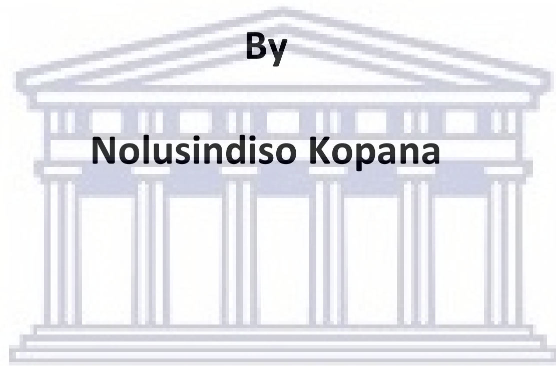
Effects of Nitric Oxide and Hydrogen Peroxide on antioxidant enzyme activity in *Zea mays* subjected to drought

By

Nolusindiso Kopana



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

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Keywords

Zea mays

Reactive Oxygen Species

Nitric Oxide

Drought stress

Antioxidant enzyme

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Abstract

Agricultural practices are significantly affected by drought. Drought is one of the most important plant stresses, causing several physiological, morphological, biochemical, and molecular changes in plants. Drought stress is of great challenge for crop growth, development and yield. *Zea mays* (maize) is one of the important crops worldwide due to the nutritional profile and other uses such as human consumption, manufacturing and animal feed. Under unfavorable conditions, plants produce high amounts of reactive oxygen species (ROS). Excessive formation of ROS is harmful for plant survival and can induce cell death. Defense mechanisms activated in response to drought in plants include antioxidant enzyme activity and proline accumulation. There is evidence of the use of nitric oxide (NO) donors and hydrogen peroxide (H_2O_2) at low concentrations to enhance the activity of antioxidant enzymes in stressed plants. Hence, the aim of the study is to examine the role of NO and H_2O_2 in regulation of antioxidant enzyme activity in maize subjected to water deficit. This was achieved by pretreatment of maize plants with a NOS inhibitor (N_{ω}-Nitro-L-arginine methyl ester, L-NAME) and H_2O_2 scavenger dimethylthiourea (DMTU) to alter the amount of NO and H_2O_2 in plant tissue under water-deprived conditions. Also, an NO donor (DETA/NO) was exogenously applied to examine NO effects in these plants. H_2O_2 was applied under well-watered conditions to assess the influence of H_2O_2 on endogenous NO levels. The effects of L-NAME and DMTU on H_2O_2 levels were evaluated. Moreover, the

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effects of DMTU on levels of NO were investigated. Lastly, effects of NO and H_2O_2 on antioxidant enzyme activity were assessed. Application of DMTU and L-NAME changed enzymatic activity of catalase, ascorbate peroxidase and superoxide dismutase. These alterations on enzymatic activities corresponded with changes in H_2O_2 levels. These results deduce that H_2O_2 acts upstream on NO generation, as suggested by the decline in NO content upon application of DMTU. The inhibition of NO production by L-NAME had no effects in accumulation of H_2O_2 and the application of H_2O_2 induced NO accumulation.

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DECLARATION

I declare that " *Effects of nitric oxide and hydrogen peroxide on antioxidant enzyme activity in Zea mays subjected to drought*" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name………………………………………….. Date…………………………………..

Signed………………………………………………

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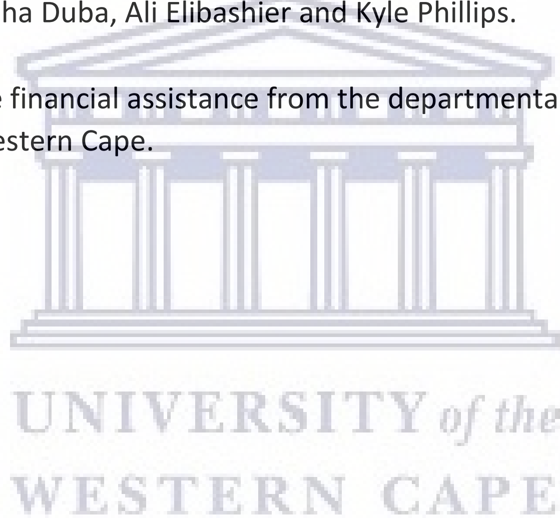

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Abbreviations

ABA	Abscisic acid
APX	Ascorbate peroxidase
Atrboh	Arabidopsis transcript levels of respiratory burst oxidase homolog
AsA	Ascorbate
AsA-GSH	Ascorbate-glutathione cycle
CAT:	Catalase
Ca^{2+}	Calcium
CEC:	Crop estimate committee
cGMP	cyclic guanosine monophosphate
CO_2	Carbon dioxide
CTK	Cytokinin
Cu/Zn-SOD	Copper/ Zinc superoxide dismutase
Cys	Cysteine
DAFF:	Department of Forestry and fishing
DETA/NO	Diethylenetriamine/ nitric oxide
DHAR	Dehydroascorbate reductase
DMTU	Dimethylthiourea
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide
FAO	Food and Agricultural Organisation of the United States
FDA	Food and Drug Administration
Fe-SOD	Ion superoxide dismutase
GA	Gibberellic acid
GDC	Glycine decarboxylase
GDP	Gross domestic product
GSH	Glutathione
GSSG	Glutathione disulfide

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GR	Glutathione reductase
HbO_2	Oxyhemoglobin
L-Arg	L-arginine
L-NAME	N(G)-Nitro-L-arginine methyl ester
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MDHAR	Monodehydroascorbate Reductase (MDHAR)
MetHb	Methemoglobin
Mn-SOD	Manganese superoxide dismutase
NAD(P)H	Reduced nicotinamide adenine dinucleotide phosphate
NiNOR	plasma membrane-bound nitrite-NO reductase
NO	Nitric oxide
NOS	Nitric oxide synthase
NOSI	Nitric oxide synthase-like
NOX	NADPH oxidase
NR	Nitrate reductase
OH.	hydroxyl radical
$^1O_2$	Singlet oxygen
PAGE	Polyacrylamide gel electrophoresis
PCD	Programmed cell dead
PSI	photosystem I
PSII	Photosystem II
PUFAs	Polyunsaturated fatty acids
PVP	Polyvinylpyrolidone
Rbohs	Respiratory burst oxidase homologs
ROS	Reactive oxygen species
RWC	Relative water content
S.A	South Africa
SNO	S-nitrosothiol

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SOD	Superoxide dismutase
SNP	Sodium Nitroprusside
TBA	Thiobarbituric
TBARS	Thiobarbituric acid reactive substance
TCA	Trichloroacetic acid
TEMED	N,N,N',N'- Tetramethylethylenediamine
TRX	Reduced thioredoxin
UQ	Ubiquinone
USA	United States of America
WD	Water deficit
WHO	World Health Organisation
WW	Well-watered
XOR	Xanthine oxido-reductase

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Chapter 1

Literature review

1.0 Introduction

Unfavorable dry weather conditions have significant harmful effects on crop physiology and agricultural yield. This may be a result of an excessive increase in temperature and a shift in rainfall patterns, especially a decline in precipitation (Belle et al., 2015). Moreover, in the semi-arid region of South Africa where three major maize producing provinces are situated, maize yield deteriorates during harsh drought resulting in explicit economic crisis. Thus, it has become a major issue to understand responses and adaptation of maize to drought. Maize is a fundamental food and forage crop (Challinor, Wheel et al., 2007). It is one of the major valuable crops worldwide. With the population expansion in South Africa and climate change, the necessity for crop supply has heightened (Challinor , Wheel et al., 2007), so it is essential to advance maize yield even under unfavourable conditions (NDMC., et al., 2012).

Water deficit promotes plant plasma membrane injury and elevates production of free radicals in plants cells (Iturbe-Ormaetxe et al., 1998). During normal plant growth, reactive oxygen species (ROS) are generated but their accumulation increases during abiotic stress (Møller et al., 2001). Examples of ROS include the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^•$) (Sharma et al., 2012). Therefore, cell death may result when the plant suffers abiotic stress (Iturbe-Ormaetxe et al., 1998). Nonetheless, ROS play an essential role in plant intracellular redox signaling (Siddiqui et al., 2010). Therefore, it is vital to control the concentration of ROS in plant cells. As a reaction to oxidative stress, antioxidant enzymes are activated to reduce oxidative damage either by detoxifying ROS or preventing their excessive accumulation (Sun et al. 2003; Wang et al. 2002; Sharma et al., 2012). This defense system includes the enzymatic antioxidants such as the superoxide dismutase (SOD) which catalyses the dismutation of O_2^- to produce H_2O_2 (Gupta et al., 1993). H_2O_2 is further scavenged to water and oxygen by enzymes

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such as catalase, glutathione peroxidase and the antioxidant enzymes of the ascorbate-glutathion cycle (Sharma et al., 2012).

In addition, the use of exogenously applied NO donors has been shown to enhance plant tolerance to several abiotic stress including drought (Garcia-Mata and Lamattina, 2001), salinity (Zhao et al., 2004) and heat (Uchida et al., 2002). NO has antioxidant properties that maintain cellular redox homeostasis and limit ROS toxicity, protecting plants from oxidative damage (Qiao and Fan, 2008). As a signaling molecule, NO regulates the expression of antioxidant genes (Qiao and Fan, 2008) that detoxify ROS and thus enhance stress tolerance. The signaling interactions between nitric oxide and the antioxidant defense system are relevant approaches used to enhance abiotic stress tolerance in plants (Laspina ., et al 2005). Due to the major impact drought has on crop production, for plant breeders and researchers it has been of great interest to elucidate plant behavior when exposed to both biotic and abiotic stresses, with the purpose to improve plant tolerance to harsh environmental conditions, and therefore minimize the decrease in crop production.

This chapter will discuss the effects of drought on maize production and yield. It will review plant responses to abiotic stress and the interaction of signaling pathways mediated by nitric oxide and ROS in plants during oxidative stress. Lastly, it will explain NO and H_2O_2 effects in the activation of antioxidant defense system in plants.

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1.1 Importance and production of maize

Our food source heavily depends on crops, yet agricultural production is greatly affected by drought (Kadam et al., 2014). With global climate change and uncertainties in precipitation patterns, food security has become more vulnerable than in the past (FAO, 2008), yet few economically viable approaches exist to support crop production under drought (Li et al., 2000). Despite ongoing breeding efforts to develop drought-resistant cultivars (Bennett et al., 2013), prolonged droughts in the food-insecure regions may cause famine, epidemics, and deaths, generate water crisis due to drying up of perennial streams, impact agriculture-based livelihood systems, food security and overall economic development (Karim and Rahman., 2011).

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Amongst many crops grown in South Africa maize (*Zea mays*) is one of the staple foods. Maize belongs to the family *Poaceae* (*Gramineae*) and the tribe *Maydeae* (Sikandar *et al*., 2007). In terms of global production, it is the third most important cereal, after wheat and rice, respectively. It is one of the staple food crops of the world and the staple cash crop of southern Africa (Burtt-Davy, 1914). About half of its global production is in developing countries, where maize flour (mealie-meal) is the staple food. It also has many diversified uses which include starch products, corn oil, baby foods and popcorn (Ranum et al., 2014).

According to the Crop Estimate Committee (CEC), the agricultural sector in South Africa contributes significantly to the economy of the country (Ranum et al., 2014). Agriculture has a high impact on the South African economy as it comes with a dual farming economy, a well-developed profitable farming as well as the substance-based production of crops within the profound rural areas (Olaleye et al., 2010). Maize farming has been one of the occupations of most indigenous people in Southern Africa. The first known proof of maize is the fossil of pollen grains which are approximately 80,000 years in age and that are nearly similar to the recent maize pollen (Ranum et al., 2014).

Most of the maize produced is consumed locally, therefore; the domestic market is very important to the industry. Maize is produced in most of South Africa (S.A) with Free State, Mpumalanga and North West provinces being the largest producers, accounting for over four-fifths of total production (BFAP, 2017). Maize is produced mostly on dry land with less than 10% produced under irrigation. The rainfall pattern and other weather conditions of a particular season determine the planting period as well as the length of the growing season. Maize is planted mainly between mid-October and mid-December. About 60% of maize produced in S.A has been white and the other 40% yellow maize (Department of Agriculture Fishing and Forestry, 2015). BFAP (2016, 2017) expects this to have changed by 2021, with farmers shifting towards yellow maize and oilseeds. Yellow maize is easy to trade globally (BFAP, 2017).

White maize is primarily for human consumption. Yellow maize is the most important ingredient in feed rations for dairy, beef, poultry and egg production (Ranum et al., 2014). The ratio between human consumption and animal feed is expected to change owing to patterns in the demand by

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animal feed, and the growing middle-class population. The maize industry is important to the economy both as an employer and earner of foreign currency because of its multiplier effects (maize also serves as a raw material for manufactured products such as paper, paint, textiles and medicine). The local consumption requirements for maize are usually around 7 million tons per year (DAFF., 2017)

The maize industry has strong linkages throughout the economy, both upstream to the input industries and downstream into milling, animal feed and food processing industries (Ranum, et al., 2014). Maize hectares vary from year to year, depending on weather and market conditions, but on average approximately 2.5 to 2.75 million hectares of hybrid maize are planted in S.A each year, as reported by the Department of Agricultural fisheries and forestry. Approximately 10 to 12 million tons of maize is produced in South Africa annually (ABSA, 2017). Illustrated on Table 1 and 2 is that South Africa's food security status has improved, according to the Global Food Security Index released by the Economist Intelligence Unit (EIU) and commissioned by DuPont. S.A is the 47th most food secure country in the world, and the highest ranked in Africa. On a ten-year average, maize for human consumption totals about 4.1 million tons, animal feed about 3.9 million tons, while starch- and glucose-manufacturing industries consume about 650 000 tons of maize annually. Feed production in S.A is estimated at more than 11 million tons per annum. On a five-year average, S.A normally has approximately 1.8 million tons surplus maize for the export markets (BFAP, 2017).

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The two main white maize-growing provinces in S.A, Free State and North West provinces, produced about 78% of the white maize harvest in 2017, whereas the Free State and Mpumalanga provinces produced about 67% of the yellow maize harvest. Drought has had a significant impact on agriculture, livelihoods and communities. Estimates are an economic loss of R5.9 billion in agriculture in the Western Cape alone, with a resultant 30 000 job losses and exports dropping 13-20%. This is due to reduced farming outputs and additional income losses as export volumes decline (DAFF., 2017). Many hectares of productive fruit trees and vineyards have been removed ahead of the normal replanting schedule due to the lack of available water as well as to prevent disease and pests from spreading. Stock farmers have also suffered heavy

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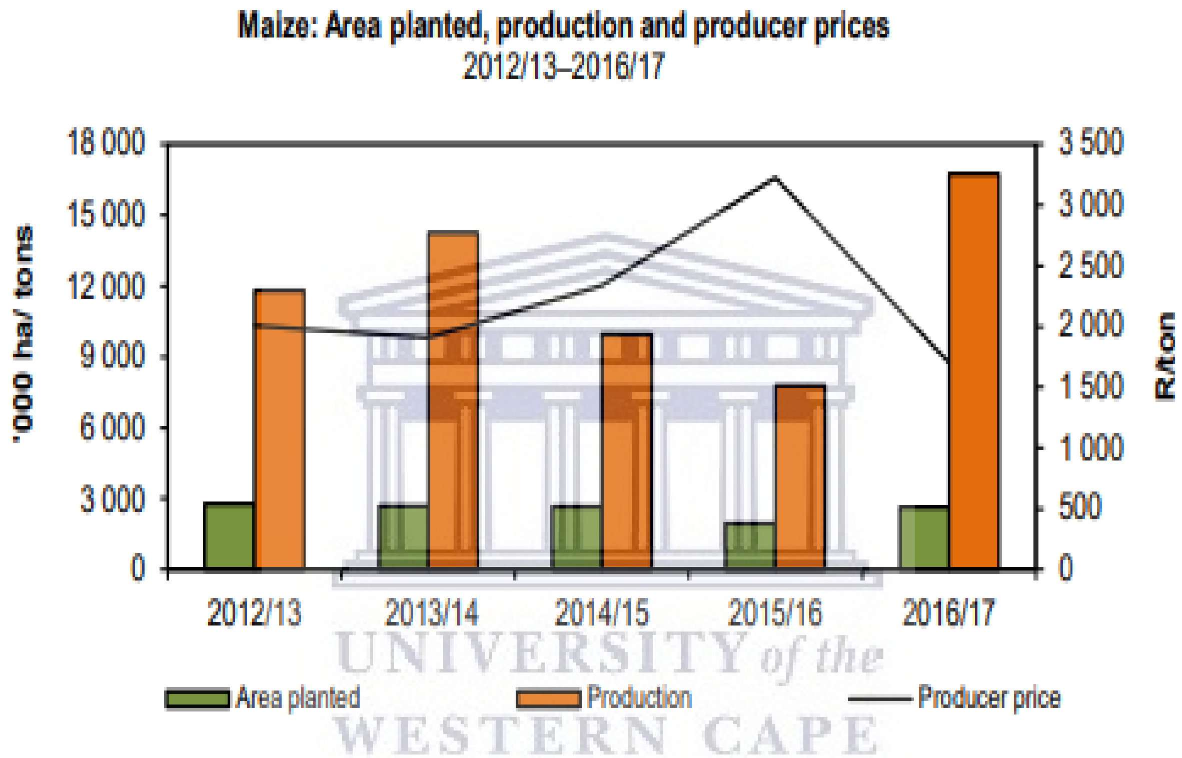
losses. As a result, many agricultural businesses risk bankruptcy. Finally, the impact of increased water tariffs is adding to the need to ensure optimal water efficiency on farms (DAFF, 2018).

a)



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b)

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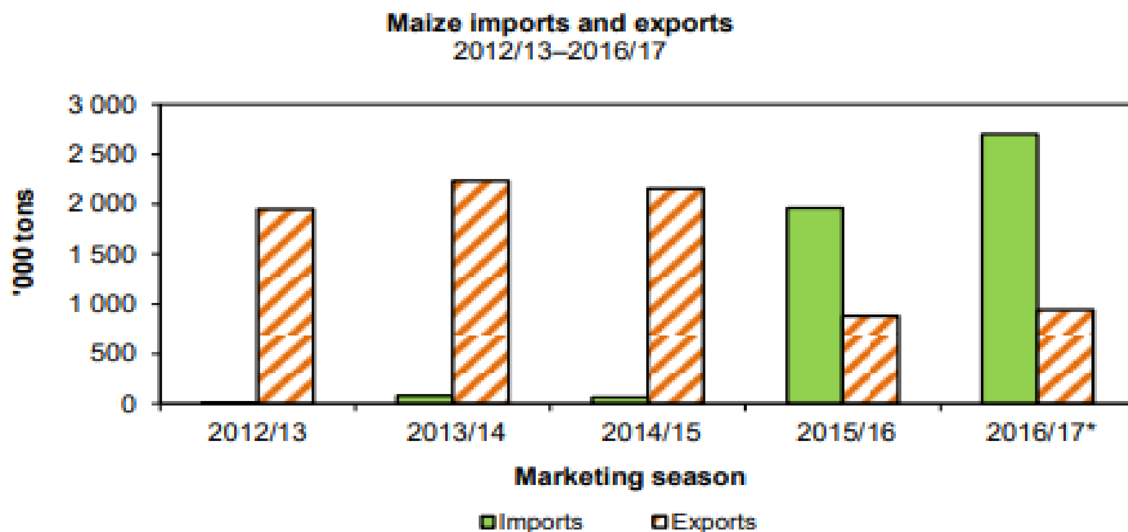

Figure 1.1 Presentation of a) maize production, area planted and producer price between 2012/13-2016/17 and b) exports and imports in South Africa in the same period, adapted from DAFF 2017 report.

1.2 Drought description and impacts

Drought is often characterized as slow-onset natural hazards whose impacts are complex and reverberate through many sectors of the economy such as water resources, agriculture, and natural ecosystems (Wilhite et al., 2007). Various definitions of drought, all relating to specific drought impacts on economic activities, ecosystems, and society and water management issues (Tate et al., 2000). A review by Dracup et al. (1980) and Wilhite and Glantz (1985) alluded to drought as a condition of insufficient moisture caused by a deficit in precipitation over some time period. According to Pereira et al (2009), drought can be defined as a temporal imbalance of water availability consisting of a persistent lower than average precipitation of uncertain frequency, duration and unpredictable severity. In Vicente-Serrano et al (2012) drought is described as a natural phenomenon which occurs when water availability is significantly below normal levels over a long period of time and cannot meet demand.

The time lag between the beginning of a period of water scarcity and its impact on socio-economic and environmental assets is referred to as the timescale of a drought (Ranum et al.,

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2014). As a result, drought indices usually consider short-term droughts (three months or less), medium-term droughts (lasting 4–9 months) and long-term droughts (12 months or more). Short-term droughts have an impact on water availability in the vadose zone and therefore are largely meteorological and agricultural droughts. On the other hand, long-term droughts also affect surface and ground water resources and therefore are hydrological drought (Strable et al., 1996)

Droughts often manifest in two folds, a shortage of soil moisture and shortage of water stored in other reservoirs. These two climate parameters are the key drivers of the hydrological cycle (Hisdal, 2000; Mniki, 2009; Olaleye, 2010). A disturbance in any one component or process of the hydrological cycle has impacts on the agricultural cycle. To this end, the immediate consequence of drought is a fall in crop production, due to inadequate and poorly distributed rainfall (Clay et al., 2003). Drought occurs in virtually all climatic regions and drought-induced crop yield loss is considered among the greatest losses in agriculture (Olaleye, 2010). Additionally, livestock production is impacted due to low rainfall, which causes poor pasture growth and may also lead to a decline in fodder supplies from crop residues.

Generally, S.A is classified as a semi-arid and water stressed country. The country's average annual rainfall is about 450 mm a year, which is below the world's 860 mm average per year (SAWS, 2018). Rainfall in S.A exhibits seasonal variability, with most rainfall occurring mainly during summer months (November through March) (DAFF, 2017). However, in the southwest region of the country, rainfall occurs in winter months (May through August). S.A experiences rainfall that varies significantly from west to east. Figure 1.2 indicates variation in rainfall received by provinces across the 6 months period in 2018. Annual rainfall in the northwest region often remains below 200 mm, whereas much of the eastern Highveld receives between 500 mm and 900 mm (occasionally exceeding 2000 mm) of rainfall per annum. The central part of the country receives about 400 mm of rain per annum, with wide variations occurring closer to the coast. Most regions receiving 400 mm of rainfall play a significant role, e.g., land to the east are suitable for growing crops while land to the west are suitable for livestock grazing as well as crop cultivation on irrigated land. The Free State and North West Provinces fall within regions that

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receive less than 500 mm of rainfall per year and play an important role towards the South African economy.

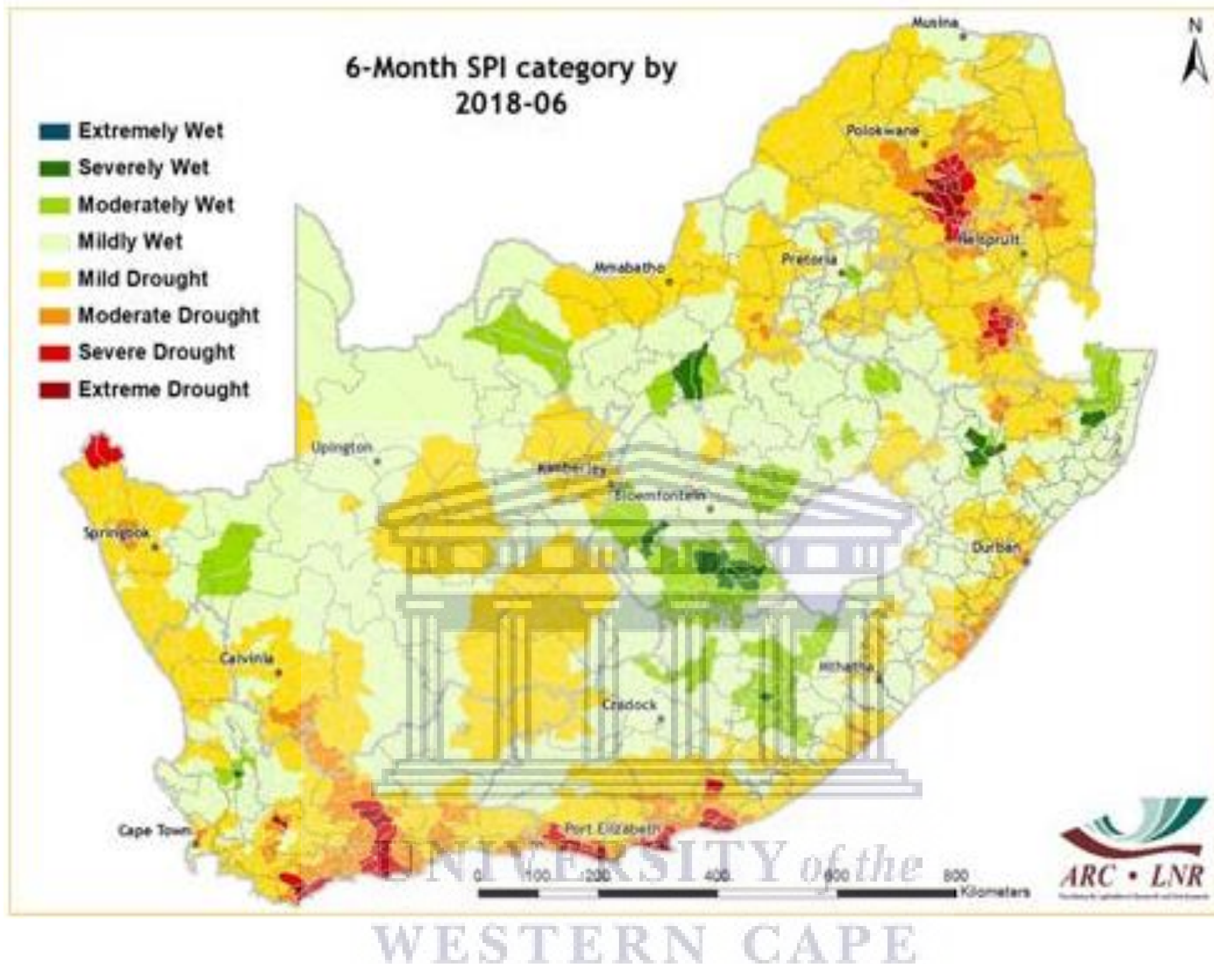

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Figure 1.2 Illustration of areas affected by drought in 2018 within a period of six month in South Africa, picture depicted from ARC report 2018.

Rainfall decrease is one of the major environmental challenges faced by SA. According to the S.A weather report, year 2016/2017 were recorded as one of the years in history that drought has been most severe. The decrease on rainfall had a huge impact on the crop production and yield which automatically affected the economy (Mniki ., 2009). Poor crop yield is a result of seasonal rainfall being below than normal, which also cause ground and dam water levels to fall critically low. When such conditions continue to occur time to recover natural resources and the economy

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is insufficient between rainfall-deficit periods. Drought had a very negative impact on commercial farming around 1991/1992 in S.A (Ngaka et al ., 2011). This was a result of low rainfall and inadequate capacity of range lands to support grazing and shortage of drinking water for livestock. Although the Agricultural sector contributes minimal to the Gross Domestic Product (GDP), it plays a very essential role in wealth creation in S.A especially in rural areas, employing more than one million people (IFRC., 2011).

Economies of many countries in sub-Saharan Africa are particularly vulnerable to the effects of drought because they depend on rain-fed agriculture, and low levels of income per capita. In 2007/2008, the government of South Africa spent R285 Million on drought relief, of which R20 Million and R25 Million were allocated to the Eastern Cape and the Free State provinces, respectively (Ngaka et al., 2011).

1.2.1 Drought effects on maize growth and development

Plant demand for water varies across the tissues and across the growth phases in the same species of a crop plant, and maize is no exception (Zhu et al., 2002). Evaluation of the maximum amount of water that plants need is essential to resolve water shortage in plants (Zhu et al., 2002). At early growth stages of maize the demand for water is low then it increases at the productive stages and during terminal growth stages water requirement decreases again (Dash et al., 2001). During reproductive growth stage, maize requires 8-9 mm of water each day per plant. Regarding water requirements, studies estimate that four weeks are most crucial, which includes two weeks before and two weeks after pollination (Pannar et al., 2012). Drought stress reduces growth rate during vegetative growth stages of maize, especially at V1–V5 stages, and prolong the vegetative growth stage. This reduces the duration of the reproductive growth stage (Pannar et al., 2012). In the mid-80s, relative water content (RWC) was introduced as an indication of plant water status which, afterwards, was used instead of plant water potential. As RWC refers to its relation with cell volume, it is able to indicate the balance between absorbed water by plant and consumed through transpiration. Osmoregulation appears to be one of the main mechanisms preserving turgor pressure in most plant species against water loss from the plant so, it causes the plant to continue water absorption and retain metabolic activities

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(Gunasekera and Berkowitz, 1992). Zlato Stoyanov (2005) found that by exerting drought stress for 14 days and reaching soil water potential to -0.9 MPa, osmotic potential and turgor pressure in the first leaf of bean was strongly decreased. Ramos et al (2003) stated that RWC of bean leaves under drought stress was significantly less than the control. Lazacano-Ferrat and Lovat (1999) subjected bean plants to drought stress and evaluated RWC of the stem and found that RWC was significantly lower compared to control plants (Gaballah et al., 2007). Plants grown under water-limited conditions undergo various adaptive mechanisms to reduce stress, including extensive root system modification to enhance water uptake, alterations to leaf size or leaf rolling, modification in cuticle permeability, developing a waxy layer or spongy tissue, and reduced stomata size to avoid water evaporation. These tolerance adaptation mechanisms are commonly classified as drought escape, drought recovery, and drought avoidance (Bartels et al., 2005).

All maize developmental stages from germination to harvest maturity including seedling establishment, vegetative growth and reproductive growth stages are prone to drought stress (Du Plessis et al., 2003). Maize grain size is greater than other cereals and therefore requires more water for maintenance of osmotic potential and conversion of stored food into consumable form and for proper germination (Gharoobi et al., 2012). Survival under stressful conditions depends on the plant's ability to perceive the stimulus, generate and transmit the appropriate signals and initiate various physiological and biochemical changes. Plants respond and adapt to water stress by altering their cellular metabolism and invoking various defense mechanisms (Zhu 2002, Boudsocq and Laurière 2005; Chae et al., 2009). The plant hormone Abscisic Acid, as a stress signal, increases as a result of water deficit stress and plays crucial roles in the regulation of plant water balance and osmotic stress tolerance (Zhu et al., 2002). The accumulation of reactive oxygen species (ROS) under abiotic stress increases during drought stress and therefore can become harmful for the plant.

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1.3 Production of Reactive Oxygen Species in Plant Cell

The production of reactive oxygen species (ROS) highly depends on the availability of oxygen. Molecular oxygen was introduced to the early reducing atmosphere of the earth about 2.7 billion

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years ago by oxygen-evolving photosynthetic organisms, causing the advent of ROS as toxic by-products (Halliwell et al., 2006).

In the past years, it has been demonstrated that ROS performs a vital signaling role in plants; processes such as growth, development and especially response to biotic and abiotic stimuli (Foyer et al., 2005). When the delicate balance between ROS production and elimination important for normal cellular homeostasis is disturbed, cellular damages are manifested in the form of degradation of biomolecules like pigments, proteins, lipids, carbohydrates, and DNA, which ultimately amalgamate in plant cellular death**.** The survival of plants therefore depends on many important factors like change in growth conditions, severity and duration of stress conditions and the capacity of the plants to quickly adapt to changing energy balance (Miller et al., 2010). The most important members of the ROS family include free radicals like superoxide anion (O_2^-), hydroxyl ion (OH) and non-radicals like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$).

Singlet oxygen ($^1O_2$) is the highly reactive, excited state of molecular oxygen that can be formed in a reaction between O_2 and the chlorophyll triplet state (Krieger-Liszkay., 2000 and Gill. 2010). Under normal conditions, $^1O_2$ is generated during photosynthesis by the photo-activation of photosensitizers, mainly chlorophylls and their precursors (Krieger-Liszkay. 2005). Singlet oxygen is also generated during senescence and under different abiotic stresses (Pospisil. 2007). Similar to other ROS, $^1O_2$ has a dual effect (Morker et al., 2011). As an oxidizing agent, it can react with various biological molecules (Albores et al., 2011), causing damage and leading to cell death. It can also play a signaling role by activating the expression of various genes (Kin et al., 2008). Singlet oxygen interacts with molecules mostly in its nearest environment because of its short half-life that ranges between 3.1 to 3.9 µs in pure water (Krasnovsky. 1998). The diffusion distance of $^1O_2$ has been calculated to be up to 10 nm in a physiologically relevant situation (Fischer et al., 2007).

One other member of the ROS family is superoxide anion. The primary source of superoxide anion in chloroplasts are Mehler reactions, during which O_2 is reduced by electrons from the photosynthetic electron transport chain (Allen et al., 1973). Generated O_2^- is then converted to

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H_2O_2, mostly by the action of Cu/ZnSOD, as illustrated in Figure 1.3 (Asada et al., 2006). Therefore, superoxide dismutase (SOD) determines the lifetime of O_2^- in cells and the probability of its involvement in biochemical processes. Furthermore, O_2^- is a moderately reactive, short-lived ROS with a half-life of approximately 2–4 µs, and it cannot cross the chloroplast membrane (Takahashi and Asada., 1983). However, chloroplasts are not the only sites of O_2^- production. In peroxisomes, O_2^- is generated by two different sources: in the peroxisomal matrix via xanthine oxidase (Sandolia et al., 1988) and by electron transport chain (ETC) in the peroxisome membrane (Del and Donaldson., 1995). Peroxisomes can be considered as an important source of signaling molecules since they have capacity to rapidly produce and scavenge H_2O_2 and O_2^- due to the presence of many antioxidants in these organelles.

Another important source of O_2^- in plant cells are NADPH oxidases (NOX), commonly known as respiratory burst oxidase homologs (Rbohs) in plants, which catalyze the production of O_2^- (Kaur and Fluhr et al., 2014). Plant Rbohs have been intensively studied recently since they play key roles in many physiological processes, such as ROS signaling and stress responses (Kwak et al., 2003). Finally, O_2^- is also produced in the cytosol by xanthine dehydrogenase and the aldehyde oxidase (Zarepour et al., 2010). Numerous studies have reported an increase in the production of O_2^- during natural and artificially induced senescence (McRae et al., 1983); however, attributing a specific signaling role to this increase is extremely difficult since the increase in most cases is accompanied by the production of other ROS and the quick conversion of O_2^- to H_2O_2.

Another ROS member is hydrogen peroxide, a moderately reactive ROS formed when O_2^- undergoes both univalent reduction as well as protonation. It can be produced both enzymatic by being dismutated to H_2O_2 under low pH conditions, or mostly by a reaction catalyzed by SOD. H_2O_2 is produced in plant cells not only under normal conditions, but also from oxidative stress (Sharma et al., 2012). Due to stomatal closure and low availability of CO_2 and its limited fixation, Ribulose 1-5-bisphosphate (RuBP) oxygenation is favored and thus photo-respiration is enhanced. This accounts for more than 70% of the H_2O_2 produced as a result of drought stress (Noctor et al., 2002). H_2O_2 in plants has a dual role as it is beneficial at low concentrations to act as signaling molecules in the regulation of various biological processes but damaging at higher

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concentrations in the cell. Due to its significantly longer half-life of 1 ms, compared to other ROS members, it can traverse longer distances and cross plant cell membranes (Bienert et al., 2007). The reaction catalysed by NADPH oxidase uses O_2 to generate superoxide (O_2^-), which is then converted into H_2O_2 by apoplastic superoxide dismutase. Exogenous application of hydrogen peroxide on stressed plants has been reported to have a positive effect depending on the concentration used, the intensity of stress on the plant, physiological conditions, and on the specificities of processes affected by H_2O_2.

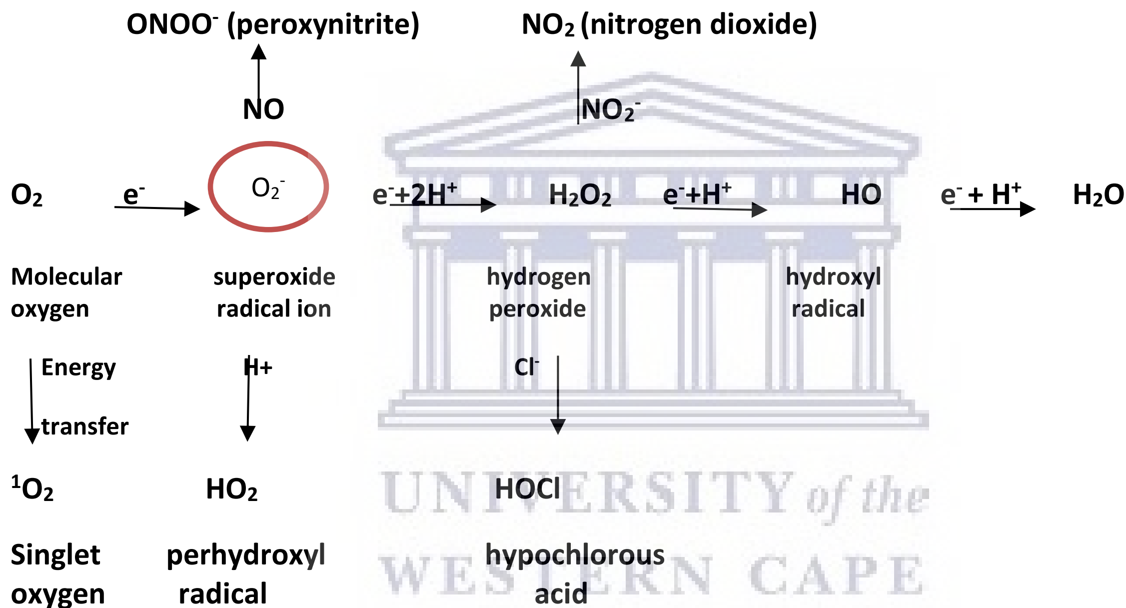

Fig. 1.3 Pathways in the univalent reduction of O_2 to water leading to the formation of various intermediate reactive oxygen species. (Noctor, 2000 and Asada 2008).

1.3.1 Exogenous application of hydrogen peroxide

The relationship between positive and negative functions performed by H_2O_2 in biological systems suggested by authors are that the application of H_2O_2 at low concentrations could improve plant tolerance to abiotic stresses such as drought, salinity and osmotic (He et al. 2009). Treatment of Arabidopsis or tobacco with H_2O_2 protects the plants from oxidative damages caused by high light intensity (Karpinski et al. 1999; Gechev et al. 2002). Tolerance to high

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temperatures was demonstrated after treatment with low concentrations of H_2O_2 in maize seedlings, *Phalaenopsis* and *Vigna radiata* (Prasad et al. 1994; Yu et al. 2002, 2003), and similarly treated potato nodal explants were found to be resistant to high temperature (Foyer et al. 1997; Lopez-Delgado et al. 1998). In these cases, it was established alterations in the activity of several antioxidant enzymes.

Many authors such as (Jing et al. 2009), (Hossain et al., 2013) and (Ashraf et al., 2014) investigated drought tolerance in various plant species after exogenous application of H_2O_2 at low concentrations and priming and found that it increased the activities of antioxidant enzymes, and the levels of AsA and GSH, resulting in lower levels of MDA, H_2O_2 and O_2^-. The dual role of H_2O_2 was confirmed in a study, in which treatment with 600 µM H_2O_2 caused an increase in the vase life of a cut Oriental × Trumpet hybrid lily Manissa, while concentrations of 800 and 1200 µM resulted in negative effects. Further evidence that the effects of H_2O_2 are dose dependent comes from a study in which wax apple trees were spray-treated with different concentrations of H_2O_2 under field conditions. Spraying wax apple fruits with 5 and 20 mM of H_2O_2 once a week produced better fruit growth and maximized the yield and quality in comparison with the control and the higher dose of 50 mM H_2O_2.

In a similar study, Ishibashi et al. (2011) demonstrated that spraying plants with H_2O_2 could lessen the symptoms of drought stress in soybean. The RWC content, photosynthetic rate and stomatal conductance of drought-stressed leaves in plants sprayed with H_2O_2 were all higher than in leaves sprayed with distilled water. During drought stress the accumulation of hydrogen peroxide occurs.

1.3.2 Abscisic acid- induced H_2O_2 production

Abscisic Acid (ABA) accumulation plays an important role in the regulation of NADPH oxidase activity in maize leaves (Jiang et al., 2002c). Moreover, various authors reported that cell wall NADH-peroxidase and diamine oxidase (Lin et al., 2001), and light reaction in chloroplasts (Zhang et al., 2001) may also contribute to ABA-induced production of ROS. Exogenous Application of ABA leads to the production of H_2O_2 in plant tissues (Guan et al. 2000, Pei et al. 2000, Jiang and

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Zhang 2001, Zhang et al. 2001, Kwak et al. 2003, Hu et al. 2005). It has been shown that there are two sources of H_2O_2 in *Vicia faba* guard cells in response to ABA, one is the light reaction in chloroplasts, which might be the main regions of H_2O_2 production, and another is the plasma membrane NADPH oxidase (Zhang et al. 2001). Using molecular genetics and cell biological analyses, studies have demonstrated that the AtrbohD and AtrbohF NADPH oxidases are required for production of H_2O_2 during ABA-induced stomatal closure in Arabidopsis guard cells (Kwak et al. 2003). In mesophyll and bundle sheath cells of maize leaves, H_2O_2 accumulation induced by ABA only occurred in the apoplast in the two types of cells, and the greatest accumulation of H_2O_2 was observed in the walls of mesophyll cells facing large intercellular spaces (Hu et al. 2005). It has been shown that ABA-induced H_2O_2 production is involved in the regulation of stomatal closure (Pei et al. 2000, Zhang et al. 2001, Kwak et al. 2003), antioxidant defense (Jiang and Zhang 2002a, Jiang and Zhang 2002b, Jiang and Zhang 2003, Hu et al. 2005, Zhang et al. 2006), and seed germination and root elongation (Kwak et al. 2003). High production of ROS disturbs the redox homeostasis in plant cells.

1.3.3 Hydrogen peroxide scavenger

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Dimethylthiourea (DMTU) is a small, highly diffusible molecule which may reach intracellular locations and reduce oxidative injury in many biological systems (Lewis et al., 1994). It is a well-known effective scavenger of harmful oxygen metabolites. DMTU scavenges H_2O_2, O_2^-, and hypochlorous acid *in vitro* (Curtis., 1988). As a result, DMTU may have value as 0_2 metabolite scavengers when used alone and/or in conjunction with SOD and/or catalase.

The problems with using DMTU as O_2 metabolite scavengers was in establishing their relative scavenging specificity and determining the mechanisms responsible for their protective effects in biological systems. It was therefore addressed by hypothesizing that reaction of DMTU with O_2 metabolites might cause the consumption of H_2O_2 and that this consumption could eventually be used to help determine the presence and toxicity of O_2 metabolites in biological systems (Parker et al., 1986). The study conducted by (Parker et al., 1986) found that the reaction of H_2O_2 caused DMTU consumption *in vitro*. Aerobic metabolism constantly generates ROS which are confined to the various plant cellular compartments, including the chloroplast, mitochondria and

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peroxisomes (Mittler 2002, Noctor et al. 2002, Foyer and Noctor 2003, Bartoli et al. 2004, Luna et al. 2004, Mittler et al. 2004).

1.3.4 Sites of ROS production in plant cells

The photosynthetic electron transport (PET) chain in the chloroplast is liable for H_2O_2 generation. The PET chains comprise a number of enzymes on the reducing side of photosystem I (PSI): Fe–S centers, reduced thioredoxin (TRX), and ferredoxin. These electron transport elements are auto-oxidizable and under conditions limiting the availability of NADP, superoxide anion radical can be formed (Dat et al., 2000; Foyer & Noctor, 2000). Mehler (1951) reported the photo-reduction of O_2 in chloroplasts and identified H_2O_2 as the reaction product. Later, O_2^- was identified as the primary product of O_2 photo-reduction in thylakoids (Asada et al., 1987). Currently, the 'Mehler reaction' is considered as the primary and the most powerful source of H_2O_2 in chloroplasts, and the rate of O_2 photo-reduction relies on environmental factors (Asada & Takahashi, 1987; Mullineaux & Karpinski, 2002; Logan et al., 2006). H_2O_2 production in chloroplasts is catalyzed by SOD forms containing copper/zinc (Cu/Zn-SOD) or iron (Fe-SOD) in the active site (Alscher et al., 2002). Water oxidation at photosystem II (PSII) and univalent photo-reduction of O_2 in PSI is coupled to both the production and destruction of H_2O_2 by APX.

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Appraisals indicate that 1–5% of the total O_2 utilized by the mitochondria is redirected against production of H_2O_2 (Miller et al., 2010**).** H_2O_2 is the leading ROS in the mitochondria, it is converted to water by APX (Sharma et al., 2012). Mitochondrial ROS production occurs at steady-state levels under normal conditions but is highly amplified under stress conditions (Pastore et al., 2007). Unfavorable conditions affect the tight coupling of ETC and ATP synthesis, leading to over-reduction of electron carriers like the ubiquinone (UQ) pool, thereby generating ROS (Rhoads et al., 2006; Blokhina and Fagerstedt, 2010) as shown in Figure 1.4. To counteract this oxidative stress in the mitochondria, two enzymes, Mitochondrial Alternative Oxidase (AOX) and Mitochondrial SOD (Mn-SOD) are very crucial. The AOX maintains the reduced state of the UQ pool and attenuates the ROS production. Its importance is evident from the fact that Arabidopsis lacking a functional AOX is hyper-sensitive to drought stress and has altered transcription profiles of different components of the antioxidant machinery (Ho et al., 2008).

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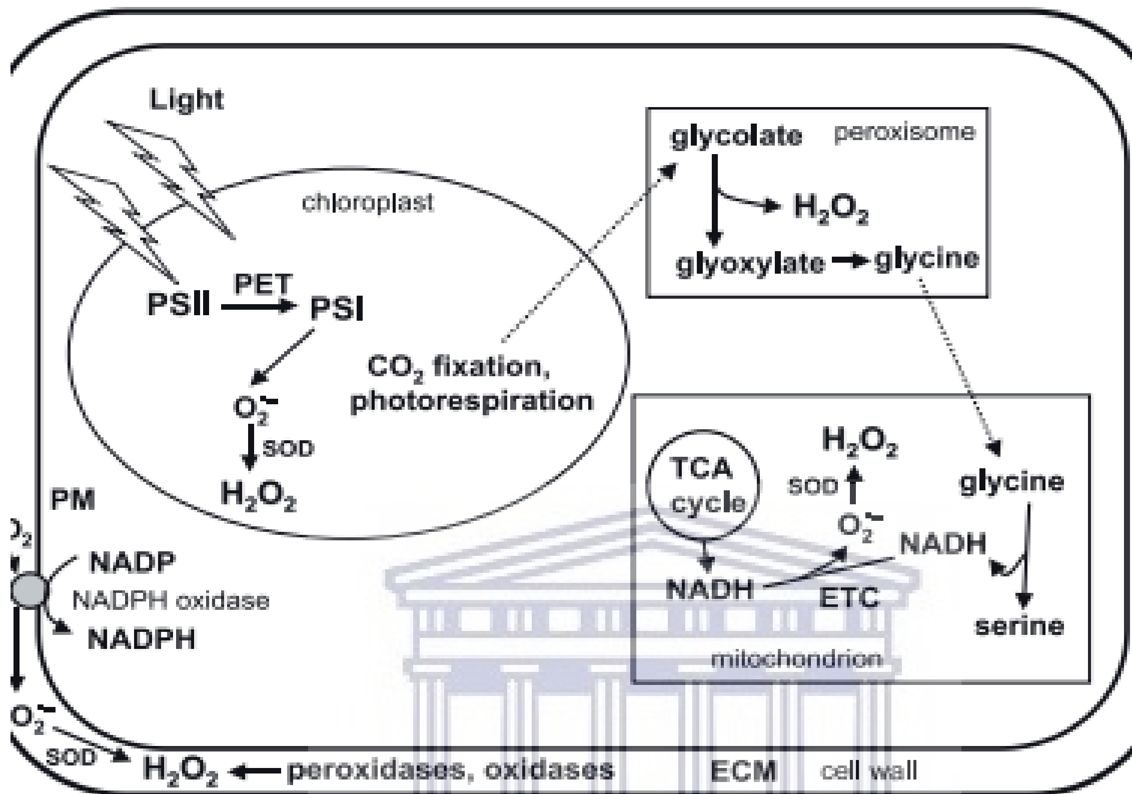

Figure 1.4 Main sources of H_2O_2 in different cellular compartments of mesophyll cells during C3 photosynthesis. The ratio of H_2O_2 production between chloroplasts and peroxisomes is 1:2.5, and between chloroplasts/peroxisomes and mitochondria: 3/5:1, these data are estimated from the rates of H_2O_2 production according to Foyer and Noctor (2003).

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Peroxisomes are single-membrane-bound spherical microbodies and are the major sites of intracellular H_2O_2 production due to their integral oxidative metabolism (Luis et al., 2006; Palma et al., 2009). They also produce O_2^-, like chloroplasts and mitochondria during various metabolic processes. O_2^- is generated at two different locations, one by the Xanthine oxidase, located in the peroxisomal matrix, which metabolizes both xanthine and hypoxanthine into uric acid and generates O_2^- as a by-product. The second is the NADPH-dependent small ETC, composed of NADH and Cytb localized in the peroxisomal membrane, which utilizes O_2 as the electron acceptor and releases O_2^- into the cytosol. During stressful conditions, when the availability of water is low and stomata remains closed, the ratio of CO_2 to O_2 reduces considerably, which causes increased

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photorespiration leading to glycolate formation. This glycolate is oxidized by glycolate oxidase in peroxisomes to release H_2O_2, making it the leading producer of H_2O_2 during photorespiration (Noctor et al., 2002). Besides, there are other supplemental metabolic processes like β-oxidation of fatty acids, the flavin oxidase pathway and the disproportionation of O_2^- radicals for peroxisomal ROS production.

The apoplast is a diffusible space around the plant cell membrane and is responsible for converting the incoming CO_2 into a soluble, diffusible form which enters the cytosol to undergo photosynthesis. At times of adverse environmental conditions, stress signals combined with abscisic acid (ABA) make the apoplast a prominent site for H_2O_2 production (Hu et al., 2006).

1.3.5 Lipid Peroxidation

When ROS level exceed a certain threshold, enhanced lipid peroxidation takes place in both cellular and organellar membranes, which, in turn, affects normal cellular functioning. Lipid peroxidation exacerbates the oxidative stress through production of lipid-derived radicals that themselves can react with and damage proteins and DNA. The level of lipid peroxidation has been widely used as an indicator of ROS-mediated damage to cell membranes under stressful conditions (Sharma et al., 2012). Increased degradation of lipids has been reported in plants growing under environmental stresses. Increased in lipid peroxidation under these stress parallels with increased production of ROS. Malondialdehyde (MDA) is one of the final products of peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage (Stadtman. 1986). Two common sites of ROS attack on the phospholipid molecules are the unsaturated double bonds between two carbon atoms and the ester linkage between glycerol and the fatty acid. The polyunsaturated fatty acids (PUFAs) present in membrane phospholipids are particularly sensitive to attack by ROS (Sminorff. 1995). A single hydroxyl radical can result in peroxidation of many polyunsaturated fatty acids because the reactions involved in this process are part of a cyclic chain reaction. The thiobarbituric acid reactive substances (TBARS) assay is a well–established method for monitoring lipid peroxidation level. MDA forms a 1:2 adduct with thiobarbituric acid (TBA) and can be estimated spectrophotometrically or fluorometrically (Hodges et al., 1999).

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1.3.6 Plant Programmed Cell death

The level of stress that a cell is exposed to can be a critical determinant of the ultimate fate of the cell. In a population of cells subjected to low level stresses, the majority will survive. When the cells are subjected to moderate levels of stress, the majority execute Programmed cell death (PCD). PCD has been defined as a sequence of events that lead to the controlled and organized destruction of the cell (Lockshin et al., 2004). It is crucial for defense responses to restrict the spread of pathogens and for proper development of multicellular organisms (Lam, 2004). PCD was initially described in very specific morphological terms (Kerr et al., 1972) and still is characterized by cell shrinkage, nuclear condensation and DNA fragmentation, and eventually the breakup of the cell into 'apoptotic bodies' (Adrain et al., 2001).

The plant cell membrane is made up of lipids and glycoproteins and acts as a physical, protective barrier. The fluidity of the cell membrane is altered when the cell is exposed to stress such as heat. Oxidative stress can damage cell membranes. The ROS associated with oxidative stress can act on membrane lipids to decrease membrane stability. A reliable Evan's blue staining technique was adapted that has been used by many researchers to assess cell death or membrane damage (Smith et al., 1982; Oprisko et al., 1990; Vemanna et al., 2017) for instantly monitoring cell death induced by stress. Evan's blue is an acidic, non-permeating exclusion dye which stains dead or damaged cells. The dye does not enter live cells with stable membranes (Gaff and O'kong'o-ogala, 1971).

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1.4 Antioxidative defense system

For optimal functioning of the plant, redox balance needs to be maintained. High concentrations of ROS are harmful for plant growth and development; hence plants carry an active antioxidant defense system to adapt to ROS-induced oxidative stress (Anjum et al., 2011b, c; Ashraf et al., 2015). Antioxidants comprise of two categories, namely enzymatic antioxidants such as ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR) and non-enzymatic, ascorbic acid (AsA), dehydroascorbate (DHA), reduced glutathione (GSH).

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These antioxidants promote stress tolerance either directly or indirectly in the case of drought (Sharma et al., 2012). Adebayo and Menkir (2015) reported that maintained harvests in maize under drought stress were directly associated with high antioxidant activities. Farooq et al. (2009) also deduced that elevated activities of enzymatic and non-enzymatic antioxidants may promote drought tolerance by minimizing ROS.

Antioxidant enzymes supply cells with a highly active system for detoxifying O_2^- and H_2O_2. The stability of SOD and the various H_2O_2-scavenging enzymes in cells is regarded important as it regulates the steady state level of O_2^- and H_2O_2 (Asada et al., 1987 ; Bowler et al. 1991). This study will mainly focus on the activities of the following three antioxidants: catalase, superoxide dismutase and ascorbate peroxidase.

1.4.1 Catalase

In plants, CAT is highly recognized as the primary enzymatic H_2O_2 remover of photosynthetic cells, which convert H_2O_2 into H_2O and O_2 (Scandalios, 1987). CAT mainly occurs in peroxisomes and catalyzes the dismutation reaction without requiring any reductant. The active CAT enzyme is a tetrameric iron porphyrin protein CAT scavenges H_2O_2 generated during mitochondrial electron transport, β-oxidation of the fatty acids, and most importantly in photo respiratory oxidation (Scandalios et al., 1997). Catalase activity is a determining factor for the protection of photosynthetic cells against oxidative stress induced during abiotic stress conditions such as chilling, drought, salt and ozone. CAT isoforms are distinguished on the basis of organ specificity and responses to environmental stress (Willekens et al., 1994a). It has been suggested that a CAT isoform in maize mitochondria (Scandalios et al., 1980), but no mitochondrial form has been reported in C_3 species (Foyer and Noctor 2000).

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Peroxisomes contain a large amount of CAT, but its features suggest that the enzyme is inefficient in removing low concentrations of H_2O_2 (Willekens et al. 1994a; Noctor et al., 2000).These enzymes allow low steady state levels to prevail in order to sustain redox signaling pathways without causing oxidative stress (Noctor et al., 1998). Catalase activities decrease under conditions that suppress photorespiration, such as increased CO_2 (Azevedo et al., 1998).

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1.4.2 Ascorbate peroxidase

Ascorbate peroxidase is the main enzyme important for H_2O_2 removal in the chloroplast, peroxisomes and mitochondria. APX utilizes ascorbate as its specific electron donor to reduce H_2O_2 to water (Asada., 1992). It mostly scavenges H_2O_2 in the cytosol and chloroplast of the plant cell. APX is broadly dispersed across the cell compartments and also has higher affinity of H_2O_2 therefore a most effective scavenger for H_2O_2, and therefore a most effective scavenger of H_2O_2 under stress. The ascorbate–glutathione cycle represents the main pathway to limit the build-up of toxic levels of H_2O_2 in photosynthetic organisms. The different affinities of the two enzymes for H_2O_2 (µm range for APX and mM range for CAT) suggest that they belong to functionally different classes of H_2O_2 scavenging enzymes.

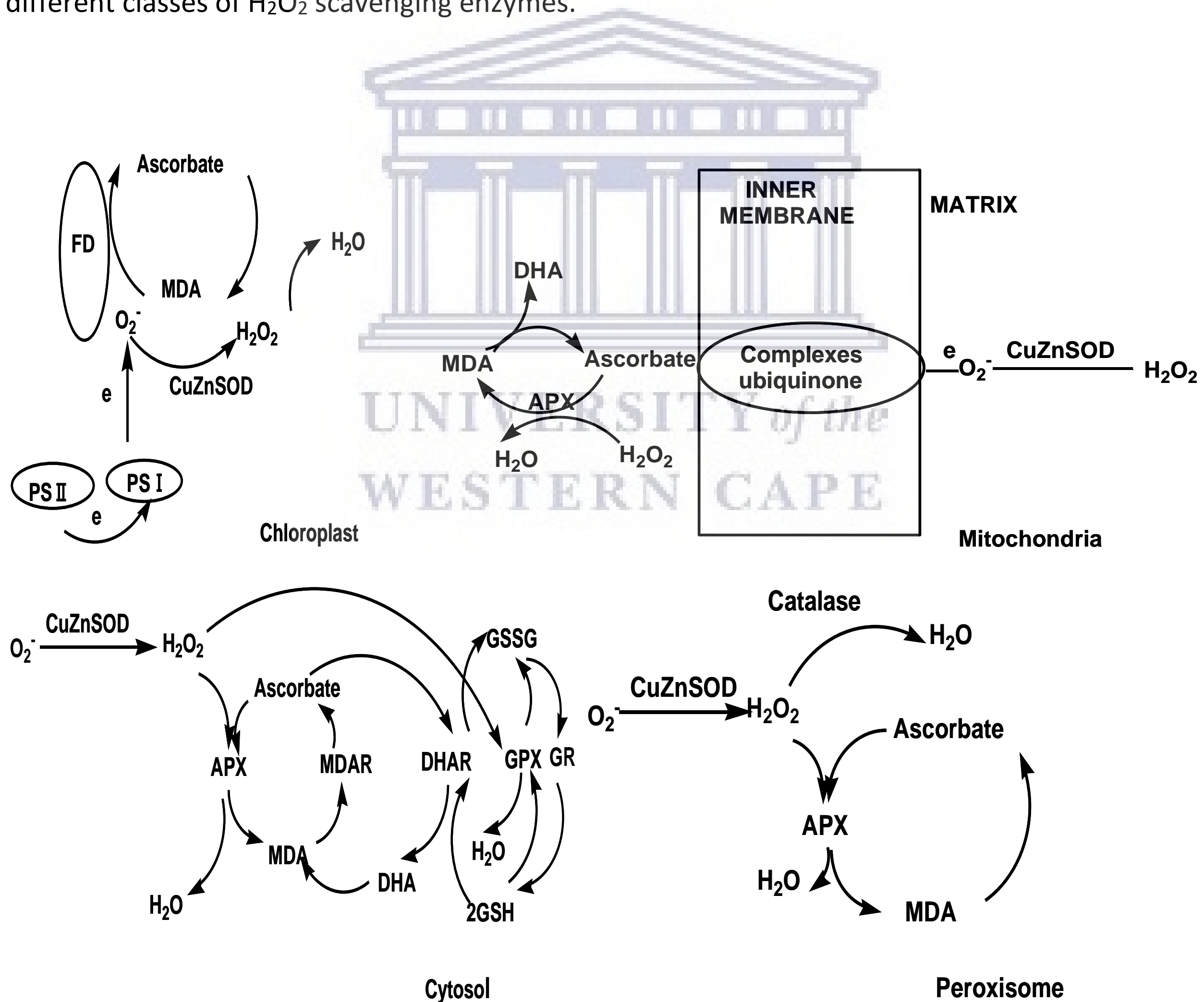

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Figure 1.5: schematic diagram representing the mechanism by which hydrogen peroxide is removed on different cellular compartments through antioxidant defense system (Guller et al., 2010).

1.4.3 Superoxide dismutase

SOD belongs to a class of metalloproteins, which catalyze the dismutation of superoxide (O_2^-) into molecular oxygen and H_2O_2 (Alscher et al., 2002) The hydrogen peroxide must then be reduced by CAT or peroxidases. SODs are classified into 3 isoforms in plants based on their metal cofactor at the active site which contains either iron, manganese or copper-zinc, and they are designated as FeSOD, MnSOD and Cu/ZnSOD (Alscher et al., 2002). Cu/ZnSOD are located in the cytosol and chloroplasts of the plant cell (Kliebestein et al., 1998). MnSODs exist in both eukaryotes and prokaryotes and occur in both the mitochondrial matrix and peroxisomes (del Río, Sandalio et al (2003). Both Cu/ZnSODs and FeSODs are dimers, whereas MnSOD in mitochondria are tetramers Abreu and Cabelli, (2010). The analysis of deduced amino acid sequences suggests that the three types of SOD fall into two phylogenetic families: the Fe-SODs together with the MnSODs separated from the Cu/ZnSODs. Mn and FeSODs are the more ancient type; they possibly evolved from the same ancestral enzyme, whereas Cu/Zn-SODs have a slight homology to Mn and FeSODs. They might have evolved separately in eukaryotes and have been selected in response to a common environmental stress.

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1.4.4 Monodehydroascorbate reductase

Monodehydroascorbate reductase (MDHAR) is a flavin adenine dinucleotide (FAD) enzyme that catalyzes the regeneration of ascorbic acid (AsA) from the MDHA radical using NADPH as the electron donor (Hossain et al., 1984). It is the only known enzyme to use an organic radical MDA as a substrate and is also capable of reducing phenoxyl radicals which are generated by horseradish peroxidase with H_2O_2. MDHAR activity is widespread in plants. The isoenzymes of MDHAR are present in several cellular compartments such as chloroplasts, cytosol, mitochondria and peroxisomes (Yoon et al., 2004). In chloroplasts, MDHAR could have two physiological functions: the regeneration of AsA from MDHA and the mediation of the photo-reduction of

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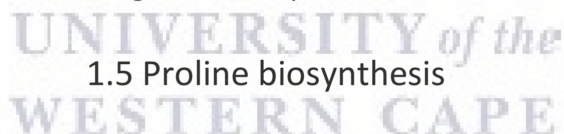
dioxygen to O_2^- when the substrate MDHA is absent (Asada et al., 1994). Characterization of membrane polypeptides from pea leaf peroxisomes also revealed MDHAR to be involved in O_2^- generation. Several studies have shown increased activity of MDHAR in plants subjected to environmental stresses.

1.4.5 Glutathione Reductase

Glutathione reductase is a NADPH-dependent enzyme which catalyzes the reduction of GSSG to GSH and, thus, maintains a high cellular GSH/GSSG ratio (Alscher., 1989, Mahmood et al. 2010). GR belongs to a group of flavoenzymes and contains an essential disulfide group. The catalytic mechanism involves two steps: first the flavin moiety is reduced by NADPH, the flavin is oxidized and a redox-active disulfide bridge is reduced to produce a thiolate anion and a cysteine (Kaminaka et al., 1998). The second step involves the reduction of GSSG via thioldisulfide interchange reactions (Rao et al., 2008). If the reduced enzyme is not reoxidized by GSSG, it can suffer reversible inactivation. Although it is in the chloroplasts, cytosol, mitochondria, and peroxisomes, around 80% of GR activity in photosynthetic tissues is accounted for by chloroplastic isoforms (Edwards et al. 1990; Mmenez et al. 1997). In chloroplasts, GSH and GR are involved in detoxification of H_2O_2 generated by Mehler reaction.



1.5 Proline biosynthesis

Proline biosynthesis in plants is one of the adaptive mechanisms developed by plants to allow them to perceive osmotic stress signals and to optimize adaptive responses for plants to overcome conditions such as water deficit stress. One of these mechanisms, osmotic adjustment, is through the accumulation of large quantities of osmolytes, as it allows plants to avoid water-deficit stress by preventing water loss. Glycine betaine, polyols, sugars and free amino acids are examples of such osmolytes (Chen et al., 2010; Slama et al., 2015). When plants are under water-deficit stress, proline is mainly synthesized from glutamate as illustrated on the flow diagram in Figure 1.7. The bifunctional pyrroline-5-carboxylate synthetase (P5CS) reduces glutamate to glutamyl-5-semialdehyde, which is spontaneously converted to pyrroline-5-carboxylate (P5C). P5C is then reduced to proline by P5C reductase (P5CR). Degradation of proline takes place in

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mitochondria via the sequential action of proline dehydrogenase (ProDH) and P5C dehydrogenase. The rate-limiting steps in proline biosynthesis and degradation are catalyzed by P5CS and ProDH, respectively.

Proline is the most common free amino acid to accumulate in plants subjected to water-deficit stress. However, proline has multifunctional roles such as stabilizing protein complexes, scavenging of free radicals and be a source of carbon and nitrogen for growth after stress relief (Szabados et al., 2010). The beneficial effect of proline on plant growth after stress is likely to be a result of changes in proline metabolism rather than the accumulation of the amino acid itself (Szabados et al., 2010; Sharma et al., 2011).The proline content of plant cells depends on tight regulation of its proline biosynthesis and catabolism. Housekeeping levels of proline biosynthesis occur in the cytosol, but stress-induced biosynthesis is thought to be localized in chloroplasts (Szekely et al., 2008).

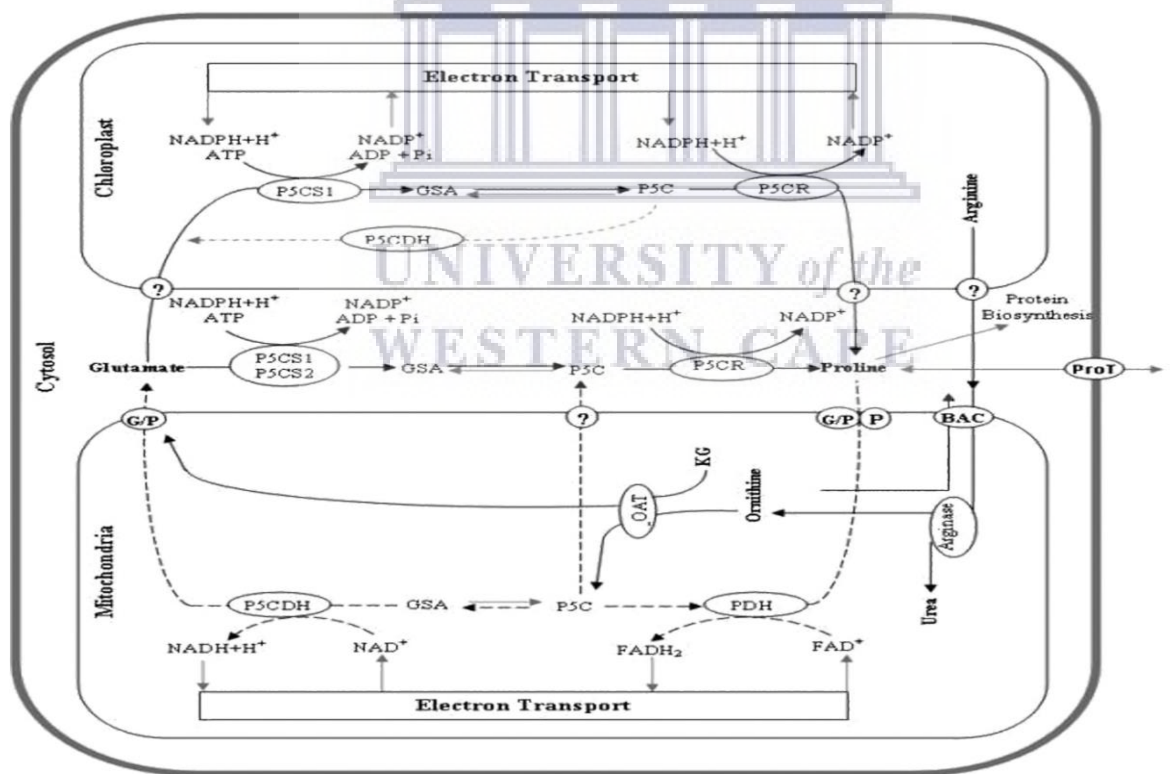

Figure 1.6 illustration of electron transportation on different cellular compartment which lead to the production of Proline in the plant cell (Verbruggen; Hermans, 2008).

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1.5.1 Proline accumulation during water stress

Proline accumulation is one of the most frequently reported modifications induced by water deficit and salt stresses in plants and is often considered to be involved in stress resistance mechanisms (Kavi Kishor et al. 1995). Proline accumulates in a diverse group of plants in response to biotic and abiotic stresses (Szabados; Savoure, 2010). The levels of proline vary from species to species and can be 100 times greater under water deficit compared to well-watered conditions (Verbruggen; Hermnas, 2008). It is suggested that proline acts in membrane and protein protection against the effects of the high concentration of inorganic ions and temperature extremes, in the stabilization of cell structures and detoxification of free radicals (Verbruggen; Hermans, 2008) and as a way to store carbon, nitrogen and energy (Hare; Cress, 1997). Studies also have shown that proline can also alter the activities of antioxidant enzymes (Campos et al., 2011).

1.6 Nitric Oxide

Nitric oxide, a molecule known to easily pass through cellular membrane due to its lipophilic nature, is involved in diverse physiological, biochemical and developmental process in plants (Krasylenk et al., 2010). NO is a reactive nitrogen species generated in different cells and its effects highly depend on its concentration, plant tissue type or age, type of stress and location (Belign et al., 1999; Kao et al., 2004). Besides proline (Kahlaoui et al., 2014), other chemicals such as sodium nitroprusside (a NO donor) are currently being applied to plants exposed to stressful conditions for improvement of growth and yield (Farooq et al. 2009). In recent years, evidence has accumulated showing that exogenous NO can alleviate the harmful effects of environmental stresses in plants (Boogar et al., 2014; Farooq et al., 2009; Liao et al., 2012). Oxidative mechanisms that lead to NO production include the production of NO from L-arginine (L-Arg) and polyamines. Reductive routes for NO production are dependent upon nitrite as the primary substrate and include reduction via nitrate reductase (NR) and a plasma membrane-bound nitrite-NO reductase (NiNOR) and mitochondrial nitrite reduction.

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1.6.1 Nitric Oxide enzymatic biosynthesis

Studies show that there are two well-known plant enzymatic systems capable of NO biosynthesis in plants, nitric oxide synthase (NOS) and nitrate reductase (NR) (Crawford, 2006). Additionally, there may be other sources that appear to involve neither of these enzymes (Arnaud et al., 2006). An apparent plant NOS (AtNOS1) was first identified in Arabidopsis (Guo et al., 2003). Several reports have confirmed that the Atnos1 mutant does indeed show reduced NO accumulation and is impaired in its ability to generate NO in response to various stimuli (Zeidler et al., 2004; Bright et al., 2006; Zhao et al., 2007; Zottini et al., 2007). However, there are many reports in which inhibitors of mammalian NOS such as Nω-nitro-L-arginine methyl ester (L-NAME) were shown, in correlation with an inhibition of NO production, to inhibit various processes in plants, and there is some biochemical evidence for the existence of plant enzymes that use L-arginine to generate NO (Neill et al., 2003; Lamotte et al., 2005; Crawford, 2006; Jasid et al., 2006).

No gene or protein with sequence similarity to known mammalian-type NOS has been found in plants (Garcia-mata, Lamattina et al.,2003; Butt, Lum et al., 2003). These findings suggest that plants have a different NOS enzyme with biochemical properties of constitutive NOS (cNOS). This has been confirmed by discovery of a plant NOS gene that is induced by viral infection and encodes a variant of the protein of glycine decarboxylase (GDC) (chandok, Ytterberg et al., 2003). This discovery has made genetic tools available to elucidate NO synthesis and action and suggests that there is a novel mechanism for making NO in plants.

The other characterized enzymatic source of NO is nitrite reductase. The primary function of NR in plants is to assimilate nitrogen by converting nitrate to nitrite. However, as shown originally in soybean (Dean et al., 1988) and also *in vitro* and *in vivo* in Arabidopsis and other species (Neill et al., 2003; Bright et al., 2006; Crawford, 2006), in an NADPH-dependent reaction, NR can also convert nitrite to NO. The peroxisomal enzyme xanthine oxido-reductase (XOR) can also reduce nitrite to NO. XOR has been shown to reduce nitrite to NO, using NADH or xanthine as the reducing substrate (Godber et al., 2000). However, this reaction only occurs under anaerobic conditions. A plasma membrane-bound, root-specific enzyme, nitrite: Ni-NOR, may function as a further source of NO. This enzyme was identified biochemically as a result of its NO-generating

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activity. Unlike NR, it does not use NADPH as a cofactor, but uses cytochrome c as an electron donor *in vitro,* and its pH optimum is more acidic than that of NR. Recent work has also suggested that, in addition to NR mediated nitrite-dependent NO production, electron transport processes in mitochondria and chloroplasts can also generate NO from nitrite (Gupta et al., 2005; Modolo et al., 2005; Planchet et al., 2005; Jasid et al., 2006). Additionally, apoplastic conversion of nitrite to NO at low pH has been demonstrated in the barley aleurone layer (Bethke et al., 2004).

1.6.2 Nitric oxide as a signaling molecule

Chief among the redox-based post-translation modifications is S-nitrosylation, the addition of a NO moiety to a reactive cysteine (Cys) thiol to form S-nitrosothiol (SNO) (Spadaro et al., 2010). This redox modification is a central route for NO bioactivity, as it can donate upon such Cys residues the ability to serve as a molecular switch, enabling the target protein to be directly responsive to changes in cellular redox status. S-nitrosylation has been shown to modulate enzyme activity. NO can react reversibly with glutathione (GSH), producing GSNO, a reservoir of NO (Liu et al., 2001; Sakamoto et al., 2002). GSNO is metabolized by GSNO reductase (GSNOR), which controls NO and nitrosothiol levels, being a key enzyme in most NO-regulated processes, such as pathogen defense, root development, and nitrogen assimilation (Feechan et al., 2005; Rustérucci et al., 2007; Frungillo et al., 2014). It has been shown that GSNO inhibits nitrate uptake and its reduction to nitrite, which would prevent NR-dependent NO production (Figure 1.7; Frungillo et al., 2014). Additionally, CO_2 elevation distinctly increased S-nitrosylated NR levels in plants grown under high-nitrate conditions, along with a significant decrease in NR activity similarly to that which occurs with chilling treatment (Cheng et al., 2015; Du et al., 2015). These results suggest that S-nitrosylation of NR may decrease NR activity. Interestingly, NR regulation in response to high CO_2 levels appears to be dependent on nitric oxide synthase-like (NOSl) activity (Du et al., 2015), pointing to a regulation between the different NO-production pathways.

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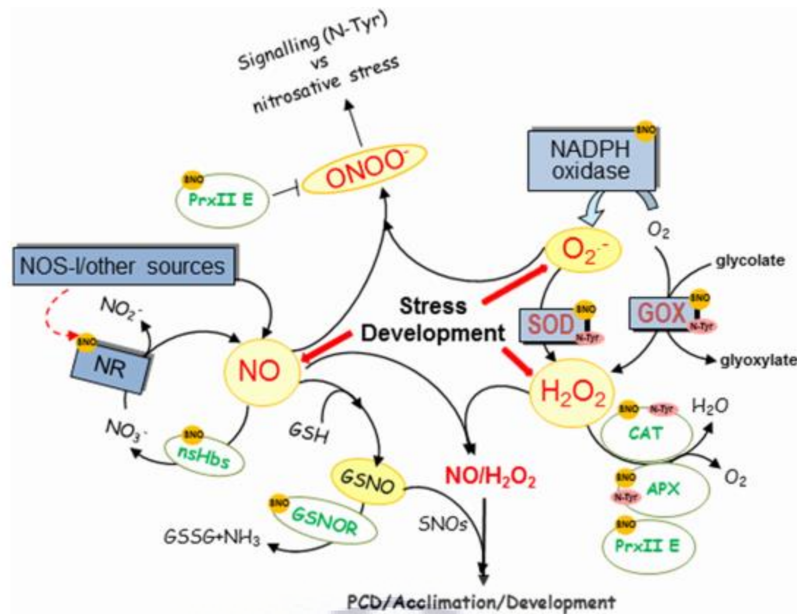

Figure 1.7 Different NO production pathways and signaling interactions between NO and H_2O_2 induced by stress development and regulation of antioxidant defense mechanism adapted from (Frungillo et al., 2014; Du et al.,2015; Cheng et al 2015)

Moreover, NO could also activate NR activity under a relatively low-nitrate concentration, the haem and molybdenum centers in NR, which enhances electron transfer during nitrate reduction (Du et al., 2008). To complete the cycle, it has also been demonstrated that NO inhibits GSNOR1 through S-nitrosylation avoiding GSNO degradation at the same time and regulating plant nitrosothiol levels as illustrated in Figure 1.7 (Frungillo et al., 2014). Thus, GSNO feedback regulates nitrogen flux through nitrite assimilation pathways and controls its bioavailability by modulating its own consumption (Frungillo et al., 2014). In the context of hypersensitive response, NO is also able to regulate the level of its own radicals, such as $ONOO^-$, through S-nitrosylation of peroxiredoxin II E (PrxII E) that inhibits its H_2O_2-reducing and peroxinitrite-detoxifying activities (Romero-Puertas et al., 2007).

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1.6.3 Defense Role of Nitric Oxide in plants

NO has become known as a vital molecule in drought tolerance of several plant species through enhancing the antioxidant systems, ROS and osmolytes metabolism (Filippou et al., 2014; Shiet al., 2014). NO may also act as a mediator to prevent water loss via ABA-induced stomatal responses through various signaling pathways such as mitogen-activated protein kinase (MAPK), cyclic guanosine monophosphate (cGMP) and Ca^{2+} (Gayatri et al., 2013). Fan et al. (2012) showed that cellular changes in plants subjected to water deficit stress were affected by NO as shown by exogenous application of SNP. NO-responsive drought-related genes include transcription factors, promoters and antioxidant-related genes (Besson-Bard et al., 2009a,b; Grun et al., 2006; Palmieri et al., 2008).Defense genes such as pathogenesis-related 1 protein and phenylalanine ammonia lyase are induced by the addition of NO donors (SNP). The same genes were induced by cGMP, further implying a guanylate cyclase pathway (Durne et al., 1998). NO plays a key signaling role during the hypersensitive response, a reactive oxygen species generating response resulting in localized cell death and limiting nutrient availability to an invading pathogen. NO, in concert with hydrogen peroxide, can induce cell death in this role (Delledonne , et al 1998).

1.6.4 Nitric oxide role in plant programmed cell death

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There are numerous and often contradictory reports concerning NO and programmed cell death (PCD). Elevated levels of NO were sufficient to induce cell death in Arabidopsis cell suspensions, independently from reactive oxygen species (ROS) (Clarke et al. 2000). The process was blocked by a specific inhibitor of guanylate cyclase, the enzyme producing cGMP, which is a well-established second messenger mediating NO responses in mammalian cells. The influence of NO and ROS donors on PCD was investigated in tobacco (de Pinto et al. 2002). The increase in either NO or ROS separately did not induce cell death, whereas the simultaneous increase of NO and ROS activated a process of cell death, with typical cytological and biochemical features of PCD (de Pinto et al. 2002). The interaction between NO and ROS in PCD induction was investigated in soybean cell suspensions (Delle donne et al. 2001), and the researchers concluded that NO by itself does not induce PCD, but the key factor determining it is the NO:-superoxide ratio (Delle donne et al. 2001). Contrary to that, in Taxus Brevifolia and Kalanchoë daigrenothtiana SNP or

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mechanical stress caused to burst, which preceded a significant increase in nuclear DNA fragmentation and cell death (Pedroso et al. 2000; Yamasaki, 2000; Romero-Puertas et al., 2004).

On the other hand, Beligni et al. (2002) provided data indicating a NO-dependent delay of developmental PCD mediated by gibberellic acid (GA) in barley aleurone layers. Although NO can inhibit PCD in GA-treated cells, it does not have a general effect on cellular metabolism and is only predicted as a specific endogenous modulator of PCD (Beligni et al. 2002).

1.6.5 Nitric Oxide and Abiotic stress

Literature shows that exogenous NO can reduce the negative effects of water stress (Farooq et al., 2009, Liao et al., 2012, Rahimian Boogar et al., 2014). Exogenous SNP application has been reported for drought stress tolerance (Hao et al., 2008) on drought-stressed hull-less barley seedlings treated with different concentrations of SNP; the results showed that drought stress seriously altered physiological functions and growth but NO application alleviated the drought enduring damages by antioxidant and ROS scavenging enzyme activity (Gan et al., 2015). NO also interacts with plant hormones and various other signaling molecules, and regulates osmoprotectants that protect against drought stress. In contrast, NO scavengers such as L-NAME (NG-nitro-L-arginine-methyl ester) reduce endogenous NO levels, leading to higher stress sensitivity (Hao et al., 2008, Xu et al., 2010a).

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Removal of NO using a combination of both chemical and genetic approaches has been shown to inhibit ABA-related stomatal responses. ABA induces NO production along with a rise in pH and H_2O_2 levels in water-stressed plants (Greco et., 2012; Wendehenne et al., 2001). NO has also been prostulated to activate mitogen-activated protein kinase (MAPK) signaling cascades which may then drive stomatal closure (Zhang et al., 2007).

1.6.6 Signaling interaction

Signaling interactions between ABA, H_2O_2, and NO occur in order to mediate plant survival under adverse conditions. ABA activates H_2O_2 generation by NADPH oxidase via a signalling pathway involving the ABA receptors, calmodulin, the OST1 protein kinase, and other unidentified

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components (Desikan et al., 1998b; Kller et al., 1998; Torres et al., 1998). H_2O_2 induces NO generation by nitrate reductase and NOS-like enzymatic activity (Lin et al., 2012). NO induces stomatal closure through a process that requires MAPKs, cGMP, and Ca^{2+} (McAinsh et al., 1996; pei et al., 2000). It is also likely that NO signaling independent of ABA and H_2O_2 can also occur to cause stomatal closure during certain conditions (Bowler and Fluhr, 2000). NO also enhances antioxidant gene and enzyme activity via MAPK and other unidentified signaling pathways (Hirt, 1997).

NO proved to be capable of regulating multiple plant responses towards a variety of biotic and abiotic stresses and alleviating some consequences provoked by oxidative stresses (Delledonne, 2005; Bligni and Lamattima, 1999a; Crawford and Guo, 2005). High levels of NO can damage membranes and cause DNA fragmentation (Pedroso et al., 2000; Yamasaki, 2000; Romero-Puertas et al., 2004) and can reduce photosynthesis in oat and alfalfa (Hill and Bennet, 1970) and reproduction in carrot cell suspensions (Zottini et al.,2002) are inhibited by NO exposure. Interestingly, NO signaling is based on interactions with plant hormones. Furthermore, cytokinin (CTK) stimulated more NO formation, probably mainly via a NR source under the conditions of drought stress in *Zea mays* (Ruixin, Kaibo, et al., 2009). An increase in NO levels alleviates drought-induced ROS damage to plants. NO prevents oxidative damage in plants by the regulation of general mechanisms for cellular redox homeostasis and by enhancing the H_2O_2-scavenging enzyme activities (Lamattina et al., 2003; Shi et al., 2007; Zheng et al., 2009). Although the NO molecule possesses antioxidant properties (Karplus et al., 1991), NO rapidly reacts with, oxygen species, hemes, thiols and proteins to produce biochemical signals that directly and indirectly regulate enzymatic activities.

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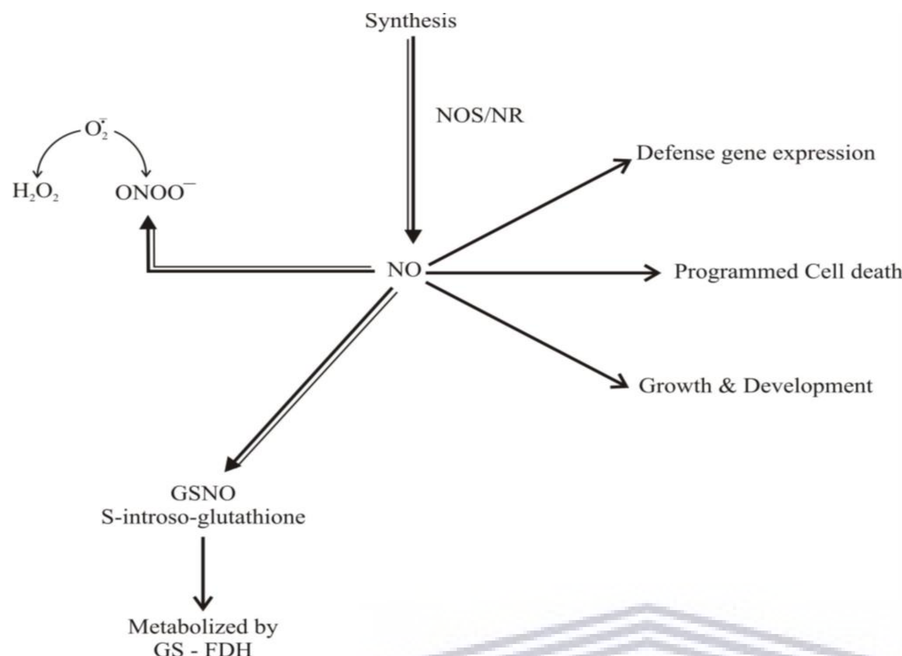

Fig. 1.8 NO signalling in plants. Double lined arrows represent potential synthesis and biochemical interactions while single lined arrows represent potential cellular effects and target sites for NO action.

1.6.7 Hydrogen peroxide and nitric oxide interactions

H_2O_2 and NO are key signaling molecules produced in cells in response to various stimuli and involved in a wide range of plant signal transduction processes. It is often observed that NO and ROS such as superoxide and H_2O_2 are generated in response to similar stimuli and with similar kinetics. NO and ROS can interact in many different ways. For example, they can interact chemically in the formation of compounds such as peroxynitrite. NO may also affect the activities of enzymes that alter ROS levels. Thus, they could both impact either negatively or positively on the related signaling pathways and thereby lead to additive and possibly synergistic responses. Stomatal closure in response to ABA is one such example where this occurs. Lum et al. (2002) observed that exogenous H_2O_2 induced NO generation *Phaseolus aureus* leaves. In a chemiluminescence-based assay, H_2O_2 induced a substantial increase in an apparent NOS-like activity. This increase was reduced by using an NOS inhibitor. Interestingly, the H_2O_2 induced NO

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generation was inhibited by the calcium channel blocker. Thus, it is possible that Ca^{2+} ions may mediate this effect of H_2O_2.

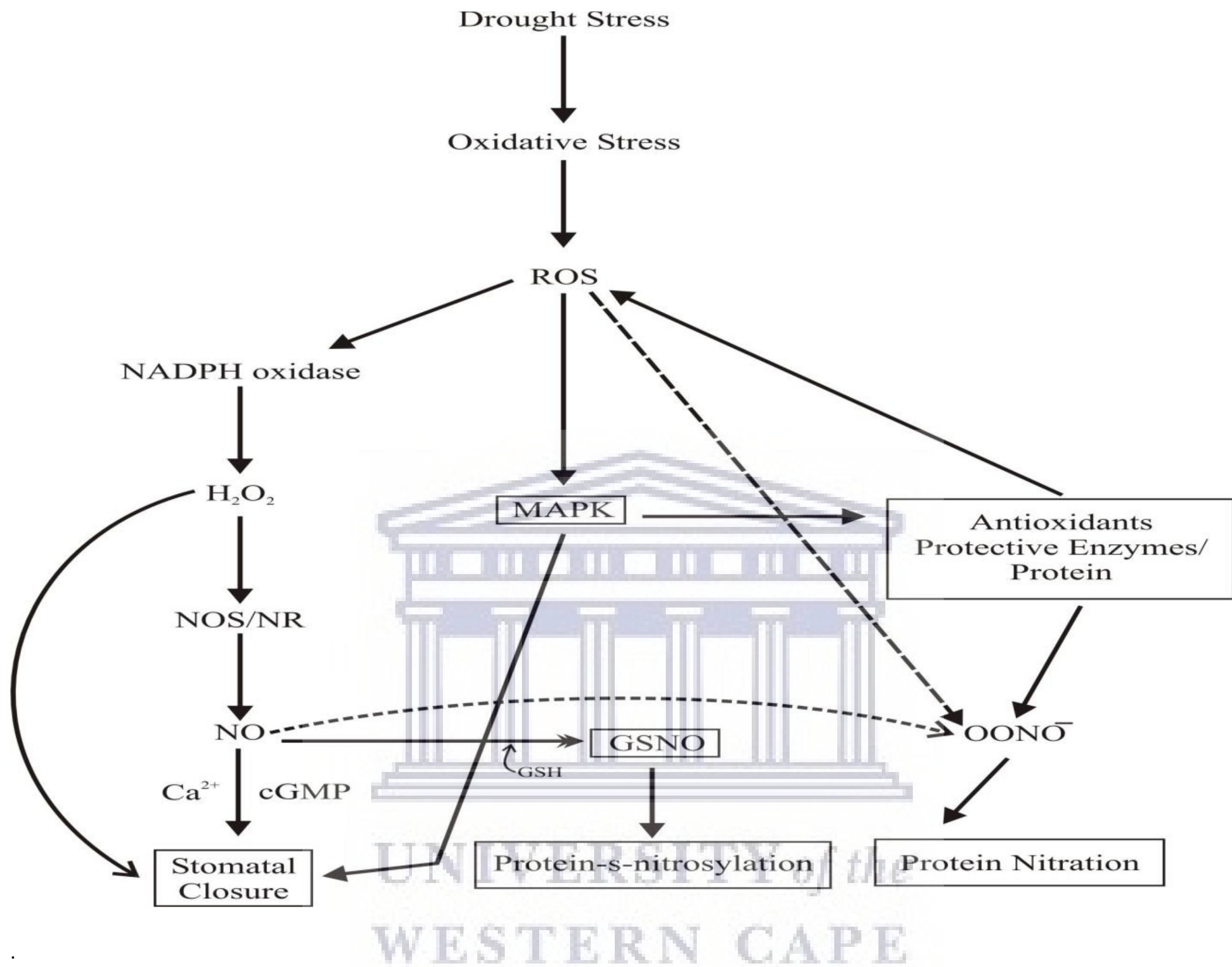

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Figure 1.9 Schematic summary of plants response on drought stress, accumulation and scavenging of ROS and interaction of ROS and nitric oxide.

Similarly, ABA-induced NO synthesis is dependent on prior H_2O_2 generation in both *V. faba* (Dong et al., 2005) and Arabidopsis (Bright et al., 2006). Removal of the H_2O_2 with antioxidants or inhibition of its synthesis by inhibiting NADPH oxidase activity prevents NO generation and stomatal closure. Similarly, removal of the NO using PTIO prevents either H_2O_2- or ABA-induced stomatal closure. Guard cells of the AtrbohD/F double mutant (Kwak et al., 2003) also fail to make NO in response to ABA. However, H_2O_2 stimulation of guard cell NO accumulation in the Atnos1 mutant was as in the wild type (Bright et al., 2006). Thus, the requirement of AtNOS1 for NO

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synthesis must be upstream of H_2O_2 and its signalling effects. It also appears that the ABA–H_2O_2–NO cascade is not restricted to guard cells.

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Chapter 2

Materials and Methods

2.1 Seed germination and growth of *Zea mays*

Commercial maize (*Zea mays*) seeds were surface sterilized for 10 minutes in a 12% bleach solution containing a final concentration of 0.35% (v/v) sodium hypochloride, and then rinsed 5 times with distilled water to remove the bleach solution. Seeds were placed on paper towel and kept in the dark until radicles emerged. Germinated seeds were planted in autoclaved Promix (one plant per 3-liter pot) and grown on regulated conditions of 25/19 °C day /night temperature cycle under a 16/8 h light/dark cycle, at a photosynthetic photon flux density of 300 µmol photons $m^{-2}.s^{-1}$ during the day phase. Seedlings were supplied with 250 ml of water every third day of a week directly to the promix around the plant. After 7 days of planting, maize seedlings reached V1 stage (when the collar of the first true leaf is visible).

2.2 Plant treatments

When plants reached the V2 stage, water deprivation commenced by watering once a week with 20 % of the initial amount (250 ml per pot), while for the well-watered plants 250 ml was supplied twice a week. Water deprivation on maize seedlings was conducted for a period of 21 days. Then pre-treatment with inhibitors was initiated at the V4 stage on water deprived seedlings with the following final concentrations: 0.5 mM L-NAME and 5 mM DMTU. On the following day, seedlings were then treated with various treatments with the following final concentrations: 50 µM DETA/NO, a combination of 5 mM DMTU + 0.5 mM L-NAME, a combination of 5 mM DMTU+50 µM DETA/NO, and with 0.5 mM H_2O_2 on well-watered plants and the untreated (control) was supplied with HEPES at pH7.3. Therefore there were a final of eight treatments including the control in the following order: Untreated, 0.5 mM H_2O_2, Water Deficit (WD), WD + 5 mM DMTU, WD + 0.5 mM L-NAME, WD+50 µM DETA/NO, WD + 5 mM DMTU + 0.5 mM L-NAME and WD+5 mM DMTU + 50 µM DETA/NO). Plants were harvested after a week of treatment and were at stage V5. Relative water content and cell viability were measured while harvesting.

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2.3 Evaluation of Cell viability on Maize Leaves

Cell viability was measured spectrophotometrically through the uptake of Evan's blue dye on untreated and treated maize leaves by the assay described by Sanevas et al (2007). The degree of dye uptake is inversely proportional to the cell viability (increased uptake of the dye is the indication of compromised cell membrane integrity). Fresh leaves of the control and treated maize leaves were harvested, then 1 cm sections from the second youngest leaf were excised. The sections were then placed individually in 0.25% Evan's blue dye for an hour. Then leaf sections were rinsed thoroughly with distilled water to ensure the removal of unbound dye. Leaf sections were incubated for an hour in 1% SDS at 55°C in order to release Evan's dye from the cells into the solution. The released Evan's blue dye was then detected spectrophotometrically by measuring the absorbance readings at 600nm for each triplicate sample. And by subtracting the sample absorbance from the blank absorbance to obtain a final A_{600}nm values of the samples.

2.4 Measurement of Relative Water Content

Relative water content was measured in duplicate using the second youngest leaf of well-watered and water-deprived plants. For determining the fresh weight, leaves detached from the second youngest leaf with cutting done from the tip of the leaf to give 10 cm sections which were weighed. The leaves were then incubated in a beaker filled with distilled water for 2 hours to allow the full uptake of water to determine the turgor weight of the leaves. Leaves were then surface dried to ensure there was no water on the surface and they were subsequently weighted. For dry weight determination, leaves were placed at 80°C in an oven to dry for 48 hours, then weighed to obtain the dry weight.

2.5 Metabolite extraction

Plant material (100 mg) was ground into a fine powder in liquid nitrogen and homogenized in 500 µl of cold 6% (w/v) trichloroacetic acid TCA, followed by centrifugation at 12 000 rpm for 30 minutes at 4°C, followed by collection of the supernatant for MDA and H_2O_2 measurements. The rest of the plant material was ground into fine powder by pestle and mortar that had been pre-cooled with the liquid nitrogen and stored at -80°C for further biochemical assays.

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2.6 Determination of Lipid Peroxidation

Lipid peroxidation was assayed indirectly via measuring Malondialdehyde (MDA) which is the by-product of lipid peroxidation. MDA was determined by mixing TCA extracts in a 1:2 ratio with a solution containing 20% TCA and 0.5% thiobarbituric acid (TBA) in a final volume of 600 µl samples were briefly vortexed for homogenation and incubated at 90°C for 20 minutes, then chilled on ice for 10 minutes to stop the reaction. Samples were then centrifuged for 5 minutes and the absorbance readings were measured in triplicates spectrophotometrically at 532 nm and 600 nm wavelength as described by (Buege et al., 1978). The amount of MDA was calculated using a molar extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.7 Determination of H_2O_2 content

H_2O_2 content was assayed by homogenizing 50 µl of TCA extract with 150 µl of reaction buffer containing 5 mM K_2HPO4, pH 5.0 and 0.5 M KI (Velikova et al., 2000). Reactions were incubated at 25°C for 20 min, followed by recording of absorbance readings in triplicate at 390 nm. H_2O_2 content was calculated using a standard curve constructed with the absorbance of H_2O_2 standards read at an absorbance of 390 nm.

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2.8 Protein Extraction and concentration determination

Plant material was ground into fine powder using mortar and pestle under cold conditions using liquid nitrogen. Cold extraction buffer 5% Polyvinylpolyoyrolidone (PVPP), 40 mM KPO_4, 1 mM EDTA pH7.4 was added to the plant material in centrifuge tube at a plant material: extraction buffer ratio of 1:5 and vigorously vortexed, then centrifuged at 13000 rpm for 30 minutes at 4°C. The supernatant was transferred to new tubes and centrifuged again under the same conditions. This step was repeated twice. The protein was then quantified using a Bradford Assay and stored at -20°C.

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2.9 Determination of Proline Content

Crude protein was extracted using a 5% PVPP buffer mixed with a prepared reaction solution (10 ml of 3% sulphosalicylic acid, 10 ml of acetic acid and 20 ml of 2.5% acid-ninhydrin) as described by (Bates et al., 1973). The protein extract (50 µl) was mixed with 1 mL of reaction solution. The homogenate was centrifuged at 13 000 rpm for 15 min in a 1.5 ml centrifuge tube. The reaction solution with 50 µl of 100 mM PBS at pH7.0 served as a reference. The reaction mixture was incubated at 95°C for 30 minutes (the color turned red after boiling) and cooled on ice for 5 minutes. For measuring the absorbance, 200 µl of the reaction solution was pipetted to the wells of the microtitter plate. A proline standard curve was prepared for quantification using L-Proline (0, 5, 10, 20, 25 and 30 µg). The absorbance of the chromophore was read spectrophotometrically at 520 nm using PBS as a blank. The proline content of samples was calculated by referring to a standard curve drawn from absorbance readings from samples containing known concentrations of proline.

2. 10 Nitric oxide measurement

Tissue extracts were obtained by weighing out ground 150 mg of plant tissue and adding 750 µl of 6% TCA (w/v) in a micro-centrifuge tube. After vortexing the mixture, the sample was spun for 20 min at 10,000 rpm. The supernatant was transferred to a clean tube and stored at -20°C. To the supernatant, 200 Units of catalase and 200 Units of superoxide dismutase were added. The mixture was mildly vortexed and incubated for 10 min. Freshly prepared oxyhemoglobin was added to a final concentration of 10 µM. The mixture was incubated for 2 min, followed by measurement of NO content spectrophotometrically at 401 and 421 nm as described by (Pasqualini et al., 2009), based on the conversion of oxyhemoglobin (HbO_2) to methemoglobin (MetHb).

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2.11 Detection of antioxidant enzyme activity in maize using native gels

2.11.1 APX activity

For determination of the response of *Zea mays* APX isoforms in the treatments, electrophoretic APX separation was carried out as previously described by Mittler and Zilinskas (1993) and non-denaturing polyacrylamide gel electrophoresis was performed at 4°C on 12% polyacrylamide mini gels. Prior to loading extracts containing 50 µg of protein into the wells, gels were equilibrated with running buffer containing 2 mM ascorbate for 30 min at 4°C. After the electrophoresis, gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 20 min and then transferred to solutions containing 50 mM potassium phosphate buffer (pH 7.8), 5 mM ascorbate and 2 mM H_2O_2 for 20 min. The gels were washed in the buffer for 1 min and submerged in a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM N,N,N′, N′-tetra-methyl ethylene-diamine and 2.5 mM nitroblue tetrazolium for 10–20 min, with gentle agitation in the presence of light. The gel images were captured and analyzed by densitometry using AlphaEase FC imaging software (Alpha Innotech Corporation**).**

2.11.2 Superoxide Dismutase activity

Protein extracts were obtained from leaf and root tissue. Tissue (100 mg) was weighed and homogenized in 500 µl of buffer [40mM K_2HPO4, pH 7.4, 1 mM ethylene diaminetetra acetic acid (EDTA), 5% (w/v) polyvinylpyrolidone (PVP) molecular weight = 40 000]. Native polyacrylamide gels (12%) were prepared and equal amounts of protein extracts from both leaves (200 µg) and roots were loaded on gels. Activity of all SOD isoforms was determined by staining with 0.5 mM riboflavin and 2.5 mM nitroblue tetrazolium, as described by Dewiret al. (2006). Densitometry was measured and used to estimate SOD activity for each sample.

2.11.3 Catalase activity

For determination of catalase activity, a 7.5% separating gel was prepared and 200 µg of protein extract for both root and leaves were loaded on the gel and electrophoresed at 60 V for 8 hours. Then, after performing all the staining steps in the dark, the gel was initially washed three times

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with distilled water for 10 minutes. Then, 0.003% of hydrogen peroxide was prepared by mixing 10 µl of 30% H_2O_2 solution with 99.99 ml of distilled water, then gels were incubated in 0.003% H_2O_2 solution for 30 minutes. Two fresh conical tubes were used, one for preparing 2% ferric chloride by weighing 0.6 g and dissolving it in a 30 ml of distilled water. On the second tube, 2% of potassium ferric cyanide was prepared by weighing 0.6 g and also dissolving it in 30 ml of distilled water. Both solutions were covered with foil to avoid exposure to the light. Ferric chloride and potassium ferric cyanide solutions were poured simultaneously to a beaker and mixed immediately, then immediately poured on the staining vessel containing the gel, but not directly on the gel. The gel was agitated on the light box until the gel darkened and chromatic bands began to form. The stain was poured off and the gel was rinsed extensively with distilled water and photographed.

2.12 Statistical Analysis

One-way analysis of variance (ANOVA) was used to evaluate statistical validity of the results and means (from three independent experiments) were compared according to the Turkey–Kramer test at 5% level of significance, using GraphPad Prism 6.01 software.

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Chapter 3: Results

3.1 Effects of the chemical treatment on water stress responses in maize seedlings.

3.1.1 Water status is altered by the various treatments.

Figure 3.1 illustrates changes in RWC observed when plants were grown under insufficient water supply. Comparison of water status in leaves upon treatment with 0.5 mM H_2O_2 well-watered maize seedlings versus the untreated well-watered seedlings shows that there was an increase of approximately ±15% in H_2O_2 content. A decrease of 58% in RWC was observed in water-deprived maize leaves compared to the untreated well-watered leaves was observed. Treatment with 5 mM DMTU caused a 38% decrease of RWC compared to untreated well-watered leaves, whereas 0.5 mM L-NAME treatment caused a decrease of approximately 47% in RWC. Treatment of water-deprived maize seedlings with 50 µM DETA/NO resulted in a 37% decrease compared to the untreated control. Treatment of water-deprived maize seedlings with 5 mM DMTU in combination with 0.5 mM L-NAME resulted in a decrease of approximately of 38% decrease in RWC compared to the untreated well-watered seedlings. Treatment of water-deprived maize seedlings with 5 mM DMTU together with 50 µM DETA/NO had no effect on the RWC.

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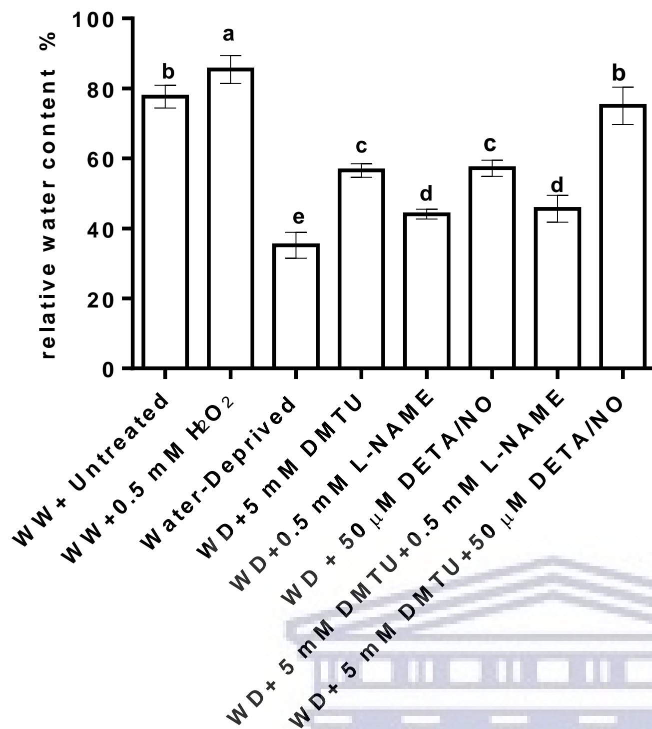

Figure 3.1 Representation of oxidative stress effects on maize seedlings under water deficit and various treatments: RWC assay was conducted on the 2nd youngest leaf of maize at V3 growth stage. The assay was done on untreated well-watered and H_2O_2-treated well-watered, water-deprived seedlings including various treatments performed in a 7 days period, with 2 days intervals (0.5 mM H_2O_2, 5 mM DMTU, 0.5 mM L-NAME and 50 µM DETA/NO). The error bars signify standard deviation, bars with the same letters are statistically similar, where *P<0.05*

3.2 DETA/NO treatments reduced cell death on maize subjected to drought stress

Presented below are the responses of maize leaves during water stress and changes in cell viability in response to the various treatments. There was no significant difference in cell death between leaves of plants treated with 0.5 mM H_2O_2 and the untreated well-watered leaves. Water-deprived leaves showed an increase of approximately 78% in cell death compared to the untreated control. Water-deprived plants treated with 5 mM DMTU had approximately a 14% increase of cell death compared to the untreated control plants. Treatment with 0.5 mM L-NAME in water-deprived leaves showed a significant increase of 57% compared to the untreated

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control. When water-deprived plants were treated with 50 µM DETA/NO, there was 17% increase of cell death in their leaves compared to the untreated control. Treatment of water stressed leaves with a combination of DMTU and L-NAME resulted in an increase of 38% in cell death compared to the control whereas the combined treatment of DMTU and DETA/NO in water deficient leaves reduced cell death to a level equivalent to the untreated control.

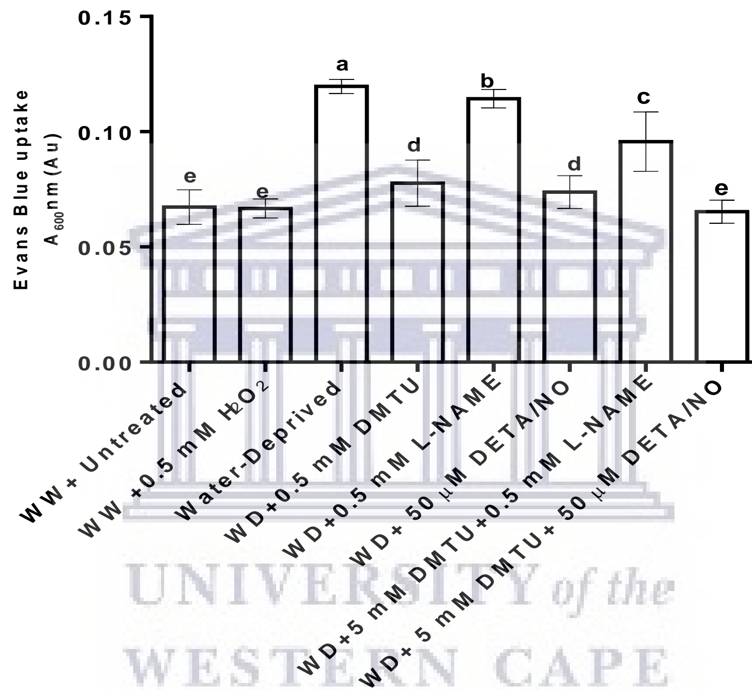

Figure 3.2: The degree of cell death in maize seedlings, supplied with water and those deprived of water. The cell death assay shows in absorbance units (Au) as a function of Evan's blue uptake. Cell death was measured on leaves from untreated well-watered maize seedlings and well-watered maize seedlings treated with H_2O_2, DMTU, LNAME and DETA/NO) . The error bars signify standard deviation, bars with the same letters are statistically similar, where *P<0.05*

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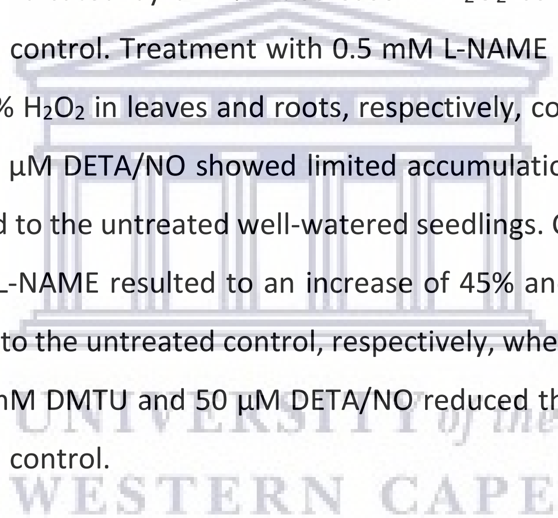
3.1.3 Exogenous application of a H_2O_2 scavenger and NO donor reduced accumulation in maize leaves during drought stress

The level of hydrogen peroxide is shown in Figure 3.4. Well-watered maize seedlings treated with 0.5 mM H_2O_2 showed an increase of 9% in leave H_2O_2 content compared to the untreated control. The well-watered roots of maize seedlings treated with H_2O_2 had an equal amount of H_2O_2 content to the untreated control. An increase of approximately 190% in H_2O_2 content in water-deprived maize leaves was observed compared to the untreated control. For roots, there was a 180% increase in H_2O_2 content under water deficit compared to the untreated well-watered roots. Exogenous application of 5 mM DMTU to water deprived maize seedlings reduced the accumulation of H_2O_2 , as indicated by a 22% decrease in H_2O_2 content in leaves and roots compared to the untreated control. Treatment with 0.5 mM L-NAME resulted in an increase of approximately 43% and 41% H_2O_2 in leaves and roots, respectively, compared to the untreated control. Treatment with 50 µM DETA/NO showed limited accumulation of H_2O_2 (25% in leaves and 23% in roots) compared to the untreated well-watered seedlings. Combined treatment with 5 mM DMTU and 0.5 mM L-NAME resulted to an increase of 45% and 47% in H_2O_2 content in leaves and roots compared to the untreated control, respectively, whereas combined treatment of maize seedlings with 5 mM DMTU and 50 µM DETA/NO reduced the levels of H_2O_2 to those equivalent to the untreated control.



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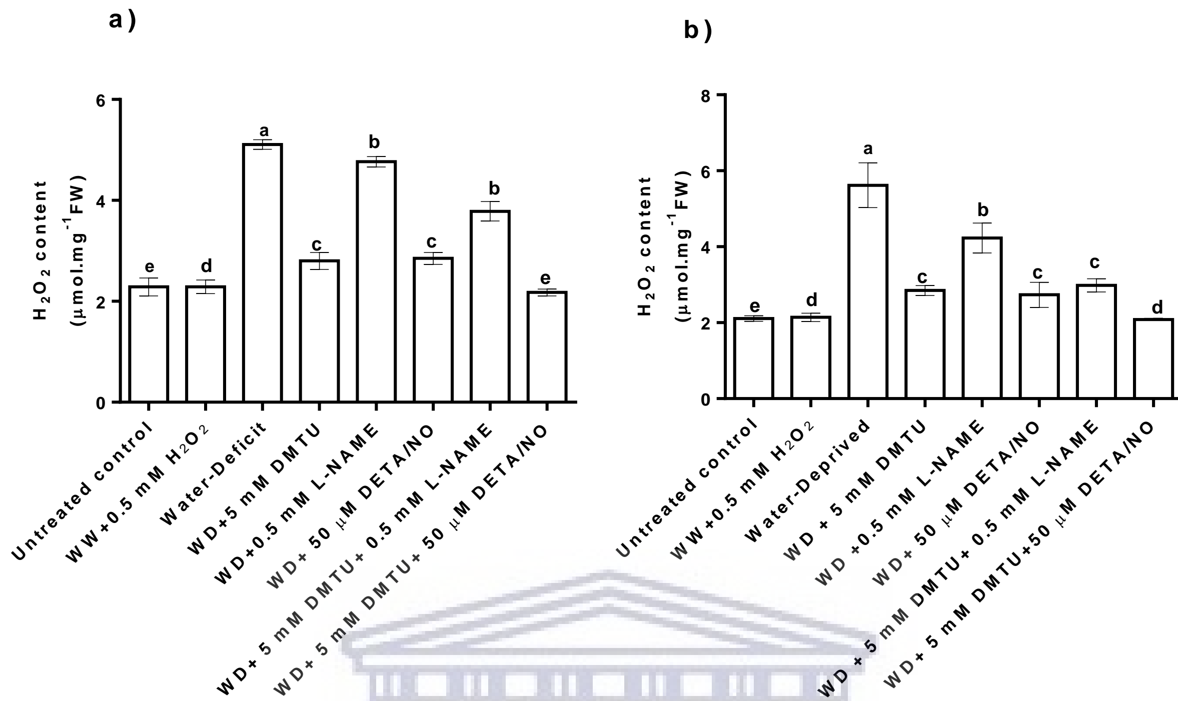

Figure 3.3: The effect of the various treatments on leaf and root H_2O_2. H_2O_2 content measured in leaves (a) and roots (b) from untreated well-watered maize seedling and well-watered maize or water-deprived maize seedling treated with H_2O_2, DMTU, L-NAME and DETA/NO). Error bars signify standard deviation, bars with the same letters are statistically similar, where *P<0.05***.**

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3.1.4 MDA accumulates higher in leaves than roots of maize seedlings subjected to drought

To determine the extent of oxidative damage induced by water stress and the effects of various treatments, MDA content was investigated in maize leaves and roots showed in Figure 3.4. Leaves from well-watered plants treated with H_2O_2 showed no significant difference in MDA content compared to the untreated control and the same trend was observed on roots. Water deprived plants showed an increase of 64% in MDA content compared to the untreated well-watered control. In roots, an increase of approximately 35% was observed compared to the untreated control. Water-deprived maize treated with 5 mM showed an increase of 14% in MDA

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content in leaves and roots compared to the untreated control. The MDA content in water-deprived maize seedlings treated with 0.5 mM L-NAME increased by 30% and 21% in leaves and roots, respectively, compared to the untreated controls. The MDA content in the 50 µM DETA/NO treatment was 14% compared to the untreated control in roots and leaves. The combined treatment with DMTU and L-NAME resulted in approximately a 28% increase in MDA content in leaves and 24% in roots compared to the untreated control, whereas the combined treatment of DMTU and DETA/NO caused no change in MDA levels.

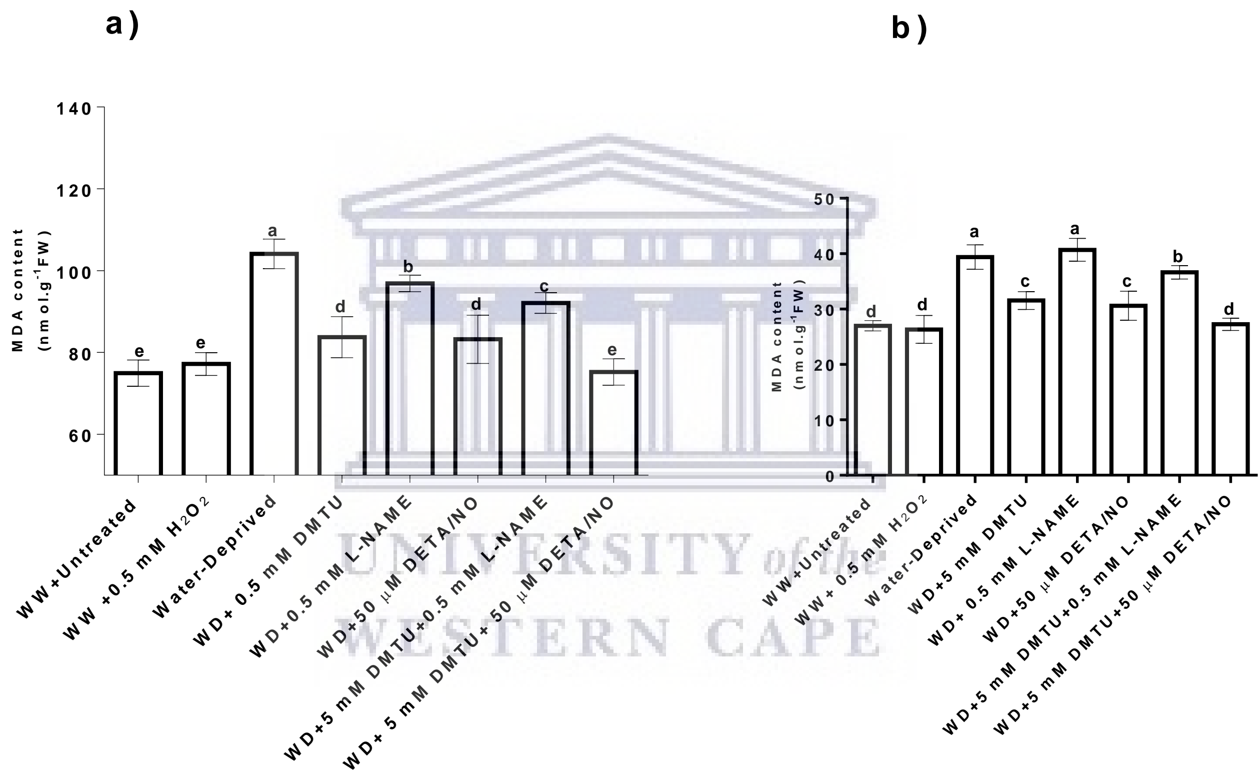

Figure 3.4 Malondialdehyde content in leaves and roots of water-deprived maize seedlings. MDA content in leaves and roots of untreated well-watered seedlings and well-watered maize seedlings treated with H_2O_2, DMTU, L-NAME and DETA/NO, together with MDA content of water-deprived maize leaves and roots. The error bars signify standard deviation, bars with the same letters are statistically similar, where *p<0.05*.

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3.1.5 Proline content during water deficit stress in maize

Figure 3.5 shows that the accumulation of proline was higher in roots than in the leaves. Proline content in leaves from well-watered maize seedlings treated with 0.5 mM H_2O_2 was the same as untreated well-watered maize leaves (control), and a similar trend was observed in the roots from seedlings treated with H_2O_2. Water deficit resulted in a 63% increase in leaf proline content when compared to the untreated well-watered plants, whereas on roots there was an increase of 51% in proline content in comparison to the untreated root control. A 15% increase in proline content was observed in response to application of 5 mM DMTU in water-deprived maize leaves compared to the untreated well-watered plants. A 30% increase in roots of proline content was observed in response to treatment with DMTU when compared to the untreated control. The 0.5 mM L-NAME treatment resulted in a 47% increase in leaf proline content and a 53% increase in root proline content in comparison to the untreated well-watered control. Treatment with 50 µM DETA/NO led to an increase in proline level, with leaves showing a 17% increase and roots showing a 23% increase compared to the untreated control. The combined treatment with 5 mM DMTU and 0.5 mM L-NAME in water-deprived seedlings resulted in an increase in proline content by 29% and 57% in leaves and roots, respectively. The proline content in water-deprived maize seedlings treated with DMTU and DETA/NO showed values similar to the untreated control in both leaves and roots.

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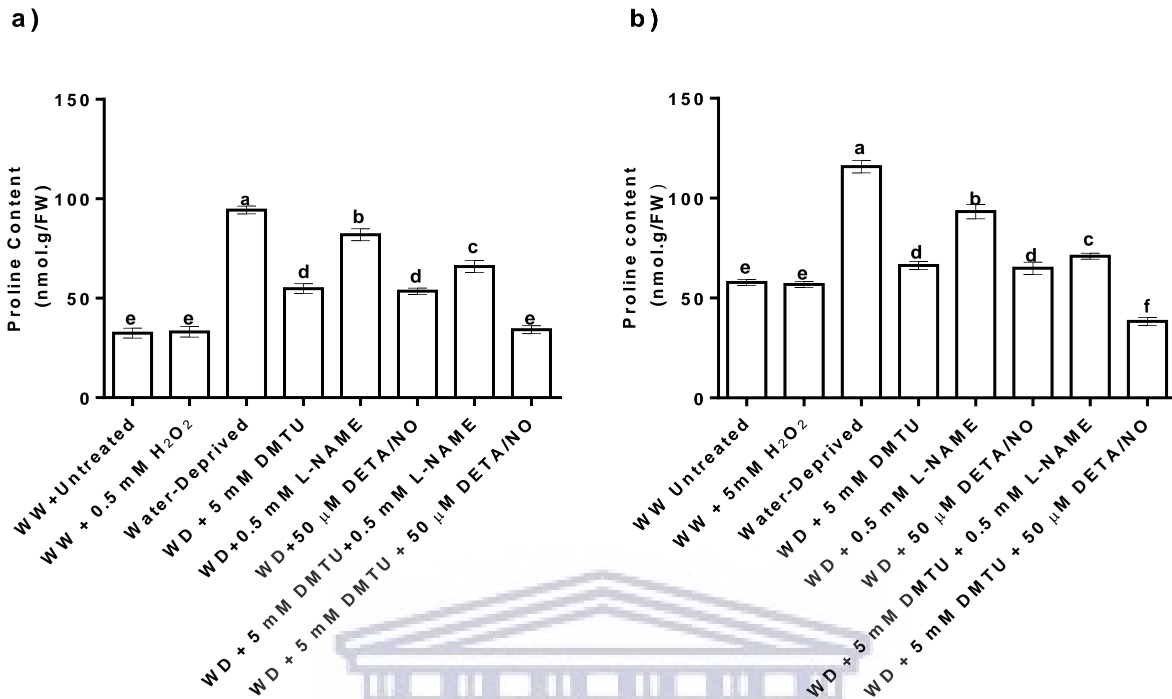

Figure 3.5 Proline levels in water-deprived maize seedlings. Proline levels were measured in untreated well-watered leaves and roots, and water-deprived leaves and roots treated with H_2O_2, DMTU, L-NAME and DETA/NO. The error bars signify standard deviation, bars with the same letters are statistically similar, where *P<0.05*.

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3.1.6 Responses of maize seedlings to exogenous application of nitric oxide and changes prompted by various treatments during water stress

NO content in leaves from plants treated with 0.5 mM H_2O_2 showed an increase of 14% compared to the untreated controls. An increase of 158% in No content in leaves of water-deprived seedlings and 166% in No content in roots was observed compared to the untreated control. Treatment with 5 mM DMTU water-deprived samples showed approximately a 50% increase in NO content in leaves and 63% in roots compared to the untreated control. In water-deprived maize seedlings treated with 0.5 mM L-NAME, NO content was reduced to a level similar to that of plants treated with 0.5 mM H_2O_2 in both leaves and roots. The treatment with DETA/NO in water-deprived maize seedlings resulted in an increase in nitric oxide content of approximately

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264% in leaves and 200% in roots. When maize seedlings were treated with both DMTU and L-NAME in water deprived conditions, there was a decrease in nitric oxide content by approximately 42% in leaves and 33% in roots compared to the untreated controls. The treatment with both DMTU and DETA/NO in water-deprived maize seedlings caused the nitric oxide content to increase by 114% in leaves and 113% in roots compared to the untreated controls.

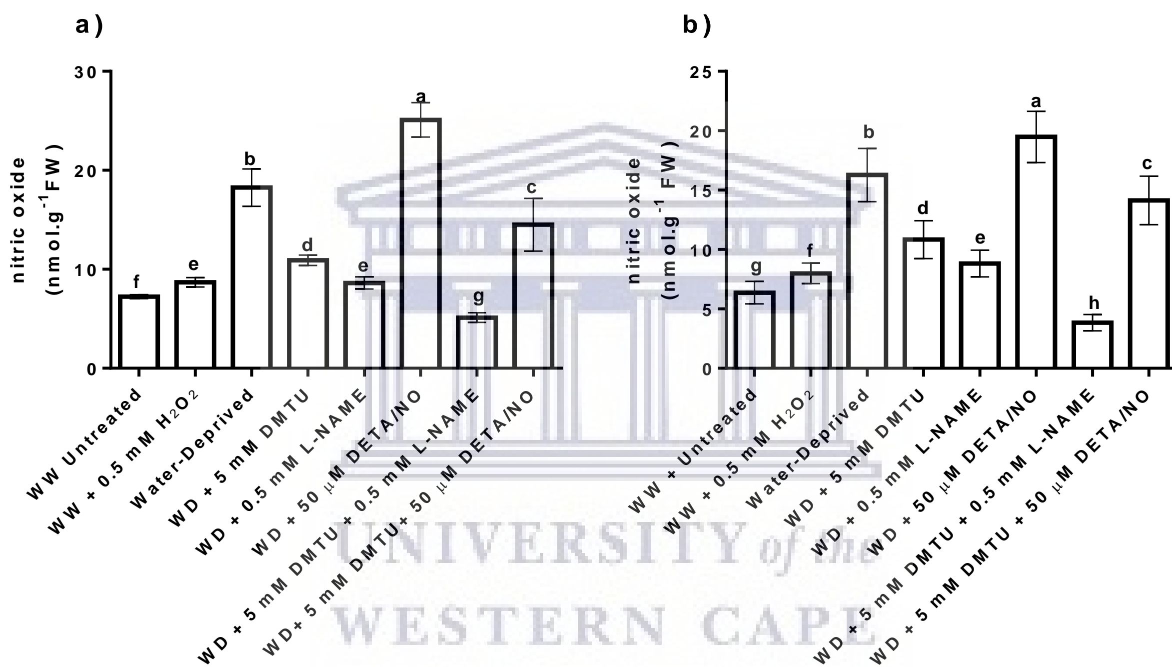

Figure 3.6 NO content in maize seedlings under various treatments. NO content was measured in untreated well-watered and untreated water-deprived maize seedlings together with corresponding treatment with H_2O_2, DMTU, L-NAME and DETA/NO. The error bars signify standard deviation, bars with the same letters are statistically similar, where *P<0.05*.

3.2 Role of NO and H_2O_2 in the regulation of antioxidant enzyme activity during water deficit stress in maize leaf seedlings

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3.2.1 Superoxide dismutase isoform activity in response to various treatments

A total of 6 SOD isoforms was observed in the native polyacrylamide gels, namely (MnSOD, Cu/ZnSOD (denoted Cu/ZnSOD1, CuZnSOD2, Cu/ZnSOD3 and Cu/ZnSOD4) and one FeSOD (Figure 3.7). Differentiation of SOD isoform profiles was done with the use of different SOD inhibitors (KCN and H_2O_2). Enzymatic activity of MnSOD was slightly increased in response to treatment with H_2O_2 compared to the untreated control plants. MnSOD isozyme activity in water-deprived plants increased. However, treatment with DETA/NO and water-deprived slightly increased MnSOD isozyme activity, a trend similar to the combined treatment with L-NAME and DMTU. Enzymatic activity of MnSOD increased in response to the combination treatment with DMTU and DETA/NO.

Enzymatic activity detected for Cu/ZnSOD1 and Cu/ZnSOD2 decreased in response to H_2O_2 in well-watered plants. Treatment of water-deprived plants with DMTU enhanced the enzymatic activity of Cu/ZnSOD1 and Cu/ZnSOD2. In response to L-NAME, DMTU, and the combined DMTU and L-NAME treatment, Cu/ZnSOD1 and Cu/ZnSOD2 activities were increased. However enzymatic activity of Cu/ZnSOD1 and Cu/ZnSOD2 decreased in the combined treatment with DMTU and DETA/NO. The activity of Cu/ZnSOD3 and Cu/ZnSOD4 decreased in all treatments. Water deficit enhanced enzymatic activity of Cu/ZnSOD3 and Cu/ZnSOD4. Cu/ZnSOD3 and Cu/ZnSOD4 activities were not affected by treatment with DMTU and DETA/NO. The enzymatic activity of FeSOD was not affected in any of the various treatments.

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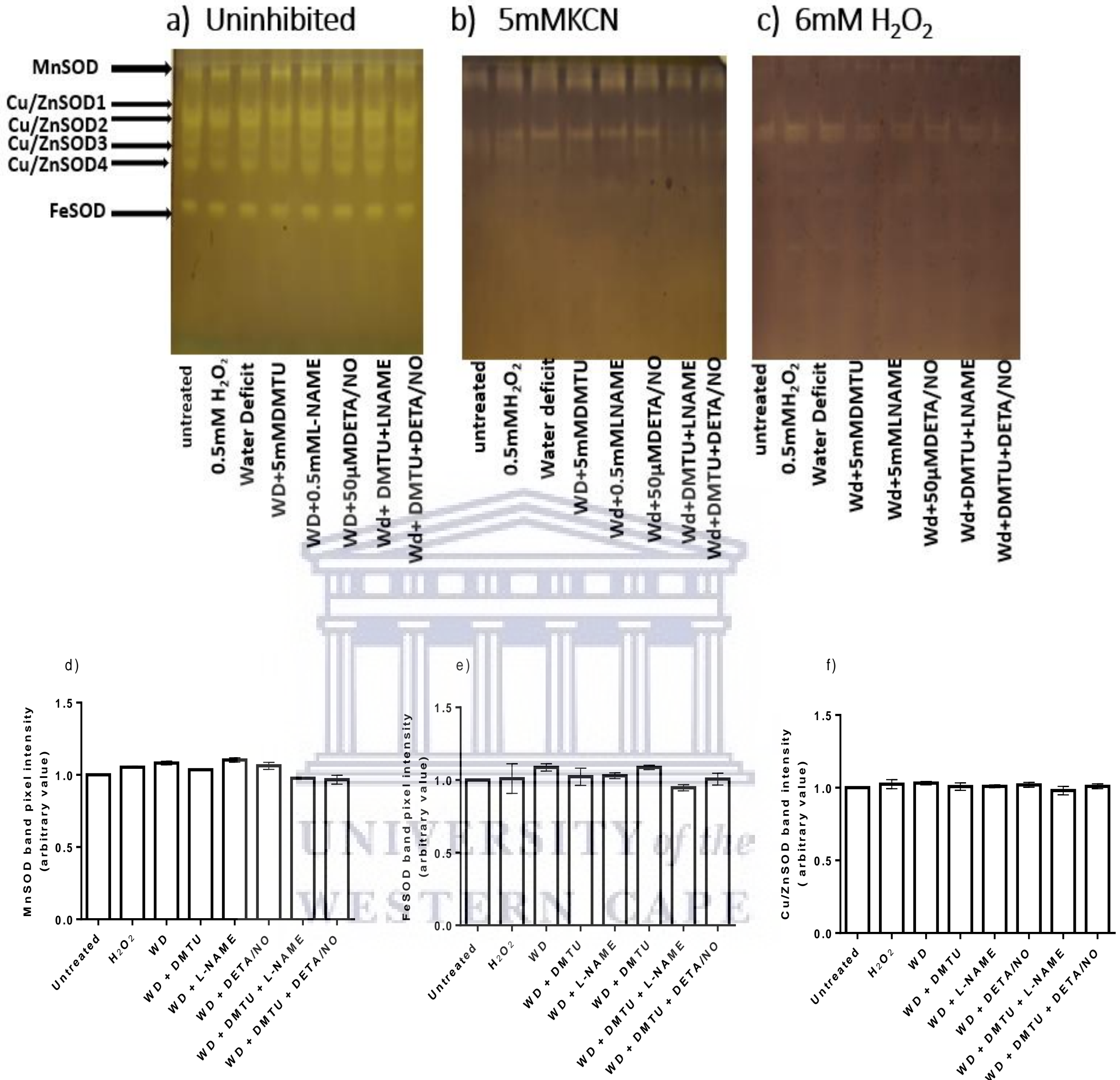

Figure 3.7 Effects of various treatments on SOD enzymatic activity in maize leaves. In-gel activities of various SOD isoforms in response to the following treatments are shown: 0.5 mM H_2O_2, 50 µM DETA/NO, 5mM DMTU, 0. 5mM L-NAME. Individual SOD isoforms were identified by incubating gels in 6 mM KCN (B) and 5 mM H_2O_2 (C), respectively from which pixel intensities

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were analyzed, (b) MnSOD, (e) FeSOD and (f) Cu/ZnSOD. The error bars signify standard deviation, bars with the same letters are statistically similar, where *P<0.05.*

3.2.2 Changes in root SOD isoforms activity in response to various treatments

Maize roots revealed a total of three SOD isoforms: Mn-SOD, Fe-SOD, and Cu/Zn-SOD (Figure 3.8). The intensity of MnSOD isozyme was enhanced in response to treatment with H_2O_2 under well-watered conditions when compared to the untreated control. Enzymatic activity of MnSOD isozyme was enhanced in water-deprived and DMTU-treated plants. The L-NAME, DETA/NO and the combined treatment with DMTU and L-NAME resulted in the similar increase in enzymatic activity of MnSOD. In response to the combined treatment with DMTU and DETA/NO, the enzymatic activity of MnSOD decreased in maize roots.

Enzymatic activity of the Cu/ZnSOD isozyme was enhanced in response to H_2O_2 in the well-watered treatment when compared to the untreated control. The untreated water-deprived treatment increased Cu/ZnSOD enzymatic activity whereas in the presence of DMTU the water-deprived treatment had decreased Cu/ZNSOD enzymatic activity. In response to L-NAME and DETA/NO in the water-deprived treatment, enzymatic activity of Cu/ZnSOD increased. Combined treatment with DMTU and L-NAME inhibited Cu/ZnSOD in maize roots whereas the combined treatment with DMTU and DETA/NO activated the enzymatic activity of Cu/ZnSOD. Enzymatic activity of FeSOD detected in all various treatment was less than the activity of other isoforms and no distinct difference was observed in response to the various treatments.

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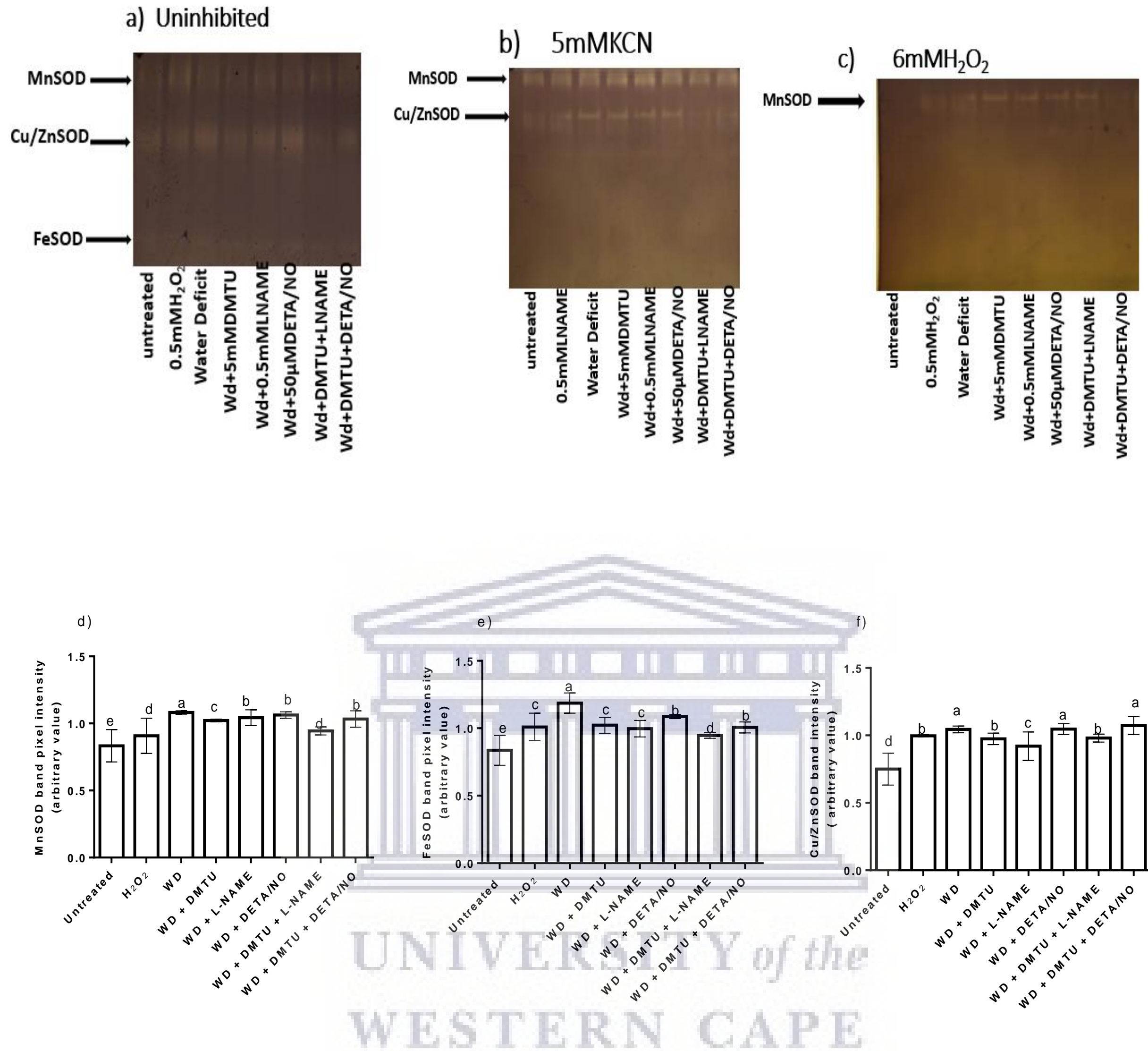

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Figure 3.8 The effect of various treatments on SOD enzymatic activity in maize roots. In-gel activities of various SOD isoforms in response to the following treatments are shown: 0.5 mM H_2O_2, 50 µM DETA/NO, 5 mM DMTU, 0.5 mM L-NAME and untreated control. Individual SOD isoforms were identified by incubating gels in 6 mM KCN (B) and 5 mM H_2O_2 (C) ,respectively from which pixel intensities were analyzed, (b) MnSOD, (e) FeSOD and (f) Cu/ZnSOD. The error bars signify standard deviation, bars with the same letters are statistically similar, where $p < 0.05$.

For better analysis of enzymatic analysis densitometry analysis was done. Quantification of SOD bands intensities by densitometry revealed that Cu/ZnSOD was the predominant isozyme in

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maize roots. Figure 3.8 Densitometry analysis indicated an increase in MnSOD enzymatic activity of about 3% in response to treatment with H_2O_2 under well-watered conditions compared to the untreated control. In response to water deprivation, MnSOD activity increased by approximately 12% compared to the untreated control. The DMTU treatment decreased MnSOD activity by approximately 6% compared to the water-deprived plants. However, in the L-NAME treatment, the enzymatic activity of root MnSOD equally increased like the untreated water deprived seedlings. In response to DETA/NO and the combined treatment with DMTU and L-NAME MnSOD enzymatic activity decreased by approximately 7%. Furthermore, combined treatment with DMTU and DETA/NO decreased MnSOD enzymatic activity by 10%.

Enzymatic activity of Cu/ZnSOD detected on maize roots slightly increased by 3% in response to H_2O_2 treatment compared to the untreated control. An increase was observed on Cu/ZnSOD activity in water-deprived roots, which was reduced by 4% in the DMTU treatment. Enzymatic activity of Cu/ZnSOD slightly decreased by 2% in response to the L-NAME treatment when compared to the water-deprived treatment, whereas on DETA/NO treatment the Cu/ZnSOD activity increased by approximately 8% compared to the water-deprived. Combined treatment with DMTU and L-NAME reduced the enzymatic activity of Cu/ZnSOD by approximately 12%. However combined treatment with DMTU and DETA/NO resulted in an 8% decrease of enzymatic activity compared to the water-deprived roots. Enzymatic activity for FeSOD did not change in response to the various treatments.

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3.2.3 Response of ascorbate peroxidase to various treatments in maize leaves

On native PAGE gels, only three APX isoforms were detected in maize leaves Figure 3. H_2O_2-treated maize leaves resulted in a slight increase in APX1 activity compared to the untreated control. APX1 isoform intensity increased in water-deprived compared to the untreated control. Treatment with DMTU under water deficit conditions caused a decrease in leaf APX1 activity compared to the water-deprived treatment. In the presence of L-NAME, APX1 activity decreased. In response to the DETA/NO treatment, the activity of APX1 increased compared to water-deprived plants. The combined treatment with DMTU and L-NAME in the water-deprived

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treatment reduced the activity of APX1. The combined treatment with DMTU and DETA/NO resulted in the increased of APX1 activity and reversed the effects of L-NAME.

Activity of APX2 in H_2O_2–treated well-watered plants showed no change when compared to the untreated control. Under water deficit, an increase in APX2 activity was observed, of which a decrease was observed in the presence of DMTU and L-NAME. The addition of DETA/NO increased the activity of APX2 to a level similar to that of water-deprived samples. However, there was a slight decrease in APX2 activity in L-NAME-treated plants, including the combined DMTU and L-NAME treatments. A similar trend was observed for the combined treatment with DMTU and DETA/NO. Similar trends in changes for the enzymatic activity of APX3 were observed under various treatments.

Densitometry analysis (b) and (c) showed no significant change in the activity of APX 1 and APX2 in response to all treatments. The activity of APX3 under water deficit increased by 15% compared to the untreated control. The H_2O_2 treatment in well-watered plants slightly increased APX3 activity compared to the untreated control. However, the L-NAME treatment reduced APX3 activity by approximately 10% compared to plants exposed to water deficit. Combined treatment with DMTU and DETA/NO showed no change in the activity of APX3 compared to the control and water deficit plants.

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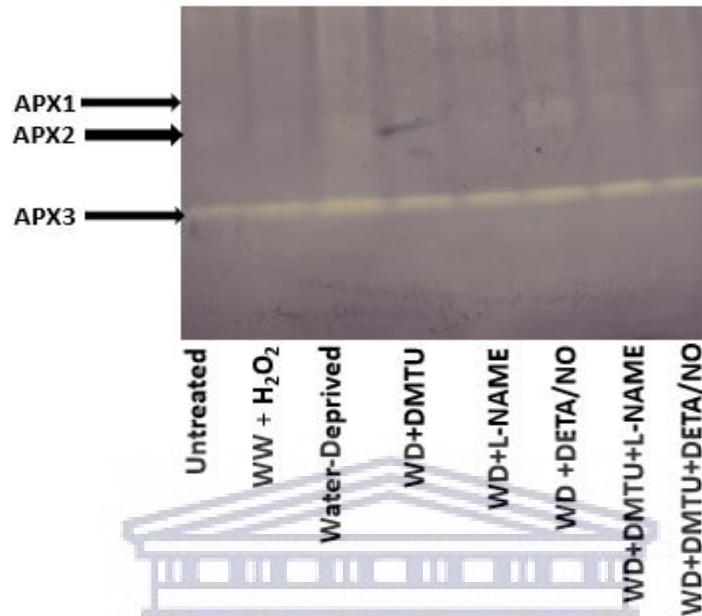

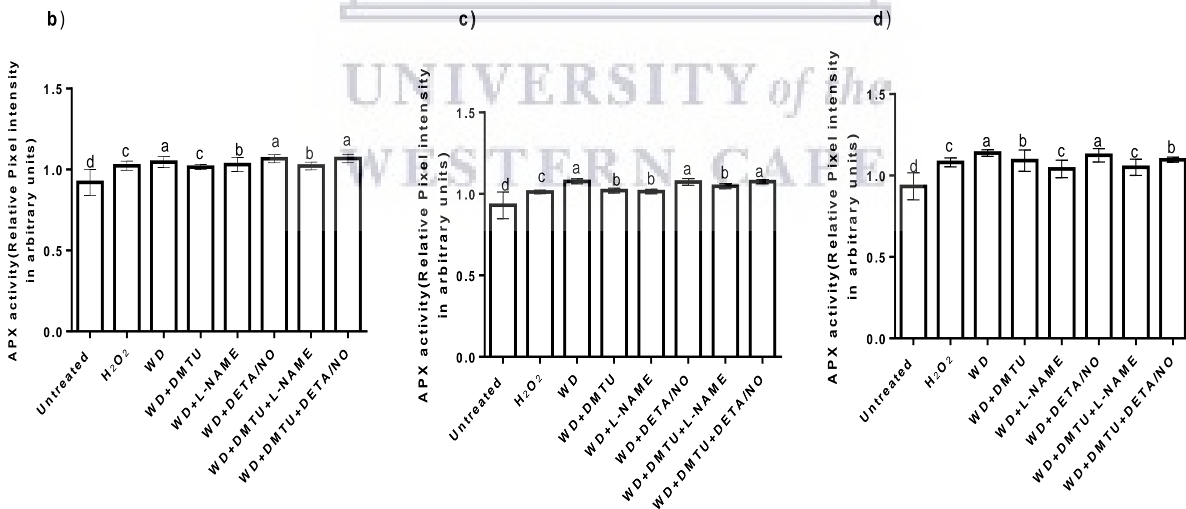

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Figure 3.9 In-gel activity for APX in response to various treatments. Assays were done on maize plants that were treated with the various compounds at the V3 stage for a period of a week. In-gel activities of various isoforms in response to treatment with 5 mM DMTU, 0.5 mM H_2O_2, 0.5

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mM LNAME, 50 µM DETA/NO, 5 mM DMTU + 0.5 mM LNAME, 5 mM DMTU + 50 µM DETA/NO are shown for APX as detected in maize leaves (A) from which pixel intensities were analyzed; (b)APX1, (c) APX2 and (d) APX3. Different letters indicate the differences between means at $p < 0.05$. Values are means ± SE (N=3).

3.4 Modulation of NO and H_2O_2 influences ascorbate peroxidase activity

In Figure 3.10 for maize roots, three APX isoforms were detected on native PAGE. The intensity of APX2 was higher than that of APX1 and APX3. APX1 intensity slightly increased in the H_2O_2-treated plants compared to the untreated control. In response to all treatments, the activity of APX showed an increase in the DETA/NO treatment and the combined treatment of DETA/NO + DMTU. In water-deprived plants, APX2 intensity increased compared to the untreated control. Addition of L-NAME in the water-deprived treatment resulted in a decrease in the intensity of the APX2 isoform as well as the combined DMTU and L-NAME treatment, whereas APX2 intensity increased in the DETA/NO treatment. APX3 isoform indicated no significant difference in all the various treatments.

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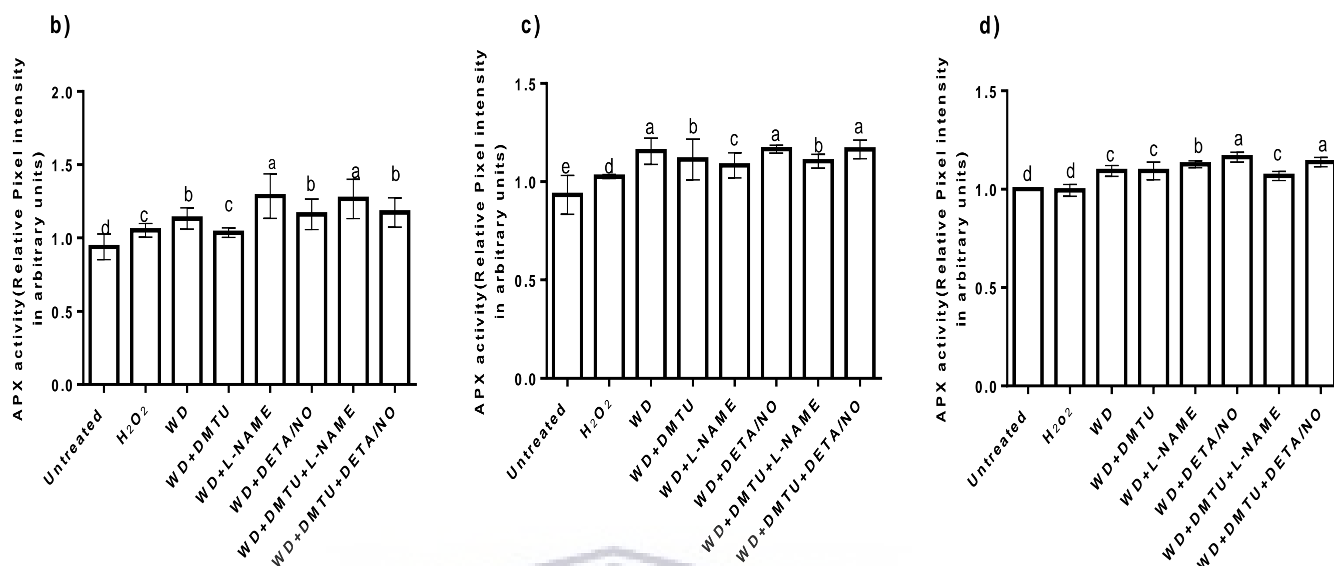

Figure 3.9 In-gel activity of APX isozymes in response to the various treatments. Assays were done on maize plants which were subjected to the various treatments at the V3 stage for a period of a week after 21 days of no watering. In-gel activities of various isoforms in response to treatment with 5 mM DMTU, 0.5 mM H_2O_2, 0.5 mM L-NAME, 50 µM DETA/NO, 5 mM DMTU + 0.5 mM L-NAME, 5 mM DMTU + 50 µM DETA/NO are shown for APX as detected in maize leaves (A) from which pixel intensities were analyzed (b)APX1, (c) APX2 and (d) APX3. The error bars signify standard deviation, bars with the same letters are statistically similar, where $p < 0.05$.

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Enzymatic activity of APX isoform determined in the pixel intensity showed an increase of 15% in response to addition of DETA/NO and DMTU, a trend observed in all three APX isoforms in response to the various treatments. Application of L-NAME increased APX enzymatic activity by approximately 5%. The combined treatment with DMTU and L-NAME reduced APX activity by 10%.

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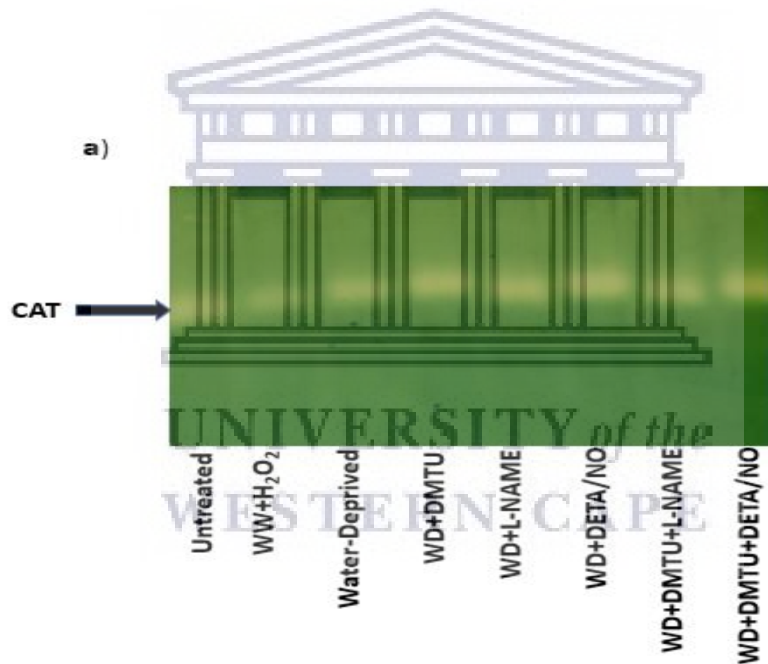
3.5 Catalase response on various treatments in maize leaves during water stress

In Figure 3.11 Changes in catalase isozymes activities were determined and only one CAT isozyme was detected on maize leaves in response to all various treatments. The activity of CAT decreased in H_2O_2-treated plants compared to the untreated control. In water deficit plants, the CAT activity was equivalent to the untreated control. The addition of DMTU to water-deprived plants resulted in an increase in CAT activity whereas treatment of water-deprived plants with L-NAME caused a slight decrease in CAT activity. Application of DETA/NO increased the activity of CAT compared to the untreated control plants. The combined treatment with DMTU and L-NAME decreased CAT activity while the combined treatment with DMTU and DETA/NO treatment slightly increased CAT activity.



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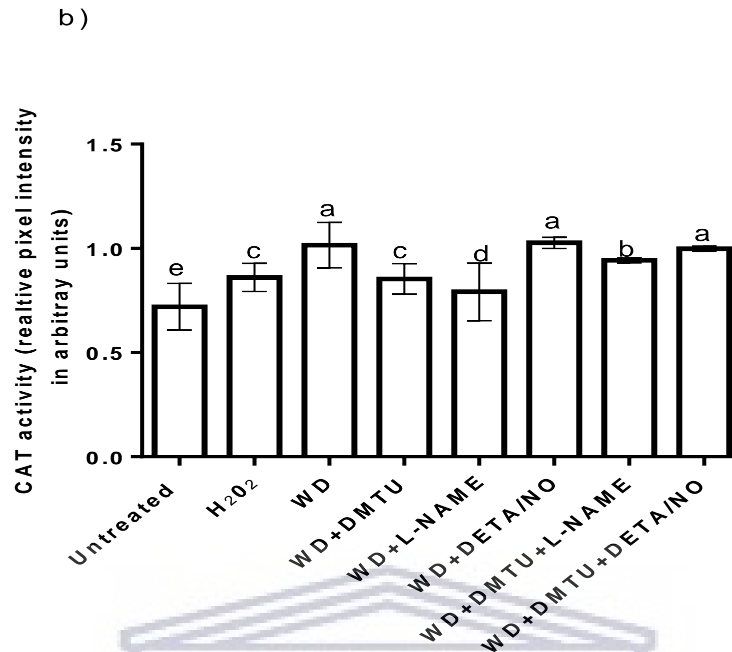

Figure 3.11: Response of catalase activity to application of H_2O_2 NO donor, H_2O_2 **scavenger and NO Inhibitors.** (a) Changes in catalase isozymes activity in maize leaves from plants that were treated with various treatments at V3 stage for a period of 7 days, equal amounts of protein was loaded for all treatments (0.5 mM H_2O_2, 0.5 mM L-NAME, 5 mM DMTU, 50 µM DETA/NO)**.** (b) Effect of various treatments on enzyme activity of catalase estimated from pixel intensities of band in native PAGE. Error bars represent the means (± SE; n= 3) of three independent experiments. Different letters above the bars indicate means that are statistically different at $p < 0.05$.

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Chapter 4: Discussion

4.1 Drought stress reduces water status in maize

During water deficit, many physiological and biochemical processes are disturbed. Understanding the multiple mechanisms by which plants respond to water stress is important in enhancing crop drought tolerance (Deikman *et al.* 2012, Juenger 2013). Leaf RWC is one of the best physiological indices used to assess the degree of water deficit in plant tissue (Alizade, et al., 2002). Low RWC in plants under drought stress was observed in this study. The reduction in leaf RWC was as a result of sensitivity of plant tissue to changes in water status (Cechin et al., 2006). Observations were consistent with many investigations which showed that when leaves are subjected to drought, leaves exhibit reductions in RWC and water potential (Kyparissis et al., 1995; Scarascia-Mugnozza et al., 1996; Li and Van Staden, 1998a, b; Decov et al., 2000; Nayyar and Gupta, 2006). Application of DMTU in water-deprived maize leaves conferred water deficit tolerance by maintaining more water than the untreated water-deprived plants. Similar observations were found on different species whereby 5 mM DMTU inhibited H_2O_2 toxic effects and therefore retaining water status in plants (Li et al., 2017; Liu et al., 2019).

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4.2 Osmolyte accumulation induced water retention on maize seedlings

During drought stress, plants accumulate osmolytes and soluble sugars to maintain the cell turgor (Gill et al., 2010). Proline is accumulated in the cytoplasm and chloroplast stroma when the RWC decreases, with their role being to stabilize cellular structures through hydrophilic interactions and hydrogen bonding (Ashraf et al., 2007). In plants, proline content increases more than other amino acids under water stress. In this study proline content in H_2O_2-treated well-watered samples was equivalent to the untreated control, therefore treatment with H_2O_2 had no effect on proline content. Water-deprived maize leaves and roots presented high accumulation of proline, which is in accordance with earlier observations made on maize (Chandrasekar et al., 2000). In the presence of DETA/NO, proline accumulation levels significantly decreased in maize seedlings under water deficit conditions, which is consistent with the studies reported by Lei et

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al (2007) whereby DETA/NO was found to act as an antioxidant during water stress conditions on plants and thus limited stress effects. Further, Lei (2007) reported that water stress tolerance was induced by exogenous NO, which was attributed to high accumulation of proline in plants. However, some studies (Zhang et al., 2008) noted that the high accumulation of proline was attributed to the effect that exogenous NO has on the activity of some key enzymes involved in the synthesis of proline. The addition of L-NAME on water-deprived plants blocked DETA/NO effects. In all the various treatments conducted in this study, proline content accumulation was higher in the roots compared to the leaves. The possible reason for this could be that roots retain water and leaves had reduced water status. Proline accumulation is considered as a symptom of a reduced water status of the plant (Hanson et al., 1979).

4.3 Oxidative stress induced under water stress conditions on maize and responses to chemical treatments

The changes that plants undergo as a result of water stress is over-production of ROS, which leads to oxidative damage. Overproduction of ROS to such an extent that the redox homeostasis of plants is disturbed results in lipid peroxidation, which eventually leads to programmed cell death. Hydrogen peroxide is one of O_2 metabolites normally produced by plants. It becomes toxic for the plant when the production levels exceed the scavenging under abiotic and biotic stress conditions, and therefore results in oxidative stress. In examination of oxidative stress on maize plants subjected water deficit, changes in H_2O_2 and MDA content were observed in response to application of various treatments. Under water-deprived conditions, increased levels of H_2O_2 and MDA content were observed both on maize roots and leaves, consistent to the report by Niedzwiedz-Siegien et al. (2004) whereby elevated lipid peroxidation levels were observed during drought stress and by Esfandiari et al., (2007) during salt stress in wheat seedlings. DMTU treatment scavenged H_2O_2 accumulation, however the accumulation of H_2O_2 content was not fully blocked. L-NAME application on water-deprived plants did not inhibit the accumulation of H_2O_2, therefore the MDA content increased. Studies showed that NO donated by the DETA/NO acts as the osmoprotectant during water stress on maize and therefore reduces H_2O_2 content (Noman, Qasim, Shafaqat et al., 2019). Similar results were observed in this study. The effects of

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H_2O_2 scavenger and NO donor were also better demonstrated when combined, which resulted in high reduction of H_2O_2 MDA content. The exogenous application of low concentration of H_2O_2 on maize leaves and roots under well-watered conditions had no effect on H_2O_2 content, instead H_2O_2 levels were equivalent to the untreated control. These findings were in agreement with Fedina et al(2009); Hossain and Fujita (2013), who reported that endogenous H_2O_2 levels were not enhanced by the addition of a low concentration H_2O_2 in seedlings for 24 or 48 h prior to drought stress or salt stress. In this study, increased levels of MDA accumulation were observed in maize leaf during water stress compared to the maize roots.

4.4 Changes in cell viability on water-deprived maize seedling under various treatments

Oxidative damages described above resulted in cell death as the primary consequent. In plants, NO enhances stress tolerance and it is expected to reverse the effects of oxidative damage. Application of H_2O_2 scavenger and NO donor on water deprived maize seedlings showed positive response towards oxidative damage suggested by the reduction of MDA and H_2O_2 accumulation levels in this study. Furthermore, determination of cell death was of importance in examining the NO inhibitor, H_2O_2 scavenger and NO donor effects on maize. Application of H_2O_2 to water-deprived maize decreased the level of cell death to the levels equivalent to the untreated control. In response to water-deprived conditions, maize leaves indicated increased levels on cell death, which was reversed by application of DMTU. The exogenous application of 50 µM DETA/NO resulted in decreased cell death levels compared to the water-deprived seedlings, which reflects the protective properties of NO in water deficit stressed plants. These protective properties were restricted when the L-NAME applied. These results were in agreement with Chung et al (2001), who reported that NO can prevent cell death in plants by acting as an antioxidant or antiapoptotic modulator. However NO can also be toxic, causing plant cell death. The toxic and protective effects of NO during water stress are concentration-dependent (Wink et al., 1998). Protective effects of NO were demonstrated in this study at 50 µM DETA/NO as it was able to reduce cell death levels induced by water deficit on maize leaves.

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4.5 NO reduces water stress effects on maize plants

To determine the relationship between NO and H_2O_2 application in reference to their role in water deficit stress in maize, an NOS inhibitor and H_2O_2 scavenger were applied. Application of H_2O_2 on well-watered plants resulted in a slight increase in NO content, thus suggesting that H_2O_2 under well-watered conditions increases endogenous NO. Restriction of water supply in maize plants resulted in increased NO content, which is possibly due to an increase in H_2O_2 content during water stress as it is known that H_2O_2 induces NO generation (Zeng, Liu et al., 2011). However, the application of DMTU on water deprived samples reduced the content of NO, were consistent to previous reports that H_2O_2 induces the accumulation of NO under-water stress conditions (Uchida et al., 2002). Therefore, the restriction of H_2O_2 accumulation resulted in the reduced levels of NO. Application of L-NAME on water-deprived seedlings reduced the NO level due to inhibition of NOS activity. Reduction of NO by using chemicals such as L-NAME reduced endogenous NO levels, leading to higher stress sensitivity (Hao et al., 2008; Xu et al., 2010a). Application of 50 µM DETA/NO significantly increased the NO level in water-deprived samples, which is consistent with studies reported by (Farooq et al., 2009; Liao et al., 2012; Rahimi and Boogar et al., 2014) that exogenous NO can reduce the negative effects of water stress.

4.6 Drought stress induced antioxidant enzyme activity in maize

Plants subjected to stress conditions use antioxidant enzymes to scavenge ROS (Wang *et al.,* 2008). In this study, an increase in ROS accumulation was demonstrated by the increase in membrane damage (as indicated by malondialdehyde content) and cell death levels under water stress in maize plants. Consequently, antioxidant enzyme activities were activated to counter the oxidative stress. Superoxide dismutase (SOD) scavenges superoxide (O_2^-) radicals (Lee and Lee, 2000), producing less harmful H_2O_2 (Tewari et al., 2006). All forms of SOD (MnSOD, Cu/ZnSOD and FeSOD) were activated in response to water stress in maize plants in this study. Maize leaves induced more SOD isoforms than the roots, which may imply that oxidative stress was more severe in the leaves than in roots. The activities of all SOD isoforms increased during drought stress. These results are in agreement with studies in *Brassica napus* L (Abedi et al., 2010), *Sesamum indicu* L cvs (Fazeli et al., 2007) and rice seedlings, where SOD activity increased in

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response to drought stress. The obtained results also show an increase in APX activity in maize leaves and roots in response to water deficit, which could be attributed to efforts to prevent oxidative damage caused by the increased levels of H_2O_2. Similar results where APX activity increased in response to water stress were reported by Zlatev et al. (2006) in bean and Chugh et al. (2011) in maize. The activity of CAT decreased in response to water stress when compared to the well-watered plants. A decrease in CAT activity was also observed in a study reported by Pan et al. (2006) in liquorice and by Bakalova et al. (2004) in wheat. Reduction of CAT activity is likely the underlying reason for the elevation of H_2O_2 in response to drought. Another interesting issue could be that CAT is confined to peroxisomes and related organelles and is not typically induced by drought (Smirnoff 1993). Its higher activity during drought exposure would rather be linked to its function probably to detoxify H_2O_2 derived from fatty acid oxidation (important source of energy in senescing tissues) (Simova-Stoilova et al. 2009).

4.7 NO and H_2O_2 inhibition reduced antioxidant enzyme activity under water stress

Increased SOD activity enhances oxidative stress tolerance (Asada, 1999) and signaling molecules such as nitric oxide (NO) are vital in enhancing SOD activity. Changes in the enzymatic activity were also observed on the obtained results under various treatments with NO donor, NOS inhibitor and H_2O_2 scavenger. The production of NO and H_2O_2 overlaps both spatially and developmentally (Asai et al 2009 and Cui et al 2011). Importantly, H_2O_2 and NO can react with each other and influence the activities of enzymes that alter each other's levels. NO donors caused a rapid decline in H_2O_2 accumulation in both leaves and roots of maize, in contrast to a study described by Pasqualini et al. (2009). An NO donor resulted in high accumulation of H_2O_2 in *N. tabacum* (Pasqualini et al., 2009). Application of H_2O_2 on well-watered plants enhanced the enzymatic activity of SOD in maize roots and leaves (Figure 3.5) Similar findings were reported by Li et at (2011) and Gondim et al (2012), where exogenous application of H_2O_2 enhanced the activities of the antioxidant enzymes SOD, CAT, and APX, and reduced the content of MDA in wheat and maize subjected to salt stress. These results were also in agreement with Jing et al., (2009) studies, who reported that the involvement of exogenous H_2O_2 could increase the

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activities of the antioxidant enzymes CAT,SOD, APX, GPOX, DHAR, GR, and the levels of the antioxidants AsA and GSH, resulting in decreased levels of endogenous H_2O_2 in plants under drought stress. Treatment with 50 µM DETA/NO during water stress in maize leaves indicated differential regulation of SOD isoforms whereas the DMTU and L-NAME treatment increased SOD activity, contradicting the findings reported in several studies whereby NO donors increased SOD activity during water stress (Tan et al., 2008) or waterlogging (Wang et al., 2011). However, in maize roots, application of NO donor increased the activity of SOD isoforms under water-deprived conditions (Figure 3.10). Application of NO enhances drought tolerance by increasing antioxidants capacity, to scavenge ROS (Arasimowicz-Jelonek et al., 2009a; Farooq et al., 2009). Application of DETA/NO on maize seedlings subjected to drought enhanced catalase and ascorbate peroxidase activity in both leaves and roots. The effects of NO were inhibited by the addition of the NOS inhibitor, L-NAME shown by the decrease antioxidant enzyme activity. This confirms that the effects seen in the NO donor (DETA/NO) treatments are as a result of NO and dependent on NOS-like activity.

4.8 Conclusion & Future Aspects

H_2O_2 and NO act in concert and strengthen the antioxidant defense system to alleviate oxidative stress arising from water deficit stress in maize. In this study, results illustrate that H_2O_2 acts upstream of NO in the water deficit stress-induced antioxidant defense. Water deficit stress induced an NO burst, which was inhibited by treatment with an H_2O_2 scavenger, suggesting that NO synthesis might be the result of H_2O_2 accumulation. Treatment with L-NAME showed no effects on water deficit stress-induced H_2O_2 generation, indicating that NOS-like activity is not required for the initial H_2O_2 accumulation. Taken together, these results suggest that water deficit stress-induced H_2O_2 triggers the production of NO and eventually regulates the antioxidant defense systems in maize plants. In view of the fact that exogenous application of NO to *Zea mays* plants led to reduced levels of H_2O_2 and improved the cell viability in NOS inhibited *Zea mays*, suggests that NO mobilized the enzymatic antioxidant defense system. For efficient scavenging of ROS in the cell, APX catalyzes the conversion of H_2O_2 into water. It is well established that both NO and H_2O_2 take an active part in the signal cascade in response to abiotic

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stress. However, a different picture emerged that NO donor acutely decreased H_2O_2 content after wounding in sweet potato, implying that NO acts in concert with H_2O_2 in response to mechanical wounding . Nevertheless, H_2O_2 and NO interact in a variety of patterns, and although some studies have shown that NO treatment can induce the production of H_2O_2, other studies have shown opposite results. The discrepancy between these studies implies that some unknown signaling pathways are yet to be explained. For example, in cucumber plants, NO acted downstream of H_2O_2 in brassinosteroid-induced abiotic stress tolerance, consistent with the findings of this study. Thus, the relationship between H_2O_2 and NO in signal transduction may be more complicated than the simple linear manner in which H_2O_2 induces NO or vice versa. In addition, H_2O_2 and NO may crosstalk differently under different stresses, species, and plant status. Therefore, the question of the relationship between H_2O_2 accumulation and NO production in maize plants exposed to water stress is partially understood. Further experiments are still needed to fully establish the relationship between H_2O_2 and NO and its possible function in different plants species under various conditions. Additional work using proteomic and molecular approaches are required to understand the detailed mechanism of NO and H_2O_2-induced water deficit stress tolerance.

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Chapter 5

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