Comparative analysis of molecular and physiological responses of two canola genotypes to drought stress

Mbukeni Andrew Nkomo

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

UNIVERSITY of the
WESTERN CAPE

Supervisor: Dr Ashwil Klein

Co-Supervisor: Prof Ndomelele Ludidi

Prof Bongani Ndimba

January 2016

How to cite:

Nkomo, M.A. (2016) Comparative analysis of molecular and physiological responses of two canola genotypes to drought stress. M.Sc. Biotechnology. *University of the Western Cape*. Available at University of the Western Cape E-Theses Online

Comparative analysis of molecular and physiological responses of two canola genotypes to drought stress

Mbukeni Andrew Nkomo

KEYWORDS

1D SDS-PAGE

2D SDS-PAGE

Ascorbate peroxidase

Canola

Drought stress

MALDI TOF-TOF MS

Proteomics

Reactive oxygen species

RuBisCO

Superoxide dismutase

ABSTRACT

Comparative analysis of molecular and physiological responses of two canola genotypes to drought stress

Mbukeni Andrew Nkomo

M.Sc. Thesis, Department of Biotechnology, University of the Western Cape

Food security has always been one of the priority concerns in Africa, and it is mostly threatened by drought stress due to climate change. Drought-induced stress is one of the serious limiting factors of plant production, and it is known to impose oxidative stress as a consequence of excessive reactive oxygen species (ROS) accumulation that lead to lipid peroxidation, which is manifested as increased cell death. Hence, this study investigated the influence of drought stress on two contrasting canola genotypes (*Agamax* and *Garnet*), by monitoring their physiological and molecular changes. The results showed that the plant growth and biomass of both genotypes were significantly affected by drought stress as a consequence of excessive ROS accumulation (manifested as H_2O_2 and $OH^·$ content). However, under drought stress conditions, the reduction in biomass and shoot length was more pronounced in the *Garnet* genotype when compared to that of the *Agamax* genotype. This was further supported by the increase in lipid peroxidation and cell death, which were shown to be significantly higher in the *Garnet* genotype when compared to the *Agamax* genotype under drought stress. Furthermore, the antioxidant capacity of the *Agamax* genotype under drought stress was significantly higher than the *Garnet* genotype, suggesting that the *Agamax* has a higher

UNIVERSITY of the
WESTERN CAPE

ROS scavenging ability which prevents oxidative stress and ultimately ROS-induced cellular damage. Hence, given the higher levels of antioxidant activity coupled with the reduction in ROS accumulation that was observed in the *Agamax* genotype, we suggest that the *Agamax* genotype might be slightly less susceptible to drought stress, when compared to the *Garnet* genotype.

Furthermore, understanding the proteomic responses of these two contrasting genotypes that showed a marked difference in response to drought stress might help in unlocking complex biological networks of proteins underlying drought stress tolerance. Hence we use two-dimensional (2D) gel electrophoresis coupled with Matrix assisted laser desorption/ionisation-time of flight/time of flight tandem mass spectrometry (MALDI TOF-TOF MS) analysis for this part of the study, in order to detect and analyze those differentially expressed proteins or proteins whose abundance levels were influenced as a consequence of drought stress. To gain additional insight into the leaf proteomes of the two canola genotypes, a protamine sulphate precipitation (PSP) method was used to remove RuBisCo and confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. A total of 55 well resolved protein spots were selected for mass spectrometry analysis of which 31 (56%) were positively identified using the selective criteria analysis (SCA). All positively identified proteins were then classified into functional categories including protein folding (3%), photosynthetic (29%), detoxification and protection (20%), and energy related proteins whereas 16% could not be classified into any functional category. Apart from spot 32 (Fe superoxide dismutase) and spot 34 (chloroplast beta-carbonic anhydrase), no further significant difference in protein expression/abundance was observed for all the identified proteins for both

UNIVERSITY of the
WESTERN CAPE

genotypes in response to drought stress. Both proteins (spots 32 and 34) have been shown to contain antioxidant activity properties which suggest that they might play a crucial role in improving drought stress tolerance in canola plants.

January 2016

THESIS SYNOPSIS

This **synopsis**, provides a brief overview of this research and summarily outlines the major aims of this study. This thesis is presented as **four chapters**. **Chapter 1** (**Literature review)** explores and coherently presents existing information around the subject under study. It further provides a justification for this research, the questions driving the research and outlines the importance of this study. **Chapter 2** describes the **Comparative analysis of the effect of drought on the antioxidant system of two contrasting canola genotypes** by monitoring the physiological and biochemical responses of two contrasting canola genotypes in response to drought stress. **Chapter 3** investigates the **Proteomic analysis of two contrasting canola genotypes in response to drought stress** by profiling the leaf proteomes of the two canola genotypes and identifying their differentially expressed proteins. This study will also observe how protein abundance is altered by the removal of RuBisCO. Finally, **Chapter 4** summarises the outcomes and the conclusions drawn throughout the thesis, while highlighting the important aspects that could open avenues towards future and further research studies.

UNIVERSITY of the
WESTERN CAPE

January 2016

DECLARATION

I declare that **Comparative analysis of molecular and physiological responses of two canola genotypes to drought stress** is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Mbukeni Andrew Nkomo

2870274

Signature……………………………

UNIVERSITY of the
WESTERN CAPE

January 2016

ACKNOWLEDGEMENTS

My appreciation goes to my friends and colleagues in the **Plant Biotech Research Group**, **Proteomic Research Group, Plant Omics Laboratory** and the **Department of Biotechnology**, specifically to my Co-Supervisors **Prof Ndiko Ludidi** and **Prof Bongani Ndimba**, I am grateful for all of their support and mentorship they gave throughout my master's degree.

My special thanks goes to my supervisor **Dr Ashwil Klein** and my mentor **Mr Anye Valentine** for provided constructive comments and never hesitated to offer their assistance in proof reading my thesis.

I greatly appreciate the care and prayers of my mother (**Nomfusi Grace Nkomo**), sisters (**Nokuthula Nkomo**), brothers (**Sello Nkomo** and **Bongani Phiri**), aunty (**Thobeka Ester Sibeko**), and a special thanks to **Segomotsi Ruth Lebatlang** (my fiancée) and the rest of our families.

Finally, I would like to thank **NRF** and **ARC** for their financial support throughout the year.

Dedicated to "my soul and air" Mabatho Emilly Nkomo.

"The person who says it cannot be done should not interrupt the person doing it"

LIST OF ABBREVIATIONS

1D	One-dimensional
2D	Two-dimensional
2D	Two-dimensional gel electrophoresis
2D-SDS-PAGE	Two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
ANOVA	Analysis of variance
AO	Amine oxidase
APS	Ammonium persulfate
APX	Ascorbate peroxidase
AsA	Ascorbic acid
ASH-GSH cycle	Ascorbic acid-glutathione cycle
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
BLASTp	Protein to protein BLAST
BSA	Bovine serum albumin
CAT	Catalase
CBB	Coomassie Brilliant Blue
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1- propanesulfonate
cm	Centimeters
CO_2	Carbon dioxide
Cu/Zn-SOD	Copper zinc superoxide dismutase

UNIVERSITY of the
WESTERN CAPE

Da	Dalton
DHAsA	Dehydroascorbic acid
DHAR	Dehydroascorbate reductase
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTNB	Nitrobenzoic acid
DTT	Dithiothreitol Cleland’s reagent
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
Fe-SOD	Iron superoxide dismutase
FW	Fresh weight
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione S transferase
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
K_2HPO_4	Dipotassium phosphate
KCN	Potassium cyanide
kDa	Kilo Daltons

UNIVERSITY of the
WESTERN CAPE

KI	Potassium iodide
L	Liter
m/z	Mass to charge ratio
MALDI-TOF	Matrix assisted laser desorption/ionisation-time of flight
MALDI-TOF-TOF	Matrix assisted laser desorption/ionisation-time of flight/time of flight tandem mass spectrometry
MASCOT	Matrix Science
MDA	Malondialdehyde
mg	Mili grams
ml	Mili liter
mM	Milli molar
Mn-SOD	Manganese superoxide dismutase
MOWSE	Molecular weight search
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSDB	Mass spectrometry protein sequence database
MTT	3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information

UNIVERSITY of the WESTERN CAPE

Nr	Non-redundant database
$^1O_2$	Singlet oxygen
O_2^-	superoxide anion
OEC	Oxygen-evolving complex
OH	Hydroxide
$OH^•$	Hydroxyl radical
PAGE	Polyacrylamide gel electrophoresis
PCD	Programmed cell death
pI	Isoelectric point
PMF	Peptide mass fingerprinting
PMS	Phenazine methosulfate
PTM	Post translational modification
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
RuBP	Ribulose-1, 5-biphosphate
RWC	Relative water content
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing Tween 20
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-Tetramethylethylenediaminese
TFA	Trifluoroacetic acid

UNIVERSITY of the
WESTERN CAPE

TOF	Time of flight
Uniprot	Universal protein resource
V	Volts
v/v	Volume to volume
Vhrs	Volt hours
w/v	Weight to volume

UNIVERSITY of the
WESTERN CAPE

LIST OF FIGURES

CHAPTER 1

Figure 1: Plant global response to cope with water deficit, high temperature and salinity.

CHAPTER 2

Figure 2.1: Measurement of canola plant growth and biomass.

Figure 2.2: Drought stress alters ROS accumulation and oxidative damage in canola plants.

Figure 2.3: Changes on SOD enzymatic activity in leaves of two contrasting canola genotypes.

Figure 2.4: Spectrophotometric determination of total SOD activity in leaves of two contrasting canola genotypes.

Figure 2.5: Measurement and detection of total APX activity in leaves of contrasting canola genotypes by spectrophotometry.

Figure 2.6: Measurements of ascorbate content and changes of individual ascorbate peroxidase (APX) isoforms in response to drought stress.

CHAPTER 3

Figure 3.1: Comparison analysis (1D-SDS PAGE) of leaves of the two canola genotypes.

UNIVERSITY of the
WESTERN CAPE

Figure 3.2: Effect of Protamine Sulphate on RuBisCO precipitation.

Figure 3.3: Leaf proteome profiles of two canola genotypes under drought stress.

Figure 3.4: Zoom in section on expression pattern of drought stress responsive proteins.

Figure 3.5: Functional classification of identified proteins according to Bevan *et al.* (1998) or as predicted based upon function.

LIST OF TABLES

CHAPTER 2

Table 2.1: Measurement of individual SOD isoforms in leaves of the two contrasting canola genotypes under drought stress.

Table 2.2: Measurement of individual APX isoform in leaves of the two contrasting canola genotype.

UNIVERSITY of the
WESTERN CAPE

CHAPTER 3

Table 3.1: A list of drought responsive proteins identified by MALDI TOF-TOF MS.

SUPPLEMENTARY DATA

Table 1, Supplementary data: Drought stress responsive proteins that either fall outside the pI range of 4-7, or having a non-significant MOWSE scores of below 70 and those spots with no significant matches.

TABLE OF CONTENTS

KEYWORDS i

ABSTRACT ii

THESIS SYNOPSIS v

DECLARATION vi

ACKNOWLEDGEMENTS vii

LIST OF FIGURES xiii

LIST OF TABLES xiv

TABLE OF CONTENTS xv

CHAPTER 1 1

Literature Review 1

1.1. Introduction 1

1.2 Significant role of canola plants and their agricultural importance 2

1.3 Sources of ROS in plants 3

1.4 ROS signalling in plant cells biochemistry 4

1.4.1 Versatile roles of H_2O_2 in plant cell signalling 5

1.4.2 Versatile roles of $OH^{\bullet}$ in lipid peroxidation 6

1.5 Defence mechanisms against ROS-induced damage 6

1.5.1 Physiological response 6

1.5.2 Biochemical responses 8

1.5.2.1 Enzymatic scavenging of ROS in plants 9

1.5 2.2 Non-enzymatic scavenging of ROS in plants 10

1.6 Proteomics 13

UNIVERSITY of the
WESTERN CAPE

1.6.1 Challenges in bi-dimensional (2D) gel electrophoresis based research 14

1.7 Thesis aims 15

CHAPTER 2 16

Comparative analysis of the effect of drought on the antioxidant system of two contrasting canola genotypes 16

2.1 Introduction 16

2.2 Materials and Methods 18

2.2.1 Plant Growth and Treatments 18

2.2.2 Analyses of plant growth parameters 19

2.2.3. Measurement of the hydroxyl radical ($OH^\cdot$) content 19

2.2.4 Measurement of the hydrogen peroxide (H_2O_2) content 20

2.2.5 Measurement of the malondialdehyde (MDA) content 20

2.2.6 Measurement of cell death 21

2.2.7 Preparation of protein extracts 21

2.2.8 Measurement of the antioxidant enzyme activity in canola leaves 21

2.2.9 Measurement of the ascorbate content 23

2.2.10 Quantitative evaluation of the antioxidant enzymatic activities 24

2.2.11 Statistical analysis 24

2.3 Results 24

2.3.1 Growth and physiological responses of canola plants exposed to drought stress. 24

2.3.2 Drought stress influences ROS accumulation and oxidative damage in canola plants 25

UNIVERSITY of the
WESTERN CAPE

2.3.3 Drought stress differentially alters SOD activity in canola plants 28

2.3.4 The effects of drought stress on ascorbate ratios and ascorbate peroxidase (APX) activity in canola leaves 31

2.4 Discussion 34

2.4.1 Drought stress alters physiological responses in canola genotypes 34

2.4.2 ROS accumulation and its response in plant growth reduction 35

2.4.3 Influence of ROS accumulation on SOD activity 36

2.4.4 Influence of ROS associated changes on ascorbate content and APX activity 37

CHAPTER 3 39

Proteomics analysis of the effect of drought stress on two contrasting canola genotypes 39

3.1 Introduction 39

3.2 Materials and Methods 40

3.2.1 Plant Growth and Treatments 40

3.2.2 Protein extraction from canola leaves for proteomic analysis 41

3.2.3 Protein quantification 41

3.2.4 One and two dimensional polyacrylamide gel electrophoresis 42

3.2.5 Protein identification by MALDI TOF-TOF MS and database search 42

3.2.6 Bioinformatics analysis 43

3.3.2 Removal of the RuBisCO subunits from canola leaf protein extracts 45

3.3.3 Detection of stress responsive protein spots from canola genotypes using 2D PAGE analysis 46

UNIVERSITY of the
WESTERN CAPE

3.3.4 Functional characterization of the differentially expressed protein spots 51

3.4 Discussion 52
3.4.1 Energy metabolism 53
3.4.2 Detoxification and protection 54
3.4.3 Photosynthesis-metabolism 56
3.4.4 Other functional categories 56
CHAPTER 4 58

CONCLUSION AND FUTURE REMARKS 58

REFERENCES 61

SUPPLEMENTARY DATA 81

UNIVERSITY of the
WESTERN CAPE

CHAPTER 1

Literature Review

1.1. Introduction

Plants are generally exposed to diverse environmental stresses which most often alter their developmental, physiology and morphology processes. Environmental stresses can be defined as any change in plant's growth condition, within the plant's natural habitat, which disrupts its metabolic homeostasis (Shulaev *et al.,* 2008). However, drought stress remains as one of the important environmental stresses known to interfere with many vital processes of the plants including photosynthesis, hormonal balance and plant nutrition (Yang *et al.,* 2001; Pinheiro *et al.,* 2011). Further exposure to drought stress have been shown to trigger internal stresses like osmotic and oxidative stresses within the plant, which often increase the production of cellular reactive oxygen species (ROS) such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hydroxyl radicals ($OH^•$) and the organic hydroperoxide (ROOH) within the cells (Mittler, 2002). Overproduction of ROS can negatively affect the plant cells by causing adverse downstream effects in plant cells, which include protein oxidation, lipid peroxidation and RNA/DNA degradation (Mittler, 2002), and thus ultimately leading to plant programmed cell death (PCD) (Gill and Tuteja, 2010).

UNIVERSITY of the
WESTERN CAPE

However, plants' response to these stresses define their survival capacity, and it is therefore important to regulate the concentration of ROS in the cells as most plants have developed complex detoxification systems and enzymatic scavenging pathways to curb ROS accumulation (Apel and Hirt, 2004; Gill and Tuteja, 2010). While it is evident that ROS are not only toxic molecules but also important key regulators of signal transduction

molecules, which are involved in mediating responses to environmental stresses (Mittler *et al.,* 2004; Torres and Dangl, 2005). Such signal transduction molecules may include the activation of drought-responsive proteins. It is also well known that proteins represent the preponderance of biologically active molecules corresponding with most cellular functions (Zhu*,* 2002). Perhaps, it is possible that the use of proteomics tools combined with other rapidly advancing molecular techniques might provide a powerful impetus, which might lead in the identification of key proteins or genes involved in drought tolerant responses. Hence, this review will be exploring the effects of drought stress on plants with emphasis on canola and its importance in the agricultural sector, as well as the roles of plant developed mechanisms to curb ROS accumulation. This review will also be looking at the implications of using proteomics tools on drought stress studies, and specifically focusing on gel based proteomics.

UNIVERSITY of the
WESTERN CAPE

1.2 Significant role of canola plants and their agricultural importance

Canola (e.g. *Brassica napus* and *Brassica rapa*) represent the family of *Brassica* plant species that are well adapted to cool seasons and sensitive to high temperate climates (Morrison 1993; Morrison and Stewart 2002). The production of canola based oil products, are well recognized and have increased worldwide due to their potential health benefits associated with a reduced risk of a number of chronic diseases, such as the coronary heart disease and cancer (Gosslau and Chen, 2004; Grispen *et al.,* 2006). Canola is also used as a good source of protein in animal feed (Grispen *et al.,* 2006). Apparently, these potential health benefits have been partly linked to compounds which possess some antioxidant activities. This compounds include vitamin C and E, carotenoids, and phenolic compounds, which mostly have been shown to contribute

towards the first line of defense against oxidative stress by quenching the- singlet oxygen (Krinsky, 2001).

Most of South Africa's canola is grown in the Western Cape Province as a winter crop and recent climate change assessments have demonstrated a significant change in rainfall pattern, resulting in water deficit which is manifested as drought stress. The effect of drought stress has long been recognized in the agricultural industry due to its negative impact onto plant growth and production (Munns, 2005). Furthermore, when plants encounter environmental stresses (such as drought) they respond by decreasing the surface area of the leaves in order to reduce evaporation and the closing up of the stomata resulting in the slowing down or inhibition of photosynthesis and transpiration processes (Gill and Tuteja, 2010). Stomatal activity can also be affected by environmental stresses, which limit the influx of carbon dioxide (CO_2) and thus impacting onto organelles such as chloroplasts, mitochondria and peroxisomes. These organelles have an intensive rate of electron flow or highly oxidizing metabolic activity, thus further exposure to environmental stresses may lead to the induction of internal stresses (osmotic and oxidative stresses), due to overproduction of reactive oxygen species (ROS).

UNIVERSITY of the
WESTERN CAPE

1.3 Sources of ROS in plants

Reactive oxygen species (ROS) are key signals in the biosynthesis of organic molecules, which are formed as by-products of biological redox reactions in the apoplastic space, chloroplast, cytosol and mitochondria (Arora *et al.,* 2002; Apel and Hirt, 2004; Asada, 2006). However in recent years, other sources of ROS have been identified in plants including amine oxidases, NADPH oxidases and the peroxisomes (Mittler, 2002). During normal growth conditions, ROS are generated at basal level by cellular processes such

as photorespiration and ß-oxidation of fatty acids, but their levels increase when plants are exposed to abiotic stress. Increase in ROS production is termed oxidative burst (Apel and Hirt, 2004). Different enzymes have been associated with oxidative burst, the most important being the plasma-membrane-bound NADPH oxidase (NOX) enzyme which is encoded by the respiratory burst oxidase homolog (*Rboh*) genes (Miller *et al.,* 2010). This enzyme is also involved in the production of superoxide by catalysing the transfer of electrons from NADP to electron acceptors such as the molecular oxygen (Sagi and Fluhr, 2006).

In addition to NADPH oxidase, which has been shown to produce ROS during pathogen attack (Liu *et al.,* 2010), other ROS producing enzymes include the amine oxidase (AO) (Allan and Fluhr, 1997), which oxidises several forms of amine to release H_2O_2 in the apoplast, and the oxalate oxidase (OXO) which catalyses the oxidation of hypoxanthine to xanthine, which is further catalysed to produce uric acid and O_2^- anion. Apparently an overproduction of ROS, such as singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^•$), during abiotic stresses, will disrupt metabolic processes such as photosynthesis, respiration and nitrogen fixation, which can some phytotoxic reactions such as lipid peroxidation (Apel and Hirt, 2004; Gill and Tuteja, 2010; Miller *et al.,* 2010).

UNIVERSITY of the
WESTERN CAPE

1.4 ROS signalling in plant cells biochemistry

An overproduction of ROS critically depends on the balance between ROS production and ROS scavenging mechanisms, which also relies on the severity and duration of environmental stresses (Farooq *et al.,* 2009). Increase in ROS production may cause a direct damage to cellular components such as membrane proteins, the photosystem II

complex, and membrane lipids (Mittler, 2002), while apart from the ROS damaging effects, plants also use them as secondary messengers in signal transduction processes (Apel and Hirt, 2004). In fact, in ROS signalling pathway, O_2^- is one of the primary ROS molecules formed by the reduction of oxygen electrons in a reaction catalysed by NADPH oxidase (Sagi and Fluhr, 2006). While under low pH conditions and in the present of superoxide dismutase (SOD) enzyme, O_2^- can be catalysed to form H_2O_2 (Hancock *et al.,* 2001), which together with $OH^•$ form the subject of the current investigation.

1.4.1 Versatile roles of H_2O_2 in plant cell signalling

Hydrogen peroxide (H_2O_2) is one the most stable ROS signalling molecules, that has the ability to diffuse across membranes thus travelling relatively large distances (Hancock *et al.,* 2001), and mediating the acquisition of tolerance to environmental stresses (Desikan *et al.,* 2003). Apparently H_2O_2 is also known to plays an important role in cell growth and development (Hancock et al., 2001; Neill, 2002), it can also act as a balance point for oxidative damage in response to environmental stresses. Hydrogen peroxide is also found to communicate with other signal molecules such as abscisic acid (ABA), nitric oxide (NO), and calcuim (Ca^{2+}), forming part of the signaling network that controls response to a wide variety of physiological phenomena throughout the downstream of H_2O_2 (Neill, 2002). Evidence also suggested that H_2O_2 production in plants induces defense genes to limit direct infection by pathogens in plant-microbe interactions (Bozsó *et al.,* 2005). In addition to the abovementioned roles, H_2O_2 also takes part in resistance mechanisms, through a reinforcement of the plant cell wall by lignification and cross-linking of the cell wall structural proteins using peroxidases as substrates (Quiroga *et al.,* 2000; Arora *et al.,* 2002).

UNIVERSITY of the
WESTERN CAPE

1.4.2 Versatile roles of $OH^{\bullet}$ in lipid peroxidation

In plants, H_2O_2 is either toxic or protective depending on its concentration and its response to the plant antioxidant system, as higher H_2O_2 can induce oxidative stress and injury to plant cells (Apel and Hirt, 2004; Miller, 2002). Several lines of research have shown that in the presence of metal ions such as copper and iron, both the O_2^- and H_2O_2 can be converted non-enzymatically through the Fenton or Harber-Weiss reactions to produce hydroxyl radicals (Apel and Hirt, 2004; Asada, 2006; Gill and Tuteja, 2010). According to studies conducted by Fischer *et al.* 2007 and Halliwell (2006), the hydroxyl radicals ($OH^{\bullet}$) downstream effects in plant cells include lipid peroxidation which causes changes in the structure and physical state of the membrane and its domains, that then leads to rigidity and leakiness. Hydroxyl radicals ($OH^{\bullet}$) are the most potent lipid oxidizing ROS, which induce oxidative stress and lipid peroxidation (Gill and Tuteja, 2010). Overall, lipid peroxidation can significantly affect membrane functionality and damage membrane proteins with sulphur containing amino acids, which eventually cause the organelles or cells to rupture (Halliwell, 2006). These responses appear to be the effect of cellular signaling induced via a necrotic or programmed cell death (PCD) pathway in plant tissues (Dat *et al.,* 2003; Epple *et al.,* 2003; Mittler *et al.,* 2004).

1.5 Defence mechanisms against ROS-induced damage

1.5.1 Physiological response

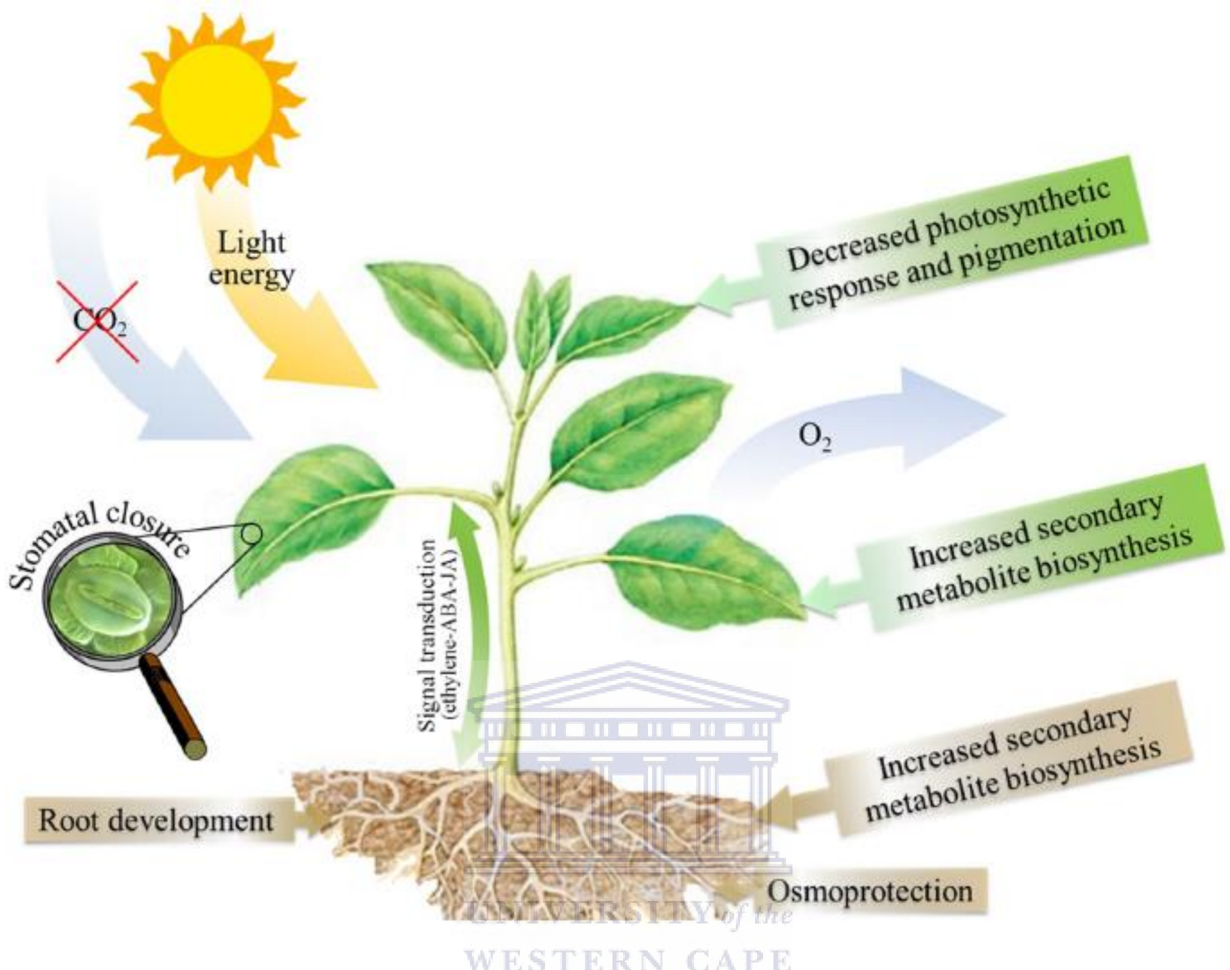
It is well known that plant growth and development can be affected by drought stress resulting in ROS-induced damage. To protect themselves from ROS-induced damage, plants activate several defense responses that are mostly governed by their physiological and biochemical alterations, which mostly determine their capacity to survive (Zingaretti

UNIVERSITY of the
WESTERN CAPE

et al., 2013). Some of the main physiological and biochemical mechanisms triggered by plants under water stress are illustrated in Figure 1 below.

Physiological alteration is often characterized by developmental and morphological traits such as root thickness, decrease in leaves surface area and the ability of roots to penetrate compacted soil layers (Pathan *et al.,* 2007), which are vital in avoiding dehydration during drought by maintaining a constant balance in solute concentration, membrane fluidity and avoiding turgor loss. A plants morphological changes under water deficit are mostly associated with hormone actions even though not discussed in detail in this study. Such hormones include abscisic acid (ABA), which is the main hormone shown to respond to water stress (Hetherington, 2001; Wilkinson and Davies, 2002). A higher concentration of ABA has also been shown to prevent the excessive accumulation of ethylene (another hormone), in order to indirectly maintaining the growth of roots and shoots (Spollen *et al.,* 2000; Sharp *et al.,* 2002). Increases in ABA levels has also been shown to be involved in preventing oxidative stress through the activation of antioxidant systems and in order to reduce ROS production (Jiang and Zhang, 2001).

UNIVERSITY of the
WESTERN CAPE



UNIVERSITY of the
WESTERN CAPE

Figure 1: Plant global response to cope with water deficit, high temperature and salinity. (Adapted from Zingaretti *et al.,* 2013).

1.5.2 Biochemical responses

The adaptation strategy with biochemical alteration is more complex as it greatly depends on the balance between the ROS production and ROS scavenging mechanisms. This mechanisms are mostly comprised of complex antioxidant systems, including an accumulation of osmoprotective solutes, increased levels of antioxidants enzymes and non-enzymatic metabolites, in order to manage the balance between the ROS production and ROS scavenging mechanisms (Apel and Hirt, 2004; Asada, 2006; Miller *et al.,* 2010).

Antioxidant enzymes and non-enzymatic metabolites have been shown to protect plants by suppressing the levels of ROS and also inhibiting the damages caused by ROS following exposure to abiotic stress (Keyster *et al.,* 2012; Keyster *et al.,* 2013). Although various scientific articles and reviews have extensively discussed the significance of ROS production and/or scavenging in plants (Apel and Hirt, 2004; Asada, 2006; Gill and Tuteja, 2010), many questions related to their mechanisms remains unanswered (Mittler, 2002). Thus, giving the impression that a high level of complexity exists in plant signaling processes when examining their response to ROS accumulation during abiotic stress conditions.

1.5.2.1 Enzymatic scavenging of ROS in plants

UNIVERSITY of the
WESTERN CAPE

Antioxidative enzymes are directly involved in scavenging ROS (Mittler, 2002). Superoxide dismutase (SOD) is the first line of defense against ROS and it catalyzes the dismutation of O_2^- to H_2O_2. Superoxide dismutase is located in various cell compartments based on the three distinct metal cofactor: Cu/ZnSOD (cytosol, peroxisomes, and chloroplasts); MnSOD (mitochondria); and FeSOD (plastids) (Bowler *et al.,* 1992; Delledonne *et al.,* 2001). In the absence of SOD, O_2^- reacts with nitric oxide to form peroxynitrite ($ONOO^-$), a highly reactive and destructive anion which is decomposed to form $OH^•$ radicals and together with H_2O_2, contributing to the plant hypersensitive cell death. The peroxynitrite ($ONOO^-$) effect can also be neutralized by non-enzymatic metabolites such as ascorbate and glutathione (Laspina *et al.,* 2005).

Furthermore, several studies have shown that H_2O_2 produced by SOD is still toxic and must be scavenged by catalase and other AsA–GSH cycle scavenging enzymes. Catalases (CAT) are iron-heme containing enzymes allocated in the peroxisomes, the

cytosol and the mitochondria but not in the chloroplast, CAT dismutates H_2O_2 into H_2O and O_2 (Asada, 1999; McKersie and Leshem, 1994), while the chloroplast H_2O_2 is eliminated by ascorbate peroxidase (APX) to produce H_2O at the expense of oxidizing ascorbate (non-enzymatic metabolites) to monohydroascorbate (MDA) (Miller *et al.,* 2010). Ascorbate is then regenerated by the monodehydroascorbate reductase (MDAR) through the utilizing of electrons from NADPH (Moller, 2001).

However, H_2O_2 is also reduce by glutathione peroxidases (GPX; EC 1.11.1.7) found in the cytosol, mitochondria, and plastids. Glutathione peroxidases (GPX), oxidize co-substrates such as ascorbate or phenolic compounds in order to eliminate H_2O_2. Thus, the cysteine oxidation in GSH by H_2O_2 generates a thiyl radical that reacts with a second oxidized glutathione molecule, forming a disulphide bond (GSSG) (Bray *et al.,* 2000). The NADPH-dependent GSSG reduction back to GSH is catalysed by a flavoenzyme glutathione reductases (GR) which also uses electrons directly from NADPH (Mittler, 2002). In addition to contributing to the protection of antioxidant enzymes involved in ROS detoxification (Moradi and Ismail, 2007), non-enzymatic metabolites such as glutathione and ascorbate also play an important role by directly limiting ROS accumulation.

UNIVERSITY of the
WESTERN CAPE

1.5 2.2 Non-enzymatic scavenging of ROS in plants

Most biochemical studies have gone some way in showing the importance of non-enzymatic metabolites (such as ascorbate, glutathione, tocopherol and ß-carotene), and to some extent, osmoprotective compounds (such as proline, sucrose and glycine betaine) in the attainment of drought stress (Moradi and Ismail, 2007; Miller *et al.,* 2010), but on understanding of their role and contribution to drought tolerant is still in its infancy.

Non enzymatic metabolites

Non enzymatic metabolites are essential for maintenance of redox homeostasis by scavenging excess ROS under normal and stressful environments. These include the water-soluble ascorbic acid (AsA) and glutathione (GSH) (Noctor and Foyer, 1998), and the lipid soluble β-carotene and tocopherols (Munne-Bosch and Alegre, 2002).

Tocopherols and β-carotene are lipid-soluble molecules involved in the stabilization of membrane structure by removing lipid peroxyl radicals and oxygen free radicals (Arango and Heise, 1998; Triantaphylides *et al.,* 2008). Tocopherols are known to be synthesized in the envelopes of plastids and are stored in the plastoglobuli of the stroma (Lichtenthaler *et al.,* 1981). On the other hand, results obtained by Trebst *et al.* (2002) suggest that the α-tocopherol (vitamine E) and the β-carotene signalling compound are located in the thylakoid membrane of chloroplasts, this position also allows α-tocopherol to directly scavenge the singlet oxygen ($^1O_2$) generated during the quenching of the triplet state of the PSII reaction center (Munne-Bosch and Alegre, 2002; Trebst *et al.,* 2002; Triantaphylides *et al.,* 2008). In addition, Munne-Bosch and Alegre (2002) reported that the ability of tocopherols to diffuse laterally in the plane of the membrane allows them to react with peroxyl radicals (form during lipid peroxidation) forming tocopheroxyl radicals.

Tocopheroxyl radicals can be reduced by the water-soluble metabolites, ascorbate and glutathione in a tocopherols regeneration process (Munne-Bosch and Alegre, 2002). Water-soluble ascorbic acid (AsA) and glutathione (GSH) mostly result in a specific increase in their levels during different types of abiotic stress, as they both play an

important role in protecting plants from oxidative stress. Both AsA and GSH are found in the chloroplast, cytosol, mitochondria, peroxisomes and apoplast, where their primary function is the detoxification of H_2O_2, although they are still capable of scavenging $^1O_2$, O_2^- and $OH^\bullet$ (Arora *et al.,* 2002; Asada, 2006).

Osmoprotective compounds

Osmoprotective compounds such as proline, sucrose and glycine betaine, play a major role in stress signalling responses (Taylor, 1996; Hare *et al.,* 1999). Osmoprotectants functions include stabilisation of the redox balance and maintenance of proper protein folding. The proline and glycine betaine were also shown to be involve in the regulation of key defence enzymes (catalase and peroxidase), which are involved in suppressing cell death during stress conditions (Banu *et al.,* 2009). However, a study by Omidi *et al.* (2010) compared two canola genotypes under drought stress, and the Okapi genotype (tolerant) was shown to have high proline content when compared to the RGS genotype (sensitive) and the mediated increase in proline content was assumed to serve as an indicator for drought tolerant (Omidi *et al.,* 2010).

Even so, other studies have reported high levels of proline content in some susceptible cultivars exposed to drought stress conditions (Premachandra *et al.,* 1990; Sundaresan *et al.,* 1995). In this context, another study by Fukutoku and Yamato (1981) suggested that proline can function as an important biochemical marker for water deficit but not a measurement of plant tolerance, which might explain the contradictory results for the levels of proline. Rizhsky *et al.* (2004) also found that under a combination of drought and heat stress, plant cells accumulated sucrose instead of proline, suggesting that sucrose plays an important role in tolerance against environmental stress. This might further be

UNIVERSITY of the WESTERN CAPE

supported by the fact that virtually all resurrection plants studied to date, accumulate sucrose during drying (Whittaker *et al.,* 2004; Farrant, 2007; Peters *et al.,* 2007), thus limiting the damaging effects of ROS.

1.6 Proteomics

Proteomics can be defined as the large scale study of the gene products i.e. proteins expressed by their tissue, cell or organism (Blackstock and Weir, 1999; Pandey and Mann, 2000). The use of proteomics tools has enabled a more direct approach in the characterization of cellular, subcellular or organismal proteins, hence providing significant insight into identification of stress-induced gene products. In general, stress-induced gene products can be classified in two categories: firstly, as genes that directly protect plants against stress and secondly, as genes that can regulate the expression of other genes (Bray, 1997; Shao *et al.,* 2007). Hence, it is importance to use proteomics techniques over transcriptomics as proteomics techniques provide additional information on gene regulation, mainly by targeting the active translated portion of the genomes (Gygi *et al.,* 1999; Ideker *et al.,* 2001). This offers an insight into protein abundance due to post-translational, which might not be detected through transcriptomics analysis and thus revealing a weak or moderate correlation between mRNA and protein levels. So far, many proteomics technologies have been employed in the field of protein biomarker discovery (Savino *et al.,* 2012). Proteomics technology can be divided into gel based and non-gel base techniques. However, this review will only be discussing the gel-based proteomics and specially focusing on the 2D gel electrophoresis.

UNIVERSITY of the
WESTERN CAPE

1.6.1 Challenges in bi-dimensional (2D) gel electrophoresis based research

Plants have many effective protection systems, allowing them to perceive an appropriate response to drought stresses (Arora *et al.,* 2002; Apel and Hirt, 2004; Asada, 2006; Halliwell, 2006). However, our understanding of these appropriate responses is incomplete because of the complexity of the drought-induced stress. Hence, many proteomics technologies are currently being used in drought stress research so as to help in unlocking the complex biological networks of proteins underlying drought stress tolerant. Gel-based approach is one of these proteomics technologies used and it includes either the mono-or bi-dimensional gel electrophoresis (1DE or 2DE) followed by identification of protein spots using either the Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) or the nano-LC/MS. Although proteome profiling (using 2D gel electrophoresis) has already been used successful in several plant species and within different tissues in response to various stress (Kim *et al.,* 2005; Wang *et al.,* 2008), one of the limitations of the 2D system is the inability to detect low abundant proteins. This is mostly due to the masking of low abundant proteins by major abundant proteins like RuBisCO (ribulose bisphosphate decarboxylase/oxygenase) in leaf samples (Abat and Deswal, 2009) and other housekeeping proteins, which are present at 10^6-10^5 order of magnitude (Gygi *et al.,* 2000; Patterson and Aebersold, 2003).

Several reports in literature have addressed the issue of RuBisCO complexity in proteomics analyses. For example, Kim and co-workers (2001) initially developed the poly-ethylene glycol (PEG) based method, to which they showed that addition of 20%

UNIVERSITY of the
WESTERN CAPE

PEG was significant in precipitating RuBisCO protein (large and small subunits) in the pellet fraction. Even though this method was successful in depleting RuBisCO it was shown to be time consuming. Hence another method was developed by Krishnan and Natarajan (2009) (using 10 mM calcium and 10 mM phytate at 42°C), which was shown to also deplete some of the heat labile proteins and only 86% of RuBisCO proteins were depleted in fraction. More recently, a protamine sulfate-based specific RuBisCO depletion method was also introduced by Kim and co-workers (2013). Following a protein depletion on soybean leaves 423 new spots were detected which were not discernible in the total fraction, thus making it the most reliable method that can be universally applied in plants (Gupta *et al.,* 2015).

1.7 Thesis aims

The first aim of this study was to investigate the influence of drought stress on the molecular and physiological responses of two contrasting canola genotypes. Furthermore, the study also explored the identification and functional classification of drought stress responsive proteins using 2D PAGE coupled with MALDI-TOF MS analysis that could be used as potential candidates for genetic engineering towards the development of drought tolerant crops.

UNIVERSITY of the
WESTERN CAPE

CHAPTER 2

Comparative analysis of the effect of drought on the antioxidant system of two contrasting canola genotypes

2.1 Introduction

Canola (*Brassica napus* L.) represent the family of plants that are the world's second most important oil-producing crops, and serve as potential model crops for the production of a diverse range of pharmaceutical products and biodegradable grease (Grombacher and Nelson,1992; Raymer, 2002; Grispen *et al.,* 2006). The consumption of canola based oil products has increased worldwide due to their potential health benefits such as reducing the risk of the coronary heart disease and other cardiovascular problems in humans (Van Duyn and Pivonka, 2000; Miller-Cebert *et al.,* 2009). Apart from the above-mentioned facts, canola is also used as a good source of protein in animal feed (Grispen *et al.,* 2006). However, the productivity of canola is mostly limited due to its susceptibility to abiotic stress conditions such as drought, salinity, chilling and flooding, which eventually lead to changes in their physiological, morphological and developmental processes, and therefore inhibiting plant growth (Munns, 2005). Exposure to these abiotic stress conditions may lead to the induction of internal stresses (osmotic and oxidative stresses) due to the overproduction of reactive oxygen species (ROS) such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radicals ($OH^•$) (Mittler, 2002).

UNIVERSITY of the
WESTERN CAPE

Both the osmotic and oxidative stresses have been shown to disrupt essential metabolic processes such as photosynthesis, respiration and nitrogen fixation, causing phytotoxic reactions such as protein oxidation, nucleic acids degradation and lipid peroxidation (Apel and Hirt, 2004; Gill and Tuteja, 2010), thus leading to the disruption of the vital cellular

metabolism in plants (Israr *et al.,* 2006). This may ultimately lead to the inhibition of cell division, and eventually causing programmed cell death (PCD) (Gill and Tuteja, 2010). However, plants activate several defense responses that are mostly governed by their physiological and biochemical alterations, which mostly determine their capacity to survive and adapt to most abiotic stresses. Physiological alteration is often characterized by developmental and morphological traits such as root thickness, surface area and the ability of roots to penetrate compacted soil layers (Pathan *et al.,* 2007), which are vital in avoiding dehydration during drought by maintaining a constant balance in solute concentration, membrane fluidity and avoiding turgor loss (Tabaeizadeh, 1998; Pathan *et al.,* 2007). While the adaptation strategy is more complex with biochemical alteration as it greatly depends on the balance between ROS production and ROS scavenging mechanisms which are mostly comprised into antioxidant enzymes, and non-enzymatic metabolites (Apel and Hirt, 2004; Asada, 2006; Gill and Tuteja, 2010; Miller *et al.*, 2010).

UNIVERSITY of the
WESTERN CAPE

Antioxidant enzymes and the non-enzymatic metabolites have been shown to protect plants by suppressing the levels of ROS and also inhibiting the damage caused by ROS following an exposure to abiotic stress (Keyster *et al.,* 2012; Keyster *et al.,* 2013; Egbichi *et al.*, 2014). Although various scientific articles and reviews have extensively discussed the significance of ROS production and/or scavenging in plants (Apel and Hirt, 2004; Asada, 2006; Gill and Tuteja, 2010; Gill *et al.*, 2011), many questions related to their mechanisms still remains unanswered (Mittler, 2002). Thus, giving the impression that a high level of complexity exists in plant signaling processes when examining responses to ROS accumulation during abiotic stress conditions. In addition, various scavenging pathways, osmoprotective compounds (such as proline, sucrose and glycine betaine) and

antioxidants such as cysteines also play an important role in limiting the ROS accumulation either directly or indirectly by contributing to the protection of antioxidant enzymes involved in the ROS detoxification (Moradi and Ismail, 2007).

Apparently a failure of these scavenging mechanisms would result in an excessive accumulation of the ROS (O_2^- and H_2O_2), which are detrimental for plant health and development. In addition, several studies have shown that in the presence of metal ions such as copper and iron, both the O_2^- and H_2O_2 can be converted to hydroxyl radicals ($OH^\cdot$) either by the Fenton or Harber-Weiss reaction (Hancock *et al.,* 2001; Apel and Hirt, 2004; Asada, 2006; Gill and Tuteja, 2010). A few lines of research have also shown that high levels of hydroxyl radicals ($OH^\cdot$) in plant cells can increase the extent of lipid peroxidation which subsequently leads to a degradation of the biological membranes and their domains, thus leading to cell wall rigidity and leakiness, which are manifested as high levels of cell death (Fischer *et al.,* 2007; Halliwell, 2006). Therefore, this study will investigate the influence of drought stress on two contrasting canola genotypes (*Agamax* and *Garnet*) by monitoring their physiological and molecular responses towards drought stress.

UNIVERSITY of the
WESTERN CAPE

2.2 Materials and Methods

2.2.1 Plant Growth and Treatments

Seeds of two *Brassica napus* (L.) genotypes (*Agamax* and *Garnet*) were obtained from Agricol (Brackenfell, South Africa) and all the chemicals were purchased from Sigma-Aldrich, unless otherwise stated.

The seeds (approximately 70 of each genotype) were surface sterilized in 0.35% (v/v) sodium hypochlorite (bleach) for 10 minutes, followed by five washes with sterile distilled water. The seeds were imbibed in sterile distilled water at room temperature for 30 minutes, and incubated in 10% (w/v) calcium sulphate for 16 hours in the presence of constant oxygen. The seeds were then germinated in 1L pots [17.5 cm x 20 cm] containing a 2:1 mixture of Landscapers choice compost soil and potting soil (Shoprite® Brackenfell, South Africa), that was pre-soaked with distilled water. The germinated seeds were allowed to grow (one plant per pot) on a 25/19°C day/night temperature cycle and a 16/8 hours light/dark regime with a photon flux density of 300 μmol photons.$m^{-2}.s^{-1}$ during the day (light) for a period of 31 days.

Drought stress was induced by withholding water over a period of 31 days whereas control plants were irrigated twice a week with distilled water for the same period. After 31 days, plants at the same developmental stage (4-leaf stage) were selected for all other subsequent experiments.

UNIVERSITY of the
WESTERN CAPE

2.2.2 Analyses of plant growth parameters

Plants were carefully removed from the soil, avoiding any loss or damage to shoots and leaves. Six plants from each treatment were divided into fresh weights (using leaves) and shoot height.

2.2.3. Measurement of the hydroxyl radical ($OH^{\cdot}$) content

For analysis of the hydroxyl radical ($OH^{\cdot}$) content in canola leaves, a modified procedure by Ahuja *et al.* (2015) was used and calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} after monitoring absorbance at 532 nm and corrected for non-specific

absorbance at 600 nm. The $OH^{\cdot}$ estimation was carried out in triplicate for all samples and expressed as nmol g^{-1} FW.

2.2.4 Measurement of the hydrogen peroxide (H_2O_2) content

Hydrogen peroxide H_2O_2 content was determined based on a method adapted from Velikova *et al.* (2000), where leaves (~100 mg) were ground to a fine powder in liquid nitrogen and homogenized in 500 µl of ice cold 5% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 13,200 X *g* for 30 minutes at 4°C to obtain the H_2O_2 extract. The reaction mixture consists of 75 µl leaves extract, 5 mM K_2HPO_4, pH 5.0 and 0.5 M KI, and samples were incubated at 25°C for 20 minutes and the absorbance measured for each sample at 390 nm. The H_2O_2 content was then calculated based on a standard curve constructed from the absorbance (390 nm) of the H_2O_2 standards.

2.2.5 Measurement of the malondialdehyde (MDA) content

UNIVERSITY of the

For MDA analysis, an aliquot (100 µl) of leaves extract (from section 2.2.4) was mixed with 400 µl of 0.5% TBA (prepared in 20% TCA). The mixture was incubated at 95°C for 30 minutes and the reaction was then stopped by placing the mixture onto ice for 5 minutes. The reaction mixture was centrifuged at 12 000 X *g* for 5 minutes at 4°C. The absorbances of each extract was then measured at 532 nm and 600 nm respectively. After subtracting the non-specific absorbance (600 nm) from each sample, the MDA concentration was then determined by its extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol.g^{-1}FW.

2.2.6 Measurement of cell death

A modified method by Sanevas *et al.* (2007) was used for the cell viability assays of each leaf extract of each treatment. Briefly, leaf material (± 100 mg per treatment) from five different plants of each of the treatments were harvested and stained at room temperature with 0.25% (w/v) Evans Blue for 15 minutes. The leaves were washed twice for 20 minutes in distilled water and incubated for 1 hour at 55°C, after which the Evans Blue stain was extracted using 1% (w/v) SDS. The absorbance of each extract was then measured at 600 nm to determine the levels of the Evans Blue up-take by the dead leaves material.

2.2.7 Preparation of protein extracts

UNIVERSITY of the
WESTERN CAPE

Cell extracts were obtained from canola leaves by grinding the leaf tissue into a fine powder in liquid nitrogen and homogenizing 500 mg of the tissue with 1 ml of the homogenizing buffer [40 mM K_2HPO_4, pH 7.4, 1 mM ethylenediaminetetra acetic acid (EDTA), 5% (w/v) polyvinylpyrrolidone (PVP) molecular weight = 40 000]. The resulting homogenates were then centrifuged at 12 000 X *g* for 15 minutes and the supernatants used for the detection of the antioxidant enzymes. Protein concentrations were determined according to the Bradford (1976) method, using bovine serum albumin (BSA) as a standard.

2.2.8 Measurement of the antioxidant enzyme activity in canola leaves

Superoxide dismutase (SOD) enzymatic activity was measured using both a spectrophotometric and using an in-gel approaches in 200 mg of leaf material. For the spectrophotometric SOD assay, a method modified from Beauchamp and Fridovich (1971) was used. For this spectrophotometric method, 190 µl of the assay buffer [50 mM

K_2HPO_4, pH 7.8, 0.1 mM EDTA, 10 mM methionine, 5 µM riboflavin, 0.1 mM Nitrotetrazolium Blue chloride (NBT)] and 10 µl of the leaf extracts were mixed. The mixture was incubated at room temperature for 20 minutes on a fluorescent light box and absorbance readings at 560 nm were recorded. The SOD activity was then calculated based on the amount of enzyme that was required to cause 50% decrease in the reduction of the NBT to blue formazan. For the detection of SOD isoforms, a native PAGE was performed at 4°C in 10% polyacrylamide mini gels using 120 µg of the protein per sample. The SOD activity was detected by staining with 0.5 mM riboflavin and 2.5 mM nitroblue tetrazolium, and as described by Beauchamp and Fridovich (1971). The associated SOD isoform patterns were determined by incubating gels in 5 mM H_2O_2 (to inhibit both Cu/ZnSOD and FeSOD), or 5 mM KCN (to inhibit only Cu/ZnSOD) (Archibald and Fridovich 1982), as MnSOD is resistant to both treatments.

UNIVERSITY of the
WESTERN CAPE

Ascorbate peroxidase (APX) isoforms were detected as described by Lee and Lee (2000). Non-denaturing PAGE was performed at 4°C in a buffer containing 2 mM ascorbate. Subsequent to electrophoresis the gel was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) and 2 mM ascorbate for a total of 20 minutes with the equilibration buffer changed every 10 minutes. This was followed by the addition of 2 mM H_2O_2 to the gel in 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM ascorbate, immediately after which the gel was incubated for 20 minutes. The gel was subsequently washed with sodium phosphate buffer (pH 7.8), 28 mM TEMED and 2.5 mM NBT, with gentle agitation for approximately 10 minutes in the presence of light, after which the reaction was stopped by a brief wash with distilled water. For the spectrophotometric determination of the ascorbate peroxidase (APX) activity, reaction were performed by mixing 10 µl of leaf

extracts with 50 mM K_2HPO_4, pH 7.0, 0.1 mM EDTA, 0.36 mM ascorbate and 0.72 mM H_2O_2 in a 200 µl reaction. The APX activity was then calculated by following the change in absorbance at 290 nm as described by Nakano and Asada (1981).

2.2.9 Measurement of the ascorbate content

The assays of total ascorbate content, including ascorbate (AsA) and dehydroascorbate (DHAsA) content was based on the formation of the red chelate between the 4.7-diphenyl-1,10-phenanthroline (bathophenanthroline) and the ferrous ion reduced from ferric ion by AsA in acid solution. Total AsA was determined by using dithiothreitol to reduce the DHAsA to AsA. The reaction mixture of the AsA assay included 0.6 M trichloroacetic acid, 3 mM bathophenanthroline, 8 mM H_3PO_4, 2 mM N-ethylmaleimide, 0.17 mM $FeCl_3$, 0.5 ml of absolute ethanol, and 0.1 ml of the enzyme extract. The initial reaction mixture of the total AsA assay included 0.15 ml of the enzyme extract, and 0.45 ml dithiothreitol solution (3.89 mM). The mixture was then left at room temperature for 15 minutes and centrifuged at 1 350 X *g* for 10 minutes. The supernatant was used in the following assay, where the reaction mixture of the total AsA assay included a 0.6 M trichloroacetic acid, a 3 mM bathophenanthroline, a 8 mM H_3PO_4, a 2 mM N-ethylmaleimide, a 0.17 mM $FeCl_3$, a 0.5 ml of absolute ethanol, and a 0.1 ml of the supernatant. The total volumes of the mixtures for the AsA assay and the total AsA assay were both 2.25 ml. After incubation at 37°C for 30 minutes, the solutions were measured at 534 nm. The DHAsA concentrations were calculated by a subtraction of the AsA content from the total AsA content (Arakawa *et al.,* 1981; Nakagawara and Sagisaka, 1984).

UNIVERSITY of the
WESTERN CAPE

2.2.10 Quantitative evaluation of the antioxidant enzymatic activities

The image acquisition densitometry analysis of all native PAGE gels, using the Spot Denso tool (AlphaEase FC imaging software, Alpha Innotech Corporation). Individual gels were scored as arbitrary values (relative enzymatic activity) of three independent gels. The enzymatic activity (for the respective antioxidants) of each isoform in the treatments was scored as an average of the relative pixel intensities from three independent gels and expressed in arbitrary units (by assigning a value of 1 for the isoform control (WW) and expressing the rest of the pixel intensities for that type of an isoform in the other treatments relative to their control isoforms).

2.2.11 Statistical analysis

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

UNIVERSITY of the
WESTERN CAPE

2.3 Results

2.3.1 Growth and physiological responses of canola plants exposed to drought stress.

Drought-induced stress negatively influenced the plant growth of both genotypes with the *Agamax* being the least affected (Figure 2.1 A and Figure 2.1 B). A significant reduction in fresh weights (± 45%) in responses to drought stress was observed for *Agamax*, whereas this reduction was more severe for *Garnet* (± 79%) when compared to their respective controls. Furthermore, no significant reduction in shoot height was observed for *Agamax* in response to drought stress, whereas a significant reduction in shoot height (± 68%) was observed for Garnet in the same treatment when compared to controls.

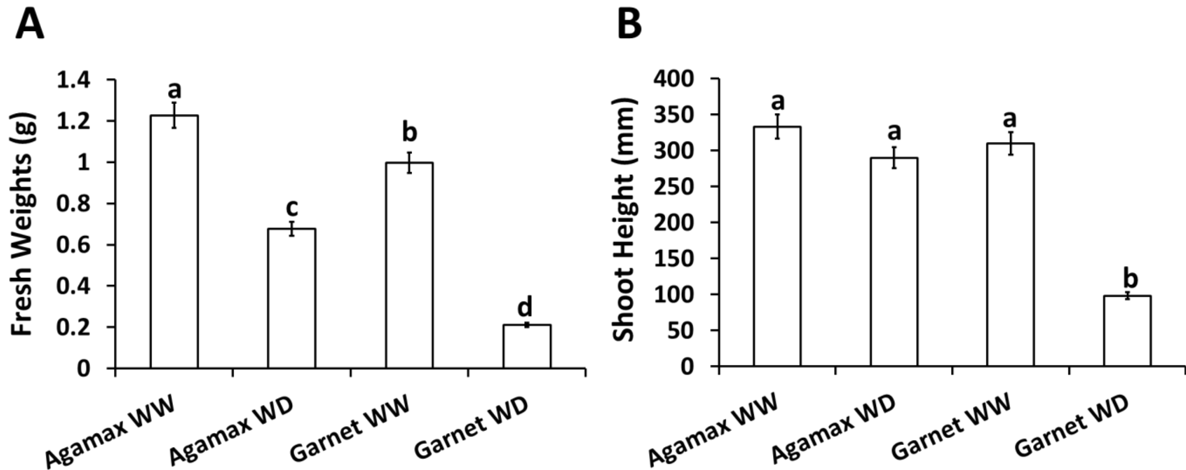


Figure 2.1: Measurement of canola plant growth and biomass. The graphs [Fresh weights (A), and shoot height (B)]. Data represent the mean (±SE) of three independent experiments per treatment. The same letters (a) above the error bars indicated that there was no significant difference between means (P< 0.05). WW represent well-watered and WD represents water-deprived plants.

UNIVERSITY of the
WESTERN CAPE

2.3.2 Drought stress influences ROS accumulation and oxidative damage in canola plants

Drought stress is known to alter normal cellular metabolism as a results of the oxidative stress due to an increase in ROS production, which may result in oxidative damage to cellular macromolecules (DNA, proteins and lipids) (Gill and Tuteja, 2010). However, plant programmed cell death (PCD) mostly results from oxidative damage and one of the known indicators of oxidative damage is lipid peroxidation assessed as the content of malondialdehyde (MDA), which is a useful indicator of oxidative damage to lipids, as a results of membranes susceptibility to the hydroxyl radical ($OH^{\cdot}$) (Foyer and Noctor, 2005). Hence in this part of the study, both the extent of malondialdehyde (MDA) content and the level of cell death were measured as an estimate of the oxidative damage due to increases in H_2O_2 and $OH^{\cdot}$ content. Under normal conditions, no significant difference was observed in the levels of H_2O_2 and $OH^{\cdot}$ contents in both genotypes (Figure 2.2 A-B).

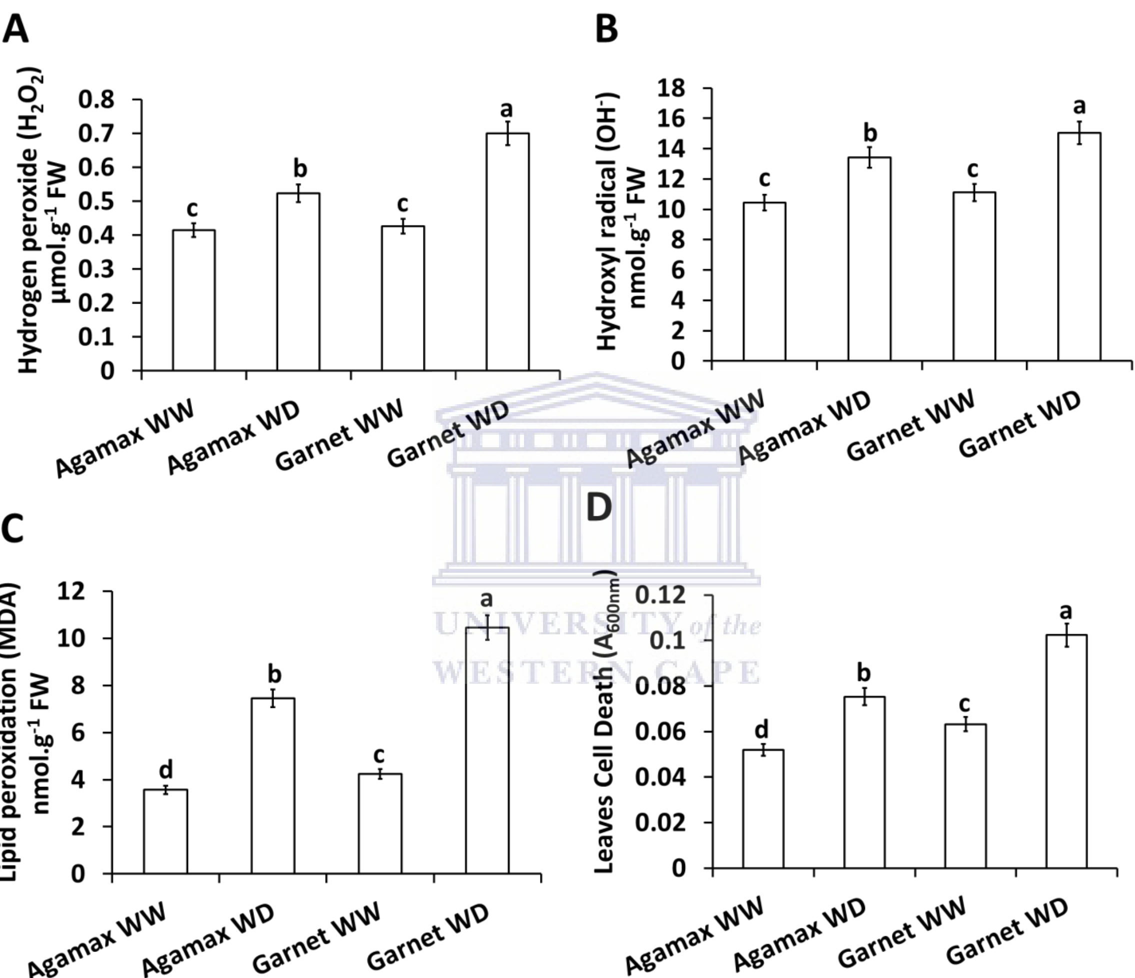
However, these levels increased significantly in both genotypes when exposed to drought stress with the highest increase observed for the *Garnet* genotype. For the *Agamax* genotype, a slight but significant increase in H_2O_2 content (± 26%) in responses to drought stress was observed, whereas the H_2O_2 content in *Garnet* increased by ± 64%. A similar trend for the $OH^{\cdot}$ radical content was also observed for both genotypes under drought stress. The results showed a significant increase in $OH^{\cdot}$ content in the *Garnet* genotype compared to the *Agamax* genotype. The $OH^{\cdot}$ content for the *Garnet* genotype was ± 35% higher in response to drought stress whereas that of *Agamax* was ± 28% when both treatments were compared to their respective controls.

For lipid peroxidation, a significant increase in malondialdehyde content was observed for both genotypes in response to drought stress, although the increase in the *Garnet* genotype was much higher than the increase in the *Agamax* genotype. Exposure to drought stress dramatically increased the level of lipid peroxidation (Figure 2.2 C) in both genotypes. However, the most significant increase in the level of lipid peroxidation was observed for the *Garnet* genotype at ± 147% with *Agamax* genotype showing an increase of ± 109% (Figure 2.2 C), compared to their respective control.

UNIVERSITY of the
WESTERN CAPE

To test whether the increase in lipid peroxidation was associated with membrane leakage and thus the ultimate loss of membrane integrity, the level of cell death on the leaves of both canola genotypes were estimated using the Evans blue stain. Figure 2.2 D shows that under normal conditions the level of cell death in the *Garnet* genotype was slight higher (± 22%) than that of the *Agamax* genotype. On the other hand, the levels of cell death for both the *Agamax* and the *Garnet* were significantly higher under drought stress when compared to their respective controls. Under drought stress, the level of cell death

for the *Agamax* genotype increased by ± 45% with an even higher increase of ± 62% for the *Garnet* genotyped.

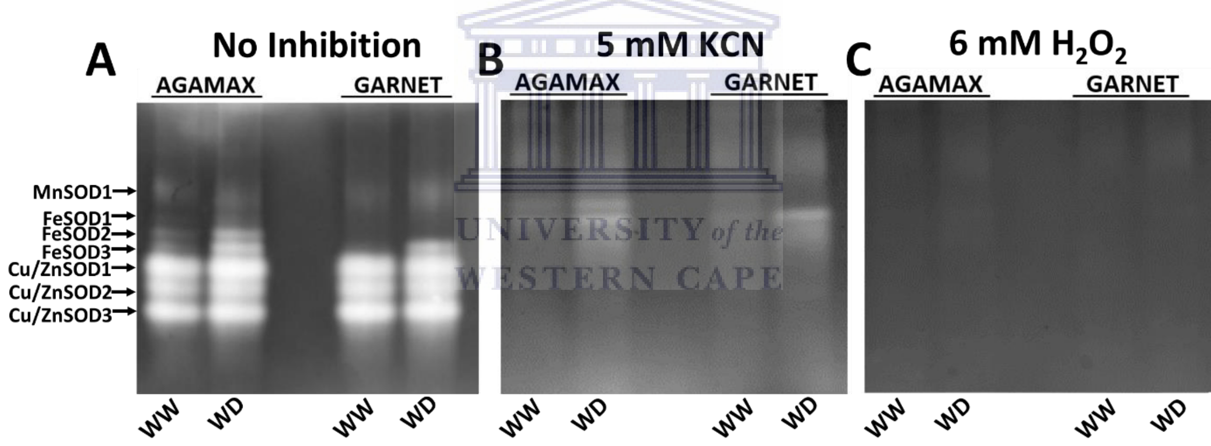


UNIVERSITY of the WESTERN CAPE

Figure 2.2: Drought stress alters ROS accumulation and oxidative damage in canola plants. Hydrogen peroxide content (A), hydroxyl radical (B), lipid peroxidation (C) and cell death (D) were measured in the leaves of two canola genotypes (at the 4 leaf stage of vegetative growth) after 31 days of treatments. WW represent well-watered and WD represents water-deprived plants. The data represent the means ± standard error (SE) from three independent experiments measured in triplicate.

2.3.3 Drought stress differentially alters SOD activity in canola plants

Individual SOD isoforms were identified and characterized by incubating them onto native polyacrylamide gels with 5 mM KCN (to inhibit only Cu/ZnSOD) (Figure 2.3 B), or with 6 mM H_2O_2 (to inhibit both Cu/ZnSOD and FeSOD) (Figure 2.3 C). Isoforms that were resistant to both H_2O_2 and KCN were identified as MnSOD (Fridovich, 1982). Upon exposure to different SOD inhibitors (6 mM H_2O_2 and 5 mM KCN), the SOD isoform profile of the canola leaves included a singlemanganese superoxide dismutase (MnSOD), three iron superoxide dismutases (FeSOD) and three copper/zinc superoxide dismutases (Cu/ZnSODs) (Figure 2.3 A).



UNIVERSITY of the
WESTERN CAPE

Figure 2.3: Changes on SOD enzymatic activity in leaves of two contrasting canola genotypes. The in-gels show the detection of SOD isoforms (A) with no inhibitors, (B) in the presence of 5 mM KCN and (C) in the presence of 6 mM H_2O_2. The WW represent well-watered and WD represents water-deprived plants.

Although the in-gel enzymatic activity might offer a more insight into individual SOD isoforms, it generally imitates the trend seen in SOD spectrophotometric assays. However, a spectrophotometric measurement of the SOD enzymatic activity showed no significant differences in the levels of the total SOD activity under normal conditions. Under drought stress, a significant increase in total SOD activity (± 40%) was observed

for the *Garnet* genotype with an even higher increase (± 100%) observed for the *Agamax* genotype when compared to their respective controls (Figure 2.4). This was further supported by the densitometry data analysis, which determined the pixel intensities of the individual SOD isoforms in Figure 2.3 A. The results in Figure 2.3 A show that drought stress differentially alters some of the SOD isoforms on both the canola genotypes.

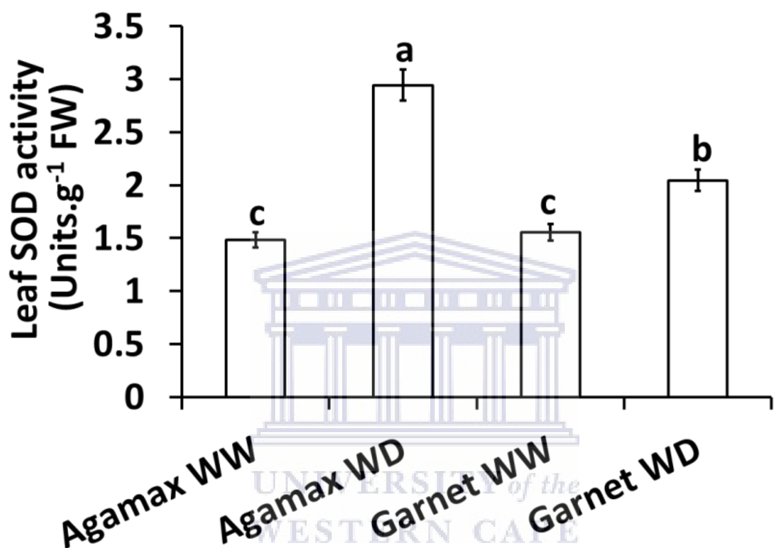


Figure 2.4: Spectrophotometric determination of the total SOD activity in leaves of two contrasting canola genotypes. Different letters on bars indicate the statistically different means ($P < 0.05$). The WW represent well-watered and WD represents water-deprived plants.

Even though a slight visual change observed in the MnSOD1 activity, according to the densitometry analysis, there was no statistical difference ($P < 0.05$) between the different treatments (Figure 2.3 A; Table 2.1). Interestingly, only one FeSOD isoform (FeSOD3) was detected for the *Garnet* genotype whereas three FeSOD isoforms (FeSOD1, FeSOD2, FeSOD3) were present in the *Agamax* genotype. Although FeSOD3 was detected in the *Garnet* genotype in response to drought stress, under normal conditions FeSOD3 was shown to be expressed at very low levels. Furthermore, no or very low SOD

activity was detected for two FeSODs (FeSOD1 and FeSOD2) in the *Garnet* control and drought stress treatments. However, FeSODs (FeSOD1 and FeSOD2) activities were detected in the *Agamax* genotype and a significant difference was observed when comparing both drought stress treatments to their controls, which resulted in an increase of approximately ± 16% for the FeSOD1 and ± 72% for the FeSOD2.

A densitometry analysis (Table 2.1) revealed that there was a slightly increase in the FeSOD3 (± 16%) activity of the *Garnet* control plants when compared to their controls. Whereas under drought stress, FeSOD3 (Figure 2.3 A) showed a significant increase in enzymatic activity on both the *Agamax* (± 65%) and *Garnet* (± 75%) when compared to their respective control. However, the activities of all copper/zinc SOD isoforms (Cu/Zn SOD1, Cu/Zn SOD2 and Cu/Zn SOD3) did not show any significant change in both genotypes (*Agamax* and *Garnet*) when compared to their respective controls in response to drought stress and the relative expression levels of these Cu/Zn SOD isoforms ranged from 1.00 ± 0.05% to 1.08 ± 0.05%.

UNIVERSITY of the
WESTERN CAPE

Table 2.1: Measurement of the individual SOD isoforms in leaves of two contrasting canola genotypes under drought stress.

Canola SOD Isoforms	TREATMENTS			
	Agamax WW	*Agamax* WD	*Garnet* WW	*Garnet* WD
Mn SOD1	1.00 ± 0.05[a]	1.13± 0.06[a]	1.00 ± 0.05[a]	1.24± 0.06[b]
Fe SOD1	1.00 ± 0.05[a]	1.16± 0.06[b]	NA	NA
Fe SOD2	1.00 ± 0.05[a]	1.72± 0.09[b]	NA	NA
Fe SOD3	1.00 ± 0.05[a]	1.65± 0.08[b]	1.00 ± 0.05[a]	1.74± 0.09[b]
Cu/Zn SOD1	1.00 ± 0.05[a]	1.10± 0.06[a]	1.00 ± 0.05[a]	1.06± 0.05[a]
Cu/Zn SOD2	1.00 ± 0.05[a]	1.08± 0.05[a]	1.00 ± 0.05[a]	1.01± 0.05[a]
Cu/Zn SOD3	1.00 ± 0.05[a]	1.08± 0.05[a]	1.00 ± 0.05[a]	1.01± 0.05[a]

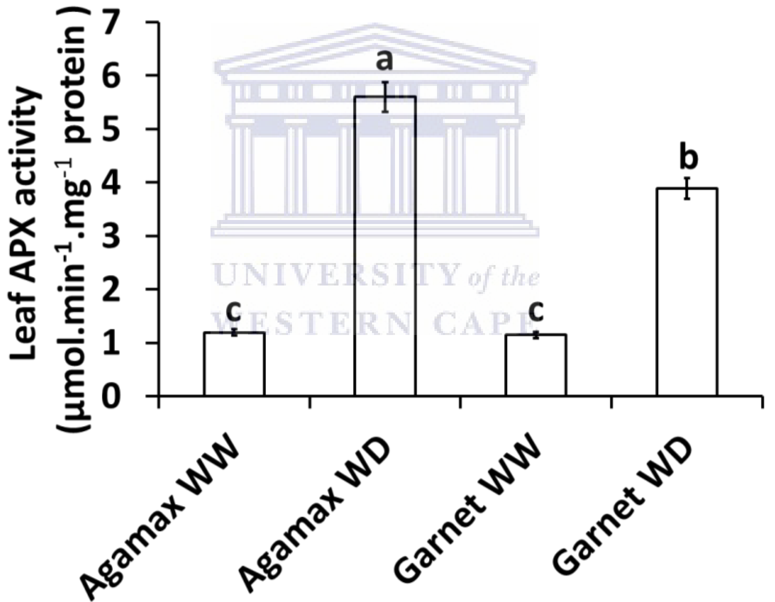
Table 2.1 represent the integrated pixel density values of the superoxide dismutase isoforms as observed on 12% native acrylamide gel (Figure 2.3 A). The relative pixel intensity values are determined using the Alpha Ease FC software and the SOD activities are expressed as arbitrary units, all SOD isoform were normalized using the control of Agamax genotype. Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance according to Tukey-Kramer test. The letters NA in the table indicate that very low or no activity was detected. Table 2.1, the same letters (a) indicated that there was no significant difference between means.

UNIVERSITY of the
WESTERN CAPE

2.3.4 The effects of drought stress on ascorbate ratios and ascorbate peroxidase (APX) activity in canola leaves

Since ascorbate peroxidase (APX) can detoxify H_2O_2 through the Halliwell-Asada pathway using ascorbate as a donor (Halliwell, 2006), it became necessary to evaluate the efficiency of total APX activity (Figure 2.5) and the activity contributed by individual APX isoforms (Figure 2.4 B). In order to determine their efficiency in utilizing the reduced ascorbate when detoxifying H_2O_2, and producing the dehydroascorbate (DHAsA), H_2O and O_2. Interestingly, for the total APX activity, both the *Agamax* and the *Garnet* controls showed no significant differences in the levels of total APX activity (Figure 2.5). A significant increase in total APX activity (± 239%) was observed for the *Garnet* genotype,

with an even higher increase (± 370%) observed for the *Agamax* genotype in response to drought stress when compared to their respective controls (Figure 2.5). On the other hand, the H_2O_2 scavenging capacity was also studied by observing the activity of the APX in-gel (Figure 2.5 B). In this study, four APX isoforms (APX1, APX2, APX3, and APX4) that were more pronounced, were identified according to their migration pattern (Figure 2.5 B), and were analyzed using a densitometry analysis of their controls (WW) and in order to normalize the treatments.



UNIVERSITY of the
WESTERN CAPE

Figure 2.5: Measurement and detection of the total APX activity in leaves of contrasting canola genotypes by spectrophotometry. Different letters on bars indicate statistically different means ($P < 0.05$).

Densitometry analysis revealed that there were no significant changes observed for the APX1 activity between the two genotypes, whereas for the APX3 and the APX4 (Table 2.2), an increase was observed under drought stress for both genotypes. Although both genotypes showed an increase in APX activity (depicted as individual isoforms) under

drought stress, there was no significant difference amongst the two genotypes for the APX3 and APX4 (Table 2.2, the same letters (b) indicated that there was no significant difference between means). However, the densitometry analysis for the APX2 revealed that there was a slight increase in the *Garnet* activity (± 79%), with an even higher increase (± 100%) observed for the *Agamax* in comparison to their respective controls (Figure 2.5 B; Table 2.2).

Table 2.2: Measurement of individual APX isoform in leaves of the two contrasting canola genotype. Table 2.2: represent the integrated pixel density values of the superoxide dismutase isoforms as observed on 12% native acrylamide gel (Figure 2.4 A). The relative pixel intensity values are determined using the Alpha Ease FC software and the APX activities are expressed as arbitrary units, all APX isoform were normalized using the control of *Agamax* genotype. Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance according to Tukey-Kramer test.

Canola APX Isoforms	TREATMENTS			
	***Agamax* WW**	***Agamax* WD**	***Garnet* WW**	***Garnet* WD**
APX1	1.00 ± 0.05^a	1.13 ± 0.06^a	1.00 ± 0.05^a	1.06 ± 0.05^a
APX2	1.00 ± 0.05^a	2.00 ± 0.10^c	1.00 ± 0.05^a	1.79 ± 0.09^b
APX3	1.00 ± 0.05^a	2.12 ± 0.11^b	1.00 ± 0.05^a	2.33 ± 0.12^b
APX4	1.00 ± 0.05^a	1.18 ± 0.06^b	1.00 ± 0.05^a	1.25 ± 0.06^b

UNIVERSITY of the
WESTERN CAPE

While the levels of total AsA increased under drought stress in comparison to their controls, no significant difference was observed between the two genotypes (Figure 2.6 A). However, results for the reduced AsA showed no significant differences across all treatments for both genotypes although the levels of DHAsA increased under drought stress condition. Even so, an increase in the DHAsA was observed to be more pronounced in the *Agamax* genotype (± 49%) than in the *Garnet* genotype (± 24%), when comparing to their respective controls.

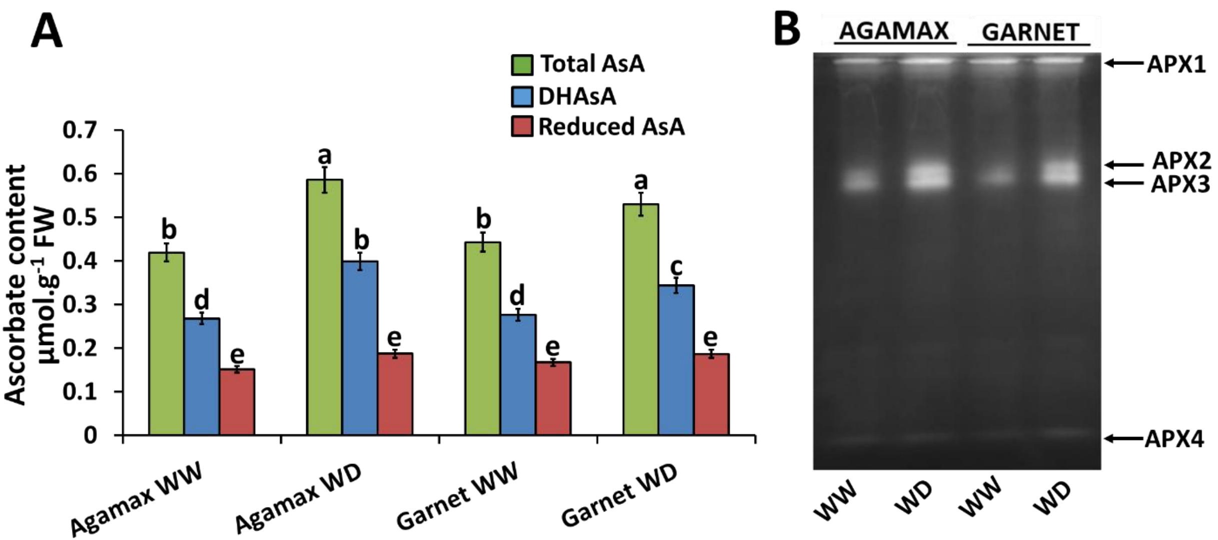


Figure 2.6: Measurements of ascorbate content and changes of individual ascorbate peroxidase (APX) isoforms in response to drought stress. Data for ascorbate content are mean ± standard error of three different plants, representing three independent experiments.

UNIVERSITY of the
WESTERN CAPE

2.4 Discussion

2.4.1 Drought stress alters physiological responses in canola genotypes

Drought is regarded as one of the main environmental stressors that has been shown to cause a reduction in plant growth and development, thus contributing to crop loss and rises in costs of limited agricultural food resources (Zhu, 2002). Many studies are in support of the fact that drought stress causes a reduction in overall plant growth as is seen in *Brassica species* (Hasanuzzaman *et al.,* 2014) and other plant species (Rizhsky *et al.,* 2002; Jaleel *et al.,* 2009). These data are in agreement with our findings, which demonstrated that drought stress does instigate a decrease in shoot height (Figure 2.1, B) and fresh weight (Figure 2.1, A) in both canola genotypes. However, it is also worth noting that when comparing the two genotypes, *Garnet* showed a significantly higher

reduction in fresh weights (Figure 2.1, A) and shoot heights (Figure 2.1 B) as compared to *Agamax* when exposed to drought stress.

2.4.2 ROS accumulation and its response in plant growth reduction

The observed reduction on plant growth in Figure 2.1 A and B could also be attributed to oxidative stress. Oxidative stress is a common effect of drought stress, which is mostly indicated by the increase in ROS accumulation (Garg and Manchanda, 2009; Hasanuzzaman *et al.,* 2012). In our experiment, both the H_2O_2 and $OH^{\cdot}$ levels significantly increased under drought stress, even though when comparing the two genotypes, the increase was more in the *Garnet* genotype (Figure 2.2 A and B). A similar relationship of an increase in ROS activity and a reduction in plant growth has also been observed in other previous research studies (Smirnoff and Wheeler, 2000; Hasanuzzaman *et al.,* 2011).

UNIVERSITY of the

Nevertheless, $OH^{\cdot}$ is one of the highly reactive compounds, which is responsible for the oxidation of polyunsaturated fatty acids (PUFA), thus producing secondary products such as malondialdehyde (MDA), which is an indicator of lipid peroxidation (Smirnoff, 1993). ROS-mediated cell death in plants can also be triggered by increases the levels of lipid peroxidation, which is manifested as increased levels of MDA (Aziz and Larher, 1998). In our results, the levels of MDA increased in both genotypes in response to drought stress (Figure 2.2 C), which partly contributed to an increase in the levels of cell death (Figure 2.2 D). Even so, when comparing the two genotypes the increase was more in the *Garnet* genotype than the *Agamax* genotype. A similar relationship of increased MDA levels and cell death was also observed in previous research findings (Keyster *et al.,* 2013; Egbichi *et al.*, 2014). Both researchers were also able to show a link between a reduction in plant

growth and the increased levels of malondialdehyde and ROS accumulation (Keyster *et al.,* 2013; Egbichi *et al*., 2014).

2.4.3 Influence of ROS accumulation on SOD activity

Having established that there is a link between an increase in the levels of ROS, lipid peroxidation and cell death, which leads to a reduction in the levels of plant growth, it became necessary to evaluate the levels of antioxidant defense system. As plants possess an antioxidant defense system, both this antioxidant enzymes and non-enzymatic metabolites playing a significant role in ROS signaling and scavenging (Mantri *et al.,* 2012). Superoxide dismutase (SOD) is one of the most abundant enzymatic antioxidants, serving as a first-line of defense and as a major contributor to the cellular redox state by protecting plants against oxidative damage (Smirnoff, 2000). From Figure 2.4, it is clear that total SOD activity increased in both genotypes under drought stress conditions, while the increase was even higher in *Agamax* genotype indicating that it has better antioxidant scavenging capacity for superoxide anion (O_2^-). Several researchers also documented an increase in SOD activity in other *Brassica* species in response to drought stress (Alam *et al.,* 2013; Hasanuzzaman *et al.,* 2014), while research by Matters and Scandalios (1986), also suggested a possible link between the increase in plant growth and the increase in SOD activity. Although other *Brassica* studies have shown an increase in total SOD activity (Alam *et al.,* 2014; Hasanuzzaman *et al.,* 2014), It is also important to note that to our knowledge, no other previous studies have been done to investigate the expression profile of individual SODs in *Brassica* species.

UNIVERSITY of the
WESTERN CAPE

The presence of seven SOD isoforms in the *Agamax* genotype and only five isoforms in the *Garnet* genotype further indicates that the *Agamax* genotype had a better scavenging

capacity for O_2^-. Moreover, it seems more likely that the two extra FeSOD (FeSOD1 and FeSOD2) could be contributing towards the higher levels of total SOD activity in the *Agamax* genotype (Figure 2.4). We further suggested that the FeSOD1 and FeSOD2 could be considered as potential candidates for drought stress tolerance as the activities of these two isoforms were enhanced under drought stress when compare to the respective *Agamax* control. This is an important discovery as no or very low activity was detected for the FeSOD1 and FeSOD2 under both control and drought stress conditions of the *Garnet* genotype. The implications of these findings and the fact that *Garnet* genotype accumulated higher H_2O_2 levels prompted us to further investigate a possible link between the H_2O_2-induced damage and ascorbate peroxidase (APX) scavenging activity.

2.4.4 Influence of ROS associated changes on ascorbate content and APX activity

UNIVERSITY of the
WESTERN CAPE

It is well established that the scavenging capacity of H_2O_2 through the Halliwell-Asada pathway is not only mediated by the ascorbate peroxidase (APX) but rather requires a coordinated participation of the ascorbate (AsA). Ascorbate is one of the most important antioxidant metabolites with a variety of functions in cellular metabolism, including the direct or indirect scavenging of H_2O_2 via the Halliwell-Asada pathway (Noctor and Foyer, 1998). In fact, the results presented here show that under drought stress, both genotypes increased their levels of the total AsA pool, which might be involved in the direct scavenging of ROS (Figure 2.6 A). However, given that the reduced levels of AsA remained the same in both genotypes while the DHAsA levels increased under drought stress with the *Agamax* genotype showing the highest increase (Figure 2.6 A), it gives, a

suggestion that the *Agamax* genotype might be having the highest efficiency for scavenging H_2O_2.

This is also further supported by the fact that under drought stress conditions (WD), the *Agamax* genotype had high levels of the total APX activity when compared to the *Garnet* genotype (Figure 2.5). This increase in APX activity might also be correlated to the APX2 isoforms (Figure 2.6 B; Table 2), which under drought stress showed that the *Agamax* genotype had the highest activity while the rest of the other isoforms showed no significant difference amongst the two genotypes. However, in consideration of the results presented in this chapter between the two canola genotypes, the *Agamax* genotype was shown to be the least prone strain or variety to drought stress. This is supported by the fact that under drought stress conditions, *Agamax* genotype showed the least oxidative damage (i.e. lowest levels of ROS (H_2O_2 and $OH^{\cdot}$), lipid peroxidation (MDA) and cell death) and had also shown the highest activity in scavenging antioxidant systems (i.e. ascorbate pool, superoxide dismutase and ascorbate peroxidase).

UNIVERSITY of the
WESTERN CAPE

CHAPTER 3

Proteomics analysis of the effect of drought stress on two contrasting canola genotypes

3.1 Introduction

Canola is one of the central oil-producing crops in South Africa and it is mostly grown as a winter crop in the Western Cape Province, partly because winter in the Western Cape is accompanied by substantial rainfalls. However, recent assessment of the Western Cape climatic conditions showed a significant reduction in rainfall and a rapid increase in surface temperatures, which brings about drought stress (Engelbrecht *et al.,* 2015). Drought stress is a serious limiting factor that alters various plant processes including growth and yield. This environmental condition poses a great threat to the sustainable food security due to shortages of crop-based food for human consumption and livestock fodder. Various plant genotypes within the same species have been shown to respond differently to drought stress (Hong-Bo *et al.,* 2006; Yildiz-Aktas *et al.,* 2009).

UNIVERSITY of the
WESTERN CAPE

Numerous studies, including the work presented in Chapter 2, have shown that plants cope with drought stress by controlling their various molecular and physiological processes (Alam *et al.,* 2013; Alam *et al.,* 2014; Hasanuzzaman *et al.,* 2014). Other studies have also identified numerous genes involved in the modulation of drought stress responses using either molecular, genetics or genomics approaches (Koh *et al.,* 2015). Although significant progress been made in plant science using proteomic approaches, the identification of drought stress responsive proteins in canola plants still remains limited.

The key in understanding the complex network of proteins involved in drought stress tolerant using gel-based proteomics analysis is mostly limited due to the masking of low abundant proteins by major abundant proteins like RuBisCO (ribulose bisphosphate decarboxylase/oxygenase) in leaf samples (Abat and Deswal, 2009; Tanou *et al.,* 2012). This has prompted much interest and thus several methods have been reported in the literature in helping to overcome this problem of the masking of low abundant proteins by RuBisCo. Although each of these methods has its own pros and cons, one of the most reliable methods is that of Kim *et al.* (2013) which uses the protamine sulfate (PS). This method has been shown to be highly efficient in the depletion of RuBisCo in leaves and the depletion of major seeds storage proteins (SSP), making it the most reliable method that can be universally applied in plants (Gupta *et al.,* 2015). Therefore, a 2D gel electrophoresis coupled with the RuBisCo depletion methods, can be very useful to reduce the high abundant proteins.

UNIVERSITY of the
WESTERN CAPE

This study was directed towards the optimization of RuBisCo depletion and the analysis of low abundant proteins to construct proteome profiles of two contrasting canola genotypes in response to drought stress. This would be done to identify changes in protein expression/abundance between the two genotypes, in order to identify putative biomarkers that can be used to enhance drought stress tolerance in canola plants.

3.2 Materials and Methods

3.2.1 Plant Growth and Treatments

Plant growth and treatment was performed as described in section 2.2.1.

3.2.2 Protein extraction from canola leaves for proteomic analysis

Total soluble proteins from canola leaves were extracted using a slightly modified Protamine sulphate precipitation (PSP) method previously described by Kim *et al.* (2013). Protein extracts were obtained by homogenizing 1 g of leaf tissue with 10 ml of ice-cold protein extraction buffer [500 mM Tris-HCl (pH 8.3), 2% v/v NP-40, and 20 mM w/v $MgCl_2$], followed by centrifugation at 12 000 X *g* for 10 minutes at 4°C. The supernatant was mixed with 5% protamine sulphate stock solution (to a final concentration of 0.24%), incubated on ice for 30 minutes and centrifuged at 12 000 X *g* for 10 minutes at 4°C. The PSP reaction mixture was pre-mixed with four volumes of 12.5% TCA/acetone to precipitate proteins at −20°C for overnight. The PSP-derived pellet was re-suspended in a protein extraction buffer (volume used was equal to that of the supernatant) followed by a thorough mixing with four volumes of 12.5% TCA/acetone and protein precipitation at −20°C overnight. After centrifugation at 16 000 X *g* for 5 minutes at 4°C, the pellet was air-dry and re-suspended in IEF rehydration buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS (3-[(3-cholanidopropyl)dimethylammonio]-1-propane sulfonate), containing 0.2% v/v Bio-Lyte 3–10 Ampholyte (Bio-Rad) and 40 mM DTT (dl-dithiothreitol)) and stored at -20°C for further analysis.

UNIVERSITY of the
WESTERN CAPE

3.2.3 Protein quantification

Protein concentration for each sample was determined according to the method of Bradford (1976). The protein concentration was calculated using a bovine serum albumin (BSA) as standard.

3.2.4 One and two dimensional polyacrylamide gel electrophoresis

A fraction of the total soluble leaf protein extracts (30 µg) was size fractionated on a 1D SDS gel to evaluate both the quality and loading quantities of each extracts prior to the 2-D SDS PAGE analysis. For the 2-D SDS PAGE analysis, protein samples (100 µg) were premixed with the Destreak rehydration solution (GE Healthcare) containing 0.2% carrier ampholytes (pH 3–10; Bio-Lyte, Bio-Rad, Hercules, CA, USA) to a final volume of 125 µl and loaded into a focusing tray. Immobilized pH gradient strips (4-7 NL, 7 cm, Bio-Rad) were passively rehydrated overnight. Isoelectric focusing (IEF) was carried out using a Protean IEF Cell system (Bio-Rad) under the following conditions: 250 V for 15 minutes with a linear ramp, 8000 V for 1 h with a linear ramp, and finally 8000 V for 35,000 V-h with a rapid ramp.

UNIVERSITY of the
WESTERN CAPE

After IEF, the strips were incubated for further 15 minutes in equilibration buffer I consisting of 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 130 mM dithiothreitol. The strips were incubated for 15 minutes in equilibration buffer II, consisting of 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 135 mM iodoacetamide. The strips were then equilibrated in SDS containing buffers and run on 12% (w/v) SDS polyacrylamide gels as previously described (Ngara and Ndimba, 2011). The gels were stained with coomassie brilliant blue (CBB) R-250 and imaged using the PharosFX plus molecular imager scanner (Bio-Rad).

3.2.5 Protein identification by MALDI TOF-TOF MS and database search

Low abundant proteins were recovered from the coomassie brilliant blue (CBB)-stained gels and an in-gel digestion was performed as previously described by Rosenfeld *et al.* (1992). The gel spots were analysed using a Bruker Ultraflex III MALDI TOF-TOF mass

spectrometer (Bruker Daltronic GmbH, Germany) and as previously described by Ngara *et al.* (2012). Protein Escape Analysis software was used for the spectral processing and generation of the peak lists for the MS and MS/MS spectra. The combined MS and MS/MS spectral data were subjected to database searching using a copy of the MASCOT ver. 2.1 (Matrix Science, London, UK) that was run locally through the BioTools interface, ver. 3.1 (Bruker). Search criteria included: enzyme trypsin, variable modifications, oxidation (M), peptide tolerance, 100 ppm (parts per million), carbamidomethyl (C) as a fixed modification, MS/MS tolerance, 0.8 Da, instrument and MALDI TOF-TOF MS. The database search was run against the National Center for Biotechnology Information non-redundant protein database NCBInr (www.ncbi.nlm.nih.gov/protein) and Swiss-Prot database (www.uniprot.org).

UNIVERSITY of the
WESTERN CAPE

3.2.6 Bioinformatics analysis

Theoretical Mr and p*I* of MS identified proteins were estimated using the Compute pI/MW tool available on ExPASy (http://expasy.org). However, to ensure quality and correct identification of proteins, a threshold criteria was established for the identified proteins. To be conceded as a positive identification protein spot, p*I* had to be within the range of 4-7 and have a MASCOT scores of above 70. Proteins were eventually grouped into functional categories and according to Bevan *et al.* (1998).

3.3 Results

3.3.1 Separation and visualisation of total soluble proteins on 1D SDS gel

The results described in Figure 3.1 shows the 1D SDS-PAGE analysis of the two contrasting canola genotypes covering the MW range of between 10 and 200 kDa. Lane

M shows the molecular weight marker. Lanes 1, represent the control sample (well-watered; WW) of the *Agamax* genotype, lane 2, represents the drought stress sample (water-deprive; WD) of the *Agamax* genotype whereas lane 3 and 4 represent the same treatments for the *Garnet* genotype. Thirty micrograms of each sample was separated and visualized on a CBB stained polyacrylamide gel. The protein samples for each treatment showed high similarity in terms of protein expression (see the red boxes representing proteins with similar expression), suggesting that protein loading was relatively uniform. The results further show good quality extracts, with no visible signs of streaking and protein degradations. However, it was observed that all treatments had a high abundance of the RuBisCO subunits (as indicated on the gel) (Figure 3.1).

UNIVERSITY of the
WESTERN CAPE

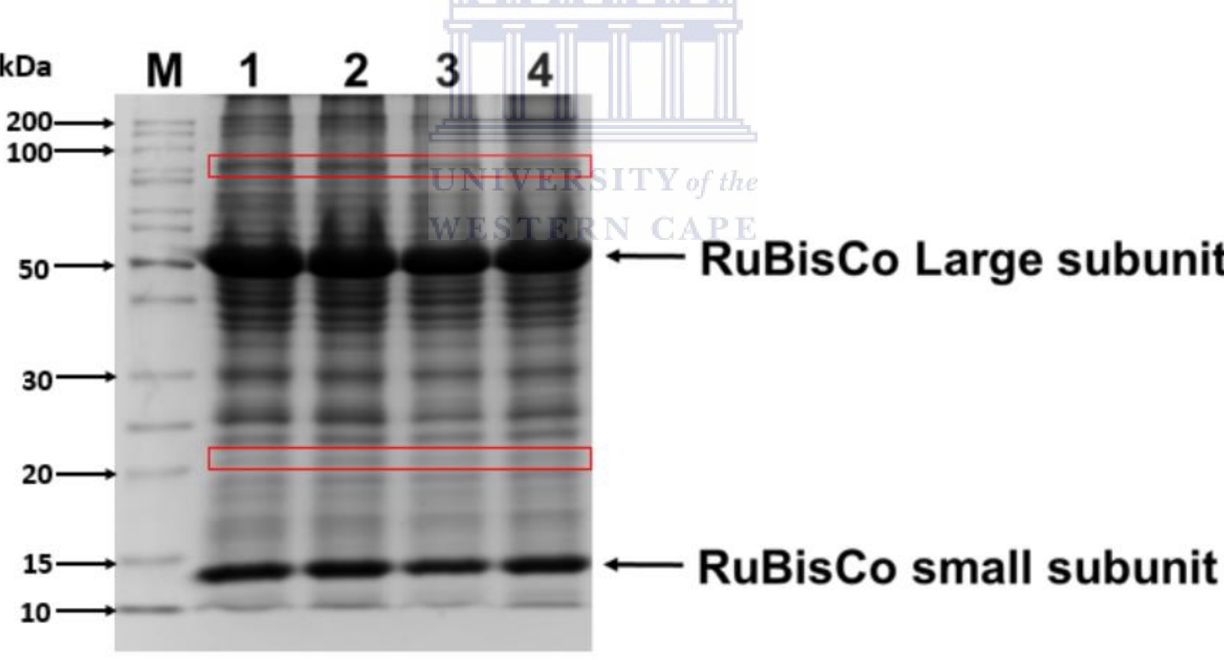


Figure 3.1: Comparative analysis of leaf protein profiles of two canola genotypes**.** Lane M, represent 200 kDa PageRule^{TM} unstained marker. A total of 30 µg total soluble protein was loaded on each lane of SDS-PAGE (12%). (1) *Agamax* control (well-watered; WW), (2) *Agamax* drought stress (water deprive; WD), (3) *Garnet* control (well-watered; WW) and (4) Garnet drought stress (water deprived; WD).

The two indicated RuBisCO subunits greatly reduced the visibility of other protein spots that co-migrated in the same vicinity on the 1D gel (Figure 3.1). In order to detect, identify

and functional categorize these low abundant proteins, all RuBisCO subunits were removed using the protamine sulphate precipitation (PSP) method described in section 3.2.2.

3.3.2 Removal of the RuBisCO subunits from canola leaf protein extracts

Here we describe the use of the PSP method to remove RuBisCO subunits from leaf extract of two contrasting canola genotype to enhance the visibility of spots that migrates in the vicinity of RuBisCO proteins. 1D-SDS polyacrylamide gels was used to show the RuBisCO-depleted sample labeled “S” (i.e 0.24% PS supernatant sample), which were compared to the corresponding total soluble sample (prior to RuBisCO depletion) labeled “T” and the pelleted sample labeled “P” that contained the RuBisCO subunits.

As previously described in section 3.3.1 the leaf protein extracts were of good quality, showing no visible signs of streaking and protein degradations. Protein extracts from leaf tissue for each genotype covered the MW range of between 10 and 200 kDa. In all instances, 10 µg of each protein sample was separated on a 12% SDS polyacrylamide gel (Figure 3.2 A and 3.2 B). The results of Figure 3.2 A and 3.2 B were able to show successful depletion or removal of RuBisCO large and small subunits from the two contrasting canola genotypes (Figure 3.2 A and 3.2 B; lanes S). After precipitation, new bands were detected that were mostly over shadowed by RuBisCO. While for lane T and P, the bands inside the red boxes represent RuBisCO large subunits and those inside the blue boxes represent RuBisCO small subunits.

UNIVERSITY of the WESTERN CAPE

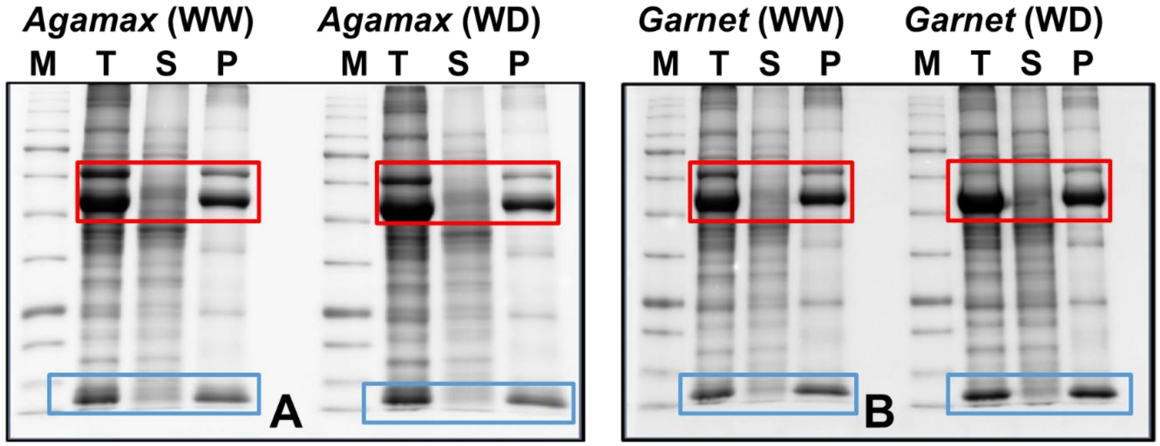


Figure 3.2: Effect of Protamine Sulphate on RuBisCO precipitation. Total leaf proteins (T) of canola were subjected to 0.24%. Both PS supernatant (S) and PS-precipitated (P) proteins (10 µg) were resolved on 12% SDS-PAGE.

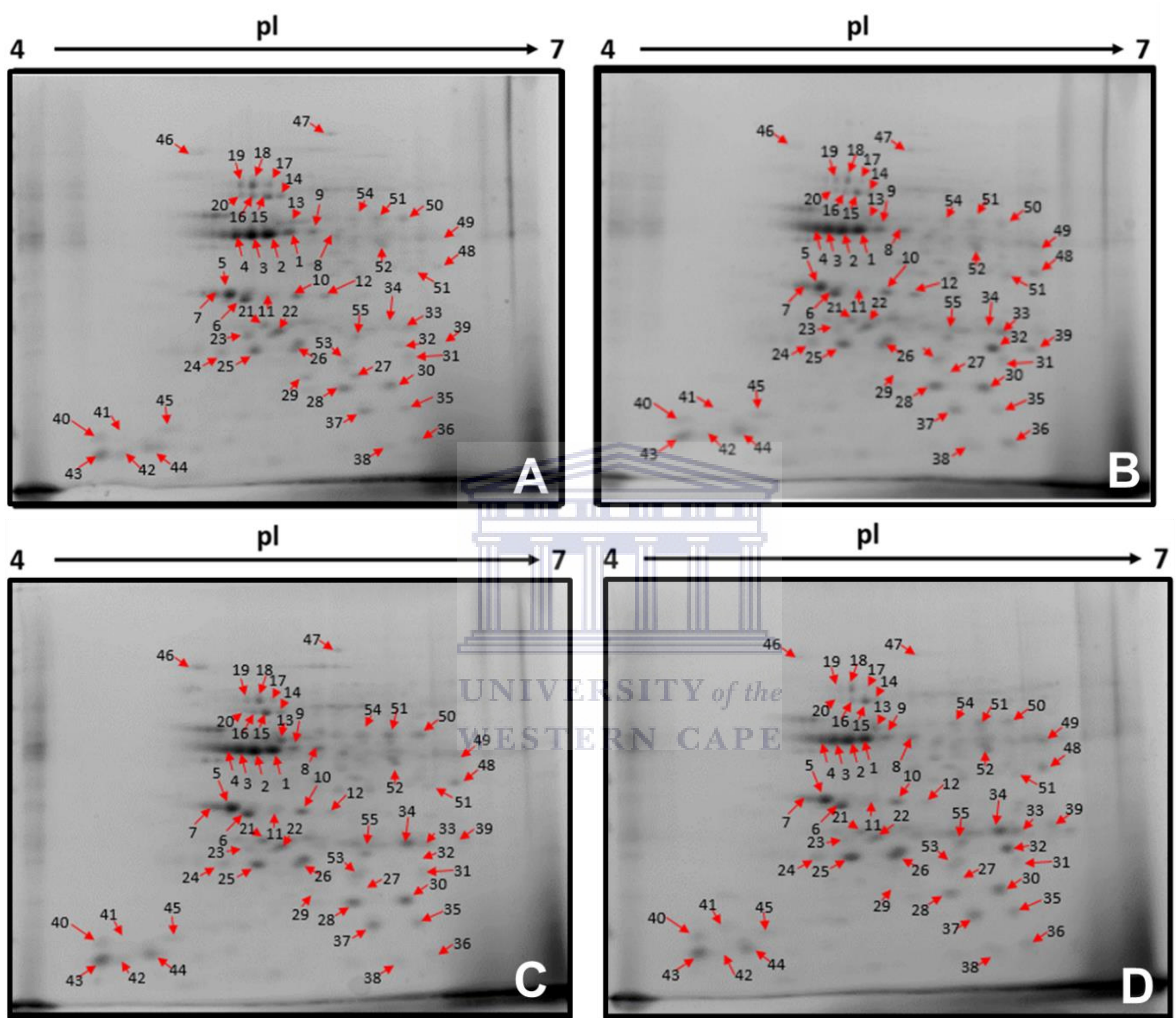
3.3.3 Detection of stress responsive protein spots from canola genotypes using 2D PAGE analysis

Prior to 2D analysis, the 1D PAGE analyses shows that there was relatively uniform loading across the two canola genotypes and the protein extracts from both genotypes (in different treatments) where RuBisCo was removed (Figure 3.2 A and 3.2 B; lanes S) were further analyzed using a 2D-SDS PAGE analysis for the detection and identification of drought stress responsive proteins.

For each sample 100 µg of leaf protein extract were passively rehydrated on 7 cm IPG strips, pH range 4-7 and focused using the BIORAD IEF machine and separated on a 12% SDS PAGE (Figure 3.3) and protein abundance between three biological replicate gels (not shown) for each sample was uniform. Indicating that 2D PAGE analysis was reproducible within an experiment. However, limited response from most of the low abundance proteins with respect to their abundance (Figure 3.3). Hence this might be

UNIVERSITY of the
WESTERN CAPE

one of the reasons why, PDQuest^{TM} software failed to detect most of the low abundant protein thus lead to manual identification and cutting of protein spots.



UNIVERSITY of the
WESTERN CAPE

Figure 3.3: Leaf proteome profiles of two canola genotypes under drought stress. A total of 100 µg total soluble protein was loaded from PS supernatant sample on the 7 cm linear IPG strips (pH 4–7 cm) in the first dimension followed by SDS-PAGE (12%) analysis. (A) *Agamax* well-watered, (B) *Agamax* water deprive, (C) *Garnet* well-watered and (D) *Garnet* water deprived. Results presented here are representative of three independent biological replicates.

As observed on the 1D gel (Figure 3.2 A and 3.2 B; lanes S), the 2D leaf proteome profiles of both genotypes were shown to cover the MW range between 10-200 kDa (Figure 3.3).

TABLE 3.1: A list of drought responsive proteins identified by MALDI TOF-TOF MS. Identified proteins were classified into functional classes according to Bevan *et al.* (1998) see (Figure 3.5). The table lists group ID of protein spots, protein names, species, accession numbers, theoretical mass (kDa)/p*I*, experimental mass (kDa)/p*I*, number of unique peptides/coverage (%), and expression cluster number.

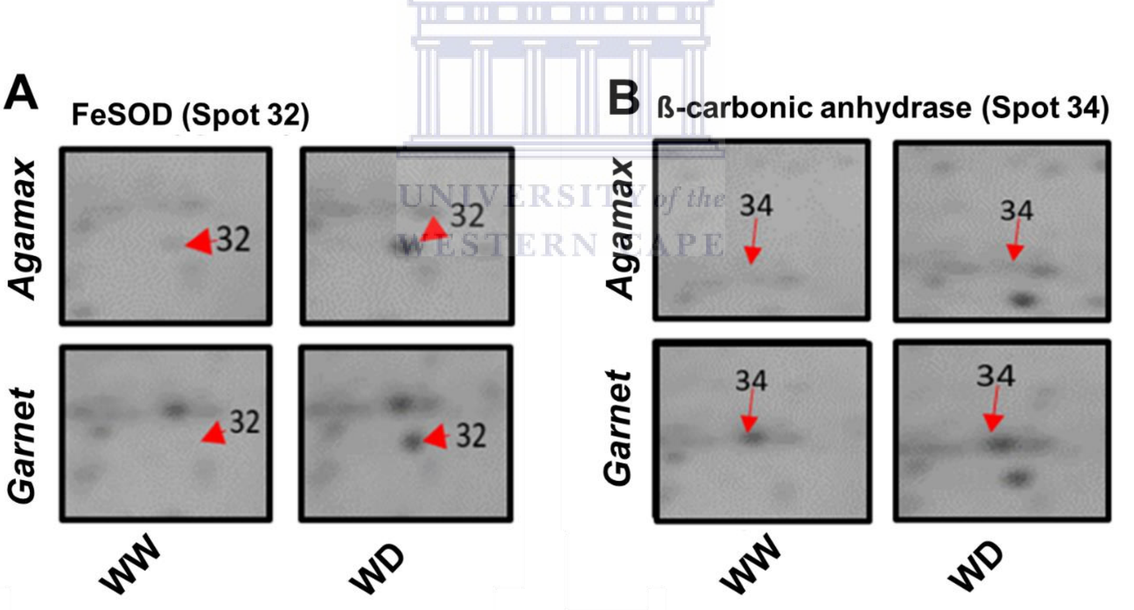
Spot	Best Match Protein	NCBI and SwissProt DATA Base	Species	MOWSE score	Exp. MW/p*I*	Matching peptides	Coverage [%]
			Photosynthesis				
5	Oxygen-evolving enhancer protein 1-1	gi|15240013	*At*	197.30	35.10/5.39	3	12.30
7	Oxygen-evolving enhancer protein 1-2	PSBO2_ARATH	*At*	91.29	35.00/5.84	3	9.70
11	Oxygen-evolving enhancer protein 1	gi|39932634		54.90	2.60/4.35	1	65.40
24	Oxygen-evolving enhancer protein 2	PSBP_BRAJU	*Bj*	72.92	23.30/4.76	1	4.60
25	Oxygen-evolving complex protein 2	gi|1076373	*At*	78.01	44.1/6.02	8	14.9
26	Oxygen-evolving complex protein 2	gi|1076373	*At*	75.19	21.5/5.87	3	15.3
40	Plastocyanin	gi|223149	*Cb*	142.69	10.40/4.05	1	24.20
43	Plastocyanin	gi|223149	*Cb*	161.18	10.40/4.05	1	24.20
			Energy related protein				
14	ATP synthase subunit beta chain	ATPB_LOBMA	*Lm*	282.63	53.90/5.59	6	17.10
15	ATP synthase subunit beta chain	ATPB_BRANA	*Bn*	134.00	53.70/5.07	17	35.10
16	ATP synthase subunit beta chain	ATPB_BRANA	*Bn*	134.00	53.70/5.07	17	35.10
18	atpA gene product	gi|383930459	*Bn*	505.98	55.30/4.99	7	19.50
19	ATPase subunit I	gi|297837977	*Al*	205.92	47.00/5.90	3	12.10
20	ATP synthase subunit beta chain	ATPB_BRANA	*Bn*	70.30	53.70/5.07	19	42.60
29	ATP synthase delta chain	SYK_SOLLC	*Sl*	39.96	67.10/5.55	1	1.40
49	Glyceraldehyde-3-phosphate dehydrogenase	gi|284177800	*No*	56.39	18.50/6.15	1	6.40
50	glyceraldehyde 3-phosphate dehydrogenase B subunit	gi|336390	*At*	74.83	42.80/5.52	2	6.50
51	Glyceraldehyde-3-phosphate dehydrogenase B subunit	G3PB_ARATH	*At*	59.76	47.60/6.36	2	5.80

Spot	Best Match Protein	NCBi and SwissProt DATA Base	Species	MOWSE score	Exp. MW/p*I*	Matching peptides	Coverage [%]
Disease/Defense							
32	Superoxide dismutase [Fe]	gi|312837924	*Br*	191.02	22.20/5.77	4	15.40
34	Chloroplast beta-carbonic anhydrase	gi|297787439	*Bn*	287.57	35.70/5.35	6	22.10
35	Superoxide dismutase [Cu/Zn]	gi|340031652	*Ci*	72.98	2.90/5.30	1	50.00
36	Superoxide dismutase [Cu/Zn]	gi|3288850	*Br*	126.49	15.20/5.63	2	16.40
37	Superoxide dismutase [Cu/Zn]	gi|340031652	*Ci*	81.85	2.90/5.30	1	50.00
55	Glutathione S-transferase	gi|87294807	*Bn*	139.65	24.70/5.78	2	11.10
Protein folding							
47	Chaperone protein	CLPC1_ORYSJ	*Os*	81.80	101.70/6.10	12	19.50
Unclassified							
1	Unnamed protein product	gi|312281705	*Th*	277.46	48.00/6.05	5	14.40
3	Hypothetical protein ARALYDRAFT_482998	gi|297827581	*Al*	233.89	51.90/5.59	5	9.30
4	Hypothetical protein ARALYDRAFT_482998	gi|297827581	*Al*	188.95	51.90/5.59	5	12.40
10	Uncharacterized protein At2g37660	gi|227204455	*At*	271.60	26.30/5.15	3	14.00
13	Os06g0668200	gi|115469436	*Os*	95.75	42.30/6.19	1	4.50

At, Arabidopsis thaliana; Al, Arabidopsis lyrata; Bj, Brassica juncea; Bn, B. napus; Bo, Brassica oleracea; Cb, Capsella bursa-pastoris; Cs, Cucumis sativus; Ci, Calophyllum inophyllum; Sl, Solanum lycopersicum; Th, Thellungiella halophila; Lm, Lobularia maritima; Os, Oryza sativa.

While, a total of 55 well-resolved protein spots were selected for mass spectrometry analysis (Figure 3.3) of which 31 were selected as positively identified when using our threshold criteria (see section 3.2.6) are listed in Table 3.1. While the rest of the proteins were either failing to match any protein on the database (NCBi and SwissProt Data Base) or were positively identified via MALDI TOF-TOF MS but fall outside of our threshold criteria and were listed in our Supplementary data under Table 1.

However, some of the proteins showed significantly difference in their levels of abundance between these genotypes and also between the control (well-watered; WW) and drought stress (water deprive; WD) experiment (Figure 3.3). Differential expression analysis between the control and the drought stress treatment for both genotypes showed that the increase in protein abundance for spot 32 was more pronounced under drought stress condition. Spot 32 was identified as an iron superoxide dismutase (Table 3.1), which is known to be involve in responses to oxidative damage (Figure 3.4 A). ß-carbonic anhydrase (spot 34) is another important protein categorized under disease/defense proteins, which was shown to be downregulated in *Agamax* and upregulated in *Garnet* genotype (Figure 3.4 B).

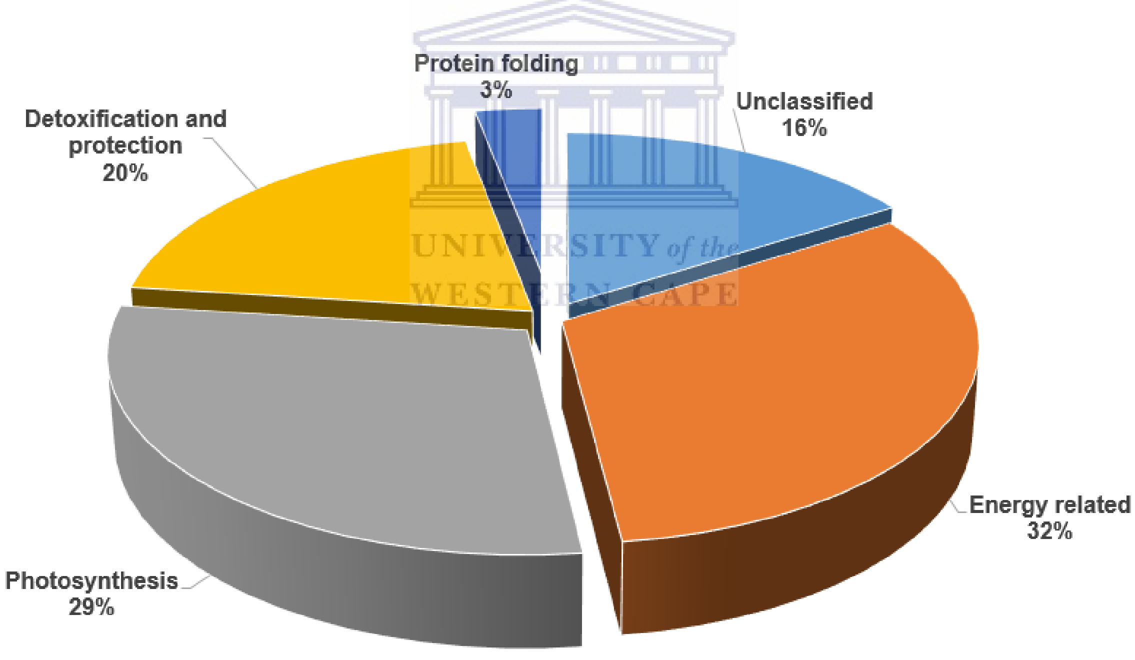


UNIVERSITY of the
WESTERN CAPE

Figure 3.4 Zoom in section on the expression pattern of drought stress responsive proteins. (A) FeSOD (spot 32), upregulated in both genotypes under drought stress condition; (B) Chloroplast ß-carbonic anhydrase (spot 34), was upregulated only in *Garnet* genotype under both control and drought stress conditions.

3.3.4 Functional characterization of the differentially expressed protein spots

As a consequence of RuBisCo depletion method seen in Figure 3.2, and the threshold criteria (see Section 3.2.6) applied for the selection of proteins after MALDI TOF-TOF MS. Only 31 proteins were positively identified from the 55 spots that were initially selected for MS analysis. Functional classification was perfomed on these 31 spots and five clusters were identified accoding to Bevan *et al.,* 1998, which included protein folding (3%), detoxification and protection (20%), photosynthetic (29%), energy related proteins (32%) and unclassified (16%) (Table 1; Figure 3.5). The major functional categories were energy and photosynthetic related proteins which mostly have interlinking functions.



UNIVERSITY of the
WESTERN CAPE

Figure 3.5. Functional classification of identified proteins according to Bevan *et al.* (1998) or as predicted based upon functions.

3.4 Discussion

This study described the comparatively gel-based proteomic analysis of two contrasting canola genotypes under drought stress. This work aimed at identifying potential protein biomarkers that could be used to enhance drought stress tolerance in economically important food/feed crops. The 1D protein profiles between the two genotypes showed a higher similarity in relation to their protein expression and banding patterns (Lanes 1-4; Figure 3.1).

Unfortunately, large amounts of RuBisCO was also detected in our gels, which masked the detection of some of the low abundant proteins (Figure 3.1). Hence PSP RuBisCO depletion method, was used to enhance the visibility of protein bands that migrated in the vicinity of the RuBisCO subunits (Figure 3.2 A and 3.2 B; Lanes "S"). This method was previously shown to deplete RuBisCO, which helps to increase the identification of low abundant proteins (Kim *et al*., 2013). In this study by Kim *et al.* (2013), there were able to shown that the addition of 0.1% PS can deplete RuBisCo below detectable limits, and this was confirmed by the use of antibodies (by western blot analysis). On the other hand, an addition of 0.1% PS did not completely remove the RuBisCO in our study (not shown), and we therefore explored higher concentration. After optimization, the addition of 0.24% PS (presented in Figure 3.2) was shown to be sufficient enough for the removal of the RuBisCO subunits. Although the PSP method did lead to a significant depletion of RuBisCO in both canola genotypes (Figure 3.2), we could not verify the complete removal of the RuBisCO using the western blot analysis, due to lack of antibodies that could help in determining the actual levels of RuBisCO proteins in our samples.

UNIVERSITY of the
WESTERN CAPE

Protein identification with 2D gel electrophoresis are known to revealed the presence of several proteins with conflicting pI ranges from those observed on in-gel (Faghani *et al.*, 2015). In order to minimize such conflicting results, a threshold criterion (section 3.2.6) was created in this study, and proteins that did not meet the stringent threshold criteria were disregarded from this study and are listed in the Supplementary Data (Table S1). Hence, from the 55 protein spots that were selected for MS analysis, only 31 were positively identified. That brings about a success rate of 56% which is good especially given the current limitation in proteome data for the canola plants in the public domain.

It is interesting to also note that from the 31 protein spots that were positively identified, only two (spot 32 and 34) were found to be differentially regulated in response to drought stress, when using the PDQuestTM analysis. The other remaining spots remained unaltered, suggesting that the limitations might be due to the 2D analysis system that tends not to allow the detection of low abundance proteins. It is also conceivable that the protein concentration used in this study could have had been overestimated and therefore below the limits established for image analysis using the PDQuest software. Furthermore, all positively identified proteins were functionally classified and had various biological processes including protein folding, detoxification and protection, photosynthetic, energy related proteins and some unknown proteins (Table 3.1). Only a brief description of the major proteins within major functional categories and with unique functions or coverages will be explained below:

UNIVERSITY of the
WESTERN CAPE

3.4.1 Energy metabolism

With regard to unique coverage, the largest group of proteins belonged to the ATP synthase subunits (spots 14, 15, 16, 18, 19, 20 and 29) followed by the oxygen-evolving

enhancer protein (spots 5, 7, 11, 24, 25 and 26), all belonging to the photosynthesis related proteins. Literature has also associated the ATP synthase proteins with the photosynthesis related processes (Lapaille *et al.*, 2010) as most of these processes do require energy from the energy-producing pathways such as glycolysis. From the identified ATP synthase subunit proteins, three of the spots (15, 16 and 20), were mapped to the same SwissProt accession number ATPB_BRANA, raising the possibility that the three spots might be the differentially spliced products of the same gene considering they were found in close proximity to one another (see Figure 3.3). It is also suggested that a single gene can code for more than three proteins, as a result of the post-translation modification, splicing or other unknown chemical entities (Wade *et al.*, 2002; Katz-jaffe *et al.*, 2005). The ATP synthase subunits are found in the mitochondria and chloroplast, their inhibition is known to be detrimental to plant physiological appearances, leading to plant cell death (Lapaille *et al.*, 2010). While the oxygen-evolving enhancer proteins are mostly involved in the protection of photosynthesis reaction center proteins from damage by oxygen radicals formed in light (Ngara *et al.,* 2012). Similar proteins were also identified in a study by Farrant (2007), which suggested that this protein could also be involved in regulating water replacement in vacuoles, so providing a mechanical stabilization role.

3.4.2 Detoxification and protection

Plant responses to drought stress also involve the expression of different proteins including a number of disease and defense related proteins, which have previously been demonstrated to be crucial components in plants defense mechanism (Xiao *et al.,* 2009). These defense mechanisms are required in the regulation of ROS levels through different pathways, which mostly require enzymatic and non-enzymatic antioxidants to scavenge

UNIVERSITY of the
WESTERN CAPE

ROS (Xiao *et al*., 2009). Hence, a tight control is needed to balance ROS activities in order to promote plant growth and avoid oxidative damage. In this study, several proteins were identified as being involved in the scavenging of ROS and one of the protein that was assess had shown higher expression levels under drought stress in both genotypes (spot 32), also see Figure 3.4 and Figure 3.5 A. FeSOD, (spot 32) is one of the essential enzymes in the functioning of the ascorbate–glutathione pathway. Furthermore, three other Cu/ZnSOD proteins (spot 35, 36, and 37) that were identified in both genotypes were not influenced by drought stress. This superoxide dismutase enzyme acts as the first line of defense in the ascorbate–glutathione cycle, by scavenging of O_2^- ions and producing H_2O_2, which

UNIVERSITY of the
WESTERN CAPE

can be scavenged further to produce water and oxygen by other enzymes such as the ascorbate peroxidase (Described in more detail in Chapter 1). Chloroplast ß-carbonic anhydrase (spot 34) is another stress responsive protein, which was found to be differentially regulated by drought stress but only in the Garnet genotype. This is in support of the evidence presented by Pereira *et al.* (2013), who have shown that sensitive plant genotypes highly accumulates ß-carbonic anhydrase under stress conditions. Emerging data have also suggested that the ß-carbonic anhydrase might have different functions or roles, depending of its location and the type of plant. Likewise, other studies have also suggested that carbonic anhydrase does participate in a broad range of biochemical processes, like carboxylation and decarboxylation reactions, inorganic carbon transport, ion transport, and water and electrolyte balance (Moroney *et al*., 2001; Fabre et al., 2007). In another study by Slaymaker *et al.* (2002) also suggested that ß-

carbonic anhydrase might be having some antioxidant activity properties, which plays a major role in the hypersensitive defense response.

3.4.3 Photosynthesis-metabolism

Photosynthesis is one of the most important processes in plants and it is mostly affected by several factors such as stomatal closure, reduction in the activity of photosynthetic enzymes and decrease in ATP synthesis (Chaves *et al.,* 2001; Chaves *et al.*, 2003). Apart from the high abundance of oxygen-evolving enhancer protein (spots 5, 7, 11, 24, 25 and 26), some of the photosynthesis related proteins that were identified in this study included two plastocyanin proteins (spots 40 and 43). Oxygen-evolving enhancer proteins were previously identified in wheat (Faghani *et al.*, 2015) and sorghum (Ngara *et al.*, 2012) and consisting of four manganese ions, calcium and possibly chloride ions, which are bound to extrinsic proteins (McEvoy and Brudvig, 2006). The oxygen evolving enhancer protein is believed to have a dual function; (i) optimizing the manganese cluster during photolysis and (ii) protecting the reaction centre proteins from damage by oxygen radical formed in light (Heide *et al.,* 2004). On the other hand, plastocyanins are copper-containing proteins that mostly known to be associated with photosynthesis and play a significant role in the electron transport process and mostly associated with the cyt b/c1 complex and the cytochrome oxidase complex in the electron transport chain.

UNIVERSITY of the
WESTERN CAPE

3.4.4 Other functional categories

Proteins that are listed as belonging to the other functional categories include those that belongs to either protein folding or unclassified categories. The protein expression levels of these candidate proteins remained unaltered for both genotypes and within treatments (Figure 3.3). A single chaperone protein (spot 47), was identified in our study and

categorized under protein folding (Figure 3.3; Table 3.1). The availability of chaperones had previously been shown to have a significant impact in plant development and growth, as they plays a key role in protein folding (Feder and Hofmann, 1999). Besides protein folding, chaperones are also known for their ability to reduce aggregation through the holding of misfolded polypeptides in their intermediate stages of folding so as to promote refolding (Freeman and Morimoto, 1996). However, a large fraction of partially characterized proteins were also identified and grouped under unclassified (Unnamed protein product; spot 1, hypothetical protein ARALYDRAFT_482998; spot 3, hypothetical protein ARALYDRAFT_482998; spot 4 and uncharacterized protein at2g37660; stop 10). Hence, future studies are needed to further understanding the molecular functional interactions of these candidate's protein in particular, in order to unravel their role and function in drought stress response.

UNIVERSITY of the
WESTERN CAPE

CHAPTER 4

CONCLUSION AND FUTURE REMARKS

Plant survival against drought stresses mostly depends on plant growth and development, which both are influenced by different physiological and molecular processes. Given the growing realization on the effect of drought stress due to global warming (Engelbrecht *et al.,* 2015), the world faces the challenge of ensuring effective growth and production of crop-based food which might have a negative impact onto plant yield. Hence, it is essential to improve plant tolerance or resistance to environmental stresses, in order to maintain food security. The study presented here focused on the physiological and molecular responses of two contrasting canola genotypes (*Agamax* and *Garnet*), in order to determine tolerance and/or sensitivity to drought stress. This study also provided a comparative overview of the proteomic profiling, in trying to unravel the network of proteins that might underpin drought stress tolerance in canola.

UNIVERSITY of the
WESTERN CAPE

In Chapter 2, it was demonstrated that drought stress triggers mechanisms that results in overproduction of ROS, which in turn induce the activity of various antioxidant enzymes and other metabolites in order to maintain redox homeostasis. Furthermore, the screening of the antioxidant enzymes (see Chapter 2), led to the identification of two novel FeSOD isoforms (FeSOD 1 and FeSOD 2) in *Agamax* genotype which were absent in the *Garnet* genotype (Figure 2.3). This suggests that *Agamax* genotype had a more efficient O_2^- scavenging capacity than *Garnet* and this was further supported by the higher levels of the total SOD activity that was observed in the *Agamax* under drought stress. While, it was also demonstrated that under drought stress, *Agamax* showed significant

accumulation of both the antioxidant enzyme activity (APX; Figure 2.6 B) and the non-enzymatic metabolites (total AsA and DHAsA; Figure 2.6 A). This suggested that the defence system of *Agamax* was more efficient than that of the *Garnet*. Overall, the results in Chapter 2 showed that *Agamax* adapted much better to drought stress than *Garnet*. This results are also in line with those of Gokul *et al.* (Unpublished results), where *Agamax* showed a better tolerant to vanadium treatment when compared to *Garnet*. In conclusion, to the results obtained in Chapter 2, we suggest that the *Agamax* genotype is a more tolerant or less susceptible variety to drought stress when compared to *Garnet* genotype.

This study also further looked at the proteomic profiling of these two genotypes in order to explore the possible underlying molecular mechanisms of drought stress tolerant (see Chapter 3). Although proteome profiling (using 2D gel electrophoresis), has already been used successful in several plants species and within different tissues in response to various stresses (Kim *et al.,* 2001; Wang *et al.,* 2008), there are still limitations associated with these systems. One of the limitations in this procedure is the masking of low abundant proteins by major abundant proteins like RuBisCO in leaves (Abat and Deswal, 2009; Tanou *et al.,* 2012). The comparison of the leaves protein extracts (using 1D gel electrophoresis) showed this drawback (masking of low abundant proteins by major subunits of RuBisCo). Nevertheless, this drawback was overcome through the refining and optimization of protamine sulfate precipitation (PSP) method for the depletion of RuBisCo (Kim *et al.,* 2013). These results pointed out a number of low abundant important proteins that were mostly over-shadowed by some of these major proteins. Based on these results, we established that a large fraction of these low abundant proteins as

UNIVERSITY of the
WESTERN CAPE

identified by MALDI TOF-TOF MS were involved in oxidative stress responses (defense protein), protein synthesis and energy related functions (ATP synthase). These results suggest that such defense-related proteins may play an important biochemical role in the adaptation of canola leaves to drought stress.

The most important aspect recognized was the initial identification of three Cu/Zn SODs in Chapter 2, and these isoforms were upregulated in all treatments (Figure 2). Although there is still not sufficient evidence, it could still be hypothesized that the three Cu/Zn SOD isoforms (spot 35, 36 and 37) that were detected in RuBisCo depleted 2D proteome profiles might be correlating to the three Cu/Zn SOD isoforms identified in Chapter 2. This is supported by the fact that the level of expression was similar in all treatment when comparing this three Cu/Zn SOD isoforms. While the proteomic profiling analysis, also led to the identification of FeSOD (spot 32), which appears to be most closely related to the FeSOD 3 (see Chapter 2). Thus to our knowledge, this is the first study that shows a possible link between biochemical and proteomic analysis of SOD isoforms. Therefore, it would also be interesting to obtain the corresponding full-length sequence of these SOD isoforms, in order to establish if their changes may also be occurring at the transcript level. However, it may also be important in future to perform genetic manipulation studies on these two novel FeSODs (Figure 2) in *Garnet*, in order to make *Garnet* a less susceptible variety to drought stress. While transgenic studies on over-expressing these novel FeSOD, may also be an important step in the production of drought stress tolerance canola genotypes, it is also important to identify other proteins like the chloroplast beta-carbonic anhydrase (spot 34) might also be candidates for future transgenic studies.

UNIVERSITY of the
WESTERN CAPE

REFERENCES

Abat, J.K. and Deswal, R. 2009, "Differential modulation of S-nitrosoproteome of Brassica juncea by low temperature: Change in S-nitrosylation of Rubisco is responsible for the inactivation of its carboxylase activity", *Proteomics,* vol. 9, pp. 4368-4380.

Ahuja, N. Singh, H.P. Batish, D.R. and Kohli, R.K. 2015, "Eugenol-inhibited root growth in Avena fatua involves ROS-mediated oxidative damage", *Pesticide Biochemistry and Physiology,* vol. 118, pp. 64-70.

Alam, M.M. Hasanuzzaman, M. Nahar, K. and Fujita, M. 2013, "Exogenous salicylic acid ameliorates short-term drought stress in mustard (*Brassica juncea* L.) seedlings by up-regulating the antioxidant defense and glyoxalase system", *Australian Journal of Crop Science,* vol. 7, pp. 1053-1063.

UNIVERSITY of the
WESTERN CAPE

Alam, M.M. Nahar, K. Hasanuzzaman, M. and Fujita, M. 2014, "Exogenous jasmonic acid modulates the physiology, antioxidant defense and glyoxalase systems in imparting drought stress tolerance in different *Brassica species*", *Plant Biotechnology Reports,* vol. 8, pp. 279-293.

Alam, M. Nahar, K. Hasanuzzaman, M. and Fujita, M. 2014, "Alleviation of osmotic stress in *Brassica napus*, *B. campestris*, and *B. juncea* by ascorbic acid application", *Biologia Plantarum,* vol. 58, pp. 697-708.

Allan, A.C. and Fluhr, R. 1997, "Two Distinct Sources of Elicited Reactive Oxygen Species in Tobacco Epidermal Cells", *The Plant Cell,* vol. 9, pp. 1559-1572.

Apel, K. and Hirt, H. 2004, "Reactive oxygen species: metabolism, oxidative stress, and signal transduction", *Annual Review of Plant Biology,* vol. 55, pp. 373-399.

Arakawa, N. Tsutsumi, K. Sanceda, N.G. Kurata, T. and Inagaki, C. 1981, "A rapid and sensitive method for the determination of ascorbic acid using 4, 7-diphenyl-l, 10-phenanthroline", *Agricultural and Biological Chemistry,* vol. 45, pp. 1289-1290.

Arango, Y. and Heise, K. 1998, "Localization of α-tocopherol synthesis in chromoplast envelope membranes of *Capsicum annuum* L. fruits", *Journal of Experimental Botany,* vol. 49, pp. 1259-1262.

Arora, A. Sairam, R. and Srivastava, G. 2002, "Oxidative stress and antioxidative system in plants", *Current Science Bangalore,* vol. 82, pp. 1227-1238.

Asada, K. 2006, "Production and scavenging of reactive oxygen species in chloroplasts and their functions", *Plant Physiology,* vol. 141, pp. 391-396.

Asada, K. 1999, "The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons", *Annual Review of Plant Biology,* vol. 50, pp. 601-639.

Aziz, A. and Larher, F. 1998, "Osmotic stress induced changes in lipid composition and peroxidation in leaf discs of *Brassica napus* L.", *Journal of Plant Physiology,* vol. 153, pp. 754-762.

Banu, M.N.A. Hoque, M.A. Watanabe-Sugimoto, M. Matsuoka, K. Nakamura, Y. Shimoishi, Y. and Murata, Y. 2009, "Proline and glycinebetaine induce antioxidant

UNIVERSITY of the
WESTERN CAPE

defense gene expression and suppress cell death in cultured tobacco cells under salt stress", *Journal of Plant Physiology,* vol. 166, pp. 146-156.

Beauchamp, C. and Fridovich, I. 1971, "Superoxide dismutase: improved assays and an assay applicable to acrylamide gels", *Analytical Biochemistry,* vol. 44, pp. 276-287.

Bevan, M. Bancroft, I. Bent, E. Love, K. Goodman, H. Dean, C. Bergkamp, R. Dirkse, W. Van Staveren, M. and Stiekema, W. 1998, "Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*", *Nature,* vol. 391, pp. 485-488.

Blackstock, W.P. and Weir, M.P. 1999, "Proteomics: quantitative and physical mapping of cellular proteins", *Trends in Biotechnology,* vol. 17, pp. 121-127.

Bowler, C. Montagu, M.V. and Inze, D. 1992, "Superoxide dismutase and stress tolerance", *Annual Review of Plant Biology,* vol. 43, pp. 83-116.

Bozsó, Z. Ott, P. Szatmari, A. Czelleng, A. Varga, G. Besenyei, E. Sárdi, É. Bányai, É. and Klement, Z. 2005, "Early Detection of Bacterium-induced Basal Resistance in Tobacco Leaves with Diaminobenzidine and Dichlorofluorescein Diacetate", *Journal of Phytopathology,* vol. 153, pp. 596-607.

Bradford, M.M. 1976, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", *Analytical Biochemistry,* vol. 72, pp. 248-254.

UNIVERSITY of the WESTERN CAPE

Bray, E.A. 1997, "Plant responses to water deficit", *Trends in Plant Science,* vol. 2, pp. 48-54.

Bray, E.A. Bailey-Serres, J. and Weretilnyk, E. 2000, "Responses to abiotic stresses", *Biochemistry and Molecular Biology of Plants,* vol. 1158, pp. 1203.

Chaves, M.M. Maroco, J.P. and Pereira, J.S. 2003, "Understanding plant responses to drought—from genes to the whole plant", *Functional Plant Biology,* vol. 30, pp. 239-264.

Chaves, M. Pantschitz, E. and Schulze, E. 2001, "Growth and photosynthetic carbon metabolism in tobacco plants under an oscillating CO_2 concentration in the atmosphere", *Plant Biology,* vol. 3, pp. 417-425.

Dat, J.F. Pellinen, R. Van De Cotte, B. Langebartels, C. Kangasjärvi, J. Inzé, D. and Van Breusegem, F. 2003, "Changes in hydrogen peroxide homeostasis trigger an active cell death process in tobacco", *The Plant Journal,* vol. 33, pp. 621-632.

Delledonne, M. Zeier, J. Marocco, A. and Lamb, C. 2001, "Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response", *Proceedings of the National Academy of Sciences,* vol. 98, pp. 13454-13459.

Desikan, R. Hancock, J.T. and Neill, S.J. 2004, "Oxidative stress signalling", in *Plant Responses to Abiotic Stress* Springer, pp. 121-149.

UNIVERSITY of the
WESTERN CAPE

Egbichi, I. Keyster, M. and Ludidi, N. 2014, "Effect of exogenous application of nitric oxide on salt stress responses of soybean", *South African Journal of Botany,* vol. 90, pp. 131-136.

Engelbrecht, J. 2015, "A system to monitor large-area surface deformation: ideas that save", *CSIR Science Scope,* vol. 8, pp. 70-71.

Epple, P. Mack, A.A. Morris, V.R. and Dangl, J.L. 2003, "Antagonistic control of oxidative stress-induced cell death in Arabidopsis by two related, plant-specific zinc finger proteins", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 100, pp. 6831-6836.

Fabre, N. Reiter, I.M. BECUWE-LINKA, N. Genty, B. and Rumeau, D. 2007, "Characterization and expression analysis of genes encoding α and β carbonic anhydrases in *Arabidopsis*", *Plant, Cell and Environment,* vol. 30, pp. 617-629.

Farooq, M. Wahid, A. Kobayashi, N. Fujita, D. and Basra, S. 2009, "Plant drought stress: effects, mechanisms and management" in *Sustainable Agriculture* Springer, pp. 153-188.

Farrant, J.M. 2007, "Mechanisms of desiccation tolerance in angiosperm resurrection plants", *Plant Desiccation Tolerance,* pp. 51-90.

Farrant, J.M. Brandt, W. and Lindsey, G.G. 2007, "An overview of mechanisms of desiccation tolerance in selected angiosperm resurrection plants", *Plant Stress,* vol. 1, pp. 72-84.

UNIVERSITY of the
WESTERN CAPE

Fischer, B.B. Dayer, R. Wiesendanger, M. and Eggen, R.I. 2007, "Independent regulation of the GPXH gene expression by primary and secondary effects of high light stress in *Chlamydomonas reinhardtii*", *Physiologia Plantarum,* vol. 130, pp. 195-206.

Fukutoku, Y. and Yamada, Y. 1981, "Sources of Proline-nitrogen in water-stressed soybean (*Glycine max* L.) I. Protein metabolism and proline accumulation", *Plant and Cell Physiology,* vol. 22, pp. 1387-1404.

Garg, N. and Manchanda, G. 2009, "Role of *arbuscular mycorrhizae* in the alleviation of ionic, osmotic and oxidative stresses induced by salinity in *Cajanus cajan* (L.) (pigeonpea)", *Journal of Agronomy and Crop Science,* vol. 195, pp. 110-123.

Gill, S.S. and Tuteja, N. 2010, "Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants", *Plant Physiology and Biochemistry,* vol. 48, pp. 909-930.

UNIVERSITY of the
WESTERN CAPE

Gosslau, A. and Chen, K.Y. 2004, "Nutraceuticals, apoptosis, and disease prevention", *Nutrition,* vol. 20, pp. 95-102.

Grispen, V.M. Nelissen, H.J. and Verkleij, J.A. 2006, "Phytoextraction with *Brassica napus* L.: a tool for sustainable management of heavy metal contaminated soils", *Environmental Pollution,* vol. 144, pp. 77-83.

Grombacher, A. and Nelson, L. 1992, "*Canola production*", *A Publication of Nebraska NebGuide University*.

Gupta, R. Wang, Y. Agrawal, G.K. Rakwal, R. Jo, I.H. Bang, K.H. and Kim, S.T. 2015, "Time to dig deep into the plant proteome: a hunt for low-abundance proteins", *Frontiers in plant science,* vol. 6, pp. 6-22.

Gygi, S.P. Rochon, Y. Franza, B.R. and Aebersold, R. 1999, "Correlation between protein and mRNA abundance in yeast", *Molecular and cellular biology,* vol. 19, pp. 1720-1730.

Gygi, S.P. Corthals, G.L. Zhang, Y. Rochon, Y. and Aebersold, R. 2000, "Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 97, pp. 9390-9395.

Hajduch, M. Casteel, J.E. Hurrelmeyer, K.E. Song, Z. Agrawal, G.K. and Thelen, J.J. 2006, "Proteomic analysis of seed filling in *Brassica napus,* developmental characterization of metabolic isozymes using high-resolution two-dimensional gel electrophoresis", *Plant Physiology,* vol. 141, pp. 32-46.

Halliwell, B. 2006, "Reactive species and antioxidants redox biology is a fundamental theme of aerobic life", *Plant Physiology,* vol. 141, pp. 312-322.

Halliwell, B. and Chirico, S. 1993, "Lipid peroxidation: its mechanism, measurement, and significance", *The American Journal of Clinical Nutrition,* vol. 57, pp. 715S-724S.

Hancock, J. Desikan, R. and Neill, S. 2001, "Role of reactive oxygen species in cell signalling pathways", *Biochemical Society Transactions,* vol. 29, pp. 345-349.

UNIVERSITY of the
WESTERN CAPE

Hare, P. Cress, W. and Van Staden, J. 1999, "Proline synthesis and degradation: a model system for elucidating stress-related signal transduction", *Journal of Experimental Botany,* vol. 50, pp. 413-434.

Hasanuzzaman, M. Hossain, M.A. Da Silva, J.A.T. and Fujita, M. 2012, "Plant response and tolerance to abiotic oxidative stress: antioxidant defense is a key factor" in *Crop stress and its management: Perspectives and strategies* Springer, pp. 261-315.

Hasanuzzaman, M. Hossain, M.A. and Fujita, M. 2011, "Selenium-induced up-regulation of the antioxidant defense and methylglyoxal detoxification system reduces salinity-induced damage in rapeseed seedlings", *Biological Trace Element Research,* vol. 143, pp. 1704-1721.

Hasanuzzaman, M. Alam, M.M. Rahman, A. Nahar, K. and Fujita, M. 2014, "Exogenous proline and glycine betaine mediated upregulation of antioxidant defense and glyoxalase systems provides better protection against salt-induced oxidative stress in two rice (*Oryza sativa* L.) varieties", *BioMed Research International,* vol. 2014, pp. 757219.

Hetherington, A.M. 2001, "Guard cell signaling", *Cell,* vol. 107, pp. 711-714.

Hong-Bo, S. Xiao-Yan, C. Li-Ye, C. Xi-Ning, Z. Gang, W. Yong-Bing, Y. Chang-Xing, Z. and Zan-Min, H. 2006, "Investigation on the relationship of proline with wheat anti-drought under soil water deficits", *Colloids and Surfaces B: Biointerfaces,* vol. 53, pp. 113-119.

UNIVERSITY of the
WESTERN CAPE

Ideker, T. Thorsson, V. Ranish, J.A. Christmas, R. Buhler, J. Eng, J.K. Bumgarner, R. Goodlett, D.R. Aebersold, R. and Hood, L. 2001, "Integrated genomic and proteomic analyses of a systematically perturbed metabolic network", *Science (New York, N.Y.),* vol. 292, pp. 929-934.

Jaleel, C.A. Manivannan, P. Wahid, A. Farooq, M. Al-Juburi, H.J. Somasundaram, R. and Panneerselvam, R. 2009, "Drought stress in plants: a review on morphological characteristics and pigments composition", *International Journal of Agricultural and Biological Engineering,* vol. 11, pp. 100-105.

Jiang, M. and Zhang, J. 2001, "Effect of abscisic acid on active oxygen species, antioxidative defence system and oxidative damage in leaves of maize seedlings", *Plant and Cell Physiology,* vol. 42, pp. 1265-1273.

UNIVERSITY of the
WESTERN CAPE

Katz-Jaffe, M.G. Linck, D.W. Schoolcraft, W.B. and Gardner, D.K. 2005, "A proteomic analysis of mammalian preimplantation embryonic development", *Reproduction (Cambridge, England),* vol. 130, pp. 899-905.

Keyster, M. Klein, A. Du Plessis, M. Jacobs, A. Kappo, A. Kocsy, G. Galiba, G. and Ludidi, N. 2013, "Capacity to control oxidative stress-induced caspase-like activity determines the level of tolerance to salt stress in two contrasting maize genotypes", *Acta Physiologiae Plantarum,* vol. 35, pp. 31-40.

Keyster, M. Klein, A. and Ludidi, N. 2012, "Caspase-like enzymatic activity and the ascorbate-glutathione cycle participate in salt stress tolerance of maize conferred by exogenously applied nitric oxide", *Plant Signaling and Behavior,* vol. 7, pp. 349-360.

Kim, S.T. Cho, K.S. Jang, Y.S. and Kang, K.Y. 2001, "Two-dimensional electrophoretic analysis of rice proteins by polyethylene glycol fractionation for protein arrays", *Electrophoresis,* vol. 22, pp. 2103-2109.

Kim, Y.J. Lee, H.M. Wang, Y. Wu, J. Kim, S.G. Kang, K.Y. Park, K.H. Kim, Y.C. Choi, I.S. and Agrawal, G.K. 2013, "Depletion of abundant plant RuBisCO protein using the protamine sulfate precipitation method", *Proteomics,* vol. 13, pp. 2176-2179.

Koh, K.D. Balachander, S. Hesselberth, J.R. and Storici, F. 2015, "Ribose-seq: global mapping of ribonucleotides embedded in genomic DNA", *Nature methods*.

Krinsky, N.I. 2001, "Carotenoids as antioxidants", *Nutrition,* vol. 17, pp. 815-817.

Krishnan, H.B. and Natarajan, S.S. 2009, "A rapid method for depletion of Rubisco from soybean (*Glycine max*) leaf for proteomic analysis of lower abundance proteins", *Phytochemistry,* vol. 70, pp. 1958-1964.

Lapaille, M. Escobar-Ramirez, A. Degand, H. Baurain, D. Rodriguez-Salinas, E. Coosemans, N. Boutry, M. Gonzalez-Halphen, D. Remacle, C. and Cardol, P. 2010, "Atypical subunit composition of the chlorophycean mitochondrial F1FO-ATP synthase and role of Asa7 protein in stability and oligomycin resistance of the enzyme", *Molecular Biology and Evolution,* vol. 27, pp. 1630-1644.

Laspina, N. Groppa, M. Tomaro, M. and Benavides, M. 2005, "Nitric oxide protects sunflower leaves against Cd-induced oxidative stress", *Plant Science,* vol. 169, pp. 323-330.

UNIVERSITY of the
WESTERN CAPE

Lee, D.H. and Lee, C.B. 2000, "Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays", *Plant Science,* vol. 159, pp. 75-85.

Lichtenthaler, H. Buschmann, C. Döll, M. Fietz, H., Bach, T. Kozel, U. Meier, D. and Rahmsdorf, U. 1981, "Photosynthetic activity, chloroplast ultrastructure, and leaf characteristics of high-light and low-light plants and of sun and shade leaves", *Photosynthesis Research,* vol. 2, pp. 115-141.

Liu, X. Williams, C.E. Nemacheck, J.A. Wang, H. Subramanyam, S. Zheng, C. and Chen, M. 2010, "Reactive oxygen species are involved in plant defense against a gall midge", *Plant Physiology,* vol. 152, pp. 985-999.

Mantri, N. Patade, V. Penna, S. Ford, R. and Pang, E. 2012, "Abiotic stress responses in plants: present and future" in *Abiotic Stress Responses in Plants* Springer, pp. 1-19.

Matters, G.L. and Scandalios, J.G. 1986, "Effect of the free radical-generating herbicide paraquat on the expression of the superoxide dismutase (SOD) genes in maize", *Biochimica et Biophysica Acta (BBA) General Subjects,* vol. 882, pp. 29-38.

McKersie, B.D. and Leshem, Y.Y. 1994, *Stress and stress coping in cultivated plants.* Kluwer Academic Publishers.

Miller, G. Suzuki, N. CIFTCI-YILMAZ, S. and Mittler, R. 2010, "Reactive oxygen species homeostasis and signalling during drought and salinity stresses", *Plant, Cell and Environment,* vol. 33, pp. 453-467.

UNIVERSITY of the WESTERN CAPE

Miller-Cebert, R.L. Sistani, N.A. and Cebert, E. 2009, "Comparative mineral composition among canola cultivars and other cruciferous leafy greens", *Journal of food composition and analysis,* vol. 22, pp. 112-116.

Mittler, R. 2002, "Oxidative stress, antioxidants and stress tolerance", *Trends in Plant Science,* vol. 7, pp. 405-410.

Mittler, R. Vanderauwera, S. Gollery, M. and Van Breusegem, F. 2004, "Reactive oxygen gene network of plants", *Trends in Plant Science,* vol. 9, pp. 490-498.

Møller, I.M. 2001, "Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species", *Annual Review of Plant Biology,* vol. 52, pp. 561-591.

Mooney, B.P. and Thelen, J.J. 2004, "High-throughput peptide mass fingerprinting of soybean seed proteins: automated workflow and utility of UniGene expressed sequence tag databases for protein identification", *Phytochemistry,* vol. 65, pp. 1733-1744.

Moradi, F. and Ismail, A.M. 2007, "Responses of photosynthesis, chlorophyll fluorescence and ROS-scavenging systems to salt stress during seedling and reproductive stages in rice", *Annals of botany,* vol. 99, pp. 1161-1173.

Moroney, J. Bartlett, S. and Samuelsson, G. 2001, "Carbonic anhydrases in plants and algae", *Plant, Cell and Environment,* vol. 24, pp. 141-153.

UNIVERSITY of the
WESTERN CAPE

Morrison, M.J. and Stewart, D.W. 2002, "Heat stress during flowering in summer *Brassica*", *Crop Science,* vol. 42, pp. 797-803.

Morrison, M. 1993, "Heat stress during reproduction in summer rape", *Canadian Journal of Botany,* vol. 71, pp. 303-308.

Munné-Bosch, S. and Alegre, L. 2002, "Plant aging increases oxidative stress in chloroplasts", *International Journal of Plant Biology,* vol. 214, pp. 608-615.

Munns, R. 2005, "Genes and salt tolerance: bringing them together", *New Phytologist,* vol. 167, pp. 645-663.

Nakagawara, S. and Sagisaka, S. 1984, "Increase in enzyme activities related to ascorbate metabolism during cold acclimation in poplar twigs", *Plant and cell physiology,* vol. 25, pp. 899-906.

UNIVERSITY of the
WESTERN CAPE

Neill, S.J. Desikan, R. Clarke, A. Hurst, R.D. and Hancock, J.T. 2002, "Hydrogen peroxide and nitric oxide as signalling molecules in plants", *Journal of Experimental Botany,* vol. 53, pp. 1237-1247.

Ngara, R. and Ndimba, BK. 2011, “Mapping and characterisation of the sorghum cell suspension culture secretome”, *African Journal of Biotechnology*, vol.10, pp. 253-66.

Ngara, R. Ndimba, R. Borch-Jensen, J. Jensen, O.N. and Ndimba, B. 2012, "Identification and profiling of salinity stress-responsive proteins in Sorghum bicolor seedlings", *Journal of Proteomics,* vol. 75, pp. 4139-4150.

Noctor, G. and Foyer, C.H. 1998, "Ascorbate and glutathione: keeping active oxygen under control", *Annual Review of Plant Biology,* vol. 49, pp. 249-279.

O'Farrell, P.H. 1975, "High resolution two-dimensional electrophoresis of proteins", *The Journal of Biological Chemistry,* vol. 250, pp. 4007-4021.

Omidi, H. 2010, "Changes of proline content and activity of antioxidative enzymes in two canola genotype under drought stress", *American Journal of Plant Physiology,* vol. 5, pp. 338-349.

Pandey, A. and Mann, M. 2000, "Proteomics to study genes and genomes", *Nature,* vol. 405, pp. 837-846.

Pathan, M.S. Lee, J. Shannon, J.G. and Nguyen, H.T. 2007, "Recent advances in breeding for drought and salt stress tolerance in *soybean*" in *Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops* Springer, pp. 739-773.

Patterson, S.D. and Aebersold, R.H. 2003, "Proteomics: the first decade and beyond", *Nature genetics,* vol. 33, pp. 311-323.

Peters, S. Mundree, S.G. Thomson, J.A. Farrant, J.M. and Keller, F. 2007, "Protection mechanisms in the resurrection plant *Xerophyta viscosa* (Baker): both sucrose and *raffinose* family oligosaccharides (RFOs) accumulate in leaves in response to water deficit", *Journal of Experimental Botany,* vol. 58, pp. 1947-1956.

Pinheiro, C. António, C. Ortuño, M.F. Dobrev, P.I. Hartung, W. Thomas-Oates, J. Ricardo, C.P. Vanková, R. Chaves, M.M. and Wilson, J.C. 2011, "Initial water deficit effects

UNIVERSITY of the
WESTERN CAPE

on *Lupinus albus* photosynthetic performance, carbon metabolism, and hormonal balance: metabolic reorganization prior to early stress responses", *Journal of Experimental Botany,* vol. 62, pp. 4965-4974.

Premachandra, G. Saneoka, H. and Ogata, S. 1990, "Cell membrane stability, an indicator of drought tolerance, as affected by applied nitrogen in *soyabean*", *The Journal of Agricultural Science,* vol. 115, pp. 63-66.

Quiroga, M. Guerrero, C. Botella, M.A. Barceló, A. Amaya, I. Medina, M.I. Alonso, F.J. de Forchetti, S.M. Tigier, H. and Valpuesta, V. 2000, "A tomato peroxidase involved in the synthesis of lignin and *suberin*", *Plant Physiology,* vol. 122, pp. 1119-1128.

Raymer, P.L. 2002, "Canola: an emerging oilseed crop", *Trends in New Crops and New Uses,* vol. 1, pp. 122-126.

UNIVERSITY of the
WESTERN CAPE

Rizhsky, L. Liang, H. Shuman, J. Shulaev, V. Davletova, S. and Mittler, R. 2004, "When defense pathways collide. The response of *Arabidopsis* to a combination of drought and heat stress", *Plant Physiology,* vol. 134, pp. 1683-1696.

Rizhsky, L. Liang, H. and Mittler, R. 2002, "The combined effect of drought stress and heat shock on gene expression in *tobacco*", *Plant Physiology,* vol. 130, pp. 1143-1151.

Sagi, M. and Fluhr, R. 2006, "Production of reactive oxygen species by plant NADPH oxidases", *Plant Physiology,* vol. 141, pp. 336-340.

Salekdeh, G.H. and Komatsu, S. 2007, "Crop proteomics: aim at sustainable agriculture of tomorrow", *Proteomics,* vol. 7, pp. 2976-2996.

Sanevas, N. Sunohara, Y. and Matsumoto, H. 2007, "Characterization of reactive oxygen species-involved oxidative damage in *Hapalosiphon species* crude extract-treated wheat and onion roots", *Weed Biology and Management,* vol. 7, pp. 172-177.

Savino, R. Paduano, S. Preianò, M. and Terracciano, R. 2012, "The proteomics big challenge for biomarkers and new drug-targets discovery", *International Journal of Molecular Sciences,* vol. 13, pp. 13926-13948.

Shao, H. Jiang, S. Li, F. Chu, L. Zhao, C. Shao, M. Zhao, X. and Li, F. 2007, "Some advances in plant stress physiology and their implications in the systems biology era", *Colloids and Surfaces B: Biointerfaces,* vol. 54, pp. 33-36.

UNIVERSITY of the
WESTERN CAPE

Sharp, R.E. LeNoble, M.E. Else, M.A. Thorne, E.T. and Gherardi, F. 2000, "Endogenous ABA maintains shoot growth in tomato independently of effects on plant water balance: evidence for an interaction with ethylene", *Journal of Experimental Botany,* vol. 51, pp. 1575-1584.

Shulaev, V. Cortes, D. Miller, G. and Mittler, R. 2008, "Metabolomics for plant stress response", *Physiologia Plantarum,* vol. 132, pp. 199-208.

Slaymaker, D.H. Navarre, D.A. Clark, D. Del Pozo, O. Martin, G.B. and Klessig, D.F. 2002, "The tobacco salicylic acid-binding protein 3 (SABP3) is the chloroplast *carbonic anhydrase*, which exhibits antioxidant activity and plays a role in the

hypersensitive defense response", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 99, pp. 11640-11645.

Smirnoff, N. 1993, "Tansley Review No. 52. The role of active oxygen in the response of plants to water deficit and desiccation", *New Phytologist*, pp. 27-58.

Smirnoff, N. and Wheeler, G.L. 2000, "Ascorbic acid in plants: biosynthesis and function", *Critical Reviews in Biochemistry and Molecular Biology,* vol. 35, pp. 291-314.

Spollen, W.G. LeNoble, M.E. Samuels, T.D. Bernstein, N. and Sharp, R.E. 2000, "Abscisic acid accumulation maintains maize primary root elongation at low water potentials by restricting ethylene production", *Plant Physiology,* vol. 122, pp. 967-976.

Sundaresan, S. and Sudhakaran, P. 1995, "Water stress-induced alterations in the proline metabolism of drought-susceptible and-tolerant *cassava* (Manihot esculenta) cultivars", *Physiologia Plantarum,* vol. 94, pp. 635-642.

Tabaeizadeh, Z. 1998, "Drought-induced responses in plant cells", *International Review of Cytology,* vol. 182, pp. 193-247.

Tanou, G. Filippou, P. Belghazi, M. Job, D. Diamantidis, G. Fotopoulos, V. and Molassiotis, A. 2012, "Oxidative and nitrosative-based signaling and associated post-translational modifications orchestrate the acclimation of citrus plants to salinity stress", *The Plant Journal,* vol. 72, pp. 585-599.

Taylor, C.B. 1996, "Proline and water deficit: Ups, downs, ins, and outs", *The Plant Cell,* vol. 8, pp. 1221.

UNIVERSITY of the
WESTERN CAPE

Torres, M.A. and Dangl, J.L. 2005, "Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development", *Current Opinion in Plant Biology,* vol. 8, pp. 397-403.

Trebst, A. Depka, B. and Holländer-Czytko, H. 2002, "A specific role for tocopherol and of chemical singlet oxygen quenchers in the maintenance of photosystem II structure and function in *Chlamydomonas reinhardtii*", *FEBS letters,* vol. 516, pp. 156-160.

Triantaphylides, C. Krischke, M. Hoeberichts, F.A. Ksas, B. Gresser, G. Havaux, M. Van Breusegem, F. and Mueller, M.J. 2008, "Singlet oxygen is the major reactive oxygen species involved in photooxidative damage to plants", *Plant Physiology,* vol. 148, pp. 960-968.

Van Duyn, M.A.S. and Pivonka, E. 2000, "Overview of the health benefits of fruit and vegetable consumption for the dietetics professional: selected literature", *Journal of the American Dietetic Association,* vol. 100, pp. 1511-1521.

Velikova, V. Yordanov, I. and Edreva, A. 2000, "Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines", *Plant Science,* vol. 151, pp. 59-66.

Wade, L.J. Ghareyazie, B. and Bennett, J. 2002, "Proteomic analysis of rice leaves during drought stress and recovery", *Proteomics,* vol. 2, pp. 1131-1145.

UNIVERSITY of the
WESTERN CAPE

Wang, W. Vignani, R. Scali, M. and Cresti, M. 2006, "A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis", *Electrophoresis-An International Journal,* vol. 27, pp. 2782-2786.

Wang, D. Naidu, S.L. Portis, A.R. Moose, S.P. and Long, S.P. 2008, "Can the cold tolerance of C4 photosynthesis in *Miscanthus x giganteus* relative to *Zea mays* be explained by differences in activities and thermal properties of Rubisco?", *Journal of Experimental Botany,* vol. 59, pp. 1779-1787.

Whittaker, A. Martinelli, T. Bochicchio, A. Vazzana, C. and Farrant, J. 2004, "Comparison of sucrose metabolism during the rehydration of desiccation-tolerant and desiccation-sensitve leaf material of *Sporobolus stapfianus*", *Physiologia Plantarum,* vol. 122, pp. 11-20.

Wilkinson, S. and Davies, W.J. 2002, "ABA-based chemical signalling: the co-ordination of responses to stress in plants", *Plant, Cell and Environment,* vol. 25, pp. 195-210.

Xiao, X. Yang, F. Zhang, S. Korpelainen, H. and Li, C. 2009, "Physiological and proteomic responses of two contrasting *Populus cathayana* populations to drought stress", *Physiologia Plantarum,* vol. 136, pp. 150-168.

Yang, J. Zhang, J. Wang, Z. Zhu, Q. and Wang, W. 2001, "Hormonal changes in the grains of rice subjected to water stress during grain filling", *Plant Physiology,* vol. 127, pp. 315-323.

UNIVERSITY of the
WESTERN CAPE

Yildiz-Aktas, L. Dagnon, S. Gurel, A. Gesheva, E. and Edreva, A. 2009, "Drought Tolerance in Cotton: Involvement of Non-enzymatic ROS-Scavenging Compounds", *Journal of Agronomy and Crop Science,* vol. 195, pp. 247-253.

Zhang, T. Zhao, X. Wang, W. Pan, Y. Huang, L. Liu, X. Zong, Y. Zhu, L. Yang, D. and Fu, B. 2012, "Comparative transcriptome profiling of chilling stress responsiveness in two contrasting rice genotypes", *PLOS One,* vol. 7, pp. e43274.

Zhu, J. 2002, "Salt and drought stress signal transduction in plants", *Annual Review of Plant Biology,* vol. 53, pp. 247.

Zingaretti, S.M. De Matos Pereira, L. Inácio, M.C. De Castro França, S. and Paz, T.A. 2013, “*Water stress and agriculture”,* INTECH Open Access Publisher.

UNIVERSITY of the
WESTERN CAPE

SUPPLEMENTARY DATA

Table 1, Supplementary data: Drought stress responsive proteins that either fall outside the pI range of 4-7, or having a non-significant MOWSE scores of below 70 and those spots with no significant matches.

Spot	Best Match Protein	NCBi and SwissProt DATA Base	Species	MOWSE score	Exp. MW/pI	Matching peptides	Coverage [%]
			Photosynthesis				
11	Oxygen-evolving enhancer protein 1;	gi|39932634		54.90	2.60/4.35	1	65.40
33	Ribulose-phosphate 3-epimerase	RPE_ORYSJ	*OS*	79.28	29.00/9.56	2	9.50
39	Ribulose-phosphate 3-epimerase	RPE_ORYSJ	*OS*	69.78	29.00/9.58	1	7.30
	Ribulose-phosphate 3-epimerase	RPE_ORYSJ	*OS*	69.78	29.00/9.58	1	7.30
			Energy related				
29	ATP synthase delta chain	SYK_SOLLC	*Sl*	39.96	67.10/5.55	1	1.40
49	Glyceraldehyde-3-phosphate dehydrogenase	gi|284177800	*No*	56.39	18.50/6.15	1	6.40
51	Glyceraldehyde-3-phosphate dehydrogenase B subunit	G3PB_ARATH	*At*	59.76	47.60/6.36	2	5.80
			Disease/Defense				
31	Germine-like protein	gi|1755154	*At*	94.30	21.80/7.73	1	8.10
38	Superoxide dismutase [Cu/Zn]	gi|66841106	*Lg*	47.69	2.40/7.90	1	57.70
53	Germine-like protein	gi|1755154	*At*	97.24	21.80/7.73	1	8.10
			Metabolism				
48	Ferredoxin-NADP reductase	gi|317456226	*Sh*	66.02	17.00/5.00	1	12.90
			Protein folding				
22	chaperonin 10	gi|3057150	*At*	264.35	26.90/9.35	5	18.90
	Unclassified						
21	F23N19.15	gi|6630456	*At*	66.31	21.40/4.79	1	5.30
23	F23N19.15	gi|6630456	*At*	66.31	21.40/4.79	1	5.30
28	Peptidyl-prolyl cis-trans isomerase	gi|255548201	*RC*	69.47	28.20/9.71	1	4.60
30	predicted protein	gi|168007785	*Php*	86.51	17.50/9.05	1	9.70
52	hypothetical protein LOC_Os11g14750	gi|62732689	*Os*	45.32	41.10/4.78	1	2.40
		Spots with no significant matches					
	2,8,12,17,27,41,42,44,45,46,54						

At, Arabidopsis thaliana; Ar, Acer rubrum; Os, Oryza sativa; Pp, Pinus pinaster Sh, Solanum habrochaites; Sl, Solanum lycopersicum; Lg, Larix gmelinii; Php, Physcomitrella patens; Rc, Ricinus communis.

UNIVERSITY of the
WESTERN CAPE