Investigation of the link between drought-induced changes in the expression of a novel sterol biosynthesis gene and drought tolerance in soybean

By

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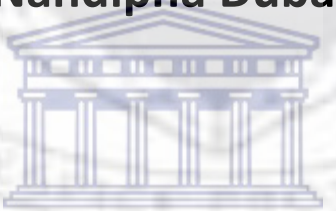

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Key words

Glycine max

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Drought stress

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Phytosterols

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Abstract

Glycine max (soybean) is an important crop species globally as it is used as a protein-rich food and feed crop and as a source of oils used in the food and biofuel industry. However, the growth and yield of soybean is adversely affected by drought. Exposure of soybean to drought leads to accumulation of reactive oxygen species (ROS) and cell membrane instability. Sterols are membrane components that regulates membrane fluidity and permeability. Besides being major components of the cell membranes, sterols such as lanosterol appear to play a role in the regulation of ROS scavenging and some are precursors to brassinosteroids that act as signaling molecules with hormonal function that regulate growth, development and responses to abiotic stresses such as drought and salinity. In this study, the involvement of plant sterols, also known as phytosterols, in the regulation of soybean responses to drought stress was investigated in *Glycine max* by determining the effects of drought on the expression of a candidate lanosterol synthase gene (Glyma08g24160) and the content of a subset of phytosterols in soybean. The effects of inhibition of sterol synthesis on ROS production and on superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and dehydroascorbate reductase (DHAR) were investigated. The concentration of hydrogen peroxide (H_2O_2) as well as superoxide ($O_2^{\cdot-}$) increased in response to drought and sterol synthesis inhibition, however, $O_2^{\cdot-}$ concentration and sterol contents declined under drought stress and sterol synthesis inhibition. Furthermore, drought stress altered the expression of

a gene encoding a lanosterol synthase, suggesting that lanosterol and phytosterols could be involved in regulating soybean responses to drought.

DECLARATION

I declare that "*Investigation of the link between drought-induced changes in the expression of a novel sterol biosynthesis gene and drought tolerance in soybean"* 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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- Firstly, I would like to thank GOD, my heavenly father for giving me life, strength, guidance and wisdom. I am where I am today because of you Lord. There were times where I wanted to give up, but you gave me strength to continue with my journey. "To God be the glory".
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Abbreviations

ABA: Abscisic acid

APX: Ascorbate peroxidase

AsA: Ascorbate

AsA-GSH: Ascorbate-glutathione cycle

BL: Brassinolide

BR's: Brassinosteroids

CAT: Catalase

CAS: Cycloartenol synthase

CO_2: Carbon dioxide

Cu/Zn-SOD: Copper/Zinc superoxide dismutase

DHAR: Dehydroascorbate reductase

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

ETC: Electron transport chain

FAO: Food and Agricultural Organization of the United Nations

FDA: Food and Drug Administration

Fe-SOD: Ion superoxide dismutase

FNSWG: Food and Nutrition Security Working Group

GDP: Gross domestic product

GSH: Glutathione

GSSG: Glutathione disulfide

GPX: Guaiacol peroxidase

GPox: Glutathione peroxidase

GR: Glutathione reductase

HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase

IPP: Isopentenyl pyrophosphate

LAS: Lanosterol synthase

MDA: Malondialdehyde

MDHAR: Monodehydroascorbate Reductase (MDHAR)

Mn-SOD: Manganese superoxide dismutase

NAD(P)H: Reduced nicotinamide adenine dinucleotide phosphate

TEMED: N,N,N',N'-Tetramethylethylenediamine

$OH^{\cdot}$**:** hydroxyl radical

$^1O_2$: Singlet oxygen

$O_2^{\cdot}$: Superoxide

OSC: Oxidosqualene Cyclase

PAGE: Polyacrylamide gel electrophoresis

PCD: Programmed cell dead

PCR: Polymerase chain reaction

PSI: Photosystem I

PSII: Photosystem II

PVP: Polyvinylpyrolidone

RWC: Relative water content

ROS: Reactive oxygen species

SOD: Super oxide dismutase

SA: South Africa

SQE: Squalene epoxidase

TCA: Trichloroacetic acid

USA: United States of America

WD: Water deficit

WDT: Water deficit and terbinafine

WHO: World Health Organization

WW: Well-watered

WWT: Well-watered and terbinafine

List of figures

List of tables

Table of contents

Aims and objectives

- To examine the effect of water deficit on soybean by measuring seedling fresh weight and length.
- To determine the effect of drought on expression of a sterol biosynthesis gene.
- To determine the impact of drought on sterol content in soybean leaves.
- To determine the impacts of changes in sterol content as a result of drought and inhibition of sterol synthesis on production of reactive oxygen species (ROS) and the activity of antioxidant enzymes.

Hypothesis

- Drought stress leads to increased expression of a candidate lanosterol biosynthesis gene and low sterol content of soybean leaves mediate ROS production and antioxidant enzyme activities.

CHAPTER 1

LITERATURE REVIEW

1.1 introduction

The *Fabaceae*, commonly known as the legumes, constitute the third largest family of economically important flowering plants (Lewis *et al.,* 2005). The legume family is unique due to its ability to fix atmospheric nitrogen, using specialized organs nodules on their roots, to ammonia in symbiosis with nitrogen fixing bacteria '*rhizobia*'. This makes legumes essential components of agricultural ecosystems as it reduces the need for nitrogen in synthetic fertilizers which cause land and water pollution (Varshney *et al.,* 2009). *Glycine max* (soybean) is amongst the important legume crops, as it is an excellent source of dietary protein, carbohydrate, vitamins and minerals for both human food and animal feed (Sakai and Kogiso, 2008). This makes soybean an important crop relevant for food security and significant in nutrition for developing countries. Soybean also has health benefits as it has been shown to contain compounds that have protective effects against cancer, menopause, diabetes and various chronic renal diseases (Friedman and Brandon, 2001). Soybean is also used in the production of biodiesel (Pimentel and Patzek, 2005).

However, the growth and development of plants is negatively affected by various biotic and abiotic stresses such as salinity, drought, fungi, bacteria and high temperatures. Drought is one of the major environmental factors that cause crop yield loss. One-third of the world's population lives in water-deficient regions and it is estimated that drought will continue being the major problem for global crop yield as it is estimated to become more frequent and severe (Cutforth *et al.,* 2007). Drought negatively affects soybean production worldwide and threatens food and protein security (Kunert *et al.,* 2016). Drought can reduce soybean yield

by approximately 40% (Specht *et al.*, 2001). Various physiological, morphological and biochemical changes are induced by drought stress in plants, and as a result normal plant growth and development is disturbed (Manavalan *et al.,* 2009). The symptoms of water deficit are generally suppressed growth, reduced photosynthetic rate, reduced transpiration rate, and leaf senescence (Chaves *et al.,* 2003).

Plants respond to drought stress using numerous mechanisms like gene expression, protein expression and accumulation of osmoprotectants (Gill and Tuteja, 2010). Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), Superoxide ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$) and the hydroxyl radical ($^{\cdot}OH$) form in plant cells as a result of normal metabolism (Ahmad *et al.,* 2008). ROS play an important role as signalling molecules in plants by controlling processes such as growth, development, response to biotic and abiotic environmental stimuli and programmed cell death (Mittler *et al.,* 2004). Furthermore, under physiological steady state conditions ROS can be scavenged by the plant's antioxidant defense system (Foyer and Noctor, 2005). However, the balance between ROS production and scavenging may be disturbed by several abiotic and biotic stresses such drought, salinity, pathogens and high temperatures; leading to overproduction of ROS. High levels of ROS in cellular components are toxic (Scandalios, 1997). ROS at these high levels can react with unsaturated fatty acids, nuclei acids and proteins and lead to peroxidation of essential membrane lipids, DNA lesions and site specific amino acid modifications (Choudhury *et al.,* 2013). Drought-induced ROS accumulation is counteracted by enzymatic antioxidant systems that include enzymes such as superoxide dismutase (SOD), ascorbate peroxidase and catalase (CAT). The antioxidant defense system protects plants cells from oxidative damage (Tang *et al.,* 2006).

Recently, plant sterols have been implicated in drought stress tolerance by regulating ROS production (Pose *et al.,* 2009, Kumar *et al.,* 2015). Plant sterols are important components of the membrane in eukaryotic organisms. They control membrane fluidity and permeability (Piironen *et al.,* 2000) and play an essential role in plant growth and development. Mutants that were defective in a sterol biosynthesis gene precursor had reduced sterol content and ROS production (Cao *et al.,* 2005; Pose *et al.,* 2009; Wang *et al.,* 2012). Additionally, *Arabidopsis* plants defective in lanosterol synthesis have an albino phenotype and have poor cell viability (Babiychuk *et al.,* 2008). There is thus a possibility that sterol biosynthesis genes may be involved in signalling processes that regulates plant responses to drought, possibly involving ROS scavenging. Therefore, in this study, we used semi-quantitative polymerase chain reaction (semi-qPCR) and quantitative polymerase chain reaction (qPCR) to determine if exposure of soybean plants to drought alters the expression of a gene encoding a candidate lanosterol synthase. This would be indicative of a potential role of the gene in regulating soybean responses to drought stress. To further understand responses of soybean to drought stress, we studied changes in sterol content on ROS accumulation and antioxidant enzyme activities.

1.2 Phytosterols

Plant sterols, also called phytosterols, include over 250 different sterols and related compounds in various plant species and marine organisms (Akihisa *et al.,* 1991). They are either synthesized *in vivo* or taken up from the environment (Piironen *et al.,* 2000). The most common phytosterols are sitosterol (which contains an ethyl group at C-24), campesterol (which contains a methyl group at C-24) and stigmasterol (an unsaturated phytosterol because of the double bond at C-22) (Suzuki *et al.,* 2006; Babiychuk *et al.,* 2008) as shown in

Figure 1.1 Although plant sterols are found in various forms, few of them are found abundantly in nature. Approximately 65% of total plant sterols is β-sitosterol, followed by campesterol at approximately 30% of the total plant sterol content (Moghadasian, 2000). Phytosterols can be found in both saturated (sterols) and unsaturated (stanols) chemical forms and occur in significant amounts in seeds, fruits and vegetable oils (Weihrauch and Gardner, 1978).

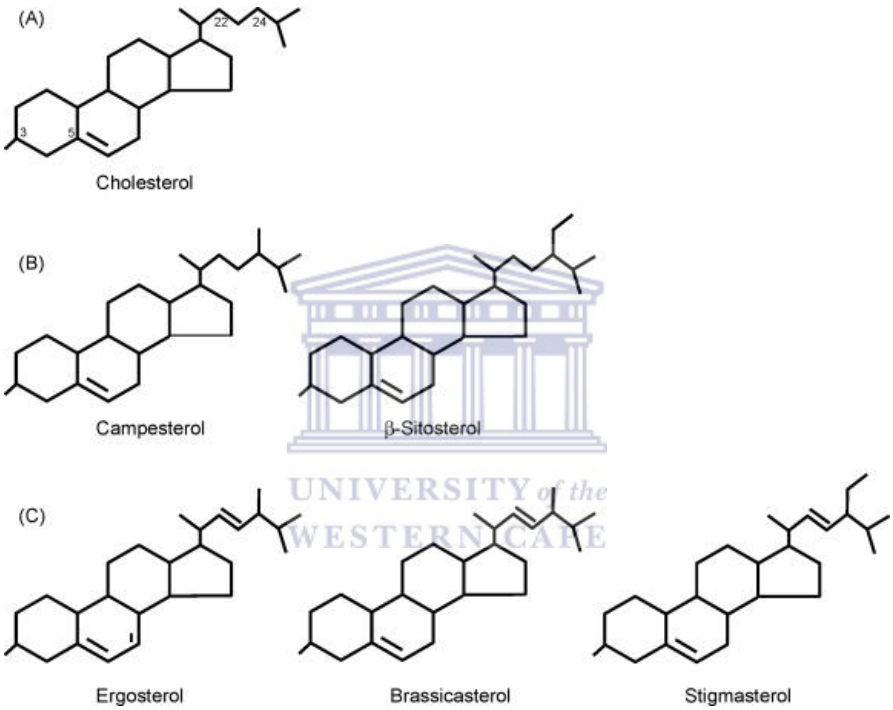


Figure 1.1: The chemical structures of cholesterol and phytosterols. Phytosterols (b) and cholesterol (a) have similar chemical structure, but differ at C-24 side chain. (c) Chemical structure of ergosterol, Brassicasterol and stigmasterol with unsaturation at C-22 in their side chains. Picture adapted from Calpe-Berdiel *et al*., 2009

In both animals and plants, sterols are highly concentrated in the plasma membrane but occur at low concentrations in the endoplasmic reticulum and mitochondria (Hartmann and Benveniste, 1987). Sterols are essential compounds in all eukaryotes (Schuler *et al.,* 1990), because they are structural components of cell membranes (Schaller, 2003; Thimmappa *et al.,* 2014). Animal cholesterol (figure 1.1 a) and plant phytosterols (figure 1.1 b) have a

common tetracylic carbon skeleton but they differ in the side chain, although their role is similar (Suzuki *et al.,* 2006). Phytosterols always contain some addition to the C-24 position on the aliphatic side chain of the sterol (Hennessey, 1992). In mammals, insects, and higher plants, sterols are converted to steroidal hormones (Ohyama *et al.,* 2009). Plant cells have distinct features when compared to fungal and animal cells. During sterol biosynthesis, plant cells consist of several pathway end-products (figure 1.2 and 1.3) such as campesterol, stigmasterol, sitosterol and isofucosterol (Corey *et al.,* 1996), whereas the sterol profile of animals is made up of cholesterol only (Suzuki *et al.,* 2006). In addition, the sterol biosynthesis scheme emanating from squalene to ergosterol in fungi and cholesterol in animals differ from the one from squalene to phytosterols in plants. In addition, the structures of sexual hormones, such as aldosterone and testosterone, biosynthesized from cholesterol in animals, differ from those of brassinosteroids (plant steroidal hormones) biosynthesized from campesterol in plants (figure 1.2) (Suzuki *et al*., 2006).

Phytosterols are used clinically as they have been reported to interfere with cholesterol absorption by reducing total serum and low-density lipoprotein cholesterol levels and have been used in hypercholesterolemic patients since the early 1950s (Ikeda and Sugano, 1983). The human diet contains about 200-300 mg per day of plant sterols. Therefore, the higher the intake of plant sterols, the higher the inhibition of cholesterol absorption and the lower the serum cholesterol levels (Ikeda *et al.,* 1988)

1.2.1 Biosynthesis of phytosterols

Plant sterols have been extensively studied in past years, with major focus on biosynthetic and biochemical aspects (Schaller, 2003). Sterol biosynthesis from acetyl-CoA have been characterized in great detail (Bach *et al.,* 1997). Most of the genes that are involved in the

biosynthetic pathway have been isolated and characterised using metabolic interference, functional complementation of yeast sterol mutants, expression in bacteria, protein purification and sequencing of the encoding genes (Lecain *et al*., 1996; Bak *et al.,* 1997; Bouvier *et al.,* 1997; Klahre *et al.,* 1998). Some of these genes have been cloned and identified mostly in *Arabidopsis*, while most have been identified in other plant species including soybean, rice and maize (Schaller, 2004). The synthesis of sterols takes place in the cytoplasm and occurs after the germination of seeds, with a gradual reduction in their synthesis as the seedlings matures (Guo *et al.,* 1995). In plants, the sterol biosynthesis pathway consists of a sequence of more than 30 enzyme-catalysed reactions involving plasma membrane-localized proteins (Piironen *et al.,* 2000). Sterols are initially synthesized through the mevalonic acid pathway, which takes place in the cytosol (Figure 1.2). The mevalonic acid pathway produces isopentenyl diphosphate (IPP), which serves as the central building block for the biosynthesis of all the terpenoids, including sterols, which are C_{30} triterpenoids (Clouse, 2002). Isopentenyl pyrophosphate is further converted to squalene, the linear 30-carbon intermediate that is considered as the first committed precursor of all cyclic triterpenoids. This is followed by oxidation of squalene to 2,3-oxidosqualene by squalene epoxidase (Pose *et al.,* 2009).

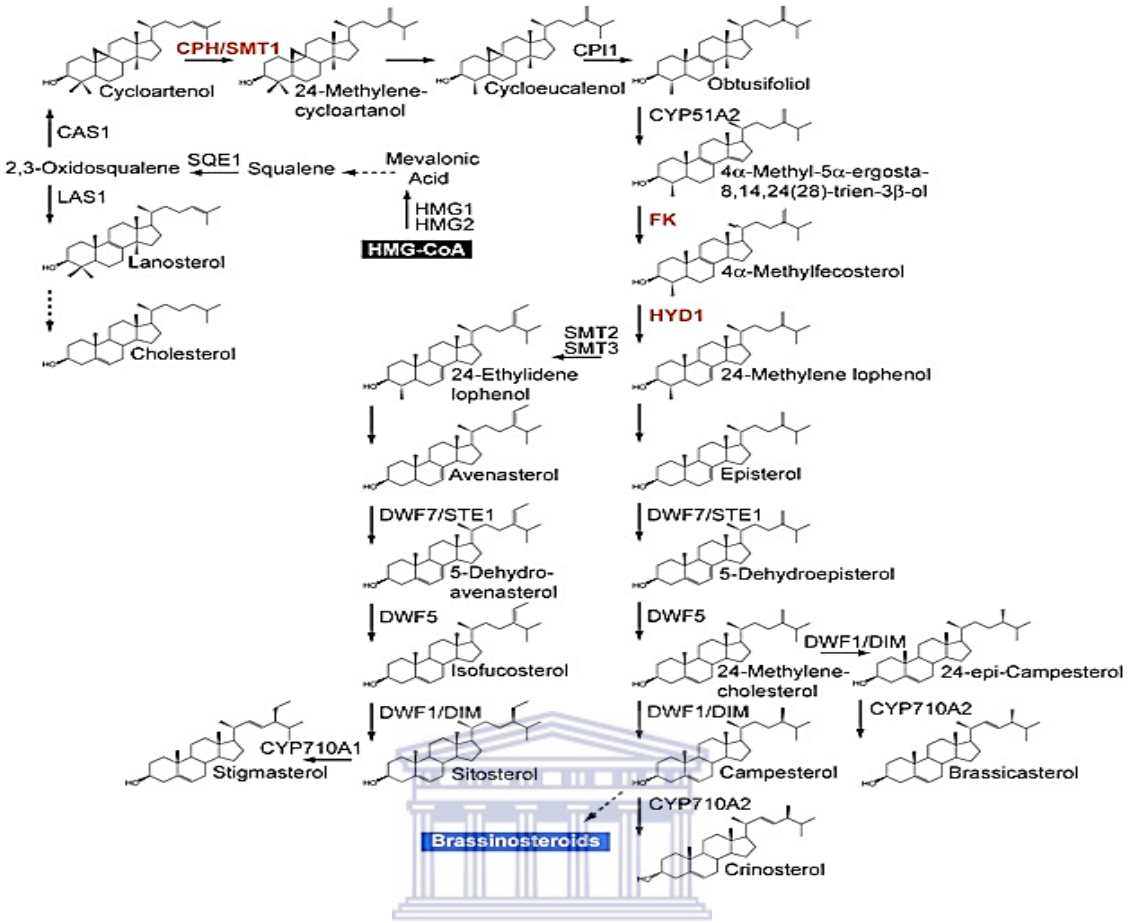


Figure 1.2: The sterol biosynthesis pathway in *Arabidopsis.* The biosynthesis consist of 17 enzymes and 22 steroid structures. HMG-CoA (black) is the precursor for sterol biosynthesis and brassinosteroids (Blue) are the synthesized from campesterol. Image source: DeBolt *et al.,* 2009

From 2,3-oxidosqualene, plant cells use a sterol biosynthetic pathway that is different from that of animals and fungi. Animals and fungi use lanosterol synthase to cyclize 1,2-oxidosqualene into lanosterol, a tetracyclic sterol precursor which is also further metabolized into cholesterol and ergosterol. However, plants use cycloartenol synthase to cyclize 1,2-oxidosualene into cycloartenol, the first cyclic intermediate of plant sterol biosynthesis (Suzuki *et al.,* 2006; Babiychuk *et al.,* 2008)**.** The pathway is linear until it reaches 24-methylene lophenol (figure 1.2). After formation of 24-methylene lophenol, there is a divergence that leads to either 24-methyl sterols which include campesterol and the brassinosteroids, or 24-ethyl sterols, which include sterols such as sitosterol and stigmasterol (Clouse, 2002).

1.2.2 Lanosterol biosynthesis in plants.

Until about a decade ago, it was believed that plant phytosterols are synthesised via the cycloartenol route only (Giner and Djerassi, 1995). However, as early as four decades ago, lanosterol was detected in higher plants (Toshihiro *et al.,* 1977; Giner *et al.,* 2000), suggesting that plants also have a biosynthetic pathway to produce lanosterol. The genes that encode lanosterol synthase in plants have been identified in dicotyledonous plants such as *Arabidopsis thaliana (*Kolesnikova *et al.,* 2006), *Panax ginseng* (Suzuki *et al.,* 2006), and *lotus japonica* (Sawai *et al.,* 2006) *(figure 1.3).*

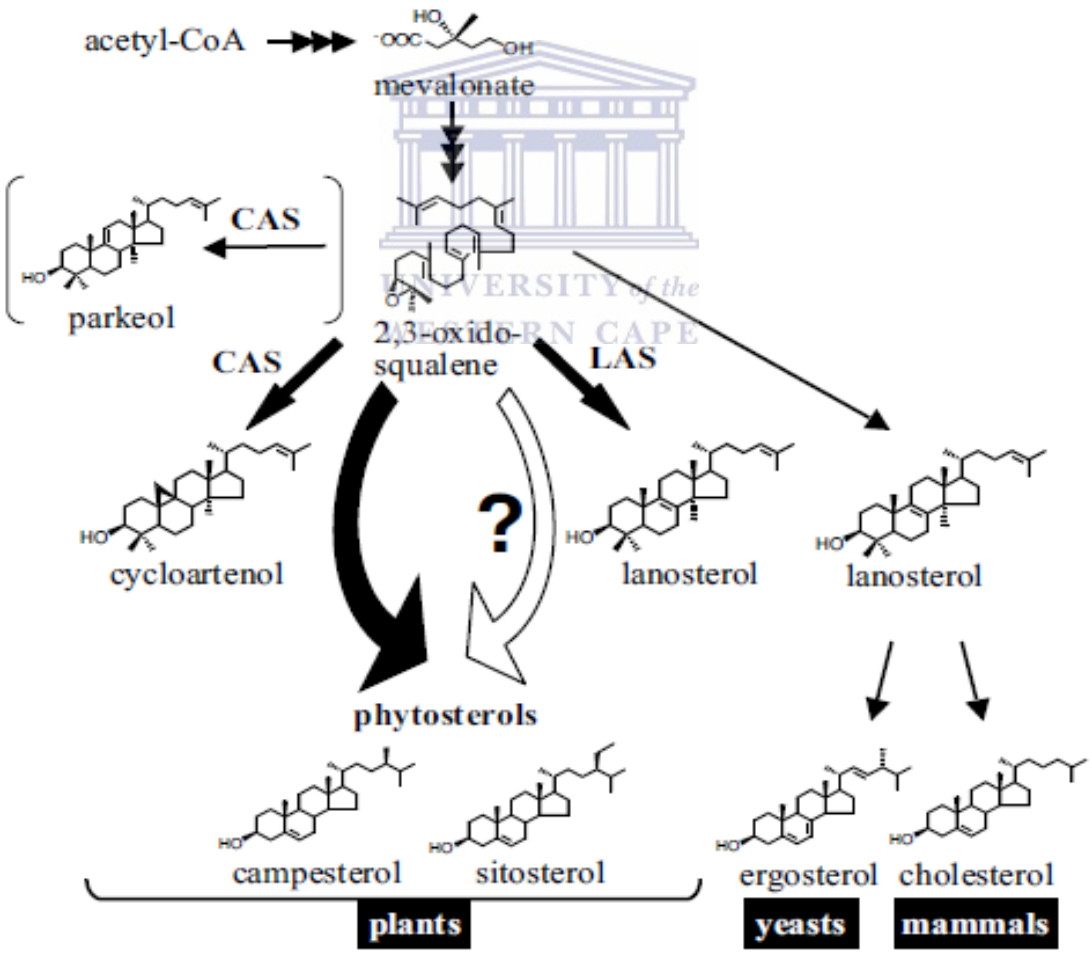


Figure 1.3: The cyclization step of oxidosqualene in yeasts, mammals and plants. Biosynthesis of phytosterols via lanosterol has been demonstrated in several plants as lanosterol genes were identified in plant species such as *Arabidopsis thaliana*, *Panax ginseng* and *Lotus japonica.* CAS, cycloartenol synthase; LAS, Lanosterol synthase. Diagram adapted from Ohyama *et al.,* 2008

An *Arabidopsis thaliana* gene, *At3g45130*, was described to encode the first functional lanosterol synthase in plants (Kolesnikova *et al.,* 2006; Suzuki *et al.,* 2006). In a study conducted by Suzuki *et al.,* (2006), they showed that *Arabidopsis* expresses the *At3g45130* gene but the expression of the gene was different in each tissue, with high expression in siliques and stems and low levels in seedlings and leaves. However, accumulation of lanosterol was low in all tissues that express *At3g45130*. Transgenic *Arabidopsis* overexpressing At3g45130 showed higher accumulation of lanosterol than in the control clones, suggesting that At3g45130 directly cyclized oxidosqualene to lanosterol in plant cells. Based on this evidence, it was concluded that At3g45130 is actually lanosterol synthase 1 (LAS1).

Sawai *et al.* (2006) also proved the existence of lanosterol in *Lotus japonicus*. In their study they showed that the cDNA for OSC7 (oxidosqualene cyclase 7) encodes lanosterol synthase (LAS) by the complementation of a LAS-deficient mutant yeast and structural identification of the accumulated lanosterol. Small amounts of phytosterols that are synthesized via lanosterol were observed in *Arabidopsis* seedlings (Ohyama *et al.,* 2009). Overexpression of LAS1 enhanced the levels of phytosterols and there were no phytosterols that are derived from lanosterol observed in LAS1-knockout plants. This proved the existence of pathway for lanosterol in plant cells.

Phylogenetic reconstruction has shown that only eudicots possess both LAS1 and CAS1, but plant lanosterol synthases evolved independently from those in animals and fungi (Kolesnikova *et al.,* 2006; Sawai *et al.,* 2006). According to Kolesnikova *et al*. (2006), plant lanosterol synthases consist of a third catalytically distinct class of lanosterol synthases. Furthermore, a phylogenetic tree based on the coding sequences suggested that LAS is more

likely to have diverged from the ancestral CAS, mainly because of capability of CAS to be converted to LAS by substitution of two amino acids (Lodeiro *et al.,* 2005; Sawai *et al.,* 2006). Therefore, Sawai *et al*. (2006) confirmed that there is no study that contradicts the idea that cycloartenol is actually the principal cyclic triterpene intermediate for sterol biosynthesis, and lanosterol in plants may act as an alternative intermediate for the sterols or a precursor of other important metabolites. Thus, the Identification of lanosterol metabolizing enzymes may be the answer in understanding the physiological roles of lanosterol in plants.

1.2.3 Biological functions of sterols in plants.

The biological functions of sterols in plant growth and development have been revealed. Sterols are recognised as important structural components of eukaryotic cell membrane that regulate membrane fluidity and permeability (Schuler *et al.,* 1990; Schaller, 2003; Thimmappa *et al.,* 2014). Although all phytosterols have an ability to regulate membrane fluidity, their efficiency in carrying this function differs. Sitosterol and campesterol are the most efficient in comparison with stigmasterol because stigmasterol possesses a trans-oriented double bond at C-22 that has a reduced ordering effect (Piironen *et al.,* 2000).

Sterols are also the biosynthetic precursors of steroid hormones in animals, insects and plants (Suzuki *et al.,* 2006; Ohyama *et al.,* 2009). Furthermore, some sterols are involved in controlling membrane-associated metabolic processes and some are precursors to brassinosteroids, which act as a signalling molecule with hormonal function that regulate growth, developmental and cellular processes (Wang *et al.,* 2009) and the accumulation of reactive oxygen species (Gong *et al.,* 2013). Sterols can also act as substrates for a variety of secondary metabolites such as the glycoalkaloids, cardenolides and saponins. They also play a role in cellular differentiation and proliferation (Pirronen *et al.,* 2000). Plant sterols play a

role in maintaining proper bulk membrane structure and may mediate cellular responses to plant hormones since they have an ability to inhibit abscisic acid-stimulated plant cell membrane permeability and ABA-induced vesicle fusion (Stillwell *et al.,* 1990).

Several lines of studies on the molecular genetics and biochemical analysis of sterol-deficient mutants in *Arabidopsis* have shown abnormalities in plant development, which includes post-embryonic seedling lethality (Kim *et al*., 2005), dwarfism caused by deficiency in brassinosteroids (Klahre *et al.,* 1998; Choe *et al.,* 1999; Suzuki *et al.,* 2004) and plant size variation (Schaeffer *et al.,* 2001; Rasbery *et al.,* 2007). These studies have shown that membrane sterols are crucial for physiological functions in plants and also act as precursors to brassinosteroids phytohormones (Clouse, 2002). A previous study conducted by Babiychuk *et al.* (2008) revealed the biological functions of sterols in plant cells. CAS1 is thought to be the major 2,3-oxidosualene cyclase that initiates post-squalene sterol biosynthesis in plants (Piironen *et al.,* 2000; Thimmappa *et al.,* 2014). The role of cycloartenol has been studied to better understand the biological functions of sterols in plant cells (Babiychuk *et al.*, 2008). This was achieved by analysing the allelic series of *cas1* mutations in *Arabidopsis thaliana.* It was observed that plants that carry the weak mutant allele *cas1–1* were viable but they developed albino inflorescence shoots because of photo-oxidation of plastids in stems that contained low amounts of carotenoids and chlorophylls. They also discovered the role of CAS1 in male gametophyte development through *cas1-2* and *cas1-3* mutant alleles which are non-transmissible through male gametes. The induced loss-of-function in *cas1-2* seedlings caused abnormal growth of leaves, arrest of development of shoot as well as root meristems. Therefore, the phenotypes observed was a result of structural defects in cellular membrane networks caused by depletion of sterols. Thus, CAS1 is crucial for plant cell viability.

However, the function of lanosterol synthases and lanosterol metabolites in plants is still unclear. Some physiological functions of lanosterol in plants have been investigated. These studies showed that there were no significant differences in sterol profiles and no visible morphological phenotypes observed between *Arabidopsis* overexpressing LAS1 and LAS1 mutant *Arabidopsis* plants under normal growth conditions (Suzuki *et al.*, 2006; Ohyama *et al.*, 2009). Moreover, overexpression of the LAS1 gene did not compensate for a CAS1 knock-out mutation allele, which was identified to be male gametophyte-lethal (Ohyama *et al.*, 2009). Based on these findings, the lanosterol pathway may not be essential for the biosynthesis of membrane sterols for cell maintenance under normal conditions. Nonetheless, these enzymes appear to be involved to a small extent in the synthesis of phytosterols and potentially steroid-derived metabolites (Kolesnikova *et al.,* 2006; Suzuki *et al.,* 2006; Ohyama *et al.,* 2009). However, methyl jasmonate and *Pseudomonas syringae* infection enhanced the expression of LAS1 (Zimmermann *et al*., 2004), suggesting that secondary metabolites that are metabolised through lanosterol pathways may be involved in defence responses.

1.2.4 Role of sterols in regulation of reactive oxygen species (ROS)

Besides being major components of the cell membrane, plant sterols appear to play a role in the regulation of ROS scavenging under abiotic stresses such as drought and salinity. The application of brassinosteroids (BRs) induced plant tolerance to diverse abiotic stresses by triggering H_2O_2 generation in cucumber leaves (Cui *et al.,* 2011). In addition, the exogenous application of brassinolide (BL) on *Zea mays* seedlings subjected to water stress caused elevation of activity of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and also ascorbic acid and carotenoids (Li and Van Staden,

1998). Nunez *et al.* (2003) also showed that rice seedlings treated with BR and exposed to salinity stress had altered activity of antioxidant enzymes. Under osmotic stress, BRs increased the activity of CAT and reduced the activity of peroxidase and ascorbic acid oxidase in *Sorghum vulgare* (Vardhini and Rao, 2003).

According to the study by Pose *et al*. (2009), sterols appear to play a role in drought tolerance and regulation ROS. In the study they identified the *Arabidopsis* drought hypersensitive/squalene epoxidase 1-5 (*dry2/sqe1-5)* mutant to be hypersensitive to drought stress. The mutant was affected by mutation in the squalene epoxidase 1 (SQE1) gene, reducing its activity (squalene epoxidase catalyses the first oxygenation step in sterol biosynthesis). Due to reduced activity of SQE1 in the mutant, developmental defects such as reduced shoot development, poor root architecture and short root length were observed. The reduced activity of SQE1 altered the sterol composition in shoots and roots and induced the enzymatic activity of NADPH oxidases, thus enhancing ROS production. This suggests that sterols have a role in the regulation of NADPH oxidases and ROS production.

The role of sterols in regulating ROS production is further supported by a study of seedling lethality in the sterol-deficient *Arabidopsis thaliana cyp51a2* mutant, which is defective in the 14a-demethylation step of the early sterol pathway. This study showed that the expression levels of genes involved in ethylene biosynthesis/signalling and detoxification of reactive oxygen species (ROS) increased in the mutant compared with the wild type. As a result, high levels of accumulation of ethylene in the sterol-deficient mutant were observed. The study also showed that the sterol-deficient mutant was under oxidative stress due to excessive production of ROS, a key factor that triggers programmed cell death (PCD) and is associated with ethylene production. The results of this study thus suggest that changes in membrane

sterol contents and composition in the *cyp51A2* mutant trigger the generation of ROS and ethylene and induces premature seedling senescence through potentiation of PCD (Kim *et al.,* 2010).

Several other studies have also revealed that phytosterols, especially β-sitosterol, are involved in plant responses to oxidative stress as they show high antioxidant activity (Weng and Wang, 2000; Wang *et al.,* 2002; Vivancos and Morena, 2005; Li *et al.,* 2007). According to Wang *et al*. (2012) overexpression of the enzyme 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS), the positive regulator of the sterol synthesis, caused up-regulation of sterol biosynthesis genes enhancing sterol content and reducing hydrogen peroxide-induced cell death, which led to stress tolerance in *Arabidopsis*.

1.3 The production of reactive oxygen species (ROS) under drought stress

Aerobic organisms use atmospheric oxygen (O_2) during cellular respiration as a terminal electron acceptor to yield high energy (Dismukes *et al.,* 2001). Oxygen is generally unreactive when it is in its ground state. However, it can rise to a reactive state such as ROS during normal metabolic activity and under various environmental factors (figure 1.5) (Takahashi and Asada, 1988; Mittler, 2002). About 1 % of O_2 consumed by plants is estimated to contribute to the production of ROS (figure 1.4) (Asa and Takahashi, 1987; Blokhina *et al.,* 2003). The generation and the protection against ROS is an essential characteristic of plant cells. ROS are localised in organelles such as chloroplasts, mitochondria and peroxisomes because these organelles possess a high rate of electron flow and highly oxidising metabolic activity (Mittler, 2002; Asada, 2006). ROS are formed as a result of reduction of one, two or three electrons in molecular oxygen (Halliwell, 2006). ROS can exist as free radicals such as superoxide ($O_2^{\cdot -}$),

hydroxyl ($^{\cdot}OH$), hydroperoxyl (HO_2^-) ions or as non-radicals such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Gill and Tuteja, 2010; Karuppanapandian *et al.,* 2011).

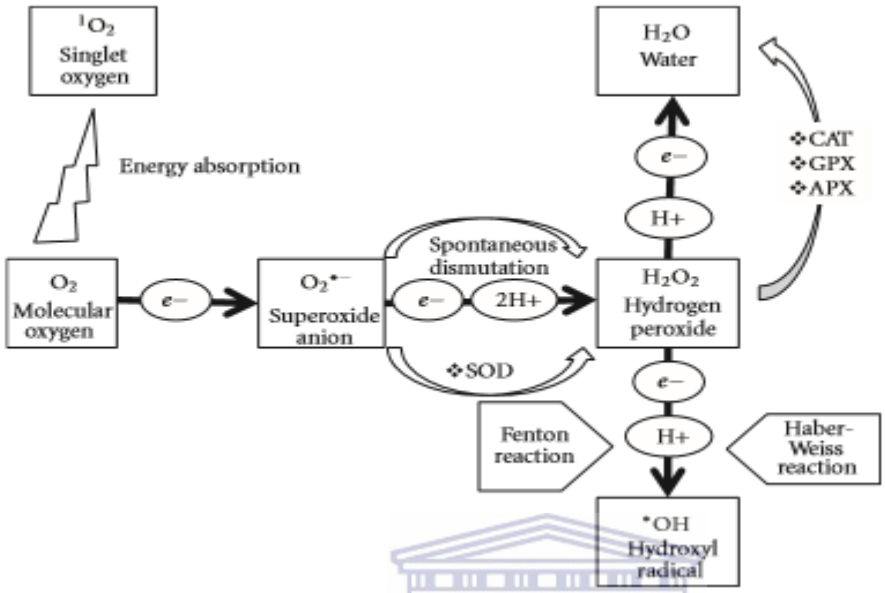


Figure 1.4: The generation of reactive oxygen species (ROS) in plants. The reduction of molecular oxygen (O_2) leads to formation of $O_2^{\bullet-}$, Hydrogen peroxide (H_2O_2) and hydroxyl ($^{\bullet}OH$). Moreover, the energy transferred to O_2 also leads to formation of $^1O_2$. Superoxide is then dismutated to H_2O_2 by superoxide dismutase. H_2O_2 is detoxified by ascorbate peroxidase (APX), catalase (CAT), and guaiacol peroxidase (GPX) to form H_2O. Diagram adapted from Sharma *et al.,* 2012.

However, ROS are recognised for playing a crucial role in plants, depending on their concentration in the plant cell (Apel and Hirt, 2004). At low concentrations, ROS act as secondary messengers involved in signal transduction pathways that regulate plant responses to stress and developmental cues (Suzuki and Mittler, 2006) and they influence expression of several genes (Miller *et al.,* 2010). Some of the ROS that are generated by plants, mainly H_2O_2, are useful in plant developmental processes such as formation of lignin or strengthening of walls in epidermal cells, regulation of physiological processes such as photosynthesis, senescence and photorespiration (Srivalli *et al.,* 2003; Peng *et al.,* 2005; Jubany-Marí *et al.* 2009).

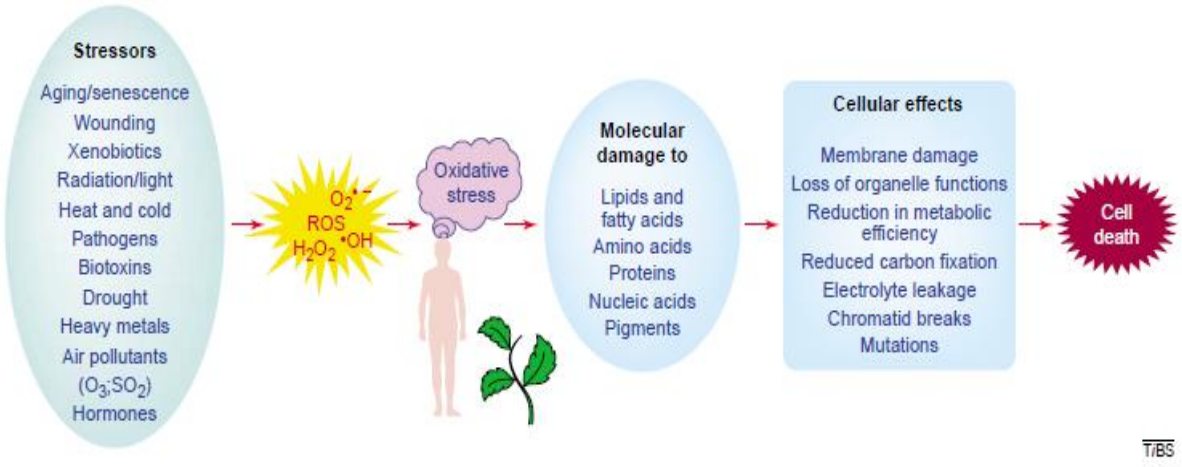


Figure 1.5: Some of the initiators (stressors) of reactive oxygen species (ROS) production and the biological consequences that can lead to cell death. Diagram adapted from Scandalios, 2002.

Furthermore, under physiological steady state conditions, ROS can be scavenged by the antioxidative defense system of the plant (Foyer and Noctor, 2005). However, the equilibrium between production and scavenging of ROS in cellular components may be disturbed by several abiotic and biotic stresses such drought, salinity, pathogens and high temperatures (Sharma *et al.,* 2012). These factors cause excessive accumulation of ROS, commonly called ''oxidative burst''. This excessive accumulation of ROS causes oxidative damage to lipids, nucleic acids and proteins, leading to cell death and low yield as shown in figure 1.5 (McCord, 2000; Karuppanapandian *et al.,* 2011). Damage to lipids occurs when ROS react with unsaturated fatty acids, causing leakage of cellular contents and resulting in cell death (Abreu *et al.,* 2001). The oxidative damage by ROS on proteins can result in site specific amino acid modifications, peptide chain modifications, aggregation of cross-linked reaction products and elevated susceptibility to proteolysis (Srivalli *et al.,* 2003). ROS has also negatively impacts DNA as it causes lesions that cause deletions, mutations and lethal genetic effects (Srivalli *et al.,* 2003;Karuppanapandian *et al.,* 2011).

1.3.1 Production of the superoxide radical ($O_2^{\cdot-}$)

The superoxide radical is regarded as the first ROS to be generated (figure 1.4) and is a moderately reactive ROS with a half-life of approximately 2-4 µs (Gechev *et al.,* 2006). Superoxide is produced mainly in the thylakoid membrane-bound primary electron acceptor of PSI. Due to drought stress, abscisic acid stimulates stomatal closure to reduce further water loss. Closure of stomata decreases CO_2 concentration in leaf mesophyll tissue and results in an accumulation of NADPH. Under such conditions, where NADP+ is a limiting factor, oxygen acts as an alternate acceptor of electrons from the thylakoid electron transport chain, resulting in the formation of superoxide radical ($O_2^{\cdot-}$) in a reaction catalysed by NADPH oxidase. Superoxide is produced as result of transfer of one electron as part of the electron transport chain components with O_2 (Dat *et al.,* 2000). Various essential metabolic enzymes containing Fe-S clusters can be inactivated by $O_2^{\cdot-}$, leading to alteration of catalytic activities (Halliwell, 2006). The generation of $O_2^{\cdot-}$ can trigger the formation of more reactive ROS such $^{\cdot}OH$ and $^1O_2$, which may cause membrane lipid peroxidation and loss of cellular integrity (Van Breusegem *et al.,* 2001).

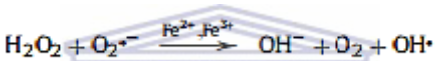
1.3.2 Production of hydrogen peroxide (H_2O_2)

Hydrogen peroxide occurs mainly in the peroxisomes and can also be produced from β-oxidation of fatty acids as a by-product (Ahmad *et al.,* 2008). H_2O_2 is moderately reactive and has relatively long half-life (1 ms) compared to other ROS such as $O_2^{\cdot-}$ and $^1O_2$, which have much shorter half-lives in the 2-4 µs range (Henzler and Steudle, 2000). The production of H_2O_2 occurs during the dismutation of $O_2^{\cdot-}$ by SOD. It has been confirmed that excessive production of H_2O_2 in plant cells leads to oxidative stress. Since H_2O_2 travels freely across

membranes, it is able to diffuse and may inactivate enzymes such as those that are components of the Calvin cycle by oxidizing their thiol groups (Halliwell, 2006). However, H_2O_2 can possibly act as a messenger in that mediates stress responses (Halliwell, 2006; Moller *et al.,* 2007).

1.3.4 Production *of the hydroxyl (˙OH) radical*

Hydroxyl (˙OH) is one of the highly reactive ROS. This ROS can be produced from the reaction of $O_2^{\cdot-}$ and H_2O_2 at neutral pH in the presence of metal ions such as Fe as part of the Haber-Weiss reaction (Kehrer, 2000).



In the reaction, $O_2^{\cdot-}$ donates an electron to iron (Fe^{3+}), producing a reduced form of iron (Fe^{2+}). (Fe^{2+}) then reduces H_2O_2, produced as a result of dismutation of $O_2^{\cdot-}$ to ˙OH by SOD. The final step, which involves the oxidation of Fe^{2+} by H_2O_2 is referred to as the Fenton reaction (Rigo *et al.,* 1977). Since ˙OH is a highly reactive ROS, it can react with many constituents of the cell (including proteins, DNA and lipids) because cells do not possess efficient enzymatic mechanism for the detoxification of ˙OH but rely on prevention of the formation of this highly reactive ROS, which includes elimination of $O_2^{\cdot-}$/ H_2O_2 and possible the metals ions that are involved in the catalyses of Haber-Weiss reaction (Mittler *et al.,* 2004; Halliwell, 2006; Hintze, and Theil, 2006). Therefore, the excess production of ˙OH in the cell leads to cell death

1.4 production of singlet oxygen ($^1O_2$)

The production of singlet oxygen ($^1O_2$) occurs mainly in the chloroplasts when energy transferred to ground state triplet oxygen ($^3O_2$) from chlorophyll triplet excited states under

light intensities. (Laloi *et al.,* 2006). $^1O_2$ is very reactive, with a half time of 3 µs, and it has damaging effects on PSI, PSII and the rest of the photosynthesis machinery (Foyer and Harbinson, 1994). It also reacts with proteins, nucleic acids and lipids, leading to cell death (Wagner *et al.,* 2004). Abiotic stresses such as drought also trigger the formation of $^1O_2$ due limitation of the CO_2 concentration in chloroplast as a result of stomatal closure (Hatz *et al.,* 2007). $^1O_2$ can activate a genetic programme that causes growth inhibition and cell lethality through the EXECUTER1 and EXECUTER2 pathways. EXECUTER1 and EXECUTER2 acts together to transfer stress-related signals from the plastid to the nucleus (Wagner *et al.,* 2004; Lee *et al.,* 2007))

1.4 Detoxification of ROS

In order to reduce the oxidative damage caused by ROS, plants have developed non-enzymatic and enzymatic antioxidant defenses. The non-enzymatic defenses include the cellular redox buffers glutathione (γ-glutamyl-cysteinyl-glycine, GSH) and ascorbic acid (AsA) as well as phenolic compounds, tocopherols and carotenoids (Sharma *et al.,* 2012). These non-enzymatic defenses play a crucial role in defenses, act as enzyme cofactors and also have influence for plant growth and development by controlling processes such as cell elongation, mitosis, senescence and cell death (De Pinto and De Gara, 2004). It has been shown that mutants perturbed in the biosynthesis of non-enzymatic antioxidants are hypersensitive to stress (Gao and Zhang, 2008).

The enzymatic antioxidative defense network includes several antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase CAT), guaiacol peroxidase (GPX) and enzymes for the ascorbate-glutathione (AsA-GSH) cycle, glutathione reductase

(GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), as shown in Figure 1.6 (Noctor and Foyer, 1998; Smirnoff, 2005). SOD is one of the major antioxidant enzymes because it serves as the frontal defense against oxidative stress and is the only enzyme that can scavenge superoxide ($O_2^{\cdot-}$) (Dat *et al.,* 2000). It is found in most subcellular compartments that generate ROS and belongs to a group of metalloenzymes that use either Cu/Zn, Mn or Fe as a co-factor to catalyze their reactions to convert $O_2^{\cdot-}$ to H_2O_2 (Gill and Tuteja, 2010). There are three forms of SOD found in plants, copper/zinc SOD (Cu/Zn-SOD), manganese SOD (Mn-SOD), and iron SOD (Fe-SOD). These isozymes are located in different cellular compartments, with Fe-SOD localized in chloroplasts, MnSOD is localized in mitochondria and Cu/Zn-SOD existing in three sub-cellular localizations (namely the cytosol, peroxisomes, chloroplast and mitochondria) (Mittler, 2002). Copper/Zinc SOD is sensitive to cyanide, whereas both MnSOD and Fe-SOD are insensitive to cyanide but Fe-SOD is sensitive to H_2O_2 (Scandalios, 1993; del Rio *et al.*, 1998). SOD catalyses the dismutation of $O_2^{\cdot-}$ to O_2 and H_2O (Moller, 2001; Arnholdt-Schmitt *et al*., 2006).

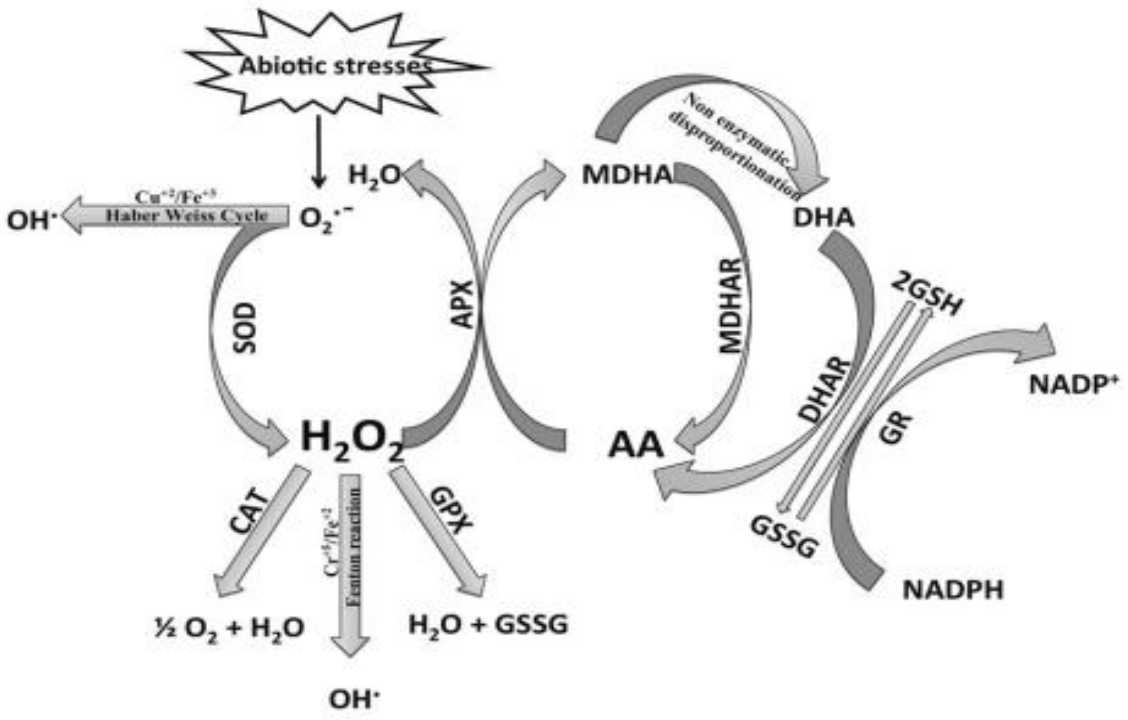


Figure 1.6: Reactive oxygen species (ROS) scavenging mechanism in plants. SOD (superoxide dismutase); APX (ascorbate peroxidase; CAT (catalase); GPox (glutathione peroxidase); MDHA (monodehydroascorbate); MDHAR (monodehydroascorbate reductase); DHA (dehydroascorbate); GSH (glutathione); GR (glutathione reductase); GSSG (glutathione oxidized. Picture Adapted from Gill and Tuteja, 2010.

Since high concentration of H_2O_2 are also toxic to plants, any excessive H_2O_2 produced should then be scavenged by CAT and several other classes of peroxidases. These peroxidases, however, have different affinities for H_2O_2 and have different cellular roles in scavenging H_2O_2 (Willekens *et al.,* 1997). Catalase is localized in the cytosol, peroxisomes and mitochondria and it catalyzes the dismutation of two molecules of H_2O_2 into H_2O and O_2 (Scandalios, 2002). It has been shown that catalases have weak activity against organic peroxides but have high specificity for H_2O_2. They are only active at high levels of H_2O_2 because of their rather low affinity for H_2O_2. Nonetheless, their catalysis rate, once they bind H_2O_2, is very fast. In essence, CATs have a very fast turnover rate but lower affinity for H_2O_2 than APX (Mittler,

2002; Gill and tuteja, 2010). Therefore, at lower levels only APX and other peroxidases remove H_2O_2. However, amongst other H_2O_2-scavanging enzymes, CATs are considered as unique as they do not require cellular reducing agents, unlike APX (which needs ascorbate as a reducing agent) to catalyze their reaction (Mittler, 2002).

Peroxidases such as APX and GPox are located throughout the cell and catalyze the reduction of H_2O_2 to H_2O. GPox is less specific for electron donor substrate and oxidizes aromatic electron donors such as guaiacol and pyragallol by decomposing H_2O_2 (Schuller, 1996). GPoxX uses glutathione (GSH) to reduce H_2O_2 to H_2O resulting in an oxidized form of glutathione (glutathione disulphide (GSSG)) which must be regenerated back to GSH by glutathione reductase (GR) using NADPH as an electron donor. GPox also plays an important role in biosynthetic processes such as ethylene biosynthesis, lignification of cell wall and wound healing (Kobayashi *et al.,* 1996). APX is the major plant peroxidase in the scavenging of H_2O_2 because isoforms of APX have higher affinity for H_2O_2 than CAT (Wang *et al.,* 1999). Unlike other peroxidases, APX requires ascorbate as an electron donor in the first step of the ascorbate–glutathione cycle (AsA-GSH cycle), which is also referred to as the Halliwell-Asada pathway (Figure 1.4.1) to break down H_2O_2 into water and two molecules of monodehydroascorbate (MDHA). This process occurs in the cytosol and chloroplasts in plants. In higher plants, there are five isozymes of APX that have been found in different subcellular localizations. These include thylakoidal, stromal, cytosolic, mitochondrial and peroxisomal APX isozymes (Apel and Hirt, 2004). Each APX isoform scavenges H_2O_2 found within the organelle where it is localized, except for the cytosolic APX which removes H_2O_2 produced in the apoplast and cytosol (Mittler and Zilinskas, 1992). Monodehydroascorbate reductase

(MDHAR) and dehydroascorbate reductase (DHAR) are involved in the regeneration of ascorbate (de Azevedo Neto, 2006).

1.5 Drought

Drought is one of the most devastating environmental conditions for plants and is associated with climate change. Drought is the term that describes water shortages over an extended period of time (Dracup *et al.,* 1980). It occurs when precipitation is below average in a given region within a given season, leading to shortages of water (Wilhite and Glantz, 1985). In many cases, drought commonly occurs together with high temperatures, increased soil salinity and elevated irradiance while associated with reduced soil nutrients and damage to plant roots in hard and hot soils (Zhu, 2002; Al-Kaisi *et al.,* 2013). Drought affects about 60 % of the world's population with about 630 million living in arid and semi-arid areas (Figure 1.5.1). A large portion of these populations depends mostly on farming for their livelihood (Ngaira, 2005). Drought has devastating economic, environment and social impacts in terms of loss of human life caused by water and food-borne diseases, food insecurity caused by reduced agricultural productivity and degradation of natural resources (Patz *et al.,* 2005; Devereux, 2007; Kang *et al.,* 2009). Drought contributes significantly to famine and is the major limitation to rain-fed agricultural production, especially in arid and semi-arid lands (Falkenmark and Widstrand, 1992). In the 1970s and 1980s, rainfall was below average in most parts of the world and this severely affected crop production (Parry, 1990). Due to increasing temperatures, climate change is estimated to increase the occurrence of drought globally, particularly in the tropics and subtropics, resulting in reduced food and feed supplies globally (Schmidhuber and Tubiello, 2007).

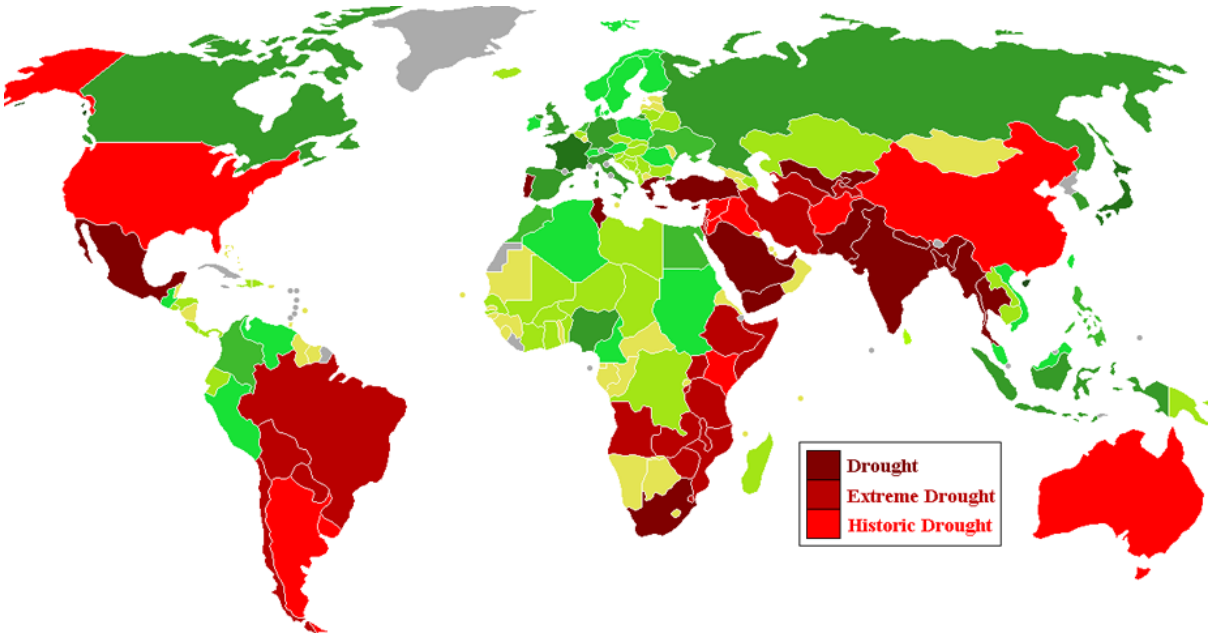


Figure 1.7: **Incidents of Drought in 2009 which Impacted Global Food Production**. The food producing countries affected by drought, resulting in destruction of crops and death of animals. Picture adapted from deCarbonnel, 2009

It has been estimated that from 1970 to 2000, land areas affected by drought has doubled worldwide (Isendahl and Schmidt, 2006). This occurrence has raised extensive concern on food supply for the increasing population worldwide, which by 2050 is estimated to reach about 9.1 billion (Sto, 2011). In 2009, countries such as China, Australia, the majority of countries in Africa and South America, together with the US, that made up two thirds of agricultural output of the world were affected by drought (figure 1.7). This resulted in destruction of crops and livestock (deCarbonnel, 2009). It has also been predicted that the world will experience a decrease of 20% to 40% in agricultural production depending on the severity and length of the current global droughts (Adhikari *et al.,* 2015). In recent years, drought has become more frequent and severe, and more areas are being affected.

1.5.1 The impacts of drought on agriculture and food security in South Africa

The South African landmass is considered to have 12 % arable land and only 3% of the land is regarded as truly fertile. Only 1.5% of the land is under irrigation produces 30% of the crops in country (AgriSA, 2016 a; Drought Task Team, 2016). This means that the agricultural land in South Africa is limited in terms of availability for crop production, although the agricultural sector plays a significant role in the South African economy as it directly contributes up to 4% per year to the national gross domestic product (GDP) and indirectly contributes almost 25% to the GDP if agro-processing and downstream industries are considered (Agri Western Cape, 2015). South African agricultural performance is affected by changes in climatic conditions, causing a decline in its contribution to the country's GDP (Grain SA, 2016). The contribution of South African agriculture to national GDP decreased by 18.5% from 1910 to recent years (Grain SA, 2016).

South Africa is the major producer of agricultural products in Africa as it produces a wide range of food commodities. The country produces an exportable excess of food to the Middle East, Africa and Europe (Grain SA, 2016). Approximately 60% of the country's water resources is used mainly in agriculture (Baleta and Pengram, 2014). About a quarter of the country's water supply is obtained from Lesotho because Lesotho receives 60 % more rainfall than South Africa (DWAF, 2014). Therefore, drought is one of the major consequences of climate change that cause constraints on water availability and agricultural production in South Africa. Southern Africa has recently experienced the worst drought in at least the last three decades in the 2015/2016 production year due to El Nino phenomenon that occurred since mid-2015 (FAO, 2016). El Nino generally brings drier conditions to Southern Africa. The 2015/16 El Nino phenomenon is considered the strongest since the past 50 years, leading to

the 2015/16 drought being the worst in Southern Africa since 1992 (Food and Agriculture Organisation of the United Nations a, 2015). In South Africa, 2015 had the lowest national annual rainfall since 1904 in all nine provinces (Agri SA b, 2016). In the past years rainfall was averaged 608 mm per annum, while in 2015 South Africa received 403 mm of rainfall, which is 66% of the annual average rainfall. The lowest rainfall received previously in the country was in 1945, with 437 mm rainfall (Agri SA b, 2016). The worst affected provinces which were declared disaster areas in 2015 are the Free State, KwaZulu Natal, North West, Limpopo and Northern Cape, as indicated in the map below (figure 1.8) (Bureau for Food and Agricultural Policy, 2016). It was reported that drought affected the level of water in the storage dams, which dropped from an average of 70% in 2015 to less than 40% in 2016 (Agri SA a, 2016). Due to current droughts, most parts of the county are experiencing water shortages in 2017 as well because the water in the storage dams has decreasing rapidly.

Drought consequently resulted in significant delays of up to 60 days in planting and poor conditions for early crop development and growth, which resulted in extensive crop failure (FAO , 2015; Agri SA a, 2016). In addition, drought resulted in reduced planting areas for summer/spring crops such as maize, soybean and wheat, leading to lower yields in many areas. Farmers that grow agricultural commodities such as maize, soybean and sunflower have consequently suffered severe losses (Agri SA a, 2016). Furthermore, the decline in grain yield resulted in increased import of maize to approximately 3-5 million tons (Agri SA a, 2016). Due to drought conditions, the total summer crop in South Africa was estimated to decrease by 24% year-on-year and production of soybean was expected to decrease by 72% year-on-year in 2016 (Grain SA, 2016).

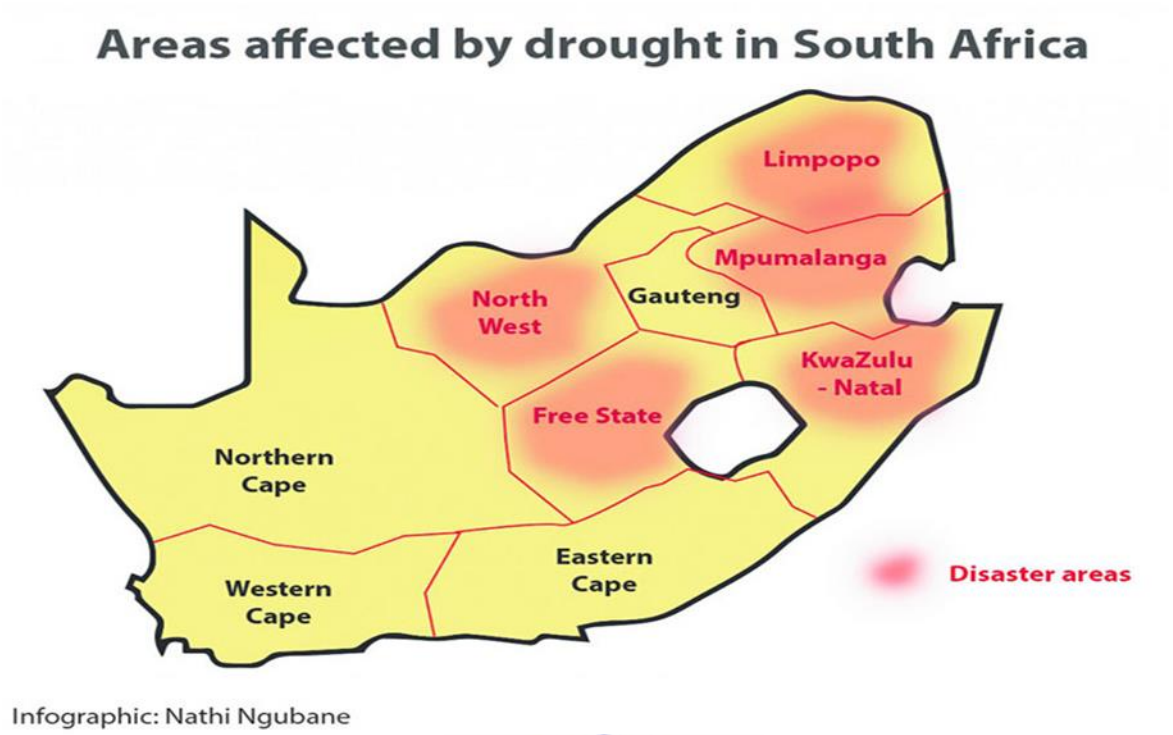


Figure 1.8: Area in South Africa in which drought has been declared disastrous in the 2015/16 growing season. Source: (Tau, 2016)

The Crop Estimate Committee has estimated the area of maize planted for the 2016 season to be at 1,95 million ha, which is the smallest area planted to maize since the 1928 season (1,926 million ha) (FAO, 2016). According to United Nations Food and Agriculture Organisation b (2015), the 2015 harvest was estimated to be 22.6 % lower than the previous season's harvest in 2014. It was not just maize growers who suffered lower harvest, instead other commodity growers such as soybean, wheat, sugar cane and livestock producers experienced similar losses (Mail and Guardian, 2015). Therefore, in 2015 the agricultural sector as a whole experienced a 14% decline and drought caused 16 billion Rand loss of profits in this sector. As a result, some foods prices such as bread and cereals increased with consumer price inflation increase of up to 4.8% (Mail and Guardian, 2015).

Since the El Niño conditions are predicted to occur more often in the upcoming decades, this will cause more frequent droughts in Southern Africa when compared to long-term historical

trends (Food and Agriculture Organisation of the United Nations b, 2015). It is also estimated that by 2025, water demand in the South Africa will exceed supply (Ashton *et al.,* 2008). This will have a negative impact on agricultural productivity including crop losses, lower yields and increased livestock deaths. This will threaten food security in terms of food availability, access, utilization and stability. Livestock farmers will experience loss of income as the animals will be deprived of grazing resources and therefore, farmers will not be able to maintain stock, and thus forced to sell and slaughter their livestock to sell meat prematurely. Meat will be cheaper in the first instance but become more expensive due to production costs, and further into the future it will have a negative impact as there will be limited livestock for meat (Shabelle, 2011). In this most likely assured scenario, South Africa will have to import meat, resulting in extremely high prices for meat. Southern African countries such as Botswana, Namibia, Lesotho, Zimbabwe, Mozambique and Zambia depend mostly on agricultural imports from South Africa and up to 40% of their food is imported from South Africa (Food and Agriculture Organisation of the United Nations a, 2015). Importantly, South African agricultural commodities considered to be the staple food in Southern Africa are the key sources of calorie and protein intake for lower income households, therefore, the reduction in production of these commodities could pose a major threat on food security (Mail and Guardian, 2015). Before the recent droughts, all of these Southern African countries were already estimated to have about 14 million people who were food-insecure (FAO, 2015). Therefore, the upcoming droughts will have a devastating impact as these countries will require more imports and there will be insufficient accessible supply to meet the demands.

Drought does not affect the agricultural sector only, but has other secondary effects where several other sectors can be affected. Since drought will decrease exports of food crops, while

increasing the need for food imports to meet local needs, this could result in increased economic pressure on the national budget. This has a severe effect on consumers, especially the poorest and more vulnerable, as food supply becomes limited. Such impact leads to a rise in food prices. In 2016 food prices in South Africa increased by 6.4%, which contributed to a sharp increase in the national inflation rate, which increased to 7% year-on-year (Grain SA, 2016). The rise in food prices will affect the majority of South African (and in deed African) homes, especially the poorest because they will not afford the food. This can impact their health and increase the risk of malnutrition (FNSWG, 2015). The impacts of drought can affect several other sectors such as food producing sectors. This will lead to income losses in many homes as the companies will be forced to retrench employees as there will be no work for them, especially those who work in farms. At a time when prices of other commodities are low, there will be increased skill shortages in the country (FAO a,b, 2015). Therefore, the development of drought-resistant crops is crucial to address this forthcoming threat to food security.

1.6 Soybean

The history of soybean is known to a limited extent due to the lack of record keeping in the early centuries. Historians suggested that the soybean plant originated from the Eastern part of the hemisphere and was introduced as a crop in Western part of the hemisphere (Hymowit *et al.,* 1984). Historical evidence showed that soybean was domesticated in the eastern half of north China during the Zhou dynasty around 11th century (Hymowitz, 1970; Guo *et al.,* 2010). The whole bean was fermented into products like paste for human consumption while also being used as treatment for swelling and wounds. The use of this crop then spread to countries such as Korea, Japan and other Southeast Asian countries. Soybean was introduced

in Europe by a German botanist, Englebert Kaempfer in 1712 (Gibson and Benson, 2005). The first scientific study on soybean plants was conducted by Carl von Linne, a Swedish botanist. In the 19th century, soybean was introduced to America by trading ships and was grown by farmers there (Hymowitz and Harlan, 1983). They used it to produce soy sauce and later used it for animal feed. In 1896, researcher George Washington Carver found new uses of soybean. He discovered soybean's ability for nitrogen fixation and potential for vegetable protein, edible oil and meal (Gibson and Benson, 2005). The United States imported about 40% of its edible oil and fats prior to World War II (American Soybean Association, 1998). When the war took place, the oil supply line was cut and, as a result, it was crucial for Americans to produce soybean to meet the need for their oil demands. This resulted in an increase in soybean production. In the 1950's, the cost of the soybean meal was very low, which in turn triggered an additional demand for soybean production (American Soybean Association, 1998). The US produced approximately 75% of the world's soybean from the 1950's through the 1970's. Soybean production in other nations, like Brazil and Argentina, also increased with increase in demand (Hartman *et al.,* 2011).

The United States ranks first in soybean production, supplying two thirds of global soybean needs. Soybean crop production has expanded and resulted in the emergence of Brazil and Argentina as major producers. These countries are regarded as the second and third most important soybean-producing countries in the world. The USA, Brazil and Argentina dominate global soybean production (Hartman *et al.,* 2011). The production of soybean in China decreased despite the fact that it is the country that domesticated the crop (Hymowitz, 1970).

In South Africa, the first record of soybean appeared in 1903 in the Cedara Memoirs. The soybean was imported from China, but it did not germinate properly. Soybean was planted in

the Research Centre in Potchefstroom in early fifties and it was often called 'Geduld' which means patience (Du Toit, 1942; DAFF, 2010). In the 1970s, South African production was 10 000 tonnes and rose to 190 000 tonnes by 2001. In recent years, South Africa has become one of the leading countries in soybean production in Africa (Grain SA, 2016). Currently soybean production ranges from 450 000-500 000 tons per annum with an average yield of 2.5 to 3 tons per hector (DAFF, 2010). It is grown throughout the country but mainly in Mpumalanga and the Free State province. Amongst other provinces, Mpumalanga produces the highest quantities of soybean (42%), followed by Free State at 22%, 15 % in KwaZulu-Natal, Limpopo at 8%, North West at 5% and Gauteng at 5% (DAFF, 2010)

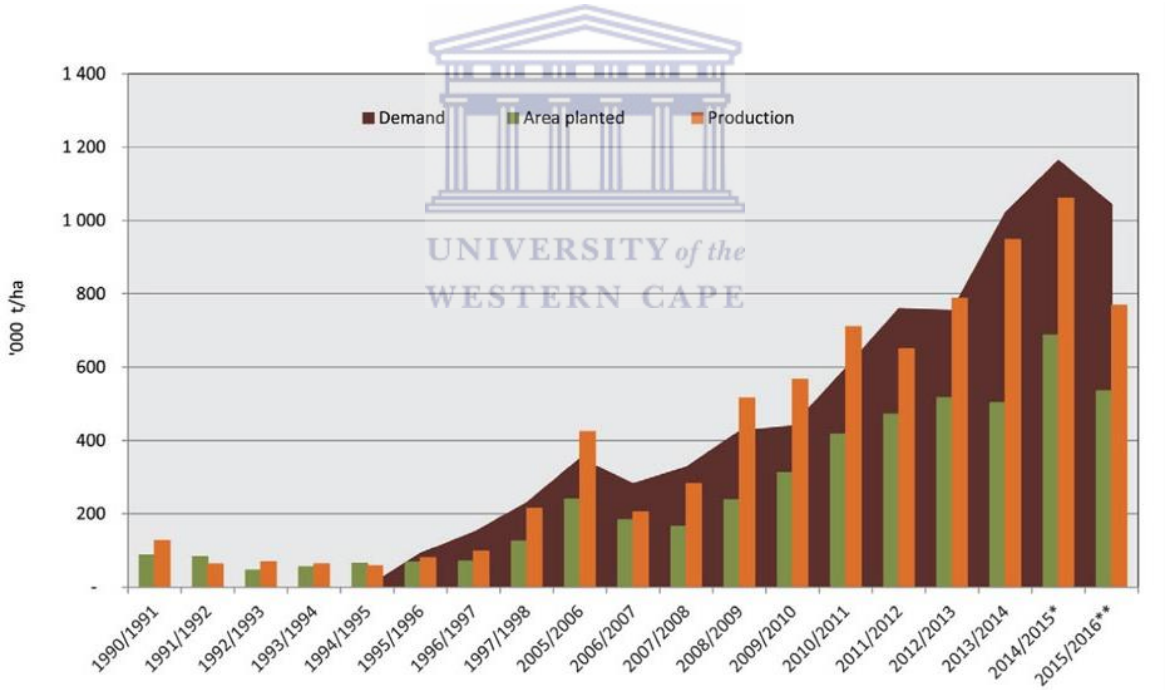


Figure 1.9: South African soybean production. Source: Grain SA, 2016

The report by Grain SA (2016) showed that soybean production between 2011/2012 to 2014/2015 production seasons increased by 63% and the area planted increased by 57% because of high demand (figure 1.9). However, soybean demand exceeded production from the 2013/2014 production season. Moreover, the current drought resulted in a decrease in

the area planted as well as production (Grain SA, 2016). On the other hand soybean production increased significantly in the past few years when compared to other crops such as sunflower, maize, and groundnuts. South Africa exports some of soybean oil to African markets. However, SA also imports significant volumes of soybean oil from Spain, Argentina, Netherlands, Romania and Brazil (Grain SA, 2016). This illustrates that soybean is significant for the South African economy.

1.6.1 The importance of soybean

Among other legumes, soybean is one of great importance worldwide and the leading oilseed crop that boosts world economy (Toorchi *et al.*, 2009). Soybean is of such extraordinary economic importance that it is used to settle oil import prices. This is due to the chemical and mineral composition of the grain (Raghuvanshi and Bisht, 2010). The first soy food products produced from soybean fermentation include sauce, tempeh, miso, and natto (Gibson and Garren Benson, 2005).

In recent decades, soybeans have become popular and regarded as an important crop because of the advanced popularity of soy foods, such as soy milk and textured vegetable protein (Phang *et al.*, 2011). Presently, about 85% of soybean globally is used as a protein-rich food and feed crop and as a source of oils used in food (Phang *et al.*, 2011). This is particularly relevant for world food security and has specific significance to nutrition in developing countries where protein deficiency is rampant. The oil extracted from soybean is used to produce some food products such as margarine, salad dressings, shortenings and cooking oil. The high-protein fibre that remains after the extraction of the oil is toasted and prepared into animal feed for livestock (Ali, 2010).

Soybean is also considered as one of the richest source of plant-based protein among other crops, equivalent to meat in terms of quality (Nwokolo, 1996). The wide variety products derived from soybean has created a huge market. The protein extracted from soybean has been rated number one by the World Health Organisation (WHO) and Food and Drug Administration (FDA) for its nutritional quality for both adults and children (Medindia, 2016). Most crops, like corn and wheat, consist of proteins that have a low percentage of essential amino acids such as tryptophan, lysine, and sulphur-containing amino acids at levels which are not sufficient for human nutritional requirements. Soybean proteins assist in balancing this nutrient deficiency of other grains. Soybean produces the essential eight amino acids than other beans and is, therefore, sufficient for animal and human nutritional requirements (Barrett, 2006). Soybean proteins can substitute meat as it contains sufficient amount of proteins (Hartman *et al.,* 2011). This is beneficial for people that do not eat meat and those that have health problems concerning meat. Soybean proteins are found in baby formula, weight-loss and sport drinks, and as a low-fat component in hamburger (Friedman and Brandon, 2001; Barrett, 2006).

Extracts of soybean are also used in the manufacture of pharmaceutical products such as antioxidants and vitamin E capsules (Barrett, 2006). Soybeans have a wealth of health benefits as they have been shown to contain compounds with ability to improve the metabolism, heart health, defend against cancer, reduce the effects of menopause, protect against birth defects, increase circulation, and decrease the risk of diabetes (Friedman and Brandon, 2001).

Soybean is not only important for food and health purposes. It is also used for industrial applications such as the production varnishes, paints, inks, soaps, lubricants and other products (Cahoon, 2003). As a legume plant, it is also recognised for its ability to enrich soil

with nitrogen (Vlahović *et al.,* 2013). Soybean has a symbiotic relationship with nitrogen-fixing bacteria *Bradyrhizobium japonium* in its roots (Marino *et al.,* 2007; Toorchi *et al.,* 2009; Sanchez *et al.,* 2011). This symbiotic nitrogen fixation plays a significant role by improving soil fertility (Zahran, 1999). This nitrogen fixation reduces the need for use of synthetic nitrogen fertilizers, thus providing an environmentally friendly crop production system since synthetic fertilizers lead to land and water pollution. It is also used in the production of biodiesel (Pimentel and Patzek, 2008; Phang *et al.,* 2011), an environmentally friendly fuel that replaces non-renewable fossil fuel products. This makes this legume crop an important agricultural commodity with direct significance on food security and environmental sustainability.

1.6.2 Soybean responses to drought stress

Drought is one of the major abiotic stresses that have devastating effects on crop yield as it affects the growth, reproduction and development of plants. Prolonged drought can cause permanent cell death, resulting in crop failure (Farooq *et al.,* 2009). In several parts of the world, impacts of water deficit can reduce crop productivity by 50% (Lisar *et al.,* 2012). Soybean is one of the crops that is negatively affected by drought. Soybean is mostly planted in rain-fed agricultural regions and is considered as one of the most susceptible crops to yield loss due to water deficit (Liu *et al.,* 2004). Soybean is considered more sensitive to water deficit during germination, flowering and seed development (Ohashi *et al.,* 2006). Like other plants, when soybeans are exposed to drought, numerous physiological, morphological and biochemical changes are induced, as a result the normal growth and development is disturbed (Manavalan *et al.*, 2009). The symptoms of water deficit include suppressed growth, reduced photosynthetic rate, reduced transpiration rate and leaf senescence (Chaves *et al.,* 2003). According to Specht *et al.* (2001), drought reduced soybean yield by about 40%. The

decreased photosynthetic rate, stomatal conductance and transpiration rate caused by water deficit in soybean reduced yield by 20% at seedling stage and by 46% at flowering stage. Under drought stress, the dry mass was reduced by 30 % when compared to well-watered control plants, with drought causing significantly decreased photosynthetic rate, stomatal conductance and transpiration rate (Ohashi *et al.,* 2006).

Under drought conditions, soybean adopts various mechanisms in order to cope with water deficit stress. This includes three mechanisms, namely drought avoidance, drought escape and drought tolerance (Verslues *et al.,* 2006). Drought avoidance is when the plant maintains tissue water during stress conditions by reducing water loss (evapotranspiration) and absorbing water as much as possible by improving uptake of water by roots (Araus *et al.,* 2002). Soybean adapts to water deficiency by stimulating growth of roots and reducing shoot growth because shoots are very sensitive to growth inhibition due to low cell turgor when compared to roots. Roots become thick in order to penetrate hard soils to absorb water at different depths of the soil. Plants also develop long taproots to absorb water located deeper in the soil. In case of severe droughts, the growth of roots may be inhibited due to low water availability. Therefore, instead of developing long taproots, the plants reduce root length growth by developing root hairs (O'toole and Bland, 1987; Manavalan *et al.,* 2009). The second mechanism, drought escape, occurs when the plant develops rapidly to complete its life cycle before drought occurs. Drought tolerance is when the plant is able to withstand drought with low water potential, by maintaining cell turgor (osmotic adjustment) and reducing evapotranspiration in leaves by accumulation of compatible solutes (Zlatev *et al.,* 2012).

During periods of drought, relative water content (RWC), water potential and turgor are reduced in the cell of the plant and this causes a rise in the concentrations of solutes (osmolytes/osmotic adjustment) such as proline, mannitol and sorbitol the in cytosol and extracellular matrices (Serraj and Sinclair, 2002). Plants accumulate these osmolytes in order to protect themselves against dehydration. When the osmolytes accumulates in plant cells, they result in decline of water loss, reduced osmotic potential and maintenance of cell turgor pressure that contributes to maintenance of physiological processes and this reduces wilting in plants. Drought decreases cell expansion and causes inhibition of growth (Serraj and Sinclair, 2002; Lisar *et al.,* 2012). Drought also causes accumulation abscisic acid (ABA). Abscisic acid is induced under drought stress conditions and acts as a signalling molecule. Abscisic acid plays a significant role in regulation of water status in plants by regulating stomatal closure in order to maintain leaf water content and water potential and inducing expression of stress-related genes that encode proteins and enzymes involved in combating water deficit stress in cells (Davies, and Zhang, 1991; Zhu, 2002). Stomatal closure can result in the limitation of gaseous exchange in terms of CO_2 assimilation. This disrupts electron requirements for photosynthesis and leads to a greater susceptibility to photo-oxidative damage. In soybean, the photosynthetic rate and water potential is decreased under drought stress in leaves, pods and leaves (Liu *et al.,* 2004). This is followed by high accumulation of ROS, which inhibits photochemical functions and activates antioxidant enzyme activities such as APX and SOD (Mittler *et al.,* 2004). Drought also causes synthesis of new proteins and mRNAs involved in water deficit stress defence (DuPont and Altenbach, 2003). Drought may also affect nutrient uptake and transport in plant organs (Al-Kaisi *et al.,* 2013). In addition, nitrogen fixation in soybean is very sensitive to drought (Sinclair *et al.,* 2007). The reduction in nitrogen fixation disrupts metabolism and causes reduction of soybean yield due to

inadequate nitrogen fixation required for production of proteins that are essential for seed production (Serraj *et al.,* 1999; Streeter, 2003; Sinclair *et al.,* 2007) and reduction of photosynthetic activity due to insufficient nitrogen accumulation in leaves (Salvagiotti *et al.,* 2008). Therefore, understating the physiological and molecular responses associated with drought tolerance in soybean is crucial to overcome the challenges of drought on soybean production and yield. Focus on breeding technologies and genetic engineering approaches in developing drought-tolerant soybean lines to improve soybean yield under drought is of importance. To achieve such desired tolerance, understanding the contribution of genetically determined metabolic components to the regulation of drought responses is crucial. For this reason, this study investigated the possible contribution of a sterol biosynthetic gene to soybean responses to drought.

CHAPTER 2

MATERIALS AND METHODS

Table 2.1: List of suppliers and chemicals

Chemicals	**Suppliers**
L-Ascorbic acid	Sigma- Aldrich
Agarose	Whitehead Scientific
Ammonium persulphate	Sigma- Aldrich
Evans blue	Sigma- Aldrich
Terbinafine	Sigma- Aldrich
Calcium sulphate	Sigma- Aldrich
Promix	Windell Hydroponics
Sodium dodecyl sulphate (SDS)	BIO-RAD
Hydrogen peroxide	Sigma- Aldrich
NBT	Sigma- Aldrich
TCA	Sigma- Aldrich
Thiobarbituric acid	Sigma- Aldrich
Potassium iodide	Sigma- Aldrich
K_2HPO_4	Sigma- Aldrich
EDTA	Sigma- Aldrich
PVP	Sigma- Aldrich
Bovin serum albumin	Sigma- Aldrich
Bradford reagent	Bio-Rad
Glycine	Sigma- Aldrich

Tris base	Sigma- Aldrich
TEMED	Bio-Rad
Ferric chloride	Sigma- Aldrich
Potassium ferricynide	Sigma- Aldrich
Riboflavin	Sigma- Aldrich
Potassium cynide	Sigma- Aldrich
DHA (bis (dehydro-l-ascorbic acid)	Sigma- Aldrich
GSH	Sigma- Aldrich
L-methionine	Sigma- Aldrich
cDNA synthesis kit	New England Biolabs
RNA mini prep kit	Zymo Research
DNase treatment kit	Thermo Fisher scientific
GelRed™ nucleic acid	Biotium
Hot start	Thermo Fisher scientific
Luminaris color HiGreen qPCR master mix	Thermo Fisher scientific
Potassium hydroxide	Sigma- Aldrich
Hexane	Sigma- Aldrich
Pyridine	Sigma- Aldrich
N,O-bis (trimethylsilyl)trifluoroacetamide (99% BSTFA)	Sigma- Aldrich
Dimethylsulfoxide (DMSO)	Pierce
KH_2PO_4	Sigma- Aldrich
Bis acrylamide	Bio-Rad

2.1 Seed germination and plant growth

Soybean (LS 6150R cultivar) seeds were surface sterilised with 10% commercial bleach for 10 min and washed with dH_2O six times. The seeds were soaked in sterile dH_2O for 10 min followed by imbibing of the seeds in 10 mM $CaSO_4$ for 16 hours. The seeds were inoculated with *Bradyrhyzobium japonicum* bacteria and were germinated for 3 days in paper towel soaked with dH_2O.

When the germinated seeds had radicles approximately 1.5 cm long, they were transplanted into 20 cm plastic pots (two plants per pot) containing 3 L of moist Promix Organic (from Windell Hydroponics; a medium made of mixture of perlite and coconut peat, formulated with an organic fertilizer that provides nutrients to growing plants, and has an ability to retain water for a longer periods than soil or other growth media). Before the seedlings were transplanted to pots, the Promix Organic had been dried at 80°C for 48 hours to remove the moisture. For control plants (WW) and 50 µM terbinafine (WWT) (sterol synthesis inhibitor) -treated plants, water was added to maintain a water potential of -0.03 MPa in both treatments. For water-deficit plants (WD) and the combination of water-deficit and 50 µM terbinafine (WDT) -treated plants, water was added to maintain a water potential of -0.41 MPa. The seedlings were grown in a growth room at 25°C during the day and 19°C at night under 16/8 hours light/dark at a photosynthetic photon flux density of 200 $\mu mol.m^{-2}.s^{-1}$ during the light phase.

2.2 Treatment of plants

At the VC stage (when unifoliate leaves fully opened) of plant growth, plants were treated with either water or terbinafine. Control plants were supplied with 50 ml of water (containing

methanol at a final concentration of 1%) for each pot every third day whereas terbinafine treatments were done by watering with 50 ml of water containing terbinafine (from a stock solution made in methanol) at a final concentration of 50 µM terbinafine such that the final methanol concentration is 1%. Plants were irrigated with the terbinafine solution every third day. Untreated and Water Deficit plants were supplied with water containing 1% methanol since the terbinafine is dissolved in methanol and the Terbinafine treatments thus contain methanol at a final concentration of 1%.

2.3 Measurements of growth parameters

Plants were harvested at V stage after 32 days of growth. Ten plants from each treatment were carefully removed from the Promix Organic. Shoots were excised and weighed as soon as possible after their excision and the length of the shoot was also measured. Leaves were harvested for further analysis and the remaining plant material was quickly frozen in a liquid nitrogen and stored at -80˚C.

2.4 Measurement of cell viability

A modified method followed for the cell viability assay (Sanevas *et al.,* 2007) was performed of freshly harvested leaf tissue. Fresh leaf tissue from the second youngest trifoliate was harvested from three different plants of each treatment and stained for 30 min with 0.25% (w/v) Evans Blue dye at room temperature. To remove surface bound dye, leaves were washed for 45 min in distilled water, followed by incubation for 1 hour at 55°C. The Evans blue stain taken by dead leaf cells was extracted using 1% (w/v) SDS. The absorbance for the level of Evans Blue up-take by the leaf tissue was measured spectrophotometrically at 600 nm.

2.5 Measurement of superoxide ($O_2^{\cdot -}$)

A modified method of Russo *et al*. (2008) was used to determine superoxide content. Superoxide concentrations were determined by submerging fresh leaf tissue from three different plants in a solution containing either 10 mM KCN (to inhibit Cu/Zn SOD) or 10 mM H_2O_2 (to inhibit Mn and Cu/Zn SOD) or 2% (m/v) SDS (to inhibit Mn and Fe SODs) or no inhibitors; together with 80 mM nitro blue tetrazolium chloride (NBT) and 50 mM potassium phosphate (pH 7.0). The leaves were incubated for 20 min within the solution after which they were homogenized and centrifuged at 10,000 × *g* for 5 min and the supernatant was spectrophotometrically analysed by reading absorbance at 600 nm. The superoxide concentration was calculated using the NBT extinction coefficient of 12.8 mM cm^{-1}.

2.6 Determination of lipid peroxidation (MDA) and Hydrogen peroxide (H_2O_2) content

2.6.1 Metabolite extraction (TCA extraction)

Metabolites were extracted using a modified method of Khan and Panda (2008). Extracts were isolated from soybean leaves by grinding them into a fine powder in liquid nitrogen. The frozen powder (100 mg) was directly homogenized in 5 volumes of 6% TCA. The resulting homogenates were centrifuged at 13,000 x g for 20 min at 4°C. The supernatants were used to assay for malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content.

2.6.2 Measurement of lipid peroxidation

Lipid peroxidation was determined by measuring the amount of MDA produced by the thiobarbituric acid reaction as described by Terzi and Kadioglu (2006). The TCA extract was mixed with the same volume of a 0.5% (w/v) thiobarbituric acid solution prepared in 20% (w/v) tricholoroacetic acid. The mixture was heated at 90°C for 30 min and then quickly cooled in an ice-bath for 10 min. The mixture was centrifuged at 13000 × *g* for 5 min and the absorbance of the supernatant was monitored at 532 and 600 nm. After subtracting the non-specific absorbance (600 nm), the MDA concentration was determined by its molar extinction coefficient (155 mM^{-1} cm^{-1}).

2.6.3 Measurement of hydrogen peroxide (H_2O_2)

Hydrogen peroxide content was determined using a method modified from Velikova *et al.* (2000). For this method, 50 µl of the supernatant from the TCA extracts was added to 50 µl of 100 mM potassium-phosphate buffer (pH 5.0) and 100 µl of 0.5M KI. The mixture was incubated at room temperature for 20 min. H_2O_2 concentration in the supernatant were evaluated by comparing its absorbance at 390 nm wavelength to a standard calibration curve. The concentration of H_2O_2 was calculated from a standard curve.

2.7 Determination of antioxidant enzyme activity

2.7.1 Native PAGE activity assays

2.7.1.1 Protein isolation for analysis of Antioxidant Enzyme activity assays

Total soluble protein was isolated from soybean leaves by grinding the leaf tissue into a fine powder in liquid nitrogen. The frozen powder (100 mg) was homogenized with homogenizing

buffer [40 mM K_2HPO_4, pH7.4, 1 mM EDTA, 5% (w/v) polyvinylpolypyrrolidone (PVPP) (molecular weight = 40 000]. The resulting homogenates were centrifuged at 13,200 rpm for 20 min at 4^oC and the supernatants were used to determine protein concentration using the Bradford assay method (Bradford, 1976) with Bovine Serum Albumin as a standard. The supernatant fractions were used as crude extract for enzyme activity assays.

2.7.1.2 Ascorbate peroxidase (APX) Activity assay

The activity of APX isoforms was determined with native polyacrylamide gel electrophoresis (PAGE) as described by Rao *et al.* (1996). The gel (5% stacking, 12% resolving) was equilibrated with a running buffer containing 2 mM ascorbate, 192 mM glycine and 24 mM Tris base (pH 7). Total protein extracts of 50 µg were loaded and the native PAGE was performed at 4^oC for 4 hours at 70 V. The APX activity was detected by incubating the gel in the dark with a solution containing 50 mM potassium phosphate buffer (KPO_4, pH 7.0) and 2 mM ascorbic acid for 10 min, followed by incubation with a solution containing 50 mM potassium phosphate buffer (pH 7.8), 2 mM H_2O_2 and 4 mM ascorbic acid for 10 min in a shaker in the dark. The gel was washed with 50 mM potassium phosphate buffer (pH 7.8) for 1 min in darkness. The gel was then stained with a solution containing 50 mM potassium phosphate buffer (pH 7.8), 28 mM N,N,N',N'- Tetramethylethylenediamine (TEMED) and 0.5 mM nitro blue tetrazolium chloride (NBT) for 10 min in the dark. After the staining, the gel was exposed to light with shaking until APX isozymes were visible.

2.7.1.3 Catalase (CAT) activity assay

A native PAGE (7.5% resolving gel and 5% stacking) gel with a thickness of 1.5 mm was prepared. The gel was equilibrated with a running buffer containing 192 mM glycine, and 24

mM Tris base (pH 7). Protein extracts (50 µg each) were loaded onto the native PAGE gel. Gel electrophoresis was performed at 4°C for 10 hours at 60 V. Catalase isozymes were visualised by firstly washing the gel with distilled water 3 times for 10 min in shaker. After washing, the gel was incubated for 10 min in a solution containing 0.003% H_2O_2 (V/V). After the incubation, the gel was rinsed twice with distilled water for 5 min. Then the gel was stained simultaneously with 2% ferric chloride (w/v) and 2% potassium ferricynide (w/v) as decribed in Yamashita *et al*. (2007). When achromatic bands begin to form, the stain was discarded and the gel rinsed with water.

2.7.1.4 *Superoxide dismutase (SOD) Activity assay*

The activity of SOD isoforms was analysed using a procedure modified from Beauchamp and Fridovich, (1971). A native PAGE (12% resolving and 5% stacking gel) with a thickness of 1.5 mm was prepared. The gel was equilibrated with a running buffer containing 192 mM glycine and 24 mM Tris base (pH 7). Protein extracts (80 µg each) were loaded onto the native PAGE gel. Electrophoresis was performed at 4°C for 6 hours at 70 V until the dye reached the bottom of the gel. The gel was then washed with 50 mM KPO_4 (pH 7.8) for 15 min in shaker. After washing, the gel was incubated for 10 min in a solution containing 50 mM KPO_4 (pH 7.8) and 0.5 mM NBT in the dark. This was followed by incubation for 10 min in a solution containing 50 mM KPO_4 (pH 7.8), 35.5 mM TEMED and 0.5 mM riboflavin in the dark. The gel was rinsed with distilled water and exposed to light to visualise the bands. In order to identify the different isozymes of SOD, 2 mM potassium cyanide (KCN) (inhibitor of Cu/ZnSOD) and 5 mM H_2O_2 (inhibitor of Cu/ZnSOD and FeSOD) were used in separate gels. MnSOD activity is resistant to both KCN and H_2O_2.

2.7.1.5 Dehydroascorbate Reductase (DHAR) activity assay

The activity of DHAR isoforms was determined by native polyacrylamide gel electrophoresis (PAGE) as described by Sgherri *et al*. (2000) with some modifications. A native PAGE gel (7.5% resolving and 5% stacking) with a thickness of 1.5 mm was prepared for the electrophoresis, together with a running buffer containing 192 mM Glycine and 24 mM Tris base (pH 7). To detect the change in activity of DHAR isozymes, total protein extracts of 50 µg from each treatment were loaded on the gel. Native PAGE was performed at 4^{o}C for 7 hours at 70 V. After electrophoresis, the gel was incubated for 20 min at room temperature in in 0.1 M potassium phosphate buffer (pH 6.4) containing 2 mM dehydro-L-ascorbate (DHA) and 4 mM glutathione (GSH). The gel was then washed with distilled water and stained with a solution of 0.125 N HCl containing 0.1 % potassium ferrocyanide and 0.1 % ferric chloride (w/v). When dark blue band were visible, the stain was discarded and the gel was rinsed with distilled water.

2.8 Transcript profiling of Glyma08g24160

Semi-quantitative RT-PCR and quantitative RT-PCR were used to assess expression levels of a soybean gene Glyma08g24160, which encodes lanosterol synthase, in control plants versus treated plants. The protein sequence of Glyma08g24160 was obtained using the key word search on Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html), with “lanosterol synthase” as the search word. This gene is predicted to encode a lanosterol synthase in soybean. Lanosterol synthase catalyses the conversion of oxidosqualene to lanosterol. To determine if glyma08g24160 is involved in soybean responses to drought stress and investigate the changes sterol content under drought stress, semi-quantitative reverse

transcription PCR (semi-qRT-PCR) was used as a tool to detect the expression levels of this genes in response to water deficit, sterol inhibition (terbinafine) and combination of water deficit and terbinafine.

2.8.1 RNA extraction and first strand cDNA synthesis

Total RNA was extracted from soybean leaves of control and treated plants using the ZR Plant RNA MiniPrep kit (Zymo Research, USA) as described in the manufacturer's manual. RNA was quantified using a spectrophotometer (NanoDrop) at 260 nm and run on 1% agarose gel electrophoresis to confirm its quality. Two micrograms of total RNA were treated with RNase-free DNase I according to the manufacturer's instructions to digest contaminating DNA. RNA (300 ng) was reverse-transcribed into first strand cDNA using ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs) as per manufacturer's instructions and an Oligo $dT_{(23)}$ primer was used to produce the cDNA.

2.8.2 Semi-quantitative R-T PCR analysis

PCR amplifications were performed in 0.2 ml thin-walled tubes using a T100™ Thermal Cycler (Bio-Rad). PCR amplifications were performed in a 25 µl reaction mixture containing 2 µl of cDNA as a template, 10 X Hot Start PCR buffer, 0.2 mM each of dNTPs, 0.5 µM of each gene-specific primer and an appropriate amount of Maxima Hot Start *Taq* DNA Polymerase that gave 1.25 U (Thermo Fisher Scientific). The PCR conditions for Glyma08g24160 were as follows: 95 °C for 2 min, 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and 25 amplification cycles. Soybean 18S rRNA was used as an internal control with the following PCR conditions; 95 °C for 2 min, 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, and 30 amplification cycles. The primers used are given in Table 2.2. Equal amount of PCR products were mixed with 1X GelRed™ nucleic

acid stain (Biotium, USA) and run on 1 % TAE agarose gel at 70 V for 1h30. The sizes of the products were estimated using a 100 bp molecular weight marker and the gel was visualized and photographed under the UV light on an AlphaEase Gel Documentation System (Alpha Innotech Inc.).

Table 2.2: Primers used in Semi-RT PCR and Quantitative RT-PCR for determination of Glyma08g24160 expression**.**

Primer Name	Primer set Sequence (5'-3')	Amplicon size (bp)	Species
Glyma08g24160	F: ACTTTCAACACAGAGGGAAGACGGT R: TTAGGGTCTCTATCCGCCTGTCCAG	159	*Glycine max*
18S rRNA	F: CTGTGAAACTGCGAATGGCTC R: CTGCCTTCCTTGGATGTGGT	350	*Glycine max*

2.8.2.1 Densitometry analysis

Densitometry analysis of PCR gels and native PAGE was conducted using AlphaEase FC Imaging software V4 (Alpha Innotech Corporation). The densitometry Software was used as described in the manufacture's manual.

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2.8.3 Quantitative R-T PCR analysis

Real-time PCR amplifications were performed in a 10 µl reaction mixture using Luminaris Color HiGreen qPCR Master Mix (Thermo Fisher Scientific) according to manufacturer's instructions. The master mix includes Hot Start Taq DNA polymerase, uracil-DNA glycosylase (UDG), double-stranded DNA (dsDNA)-binding dye SYBR® Green I and dNTPs in an optimized PCR buffer. The thermal cycling was performed using the following conditions: 95°C for 10 min, 95°C for 10 s, 58°C for 10s, 72°C for 10s, with 40 amplification cycles. The cycling conditions of Glyma08g24160 were the same as of 18S rRNA and the primers used are provided in Table 2.2.

The SYBR Green dye used in the real-time PCR reaction to detect amplification of glyma08g24160, binds to any double-stranded DNA, including non-specific PCR amplification products. Therefore, to exclude the detection of no-specific PCR amplification, a melting curve was run after the real-time PCR with the following thermal conditions: 95°C for 5 sec and 58°C for 1 min. Relative gene expression quantification was analyzed using the Delta $(2^{-\Delta\Delta Ct})$ method (Livak & Schmittgen, 2001). The Delta delta method requires the use of internal control which is uniformly expressed in all samples. In this study 18S rRNA were used since their expression does not change in response to variety of treatment conditions (Nicot *et al*., 2005). The amplification of glyma08g24160 between the control and treated plants was performed three times. The mean Cp values were determined and the expression of glyma08g24160 was compared relative to the internal control 18S rRNA gene.

2.9 Determination of sterol content in soybean

2.9.1 Extraction of soybean sterols

Sterol profiles of soybean leaves and roots was determined using the method described by Du and Ahn, (2002) with some modifications. Leaves and roots of soybean (100 mg each) were ground into fine powder in liquid nitrogen and carefully weighed into a 2ml screw-cap microcentrifuge tube, then 1 mL of saponification reagent [prepared by mixing absolute ethanol and 33% (w/v) KOH solution at a ratio of 94:6, 0.5 ml of 20% ascorbic acid (to prevent oxidation of tocopherols during saponification)] and 50 µl of 5-α-cholestane solution (1 µg/µl in hexane) were added immediately. The sample was homogenized for 5s at full speed, capped, and then incubated for 1 hour at 50^{o}C. After cooling on ice water for 10 min, 0.5 mL of hexane was added. Tubes were capped tightly and then the contents were mixed thoroughly by shaking. After 15 hours for phase separation, the hexane layer containing unsaponifiables was carefully transferred to a centrifuge tube and dried using centrifugation under vacuum (SpeedVac) for 1h. To the dried sample, 200 µl of pyridine and 100 µl N,O-bis(trimethylsilyl) trifluoroacetamide (99% BSTFA) were added. The sample was derivatized at 50^{o}C in an oven for 1 hour, and then analysed using a GC-MS (6890N gas chromatograph, Agilent). External standards of sterols were prepared the same way as the samples for calibration.

2.9.2 Gas- Chromatography Mass Spectrophotometry analysis

Compounds were identified by GC-MS as described in Rahier and Benveniste, (1989) using a 6890N gas chromatograph (Agilent) equipped with a DB-225 MS column (J&W 122-2232 model ; 30 m length; 250 µm diameter; 0.25 µm thickness) and coupled to a CTCPAL mass

analyser (Agilent). The injection volume was 1 µl, using a 10 µl syringe. The temperature program of ovens was a gradual 7°C/min increase from 200°C to 325°C. The inlet to the column was set to splitless mode, at an initial temperature of 250°C, 131.1 kPa pressure and the purge flow was 50.0 mL/min. The flow rate at the column was set to 1.2 mL/min (constant flow), the pressure was at 131.2 kPa and the speed at 42 cm/sec. Sterols were unequivocally identified by coincidental retention time and identical EI-MS spectra at 70 eV like reference compounds.

2.10 Statistical analyses

Eight plants were used from each treatment for growth parameters; and for molecular and biochemical analysis, the plants were pooled into one sample per treatment and each experiment was repeated 3 times. Statistical analysis was performed using the Duncan's multiple range test, with a significance represented by a *P value* < 0.05.

CHAPTER 3

RESULTS

3.1 Physiological and morphological responses of soybean to drought stress and inhibition of sterol biosynthesis.

3.1.1 Drought and sterol biosynthesis inhibition causes reduction in growth of soybean.

The effect of water deficit and inhibition of sterol biosynthesis on soybean growth was evaluated by looking at physiological responses including size of the trifoliate leaves (figure 3.2), shoot fresh weights (figure 3.1 a) and shoot lengths (figure 3.1 b). The shoot fresh weights and shoot length were measured at V4 stage of growth. Terbinafine reduced the weight of the shoots significantly by approximately 20% when compared to well-watered plants (figure 3.1 a). However, shoot fresh weight was 2 times lower in water deficit conditions when compared to well-watered shoot weights. The combination of drought and terbinafine treatment decreased the shoot weight by 40% when compared to water deficit shoots. The shoot length (figure 3.1 b) showed a similar trend as shoot weight, albeit with less pronounced decrease in lengths than in weights. The lengths of soybean shoots under drought stress were approximately 40% lower than shoot lengths under well-watered conditions. Both terbinafine treatment and combination of terbinafine and drought also decreased the length of shoot length.

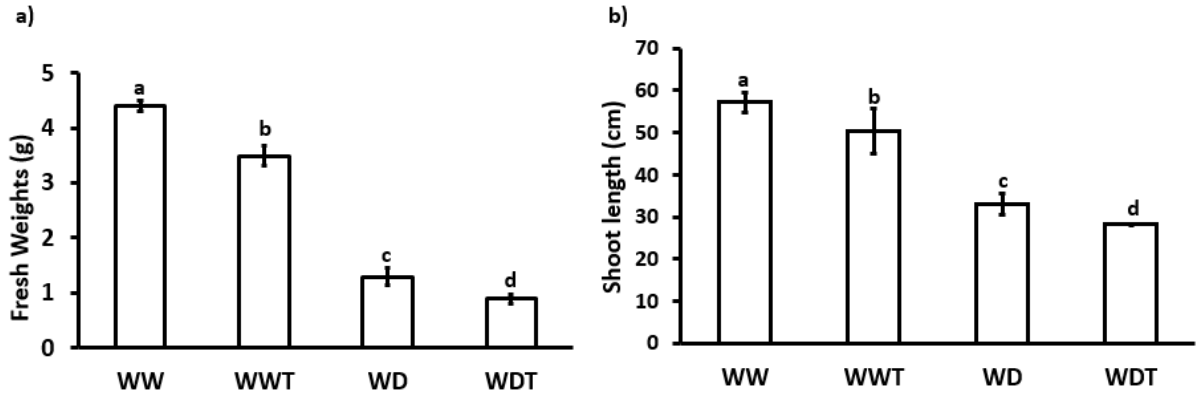


Figure 3.1: The effect of water deprivation on shoot weights (a) and shoot length (b) of soybean at the V4 stage of vegetative growth. Values are means ± SE of 10 plants from three independent experiments, $p \leq 0.05$. WW (Well-watered); WWT (terbinafine treatment); WD (water deficit); WDT (Water deficit and terbinafine).

In addition, plants that were subjected to drought and terbinafine treatment have been shown to have decreased growth in terms of size and area of trifoliate leaves (figure 3.2). Terbinafine treatment (WWT) decreased the growth of soybean leaves when compared to well-watered plants. In addition, Drought stress (WD) decreased the growth of soybean, and the combination of drought with terbinafine treatment (WDT) caused much lower growth when compared to drought treated plants without terbinafine.

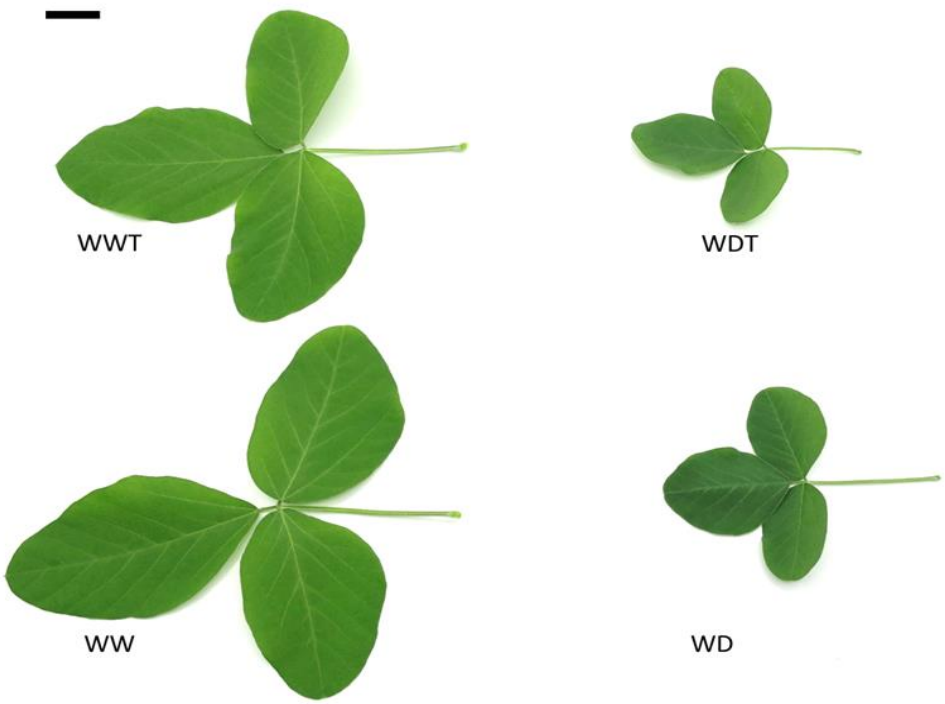


Figure 3.2: The effect of water deficit and terbinafine on soybean growth. The trifoliate leaves of the second youngest leaves were captured at the V4 stage of vegetative growth. Black bar = 2 cm. WW (Well-watered); WWT (terbinafine treatment under WW conditions); WD (water deficit); WDT (Terbinafine treatment under Water deficit conditions).

3.2 The effects of drought and inhibition of sterols on H_2O_2, $O_2^{\cdot-}$, MDA and Cell viability.

This part of the study investigated the effects of changes in sterol content caused by terbinafine on lipid peroxidation and cell viability. Drought and terbinafine treatment caused high accumulation of H_2O_2. Hydrogen peroxide content in terbinafine-treated plants was approximately 50% higher than the well-watered plants, and similar trend was discovered between the water deficit and the combination of water deficit and terbinafine treatment (Figure 3.3 a).

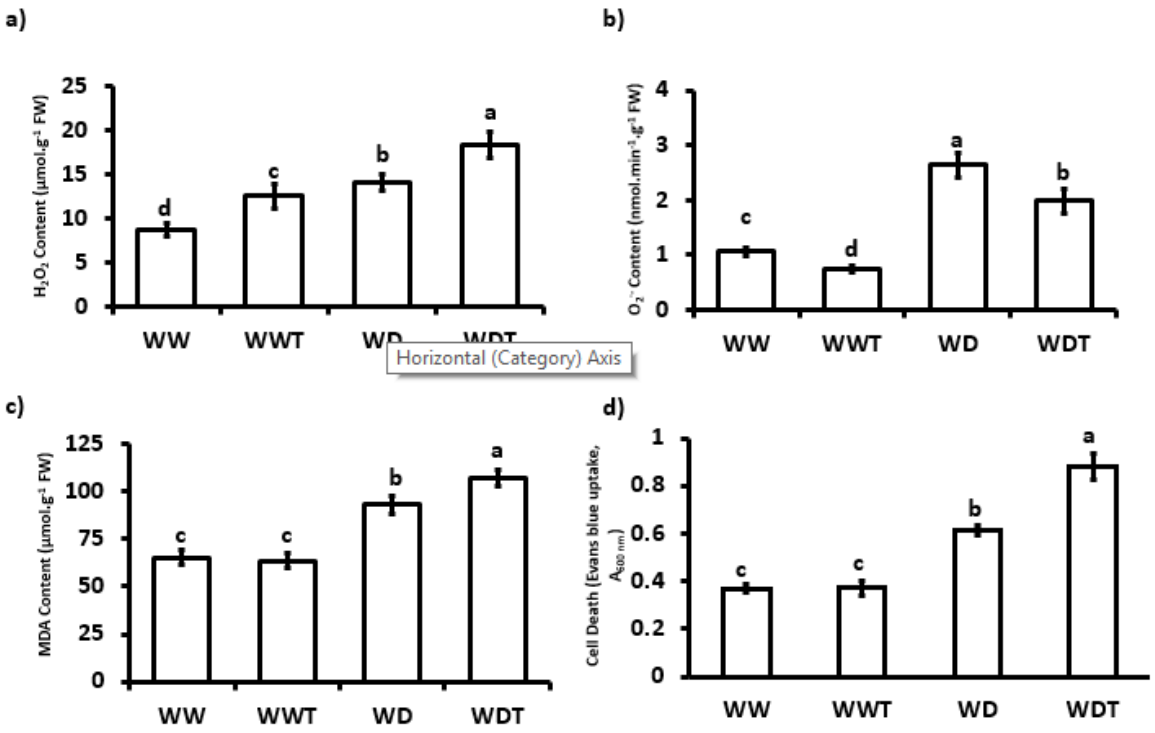


Figure 3.3: The effects of drought stress and terbinafine on lipid peroxidation (MDA), H_2O_2 and $O_2^{\cdot-}$ content. Hydrogen peroxide (H_2O_2) (a) and $O_2^{\cdot-}$ (b) were measured as the indication of ROS accumulation, and lipid peroxidation (c) and cell viability (d) were measured as the indication of oxidative stress. Error bars represent the means ± SE; n= 3.. Different letters indicate the difference between means at p<0.05. WW (Well-watered); WWT (terbinafine treatment); WD (water deficit); WDT (Water deficit and terbinafine).

Superoxide produced contrasting results in terbinafine-treated plants when compared to hydrogen peroxide (Figure 3.3b). High accumulation of $O_2^{\cdot-}$ was observed in the presence water deficit plants. Superoxide content in water deficit plants was approximately 175% higher than well-watered plants. Terbinafine treatment caused reduction of $O_2^{\cdot-}$ content by approximately 25% when compared to well-watered plants. A similar trend was observed under water deficit when it was compared to water deficit in the presence of terbinafine. The combination of drought and terbinafine treatment caused a decrease in superoxide content when compared to accumulation of superoxide under water deficit, but it was increased by approximately 100% when compared to well-watered plants. The oxidative damage to leaves of soybean was determined by measuring levels of lipid peroxidation and cell viability. The

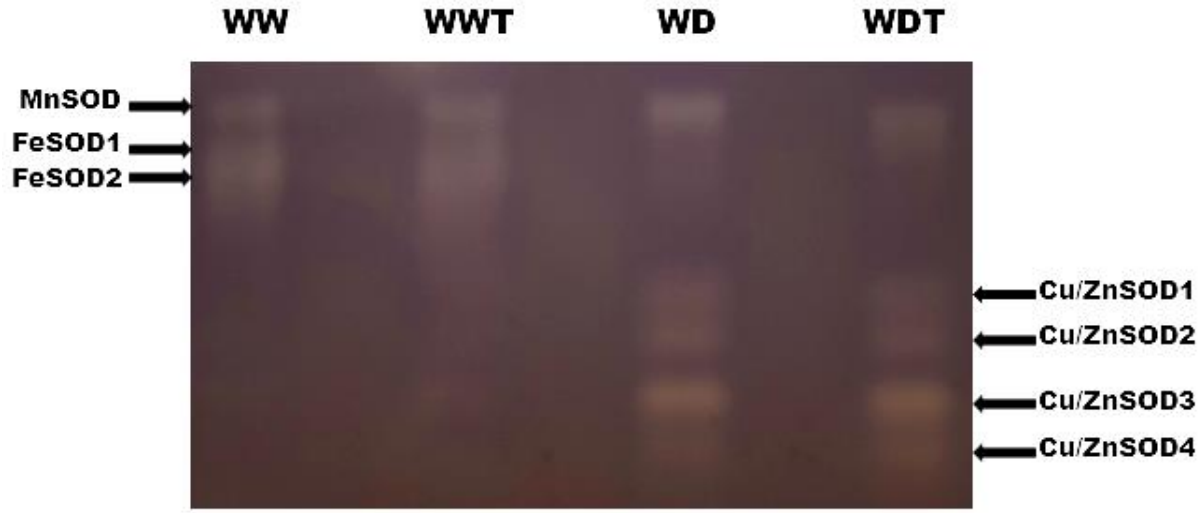
level of lipid peroxidation was determined by measuring MDA content (Figure 3.3c), a major reactive aldehyde formed as result of peroxidation of lipids. Terbinafine treatment had no effects in terms of lipid peroxidation as there was no change in the level of MDA content between the well-watered leaves and terbinafine treated leaves. However, there was a high level of MDA content in leaves of drought-treated plants and in the combination of terbinafine and drought-treated plant leaves. The levels of MDA content in the combination treatment of water deficit and terbinafine was significantly higher by approximately 20% when compared to MDA content under water deficit. Cell death was determined by measuring the level of Evans Blue up-take by dead leaf cells. Cell death showed similar results as in lipid peroxidation (Figure 3.3 d). The level of cell death in terbinafine-treated plants was the same as in control plants (well-watered plants). Nevertheless, under water deficit conditions, cell death was 50% higher than under well-watered conditions. The combination of water deficit and terbinafine treatment increased cell death by approximately 40% compared to water deficit only, and 2 times higher when compared to control and terbinafine treated plants.

3.3 The effects of water deficit and sterol synthesis inhibition on the activity of antioxidant enzymes in soybean leaves.

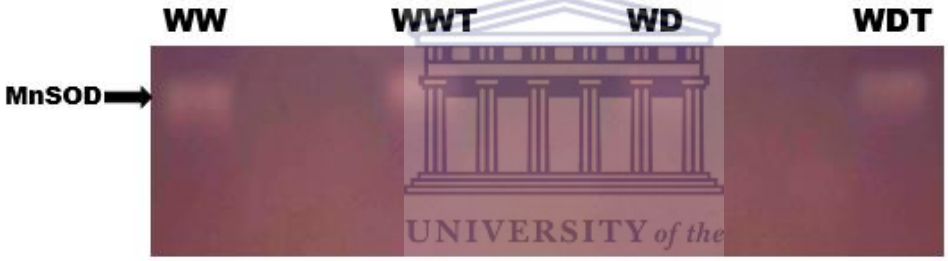
3.3.1 The effects of water deficit and terbinafine on activity of superoxide dismutase isozymes

In the study, we examined the effects of water deficit and sterol inhibition (terbinafine treatment) on the activity of SOD isozymes. There were 7 SOD isozymes detected in the leaves of soybean. The SOD isozymes were then identified by using inhibitors. There was one MnSOD, two Fe-SOD, and three Cu/Zn SOD isoforms detected. The enzymatic activity of MnSOD was enhanced by both the treatment of terbinafine and water deficit, however, the combination of water deficit and terbinafine inhibited the activity of MnSOD. Enzymatic activity detected for Fe-SOD 1 and Fe-SOD2 increased in response to terbinafine treatment. On the other hand, Fe-SOD1 and Fe-SOD2 activity was inhibited by water deficit, just as it was in the combination of water deficit and terbinafine. The enzymatic activity of Cu/Zn SOD was undetectable in control plants; however, terbinafine slightly activated the activity of Cu/Zn SOD1, 2, 3 and water deficit activated all four Cu/Zn SOD (Figure 3.4a). Cu/Zn SOD 1 and Cu/Zn SOD2 were down-regulated by combination of water deficit and terbinafine compared to water deficit alone, and there was no difference between Cu/Zn SOD 3 and Cu/Zn SOD 4 in response to combination of water deficit and terbinafine, when compared to water deficit treated plants.

a)



b)



c)

Figure 3.4: In-gel assay for SOD activity in response to water deficit and terbinafine in leaves of soybean. Assays were done on leaves of soybean plants that were harvested at V4 stage of vegetative growth. The in-gel assays show the types of SOD isoforms present in leaves of soybean (a) with no inhibitors, (b) in the presence of 6 mM H_2O_2 and (c) with 5 mM KCN. Different letters indicate the difference between means at $p<0.05$. Values are means ± S.E (N=3). WW (Well-watered); WWT (terbinafine treatment); WD (water deficit); WDT (Water deficit and terbinafine).

3.3.2 Water deficit and terbinafine alters the activity of ascorbate peroxidase isozymes in soybean leaves.

On native PAGE activity gels, only two APX isozymes were detected in the leaves of soybean (Figure 3.5 a). The activity of APX isozymes was increased and decreased in response to various treatments. APX 1 showed a slight increase in intensity in the water deficit leaves when compared to control plants. The addition of terbinafine caused a decrease in APX1 activity both in the absence drought (WWT) and in the presence of drought (WDT) when compared to control plants and drought treated plants. There was no change in the intensity of APX 2 under water deficit and in the combination of water deficit with terbinafine when compared to control plants. However, there was a slight increase in APX 2 activity in terbinafine-treated plants and a slight decrease in a combination of water deficit and terbinafine.

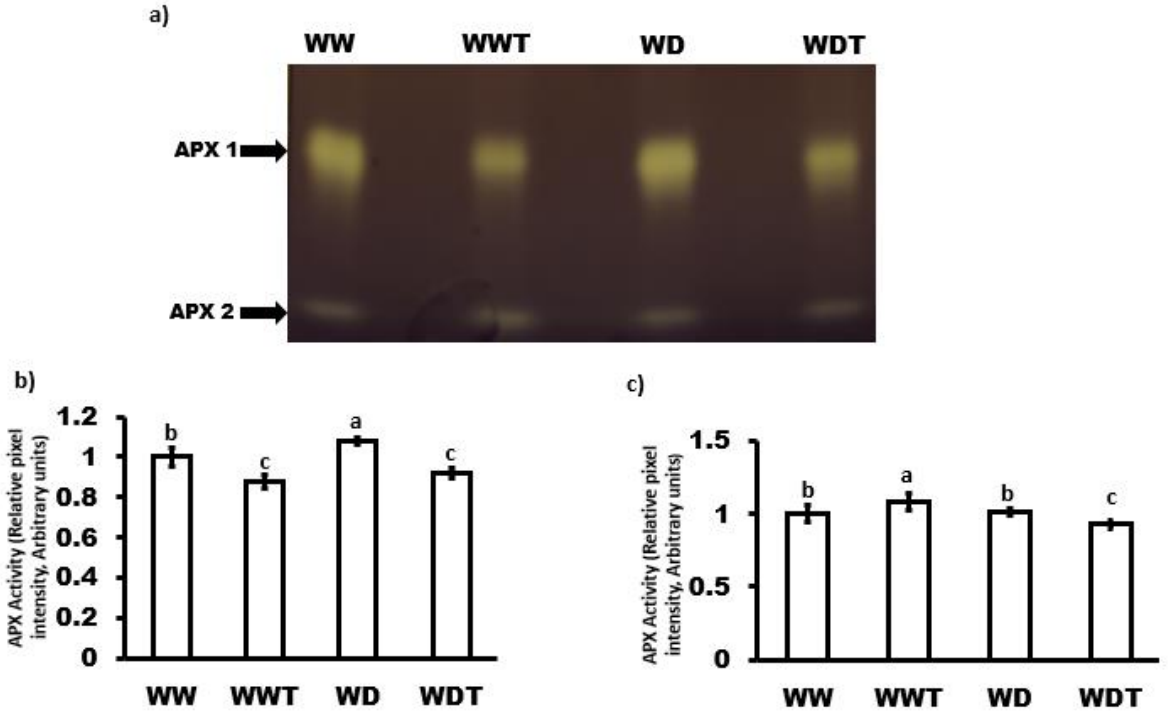


Figure 3.5: In-gel activity assays for APX activity in response drought and terbinafine treatment. Soybean leaves that were treated with 50 µM terbinafine and combination of water deficit with terbinafine were harvested at V4 stage of vegetative growth. The in-gel activity assay of APX isoforms in response various treatments is shown (A), from which pixel intensities of APX1 (B) and APX2 (C) were determined. Different letters indicate the differences between means at $p < 0.05$. Values are means ± SE (N=3). Abbreviations in the figure are as follows: WW (Well-watered); WWT (terbinafine treatment); WD (water deficit); WDT (Water deficit and terbinafine).

Densitometry analysis showed that terbinafine treatment decreased the activity of APX 1 by approximately 10% when compared to control plants (figure 3.5b). However, water deficit caused an increase of approximately 10% when compared to control plants. There was no change in APX 1 activity between terbinafine-treated plants and the combination of terbinafine with water deficit. However, the combination of water deficit with terbinafine reduced APX 1 activity by approximately 20% when compared to plants exposed to water deficit. Densitometry analysis (b) showed no significant change in the activity of APX2 in response all treatments.

3.3.3 Catalase activity in soybean leaves is differentially regulated by water deficit and terbinafine treatment.

Changes in catalase isozyme activities were determined and only one catalase isozyme was detected (zones of clearing in a green background) in response to various treatments. However, there were also dark bands detected and these dark bands could be non-specific peroxidase activity (Figure 3.6).

The enzymatic activity of catalase was inhibited in response to terbinafine treatment and water deficit. Terbinafine decreased catalase activity by 40% when compared to control plants. However, water deficit decreased the activity of catalase by 20% when compared to control plants. Nonetheless, the inhibition caused by water deficit and terbinafine was reversed by the combination of water deficit with terbinafine as the activity of catalase was enhanced in this combination treatment when compared to other treatments.

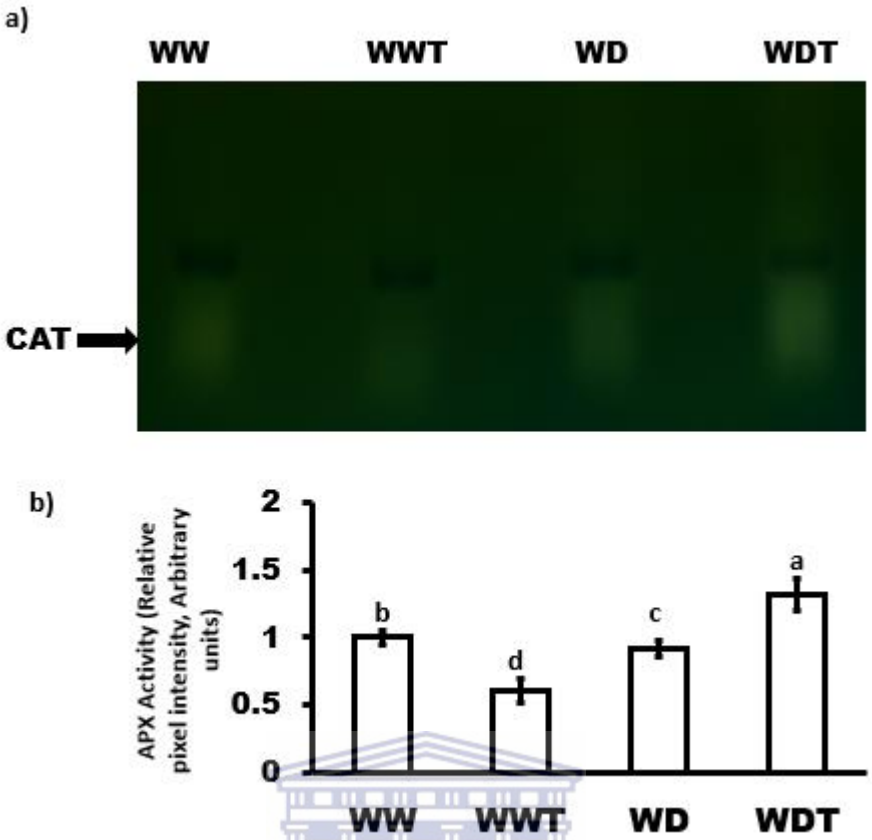


Figure 3.6: In-gel activity assays for catalase in response water deficit and terbinafine treatment. The assay was done on soybean leaves that were treated with 50 µM terbinafine, water deficit and combination of water deficit and 50 µM terbinafine. The activity of APX isoforms in response to various treatments are shown for in-ge- assay (A) and pixel intensity (B) of soybean. The different letters indicate the difference between means at $p<0.05$. Values are means ± S.E (N=3). WW (Well-watered); WWT (terbinafine treatment); WD (water deficit); WDT (Water deficit and terbinafine).

3.3.4 Terbinafine and water deficit causes changes in dehydroascorbate reductase activity in the leaves of soybean.

Dehydroascorbate reductase (DHAR) is involved the regeneration of ascorbate, the electron donor for APX. Therefore, changes in enzymatic activity of DHAR isozymes in response to various treatments were investigated. One DHAR isozyme was detected in response to the various treatments (Figure 3.7 a and b). Enzymatic activity of DHAR in the terbinafine treatment increased by approximately 25% when compared to control plants. However,

water deficit and the combination of drought inhibited the activity of DHAR. Water deficit decrease the activity of DHAR by approximately 25% when compared to the control plants. Nonetheless the combination of water deficit and terbinafine caused a significant decrease when compared to all other treatments.

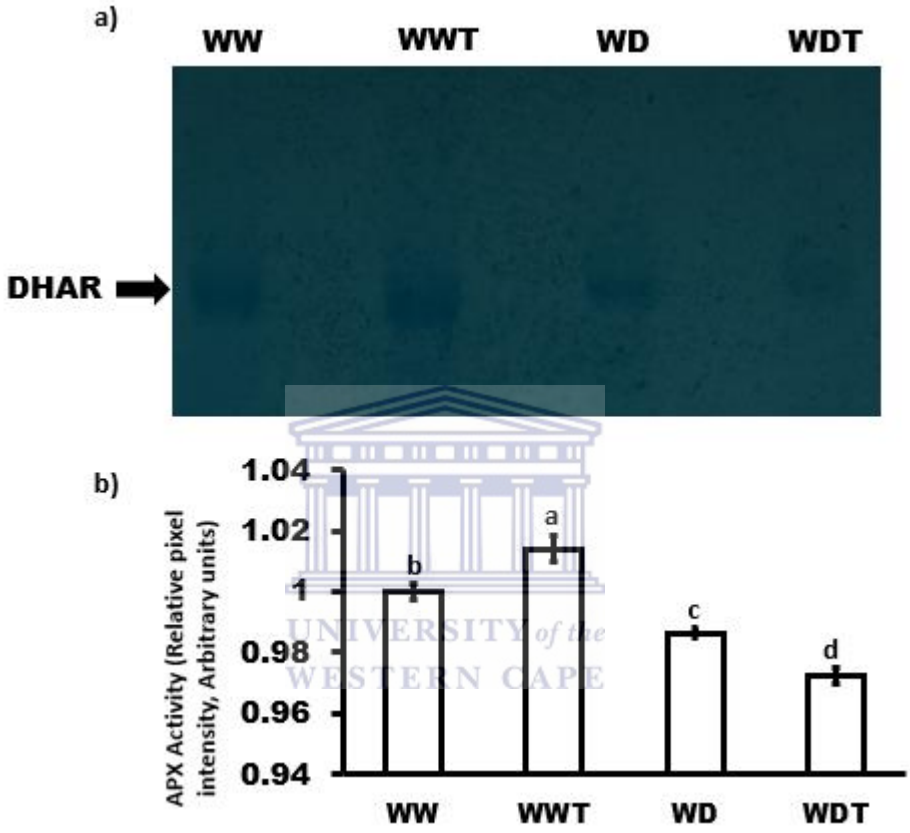


Figure 3.7: In-gel activity assays for dehydroascorbate reductase in response water deficit and terbinafine treatment. The assay was done on soybean leaves that were treated with 50 µM terbinafine, water deficit and combination of water deficit with 50 µM terbinafine. The activity of APX isoforms in response to various treatments are shown for soybean leaves using an in-gel assay (A), from which pixel intensities (B) were determined. Different letters indicate the differences between means at $p < 0.05$. Values are means ± SE (N=3). The abbreviations used are as follows: WW (Well-watered); WWT (terbinafine treatment); WD (water deficit); WDT (Water deficit with terbinafine).

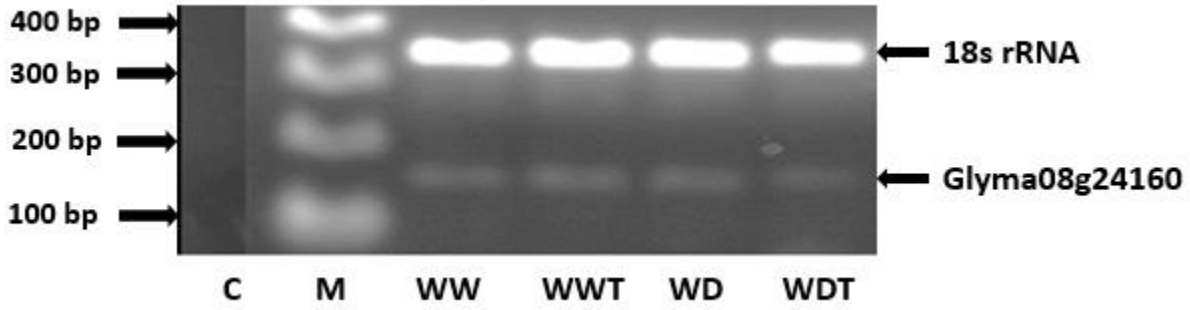
3.4 The effect of drought on the expression of a candidate lanosterol synthase gene (Glyma08g24160)

The results for gene expression quantification from semi-quantitative PCR are shown in Figure 3.8. The 18S rRNA gene was used as reference gene because its expression does not change significantly in response to a variety of treatment conditions. The results obtained from semi-qRT-PCR showed that the expression of glyma08g24160 was increased and decreased by the various treatments (Figure 3.8 a) and all the amplicons produced the same size of the gene on agarose gel as the predicted size based on the sequence of the PCR product that in generated from the designed primers.

A slight up-regulation of glyma08g24160 was observed in water deficit and terbinafine treated plants, but there was no significant difference between the expression levels in terbinafine treated leaves and in water deficit leaves (Figure 3.8 b). The combination of water deficit with terbinafine caused a significant down-regulation of the gene. To validate the results obtained from the semi-quantitative RT-PCT, quantitative PCR (qPCR) was conducted because qPCR is sensitive that semi-quantitative PCR. Quantitative PCR (Figure 3.9) showed a similar trend for gene expression as in semi-PCR although there were some differences in the level of the expression of the gene in different treatments. The level of gene expression between water deficit and terbinafine was different when compared to the semi quantitative PRC. The up-regulation of the gene was 2 times higher than in the well-watered leaves. Although gene expression was ± 20% higher in terbinafine-treated plants than in the well-watered leaves. Drought and terbinafine treatments caused the up-regulation of the gene,

but the combination of water deficit with terbinafine down-regulated the gene to the same level as in well-watered leaves.

a)



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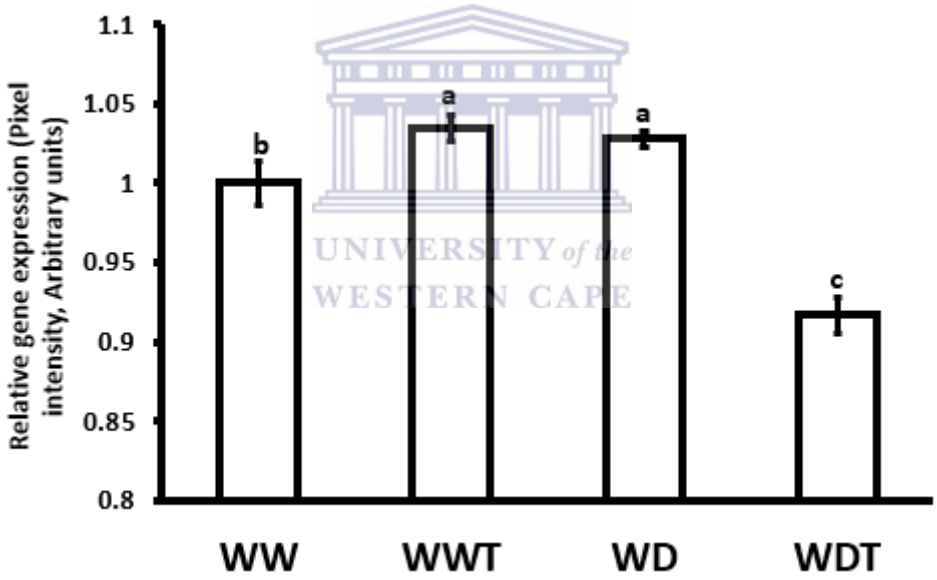

Figure 3.8: Semi-quantitative R-T PCR for gene expression levels of Glyma08g24160 in response to water deficit and terbinafine in the leaves of soybean. The effects of water deficit and terbinafine on the expression of the sterol biosynthesis gene (glyma08g24160; 159 bp in size) expression was measured in the leaves of soybean plant using semi-quantitative RT-PCR (a). Soybean 18S rRNA (350 bp) was used as a reference gene. All PCR products were size-fractionated with a DNA size marker (M) on a 1% agarose gel. The intensity of the PCR bands (b) was analysed using densitometry (expressed as relative pixel intensity). Error bars represent the means (± SE; n= 3) of three independent experiments. Different letters above the bars indicate means that are statistically different at $p < 0.05$. Abbreviations used are as follows: C; control. WW (Well-watered); WWT (terbinafine treatment); WD (water deficit); WDT (Water deficit with terbinafine treatment).

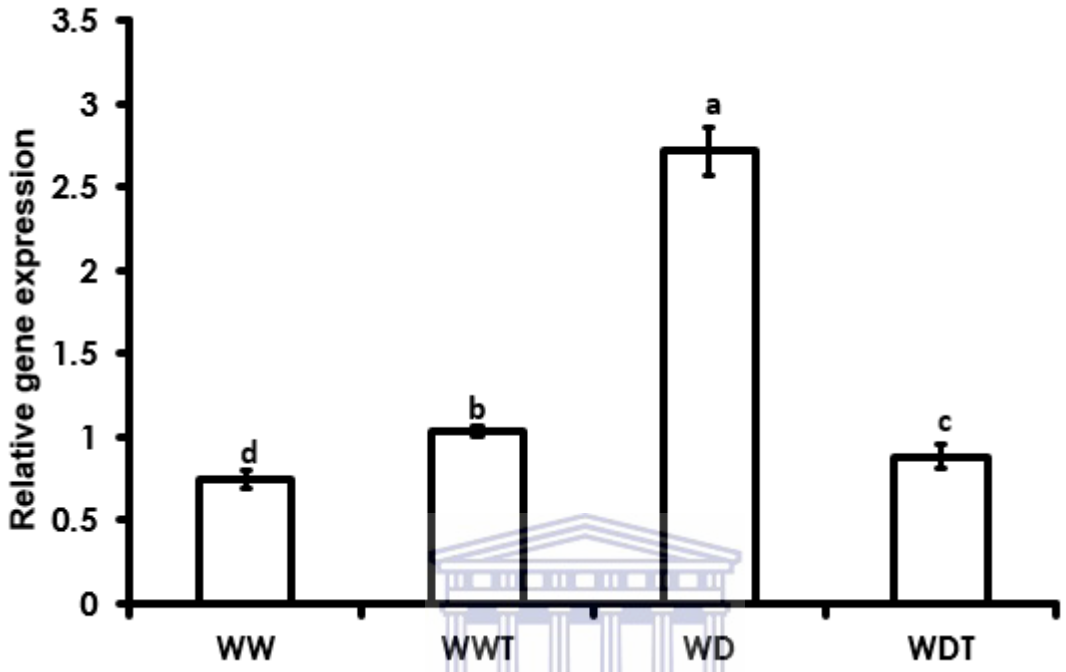

Figure 3.9: Quantitative R-T PCR for gene expression levels of Glyma08g24160 t in response to water deficit and terbinafine in the leaves of soybean. The effects of water deficit and terbinafine on sterol biosynthesis gene (glyma08g24160) expression was measured in the leaves of soybean plant using quantitative RT-PCR. Soybean 18S rRNA was used as a reference gene. Error bars represent the means (± SE; n= 3) of three independent experiments. The different letters indicate the difference between means at $p<0.05$. WW (Well-watered); WWT (terbinafine treatment); WD (water deficit); WDT (Water deficit and terbinafine).

3.5 The effects of water deficit and terbinafine treatment on sterol content of soybean leaves

This part of the study investigates the role of phytosterols in drought responses in soybean and the effects of terbinafine on changes in sterol content. The amount of stigmasterol, sitosterol and cycloartenol was reduced in response to terbinafine treatment (table 3.1). However, lanosterol statistically remained the same as in well-watered leaves but water

deficit reduced the amount of lanosterol. Water deficit resulted in more significant reduction of sitosterol, stigmasterol, cycloartenol and lanosterol when compared to terbinafine treatment. The combination of water deficit and terbinafine reversed the effect of water deficit by increasing the amount of stigmasterol, cycloartenol and lanosterol, but there was no significant change in sitosterol levels when compared to water deficit. Nevertheless, the amount of sitosterol, stigmasterol, cycloartenol and lanosterol was lower than in well-watered leaves and terbinafine leaves.

Table 3.1: Sitosterol, stigmasterol, cycloartenol and lanosterol responses to water deficit and sterol inhibition

	Well-watered leaves	Well-watered leaves and terbinafine	Water deficit in leaves	Water deficit in leaves treated with terbinafine
Sitosterol ($\mu g.mg^{-1}$ FW)	0.33 ± 0.025 [a]	0.27 ± 0.022 [b]	0.18 ± 0.019 [c]	0.21 ± 0.023 [c]
Stigmasterol ($\mu g.mg^{-1}$ FW)	0.26 ± 0.021 [a]	0.21 ± 0.019 [b]	0.14 ± 0.013 [d]	0.18 ± 0.013 [c]
Cycloartenol ($\mu g.mg^{-1}$ FW)	0.18 ± 0.014 [a]	0.15 ± 0.012 [b]	0.08 ± 0.006 [c]	0.09 ± 0.008 [c]
Lanosterol ($\mu g.mg^{-1}$ FW)	0.13 ± 0.016 [a]	0.12 ± 0.009 [a]	0.04 ± 0.003 [b]	0.11 ± 0.008 [a]

Data represent means ± standard error of three biological replicates (± SE; n= 3). Different letters indicate statistically different means at $p < 0.05$. Abbreviations in the table refer to the following: WW (Well-watered); WWT (terbinafine treatment); WD (water deficit); WDT (Water deficit and terbinafine).

Chapter 4

4. Discussion

4.1 Drought reduced growth of soybean

The growth of the plants is achieved through cell division, enlargement and differentiation and these processes involve the physiological, genetic and morphological events and their complex interactions. Cell growth is one of the physiological processes that is mostly sensitive to drought due to reduction of turgor pressure. Water deficit can inhibit cell elongation and expansion, and damage mitosis; resulting in reduced plant height and leaf area, accompanied by poor quality and quantity of plant growth (Nonami, 1998; Anjum *et al.,* 2011) as observed in this study.

The goal of this study was to investigate the role of a candidate lanosterol biosynthesis gene (which acts as a precursor to phytosterols synthesis) and phytosterols in soybean responses to drought stress. The effects of drought on soybean growth were determined based on the size of the trifoliate leaves, shoot fresh weights and shoot lengths between the control plants and water deprived plants (Figure 3.1 and Figure 3.2). The plants that were grown under water deficit had small leaves and reduced leaf area. Shoot length and shoot weight were also reduced by drought. These findings are in agreement with the results of Zeid and Shedeed (2006), where drought stress reduced the fresh and dry masses of shoots in alfalfa (*Medicago sativa* L.). Drought stress reduced shoot length, shoot weight and leaf area in *Gossypium hirsutum* L. (Pace *et al.,* 1999) and *Zea mays* (Khan *et al.,* 2001; Kamara *et al.,* 2003). The reduction of leaf area, shoot length and weight observed in this study may be associated with reduction in cell division and cell elongation due to a reduced water potential gradient

between the xylem and the growing cells, which prevents the movement of water into the growing area, limiting leaf water potential and growth of plants. This may be because of the fact that, under water deficit conditions, roots induce signal cascades, such as ABA, to the shoots via the xylem (Davies, and Zhang, 1991). ABA stimulates the efflux of K^+ ions from guard cells and results in loss of turgor pressure, leading to stomatal closure, which is an adaptation strategy of the plant to limited water supply to reduce leaf water loss through transpiration (Guerrero and Mullet, 1986). The reduction in turgor pressure results in reduced cell growth, followed by reduction of leaf area and size and thereby reducing plant height and growth (Rucker *et al.,* 1995).

4.2 Drought induces oxidative stress in soybean.

Several studies have revealed that oxidative stress is one of the main causes of reduction in growth and development of different plant species. Oxidative stress, defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defences. Oxidative stress is caused by changes in environmental conditions such as drought, which cause high accumulation of ROS such as $O_2^{\cdot -}$ and H_2O_2 and these reactive oxygen species attack biological molecules in cells and impair their functions, leading to cell death and affecting crop yield. In this study, drought caused high accumulation of H_2O_2 and $O_2^{\cdot -}$. Since ROS are toxic to plants (including soybean) high ROS content induced oxidative damage to lipids, as indicated by high MDA content (a marker for lipid peroxidation) in leaves of soybean subjected to such treatment. This was associated with increased cell death and reduction in growth in soybean. It can thus be suggested that drought caused overproduction of ROS, which led to lipid peroxidation and cell death. These findings are in agreement with

the study by Maraghni *et al.* (2014) where PEG-induced drought stress on wild jujube (*Ziziphus lotus*) increased the level of lipid peroxidation. Drought stress also increased superoxide ($O_2^{\cdot-}$) production of leaves and hydrogen peroxide (H_2O_2) content of the leaves and roots of Kentucky bluegrass (*Poa pratensis L*.) which lead to increased lipid peroxidation (Bian and Jiang, 2009).

4.3 Drought stress increases the activity of antioxidant enzyme activity

During oxidative stress caused by ROS, plants induce the activity of antioxidant enzymes in order to scavenge ROS to prevent their destructive excessive accumulation, thus contributing to ensuring their survival. In this study, there was high accumulation of ROS (H_2O_2 and O_2) which are toxic to plant cells. As a result activated, drought activated antioxidant enzyme activities. Superoxide dismutase (SOD) serves as prime major scavenger of O_2^- by converting it to H_2O_2 and O_2. All three forms of SOD (FeSOD, MnSOD and Cu/ZnSOD) increased in response to drought stress due to high accumulation of O_2^-. These results are consistent with the other studies in rice seedlings (Sharma and Dubey, 2005), *Brassica napus* L. (Abedi and Pakniyat, 2010.) and *Sesamum indicum L.* cvs (Fazeli *et al.,* 2007); where total SOD and SOD isoform activity increased in response to drought stress. The results presented here also indicate an increase in activity of APX, the scavenger of H_2O_2. The increase in APX activity under drought stress could be attributed to efforts to prevent oxidative damage as a protective measure in response to increased levels of H_2O_2 under drought conditions. Similar results where APX activity increased in response to drought stress were observed in a study by Sofo *et al.* (2005) on olive trees and Chugh *et al*. (2010) on *Zea mays*.

The activity of CAT decreased in response to water deficit in comparison to well-watered plants. These results are in agreement with Bakalova *et al*. (2004) in wheat and with Abedi

and Pakniyat (2010) in *Brassica napus*, where the activity of CAT declined in response to drought stress. Dehydroascorbate reductase is responsible for the regeneration of ascorbate (AsA) (an electron donor for APX) from an oxidized state (Chen and Gallie, 2006). In this study the activity of DHAR also decreased in response to drought stress. Similar results were found in the roots of *Poa pratensis* L. (Bian and Jiang, 2009) and in pea nodules (Gogorcena *et al.,* 1995). The decrease in DHAR activity might be due to oxidation and inactivation of the enzyme by enhanced ROS production (Sharma and Dubey, 2005).

4.4 Drought and sterol synthesis inhibition reduces sterol content and impacts on ROS production and antioxidant enzyme activity.

The effects of drought on sterol composition was determined in order to elucidate the role of phytosterols in soybean responses to drought stress. Drought stress decreased sitosterol, stigmasterol, cycloartenol and lanosterol content. The decrease in sterol content potentially resulted in membrane disruption as indicated by high levels of lipid peroxidation and decreased cell viability in response to inhibition of sterols biosynthesis under drought conditions, since sterol are also crucial for membrane integrity because plant sterols have been shown to maintain proper bulk membrane structure (Stillwell *et al*., 1990). The reduction in sterol content occurred concomitantly with overproduction of ROS as indicated by increased production of hydrogen peroxide (H_2O_2) and superoxide (O_2^-), which lead to increased lipid peroxidation and decreased cell viability. As a result, the reduction in sterol content could have signaled the activation of antioxidant enzymes such as APX and SOD, which increased in response to drought stress. Similar findings were observed in a study by Pose *et al*. (2009) where an *Arabidopsis* mutant that is hypersensitive to drought stress had reduced sterol content and excessive accumulation ROS.

The effects of changes in sterol content was also investigated using a sterol biosynthesis inhibitor under well-watered and drought conditions in order to determine the impact of inhibition of sterol biosynthesis on ROS production and antioxidant enzyme activities. The inhibition of sterol synthesis resulted in reduced sterol content. This study contradicts the finding of Kumar *et al.* (2015) where drought stress increased the accumulation phytosterols in rice. The reduction in sterol content increased the production of ROS. In this study, there was an increase in H_2O_2 and decrease in $O_2^{\cdot-}$ content in response to the sterol biosynthesis inhibitor. The high accumulation of H_2O_2 could be explained by decrease in APX and CAT activities, the scavengers of H_2O_2 in plants. Decreased sterol content caused by sterol biosynthesis inhibitor is logical since this is the function of the inhibitor. However, increased CAT activity when sterol biosynthesis was inhibited in the presence of drought suggests that the excessive H_2O_2 potentially triggers activation of CAT in an effort to scavenge the excessive H_2O_2. The decrease in $O_2^{\cdot-}$ may be caused by high activity of SOD, although sterol synthesis inhibition in the presence of drought decreased the activity of SOD. The activity of CAT and DHAR also decreased in the presence of the sterol biosynthesis inhibitor. This could be because terbinafine (the sterol synthesis inhibitor) may be interfering with activity of these antioxidant enzyme in response to drought in soybean. This is supported by the increase in lipid peroxidation and cell death which lead to decrease in soybean growth.

Therefore, a general explanation is that there is a possibility of involvement of the phytosterols in the signalling network that regulates soybean responses to drought stress which involves ROS scavenging mediated by antioxidant enzyme activity.

4.5 The expression of glyma08g24160 gene is altered by drought stress

To further examine the relationship between drought and phytosterols, we examined the expression of sterol biosynthesis gene, glyma08g24160, which encodes lanosterol synthase. Expression of this gene was investigated in response to drought stress in the leaves of soybean using quantitative and semi-quantitative PCR in the presence or absence of the sterol biosynthesis inhibitor (terbinafine). Glyma08g24160 was up-regulated by drought stress. Since this gene encodes lanosterol synthase, which catalyses the production of lanosterol, the precursor to sterol synthesis, the reduction of lanosterol and other phytosterols could have triggered the up-regulation of this gene in an effort produce of more lanosterol to compensate for the drought-mediated suppression of sterol biosynthesis. On the other hand, sterol synthesis inhibition and the combination of drought and inhibition of sterol synthesis also caused up-regulation of the gene. However, the response pattern between the two treatments were different, as it was more up-regulated in sterol synthesis-inhibited plants in the absence of drought than in the combination of drought and sterol inhibition. This may be also triggered by differential decrease in sterol contents observed between these treatments.

4.6 Conclusions and future prospects

The work described in this thesis explored how soybean is affected by drought by looking at the physiological, biochemical parameters of soybean in relation to sterol biosynthesis. We also determined the impacts of drought on the expression of sterol biosynthesis and then determined the roles of phytosterols as signaling molecules in soybean responses to oxidative stress. Drought stress induced the expression of Glyma08g24160, a candidate lanosterol synthase gene as a result of reduced sterol content in soybean leaves. The reduced sterol content triggered accumulation of reactive oxygen species coupled with increased lipid peroxidation, cell death, and reduction in growth, together with activation of the antioxidant

defense system. In order to make sure that the accumulation of ROS and activation of antioxidant enzyme activities was regulated by altered sterol content, but not drought alone, we used a sterol biosynthesis inhibitor in the absence and in the presence of drought to determine the role of phytosterols on ROS production and activities of antioxidant enzymes. The reduction of sterol content caused by sterol synthesis inhibition altered ROS production and antioxidant enzyme activities. Although the antioxidant enzymes were activated, the induction of these enzymes was not sufficient to efficiently scavenge ROS. The expression of glyma08g24160 increased with increased need for biosynthesis of phytosterols, and consequently with the elevated ROS production and antioxidant enzyme activities. This implies that sterol biosynthesis and phytosterols have a role in plant tolerance to drought stress. These results supports the study hypothesis. However, more studies should be done on other organs such roots and also determine the role of other phytosterols that were not included in the study, and also the role of each sterol in soybean responses to drought stress. It will be important to further investigate possible roles of the candidate lanosterol synthase gene in soybean drought tolerance through silencing/knock-out of the gene.

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