Evaluation of agronomic and drought response traits in two cultivars of soybean for cultivation in the Transkei region of the Eastern Cape

BY

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Keywords

Antioxidant activity

Cell death

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Leaf rolling

Osmolytes

Reactive Oxygen Species

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Yield components

Abstract

Drought is a major stress that affects the productivity of plants across the globe. The consequences of drought are posed to continue reducing agricultural productivity as world climate changes. This makes it imperative to improve tolerance of staple food crops to drought. Under drought stress, reactive oxygen species (ROS) accumulate, which may lead to oxidative damage of DNA, proteins and lipids. However, plants have evolved antioxidant defence machinery to scavenge the oxygen radicals produced to counter this damage. Soybean (*Glycine max*) is the most widely cultivated legume in the world, contributing approximately 50% of the global growing area of leguminous crops. However, there is a projected drastic climatic change and doubling in the population of Sub-Saharan Africa in the next 40 years. This calls for an increase in crop productivity to meet food demand. Soybean possesses a high-quality protein source for human and animal nutrition. The plant is produced in different provinces of South Africa, with the Eastern Cape producing the least soybean. This research aimed to evaluate the agronomic potential of two soybean cultivars LS 6164R and DM 5953 RSF in the Transkei region of the Eastern Cape, specifically in the King Sabatha Dalindyebo local municipality. Drought responses of the two cultivars were assessed to determine the more drought-tolerant cultivar. The agronomic study was conducted in the field with an assessment of the soybean yield and yield components. The physiological, biochemical and molecular responses of the cultivars to drought were determined based on changes in growth, relative water content, chlorophyll content, cell death, reactive oxygen species content, lipid peroxidation, enzymatic and non-enzymatic antioxidant assays. The agronomic study illustrated that both cultivars were able to adapt to the cultivation environment and able to produce viable soybean grain. The LS 6164R cultivar produces a higher yield than DM 5953 RSF under these conditions. The drought study shows that water deficit stress upregulated ROS levels, inducing lipid peroxidation which caused oxidative damage to the soybean tissues. This resulted in stunted growth, chlorophyll pigment disruption and leaf-rolling. However, the damage was less pronounced in the LS 6164R cultivar than the DM 5953 RSF cultivar. There were significant changes in the antioxidant systems as both cultivars were able to increase the activity of antioxidants such as SOD, CAT and APX together with the osmolyte proline. These molecular changes were not effective in alleviating cellular damages in the DM 5953 RSF cultivar. The observation obtained from these findings shows that LS 6164R cultivar produces more yield than DM 5953 RSF, and also performs better under drought than DM 5953 RSF. Results from this study serve as valuable knowledge on comprehensively understanding the soybean genotype with better

agronomic traits under the cultivation environment of the King Sabatha Dalindyebo local municipality and performance under drought conditions.

DECLARATION

I declare that "Evaluation of agronomic and drought response traits in two cultivars of soybean for cultivation in the Transkei region of the Eastern Cape" is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Musa Oyebowale Akanbi

Signature

December 2019

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My profound gratitude first goes to Almighty Allah for protecting and guiding me throughout my 25 years of life. I give thanks to him for granting me strength, ability, endurance and knowledge to complete this study. In moments of difficulties and challenges, I held steadfast to his word in Quran 94:5/6 “Verily with every difficulty, there comes relief”.

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List of Abbreviations

ABA:	Abscisic acid
ANOVA:	Analysis of variance
APX:	Ascorbate peroxidase
APS:	Ammonium persulphate
ASA:	Ascorbate
ATP:	Adenosine triphosphate
BSA:	Bovine serum albumin
CAT:	Catalase
Cd:	Cadmium
Chl:	Chlorophyll
DHAR:	Dehydroascorbate reductase
EDTA:	Ethylenediaminetetraacetic acid
ETC:	Electron Transport Chain
GPX:	Glutathione peroxidase
GR:	Glutathione reductase
GSH:	Glutathione
KCN:	Potassium cyanide
LPO:	Lipid peroxidation
MDA:	Malondialdehyde
MDHAR:	Monodehydroascorbate reductase
NaCl:	Sodium Chloride
NADP:	Nicotinamide adenine dinucleotide phosphate, oxidized
NADPH:	Nicotinamide adenine dinucleotide phosphate, reduced
NBT:	Nitrotetrazolium Blue chloride
P5SCR:	Pyrroline-5-carboxylate reductase
PAGE:	Polyacrylamide gel electrophoresis
PCD:	Program Cell Death
PEP:	Phosphoenolpyruvate
PSI:	Photosystem I
PSII:	Photosystem II
PUFA:	Poly-unsaturated fatty acid
PVPP:	Polyvinylpolypyrrolidone

ROS:	Reactive Oxygen Species
RWC:	Relative water content
SDS:	Sodium dodecyl sulfate
SOD:	Superoxide dismutase
TBA:	Thiobarbituric Acid
TCA:	Trichloroacetic acid
TEMED:	N, N, N', N'-Tetramethylethylenediaminese
VE:	Vegetative emergence
WD:	Water-deprived
WW:	Well-watered

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1 Chapter One

Literature Review

1.1 Introduction

There is a need for an increase in food production as sub-Saharan Africa's population is projected to double in the next 40 years (Cleland, 2013). Due to limited viable agricultural land in many areas having high population densities, it is of the utmost importance to intensify sustainable agricultural production (Garnett *et al*., 2013).

Soybean is an important legume crop with a high value, it occupies a vital position among protein foodstuffs (Thoenes, 2007). They are a small but vital and growing constituent of the agricultural economy in Southern Africa (de Beer and Prinsloo, 2013).

South Africa is one of the largest producers of soybean in Sub-Saharan Africa. It is produced in different provinces of South Africa, but the Eastern Cape contributes the least in terms of productivity (DAFF, 2017). The country's economic stability and vitality depend upon a healthy agricultural sector (Nompozolo and Igodan, 2000), and there has been a large demand of soybean recently from the crushing and processing industries (DAFF, 2017). Hence, small-scale and rural farmers must be given the opportunity to enhance their knowledge of soybean traits and cultivation requirements.

Plants have been faced with environmental challenges in the form of biotic and abiotic stress factors. These factors negatively affect the growth and development of plants depending on the level of exposure and duration of the stress. Food stability is being frightened globally by abiotic stresses such as drought and salinity, as the yield and growth of plants are being reduced during these conditions (Kapanigowda *et al*., 2013).

Drought is a major stress that affects the productivity of plants across the globe, with South Africa experiencing the worst drought recorded from 2015 to 2016 (SAWS, 2016). In a period of approximately 30 years, from the year 1970 to 2000, the proportion of land exposed to extreme drought conditions increased by more than 2-fold. This has led to subsistence and commercial crop production being threatened by drought stress worldwide (Challinor *et al.*, 2010).

Different morphological, biochemical, and molecular changes occur in plants as a result of varying stress conditions and these disturb plant growth, development and productivity (Upadhyaya *et al.*, 2013). Abiotic stress might result in the overproduction and accumulation of reactive oxygen species (ROS) in plant cells. These ROS are toxic and reactive, causing oxidative damages to lipids, proteins, DNA and carbohydrates in cells (Jha and Subramanian, 2016). To counteract these ROS and keep them in check, the plant stimulates its antioxidant machinery that protects its cells from damage through reduction of the ROS. ROS influence the expression of some genes that enable control of a series of processes in the cell cycle, programmed cell death, pathogen defence, systemic signalling and development (Upadhyaya *et al*., 2013). Antioxidant enzymes involved in ROS detoxification include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), guaiacol peroxidase, glutathione-S- transferase (GST). Non-enzymatic antioxidant includes ascorbic acid (ASA), glutathione (GSH), proline, glycine betaine, phenolic compounds, alkaloids, non-protein amino acids and alpha-tocopherol. This defence system works in a coordinated manner to regulate reactions involved in ROS metabolism and protection to plant cells against oxidative damages by dismutation and scavenging of ROS (Gill and Tuteja, 2010).

It is vital for South Africa to have a considerable production of staple food crop rich in protein such as soybean. This is important to curb the incidence of undernutrition and malnutrition, most especially in rural households and also to increase the country's export earnings. Production of soybean is very low in the Eastern Cape, and prospects for increased production are worsened by the drought crisis that has been persistent in South Africa. This prompted the agronomic study and comparative drought analysis of two soybean cultivars, namely LS 6164R and DM 5953 RSF.

Therefore, our aims were to evaluate the agronomic potential of two soybean cultivars (LS 6164R and DM 5953 RSF) in King Sabatha Dalindyebo local municipality of the Transkei region of Eastern Cape and to also assess their drought responses to determine the more tolerant cultivar. The objectives were as follows:

- Evaluation of the yield components of the two soybean cultivars (LS 6164R and DM 5953 RSF) under field conditions by recording the shoot length, number of branches, number of pods, weight per pods and the total weight of the plant.
- Assessing the yield by measurement of soybean grain produced in tonnes/hectare.

- Understanding the morphological responses of both soybean cultivars under water deficit via capturing the digital image of the whole plant and trifoliate leaves.
- Determining the physiological responses of the two soybean cultivars by measuring the shoot length, shoot weight, root length, relative water content (RWC), chlorophyll content and cell viability.
- Determining some of the molecular response to drought in the two soybean cultivars via assessing their level of ROS (H_2O_2 and O_2^-), lipid peroxidation (MDA), enzymatic (SOD, CAT, APX) and non-enzymatic antioxidant/osmolyte (proline).

1.2 Origin of soybean

Soybean (*Glycine max*) originated in Southeast Asia and was first domesticated by Chinese farmers around 1100 BC. It was cultivated in Japan and many other non-African countries by the first century AD and was introduced to the Americas and Europe by the 18th century (Shurtleff and Aoyagi, 2009). Soybean was introduced to Africa from China with Northern African countries being the first to cultivate soybean in mass, with Egypt leading the line in 1858, followed by Algeria in 1860, and then Tunisia in 1873 (Matagrin, 1939). The first record of soybean cultivation in Southern Africa occurred in 1903. Soybean was cultivated in the Natal and Transvaal regions of South Africa (Burtt-Davy, 1905). The plant was first grown in Zimbabwe in the year 1906 before being introduced to Mauritius by P. Boname in the year 1907. Since then, it has spread throughout the continent from Western Africa to East Africa (Shurtleff and Aoyagi, 2009).

1.3 Biology of soybean

Glycine max belongs to the legume family, with the botanical name *Leguminosae* (Sauer, 2017). The family belongs to the *Dicotyledonae* class which is distinguished by two embryonic or seed leaves. Soybeans, green beans, peas, chickpeas, shelling beans, lupines, lentils, clover and alfalfa all belongs to the legume family (Kurlovich *et al*, 1995). The anatomy of soybean, with the shoot and root system, is illustrated in Figure 1.1.

1.4 Vegetative stage and development

Soybean seeds begin germination by absorbing water in quantity equal to about 50% of its weight (Ritchie *et al*., 1985). The first to grow is the radicle or primary root, which grows from the swollen seed where it elongates downward and anchors itself in the soil. Quickly after initial primary root growth, the hypocotyl (a small section of the stem between the cotyledonary node and the primary root) starts elongation toward the soil surface, pulling the cotyledons (seed leaves) with it (Ritchie *et al*., 1985). The anchored primary root and elongating hypocotyl provide leverage for pulling the cotyledons to the soil surface for vegetative emergence or VE. VE commonly occurs 1 to 2 weeks after sowing, which depends on the plant depth, moisture and temperature of the soil (Hanway and Thompson 1967). After the VE, lateral roots start to grow from the primary root (Fehr and Caviness 1977) as shown in Figure 1.1.

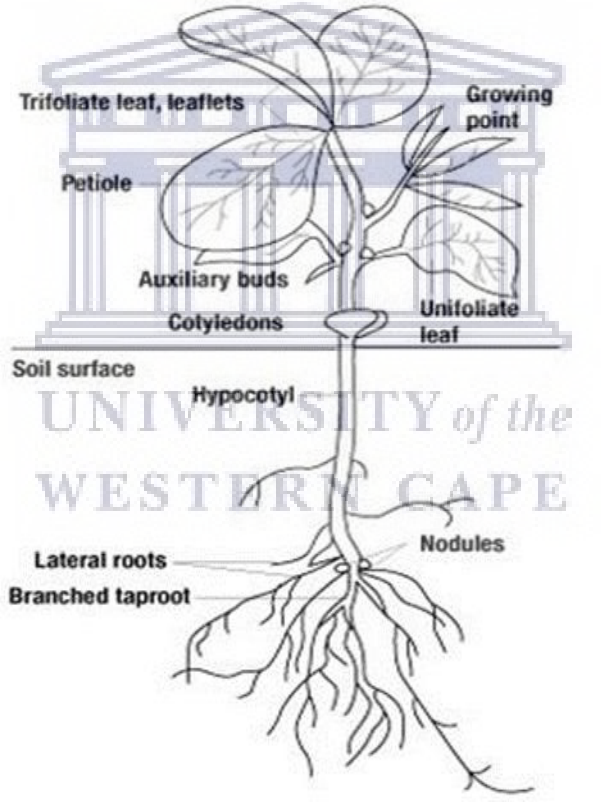

Figure 1.1: Diagram showing the botanical part of a soybean plant
Vegetative features showing the shoot and the root system of Glycine max (Amnh, 2018).

1.5 World soybean production

Production of soybean worldwide has increased to more than 100 million metric tonnes per year over the past 30 years with global production forecasted to be over 337 million tonnes per year by 2018-2019 (USDA, 2018). The United States, Brazil and Argentina contribute more

than 80% of global soybean production, making them the world's largest soybean producers (FAOSTAT, 2015; USDA, 2016). In Sub-Saharan Africa, 1.4 million tonnes of the crop is produced per year and production is projected to increase at a compound growth annual rate of 4.1% per year to reach 2.5 million tonnes by 2025, with Nigeria as the largest producer followed by South Africa (FAOSTAT, 2015; USSEC, 2011).

1.6 Soybean production in South Africa

The Bureau for Food and Agricultural Policy Project (2013) estimated that 605 000 hectares of South Africa landmass would have been used for soybean plantation by the year 2020. This is due to the processing of soybean to consumable products and growing demand for animal protein. This is projected to result in soybean productivity tripling to 1.62 million tons by 2020 (BFAP, 2013). For this target to be reached, there needs to be all-round production of the crop in different viable regions in South Africa. Soybean is significantly produced in the Mpumalanga (42%) and Free State (22%) provinces, while KwaZulu-Natal produces 8%, Limpopo 8%, North West 5% and Gauteng 2%, as shown in Figure 1.2. The four provinces of Mpumalanga, Free state, Limpopo, and KwaZulu-Natal accounts for more than 80% of total soybean production in South Africa (DAFF, 2017). Table 1.1 shows soybean production by tons from the period of 2012 to 2016 in all nine Province in South Africa.

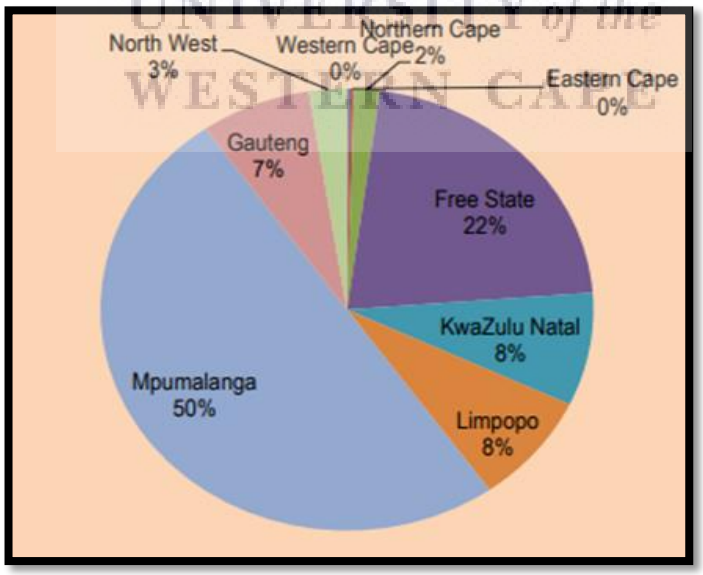

Figure 1.2: Province percentage contribution to soybean export
Chart showing Mpumalanga province with 50% production followed by Free State (22%), KwaZulu Natal (8%), Limpopo (8%), Gauteng (7%), Northern Cape (2%), Western Cape and Eastern Cape with 0% (DAFF, 2017).

Table 1-1: Production of soybean in tons from 2012 to 2016.

PROVINCE	Production (tons) 2012	Production (tons) 2013	Production (tons) 2014	Production (tons) 2015	Production (tons) 2016	Mean
Western Cape	0	0	0	1600	1200	560
Eastern Cape	800	800	3600	2100	2100	1880
Northern Cape	1500	7000	14000	14000	12400	9780
Free State	192500	249500	352000	366000	156600	263320
KwaZulu-Natal	81600	82000	98000	102900	60 000	68900
Limpopo	50600	53500	66000	72000	58800	60180
Mpumalanga	263100	335000	335000	389900	363000	337200
Gauteng	28500	34000	47400	69000	52500	46280
North West	31500	22700	32000	52500	18000	31340

Table showing soybean production in tons from 2012 to 2016 in different provinces of South Africa produces soybean, with Western Cape and Eastern Cape having the lowest contribution to production in recent years (DAFF, 2017).

The report from DAFF (2017) shows that the Western and Eastern Cape Provinces have the lowest productivity of soybean with 0.00% contribution towards the country's total soybean exports in 2016.

Eastern Cape Province has a reasonable amount of maize and sorghum production (Nompozolo and Igodan, 2010) but with lower productivity in legumes. The province has the same moderate (18%) rainfall necessary for soybean productivity like the North West and Free State provinces as shown in Figure 1.3 (Dlamini *et al.*, 2014) but produces far less soybean (Figure 1.2 and Table 1-1). This indicates that constraints to soybean production in the Eastern Cape is not to be related to rainfall. Subsistence farmers in the region may be operating under a complex farming environment (Nompozolo and Igodan, 2000), in which different factors influence their choice to grow soybean. Such deciding factors may involve soybean growth conditions and the availability of the desired cultivar. Soybean cultivars accessible to the small-scale farmers in Eastern Cape most especially the Transkei region might not be feasible for cultivation in the region, variations in beliefs, political factors as well as the local climatic condition and market access may also influence the decision to grow soybean. Therefore, cultivars that can perform better with adequate growth factors such as temperature, soil composition, climate, and rainfall pattern in the region needs to be evaluated.

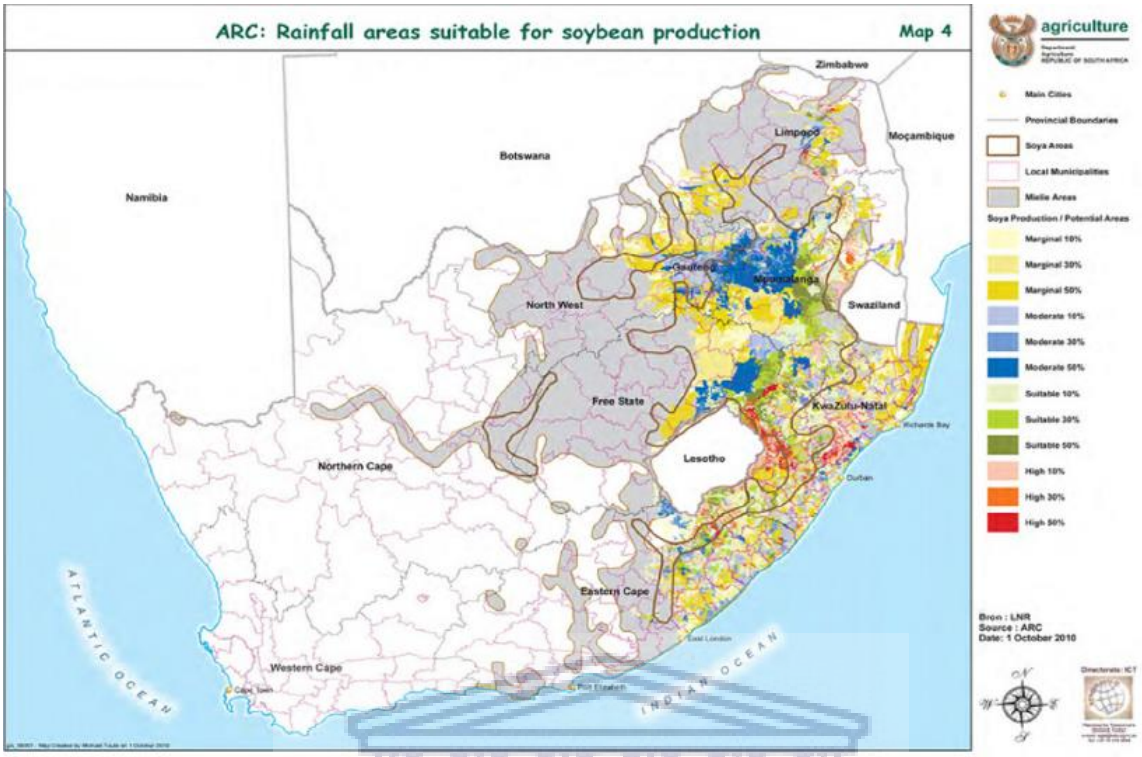

Figure 1.3: Rainfall area suitable for soybean productivity by province
Map showing the suitability of soybean production in all province in South Africa. North West, Free State, Limpopo, and Eastern Cape have a moderate (18%) rainfall suitability, with Mpumalanga having moderate of 58% and Kwazulu Natal having a suitable rainfall pattern (Dlamini *et al*., 2014).

1.7 Economic and nutritional impact of soybean

Despite soybean's magnificent qualities, it forms a very small percentage of the average household's diet in South Africa (Joubert and Jooste, 2013). The main uses of the crop include soybean oil, soybean cake and soybean products for human consumption. Soybeans are majorly consumed by the livestock sector. The consumption of soybean for oil and protein is at 25%, whilst 60% is for animal feed (DAFF, 2017). The largest consumer of soybean-derived protein in the country is largely the poultry industry (Joubert and Jooste, 2013). Human consumption constitutes about 15% intake, with a variety of over the counter foodstuffs such as soya sauces and mince/soups. Also, various nutritious breakfast foods such as yoghurt and flavoured soymilk products are all included in the 15% (DAFF, 2017). Direct consumption of the legume is low. In a few cases, soymilk is consumed by people who are intolerant to lactose (McCray, 2003). To reduce the incident of malnutrition and undernutrition prevalent in Southern Africa, soybean needs to be incorporated into the household diet. Although South African soybean exports were not competitive in world terms in early 2000, there was an improvement over the

preceding years (DAFF, 2017). Forex is earned when soybean is exported, which boosts the economy of the country. However, the crop exports significantly declined during the year 2013 and 2014 due to low productivity caused by the drought crisis during this period (DAFF, 2017), and productivity has declined ever since and expected to continue dropping as drought is prolonged.

1.8 Constraints to soybean production

Production of leguminous crops have not kept at pace with cereal crops. Short growing seasons, exposure to intermittent or terminal drought together with progressive soil degradation by chemical and physical means, are limiting factors reducing legumes productivity (Graham and Vance, 2003). These are some of the environmental factors that affect soybean productivity.

1.8.1 Drought

The increment in the frequency of drought is a vital distinction of forecast change in climate and is among the most important abiotic factors limiting the yield of the crops worldwide (Meehl *et al*.,2007). Drought, which can be defined as lack of sufficient precipitation which results in insufficient rainfall over an extended period, causes limited up-take of water by roots and excessive loss of water via transpiration in the leaves. It is exacerbated by increased evapotranspiration caused by low air humidity and high rates of radiation (Yoshida, 1981).

Photosynthesis decreases in drought-stressed plants as these plants have reduced stomatal conductance to preserve water. Due to this, fixing of CO_2 becomes limited, which results in the photosynthetic rate deduction. Boyer (1970) argued that the main factor limiting photosynthesis in drought conditions is the entrance resistance of CO_2 diffusion into stomata. Undoubtedly in moderate drought conditions, closure of stomata is the main factor for reduced levels of photosynthesis as it results in reduced leaf internal CO_2 concentration. However, Iturbe-Ormaetxe *et al*, (1998) noticed that under severe drought stress changes occur in the chlorophyll content, which disturbs and inhibits photosynthesis. It was also shown that drought stress causes a negative impact in nodule formation via disturbing the elegant mechanisms of the control of oxygen in nodules which is vital for active nitrogen fixation (Zimmer *et al*., 2016).

Globally huge economic losses in agriculture are largely caused by drought, which is projected to increase as global climate change progresses (*Keane et al.*, 2009). World surface temperature is anticipated to increase in the range of +1.5 to +5.9°C towards the end of the 21st century.

The Southwest and Southern Plains of the United States lost more than $5 billion in agricultural products due to drought and heatwaves in 2011. Russian wheat production also had more than 30% reduction in productivity in 2010 due to the drought and heatwaves experienced in agricultural regions (USDA, 2016).

South Africa continues to experience the worst drought since 2015, with the country receiving the lowest rainfall since rainfall recording started in 1904 (SAWS, 2016). Many farmers lost much of their crops and livestock due to water scarcity. Important dams such as Pongolapoort used for irrigation lost 20% of its water content in a single year (Fernandes, 2016). Due to the consequent loss of available water for agriculture during periods of drought, it is important to increase the tolerance of staple food crops to drought. Hence, understanding the molecular mechanisms of how different varieties/lines of a particular plant species respond in drought stress conditions would contribute to the development of varieties with higher drought tolerance, which would increase crop productivity under water deficit. Water-deficit areas of the world are projected to reach more than 50 countries accommodating 3 billion people by 2030 (Graham and Vance, 2003). Progression of drought increases the salt concentration of soil, which makes the soil more saline (Munns and Tester, 2008).

1.8.2 Salinity

Salinity is another vital abiotic factor that decreases agricultural yield. Salinity has destroyed more than 45 million hectares of irrigated land globally, with about 1.5 million hectares of farmland damaged yearly and rendered unsuitable for farming (Hasegawa *et al*., 2000). Under extreme soil salinity, plant growth is reduced due to oxidative damage, membrane disorganization, ion toxicity, water stress, genotoxicity and reduction of cell division and expansion (Munns and Tester, 2008). During initial exposure to salt stress, plants encounter osmotic stress which leads to a reduction in leaf expansion, this ultimately leads to stomatal closure and inhibition of cell division (Munns, 2002). Munns (2002) observed that salinity decreases the plant's ability to take up water, resulting in a pronounced reduction in growth rates, and metabolic changes similar to water stress.

1.9 Reactive oxygen species (ROS) overview

Molecular oxygen was introduced into the environment over 2.7 billion years ago by O_2-evolving photosynthetic organisms. This resulted in reactive oxygen species production which is a nonessential collaborator of aerobic life (Halliwell, 2006). The possession of dual unpaired electrons by an O_2 molecule makes it have an equal spin quantum number. ROS is formed

through the spin limitation that enables O_2 to receive one electron at a time. However, ROS are also produced as metabolic pathway by-products, through processes occurring in various subcellular locations such as peroxisomes, chloroplasts and mitochondria (Navrot *et al*., 2007). However, ROS are degraded under steady-state situations by various antioxidant defence mechanisms (Foyer and Noctor, 2005). Biotic and abiotic factors such as drought, salinity, temperature, air pollution, herbicides, nutrient deficiency, heavy metals, chemicals and pathogen attack discompose the equilibrium between the formation and scavenging of ROS. If the equilibrium is destabilized, ROS are overproduced and cause oxidative damage to the cell. These ROS include hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), superoxide (O_2^-), hydroperoxyl radical (HO_2^-), alkyl hydroperoxide radical (ROO^-) and alkoxyl radical (RO^-). ROS are toxic at high concentrations because of their reactivity with organic compounds in cells, causing damage to carbohydrates, proteins and DNA. This ultimately leads to programmed cell death (PCD) (Mittler, 2002). ROS overproduction in unfavourable conditions, such as drought, leads to loss of crop productivity (Gill and Tuteja, 2010).
Through the disruption of the cell membrane, many functions of the cells are negatively affected by lipid peroxidation (LPO) caused by excessive ROS levels (Gill and Tuteja, 2010). The spatial and temporal production, together with the quantity of ROS produced, determine if ROS will perform signalling (likely protective) function or a damaging effect. ROS production may trigger the expression of genes encoding protective enzymes that scavenge the ROS. Among the defence mechanism is the production of enzymatic or non-enzymatic antioxidant which dismutase the ROS formed, and ensure ROS accumulation is kept in check. These antioxidants are found in many compartments of the cell, signifying their importance in detoxification of ROS for the cell to survive (Foyer and Noctor, 2005)

Many studies have postulated that expression of several genes and signal transduction pathways are regulated by ROS. This has developed a mechanism in which plants use ROS as signals to regulate various genetic responses to stress (Gill and Tuteja, 2010). Such signalling mechanisms underlie the coordination of some physiological processes associated with responses to biotic and abiotic stresses (Foyer and Noctor, 2005).

1.10 The chemistry of the ROS

The ability of phototrophs to convert light to biological energy is vital for life on Earth. Photosynthesizing organisms are at possible risk of oxidative damage due to their bioenergetic lifestyle and the enormous amount of photosensitizers and polyunsaturated fatty acids

(PUFA) in the chloroplast envelope (Gill and Tuteja, 2010). ROS formation in cells occurs as a result of O_2 present in the atmosphere, allowing respiratory metabolism and effective energy generation systems utilizing O_2 as the final electron acceptor. Even at the non-reactive state, atmospheric oxygen can give rise to ROS (O_2^-, H_2O_2, •OH and $^1O_2$) as shown in Figure 1.4. A single reduction of O_2 produces O_2^-, which later dissociates, with one O_2^- giving up an added electron to another O_2^-. H_2O_2 is produced by protonation and HO_2^- can be generated through O_2^- protonation (Temple *et al*., 2005). Further reactions occur in the presence of transition metals such as copper and iron via the Haber-Weiss mechanism or Fenton reaction, which liberates •OH, the most reactive ROS. The reaction of O_2^- with an important signalling free radical species NO results in the forming of the production of peroxynitrite ($OONO^-$). Also, the reaction of O_2^- with chlorophyll photoexcitation forms $^1O_2$ (Temple *et al*., 2005).

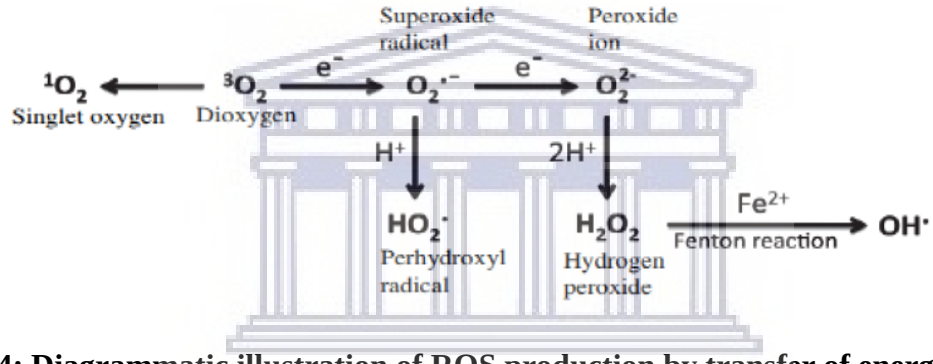

Figure 1.4: Diagrammatic illustration of ROS production by transfer of energy
ROS such as O_2^-, H_2O_2, •OH and $^1O_2$ are formed from atmospheric oxygen (Gill and Tuteja, 2010).

1.10.1 Superoxide radicals (O_2^-)

Superoxide is produced after the reaction of O_2 with other components of the mitochondrial and chloroplast electron transport chain (ETC). This results in the transfer of one electron instead of reacting with terminal oxidases-cytochrome *c* oxidase and alternative oxidase, which usually releases H_2O_2 after transferring four electrons (Apel and Hirt, 2004). O_2^- is the first ROS produced and it has a half-life between 2-4 µs. It is formed when around 1-2% of O_2 is utilized in plant tissues (Sharma, 2016). Also, the reduction in O_2 during electron transport via the non-cyclic route at the various cell compartments, including the ETC of the chloroplast, results in O_2^- formation. O_2 reduction to O_2^- takes place at the PS1 level in the ETC, leading to the formation of other ROS such as •OH and $^1O_2$, which cause lipid membrane peroxidation and cell weakening (Sharma, 2016). Protonation of O_2 results in the production of a strong oxidizing agent, HO_2^-, at the negatively charged surface of the membrane. These changes lead

to attack on polyunsaturated fatty acids (PUFA) by HO_2^- Furthermore, the electron can be donated to iron (Fe^{3+}) by O_2^- to produce a reduced form of iron (Fe^{2+}) that has the ability to reduce the H_2O_2 produced due to SOD dismutation of O_2^- to •OH (Gill and Tuteja, 2010).

1.10.2 Hydrogen peroxide (H_2O_2)

H_2O_2 is produced by the univalent reduction of O_2^- but possesses a long half-life (1 millisecond) than the other ROS (which have a relatively short half-life of 2-4 microseconds). Augmented production of H_2O_2 in plant cells results in oxidative stress whereby ROS inactivates enzymes by oxidation of their thiol group (Gill and Tuteja, 2010).

At low concentrations, H_2O_2 is an important acclamatory signal that elicits different biotic and abiotic tolerance responses by acting as a signalling molecule. At high concentrations, H_2O_2 can result in PCD (Varindra and Sekhon, 2006). A number of studies have ascertained the role played by H_2O_2 as a regulatory molecule in a broad range of physiological processes such as photorespiration, senescence, stomata movement, photosynthesis, cell cycle, growth and development (Gill and Tuteja, 2010). The high permeability of H_2O_2 across the membrane and its relatively long half-life allows its actions as a secondary messenger for ROS-mediated signals (Foreman *et al*., 2003; Varindra and Sekhon, 2006).

1.11 Production of ROS in cellular compartments

Under light energy, ROS are produced in the chloroplast and peroxisomes, while in the darkness they are being produced in the mitochondria. Although there are various sites for ROS generation in the plant cell, these aforementioned sites form the greater part of comprehensive intracellular ROS production (Gill and Tuteja, 2010).

1.11.1 Mitochondria and ROS generation

Plant mitochondria are referred to as "energy factories" and are the main site for the generation of ROS. Mitochondria have a unique ETC compartment with roles in photorespiration and other processes which are not the same as those in animals. The ability of plants to photosynthesize enables the cellular environment of plant mitochondria to be distinctive, this results in an environment rich in O_2 and carbohydrates (Almeras *et al*., 2003). It has been well documented that about 1-5% of O_2 utilization of isolated mitochondria results in ROS production (Foyer and Noctor, 2003). ROS generation in the mitochondria is possible as the

ETC conceals electrons with sufficient free energy, allowing O_2 to be reduced. Under various stress situations, ROS are produced at high levels in the mitochondria, although ROS are present at homeostatically controlled levels under typical respiration conditions (Murphy, 2009).

Complex I and III of the ETC are the main site for O_2^- production in the mitochondria, as shown in Figure 1.5. The reactive O_2^- can as well undergo dismutation to be reduced to H_2O_2. A highly toxic uncharged •OH is formed when H_2O_2 reacts with Fe^{2+} and Cu^{2+}. This uncharged ROS can penetrate the mitochondrial membranes, thus escaping the mitochondrion (Gill and Tuteja, 2010). A hydrogen atom reduced by •OH leads to peroxidation of membrane PUFA and causes the accumulation of Malondialdehyde (MDA), cytotoxic lipid, 4-hydroxy-2-nonenal, hydroxyalkenals and alkenals. Accumulation of this lipid peroxidation (LPO) products leads to cell destruction via incorporation of nucleic acids, proteins and other lipids. Oxylipins and lipid-derived reactive electrophile species are also formed from LPO (Noctor *et al*., 2007)

Bioenergetics of plant cells is influenced by abiotic factors. Through energy-dissipating systems, mitochondria in plant cells can monitor ROS generation. Intrinsically, the mitochondria may play a major role in the adjustment of the cell under abiotic stress which activates oxidative stress at the cellular level (Gill and Tuteja, 2010).

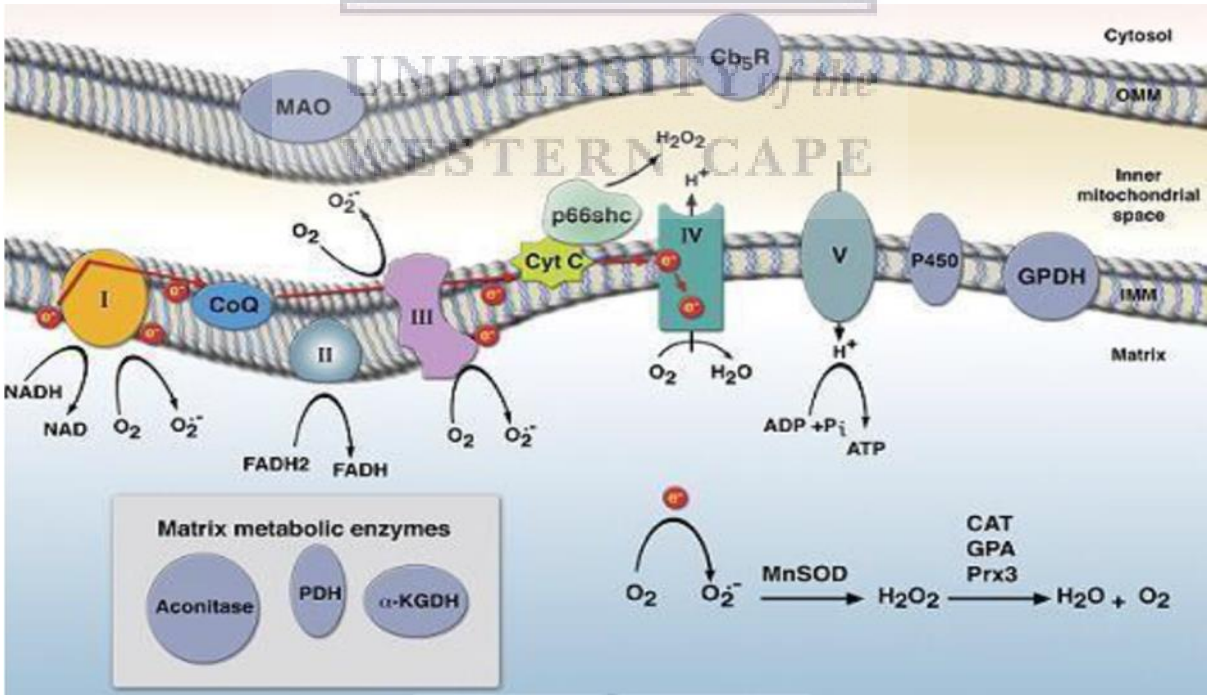

Figure 1.5: Mitochondria formation of ROS
O_2 and H_2O_2 are formed at different sites inside the mitochondrion. The main site for O_2 production is complex I and III while the site for H_2O_2 generation is Mn-SOD in the matrix

and p66shc in the inner mitochondrial space. The red arrows in the illustration represent electron flow from complex I through IV (Finkel, 2011).

1.11.2 Chloroplasts and ROS generation

Chloroplasts are the site for photosynthesis in higher plants. It is composed of a coordinated membrane system which covers essential compost of light-capturing photosynthesis machinery, and thus, enabling accessibility of all structural characteristic for excellent light-harvesting (Pfannschmidt, 2003). O_2^- is formed by oxygen produced in the chloroplast during photosynthesis, which accepts electrons moving through the photosystems. The availability of ROS production centres such as triplet Chl, ETC in PSI and PSII (figure 1.6) makes the chloroplast the main site for the production of ROS (Asada, 2006). Under stressful conditions, elevated ROS formation occurs, contrary to normal circumstances where electrons flow from the excited photosystem centres towards NADP to produce NADPH (Diaz-Vivancos *et al*., 2008). This reduces CO_2, the final electron acceptor, following insertion into the Calvin cycle (Gill and Tuteja, 2010). In circumstances of ETC overload, part of the flow of electron is rechannelled from ferredoxin to O_2, which is reduced to O_2^- via the Mahler reaction (Pfannschmidt, 2003).

The oxygen condition, as well as the reduction of polyunsaturated fatty acids (PUFA) and photosensitizers in the chloroplast envelope of photosynthesizing plants, pose them to extreme risk of oxidative damage (Foyer and Noctor, 2003). Studies have associated chloroplast-produced ROS with the hypersensitive response, as illustrated by Gray *et al.,* (2002) to show that chloroplast-produced ROS have the capability to transmit the spread of wound-induced PCD. Chloroplasts were also shown to be sources of oxidative stress in diseases development in pea leaves, where the disproportion in antioxidants and boundless production of ROS in the chloroplast were evident, presumably induced by ETC convulsion (Diaz-Vivancos *et al.,* 2008).

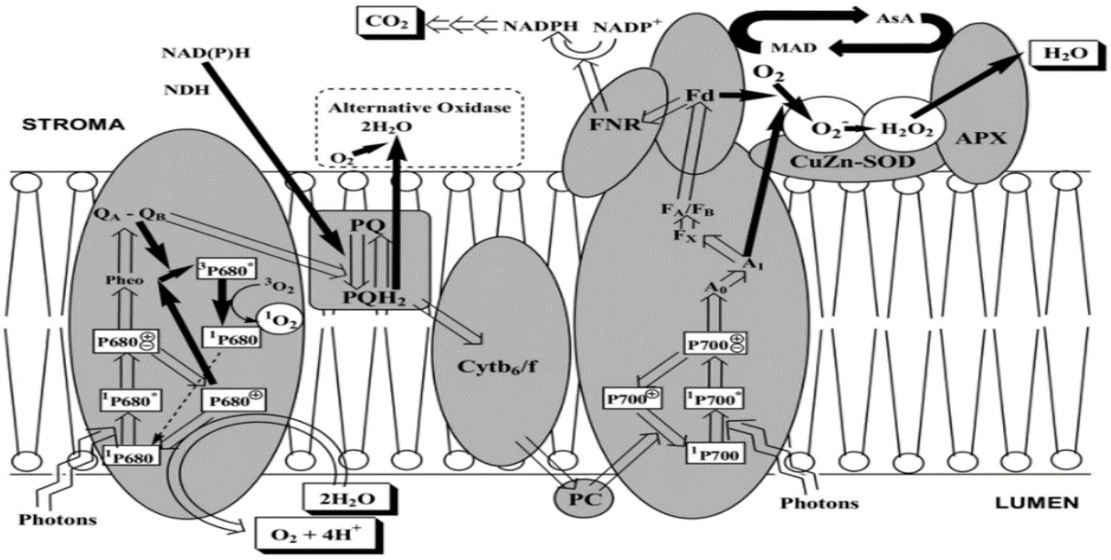

Figure 1.6: Chloroplast formation of ROS.
Singlet oxygen ($^1O_2$) produced at photosystem ll (PS II), superoxide (O_2^-) and hydrogen peroxide (H_2O_2) produced at photosystem I (PSI). The grey arrows indicate the flow of electrons through PSI and II. The electron flow is directed to produce ROS (illustrated as black arrows) when photosystems are overloaded with electrons due to the presence of excess light and insufficient photosynthetic capacity (Asada, 2006).

1.11.3 Peroxisomes and ROS generation

Peroxisomes are called 'microbodies' and are proportionately small organelles present in most eukaryotic cells. Their main function is the catabolism of long-chain fatty acids through beta-oxidation (Corpas *et al*., 2001). This important oxidative metabolism they undergo makes them another spot for intracellular production of ROS. O_2^- radicals are formed at this site (Figure 1.7) due to their normal metabolism just as it occurs in chloroplasts and mitochondria (Del Rio *et al*., 2002).

Two sites in the peroxisomes are responsible for O_2^- production. The peroxisome matrix is the first site of its production with the enzyme xanthine oxidase (XOD) catalyzing xanthine and hypoxanthine oxidation to uric acid (Gill and Tuteja, 2010). The peroxisome membrane is the second spot which depends on NADPH. This membrane is composed of flavoprotein, NADH and cytochrome b, allowing O_2^- production by the peroxisome ETC as shown in Figure 1.6 (Corpas *et al*., 2001). Besides O_2^-, H_2O_2 is also formed in the peroxisomes. Generation of H_2O_2 also occurs via glycolate oxidase and flavin oxidase activities in peroxisomes. This is as a result of beta-oxidation of fatty acids and O_2^- disproportionation (Liang and Huang, 1983) as illustrated in Figure 1.7.

Induced level of H_2O_2 and O_2^- production in the peroxisomes causes oxidative damage, although a small amount of the ROS performs a signalling function that alleviates pathogen-induced cell PCD (Gill and Tuteja, 2010). As a result, it was contemplated that peroxisomes should be considered a cellular compartment with the ability to generate and let-off important signal molecules such as O_2^-, H_2O_2, and NO into the cytosol. This serves as a substantial benefaction to an interspersed intercommunication system with cell compartments (De Río *et al*., 2002).

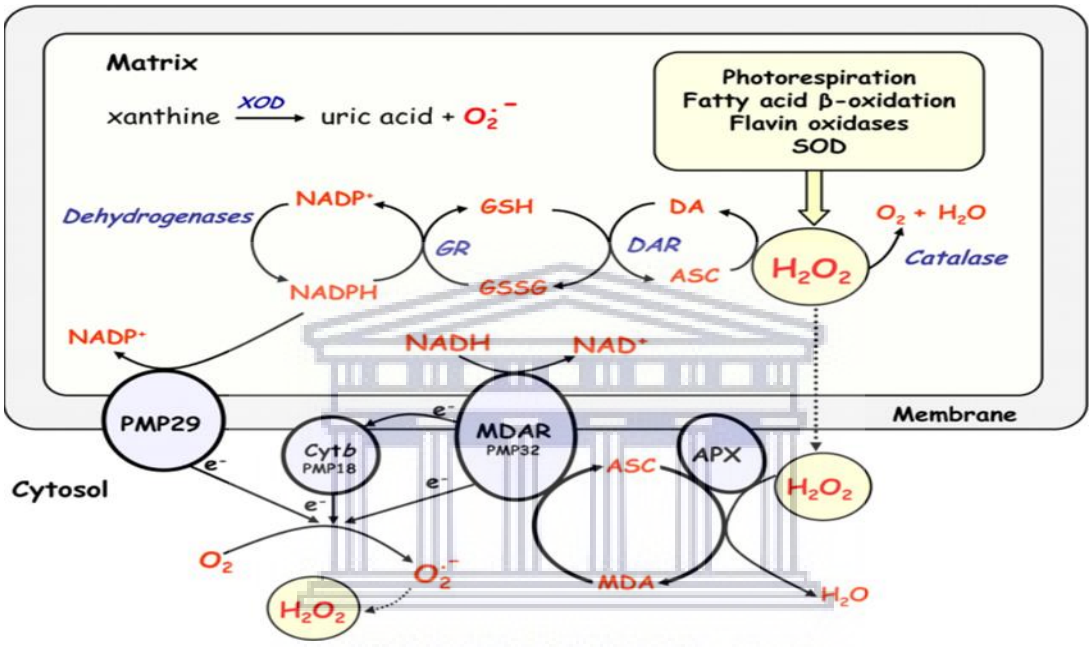

Figure 1.7: Peroxisome formation of ROS
The matrix and cytosol of peroxisome are sites for O_2^- and H_2O_2 production. O_2 is produced in the matrix through the activity of xanthine oxidase, and free O_2 is reduced to O_2^- in the cytosol via electron transfer from the ETC. Different reactions in the matrix; such as photorespiration, β-oxidation, flavin oxidases and SOD activity; produce H_2O_2. The disproportionation of O_2^- and H_2O_2 diffusion from the matrix enable a pool of H_2O_2 to present in the cytosol (Corpas *et al*., 2001).

1.12 Biochemical influence of ROS overproduction

ROS in plant cells under nominal conditions serve as signalling molecules that trigger responses effective for normal cell function. However, an increased ROS production due to various stress levels can lead to oxidative stress, which causes damages to lipids, DNA and proteins.

1.12.1 Oxidation of Proteins

Proteins are fundamentally the major working constituent of cells and are largely classified into two groups, functional and structural proteins. Structural proteins help in providing rigidity and integrity to the cell through vital organization and formation of complexes within the cells, while functional proteins undergo unique enzymatic reactions or help biochemical processes within cells (Buxbaum, 2007).

Protein oxidation is the covalent modification of proteins which is induced by ROS (Gill and Tuteja, 2010). Protein oxidation is mostly non-reversible, except for few reversible reactions involving sulphur-containing amino acids. Protein carbonylation is the marker for oxidation of proteins (Møller *et al*., 2007). His, Arg, Lys, Thr, Pro, and Trp are amino acids that are highly predisposed to oxidation. This is due to the presence of carbonyl free groups that may cause inhibition or changes in their activities and activate susceptibility towards proteolytic attack (Gill and Tuteja, 2010). Incessant degradation of amino acids results in carbonylation of amino acids, for example, arginine and proline to g-glutamyl semialdehyde (Møller *et al*., 2007). Sulphur-containing amino acids and thiol groups are violated by ROS. Met and Cys are both reactive with $^1O_2$ and •OH. Activated oxygen can form methionine sulphoxide derivatives when added to methionine residue, or extract an H atom from Cys residue to form a thiyl radical bonding to a second radical. This results in the formation of disulphide bridges (Gill and Tuteja, 2010). Various abiotic stresses result in protein carbonylation (Hancock *et al*., 2006).

1.12.2 Damage to DNA

DNA is the genetic make-up of living organisms responsible for coding thousands of proteins important for life. Due to its important role as a carrier of hereditary genetic information, damage to DNA such as double-stranded breaks are mostly regarded as the most disastrous group of molecular damage to occur within living cells (Fattah *et al*., 2010).

It is a well-established fact that both biotic and abiotic stresses damage the plant genome, causing genotoxic stress. Spontaneous DNA damage is caused when there is endogenous production of reactive metabolites which cause damage to DNA. High levels of ROS generation result in damage to lipids, structural proteins and nucleic acids in plant cells (Valko *et al*., 2006). Studies have shown that the ROS that causes the most damage to DNA is •OH. This is established by its reaction with purine and pyrimidine bases and the deoxyribose backbone (Tuteja *et al*., 2001). Guanine is majorly attacked by $^1O_2$ while H_2O_2 and O_2^- don't react with DNA (Wiseman and Halliwell, 1996).

DNA damage encompasses base deletion, cross-links, pyrimidine dimers, base modification and strand breaks caused by oxidation and alkylation (Tuteja *et al*., 2001). Plant growth and development is impaired upon damage to their DNA due to various physiological effects like a reduced synthesis of proteins, the disintegration of the cell membrane and photosynthetic protein damage. Replication errors, transcription induction, signal transduction induction, genomic instability and cell membrane can also result from damage to DNA (Gill and Tuteja, 2010).

1.13 Plant antioxidant defence mechanism

Plant cells and their organelles such as mitochondria, peroxisomes and chloroplasts protect themselves against ROS through the evolvement of antioxidant defence mechanisms (Varindra and Sekhon, 2006). Various studies have reported that the induction of enzymatic and non-enzymatic antioxidant machinery is important for the protection of plants under ROS-induced oxidative stress (Figure 1.8). Although when plants are in an unstressed condition the ROS formed are substantially scavenged by an enzymatic and non-enzymatic antioxidant, under drought and salinity the amount of ROS produced can supersede the capacity of the antioxidant system (Mittler *et al*., 2011), which in turns leads to oxidative stress as depicted in Figure 1.8.

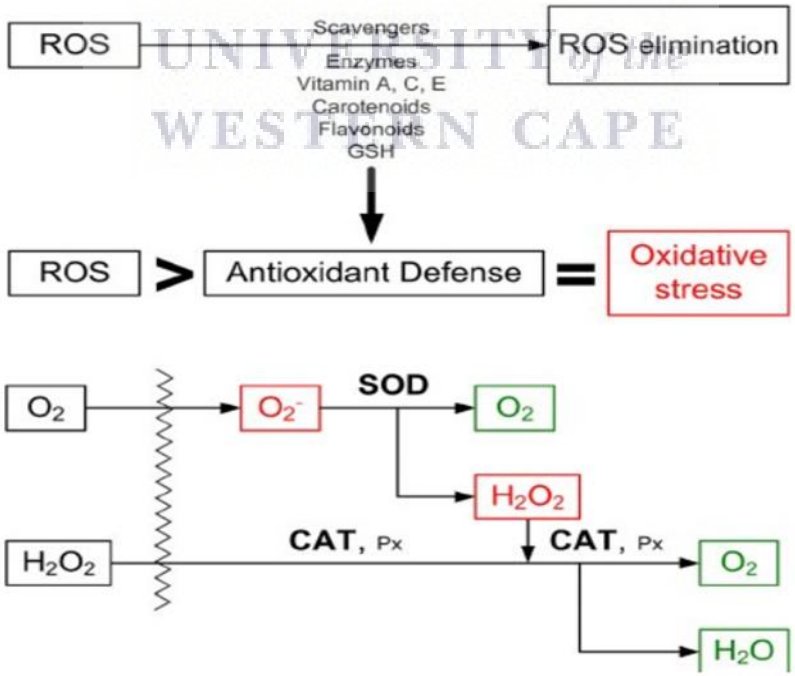

Figure 1.8: ROS elimination in the plant cell.
Antioxidant defence system acting to scavenge ROS produced under abiotic stress. Oxidative stress results due to overproduction of ROS beyond the antioxidant capacity (Puač *et al*., 2014).

The enzymatic antioxidant defence system is made up of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), glutathione peroxidase (GPX) and glutathione reductase (GR), while glutathione, some compatible solutes such as proline and glycine betaine, tocopherols, and carotenoids are examples of non-enzymatic antioxidants (Mittler, 2002).

All antioxidants serve a crucial role in the detoxification of ROS. The section below focuses more on the importance of SOD, APX and CAT enzymatic antioxidants.

1.13.1 Superoxide dismutase

SOD enzyme is effective in detoxification of superoxide-mediated oxidative stress. The enzyme is important in plant stress tolerance as it propels the disjunction of O_2^- molecules to H_2O_2 and O_2 via one O_2^- reduction to H_2O_2, with the other O_2^- oxidized to O_2 (Puač *et al*., 2014) (Figure 1.9). This helps to reduce the risk of generation of •OH from O_2^- via the metal catalysed Haber-Weiss-type reaction (Gill and Tuteja, 2010). This dismutation reaction happens at a rapid reaction rate 10000 times faster than the steady-state of the enzyme (Pang and Wang, 2008).

Plant cells house three families of SOD isoforms that are grouped based on their cofactors; the iron (Fe-SOD), the copper/zinc (Cu/Zn-SOD), and the manganese (Mn-SOD) forms. Cu/Zn-SOD is the most abundant amidst the metalloproteins, it is confined to the chloroplast, peroxisome and cytosol. Mn-SOD is constrained to the matrix of the mitochondria while Fe-SOD is present in a modest concentration in the chloroplast as shown in Figure 1.9 (Pang and Wang, 2008).

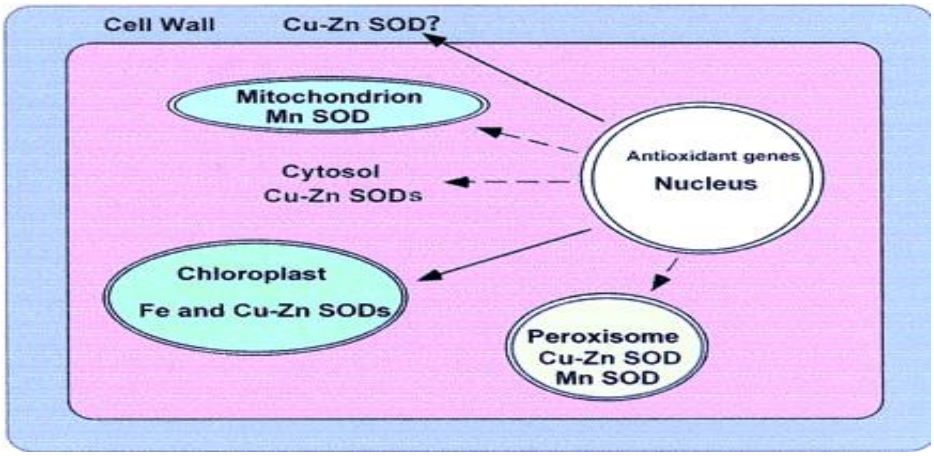

Figure 1.9: Localization of the SOD metalloproteins in the plant cell. Distribution of SOD isoenzymes within the mitochondrion, chloroplast and peroxisomes (Alscher *et al*., 2002).

The level of SOD enzymatic activity and SOD gene expression increases under abiotic stresses such as water deficit (Gill and Tuteja, 2010). A previous study conducted by Gupta *et al.*, (1993) showed how SOD overexpression safeguarded transgenic tobacco plants that overexpressed the pea Cu/Zn SOD under oxidative stress. Furthermore, the effect of heavy metals on plants was illustrated when treatment with aluminium on root tips of soybean increased SOD activity (Cakmak and Horst, 1991). Excess copper induces an increase in Cu/Zn SOD in soybean roots (Chongpraditnun *et al.*, 1992). Furthermore, pea MnSOD overexpressed in rice was able to confer tolerance to drought and reduce oxidative stress (Wang *et al.*, 2005). This reflects the role of the two families of SOD (Cu/Zn SOD and Mn-SOD) in alleviating abiotic stress in plants.

1.13.2 Ascorbate peroxidase

APX performs one of the biggest roles in ROS detoxification. Scavenging of H_2O_2 in chloroplasts and the cytosol of the plant is made possible by APX (Gill and Tuteja, 2010). The family of APX consists of four different isoforms, namely glyoxysome membrane (gmAPX), thylakoid (tAPX), cytosolic (cAPX), and chloroplast stromal soluble (sAPX) forms (Noctor and Foyer, 1998). The enzyme has a greater affinity (µM range) for H_2O_2 than catalase (CAT) and peroxidase (mM range) (Smirnoff, 2005). The detoxification system for H_2O_2 under abiotic stress in plants is part of the ascorbate-glutathione cycle in which the isoenzymes APX make use of ascorbate as a specific electron donor to break up the H_2O_2 into H_2O (Sofo *et al.*, 2015) as shown in Figure 1.10.

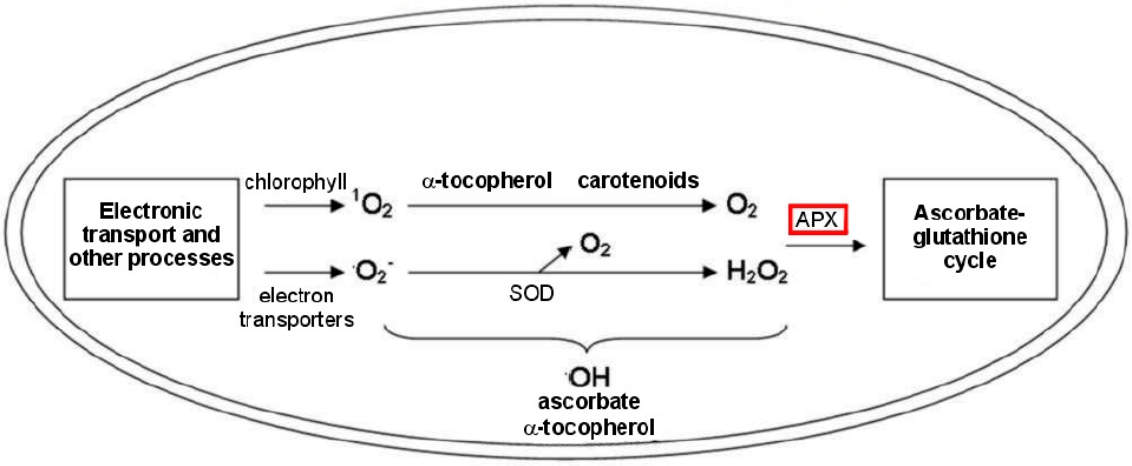

Figure 1.10: Plant chloroplast ROS production and breakdown
ROS generation and scavenging in chloroplasts exposed to abiotic stress, exhibiting the main role of APX in scavenging of ROS (Sofo *et al.*, 2015).

Altered growth and physiology occur in plants which have mutated *APX* genes, including modification in antioxidant metabolism, confirming the role of the enzyme in regulating plant developmental processes (Ashraf, 2009). APX gene expression is regulated in response to abiotic stress and involved in preserving the homeostasis of ascorbate (ASA) and glutathione (GSH) (Sofo *et al*., 2015). ASA and GSH are non-enzymatic antioxidants involved in redox homeostasis and associated with sustaining photosynthesis under unfavourable environmental conditions (Sofo *et al*., 2015).

Tobacco plants overexpressing chloroplastic APX show increased tolerance to salt and water stress (Ashraf, 2009). Kausar *et al,* (2012) also noticed an increase in the expression of APX in soybeans exposed to drought. Cd and salt stress also elevate APX activity in *Ceratophyllum demersum* (Arvind and Prasad, 2003) and *Raphanus staivus* (Lopez *et al*., 1996).

1.13.3 Catalase

Catalase (CAT) is a tetrameric heme antioxidant enzyme which has the capability of dissociating H_2O_2 to produce H_2O and O_2. This antioxidant enzyme has a maximum turnover estimate of 6 million molecules of H_2O_2 converted to H_2O and O_2 per minute by one molecule of CAT (Lee *et al*., 2007). The enzyme is important in ROS detoxification under stress conditions (Mittler, 2002). It works by dissociating H_2O_2 formed in the peroxisomes by oxidases responsible for β-oxidation of fatty acids or produced as a result of photorespiration and purine catabolism (Varindra and Sekhon, 2006).

CAT possesses three isoforms which are iron porphyrin enzymes whose regulation and expression vary (Mittler, 2002). Peroxisomes and the cytosol are the sites for localization of *CAT1* and *CAT2* respectively, while *CAT3* is localized in the mitochondria (Scandalios, 1990). Environmental stresses such as cold, salinity, heat shock and water deficit can either reduce or induce CAT turnover rate and this is important in plant adaptation to oxidative stress (Mittler, 2002). Some authors have concentrated on the role of CAT during drought and salt stress, due to the important role of CAT in scavenging H_2O_2 during photorespiration. Sustaining CAT activity in drought-stressed leaves possibly allows photorespiratory H_2O_2 formed under water deficit to be eliminated. CAT activity under harsh stress conditions aids in delaying oxidative stress (Bauwe *et al.*, 2012). Photorespiration works as an energy sink in stress conditions to prevent photoinhibition of the electron transport chain (De Pinto *et al.*, 2013). With this, the role of CAT in preventing ROS overproduction during photorespiration is vital (Bauwe *et al.*, 2012; Voss *et al.*, 2013).

In contrast to APX and SOD for which studies have shown an increase in their activity during stress, some studies have shown a decrease in CAT activity under certain stress conditions (Radotić *et al.*, 2000). The reduction in CAT activity would lead to overproduction of ROS. In a study of transgenic tobacco with a repressed level of CAT activity under abiotic stress, an increase in ROS levels was noticed (Willekens *et al.*, 1997). In another study, it was shown that, when the activity of CAT is repressed under abiotic stress in plants, APX and GPX activity were upregulated (Apel and Hirt, 2004). Hence, a balanced regulation of SOD, CAT and APX activity is vital in alleviating ROS-induced oxidative stress. Inability to induce the activity of these enzymes would result in susceptibility of plants to oxidative stress (Rizhsky *et al*., 2002).

1.14 Osmolyte involvement in abiotic stress

1.14.1 Proline

The amino acid proline accumulates in higher plants under stress (Hsu *et al*., 2003). Aside from involvement in osmotic adjustment, proline helps to stabilize the sub-cellular structure and scavenge free radicals (Kavi Kishor *et al*., 2005). Accelerated disintegration of proline upon stress relief can contribute to adequate reducing agents that guide mitochondrial oxidative phosphorylation and ATP production for recovery from stress and stress-induced deterioration (Ashraf and Foolad, 2007). Biosynthesis of proline in plants is made possible by two enzymes, pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) (Delauney and Verma, 1993), as exhibited in Figure 1.11.

Under drought or salinity stress in plants, the build-up of proline usually takes place in the cytosol where it accumulates as part of cytoplasmic osmotic adjustment (*Ketchum et al*., 1991). As noticed in *Distichlis spicata* cells treated with 200 mM NaCl, the cytosolic concentration of proline increased to more than 230 mM (Voetberg and Sharp, 1991). A similar result was noticed in maize roots with reduced water potential (Ober and Sharp, 1994).

Proline accumulation in various plants has been shown to correspond to stress tolerance, where proline concentration is generally greater in stress-tolerant plants than in stress-sensitive plants. Salt tolerant alfalfa, for example, showed double the accumulation of proline in the root under salt stress than salt-sensitive alfalfa (Petrusa and Winicov, 1997). However, this theory may not be global as studies by Lutts *et al*, (1990) and de-Lacerda *et al*, (2003) on rice and sorghum showed that proline accumulation in salt-stressed leaves was as a result of salt injury symptom rather than an indication of salt tolerance. Nonetheless, more studies are required to determine

if the accumulation of proline concerning stress tolerance is species-specific or depends on experimental conditions.

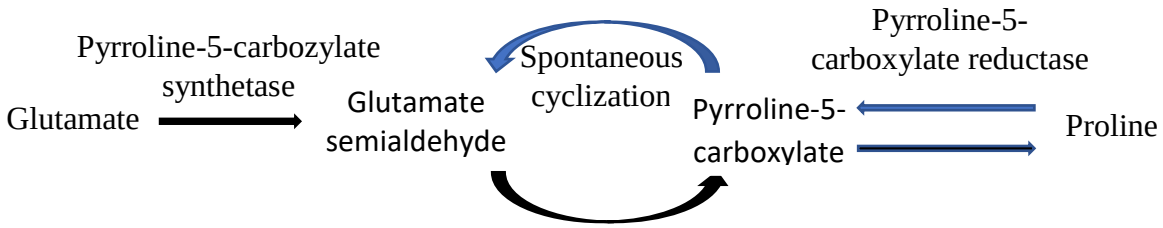

Figure 1.11: Proline biosynthesis in higher plants
Synthesis of proline showing key enzyme P5CS in the synthesis of proline, and P5SCR that breaks down the proline accumulated following relief from stress conditions.

Zhu (2002) reported that ABA-dependent and ABA-independent signalling pathways mediate the accumulation of proline. Under environmental stress, ABA is generally known to mediate signals which lead to the expression of stress-related genes. This following synthesis of compatible osmolytes like proline (Kavi Kishor *et al*., 2005). Besides, the accumulation of ABA in plant cells under osmotic stress has been established to regulate P5CS gene expression (Xiong *et al.*, 2001). However, it has been shown that ABA only is not sufficient for P5CS transcript induction. Calcium involvement in ABA-induced P5C5 gene expression during stress response has been shown (Knight *et al*., 1997). Thiery *et al*, (2004) illustrated the role of a signalling protein, phospholipase D, together with calcium and ABA in the regulation of proline biosynthesis. There appears to be a relationship between MAP kinases and proline biosynthesis, although this is yet to be deciphered in terms of signalling components that regulate the process (Kavi Kishor *et al*., 2005).

In genetic engineering of crops for osmolyte (e.g. proline) over-production, the level of substrate availability should be considered. An alternative way to such genetic engineering is the exogenous application of the osmolyte proline to plants, which has been shown to alleviate oxidative damage in the cell and enhance tolerance (Ashraf and Foolad, 2000). It will be worthy to note how changes in osmolyte concentration under drought stress relate in terms of crop tolerance or sensitivity in different cultivars.

1.15 Rationale and justification

Various cultivars of soybean have a differing adaptation to specific geographical areas. Therefore, the best-adapted cultivar will be suitable in the long run to give the best yield and quality for a specific locality within a geographical area. Recent statistics from the Department of Agriculture, Forestry and Fisheries, (DAFF) in South Africa shows that the Eastern Cape Province has one of the lowest production of soybean. There may be unavailability of resources, constraints related to soil quality, unsuitable climate or adaption of existing cultivars in this province. Therefore, the need arises to evaluate and choose cultivars with high yield in parts of the province. In addition to this, soybean cultivars need to be assessed to determine which cultivar has better tolerance to water deficit, as drought frequently occurs not only in the Eastern Cape but in many parts of the country. Results from such studies can serve as a template basis for developing soybean varieties that are better adapted to drought stress.

2 Chapter Two

Materials and methods

Table 2-1: List of chemicals and their suppliers

CHEMICALS	SUPPLIERS
2-Thiobarbituric acid	Sigma-Aldrich
40 % Acrylamide/Bis-acrylamide solution	BIO-RAD
5- sulfosalicylic acid dehydrate	Sigma-Aldrich
Acetone	Sigma-Aldrich
APS	Sigma-Aldrich
Bradford reagent	Bio-RAD
BSA	Sigma-Aldrich
EDTA	Sigma-Aldrich
Ethanol	Sigma-Aldrich
Evans Blue	Sigma-Aldrich
Ferric chloride	Sigma-Aldrich
Glacial acetic acid	Sigma-Aldrich
Glycine	Sigma-Aldrich
Hydrogen peroxide	Sigma-Aldrich
K_2HPO_4	Sigma-Aldrich
KH_2PO_4	Sigma-Aldrich
L-Ascorbic acid	Sigma-Aldrich
L-Glutathione reduced	Sigma-Aldrich

L-Proline	Sigma-Aldrich
NBT	Sigma-Aldrich
Ninhydrin	Sigma-Aldrich
Polyacrylamide 40%	Sigma-Aldrich
Potassium cyanide	Sigma-Aldrich
Potassium ferricyanide	Sigma-Aldrich
Potassium hydroxide	Sigma-Aldrich
Potassium iodide	Sigma-Aldrich
Promix	Windel Hydroponics (South Africa)
PVPP	Sigma-Aldrich
Riboflavin	Sigma-Aldrich
SDS	BIO-RAD
Sodium hydroxide	Sigma-Aldrich
Soybean seeds	ARC (Potchefstroom, South Africa)
TCA	Sigma-Aldrich
TEMED	BIO-RAD
Tris base	Sigma-Aldrich

2.1 Plant growth in the field for agronomic study

An agronomy study was conducted on soybean (*Glycine max*) cultivars LS 6164R and DM 5953 RSF on a 1-hectare field (Mqhekezweni great place, Mthatha, Eastern Cape, GPS coordinates 31°48'37.3"S 28°33'13.0"E) for each cultivar. To generate replicate, each hectare was divided into 0.3-hectare portion. For each cultivar, planting was done one week apart for each of the 0.3-hectare portions. This gave 3 blocks of 0.3 hectares for each cultivar where

each block is a single experiment so that each experiment is 1 week apart from the others. Therefore, 3 blocks were planted with LS 6164R and DM 5953 RSF in a randomized manner. The seeds were rinsed thoroughly with water following which they were placed between paper towels with adequate water for germination. After 72 hours the seeds with emerged radicles were sowed by hand in the field at a spacing of 15 cm between seeds and 45 cm between rows. After 60 days of growth, yield component parameters such as shoot length (excluding pods), number of soybean pods and branches, and average weight per pod were recorded from thirty randomly selected plants from each cultivar. The length of the shoot was measured from the plants using a measuring tape. The total number of branches per plant, number of pods per plant and number of pods per branch were counted and recorded. The pods from each plant were harvested, weighed and recorded. The average weight of the pods was estimated by dividing the total weight of pods in each plant by the numbers of pods in the plant after which the shoot weight (excluding the pods) was measured and recorded.

Another randomly selected set of thirty plants from each cultivar were harvested 72 days after planting (Appendix Fig 3.16B) and the yield of each cultivar was calculated and recorded in tonnes of seeds per hectare.

2.2 Plant growth for water deficit study

2.2.1 Seed sterilization

For the drought response study, soybean seeds from the harvest in section 2.1 were sterilized by placing the seeds of both cultivars in 15 × 100 mm Petri plates. A beaker containing 100 ml bleach (0.35% (v/v) sodium hypochlorite) were placed in the desiccator together with the open petriplate containing the seeds. Concentrated hydrochloric acid (4 ml) was added dropwise along the side of the bleach-containing beaker. The desiccator was closed and allowed to stand for 16 hours.

2.2.2 Seed germination, planting and treatment

Germination was done for 3 days with the sterilized seeds placed in a moist paper towel and kept in the dark. Seeds were allowed to germinate and seedlings to grow until the radicle was approximately 0.5 cm in length. The germinated seeds were transplanted into vermiculite (Windell Hydroponics) in a plastic container and allowed to grow for 3 days with adequate nutrient solution [1 mM K_2SO_4, 2 mM $MgSO_4$, 3 mM $CaCl_2$, 1 mM K_2HPO_4 buffer at pH 7.3, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 2 µM $CuSO_4$, 2 µM Na_2MoO_4, 0.1 µM $CoSO_4$, 50

μM Fe-EDTA and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.3] supplied to enable selection of good looking seedlings before transferring to pots.

At the VC stage, the seedlings were transferred into 20 cm diameter plastic pots (one plant per pot) containing 6 L of Promix Organic (Windell Hydroponics) adjusted with water to be either -0.02 MPa or -0.4 MPa. Promix is a combination of perlite, coco peat and other components with the ability to maximise root aeration and water retention better than soil or other growth media. To prepare the Promix, the nutrient solution was added until the Promix was thoroughly moist, then it was oven-dried at 80˚C in a single layer (approximately 1 cm) until it was completely dry (at least 72 hours). Water was added to eight pots of Promix for control plants (Well-Watered) to make the water potential of the Promix -0.02 MPa, while Promix for experimental plants (eight pots for Water-Deprived) was adjusted with water to be at a water potential of -0.4 MPa. The well-water plants received 200 ml of water once a week while the water-deprived plants received no water. The plants were grown at a temperature of 25˚C during the day and 19˚C at night under a 16/8 hours light/dark cycle at a photosynthetic photon flux density of 400 $\mu mol.m^{-2}.s^{-1}$.

2.2.3 Harvesting and growth parameters measurements

The well-watered (WW) plants were harvested 15 days after planting at stage V4, while the water-deprived (WD) plants were harvested on the 25 days after planting when no further growth was noticed and the trifoliate leaves had begun rolling. Soybean plants were photographed after harvesting. The shoot weight, shoot length and root length were measured and recorded. Only the trifoliate leaves and with the roots were harvested and frozen immediately in liquid nitrogen and stored at -80˚C for subsequent analysis, while the 2nd youngest trifoliate leaves were used for measuring the relative water content, cell viability and superoxide content immediately upon harvesting without freezing.

2.3 Measurement of relative water content (RWC)

The 2nd youngest leaves were weighed at harvest to determine the fresh weight (FW). The leaves were immersed in distilled water for 24 hours at room temperature in a dark cupboard. Turgid weights (TW) of the leaves were measured and recorded before measuring dry weight (DW) after incubation at 72˚C for 48 hours. The RWC was estimated by a method adapted from Barrs and Weatherly (1962), which relates FW, TW and DW in which RWC (%) = [(FW – DW) / (TW – DW)] X 100.

2.4 Measurement of cell viability

A modified method based on Sanevas *et al*, (2007) was used to quantify the cell viability using the 2nd youngest trifoliate leaves. The leaves were stained with 0.25% (w/v) Evans Blue dye for 30 minutes at room temperature. The leaves were washed thrice with distilled water for 20 minutes to remove the unbound dye, followed by incubation in a water bath for 1 hour at 55˚C. SDS (1% w/v) was used to extract the Evans Blue stain taken up by the dead cells in the leaves. Absorbance was measured at 600 nm to quantify the level of Evans Blue uptake by the leaf tissue.

2.5 Measurement of superoxide (O_2^-)

The superoxide content was estimated using a modified method from Russo *et al*, (2008). The fresh 2nd youngest trifoliate leaves were separately submerged in separate solution containing 10 mM KCN (to inhibit Cu/Zn SOD), 10 mM H_2O_2 (to inhibit Mn and Cu/Zn SOD), 10% (m/v) SDS (to inhibit Mn and Fe SODs); which contained 80 mM NBT and 50 mM potassium phosphate (pH 7.0). The leaves were incubated in the various solutions at room temperature for 20 minutes before homogenizing. The homogenized solution was centrifuged at 10 000 × *g* for 10 minutes before collecting the supernatant and absorbance was subsequently measured at 600 nm. The concentration of the superoxide was estimated using the extinction coefficient of NBT at 12.8 mM cm^{-1}.

2.6 Measurement of chlorophyll content

Chlorophyll *a* and *b* were determined using a method modified from Oancea *et al*, (2005). The assay was quantified on the frozen ground plant leaf (100 mg). The leaf material was added to a 1.5 ml Eppendorf tube wrapped with foil to block break down of chlorophyll species. The sample tube was filled with ten volumes of plant material (1000 µl) of 100% (v/v) acetone and vortexed briefly. Samples were loaded into a glass microtitre plate in triplicate and read spectrophotometrically at an absorbance of 662 nm and 644 nm respectively. The optical reading was used to estimate the concentration of the different chlorophyll.

2.7 Metabolite extraction (Trichloroacetic acid extraction)

A method described by Khan and Panda (2008) was used to extract metabolites. The soybean leaf and roots samples were ground into fine powder in liquid nitrogen. To isolate the extracts,

100 mg of the frozen samples were homogenized in 500 µl of cold 6% trichloroacetic acid (TCA). This was followed by centrifugation of the homogenates at 13 000 x *g* for 30 minutes at 4˚C. Hydrogen peroxide and malondialdehyde assays were done using the resulting supernatant.

2.8 Measurement of hydrogen peroxide (H_2O_2)

The supernatant from the TCA extract (50 µl) in section 2.7 was used to determine the hydrogen peroxide content using a method modified from Velikova *et al*, (2000). The extract was added to 50 µl of 100 mM potassium-phosphate buffer (pH 5.0) and 100 µl of 0.5M potassium iodide to make a final volume of 200 µl. The H_2O_2 content of the samples was determined using a standard curve established from the absorbance of H_2O_2 standards at 390 nm after incubating the reaction at room temperature (25˚C) for 20 minutes.

2.9 Measurement of malondialdehyde (MDA) content

The TCA extracted supernatant in section 2.7 was used to estimate the level of lipid peroxidation based on the estimation of the MDA content as described by Zhang *et al*, (2007). An aliquot of 200 µl was mixed with 400 µl of 0.5% (w/v) thiobarbituric acid solution (prepared in 20% (w/v) trichloroacetic acid) in 1.5 ml centrifuge tubes. The resulting mixture was incubated at 90 ˚C for 30 minutes and the reaction was stopped by placing the tubes on ice for 10 minutes. The mixture was centrifuged at 13 000 x *g* for 10 minutes at 4˚C and the absorbance was measured at 532 nm and 600 nm. The MDA concentration of the extract was estimated by its molar extinction coefficient (155 Mm^{-1} cm^{-1}) after subtraction of the non-specific absorbance of 600 nm.

2.10 Determination of free proline content

Proline was quantified using a modified colourimetric method based on acid-ninhydrin according to Bates *et al*, (1973). Frozen plant tissue (500 mg) was homogenized in 10 ml of 3% aqueous sulfosalicylic acid. The supernatants (2 ml each) were collected from the homogenate after centrifugation at 10 000 x *g* for 5 minutes. Acid-ninhydrin was freshly prepared by warming 1.25 g of ninhydrin in 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid with gentle agitation until it was dissolved. The supernatant from the earlier homogenate was reacted with 2 ml of prepared acid-ninhydrin and 2 ml of glacial acetic acid

in a 15 ml tube at 95˚C for 60 minutes. The mixture was cooled on ice before extracting the mixture with 4 ml of toluene vigorously for 20 seconds. The chromophore that contains toluene was aspirated from the aqueous phase and absorbance was measured at 520 nm. The concentration of proline was estimated using a standard curve of L-proline and calculations were made based on the fresh weight as described by Bates *et al*, (1973).

2.11 Protein quantification for enzymatic antioxidant assays

Protein was extracted from the soybean leaves and roots by grinding the tissue in liquid nitrogen before homogenizing 200 mg of the ground tissue in 400 µl homogenizing buffer [40 mM K_2PO_4, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), and 5% (w/v) polyvinylpolypyrrolidone (PVPP) molecular weight = 40 000]. Bradford assay method (Bradford, 1976) with bovine serum albumin (BSA) as standard was used to quantify the homogenate supernatant after centrifuging at 10 000 x *g* for 30 minutes at 4˚C. This supernatant forms the basis for subsequent enzymatic activity assays.

2.12 Quantification of ascorbate peroxidase (APX) antioxidant activity

2.12.1 APX native PAGE in-gel activity

The activity of various APX isoforms was determined as described by Rao *et al*, (1996) using native polyacrylamide gel electrophoresis (PAGE). The gel, made of 5% (v/v) stacking and 12% (v/v) separating gels, was equilibrated with a running buffer containing 2 mM ascorbic acid, 192 mM glycine and 24 mM Tris base. Extracts containing 100 µg of protein was mixed with loading dye and loaded onto the native gel. The gel was electrophoresed at 80 volts at 4˚C until the dye had reached the bottom edge of the gel. The gel was removed from the chamber and incubated in a solution containing 50 mM potassium phosphate buffer (KPO_4, pH 7.0), and 2 mM ascorbic acid for 10 minutes in the dark, following which it was incubated in a solution containing KPO_4 buffer (50 mM, pH 7.8), 2 mM H_2O_2 and 4 mM ascorbic acid on a shaker placed in the dark for 10 minutes. The solution was discarded and the KPO_4 buffer (50 mM, pH 7.8) was used to wash the gel in the darkness for 1 minute following which it was stained with a solution containing KPO_4 (50 mM, pH 7.8), 28 mM TEMED (N,N,N′,N′-Tetramethylethylenediamine) and 0.5 mM NBT (Nitro blue tetrazolium chloride) in the dark for 15 minutes. The gel was exposed to light, with gentle agitation, and the activity of the isoforms was documented by photographing the gel after washing with distilled water.

2.12.2 APX spectrophotometry analysis

The total APX activity was measured spectrophotometrically using the extracted quantified protein. The analysis was done using a modified method based on Nakano and Asada (1981). The reaction was initiated by mixing 10 µl of the protein extract with K_2HPO_4 (50 mM, pH 7.0), 0.1 mM EDTA, 0.36 mM ascorbate and 0.72 mM H_2O_2 in a 200 µl reaction. The extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to estimate the activity of APX by following the change in absorbance at 290 nm.

2.13 Quantification of catalase (CAT) antioxidant activity

2.13.1 CAT native PAGE in-gel activity

A native PAGE gel made up of 5% (v/v) stacking and 7.5% (v/v) separating gels were equilibrated with a running buffer containing 192 mM glycine and 24 mM Tris base (pH 7). Protein extracts (100 µg) were loaded onto the gel together with loading dye. The gel was electrophoresed at 60 Volts for 8 hours at 4˚C. The gel was removed and rinsed thrice with distilled water for 10 minutes before incubation in 0.003% hydrogen peroxide solution (v/v) for 30 min. Two solutions of 2% ferric chloride (w/v) and 2% potassium ferricyanide (w/v) were prepared independently in the dark to minimize light exposure. The gel was stained simultaneously with the two solutions and agitated in the light as described by Yamashita *et al*. (2007). Once the gel darkened and chromatic bands began to show, the stain was poured out and the gel was rinsed extensively with distilled water, followed by photographing of the gel.

2.13.2 CAT spectrophotometry analysis

The total CAT activity was quantified using a method illustrated by Aebi (1984). A reaction mixture (1 ml) was prepared to contain 100 mM KPO_4 (pH 7), 0.5 mM EDTA, 1 mM H_2O_2 and 20 µg protein. The CAT activity was determined using its extinction coefficient of 39.4 $mM.cm^{-1}$ after the reaction absorbance was read at 240 nm.

2.14 Quantification of superoxide dismutase (SOD) antioxidant activity

2.14.1 SOD native PAGE in-gel activity

SOD isoform activity was determined using a method modified from Beauchamp and Fridovich, (1971). Native gels made up of 12% separating and 5% stacking gel phases were prepared and equilibrated with running buffer containing 192 mM glycine and 24 mM Tris

base (pH 7). Protein extracts of 100 µg each were loaded onto the gel after mixing with the loading dye. The gel was electrophoresed at 70 volts at 4˚C until the loading dye reached the bottom of the gel. The gel was removed from the chamber and washed with 50 mM KPO_4 (pH 7.8) on a shaker for 15 minutes before it was subsequently incubated in a solution containing 50 mM KPO_4 (pH 7.8) and 0.5 mM NBT in the dark for 15 minutes. The SOD isoform patterns were stained as explained by Beauchamp and Fridovich, (1971) through the incubation of one gel in 5 mM H_2O_2 (to inhibit both Cu/ZnSOD and FeSOD), and another in 5 mM KCN (to inhibit only Cu/ZnSOD) as MnSOD is resistance to both KCN and H_2O_2.

2.14.2 SOD spectrophotometry analysis

The enzymatic activity of SOD was quantified spectrophotometrically using a method described by Beauchamp and Fridovich, (1971). Quantified protein extract of 10 µl was loaded into a microtiter plate after dilution to 1 $mg.ml^{-1}$. The plate contained a solution of 20 mM KPO_4 (pH 7.5), 0.1 mM nitrotrezolium blue chloride (NBT), 0,005 mM riboflavin, 10 mM methionine and 0.1 mM EDTA, and made to a final volume of 200 µl using distilled water. The mixture was incubated on a lightbox at room temperature for 20 minutes following which the absorbance was measured and recorded at 560 nm. The activity of SOD was estimated based on the enzyme required to cause a 50% reduction in the decrease of NBT to blue formazan.

2.15 Densitometry analysis

The AplhaEase FC imaging software (Alpha Innotech Corporation) was used to analyse the densitometry of the in-gel (native PAGE) results for APX, CAT and SOD, according to instructions in the manual from the manufacturers. The relative enzymatic activity (arbitrary values) was recorded from the individual gel for each isoform in each sample. The respective isoforms of each treatment were scored as an average of the relative pixel intensities of three independent gel bands. It was expressed in arbitrary units via assigning the value of 1 for the control isoform (WW) and other experimental isoforms (WD) relative to their control isoforms.

2.16 Statistical analysis

For the agronomy study, 30 plants were used for the yield component measurement and another 30 plants for estimating the yield in tonne/hectares. The block of three 0.3 hectares for each cultivar gave replicates to the experiment. The water deficit experiment was performed three

times independently, with 8 plants used for each experiment for each treatment (WW or WD). Statistical analysis for both agronomic and drought study was done using one-way analysis of variance (ANOVA) and was tested for significance using the GraphPad Prism 6.01 software via the application of the Tukey-Kramer test at 5% level of significance.

3 Chapter Three

Results

3.1 Evaluation of agronomic traits of field-cultivated *Glycine max*

3.1.1 Morphological dissimilarities between the two soybean cultivars

The soybean seeds of the cultivar LS 6164R and DM 5953 RSF were cultivated to determine if they differ in their growth characteristics when cultivated in the designated experimental site. Measurement of shoot length showed that LS 6164R achieves a shoot length of approximately 60 cm while the DM 5853 RSF counterpart achieves an average shoot length of 90 cm (Fig 3.1A). This relates to over 50% increase in the length of DM 5853 RSF over the LS 6164R.

3.1.2 Assessment of the yield component between the two soybean cultivars

Yield components of soybean such as number of branches, number of pods, pods per branch, weight per pod, and the shoot weight (excluding pods) of the plants were assessed. The average number of pods per plant for the LS 6164R and DM 5853 RSF were 263 and 305 respectively (Fig 3.1C). There was an average of 7 branches in total in the LS 6164R cultivar, while DM 5853 RSF had approximately 16 branches in total (Fig 3.1B). However, there were approximately 38 pods per branch in LS 6164R and 18 pods on average in the DM 5853 RSF cultivar (Fig 3.3D). The average weight of the LS 6164R pod was 1.34g compared to 0.93g of the DM 5853 RSF. The shoot weight (excluding pods) showed that the LS 6164R achieved an average weight of 84g while DM 5853 RSF weighed approximately 88g (Fig 3.1F).

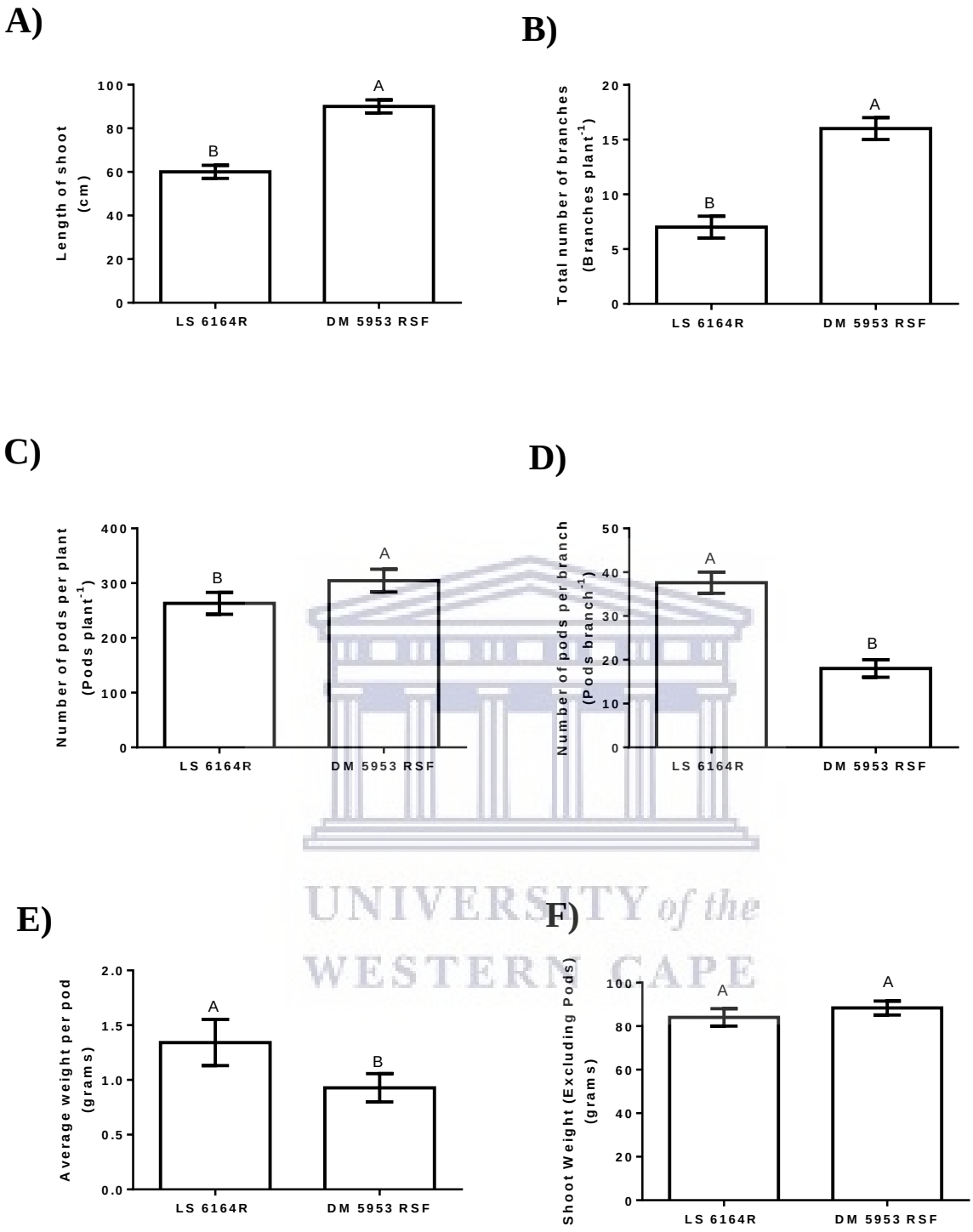

Figure 3.1: Graphical illustration of the yield components of the soybean cultivars after harvest

Assessment of the yield components for the two cultivars grown in the field. The figure shows the (A) shoot length, (B) the total number of branches, (C) numbers of pods per plant, (D) numbers of pods per branches, (E) average weight per pod and (F) shoot weight of the plant. Data represent the mean ± standard error of three independent experiments, were bars with the same letters signifying statistically similar values at $P < 0.05$.

3.1.3 Assessing the yield of soybean

Plant's yield may be estimated in kilograms/hectare, tonnes/hectare or metric tons/hectare. The yield of each cultivar in this study was estimated in tonnes/hectare and the result is illustrated in Figure 3.2. LS 6164R achieved a yield of approximately 3 tonnes/hectare, implying a two-fold double increase in productivity over the DM 5953 RSF which produced 1.5 tonnes/hectare.

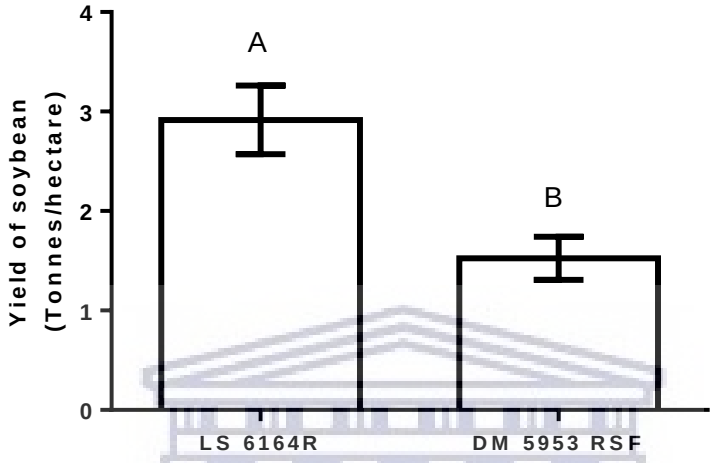

Figure 3.2: Graphical illustration of the yield of the soybean cultivars.
The yield of the soybeans assessed at harvest. Bars represent the mean ± standard error of three independent experiments with bars of same letters representing statistically similar values where $P < 0.05$.

3.2 Physiological and morphological response of *Glycine max* to water-deficit stress

3.2.1 Water deficit stress results in reduced growth in the two soybean cultivars

A number of indicators have been used to assess how plants adapt to varying environmental conditions. A few of these indicators include analysing plant morphology, biomass, leaf area and height (Acosta-Motos *et al.*, 2017). The morphological differences between the two soybean cultivars under well-watered and water-deprived conditions were examined after harvest. The plants were harvested from their respective pots and photographed as shown in Figure 3.3. Stunted growth was observed under water-deprived conditions for both cultivars, with water deprivation resulting in LS 6164R achieving growth to early V4 (LS 6164R was starting early V5 under well-watered conditions) whereas water deprivation resulted in DM 5953 RSF reaching the early V3 stage (DM 5953 RSF was starting early V5 under well-watered conditions) (Figure 3.3).

Figure 3.3: Comparison based on the morphology of LS 6164R and DM 5953 RSF soybean cultivars under well watered (WW) and water deficit (WD) conditions.
Representative image of (A) LS 6164R and (B) DM 5953 RSF.

The length of the shoots and the roots of the plants were measured and recorded in three independent experiments (Figure 3.4). A 53% reduction in shoot length in the LS 6164R was observed in response to water deprivation, whereas a 38% reduction was observed in the water-deprived DM 5953 RSF cultivar, in relation to their well-watered state (Figure 3.4A). There was no significant difference in the length of the root in both cultivars as shown in Figure 3.4B. The shoot weight shows approximately 75% reduction in both the water-deprived cultivars compared to their control (Fig 3.4C).

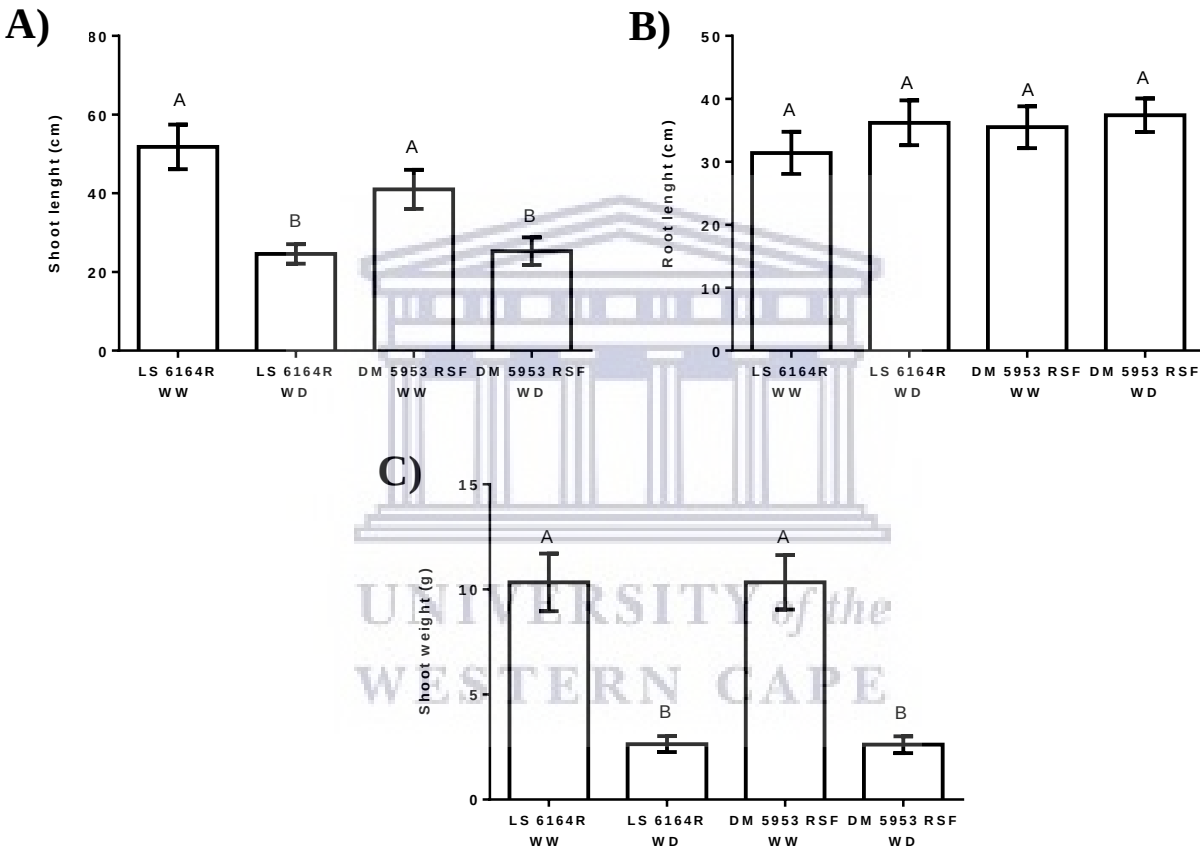

Figure 3.4: The effect of water deficit stress on soybean growth parameters and biomass. (A) Shoot length, (B) root length and (C) shoot weight of the two soybean cultivars under well-watered and water-deprived condition. Bars represent the mean ± standard error of three independent experiments, where bars with the same letters indicate statistically similar values at $P < 0.05$.

3.2.2 Water deficit stress disrupts the growth of soybean trifoliate leaves

The morphology of the youngest fully expanded and 2nd youngest trifoliate leaves shows that the LS 6164R cultivar rolled less and was larger under water deficit than the DM 5953 RSF cultivar (Fig 3.5).

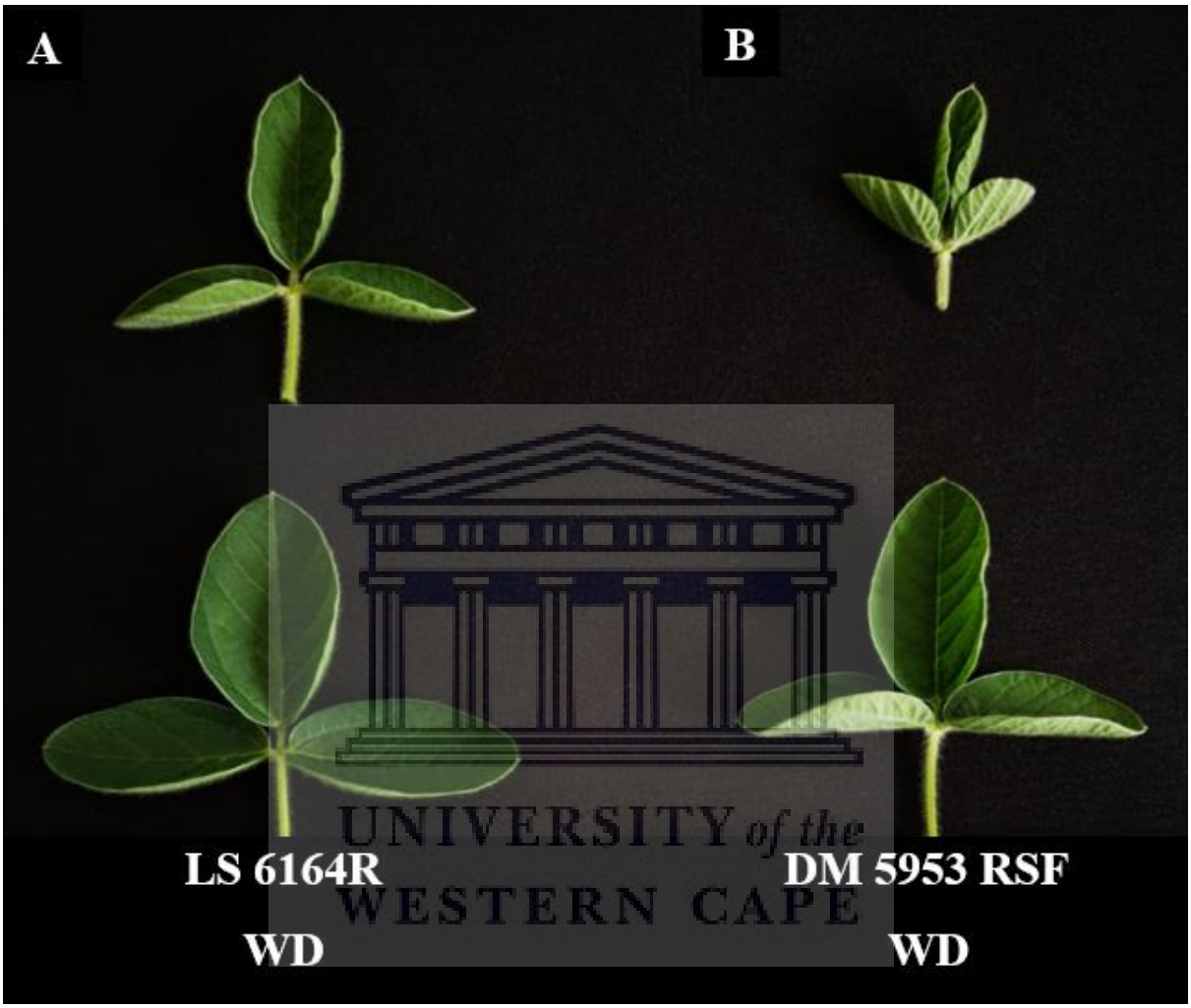

Figure 3.5: The effect of water deficit stress on the morphology of soybean trifoliate leaves
Digital imaging of the youngest fully expanded trifoliate (top) and 2nd youngest trifoliate leaves (bottom) of the (A) LS 6164R (left) and (B) DM 5953 RSF (right) water-deprived soybean cultivar.

3.3 Physiological and biochemical response of *Glycine max* to water deficit stress

3.3.1 Quantification of stress-induced changes via measuring the relative water content and cell viability of soybean leaves

Relative water content (RWC) is one of the most widely used physiological methods to determine plant water status. Water deficit stress reduced the RWC of both soybean cultivars, as shown in Figure 3.6A. Both LS 6164R and DM 5953 RSF had a RWC of approximately

93% under well-watered conditions, but their water-deprived counterparts only had a RWC of approximately 74% and 68% respectively. This equates to an approximately 20% and 27% reduction in RWC in the LS 6164R and DM 5953 RSF, respectively in relation to their well-watered controls. Although there was no statistically significant difference in the RWC of the two cultivars, the figure suggests that LS 6164R fares better than DM 5953 RSF in terms of its water status under low water availability.

Measurement of cell viability is often used as a physiological indicator for quantifying damage to plant tissues. Evans Blue can only infiltrate and be retained by dead cells and its uptake by cells is thus a good indicator of loss of cell viability. This cell viability assay was conducted on both soybean cultivars (Figure 3.6B). Water-deprived LS 6164R showed an approximately 54% increase in Evans Blue uptake compared to its well-watered control, whereas water-deprived DM 5953 RSF showed an increase of 73% in Evans Blue uptake when compared to its control. This indicates a significant difference in cell death in response to water deficit between the two cultivars.

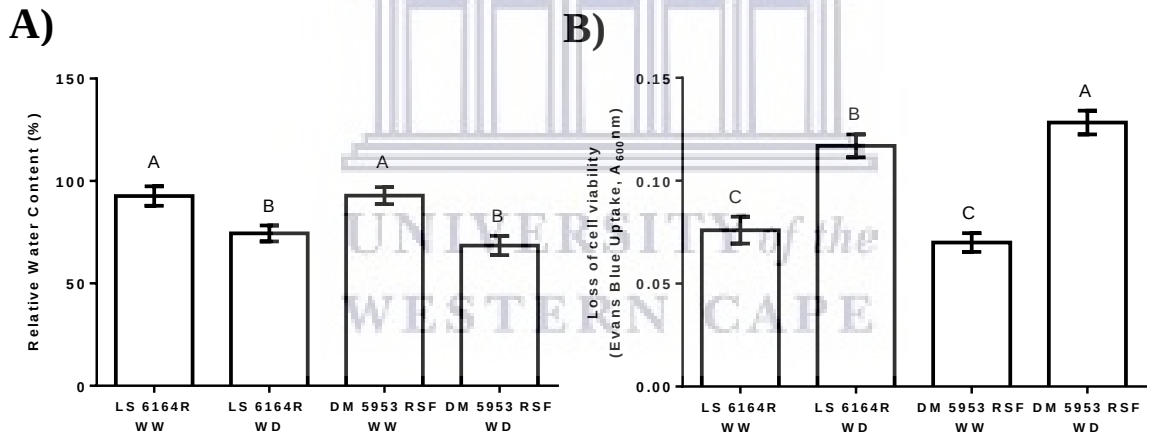

Figure 3.6: Relative water content and loss of cell viability of the soybean cultivars under well-watered and water-deprived conditions

Graphical representation of the (A) RWC and (B) loss of cell viability using the 2nd youngest leaves under adequate water supply and water deprivation. Bars represent the mean ± standard error of three independent experiments. Bars with the same letters signify statistically similar values, where $P < 0.05$.

3.3.2 Water deficit stress reduces chlorophyll *a* and *b* content in soybean plants

Chlorophyll assay can be used as a potential indicator to quantify the sensitivity of plants to environmental stress such as drought. Due to this possibility, this study measured the chlorophyll content of the cultivars and the results were tabulated in Table 3.1.

Results showed that there were no significant differences in both cultivars in terms of their normal regulation of total chlorophyll under unstressed conditions (well-watered). However, the cultivars respond differently to water deprivation in terms of chlorophyll content when compared to their response under well-watered conditions. There were significant reductions in both chlorophylls *a* and *b* between the water-deprived plants and the well-watered controls. In reference to this, water-deprived LS 6164R showed a 33% reduction in the chlorophyll *a* content in relation to its well-watered control, whereas water-deprived DM 5953 RSF showed a 40% reduction in chlorophyll *a* content. A similar repression pattern was also noticed in the chlorophyll *b* content, where the LS 6164R and DM 5953 RSF plants under water deficit showed a 21% and 24% reduction in chlorophyll *b* content, respectively, compared to their well-watered controls. Since the total chlorophyll content is expressed from the addition of chlorophyll *a* and *b*, it is no surprise that total chlorophyll (*a* + *b*) was also reduced in the water-deprived plants, with 25% and 30% reduction observed in the LS 6164R and DM 5953 RSF respectively (Table 3.1). These reductions were statistically different between WW and WD but not statistically different between cultivars.

Table 3-1: Consequence of water deprivation on the chlorophyll content of the two soybean cultivars

Soybean cultivar + Treatment	Chlorophyll *a* ($\mu g.g^{-1}$)	Chlorophyll *b* ($\mu g.g^{-1}$)	Total chlorophyll (*a* + *b*) ($\mu g.g^{-1}$)
LS 6164R WW	0.279 ± 0.005^{a}	0.584 ± 0.006^{a}	0.864 ± 0.007^{a}
LS 6164R WD	0.188 ± 0.015^{b}	0.459 ± 0.052^{b}	0.646 ± 0.021^{b}
DM 5953 RSF WW	0.272 ± 0.002^{a}	0.570 ± 0.008^{a}	0.842 ± 0.018^{a}
DM 5953 RSF WD	0.164 ± 0.0010^{b}	0.433 ± 0.005^{b}	0.597 ± 0.010^{b}

Different letters indicate significant differences between means at $P<0.05$. Values represent the mean ± standard error of three independent experiments (n=3). WW and WD represent well-watered and water-deprived, respectively.

3.3.3 Water deficit stress influences the accumulation of ROS in the soybean plant

This study showed that there was a 138% and 82% increase superoxide content in the LS 6164R and DM 5953 RSF leaves, respectively, in response to water deprivation (Figure 3.7A). Water-deprived LS 6164R showed a 30% increase in leaf hydrogen peroxide content compared to its well-watered control, whereas water-deprived DM 5953 RSF showed a 40% increase in leaf hydrogen peroxide content when compared to its well-watered control (Figure 3.7B). An increase of 30% in hydrogen peroxide content was noticed in the roots of water-deprived LS 6164R whereas the increase was 60% in water-deprived DM 5953 RSF in comparison to the well-watered controls (Figure 3.7C). Statistically, the increase in leaf hydrogen peroxide content was significant only in DM 5953 RSF in response to water deficit whereas it was significant in roots in both cultivars.

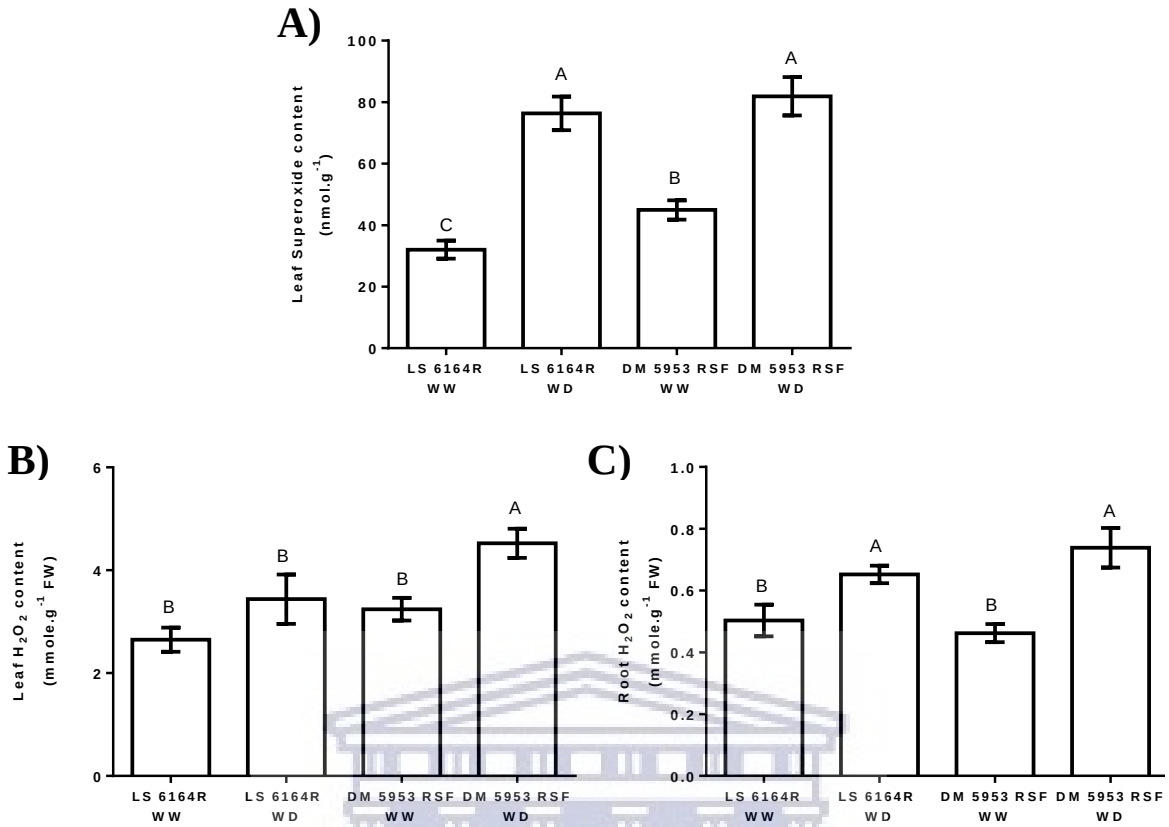

Figure 3.7: Levels of ROS in the two soybean cultivars under well-watered and water-deprived conditions

(A) Superoxide content of the 2nd youngest trifoliate leaves, (B) Hydrogen peroxide content of the 2nd youngest trifoliate leaves and (C) roots of LS 6164R and DM 5953 RSF under well-watered and water deficit conditions. Bars represent the mean ± standard error from three independent experiments, with bars of same letters signifying statistically similar values at $P < 0.05$

3.3.4 Water deficit stress increases lipid peroxidation (MDA) in soybean plants

The damage to cell membrane resulting from ROS can be quantified by measuring malondialdehyde content, a product of peroxidation of PUFA. This is routinely used as a biochemical indicator of oxidative stress. With the increased level of superoxide and hydrogen peroxide in the water-deprived plants in the sub-section above, it became necessary to assess the level of damage these ROS may have caused to the lipids of the cell membrane. A significant increase in MDA (77% and 174%) was noticed in the leaves of water-deprived LS 6164R and DM 5953 RSF, respectively, in relation to the well-watered controls (Figure 3.8A). In addition, the root tissues show an increase of 79% in water-deprived LS 6164R, while it was a 212% increase in water-deprived DM 5953 RSF when compared to their well-watered

controls (Figure 3.8B). This suggests that the ROS results in more cell membrane damage to the DM 5953 RSF cultivar than LS 6164R.

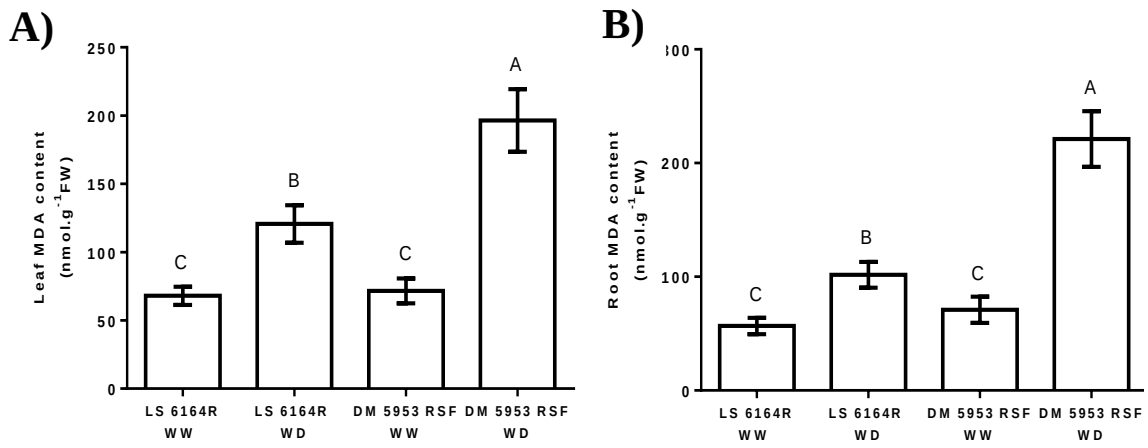

Figure 3.8: Lipid peroxidation in the soybean cultivars in response to water deprivation MDA content measured in (A) the 2nd youngest trifoliate leaves and (B) roots of the soybean cultivars under well-watered (WW) and water deficit (WD) conditions. Bars represent the mean ± standard error of three independent experiments. Bars with the same letters signify statistically similar values at $P < 0.05$

3.3.5 Proline level increases in response to water deficit stress in soybean plants

Due to previous documentation on proline accumulating under various environmental stress conditions, it became vital to determine if the two soybean cultivars differ in their proline content under water deprivation. There was an increase of approximately 32-fold and 52-fold, respectively, in LS 6164R and DM 5953 RSF leaves in response to water deficit when compared to their well-watered controls (Figure 3.9A). However, roots showed a 6.3-fold and 8.1-fold increase, respectively, in proline content in LS 6164R and DM 5953 RSF in response to water deficit compared to their respective well-watered controls (Figure 3.9B).

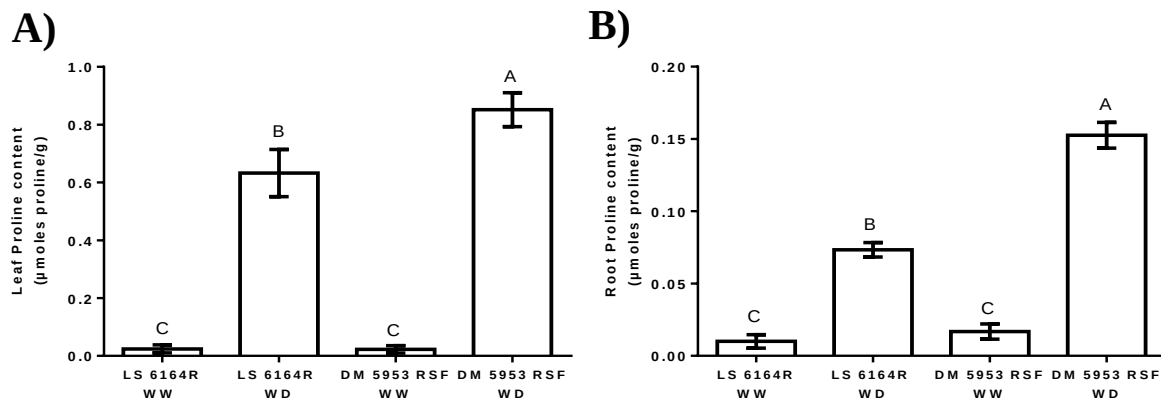

Figure 3.9: Proline accumulation in soybean cultivars in response to water deprivation
Free proline content in (A) the 2nd youngest trifoliate leaves and (B) roots of the soybean cultivars under well-watered (WW) and water deficit (WD) conditions. Bars represent the mean ± standard error of three independent experiments, where bars with the same letters indicate statistically similar values at $P < 0.05$.

3.3.6 SOD activity enhanced in soybean in response to water deficit stress

Hence, it became important to assess the response of SOD to water deprivation in the two soybean cultivars. In this study, native PAGE analysis detected 6 isozymes of SOD in soybean leaves while only 5 were detected in the root tissue (Figure 3.10). Using inhibitors that block the activity of a particular SOD isoform (Figure 3.10b and c), each isozyme was identified (Figure 3.10a).

The presence of one manganese superoxide dismutase (MnSOD), and four copper/zinc superoxide dismutase (Cu/ZnSOD) was detected in the leaves and roots of both cultivars. An iron superoxide dismutase (FeSOD) isoform was only noticed in the leaves of the cultivars, while there seems to be little or no activity of FeSOD in the any of the cultivar's roots. This visual representation of the in-gel activities of SOD isozymes indicates that the activities of most of the isoforms detected were upregulated in response to water deprivation. For example, the MnSOD1 and most families of Cu/ZnSOD in the water-deprived DM 5953 RSF leaves were more upregulated than its water-deprived LS 6164R counterpart, with no differences in their activities under well-watered conditions.

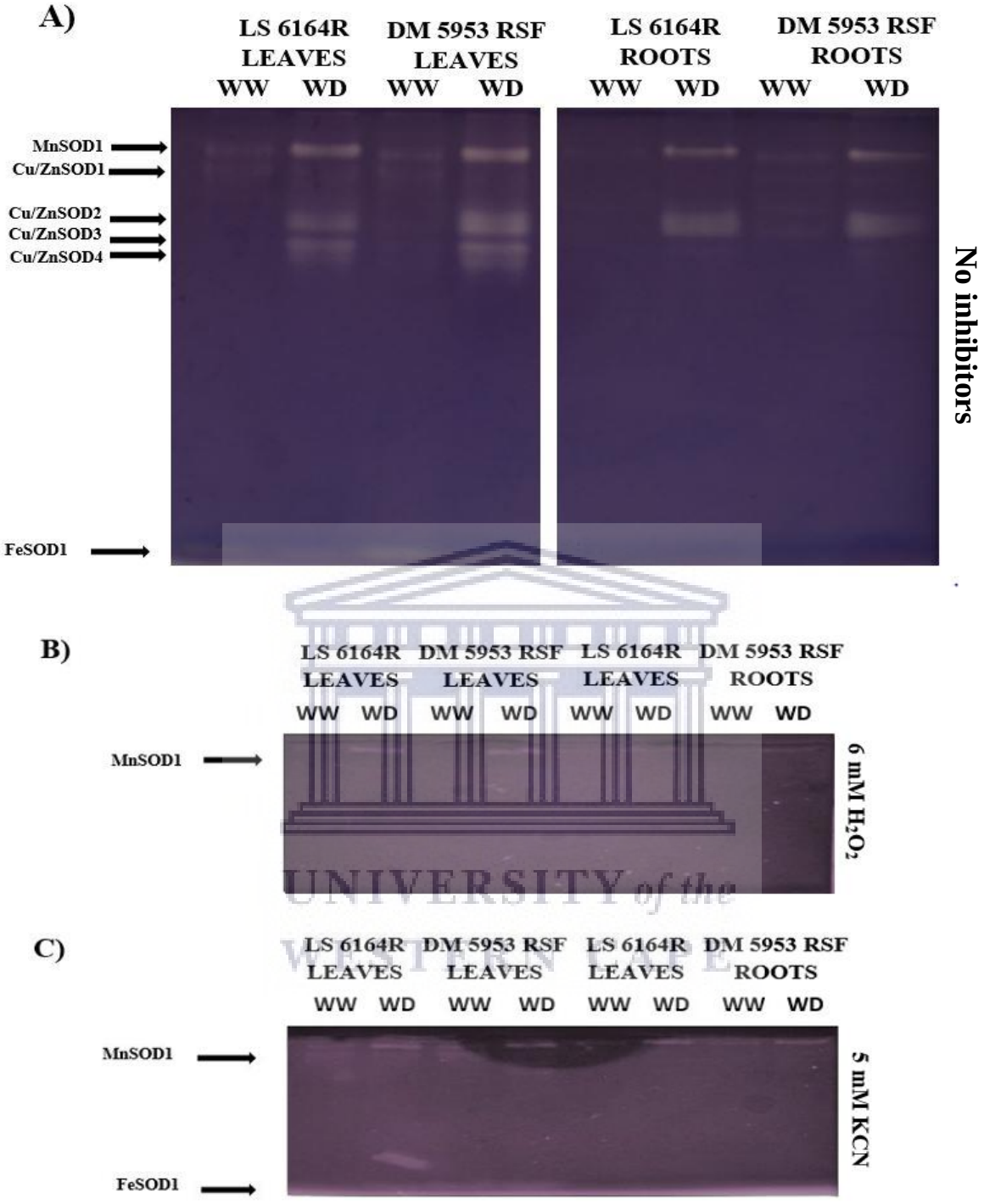

Figure 3.10: Superoxide dismutase in-gel enzymatic activity of the two soybean cultivars under well-watered and water-deprived conditions

The In-gel assay shows the detention of isoforms of SOD (A) in the absence of no inhibitors, (B) 6 mM H_2O_2 and (C) 5 mM KCN. 100 microgram of protein sample loaded in each well. WW and WD represent well-watered and water-deprived respectively.

To ascertain the actual change in each isoform activity (as represented by band intensity), densitometry analysis was performed and the results are tabulated in Table 3.2. The activity of MnSOD1 was significantly increased in the leaves and roots of both cultivars in response to water deprivation, with an increase of 36% LS 6164R and 43% in DM 5953 RSF compared to their well-watered controls in leaves (Table 3.2a). Root MnSOD1 showed a 27% increase in LS 6164R and a 43% increase in DM 5953 in response to water deprivation, respectively (Table 3.2b).

There were no significant differences between the activities of the Cu/ZnSOD1 isoforms in neither the leaves nor roots of the two cultivars irrespective of the level of water supply (WW or WD). The Cu/ZnSOD2 isoform in the leaves showed an increase of 43% in water-deprived LS 6164R while a 63% increase was observed in water-deprived DM 5953 RSF when compared to their respective controls (Table 3.2a). Root Cu/ZnSOD2 showed a 43% and 27% increase in the LS 6164R and DM 5953 RSF, respectively, in response to water deficit (Table 3.2b). There was a 39% and 54% increment in Cu/ZnSOD3 isoform activity in the water-deprived leaves of LS 6164R and DM 5953 RSF respectively (Table 3.2a), while only a 33% and 16% increase respectively occurred in the roots of LS 6164R and DM 5953 RSF (Table 3.2b). The Cu/ZnSOD4 isoform activity in the leaves was elevated by approximately 25% and 30% in the water-deprived LS 6164R and DM 5953 RS, respectively (Table 3.2a). However, there were no significant differences in the Cu/ZnSOD4 isoform activity in response to water deficit in the roots of the cultivars (Table 3.2b).

Furthermore, the activity of the FeSOD1 isoform was similar in water-deprived leaves of both cultivars (Table 3.2a), while there was no visible band detected in the root samples for this isoform (Table 3.2b).

Table 3-2: Densitometry measurement of the individual SOD isoforms present in the two soybean cultivars

A)

SOD isoforms (Leaf)	Soybean cultivar/Treatment			
	LS 6164R		DM 5953 RSF	
	WW	WD	WW	WD
MnSOD1	1.00 ± 0.02^{a}	1.36 ± 0.03^{b}	1.00 ± 0.02^{a}	1.43 ± 0.02^{b}
Cu/ZnSOD1	1.00 ± 0.02^{a}	1.02 ± 0.05^{a}	1.00 ± 0.02^{a}	0.98 ± 0.09^{a}
Cu/ZnSOD2	1.00 ± 0.02^{a}	1.43 ± 0.04^{b}	1.00 ± 0.02^{a}	1.63 ± 0.09^{c}
Cu/ZnSOD3	1.00 ± 0.02^{a}	1.39 ± 0.11^{b}	1.00 ± 0.02^{a}	1.54 ± 0.02^{c}
Cu/ZnSOD4	1.00 ± 0.02^{a}	1.25 ± 0.14^{b}	1.00 ± 0.02^{a}	1.30 ± 0.14^{b}
FeSOD1	1.00 ± 0.02^{a}	1.00 ± 0.07^{a}	1.00 ± 0.02^{a}	0.93 ± 0.12^{a}

B)

SOD isoforms (Root)	Soybean cultivar/Treatment			
	LS 6164R		DM 5953 RSF	
	WW	WD	WW	WD
MnSOD1	1.00 ± 0.02^{a}	1.27 ± 0.03^{b}	1.00 ± 0.02^{a}	1.11 ± 0.09^{b}
Cu/ZnSOD1	1.00 ± 0.02^{a}	1.00 ± 0.04^{a}	1.00 ± 0.02^{a}	0.94 ± 0.04^{a}
Cu/ZnSOD2	1.00 ± 0.02^{a}	1.43 ± 0.03^{c}	1.00 ± 0.02^{a}	1.27 ± 0.08^{b}
Cu/ZnSOD3	1.00 ± 0.02^{a}	1.33 ± 0.05^{b}	1.00 ± 0.02^{a}	1.16 ± 0.09^{b}
Cu/ZnSOD4	1.00 ± 0.02^{a}	1.04 ± 0.05^{a}	1.00 ± 0.02^{a}	1.02 ± 0.01^{a}
FeSOD1	N/A	N/A	N/A	N/A

The table illustrates the pixel intensity values of the individual SOD isoforms detected in Figure 3.9A. Table (A) represent the leaf while (B) represent the root samples. The data illustrated in the table are the means ± standard error of three independent experiments (n=3). Different letters next to the means in a similar row for a particular isoform represent a significant difference between the treatments while the same letters represent no significant difference (P<0.05) in accordance with the Tukey-Kramer test. The letters WW and WD represent well-watered and water-deprived respectively, while N/A indicates that there was no detected isoform.

In as much as the above analyses (Figure 3.10 & Table 3.2) have quantified individual SOD isoforms present in the tissues, there was a need to analyze the total SOD activity in each sample since densitometry may also be prone to error. This is because some isoforms might not be visible in the gel despite having activity in the cell. A spectrophotometry assay was employed to determine the total SOD activity.

The total SOD activity shows no significant differences in both leaves and root tissues of the soybean cultivars under water treatment (Figure 3.11). However, in water deficit conditions in the leaves, there was a 39% increase in SOD activity in the LS 6164R cultivar and a 46% increase in the SOD activity in the DM 5953 RSF (Figure 3.11A). The roots showed an increase of 53% in total SOD activity in the LS 6164R and an increase of 26% in the DM 5953 RSF cultivar in response to water deficit (Figure 3.11B).

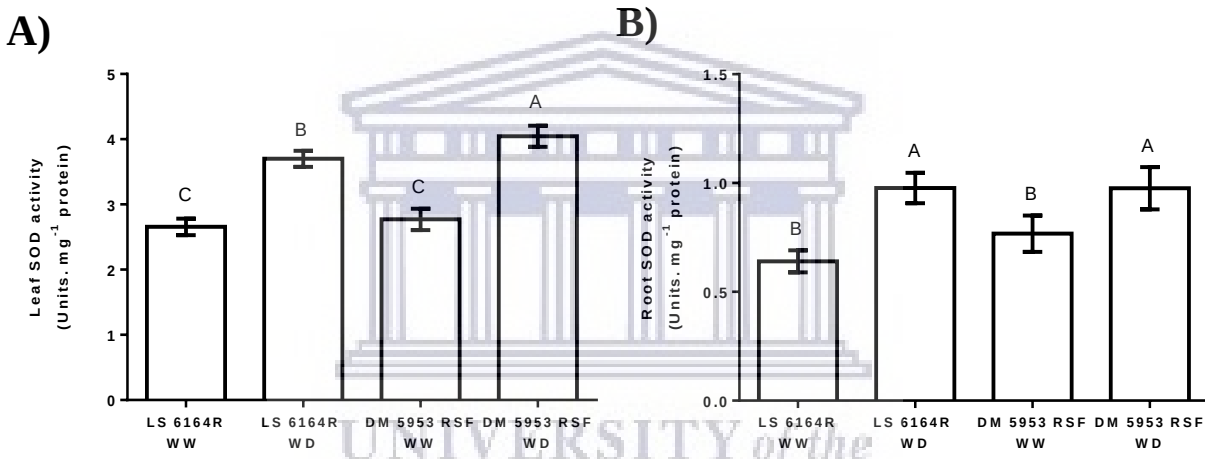

Figure 3.11: Spectrophotometry analysis of SOD activity in the soybean cultivars under well-watered and water-deprived conditions
(A) The SOD activity of the 2nd youngest trifoliate leaves and (B) roots of the soybean cultivars. Bars represent the mean ± standard error of three independent experiments, where bars with the same letters indicate statistically similar values at $P < 0.05$. WW and WD represent well-watered and water-deprived, respectively.

3.3.7 APX activity enhanced in soybean in response to water deprivation stress

APX is an important enzyme that dissociates the hydrogen peroxide formed under abiotic stress to water and oxygen using ascorbate as an electron donor (Ullah *et al*., 2016). Since this enzyme is vital in detoxifying ROS, it became vital to determine its activity in response to water deficit in the soybean cultivars.

The result of the APX native PAGE activity showed the presence of three isoforms in both the leaves and roots of the soybean tissue (Figure 3.12). The APX 1 and 2 isoforms in both the LS 6164R and DM 5953 RSF water-deprived cultivars showed an elevation in both the leaves and roots tissue in comparison to their respective controls (Figure 3.12A and B). However, the APX3 isoform showed suppression in only the leaves of the water-deprived cultivars (Figure 3.12A), while root APX3 activity increased in both the water-deprived cultivars (Figure 3.12B).

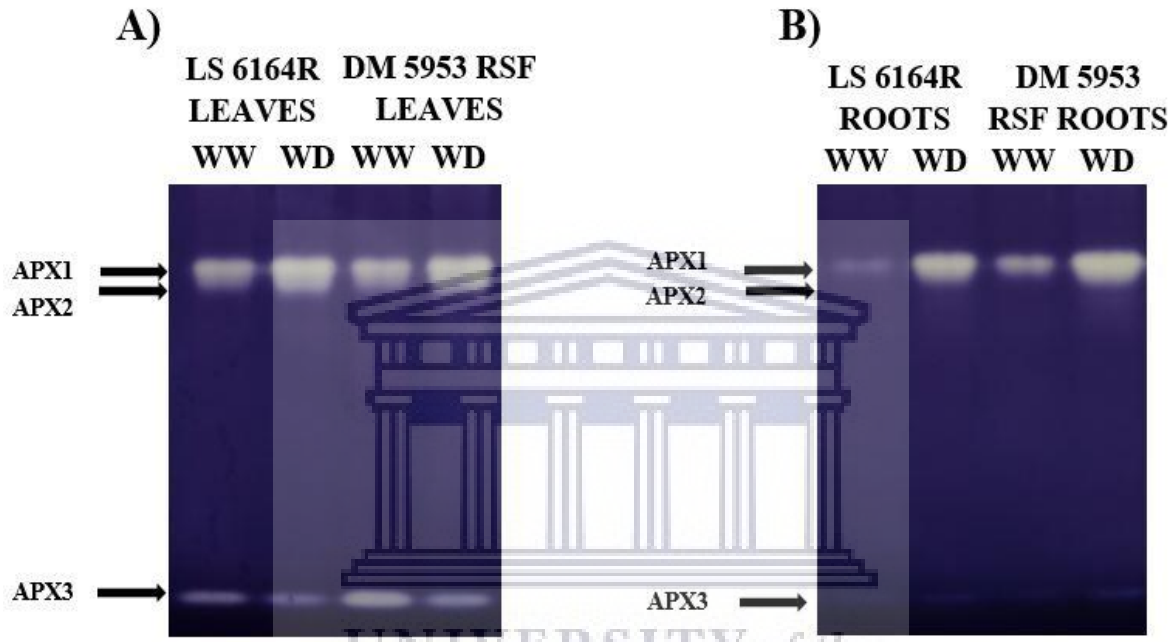

Figure 3.12: Ascorbate peroxidase in-gel enzymatic activity of the two soybean cultivars under well-watered and water-deprived conditions
APX in-gel assay of LS 6164R and DM 5953 RSF (A) trifoliate leaves and (B) roots under well-watered and water-deprived conditions. Protein samples (100 micrograms for each lane) were loaded for each treatment. WW and WD represent well-watered and water-deprived conditions, respectively.

The pixel intensity of the APX isoforms was quantified and tabulated accordingly as shown in Table 3.3. There was a significant upregulation of 18% and 20% in the APX1 activity from water-deprived leaves of LS 6164R and DM 5953 RSF respectively (Table 3.3A). However, in the roots, APX 1 shows a pronounced increase of 143% in the LS 6164R cultivar in response to water deficit whereas an increase of 46% occurred in water-deprived DM 5953 RSF (Table 3.3B). The APX 2 isoform showed an increase of 38% and 51% in leaves of water-deprived LS 6164R and DM 5953 RSF, respectively (Table 3.3A). The activity of APX 2 in the roots was upregulated by 128% and 132% in LS 6164R and DM 5953 RSF, respectively, in response to water deficit (Table 3.3B). The activity of the APX 3 isoform in the leaves was reduced by

31% and 33%, respectively, in the LS 6164R and DM 5953 RSF in response to water deficit (Table 3.3A). However, there was an upregulation in the activity of the APX 3 isoform in the water-deprived roots, where the upregulation was more pronounced in DM 5953 RSF (28%) than LS 6164R (11%) (Table 3.3B).

Table 3-3: Densitometry measurement of APX isoforms detected in the two soybean cultivars

A)

APX isoforms	Soybean cultivar + Treatment			
(Leaf)	LS 6164R		DM 5953 RSF	
	WW	WD	WW	WD
APX1	1.00 ± 0.02^{a}	1.18 ± 0.06^{b}	1.00 ± 0.02^{a}	1.20 ± 0.07^{b}
APX2	1.00 ± 0.02^{a}	1.38 ± 0.05^{b}	1.00 ± 0.02^{a}	1.51 ± 0.03^{c}
APX3	1.00 ± 0.02^{a}	0.69 ± 0.08^{b}	1.00 ± 0.02^{a}	0.67 ± 0.09^{b}

B)

APX isoforms	Soybean cultivar + Treatment			
(Root)	LS 6164R		DM 5953 RSF	
	WW	WD	WW	WD
APX1	1.00 ± 0.02^{a}	2.43 ± 0.03^{c}	1.00 ± 0.02^{a}	1.46 ± 0.03^{b}
APX2	1.00 ± 0.02^{a}	2.28 ± 0.07^{b}	1.00 ± 0.02^{a}	2.32 ± 0.04^{b}
APX3	1.00 ± 0.02^{a}	1.11 ± 0.04^{b}	1.00 ± 0.02^{a}	1.28 ± 0.04^{c}

The table illustrates the pixel intensity values of the individual APX isoforms detected in Figure 3.11. Pixel intensities are for activities in leaves (A) and roots (B). The data illustrated in the table are the means ± standard error of three independent experiments (n=3). Different letters next to the means in a similar row for a particular isoform represent a significant difference between the treatments while the same letters represent no significant difference ($P<0.05$) in accordance with the Tukey-Kramer test. WW and WD represent well-watered and water-deprived, respectively.

A spectrophotometry assay was employed to determine the total APX activity under both treatments (Figure 3.13). It was observed that there was a significant increase of approximately 22% in the total APX activity of leaves from water-deprived LS 6164R in comparison to its well-watered control, and an elevation of 48% was noticed in the leaves from water-deprived

DM 5953 RSF (Figure 3.13A). The well-watered leaves show a similar level of APX enzyme activity. However, the roots show dissimilar activity between the two cultivars in both well-watered and water-deprived conditions. Elevation of APX activity in response to water deficit was 46% and 45% in the LS 6164R and DM 5953 RSF roots, respectively, when compared to their well-watered state (Figure 3.13B).

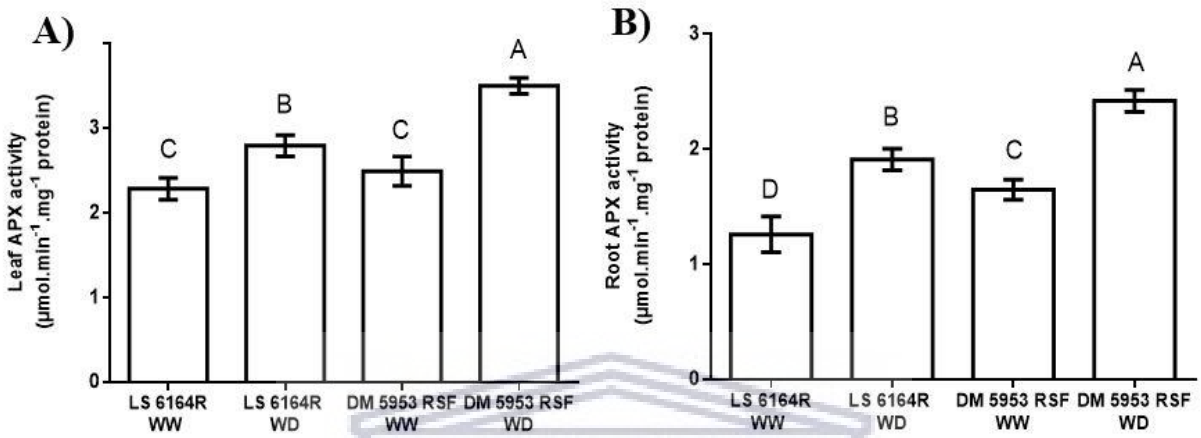

Figure 3.13: Spectrophotometry analysis of APX activity in the soybean cultivars under well-watered and water-deprived conditions
APX activity of the (A) trifoliate leaves and (B) roots of the soybean cultivars. Bars represent the mean ± standard error of three independent experiments. Bars with the same letters indicate statistically similar values at $P < 0.05$. WW and WD represent well-watered and water-deprived conditions, respectively.

3.3.8 CAT activity enhanced in soybean in response to water deprivation stress

Catalase (CAT) is well known to be a scavenging enzyme responsible for the dissociation of hydrogen peroxide to water and oxygen as mentioned earlier in literature. Although APX has a higher affinity for hydrogen peroxide, CAT binds more hydrogen peroxide because of its high turn-over rate (Smirnoff, 2005). The in-gel activity shows two isoforms of CAT detected in leaf tissue (Figure 3,14A), while only CAT 1 was detected in roots (Figure 3.14B). There was an increase in all CAT 1 isoforms under all water-deprived cultivars in both leaves and roots but the CAT 2 isoform was only visible in the leaves, where the CAT 2 isoform showed downregulation under water deprivation in the two cultivars. The CAT 1 isoforms in both leaves and roots tissues were named according to their position in the gel and the naming does not imply CAT 1 and CAT 2 in leaves are the same as CAT 1 and CAT 2 in roots.

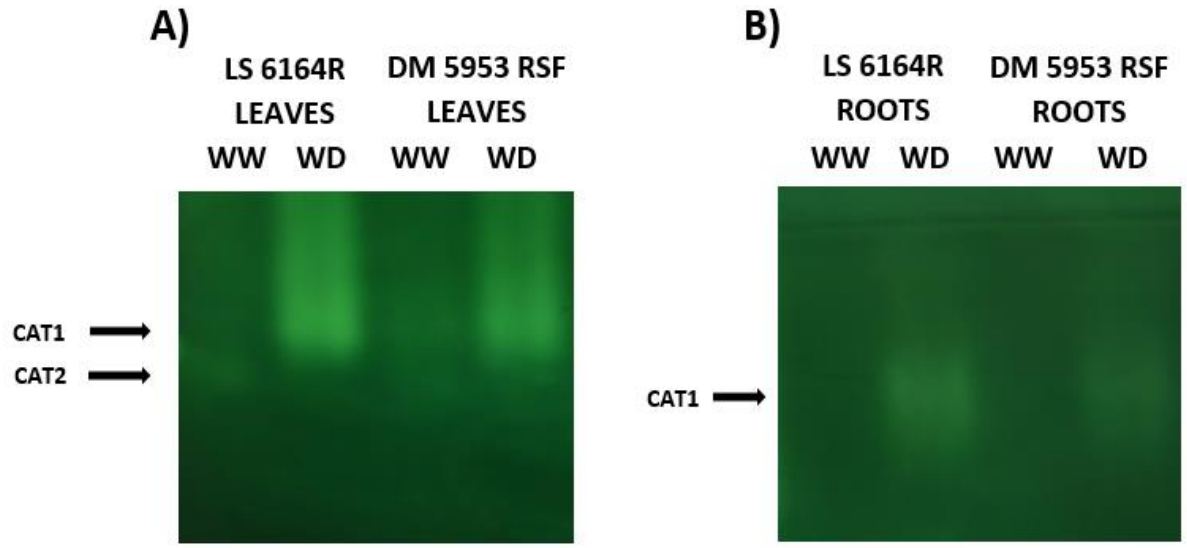

Figure 3.14: Catalase in-gel enzymatic activity of the two soybean cultivars under well-watered and water-deprived conditions
CAT in-gel assay of LS 6164R and DM 5953 RSF (A) trifoliate leaves and (B) roots under well-watered and water-deprived conditions assayed using 100 micrograms of protein in each well. WW and WD represent well-watered and water-deprived, respectively.

The relative pixel intensity of the individual isoforms was determined and results are shown in Table 3.4. There was an upregulation in the CAT 1 isoform in leaves and roots of both cultivars. In comparison to their well-watered state, the LS 6164R cultivar shows a 78% increment in leaves from the water-deprived plants, with 43% increase occurring in leaves from the DM 5953 RSF cultivar (Table 3.4A). The roots show a 50% and 26% increase in the activity of CAT 1 for the LS 6164R and DM 5953 RSF water-deprived samples, respectively, compared to their well-watered controls (Table 3.4B). There was no significant difference between the two treatments in both cultivars in the activity of CAT 2.

Table 3-4: Densitometry measurement of the individual CAT isoforms detected in the two soybean cultivars

A)

CAT isoforms	Soybean cultivar + Treatment			
(Leaf)	LS 6164R		DM 5953 RSF	
	WW	WD	WW	WD
CAT1	1.00 ± 0.02^{a}	1.78 ± 0.06^{c}	1.00 ± 0.02^{a}	1.43 ± 0.07^{b}
CAT2	1.00 ± 0.02^{a}	0.95 ± 0.04^{a}	1.00 ± 0.02^{a}	0.91 ± 0.04^{a}

B)

CAT isoforms	Soybean cultivar + Treatment			
(Root)	LS 6164R		DM 5953 RSF	
	WW	WD	WW	WD
CAT 1	1.00 ± 0.02^{a}	1.50 ± 0.03^{c}	1.00 ± 0.02^{a}	1.26 ± 0.03^{b}

Table 3.3 illustrates the pixel intensity values of the individual CAT isoforms detected in Figure 3.13. Table (A) represents the activities detected in leaves while (B) represents activities in roots. The data illustrated in the table are the means ± standard error of three independent experiments (n=3). Different letters next to means in a similar row for a particular isoform represent significant differences between the treatments while the same letters represent no significant difference (P<0.05) in accordance with the Tukey-Kramer test. WW and WD represent well-watered and water-deprived, respectively.

Quantification of the total CAT activity was conducted to support the above in-gel and densitometry results. Spectrophotometry analysis showed that there was 77% and 44% increase in total catalase activity in the LS 6164R and DM 5953 RSF water-deprived leaves, respectively, in comparison to their well-watered state (Figure 3.15A). The roots also showed a similar trend pattern with approximate 73% increment in the LS 6164R and 35% increment in the DM 5953 RSF water-deprived samples in comparison to their well-watered treatment (Figure 3.15B).

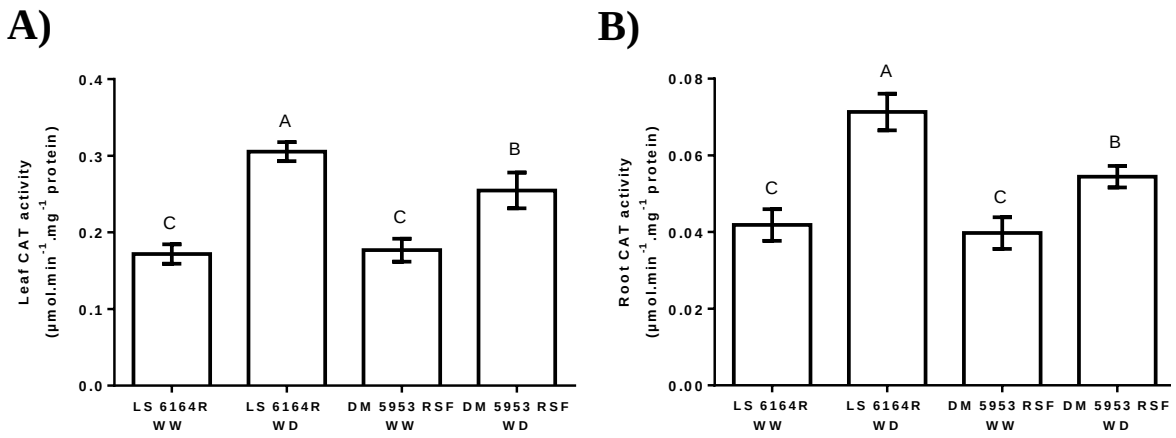

Figure 3.15: Spectrophotometry analysis of CAT activity in the soybean cultivars under well-watered and water-deprived conditions

CAT activity of the trifoliate leaves (A) and roots (B) of the soybean cultivars. Bars represent values of the mean ± standard error of three independent experiments. Bars with the same letters indicate values that are statistically similar at $P < 0.05$. WW and WD represent well-watered and water-deprived respectively.

4 Chapter Four

Discussion

4.1 Evaluation of the agronomic trait in two cultivars of *Glycine max*

4.1.1 Morphological differences observed in the LS 6164R and DM 5953 RSF soybean

Plant morphology is associated with evolutionary aspects of a particular plant species and can impact agronomic traits (Ackerly *et al*., 2000). This can be exploited for crop breeding to develop varieties with desired morphological traits. Shoot length is one such trait, for which the LS 6164R cultivar was found to be a shorter cultivar compared to the DM 5953 RSF cultivar. Shorter soybean cultivars may pose challenges for machine-based harvesting since pods may be below the lowest level to which the harvest can reach, leaving pods on the lower portion of the shoots unharvested. Furthermore, if the shortness leads to many pods being close to the ground, soiling of the pods can increase disease incidence. On this basis, DM 5953 RSF may be a desirable cultivar than LS 6164R.

4.1.2 Deviation in the yield of LS 6164R and DM 5953 RSF soybean

Measurement of the yield components is vital as they interact with one another to achieve a given yield (Fageria, 2016). Assessing yield and yield potential is important in plant breeding for crop production in order to select/breed for high yielding varieties (Kravchenko and Bullock, 2000; Van Roekel *et al*., 2015). The main metrics used to ascertain the efficiency of food production is crop yield. It relates to the quantity of harvested crop per area of land (Lobell *et al.*, 2009). Yield is a complicated attribute of plants which is altered by a series of yield components and their synergy (Flanagan, 2017). In legumes, increase in grain yield is associated with components such as the number of seeds per pod, number of pods per unit area, seed weight, grain harvest index, and shoot dry weight amongst others (Flanagan, 2017).

In this study, the number of branches and number of pods per plants in the LS 6164R cultivar were fewer in comparison with the DM 5953 RSF cultivar. However, the number of pods per branch in LS 6164R was greater than in DM 5953 RSF as the pods were densely populated in the fewer LS 6164R branches but sparsely distributed among the numerous DM 5953 RSF branches. LS 6164R has less biomass than DM 5953 RSF, which should be because of the higher height and more branches in DM 5953 RSF than LS 6164R. However, in terms of market value and crop yield, the weight of the seeds is the most important. The pods in LS 6164R

weighed more than those in DM 5953 RSF. It is thus clear that plant height and number of branches in these cultivars is not an indication of yield potential.

Quantification of total yield in tonnes/hectare clearly showed that the yield of LS 6164R is approximately double that of DM 5953 RSF.

4.2 Evaluation of physiological and biochemical drought traits in LS 6164R and DM 5953 RSF *Glycine max* cultivars.

4.2.1 Water deficit stress reduces growth and biomass in soybean

Omir and Samira, (2012) observed a reduced soybean yield under water-stressed conditions. Yield reduction differs depending on the time of onset of drought and the growth stage at which the drought occurs (Farooq *et al*., 2017). For example, soybean has a yield reduction of up to 40% if drought commences at the grain filling stage (Maleki *et al*., 2013) but between 45 and 50% reduction during the podset (Kobraee *et al*., 2011). A study in common bean shows that drought-tolerant lines significantly produced better yield than sensitive ones (Beebe *et al*., 2008). Limited water supply is one of the main causes of crop loss globally, reducing crop yield and quality (Zandalinas *et al*., 2018). Morphological, biochemical and physiological changes arise in plants in response to drought stress (Casaretto *et al*., 2016). These changes result in the disruption of plant growth by altering the division, enlargement and differentiation of cells (Ullah *et al*., 2016a). A study from Anjum *et al*., (2016) and Wang *et al*., (2016) illustrated that plant height is an agronomic parameter that is influenced by various stress conditions. Plant height and biomass are other traits that are reduced in response to drought (Anjum *et al.*, 2017).

In this study, water deprivation stress in both soybean cultivars resulted in stunted growth, reduced shoot length and reduced shoot weight. This is in agreement with the result from Ullah *et al.*, (2016b) where drought caused a major reduction in tomato shoot height and biomass. Other similar findings such as observations in switchgrass (Hui *et al.*, 2018), rice (Pandey and Shukla, 2015), maize (Kamara *et al*., 2003; Khan *et al*., 2001), sugarcane (Vasantha *et al.*, 2005) and canola (Shafiq *et al*., 2014) provide supporting evidence for above. The reduction in the shoot length might have been due to reduce cell division, decreased cell elongation/expansion which caused reduced growth (Earl and Davis, 2003; Jaleel *et al*., 2009). Furthermore, it might also be as a result of reduced turgor pressure consequent to water deficit (Taiz and Zeiger, 2006). Reduction in shoot length in LS 6164R was more pronounced than in

DM 5953 RS. This could be as a mechanism to conserve water by reducing its growth via lesser cell elongation or division (Jaleel *et al*., 2009).

Interestingly, there were no significant differences in the soybean root length whether the cultivars were deprived of water or not. Under drought, Anjum *et al*., (2017) argued that the growth and development of a plant are more pronounced in the leaf than the root, but the root is less sensitive to drought than the leaf. The similarities in the root length between the two cultivars under water deficit contradict studies from plants such as sorghum (Nxele *et al.*, 2017), sunflower (Manivanna *et al*., 2007) and alfalfa (Zeid and Shedeed, 2006); which show a significant reduction in root length when grown under conditions of limited water availability. It is likely that different levels of severity of drought in the different experiments account for this contradiction and this highlights the importance of measuring the water potential of the growth medium in drought experiments to ensure that results are comparable.

Biomass reduction has been described in soybean (Specht *et al*., 2001), bitter orange (Wu *et al*., 2008), parsley (Petropoulous *et al*., 2008) and sunflower (Tahir and Mehid, 2001) in response to water deficit. Farooq *et al*., (2009) explained that the effect of drought stress largely affects the fresh and dry weight of plants. A significant reduction in shoot weight was noticed in both of the water-deprived cultivars of soybean. This is likely due to a reduction in cell division or cell elongation due to water deprivation (Shao *et al*., 2008). According to Mohammadian *et al*., (2005), plant tolerance to stress in terms of its biomass may differ between different genotypes of the same species. However, both cultivars of soybean under this study have a similar level of biomass loss. Therefore, for these cultivars, biomass is not the differentiating factor in drought response at this level of water deficit. It is possible that higher levels of water deficit may affect the two cultivars differently, which may have to be explored in future.

4.2.2 Water deficit stress results in the rolling of soybean trifoliate leaves

Since it has been ascertained earlier that deprivation of water from water deficit in crops causes decreased cell division and differentiation, it is no surprises that in the long run the stress might reduce leaf size. Leaf rolling is one of the morphological responses by which plants reduce the leaf area (Anjum *et al.*, 2017). The extent of drought tolerance can be inferred from the extent of leaf rolling (Panda and Shukla, 2015). Leaf rolling allows the plant to achieve some reduction in water loss, also the area of leaf exposed to light radiation and heat becomes

reduced (Kiriga *et al*., 2016). Due to this, leaf-rolling is seen as a trait that can be used in evaluating drought tolerance (Baret *et al.*, 2018).

Studies have shown that under severe water deficit, leaf rolling in soybean occurs via its trifoliate leaves clamping together, the middle leaflet sandwiched between the two outer leaflets (Licht and Archontoulis, 2017). This morphological change was noticeable in the current study. The DM 5953 RSF cultivar was the first to undergo leaf rolling, whereas LS 6164R leaf rolling occurred only a week later. Several studies have tried to explain some biomolecular aspects of leaf rolling in maize (Baret *et al*., 2018), wheat (Willick *et al*., 2018), rice (Pandey and Shukla, 2015), and sorghum (Sezgin *et al*., 2018). Rolling of the leaves in the two water-deprived soybean cultivars would contribute to the maintenance of its water status as documented by Kadioglu *et al*., (2012). This was shown to be the case in rice seedlings (Ha, 2014). Keeping a balance between the rate of evapotranspiration and water uptake also determines the water status of the plant and has an influence on the onset and extent of leaf rolling (Baret *et al*., 2018). Moreover, the accumulation of specific phytohormones can influence rolling (Talaat and Shawky, 2012). Additionally, changes in the concentration of organic acids have been implicated in leaf rolling (Saglam *et al*., 2010) and could have contributed to the rolling observed in the two soybean cultivars in this study.

A study in rice by Cal *et al*., (2019) demonstrated that drought tolerance may not be correlated with levels of leaf rolling, rather the predominance of a constitutive trait such as plant type and genotype over a morphological responsive trait such as leaf rolling as a result of leaf water status. It is thus likely that the onset and extent of leaf rolling in response to drought is an indicator, and not determinant, of the extent of drought sensitivity in plants.

4.2.3 Water deficit stress reduces the relative water content (RWC) in soybean

RWC is widely used to quantify plant water status (Tanentzap *et al.*, 2015; Ullah *et al.*, 2016a). Leaf relative water content indicates the balance between the supply of water to the leaf tissue and the rate of transpiration (Soltys-Kalina *et al.*, 2016).

This study shows that water-deprived leaves have a significant reduction in their RWC in comparison to their well-watered controls. This decrease is in agreement with studies of different plants under increasing water deficit; *e.g.* tomato (Zgallai *et al.*, 2005; Ullah *et al*., 2016b), peanut (Shivakrishna *et al*, 2018), barley (Yuan *et al*., 2005), and rice (Hsu and Kao, 2003). Furthermore, RWC has been used to distinguish between sensitive and tolerant cultivars in common bean (Korir *et al.*, 2006) and wheat (Keyvan, 2010). However, in this study, it

appears the RWC of the two soybean cultivars does not vary significantly from one another under water stress. The fact that the two cultivars had different responses to the same level of water deficit at which they had similar RWC and similar biomass shows that their molecular responses to drought are different. This is supported by the fact that their leaf rolling behaviour is different despite having similar RWC and similar biomass at the same level of water deficit. This implies that one (LS 6164R) is more drought tolerant than the other (DM 5953 RSF).

4.2.4 Water deficit stress reduces the chlorophyll content in soybean

Chlorophyll is one of the vital pigments of the photosynthetic apparatus that absorbs light and transfer its energy to the reaction centre of the photosystem (Hörtensteiner and Kräutler, 2011). Drought stress causes physiological changes such as chlorophyll degradation, disruption of photosystem activity, reduced photosynthetic rate, reduced stomatal conductance and increased abscisic acid (ABA) content (Pandey and Shukla, 2015). The leaf chlorophyll content is related to the degree of photosynthetic activity (Arjenaki *et al*., 2012). Various studies have shown that water deprivation in plants disrupts total chlorophyll generation through the inhibition of chlorophyll *a* and *b* synthesis (Arjenaki *et al*., 2012).

This study shows a significant reduction in chlorophyll *a*, *b* and *a+b* in the soybean water-deprived cultivars in comparison with the well-watered. It agrees with comparison to previous soybean studies (De Souza *et al.*, 1997; Ohashi *et al.*, 2006). Fotovat *et al.*, (2007) noticed a significant decrease in wheat leaf chlorophyll content under drought, and a similar trend was noticed in leaves of rice (Sikuku *et al.*, 2012; Chozin *et al.*, 2014), sunflower (Manivannan *et al.*, 2007), peanuts (Shivakrishna *et al.*, 2018) and bean (Zlantko, 2005). However, this study does not correlate with results from Schlemmer *et al.*, (2005) where there was no significant difference noticed in the chlorophyll content of the maize water-deprived leaves. A logical justification for this reduction in chlorophyll content is stress-induced impairment of the pigment biosynthesis pathway (Pandey and Shukla, 2015) and thus depletion of chlorophyll. This has also been linked to ROS-induced lipid peroxidation (Shivakrishna *et al*., 2018).

Arjenaki *et al.*, (2012) and Mafakheri *et al.*, (2010) used the chlorophyll parameter as a screening tool to select drought-tolerant wheat and chickpea cultivars respectively. They both independently studied drought in relation to total chlorophyll in varying cultivars as a gauge for identifying more tolerant cultivars. As a study by Toscano *et al.*, (2016) explained, tolerant cultivars reduce the rate of photosynthesis mainly due to stomata closure to minimize water loss. In sensitive cultivars, reduce the photosynthetic rate is due to water unavailability

resulting in severe damages to cells. Although this study shows that there was more reduction in chlorophyll content in the water-deprived DM 5953 RSF cultivar than LS 6164R, however, the difference was only 5%. This shows that other indicators of drought stress have to be exploited to arrive at a more pragmatic conclusion.

4.2.5 Water deficit stress elevates loss of cell viability in soybean

In the course of ontogenesis, plants are exposed to varying abiotic stress. These stresses may destroy plant tissue, threatening plant survival (Petrov *et al.*, 2015). Cell death occurs during drought-induced stress in plants, which is associated with programmed cell death or necrosis. Cell death is related to damage caused to DNA, cell membrane and lipids (Sharma *et al.*, 2016: Zhang *et al.*, 2015). Therefore, assays for cell viability assays are indicators of cellular injury imposed by drought stress.

Loss of cell viability was substantially elevated in leaves from the water-deprived plants compared to their respective controls. The observed loss of viability agrees with a study from Nxele *et al.*, (2017) where an elevation of cell death was noticed in both drought and salinity-induced stresses in sorghum. In addition, research by Basset and Matsumoto, (2008) illustrates similar findings where aluminium-induced stress results in disruption of the membrane, thus, leading to increase cell death. In light of this, it can be concluded that the elevation of cell death (loss in cell viability) in our study is related to damage to the cell membrane and other organelles within the plant due to the accumulation of ROS under water deprivation (Zhang *et al.*, 2015). A more increase in loss of cell viability was observed in the DM 5953 RSF cultivar than LS 6164R, indicating that the water deprivation resulted in more cellular damage in the DM 5953 RSF than LS 6164R cultivar. The difference may be attributed to the ability of LS 6164R to tolerate water deficit better than the DM 5953 RSF.

4.2.6 Water deficit stress increases ROS production in soybean

Plants, as other aerobes, are regularly exposed to reactive oxygen species. The ROS constitute molecules like hydrogen peroxide (H_2O_2) or ions such as superoxide (O_2^-) (Jha and Subramanian, 2016). If excessively accumulated, ROS damage DNA, disrupt membrane lipids and/or denature proteins (Munne-Bosch and Pinto-Marijuan, 2016), and ROS regulation under drought has been reported in plants (Chan *et al.*, 2016). It is important to measure the production of ROS in response to drought because an increase in ROS has been shown to indicate stress and consequently leads to damage of macromolecules of the cell (Gill and

Tuteja, 2010). However, ROS like superoxide and hydrogen peroxide at appropriate levels have signalling roles in plants (Wang *et al*., 2016).

Although superoxide (O_2^-) has a relatively short half-life, its dismutation by superoxide dismutase (SOD) promotes H_2O_2 generation. A pronounced elevation of O_2^- was noticed in the leaves of the water-deprived cultivars. The increase in H_2O_2 content was significant between water-deprived and well-watered tissues. This correlates with the study of Zhang *et al*, (2010) where drought resulted in an elevation of O_2^- and H_2O_2 in soybean seedlings. Comparable findings where increases in ROS due to drought were observed include sorghum (Nxele *et al.*, 2017) and wheat (Caverzan *et al.*, 2016). The excessive increase in ROS in this study might have been due to increasing rates of Mehler reactions, due to the reduction of Fe-S centre in PSI, making it lose its ability to accept electrons from the thylakoid electron transport chain. As a result, the oxygen molecules accept the electrons and results in highly reactive superoxide radicals and H_2O_2 (Puthur, 2016). The increased level of photorespiration under water deficit might also have led to ROS increase in the peroxisomes (Sharma *et al.*, 2012). There was more production of H_2O_2 in the leaves than in the root tissues. This might be due to the occurrence of more organelles per tissue in the leaves as ROS are mainly produced by organelles such as mitochondria, chloroplast and peroxisomes (Caverzan *et al.*, 2016). Considering that both the leaves and roots of the DM 5953 RSF cultivar have a greater increase in H_2O_2 than LS 6164R cultivar, it is logical to understand that there was more damage in the DM tissues and its antioxidant system was not as effective in scavenging these species. Nevertheless, a larger increase of O_2^- in LS 6164R than DM 5953 RSF tells us that more O_2^- was accumulated in LS 6164R.

4.2.7 Water deficit induces the accumulation of MDA in soybean

Studies have shown that the aftermath of over-generation of ROS due to water deprivation is the peroxidation of lipids (Gontia-Mishra *et al.*, 2016). ROS contributes to the destruction of polyunsaturated fatty acids (PUFA), which may destroy the cell membrane since PUFA is a major component of cell membranes (Wahsha *et al*., 2012).

Oxidative stress is measured as malondialdehyde (MDA), the final product of lipid peroxidation (Toscano *et al*, 2016; Murtaza *et al*, 2016). Since an increase of H_2O_2 and O_2^- was noticed in the stressed tissues, it was paramount to quantifying the damage in terms of lipid membrane peroxidation levels. Increased levels of MDA in each of the water-deprived cultivars in relation to its respective control imply that drought caused lipid peroxidation in these

cultivars. Reports from maize (Talaat *et al.*, 2015; Ye *et al.*, 2016), potato (Li *et al.*, 2017) and sorghum (Gurpreet and Nidhi, 2013; Nxele *et al.*, 2017) also show increased MDA levels under drought, which agrees with this study. The increase in MDA content in water-deprived tissues should be as a result of the accumulation of the ROS, leading to oxidative damage to the lipid membrane (Zhang *et al.*, 2019).

Moreover, when comparing the extent of increase of MDA across the two water-deprived cultivars, DM 5953 RSF had higher levels than LS 6164R in both the leaf and root tissues. This suggests that LS 6164R copes better with drought-induced oxidative stress and thus tolerates drought better than DM 5953 RSF (Talaat *et al.*, 2015). In studies on maize (Anjum *et al.*, 2016), barley (Zahedi *et al.*, 2016), wheat (Abid *et al.*, 2016,) faba bean (Siddiqui *et al.*, 2015) and alfalfa (Zhang *et al.*, 2019), a comparison was made on different cultivars to identify those with better tolerance to drought. It was observed that MDA levels were reduced in the tolerant cultivars than the sensitive ones. This supports the notion in this study that LS 6164R better adapts to water deficiency than the DM 5953 RSF.

4.2.8 Proline accumulates in response to water deficit stress in soybean

Plants make use of organic and inorganic solutes to improve their osmotic pressure in order to maintain both turgor and the osmotic gradient for effective uptake of water. Among these important osmolytes is proline (Abbas *et al.*, 2014). Through osmotic adjustment, plants cells adapt physiologically to water deprivation. An increase in free proline content lowers the water potential in the cell, allowing retention of water potential within the cell (Borgo *et al.*, 2015).

Previous studies have correlated accumulation of these osmolytes to the response of the plant to varying environmental stresses (Hamdia and Shaddad, 2010). A significant upregulation of proline in the two water-deprived cultivars was observed in this study. Similar findings were noticed in citrus plants (Zabdalinas *et al.*, 2016), cowpea (Zegaoui *et al.*, 2017), sorghum (Nxele *et al.*, 2017) and sugarcane (de Silva Vantini *et al.*, 2016). The accumulation of proline in the soybean water-deprived tissues might have been related to a regulatory mechanism that increases cell osmolarity, which helps prevent water loss and alleviate damages (de Silva Vantini *et al.*, 2016). Interestingly, an increase in the proline was more pronounced in the DM 5953 RSF than LS 6164R water-deprived tissues. This suggests that the LS 6164R might not have had the need to greatly overexpress proline levels compared to the DM 5953 RSF. This could be related to the suggestion that LS 6164R is more tolerant to drought than DM 5953.

The upregulation of proline in both tissues might also be a reflection of the degree of injury symptoms rather than drought tolerance as experimented by de-Lacerda *et al*., (2003) on sorghum. Therefore, the cultivar with more injury will upregulate more proline than the other.

4.2.9 Water deficit alters the enzymatic antioxidant activities in soybean

In previous sections, an increased level of cell death, ROS and lipid peroxidation were associated with reduced soybean growth under water deficit. Antioxidant systems are evolved by plants to effectively scavenge ROS and alleviate oxidative damages (Puthur, 2016). Various studies have used antioxidant capacity to gauge plant tolerance to drought (Aldesuquy *et al*., 2013).

4.2.9.1 The efficiency of SOD activity in alleviating water-deficit stress in soybean

The SOD enzyme is the first line of defence against damage caused by ROS, essential in the dismutation of O_2^- to H_2O_2 (Caverzan *et al*., 2016; Liu *et al*., 2016). Through the breaking down of superoxide, the detoxification of superoxide by SOD becomes possible (Wang *et al*., 2016).

This study shows a significant increase in total SOD activity in both the leaves and roots of the water-deprived soybean, with more intense increase noticed in the DM 5953 RSF than in the LS 6164R leaves. Upregulation of SOD enzyme under drought stress has been observed in peanut (Furlan *et al.*, 2016) and tomato (Celik *et al.*, 2017), which agree with this study. However, a contradicting result in wheat showed decreased activity of SOD (Ibrahim, 2014; Niari and Najaphy, 2018). The increase in total SOD activity in the water-stressed soybean was expected as there was an increase in the O_2^- content, therefore upregulation of SOD to catalyse its dismutation to H_2O_2 became vital for the survival of the water-deprived plants. Interestingly, the stressed leaf SOD activity in the DM 5953 RSF appears to have superseded that of the LS 6164R, this could explain why there was lower O_2^- in DM 5953 RSF. However, the SOD enzyme breakdown of superoxide to H_2O_2 might not have been effective enough in DM 5953 RSF like it was in LS 6164R.

Mn-SOD, Cu/Zn-SOD and Fe-SOD are co-factors of the SOD enzyme, and they are differentially influenced by abiotic stress (Szollosi, 2014). One Mn-SOD, four Cu/Zn-SOD and one Fe-SOD isoenzyme were noticed in the leaves of both cultivars of soybean, whereas all were present in the roots except Fe-SOD. The increase in the MnSOD and four Cu/Zn-SOD isoenzymes under water deprivation signify that the mitochondria and chloroplast were involved in the detoxification of superoxide (since these isoforms occur in these respective

organelles) (Celik *et al*., 2017). Since literature has revealed that the two isoenzymes were localized in that specific subcellular compartment (Pang and Wang, 2008), and were upregulated in our study, we may conclude that these organelles are defence centres against superoxide. Previous studies directly agree with this study, as is the case with cotton (Sekmen *et al*., 2014), tomato (Celik *et al.*, 2017), and sugarcane (Boarettto *et al.*, 2014). Although FeSOD isoenzyme was present in the leaves, no significant differences were observed in the two treatment conditions for this isozyme. This agrees with other reports showing little or no activity of the Fe-SOD isoenzyme in response to abiotic stress (Abedi and Pakniyat, 2010), indicating little relevance under the influence of drought for Fe-SOD, irrespective of plant species.

4.2.9.2 The efficiency of APX activity in alleviating water-deficit stress in soybean

The H_2O_2 produced under abiotic stresses is broken down by APX and CAT enzymatic antioxidant to H_2O, with APX using ascorbic acid for this function (Sofo *et al*., 2015). Significant upregulation was observed in the total APX activity of the water-deprived soybean. This was similar to reports for common bean (Zlatev *et al.*, 2005), maize (Chugh *et al.*, 2011), rice (Srivalli *et al*., 2003), peanut (Furlan *et al.*, 2016) and tomato (Celik *et al*., 2017) which show increased APX activity under drought stress.

Significant upregulation of the APX 1 and APX 2 isoforms was noticed in both the leaves and roots under water deprivation. This may signify their crucial role in raising the leaf total APX activity for effective H_2O_2 breakdown. However, downregulation of APX 3 isoform was observed in the leaf tissue under stress.

Although DM 5953 RSF induces more of its APX enzyme than LS 6164R, this response was not enough to relieve the plant from excessive H_2O_2 and alleviate the associated oxidative damage. The higher APX activity in DM 5953 may thus be a response to higher H_2O_2 content in this cultivar rather than an indicator of tolerance. Thus, insight into the other H_2O_2-detoxifying enzyme, CAT, was needed for broadening our perspective.

4.2.9.3 CAT activity in alleviating water-deficit stress-induced oxidative damage

CAT converts H_2O_2 into H_2O and O_2 without any complex ascorbate or glutathione regulatory system, and as a consequence, it is involved in detoxifying more H_2O_2 than APX (Sofo *et al.*, 2015). This study shows that total CAT activity was significantly upregulated in both leaf and root tissues of water-deprived soybean. This correlates with a study in tomato (Celik *et al*.,

2017), alfalfa (Wang *et al.*, 2009), maize (Chugh *et al.*, 2011) and wheat (Luna *et al.*, 2005; Chakraborty and Pradhan, 2012). However, a few studies also show a decrease in CAT activity under drought (Apel and Hirt, 2004), which was related to the degree of sensitivity among varying plant species. The upregulation of CAT by LS 6164R suggests that this cultivar is more efficient in detoxifying H_2O_2 than the DM 5953 RSF cultivar. Roy *et al.*, (2009) and Nair *et al.*, (2008) have associated high CAT activity with drought tolerance among genotypes of rice and cowpea respectively.

The increase in SOD, CAT, and APX activity in water-deprived plants might have been due to enhancement of free proline levels, as an experiment by Osman, (2015) directly related upregulation of proline under drought with an increase in the level activity of these antioxidant enzymes, an effect also seen for glycine betaine (another osmolytes), which has been shown to increase APX and CAT activity under drought stress (Cruz *et al*., 2013).

5 Chapter Five

Conclusion and future prospects

In light of climate change and the growing world population, food security is one of the biggest concerns for the 21st century. This growing concern is far worse in Sub-Saharan Africa which accounts for a large but poor population that is expected to increase even more by 2050, thus increasing the productivity of agriculture is paramount to the sustainability of food security for the African and global population (OECD/FAO, 2016). Due to environmental stresses such as drought, heat and salinity, food security has decreased over the past few years (Hadebe *et al.*, 2017), with the rural community at greater risk of food shortage. Therefore, it becomes vital for researchers to enhance the tolerance of plants to abiotic stress. Here, we evaluated two cultivars of soybean (*Glycine max)*, LS 6164R and DM 5953 RSF.

The agronomy study reveals that the yield of both cultivars when they are grown in the experimental region was on par with the national average. We confirm that cultivation of these soybean cultivars is feasible to the region. Furthermore, we conclude that lack of production of soybean in the Eastern Cape compared to other provinces might have been due to political factor or unavailability of resources, rather than the physiological aspects concerning conditions of growth (climate) in the region.

It was worthy to note that the LS 6164R cultivar has a higher yield than DM 5953 RSF in the experimental location and could thus be the cultivar of choice for this location. In the immediate future, we intend to inoculate both cultivars with *Rhizobium japonicum* to assess the effect of inoculum on yield. In terms of responses to drought, both cultivars show similar changes in biomass and length. However, the DM 5953 RSF cultivar was the first to initiate leaf rolling, indicating that this cultivar may be more sensitive to water deficit than LS 6164R. These differences may be due to differences in antioxidant activities because ROS accumulation and lipid peroxidation were more pronounced in the DM 5953 RSF than the LS 6164R cultivar under water stress. The protein profiling needs to be undertaken in future studies using proteomics to reveal differences between the protein patterns of the two cultivars. Also, it will be important to analyze gene expression via technologies such as RNA sequencing to identify genes responsible for the observed differences in drought tolerance in these two cultivars. Such future studies will aid in breeding soybean with better drought tolerance. The yield potential

should also be taken into account and this would be imperative to plant breeders to generate a better soybean genotype that not only has better adaptability towards drought condition but also has a higher yield.

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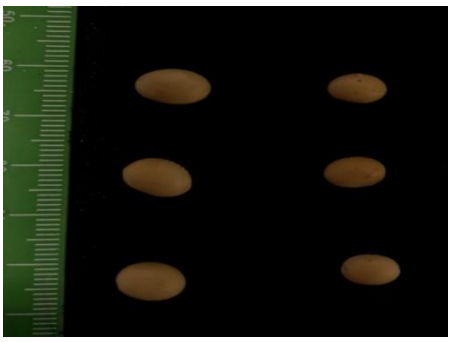
Appendix

A)

LS 6164R DM 5953 RSF



B)

LS 6164R **DM 5953 RSF**

C)

LS 6164R **DM 5953 RSF**

Figure 3.16: Digital imagining of soybean LS 6164R and DM 5953 RSF harvested from the field.

Digital imaging of the (A) soybean seed (B) soybean plant at harvest (after 72 days of planting) and (C) soybean pods/plant at harvest. Seeds and plants were selected at random for photographing.