Polyethylene glycol (PEG) induced water stress alters the physiological and molecular responses of chia plants

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WESTERN CAPE

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KEYWORDS

Ascorbate peroxidase

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Water stress

ABSTRACT

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

Water deficit is known to be one of the most detrimental environmental factors to affect crop production and growth in South Africa. This factor has become more apparent with increasing cases of drought in the country. It was therefore important in this study to explore an alternative food crop that encompasses all the nutritional benefits and one that is able to grow optimally in arid and semiarid environmental conditions. Chia is native to Mexico and it is thus able to grow very well in drought prone areas. However, there is very limited research on the agronomical responses of chia to environmental stresses. Therefore, study aims to elucidate the influence of PEG-induced water stress on the physiological and molecular responses of chia. The study explored the impact of increasing concentrations of PEG 8000 (0%, 5%, 10% and 20%) on the seed germination, the growth parameters, ROS accumulation and the antioxidant capacity of chia plants. The results obtained in this study showed that with increasing PEG 8000 concentrations germination was significantly reduced. The decrease in water potential with increasing PEG concentrations reduced

physiological parameters including root, shoot length and plant biomass. Photosynthetic metabolism was compromised due to a reduction in water potential associated with increasing PEG concentrations. PEG-induced water stress enhanced ROS accumulation in chia leaves, with the highest accumulation observed in higher concentrations (10% and 20%). The increase in ROS molecules (as seen for superoxide and hydrogen peroxide) resulted in enhanced oxidative damage manifested as increase lipid peroxidation and ultimately cellular death. ROS scavenging antioxidant enzyme activities (ascorbate peroxidise and superoxide dismutase) were differentially regulated in response to PEG 8000 treatment. Superoxide dismutase (SOD) activity was upregulated at lower PEG concentrations (5% and 20%), whereas a significant reduction was observed at higher concentrations (20%). Contrasting results were observed for hydrogen peroxide metabolising enzymes (ascorbate peroxidase; APX, catalase; CAT and glutathione reductase; GR) to different treatments of PEG. The results suggest that chia can withstand low concentrations of PEG but compromised when concentrations exceed 10%.

December 2017

DECLARATION

I declare that "**Polyethylene glycol (PEG) induced water stress alters the physiological and molecular responses of chia plants**" is my own work, that has not been submitted for any degree or examination in any other university, and that all sources I have used or quoted have been indicated and acknowledged by complete references.

Sinazo Bali December 2017

Signed …… ……

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LIST OF ABBREVIATION

ANOVA	analysis of variance
APX	ascorbate peroxidise
BSA	bovine serum albumin
CAT	catalase
Cu/Zn – SOD	copper zinc superoxide dismutase
EDTA	ethylenediaminetetraacetic acid
Fe – SOD	iron superoxide dismutase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidised glutathione
IDV	integrated density value
MDA	malondialdehyde
Mn – SOD	manganese superoxide dismutase
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitrotetrazolium blue chloride
PAGE	polyacrylamide gel electrophoresis
PVP	polyvinylpyrrolidone
ROS	reactive oxygen species
SOD	superoxide dismutase
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TEMED	N,N,N',N tetramethylethylenediamine

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CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

Environmental stress conditions in plants can be defined as changes in growth and developmental conditions, which occur within the plants natural habitat, thus leading to the alteration or disruption of the plants metabolic homeostasis (Shulaev *et al.,* 2008). These environmental stresses include both abiotic and biotic factors. However, water deficit otherwise termed as drought stress, remains one of the most fundamental stresses that are currently affecting plant growth and development in South Africa (van der Berg and Zeng, 2006). Constant exposure to drought stress, results in physiological and molecular alterations in plants (Kaur and Gupta, 2005). Plants often alternate to various adaptive mechanisms during abiotic stress conditions (Halliwell, 2006; Singh *et al*., 2014).

Furthermore, when plants are continuously exposed to such unfavourable conditions, the result is an imbalance between the reactive oxygen species (ROS) accumulation and alteration of the ROS scavenging antioxidant capacity (Gill and Tuteja, 2010; Junglee *et al*., 2014). The imbalance occurs when there is an excessive accumulation of the ROS molecules, thus leading to the inability of the ROS scavenging antioxidants to detoxify the toxic ROS radicals and non-radicals (Gill and Tuteja, 2010). This is due to the fact that

ROS molecules production surpasses the ability of their 'protective defence mechanisms' capacity to detoxify them.

Currently, there has been an increase in abiotic factors such as water stress which is a consequence of climatic changes in the environment (Ramakrishna and Ravishankar, 2013). Water stress encompass drought, the accumulation of salts in soils and other factors that lead to water deficit in plants (Le Gall *et al*., 2015; van der Berg and Zeng, 2006). These osmotic stresses impact the agricultural development and production which thus threatens food security. Therefore, it is vital that alternative food crops such as pseudocereals, which encompass high nutritional value and are found to grow optimally in these arid environments, are explored. One of the most nutritional pseudocereals, which is considered a superfood and has recently increased commercially, is *Salvia hispanica* commonly known as chia (Daniells, 2013). Given the absence of comprehensive information in the public domain regarding chia's interactions with abiotic/biotic stress conditions, it is imperative to explore these aspects.

This review will focus on the challenges associate with food security and the impact of water stress conditions such as drought on sustainable food security. This review will further explore the importance of investigating alternative food crops such as pseudocereals with high nutritional value, which are able to adapt to scarce arid environments. Finally, this review will

explore the effects of water stress on the physiological and molecular parameters in plants as a whole.

1.2 Food security

Food security can be defined as the state at which all people of a particular region have access to sufficient, safe and nutritional food, needed to maintain a healthy lifestyle (Maxwell, 1996; Slama, 2015). The definition of a holistic food secure population comprises of four key dimensions which include: food utilisation, food accessibility, food sustainability and the availability of food (Schmidhuder and Tubiello, 2007; Tibuello *et al*., 2007). Figure 1.1 illustrates how these four dimensions are the building blocks for the framework of food security, their respective functions and what influences these dimensions (Klennert, 2009). Ideally, these dimensions should be maintained at all times for a community to be considered food secure. However, there are multiple factors that play a key role in limiting food security, thus leading to food insecure communities, especially in developing countries.

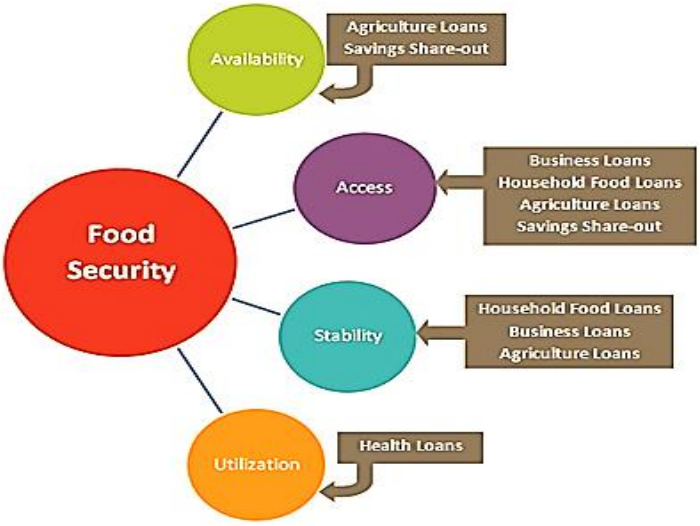

Figure 1.1: Dimensions of food security and their functions. Adapted from FOA 2005 and revised by Klennert, 2009.

Agricultural factors play a critical role in maintaining the status of food security (Abdu-Radeem and Worth, 2011). This includes crop-based plants, which encompass legumes, cereal and pseudocereal crops. Cereals and pseudocereals have served as food supply to humans and are also utilised as animal feed for centuries (Mergoum *et al*., 2009). Thus, these crop plants have been exploited for their nutritional and medicinal properties (Mohammad and Amusa, 2005). Consequently, food crops serve as an important factor in maintaining food security. However, changes in climatic conditions, such as; accumulation of salts, heavy metal, and increase in fungal and bacterial pathogens affect the state of food security and thus leading to a state of food insecurity (Ali *et al*., 2017; Bwalya, 2013; Masipa, 2017).

Food insecurity is exponentially increasing and this is more prominent in developing countries (Igram *et al*., 2012; Masipa, 2017). Despite advances made over the past 10 years, there is an increase in household food insecurity (Igram *et al*., 2012; van der Berg, 2006). The country is still plagued with high unemployment and poverty which further contributes to food insecurity (Binswanger-Mkhize, 2009; McMichael, 2000). Food insecurity in South Africa greatly affects the low income households, especially individuals living in rural areas and informal settlements of townships, who solely dependent on the government grant system (Oldewage-Theron *et al*., 2006; Sasson, 201; Naicker *et al*., 2015). Moreover, South Africa has been deemed to be food secure. However, data released by STATS SA (2017) suggested that the prevalence of poverty by headcount has increased since 2011 (Figure 1.2). Furthermore, Drimie and McLachlan (2013) amended the definition of the state of food security in South Africa to accommodate regions that still remain food insecure. Thus illustrating that South Africa is not a food secure nation and further research needs to be implemented to improve the state of food security in the country.

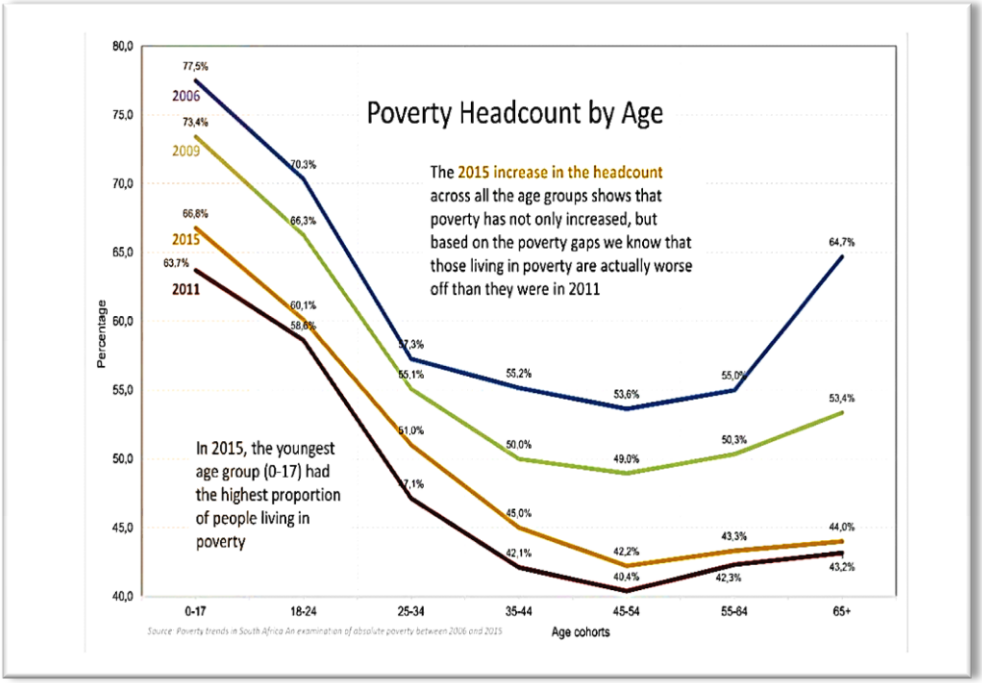

Figure 1.2: Poverty headcount by age in South Africa adopted from a study showing poverty trends in South Africa between 2006 and 2015 http://www.statssa.gov.za/?p=10341.

South Africa is an exponentially fast growing population, thus maintaining and improving the food security proves to be rather challenging. Various abiotic and biotic stress conditions associated with climate change have negatively affected food security in South Africa and globally (Strange and Scott, 2005; Reynolds *et al*., 2015. These stress conditions limit agricultural growth and production and ultimately reduce food security (Godfrey *et al*., 2005; Gregory *et al*., 2010; Knox *et al*., 2012). One of the most intricate aspects that have major impact on food insecurity in terms of agriculture is abiotic stresses such as drought (Alemayehu *et al*., 2015; Gregory *et al*., 2017).

With low rainfall and an increase in temperatures, the country is experiencing drought, thus putting the economical and the agricultural sectors at a drawback, which further makes food security vulnerable (Barrett, 2010). At present, global crop production is dependent on nine crops to supply more than 75% of the global plant originating energy. Furthermore, only three staple cereals, wheat, rice and maize contribute more than half of the total food production (FAO, 2015; Nuss 2012,). The production levels of the major staple food crops are not sufficient to fulfil the projected global food requirements (Godfrey, 2014). Efforts placed on maximising the potential of yields of these main staple crops are deemed to have reached a plateau, whereby yield increases are below what is actually needed (FOA, 2015; Godfrey, 2014; Tilman *et al*., 2001). Currently, plant science research is largely focused on major crops worldwide, whereas minor crops that encompass unique advantages for issues concerning global food security and the mitigation of climate changes are not the main focus.

There are a variety of plants which can potentially be used for human consumption and encompass a variety of nutritional benefit (Gordon, 2006). However, very few of these crop plants are being used. The focus tends to be on the three major cereal crops that are most predominantly used globally which include; maize (*Zea mays*), wheat (*Triticum aestivum* and other wheat species) and rice (*Oryza sativa*) (Gordan, 2006; Berghofer and Schoenlencher, 2012). However, this tendency on the reliance on these cereals cannot be seen as positive for many reasons including the sensitivity

of many of these cereal crops to water deficit conditions, which had negative impacts on food security (Pimintel *et al*., 2017; Valcárcel-Yamani *et al*., 2012). However, there are other alternative food crops (deemed as forgotten crops) that have not been extensively studied and these include pseudocereal crops (Fita *et al*., 2015; Valcárcel-Yamani *et al*., 2012; Williams *et al*., 2002).

1.3 Pseudocereals as alternative food crops

Pseudocereals are dicotyledonous species that are not closely related to each other or to monocotyledonous cereal species (Alvarez-Jubete *et al*., 2010; Valcárcel-Yamani *et al*., 2012). The name pseudocereal derives from their production of small grain-like seeds that resemble in function and composition those of cereals (also referred to as grasses) (Gordan, 2006; Jakop *et al*., 2009). This group comprises three crops; amaranth spp, quinoa and buckwheat (Valcárcel-Yamani *et al*., 2012). They are known to be evolutionary distinct from cereal crops, however, they produce grains that are used in the same manner as for cereal crops (Berti *et al*., 2005; Tang *et al*., 2016). Pseudocereals contain health and nutritional benefits that are significantly greater compared to those of cereals, as they contain a higher nutrition profile and are thus classified as superfoods (Galoa *et al*., 2015, Jubete *et al*., 2010). With increasing reports on celiac disease (CD) (a gluten-based disorder due to sensitivity) there is a demand for foods that do not

contain these hyper allergens (Tang *et al*., 2016). Therefore, plant science research studies have also shown an interest in the gluten- free crops. Three of the most extensively studied pseudocereals include quinoa (*Chenopodium quinoa*), buckwheat (*Fagipyrum escelentum and F. tartaricum*) and amarantus (*Amarantus sp*.) (Jubete *et al*., 2010). However, a fourth pseudocereal crop namely chia (*Salvia hispanica* L.) has entered the fray although limited research on this crop have been performed.

1.4 The importance of chia as an alternative food crop

Chia *(Salvia hispanica* L.), is a pseudocereal plant that is a member of the *Labiatae* (mint) family (Ali *et al*., 2012). The physiological structure of the plant consists of broad leaves and produces purple and white flowers (Figure 1.3) (Bueno *et al*., 2012). The plant contains both black and white seeds, produced by the same plant, with the black seeds being the most dominant in terms of quantity (Ayerza and Coates, 2011). Chai is an annual crop that it takes a year to complete its biological lifecycle (Ayezra *et al*., 1993; Ali *et al*., 2012,).

Furthermore, this plant is native to southern and central Mexico and Guatemala (Ixtiana et al., 2008). The history of chia dates back to its native land of South America in pre-Columbian and Aztec eras, where it was locally utilised for its medicinal purposes (Ali, *et al*, 2012). One of the key hallmarks of chia is the seeds and their oil content that allows it to have multiple health

and nutritional benefits (although the entire plant can be consumed and the stems and roots can be used as biomass for biofuel production). To add, the seeds contain a variety of active ingredients including essential fatty acids and phenolic compounds that have been identified, which contribute its health benefits (Ali *et al*., 2012; Ayezra, 2016).

Figure 1.3: *Salvia hispanica* (chia) plant with purple flowers and broad leaves: adopted from di Sapio e*t al*., (2009).

1.4.1 Nutritional composition of *Salvia hispanica*

Chia seeds (Figure 1.4) are composed of multiple nutritionally important polyunsaturated fatty acids (PUSFA) (Ayezra and Coates, 2005; Ali *et al*.,

2012). Comprising of approximately 25 to 40% oil content, of which 60% and 20% is w-3 alpha linolenic and w-6 alpha linoleic acids respectively (Ali *et al*., 2012). These are both essential fatty acids that cannot be synthesized artificially and are needed by the human body. The amount of the essential fatty acids that are found in the chia are found to be more superior than that of those found in most true cereals such as maize and sorghum combined (Poudyal *et al*., 2012, Ayezra, 2016).

Figure 1.4: Morphology of chia seeds. The image was adopted from Lin *et al*., (1994).

Due to the vast health and medicinal properties found in chia seeds, it has been shown to lower the triglycerides (TG) and cholesterol levels, which in turn lowers the risk of cardiovascular related diseases and lowers blood pressure (Heuer *et al*., 2002; Ali *et al*., 2012). According to Ayerza and Coates (2012) and Fernandez *et al.,* (2014) the effects of chia seed feeding

on rat plasma, indicated that serum TG and low density lipoprotein (LDL) were notably decreased and high density lipoprotein and the ω-3 polyunsaturated fatty acids where increased (Ixtaina, 2008).

Several crop seeds including sunflower seeds, rapeseed, soybean seed, and evening primrose are commercially recognised as being good sources of oil for dietary needs (Ayezra, 1994). However, Antruejo *et al.* (2011) conducted a comparative study that proved chia seeds has superior nutritive qualities to other major crops (Figure 1.5) and that there is a potential market for chia in South Africa.

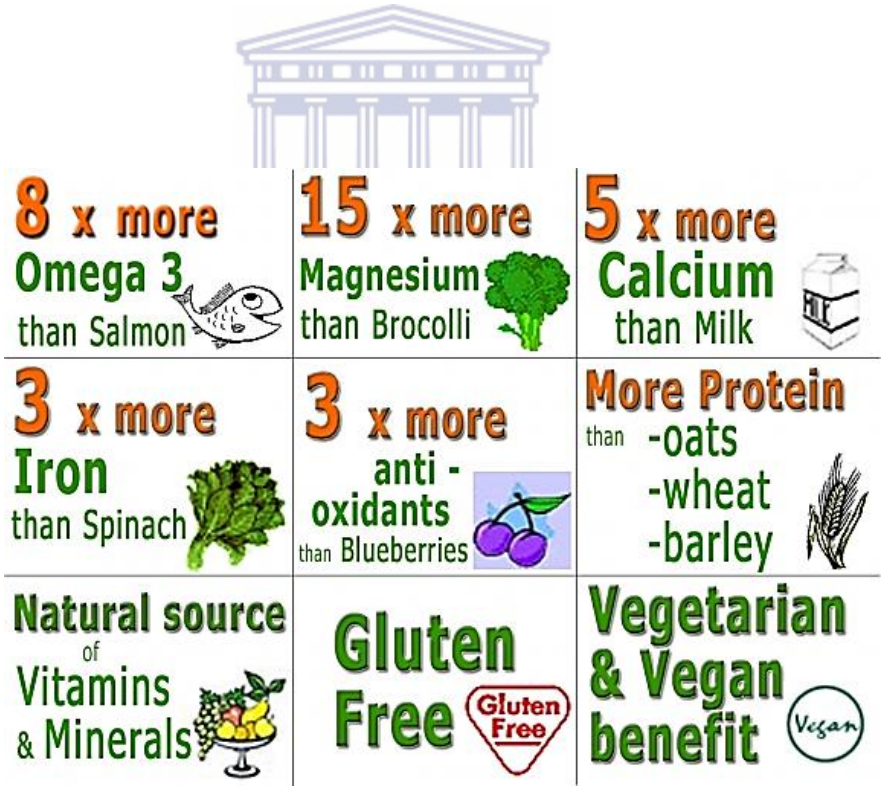

Figure 1.5: Nutritional value and benefits of chia compared to other food crops. Image was adopted from: http://www.buychia seeds.info/html/chia_seeds_benefits.html.

1.4.2 Cultivating of chia plants

Chia has recently gained its popularity by becoming one of the main oil sources that contains high levels of polyunsaturated fatty acids. This crop has now been widely cultivated and commercialised for its ω-3 alpha linolenic acids content and antioxidant properties (Ayerza, 2005). Its cultivation today is not only limited to South America but has extended to Bolivia, Australia, Ecuador, Italy and Southern Asia (Joseph, 2004; Crawford *et al*., 2012; de Freista *et al*., 2016). Chia production is a major contributor to the Argentine economy, accounting for 24% of the agricultural industry (Ayerza, 2016). In 2008 Argentina contributed approximately 4% of the world grain production (Ayerza, 2016). Although it is native to Mexico, Australia is currently the largest producers of chia seeds (Crawford *et al*., 2016; Timilsena, 2016,).

Therefore, there is a need to establish a suitable crop that can be deemed to be drought tolerant and is able to withstand such environments outside of the scope of cereals, but also pseudocereals. The promotion of the consumption and cultivation of chia could be of value due to its unique nutrient composition and its inherited tolerance to drought and other stress factors. Chia has been deemed to be drought tolerant, although there is lack of research on the physiological and molecular responses of this crop to drought stress.

1.5 Effects of abiotic stresses on plants

Plants are constantly faced with environmental challenges such as abiotic stresses, which lead to strenuous conditions on their metabolic functioning, frequently impaired growth and development (Mourato *et al*., 2012). The end result is the drastic reduction in yields, which further affects the world food supply (Tester and Bacic, 2005). Furthermore, a demonstration of the reduction in the cereal production has been projected by about 2% in Africa as a result of climate changes (van der Berg and Zeng, 2006). The consequence of these climate conditions leads to the generation of abiotic stresses such as water stress (Ngara, 2009).

The impact of these abiotic stresses in plants leads to the production of reactive oxygen species in plants. Under normal conditions these oxygen molecules act as signalling molecules of various biochemical pathways (Klein *et al*., 2012, Tuteja and Gill, 2010). However, if not regulated these molecules lead to oxidative stress and ultimate cellular death as demonstrated in Figure 1.6 (Mahaja and Tuteja, 2012).

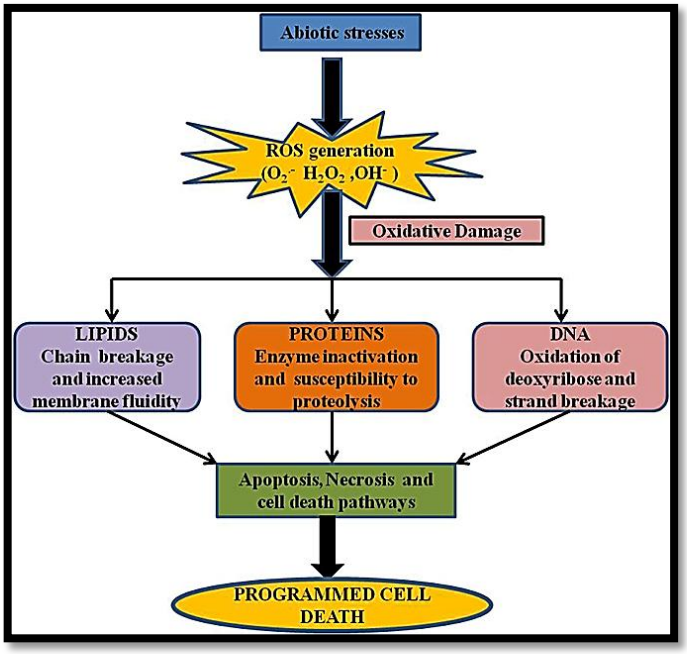

Figure 1.6: Role of abiotic stress in plants. Abiotic stress such as water stress induces enhanced ROS accumulation, which leads to oxidative damage and ultimately programmed cell death. Image adopted from Mahaja and Tuteja, (2012).

1.5.1 Water stress in plants

Water is an essential requirement that is needed throughout all forms of life (Xiong *et al*., 2002). For vegetative growth of plants to occur, there needs to be a certain degree of water status (Hirt and Shinozaki, 2003; Szilgyi, 2003). The water status can be measured as the free energy state of water molecules including water potential (Jones, 2006). In a plant cell, the water potential consists mainly of the osmotic potential and pressure (Fricke 1997; Nonami, 1998). Changes in the water potentials in the environment can impose stress to plants, thus disrupting the normal metabolic activities. During water stress conditions, plants will tend to water lose to the external

environment, this leads to a reduction in turgor pressure (Skirycz and Inzé, 2010). Water stress in plants can thus be characterised as the turgor pressure loss, a decrease in leaf water potential, stomatal closure, diminishing of cell expansion and growth and alteration of enzymatic activity (Fricke, 1997; Blum 2017).

1.5.2 Polyethylene glycol induced water stress in plants

Osmotic solutions are used to impose water stress in plant related studies, reproducibly under *in vitro* conditions (Pandey and Agarwal, 1998). Polyethylene glycol (PEG) is mainly used for the determination of water deficit stress related information from plants (Turkan *et al*., 2005; Landjeva *et al*., 2008). PEG molecules of are non-ionic and virtually impermeable (Hohl and Peter, 1991). Rubinstein (1982) was the first to show that PEG does not enter cell was and the PEG molecules with molecular weights greater than 3000 are not absorbed by the plant (Tarkow *et al*., 1996). Thus, because PEG does not enter the apoplast, water is withdrawn from the cell and the cell wall. Molecules of PEG 8000 are small enough to influence osmotic potential, but they are also large enough to be absorbed by plants. Therefore, PEG solutions mimic dry soil more closely than solutions of low M_r osmotic, which infiltrate the cell wall with solutes (Verslues *et al*., 1998).

1.6 The role of stress-induced ROS production in plants

When plants are constantly exposed to harsh environmental conditions due to their immobile nature, this results in the over accumulation of ROS molecules (Gill and Tuteja, 2010; Zorov *et al*., 2014). These ROS molecules are known to be intermediates that are formed due to the reduction of atmospheric oxygen which comprises of: superoxide radicals, hydroxyl radicals and alkoxyl (Figure 1.7) (Bhattachrjee, 2005; Bashan *et al*., 2009; Zorov *et al*., 2014). Under favourable conditions, ROS molecules are produced as byproducts of multiple metabolic pathways that are mainly found in the chloroplast, peroxisomes and the mitochondria (Foyer and Noctor, 2005). Moreover, they are unable to cause damage when they are being scavenged by their antioxidant mechanisms (Colín-González *et al*., 2012; Das and Roychoudhury, 2014; Kasote *et al*., 2015). The two main ROS molecules that this study focuses on are superoxide and hydrogen peroxide.

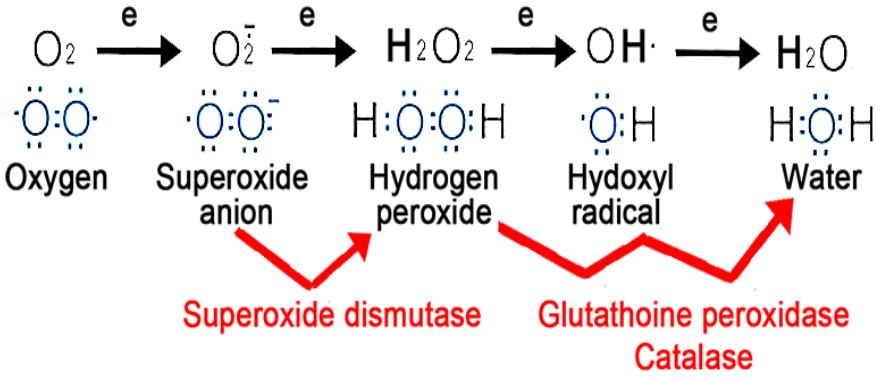

Figure 1.7: The electron structures of selected ROS molecules and their respective detoxifying enzymes. These reactions indicate the unpaired nature of reactive oxygen species. Image was adopted from Bashan *et al*., 2009.

1.6.1 The role of superoxide in plants under stress

The superoxide radical is produced mainly in the thylakoid membrane during the electron transport chain (Halliwell, 2006). This is the first ROS molecule that is produced under stress conditions and is also one that is moderately reactive, with a half-life of 2-4 microseconds (Gill and Tuteja, 2010; Hossain *et al*., 2011; Mourant *et al*., 2012). The consequent production of this radical may trigger the formation of highly reactive ROS (OH and $^1O_2$) which can lead to the peroxidation and weakening of the cell structure (Gill and Tuteja, 2010). Due to the fact that this radical cannot diffuse across the cell, mechanisms are therefore needed to be in place to effectively scavenge this radical to hydrogen peroxide and water, which is the function of the SOD (Mourant *et al*., 2012).

1.6.2 The role of hydrogen peroxide in plants

In plants, hydrogen peroxide (H_2O_2) can either be toxic or protective depending on the quantity levels and its response to the plant antioxidant system, as high levels of H_2O_2 can induce oxidative stress and injury to plant cells (Apel and Hirt, 2004; Miller, 2002). Hydrogen peroxide is known to be the most moderate ROS molecule, as it has a longer half-life (1 ms) compared to other ROS (Gill and Tuteja, 2010). Although moderate and having a much longer half live than other radicals, it has been established that an excess of H_2O_2 in the plant cells leads to the occurrence of oxidative stress (Gill and Tuteja, 2010). The effect of excess H_2O_2 in plant cells may consequently lead to the inactivation of enzymes, by oxidising their thiol groups. Furthermore, H_2O_2

plays a double role in plants; whereby at low concentrations it acts as a signalling molecule that is involved in the regulatory signal that is important in the physiological processes such as plant senescence, stomatal movement as well as growth and development parameters (Noctor *et al*., 2002, Mittler, 2002). The H_2O_2 molecule as also been shown to act a s a key regulator in a broad range of physiological processes, such as senescence, photosynthesis, stomatal movement as well as cell growth and development of some plants (Peng *et al*, 2005).

The different ROS molecules in plants are known to result in damages to biomolecules, including lipids, proteins and DNA under various environmental stresses, (Noctor and Foyer, 1998). Damages to these compartments can be detrimental to the plants physiological state as well as the molecular damage (Noctor and Foyer, 1998; Bright *et al*., 2006; Quan *et al*., 2008). The damage that occurs to lipids, DNA and proteins ultimately results in cell death. The damage to DNA results in various physiological effects to plants, which includes reduced protein synthesis, damage to photosynthetic proteins which thus affects the growth and the development of the entire plant and cell membrane destruction (Millar and Leaver, 2000). Lipid peroxidation has been well thought-out to be the major determinant of seed viability loss and inevitably crop loss (Gill and Tuteja, 2010).

1.6.3 Lipid peroxidation and its detrimental effects in plants

The peroxidation of lipids is considered to be the most detrimental process that is known to occur in every living organism (Yadav *et al*., 2010; Skorzysnka-Polit, 2007). Lipid peroxidation has been associated with damages that are evoked by some environmental stresses in plants including osmotic stress. Membrane damage is sometimes taken as a single parameter to determine the level of lipid destruction under various stresses (Ahsan *et al*., 2003). This is elucidated when there is an over accumulation of ROS in a plant due to unfavourable conditions. Moreover, the excessive accumulation of ROS, cause disruption of polyunsaturated fatty acids, thereby causing lipid peroxidation (Yadav *et al*., 2010). Malondialdehyde (MDA) is often indicative of oxidative stress since it is a decomposition product of fatty acid degradation (Hossain *et al*., 2013)

However, there are mechanisms that the plants utilises in order to inhibit or slow the effect of DNA damage, lipid peroxidation as well as damage to other cellular compartments. These mechanisms encompass activating antioxidant enzymes as well as non-enzymatic antioxidants that are able to scavenge the ROS molecules, thus reducing of oxidative damage under abiotic stresses that the plant may encounter.

1.7 The significant role of antioxidant enzymes in plants under stress

ROS are known to interact with multiple cellular components, leading to membrane, cellular damage and ultimately inhibition of plant growth. However, plants employ antioxidant defence systems to counter the damage caused by the accumulation of ROS (Khan and Fatma, 2008; Misra *et al*, 2009). ROS scavenging mechanisms include several enzymatic enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase MDHAR (Blokhina *et al*., 2003; Sharma *et al*., 2012; Carvezan *et al*., 2016) (Table 1.1). However, detoxification of ROS is not limited to enzymatic antioxidants, there are also metabolites that are involved in the detoxification of ROS which are non-enzymatic such as; ascorbic acid (AsA), glutathione (GSH) as well as various phenolic compounds (Blokhina *et al*., 2003).

Table 1.1: The mechanisms of ROS scavenging enzymes (Blokhina *et al*., 2003)

Enzyme	EC	Reaction catalysed
Superoxide dismutase	1.15.1.1	$2O_2$- + $2O_2$/ 2H-↔ $2H_2O_2$ + O_2
Ascorbate peroxidase	1.11.1.11	H_2O_2 ↔ DHA +$2H_2O$
Monodehydroascorbate reductase	1.6.5.4	NADH + 2MDHA ↔ NAD^+ + 2AA
Dehydroascorbate reductase	1.8.5.1	2GSH + DHA ↔ GSSG + AA
Glutathione reductase	1.6.4.2	NADPH + GSSG ↔ $NADP^+$ + 2GSH

1.7.1 Superoxide dismutase in regulating abiotic stress in plants

Superoxide dismutase is described by Buetter (1998) as a primary antioxidant enzyme due to the fact that it is the only known enzyme that acts directly on the radical. Therefore, SOD is known to be the catalytic component for the dismutation of superoxide radicals to H_2O_2 and H_2O (Table 1.1) and hence reduces the formation of hydroxyl radical via the Haber-Weiss-type reaction in plants under drought stress (Kleibenstein *et al*., 1999; Hallwell, 2006). There are three main SOD isoenzymes classified as; Copper/zinc (Cu/Zn- SOD), the manganese (Mn- SOD) and the iron (Fe- SOD) (Chaudhary *et al*., 2009). Previous studies have shown that SOD is most likely to be up-regulated under abiotic stress conditions (Boguszewska *et al*., 2010). An increase in the activity of SOD was detected in response to drought stress in different cultivars of *Phaseolus vulgaris* (Zlatev et al., 2006).

1.7.2 Catalase (CAT)

Catalase (CAT) (EC: 1.11.1.6) enzymes are known as tetrameric heme containing enzymes that has the ability of directly scavenging H_2O_2 into H_2O and O_2 (Table 1.1) (Blokhina *et al*., 2003). CAT is considered to have the highest turnover rate for all the enzymes, as one CAT molecule can convert approximately six million molecules H_2O_2 to H_2O and O_2 per minute (Polidoros *et al*., 1999; Mishra and Das, 2003; Ray *et al*., 2012). CAT isoenzymes have been identified and studied in higher plants. These CAT isoenzymes are localised in different cellular compartments with CAT1 and CAT2 being located in the peroxisomes and cystol, on the other hand CAT3 is found to be located in the mitochondria (Kirkman and Gaetani, 2007; Gill and Tuteja 2010; Ray *et al*., 2012). These CAT isozymes have been illustrated to be regulated temporally and spatially, thus responding differently to light (Willekens *et al*., 1994; Skadsen *et al*., 1995). In response to stress in plants, the activity of CAT has been found to increase in response to some drought-susceptible wheat cultivars (Simova-Stoilova *et al*., 2010; Zamocky *et al*., 2008).

1.7.3 Ascorbate peroxidase

Ascorbate peroxidase (APX) (Table 1.1) is a key enzyme that regulates ROS levels acting in different subcellular compartments (Noctor and Foyer, 2006; Gill and Tuteja, 2010). APX is found to be regulated in multiple cellular

organelles including; the mitochondria, peroxisomes, the cystol as well as the chloroplast (Noctor and Foyer, 2006). Moreover, the APX enzyme mainly functions to scavenge and maintain the levels of H_2O_2 (Table 1.1) in conjunction with CAT (Gill and Tuteja, 2012). Previous studies have demonstrated that during various abiotic as well as biotic stresses, there is an enhanced expression of APX in plants. Furthermore, it has been well documented that the overexpression of APX in chloroplast of some plants also enhances the tolerance to salt and water deficit in plants (Sharma and Dubey, 2005; Sinha and Saxena, 2006).

1.7.4 Non-enzymatic detoxification mechanisms in plants

Antioxidant mechanisms for the scavenging of ROS molecules are not limited to enzymatic antioxidants solely; there are non-enzymatic antioxidants that forms part of the antioxidant machinery (Das and Roychouthury, 2014). Non-enzymatic antioxidant defence systems consist of ascorbic acid (AA), carotenoids, flavonoids, reduced glutathione (GSH), phenolics, and osmlytes (Athar, 2005; Smirnoff, 2005; Gill and Tuteja, 2010). These non-ezymatic antioxidants are known to be involved in protecting the plants from cellular damage and also play a critical role in plant growth and development.

1.7.4.1 The role of osmolytes in plant protection

One of the key metabolic consequences of water stress is the accumulation of osmolytes. Osmolytes are low molecular weight organic compounds, also known to be compatible solutes which are highly soluble and do not interfere with normal metabolic reactions because they are non-toxic even at high cellular concentrations (Flowers *et al*., 1977; Soshinkova *et al*., 2013; Yancey, 2005). The primary function of osmolytes is accepted to be osmotic adjustment in plants (Hasegawa *et al*., 2000), but this is not always the case, especially in glycophytes exposed to saline conditions (Slama *et al*., 2015). A wide range of osmoprotective compounds have been identified including mono-, di-, oligo- and polysaccharides, sugar alcohols (polyols) and amino acids (Rhodes *et al*., 2002; Ashraf and Foolad, 2007; Slama *et al*., 2015). Over-expression of genes for the synthesis of these different osmolytes enable the plants to cope better with stress due to enhanced accumulation of the concerned osmolytes.

1.7.4.2 Flavonoids and polyphenolic compounds

Flavonoids and polyphenolics are antioxidant compounds that are widely distributed in the plant kingdom. These flavonoids and phenolic acids are considered as secondary ROS scavenging systems when plants are exposed to abiotic or biotic stresses (Das and Roychoudhury, 2010). They play a role in scavenging singlet oxygen radicals and also repairing the oxidative damage caused in the chloroplastic membrane induced by abiotic stresses (Vierstra

et al., 1982; Loreta *et al*., 2004). Previous studies have shown that water deficit induced by PEG treatment, increased the flavonoids and phenolics (Basu *et al*., 2010). Therefore, plants that contain these phenolics and flavonoids in abundance as compared to others are more likely to thrive under stress conditions (Das and Roychoudhury, 2010). Moreover, chia contains high contents of phenolic acids and therefore could be the reason why this plant thrives in arid environments (Ali *et al*., 2012).

JUSTIFICATION

Drought is known to be one of the most detrimental abiotic stresses to affect agricultural growth and production. Water deficit not only limits crop production and yield, however it has an impact on every single business sector that is expected to render a profit. Furthermore, due to the fact that plants are immotile, they will be constantly affected by drought conditions and other abiotic stress conditions. Constant exposure to these environments leaves the plant vulnerable and due to over accumulation of ROS molecules. This results in the disruption of lipids at the membranes, ultimately leads to cellular death and thus death in crops. However, plants do have measures that they use in order to avoid or withstand the stress conditions, and this is done through a cascade of molecular responses which can make the plant somewhat tolerant to environmental stresses.

Although South Africa has adapted genetically modified maize since the 1997, there is still no evidence that it is drought resistance. Moreover, with increase poverty, food insecurity and malnutrition in the country, it has become imperative to explore alternative food crops that encompass all the nutritional benefits and also able to grow optimally in drought prone soils. *Salvia hispanica*, commonly known as chia, is known to be a superfood due to its nutritional benefits. This crop can be used entirely, from the seeds, being used as pseudocereals and can be used as an oil seed due to its extremely high quality and quantity of omega 3 and 6 fatty acids, which will be explored in Chapter 1. The leaves of chia can be utilised in salads and the roots and stems can be used as biomass for biofuel production. This plant can be beneficial in improving food insecurity in the country.

Finally, the response of chia to abiotic and biotic stresses is limited in the public domain, with most studies focusing on the phytochemical properties of the seeds. Therefore, this study will for part of a bigger stop to gather as much knowledge as possible of chia in order to try to make this crop a commercial one in the country.

AIMS AND OBJECTIVES OF STUDY

The aim of this study is determine the impact of water stress modulated by increasing PEG 8000 concentration (0%, 5%, 10% and 20%) on the physiological and molecular responses of chia plants. The effect of water stress on cereal crops have been well documented, with only limited studies on pseudocereal crops and to date there is no published data on chia. Therefore, this study aims to monitor the effect of increasing water stress on the germination rate and morphology of chia seeds. Furthermore, this study aims to achieve the effects of increasing water stress, elucidated by PEG on the physiological responses of chia by monitoring the growth parameters. These growth parameters include: (i) root and shoot length, (ii) biomass production and (iii) chlorophyll contents. Moreover, this also aims to explore the molecular responses of chia by investigating the accumulation of ROS molecules (O_2^- and H_2O_2), lipid peroxidation and cell death accounted under water stress conditions. Finally, the study will investigate the relationship between the physiological changes and ROS accumulation on the antioxidant enzymes capacity that are responsible for the detoxification of ROS (SOD, APX, CAT and GR).

CHAPTER 2
MATERIALS AND METHODS

2.1 Chemical reagents and suppliers

Table 2.1: List of chemical reagents used in this study

Chemical	Supplier
Acetone	Merck Millipore
Acrylamide/Bis (40%)	BIO –RAD
Ammonium acetate ($C_2H_3O_2NH_4$)	Sigma Aldrich
Ammonium Persulfate (APS)	BIO –RAD
Ascorbic acid / Ascorbate	Sigma Aldrich
Bovine Serum Albumin (BSA)	Roche
Bradford Reagent (1X)	BIO –RA
Calcium chloride ($CaCl_2$)	Sigma Aldrich
Cobalt (II) chloride ($CoSO_4$)	Sigma Aldrich
Copper (II) sulfate ($CuSO_4$)	Sigma Aldrich
5,5-Dithiobis(2-nitrobenzoic acid) (DTNB)	Sigma Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich
Ethylenediaminetetraacetic acid ferric sodium salt (Fe-Na EDTA)	Sigma Aldrich
Evans Blue	Sigma Aldrich
Glacial acetic acid	Merck Millipore
Glutathione disulfide (GSSG)	Sigma Aldrich
Glycerol	Merck Millipore
Glycine	BIO-RAD

Hydrochloric acid (HCl)	Merck Millipore
Hydrogen peroxide (H_2O_2)	Merck Millipore
Magnesium sulfate (MgSO)	Sigma Aldrich
Manganese (II) sulfate ($MnSO_4$)	Sigma Aldrich
2-(N-Morpholino)ethanesulfonic acid (MES) hydrate	Sigma Aldrich
Methanol	Merck Millipore
Methionine	Sigma Aldrich
Methylthiazolyldiphenyl-tetrazolium bromide (MTT)	Sigma Aldrich
B-nicotinamide adenine dinucleotide (NADH)	Sigma Aldrich
Nitrotetrazolium blue chloride powder (NBT)	Sigma Aldrich
Phenazine methosulfate (PMF)	Sigma Aldrich
Polyvinylpyrrolidone (PVP) MW: 40 000	Sigma Aldrich
Potassium cyanide (KCN)	Sigma Aldrich
Potassium hydroxide pellets	Merck Millipore
Potassium iodide (KI)	Sigma Aldrich
Potassium nitrate (KNO_3)	Sigma Aldrich
Potassium phosphate monobasic (KH_2PO_4)	Sigma Aldrich
Potassium phosphate dibasic (K_2HPO_4)	Sigma Aldrich
Potassium sulfate (K_2SO_4)	Sigma Aldrich
Promix	Stodels
Propan-2-ol (isopropanol)	Merck Millipore
Riboflavin	Sigma Aldrich

Sodium hydroxide (NaOH)	Merck Millipore
Sodium molybdate (Na_2MoO_4)	Sigma Aldrich
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma Aldrich
Thiobarbituric acid (TBA)	Sigma Aldrich
Trichloroacetic acid (TCA)	Merck Millipore
Tris(hydroxymethyl)-aminethane	BIO-RAD
Zinc sulfate monohydrate ($ZnSO_4$)	Sigma Aldrich

2.2 Seed germination

Salvia hispanica (chia) seeds (thousand seeds per treatment) (purchased from Faithful to Nature, Cape Town) were germinated on PEG 8000 pre-treated (5%, 10% and 20%) moist filter paper at room temperature (in the dark) for 7 days. Germinated seeds were counted every 24 hours to determine germination percentage per day as well at the state of the seed morphology.

2.3 Plant growth and treatments

After 7 days of germination, seeds were transplanted (one plant per pot) in moist (distilled water) promix growth medium (purchased from Stodels Garden Center, Brackenfell, South Africa) and allowed to grow until the first fully expanded leaves had emerged in a 25/19 ^{o}C day/night temperature cycle under a 16/8 hours light/dark cycle.

Seedlings were supplemented with 0.5 X nutrient solution [1 mM K_2SO_4, 2 mM $MgSO_4$, 5 mM $CaCl_2$, 5 mM KNO_3, 10 mM NH_2NO_3, 1 mM K_2HPO_4 buffer (at pH 6.4), 5 µM H_3BO_3, 5 µM $MnSO_4$, 1 µM $ZnSO_4$, 1 µM $CuSO_4$, 2 µM Na_2MoO_4, 1 µM $CoSO_4$, 100 µM Fe-NaEDTA and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)] at pH 6.5 every second day. Nutrient solution was supplemented to control plants and 5%, 10% and 20% PEG 8000 plants for treatments. Nutrient or PEG 8000 solutions (50 ml) were exogenously applied to each plant at the base of the stem of the plant every second day for a period of 14 days.

2.4 Measuring of PEG 8000 water potential

Water potential of PEG 8000 (5%, 10% and 20%) solutions were determined with an isopietic thermocouple psychrometer using a modified methodology adopted from Michel and Kaufmann (1972). The water potential for each solution was calculated using formula:

$$\textit{Water potential} = (RT/Mw)$$

Where R is the gas constant, T is temperature (K), and Mw is the molecular weight of water.

2.5 Analysis of physiological parameters

After 14 days of treatment, plants were carefully removed from their growth medium. Growth analysis on chia plants was performed by measuring plant

length (shoot and root), fresh weights (shoot and root) and dry weights (shoot and root). There parameters were measured as previously described by Lokhande *et al*. (2010). For measuring dry weight; shoot and root tissues were dried in an oven at 60 °C for 72 hours.

2.6 Leaf chlorophyll content

Chlorophyll content was estimated using a modified method described by Harborne (1973). The chlorophyll pigments were determined by homogenising 200 mg of leaf tissue with 100% (v/v) acetone. The mixture was vortexed and centrifuged at 13000 x g for 10 minutes. This process was repeated until a clear pellet was observed. The absorbance of the different fractions (200 µl) was recorded at 662 and 644 nm respectively using the spectrophotometer. Chlorophyll content was calculated using the following formulas:

*Chlorophyll **a** (µg/mL) = 12.21 A 662 nm– 2.81 A 646 nm*

*Chlorophyll **b** (µg/mL) = 20.13 A 646 nm – 5.03 A 66 2nm*

Total chlorophyll content (µg/mL) = 17.30 A 646 nm – 7.18 A 662 nm.

2.7 Measurement of cell death (Evans Blue uptake assay)

To determine the cellular death in chia leaves, a method adopted from Sanevas *et al.* (2007) was used with slight modifications. Fresh leaf tissue

(200 mg) from the third youngest leaves (from three different plants per treatment) was stained using 0.25% (w/v) Evans blue and incubated at room temperature for 1 hour. The leaves were washed five times with distilled water to remove all surface-bound dye. The dye absorbed by the dead leaf cells were extracted using 1% (w/v) SDS. This was incubated for 1 hour at 55 °C. Absorbance of each extract was measured at 600 nm to determine the level of Evans Blue uptake and thus the level of cell death in the tissue.

2.8 Superoxide content

Superoxide content in the leaves of chia plants were estimated using a method adopted from Gokul *et al.* (2016). Leaf samples from each treatment were incubated at room temperature for 20 minutes in a buffer containing: 10 mM potassium cyanide (KCN), this was used for the inhibition of the Cu/Zn SODs, 10 mM H_2O_2, which was used for the inhibition of the Cu/ZN as well as the Mn-SODs, 80 µM of NBT and 2% SDS and this was used for the inhibition of Fe- and Cu/Zn- SOD and 50 mM potassium phosphate buffer at pH 7. Following the incubation, the leaf pieces within the solution were crushed using a miniature pestle, this was done to release the superoxide within them. The crushed samples were centrifuged at 13 000 x g for 10 minutes Absorbance readings and absorbance measured at 600nm. Superoxide content was calculated using the extinction coefficient if 12.8 $mM^{-1.}cm^{-1}$.

2.9 Protein extraction for biochemical analysis

Leaf tissue from all treatments were harvested and ground into a fine powder using liquid nitrogen. Leaf material (200 mg) was homogenized in 1 ml of 6% (w/v) trichloroacetic acid (TCA) for analysis of H_2O_2 content and lipid peroxidation or in 1 ml of homogenizing buffer (PVP) for the measurement of antioxidant enzymatic activities. Protein concentrations for all assays were measured in extracts as described by the manufacturer for the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories, Inc., Hercules, CA).

2.10 Measurement of the H_2O_2 content assay

For the measurement of H_2O_2 content in the leaf tissues of chia plants, a method previously described by Volikova *et al*. (2000) was used. The reaction mixture contained 50 µl of the TCA extract (section 2.8), 1 M KI and 20 mM K_2HPO_4, pH 5.0. The sample mixture was incubated at 25 ^{o}C for 20 minutes and the absorbance readings recorded at 390 nm. Hydrogen peroxide content was calculated using a standard curve based on the absorbance (390 nm) of H_2O_2 standards.

2.11 Estimation of lipid peroxidation

Lipid peroxidation was determined using a method previously described by Vos *et al*. (1991). TCA extracts (200 µl) from each treatment was mixed with 400 µl of 0.5% TBA (dissolved in 20% TCA) and incubated at 95 $^{\circ}$C (waterbath)

for 1 hour. After incubation, samples were immediately placed on ice for 10 minutes and centrifuged (13 000 x g) for 20 minutes. Absorbance readings of supernatants were measured at 600 nm and 532 nm respectively. To adjust the non-specific turbidity the value obtained at 600 nm was subtracted from the 532 nm reading. The concentration of MDA was calculated using a molar extinction coefficient (155 $mM^{-1}.cm^{-1}$).

2.12 Determination of total superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activities were measured using a modified method described by Samantary (2002). The reaction mixture consisted of 10 PVP protein extracts (section 2.8) with 190 µl of SOD buffer (0.1 mM NBT, 0.005 mM riboflavin, 10 mM methionine and 0.1 mM EDTA). Reaction mixtures were incubated under light at room temperature for 15 minutes to initiate the reaction. Absorbance readings were recorded at 560 nm and SOD activity calculated based on the amount of SOD enzyme that was required to reduce 50% of NBT to formazan.

2.13 Ascorbate peroxidase total activity

APX activity was determined using a modified method described by Asada (1984). Each reaction mixture contained 10 µl PVP leaf extract (section 2.8) and 190 µl reaction buffer (50 mM K_2HPO_4 at pH 7.0, 0.2 mM EDTA and 0.25

mM ascorbic acid). The reaction was initiated by the addition of 90 µM H_2O_2. The APX activity was calculated based of the changes in absorbance at 290 nm, using the extinction co-efficient of 2.8 $Mm^{-1}cm$ (Teksi *et al*., 2016).

2.14 Catalase activity total activity

Catalase (CAT) activity was measured as previously described by Cakmak and Marschner (1992) and Monnet *et al.* (2006) with slight modifications. The reaction mixture contained 100 mM potassium phosphate buffer pH 7.0 (900 µl) and 100 µl of enzyme extract. The reaction was initiated with 6 mM H_2O_2. The rate of decomposition of H_2O_2 was recorded at 240 nm for every 30 seconds for 3 minutes. One enzymatic unit of CAT can be defined as the amount of enzyme required to decompose 1.0 µmol of H_2O_2 at pH 7.0 with the concentration of H_2O_2 decreasing from 10.3 mM to 9.2 mM (Caliborne 1985, Prabharkar *et al*., 2007). CAT was calculated using the extinction co-efficient of 43.6 $M^{-1}cm^{-1}$.

2.15 Glutathione reductase total activity

Glutathione reductase (GR) activity was determined using a modified method described by Esterbauer and Grill (1978) by following the rate of NADPH oxidation at 340 nm. The reaction buffer consisted of: 0.2 mM NADPH, 0.5 mM GSSG, 1 mM EDTA in 100 mM K_2HPO_4 pH 7.8. The reaction

buffer (190 µl) was pre-mixed with10 µl of PVP protein extract (Section 2.8) in a final volume of 200 µl. GR activity was calculated based on the oxidation of NADPH, using the extinction coefficient of 6.2 $mM^{-1}.cm^{-1}$.

2.16 Detection of ROS detoxifying antioxidant isoforms

2.16.1 Superoxide dismutase isoforms

Native gels were first separated on a 12% acrylamide gels (Laemmli, 1970). SOD isoforms then were detected in chia leaf samples as described by Beauchamp and Fridovich (1971) with slight modifications using 90 µg of PVP protein extracts. SOD activity was detected by staining with 0.5 mM riboflavin and 2.5 mM NBT.

2.16.2 Ascorbate peroxidase isoforms

For the detection of APX isoezymes electrophoretic separation was performed as described by Seckin *et al*. (2010). Native PAGE was performed at 4°C in 12% polyacrylamide gels (Laemmli, 1970). The gels were washed with 50 mM potassium phosphate buffer (pH 7.8) containing, 4 mM ascorbic acid and 2 mM H_2O_2 for 10 minutes. The gel was then stained with 50 mM KPO_4 (pH 7.8), 16 mM TEMED and 2.5 mM NBT for 10-20 minutes with gentle agitation in the presence of light.

2.16.3 Catalase isoforms

The CAT in-gel activity was based primarily on a method derived from Chandlee and Schandalios (1983). Gels were soaked in 5 mM H_2O_2 for 10 minutes. Gels were then incubated in 1% ferric chloride (w/v) and 1% potassium ferricyanide solution.

2.16.4 Glutathione reductase isoforms

GR isoforms were detected using a modified method described by Lee and Lee (2000). GR was visualized on the native PAGE gel by incubation in 50 ml of 0.25 M Tris–HCl buffer (pH 7.9) containing 4.0 mM glutathione disulfide (GSSG), 1.5 mM NADPH, and 2 mM DTNB for 20 minutes. The activity was negatively stained in the dark with a solution containing 1.2 mM MTT and 1.6 mM PMS for 10 minutes at 30 °C.

2.17 Densitometry analysis of ROS scavenging enzymes

The native PAGE gels (SOD, APX, CAT and GR) were analysed after image acquisition, using the Spot Denso tool (AlphaEase FC imaging software V4, Alpha 88 Innotech Corporation). The enzymatic activity (for the respective antioxidant enzymes) of each isoform in the treatments was given a score of an average of the relative pixel intensities. This was achieved by allocating a value of 1 for the isoform with the least pixel intensity in that type of isoform

and expressing the rest of the pixel intensities for that type of isoform in the other treatments relative to this isoforms.

2.18 Statistical analysis

The data obtained in this study was analysed using one–way analysis of variance (ANOVA) test and validated for significant differences by the Tukey-Kramer test at 95% confidence interval. All measurements represent the means and standard errors (± SE) of three replicates.

CHAPTER 3

PEG- INDUCED WATER STRESS ALTERS THE PHYSIOLOGICAL RESPONSES OF CHIA PLANTS

3.1 Introduction

Chia (*Salvia hispanica* L.) is a member of the mint family and represents one of a group of superfood crops that fall under the class of pseudocereals (Ali *et al*., 2012). Combined with its medicinal and nutritional benefits, chia is an ideal candidate for more concerned crop improvement and ultimately food security. The physiochemical properties of chia associated with its high nutritional composition have been extensively studied. On the other hand, the mechanisms involved in its ability to cope with any abiotic stress remains stagnant. Moreover, chia has been deemed to be drought tolerant, owning to the fact that it can grow optimally in arid soils. This is because it is cultivated in central and southern Mexico and thus it can be used as an alternative food crop (Ayezra and Coates, 2009; Ali *et al*., 2012). However, studies on chia are limited to the phytochemical properties of the plant. Elucidating chia to water stress conditions is important in understanding the complex mechanisms of response at various degrees of water stress.

Water availability is an important aspect for progressive biochemical and physiological activities in all forms of life. Water deficit also referred to as

drought or osmotic stress is one of the most detrimental abiotic stress factors that limit crop production globally (Ngara, 2009). Osmotic stress causes plants to lose water to the external environment and result in a decrease in turgid pressure (Shao *et al*., 2007). As a consequence, decrease in turgor pressure affects the cellular processes such as cell expansion and division (Xiong and Zhu, 2002). Water stress can be mimicked by adding osmotic polymers such as polyethylene glycol (PEG). Polyethylene glycol has been used in a number of studies to impose water stress on plants due to their properties. The effect of PEG has been shown to lead to negative effects on plant physiology and hormone balance.

When plants are exposed to water stress conditions, they respond using a combination of morphological, physiological and molecular mechanisms (Xiong and Zhu, 2002; Altman, 2003; Pathan *et al*., 2007). Germination of seeds is one of the most fundamentally crucial processes that need to occur in order for plant growth and production to proceed. Seed germination and seedling growth has been found to be negatively affected by water deficit in many studies (Davidson and Chevalier, 1987; Kiem and Krostad, 1981; Owen, 1972; Passioura, 1988). Germination of seeds can be determined by inherent characteristics and outer surroundings. One the most important factors limiting seed germination are environmental stresses and the most detrimental of them is the lack of water or water deficit. This has become more apparent in recent years more especially in Africa due to increasing drought. Water deficit is known to limit germination of seeds and thus, there

is a need to look at alternative food crops that can adapt to arid and semi-arid climates like chia and to study their tolerance mechanisms to increase crop productivity.

In this frame, the effects of reduced PEG 8000 water potential of seed germination and morphology will be evaluated. This chapter will also go on to demonstrate the effects of PEG-induced water stress on the physiological parameters of chia.

3.2 Results

3.2.1 Water stress influences the rate of germination

Chia seeds were germinated under different PEG 8000 concentrations (5%, 10% and 20%) for a period of 7 days as described in section 2.2. The number of seeds germinated for each treatment was counted every day for 1 week and the results collated (Figure 3.2A). Approximately 80% of seeds were germinated in both control and the 5% treatment after day one. The results showed that with increasing PEG concentrations, there was a decrease in water potential that resulted in a decrease in seed germination (Figure 3.1). The decrease in water potential significantly reduced seed germination. Based on the results presented here, there seems to be a direct correlation between decreased water potential and the rate of seed germination. This demonstrates that with increased PEG concentrations, seed germination and

the rate at which seed germinate was significantly reduced (Figure 3.2A-B). Under low water potential (at -0.5 MPa; 20%), the rate of germination was reduced by ± 80%, whereas this reduction was less severe (± 22%) under moderately low water potential (at -0.1 MPa; 10%) when compared to the control. Furthermore, no significant reduction in germination rate was observed in the 5% treatment. A similar trend was observed for root radical emergence under different treatment conditions (Figure 3.2C).

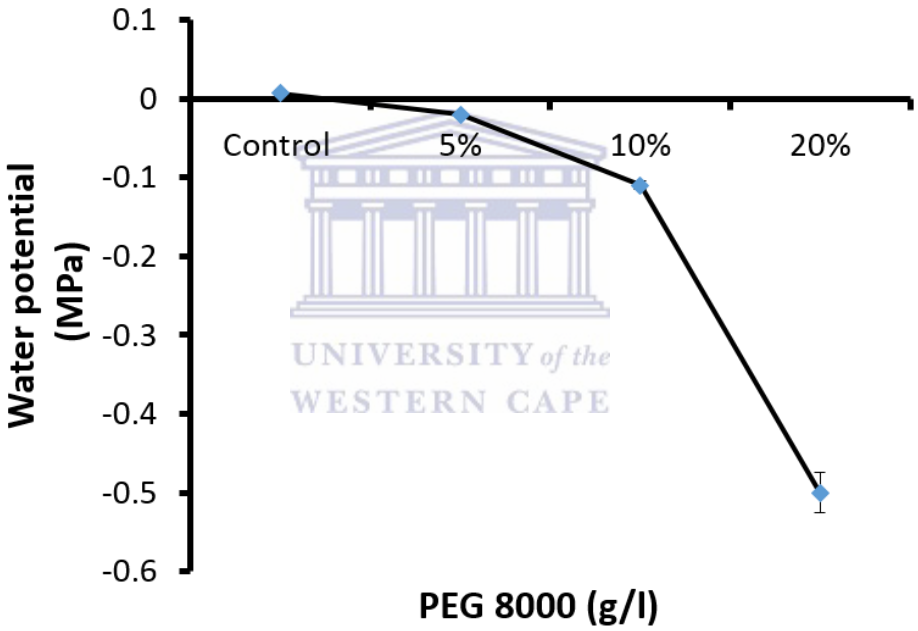

Figure 3.1: Determination of water potential of different PEG 8000 treatments.

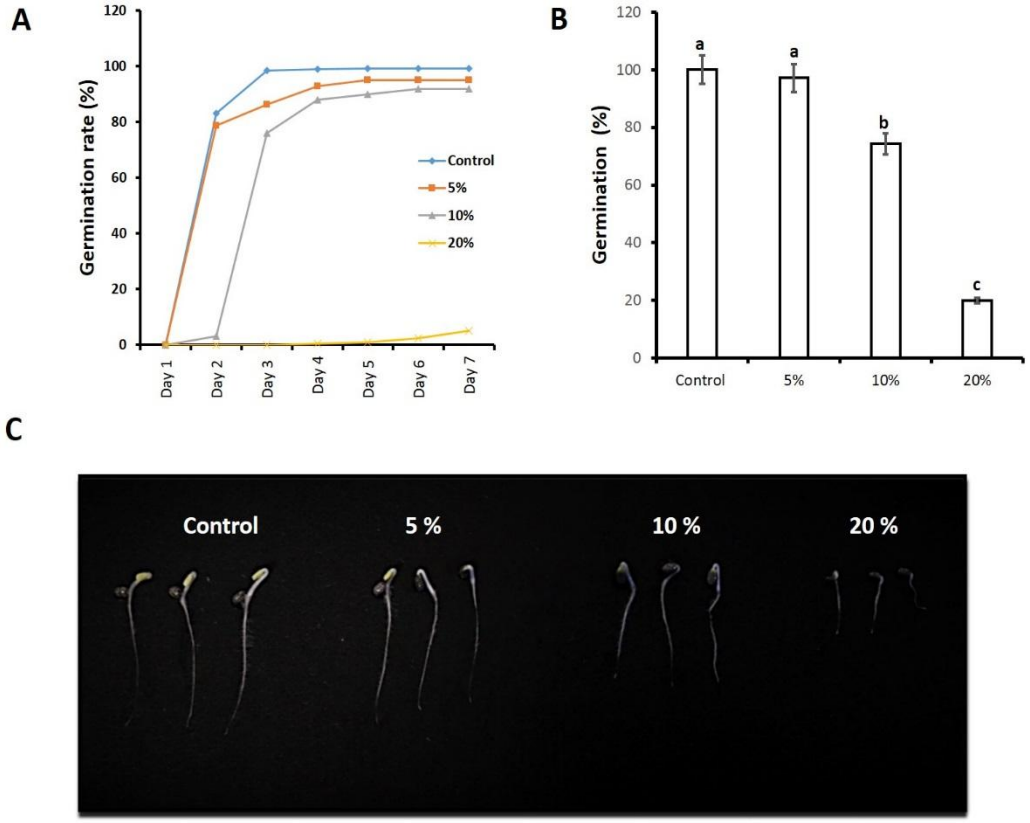

Figure 3.2: The influence of PEG-induced water stress on the rate of seed germination. Seeds were germinated on sterilized filter paper pre-treated with different concentrations of PEG 8000. Control seeds were germinated on filter paper moist with distilled water. Once the radical of the seeds had appeared after 7 days of germination, the percentage was statistically calculated. **(A)** Percentage of germinated seeds each day, **(B)** Total germinated seeds at day 7, (**C)** Morphology of germinated seeds at day 7. The error bars are representative of the mean (±SE) of three independent experiments from 100 seeds treatment in each experiment. The same letters (a) above the error bars indicated that there was no significant difference between means ($p < 0.05$).

3.2.2 The effect of PEG-induced water stress on the growth parameters of chia plants

Growth is known to be one of the most water stress sensitive physiological processes due to the reduction of turgor pressure. Therefore, water stress greatly limits the cell expansion as well as the cell growth due to low turgor pressure. This result shows that at higher concentrations of PEG 8000 there

was a significant reduction in plant growth and development (Figure 3.3 A and B).

Interestingly, no significant reduction in shoots length was observed for 5% and 10% PEG treatments, while on the other hand, shoots length for 20% PEG treatment showed a slight reduction when they were compared to the control. While roots length analysis (Figure 3.3; B) revealed that there was a slightly decrease in the 5% of ± 6%. An additional reduction was found in 10% PEG treated plants at ± 16% and 20% PEG treated plants showed a reduction of ± 16%, in comparison to their control. The trend in these results can also be correlated to the osmotic potential of the PEG 8000 solutions (Figure 3.1), where a decrease in the osmotic potential conferred a decrease in the germination rate and poor radical formation (Figure 3.2 A-C). Therefore, decrease in the osmotic potential of increasing PEG 8000 solutions also conferred a decrease in the shoot and root biomass as well as the shoot and root length.

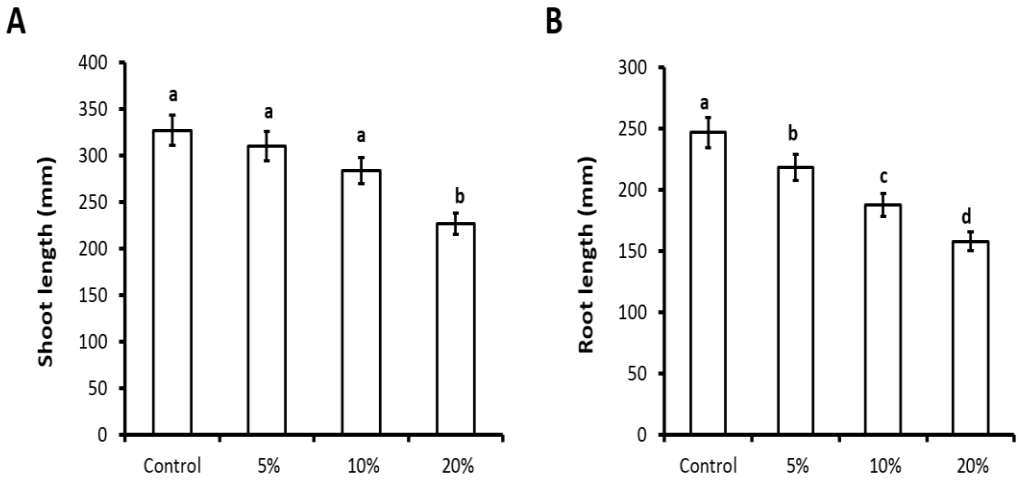

Figure 3.3: The influence of PEG-induced water stress on the growth parameters of chia plants. Plant growth parameters include: (**A)** Shoot length and **(B)** Root length at the end of the treatment period. The error bars are representative of the mean (±SE) of three independent experiments from five plants per treatment in each experiment. Means with different letters are statistically different from each other ($p < 0.05$).

3.2.3 The influence of PEG-induced water stress on chia biomass

The application of water stress induced by increasing concentrations PEG 8000 decreased shoot biomass (Figure 3.4 A and B). In response to treatment with 20% PEG, shoot fresh weight was reduced by ± 106% whereas the reduction in the 10% treatment was less severe (± 45%) (Figure 3.4 A). The least reduction in shoot fresh weight was observed in the 5% PEG treatment compared to the control plants. Treatment with 5% reduced shoots dry weight by ± 36%, when compared to the control. A similar phenomenon trend was also observed for shoot dry weight where treatment with 5%, 10% and 20% PEG treatment reduced the shoot dry weight by ± 27%, ± 57% and± 60%, respectively to the control (Figure 3.4 B).

The same phenomenon was observed in the root fresh and dry weights (Figure 3.4 C and D). The root fresh weight was reduced by ± 35% for the 5% water stress treated plants. There was a higher reduction of ± 52% and ± 67% for the ± 10% and 20% PEG 8000 treated chia plants, respectively when compared to the untreated chia plants. In addition, roots dry weights expressed a similar trend to the shoot dry weights presenting a ± 15%, ± 63% and ± 70% (5%, 10% and 20% PEG treatments) reduction.

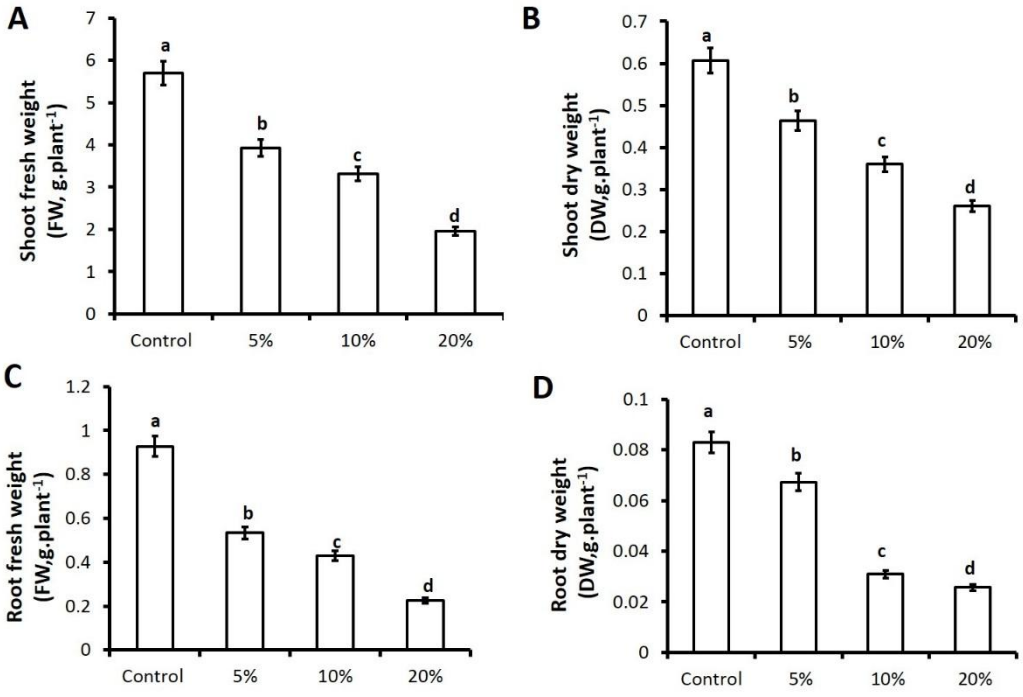

Figure 3.4: The effect of PEG-induced water stress on the shoot biomass of chia plants. Plant biomass is represented by **(A)** shoot fresh weight, **(B)** shoot dry weight, **(C)** root fresh weight and **(D)** root dry weight. Error bars are representative of the mean (±SE) of three independent experiments from five plants per treatment in each experiment. Means with different letters are statistically different from each other ($p < 0.05$).

3.2.4 PEG-induced water stress alters photosynthetic pigments in chia plants

Chlorophyll is an important photoreceptor and is involved in photosynthesis. The results showed that plants treated with 5% PEG slightly reduced (± 7.2%) total chlorophyll content. However, a more significant decrease in total chlorophyll content was observed with an increase in PEG concentrations. The reduction in chlorophyll content observed in both 10% and 20% treatments was approximately 22% when compared to the control plants. Chlorophyll a and b results showed a similar pattern of results, with the highest decrease found in the 10% and 20% treated plants. Interestingly, there were no subsequent differences found between the 10% and 20% treated plants across all the photosynthetic pigments.

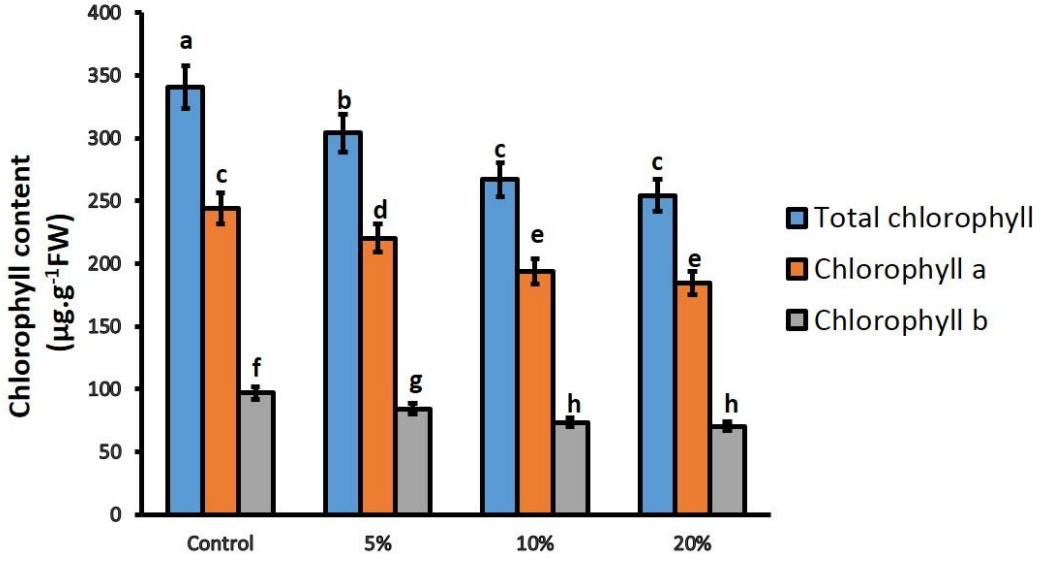

Figure 3.5: Influence of PEG-induced water stress on the chlorophyll content of chia plants. Plants were exposed to different concentrations of PEG 8000 (5%, 10% and 20% every second day) for a period of 14 days. The total chlorophyll content was measured in chia leaf tissue. The error bars are representative of the mean (±SE) of three independent experiments from five plants per treatment in each experiment. The same letters above the error bars indicated that there was no significant difference between means ($p < 0.05$).

3.3 Discussion

The challenges associated with abiotic stress on plant growth are evident among the emerging impact of climatic changes (Bellard *et al.*, 2012). The constraints to crop production are exacerbated with increasing population competing for natural resources (Wallace *et al.*, 2003). Nevertheless, molecular studies on plant responses to abiotic stresses have indicated that drought imposes water stress which can be induced by compounds such as sorbitol and polyethylene glycol (PEG) during (Ngara, 2012). This consequently, helps in understanding the way that drought stress affects

plants. This chapter investigated the influence of PEG-induced water stress on seed germination, growth and biomass production of chia plants.

3.3.1 Increasing PEG concentrations decrease germination rate

Water stress is a multifaceted stress that poses detrimental effects on yield limitations depending on the plant growth stage, duration and severity of the stress (El-Kablawy and AL-Rawai, 2005). Germination is known to be the most crucial and sensitive stage in the life cycle of plants more importantly in arid and semi-arid environments (El-Kablawy and AL-Rawai, 2005; Ahmad *et al*., 2009). Seed germination can be affected by both seed genetic characteristics (such as dormancy and seed coat thickness) and also by environmental conditions (Luzuriaga *et al*., 2006; Finch-Savage and Leubner-Metzger, 2006). Seeds that are exposed to unfavourable conditions such as water stress may compromise the subsequent radical formation and seedling. Water potential is known to be one of the most important external factors affecting seed germination and percentage (Alvarado and Bradford 2002; Norsworthy ad Oliveira 2006).

The results presented in this chapter highlighted drastic reduction in seed radical formation germinated in higher PEG concentration (10% and 20%) (Figure 3.2 C). A similar trend was observed when evaluating the rate at which seeds germinated under the different treatment conditions (Figure 3.2 B). This reduction observed here can be correlated to a study by Kaufmann

and Eckard (2008) that showed a decrease in water potential decrease the germination percentage of *Pinus contorta* and *Picea engelmaii* seeds.

This outcome was further supported by Muscolo *et al.* (2013), who illustrated that high concentrations of PEG reduced the final germination percentages in four lentil genotypes (Eston, Castellucio, Ustica and Pantelleria). We suggest that the reduction in seed germination could be as a consequence of a reduction in water potential caused by PEG. A study by Toscano *et al*. (2017), showed that ornamental sunflower seeds imbibed in low water potentials of PEG delayed the rate of germination, which is in support of our study.

3.3.2 Impact of PEG- induced water stress on the physiological responses of chia

Changes in plant morphology is most likely one of the first indicators when plants experience stress conditions (Ahuja *et al*., 2005; Yadav *et al*., 2005). During water stress conditions, plants' may exhibit visual alterations including leaf rolling, stunted shoot and root development and yellow colour progression on leaves (Cardoza and Steward, 2006). The extent of morphological damage which is cause by the stress can thus be determined by measuring parameters such as shoot and root growth, as well as shoot and root biomass (Ahuja *et al*., 2005).

Water stress in plants has been found to stunt plant growth which was found in this study, where the application of PEG was found to have adverse reduction in shoot and root length (Reddy *et al*., 2003; Ullah *et al*., 2017). The same phenomenon was presented in this study, where high concentration of PEG (10% and 20%) reduced shoot and root length (Figure 3.3). This phenomenon has not been demonstrated in previous studies of chia however, Saleh *et al*. (2015) showed a decrease in *Oryza sativa* growth parameters when treated with 30% PEG. The influence of the PEG osmotic solution was found to have negative effects on the plant biomass production whereby results showed that with the increasing PEG concentration there was significant decrease in the plant biomass as compared to that of the control (Figure 3.4). This impact has been well presented in other previous studies under stress conditions by Murthy *et al*. (2010) and more recently in study by Suis *et al*. (2015).

3.3.3 Reduction in chlorophyll content with an increase in PEG-induced water stress

High chlorophyll content is a desirable trait because it indicates a low degree of photo-inhibition of photosynthetic apparatus, thereby reducing carbohydrate losses for plant growth (Farquhar *et al.,* 1989; Saud *et al*., 2010). According to Chaves *et al*. (2011), water stress conditions caused a reduction in the chlorophyll content. One of the primary characteristics of PEG is its ability to reduce the water potential and thus leading into

progressive reduction in photosynthetic systems (Kaur and Gupta, 2005; Singh *et al.,* 2014; Shivakrishna *et al.,* 2017).

Chlorophyll a and b fluorescence, though corresponding to a very small fraction of dissipated energy from the photosynthetic apparatus, is generally accepted as providing access to the understanding of its function (Genty *et al*., 1989; Lotfi *et al*., 2015). The results presented in this study showed significant decrease in chlorophyll a, chlorophyll b levels which subsequently led to a significant reduction in the total chlorophyll content, with the most significant reduction observed in plants treated with 20% PEG 8000. This reduction could be ascribed to an increase in chlorophyll degradation as seen for the reduction in the levels of chlorophyll a and b (Figure 3.6). The reduction in green chlorophyll pigments and total chlorophyll content have been reported in various plant species including sunflower (Arfan *et al*., 2007), castor bean Pinheiro *et al*., 2008) and *Thymus citriodorus* (Suis *et al*., 2008) in response to water stress induced by PEG. A study done by Ashraf and Harris (2013), suggests that during the early stages of drought stress closure of the stomata is stimulated, thereby inhibiting the entry of carbon dioxide and consequently leading to the accumulation of oxygen molecules.

CHAPTER 4

WATER STRESS MODULATES THE ACCUMULATION ROS AND ALTERS ANTIOXIDANT CAPACITY IN CHIA

4.1 Introduction

Chia is an oleagonios native to the region extending from west-central Mexico to northern Gautemala. This plant stands out for its adaptations to regions of tropical and subtropical climates (Capitani *et al*., 2012). Recently, *S. hispanica* has become an important source of nutrition for humans, providing health benefits such as reduction of cardiovascular diseases, bowel regulation, cholesterol, obesity and prevention of type 2 diabetes (Jin *et al*., 2012, Poudyal *et al*., 2012). The seeds of chia have been under much investigation due to their high levels of antioxidants, protein, dietary fibre and most importantly their oil quantity and quality (Ixtaina *et al*., 2011).

Chia has been extensively studied with regards to its medicinal benefits, nutritional composition and/or phytochemical properties. However, regarding agronomical aspects, experimental data is still very scant especially in the scope of plant physiology and biochemistry in response to abiotic stress conditions of chia. During plant growth and development, there are intrinsic factors (phenotypic, genotypic) and extrinsic factors (light, temperature, drought, excess water, salinity, heavy metals) that influence

how the plant will adapt to survive to that environment (Zhang *et al*., 2010; Obidiegwu *et al*., 2015). When plants experience constant exposure to such harsh environments there is an overproduction of reactive oxygen species (ROS). ROS can have detrimental effects on the physiological and biochemical state of the plant and under severe conditions; it causes oxidative stress, DNA and protein damage and ultimate cell death (Birben *et al*., 2012; Ahamd *et al*., 2017). Plants have enzymatic and non-enzymatic antioxidant mechanisms, which are able to detoxify these toxic ROS molecules, attempting to bring the homeostasis of the plant back in balance (Most and Papenbrock, 2015). Some of these antioxidant enzymes include: superoxide dismutase (SOD), acscorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR).

This chapter will focus on the influence of PEG-induced water stress on the biochemical responses of chia plants by monitoring changes in ROS accumulation, the extent of lipid peroxidation (manifested as cellular death) and antioxidant capacity.

4.2 Results

4.2.1 Stress-induced ROS accumulation

Chia plants were grown and treated as described in section 2.3. The effect of increasing PEG concentrations on ROS accumulation (superoxide and hydrogen peroxide) in the leaf tissue of chia plants was investigated.

The superoxide radical was the first ROS molecule evaluated in this study. The results showed that with increasing PEG 8000 concentrations and subsequent decrease in water potential, an increase in superoxide content was observed (Figure 4.1 A). For plants treated with 5% PEG, the superoxide content in chia leaves was measured at ± 45% with ± 52% observed in the 10% PEG treatment. However, the highest increase in superoxide content was observed in the 20% PEG treatment (± 80%). The outcome presented here suggest that with increased PEG concentration there was a noticeable increase in superoxide content in chia leaves. The scavenging of superoxide molecules (via superoxide dismutase) results in the accumulation of H_2O_2 molecules which is detrimental to plant growth and development if not controlled. However, H_2O_2 can also serves as a signalling molecule for various enzymes under optimal conditions.

For H_2O_2 analysis in this study, the results showed that high concentrations (20%) of PEG 8000 augment H_2O_2 content in chia leaves whereas the lower PEG concentrations (5% and 10%) does not significantly affect H_2O_2 content relative to the control (Figure 4.1 B). The most notable increase in H_2O_2

content was observed in the leaves of chia plants treated with 20% PEG. This increase stood at approximately 35% when compared to the H_2O_2 content of the control plants (Figure 4.1 B).

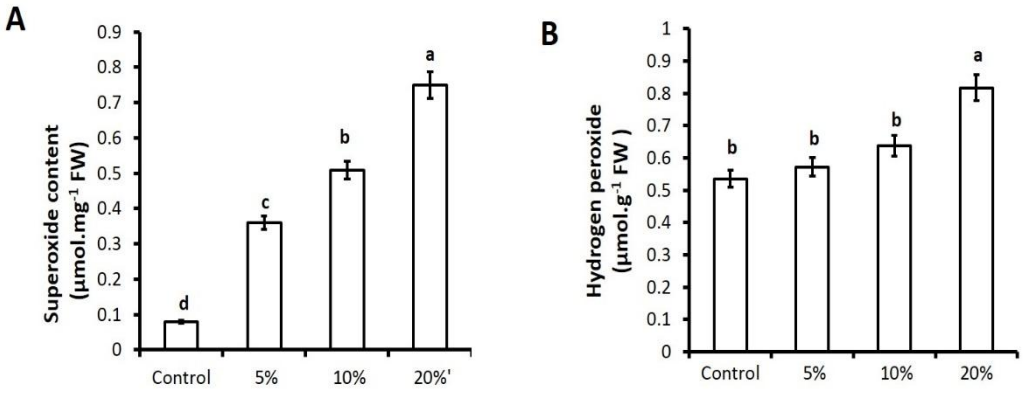

Figure 4.1: Changes in ROS accumulation regulated by different concentration s of PEG 8000. Measurement of **(A)** superoxide levels and **(B)** hydrogen peroxide content in leaf samples of chia plants treated with different PEG 8000 concentrations (0%, 5%, 10% and 20 %) for a period of 14 days. The error bars are representative of the mean (±SE) of three independent experiments from chia leaves. Bars with different letters signify that they are statistically different ($p < 0.05$).

4.2.1 PEG-induced water stress enhanced oxidative stress in chia plants

Malondialdehyde (MDA) is a useful indicator of oxidative damage to lipids, as a result of membranes susceptibility to reactive oxygen species (ROS). This is an imperative indicator for the level of stress induced and thus effects on plant cells as well as tissues, under stress conditions. The results showed a significant increase (± 47%) in MDA detected in the 20% PEG treatment as compared to the controls (Figure 4.2 A). No significant changes in MDA levels were observed in the leaves of plants treatment with in the 5% and 10% PEG

8000 respectively (Figure 4.2 A). Although there was an increase in the levels of MDA in the 5% and 10% treatments as compared to the control, the results of these treatments are statistically similar. In order to verify whether the increase in membrane leakage and the ultimate loss of membrane integrity due to lipid peroxidation results in cellular death, we determined the levels of cell death using Evans Blue assay.

Cell death occurs as a result of oxidative damage which can be characterized by cellular effects such as lipid peroxidation and nucleic acid damage (Foyer and Noctor, 2005). In our study on differential PEG treatment, we demonstrated a strong correlation between the reduction in shoot and roots dry weight biomass (Figure 3.3) and increased in level of cellular death (Figure 4.2 B). The results given presented in Figure 4.2 B indicated that an increase in all PEG concentrations (5%, 10% and 20%) resulted in an increase in levels of cell death with highest level of cell death observed in the 20% PEG treatment (± 60%). Cell death in the 5 % and 10 % PEG treatment was increased by ± 27% and ± 57%, respectively when compared to the control samples (Figure 4.2 B).

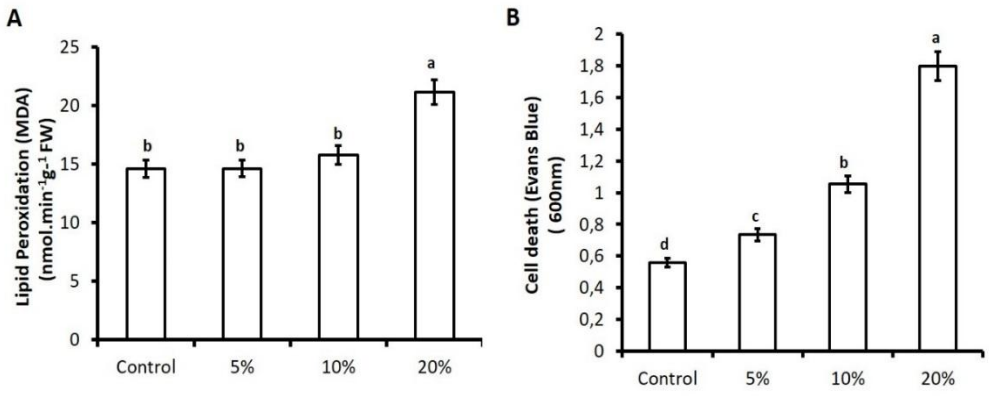

Figure 4.2: ROS-induced oxidative stress augments oxidative damage manifested as increased lipid peroxidation and cellular death. Different PEG 8000 concentration alters **(A)**: lipid peroxidation and **(B)**: cell death in leaf samples of chia plants. The error bars are representative of the mean (±SE) of three independent experiments from Chia leaves. Different letters represent statistical differences ($p < 0.05$).

4.2.2 PEG-induced water stress modulates antioxidant capacity in chia plants

4.2.2.1 Changes in superoxide dismutase activity

Superoxide dismutase (SOD) is the first defensive enzyme involved in the antioxidant process (Lee *et al.,* 2001). This antioxidative enzyme is involved in the conversion of the superoxide radical to hydrogen peroxide and oxygen (Libik- Konieczny *et al*., 2012). Total SOD activity was differential regulated by changes in PEG 8000 concentration (Figure 4.3). Chia plants treated with 5% PEG increased total SOD activity in leaf tissue by ± 33% when compared to control plants. Similarly, total SOD activity in the 10% treatment was increased by ± 68% compared to the control plants. Interestingly, total SOD

activity was reduced (± 14%) in the 20% treatment which was significantly lower than observed for both lower PEG concentrations (Figure 4.3).

Similarly to what was observed for MnSOD 1 and MnSOD 2, the enzymatic activity for MnSOD 3 was differentially regulated in the PEG treatments (Figure 4.4 A-B). The highest increases in SOD activity was detected in the 5% and 10% PEG treatments. No significant changes in SOD activity was detected in the 20% PEG treatment (Figure 4.4 A and B).

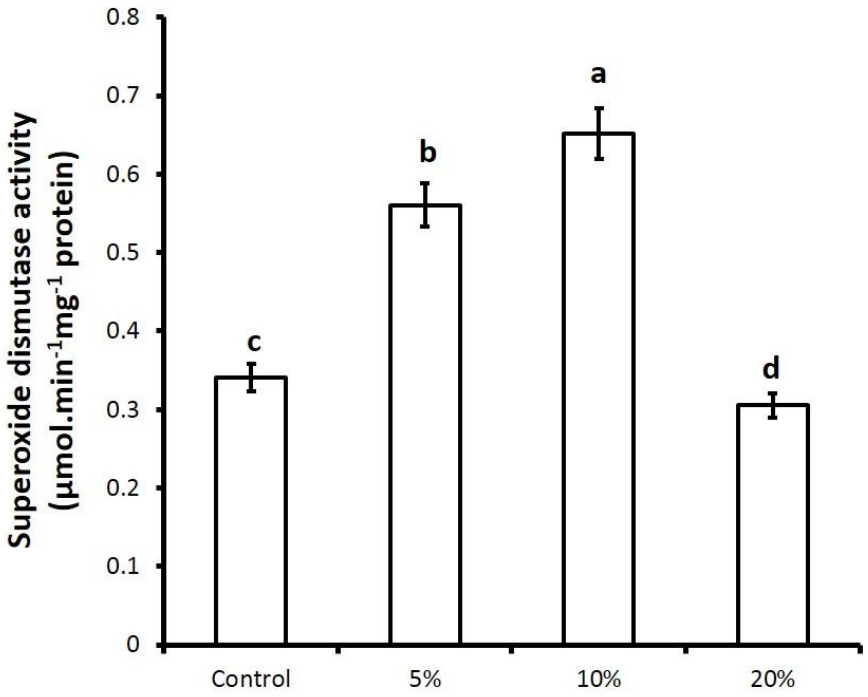

Figure 4.3: Spectrophotometric determination of the total SOD activity in leaves of chia plants. Total SOD activity was differentially altered by PEG concentrations (5%, 10% and 20%). The error bars are representative of the mean (±SE) of three independent experiments from Chia leaves. Different letters on each bar indicate the statistically different means ($p < 0.05$).

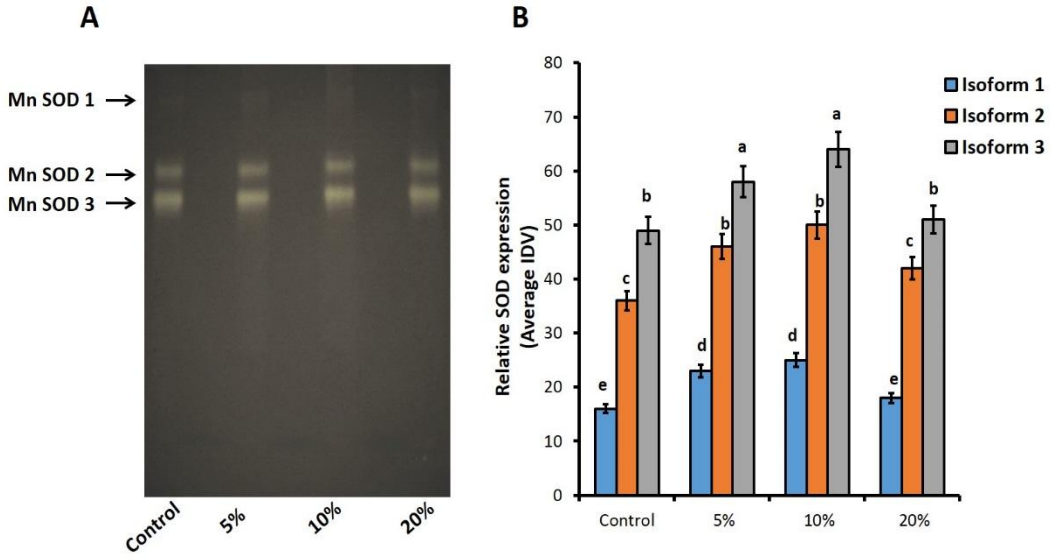

Figure 4.4: SOD isoforms are differentially regulated in response to different PEG treatments (5%, 10% and 20%). SOD activity was detected in chia leaf samples using in-gel activity assays (A) Chia plants were treated with PEG 8000 (5%, 10% and 20%) for a period of 14 days. Individual SOD isoforms were identified using differential staining (B) Relative SOD expression represented by the average IDV. The error bars are representative of the mean (±SE) of three independent experiments from Chia leaves. Different letters on bars indicate statistically different means ($p < 0.05$).

4.2.2.2 The influence of PEG-induced waster stress on ascorbate peroxidase activity

Ascorbate peroxidase (APX) is an important antioxidant enzyme that is highly responsive to abiotic stress conditions and plays a major role in the detoxification of H_2O_2 in plants. This section will focus on the impact of PEG-induced water stress on APX activity (total and individual isoforms). The results showed that total APX activity was increased in all PEG treatments compared to that of the control with the highest increase of ± 79% observed in the 10% PEG treatment (Figure 4.5). Although significant increases in APX

activity was observed in the 5 % and 20% PEG treatments, these increase were not as pronounced as observed in the 10% PEG treatment.

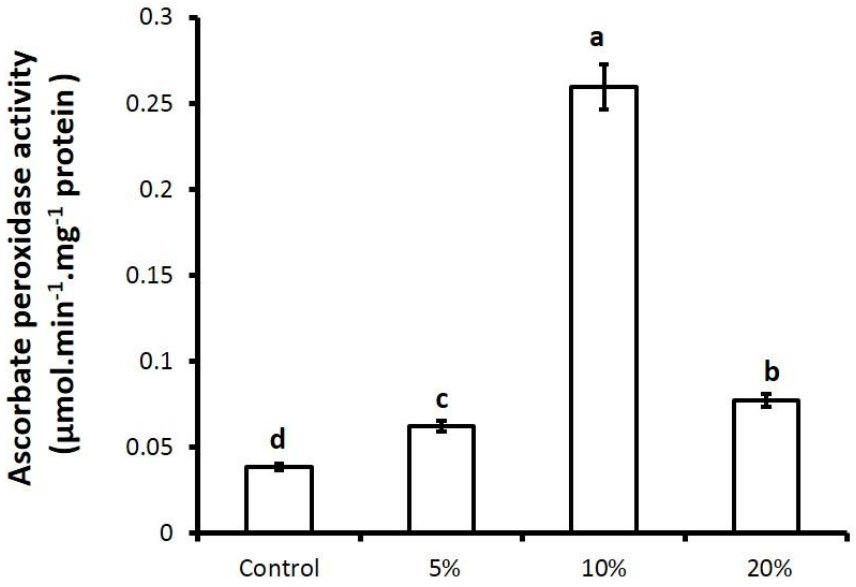

Figure 4.5: Total APX activity in chia leaves in response to PEG-induced water stress. Chia plants were treated with different concentrations of PEG 8000 (5%, 10%, 20%) for a period of 14 days treated every second day. The different letters show statistical differences between treatments ($p < 0.05$).

Ascorbate peroxidase isoforms were detected using native PAGE as described in section 2.16.2 Three APX isoforms (APX 1-3) were detected in this study and each isoform was differentially expressed by the different PEG treatments.

Densitometry analysis of APX 1 showed that the enzymatic activity was significantly increased with an increase in PEG concentration. In response to

treatment with 5% PEG, APX 1 activity was increased by ± 16% whereas 10% PEG increased APX 1 activity by ± 21% and 19% PEG presented an increase of ± 24% when compared to the control (Figure 4.6 A-B).

For APX 2, very low activity was detected in the control and the 5% PEG treated samples. APX 2 activities were detected in the 10% and 20% PEG treated samples. This suggests that APX 2 activity is PEG-induced (only at high concentrations). The activity detected for APX 3 is PEG-induced as all treatments increased the enzymatic activity of the isoform. Statistically there was no significant difference in APX activity amongst the different treatments for APX 3 (Figure 4.6 A-B).

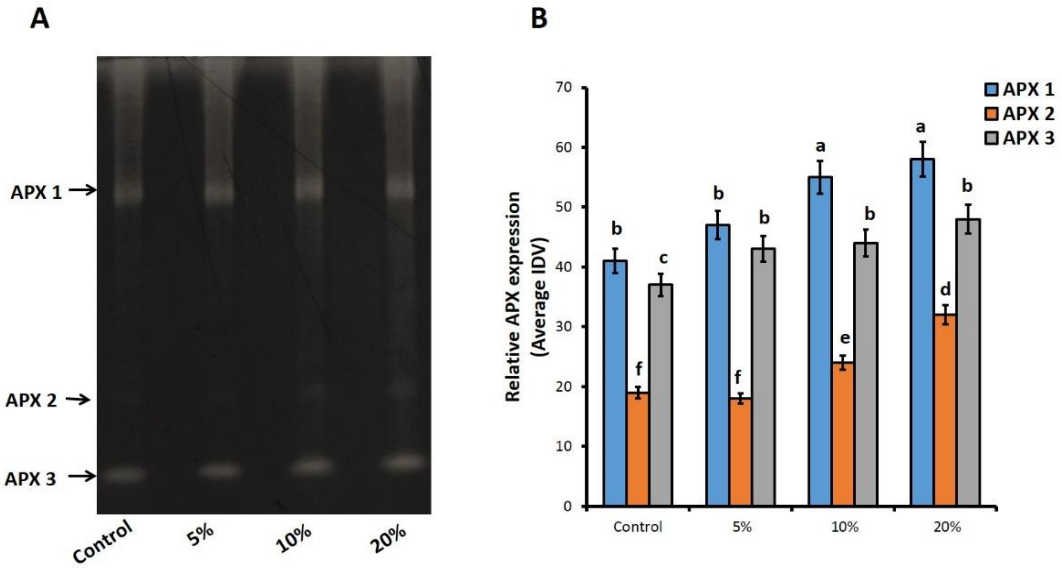

Figure 4.6: APX isoforms are differentially regulated in response to different PEG treatments (5%, 10% and 20%). APX activity was detected in chia leaf samples using in-gel activity assays Chia plants were treated with PEG 8000 (5%, 10% and 20%) for a period of 14 days. (**A)** Individual APX isoforms **(B)** relative APX expression. The error bars are representative of the mean (±SE) of three independent experiments from Chia leaves. Different letters on bars indicate statistically different means ($p < 0.05$).

4.2.2.3 Detection of catalase activity in chia plants exposed to PEG-induced water stress

CAT, like APX is an important catalyers of H_2O_2, specifically in the peroxisome and thus a typical reaction is the dismutation of two molecule of H_2O_2 to water and O_2 (Table 1.1) (Gill and Tuteja, 2010). This section will focus on the impact of PEG-induced water stress on CAT activity (total and individual isoforms).

PEG- induced water stress in chia plants showed an increase in CAT activity with increasing PEG concentrations. No significant changes were observed in the 5% PEG treated plants when compared to the control. An increase of ± 19% and ± 90% observed in chia plants supplemented with 10% and 20% PEG 8000 respectively.

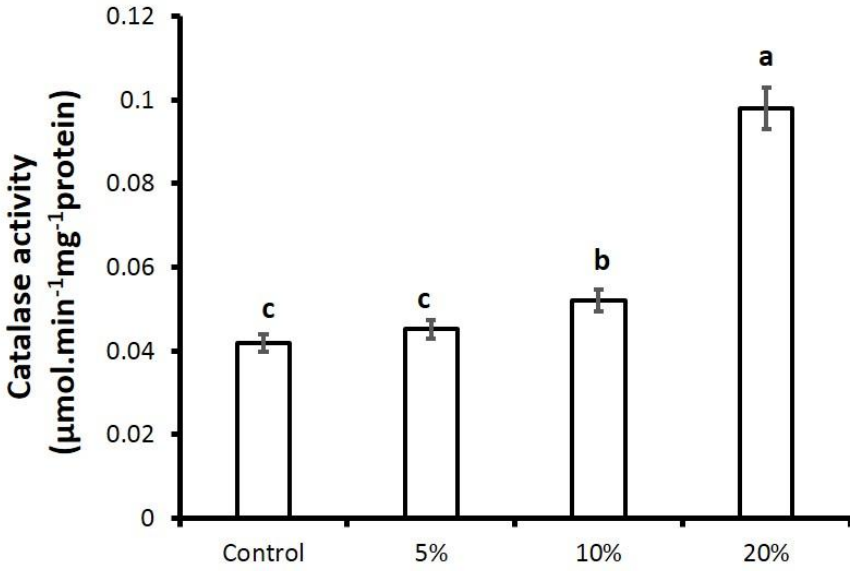

Figure 4.7: Total CAT activity in chia leaves in response to PEG-induced water stress. Chia plants were treated with different concentrations of PEG 8000 (5%, 10%, 20%) for a period of 14 days treated every second day. The different letters show statistical differences between treatments ($p < 0.05$).

In total two CAT isoforms (CAT 1-2) were detected and the activity of these isoforms was regulated by the different concentrations of PEG 8000. For CAT 1, both treatments with 5% and 10% PEG did not alter enzymatic activity (Figure 4.8). However, treatment with 20% PEG increased CAT 1 activity by ± 79% when compare to the control and lower PEG treatments. This observation was supported by the densitometry analysis of CAT 1 activity (Figure 4.8 B).

For CAT 2, no or very little activity was detected in the control sample and the 5% PEG treatment. The results suggest that CAT 2 activity is PEG dependent (high concentration) with noticeable activity detected in the 10% PEG treatment but significantly higher activity detected in the 20% PEG

treatment (Figure 4.8 C). The enzymatic activity detected in the 20% treatment was measured at ± 77% using densitometry analysis.

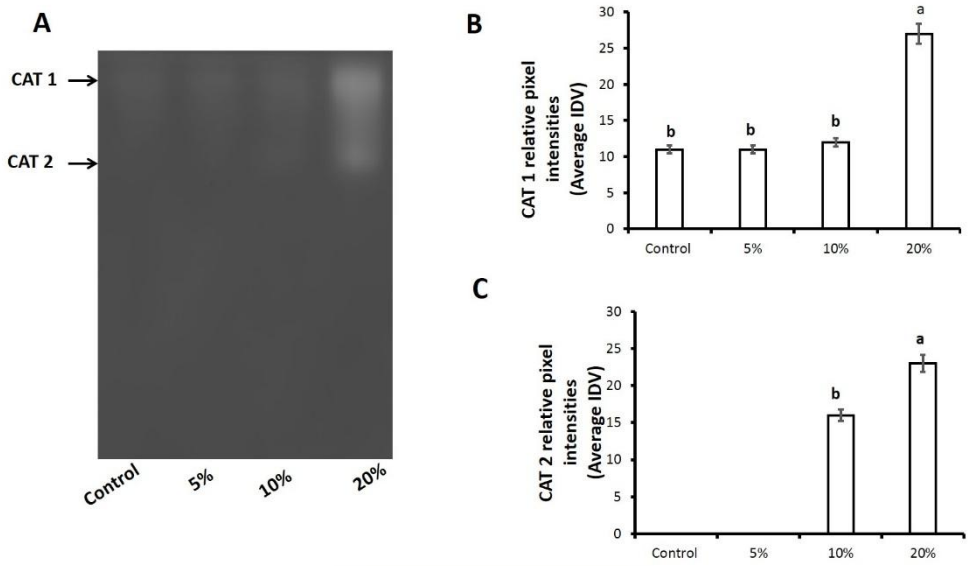

Figure 4.8: The effect of water stress on CAT activity. In-gel active assays were performed on chia leaf tissues treated for 14 days. The data represents the mean (± SE) of 3 independent experiments from 3 biological repeats in each experiment. Means with different letters are statistically different from each other ($p < 0.05$).

4.2.2.4 Measurement and detection of glutathione reductase (GR) activity in chia leaves

Glutathione reductase (GR), like APX and CAT is important in detoxifying GR to maintain the redox state of ascorbate and glutathione. GR catalyses the NADPH dependent reaction –SH of GSSG, which is important in maintaining the GSH and thus control the redox state of plant cells. The results of this study showed that there was an a significant increase in the GR activity of

Chia leaves in the 20% PEG 8000 treated, presenting an increase of ± 59% as compared to the control (Figure 4.9). In contrast, a reduction of ± 7% and ± 5% observed in 5% and 10% PEG treated plants, respectively when compared to the control.

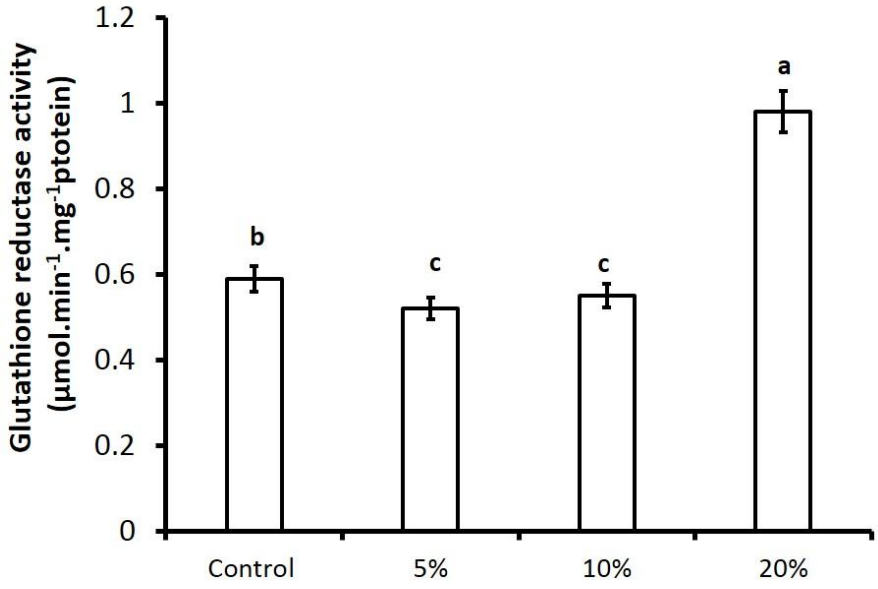

Figure 4.9: Glutathione reductase activity is altered in Chia leaves in response to water stress treatments. Chia plants were treated with different concentrations of PEG 8000 (5%, 10%, 20%) for a period of 14 days treated every second day. The different letters show statistical differences between treatments (p<0.05).

Three GR isoforms were detected (GR 1, GR 2 and GR 3) after specific staining described in section 2.16.4 (Figure 4.10 A). Densitometry analysis revealed that water stress differentially altered the GR activity across all treatments. For GR 1, as significant increase of ± 17% and ± 23% was observed in the 5% and 10% PEG 8000 treatment, respectively. A further

increase of ± 67% was observed in the 20% PEG 8000 treated chia plants (Figure 4.10 B).

For GR 2, no significant changes were detected in the 5% and 10% PEG-induced water stress plants (Figure 4.10 B). On the contrary, there was an increase of approximately 45% observed for the 20% PEG 8000 treated plants (Figure 4.10 B). A similar trend was observed for GR 3, where no changes were detected for the 5% and 10% PEG treated chia plants and an increase of approximately 49% observed for the 20% PEG 8000 treated plants (Figure 4.10 B).

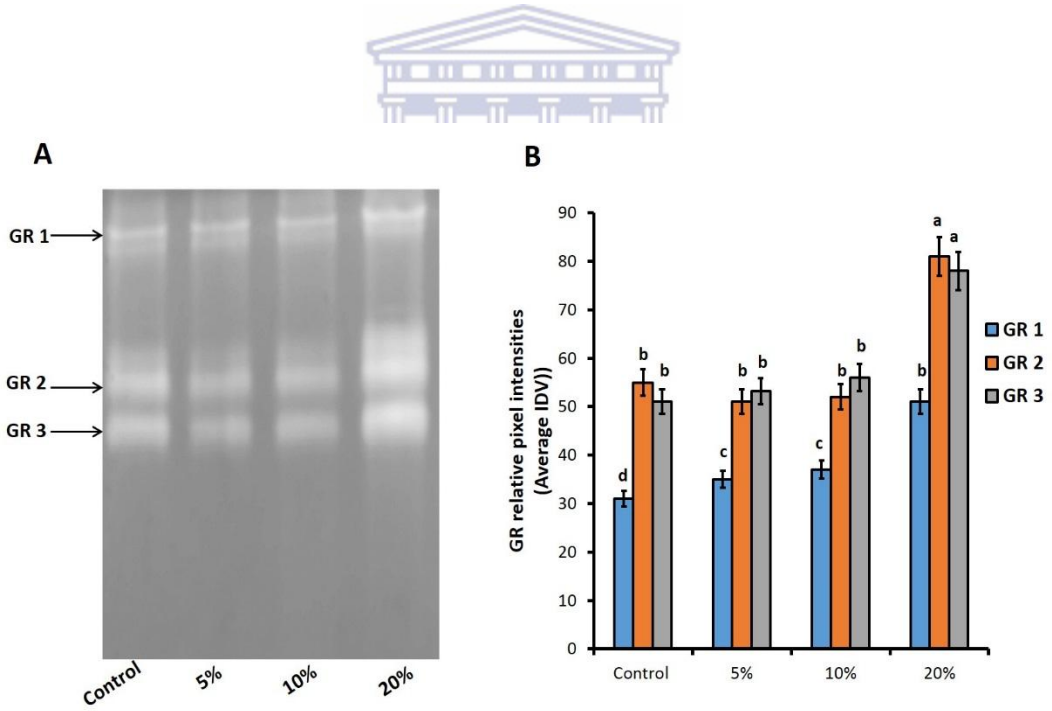

Figure 4.10: The effect of water stress on GR activity in chia leaves. In-gel active assays were performed on chia leaf tissues treated for 14 days. **(A)** Individual GR isoforms **(B)** densitometry analysis of relative GR expression. The data represents the mean (± SE) of 3 independent experiments from 3 biological repeats in each experiment. Means with different letters are statistically different from each other ($p < 0.05$).

4.3 Discussion

The work presented in this chapter analysed the influence of PEG-induced water stress (imposed by different concentration of PEG 8000) on the biochemical responses of chia plants. We analysed changes in ROS accumulation under stress conditions and evaluated the antioxidant capacity of chia plants to mitigate the negative effects caused by these ROS molecules.

4.3.1 PEG- induced water stress influences the ROS accumulation and oxidative damage

Reactive oxygen species (ROS) production/accumulation has been documented as a natural occurring consequence of cellular metabolism (Halliwell, 2006; de Rio *et al*., 2006). ROS accumulation as a result of environmental stresses is one of the leading factors that limits crop production worldwide (Gill *et al*., 2010, Ahsan *et a*l., 2003). This study focused primarily on two ROS biomarker, namely superoxide and hydrogen peroxide and their role in chia plants under PEG-induced water stress.

The results presented in this chapter showed that with increased PEG concentrations there was an exponential increase in O_2^- content (Figure 4.1 A). This observation was also documented in a study by Kapoor *et al*. (2011) the authors demonstrated that accumulation of O_2^- was impacted by water stress in different rice cultivars. The scavenging of O_2^- by superoxide

dismutase results in the formation/production of hydrogen peroxide and if not controlled can lead to oxidative damage and ultimate cellular death manifested as reduced plant biomass as observed in chapter 3 (Figure 3.4).

Similar to what was observed for superoxide accumulation under stress treatments there was a significant increase in hydrogen peroxide accumulation (Figure 4.1 B), partly from the scavenging of superoxide anions but also via other mechanisms. However, under optimal conditions hydrogen peroxide can serve a signalling molecule to aid in plants' defence against abiotic stress at intermediate levels (Cheeseman *et al*., 2007). The results showed that under low and moderately high PEG concentrations (5% and 10%) hydrogen peroxide levels were statistically similar to that of the control (Figure 4.1 B). However, under high PEG concentrations, hydrogen peroxide levels were significantly enhanced which resulted in increased oxidative damage. This effect has been observed in various other studies (Hernandez *et al*., 2010; Smirnoff and Wheeler, 2002). An excessive accumulation of these ROS molecules can lead to detrimental effects on the plants like the peroxidation of lipids and ultimately cellular death in severe conditions.

The peroxidation of lipids is considered the most detrimental processes that are known to occur in living organisms (Garg and Manchanda, 2009). Literature suggests that lipid peroxidation is a precursor of ROS overproduction and accumulation, by peroxidation of membrane lipids (Ahuja *et al*., 2015). The increase in ROS accumulation resulted in enhanced

oxidative stress manifested as increased lipid peroxidation (Figure 4.2 A). The increase in lipid peroxidation may be attributed to the lack of antioxidant capacity to ROS resulting from stress conditions (Boughalleb *et al*., 2015). This phenomenon was supported by Mirzee *et al*. (2013), who showed that higher PEG concentrations increased lipid peroxidation (denoted as MDA content) in Canola plants. The excessive accumulation of ROS in plants causes damage to important cellular compartments that are involved in important metabolic functions required for plants survival, such as the chloroplast, pigments and nucleic acids. Damage to these important cellular components ultimately results in cell death (Figure 4.2 B).

Therefore, observed increase in cell death in response to water stress imposed by PEG 8000 (Figure 4.2 B), can be associated with the conferred increase in lipid peroxidation. In a study by Wang and colleagues (2010) gave an indication that the cell death observed in this study is associated with programmed cell death pathway. This increase in cell death may be associated with the increase in the accumulation of ROS which subsequently led to increase in lipid peroxidation (Wang *et al*., 2010). Increase in cell death means that most or all of the cellular compartments will not be functional and therefore certain important metabolic processes will not take place, one of the most important for plant survival being photosynthesis which was discussed in chapter 3 (Figure 3.5). Plants do however have mechanisms to cope with the detrimental effects of oxidative stress when under extreme stress conditions. Plants have developed antioxidant defence systems, which

include antioxidant enzymes such as SOD, APX, CAT and GR (Wang *et al*., 2010).

4.3.2 Water stress modulates antioxidant enzymatic activity in chia plants

In this study a clear link has been established between the decreases in plant biomass as well as excessive accumulation of ROS molecules which results in lipid peroxidation and ultimately cellular death. Therefore, an assessment of the antioxidant defence system was evaluated. The antioxidant system of chia was differentially regulated in response to water stress induced by the different PEG treatments. In this study, measurements of the enzymatic activities (total enzymatic activity and the individual isoform intensities) of SOD, CAT, APX and GR were analysed.

4.3.2.1 SOD activity was differentially altered in response to PEG 8000

Changes in superoxide dismutase activity were monitored to establish the rate at which superoxide molecules are scavenged. Superoxide dismutase (SOD) is responsible for the dismutation of the highly toxic O_2^- anion to less toxic form H_2O_2 (Gill and Tuteja, 2010). Superoxide dismutase serves as the first defence against uncontrolled oxidation during unfavourable conditions.

As reported by Cruz de Carvalho (2008), SOD includes the conversion of singlet oxygen molecules which are highly reactive into more stable hydrogen peroxide.

In this present study, SOD activity was increased by both 5% and 10% PEG 8000 treatments with a significant decrease observed in the 20% treatment (Figure 4.3). The decrease in the SOD activity observed in chia might be indicative of the overproduction of ROS that inactivated SOD proteins as reported for other plant species (Suis *et al*., 2015). However, the increase in SOD activity observed in the lower PEG concentrations (5% and 10%) (Figure 4.3) could be a direct consequence of the superoxide accumulation. Thereby, supporting the notion that the excessive increase in the superoxide (Figure 4.1 A) lead to the inactivation of the SOD proteins and thus the decrease in activity as observed in the 20% treatment (Figure 4.3).

Subsequently, chia leaves were subjected to native PAGE analysis in order to identify potential isoforms contributing to the total SOD activity (Figure 4.4). Three MnSOD isoforms were identified in the samples supplemented with different PEG concentrations (5%, 10% and 20%). The presence of enhanced expression of MnSOD isoforms in 5% and 10% PEG 8000 treated plants indicates that at low to moderated levels of water stress chia has better scavenging capacity for O_2^-. Moreover, it can be suggested from the results that the increased expression of MnSOD 3 could be contributed to the higher increased levels of the total SOD activity in the 10% PEG treated plants. This

further suggests that MnSOD 3 could be considered as potential candidate for water stress tolerance, due to the enhanced activity of this isoform. The expression of all three MnSOD isoforms were reduced in 20% PEG treated chia leaves. This further correlated to the reduced total SOD activity and it can thus be implicated that at this PEG concentration there was a degradation of mitochondria and/or peroxisomes as MnSOD is found to be localised in these cellular compartments.

These findings and the fact that at 20% PEG chia plants accumulated higher levels of H_2O_2 lead to further the investigation of the antioxidant enzyme (ascorbate peroxidase) that has a high affinity for detoxifying H_2O_2.

4.3.2.2 Water stress modulates hydrogen peroxide scavenging antioxidant enzyme activity

APX activity is differentially altered by PEG-induced water stress in chia plants

Ascorbate peroxidase (APX) is described as one of the most important ROS-scavenging antioxidant enzymes, which are involved in plants defence mechanisms by detoxifying H_2O_2 to dehydroascorbate and H_2O using ascorbate (AsA) as an electric donor (Shigeoka *et al*., 2002; Caverzan *et al*., 2016). The importance of APX is not restricted to one cellular organelle; it plays a role in ROS scavenging in the chloroplast, cytosol, mitochondria and peroxisomes (Asada, 1992; Noctor and Foyer, 1998; Mittler *et al*., 2004). The

role of APX 1 has been widely studied in plants and most results have demonstrated that, in response to environmental stress. According to Shigeoka *et al*. (2002) APX activity generally increases in response to drought stress, this increase was also documented in this report across all treatments with 10% PEG treated plants having the highest increase (Figure 4.5).

In parallel with the results obtained for the total APX activity, three APX isoforms where detected using native PAGE (Figure 4.6 A). The three APX isoforms that were identified in chia (APX 1, APX 2 and APX 3) were not further classified due to limited sequence information available in the public domain for chia. Interestingly, a fourth APX isoform was found in the 10% PEG treated samples (Figure 4.6 A). The presence of the additional fourth APX isoform in 10% PEG treated chia plants can be correlated to enhanced APX total activity (Figure 4.5). It can be further stipulated that a moderate levels of water stress, chia shows enhanced mechanisms of tolerance.

Furthermore, no significant changes were observed for 5% PEG treated plants in APX 1, however, there were slight increases observed for APX 2 and APX 3 (Figure 4.6 B). These low changes can be interlinked to low levels of hydrogen peroxide that were detected (Figure 4.1 B). This is also further supported by the fact that at lower concentrations of PEG (5%), chia showed the least oxidative damage (indicated by ROS accumulation, lipid peroxidation and cell death). Moreover, although there were increases

across all APX isoforms in the 20% PEG treated chia plants, (which was also reflected in the total APX activity) this increase was inefficient to counter the increase of H_2O_2 and all other oxidative stress markers.

CAT activity is differentially altered by PEG- induced water stress in chia plants

Analysis of recent literature suggested that an increase in CAT activity is generally positively related to the degree of water deficit experienced by plants (Piheiro et al., 2010; Faize and Chaves, 2011). For CAT activity, no significant changes was observed when plants were treated with low to moderately high concentration of PEG 8000. However, a significant increase in CAT activity was observed in the high PEG 8000 (20%) treatment (Figure 4.7 A). This implicated that CAT has a higher affinity than APX for the removal of photorespiratory H_2O_2 produced in chia plants when subjected to water stress, especially under severe degrees of water stress (Sofo *et al*., 2015).

In contrast to APX isoforms, CAT isoforms are limited to removal of H_2O_2 in the peroxisomes by photorespiration (Noctor *et al*., 2000). Therefore, the results presented in this study illustrated the detection of two CAT isoforms. The densitometry analysis (Figure 4.8 B) showed that increases in CAT 1 activity were only detected in 20 % PEG treated chia leaves. This subsequent increase was supported by result by Kim *et al*., 2005 and Zhang *et al*., 2012.

Conversely, CAT 2 was only detected on 10% and 20% PEG treatments. This suggests that this isoform could serve as a CAT biomarker for chia plants experiencing water stress. This could further suggest that at lower concentrations of water stress (5%) chia limits the demand for active enzymatic CAT in the peroxisome given the reduction in ROS molecules present in the plant at this concentration (Figure 4.1).

4.3.2.3 *Glutathione reductase activity is altered by PEG-induced water stress in chia leaves*

Glutathione reductase (GR) is the last enzyme in the ascorbate-glutathione cycle and is also crucial for H_2O_2 detoxification in green leaves (Foyer and Harbinson, 1994). GR has a central role of maintaining the reduced glutathione (GSH) pool during stress conditions (Hossain *et al*., 2012). Therefore, it was important to evaluate the regulation of this enzyme in chia plants when treated with the varying concentrations of PEG 8000. The GR total was increased in 20% PEG treated chia leaves, which is in supported by Lai *et al*., (2007), the authors demonstrated an enhanced GR activity in leaves treated with 40% PEG. On the contrary, there were slight reductions in the 5% and 10% PEG treated plants respectively. This slight reduction has been suggested to occur at low to moderate levels of water stress imposed by PEG (Cruz de Carvalho, 2008). Furthermore, it is likely that the enhanced expression levels of SOD and APX could be directly linked to the levels ROS

molecules present in chia plants, thus the lower expression of GR at these concentrations.

This significant reduction in the 5% and 10% PEG treated plants was further correlated with detected three GR isoforms (Figure 4.10). Although there were enhanced levels of GR in the 20% treated plants, however the results suggest that this increase in the GR activity as insufficient for the regeneration of GSH in plants. Thus, the end result of insufficient GSH regeneration in plants is what led to the accumulation of ROS and the incredibly high cell death that was reported in the 20% treated plant (Figure 4.1 and 4.2).

CONCLUSION AND FUTURE REMARKS

Water deficit stresses manifested as water stress have detrimental effects on the human population globally. These effects pose more threats on the agricultural sector of developing countries especially in arid and semi-arid environments. Despite the well-studied effects of drought on cereals such as maize, there is yet to be any molecular work on chia that investigates this plant as an alternative food crop. Thus this work aims to form part of a long term vision that will have an impact on the food insecurity, malnutrition and poverty problems that Africa faces.

This work was described in two research chapters. Chapter three described the effects of increasing concentrations of water stress on chia germination rate, growth parameters, biomass production and on the photosynthetic metabolism. Increasing water stress induced by PEG 8000 concentrations conferred a reduction in the germination rate and morphology of seeds (Figure 3.2). Elevated concentrations of PEG 8000 (20 %) further reduced physiological parameters marker, presenting a reduction in plant growth (Figure 3.3), biomass (Figure 3.4) and increased photo-inhibition (Figure 3.5). This study showed correlations between the decrease osmotic potential of the PEG 8000 solutions (Figure 3.1) and the reduction in germination rate and morphology and the physiological parameters described. The reduction of physiological parameters at high PEG concentrations was also supported

by the accumulation of ROS and the alteration of the antioxidant enzyme capacity that was investigated in Chapter 4.

Chapter four analysed the biochemical responses of chia to PEG-induced water stress, by monitoring ROS metabolism and antioxidant capacity. The molecular responses of chia to water stress are yet to be described in literature and thus this study was the first to describe the effects of increasing PEG-induced stress on the biochemical responses of chia. Water stress significantly enhanced ROS biomarkers in chia leaves, a phenomenon that is well documented in literature (Figure 4.1 and 4.2). These results further implicated that deleterious effects observed in the plant physiological parameters were caused by the enhanced levels of ROS molecules. Overproduction of ROS is known to lead to oxidative damage and ultimately cell death. Hence, there was an increase in the lipid peroxidation which was correlated to the increase in the cell death (Figure 4.2 A-B) with the most significant increase in the 10% and 20% treated plants. However, plants have detoxification mechanisms and in this study we evaluated how the antioxidant enzymes (SOD, APX, CAT and GR) are regulated in chia plants induced with osmotic stress. The differential regulation of antioxidant enzyme activity (as seen for changes in individual isoforms) in this study suggest these enzyme (individual isoforms) can be potential biomarkers to improve water stress tolerance in chia plants.

Therefore, it can be concluded from this study that chia has some level of tolerance to water stress at low to moderate concentrations but shows signs of sensitivity at higher concentrations. However, further research is required to support this hypothesis. Therefore, a comparative analysis of the leaf and root proteome will be analysed using gel based proteomics. This will allow us to identify and functionally characterise differentially expressed proteins under various treatment conditions that could serves as potential biomarkers to enhance stress tolerance in chai plants. To note, the work presented in this thesis is the first of its kind that evaluated the influence of PEG-induced water stress on physiological and biochemical responses of chia plants.

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