old honeybush (*C. subternata*) seedlings were transplanted to one plant per pot. The plants were well-watered for 5 weeks to ensure good establishment before introducing the different irrigation treatments.

3.2.3 Irrigation and weed control.

From the 6th week after transplanting (WAT), *C. subternata* plants were subjected to 3 different irrigation treatments for 105 days (September–December 2020). The well-watered treatment (control) received 500 mL of water 3 times a week, semi-stressed received the same

quantity of water twice a week while the stressed treatment received 500 mL of water once a week until the end of the study. The plants were hand irrigated with an Erlenmeyer flask. Weeds that emerged in the pots during the trial period were mostly broad-leaved plants. The weeds were either hand-pulled or manually removed using a garden fork immediately after irrigation when the soil was still wet.

3.2.4 Data collection

3.2.4.1 Growth parameters

Measurement of growth parameters commenced at 6 WAT on a monthly basis, until the trial was terminated in December 2020. Plant height was measured from the soil surface to the tip of the longest shoot, using a tape measure, stem diameter was measured with a digital Vernier caliper while the stem circumference was calculated from stem diameter values using the following formula:

$C = \pi d$

where π = 3.14 and d = diameter

3.2.4.2 Total yield (shoot and root biomass)

At the end of the study (20 WAT), the above-ground biomass (shoot) was cut just above the soil surface using a pruning shear, placed in a labelled paper bag and then weighed using a sensitive weighing balance to obtain the fresh mass of the shoot. The fresh shoot was oven-dried at 70°C for 24 h. The dried samples were also weighed and recorded using a sensitive digital scale to 4 decimal places. The root biomass was determined by washing plant roots from each pot under running water using a 0.053 mm sieve in order to separate the roots from the soil and prevent loss of fine roots. The washed roots were air-dried overnight and weighed using a sensitive scale. Total plant yield is the combined fresh weight of the above-ground biomass and the air-dried root biomass.

3.2.4.3 Estimation of proline content using the colorimetric method

Determination of the proline content of *C. subternata* commenced at 6 WAT using the modified method of Ábrahám *et al*. (2010). Leaf samples were collected at 2-week intervals during the growth period. The extraction procedure was done by crushing 0.1 g of fresh frozen leaves in 0.5 mL of 3% sulfosalicylic acid (w/v), using a plastic test tube and pestle. The homogenised extracts were centrifuged for 5 min at a speed of 13 500 r/min. A reaction mixture of 0.1 mL of 3% sulphosalicylic acid, 0.2 mL of glacial acetic acid, 0.2 mL of acid ninhydrin buffer (1.25 g ninhydrin, 30 mL glacial acetic acid, and 20 mL of 6 M phosphoric acid) and 0.1 mL of centrifuged sample extract was made in a test tube with a pipette. The mixture was boiled for 30 min at 100°C then terminated in an ice bath at room temperature. After complete cooling,

1 mL of toluene was added to the mixture and mixed thoroughly, then placed on the bench for 5 min to allow separation of chromophore. The absorbance was read at 520 nm on the UV-visible spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, Waltham MA, USA) with a 10 mm quartz glass cuvette. From the proline standard curve, the proline concentrations of the *C. subternata* samples were determined. Proline content was calculated using the formula:

$$mmoles\ per\ g\ tissue = \frac{mg\ proline/mL \times mL\ toluene}{115.5} \times \frac{5}{g\ sample}$$

where 115.5 = molecular mass of proline

3.2.4.4 Determination of relative water content (RWC)

An improved version of the method of Sade *et al*. (2015) was used to determine the RWC of *C. subternata* leaves. Leaf samples were collected fortnightly at midday (12:00) where 5 top-most leaves per plant were collected, cut into two halves and immediately stored in pre-weighed, labelled glass vials to minimize humidity or vapour loss. The samples were preserved on ice during sampling and quickly transported to the laboratory for RWC determination. Fresh weight (FW) of each sample was determined using a sensitive weighing scale. 2 mL of distilled water was added to each vial, kept in a dark cupboard at room temperature for 4 h to facilitate re-hydration. Thereafter, the turgid leaf samples were removed from the vials and slightly blotted with a paper towel to remove excess water. The blotted leaves were weighed to determine the turgid weight (TW) and later oven-dried at 70°C for 48 h. The dried samples were later weighed to determine the dry weight (DW). Relative water content was calculated using the formula shown below:

$$RWC = \frac{(FW-DW)}{(TW-DW)} x\ 100$$

3.2.4.5 Statistical analysis

Data were analysed with the randomised block factorial ANOVA using SAS statistical software (version 9.4, SAS Institute Inc., Cary, NC, USA, 2013). ANOVA was used for each observation time (harvest/month) separately, as well as with time as sub-plot factor (Little, 1972). The Shapiro-Wilk test was utilized in testing for deviation from normality (Shapiro and Wilk, 1965). Fisher's least significant difference was calculated at the 5% level to compare treatment means (Ott, 1998). A probability level of 5% was considered significant for all tests.

3.3 Results and discussion

3.3.1 Physical and chemical characteristics of the soils

The results of the baseline physicochemical analysis of the soils on which the *C. subternata* plants were grown are shown in Tables 3.1 and 3.2. Stellenbosch granite soil had the highest coarse sand levels (0.5–2 mm) while the lowest was found in Stellenbosch shale. Stellenbosch clovelly had more clay content, with Stellenbosch granite having the lowest (Table 3.1). The textural classes for Stellenbosch granite, Stellenbosch clovelly and Stellenbosch shale fall within the coarse sandy loam, fine sandy clay loam and sandy clay loam, respectively. Soil pH and other soil nutrients were within the range for normal growth of most plants.

Table 3.1: Baseline physical characteristics of the three types of soil used in the study.

Physical characteristics	Stellenbosch granite	Stellenbosch shale	Stellenbosch clovelly
Clay (<0.002 mm)	13	20	23
Silt (0.002–0.02 mm)	17	13	6
Fine sand (0.02–0.2 mm)	33	50	37.8
Medium sand (0.2–0.5 mm)	3	5	13.0
Coarse sand (0.5–2 mm)	35	12	20.4
Stone volume (%)	0.22	7.72	0.00
Soil textural class	Coarse sandy loam	Fine sandy clay loam	Sandy clay loam

Table 3.2: Baseline chemical composition of the three soil types.

Soil type	Ex. Cations (cmol (+)/ kg				Macronutrients				pH (KCl)	Resistance (Ohms)	K (%)	Ca (%)	Na (%)	Mg (%)	Acid saturation (%)
	Na	K	Ca	Mg	NO3	P	NH4	K							
SG	0.14	0.52	4.4	1.6	31.3	23.9	3.2	203	5.3	800	6.97	58.99	1.88	21.45	10.71
SC	0.13	0.52	4.7	1.2	39.7	29.6	3.3	205	5.5	910	7.17	64.82	1.79	16.55	9.67
SS	0.07	0.32	2.8	0.59	10.6	16.9	13.4	124	5.5	1400	7.59	66.38	1.66	13.99	10.39

*SG=Stellenbosch granite; SC= Stellenbosch clovelly; SS= Stellenbosch shale.

3.3.2 Growth parameters

In general, water stress and soil type had no significant influence ($p > 0.05$) on plant height, stem diameter or stem circumference throughout the period of the trial (Figure 3.1 (A-C), Table 3.3). A summary of *p*-values for separate ANOVAs of growth parameters per month is presented in Table 3.4.

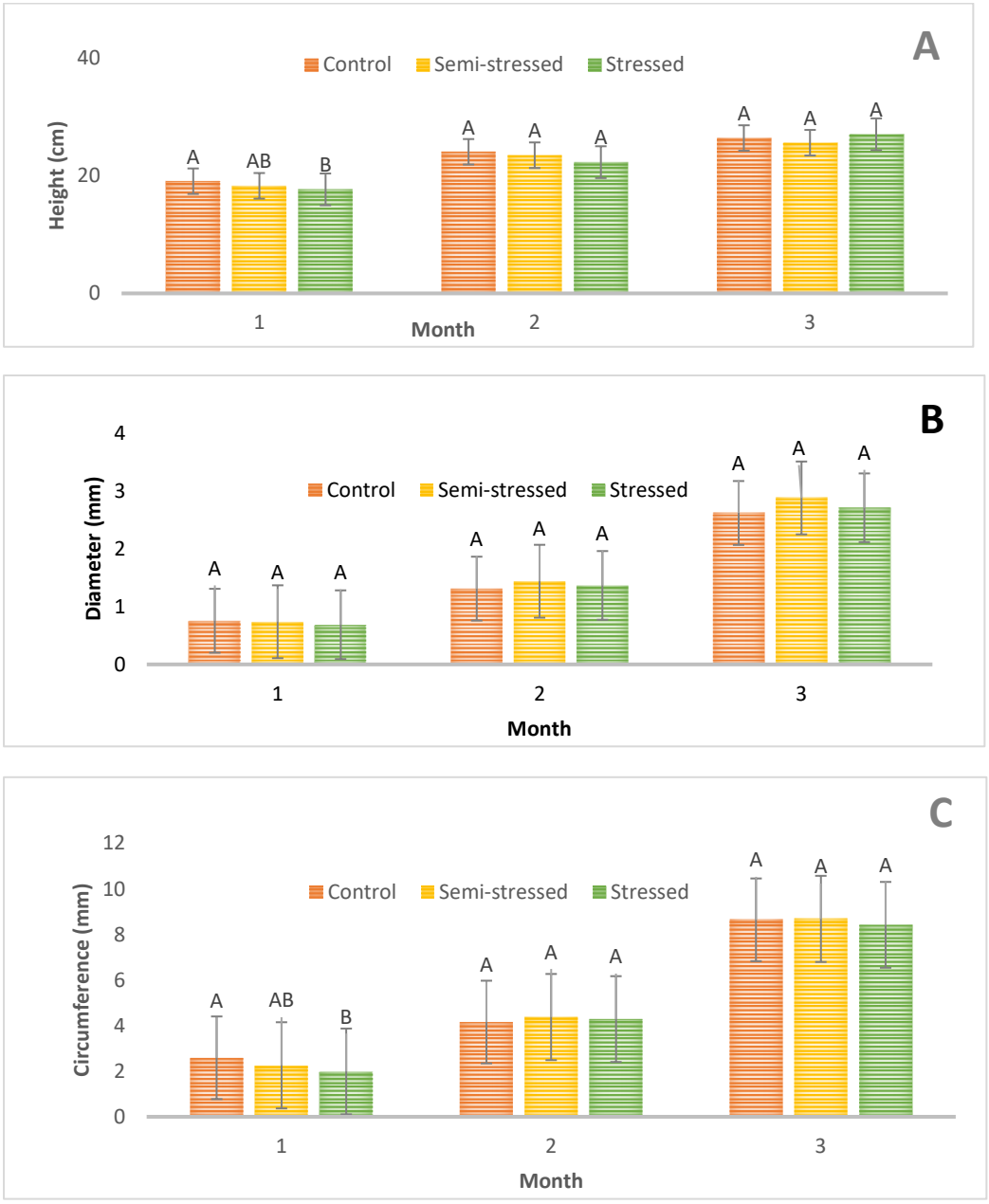


Figure 3.1 (A-C). Effects of different irrigation levels on plant height, stem diameter and stem circumference of *C. subternata* at different months. Means with the same letters are not significantly different (p≤0.05). Whiskers= standard error bars.

Table 3.3. Mean growth of *C. subternata* established on three different types of soil at different sampling times.

Sampling time	Soil type	Plant height (cm)	Stem diameter (mm)	Stem circumference (mm)
1	Stellenbosch granite	17.9083 ± 1.79 [a]	0.69659 ± 0.10 [a]	2.2632 ± 0.41 [a]
	Stellenbosch shale	18.4510 ± 1.99 [a]	0.69737 ± 0.09 [a]	2.4456 ± 0.63 [a]
	Stellenbosch clovelly	18.6031 ± 1.52 [a]	0.78639 ± 0.23 [a]	2.1376 ± 0.39 [a]
	LSD	**1.38**	**0.13**	**0.36**
2	Stellenbosch granite	23.248 ± 2.58 [a]	1.3674 ± 0.26 [a]	4.3648 ± 0.54 [a]
	Stellenbosch shale	22.801 ±2.75 [a]	1.3098 ± 0.25 [a]	4.3931 ± 0.86 [a]
	Stellenbosch clovelly	23.729 ± 3.45 [a]	1.4395 ± 0.23 [a]	4.0709 ± 0.86 [a]
	LSD	**2.51**	**0.22**	**0.61**
3	Stellenbosch granite	26.557 ± 4.54 [a]	2.7348 ± 0.52 [a]	8.8084 ± 1.12 [a]
	Stellenbosch shale	25.897 ± 3.75 [a]	2.6197 ± 0.50 [a]	8.6203 ± 1.66 [a]
	Stellenbosch clovelly	26.515 ± 5.61 [a]	2.8529± 0.41 [a]	8.3426 ± 1.71 [a]
	LSD	**4.25**	**0.43**	**1.22**

There is no significant difference ($p \geq 0.05$) among treatments per sampling time. **N**= 12; **LSD**= Least significant difference. Data are mean ± standard deviation.

Table 3.4: Summary of p-values for separate ANOVAs on growth parameters per month.

		Plant height			Stem diameter			Stem circumference		
Effect	df	1	2	3	1	2	3	1	2	3
Rep	3	0.6831	0.3165	0.5993	0.1744	0.6363	0.5498	0.1988	0.3037	0.1943
Irrigation	2	0.1358	0.3525	0.7881	0.5454	0.4991	0.4634	0.0084	0.75500	0.8855
Soil	2	0.5587	0.9875	0.9375	0.2916	0.4898	0.5370	0.2335	0.4934	0.7318
Irrigation x Soil	4	0.0948	0.6838	0.6838	0.7972	0.7724	0.7989	0.7940	0.0957	0.1535

* **1, 2, 3** = Sampling months; **N**= 12

3.3.3 Yield (shoots and root biomass)

The results of the effect of irrigation treatments and soil type on the yield of *C. subternata* are presented in Figure 3.2 and Table 3.5 respectively. A summarised presentation of p-values for separate ANOVAs on shoots and roots biomass is shown in Table 3.6.

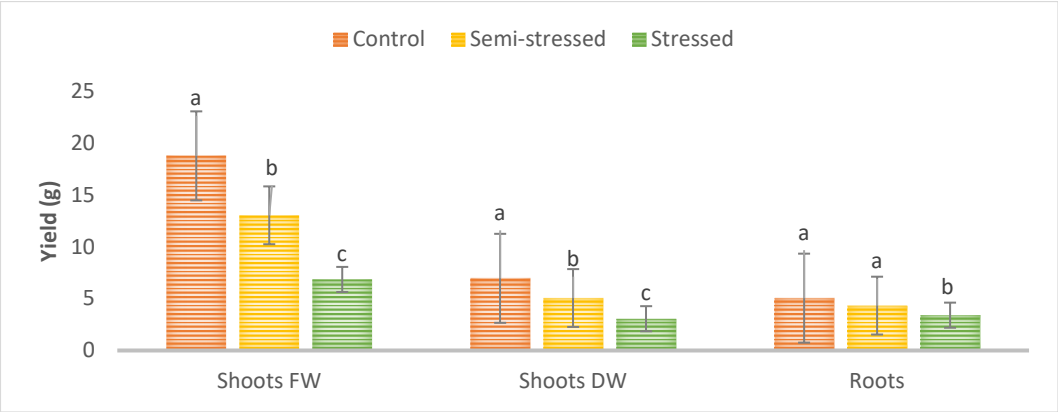


Figure 3.2. Effect of diverse water stress levels on the root and shoot biomass of *C. subternata*. FW= fresh weight; DW= dry weight. Means with the same letter are not significantly different (p≤0.05). Whiskers= standard error bars.

All three irrigation treatments significantly affected the yield (fresh and dry shoots) (p≤0.05). Highest shoot and root yields were recorded in the control treatment on Stellenbosch shale soil, with a progressive yield decline observed with increase in stress level. However, there were no significant differences (p> 0.05) in the root yield of the well-watered (5.05 g) and the semi-stressed (4.33 g) treatments. Stellenbosch clovelly consistently had poor shoot and root yields among the three soil types while there were no significant differences (p> 0.05) between the biomass yields from Stellenbosch granite and Stellenbosch shale.

Table 3.5: Effect of different soil types on the yield of *C. subternata.*

Soil type	Shoot (g)		Roots (g)
	Fresh weight	Dry weight	
Stellenbosch granite	14.048 ± 6.20 [a]	5.0531 ± 2.12 [ab]	4.3146 ± 1.63 [ab]
Stellenbosch shale	15.625 ± 7.43 [a]	6.2229 ± 2.57 [a]	4.8604 ± 1.37 [a]
Stellenbosch clovelly	9.022 ± 5.27 [b]	3.8219 ± 2.03 [b]	3.6188 ± 1.38 [b]
LSD	**3.41**	**1.39**	**0.81**

FW= fresh weight; **DW**= dry weight. **N**= Number of observations (12); **LSD**= Least significant difference
Means with the same letter are not significantly different (p≤0.05). Data are mean ± standard deviation.

Table 3.6: Summary of p-values for separate ANOVAs on shoots and roots biomass.

		Shoots (FW)	Shoots (DW)	Roots
Effect	df			
Rep	3	0.7772	0.7974	0.0002
Irrigation	2	<.0001	<.0001	0.0013
Soil	2	0.0014	0.0061	0.0145
Irrigation x Soil	4	0.3941	0.5658	0.3069

N= 12**; FW=** fresh weight; **DW=** dry weight

3.3.4 Relative water content (RWC)

Blum (2011) defined RWC as “the percentage of water present in the leaf as a fraction of the total volumetric water that the leaf can hold at full turgor”. It has direct connection with soil water content and mostly used as an indicant of water stress in plant leaves. In this study, changes in the leaf RWC due to the different irrigation levels are depicted in Figure 3.3. At both sampling times, the well-watered treatment consistently had significantly higher (p≤0.05) RWC (87% and 86%, respectively), while the stressed treatment recorded the lowest values (79% and 76% respectively). Although, there was no significant difference between the semi-stressed (81% and 82%) and the stressed treatments (p>0.05).

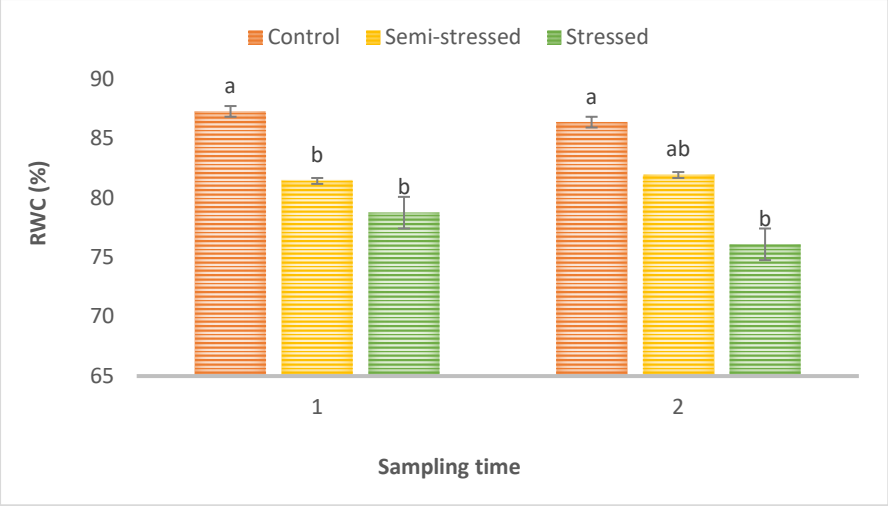


Figure 3.3. Relative water content of *C. subternata* in response to three different irrigation treatments at different sampling times. RWC= relative water content. Means with the same letter are not significantly different (p≤0.05). Whiskers=standard error bars.

For the three soil types, in the first sampling period, there were no significant differences in the leaf RWC of *C. subternata* grown on granite and clovelly soil types (p>0.05). Same observation was reported between granite and shale derived soils. However, water stress significantly decreased (p≤0.05) the relative water content of Stellenbosch clovelly when compared with Stellenbosch shale (Table 3.7). In contrast, the second sampling time showed no significant differences among the treatments. Summary of p-values for ANOVAs on the relative water content per period is presented in Table 3.8.

Table 3.7. Effect of soil type on relative water content of *C. subternata* at different sampling times.

Sampling time	Soil type	RWC (%)
1	Stellenbosch granite	82.766 ± 7.31^{ab}
	Stellenbosch shale	86.362 ± 7.66^{a}
	Stellenbosch clovelly	78.400 ± 9.81^{b}
	LSD	5.16
2	Stellenbosch granite	84.764 ± 8.24^{a}
	Stellenbosch shale	80.241± 7.57^{a}
	Stellenbosch clovelly	79.450 ± 9.65^{a}
	LSD	5.99

RWC= relative water content; **N**= Number of observations (12); **LSD**= Least significant difference. Means with the same letter are not significantly different (p≤0.05). Data are mean ± standard deviation.

Table 3.8: Summary of p-values for ANOVA on the relative water content per month period.

		RWC (%)	
Effect	df	1	2
Rep	3	0.0445	0.4147
Irrigation	2	0.0072	0.0062
Soil	2	0.0145	0.1639
Irrigation x Soil	4	0.0146	0.1311

***1,2**= Sampling time; **N**= 12, **RWC**= relative water content

3.3.5 Proline

In this study, Figure 3.4 shows that the stressed *C. subternata* plants consistently had significantly higher proline contents in all sampling periods (39.55 µmol/g FW, 33.38 µmol/g FW, and 39.78 µmol/g FW), while the least was found in the well-watered ($p \leq 0.05$). Significantly higher proline contents were observed in the stressed plants compared to the other two treatments in the first sampling period. However, no significant difference was observed among all treatments in the second and third sampling periods ($p > 0.05$).

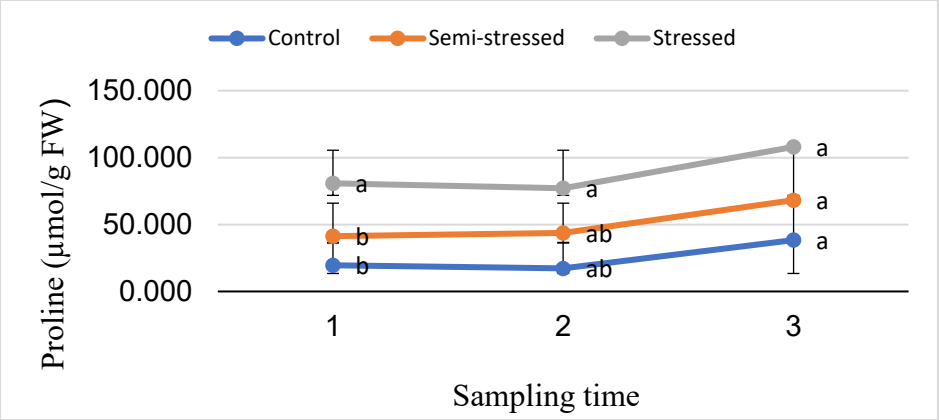


Figure 3.4. Effects of three irrigation levels on proline content of *C. subternata* at different sampling times. Means with the same letter are not significantly different ($p \leq 0.05$). Whiskers=standard deviation bars.

Soil type did not have any significant effect ($p > 0.05$) on the proline contents of the plants (Table 3.9). Summary of p-values for ANOVAs on the accumulation of proline per month is presented in Table 3.10.

Table 3.9: Proline content of *C. subternata* cultivated on three different types of soil at different sampling times.

Sampling time	Soil type	Proline (µmol/g FW)
1	Stellenbosch granite	25.818 ± 26.42
	Stellenbosch shale	21.996 ± 19.42
	Stellenbosch clovelly	33.053 ± 26.18
	LSD	**17.95**
2	Stellenbosch granite	24.258 ± 9.89
	Stellenbosch shale	22.562 ± 16.08
	Stellenbosch clovelly	30.284 ± 19.57
	LSD	**11.61**
3	Stellenbosch granite	37.616 ± 21.31
	Stellenbosch shale	31.220 ± 16.90
	Stellenbosch clovelly	39.156 ± 26.20
	LSD	**18.77**

There is no significant difference (p≥0.05) among treatments per sampling time. **N**= Number of observations (12); **LSD**= Least significant difference. Data are mean ± standard deviation.

Table 3.10: Summary of p-values for ANOVA on the accumulation of proline per period

		Proline (µmol/g FW)		
Effect	df	1	2	3
Rep	3	0.0724	0.135	0.2323
Irrigation	2	0.0595	0.0287	0.5083
Soil	2	0.4465	0.3688	0.6566
Irrigation x Soil	4	0.3799	0.3793	0.8046

*__1,2,3__= Sampling months; **N**=12

3.4 Discussion

3.4.1 Growth parameters

When compared to the stressed plants, the well-watered (control) treatment had significantly taller plants with greater stem circumference in the first sampling month on Stellenbosch clovelly soil. Thereafter, growth and development of plants did not differ significantly among treatments. The results for the growth parameters of *C. subternata* in this study contrast with the findings of Tshikhudo *et al*. (2019) where plant height, stem diameter and the number of leaves of bush tea (*Athrixia phylocoides* DC). increased with increase in rainfall. Stress experienced by crops during growth has a cumulative effect, which ultimately reduces the final biomass production (Kamara *et al*., 2003). This may be the reason why there was generally

no significant difference in the growth of *C. subternata* grown in this trial while the cumulative effects of water stress were only seen in the harvested biomass (Table 3.5 and Figure 3.2). However, a study by Habibi (2018) on *Aloe vera* demonstrated that short-term water deficit had no significant effect on the leaf biomass. The short duration of the present study may be responsible for the non-significant differences observed in the growth of both the drought-stressed (semi-stressed and stressed) and the well-watered (control) plants.

3.4.2 Yield (shoots and root biomass)

Eziz *et al*. (2017) noted that plant growth and biomass production generally decrease with decrease in water availability. However, plants may behave contrary to this, where the cumulative effect of stress during growth may only be visible in the reduced biomass yield (Kamara *et al*., 2003); which was also observed in this study. According to Khan *et al*. (2018), water stress can cause a severe reduction in crop yield and both the severity and duration of the stress are critical. Water availability is a key factor for sustainable crop production. Its scarcity can have an adverse effect on the physiological and biochemical processes of the plants, thereby causing low yield. The drought-induced yield (root, fresh and dry shoot) decline was comparable to the findings of Zhao *et al*. (2006) who found that there was a severe reduction in the fresh and dry weights of *Brassica napus* under water-limiting conditions. Stress at vegetative stage of plants may lead to reduced stomatal conductance, net photosynthesis, and yield (Kerepesi and Galiba, 2000; Fathi and Tari, 2016). The observed yield reduction due to water stress in this study, may therefore, be attributed to impairment of physiological and biochemical processes like photosynthesis, respiration, translocation, ion uptake carbohydrate and nutrient metabolism (Ali and Anjum, 2016) during growth. During the period of stress, plants adopt coping mechanisms such as stomatal closure. However, stomatal closure prevents the intake of CO_2 into the plant cells, thereby, interfering with Calvin-cycle, which will eventually reduce the potential yield of the crop (Ali and Anjum, 2016).

3.4.3 Relative water content (RWC)

Mabizela (2020) reported similar results, where the stressed and semi-stressed plants had lower RWC compared to the well-watered treatment with the variance showing evidently from the third day after stress initiation. The low RWC in the stressed treatment indicates a stressed plant population compared with the control. Lower RWC in stressed *C. subternata* leaves used in this study is in accordance with the findings of the studies on different plants (Arjenaki, Jabbari & Morshedi, 2012; Kabbadj *et al*., 2017). Another study on olives supports the outcome of this research, where the lowest RWC values were reported for severely water-

stressed olives (Boussadia et al., 2008). Higher RWC in plant leaves means that the plants have the least water strain and vice versa.

During the onset of drought, a reduction in stomatal conductance can reduce availability of CO_2 for photosynthesis, subsequently leading to inhibition of underlying biochemical processes such as Rubisco carboxylation and electron transport activity, relative water content and even pigment content (Khalil *et al*., 2020). The reduction in the leaf RWC due to the strain caused by limited water availability may be attributed to reduction in stomatal conductance after stomatal closure in response to drought stress. As a result of this, there is an observed decrease in the RWC of the stressed *C. subternata* plants compared to the well-watered plants (Boussadia *et al*., 2008). Soil texture is highly influential for water uptake, and may impede root elongation, availability of water, oxygen and nutrient (Khalil et al., 2020).

High percentage of clay in clovelly soil may be responsible for the low leaf RWC in the first sampling period. High clay content in soil increases soil hardness and strength when soil is drying out. As soil strength increases, the more difficult it is for plant roots to access water and nutrients, hence, the lowest RWC in the leaves of *C. subternata* plants growing on clovelly soil. However, in the second sampling period, since this was a pot experiment, the packaging of the soil might have altered the actual field structure, allowing more macropores in the soils with high clay contents, than likely to exist in the field (Khalil *et al*., 2020). The presence of these macropores may have contributed to the non-significant effects observed among all treatments in response to the water treatment.

3.4.4 Proline

Several abiotic factors such as water stress, high temperatures and salinity can cause protein modification, membrane injury and osmotic stress in plants (Meena *et al*., 2019). Plants respond to water stress by building-up osmolytes such as proline, glycine betaine, glycerol and many more, in order to minimize and tolerate cell injury (Sharma *et al*., 2019). The obtained results are comparable to those reported by Mabizela (2020) on proline contents of *C. subternata*, where proline concentration increased massively in stressed treatments, slightly in semi-stressed and constant in control treatments. Higher proline contents were also reported in wheat, *Amaranthus* species and *Achillea* species after being subjected to water stress (Keyvan, 2010; Slabbert & Krüger, 2014; Gharibi *et al*., 2016). Low proline content in plants indicates minimum water stress and vice-versa. The high and significant levels of proline that was observed between treatments during the third sampling period may be attributed to the plants having reached reproductive stage and had started flowering. Proline can accumulate in plants both under stress and non-stress conditions, although, produced at

low levels in all tissues in unstressed conditions (Kavi Kishor *et al*., 2015). As a metabolite and signal molecule, proline plays a crucial role in synthesis of protein and the response of plant cells to environmental stresses (Mattioli et al., 2009). Proline levels may increase during wounding and pathogen attack in some tissues, different stages of plant growth and development, nodule formation, fertilization, cytokinesis, apoptosis, senescence, and cell wall lignification. Under normal physiological (un-stressed) conditions, plants accumulate high amount of proline during the transition to flower *initiation* (Kavi Kishor *et al*., 2015). Thus, suggesting that proline may have a role to play in flower initiation and its subsequent development.

3.5 Conclusion

From this study, it is evident that different deficit irrigation levels and soil type had no significant effects on growth parameters of *C. subternata*. Likewise, soil type had no impact on the proline, RWC and the yield of the plants. Water stress increased the proline content, resulting in lower RWC. However, deficit irrigation had significant effect on the yield (root, fresh and dry shoot biomass). The higher the water stress, the lower the shoot and root biomass yield and vice-versa. Although, the well-watered and the semi-stressed plants gave higher shoot yield, more research is still needed to determine the tea quality of the stressed and unstressed *C. subternata* plants.

REFERENCES

Ábrahám, E., Hourton-Cabassa, C., Erdei, L. and Szabados, L., 2010. Methods for determination of proline in plants. Plant stress tolerance: methods and protocols, pp.317-331.https://doi.org/10.1007/978-1-60761-702-0_20

Ali, N. and Anjum, M.M., 2016. Drought stress: major cause of low yield and productivity. Austin Environ. Sci. 1 10–12.

Blum, A., 2011. Plant breeding for water-limited environments. Springer: New York.

Čereković N, Pagter M, Kristensen Hl, Pedersen Hl, Brennan R And Chandrasekhar, K., Dileep, A., Lebonah, D.E. and Pramoda Kumari, J., 2014. A short review on proteomics and its applications. International Letters of Natural Sciences, 12(1).

. https://doi.org/10.1016/j.scienta.2013.08.026

Chakhchar, A., Lamaoui, M., Wahbi, S., Ferradous, A., El Mousadik, A., Ibnsouda-Koraichi, S., Filali-Maltouf, A. and El Modafar, C., 2015. Leaf water status, osmoregulation and secondary metabolism as a model for depicting drought tolerance in Argania spinosa. *ActaPhysiologiae Plantarum*, *37*, pp.1-16. https://doi.org/10.1007/s11738015-1833-8

Connor, R., 2015. The United Nations world water development report 2015: water for a sustainable world (Vol. 1). UNESCO publishing.

Du Toit, J., Joubert, E. and Britz, T.J., 1998. Honeybush tea–a rediscovered indigenous South African herbal tea. Journal of Sustainable Agriculture, 12(2-3), pp.67-84.

Eziz, A., Yan, Z., Tian, D., Han, W., Tang, Z. and Fang, J., 2017. Drought effect on plant biomass allocation: A meta-analysis. Ecol. Evol. 7 (24) 11002–11010. https://doi.org/10.1002/ece3.3630

Fathi, A. and Tari, D.B., 2016. Effect of drought stress and its mechanism in plants. Int. J. Life Sci. 10 (1) 1–6.

Gharibi, S., Tabatabaei, B.E.S., Saeidi, G. and Gol,i S.A.H., 2016. Effect of drought stress on total phenolic, lipid peroxidation, and antioxidant activity of Achillea species. Appl. Biochem. Biotechnol. 178 (4) 796–809. https://doi.org/10.1007/s12010-015-1909-3

Habibi, G., 2018. Effects of mild and severe drought stress on the biomass, phenolic compounds production and photochemical activity of Aloe vera (L.) Burm. f. Acta. Agric. Slov. 111 (2) 463–476 .https://doi.org/10.14720/aas.2018.111.2.19

Harb, A., Krishnan, A., Ambavaram, M.M. and Pereira, A., 2010. Molecular and physiological analysis of drought stress in *Arabidopsis* reveals early responses leading to acclimation in plant growth. Plant physiology, 154(3), pp.1254-1271.

Horn, A., 2019. Honey Bush Tea - Small Industry, big potential. 36 - 38.

Jones, H.G., 2007. Monitoring plant and soil water status: established and novel methods revisited and their relevance to studies of drought tolerance. *Journal of experimental botany*, *58*(2), pp.119-130.

Joubert, E., Joubert, M.E., Bester, C., De Beer, D. and De Lange, J.H., 2011. Honeybush (*Cyclopia* spp.): From local cottage industry to global markets—The catalytic and supporting role of research. South African Journal of Botany, 77(4), pp.887-907. https://doi.org/10.1016/j.sajb.2011.05.014

Joubert, E.D.B.D. and de Beer, D., 2011. Rooibos (*Aspalathus linearis*) beyond the farm gate: From herbal tea to potential phytopharmaceutical. South African Journal of Botany, 77(4), pp.869-886.

Kabbadj, A., Makoudi, B., Mouradi, M., Pauly, N., Frendo, P. and Ghoulam, C., 2017. Physiological and biochemical responses involved in water deficit tolerance of nitrogen-fixing Vicia faba. PloS One 12 (12) p.e0190284. https://doi.org/10.1371/journal.pone.0190284

Kamara, A.Y., Menkir, A., Badu-Apraku B. and Ibikunle, O., 2003. The influence of drought stress on growth, yield, and yield components of selected maize genotypes. J. Agri. Sci. 141 (1) 43–50. https://doi.org/10.1017/s0021859603003423

Kardile, P.B., Dahatonde, K.N., Rakshe, M.V. and Burondkar, M.M., 2018. Effect of moisture stress on leaf Relative Water Content (RWC) of Four Cowpea (*Vigna unguiculata L.* walp.) genotypes at different stages of growth. *Int. J. Curr. Microbiol App Sci.* **7** 2645-2649. https://doi.org/10.20546/ijcmas.2018.704.301

Kavi Kishor, P. B., Hima Kumari, P., Sunita, M. S. L., & Sreenivasulu, N., 2015. Role of proline in cell wall synthesis and plant development and its implications in plant ontogeny. Frontiers in Plant Science, 6, 544. https://doi.org/10.3389/fpls.2015.00544

Kerepesi, I. and Galiba, G., 2000. Osmotic and salt stress-induced alteration in soluble carbohydrate content in wheat seedlings. Crop Sci. 40 (2) 482–. https://doi.org/10.2135/cropsci2000.402482x

Keyvan, S., 2010. The effects of drought stress on yield, relative water content, proline, soluble carbohydrates and chlorophyll of bread wheat cultivars. J. Anim. Plant Sci, 8(3), pp.1051-1060.

Khalil, A.M., Murchie, E.H. and Mooney, S.J., 2020. Quantifying the influence of water deficit on root and shoot growth in wheat using x-ray computed tomography. AoB Plants. 12 (5) 36 pp. https://doi.org/10.1093/aobpla/plaa036

Khan, A., Pan, X., Najeeb, U., Tan, D.K.Y., Fahad, S., Zahoor, R. and Luo, H., 2018. Coping with drought: stress and adaptive mechanisms, and management through cultural and molecular alternatives in cotton as vital constituents for plant stress resilience and fitness. Biol. Res. 51. https://doi.org/10.1186/s40659-018-0198-z.

LI-Ping, B.A.I., Fang-Gong, S.U.I., Ti-Da, G.E., Zhao-Hui, S.U.N., Yin-Yan, L.U. and Guang-Sheng, Z.H.O.U., 2006. Effect of soil drought stress on leaf water status, membrane permeability and enzymatic antioxidant system of maize. *Pedosphere*. **16** (3) 326-332. https://doi.org/10.1016/s1002-0160(06)60059-3

Little, T.M. and Hills, F. J., 1972. Statistical Methods in Agricultural, University of California, Davis, California 95616, pp 93-101.

Long, K. and Pijanowski, B.C., 2017. Is there a relationship between water scarcity and water use efficiency in China? A national decadal assessment across spatial scales. Land Use Policy, 69, pp.502-511. https://doi.org/10.1016/j.landusepol.2017.09.055

Lugojan, C., & Ciulca, S., 2011. Evaluation of relative water content in winter wheat. Journal of Horticulture, Forestry and Biotechnology, 15(2), 173-177.

Mabizela, G.S., 2020. 'Metabolic and quality profiling of *Cyclopia subternata* and *C. genistoides* in response to seasonal variation and drought stress'. Doctral dissertation, Tshwane University of technology.

Mattioli, R., Falasca, G., Sabatini, S., Altamura, M.M., Costantino, P. and Trovato, M., 2009. The proline biosynthetic genes P5CS1 and P5CS2 play overlapping roles in Arabidopsis flower transition but not in embryo development. Physiologia Plantarum, 137(1), pp.72-85.

McGaw, L.J., Steenkamp, V. & Eloff, J.N., 2007. Evaluation of *Athrixia* bush tea for cytotoxicity, antioxidant activity, caffeine content and presence of pyrrolizidine alkaloids. Journal of Ethnopharmacology, 110(1), pp.16-2 https://doi.org/10.1016/j.jep.2006.08.029

Meena, M., Divyanshu, K., Kumar, S., Swapnil, P., Zehra, A., Shukla, V., Yadav, M. and Upadhyay. R.S., 2019. Regulation of L-proline biosynthesis, signal transduction, transport, accumulation and its vital role in plants during variable environmental conditions. Heliyon 5 (12) p.e02952.

National Gazette of South Africa, "National Gazette NO. 38924, 03 JULY 2015, VOL 601", 2015

Obidiegwu, J.E., Bryan, G.J., Jones, H.G. and Prashar, A., 2015. Coping with drought: stress and adaptive responses in potato and perspectives for improvement. *Front. Plant Sci.* **6** 542 pp. https://doi.org/10.3389/fpls.2015.00542

Ott, R.L.,1998. An Introduction to Statistical methods and data analysis. Belmont, California: Duxbury Press: pp 807-837 (pp 1-1051)

Per, T.S., Khan, N.A., Reddy, P.S., Masood, A., Hasanuzzaman, M., Khan, M.I.R. and Anjum, N.A., 2017. Approaches in modulating proline metabolism in plants for salt and drought stress tolerance: Phytohormones, mineral nutrients and

transgenics. *Plant.Physiol.Biochem.* **115**126-https://doi.org/10.1016/j.plaphy.2017.03.018

Rampedi, I. & Olivier, J., 2005. The use and potential commercial development of *Athrixia phylicoides*. Acta Academica, 37(3), pp.165-183.

Sade, N., Galkin, E. and Moshelion, M., 2015. Measuring Arabidopsis, tomato and barley leaf relative water content (RWC). Bio-protocol 5(8): e1451. https://doi.org/10.21769/bioprotoc.1451

SAS INSTITUTE, INC., 2013. Statistical Analysis Software. Users' Guide Statistics Version 9.4. SAS Institute Inc., Cary, North Carolina.

Shapiro, S.S. and Wilk, M.B., 1965. An analysis of Variance Test for Normality (complete samples), Biometrika 52, 591-611. https://doi.org/10.1093/biomet/52.3-4.591

Sharma, S., Villamor, J.G. and Verslues, P.E., 2011. Essential role of tissue-specific proline synthesis and catabolism in growth and redox balance at low water potential. Plant physiology, 157(1), pp.292-304.

Siddique, A. and Dubey, A.P., 2017. Phyto-toxic effect of heavy metal ($CdCl_2$) on seed germination, seedling growth and antioxidant defence metabolism in wheat (*Triticum aestivum L.*) variety HUW-234. *Int. J. Bio-resour.* **8** (2) 261-267. https://doi.org/10.23910/ijbsm/2017.8.2.1684

Slabbert, M.M. and Krüger, G.H.J., 2014. Antioxidant enzyme activity, proline accumulation, leaf area and cell membrane stability in water stressed *Amaranthus* leaves. S. Afr. J . Bot. 95 123–128 .https://doi.org/10.1016/j.sajb.2014.08.008

Soltys-Kalina D, Plich J, Strzelczyk-Żyta D, Śliwka J And Marczewski W., 2016. The effect of drought stress on the leaf relative water content and tuber yield of a half-sib family of 'Katahdin'-derived potato cultivars. Breed Sci. 66 (2) 328-331.

Soni, R.P. Katoch, M., Kumar, A., Ladohiya, R. & Verma, P., 2015. Tea: Production, Composition, Consumption and its Potential as an Antioxidant and Antimicrobial Agent. International Journal of Food and Fermentation Technology, 5(2). https://doi.org/10.5958/2277- 9396.2016.00002.7

Surendar, K. K., Devi, D. D., Ravi, I., Jeyakumar, P., & Velayudham, K., 2013. Water stress affects plant relative water content, soluble protein, total chlorophyll content and yield of ratoon banana. International Journal of Horticulture, 3. https://doi.org/10.5376/ijh.2013.03.0017

Tshikhudo, P.P., Ntushelo, K., Kanu, S.A. and Mudau, F.N., 2019. Growth response of bush tea (*Athrixia phylicoides DC*) to climatic conditions in Limpopo Province, South Africa. S. Afr. J. Bot. 121 500–504. https://doi.org/10.1016/j.sajb.2018.12.012

Van Wyk, B.E., & Gericke, N., 2000. People's Plants. A Guide to Useful Plants of South Africa. First Ed. Briza Publication, Pretoria, South Africa, p. 102.

Velasco-Muñoz, J.F., Aznar-Sánchez, J.A., Belmonte-Ureña, L.J. and López-Serrano, M.J., 2018. Advances in water use efficiency in agriculture: A bibliometric analysis. Water, 10(4), p.377. https://doi.org/10.3390/w10040377

WWAP (United Nations World Water Assessment Programme)., 2012. The United Nations World Water Development Report 4: Managing Water under Uncertainty and Risk. UNESCO, Paris. https://doi.org/10.18356/cda9571e-en

WWF., 2018. Agricultural Water File: Farming for a drier future. http://awsassets.wwf.org.za/downloads/wwfwaterfiles19july2018.pdf

Zhao, T.J., Sun, S., Liu, Y., Liu, J.M., Liu, Q., Yan, Y.B. and Zhou, H.M., 2006. Regulating the drought-responsive element (DRE)-mediated signaling pathway by synergic functions of trans-active and transinactive DRE binding factors in Brassica napus. J. Biol. Chem. 281 (16) 10752–10759. https://doi.org/10.1074/jbc.m510535200

CHAPTER FOUR

RESPONSE OF *CYCLOPIA SUBTERNATA* TO WATERING FREQUENCY: STOMATAL CONDUCTANCE, PROLINE, AND RELATIVE WATER CONTENT

(This Chapter consist of field and pot study. The pot study has been published in the proceeding of the 35th International Conference on "Chemical, Biological and Environmental Engineering" (ICCBEE-22) Nov. 28-29, 2022, Johannesburg (South Africa). DOI: https://doi.org/10.17758/IICBE4.C1122203 . Please refer to appendix 2).

Abstract— *Cyclopia*, commonly referred to as honeybush, is an indigenous tea plant native to the Eastern and Western Cape provinces of South Africa and is known for its sweet taste and honey-like aroma. The tea is famous for its antioxidants and can be used in value-added products such as cosmetics and other food ingredients. It is estimated that there are 23 *Cyclopia* species in South Africa, but only six are used commercially. Studies on abiotic stresses in honeybush are limited and this study helped to investigate the response of the species to water stress mechanisms, which is of utmost importance for the development of drought resistant lines for this highly sought-after tea plant. A pot experiment was conducted on a Stellenbosch granite soil in which *Cyclopia subternata* plants were subjected to three different watering frequencies (thrice, twice and once a week). More frequent watering (control) showed highest percentage of plant growth than plants subjected to other watering treatments in all the three growth parameters investigated. Higher proline concentrations and lower relative water content were observed in the water stressed plants (watering twice and once a week). Stomatal conductance was generally lower in stressed plants and highest in well-watered plants. The drop in stomatal conductance in the stressed plants is due to the induction of stomatal closure which is a coping mechanism to aid survival by reducing transpiration rate.

.

4.1 Introduction

It is said that tea is the second most consumed beverage in the world after water. The type of tea (oolong, green, black, and herbal) usually depends on the post-harvest treatment (Soni *et al*., 2015). Three different types of tea are grown in South Africa: Rooibos (*Aspalathus linearis*), bush (*Athrixia phylicoides*) and honeybush (*Cyclopia* species). While bush tea is still relatively unknown as a commercial product, honeybush has gained popularity, with rooibos being the best known and most established (Van Wyk and Gericke, 2000; Joubert *et al*., 2008; Joubert *et al*., 2011). The market for honeybush is expected to grow due to the health benefits derived from it. Polyphenols, the antioxidants in honeybush tea, have beneficial effects on

human health (Soni *et al*., 2015). Traditionally, honeybush has been used to treat disorders such as heartburn, ulcers, colic in infants, chronic tonsillitis, lung infections, nausea and mucus build-up in the respiratory tract or body cavities (Joubert *et al*., 2008; Marnewick *et al*., 2000; Joubert *et al*., 2019; Mabizela, 2020). There are 23 species of *Cyclopia* in the Cape Floristic Region of South Africa, of which only six are used commercially, among the six is *Cyclopia subternata*. About 82% of honeybush is still harvested in the wild (Bester, 2012; Bester *et al*., 2014; DAFF, 2016). These species have a very limited range and rare habitat requirements.

Abiotic stressors such as drought are undoubtedly one of the most limiting factors for plant growth (Atkinson and Urwin, 2012; Krasensky and Jonak, 2012). Plant growth is mostly limited by the unavailability of water and climate change is expected to increase the extent of water stress on agricultural soils (Martorell, 2014; Brilli *et al*., 2019). Plants accumulate proline and carbohydrates as a coping mechanism for water stress (Mabizela, 2020). Though some authors question the direct relationship between proline accumulation and stress adaptation (Mattioli *et al*., 2009), others concluded that proline as a multifunctional molecule can serve as an osmolyte and radical scavenger by responding to a variety of abiotic and biotic stressors, or as a source of energy for regrowth by degrading in response to stress (Szepesi and Szőllősi, 2018).

A plant's response to water stress is largely determined by the regulation of stomatal conductance. Water scarcity leads to stomatal closure, which is one of the first responses to water shortage (Flexas *et al*., 2014). The relative water content (RWC) of a plant can be used to determine how well or poorly it absorbs water and the extent of stress (Surendar *et al*., 2013; Soltys-Kalina *et al*., 2016). RWC is defined as "the percentage of water present in the leaf as a fraction of the total volumetric water that the leaf can hold at full turgor" (Blum, 2011). Under drought conditions, RWC is said to be a more accurate indicator of water status than any other metric of water potential. Leaf water supply and transpiration rate are closely linked and can give a good indication of the balance between these two variables (Lugojan and Ciulca, 2011). Therefore, farmers need to understand how water use affects plant growth to maximize their productivity (Harb *et al*., 2010).

South Africa's arid climate, characterized by hot, dry conditions and low relative humidity, resulting in uneven distribution of rainfall and high evapotranspiration often leads to water stress (Bennie and Hensley, 2001). There is very little research on how honeybush responds to drought stress in the Mediterranean climate of South Africa, where there is persistent drought during summer periods (Lionello *et al*., 2006; Mabizela, 2020). Therefore, the aim of

this study was to evaluate the physiological and morphological responses of *C. subternata* to different watering frequencies.

4.2 Materials and Methods

4.2.1 Study site and experimental design

A field and glasshouse pot trials were conducted at separate sites at the Agricultural Research Council (ARC), Infruitec and Nietvoorbij (-33.925920º, 18.874259° and -33.925920º, 18.874259°) respectively, in Stellenbosch, South Africa, to assess the effect of water deficit stress on potted and field grown *C. subternata*. The trial sites are presented in Figure 6.1 (A= Nietvoorbij field) and (B= Infruitec greenhouse). The pot trial was a randomized block design (RBD) with one soil type (Stellenbosch granite), three irrigation treatments [watering thrice (control), twice (semi-stressed) and once a week (stressed)] and eight replicates. The experiment was conducted for 112 days (from mid-May until early September 2021). The field trial consisted of three irrigation treatments [three irrigations from January to harvest (well-watered or control), two irrigations from January to harvest (semi-stressed) and one irrigation from January to harvest (stressed)], replicated eight times, and laid out in a randomised complete block design (RCBD). Prior to field planting, seeds of *C. subternata* were sown in March 2019 and were transplanted to the field on 19 December 2019. Nine months old plants were spaced 1 meter apart both within and between the rows. Each treatment consisted of three rows, with each row having 12 plants. Each replication or block was separated from each other by a 2 m spacing while each treatment within a block was also separated by a spacing of 2 m. Therefore, 864 plants per 1110 m^2 plot were planted, giving a total of 7783.78 plants/ha. 10 of the middle row plants were the data plants while the two side rows constituted the border plants.

Figure 4.1: An illustration of ARC (Infruitec-Nietvoorbij) maps in Stellenbosch (Google Earth, 2022).

4.2.2 Soil collection and planting

4.2.2.1 Pot trial

The soil (Stellenbosch granite) was collected from the ARC Nietvoorbij Research Farm and sieved to remove plant debris and larger fragments, in preparation for transplanting. Soil samples were collected for physicochemical analysis. Each pot was filled with14 kg of soil in a 30 cm (top diameter) plastic pot. The soil in each pot was irrigated to pot capacity (PC) before transplanting nine months old *C. subternata* seedlings, one plant per pot.

4.2.2.2 Field trial

Prior to transplanting, activities such as site preparation, which include herbicides application, ripping, discing of the soil and installation of irrigation system were carried out. Nine months old seedlings were transplanted from the glasshouse to the field.

4.2.3 Watering treatments

4.2.3.1 Pot trial

All plants were watered uniformly with 300 ml of water for the first 81 days after transplanting (DAT) to ensure uniformity and strong root growth, before treatment application. Thereafter, *C. subternata* plants were subjected to three irrigation treatments (from early August to early September 2021) until the study was terminated at 112 DAT. The watering treatments were irrigating thrice (3 days/week), twice (2 days/week) and once (1 day/week). 300 ml of water was applied per pot, at every irrigation.

4.2.3.2 Field trial

All the plants received the same irrigation from transplanting until treatments (different irrigation levels) were applied from January to March 2022. Irrigation water was applied through the drip irrigation system with a maximum pressure of 3.5 bar (350 kPa). The dripper lines had a flow rate of 2.30 L/H, with each irrigation cycle running for 24 hours. In January, all treatments were fully irrigated. For February irrigation, only the well-watered (control) and semi-stressed treatments were irrigated, while the stressed treatments were excluded. The last irrigation before harvest was in March where only the control treatment plants were irrigated. Irrigation was done once per month for the corresponding treatment.

Water and tensiometer readings

Four tensiometers with different depths (30, 60, 90 and 120 cm) were installed close to the neutron probes in the field. The readings on the tensiometers were recorded simultaneously with neutron probe data collection. Water meters were used to measure the amount of water that passed through the irrigation pipes that supplied water to different treatments. The readings were generated automatically when water passed through the pipes and recorded from November 2020 until June 2022 depicted in Figure 4.2.

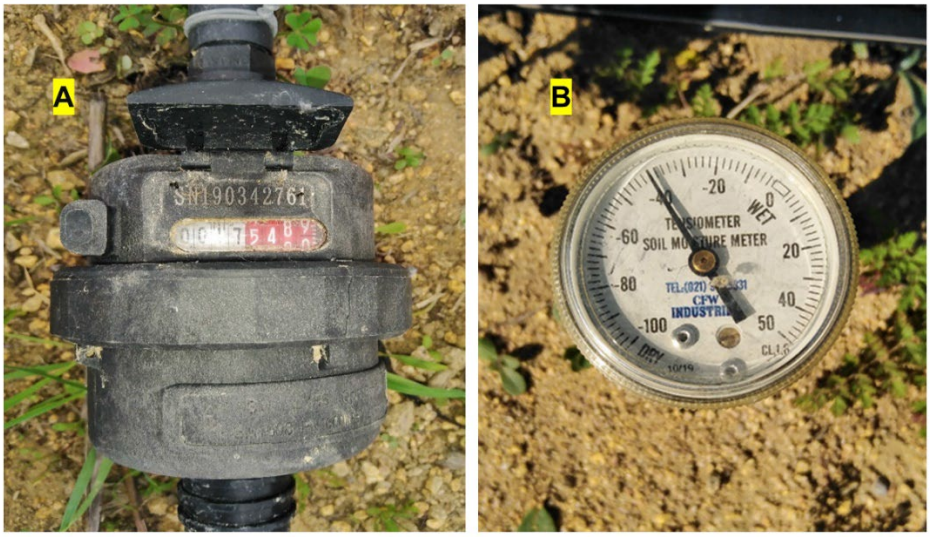


Figure 4.2: The water meter (A) fitted on the water supply pipes and tensiometer (B) placed next to the *C. subternata* plants.

Neutron probe readings

Neutron probe count values in the field were measured at weekly intervals (October 2020 - February 2022) at 30, 60, 90 and 120 cm soil depths. A “water standard” measurement was taken before each neutron measurement session to obtain a complete water saturated neutron measurement.

4.3 Data collection

3.3.1 Growth parameters

Growth parameters for the pot trial were measured weekly from the second week of August until the second week of September 2021, when the study was terminated. Measurements commenced shortly before the introduction of irrigation treatments. Plant height, stem diameter, stem circumference measurement methods are detailed in section 3.2.4.1 (Chapter 3).

4.3.2 Stomatal conductance

Stomatal conductance was measured with an SC-1 leaf porometer (Decagon Devices, Pullman, USA). The equipment measures the rate of passage of water vapour or carbon dioxide (CO2) through the leaf stomata. Measurement was done at weekly intervals from 09 August to 06 September 2021 for the pot trial while the field measurement took place from January to March 2022. Measurements were taken on the abaxial (bottom) side of the leaf at mid-day, which corresponds with the peak period of the environmental factors.

4.3.3 Relative water content (RWC)

The leaves of *C. subternata* were sampled weekly for the pot trial and only once (at the end of the study) for the field trial. It was determined using an improved version of the method of Sade *et al.* (2015) which is detailed in section 3.2.4.4 (Chapter 3).

4.3.4 Estimation of proline content using the colorimetric method

Proline content of *C. subternata* was determined using the modified method of Ábrahám *et al.* (2010) which is reported in section 3.2.4.3 (Chapter 3). Leaf samples were collected at weekly intervals for the pot trial and only once (at the end of the study) for the field trial. Figure 4.3 shows the extraction of centrifuged samples and preparation of reaction mixture into the test tubes using a pipette.

Figure 4.3: Pipetting a reaction mixture into test tubes during the determination of proline in *C. subternata* leaves.

4.3.5 Shoots yield and harvesting

4.3.5.1 Pot trial

Shoot yield was determined by cutting the shoot just above the soil surface, using a pruning shear (Figure 4.4). The harvested shoots were placed in a labelled paper bag and weighed

with a sensitive scale, followed by oven-drying at 70ºC for 24 hours. The dried weights were also recorded.

Figure 4.4: Cutting above ground shoots of *C. subternata* plants with a pruning shear.

4.3.5.2 Field trial

The middle row plants in the field trial were trimmed on the sides using a pruning shear before cutting at knee level (07 April 2022). A composite bulk was pooled from each treatment and harvested into crates before weighing, using a weighing balance. The harvested plants were tied into bunches shortly after weighing then transported to the processing facilities for drying and other post-harvest activities.

4.3.6 Statistical analysis

Data were subjected to analysis of variance (ANOVA) using SAS statistical software (version 9.4, SAS Institute Inc., Cary, NC, USA, 2000), utilizing time as a sub-plot component for each observation time (sampling/week) separately (Little and Hills, 1972). The Shapiro-Wilk test was used to test for deviation from normality (Shapiro and Wilk, 1965). To compare treatment means, Fisher's least significant difference was determined at the 5% level (Ott and Longnecker, 2015). For all tests, a probability level of 5% was considered significant.

4.4 Results

4.4.1 Physical and chemical properties of the soil

Table 4.1 presents the physical and chemical properties of the soil medium used in this trial. The soil was classified as coarse sandy loam with a slightly acidic pH of 5.3 and a stone volume of 0.22 %. The nutrients found in the soil were within normal ranges for plant growth.

Table 4.1: Chemical and physical properties of the soil medium used in the study.

Physical properties		Values
Clay (<0.002 mm)		13
Silt (0.002–0.02 mm)		17
Fine sand (0.02–0.2 mm)		33
Medium sand (0.2–0.5 mm)		3
Coarse sand (0.5–2 mm)		35
Stone volume (%)		0.22
Soil textural class		Coarse sandy loam
Chemical properties		
Ex. Cations (cmol (+)/ kg	Na	0.14
	K	0.52
	Ca	4.4
	Mg	1.6
Macronutrients (mg/kg)	NO3	31.3
	P	23.9
	NH4	3.2
	K	203
Base saturation (%)	K	6.97
	Ca	58.99
	Na	1.88
	Mg	21.45
Acid saturation		10.71
pH (KCl)		5.3
Resistance (Ohms)		800

4.4.2 Growth parameters

4.4.2.1 Pot trial

As shown in Table 4.2, in general, the three watering frequencies had no significant effect ($p>0.05$) on plant height, although significant effects on stem diameter and stem circumference were observed during the first two weeks of the observation period. However, the plants watered thrice weekly, having significantly lower stem diameter and stem circumference quickly caught up with the less frequently watered plants from the third week of treatment application with no significant difference among all treatments. This could be attributed to better growth in the well-watered treatments. In the last week of observation, irrigation once a week had the poorest performance in terms of plant height, stem diameter and circumference.

Table 4.2: Weekly growth of *C. subternata* as influenced by watering frequency.

Sampling time (week)	Irrigation	Plant height (cm)	Stem diameter (mm)	Stem circumference (mm)
1`	Control	22.99 a	1.96 b	6.14 b
	Twice a week	23.75 a	2.19 a	6.88 a
	Once a week	22.57 a	2.09 ab	6.57 ab
2	Control	23.09 a	2.0 b	6.26 b
	Twice a week	24.30 a	2.24 a	7.05 a
	Once a week	22.67 a	2.13 ab	6.69 ab
3	Control	26.49 a	2.23 a	7.02 a
	Twice a week	26.63 a	2.21 a	6.95 a
	Once a week	25.31 a	1.99 a	6.24 a
4	Control	27.98 a	2.25 a	7.08 a
	Twice a week	28.35 a	2.25 a	7.07 a
	Stressed	26.28 a	2.00 a	6.28 a
5	Control	31.00 a	2.45 a	7.69 a
	Twice a week	30.10 ab	2.36 a	7.43 a
	Once a week	27.74 b	2.08 b	6.53 b

Means with the same letter are not significantly different (p≤0.05).

The percentage change in plant height, stem diameter and stem circumference as influenced by watering frequency (Table 4.3) highlight the average mean difference in growth parameters from the start of the treatments to the termination of the trial.

Table 4.3: Percentage change in *C. subternata* growth in response to different watering frequencies.

Growth parameter	Irrigation	Percentage change (%)
Plant height (cm)	Control	34.87
	Twice a week	26.74
	Once a week	22.91
Stem diameter (mm)	Control	25.20
	Twice a week	7.96
	Once a week	0.57
Circumference (mm)	Control	25.20

	Twice a week	7.97
	Once a week	0.58

The well-watered plants (control) showed the highest percentage growth in all three parameters, while plants under water deficit stress (twice and once a week) showed the least growth. As the irrigation frequency increased, the percentage growth also increased and vice-versa.

4.4.2.2 Field trial

Generally, there was no significant difference ($p \geq 0.05$) observed between the control and semi-stressed treatments throughout the study period (Table 4.4). However, stressed plants consistently had the lowest growth in terms of plant height, stem diameter and stem circumference. The influence of different water stress levels on all three irrigation levels in this study was notably visible between the control and severely stressed treatments ($p \leq 0.05$). From the 11th week until the end of the study, the control irrigation treatment was neither different ($p=0.05$) from semi-stressed nor severely stressed populations in terms of plant height. Whereas the same plants under observation were significantly ($p \leq 0.05$) superior in terms of stem diameter and stem circumference when the control and stressed plants are compared. At the end of the study, all three irrigation treatments did not have any significant influence on plant height. However, stem diameter and stem circumference in both stressed (partially and severely) populations were not statistically different from each other ($p \geq 0.05$).

Table 4.4: The response of field-grown *C. subternata* to water deficit stress.

Sampling time (week)	Irrigation treatments	Plant height (cm)	Stem diameter (mm)	Stem circumference (mm)
1	Control	82.315 a	16.914 a	53.109 a
	Semi-stressed	85.492 a	17.719 a	55.638 a
	Stressed	63.403 b	13.824 b	43.406 b
3	Control	87.079 a	19.426 a	60.999 a
	Semi-stressed	88.763 a	19.305 a	60.617 a
	Stressed	68.801 b	15.938 b	50.044 b
5	Control	92.550 a	22.879 a	71.838 a
	Semi-stressed	93.705 a	21.315 ab	66.930 ab
	Stressed	73.339 b	18.359 b	57.648 b
7	Control	96.963 a	25.682 a	80.642 a
	Semi-stressed	98.568 a	22.426 ab	70.416 ab
	Stressed	78.423 b	19.798 b	62.167 b
9	Control	101.481 a	29.279 a	91.937 a
	Semi-stressed	103.532 a	24.363 b	76.500 b
	Stressed	82.052 b	21.430 b	67.291 b
11	Control	104.979 ab	31.545 a	99.051 a
	Semi-stressed	110.392 a	25.913 ab	81.366 ab
	Stressed	88.063 b	23.306 b	73.181 b
13	Control	107.343 ab	33.475 a	105.111 a
	Semi-stressed	113.104 a	27.071 b	82.003 b
	Stressed	90.528 b	24.933 b	78.290 b
15	Control	109.305 ab	34.436 a	108.129 a
	Semi-stressed	114.470 a	27.884 b	87.556 b
	Stressed	92.589 b	25.770 b	80.917 b
17	Control	109.990 ab	36.161 a	113.54 a
	Semi-stressed	116.571 a	28.823 b	90.50 b
	Stressed	96.548 b	27.005 b	84.80 b
19	Control	113.058 a	37.959 a	119.19 a
	Semi-stressed	120.370 a	30.215 b	94.88 b

Stressed	99.399 a	28.378 b	89.11 b

Means with the same letter are not significantly different (p≤0.05).

4.4.3 Shoot yield

4.4.3.1 Pot trial

The results of the effect of irrigation treatments on the shoot of *C. subternata* are shown in Table 4.5. No significant difference (p>0.05) was found in the fresh and dry shoot yield in all treatments.

Table 4.5: Average shoot yield of *C. subternata* in response to different irrigation frequencies.

Irrigation	Shoot	
	Fresh weight (g)	Dry weight (g)
Control	4.0513	1.3833
Twice a week	4.4960	1.5198
Once a week	4.5798	1.5775

There is no significant difference (p≥0.05) among treatments during sampling time.

4.4.3.2 Field trial

Figure 4.5 presents the average yield of field cultivated *C. subternata* after harvest. The yield response of *C. subternata* to different irrigation treatments shows significant differences among treatments (p<0.05). The average yield in kg was the greatest in frequently watered treatment compared to the least watered treatment. Control and semi-stressed treatments did not have statistical difference amongst each other (p>0.05). Semi-stressed and stressed treatments did not have a statistically influence amongst the two (p>0.05).

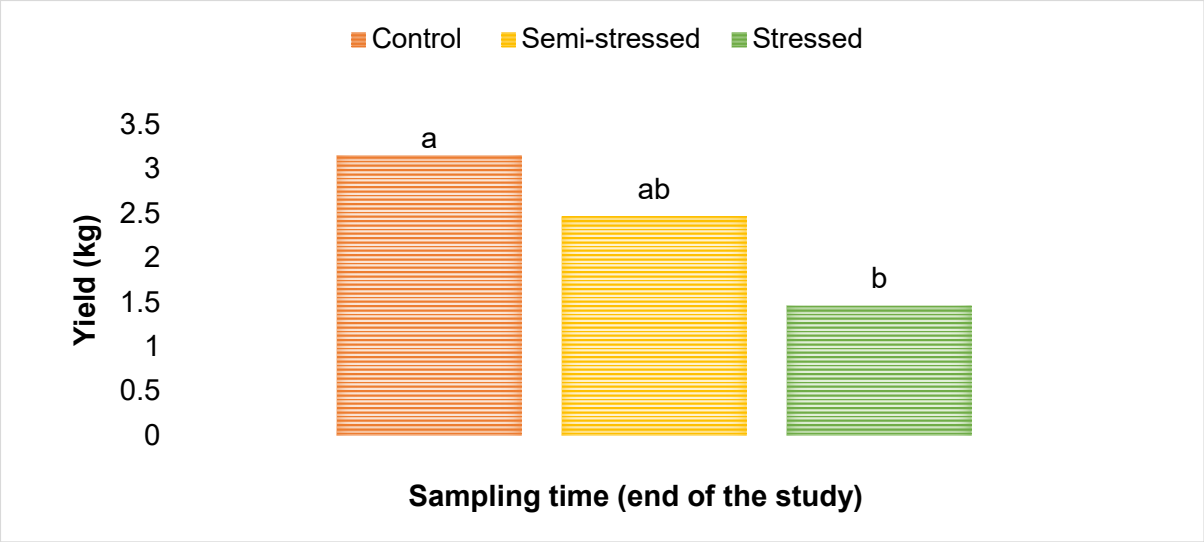


Figure 4.5: The RWC of *C. subternata* in response to different irrigation treatments in the field. RWC= relative water content. Means with different letters shows significant difference (p≤0.05).

Figure 4.6 shows the effect of irrigation treatments on soil water content of field-grown *C. subternata* for 2020/21 growing season. The soil was equally moistened throughout the season (day 120- 250) until treatment application commenced. As expected, treatment three (stressed plants) had drier soil compared to the other two irrigation treatments. The second season (2021/22) had similar trend with drier soils in less irrigated plots and greater soil-water content in well-watered plots (data not shown).

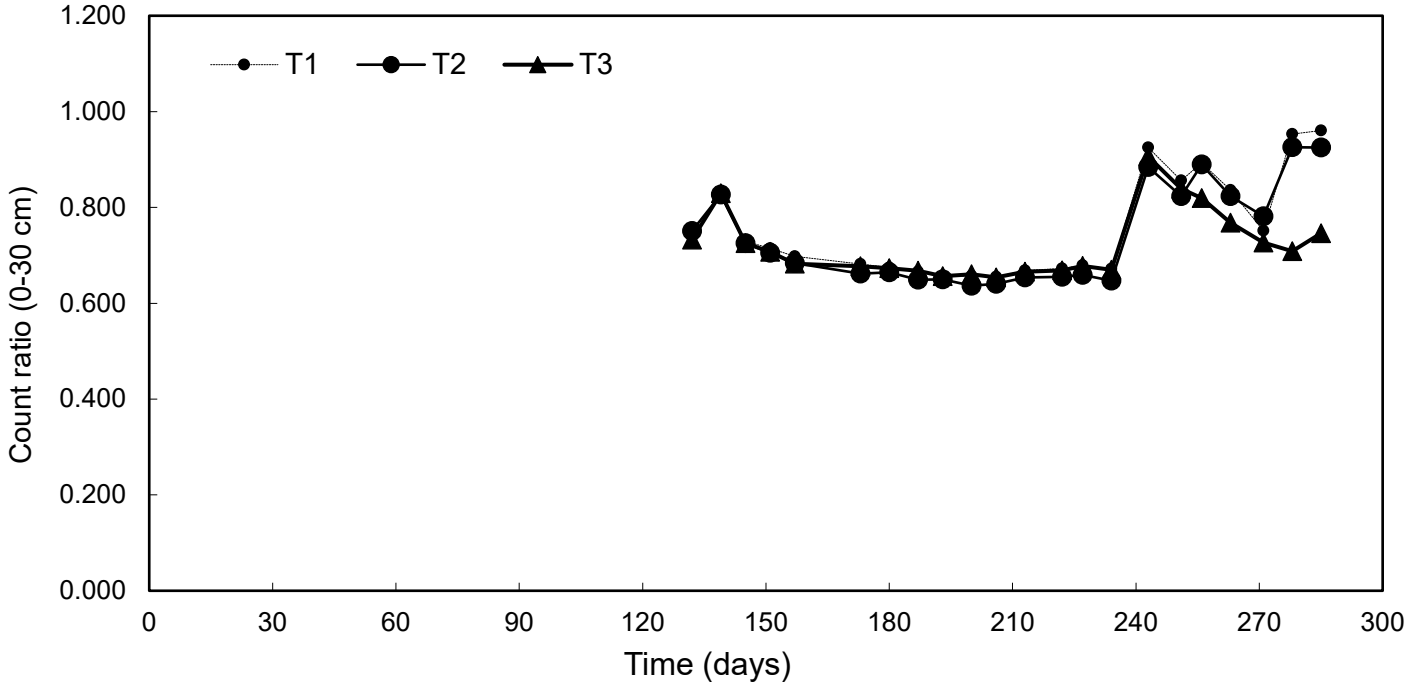


Figure 4.6: The effect of different irrigation treatments on field-grown *C. subternata* plants on soil-water content. T1= treatment 1/ well-watered; T2= treatment 2/ semi-stressed; T3= treatment 3/ stressed plants.

4.4.4 Relative water content (RWC)

4.4.4.1 Pot trial

Figure 4.7A shows differentiation of RWC in *C. subternata* leaves when plants were subjected to different water deficit stress over a period of five weeks. From the second week onwards, watering frequency had a significant effect ($p < 0.05$) on the treatments. Well-watered plants consistently had higher RWC than plants watered once or twice a week, with irrigation once a week having the least RWC.

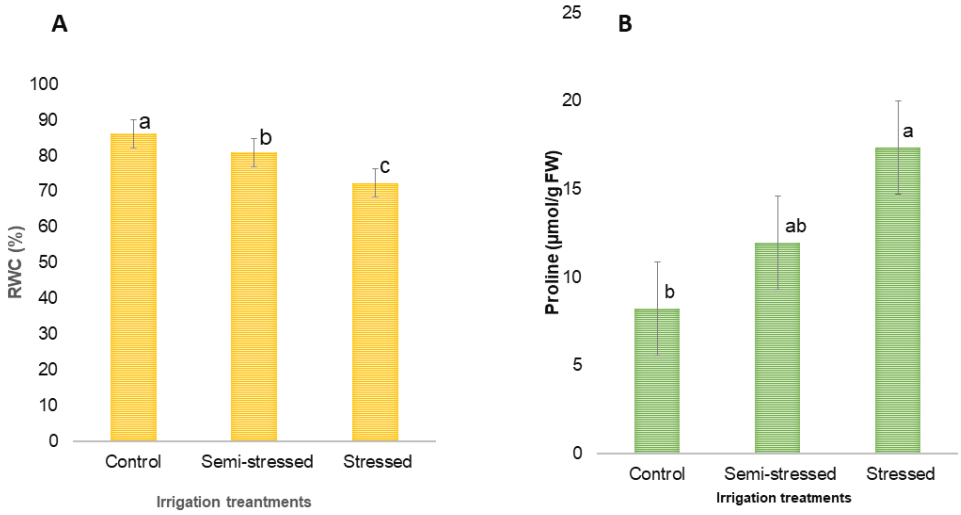


Figure 4.7: Effect of diverse water stress levels on relative water content (A) and proline concentration (B) of C. subternata.

4.4.4.2 Field trial

The difference in the RWC of the *C. subternata* plants at the end of the field study is depicted in Figure 4.8. The irrigation treatment that received the most water had the highest RWC (92.93%) while the stressed treatment reported the lowest with a percentage of 85.11%.

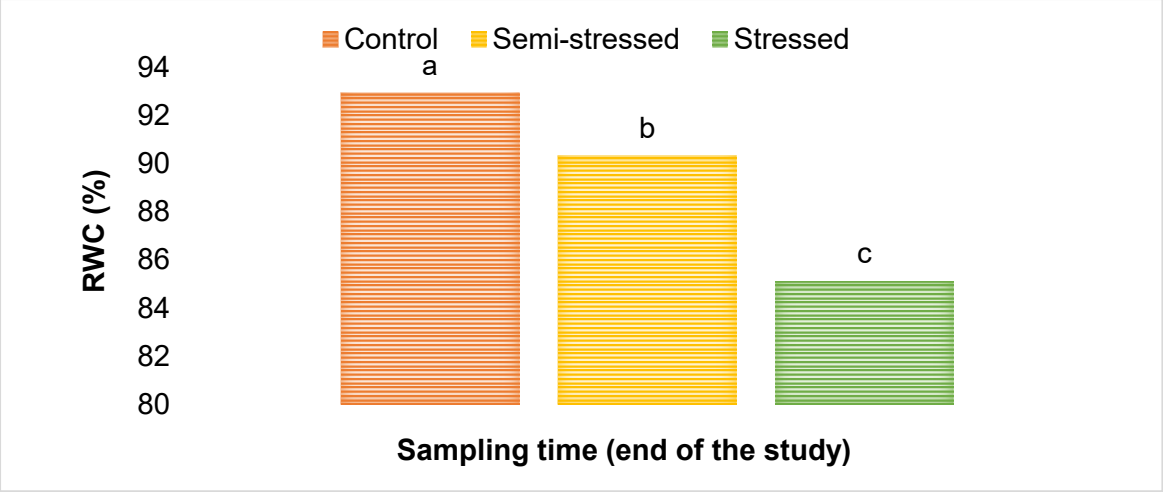


Figure 4.8: The RWC of *C. subternata* in response to different irrigation treatments in the field. RWC= relative water content. Means with different letters shows significant difference (p≤0.05).

4.4.5 Proline

4.4.5.1 Pot trial

Water stress in plants increases metabolite levels and stimulates metabolism (Sharma *et al*., 2019). In the first sampling week, no significant difference ($p > 0.05$) was observed among all the three treatments. Thereafter, the highest proline contents were consistently observed in plant populations that received irrigation once a week as shown in Figure 4.7B. Whereas the well-watered *C. subternata* plants had significantly lowest proline accumulation throughout the observation period.

4.4.5.2 Field trial

The difference in the proline accumulation of the *C. subternata* plants at the end of the field trial is depicted in Figure 4.9. The proline content in the well-watered treatment was the lowest (13.325 µmol/g FW) while the semi-stressed plants had a slightly higher proline accumulation of 16.807 µmol/g FW. The highest proline accumulation was found in the stressed treatment (25.513 µmol/g FW), indicating higher water stress. Water stress had a significant influence ($p<0.05$) on the proline contents of the cultivated honeybush. The higher the water stress, the higher the accumulation of proline content in the plants and vice versa.

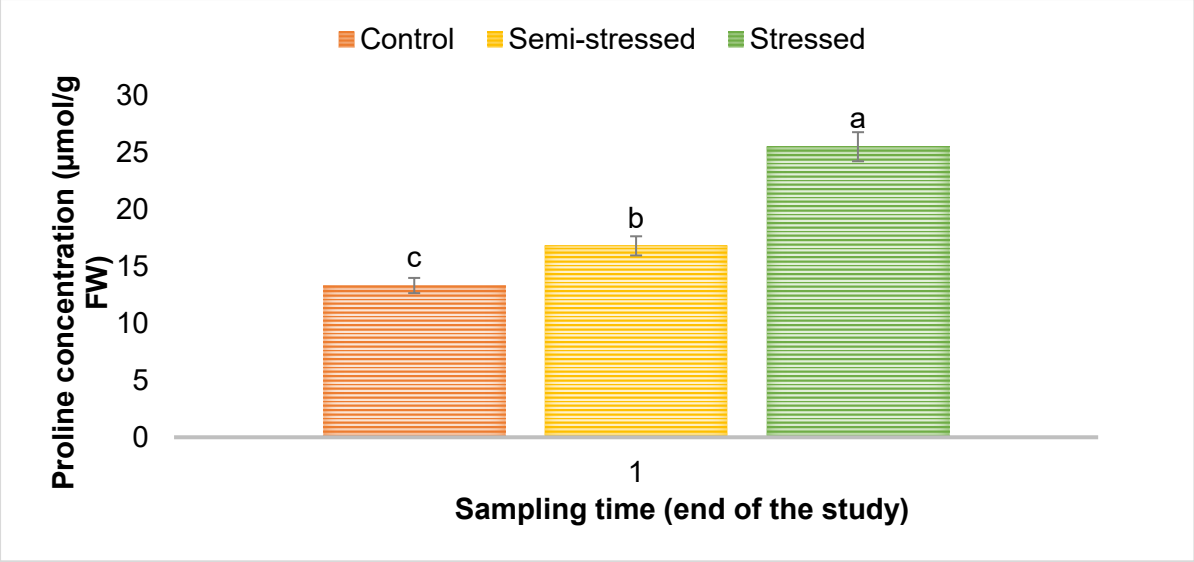


Figure 4.9: The concentration of proline in field cultivated *C. subternata* plants in response to different irrigation treatments. Means with different letters are significantly different (p≤0.05).

4.4.6 Stomatal conductance

4.4.6.1 Pot trial

In this study, the average stomatal conductance of *C. subternata* as influenced by watering frequency is presented in Figure 4.10. No significant difference ($p>0.05$) was observed in the first three weeks of sampling dates for all irrigation treatments. The reason for this may be because the plants were yet to reach a threshold where stomatal closure is triggered as leaf water potential reaches a critical stress level due to deficit irrigation (Mofokeng *et al*., 2015). However, the stressed plants (watering twice and once a week) generally had lower stomatal conductance, which became significantly lower ($p<0.05$) in the last two weeks of the study. Although, the two water stressed treatments did not differ significantly from each other during this period. This result is in accordance with the findings of Chowdhury *et al*. (2016), where a greater reduction in photosynthesis and stomatal conductance was observed in the water stressed genotypes of soybean.

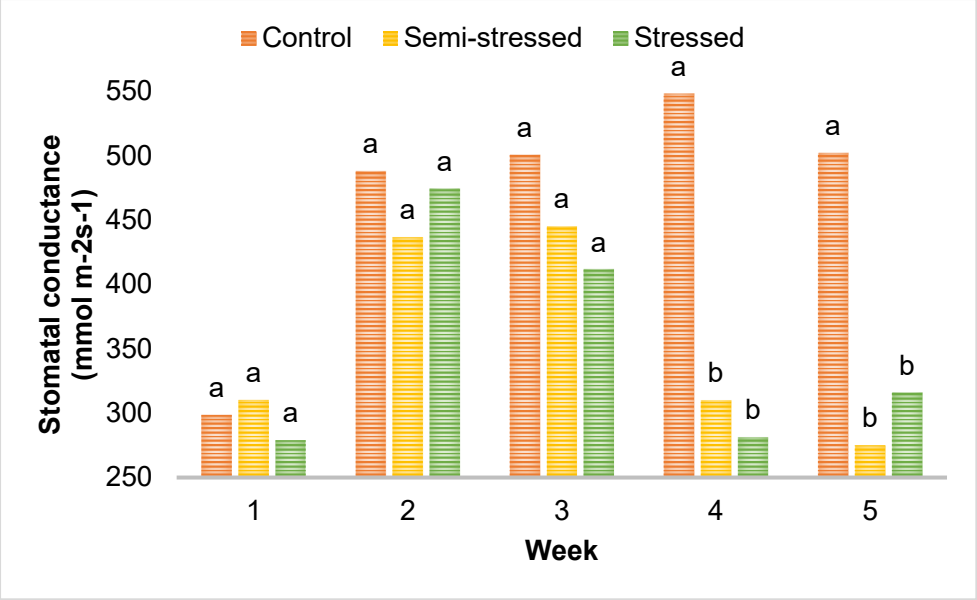


Figure 4.10: Effects of irrigation treatments on the stomatal conductance of *C. subternata* leaves.

4.4.6.2 Field trial

Generally, no significant difference ($p>0.05$) was observed on the stomatal conductance of *C. subternata* as a response to different irrigation treatments as shown in Figure 4.11. The stomatal conductance for well-watered, semi-stressed and stressed treatments ranged from 37.55- 359.813 $mmol^{-1}s^{-1}$, 53.85- 341.313 $mmol^{-1}s^{-1}$ and 38.25-395.15 $mmol^{-1}s^{-1}$ respectively. Stressed plants had the lowest conductance readings compared to the other two treatments throughout the observation period. In the first sampling week, no water influence was observed amongst the three irrigation treatments. In the second and third week, the control treatment had the highest stomatal conductance which then dropped in the fourth week and picked up again in the fifth and sixth observation week. Significant effect of water stress between the control and stressed plants was only observed in week two, four and five ($p<0.05$). Semi-stressed treatment was neither different from control nor stressed at week four.

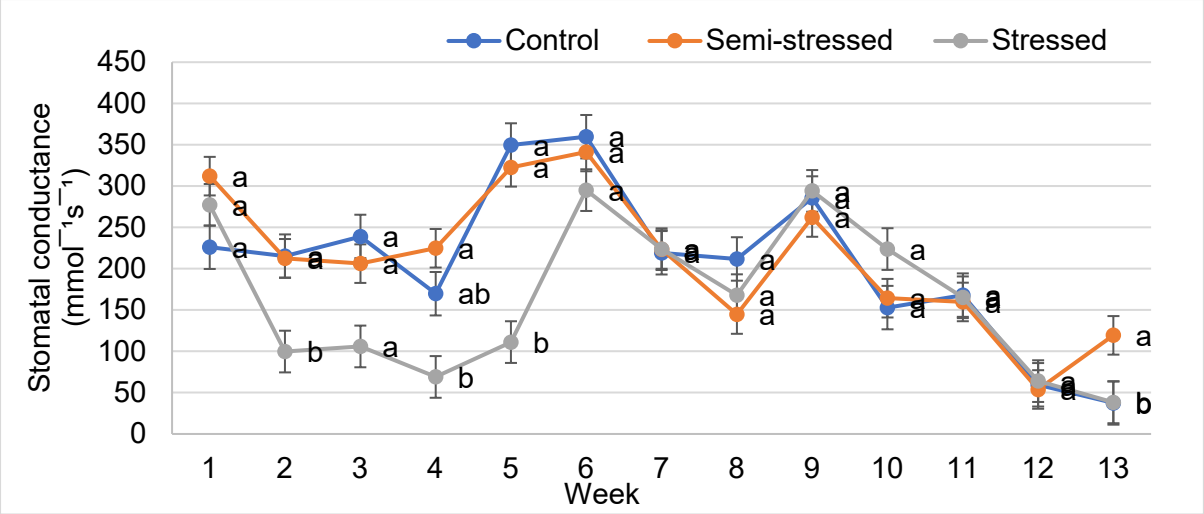


Figure 4.11: Stomatal conductance of *C. subternata* plants as a result different water stress level. Means with the same letter are not significantly different (p≤0.05).

4.5 Discussion

4.5.1 Growth parameters

The results obtained on the growth parameters of *C. subternata* in this study agree with the findings of Tshikhudo *et al*. (2019) who reported that the growth parameters of bush tea increased with increasing rainfall. As a thermophilic evergreen woody species, tea plant is very sensitive to low temperatures which affects its productivity. In response to low temperatures, the plants adapt to the cold by going into dormancy to survive potentially damaging weather conditions (Hao *et al*., 2018). Therefore, the reason why there was generally no significant effect on the growth of *C. subternata* in this study, especially in plant height, may be due to the fact that this trial was carried out in winter. Hence, the winter season and the short duration of the experiment might have contributed to the findings of this study. Water is a crucial element in the environment that plants need to grow and develop, yet both too much and not enough water can cause water stress (Mahajan and Tuteja 2005). Tea plants can withstand a variety of stresses. However, different cultivars have different levels of tolerance (Safaei *et al*., 2019). In this study, only *C. subternata* was investigated. Different morphological, physiological, and molecular responses can be induced in tea plants due to abiotic conditions like drought (Rahimi *et al*., 2018). According to Akhtar and Nazir (2013), plant growth is inhibited by osmotic and water stress. As a result, the amount of cytokinin that moves from the roots to the shoots drops, while abscisic acid levels in the leaves rise. This may explain why water deprived plants in this study continuously had the least growth. The control treatment had the highest stem diameter and stem circumference in all treatments and observation times. Climate can affect the growth and development of a plant (Tshikhudo *et al*., 2018). However, the degree to which the climatic factors (rainfall, relative humidity, and

temperature) affect the growth and development of the plant may vary (Bareja, 2011). These results are comparable to those reported by Tshikhudo *et al*. (2018) on bush tea.

4.5.2 Harvesting and shoots yield

The study aimed at assessing the effect of irrigation at three different levels on the water use efficiency of *C. subternata*. These data (pot experiment) agree with (Habibi, 2018) who found that there was no significant effect on the leaf biomass of Aloe vera under short-term water deficit. However, the findings of this study contrasts Eziz *et al.* (2017) who reported that water availability generally increases plant growth and biomass, and vice versa. According to Zhao *et al*. (2006), a significant decrease in plant biomass was observed in Brassica napus grown under water deficit conditions. The reason for the results in this study may be influenced by the accumulation of proline as a defense mechanism against drought. The short observation period might also have contributed to the results. A plant's water status determines its growth and development, and therefore its productivity in agricultural systems, and its survival in natural systems (Gimenez *et al*., 2013). The soil water content in the experimental orchard was measured using the neutron scattering technique as often as logistically possible. Soil water content plays a huge role in dictating agronomic, geological, and biological characteristics of the soil mass (Su *et al*., 2014) and important for plant growth (Baver, 1956). The soil becomes increasingly dry as soil water potential decreases, resulting in a decrease in available soil water for root absorption. Cell enlargement is directly linked to the level of cell turgor, while photosynthesis, on the other hand, is directly inhibited by the lack of water in the cells (Gimenez *et al*., 2013). This may be the reason why the most water stressed plants in this study had the least yield.

4.5.3 Relative water content (RWC)

Other studies on olives, potatoes and *C. subternata* came to similar conclusions as this study, where water-stressed plants had the lowest RWC values (Boussadia et al., 2008; Soltys-Kalina *et al*., 2016; Mabizela, 2020). The ability of a plant to tolerate water stress depends on several factors, including its morphology, physiology and biochemistry (Soltys-Kalina *et al*., 2016). Water stress and its effects on plant metabolites using RWC as a guide, give us an insight into the internal water relations of honeybush plants. Therefore, the slight decrease in RWC in plants irrigated twice a week may indicate that *C. subternata* has tolerance for mild to moderate water deficit stress. Drought tolerance can be enhanced by understanding how plants respond to water stress (Deikman *et al*. 2012; Juenger, 2013). According to Soltys-Kalina *et al*. (2016), although many physiological and biochemical processes are disturbed during stressful water conditions, plants' protoplasts have evolved the acclimatisation and

adaptation mechanisms. All three diverse irrigation treatments in this study (field) had a significant effect ($p<0.05$) on the plants, thus, significantly different from each other. M'barki *et al*., 2019 reported similar results on the cultivar dependent impact of soil amendment with water retaining polymer on olive under two water regimes. According to their research, there is a significant decline in the RWC with the reduction in water availability. Due to these changes, RWC may be used as a tool to identify the level of water stress in honeybush plants. Even though water constitutes the largest component of plants, its volume within the plant is very small compared to the total volume transpired. Water status for plants is determined by root uptake of soil water, which is in balance with the atmospheric demand. Water deficits can occur when the plant cannot absorb enough water (Gimenez *et al*., 2013). The RWC method allows us to compare the effects of water stress on plant metabolites and to gain a better understanding of the internal water relations within honeybush. The RWC method allows us to compare the effects of water stress on plant metabolites and to gain a better understanding of the internal water relations within honeybush plant. Using this method can be effective in both crop improvement programs and drought-stressed honeybush cultivation.

4.5.4 Proline

Proline accumulation can occur in plants regardless of stress or non-stress conditions but is relatively low under optimal irrigation conditions (Kavi Kishor *et al*., 2015). In this study, an increase in proline content in *C. subternata* plants indicates higher water stress and vice-versa. In general, as the irrigation treatments progressed, the proline concentration in the control treatment appears to be decreasing while that of the plants under less frequent irrigation appears to be increasing with time. Mabizela (2020) reported similar results in *C. subternata*, where there were massive and slight increases in proline contents in the stressed and semi-stressed treatments respectively. Although some authors question the link between proline accumulation and stress adaptation, it is generally accepted that plant cells benefit from an increase in proline content after injury (Mattioli *et al*., 2009). Various environmental stresses trigger the cytosol to synthesize L-proline and accumulate it in the chloroplasts of plants. Plants accumulate L-proline because of osmotic stress caused by salinity, drought, and other abiotic factors (Meena *et al*., 2019). There are multiple enzymes that participate in the pathways involved in the synthesis and degradation of proline. Plant chloroplasts and cytoplasm synthesize proline, while mitochondria degrade it (Szepesi *et al*., 2018). According to Shinde *et al*. (2016), mutants with altered lipid metabolism accumulate more proline under stress, proline dehydrogenase can load electrons into the mitochondrial electron transport chain regulating the cellular redox state. This increases proline accumulation under stress. In this study, proline accumulation may be an adaptive response to water stress to reduce cell damage and improve survival of the *C. subternata* plants.

4.5.5 Stomatal conductance

According to a study by Atteya (2003), drier soils resulted in lower stomatal conductance. As noted by Makbul *et al*., (2011), water stress also decreased stomatal conductance in another study of soybean, in which a 42% decrease in stomatal conductance was observed in drought-stressed leaves compared to non-stressed leaves. Another study reported that, soybean leaves adjust their stomatal conductance to maximize water retention during an extended drought, to prevent losing excessive water (Ku *et al*., 2013). Stomatal closure due to water stress in *C. subternata* leaves resulted in lower stomatal conductance. To ensure survival, the *C. subternata* plants under water stress in this study showed a progressive decline in stomatal conductance, as water deficit stress intensified. The decrease in stomatal conductance in the stressed plants, especially towards the end of the study is an indication of vapor pressure deficit. Increases in vapor pressure deficit between leaf and air lead to the partial closure of stomata, thus, decreasing stomatal conductance to prevent excessive dehydration and physiological damage (Mofokeng *et al*., 2015). Stressed plants had the lowest conductance readings compared to the other two treatments in most observation period. These results are supported by Hernandez-Santana *et al*. (2016) who reported that one of the primary responses of fruit trees to water shortages is a decrease in stomatal conductance. A leaf's water status and atmospheric evaporative demand dictate stomatal opening primarily through light conditions and low CO_2 partial pressure in substomatal cavities. When stomata are fully open during daylight, maximum CO_2 assimilation occurs, resulting in optimal photosynthesis (Gimenez *et al*., 2013). This may be the reason why the least irrigated plants in this study consistently had the lowest values. For improved honeybush irrigation management, it is crucial to gain a thorough understanding of the mechanisms that govern the stomatal closure process in response to imposed water stress.

4.6 Conclusion

Although the irrigation treatments had no significant effect on the overall growth of the *C. subternata* plants, the percentage growth was significantly higher in the well-watered plants compared to the stressed plant populations, indicating better growth and development. Proline concentrations were significantly higher in plants receiving less water than in well-watered plants, which is an indication of water stress. Higher RWC was found in the well-watered plants, followed by plants watered twice a week while the least RWC was observed in the most stressed plants. This proved that proline concentration increases with decreasing RWC and vice versa. Stomatal conductance of plants in this study generally increased with increasing watering frequency and vice versa. The honeybush industry is unable to meet the

huge foreign demand that outweighs local supply. Therefore, the cultivation of *Cyclopia* species would not only guarantee the species' sustainability and conservation but will also enhance the standard of living for rural harvesters as well as commercial growers. Irrigation guidelines are critical for this crop as *Cyclopia* species is a niche tea produce.

REFERENCES

Ábrahám, E., Hourton-Cabassa, C., Erdei, L. and Szabados, L., 2010. Methods for determination of proline in plants. In Plant stress tolerance (pp. 317-331). *Humana Press*.

Akhtar, I. and Nazir, N., 2013. Effect of waterlogging and drought stress in plants. International Journal of water resources and environmental sciences, 2(2), pp.34-40.

Atkinson, N.J. and Urwin, P.E., 2012. The interaction of plant biotic and abiotic stresses: from genes to the field. *Journal of experimental botany*, 63(10), 3523-3543.

Atteya, A.M., 2003. Alteration of water relations and yield of corn genotypes in response to drought stress. Bulg. *J. Plant Physiol*, 29(1-2), 63-76.

Atteya, A.M., 2003. Alteration of water relations and yield of corn genotypes in response to drought stress. Bulg. *J. Plant Physiol*, 29(1-2), 63-76.

Bareja, B.G., 2011. Climatic factors can promote or inhibit plant growth and development. Crops Review 2010– 2016.

Baver, L.D., 1956. Soil physics (Vol. 81, No. 4, p. 337). LWW.

Bennie, A.T.P. and Hensley, M., 2001. Maximizing precipitation utilization in dryland agriculture in South Africa—a review. *Journal of Hydrology*, 241(1-2), 124-139.

Bester, C., 2012. A model for commercialisation of honeybush tea, an indigenous crop. In II All Africa Horticulture Congress 1007 (pp. 889-894).

Bester, C., Joubert, M.E. and Joubert, E., 2014. A breeding strategy for South African indigenous herbal teas. In XXIX International Horticultural Congress on Horticulture: Sustaining Lives, Livelihoods and Landscapes (IHC2014): 1127 (pp. 15-22).

Blum, A., 2011. Plant water relations, plant stress and plant production. In Plant breeding for water-limited environments (pp. 11-52). Springer, New York, NY.

Boussadia, O., Mariem, F.B., Mechri, B., Boussetta, W., Braham, M. and El Hadj, S.B., 2008. Response to drought of two olive tree cultivars (cv Koroneki and Meski). *Scientia Horticulturae*, 116(4), 388-393.

Brilli, F., Pollastri, S., Raio, A., Baraldi, R., Neri, L., Bartolini, P., Podda, A., Loreto, F., Maserti, B.E. and Balestrini, R., 2019. Root colonization by Pseudomonas chlororaphis primes tomato (*Lycopersicum esculentum*) plants for enhanced tolerance to water stress. *Journal of plant physiology*, *232*, pp.82-93.

Chowdhury, J.A., Karim, M.A., Khaliq, Q.A., Ahmed, A.U. and Khan, M.S.A., 2016. Effect of drought stress on gas exchange characteristics of four soybean genotypes. *Bangladesh Journal of Agricultural Research*, 41(2), 195-205.

Deikman, J., Petracek, M. and Heard, J.E., 2012. Drought tolerance through biotechnology: improving translation from the laboratory to farmers' fields. Current opinion in biotechnology, 23(2), pp.243-250.

Department Of Agriculture, Forestry & Fisheries (Daff). 2016. Honeybush tea, production guidelines. [Online]. Available from: https://www.dalrrd.gov.za/Portals/0/Brochures%20and%20Production%20guidelines/Honeybush%20tea%20production%20guideline.pdf [Accessed: 01/05/2022].

Eziz, A., Yan, Z., Tian, D., Han, W., Tang, Z. and Fang, J., 2017. Drought effect on plant biomass allocation: A meta-analysis. *Ecology and evolution*, 7(24), 11002-11010.

Flexas, J., Diaz-Espejo, A., Gago, J., Gallé, A., Galmés, J., Gulías, J. and Medrano, H., 2014. Photosynthetic limitations in Mediterranean plants: a review. *Environmental and Experimental Botany*, 103, 12-23.

Gimenez, C., Gallardo, M. and Thompson, R.B., 2013. Plant–water relations.

Habibi, G., 2018. Effects of mild and severe drought stress on the biomass, phenolic compounds production and photochemical activity of *Aloe vera (L.)* Burm. f. *Acta Agriculturae Slovenica*, 111(2), 463-476.

Hao, X., Wang, L., Zeng, J., Yang, Y. and Wang, X., 2018. Response and adaptation mechanisms of tea plant to low-temperature stress. In Stress physiology of tea in the face of climate change (pp. 39-61). Springer, Singapore.

Harb, A., Krishnan, A., Ambavaram, M.M. and Pereira, A., 2010. Molecular and physiological analysis of drought stress in Arabidopsis reveals early responses leading to acclimation in plant growth. *Plant physiology*, 154(3), 1254-1271.

Hernandez-Santana, V., Rodriguez-Dominguez, C.M., Fernández, J.E. and Diaz-Espejo, A., 2016. Role of leaf hydraulic conductance in the regulation of stomatal conductance in almond and olive in response to water stress. Tree Physiology, 36(6), pp.725-735. https://doi.org/10.21769/bioprotoc.1451

Joubert, E., De Beer, D., Malherbe, C.J., Muller, M., Louw, A. and Gelderblom, W.C.A., 2019. Formal honeybush tea industry reaches 20-year milestone–progress of product research targeting phenolic composition, quality and bioactivity. *South African Journal of Botany*, 127, 58-79

Joubert, E., Gelderblom, W.C.A., Louw, A. and de Beer, D., 2008. South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phylicoides*—A review. *Journal of ethnopharmacology*, 119(3), 376-412.

Joubert, E., Joubert, M.E., Bester, C., De Beer, D. and De Lange, J.H., 2011. Honeybush (*Cyclopia* spp.): From local cottage industry to global markets—The catalytic and supporting role of research. *South African Journal of Botany*, 77(4), 887-907.

Juenger, T.E., 2013. Natural variation and genetic constraints on drought tolerance. Current opinion in plant biology, 16(3), pp.274-281.

Kavi Kishor, P.B., Hima Kumari, P., Sunita, M.S.L. and Sreenivasulu, N., 2015. Role of proline in cell wall synthesis and plant development and its implications in plant ontogeny. *Frontiers in Plant Science*, 6, 544.

Krasensky, J. and Jonak, C., 2012. Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *Journal of experimental botany*, 63(4), 1593-1608.

Ku, Y.S., Au-Yeung, W.K., Yung, Y.L., Li, M.W., Wen, C.Q., Liu, X. and Lam, H.M., 2013. Drought stress and tolerance in soybean. A comprehensive survey of international soybean research—Genetics, physiology, agronomy and nitrogen relationships, 209-237.

Lionello, P., Malanotte-Rizzoli, P., Boscolo, R., Alpert, P., Artale, V., Li, L., Luterbacher, J., May, W., Trigo, R., Tsimplis, M., Ulbrich, U. and Xoplaki, E., 2006. Mediterranean climate, an overview of the main characteristics and issues.

Little T.M and Hills F.J., 1972. Statistical Methods in Agricultural Experimentation. University of California, Davis. California 95616, pp 93-101.

Lugojan, C. and Ciulca, S., 2011. Evaluation of relative water content in winter wheat. *Journal of Horticulture, Forestry and Biotechnology*, 15(2), 173-177.

M'barki, N., Aissaoui, F., Chehab, H., Dabbaghi, O., del Giudice, T., Boujnah, D. and Mechri, B., 2019. Cultivar dependent impact of soil amendment with water retaining polymer on olive (*Olea europaea* L.) under two water regimes. *Agricultural Water Management*, *216*, pp.70-75.

Mabizela, G.S., 2020. Metabolic and quality profiling of *Cyclopia subternata* and *C. Genistoides* in response to seasonal variation and drought stress. Doctoral dissertation, Tshwane University of Technology.

Mahajan, S. and Tuteja, N., 2005. Cold, salinity and drought stresses: an overview. Archives of biochemistry and biophysics, 444(2), pp.139-158.

Makbul, S., Saruhan Güler, N., Durmuş, N. and Güven, S., 2011. Changes in anatomical and physiological parameters of soybean under drought stress [Kuraklık stresi altındaki soya fasülyesinin anatomik ve fizyolojik parametrelerindeki değişimler].

Marnewick, J.L., Gelderblom, W.C. and Joubert, E., 2000. An investigation on the antimutagenic properties of South African herbal teas. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 471(1-2), 157-166.

Martorell Lliteras, S., 2014. Understanding the regulation of leaf and plant gas exchange under water stress with a process-based model of stomatal conductance.

Mattioli, R., Falasca, G., Sabatini, S., Altamura, M.M., Costantino, P. and Trovato, M., 2009. The proline biosynthetic genes P5CS1 and P5CS2 play overlapping roles in Arabidopsis flower transition but not in embryo development. *Physiologia Plantarum*, 137(1), pp.72-85.

Meena, M., Divyanshu, K., Kumar, S., Swapnil, P., Zehra, A., Shukla, V., Yadav, M. and Upadhyay. R.S., 2019. Regulation of L-proline biosynthesis, signal transduction, transport, accumulation and its vital role in plants during variable environmental conditions. Heliyon 5 (12) p.e02952.

Mofokeng, M. M., Steyn, J. M., Du Plooy, C. P., Prinsloo, G., and Araya, H. T., 2015. Growth of *Pelargonium sidoides DC*. in response to water and nitrogen level. *South African Journal of Botany*, 100, 183-189.

Ott, R.L.,1998. An Introduction to Statistical methods and data analysis. Belmont, California: *Duxbury Press*: pp 807-837 (pp 1-1051)

Rahimi, M., Mojtaba, K, and Mojtaba, M., 2019. Evaluation of tea (*Camellia sinensis L.*) biochemical traits in normal and drought stress conditions to identify drought tolerant clones. Physiology and Molecular Biology of Plants.

Sade, N., Galkin, E. and Moshelion, M., 2015. Measuring Arabidopsis, tomato and barley leaf relative water content (RWC). *Bio-protocol* 5(8): e1451.

Safaei C.S., Roofigari H.S., Marzvan, S. and Azadi, R., 2019. Responses of some Iranian tea *[Camellia sinensis* (*L.*) O. Kuntze] clones to drought stress. Crop Breeding Journal, 9(2), pp.45-60.

Shapiro, S.S. and Wilk, M.B., 1965. An analysis of variance test for normality (complete samples). *Biometrika*, 52(3/4), 591-611.

Sharma, H.S., Feng, L., Muresanu, D.F., Castellani, R.J. and Sharma, A., 2019. Neuroprotective effects of a potent bradykinin B2 receptor antagonist HOE-140 on microvascular permeability, blood flow disturbances, edema formation, cell injury and nitric oxide synthase upregulation following trauma to the spinal cord. *In International review of neurobiology* (Vol. 146, pp. 103-152). Academic Press.

Shinde, S., Villamor, J.G., Lin, W., Sharma, S. and Verslues, P.E., 2016. Proline coordination with fatty acid synthesis and redox metabolism of chloroplast and mitochondria. Plant Physiology, 172(2), pp.1074-1088.

Soltys-Kalina, D., Plich, J., Strzelczyk-Żyta, D., Śliwka, J. and Marczewski, W., 2016. The effect of drought stress on the leaf relative water content and tuber yield of a half-sib family of 'Katahdin'-derived potato cultivars. *Breeding science*, 66(2), 328-331.

Soni, R.P., Katoch, M., Kumar, A., Ladohiya, R. and Verma, P., 2015. Tea: production, composition, consumption and its potential as an antioxidant and antimicrobial

agent. *International Journal of Food and Fermentation Technology*, 5(2), 95-106.

SU, S.L., Singh, D.N. and Baghini, M.S., 2014. A critical review of soil moisture measurement. Measurement, 54, pp.92-105.

Surendar, K.K., Devi, D.D., Ravi, I., Jeyakumar, P., and Velayudham, K., 2013. Water stress affects plant relative water content, soluble protein, total chlorophyll content and yield of ratoon banana. International Journal of Horticulture, 3.

Szepesi, Á. and Szőllősi, R., 2018. Mechanism of proline biosynthesis and role of proline metabolism enzymes under environmental stress in plants. In Plant metabolites and regulation under environmental stress (pp. 337-353). Academic Press.

Szepesi, Á. and Szőllősi, R., 2018. Mechanism of proline biosynthesis and role of proline metabolism enzymes under environmental stress in plants. In Plant metabolites and regulation under environmental stress (pp. 337-353). Academic Press.

Tshikhudo, P.P., Ntushelo, K., Kanu, S.A. and Mudau, F.N., 2019. Growth response of bush tea (*Athrixia phylocoides* DC.) to climatic conditions in Limpopo Province, South Africa. *South African Journal of Botany*, 121, 500-504.

Van Wyk, B.E. and Gericke, N., 2000. People's plants: A guide to useful plants of Southern Africa. Briza publications.

Zhao, T.J., Sun, S., Liu, Y., Liu, J.M., Liu, Q., Yan, Y.B. and Zhou, H.M., 2006. Regulating the drought-responsive element (DRE)-mediated signaling pathway by synergic functions of trans-active and trans-inactive DRE binding factors in Brassica napus. *Journal of Biological Chemistry*, 281(16), 10752-10759.

CHAPTER FIVE

ANALYSIS OF THE DIFFERENTIALLY EXPRESSED PROTEINS AND METABOLIC PATHWAYS OF HONEYBUSH (*CYCLOPIA SUBTERNATA*) IN RESPONSE TO WATER DEFICIT STRESS

(This chapter has been published in *Plants* 2023, 12, 2181. https://doi.org/10.3390/plants12112181 . Please refer to appendix 3).

Abstract: Honeybush (*Cyclopia* spp.) is a rich source of antioxidant properties and phenolic compounds. Water availability plays a crucial role in plant metabolic processes, and it contributes to overall quality. Thus, this study aimed to investigate changes in molecular functions, cellular components, and biological processes of *Cyclopia* subternata exposed to different water stress conditions, which include well-watered (as Control, T1), semi-water stressed (T2), and water deprived (T3) potted plants. Samples were also collected from a well-watered commercial farm first cultivated in 2013 (T13) and then cultivated in 2017 (T17) and 2019 (T19). Differentially expressed proteins extracted from *C. subternata* leaves were identified using LC-MS/MS spectrometry. A total of 11 differentially expressed proteins (DEPs) were identified using Fisher's exact test ($p < 0.00100$). Only α-glucan phosphorylase was found to be statistically common between T17 and T19 ($p < 0.00100$). Notably, α-glucan phosphorylase was upregulated in the older vegetation (T17) and downregulated in T19 by 1.41-fold. This result suggests that α-glucan phosphorylase was needed in T17 to support the metabolic pathway. In T19, five DEPs were upregulated, while the other six were downregulated. Based on gene ontology, the DEPs in the stressed plant were associated with cellular and metabolic processes, response to stimulus, binding, catalytic activity, and cellular anatomical entity. Differentially expressed proteins were clustered based on the Kyoto Encyclopedia of Genes and Genomes (KEGG), and sequences were linked to metabolic pathways via enzyme code and KEGG ortholog. Most proteins were involved in photosynthesis, phenylpropanoid biosynthesis, thiamine, and purine metabolism. This study revealed the presence of trans-cinnamate 4-monooxygenase, an intermediate for the biosynthesis of a large number of substances, such as phenylpropanoids and flavonoids.

Keywords: *Cyclopia subternata*; differentially expressed proteins (DEPs); water deficit stress; carbon fixation; proteomic analysis

5.1. Introduction

Honeybush is the general term used to classify all known types of *Cyclopia* species, a genus in the leguminous family (Schutte *et al*., 1998). It is native to the fynbos biome in the Western and Eastern Cape Provinces of South Africa, and its tea has a sweet, honey-like taste (Ntlhokwe, 2016). *Cyclopia* species are among the few wild plants that have been turned into commercial commodities in South Africa. Although 23 species grow in the wild, only a few are currently used to make tea, including *C. subternata, C. intermedia, C. genistoides, C. maculata, C. longifolia*, and *C. sessiliflora* (Mabizela, 2020; Koen *et al*., 2020). The popularity of honeybush can also be attributed to its low tannin content, the absence of caffeine, and the presence of antioxidants. The tea from honeybush is usually enjoyed in its fermented state, although the unfermented (green) one is also marketed (Le Roux *et al*., 2012). These herbal teas are famous for their rich organic antioxidant properties and phenolic compounds, which are helpful in treating colon, throat, and lung ailments, heartburn, ulcers, nausea, and urinary tract infections (Soni *et al*., 2015; Joubert *et al*., 2019). Polyphenols in the unfermented honeybush tea have been broadly perceived as having anti-cancerous properties (Jankun *et al*., 1997; Kanwar *et al*., 2012). Increasing awareness of the health benefits of *Cyclopia* species is one of the factors contributing to the market growth of the tea. Extracts of honeybush teas are also utilized in food and other aesthetic products (Joubert *et al*., 2011). During the last two decades, the export of South African honeybush has expanded due to an increase in demand. It is estimated that about 90% of the honeybush tea crop is exported, with the largest share to the Netherlands (44%), Germany (30%), United Kingdom (8%), and the United States (7%), while the remaining percentage is packaged for local consumption in South Africa (McGregor, 2017; Karsen *et al*., 2022).

Plants are continually exposed to biotic and abiotic stresses in their natural and agricultural environments, which can threaten their survival and growth (Zipfel and Oldroyd, 2017). Stressful conditions could result in delayed seed germination, reduced plant growth, and lower crop yield (Komatsu *et al*., 2013; Zahid *et al*.,2 023). Furthermore, a water deficit could inhibit essential processes such as photosynthesis, respiration, and transpiration, thus restraining the growth and development of plants (Henry and Krutz, 2016; Farooqi *et al*., 2020). Lack of water can alter plant chemistry and negatively influence their growth by disrupting metabolic processes and pigment formation, thereby decreasing photosynthesis, and thus preventing food production (Metwaly *et al*., 2022; Farouk and Al-Huqail, 2020; Farouk and Al-Ghamdi, 2021). Heat and water stress have the potential to compromise sustainable crop production on a global scale (Mittler, 2006; Zhou *et al*., 2017).

Plants can be affected by water stress in two different ways, drought (too little water) or waterlogging (too much water), which reduce oxygen in the soil and impair nutrient uptake

(Osakabe *et al*., 2014; Wojtyla *et al*., 2020). There are numerous molecular programs in plants such as drought avoidance, drought tolerance, drought escape, proline accumulation, synthesis of proteomes, accumulation of osmolytes, and closure of stomatal conductance, which have evolved in response to these changes in available water. These molecular programs quickly detect and adapt to environmental changes and other stresses (Mabizela, 2020; Belay *et al*., 2022; dos Santos *et al*., 2022). Plant proteins play a crucial role in both biotic and abiotic stress responses because they regulate physiological characteristics directly to adapt to changes in the environment. They are also crucial executors of cellular mechanisms and key players in cellular homeostasis (Liu *et al*., 2019). The scarcity of genomic information has hampered crop proteomics applications. However, within the past decade and due to the advancement in analytical tools, the use of proteomics tools for crop plant analysis has amplified drastically. With the successful development of “next generation” sequencing technology, the identification and annotation of proteins and their isoforms in a specific crop species are becoming considerably easier (Komatsu *et al*., 2013).

Proteomics analysis aims to provide an inclusive profile of various proteins found in a specified organism in response to different biotic or abiotic stresses (Mabizela, 2020; Jorrin-Novo *et al*., 2019; Chandrasekhar *et al*., 2014). Plant resistance mechanisms (Liu *et al*., 2019; Lyu *et al*., 2020; Ma *et al*., 2020), responses to water stress (Wang *et al*., 2018; Katam *et al*., 2020; Haddoudi *et al*., 2021) and other exogenous stimuli (Gokul *et al*., 2021) have been investigated using proteomics tools in several studies. For example, Haddoudi *et al*. (2021) investigated the morpho-physiological, biochemical, and molecular responses to water deficit stress in four contrasting *Medicago truncatula* lines (TN6.18, JA17, TN1.11, and A10). The study showed that line TN6.18 was most resistant to water deficit stress with the highest root biomass production, a significantly higher increase in soluble sugar and its total protein contents, and lower levels of lipid peroxidation with greater cell membrane integrity. Furthermore, RT-qPCR revealed that the DREB1B gene had a higher induction rate in roots of TN6.18 and JA17 than in A10 roots. The authors suggested that DREB1B plays a key role in the water deficit tolerance of *M. truncatula*. However, there is a limited understanding of how potted and commercially farmed *C. subternata* plants would respond to induced/natural water stress at the proteomic level. Therefore, the aim of the present study was to compare the effect of water deficit stress on molecular functions, cellular components, and biological processes of potted and commercially farmed *C. subternata* plants. This study provides insights into the proteomic basis of drought tolerance mechanisms mediated by the key regulators.

5.2 Materials and Methods

5.2.1 Plant Material and Sample Collection

To assess the effect of water deficit stress, a full-scale experiment was set up with potted *C. subternata* plants in a controlled glasshouse at the Agricultural Research Council (ARC), Infruitec, Stellenbosch (−33.925920°, 18.874259°), South Africa. The experiment was conducted for 112 days (from mid-May until early September 2021). All plants were watered uniformly with 300 mL of water for the first 81 days after transplanting (DAT) to ensure uniformity and strong root growth before treatment application. Thereafter, *C. subternata* plants were subjected to three irrigation treatments (from early August to early September 2021) until the study was terminated at 112 DAT. The watering cycles were irrigating thrice (3 days/week), twice (2 days/week), and once (1 day/week), which is described further as T1 = Nietvoorbij well-watered plant sample (Control), T2 = Nietvoorbij semi-stressed plant sample, and T3 = Nietvoorbij water-deprived plant sample (Figure 5.1). At every irrigation time, 300 mL of water was applied per pot. Each treatment was replicated eight times. The water needed per potted plant (300 mL) was based on extensive in-house preliminary trials.

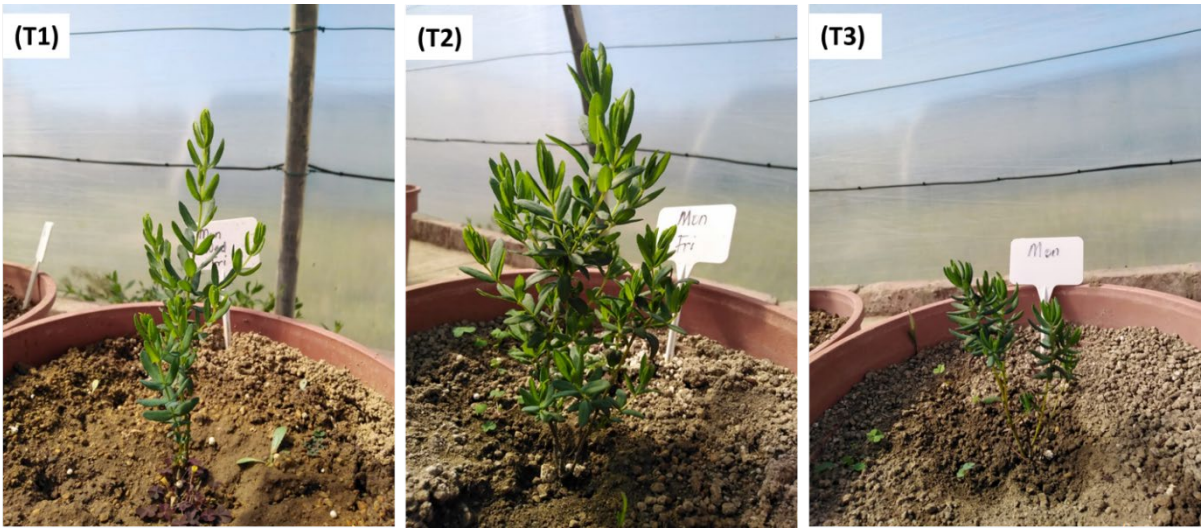


Figure 5.1: Photos of irrigated and stressed *Cyclopia subnernata*, (T1) = Nietvoorbij well-watered plant sample (3 days/week, Control), (T2) = Nietvoorbij semi-stressed plant sample (watered 2 days/week), and (T3) = Nietvoorbij water-deprived plant sample (watered 1 day/week).

Under semi-/mild-stressed water conditions, plants showed early vigor or maturity development (Figure 5.1, T2). Under this water stress condition, the plant undergoes low evapotranspiration to optimize water use efficiency and limit the loss of water due to direct evaporation from the soil surface. The early vigor enables water to be stored and made available later for developmental processes as the soil water becomes gradually depleted (Tuberosa, 2012). Furthermore, according to Seleiman *et al*. (2021), plants have developed

diverse adaptive strategies through evolution that make them more tolerant to the adverse effects of water stress. This survival strategy includes drought stress escape, avoidance, and tolerance approach. Based on the observed morphological changes in the semi-stressed *C. subternata,* escape and tolerance water stress survival strategy responses could be hypothesized. However, further investigation would be needed to validate this hypothesis.

Furthermore, the glasshouse experiment was compared to a commercially managed honeybush farm (Napier farm in Cape Agulhas, South Africa). The commercial farm was managed under good agricultural practice (GAP) with regular irrigation regimes, and the plants were never under any water stress. Samples were collected from the farm in May 2021, and batches were collected based on the year the honeybush was cultivated between 2013 and 2019; T13 = Napier well-watered plants cultivated in 2013, T17 = Napier well-watered plants cultivated in 2017, and T19 = Napier well-watered plants cultivated in 2017. Leaf samples were collected into pre-marked centrifuge tubes and immediately stored in liquid nitrogen. Samples were immediately sent to the Proteomics Research & Services Unit of the University of the Western Cape for protein extraction, 1D- SDS PAGE analysis, HILIC digestion, and other analysis.

5.2.2 Sample Preparation

Frozen samples were ground to a fine powder in liquid nitrogen using a mortar and pestle. Ground samples were stored at −20 °C until used. Ground tissue (0.5 g) containing 0.025 g polyvinylpolypyrrolidone (PVPP) was resuspended in 2 mL of 10% TCA/acetone. Polyvinylpolypyrrolidone (PVPP) has a high capacity to bind to polyphenols, and it is effective for the removal of phenolic impurities from plant tissue extracts (Ranatunge *et al*., 2017). The samples were thoroughly vortexed for 30 s and centrifuged at 16,000× *g* for 3 min at 4 °C. The resultant pellet was further rinsed with 2 mL of cold acetone and centrifuged for 3 min at 16,000× *g* at 4 °C. Acetone rinses were repeated until a white pellet was obtained. The protein pellet was air-dried at room temperature and further used for protein extraction.

5.2.3 Protein Extraction and Pellet Solubilization

Proteins were extracted using the phenol/SDS extraction protocol described previously by Wang *et al*. (2006) with slight modifications. The protein pellet was resuspended in 0.7 mL SDS extraction buffer (30% sucrose, 2% SDS, 100 mM Tris-HCl, pH 8.0, 2.5% 2-mercaptoethanol, 1 mM PMSF) and 0.7 mL phenol (Tris-buffered, pH 8.0; Sigma-Adrich, St. Louis, MO, USA). The mixture was vortexed for 20 min and the phenol phase was separated by centrifugation at 16,000× *g* for 15 min at 4 °C. The phenol phase was back-extracted with an equal volume of SDS extraction buffer for 3 min followed by centrifugation at 16,000× *g* for

10 min at 4 °C. At least 5 volumes of cold methanol containing 0.1 M ammonium acetate was added to the phenol phase, and the mixture was stored at −20 °C for 16 h. Precipitated proteins were recovered at 16,000× *g* for 20 min at 4 °C and washed with cold methanol once, followed by two 80% acetone washes. The final pellet was air-dried and dissolved in 100 µL protein solubilization buffer (4 M urea, 2% SDS, 50 mM Tris-HCl, pH 8.0), and the protein concentration was quantified using the Pierce microplate BCA protein assay kit (Thermo Scientific, USA) according to the manufacturer's instructions with bovine serum albumin used as a standard.

5.2.4 Quality Control Using SDS–PAGE Analysis

The purity and quality of the extracted proteins were evaluated using SDS-PAGE analysis. Briefly, proteins (10 µg) were prepared in a 1:3 ratio with 4 *x* Laemmli SDS-PAGE buffer (250 mM Tris-HCl, pH 6.8; 4% SDS; 30% glycerol; 350 mM β-mercaptoethanol; 0.02% bromophenol blue) and boiled at 95 °C for 3 min. The proteins were then resolved according to their molecular weight on 12% polyacrylamide gels under constant 100 V with the aid of the Mini—Protean III® Cell gel casting system (Bio-Rad Laboratories Ltd., Rosebank, Johannesburg, South Africa) until bromophenol blue reached the bottom of the gel. After electrophoresis, proteins were visualized using the Acqua Stain protein gel dye, and the gels were processed using Quantity One software on the Molecular Imager PharosFX Plus System (Bio-Rad Laboratories Ltd., Rosebank, Johannesburg, South Africa).

5.2.5 Protein Pellet Solubilization

All protein pellets were first solubilized in 50 mM Tris containing 2% SDS (Sigma-Adrich, St. Louis, MO, USA) and 4 M urea (Sigma-Adrich, St. Louis, MO, USA) by vortexing for 30 min. Samples were quantified using the Thermo-Fischer BCA kit following the manufacturer's instructions. Approximately 50 µg of protein was aliquoted for trypsin digestion.

5.2.6 On-Bead Digest

All reagents used are of analytical grade or equivalent. Samples were resuspended in 50 mM ammonium bicarbonate (Sigma-Adrich, St. Louis, MO, USA) before reduction with 10 mM dithiothreitol (DTT) (Sigma) for 30 min at room temperature. This step was followed by alkylation with 30 mM iodoacetamide at room temperature in the dark. After the reduction and alkylation of the protein samples, the samples were diluted with an equal volume of binding buffer (200 mM sodium acetate, 30% acetonitrile, pH 4.5). The protein solution was added to MagResyn HILIC magnetic particles (Resyn Biosciences (Pty), Ltd. Gauteng, South Africa) prepared according to the manufacturer's instructions and incubated overnight at 4°C. After binding, the supernatant was removed, and the magnetic particles were washed twice with

washing buffer (95% acetonitrile). After washing, the magnetic particles were suspended in 50 mM ammonium bicarbonate containing trypsin (New England Biolabs®, Ipswish, UK) to a final ratio of 1:50. After overnight incubation at 37°C, the peptides were removed from the beads and collected in a fresh tube. The adsorbed peptides were removed by incubating them for 3 min at room temperature in 20 µL 1% trifluoroacetic acid (TFA). Residual digest reagents were removed using Empore Octadecyl C18 extraction discs (SupelcoTM Analytical, Sigma-Adrich, St. Louis, MO, USA) as the C18 stage tip. The samples were loaded onto the stage tip after methanol (30 µL) equilibrated with 2% acetonitrile: water, 0.05% TFA (30 µL), was used to activate the C18 membrane. The bound sample was washed with 2% acetonitrile: water, 0.1% TFA (30 µL), and thereafter eluted with 50% acetonitrile: water 0.05% TFA (30 µL). The eluate was evaporated to dryness, and the dried peptides were dissolved in 2% acetonitrile: water, 0.1% FA, for LC-MS analysis.

5.2.7 LC–MS/MS Analysis—Dionex Nano-RSLC

The method for LC–MS/MS analysis was adapted from Hooijberg *et al*. (2018). Thermo Scientific Ultimate 3000 RSLC equipped with a 5 mm x 300 µm C18 trap column (Thermo Scientific, USA) and a Charged Surface Hybrid (CSH) 25 cm × 75 µm of a 1.7 µm particle size C18 analytical column (WatersTM, Microsep Pty Ltd., Johannesburg, South Africa) was used for liquid chromatography. The loading solvent system employed was 2 % acetonitrile: water, 0.1% FA; Solvent A: 2% acetonitrile: water, 0.1% FA; and Solvent B: 100% acetonitrile: water. The samples were loaded onto the trap column using a loading solvent at a flow rate of 2 µL/min from a temperature-controlled autosampler set at 7°C. Loading was performed for 5 min before the sample was eluted onto the analytical column. The flow rate was set to 250 nL/min, and the gradient was generated as follows: 5–35% solvent B over 60 min and 35–50% solvent B from 60 to 75 min. Chromatography was performed at 40 °C, and the outflow was delivered to the mass spectrometer through a stainless-steel nano-bore emitter.

5.2.7.1 Mass Spectrometry

Mass spectrometry was performed using a Thermo Scientific Fusion MS fitted with a Nanospray Flex ionization source. The sample was introduced through a stainless-steel emitter. Data were collected in positive mode with spray voltage set to 1.8 kV and ion transfer capillary set to 280°C. Spectra were internally calibrated using polysiloxane ions at m/z = 445.12003 and 371.10024. MS1 scans were performed using the orbitrap detector set at 120,000 resolutions over the scan range 350 to 1650 with an AGC target at 3 E5 and a maximum injection time of 50 min. Data were acquired in profile mode. The MS2 acquisitions were performed using monoisotopic precursor selection for ions with charges +2 to +7 with

error tolerance set to ±10 ppm. Precursor ions were excluded from fragmentation once for a period of 60 s. Precursor ions were selected for fragmentation in HCD mode using the quadrupole mass analyzer with HCD energy set to 30%. Fragment ions were detected in the orbitrap mass analyzer set to 30,000 resolutions. The AGC target was set to 5 E4 and the maximum injection time to 80 min, and data were acquired in centroid mode.

5.2.7.2 MS Data Analysis

The raw files generated via MS were imported into Proteome Discoverer v1.4 (Thermo Scientific, USA) and processed using the Sequest and Amanda algorithms. Database interrogation was performed against a concatenated database created using the Uniprot "Fabaceae-reviewed" (accessed in December 2021). Semi-tryptic cleavage with 2 missed cleavages was allowed. Precursor and fragment mass tolerance was set to 10 ppm and 0.02 Da, respectively. The deamidation (NQ), oxidation (M), and acetylation of protein N-terminal were allowed as dynamic modifications and thiomethyl of C as a static modification. Using the Target-Decoy PSM validator node, the peptides were validated. The search results were imported into Scaffold Q+ for further validation (www.proteomesoftware.com).

5.2.8 Gene Ontology and KEGG Analysis Pipeline

The proteins identified were mapped to Universal Protein Resource (UniProt https://www.uniprot.org/idmapping, accessed 22 August 2022) to assess their function. Functional annotations of differentially abundant proteins (DAPs) were performed using Panther for gene ontology analysis (http://www.pantherdb.org, accessed 17 February 2023). The proteins were classified by Gene Ontology annotation based on three categories: biological process, cellular component and molecular function (Mi *et al*., 2013). Moreover, the DAPs sequences were uploaded into EggNog-Mapper genome-wide functional annotation (http://eggnog-mapper.embl.de, accessed 18 February 2023). Sequences were assigned to various metabolic pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis via EggNog-Mapper (Cantalapiedra *et al*., 2021).

5.2.9 Experimental Design and Statistical Analysis

A full factorial experimental layout design was used in this study. The treatments were set up in a randomized block design (RBD) with three different irrigation treatments. All proteomics analyses were conducted using independent replicates in triplicate (n = 3). To test for normality, the Shapiro–Wilk test was performed. Data obtained were analyzed using ANOVA at $p \leq 0.05$, and mean values were tested according to Tukey's multiple comparison test at $p \leq 0.05$, with F-values considered significant at $p \leq 0.01$. Protein quantitation was performed using Fischer's exact test at ($p < 0.001$) on the paired data with the Benjamini–Hochberg

correction applied. Protein identifications were accepted if they could be established at greater than 95% probability, with a protein threshold of 1% false discovery rate, and contained at least two unique identified peptides.

5.3 Results

5.3.1. 1D-SDS-PAGE of *C. subternata* Protein Samples

One-dimensional gel electrophoresis showing the responses of *C. subternata* protein samples to different water stress levels is presented in Figure 5.2. Protein loading was relatively uniform, and no streaking of proteins was observed in the leaf protein extracts. The information integrates the molecular weight and the amount of protein extract per treatment. Protein bands from all treatments covered a molecular weight range of 10 to 150 kDa. The youngest commercial farm samples (T19) have a missing band between 37 and 50 kDa. In contrast, an extra protein that differentiates T1 and T17 from the other treatments was located between 15 and 20 kDa. Similarly, at 15 kDa, the two water-stressed potted plants had lower band intensity (Figure 5.2).

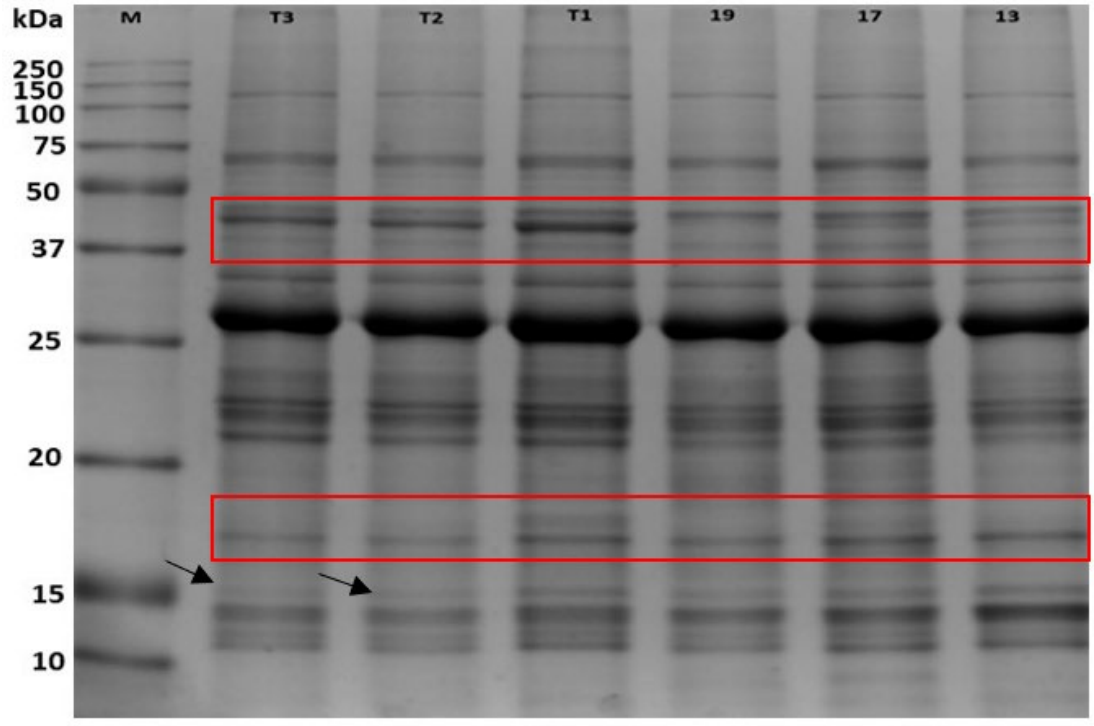


Figure 5.2: A representative one-dimensional electrophoresis gel profile of *Cyclopia subternata* leaf protein in response to different water stress treatments. Approximately 20 _g of protein samples were loaded on a 12% SDS-PAGE electrophoresis gel. M = Molecular weight marker (ladder); T1 = Nietvoorbij well-watered plant sample; T2 = Nietvoorbij semi-stressed plant sample; T3 = Nietvoorbij water-deprived plant sample; and T-19, -17, and -13 = Napier well-

watered plants cultivated in 2019, 2017, and 2013, respectively. The red box and black annotated arrows indicate marked changes in protein intensity.

5.3.2 Identification of Induced Proteins in *C. subternata* Using LC-MS/MS Analysis

Based on known proteins, over 600 proteins were identified across all the treatments; however, after the four filter standards (Fisher's exact test at $p < 0.00100$, peptide threshold (95%), protein threshold (1% false discovery rate (FDR), and a minimum of two peptides) used, the results showed that only 11 differentially expressed proteins (DEPs) were found to be significant ($p < 0.00100$). Table 5.1 presents significantly different proteins identified from stressed and well-watered treatments in *C. subternata* leaves. For example, a total number of 269 proteins were identified from the broad comparison of both well-watered *C. subternata* plants (T17 and T19) that were cultivated commercially. However, only one enzyme (α-glucan phosphorylase) was found to be statistically common between T17 and T19 ($p < 0.00100$) using Fisher's exact test. Notably, the enzyme (α-glucan phosphorylase) was upregulated in T17 (older vegetation) and downregulated in T19 by 1.41-fold. This result suggests that α-glucan phosphorylase was produced less in T19 and more in T17 as needed.

Table 5.1: List of proteins identified from *C. subternata* plants grown under well-watered and water-stressed conditions.

Protein Name	Cellular Component	Molecular Functions	Biological Processes	MW (KDa)	Tax. ID	FET	*FC (log2)	FC	Expression Change
Alpha-glucan phosphorylase, H isozyme	Cytoplasm	Glycogen phosphorylase activity, pyridoxal phosphate binding, SHG alpha-glucan phosphorylase activity, linear malto-oligosaccharide phosphorylase activity	Carbohydrate metabolism	95.9	3906	0.00070	0.5	1.41	Upregulated in **T17** and downregulated in **T19**
Ribulose bisphosphate carboxylase large chain	Plastid, Chloroplast	Magnesium ion binding, monooxygenase activity, ribulose-bisphosphate carboxylase activity	Photorespiration, reductive pentose-phosphate cycle	50	49830	0.00070	1.1	2.14	Upregulated in **T1** and downregulated in **T19**
Trans-cinnamate 4-monooxygenase	Integral component of membrane	Heme binding, iron ion binding, trans-cinnamate 4-monooxygenase activity,	Lignin metabolic process	58	3847	0.00029	0.0	1.00	Downregulated in **T1** and upregulated in **T19**
Probable UDP-arabinopyranose mutase 1	Extracellular region (Secreted, cell wall, cell junction, plasmodesma, Golgi apparatus)	UDP-arabinopyranose mutase activity	Cell wall organization, cellulose biosynthetic process, plant-type cell wall organization or biogenesis, protein glycosylation	-	3888	0.00081	0.1	1.07	Downregulated in **T1** and upregulated in **T19**
Probable cinnamyl alcohol dehydrogenase	Stem, hypocotyl, root tissue	Cinnamyl-alcohol dehydrogenase activity, sinapyl alcohol dehydrogenase activity, zinc ion binding	Lignin biosynthetic process	-	3879	0.0037	0.09	1.06	Downregulated in **T1** and upregulated in **T19**
Ribulose bisphosphate carboxylase large chain	Plastid, Chloroplast	Magnesium ion binding, monooxygenase activity, ribulose-bisphosphate carboxylase activity	Photorespiration, reductive pentose phosphate cycle	50	49830	0.00074	1.2	2.30	Downregulated in **T19** and upregulated in **T3**
Ribulose bisphosphate carboxylase large chain	Plastid, Chloroplast	Magnesium ion binding, monooxygenase activity, ribulose-bisphosphate carboxylase activity	Photorespiration, reductive pentose phosphate cycle	50	49830	0.00081	1.2	2.30	Downregulated in **T19** and upregulated in **T3**

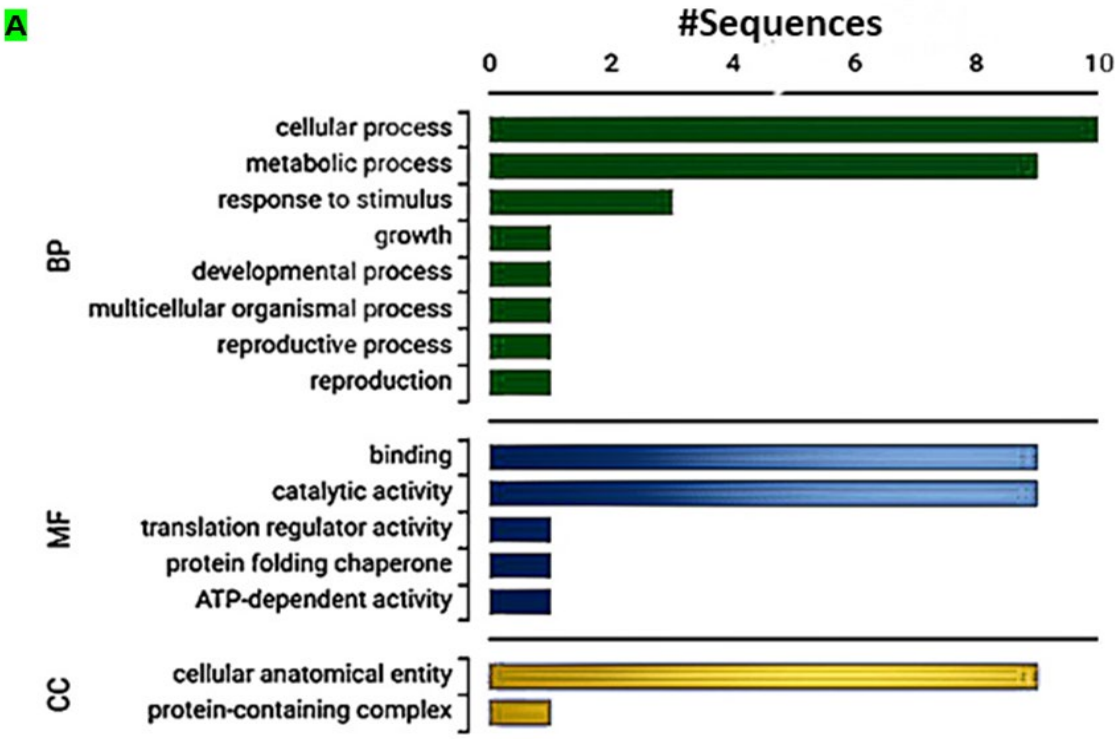
(Continued): List of proteins identified from *C. subternata* plants grown under well-watered and water-stressed conditions.

Protein Name	Cellular Component	Molecular Functions	Biological Processes	MW (kDa)	Tax. ID	FET	*FC (log2)	FC	Expression Change
Ribulose bisphosphate carboxylase large chain	Plastid, Chloroplast	Magnesium ion binding, monooxygenase activity, ribulose-bisphosphate carboxylase activity	Photorespiration, reductive pentose-phosphate cycle	53	49830	0.0015	1.2	2.30	Downregulated in **T19** and upregulated in **T3**
Glutamine synthetase nodule isozyme	Cytoplasm	ATP binding, glutamate-ammonia ligase activity	Glutamine biosynthetic process	39	3918	0.0018	0.7	1.62	Upregulated in **T19** and downregulated in **T3**
Elongation factor 1-alpha	Cytoplasm	GTP binding, translation elongation factor activity, GTPase activity	-	49	3918	0.0037	1.6	3.03	Downregulated in **T19** and upregulated in **T3**
Chlorophyll a-b binding protein, chloroplastic	Chloroplast thylakoid membrane, integral component of membrane, photosystem I, photosystem II	Chlorophyll binding, metal ion binding	Photosynthesis, light harvesting in photosystem I, response to light stimulus	26	3847	0.0041	0.6	1.52	Upregulated in **T19** and downregulated in **T3**

T1 = well-watered Nietvoorbij field plants; T3 = water stressed Nietvoorbij field plants; T17 = well-watered Napier field plants cultivated in 2017; T19 = well-watered Napier field plants cultivated in 2019 (UniProt https://www.uniprot.org, accessed 22 August 2022). * FC = Fold change, FET = Fisher's Exact Test, MW = Molecular weight, Tax. ID = Taxonomic identifier

5.3.3 GO Analysis of Gene Ontology Enrichment

Differentially expressed proteins involved in combined water deficit with heat stress were mapped using Panther for gene ontology analysis (http://www.pantherdb.org, accessed on 22 August 2022). Based on the total number of proteins listed above in Table 5.1, the Panther analysis annotated into three categories of GO distribution by level 1, namely, biological process (BP), molecular function (MF), and cellular component (CC). The proteins involved in these activities include α-glucan phosphorylase, ribulose bisphosphate carboxylase, trans-cinnamate 4-monooxygenase, UDP-arabinopyranose mutase 1, cinnamyl alcohol dehydrogenase, glutamine synthetase nodule isozyme, elongation factor 1-α, and chlorophyll a-b binding protein. This was to improve the understanding of biological functions associated with water stress in *C. subternata*. Figure 5.3A summarizes the gene ontology distribution of proteins obtained from treated (water-stressed) and non-treated (well-watered) *C. subternata* leaf samples.



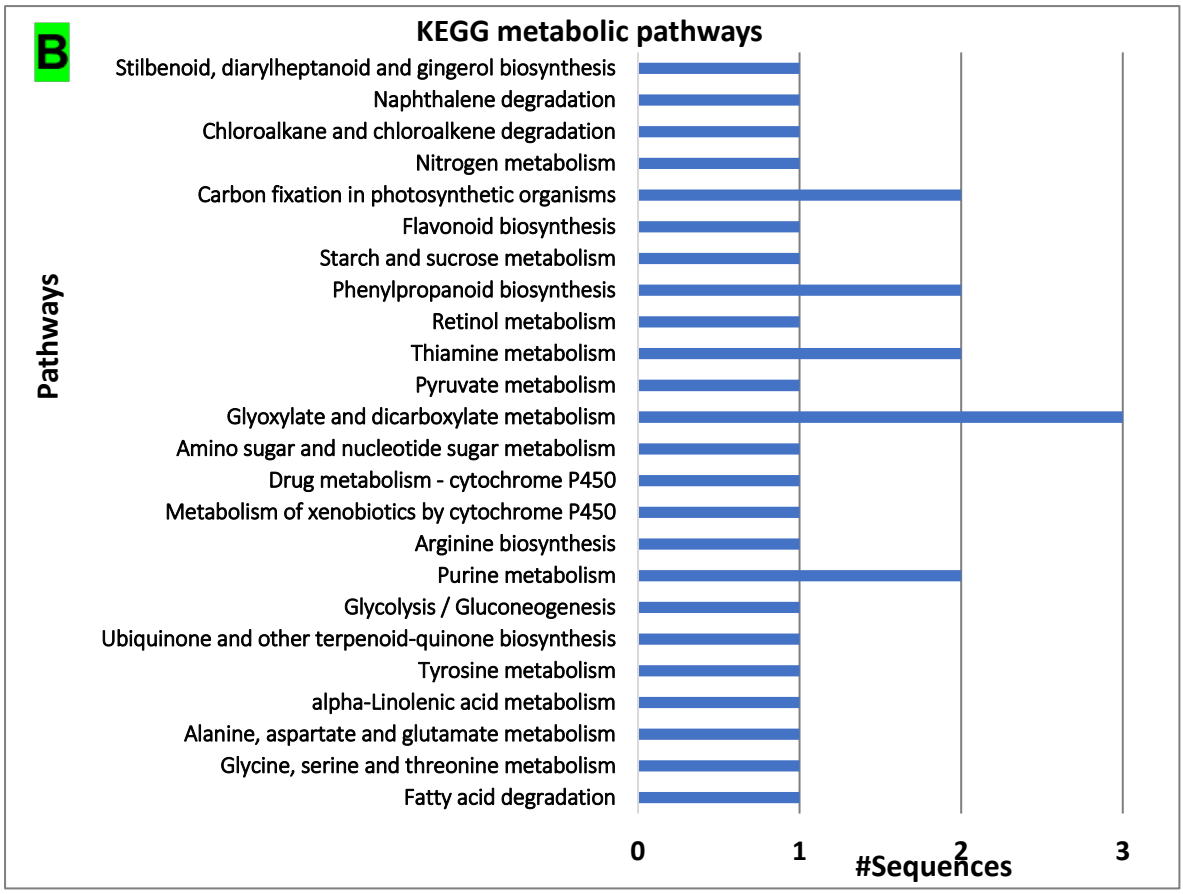


Figure 5.3: (A) Gene ontology (GO) analysis of common (shared) proteins differentially regulated in *Cyclopia subternata* leaf under water deficit stress and regularly watered as determined by Panther (http://www.pantherdb.org, accessed on 22 August 2022) (according to GO distribution by level 1. BP = biological processes; MF = molecular functions; CC = cellular components. (B) An illustration of metabolic pathways linked to differentially expressed proteins in the leaves of *C. subternata* under water deficit stress as well as regularly watered as retrieved by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis via EggNog-Mapper.

5.3.4 Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

Proteins were mapped and assigned to different metabolic pathways according to the KEGG pathway database for a better understanding of the roles of DEPs in *C. subternata* leaves (Figure 5.3B). Sequences were linked to pathways via both enzyme code (EC) and KEGG ortholog (KO). According to the results of this study, a total of 30 sequences were associated with 24 KEGG pathways, where only five were differentially expressed. The KEGG pathway database was used to sort the identified metabolic pathways into major categories.

5.4 Discussion

5.4.1 1D-SDS-PAGE and LC-MS/MS Analysis

Based on protein fragmentation via SDS-PAGE, similar banding patterns or fragmentation with varying abundance or intensity were observed across the different water treatments for *C.*

subternata. However, as indicated by the intensity of the bands, the treated samples from the Nietvoorbij farm glasshouse (T1–T3) had greater protein abundance between 37 and 50 kDa compared with all other treatments (Figure 5.2). In contrast, band intensity at the lower molecular weight range between 10 and 15 kDa was more expressed in commercial farm samples (T13 to T19) than in other treatments. However, the youngest commercial farm samples (T19) have a missing band between 37 to 50 kDa, while T1 had the highest intensity in one of the bands within this range. In contrast, an extra protein that differentiates T1 and T17 from the other treatments was located between 15 and 20 kDa, but the intensity of this band also differed between the treatments. Similarly, at 15 kDa the two water-stressed potted plants had lower band intensity (Figure 5.2). The differences in fragmented protein abundance suggest that there were possible events of either up- or downregulation of various proteins within the molecular weight range (Nsumpi *et al*., 2020).

According to Ubiparip *et al*. (2018), alpha-glucan phosphorylases play an essential role in catalyzing reversible phosphorolysis and storing polysaccharides such as glycogen, starch, and maltodextrins. This could suggest that T17, which is the older plant, is well adapted for the storing of available/excess polysaccharides compared to the younger plants. The reason for the presence of α-glucan phosphorylase in these samples alone may be because they were irrigated at equal rates. Furthermore, the comparison of the total number of proteins identified between well-watered T1, cultivated in the glasshouse, and that well-watered and commercially farmed (T19) was 271. However, only four proteins (i.e., ribulose bisphosphate carboxylase large chain, (RubisCO), trans-cinnamate 4-monooxygenase, probable UDP-arabinopyranose mutase 1, and cinnamyl alcohol dehydrogenase) were found to be significantly common between both samples ($p < 0.00100$). The cluster of one RubisCO large subunit protein was found to be 2.41-fold higher in T1 than in T19. RubisCO is the key enzyme that facilitates the process of carbon fixation in the Calvin cycle. The fold increase under T1 (well-irrigated) could suggest that there was a higher photosynthetic carbon flux in the Calvin cycle under the glasshouse conditions. The results found in this study contradict those in the study by Haworth *et al*. (2018) on olives, where heat stress stimulated a deterioration in photosynthesis with reduced RubisCO activity.

Furthermore, the downregulation of RubisCO subunits under T19 could suggest nitrogen deficiency in the leaf (Kang *et al*., 2022). Abiotic stresses such as drought reduce the rate of photosynthesis by disturbing the cell homeostasis and affecting the photosynthetic pigments, soluble proteins, proteins in thylakoid membranes, the electron transport chain, photophosphorylation, and carbon dioxide (CO2) fixation (Morales *et al*., 2019). During drought conditions, crop growth and yields are seriously impaired, and photosynthesis is

hindered (Wang *et al*., 2022). Based on the results obtained from T1 and T19 protein profiles, the impact of abiotic stress due to water deficit/stress could not be established. Probable cinnamyl alcohol dehydrogenase (CAD) was downregulated in T1 and upregulated in T19 by 1.06-fold. According to reports, cinnamyl alcohol dehydrogenase catalyzes the last stages of the production of monolignol (Mansell *et al*., 1974). Lignins are complex polymers that play a role in the alteration of biofuel and ensure good leaf quality in plants (Liu *et al*., 2018). They also play an essential role in plant defense, mechanical support, and water retention (Chun *et al*., 2019). Different plants have responded differently to CAD downregulation in terms of lignin content (Mansell *et al*., 1974). Therefore, the downregulation of CAD enzyme in this study may suggest that a low amount was needed in T1 (Nietvoorbij well-watered plants) compared to T19 (Napier commercial farm plants). The fold change in probable UDP-arabinopyranose mutase 1 was reported to be 1.07 times lower in T1 than in T19.

Saqid *et al*. (2019) stated that L-Arabinofuranose is an omnipresent component produced from the cytosolic UDP arabinopyranose (UDP-Arap mutase 1). In contrast, trans-cinnamate 4-monooxygenase had a fold change of 1, with T1 being lower and T19 being higher. Trans-cinnamate is a naturally occurring aromatic compound in plants, and it could serve as a central intermediate for the biosynthesis of a large number of substances, such as phenylpropanoids, coumarins, and flavonoids (Otto *et al*., 2019). Enzymes such as trans-cinnamate 4-monooxygenase (CYP73A) were identified in the 'phenylpropanoid biosynthesis' pathway in (Shu *et al*., 2022).

Based on known proteins and the four filter standards used, the results showed that only six differentially expressed proteins (DEPs) were found between the T19 (well-irrigated treatment) and T3 (stressed treatment from Nietvoorbij) groups. This included the four enzymes (three large chain ribulose bisphosphate carboxylase subunits and glutamine synthetase (GS) nodule isozyme) and two proteins (chlorophyll a/b binding protein, chloroplastic and elongation factor 1-alpha). Glutamine synthetase plays an important role in the metabolism of nitrogen by catalysing the reaction of condensation of glutamate and ammonia to form glutamine (van Heeswijk *et al*., 2013). The GS enzyme was downregulated in the stressed treatment (T3) by 1.62-fold compared to the well-watered treatment (T19) in response to water stress in this study. The findings are comparable to those reported by Mabizela (2020) on *C. subternata*. During drought stress, nodule function and the growth of honeybush are directly affected when plants receive less water during summer periods, as honeybush are rain-fed plants. Furthermore, drought decreases the rate of photosynthesis and lowers the level of photosynthates needed by the bacteria for nitrogen fixation (Brink *et al.*, 2017). This finding

may suggest that there was a positive nitrogen metabolism, which may be true as *Cyclopia* species are known to fix their own nitrogen (Bester, 2013; Postma *et al*., 2016).

Chloroplast is responsible for both light and dark reactions during photosynthesis, but it is highly sensitive to various abiotic stresses (Ashraf and Harris, 2013). Drought disrupts cellular homeostasis and affects the photosynthetic pigments, soluble proteins, proteins in the thylakoids membranes, the electron transport chain, photophosphorylation, and carbon dioxide (CO2) fixation, which reduces the rate of photosynthesis. The closure of stomata also decreases photosynthesis, increasing chloroplast and sub-stomatal CO2 concentration and decreasing CO2 assimilation (Morales *et al*., 2019). There was a downregulation of chlorophyll a/b binding proteins in the stressed treatment (T3) compared to T19. Drought causes leaf stomatal closure, which hinders CO2 entry into the mesophyll cells, thus reducing photosynthesis (Xu *et al*., 2016). This is supported by a study conducted by Mabizela (2020), where the amount of chlorophyll a/b-binding proteins in plants increased under drought conditions. In agreement, Benešová *et al*. (2012) reported an increase in chlorophyll a/b-binding protein levels in a tolerant genotype of *Zea mays*, leading to open stomata and efficient transpiration. According to the proteomic findings, *C. subternata's* photosynthesis-related proteins are regulated during drought stress, which may have crucial implications for plant tolerance research.

5.4.2 Gene Ontology Enrichment and KEGG Pathway Analysis

The reported DEPs were mostly found in the biological processes of cellular process, metabolic process, and response to a stimulus. Additionally, the biological processes involved are growth, developmental, multicellular organismal, reproductive, and reproduction processes. In terms of molecular functions, most of the differently expressed proteins identified were involved in binding and catalytic activities in leaves. Some of the observed molecular functions include translation regulator activity, protein folding chaperone, and ATP-dependent activity. In terms of the cellular component category, the most differentially
expressed proteins identified were in the cellular anatomical entity followed by the protein-containing complex. The enriched KEGG pathways in Figure 5.3B demonstrate that the greatest number of enzymes annotated in the leaves of *C. subternata* were linked to the glyoxylate and dicarboxylate metabolism. This may suggest that the activation of this pathway plays a crucial role in the mechanisms of water stress responses in *C. subternata*. Some of the observed metabolic pathways that were also identified in the leaves are carbon fixation in photosynthetic organisms, phenylpropanoid biosynthesis, thiamine metabolism, and purine metabolism.

5.4.3 Regulation of Biosynthesis of Secondary-Metabolites-Related Pathways

5.4.3.1 Phenylpropanoids Pathway

Environmental stresses have the tendency to hinder plant growth and productivity by altering the metabolism of reactive oxygen and nitrogen species (Chaki *et al*., 2020). Plants under water stress experience oxidative stress, which changes how phenylpropanoids, flavonoids, and other secondary metabolites are synthesized. To detoxify ROS, plant cells evolve an antioxidant enzymatic defense system that uses both enzymatic and non-enzymatic antioxidants (Chaki *et al*., 2020). One of the most widely studied metabolic pathways among secondary metabolites is the phenylpropanoids pathway (Biala and Jasiński, 2018). Metabolites from phenylpropanoid pathways are important for plant development, structural support, and responsiveness to internal and external stimuli. These metabolites are crucial mediators of plants' interactions with other organisms and play a significant role in stress response to light variations (Yang *et al*., 2018) and mineral scarcity (Clemens and Weber, 2015). The phenylpropanoids pathway (ko00940) in the leaves of *C. subternata* contains the largest concentration of differentially expressed proteins in secondary metabolism (Figure 5.4a). From this pathway, two proteins were identified, namely, cinnamyl-alcohol dehydrogenase (EC:1.1.1.195), which is the most dominant, and heme-thiolate (EC:1.14.14.91). These results are comparable to studies by Yu *et al*. (2020), where increased cinnamic acid accumulation and tolerance were imparted to millet through the regulation of genes involved in the phenylpropanoid biosynthesis pathway under
drought stress.

5.4.3.2 Carbon Fixation in Photosynthetic Organism

Nearly all biological processes on earth rely on autotropic CO2 fixation, which has created prehistoric carbon reserves that are used today to meet more than 80% of the world's energy needs (Ducat and Silver, 2012). The most biologically prevalent and commercially significant method for carbon fixation is the reductive pentose phosphate pathway (Calvin cycle), which has attracted most of the research attention (Stitt *et al*., 2010). Since ancient times, many plant species have been bred to increase their agricultural and commercial value. However, instead of increasing photosynthetic efficiencies, these methods typically produce varieties with a higher percentage of biomass directed towards a given product such as edible seeds or fruits (Ducat and Silver, 2012).

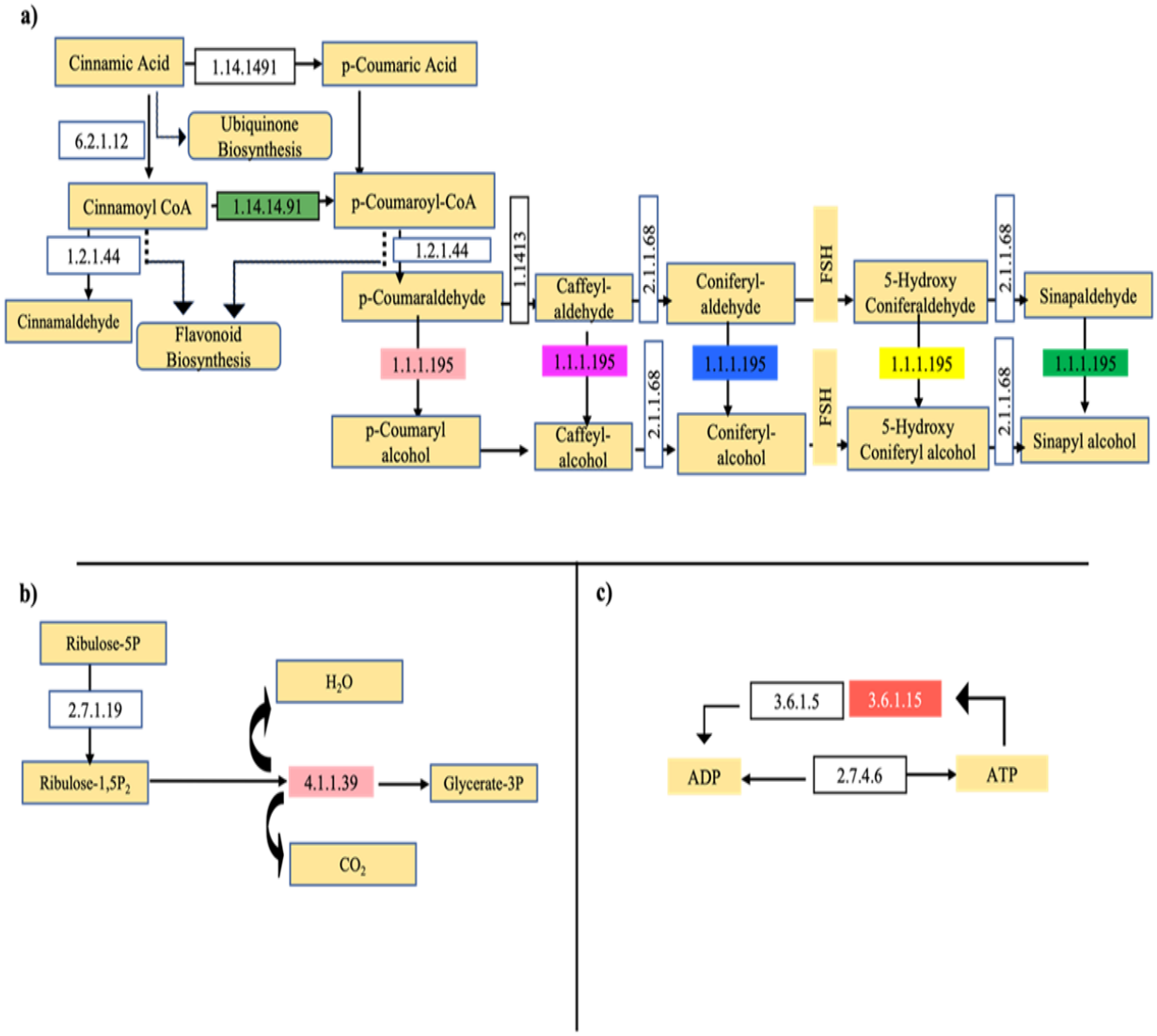


Figure 5.4: KEGG pathway analysis revealing proteins differentially regulated in water deficit plants of *C. subternata*. (A) Differentially expressed proteins identified, i.e., E.C 1.14.14.91 (transcinnamate4-monooxygenase) and E.C 1.1.1.195 (cinnamyl-alcohol dehydrogenase), affecting the phenylpropanoid biosynthesis pathway are shown by coloured blocks. (B) The enzyme (E.C.4.1.1.39—ribulose-bisphosphate carboxylase) shown by coloured block affecting the conversion of ribulose-1,5P2 to glycerate-3P in the carbon fixation in photosynthetic organism pathway. (C) In purine metabolism pathway, the enzyme identified is shown by red coloured block, with relevant enzyme code E.C 3.6.1.15, nucleoside-triphosphate phosphatase in conjunction with E.C. 3.6.1.5 (ATP-diphosphatase) affecting the conversion of ATP to ADP.

During the onset of drought, a reduction in stomatal conductance can reduce the availability of CO2 for photosynthesis, subsequently leading to the inhibition of underlying biochemical processes such as Rubisco carboxylation and electron transport activity, relative water content, and even pigment content (Khalil *et al*., 2020). In this study, analyses of proteins related to the carbon fixation in photosynthetic organism pathway (ko00710) showed that the enzyme ribulose biosphosphate caroboxylase (EC:4.1.1.39) was differentially expressed in

the leaves of C. subternata that were cultivated under different water stress conditions (Figure 5.4b). The most prevalent enzyme in the biosphere, rubisco, is one of the commonly known enzymes; it is the main carboxylase of the photosynthetic process. Carboxylation of ribulose bisphosphate is the initial step in the photosynthetic carbon reduction cycle, which results in the uptake of CO2 (von Caemmerer, 2020).

5.4.3.3 Purine Metabolism Pathways

Purine and pyridine serve as building blocks to produce nucleic acids and as an energy source. Therefore, nucleic acid biosynthesis and metabolism play a key role in the growth and development of plants (Stasolla *et al*., 2003). The salvage process is used to use preformed purine bases and nucleosides for nucleotide synthesis. In some cases, purine nucleosides come from exogenous sources as catabolic products of nucleic acids in decomposing cells. Purine nucleosides result from the intercellular breakdown of unstable RNA (Wasternack, 1982; Bray, 1983). In this study, analyses of proteins related to the purine metabolism pathway (ko00230) showed that an enzyme nucleoside triphosphate phosphatase (EC: 3.6.1.15) was differentially expressed in the leaves of *C. subternata* that were cultivated under different water stress conditions (Figure 5.4c). This enzyme is in the plasma membrane and plays a role in breaking down extracellular triphosphate nucleotides (Beukers *et al*., 1993).

5.5 Conclusion

This study demonstrated that water deficit stress had a critical influence on the biological processes, molecular activities, and cellular compartments of *C. subternata*. Only 11 differentially expressed proteins (DEPs) were found to be unique across all the treated and control *C. subternata*. The DEPs identified were associated with the biological processes linked to the cellular process, metabolic process, and response to a stimulus. Based on the KEGG analysis, five molecular pathways were identified, namely, phenylpropanoid biosynthesis, glyoxylate and dicarboxylate metabolism, purine metabolism, thiamine metabolism, and carbon fixation in a photosynthetic organism. Within these pathways, five enzymes (ribulose biphosphate carboxylase, glutamine synthetase, nucleoside-triphosphate phosphatase, heme-thiolate, and cinnamyl alcohol dehydrogenase) were identified. Overall, the use of proteomic tools helped identified proteins in honeybush plants that are water stress-relevant. Future investigations to assess the mechanisms responsible for making *C. subternata* water-stress-tolerant compared to other crop species are required.

REFERENCES

Ashraf, M. and Harris, P.J.C., 2013. Photosynthesis under stressful environments, an overview. *Photosynthetica*, 51(2):163-190.

Belay, Z.A. and Caleb, O.J., 2022. Role of integrated omics in unravelling fruit stress and defence responses during postharvest: A review. *Food Chemistry: Molecular Sciences*, p.100118.

Benešová, M., Holá, D., Fischer, L., Jedelsky, P.L., Hnilicka, F., Wilhelmova, N., Rothova, O., Kocova, M., Prochazkova, D., Honnerova, J., Fridrichova, L. & Hnilickova, H., 2012. The physiology and proteomics of drought tolerance in maize early stomatal closure as a cause of lower tolerance to short-term dehydration. *Plos One*, 7(6):1-17.

Bester, C., 2013. A model for commercialisation of honeybush tea, an indigenous crop. *Acta Horticulturae*, 1007, 889-894).

Beukers, M.W., Pirovano, I.M., van Weert, A., Kerkhof, C.J., Ijzerman, A.P. and Soudijn, W., 1993. Characterization of ecto-ATPase on human blood cells: a physiological role in platelet aggregation. *Biochemical pharmacology*, 46(11), pp.1959-1966.

Biała, W. and Jasiński, M., 2018. The phenylpropanoid case–it is transport that matters. *Frontiers in Plant Science*, 9, p.1610.

Bray, C.M .1983. Nitrogen Metabolism in Plants. Longman, London.

Brink, C., Postma, K. Jacobs, K. 2017. Rhizobial diversity and functions in rooibos (Asphalathus linearis) and honeybush (*Cyclopia* spp.) plants, a review. *South African Journal of Botany*, 110:80-86.

Cantalapiedra, C.P., Hernández-Plaza, A., Letunic, I., Bork, P. and Huerta-Cepas, J., 2021. eggNOG-mapper v2: functional annotation, orthology assignments, and domain prediction at the metagenomic scale. *Molecular biology and evolution*, 38(12), pp.5825-5829.

Chaki, M., Begara-Morales, J.C. and Barroso, J.B., 2020. Oxidative stress in plants. *Antioxidants*, 9(6), p.481.

Chandrasekhar, K., Dileep, A., Lebonah, D.E. and Pramoda Kumari, J., 2014. A short review on proteomics and its applications. *International Letters of Natural Sciences*, 12(1).

Chun, H.J., Baek, D., Cho, H.M., Lee, S.H., Jin, B.J., Yun, D.J., Hong, Y.S. and Kim, M.C., 2019. Lignin biosynthesis genes play critical roles in the adaptation of Arabidopsis plants to high-salt stress. *Plant signaling & behavior*, 14(8), p.1625697.

Clemens, S., and Weber, M., 2016. The essential role of coumarin secretion for Fe acquisition from alkaline soil. *Plant Signal. Behav*. 11:e1114197.

dos Santos, T.B., Ribas, A.F., de Souza, S.G.H., Budzinski, I.G.F. and Domingues, D.S., 2022. Physiological Responses to Drought, Salinity, and Heat Stress in Plants: *A Review*. Stresses,2(1), pp.113-135.

Ducat, D.C. and Silver, P.A., 2012. Improving carbon fixation pathways. *Current opinion in chemical biology*, 16(3-4), pp.337-344.

Farooqi, Z.U.R., Ayub, M.A., ur Rehman, M.Z., Sohail, M.I., Usman, M., Khalid, H. and Naz, K., 2020. Regulation of drought stress in plants. In Plant life under changing environment (pp. 77-104). Academic Press.

Farouk, S. and Al-Ghamdi, A.A.M., 2021. Sodium nitroprusside application enhances drought tolerance in marjoram herb by promoting chlorophyll biosynthesis and enhancing osmotic adjustment capacity. *Arabian Journal of Geosciences*, 14, pp.1-13.

Farouk, S. and Al-Huqail, A.A., 2020. Sodium nitroprusside application regulates antioxidant capacity, improves phytopharmaceutical production and essential oil yield of marjoram herb under drought. *Industrial Crops and Products*, 158, p.113034.

Gokul, A., Fahiem Carelse, M., Niekerk, L.A., Klein, A., Ludidi, N., Mendoza-Cozatl, D. and Keyster, M., 2021. Exogenous 3, 3′-Diindolylmethane Improves Vanadium Stress Tolerance in *Brassica napus* Seedling Shoots by Modulating Antioxidant Enzyme Activities. *Biomolecules*, 11(3), p.436.

Haddoudi, L., Hdira, S., Hanana, M., Romero, I., Haddoudi, I., Mahjoub, A., Ben Jouira, H., Djébali, N., Ludidi, N., Sanchez-Ballesta, M.T. and Abdelly, C., 2021. Evaluation of the Morpho-Physiological, Biochemical and Molecular Responses of Contrasting *Medicago truncatula* Lines under Water Deficit Stress. *Plants*, *10*(10), p.2114.

Haworth, M., Marino, G., Brunetti, C., Killi, D., De Carlo, A. and Centritto, M., 2018. The impact of heat stress and water deficit on the photosynthetic and stomatal physiology of olive (*Olea europaea L.*)—A case study of the 2017 heat wave. *Plants*, 7(4), p.76.

Henry, W.B. and Krutz, L.J., 2016. Water in agriculture: Improving corn production practices to minimize climate risk and optimize profitability. *Current Climate Change Reports*, 2, pp.49-54.

Hooijberg, E.H., Miller, M., Cray, C., Buss, P., Steenkamp, G. and Goddard, A., 2018. Serum protein electrophoresis in healthy and injured southern white rhinoceros (*Ceratotherium simum simum*). *Plos one*, *13*(7), p.e0200347. https://doi.org/10.1016/j.sajb.2011.05.014

Jankun, J., Selman, S.H., Swiercz, R. and Skrzypczak-Jankun, E., 1997. Why drinking green tea could prevent cancer. *Nature*, 387(6633), pp.561-561.

Jorrin-Novo, J.V., Komatsu, S., Sanchez-Lucas, R. and de Francisco, L.E.R., 2019. Gel electrophoresis-based plant proteomics: Past, present, and future. Happy 10th anniversary Journal of Proteomics. *Journal of proteomics*, 198, pp.1-10.

Joubert, E., De Beer, D., Malherbe, C.J., Muller, M., Louw, A. and Gelderblom, W.C.A., 2019. Formal honeybush tea industry reaches 20-year milestone–progress of product research targeting phenolic composition, quality and bioactivity. *South African Journal of Botany*, 127, 58-79

Joubert, E., Joubert, M.E., Bester, C., De Beer, D. and De Lange, J.H., 2011. Honeybush (*Cyclopia* spp.): From local cottage industry to global markets—The catalytic and supporting role of research. *South African Journal of Botany*, 77(4), pp.887-907.

Kang, J., Chu, Y., Ma, G., Zhang, Y., Zhang, X., Wang, M., Lu, H., Wang, L., Kang, G., Ma, D. and Xie, Y., 2023. Physiological mechanisms underlying reduced photosynthesis in wheat leaves grown in the field under conditions of nitrogen and water deficiency. *The Crop Journal*, 11(2), pp.638-650.

Kanwar, J., Taskeen, M., Mohammad, I., Huo, C., Chan, T.H. and Dou, Q.P., 2012. Recent advances on tea polyphenols. *Frontiers in bioscience* (Elite edition), 4, p.111.

Karsen, P.A., Lötze, E., Valentine, A.J. and Hoffman, E.W., 2022. Propagation and cultivation practices of honeybush (*Cyclopia* spp.) for the sustainable production of an export quality indigenous South African tea. *Crop Science*, 62(5), pp.1702-1733.

Katam, R., Shokri, S., Murthy, N., Singh, S.K., Suravajhala, P., Khan, M.N., Bahmani, M., Sakata, K. and Reddy, K.R., 2020. Proteomics, physiological, and biochemical analysis of cross tolerance mechanisms in response to heat and water stresses in soybean. *Plos one*, 15(6), p.e0233905.

Khalil, A.M., Murchie, E.H. and Mooney, S.J., 2020. Quantifying the influence of water deficit on root and shoot growth in wheat using x-ray computed tomography. *AoB Plants*. 12 (5) 36 pp. https://doi.org/10.1093/aobpla/plaa036

Koen, J., Slabbert, M.M., Booyse, M. and Bester, C., 2021. Honeybush (*Cyclopia* spp.) anther-stigma distance and intraspecies cross compatibilities. *South African Journal of Plant and Soil*, 38(1), pp.13-18.

Komatsu, S., Mock, H.P., Yang, P. and Svensson, B., 2013. Application of proteomics for improving crop protection/artificial regulation. *Frontiers in Plant Science*, 4, p.522.

Le Roux, M., Cronje, J.C., Burger, B.V. and Joubert, E., 2012. Characterization of volatiles and aroma-active compounds in honeybush (*Cyclopia subternata*) by GC-MS and GC-O analysis. *J. Agric. Food Chem*. 60, 2657–2664. https://doi.org/10.1021/jf2048383

Liu, Q., Luo, L. and Zheng, L., 2018. Lignins: biosynthesis and biological functions in plants. *International journal of molecular sciences*, 19(2), p.335.

Liu, Y., Lu, S., Liu, K., Wang, S., Huang, L. and Guo, L., 2019. Proteomics: a powerful tool to study plant responses to biotic stress. *Plant Methods*, 15(1), pp.1-20.

Lyu, S., Gao, L., Zhang, R., Zhang, C. and Hou, X., 2020. Correlation analysis of expression profile and quantitative iTRAQ-LC-MS/MS proteomics reveals resistance mechanism against TuMV in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*). *Frontiers in Genetics*, 11, p.963.

Ma, Z., Wang, L., Zhao, M., Gu, S., Wang, C., Zhao, J., Tang, Z., Gao, H., Zhang, L., Fu, L. and Yin, Y., 2020. iTRAQ proteomics reveals the regulatory response to Magnaporthe oryzae in durable resistant vs. susceptible rice genotypes. *PLoS One*, 15(1), p.e0227470.

Mabizela, G.S., 2020. 'Metabolic and quality profiling of *Cyclopia subternata* and *C. genistoides* in response to seasonal variation and drought stress'. Doctral dissertation, Tshwane University of technology.

Mansell, R.L., Gross, G.G., Stöckigt, J., Franke, H. and Zenk, M.H., 1974. Purification and properties of cinnamyl alcohol dehydrogenase from higher plants involved in lignin biosynthesis. *Phytochemistry*, 13(11), pp.2427-2435.

McGregor, G., 2017. An overview of the honeybush industry. Department of Environmental Affairs and Development Planning, Cape Town. pg, 2(1), p.3

Metwaly, E.S.E., Al-Yasi, H.M., Ali, E.F., Farouk, H.A. and Farouk, S., 2022. Deteriorating harmful effects of drought in cucumber by spraying glycinebetaine. *Agriculture*, 12(12), p.2166.

Mi, H., Muruganujan, A., Casagrande, J.T. and Thomas, P.D., 2013. Large-scale gene function analysis with the PANTHER classification system. *Nature protocols*, 8(8), pp.1551-1566.

Mittler, R., 2006. Abiotic stress, the field environment and stress combination. *Trends in plant science*, 11(1), pp.15-19.

Morales, F., Ancín, M., Fakhet, D., Gonzaléz-Torralba, J., Gámez, A.L., Seminario, A., Soba, D., Mariem, S.B., Garriga, M. and Aranjuelo, I., 2019. Photosynthetic metabolism under stressful growth conditions as a base for crop breeding and yield improvement. *Plants*, 9(88):1-23.

Nsumpi, A.N., Belay, Z.A. and Caleb, O.J., 2020. Good intentions, bad outcomes: Impact of mixed-fruit loading on banana fruit protein expression, physiological responses and quality. *Food Packaging and Shelf Life*, *26*, p.100594.

Ntlhokwe, G.E., 2016. Application of comprehensive two-dimensional gas chromatography for the characterisation of the volatile composition of Honeybush tea (Doctoral dissertation, Stellenbosch: Stellenbosch University).

Osakabe, Y., Osakabe, K., Shinozaki, K. & Tran, L.S.P., 2014. Response of plants to water stress. *Frontiers in Plant Science*, 5(86):1-8.

Otto, M., Wynands, B., Lenzen, C., Filbig, M., Blank, L.M. and Wierckx, N., 2019. Rational engineering of phenylalanine accumulation in Pseudomonas taiwanensis to enable high-yield production of trans-cinnamate. *Frontiers in Bioengineering and Biotechnology*, 7, p.312.

Postma, A., Slabbert, E., Postma, F. & Jacobs, K. 2016. Soil bacterial communities associated with natural and commercial *Cyclopia* spp. *FEMS Microbiology Ecology,* 92:1-5.

Ranatunge, I., Adikary, S., Dasanayake, P., Fernando, C.D. and Soysa, P., 2017. Development of a rapid and simple method to remove polyphenols from plant extracts. *International journal of analytical chemistry*, *2017*.

Saqib, A., Scheller, H.V., Fredslund, F. and Welner, D.H., 2019. Molecular characteristics of plant UDP-arabinopyranose mutases. *Glycobiology*, 29(12), pp.839-846.

Schutte, A.L. and Van Wyk, B.E., 1998. Evolutionary relationships in the Podalyrieae and Liparieae (Fabaceae) based on morphological, cytological, and chemical evidence. *Plant Systematics and Evolution*, 209(1), pp.1-31.

Seleiman, M.F., Al-Suhaibani, N., Ali, N., Akmal, M., Alotaibi, M., Refay, Y., Dindaroglu, T., Abdul-Wajid, H.H. and Battaglia, M.L., 2021. Drought stress impacts on plants and different approaches to alleviate its adverse effects. *Plants*, *10*(2), p.259.

Shu, B., Hu, Y. and Luo, C., 2022. The metabolites involved in phenylpropanoid biosynthesis increase the susceptibility of octoploid strawberry to crown rot caused by Colletotrichum siamense. *Scientia horticulturae*, *306*, p.111447.

Soni, R.P., Katoch, M., Kumar, A., Ladohiya, R. and Verma, P., 2015. Tea: production, composition, consumption and its potential as an antioxidant and antimicrobial agent. *International Journal of Food and Fermentation Technology*, 5(2), 95-106.

Stasolla, C., Katahira, R., Thorpe, T.A. and Ashihara, H., 2003. Purine and pyrimidine nucleotide metabolism in higher plants. *Journal of plant physiology*, 160(11), pp.1271- 1295.

Stitt M., Lunn J. and Usadel B., 2010. Arabidopsis and primary photosynthetic metabolism — more than the icing on the cake [SINGLE]. *Plant J*, 61:1067-1091.

Tuberosa, R., 2012. Phenotyping for drought tolerance of crops in the genomics era. *Frontiers in physiology*, *3*, p.347.

Ubiparip, Z., Beerens, K., Franceus, J., Vercauteren, R. and Desmet, T., 2018. Thermostable alpha-glucan phosphorylases: characteristics and industrial applications. *Applied microbiology and biotechnology*, 102(19), pp.8187-8202.

van Heeswijk, W.C., Westerhoff, H.V. & Boogerd, F.C. 2013. Nitrogen assimilation in Escherichia coli, putting molecular data into a systems perspective. *Microbiology and Molecular Biology Reviews*, 77(4):628-695.

Von Caemmerer, S., 2020. Rubisco carboxylase/oxygenase: from the enzyme to the globe: a gas exchange perspective. *Journal of Plant Physiology*, 252, p.153240.

Wang, L.F., Lu, K.K., Li, T.T., Zhang, Y., Guo, J.X., Song, R.F. and Liu, W.C., 2022. Maize phytomelatonin receptor1 functions in plant tolerance to osmotic and drought stress. *Journal of Experimental Botany*, 73(17), pp.5961-5973

Wang, W., Vignani, R., Scali, M., Cresti, M., 2006. A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. *Electrophoresis* 27, 2782–2786. https://doi.org/10.1002/elps.200500722

Wang, X. and Komatsu, S., 2018. Proteomic approaches to uncover the flooding and drought stress response mechanisms in soybean. *Journal of proteomics*, *172*, pp.201-215.

Wasternack, C. 1982 Metabolism of pyrimidines and purines. In: Pirson A, Zimmermann MH (eds) Encyclopedia of Plant Physiology. New Ser, Vol 14B, Springer, Berlin pp 263–301

Wojtyla, L., Paluch-Lubawa, E., Sobieszczuk-Nowicka, E. & Garnczarska, M., 2020. Drought stress memory and subsequent drought stress tolerance in plants. Chapter 7. In. Priming-mediated stress and cross-stress tolerance in plants.

Xu, Z., Jiang, Y., Jia, B. and Zhou, G., 2016. Elevated-CO2 response of stomata and its dependence on environmental factors. *Frontiers in Plant Science*, 7(657):1-15.

Yang, L., Wen, K. S., Ruan, X., Zhao, Y. X., Wei, F. and Wang, Q., 2018. Response of plant secondary metabolites to environmental factors. *Molecules* 23:E762.

Yu, A., Zhao, J., Wang, Z., Cheng, K., Zhang, P., Tian, G., Liu, X., Guo, E., Du, Y. and Wang, Y., 2020. Transcriptome and metabolite analysis reveal the drought tolerance of foxtail millet significantly correlated with phenylpropanoids-related pathways during germination process under PEG stress. *BMC plant biology*, 20(1), pp.1-17.

Zahid, G., Iftikhar, S., Shimira, F., Ahmad, H.M. and Kaçar, Y.A., 2023. An overview and recent progress of plant growth regulators (PGRs) in the mitigation of abiotic stresses in fruits: A review. *Scientia Horticulturae*, *309*, p.111621.

Zhou, R., Yu, X., Ottosen, C.O., Rosenqvist, E., Zhao, L., Wang, Y., Yu, W., Zhao, T. and Wu, Z., 2017. Drought stress had a predominant effect overheat stress on three tomato cultivars subjected to combined stress. *BMC plant biology*, 17(1), pp.1-13.

Zipfel, C. and Oldroyd, G.E., 2017. Plant signalling in symbiosis and immunity. *Nature*, 543(7645), pp.328-336.

CHAPTER SIX

THE EFFECT OF DIFFERENT WATER STRESS LEVELS ON THE QUALITY OF FIELD GROWN *C. SUBTERNATA* TEA

6.1 Introduction

Honeybush is the general term used to classify all the known types of *Cyclopia* species, a genus in the leguminous family (Schutte and Van Wyk, 1996). It is native to the fynbos biome in the Western and Eastern Cape Provinces of South Africa and its tea has a sweet, honey-like taste (Du Toit and Joubert, 1998). In-depth analysis of the sensory profile of honeybush identified additional attributes (Theron *et al*., 2014). A follow-up study confirmed that floral, fruity and sweet-associated aroma notes, including honey, are characteristic of honeybush tea (Du Preez *et al*., 2020). Honeybush tea is usually enjoyed in its oxidised ("fermented") state, although the unfermented (green) one is also marketed (Le Roux *et al*., 2012). *Cyclopia* species are among the few wild plants that are turned into commercial commodities in South Africa (Joubert *et al*., 2008). Although 23 *Cyclopia* species grow in the wild, only a few, mainly, *C. subternata, C. intermedia, C. genistoides and C. longifolia,* are used to make to make tea. Others used in smaller quantities are *C. maculata, C. sessiliflora* and *C. plicata.* (De Villiers and McGregor, 2017; Mabizela, 2020; Koen *et al*., 2021). The popularity of honeybush cannot only be attributed to its unique sensory profile, but also to its low tannin content, the absence of caffeine, and the presence of antioxidants (Joubert *et al*., 2019; Stander *et al*., 2019). With consumers gradually being more wellbeing conscious, there is now a bigger appreciation for herbal products including herbal teas (Payne *et al*., 2014).

As much as consumers prefer healthy food, they are mostly not eager to compromise on taste and other sensory properties (Verbeke and Ward, 2006; Grunert, 2011). Studies have proven that water stress has various effect on the sensory quality of different plant food products. Hence, Griñán *et al*. (2019) reported that water deficit conditions had a significant effect on 11 out of 22 sensory attributes of quince fruit, while Lipan *et al*. (2019) observed no significant differences in 12 out of 17 sensory attributes used to describe the quality of almonds under water deficit conditions. Mabizela (2020) showed that drought stress for a short period of time did not affect the intensities of the various sensory attributes of *C. subternata* herbal tea, however, the effect of an extended period of drought-stress is not known. Therefore, the aim of the present study was to determine the effect of different irrigation rates of *C. subternata* plants on the sensory profile of its herbal tea infusions, using descriptive sensory analysis.

6.2 Materials and Methods

Ethical clearance (Project ID no. 9204) was obtained from the Research Ethics Committee for Social, Behavioural and Education Research at Stellenbosch University (SU).

6.2.1 Samples and infusion preparation

6.2.1.1 Processing of plant material

Cyclopia subternata seedlings (9 months old), planted at ARC Nietvoorbij Research farm in Stellenbosch, South Africa, were subjected to well-watered (T1; n = 8), semi-stressed (T2; n = 8) and stressed (T3; n = 6) irrigation treatments. The plant material of two replicates of Treatment 3 was pooled as limited yield was obtained for tea processing. The plant material was processed according to a standardised protocol as described by Robertson *et al*. (2018). Briefly, the plant material was mechanically cut into small pieces, moistened to ca. 65% moisture content, and oxidised ("fermented") at 90°C for 16 h in a temperature-controlled laboratory oven. The oxidised plant material was then spread in a thin layer on drying mesh trays and dried (40°C/6 h) in a crossflow drying tunnel. The dried plant material was mechanically sieved and the "tea-bag-cut" fraction (<12 mesh and >40 mesh; equalling <1.68 mm and >0.42 mm) collected for sensory analysis.

6.2.1.2 Infusion preparation

Infusions were prepared at 'cup-of-tea' strength using deionised water according to a standard protocol (Erasmus *et al*., 2017). Briefly, this entailed infusing 12.5 g of the sieved, dried plant material for 5 min in 1000 g freshly boiled distilled water. The infusion was strained directly into a pre-heated 1-litre stainless steel vacuum flask, whereafter ca 70 mL aliquots were served in preheated (70°C) white porcelain mugs labelled with a three-digit code. Each mug was covered with a plastic lid to prevent the loss of volatiles and placed in temperature-controlled (65°C) water baths (Severin Elektrogeräte GmbH, Sundern, Germany) to keep the infusions at a constant temperature during sensory analysis.

6.2.2 Descriptive sensory analysis

Descriptive sensory analysis was performed by a trained panel (n = 10; Department of Food Science, SU) with extensive experience in sensory analyses of honeybush tea. The assessors were trained according to the consensus method (Lawless & Heymann, 2010). The aroma, flavour, taste and mouthfeel attributes, as defined by Du Preez et al. (2020), were used for the sensory profiling of the infusions. Aroma refers to odours perceived through orthonasal analysis, while flavour refers to the retronasal perception of aromas in the mouth. Similar to flavour, the basic taste modalities, i.e., sweet, sour, and bitter and the mouthfeel attribute, astringency, are perceived in the oral cavity (Lawless & Heymann, 2010). Astringency is described as the tactile sensation that occurs in the oral cavity due to the precipitation of

salivary proteins (Green, 1993). The training was conducted in six 1-hour sessions over three consecutive days.

Analyses (testing) of the samples (n = 22) were conducted in triplicate over a total of six consecutive days, i.e. three sensory replicates were performed. The sample set (n = 22) of each sensory replicate were randomised, and five or six samples were analysed per session. Coded samples were served in a heated water bath in a random order per assessor, as generated by the Compusense20® software programme (Compusense®, Guelph, Canada). Attribute intensities were rated on unstructured line scales (0 = none; 100 = extremely high). Panellists were assigned individual booths (light and temperature controlled; ca 21°C). Unsalted water biscuits (Woolworths, South Africa) and deionised water (Department of Food Science, SU) were used as palate cleansers between samples.

6.2.3 Statistical analysis

DSA data were subjected to various statistical analyses to confirm panel reliability (Næs et al., 2010) and normality. Subsequent statistical analyses were conducted on means over assessors. DSA and composition data were subjected to analysis of variance (ANOVA) to test for treatment differences using SAS software (Version 9.2, SAS Institute Inc., Cary, USA). Fisher's least significant difference (LSD) was calculated (5% level) to compare treatment means. P values < 0.05 were considered significant. Principal component analysis (PCA), using the correlation matrix, was performed to indicate the association between samples and attributes, using XLStat (Version 2022, Addinsoft, Paris, France).

6.3 Results

The association between the sensory attributes and the herbal tea samples of field grown *C. subternata* (n = 22) is presented by the principal component analysis (PCA) biplot as shown in Figure 6.1.

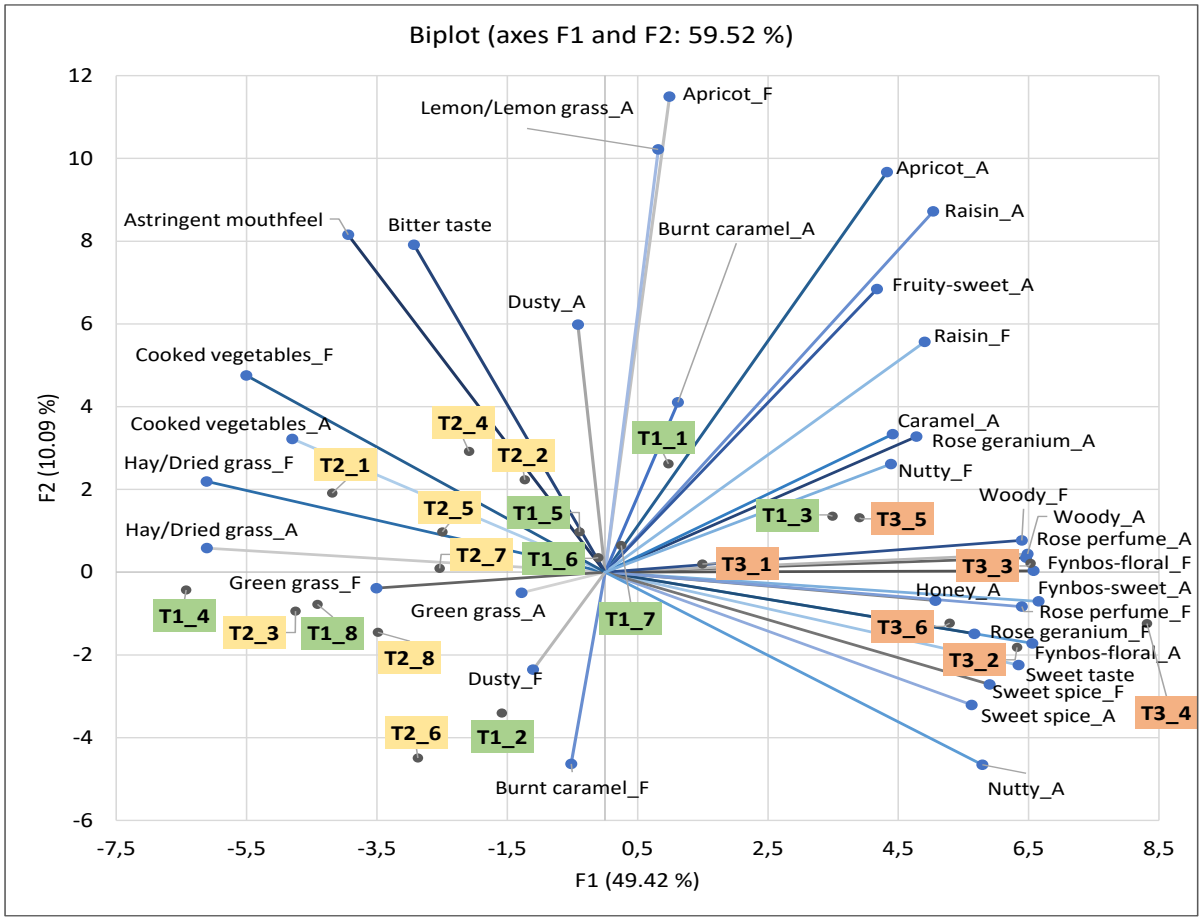


Figure 6.1: PCA biplot of the aroma, taste and mouthfeel attributes and the herbal tea samples of field grown C. subternata under different water stress levels. The plant material was processed according to a standard protocol as described by Robertson et al. (2018). T1= treatment 1/ well-watered; T2= treatment 2/ semi-stressed; T3= treatment 3/ stressed; A and F after each descriptor refer to 'aroma' and 'flavour', respectively.

The first two components explained 49.42% and 10.09% of the variance, respectively. The positive and negative aroma attributes were separated on F1 of the PCA plot. The infusions from the well-watered and semi-stressed treatments (Treatment 1 and 2) were positioned generally more on the left on F1, associating with negative aroma attributes such as 'cooked vegetables', 'hay/ dried grass', and 'dusty'. Positive aroma attributes like 'fynbos sweet', 'rose perfume' and 'sweet spice' associated with the stressed treatment (Treatment 3).

The ANOVA results are depicted in Figure 6.2 and 6.3, showing the intensities of the major positive aroma attributes (Figure 6.2A), secondary positive aroma attributes (Figure 6.2B, positive flavour, taste and mouthfeel attributes (Figure 6.2C) and negative aroma and flavour attributes (Figure 6.3), perceived in the infusions of *C. subternata* grown under different water conditions.

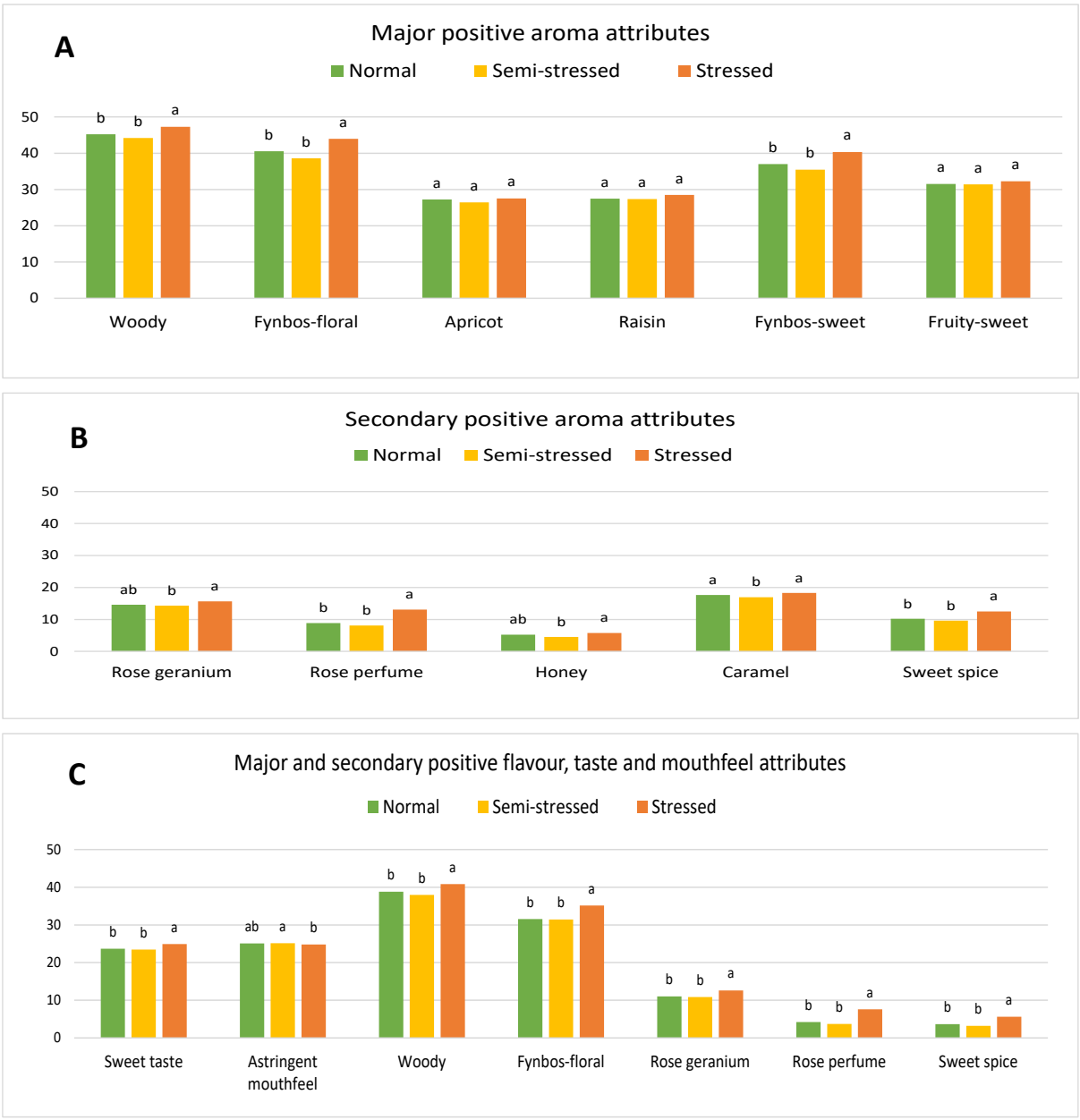


Figure 6.2: Mean intensities of the A) major positive aroma attributes, B) secondary positive aroma attributes, and C) major and secondary flavour, taste and mouthfeel attributes of the herbal tea infusions of *C. subternata* grown under different water conditions. Means with the same letter are not significantly different (p≤0.05).

The different irrigation frequencies significantly ($p<0.05$) affected major and secondary positive aroma attributes. Infusions prepared from stressed plant materials had higher intensities of 'woody', 'fynbos-floral', 'fynbos-sweet' (major positive aroma attributes) and 'rose perfume' and 'sweet spice' (secondary positive aroma attributes) than the normal (well-watered) and semi-stressed plant materials. The intensities of these attributes in infusions prepared from normal and semi-stressed plant materials were not significantly different ($p>0.05$). Other aroma attributes, i.e., 'apricot', 'raisin', 'fruity-sweet', 'rose-geranium' and 'honey' were not affected by treatment ($p\geq0.05$). 'Caramel' intensity was significantly lower

(p<0.05) for the semi-stressed treatment. The major and secondary flavour, taste, and mouthfeel attributes of *C. subternata* infusions were also significantly affected (p<0.05) by the different irrigation frequencies of the plants (Figure 6.2C). The infusions made from stressed plant material coherently had high intensities (p<0.05) than the other two infusions that were prepared from normal and semi-stressed plant materials. No significant difference (p>0.05) was observed between infusions prepared from normal and semi-stressed plant materials. For all three treatments, the intensities of 'lemon/lemon grass' and 'nutty' aroma and 'apricot', 'raisin', and 'nutty' flavour were < 5 (on a 100-point scale), i.e., 'barely perceptible'. Therefore, these results are not reported.

The ANOVA results for the negative aroma and flavour attributes are presented in Figure 6.3.

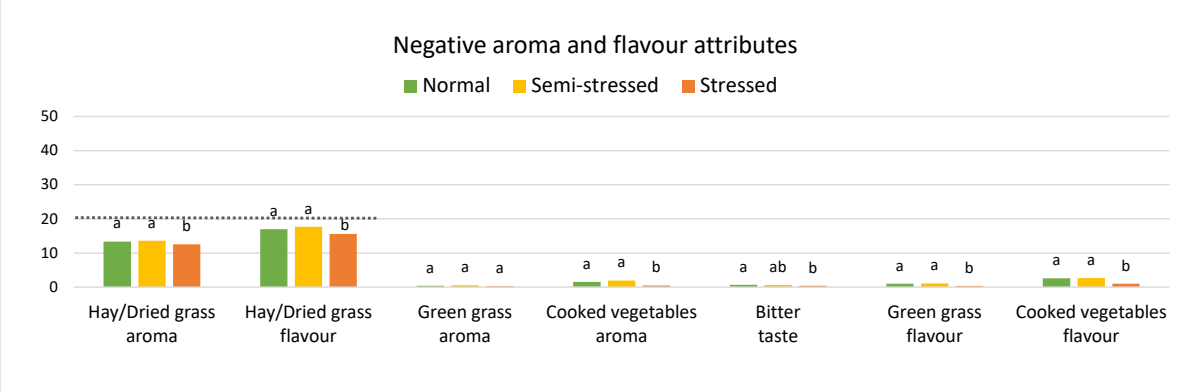


Figure 6.3: Mean intensities for the negative aroma and flavour attributes observed for the herbal tea infusions of *C. subternata* grown under different water conditions. The dotted line signifies that the mean intensities were less than < 20. Means with the same letter are not significantly different (p≤0.05).

Although 'green grass' and 'cooked vegetable' aroma and 'green grass' and 'cooked vegetable' flavour notes as well as bitter taste, were reported by the panel, they are regarded as barely perceptible because of their low intensities (< 5). Even though they had extremely low intensities, it does not automatically disqualify them from being part of the profile as they could still have a crucial contribution to the final sensory profile of the infusions (Theron *et al*., 2014). A mean intensity of 20 is used as a baseline for 'hay/dried grass' aroma and 'hay/dried grass' flavour. If less than 20, 'hay/dried grass' is considered to be a positive attribute, but when >20 then it becomes too prominent and thus a negative attribute. Bergh *et al*. (2017) suggested that consumer testing is required to clarify to what extent this attribute is perceived as negative, i.e., what intensity should be the cut-off point for acceptability. For all the negative attributes, except 'green grass' aroma, the normal and semi-stressed treatments produced infusions with significantly higher (p<0.05) intensities than the infusions from stressed plant materials. Treatment had no significant effect (p≥0.05) on 'green grass' aroma intensity.

Although some of the negative attributes with intensities < 5 forms part of the sensory profile, they may not necessarily be regarded as 'characteristic' (Theron *et al*., 2014).

6.4 Discussion

The characteristic aroma, taste, and mouthfeel attributes of honeybush tea are formed during the 'fermentation' process (Du Toit and Joubert, 1998), with the flavour changing from a predominantly green, grassy and hay-like to floral, fruity and sweet. This indicates that the fermentation process release aroma-impact volatile organic compounds (VOCs) and/or form such compounds from non-volatile precursors. Since all the treatments were processed under the same conditions, differences in their sensory profiles are the result of treatments.

The positive effect of drought stress on the sensory profile of the infusions could be due a "concentration effect" achieved by the low relative water content of the leaves of the water-stressed plants. This may suggest that *C. subternata* flourishes under water stress conditions since it grows naturally in the wild and survive the dry summers of their natural habitat. Another explanation may be that changes in the biochemical pathways of the plants occurred due to water stress, which increased the production of VOCs and/or their precursors.

According to Pevicharova *et al*. (2018), limited studies have been conducted on the influence of water deficit on the sensory properties of tomatoes, however, reduced irrigation has proven to increase total soluble solids like sugar, amino acids and organic acids in the fruits (Shinohara *et al*., 1995; Nuruddin *et al*., 2003). It has been reported that honeybush tea, like many other natural products, contains many VOCs that have diverse properties both physiologically and chemically (Ntlhokwe, 2016). No information is, however, available on their precursors. Studies on *Camellia sinensis* tea have shown that many of the volatiles are stored in the plant as glycosides. During processing, hydrolysis of the glycosidic bond takes place, releasing the bound volatile compounds (Ho *et al*., 2015). Examples of such released volatile compounds are linalool and geraniol, both present in the volatile fraction of *C. subternata* (Le Roux *et al*., 2012).

Mabizela *et al*. (2020) reported that the major positive aroma attributes were observed at higher intensities in the infusions prepared from plant materials that were harvested in summer and winter (*C. subternata*) than those harvested in autumn. In the case of *C. genistoides*, autumn harvesting produced tea with higher intensities of the positive aroma attributes (Mabizela *et al*., 2022). In contrast to the findings of this study, a potted study on the effect of drought stress on the sensory profile of *C. subternata* by Mabizela (2020) shows that no effect was observed, which could be due to shorter water stress period. Another study on the impact of water deficit on the sensory profile of tomato shows that the sweetness was expressed

better in water stressed fruits. However, the limited water supply did not influence the aroma and external colour of the tomato fruits (Pevicharova *et al*., 2018). While lack of water due to the dry summer months was indicated to improve the sensory profile of *C. subternata* infusions, lack of water can also have a negative effect on the plant by altering the plant chemistry, disrupting metabolism and pigments, thereby decreasing photosynthesis (Foyer and Noctor 2000, Fu and Huang, 2001; Ahmad *et al*., 2018; Hussain *et al*., 2018). However, water stress in this study has proven to be beneficial for tea quality considering the increased intensity of the positive sensory attributes. Therefore, *C. subternata* growers can irrigate mainly for the survival of the plants and cut down the water supply when they are approaching the harvest season to improve tea quality.

6.5 Conclusion

This study was carried out to investigate the effect of water stress on the sensory profile of herbal tea infusions of *C. subternata.* It is evident that the three water treatments had a significant effect on the positive aroma and flavour attributes, as well as taste and mouthfeel where infusions from stressed plant materials had higher attribute intensities than the treatments which received more water. Therefore, it can be concluded that severe water-deficit stress seems to boost the sensory profile of the infusions, in particular the 'woody', 'fynbos-floral', 'rose perfume', 'fynbos-sweet' and 'sweet spice' aromas as well as a sweet taste. Other major positive aroma attributes, i.e. 'apricot', 'raisin' and 'fruity-sweet', and astringency were also affected by the treatments. These findings may also suggest that *C. subternata* plants do not necessarily require excessive water supply to produce a better-quality tea as volatile compounds seem to be highly concentrated in stressed plants due to changes in the biochemical pathways. However, more studies need to be conducted on the effect of water stress to get better and clear understanding on this topic as this was the first time that the effect of a prolonged water stress of *C. subternata* on its tea quality could be demonstrated.

REFERENCES

Ahmad, H.M., Kamal, M.S. and Al-Harthi, M.A., 2018. High molecular weight copolymers as rheology modifier and fluid loss additive for water-based drilling fluids. Journal of Molecular Liquids, 252, pp.133-143.

Bergh, A.J., Muller, M., Van der Rijst, M., Joubert, E., 2017. Optimisation and validation of high-temperature oxidation of *Cyclopia intermedia* (honeybush) – from laboratory to factory. South African Journal of Botany 110, 152–160.

De Villiers, C. and McGregor, G.K. 2017. Review of the regulatory and policy framework relating to the harvesting of wild honeybush (*Cyclopia* spp.) Department of Environmental Affairs and Development Planning, Cape Town.

Du Preez, B.V.P., De Beer, D., Moelich, E.I., Muller, M. and Joubert, E., 2020. Development of chemical-based reference standards for rooibos and honeybush aroma lexicons. Food Research International, 127, p.108734.

Du Toit, J. and Joubert, E., 1998. The effect of pre-treatment on the fermentation of honeybush tea (*Cyclopia maculata*). Journal of the Science of Food and Agriculture, 76(4), pp.537- 545.

Erasmus, L.M., Theron, K.A., Muller, M., Van der Rijst, M. and Joubert, E., 2017. Optimising high-temperature oxidation of *Cyclopia* species for maximum development of characteristic aroma notes of honeybush herbal tea infusions. South African Journal of Botany, 110, pp.144-151.

Foyer, C.H. and Noctor, G., 2000. Tansley Review No. 112 Oxygen processing in photosynthesis: regulation and signalling. The New Phytologist, 146(3), pp.359-388.

Fu, J. and Huang, B., 2001. Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. Environmental and Experimental Botany, 45(2), pp.105-114.

Green, B.G., 1993. Oral astringency: a tactile component of flavor. Acta psychologica, 84(1), pp.119-125.

Griñán, I., Galindo, A., Rodríguez, P., Morales, D., Corell, M., Centeno, A., Collado-González, J., Torrecillas, A., Carbonell-Barrachina, A.A. and Hernández, F., 2019. Volatile composition and sensory and quality attributes of quince (Cydonia oblonga Mill.) fruits as affected by water stress. *Scientia Horticulturae*, *244*, pp.68-74.

Grunert, K.G., 2011. Sustainability in the food sector: A consumer behaviour perspective. *International Journal on Food System Dynamics*, *2*(3), pp.207-218.

Ho, C.T., Zheng, X. and Li, S., 2015. Tea aroma formation. Food Sci. Hum. Wellness 4, 9–27. https://doi.org/10.1016/j.fshw.2015.04.001

Hussain, H.A., Hussain, S., Khaliq, A., Ashraf, U., Anjum, S.A., Men, S. and Wang, L., 2018. Chilling and drought stresses in crop plants: implications, cross talk, and potential management opportunities. Frontiers in plant science, 9, p.393.

Joubert, E., De Beer, D., Malherbe, C.J., Muller, M., Louw, A. and Gelderblom, W.C.A., 2019. Formal honeybush tea industry reaches 20-year milestone–progress of product

research targeting phenolic composition, quality and bioactivity. *South African Journal of Botany*, *127*, pp.58-79.

Joubert, E., Gelderblom, W.C.A., Louw, A. and De Beer, D., 2008. South African herbal teas: Aspalathus linearis, *Cyclopia* spp. and Athrixia phylicoides—A review. Journal of ethnopharmacology, 119(3), pp.376-412.

Koen, J., Slabbert, M.M., Booyse, M. and Bester, C., 2021. Honeybush (*Cyclopia* spp.) anther-stigma distance and intraspecies cross compatibilities. South African Journal of Plant and Soil, 38(1), pp.13-18.

Lawless, H.T. and Heymann, H., 2010. Sensory evaluation of food: principles and practices (Vol. 2). New York: Springer.

Le Roux, M., Cronje, J.C., Burger, B.V. and Joubert, E., 2012. Characterization of volatiles and aroma-active compounds in honeybush (*Cyclopia subternata*) by GC-MS and GC-O analysis. J. Agric. Food Chem. 60, 2657–2664. https://doi.org/10.1021/jf2048383

Lipan, L., Cano-Lamadrid, M., Corell, M., Sendra, E., Hernández, F., Stan, L., Vodnar, D.C., Vázquez-Araújo, L. and Carbonell-Barrachina, Á.A., 2019. Sensory profile and acceptability of hydrosostainable almonds. *Foods*, *8*(2), p.64.

Mabizela, G.S., 2020. 'Metabolic and quality profiling of *Cyclopia subternata* and *C. genistoides* in response to seasonal variation and drought stress'. Doctral dissertation, Tshwane University of technology.

Mabizela, G.S., du Preez, B. von P., Human, C., Muller, M., de Beer, D., van der Rijst, M., Slabbert, M.M., Bester, C. and Joubert, E., 2022. A balancing act – Optimising harvest season of *Cyclopia genistoides* (honeybush tea) for enhanced phenolic content and acceptable sensory profile. J. Food Compos. Anal. 110, 104583. https://doi.org/10.1016/j.jfca.2022.104583

Mabizela, G.S., Muller, M., de Beer, D., van der Rijst, M., Slabbert, M.M., Joubert, E. and Bester, C., 2020. Effect of genotype and harvest season on quality characteristics of *Cyclopia subternata:* Phenolic content and sensory profile. South Afr. J. Bot. 132, 491–501. https://doi.org/10.1016/j.sajb.2020.06.010

Næs, T., Brockhoff, P. B., & Tomic, O. (2010). Statistics for Sensory and Consumer Science. Chichester: John Wiley & Sons Limited. https://doi.org/https://doi.org/10.1002/9780470669181

Ntlhokwe, G.E., 2016. Application of comprehensive two-dimensional gas chromatography for the characterisation of the volatile composition of Honeybush tea (Doctoral dissertation, Stellenbosch: Stellenbosch University).

Nuruddin, M.M., Madramootoo, C.A. and Dodds, G.T., 2003. Effects of water stress at different growth stages on greenhouse tomato yield and quality. HortScience, 38(7), pp.1389-1393.

Payne, C.R., Niculescu, M. and Barney, C.E., 2014. Consumer consumption intentions of smaller packaged snack variants. *International Journal of Consumer Studies*, *38*(3), pp.238-242.

Pevicharova, G.T., Ganeva, D.G. and Grozeva, S.Y., 2018. Impact of water deficit on sensory profile of tomato (*Solanum lycopersicum L.*) grown under hot summer conditions in Bulgaria. Agric. Food, 6, pp.122-137.

Robertson, L., Muller, M., De Beer, D., Van der Rijst, M., Bester, C. and Joubert, E., 2018. Development of species-specific aroma wheels for *Cyclopia genistoides, C. subternata* and *C. maculata* herbal teas and benchmarking sensory and phenolic profiles of selections and progenies of *C. subternata*. South African Journal of Botany, 114, pp.295-302.

Schutte, A.L. and Van Wyk, B.E., 1998. Evolutionary relationships in the Podalyrieae and Liparieae (Fabaceae) based on morphological, cytological, and chemical evidence. Plant Systematics and Evolution, 209(1), pp.1-31.

Shinohara, Y., Akiba, K., Maruo, T. and Ito, T., 1994. Effect of water stress on the fruit yield, quality and physiological condition of tomato plants using the gravel culture. Hydroponics and Transplant Production 396, pp.211-218.

Stander, M.A., Joubert, E. and De Beer, D., 2019. Revisiting the caffeine-free status of rooibos and honeybush herbal teas using specific MRM and high resolution LC-MS methods. *Journal of Food Composition and Analysis*, *76*, pp.39-43.

Theron, K.A., Muller, M., Van Der Rijst, M., Cronje, J.C., Le Roux, M. and Joubert, E., 2014. Sensory profiling of honeybush tea (*Cyclopia* species) and the development of a honeybush sensory wheel. Food research international,66, pp.12-22.

Verbeke, W. and Ward, R.W., 2006. Consumer interest in information cues denoting quality, traceability and origin: An application of ordered probit models to beef labels. *Food quality and preference*, *17*(6), pp.453-467.

CHAPTER SEVEN
GENERAL CONCLUSION AND RECOMMENDATIONS

The honeybush industry is unable to meet the huge foreign demand that outweighs local supply. Therefore, the cultivation of *Cyclopia* species would not only guarantee the species' sustainability and conservation but will also enhance the standard of living for rural harvesters as well as commercial growers. It was evident that different soil type had no significant influence on growth parameters, proline, relative water content of *C. subternata*. However, water deficit stress increased the proline concentrations, decreased the relative water contents and lower yields. Higher proline concentrations which is an indication of higher water stress may also suggest that *C. subternata* are drought tolerant. Stomatal conductance of both potted and field-grown plants generally increased with increasing watering frequency and vice versa. This phenomenon has also been reported in other crop species. Although more than 600 proteins were identified amongst all the water treatments, only 11 produced significant results based on three filter parameters. Stress response related protein was also identified, where photosynthesis proteins were the most dominant. Gene ontology distribution of *C. subternata* leaf protein in response to different water stress treatments for differentially expressed proteins were mostly found in the biological processes of cellular process, metabolic process, and response to stimulus, respectively. This study suggests that proteomics contributed to the identification of crucial stress-responsive proteins in *Cyclopia* species. The use of proteomic tools can also help in propagation of honeybush plants that are water stress-tolerant, as well as identifying protein-encoding genes that can be selected for maximum yields. It can also be concluded that severe water-deficit stress seems to boost the sensory profile of the infusions, in particular the ‘woody’, ‘fynbos-floral’, ‘rose perfume’, ‘fynbos-sweet’ and ‘sweet spice’ aromas as well as a sweet taste. Therefore, *C. subternata* plants do not necessarily require excessive water supply to produce a better-quality tea.

Recommendations

- Research may be needed in future to assess the mechanisms responsible for making *C. subternata* plants more stress-tolerant compared to other important crop species.

- More studies need to be conducted on the effect of water stress on the sensory profile to get better and clear understanding on this topic as this was the first time that the effect of a prolonged water stress of *C. subternata* on its tea quality was demonstrated.

APPENDICES

Water SA 49(1) 64–72 / Jan 2023
https://doi.org/10.17159/wsa/2023.v49.i1.3988

Research paper

Cyclopia subternata growth, yield, proline and relative water content in response to water deficit stress

MS Mahlare[1], MN Lewu[2], FB Lewu[1] and C Bester[2]

[1]*Department of Agriculture, Faculty of Applied Sciences, Cape Peninsula University of Technology, Private Bag X8, Wellington 7654, South Africa*
[2]*ARC Infruitec-Nietvoorbij, Private Bag X5026, Stellenbosch 7599, South Africa*

Cyclopia, generally known as honeybush, and belonging to the *Fabaceae* family, originates from the Cape Floristic Region of the Eastern Cape and Western Cape provinces of South Africa. Currently, 6 honeybush species are commercially cultivated but, to date, there have been limited trials attempting to study their agronomic water demand. A pot trial was conducted where *Cyclopia subternata* plants were cultivated on different soil types (Stellenbosch granite, Stellenbosch shale and Stellenbosch clovelly) and subjected to three different water-deficit stress levels (well-watered, semi-stressed and stressed). Remarkably, irrigation treatments and soil types did not significantly affect the growth of the plants. However, the well-watered treatment consistently had higher yields compared to the other two treatments. The water-stressed (semi-stressed and stressed) treatments had lower relative water contents (RWC) with higher concentrations of proline, which signify water stress, compared to the control treatment. Higher proline and lower RWC contents found in this study are indications of water stress.

CORRESPONDENCE
FB Lewu

EMAIL
LewuF@cput.ac.za

DATES
Received: 9 March 2022
Accepted: 4 January 2023

KEYWORDS
honeybush
proline content
relative water content
tea plant
water deficit stress

INTRODUCTION

South Africa, a drought-prone country, is home to rooibos (*Aspalathus linearis*), bush (*Athrixia phylicoides*) and honeybush (*Cyclopia* species) teas (Joubert et al., 2011). The teas are sold as either black or green (fermented or unfermented, respectively) (Horn, 2019). Even though the commercialization of some of these remedial teas is still in its infancy stage, honeybush has gained recognition, while rooibos is the most well-known and well established in the industry (Van Wyk and Gericke, 2000; Joubert et al., 2008; Joubert et al., 2011).

Studies state that these South African indigenous tea species have essential nutrients (iron, calcium, magnesium, copper, and potassium) that can improve wellbeing and/or prevent diseases, and have economic potential (Rampedi and Olivier 2005; McGaw et al., 2007). These herbal teas are famous for their rich caffeine-free and organic antioxidant properties, which are helpful in colon, throat and lung illnesses, prevention of urinary stone and tooth caries and other medical problems (Soni et al., 2015).

The demand for honeybush tea has prompted concerns of over-exploitation of natural populations of the *Cyclopia* species. The increased rate of wild harvesting diminishes the natural population, thus making the exploitation of *Cyclopia* species unsustainable. Harvesting practices have contributed to the decrease and even disappearance of populations of the wild *Cyclopia* species (Du Toit et al., 1998). Other factors threatening the growth of the honeybush industry include drought and veld fires. To ensure sustainable production, commercial honeybush plantations have been established (Joubert et al., 2011).

Commercial production is therefore becoming increasingly important to save the natural populations from decline while ensuring consistent supply. Cultivation of *Cyclopia* species will not only contribute to sustainability and conservation of the species but will also improve the livelihoods of rural harvester communities. Although cultivated honeybush plants receive water through irrigation in addition to rainfall, irrigation volume is at the discretion of farmers, without any understanding of the water requirements of the species. Presently, the shortage of water has massively increased in some parts of the world, including some regions in South Africa, due to a variety of reasons such as an ever-increasing population, industrialization, water pollution and poor management, climate change and others (WWAP, 2012; Connor, 2015; Long and Pijanowski, 2017).

In addition, the South African Department of Water and Sanitation (DWS) has reduced agricultural allocations significantly, and irrigation volume for the agricultural sector is unlikely to increase anytime soon. For example, in 2015, DWS restricted an irrigation water allocation in KwaZulu-Natal by 40–100% due to a water shortage caused by insufficient rain (RSA, 2015). Also, agriculture in the Western Cape has had to cut its water use by 60% since 2017 (WWF, 2018). As a result, research that focuses on the sustainability of water-use in agriculture is gaining huge interest (Velasco-Muñoz et al., 2018). Environmental factors, including water stress, tend to interfere with crucial physiological processes and biochemical mechanisms; resulting in yield loss (Per et al., 2017).

Response of *Cyclopia Subternata* to Watering Frequency: Stomatal Conductance, Proline, and Relative Water Content

Mary-Jane S. Mahlare[1], Muinat N. Lewu [2], Francis B. Lewu [1*] and Cecilia Bester[2]

Abstract**—** *Cyclopia*, commonly referred to as honeybush, is an indigenous tea plant native to the Eastern and Western Cape provinces of South Africa and is known for its sweet taste and honey-like aroma. The tea is famous for its antioxidants and can be used in value-added products such as cosmetics and other food ingredients. It is estimated that there are 23 *Cyclopia* species in South Africa, but only six are used commercially. Studies on abiotic stresses in honeybush are limited and this study helped to investigate the response of the species to water stress mechanisms, which is of utmost importance for the development of drought resistant lines for this highly sought-after tea plant. A pot experiment was conducted on a Stellenbosch granite soil in which *Cyclopia subternata* plants were subjected to three different watering frequencies (thrice, twice and once a week). More frequent watering (control) showed highest percentage of plant growth than plants subjected to other watering treatments in all the three growth parameters investigated. Higher proline concentrations and lower relative water content were observed in the water stressed plants (watering twice and once a week). Stomatal conductance was generally lower in stressed plants and highest in well-watered plants. The drop in stomatal conductance in the stressed plants is due to the induction of stomatal closure which is a coping mechanism to aid survival by reducing transpiration rate.

Keywords**—** *Cyclopia*, stomatal conductance, proline content, relative water content, water stress

I. INTRODUCTION

*I*T is said that tea is the second most consumed beverage in the world after water. The type of tea (oolong, green, black, and herbal) usually depends on the post-harvest treatment [1]. Three different types of tea are grown in South Africa: Rooibos (Aspalathus linearis), bush (Athrixia phylicoides) and honeybush (*Cyclopia* species). While bush tea is still relatively unknown as a commercial product, honeybush has gained popularity, with rooibos being the best known and most established [2]-[4]. The market for honeybush is expected to grow due to the health benefits derived from it. Polyphenols, the antioxidants in honeybush tea, have beneficial effects on human health [5], [1]. Traditionally, honeybush has been used to treat disorders such as heartburn, ulcers, colic in infants, chronic tonsillitis, lung infections, nausea and mucus build-up in the respiratory tract or body cavities [3], [6]-[8]. There are 23 species of *Cyclopia* in the Cape Floristic Region of South Africa, of which only six are used commercially, among the six is *Cyclopia subternata*. About 82% of honeybush is still harvested in the wild [9]-[11]. These species have a very limited range and rare habitat requirements.

Abiotic stressors such as drought are undoubtedly one of the most limiting factors for plant growth [12], [13]. Plant growth is mostly limited by the unavailability of water and climate change is expected to increase the extent of water stress on agricultural soils [14], [15]. Plants accumulate proline and carbohydrates as a coping mechanism for water stress [8]. Though some authors question the direct relationship between proline accumulation and stress adaptation [16], others concluded that proline as a multifunctional molecule can serve as an osmolyte and radical scavenger by responding to a variety of abiotic and biotic stressors, or as a source of energy for regrowth by degrading in response to stress [17].

A plant's response to water stress is largely determined by the regulation of stomatal conductance. Water scarcity leads to stomatal closure, which is one of the first responses to water shortage [18]. The relative water content (RWC) of a plant can be used to determine how well or poorly it absorbs water and the extent of stress [19], [20]. RWC is defined as "the percentage of water present in the leaf as a fraction of the total volumetric water that the leaf can hold at full turgor" [21]. Under drought conditions, RWC is said to be a more accurate indicator of water status than any other metric of water potential. Leaf water supply and transpiration rate are closely linked and can give a good indication of the balance between these two variables [22]. Therefore, farmers need to understand how water use affects plant growth to maximize their productivity [23].

South Africa's arid climate, characterized by hot, dry conditions and low relative humidity, resulting in uneven distribution of rainfall and high evapotranspiration often leads to water stress [24]. There is very little research on how honeybush responds to drought stress in the Mediterranean climate of South Africa, where there is persistent drought during summer periods [25], [5], [8]. Therefore, the aim of

Mary-Jane S. Mahlare1 and Francis B. Lewu1 are with the Department of Agriculture, Faculty of Applied Sciences, Cape Peninsula University of Technology, Private Bag X8, Wellington 7654, South Africa

Muinat N. Lewu 2and Cecilia Bester2 are with ARC Infruitec-Nietvoorbij, Private Bag X5026, Stellenbosch 7599, South Africa

https://doi.org/10.17758/IICBE4.C1122203

plants

MDPI

Article

Analysis of the Differentially Expressed Proteins and Metabolic Pathways of Honeybush (*Cyclopia subternata*) in Response to Water Deficit Stress

Mary-Jane S. Mahlare [1,2], Lizex Husselmann [3], Muinat N. Lewu [1,*], Cecilia Bester [1], Francis B. Lewu [2,*] and Oluwafemi James Caleb [4,5,*]

1 Agricultural Research Council (ARC) Infruitec-Nietvoorbij, Stellenbosch 7599, South Africa
2 Department of Agriculture, Faculty of Applied Sciences, Cape Peninsula University of Technology, Wellington Campus, Private Bag X8, Wellington 7654, South Africa
3 Department of Biotechnology, University of the Western Cape, Bellville 7535, South Africa
4 Africa Institute for Postharvest Technology, Faculty of AgriSciences, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa
5 Department of Horticultural Science, Faculty of AgriSciences, Stellenbosch University, Matieland 7602, South Africa
* Correspondence: lewum@arc.agric.za (M.N.L.); lewuf@cput.ac.za (F.B.L.); caleboj@sun.ac.za (O.J.C.)

Abstract: Honeybush (*Cyclopia* spp.) is a rich source of antioxidant properties and phenolic compounds. Water availability plays a crucial role in plant metabolic processes, and it contributes to overall quality. Thus, this study aimed to investigate changes in molecular functions, cellular components, and biological processes of *Cyclopia subternata* exposed to different water stress conditions, which include well-watered (as Control, T1), semi-water stressed (T2), and water-deprived (T3) potted plants. Samples were also collected from a well-watered commercial farm first cultivated in 2013 (T13) and then cultivated in 2017 (T17) and 2019 (T19). Differentially expressed proteins extracted from *C. subternata* leaves were identified using LC-MS/MS spectrometry. A total of 11 differentially expressed proteins (DEPs) were identified using Fisher's exact test ($p < 0.00100$). Only α-glucan phosphorylase was found to be statistically common between T17 and T19 ($p < 0.00100$). Notably, α-glucan phosphorylase was upregulated in the older vegetation (T17) and downregulated in T19 by 1.41-fold. This result suggests that α-glucan phosphorylase was needed in T17 to support the metabolic pathway. In T19, five DEPs were upregulated, while the other six were downregulated. Based on gene ontology, the DEPs in the stressed plant were associated with cellular and metabolic processes, response to stimulus, binding, catalytic activity, and cellular anatomical entity. Differentially expressed proteins were clustered based on the Kyoto Encyclopedia of Genes and Genomes (KEGG), and sequences were linked to metabolic pathways via enzyme code and KEGG ortholog. Most proteins were involved in photosynthesis, phenylpropanoid biosynthesis, thiamine, and purine metabolism. This study revealed the presence of trans-cinnamate 4-monooxygenase, an intermediate for the biosynthesis of a large number of substances, such as phenylpropanoids and flavonoids.

Keywords: *Cyclopia subternata*; differentially expressed proteins (DEPs); water deficit stress; carbon fixation; proteomic analysis

Citation: Mahlare, M.-J.S.; Husselmann, L.; Lewu, M.N.; Bester, C.; Lewu, F.B.; Caleb, O.J. Analysis of the Differentially Expressed Proteins and Metabolic Pathways of Honeybush (*Cyclopia subternata*) in Response to Water Deficit Stress. *Plants* **2023**, *12*, 2181. https://doi.org/10.3390/plants12112181

Academic Editor: Inmaculada Pascual

Received: 2 March 2023
Revised: 10 May 2023
Accepted: 13 May 2023
Published: 31 May 2023

1. Introduction

Honeybush is the general term used to classify all known types of *Cyclopia* species, a genus in the leguminous family [1]. It is native to the fynbos biome in the Western and Eastern Cape Provinces of South Africa, and its tea has a sweet, honey-like taste [2]. *Cyclopia* species are among the few wild plants that have been turned into commercial commodities in South Africa. Although 23 species grow in the wild, only a few are currently used to make tea, including *C. subternata*, *C. intermedia*, *C. genistoides*, *C. maculata*, *C. longifolia*, and *C. sessiliflora* [3,4]. The popularity of honeybush can also be attributed to its low tannin