

3,3'-diindolylmethane improves drought tolerance of *Zea mays* through enhancing antioxidant activity

Gerhard Leroy Basson



UNIVERSITY *of the*
WESTERN CAPE

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

Supervisor: Prof Ndiko Ludidi

Co-supervisor: Dr Marshall Keyster & Dr Arun Gokul



**UNIVERSITY of the
WESTERN CAPE**

University of the Western Cape
Private Bag X17, Bellville 7535, South Africa
Telephone: ++27-21- 959 2255/959 2762
Fax: ++27-21- 959 1268/2266
Email: jvanbeverdonker@uwc.ac.za

FACULTY OF NATURAL SCIENCE

GENERAL PLAGIARISM DECLARATION

Name: Gerhard Leroy Basson
Student number: 3373827


I hereby declare that I know what plagiarism entails, namely to use another's work and to present it as my own without attributing the sources in the correct way. (Refer to University Calendar part 1 for definition)

I know that plagiarism is a punishable offence because it constitutes theft.

I understand the plagiarism policy of the Faculty of Natural Science of the University of the Western Cape.

I know what the consequences will be if I plagiarise in any of the assignments for my course.

I declare therefore that all work presented by me for every aspect of my course, will be my own, and where I have made use of another's work, I will attribute the source in the correct way.



Signature

20/12/2018

Date

Acknowledgements

I would like to express my gratitude to my principal investigator **Prof. Ndiko Ludidi** for all the guidance he has offered. I would also like to thank you for providing and encouraging me to build international relations to further benefit my career. Prof. Ndiko Ludidi has also involved me in multiple projects, some of which do not align with my research project and thereby allowing me to further broaden my understanding and acquire more skills. For this and all the above-mentioned reasons I am most grateful.

I would also like to thank my co-supervisor's **Dr Marshall Keyster** and **Dr Arun Gokul** for their continuous support and guidance. The skills and knowledge I have acquired from them has made me a better individual in my professional career as well as a better person. I would also like to thank them for driving me to strive for better and for always accommodating my scientific ideas and supporting them. They have played an important role in helping me to develop my potential as a young scientist. Driving me to ask the scientific questions that few would tend to ask.

To my parents **Lindsay** and **Chantal Basson**, I would like to express my deepest gratitude for their hard work and the sacrifices they made in order to provide me with opportunities they never had. I wouldn't be where I am today were it not for their love and support. I would also like to thank my sisters **Bokeem** and **Brittney Basson** for their words of encouragement and the part they played at home while growing up.

I would also like to say thank you to a very special **Ms Jamey Bester**. You were present from the start of my studies in 2013 and you are still here. Thank you for the encouragement over the years and the continuous support. You have played a key role in my development as a person and I am eternally grateful for your continuous love and support.

To my friends and colleague's **Dr Kyle Phillips**, **Dr Arun Gokul**, **Mr Fahiem Carelse**, **Ms Lee-Ann Niekerk**, **Mr Ali Ali** I would like to extend a special thanks for your friendship as well as for providing a good working environment.

Furthermore, I would like to thank the **Plant Biotechnology Research Group** and the **University of the Western Cape** for providing the necessary work environment.

Lastly, I would like to thank **MERSETA**, **FoodBev**, **MERSETA** and the **National Research Foundation (NRF)** for funding for my work.

List of Abbreviations

DIM	3, 3' Diindolylmethane
RWC	Relative water content
FW	Fresh weight
TW	Turgid weight
DW	Dry weight
MDA	Malondialdehyde
TCA	Trichloroacetic acid
TBA	Thiobarbituric acid
EDTA	Ethylenediaminetetraacetic acid
APX	Ascorbate peroxidase
CAT	Catalase
TEMED	N, N, N, N'- Tetramethylethylenediamine
Chl a	Chlorophyll a
Chl b	Chlorophyll b
WW	Well-watered
WD	Water-deprived
ROS	Reactive oxygen species
NBT	Nitro blue tetrazolium chloride



List of Figures

Chapter 1

Figure 1: South African maize area planted, production and the producer's price.....	3
Figure 2: Diagram showing Generation of ROS by energy transfer adapted from.....	5
Figure 3: Proline serves as a multifunctional amino acid in plant adaptive responses as a protective osmolyte.....	10
Figure 4: Represents the biosynthesis of the glucosinolate core structure.....	13

Chapter 3

Figure 3.1.1: The effect of water deprivation on the water status of seedlings in presence and absence of DIM.....	26
Figure 3.2: The effect of water deprivation on the proline accumulation of seedlings in presence and absence of DIM.....	28
Figure 3.3: The effect of water deprivation on the Hydrogen peroxide and Malondialdehyde accumulation of seedlings in presence and absence of DIM.....	30
Figure 3.4.1: In-gel activity assays for APX activity in <i>Zea mays</i> leaves in response to water-deprived and DIM treatments.....	32
Figure 3.4.2: In-gel activity assays for APX activity in <i>Zea mays</i> roots in response to water-deprived and DIM treatments.....	33
Figure 3.5.1: In-gel activity assays for CAT activity in <i>Zea mays</i> leaves in response to water-deprived and DIM treatments.....	34
Figure 3.5.2: In-gel activity assays for CAT activity in <i>Zea mays</i> roots in response to water-deprived and DIM treatments.....	36

List of Tables

Table 2.1: List of chemicals and suppliers.....	17
Table 2.2: List of equipment.....	18
Table 3.1.2: The effect of DIM, water deprivation and their combination on plant chlorophyll ($\mu\text{g}\cdot\text{g}^{-1}$) a and b.....	27



UNIVERSITY *of the*
WESTERN CAPE

3,3'-diindolylmethane improves drought tolerance of *Zea mays* through enhancing oxidative capacity

Gerhard Leroy Basson

Keywords

Antioxidant enzymes

Ascorbate peroxidase (APX)

Catalase (CAT)

Chlorophyll

Drought

Hydrogen peroxide (H₂O₂)

Lipid peroxidation

Maize

Oxidative capacity

3,3' Diindolylmethane

Primed

Reactive oxygen species (ROS)

Water-deprivation



3,3'-diindolylmethane improves drought tolerance of *Zea mays* through enhancing oxidative capacity

Gerhard Leroy Basson

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

Abstract

Maize is one of the most abundantly produced cereals and contributes to about 40% of the global cereal production. This figure will have to increase in order to feed the ever-growing human population. One of the major environmental constraints that impact maize production is drought. Plants use antioxidant defences to cope with drought stress. Understanding and improving these defence mechanisms will be important to improve overall drought tolerance. A previous study done by Gokul and authors in 2016 showed that 3,3'-diindolylmethane (DIM) improves both seed germination and seedling shoot growth in *Brassica napus*. Plants belonging to the Brassicaceae family have the metabolic machinery to synthesize glucosinolates such as DIM, which play vital roles in physiological and stress responses. These responses have not been investigated in plants such as maize, which lack the machinery to produce DIM. Therefore, this study investigated the effects of exogenously applied DIM on the physiological and biochemical responses of maize under drought stress. Physiological parameters such as relative water content, chlorophyll content and lipid peroxidation, were determined in order to understand how drought and DIM, as separate or combined treatments, affected the plants. Additionally, proline accumulation was also assessed because free proline plays a role as an osmoprotectant during stress. The accumulation of ROS, namely hydrogen peroxide, was measured using spectrophotometric assays to determine how the above treatments affect ROS accumulation in maize. As a result of changes in the ROS content in due to the treatments, it would only be natural to investigate the changes in antioxidants as well. Given that hydrogen peroxide was the ROS to be measured, we therefore investigated the antioxidant enzymatic activities responsible for hydrogen peroxide scavenging. Therefore, changes in Ascorbate peroxidase (APX) and catalase (CAT) were assessed. An improved drought response was observed in maize plants treated with DIM as these plants had better ability to maintain their water status than when no DIM was applied. This is indicated by water-deprived plants treated with DIM having a higher RWC than water-deprived plant without DIM. Chlorophyll content was also

determined since a negative effect on chlorophyll content would impair photosynthesis because of the key role of chlorophyll on light absorption for photosynthesis. Drought significantly, altered the chlorophyll a/b ratio, which would likely impact the normal photosynthetic activity of the plants. However, water-deprived leaves treated with DIM had a chlorophyll a/b ratio closer to that of the control plants, suggesting that DIM aided in the maintenance of chlorophyll content, and thus likely the photosynthetic activity of maize, under water-deprivation stress. In response to stress, plants usually accumulate compatible solutes such as free proline as a form of an adaptive response. An inversely proportional relationship between RWC and proline content was observed. Therefore water-deprived plants which showed a reduction in RWC had an increase in proline content. Additionally, it was observed that water deprivation caused a significant increase in H₂O₂ in leaves and roots, which correlated with an increase lipid peroxidation. However, water-deprived leaves treated with DIM showed a significant decrease in H₂O₂ accumulation when compared to water deprivation alone and therefore experienced less lipid peroxidation. Additionally, DIM-treated roots showed an increase in H₂O₂ accumulation which did not result in an increase in lipid peroxidation. This suggest that that DIM might play a role in H₂O₂-dependant signalling pathways. An increase was observed in APX and CAT activity in plants treated with DIM. This suggest that DIM primed the plants through increasing the activity of these antioxidant enzymes and therefore would allow for better hydrogen peroxide scavenging ability. It was also observed that water-deprived plants treated with DIM showed lower antioxidant activity when compared to water-deprived plants without DIM. This suggests that treatment with DIM under drought stress suppresses hydrogen peroxide hyper-accumulation, which would not trigger elevated antioxidant enzyme activity since they would not be needed in high levels when there is less hydrogen peroxide. These results indicate that exogenous DIM improves drought stress responses even in plants that lack the metabolic machinery to synthesize DIM. In conclusion exogenous DIM treatment could serve as a possible route to improve drought stress tolerance, through the activation of reactive oxygen species (ROS) signalling pathways without causing oxidative damage in Maize. This activation maintains a steady-state balance between ROS and antioxidant enzyme activity as these plants will have enhanced antioxidative capacity.

Table of Contents

General plagiarism declaration.....	ii
Acknowledgements.....	iii
List of Abbreviations.....	iv
List of Figures.....	v
List of Tables.....	vi
Keywords.....	vii
Abstract.....	viii
Chapter 1:	1
Literature review	
1.1 Introduction.....	1
1.2 Maize in South Africa.....	2
1.3 Abiotic stress induces the accumulation of ROS in plants.....	4
1.3.1 Singlet Oxygen ($^1\text{O}_2$)	5
1.3.2 Superoxide anion (O_2^-).....	6
1.3.3 Hydrogen Peroxide (H_2O_2).....	6
1.3.4 Hydroxyl Radical ($\cdot\text{OH}$).....	6
1.4 ROS accumulation affects plant physiology and biochemistry.....	7
1.4.1. Lipid peroxidation.....	7
1.4.2. Leaf senescence induced by stress.....	8
1.5 ROS-scavenging Mechanisms.....	9
1.5.1. Non-enzymatic ROS-scavenging mechanisms.....	9
1.5.1.1. Plants accumulate osmolytes in response to abiotic stresses.....	9
1.5.2. Enzymatic ROS-scavenging mechanisms.....	11
1.5.2.1. Superoxide Dismutase (SOD).....	11
1.5.2.2. Catalase (CAT).....	11
1.5.2.3. Ascorbate Peroxidase (APX).....	12
1.6 Glucosinolates.....	13
1.6.1. 3, 3-Diindolylmethane (DIM).....	14
1.7 Justification.....	15
1.8 Objectives.....	16
Chapter 2.....	17
Methods	
2.1 Preparation of DIM solution.....	18
2.2 Plant growth and treatments.....	19
2.3 Leaf relative water content (RWC).....	20
2.4 Determination of “chlorophyll a and b” in <i>Zea mays</i> leaves.....	21

2.5. Trichloroacetic (TCA) acid extraction.....	22
2.5.1. Determination of Lipid peroxidation. in <i>Zea mays</i> leaves and roots.....	22
2.5.2. Determination of H ₂ O ₂ in <i>Zea mays</i> leaves and roots.....	22
2.6. Determination of proline content in <i>Zea mays</i> leaves and roots.....	23
2.7. Protein Extraction.....	22
2.8. Ascorbate peroxidase (APX) activity in <i>Zea mays</i> leaves and roots.....	24
2.9. Catalase (CAT) activity in <i>Zea mays</i> leaves and roots.....	25
2.10. Statistical analysis.....	25
Chapter 3.....	26
Results	
3.1. Exogenous DIM improves physiological responses of <i>Zea mays</i> seedlings under water deprivation.....	26
3.1.1 Exogenous DIM maintains maize leaf RWC under water-deprived conditions.....	26
3.1.2. Exogenous DIM mitigates the negative effects of drought on the chlorophyll a/ b Chl (a/b) ratio	27
3.2. The effects of water-deprivation and DIM on proline accumulation.....	28
3.3. The effects of water-deprivation and DIM on H ₂ O ₂ and MDA.....	29
3.4. Ascorbate peroxidase (APX) activity is altered in <i>Zea mays</i> leaves and roots under water-deprivation and DIM.....	31
3.5. Catalase (CAT) activity is altered in <i>Zea mays</i> leaves and roots under water-deprivation and DIM.....	34
Chapter 4.....	37
Discussion and conclusion	
4.1 Exogenous DIM mitigates the reduction of leaf water status under water-deprivation.....	37
4.2. Exogenous DIM improves maintenance of photosynthetic pigments under water-deprivation.....	38
4.3 Water deprivation increases proline.....	39

4.4 DIM improves plant responses to water-deprivation stress by regulating ROS (H ₂ O ₂)	40
4.5 DIM increases plant adaptive responses to water deprivation through the activation of antioxidant enzymes.....	41
4.6 Conclusion and Future work.....	43
Chapter 5.....	45
References.....	44



UNIVERSITY *of the*
WESTERN CAPE

Chapter 1

LITERATURE REVIEW

1.1. Introduction

Drought is a climatic phenomenon which has detrimental effects on a global scale. Persisting drought has major environmental implications such as land degradation, aridity and desertification (Masih, et al., 2014). Agricultural drought is seen as a deficiency in soil moisture which compromises crop production (Edossa, et al., 2016). In response to stresses, plants accumulate reactive oxygen species (ROS). These ROS are oxygen containing radicals which react with DNA, lipids and proteins and play an important role as signalling molecules at basal levels. However, during stress these molecules are overproduced, resulting in the plant suffering from oxidative stress. In order to maintain steady state ROS levels, plants have mechanisms in place which maintain ROS homeostasis. These mechanisms can be either enzymatic or non-enzymatic. Identifying methods of maintaining optimal crop production during drought will be crucial in improving food security. Investigating how plants respond to drought at a physiological and molecular level will eventually lead to finding ways of mitigating the stress, thereby developing more resilient crops. Methods to improve plant tolerance to abiotic stress includes the exogenous addition of signalling molecules such hormones, growth regulators etc., which play a vital role in their growth and development. This could in turn improve plant adaptive responses to cope with abiotic stresses possibly through the activation of ROS-scavenging mechanisms. The molecule 3,3'-diindolylmethane (DIM) is a compound which naturally occurs in the cruciferous plant family and abundantly produced in the genus Brassica (Le, et al., 2003). DIM is a potent

antioxidant agent in mitigating the effects of oxidative stress in cancer in mammals (Lu, et al., 2016). A previous study done by Gokul et al. (2016) was the first to show that the application of DIM acted as a growth promoter and improved antioxidant capacity of *B. napus* seedlings via the induction of hydrogen peroxide and superoxide. This thesis aims to investigate the signalling roles of DIM in plants (maize) that lack the molecular/metabolic machinery to endogenously produce this compound, with emphasis on the effects of DIM on the plants under water-deprived conditions. Therefore, this literature review will discuss potential signalling pathways for DIM as well as general background of the study.

1.2. Maize in South Africa

In South Africa, maize is one of the most important cereal crops for most of the population, serving as both a staple food and feed for livestock. The majority of maize that is produced in South Africa is mostly consumed locally, which makes the domestic market a crucial influential factor in the industry. It is estimated that approximately 50% of the maize produced is consumed by humans, 40% used by the animal feed industry and the rest used for seed and industrial uses (10%) (Mogala, 2012). Additionally, maize has also been the biggest contributor to the gross value of field crops for the past five seasons, contributing approximately 48 %. This amounted to a gross value of R27 556 million during the 2015/16 year. In South Africa, approximately 43% white maize and is used primarily for human consumption and the remaining 57% is yellow maize which is mainly used for animal feed production (2016). Free State and North West provinces are the two main provinces which produce white maize, while Mpumalanga and the Free State are the major yellow maize producers. During 2016 the Free State and North West provinces experienced one of its

poorest harvest of white maize as a result of an El Niño-induced drought causing crop failures due to the lack of rain. South African is currently battling one of its worst droughts which had started as early as 2015. During the 2015/2016 season commercial maize producers planted roughly 26% less maize than they had the previous year as many of the major maize production areas were impacted by the severe drought (DAFF, 2016). During the 2013 to 2016 period South Africa has seen a decrease in the production for all field crops. The decrease was mainly attributed to low productions of crops such as wheat and canola, soya beans, groundnuts maize and sorghum. Through the duration of the 2014/2015 period the production of wheat decreased by 17.7% and canola by 22.5%. Soya bean had a 29.9% decrease in production and groundnut a 53% decrease. Maize production had decreased by 27.6% while sorghum had a 26.6% decrease in production (Figure 1).

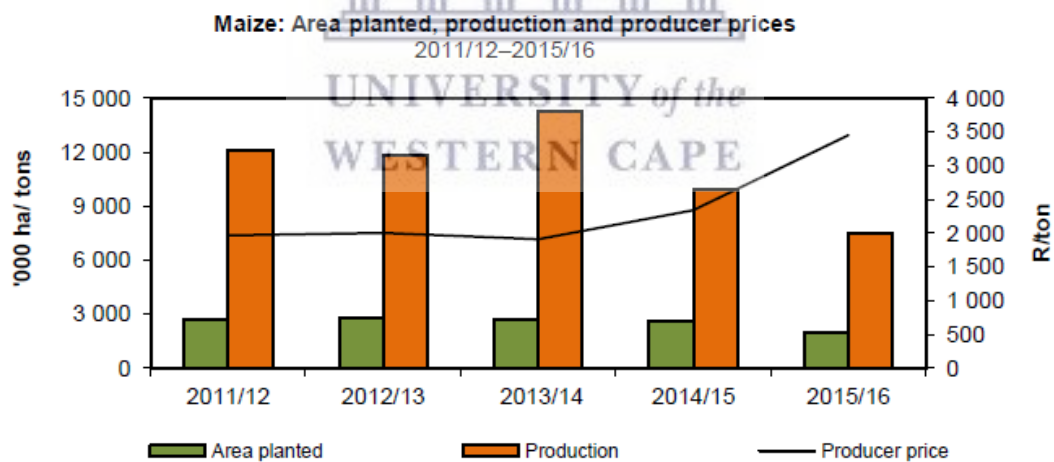


Figure 1: South African maize area planted, production and the producer's price. Area of maize planted, production as well as the producer prices in South Africa. The area planted is depicted as green, production in orange and the producer prices depicted as the black line. This figure was adapted from the Department of Agriculture, Forestry and Fisheries (DAFF, 2016).

1.3. Abiotic stress induces the accumulation of ROS in plants

In the absence of stress, reactive oxygen species (ROS) play an important role as signalling molecules in vital biological processes. These processes include the regulation of growth, cell cycle, hormone signalling, programmed cell death as well as plant development (Hancock, et al., 2001). ROS levels are tightly controlled by production and scavenging rates. Various developmental and environmental signals influence the ROS signalling network and maintain ROS homeostasis either in cell-specific or compartment-specific manner. ROS levels are perceived by different enzymes, proteins or receptors which modulate various metabolic, developmental as well as defence pathways. ROS are generated by an array of enzymatic activities and removed by ROS-scavenging mechanisms which can be enzymatic or non-enzymatic (Hancock, et al., 2001). The ROS-producing and scavenging pathways the impact the amount of ROS generated as well as its duration. These interactions form the feedback mechanism which maintain low steady-state levels of ROS to be used for signalling (Ashraf, 2009).

Abiotic stress conditions such as drought, UV radiation, intense light, chilling, wounding and pathogen infection causes an increase in the production and accumulation of ROS, resulting in oxidative stress (Gill & Tuteja, 2010). ROS forms part of a class of reactive molecules which are free radicals that result from reduction of molecular oxygen during aerobic respiration (Hancock, et al., 2001). These metabolites are produced at multiple sites within plant cells which include organelles such as the chloroplast, mitochondrion, peroxisomes, endoplasmic reticulum as well as the plasma membrane. This suggests that in order to produce ROS the “location” has to be rich in oxygen as well as reductants (Oukarrouma, et al., 2015). In these locations, molecular oxygen (O₂)

is reduced to either superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) or hydroxyl radical ($HO\cdot$) (Figure 2) most of which are highly toxic through their oxidizing reactivity (Mittler, et al., 2004).

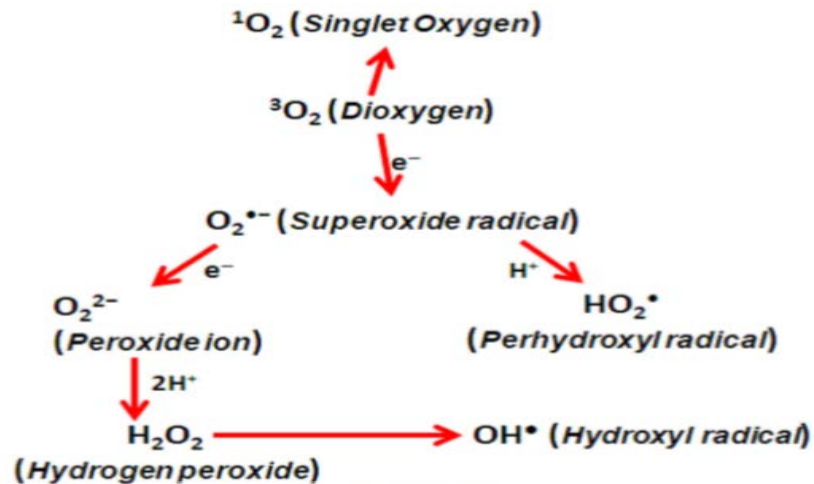


Figure 2: Diagram showing generation of ROS by energy transfer. Adapted from (Das & Roychoudhury, 2014)



1.3.1. Singlet Oxygen (1O_2)

Singlet oxygen (1O_2) is primarily generated in the chloroplast when the absorption of energy from sunlight exceeds the usage of this energy during photosynthesis. The additional energy reacts with molecular oxygen and forms 1O_2 . Despite its short half-life, 1O_2 is a very reactive molecule and the over accumulation thereof results in damage of proteins involved in photosystem II. Furthermore, 1O_2 is the major contributor to light-induced loss in photosystem II which contributes to cell death (Das & Roychoudry, 2014).

1.3.2. Superoxide Radical (O_2^-)

Superoxide (O_2^-) is produced as a result of the reduction of singlet oxygen. This usually occurs during the photosynthetic electron transport chain and during the transport of electrons in the mitochondria during respiration. Superoxide has a short half-life, is highly reactive and is unable to diffuse. This, however does not prevent it from causing damage as it can still cause lipid peroxidation of the lipid membranes of cells (Szollosi, 2014). Additionally, O_2^- has an inhibitory effect on peroxidase enzymes as well as ribonucleotide reductases activity resulting in their peroxidation (Das & Roychoudry, 2014)

1.3.3. Hydrogen Peroxide (H_2O_2)

When a superoxide anion (O_2^-) is univalently protonated as well as reduced, hydrogen peroxide (H_2O_2) is produced. Hydrogen peroxide is moderately reactive and is produced in plant cells under normal conditions. This compound has a relatively long half-life and has the ability to diffuse across cellular membranes. This attribute makes it a suitable signalling molecule at low concentration, however at the same time these exact traits make it dangerous at high concentrations (Petrov & Van Breusegem, 2012). H_2O_2 has the ability to reduce enzyme function and can even cause complete inactivation at high concentrations. This is due to its ability to oxidize the thiol groups present on the enzymes (Sharma, et al., 2012). In order to maintain steady state levels of H_2O_2 , plants have various peroxidase enzymes which detoxify the H_2O_2 .

1.3.4. Hydroxyl Radical ($\cdot OH$)

The hydroxyl radical ($\cdot OH$) is the result of the reaction of O_2^- with H_2O_2 which is further catalysed by transition metals via the Fenton reaction (Saed-Moucheshi,

et al., 2014). Plants take up a variety of transition metals which are essential for various metabolic activities. The constant uptake of these metals could result in an over production of $\cdot\text{OH}$ which would result in detrimental effects. Although $\cdot\text{OH}$ has a short half-life, it is the most destructive of the all ROS due to its high reactivity. In chloroplasts, if its concentration increases to just 10 μM the resulting effect causes more than a 50% decrease in photosynthetic activity (Das & Roychoudry, 2014). Hydroxyl causes damage to proteins as well as the peroxidation of membrane lipids. Unfortunately, cells do not have the metabolic machinery to detoxify $\cdot\text{OH}$ and therefore rely on mechanisms which would prevent its formation.

1.4. ROS accumulation affects plant physiology and biochemistry

1.4.1. Lipid peroxidation

Cellular membranes are rich in polyunsaturated fatty acids which contain methylene $-\text{CH}_2-$ groups containing hydrogen. These hydrogen groups are the primary targets of oxidative reactions and are therefore sensitive to oxidative attacks (Lü, et al., 2010). When there is an over-accumulation of ROS, which usually occurs under stress, it results in the peroxidation of these membrane lipids (Jajic, et al., 2015). Hence measuring the amount of lipid peroxidation is a widely used technique as an indicator of oxidative damage. Determining the degree of lipid peroxidation using primary lipid hydroperoxides is difficult due to their instability and reactivity. Therefore, it is more effective to determine the degree of lipid peroxidation by measuring the concentration of by-products produced by the breakdown of the primary hydroperoxides (Davey, et al., 2005). These by products are secondary products and mostly consist of aldehydes which have the ability to further intensify oxidative damage. The damaging

effects are due to the reactivity and durability as well as the ability to act both intra- and extracellularly. They interact with proteins as well as nucleic acids, often causing irreversible damage which then disrupts normal cellular function. One of the aldehydes produced as a result of lipid peroxidation is Malondialdehyde (MDA). MDA is one of the more commonly studied by-products and its quantification can therefore be used as an indicator of the degree of lipid peroxidation (Del Rio, et al., 2005).

1.4.2. Leaf senescence induced by stress

Leaves obtain their green colour due to a pigment called chlorophyll. Chlorophyll plays an essential role in the production of energy through the process of photosynthesis. Plants use this energy to grow and carry out various metabolic activities. When plants experience stress as a result of water deprivation, stomatal conductance is reduced as a means of water conservation. This results in a reduction in internal leaf carbon dioxide (CO₂) concentrations resulting in a reduced photosynthetic rate (Mafakheri, et al., 2010). Studies have shown a link between a reduction in chlorophyll and drought. A study done in 1988 by Conroy and authors showed that drought decreased chlorophyll in sunflower (Conroy, et al., 1988). The decrease in chlorophyll under drought stress is mainly the result of damage to chloroplasts caused by reactive oxygen species. Although energy is still being produced from light the rest of the photosynthetic processes are not able to continue due to the reduction of internal CO₂. This additional energy is passed on to molecular oxygen forming ¹O₂ which will eventually lead to the production of other ROS such as superoxide, hydrogen peroxide and hydroxyl resulting in the induction of photo-oxidative stress. Prolonged photo-oxidative stress damages chloroplasts, ultimately reducing yield (Pintó-Marijuan & Munné-Bosch, 2014).

1.5 ROS-scavenging Mechanisms

Plants have evolved a number of defence/antioxidant mechanisms to maintain the steady state levels of ROS by means of both non-enzymatic and enzymatic mechanisms during stress. Non-enzymatic mechanisms include glutathione, ascorbic acid, proline, etc.

1.5.1. Non-enzymatic ROS-scavenging Mechanisms

Non-enzymatic approaches include vitamin C or ascorbic acid and vitamin E as they are capable of reducing ROS (Lobo, et al., 2010). One of the most important aspects of the non-enzymatic defences is glutathione as it provides intra-cellular defence against the deleterious effects of ROS. Glutathione is made up of three peptides (glutamyl, cysteinyl and glycine) which provides a free sulphhydryl group. This free group provides an abundant target for attack as ROS molecules oxidize glutathione, however the reduced form can be regenerated in a redox reaction by an NADPH-dependent reductase (Apel & Hirt, 2004).

1.5.1.2. Plants accumulate osmolytes in response to abiotic stresses.

Plants are regularly challenged by many inimical environmental conditions such as drought, heavy metals and salinity to name a few. In response to drought, plants tend to accumulate compatible solutes known as osmolytes. These are low-molecular weight organic compounds that are water soluble and are non-toxic at millimolar concentrations. These compatible solutes play an important role in adaptive stress responses as they serve in cellular redox balance and stabilize protein structures (Suprasanna, et al., 2015). Furthermore, the

accumulation of these osmolytes minimizes water loss as they adjust the osmotic potential and maintain cellular turgidity, thereby reducing wilting. Common osmolytes include glycine betaine, some sugars and proline.

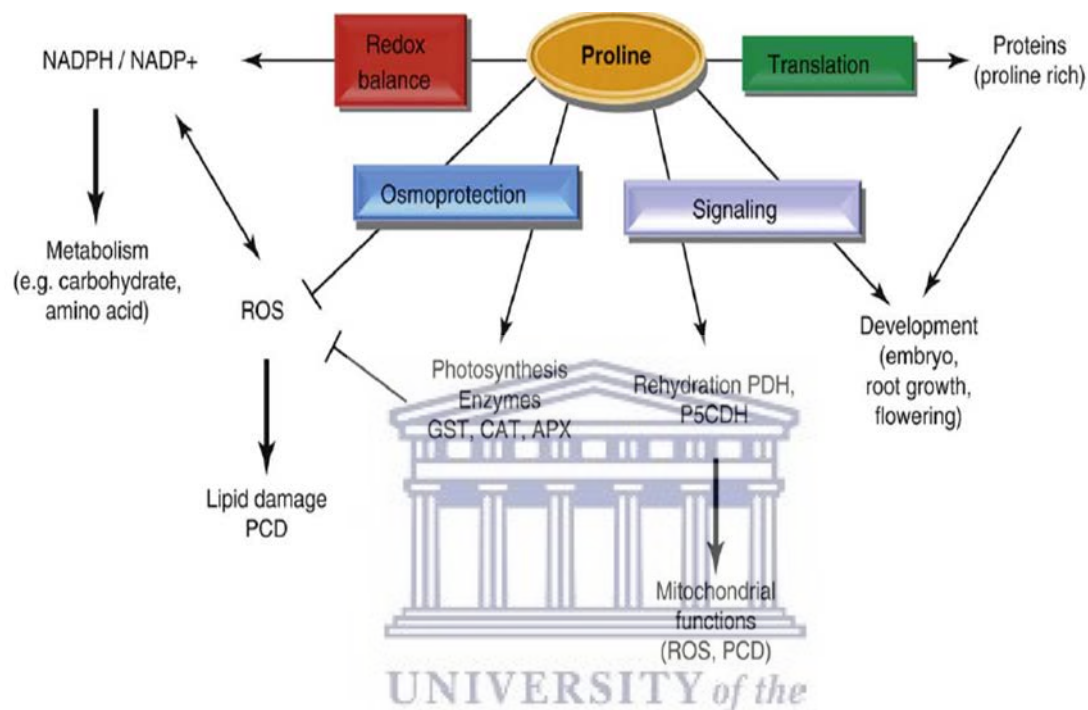


Figure 3: Proline serves as a multifunctional amino acid in plants. Proline is an amino acid and as such plays a role as a building block during protein synthesis. In addition to this it also forms part of a signalling network involved in mitochondrial functions as well as development. Furthermore, proline plays a significant role in plant adaptive responses as a protective osmolyte. These adaptive responses include regulation of photosynthesis, maintaining redox-balance through ROS scavenging mechanisms (enzymatically and non-enzymatically) as well as post-stress recovery. Abbreviations: APX, ascorbate peroxidase; CAT, catalase; PCD, programmed cell death; ROS, reactive oxygen species. Adapted from Szabados & Savoure (2010)

Proline is a proteinogenic amino acid with a unique conformational rigidity and is essential for primary metabolism. Proline plays an important role in combating stress (Figure 3) as it can serve as a signalling molecule, free radical scavenger, chelator, cellular redox balancer, cytosolic pH buffer, compatible solute as well as a stabilizer of subcellular structures and membranes involved in photosystems II (PS II) (Hossain, et al., 2014).

1.5.2. Enzymatic ROS-scavenging Mechanisms

Enzymatic antioxidants are localized in different compartments of the cell. These include superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase activity.

1.5.2.1. Superoxide Dismutase (SOD)

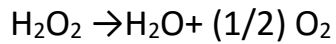
SOD forms part of the initial defence against ROS damages as a result of environmental stress. It belongs to the family of metalloenzymes, which is ubiquitous to all aerobic organisms. SOD is responsible for catalysing the removal of O_2^- via dismutation to form H_2O_2 and O_2 and in doing so prevents the possibility of $\cdot OH$ formation (Scandalios, 1993). SOD has isoforms which are classified according to metallic ion it binds to such as manganese-SOD (which is localized in mitochondria), iron-SOD (in chloroplasts) and copper/zinc-SOD (in the cytosol, peroxisomes as well as chloroplasts) (Mittler, 2002). In response to abiotic stress, plants up-regulate SOD activity. The dismutation of O_2^- to H_2O_2 can be represented by the following chemical reaction:



1.5.2.2. Catalase (CAT)

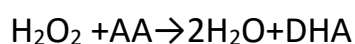
Catalase (CAT) is localized in peroxisomes and mitochondria and is comprised of a tetrameric heme-containing enzyme. The role of CAT is to catalyse the conversion of H_2O_2 produced by SOD and other processes into water (H_2O) and oxygen O_2 (Gill & Tuteja, 2010). Although CAT has high affinity for H_2O_2 , it has reduced specificity for organic peroxides (R-O-O-R). CAT has quite a high turnover rate, converting approximately 6×10^6 molecules of H_2O_2 to H_2O and O_2

min^{-1} without needing a reducing correspondent, singling it out from other enzymatic antioxidants. In stressful conditions, there is an increase in the demand for generating and expending energy within the cell. This results in an increase in catabolism, generating H_2O_2 . The CAT enzyme then eliminates the H_2O_2 produced (Das & Roychoudry, 2014).



1.5.2.3. Ascorbate Peroxidase

Ascorbate peroxidase (APX) is a fundamental component of the Ascorbate-Glutathione (ASC-GSH) cycle and catalyses the same reaction as CAT. However, CAT primarily scavenges H_2O_2 among other compounds in peroxisomes, while APX scavenges H_2O_2 in both the cytosol as well as the chloroplast. APX reduces H_2O_2 to H_2O and Dehydroascorbate (DHA), by making use of ascorbic acid (AA) as the reducing agent (Shigeoka, et al., 2002). The APX family consist of five currently observed isoforms which are characterized based on their amino acid sequences and locations such as plastid (consisting of the stromal and thylakoidal), peroxisomal, mitochondrial as well as cytosolic (Caverzan, et al., 2012). Compared to CAT, APX is better at scavenging H_2O_2 under stressful conditions as it is distributed more widely and has a higher affinity for H_2O_2 . The APX-catalysed conversion of H_2O_2 to H_2O can be represented as the following reaction:



1.6. Glucosinolates

Glucosinolates are sulphur- and nitrogen-containing secondary metabolites produced by members of the *Brassicaceae* family. Members of the *Brassica* family include vegetables such as Brussels sprouts, cauliflower, broccoli and cabbage. The biosynthesis of glucosinolates is carried out in three phases namely (1) the elongation of an amino acid chain, during which additional methylene groups are introduced into the side chain, (2) the amino acid moiety is converted into the glucosinolates core structure, and (3) the later modification of the side chain (Figure 4).

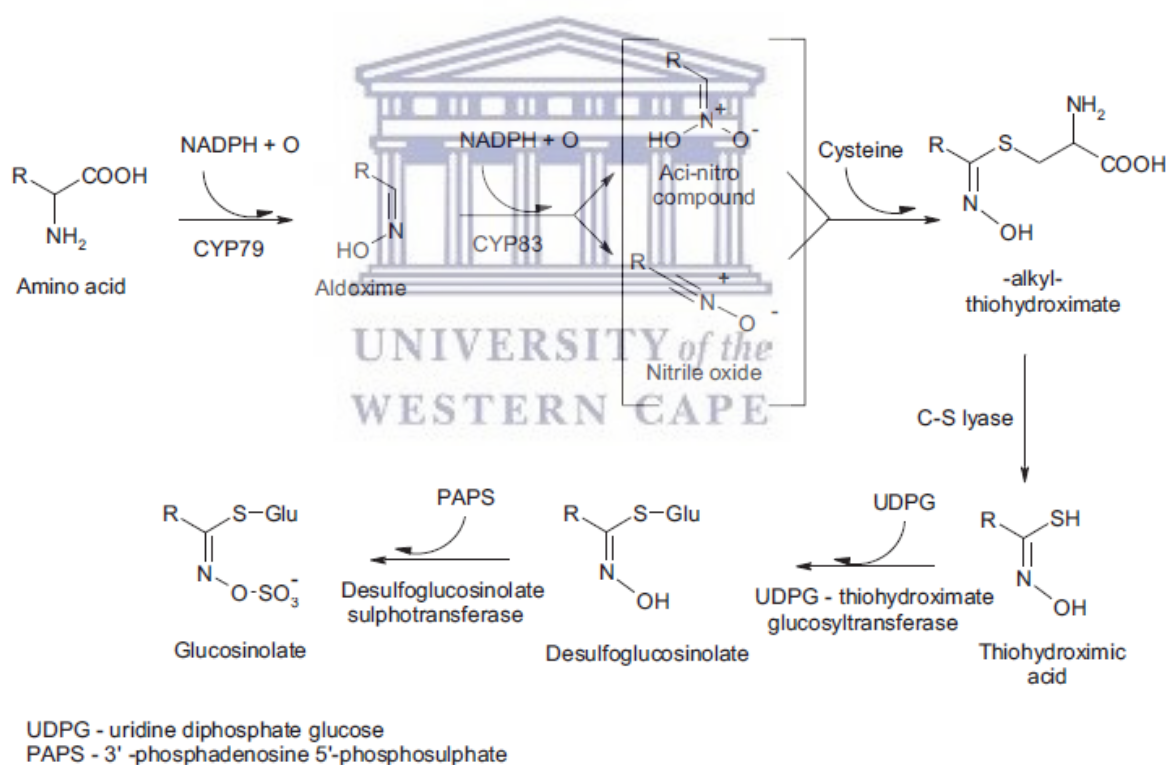


Figure 4: Represents the biosynthesis of the glucosinolate core structure. Biosynthesis starts with the conversion of an amino acid precursor to aldoximes by enzymes belonging to the Cytochrome P450 family such N-hydroxylating multifunctional cytochrome P-450 (CYP79). A cytochrome P450 monooxygenase (CYP83) then oxidizes the aromatic and aliphatic aldoximes to form an unstable aci-nitro intermediate. This intermediate then conjugates with the thiol group of cysteine via the α -carbon atom to form S-alkylthiohydroximate. C-S lyase then cleaves the C-S bonds to form thiohydroxamic acid. The enzymes uridine diphosphate thiohydroximate glucosyltransferase (UDPG) and 3'-phosphoadenosine 5'-phosphosulfate: desulfoglucosinolate sulfotransferase (PAPS) carry out

the glycosylation of thiohydroxamic acid to form the core glucosinolate structure. Abbreviations NADPH, Nicotinamide adenine dinucleotide phosphate; C-S lyase. Cysteine lyase; UDPG, uridine diphosphate thiohydroximate glucosyltransferase; PAPS, 3'-phosphoadenosine 5'-phosphosulfate Figure adapted from Redovniković, et al., (2008)

Additionally, most plants that produce and store glucosinolates also possess a β -thioglucosidase known as myrosinase, which hydrolyses the glucosinolates to form various compounds with a wide range of activities. This hydrolysis however only happens when plant tissues are disrupted, most likely due to wounding from either insect or herbivore attack. When insects or herbivores prey on these plants they damage the cell walls of myrosin cells, releasing myrosinase (β -thioglucosidase). These conditions cause the enzyme and the substrate to come into contact, resulting in the hydrolysis of the glucosinolates to form compounds such as Indole-3 carbinol (I3C) and other isothiocyanates. Isothiocyanates are compounds which exhibit a degree of toxicity and serves as a deterrent to prevent insects and herbivores from further preying upon the plant. This hydrolysis reaction will not always yield the same products as the chemical nature of the products produced by this reaction influenced by a variety of factors. These include the side chain present on the glucosinolate molecule, the plant species as well as conditions such as pH, under which the reaction is carried out. In some cases, the products produced from this hydrolysis reaction conjugate to form new products such as 3,3-diindolylmethane (DIM).

1.6.1. 3, 3-Diindolylmethane (DIM)

As previously mentioned, the enzymatic hydrolysis of glucosinolates by myrosinase yields indole-3-carbinol (I3C). Under appropriate conditions (mostly acidic), this indole compound can undergo self-condensation, resulting in the formation of DIM (Busbee, et al., 2015). DIM has gained a considerable amount of interest in the medical sector due to its anticancer activity where cancer cells

treated with DIM showed an arrest of the G1 phase, thereby preventing the further proliferation of the cancerous cell (Vivar, et al., 2009). Furthermore, indole (I3C) or polymers of indole (DIM) products have been shown to have radical scavenging ability (Arnao, et al., 1996). DIM has been shown to improve both germination and growth of *Oryza sativa* (Pal, et al., 2007). Furthermore, a study done by Gokul et al. (2016) showed that DIM increased both the germination and growth of *Brassica napus* potentially through the modulation of reactive oxygen species signalling.

1.7. Justification

Currently the world is facing a major climate change. This will have a large impact on agriculture, challenging the ability to produce sufficient crops under the ever-changing environment. These conditions will vary based on the geographical location but nonetheless have the potential to compromise food security. Drought is one of the major environmental factors that limit crop production. Due to the negative impacts of drought, it is imperative to investigate ways of mitigating the damage drought induces on crop plants and in so doing improve food security. For the purpose of this study, focus will solely be on the water deprivation aspect, whereby it will investigate the exogenous application of DIM and the effects thereof on water deficit responses in maize. This study will aim at determining if DIM can mitigate the effects of water deprivation by inducing stress activated pathways in plants outside of the *Brassicaceae* family, improving maize drought stress responses, thereby enhancing maize tolerance to drought. Enhanced tolerance to drought would sustain crop yield, resulting in the maintenance of food security, which would benefit the country.

1.8. Objectives of the study

This study focuses on investigating whether micromolar amounts of DIM can salvage *Zea mays* seedlings from water deprivation stress as well as its physiological and biochemical effects. In doing this project, the study will identify the strain water deprivation puts on plants as well as the mechanisms induced by DIM to improve tolerance to drought. The aim of this study is to firstly investigate whether DIM affects the water status of *Zea mays* seedlings. Additionally, it will also investigate how plants respond to water-deprivation and DIM treatments in terms of proline accumulation. ROS accumulation as well as the damage to lipid membrane as a result of ROS will also be investigated. Lastly, ROS-scavenging mechanisms such as antioxidant enzyme activities will also be investigated to understand how DIM would affect them. The knowledge gained from this study will shed light on the activity of DIM in plants such as *Zea mays* that lack the metabolic machinery to produce it as well as identifying whether DIM maintains its protective effects in plants other than *Brassica napus*.



UNIVERSITY of the
WESTERN CAPE

Chapter 2

METHODS

Table 2.1: List of chemicals and suppliers

2- Thiobarbituric acid (TBA)	Sigma-Aldrich
3,3'-Diindolylmethane (DIM)	Sigma-Aldrich
4-Nitro blue tetrazolium chloride (NBT)	Sigma-Aldrich
5-sulfosalicyclic acid dihydrate	Sigma-Aldrich
Acetone	Sigma-Aldrich
Aquarium air pump (ASP100)	Tetra
Bovine serum albumin (BSA)	Sigma-Aldrich
Bradford reagent	Sigma-Aldrich
Calcium sulphate dihydrate	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Ferric chloride	Sigma-Aldrich
Glacial Acetic acid	Merck
Glycine 99 %	Sigma-Aldrich
Hydrogen peroxide	Sigma-Aldrich
L-Ascorbic acid	Sigma-Aldrich
L-Proline	Merck
Ninhydrin	Merck
N, N, N', N'-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Polyacrylamide 40%	Sigma-Aldrich
Polyvinylpolypyrrolidone (PVPP)	Sigma-Aldrich
Potassium ferric cyanide	Sigma-Aldrich
Potassium phosphate dibasic	Merck
Potassium phosphate monobasic	Merck
Promix	Windell Hydroponics
Trichloroacetic acid 99%	Sigma-Aldrich
Tris (hydroxymethyl	Sigma-Aldrich
TWEEN 80	Sigma-Aldrich

Table 2.2: List of equipment

Instruments	Model	Company
Centrifuge	5415D	Eppendorf
Gel electrophoresis tank	Mini-Protean Tetra Cell	BIO RAD
Freeze drier	Freezone Plus 2.5 Litre	Labconco
Heating block	Block heater	Stuart Scientific
Mass balance	AS 220R2	Radwag
pH meter	pH 8 + DHS-S/N	ACCSEN
Power supply	MP 250V	Cleaver Scientific
Shaker	Mini orbital shaker (SSM1)	Stuart Scientific
Spectrophotometer (Microtitre plate reader)	POLARstar	Omega



2.1. Preparation of DIM solution

A stock solution of 2 mM DIM [sigma; $\geq 98\%$ (HPLC)] was prepared by dissolving 10 mg of DIM in 235 μl Tween 80 (sigma; viscous liquid, cell culture tested). The solution was then made up to 10 ml using 99.8 % (v/v) ethanol [sigma; absolute, (GC)], snap-frozen in liquid nitrogen followed by freeze drying at -41°C overnight using a Labconco benchtop freeze drier (FreeZone 1). Deionised water (19.77 ml) was then added to the DIM resulting in a 2 mM stock solution [containing 1.18% (v/v) Tween 80] as described by (Gokul, et al., 2016). The preparation of the control was done in the same manner as mentioned for DIM preparation with the absence of the DIM powder (Tween 80 final concentration of 1.18%).

2.2. Plant growth and treatments

Plant growth media (Promix[®] Organic; Windell Hydroponics, Cape Town, South Africa) was used for plant growth experiments. The media was prepared by drying at 80°C until the growth media was completely dry, followed by the addition of 1% nutrient pellets [Talborne organics, South Africa]. A calculated volume of water [200% field capacity] was added to the growth media to simulate well-watered conditions. Water-deprived treatments received 10% of the volume applied in well-watered conditions.

Zea Mays (Nelsons choice) seeds were surface-sterilized in 0.35% (v/v) sodium hypochlorite (Sigma-Aldrich, St Louis, MO, USA) for 10 minutes. After sterilization seeds were thoroughly washed with sterile distilled water to remove the sodium hypochlorite. Seeds were then primed by immersion in 10 mM CaSO₄ (Sigma-Aldrich) solution for 16 hours under continuous aeration using an aquarium air pump (Tetra ASP100). This was done as halopriming has been shown to improve the rate of germination (Abbasdokht, et al., 2010). Thereafter, seeds were allowed to germinate between two sheets of moist paper towel in a plastic container. Germination proceeded in the absence of light at room temperature and was monitored regularly. Seeds with a radicle of approximately 1 cm were then transferred to pots containing the plant growth media described previously.

To execute plant growth experiments, 20 cm diameter pots containing the well-watered plant growth media was used. Plants were grown under 25/19°C day/night temperature cycle with a 16/8 h light/dark cycle, at a photosynthetic photon flux density of 300 mol photons.m⁻²s⁻¹ (during the day phase), in a randomized design. Plants were watered with 200 ml of water every 72 hours. Seedlings were grown under well-watered conditions until the V2 stage was

reached. Treatment was initiated at the V2 stage which consisted of: well-watered, 15 μM DIM, water-deprived and water-deprivation in combination with 15 μM DIM. Well-watered and DIM plants were treated twice a week with 200 ml of 0.009% (v/v) Tween 80 and 15 μM DIM containing 0.009% (v/v) Tween 80 respectively. Water-deprived and water-deprived DIM seedlings were treated with 20 ml of 0.009% (v/v) Tween 80 and 15 μM DIM containing 0.009% (v/v) Tween 80 respectively once a week for 21 days. After 21 days plants were harvested whereby 5 cm cuttings of the second youngest leaves from all 4 independent experiments was obtained to be used for determining the relative water content. The rest of the remaining plant material (leaves and roots) was harvested separately, snap-frozen in liquid nitrogen and ground up using a mortar and pestle before being stored at -80°C .



2.3 Leaf relative water content (RWC)

Relative water content was determined using a modified method of Hellmuth (1972). RWC was determined for well-watered, DIM, water-deprived, water-deprived and DIM treatments using the 2nd youngest leaf. A 5 cm section was cut from the tip of each leaf using a pair of scissors. The fresh weight (FW) was then determined by weighing the cuttings. The leaves were then placed in 15 ml tubes and fully submerged in distilled H_2O and incubated for 4 hours in order to allow water uptake to full turgidity. The leaves were then blotted with paper towel to remove excess surface water and weighed to determine the turgid weight (TW). Thereafter leaves were dried in an oven at 80°C for 48 hours, and then weighed to determine their dry weights (DW). This was all done in triplicate. After obtaining the FW, TW and DW, the RWC was then calculated using the formula below (Barr & Weatherley, 1962).

$$RWC = \{(FW - DW)/(TW - DW)\} * 100$$

2.4. Determination of “chlorophyll a and b” in *Zea mays* leaves

In order to determine changes in chlorophyll content, a modified method of (Oancea, et al., 2005) was followed. This assay was done for three independent experiments. Frozen leaf material (100 mg) was weighed out in a 1.5 ml Eppendorf tube. To prevent the degradation of the chlorophyll, tubes were wrapped in foil. A 10X volume of 100 % (v/v) acetone was added to the plant material and vortexed briefly. The mixed samples were then centrifuged at 13 000 rpm for 5 minutes and the supernatant added to a glass-coated microtiter plate. This was then read on a spectrophotometer at the wavelengths 662 nm and 644 nm. The concentrations of the different chlorophyll species concentrations were then determined using the absorbance readings in conjunction with the calculation:

$$\text{Chl a} = (9.784 * E_{662} - 0.99 * E_{644}) * V / m$$

$$\text{Chl b} = (21.462 * E_{644} - 4.65 * E_{662}) * V / m$$

Where E_{662} and E_{644} , denote the absorbance, V is the volume of the solvent and m is the tissue mass in grams.

2.5. Trichloroacetic (TCA) acid extraction

To obtain protein-free extracts to be used in determining malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), a trichloroacetic acid (TCA) extraction was performed using a modified method from Velikova et al., (2000). Frozen plant material (100 mg) was added to 2 ml tubes. A volume of (5 times) of 6 % (w/v) trichloroacetic acid (TCA) was added to the tubes. The samples were

homogenised using a vortexer followed by centrifugation at 13000 x g for 10 minutes to pellet the plant material. The supernatant was then transferred to sterile tubes to be used for subsequent experiments.

2.5.1. Determination of Lipid peroxidation in *Zea mays* leaves and roots

In order to determine the level of lipid peroxidation, a modified MDA assay was performed. A volume of 200 μ l of TCA extract was transferred to a sterile 2 ml tubes, to these tubes 400 μ l of 0.5 % 2-thiobarbituric acid (w/v) prepared in 20 % TCA was added. The homogenate was then vortexed briefly before being incubated for 30 min at 95°C and thereafter incubated on ice for 10 min. Following the incubation on ice, samples were then centrifuged at 13000 x g for 5 minutes. Thereafter, 200 μ l of supernatant was loaded on a 96-well microtiter plate and read at 532 nm and 600 nm on a spectrophotometer to determine the presence of thiobarbituric acid reactive substances (TBRAS), which is a reflective of MDA. The absorbance at 600 nm was subtracted from the absorbance at 532. The extinction co-efficient of 155 mM.cm⁻¹ was used to determine MDA concentrations.

2.5.2. Determination of H₂O₂ in *Zea mays* leaves and roots

Hydrogen peroxide (H₂O₂) content was determined by homogenising 50 μ l of TCA extract with 150 μ l of reaction buffer which contained 5 mM dipotassium hydrogen phosphate (K₂HPO₄), pH 5.0 and 0.5 M potassium iodide (KI). Thereafter samples were incubated at 25°C for 20 min. A H₂O₂ standard ranging from 0 nM, 500 nM, 1000 nM, 1500 nM, 2000 nM to 2500 nM were prepared by diluting H₂O₂ in deionised water. Following the incubation 200 μ l of both

samples and standard curve was loaded in triplicate and read at an absorbance of 390 nm on a spectrophotometer. Hydrogen peroxide content was then calculated using a standard curve constructed with the absorbance of H₂O₂ standards read at an absorbance of 390 nm.

2.6. Determination of Proline content in *Zea mays* leaves and roots

A modified method of Chen et al., (2015) was used to determine proline content. A reaction solution consisting of 0.75 % sulphosalicylic acid, 25 % acetic acid and 1.25 % acid-ninhydrin was prepared. 1 ml of the reaction solution was added to a 2 ml tube to which 50 µl of crude protein extract (in section 2.7) was added and mixed by vortexing. Following mixing, samples were boiled at 95°C in a water bath for 15 minutes and then cooled down on ice for 5 minutes. Proline standards ranging from 0 µg, 5 µg, 10 µg, 15 µg, 20 µg, 25 µg, 30 µg were prepared by diluting L-proline in deionised water. Standards and samples were then loaded on a 96-well plate in triplicate and read on a spectrophotometer at 520 nm. Proline content was then calculated using a standard curve constructed with the absorbance of L-proline standards and normalized to protein content in the sample with the unit µg/mg of protein.

2.7. Protein Extraction

Protein was extracted from both the leaves and roots by homogenizing 100 mg of plant tissue (in section 2.2) 0.5 ml of protein extraction buffer was containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 5% (w/v) Polyvinylpolypyrrolidone (PVPP) dissolved in 40 mM potassium phosphate buffer (KPO₄), pH 7.4]. The sample was mixed by vortexing and the homogenate was then pelleted by centrifugation at 13000 x g for 30 minutes. Thereafter, the

supernatant was carefully transferred to a new sterile tube to be used for subsequent experiments whereas the pellet was discarded. The protein concentration was then quantified using the Bradford assay. Bradford reagent was purchased from Sigma-Aldrich and assay was done as per manufacturer's instruction using bovine serum albumin as a standard. Protein samples were stored at -20 °C for future experiments.

2.8. Ascorbate peroxidase (APX) activity

Ascorbate peroxidase (APX) activity was determined using in-gel activity-based staining. Protein extract (50 µg) (in section 2.7) was loaded on a non-denaturing 10 % polyacrylamide gel and then subjected to native polyacrylamide gel electrophoresis (PAGE) at 4°C. The gels were run in an APX running buffer consisting of [192 mM glycine, 25 mM Tris (hydroxymethyl) aminomethane (Trismabase) and 2 mM L-Ascorbic acid]. Following electrophoresis, gels were equilibrated in 50 mM potassium phosphate buffer (KPO₄, pH 7) containing 2 mM L-Ascorbic acid for 20 minutes. Thereafter a second incubation was carried out in 50 mM KPO₄ (pH 7.8) buffer containing 4 mM L-Ascorbic acid and 2 mM H₂O₂ for 20 minutes, followed by an incubation in 50 mM KPO₄ (pH 7.8) for 1 minute. The gel was then stained with a solution containing of 50 mM KPO₄ (pH 7.8), 28 mM (N, N, N', N'-Tetramethylethylenediamine) TEMED and 0.5 mM 4-Nitro blue tetrazolium chloride (NBT) for 20 min in the absence of light. After the incubation, gels were exposed to light and APX activity was detected by the formation of achromatic bands against the darker purple background. At this point the reaction was stopped by the removal of the staining solution and addition of deionized water. Additionally, pixel intensity ratio was also determined using the Alpha Ease FC Software to improve the analysis of APX isoforms. This was done by comparing the intensity of the achromatic bands

against the darker purple background.

2.9. Catalase Activity (CAT) activity

Catalase (CAT) activity was determined using in-gel activity staining. The method entailed using a non-denaturing 7.5% polyacrylamide gel, subjected to native polyacrylamide gel electrophoresis (PAGE) at 4°C. Protein extracts (200 µg) (in section 2.7) were loaded on the gel and run in a running buffer containing [192 mM glycine, 25 mM Tris (hydroxymethyl) aminomethane (Trismabase)]. Following electrophoresis gels were incubated in 0.003 % H₂O₂ for 20 minutes in the absence of light. Thereafter gels were stained with 2 % ferric chloride and 2 % potassium ferric cyanide simultaneously and exposed to light. CAT activity was detected by the formation of achromatic bands against a blue/green background. The reaction was then stopped by the removal of the staining solution and addition of deionized water. Pixel intensity ratio was also determined using the Alpha Ease FC Software, to improve the analysis of CAT isoforms. This was done by comparing the intensity of the achromatic bands against the darker purple background.

2.10. Statistical analysis

All data statistically validated using One –way analysis of variance (ANOVA) using GraphPad Prism 6.01 software. The Tukey-Kramer test at 5% level of significance was done to compare the means.

Chapter 3

RESULTS

3.1. Exogenous DIM improves physiological responses of *Zea mays* seedlings under water deprivation.

3.1.1. Water deprivation decreases leaf relative water content (RWC), however plants supplemented with DIM supplementation maintain their RWC under water-deprived conditions.

The RWC in leaves of well-watered maize seedlings was approximately 92% whereas in the leaves of maize seedlings deprived of water the RWC was approximately 47%, hence under water deprived conditions a decrease of approximately 45% in the RWC of leaves was observed compared to well-watered conditions (Figure 3.3.1). DIM-treated seedlings had a RWC of 98% whereas water-deprived seedlings treated with DIM had a RWC of 82%. A 35% increase in RWC was observed when comparing water-deprived and DIM to solely water-deprived seedlings.

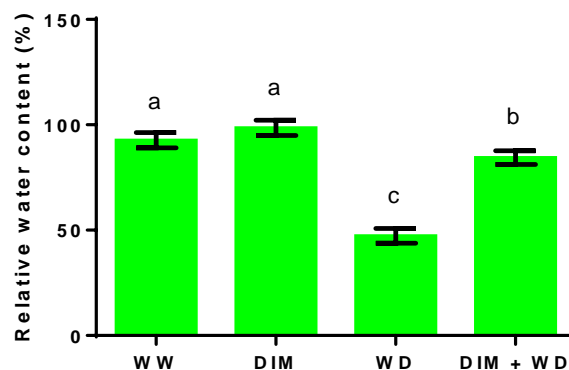


Figure 3.1.1: The effect of water deprivation on the water status of seedlings in presence and absence of DIM. Relative water content (RWC) of the 2nd youngest of maize seedlings grown under well-water and water deficit conditions in the presence and absence of 15 μ M DIM. Data presented

are means (\pm SE) of three independent experiments (n=3). Error bars denote standard deviation, where bars with the same letters are statistically similar and varying letters indicated statistical differences where $P < 0.05$. Abbreviations in the figure are as follows: WW (Well-watered); DIM (15 μ M DIM treatment); WD (water-deprived); DIM + WD (15 μ M DIM and water-deprivation).

3.1.2. Drought decreases chlorophyll a/ b Chl (a/b) ratio *but* when supplemented with DIM reduction is minimized.

Drought has been shown to negatively impact photosynthesis by altering the chlorophyll content, damaging the photosynthetic machinery as well as the chlorophyll components (Jaleel, et al., 2009). Hence, chlorophyll content was determined to assess the physiological state of water-deprived *Zea mays*. In this study, water deprivation resulted in a decrease of $\sim 26\%$ in Chlorophyll a (Chl a) and a $\sim 93\%$ increase in Chlorophyll b (Chl b). Water-deprived plants treated with DIM only had an 11% decrease in Chl a and 48% increase in Chl b. DIM treatment resulted in an increased of $\sim 47\%$ in Chl b when compared to the well-water plants, no significant changes was observed in Chl a. Furthermore, water-deprivation resulted in a decrease of $\sim 63\%$ in the Chl (a/b) ratio compared to the well-watered plants. DIM treatments resulted in a $\sim 33\%$ reduction in the Chl (a/b) ratio, statistically similar to water-deprived with DIM, when compared to the well-water plants. Additionally, DIM, water-deprived and DIM with water-deprived treatments resulted in an increase of $\sim 19\%$, $\sim 24\%$ and $\sim 12\%$ respectively when compared to well-watered plants (Table 3.1.2).

Table 3.1.2: The effect of DIM, water deprivation and their combination on plant chlorophyll ($\mu\text{g}\cdot\text{g}^{-1}$) a and b.

	Chl a	Chl b	Chl a+b	Chl (a/b) ratio
WW	0,309 \pm 0,011a	0,225 \pm 0,011a	0,534 \pm 0,011a	1,383 \pm 0,011a
DIM	0,307 \pm 0,009a	0,332 \pm 0,011b	0,639 \pm 0,011b	0,925 \pm 0,011b
WD	0,228 \pm 0,009b	0,435 \pm 0,011c	0,663 \pm 0,011b	0,524 \pm 0,011c
DIM + WD	0,274 \pm 0,010c	0,333 \pm 0,011b	0,607 \pm 0,011c	0,829 \pm 0,011b

Different letters indicate significant differences between means at $P < 0.05$. Values means \pm S.E (N=4). Abbreviations in the figure are as follows: WW (Well-watered); DIM (15 μ M DIM treatment); WD (water-deprived); DIM + WD (15 μ M DIM and water-deprivation).

3.2. The effects of water-deprivation and DIM on proline accumulation.

Plants accumulate osmolytes such as proline during abiotic stress in order to adjust the osmotic potential, maintain cellular redox state as well as turgidity. Hence, measuring proline accumulation was used as a biochemical indicator of stress. According to Figure 6 (A) proline accumulation in leaves which were water-deprived resulted in an increase of $\sim 44\%$ when compared to well-watered leaves, with the supplementation of DIM the water-deprived maize a similar observation was made. It was observed in Figure 6 (B), The roots which were water-deprived had a major increase of $\sim 216\%$ when compared to well-watered roots. However, water deprived roots supplemented with DIM had an increase of $\sim 160\%$ when compared to the well-watered roots.

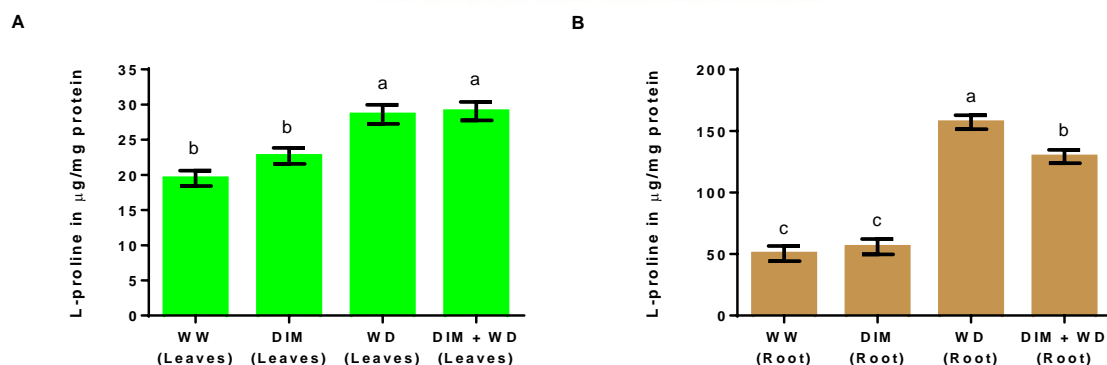


Figure 3.2: The effect of water deprivation on the proline accumulation of seedlings in presence and absence of DIM. Proline content of the leaves (A) and roots (B) of maize seedlings grown under well-water and water deficit conditions in the presence and absence of 15 μ M DIM. Data presented are means (\pm SE) of three independent experiments (n=3). Error bars denote standard deviation, where bars with the same letters are statistically similar and varying letters indicated statistical differences where $P < 0.05$. Abbreviations in the figure are as follows: WW (Well-watered); DIM (15 μ M DIM

treatment); WD (water-deprived); DIM + WD (15 μ M DIM and water-deprivation).

3.3. The effects of water-deprivation and DIM on H₂O₂ and MDA.

The accumulation of ROS is most often used as a biochemical indicator of stress due to the damage it inflicts on cellular structures such as the cell membrane as well as the oxidative effects on thiol groups present on enzymes, rendering them inactive. Of the various ROS species, we focused on hydrogen peroxide (H₂O₂) as it has one of the longest half-lives of all other ROS as well as the ability to diffuse across cellular membranes thereby causing a wider array of damage. Additionally, it is these exact same characteristics which make H₂O₂ such a suitable signalling molecule in plants. However, the signalling roles of H₂O₂ as with all other ROS work in a concentration-dependant manner. According to Figure 3.3 (A) an increase of $\sim 35\%$ was observed in leaves under water-deprived conditions when compared to well-watered leaves. Furthermore, water-deprived leaves supplemented with DIM had a $\sim 52\%$ decrease in H₂O₂ when compared to well-watered samples. No statistical differences were observed when comparing DIM treated leaves to well-watered leaves. Figure 3.3(B) illustrated the MDA content in leaves and a similar trend was observed across treatments, whereby water-deprivation showed a $\sim 85\%$ increase in MDA relative to the well-watered samples. However, water-deprived leaves supplemented with DIM statistically had the same level of lipid peroxidation as the well-watered. Figure 3.3(C) showed an overall increase in roots for DIM and water-deprivation. An increase of $\sim 26\%$ and $\sim 45\%$ in H₂O₂ was observed respectively. H₂O₂ content under water-deprivation and water-deprivation supplemented with DIM was statistically the same. Furthermore, it was observed in Figure 3.3 (D) that MDA content had an increase of $\sim 80\%$ under water-deprivation relative to well-watered roots. DIM treated roots were

statistically similar to well-watered roots and water-deprived roots were statistically similar to water-deprived supplemented with DIM roots in MDA content.

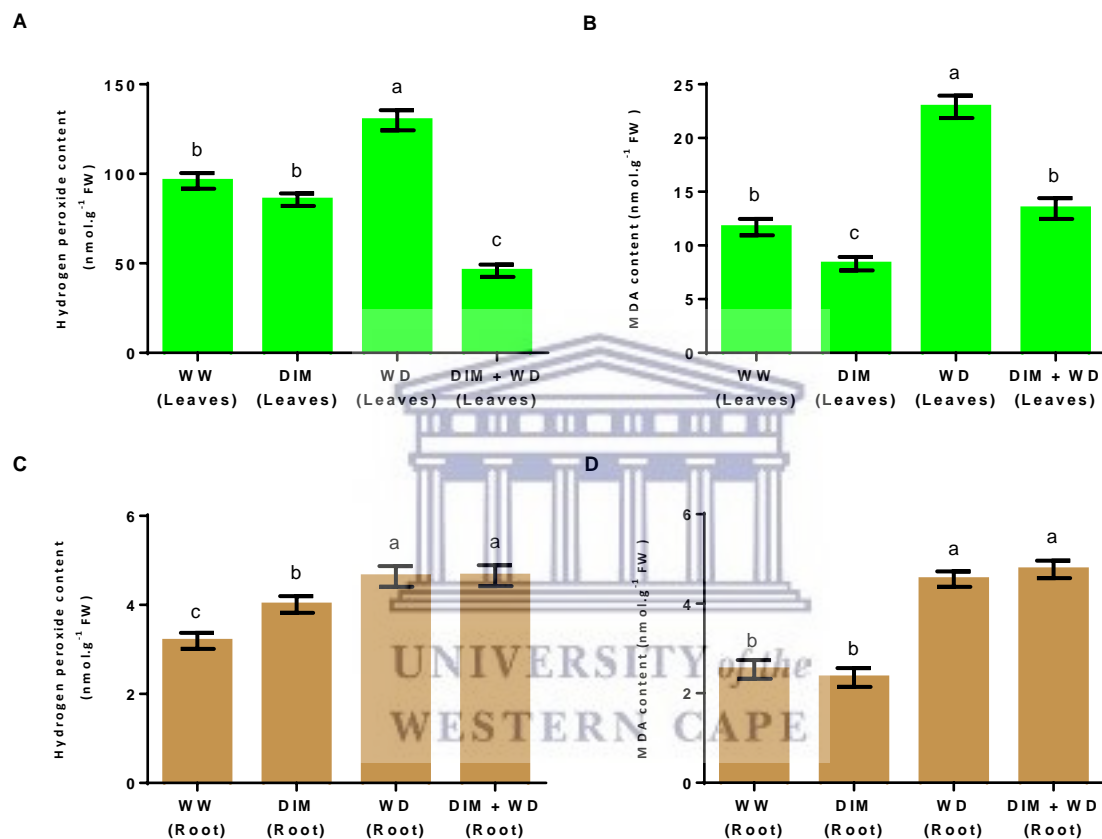
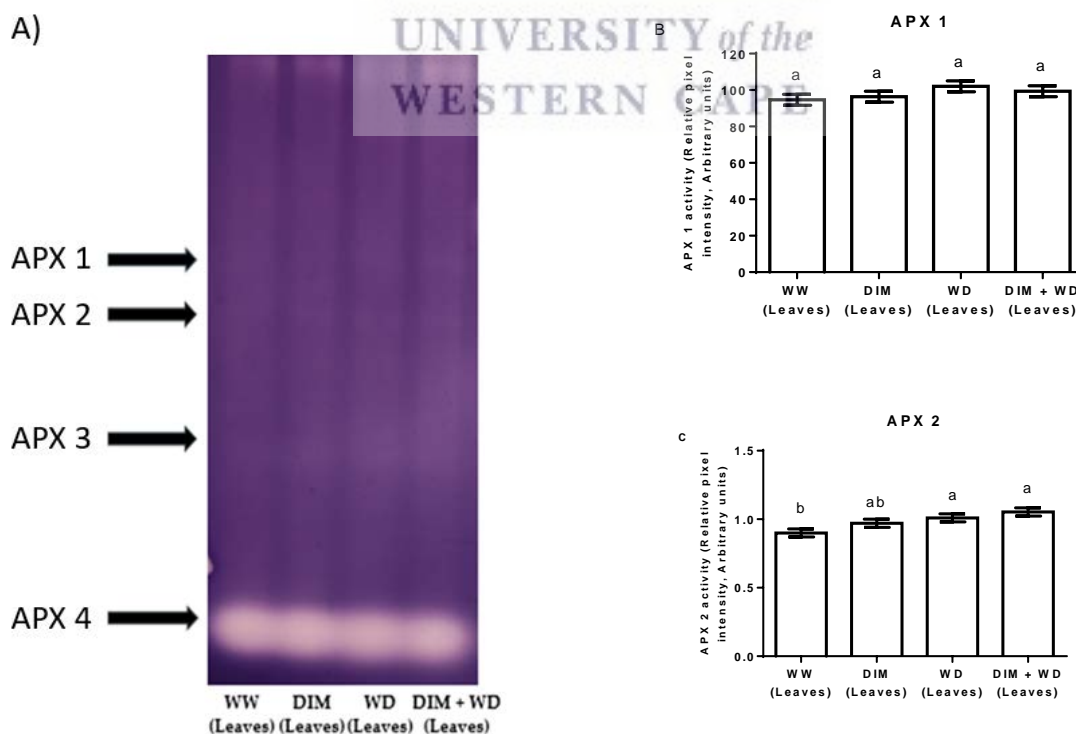


Figure 3.3: The effect of water deprivation on the Hydrogen peroxide and Malondialdehyde accumulation of seedlings in presence and absence of DIM. Hydrogen peroxide in leaves (A) and roots (C) and Malondialdehyde content in leaves (B) and roots (D). These contents were observed in maize seedlings grown under well-water and water deficit conditions in the presence and absence of $15\ \mu\text{M}$ DIM. Data presented are means ($\sim\text{SE}$) of three independent experiments ($n=3$). Error bars denote standard deviation, where bars with the same letters are statistically similar and varying letters indicated statistical differences where $P < 0.05$. Abbreviations in the figure are as follows: WW (Well-watered); DIM ($15\ \mu\text{M}$ DIM treatment); WD (water-deprived); DIM + WD ($15\ \mu\text{M}$ DIM and water-deprivation).

3.4. Ascorbate peroxidase (APX) activity is altered in *Zea mays* leaves and roots under water-deprivation and DIM.

APX is one of the antioxidant enzymes which scavenges H₂O₂. This enzyme reduces H₂O₂ to water using ascorbate as an electron donor. In this study four isoforms of APX were identified in leaves (Figure 3.4.1. A). There were no statistical differences observed for APX 1 in leaves across treatments (Figure 3.4.1. B). An increase of ~ 7 %, 11 %, and 15% was observed in APX 2 for DIM, water-deprived and water-deprived in combination with DIM respectively, when compared to well-watered leaves (Figure 3.4.1. C). Furthermore, an increase in of ~ 12 %, 21 % and 25 % was observed for APX 3 in DIM, water-deprived and water-deprived in combination with DIM respectively (Figure 3.4.1. D)). Additionally, an increase of ~ 13 %, 14 % and 11 % was observed for APX 4 in DIM, water-deprived and water-deprived in combination with DIM leaves respectively (Figure 3.4.1. E).



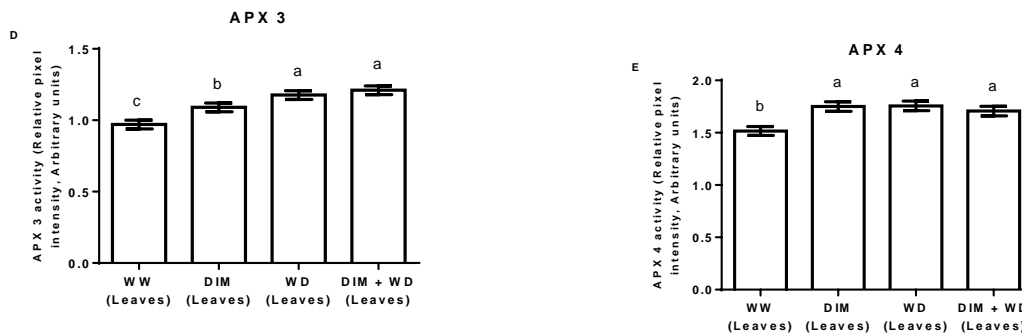


Figure 3.4.1: In-gel activity assays for APX activity in *Zea mays* leaves in response to water-deprived and DIM treatments. The in-gel activity assay of APX isoforms in response to the various treatments is represented in (A), from which pixel intensities of APX 1 (B) APX 2 (C), APX 3 (D) and APX 4 (E) were determined. Different letters indicate the differences between means at $p < 0.05$. Values are means \pm SE (N=3). Abbreviations in the figure are as follows: WW (Well-watered); DIM (15 μ M DIM treatment); WD (water-deprived); DIM + WD (15 μ M DIM and water-deprivation).

An overall increase in APX activity was observed in roots (Figure 3.4.2) across treatments when compared to the well-watered roots (A). Water-deprivation elicited the highest increase in activity of all APX isoforms. An increase of ~ 12 %, 21 % and 25 % was observed for APX 1 (B), 2 (C), 3 (D) respectively, when comparing water-deprived roots to well-watered. Furthermore, DIM treated roots showed an increase of ~ 17 %, 35 % and 9 % APX 1 (B), 2 (C), 3 (D) respectively, when compare to well-watered roots. Water-deprived roots supplement with DIM had the same activity as well-watered roots for APX 1 and 3.

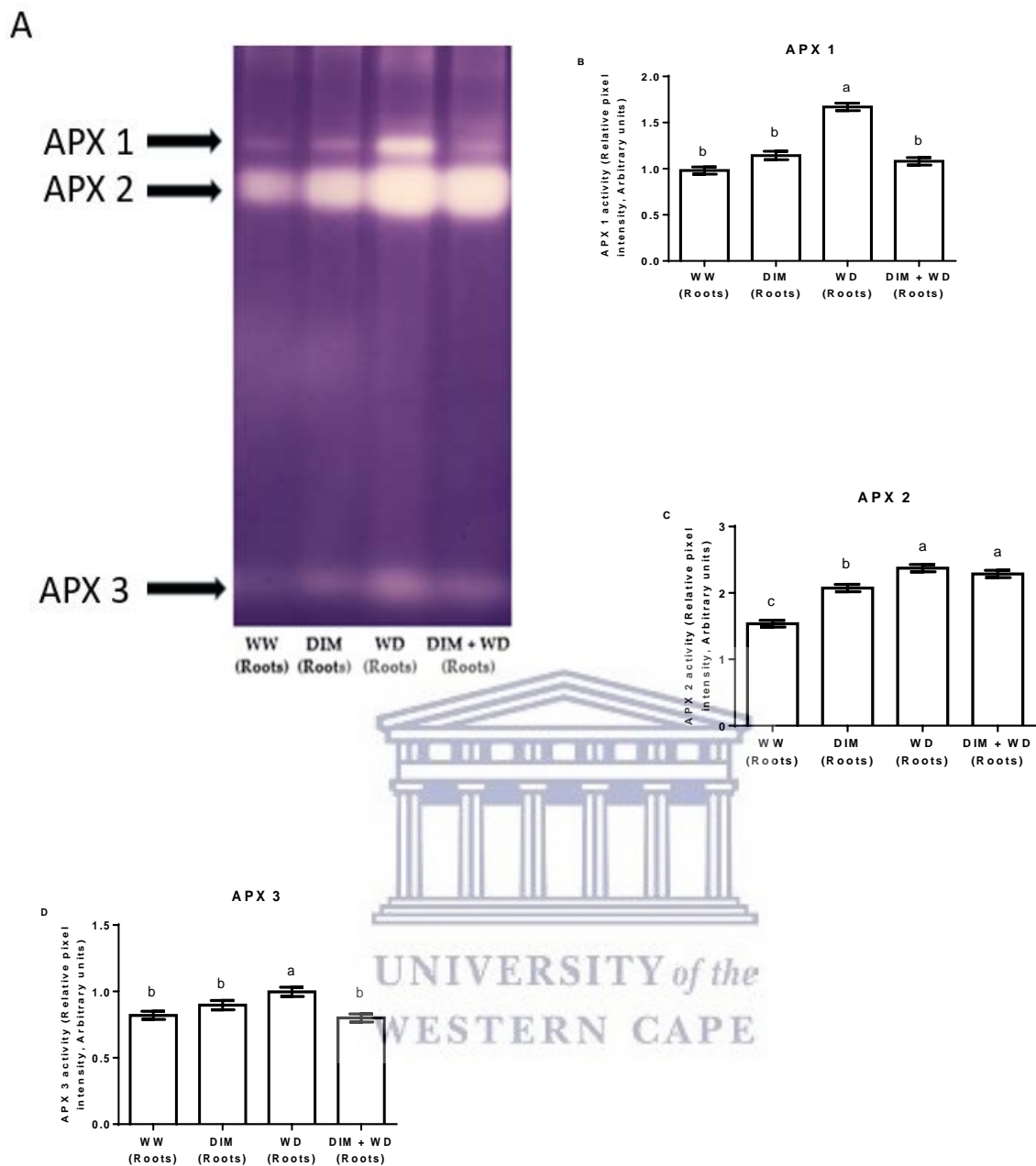


Figure 3.4.2: In-gel activity assays for APX activity in *Zea mays* roots in response to water-deprived and DIM treatments. The in-gel activity assay of APX isoforms in response to the various treatments is represented in (A), from which pixel intensities of APX 1 (B) APX 2 (C), APX 3 (D) and APX 4 (E) were determined. Different letters indicate the differences between means at $p < 0.05$. Values are means \pm SE (N=3). Abbreviations in the figure are as follows: WW (Well-watered); DIM (15 μ M DIM treatment); WD (water-deprived); DIM + WD (15 μ M DIM and water-deprivation).

3.5. Catalase (CAT) activity is altered in *Zea mays* leaves and roots under water-deprivation and DIM.

Catalase (CAT) is a tetrameric heme-containing enzyme that catalyses the conversion of H₂O₂ produced by SOD and other processes into water (H₂O) and oxygen O₂. In this study two CAT isoforms were identified in leaves (Figure 3.5.1 A). No statistical differences were observed in leaves treated with DIM for CAT 1 and 2 activity when compared to well-watered leaves. Under water-deprivation stress, a decrease of 32 % and 30 % was observed in CAT 1 and 2 respectively when compared to well-watered leaves (Figure 3.5.1 B and C). Furthermore, water-deprived leaves treated with DIM showed a 5 % and 13 % decrease in CAT 1 and 2 respectively (Figure 3.5.1 B and C).

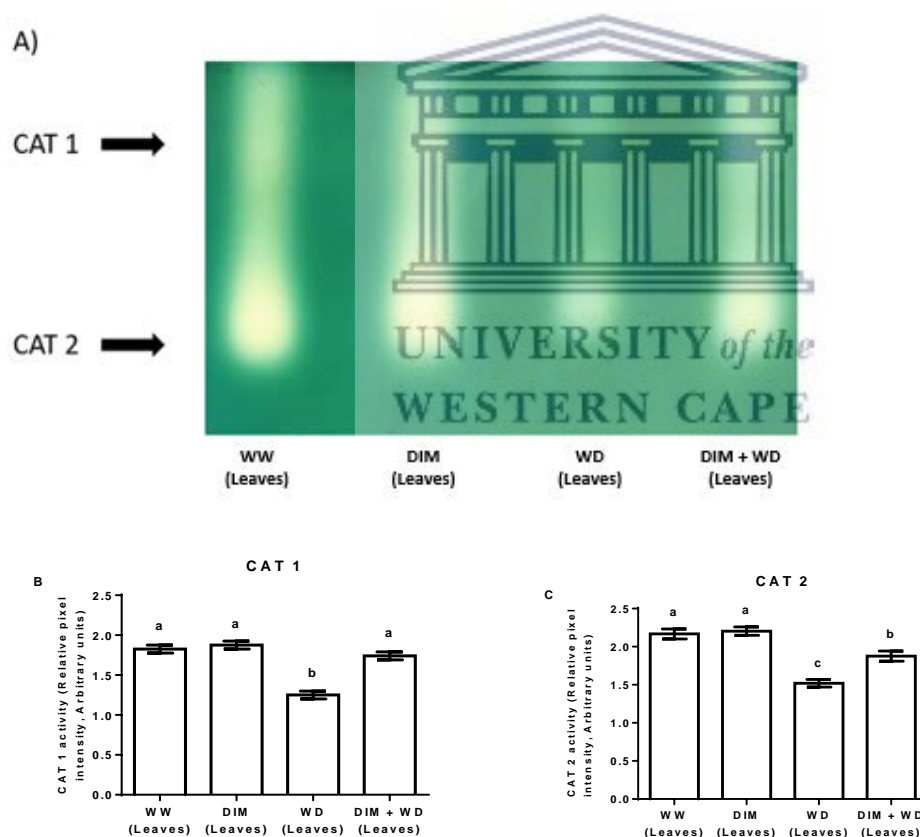
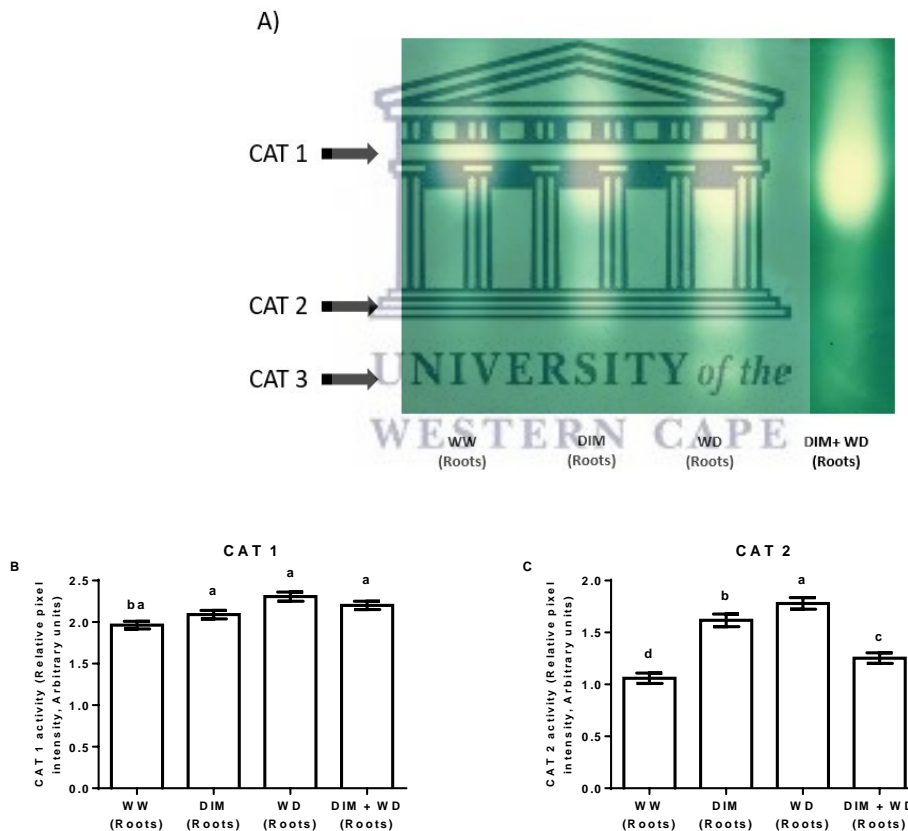


Figure 3.5.1: In-gel activity assays for CAT activity in *Zea mays* leaves in response to water-deprived and DIM treatments. The in-gel activity assay of CAT isoforms in response to the various treatments is represented in (A), from which pixel intensities of CAT 1 (B) CAT 2 (C), was determined. Different letters indicate the differences between means at $p < 0.05$. Values are means \pm SE (N=3). Abbreviations in the figure are as follows: WW (Well-watered); DIM (15 μ M DIM treatment); WD (water-deprived);

DIM + WD (15 μ M DIM and water-deprivation).

Three CAT isoforms were identified in *Zea mays* roots (Figure 3.5.2 A). DIM treated roots an increase of 6 %, 36 % and 16% was observed in CAT 1, 2, 3 respectively when compared to well-watered roots (Figure 3.5.2. B, C, D). Under water-deprivation stress an increase of 15 %, 49 % and 15% was observed in CAT 1, 2 and 3 respectively when compared to well-watered roots (Figure 3.5.2. B, C, D). Additionally, water-deprived roots treated with DIM showed an increase of 11 %, 15 %, and 23 % in CAT 1, 2 and 3 respectively when compared to well-watered roots (Figure 3.5.2. B, C, D)



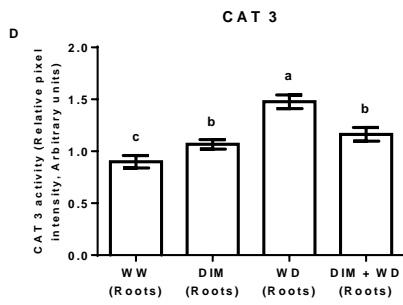


Figure 3.5.2: In-gel activity assays for CAT activity in *Zea mays* roots in response to water-deprived and DIM treatments. The in-gel activity assay of CAT isoforms in response to the various treatments is represented in (A), from which pixel intensities of CAT 1 (B) CAT 2 (C) and CAT 3 (D) was determined. Different letters indicate the differences between means at $p < 0.05$. Values are means \pm SE (N=3). Abbreviations in the figure are as follows: WW (Well-watered); DIM (15 μ M DIM treatment); WD (water-deprived); DIM + WD (15 μ M DIM and water-deprivation).



Chapter 4

DISCUSSION AND CONCLUSION

4.1 Exogenous DIM mitigates the reduction of leaf water status under water-deprivation

Plants grow through the process of cellular division, enlargement and differentiation. Drought or water deprivation reduces the turgor pressure inside cells. Therefore, determining leaf relative water content serves as a good indicator of the intensity of drought stress. In this study, water-deprivation significantly decreased the RWC relative to the well-water leaves. This is indicative of a reduction in osmotic potential in the plant due to a decrease in soil water potential as a result of drought (Lugojan & Ciulca, 2011). However, plants treated with DIM showed a smaller decrease than the water-deprived plants when compared well-water leaves. This suggests that DIM plays a role in the facilitation of osmotic regulation and therefore water-deprived plants treated with DIM had higher RWC than water-deprived plant without DIM. Additionally, these findings also suggest that DIM improved the plant's drought avoidance mechanisms. Drought avoidance is defined as the ability to maintain water content in higher tissues even though water content in the soil is reduced (Basu, et al., 2016). It has also been found that plants which are more tolerant are able to maintain RWC under osmotic stress. This is further supported by a study done by Ma and authors in 2018 where tolerant rice genotypes showed better RWC maintenance when compared to sensitive genotypes under salinity stress (Ma, et al., 2018).

4.2. Exogenous DIM improves maintenance of photosynthetic pigments under water-deprivation

Plants, like all organisms, require energy for growth and development. They produce this energy through the process of photosynthesis. During this process chlorophyll captures light to catalyse conversion of water and carbon dioxide to form nutritional organic substances and oxygen (Hoefnagels, 2017). In response to drought, plants close their stomata to avoid water loss from transpiration pathways. This results in a reduction in intercellular carbon dioxide concentrations leading to a decrease in photosynthetic activity (Anosheh, et al., 2016). To determine the impact of drought on photosynthetic activity total chlorophyll content was determined. In this study drought significantly, decreased chlorophyll a (Chl a) and increased chlorophyll b (Chl b). These findings agree with a study done by Guo et.al, whereby drought decreased Chl a species in *Lycium ruthenicum* Murr. seedlings (GUO, et al., 2016). Furthermore, a study done by Teixeira and authors in 2016, found that drought and heat resulted in the impairment of Chl b reductase in a susceptible soybean cultivar. Chl b reductase is involved in chlorophyll degradation and catalyses the conversion of Chl b to Chl a (Teixeira, et al., 2016). The disruption of Chl b reductase activity could prevent the formation of Chl a as well as preventing the degradation of Chl b. This could be the reason for the increase in Chl b under water-deprivation. Additionally, a reduction in the chlorophyll a/b ratio was also observed in water deprived leaves as a result of water deprivation. The decrease in Chl a/b ratio agree with a study done by Sharifi and Mohammadkhani in 2015 where drought treated maize showed a decrease in the Chl a/b ratio (Sharifi & Mohammadkhani, 2015). Water-deprived leaves treated with DIM had a less

significant decrease in Chl a and an increase in Chl b when compared to well water-watered leaves. This suggest that DIM played a role in the maintenance of photosynthetic pigments under water-deprivation stress. Furthermore, it also observed that water deprived leaves treated with DIM showed a smaller decrease in Chl a/b ratio when compared to well-watered leaves. This improvement could be as a result of DIM potentially improving the osmoregulatory responses in plants under water-deprivation stress. This idea is further supported by the fact that water deprived leaves treated with DIM were able to better retain their RWC.

4.3 Water-deprivation increases proline

As previously mentioned, drought causes a reduction in cellular turgidity. In order to minimize this reduction plants, accumulate compatible solutes as a form of adaptive response. This includes the accumulation of free proline, which adjusts the osmotic potential in the cytoplasm and its content can be used as a physiological marker or indicator of osmotic stress (Verslues, et al., 2006). In this study, water-deprivation significantly increased proline accumulation in leaves and roots. This correlates with the reduction in RWC observed in water-deprived leaves as proline plays an important role as an osmotic adjustor to maintain cellular turgidity. These findings agree with a study done by Tayebimeigooni et al., (2012) where an inversely proportional relationship was observed between RWC and proline content in salinity stressed Chinese kale (Tayebimeigooni, et al., 2012). Furthermore, water-deprived roots treated with DIM showed lower accumulation of proline than water-deprived roots without DIM. This suggest that these roots experienced lower levels of stress compared to their water-deprived counterparts. This is further supported by the fact that in water-deprived plants, DIM allowed for better water retention in water-deprived

leaves as well as well as maintaining the chlorophyll a/b ratio when compared to water-deprived solely.

4.4 DIM improves plant responses to water-deprivation stress by regulating ROS (H₂O₂)

In response to stresses, plants accumulate reactive oxygen species (ROS) which, when over-accumulated result in the plant suffering from oxidative stress. Although there are various ROS species, for the purpose of this study we focused on hydrogen peroxide (H₂O₂). At basal levels H₂O₂ serves as an important role as a signalling molecule due to its ability to diffuse across membranes. Additionally, H₂O₂ also has a longer half-life than the other ROS (Helena & de Carvalho, 2008). It was observed that water-deprivation caused a significant increase in H₂O₂ in leaves and roots (Figure 3.3 A and C) when compared to their respective well-watered counter parts. These findings agree with a study done by Moussa et.al in 2008, where both sensitive and tolerant maize cultivars showed an accumulation of H₂O₂ in response to drought stress (Moussa & Abdel-Aziz, 2008). There is a positive correlation between H₂O₂ accumulation and lipid peroxidation as the over accumulation of ROS as H₂O₂ often results in an increase in lipid peroxidation (Jajic, et al., 2015). It was also observed that water-deprived leaves treated with DIM showed a significant decrease in H₂O₂ accumulation when compared to water deprivation solely (Figure 3.3 A). This result agrees with the findings in Figure 3.1.1 as DIM improved water retention in water-deprived leaves and therefore these plants appear to experience less stress than their water-deprived counterparts. This notion is further supported by the fact that water deprived leaves treated with DIM experience less cellular damage, as indicated by a reduction lipid peroxidation, (Figure 3.3 B), than water-deprived plants without DIM. Additionally, although DIM treated roots

showed an increase in H₂O₂ accumulation (Figure 3.3 A) this did not result in an increase in lipid peroxidation (Figure 3.3 B). This suggest that DIM could be playing a role in H₂O₂ dependant signalling pathways. These include the pre-activation of H₂O₂ dependant antioxidants for an improved scavenging ability. A similar phenomenon was observed by Gokul and authors whereby DIM increased levels of H₂O₂ in *Brassica napus* seedlings without causing damage to membrane lipids (Gokul, et al., 2016).

4.5 DIM increases plant adaptive responses to water deprivation through the activation of antioxidant enzymes

To prevent damage as a result of oxidative stress induce by ROS plants activate ROS-scavenging mechanisms such as antioxidants. These antioxidants are meant to remove the otherwise toxic ROS and thereby maintain homeostasis. In this study we focused on H₂O₂ and therefore it would only be fitting to focus on ascorbate peroxidase (APX) and catalase (CAT) as they can scavenge this ROS molecule. APX is a fundamental component of the Ascorbate Glutathione (ASC-GSH) cycle and reduces H₂O₂ to H₂O and Dehydroascorbate (DHA). In this study an increase in APX activity was observed in water-deprived when leaves and roots compared to its well-watered counterparts (Figure 3.4.1 A and 3.4.2 A). This increase in APX activity is most-likely an effort to prevent oxidative damages as a result of H₂O₂ accumulation. Additionally, in roots a significant increase in APX activity was observed in DIM treated roots when compared to well-watered roots. Furthermore, water-deprived roots treated with DIM showed less APX activity when compared to water-deprived roots in absence of DIM. This suggest that DIM alleviated some of the stress and therefore less activity was required. Alternatively, this could also indicate that DIM primed the plant and activated

stress related pathways and as a result thereof these primed plants were better able to deal with the stress. The effects of exogenously applied compounds for priming has been highlighted in a review by Kissoudis and authors in 2014 (Kissoudis, et al., 2014). In a study done by Li, et al in 2014 exogenous spermidine (Spd) increased APX activity in *Trifolium repens* L.. The authors concluded that Spd played an important role as either a stress-protective compound or a physiological activator (Li, et al., 2014). Therefore, the DIM could be performing a similar function.

A reduction in catalase activity was observed in water-deprived leaves when compared to well-watered leaves. These results agree with a study done by Abedi and Pakniyat where water-deprivation resulted in a reduction in CAT activity in *Brassica napus* (Abedi & Pakniyat, 2010). The reduction of CAT activity could account for the increase in APX activity in water-deprived leaves (Figure 3.5.1 A) as both antioxidants scavenge H₂O₂. No significant differences were observed in DIM treated leaves when compared to well-watered leaves. Additionally, in roots a significant increase was observed in water-deprived roots when compared to well-watered roots. The increase in CAT is most-likely in response the increase in H₂O₂ (Figure 3.3 C). These findings agree with a study Guo and authors where drought caused an increase in CAT activity in roots *Lycium ruthenicum* (Guo, et al., 2018). It was also observed that water deprived roots treated with DIM exhibited a less significant increase in CAT activity when compared to water-deprived roots solely. This could be as a result of DIM increasing H₂O₂ activity (Figure 3.3 C) in roots which caused an increase in CAT activity (Figure 3.5.2 A). As a result of DIM priming the plant potentially through activation CAT via oxidative burst, when the stress occurred the plant was better prepared and therefore it was not required to increase CAT activity that much.

The effects of priming were highlighted in a study done by Ananieva and authors in 2003, whereby the exogenous application of salicylic acid increased the activity of antioxidant enzymes such as CAT in *Hordeum vulgare* L. (Ananieva, et al., 2003). In another study done by Latef & Tran in 2016, maize seeds primed with silicon showed and improved tolerance to alkaline stress. The authors concluded that priming with silicon enhanced leaf relative water content as well as the activity of antioxidant enzymes such as CAT (Latef & Tran, 2016).

4.6 Conclusion and Future work

This study aimed at investigating the physiological and biochemical effects of exogenous DIM on *Zea mays* under drought. To our knowledge this is the first study that investigates the effects of DIM not only in plants that lack the metabolic machinery to produce it, but also under water-deprivation stress. In the study it was observed that DIM improved the water status of water-deprived *Zea mays* seedlings under water-deprivation and improved the RWC almost to that of the well-watered seedlings. Additionally, it was also observed that water-deprivation decreased the “chlorophyll a and b” ratio but when supplemented with DIM chlorophyll the ratio improved. Suggesting that water-deprived plants supplemented with DIM would be able to better carry out photosynthesis than those without DIM. It was also observed Water deprived seedlings treated with DIM showed a decrease in lipid peroxidation. This is as a result of DIM allowing for better retention of water under water-deprivation and therefore these plants experienced more moderate stress. It is evident that DIM could serve as a priming agent as it increased CAT and APX activity prior to stress thereby inducing a basal defence allowing for better ability to cope with water-deprivation stress. The ability to improve plant adaptive responses to stress is

important in the development of more tolerant crops and based on the results above DIM could be a potential candidate in that process. It is also important to note that DIM appears to maintain its beneficial/ protective effects even in plants that lack the metabolic machinery to produce it. The future work from this study will investigate how DIM affects the other ROS-species (O_2^- and $\cdot OH$) as well as their respective antioxidants. We will also be looking at alteration in gene expression of these antioxidants as well looking at genes involved in both chlorophyll biosynthesis as well as degradation. Knowledge on DIM and its role in plants is still limited and this study aims at attempting to change that.



Chapter 5

REFERENCES

- Abbasdokht, H., Edalatpishhe, M. & Gholami, A., 2010. The Effect of Hydropriming and Halopriming on Germination and Early Growth Stage of Wheat (*Triticum aestivum* L.). *International Journal of Agricultural and Biosystems Engineering*, 4(8), pp. 551-555.
- Abedi, T. & Pakniyat, H., 2010. Antioxidant Enzyme Changes in Response to Drought Stress in Ten Cultivars of Oilseed Rape (*Brassica napus* L.). *Czech Journal of Genetics and Plant Breeding*, 46(1), pp. 27-34.
- AgriSA, 2016. "A raindrop in the drought" Report to the Multi-stakeholder task team on the drought: Agri SA's status report on the current drought crisis.
- Ananieva, E., Christov, K. & Popova, L., 2003. Exogenous treatment with Salicylic acid leads to increased antioxidant capacity in leaves of barley plants exposed to Paraquat. *Journal of Plant Physiology*, Volume 161, pp. 319-328.
- Anosheh, H., Moucheshi, S., Pakniyat, H. & Pessarakli, M., 2016. Stomatal responses to drought stress. In: P. Ahmad, ed. *Water Stress and Crop Plants Sustainable Approach*. Tuckson: John Wiley & Sons, pp. 24-40.
- Apel, K. & Hirt, H., 2004. Reactive Oxygen Species: Metabolism, Oxidative Stress and Signal Transduction. *Annual review of plant biology*, Volume 55, pp. 373-99.
- Arnao, M., Sanchez-Bravo, J. & Acosta, M., 1996. INDOLE-3-CARBINOL AS A SCAVENGER OF FREE RADICALS. *BIOCHEMISTRY and MOLECULAR BIOLOGY INTERNATIONAL*, 39(6), pp. 1125-1134.
- Ashraf, M., 2009. Biotechnological approach of improving plant salt tolerance using antioxidants as markers. *Biotechnology Advances*, 27(1), pp. 84-93.
- Basu, S., Ramegowda, V., Kumar, A. & A, P., 2016. Plant adaptation to drought stress. *F1000Research*, Volume 5, pp. 1-10.
- Busbee, P., Nagarkatti, M. & Nagarkatti, P., 2015. Natural Indoles, Indole-3-Carbinol (I3C) and 3,3'-Diindolylmethane (DIM), Attenuate Staphylococcal Enterotoxin B-Mediated Liver Injury by Downregulating miR-31 Expression and Promoting Caspase-2-Mediated Apoptosis. *PLOS ONE*, pp. 1-20.
- Caverzan, A. et al., 2012. Plant responses to stresses: Role of ascorbate peroxidase in the antioxidant protection. *Genetics and Molecular Biology*, 35(4), pp. 1011-1019.
- Coetzee, K., 2016. *Drought and its effects on the agricultural industry*. [Online] Available at: <https://www.farmersweekly.co.za/tax-and-management/global-farming/drought-and-its-effects-on-the-agricultural-industry/>

[Accessed 30 05 2018].

Conroy, J., Virgona, J. & Smillie, R., 1988. Influence of drought acclimation and CO₂ enrichment on osmotic adjustment and chlorophyll a fluorescence of sunflower during drought. *Plant Physiology*, Volume 86, pp. 1108-1115.

DAFF, 2016. *National Department of Agriculture*. [Online] [Accessed 15 May 2018].

Das, K. & Roychoudry, A., 2014. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *frontiers in Environmental Science*, Volume 2.

Davey, M. et al., 2005. High-throughput determination of malondialdehyde in plant tissues. *Analytical Biochemistry*, Volume 47, p. 201–207.

Del Rio, D., Stewart, A. & Pellegrini, N., 2005 . A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutrition, Metabolism, and Cardiovascular Diseases*, 15(4), pp. 316-28.

Edossa, D., Woyessa, Y. & Welderufael, W., 2016. Spatiotemporal analysis of droughts using self-calibrating Palmer's Drought Severity Index in the central region of South Africa. *Theoretical and Applied Climatology*, 126(3), p. 643–657 .

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

Gokul, A., Roode, E., Klein, A. & Keyser, M., 2016. Exogenous 3,3'-diindolylmethane increases Brassica napus L. seedling shoot growth through modulation of superoxide and hydrogen peroxide content. *Journal of Plant Physiology*, Volume 196-197, pp. 93-98.

GUO, Y. et al., 2016. Effects of drought stress on growth and chlorophyll fluorescence of Lycium ruthenicum Murr. seedlings. *PHOTOSYNTHETICA*, 55(4), pp. 524-532.

Guo, Y. et al., 2018. Effect of Drought Stress on Lipid Peroxidation, Osmotic Adjustment and Antioxidant Enzyme Activity of Leaves and Roots of Lycium ruthenicum Murr. Seedling. *Russian Journal of Plant Physiology* , 65(2), pp. 244-250.

Hancock, J., Desikan, R. & Neill, S., 2001. Role of reactive oxygen species in cell signalling pathways.. *Biochemical Society Transactions*, 29(2), pp. 345-350.

Helena, M. & de Carvalho, C., 2008. Drought stress and reactive oxygen species Production, scavenging and signaling. *Plant Signaling & Behavior*, 3(3), pp. 156-165.

Hellmuth, E., 1970. Measurement of Leaf Water Deficit with Particular Reference to the Whole Leaf Method. *Journal of Ecology*, 58(2), pp. 409-417.

Hoefnagels, M., 2017. Photosynthesis . In: *Biology: Concepts and Investigations 4th Edition*. New York: McGraw-Hill Education, pp. 88-103.

Hossain, M., Hoque, M. B. D. & Fujita, M., 2014. Proline Protects Plants Against Abiotic Oxidative Stress: Biochemical and Molecular Mechanisms. In: K. Gomez, ed. *Oxidative Damage to Plants: Antioxidant Networks and Signaling*. San Diego: Academic Press, pp. 477-522.

Jajic, I., Sarna, T. & Strzalka, K., 2015. Senescence, stress, and reactive oxygen species. *Plants*, Volume 4, pp. 393-411.

- Jaleel, C. et al., 2009. Drought Stress in Plants: A Review on Morphological Characteristics and Pigments Composition. *INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY*, Volume 11, p. 100–105.
- Kissoudis, C., van de Wiel, C., Visser, R. & van der Linden, G., 2014. Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk. *Frontiers in PLANT SCIENCE*, 5(207), pp. 1-20.
- Latef, A. & Tran, L., 2016. Impacts of Priming with Silicon on the Growth and Tolerance of Maize Plants to Alkaline Stress. *frontiers in Plant Science*, Volume 243, pp. 1-10.
- Le, H., C.M, S., Firestone, G. & Bjeldanes, L., 2003. *THE JOURNAL OF BIOLOGICAL CHEMISTRY*, 278(No. 23), p. 21136–21145.
- Li, Z. et al., 2014. Exogenous Spermidine Improves Seed Germination of White Clover under Water Stress via Involvement in Starch Metabolism, Antioxidant Defenses and Relevant Gene Expression. *Molecules*, Volume 19, pp. 18003-18024.
- Lobo, V., Patil, A., Phatak, A. & Chandra, N., 2010. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews*, 4(8), p. 118–126..
- Lugojan, C. & Ciulca, S., 2011. Evaluation of relative water content in winter wheat. *JOURNAL of Horticulture, Forestry and Biotechnology*, 15(2), pp. 173- 177.
- Lü, J., Lin, P., b, Y. Q. & Chen, C., 2010. Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. *Journal of Cellular and Molecular Medicine*, 14(4), pp. 840-860.
- Lu, L., Dong, J., Li, D. & Zhang, J. F. S., 2016. 3,3'-diindolylmethane mitigates total body irradiation-induced hematopoietic injury in mice. *Free Radical Biology and Medicine*, Volume 99, pp. 463-471.
- Mafakheri, A. et al., 2010. Effect of drought stress on yield, proline and chlorophyll contents in three chickpea cultivars. *Australian Journal of Crop Sciences*, 4(8), pp. 580-585 .
- Ma, N. et al., 2018. Susceptibility and tolerance of rice crop to salt threat: Physiological and metabolic inspections. *PLoS One*, 13(2), pp. 1-17.
- Masih, I., Maskey, S., Mussá, F. & Trambauer, P., 2014. A review of droughts on the African continent: a geospatial and long-term perspective. *Hydrology and Earth System Sciences*, Volume 18, p. 3635–3649.
- Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science*, 7(9), pp. 405-410.
- Mittler, R., Vanderauwera, S., Gollery, M. & Van Breusegem, F., 2004. Reactive oxygen gene network of plants. *TRENDS in Plant Science*, 9(10), pp. 490-498.
- Mogala, M., 2012. MAIZE MARKET VALUE CHAIN PROFILE. *Department of Agriculture, Forestry & Fisheries* , Issue , pp. 1-44.
- Moran, R., 1982. Formulae for determination of chlorophyllous pigments extracted with N, N-dimethylformamide. *Plant Physiology*, Volume 69, pp. 1376-1381.
- Moussa, H. & Abdel-Aziz, S., 2008. Comparative response of drought tolerant and drought sensitive maize genotypes to water stress. *Australian Journal of Crop Sciences*, 1(1), pp. 31-36.

- Oancea, S., Foca, N. & Airinei, A., 2005. Effects of heavy metals on plant growth and photosynthetic activity. *Analele Științifice Ale Universității "AL. I. CUZA" Iasi*.
- Oukarrouma, A., Bussotti, F., Goltsev, V. & Kalajid, H., 2015. Correlation between reactive oxygen species production and photochemistry of photosystems I and II in *Lemna gibba* L. plants under salt stress. *Environmental and Experimental Botany*, Volume 109, pp. 80-88.
- Pal, C. et al., 2007. Eco-friendly synthesis and study of new plant growth promoters: 3,30-Diindolylmethane and its derivatives. *Bioorganic & Medicinal Chemistry Letters*, Volume 17, p. 4924–4928.
- Petrov, V. & Van Breusegem, F., 2012. Hydrogen peroxide—a central hub for information flow in plant cells. *AoB PLANTS*, Volume 2012, pp. 1-13.
- Pintó-Marijuan, M. & Munné-Bosch, S., 2014. Photo-oxidative stress markers as a measure of abiotic stress-induced leaf senescence: advantages and limitations. *Journal of Experimental Botany*, 65(14), p. 3845–3857.
- Rendovnikovic, I., Gliveti, T., Delonga, K. & Vorkapi-Fura, J., 2008. Glucosinolates and their potential role in plant. *PERIODICUM BIOLOGORUM*, 110(4), p. 297–309.
- Saed-Moucheshi, A., Pakniyat, H., Pirasteh-Anosheh, H. & Azooz, M., 2014. Chapter 20 - Role of ROS as Signaling Molecules in Plants. In: *Oxidative Damage to Plants Antioxidant Networks and Signaling*. India: Academic Press, pp. 585-620.
- Scandalios, J., 1993. Oxygen Stress and Superoxide Dismutases. *Plant Physiology*, 101(1), pp. 7-12.
- Sharifi, P. & Mohammadkhani, N., 2015. Effects of Drought Stress on Photosynthetic Factors in Wheat Genotypes during Anthesis. *Cereal Research Communications*, 44(2), pp. 1-11.
- Sharma, P., Jha, A., Dubey, R. & Pessarakli, M., 2012. Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. *Journal of Botany*, Volume 2012, pp. 1-26.
- Shigeoka, S. et al., 2002. Regulation and function of ascorbate peroxidase isoenzymes. *Journal of Experimental Botany*, 53(372), pp. 1305-1319.
- Suprasanna, P., Nikalje, G. & Rai, A., 2015. Osmolyte Accumulation and Implications in Plant Abiotic Stress Tolerance. In: N. Iqbal, ed. *Osmolytes and Plants Acclimation to Changing Environment: Emerging Omics Technologies*. Mumbai: Springer, pp. 1-10.
- Szabados, L. & Savoure, A., 2010. Proline: a multifunctional amino acid. *Trends in Plant Science*, 15(2), pp. 89-97.
- Szollosi, R., 2014. Chapter 3 - Superoxide Dismutase (SOD) and Abiotic Stress Tolerance in Plants: An Overview. In: P. Ahmad, et al. eds. *Oxidative Damage to Plants Antioxidant Networks and Signaling*. India: Academic Press, pp. 89-129.
- Tayebimeigooni, A. et al., 2012. Leaf water status, proline content, lipid peroxidation and accumulation of hydrogen peroxide in salinized Chinese kale (*Brassica alboglabra*). *Journal of Food, Agriculture & Environment*, 10(2), pp. 371-374.
- Teixeira, R., Ligterink, W., Franca-Neto, J. H. H. & da Silva, E., 2016. Gene expression profiling of the green seed problem in Soybean. *BMC Plant Biology*, 16(37), pp. 1-15.

Velikova, V., Yordanov, I. & Edreva, A., 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants Protective role of exogenous polyamines. *Plant Science*, 51(1), pp. 59-66.

Verslues, P. et al., 2006. Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *The Plant Journal* , Volume 45, p. 523–539.

Vivar, O., Lin, C., Firestone, G. & Bjeldanes, L., 2009. 3,3'-Diindolylmethane Induces a G1 Arrest in Human Prostate Cancer Cells Irrespective of Androgen Receptor and p53 Status. *Biochemical Pharmacology*, 78(5), pp. 469-476.

