A BIOCHEMICAL AND PROTEOMIC ANALYSIS OF SUGARGRAZE SORGHUM UNDER HYPEROSMOTIC STRESS

Miss Xolisa Nxele

A thesis submitted in partial fulfilment of the requirements for the degree of Magister *Scientiae*, in the Department of Biotechnology, University of the Western Cape.

Supervisor: **Prof. Bongani K. Ndimba**

Co-supervisor: **Dr. Ashwil Klein**

February 2015

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KEYWORDS

2D SDS-PAGE

Ascorbate peroxidase

Drought stress

Glutathione reductase

MALDI-TOF-TOF MS

Proteomics

Reactive oxygen species

Salinity stress

Sorghum

Superoxide dismutase

ABSTRACT

A BIOCHEMICAL AND PROTEOMIC ANALYSIS OF SUGARGRAZE SORGHUM UNDER HYPEROSMOTIC STRESS

X. Nxele

MSc Thesis, Department of Biotechnology, University of the Western Cape.

Sugargraze is a moderately drought tolerant sweet sorghum hybrid which is ideal for grazing, winter stand over and pit silage. A major advantage that Sugargraze has over other forages is its very high sugar content which improves feed quality thus increasing palatability and results in significantly reduced feed wastage. This study explored the influence of hyperosmotic stress on plant development, ROS accumulation, antioxidant capacity and the extent of cell death. Heat shock protein (Hsp70) expression immunoblotting assays were used to demonstrate whether the various treatment conditions induced stress within natural physiological parameters for the experimental material. This was coupled with the separation, visualization and identification of abundant proteins in Sugargraze leaves in response to hyperosmotic stress using two-dimensional gel electrophoresis (2-DE) in combination with mass spectrometry (MS). The results showed that hyperosmotic stress significantly influences plant development by reducing plant biomass and increasing the levels of ROS accumulation, proline content and subsequently reducing total chlorophyll content. An over accumulation of ROS in the form of hydrogen peroxide and lipid peroxidation was observed in the stressed plants which was supported by the extent of cell death. Although an increase in antioxidant enzyme activity (in the form of total enzymatic activity or individual isoform activity) in response to hyperosmotic stress was observed, this increase was not sufficient to counter the deleterious effects caused by the stress

conditions hence the decrease in plant biomass and increase in cell death. Western blotting analysis of Sugargraze leaf tissues using Hsp70 antibodies showed that hyperosmotic stress induced Hsp70 expression to levels significantly higher than observed for the control plants. A total of thirteen CBB stained spots were selected for mass spectrometric identification, owing to their good resolution and abundance levels, and of these, nine were positively identified. Identified proteins were divided into functional categories including both known and novel/putative stress responsive proteins. Molecular and physiological functions of some of the proteins of interest identified will be subjected to further investigation via bioinformatic and molecular biology approaches.

DECLARATION

I declare that **A BIOCHEMICAL AND PROTEOMIC ANALYSIS OF SUGARGRAZE SORGHUM UNDER HYPEROSMOTIC STRESS** 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.

Xolisa Nxele February 2015

Signed ……

ACKNOWLEDGEMENTS

To God, my father, if it were not for your mercy, grace and strength you gave me through this degree, I would not be here. Thank you for leading and guiding me.

I thank my family, for being a pillar of strength, undying love, support and encouragement. To my grandmother, Mrs Gladys Duba, for being a role model in my life. I look at you and wonder how you do it. You are a blessing to my sister and me. Ms. Akhona Nxele, I always thought I was the older and wiser one, thank you for knowing what to say each time I called to complain. My uncle, Mr. Madodana Stuurman, I know I can always count on you. To my sons, the Ondahs, you are an encouragement and inspiration to me, I love you.

Special thanks to all of my friends, you guys made this journey worthwhile. We did a lot of complaining together, but we made it!

I would like to express my sincere gratitude to my supervisors Prof. Bongani K. Ndimba and Dr. Ashwil Klein for their guidance throughout the course of the study and their patience with me. Prof. Ndimba, thank you for allowing me to be in the group and for making sure everything that was needed in my research was available in time. Dr. Klein, you started co-supervising me in my second year and with your help I submitted in time. Thank you for letting me share your office space and transforming all my “nonsense” into sense.

Many thanks to the proteomics group members for their support and input during the course of this study. Everyone in the Biotechnology department, fellow students and staff members for the friendly atmosphere and smiles.

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LIST OF ABBREVIATIONS

µg	Micrograms
µl	Microliters
1D	One-dimensional
2D	Two-dimensional
2DE	Two-dimensional gel electrophoresis
2D-SDS-PAGE	Two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
3D	Three-dimensional
APS	Ammonium persulfate
APX	Ascorbate peroxidase
ASC	Ascorbic acid
ASH-GSH cycle	Ascorbic acid-glutathione cycle
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
BLASTp	Protein to protein BLAST
BSA	Bovine serum albumin
CAT	Catalase

CBB	Coomassie Brilliant Blue
CDD	Conserved domains database
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1- propanesulfonate
Chl_a	Chlorophyll a
Chl_b	Chlorophyll b
cm	Centimeters
CO_2	Carbon dioxide
Cu/Zn-SOD	Copper zinc superoxide dismutase
Da	Dalton
dH_2O	Distilled water
DHA	Dehydroascorbic acid
DHAP	Dihydroxyacetone phosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTNB	Nitrobenzoic acid
DTT	Dithiothreitol Cleland's reagent
DW	Dry weight

ECM	Extracellular matrix
ER	Endoplasmic reticulum
ESTs	Expressed sequence tags
Fe-SOD	Iron superoxide dismutase
FW	Fresh weight
GAP	Glyceraldehyde 3 phosphate
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulphide
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
Hsc70	Heat shock cognate 70
Hses	Heat shock elements
Hsfs	Heat shock transcription factors
Hsp70	Heat shock protein 70
Hsps	heat shock proteins

IEF	Isoelectric focusing
IPG	Immobilized pH gradient
K_2HPO_4	Dipotassium phosphate
KCN	Potassium cyanide
kDa	Kilo Daltons
KI	Potassium iodide
L	Liter
LEA	Late-embryogenesis abundant protein
LPO	Lipid peroxidation
m/z	Mass to charge ratio
MALDI-TOF	Matrix assisted laser desorption/ionisation-time of flight
MALDI-TOF-TOF	Matrix assisted laser desorption/ionisation-time of flight/time of flight tandem mass spectrometry
MASCOT	Matrix Science
MDA	Malondialdehyde
mg	Mili grams
ml	Mili liter
mM	Milli molar

Mn-SOD	Manganese superoxide dismutase
MOWSE	Molecular weight search
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTT	3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
MW	Molecular weight
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
Nr	Non-redundant database
O_2	Singlet oxygen
O_2^-	superoxide
OEC	Oxygen-evolving complex
OH	Hydroxide

OH^-	Hydroxide ion
PAGE	Polyacrylamide gel electrophoresis
PCD	Programmed cell death
pI	Isoelectric point
PM	Plasma membrane
PMF	Peptide mass fingerprinting
PMS	Phenazine methosulfate
PPase	Pyrophosphatases
PTM	Post translational modification
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
RuBP	Ribulose-1, 5-biphosphate
RWC	Relative water content
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing Tween 20

TC	Total chlorophyll
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TOF	Time of flight
Uniprot	Universal protein resource
UV	Ultraviolet
V	Volts
v/v	volume to volume
Vhrs	Volt hours
w/v	Weight to volume

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CHAPTER 1

LITERATURE REVIEW

1.1 The importance of cereal crops

All cereal crops belong to the taxonomic family known as *Gramineae* (*Poaceae*). Cereal grains are the seeds of cultivated grasses that include wheat, corn, oats, barley, rye, rice, sorghum, and millet (Harlan et al., 1973). Cereals such as rice, wheat and maize are particularly important to humans because of their role as staple food crops in many areas of the world (Nadeem et al., 2010). Cereals are also used to produce animal feed, oils, starch, flour, sugar, syrup, processed foods, malt, alcoholic beverages, gluten and renewable energy (Pomeranz and Munck 1981). According to research conducted by Nadeem et al. (2010) about 50% of the world's calories are provided by rice, wheat and maize, whereas in many parts of Africa and Asia, people rely mainly on grains such as sorghum or millet. Sorghum, maize and barley are important sources of livestock feed with sorghum, barley and rice also being used in the brewing industry (Chopra, 2002).

1.2 Sorghum as a cereal crop

The name *Sorghum bicolor* (L.) Moench was proposed by Clayton in 1961 as the correct name for the cultivated sorghum which is currently in use (Spangler, 2003). Sorghum is the world's fifth major cereal in terms of production and acreage (Rao et al., 2013) It is a staple food crop for millions of the poorest and most food-insecure people in the semi-arid tropics of Africa, Asia and Central America (Conway and Toenniessen 1999). The crop is genetically suited to hot and dry agro-ecologies where it is difficult to grow other food grains (Mekbib, 2007). These areas are frequently drought-prone and characterized by fragile environments. In many of these areas, sorghum is a dual-purpose crop. Their grain provides food for humans and their residues are used

as feed for livestock (Herrero et al., 2010). Sorghum is grown in two broad groups of countries. The first group consists primarily of African and Asian countries where production is traditional, subsistence-based and small-scale with sorghum being used for food. Yields from these countries are generally low and can vary considerably from year to year. Both yield and quality are affected by a wide range of biotic (pests and diseases) and abiotic (drought and salinity) stresses. The second group include more industrialized countries where production is modern, mechanized, and sorghum is primarily used for animal feed with significantly higher yields.

1.2.1 Origin and use

Sorghum originated in north-eastern Africa, with domestication having taken place there around 5,000–8,000 years ago (Mann et al., 1983; Wendorf et al., 1992). The largest diversity of cultivated and wild sorghum is also found in this part of Africa (de Wet, 1977; Doggett, 1988; Kimber, 2000). The secondary center of origin of sorghum is the Indian Subcontinent, with evidence for early cereal cultivation dating back about 4,500 years (Vavilov, 1992; Damania, 2002).

Traditional foods made from sorghum include unfermented and fermented breads, porridges, couscous and snacks, as well as alcoholic beverages. Sorghum blended with wheat flour has been used over the last two decades to produce baked products, including yeast-leavened pan, hearth and flatbreads, cakes, cookies, and flour tortillas (Badi et al., 1990). Malt drinks and malt cocoa-based weaning food and baby foods are popular in Nigeria. Hard endosperm sorghum is used extensively in south-east Asia for noodles. Sorghum grain is one of the major ingredients in swine, poultry and cattle feed in the western hemisphere, China and Australia (Bramel-Cox et al., 1995) Sorghum is also grown for forage; in northern India it is very common and fed to animals fresh or as silage or hay. Sweet sorghum is used to a limited extent in producing sorghum syrup

and ‘jaggery’ (raw sugar) in India and has recently gained importance in ethanol production (Rao et al., 2009).

1.2.2 Nutritional value of sorghum

Sorghum is regarded as a substitute for wheat and is great for those requiring a gluten-free diet (Awik et al., 2004). White, food grade sorghums can be milled directly into whole-grain flour to produce foods such as cookies, cakes, brownies, breads, pizza dough, pastas, cereals, pancakes and waffles (Iva, 2012). The Japanese have used white, food grade sorghums in a variety of extruded snack food products, thus providing many nutritional benefits. It is commonly eaten with the hull (the outer layer of the grain), which retains the majority of the nutrients. Sorghum grain contains 11.3% protein, 3.3% fat and 56–73% starch (Iva, 2012). It is relatively rich in iron, zinc, phosphorus and B-complex vitamins. Tannins, found particularly in red-grained types, contain antioxidants that protect against cell damage, a major cause of diseases and aging. The protein and starch in sorghum grain are more slowly digested than those from other cereals, and slower rates of digestibility are particularly beneficial for people with diabetes (Iva, 2012). Sorghum starch is gluten-free, making sorghum a good alternative to wheat flour for individuals suffering from celiac disease (Ekpebegh, 2003).

1.2.3 Sweet sorghum

Sweet sorghum is a C4 plant species (Figure 1.1), indigenous to Africa having wide flat leaves and a round or elliptical head, full of grain at the stage of maturity (Bryan, 1990). Similar to grain sorghum, it has been traditionally under cultivation for nearly 3000 years. It can be grown successfully in semi-arid tropics, where other crops fail to thrive and are highly suitable for cultivation in harsh dry land growing areas. Sweet sorghum is characterized by the high sugar content, mainly sucrose, fructose, and glucose in the juice of the stalk from which ethanol can be

easily produced and used as biofuel (Vasilakoglou et al., 2011). Based on these characteristics, sorghum has become a popular energy plant worldwide (Matrorilli et al., 1995).

Figure 1.1 Sweet sorghum cultivar. The figure shows a matured sweet sorghum cultivar grown in the field.

The name "sweet sorghum" is used to identify sorghum varieties which have juicy and sweet stalks. Sweet sorghum is mainly cultivated for syrup production (Hunter and Anderson 1997) or forage, whereas other sorghum varieties such as kafirs and milos are primarily cultivated for human consumption (Ritter et al., 2007). After harvest, the stalks are squeezed for the sweet juice, which can be turned into sugar or fermented to ethanol. The stalk material remaining after the sugar juice has been squeezed out is called the bagasse and can be used as animal feed or pre-treated, hydrolyzed and fermented to ethanol. Sweet sorghum is therefore considered to be a promising crop for low cost production of ethanol (Barbanti et al., 2006; Yung-Long et al., 2006; Wang and Lui, 2009).

1.2.3.1 Sugargraze as a sweet sorghum variety

Sugargraze is a top quality 3-way cross (sorghum x sorgho x sudan-grass) hybrid designed for grazing, greenchop or hay. Sugargraze grows off rapidly and its sweet succulent stalks and broad deep green leaves result in an excellent quality forage (Figure 1.2). Sugargraze is widely adapted to various growing conditions. Its inherent massive root system results in drought tolerance, which makes Sugargraze highly suitable for limited rainfall areas. This hybrid will also respond to adequate moisture with the ability to produce superior yields. In general, higher planting rates are recommended for overall premium quality forage.

Figure 1.2 Sugargraze as an excellent forage crop. The figure shows a cow grazing on mature sugargraze plant.

1.3 The influence of abiotic stress on plants

Abiotic stress conditions such as drought and salinity can induce a number of responses in plants ranging from readjustment of transport and metabolic processes thus leading to growth inhibition (Debnath et al., 2011). Abiotic stress cause ion imbalance and hyperosmotic stress that influence plant growth and leads to ultimate death (Mahajan and Tuteja, 2005). A direct result of these primary effects is the enhanced accumulation of reactive oxygen species (ROS), which is harmful to the plant cells at higher concentrations. Oxidative stress occurs due to a serious

imbalance in ROS production and antioxidant defense in cell compartment thus leading to significant physiological challenges (Reginato et al., 2014). The excess ROS in turn causes damage to proteins, lipids, carbohydrates, DNA, whilst also changing the plant's physiology and metabolic content thus resulting in ultimate cell death (Figure 1.3). A few lines of research have shown that there are cross links between the components of drought and salt stress as both these stresses ultimately result in cell dehydration and osmotic imbalance (Liu and Zhu, 1998).

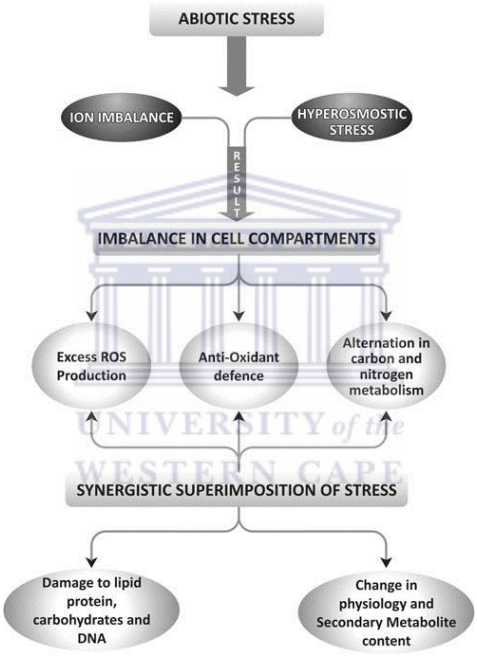

Figure 1.3 Plant's responses to abiotic stress conditions. Figure was adapted from Debnath et al. (2011).

1.3.1 Drought

Drought or water deficit is an important environmental condition causing reduction in plant growth and development as well as plant productivity and crop yields (Hsiao and Acevedo, 1974). The effect of water deficit can be manifested in many ways, as varied morphological, physiological, and biochemical changes in plants under different drought-induced stress

conditions. A few lines of literature have shown that water stress as a result of water deficit alter leaf morphology (Hsiao and Acevedo, 1974), affects shoot and root growth and development, restricting photosynthetic activity by decreasing CO_2 influx, reduce carboxylation reactions, thus influencing the electron transport chain activities of the chloroplasts (Akinci, 1997). Furthermore, research conducted by Davis, (1995) has shown that water deficit negatively influences metabolic pathways, mineral uptake and membrane structures.

1.3.2 Salinity

Salinity better known as salt stress is regarded as one of the most serious factors limiting agricultural crop productivity, with detrimental effects on seed germination, plant strength and crop yield (Munns and Tester 2008). During the onset and development of salt stress within a plant system, all major molecular and metabolic processes including lipid metabolism are affected (J. Parker, 1968). Salinity or salt-induced stress negatively influence plant growth and survival, development and biomass production which in turn triggers a series of morphological, physiological, biochemical, and molecular changes in plants. However, the most noticeable effect of salt stress on crop plants is only visible in grain yield upon harvest (Grover et al., 1998).

1.4 Stress-induced ROS production in plants

ROS are important cues used by plants in defense against abiotic and biotic stresses, polymerization of cell wall components and in the biosynthesis of organic molecules. In unstressed plants they are needed at basal levels for normal metabolic processes and their production is increased when a plant is stressed. When plants are exposed to stress, the production of ROS such as H_2O_2, O_2^-, O_2 and OH^- is enhanced. A number of studies have shown that surplus ROS cause phytotoxic reactions like, lipid peroxidation, protein degradation and DNA damage (McCord, 2000; Vinocur B, 2005; Klein, 2012). In plant cells, ROS, particularly

H_2O_2, O_2^-, and OH^- are generated in the cytosol, chloroplasts, mitochondria, peroxisomes and the apoplastic space (Mittler, 2002).

ROS have been shown to play a pivotal role in plants as signal transduction molecules involved in mitigating pathogen infections, environmental stresses, programmed cell death (PCD) and developmental stimuli (Mittler et al., 2004; Klein, 2012). Stress induced membrane injuries are linked to increased ROS production. Thus, elevated ROS level production may lead to stomatal closure, causing a decrease in CO_2 concentration inside the chloroplasts. This leads to decreases in $NADP^+$ concentration with generation of ROS (Noctor and Foyer, 1998). As ROS concentration increases, the D1 protein of photosystem II is damaged, thus inhibiting photosynthesis. Stress proliferated photorespiration and NADPH activity also plays a role in enhancing H_2O_2 accumulation, which may inactivate enzymes by oxidizing their thiol groups. During this reaction, H_2O_2 works concurrently with a metal reductant to create the hydroxyl radical that has the ability to react with all biological molecules, due to its high reactivity (Foyer and Halliwell, 1976). ROS scavenging enzymes such as SOD, APX, GPX, catalase and GR are activated to remediate the toxic effects of ROS in stressed plants.

1.5 Mechanisms of ROS scavenging in plants

ROS are likely to interact with numerous cellular components, leading to significant damage to membranes, other cellular structures and growth inhibition (Verma, 2005; Klein, 2012; Gao, 2008). Certain ROS are very toxic and must be detoxified using cellular responses in order for plants to survive and grow (Gratão et al., 2005). ROS scavenging uses a detoxification mechanism, which may be as a result of simultaneous or sequential action of a few antioxidant enzymes, including CAT, GPX, SOD and APX. Parida (2005) showed that plants with high constitutive and induced antioxidant level have a higher probability to resist damage mediated by

ROS overproduction and can therefore withstand oxidative stress. ROS scavenging is used by plants against abiotic stresses as part of the defense responses (Vranova and Inze 2002). The extent of damage by ROS depends on the ratio between ROS production and removal by antioxidant systems (Demiral, 2005; Khan and Panda 2008). Azooz et al. (2009) showed a correlation between antioxidant enzyme activity and salinity tolerance by comparing a tolerant cultivar to a sensitive maize cultivar. The antioxidant enzymes were demonstrated to increase under drought and salinity stress and closely related to salinity tolerance of numerous plants (De Azevedo et al., 2006); Koca et al., 2007; Athar et al., 2008).

1.5.1 Enzymatic scavenging of ROS in plants

Superoxide dismutase is found in certain cell compartments and is a major scavenger of superoxide (O_2^-). SOD converts O_2^- to H_2O_2, which is eliminated by ascorbate peroxidase (APX; EC 1.11.1.7) at the expense of oxidizing ascorbate to monohydroascorbate (Klein, 2012). Hydrogen peroxide is also scavenged by catalase (CAT; EC 1.11.1.6) and peroxidase (POD) to form water and oxygen (Mittler, 2002; Chaparzadeh et al., 2004).

1.5.1.1 Superoxide Dismutase

Superoxide dismutases are a group of metallo-proteins that catalyze the dismutation of superoxide (O_2^-) to molecular oxygen and hydrogen peroxide (H_2O_2). In response to environmental stresses, plants produce increased levels of ROS and SOD provides the first line of defense and is thus important in plant stress tolerance. O_2^- is removed by dismutating one O_2^- and reducing it to H_2O_2 while another oxidized to molecular oxygen (O_2) (Table 1).

Table 1.1 Superoxide dismutation reaction using Superoxide as a substrate

Enzymatic antioxidant	**Enzyme code**	**Reaction catalyzed**
Superoxide dismutase (SOD)	EC 1.15.1.1	$O_2^- + O_2^- + 2H \longrightarrow 2H_2O_2 + O_2$

SOD removes O_2^- consequently decreasing the risk of OH formation using the metal catalyzed Haber-Weiss-type reaction, which is 10 000 times faster than spontaneous dismutation. These metalloenzymes are classified into three known types by their metal cofactors: copper/zinc (Cu/Zn-SOD), manganese (Mn-SOD) and the iron (Fe-SOD) and is localized in different cellular compartments (Mittler, 2002). Mn-SOD is found in peroxisomes and mitochondria; Cu/Zn-SOD isozymes are located in higher plant chloroplasts and cytosolic fractions (del Rio et al., 2003). Although Fe-SOD isozymes are rarely detected in plants, they are linked to the chloroplasts when available (Alscher et al.,2002) (Table 2). SOD isozyme activity can be detected by negative staining and identification is made on KCN and H_2O_2 sensitivity. The Mn-SOD is resistant to both inhibitors; Cu/Zn-SOD is sensitive to both inhibitors while; Fe-SOD is resistant to KCN and sensitive to H_2O_2. Mn-SOD of mitochondria are tetramers while the prokaryotic Mn-SOD and Fe-SOD, and the eukaryotic Cu/Zn-SOD enzymes are dimers.

Table 1.2 Localization of SOD isozymes

SOD isozymes	**Location**	**Resistant to**	**Sensitive to**
Fe-SOD	Chloroplast	KCN	H_2O_2
Mn-SOD	Mitochondria and Peroxisomes	KCN and H_2O_2	-
Cu/Zn-SOD	Chloroplast and cytosol	-	KCN and H_2O_2

1.5.1.2 Ascorbate peroxidase

Ascorbate peroxidase is the most important antioxidant enzyme in the chloroplast. It uses ascorbate as the reductant to scavenge H_2O_2 in a process that forms water as a by-product and dehydroascorbate (DHA) as the final product (Moller, 2001). A certain level of ascorbate has to be maintained in the cell and this is done by dehydroascorbate reductase (DHAR; EC 1.8.5.1) via recycling of DHA back into ascorbate by oxidizing reduced glutathione (GSH) (Moller, 2001). APX scavenges ROS and protects cells in algae, higher plants, euglena and other organisms (Gill and Tuteja 2010). It is involved in H_2O_2 scavenging in water-water and ASG-GSH cycles, using ASC as an electron donor. The APX family is composed of five known isoforms, such as, thylakoid (tAPX) and glyoxisome membrane forms (gmAPX), chloroplast stromal soluble form (sAPX) and the cytosolic form (cAPX) (Noctor and Foyer, 1998). In comparison to CAT and POD (mM range), APX has a higher affinity for H_2O_2 (μM range) and could have an important role in ROS management during stress.

1.5.1.3 Catalase

Catalases are tetrameric heme containing enzymes that are vital for ROS detoxification during stress conditions. The CAT-mediated detoxification process is characterized by the direct dismutation of H_2O_2 into water and molecular oxygen and O_2 (Garg, 2009). This group of enzymes has been identified in plants and is very heterogeneous. One CAT molecule is able to convert about 6 million molecules of H_2O_2 to H_2O and O_2 per minute. CAT is essential in removing H_2O_2 generated in peroxisomes using oxidases involved in β-oxidation of fatty acids, photorespiration and purine catabolism.

1.5.1.4 Glutathione Peroxidase

Glutathione peroxidases (GPX; EC 1.11.19) are a large family of isozymes, protect plant cells from oxidative stress by using GSH to reduce H_2O_2 and organic and lipid hydro peroxides (Noctor and Gomez, 2002). GPXs are a family of multiple isozymes involved in scavenging hydrogen peroxide enzymes and is characterized by the presence of three conserved cysteine residues in the coding region (Rouhier and Jacqout, 2005). The GPX enzymes found in plants are both structurally and functionally different from the GPXs found in animals. Animal GPX uses glutathione only as a reductant while the plant GPX shows an alternative pathway. This is evidenced by glutathione peroxidase proteins identified in *Arabidopsis thaliana* and Chinese cabbage that had glutathione-dependent peroxidase activity and showed high affinity to thioredoxin as a reductant (Mittler, 2002). These enzymes are localized in all the plant parts and different cell compartments (Rouhier and Jacqout, 2005; Ashraf, 2009). Plant GPXs are responsive to ROS accumulation induced by both biotic and abiotic stress.

1.5.1.5 Glutathione Reductase

Glutathione reductase (GR) belongs to a group of flavoenzymes and contains an essential disulfide group; where, one mole of NADPH is required to reduce GSSG to GSH for every mole of GSSG. Glutathione is a flavor-protein oxidoreductase that is harbored in prokaryotes and eukaryotes (Romero-Puertas et al., 2006). GR plays important role in ROS detoxification, GSH regeneration and confers abiotic stress tolerance in plants (Hasanuzzaman et al., 2012; Hasanuzzman et al.,2010). It is found in mitochondria, cytosol and chloroplasts. Increased GR activity confers stress tolerance and has the ability to alter the redox state of important components of the electron transport chain. The major involvement of GR in conferring stress

tolerance is the recycling of GSH and the maintenance of GSH/GSSG ratio in plant cell (Hasanuzzman et al., 2010).

1.5.2 Non-enzymatic detoxification of ROS in plants

1.5.2.1 Ascorbic acid

Ascorbic acid is a potent, ubiquitous and water soluble antioxidant laboring to prevent or minimize ROS mediated damage in plants (Smirnoff, 2000; Athar et al., 2008). It is found in all plant tissue, although its quantities are more in photosynthetic cells and meristems. Additionally, it has been found to be high in concentration in mature leaves that have high chlorophyll and fully developed chloroplasts. Under homeostatic conditions, ASC is available in its reduced form in the leaves and chloroplast (Smirnoff, 2000). Stromal concentration of ascorbate is in the region of 50 mM and approximately 30-40% of total ascorbate is located in chloroplast (Foyer, 2005). Plant mitochondria make ASC, metabolize and regenerate its oxidized forms (Szarka et al., 2007). ASC is regarded as a potent ROS scavenger due to its ability to donate electrons in enzymatic and non-enzymatic reactions. It protects membranes by directly scavenging O_2^- and OH and generating α-tocopherol from tocopheroxyl radical. Chloroplastic ASC poses as a cofactor for violaxanti de-epoxidase and therefore sustains the dissipation of excess excitation energy. The ASC-GSH cycle is additionally important as it functions in the preservation of enzymes containing prosthetic transition metal ions (Noctor and Foyer, 1998).The ASC redox system consists of L-ascorbic acid, MDHA and DHA.

1.5.2.2 Glutathione

GSH/tripeptide glutathione is considered one of the significant metabolites in plants and is crucial in intracellular defense against ROS induced oxidative damage. It is found ubiquitously

in its reduced form (GSH) in plant tissues and in all cell compartments (Mittler and Zilinskass, 1992; Herna et al., 1998). Additionally, it plays an important role in a number of physiological processes, such as sulfate transport regulation, signal transduction, metabolite conjugation, the expression of stress responsive genes and the detoxification of xenobiotics (Xiang et al., 2001; Mullineaux and Rausch, 2005). GSH plays a pivotal role in plant growth and development procedures, like cell differentiation, senescence and death, pathogen resistance and enzymatic regulation (Rausch and Wachter, 2014). GSH is crucial for the maintenance of a normal reduced cell state thereby counteracting the inhibitory effects of ROS induced oxidative stress (Meyer, 2008). It scavenges O_2^-, H_2O_2 and OH. Furthermore, it regenerates ASC via the ASC-GSH cycle, providing an antioxidative defense system (Foyer and Halliwell, 1976). It has been shown, that GSH concentrations decline and redox state becomes more oxidized when stress levels are elevated, causing a deterioration of the system (Tausz et al., 2004).

1.5.2.3 Proline

In addition to functioning as an osmolyte, proline is an antioxidant, protein stabilizer, metal chelator, LPO inhibitor and OH and O_2^- scavenger and potential inhibitor of PCD. Proline is now known as a nonenzymatic antioxidant that lessens the deleterious effects of ROS needed by animals, microbes and plants (Chen and Dickman, 2005). Plants under stress contain approximately 5% of proline among other free amino acids (Soshinkova et al., 2013). Proline has been shown to scavenge both hydroxyl and superoxide radicals (Shevyakova et al., 2009). Proline improves stress tolerance through up-regulating certain proteins, decreasing lipid peroxidation and preventing photo-inhibition (Islam et al., 2009).

1.6 Proteomics

Proteomics has been defined as the large scale study of proteins in terms of their structure and function at organism, tissue or cellular levels (Graves and Haystead, 2002). Biologically, proteomics is derived from the studies of genomics. The term proteome was created by joining together the words "protein" and "genome". Proteomics studies proteins on a global and integrated way thus creating a 3 dimensional map (3D) of the cell, illustrating where proteins are found (Blackstock and Weir, 1999). The field of proteomics is composed of many areas of study, such as protein-protein interaction, protein function, modifications and localization. It integrates different disciplines like, biochemistry, molecular biology and bioinformatics.

1.6.1 Why use proteomics?

The proteome of an organism is subjected to spatial and temporal alterations; therefore proteomics has become vital tool to study comparisons between diseased and healthy, stressed and unstressed systems, which are impossible when explored from the genome angle. With the aid of protein studies, protein alterations can be characterized and drug targets identified. Genome annotation can be achieved by integrating data obtained from a protein to confirm the existence of a particular gene, as it is impossible to accurately predict exon-intron structures of most genes (Dunham et al., 1999). Ascribing function to genome sequences has proven its difficulties, now protein identification within a genome will help infer function to genomes, as 3D protein structure is known (Burley et al., 1999). Proteomics facilitates the analysis of post-translational modifications. Using proteomics, a comprehensive view of the protein-protein interaction in a two-hybrid analysis of *Saccharomyces cerevisiae* was achieved. Similar studies can be done to study cell growth, death and other cellular cycles (Uetz et al., 2000).

1.6.2 Strengths and limitations of proteomics

Proteins are separated in the first dimension (1D) to resolve protein mixtures according to their molecular mass. 1D is reproducible, separates proteins between 10 and 300 kDa, and is simple to perform. However, with 1D, proteins can only be resolved in their purified form, which is the limitation of this technology. When crude samples are analyzed, the second dimension (2D) electrophoresis is used. 2D separates proteins according to their net charge and molecular mass, in the first and second dimensions respectively (Graves and Haystead 2002). This technique has the ability to resolve proteins that have gone through post-translational modification. The primary application of the 2D system is expression profiling, where samples qualitatively and quantitatively. Different types of samples (cell lines, whole organisms, tissues or body fluids like urine) can be used in this system. Over the years the 2D technique has been improved and one such improvement is the introduction of immobilized pH gradients that helped enhance the reproducibility of 2D gels (Görg et al., 2000). 2D separation speed has been enhanced and minigel formats can be used. Furthermore, automated efforts have also been made, from gel running to spot picking (Traini et al., 1998). Computers, scanners and software program use have helped in the detection, storage and annotation of images and identification and quantification of protein spots respectively. Despite its advantages, 2D analysis is time consuming and labor intensive. A single experiment spans over two days and one sample can be electrophoresed per gel. Proteins that are in the pH extremes are not represented, hydrophobic and large proteins are unable to enter the first dimension. 2D does not detect low-copy proteins in total-cell lysates, whereas in crude cell extracts abundant proteins dominate the gel (Graves and Haystead 2002).

1.6.3 Plant proteomics

In the past, although proteomics had been used to study agriculturally important crops, most of the studies generated in regard to developmental processes and stress response mechanisms were gained from Arabidopsis and rice (van Wijk, 2001), due to the availability of their completed genome sequences. Genome sequences are invaluable resources in proteomics, as they provide protein identification tools. In instances where fully annotated sequences are unavailable, protein identification is done using homologous proteins of closely related species (Carpentier et al., 2008). Otherwise, expressed sequence tags (ESTs), that represent a segment of a gene sequence can also be used (Aebersold and Goodlett, 2001). For plant species with insignificant amounts of readily available genomic DNA or EST sequences, success rates in protein identification are lowered, meaning there is limited proteomic data. Rice, maize and sorghum are the only cereal crops that are fully sequenced (Graves and Haystead, 2002). The latter offer priceless tools for gene and protein identification that could be applied in plant breeding approaches for abiotic and biotic stress tolerance and yield increase (Salekdeh and Komatsu, 2007).

1.6.4 Proteomics as a tool to study stress responses in plants

Although numerous genomes have been sequenced, the information supplied is insufficient to provide details regarding plants ability adapt to stress, regulatory biology and gene function. It is therefore imperative to use tools that tackle analysis both quantitatively and qualitatively (Timpero et al., 2008). These are commonly done at transcriptomic, metabolic and proteomic levels. Abiotic stresses that yield cellular dehydration like, freezing, salt and water stress have a cross talk in regard to signaling pathways and have similar changes in plant gene expression and metabolism (Seki et al., 2001; Kreps et al., 2002; Rabbani et al., 2003; Cook et al., 2004; (Sung et al., 2001)Since the amount of mRNA produced is inversely proportional to that of cellular

proteins; taking into account post translational modification of proteins, this makes proteomics the choice of study (Futcher et al., 1999; Gygi et al., 1999). Additionally, changes in proteins are rapid within cells/tissues as influenced by certain modifications like, cell cycle, external stimuli, physiological states and tissue studied. Owing to these, proteomics is thus the tool of choice when analyzing biochemical pathways and the complex response of plants to environmental stimuli. Using proteomics to interrogate plants comparatively in regard to stress response, using the proteome, a link between the transcriptome and metabolome is established (Cook et al., 2004; Gray and Heath, 2005). A study investigating the response of rice leaves to high temperature stress obtained 48 differentially expressed proteins, from samples obtained after 12 or 24 hours exposure, from these, 18 were heat shocked proteins (Lee et al., 2007). A 2D comparative drought stress and recovery study was done using tall wheatgrass at its vegetative stage. In this study 58 of 600 reproducible and significantly changed proteins following stress were identified. Of these, 7 were chaperones and oxidative defense enzymes, while a new protein was identified as a heat shock protein.

1.7 Production of heat shock proteins in response to abiotic stress

Heat-shock proteins (HSP) and late embryogenesis abundant (LEA) proteins, commonly respond to desiccation tolerance as a result of stresses such as salinity, flooding, cold, high temperature stress and drought (Gazanchian et al., 2007). It is widely known that HSPs are molecular chaperones, which have strong cytoprotective effects and maintaining functional proteins in their functional conformations thus preventing aggregation of non-native proteins. HSPs are located in different places within the plant cell and different classes are expressed in response to varying stresses (Figure 1.4).

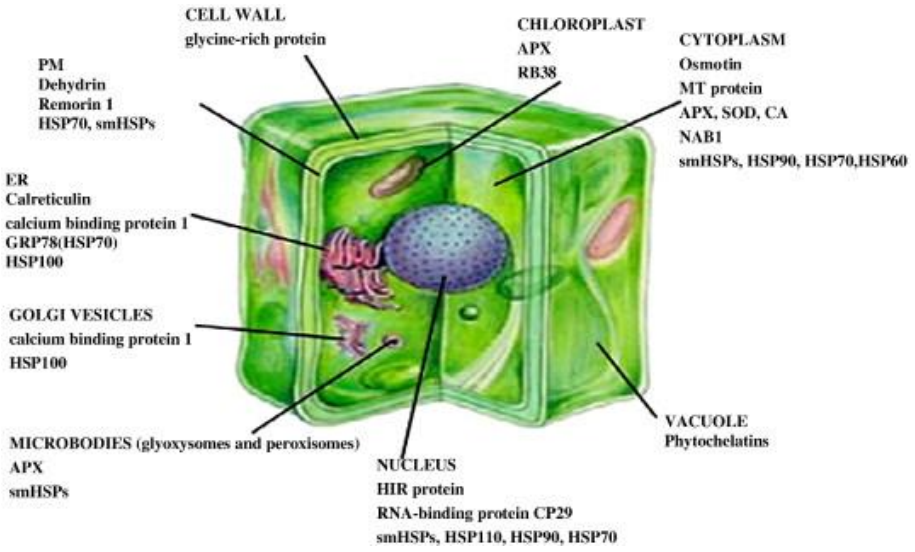

Figure 1.4 Locations of major stress-related proteins in the plant cell. Figure adapted from Timperio et al. (2008).

Hsp70 members are expressed in response to abiotic stresses like heat, cold, chemicals and drought (Guy and Li, 1998; Timperio et al., 2008) and function as molecular chaperones. They are composed of two prominent functional domains, an N-terminal portion with an ATPase domain (40 kDa) and a peptide binding domain (25 kDa) in the C terminus. These domains are divided by a hinge region that is protease cleavage susceptible. Since Hsp70s are found in the mitochondria, plastids, cytosol and endoplasmic reticulum (ER), they have a transit peptide in the N-terminus, which is a precursor for members that are to be imported into organelles. Other HSP70s have a C-terminal subdomain of approximately 5 kDa that is used for co-chaperone interactions (Sung et al., 2001). Heat is induced by the binding of heat shock transcription factors (Hsfs) to heat shock elements (Hses) in the promoter region. The Hsf/Hsp70 autoregulates the expression of Hsp70 and other heat shock genes in response to heat shock. During unstressed conditions, Hsp70 may remain unbound or binds to inactive Hsf, while in heat shock conditions, free Hsp70s are exhausted after binding to heat denatured proteins, thus freeing HSF from

Hsp70. Nucleic Hsf activates the transcription of Hsp70, causing an increase in Hsp70 protein. Surplus Hsp70 proteins capture newly made HSF before it gets to the nucleus and form a trimeric structure which lessens the heat induced expression of Hsp70 and other heat shock proteins (Sung et al., 2001).

HYPOTHESIS

Hyperosmotic stress caused by drought and salinity treatments would alter Sugargraze sorghum physiology and mediate changes in biochemical parameters and proteome profile.

AIMS OF THIS STUDY

The aim of this study was to investigate the influence of hyperosmotic stress (as a consequence of drought and salinity treatments) on the physiological and biochemical parameters of Sugargraze sorghum plants. Furthermore, the study also explored the identification of stress responsive proteins following drought and salinity treatments using 2-DE PAGE analysis coupled with mass spectrometry that could be used as potential candidates for genetic engineering towards the development of drought and salt stress tolerant crops.

CHAPTER 2

MATERIALS AND METHODS

2.1 General chemicals and suppliers

Table 2.1 List of chemicals used in this study

Chemicals	Suppliers
Acetic acid	KIMIX
Acetone	Merck
Acetonitrile	Merck
Acrylamide/Bis	BIO-RAD
Agarose	White Scientific
Ammonium Bicarbonate	Merck
Ammonium Persulfate	BIO-RAD
Bovine Serum Albumin (BSA)	Roche
Bradford Reagent	BIO-RAD
Bromophenol blue	Sigma Aldric
Carrier Ampholytes	BIO-RAD
3-[(3-Cholamidopropyl)dimethylammonio]-1-Propanesulfonate CHAPS	Sigma Aldrich
Coomassie Brilliant Blue	Sigma
Dithiothreitol (DTT)	Fermentas
Dipotassium phosphate	Sigma Aldrich
Dimethylsulfoxide (DMSO)	Pierce biotechnology
Ethanol	KIMIX
Ethylenediaminetetraacetic acid	Sigma
Glucose	Merck

Glycerol	Merck
Glycine	BIO-RAD
Hydrocloric acid (HCl)	Merck
Hydrogen peroxide	Sigma
Iodoacetamide (Iodo)	Sigma
Isopropanol	KIMIX
Mineral oil	BIO-RAD and GE health care
Molecular weight marker	Fermentas
Ninhydrin	Pierce biotechnology
Potassium cyanide	Sigma
Riboflavin	Merck
Sodium chloride (NaCl)	Merck
Sodium dodecyl sulphate (SDS)	BIO-RAD
N,N,N',N'Tetramethylethylenediamine (TEMED)	Sigma
Thiourea	Sigma Aldrich
Tris-HCl	Aldrich
Urea	Sigma

2.2 General Stock Solutions and Buffers

80% acetone: 80% (v/v) acetone in distilled water.

0.5% agarose sealing solution: 0.5% (w/v) agarose prepared in 1X SDS-PAGE running buffer with a tint of bromophenol blue.

10% APS: 10% (w/v) APS in distilled water. The solution was freshly prepared before use.

0.5% blocking solution: 0.5% (w/v) Elite fat free instant milk powder in TBS.

1% blocking solution: 1% (w/v) Elite fat free instant milk powder in TBS.

Bradford reagent: 1 part BIO-RAD Protein Assay dye reagent concentrate diluted with 4 parts distilled water.

5 mg/ml BSA stock solution: 5 mg/ml BSA in urea buffer.

1.25% (w/v) CBB stock solution: 1.25% (w/v) CBB R-250 in distilled water.

CBB staining solution I: 50 ml of 1.25% (w/v) CBB stock solution, 10% (v/v) glacial acetic acid and 25% (v/v) propan-2-ol in distilled water.

CBB staining solution II: 6.25 ml of 1.25% (w/v) CBB stock solution, 10% (v/v) glacial acetic acid and 10% (v/v) propan-2-ol in distilled water.

CBB staining solution III: 6.25 ml of 1.25% (w/v) CBB stock solution and 10% (v/v) glacial acetic acid in distilled water.

Destaining solution: 10% (v/v) acetic acid and 1% (v/v) glycerol in distilled water.

Displacing solution: 0.375 M Tris-HCl, pH 8.8, 50% (v/v) glycerol with a tint of bromophenol blue.

50% DTT: 50% (w/v) DTT in urea buffer.

Evans blue stock solution: 0.5% (w/v) in distilled water.

Equilibration base buffer: 6 M urea, 2% SDS, 0.05 M Tris-HCl, pH 8.8 and 20% (v/v) glycerol in distilled water.

70% ethanol: 70% (v/v) ethanol in distilled water.

0.1 M HCl: 0.1 M HCl in distilled water.

100 mM NaCl: 100 mM NaCl in distilled water.

Seed sterilization solution: 0.35% commercial bleach

2X SDS sample loading buffer: 60 mM Tris pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 200 mM DTT, 0.025% (w/v) bromophenol blue.

1X SDS-PAGE running buffer: 25 mM Tris, 192 mM glycine containing SDS.

10% TCA: 10% (w/v) TCA in acetone.

Tris-buffered saline (TBS): 50 mM Tris and 150 mM NaCl, pH 7.5.

TBST: TBS containing 0.1% (v/v) Tween 20.

Transfer buffer: 25 mM Tris, 192 mM glycine and 20% (v/v) methanol in distilled water.

0.5 M Tris-HCl, pH 6.8: 0.5 M Tris in distilled water adjusted to pH 6.8 with concentrated HCl.

1.5 M Tris- HCl, pH 8.8: 1.5 M Tris in distilled water adjusted to pH 8.8 with concentrated HCl.

Urea buffer: 9 M urea, 2 M thiourea and 4% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS). The solution was kept at -20ºC.

2.3 Plant growth

Sugargraze (*Sorghum bicolor (L) Moench*.) seeds obtained from Klein Karroo, Outshoorn, South Africa were surface-sterilized in 0.35% sodium hypochlorite for 10 minutes and then rinsed four times with sterile distilled water. Seeds were imbibed in sterile distilled water for 30 minutes and sown in 2 litres of pre-soaked (distilled water) nutrient rich, weed-free potting soil, in 20 cm diameter plastic pots. The soil was kept moist by irrigation with distilled water during germination. Germinated seedlings (one plant per pot) were grown on a 25/19 °C day/night temperature cycle under a 16/8 hours light/dark cycle, at a photosynthetic photon flux density of 300 μmol photons.m^{-2}.s^{-1} during the day phase. All plants were irrigated with distilled water until the V3 stage (four fully expanded leaves and one emerging leaf). Plants at the same stage (V3) of development and similar height were selected for all subsequent experiments.

2.4 Treatment of plants

Treatments were initiated on plants when they reached the V3 stage of vegetative growth. Control plants (referred to as Untreated) at the V3 stage was supplied with distilled water at intervals of three days for a total of 16 days. For the NaCl treatments (over a period of 16 days); plants at the V3 stage were supplied with distilled water that was supplemented with 100 mM NaCl (NaCl, regarded as salt stress) at intervals of three days for a total of 16 days. For the drought treatments (water deficit), water was withheld from the plants at the V3 stage for the duration of the experiment (over a period of 16 days).

2.5 Analysis of growth

After 16 days of treatment, 10 plants from each treatment were carefully removed from the soil to avoid any loss of material and scored for fresh and dry weights of the leaves.

2.6 Proline content analysis

Proline content was estimated using a modified method described by Khare et al. (2012). Fresh leaf samples from Sugargraze (0.1 g each) were ground to a fine powder using a mortar and pestle and homogenized in 0.5 ml of 3% (w/v) sulphosalicylic acid. The homogenate was centrifuged at 6,000 rpm for 15 minutes. About 0.2 ml of each homogenate was combined with 0.2 ml of glacial acetic acid to which 0.2 ml of ninhydrin was added. The reaction mixture was boiled in a water bath at 100°C for 30 minutes and immediately cooled in an ice bath. After cooling the reaction mixture, 0.4 ml of toluene was added. After thorough mixing, the chromophore containing toluene was separated and the absorbance measured at 520 nm against a toluene blank using a UV-visible spectrophotometer (PolarStar omega, BMG Labtech).

2.7 Measuring relative water content (RWC)

The leaves used in this assay were at the same developmental stage (V3) as those leaves collected for cell viability. The fresh weight (FW) of the leaves was determined before and after overnight incubation in a 1L water-filled glass cylinder (closed) at room temperature. Leaves were then dried for 72 hours at 70°C in an oven, to determine the dry weight (DW). The relative water content of leaves for the different treatments was measured using the formula described by Claussen (2005).

2.8 Chlorophyll content analysis

Estimation of chlorophyll content was based on a modified method from Hiscox and Israelstam (1980). Freshly harvested Sugargraze leaves were cut into pieces of approximately 0.5 cm^{-2}. Leaf tissue (100 mg per plant) was mixed with 0.5 ml of dimethylsulfoxide (DMSO) and incubated at 65°C for 3 hours. The absorbance of an aliquot of the leaf-DMSO extract (200 µl) was read at 645 nm and 663 nm, with DMSO being used as a blank. The chlorophyll content was calculated according to the formula described by Arnon (1949).

2.9 Evaluation of cell viability

A method described by Sanevas et al. (2007) was used to measure cell viability 16 days after treatment. Fresh leaf and root tissue (0.1 g per plant from three different plants of each treatment) were stained using 0.25% (w/v) Evans Blue dye at room temperature for of 15 minutes. The leaf and root tissue were washed for 30 minutes in distilled water to remove surface-bound dye. The dye absorbed by the dead leaf and root cells was extracted using 1% (w/v) SDS for 1 hour at 55°C. Absorbance of the extracts was measured at 600 nm to determine the level of Evans Blue up-take by the dead leaf and root tissue.

2.10 Preparation of protein extracts for biochemical analysis

Protein extracts were obtained from leaf, stem and root tissues, that were ground to a fine powder using liquid nitrogen and 0.1 g of tissue was homogenized in 1 ml of buffer [40 mM K_2HPO_4, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 5% (w/v) polyvinylpyrrolidone (PVP) molecular weight = 40 000] for determination of antioxidant enzymatic activities, or 1 ml of 10% trichloroacetic acid (TCA) for H_2O_2 content, lipid peroxidation levels. The homogenates were centrifuged at 12 000 rpm for 15 minutes and the supernatants were used for the respective biochemical assays. Protein concentrations were determined according to Bradford, (1976), using bovine serum albumin (BSA) as a standard.

2.11 Measurement of H_2O_2 content

H_2O_2 content was determined based on a method described by Velikova et al. (2000). The reaction mixture consisted of 75 µl of the TCA extract, 5 mM K_2HPO_4, pH 5.0 and 0.5 M KI. Samples were incubated at 25°C for 20 minutes and absorbance readings of samples were measured at 390 nm. H_2O_2 content was calculated based on a standard curve constructed from the absorbance (A390 nm) of different H_2O_2 concentrations.

2.12 Measurement of lipid peroxidation

Products of lipid peroxidation (reflective of MDA content) were estimated as described by Buege and Aust (1978). For these measurements, 1 ml of TCA extract was mixed with 4 ml of 0.5% TBA (dissolved in 20% TCA). The mixture was heated for 30 minutes at 95°C and then cooled in an ice bath for 10 minutes. The specific absorbance of products was read at 532 nm and nonspecific background-absorbance at 600 nm was subtracted from the readings. The concentration of MDA was calculated using a molar extinction coefficient 155 mM^{-1} cm^{-1}.

2.13 Measuring APX and GR enzymatic activity

For all antioxidant enzyme activity assays, proteins were prepared using the homogenizing buffer described in section 2.10. APX activities were measured using a modified method described by Asada (1984). The leaf and root extracts (extracts supplemented with ascorbate to a final concentration of 2 mM) were added to the assay buffer (50 mM K_2HPO_4, pH 7.0, 0.1 mM EDTA, and 50 mM ascorbate). The reaction was initiated with 1.2 mM H_2O_2 in a final reaction volume of 200 µl and APX activity was calculated based on the change in absorbance at 290 nm using the extinction co-efficient of 2.8 $mM^{-1}cm^{-1}$.

GR activity was determined using a slightly modified method described by Esterbauer and Grill (1978) by following the rate of NADPH oxidation at 340 nm. The assay mixture contained: 0.2 mM NADPH, 0.5 mM GSSG, 1 mM EDTA in 100 mM K_2HPO_4 pH 7.8 and 50 µg of enzyme extract in a 200 µl reaction. GR activity was calculated based on the oxidation of NADPH in the reaction, using the extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

2.14 Detection of antioxidant isoforms in response to abiotic stress

2.14.1 Superoxide dismutase (SOD)

SOD isoforms were detected in sorghum leaves, roots and stems using native PAGE in 12% acrylamide mini gels using 80 µg of protein per sample. SOD activity was detected by staining with 0.5 mM riboflavin and 2.5 mM nitroblue tetrazolium, as described by Beauchamp and Fridovich (1971). SOD isozyme bands were determined by incubating the gels with 5 mM H_2O_2 to inhibit both Cu/ZnSOD and FeSOD, or with 5 mM KCN to inhibit only Cu/ZnSOD (Archibald and Fridovich, 1982). MnSOD activity is resistant to both treatments.

2.14.2 Ascorbate peroxidase (APX)

Electrophoretic APX separation and detection was carried out as described by Seckin et al. (2010) and native PAGE was performed at 4°C in 10 % polyacrylamide mini gels containing 10% glycerol. Prior to sample (60 µg) loading, gels were equilibrated with running buffer containing 2 mM ascorbate for 30 minutes at 4°C. Following electrophoresis, gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 20 minutes and then transferred to solutions containing: 50 mM potassium phosphate buffer (pH 7.8), 4 mM ascorbate and 2 mM H_2O_2 for 20 min. The gels were transferred to a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 16 mM N,N,N′,N′-Tetramethylethylenediamine (TEMED) and 2.5 mM NBT for 10-20 minutes with gentle agitation in the presence of light.

2.14.3 Glutathione reductase (GR)

GR isoforms was detected using a modified method described by Lee and Lee (2000). GR was visualized on the native PAGE gel by incubation in 50 ml of 0.25 M Tris–HCl buffer (pH 7.9) containing 4.0 mM glutathione disulfide (GSSG), 1.5 mM NADPH, and 2 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) for 20 minutes. The activity was negatively stained in the dark with a solution containing 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 1.6 mM phenazine methosulfate (PMS) for 5-10 minutes at 30°C.

2.14.4 Densitometry analysis

Densitometry analysis was done on all the native PAGE gels after image acquisition, using the Spot Denso tool (AlphaEase FC imaging software V4, Alpha 88 Innotech Corporation). The enzymatic activity (for the respective antioxidant enzymes) of each isoform in the treatments was

scored as an average of the relative pixel intensities (by assigning a value of 1 for the isoform with the lowest pixel intensity in that type of isoform and expressing the rest of the pixel intensities for that type of isoform in the other treatments relative to this isoform, for example: if MnSOD1 has the lowest pixel intensity in the NaCl treatment, then this pixel intensity was assigned a value of 1 and all the pixel intensities for MnSOD1 in the rest of the treatments will be expressed relative to the pixel intensity of MnSOD1 in the NaCl treatment) from three independent gels and expressed in arbitrary units.

2.15 Profiling the leaf proteome of Sugargraze sorghum

2.15.1 Sample preparation for proteomics analysis

Sorghum leaves were ground to a fine powder with mortar and pestle and the protein was precipitated in 10% (w/v) TCA, the homogenate was then briefly vortexed and centrifuged at 13 400 rpm for 10 minutes. Thereafter, 80% (v/v) ice cold acetone was used to wash the homogenate thrice. The homogenate was then vortexed and centrifuged at 13 400 rpm. The pellet was air dried for about 15 minutes at room temperature and re-suspended in urea buffer (9 M urea, 2 M thiourea and 4% CHAPS), for an hour or more while vortexing vigorously at room temperature. Protein concentrations were determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard.

2.15.2 One dimensional polyacrylamide gel electrophoresis (1D-PAGE)

Proteins were prepared using a 1:1 ratio of protein and 2x reducing buffer. Samples were then heated at 95°C for 3 minutes and separated by 1D PAGE as described in the section below.

1D PAGE was used to resolve proteins according to their molecular weight with the aid of the Mini-Protean III® Cell gel casting system (Bio-Rad). Resolving 12% gels (40% Acrylamide/Bis

stock solution (37:5:1); 1.5 M Tris-HCl, pH 8.8; 0.5 M Tris-HCl, pH 6.8; 10% SDS; 10% APS, 16 mM TEMED) were gently poured between two glass plates according the manufacturer's manual (Bio-Rad). The liquid gel was overlaid with isopropanol and allowed to polymerize for about 30 minutes. When this time had elapsed, the isopropanol was decanted and the plates rinsed with dH_2O. A 5% stacking gel (40% Acrylamide/Bis stock solution (37:5:1); 0.5 M Tris-HCl, pH 6.8; 10% SDS; 10% APS, 16 mM TEMED) was prepared and poured on top of the resolving gel and a 10-well comb was inserted to form wells necessary to load the samples. The gel was allowed to polymerise for about 15 minutes. Gels were placed into the buffer tank according to the manufacturer's guide (Bio-Rad). Upon polymerisation, the combs were removed, the tank was filled up with 1X running buffer and the samples loaded. Gels were electrophoresed at 120V until the dye reached the bottom of the gel for approximately 90 minutes.

2.15.3 Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

2.15.3.1 Sample preparation for rehydration of IPG strips

2D-PAGE was used to resolve proteins according to their isoelectric point (pI) and their molecular weight (O'Farrell, 1975). Prior to 2D-PAGE analysis, protein samples (100 µg) were mixed with 1.25 µl of 10X ampholytes and 0.2% (w/v) DTT and made up to a final volume of 125 µl with urea lysis buffer. Samples were then loaded onto a re-swelling tray. Forceps were then used to gently position the IPG strip (7cm long, pH range 4-7) on top of the sample, avoiding the formation of air bubbles. The strip was overlaid with mineral oil to prevent evaporation of the sample and allowed to passively rehydrate overnight at room temperature.

2.15.3.2 Isoelectric focusing (IEF) of the IPG strip

After rehydration, the IPG strips were rinsed with dH_2O, blotted on tissue paper to remove excess water and each placed on a focussing platform, with the gel side facing downwards. Pre-wet electrode pads were positioned at each end of the strips. The strips were overlaid with mineral oil and focused with the IEF program as described in Table 2.2.

Table 2.2 Isoelectric focusing parameters for 7 cm IPG strips.

Step	**Voltage**	**Duration**
Step 1	250	0.15h
Step 2	4000	1:00h
Step 3	4000	12,000 Vhrs

2.15.3.3 Equilibration of IPG Strips

After IEF, IPG strips were equilibrated in SDS-containing buffers in order to solubilise focused proteins and allow SDS binding prior to second dimension SDS-PAGE. The focused IPG strips were incubated gel side up in re-swelling tray channels containing 2.5 ml equilibration buffer [6 M urea, 2% (w/v) SDS, 50 mM Tris/HCl, pH 8.8 and 20% (v/v) glycerol], firstly containing 2% (w/v) DTT for 15 minutes followed by 2.5% (w/v) iodoacetamide for another 15 minutes with gentle agitation at room temperature. After equilibration, the isoelectric focused proteins were ready for separation on second dimension SDS-PAGE as described in section 2.15.3.

2.15.3.4 Second dimension by SDS-PAGE analysis

The gels were prepared as outlined at section 2.15.2, whereas the stacking gel was omitted. Each strip was then placed on top of the SDS-PAGE and overlaid with melted sealing agarose gel. The

agarose was allowed to settle prior to placing the gels into the buffer tank. Gels were electrophoresed at 200V for 35 minutes, until the bromophenol blue dye (indicator dye) reached the bottom of the gel.

2.16 Coomassie Brilliant Blue (CBB) staining

The gels were carefully removed from the plates and placed into storage boxes. In these, Coomassie staining solution I was added and left overnight. The following day, Coomassie staining solution I was decanted and the gels were immersed in Coomassie staining solution II, this was left for 30 minutes, decanted and Coomassie staining solution III was added to the gels. This step was also done for 30 minutes. After this, the third stain was decanted and the Coomassie destaining solution was added to the gels. The destaining solution was maintained until the protein versus background ratio appropriate for visualization was obtained. Gels were imaged using a PharosFX plus molecular imager scanner (Bio-Rad).

2.17 2D SDS PAGE comparative analysis

2D SDS-PAGE analysis was done using PDQuest™ Advanced 2D analysis software version 8.0.1 Build 055 (BIO-RAD). 2D gels were imaged using the Molecular Imager PharosFX Plus System (BIO-RAD) and analysed according to the PDQuest™ Advanced 2D Analysis Software user manual (BIO-RAD). All analyses in experiments were made using three biological replicates per treatment group. The gels were normalised with the aid of the local regression model compensating for gel to gel differences in spot quantities due to non-expression related variations. Before differential protein expression was done, spots were manually edited using the consensus tool to obtain spot expression consensus across all biological replicates in treatment groups. Differentially expressed protein spots were statistically significant using the Student's t-

test at a 95% significance level. Protein spots of interest were manually picked using pipette tips for identification by MS/MS.

2.18 Western Blot analysis for Heat Shock Protein 70 (Hsp 70)

Western Blot analysis for Hsp70 on Sugargraze sorghum leaf protein extracts separated on a 1-DE gel were performed as described by Ndimba et al. (2010). The 1-DEgel were not stained with CBB, instead it was pre-equilibrated overnight in cold transfer buffer at 4°C.

A 7 cm by 9 cm polyvinylidenedifluoride (PVDF) membrane (Hybond-P PVDF membrane, GE Healthcare) was activated by immersion in 100% isopropanol for 30 seconds. The PVDF membrane was then immersed in cold transfer buffer with six 7 cm x 9 cm pieces of filter paper and incubated at room temperature for 15 minutes. A gel-membrane sandwich was prepared by placing three pieces of the filter paper on the electrode cassette of the Transblot® Electrophoresis Transfer Cell (BIO-RAD). The PVDF membrane was then placed on top of the filter paper. The 1DE SDS gel was placed on top of the membrane and three layers of filter paper placed on top of the gel. Bubbles were eliminated by rolling the sandwich with a 2 cm stripette. The transfer was performed at 24V for 20 minutes.

After protein transfer, the membrane was placed in 1% (w/v) blocking buffer (Elite fat free instant milk powder in 1X TBS) for 1 hour. The blocking solution was removed and the membrane incubated in primary antibody (Human HeLa cells anti-Hsp 70 monoclonal antibody raised in mouse; Biomol International LP) diluted 1:1000 in 1% (w/v) blocking solution. The membrane was washed three times with agitation for 5 minutes with TBST (1X TBS containing 0.1% (v/v) Tween 20). After the wash, the membrane was incubated for one hour with the secondary antibody (goat anti-mouse IgG (H and L) Horseradish peroxidase conjugated

(Invitrogen corp., Carlsbad, CA, USA) diluted 1:1000 in 1% (w/v) blocking solution. After incubation, the membrane was washed three times in TBST for 10 minutes per wash with slight agitation at room temperature. The heat shock proteins were detected with Clarity™ Western ECL Substrate Lumino/enhancer and Clarity™ Western ECL peroxide solution (BIO-RAD). The two solutions were prepared in a 1:1 ratio and 2 ml of the mixture was transferred onto the membrane. The membrane was imaged with the UVP BioSpectrum® Imaging System (Ultra Violet Productions, Cambridge, UK).

2.19 Statistical analysis

The data was analyzed using the one-way analysis of variance (ANOVA) and tested for significance by the Tukey-Kramer test at 5% level of significance, using GraphPad Prism 5.03 software.

CHAPTER 3

HYPEROSMOTIC STRESS MODULATES THE ACCUMULATION OF ROS, PROLINE AND ANTIOXIDANT CAPACITY IN SUGARGRAZE SORGHUM PLANTS

3.1 Introduction

Hyperosmotic stress as a result of drought and salinity are one of the major environmental factors that limit worldwide productivity and distribution of cereal crops. During plant growth, increased salt stress levels have been shown to induce water stress caused by an increase in osmotic potential. This also resulted in increased levels of ions or other plant toxins in both soil and plant tissues (Hale and Orcutt, 1987; Howard and Mendelssohn, 1999). Salt stress can also trigger various interacting events including the inhibition of enzyme activities in metabolic pathways (Binzel and Reuveni, 1994; Tsugane et al., 1999). However, the complete response of plants to salt stress has not been systematically elucidated. Drought and water stress in plants triggered by salt stress result in the generation and accumulation of reactive active oxygen species, such as superoxide (O_2^-), hydroxyl radicals (·OH), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2). These oxygen species are thought to play an important role in inhibiting plant growth, and to maintain normal growth therefore these oxygen species have to be carefully regulated. The regulation of ROS accumulation may be modulated by non-enzymatic and enzymatic antioxidant constituents. Proline is considered a non-enzymatic compatible solute that accumulates in many plant species under a broad range of stress conditions such as water shortage, salinity, extreme temperatures, and high light intensity (Aspinall and Paleg, 1981; Hare et al., 1999; Mansour, 2000). It has been shown to play a major role in osmotic adjustment in plants such as in potato (Büssis and Heineke, 1998) whereas in species like tomato (Pérez-Alfocea et al., 1993) it accounts for only a small fraction of the total concentration of

osmotically active solutes. There is still some controversy surrounding the contribution of proline to osmotic adjustment and tolerance of plants that are exposed to unfavorable environmental condition (Molinari et al., 2007).

Alternatively, enzymatic constituents such as SOD, APX and GR play a vital role in the antioxidant defense system to neutralize the harmful effects caused by overproduction of active oxygen species (Foye et al., 1994). The primary scavenger in the detoxification of active oxygen species in plants is SOD, which protects plant cells against superoxide-induced oxidative stress (Asada and Kiso, 1973; Fridovich, 1986). Detoxifying cellular toxic products of SOD, such as H_2O_2, through the activity of the Asada-Halliwell scavenging cycle becomes important in the defense mechanisms against active oxygen species. The cycle found in the chloroplast and cytosol involves the oxidation and reduction of ascorbate and glutathione by the activation of APX and GR (Alscher and Hess, 1993; Foyer and Mullineaux, 1994). APX catalyzes the elimination of the toxic by-product of SOD at the expense of oxidizing ascorbate to monodehydroascorbate, and GR catalyzes the regeneration of ascorbic acid (Smirnoff, 1993).

Although several biochemical and physiological changes have been shown to be involved in the hyperosmotic acclimation process in sorghum plants, little is known about the responses of antioxidant enzymes against hyperosmotic stress (caused by drought and salinity) that induces the overproduction of active oxygen species. Therefore, in order to clarify the tolerant mechanism of antioxidant enzymes against hyperosmotic stress, we describe the changes of H_2O_2 and MDA contents as well as the physiological and biochemical changes in the amounts and activities of antioxidant enzymes in Sugargraze sorghum plants exposed to drought and salinity stress.

3.2 Hyperosmotic stress influences Sugargraze physiological and biochemical parameters

Sugargraze plants were grown and treated as described in section 2.3 and 2.4 respectively. The results showed that plants exposed to hyperosmotic stress caused by salinity and drought treatments resulted in a significant reduction of fresh and dry weights (Figure 3.1a-b). However, the reduction in biomass was more significant in the salinity treated plants when compared to the untreated control plants. The results were further supported by shoot length and root length measured in this study (Figure 3.1c-d).

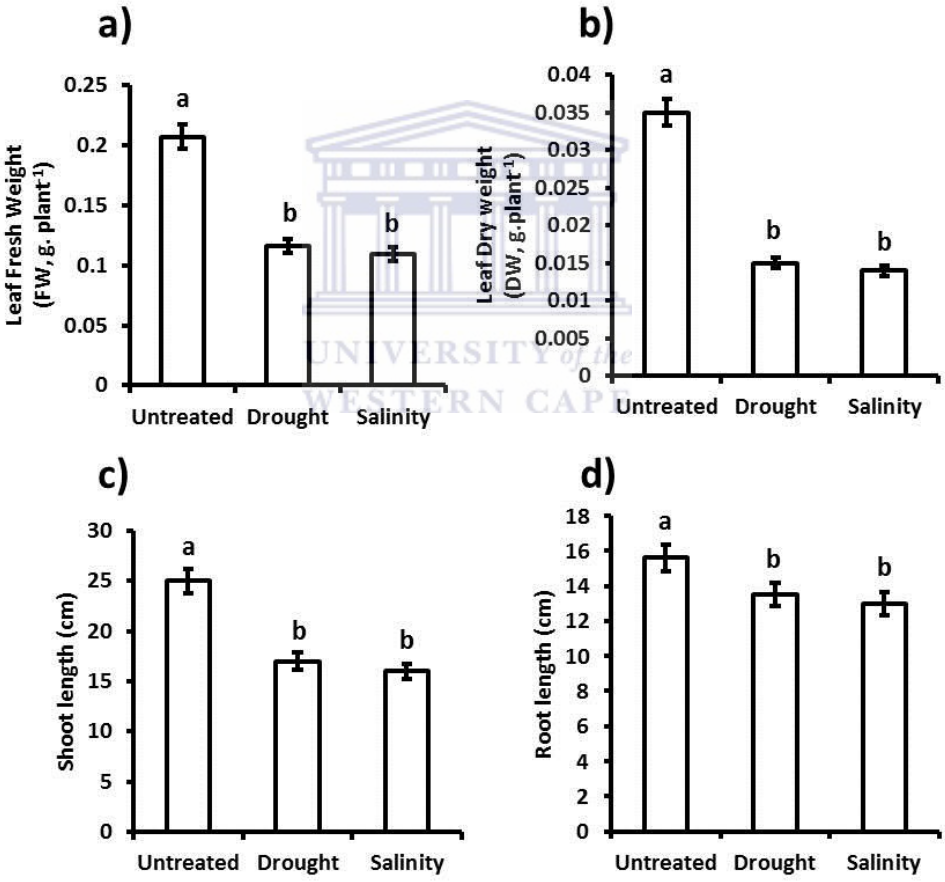

Figure 3.1 Hyperosmotic stress inhibits Sugargraze biomass. Measurements were done on Sugargraze sorghum plants treated at the V3 developmental stage for a period of 16 days. Biomass was evaluated by measuring **a)** leaf fresh weight, **b)** leaf dry weight coupled with **c)** shoot length and **d)** root length at the end of the treatment period. The error bars are representative of the mean (±SE) of three independent experiments from three plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

When compared to control plants, salinity and drought treated plants showed significant reduction in shoot and root length (Figure 3.1c-d). Similar patterns were observed in terms of relative water content (RWC), proline accumulation and chlorophyll content (Figure 3.2). These biochemical parameters were evidently affected as a result of hyperosmotic stress.

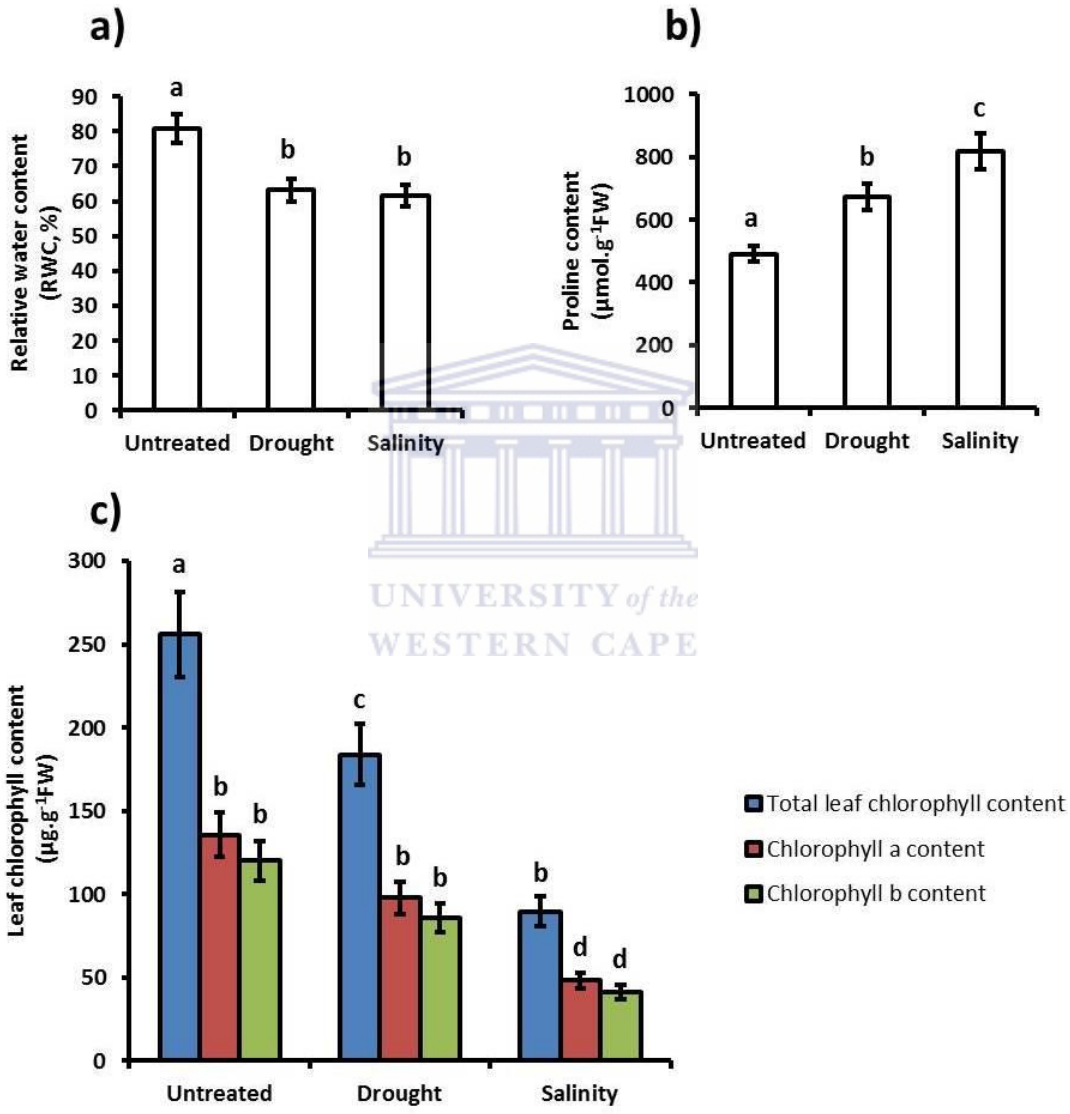

Figure 3.2 Drought and salinity influence the biochemical parameters of Sugargraze sorghum plants. Plants were exposed to water stress (drought) and salinity stress (100 mM NaCl; every third day) for a period of 16 days. **a)** Relative water content, **b)** proline content and **c)** total chlorophyll content was measured in Sugargraze leaf tissue. Error bars are representative of the mean (±SE) of three independent experiments from 6 plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

The RWC observed for Sugargraze leaves in response to hyperosmotic stress (caused by drought and salinity treatments) were approximately 22% lower than the untreated plants, with no significant difference observed between the treatments (Figure 3.2a). Proline, which is considered as a major osmoregulator in plants under various stresses and sought after compatible osmolyte, was also measured in this study. Both drought and salinity stress caused a significant increase in proline content with the highest increase being observed in the salinity treated plants (Figure 3.2b). The proline content in plants exposed to drought stress was 37% higher when compared to the untreated plants with an even higher increase of 66% observed in the salinity treated plants (Figure 3.2b). Chlorophyll pigments were drastically reduced in the treated plants (drought and salinity treatments) when compared to the untreated control plants (Figure 3.2c). The chlorophyll content in plants exposed to water stress condition showed a significant reduction of about 28% with an even higher reduction of 65% observed in the salinity treated plants (Figure 3.2c).

3.3 Hyperosmotic stress enhances oxidative damage in Sugargraze sorghum plants

The role of hyperosmotic stress (as a direct consequence of drought and salinity treatment) on ROS production, lipid peroxidation and the extent of cell death in Sugargraze sorghum plants were investigated. Previous literature has shown that drought and salinity-induced stress conditions have resulted in an increase in ROS accumulation that leads to oxidative damage in plants. This part of the study will illustrate how osmotic stress caused by drought and salinity treatments significantly increase the level of cell death, as a direct result of oxidative damage in the form of increased H_2O_2 content and elevated MDA levels (Figure 3.3). Hydrogen peroxide content and lipid peroxidation levels were measured as illustrated in section 2.11 and 2.12 respectively. The result showed that H_2O_2 content in both leaves and roots of Sugargraze

sorghum were significantly higher in the treated plants when compared to the untreated plants (Figure 3.3a-b). However, the leaves displayed a higher increase in H_2O_2 content compared to the roots when treated samples are compared to the control plants. The H_2O_2 content in Sugargraze leaves in response to drought and salinity were increased by approximately 112% and 124% respectively when compared to the control plants (Figure 3.3a).

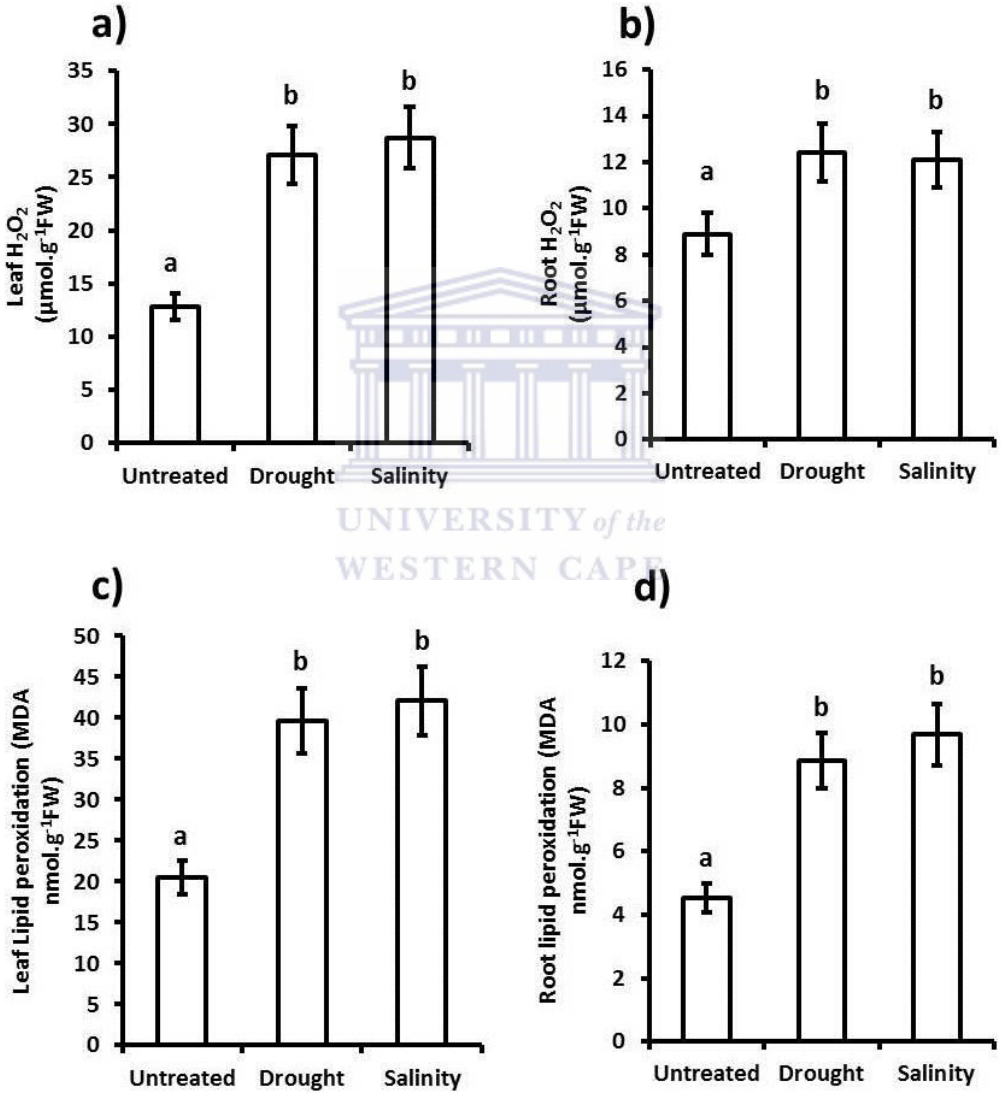

Figure 3.3 The extent of oxidative damage in Sugargraze leaves and roots in response to hyperosmotic stress. Experimental plants were exposed to water stress (drought) and salinity stress (100 mM NaCl; every third day) for a period of 16 days. H_2O_2 content **(a-b)** and lipid peroxidation levels **(c-d)** was measured in Sugargraze leaf and root tissue. Error bars are representative of the mean (±SE) of three independent experiments from 6 plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

The H_2O_2 content observed in the roots were about 39% for the drought treated plants and 36% for salinity treated plants (Figure 3.3b). These results were supported by the extent of lipid peroxidation (measured as MDA content) in response to drought and salinity treatments in this study (Figure 3.3c-d). The MDA content was monitored as an indicator of the level of peroxidation of lipids, which is considered as an important sign for the level of stress induced deleterious effects on plant cells and tissues, under stress conditions (Kumar et al., 2010). The MDA content in leaves was significantly influenced by the osmotic effect caused by drought and salinity treatments (Figure 3.3c-d). Leaf MDA levels were enhanced by approximately 94% in the drought treated plants compared to the control plants, whereas an even higher increase in MDA levels of approximately 106% was observed in the salinity treated plants (Figure 3.3c). A similar trend was observed in the roots of Sugargraze sorghum. Root MDA levels was approximately 98% higher in drought treated plants as opposed to the even higher increase (approximately 116%) measured in the salinity treated plants (Figure 3.3d).

3.4 Cell death is influenced by drought and salinity treatments in Sugargraze sorghum plants

The effect of the various treatments on Sugargraze leaf and root cell death was investigated because abiotic stress conditions have been shown to induce PCD and such PCD is linked to enhanced lipid peroxidation as illustrated in section 3.3, Figure 3.3c-d. For evaluating cell death, the extent of Evans Blue uptake (indicative of dead cells that take up the Evans Blue stain because of ruptured cell membranes, since living cells with intact cell membranes do not take up the stain) was measured in the leaf and root samples at the end of the 16 day treatments. The results showed that cell death evaluated in the drought treated leaf sample was increased by approximately 101% whereas an even higher increase of approximately 120% was observed in

the salinity treated leaf sample (Figure 3.4a). A similar trend in cell death was observed in the treated root samples (Figure 3.4b). The drought treatment increased cell death in Sugargraze roots by approximately 53% whereas the salinity treatment increased the extent of root cell death to approximately 56% when compared to the untreated control plants (Figure 3.4b).

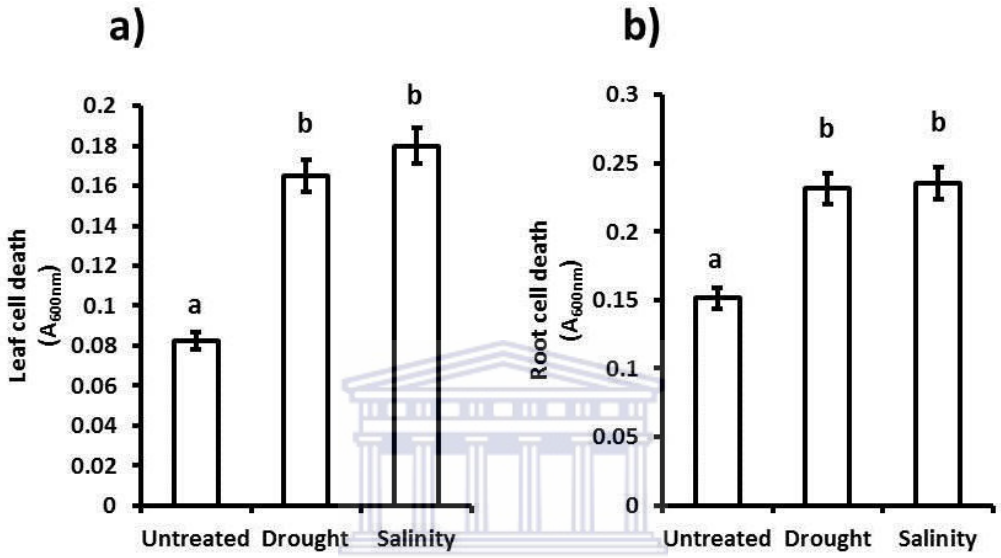

Figure 3.4 The extent of cell death in Sugargraze leaves and roots in response to hyperosmotic stress. Plants used in this assay was deprived of water (drought) and exposed to 100 mM NaCl (every third day) for a period of 16 days. The error bars are representative of the mean (±SE) of three independent experiments from 3 plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

3.5 Hyperosmotic stress modulates antioxidant enzyme activity in Sugargraze sorghum plants

3.5.1 The influence of hyperosmotic stress on SOD enzymatic activity

Since the discovery of SOD by McCord and Fridovich (1969), the enzyme has attracted the attention of many researchers because it is an essential component in an organism's defense mechanism against oxidative stress (Hamid et al., 2004). SOD is the first enzyme involved in the antioxidative process (Lee et al., 2001; Rubio et al., 2002). This enzyme converts the superoxide

radical (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Mhadhbi et al., 2004). This part of the study explores the influence of drought and salinity treatments on SOD enzymatic activity (different isoforms) in Sugargraze leaf and root samples. A total of 100 µg of leaf, stem and root protein for each sample was separated on a 12% non-denaturing polyacrylamide gel.

3.5.1.1 Leaf SOD activity

The existence of different SOD isoforms in Sugargraze leaf, stem and root samples was determined by using two SOD inhibitors (H_2O_2 and KCN) (Figure 3.5-3.7).

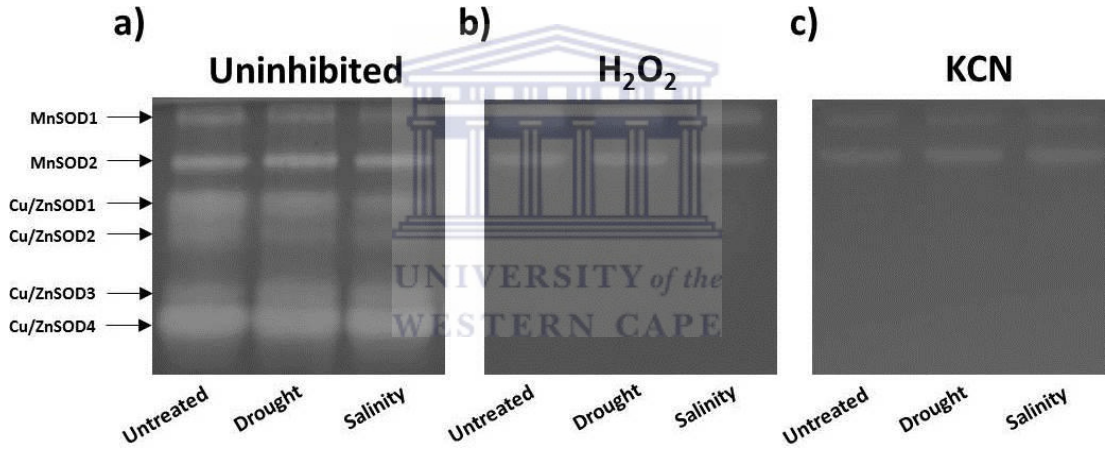

Figure 3.5 Drought and salinity stress differentially regulate SOD isoforms in Sugargraze leaves. Identification of SOD enzymatic activity in Sugargraze leaf samples using in-gel activity assays. Drought stressed plants were water deprived and salinity stress treated with 100 mM NaCl (every third day) for a period of 16 days. Individual SOD isoforms were identified by incubating native PAGE gels in **b)** 6 mM H_2O_2 (for FeSODs) and **c)** 6 mM KCN (for Cu/ZnSODs).

Analysis of these results suggests the existence of two MnSOD isoforms, four Cu/ZnSOD isoforms and no FeSOD isoforms in Sugargraze leaf samples. The enzymatic activity of the first two SOD isoforms was resistant to both H_2O_2 and KCN, which suggests that they are MnSOD isoforms, hence they were named MnSOD1 and MnSOD2 respectively (Figure 3.5a-c). The SOD activity of isoforms 4 to 6 (numbering from the top of the gel) was sensitive to both H_2O_2

and KCN, suggesting that they are Cu/ZnSOD isoforms; hence they were named Cu/ZnSOD1, Cu/ZnSOD2, Cu/ZnSOD3 and Cu/ZnSOD4 respectively. The activities of these isoforms were differentially regulated as illustrated by Figure 3.5. Densitometry analysis revealed that most of the isoforms detected in this study were unaffected in response to drought and salinity treatments (Table 3.1). However, the activity of four isoforms was significantly influenced by the various treatments (Table 3.1). These isoforms are MnSOD1 and Cu/ZnSOD1-3.

Table 3.1 Relative SOD activity in Sugargraze leaves

Relative leaf SOD activity (Arbitrary values)	Plant organs	Treatments	Sorghum SOD isoforms					
			1	**2**	**3**	**4**	**5**	**6**
	Leaves	**Untreated**	1.00 ± 0.05^{a}	1.00 ± 0.05^{a}	1.00 ± 0.05^{a}	1.00 ± 0.05^{a}	1.00 ± 0.05^{a}	1.00 ± 0.05^{a}
		Drought	0.9 ± 0.05^{a}	0.92 ± 0.05^{a}	0.95 ± 0.05^{a}	0.85 ± 0.04^{b}	1.10 ± 0.06^{b}	0.91 ± 0.05^{a}
		Salinity	0.81 ± 0.04^{b}	0.92 ± 0.05^{a}	0.80 ± 0.04^{b}	0.82 ± 0.04^{b}	1.09 ± 0.06^{b}	0.90 ± 0.05^{a}

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with the different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance according to Tukey-Kramer test.

The analysis showed that salinity inhibited the activity of MnSOD1 by approximately 19% whereas the drought treatment did not significantly influence the enzymatic activity of the isoform. A similar trend was observed for Cu/ZnSOD1, with a 20% reduction in isoform activity, whereas the drought treatment did not significantly influence Cu/ZnSOD1 activity when compared to the untreated plants. However, both drought and salinity treatments altered the enzymatic activity of Cu/ZnSOD2 by an approximately 15% reduction compared to the untreated plants. Amongst the six isoforms detected, in the leaf sample, Cu/ZnSOD3 was the only isoform which showed an increase activity in response to the various treatments. The enzymatic activities

of the remaining isoforms (MnSOD1 and Cu/ZnSOD4) were not influenced by the various treatments.

3.5.1.2 Stem SOD activity

Similar to what has been observed in the leaf sample the stem also produced six SOD isoforms. Due to sensitivity or resistance to H_2O_2 and KCN, isoforms identified were described as manganese (MnSOD1 and MnSOD2) and copper/zinc (Cu/ZnSOD1, Cu/ZnSOD2, Cu/ZnSOD3, Cu/ZnSOD4) (Figure 3.6a-c).

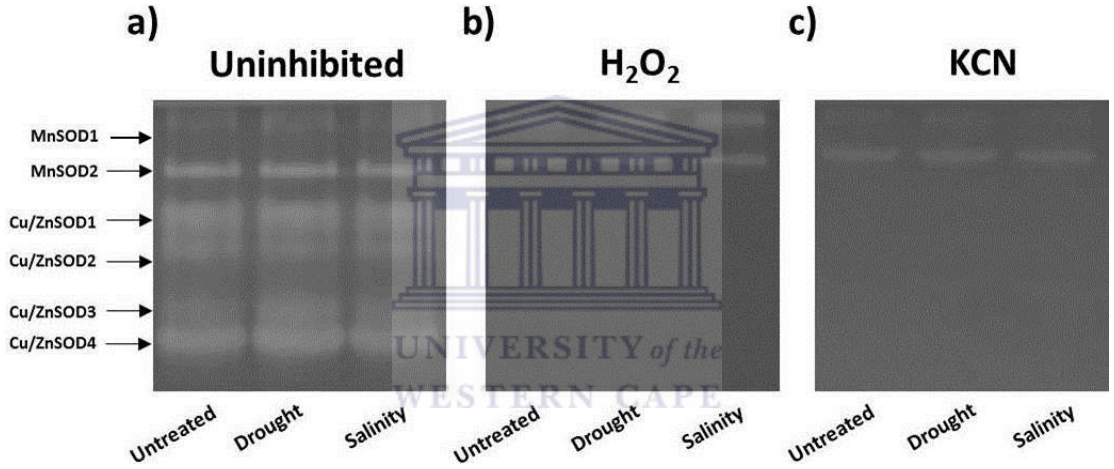

Figure 3.6 Sugargraze stem SOD isoforms detected in response to drought and salinity treatments. SOD activity was detected in Sugargraze stem samples using in-gel activity assays. Drought stressed plants were water deprived and salinity stress treated with 100 mM NaCl (every third day) for a period of 16 days. Individual SOD isoforms were identified by incubating native PAGE gels in **b)** 6 mM H_2O_2 (for FeSODs) and **c)** 6 mM KCN (for Cu/ZnSODs).

Densitometry analysis revealed that two SOD isoforms (MnSOD1 and Cu/ZnSOD3) were altered in response to drought treatment whereas salinity treatment did not influence the activities of these isoforms. A slight but significant increase in activity of MnSOD1 (9%) was observed whereas Cu/ZnSOD3 activity was increased by 13% as a result of the drought treatment. The remaining isoforms were not significantly influenced by either drought or salinity treatments (Table 3.2).

Table 3.2 Relative SOD activity in Sugargraze stem

Relative stem SOD activity (Arbitrary values)	Plant organs	Treatments	Sorghum SOD isoforms					
			1	2	3	4	5	6
	Stem	Untreated	1.00 ± 0.05[a]	1.00 ± 0.05[a]	1.00 ± 0.05[a]	1.00 ± 0.05[a]	1.00 ± 0.05[a]	1.00 ± 0.05[a]
		Drought	1.09 ± 0.06[b]	0.9 ± 0.05[a]	1.02 ± 0.05[a]	1.01 ± 0.05[a]	1.13 ± 0.06[b]	1.02 ± 0.05[a]
		Salinity	0.99 ± 0.05[a]	0.97 ± 0.05[a]	1.02 ± 0.05[a]	0.99 ± 0.05[a]	0.99 ± 0.05[a]	1.00 ± 0.05[a]

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with the different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance according to Tukey-Kramer test.

3.5.1.3 Root SOD activity

Separation of 100 µg of root sample from each treatment on a 12% non-denaturing polyacrylamide gel revealed the presence of only five SOD isoforms, which is less than what was observed in both leaf and stem samples (Figure 3.7a-c). Based on sensitivity and resistance to H_2O_2 and KCN, these isoforms were identified as two manganese (MnSOD1 and MnSOD2) and three copper/zinc (Cu/ZnSOD1, Cu/ZnSOD2 and Cu/ZnSOD3) SOD isoforms (Figure 3.7).

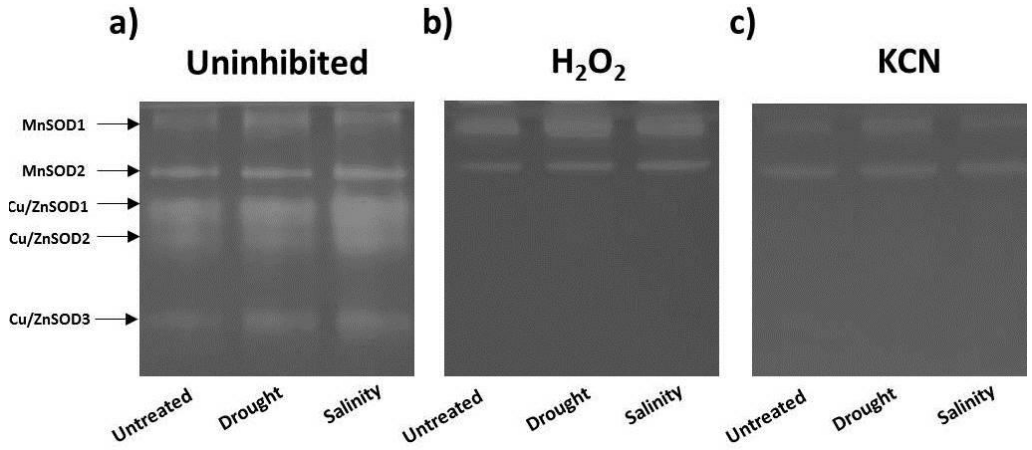

Figure 3.7 Sugargraze root SOD isoforms in response to drought and salinity treatments. Detection of SOD isoforms in Sugargraze roots using in-gel activity assays. Drought stressed plants were water deprived and salinity stress treated with 100 mM NaCl (every third day) for a period of 16 days. Individual SOD isoforms were identified by incubating native PAGE gels in **b)** 6 mM H_2O_2 (for FeSODs) and **c)** 6 mM KCN (for Cu/ZnSODs).

Contrary to what was observed in both leaf and stem samples, the various treatments seemed to significantly influence the enzymatic activity of SOD isoforms. The enzymatic activity of all five isoforms was enhanced in the treated samples when compared to the untreated plants.

Table 3.3 Relative SOD activity in Sugargraze roots

Relative root SOD activity (Arbitrary values)	Plant organs	Treatments	Sorghum SOD isoforms					
			1	**2**	**3**	**4**	**5**	**6**
	Roots	**Untreated**	1.00 ± 0.05[a]	1.00 ± 0.05[a]	1.00 ± 0.05[a]	1.00 ± 0.05[a]	1.00 ± 0.05[a]	
		Drought	1.20 ± 0.06[b]	1.01 ± 0.05[a]	1.01 ± 0.06[a]	1.04± 0.05[a]	1.14 ± 0.06[b]	
		Salinity	1.13 ± 0.06[b]	1.09 ± 0.06[b]	1.11 ± 0.06[b]	1.11 ± 0.06[b]	1.15 ± 0.06[b]	

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with the different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance according to Tukey-Kramer test.

The activity of MnSOD1 was increased by approximately 20% in response to drought whereas salinity showed a slight but significant increase in activity of approximately 13% when compared to the untreated plants. For MnSOD2, the enzymatic activity remained unchanged in response to drought treatment whereas salinity increased the activity by approximately 9% when compared to the untreated plants. Drought treatment did not influence the enzymatic activity of Cu/ZnSOD1, whereas salinity enhanced the activity by approximately 11%, compared to the untreated plants. A similar trend was observed for Cu/ZnSOD2 (Table 3.3). A significant increase in activity was observed for Cu/ZnSOD3 in response to both treatments. The activity of Cu/ZnSOD3 was increased by 14% in the drought treatment with an even higher increase of 15% observed in the salinity treatment.

3.5.2 Hyperosmotic stress alters the enzymatic activity of APX in Sugargraze plants

APX is highly responsive to various abiotic stresses and plays an important role in the scavenging of ROS in plants. This section will analyze the effect of drought and salinity stress on APX activity (total activity and individual isoforms) of Sugargraze leaf, stem and root samples using in-gel enzymatic activity assays (Figures 3.8-3.10) coupled with densitometry analyses (Figure 3.8-3.10). For each treatment, 60 µg of protein sample was separated on a 12% non-denaturing polyacrylamide gel.

3.5.2.1 Leaf APX activity

For Leaf APX activity, the results showed that four APX isoforms was detected in both untreated and drought treated samples with only three isoforms being present in the salinity treated samples. The results further show that the activity (as band intensities) of the different isoforms was differentially regulated by the various treatments (Figure 3.8a, c-f).

Densitometry analysis showed that water stress (drought) increased the enzymatic activity of three isoforms (APX1, APX3 and APX4) with a slight reduction observed in APX2 when compared to the untreated samples. The enzymatic activity of APX 1 and APX4 was increased by approximately 27%, with a slight but significant increase of 10% observed for APX3. However, the activity of APX2 was reduced by approximately 14% when compared to the untreated samples. Salinity, on the other hand significantly reduced total APX activity to a level significantly lower than the untreated samples (Figure 3.8b). This is supported by the reduction in enzymatic activity of APX2 (46%) and APX3 (no activity detected) (Figure 3.8d-e) coupled with the unchanged activity for APX1 (Figure 3.8c). APX4 was the only isoform whose activity was enhanced by approximately 26% in response to treatment with salinity (Figure 3.8f).

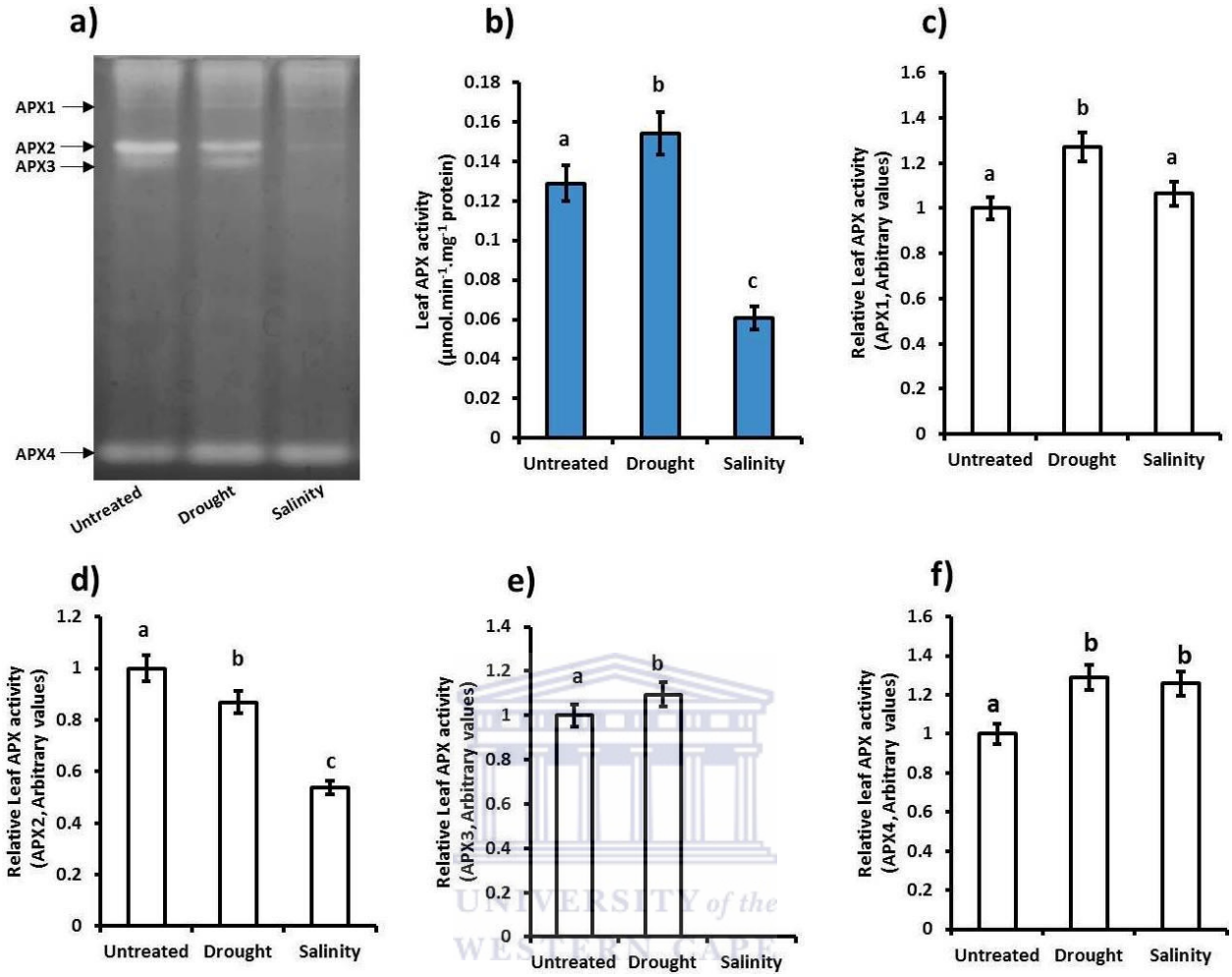

Figure 3.8 Sugargraze leaf APX activities in response to hyperosmotic stress caused by drought and salinity. Assays were done on Sugargraze sorghum leaves taken from plants that were exposed to water stress (drought) and salinity stress (100 mM NaCl; applied every third day) at the V1 stage for a period of 16 days (they were at the V3 stage at time of harvest).

3.5.2.2 Stem APX activity

For stem APX activity (Figure 3.9), salinity did not influence total APX activity when compared to the untreated samples. Apart from the 29% increase in activity for isoform 2 (Figure 3.9d), the other isoforms remain unchanged compared to the untreated samples (Figure 3.9c, e). However, drought treatment seems to significantly influence total APX activity. Drought reduced total APX activity by approximately 34% compared to the untreated and salinity treated samples.

However, the activity of only 1 isoform (APX1) was reduced by approximately 16% in response to treatment with drought whereas the remaining isoforms were unaffected.

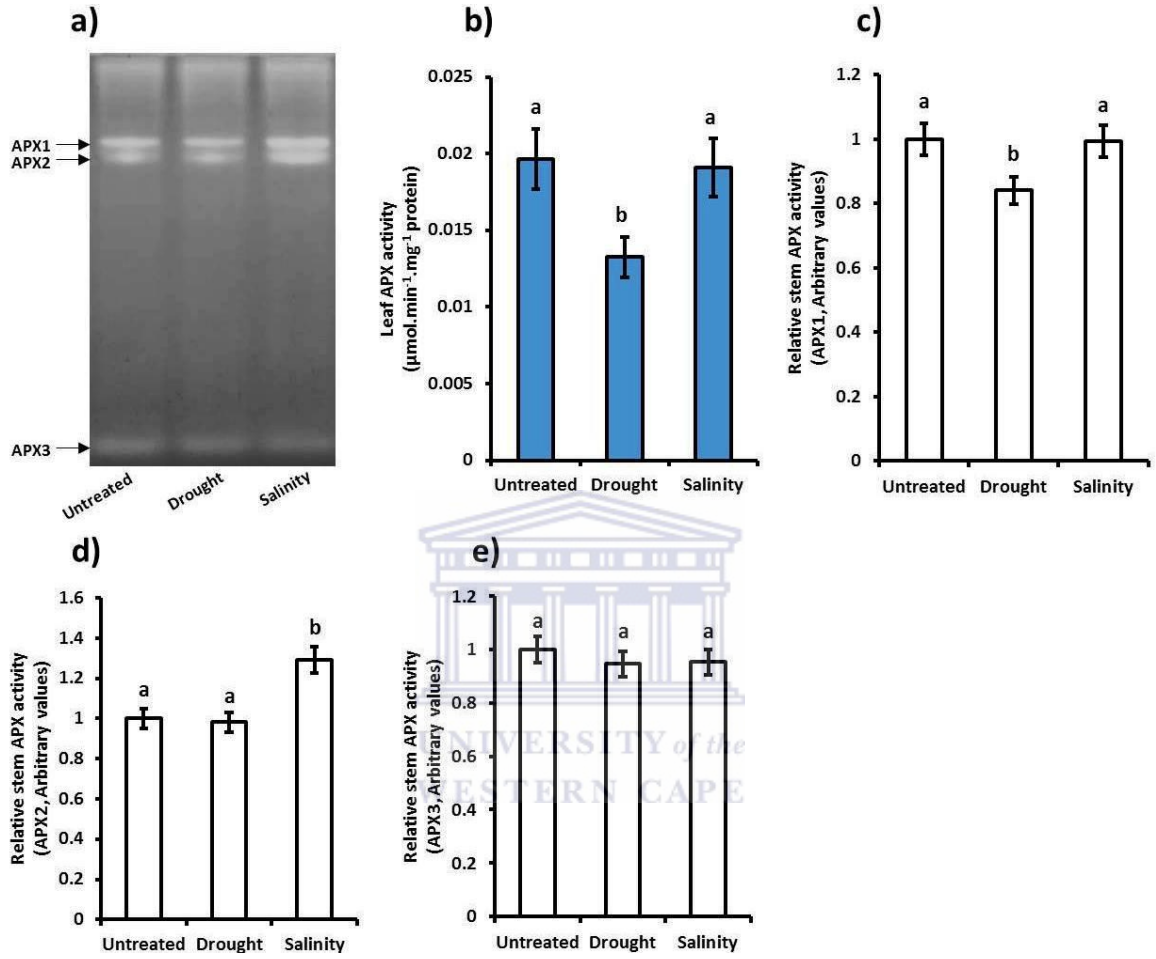

Figure 3.9 APX activity of Sugargraze stem in response to drought and salinity treatments. Assays were done on Sugargraze sorghum stem taken from plants that were exposed to water stress (drought) and salinity stress (100 mM NaCl supplied every third day) at the V1 stage for a period of 16 days (they were at the V3 stage at time of harvest).

3.5.2.1 Root APX activity

Root APX activity (as total activity and individual isoforms) was significantly influenced by drought and salinity treatments (Figure 3.10).

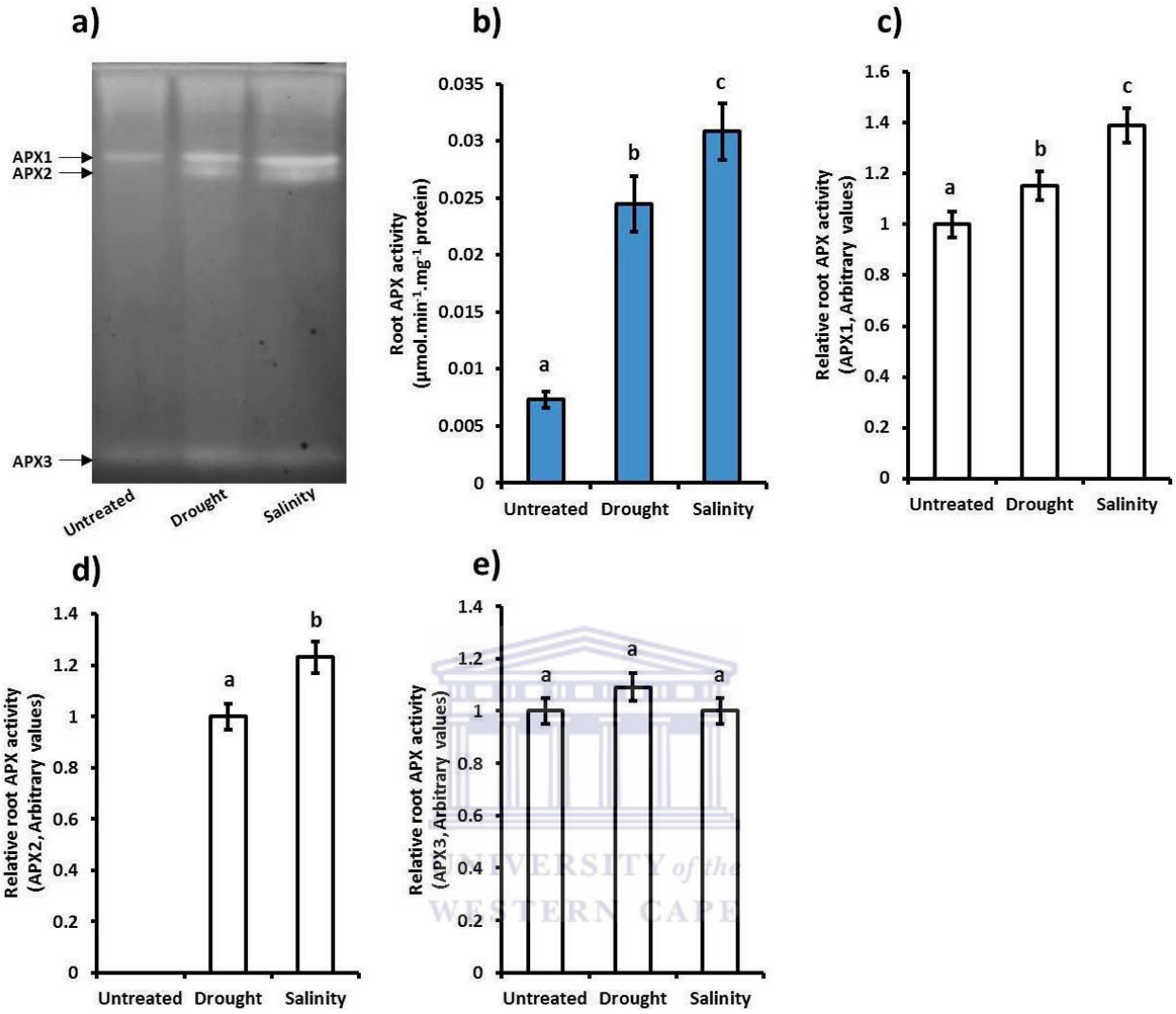

Figure 3.10 The effect of hyperosmotic stress on root APX activity. Assays were done on Sugargraze sorghum roots taken from plants that were exposed to water stress (drought) and salinity stress (100 mM NaCl supplied every third day) at the V1 stage for a period of 16 days (they were at the V3 stage at time of harvest).

In response to drought treatment root APX activity (total activity) was enhanced by approximately 243% with an even higher increase of 328% observed in response to salinity. The increase in total APX activity observed in both treatments was supported by the increase in activity of some APX isoforms (APX1 and APX2). In both treatments the enzymatic activity of APX3 was not significantly influenced when compared to the untreated samples.

3.5.3 GR enzymatic activity is influenced by drought and salinity treatments in Sugargraze plants

Although APX plays an important role in the H_2O_2 scavenging system, GR is also an essential contributor in the removal of H_2O_2 in order to maintain the redox state of ascorbate and glutathione (Foyer et al, 1994). In order to analyze the enzymatic changes of GR (as total activity and individual isoforms) in response to drought and salt stress (NaCl), leaf, stem and root protein extracts from Sugargraze sorghum plants were subjected to native polyacrylamide gel electrophoresis (PAGE) and densitometry analysis (Figure 3.11-3.13).

3.5.3.1 Leaf GR activity

Hyperosmotic stress as a result of drought and salinity treatments significantly influenced total GR activity in Sugargraze leaves (Figure 3.11b). Water stress (drought treatment) increased total leaf GR activity by approximately 28% when compared to the activity observed for untreated plants.

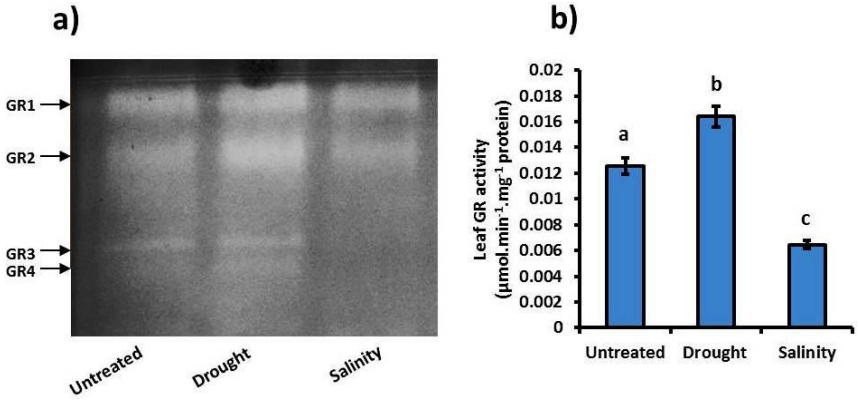

Figure 3.11 GR activity in Sugargraze leaves in response to drought and salinity treatments. Sugargraze leaf samples were used to detect GR activity using enzymatic and in-gel activity assays. Plants were water deprived (drought stress) and exposed to salinity stress (100 mM NaCl; every third day) for a period of 16 days.

Unlike water stress, plant treated with salt stress significantly reduced total GR activity by 49% compared to the untreated plants (Figure 3.11b). The result further showed that four GR isoforms were identified in the various treatments as illustrated on the gel image (Figure 3.11a).

Based on densitometry analysis these isoforms were differentially regulated by the various stress conditions over the period of 16 days (Table 3.4). The enzymatic activity of GR1 was enhanced by 27% in the drought treatment where a small but insignificant increase of 7% was observed in the salinity treatment. An even higher increase in GR2 activity (45%) was observed whereas a slight but insignificant decrease was observed in response to treatment with salinity. For GR3 and GR4, no isoform was detected in the salinity treatment whereas drought augmented GR activity in both isoforms by approximately 25% compared to the untreated plants (Table 3.4). The loss of activity in the salinity treatment could be attributed to the absence of the two isoforms (GR3 and GR4).

Table 3.4 Relative GR activity in Sugargraze leaves

Relative leaf GR activity (Arbitrary values)	Plant Organs	Treatments	Sorghum GR isoforms					
			1	2	3	4	5	6
	Leaves	Untreated	1.00 ± 0.05[a]	1.00 ± 0.05[a]	1.00 ± 0.05[a]	1.00 ± 0.05[a]		
		Drought	1.27 ± 0.06[b]	1.45 ± 0.07[b]	1.25 ± 0.06[b]	1.24 ± 0.06[a]		
		Salinity	1.07 ± 0.05[a]	0.97 ± 0.05[a]	-	-		

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with the different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance according to Tukey-Kramer test.

3.5.3.2 Stem GR activity

After staining the native PAGE gels for GR activity, five GR isoforms was detected in the drought treatment with only four isoforms detected in the salinity and untreated plants (Figure

3.12a). Total GR activity in Sugargraze stem samples was altered by drought treatment whereas salt stress had no significant influence (Figure 3.12b). Total GR activity in response to drought stress was increased by 28% compared to untreated plants. These isoforms were differentially regulated by the various treatments (Figure 3.12a).

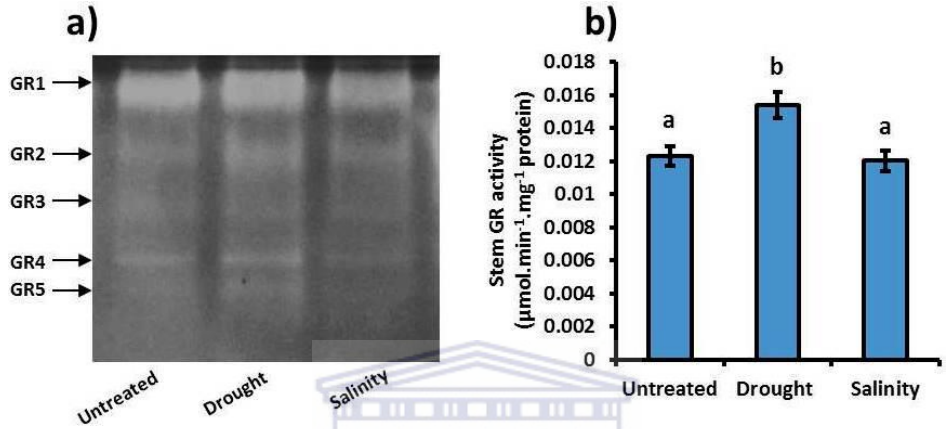

Figure 3.12 Stem GR activity is altered by drought and salinity stress. GR activity was measured in Sugargraze stem using enzymatic and in-gel activity assays. Drought stressed plants were water deprived while salinity stressed plants were treated with 100 mM NaCl every third day for a period of 16 days.

Densitometry analysis revealed that drought increased GR1 activity by 11% with a slight but insignificant reduction observed in the salinity treatment when compared to the untreated plants (Table 3.5). The enzymatic activity of GR2 was augmented by approximately 32% in the drought treatment with no significant change observed in the salinity stress plants. For GR3, both treatments did not influence GR activity when compared to the untreated plants. A significant increase in enzymatic activity was observed for GR4 in response to the drought treatment with salinity exhibiting a slight but insignificant decrease in GR activity. Enzymatic activity for GR5 detected in the drought treatment was about 70% higher that the activity observed for the untreated plants (Table 3.5). Salinity on the other-hand showed a slight decrease compared to the untreated plants. We can therefore hypothesize that three GR isoforms (GR2, GR4 and GR5)

contribute to total GR activity in the response to drought treatment. It is evident that salt stress did not alter total GR activity as the activity observed for the individual isoforms were either similar or less then what was observed for the untreated plants.

Table 3.5 Relative GR activity in Sugargraze stem

Relative stem GR activity (Arbitrary values)	Plant organs	Treatments	Sorghum GR isoforms					
			1	**2**	**3**	**4**	**5**	**6**
	Stem	**Untreated**	1.00 ± 0.05[a]	1.00 ± 0.05[a]	1.00 ± 0.05[a]	1.00 ± 0.05[a]	1.00 ± 0.05[a]	
		Drought	1.11 ± 0.06[b]	1.32 ± 0.07[b]	1.03 ± 0.05[a]	1.79 ± 0.09[b]	1.70 ± 0.09[b]	
		Salinity	0.95 ± 0.05[a]	0.99 ± 0.05[a]	1.02 ± 0.05[a]	0.98 ± 0.05[a]	0.95 ± 0.05[a]	

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with the different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance according to Tukey-Kramer test.

3.5.3.4 Root GR activity

A similar outcome to stem GR activity was observed for total root GR activity. Treatment with salt stress did not influence the root GR activity when compared to the untreated plants, whereas the drought treatment significantly altered total root GR activity (Figure 3.13b). The increase in GR activity observed in response to drought treatment was about 30% higher than was present in the untreated plants (Figure 3.13). A total of six GR isoforms was detected in the roots of Sugargraze plants. Like all the isoforms identified in the other tissues (leaf and stem) of Sugargraze, these isoforms were differentially regulated in response to the various treatments (Figure 3.13a). The activities of these isoforms were quantified as band intensities using densitometry analysis (Table 3.6). The enzymatic activity of root GR1 was altered by either drought or salinity treatments. In response to drought, GR1 activity was increased by 54%, whereas salinity enhanced GR1 activity by 16% compared to the untreated plants.

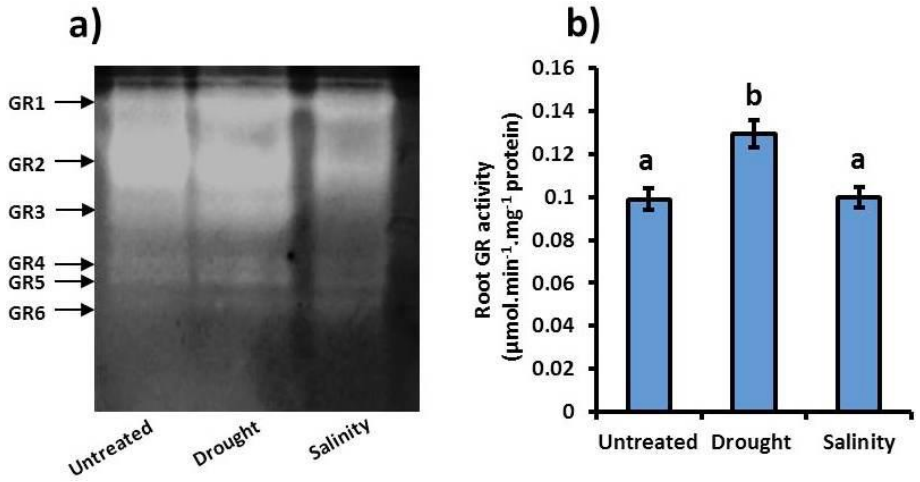

Figure 3.13 Root GR activities as a consequence of drought and salinity treatments. Detection of GR activity in Sugargraze roots using enzymatic and in-gel activity assays. Plants were water deprived (drought stress) and exposed to salinity stress (100 mM NaCl supplied every third day) for a period of 16 days.

Contrary to what was observed for GR1, the enzymatic activity for GR2 was significantly reduced in the various treatments. Drought stress resulted in inhibition of GR2 activity by approximately 26% while inhibition observed in the salinity treatment was more severe (45%) when compared to the untreated plants.

When compared to the untreated plants, there was no significant change in root GR4 activity in the drought and salinity treated plants. Although salinity treatment did not influence the enzymatic activity of GR5, a significant increase of 23% was observed in the drought treatment when compared to the untreated plants. For GR6, both drought and salinity treatments increased the enzymatic activity by 39% compared to the untreated plants. Based on the results obtained in this section it is evident that four isoforms (GR1, GR3, GR5 and GR6) significantly contributed to total GR activity in Sugargraze roots in response to drought treatment.

Table 3.6 Relative GR activity in Sugargraze roots

Relative root GR activity (Arbitrary values)	Plant Organs	Treatments	Sorghum GR isoforms					
			1	2	3	4	5	6
	Roots	Untreated	1.00 ± 0.05^{a}	1.00 ± 0.05^{a}	1.00 ± 0.05^{a}	1.00 ± 0.05^{a}	1.00 ± 0.05^{a}	1.00 ± 0.05^{a}
		Drought	1.54 ± 0.08^{b}	0.74 ± 0.04^{b}	1.75 ± 0.09^{b}	1.00 ± 0.05^{a}	1.23 ± 0.06^{b}	1.39 ± 0.07^{b}
		Salinity	1.16 ± 0.06^{c}	0.55 ± 0.03^{c}	-	0.96 ± 0.05^{a}	1.04 ± 0.05^{a}	1.39 ± 0.07^{b}

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with the different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance according to Tukey-Kramer test.

3.6 Discussion

Here, we have analysed the physiological and biochemical responses of Sugargraze sorghum to long-term (16 days) exposure to drought stress and salinity stress. The ability of Sugargraze sorghum plants to grow under drought and salt stress conditions was severely compromised with a significant reduction in leaf biomass (Figure 3.1a-b) coupled with stunted shoot and root growth (Figure 3.1c-d). This result is in agreement with a study by Jamal et al. (2014) that illustrate how cotton growth was influenced in response to hyperosmotic stress. The poor growth performance of Sugargraze under hyperosmotic stress can be attributed to excessive accumulation of ROS because H_2O_2 levels increased drastically in response to drought and salt stress and this corresponded to reduced dry weights in plants subjected to hyperosmotic stress (Figure 3.3a-b). This view is supported by the observation that extensive cell death occurred in response to drought and salinity treatment and this trend observed for cell death corresponded to that seen for lipid peroxidation (Figure 3.3c-d). It is thus plausible to suggest that hyperosmotic stress induces excessive accumulation of H_2O_2 and the resulting excessive ROS levels destabilize the cell membrane, leading to cell death and loss of biomass (Figure 3.1a-b). The over-production of ROS triggers the activation of the plant's defence mechanism in the form of

antioxidant enzymes. These enzymes are responsible for the scavenging of stress induced ROS molecules. The results have shown that SOD activity was increased in response to drought and salinity treatments. A few lines of research have shown that an increase in SOD activity would result in a decrease in superoxide content and vice versa as SOD is responsible for the detoxification of superoxide anions (Foyer and Noctor, 2005; Mittler, 2002). SOD dependent superoxide detoxification in-turn would result in increased H_2O_2 content (as H_2O_2 is a by-product of O_2- scavenging reaction). The stress-induced changes in SOD enzymatic activity corresponded with altered H_2O_2 content in this study (Figure 3.3a-b). A similar trend was observed in rice (Lee et al., 2001). In response to drought and salinity treatments H_2O_2 levels was significantly higher than was observed in the untreated plants. This in-turn triggered the activation of important H_2O_2 scavenging enzymes such as APX and GR. The efficiency by which APX and GR scavenge H_2O_2 and regenerated glutathione would be important in determining stress tolerance (higher APX activity may result in more efficient removal of H_2O_2). The result showed that APX activity in roots was significantly higher in response to hyperosmotic stress when compared to the untreated control (Figure 3.10b-d). This phenomenon was not observed in leaf and stem samples (Figure 3.8 and 3.9). Given that H_2O_2 levels remained high in both treatments, together with a slight but significant increase in APX activity in the these treatments, it is hypothesized that the increase in APX activity in response to drought and salinity stress is inadequate to counteract the excessively high levels of H_2O_2 that accumulate in response to the stress conditions. A significant reduction in total GR activity was observed under salt stress conditions whereas the individual isoforms detected in all tissues remained unaltered. However the drought treatments significantly enhanced GR activity to levels much higher than the untreated control plants. Although GR activity was significantly enhanced in response to

drought treatment it was still insufficient to cater for efficient regeneration of GSH. The result of such inefficiency in the antioxidant system is accelerated cell death under stress conditions.

A significant reduction in chlorophyll a (Chl_a), chlorophyll b (Chl_b) and total chlorophyll (TC) contents in drought and salt-stressed plants were observed compared to the control plants which is positively correlated with the relative water content and negatively correlated with proline accumulation (Figure 3.2a-c). The accumulation of proline in plants in response to abiotic stress is well documented and is considered to play an important role in osmotic adjustment (de Lacerda et al., 2003; Kumar et al., 2003; de Lacerda et al., 2005; Demiral and Turkan, 2005; Mansour et al., 2005; Misra and Gupta, 2005; Desingh and Kanagaraj, 2007; Koca et al., 2007; Veeranagamallaiah et al., 2007). Proline accumulation in plants might have a scavenger function (Hare et al., 1999) and act as an osmolyte (Parida and Das, 2005). Also, Hare et al. (1999) and Martínez et al. (2007) stated that proline is one of the compatible solutes that accumulate in response to drought stress, and the accumulation of these osmolytes represents an important adaptive response to abiotic stress.

CHAPTER 4

IDENTIFICATION OF DROUGHT AND SALT STRESS RESPONSIVE PROTEINS IN SWEET SORGHUM (SUGARGRAZE) LEAVES

4.1 Introduction

Drought and salinity are the major environmental factors which negatively influence plant development and productivity. They are the main cause of extensive agricultural production losses worldwide (Barnabas et al., 2008; Athar and Ashraf, 2009). They are regarded as major environmental constraints that modern agriculture has to cope with. It has been estimated that drought and salinity may be responsible for over 50% yield reduction in major agricultural crop plants. However, severity of crop reduction depends on the plant development stage at which the stress occurs including stress intensity and duration (Bray et al., 2000; Ashraf, 2009; Atteya, 2003; Monneveux, et al., 2006; . Plant responses towards abiotic stress conditions have been the subject of studies for decades. Initial focus on the model plants has now moved to various crop plants like wheat, barley, rice, maize, and sorghum.

Sorghum is one of the most drought tolerant crop species and is an important model system for studying physiological and molecular mechanisms underlying drought tolerance (Sabadin et al., 2012). Sorghum is also considered to be moderately salt tolerant, being particularly more tolerant than maize (Krishnamurthy et al., 2007), the most widely produced grain crop worldwide. Therefore, sorghum offers great potential as a food source in dry and relatively saline regions. Several studies have been reported on the large scale screening of sorghum varieties for stress tolerance (Krishnamurthy et al., 2007), including transcriptome changes in response to dehydration and high salinity levels (Buchanan et al., 2005). The development of the 'omics' technologies (genomics, transcriptomics, proteomics, metabolomics) has revolutionized plant

science research and has enabled more in-depth studies of interactions among biological components using models and/or networks to integrate genes, metabolites, and proteins (Yuan et al., 2008). Proteome and metabolome analyses have become powerful tools to monitor changes in response to various environmental stimuli. Proteomics, the large-scale analysis of proteins from a particular organism at a given time (Pandey and Mann, 2000; van Wijk, 2001) has been used to study stress responsive protein expression in crops such as rice (Parker et al., 2006), potato (Aghaei et al., 2008), foxtail millet (Veeranagamallaiah et al., 2008) and more recently sorghum (Ngara et al., 2012). Some of these studies have been reviewed by Sobhanian et al., (2010) thus giving a description of unique proteome changes of several other economically important crops under abiotic stress conditions. Despite these reported proteomics, advances in important crops under abiotic stress conditions, similar studies on sorghum, regarded as one of the most stress tolerant commercial crops are still limited. Therefore, this chapter will explore proteomics (2DE and MS) as a tool to identifying drought and salt stress responsive proteins in Sugargraze sorghum leaf extracts. The results obtained here will enhance the understanding of how sorghum, a potential fail-safe crop grown in arid regions, copes with drought and salinity stress when cultivated in these environments.

4.2 Separation and visualisation of Sugargraze leaf samples on 1D PAGE

Total protein for each treatment was extracted (section 2.15.1) from Sugargraze sorghum leaves, and quantified (section 2.15.2) and separated on 1D PAGE to evaluate the quality and loading quantities prior to 2D PAGE. For each sample, thirty microgram of protein was separated and visualised on a CBB stained polyacrylamide gel (Figure 4.1). The protein samples for each treatment showed high similarity in terms of protein expression, abundance and banding patterns which suggests that protein loading was relatively uniform. It was also observed that the quality

of the leaf protein extracts were good, showing no visible signs of streaking and protein degradations.The results further show that protein extracts from all treatments covered the MW range of between 10 and 110 kDa in which some bands were more expressed than others. It is evident in the drought treated sample (indicated by red arrows) where the expression of the bands was higer. Although higher expression is observed in these bands, there could be more than one protein separating as a single band. This is one illustration of a limitation of 1D SDS PAGE analysis, thus separating protiens in the second dimension, could aid in protein identification, since they would be individual spots.

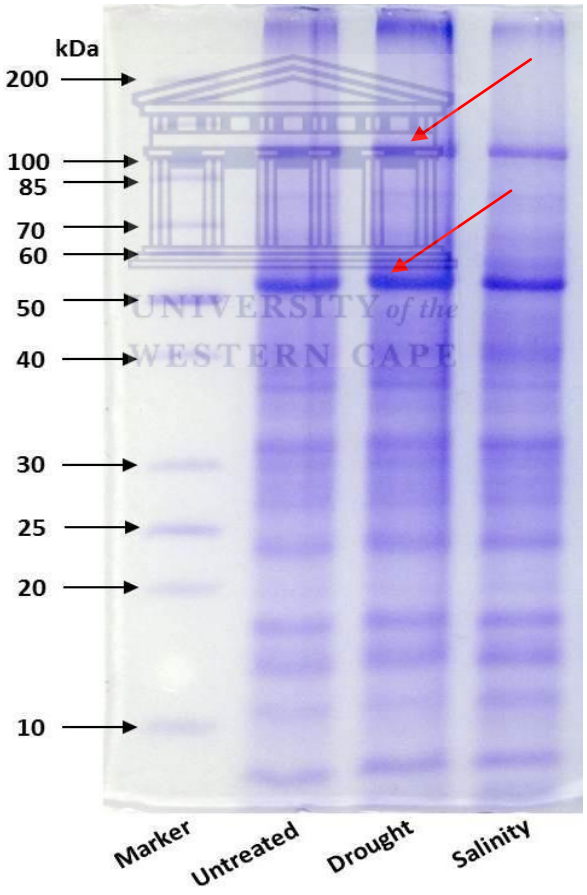

Figure 4.1 The 1D profile of Sugargraze leaf proteome in response to hyperosmotic stress. Experimental plants were exposed to water stress (drought) and salinity stress (100 mM NaCl; every third day) for a period of 16 days.

4.3 Hyperosmotic stress influences Hsp70 expression in Sugargraze sorghum leaves

For plant cells to adapt to abiotic stress conditions they may alter their gene expression to differentially regulate some stress responsive proteins (Sachs and Ho, 1986; Ho and Sachs, 1989; Seki et al., 2003; Shinozaki et al., 2003). To establish whether the stresses (drought and salinity) imposed on the plants in this study was within physiological range, the expression of HSP70 (a stress responsive protein) were investigated.

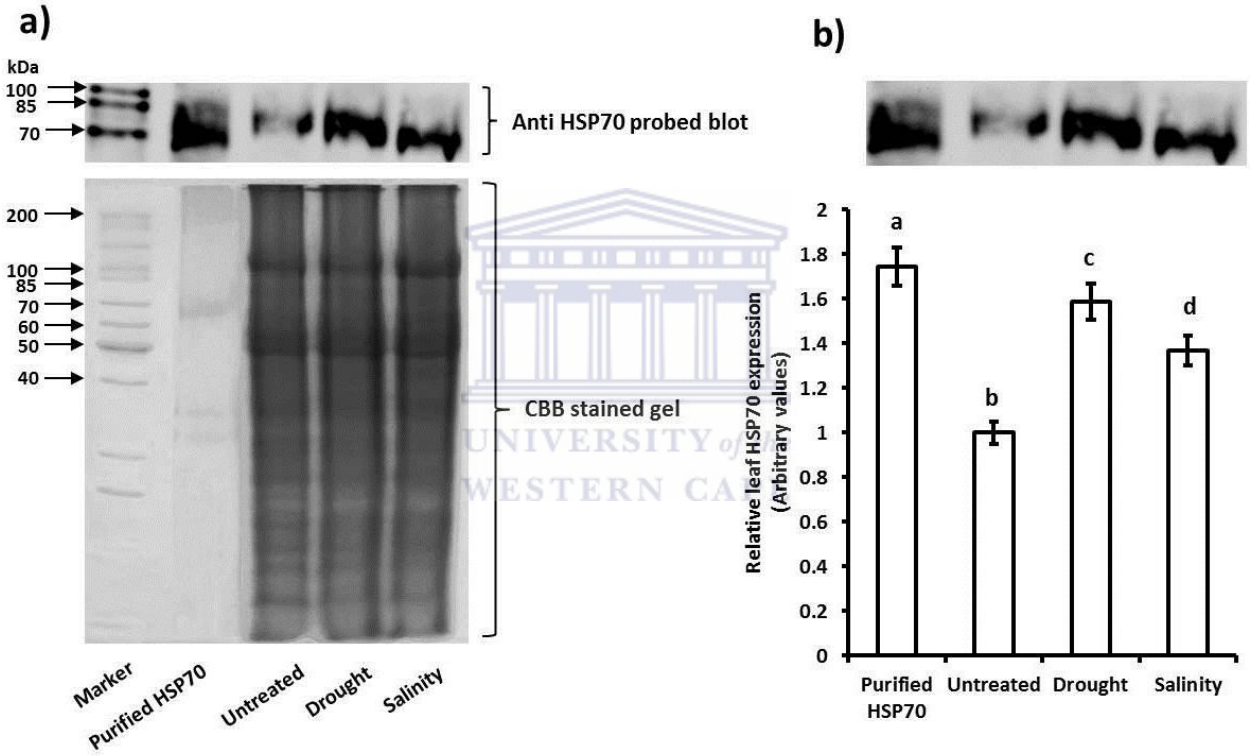

Figure 4.2 Western Blot analysis of Hsp70 expression in Sugargraze sorghum leaves in response to hyperosmotic stress. Hsp70 expression levels were detected **(a)** using human HeLa cells anti-Hsp70/Hsc70 monoclonal antibody and analysed **(b)** using densitometry analysis. 60 µg of protein was used in each reaction for each treatment.

Western Blot analysis was done on protein extracts from untreated, drought treated (water deprived) and salt treated (100 mM NaCl) Sugargraze sorghum leaves using human HeLa cells anti-Hsp70/Hsc70 monoclonal antibody as described in section 2.18. The results showed an

increase in Hsp70 expression in both drought and salinity treated plants when compared with the untreated control (Figure 4.2). The increase in Hsp70 observed for the drought treated leaves was significantly higher than the salt treated leaves. The result was supported by the densitometry analysis performed on the Hsp70 western blot gel shown in Figure 4.2. Based on densitometry analysis, drought and salinity treatments increased relative to Hsp70 expression by approximately 59% and 37% respectively, when compared to the untreated plants.

A purified Hsp70 expressed (by Dr. Takalani Maluadzi-Masuku; unpublished) from sorghum leaves was also included in the analysis as positive control to illustrate that plants do express the protein under normal physiological conditions (Figure 4.2a-b). Hsp70 is known for its expression in response to abiotic stress conditions such as a salinity, drought, heat, cold and oxidative stress (Wang et al., 2004). Like other heat shock proteins, Hsp70 acts as a molecular chaperone thus preventing the aggregation of stress-denatured proteins, by refolding them to restore their original biological role (Miemyk, 1997; Sung et al., 2001). This result demonstrates that the stress treatment imposed here was within physiological parameters, and could therefore induce known stress responses. Furthermore, it indicates that our system was sufficient for application in downstream analysis.

4.4 Two dimensional electrophoresis analysis of stress-responsive protein in Sugargraze leaf samples

Separation of protein samples using 1D PAGE analysis showed that protein expression, abundance and loading across all treatments was relatively uniform (Figure 4.1). 2-DE analysis was used for the detection and identification of drought and salinity stress responsive protein in Sugargraze sorghum leaf samples.

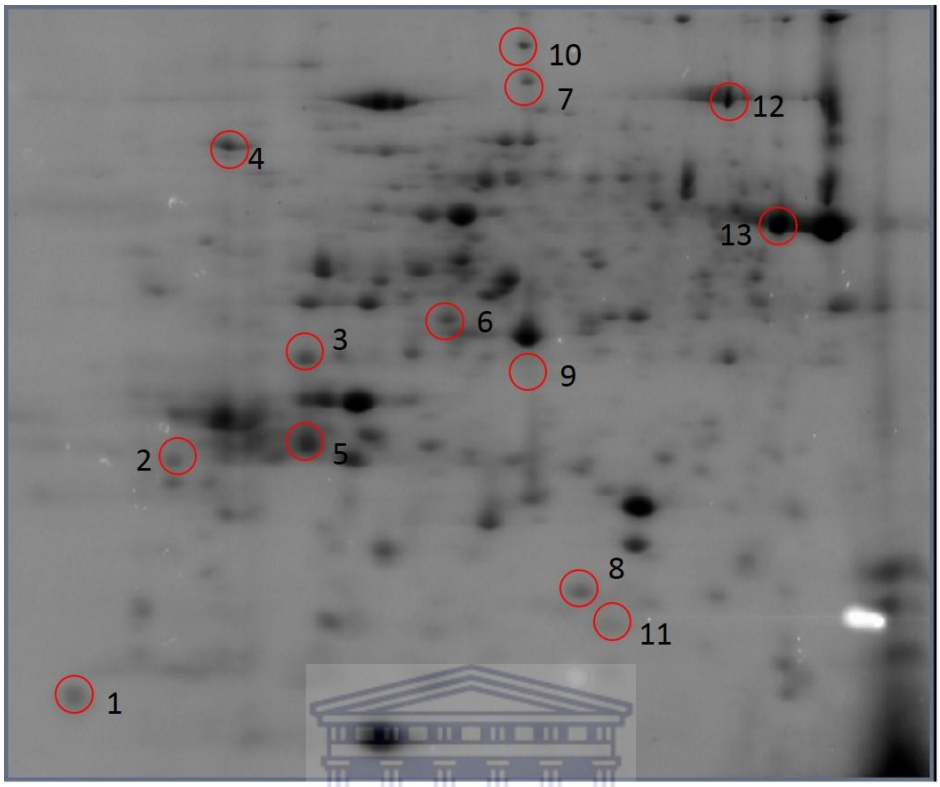

Figure 4.3 Two dimensional electrophoresis analyses of Sugargraze sorghum leaf proteins. The large format gel shows a total of 13 annotated sorghum drought and salinity stress responsive proteins that were selected for MS/MS analysis. About 100 µg of the leaf protein extract was separated in the first dimension by IEF using 7 cm linear IPG strips, pH range 4-7; and 12 % SDS PAGE gels in the second dimension. Numbered spots (1-13) were selected for identification using a combination of MALDI TOF MS and database searches.

One hundred micrograms of leaf protein extracts from three independent biological and technical replicates of stressed and unstressed Sugargraze sorghum plant leaves were resolved on 7cm IPG strips of pH range 4-7 and 12% (v/v) SDS PAGE gels. The resolved proteins were visualised, CBB stained and imaged as described in section 2.15.1; 2.15.2. The Sugargraze sorghum leaf proteome in response to drought and salinity stress is illustrated in Figure 4.3. The result further shows that most protein spots are confined in the 30-200 kDa range with an experimental IEF pH restriction of 4-7. 2-DE separation of protein spots is known for its physical limitation in separating proteins, where extreme pIs and Mr are excluded from the 2-DE profiles (Ngara et al., 2012).

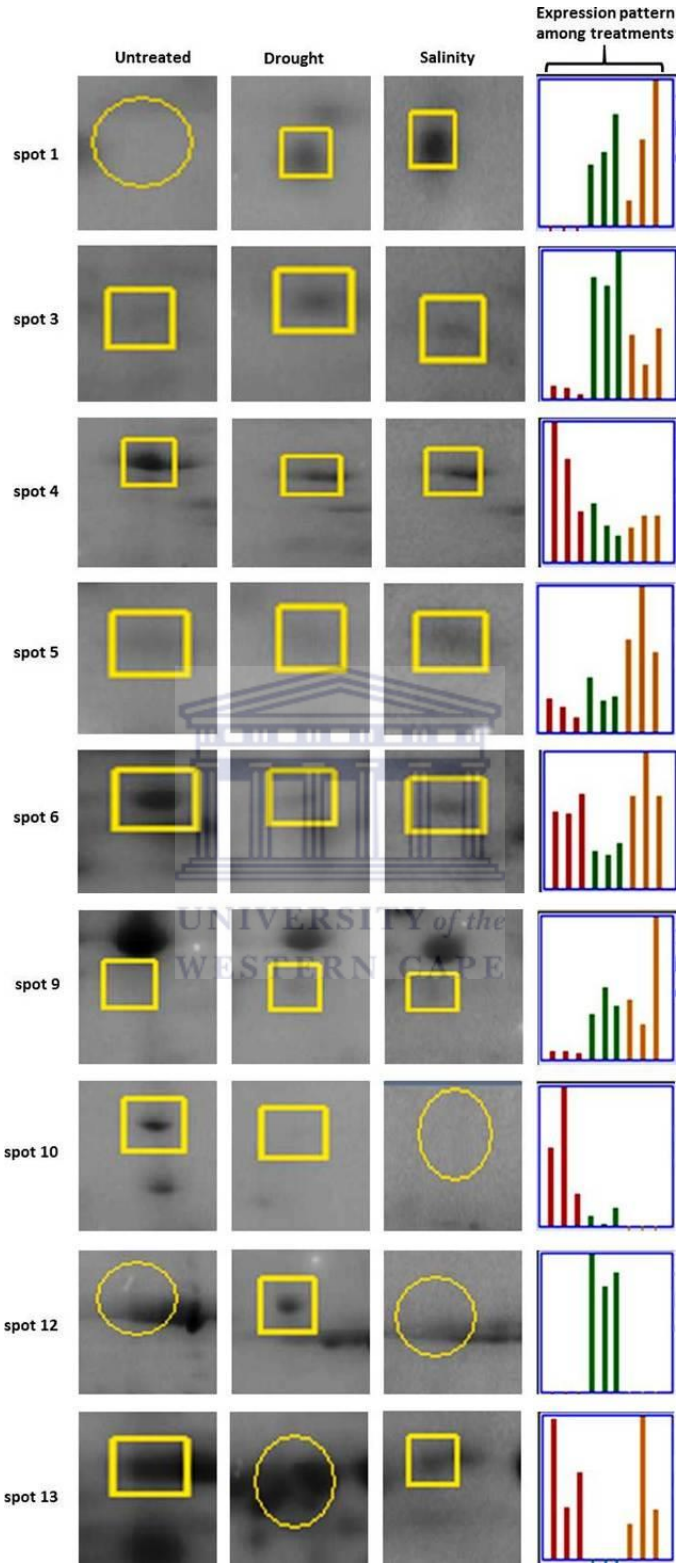

Figure 4.4 Zoomed in gel sections of representative spots showing differential expression patterns following drought and salinity stress.

To ascertain that the spots detected on the 2-DE PAGE gels are reproducible, three biological and technical replicates were included for each treatment (results not shown).

Differential expression between the control and treatment groups (drought and salinity) was assessed using PDQuest™ software. This resulted in the visualization of 201, 277 and 244 CBB stainable leaf protein spots in the untreated, drought and salt treated groups respectively. From the analysis of differential protein expression between the control, drought and salt treatment groups, a total of 117 protein spots were statistically significant using the Student's t-test at a 95% confidence interval.

4.5 Identification of stress responsive proteins in Sugargraze sorghum leaves using MS/MS

Of the 117 differentially expressed protein spots that were identified and found to be statistically significant as determined using PDQuest ($p<0.05$), 13 well resolved, reproducible, abundant spots (Figure 4.4d) were excised and identified using MALDI M/S. From the 13 spots that were selected, 9 spots gave positive hits, 3 of which produced significant results according to their MASCOT MOWSE scores. These include spots 1, 3 and 5, identified as pathogenesis protein 1, Thaumin-like protein pathogenesis related protein 4 precursor and soluble inorganic pyrophosphatase respectively. The results for these proteins spots are tabulated in Table 4.1. Table 4.1 shows spot numbers, their protein identities, number of matching peptides, theoretical Mr/p*I* and their abundance changes.

Table 4.1 List of sorghum proteins identified by MALDI M/S and data base searching

Spot number	Best protein match	Plant species	Accession	Accession	Score	Expression change	Theoretical Mr/pI	Matching peptides
	Energy/metabolism							
5	Soluble inorganic pyrophosphatase	Sorghum	04g034340	gi|242063194	153.93	Up regulated	5.71	5
	Oxygen evolving protein 1	Abies alba	-	gi|376339352	54.21		4.94	1
6	Fructose aldolase	Zea mays	-	gi|195622374	54.21	Up regulated	3061.58	1
10	Fructose aldolase biphosphate aldolase chloroplast precursor	Sorghum	08g004500	gi|242084963	46.32	Up regulated	6.46	2
12	ATP synthase subunit alpha chloroplastic OS	Oryza nivara	-	gi|2014364	18.39	Up regulated	1013.56	2
13	Ribulose biphosphate carboxylase	Cibotium barometz	RBL_CIBBA	-	24.41	Up regulated	1021.52	1
	Disease/defence							
1	Pathogenesis related protein 1	Sorghum	02g002150	gi|242042878	76.64	Up regulated	4.93	2
	Unknown protein 12	Psuedotsuga menziessi	UP12_PSEMZ	-	66.98		11	2
	NAC transcription factor	Populus tremula	-	gi|429345851	50.70		9	1
3	Similar to Thaumin-like pathogenesis related protein 4 precursor	Sorghum	-	gi|242084316	85.60	Down regulated	6.01	2
4	Stromal 70 kDa heat shock related protein	Pisum sativum	-	gi|357514861	47.94	Down regulated	5.08	3
	Protein gamma response	Medicago truncatula	-	gi|357514861	30.66		5.75	1
9	Putative LRR disease resistance protein/transmembrane receptor kinase PS4		PRO_0000240613		39	Up regulated	989.09	4

Identified spots were classified in two groups, energy/metabolism and disease/defence. Drought and salinity induced proteins might possibly contribute towards a plant's tolerance mechanism and thus could be useful candidates for further investigation of their molecular mechanisms of action. The 13 protein spots (Figure 4.3) whose abundance changed with a ≥95% statistical level were picked from CBB stained gels, trypsinised and analysed using MALDI TOF MS and LIFT MS spectra were searched by MASCOT against NCBI nr protein database [taxonomy: *Viridiplantae* (green plants) to identify the proteins according to their criteria stated in chapter 2 section 2.18. Of the 13 stress responsive protein spots that were selected for identification by MS (Figure 4.4d), 9 gave hits in the searched sequence database, matching those from sorghum (*S.*

bicolor) and orthologs from other monocotyledons and other green plants like *Oryza nivara* (wild rice) and *Pisum sativum* (green pea) to name a few.

4.7 Discussion

Environmental stress conditions such as drought and salinity stress poses serious threats to the human population on a global scale especially the poor rural farmers in the developing world. Despite its well-known natural stress tolerance, sorghum remains understudied at molecular level. This work forms part of our long-term vision that seeks to elevate this African crop as an important model plant to study stress avoidance and tolerance mechanisms in important crop species. The extent of hyperosmotic stress (as a result of drought and salinity treatments) imposed on Sugargraze plants was found to be physiologically significant based on the Hsp70 expression levels observed in this study. Proteins that were separated and visualised using 1-DE and 2-DE PAGE analysis and identified using mass spectrometry were functionally classified into the energy/metabolism and disease/defence categories. Of the 13 spots that were submitted, three proteins were positively identified in relation to their MOWSE scores. These proteins include pathogenesis related protein 1, Thaumin-like pathogenesis related protein 4, that function in antifungal response and the oxygen evolving protein 1 that stabilizes the manganese cluster in photosystem II. These proteins were all up-regulated under drought and salinity stress which illustrates their importance in the plant's ability to survive under these stress conditions. The oxygen evolving protein that aids in photosynthesis could be a potential target for use in transgenic studies plants thus improving the plants photosynthetic efficiency under abiotic stress. Current and future studies are aimed at further understanding the molecular functional interactions of our protein candidates, particularly those that show sorghum unique features, and

towards evaluating the mechanisms that make this crop more stress tolerant in comparison to other important crop species.

CHAPTER 5

DISCUSSION AND CONCLUDING REMARKS

5.1 Introduction

Economically important crop plants are often exposed to unfavourable environmental conditions that play a major role in determining productivity of crop yields (Boyer, 1982). These abiotic factors that cause abiotic stress influence the differential distribution of the plants species across different types of environment (Chaves et al., 2003). Some examples of abiotic stresses that a plant may face include decreased water availability, extreme temperatures, decreased availability of soil nutrients and high saline environments. The ability of plants to adapt and/or acclimate to different environments is related to the plasticity and resilience of photosynthesis, in combination with other processes that determine plant growth and development (Chaves et. al., 2011). A remarkable feature of plant adaptation to abiotic stresses is the activation of multiple responses involving complex gene interactions and crosstalk with many molecular pathways (Umezawa et al., 2006). Abiotic stresses stimulate complex cellular responses that have been elucidated in exploring and understanding plant abiotic responses at the whole-plant, physiological, biochemical, cellular and molecular levels (Grover et al., 2001).

The aim of this study was to investigate how drought and salinity treatments influence the physiological and biochemical parameters of Sugargraze sorghum plants. This study further presented information regarding identification of stress responsive proteins associated with hyperosmotic stress (as a consequence of drought and salinity treatments) using 2-DE PAGE analysis coupled with mass spectrometry. The information gathered here will aim to improve our understanding of the biology of abiotic stress responses in plants and give insight into possible approaches to develop plants better adapted to face the environmental constraints.

5.2 Hyperosmotic stress influences physiological and biochemical parameters of Sugargraze sorghum plants

The influence of hyperosmotic stress was reported to influence plant growth by significantly reducing the shoot and root length which is in agreement to what was documented by Shao et al. (2008). This reduction was supported by the reduction in plant biomass in the treatments compared to the untreated control plants. This reduction in plant growth and biomass could be attributed to the increased in oxidative stress that leads to increase levels of lipid peroxidation and ultimate cell death. The observed increase in cell death in response to drought and salinity treatments can either be necrotic death or programmed cell death and this remains to be investigated. However, the fact that strong evidence exists for the involvement of programed cell death in plant responses to salt stress (Katsuhara, 1997; Wang et al., 2010) implies that it is highly likely that the cell death observed here could be a consequence of a programmed cell death pathway. All the oxidative markers were significantly enhanced in response to drought and salinity treatments. This phenomenon was previously also reported in studies that investigated the influence of drought (Jiang and Huang, 2001) and salinity (Shalata and Tal, 1998) on the oxidative parameters in plants. To counter the negative effects caused by drought and salinity treatments, plants will activate various defense mechanisms to suppress the extent of oxidative damage caused by the treatments. Osmo-protectant molecules will accumulate as an indication of stress levels in the plant systems. Proline is one such molecule whose levels significantly increased in the treated plants when compared to the untreated plants. Similar outcomes have been observed in numerous studies that involve abiotic stress conditions (Yoshiba et al., 1997; Ghoulam et al., 2002; Demiral and Türkan, 2005). We have also analyzed the influence of hyperosmotic stress on total chlorophyll content. In our observation, drought and salinity treatments imposed on plants significantly altered chlorophyll content as illustrated in Figure

3.2c. This observation was supported by the reduction in relative water content in these treatments when compared to the untreated plants. The reduction in water retention was evident in the treated plants as some level of leaf discoloration (with visibility of leaf veins) was observed (Result not shown). The antioxidant system of Sugargraze plants was differentially regulated in response to the hyperosmotic effect caused by drought and salinity treatments. In this study we measured the enzymatic activities (as total enzymatic activity and individual isoform intensities) of SOD, APX and GR in Sugargraze leaf, stem and root tissues. The result showed that six SOD isoforms were detected in Sugargraze leaves and stem samples with only five present in the root sample. These isoforms were differentially regulated in the various treatments which could be as a direct consequence of superoxide accumulation (result not shown). Previous literature have shown that an increase in SOD activity would result in a decrease in superoxide content and vice versa as SOD is responsible for the detoxification of superoxide anions (Beyer and Fridovich, 1987; Foyer and Noctor, 2005; Mittler, 2002). SOD dependent superoxide detoxification in-turn would result in increased H_2O_2 content (as H_2O_2 is a by-product of O_2^- scavenging reaction). The stress-induced changes in SOD enzymatic activity corresponded with altered H_2O_2 content in this study (Figure 3.3a-b). This was also observed in rice (Lee et al., 2001).

APX and GR are important in H_2O_2 detoxification and glutathione regeneration and the efficiency with which the H_2O_2 is scavenged would be important in the determination of stress tolerance (higher APX activity may result in more efficient removal of H_2O_2). The involvement of APX in the response to salt stress was also demonstrated in rice, in which salt treatment resulted in greater APX activity, and certain isoforms were preferentially induced (Lee et al., 2001). A similar observation was made in the root sample, where APX activity was significantly

higher in response to salt stress (Figure 3.10b-d). However, this phenomenon was not observed in leaf and stem samples (Figure 3.8 and 3.9). Given that H_2O_2 levels remained high both treatments together with a slight but significant increase in APX activity in the these treatment, it is hypothesized that the increase in APX activity in response to drought and salinity stress is inadequate to counteract the excessively high levels of H_2O_2 that accumulate in response to the stress conditions. A significant reduction in total GR activity was observed under salt stress conditions whereas the individual isoforms detected in all tissues remained unaltered. However the drought treatments significantly enhanced GR activity to levels much higher than the untreated control plants. Although GR activity was significantly enhanced in response to drought treatment it was still insufficient to cater for efficient regeneration of GSH. The result of such inefficiency in the antioxidant system is accelerated cell death under stress conditions.

We thus conclude that antioxidant capacity (i.e. the extent to which antioxidant enzymes detoxify/scavenge ROS) and proline accumulation play a crucial role in regulating plant tolerance against abiotic stress conditions.

5.3 Identification of stress responsive protein in Sugargraze leaf proteome in response to hyperosmotic stress

Prior to protein identification using 2-DE PAGE analysis and mass spectroscopy we had to demonstrate that our stress treatment conditions were within the physiological parameters of our experimental system to induce known stress responses. Therefore, a western blot analysis was performed to determine Hsp70 expression in the different treatments. We have also included a purified Hsp70 from sorghum plants as a control in this study. Based on the Hsp70 expression levels observed in this study we concluded that the extent of hyperosmotic stress (as a result of drought and salinity treatments) imposed on Sugargraze plants was found to be physiologically significant. For protein identification in Sugargraze sorghum, 13 proteins were selected using

PDQuest™ analysis. Proteins were identified using mass spectrometry coupled with the MASCOT database search in accordance with sequence similarity with previously characterized proteins from the Uniprot dataset. A total of nine protein spots were successfully identified of which 7 spots were up regulated. These include the oxygen evolving protein 1, fructose aldolase and pathogenesis related protein 1, to name a few. The remaining 2 proteins were down regulated including the heat shock protein (Hsp70) and the Thaumin-like pathogenesis related protein 4 precursor.

The identified proteins were subdivided into the metabolism/energy and disease/defense categories respectively. From the 9 identified proteins, 5 fall within the metabolism/energy category whereas the remaining 4 were categorized as disease/defense related proteins. When considering the MASCOT MOWSE scores, only 3 of the 9 protein spots were positively identified. These include the oxygen evolving protein 1/soluble inorganic pyrophosphatase (spot 5) with a MOWSE score of 153.93, the pathogenesis related protein 1 (spot 1) with a MOWSE score 76.64 and the Thaumin-like pathogenesis related protein precursor 4 (spot 3) with a MOWSE score of 85.60 (Table 4.1).

The oxygen evolving protein is a 33 kDa subunit of the oxygen evolving complex in photosystem II (Murakami et al., 2002). This protein functions to stabilize the manganese cluster in photosystem II, the primary water splitting site. This protein was also identified in a similar study by Ngara et al. (2012) on sorghum seedlings under salinity stress. Soluble inorganic pyrophosphatase is involved in the catalysis of pyrophosphate into two phosphate ions and assimilation of mineral nutrients (Jardin et al., 1995). Both the pathogenesis related protein 1 and Thaumin-like pathogenesis related protein precursor 4 are associated with fungal defense in plant systems (Lin et al.,1996; Niderman et al., 1995).

5.4 Functional characterisation of identified Sugargraze sorghum stress responsive proteins

The nine positively identified proteins were grouped into two functional categories using a combination of similarity searches on UniProt, literature sources and information available on the CDD entries of the NCBI protein database for hypothetical proteins. The proteins in each functional category are listed in Table 4.1. A brief account of the possible biological roles of some of these proteins in response to drought and salinity stress in sorghum leaves is described below.

Energy

Most of the Sugargraze stress responsive leaf proteins (5) identified were energy related (Table 4.1). These include proteins that are involved in photosynthesis-like, ribulose carboxylase bisphosphate (spot 13).Ribulose carboxylase bisphosphate (RuBisco) is involved in carbon fixation, when energy rich molecules such as glucose are produced by plants by fixing atmospheric carbon dioxide (CO_2) (Whitney and Andrews, 2001). Oxygen evolving protein 1 (spot 5), stabilizes the cluster involved in water splitting. However, spot 5 was also identified as a soluble inorganic pyrophosphatase. Fructose aldolase (spot 6) and fructose bisphosphate aldolase (spot 10) which are involved in splitting fructose 1,6 bisphosphate into glyceraldehyde 3 phosphate (GAP) and dihydroxyacetone phosphate (DHAP) in the Calvin cycle, gluconeogenesis and glycolysis. ATP synthase subunit alpha (spot 12) that is involved in ATP synthesis was identified. In accordance with the proteins identified, a similar trend was observed in Ngara et al., (2012) in sorghum seedlings under salinity stress. Additionally, in a study on wheat leaves by Donnelly et al., (2005), 40% of the proteins identified were involved in energy, primary or secondary metablolism. Ahsan et al., (2007), also identified proteins related to energy under water logging in tomato leaves.

Disease/defence

Abiotic stresses like heat, cold, salinity and drought are interrelated and all yield secondary stresses like osmotic and oxidative stresses. The plant then responds to these primary and secondary stresses by regulating the expression of certain proteins, in certain instances, a single protein can respond to different stresses. Additionally, abiotic stress inducible genes produce chaperones, enzymes and osmo-protectants that protect the plant. Here we report on 4 disease/defence related proteins that were shown to be stress responsive in the Sugargraze sorghum leaf proteome. Hsp70 (spot 4), a molecular chaperone was also identified among these proteins. Spot 4 was also identified as a gamma response protein, which is involved in cell cycle arrest before mitosis in response to damage of in deoxyribonucleic acid (DNA). According to Ebrahim et al., (2011), pathogen related proteins are secreted in response to both abiotic and biotic stresses, including, cold, heavy metals, drought, salinity, air pollutants and pathogen attacks. Pathogenesis related protein 1 (spot 1) and spot 3 the Thaumin like pathogenesis related protein 4 precursor are an antifungal proteins. The roles of the NAC transcription factors span from disease/defence to response to abiotic and biotic stress (spot 1) (Puranik et al., 2012). This spot was identified in the drought and salinity treatments and was not present in untreated plants. The putative Leucine rich repeat (LRR) disease resistance protein transmembrane receptor kinase PS4 (spot 9) has been isolated in plants like Arabidopsis (Fontes et al., 2004).

5.4 Concluding remarks

The work described in this thesis is two-fold. Firstly, we explore the role of hyperosmotic stress on the physiological and biochemical parameters of Sugargraze sorghum plants. Secondly, we identify stress responsive proteins associated with hyperosmotic stress (as a consequence of drought and salinity treatments) using 2-DE PAGE analysis coupled with MALDI-TOF MS. We

hypothesized that hyperosmotic stress would influence biochemical pathways and protein abundance. As was previously described by various researchers, that drought and salinity influence the plant growth and development. A similar trend was observed in this study. This reduction in plant growth was supported by the increased levels of ROS and proline accumulation coupled with the reduction in chlorophyll content and the extent of cell death. Although the antioxidant defense system was activated due to the increase in ROS production it was not adequate to scavenge ROS to a level less toxic to the plants. However, a few isoforms were significantly enhanced in response to treatment with drought and salinity stress and could the potential candidates for further analysis to improve plants response under abiotic stress conditions. Using 2-DE PAGE in combination with mass spectrometry allowed for the separation, visualization and identification of stress responsive proteins in the Sugargraze leaf proteome. Although only nine differentially expressed proteins were identified, this study is indicative that proteomic tools used here is able to separate and allow for the detection of qualitative proteins in Sugargraze sorghum. These proteins could be potential markers of which their associated genes can be linked to various phenotypic characteristics to be used in genetic breeding programs to improve abiotic stress tolerance an economically important crop. Overall the observations made in this thesis could be used as a platform to bridge the gap in sorghum biochemistry and proteomics under abiotic stress conditions and pave the way crop improvement.

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