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BIO TECHNOLOGY

Association between antioxidant activities and drought responses of two contrasting sugar beet genotypes

by

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Association between antioxidant activities and drought responses of two contrasting sugar beet genotypes

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Native-PAGE

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Reactive oxygen species

Sugar beet

ABSTRACT

Sugar beet (*Beta vulgaris L*), which belongs to the Amaranthaceae family is a biennial crop widely used as a source of domestic sugar in the European countries, United States, China and Japan. However, in South Africa, sugar cane is the sole source of natural sugar and therefore sugar beet is a potential source of bioethanol since it would not be in direct competition with a food crop for sugar production. Thus, sugar beet can be utilized as a renewable source of energy, reducing the need and use of fossil fuels (such as petrol) that produce greenhouse gases and consequently cause global warming and climate change. Nonetheless, drought stress is one of the major factors limiting the growth of sugar beet, resulting in sugar beet yield reductions worldwide.

The responses of two sugar beet cultivars with contrasting sensitivity to drought were investigated in an attempt to identify potential biological markers for the development of drought tolerant sugar beet cultivars. Physiological and biochemical parameters such as fresh weight, hydrogen peroxide (H_2O_2) concentration, malondialdehyde (MDA) content (as a measure of lipid peroxidation) as well as cell death, were determined. Enzymatic activities of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), dehydroascorbate reductase (DHAR) and glutathione peroxidase (GPX) were examined in the leaves of the two sugar beet cultivars under both water regimes.

The results on physiological analysis showed that drought stress significantly reduced growth of sugar beet cultivars and this reduction corresponded with excessive ROS (H_2O_2) generation, with SY1 (drought tolerant cultivar) showing less biomass reduction compared to SY2 (drought sensitive cultivar). This excessive H_2O_2 accumulation under drought stress conditions was corroborated by increased levels of lipid peroxidation and cell death, with more pronounced increase in SY2 compared to SY1. Drought stress enhanced antioxidant enzyme activities of SOD, APX, DHAR and GPX, with SY1 showing higher enzyme activities than

SY2, which is thought of as key in the detoxification of ROS and therefore minimizing oxidative damage. However, there was a reduction in CAT activity under drought stress in both sugar beet cultivars especially on SY1, which is considered a common phenomenon especially under APX activation. Based on these results, the study suggests that the capacity to scavenge ROS through enhanced enzymatic antioxidants under drought conditions reduces oxidative damage and can be associated with drought tolerance. Based on comparison of biomass, accumulation of H_2O_2, level of lipid peroxidation and cell death as well as level of antioxidant enzyme activities, the study concludes that SY1 is less susceptible to drought than SY2 and this response is in part regulated by better ROS scavenging in SY1 than in SY2.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AOS	Active oxygen species
APS	Ammonium persulfate
APX	Ascorbate peroxidase
ASA	Ascorbic acid
ASH-GSH cycle	Ascorbate-glutathione cycle
BSA	Bovine serum albumin
Car	Carotenoids
CAT	Catalase
Chl	Chlorophyll
cm	Centimetres
CO_2	Carbon dioxide
Cu/Zn-SOD	Copper zinc superoxide dismutase
dH_2O	Distilled water
DHAR	Dehydroascorbate reductase
DHA	Dehydroascorbate
DNA	Deoxyribonucleic acid
DTNB	Nitrobenzoic acid
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
$FeCl_3$	Iron (III) chloride
Fe-SOD	Iron superoxide dismutase
FW	Fresh weight
GPX	Glutathione peroxidase

GSH	Glutathione
GSSG	Glutathione disulphide
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
K_2HPO_4	Dipotassium phosphate
$K_3[Fe(CN)_6]$	Potassium ferricyanide
KCN	Potassium cyanide
KI	Potassium iodide
MDA	Malondialdehyde
mg	Milligrams
ml	Millilitre
mM	Millimolar
Mn-SOD	Manganese superoxide dismutase
MTT	(3-(4, 5-Dimethylthiazol-2-yl)-2, 5- Diphenyltetrazollium Bromide)
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium chloride
$^1O_2$	Singlet oxygen
O^{-2}	Superoxide anion
OH	Hydroxide
OH•	Hydroxyl radical
PAGE	Polyacrylamide gel electrophoresis
PCD	Programmed cell death
PMS	Phenazine methosulfate
POD	Guaiacol peroxidase

PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RWC	Relative water content
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TEMED	N,N,N′,N′-Tetramethylethylenediamine
w/v	Weight by volume

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Literature Review

1.1. Introduction

The world population is rising at an increasing rate and is expected to be approximately 9 billion in 2050 (Connor 2015; Shanker & Venkateswarlu 2011). On the other hand, agricultural productivity is not increasing at the required rate to meet up with food security needs (Farooq et al. 2009). This is compounded in a major way by abiotic stresses, which refer to non-living factors that have a negative impact on the environment, especially in plant physiology and metabolism, such as drought, extreme temperature and salinity (Cattivelli et al. 2008). Drought stress is the most predominant stress among all the abiotic stresses which causes huge reductions in the production and growth of agricultural crops around the world (Shanker & Venkateswarlu 2011; Hajheidari et al. 2005). This is because water stress is normally accompanied by other abiotic stresses like nutrient deficiency, salinity and extreme temperatures (Shanker & Venkateswarlu 2011). Moreover, several predictions project an increase in atmospheric carbon dioxide and low precipitation resulting in reduced fresh water availability and consequently more frequent droughts (Shanker &Venkateswarlu 2011).

The lack of highly tolerant crop genotypes and the complexity in physiological and genetic traits that control drought responses are the major constraints toward developing drought tolerant crops (Ren et al. 2016). Understanding the genetic factors that contribute to drought tolerance and the mechanisms of drought tolerance could produce cultivars with improved drought tolerance. There exist great differences in drought tolerance amongst plant species and even between genotypes of the same species, which allows them to adapt and grow under different water availability conditions (Ren et al. 2016). Investigation of genetic factors of

tolerance or sensitivity to drought is a promising tool for the identification of potential biological markers conferring drought tolerance (Ren et al. 2016). Characterization of two contrasting genotypes within the same species can generate extensive data suitable for the identification of genetic factors that confer the phenotypic differences of the particular plant in respect of responses to drought. A potentially useful avenue for improvement of crop productivity in drought-prone regions like South Africa is the development of varieties with increased drought tolerance through genetic improvement facilitated by identification of genes responsible for drought tolerance. After identification of these genes, the latter would be tested for efficiency in conferring drought tolerance and therefore be included in future breeding programs.

Beta vulgaris L. (cultivated sugar beet) is a biennial crop that was established as a cultivated crop in Europe during the 18th century from white fodder beets. The beets produce sugar through the process of photosynthesis and accumulates the sugar in roots, which serve as storage for sugar reserves (FAO 2009). When grown for sugar production, sugar beet roots are harvested at the end of the first growing season while still in the vegetative phase. However, sugar beet seeds are produced during the second year of the growing season when the plant is in its flowering phase, usually in summer (Cattanach et al. 2016).

Sugar beet has adapted to a wide range of climatic conditions, but grows well in temperate regions (Monsanto Company 2010). The growth of sugar beet plant usually takes 60-70 days after emergence to reach maturity under optimum day temperatures of 18-26°C. Regions with long day length are more suitable for sugar beet growth and produce maximum yields of sucrose and by-products (Monsanto Company 2010). Thus, areas that have bright, sunny days with temperatures of 18-26°C followed by night-time temperatures of 4-10°C from 90 days after emergence to harvest are more favorable for sugar beet production. South Africa exhibits a wide range of environmental conditions, long summer days (Western Cape) and summer

rainfall (Eastern Cape) with temperatures that are suitable for sugar beet growth. Sugar beet possesses a unique requirement of soil fertility. When soil nutrients such as nitrogen, phosphorus and potassium are too high, more impurities are produced in the roots, which reduce sucrose content during extraction. At the same time, insufficient soil nutrients result in premature leaves and reduced sucrose amount. The quality of sugar beet primarily depends on the amount of sucrose that is stored in the roots and the degree of impurities. Therefore, it is important to manage soil fertility for sugar beet crop production. The percentage of sucrose produced from sugar beets range from 13-22% depending on the cultivar and the environment (Monsanto Company 2010).

Due to a high concentration of sucrose in the roots, sugar beet is the second main source of sugar after sugar cane, with approximately 20% of the world's sugar production while the remaining 80% is derived from sugar cane. The beets are primarily grown for the production of sugar due to the amount of sucrose in the roots. Sugar, a pure high energy food, is a vital component of most living systems (FAO 2009). During sugar extraction from the beets, some useful by-products are produced. The dry matter of sugar beet contains pulp, which is insoluble in water and mainly composed of cellulose, hemicellulose, lignin and pectin (FAO 2009). The pulp is widely used for animal feed as it provides essential fiber in rations and increases the palatability of feed (Monsanto Company 2010; FAO 2009). During the crystallization step of sugar extraction, a high viscous syrup known as molasses is produced. Molasses is widely used in biochemical transformation such as alcohol fermentation, production of baker's yeast as well as in the manufacturing of pharmaceutical products (Monsanto Company 2010; FAO 2009).

Sugar beet is mainly cultivated in developed countries such as the European Union, United States, Japan and China, while Sugar cane is mainly cultivated in the tropical regions of developing countries such as South Africa, Brazil, Thailand and India. Since South Africa is

currently one of the drought-prone areas, efficient approaches are required for developing high yielding drought tolerant sugar beet genotypes. However, identification of attributes suitable for the process of screening genotypes for drought tolerance is a major challenge to plant breeders (Habibi et al. 2011), and this is due to the multi-trait nature of plant responses to drought. Thus, a comprehensive analysis of differences (from a morphological, physiological and genetic point of view) between drought tolerant and drought sensitive lines of the crop species (in this case, sugar beet) could be used as drought tolerant indexes in future elite germplasm and eventually tackle the challenge of drought tolerance in plants.

1.2. Hypothesis

The variations observed in drought tolerant (SY1) and drought sensitive (SY2) sugar beet cultivars are positively associated with ROS scavenging antioxidant enzyme activities.

1.3. Aim and Objectives

The aim of this study is to establish molecular differences between two contrasting sugar beet cultivars in response to drought in an attempt to identify potential biological markers of drought tolerant sugar beet cultivars. The objectives of this study include evaluation of physiological and molecular responses, such as:

a) Biomass measurements between drought tolerant and drought sensitive sugar beet cultivars.

b) Determination of hydrogen peroxide and lipid peroxidation contents.

c) Measurements of cell viability

d) Evaluation of antioxidant enzyme activities of SOD, APX, CAT, DHAR and GPX using both in-gel activity staining and spectrophotometric assays.

1.4. Effects of Drought on Sugar Beet

The production of agricultural crops around the world is mainly limited by abiotic stresses (Leufen et al. 2013). Drought is of particular importance, since it is the main abiotic factor that causes the highest yield losses worldwide (Leufen et al. 2013; Prasad et al. 2008). The effects of drought on sugar beet at morpho-physiological and molecular level are discussed below.

1.4.1. Morpho-physiological Responses

The impact of drought stress depends on the duration of stress, type of cultivar and available mechanisms of tolerance. Under severe drought stress, wilting of leaves is one of the first signals that is frequently observed in sugar beet when soil water is gradually depleted (Choluj et al. 2004; Hajheidari et al. 2005; Shaw et al. 2002; Shanker &Venkateswarlu 2011). Sugar beet leaf wilting is typically accompanied by color change (usually from green to yellow) and the leaves tend to lie uniformly on the soil surface when drought stress intensifies. Constant water deficiency increases leaf temperature due to reduced transpiration rate, resulting in leaf death (Mohammadian et al. 2005). Moreover, relative water content and the growth rate of leaves also declines in response to drought stress (Graves 2012; Hajheidari et al. 2005). Water deficit also accelerates senescence of leaves and therefore reduce leaf longevity (Mohammadian et al. 2005). Photosynthesis has been proven to be highly sensitive to drought, since the photosynthetic efficiency decreases with increasing water deficiency (Leufen et al. 2013; Prasad et al. 2008; Clover et al. 1999). Drought stress reduces soil water content which then triggers stomatal closure resulting in lower internal carbon dioxide uptake (Clover et al. 1999). Carbon dioxide is a key reactant required in photosynthesis, therefore lower internal CO_2 concentration limits photosynthesis (Leufen et al. 2013; Prasad et al. 2008; Clover et al. 1999). Drought stress can also reduce photosynthetic activity by altering the activity of enzymes that participate in the Calvin cycle (Clover et al. 1999).

1.4.2. Molecular Responses and ROS

Reactive oxygen species (ROS), also called active oxygen species (AOS) or reactive oxygen intermediates (ROI), are continuously produced during aerobic metabolism as by-products of different metabolic pathways which are localized in cellular compartments that possess a highly oxidizing metabolic activity or high rates of electron flow such as chloroplast, mitochondria and peroxisomes (Ren et al. 2016; Boguszewska & Zagdańska 2012). The production of ROS is one of the common responses to abiotic stress factors (Habibi et al. 2011; Gill & Tuteja 2010; Sayfzadeh & Rashidi 2010). The most common ROS produced during abiotic stress are hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot -}$), hydroxyl radicals (OH•) and singlet oxygen ($^1O_2$) as shown in Fig. 1.1 (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). A major source of ROS production in plant cells is well established and involves the aforementioned organelles, each possessing a highly oxidizing metabolic activity (Boguszewska & Zagdańska 2012). Peroxisomes and chloroplasts appear to be the main sources of ROS during light, while the mitochondrion produces ROS mainly under dark conditions (Gill & Tuteja 2010).

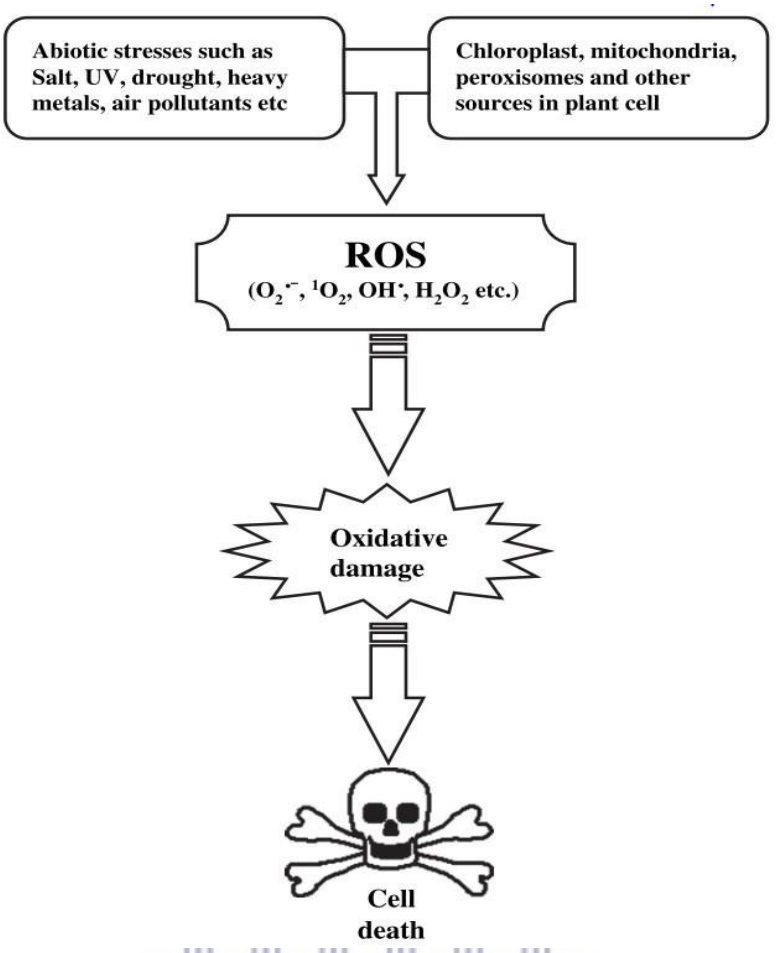

Figure 1.1: The production of ROS through abiotic stresses in different cellular compartments induces cell death. (Adapted from Gill & Tuteja 2010).

Finely controlled levels of ROS are essential in plant defense as they can act as secondary messengers in signal transduction pathways (Genet et al. 2010). However, excessive production of ROS is detrimental because they initiate destructive oxidative processes and consequently injure biological molecules such as lipids, proteins and nucleic acids (Habibi et al. 2011; Genet et al. 2010; Gill & Tuteja 2010).

The accumulation of ROS can be seen as an imbalance between ROS production and the antioxidant system, thus, if ROS production exceeds the antioxidant capacity, oxidative stress occurs. Due to the abundance of the photosensitizers and their oxygenic conditions, plants face a risk of oxidative damage, which can ultimately promote cell death (Gill & Tuteja 2010). The production of ROS through energy transfer is shown in Fig. 1.2. The reduction of a single

electron from O2 yields superoxide ($O2^{\cdot -}$), which results in the production of hydrogen peroxide (H2O2) under low pH conditions. Furthermore, the presence of transition metals (such as copper and iron) may generate hydroxyl radicals (OH•) through the HabereWeiss mechanism or the Fenton reaction (Gill & Tuteja 2010). The role and source of each ROS are discussed below.

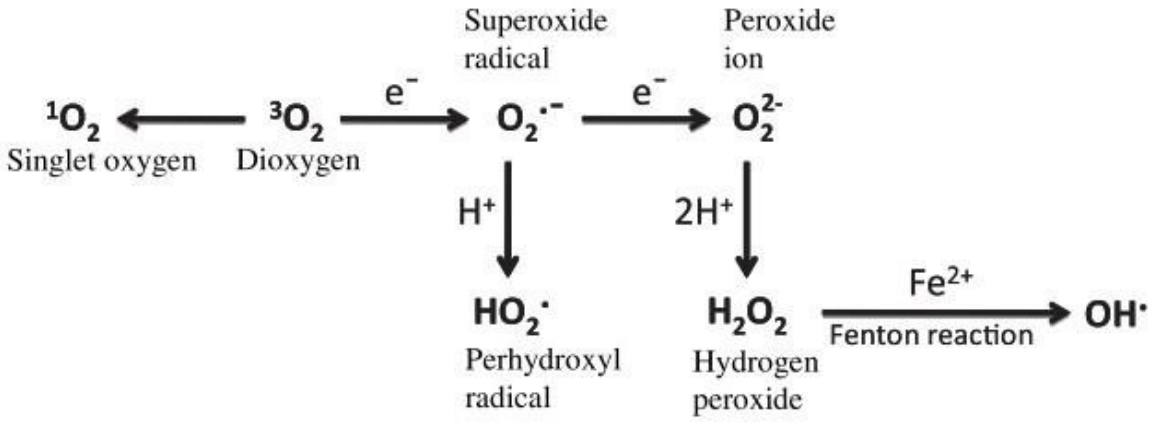

Figure 1.2: The production of ROS through energy transfer. (Adapted from Gill & Tuteja 2010).

1.4.2.1. Hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) is one of the most abundant amongst ROS in aerobic biological systems in with a highly reactive and toxic effect (Caverzan et al. 2016). Normally; ROS are produced as by-products during aerobic metabolism. At low concentrations, ROS, specifically H_2O_2, act as a signalling molecule where it plays a major role in acclimation against biotic and abiotic factors. H_2O_2 is also involved in various processes such senescence, stomatal movement, photosynthesis, photorespiration, growth and development where it acts as a key regulator. When compared to other ROS, H_2O_2 has a long half-life and high cell membrane permeability, which enables it to be transported to other compartments, where it can act as a signalling molecule that mediates responses to various biotic and abiotic stresses (Caverzan et al. 2016; Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). Moderate concentrations of H_2O_2 increase seed germination, seedling growth

and resistance to abiotic stresses (Caverzan et al. 2016; Gill & Tuteja 2010). Furthermore, pre-treatment of wheat seeds with H_2O_2 has been reported to enhance drought tolerance of seedlings (Caverzan et al. 2016). Maize and rice seeds treated with H_2O_2 also showed increased tolerance to salt stress (Caverzan et al. 2016). However, under severe stress conditions, excessive accumulation of H_2O_2 may inactivate some enzymes by oxidizing their thiol groups, leading to oxidative damage (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). Therefore, H_2O_2 plays a dual effect under abiotic stress conditions, one as a signalling molecule in which it triggers stress defense or acclimation responses and the other as a harmful chemical where it initiates uncontrolled oxidative cascades that damage cellular membranes and other cellular components resulting in oxidative stress and eventually cell death (Caverzan et al. 2016; Boguszewska & Zagdańska 2012; Gill & Tuteja 2010).

1.4.2.2. Superoxide ($O_2^{\cdot-}$)

Superoxide ($O2^{\cdot-}$) is continuously produced in the reaction centres of photosystem I (PSI) and photosystem II (PSII) by partial reduction of oxygen molecules (O2) during the process of photosynthesis (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). Complex I, II and III in the electron transport chain (ETC) within the mitochondrion also contributes in the production of superoxide radical (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010).This moderately reactive ROS has a half-life of 2-4 µs and is one of the first ROS molecules to be produced (Gill & Tuteja 2010). It has been noted that in plants, approximately 1-2% of O2 consumption leads to the production of $O2^{\cdot-}$ (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). The production of $O2^{\cdot-}$ may also induce the formation of highly reactive ROS molecules (Fig. 1.2), such as hydroxyl radical (OH•) and singlet oxygen ($^1O2$), each causing degradation of membrane lipids and cellular dysfunction (Gill & Tuteja 2010). The involvement of this

molecule in response to stress has also been reported. A study in *Arabidopsis thaliana* suggested that $O2^{\cdot -}$, which is produced in the photosynthetic pigments, leads to the activation of genes involved in signalling pathways (Gill & Tuteja 2010)

$$O_2^{\cdot -} + Fe^{3+} \Longrightarrow {}^1O_2 + Fe^{2+}$$

$$2O_2^{\cdot -} + 2H \xrightarrow{SOD} O_2 + H_2O_2Fe^{3+}$$

$$Fe^{2+} + H_2O_2 \Longrightarrow Fe^{3+} + OH^- + OH\bullet \text{ (Fenton reaction)}$$

Figure 1.3: The redox reactions of iron that generate hydroxyl radicals. The reactions in which $O_2^{\cdot -}$, H_2O_2 and iron produce $OH\bullet$ is known as the Haber-Weiss reaction, while the final step which involves the oxidation of Fe^{2+} by H_2O_2 is referred to as the Fenton's reaction. (Adapted from Gill & Tuteja 2010).

1.4.2.3. Hydroxyl radicals (OH•)

Hydroxyl radicals (OH•) are produced from $O_2^{\cdot -}$ and H_2O_2 by an iron-catalyzed reaction (fig. 1.3) known as Fenton reaction at ambient temperature and neutral pH (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). The first part of the reaction involves $O_2^{\cdot -}$ that donates an electron to Fe^{3+} in order to produce Fe^{2+} which reduces H_2O_2 that is produced by SOD through dismutation of $O_2^{\cdot -}$ to form OH• These $OH^{\bullet}$ molecules are responsible for regulating oxygen toxicity within the plants (Gill & Tuteja 2010). Furthermore; $OH\bullet$ has an ability to react with almost all biological macromolecules (such as lipids, nucleic acids and proteins), damaging the constituent of cells and eventually promoting cell death (Gill & Tuteja 2010).

$$H_2O_2 + O_2^{\cdot -} \xrightarrow{Fe^{2+},\ Fe^{3+}} OH^- + O_2 + OH\bullet \text{ (Fenton reaction)}$$

Figure 1.4: The production of hydroxyl radicals by the Fenton reaction using iron metals. (Adapted from Gill & Tuteja 2010).

1.4.2.4. Singlet oxygen ($^1O_2$)

Singlet oxygen ($^1O_2$) is generated through the chlorophyll (Chl) triplet state reaction with $^3O_2$ and is the first excited electronic state of O_2 (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). It has been reported that formation of this molecule during photosynthesis largely contributes to the damage of PSI and PSII as well as the entire machinery of photosynthesis (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). Moreover; abiotic conditions such as drought and salinity may induce stomatal closure resulting in low carbon dioxide intake, therefore, promoting formation of $^1O_2$. This oxidizing agent has been determined to have a life time of 3 μs and can react with proteins, lipids, nucleic acids and pigments which eventually result in cell death (Gill & Tuteja 2010). Even though there is limited information on $^1O_2$ as signalling molecule against stress, a study in *Arabidopsis* showed that $^1O_2$ plays a vital role in stimulating distinct groups of early stress-response genes that are different from those activated by H_2O_2 and $O_2^{\cdot -}$ (Gill & Tuteja 2010). Later, it was suggested that $^1O_2$ does not only damage cells but can also act as a signal that activates several stress-response pathways (Gill & Tuteja 2010). Moreover, it has been noted that some plant species produce different types of secondary metabolites through the use of $^1O_2$ to enhance their effectiveness as antimicrobial agents (Gill & Tuteja 2010).

In conclusion, it is well documented that drought and other abiotic stresses stimulate the production of ROS. It is important to control the concentration of ROS as they play a dual role in cells under various abiotic conditions. Moderate levels of ROS play a significant role as key regulatory molecules that are essential for cell defense against abiotic stress. However, over-production of ROS exerts toxic effects to the cells, which may cause oxidative damage to the membrane, proteins and nucleic acids making these components nonfunctional and ultimately induce cell death. Thus, the positive or toxic effects of ROS are determined by the balance between ROS generation and antioxidant system.

1.4.3. ROS and cell biochemistry

1.4.3.1. Lipid peroxidation (LPO)

Lipid peroxidation (LPO) appears to be the most devastating in damaging plant cellular processes (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). It is well established that during lipid peroxidation, various products such as aldehydes (MDA), lipid epoxides, hydroperoxides, glycol and ketones are produced (Gill & Tuteja 2010). Malondialdehyde (MDA) is one of the toxic products that react with thiobarbituric acid (TBA) to form colored products called thiobarbituric acid reactive substances (TBARS) and is therefore used to measure the degree of lipid peroxidation in plant tissue (Gill & Tuteja 2010). The entire process of lipid peroxidation can be categorized into three distinct steps; such as initiation, propagation and termination as depicted in Fig. 1.5. The overall products produced to each step reduce the fluidity of membranes and permit the exchange of phospholipids between the two halves of the bilayer (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). These effects subsequently elevate the permeability of the membrane to molecules that do not normally cross it rather than through specific channels (Gill & Tuteja 2010). Eventually, membrane proteins are damaged and the receptors, enzymes, and ion channels become inactive (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). It has been reported that plants exposed to abiotic stresses show an increased degree of lipid peroxidation as a result of excessive ROS accumulation (Gill & Tuteja 2010). Furthermore, Zlatev et al. (2006) reported an increased LPO in the leaves of *Phaleolus vulgaris* plants under drought stress.

Initiation step:

RH + OH• (Lipid) ⟹ R• + H_2O (Lipid alkyl radical)

Propagation step:

R• + H_2O ⟹ ROO• (Lipid peroxy radical) + RH

ROO• + RH ⟹ ROOH + R•

ROOH ⟹ RO• (Epoxide, hydroperoxide, glycerol and aldehydes)

Termination step:

R• + R• ⟹ R + R (Fatty acid dimer)

R• + ROO• ROOR (Peroxide bridged dimer)

ROO• + ROO• ROOR + O_2 (Peroxide bridged dimer)

Figure 1.5: Three major steps of lipid peroxidation (adapted from Boguszewska & Zagdańska 2012; Gill & Tuteja 2010)

1.4.3.2. Protein oxidation

Protein oxidation accounts for approximately 68% of the oxidized molecules within cells due to ROS production (Boguszewska & Zagdańska 2012). Oxidation of proteins occur as covalent modification of a protein induced by ROS or by-products of oxidative stress and are mostly irreversible except for a few sulfur-containing amino acids (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). Regardless of the source and action of ROS, sulfur-containing cysteine (Cys) and methionine (Met) are more susceptible to oxidation and may react with $^1O_2$ and OH•, which can be reversible (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). Inactivation of the small heat shock protein in chloroplasts is an example of reversible oxidation of methionine that is reactivated by thioredoxin in reaction catalyzed by methionine sulphoxide reductase (Boguszewska & Zagdańska 2012). Protein carbonylation is the mostly commonly occurring protein modification after oxidation of sulfur-containing residues and is used as a marker of protein oxidation (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). It has been noted that various abiotic stresses increase carbonylation of proteins due to high ROS concentration. Furthermore, LPO products like 4-hydroxy-2-nonenal (HNE) may react with proteins under oxidative conditions, therefore inhibiting the structure and function of proteins (Gill & Tuteja 2010).

1.4.3.3. DNA damage

Biotic and abiotic stress factors may damage DNA and constituents as a result of excessive ROS accumulation (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). HO· is considered to be the highly reactive ROS that is responsible for damaging all DNA components such as pyrimidines, purines and the deoxyribose backbone. Various physiological effects such as reduced protein synthesis, cell membrane destruction and damage to photosynthetic proteins can be observed as a result of DNA damage, which affects growth and development of the whole plant (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). Thus, oxidation of biological molecules induces cell death, leading to reduced growth and development in a plant. This always reduces crop yield and under severe drought stress conditions, may significantly diminish crop yield resulting in no yield at all.

1.5. Plant Responses to Drought

1.5.1. Physiological Responses

Plants like sugar beet have evolved mechanisms to overcome and escape the negative effects caused by abiotic and biotic stress factors (Habibi et al. 2011; Hajheidari et al. 2005). Sugar beet response to drought stress follows Le Chatelier's Principle, which states that when an equilibrium is disturbed by altering the conditions, the system tends to respond in such a way as to minimize the effect of perturbation and re-establish an equilibrium (Ober & Luterbacher 2002; Shaw et al. 2002). Sugar beet exhibits a large and deep root system, which enables it to tap into deep water reserves and thus avoid water deficiency (FAO 2009). Recent studies on sugar beet have shown that root growth is less susceptible than shoot growth in response to drought stress (Shaw et al. 2002). Drought escape is another mechanism of avoidance in which sugar beet completes its life cycle before the detrimental effects of drought stress accumulate.

1.5.2. Molecular Responses and ROS scavenging antioxidant defense mechanisms

The accumulation of ROS which has been discussed is a common phenomenon in sugar beet under drought conditions, causing significant damage to cellular structures and affect normal cellular functions under severe stress conditions (Gill & Tuteja 2010; Farooq et al. 2009; Bano& Aziz 2004). Fortunately, plants have evolved various protective mechanisms that include enzymatic and non-enzymatic antioxidants defense systems as shown in Fig. 1.6. These detoxification systems function as an efficient cooperative system to counter the toxic effects of ROS (Sen & Alikamanoglu 2012; Farooq et al. 2009).

The enzymatic antioxidants include a variety of ROS scavengers such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), dehydroascorbate reductase (DHAR) and glutathione reductase (Leufen et al. 2013; Sen & Alikamanoglu 2012; Gill & Tuteja 2010).

The non-enzymatic antioxidants include ascorbate (ASH), glutathione (GSH), flavonoids, carotenoids and α-tocopherol (Sen & Alikamanoglu 2012; Shanker &Venkateswarlu 2011). These antioxidants are located in almost all cellular compartments, indicating the significance of ROS scavenging to cellular viability (Sen & Alikamanoglu 2012; Shanker &Venkateswarlu 2011; Farooq et al. 2009). Our study will focus on the enzymatic antioxidants since they are the most effective mechanism in scavenging ROS under various stress conditions (Boguszewska & Zagdańska 2012).

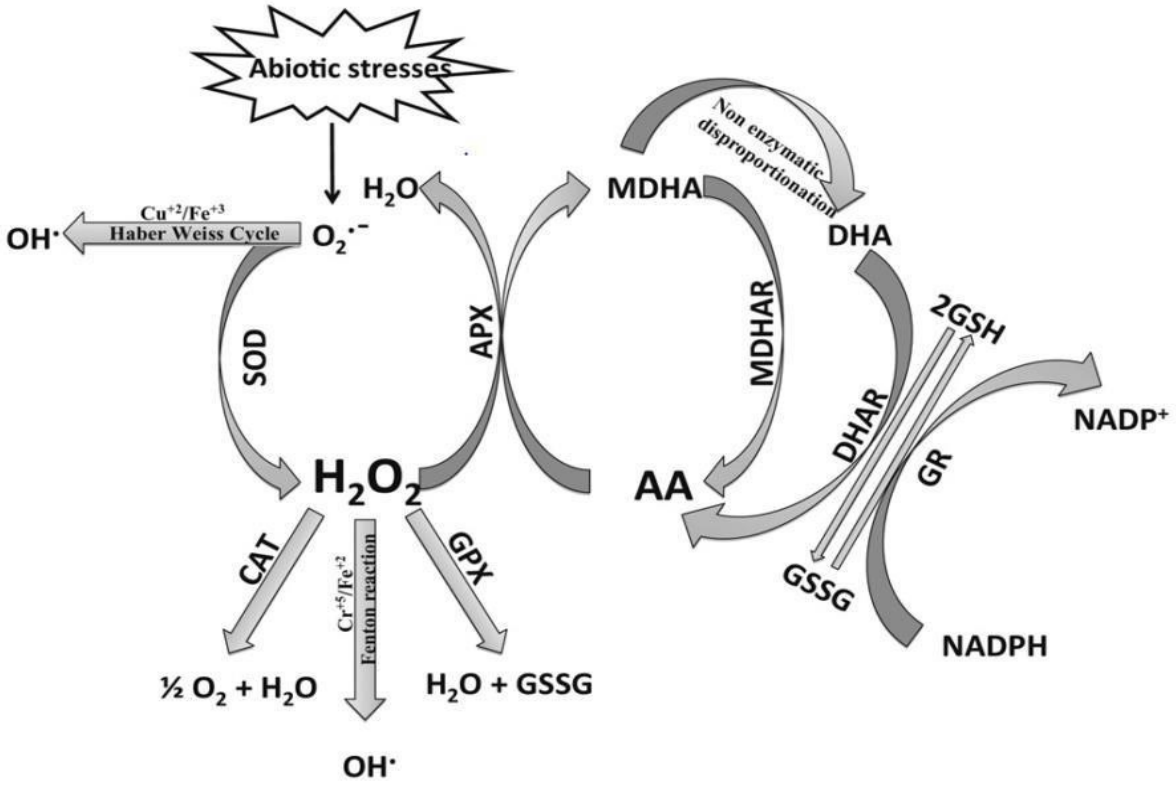

Figure 1.6: The production of reactive oxygen intermediates and antioxidant defense system. Some enzymes are essential for regenerating other antioxidant enzymes in order to maintain minimum levels of ROS. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) and non-enzymatic antioxidants are glutathione (GSH), ascorbic acid (AA), which are both soluble water. (Adapted from Gill & Tuteja 2010).

1.5.2.1. Superoxide dismutase (SOD)

Superoxide dismutase (SOD) enzymes are a group of metalloenzymes which are localized in all cellular compartments susceptible to ROS-induced oxidative stress. SOD constitutes the first line of antioxidant defense and is an essential enzymatic system for scavenging ROS (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010; Cruz de Carvalho 2008). The enzyme catalyzes the dismutation of superoxide ($O_2^{\cdot -}$), the first ROS to be produced, one molecule of $O_2^{\cdot -}$ is reduced to hydrogen peroxide (H_2O_2) and another to oxygen (Sen &

Alikamanoglu 2012). The elimination of $O_2^{\cdot-}$ also reduces the formation of hydroxyl radical (OH•), a highly reactive active oxygen species (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). Plants possess three SOD isoforms which are categorized based on their metal cofactors, such as the copper/zinc SOD (Cu/Zn-SOD), the manganese SOD (Mn-SOD) as well as the iron SOD (Fe- SOD) and are located in different cellular compartments within cells (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). The Cu/Zn-SOD predominates in the chloroplasts in higher plants as well as in the cytosolic fraction. In eukaryotic cells, the Mn-SOD is located in the mitochondrion and also in peroxisome. The Fe-SOD are rarely found in plants but have been associated with the chloroplast (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010).

The SOD isoforms can be detected by negative staining and identified on the basis of their sensitivity to H_2O_2 and KCN inhibitors as shown in table 1.1 (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). The Cu/Zn-SOD is sensitive to both inhibitors, while Mn-SOD is resistant to both inhibitors and Fe-SOD is sensitive to H_2O_2 but tolerant to KCN (Gill & Tuteja 2010). The activity of SOD varies considerable among various plant species and even between cultivars of the same species in response to environmental stresses. Nonetheless, several studies have demonstrated that an upregulation in SOD activity plays a critical role in combating oxidative stress that is caused by biotic and abiotic stress conditions (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). Ren et al. (2016) conducted an experiment of two contrasting *Cerasus humilis* genotypes in response to drought stress and observed significant increase in SOD activity of drought tolerant seedlings, while no significant increase was observed in the activity of drought sensitive plants in response to drought. Drought tolerance is strongly correlated with enhanced antioxidant enzyme activities due to increased capabilities of scavenging ROS that maintains a low steady state, resulting in minimum oxidative injury. Furthermore, Boguszewska & Zagdańska (2012) reported increased SOD activity in potato cultivars under drought stress. Therefore, SODs plays a

crucial role in ensuring plant survival against environmental stress conditions.

Table 1.1: The localization of SOD isozymes in different cellular compartments. (Adapted from Gill & Tuteja 2010)

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	–	H_2O_2 and KCN

1.5.2.2. Ascorbate peroxidase (APX)

Ascorbate peroxidase (APX) is a crucial enzyme in the glutathione-ascorbate cycle for H_2O_2 detoxification during oxidative stress. The H_2O_2 produced by SOD is converted into water and oxygen through the activity of APX and/or CAT (Shanker &Venkateswarlu 2011). The five different isoforms of APX that have been identified include the thylakoid (tAPX), glyoxysome membrane form (gmAPX), chloroplast stromal soluble form (sAPX) and cytosolic form (cAPX) and function independently in scavenging intracellular H_2O_2 (Boguszewska & Zagdańska 2012; Cruz de Carvalho 2008).

The first step in the GSH/ASH pathway involves reduction of H_2O_2 to water by APX using ascorbate as an electron donor (Gill & Tuteja 2010). The oxidized ascorbate (monodehydroascorbate) is regenerated by monodehydroascorbate reductase (MDAR). Because monodehydroascorbate is a radical, it can undergo disproportionation producing ascorbate and dehydroascorbate (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). Subsequently, dehydroascorbate is reduced to ascorbate by dehydroascorbate reductase (DHAR) at the expense of GSH, producing oxidized glutathione (GSSG). Ultimately, GSSG is reduced back to GSH by glutathione reductase (GR) using NADPH as an electron donor as illustrated in Fig. 1.6. Therefore, concomitant increase in these antioxidant components is

necessary in maintaining the steady-state level of ROS under unfavorable conditions.

The APX family possesses a higher affinity for H_2O_2 than CAT and POD, making it a key enzyme in regulating ROS concentrations during biotic and abiotic stress conditions (Gill & Tuteja 2010). The expression of APX has been detected to be enhanced in various plants in response to different stress conditions. An increase of cytosolic APX was reported in pea plants following drought stress (Cruz de Carvalho 2008). Zlatev et al. (2006) reported a significant increase in APX activity under drought stress in three cultivars of *Phaseolus vulgaris L.* Furthermore, enhanced APX activity was also observed in *Anabaena doliolum* under salt stress (Gill & Tuteja 2010). In a study by Arvind & Prasad (2003), increased APX activity was observed in *Ceratophyllum demersum* following Cd stress. It is evident that overexpression of APX is crucial against oxidative stress as it eliminates H_2O_2, which is mainly produced by SOD as a result of scavenging superoxide radical, and its higher concentration is injurious to cells, resulting in a localized oxidative damage, lipid peroxidation, and disruption of metabolic function as well as weakening cellular integrity (Zlatev et al. 2006).

1.5.2.3. Catalase (CAT)

Catalase (CAT) can be defined as enzymatic antioxidant that possesses a tetrameric heme group with an ability to directly detoxify H_2O_2 (Gill & Tuteja 2010). This heme-containing enzyme catalyzes the dismutation of two H_2O_2 molecules into H_2O and O_2 (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). CAT predominates in the peroxisomes, but have been identified in the cytosol as well as in the mitochondria (Gill & Tuteja 2010). The number of CAT isoforms varies among plant species and are differentially expressed. For example, three CAT isoforms have been identified in maize (*Zea mays)*, where *CAT1* and *CAT2* are located in the peroxisomes and cytosol, while *CAT3* resides in the mitochondrion (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). CAT isoforms have been shown

to have different response to light and are regulated spatially and temporally (Gill & Tuteja 2010).

It has been noted that one molecule of CAT can convert approximately six million molecules of H_2O_2 to H_2O and O_2 per minute, resulting in the highest turnover rate of all enzymes (Gill & Tuteja 2010). This demonstrates the importance of this enzyme in ROS detoxification during biotic and abiotic stresses. However, CAT has been observed to be a less scavenging enzyme than APX in terms of oxidative stress (Genet et al. 2010; Gill & Tuteja 2010). In fact various reports indicate that CAT activity is strongly stimulated under severe drought stress, whereas under moderate drought stress H_2O_2 scavenging is accomplished by ascorbic acid via the ASH/GSH cycle (Cruz de Carvalho 2008). Furthermore, CAT has a lower affinity than APX for scavenging H_2O_2 which suggest its role in counteracting excessive H_2O_2 accumulation under severe stress conditions (Gill & Tuteja 2010; Cruz de Carvalho 2008). In addition, APX activity may be inhibited by excessive accumulation of H_2O_2. Therefore CAT activity is essential in maintaining minimum concentrations of H_2O_2 under severe stress environments (Cruz de Carvalho 2008).

Several authors have reported that CAT activity may increase, decrease or remain unchanged depending on the plant species (or cultivar), length and intensity of stress as well as developmental stage (Habibi et al. 2011; Gill & Tuteja 2010). An increase and unchanged CAT activity has been observed in response to water deficit between drought sensitive and drought tolerant *Solanum tuberosum (*potato) cultivars, respectively (Boguszewska & Zagdańska 2012). Moreover, a decrease in CAT activity has been noted in *Glycine max* (soybean), *Phragmites australis* (common reed) and *Capsicum annuum* (pepper), while there was an upregulation in the activities of *Oryza sativa* (rice), *Triticum aestivum* (wheat) and *Brassica juncea*, (mustard greens) under Cd stress (Gill & Tuteja 2010). These reports suggest a variable response of CAT activity under abiotic stresses among several plant

species.

1.5.2.4. Glutathione reductase (GR)

Glutathione reductase (GR) is a flavo-protein oxidoreductase that uses NADPH as a reductant to catalyze the reduction of glutathione disulphide (GSSG) to the sulfhydryl form (GSH), which is fundamental in plant defence against ROS-induced oxidative stress (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). GR predominates in the chloroplast with a relatively small amount localized in the mitochondrion, peroxisome and cytosol (Yousuf et al. 2012; Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). GR and GSH play a significant role in plant defence against oxidative stress and in regulating several metabolic regulatory and antioxidative processes (Gill & Tuteja 2010). Furthermore, GR plays an essential role in maintaining GSH pool in the ASH/GSH cycle, which is central in ROS detoxification. It has been noted that GR and GSH play a fundamental role in determining plant tolerance under various stresses (Gill & Tuteja 2010).

Several studies have reported enhanced activity of GR in response to different environmental stresses. Increased GR activity has been reported in *Cypripedium arietinum* following salt stress (Gill & Tuteja 2010). Yang et al. (2008) observed significant increase in GR activity of *Picea asperata* seedlings under high light drought conditions, while there was no significant change in response to low light conditions.

Moreover, enhanced GR activity in *Oryza sativa* seedlings has been demonstrated under drought stress conditions (Sharma & Dubey 2005). Thus, overexpression of GR and subsequent enzymes are considered to be vital as protective antioxidants, with a crucial role in regulating ROS detoxification during stress conditions.

1.5.2.5. Dehydroascorbate reductase (DHAR)

Dehydroascorbate reductase (DHAR) is another enzyme with a crucial role in the Foyer-Halliwell-Asada pathway. DHAR restores ascorbate from the oxidized state and ensures the

regulation of cellular ascorbate redox state, which is essential for maintaining ROS under severe stress conditions (Boguszewska & Zagdańska 2012). The enzyme uses glutathione to reduce dehydroascorbate to ascorbate. It has been noted that suppression of DHAR expression leads to enhanced concentration of H_2O_2 in response to drought stress (Boguszewska & Zagdańska 2012). Furthermore, suppression of DHAR expression showed decreased rate of CO_2 assimilation which resulted in reduced plant growth and biomass accumulation (Boguszewska & Zagdańska 2012).

In contrast, overexpression of DHAR activity has been found to enhance plant tolerance against various biotic and abiotic factors. Eltayeb et al. (2006) showed that over-expression of DHAR enhanced drought tolerance in tobacco. Plants with enhanced DHAR expression also showed tolerance to ozone by maintaining high ascorbate levels (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). Therefore, plant species that exhibit high potential for the expression of DHAR have an ability to withstand severe environmental conditions.

1.5.2.6. Glutathione Peroxidase (GPX)

Glutathione peroxidase (GPX) is a large family of diversified isozymes that play a major role in controlling oxidative damage (Gill & Tuteja 2010). GPX utilizes GSH to reduce H_2O_2 and lipid hydroperoxides and therefore, ensuring plant survival during oxidative stress (Gill & Tuteja 2010). It has been noted that several plant species preferably use thioredoxin as a reductant in ensuring protection of biological membranes under oxidative stress (Cruz de Carvalho 2008). A total of seven GPX isoforms (*AtGPX1- AtGPX7*) have been identified in *Arabidopsis* in the chloroplast, cytosol, mitochondria and endoplasmic reticulum (Gill & Tuteja 2010).

The activity of GPX also follows a heterogeneous pattern in response to abiotic stress factors. An upregulation in GPX activity has been shown to enhance abiotic stress tolerance in various plants (Gill & Tuteja 2010). Gapinska et al. (2008) reported that 150 mM NaCl stress enhanced

GPX activity in tomato plants. A reduction in GPX activity has been reported in the roots of *Pisum sativum* while there was no significant change in the leaves following Cd stress (Leon et al. 2002). GPX function in the scavenging of lipid peroxides is vital in maintaining oxidative stress. Efficient antioxidant defense system results in efficient detoxification of ROS and thus a better crop yield.

Table 1.2: Plant enzymatic antioxidants in response to drought stress. (Adapted from Cruz de Carvalho 2008)

Drought Responses	Plant Source
Scavenging enzyme activity increase	
SOD	Wheat[T]
	Pea
	Common bean Olive tree Rice[T]
GR	Cotton
	Maize Wheat[T]
APX	Rice[T]
	Cotton Maize
Scavenging enzyme activity decrease/unchanged	
SOD	Wheat[S]
	Sunflower Rice
GR	Wheat[S]
	Pea Cowpea
CAT	Rice
	Sorghum

NB: Tolerant cultivars are represented with [T] while [S] denotes sensitive cultivars.

1.5.3. Non-enzymatic antioxidants against abiotic stress

Non-enzymatic antioxidants can be defined as vitamins, secondary metabolites and phytochemicals that have a biological activity of scavenging ROS during biotic and abiotic stress conditions (Gill & Tuteja 2010). The non-enzymatic antioxidants are classified into two classes, the water-soluble reductants such as ascorbic acid (vitamin C) and glutathione (GSH), and the lipid-soluble membrane-associated antioxidants such as alpha tocopherols (Vitamin E) and carotenoids (Boguszewska & Zagdańska 2012). These antioxidants predominate in the photosynthetic tissues (Karim et al. 2011). Accumulation of these non-enzymatic antioxidants is correlated with increased tolerance to drought stress in several plant species (Cruz de Carvalho 2008).

1.5.3.1. Ascorbic acid (Vitamin C)

Ascorbic acid (vitamin C) is the most dominant and influential antioxidant that occurs in all plant species with its abundant activity in photosynthetic cells and meristems (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). This water soluble antioxidant has an ability to directly scavenge $O_2^{\cdot -}$ and OH• and to regenerate α-tocopherol from tocopheroxyl radical, providing defence to the membrane (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). The capability of ASH to donate electrons in several enzymatic and non-enzymatic reactions contributes to its ability to act as the most powerful scavenger of ROS (Gill & Tuteja 2010). Moreover, ASH has also been reported to play an essential role in the ASH-GSH cycle by stabilizing the activities of enzymes that contain prosthetic transition metal ions (Gill & Tuteja 2010). It has been noted that abiotic stresses like drought stimulate the activity of ASH which minimizes damage caused by ROS. The concentration of ASH was significantly enhanced in turf grass following drought stress (Boguszewska & Zagdańska 2012). A study by Demirevska- Kepova et al. (2006) reported increased content of oxidized ascorbate during Cd exposure in barley plants. Similar results were reported by Yang et al.

(2008) in *Picea asperata* plants under high light and drought conditions.

1.5.3.2. Glutathione

Glutathione is a tripeptide (α-glutamyl-cysteinyl-glycine), localized in all cellular components like chloroplast, mitochondrion, peroxisomes, endoplasmic reticulum, vacuole, apoplast and cytosol (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). In plant tissues, glutathione occurs abundantly in reduced (GSH) form and is considered to be the most crucial intracellular defense against oxidative stress that is induced by ROS (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). The production of GSH is accomplished in two steps which are ATP-dependent. The first step involves synthesis of γ-glutamylcysteine from Cys and Glu, catalyzed by glutamate-cysteine ligase (GCL) and is the rate limiting step in the pathway (Gill & Tuteja 2010). The second step involves addition of Gly to γ-glutamylcysteine by glutathione synthetase to produce GSH (Gill & Tuteja 2010). The synthesized GSH serves as a substrate or co-factor for several reactions that produce oxidized glutathione (GSSG), which are two molecules of glutathione joined together by a disulfide bond (Gill & Tuteja 2010). The GSH/GSSG ratio is crucial in sustaining cellular redox status (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010).

GSH has been found to play a pivotal role in physiological processes like regulation of signal transduction pathways, conjugation of metabolites and expression of stress-responsive genes (Gill & Tuteja 2010). The role of GSH in various growth and developmental processes such as cell differentiation, and senescence as well as enzymatic regulation has been established in plants (Gill & Tuteja 2010). GSH is also responsible for regenerating ASH in the ASH-GSH cycle, which is central for antioxidative defence system (Gill & Tuteja 2010). The contribution of GSH in scavenging excessive $O_2^{\cdot -}$, OH• and H_2O_2 accumulation is essential in plant chloroplast as it provides protection to the photosynthetic apparatus from oxidative damage (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). It is important to note that the

concentration of GSH varies in response to various abiotic stresses. A significant increase in the content of GSH has been reported in *Anabaena doliolum* following salt stress (Srivastava et al. 2005). Metwally et al. (2005) reported increased concentration of GSH under high cadmium (Cd) concentration in pea (*Pisum sativum*) plants. It has also been noted that plants with low concentration of GSH are more sensitive to oxidative damage (Gill & Tuteja 2010). Therefore, enhanced concentration of GSH is central in protecting plant tissues against ROS-induced oxidative damage.

1.5.3.3. Alpha -Tocopherols (Vitamin E)

Tocopherols are lipid soluble antioxidants that function in bio-membranes in which they efficiently scavenge ROS and lipid radicals (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). Tocopherols are located in the thylakoid membrane of chloroplasts where they mainly protect membrane stability and detoxify oxygen species like $^1O_2$ (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). Alpha-Tocopherols (Vitamin E) are the most crucial isomers among tocopherols (such as β-, γ-, δ-) with the presence of three methyl groups in molecular structure, which provide high antioxidative activity (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010).

Considerable amounts of α-Tocopherols have been reported in the leaves of several plant species including *Arabidopsis* (Gill & Tuteja 2010). An induction in the contents of α-tocopherols and ASH in tomato plants has been observed following chilling stress (Gill & Tuteja 2010). A study by Srivastava et al. (2005) reported increased α-Tocopherols content in *Anabaena doliolum* under salt and Cu^{2+} stress. Another study by Giacomelli et al. (2007) in *Arabidopsis* revealed high concentrations of α-tocopherols, ASH and GSH in response to high light. It is evident that α-tocopherols play a crucial role in plant defence system by scavenging ROS-induced oxidative stress which impair normal cellular activities.

1.5.3.4. Carotenoids

Carotenoids (Car) are photosynthetic pigments that occur in plants and some bacteria and over 6000 carotenoids have been established (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010). Car exhibit a multitude role in plant species with three distinctive functions that are crucial to survive harsh environmental conditions (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010; Zhang & Kirkham 1996). The first function involves an accessory light-harvesting role, where Car absorb light between 400 and 550nm which is transferred to the chlorophyll (Chl). The second function involves an antioxidant role in which they provide defence to photosynthetic apparatus by scavenging a triplet sensitizer (Chl3), $^1O_2$ and other toxic free radicals. The third function encompasses a structural role where they provide stability to the thylakoid membrane and for the stability of light harvesting complex proteins (Boguszewska & Zagdańska 2012; Gill & Tuteja 2010).

The contents of Car have been reported in several plant species under different abiotic conditions. Decreased Car contents have been observed by Rai et al. (2005) in *Phyllanthus amarus* with increasing concentration of Cd. A similar trend was reported by Demirevska-Kepova et al. (2006) in barley following Cd stress. Unchanged concentration of Car was also reported in sorghum and sunflower following drought stress (Zhang, J. & Kirkham 1996).

1.5.4. Compatible and Inorganic Solutes as defence mechanism

Sugar beet is a glycophytic plant that has a high ability for osmotic adjustment (Choluj et al. 2004). Accumulation of organic compounds such as proline and glycine betaine are getting important consideration in modern agricultural research in response to drought stress among plant species (Raza et al. 2014). Plants supplied with these compounds maintain water within cells and protect cellular compartments from injury caused by dehydration, therefore balancing turgor pressure during drought stress (Raza et al. 2014; Chołuj et al. 2004). Furthermore, overexpression of these solutes stabilizes membrane integrity, protect protein structure and activity of enzymes in the cells (Raza et al. 2014; Chołuj et al. 2004). Under abiotic stress

conditions, application of glycine betaine has been shown to enhance the seedling and germination stage development in various plants.

Accumulation of inorganic ions such as potassium, sodium and chloride in the leaves and roots of sugar beet have been reported. These inorganic ions are effective in osmotic adjustment induced by salinity or drought (Chołuj et al. 2004). Potassium is one of the essential elements required by plants in large quantities for maintaining the osmotic balance, opening and closing of stomata and is a cofactor of pyruvate kinase (Raza et al. 2014). The element has an ability to adjust the activity of SOD in plants, thus, alleviating oxidative injury of reactive oxygen species caused by biotic and abiotic stress factors (Raza et al. 2014). Therefore, the ability of potassium to retain water in plant cells could be used to strengthen drought tolerance of plants. Nevertheless, soluble non-sucrose constituents of sugar beet storage root, which accumulate subsequent to stress reduce sucrose crystallization during sugar extraction processes (Choluj et al. 2004). Hence, the quality of sugar beet is determined by the ratio of sucrose to total soluble solid in roots.

1.6. Bioethanol production in South Africa

The phenomenon of global warming and rapid increase in fuel prices have led many countries to consider alternative ways of energy generation (Shapouri & Salassi 2006; Meyer et al. 2005). Several countries including United States, Brazil and the European Union as leading producers have opted to bioethanol as a renewable source of energy, reducing the need and use of fossil fuels (such as petrol and diesel) that produce greenhouse gases and consequently cause global warming. Bioethanol production is mostly accomplished through the fermentation of starch and cane sugar (Miao et al. 2012). Maize and sugar cane are the most common feedstocks for the production of ethanol. It has been reported that one litre of ethanol reduces net emissions of carbon dioxide by more than 90%, which is equivalent to 2.2 kg CO_2 reduction (Miao et al. 2012). Furthermore, ethanol also reduces several carcinogenic emissions from

burning of fossil fuel and therefore preventing additional pollutants in the air.

In South Africa, analysis of prospective feedstocks for bioethanol production shows that sugar cane and sweet sorghum are most favorable (Miao et al. 2012). The use of maize for ethanol production has been banned in South Africa since it is utilized as a source of food for humans. The extensive production of sorghum in South Africa declined since the demand for this crop decreased. Sugar cane, which is mostly cultivated in Kwazulu-Natal and Mpumalanga is a sole source of natural sugar that is mainly used for human consumption. Thus, the potential for extensive cultivation of sugar cane is limited and its high water requirements are a constraint in a drought-prone country like South Africa (Miao et al. 2012). Sugar beet, specifically tropical sugar beet has been proposed as a potential source of feedstock for bioethanol production since it would not be in direct competition with a food crop for sugar production. Due to the lack of public domain information available on sugar beet in South Africa, any attempt to construct a rigorous economic model based on sugar beet as feedstock would be hypothetical (Miao et al. 2012), thus continued research and development of tropical sugar beet is useful and the crop may prove to be a competitive feedstock source in the future.

Recent trials in the Eastern Cape region have shown that sugar beet can be produced in South Africa to the same yield levels as other major sugar beet producing countries. As part of the commitment of the national government towards sustainable and green energy, the government has invested billions of Rands in establishing bioethanol production facilities. This is where sugar beet and sweet sorghum would be used as feedstock for bioethanol production industry, making a strong case for cultivation of sugar beet. However, sugar beet is sensitive to drought, which presents a challenge since South Africa is a semi-arid country that is currently experiencing drought. The frequency and severity of drought conditions in South Africa are projected to increase, it is therefore essential to understand how sugar beet responds to drought at the molecular level in order to develop drought tolerant sugar beet varieties.

Towards this effort, our study aims to study physiological and genetic responses of sugar beet under drought conditions, by evaluating changes in the expression of genes involved in the detoxification of reactive oxygen species (ROS), since ROS are some of the major causes of cell death in plants during drought. Understanding these mechanisms would help to develop genetically improved sugar beet varieties with superior tolerance to drought through the use of molecular marker-assisted breeding. Such superior varieties will lead to improved crop yield in sugar beet, which will facilitate sustained supply of plant biomass as feedstock that is required for bioethanol production.

1.7. Economic impact of Drought

Drought is one of the main abiotic factors that greatly limits the production and distribution of agricultural crops around the world. South Africa is currently experiencing one of the severest droughts of the past century and this significantly reduces production of agricultural crops (Pretorius & Smal 2014). The agricultural sector is one of the main sectors that plays a prominent role in South African economy. Nonetheless, the relative percentage contribution of the agricultural sector to the gross domestic product (GDP) continues to decline as seen in 2015/2016 production year.

The agricultural sector also indirectly affects the economy through the forward and backward linkages with other sectors of the economy (Pretorius & Smal 2014). The forward linkages initiate when the agricultural sector provides the delivery of various raw material to the secondary industries to be processed further (Pretorius & Smal 2014). The backward linkages originate when the agricultural sector purchases goods and services from other sectors (Pretorius & Smal 2014). Drought reduces crop yield of agricultural products, resulting in a major loss of money for farmers and farm workers. Moreover, the manufacturing industries of fertilizers, pesticides, machinery, etc. are dependent on sales for farmers, which therefore means if the farmers lose these industries lose as well. As a

consequence, workers will have to take pay cuts or even be laid off until further notice. It has been reported that approximately 69 000 jobs could be terminated due to drought (Pretorius & Smal 2014), which would negatively impact South Africa's national economy.

Drought conditions poses a negative impact on the production of bioethanol because the amount of ethanol produced is depended on the yield of sugar beet crops (Miao et al. 2012). Insufficient amounts of sugar beet will rapidly increase the price of ethanol, therefore promoting the use of fossil fuels that present a problem to the environment. Moreover, drought conditions in Brazil raised the price of sugar which is their main feedstock for ethanol production, causing significant losses during the season of 2011 (Adonzio & Royales 2012). Although significant improvements have been made for drought tolerance in major agricultural crops, only recently has the impact of drought been recognized as one of the largest, single causes of yield reductions in sugar beet production (Ober & Luterbacher 2002).

In dry areas, sugar beet production is accomplished through the summer rainfall or use of irrigation systems. However, in most regions of the world the amount of summer rainfall is unpredictable and is usually not sufficient for sugar beet growth requirements (Ober & Luterbacher 2002). Moreover, the effects of climate change have increased the frequency and severity of drought. This statement can be supported by South Africa's 2015/2016 production year, which is probably the most devastating drought experienced in South Africa in the past centuries (Agri SA 2016). This will see South Africa being highly dependent on imported crops and a sharp increase in food prices which will impact the economy of South Africa. Therefore, the development of drought tolerant plants could play a crucial role in solving the crisis that is currently being experienced by South Africa.

Chapter 2

Methods and Material

Table 2.1: List of chemicals and suppliers.

(3-(4,5-Dimethylthiazol-2-yl)- 2,5-Diphenyltetrazollium Bromide)	Sigma- Aldrich
2- Thiobarbituric acid	Sigma- Aldrich
30 % acrylamide solution	Sigma- Aldrich
5- sulfosalicylic acid dehydrate	Sigma- Aldrich
Acetone	Sigma- Aldrich
Ammonium persulfate	Sigma- Aldrich
Ethanol 200 proof	Sigma- Aldrich
Evans blue	Sigma- Aldrich
Pro-mix	Windel Hydroponics
Sugar beet Seeds	Syngenta BV
Glycine 99%	Sigma- Aldrich
L- Ascorbic acid	Sigma- Aldrich

L- Glutathione reduced	Sigma- Aldrich
Nitroblue tetrazolium chloride	Sigma- Aldrich
Phenazine methosulfate	Sigma- Aldrich
Hydrogen Peroxide	Sigma- Aldrich
Potassium Bromate	Sigma- Aldrich
Potassium hydroxide	Sigma- Aldrich
Potassium phosphate dibasic	Sigma- Aldrich
Potassium phosphate monobasic	Sigma- Aldrich
Quick start 1X Bradford dye reagent	Bio-Rad
Sodium dodecyl sulfate	Bio-Rad
Sodium metavanadate	Sigma- Aldrich
Trichloroacetic acid 99%	Sigma- Aldrich
Tris (hydroxymethyl) amino-methane	Sigma- Aldrich

2.1. Plant Growth

Sugar beet (*Beta vulgaris*) seeds, drought tolerant (SY1) and drought sensitive (SY2) supplied by Syngenta BV were used in this study. Sugar beet seedlings were grown in a greenhouse (randomized complete block design) located at the University of Western Cape. Seeds were surface sterilized in 0.35% (v/v) sodium hypochlorite (bleach) for a maximum of 10 minutes and then washed five times with distilled water. Seeds were then imbibed for 16 hours in aerated 10 mM calcium sulphate ($CaSO_4$). After rinsing with distilled water, the seeds were then incubated in the dark (plastic container covered with foil) at room temperature in a germination chamber for 3 days to allow most seeds to emerge radicles. The growth medium (Pro-Mix Organic) was moistened evenly with nutrient solution [comprised of macronutrients (0.5 mM K_2SO_4, 0.5 mM, 8 mM KH_2PO_4, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM KNO_3 and 2 mM NH_4NO_3) and micronutrients (30 μM H_3BO_3, 10 μM $MnSO_4$, 0.7 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 1 μM $NaMoO_4$, 0.05 μM $CoCl_2$ and 50 μM Fe EDTA)] for 4 days prior sowing. Germinated seeds were sown in 20 cm diameter plastic pots containing 3 L of Promix Organic (Windel Hydroponics, South Africa) under controlled environmental conditions (25/19°C day/night temperature cycle under a 16/8 h light/dark cycle) in a greenhouse. Plants were maintained with 200 ml dH_2O until they had three fully developed leaves.

2.1.1. Treatment of Sugar Beet Plants

Sugar beet plants (SY1 and SY2) which were of similar height and had three fully developed leaves were selected for all experiments. The well-watered plants (control) of both SY1 and SY2 cultivars were continuously supplied with 200 ml distilled H_2O for every second day until the day of harvest and the same amount of distilled H_2O was applied in water-deprived (WD) plants once a week until the development of five fully expanded leaves. At this stage, the water-deprived plants of SY1 and SY2 were supplied with 200 ml dH_2O

once in two weeks until the effects of drought stress were observable (mild wilting of the oldest leaves). The plants were immediately harvested in 35 days after emergence and the fresh weights were recorded.

2.1.2. Storage of Plant Material

The leaves of sugar beet were immediately frozen in liquid nitrogen and ground into a fine powder using a pestle and mortar. The samples were then stored at -80°C for subsequent experiments.

2.2. Evaluation of Cell Viability

The cell death assay was performed using a modified method of Sanevas et al. (2007). The third youngest leaf from both well-watered and water-deprived plants was used for cell death measurement. A portion of 1 cm^2 leaf material was transferred into a 15 ml conical tube covered with foil and filled with 0.25% Evans Blue dye. Following 30 minutes incubation at room temperature, the leaf material was rinsed with distilled water to remove Evans Blue dye that did not penetrate into plant cells. The samples were then transferred to a clean tube containing distilled H_2O to completely remove any present dye and incubated overnight at room temperatures. After draining off the water, 1.5 ml of 1% SDS was added into the samples and incubated at 55°C for 1 hour. The samples were then centrifuged at 2000 x g for 5 minutes and 200 µl of the resulting supernatant was added in triplicates to a microtiter plate in order to measure absorbance readings at 600 nm.

2.3. Trichloroacetic Acid (TCA) Extraction

Trichloroacetic acid (TCA) extraction was performed in order to obtain protein-free extracts for use in determining the level of malondialdehyde (MDA) as a measure of lipid peroxidation and hydrogen peroxide (H_2O_2) content. Plant material (100 mg) was homogenized in 5X the volume of 6% TCA. The mixture was briefly vortexed and centrifuged at 13000 x *g* for 15 minutes at 4˚C. The supernatant was carefully separated from the pellet, transferred to a sterile

Eppendorf tube and stored at -20°C for subsequent experiments (MDA and H_2O_2 assays).

2.4. Malondialdehyde (MDA) Content

Malondialdehyde (MDA) is the product of lipid peroxidation which occurs naturally, thus it is used to estimate the degree of lipid peroxidation. The degree of lipid peroxidation was measured on sugar beet leaves using the method of Dhindsa et al. (1981). The supernatant from TCA extraction was used in which 200 µl was vigorously mixed with 400 µl of 0.5% thiobarbituric acid (TBA). Following boiling at 95˚C for 20 minutes, the mixture was then chilled on ice for 10 minutes and centrifuged at 13000 x *g* for 5 minutes at 4˚C. After centrifugation, 200 µl of the solution was loaded in triplicates into a microtiter plate to measure the absorbance readings at 532 nm and 600 nm wavelengths. The value for the non-specific absorption at 600nm was subtracted from the 532 nm reading and an extinction coefficient of 155 $mM.cm^{-1}$ was used to calculate the concentration of MDA.

2.5. Hydrogen Peroxide (H_2O_2) Assay

A standard curve with known concentrations of H_2O_2 was prepared in order to determine the amount of hydrogen peroxide in sugar beet leaves. An amount of 10 µM H_2O_2 was mixed with varying quantities of dH_2O, 1 M potassium iodide (KI) and 20 mM potassium phosphate (KH_2PO_4), pH 5 to a final volume of 200 µl. Both standards and samples were done in triplicates. A master-mix was made for the samples in which 50 µl of TCA extract was mixed with 50 µl KH_2PO_4 and 100 µl KI to make a final volume of 200 µl. The absorbance at 390 nm was measured and H_2O_2 content was determined using the extinction coefficient 39.4 mM^{-1} cm^1.

2.6. Protein Extraction

The protein was extracted from sugar beet leaves by homogenizing 400 mg of plant tissue with 1.4 ml of extraction buffer [40 mM K2HPO4 at pH 7.4; 1 mM EDTA and 5% (w/v) Polyvinylpolypyrrolidone (PVPP)]. After homogenization, the resulting homogenates were

centrifuged at 13000 x g for 30 minutes at 4˚C. The supernatant was carefully separated from the pellet and stored at -20˚C. Protein concentration of the extracts was quantified in all assays by the Bio-Rad protein assay using bovine serum albumin as standard.

2.7. Protein Quantification (Bradford assay)

The Bradford assay was performed in order to determine the protein concentration of the samples (Bradford 1976). Protein standards were made using 1mg/ml of bovine serum albumin (BSA), distilled water and 1X Bio-Rad protein assay dye in order to create a standard curve. A master-mix was made composed of 1 µl protein extract for each protein sample, 9 µl dH_2O and 190 µl dye to make up a final volume of 200 µl. The mixture was loaded into a microtiter plate and the absorbance readings were measured at 595nm after 10 minutes incubation at room temperatures. All standards and samples were done in triplicates and the protein concentration of the samples was then determined from the standard curve.

2.8. Superoxide Dismutase (SOD, EC 1.11.1.5) Activity

Total superoxide dismutase (SOD) activity was measured spectrophotometrically using the method of Beauchamp & Fridovich (1971) with slight modification. The reaction mixture contained 50 µg protein extract, 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM L-methionine, 2 µM riboflavin and 75 µM nitroblue tetrazolium (NBT). The reaction was initiated by incubating the mixture in a light box for 10 minutes. Following incubation, the formation of blue color change was measured at 590 nm and one unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT and the specific activity of plant extracts was expressed as units per mg of protein.

The native-PAGE of SOD activity staining was performed following the method of Beauchamp & Fridovich (1971). Subsequent to electrophoresis, the gels were incubated in 50 mM potassium phosphate (pH 7.8) buffer containing 2.5 M NBT for 20 minutes in the dark. The gels were then stained on a light box with 50 mM potassium phosphate (pH 7.8) buffer

containing 28 mM riboflavin and 28 mM N,N,N,N-tetramethylethylenediamine (TEMED) until SOD isoforms became visible. A total of two SOD isoforms (Mn-SOD and Cu-Zn SOD) were identified using H_2O_2 and KCN inhibitors. The gel was incubated for 20 min in 50 mM K-phosphate buffer, pH 7.8, containing either 3mM KCN or 5mM H_2O_2 prior staining for activity. The Cu/ Zn-SODs were inhibited by KCN and H_2O_2; and Mn-SODs were resistant to both inhibitors.

2.9. Ascorbate Peroxidase (APX, E.C. 1.11.1.11) Activity

A modified method of Nakano & Asada (1981) was used in this study to measure total ascorbate activity between two sugar beet cultivars. The reaction mixture contained 50 µg protein extract, 2 mM ascorbate which was dissolved in 50 mM potassium phosphate buffer (pH 7) and 0.1 mM EDTA. The reaction was started by adding 10 mM H_2O_2 and the absorbance reading was immediately measured at 290 nm for 1 min. APX activity was calculated using the extinction coefficient 2.8 mM^{-1} cm^{-1} and was expressed as units (µmol of ascorbate oxidized per minute) per mg of protein.

In-gel APX activity staining was performed using the method of Seckin et al. (2010) in order to determine APX isoforms between the two cultivars of sugar beet in response to drought stress. Native polyacrylamide gel electrophoresis (PAGE) was performed at 4°C with a running buffer that was composed of 192 mM glycine, 25 mM TRIS and 2 mM ascorbate. Subsequent to electrophoresis, the gel was equilibrated in 50 mM potassium phosphate (KPO_4) buffer (pH7) containing 2 mM ascorbate for 20 minutes. The gel was incubated for the second 20 minutes in a new solution of 50 mM KPO_4 (pH 7.8) buffer containing 4 mM ascorbate and 2 mM H_2O_2. Finally, the gel was stained in a solution containing 50 mM KPO_4 (pH 7.8), 28 mM TEMED and 0.5 mM NBT, with exposure to light. After the formation of achromatic bands, the stain was discarded and the gel was rinsed with dH_2O to stop any reaction.

2.10. Catalase (CAT, EC 1.11.1.6) Activity

Total catalase (CAT) activity was determined according to the method of Luck (1965) with minor modifications. The method is based on the consumption of H_2O_2, measuring a decrease in absorbance at 240 nm ($\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$). The reaction mixture (1 ml final volume) was made up of 50 mM potassium phosphate (pH 7) buffer containing 50 µg protein extract and 1.5 mM EDTA. The reaction was initiated by adding 1 mM H_2O_2 and the absorbance was measured immediately. CAT activity was expressed as units per mg of protein, one unit being the amount of enzyme which liberates half the peroxide in 100 s at 25°C (Luck 1965).

CAT in-gel activity staining was performed in this study as reported by Gara et al (1997). After electrophoresis, the gels were rinsed extensively with dH_2O, followed by incubation in 0.003% H_2O_2 for 20 minutes in the dark. The gels were then stained in a light box with 2% ferric chloride and 2% potassium ferricyanide until the formation of CAT bands. Proteins representing CAT activity were observed as clear bands on a blue background.

2.11. Glutathione Peroxidase (GPX, EC 1.11.1.9) Activity

A modified method of Nagalakshmi & Prasad (2001) was used to determine total activity of glutathione peroxidase (GPX) between the two sugar beet genotypes in response to drought. The reaction mixture was made up of 0.2 M potassium phosphate (pH 7.0) buffer containing 50 µg protein extract, 10 mM EDTA, 1.14 M NaCl, 10 mM GSH, 2 mM NADPH and 2.5 mM H_2O_2. GPX activity was determined by measuring the disappearance of NADPH at 340 nm for 1 minute ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$).

In-gel activity staining for GPX was performed using a modified method of Seckin et al. (2010). The gels were incubated for 20 minutes in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM glutathione immediately after electrophoresis. Subsequently, the gels were soaked for 20 minutes in 50 mM potassium phosphate buffer (pH 7.8) containing 4 mM glutathione and 2 mM cumin hydroperoxide. GPX isoforms were visualized by incubating the gels in a light box in 50 mM potassium phosphate buffer (pH 7.8) containing 1.2 mM [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazollium Bromide] (MTT) and 1.6 mM phenazine

methosulfate (PMS). GPX isoforms appeared as colorless bands in a purple background and the gels were rinsed with dH_2O.

2.12. Dehydroascorbate Reductase (DHAR, E.C. 1.8.5.1) Activity

A method of De Tullio et al (1998) was employed in this study to determine the total activity of dehydroascorbate reductase (DHAR) between the two contrasting lines of sugar beet. The reaction mixture contained 50 µg protein extract, 0.1 M KPO_4 (pH 6.2) and 2 mM GSH. The reaction was initiated by adding 1 mM dehydroascorbate (DHA) and the absorbance was recorded immediately. The assay measured the production of ascorbate at 265 nm using the extinction coefficient 14 $mM^{-1}cm^{-1}$ and the changes in absorbance were followed for 1 minute. The rate of non-enzymatic DHA reduction was corrected by subtracting the values obtained in the absence of enzyme extract.

In-gel activity staining was performed using 7.5% native-PAGE according to De Tullio et al (1998). Subsequent to electrophoresis, the gel was incubated for 20 minutes with shaking in a 0.1 M KPO_4 (pH 6.2) buffer containing 4 mM GSH and 2 mM DHA. Following washing with dH_2O, the gel was then stained in a light box with 0.1 M hydrochloric acid (HCl) solution containing 0.1% (w/v) ferric chloride and 0.1% (w/v) potassium ferricyanide until the formation of achromatic bands. Upon the formation of achromatic bands, the reaction was stopped by rinsing the gel with dH_2O and DHAR activity was observed as dark blue bands against a light blue background.

2.13. Native-PAGE

All in-gel activity staining (such as SOD, APX, CAT, GR, GPX and DHAR) performed in this study were electrophoresed on a native-PAGE composed of 12% separating gel, [except CAT and DHAR (7.5%)] and 5% stacking gel. The separating gel contained 40% polyacrylamide, 1.5 M TRIS (pH 8.8), 10% ammonium persulfate (APS) and 200 µl TEMED in a total volume of 20 ml. The stacking gel contained the same concentrations except TRIS

(1 M at pH 6.8) and TEMED (80 µl) in a total volume of 8.8 ml. After gel solidification, 50 µg protein extract was mixed with 4x protein loading dye and the mixture was then loaded in the wells. The gels were then electrophoresed under cold conditions (4˚C) at 80 mV until the loading dye (with the protein extract) reached the bottom part of the gel and subsequently stained for specific activity.

2.14. Statistical Analyses

Graphs were generated from mean values (from three independent experiments) obtained from each experiments. The mean values were analyzed using one-way analysis of variance (ANOVA) and tested at the 5% level of significance based on the Tukey-Kramer test. The statistical analyses and drawing of the figures were done using GraphPad Prism 5.03 software.

Chapter 3

Results

3.1. Drought stress reduces the biomass of two contrasting sugar beet cultivars.

Plant biomass is one of the key parameters that indicate plant growth, making it essential in determining plant responses to drought stress. Evaluation of biomass between two sugar beet genotypes, SY1 (drought tolerant) and SY2 (drought sensitive) shows variation in response to drought. Drought stress significantly reduced the fresh weights of both cultivars

when compared with their controls (Fig. 3.1). The drought sensitive cultivar displayed a reduction of ± 54% in biomass while the drought tolerant cultivar showed ± 52% reduction in biomass under drought stress conditions. It is also worth noting that wilting of the oldest leaf occurred one week in SY2 before it occurred in SY1, indicating that SY2 is more sensitive to drought than SY1. The extent of loss of biomass is indicative of the degree of sensitivity to drought.

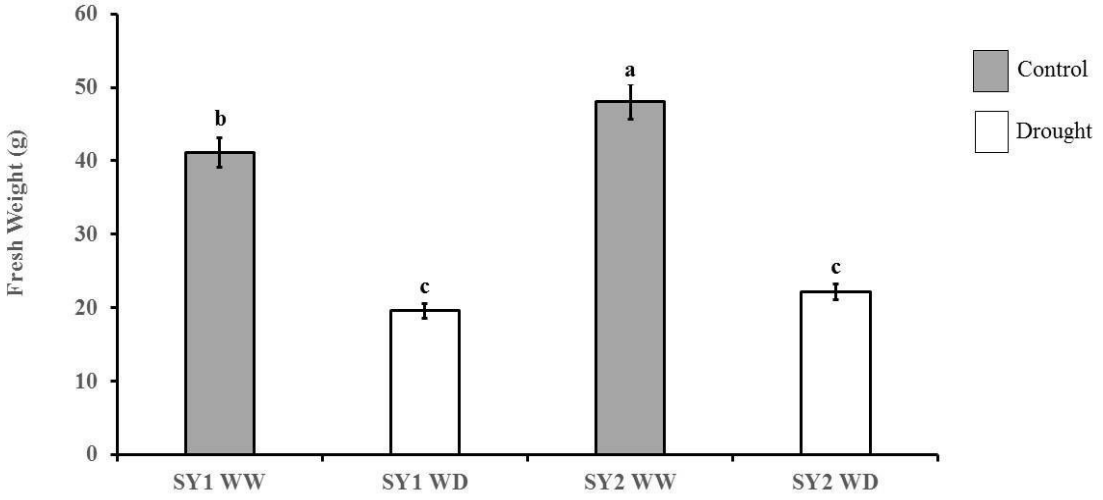

Figure 3.1: Drought stress reduces the biomass of two contrasting sugar beet genotypes. The fresh weights (g) of well-watered and water-deprived sugar beet plants were determined immediately after 35 days of drought stress. Data presented are means (±SE) of ten replicates (n = 10). Different letters denote significant difference between means at $P < 0.05$.

3.2. Drought stress enhances the production of hydrogen peroxide in the leaves of two sugar beet cultivars.

The major consequence of drought stress is the accumulation of reactive oxygen species which disrupt cell compartments leading to cellular dysfunction and damage. It therefore becomes imperative to study the influence of ROS, particularly hydrogen peroxide (H_2O_2) in relation to physiological and molecular responses under drought stress conditions. The concentration of hydrogen peroxide is one of the essential indicators in assessing oxidative damage. Thus, the effects of drought stress on hydrogen peroxide content were investigated between two contrasting sugar beet cultivars. Analysis of hydrogen peroxide concentration in the leaves of sugar beet cultivars is shown in Fig. 3.2. There was no significant change in the concentration of H_2O_2 under normal conditions for both SY1 and SY2 cultivars. However, drought stress augmented the concentration of H_2O_2 with the drought tolerant cultivar displaying the least increase (± 19%) compared to the drought sensitive cultivar (SY2) which produced ± 34% accumulation of H_2O_2.

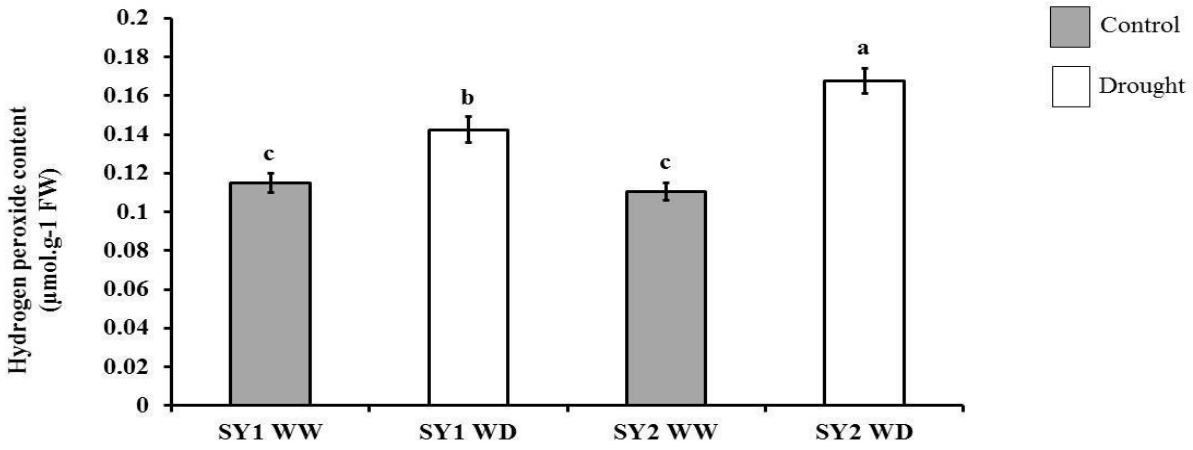

Figure 3.2: Drought stress alters hydrogen peroxide content between two sugar beet cultivars. Data presented are means (±SE) of three independent experiments (n=3). Different letters above error bars denote mean values that are significantly different at $P < 0.05$.

3.3. Drought stress induces cell death within the leaves of sugar beet cultivars.

Drought stress triggers the generation of active oxygen species which induce oxidative damage and ultimately result in cell death, thus the latter can be utilized to assess oxidative damage in plant cells. In our study, cell viability was determined through the use of Evans blue dye, which only stains dead plant cells. Under normal conditions, a higher reduction in cell death was observed on the drought sensitive cultivar. Plant exposure to drought conditions negatively affected the growth of both sugar beet cultivars. However, the drought tolerant cultivar was the least (± 48% increase in Evans Blue uptake) affected, whereas drought sensitive cultivar was more affected (± 56% increase in Evans Blue uptake) by drought in relation with the controls (Fig. 3.3).

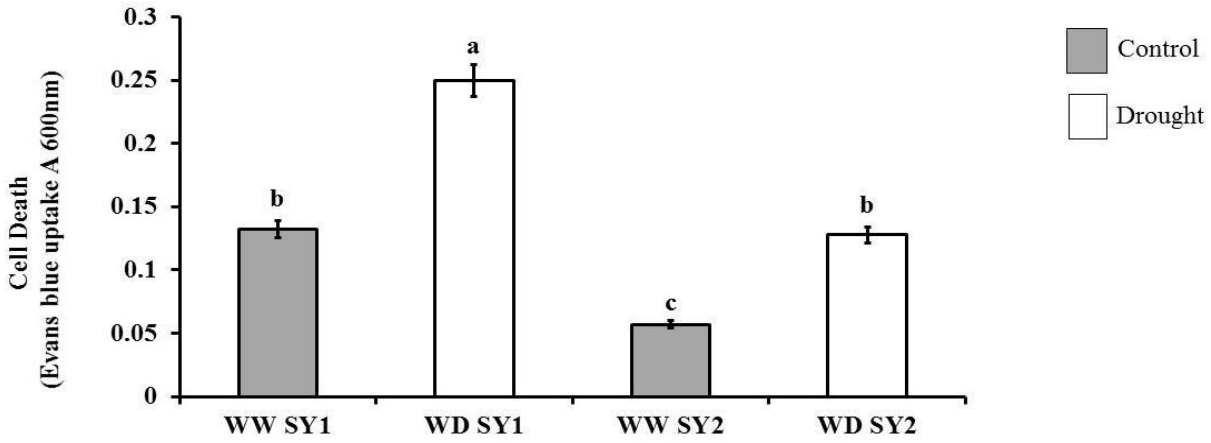

Figure 3.3: The effect of drought stress on the cell viability of two sugar beet cultivars. Cell death was determined using Evans blue dye, immediately after harvesting the plants. The results presented are means (±SE) of 3 replicates (n = 3). Different letters indicate significant difference between means at $P < 0.05$.

3.4. Lipid peroxidation increases between two sugar beet cultivars in response to drought stress.

Malondialdehyde (MDA), a product of lipid peroxidation can be used as an indicator to assess the degree of oxidative damage and membrane damage due to the susceptibility of membranes to attack by ROS (Sinha & Saxena 2006). The effect of drought stress on lipid peroxidation was studied in the leaves of two contrasting sugar beet genotypes. Evaluation of lipid peroxidation between two controls of sugar beet revealed no significant change in MDA content. Drought stress significantly increased MDA content for both SY1 and SY2 with a percentage increase of ±17% and ±18% respectively, in comparison to their controls (Fig. 3.4).

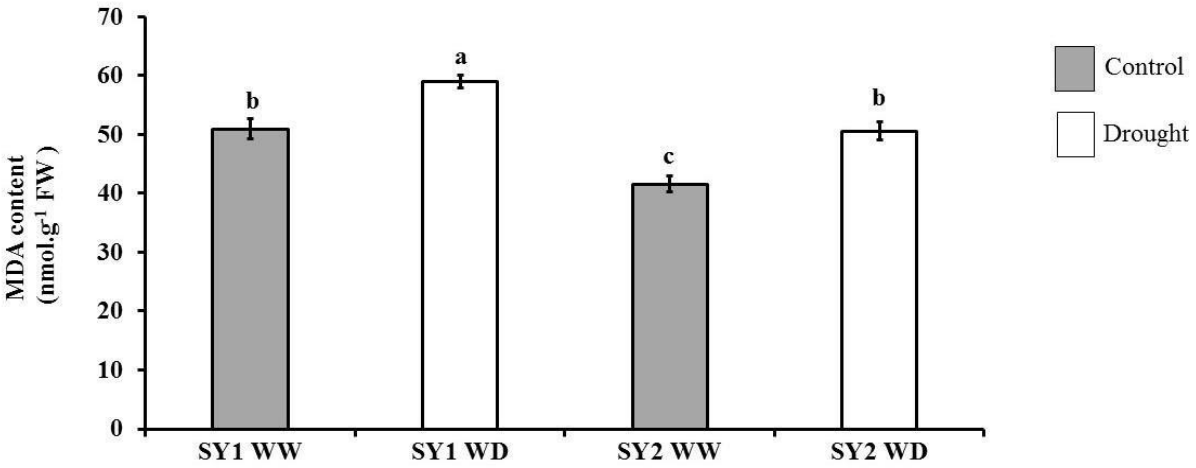

Figure 3.4: The production of MDA between two sugar beet genotypes in response to drought stress. Data presented are means (±SE) of three independent experiments (n=3). Different letters above error bars denote mean values that are significantly different at $P < 0.05$.

3.5. Drought stress enhances the activity of superoxide dismutase (SOD) in the leaves of two sugar beet cultivars.

In order to maintain redox homeostasis and keep ROS at constant level within the plant, the activities of diverse antioxidant enzymes are up-regulated in response to abiotic stresses such as drought. Superoxide dismutase (SOD) is the first line of defence against detoxification of ROS, particularly superoxide ($O_2^{\bullet-}$). Therefore, the activity of SOD in response to drought stress was investigated between two contrasting sugar beet cultivars. The activity of SOD was determined using in-gel activity staining (qualitative) and through spectrophotometric assay (quantitative) as shown in Fig. 3.5.

In order to identify SOD isoforms on a native PAGE in the leaves of sugar beet, two inhibitors (H_2O_2 and KCN) were used as described in chapter 2. A total of two SOD isoforms, such as manganese SOD (Mn-SOD) and copper zinc-SOD (Cu/Zn-SOD) were detected under both water regimes (Fig. 3.5a). The pixel intensity ratio was also performed using the Alpha Ease FC Software for better analysis of SOD isoforms as shown in Fig. 3.5b (i-iv). The

activity bands of Mn-SOD increased on SY1 in relation to SY2 under normal conditions. However, drought stress did not significantly change Mn-SOD activity on SY1 cultivar, whereas there was an upregulation on SY2 cultivar with respect to their controls as depicted in Fig. 3.5b.i and Fig. 3.5b.ii, respectively. Drought stress enhanced the activity of Cu/Zn-SOD for both sugar beet genotypes with SY1 displaying the highest activity for this isoform compared to the controls (Fig. 3.5b.iii-iv).

Total activity of SOD isozymes showed variation between the control and water-deprived plants. Plant exposure to drought stress conditions increased total SOD activity in both cultivars of sugar beet (Fig. 3.5c). The maximum increase of total SOD activity was observed on SY1 cultivar which was probably due to high Cu/Zn-SOD activity since the activity of Mn-SOD remained unchanged in response to drought stress conditions.

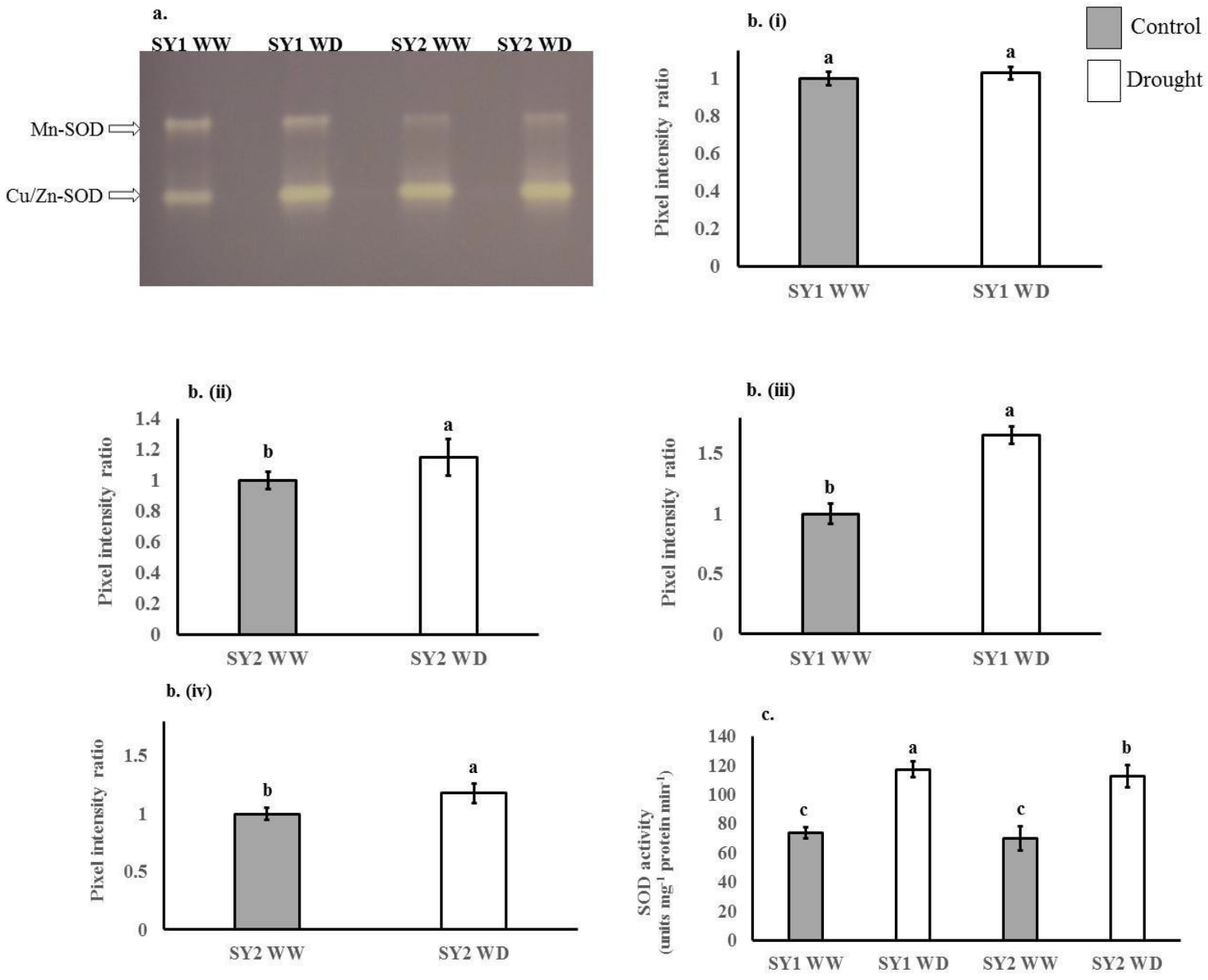

Figure 3.5: Effect of drought stress on SOD activity between two sugar beet genotypes. Figure 3.5a represents the native PAGE for the determination of SOD isoforms, while b(i) and b(ii) represent Mn-SOD pixel intensity ratio of SY1 and SY2 respectively. Relative pixel intensity ratio of Cu/Zn-SOD is illustrated in b(iii) for SY1 and b(iv) for SY2 and c shows total SOD activity. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction at 560nm. Data presented are means (±SE) of three independent experiments (n=3). Different letters above error bars denote mean values that are significantly different at $P < 0.05$.

3.6. The activity of ascorbate peroxidase (APX) increases under drought stress in two sugar beet cultivars.

Ascorbate peroxidase (APX) is one of the essential enzymes involved in ROS detoxification in which it facilitates the conversion of H_2O_2 to H_2O and O_2. Therefore, the role of this enzyme was investigated between two sugar beet cultivars in response to drought stress. APX activity was determined using both in-gel activity staining and spectrophotometric assays.

The results on a native PAGE revealed two APX isoforms (*APX1 and APX2*) as shown in Fig. 3.6a. The activity of *APX1* only appeared on SY2 cultivar under normal conditions while *APX2* appeared in both water regimes of SY1 and SY2 cultivars. There was no significant difference in the activity of *APX2* between the two cultivars under normal conditions. However, the level of expression for *APX2* was upregulated for both cultivars (even higher on SY2) in response to drought stress when compared to their respective controls (Fig. 3.6b).

Analysis for total APX activity revealed a pattern similar to the native PAGE as depicted in Fig. 3.6c. Drought stress enhanced total APX activity of sugar beet cultivars and a maximum increase was observed on SY2, demonstrating the need for scavenging H_2O_2 that is induced by drought.

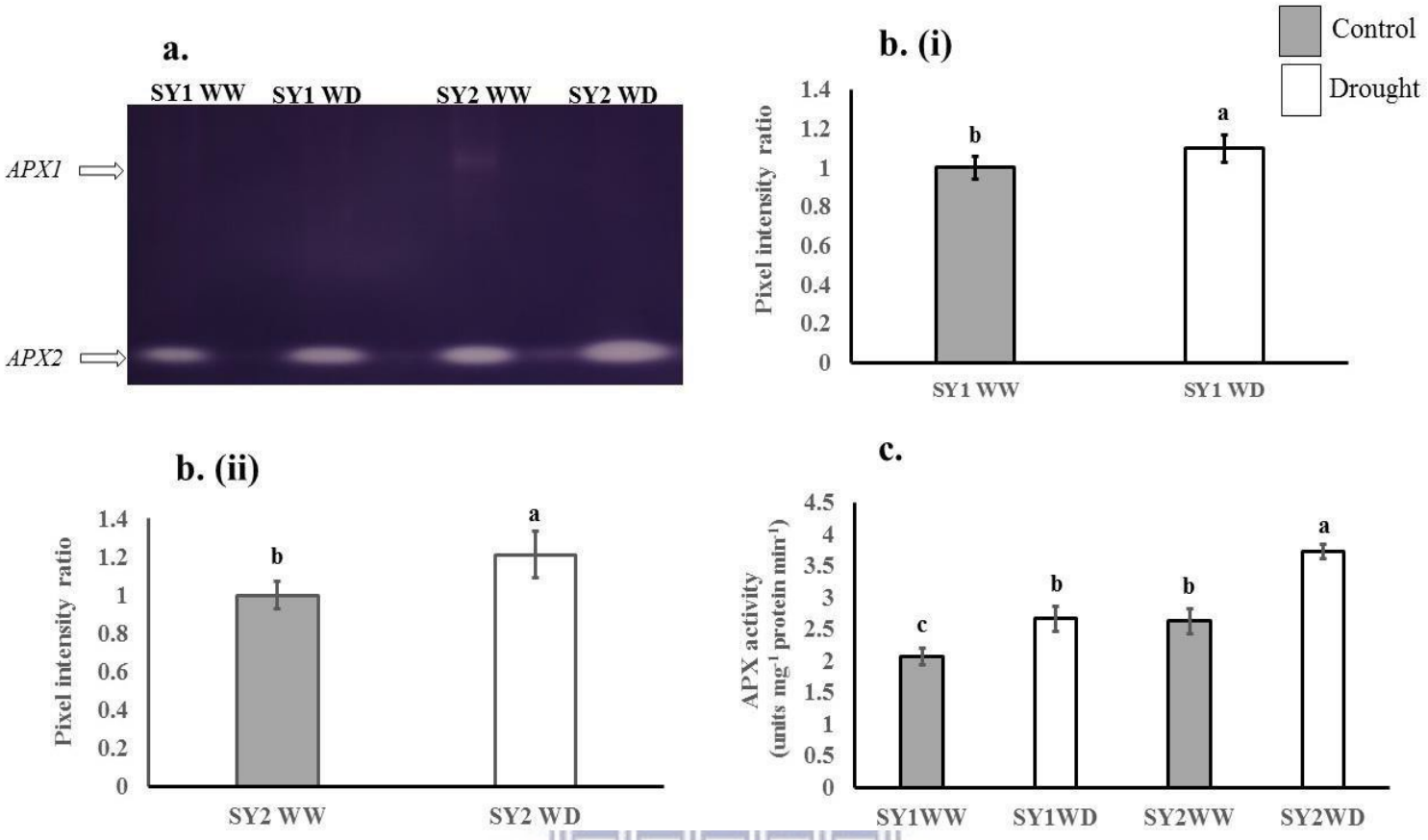

Figure 3.6: Ascorbate peroxide activity between two sugar beet genotypes changes in response to drought stress. Fig. 3.6a, b (i, ii) and c represent native PAGE, relative APX pixel intensity ratio and total APX activity, respectively. Data presented are means (±SE) of three independent experiments (n=3). Different letters above error bars denote mean values that are significantly different at $P < 0.05$.

3.7. Drought stress decreases catalase activity in the leaves of two sugar beet cultivars.

Catalase (CAT) is also vital for the decomposition of H_2O_2, therefore, CAT activity was investigated in the leaves of two sugar beet cultivars through in-gel activity staining and spectrophotometric assay. The native PAGE analysis revealed two CAT isoforms, which are indicated by a clear zone against a dark blue background (Fig. 3.7a). A darker blue background was also observed, which is indicative of non-specific peroxidase activity. Comparison between the two cultivars under normal conditions revealed no significant change in CAT activity bands. However, in water-deprived plants, CAT activity bands decreased for both sugar beet cultivars, more on SY1 than on SY2 cultivar as shown in Fig. 3.7b (i-iv).

The same trend was observed for total CAT activity, where maximum CAT activity prevails under normal conditions and decreases for both SY1 and SY2 in response to drought stress conditions (Fig. 3.7c).

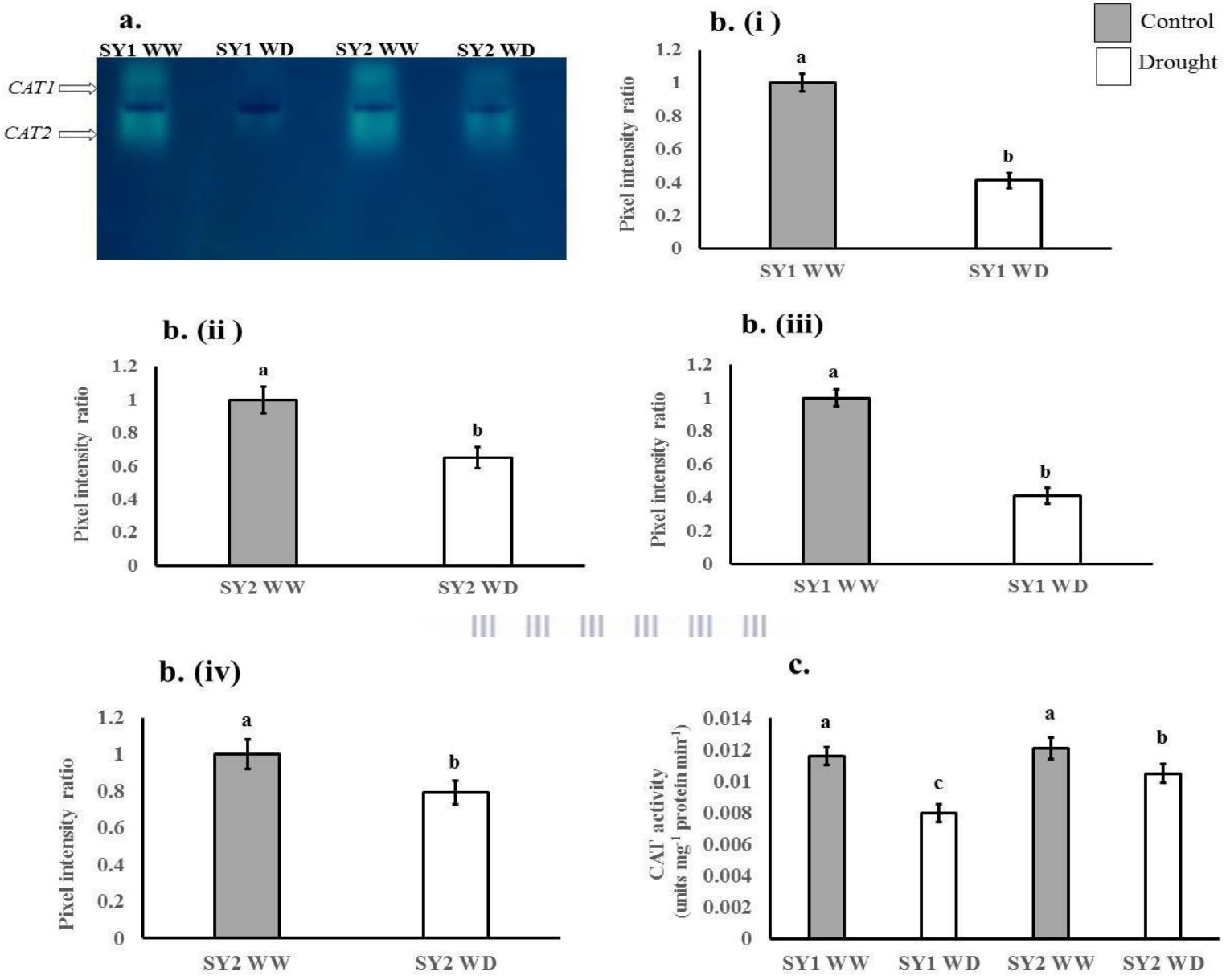

Figure 3.7: Catalase activity between two contrasting sugar beet genotypes decreases in response to drought stress. A clear zone against a blue background on a native PAGE (Fig. 3.7a) indicates CAT activity. The pixel intensity ratio of the generated isoform is represented in Fig. 3.7b (i-iv) and Fig. 3.7c shows the total CAT activity. Data presented are means (±SE) of three independent experiments (n=3). Different letters above error bars denote mean values that are significantly different at $P < 0.05$.

3.8. Drought stress enhances the activity of certain dehydroascorbate reductase (DHAR) isoforms in the leaves of sugar beet cultivars.

Dehydroascorbate reductase plays an important role against oxidative damage by regenerating ascorbate which is required for ROS detoxification. The role of this enzyme was investigated in the leaves of two sugar beet cultivars in response to drought stress. DHAR activity was evaluated by using in gel activity staining and spectrophotometry.

In gel activity staining produced one DHAR isoform which is present in both water regimes of SY1 and SY2 cultivars (Fig. 3.8a). The activity band of DHAR was upregulated in the drought tolerant cultivar as shown in Fig. 3.8b (i), while there was downregulation in the drought sensitive cultivar in response to drought stress as depicted in Fig. 3.8b (ii).

Spectrophotometric results for total DHAR activity displayed maximal activity in the drought tolerant cultivar under water-deprived conditions response as shown in Fig. 3.8c. However, a reduction in DHAR activity was observed on the drought sensitive cultivar in response to drought stress.

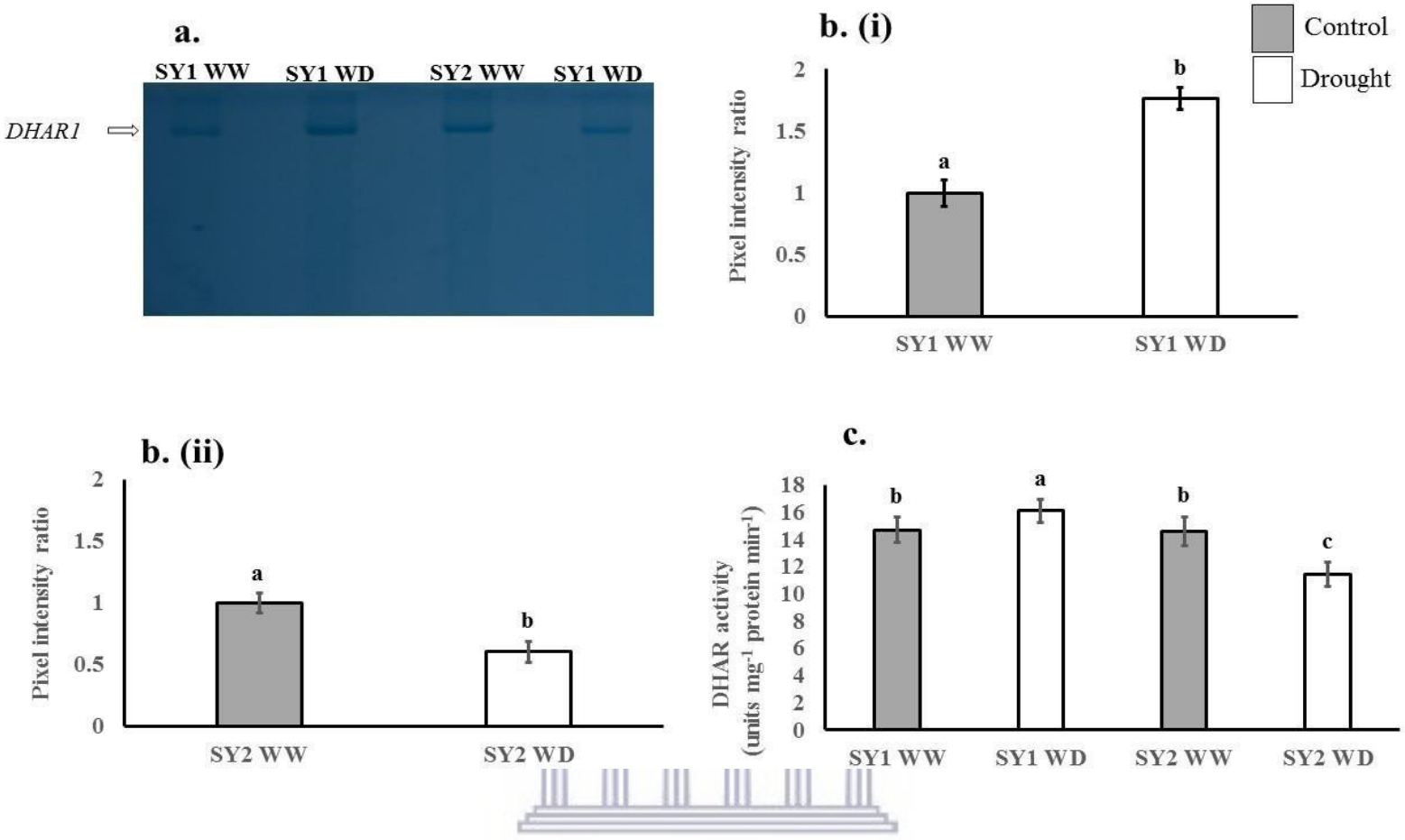

Figure 3.8: Drought stress alters the activity of dehydroascorbate in sugar beet cultivars. Fig. 3.8a, b (i-ii) and c represent the native PAGE, relative DHAR pixel intensity ratio and total DHAR activity respectively. The dark blue bands on a blue background indicate DHAR activity. Data presented are means (±SE) of three independent experiments (n=3). Different letters above error bars denote mean values that are significantly different at $P < 0.05$.

3.9. Drought stress alters the activity of glutathione peroxidase (GPX) in the leaves of two sugar beet cultivars.

Glutathione peroxidase (GPX) plays a critical role in the survival of plants against oxidative stress by utilizing GSH to reduce H_2O_2 and lipid hydroperoxides. GPX activity was assessed in the leaves of two contrasting sugar beet cultivars in response to drought stress, using both in-gel activity staining and spectrophotometric assay. The results on native-PAGE revealed three GPX isoforms (*GPX1, GPX2 and GPX3*) present in both water regimes of sugar beet cultivars with varying activity bands (Fig. 3.9a).

The expression level of GPX isoforms displayed no significant change in band intensities under normal conditions as shown by pixel intensity ratio in Fig. 3.9b (i-vi). However, drought stress enhanced the activity of all GPX isoforms in the drought tolerant cultivar, whereas the drought sensitive cultivar showed a significant increase in the activity of *GPX3* (Fig. 3.9b.vi), while there was a gradual decrease on *GPX1* (Fig. 3.9b.ii) and *GPX2* activity remained unchanged in relation to normal conditions (Fig. 3.9b.iv).

Total GPX activity displayed an increase in both cultivars of sugar beet (even higher on SY1) under drought stress conditions compared to their controls (Fig. 3.9c). An increase in total GPX activity that was observed on SY2 cultivar can most likely be attributed to the induction of the *GPX3* isoform since the expression of *GPX1* and *GPX2* were not upregulated under water deprived conditions. There was no significant difference in total GPX activity of SY1 and SY2 cultivars under normal conditions, which resembles a similar pattern that was observed on the native PAGE.

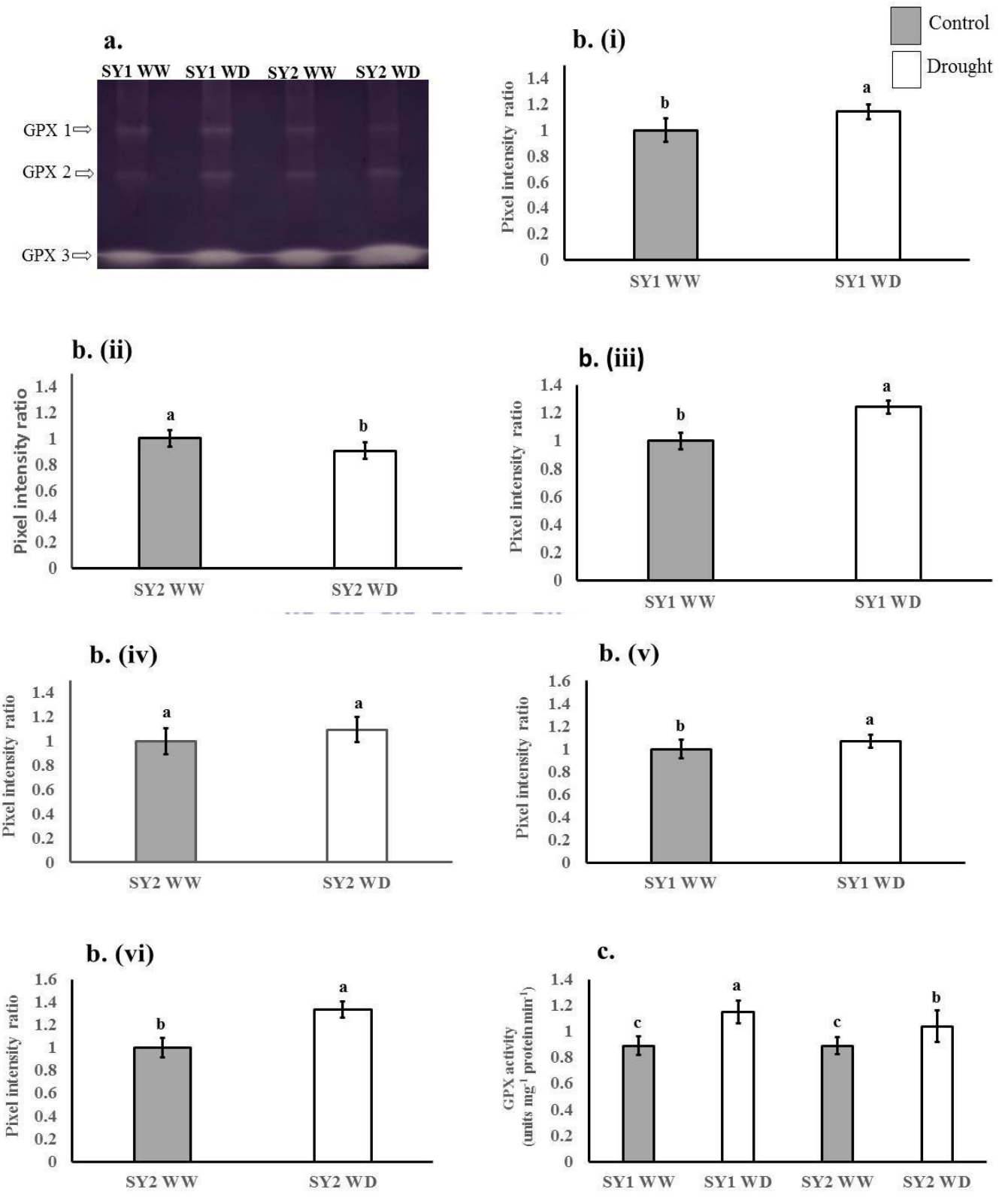

Figure 3.9: The effect of drought stress enhances the activity of glutathione peroxidase in sugar beet plants. Fig. 3.9a shows the native PAGE for determination of GPX isoforms, while b (i-vi) and c represent the relative GPX pixel intensity ratio of the detected isoforms and total GPX activity

respectively. Data presented are means (±SE) of three independent experiments (n=3). Different letters above error bars denote mean values that are significantly different at $P < 0.05$.

Chapter 4

Discussion

4.1. Drought stress reduces the biomass of two contrasting sugar beet cultivars.

Among abiotic stresses, drought stress is considered to be the most detrimental factor limiting the growth and yield of agricultural crops. In an effort to stabilize agricultural production, it is imperative to identify genetic sources for drought tolerance. The effect of drought stress on sugar beet biomass was investigated in the present study to elucidate the physiological mechanism of two contrasting sugar beet cultivars by comparatively examining growth parameters. Drought stress is accompanied by increased oxidative stress as a result of excessive accumulation of ROS, particularly hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\cdot-}$) in specialized organelles such as mitochondrion, chloroplast and peroxisomes (Genet et al. 2010). This significantly reduces plant growth, as reported in several plant species under abiotic stress (Shanker &Venkateswarlu 2011; Sayfzadeh & Rashidi 2010; Hajheidari et al. 2005). Cessation of irrigation for a period of 30 days after emergence of the seedlings of the two sugar beet cultivars induced drought stress, which resulted in reduced biomass in the cultivars.

Several studies have reported that constant water deficit reduces leaf water potential, relative water content as well as root mass and these factors contribute to plant biomass (Hajheidari et al. 2005). A study by Hajheidari et al. (2005) on two genotypes of sugar beet differing in genetic background showed a reduction in biomass under drought stress conditions. The fresh weight of drought stressed plants was decreased by approximately 50% of the irrigated controls in both sugar beet genotypes (Hajheidari et al. 2005). Moreover, Shaw et al. (2002) reported that drought tolerant cultivars had the least reduced growth rate compared to the sensitive cultivars. These findings were in agreement with the data presented in this thesis, which demonstrated

a reduction in biomass between two cultivars of sugar beet under drought stressed conditions. Analysis of the two cultivars in relation to each other showed maximum biomass on the drought sensitive cultivar under normal conditions (Fig. 3.1). However, plant exposure to water deficit significantly reduced the biomass of drought sensitive cultivar (SY2) more than that of drought tolerant cultivar (SY1). This may suggest that the drought tolerant cultivar possesses a mechanism of retaining water under severe stress by reducing the surface area of the leaves. A reduction in biomass following drought stress may be attributed to the plant using its energy to activate antioxidant enzymes as a defence mechanism against activated oxygen species. Based on these findings, it appears that SY1 plants exhibit a greater adaption to water deficit than SY2 plants, indicating that SY1 may be resilient to drought stress while SY2 is more sensitive.

4.2. Drought stress enhances the production of hydrogen peroxide in the leaves of two sugar beet genotypes.

When plants are exposed to drought stress, accumulation of reactive oxygen species such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), superoxide anion radicals (O_2^-) and many more prevail (Genet et al. 2010; Sayfzadeh & Rashidi 2010). The effect of drought stress on the accumulation of H_2O_2, as one of the important oxygen species, was investigated in the leaves of the two sugar beet cultivars. H_2O_2 is a strong oxidant which is mainly produced through the scavenging of superoxide radicals (by superoxide dismutase) and may diffuse across relatively long distances, with high permeability across membranes (Gill & Tuteja 2010; Zlatev et al. 2006). Under normal conditions or low concentrations, H_2O_2 serves as a secondary messenger involved in signal transduction pathways which may trigger an antioxidative response and therefore contributing to plant defence against oxidative stress (Boguszewska & Zagdańska 2012; Genet et al. 2010; Zlatev et al. 2006). Nonetheless, the major consequence of excessive H_2O_2 accumulation is the disruption of macromolecules such as proteins, lipids

and nucleic acids which may cause oxidative damage, impairing normal functions of cells (Boguszewska & Zagdańska 2012; Genet et al. 2010; Sayfzadeh & Rashidi 2010; Farooq et al. 2009; Karim et al. 2011). It is important to distinguish between conditions where ROS will act as signalling, protective or damaging factors, depending on the equilibrium between the production of ROS and ROS scavenging enzymatic antioxidants (Boguszewska & Zagdańska 2012). In this study, exposure of the two sugar beet cultivars to drought stress significantly increased the concentration of H_2O_2 within leaves (Fig. 3.2). The drought sensitive cultivar displayed higher accumulation of H_2O_2, which may indicate oxidative stress. A moderate increase of H_2O_2 in drought tolerant plants under drought stress suggests that enzymatic antioxidants play a crucial role in minimizing ROS concentrations. The high H_2O_2 content produced in the drought sensitive cultivar under drought stress strongly correlates with the high amount of cell death and lipid peroxidation as shown in Fig. 3.3 and Fig. 3.4 respectively.

A study by Zlatev et al. (2006) on the comparison of resistance to drought of three bean cultivars reported increased production of H_2O_2 content in the leaves of stressed plants under drought stress. The increase was more severe on drought sensitive cultivar, causing a decrease in membrane stability and high lipid peroxidation (Zlatev et al. 2006). Furthermore, Ren et al. (2016) reported enhanced accumulation of ROS (particularly H_2O_2 and $O_2^{\cdot-}$) in the leaves of two *Cerasus humilis* cultivars. They observed that drought sensitive *Cerasus humilis* cultivar namely Nongda4 (ND4) showed a more significant accumulation of H_2O_2 than drought tolerant cultivar Huai'rou (HR) and this is in agreement with our findings in the study reported in this thesis.

4.3. Drought stress induces cell death in sugar beet plants

Water deficit is considered one of the most eminent threats to plants as a result of its multiple damaging effects (Nahar et al. 2015). Prolonged water deficit will inevitably induce accumulation of active oxygen species (AOS)/ reactive oxygen species (ROS) within plant cells, which can cause severe oxidative stress by oxidizing multiple cellular components (such as membrane lipids, nucleic acids and proteins as well as the photosynthetic pigments) and disrupting the enzymatic functions (Caverzan et al. 2016; Genet et al. 2010; Cruz de Carvalho 2008).

Efficient ROS detoxification and reduced cell death is correlated with plant tolerance to drought stress (Genet et al. 2010). Therefore, the viability of cells in the leaves of two sugar beet cultivars was investigated in response to drought. Cell death measurement was done using Evans blue assay, which only stains dead cells. The water-deprived plants of both sugar beet cultivars showed high amount of dead cells in comparison to the controls. Cell death increase was more pronounced on drought sensitive cultivar which may indicate that accumulation of AOS prevailed and this is also supported by higher MDA content on drought sensitive cultivar than drought tolerant cultivar. Our findings are in agreement with those from other studies which reported increased cell death under unfavorable conditions.

4.4. Lipid peroxidation increases between two sugar beet genotypes in response to drought stress.

Lipid peroxidation has been found to be one of the most detrimental processes in all living organisms (Gill & Tuteja 2010). The effect of drought stress on lipid peroxidation was studied in this thesis in the leaves of two contrasting sugar beet genotypes. Oxidative stress which results from the accumulation of ROS can damage polyunsaturated fatty acids that form the lipid bilayer through lipid peroxidation (Wahsha et al. 2012; Foyer & Noctor 2005). The peroxidation of lipids produces various products such as malondialdehyde (MDA) which can be used to assess the degree of lipid peroxidation (Gill & Tuteja 2010).

In this thesis, as an indicator of lipid peroxidation, the MDA content in the leaves of drought sensitive sugar beet plants was more pronounced than on drought tolerant plants upon exposure to drought stress conditions, suggesting that SY1 exhibits less sensitivity than SY2. Moreover, several reports have indicated that H_2O_2 and other oxygen species are the driving factors of lipid peroxidation. Hence, the high amount of lipid peroxidation that is observed in the leaves of the SY2 cultivar under drought stress appears to be due to high accumulation of H_2O_2, which degrades polyunsaturated fatty acids that form the base of lipid membranes (Wahsha et al. 2012). Many authors have shown that plants exposed to abiotic stresses exhibit high lipid peroxidation due to increased production of ROS (Gill & Tuteja 2010). Khan & Panda (2008) reported a significant elevation in the level of lipid peroxidation when assessing the response of *Oryza sativa* (rice) cultivars following salt stress. An increased MDA content has been noted in the leaves of *Phaleolus vulgaris* plants under drought stress conditions (Zlatev et al. 2006). Simova-Stoilova et al. (2010) reported that leakiness of membranes as a consequence of lipid peroxidation was more severe in sensitive cultivars of wheat plants in response to drought stress conditions than tolerant ones. In addition, Pastori & Trippi (1992) as well as Sairam et al. (1998) reported reduced lipid peroxidation and higher membrane stability in

drought tolerant genotypes of maize and wheat, respectively.

These findings are in agreement with our results as presented in this thesis, which display a pronounced oxidative damage on drought sensitive sugar beet cultivar than drought tolerant cultivar. Hence, lipid peroxidation may be used as an important marker of physiological mechanisms in selecting cultivars tolerant to drought stress. There is also a positive correlation in the results of the present study in terms of plant biomass, ROS generation, cell death and lipid peroxidation.

4.5. ROS scavenging enzymatic antioxidants

When plants are exposed to unfavorable environmental conditions such as drought stress, production of ROS increases (Gill & Tuteja 2010; Sayfzadeh & Rashidi 2010). In such conditions, plants have evolved protective mechanisms of defense in order to combat the toxic effects of these oxygen intermediates and escape from oxidative damage (Genet et al. 2010; Gill & Tuteja 2010). The enzymatic antioxidant (such as superoxide dismutase, ascorbate peroxidase, catalase, dehydroascorbate reductase and glutathione peroxidase) defence system has been established as one of the most effective machineries for protection against various abiotic stresses, including drought (Gill & Tuteja 2010; Sayfzadeh & Rashidi 2010). Several papers have noted that whether plants undergo oxidative damage or signalling depends on the balance between ROS production and activities of enzymatic antioxidants (Genet et al. 2010). The activities of enzymatic antioxidants under drought stress varies among plant species and even between cultivars of the same species (Sayfzadeh & Rashidi 2010). The ability of reducing oxidative damage through ROS detoxification is typically regarded as an indicator of plant tolerance to drought stress (Romano et al. 2013; Genet et al. 2010). Moreover, several authors reported increased antioxidant enzyme activity in the drought tolerant plants, whereas drought sensitive cultivars displayed low antioxidant enzyme activity (Sayfzadeh & Rashidi 2010). Therefore, the effect of drought stress on enzymatic antioxidants (SOD, APX, CAT, DHAR and GPX) was investigated in the leaves of two contrasting sugar beet cultivars. In our knowledge, this is a first study in South Africa that is based on characterization of the influence of drought on the antioxidant system of contrasting sugar beet cultivars in relation to their sensitivity to drought using both in-gel and spectrophotometric antioxidant activity assays.

4.5.1. Drought stress stimulates activity of superoxide dismutase (SOD) in the leaves of two sugar beet cultivars

Superoxide dismutase (SOD) is the most effective intracellular antioxidant enzyme which is abundant in all aerobic organisms (Gill & Tuteja 2010). SOD is considered as the first line of defense against ROS mediated oxidative stress, where it catalyzes the dismutation of two superoxide molecules into H_2O_2 and O_2 which is the first step in ROS detoxification (Genet et al. 2010; Gill & Tuteja 2010; Sayfzadeh & Rashidi 2010; Farooq et al. 2009). This also reduces the production of hydroxyl radical by the metal catalyzed Haber-Weiss reaction (Gill & Tuteja 2010). It is well established that the induction of SOD activity in plants and subsequent upregulation of other antioxidants play an indispensable role in maintaining minimum levels of ROS generation (Genet et al. 2010). In this study, the activity of SOD isoforms was detected through negative staining and identified based on their sensitivity using two inhibitors (H_2O_2 and KCN) as described in Chapter 2. The use of in-gel activity staining is essential because it analyses individual SOD isozymes and therefore aids in understanding the effects of stress in different subcellular compartments (Genet et al. 2010). Moreover, detoxification of superoxides in plant cells occurs through differential expression or regulation of SOD isozymes, hence research on the expression of individual SOD isozymes is crucial in order to reveal their role under unfavorable environmental conditions like drought stress (Karim et al. 2011). Nonetheless, several studies reflect the effects of stress on total enzymatic activity, hence our study focused on both in-gel activity staining (which detects the activity of individual isoforms) and spectrophotometric assays (for assessing total enzyme activity). As different SOD isozymes contribute to total enzyme activity, it is essential to assess the contribution of each isoform in response to drought stress. After analysis of SOD native PAGE (Fig. 3.5a), the manganese SOD (Mn-SOD) which is mostly found in mitochondrion and the copper zinc-SOD (Cu/Zn-SOD) which predominates in the chloroplast, cytosol, and peroxisome were detected under both water regimes in the leaves of SY1 and SY2 cultivars. The SY1 cultivar showed

high intensity band of Mn-SOD (which indicates high activity) compared to SY2 cultivar under normal conditions, but the Cu/Zn-SOD activity band was upregulated by SY2 to a greater extent than SY1 under watered regime (Fig. 3.5b.i-iv). However, drought stress significantly enhanced activity bands of these two isoforms, with a remarkable increase of Cu/Zn-SOD on SY1 compared to SY2 with respect to their controls. These results suggest that Mn-SOD and Cu/Zn-SOD could play a pivotal role in the detoxification of ROS and that mitochondrion, peroxisome, chloroplast as well as a fraction of cytosolic compartments are crucial in SOD against the production of superoxide when sugar beet responds to drought stress (Ren et al. 2016). Furthermore, the decrease of Cu/Zn-SOD activity that is observed on SY2 compared to SY1 upon exposure to drought stress is likely indicative of the role of SOD as a key determinant of responses to drought. Similar results have been reported by Ren et al. (2016), where drought stress significantly enhanced the activity of SOD isoforms, especially on drought tolerant plants.

An overall increase in total SOD activity that was observed in the leaves of both SY1 and SY2 was due to activities of Mn-SOD and Cu/Zn-SOD (since Fe-SOD was not detected), especially Cu/Zn-SOD which proved to be the main isozyme responsive to drought stress (Fig. 3.5c). The maximum SOD activity was observed in the drought tolerant cultivar (SY1), which might lead to its high defense mechanism against ROS-mediated oxidative stress and this is supported by less elevation in H_2O_2 content (likely as a result of enhanced APX activity), lipid peroxidation and cell death. However, when comparing these two cultivars in response to water deficit, drought sensitive plants (SY2) showed a decrease in SOD activity compared to drought tolerant plants, which may suggest a low dismutating ability for SY2 to scavenge $O_2^{\cdot -}$, resulting in high lipid peroxidation and consequently more cell damage (as revealed by cell death in Fig. 3.3). Furthermore, the induction of SOD activity in the leaves of sugar beet cultivars under drought stress may be attributed to high generation of reactive oxygen species (ROS) as substrates that stimulate the expression of genes encoding SOD (Genet et

al. 2010). The results reported in this thesis are in agreement with other studies which show enhanced SOD activity in response to environmental stresses. Such response was observed between two cultivars of *Crithmum maritimumm L.* following salt stress (Karim et al. 2011). Moreover, significant increase in activities of Mn-SOD and Cu/Zn-SOD isoforms were observed following salt stress (Gill & Tuteja 2010). Zlatev et al. (2006) observed enhanced activity of SOD in three bean cultivars in response to drought stress. Enhancement of SOD activity has been reported in the leaves of white clover following drought stress (Wang & Li 2008). It is evident that high expression levels of SOD isozymes play a crucial role as key components of cellular defence against ROS- induced oxidative stress, ensuring plant survival under severe environmental conditions.

The results of the present study support the hypothesis that prolonged drought stress induces oxidative injury as a result of excessive ROS accumulation, causing oxidation of membrane lipids and ultimately cell death. Thus, the production of ROS and ROS scavenging capacity is recognized as one of the key determinants of stress tolerance.

4.5.2. The activity of ascorbate peroxidase (APX) increases under drought stress in the leaves of two sugar beet cultivars

Ascorbate peroxidase (APX), which is located in chloroplast and cytosol plays the most essential role in maintaining minimum levels of ROS (Gill & Tuteja 2010). APX reduces H_2O_2 (which is produced by SOD in different cellular compartments) using ascorbate (ASH) as an electron donor or substrate in ascorbate-glutathione (ASH-GSH) cycle, which is also known as Foyer-Halliwell-Asada pathway (Gill & Tuteja 2010; Zhang & Kirkham 1996). APX has also been noted to have a higher affinity for H_2O_2 than other H_2O_2 scavenging enzymes (such as CAT and POD), which makes it crucial against ROS detoxification during oxidative stress (Gill& Tuteja 2010).

The role of this enzyme was investigated in the leaves of two sugar beet genotypes in response

to drought stress using both in-gel activity staining and spectrophotometric assay. Analysis of APX isozyme on a native PAGE revealed the presence of two APX isoforms, *APX1* was identified only on SY2 under normal conditions while *APX2* appeared on SY1 and SY2 under both water regimes (Fig. 3.6a). Activation of *APX1* which was only present on SY2 under normal conditions is unclear, and perhaps identifying the localization of these isoforms could provide a better explanation. Even though *APX2* activity seemed to be high on SY2 than SY1 under normal conditions, statistically there was no significant difference which may indicate similar dismutating capabilities under normal conditions (Fig. 3.6b). However, drought stress enhanced *APX2* activity within these two cultivars. The SY2 cultivar showed maximum activity for this enzyme, indicating a greater need to eliminate excessive H_2O_2 compared to the SY1 cultivar. As shown previously in Chapter 3, the SY2 cultivar displayed high H_2O_2 content, increased lipid peroxidation and high levels of cell death, which indicates more oxidative damage. Therefore, increased APX activity observed in SY2 can be associated with the plant's response to eliminate excessive oxidative stress. On the other side the gradual increase in APX activity on SY1 was enough to eliminate H_2O_2 as the latter did not experience more oxidative damage than SY2, therefore saved its energy for activation of other detoxification mechanism. The results on spectrophotometric assay for total enzyme activity revealed a similar pattern which demonstrated high total APX activity in response to water deficit (Fig. 3.6c). These results are in accordance with those from other studies reporting similar patterns under different environmental stresses. Significant increase in APX activity has been reported in three bean cultivars following water stress (Zlatev et al. 2006). Sharma & Dubey (2005) conducted a study on the modulation of nitrate reductase activity in rice seedlings under aluminium toxicity and water stress and found enhanced expression of APX activity. Enhanced APX activity has also been reported in *Anabaena doliolum* following salt stress, which was crucial for H_2O_2 decomposition (Srivastava et al. 2005). Furthermore, it has been noted that simultaneous up- regulation of SOD and APX

expression levels provide a more efficient protection strategy against oxidative stress (Boguszewska & Zagdańska 2012; Genet et al. 2010; Gill & Tuteja 2010). Therefore, APX activity has a pivotal role in H_2O_2 decomposition, reducing the risk of oxidative stress.

4.5.3. Drought stress decreases catalase activity in the leaves of two sugar beet cultivars

The role of catalase (CAT) was assessed in the leaves of two sugar beet cultivars in response to drought stress. CAT, which resides in the peroxisomes and glyoxysomes, aids in the breakdown of H_2O_2, reducing the latter to $2H_2O$ (Caverzan et al. 2016; Gill & Tuteja 2010; Zhang & Kirkham 1996). This enzyme is considered to possess the highest turnover rate of all enzymatic antioxidants (Gill & Tuteja 2010). However, it has been noted that CAT has a relatively poor affinity for its substrate, which limits the protective mechanism of this enzyme (Zhang & Kirkham 1996). It should be noted that CAT responds to water deficit in various ways (i.e. may increase, decrease or remain the same) depending on the severity and duration of stress as well as the plant species or even between cultivars of the same species (Cruz de Carvalho 2008; Zhang & Kirkham 1996). A study on antioxidant responses to drought in sunflower and sorghum seedlings reported unchanged CAT activity under drought stress conditions (Zhang & Kirkham 1996).

A decrease in CAT activity was reported by Song et al. 2014 in two cultivars of tomato under chilling stress. Furthermore; Sayfzadeh & Rashidi 2010 conducted a study on the effect of drought stress on antioxidant enzyme activities and root yield of sugar beet and observed significant increase in CAT activity. Therefore, it is evident that CAT activity varies between plant species and cultivars and these variations are dependent on the above mentioned factors as well as environmental conditions.

Evaluation of CAT activity in the leaves of two sugar beet cultivars on native PAGE revealed two CAT isoforms which were differently expressed under well-watered and drought stressed

conditions (Fig. 3.7a). Even though CAT activity bands were differently expressed under well-watered and water-deprived conditions, there was no significant difference between *CAT1* and *CAT2* in terms of expression levels. CAT activity bands were significantly enhanced in both cultivars under well-watered conditions. Even though SY2 seemed to have high intensity bands compared to SY1, statistically there was no significant difference in CAT activity between these two cultivars under normal conditions. However, drought stress significantly decreased CAT activity (both *CAT1* and *CAT2*), especially on the SY1 cultivar (Fig. 3.7b). The decrease in CAT activity is seen as a general response under drought stress conditions, especially when APX activity is up-regulated. Moreover, the reduction in CAT activity under drought stress conditions can be associated with inhibition of enzyme synthesis or change in the assembly of enzyme subunits under drought stress conditions (Genet et al. 2010). It has also been suggested that CAT is a less susceptible scavenging enzyme than APX concerning oxidative stress (Cruz de Carvalho 2008). Several authors have observed that CAT activity is highly enhanced when plants experience severe water stress and its lower affinity for scavenging H_2O_2 than APX may suggest its role in counteracting excessive H_2O_2 production (Cruz de Carvalho 2008). Interestingly; our results show high accumulation of H_2O_2, lipid peroxidation and cell death on the drought sensitive cultivar suggesting that this cultivar experienced more water loss than the drought tolerant cultivar. As such, the drought sensitive cultivar increased its CAT activity to a greater degree compared to drought tolerant cultivar, which can be interpreted as an attempt of the plant to stabilize excessive H_2O_2 production.

Our results show a pattern similar to those from various studies under abiotic stress conditions. A study by Omar et al. (2012) on natural and artificial dehydration of *Jatropha curcas* seeds revealed one CAT isoform which was present in both endosperm and embryo. Analysis of total CAT activity displayed a pattern similar to that of in-gel activity staining observed in this study in both cultivars under normal and water-deprived conditions, in which

downregulation of CAT activity prevailed more on SY1 than SY2 (Fig. 3.7c) in response to drought stress. Such decrease may indicate that H_2O_2 concentration in drought tolerant cultivar has been limited to the level that was not able to efficiently activate CAT. Furthermore, recent reports have noted that increased CAT activity is positively correlated with the degree of drought stress experienced by a plant (Genet et al. 2010).

A decrease in CAT activity was also reported in the leaves of two ecotypes differing in genetic background in response to salt stress (Karim et al. 2011). Likewise, a study on the antioxidant enzyme changes in response to drought stress in ten cultivars of oilseed rape (*Brassica napus* L.) reported decreased CAT activity (Genet et al. 2010). Even though some studies observed significant increase in the levels of CAT (Sayfzadeh & Rashidi 2010), the lack of its contribution to drought tolerance has been reported in many studies (Sofo et al. 2005), suggesting a limited role for this enzyme in maintaining minimum levels of ROS during severe drought stress conditions (Romano et al. 2013).

4.5.4. Drought stress enhances the activity of certain dehydroascorbate reductase (DHAR) isoforms in the leaves of sugar beet cultivars

APX is the first enzyme in the ascorbate-glutathione cycle and its activity should be maintained continuously to ensure plant survival against oxidative stress. Dehydroascorbate reductase (DHAR), which predominates in chloroplast is another enzymatic antioxidant that plays a critical role in ascorbate-glutathione cycle by regenerating ascorbate from the oxidized state (Gill & Tuteja 2010; Kusvuran et al. 2016; Zhang & Kirkham 1996). The regulation of ascorbate by DHAR has been found to be crucial for tolerance against various environmental stresses (Gill & Tuteja 2010).

Due to APX requiring constant supply of ascorbic acid, concurrent increase of these two enzymes is essential to increase the protective mechanism of plants against active oxygen species, particularly H_2O_2. The effect of drought stress was therefore investigated in the leaves of two sugar beet cultivars using both in-gel activity staining and spec-assay. The results on a 7.5% native PAGE produced one DHAR isozyme, which is indicated by dark blue bands on a light blue background (Fig. 3.8a). DHAR activity bands showed no significant difference between the two cultivars under normal conditions. Nonetheless, drought stress increased DHAR activity in drought tolerant cultivar, while a decrease was observed in drought sensitive plants as depicted in Fig. 3.8b (i) and Fig. 3.8b (ii), respectively. The decrease of DHAR activity that is observed on drought sensitive cultivar may be attributed to excessive ROS accumulation, which resulted in the inhibition of enzymes involved in ROS-scavenging system. Increased DHAR activity on drought tolerant cultivar may be attributed to the regeneration of ascorbate, which is considered to be a crucial ROS detoxification machinery of ASH/GSH pathway. Moreover, increased expression of DHAR along with enhanced APX activity suggest that the two antioxidants work in a coordinated manner and were regulated at transcript levels (Ren et al. 2016; Gill & Tuteja 2010).

Analysis on total DHAR activity showed that drought stress enhanced expression of DHAR on drought tolerant plants, whereas a decrease was observed on drought sensitive cultivar (Fig. 3.8c). Our results display a pattern similar to that of Ren et al. (2016), who observed increased DHAR activity in drought tolerant cultivar of *Cerasus humilis* plants following drought stress. Several authors have reported that over-expression of DHAR activity increased plant tolerance to various abiotic stresses by maintaining high levels of ascorbate in the ascorbate-glutathione cycle (Gill & Tuteja 2010). Furthermore, Yin et al. (2010) showed that transgenic tobacco plants with increased expression of DHAR confer tolerance to aluminium stress. A study by Ushimaru et al. (2006) on transgenic *Arabidopsis* plants expressing rice DHAR gene also confer resistance following salt stress. Enhanced tolerance to drought and ozone stresses in transgenic tobacco overexpressing DHAR has also been reported (Eltayeb et al. 2006). Therefore, it is clear that plants with increased expression of DHAR show tolerance to stresses by recycling ascorbic acid which is required for scavenging H_2O_2.

4.5.5. Drought stress alters the activity of glutathione peroxidase (GPX) in the leaves of two sugar beet cultivars

Glutathione peroxidase (GPX) is a large group of isozymes that aids in oxidative stress by using reduced glutathione (GSH) as a reductant to scavenge H_2O_2, organic and lipid hydroperoxides converting them to harmless products like water (Caverzan et al. 2016; Gill & Tuteja 2010; Cruz de Carvalho 2008). It has also been noted that plants preferably use thioredoxin as a reducing agent (Cruz de Carvalho 2008). GPX isozymes are located in the mitochondrion, chloroplast and in the cytosol (Caverzan et al. 2016). It is worth noting that the balance of H_2O_2 scavenging enzymes (such as GPX, APX and CAT) is vital for the suppression of high H_2O_2 concentration under abiotic stresses. Moreover, studies on GPX activity under drought stress are also heterogeneous. Therefore, our study investigated the role of GPX in the

leaves of two sugar beet cultivars in response to drought stress through in-gel activity staining and spec assays.

The results on 12% native PAGE revealed a total of three GPX isoforms (*GPX1*, *GPX2* and *GPX3*) which were present in both water regimes of SY1 and SY2 with differential expression levels (Fig. 3.9c). There was no significant change in the activity of all three isoforms for both SY1 and SY2 under normal conditions. Interestingly, drought stress enhanced the activity of all three isozymes in the drought tolerant cultivar with respect to the control as shown in Fig. 3.9b (i, ii, v). However, only *GPX3* was up-regulated on drought sensitive cultivar as shown in Fig. 3.9b (vi), while there was a decrease in *GPX1* and the activity of *GPX2* remained the same in relation with the control. The results on total GPX activity followed a similar trend, where maximum activity was observed under drought stress conditions especially on drought tolerant cultivar (Fig. 3.9c). The up-regulation in total GPX activity that is observed in water-deprived plants can be associated with enhanced activity of individual GPX isoforms. This upregulation, which predominates on drought tolerant cultivar suggests its ability in utilizing GSH to minimize the inhibitory effects of ROS-induced oxidative stress. Increased GSH content has also been reported to enhance enzymatic components of other antioxidants, reducing oxidative damage under detrimental conditions (Nahar et al. 2015).

Increased GPX activity has been reported in the leaves of sugar beet genotypes following drought stress (Sayfzadeh & Rashidi 2010). Romano et al. (2013) conducted a study on morpho-physiological responses of sugar beet genotypes to drought stress and observed no significant changes in the leaves of sugar beet genotypes. Furthermore, Tohidi-Moghaddam et al. (2009) reported that water deprived plants displayed enhanced GPX activity in the leaves of canola (*Brassica napus L.*) cultivars. These findings support the hypothesis that antioxidant enzyme (such as GPX) activity responds to abiotic stresses in a heterogeneous manner. As such, different antioxidant enzyme activities in different genotypes could be related to different genetic behavior for drought tolerance (Sayfzadeh & Rashidi 2011).

In brief conclusion, the data presented in this thesis confirms that activation of individual isoforms which contribute to total enzyme activity during stress are highly dependent on plant genotype, and that oxidative stress between two sugar beet genotypes is differentially expressed in response to drought, with higher tolerance exhibited by SY1.

6. Conclusion and future prospects

The results of the present study demonstrate that drought stress is a major yield limiting factor that primarily reduces sugar beet growth and production. It is therefore imperative to identify sugar beet genetic resources that would confer drought tolerance in order to increase sugar beet production. The physiological and molecular responses of two contrasting sugar beet cultivars in response to drought stress have been established in this study. Drought stress adversely influenced the growth of sugar beet cultivars and this was observed on the reduction of biomass. When assessing drought tolerance/sensitivity of these two cultivars based on biomass, SY1 seemed to be more tolerant than SY2 as SY1 sustained leaf biomass which is considered one of the vital factors in evaluating drought stress tolerance. Drought stress induced production of hydrogen peroxide, which resulted in significant increase in MDA content measured as a by-product of lipid peroxidation. Similarly, significant enhancement in the concentration of H_2O_2 and MDA content were observed on SY2 compared to SY1, suggesting that the latter is better protected to oxidative damage which might confer drought tolerance. Although low concentration of ROS function in signal transduction pathways that involves H_2O_2 as a secondary messenger in triggering defence responses, overproduction of ROS will overcome the scavenging capacity of antioxidant enzyme resulting in extensive cellular damage and ultimately cell death. Thus, the high accumulation of H_2O_2 in plants exposed to drought is kept at equilibrium by a versatile and cooperative antioxidant system that controls the intracellular concentration of ROS and sets the redox-status of the cell. Enhanced antioxidant enzyme activities of SOD, APX, DHAR and GPX that are observed in the leaves of two sugar beet cultivars under drought stress conditions, were key in the detoxification of ROS. The drought tolerant cultivar showed higher antioxidant enzyme activities in response to water deficit compared to the drought sensitive cultivar which might explain its scavenging ability and adaptation to drought. However, there was a reduction in CAT activity under drought stress

conditions in both sugar beet cultivars especially on drought tolerant plants, which is considered a common phenomenon especially under APX activation. These results confirm our hypothesis that the variations observed in drought tolerant (SY1) and drought sensitive (SY2) sugar beet cultivars are determined by ROS scavenging antioxidant defence system and the induction of individual enzymatic activities during drought stress were therefore dependent on sugar beet cultivar. In essence, different sugar beet cultivars exhibit different responses to drought stress in terms of antioxidant enzyme activities and physiological parameters, with SY1 showing higher tolerance for drought than SY2. These results can be utilized as practical biochemical parameters for drought tolerance when selecting drought tolerant sugar beet cultivars for breeding in semi-arid regions like South Africa. The SY1 cultivar which displays enhanced antioxidant enzyme activities that corresponds with its capacity for better protection mechanisms against oxidative injury can be introduced to farmers as a drought tolerant cultivar to plant in dry regions.

The use of sugar beet is widely established in the European countries as a major source of natural sugar. However, in South Africa, sugarcane is the sole source of natural sugar and therefore sugar beet is a potential source of bioethanol since it would not be in direct competition with a food crop for sugar production. Thus, it can be used as a renewable source of energy, reducing the need and use of fossil fuels (such as petrol) that produce greenhouse gases and consequently cause global warming or climate change. As part of the commitment of the national government towards sustainable and green energy, the government has invested billions of Rands in establishing bioethanol production facilities. This is where sugar beet would be used as feedstock for bioethanol production industry, making a strong case for cultivation of sugar beet.

Because of the multigenic nature of the responses of plants to drought stress, which presents a challenge in developing drought resilient cultivars, future prospects of this study may involve the use of omics (i.e. genomics, transcriptomics, proteomics and metabolomics)

analyses to identify and correlate proteins, metabolites and genes that display variations in their levels of expression between the contrasting sugar beet cultivars. This will enable identification of several biological markers which would be coupled with phenotyping in order to link the omics to the phenome, therefore accelerating the development of drought tolerant cultivars. Ultimately, the candidate genes would be introduced onto locally adapted cultivars through marker assisted selection (MAS) or conventional breeding to enhance drought tolerance of sugar beet cultivars. The results could also be used as basis in developing other drought tolerant crops like soybean, canola, maize, and cowpea and this could have a positive impact in South Africa by stabilizing agricultural productivity.

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