Plant growth, stress tolerant traits and regulation of heat activated proteins in *Aspalathus linearis* (Burm. f.) R. Dahlgren exposed to elevated temperature and drought

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Supervisors

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Flowering plant of *Aspalathus linearis*, rooibos (Photo: D MacAlister)

"It's full of stars"

Dr Dave Bowman
2001: A Space Odyssey

Declaration

I, Dunja MacAlister, know the meaning of plagiarism and declare that all the work in the document, save for that which is properly acknowledged, is my own. The thesis is submitted for the degree of PhD in the Department of Biological Sciences, University of Cape Town. It has not been submitted for any degree or examination at any other university.

I confirm that I have been granted permission by the University of Cape Town's Doctoral Degrees Board to include the following published paper and one submitted manuscript in my PhD thesis. My supervisors, A. Muthama Muasya, Olivier Crespo and Samson B.M. Chimphango, and collaborators, John B.O. Ogola, Sipho T. Maseko, Alex J. Valentine, Carl-Otto Ottosen and Eva Rosenqvist, have agreed that I may include the publications:

1. MacAlister D, Muasya AM, Crespo O, Ogola JBO, Maseko ST, Valentine AJ, Ottosen C-O, Rosenqvist E, Chimphango SBM. 2020. Effect of temperature on plant growth and stress tolerant traits in rooibos in the Western Cape, South Africa. Scientia Horticulturae. 263, 109137.
2. MacAlister D, Muasya AM, Crespo O, Ogola JBO, Maseko ST, Valentine AJ, Ottosen C-O, Rosenqvist E, Chimphango SBM. Stress tolerant traits and root proliferation of *Aspalathus linearis* (Burm.f.) R. Dahlgren grown under differing moisture regimes and exposed to drought. 2020. South African Journal of Botany. 131, 342-350.

My supervisors and collaborators have testified that I have made substantial contributions to the conceptualisation and design of the papers, and that I independently ran the experiments and wrote the manuscripts, with their guidance in the form of comments and suggestions (Appendix A).

Signed by candidate

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Abstract

Climate change is increasingly becoming a concern on plant growth, as seen in the increased number of warmer days and nights as well as an increased occurrence of heat waves, and drought periods globally. The Intergovernmental Panel on Climate Change has stated that global surface temperatures are constantly increasing and are likely to exceed 2 °C, compared to average temperatures in 1900, by the end of the 21st century. Changes in precipitation will also become more erratic, with high latitude and mid-latitude areas expected to have increases and decreases in rainfall respectively, while already dry areas will have increased frequencies of drought. Regions with Mediterranean climates, such as the Western Cape in South Africa, are particularly vulnerable to these climate impacts, with models and studies showing that there are already significant increases in temperatures, shifts in later winter rainfall and an increased severity of flooding. These climatic changes will impact both natural and agricultural plant species growth and distribution due to the changes in suitable growing conditions and regions. Plants are already exposed to a wide variety of environmental factors, each of which influences the growth, and deviations from the optimal conditions is considered abiotic stress and negatively affects plant growth. Plants in the field are rarely affected by only one stress as they are frequently exposed to a combination of abiotic stresses and with the changes in climate, plants will likely be experiencing abiotic stress such as heat and drought stress simultaneously. The aim of this thesis was to determine the effects of heat and drought on the plant growth and physiological performance of one of the most important indigenous commercial crops in South Africa, *Aspalathus linearis* (Burm.f.) R. Dahlgren, better known as rooibos tea, known for its many health benefits. This was achieved by focusing on three objectives: (1) determining the effects of temperature on plant growth and identifying the thermotolerant traits of the plants grown in the field along a temperature gradient, (2) determining the heat activated proteins and associated mechanisms for heat

tolerance in field grown plants and (3) determining the physiological and morphological responses of *A. linearis* grown under two moisture regimes and later exposed to drought.

The results for objective one are presented in chapter two, where a field study was conducted to observe the effects of temperature on the growth and stress tolerant traits of *A. linearis* grown at four farms sites in the Cederberg, South Africa along a temperature gradient. The four sites represent the rooibos farming area, from coolest to warmest respectively; Aurora (alt. 93 m), Citrusdal (alt. 588 m), Clanwilliam (alt. 312 m) and Uitsig (alt. 344 m). Aurora was also situated closest to the coastline, ~18 km, compared to the other farms. The traits observed were changes in gas exchange, carbohydrate concentrations, phenolics and pigments, along with biomass, over a two-year period. *Aspalathus linearis* plants showed evidence of transpirational leaf cooling during summer and this, combined with lower chlorophyll and high phenolic content, could be considered acclimatized adaptive changes allowing the plants to mitigate the heating effects of elevated temperatures.

Chapter three presented the results for objective two where the proteome of *A. linearis* was analysed from field plants along a temperature gradient. Protein samples were collected from the plants concurrently with the physiological samples for the previous chapter. These protein samples were quantified and then functionally annotated using the OrthoDB and UniProt databases. Overall, a total of 180 proteins were differentially expressed in the plants during exposure to high temperatures in the field. Of these 180 proteins, 113 were more upregulated in the cooler sites, Aurora and Citrusdal, and 67 proteins were more upregulated in the hotter sites, Clanwilliam and Uitsig thus indicating that with increasing temperatures there is a downregulation of proteins expressed during heat stress. From the 180 proteins, there were six main proteins involved in photosynthesis or light harvesting in *A. linearis*, with four of

the six proteins upregulated in plants grown at Aurora, the cooler site, and in the hottest site, Uitsig. This agrees with results from chapter two, where plants from Aurora had superior photosynthetic rates compared to the other plants therefore allowing them to grow and produce better biomass. The hotter sites upregulated heat shock proteins more than the cooler sites, suggesting that their expression could be enhancing the thermotolerance of *A. linearis* plants through their chaperone activity where they protect other proteins against denaturation. There were also numerous proteins expressed in the plants which were related to oxidation-reduction processes and antioxidants, most of which were expressed in the hottest site, Uitsig. One of the main concerns for plants during heat stress is the oxidative damage brought on by reactive oxygen species, and the expression of these proteins indicates that these proteins are contributing to the plants' thermotolerance through the production of antioxidant phenolic compounds as was seen in chapter two.

In chapter four, a glasshouse study was conducted where plants were grown at two different moisture regimes (field capacities, FC) and then exposed to drought and both physiological and morphological parameters were measured. Morphological parameters measured included plant biomass, root/shoot ratios, total root length, average root diameter, total root surface area and specific root length. Physiological parameters measured were gas exchange, carbohydrate and phenolic concentrations, pigment concentrations, leaf relative water content and water potential. During drought, the gas exchange, relative water content and non-structural carbohydrates in leaves were all reduced, while chlorophyll concentrations remained constant. *Aspalathus linearis* plants also had reduced stomatal conductance and transpiration, increased root/shoot ratios, root length and antioxidants such as polyphenol in leaves under drought conditions.

Overall, changes in soil nutrients, including boron, available phosphorus, manganese and copper, and increasing temperatures had a negative impact on crop biomass, however, the phenolic content, which is a measure of tea quality, did not vary with sites. This suggests that farmers who are planning on shifting their rooibos farming further south of Cederberg, could still achieve good growth and high yields without compromising the quality of the tea. It was also seen that *A. linearis* plants upregulated heat shock proteins, along with proteins involved in antioxidant compounds particularly in the hotter sites thereby playing a critical role in their acquired heat-stress tolerance. Plants in the cooler sites upregulated proteins involved in photosynthesis and chlorophyll production, therefore allowing them to have higher photosynthetic activity and subsequently higher productivity. The up and down regulation was based on comparing the warmer sites (heat-stressed) to the cooler sites (control). The plants grown at lower FC and then droughted, exhibited drought tolerant mechanisms which included higher root/shoot ratios as well as thinner roots, both of which are effective for water and nutrient uptake. Overall, plants in the 30 % FC treatment recorded lower P_{max}, g_s and E after three days in the drought conditions while 70 % FC plants were only affected after five days. Furthermore, plants grown under low moisture (30 % FC) conditions produced 50 % lower biomass compared to plants grown under adequate moisture (70 % FC) conditions. This implies that low rainfall and the occurrences of dry spells and drought, associated with climate change are likely to reduce the production of *A. linearis* in the Cederberg area. The combination of both field work and glasshouse studies have provided insight into how these plants are affected by both heat and drought stress, as well as declining soil nutrients such as calcium, magnesium, manganese iron, copper and potassium. *Aspalathus linearis* is tolerant to high temperatures as well as dry conditions, however, more needs to be explored with regards to their thresholds particularly since climate change is

likely to continue in the near future and eventually moving farming south will no longer be an option for farmers.

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A big thank you goes to my supervisors, Dr Samson BM Chimphango, Prof. A Muthama Muasya and Dr Olivier Crespo for providing me with the inspiration and guidance to get this project going. Another big thank you goes to the collaborators on this entire project, John BO Ogola, Sipho T Maseko, Alex J Valentine, Carl-Otto Ottosen, Eva Rosenqvist and Suhail Rafudeen, for all their specialist inputs along the way as well as their incredibly insightful and helpful scrutinization of my work. I am also immensely grateful to Hawwa Gabier, who took the time to help me through my protein data, while working on her own thesis.

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was amazing, particularly when I was stuck in the sand at one or two of the farms. I am very thankful to the Agricultural Research Council's Climate Monitoring Services (ARC-ISCW, Pretoria, South Africa) for the use of their weather stations and for providing us with data that were essential to this thesis. The research group from the University of the Western Cape, led by Dr Uljanna Hesse, need to be thanked for allowing us to use their rooibos protein database, which is not public as of yet, to analyse my results from CPGR, without which it wouldn't have been possible to do. This project would not have been possible without the financial support of the National Research Foundation (NRF), South Africa and the University of Cape Town.

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Table of Contents

Chapter 1

General Introduction

1.1 Background

Plants are exposed to a variety of biotic factors on a continuous basis and abiotic factors such as temperature, relative humidity, light, water and nutrient availability, determine how well plants grow (Mittler 2006, Ahmad and Prasad 2012, Źróbek-Sokolnik 2012), the effect of each being dependent on intensity and quantity. A certain quantity or condition of each abiotic factor is needed for optimal growth in plants, however, deviations from these conditions can be considered abiotic stress and can adversely affect a plant's growth and development (Bray et al. 2000). One of the main reasons for crop loss around the world is due to abiotic stresses on the plants, reducing the average yields for most crop plants by more than 50% (Boyer 1982, Wang et al. 2003, Mittler 2006). These stresses, and the combinations of them, lead to a wide range of responses from plants including changes in physiology, morphology, biochemical and molecular changes (Boyer 1982, Zandalinas et al. 2018). The Intergovernmental Panel on Climate Change (IPCC; Hoegh-Guldberg et al. 2018) has stated that, relative to 1850-1900, global surface temperatures are likely to exceed 2 °C by the end of the 21st century and have indicated that the number of warmer days and nights have increased all around the world as have the occurrence of heat waves. Furthermore, the changes in precipitation will be more erratic with increases in rainfall in high latitude and tropical regions, decreases in rainfall in mid-latitude and sub-tropical regions and frequencies of drought increasing in already dry areas (Hoegh-Guldberg et al. 2018). All this will have a negative impact on global crop productivity as well as yield (Long and Ort 2010, Casaretto et al. 2016, Hoegh-Guldberg et al. 2018).

1.1.1 Climate change in South Africa

Data compiled from Berkeley Earth by scientist Ed Hawkins, show very well the increasing average temperatures (more red stripes) around the world with South Africa showing the same pattern (Figure 1.1; Hawkins 2018). Climate models have also indicated that maximum temperatures could increase at double the global rate in South Africa while at the same time rainfall will continue to decrease (Engelbrecht et al. 2009, Malherbe et al. 2013). Many climate model studies have predicted an increase in the occurrences of extreme events leading to more intense and frequent droughts or floods in certain areas (Midgley et al. 2005, Hewitson and Crane 2006, New et al. 2006, Gizaw and Gan 2017). All of this will impact many agricultural crops as well as natural species due to changes in the suitable growing regions and conditions (Bita and Gerats 2013, Hoegh-Guldberg et al. 2018).

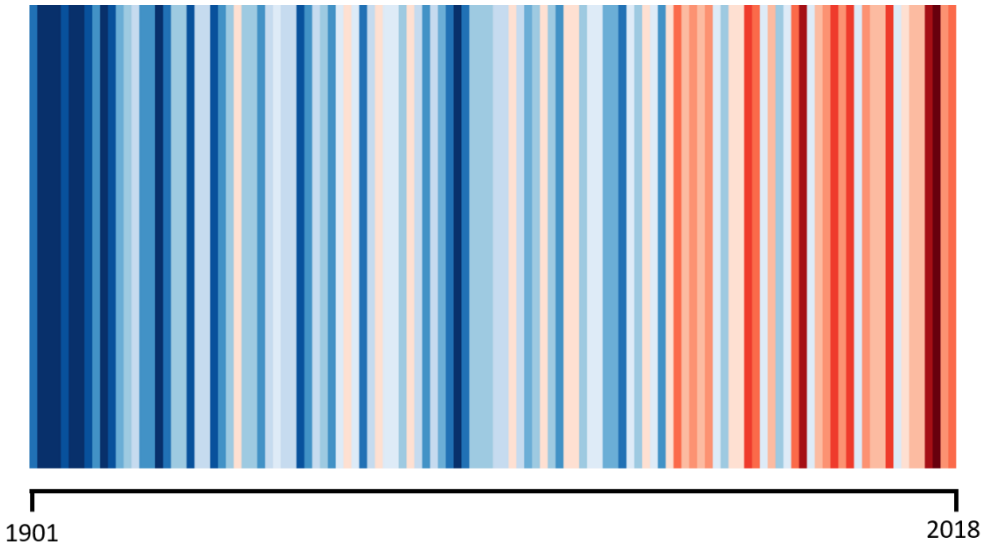

Figure 1.1. Annual average temperatures for South Africa from the period 1901 – 2018 using data from Berkeley Earth. Blue stripes indicate cooler years while red indicate warmer years (Hawkins 2018).

Within South Africa is the Western Cape, which has a Mediterranean climate and as such experiences hot, dry summers and cold, wet winters (Midgley et al. 2005). The IPCC has reported that Mediterranean ecosystems are particularly vulnerable to the impacts of climate change and indeed global climate models predict that Mediterranean climate regions will become warmer and drier, particularly during winters, and as a result these regions will shrink by as much as 7.2 % (Klausmeyer and Shaw 2009). Analyses done on the historical climate trends of the Western Cape show an increase in temperature extremes over most of the region as well as increases in the duration of warm spells while regional total precipitation had no significant changes (New et al. 2006, Kruger and Sekele 2013, MacKellar et al. 2014). It has been suggested that the frequency and intensity of rainfall events in the Western Cape will shift towards later winter rainfall as well as an increase in the severity of flood events (Midgley et al. 2005, Meadows 2006). During 2015 – 2017 the Western Cape experienced lower than usual rainfall while evaporation remained the same as previous years and both municipal and agricultural water use increased and as a result there was a water shortage (Wolski 2018).

1.1.2 Climate change in the Cederberg

In the Western Cape Mediterranean region, the Cederberg is home to *Aspalathus linearis* (Burm.f.) R. Dahlgren, better known globally as rooibos tea (Figure 1.2). The Cederberg region where rooibos farming takes place, experiences very hot dry summers (December – February) and cold, wet winters (June – August; Figure 1.3). A downscaled climate scenario showed that the likelihood of exceeding temperature thresholds of 32 °C was high and was likely to alter the spatial distribution patterns of wild and cultivated *A. linearis* (Archer et al. 2009, Lötter and le Maitre 2014). Several publications (e.g. Archer et al. 2008, Archer and Tadross 2009, Malgas et al. 2010) have looked at the projected climate impacts on *A. linearis*

distribution and farming, all of them indicating a trend in moving farming south where temperatures are cooler, and the areas are wetter. This has been partially supported by anecdotal evidence by Archer et al. (2008) as well as personal communications with Managing Director of Rooibos Ltd in Clanwilliam, Mr Martin Bergh, and Managing Director of Cape Natural, Mr Dawie de Villiers (Figure 1.4). Work by Archer et al. (2008) reported on the effects of drought in South Africa where the late winter rains in combination with possible heat stress have caused reduced yield and poor-quality *A. linearis*. Farmers have had to adapt their farming methods to sustain cultivation of *A. linearis* under these changing conditions, some of which include earlier ground preparation, deeper ploughing, ploughing fields more than once, harvesting at different times during the year, retaining bush strips on the land and planting wind breaks to prevent wind erosion (Archer et al. 2008).

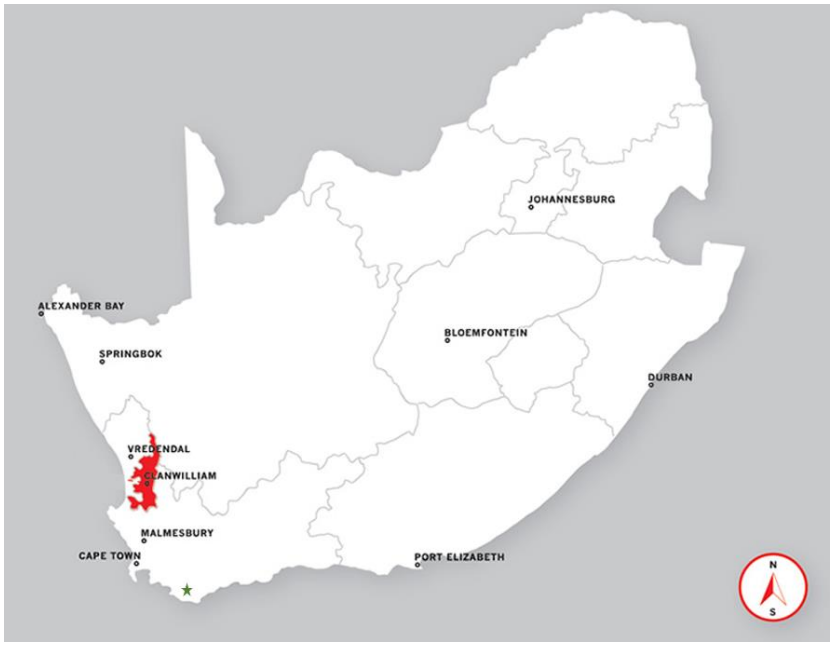

Figure 1.2. Distribution of *A. linearis* farming in South Africa (Rooibos Ltd 2019). The green star indicates where *A. linearis* farming is taking place in the Elim area in the Overberg district, Western Cape.

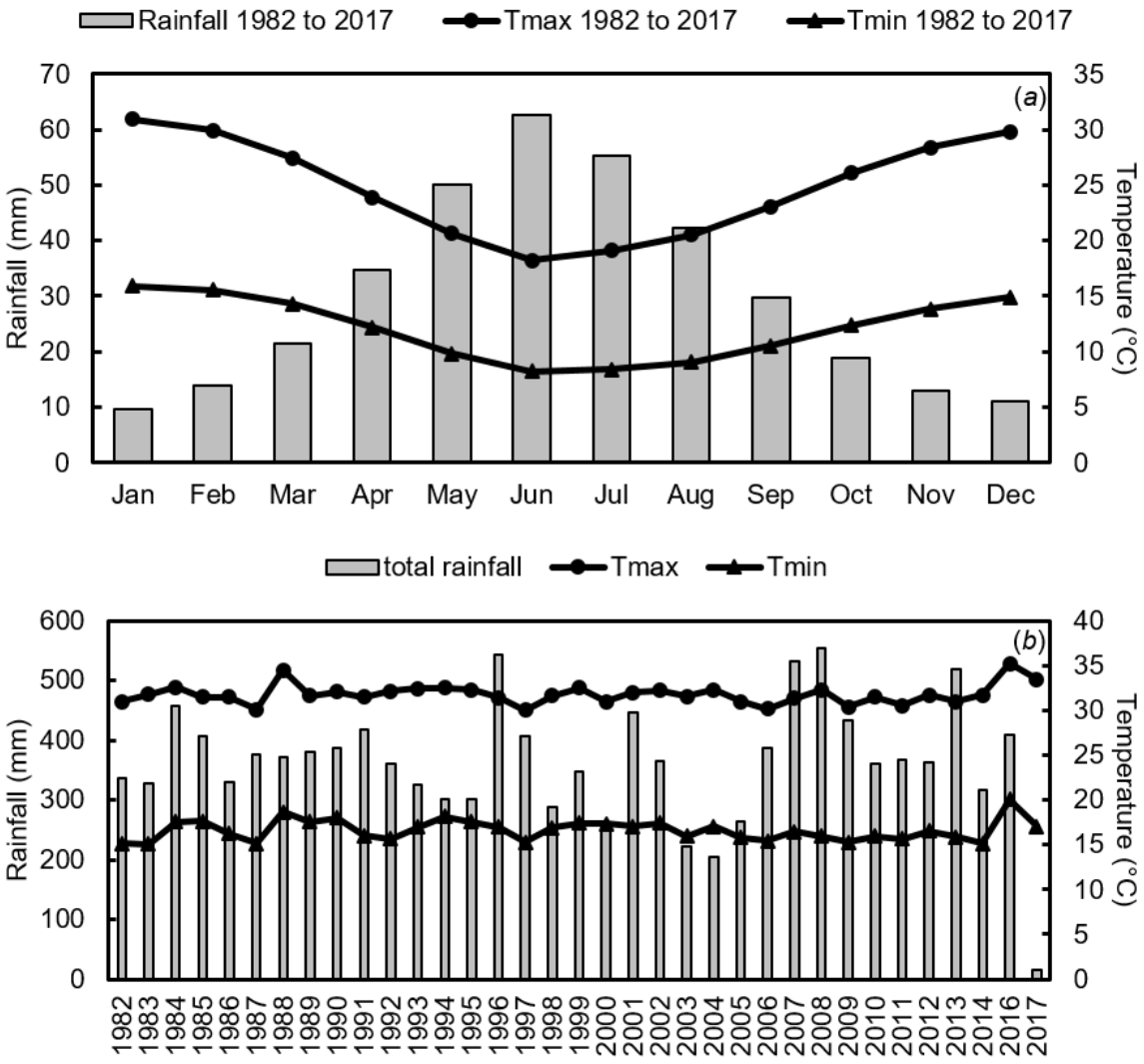

Figure 1.3. (a) Monthly seasonality showing the long term (1982 to 2017) monthly climatology of rainfall totals and monthly averaged minimum and maximum temperatures and (b) annual time series of temperature and rainfall in the Cederberg region. Data combined from two weather stations situated near Citrusdal and near Uitsig.

1.2 Rooibos – a brief overview on biology

Aspalathus linearis is a N_2-fixing shrub species endemic to the west coast of Southern Africa (Dahlgren 1968, Muofhe and Dakora 1999), the natural distribution of which can be seen in Fig. 1. (A) in Malgas et al. (2010). It is part of the Fabaceae, the second largest family in the Cape flora, with *Aspalathus* being the second largest genus in the Cape flora with 278

species, 254 of which are endemic (Edwards et al. 2008, Manning and Goldblatt 2012). *Aspalathus* spp. have an important ecological role to play in the post-fire environment where they contribute significantly to the total N capital of the soil through N_2-fixation (Cocks and Stock 2001). *Aspalathus linearis* is adapted to deep, well-drained oligotrophic and acidic sands (Muofhe and Dakora 2000) and its climatic distribution is determined by hot, dry summers and winter rainfall with an annual rainfall of 300 – 350 mm (Dahlgren 1968). The plants are also able to regenerate by re-seeding (cultivated) or re-sprouting (wild; van der Bank et al. 1999, Lötter and le Maitre 2014). The commercial cultivation of *A. linearis* is restricted to a geographical area (Figure 1.2) and any attempts to cultivate elsewhere have been met with failure due to *A. linearis*' unique soil and climatic requirements as well as its associations with other biotic components in the ecosystem (Morton 1983, Graaff et al. 2009). However, during this research, it was discovered that *A. linearis* cultivations had been done towards the south-most tip of Africa at Elim, in the Western Cape and *A. linearis* has been successfully growing there for the last ten years (Figure 1.2).

1.2.1 Production and demand

The first reported use of rooibos was in 1772 by botanist Carl Thunberg, who observed the native Khoisan using it as beverage (Thunberg 1795). It was much later, in the 20th century, that it was listed as a medicinal plant in South Africa, however, no specific applications were mentioned (Watt and Breyer-Brandwijk 1932). Marketing and small-scale domestication of *A. linearis* began in 1902, however, its economic value was not exploited until the 1930's, when research was put into its cultivation, leading to the development of the rooibos industry as it is modelled today (Morton 1983, Lötter and le Maitre 2014, Stander et al. 2019). Rooibos tea, which is made from the stem and leaves of *A. linearis*, is a well-known, mild-tasting herbal tea enjoyed in over 60 countries around the world (Joubert and de Beer 2011,

South African Rooibos Council 2019a). The tea is associated with important health benefits and great medicinal value since it contains no caffeine, very little tannins and a large amount of antioxidants (van Heerden et al. 2003, Joubert and de Beer 2011). It is one of the most important commercially cultivated indigenous crops in South Africa and contributes greatly to the welfare and cultural heritage of the local communities of the Western Cape (van Der Bank et al. 1995, Hawkins et al. 2011). There is an increasing world demand for rooibos tea that has contributed to an estimated R129 million in foreign earnings along with a domestic estimated retail sale value of R429 million in 2010 (Joubert and de Beer 2011). In 2018, the biggest importers of rooibos tea were Japan, Germany and the Netherlands with 29.42, 27.97 and 11.24 % of total exports going to these countries respectively (South African Rooibos Council 2019a). The rooibos industry has over 500 farmers that are producing rooibos at both a commercial and smallholder scale (South African Rooibos Council 2019a). In addition, wild rooibos is traditionally cultivated in the area by low-income people without land and it is not frequently harvested compared to the cultivated variety (Archer et al. 2008). The Heiveld Co-operative was formed in 2001, with the assistance of the Environmental Monitoring Group (a Non – Governmental Organisation), to help small-scale farmers in the area produce and market both wild and cultivated rooibos tea (Malgas and Oettlé 2007). With the Co-op's help, approximately 40 tons of wild rooibos is exported annually to several countries (Archer et al. 2008). There are a variety of wild forms and tea types of rooibos, including red, black, red-brown and grey. Only one of the red types, the Rocklands type, is used for commercial cultivation (van Der Bank et al. 1995).

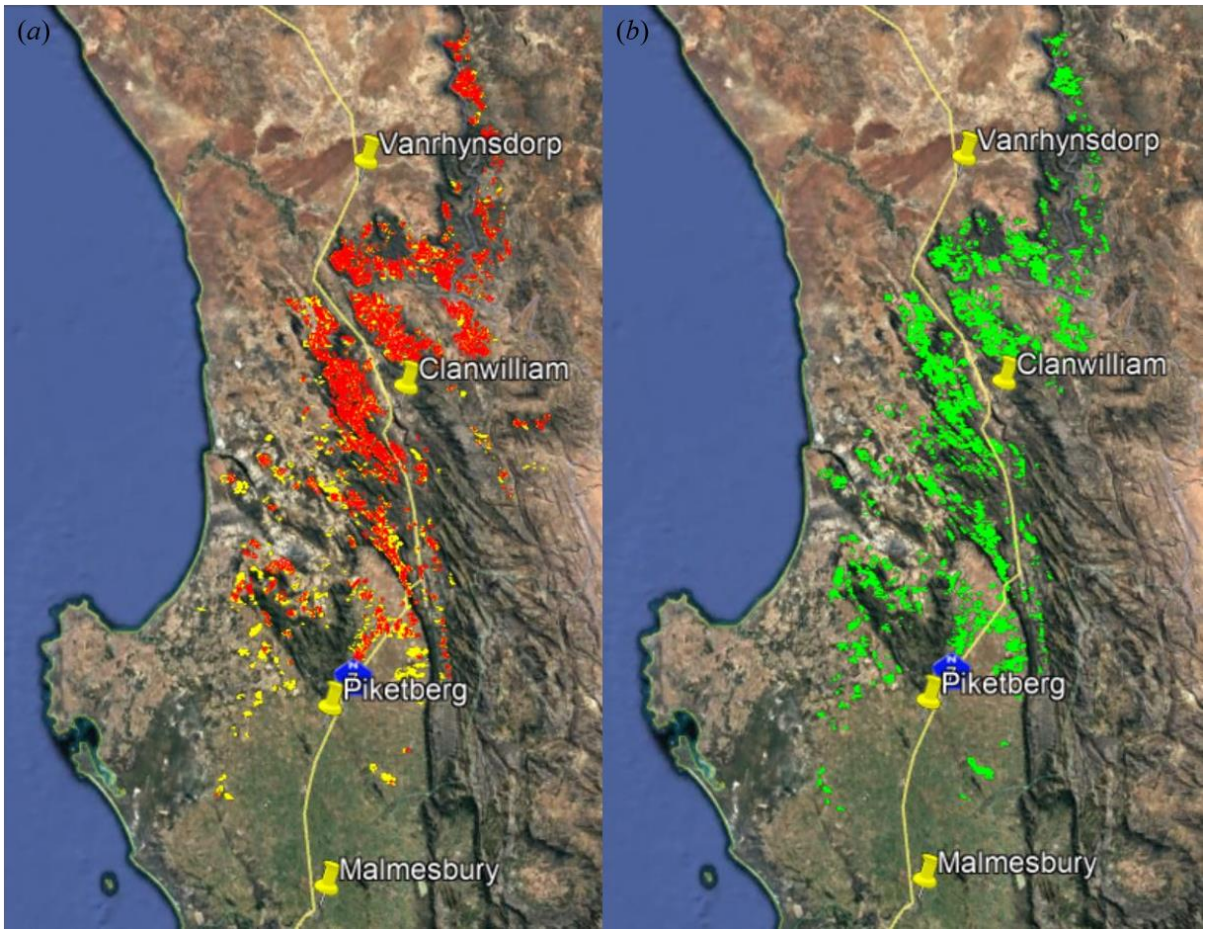

Figure 1.4. The distribution and movement of *A. linearis* farming during (a) 2016 (red area) and 2017 (yellow area) and (b) 2018 (green area; pers. comm. with Mr Dawie de Villiers).

1.2.2 Growth and physiology

One of the most important factors limiting the growth, development and yield of *A. linearis* is drought stress during the summer drought period as the plants are solely dependent on any rainfall they receive (Boyer 1982). The *A. linearis* plant is able to grow up to 2 m in height and is likely surviving the summer drought periods due to the presence of a deep taproot, which can extend as far down as 2 – 3 m (DAFF 2016, Ayeleso et al. 2017, Smith et al. 2018). As a legume, *A. linearis* is able to form symbiotic relationships with rhizobial bacteria and can nodulate with different groups of rhizobia belonging to α and β-Proteobacteria as well as rhizobia from the genus *Bradyrhizobium* (Hassen et al. 2012). As a result, *A. linearis*

plants can fix over 100 kg N ha^{-1} annually and thus minimize the need for fertilizing with N commercially (Muofhe and Dakora 1999, Sprent et al. 2010). A study by Kemp et al. (2018) looked at the seasonal variation of N_2-fixation, microbial activity in the soils and N cycling strategies of both wild and cultivated *A. linearis* and it was found that both N_2-fixation and N uptake by the plant roots are influenced and limited by the availability of water as well as soil nutrients such as N, P and C. Added to this, *A. linearis* is able to form symbiotic relationships with mycorrhizal fungi and produce cluster roots, both aiding in the uptake of nutrients, particularly P, from surrounding soils (Allsopp and Stock 1992, Sprent et al. 2010). Studies have also shown that *A. linearis* has superior growth and nodulation at low P compared to other legumes (Maistry et al. 2013) and that it is superior in producing extracellular acid phosphatase in its roots to aid P uptake (MacAlister et al. 2018). Numerous studies have been done on *A. linearis* due to its health benefits as a tea, mainly due to its high phenolic content (Ferreira et al. 1995, Joubert et al. 2008, Joubert and de Beer 2011, Ayeleso et al. 2017, Muller et al. 2018) with some recent work showing that it has a pool of both enolic acids and dihydrochalcone glycosides, not seen in other plants (Stander et al. 2017). With such an emphasis on its health properties, the South African Rooibos Council (SARC) will invest R4.5 million, between now and 2022, into the research of how rooibos can help tackle some of today's prevalent health issues such as allergies, heart disease, diabetes and skin cancer (South African Rooibos Council 2019b).

1.3 Heat tolerance

As a result of climate change and global warming, rising temperatures and heat stress are becoming a problem in agricultural crops all around the world (Hall 2001), including in *A. linearis* as is seen by the expansion of farming to cooler and wetter regions (Figure 1.4). Heat stress or heat shock is defined as the rise in temperature beyond a crop threshold for a period

of time that damages the plants' cells and negatively affects productivity (Wahid et al. 2007). Heat tolerance is therefore defined as a plants' ability to manage these higher than normal temperatures and acquire thermotolerance rapidly to survive the otherwise lethal temperatures (Vierling 1991, Coleman et al. 1995) and to be able to grow and produce economic yields (Wahid et al. 2007, Źróbek-Sokolnik 2012). As a result, heat stress may become a major limiting factor in field crop production and therefore an assessment of high temperature sensitivity is important (Wahid et al. 2007). High temperatures cause a range of morphological, anatomical, physiological and biochemical changes in plants which affects their growth and development and ultimately their yields (Wahid et al. 2007). These changes can include and are not limited to: accumulation of a variety of osmolytes, changes in photosynthesis, partitioning of assimilates, cell membrane thermostability, hormonal homeostasis and stability changes, induced biosynthesis of phenolics and suppressed oxidation of phenolics (Dias and Lidon 2010, Bita and Gerats 2013, Li et al. 2013, Shanmugam et al. 2013, Haque et al. 2014). Plants also exhibit responses such as the expression of stress proteins like heat shock proteins (HSPs; Wahid et al. 2007).

1.3.1 Gas exchange

At high temperatures, any constraints on photosynthesis can lead to limited growth of plants, therefore variations of different photosynthetic attributes, under heat stress, are good indicators of plant thermotolerance (Wahid et al. 2007). Both stomatal conductance and photosynthetic rates can increase with moderate increases in temperature, however, their activity could be inhibited if temperatures increase above the optimal threshold in many plant species due to the decrease in rubisco activation (Morales et al. 2003) while heat tolerant plants are able to maintain their photosynthetic rates, along with high stomatal conductance during hot spells (Sharma et al. 2015). This ability of plants to sustain their gas exchange

during periods of heat stress has a direct relationship with thermotolerance (Wahid et al. 2007, Sharkey and Zhang 2010). Another effect of elevated temperatures is the imbalance of photosynthesis and respiration, since photosynthesis decreases while respiration increases with increasing temperatures, causing a need for more carbon fixation to sustain plant growth and survival (Liu and Huang 2000, Wahid et al. 2007). Within the photosynthetic system, one of the most sensitive components is considered to be photosystem II (PSII) due to its ability to limit photochemistry in response to environmental stresses such as heat and light (Baker and Rosenqvist 2004). The measurement of chlorophyll fluorescence (F_v/F_m) reflects the maximum quantum efficiency of PSII photochemistry in dark-adapted leaves (Baker and Rosenqvist 2004). This has become a common measure of stress responses in plants and has been used to detect and quantify damage in PSII in response to heat stress in several crop plants including legumes (Herzog and Chai-Arree 2012), *Zea mays* (maize; Sinsawat et al. 2004) and *Triticum aestivum* (wheat; Sharma et al. 2012) where a decrease in F_v/F_m correlates with a linear decrease in the maximum quantum yield of photosynthesis.

1.3.2 Photosynthetic pigments

Pigments, such as chlorophyll and carotenoids, are integrally related to the physiological functioning of leaves (Sims and Gamon 2002) and as such are one of the best ways to understand the physiological and biochemical functioning in leaves during stress. Both chlorophyll and carotenoid molecules absorb light energy and transfer it to the photosynthetic centres in leaves (Sims and Gamon 2002), however carotenoids also have a secondary role in photoprotection (Young 1991). During stress, when the incident light exceeds that needed during photosynthesis, the excess energy causes the production of reactive chemical and oxygen species such as triplet chlorophyll, singlet oxygen and hydroxyl radicals (Niyogi 1999). These reactive species have detrimental effects on chlorophyll and photosynthetic

systems through oxidative damage (Camejo et al. 2006) and carotenoids dissipate the harmful excess energy therefore avoiding damage to the photosynthetic system (Demmig-Adams and Adams III 1996). The genetic variability in acquired thermotolerance has successfully been characterised through chlorophyll assays in crop species such as *Arabidopsis thaliana* (arabidopsis; Burke et al. 2000), *Glycine max* (soybean; Burke 1998), *T. aestivum* (O'Mahony et al. 2000, Dash and Mohanty 2001, Ibrahim and Quick 2001), *Lycopersicon esculentum* (tomato; Camejo et al. 2005) and *Arachis hypogaea* (peanut; Selvaraj et al. 2011). By decreasing the amount of photosynthetic pigments in the plant during heat stress, both photosynthetic and respiratory activity is reduced (Sharkey and Zhang 2010). For example, in *T. aestivum*, chlorophyll loss is closely linked to the heat-stress induced damage of the thylakoid membrane, and as a result, the loss of chlorophyll can be a high throughput screening method for thermotolerance (Shah and Paulsen 2003).

1.3.3 Phenolic content

The onset of heat stress induces the production of secondary metabolites such a polyphenols, flavonols and anthocyanins, suggesting an acclimation mechanism in plants against thermal stress and damage through antioxidant abilities (Rivero et al. 2001, Bita and Gerats 2013). The combination of enhanced synthesis of carotenoids and phenolics under stress conditions protects the cellular structures of the plants from oxidative damage (Close and McArthur 2002, Winkel-Shirley 2002, Wahid and Ghazanfar 2006). Anthocyanins, which are controlled in plant tissues by the prevailing temperatures and high visible light levels, protect sensitive tissues in the plant by acting as UV screens (Dixon and Paiva 1995, Mendez et al. 1999, Singh et al. 1999). The accumulation of phenolic compounds, under heat stress, has been reported in *L. esculentum*, *Citrullus lanatus* (watermelon; Rivero et al. 2001), *Z. mays* (Singh et al. 1999) and *Pinus banksiana* (jack pine seedlings; Nozzolillo et al. 1990). As mentioned

previously, rooibos tea is well known for its suggested health-promoting benefits, largely due to the phenolic composition of plants (Dai and Mumper 2010, Beelders et al. 2012). However, there is a natural variation in the phenolic concentration of the plants in nature (Joubert and Schulz 2012) possibly between cultivars. Due to most phenolics being glycosylated and stored in vacuoles, an equivalent amount of carbohydrates are needed for phenolics, therefore increases in total soluble sugars, parallels an increase in phenolics (Nozzolillo et al. 1990).

1.3.4 Carbohydrates

Many metabolites, osmolytes, accumulate in response to heat stress, some of which include proline, glycine betaine and soluble sugars (Wahid 2007). High temperatures modify the activity of enzymes responsible for carbon metabolism through the down-regulation of associated genes affecting sucrose synthesis and starch accumulation (Ruan et al. 2010). The production of osmolytes during heat stress has numerous uses such as increasing the stability of proteins, stabilizing the structure of membrane bilayers, regulating osmotic activities, maintaining cell water balance and buffering cellular redox potential (Sung et al. 2003, Farooq et al. 2008, Mirzaei et al. 2012). It has been suggested that high carbohydrate availability during periods of heat stress is an important physiological trait associated with thermotolerance (Liu and Huang 2000, Makonya et al. 2019). Reductions in the carbon accumulation in plants could result from higher levels of respiration compared to photosynthesis, which would subsequently increase the carbon consumption to production ratio during heat stress (Huang and Gao 2000). Indeed, sugars, particularly sucrose, are important metabolic signals in plants that aid in the regulation of plant development and response to stresses through carbon allocation and sugar signalling (Roitsch and González 2004). Carbohydrates also act as antioxidants in plants (Lang-Mladek et al. 2010) where at

high concentrations, sucrose becomes a reactive oxygen species (ROS) scavenger (Sugio et al. 2009).

1.3.5 Heat shock proteins

Apart from the physiological mechanisms mentioned previously, one of the most important molecular mechanisms involved in thermotolerance is the production of HSPs which have a wide range of molecular masses (Vierling 1991, Wahid and Close 2007, Bita and Gerats 2013). There are several classes of HSPs designated by their molecular weights as HSP110, HSP90, HSP70, HSP60 and small HSPs (sHSPs; Vierling 1991). Heat shock proteins have an important role in thermotolerance; they act as molecular chaperones and prevent the denaturation or aggregation of target proteins and they assist in protein refolding (Vierling 1991, Ahuja et al. 2010, Scharf et al. 2012). A proteome study on three *Oryza sativa* (rice) varieties, each with a different temperature tolerance, showed that more small HSPs accumulated in the *O. sativa* variety that was most tolerant to high temperature inferring a link between the accumulation of sHSPs and heat tolerance (Jagadish et al. 2010). Similarly, a study on heat tolerant *Vitis vinifera* (grape) genotypes showed higher expressions of HSP70, as well as genes related to stress protection, at elevated temperatures (Zhang et al. 2005). A study by Heckathorn et al. (1996) found that HSP production increased with increasing additions of N, indicating that these proteins have a high N demand. This suggests that the production of HSPs is resource-limited and could be costly in terms of resources for the plant, however, for plants like *A. linearis*, which fix their own N atmospherically, this could be an advantage during heat stress.

1.4 Drought stress

Many agricultural activities are dependent on the Mediterranean climate of the Western Cape and increases in future droughts will put them at risk (Archer and Tadross 2009). Drought stress in plants is considered to be a loss of water that leads to certain physiological and morphological changes which include stomatal closure and subsequently restricted gas exchange, reduced water content, lower leaf water potential and loss of leaf turgor (Chaves et al. 2002, Jaleel et al. 2009). The significance of the changes is dependent on the length and severity of the drought stress period that the plants are exposed to, ultimately affecting the establishment and growth and yield of the plants (Farooq et al. 2009, Jaleel et al. 2009). There are a variety of natural strategies that plants express for drought avoidance and tolerance seen in Mediterranean ecosystems where drought-avoiding, deep-rooted perennials and winter/spring annuals coexist with drought-tolerant sclerophyllous plants (Chaves et al. 2002). Drought can have numerous impacts on plants, some of which include water relations, osmotic adjustment, pigment content, photosynthetic activity, growth and yield (Benjamin and Nielsen 2006, Praba et al. 2009). Plants have several root and shoot traits that are known to improve resistance to drought and plant survival in these conditions (Araújo et al. 2015). Drought stress often enhance the allocation of biomass to the roots, which leads to increases in root length, density and depth. Having the ability to grow deep roots to access water stored in deep soils increases the total amount of water that is available to plants and contributes to the final yield of the plant in drought stressed environments (Farooq et al. 2009, Jaleel et al. 2009, Lynch 2013). Larger root biomass, greater root surface area, increased water uptake through enhanced transpiration and greater associations with N_2-fixing organisms are some of the characteristics associated with the proposed root ideotype for the optimization of water uptake, the “steep, cheap and deep” (Lynch 2013, Rao et al. 2016).

1.5 Problem statement

Despite all the information and studies on *A. linearis* with regards to its health benefits, there is not much known about the effects of heat and drought stress on the plants' physiology and tolerance mechanisms. Work by Lötter et al. (2014a) showed that *A. linearis* was negatively affected by drought and exhibited significant decreases in net photosynthesis, stomatal conductance and transpiration which all resulted in a decline in biomass as well as nutrient assimilation. It was also found that the seedlings of *A. linearis* had some mechanisms to enable them to cope with the climate change conditions. The seedlings were able to increase their water use efficiency, altering the allocation of photosynthates and developing a higher level of sclerophylly, all to compensate for the effects of drier conditions, allowing them to survive but with lower biomass (Lötter et al. 2014b). Wild *A. linearis* was found to be more resilient to the impacts of drought than the cultivated variety (Archer et al. 2008) due to its ability to increase water use efficiency and maintain higher levels of N_2 fixation during summer drought (Lötter et al. 2014a). While these reports mainly focussed on the aboveground physiology of the plant, there is no literature on the structure and morphology of *A. linearis* roots when exposed to drought conditions, nor is there information of the effects of growing *A. linearis* at different moisture regimes and then exposure to drought.

Some work has been done on the modelling of the distribution of *A. linearis* with regards to climate change (Lötter and le Maitre 2014), however, at present, there is not much known about the effect of high temperatures on the produced quantity and quality of *A. linearis* nor on the thermotolerant mechanism of these plants in response to high temperatures. Furthermore, both heat and drought stresses are likely to occur simultaneously further reducing the yield and quality of crop plants around the world, including *A. linearis* (Mittler and Blumwald 2010, Awasthi et al. 2014). There are also no published works on the

proteome of *A. linearis* in response to heat stress, therefore, considering the economic importance of *A. linearis* and the shifting cultivation areas associated with climate change this thesis was needed to determine whether *A. linearis* is indeed heat stressed in the field and what its thermotolerant mechanisms are. This would enable us to understand how *A. linearis* will react to future climate changes. It was hypothesized that plants growing in cooler areas, would have better biomass accumulation, higher photosynthetic activity, higher concentrations of chlorophyll in their tissues and lower phenolic concentrations than plants in hotter areas. While there was no hypothesis for the protein chapter, the aim was to determine whether heat activated proteins, along with other protective proteins, were upregulated in *A. linearis* plants during heat stress. Lastly, it was hypothesized that plants grown under low moisture conditions would be more tolerant to drought than those receiving adequate moisture.

1.6 Objectives of the thesis

The main objectives of this thesis are:

1. To determine the effects of temperature on the growth of *A. linearis* plants and identify the thermotolerant traits of the plants grown in the field along a temperature gradient.
2. To determine the proteomic changes of the plants in response to temperature conditions.
3. To determine the stress tolerant traits and root response of *A. linearis* plants grown at two water regimes (high and low moisture) and then exposed to drought.

Chapter 2

Effect of temperature on plant growth and stress tolerant traits in *Aspalathus linearis*

2.1 Introduction

Increases in temperatures around the world due to global warming and climate change are becoming an increasing problem for agricultural productivity, by causing a range of morphological, anatomical, physiological and biochemical changes in plants thereby affecting their growth and development, ultimately causing reduced yields (Wahid et al. 2007). For instance, studies suggest that climate change will lead to a reduction in both the geographic ranges as well as species richness in the Core Cape Region of South Africa (Midgley et al. 2002), with the fynbos biome potentially losing between 51 % and 65 % of its area, depending on the warming scenario (Lötter and le Maitre 2014). Possible scenarios under future climate change indicate that both wild and cultivated *A. linearis* plants will have a shift in their cultivation ranges south-eastward and upslope, as well as significant range contractions (Lötter and le Maitre 2014). Furthermore, up to 57 % of the climatically suitable range for *A. linearis* could be lost under climate-change conditions (Lötter et al. 2014b).

Most studies on heat stress have looked at model species such as *A. thaliana* (Bokszczanin and Fragkostefanakis 2013) and important crop species such as *Vigna unguiculate* (cowpea), *O. sativa* and *T. aestivum* (Wahid et al. 2007). However, there is a lack of understanding on certain local crops and how they have adapted to the increasing temperatures they are being exposed to in their natural environments. The nutritional and physiological responses of *A. linearis* to increased aridity have been documented albeit to a limited extent (Archer et al.

2008, 2009, Lötter and le Maitre 2014, Lötter et al. 2014b, 2014a). For example, wild cultivars of *A. linearis* were found to be more resilient to drought impacts than the cultivated variety due to their ability to increase water use efficiency and maintain higher levels of N_2-fixation during the dry summer periods (Archer et al. 2008, Lötter et al. 2014a) indicating higher physiological plasticity. In a glasshouse study, *A. linearis* exposed to drought for six weeks exhibited significant decreases in photosynthesis, stomatal conductance and transpiration, biomass accumulation and nutrient assimilation (Lötter et al. 2014b). However, it was shown that seedlings of *A. linearis* can cope with dry conditions through increased water use efficiency, altered allocation of photosynthetic products and more sclerophyllous leaves (Lötter et al. 2014b). Some modelling studies have looked at the impacts of climate change on the distribution of *A. linearis* farming (Archer et al. 2008, Lötter and le Maitre 2014). However, to the best of our knowledge no studies have examined the effects of high temperatures on the growth and thermotolerant traits of *A. linearis*.

From the foregoing and considering the economic importance of *A. linearis* and the shift in cultivation areas due to climate change and subsequent increased temperatures, this chapter assessed the effects of temperature on the growth and stress tolerant traits, such as changes in gas exchange, and concentration of carbohydrates, phenolics and pigments, of cultivated *A. linearis* over two years. Biomass and physiological measurements were taken in the field along a temperature gradient during summer in 2017, a particularly hot and dry year (Wolski 2018), and 2018 at four sites in the Cederberg. It was hypothesized that plants grown in the cooler areas, in Citrusdal and Aurora, would have better biomass accumulation, higher photosynthesis and stomatal conductance, and subsequently higher transpiration, as well as higher tissue chlorophyll concentrations and lower phenolic concentrations than those in Clanwilliam and Uitsig.

2.2 Material and methods

2.2.1 Site selection

Aspalathus linearis seeds, kindly supplied by Rooibos Ltd (Clanwilliam), were germinated in nursery beds in Clanwilliam from February 2016 and seedlings were transplanted into the fields at the start of the winter rains, June – July 2016 where they were left to grow without irrigation or fertilization. Plants were selected in a plot of 10 m × 10 m, located in a rooibos field of approximately 10 ha, with a planting density of approximately 10 000 plants per ha. Four sites, representing the rooibos farming area, were chosen in the Cederberg, Western Cape, South Africa, along a mean annual temperature (MAT) gradient from coolest to warmest respectively (Figure 2.1*a*); Aurora (32.685200S, 18.438050E, alt. 93 m, MAT 25.7 °C), Citrusdal (32.638367S, 18.958433E, alt. 588 m, MAT 26.0 °C), Clanwilliam (32.161417S, 18.777350E, alt. 312 m, MAT 28.2 °C) and Uitsig (31.920800S, 19.071833E, alt. 344 m, MAT 27.6 °C). Aurora was also situated closest to the coastline, ~18km, compared to the other farms. *Aspalathus linearis* plants are perennial and have an average lifespan of six years (Chimphango et al. 2016). Farmers harvest *A. linearis* plants during peak vegetative growth in summer (January – March; Chimphango et al., 2016) hence physiological parameters were measured on *A. linearis* in summer (February – March). Physiological parameters were also measured during winter (July – August) as a comparison between seasons. Measurements were taken one year after germination (1Y; summer and winter 2017) and two years after germination (2Y; summer 2018). At each site, measurements were done on six plants randomly selected from three rows in the field, by picking two adjacent plants in every second row, and avoiding the boundary plants.

2.2.2 Climate data

Hourly weather data was collected for each of the four sites via the Agricultural Research Council's Climate Monitoring Services (ARC-ISCW, Pretoria, South Africa), from stations ranging 12 to 16 km away from the experimental sites, with no major topological features in between, i.e. the weather stations and field sites were situated in the same altitude. Data collected included hourly mean temperature, rainfall, relative humidity (RH), radiation, potential evapotranspiration, wind speed and direction. The daily light integral (DLI; mol m^{-2} d^{-1}), the number of photons in the photosynthetic range which are integrated over the day (Poorter et al., 2016) was calculated by converting the global radiation (W m^{-2}) to photosynthetically active radiation (PAR; µmol m^{-2} s^{-1}) by multiplying radiation by 2.3. The vapor pressure deficit (VPD; kPa) was calculated using the temperature and RH data.

2.2.3 Soil analysis

Soil samples (four at each site) were collected, from the top 20 cm of soil using an auger or a garden trowel, in March 2016 prior to transplanting the seedlings in the field and in March 2018. Samples were air dried and sieved through a 2 mm mesh to remove debris. Soil pH, concentrations of carbon (C), potassium (K), sulphur (S), calcium (Ca), magnesium (Mg), iron (Fe), boron (B), copper (Cu), manganese (Mn), sodium (Na), zinc (Zn) and the percentage of sand, silt and clay, were analysed at the Plant Sciences Laboratory, Department of Agriculture Western Cape, Elsenberg, South Africa following standard soil analyses methods (Non-Affiliated Soil Analysis Work Committee 1990). Soil pH was analysed with a Metrohm Robotic Titrosampler after ion extraction with KCl. The concentrations of K, S, Ca, Mg, Fe, B, Cu, Mn, Na and Zn were determined by ICP-OES (Thermo Fisher Scientific Inc., MA, USA) and C was determined by the Walkley-Black method (Walkley and Black 1934). Available P was determined using the Bray II method (Bray and Kurtz 1945) and total P was

determined with ICP-OES (Varian, United States) adapted from Sommers and Nelson (1972) at the analytical laboratory Bemlab (Pty) Ltd. (Somerset West, South Africa). Total N concentrations in the soils were measured using mass spectrometry at the Archaeology Department, University of Cape Town. Soil samples were combusted in a Flash 2000 organic elemental analyser and the gases were passed to a Delta V Plus isotope ratio mass spectrometer (IRMS), via a Conflo IV gas control unit (all three items were Thermo Scientific, Bremen, Germany).

2.2.4 Plant biomass

During summer field measurements and sample collection, six plants from each farm site, were harvested for their aboveground shoot biomass. Leaf samples were collected to determine chlorophyll and carotenoid concentrations, secondary metabolites, carbohydrate concentrations, and for leaf C and N concentrations. The remaining biomass was oven dried at 60 °C for 72 hrs and their dry weight (DW) recorded.

2.2.5 Gas exchange measurements

Maximum photosynthetic rates (P_{max}), stomatal conductance (g_s) and transpiration (E) were measured using a LI-6400XT Portable Photosynthesis System (LI-COR Biosciences, Lincoln, NE, USA) on six plants at each farm site. The reference CO_2 concentration was maintained at 400 ppm while the flow rate was 400 μmol s^{-1} and the photosynthetic photon flux density (PPFD) inside the chamber was set to 1500 μmol photons m^{-2} s^{-1} (light saturation value derived from previous light curves, data not shown). Air temperatures in the chamber were set to ambient. The youngest fully-grown leaves were used for measurements which took place mid-morning on sunny, cloudless days. The IRGA head was clamped down on a portion of leaves (four – six leaves) and measurements were taken every ten seconds for a

period of five minutes, with the last ten measurements, averaged and used for the results as they were considered stable. Photosynthetic water-use efficiency (PWUE; μmol CO_2 mmol H_2O^{-1}) was calculated by dividing P_{max} by E. The leaves that were within the head of the LI-COR photosynthetic system were removed and scanned for total surface area with a STD4800 scanner and WinRHIZO version 2013a program (Regent Instruments, Quebec, Canada) to express gas exchange measurements per leaf area.

2.2.6 Chlorophyll and carotenoid concentration

At sample collection, four youngest fully-grown leaves from six plants, from each farm site, were collected and inserted into a vial with 10 ml of 95 % ethanol and left to auto-extract for 24 hr in the dark (Chappelle et al. 1992). After the 24-hr period, the leaves were removed, and their surface area was determined using a STD4800 scanner and WinRHIZO version 2013a program (Regent Instruments, Quebec, Canada). The light absorptions of the solutions were measured at specific wavelengths (664, 648 and 470 nm) on a Thermo Helios Epsilon spectrophotometer (Thermo Scientific, MA, USA) and chlorophyll and carotenoid concentrations were calculated using equations by Lichtenthaler (1987) for 95 % ethanol.

2.2.7 Secondary metabolites

Approximately 5 g of the youngest fully-grown leaves were collected from six plants from each farm site for total phenol, flavonol and anthocyanin analyses, immediately frozen in liquid N and kept frozen until they were lyophilized in a freeze drier (VirTis United Scientific, Cape Town, South Africa) and thereafter milled to a fine powder using a Hammer Mill (United Scientific Pty Ltd, Cape Town, South Africa). Crude extracts for total polyphenol concentrations and flavonol concentrations were prepared by stirring 0.01 g of the powdered leaves in 10 ml of 80 % (v/v) ethanol. Samples were then centrifuged at 3,220 × *g*

for five minutes. The supernatants were used for measurement of total polyphenol and flavonol concentration as described by Daniels et al. (2011) and read using a Thermo Helios Epsilon spectrophotometer (Thermo Scientific, MA, USA) at 765 nm and 360 nm respectively. For total polyphenol concentration, gallic acid was used to make the calibration curve using 0, 20, 50, 100, 250 and 500 mg L^{-1} solutions of gallic acid in 10 % (v/v) ethanol and total phenol values were expressed as mg g^{-1} of dry mass per gallic acid equivalent. For flavonol concentration, quercetin was used to make the calibration curve using 0, 5, 10, 20, 40 and 80 mg L^{-1} solutions of quercetin in 95 % ethanol and values were expressed as mg g^{-1} of dry mass per quercetin equivalent.

Approximately 0.1 g of powdered freeze-dried leaves were placed in vials with 10 ml of methanol: water: concentrated HCl (79:20:1) for anthocyanin (A) determination according to Lindoo and Caldwell (1978) and extracts were read using a Thermo Helios Epsilon spectrophotometer (Thermo Scientific, MA, USA) at 530 and 657 nm. The concentration of anthocyanin was determined using the following formula (Lindoo and Caldwell 1978):

$$\text{Anthocyanin (A/g DW)} = A_{530} - (0.333 \times A_{657}) \quad (1)$$

2.2.8 Non-structural carbohydrates

Samples were collected from the youngest fully-grown leaves (approximately 5 g) from each plant at each farm site at midday (Zhao et al. 2010), immediately put on ice, and transported to the laboratory, within three hours, where they were dried for 48 hrs at 60 °C. The dried samples were finely ground using a Hammer Mill (United Scientific Pty Ltd, Cape Town, South Africa) and glucose, fructose, sucrose and starch were assayed using the enzymatic method described by Zhao et al. (2010). Briefly, after the extraction process, glucose was assayed using a test-tube-scale glucose kit (GAHK-20). Fructose was quantified by the

addition of phosphoglucose isomerase (Sigma P9544) to the glucose aliquots and the subsequent determination of the amount of glucose released. The total glucose + fructose concentration was measured by adding the enzyme invertase (Sigma I4504) to hydrolyse sucrose in the aliquots. Sucrose concentration was determined with the following equation:

$$\text{Sucrose} = (\text{overall sum of glucose equivalents} - (\text{glucose} + \text{fructose}))\ 0.96 \qquad (2)$$

where 0.96 accounts for a water molecule that is added when sucrose is hydrolyzed. The determination of starch was done by enzymatically hydrolysing the starch in the pellet that remained from the previous extraction with alpha-amylase (Sigma A3403) and amyloglucosidase (Sigma A7095) and quantifying the amount of glucose released. All absorbances were obtained spectrophotometrically at 340 nm on a Thermo Multiskan Plate reader (Thermo Scientific, USA).

2.2.9 Leaf C and N concentrations

Concentrations of C and N in the plant leaves were measured at the Archaeology Department at the University of Cape Town using mass spectrometry (see description for soil N).

2.2.10 Statistical analysis

Canonical discrimination analysis (CDA) examined the multivariate biogeochemical differences between farm sites in the Cederberg to allow for separation of site and age groups. Further, Two-Way ANOVA tests were done to compare nutrients and physiological variables between sites and age of plants. Where the ANOVA tests showed significant differences ($P < 0.05$), the means were separated using a Tukey HSD *post hoc* test. Letters indicate significant differences between sites and age. Collinearity in soil variables (total P,

available P, pH, Ca, Mg, Na, K, Cu, Zn, Mn, B, S, C, Fe) and climate variables (wind direction, wind speed, maximum and minimum temperature, maximum and minimum RH, PAR, DLI, total evaporation and total rain) was determined through correlations where r values above 0.7 was considered collinearity (Dormann et al. 2013). The ecological importance of collinear variables was considered before the inclusion or exclusion of the variables in the subsequent regression (Dormann et al. 2013). A forward stepwise regression was used to determine which soil nutrients (total P, available P, Ca, Mg, Na, K, Cu, Zn, Mn, B, S, C, Fe) and/or climate variables (temperature, RH, DLI, total evaporation and total rain) significantly influenced the accumulation of aboveground biomass of *A. linearis* under field conditions. All statistical work was done in Statistica 13 (TIBCO Software Inc., CA, USA).

2.3. Results

2.3.1 Climate and soil

Clanwilliam and Uitsig sites had the higher average temperatures, both maximum and minimum, throughout the year compared to Aurora and Citrusdal (Figure 2.1*a*). For instance, during the growing time for *A. linearis*, September – February, the monthly average of maximum and minimum temperatures ranged from 20 – 40 °C in Clanwilliam and 17 – 37 °C in Aurora. Rainfall was highest in Citrusdal during 2016 with a total of 598 mm, followed by Aurora, Clanwilliam and Uitsig which had 292, 236 and 220 mm of rain respectively (Figure 2.1*b*). During 2017 rainfall decreased drastically for all sites, with Clanwilliam, Aurora, Uitsig and Citrusdal receiving 12, 82, 120 and 263 mm of total rain respectively (Figure 2.1*b*). In 2018, the amount of rainfall increased relative to 2017 with Clanwilliam, Aurora, Uitsig and Citrusdal receiving 169, 222, 302 and 326 mm of rainfall respectively (Figure 2.1*b*). Clanwilliam and Uitsig had almost three times as many days where temperatures were

over 35 °C, a potentially damaging threshold for most physiological processes (Ladjal et al., 2000), compared to Aurora and Citrusdal, particularly in the summer months (Figure 2.2). Daily light integral was highest in Aurora across all three years, particularly during the summer months; December – February (Figure 2.1*c*). The DLI ranged from 36 – 76 mol m^{-2} d^{-1} in Aurora and 30 – 69 mol m^{-2} d^{-1} in Clanwilliam during the growing period. Total evapotranspiration was highest at Aurora and Uitsig throughout the years, with peak levels of evapotranspiration occurring during the summer months of December and January (Figure 2.3*a*). Aurora had the highest windspeeds throughout the years, with the highest speeds recorded during the summer months, and lowest during the winter months (Figure 2.3*b*). The windspeed ranged from 3.9 – 8.4 m s^{-1} in Aurora and 2.0 – 5.1 m s^{-1} in Clanwilliam during the growing period. Citrusdal and Uitsig had similar and lower wind speeds than Aurora, while Clanwilliam had the lowest windspeed throughout the years (Figure 2.3*b*). Wind direction was predominantly SSW at Aurora, Clanwilliam and Uitsig sites while for Citrusdal it was WNW (data not shown). Aurora had the highest average maximum and minimum relative humidity (RH) compared to the other sites across the years while Clanwilliam and Uitsig had similar and lower values (Figure 2.3*d*). During the growing period, the RH ranged from 33 – 87 % in Aurora, 27 – 79 % in Citrusdal, 21 – 73 % in Clanwilliam and 20 – 71 % in Uitsig. Both Clanwilliam and Uitsig had high VPD throughout the year compared to Aurora and Citrusdal (Figure 2.3*d*). During the growth period, the average maximum VPD for Clanwilliam and Uitsig was 4.0 and 3.9 kPa respectively and 3.0 and 2.9 kPa for Citrusdal and Aurora respectively.

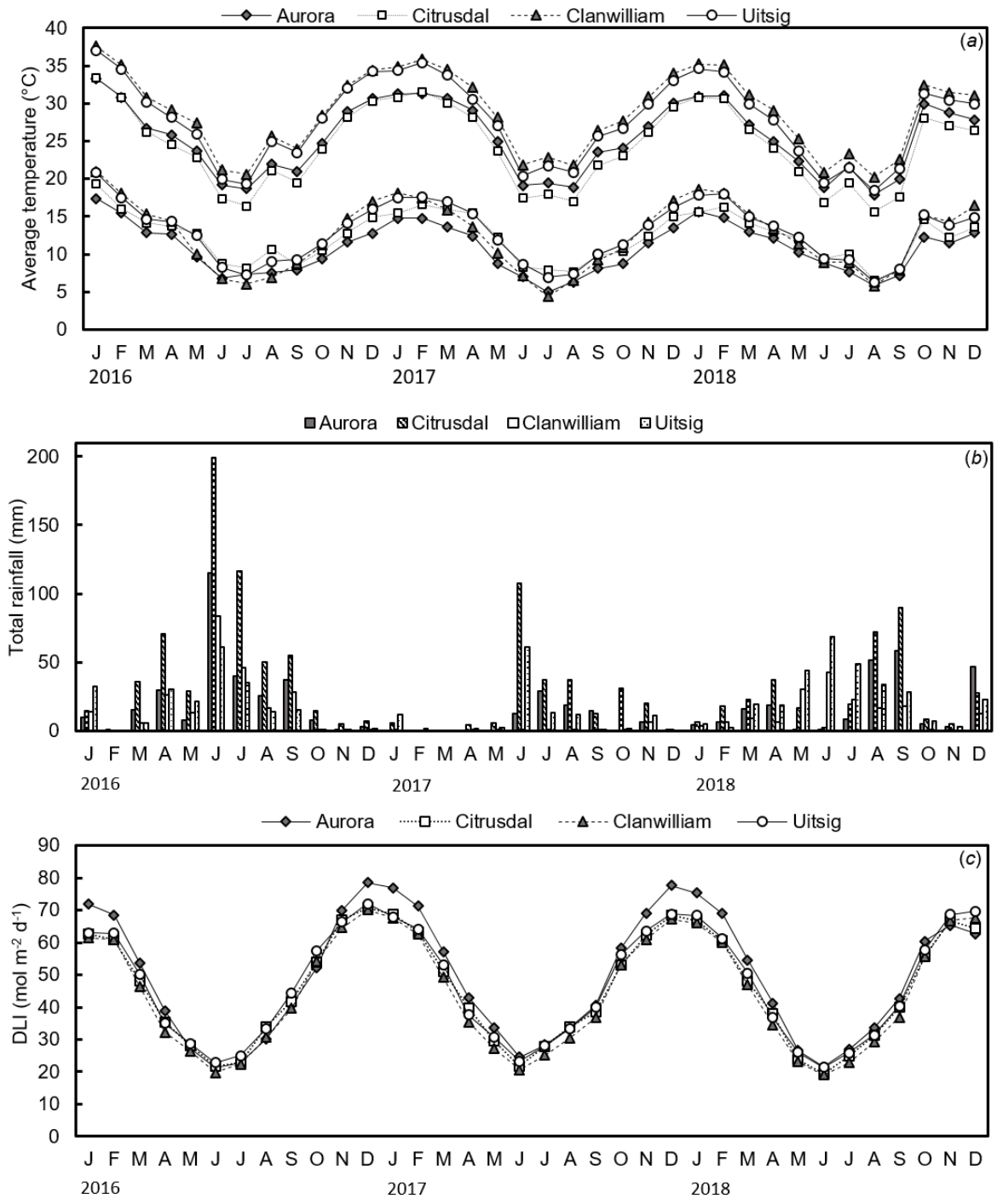

Figure 2.1. January 2016 to December 2018 in the Cederberg region, at the four sites; Aurora (diamond), Citrusdal (square), Clanwilliam (triangle) and Uitsig (circle); (*a*) Average maximum and minimum monthly temperatures, (*b*) total monthly rainfall and (*c*) average daily light integral.

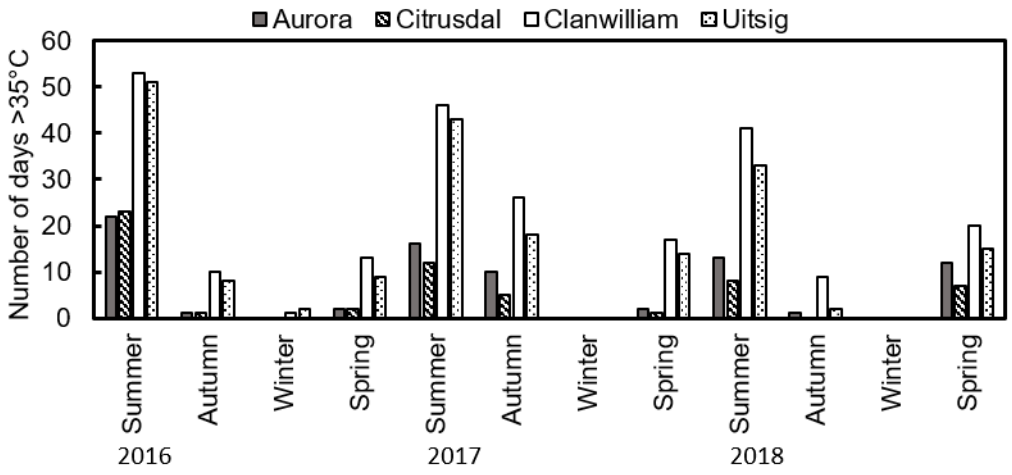

Figure 2.2. Total number of days in each season with maximum temperatures > 35 °C from Summer 2016 to Spring 2018 in the Cederberg region, at the four sites; Aurora, Citrusdal, Clanwilliam and Uitsig.

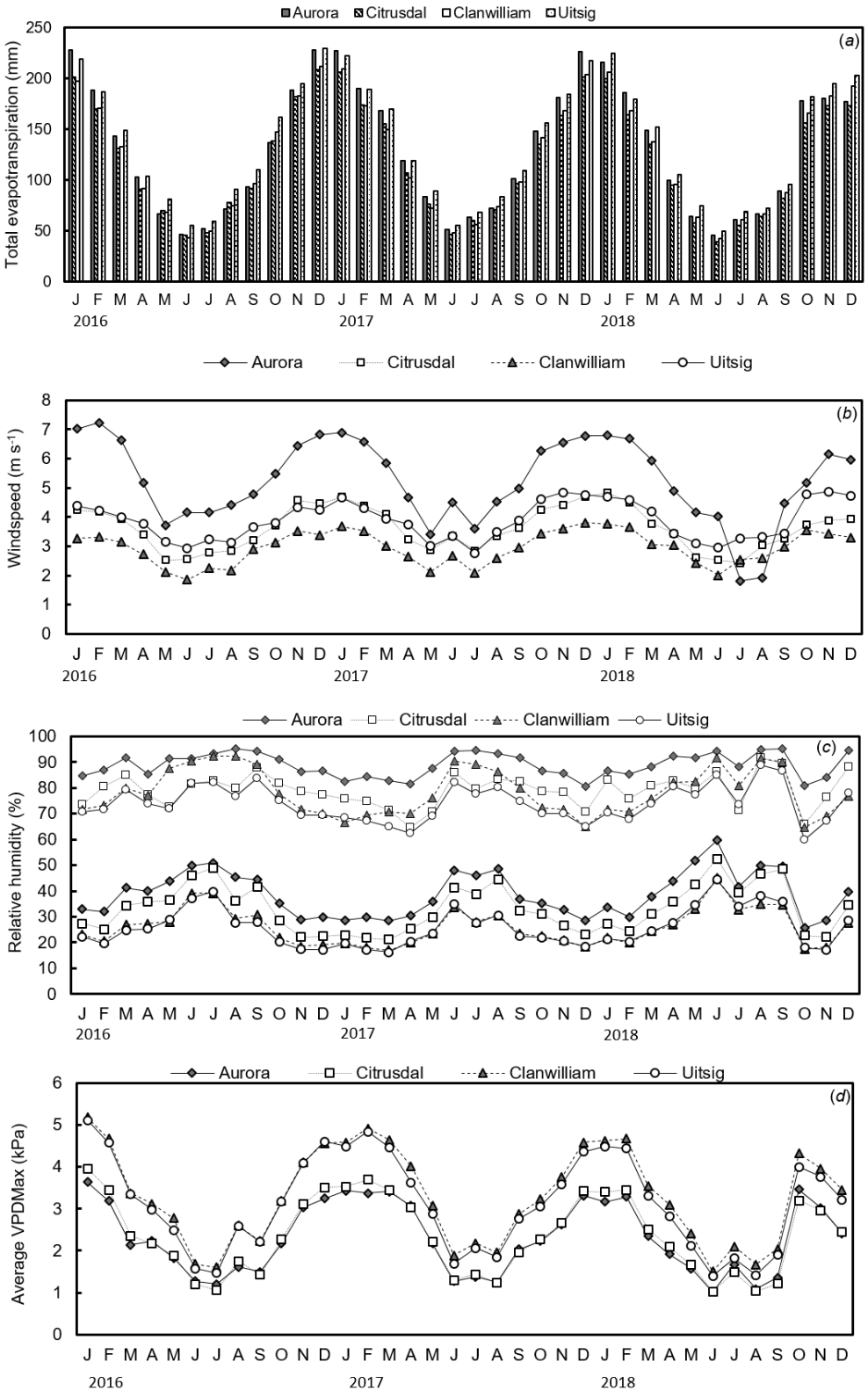

Figure 2.3. January 2016 to December 2018 in the Cederberg region, at the four sites; Aurora (diamond), Citrusdal (square), Clanwilliam (triangle) and Uitsig (circle); (*a*) total evapotranspiration, (*b*) average maximum windspeed, (*c*) average maximum and minimum relative humidity and (*d*) average maximum vapor pressure deficit.

Canonical discriminant analysis of the soil nutrient concentrations (total P, available P, Ca, Mg, Na, K, Cu, Zn, Mn, B, S, C and Fe) and the pH allowed for the sites to be split into sites and year groups. The scatterplot of the canonical scores of soil nutrients grouped together the soils sampled in 2016 separately from soils collected in 2018 along the covariate variable (CV) one (Figure 2.4). The Aurora site was also separated from the other sites in both years along the CV 2. The Squared Mahalanobis distances (Table 2.1), interaction between CV 1 and CV 2 showed that the separation was statistically significant in each grouping of site and year ($P < 0.05$ and 0.001). Covariate variable 1 and CV 2 accounted for 69.1 and 14.6 % of the variation between the soils respectively (Table 2.2). The standardized coefficients along CV 1 indicated that B, Bray II P, and S contributed the most to the separation of the soils into the groups, whereas pH, Cu, C and Mg contributed the most in CV 2 (Table 2.2).

Table 2.1. Squared Mahalanobis distance between the soils collected during 2016 (a) and 2018 (b; $n = 4$). Distances are given below the diagonals and *F*-statistics (7,13) are given above the diagonals and are significantly different as follows: *, $P < 0.05$; ***, $P < 0.001$.

Site	Aur a	Cit a	Cla a	Uit a	Aur b	Cit b	Cla b	Uit b
Aur 2016	-	9.4***	10.0***	15.4***	24.5***	44.5***	46.9***	25.5***
Cit 2016	103.9	-	6.7***	10.0***	51.0***	53.9***	66.6***	39.3***
Cla 2016	111.2	74.6	-	3.3*	46.5***	49.6***	50.8***	26.7***
Uit 2016	170.6	110.6	36.8	-	57.3***	66.9***	63.2***	32.1***
Aur 2018	271.5	565.1	514.9	634.8	-	20.2***	23.9***	14.6***
Cit 2018	493.1	597.1	549.5	741.2	223.4	-	13.9***	18.5***
Cla 2018	519.5	737.9	563.1	699.6	265.1	153.5	-	13.0***
Uit 2018	282.7	435.9	295.7	356.0	161.3	205.4	144.1	-

Table 2.2. Standardized coefficients of soil variables along canonical variates one and two showing their contribution to the discriminant function for the separation of the soils collected during 2016 and 2018 (n = 4). Eigenvalues provide a relative indication of how well each canonical variate (i.e. function) differentiates the groups. Among group variance indicates the proportion of the total variation accounted for each canonical variate. Wilk's Lambda values at ***, P<0.001.

Variable	Canonical variate 1	Canonical variate 2
B	-2.240	-0.064
pH	0.569	-1.233
Mn	-0.332	0.782
Av. P Bray II	1.514	0.142
K	0.120	-0.017
C	0.933	-0.879
Cu	0.643	-1.113
S	1.405	-0.265
Zn	-0.792	0.285
Ca	-0.547	-0.063
Fe	-0.536	0.353
Mg	0.307	0.742
Eigenvalue	144.15	30.50
Among group variance (%)	69.1 %	14.6 %
Wilk's Lambda	<0.001***	<0.001***

Soils at all sites were mainly sandy and acidic. Soil pH, Mg, Fe, Cu and Zn were only significantly different between sites while C, total P, Bray II P, K, S, Ca, B, Mn and Na were significantly different in their interaction between sites and years (Table 2.3). Soils at Aurora and Citrusdal had higher pH levels than Clanwilliam and Uitsig, while Aurora had the highest levels of Mg, Fe, Cu and Zn compared to the other sites (Table 2.3). Soil N concentrations increased substantially from 2016 to 2018 but had no differences between the sites within the

years (Table 2.3). Both total P and B concentrations increased in the soils from 2016 to 2018 at all the sites (Table 2.3). Soil C increased in the soils in Uitsig from 2016 to 2018, while soil Ca increased in Citrusdal soils only (Table 2.3). Soil Mn was similar across the years at the sites except in Clanwilliam where it decreased from 2016 to 2018 (Table 2.3). There were no differences in soil S between sites or years (Table 2.3) while soil Na decreased in Uitsig soils from 2016 to 2018 and K decreased in Clanwilliam soils only in 2018 (Table 2.3).

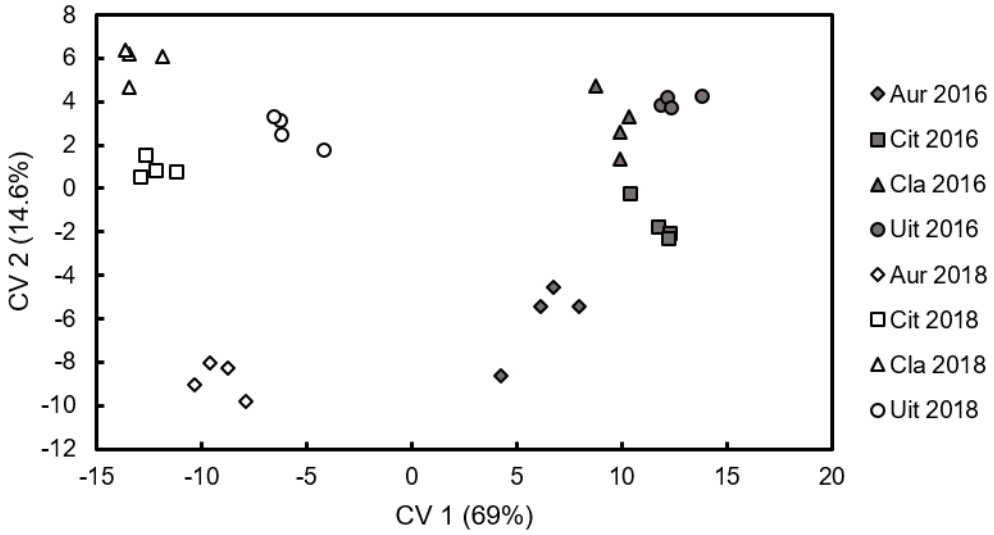

Figure 2.4. Scatterplot of canonical scores separating farm sites according to their year of soil collection; 2016 (soil collected before *A. linearis* planted in fields) and 2018 (soil collected from 2Y plant sites). Soil nutrients included total P, available P, Ca, Mg, Na, K, Cu, Zn, Mn, B, S, C, Fe and pH.

Table 2.3. Soil data collected from the four farm sites; Aurora (Aur), Citrusdal (Cit), Clanwilliam (Cla) and Uitsig (Uit), all sandstone derived soils, from the Cederberg in 2016 and 2018. Means ± s.e. (n = 4) followed by capital letters show significant differences between sites only or between year only, while small letters indicate significant differences between site and year interactions as follows: *, P<0.05; **, P<0.01; ***, P<0.001.

Variable	2016				2018				*F*-ratio		
	Aur	Cit	Cla	Uit	Aur	Cit	Cla	Uit	Year	Site	Yr+Site
pH	6.15 ± 0.19 C	5.80 ± 0.17 C	5.00 ± 0.29 B	4.05 ± 0.03 A	6.28 ± 0.06 C	5.83 ± 0.05 C	4.43 ± 0.1 B	4.13 ± 0.09 A	0.7	86.4***	2.4
C (%)	0.23 ± 0.02 ab	0.25 ± 0.02 ab	0.19 ± 0.04 a	0.13 ± 0.01 a	0.64 ± 0.24 bc	0.38 ± 0.03 ab	0.18 ± 0.03 a	0.83 ± 0.09 c	21.4***	4.0*	5.6**
N (%)	0.018 ± 0.002 A	0.019 ± 0.001 A	0.014 ± 0.004 A	0.013 ± 0.000 A	2.456 ± 0.123 B	2.459 ± 0.471 B	1.788 ± 0.339 B	1.801 ± 0.314 B	116.3***	1.0	0.9
Total P (mg kg^{-1})	39.4 ± 2.1 a	32.7 ± 3.1 a	40.7 ± 1.8 ab	35.0 ± 1.6 a	98.5 ± 8.5 d	114.0 ± 12.3 d	68.3 ± 3.7 bc	87.8 ± 5.3 cd	170.9***	3.9*	6.8**
Available P (mg kg^{-1})	22.1 ± 2.3 abc	25.9 ± 3.2 bc	14.2 ± 3.8 ab	17.8 ± 1.4 ab	35.7 ± 4.6 c	28.6 ± 4.6 bc	8.3 ± 0.6 a	21.5 ± 1.7 abc	2.5	13.5***	3.3*
K (mg kg^{-1})	44.0 ± 5 ab	45.5 ± 3.2 ab	67.5 ± 16 b	40.5 ± 5 ab	43.3 ± 7.7 ab	61.3 ± 6.3 b	27.8 ± 1.7 a	39.3 ± 1.8 ab	2.0	1.2	5.9**
S (mg kg^{-1})	2.68 ± 0.09 a	2.73 ± 0.11 a	2.95 ± 0.22 a	2.98 ± 0.05 a	3.10 ± 0.34 a	2.80 ± 0.27 a	2.25 ± 0.06 a	2.28 ± 0.07 a	3.0	1.1	4.8**
Ca (cmol kg^{-1})	1.08 ± 0.09 bc	1.53 ± 0.11 c	0.66 ± 0.19 ab	0.21 ± 0.01 a	1.72 ± 0.31 cd	2.27 ± 0.15 d	0.52 ± 0.13 ab	0.26 ± 0.03 a	8.5**	47.6***	3.9*
Mg (cmol kg^{-1})	0.42 ± 0.01 C	0.26 ± 0.03 B	0.31 ± 0.12 B	0.11 ± 0 A	0.51 ± 0.04 C	0.36 ± 0.04 B	0.24 ± 0.09 B	0.15 ± 0.01 A	1.5	18.3***	1.2
Fe (mg kg^{-1})	73.0 ± 7.7 B	25.7 ± 1.4 A	33.3 ± 2.2 A	32.3 ± 1.6 A	76.4 ± 7.9 B	24.3 ± 0.3 A	50.3 ± 11.5 A	37.0 ± 5 A	2.1	29.3***	0.9
B (mg kg^{-1})	0.105 ± 0.012 b	0.045 ± 0.005 a	0.053 ± 0.014 a	0.023 ± 0.003 a	0.323 ± 0.02 d	0.280 ± 0.007 d	0.215 ± 0.012 c	0.213 ± 0.005 c	648.0***	28.5***	4.0*
Cu (mg kg^{-1})	0.45 ± 0.048 C	0.30 ± 0.055 B	0.19 ± 0.013 A	0.16 ± 0.005 A	0.42 ± 0.066 C	0.17 ± 0.015 B	0.04 ± 0.003 A	0.04 ± 0.004 A	19.0***	37.9***	1.0
Mn (mg kg^{-1})	2.82 ± 0.2 ab	6.37 ± 1.09 cd	5.04 ± 0.48 bc	2.12 ± 0.18 a	2.01 ± 0.06 a	8.64 ± 0.44 d	2.32 ± 0.37 a	2.39 ± 0.5 a	0.5	45.7***	8.3***
Na (mg kg^{-1})	14.3 ± 1.9 bc	13.0 ± 2.0 abc	12.7 ± 0.9 abc	13.8 ± 0.8 bc	18.7 ± 0.7 c	9.0 ± 0.4 ab	8.0 ± 0.7 a	7.8 ± 0.5 a	8.9**	10.8***	7.3**
Zn (mg kg^{-1})	0.99 ± 0.16 C	0.78 ± 0.16 B	0.42 ± 0.13 A	0.32 ± 0.03 A	0.90 ± 0.09 C	0.51 ± 0.07 B	0.29 ± 0.02 A	0.27 ± 0.00 A	3.6	17.8***	0.5

2.3.2 Aboveground biomass

Aspalathus linearis plants had 20-times more biomass in their second year of growth compared to their first-year counterparts (Figure 2.5). The two-year-old (2Y) plants at Aurora had higher biomass compared with Citrusdal (statistically nonsignificant), Clanwilliam and Uitsig (Figure 2.5). A forward stepwise regression identified both soil (B, Bray II P, Mn and Cu) and climate variables (RH and total evapotranspiration) as major significant predictors of aboveground biomass in *A. linearis* (Table 2.4).

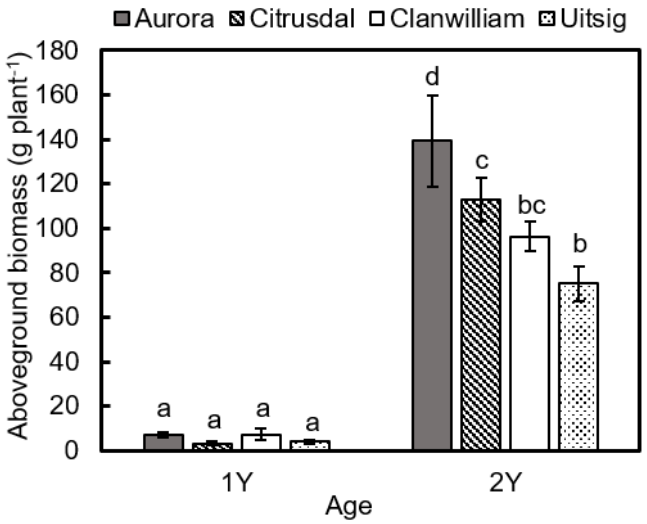

Figure 2.5. Aboveground biomass of 1Y and 2Y *A. linearis* grown at four sites; Aurora, Citrusdal, Clanwilliam and Uitsig. Lowercase letters indicate significant differences between farm sites and age of plants by Tukey's HSD *post hoc* test ($P<0.01$). Vertical bars denote ± s.e. ($n = 6$).

Table 2.4. The regression summary for the dependent variable; aboveground biomass of *A. linearis*. Results were significant as follows: *, $P<0.05$; ***, $P<0.001$.

Variable	Multiple R	Multiple R^2	R-square	*F* ratio
B	0.923	0.851	0.851	172.0***
Available P	0.990	0.980	0.004	4.4*
Mn	0.983	0.966	0.009	5.9*
Cu	0.986	0.973	0.007	5.6*
RH	0.961	0.923	0.015	4.8*
Total evap.	0.978	0.957	0.034	19.2***
C	0.931	0.867	0.016	3.5
Mg	0.939	0.881	0.014	3.3
Total P	0.946	0.895	0.014	3.6
K	0.992	0.985	0.002	2.6
Ca	0.993	0.987	0.002	2.8
Zn	0.994	0.988	0.001	1.5
DLI	0.953	0.908	0.013	3.8
Total rain	0.988	0.975	0.002	1.9
Temperature	0.991	0.982	0.003	2.8

2.3.3 Gas exchange

The highest P_{max} was evident in one-year-old (1Y) Clanwilliam plants overall (Figure 2.6*a*). Aurora and Citrusdal 1Y and 2Y plants had similar P_{max} which was lower than that of 1Y Clanwilliam plants (Figure 2.6*a*). The 2Y Aurora and Citrusdal plants had higher P_{max} compared to the 2Y Clanwilliam and Uitsig plants, while Uitsig plants had the lowest P_{max} overall (Figure 2.6*a*). The g_s was two times higher in the 1Y summer plants, except Citrusdal plants, compared to g_s in the 2Y summer plants (Figure 2.6*b*). The 1Y winter plants all had low g_s while the 2Y summer Aurora plants showed higher g_s than the 2Y summer Clanwilliam and Uitsig plants (Figure 2.6*b*). Also, 2Y summer Aurora and Citrusdal plants

had similar and higher E than 2Y Clanwilliam and Uitsig plants (Figure 2.6*c*). All the 1Y winter plants had low E, while the 1Y summer plants, Aurora and Citrusdal had similar and lower E compared to 1Y summer Clanwilliam plants which had the highest (Figure 2.6*c*). Photosynthetic water use efficiency was similar across all four sites during summer 2017 and summer 2018 while in winter 2017, the 1Y Uitsig plants had the highest PWUE, followed by that at Aurora, Clanwilliam and Citrusdal (Figure 2.6*d*). Plants had positive ΔT values during summer, indicating transpirational leaf cooling, and negative ΔT values during winter indicating a lack of transpirational cooling (Figure 2.6*e*). During summer 2017, 1Y plants from Clanwilliam had more cooling occurring than 1Y plants in Aurora and similarly in summer 2018, the 2Y Clanwilliam plants had higher levels of leaf cooling compared to the other sites (Figure 2.6*e*). During winter, there was more leaf heating occurring in Aurora and Citrusdal compared to Clanwilliam and Uitsig (Figure 2.6*e*).

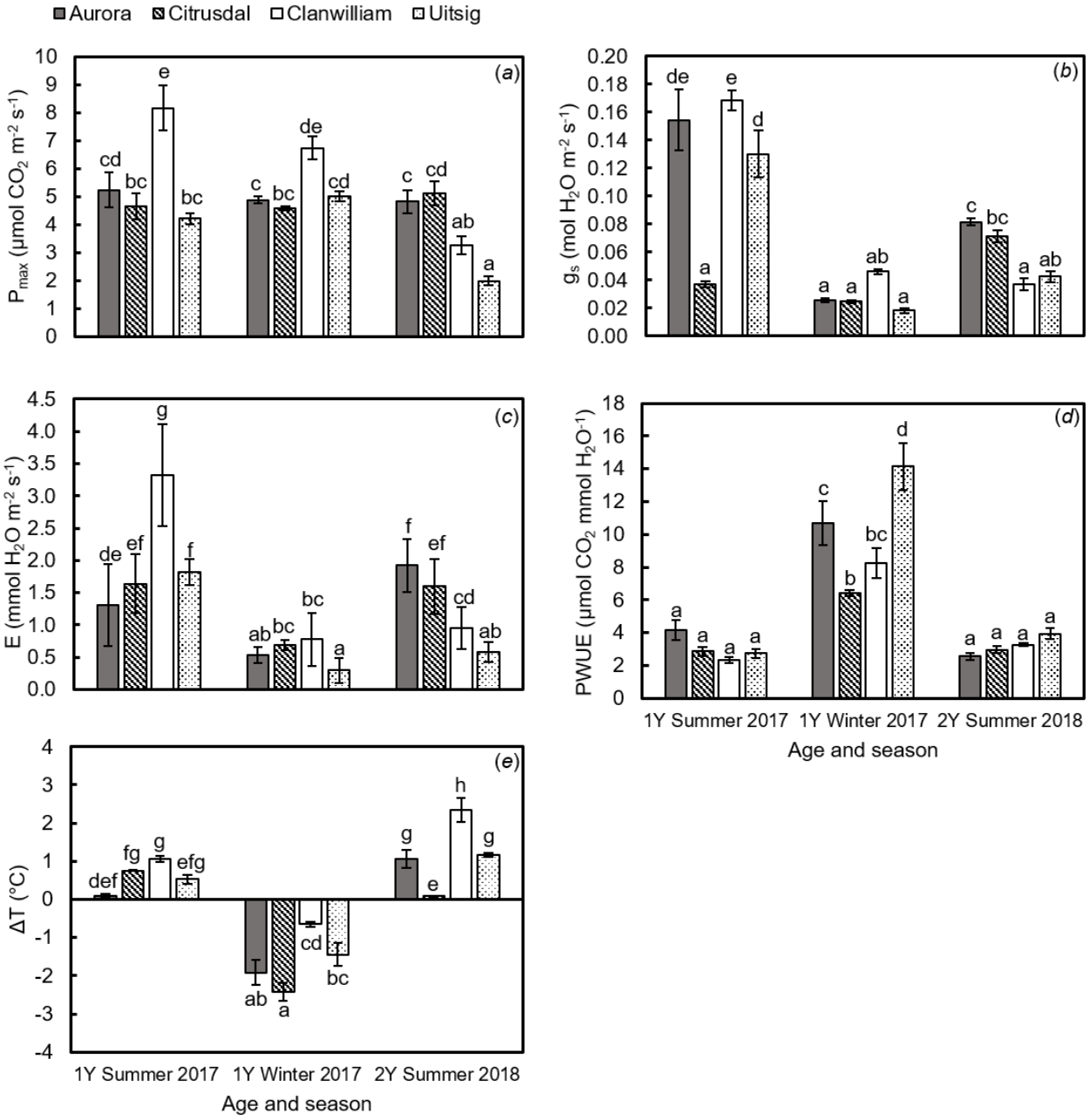

Figure 2.6. (*a*) Maximum photosynthesis (P_{max}), (*b*) stomatal conductance (g_s), (*c*) transpiration (E), (*d*) photosynthetic water use efficiency (PWUE) and (*e*) ΔT (air temperature – leaf temperature) of 1Y *A. linearis* plants sampled in summer and winter 2017 and 2Y *A. linearis* plants sampled in summer 2018, grown at four farm sites; Aurora, Citrusdal, Clanwilliam and Uitsig. Plants were measured at a PPFD of 1 500 µmol m^{-2} s^{-1} and ambient leaf temperatures. Lowercase letters indicate significant differences between farm sites and age of plants by Tukey's HSD *post hoc* test (P<0.001). Vertical bars denote ± s.e. (n = 6).

2.3.4 Chlorophyll and carotenoid concentrations

Total chlorophyll was higher in winter compared to the summer months, with Clanwilliam producing significantly more chlorophyll than Citrusdal (Figure 2.7*a*). Total chlorophyll was higher in 1Y plants at Clanwilliam compared to 1Y Aurora and Citrusdal plants during summer 2017 (Figure 2.7*a*). Total chlorophyll was significantly higher in 2Y summer Aurora and Citrusdal plants compared to 1Y summer Aurora and Citrusdal plants (Figure 2.7*a*). There were no differences between sites in chlorophyll a/b ratios during winter 2017 and during summer 2018, however in summer 2017, 1Y Aurora plants had lower chlorophyll a/b ratios compared to the other sites (Figure 2.7*b*). Chlorophyll a/b ratios were higher in 2017 compared to 2018 which showed the lowest chlorophyll a/b ratios (Figure 2.7*b*) indicating increasing antenna size. Carotenoid concentrations were highest during winter with Aurora plants producing more carotenoids compared to Citrusdal and Uitsig (Figure 2.7*c*). There were no significant differences in carotenoid concentrations in the plants between sites for summer 2017 and 2018 except in Clanwilliam plants where carotenoid concentrations were higher in the 1Y summer plants compared to the following year (Figure 2.7*c*). The chlorophyll/carotenoid ratio showed no differences between sites for any of the seasons, however, the 2Y summer plants in 2018 had significantly higher chlorophyll/carotenoid ratios than 1Y plants (Figure 2.7*d*).

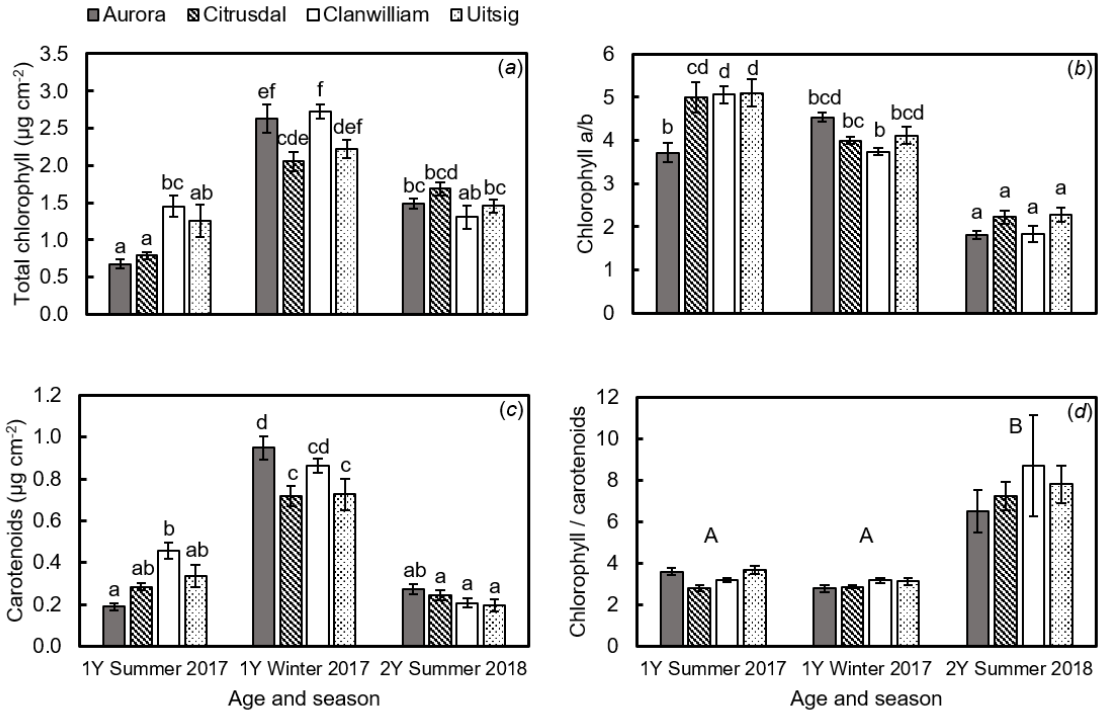

Figure 2.7. (*a*) Chlorophyll a+b concentration, (*b*) chlorophyll a/b ratio, (*c*) carotenoid concentration and (*d*) chlorophyll/carotenoid ratio of 1Y *A. linearis* plants sampled in summer and winter 2017 and 2Y *A. linearis* plants sampled in summer 2018, grown at four farm sites; Aurora, Citrusdal, Clanwilliam and Uitsig. Capital letters indicate significant differences between age of plants only and lowercase letters indicate significant differences between farm sites and age of plants by Tukey's HSD *post hoc* test ($P<0.05$). Vertical bars denote ± s.e. ($n = 6$).

2.3.5 Secondary metabolites

While there were no differences in total phenolics between sites overall, the 2Y summer plants produced two and seven times more phenolics than the 1Y summer and 1Y winter plants respectively (Figure 2.8*a*). Similarly, there were no differences in flavonol concentrations between sites, but 2Y summer plants produced significantly more flavonol

than 1Y summer and winter plants (Figure 2.8*b*). The 1Y summer plants produced almost three-times more anthocyanin than the 1Y winter plants and the 2Y summer plants, with the highest values in 1Y Aurora, Citrusdal and Clanwilliam plants (Figure 2.8*c*). Anthocyanin concentrations did not vary much between sites; however, it did increase in Clanwilliam plants from winter 2017 to summer 2018 (Figure 2.8*c*).

2.3.6 Non-structural carbohydrates

The 1Y plants, both summer and winter, had more than double the amount of glucose present in their leaf tissues compared to the 2Y summer plants (Table 2.5). Plants from Citrusdal and Clanwilliam (1Y) had the highest levels of glucose present, followed by 1Y Aurora and Uitsig plants in summer 2017 while there were no site differences in winter 2017 nor in summer 2018 (Table 2.5). The Citrusdal plants had the highest fructose concentrations compared to the other sites during summer 2017, however during winter 2017 there were no differences in fructose concentration between sites (Table 2.5). In the 2Y plants, Aurora plants had significantly higher fructose than Clanwilliam plants (Table 2.5). Both 1Y summer Aurora and Uitsig plants had the highest levels of sucrose present in leaf tissue while there were no other discernible differences between plant age and site and season for the other plants (Table 2.5). Citrusdal plants produced no discernible sucrose during summer 2017 (Table 2.5). Starch concentrations were variable and increased for Citrusdal plants from 1Y to 2Y plants while for Uitsig plants starch concentrations were the same across the years and seasons (Table 2.5). For both Aurora and Clanwilliam plants, starch decreased from summer 2017 to summer 2018 (Table 2.5).

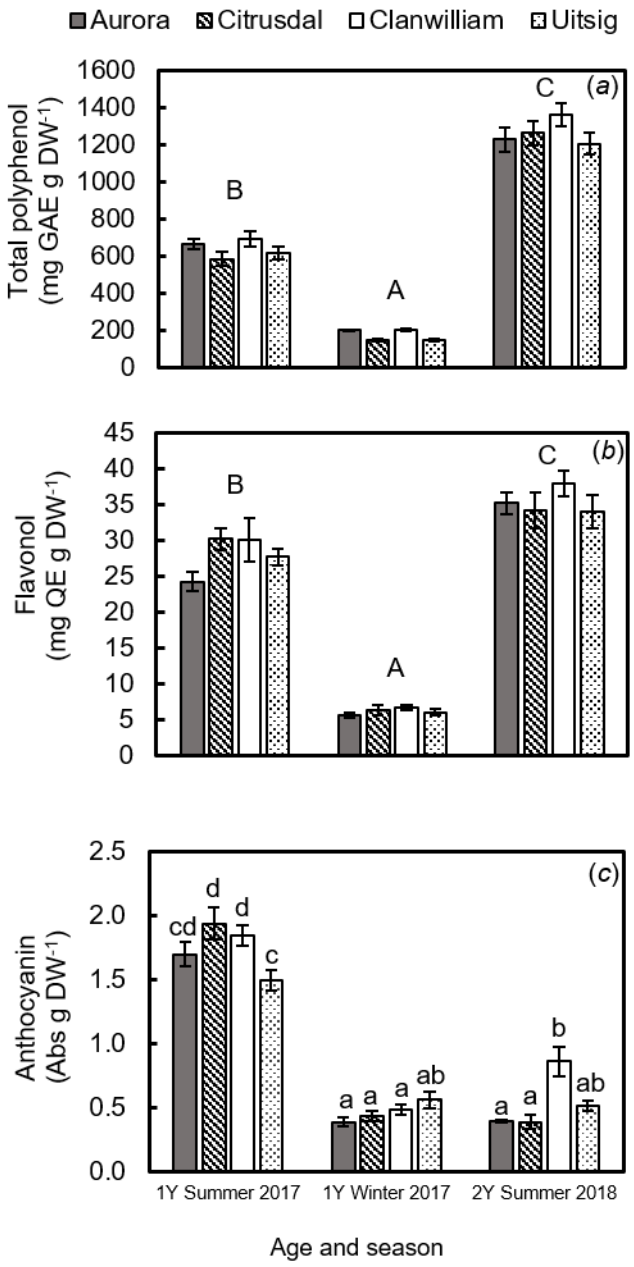

Figure 2.8. (*a*) Total polyphenol concentration, (*b*) flavonol concentration and (*c*) anthocyanin concentration of 1Y *A. linearis* plants sampled in summer and winter 2017 and 2Y *A. linearis* plants sampled in summer 2018, grown at four farm sites; Aurora, Citrusdal, Clanwilliam and Uitsig. Capital letters indicate significant differences between age of plants only and lowercase letters indicate significant differences between farm sites and age of plants by Tukey's HSD *post hoc* test ($P<0.05$). Vertical bars denote ± s.e. ($n = 6$).

Table 2.5. Leaf carbohydrate concentrations, leaf C, leaf N and leaf C/N ratios of *A. linearis* plants. Samples were collected in summer and winter 2017 and in summer 2018 from plants grown at four farm sites; Aurora, Citrusdal, Clanwilliam and Uitsig. Means ± s.e. (n = 6) followed by different letters in a column are significantly different as follows: **, P<0.01; ***, P<0.001.

Age	Season	Site	Glucose (mg g DW^{-1})	Fructose (mg g DW^{-1})	Sucrose (mg g DW^{-1})	Starch (mg g DW^{-1})	C (%)	N (%)	C/N
1Y	Summer 2017	Aurora	143.2 ± 19.5 cd	5.7 ± 2.3 ab	24.1 ± 2.2 b	76.0 ± 3.4 cd	44.3 ± 0.5 a	1.30 ± 0.07 ab	34.3 ± 1.8 cd
		Citrusdal	182.6 ± 9.3 d	23.2 ± 2.2 c	-	45.5 ± 3.7 b	45.7 ± 0.1 ab	1.37 ± 0.06 abc	33.5 ± 1.4 c
		Clanwilliam	175.7 ± 11.0 d	3.2 ± 1.3 a	3.9 ± 1.4 a	74.7 ± 5.7 cd	46.7 ± 0.3 abc	1.80 ± 0.09 bcde	26.1 ± 1.1 abc
		Uitsig	110.0 ± 8.4 bc	6.7 ± 0.4 ab	21.5 ± 2.0 b	45.8 ± 3.6 b	46.7 ± 0.2 abc	1.87 ± 0.09 cdef	25.1 ± 1.4 abc
	Winter 2017	Aurora	135.0 ± 2.6 c	9.6 ± 1.5 ab	3.9 ± 0.7 a	44.3 ± 1.7 b	46.3 ± 0.1 ab	2.11 ± 0.08 def	22.1 ± 0.8 ab
		Citrusdal	135.3 ± 2.6 c	12.8 ± 0.4 abc	4.5 ± 2.0 a	44.1 ± 6.1 b	45.8 ± 0.1 a	2.18 ± 0.11 ef	21.3 ± 1.2 a
		Clanwilliam	122.2 ± 3.5 c	3.1 ± 0.6 a	3.4 ± 1.0 a	61.8 ± 5.3 bc	49.0 ± 0.2 de	1.76 ± 0.06 bcd	28.0 ± 0.9 bc
		Uitsig	117.2 ± 3.6 bc	6.6 ± 0.9 ab	4.6 ± 2.7 a	44.4 ± 3.4 b	47.8 ± 0.2 bcd	2.33 ± 0.10 f	20.7 ± 0.9 a
2Y	Summer 2018	Aurora	52.0 ± 3.4 a	15.9 ± 6.5 bc	4.6 ± 0.5 a	21.9 ± 0.5 a	50.6 ± 0.9 ef	1.59 ± 0.09 abc	32.4 ± 2.1 c
		Citrusdal	55.4 ± 5.1 a	11.6 ± 3.9 ab	3.7 ± 0.4 a	84.0 ± 5.5 d	48.2 ± 0.2 cd	1.64 ± 0.06 abc	29.5 ± 0.9 c
		Clanwilliam	76.0 ± 3.2 ab	2.7 ± 0.6 a	9.0 ± 0.7 a	46.7 ± 2.8 b	52.2 ± 0.5 f	1.27 ± 0.08 a	41.9 ± 2.5 d
		Uitsig	42.3 ± 3.1 a	9.2 ± 1.1 ab	4.1 ± 0.6 a	44.4 ± 2.5 b	48.2 ± 0.1 cd	1.65 ± 0.06 bc	29.4 ± 1.0 c
F-ratio			4.07**	4.12**	17.60***	23.24***	8.40***	5.48***	7.47***

2.3.7 Leaf C and N concentrations

In Aurora and Citrusdal plants, leaf C was similar during summer and winter 2017 but increased in summer 2018, while in Clanwilliam plants leaf C increased significantly from summer to winter 2017 and again from winter 2017 to summer 2018 (Table 2.5). Uitsig plants had no change in leaf C over the years and seasons (Table 2.5). The highest leaf C values were in 2Y Aurora and Clanwilliam plants (Table 2.5). Leaf N increased for Aurora and Citrusdal plants from summer to winter 2017 and then decreased in summer 2018 (Table 2.5). There were no differences between Clanwilliam leaf N values from summer to winter 2017, however in summer 2018, leaf N decreased in Clanwilliam plants (Table 2.5). Leaf N in Uitsig plants was similar for summer and winter in 2017 and then decreased in summer 2018 (Table 2.5).

2.4 Discussion

Plant performance is strongly linked to environmental conditions (Poorter et al. 2016) and therefore it is not surprising that with recently observed climate change, *A. linearis* farmers are shifting to sites further south of the Cederberg area, with cooler temperatures and more rainfall, which are apparently more suitable for *A. linearis* production (Archer et al. 2008). This is supported by the results which showed that *A. linearis* grown at Aurora and Citrusdal, which had the lowest maximum temperatures and fewer days where temperatures went over 35 °C throughout the year, had greater aboveground biomass than plants grown at the warmer and lower rainfall sites. Both the Clanwilliam and Uitsig sites had higher temperatures, on average, and more than double the number of days above 35 °C compared to the cooler sites which most likely contributed to the lower biomass seen at these sites compared to Aurora and Citrusdal. During dry summer periods, temperatures can reach damaging thresholds for physiological processes, particularly when stomatal activity is decreased due to drought-

induced stomatal closure which limits the ability of plants to lower their leaf temperatures via transpirational cooling (Figure 2.6; Ladjal et al., 2000). The 2Y plants at Aurora and Citrusdal had higher rates of g_s and E compared to plants grown at Clanwilliam and Uitsig, indicating that there was likely less heat stress at these sites, therefore allowing the plants to maintain higher photosynthesis along with higher transpirational rates. Plants at all sites showed evidence of leaf cooling effects during summer, with plants in Clanwilliam recording the highest cooling effect. The plants at Clanwilliam and Uitsig probably reached a temperature threshold in their tolerance, therefore the activation state of rubisco is decreased (Morales et al. 2003) and maximum quantum efficiency is low (Sharma et al. 2015) leading to the plants no longer being able to maintain biomass production (Hatfield and Prueger 2015, Sharma et al. 2015). Studies have found that photosynthesis is sensitive to heat stresses and in the case of *Quercus ilex* (oak), photosynthesis, along with g_s and E, increased with increasing leaf temperatures but decreased when leaf temperatures were below 6 °C or above 37 °C (Gratani et al. 2000). Similarly, high temperature stresses caused reductions in photosynthesis in *Festuca arundinacea* (fescue) cultivars mainly due to the reductions in g_s (Cui et al. 2006, Wang et al. 2009). Field measurements indicate that *A. linearis* leaves were actively transpiring during summer months and subsequently cooling the plants (Figure 2.6*c, e*). Evaporative cooling is an effective mechanism for protecting the photosynthetic machinery (Dias et al. 2009, Wang et al. 2009, Sharkey and Zhang 2010) though in some plants it is the heat stability of the photosynthetic apparatus and the accompanying demand for CO_2 that controls the high stomatal conductance and leaf cooling during heat stress (Sharma et al. 2015). Previous studies have shown that plants that are able to maintain high levels of photosynthesis and carbohydrate assimilation during times of high temperatures are tolerant to heat stress (Camejo et al. 2005, Wahid et al. 2007, Wang et al. 2009, Bita and Gerats 2013, Sharma et al. 2015) and it was therefore concluded that *A. linearis* plants were

similarly tolerant to heat stress for extended periods due to transpirational cooling that aided in maintaining high photosynthetic rates. Plants at all sites showed evidence of leaf cooling effect during summer with plants in Clanwilliam recording the highest cooling effect. Therefore, transpirational cooling can be considered an important mechanism for heat stress tolerance (Ladjal et al. 2000, Wahid et al. 2007, Bita and Gerats 2013) in *A. linearis.* However, the expected patterns as seen in reports on *Z. mays*, *L. esculentum*, *C. lanatus* and *Saccharum officinarum* (sugarcane) which showed increases in soluble sugars and chlorophyll a/b ratios, decreases in chlorophyll/carotenoid ratios and increases in secondary metabolites in the tissues of plants under heat stress (Rivero et al. 2001, Camejo et al. 2005, Wahid et al. 2007) were not observed in the measured carbohydrates, pigments and secondary metabolites in *A. linearis*.

There were probably other factors that promoted biomass production of *A. linearis* at Aurora as its temperature was similar to that at Citrusdal (Figure 2.1*a*, Figure 2.5). Aurora was characterized by higher DLI, particularly in the summer months, compared to the other sites. The amount of light energy, expressed here as DLI, is the main driver of photosynthesis and biomass accumulation in plants (Liu and Heins 2002). Aurora had a combination of average maximum temperatures that were below 30 °C and high DLI during the summer months; this combination was perhaps responsible for the greater biomass in *A. linearis* in Aurora. Similar results have been reported in crops such as *Z. mays*, *G. max* and *Gossypium sp.* (cotton) where plant yields increased with temperatures between 29 °C and 32 °C but decreased sharply with further increases in temperature beyond this threshold (Schlenker and Roberts 2009). Indeed, root growth, accumulation of biomass, crop productivity and quality of plants increases with increases in DLI (Monteith 1977, Poorter and Van der Werf 1998, Lopez and Runkle 2008). On another point, higher wind speeds dry off areas by decreasing RH and

increasing VPD (Roderick et al. 2007, Archer et al. 2009) which was not the case in Aurora, which had high windspeeds but also high RH and low VPD. This is due to Aurora being in close proximity to the ocean, and therefore experiencing additions of non-rainfall moisture (Brown et al. 2008) which can include dew, fog and mist, all of which are important inputs of water for plants in arid systems (Dawson 1998). The regression analysis showed that the climate variables RH and total evapotranspiration significantly influenced plant biomass in *A. linearis*. Relative humidity and VPD have been shown to affect the growth and rate of growth in plants, where very dry air (low RH and high VPD) can cause excessive transpiration thereby inducing water deficits in plants and restricting growth, if moisture supply or the root system are inadequate (Ford and Thorne 1974). However, very moist air (high RH and low VPD) can also be detrimental to plants where the decreased transpiration could lead to impairment of nutrient uptake via mass flow (Lihavainen et al. 2016). Generally, increases in RH, and parallel decreases in VPD, correlated positively with increases in biomass accumulation in most plant species (Ford and Thorne 1974, Mortensen 1986, Lihavainen et al. 2016). Noteworthy, both RH and evapotranspiration are governed by the environment's moisture/rainfall and temperature (Ford and Thorne 1974, Mortensen 1986, Kim and Beard 1988).

The Aurora site was separated from the other sites based on soil nutrient concentrations (Figure 2.4) with the highest concentrations of Mg, Fe, Cu, Na and Zn, during 2018 compared to the other sites. Aurora also had high pH and concentrations of B in the soil, similar to Citrusdal. Higher concentrations of certain nutrients are beneficial to *A. linearis* such as P which, along with N, co-limited growth in *A. linearis* (Maistry et al. 2015). The depletion of basic cations such as Ca, Mg, Na and K, leads to decreases in *A. linearis* biomass (Smith et al. 2018). More acidic soils lead to lower soil quality and plant productivity due to

their generally high levels of Al, Mn and Fe, which are toxic to plants, and generally low levels of N, P, K, Ca, Mg, Mo and Zn availability which are needed for plant growth (Baligar et al. 1997, 1998). Furthermore, increased levels of metals like Mg, Mn and Fe are vital for plants due to their role in photosynthesis and CO_2 fixation where deficiencies, of Fe in particular, negatively affect photosynthesis (Eberhard et al. 2008, Briat et al. 2015). The micronutrient B has important roles in plant growth, the establishment of symbioses between legumes and rhizobia as well as the development and functioning of these nodules (Pilbeam and Kirkby 1983, Bolaños et al. 1994). The regression analysis showed that soil nutrients Mn, B, Cu and available Bray II P significantly influenced plant biomass. Soils differed between the years and it was evident through discrimination analysis that soils collected before *A. linearis* planting differed from the soils that were collected under the 2Y plants as expected. Over the course of two years, from 2016 to 2018, there was an increase in certain soil nutrients at Aurora; C, Bray II P, Ca, and Na, while Citrusdal soils had an increase in Ca and Mn and Uitsig soils had an increase in C. All the soils had increased levels of total P and B over the two-year period. Soil organic matter, measured by soil C, is a major source of plant nutrients (Manlay et al. 2007) and is generally positively correlated with concentrations of K, Ca, Mg and Na (Chimphango et al. 2016). Therefore, the increases in some of the nutrients at Aurora could be attributed to mineralization of soil organic matter, however, it could also be due to nutrient deposition which could lead to a build-up of minerals in the soils (Soderberg and Compton 2007, Wilson et al. 2009). Smith et al. (2018) showed that soil quality played a significant role in biomass accumulation in *A. linearis* where declines in soil organic matter and basic cations, particularly K, strongly correlated with declines in the biomass of *A. linearis* in the Clanwilliam region. The results suggest that the growth of *A. linearis* plants in the Cederberg region was affected by both soil nutrients (Smith et al. 2018), and environmental effects. This suggests that over time, soil degradation associated with *A.*

linearis farming practices including lack of cover crops, crop rotations or fertilization, coupled with enhanced temperatures and drought conditions, due to climate change, *A. linearis* farming could be negatively affected.

One of the most noticeable differences in the results was that crop biomass increased over tenfold in the space of a year at all the sites. This is similar to growth observed in numerous plants such as *Malus domestica* (apple), *L. esculentum*, *Persea americana* (avocado), *Pyrus sp.* (pear; Coombe 1976), various tree species (Kramer 1943) and in some Mediterranean shrubs where the relative growth rate increases exponentially, up to 1000 mg g^{-1} D^{-1} (El Aou-Ouad et al. 2015) until it reaches a constant, maximum rate (Salisbury and Ross 1992). This was followed with a general trend of 2Y plants having lower chlorophyll a/b ratios, higher chlorophyll/carotenoid ratios, higher total polyphenol and flavonoid content and lower anthocyanin contents. These secondary metabolites play a variety of roles in plants' tolerance to stresses and hence plant growth and biomass accumulation under stressful conditions (Rivero et al. 2001, Wahid and Ghazanfar 2006, Bita and Gerats 2013). The *A. linearis* plants are pruned to promote branching after the first year of growth in the field; subsequently they are harvested annually during the summer months at the start of the year (Chimphango et al. 2016) which coincides with maximum phenolic content in the plant leaves and thus ensuring good quality and healthy tea at harvest (Dai and Mumper 2010). The younger plants had chlorophyll a/b ratios ranging from 4 – 5, almost double the amount found in the older plants, indicating that the younger plants were producing chlorophyll with less light-harvesting proteins to alleviate the stress of absorbing high amounts of light energy therefore protecting the chloroplasts, leaves and plant (Lichtenthaler et al. 1981, Camejo et al. 2005). Along with this, these sun-adapted chloroplasts are also known to have higher carotenoid content per chlorophyll content (Lichtenthaler et al. 2000) which was seen in the chlorophyll/carotenoid

ratios here, where the younger *A. linearis* plants had lower ratios compared to the older plants. Carotenoids primarily act as accessory light harvesting pigments, extending the range of light absorbed for photosynthesis, and secondly, they have photoprotective abilities, protecting the plant under stress conditions by allowing a higher level of energy dissipation via photosynthesis through the quenching of triple state chlorophyll molecules (Young 1991, Wahid and Ghazanfar 2006). It is likely that the carotenoids are used primarily as accessory light harvesting pigments particularly during winter, with an added role in photoprotection during summer. In a similar role in photoprotection, the younger *A. linearis* plants produced three times more anthocyanin than the older plants. The production of these secondary metabolites, polyphenols, flavonols and anthocyanins, are induced through various environmental stresses including heat stress, as an acclimation mechanism against thermal damage (Singh et al. 1999, Rivero et al. 2001, Bita and Gerats 2013). They alleviate the stress on the plants through various mechanisms including having light absorptive properties, light harvesting for photosynthesis and protecting the cells from damage from high light environments as well as having antioxidant properties allowing them to scavenge for various oxidizing species (Harborne and Williams 2000). Anthocyanins in particular are controlled by prevailing temperatures and protect the plant leaf tissue by acting as UV screens reducing the effects of UV radiation (Singh et al. 1999, Harborne and Williams 2000). The younger plants at both Clanwilliam and Uitsig produced more chlorophyll than the plants at Aurora and Citrusdal, in their first year during summer, which could be a direct result of the temperature differences between the sites, resulting in a more photoprotective role of these compounds in the plants exposed to higher temperatures (Camejo et al. 2005, Sarijeva et al. 2007).

Another important factor in thermotolerance in plants is the availability of soluble sugars during heat stress periods (Liu and Huang 2000). These reducing sugars act as antioxidants which scavenge ROS produced during heat stress periods (Lang-Mladek et al. 2010). Therefore, the twofold greater glucose content in the leaf tissue of the younger *A. linearis* plants compared to the older plants that was observed was likely to protect the younger plants, as an antioxidant, during the summer season. Reductions in carbohydrates often come about due to an imbalance of photosynthesis and respiration during heat stress where photosynthesis decreases and respiration increases (Sharkey and Zhang 2010). Overall, sucrose was much lower in *A. linearis* leaves than starch, with *A. linearis* from Citrusdal producing no discernible sucrose in their leaves during summer which could be due to the plants preferring to store C as starch rather than sucrose or the sucrose was exported out the leaf to sink organs (Huber 1989, Ruan 2014). Another reason for the lower sucrose could be due to the sucrose being split into hexoses, such as glucose and fructose, through greater invertase activity and as a result, contributing to the lowering of the plant's osmotic potential, thereby protecting the plant from abiotic stresses (Huber 1989, Ruan et al. 2010). Compared to the younger plants, the older plants had lower carbohydrate contents, however respiration and photosynthetic rates were similar across all the plants indicating that the photosynthate produced in the older plants was likely channelled to both plant growth and development, as well as a possible dilution effect occurring in the older plants.

2.4.1 Conclusion

The aim of this paper was to determine the effects of the environment, particularly temperature on the growth and stress tolerant traits of *A. linearis* over a two-year period. One of the most important heat tolerance mechanisms found was the ability of the plants to actively lower the temperature of the leaves through transpirational cooling, during summer.

Therefore, transpirational cooling combined with low chlorophyll and high phenolic concentrations, could be considered as acclimatized adaptive changes allowing the plants to mitigate the heating effects of light absorption and subsequently maintaining high photosynthetic and carbohydrate levels for growth during summer periods. The results also show that changes in soil quality, particularly the decrease in nutrients such as Ca, Mg, Mn, Fe, Cu and K, along with increasing temperatures have a negative effect on the biomass production of *A. linearis.* Moreover, the phenolic content (a measure of tea quality; Joubert and de Beer, 2011) did not vary among sites. Therefore, a shift in *A. linearis* farming further south, to cooler areas as is already happening, would enhance biomass production without any penalty on the quality of tea produced, however, the soils need to be considered as well. Also, it was observed that the younger plants invested relatively more photosynthate, energy and nutritional resources than the older plants, as was seen in their higher carotenoid, anthocyanin and glucose concentrations as well as higher chlorophyll a/b ratios, to ensure their protection and survival during the summer months allowing them to persist into the following years. Further studies need to be done to examine possible expansion of *A. linearis* cultivation to cooler wetter areas within the Western Cape as well as the temperature thresholds of the plants. This chapter suggests that there are significant effects on plant production related to both soil properties and climate, including non-precipitation moisture additions into the system, which are all dependent on local geography and topology.

Chapter 3

Proteomic responses of *Aspalathus linearis* to heat stress

3.1 Introduction

During exposure to stress, plant tissue can potentially be damaged, but can also evoke a wide range of adaptive responses which include morphological, physiological, molecular and biochemical changes (Wang et al. 2003, Wahid et al. 2012, Zandalinas et al. 2018). Various sensors in plants pick up on the stress stimuli thereby activating signalling pathways which involve secondary messengers, phytohormones, signal transducers and transcription factors (Danquah et al. 2014, Gilroy et al. 2014). Along with these signalling responses, plants also have cellular responses to stress which includes the accumulation of solutes, up-regulation of antioxidants and the production of molecular chaperones (Vierling 1991, Baniwal et al. 2004, Kotak et al. 2007). Molecular chaperones contribute to cellular homeostasis during both optimal and stress conditions in plants (Wang et al. 2004). These chaperones are responsible for the prevention of protein denaturation, misfolding of newly synthesized proteins and maintaining membrane stability (Wang et al. 2004, Kotak et al. 2007). Of the molecular chaperones, many are stress proteins and when originally identified were labelled as HSPs (Lindquist 1986). The accumulation of these HSPs has been observed to play a key role in thermotolerance in plants as well as a having a major role in the heat stress response (Kotak et al. 2007).

Several genes have been identified in the involvement of the regulation and acclimation of plants to multiple abiotic stresses through transcriptomic and proteomic studies (Koussevitzky et al. 2008, Suzuki et al. 2016). Heat stress and proteomic responses have been investigated in several food crops such as *T. aestivum*, *Hordeum vulgare* (barley) and

Medicago sativa (alfalfa), where many stress proteins have been identified (Süle et al. 2004, Li et al. 2013a, Wang et al. 2015), however studies on legumes have been scarce with proteome profiling occurring mainly on model species such as *Medicago truncatula* (barrel medic) and *Lotus japonicus* (bird's-foot trefoil) and model crops, *G. max* and *Cicer arietinum* (chickpea; Ramalingam et al. 2015).

With an increasing number of plant genomes being sequenced and released, proteomics has taken the lead in plant biological research and stress responses (Jorrín-Novo et al. 2015). The main aim of proteomics has been defined as the study of when, where and how individual protein forms interact with each other as well as other molecules and how they can be modified in order to fit in with the growth and development of organisms in response to biotic and abiotic interactions (Jorrín-Novo et al. 2015). In order for plants to complete their biological cycles which includes surviving and being productive, they must be able to adapt to environmental cues, either by resisting or tolerating them (Neilson et al. 2010, Jorrín-Novo et al. 2015). The plant's perception of these stresses activates cellular signals which lead to gene expression reprogramming as well as metabolic and protein changes ultimately leading to resistance or tolerance of a stress, which is of particular interest (Jorrín-Novo et al. 2015). Thus, proteomics is contributing to the understanding of the elements involved in stress perception and transduction as well as deciphering which genes are involved in resistance and tolerance (Neilson et al. 2010, Jorrín-Novo et al. 2015). No work has been done on the proteome of *A. linearis* and its response to heat. The identification of heat-responsive proteins and the characterization of the proteome will help in the understanding of heat tolerance mechanisms in *A. linearis.* The aim of this chapter is to determine the effects of heat on the expression of proteins involved in heat tolerance in *A. linearis* through proteomic analyses.

The main objective is to determine whether heat activated proteins are upregulated in response to heat stress in *A. linearis* and their role in tolerance mechanisms.

3.2 Materials and methods

Details on the field study area and cultivation of experimental plants are described in chapter two, however, protein sample collection, analysis and results are presented below. The heat activated proteins in the field were investigated for all sites, Aurora, Citrusdal, Clanwilliam and Uitsig, during summer 2017 and samples were also taken during winter 2017 at Clanwilliam and Citrusdal. Leaf samples were collected between 11am and 12pm, and immediately frozen in liquid nitrogen, stored on ice and transferred to a -80 °C freezer until further analysis. The determination of whether heat responsive proteins were either up or down-regulated, a comparison was done between the warmer sites (Clanwilliam and Uitsig, heat-stressed) and the cooler sites (Aurora and Citrusdal, control).

3.2.1 Protein extraction

Total protein was extracted from summer leaf samples as described by Faurobert et al., (2007) and heat stable proteins were extracted from the winter leaf samples as described by Isaacson et al., (2006), with an added heating step for the heat stable protein extraction. The final pellets produced were stored at -80 °C and sent to the Centre for Proteomic and Genomic Research (CPGR, Observatory, South Africa) for analyses of the protein profiles. Pilot samples were initially sent to test the solubilization and quantification before all the field samples.

3.2.2 Protein solubilization and quantification

Samples were resuspended in 5 % SDS 50 mM TEAB (buffer determined from pilot study), placed in 95 ℃ for ten minutes and thereafter clarified by centrifuging at 10,000 × g for ten minutes at room temperature. Quantification was performed using the QuantiPro BCA assay kit (Sigma QPBCA) according to the manufacturer's instructions.

3.2.3 On-bead HILIC digest

In preparation for the hydrophilic interaction liquid chromatography (HILIC) magnetic bead workflow, HILIC beads (ReSyn Biosciences, HLC010) were aliquoted into new tubes and shipping solution removed. Beads were then washed twice with 250 µl of buffer (15 % CAN, 100 mM ammonium acetate (Sigma 14267), pH 4.5) for one minute. The beads were then resuspended in a loading buffer (30 % acetonitrile (ACN), 200 mM ammonium acetate, pH 4.5) to a concentration of 5 mg ml^{-1}. A total of 50 µg of resuspended protein from each sample was transferred to a protein LoBind plate (Merck, 0030504.100). The protein was reduced with tris (2-carboxyethyl) phosphine (TCEP; Sigma 646547) which was added to a final concentration of 10 mM TCEP and incubated at 60 °C for one hour. Samples were cooled to room temperature and alkylated with methylmethanethiosulphonate (MMTS; Sigma 208795), added to a final concentration of 10 mM MMTS, and incubated at room temperature for 15 minutes. The HILIC magnetic beads were added at an equal volume to that of the sample with a ratio of 5:1, beads: total protein. The plate was then incubated on the shaker at 900 rpm for 30 minutes at room temperature for binding of protein on beads. After binding, the beads were washed four times with 500 µl 95 % ACN for one minute. For digestion, trypsin (Promega PRV5111), made up in 50 mM TEAB, was added at a ratio of 1:10 total protein and the plate was incubated at 37 ℃ on the shaker for four hours. After

digestion, the supernatant containing the peptides was removed and dried down. Samples were then resuspended in LC loading buffer (0.1 % formic acid (FA), 2.5% ACN).

3.2.4 LCMS analysis

Liquid chromatography mass spectrometry (LCMS) analysis was conducted with a Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) coupled with a Dionex Ultimate 3000 nano-UPLC system. Data was acquired using Xcalibur v4.1.31.9, Chromeleon v6.8 (SR13), Orbitrap MS v2.9 (build 2926) and Thermo Foundations 3.1 (SP4). The resuspended peptides were dissolved in 0.1% FA (Sigma 56302), 2 % ACN (Burdick and Jackson BJLC015CS) and loaded on a C18 trap column (PepMap100, 9027905000, 300 μm x 5 mm x 5 μm). Samples were trapped onto the column and washed for three minutes before the valve was switched and peptides eluted onto the analytical column. Chromatographic separation was performed with a Waters nanoEase (Zenfit) M/Z Peptide CSH C18 column (186008810, 75 μm x 25 cm x 1.7 μm). The solvent system employed was solvent A: LC water (Burdick and Jackson BJL365), 0.1 % FA and solvent B: ACN, 0.1 % FA. The multi-step gradient for peptide separation was generated at 300 nL min^{-1} as follows: time change five minutes, gradient change: 2 – 5 % solvent B, time change 40 minutes, gradient change 5 – 18 % solvent B, time change ten minutes, gradient change 18 – 30 % solvent B, time change two minutes, gradient change 30 – 80 % solvent B. The gradient was then held at 80% solvent B for ten minutes before returning it to 2 % solvent B and conditioning the column for 15 minutes. All data acquisition was obtained using Proxeon stainless steel emitters (Thermo Fisher TFES523). The mass spectrometer was operated in positive ion mode with a capillary temperature of 320 °C. The applied electrospray voltage was 1.95kV.

3.2.5 Proteomic data analysis

Relative, label free quantification was conducted using Progenesis QI for Proteomics v2.0 (Nonlinear Dynamics, UK). Data processing included peak picking, run alignment and normalisation (singly charged spectra were removed from the processing pipeline). Relative quantification was based on four biological replicates per condition using non-conflicting peptides. A protein with a fold change ≥ 2 with a corresponding q-value <0.05 was considered regulated. Currently there are no public genomic, transcriptomic or proteomic databases for *A. linearis*, however, a research group at the University of the Western Cape, led by Dr Uljana Hesse, are in the process of creating a database based on genomic and transcriptomic data (pers. comm.). Access to their database was allowed for the protein data analysis after a Data Transfer Agreement was signed. Database interrogation was performed with Byonic Software v2.6.46 (Protein Metrics, USA) using this *A. linearis* database.

The Label free identified proteins were functionally annotated using the OrthoDB (https://www.orthodb.org/) and UniProt (http://www.uniprot.org) databases. The databases were used to search for the Gene Ontology (GO) analysis using three key terms, i.e. biological processes, molecular processes and the cellular component using Blast2GO 5.2.5 (Götz et al. 2008).

3.3 Results

3.3.1 Protein profile of field grown plants

A total of 1588 and 180 proteins were identified in the plants in the field, across all four sites during summer and winter respectively. Of the 1588 proteins, 180 were differentially expressed across the four sites during summer. No proteins were up or downregulated in the

winter samples. Of the 180 regulated proteins during summer, 87 (48.3 %), 26 (14.4 %), 10 (5.6 %) and 57 (31.7 %) were upregulated and 53 (29.4 %), 51 (28.3 %), 17 (9.4 %) and 59 (32.8 %) were downregulated in Aurora, Citrusdal, Clanwilliam and Uitsig respectively. The max fold ranged from 22.555 to 2.002 (upregulated) and -2.007 to -6.045 (downregulated) in plants grown at Aurora. For plants grown in Citrusdal, the max fold ranged from 6.045 to 2.007 (upregulated) and -2.032 to -12.023 (downregulated) while for plants grown in Clanwilliam the max fold ranged from 5.720 to 2.094 (upregulated) and -2.028 to -4.322 (downregulated). The upregulated protein max fold range in plants grown at Uitsig was 8.135 to 2.011 and the downregulated max fold ranged from -2.002 to -22.555 (Tables A.1 to A.4).

3.3.2 Molecular, biological and cellular components of proteins

All the up and downregulated proteins were GO annotated and classified into molecular (Figure 3.1), biological (Figure 3.2) and cellular (Figure 3.3) components. Of the proteins identified, 13.6 % and 6.0 %, were not predicted for molecular components in control sites and heat stressed plants respectively. Proteins involved in binding activities comprised 30.5 % in control and 34.0 % in the heat stressed sites, which included, but was not limited to ATP, heme, general protein, chlorophyll, FAD, calcium ion, carbohydrate, GTP, heat shock protein, lipid, metal ion and nucleic acid binding (Figure 3.1). There were more proteins involved in oxidoreductase activity expressed in the heat stressed sites (8.0 %) compared to the controls (6.8 %), while many other proteins which included, but was not limited to cysteine synthase activity, glutathione peroxidase activity, hydrolase activity, pectinesterase activity and protein serine/threonine kinase activity, comprising a total of 42.0 % of protein in the heat stressed sites, were only expressed in the heat stressed sites compared to the controls (Figure 3.1).

A wide range of biological components were also identified for the proteins, however 24 % and 18.6 % of the proteins had no predicted biological components in heat stressed sites and controls respectively (Figure 3.2). The largest proportion of proteins were involved in oxidation-reduction processes,15.3 % in control sites and 14.0 % in heat stressed sites, while 6.8 % of proteins in control sites were involved in proteolysis compared to 2.0 % in the heat stressed sites (Figure 3.2). The amount of proteins involved in the glycolytic process were similar in control sites (3.4 %) compared to the heat stressed sites (4.0 %), while proteins involved in defence (4 %) and responses to heat (6 %) were only expressed in heat stressed sites (Figure 3.2). A total of 48 % of the proteins involved in biological components were expressed in heat stressed sites only, while 45.8 % were expressed in control sites only, however most were involved in small proportions (< 2 %; Figure 3.2).

There were no cellular predictions for 24.0 % of the proteins in heat stressed sites and 13.8 % of the proteins in the control sites (Figure 3.3). The most highly represented cell components in heat stressed sites included cytosol (14.0 %), chloroplast (12.0 %), cytoplasm (12.0 %) and integral component of membrane (10 %; Figure 3.3). The most highly represented cell components of control sites were membrane (13.8 %), chloroplast (12.1 %), cytoplasm (6.9 %), integral component of membrane (6.9 %) and cytosol (5.2 %; Figure 3.3). A total of 6 % of proteins representing the cellular component, namely cell wall (2 %), phosphopyruvate hydratase complex (2 %) and plasma membrane (2 %) were expressed in heat stressed sites only, while 17.2 % of the proteins representing the cellular component were expressed in control sites only (Figure 3.3).

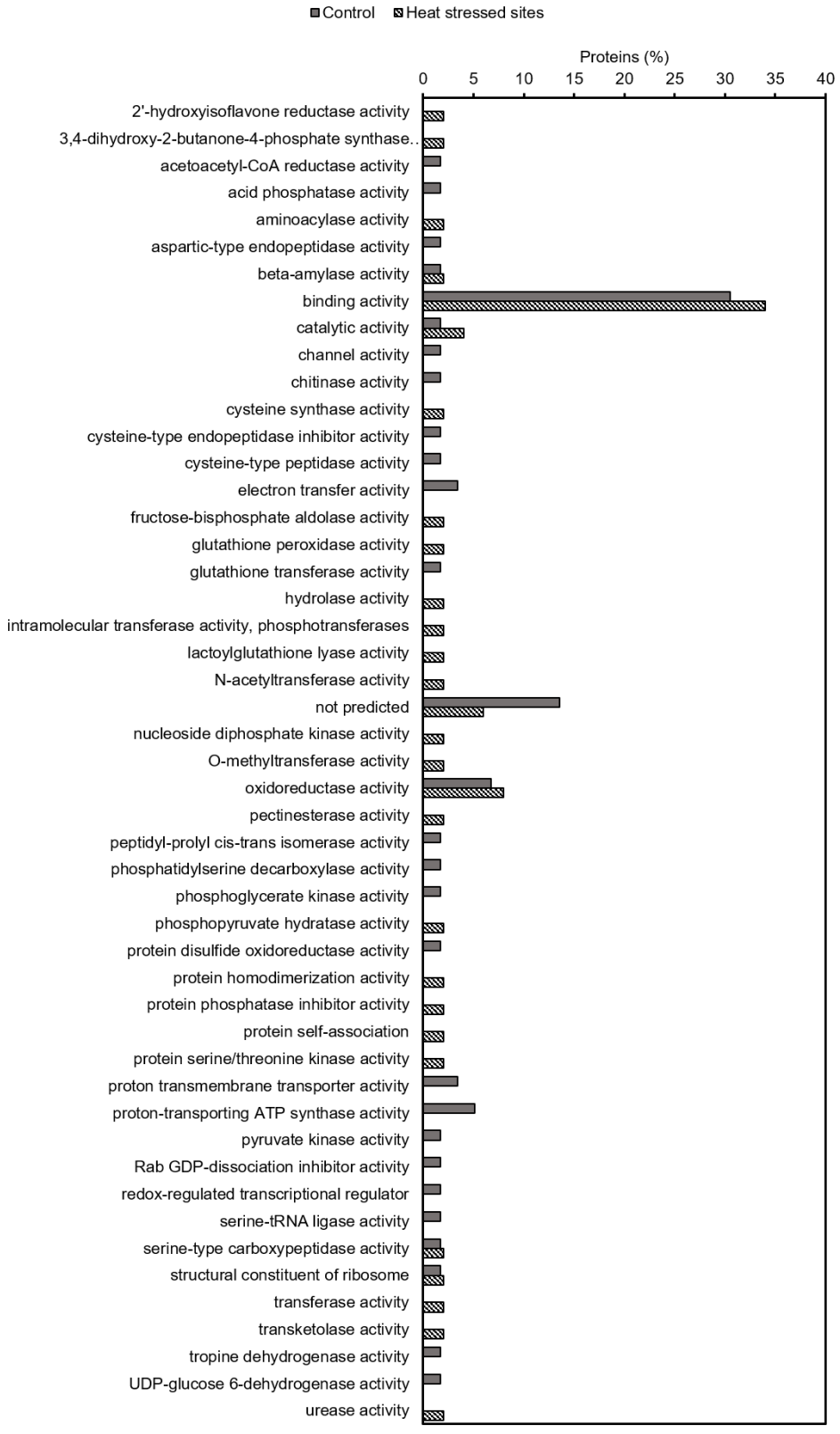

Figure 3.1. Molecular component predictions of the identified heat stress response proteins based on the GO annotations for plants in the heat stressed sites (Clanwilliam and Uitsig) compared to the control (Aurora and Citrusdal).

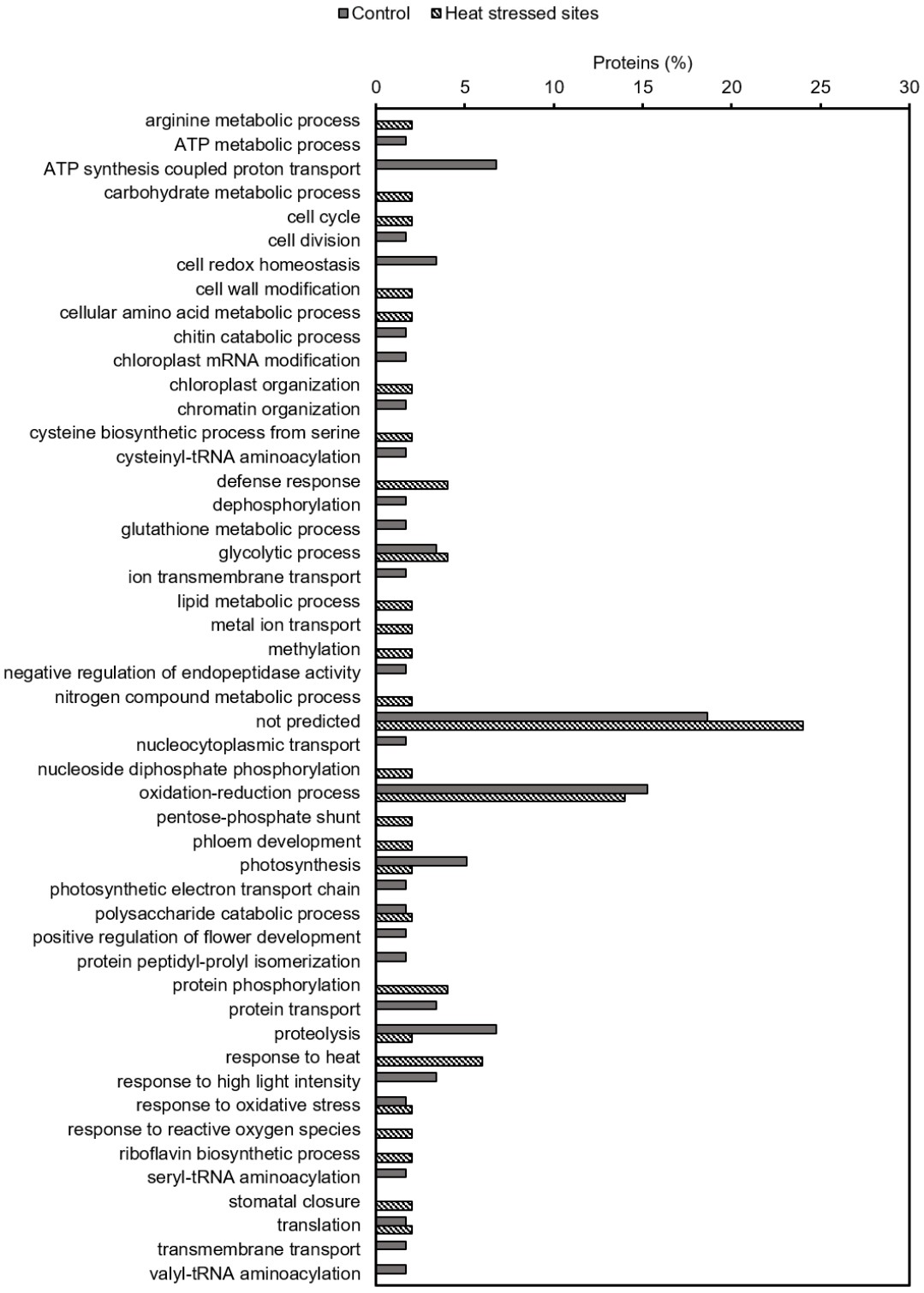

Figure 3.2. Biological component predictions of the identified heat stress response proteins based on the GO annotations for plants in the heat stressed sites (Clanwilliam and Uitsig) compared to the control (Aurora and Citrusdal).

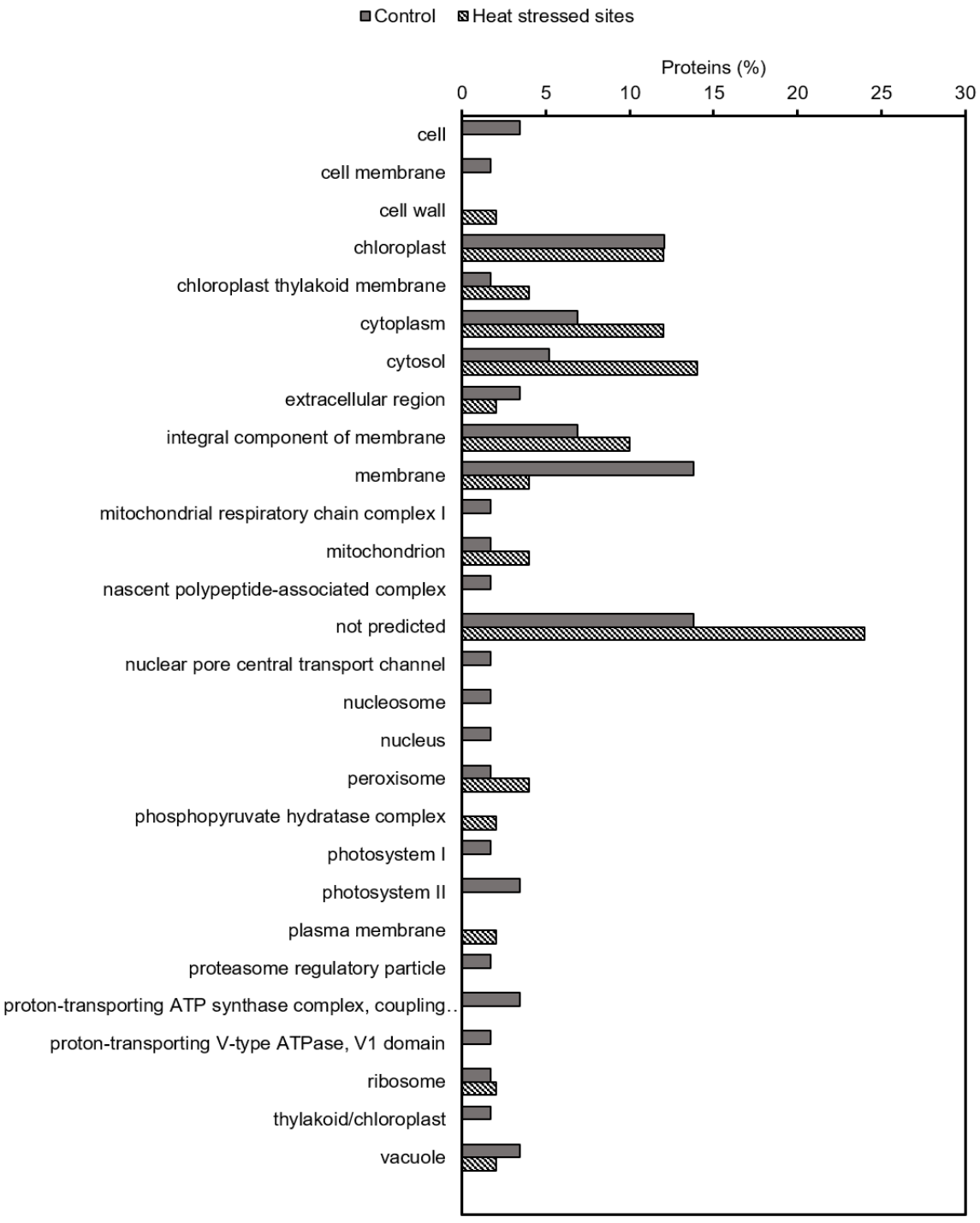

Figure 3.3. Cellular component predictions of the identified heat stress response proteins based on the GO annotations for plants in the heat stressed sites (Clanwilliam and Uitsig) compared to the control (Aurora and Citrusdal).

3.3.3 Functional categories of proteins

Based on the functional categories as described by Bevan et al. (1998) plants in the control sites had eleven functional categories of proteins compared to ten in the plants in the heat stressed sites (Figures 3.4). The heat stressed sites had higher proportions of proteins in their functional groups, except for metabolism, transporters and cell growth/division which were higher in the control sites (Figure 3.4). The highest proportion of proteins in plants grown in the heat stressed sites were proteins involved in metabolism (30.8 %) followed by signal transduction (16.1 %), energy (14.0 %), cell structure (10.5 %), protein destination and storage (10.5 %) and defence (6.3 % Figure 3.4). Proteins involved in protein synthesis, transcription, intracellular traffic and cell growth/division constituted a total of 11.9 % of proteins in these plants (Figure 3.4). The plants grown at the control sites had most of their proteins involved in metabolism (42.4 %), followed by signal transduction (13.4 %), energy (10.1 %), protein destination and storage (9.7 %), cell structure (8.8 %) and defence (6.9 % Figure 3.4). Proteins involved in transporters, protein synthesis, intracellular traffic, transcription and cell growth/division constituted a total of 8.8 % of the remaining proteins in these plants (Figure 3.4).

Of the 180 proteins identified in all the plants across the four sites, 87 of them were upregulated in plants from Aurora, 26 in Citrusdal plants, 10 in Clanwilliam plants and 57 in plants from Uitsig (Tables A.1 – A.4). Along with that, 53 proteins were downregulated in plants from Aurora, 51 in Citrusdal plants, 17 in Clanwilliam plants and 59 in plants from Uitsig (Tables A.1 – A.4). Plants in Aurora were the only ones to have more upregulated proteins than more downregulated proteins (Table A.1).

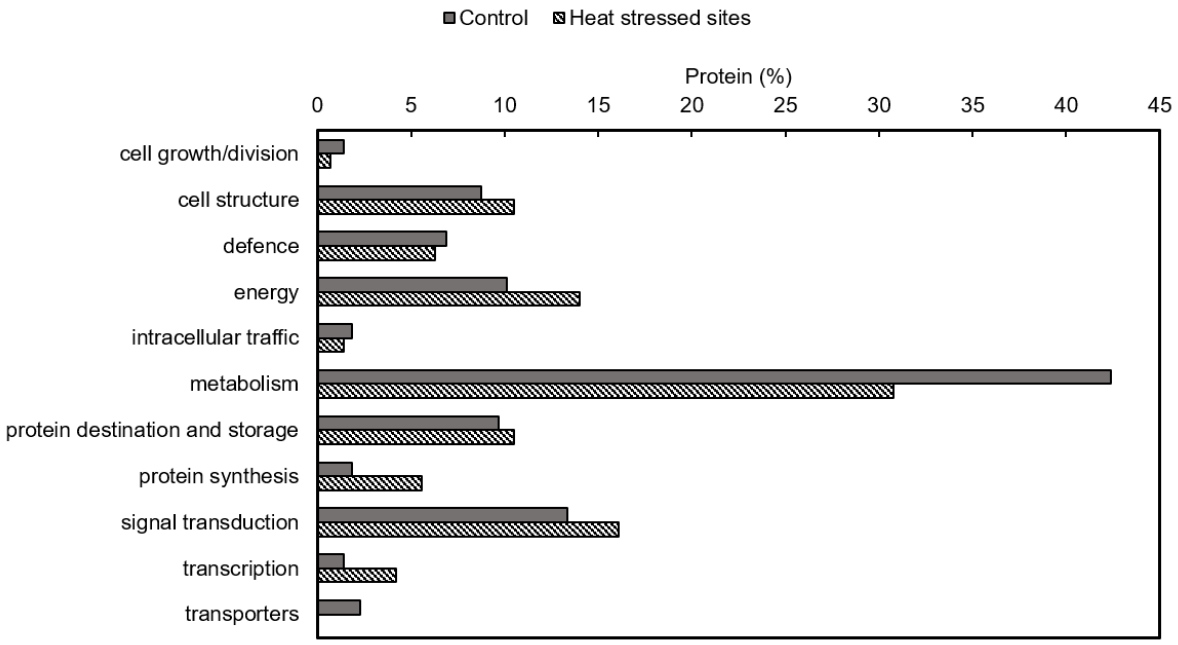

Figure 3.4. Functional classification of the upregulated proteins found in plants in the heat stressed sites (Clanwilliam and Uitsig) compared to the control (Aurora and Citrusdal).

3.4 Discussion

From the 180 proteins expressed in *A. linearis* plants, 113 proteins (63 %) were upregulated in the cooler sites, Aurora and Citrusdal, while only 67 proteins (37 %) were more upregulated in the hotter sites, Clanwilliam and Uitsig. Numerous genes have been identified and shown to be either overexpressed or repressed in response to heat stress (Rizhsky et al. 2002, 2004, Larkindale and Vierling 2008). Homeostasis in the proteome, however, could indicate that plants have reached an acclimation to their stress (Harb et al. 2010). Half of the identified proteins were related to energy (12 %) and metabolic pathways (38 %) and were up and down regulated in the various sites suggesting that these pathways are highly affected by heat stress. There were six main proteins involved in photosynthesis or light harvesting in *A. linearis*. Four of these proteins were upregulated the most in plants grown at Aurora, the cooler site, and downregulated the most in the hottest site, Uitsig, agreeing with results from chapter two, where plants from Aurora had superior photosynthetic rates compared to the

other plants. Two proteins were involved in the biological responses to high light intensity in *A. linearis* plants, and both of the proteins, light-harvesting complex-like protein OHP2, chloroplastic and protein proton gradient regulation (PGR) 5, chloroplastic were expressed the most in plants from Aurora, which also is in agreement with results in chapter two showing that Aurora had the highest DLI of the four sites. Both light-harvesting complex-like protein OHP2 and protein PGR 5, have a protective role within PSI in response to light stress in plants (Andersson et al. 2003, Kawashima et al. 2017) indicating that plants at Aurora are upregulating these proteins in response to the high light conditions, therefore protecting their photosystems from damage. Similar to this, other photosynthetic proteins such as Rubisco and phosphoribulokinase have been identified as down-regulated proteins in response to heat treatments and other stresses (Bose and Ghosh 1995, Chen et al. 2004, Lee et al. 2007), indicating that carbon fixation and assimilation is disturbed in these plants during times of heat stress. Two of the proteins involved in photosynthesis were upregulated in Clanwilliam and Uitsig more than at the cooler sites, those proteins being light-harvesting complex-like protein 3 isotype 2, chloroplastic and chlorophyll a-b binding protein of LHCII type 1. Both proteins play a role in regulating chlorophyll biosynthesis and binding under light stress as well as under normal conditions (Lohscheider et al. 2015), which was further supported by the increased chlorophyll concentrations in plants in Clanwilliam and Uitsig during this time (Figure 2.7; chapter two).

Six of the 180 proteins differentially expressed in *A. linearis* were involved in protein synthesis, three of which were expressed more in plants from Aurora and the other three expressed more in plants from Uitsig. These results indicate that protein biosynthesis is sensitive to heat stress and is severely impacted in the presence of this stress. Similar results were seen in *O. sativa* (Zou et al. 2011). One protein in particular, protein grpE, which was

related to protein folding, was upregulated in the hot site of Uitsig, and acts as a nucleotide-exchange factor for HSP70 proteins (Hu et al. 2012), and as such, it confers a thermotolerance to long-term exposure to heat stress in plants such as might be seen in plants grown at Uitsig. Furthermore, two transport proteins, linoleate 13S-lipoxygenase 2-1, chloroplastic and copper transport protein ATX1 were upregulated in plants at Uitsig similar to transport proteins upregulated at 44 °C in rice (Gammulla et al. 2010). Many proteins which are involved in metabolic processes are also involved in proteolysis. During abiotic stresses, protein unfolding and misfolding generally occur which leads to protein denaturation or aggregation. While the role of protein chaperones is to prevent the accumulation of newly produced polypeptides and unfolded proteins (Saibil and Ranson 2002), proteases are the ones that remove the unfolded, denatured proteins and release amino acids for recycling (van der Hoorn and Jones 2004). A number of these proteins were expressed in *A. linearis* plants, which included several aspartic, serine and cysteine proteases, and most of which were upregulated in plants at Aurora and Citrusdal, but also a few in the hottest site, Uitsig (Table A.1 – A.4). Similarly, in cultured *O. sativa* cells, several serine and cysteine proteases were expressed when cells were exposed to temperatures of 44 °C (Gammulla et al. 2010). Cysteine proteases as mentioned previously, are induced through a variety of stresses (Zhang et al. 2008, Gammulla et al. 2010) and play a role in signalling and executing programmed cell death (PCD; Lam and Del Pozo, 2000) therefore the up-regulation of this protein may promote PCD and leaf senescence in heat stressed plants (Gammulla et al. 2010). It is interesting to note that these proteins are down-regulated in the hotter sites of Clanwilliam and Uitsig therefore possibly protecting the plants from cell death during heat stress, which was also seen in *A. thaliana* at high temperatures (Larkindale and Vierling 2008).

Five main proteins that were related to HSPs were expressed in *A. linearis* plants, three of which were sHSPs and two associated with HSP70. While all these HSPs were present in all the plants across the four farm sites, they were more upregulated in the hotter sites, Clanwilliam and Uitsig than the cooler sites, Aurora and Citrusdal, except for small heat shock protein C2 which was highest in Citrusdal (Table A.2). Heat shock proteins, particularly sHSPs, play an important role in the acquired thermotolerance of plants (Kotak et al. 2007). Heat shock 22kDa protein, mitochondrial isoform X1 was expressed two times more in Clanwilliam plants than at the other sites (Table A.3), while 17.3 kDa class I HSP was expressed eight times more in plants grown at Uitsig, the hottest site (Table A.4). These proteins are also known for acting as chaperones that protect other proteins against denaturation and aggregation (Jakob and Buchner 1994). The expression of these sHSPs could be enhancing the thermotolerance of *A. linearis* plants in the hotter sites, similarly to the enhanced thermotolerance of tobacco through the overexpression of mitochondrial sHSPs (Sanmiya et al. 2004). Proteins belonging to the HSP70 family of proteins are all involved in binding ATP as well as the folding and oligomerization of new polypeptides (Nelson et al. 1992). Proteins in the HSP70 family can also be expressed during non-stress conditions (Miernyk 1999), which was supported by the presence in all *A. linearis* plants across the four sites, however they were expressed four and two times more in the hotter sites, Clanwilliam (Table A.3) and Uitsig (Table A.4) respectively. The protein, 17.3kDa class I HSP was the only defence protein expressed in *A. linearis* plants that had a biological process of responding to ROS (Table A.1) Two other defence classified proteins had biological processes that included a response to oxidative stress, with photosynthetic NAD(P)H dehydrogenase (NDH) subunit of subcomplex B 5, chloroplastic expressed two times more in plants from Aurora, while the other protein, probable phospholipid hydroperoxide glutathione peroxidase, was expressed two times more in Uitsig plants. One of the main concerns for

plants during heat stress is oxidative damage which can be brought on by ROS (Lang-Mladek et al. 2010). It has been thought that the evolution of chloroplast NDH in land plants was for the purposes of alleviating oxidative stress in chloroplasts (Peng et al. 2011) while the main function of phospholipid hydroperoxide glutathione peroxidase is to protect cells against the damaging effects of ROS such as hydroxyperoxides, via the reduction of these ROS by glutathione (Barnett and King 1995). A number of other proteins are also involved in antioxidant activities in *A. linearis* plants such as peroxidase 15, probable glutathione S-transferase and thioredoxin H-type-like, which were upregulated the most in plants from Aurora, while others such as peroxidase 17, peroxidase P7-like and glutathione S-transferase L3 were upregulated the most in plants from Uitsig. Two other protection proteins, cysteine proteinase inhibitor-like and cysteine proteinase 15A-like, were also expressed, however they were more upregulated in Aurora and Citrusdal respectively. These protective proteins have been shown to be uniquely expressed in *O. sativa* during high temperatures (Gammulla et al. 2010) however they can be expressed during cold, drought, salt and oxidation stress as well (Zhang et al. 2008).

A number of the proteins expressed in *A. linearis* plants had a biological function of oxidation-reduction processes (Figure 3.2). Several proteins that are involved in these processes have been upregulated in *A. linearis* plants, such as glutathione S-transferase, thioredoxin H-type, delta(24)-sterol reductase and FAD binding, zeaxanthin epoxidase have been reported in *O. sativa* (Agrawal et al. 2001, Han et al. 2009, Gammulla et al. 2010) and *A. thaliana* (Park et al. 2008) during times of heat and osmotic stress. Most of these proteins were upregulated in the hotter site, Uitsig (Table A.4). Zeaxanthin epoxidase was upregulated in Uitsig and Citrusdal plants and is an important protein in the xanthophyll cycle as well as abscisic acid biosynthesis and as such plays an important role in resistance to osmotic and

drought stress (Park et al. 2008). Glutathione S-transferases are involved in the detoxification of reactive electrophilic compounds (Zou et al. 2011) as well as in catalysing the reduction of S-glutathionylquercetin to quercetin (Dixon and Edwards 2010) which is an important flavonoid that acts as an antioxidant in plants (Wahid and Ghazanfar 2006, Bita and Gerats 2013). Thioredoxin H-type serves as a general protein disulphide oxidoreductase and participates in various oxidation-reduction processes (Holmgren 1985). Delta(24)-sterol reductase proteins are involved in the reduction of 24-methylenecholestrol to campesterol (Choe et al. 1999) which is then used in the biosynthesis of brassinosteroids in plants such as *A. thaliana*, *Pisum sativum* (pea) and *L. esculentum* and now *A. linearis* (Fujioka and Yokota 2003). These proteins are all important in plant antioxidant systems and many other studies showed that they were upregulated during abiotic stresses and therefore contribute to the plant's tolerance to these stresses (Fujioka and Yokota 2003, Kocsy et al. 2004, Lan et al. 2009, Lo Piero et al. 2009, Zou et al. 2011).

High temperatures have a strong negative effect on glycolytic pathways as well as the generation of energy as was evidenced by the greater upregulation of proteins involved in glycolytic processes and proteins involved in energy functions, in the cooler sites of Aurora and Citrusdal compared to the hotter sites. Similar results were observed in *O. sativa* where two enzymes involved in the glycolytic pathway were low in abundance at 44 °C (Gammulla et al. 2010). There were six proteins involved in glycolytic processes, based on their GO biological process terms, expressed in *A. linearis*, four of which were upregulated in plants from Aurora (Table A.1), and two which were upregulated in plants from Uitsig (Table A.4). Proteins such as acetyl-CoA carboxylase 1-like (Table A.1) and transketolase (Table A.4), which are involved in the Krebs cycle, or pentose phosphate pathway and Calvin cycle, were upregulated the most in Aurora and Uitsig respectively. Transketolase is one of the main

enzymes for the synthesis of sugar-phosphate intermediates in the pentose phosphate pathways while the main function of acetyl-CoA carboxylase is to deliver acetyl groups to the Krebs cycle to be oxidised for energy production. With an upregulation of enzymes involved in glycolytic pathways, more glycolysis occurs, therefore lowering glucose concentrations (Gammulla et al. 2010) which was observed in the slightly lower levels of glucose in plants at Aurora and Uitsig (Table 2.5; chapter two).

3.4.1 Conclusion

A total of 180 proteins were differentially expressed in the plants during exposure to high temperatures in the field. Of these 180 proteins, 113 were more upregulated in the cooler sites, Aurora and Citrusdal, and 67 proteins were more upregulated in the hotter sites, Clanwilliam and Uitsig thus possibly indicating that with increasing temperatures there is a downregulation of proteins expressed during heat stress. Heat shock proteins were present in *A. linearis* plants across all the four sites however, they were more upregulated in the hotter sites, Clanwilliam and Uitsig than the cooler sites, Aurora and Citrusdal. Some of the HSPs such as heat shock 22kDa protein, mitochondrial isoform X1 and 17.3 kDa class I HSP are known for acting as chaperones that protect other proteins against denaturation and aggregation thereby enhancing the plants' thermotolerance. Furthermore, protein grpE, that was upregulated in the hot site, Uitsig, is related to protein folding and acts as a nucleotide-exchange factor for HSP70 proteins and also confers thermotolerance to long-term exposure to heat stress in plants. Light-harvesting complex-like protein OHP2 and protein PGR 5, that have a protective role within PSI in response to light stress, were expressed the most at Aurora which had the highest DLI. This observation indicates that the photosystems of the plants at Aurora were protected from damage as evidenced by their superior photosynthetic rates and biomass accumulation. Plants at Clanwilliam and Uitsig were shown to have higher

chlorophyll concentrations than the cooler sites (chapter two) and this was reflected by the upregulation of the proteins light-harvesting complex-like protein 3 isotype 2, chloroplastic and chlorophyll a-b binding protein of LHCII type 1, both of which play a role in regulating chlorophyll biosynthesis. Of further interest was the downregulation of cysteine proteases, which are related to PCD, in the hotter sites therefore likely protecting the plants from cell death during heat stress. Overall, the heat tolerance of *A. linearis* may be linked to both the up and down regulation of certain proteins at a site.

Chapter 4

Stress tolerant traits and root proliferation of *A. linearis* grown under two water regimes in response to drought

4.1 Introduction

Mediterranean ecosystems are particularly vulnerable to climate change (Hoegh-Guldberg et al. 2018) since temperatures are increasing during summer months and winter months are becoming drier (Klausmeyer and Shaw 2009). Indeed, climatic model projections for the Western Cape of South Africa indicate a drying trend from west to east, less rainfall during winter, more irregular occurrences of extreme rainfall events which could lead to flooding, and general increases in temperatures all across the region (Gizaw and Gan 2017). Added to this are the occurrences of natural fires in the fynbos region which are a driving force in fynbos dynamics and have played an important role in the diversification of the Cape flora (Cowling 1987). These fires are dependent on vegetation conditions, such as sufficient fuel to burn, and climate and weather conditions, where warm, dry conditions provide easy ignition conditions (Cowling 1987, Turco et al. 2017). With increases in temperatures and the occurrences of drought, there will likely be an increase in fire activity in Mediterranean regions as well (Turco et al. 2017, Hoegh-Guldberg et al. 2018).

While global warming is one of the most obvious products of climate change and is able to be predicted with a high degree of certainty, rainfall patterns are more difficult to project due to the high variability in every region (Midgley et al. 2005). However, with the increases in temperature comes an inherent increase in evaporative demand on the soil thus leading to depletion of groundwater and drying trends even with no changes in precipitation (Midgley et

al. 2005). Climate projections for the Western Cape of South Africa have shown that there will be an increased variability in precipitation, along with possible increases in dry spells and late winter rains (Midgley et al. 2005, Hewitson and Crane 2006, Hoegh-Guldberg et al. 2018). While the Cederberg area mainly receives its rain during the winter months, there are also some occurrences of summer showers, however these are interspersed with frequent dry spells (Louw 2006) which are likely to become worse with climate change. Dry spells are defined as a period of consecutive days without significant rainfall (Barron et al. 2003) while droughts are defined as periods or seasons of abnormally dry weather which can range from weeks to decades (Passioura 2002, Araya and Stroonsnijder 2011).

Aspalathus linearis rely on soil moisture from rainfall received during the winter months and as such, one of the most important factors limiting the growth and production of these plants is water availability during the summer months (South African Rooibos Council 2019a). Considering the economic importance of *A. linearis* and climatic conditions under which it is grown, the limited information in literature on its response to changes in water availability is quite surprising. The available studies have reported that wild genotypes of *A. linearis* had higher water use efficiency, were more reliant on N via N_2-fixation and had higher C/N ratios, implying enhanced sclerophylly, while the cultivated *A. linearis* genotypes had lower water use efficiency, tended to retain their N and had lower C/N ratios (Hawkins et al. 2011, Lötter et al. 2014a, Kemp et al. 2018). It has been suggested that mixing commercial *A. linearis* with the wild ecotypes would be beneficial in the long term as they would lessen the impact of increasing temperatures and variable rainfall in a climate change context (Hawkins et al. 2011). Lötter et al. (2014b) found that drought stressed plants had higher root/shoot ratios, lower plant biomass overall, higher C/N ratios and lower photosynthesis, stomatal conductance and transpiration compared to controls when grown under glasshouse conditions

in quartz sand and water was withheld from them for a period of six weeks. Van Schalkwyk, (2018) examined plant development of *A. linearis* in shallow and deep soils in Clanwilliam in the Western Cape and found that *A. linearis* growing on deeper soils had higher shoot biomass compared to plants on shallower soils which may have restricted root growth and aboveground biomass. A few adaptations against drought stresses include increases in root:shoot ratios, regulation of leaf water content as well as carbohydrates, along with lower stomatal conductances and decreased photosynthesis (Benjamin and Nielsen 2006, Praba et al. 2009, Araújo et al. 2015).

While some work has been done on the drought effects on *A. linearis* with emphasis on the aboveground physiology, there is no evidence in literature on the effects of drought on the morphology and structure of roots of plants grown under well-watered and low moisture conditions. *Aspalathus linearis* plants can grow up to 2 m in height and are probably able to survive the summer drought periods in the field by producing a deep taproot that extends as deep as 2 m (Smith et al. 2018). The objective of this chapter was to determine the physiological and morphological responses of *A. linearis* grown under two soil moisture regimes, one simulating adequate water supply (high rainfall conditions) and one simulating low rainfall conditions, and later exposed to drought. It was hypothesized that plants grown under low moisture conditions will be more tolerant to drought than those receiving adequate moisture.

4.2 Methods and materials

The root morphology and physiology of *A. linearis* plants was investigated in a potted soil experiment in a well-ventilated glasshouse situated at the University of Cape Town (33.955889S, 18.462111E) with a temperature range of 15 – 39 °C and an average

temperature of 24 °C from July 2018 to April 2019. Soils were collected from an *A. linearis* farm in Clanwilliam (32.161417S, 18.777350E) and passed through a 2 mm sieve to remove any large debris. *Aspalathus linearis* seeds were germinated in trays of their habitat soil prior to three seedlings being transplanted into 20 tube pots, 11 cm × 50 cm, containing 5 kg of soil to allow for deeper root growth in the young plants. After a month, the seedlings were reduced to one per pot and half the pots (ten plants) were maintained at 70 % of field capacity (FC, i.e. high rainfall conditions) and the other half (ten plants) were maintained at 30 % FC to simulate low rainfall conditions. The FC's were determined from previous potted glasshouse experiments where *A. linearis* plants were grown in 20 cm diameter pots, in natural soils, at various FC's (unpublished). The field capacity of soil was measured by completely drying the soil (i.e. no more change in weight) before being weighed into pots. Field capacity was then determined by watering the pots until oversaturated, left over night for the excess water to drip off and then reweighed to get 100 % FC. After six months of growth in the long tube pots, the plants were moved into a phytotron to maintain a stable environment for gas exchange measurements. The photoperiod was set to 14 hrs with a light intensity of 800 µmol m^{-2} s^{-1} while temperatures ranged from 22.6 °C (night) – 28.6 °C (day) with maximum temperatures reaching 33.7 °C during the day. The VPD ranged from 1.16 kPa (night) – 2.34 kPa (day) with a maximum VPD of 3.51 kPa during the day. Half of each of the FC treatments (five plants) were then subjected to drought where water was withheld from pots, to determine how long *A. linearis* can cope without watering, until wilting point (at seven days of drought) when the plants were then harvested. Preliminary experiments determined the permanent wilting point to be after seven days when soil moisture dropped to below 5 % FC, stomatal conductance decreased by over 90 % and the relative water content (RWC) decreased to ~50 % of control plants.

4.2.1 Soil properties

The soil from Clanwilliam was primarily sandy and acidic with low C and N but high total P and while the micronutrients were mostly in low abundances in the soil, Fe was high (Table 2.3; chapter two).

4.2.2 Biomass and morphology

On the seventh day of drought, when over 50 % of the plants were starting to show signs of wilting, plants were harvested and samples were taken for determination of phenolic and carbohydrate concentrations, leaf water potential (Ψ_L), and root morphological analyses. The remaining biomass was separated into leaf, stem and root and oven dried at 60 °C for three days and DW recorded. At harvest, approximately 15 % of the total root mass was collected for root morphological analyses (Vandamme et al. 2013) and stored in 10 % ethanol at 4 °C. Roots were stained with 2 % gentian violet to allow for better visualisation on the scanner and stored in ethanol prior to scanning. Root morphological parameters; total root length (m), average root diameter (mm) and total root surface area (SA; m^2) were measured with a STD4800 scanner and WinRHIZO version 2013a (Regent Instruments, Canada). The root samples were dried in the oven for three days at 60 °C and weighed. Results were expressed as whole-root results by multiplying with the relevant conversion factors to 100 % root, using the dry weights of the root sample that was scanned and the whole root system. The specific root length (SRL) was calculated by dividing total root length (m) by total root DW (g).

4.2.3 Gas exchange

During the drought period, leaf gas exchange rates, chlorophyll content and RWC were measured every second day until harvest. The rates of P_{max}, g_s, E and the ratios of intercellular CO_2 to ambient CO_2 (C_i/C_a) were measured using a LI-6400XT Portable

Photosynthesis System (LI-COR Biosciences, Lincoln, NE, USA). The reference CO_2 concentration was maintained at 400 ppm while the flow rate was 400 μmol s^{-1} and the light inside the chamber was set to 1500 μmol photons m^{-2} s^{-1} (light saturation value derived from previous light response curves; data not shown). The PWUE (μmol CO_2 mmol H_2O^{-1}) was calculated by dividing P_{max} by E. The youngest fully-grown leaves were used for measurements and the leaves in the chamber (four – six leaves) were removed and scanned for total SA to express all measurements per leaf area.

4.2.4 Non-structural carbohydrates

During harvest, approximately 5 g of leaf samples of the youngest fully-grown leaves were collected and dried for 48 hrs at 60 °C. These samples were then finely ground using a Hammer Mill (United Scientific Pty Ltd, Cape Town, South Africa) and hexose (glucose and fructose), sucrose and starch were assayed using the enzymatic method described by Zhao et al. (2010).

4.2.5 Phenolic concentrations

Approximately 5 g of the youngest fully-grown leaves were taken at harvest, for total phenol, flavonol and anthocyanin analyses, immediately frozen in liquid N and kept frozen until they were lyophilized in a freeze drier (VirTis United Scientific, Cape Town, South Africa) and thereafter milled to a fine powder using a Hammer Mill (United Scientific Pty Ltd, Cape Town, South Africa). Crude extracts for total polyphenol and flavonol analyses were prepared by stirring 0.01 g of the powdered leaves in 10 ml of 80 % (v/v) ethanol. Samples were then centrifuged at 3,220 × *g* for five minutes. The supernatants were used for measurement of total polyphenol and flavonol concentration as described by Daniels et al. (2011) in a 24 well plate. Gallic acid was used to make the calibration curve by using 0, 20,

50, 100, 250 and 500 mg L^{-1} solutions of gallic acid in 10 % (v/v) ethanol and values of total polyphenol were expressed as mg g^{-1} DW per gallic acid equivalent. For flavonol concentrations, quercetin was used to make the calibration curve using 0, 5, 10, 20, 40 and 80 mg L^{-1} solutions of quercetin in 95 % ethanol and values were expressed as mg g^{-1} DW per quercetin equivalent. Anthocyanin measurements were done by placing 0.1 g of powdered freeze-dried leaves in vials with 10 ml of methanol: water: concentrated HCl (79:20:1) and put on a shaker in the dark at 2 °C for 48 hr. The extracts were filtered through Whatman No. 1 filter paper, topped up to 12 ml and the absorbances read at 530 and 657 nm. The concentration of anthocyanin was determined using the equation in Lindoo and Caldwell (1978).

4.2.6 Soil moisture, relative water content and water potential

Soil moisture was measured in each pot every second day of the drought treatment using a portable soil moisture probe (ML2X Moisture Meter, WET Sensor, Delta-T Devices, Cambridge, England). However, it was only measured in the first 10 cm of the pot as the probe could not go further than that, therefore deeper in the pot there was likely more moisture that wasn't picked up by the probe. As a result, the soil moisture data was not presented because it was deemed not informative. Prior to the induction of drought stress, a combination of weighing the pots and using the soil moisture probe was used to maintain the soil at the required field capacities. The RWC was used to determine *A. linearis* plants' capacity to hold water during their exposure to drought. Leaf RWC was measured on two leaves per plant every second day of drought treatment. Leaves were detached and weighted immediately for fresh weight (FW), saturated for four hours in distilled water at room temperature and reweighed to obtain turgor weight (TW; Zhou et al., 2017). Samples were

then placed in the oven at 60 °C for 72 hrs to obtain their DW. Relative water content was calculated as:

$$RWC = (FW - DW) / (TW - DW) \times 100 \quad (3)$$

The Ψ_L was measured, together with gas exchange parameters, on a branch from each plant using a portable Scholander pressure chamber (PMS Instrument Company, Albany, OR, USA).

4.2.7 Chlorophyll and carotenoid concentration

Every two days during the drought period, the two youngest fully-grown leaves were collected and inserted into a vial with 10 ml of 95 % ethanol (Lichtenthaler 1987) and left to auto-extract for 24 hr in the dark. The leaves were removed, and their surface area was determined using a STD4800 scanner and WinRHIZO version 2013a program (Regent Instruments, Quebec, Canada). The light absorptions of the solutions were measured at specific wavelengths (664, 648 and 470 nm) on a Thermo Helios Epsilon spectrophotometer (Thermo Scientific, MA, USA) and chlorophyll and carotenoid concentrations were calculated using equations by Lichtenthaler (1987).

4.2.8 Statistical analysis

For all the variables, nested ANOVA tests were done with soil moisture as the random factor and the drought treatment nested in FC. Where the tests showed significant differences ($P<0.05$), means were separated using a Tukey HSD *post hoc* test. Letters indicate significant differences between FC and drought treatment. All statistical work was done in Statistica 13 (TIBCO Software Inc., CA, USA).

4.3 Results

4.3.1 Biomass and root morphology

Plants grown at 70 % FC had double the amount of total aboveground biomass than plants grown at 30 % FC, and the drought treatment did not affect aboveground biomass in both groups of plants (Figure 4.1*a*). This pattern was consistent with leaf, stem as well as total biomass (Table 4.1) where the biomass was higher in 70 % FC plants compared to the 30 % FC plants. Root biomass showed no differences between FC and drought treatment (Table 4.1), however the root/shoot ratios increased in 30 % FC plants under drought conditions by ~30 % compared to control plants (Figure 4.1*b*). In plants exposed to drought, total root length was nearly doubled in seven days compared to the control plants (Figure 4.2*a*), and the roots of plants grown at 30 % FC were significantly thinner than the control plants' roots (Figure 4.2*b*). There were no significant differences in total root surface area between drought treatments for both 70 % and 30 % FC plants nor were there differences between FCs or drought treatment with regards to SRL (data not shown).

4.3.2 Gas exchange

In the 70 % FC droughted plants, and after one day with no water, P_{max} decreased by ~30 % compared to the control and remained at this level on day three. By day five, P_{max} decreased by 96 % compared to the control and remained at that level until harvesting on day seven (Figure 4.3*a*). In the 30 % FC plants, and after one day with no water, P_{max} remained the same as the control plants but from day three, P_{max} decreased by ~90 % in the drought plants and remained at that level until harvest on day seven (Figure 4.3*a*). In the 70 % FC plants, the g_s decreased by 24 % after one day of no water. At day three, five and seven, the g_s decreased by 43, 88 and 98 % respectively compared to the controls (Figure 4.3*b*). In the 30 % FC

plants, the g_s was similar between the drought and control after one day of no water, however, at day three the g_s of the plants under drought decreased by over 90 % and remained that way until harvest on the seventh day (Figure 4.3*b*). The results for E had similar patterns as g_s (Figure 4.3*c*). The PWUE was highest in the 30 % FC plants compared to the 70 % FC on the first day of drought and on the third day of no watering, both 70 % and 30 % FC plants subjected to drought had higher PWUE compared to their controls (Figure 4.3*d*). The PWUE was highest on the seventh day of no watering in the 30 % FC droughted plants (Figure 4.3*d*). The C_i/C_a ratios remained constant throughout the drought period, and values were similar between droughted and control plants for both FC treatments (Fig. 3*e*). There were very high C_i/C_a ratios during the fifth day of drought in both 30 % and 70 % FC plants, and negative values on the seventh day of drought in 30 % FC plants (Fig. 3*e*). The increase in C_i/C_a on the fifth day of drought was likely due to the inaccuracies of C_i calculations under drought, where cuticular transpiration and heterogeneous stomatal closures overestimate C_i (Cornic and Massacci 1996, Flexas and Medrano 2002). Similarly, on the seventh day of drought, in the 30 % FC plants, there were negative C_i/C_a ratios due to g_s and E being very low and therefore the calculation of C_i becomes unreliable (Cornic 2000).

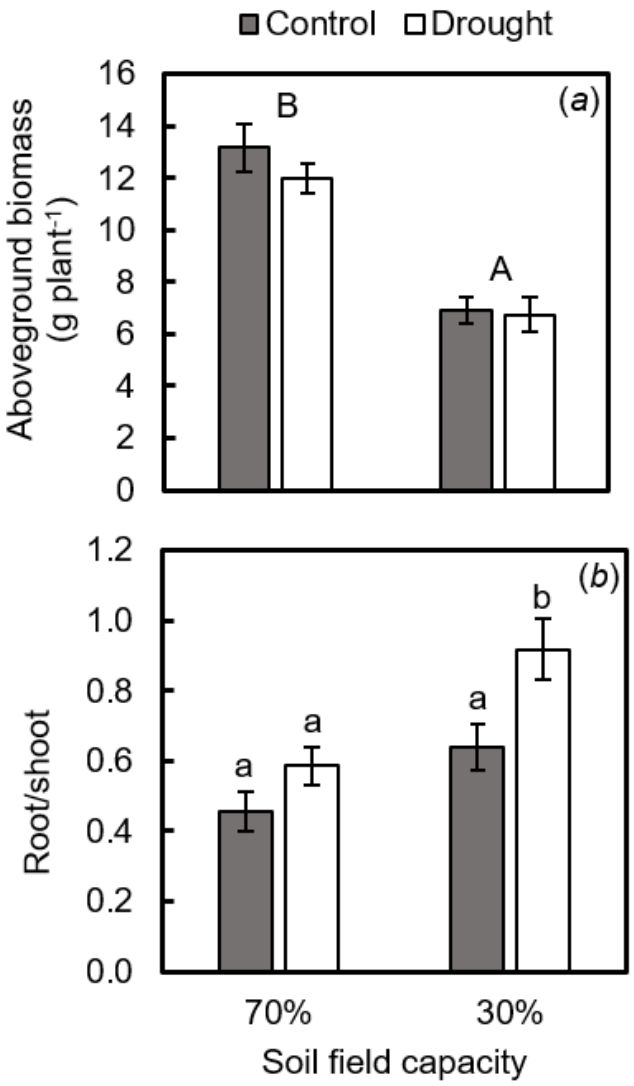

Figure 4.1. (*a*) Total aboveground biomass and (*b*) root/shoot ratio of *A. linearis* plants grown under two different soil field capacities: 70 % and 30 % FC, and then exposed to a seven-day drought. Uppercase letters show significant differences between FC only and lowercase letters indicate differences between the interaction of FC and drought treatment by Tukey's HSD *post hoc* test ($n = 5$; $P < 0.001$ and $P < 0.05$ respectively). Vertical bars denote ± s.e.

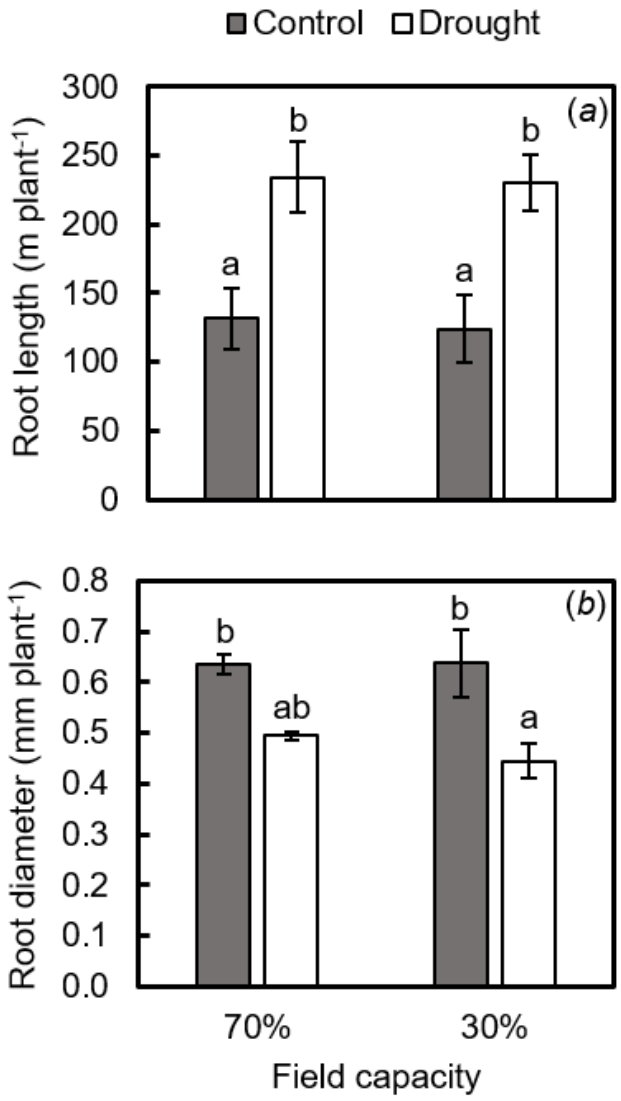

Figure 4.2. (*a*) Total root length and (*b*) average root diameter of *A. linearis* plants grown under two different soil field capacities: 70 % and 30 % FC, and then exposed to a seven-day drought. Lowercase letters indicate differences between the interaction of FC and the drought treatment by Tukey's HSD *post hoc* test ($n = 5$; $P < 0.01$). Vertical bars denote ± s.e.

Table 4.1 Biomass, carbohydrate, phenolic and leaf water potential (Ψ_L) results of *A. linearis* plants (*n* =5) grown under two different field capacities; 70 % and 30 % FC, and then exposed to a seven-day drought. Means ± s.e. followed by uppercase letters in the rows indicate significant differences between FC only and lowercase letters indicate the interaction between FC and drought treatment by Tukey's HSD *post hoc* test as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Variable	70% FC		30% FC		*F*-ratio	
	Control	Drought	Control	Drought	FC	Drought
Leaf (g plant^{-1})	6.09 ± 0.62 B	6.04 ± 0.65 B	3.46 ± 0.40 A	3.16 ± 0.26 A	30.62***	0.097
Stem (g plant^{-1})	7.09 ± 0.60 B	5.96 ± 0.31 B	3.47 ± 0.28 A	3.60 ± 0.44 A	54.77***	1.883
Root (g plant^{-1})	5.12 ± 0.77	6.18 ± 0.92	4.51 ± 0.66	5.99 ± 0.31	0.37	2.12
Total biomass (g plant^{-1})	17.62 ± 1.84 B	18.93 ± 1.79 B	11.43 ± 1.12 A	12.75 ± 0.73 A	20.72***	0.48
[Hexose] (mg g DW^{-1})	48.4 ± 2.6 a	72.2 ± 4.9 b	45.5 ± 0.6 a	48.8 ± 4.6 a	13.31**	11.03**
[Sucrose] (mg g DW^{-1})	13.6 ± 1.2 B	11.0 ± 2.4 B	9.0 ± 1.9 A	4.9 ± 0.4 A	12.84**	2.57
[Starch] (mg g DW^{-1})	144.9 ± 5.8 c	57.8 ± 5.5 b	155.8 ± 11.0 c	13.8 ± 3.3 a	4.98*	120.89***
[Hexose] / [Sucrose]	3.9 ± 0.4 a	5.8 ± 0.8 a	5.3 ± 1.1 a	11.5 ± 1.8 b	9.40**	7.8**
[Total polyphenol] (mg GAE g DW^{-1})	586.6 ± 20.8 a	735.1 ± 22.1 b	608.8 ± 43.2 ab	660.4 ± 26.2 ab	0.74	6.36*
[Flavonol] (mg QE g DW^{-1})	18.22 ± 0.50	22.16 ± 1.69	19.53 ± 0.78	21.63 ± 1.93	0.09	3.08
[Anthocyanin] (Abs g DW^{-1})	0.91 ± 0.15 B	1.15 ± 0.08 B	0.74 ± 0.05 A	0.78 ± 0.08 A	7.77*	1.55
Ψ_L (MPa)	-0.78 ± 0.05 b	-4.53 ± 1.10 a	-0.91 ± 0.07 b	-4.89 ± 0.67 a	0.14	17.91***

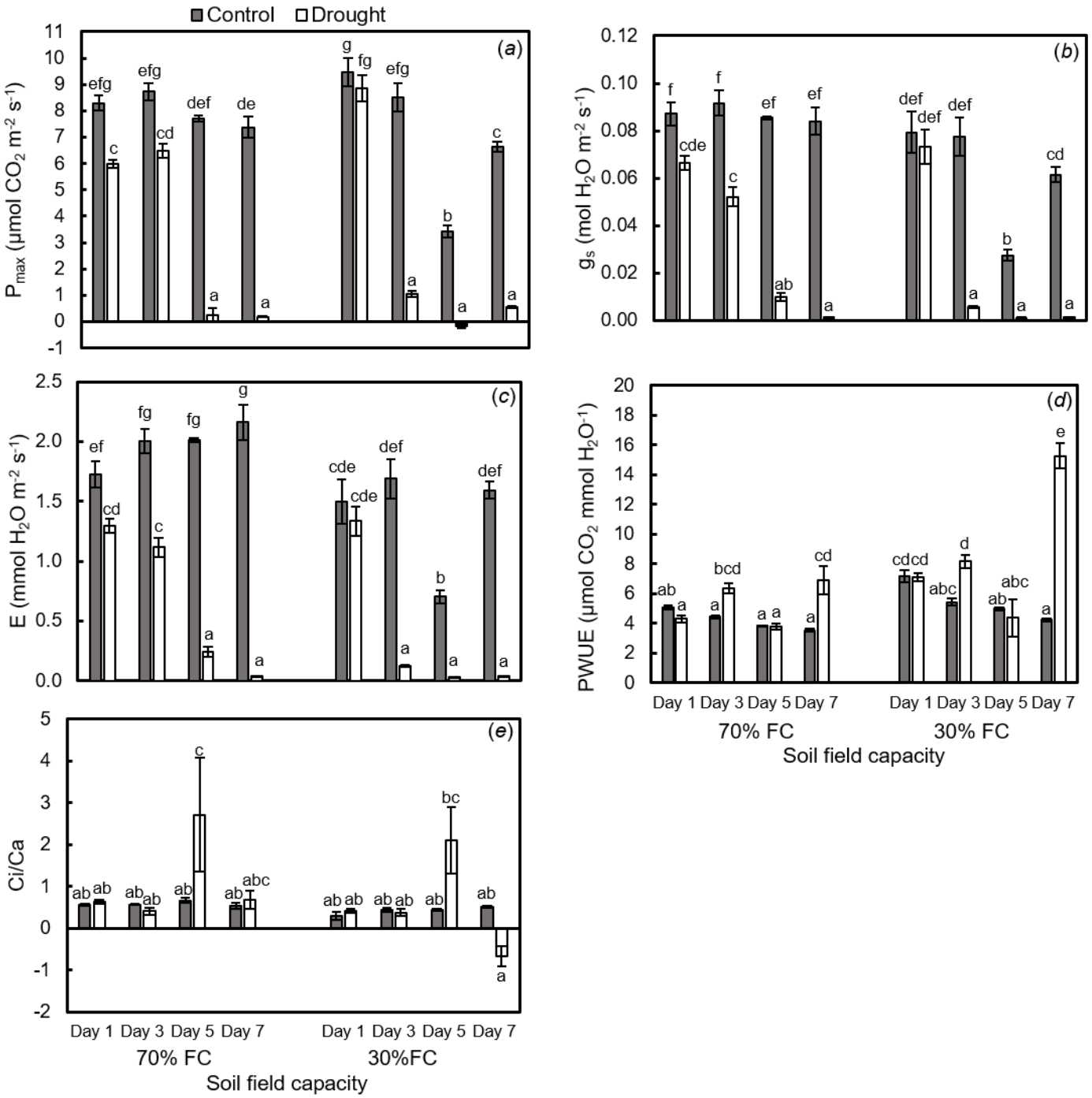

Figure 4.3. (*a*) Maximum photosynthesis, (*b*) stomatal conductance, (*c*) transpiration, (*d*) photosynthetic water use efficiency and (*e*) Ci/Ca ratios of *A. linearis* plants grown under two different soil field capacities; 70 % and 30 % FC, and then exposed to a seven-day drought. Lowercase letters indicate differences between the interaction of FC and the drought treatment by Tukey's HSD *post hoc* test ($n = 5$; $P < 0.001$). Vertical bars denote ± s.e.

4.3.3 Non-structural carbohydrates

Drought had no effect on hexose concentrations in the 30 % FC plants, however the 70 % FC droughted plants had 1.5× the amount of hexose in their leaves compared to the controls (Table 4.1). Drought treatment had no effect on the sucrose concentrations, however, the plants grown at 30 % FC had significantly lower concentrations of sucrose compared to the plants grown at 70 % FC (Table 4.1). Starch concentrations were ~60 and 90 % lower in the 70 % and 30 % FC plants during drought respectively at harvest (Table 4.1). The 30 % FC drought plants had 2× higher hexose / sucrose compared to the control plants and the 70 % FC plants (Table 4.1).

4.3.4 Phenolic concentrations

There were no differences in total phenolic content in the 30 % FC plants, however, the 70 % FC droughted plants had a 20 % increase in total phenolics compared to their control plants (Table 4.1). There were no differences in flavonol concentrations between the FC treatments or water treatments (Table 4.1). Anthocyanin concentrations were, on average, 26 % lower in the 30 % FC plants compared to the 70 % FC and the effects of drought were not significant (Table 4.1).

4.3.5 Relative water content and leaf water potential

Plants exposed to drought had an 82 % lower Ψ_L than the controls and the effects of growing them in different FC had no significant effect (Table 4.1). Both the 70 % and 30 % FC droughted plants had high RWC on the first and third day of drought, however, by the fifth day, and up until the seventh day of drought, RWC decreased by about 20 % (Table 4.2).

4.3.6 Chlorophyll and carotenoid concentration

There were no differences in total chlorophyll and carotenoid concentrations for the 70 % and 30 % FC control plants throughout the experiment, however, the 30 % FC droughted plants had higher concentrations of total chlorophyll and carotenoids on the seventh day of drought compared to the first day. There were no differences in the chlorophyll a/b ratios for any of the treatments (Table 4.3). There were no differences in the chlorophyll/carotenoid ratios for the 70 % FC control and drought plants, however, the 30 % FC droughted plants had lower chlorophyll/carotenoid ratios on the seventh day of drought compared to the first day (Table 4.3).

Table 4.2. Leaf RWC results of *A. linearis* plants (*n* =5) grown under two different soil field capacities; 70% and 30% FC, and then exposed to a seven-day drought. Means ± s.e. followed by lowercase letters, in the column, indicate differences between the interaction of FC and the days without watering (DWW) with control and drought by Tukey's HSD *post hoc* test as follows: **, $P < 0.01$; ***, $P < 0.001$.

FC	Treatment	DWW	RWC (%)
70%	Control	Day 1	70.2 ± 3.4 bcd
		Day 3	83.4 ± 1.6 de
		Day 5	75.4 ± 2.4 bcde
		Day 7	76.3 ± 1.5 bcde
	Drought	Day 1	71.6 ± 4.3 bcde
		Day 3	82.3 ± 3.4 de
		Day 5	60.3 ± 5.2 ab
		Day 7	60.0 ± 3.2 ab
30%	Control	Day 1	91.3 ± 8.2 e
		Day 3	87.2 ± 1.5 e
		Day 5	79.5 ± 3.3 cde
		Day 7	84.5 ± 2.5 de
	Drought	Day 1	84.9 ± 3.4 de
		Day 3	76.2 ± 4.4 bcde
		Day 5	49.8 ± 3.4 a
		Day 7	62.4 ± 3.0 abc
F-ratio		FC	10.24**
		Treatment	12.31***

Table 4.3. Chlorophyll and carotenoid results of *A. linearis* plants (*n* =5) grown under two different soil field capacities; 70% and 30% FC, and then exposed to a seven-day drought. Means ± s.e. followed by lowercase letters, in the columns, indicate differences between the interaction of FC and the days without watering (DWW) with control and drought by Tukey's HSD *post hoc* test as follows: **, $P < 0.01$; ***, $P < 0.001$.

FC	DWW	Treatment	Chl a+b ($\mu g\ cm^{-2}$)	Chl a/b	Car ($\mu g\ cm^{-2}$)	Chl/car
70%	Day 1	Control	3.89 ± 0.09 ab	3.69 ± 0.05	0.83 ± 0.03 ab	4.68 ± 0.07 b
		Drought	2.88 ± 0.12 ab	3.96 ± 0.07	0.62 ± 0.02 a	4.65 ± 0.05 b
	Day 3	Control	2.97 ± 0.22 ab	3.84 ± 0.12	0.68 ± 0.06 a	4.34 ± 0.09 ab
		Drought	3.12 ± 0.21 ab	4.08 ± 0.11	0.70 ± 0.05 a	4.46 ± 0.09 b
	Day 5	Control	3.17 ± 0.21 ab	3.96 ± 0.09	0.73 ± 0.05 a	4.34 ± 0.06 ab
		Drought	3.10 ± 0.27 ab	4.22 ± 0.19	0.72 ± 0.05 a	4.30 ± 0.12 ab
	Day 7	Control	3.71 ± 0.42 ab	3.96 ± 0.09	0.85 ± 0.09 ab	4.34 ± 0.06 ab
		Drought	4.20 ± 0.68 bc	4.22 ± 0.19	1.05 ± 0.08 b	4.26 ± 0.15 ab
30%	Day 1	Control	3.17 ± 0.21 ab	3.71 ± 0.12	0.71 ± 0.05 a	4.46 ± 0.17 b
		Drought	3.32 ± 0.19 ab	3.89 ± 0.12	0.72 ± 0.03 a	4.60 ± 0.10 b
	Day 3	Control	3.30 ± 0.07 ab	3.90 ± 0.08	0.78 ± 0.03 a	4.38 ± 0.04 b
		Drought	2.74 ± 0.20 a	4.33 ± 0.24	0.70 ± 0.04 a	4.16 ± 0.10 ab
	Day 5	Control	2.90 ± 0.16 ab	4.17 ± 0.13	0.67 ± 0.05 a	4.36 ± 0.08 ab
		Drought	2.79 ± 0.18 a	3.65 ± 0.15	0.72 ± 0.05 a	3.89 ± 0.10 a
	Day 7	Control	3.40 ± 0.21 ab	4.17 ± 0.13	0.79 ± 0.06 ab	4.36 ± 0.08 ab
		Drought	5.28 ± 0.23 c	3.87 ± 0.25	1.38 ± 0.08 c	3.89 ± 0.10 a
F-ratio		FC	0.02	0.14	1.79	9.49**
		Treatment	7.48***	1.82	11.35***	4.86***

4.4 Discussion

The main objective of this chapter was to determine the physiological and morphological responses of *A. linearis* grown under two moisture regimes, one simulating adequate water supply (high rainfall) and one simulating low moisture conditions (low rainfall), and later exposed to drought. As was mentioned previously, the variations in rainfall as well as longer dry spells during the growing period of the plants are major yield-limiting factors associated

with climate change (Midgley et al. 2005, Archer et al. 2008). It was hypothesized that plants grown under low moisture conditions initially would be more tolerant to drought than those who had received adequate moisture; however, this was not the case. The gas exchange parameters were more affected in plants grown under low moisture conditions compared to in plants grown under adequate conditions and then droughted as was observed in the reduction of P_{max}, g_s and E. The plants in the 30 % FC treatment recorded lower P_{max}, g_s and E after five days in phytotron conditions, also indicating the negative effects of low moisture conditions which explains the observation that plants grown under low moisture conditions had almost 50 % less biomass than plants grown under adequate moisture. This implies that low rainfall and the occurrences of dry spells and drought, associated with climate change (Hewitson and Crane 2006), are likely to reduce the production of *A. linearis* in the Cederberg area. This is consistent with previous field work which showed that climatic and soil variables were important predictors of *A. linearis* yield (chapter two).

Decreases in photosynthesis during drought can be either though stomatal closure or metabolic impairments (Cornic and Massacci 1996, Flexas and Medrano 2002). Numerous experiments have shown that the closure of stomata is more closely linked to soil moisture content than leaf water status, suggesting that the stomata are responding to chemical signals from the dehydrating roots, while the leaf water status is kept constant (Farooq et al. 2009). In general, during drought as stomata close, the C_i initially decreases with increasing stress indicating that stomatal limitations to photosynthesis dominate (Cornic and Massacci 1996, Flexas and Medrano 2002). In the *A. linearis* plants, the C_i/C_a values remained relatively constant in both the drought and control plants during the drought period, even while g_s decreased substantially, thereby indicating that there was a predominance of non-stomatal limitations to photosynthesis (Cornic and Massacci 1996, Flexas and Medrano 2002). At a

given VPD, the ratio of PWUE is inversely related to C_i/C_a (Condon et al. 2004), however, as was seen in this chapter, this was not the case for *A. linearis* plants where both PWUE and C_i/C_a generally remained unchanged during the drought period. Similar results were seen in plants such as rice (Zhao et al. 2004, Giuliani et al. 2013), tomato (Martin and Thorstenson 1988) and soybean (Bunce 2019). This implies that other physiological processes such as variable mesophyll conductance that influence the diffusion of CO_2 to chloroplast (Flexas and Medrano 2002), and decreases in ATP synthesis and ribulose 1,5 biphosphate (RuBP) regeneration (Tezara et al. 1999) are limiting the photosynthetic process thereby disrupting the correlations between PWUE and C_i/C_a (Warren and Adams 2006).

The low moisture droughted plants exhibited drought tolerant mechanisms which included higher root/shoot ratios compared to the other plants as well as thinner roots, both of which are effective for water and nutrient uptake (MacAlister et al. 2018). Increases in root/shoot ratios allow the plants to increase the proportion of biomass which takes up water relative to the aboveground biomass which uses the water (Lei et al. 2006). However, for each unit of leaf area there is more non-photosynthetic tissue to sustain, ultimately leading to reduced growth rates (Poorter and Remkes 1990). The plants under stress conditions reacted to the drought swiftly and produced double the length of roots compared to the non-stressed plants in the space of seven days. It is clear that *A. linearis* is particularly sensitive to soil drying and can respond quickly to these conditions by the production of longer roots thereby enabling the plants to explore deeper soil profiles for water sources as was evidenced by their relatively high RWC and g_s under field conditions (chapter two) compared to the plants grown in the glasshouse (chapter 4). Increases in root length in response to drought have similarly been reported in sclerophyllous woody species such as *Quercus suber* (cork oak; Puértolas et al. 2008) and *Abies fabri* (Faber's fir; Yang et al. 2013).

Other tolerant mechanisms evident in *A. linearis* include the absence of differences in pigment content except under extremely low moisture conditions (last day of drought) as well as the maintenance of high RWC well into drought. The significantly higher RWC in 30 % control plants compared to the 70 % control plants, as well as the higher amount of chlorophyll in day seven in 30 % drought plants, was surprising however, there is no appropriate explanation. *Aspalathus linearis* had no change in chlorophyll content, carotenoid content or chlorophyll/carotenoid ratios during drought. This observation supports the idea that leaf photochemistry in *A. linearis* is resistant to water deficits and that electron transport chain efficiency is maintained during drought stress (Nunes et al. 2008). Leaf RWC has been shown to be a stable parameter as water availability declines, due to plants trying to maintain it at a certain level, and as such it has been suggested that this parameter may be useful in predicting plant tolerance to water stress (Martinez-Vilalta et al. 2019). Similar to other drought tolerant plants when exposed to drought stress (Korir et al. 2006, Farooq et al. 2009, Martinez-Vilalta et al. 2019), *A. linearis* maintained unchanged RWC way into the drought for five days under controlled conditions. Furthermore, the 30 % FC and 70 % FC control plants had similar RWC's indicating that *A. linearis* is resistant to mild water deficits, when grown under these low moisture conditions, suggesting that they are able to avoid leaf dehydration (Nunes et al. 2008). *Aspalathus linearis* plants were able to survive seven days without water, before reaching wilting point, in pots and under a closed environment, while in preliminary experiments seedlings were able to survive drought for eleven days. Therefore, it is highly likely that *A. linearis* plants are accessing water deep in the soils to survive the dry spells they experience in the field. In support of this, it was observed in the field study (chapter two) that *A. linearis* plants had high g_s and E during summer months, indicating that they were able to access deep soil moisture. Species that are drought-tolerant can maintain

their water-use efficiencies by reducing the amount of water lost through E (Farooq et al. 2009) which was seen here where the drought plants had higher values of PWUE compared to the control plants throughout the drought period. Leaves with higher levels of sclerophylly, as is seen in *A. linearis*, are able to maintain their internal moisture levels even during times of stress such as drought (Turner 1994, Bussotti et al. 2002). Noteworthy is that a decrease in g_s in the drought stressed *A. linearis* showed no associated changes in leaf RWC indicating that leaf RWC is independent of g_s (Bennett et al. 1987).

One major drawback from stomatal closure is the limitation of CO_2 fixation, which increase the production of ROS in chloroplasts due to an excess of excitation energies which are not dissipated by protective mechanisms (Asada 1999). An imbalance between the antioxidant defences and the build-up of ROS, such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals, results in oxidative stress on the plant which is manifested through protein degradation, DNA nicking and cell death (Beligni and Lamattina 1999, Munné-Bosch and Peñuelas 2003). Plants have antioxidant systems to keep the amount of active oxygen species under control, which include the production of metabolites and enzymatic scavengers (Asada 1999, Jaleel et al. 2009). The status of *A. linearis* as a healthy beverage is mainly due to its naturally high levels of phenolic compounds (Joubert and de Beer 2011, Beelders et al. 2012). *Aspalathus linearis* plants that were grown under adequate moisture conditions and then droughted, had a huge increase in their total polyphenolic content, indicating the importance of polyphenols in drought stress. Similar results were observed in Mediterranean plants such as *Q. suber* and *Rosmarinus officinalis* (rosemary) where high concentrations of antioxidants were found during summer drought periods, thereby being implicated in drought tolerant roles (Faria et al. 1996, Munné-Bosch et al. 1999). There were no differences in the total phenolics in the plants grown at low and high moisture conditions, except when the

adequately watered plants were droughted, indicating that stress occurrences trigger their production.

Other metabolic changes were evident in drought stressed *A. linearis* when grown at an adequate moisture level. These well-watered and drought stressed plants accumulated more hexose in the leaf tissues indicating that the monosaccharides were not being used in the production of starch, as was evidenced by the reduced starch concentrations in the leaves, but were likely being directed to the production of substrates such as phosphoenolpyruvate (PEP) and erythrose-4-P (E4P) for use in the shikimate pathway (Herrmann and Weaver 1999). This indicates that drought stressed *A. linearis* plants were likely using their hexose for the synthesis of phenols in the shikimate pathway for protection, rather than starch synthesis (Herrmann and Weaver 1999) for growth and other metabolic processes.

4.4.1 Conclusion

Overall, the plants grown at 30 % FC fared worse compared to plants grown under adequate moisture conditions, as was evidenced in their almost 50 % lower biomass production. This implies that low rainfall and the occurrences of dry spells and drought, associated with climate change, are likely to reduce the production of *A. linearis* in the Cederberg area. The plants were sensitive to soil drying and utilized drought tolerance mechanisms such as the production of long roots, increased root/shoot ratios, maintenance of high RWC, and production of polyphenols. Empirical evidence was presented that *A. linearis* plants increase their root length within few days in response to drying soils which is likely an inherent adaptation to the tolerance of dry spells during the dry summers of the Cederberg area.

Chapter 5

General discussion and synthesis

Aspalathus linearis is one of the most important commercially cultivated crops in South Africa due to its contributions to the welfare and heritage of the local communities (van Der Bank et al. 1995, Hawkins et al. 2011) and due to its increasing demand all around the world (Joubert and de Beer 2011, South African Rooibos Council 2019a). The cultivation of *A. linearis* is restricted to the Western Cape, South Africa and attempts to cultivate it outside of this region, globally, have been met with failure, likely due to its unique climatic and soil requirements and its associations with other biotic factors in the ecosystem (Morton 1983, Graaff et al. 2009). The plants grow in a Mediterranean climate region with hot, dry summers and cold, wet winters rely solely on the rains during winter for their growth (Boyer 1982). Global climate models are predicting that Mediterranean climate regions will become warmer during summer and drier during winter, and that there will be an increase in dry spells during the seasons (Midgley et al. 2005, Hewitson and Crane 2006, Hoegh-Guldberg 2018), therefore it is vital to understand how this will impact the growth and production of *A. linearis*. Despite the wealth of information on *A. linearis* and its health benefits, not much has been done to assess the effects of heat and drought stress on the plants. While some work has been done on the aboveground effects of drought in potted, glasshouse experiments (Lötter et al. 2014b), there is no literature on the effects of drought on the root structure and morphology, especially for plants under differing moisture regimes. A distribution model of *A. linearis* has shown that with current climate change the geographic range of this crop plant will decrease (Lötter and le Maitre 2014); however, no work has looked at the effects of increasing temperatures on the plants themselves.

5.1 Heat and drought stress

In chapter two, a field experiment was conducted where the effects of temperature on the growth and stress tolerant traits of *A. linearis* were determined along a temperature gradient in the Cederberg. It was found that *A. linearis* was able to tolerate the summer heat mainly through the use of transpirational cooling, high phenolic content and low chlorophyll concentrations in the leaves. In chapter four, a potted experiment was conducted to determine the stress tolerant traits of *A. linearis* grown at different water regimes to drought. Plants grown at lower moisture levels had 50 % less biomass than plants grown at adequate moisture. *Aspalathus linearis* plants are able to tolerate drought stress through the production of long roots in search of deeper water in the soil column and through the closing of stomata to limit water loss.

Aspalathus linearis has differentially expressed traits for heat and drought stress where during drought stress stomata are closed to reduce water loss, while during heat stress in the field, stomata are open to allow for transpirational cooling of the leaves, likely made possible by underground soil water reserves. If these underground soil reserves were to start declining, that would become problematic for *A. linearis* as then they wouldn't be able to keep their stomata open for cooling, which would essentially see the plant shut down production until either favourable conditions return, or plant death. The production of chloroplasts with less light harvesting proteins, i.e. higher chlorophyll a/b ratios, was seen in the younger plants thereby allowing them to alleviate stress caused by absorbing high amounts of light energy. In drought plants, however, there was no change in pigments during the drought period, except the very last day, therefore supporting the idea that leaf photochemistry in *A. linearis* is resistant to water deficits and that the plants are able to maintain a good electron transport chain (Nunes et al. 2008). The naturally high phenolic content in *A. linearis* seems to confer a

stress tolerance for both heat and drought stress. It is of great importance to understand plant responses to the combination of stresses in the field especially as these stresses usually occur simultaneously and are very likely to be exacerbated by climate change (Mittler and Blumwald 2010, Suzuki et al. 2014). During drought, photosynthesis decreases either through stomatal limitations or metabolic impairments (Cornic and Massacci 1996, Flexas and Medrano 2002). As was evidenced in this work, *A. linearis* plants, both drought stressed and control, were able to maintain constant C_i/C_a values throughout the drought, while stomatal conductance was decreased, therefore indicating non-stomatal limitations in photosynthesis (Cornic and Massacci 1996). Soil moisture content was severely reduced when drought was induced to the potted plants, however, RWC did not change until extreme drought conditions. For instance, after one day of no water, the plants exposed to adequate water during growth, exhibited an immediate reduction in their stomatal conductance, while RWC in the leaves was not reduced until day 5 of drought. This is in agreement with Medrano et al., (2002) who suggested that stomatal conductance was a better indicator for water deficits than RWC in the leaves as there is a root-to-leaf signal which is promoted by drying soil.

When heat stress co-occurs with high light and water stress, some of the stress can be exerted through oxidative damage in plants which is likely associated with an increase in the Mehler reaction where chloroplasts reduce oxygen to form ROS (Haupt-Herting and Fock 2002). Therefore, the naturally high concentrations of phenols in *A. linearis* as was seen during environmental stresses such as possible heat stress (chapter two) and drought stress (chapter four) indicates that these phenolics play an important role in the protection of photosynthetic machinery against the excess excitation energy that was not dissipated via PSII or other processes during heat and drought stress (Wingler et al. 1999). In general, unfermented *A. linearis* has a higher antioxidant and scavenging ability compared to fermented *A. linearis* as

during the fermentation process many phenolic compounds are oxidized into other substances (von Gadow et al. 1997, Joubert et al. 2008). When compared to other African teas, *A. linearis* ranked about midway in terms of total phenolic content, however, the content can be influenced by environmental stress and species age (Bhebhe et al. 2015). In accordance with this, transcriptomic and proteomic analyses have highlighted the importance of antioxidant systems in several plant species exposed to different stress combinations (Zandalinas et al. 2018). Plants with higher antioxidant capacities and lower ROS accumulation generally have an increased tolerance to combinations of stress (Koussevitzky et al. 2008, Suzuki et al. 2014).

5.2 Drought tolerance and avoidance

Drought stress is considered to be a loss of water that leads to certain physiological and morphological changes in the plants which include stomatal closure and subsequently restricted gas exchange, reduced water content, lower leaf water potential and loss of leaf turgor (Chaves et al. 2002, Jaleel et al. 2009). The significance of the changes is dependent on the length and severity of the drought stress period that the plants are exposed to, ultimately affecting the establishment and growth and yield of the plants (Farooq et al. 2009, Jaleel et al. 2009). There are a variety of strategies for drought avoidance and tolerance seen in Mediterranean ecosystems where drought-avoiding deep-rooted perennials and winter/spring annuals coexist with drought-tolerant sclerophyllous plants (Chaves et al. 2002). However, *A. linearis* plants are not well adapted to drought stress but can avoid it through the production of longer and thinner roots to access soil moisture from deep soil layers. As was stated previously, *A. linearis* plants are surviving the heat in the field through the opening of stomata and the use of transpirational cooling to alleviate heat stress on the plants. They are therefore likely able to maintain open stomata, even through the summer dry

spells through the use of deep tap roots which allow them to access deep underground soil water (Ayeleso et al. 2017, Smith et al. 2018). A similar result was shown in two Mediterranean tree species, *Q. ilex* and *Q. suber* in Portugal, where under sufficient soil moisture there were no differences in net carbon assimilation for the two species, however by the end of the hot, dry summer, gas exchange for *Q. suber* was lower than that of *Q. ilex* (Faria et al. 1998). It was hypothesized that *Q. ilex* was able to tap into water from deeper soil layers therefore allowing it to maintain high water influx and subsequently higher leaf carbon assimilation rates compared to *Q. suber* (Chaves et al. 2002).

Just as in this thesis, it has been found in *Nerium oleander* (oleander; Gollan et al. 1985) and *Trifolium subterraneum* (clover; Socias et al. 1997) that stomata respond to drought before any changes in leaf water potential and / or leaf water content can be detected, showing that there is a drought induced root to leaf signal promoted by soil drying. Similarly, many studies have shown that stomatal responses are linked to the soil moisture rather than the leaf water status suggesting that stomata are responding to chemical signals received from dehydrating roots, while at the same time maintaining a constant leaf water content (Gowing et al. 1990, Davies and Zhang 1991). While most of the evidence for this comes from controlled, potted experiments (Davies and Zhang 1991, Jackson et al. 1995) including this thesis, there are a few field studies that support this as well, such as those on *Z. mays* (Tardieu et al. 1991), *V. vinifera* (Stoll et al. 2000) and *T. subterraneum* (Socias et al. 1997).

5.3 Soil moisture in the Cederberg and implications on other plants

Evaporation is one of the biggest causes of water loss in semi-arid and arid environments (Bach 1992) with the demand in evaporation generally higher than the ability of the soil to conduct water (Rose et al. 2005). Soil water content is therefore replenished by rainfall and

decreased through evaporation and soil drainage (Remson et al. 1960) particularly in semi-arid and arid regions where soil water content is dependent on winter rains (De Vita et al. 2007). Therefore, deep soils with high soil water storage capacities are vital reservoirs which regulate water supply to plants (Zhang et al. 2013). A study on the soil water balance on an *A. linearis* farm showed that the deeper soils had higher soil water contents and were able to store significantly more water than shallower soils and plants grew better on deeper soils, with better root growth and aboveground biomass production (van Schalkwyk 2018). In a study on evapotranspiration in dryland *T. aestivum*, in Voëlvlei Nature Reserve, South Africa, it was shown that evapotranspiration was higher in winter compared to summer due to water deficits in summer and subsequent stress in the crops (Jovanovic et al. 2011). In another study, on planted and unplanted plots, it was found that planted plots had higher levels of evapotranspiration than unplanted plots (Chazarenc et al. 2010). Therefore, the high evapotranspiration seen in the Cederberg during summer months (Figure 2.3*a*), which encompasses not only *A. linearis* but all the other plants in the ecosystem, indicates that the plants in the area are able to tap into deep water sources to allow for high evapotranspiration and maintain growth and development. A study on *A. linearis* has shown that the plants utilize transpiration and hydraulic redistribution to increase their acquisition of both shallow and deep soil nutrients (Matimati et al. 2014), which is likely aiding plants in the area around them as well by bringing deeper waters to the surface as well as nutrients.

An evergreen habitat has been postulated to be the most advantageous in a Mediterranean-type climate as this allows plants to take advantage of all the environmentally favourable opportunities for carbon gain and plant growth (Larcher 2000), as is seen by the fynbos vegetation in the Cederberg (Rebelo et al. 2006). However, these plants with long-lived leaves have to be able to survive periods with hostile conditions which requires an expanse of

protective mechanisms. These protective mechanisms range from anatomical and morphological characteristics such as sclerophylly, which is good for both resisting climate events and herbivory (Turner 1994), dense trichome layers as is seen in *Olea europaea* (olive) for increasing reflectance (Larcher 2000) or steep leaf angles to disperse excess light and protect photochemistry as is seen in macchia shrubs (Werner et al. 1999), and biochemical mechanisms such as those that target the dissipation of excess radiant energy (Demmig-Adams and Adams III 1996). Evergreen plants (e.g. *A. linearis*) tend to have a high degree of sclerophylly and as such, these small and thick leaves are well suited to the high light, high temperature environments that dominate most arid regions as they allow for the most carbon gain over transpirational loss (Turner 1994). While shrubs in Mediterranean-type climates have developed strategies to tolerate and avoid drought stress, annuals and herbs in these regions rely mostly on rapid growth to escape the summer stresses as well as fast responses to early stress signs (Chaves et al. 2002). Added to this was the evidence in proteomic work (chapter three) that showed *A. linearis* plants were up and downregulating proteins in response to environmental variables, most likely heat stress, which translated to the physiological changes seen in the plants (chapter two), and this is likely occurring in the plants in the area as well.

5.4 Shifting *Aspalathus linearis* production

One of the major finds in this thesis was that declining soil quality, supported by Smith et al. (2018), along with increasing temperatures are having a negative effect on the biomass and production of *A. linearis*. Therefore, the current expansion in *A. linearis* farming, going south of the Cederberg to cooler and wetter sites, was rewarding with plants producing more biomass and maintaining high levels of phenolics in the tissue, similar to plants at the hotter sites. As such, a shift in farming south will be beneficial to farmers as they can get more

biomass yet still retain the same health benefits of the tea through the phenolic content. While *A. linearis* is naturally distributed in the wild from Niewoudtville, in the Northern Cape, to south of Piketberg, towards Cape Town, (Mason and Du Plessis 1972, Hawkins et al. 2011, Lötter and le Maitre 2014) and even to Elim in the southern tip of the Western Cape, the large-scale commercial cultivation of *A. linearis* has been focused in the Cederberg area primarily due to the favourable conditions for processing the biomass into tea, and this being the home of *A. linearis* (Graaff et al. 2009, Hawkins et al. 2011). The commercial cultivation of *A. linearis* on farms near Elim, Bredasdorp, about 250 km south-east of the Cederberg region, has been going on for the last ten years. The vegetation types around Elim, where *A. linearis* is being farmed, is predominantly Agulhas sand fynbos and Overberg sandstone fynbos, with some patches of Cape inland salt pans (Rebelo et al. 2006). This area has a mean annual rainfall ranging between 475 - 585 mm and average maximum temperatures of 25 °C during summer (Rebelo et al. 2006) which is in stark contrast to the Cederberg where temperatures frequently rise above 35 °C in summer (chapter two) and rainfall ranges from 200 – 300 mm . Even so, farmers have been reported bountiful yields in Elim grown *A. linearis*, rivalling that from the Cederberg. The combination of cooler temperatures and wetter conditions, as well as fertile soils (Rebelo et al. 2006), are likely allowing the *A. linearis* to flourish in Elim and as such, more work needs to be done on addressing the differences between the Cederberg and Elim, and the likelihood of expanding *A. linearis* production in the southern part of the Cape. However, the biomass yield of green *A. linearis* in Elim is transported to the Cederberg area for processing as the climatic conditions in Elim are unfavourable for the drying out and fermenting processes (Morton 1983)

5.5 Contributions and the way forward

This thesis has contributed and expanded our knowledge of heat and drought stress in *A. linearis* in several ways. Through field experiments and data collection over two years, it is evident that *A. linearis* actively cools itself down during the hot summer months through transpirational cooling, indicating that the plants are tapping into deep soil layers to access water thereby also allowing them to avoid drought stress. *Aspalathus linearis* plants are well adapted to heat stress as was seen by their chlorophyll and phenolic content changes between summer and winter which allows them a degree of protection during summer stress periods. The proteomic work done on *A. linearis* has shown that on a molecular level, the plants are responding to heat stress in the field and are upregulating and downregulating certain proteins that will enable their survival. Plants in the cooler and wetter sites had higher biomass but retained their high phenolic levels indicating that farmers could move further south and have high biomass yields of tea while still maintaining the health aspect of it through high phenolics. Through the potted, drought experiment it was found that *A. linearis* was not well adapted to drought, but could avoid it for a period, through the production of roots to search for more water, before the plants started wilting.

Future studies should include the combination of heat and drought stress on the plants, particularly because it was difficult to ascertain the differences between the stresses in the field since plants had access to deep waters in the soils through their tap roots. Another aspect to consider is the temperature thresholds of the plants and how long they can maintain physiological activity during these thresholds and what the rate of recovery is after them. Future studies will also need to incorporate wild *A. linearis* plants in association with the cultivated variety and use proteomics to determine the molecular responses of all the plants at different time intervals of stress. This could lead to the creation of a possible transgenic *A.*

linearis species, with the health benefits of the cultivated variety but with the added benefit of better adaptations to stress as is seen in the wild types (Hawkins et al. 2011).

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Appendices

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31 January 2020

TO WHOM IT MAY CONCERN

Re: Contribution to co-authored publications

Madam, Sir,

I have had the pleasure to be involved through targeted engagements to the following publications, of which Dunja MacAlister is the first author.

1. MacAlister D, Muasya AM, Crespo O, Ogola JBO, Maseko ST, Valentine AJ, Ottosen C-O, Rosenqvist E, Chimphango SBM. 2020. Effect of temperature on plant growth and stress tolerant traits in rooibos in the Western Cape, South Africa. Scientia Horticulturae, 263 (Online). doi.org/10.1016/j.scienta.2019.109137
2. MacAlister D, Muasya AM, Crespo O, Ogola JBO, Maseko ST, Valentine AJ, Ottosen C-O, Rosenqvist E, Chimphango SBM. Stress tolerant traits and root proliferation of *Aspalathus linearis* (Burm.f.) R. Dahlgren grown under differing moisture regimes and exposed to drought. South African Journal of Botany (under review).

I would like to confirm that in both cases Mrs NacAlister was the lead researcher, and that the papers are directly reflecting Mrs MacAlister's research work, including conception, design, data collection, experiments and data analysis. She independently wrote the manuscripts, integrating comments and suggestions from the co-authors, and my particular involvement is one of a supervisor to a well capable PhD candidate.

Sincerely

Samson BM Chimphango

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3 February 2020

TO WHOM IT MAY CONCERN

Re: Contribution to co-authored publications

Dear Madam/Sir,

I confirm my participation in the following publication, via targeted engagements a PhD co-supervisor, of which Dunja MacAlister is the first author:

1. MacAlister D, Muasya AM, Crespo O, Ogola JBO, Maseko ST, Valentine AJ, OttosenC-O, Rosenqvist E, Chimphango SBM. 2020. Effect of temperature on plant growth and stress tolerant traits in rooibos in the Western Cape, South Africa. Scientia Horticulturae, 263 (Online). doi.org/10.1016/j.scienta.2019.109137
2. MacAlister D, Muasya AM, Crespo O, Ogola JBO, Maseko ST, Valentine AJ, OttosenC-O, Rosenqvist E, Chimphango SBM. Stress tolerant traits and root proliferation of Aspalathus linearis (Burm.f.) R. Dahlgren grown under differing moisture regimes and exposed to drought. South African Journal of Botany (under review).

I would like to confirm that in both cases Mrs MacAlister was the lead researcher, and that the papers are directly reflecting Mrs MacAlister's research work which includes the conception, design, experiments, data collection and data analysis. She independently wrote the manuscripts, integrating comments and suggestions from the co-authors, and my particular involvement is one of a co-supervisor.

Sincerely,

Prof A. Muthama Muasya

Research Officer

Olivier CRESPO

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Cape Town, February 03, 2020

TO WHOM IT MAY CONCERN

Re: Contribution to co-authored publications

Madam, Sir,

I have had the pleasure to be involved through targeted engagements to the following publications, of which Dunja MacAlister is the first author.

1. MacAlister D, Mnasya AM, Crespo O, Ogola JBO, Maseko ST, Valentine AJ, Ottosen C-O, Rosenqvist E, Chimphango SBM. 2020. Effect of temperature on plant growth and stress tolerant traits in rooibos in the Western Cape, South Africa. Scientia Horticulturae, 263 (Online). doi.org/10.1016/j.scienta.2019.109137
2. MacAlister D, Mnasya AM, Crespo O, Ogola JBO, Maseko ST, Valentine AJ, Ottosen C-O, Rosenqvist E, Chimphango SBM. Stress tolerant traits and root proliferation of *Aspalathus linearis* (Burm.f.) R. Dahlgren grown under differing moisture regimes and exposed to drought. South African Journal of Botany (under review).

I would like to confirm that in both cases Mrs MacAlister was the lead researcher, and that the papers are directly reflecting Mrs MacAlister's research work. She independently wrote the manuscripts, integrating comments and suggestions from the co-authors, and my particular involvement is one of a co-supervisor expert in climate and agriculture, and encouraging a candidate to further question and leverage these aspects in her own research.

Yours sincerely,

Olivier Crespo

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31 January 2020

TO WHOM IT MAY CONCERN

Re: Contributon to co-authored publications

Madam, Sir,

On behalf of the research collaborators namely John BO Ogola, Sipho Maseko, Alex J. Valentine, Carl-Otto Ottosen and Eva Rosenqvist, I confirm that the collaborators had targeted engagement in the project and the following publications, of which Mrs Dunja MacAlister is the first author.

1. MacAlister D, Muasya AM, Crespo O, Ogola JBO, Maseko ST, Valentine AJ, Ottosen C-O, Rosenqvist E, Chimphango SBM. 2020. Effect of temperature on plant growth and stress tolerant traits in rooibos in the Western Cape, South Africa. Scientia Horticulturae. 263 (Online). doi.org/10.1016/j.scienta.2019.109137
2. MacAlister D, Muasya AM, Crespo O, Ogola JBO, Maseko ST, Valentine AJ, Ottosen C-O, Rosenqvist E, Chimphango SBM. Stress tolerant traits and root proliferation of *Aspalathus linearis* (Burm.f.) R. Dahlgren grown under differing moisture regimes and exposed to drought. South African Journal of Botany (under review).

The collaborators confirm that in both cases Mrs MacAlister was the lead researcher, and that the papers are directly reflecting her research work, including conception, design, data collection, experiments and data analysis. She independently wrote the manuscripts, integrating comments and suggestions from the co-authors and their involvement was that of co-authors to a well capable PhD candidate.

Sincerely,

Samson BM Chimphango (on behalf of the co-authors)

Table A.1: List of heat stress responsive proteins from plants grown at Aurora. Samples were identified using the label free quantification and database searches. Positive max fold values indicate upregulated proteins, while negative max fold values indicate downregulated proteins.

Accession	Function	Protein name	Score[C]	q Value	Max fold
26_2_21101|m.21088	Cell growth/division	patellin-3	22.025	0.021	-2.661
26_2_73501|m.73388	Cell growth/division	RNA helicase	38.994	0.045	2.580
26_2_30879|m.30847	Cell structure	flowering locus K homology domain	3.885	0.031	2.591
26_2_19304|m.19292	Cell structure	multicopper oxidase LPR2-like	3.945	0.034	3.408
26_2_53491|m.53437	Cell structure	plastid-lipid-associated protein 6, chloroplastic	123.567	0.010	-2.200
26_2_52359|m.52308	Cell structure	probable 2-carboxy-D-arabinitol-1-phosphatase	32.154	0.029	-2.435
26_2_47556|m.47495	Cell structure	protein SLOW GREEN 1, chloroplastic	32.091	0.025	-2.537
26_2_37447|m.37411	Cell structure	red chlorophyll catabolite reductase, chloroplastic	49.808	0.035	-2.440
26_2_60686|m.60604	Cell structure	serine hydroxylmethyltransferase	67.386	0.007	6.573
26_2_62408|m.62350	Cell structure	Small heat shock protein C2	11.656	0.010	-2.147
26_2_568|m.540	Cell structure	uncharacterized protein LOC109337971 isoform X1	21.471	0.008	2.105
26_2_65980|m.65878	Cell structure	uncharacterized protein LOC113847216 isoform X2	14.909	0.028	2.626
26_2_41331|m.41287	Defence	ABA-responsive protein ABR17	102.619	0.004	-6.045
26_2_50641|m.50594	Defence	Alcohol dehydrogenase class-3	42.941	0.015	2.301
26_2_27496|m.27468	Defence	fructose-bisphosphate aldolase, cytoplasmic isozyme 1	2.824	0.041	-2.022

26_2_57448|m.57400	Defence	light-harvesting complex-like protein OHP2, chloroplastic	38.615	0.021	3.107
26_2_70687|m.70568	Defence	Major allergen Pru ar 1	120.665	0.003	4.464
26_2_50469|m.50429	Defence	photosynthetic NDH subunit of subcomplex B 5, chloroplastic	6.695	0.040	2.222
26_2_20439|m.20402	Defence	probable glutathione S-transferase	12.156	0.025	2.326
26_2_62979|m.62903	Defence	probable phospholipid hydroperoxide glutathione peroxidase	25.899	0.025	-2.869
26_2_13047|m.13028	Defence	tropinone reductase homolog	16.048	0.026	3.427
26_2_9842|m.9831	Energy	alpha/Beta hydrolase fold	25.146	0.042	-2.286
26_2_28266|m.28240	Energy	ATP synthase CF0 subunit IV	16.544	0.024	5.576
26_2_45353|m.45320	Energy	ATP synthase delta chain, chloroplastic-like	93.022	0.018	3.033
26_2_13753|m.13750	Energy	ATP synthase subunit O, mitochondrial	52.633	0.013	2.619
26_2_16553|m.16512	Energy	ATP-citrate synthase beta chain protein 1	82.193	0.022	2.075
26_2_44071|m.44029	Energy	chlorophyll a-b binding protein CP24 10A, chloroplastic	52.299	0.018	5.266
26_2_28446|m.28419	Energy	cytochrome c oxidase subunit 2	20.579	0.011	2.120
26_2_24276|m.24242	Energy	delta(24)-sterol reductase	14.830	0.015	-3.398
26_2_49000|m.48932	Energy	glutaredoxin-C4	14.958	0.026	2.803
26_2_22191|m.22183	Energy	light-harvesting complex-like protein 3 isotype 2, chloroplastic	13.992	0.025	-2.435
26_2_1253|m.1241	Energy	photosystem I reaction center subunit V, chloroplastic	35.042	0.017	4.178

26_2_4876|m.4814	Energy	photosystem II cytochrome b559 alpha subunit	26.870	0.017	3.596
26_2_64554|m.64476	Energy	Protein PROTON GRADIENT REGULATION 5, chloroplastic	13.751	0.035	3.104
26_2_18661|m.18642	Energy	pyruvate kinase, cytosolic isozyme	25.359	0.025	2.534
26_2_11529|m.11522	Energy	V-type proton ATPase subunit F	13.742	0.009	2.293
26_2_53447|m.53396	Intracellular traffic	ADP-ribosylation factor	34.729	0.017	2.028
26_2_33133|m.33102	Intracellular traffic	UMP-CMP kinase 3	9.549	0.008	5.475
26_2_14489|m.14463	Metabolism	1,4-alpha-glucan-branching enzyme 1, chloroplastic/amyloplastic	27.278	0.038	-3.653
26_2_2203|m.2196	Metabolism	2,3-dimethylmalate lyase isoform X2	27.792	0.019	-2.454
26_2_4044|m.4034	Metabolism	2-alkenal reductase (NADP(+)-dependent)	30.209	0.009	3.424
26_2_27520|m.27489	Metabolism	2-methylene-furan-3-one reductase	120.044	0.010	-2.396
26_2_34386|m.34329	Metabolism	3-hydroxyisobutyryl-CoA hydrolase-like protein 5	12.372	0.011	8.474
26_2_9473|m.9459	Metabolism	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	50.554	0.038	2.266
26_2_234|m.212	Metabolism	acetyl-CoA carboxylase 1-like	82.644	0.023	2.309
26_2_2462|m.2463	Metabolism	aconitate hydratase 1	17.328	0.048	2.072
26_2_52617|m.52558	Metabolism	acyl-CoA-binding protein	8.465	0.004	22.555
26_2_27624|m.27592	Metabolism	Alpha-galactosidase isoform A	6.756	0.009	-3.583

26_2_30458|m.30436	Metabolism	aminoacylase-1	11.433	0.014	-2.675
26_2_15590|m.15565	Metabolism	BAG family molecular chaperone regulator 7	11.296	0.018	2.812
26_2_35971|m.35937	Metabolism	BAHD acyltransferase DCR	46.328	0.008	4.317
26_2_45434|m.45405	Metabolism	benzyl alcohol O-benzoyltransferase	3.296	0.026	5.145
26_2_23431|m.23418	Metabolism	clavaminate synthase-like protein At3g21360	17.484	0.020	-2.924
26_2_32343|m.32310	Metabolism	cytochrome b5	24.289	0.003	3.971
26_2_31552|m.31531	Metabolism	endochitinase PR4	18.743	0.025	3.001
26_2_30332|m.30305	Metabolism	flavonoid 3'-monooxygenase	57.787	0.015	3.631
26_2_42683|m.42629	Metabolism	fructose-bisphosphate aldolase 3, chloroplastic	39.190	0.009	-2.106
26_2_25927|m.25911	Metabolism	fructose-bisphosphate aldolase 6, cytosolic	54.358	0.013	2.404
26_2_3550|m.3519	Metabolism	GDSL esterase/lipase At2g04570-like	16.339	0.006	2.254
26_2_4835|m.4779	Metabolism	GDSL esterase/lipase At5g33370-like	20.434	0.014	2.417
26_2_41586|m.41547	Metabolism	guanosine nucleotide diphosphate dissociation inhibitor 2	32.964	0.017	2.604
26_2_25913|m.25895	Metabolism	Monodehydroascorbate reductase	88.930	0.026	-2.267
26_2_1361|m.1345	Metabolism	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2	9.859	0.009	2.829
26_2_70316|m.70232	Metabolism	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6	20.476	0.023	2.186
26_2_13211|m.13204	Metabolism	NADP-dependent malic enzyme	62.289	0.020	-3.128

26_2_65926|m.65825	Metabolism	NADPH-dependent aldo-keto reductase, chloroplastic	62.782	0.009	2.536
26_2_41097|m.41054	Metabolism	peroxidase 15	16.728	0.025	2.925
26_2_71870|m.71717	Metabolism	Peroxidase 17	8.237	0.008	-2.329
26_2_58562|m.58519	Metabolism	phospho-2-dehydro-3-deoxyheptonate aldolase 1, chloroplastic-like	23.579	0.006	12.023
26_2_40578|m.40538	Metabolism	phosphoglucan phosphatase DSP4, amyloplastic	31.364	0.033	-2.756
26_2_46553|m.46491	Metabolism	Phosphomannomutase/phosphoglucomutase protein	17.723	0.035	-2.295
26_2_34761|m.34698	Metabolism	probable aldehyde dehydrogenase	26.510	0.009	-2.632
26_2_59551|m.59490	Metabolism	probable aminotransferase TAT2	10.541	0.012	4.825
26_2_27700|m.27669	Metabolism	probable fructokinase-4	21.960	0.008	6.315
26_2_17071|m.17051	Metabolism	protein CURVATURE THYLAKOID 1A, chloroplastic	14.162	0.047	2.069
26_2_40857|m.40812	Metabolism	protein EDS1L-like	13.890	0.047	-2.091
26_2_34895|m.34826	Metabolism	protein SIEVE ELEMENT OCCLUSION B	19.709	0.027	-3.263
26_2_48309|m.48259	Metabolism	protein SIEVE ELEMENT OCCLUSION B	29.097	0.041	-5.720
26_2_15584|m.15559	Metabolism	Purple acid phosphatase	10.323	0.035	3.531
26_2_31970|m.31959	Metabolism	Purple acid phosphatase	6.751	0.032	4.243
26_2_15586|m.15561	Metabolism	Purple acid phosphatase	9.397	0.016	2.796
26_2_78338|m.78228	Metabolism	putative bark agglutinin LECRPA3	39.434	0.022	-2.630

26_2_42755|m.42695	Metabolism	putative lactoylglutathione lyase	114.103	0.008	-2.476
26_2_57402|m.57355	Metabolism	pyridoxal phosphate homeostasis protein	21.978	0.029	-2.667
26_2_25365|m.25340	Metabolism	pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit alpha	53.510	0.007	2.256
26_2_12539|m.12542	Metabolism	pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit beta	23.352	0.022	3.312
26_2_43842|m.43797	Metabolism	ricin B-like lectin R40G3	19.818	0.026	-2.011
26_2_19649|m.19650	Metabolism	S-adenosylmethionine synthase 1	68.081	0.000	11.609
26_2_59787|m.59714	Metabolism	selenium-binding protein 1	38.104	0.033	4.051
26_2_35073|m.35040	Metabolism	sulfite oxidase	3.130	0.009	-2.482
26_2_19335|m.19323	Metabolism	tocopherol cyclase, chloroplastic	4.301	0.026	-2.027
26_2_301|m.274	Metabolism	transketolase, chloroplastic	15.169	0.014	-2.053
26_2_67292|m.67208	Metabolism	translocon-associated protein subunit alpha	15.227	0.033	2.065
26_2_33847|m.33810	Metabolism	UDP-glucose 6-dehydrogenase 1	35.210	0.042	2.036
26_2_8880|m.8860	Metabolism	urease	3.105	0.013	-2.700
26_2_3488|m.3457	Metabolism	valine--tRNA ligase, mitochondrial 1 isoform X2	7.165	0.027	2.136
26_2_47047|m.47006	Protein destination and storage	26S proteasome non-ATPase regulatory subunit 8 homolog A	6.002	0.016	7.841

26_2_40937|m.40890	Protein destination and storage	aspartyl protease AED3-like	18.759	0.022	4.516
26_2_74534|m.74413	Protein destination and storage	aspartyl protease AED3-like	24.921	0.013	3.925
26_2_7542|m.7533	Protein destination and storage	calnexin homolog	19.687	0.013	2.315
26_2_12194|m.12193	Protein destination and storage	cysteine proteinase inhibitor-like	16.976	0.012	5.814
26_2_32494|m.32456	Protein destination and storage	cysteine synthase	34.240	0.031	-3.949
26_2_34185|m.34136	Protein destination and storage	Serine carboxypeptidase II-2	17.389	0.035	3.418
26_2_58698|m.58652	Protein destination and storage	serine carboxypeptidase-like 50	12.394	0.034	-2.106
26_2_18033|m.18017	Protein destination and storage	thioredoxin H-type-like	12.602	0.004	5.793
26_2_5966|m.5889	Protein destination and storage	tripeptidyl-peptidase 2-like isoform X2	10.657	0.029	-2.263
26_2_63503|m.63425	Protein destination and storage	upstream activation factor subunit UAF30	20.221	0.020	3.380
26_2_72648|m.72519	Protein synthesis	50S ribosomal protein L28, chloroplastic	2.783	0.026	2.768
26_2_65519|m.65431	Protein synthesis	60S ribosomal protein L10	11.990	0.041	-2.514
26_2_5383|m.5371	Protein synthesis	organelle RRM domain-containing protein 6, chloroplastic	7.542	0.014	7.311
26_2_41549|m.41505	Protein synthesis	prohibitin-3, mitochondrial	31.033	0.039	2.043
26_2_50879|m.50817	Signal transduction	14-3-3-like protein D	6.816	0.008	-2.600
26_2_2649|m.2644	Signal transduction	3-phosphoglycerate kinase	16.125	0.029	3.035
26_2_2958|m.2953	Signal transduction	annexin D2	19.297	0.018	8.913

26_2_89869|m.89492	Signal transduction	ATOZI1, putative	16.030	0.004	4.540
26_2_72550|m.72434	Signal transduction	ATP synthase subunit b', chloroplastic	95.518	0.021	2.810
26_2_64184|m.64126	Signal transduction	bifunctional epoxide hydrolase 2-like	20.551	0.035	6.699
26_2_64731|m.64643	Signal transduction	cysteine--tRNA ligase, chloroplastic/mitochondrial isoform X3	10.239	0.035	2.543
26_2_23055|m.23030	Signal transduction	heat shock 70 kDa protein	112.816	0.025	-4.734
26_2_35378|m.35351	Signal transduction	Heme-binding protein	14.322	0.029	-2.571
26_2_8058|m.8047	Signal transduction	heme-binding-like protein At3g10130, chloroplastic	36.534	0.007	-2.599
26_2_27606|m.27575	Signal transduction	mannose-1-phosphate guanyltransferase alpha-like	8.360	0.035	-2.007
26_2_84274|m.84157	Signal transduction	mitochondrial ATP synthase	4.799	0.029	3.582
26_2_10048|m.10039	Signal transduction	nuclear transport factor 2B	8.322	0.014	7.065
26_2_50311|m.50267	Signal transduction	nucleoside diphosphate kinase 2, chloroplastic	29.602	0.012	-2.302
26_2_55408|m.55344	Signal transduction	probable histone H2A.3	26.693	0.039	2.074
26_2_60123|m.60070	Signal transduction	probable plastid-lipid-associated protein 13, chloroplastic isoform X1	61.726	0.023	-2.568
26_2_33685|m.33640	Signal transduction	protein BOLA4, chloroplastic/mitochondrial	11.734	0.008	3.684
26_2_51533|m.51467	Signal transduction	pto-interacting protein 1	6.295	0.037	-2.233
26_2_46491|m.46432	Signal transduction	thylakoid lumenal 19 kDa protein, chloroplastic	18.788	0.048	3.466

26_2_51824|m.51756	Signal transduction	uncharacterized aarF domain-containing protein kinase At5g05200, chloroplastic	20.380	0.035	-2.037
26_2_43605|m.43554	Signal transduction	very-long-chain 3-oxoacyl-CoA reductase 1-like	6.246	0.014	2.535
26_2_64125|m.64074	Signal transduction	thylakoid lumenal 15 kDa protein 1, chloroplastic	35.313	0.048	-2.542
26_2_61807|m.61753	Transcription	caffeic acid 3-O-methyltransferase	10.862	0.026	-2.086
26_2_6181|m.6173	Transcription	histone H2A	44.041	0.037	2.306
26_2_54795|m.54726	Transcription	nascent polypeptide-associated complex subunit alpha-like protein	9.778	0.046	2.125
26_2_56455|m.56398	Transporters	copper transport protein ATX1	4.721	0.046	-2.627
26_2_25106|m.25079	Transporters	linoleate 13S-lipoxygenase 2-1, chloroplastic	67.635	0.025	-2.817
26_2_55500|m.55434	Transporters	probable aquaporin PIP-type 7a	28.362	0.026	2.002
26_2_58777|m.58729	Transporters	tubulin beta-5 chain	129.817	0.014	3.224

Table A.2: List of heat stress responsive proteins from plants grown at Citrusdal. Samples were identified using the label free quantification and database searches. Positive max fold values indicate upregulated proteins, while negative max fold values indicate downregulated proteins.

Accession	Function	Protein name	Score[C]	q Value	Max fold
26_2_61265|m.61208	Cell growth/division	RNA helicase	38.994	0.045	-2.580
26_2_35971|m.35937	Cell structure	heat shock 22 kDa protein, mitochondrial isoform X1	19.652	0.029	-2.094
26_2_51824|m.51756	Cell structure	multicopper oxidase LPR2-like	3.945	0.034	-3.408
26_2_65926|m.65825	Cell structure	plastid-lipid-associated protein 6, chloroplastic	123.567	0.010	2.200
26_2_4835|m.4779	Cell structure	probable 2-carboxy-D-arabinitol-1-phosphatase	32.154	0.029	2.435
26_2_33133|m.33102	Cell structure	putative pectinesterase/pectinesterase inhibitor 40	23.003	0.017	-2.117
26_2_25927|m.25911	Cell structure	red chlorophyll catabolite reductase, chloroplastic	49.808	0.035	2.440
26_2_2606|m.2600	Cell structure	serine hydroxylmethyltransferase	67.386	0.007	-6.573
26_2_19304|m.19292	Cell structure	serine--tRNA ligase, chloroplastic/mitochondrial isoform X1	25.222	0.025	2.281
26_2_73501|m.73388	Cell structure	Small heat shock protein C2	11.656	0.010	2.147
26_2_58562|m.58519	Defence	17.3 kDa class I heat shock protein	35.837	0.008	-8.135
26_2_40357|m.40327	Defence	ABA-responsive protein ABR17	102.619	0.004	6.045
26_2_9312|m.9302	Defence	Alcohol dehydrogenase class-3	42.941	0.015	-2.301
26_2_34386|m.34329	Defence	isoflavone reductase-like protein	11.039	0.023	-2.469

26_2_45798|m.45750	Defence	Major allergen Pru ar 1	120.665	0.003	-4.464
26_2_70687|m.70568	Defence	MLP-like protein 423	22.138	0.007	-4.044
26_2_74534|m.74413	Energy	aldehyde dehydrogenase family 3 member H1-like isoform X1	22.676	0.029	3.208
26_2_55777|m.55708	Energy	Alpha/Beta hydrolase fold	25.146	0.042	2.286
26_2_34895|m.34826	Energy	ATP-citrate synthase beta chain protein 1	82.193	0.022	-2.075
26_2_27764|m.27731	Energy	cytochrome c oxidase subunit 2	20.579	0.011	-2.120
26_2_31970|m.31959	Energy	dihydropyrimidine dehydrogenase (NADP(+)), chloroplastic	10.521	0.042	2.007
26_2_19649|m.19650	Energy	enolase 1, chloroplastic	12.565	0.034	-4.139
26_2_58777|m.58729	Energy	zeaxanthin epoxidase, chloroplastic-like	4.997	0.009	2.521
26_2_45434|m.45405	Intracellular traffic	bifunctional riboflavin biosynthesis protein RIBA 1, chloroplastic	6.642	0.013	-2.054
26_2_55408|m.55344	Intracellular traffic	UMP-CMP kinase 3	9.549	0.008	-5.475
26_2_3550|m.3519	Metabolism	1,4-alpha-glucan-branching enzyme 1, chloroplastic/amyloplastic	27.278	0.038	3.653
26_2_11541|m.11534	Metabolism	3-hydroxyisobutyryl-CoA hydrolase-like protein 5	12.372	0.011	-8.474
26_2_34761|m.34698	Metabolism	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	50.554	0.038	-2.266
26_2_37447|m.37411	Metabolism	acetyl-CoA carboxylase 1-like	82.644	0.023	-2.309
26_2_40937|m.40890	Metabolism	acetylornithine aminotransferase, mitochondrial	31.933	0.016	-2.032

26_2_14489|m.14463	Metabolism	aconitate hydratase 1	17.328	0.048	-2.072
26_2_25365|m.25340	Metabolism	Alpha-galactosidase isoform A	6.756	0.009	3.583
26_2_68692|m.68603	Metabolism	BAG family molecular chaperone regulator 7	11.296	0.018	-2.812
26_2_15016|m.15002	Metabolism	BAHD acyltransferase DCR	46.328	0.008	-4.317
26_2_2462|m.2463	Metabolism	benzyl alcohol O-benzoyltransferase	3.296	0.026	-5.145
26_2_44140|m.44098	Metabolism	flavonoid 3'-monooxygenase	57.787	0.015	-3.631
26_2_52359|m.52308	Metabolism	fructose-bisphosphate aldolase 6, cytosolic	54.358	0.013	-2.404
26_2_13211|m.13204	Metabolism	GDSL esterase/lipase At2g04570-like	16.339	0.006	-2.254
26_2_51663|m.51592	Metabolism	GDSL esterase/lipase At5g33370-like	20.434	0.014	-2.417
26_2_27700|m.27669	Metabolism	Glycerol-3-phosphate 2-O-acyltransferase 6	24.367	0.035	-2.135
26_2_39120|m.39086	Metabolism	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6	20.476	0.023	-2.186
26_2_50641|m.50594	Metabolism	NADP-dependent malic enzyme	62.289	0.020	3.128
26_2_9842|m.9831	Metabolism	NADPH-dependent aldo-keto reductase, chloroplastic	62.782	0.009	-2.536
26_2_53491|m.53437	Metabolism	peroxidase 15	16.728	0.025	-2.925
26_2_70316|m.70232	Metabolism	phospho-2-dehydro-3-deoxyheptonate aldolase 1, chloroplastic-like	23.579	0.006	-12.023
26_2_234|m.212	Metabolism	phosphoglucan phosphatase DSP4, amyloplastic	31.364	0.033	2.756
26_2_7542|m.7533	Metabolism	probable aldehyde dehydrogenase	26.510	0.009	2.632

26_2_67292|m.67208	Metabolism	probable aminotransferase TAT2	10.541	0.012	-4.825
26_2_16553|m.16512	Metabolism	probable fructokinase-4	21.960	0.008	-6.315
26_2_9473|m.9459	Metabolism	protein SIEVE ELEMENT OCCLUSION B	19.709	0.027	3.263
26_2_5966|m.5889	Metabolism	Purple acid phosphatase	9.397	0.016	-2.796
26_2_50454|m.50414	Metabolism	Purple acid phosphatase	6.751	0.032	-4.243
26_2_59787|m.59714	Metabolism	putative BPI/LBP family protein At1g04970	36.640	0.035	2.384
26_2_40578|m.40538	Metabolism	pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit alpha	53.510	0.007	-2.256
26_2_14315|m.14298	Metabolism	pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit beta	23.352	0.022	-3.312
26_2_11097|m.11082	Metabolism	S-adenosylmethionine synthase 1	68.081	0.000	-11.609
26_2_27606|m.27575	Metabolism	selenium-binding protein 1	38.104	0.033	-4.051
26_2_15590|m.15565	Metabolism	tocopherol cyclase, chloroplastic	4.301	0.026	2.027
26_2_41331|m.41287	Metabolism	translocon-associated protein subunit alpha	15.227	0.033	-2.065
26_2_17326|m.17309	Protein destination and storage	aspartic proteinase	50.577	0.036	2.028
26_2_19335|m.19323	Protein destination and storage	aspartyl protease AED3-like	24.921	0.013	-3.925
26_2_25683|m.25657	Protein destination and storage	aspartyl protease AED3-like	18.759	0.022	-4.516

26_2_42907|m.42853	Protein destination and storage	calnexin homolog	19.687	0.013	-2.315
26_2_30332|m.30305	Protein destination and storage	cysteine proteinase 15A-like	38.342	0.034	2.120
26_2_67509|m.67421	Protein destination and storage	glutathione S-transferase L3	48.775	0.011	-2.463
26_2_12539|m.12542	Protein destination and storage	peptidyl-prolyl cis-trans isomerase, chloroplastic	5.608	0.037	2.438
26_2_17612|m.17586	Protein destination and storage	protein C2-DOMAIN ABA-RELATED 7-like	13.200	0.025	2.254
26_2_9482|m.9466	Protein destination and storage	tripeptidyl-peptidase 2-like isoform X2	10.657	0.029	2.263
26_2_60686|m.60604	Protein destination and storage	uncharacterized protein LOC109338437	3.501	0.026	-2.229
26_2_28446|m.28419	Signal transduction	annexin D2	19.297	0.018	-8.913
26_2_2958|m.2953	Signal transduction	beta-amylase	13.404	0.033	-3.889
26_2_1888|m.1805	Signal transduction	beta-amylase-like isoform X1	6.088	0.018	3.190
26_2_59551|m.59490	Signal transduction	heat shock cognate 70 kDa protein 2-like	213.233	0.021	-2.039
26_2_41097|m.41054	Signal transduction	mannose-1-phosphate guanyltransferase alpha-like	8.360	0.035	2.007
26_2_27624|m.27592	Signal transduction	probable histone H2A.3	26.693	0.039	-2.074
26_2_15586|m.15561	Signal transduction	uncharacterized aarF domain-containing protein kinase At5g05200, chloroplastic	20.380	0.035	2.037
26_2_62408|m.62350	Transporters	tubulin beta-5 chain	129.817	0.014	-3.224

Table A.3: List of heat stress responsive proteins from plants grown at Clanwilliam. Samples were identified using the label free quantification and database searches. Positive max fold values indicate upregulated proteins, while negative max fold values indicate downregulated proteins.

Accession	Function	Protein name	Score[C]	q Value	Max fold
26_2_75679|m.75533	Cell structure	cytochrome b-c1 complex subunit 9-like	4.285	0.038	2.253
26_2_2606|m.2600	Cell structure	heat shock 22 kDa protein, mitochondrial isoform X1	19.652	0.029	2.094
26_2_32807|m.32773	Cell structure	ribosome-binding factor PSRP1, chloroplastic	7.336	0.012	-3.628
26_2_20837|m.20794	Cell structure	serine hydroxymethyltransferase, mitochondrial	111.876	0.044	-2.285
26_2_61265|m.61208	Cell structure	serine--tRNA ligase, chloroplastic/mitochondrial isoform X1	25.222	0.025	-2.281
26_2_47060|m.47019	Cell structure	synechocystis YCF37-like protein	19.790	0.035	2.191
26_2_1888|m.1805	Defence	MLP-like protein 423	22.138	0.007	4.044
26_2_11137|m.11118	Energy	chlorophyll a-b binding protein of LHCII type 1	15.509	0.014	3.102
26_2_18993|m.18975	Energy	zeaxanthin epoxidase, chloroplastic	10.627	0.022	-2.381
26_2_9312|m.9302	Energy	zeaxanthin epoxidase, chloroplastic-like	4.997	0.009	-2.521
26_2_16528|m.16489	Metabolism	alpha-amylase 3, chloroplastic isoform X1	65.992	0.013	2.293
26_2_73524|m.73410	Metabolism	dirigent protein 22-like	9.679	0.028	-2.139
26_2_41841|m.41806	Metabolism	oxalate--CoA ligase-like	47.240	0.015	-2.184
26_2_42286|m.42243	Metabolism	peroxidase P7-like	52.091	0.038	-3.493

26_2_42635|m.42580	Metabolism	Phospholipase A1-IIgamma	46.384	0.037	2.957
26_2_11507|m.11499	Metabolism	probable carotenoid cleavage dioxygenase 4, chloroplastic	52.950	0.010	-2.776
26_2_38265|m.38232	Metabolism	probable ribose-5-phosphate isomerase 4, chloroplastic	7.130	0.033	-4.322
26_2_48309|m.48259	Metabolism	protein SIEVE ELEMENT OCCLUSION B	29.097	0.041	5.720
26_2_33847|m.33810	Metabolism	UDP-glucose 6-dehydrogenase 1	35.210	0.042	-2.036
26_2_44140|m.44098	Protein destination and storage	aspartic proteinase	50.577	0.036	-2.028
26_2_14315|m.14298	Protein destination and storage	cysteine proteinase 15A-like	38.342	0.034	-2.120
26_2_42907|m.42853	Protein destination and storage	peptidyl-prolyl cis-trans isomerase, chloroplastic	5.608	0.037	-2.438
26_2_48497|m.48438	Protein destination and storage	Protein grpE	50.715	0.025	-2.479
26_2_15409|m.15385	Protein synthesis	asparagine--tRNA ligase, cytoplasmic 1	13.793	0.029	-2.157
26_2_52593|m.52535	Protein synthesis	ribosomal protein S8	31.063	0.030	-2.123
26_2_23055|m.23030	Signal transduction	heat shock 70 kDa protein	112.816	0.025	4.734
26_2_32527|m.32488	Signal transduction	Rossmann-like alpha/beta/alpha sandwich fold	13.199	0.018	3.136

Table A.4: List of heat stress responsive proteins from plants grown at Uitsig. Samples were identified using the label free quantification and database searches. Positive max fold values indicate upregulated proteins, while negative max fold values indicate downregulated proteins.

Accession	Function	Protein name	Score[C]	q Value	Max fold
26_2_21101|m.21088	Cell growth/division	patellin-3	22.025	0.021	2.661
26_2_75679|m.75533	Cell structure	cytochrome b-c1 complex subunit 9-like	4.285	0.038	-2.253
26_2_30879|m.30847	Cell structure	flowering locus K homology domain	3.885	0.031	-2.591
26_2_47556|m.47495	Cell structure	protein SLOW GREEN 1, chloroplastic	32.091	0.025	2.537
26_2_11541|m.11534	Cell structure	putative pectinesterase/pectinesterase inhibitor 40	23.003	0.017	2.117
26_2_32807|m.32773	Cell structure	ribosome-binding factor PSRP1, chloroplastic	7.336	0.012	3.628
26_2_20837|m.20794	Cell structure	serine hydroxymethyltransferase, mitochondrial	111.876	0.044	2.285
26_2_47060|m.47019	Cell structure	synechocystis YCF37-like protein	19.790	0.035	-2.191
26_2_568|m.540	Cell structure	uncharacterized protein LOC109337971 isoform X1	21.471	0.008	-2.105
26_2_65980|m.65878	Cell structure	uncharacterized protein LOC113847216 isoform X2	14.909	0.028	-2.626
26_2_67509|m.67421	Defence	17.3 kDa class I heat shock protein	35.837	0.008	8.135
26_2_27496|m.27468	Defence	fructose-bisphosphate aldolase, cytoplasmic isozyme 1	2.824	0.041	2.022
26_2_9482|m.9466	Defence	isoflavone reductase-like protein	11.039	0.023	2.469
26_2_57448|m.57400	Defence	light-harvesting complex-like protein OHP2, chloroplastic	38.615	0.021	-3.107

26_2_50469|m.50429	Defence	photosynthetic NDH subunit of subcomplex B 5, chloroplastic	6.695	0.040	-2.222
26_2_20439|m.20402	Defence	probable glutathione S-transferase	12.156	0.025	-2.326
26_2_62979|m.62903	Defence	probable phospholipid hydroperoxide glutathione peroxidase	25.899	0.025	2.869
26_2_13047|m.13028	Defence	tropinone reductase homolog	16.048	0.026	-3.427
26_2_27764|m.27731	Energy	aldehyde dehydrogenase family 3 member H1-like isoform X1	22.676	0.029	-3.208
26_2_28266|m.28240	Energy	ATP synthase CF0 subunit IV	16.544	0.024	-5.576
26_2_45353|m.45320	Energy	ATP synthase delta chain, chloroplastic-like	93.022	0.018	-3.033
26_2_13753|m.13750	Energy	ATP synthase subunit O, mitochondrial	52.633	0.013	-2.619
26_2_44071|m.44029	Energy	chlorophyll a-b binding protein CP24 10A, chloroplastic	52.299	0.018	-5.266
26_2_11137|m.11118	Energy	chlorophyll a-b binding protein of LHCII type 1	15.509	0.014	-3.102
26_2_24276|m.24242	Energy	delta(24)-sterol reductase	14.830	0.015	3.398
26_2_50454|m.50414	Energy	dihydropyrimidine dehydrogenase (NADP(+)), chloroplastic	10.521	0.042	-2.007
26_2_17612|m.17586	Energy	enolase 1, chloroplastic	12.565	0.034	4.139
26_2_49000|m.48932	Energy	glutaredoxin-C4	14.958	0.026	-2.803
26_2_22191|m.22183	Energy	light-harvesting complex-like protein 3 isotype 2, chloroplastic	13.992	0.025	2.435
26_2_1253|m.1241	Energy	photosystem I reaction center subunit V, chloroplastic	35.042	0.017	-4.178
26_2_4876|m.4814	Energy	photosystem II cytochrome b559 alpha subunit	26.870	0.017	-3.596

26_2_64554|m.64476	Energy	Protein PROTON GRADIENT REGULATION 5, chloroplastic	13.751	0.035	-3.104
26_2_18661|m.18642	Energy	pyruvate kinase, cytosolic isozyme	25.359	0.025	-2.534
26_2_11529|m.11522	Energy	V-type proton ATPase subunit F	13.742	0.009	-2.293
26_2_18993|m.18975	Energy	zeaxanthin epoxidase, chloroplastic	10.627	0.022	2.381
26_2_53447|m.53396	Intracellular traffic	ADP-ribosylation factor	34.729	0.017	-2.028
26_2_25683|m.25657	Intracellular traffic	bifunctional riboflavin biosynthesis protein RIBA 1, chloroplastic	6.642	0.013	2.054
26_2_2203|m.2196	Metabolism	2,3-dimethylmalate lyase isoform X2	27.792	0.019	2.454
26_2_4044|m.4034	Metabolism	2-alkenal reductase (NADP(+)-dependent)	30.209	0.009	-3.424
26_2_27520|m.27489	Metabolism	2-methylene-furan-3-one reductase	120.044	0.010	2.396
26_2_15016|m.15002	Metabolism	acetylornithine aminotransferase, mitochondrial	31.933	0.016	2.032
26_2_52617|m.52558	Metabolism	acyl-CoA-binding protein	8.465	0.004	-22.555
26_2_16528|m.16489	Metabolism	alpha-amylase 3, chloroplastic isoform X1	65.992	0.013	-2.293
26_2_30458|m.30436	Metabolism	aminoacylase-1	11.433	0.014	2.675
26_2_23431|m.23418	Metabolism	clavaminate synthase-like protein At3g21360	17.484	0.020	2.924
26_2_32343|m.32310	Metabolism	cytochrome b5	24.289	0.003	-3.971

26_2_73524|m.73410	Metabolism	dirigent protein 22-like	9.679	0.028	2.139
26_2_31552|m.31531	Metabolism	endochitinase PR4	18.743	0.025	-3.001
26_2_42683|m.42629	Metabolism	fructose-bisphosphate aldolase 3, chloroplastic	39.190	0.009	2.106
26_2_11097|m.11082	Metabolism	Glycerol-3-phosphate 2-O-acyltransferase 6	24.367	0.035	2.135
26_2_41586|m.41547	Metabolism	guanosine nucleotide diphosphate dissociation inhibitor 2	32.964	0.017	-2.604
26_2_25913|m.25895	Metabolism	Monodehydroascorbate reductase	88.930	0.026	2.267
26_2_1361|m.1345	Metabolism	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2	9.859	0.009	-2.829
26_2_41841|m.41806	Metabolism	oxalate--CoA ligase-like	47.240	0.015	2.184
26_2_71870|m.71717	Metabolism	Peroxidase 17	8.237	0.008	2.329
26_2_42286|m.42243	Metabolism	peroxidase P7-like	52.091	0.038	3.493
26_2_42635|m.42580	Metabolism	Phospholipase A1-IIgamma	46.384	0.037	-2.957
26_2_46553|m.46491	Metabolism	Phosphomannomutase/phosphoglucomutase protein	17.723	0.035	2.295
26_2_11507|m.11499	Metabolism	probable carotenoid cleavage dioxygenase 4, chloroplastic	52.950	0.010	2.776
26_2_38265|m.38232	Metabolism	probable ribose-5-phosphate isomerase 4, chloroplastic	7.130	0.033	4.322
26_2_17071|m.17051	Metabolism	protein CURVATURE THYLAKOID 1A, chloroplastic	14.162	0.047	-2.069
26_2_40857|m.40812	Metabolism	protein EDS1L-like	13.890	0.047	2.091

26_2_15584|m.15559	Metabolism	Purple acid phosphatase	10.323	0.035	-3.531
26_2_78338|m.78228	Metabolism	putative bark agglutinin LECRPA3	39.434	0.022	2.630
26_2_51663|m.51592	Metabolism	putative BPI/LBP family protein At1g04970	36.640	0.035	-2.384
26_2_42755|m.42695	Metabolism	putative lactoylglutathione lyase	114.103	0.008	2.476
26_2_57402|m.57355	Metabolism	pyridoxal phosphate homeostasis protein	21.978	0.029	2.667
26_2_43842|m.43797	Metabolism	ricin B-like lectin R40G3	19.818	0.026	2.011
26_2_35073|m.35040	Metabolism	sulfite oxidase	3.130	0.009	2.482
26_2_301|m.274	Metabolism	transketolase, chloroplastic	15.169	0.014	2.053
26_2_8880|m.8860	Metabolism	urease	3.105	0.013	2.700
26_2_3488|m.3457	Metabolism	valine--tRNA ligase, mitochondrial 1 isoform X2	7.165	0.027	-2.136
26_2_47047|m.47006	Protein destination and storage	26S proteasome non-ATPase regulatory subunit 8 homolog A	6.002	0.016	-7.841
26_2_12194|m.12193	Protein destination and storage	cysteine proteinase inhibitor-like	16.976	0.012	-5.814
26_2_32494|m.32456	Protein destination and storage	cysteine synthase	34.240	0.031	3.949
26_2_55777|m.55708	Protein destination and storage	glutathione S-transferase L3	48.775	0.011	2.463
26_2_68692|m.68603	Protein destination and storage	protein C2-DOMAIN ABA-RELATED 7-like	13.200	0.025	-2.254
26_2_48497|m.48438	Protein destination and storage	Protein grpE	50.715	0.025	2.479
26_2_34185|m.34136	Protein destination and storage	Serine carboxypeptidase II-2	17.389	0.035	-3.418

26_2_58698|m.58652	Protein destination and storage	serine carboxypeptidase-like 50	12.394	0.034	2.106
26_2_18033|m.18017	Protein destination and storage	thioredoxin H-type-like	12.602	0.004	-5.793
26_2_40357|m.40327	Protein destination and storage	uncharacterized protein LOC109338437	3.501	0.026	2.229
26_2_63503|m.63425	Protein destination and storage	upstream activation factor subunit UAF30	20.221	0.020	-3.380
26_2_72648|m.72519	Protein synthesis	50S ribosomal protein L28, chloroplastic	2.783	0.026	-2.768
26_2_65519|m.65431	Protein synthesis	60S ribosomal protein L10	11.990	0.041	2.514
26_2_15409|m.15385	Protein synthesis	asparagine--tRNA ligase, cytoplasmic 1	13.793	0.029	2.157
26_2_5383|m.5371	Protein synthesis	organelle RRM domain-containing protein 6, chloroplastic	7.542	0.014	-7.311
26_2_41549|m.41505	Protein synthesis	prohibitin-3, mitochondrial	31.033	0.039	-2.043
26_2_52593|m.52535	Protein synthesis	ribosomal protein S8	31.063	0.030	2.123
26_2_50879|m.50817	Signal transduction	14-3-3-like protein D	6.816	0.008	2.600
26_2_2649|m.2644	Signal transduction	3-phosphoglycerate kinase	16.125	0.029	-3.035
26_2_89869|m.89492	Signal transduction	ATOZI1, putative	16.030	0.004	-4.540
26_2_72550|m.72434	Signal transduction	ATP synthase subunit b', chloroplastic	95.518	0.021	-2.810
26_2_17326|m.17309	Signal transduction	beta-amylase	13.404	0.033	3.889
26_2_39120|m.39086	Signal transduction	beta-amylase-like isoform X1	6.088	0.018	-3.190
26_2_64184|m.64126	Signal transduction	bifunctional epoxide hydrolase 2-like	20.551	0.035	-6.699

26_2_64731|m.64643	Signal transduction	cysteine--tRNA ligase, chloroplastic/mitochondrial isoform X3	10.239	0.035	-2.543
26_2_45798|m.45750	Signal transduction	heat shock cognate 70 kDa protein 2-like	213.233	0.021	2.039
26_2_35378|m.35351	Signal transduction	Heme-binding protein	14.322	0.029	2.571
26_2_8058|m.8047	Signal transduction	heme-binding-like protein At3g10130, chloroplastic	36.534	0.007	2.599
26_2_84274|m.84157	Signal transduction	mitochondrial ATP synthase	4.799	0.029	-3.582
26_2_10048|m.10039	Signal transduction	nuclear transport factor 2B	8.322	0.014	-7.065
26_2_50311|m.50267	Signal transduction	nucleoside diphosphate kinase 2, chloroplastic	29.602	0.012	2.302
26_2_60123|m.60070	Signal transduction	probable plastid-lipid-associated protein 13, chloroplastic isoform X1	61.726	0.023	2.568
26_2_33685|m.33640	Signal transduction	protein BOLA4, chloroplastic/mitochondrial	11.734	0.008	-3.684
26_2_51533|m.51467	Signal transduction	pto-interacting protein 1	6.295	0.037	2.233
26_2_32527|m.32488	Signal transduction	Rossmann-like alpha/beta/alpha sandwich fold	13.199	0.018	-3.136
26_2_46491|m.46432	Signal transduction	thylakoid lumenal 19 kDa protein, chloroplastic	18.788	0.048	-3.466
26_2_43605|m.43554	Signal transduction	very-long-chain 3-oxoacyl-CoA reductase 1-like	6.246	0.014	-2.535
26_2_64125|m.64074	Signal transduction	thylakoid lumenal 15 kDa protein 1, chloroplastic	35.313	0.048	2.542
26_2_61807|m.61753	Transcription	caffeic acid 3-O-methyltransferase	10.862	0.026	2.086
26_2_6181|m.6173	Transcription	histone H2A	44.041	0.037	-2.306

26_2_54795|m.54726	Transcription	nascent polypeptide-associated complex subunit alpha-like protein	9.778	0.046	-2.125
26_2_56455|m.56398	Transporters	copper transport protein ATX1	4.721	0.046	2.627
26_2_25106|m.25079	Transporters	linoleate 13S-lipoxygenase 2-1, chloroplastic	67.635	0.025	2.817
26_2_55500|m.55434	Transporters	probable aquaporin PIP-type 7a	28.362	0.026	-2.002