Signalling molecule "*calcium*" improves germination and growth of *Sorghum bicolor* seedlings under salt stress

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ABSTRACT

Abiotic stress, mainly in the form of extreme temperatures, drought and salinity has caused major crop losses worldwide, putting a severe strain on agriculture. Salinity severely limits plant growth and productivity and affects all aspects of the plant's development including the most crucial stage; germination. This study investigated the effect of salt (NaCl) stress on *Sorghum bicolor* seedlings and the role of exogenously applied calcium (Ca^{2+}) to ameliorate the effects of salt stress during germination.

Sorghum seeds were germinated in the presence and absence of various NaCl (100, 200 and 300 mM) and Ca^{2+} (5, 15 and 35 mM) concentrations. Several assays including physiological (germination and growth assays), biochemical (osmolytes and oxidative stress markers), anatomical (epidermal and xylem layers) and expression profiles of key genes [antioxidant (*SbSOD*, *SbAPX2* and *SbCAT3*), Salt Overly Sensitive (*SbSOS1*, *2* and *3*) pathway enzymes and the *vacuolar* Na^+/H^+ *exchanger antiporter2* (*SbNHX2*)] were investigated.

Salt stress delayed germination and negatively affected growth as observed by the reduced root and shoot length and decreased fresh and dry weight. There was an increase in proline content and oxidative stress markers (H_2O_2 and MDA) under salt stress. Oxidative stress resulted in damage to the epidermal and xylem layers as observed on Scanning Electron Microscopy (SEM) images. Quantitative real-time polymerase chain reaction revealed that salt stress induced the expression of *SbAPX2*, *SbCAT3* and *SbSOS1* genes, whereas *SbSOD4A*, *SbSOS2, SbSOS3 and SbNHX2* genes were not affected by salt.

Exogenous application of Ca^{2+} counteracted the harmful effects of salt stress by improving germination efficiency, promoting seedling growth, reducing oxidative damage and the Na^+/K^+ ratio, indicating the protective effect. Ca^{2+} also effectively protected the epidermis and xylem layers from the severe damage caused by salt stress. In the presence of Ca^{2+} the expression of

SbAPX2 and *SbCAT3* was reduced except for the *SbNHX2* gene, which increased by 65-fold compared to the control. The results obtained suggests that sorghum is able to respond to salt stress by inducing osmolytes, the antioxidant defence system as well as the SOS pathway. Furthermore, 5 mM Ca^{2+} was determined as the optimum Ca^{2+} concentration required to enhance sorghum's tolerance to salt stress.

Keywords: Ca^{2+}; Epidermal layer; Germination; NaCl; Osmolytes; Oxidative stress; Salt stress; *Sorghum bicolor*; SOS pathway; Quantitative real-time polymerase.

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Calcium Improves Germination and Growth of *Sorghum bicolor* Seedlings under Salt Stress

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Abstract: Salinity is a major constraint limiting plant growth and productivity worldwide. Thus, understanding the mechanism underlying plant stress response is of importance to developing new approaches that will increase salt tolerance in crops. This study reports the effects of salt stress on *Sorghum bicolor* during germination and the role of calcium (Ca^{2+}) to ameliorate some of the effects of salt. To this end, sorghum seeds were germinated in the presence and absence of different NaCl (200 and 300 mM) and Ca^{2+} (5, 15, or 35 mM) concentrations. Salt stress delayed germination, reduced growth, increased proline, and hydrogen peroxide (H_2O_2) contents. Salt also induced the expression of key antioxidant (*ascorbate peroxidase and catalase*) and the *Salt Overlay Sensitive1* genes, whereas in the presence of Ca^{2+} their expression was reduced except for the *vacuolar Na^+/H^+ exchanger antiporter2* gene, which increased by 65-fold compared to the control. Ca^{2+} reversed the salt-induced delayed germination and promoted seedling growth, which was concomitant with reduced H_2O_2 and Na^+/K^+ ratio, indicating a protective effect. Ca^{2+} also effectively protected the sorghum epidermis and xylem layers from severe damage caused by salt stress. Taken together, our findings suggest that sorghum on its own responds to high salt stress through modulation of osmoprotectants and regulation of stress-responsive genes. Finally, 5 mM exogenously applied Ca^{2+} was most effective in enhancing salt stress tolerance by counteracting oxidative stress and improving Na^+/K^+ ratio, which in turn improved germination efficiency and root growth in seedlings stressed by high NaCl.

Keywords: antioxidant; Ca^{2+}; gene expression; germination; ion homeostasis; NaCl; salt stress; oxidative stress; *Sorghum bicolor*

1. Introduction

Plants are affected by environmental factors throughout their life cycle and seed germination is the most critical stage that determines plant growth and productivity [1]. Seed germination is a complex physiological process, which begins when water is taken up by the seed and is completed by the appearance of the radicle [2]. Among other abiotic factors, such as high temperatures, heavy metals, and drought, salinity is one of the detrimental abiotic factors, causing up to 50%

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AA	Amino acid
ABA	Abscisic acid
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
ASC	Ascorbate
At %	Atomic percentage
ATP	Adenosine triphosphate
BADH	Betaine aldehyde dehydrogenase
BP	Base pair
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CaM	Calmodulin
cAPX	Cytosolic ascobate peroxidase
CAT	Catalase
CAX1	Cation exchanger 1
cDNA	Copy DNA
CDPK	Calcium dependent protein kinase
CDS	Coding DNA sequence
CBL-CIPK	Calcineurin B-like-interacting protein kinase

Cl^-	Chloride
CMO	Choline monooxygenase
CO_2	Carbon dioxide
Cu/Zn SOD	Copper/zinc superoxide dismutase
dH_2O	Distilled water
DHAR	Dehydroascorbate reductase
DNA	Deoxyribonucleic acid
DW	Dry weight
EC	Electrical conductivity
EC_e	Saturated extract
EDX	Energy dispersive X-ray spectroscopy
ER	Endoplasmic reticulum
ETC	Electron transport chain
Fe	Iron
Fe-SOD	Iron superoxide dismutase
FW	Fresh weight
GA	Gibberellin
GB	Glycine betaine
GI	Germination index
GmAPX	Glyoxisomal ascorbate peroxidase
GP	Germination percentage

GR	Glutathione reductase
GSE	Reduced glutathione
GSH	Glutathione
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
H_3PO_4	Phosphoric acid
K^+	Potassium
kDA	Kilo dalton
Li^+	Lithium
LRP	Lateral root primordia
MAPK	Mitogen-activated protein kinase
MDA	Malondealdehyde
MDHAR	Monodehydroascorbate dehydrogenase
MGT	Mean germination time
Mn-SOD	Manganese superoxide dismutase
mRNA	Messanger RNA
Na^+	Sodium ion
Na^+/H^+	Sodium/hydrogen ion
NaCl	Sodium chloride ion
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate

NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NAF/FISL	Asparginine (N), alanine (A), phenylalanine (F), isoleucine (I), serine (S) and leucine (L)
NHX	Vacuolar Na^+/H^+ antiporter
NOX-RBOH	Respiratory burst oxidase homolog
O_2	Singlet oxygen
$O_2^{\bullet-}$	Superoxide radical
$OH^{\bullet}$	Hydroxyl radical
OPP	Oxidative pentose phosphate
P5CS1	Δ'-pyrroline-5-carboxylate synthetase 1
P5SCR	Δ'-pyrroline-5-carboxylateΔ'-pyrroline-5-carboxylate reductase
PA	Phosphatidic acid
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PEG	Polyethylene glycol
pI	Isoelectric point
PLD	Phospholipase D
POD	Peroxidase
PS I	Photosystem I
PS II	Photosystem II

qRT-PCR	Quantitative real time PCR
ROS	Reactive oxygen species
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RWC	Relative water content
sAPX	Stromal ascorbate peroxidase
SCABP 8	SOS3-like Calcium Binding Protein8
SE	Secondary electrons
SEM	Scanning electron microscopy
SEM-EDX	Scanning electron microscopy- energy dispersive X-ray spectroscopy
SNF1	Sucrose non fermenting 1
SOD	Superoxide dismutase
SOS	Salt Overly Sensitive
SOS1	Salt Overly Sensitive 1
SOS2	Salt Overly Sensitive 2
SOS3	Salt Overly Sensitive 3
tAPX	Thylakoid ascorbate peroxidase
TBA	Thiobarbutric acid
TCA	Trichloroacetic acid
TG	Total germination
UBQ	Ubiquitin

UTR	Untranslated region
V-ATPase	Vacuolar proton ATP
Wt %	Weight percentage
XOD	Xanthine oxidase

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LIST OF TABLES

UNIVERSITY of the
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LIST OF FIGURES

CHAPTER 1

1.1 INTRODUCTION

The success of plant growth and development is heavily dependent on the plant's ability to withstand biotic and abiotic factors (Gao *et al.,* 2008). Biotic factors include all living organisms in an environment such as pathogens, herbivores and parasites, whereas abiotic factors include all non-living components in an environment such as resources as well as parameters that determine plant growth and these include minerals, nutrients, temperature, light, water availability, carbon dioxide, pollutants, ionizing radiation and wind. Any alteration in the equilibrium of these factors results in stress (Schulze *et al.,* 2005). Abiotic factors such as water logging, extreme temperatures, drought, soil nutrient deficiencies, and salinisation affects plant growth and productivity negatively (Bray *et al.,* 2000). Although all abiotic factors are a concern to plant growth and development, it is of great importance to determine their individual effects (Acosta-Motos *et al.,* 2017). Salinity stress is one of the most significant environmental stresses threatening the agricultural sector by limiting crop productivity as well as reducing the quality of crops (Shrivastava and Kumar, 2015). Salinity stress has affected ~800 million hectares of land, which is equivalent to more than 6 % of the world land area (Flowers *et al.,* 2010; FAO, 2008). Plants that are sensitive to soils with high salt content are termed glycophytes, and they include the majority of plant species whereas halophytes are plants that are able to grow in soils of high salt content (Glenn *et al.,* 1999; Cheeseman, 2014; Ali and Yun, 2017). Although glycophytic and halophytic plant species have different salt tolerant responses, salinity stress induces a similar response at the seed level by causing a delay in the germination process (Almansouri *et al.,* 2001).

Germination is a fundamental stage for plant growth, which requires favourable environmental conditions for a seed to germinate (Craufurd *et al.,* 1996). Most studies done on various plant

species namely; *Oryza sativa* L. (Hakim *et al*., 2010), *Zea mays* L. (Khayatnezhad and Gholamin, 2011), *Vigna unguiculata* L. (Walp) (El-Shaieny, 2015) and *Brassica juncea* L. (Pandey and Penna, 2017) have indicated that germination is negatively affected by salt stress. Salt stress delays and hinders seed germination by primarily lowering the osmotic potential, which reduces water up-take by the roots and causes the plant to be exposed to osmotic stress. Osmotic stress is the first effect of salinity, which results in cell dehydration and loss in turgidity, and hence cell death (Akram *et al.,* 2018).

In addition, salt stress also causes ionic stress in plants, which is due to the increased levels of sodium (Na^+) and chloride (Cl^-) ions (Tobe *et al.,* 2004; El-Shaieny, 2015). The accumulation of Na^+ and Cl^- in soils causes an imbalance of other essential ions such as potassium (K^+) and calcium (Ca^{2+}) amongst others (Niu *et al.,* 1995; Machado and Serralheiro, 2017). Consequently, the similarity in the physiochemical properties between Na^+ and K^+ result in their competition for major binding sites in key metabolic processes in the cytoplasm (Yermiyahu *et al*., 1997; Tester and Davenport, 2003; Almeida *et al*., 2017). In addition, Na^+ displaces membrane-bound Ca^+ resulting in the disruption of membrane integrity and an increased Na^+/ K^+ ratio (Cramer *et al*., 1985; Bhandal and Malik, 1988; Yermiyahu *et al*., 1997; Blaha *et al*., 2000; Wu and Wang, 2012). These primary stress effects further result in nutrient imbalances, alter membrane function, decrease photosynthetic rate, and hinders the ability of antioxidant enzymes to detoxify reactive oxygen species (ROS). Thus the uncontrolled over production of ROS causes oxidative damage, which is considered as a secondary stress. ROS are chemical species that contain oxygen and they include superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and singlet oxygen (O_2) (Sharma *et al*., 2012). ROS are highly toxic to plants when produced at high levels causing oxidative membrane damage, peroxidation of lipids, and degradation of nucleic acids and proteins ultimately leading to cell death (Gill and Tuteja, 2010). ROS are produced under normal metabolic processes such as

respiration, photosynthesis and photorespiration whereas under stress conditions, they are produced at high levels. At high levels, ROS are detrimental to plant growth and are kept at equilibrium by enzymatic and non- enzymatic antioxidants, which serve as a means of stress tolerance (Apel and Hirt, 2004; Rossatto *et al.,* 2017; Filiz *et al.,* 2018). Enzymatic antioxidants include, the superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and enzymes of the ascorbate (ASC)-glutathione (GSH) cycle [ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate dehydrogenase (MDHAR) and dehydroascorbate reductase (DHAR)]. Non-enzymatic antioxidants include flavonoids, phenolics, tocopherols, ASC and reduced GSH (Foyer and Nocter, 2011; Chawla *et al*., 2013). ROS are scavenged by different ROS generating and scavenging systems that are present in each of the different cellular compartments (Rasool *et al.,* 2013).

Other physiological and biochemical mechanisms to cope with osmotic and ionic stress include the high accumulation of organic ions or low molecular weight organic solutes such as compatible solutes (Liang *et al.,* 2017; Wang *et al.,* 2003). Compatible solutes include sugars (glucose, sucrose, fructose, fructans, raffinose and trehalos), sugar alcohols (glycerol, methylated inositols and mannitol), amino acid derivatives (proline, prolinebetaine, β-alaninebetaine and glycinebetaine) and sulfonium compounds (dimethylsul-phoniopropionate and choline-*O*-sulphate) (Koyro *et al.,* 2012). These compounds protect cells from oxidative stress through stabilisation of proteins, enzymes and nucleic acids and improve the osmotic balance by increasing water absorption (Surender *et al*., 2015). Their contribution to higher plants grown under saline condition vary among species, cultivars and between different cellular compartments (Ashraf and Harris, 2004).

Furthermore, when Na^+ levels increase through salt stress, plants aim to maintain normal Na^+ homeostasis either through Na^+ sequestration or Na^+ exclusion (Wu *et al*., 2018). Na^+ exclusion involves a coordinated action of calcium ion (Ca^{2+}) sensors, various pumps, as well as certain pathways including the Salt Overly Sensitive (SOS) pathway. Calcium ions are one of the major signalling molecules that play an important role in the development and growth of plants under both normal and stressful environments. Membrane receptors perceive extracellular stress signals, which then results in the activation of various internal complex signalling cascades including Ca^{2+}. The activated cytosolic Ca^{2+} will then initiate signalling pathways to exclude Na^+ from the cytosol as a way to mitigate stress tolerance (Mahajan *et al.,* 2008).

In order to gain an understanding of the effect of stress on plants, it is important to investigate both the internal and external effects that stress imposes on the plant. Scanning electron microscopy (SEM) is one of the ideal techniques that is used to analyse plant surfaces at high resolutions (Jerosch and Reichelt, 1997). Additionally, the detection of X-ray emissions from the sample with the use of SEM also allows for the determination of element compositions (Egerton, 2005). The former mentioned is the principle of energy-dispersive X-ray spectroscopy (EDX) (Goldstein *et al*., 2003).

Thus this study was conducted to unpack the mechanisms of salt stress tolerance in *Sorghum bicolor* during germination and further determine the ability of exogenously applied calcium to alleviate the effects of salt stress. A few studies have demonstrated the effects of salt stress in sorghum (Alves Da Costa, 2005; El Omari *et al*., 2016), however none investigated the effects of high salt (200 - 300 mM NaCl). We believe that it is important to investigate sorghum's response to salt at concentrations above the physiological range since sorghum is known to be a moderately drought and salt tolerant cereal crop (Igartua *et al*., 1995; Massacci *et al*., 1996; Krishnamurthy *et al*., 2007).

Literature review

1.2 Sorghum bicolor

Sorghum bicolor (L.) Moench commonly known as sorghum, is a drought tolerant crop that belongs to the grass family Poaceae. Sorghum is a C4 plant that has high photosynthetic efficiency and productivity (Smith and Frederiksen, 2000). It is a cereal crop ranked the 5th most important in the world after wheat (*Triticum sp*.), rice (*Oryza sativa*), maize (*Zea mays*) and barley (*Hordeum vulgare*) and grows well in arid and semi-arid areas (Smith and Frederiksen, 2000; Woldesemayat and Ntwasa, 2018). Sorghum originates from the southern region of the Sahara Desert in Africa and has a diverse array of cultivated genotypes including grain sorghum, sweet sorghum, forage sorghum and biomass sorghum (Legwaila *et al*., 2003).

Sweet sorghum has attracted much attention as a promising energy crop in the USA and has also shown to be a good crop for biofuel production, which is recognised in countries such as Mexico, Australia, India and Nigeria (Berenji and Dahlberg, 2004; Almodares and Hadi, 2009). The plant has several of commercial advantages, including its use as a source of food, bioethanol, paper and methane production, while the organic by-products can be used as fertilizers (Tari *et al*., 2017). The drought tolerant nature of sorghum is attributed to its finely branched root system that absorbs water efficiently, its small leaf area that reduces transpiration, the waxy layer on the leaf epidermis that helps improve water use efficiency and its ability to limit water loss by the rapid closure of the stomata (Buchanan *et al*., 2005).

Sorghum's adaptions to drought and salt stresses as well as its small genome size (730 Mb) makes sorghum a good model plant for genomic and functional studies under abiotic stress (Baillo *et al*., 2020). It is also an ideal model crop for studies aimed at improving tolerance or resistance to stresses in other plant species (Johnson *et al*., 2014). Moreover, sorghum is easy

to germinate and grow and emerges within a period of 3-10 days when grown under warm temperatures (16- 18 °C) and adequate soil moisture (Moore *et al*., 2014).

1.3 Germination

Although an important stage in a plant's life cycle, germination is not immune to effects of stresses, in particular abiotic stresses. Germination is the process that occurs when a dry, non-dormant seed takes up water by imbibition and is completed with the protrusion of the radicle (Bewly, 1997). The uptake of water by the seed is a fundamental requirement for both the initiation and the completion of seed germination and it occurs in three phases (Figure 1). The first phase involves a rapid uptake of water (phase I), which occurs as a result of dry seeds having a low water potential (Krishnan *et al*., 2004). The second phase of water uptake involves water content reaching a plateau (phase II) and the last phase (phase III) occurs after germination and involves an increase in water uptake as a result of the embryonic axis elongating. However, dormant seeds do not enter the post germination phase of water uptake (phase III) because they do not complete germination (Bethke *et al*., 2007). The inability to enter into the post germination phase of water uptake is due to inhibition by the hormone Abscisic acid (ABA) (Gimeno-Gilles *et al*., 2009). Abscisic acid prevents germination by hindering the loosening of the radicle cell wall (Bewly, 1997). A study done in sunflowers supports this by demonstrating that the temporary application of ABA to the embryo only, prevents radicle extension and once removed allows for the completion of germination (LePage-Degivry and Garello, 1992). Additionally, the inhibiting effects of ABA to germination is counteracted by gibberellins (GAs) and ethylene (ET). Gibberellins play a role in counteracting ABA inhibition during the early and late phase of germination. In contrast, ET counteracts ABA inhibition during the late phase of germination by interfering with ABA signalling, although ABA contents are not affected (Linkies *et al*., 2009).

The fast rehydration of the seed during imbibition results in damage to the seed membrane and cellular compartments, which causes the leakage of cellular solutes (Powell and Matthews, 1978). However, the leakage accelerates germination by lowering the concentration of inhibitors in the seed (Matilla *et al*., 2005). In response to the damage imposed by rehydration of the seed, the seed activates various repair mechanisms during imbibition such as DNA and mitochondria repair, *de novo* mRNA and protein synthesis from stored mRNA, the activation of amino acid metabolism as well as the activation of respiration/ energy production (Figure 1) (Weitbrecht *et al*., 2011). Once the seed progresses past phase II of water uptake and it enters phase III, the endosperm ruptures and seedling growth commences (Bewly, 1997). This stage of the plant development is associated with high mortality, therefore, the seedling must develop quickly to overcome it (Lai *et al*., 2019). However, seedling survival is threatened by abiotic and biotic stresses (Moles and Westoby, 2004).

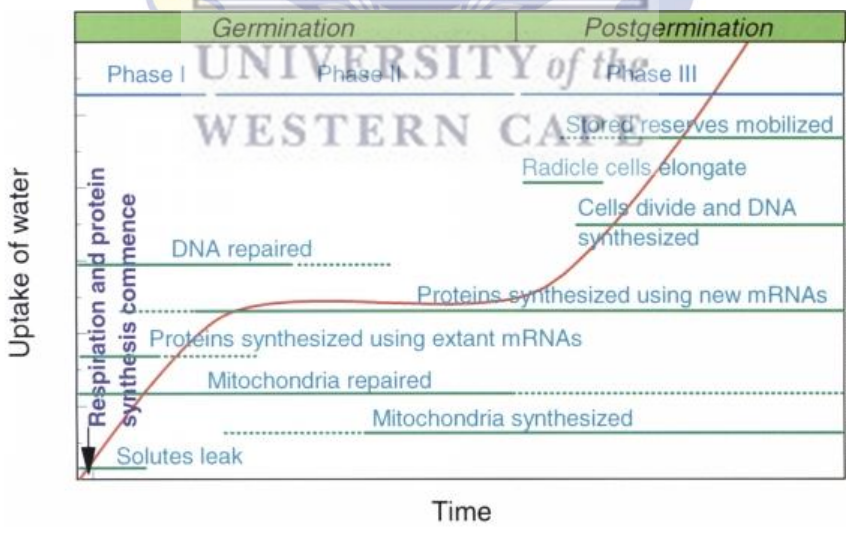

Figure 1.1. Physical and metabolic events during germination and post germination. The time it takes for these events to occur varies between species and depends on the germination conditions. The curve shows a stylised curve of water uptake (adapted from Bewly, 1997).

1.4 Abiotic stress in plants

The plant's developmental stages including growth and productivity of major crop species are affected by abiotic stresses, mainly extreme temperatures, drought and salinity (Singhal *et al*., 2016). Crop yields have been reduced by more than 50 % as a result of these stresses (Bray *et al*., 2000). The most common abiotic stress that limits crop growth is water supply or drought (Boyer *et al*., 1982). Drought is simply defined as a period without rainfall due to climate change. Drought further impacts the environment by causing salts to accumulate in the soil due to insufficient precipitation that is unable to leach ions from the soil resulting in salt stress (Shrivastava and Kumar, 2015). Salt stress is considered the main factor reducing crop yields and it has been predicted that it will affect at least 50 % of all arable land by 2050 (Wang *et al*., 2001; FAO, 2009). In addition, temperature stress is becoming a growing problem to the environment due to climate change. Temperature stress is classified into heat and cold stress, which both severely hinders plant growth. These stressors adversely affect growth by eliciting morphological, physiological, biochemical and molecular changes in plants (Wang *et al*., 2003).

1.4.1 Heat stress

High temperature stress also known as heat stress affects plant growth and metabolism, crucial processes that require optimum temperature (Watanabe and Kume, 2009). Heat stress occurs as a result of climate change and is defined as a rise in temperature, which exceeds the threshold for a period of time that causes irreversible damage to the development and growth of plants (Wahid, 2007). Heat stress has been found to decrease germination within the early stages by causing cell death and embryo damage (Essemine *et al*., 2010). High temperatures also inhibited the root and shoot lengths of *Phaseolus aureus* seedlings (Kumar *et al*., 2011).

Furthermore, significant reductions in plant height, leaf size and weight of *Sorghum bicolor* were observed due to heat (Prasad *et al*., 2006). Heat stress also causes a decline in photosynthesis by deactivating one of the primary enzymes involved in photosynthesis, ribulose-1,5-bisphophate carboxylase/oxygenase (RuBisCO) (Haldimann, 2004). In addition, plant fertility is reduced as a result of heat stress, notably for heat sensitive crops like tomato, which produce no fruit under high temperatures (Abdul-baki and Stommel, 1995).

1.4.2 Cold stress

On the other hand another major temperature stress is low temperature stress or cold stress. This kind of stress is divided into chilling stress (0 - 15°C), which are cool temperatures that cause injury without forming ice crystals in plant tissues and freezing stress (< 0 °C), which imposes damage to the plant tissues by the formation of ice crystals (Sanghera *et al*., 2011). Cold stress has various inhibitory effects on plant growth and morphology and initially causes a reduction in germination by stunting seedling growth, resulting in seedling discolouration, leaf whitening and yellowing, and withering after transplantation (Nahar *et al*., 2012). The phenotypic symptoms of cold stress include chlorosis, necrosis, desiccation, wilting, reduced leaf expansion as well as tissue breakdown (Solanke and Sharma, 2008). Cold stress further inhibits plant growth by impairing photosynthesis, which results in an indirect loss in yield due to the reduction in the availability of carbohydrates for grain production (Takeoka *et al*., 1992). Cold stress damages the cell membrane by disrupting protein lipid structure and denatures proteins (Wang and Li, 2006). This cold stress induced damage to the membrane causing it to become permeable to undesired nutrients and ions and ultimately causes ion leakage (Salinas, 2002). Plants may also suffer from dehydration as a result of the freezing of cell constituents and water (Yadav, 2010).

1.4.3 Drought stress

Drought as defined from an agricultural and physiological viewpoint occurs when there is a decrease in water availability for plants due to low soil moisture for a period of time (Keyvan, 2010; Dai, 2012). Drought occurs due to several factors namely; a long term deficit of rainfall, a shortage of soil water storage in the surface or subsurface area of soils, a deficit in river flow or due to competing of water use with social recreational needs (Manakou *et al*., 2011). Drought imposes its harmful effects on plants by delaying germination and impairing seedling establishment (Kaya *et al*., 2006). Several studies done in pea (*Pisum sativum* L.), alfalfa (*Medicago sativa* L) and rice (*Oryza sativa* L.) demonstrated how drought reduced germination by having a negative impact on early seedling growth, root and shoot dry weight, the hypocotyl length and vegetative growth (Okcu *et al*., 2005; Manikavelu *et al*., 2006; Zeid and Shedeed, 2006). Drought also impairs major processes involved in plant growth such as mitosis and cell elongation (Hussain *et al*., 2008). Drought causes a reduction in relative water content (RWC), which results in a decrease in leaf water potential and leads to stomatal closure (Farooq *et al*., 2009; Bhargava and Sawant, 2013). The closing of the stomata leads to a decrease in the transpiration rate, which causes an increase in leaf temperature. This increase in leaf temperature further leads to the denaturation of enzymes (Farooq *et al*., 2009; Arbona *et al*., 2013).

1.4.4 Salt stress

Soil salinity can be defined by soils having high concentrations of soluble salts. It is estimated by electrical conductivity (EC) of saturated extracts (EC_e) in the root zone by which soils are considered to be salt affected in the EC_e range between 2 – 4 desi Siemans per meter (dS m^{-1}), which is equivalent to ~20 mM – 40 mM NaCl at 25˚C, which could restrict the yields of

sensitive crops (Jamil *et al.,* 2011; Srivastava *et al.,* 2015; Sharma *et al.,* 2016). Soil salinity largely transpires from natural causes, which results from salts accumulating in arid and semi-arid areas over long periods of time (Sharma *et al*, 2016). Natural causes of salinity stress occur from the intrusion of sea water specifically in areas along the coast, salt laden sand blown by the sea wind, impeded drainage and lastly the weathering of rocks, which release an array of soluble salts such as chlorides of sodium, magnesium, calcium and in a lower degree, sulfates and carbonates (Munns and Tester, 2008). In recent year's salinisation is caused by irrigation systems using saline water, overuse of fertilisers as well as low precipitation and poor cultural practices, which then affect crop production (Jamil *et al*, 2011).

1.5 The effect of salt stress on plants

Salinity is considered a serious threat to crop productivity and is predicted to continue to worsen as a result of global climate change (AbdElgawad *et al*., 2016). The initial injurious effect that plant cells experience through salinity is osmotic stress. Osmotic stress occurs due to the difficulty for roots to extract water from the soil as a result of the high concentrations of salts. Ionic stress is the second effect of salinity that develops over time in which salts accumulate to a toxic level in the shoot that cannot be tolerated by the plant (Munns and Tester, 2008). Moreover, the stomatal conductance is immediately affected by the disturbed water relations which has a direct negative effect on photosynthetic processes. Furthermore, the reduced rate of photosynthesis leads to an increase in ROS formation causing oxidative stress (Sharma *et al*., 2012).

1.5.1 Reactive oxygen species in plants

ROS are chemically reactive species produced as a result of aerobic metabolism (Sharma *et al.,* 2012). They are commonly produced by electron leakage to O_2 from the electron transport activities of the mitochondria, peroxisomes, plasma membrane and chloroplast (Apel and Hirt, 2004). ROS consist of free radicals such as superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$), non-radicals such as singlet oxygen (O_2) and hydrogen peroxide (H_2O_2) ions (Trchounian *et al.,* 2016). Under normal conditions the level of ROS is maintained by various antioxidant components that are found in specific cellular compartments (Alscher *et al.,* 1997). However, environmental factors disturb the equilibrium between the production and scavenging of ROS, resulting in their increase (Apostol *et al.,* 1989).

1.5.1.1 Superoxide radical

Superoxide radical ($O_2^{\bullet-}$) is the initial ROS produced in plant cells and is responsible for the generation of other ROS either directly or indirectly. Photosystem I (PS I), that is localised in the thylakoid is the main producer of $O_2^{\bullet-}$ during non-cyclic electron transport chain (ETC). This ROS is also produced in different cellular compartments namely; peroxisomes, endoplasmic reticulum (ER), chloroplast, apoplast, the mitochondrial electron transport chain and the plasma membrane (Halliwell, 2006). The NADPH oxidase, known as respiratory burst oxidase homologues (NOX-RBOHs) in higher plants are another source of $O_2^{\bullet-}$ (Jajic *et al.,* 2015). The $O_2^{\bullet-}$ has a half-life of ~1 µs and is moderately reactive. It forms H_2O_2 by accepting the addition of one electron and two protons. Furthermore, $O_2^{\bullet-}$ can also form H_2O_2 through dismutation by SOD catalysed reactions (Jajic *et al.,* 2015).

1.5.1.2 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is the most stable ROS and is moderately reactive as well as $O_2^{\bullet-}$. H_2O_2 is produced via two main pathways in plants; either by the dismutation of $O_2^{\bullet-}$ catalysed by SOD or via the reaction catalysed by oxalate oxidases and amino acids (Hu *et al.,* 2003; Asada, 2006). The main sources of H_2O_2 in plants are from the mitochondria, plasma membrane, ETC of chloroplast, endoplasmic reticulum, β-oxidation of fatty acids and finally through photorespiration (Asada, 2006). In addition, H_2O_2 can also be produced by NADPH oxidase and xanthine oxidase (XOD) (Wang *et al.,* 2013). It has the unique ability to diffuse across membranes, which can be beneficial and detrimental (Perez and Brown, 2014). This attribute could be beneficial because H_2O_2 can serve as a signalling molecule at low intracellular levels and detrimental at high intracellular levels because this allows for H_2O_2 to cause oxidative stress at sites far from where it is formed (Ahmad *et al.,* 2010). Its beneficial roles as a signalling molecule involve regulating biological processes such as photorespiration and photosynthesis (Noctor *et al.,* 2002), as well as cell cycle (Tanou *et al.,* 2009) and stomatal movement (Bright *et al.,* 2006). On the other hand when H_2O_2 levels are high it oxidizes amino acids (cysteine and methionine), inactivates enzymes (Cu/Zn-SOD and Fe-SOD) as well as disrupts 50 % enzyme activity (fructose 1,6 bisphosphatase, phosphoribulokinase and sedoheptulose 1,7 bisphosphatase) (Dat *et al.,* 2000; Halliwell, 2006). The enzymatic antioxidants, catalase (CAT) and ascorbate peroxidase (APX) will then detoxify and convert H_2O_2 to water and oxygen (Sofo *et al*., 2015).

1.5.1.3 Singlet oxygen

Singlet oxygen (O_2) is mainly found in the chloroplast and is constantly produced in the photosystem II (PS II) reaction centre as a consequence of oxygen reacting with triplet

chlorophyll (Macpherson *et al.,* 1993; Fischer *et al.,* 2013). At low/moderate concentrations, O_2 is involved in protecting cells from photo-oxidative stress by upregulating the correct genes involved (Krieger-Liszkay *et al.,* 2008). Alternatively, under abiotic stress, O_2 production is increased as a result of the lowering of intracellular CO_2 in the chloroplast due to stomatal closure. O_2 has been found to have a half-life of ~3 µs and can diffuse over a distance of 100 nm (Hatz et al., 2007; Wagner *et al.,* 2004). Within its short life span it is able to interact with and damage proteins, lipids, nucleic acids, pigments and can elicit cellular death by causing loss of activity of PS II. Plants have built a defence against O_2 which, involves the help of β–carotene, plastoquinone and tocopherol (Krieger-Liszkay *et al.,* 2008).

1.5.1.4 Hydroxyl radical

Hydroxyl radical ($OH^{\bullet}$) is the most toxic and reactive among the different ROS. It is formed by the Fenton reaction from H_2O_2 and $O_2^{\bullet-}$, which are catalysed by transition metals such as Fe (Fe^{2+}, Fe^{3+}). In addition, $OH^{\bullet}$ is extremely harmful to biological molecules and causes cellular damage such as lipid peroxidation, membrane destruction, protein and DNA damage. Damage by $OH^{\bullet}$ ultimately leads to cell death since enzymatic mechanisms to eliminate $OH^{\bullet}$ do not exist (Gill and Tuteja, 2010).

1.6 Mechanisms of stress tolerance by plants

1.6.1 Antioxidants

The antioxidant enzyme system is one of the many survival mechanisms that plants have developed against environmental stresses (Prasad, 1997). This effective system manages the routinely formed ROS by keeping them at low levels to avoid toxicity (Shi and Zhu, 2008). It consists of a wide network of enzymatic antioxidants namely; superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) amongst others and non-enzymatic antioxidant

components, which include but are not limited to, reduced glutathione (GSE), ascorbic acid (AsA) and vitamin E (Verma *et al.,* 2015).

1.6.1.1 Superoxide dismutase

SOD is known to be the first antioxidant mechanism against ROS. It is ubiquitous in aerobic organisms and maintains cellular defence against ROS. SOD controls $O_2^{\bullet -}$ and H_2O_2 reactivity amounts and limits the formation of $OH^{\bullet}$ radicals (Ahmad *et al.,* 2010). This metalloenzyme was first discovered in maize and consisted of six isozymes that differed genetically and biochemically. Based on the metal ion in their active group, SOD is classified as; copper and zinc SOD (Cu/Zn-SOD), iron SOD (Fe-SOD) or manganese SOD (Mn-SOD) (Ahmad *et al.,* 2010; Kim *et al.,* 1996). Plants are the only organisms with multiple forms of each type of SOD, which is a reflection of their complex antioxidant defence system (Ahmad *et al.,* 2010). Various studies have indicated that an increase in SOD enhances stress tolerance (Koyro *et al.,* 2012).

1.6.1.2 Catalase

Catalases are tetrameric haem-containing enzymes that convert $2H_2O_2$ to $O_2 + 2H_2O$ and can be found in peroxisomes (Ben Amor *et al.,* 2005). Many isozymes of catalase exist in plants, two can be found in castor bean while six are present in *Arabidopsis* (Frugoli *et al.,* 1996). There are three classes of catalases found in plants; class 1 catalases, are found in photosynthetic tissue, produced during photorespiration and become active to dismutate H_2O_2. The 2nd class of catalases are produced in vascular tissue and lastly, the 3rd class is produced in seeds and young plants and they scavenge H_2O_2 that is produced during fatty acid degradation (Willekens *et al.,* 1994). Moreover, CAT is a unique antioxidant since it does not rely on a

reducing agent equivalent to degrade H_2O_2 (Scandalios *et al*., 1997). Thus, CAT is the most energy efficient antioxidant to remove H_2O_2 and it has a quick turnover time of 6 x 10^6 molecules of H_2O_2 to H_2O and O_2 per min^{-1}(Scandalios *et al*., 1997; Mittler, 2002).

1.6.1.3 Ascorbate peroxidase

Ascorbate peroxidase belongs to the class I heme-peroxidases and is found in higher plants, chlorophytes (Takeda *et al*., 2000), red algae (Sano *et al*., 2001) and in certain members of the protista kingdom (Shigeoka *et al*., 1980). Unlike other peroxidases, APX depends on ascorbate for a source of reducing power to reduce H_2O_2 to water and becomes unstable in its absence (Shigeoka *et al*., 2002; Raven, 2003). It is the key enzyme in the ascorbate glutathione cycle which is the main H_2O_2 detoxification system in plant chloroplasts. The importance of APX in ROS scavenging is not just restricted to the chloroplast but also plays a vital role in scavenging ROS in the cytosol, mitochondria and peroxisomes (Noctor and Foyer, 1998). There are various APX isoforms that exist and these include glyoxisomal (gmAPX), cytosolic (cAPX), stromal (sAPX) and thylakoid (tAPX) APX (Shigeoka *et al*., 2002). APX isoforms have different structural and kinetic properties and possess specific conserved domains and signal peptides (Huseynova *et al*., 2014). The four isoforms play their part in removing H_2O_2 that are generated through the various metabolic processes, for instance, gAPX removes H_2O_2 generated by fatty acid β-oxidation and photorespiration, whereas cAPX is likely involved in pathogen response (Bunkelmann and Trelease, 1996; Mittler *et al*., 1998). On the other hand, sAPX and tAPX isoforms scavenge H_2O_2 generated by photosynthesis (Nakano and Asada, 1981). Although both APX and CAT scavenge H_2O_2, APX is known to have a higher affinity (Km= 3 x 10^{-2}) towards H_2O_2 compared to CAT (Km= 0.047 x 10^3) and is thus a better scavenger of H_2O_2

compared to CAT under stressful conditions (Mittler and Poulos, 2005; Das and Roychoudhury, 2014; Anjum *et al*., 2016).

1.6.2 Osmoprotectants

Plants experience osmotic stress immediately after the salt concentrations reaches threshold level around the roots, which directly correlates with a reduction in the shoot growth (Munns and Tester, 2008). Osmotic stress hinders growth and productivity of plants at the molecular, biochemical, physiological and morphological level by lowering the water potential of the plant cell (Huang *et al.,* 2011). Plants respond to osmotic stress by accumulating either high concentrations of organic ions or osmoprotectants or compatible osmolytes such as proline, soluble sugars, betaines, sugar alcohols and polyols to overcome osmotic stress. Compatible solutes are neutral in the physiological pH range, highly soluble in water and do not possess inhibitory effects to enzymes at high concentrations (Rhodes *et al.,* 2002). They work to protect plants by protecting membrane integrity, stabilising enzymes/proteins, detoxifying ROS and by cellular osmotic adjustment (Ashraf and Foolad, 2007; Yancey, 1994). Furthermore despite the fact that each osmoprotectant falls in a different biochemical group, their role in plant protection have been shown to be similar against stress (Giri, 2011).

1.6.2.1 Proline

The role of proline as an osmoprotectant is vast since it serves to maintain protein integrity through its ability to act as a molecular chaperone. It has also been found to protect and prevent the denaturation of proteins and membranes by joining with water molecules that attach to proteins and membranes (Koca *et al.,* 2007). Proline has also been reported to function as an antioxidant by scavenging ROS and being a singlet oxygen quencher (Smirnoff and Cumbes,

1989; Matysik *et al*., 2002). Finally proline is also able to alleviate cytoplasmic acidosis by maintaining metabolism levels through sustaining $NADP^+$ /NADPH ratios (Hare and Cress, 1997). Proline is synthesised via two pathways in plants; the ornithine pathway and the glutamate pathway (Hayat *et al.,* 2012). During osmotic stress, proline accumulation is owed to the glutamate pathway (Strizhov *et al.,* 1997; Armengaud *et al*., 2004). Synthesis of proline by the glutamate pathway begins with glutamic acid, which is catalysed by Δ'-pyrroline-5-carboxylate synthetase (P5CS) and Δ'-pyrroline-5-carboxylateΔ'-pyrroline-5-carboxylate reductase (P5CR) (Hayat *et al.,* 2012). Most plant species have two genes, which encode the P5SC and the P5CR. The mitochondria is the main site of proline catabolism by which proline dehydrogenase or proline oxidase produces P5C from proline, which is further converted to glutamate by P5C dehydrogenase (Hayat *et al.,* 2012). Proline degradation during osmotic stress is prevented by pyruvate dehydrogenase (PDH) transcription that gets activated by rehydration and repressed by dehydration (Kiyosue *et al.,* 1996; Verbruggen *et al.,* 1996).

1.6.2.2 Betaines

Betaines are quaternary ammonium compounds that include glycine betaine (GB), proline betaine, β-alaninebetaine, choline-*O*-sulphate, pipecolatebetaine and hydroxyprolinebetaine (Ashraf and Harris, 2004; Kumar *et al.,* 2018). The most abundant occurring betaine is glycine betaine, which is regulated in response to abiotic stress (Koyro *et al.,* 2012). However, some higher plants such as rice, Arabidopsis, tobacco and tomato plants are unable to accumulate GB (McCue and Hanson, 1990). The advantage of GB accumulation in plants is its ability to stabilise the quaternary structures of proteins as well as the maintenance of cellular osmotic balance (McCue and Hanson, 1990; Papageorgiou and Murata, 1995). The synthesis of GB occurs via several pathways and depends on their enzymes and precursors (Sakamoto and Murata, 2000). Glycine betaine is synthesised by a two-step oxidation of choline whereby choline is oxidized by choline monooxygenase (CMO) in the first step, which is the rate

limiting step (Bhuiyan *et al*., 2007). Betaine aldehyde dehydrogenase (BADH) mediates the second step of GB synthesis and relies on NAD^+ as an energy source (Fitzgerald *et al*., 2009). Studies have shown that various abiotic stresses such as salinity (McCue and Hanson, 1992), heat (Alia *et al*., 1998; Mitsuya *et al*., 2011), cold (Xing and Rajashekar, 2001) and drought stress (Li *et al*, 2016) induces the expression of CMO and BADH genes in different organisms.

1.6.2.3 Soluble sugars

Sugars are essential biomolecules that play vital roles in the metabolism of both plants and animals (Zhang *et al.,* 2014). Sugar is obtained by plants through photosynthesis and they form part of most biologically active compounds (Pego *et al*., 2000). They are classified into monosaccharides, disaccharides and polysaccharides based on the number of monomers they possess (Ahmad, 2019). These different classes of sugars serve different purposes for instance monosaccharides (*e.g* glucose) are involved in sensory metabolites, whereas disaccharides (tetrahalose, sucrose, raffinose and fructans) play a role in stress response and are also said to interact with the ROS signalling pathway (Couèe *et al.,* 2006). Soluble sugars have two roles with regards to ROS; they are either involved in ROS producing pathways or they are involved in ROS scavenging. On the one hand, excess sugars produced in the leaves due to increased photosynthesis, result in an excess production of cytosolic H_2O_2 (Keunen *et al*., 2013). On the other hand, soluble sugars can feed the oxidative pentose phosphate pathway (OPP), which generates NADPH that contributes to the production of glutathione (GSH), a H_2O_2 scavenger (Bolouri-Moghaddam *et al*., 2010). Soluble sugars have been found to accumulate under salt stress and research shows that their increase specifically glucose, sucrose and fructose play a role in carbon storage, osmotic homeostasis, osmoprotection and scavenging of free radicals (Rosa *et al*., 2009). Sugars play a major role in signalling within plants to modulate their sensitivity, which ultimately helps in cell response (Khan and Khan, 2014).

1.6.3 Signalling under abiotic stress

In order for plants to survive stress it is crucial for them to receive a constant signal of their external environment since they are sessile organisms (Wang *et al.,* 2003). Plants have thus developed an intricate network of signalling, which respond to these external stresses either specifically or with more integrated signalling cascades (Bray *et al.,* 2000). When a plant experiences stress, receptors (G-protein-coupled receptors, receptor like kinase, ion channel or histidine kinase) on the membrane will perceive the stress signal, which then allows for the activation of signalling molecules such as inositol phosphates, hormones, ROS and Ca^{2+}. The signal will then reach the nucleus where stress responsive genes are induced and ultimately leads to stress tolerance. There are two types of stress responsive genes, early genes, which are induced within minutes and delayed induced genes that are induced by the products of the early induced genes (Tuteja, 2007). Signalling molecules, including hormones, proteins, nucleotides, amino acids and growth regulators, amongst others are crucial for plant survival since they allow for the activation of the antioxidant defence system against stresses (Dmitriev, 2003). In the case of stressful conditions signalling molecules are synthesised and interact with other molecules such as osmolytes as well as phytohormones and other metabolites that accumulate and interact with one another (Saed-Moucheshi *et al*., 2014).

1.6.3.1 Calcium signalling in plants

Calcium is an essential ubiquitous nutrient in plants, a structural component of cell walls and membranes and it mainly serves as a second messenger in various processes (Thor, 2019). These processes include but are not limited to response to stress (abiotic and biotic), growth of root or pollen and fertilization (Zhang *et al.,* 2017). The plasma membrane contains Ca^{2+}

permeable channels through which Ca^{2+} enters the plant cells (White, 2000). High concentrations of cytosolic Ca^{2+} are toxic to plants and their levels are maintained by Ca^{2+}-ATPases and H^+ /Ca^{2+}-antiporters in unstimulated cells (Sze *et al.,* 2000; Hirsch, 2001; Tuteja, 2009). Plants are exposed to various external stimuli through abiotic and biotic factors, which results in changes in cytoplasmic Ca^{2+} concentrations and ultimately cause the regulation of a variety of responses (Maathuis, 2009). Cell walls and organelles contain high levels of Ca^{2+} ranging from 0.1- 80 mM whereas cytoplasmic levels of Ca^{2+} are ~100 nM (White and Broadley, 2003; Stael *et al*., 2011). According to Epstein (1997) plants require a constant supply of Ca^{2+} at 1-10 nM for normal growth and development. Moreover, Ca^{2+} must be tightly regulated in plants since a deficiency or high concentrations may result in localised cell death (Ng and Mcainsh, 2003). In the event of Ca^{2+} deficiency, plants are more at risk to plant pathogens and may experience a reduction in growth of apical meristems, chlorotic leaves and cell wall breakdown (White and Broadley, 2003).

1.6.3.2 The effect of calcium under salt stress

Calcium uptake is severely affected by salinity stress and this is evident in shoots since they show symptoms of calcium deficiency under salt stress, which is particularly displayed in salt sensitive genotypes (Grieve and Maas, 1988; Cramer *et al.,* 1987). Under salt stress, Na^+ concentrations and uptake increase while Ca^{2+} concentrations and uptake decrease (Blumwald *et al.,* 2000; Hadi *et al.,* 2008). The same effect is observed when the concentration of the external Ca^{2+} concentrations increase, and the Na^+ concentration and uptake decreases (Lazof and Bernstein, 1999; Ashraf and Orooj, 2006). Increased salinity also results in a reduction of Ca^{2+} at the plasma membrane of root hairs as observed in *Gossypium hirsutum* (Cramer *et al.,* 1985). Similarly a reduction in plasma membrane bound Ca^{2+} was observed in *Zea mays*, *Triticum aestivum* and *Hordeum vulgare* protoplast under salt stress (Lynch and lauchli, 1988). On the other hand, exogenous application of Ca^{2+} has shown to have ameliorative effects on

the harmful effects of salt stress by maintaining the toxic effects of Na^+ ions, which are associated with salt stress (Bliss *et al.,* 1986). It was suggested that Ca^{2+} may alleviate stress by maintaining the plasma membrane in roots and shoots (Läuchli, 1990; Läuchli and Schubert, 1989). Exogenous Ca^{2+} has also shown to reduce Na^+ binding to cell walls of intact *Hordeum vulgare* roots (Stassart *et al.,* 1981). Similarly exogenous Ca^{2+} reduced Na^+ from binding to the plasma membrane of intact cotton root hairs (Cramer *et al*., 1985).

1.6.3.3 Calcium influence potassium levels under salt stress

Ion homeostasis is essential for the proper functioning and the physiology of living cells (Muhling and Lauchli, 2002). Thus, it important to maintain the levels of essential ions by keeping the toxic ions at low concentrations (Hadi and Karimi, 2012). Potassium (K^+) is one of the three important nutrients that plants require and is the most abundant inorganic cation found in the cytoplasm of plants and animals (Demidchik *et al.,* 2014). Unlike K^+, Na^+ is not a vital nutrient for plants (Assaha *et al.,* 2017) rather its accumulation affects ion homeostasis. Potassium plays many important physiological roles in cells including controlling tugor pressure, cell elongation and cell movement (Shabala and Pottosin, 2014). It also plays a direct and indirect role in the regulation of photosynthesis, protein synthesis and enzyme activities, as well as maintaining cytoplasmic pH homeostasis (Wang *et al.,* 2009). Under salt stress K^+ levels were reduced in the roots and leaves of durum wheat due to increased Na^+ concentration (Hadi *et al.,* 2008). This is because excess Na^+ causes a nutritional imbalance in plant cells by competing with K^+ to enter plant root cells (Shabala *et al.,* 2006). This ultimately results in plant death since the low K^+/Na^+ ratio becomes toxic (Hadi *et al*., 2008). Adequate concentrations of Ca^{2+} can then change the K^+/Na^+ ratio by increasing the K^+ levels while decreasing Na^+ (Shabala *et al.,* 2006). An improvement in Ca^{2+} results in an improvement of

membrane integrity and in turn reduces the leakage of K^+ from the roots (Cachorro *et al.,* 1993). Plants respond to salt stress by an increase in cytosolic free Ca^{2+}, however, the receptor that initially senses salt stress is unknown (Knight *et al.,* 1997). This increase in Ca^{2+} levels serves as a second messenger, which activates machineries that are involved in controlling K^+/Na^+ discrimination and K^+ uptake (Parre *et al.,* 2007). Liu *et al,* suggested that the Ca^{2+} binding protein "*Salt Overly Sensitive 3 (SOS3)"* is a sensor for the salt induced Ca^{2+} signature, which was identified in *Arabidopsis* (Liu *et al*., 2000).

1.6.4 The SOS pathway

An array of signalling pathways have been discovered over the years in order to understand the molecular mechanisms of salt tolerance in plants (Pandey *et al.,* 2010). The SOS signalling pathway is well defined and is involved in ion homeostasis by the extrusion of Na^+ from the cytosol (Shi *et al.,* 2000). Investigations of salt tolerance started in the 1990s by performing mutational analysis on the glycophyte Arabidopsis and its responses to salt stress (Zhu *et al.,* 1998). As a consequence of these investigations the main components of the SOS pathway were identified, which comprise of SOS1, SOS2 and SOS3 (Wu *et al.,* 1996). In the presence of salt stress the SOS pathway is activated by a transient spike in Ca^{2+} (Yang *et al.,* 2009; Ji *et al.,* 2013). SOS3 encodes a Ca^{2+} binding protein that senses the spike in Ca^{2+} and activates SOS2, a serine/threonine kinase (Ji *et al.,* 2013). Then the SOS3-SOS2 complex phosphorylates and activates SOS1 through the SOS2 kinase. The SOS1 is the plasma membrane Na^+/H^+ antiporter, which allows for the extrusion of excess Na^+ from the cytosol (figure 2) (Ma *et al.,* 2014).

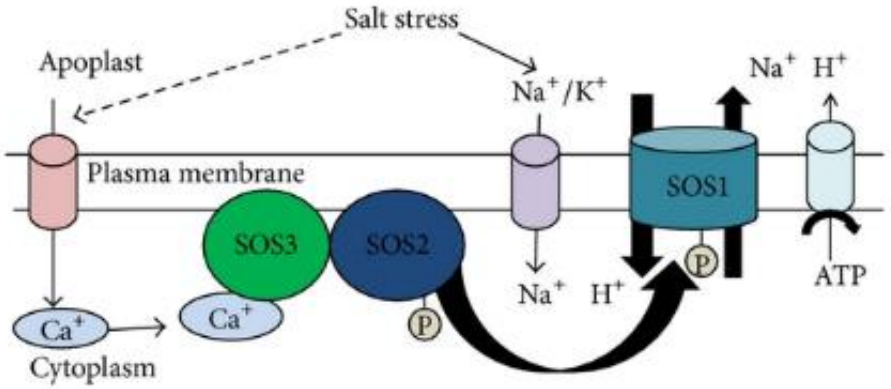

Figure 1.2. The SOS pathway in response to salt stress. Salt stress causes a spike in Ca^{2+} levels in the cytoplasm, which activates the SOS pathway. Ca^{2+} binds to and activates SOS3, which then interacts and binds to SOS2 releasing it from its self-inhibition and activates the kinase. The SOS3-SOS2 complex is recruited to phosphorylate SOS1 at the plasma membrane, which results in the efflux of Na^+ from the cytoplasm (adapted from Gupta and Huang, 2014).

1.6.4.1 SOS1

Ion homeostasis is crucial for plant growth and adaption and plants rely on Na^+/H^+ antiporters to maintain Na^+ homeostasis in the cytosol (Agarwal *et al*., 2013). There are two mechanisms used by plants to remove excess Na^+ from the cytosol, namely; the plasma membrane Na^+/H^+ antiporters (SOS1) and the vacuolar Na^+/H^+ antiporters (vacuolar NHX) (Shi *et al*., 2003). SOS1 is involved in the efflux of Na^+ from the cytoplasm whereas vacuolar NHX is involved in transporting Na^+ into the vacuole (Chinnusamy *et al*., 2004; Apse and Blumwald, 2007). The sequestration of Na^+ into the vacuole not only contributes to lowering the Na^+ in the cytosol but it also contributes to cellular osmotic water uptake as well as cell turgor and expansion (Chinnusamy *et al*., 2004; Horie and Schroeder, 2004). Additionally, extensive research has been done on the plasma membrane Na^+/H^+ antiporter or the SOS1. It was determined that SOS1 encodes a polypeptide with 1146 amino acid residues and a protein size of 127 kDa. The N-terminal region of SOS1 is highly hydrophobic with 12 predicted transmembrane domains. Blast analysis revealed strong similarities between SOS1 and the Na^+/H^+ antiporter

transmembrane regions of microbial and animal origins (Mahajan *et al.,* 2008). The C-terminal domain of the SOS1 has a long hydrophilic tail, which is unique with no similarities with other antiporters. Furthermore, a variety of genetic, biochemical and physiological measures have been explored to determine how ion homeostasis is controlled through the SOS1 mechanism (Ji *et al.,* 2013).

An investigation done in yeast mutant strains, using highly purified isolated plasma membrane vesicles was the first to show the ability of SOS1 to transport Na^+ out of cells in the presence of salt stress (Qui *et al.,* 2002; Shi *et al*., 2002). This was followed by further investigations that led to the isolation of *sos1* mutants from *Arabidopsis* using a root-bending assay (Wu *et al*., 1996). The mutants showed salt sensitivity and more growth inhibition to NaCl compared to the wild type (Wu *et al*., 1996). According to Zhu *et al*., *sos1* mutants are more sensitive to Na^+ and Li^+ ions compared to *sos2* and *sos3* mutants (Zhu *et al.,* 1998). In addition SOS1 can also be activated by the phospholipase D (PLD) signalling pathway (Shabala *et al.,* 2005; Yu *et al.,* 2010). Upon exposure to salt stress PDLα1 enzyme activity is increased in Arabidopsis, which causes a transient increase in phosphatidic acid (PA), a lipid second messenger. PA activates Mitogen-Activated Protein Kinase 6 (MAPK6), which then phosphorylates SOS1 (Yu *et al.,* 2010). Interestingly a study found that a 50 % reduction in the expression of SOS1 through RNA interference in the halophytic *Thellungiella salsuginea* resulted in ion accumulation causing a loss in its halophytism (Oh *et al.,* 2009).

1.6.4.2 SOS2

The most vital regulatory mechanisms in plant signalling cascades is phosphorylation by protein kinases such as mitogen-activated protein kinases (MAPK), calcineurin B-like-interacting protein kinases (CBL-CIPKs), calcium dependent protein kinases (CDPKs) as well

as other kinases (Luan *et al.,* 2002; Batistic and Kudla, 2004). SOS2 plays an important role in maintaining salt tolerance through Na^+ and K^+ ion homeostasis (Liu and Zhu, 1998). SOS2 encodes a Serine/ Threonine protein kinase of 446 amino acid with a protein size of 51 kDa (Zhu, 2000; Mahajan *et al.,* 2008). The N-terminal region of SOS2 contains a kinase catalytic domain and the C-terminal comprises its regulatory domain. The N-terminal domain is similar to that of yeast sucrose non fermenting 1 (SNF1) and mammalian-activated protein kinase (Pandey *et al.,* 2010), whereas the C-terminal domain is unique to the plant CIPKs.

In the transient increase of Ca^{2+} under salt stress, SOS3/CBL4 activates the phosphorylation activity of SOS2/CIPK24 by interacting with the NAF/FISL motif in the regulatory domain of SOS2 (Halfter *et al.,* 2000). All CIPKs contain a highly conserved FISL motif named the NAF motif at the C-terminal regulatory domain (Li *et al*., 2009). The NAF motif consists of 21 amino acid residues and is named after its highly conserved amino acids; Asparagine (N), alanine (A), phenylalanine (F), Isoleucine (I), serine (S) and leucine (L) which is required for CBL binding (Mao *et al*., 2016). Additionally, SOS2 was found to modulate tonoplast proteins including the Ca^{2+} /H^+ antiporter Cation Exchanger1 (CAX1) from Arabidopsis as well as the vacuolar proton ATPase (V-ATPase) (Ji *et al*., 2013). According to Ji *et al* (2013) this finding suggests that SOS2 plays a central role in the regulation of transport activities (Cheng *et al.,* 2004; Batelli *et al.,* 2007).

1.6.4.3 SOS3

As a consequence of salt stress, Ca^{2+} signatures are generated, which can be sensed by various Ca^{2+} sensors including calmodulin (CaM), CaM-like proteins, CDPKs as well as novel CBLs recently discovered in *Arabidopsis* (Luan, 2009). CBL proteins have been found to function in salt stress signalling via the SOS pathway or via SOS-like pathways. Additionally, SOS3 are Ca^{2+} sensors that encodes an EF hand type Ca^{2+} sensor with an *N*- myristoylation motif (Liu and Zhu, 1998). The functioning of SOS3 is dependent on calcium binding activity and *N*-

myristoylation (Ishitani *et al*., 2000). A SOS3 homolog CBL 10 was reported by Quan *et al*., as being the main mediator in salt tolerance and is involved in regulating the SOS pathway in *Arabidopsis* (Quan *et al.,* 2007). The SOS3 homolog, SOS3-like calcium binding protein 8 (SCABP8)/ CLB10 responds to salt toxicity mainly in the shoots whereas SOS3 functions mainly in the roots under salt stress. The *sos3* mutants showed a reduction in root growth while *scabp8* mutants showed reduction in the shoots. Additionally, when SCABP 8 was overexpressed in *sos3* mutants, its salt sensitive phenotype was partially protected, whereas the overexpression of SOS3 did not show protection in *scabp8* mutants. Although CBL 10 and SOS3 together modulate the activity of SOS2 and protect the roots and shoots under salt stress, they each have additional unique roles in maintaining salt tolerance in plants (Du *et al.,* 2011). Finally SOS3 was found to play a role in the regulation of developmental plasticity of lateral roots in *Arabidopsis* under low salt stress. The *sos3* mutant negatively affected the activation in lateral root primordia (LRP) (Zhao, 2011).

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1.5 Gene expression analysis

Gene expression dictates phenotypic alterations, which maintains the complexity of the organism (Das and Bansal, 2019). The variation of gene expression is determined by either measuring the number of messenger RNA (mRNA), generally known as the level of expression or by measuring the number of tissues in, which the genes are expressed, generally known as the breadth of expression (Khaitovich *et al*., 2005). Both are able to reveal the diversity as well as the development of specific tissues (Rosa *et al*., 2014). In human, chicken and other metazoans the compactness of genes, coding DNA sequence (CDS) length, gene length including parameters involved in the intron size are negatively related to the level of expression and expression breadth (Castillo-Davis *et al*., 2002). However, in plants, specifically Arabidopsis and rice, highly expressed genes have longer introns, CDS and untranslated region (UTR) in comparison to genes that are expressed at lower levels (Ren *et al*., 2006). When plants experience abiotic stress many genes are activated on the transcriptional level, which then regulate downstream genes as well as produce vital metabolic proteins that will provide stress tolerance (Kavar *et al*., 2007). Thus, several studies utilise transcript profiling to characterise stress responsive genes in plants (Bohnert *et al*., 2009).

To this end, many studies are able to understand plant stress response through investigating the transcript levels of various stress response genes. A recent study by Ma *et al*., conducted an expression analysis of nine SOS genes in response to salt and PEG stress in *Vitis vinifera* (grapevine) and found that all except one *VviSOS3* gene was upregulated in the leaves in response to both salt and PEG stress. Similarly in the roots majority of the *VviSOS3* genes except one were upregulated in the roots in response to PEG stress (Ma *et al*., 2019). Additionally, a study by Gálves *et al*., investigated the gene expression of four vacuolar NHX antiporter isoforms in salt sensitive (*Solanum lycopersicum* L. cv. Volgogradskij) and salt tolerant (*Solanum pimpinelifolium* L.) tomato species. The isoforms included; *LeNHX1,*

LeNHX2, LeNHX3 and *LeNHX4* and displayed varying expression patterns under salt stress. The study reported that in the absence of salt stress the transcript level for *LeNHX2, 3* and *4* were similar in both species, while *LeNHX1* had a higher transcript level in the tolerant species. In both species *LeNHX2* and *LeNHX3* had similar expression levels in the root, stem and leaves whereas, *LeNHX4* was highly expressed in the stem and fruit tissues. In the presence of salt stress all isoforms were induced especially *LeNHX3* and *LeNHX4* isoforms in the salt tolerant species (Gálves *et al*., 2011).

Moreover several studies have been conducted on the response of antioxidant genes to stresses in various plant species. A recent study by Soleimani *et al*., reported on the antioxidant response of *Cuminum cyminum* L. to salt stress. It was determined that at 50, 100 and 150 mM NaCl, the activity of the antioxidant enzymes "*SOD, APX and CAT*" increased. These same conditions induced the expression of Fe-SOD and CAT (Soleimani *et al*., 2017). A more recent study by Jalali *et al*., investigated the response of the antioxidant system of two *Oryza sativa* cultivars to salt stress (Jalali *et al*., 2020). The study corroborated the results obtained in *Cuminum cyminum* L. as demonstrated previously (Soleimani *et al*., 2017). Furthermore, novel genes and pathways for salt stress tolerance in *Suaeda salsa* were identified through transcriptomic analysis (Zhang *et al*., 2020).

1.6 Scanning electron microscopy (SEM) and Energy-Dispersive X-Ray (EDX) spectroscopy

Scanning Electron Microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX), combined or individually, are valuable techniques that can be used to examine changes on the plant cell epidermis and xylem walls as well as the element distribution in the plants (Michalak *et al*., 2014). Scanning electron microscopy produces images using a focused beam of electrons

that react with a sample (Egerton, 2005). Once the focused beam of electrons make contact with the sample it produces secondary electrons (SEs), backscattered electrons and characteristic X-ray, which gets detected by specific detectors and finally creates an image that gets displayed on a monitor (Zhou *et al*., 2007). Moreover, EDX is used to detect the composition of elements in a substance by using SEM. EDX detects elements that have higher atomic number than Boron elements at concentrations as low as 0.1 %. The characteristic X-rays produced during SEM are differentiated and their concentrations are measured on the basis that none of the elements have the same X-ray emission spectrum (Goldstein *et al*., 2003). The X-ray results from the interaction between the primary beam of electrons with the nucleus of the sample atom (Kalinski *et al*., 2012). This technique has been widely used to measure elements in several species including the green macroalga "*Vaucheria sessilis"* (Michalak *et al*., 2014), *Psychotria sarmentosa* (Van Der Ent *et al*., 2018) and four different cultivars of turfgrasses including; *Cynodon* ssp., *Zoysia* ssp., *Stenotaphrum secundatum* and *Paspalum vaginatum* (Chavarria *et al*., 2020).

1.7 Problem Statement

As the world population is increasing at an alarming rate, agriculture faces the challenge to increase food production by at least 70 % in 2050 (FAO, 2017). Abiotic stresses mainly extreme temperatures, drought and salinity are the leading factors contributing to 50 % of the world crop loss. Soil salinity has been estimated to affect 20 % of cultivated land and 33 % of irrigated agricultural land worldwide (Shrivastava and Kumar, 2015). Soil salinity mainly hinders plant growth and development through osmotic and ionic imbalances which affects many physiological and metabolic processes. Moreover, because crops respond differently to salinity stress, it is of importance to monitor these responses at an early growth stage. Signalling

molecules such as calcium (Ca^{2+}) triggers a series of physiological reactions in plants, leading to stress tolerance.

1.8 Aims and Objectives

The aim of the study is to understand the effect of salinity on the germination and growth of *Sorghum bicolor* and elucidate the mechanism by which Ca^{2+} signalling can ameliorate the effect of salt stress. The aim will be achieved through the following objectives:

- To germinate sorghum seedlings under salt stress in the absence (untreated) and presence of Ca^{2+} (treated).
- To assay the physiological response of sorghum to salt and Ca^{2+} by performing germination and growth assays.
- To measure the biochemical response of sorghum to salt and Ca^{2+} by assaying the osmolyte and oxidative stress markers.
- To study the anatomy and element distribution of the sorghum seedlings in response to salt and Ca^{2+} using Scanning Electron Microscopy-Energy dispersive X-ray spectroscopy (SEM-EDX).
- To analyse the expression patterns of selected antioxidant enzymes , salt overly sensitive pathways genes and the plasma Na^+/H^+ exchanger antiporter using quantitative Real-time PCR.

CHAPTER 2

MATERIALS AND METHODS

2.1 Germination and growth media

The medium used for the germination included different solutions prepared in autoclaved distilled H_2O. The solutions included the salt stress control; 0 mM NaCl (autoclaved dH_2O only), various sodium chloride concentrations; 100 mM, 200 mM and 300 mM NaCl and various calcium chloride concentrations; 5 mM, 15 mM and 35 mM $CaCl_2$.

2.2 Seed preparation and growth conditions

Red sorghum (*Sorghum bicolor*) seeds were purchased from Agricol, Brackenfell Cape Town, South Africa. In order to maintain uniformity in the study, seeds were chosen based on possessing an identical size and colour. Seeds were decontaminated as previously described (Mulaudzi-Masuku *et al*., 2015). Briefly, seeds were soaked for 1 minute in 70 % ethanol while shaking at 600 rpm, followed by three washes with autoclaved dH_2O. In the final step, seeds were soaked in 5 % bleach for 1 hour with shaking at 600 rpm, followed by three washes with autoclaved dH_2O. After decontamination, seeds were imbibed overnight in autoclaved dH_2O at 25 °C with shaking at 600 rpm. Seeds were then dried under the laminar flow and five seeds were sown on a BioPa MN 218 B blotting paper placed on sterilised petri dishes containing 4 ml of the growth medium described in section 2.1. Petri dishes containing the seeds and medium were placed at 25 °C and seeds were germinated for 7 days in the dark. Seeds were inspected daily and the number of germinated seed (>2 mm radicle) were counted. On day 3 only the root lengths were measured, whereas both root and shoot lengths were measured on day 7. Seedlings were harvested on day 7, rinsed thoroughly to remove excess salts and used immediately or stored at -80 °C for future use.

2.3 Physiological analysis

Germination assays including germination percentage, mean germination time, germination index and total germination were calculated according to the equations shown below:

2.3.1 Germination percentage (GP): n/N x 100, where n: total number of seeds germinated and N: the total number of seeds sown (Pandey and Penna, 2017). Formula 1.

2.3.2 Mean Germination Time (MGT): $\sum f * x / \sum f$, where f: number of seeds germinated on day x (Kader, 2005). Formula 2.

2.3.3 Germination index (GI): ∑ (n1 x d7) + (n2 x d6) + (n3 x d5)…… (n7 x d7) where n1: number of seeds germinated on day 1 and d7: number of seeds germinated on day 7. According to Kader, 2005, where a heavier weight is given to seeds germinated on the first day and less weight given to those germinated on the last day. Formula 3.

2.3.4 Total germination (TG): d7/N x 100, where d7 total number of seeds germinated on the final day (day 7) and N: the number of seeds germinated (Kader, 2005). Formula 4.

2.3.5 Growth attributes: Seedling lengths including roots and shoots were measured with a ruler (mm scale) on day 7.

Fresh weights were measured by weighing the whole seedling on day 7. Dry weights were obtained after oven-drying seedlings at 80°C overnight or until constant weight.

2.4 Biochemical analysis

Biochemical assays conducted in this study included measurements of osmolytes (Proline content and total soluble sugars), ROS (Hydrogen peroxide), and lipid peroxidation

(Malondialdehyde content). Plant material was grounded in liquid nitrogen using a motor and pestle for all the methods to follow.

2.4.1 Proline content

Proline content was determined as previously described (Pandey and Penna 2017), with slight modifications. About 0.5 g of grounded plant material was homogenised with 2 ml of 3 % sulfosalicylic acid. The homogenised samples were then centrifuged at 5000 rpm for 5 minutes and 0.5 ml of the supernatant was transferred to a clean tube containing 0.5 ml acetic acid and 0.5 ml ninhydrin acid reagent (1.25 g of ninhydrin dissolved in 30 ml of 99 % acetic acid and 20 ml of 6 M H_3PO_4) and boiled for 45 minutes in a water bath. Samples were then allowed to cool on ice completely before adding equal volumes of toluene and reading the optical density of the samples at 520 nm using the Helios® Epsilon visible 8 nm bandwidth spectrophotometer (Thermo Fisher Scientific, USA).

2.4.2 Total soluble sugars

Total soluble sugars were determined as previously described (Watanabe *et al.,* 2000) with slight modifications. About 0.1 g grounded plant material was homogenised in 10 ml of 80 % ethanol. The mixture was centrifuged at 10 000 rpm for 10 minutes and 1 ml of the supernatant was combined with 3 ml of anthrone reagent (0.15 g anthrone, 100 ml of 96 % H_2SO_4). Samples were further placed in a boiling water bath for 15 minutes, which was followed by cooling the reaction on ice until cold. The optical density was read at 620 nm using the Helios® Epsilon visible 8 nm bandwidth spectrophotometer (Thermo Fisher Scientific, USA). The total soluble sugar content was determined by generating a standard curve using glucose as a standard sugar and the content was expressed as mg g^{-1} FW.

2.4.3 Hydrogen peroxide content

Hydrogen peroxide (H_2O_2) was assayed following the optimised method by Junglee *et al* (2014). About 0.15 g ground plant material was homogenised in 0.25 ml trichloroacetic acid (TCA) (0.1 % w/v), 0.5 ml potassium iodide (1 M) and 0.25 ml potassium phosphate buffer (10 mM, pH 6). Tubes were vortexed and centrifuged for 15 minutes at 10 000 rpm (4 °C). Samples were transferred to a 96 well microtiter plate and were allowed to incubate at room temperature for 20 minutes. Absorbance was read at 390 nm using the FLUOstar® Omega microtiter plate reader (BMG LABTECH, Ortenberg, Germany). H_2O_2 was quantified by generating a standard curve with a H_2O_2 solution.

2.4.4 Malondialdehyde determination

Malondialdehyde (MDA) was estimated as described previously (Dhindsa *et al.,* 1981) using the colorimetric assay. About 0.1 g grounded plant material was mixed with 1 ml of 0.1 % TCA and centrifuged at 13 000 rpm under 4 °C conditions for 10 minutes. About 1 ml of 0.5 % thiobarbutric acid (TBA), which was prepared in 20 % TCA was added to 0.4 ml of the supernatant. Tubes were punctured with a needle and incubated in a water bath that was set at 80 °C for 30 minutes and immediately placed on ice. Tubes were centrifuged for 5 minutes at 13 500 rpm under 4 °C. Absorbance readings were measured at 532 nm and 600 nm with the Helios® Epsilon visible 8 nm bandwidth spectrophotometer (Thermo Fisher Scientific, USA). MDA content was further calculated using Formula 5.

$$nmol\ MDA/gFW = \frac{\Delta A_{corrected} \times 3.5 \times x1000}{\varepsilon \times b \times y} \quad \text{(Formula 5).}$$

Where: $\Delta A_{corrected}$= A532 – A600 corrected with $\Delta A_{corrected}$of the blank, b = light path length (0.56 cm for 200 µl), ε = millimolar extinction coefficient (155 mM^{-1}), 3.5 (dilution factor from 400 µl extract + 1 ml TBA/TCA solution), *x* (ml) = TCA 0.1 % used from extraction (1 ml), y (g) = fresh weight (FW) used for extraction and 1000 conversion factor.

2.5 Anatomical and element analysis

Element and anatomical analysis was done using the Scanning Electron Microscopy-Energy dispersive X-ray spectroscopy *(SEM-EDX)* system. Analysis were undertaken of *Sorghum bicolor* root seedlings that were grown in the absence (0 mM) and presence of 200 and 300 mM NaCl and treated with 5 mM $CaCl_2$. A small amount of grinded oven dried sample were placed on aluminium stubs coated with conductive carbon tape. Samples were then coated with a thin layer of carbon using an EMITECH-K950x carbon coater. Samples were imaged using a Zeiss Auriga field emission gun Scanning Electron Microscope, operated at an accelerating voltage of 5 kV using an in-lens secondary electron detector and element analysis were collected with an Oxford X-Max silicon solid-state drift detector at an accelerating 20 kV for 1 minute to ensure proper x-ray detection. All spectra were analysed using the built in Oxford Azetec software electron detector at the electron microscope unit, Physics Department, University of the Western Cape.

2.6 Sequence retrieval and analysis of the physiochemical parameters

Sorghum bicolor gene sequences analysed in this study included sequences coding for antioxidants, the Sodium/hydrogen (Na^+/H^+) exchanger antiporter enzymes and members of the Salt Overly Sensitive (SOS) pathway. Antioxidants included; *Superoxide dismutase* (*SbSOD4A*) annotated as *Sorghum bicolor* superoxide dismutase [Cu-Zn] 4A (Accession

numbers: LOC110431820, transcript variant X1, mRNA sequence ID: XM_021451466.1), *Ascorbate peroxidase* (*SbAPX2*) annotated as *Sorghum bicolor* L-ascorbate peroxidase 2, cytosolic (Accession numbers: LOC8077530, mRNA Sequence ID: XM_002463406.2), and catalase (*SbCAT3*) annotated as *Sorghum bicolor* catalase isozyme 3 (Accession numbers: LOC8068221, mRNA Sequence ID: XM 021460018.1). The Na^+/H^+ exchanger antiporter enzymes included the plasma Na^+/H^+ exchanger antiporter, also known as the SOS1 annotated as *Sorghum bicolor* sodium/hydrogen exchanger 8 (Accession numbers: LOC8074408, mRNA Sequence ID: XM 002443629.2), and the vacuolar Na^+/H^+ exchanger antiporter annotated as *Sorghum bicolor* sodium/hydrogen exchanger 2 (Accession numbers: LOC8074408, mRNA Sequence ID: XM 002461123.2). The SOS2 (*SbSOS2*) annotated as *Sorghum bicolor* CBL-interacting protein kinase 8 (Accession numbers: LOC8074979, mRNA sequence ID: XM_002455707.2) and SOS3 (*SbSOS3*) annotated as *Sorghum bicolor* calcineurin B-like protein 7 (Accession numbers: LOC8081639, mRNA sequence ID: XM_002453683.2). All sequences were obtained in FASTA format from the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Properties such as CDS, protein length, molecular weight (M_w), and theoretical isoelectric point (*pI*) were computed using the ExPASy Proteomic server (http://www.expasy.org/) (Gasteiger *et al*., 2005).

2.7 RNA extraction and cDNA synthesis

Total RNA was extracted from 0.1 g of the roots and shoots of 7 day old sorghum seedlings, using the Favorgen plant mini RNA extraction kit (Favorgen Biotech Corp, Ping-Tung, Taiwan) as instructed by the manufacturer. Genomic DNA was removed by treating the RNA with the RNase-free DNase reaction solution (New England Biolabs, Massachusetts) and its quality was determined by analysing on a 1 % agarose gel. The concentration and purity were determined using a NanoDrop spectrophotometer (Thermo Scientific, USA). Copied DNA

(cDNA) was synthesised from 1 µg of total RNA using the Superscript™ III First-Strand Synthesis kit (Invitrogen, Carlsbad, California, USA) as instructed by the manufacturer.

2.8 Quantitative real time PCR

Quantitative real-time PCR (qRT-PCR) was used to analyse the expression profiles of sorghum antioxidant genes namely; *SbSOD4A*, *SbAPX*2 and *SbCAT*3, the *SOS* pathway genes namely; *SbSOS1, SbSOS2* and *SbSOS3* and the *vacuolar SbNHX2* genes. Reference genes included *Beta actin* and *Ubiquitin* (*UBQ*) as previously described (Mulaudzi-Masuku *et al*., 2015). The primer information and accession numbers of the target and reference genes can be found in Table 1. The reaction mixture contained 1 µl template cDNA, 5 µl 2x SYBR Green I Master Mix (Roche Applied Science, Germany), varying concentrations of each primer and ddH_2O added to a final volume of 10 µl. The reactions were subjected to denaturation at 95 °C for 10 minutes, 45 cycles at 95 °C for 10 seconds, 55 °C (*SbSOD4A*, *SbCAT3, SbAPX2*); 58 °C (*SbSOS1, SbSOS2, SbSOS3*) and 60 °C (*SbNHX2*); for 10 seconds, and 72 °C for 20 seconds. A melting curve analysis was also performed using default parameters on the LightCycler® 480 instrument (Roche Applied Science, Germany). The expression levels of the target genes were normalized to the reference genes and analysed using the LightCycler® 480 SW (version 1.5) data analysis software. The expression was quantified by relative quantification method using a standard curve of serially diluted cDNA templates. Each qRT-PCR reaction was done in triplicate and three non-template controls were included. Data represent the average of three independent experiments.

Table 1.1. Target genes and their accession numbers used to design primers for qRT-PCR.

TARGET GENE	FORWARD PRIMER	REVERSE PRIMER	ACCESSION NUMBER
SbSOD4A	5'-TGG ACT CCA GGG CTG AAA AT-3'	5'-TCG GTT GCT ACA GGT GCT TA-3'	XM_021451466.1
SbAPX2	5'-AGTCGTGGCAGTTGAGGTAA-3'	5'-ATCCTTGTGGCATCTTCCCA-3'	XM_002463406.2
SbCAT3	5'-GGTTCGCCGTCAAGTTCTAC-3'	5'-AAGAAGGTGTGGAGGCTCTC-3'	XM_021460018.1
SbSOS1	5'-ACACGGGAGAGAGAGAGAGT-3'	5'-TCCAGCTCCAAGTTTGCCTA-3'	XM_002443629.2
SbSOS2	5'-AACCATCACGAATCCCAGGT-3'	5'-AACCATCACGAATCCCAGGT3'	XM_002455707.2
SbSOS3	5'-GGGAAGGAACACTGGTCACT-3'	5'-TCCTTCAGTGCGTTGTCTCT-3'	XM_002453683.2
SbNHX2	5'-ACTACTGGCGCAAGTTCGAT-3'	5'-TGTCGGCAACACAAAAACAT-3'	XM_002461123.2
BETA ACTIN	5'-CCTTACCGACTACCTCAT-3'	5'-ATGATCCTTCCTAATATCCA-3'	AF369906.1
UBQ	5'-GCCAAGATTCAGGATAAG-3'	5'-TTGTAATCAGCCAATGTG-3'	XM_002452660

2.9 Statistical analysis

All assays conducted in this study included four biological replicates (germinating seedlings from four independent experiments conducted at different times) and three technical replicates. The data was statistically analysed by the one-way or two-way ANOVA test where appropriate, using GraphPad Prism version 8.4.2 (2020). Statistical significance between control and treated plants were determined by the Bonferroni multiple comparison test and represented as *** = $p \leq 0.001$, ** = $p \leq 0.01$, and * = $p \leq 0.05$.

CHAPTER 3

PHYSIOLOGICAL AND BIOCHEMICAL ANALYSIS OF THE EFFECTS OF NACL AND CA^{2+} ON THE GROWTH OF *SORGHUM BICOLOR*

ABSTRACT: Salinity severely limits plant growth and productivity and affects all aspects of the plant's development including the most crucial stage; germination. This study investigated the effect of salt (NaCl) stress on *Sorghum bicolor* seedlings and the role of exogenously applied calcium (Ca^{2+}) to ameliorate the effects of salt stress. Sorghum seeds were germinated in the presence and absence of various NaCl (100, 200 and 300 mM) and $CaCl_2$ (Ca^{2+}) (5, 15 and 35 mM) concentrations. Salt stress delayed germination, reduced root and shoot length, decreased fresh and dry weight, increased proline content and maintained soluble sugar content. There was also an increase in oxidative stress markers, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents, which resulted in damage to the epidermal and xylem layers as observed on Scanning Electron Microscopy (SEM) images. Ca^{2+} counteracted the harmful effects of salt stress by improving germination efficiency, promoting seedling growth, reducing oxidative damage and the Na^+/K^+ ratio, indicating the protective effect. Ca^{2+} also effectively protected the epidermis and xylem layers from the severe damage caused by salt stress. The results obtained suggest that sorghum is able to respond to salt stress through inducing osmolytes. Furthermore, 5 mM Ca^{2+} was determined as the optimum Ca^{2+} concentration required to enhance sorghum's tolerance to salt stress.

Keywords: Ca^{2+}; Germination; NaCl; Na^+/K^+ ratio; Osmolytes; Salt stress; *Sorghum bicolor*

3.1 RESULTS

3.1.1 Physiological analysis of the effect of NaCl and Ca^{2+} on the germination and growth of Sorghum bicolor

The effect of NaCl and Ca^{2+} on the germination and growth of sorghum was demonstrated by studying three parameters including germination (germination percentage, mean germination time, germination index and total germination), growth (root and shoot length) and biomass (fresh and dry weights) assays.

3.1.1.1 Germination assays

3.1.1.1.1 Germination percentage.

Germination percentage is a critical stage in a plant's life cycle that qualitatively measures the viability of a population of seeds during germination (Kader, 2005). *Sorghum bicolor* seeds were germinated in the absence and presence of various NaCl (100, 200 and 300 mM) and Ca^{2+} (5, 15 and 35 mM) concentrations for 7 days (Figure 3.1). Germination peaked on day 1 in the absence of NaCl (0 mM NaCl) with a mean germination percentage of 95 % while a significant decrease in germination was observed at 100 mM ($p \leq 0.05$), 200 mM ($p \leq 0.01$) and 300 mM NaCl ($p \leq 0.001$) by ~10, ~25 and ~60 % respectively. On days 3 and 7, sorghum seedlings grown under 100 mM NaCl reached a peak germination percentage of ~98 %. Furthermore, the germination of sorghum seeds remained significantly delayed under 300 mM NaCl ($p \leq 0.05$) on day 3 and 7 reaching a germination percentage of 80 and 91.5 % on the respective days. Germination of seedlings grown in the absence of NaCl reached a maximum germination percentage of 100 % on day 1 when treated with different Ca^{2+} concentrations (5, 15, 35 mM)

only (Figure 3.1B). Seeds stressed with 200 mM NaCl in the presence of 5 mM Ca^{2+} ($p \leq 0.05$) showed significant improvement in germination, which resulted in a mean germination percentage of ≥70 % with a slight increase in germination in the presence of 15 mM Ca^{2+} on day 1 compared to the control (200 mM NaCl only) (Figure 3.1C). The germination percentage of seedlings germinating under 300 mM NaCl reached up to 56 % on day 1 when treated with 5 mM Ca^{2+}.

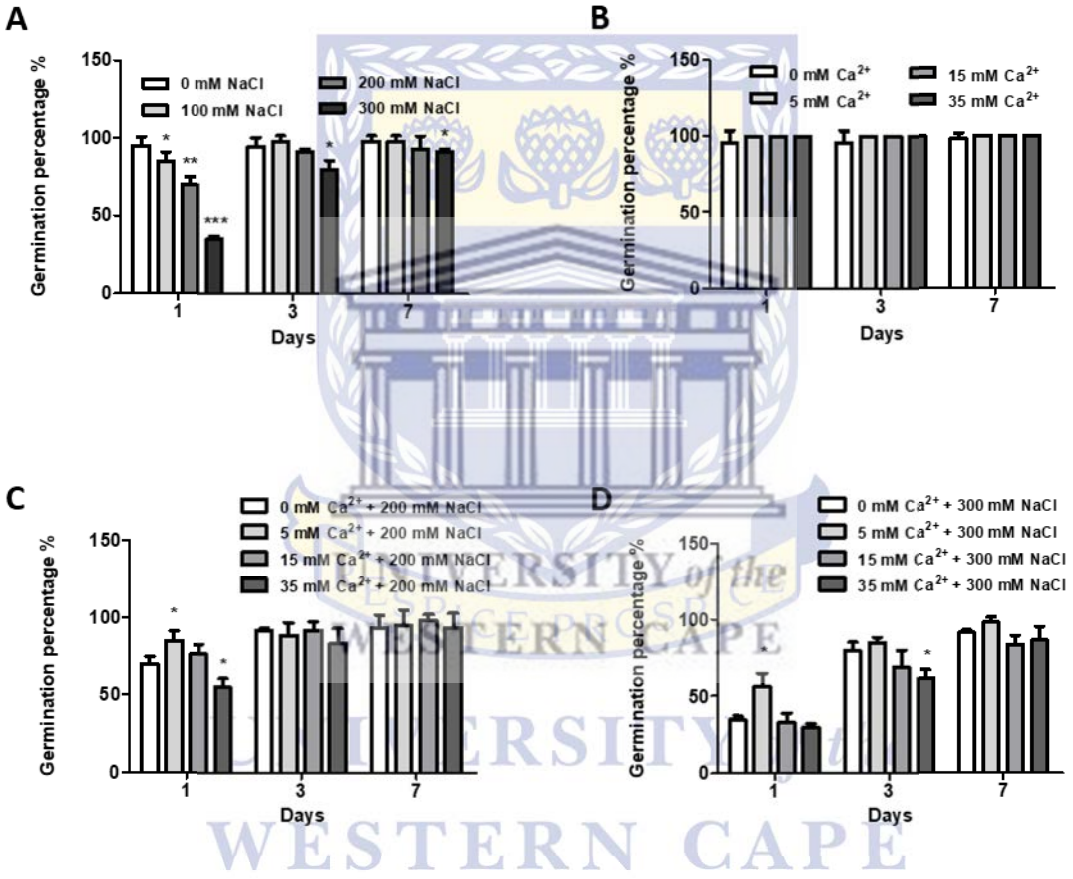

Figure 3.1. Effect of NaCl stress and Ca^{2+} on the germination percentage of sorghum seedlings. (**A**) Seedlings germinated under different NaCl concentrations only. (**B**–**D**) Seedlings germinated under different NaCl and Ca^{2+} (5, 15 and 35 mM) concentrations, (**B**) 0 mM, (**C**) 200 mM NaCl and (**D**) 300 mM NaCl. Error bars represent the SD calculated from three biological replicates. Statistical significance between control and treated plants was determined using two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as *** = $p \leq 0.001$, ** = $p \leq 0.01$, and * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

3.1.1.1.2 Mean germination time

Mean germination time is a measure of the time it takes for the seed to germinate, focusing on the day at which most seeds have germinated (Kader, 2005). Mean germination time decreased significantly ($p \leq 0.001$) for seedlings under 300 mM NaCl (Table 3.1). Application of Ca^{2+} had no significant effect on the mean germination time of control seedlings (Table 3.1), but 35 mM Ca^{2+} significantly ($p \leq 0.05$) decreased the mean germination time of seedlings under 300 mM NaCl by 0.65 fold.

3.1.1.1.3 Germination index

Germination index (GI) is a parameter that combines the percentage and speed of germination, thus, a high germination index is an indication of how fast a seed lot has germinated (Kader, 2005). A steady decrease in GI was observed when NaCl concentration increased (Table 3.1). The lowest GI observed in seedlings under 300 mM NaCl, was 95.66 units compared to 136.15 units of the control (without added NaCl). Ca^{2+} had no significant effect on the GI of control seedlings (without added NaCl). Seedlings under 300 mM NaCl treated with 5 mM ($p \leq 0.01$) and 35 mM ($p \leq 0.001$) Ca^{2+} resulted in a significant increase (108.25 units) and decrease in GI (77.83 units) respectively.

3.1.1.1.4 Total germination

Total germination (TG) of seedlings was negatively affected by increasing NaCl concentrations compared to the control (without added NaCl) and all seeds showed an approximate total TG above 90 %. Ca^{2+} increased the TG of control seedlings (without salt) from 98.25 % to 100 %. Ca^{2+} also improved the TG of salt-stressed seedlings except for 35 mM Ca^{2+}, which showed inhibitory effects (Table 3.1).

Table 3.1. Effect of Ca^{2+} on the germination attributes of sorghum seedlings in the absence (0 Mm) and presence of NaCl (200 and 300 mM). Data represented are mean ± S.D.

Ca^{2+} (mM)	NaCl (mM)	Mean germination time	Germination index (GI)	Total germination (TG)
0	**0**	12.59 ± 0.74	136.15 ± 7.70	98.25 ± 3.50
	100	11.99 ± 1.28	132.00 ± 10.86	100.00 ± 0.00
	200	11.28 ± 1.29	120.58 ± 2.23	94.25 ± 4.92
	300	7.92 ± 1.02	95.66 ± 3.93	91.50 ± 1.73
5	**0**	12.96 ± 0.00	140.00 ± 0.00	100.00 ± 0.00
	200	11.49 ± 1.84	125.33 ± 5.42	95.00 ± 5.77
	300	9.41 ± 1.36	108.25± 4.64**	98.25 ± 1.73
15	**0**	12.96 ± 0.00	140.00 ± 0.00	100.00 ± 0.00
	200	11.22 ± 1.50	124.41 ± 2.38	98.25 ± 3.50
	300	6.92 ± 0.76	85.4 ± 4.80*	83.50 ± 4.04
35	**0**	12.96 ± 0.00	140.00 ± 0.00	100.00 ± 0.00
	200	9.59 ± 2.29	109.17± 5.98**	93.25 ± 4.72
	300	5.19 ± 1.62	77.83± 4.26***	84.00 ± 4.24

(* and **) indicate significant differences at $p \leq 0.05$ and $p \leq 0.01$ respectively.

3.1.1.2 Root and shoot length

Root length was measured on day 3 (Figure 3.2A) while both root and shoot lengths were measured on day 7 (Figure 3.3A). On day 3, the root length of seedlings germinated under various NaCl concentrations and showed significant decrease compared to that of the control

(without added NaCl). Both 15 mM and 35 mM Ca^{2+} significantly ($p \leq 0.05$) reduced root length while 5 mM Ca^{2+} maintained root length close to that of the control (without added Ca^{2+}) (Figure 3.2B). There was no significant effect on the root lengths of seedlings under 200 mM and 300 mM NaCl in the presence of 5 mM or 15 mM Ca^{2+} (Figure 3.2C). However, 35 mM Ca^{2+} significantly reduced the root length of seedlings under 200 mM ($p \leq 0.001$) and 300 mM ($p \leq 0.05$) NaCl, which had a root length of 4.18 mm and 2.17 mm respectively compared to their controls (NaCl only), which had a root length of 8.83 mm and 4.7 mm respectively.

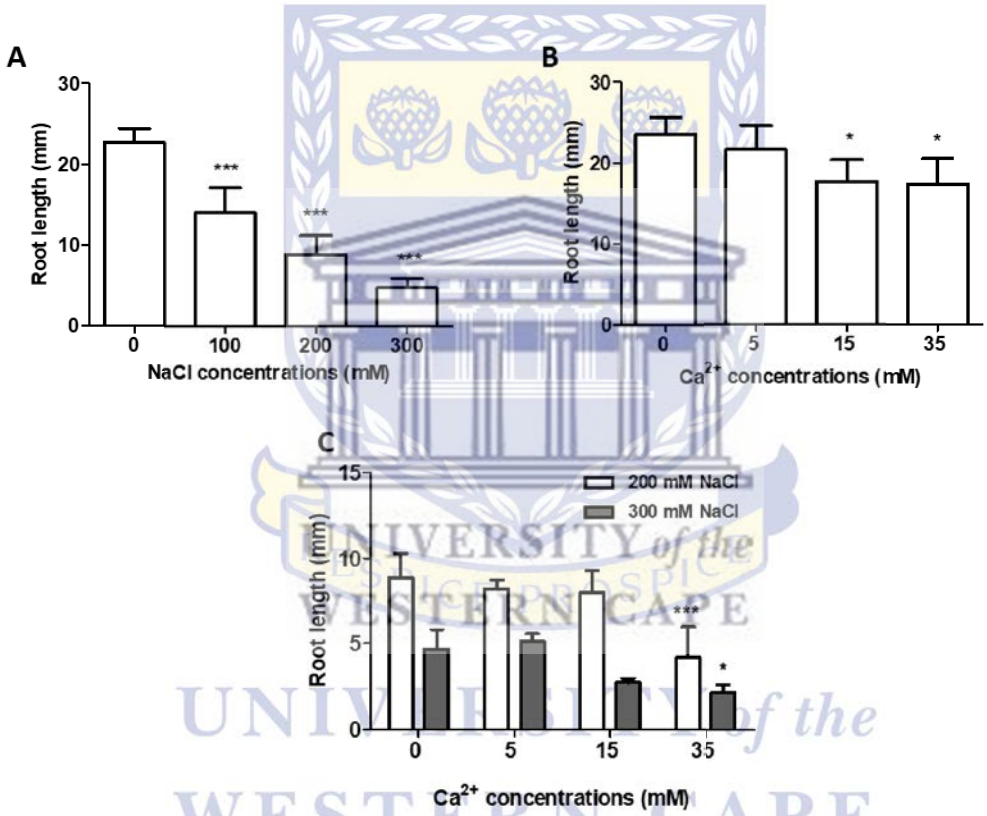

Figure 3.2. Effect of NaCl stress and Ca^{2+} on the root length of 3 day old sorghum seedlings. (**A**) Root and shoot length of sorghum seedlings in the presence of different NaCl only. (**B-C**) Seedlings under different NaCl and Ca^{2+} (5, 15 and 35 mM) concentrations, (**B**) 0 mM NaCl, (**C**) 200 mM NaCl and 300 mM NaCl. Error bars represent the SD calculated from three biological replicates and significance differences between control and treated plants were determined using one-way ANOVA shown as ** = $p \leq 0.01$, and * = $p \leq 0.05$.

The root and shoot length showed a significant decrease in the presence of increasing NaCl concentration on day 7 (Figure 3.3A). As expected the highest root (70.5 mm) and shoot (52.5 mm) lengths were observed in control (without added NaCl) seedlings, while the lowest root and shoot lengths were observed in seedlings treated with 300 mM NaCl (Figure 3.3A). The low Ca^{2+} concentrations (5 mM and 15 mM) had no significant effect on control seedlings (without added NaCl), whereas the highest Ca^{2+} concentration (35 mM) significantly ($p \leq 0.05$) decreased root length (Figure 3.3B). A similar effect of Ca^{2+} was observed for seedlings under 200 mM NaCl, where low Ca^{2+} concentrations (5 mM and 15 mM) showed no significant effect on the mean root and shoot length whereas a significant decrease was observed at 35 mM Ca^{2+} for both roots ($p \leq 0.001$) and shoots ($p \leq 0.01$) (Figure 3.3C). For seedlings under 300 mM NaCl, the highest root length (15 mm) was observed for 5 mM Ca^{2+} and the lowest (7.5 mm) was observed for 35 mM Ca^{2+} compared to the control (300 mM NaCl only) (Figure 3.3D). A similar growth pattern was observed in the shoots (Figure 3.3D).

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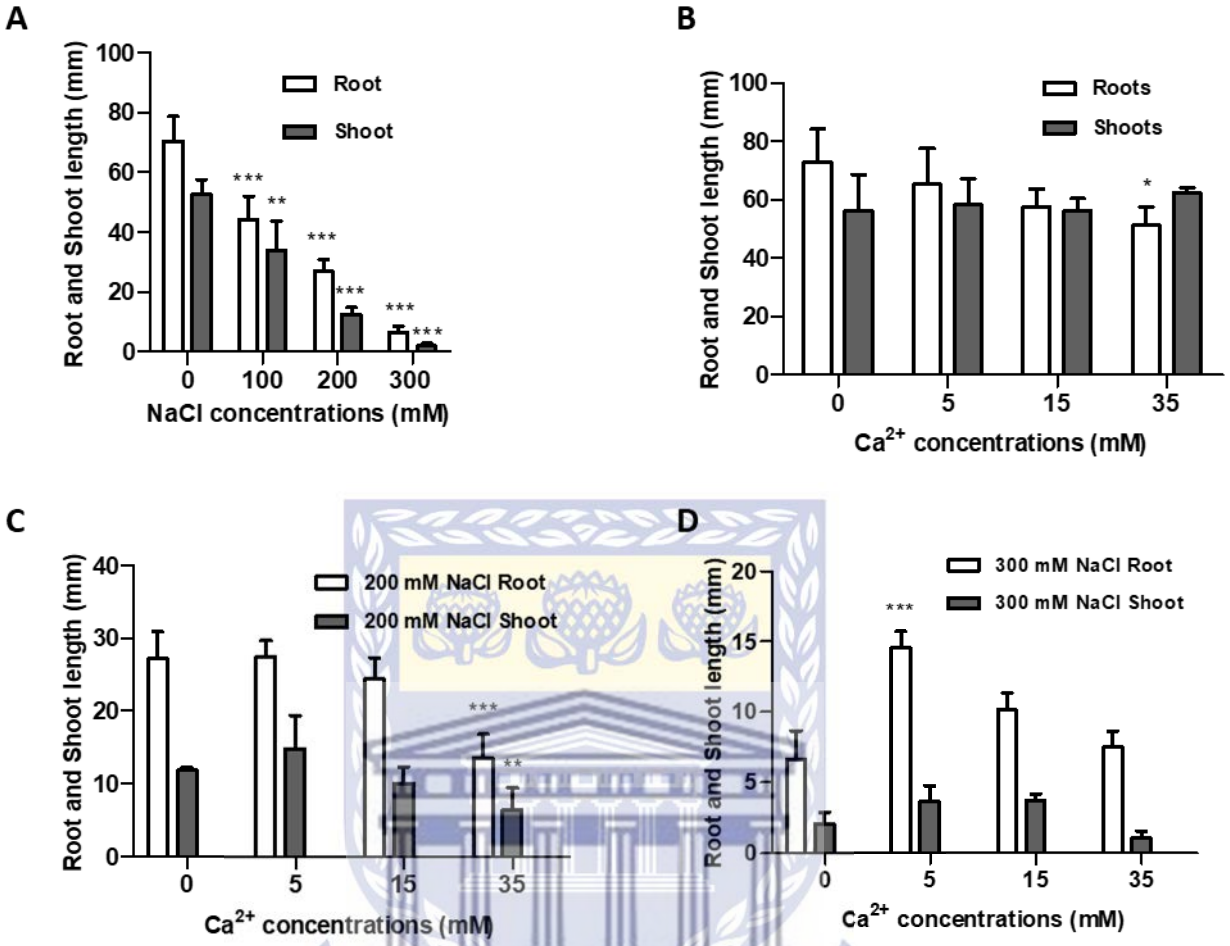

Figure 3.3. Effect of NaCl stress and Ca^{2+} on the root and shoot length of 7 day old sorghum seedlings. (**A**) Seedlings germinated in the presence of different NaCl concentrations only. (**B**–**D**) Seedlings germinated under different NaCl and Ca^{2+} (5, 15 and 35 mM) concentrations; (**B**) 0 mM, (**C**) 200 mM and (**D**) 300 mM NaCl. Error bars represent the SD calculated from three biological replicates. Statistical significance between control and treated plants was determined using two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as ** = $p \leq 0.01$, and * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

3.1.1.3 Fresh and dry weight

Fresh and dry weights were measured at day 7 of germination and seedlings treated with high NaCl showed a reduction (Table 3.2). A significant decreased was observed in fresh weights under 200 mM ($p \leq 0.01$) and 300 mM NaCl ($p \leq 0.001$) compared to the control (without added NaCl). The highest fresh weight was observed for control seedlings (0.57 g) and the

lowest was 0.29 g for seedlings under 300 mM NaCl. There was no significant effect in the fresh and dry weight of control seedlings (without added NaCl) and NaCl stressed seedlings when treated with Ca^{2+}.

Table 3.2: Effect of NaCl and Ca^{2+} on the fresh and dry weights of sorghum seedlings in the absence (0 mM) and presence of (200 and 300 mM) NaCl. Data represented are mean ± S.D.

$CaCl_2$ (mM)	NaCl (mM)	Fresh weight	Dry weight
0	**0**	0.57 ± 0.07	0.14 ± 0.09
	100	0.425 ± 0.05	0.10 ± 0.071
	200	0.33 ±0.07***	0.11 ± 0.06
	300	0.29 ±0.07***	0.14 ± 0.08
5	**0**	0.52 ± 0.17	0.15 ± 0.02
	200	0.33 ± 0.08	0.11 ± 0.06
	300	0.27 ± 0.09	0.11 ± 0.07
15	**0**	0.62 ± 0.13	0.14 ± 0.03
	200	0.31 ± 0.06	0.10 ± 0.08
	300	0.27 ± 0.06	0.12 ± 0.07
35	**0**	0.57 ± 0.07	0.16 ± 0.01
	200	0.33 ± 0.09	0.11 ± 0.07
	300	0.26 ± 0.07	0.12 ± 0.06

(*, ** and ***) indicate significant differences at $p \leq 0.05$, $p \leq 0.001$ and $p \leq 0.01$ respectively.

3.1.2 The effect of NaCl and Ca^{2+} on the biochemical and anatomical response of Sorghum bicolor

3.1.2.1 The effect of NaCl and Ca^{2+} on osmolyte accumulation

Osmolytes including proline and total soluble sugars were measured to determine the capacity of osmotic balance by sorghum in the presence of NaCl and Ca^{2+}. Proline content increased significantly ($p \leq 0.001$) with an increase in NaCl concentration (Figure 3.4A). The lowest proline content of 106.04 µM was observed in control seedlings (without added NaCl) compared to seedlings under 300 mM NaCl which displayed a very high proline content of 669.25 µM representing a ~6 fold increase (Figure 3.4A). Low Ca^{2+} concentration had no effect

on proline content of control seedlings (no added NaCl) whereas 15 and 35 mM Ca^{2+} increased proline content (Figure 3.4B). No significant change was observed in the proline content of seedlings under 200 and 300 mM NaCl in the presence of different Ca^{2+} concentrations (Figure 3.4C).

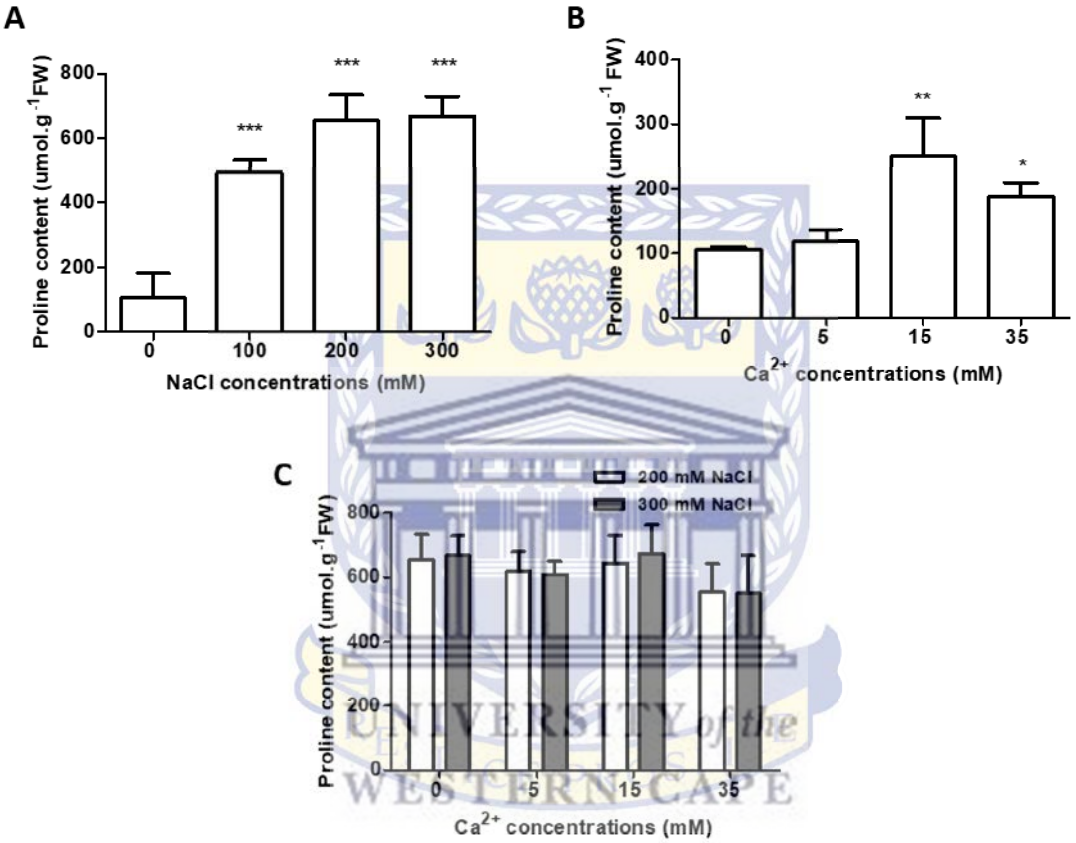

Figure 3.4. Effect of NaCl stress and Ca^{2+} on proline content in sorghum seedlings. Proline content measured on (**A**) seedlings germinated in the presence of different NaCl concentrations only. (**B**, **C**) Seedlings germinated under different NaCl and Ca^{2+} (5, 15 and 35 mM) concentrations; (**B**) 0 mM, (**C**) 200 mM and 300 mM NaCl. Error bars represent the SD calculated from three biological replicates. Statistical significance between control and treated plants was determined using two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as *** = $p \leq 0.001$, ** = $p \leq 0.01$, and * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

No significant effect was observed in the total soluble sugar content of seedlings under various NaCl concentrations (Figure 3.5A) and control seedlings (without added NaCl) treated with

different Ca^{2+} concentrations (Figure 3.5B). All Ca^{2+} concentrations showed significant decrease in total soluble sugars for seedlings under 200 mM NaCl (Figure 3.5C).

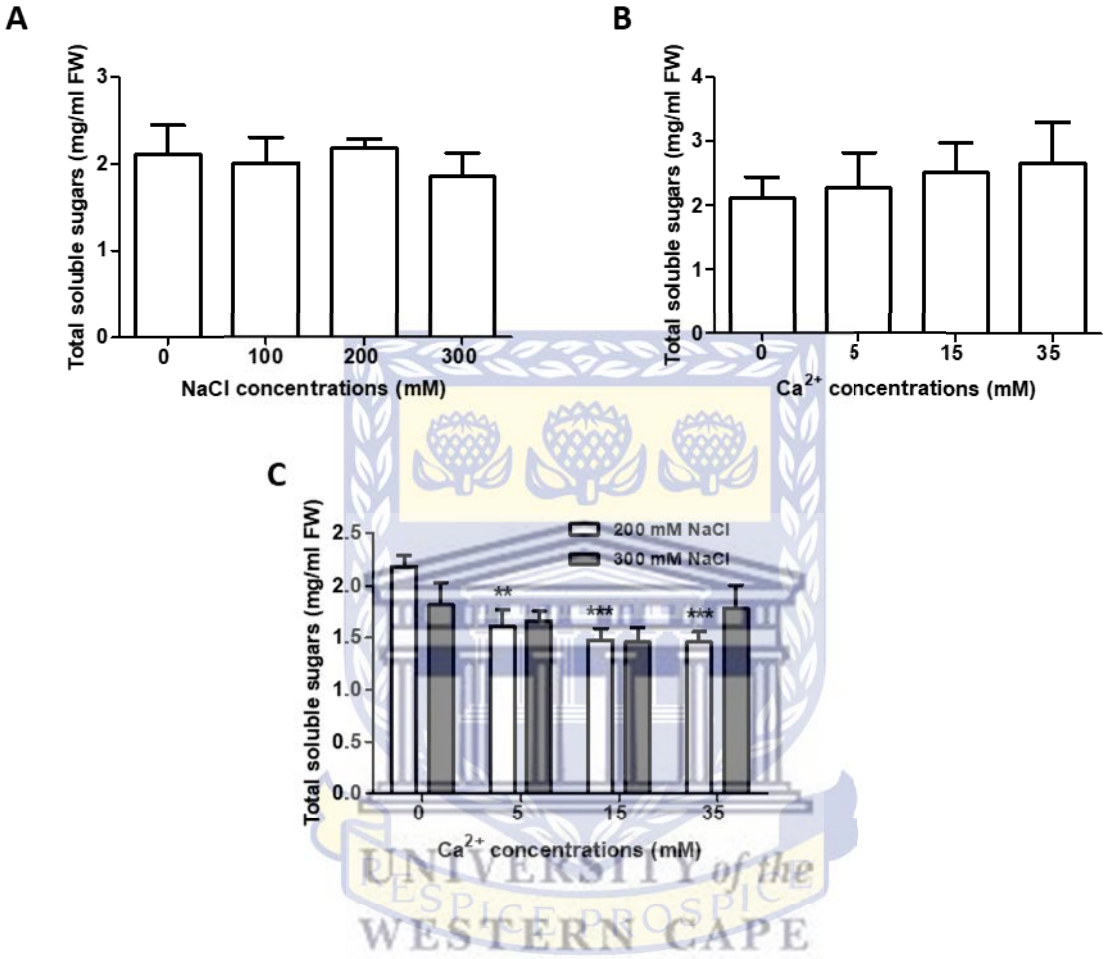

Figure 3.5. Effect of NaCl stress and Ca^{2+} on total soluble sugar content in sorghum seedlings. Soluble sugar content measured on (**A**) seedlings germinated in the presence of different NaCl concentrations only. (**B**, **C**) Seedlings germinated under different NaCl and Ca^{2+} (5, 15 and 35 mM) concentrations; (**B**) 0 mM, (**C**) 200 mM and 300 mM NaCl. Error bars represent the SD calculated from three biological replicates. Statistical significance between control and treated plants was determined using two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as *** = $p \leq 0.001$, ** = $p \leq 0.01$, and * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

3.1.2.2 The effect of NaCl and Ca^{2+} on oxidative stress

Oxidative stress in plants can be measured by assessing various factors, however, this study assayed hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents. H_2O_2 content remained low at 100 and 200 mM NaCl concentrations but showed a significant ~4 fold increase at 300 mM NaCl ($p \leq 0.001$) (Figure 3.6A). High Ca^{2+} concentrations (15 and 35 mM)

showed a gradual increase in H_2O_2 content for control seedlings (without added NaCl) (Figure 3.6B). Ca^{2+} had different effects on the H_2O_2 content of seedlings under 200 mM NaCl. About 5 mM Ca^{2+} significantly increased H_2O_2 content whereas 15 mM Ca^{2+} significantly decreased H_2O_2 content compared to the control (200 mM NaCl only) (Figure 3.6B). All Ca^{2+} concentrations significantly decreased ($p \leq 0.001$) H_2O_2 content for seedlings under 300 mM NaCl (Figure 3.6C). The lowest level of H_2O_2 content was observed for 35 mM Ca^{2+} treated seedlings showing a similar level as that observed in control (without added NaCl or Ca^{2+}) seedlings (Figure 3.6A).

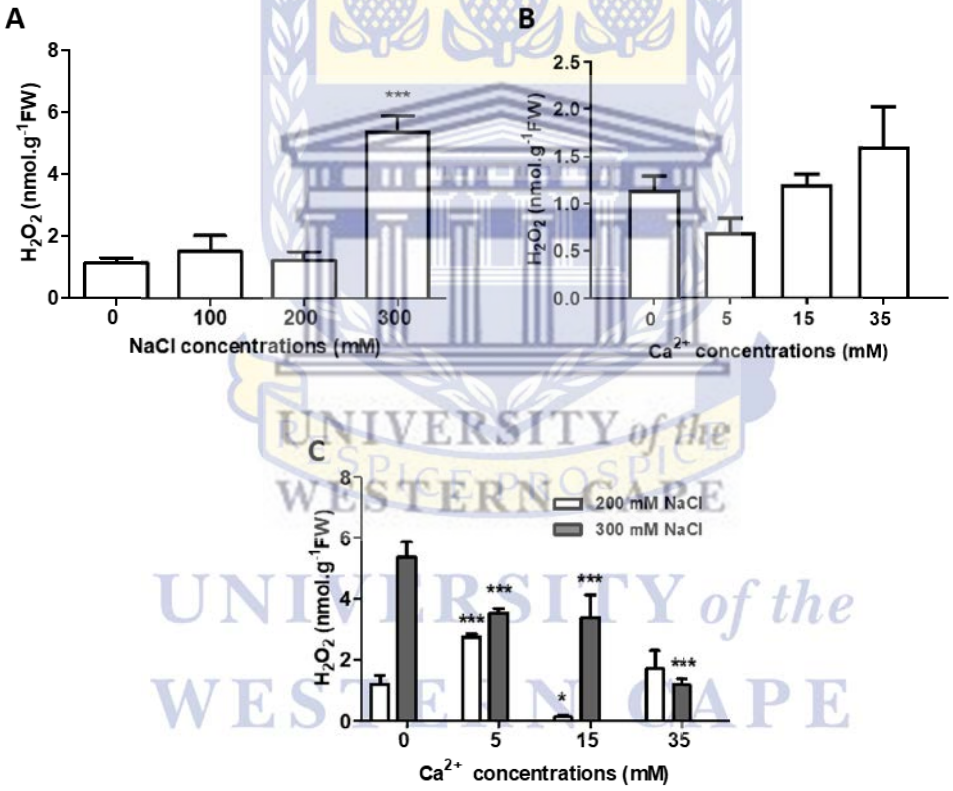

Figure 3.6. Effect of NaCl stress and Ca^{2+} on H_2O_2 content in sorghum seedlings. (**A**) H_2O_2 content measured from seedlings germinated in the presence of different NaCl concentrations only. (**B**,**C**), Seedlings under different NaCl and Ca^{2+} (5, 15 and 35 mM) concentrations; (**B**) 0 mM, (**C**) 200 and 300 mM NaCl. Error bars represent the SD calculated from three biological replicates. Statistical significance between control and treated plants was determined using two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as *** = $p \leq 0.001$ and * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

MDA content showed a significant increase under 100 mM NaCl ($p \leq 0.05$) and a gradual decrease from 200 to 300 mM NaCl (Figure 3.7A). Seedlings under 300 mM NaCl showed the lowest level of MDA content (8.06 nmol.g^{-1}FW) when compared to the control (without added NaCl). Ca^{2+} concentrations showed no significant difference in the MDA content of control seedlings (without added NaCl). The lowest Ca^{2+} concentration (5 mM) showed a slight increase in MDA for seedlings under 200 mM NaCl whereas the higher Ca^{2+} concentrations decreased MDA content. All Ca^{2+} concentrations showed no significant difference for seedlings under 300 mM NaCl.

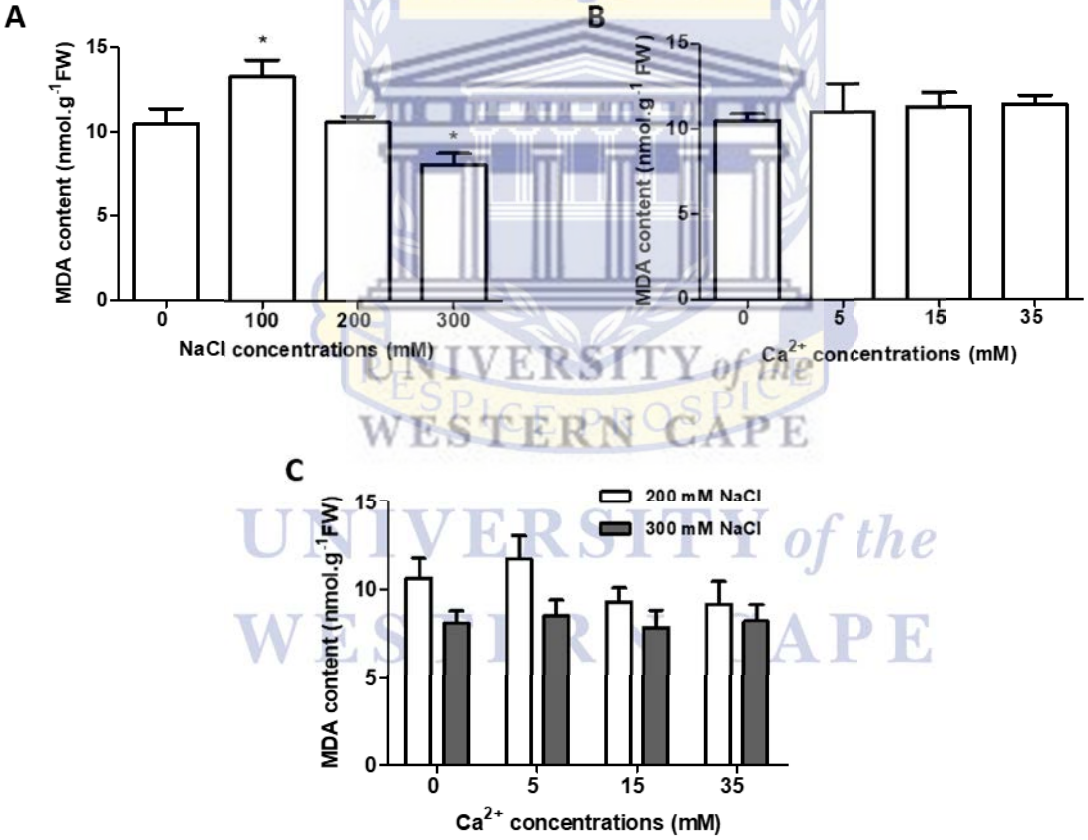

Figure 3.7. Effect of NaCl stress and Ca^{2+} on MDA content in sorghum seedlings. (**A**) MDA content measured from seedlings germinated in the presence of different NaCl concentrations only. (**B**,**C**), Seedlings under different NaCl and Ca^{2+} (5, 15 and 35 mM) concentrations; (**B**) 0 mM, (**C**) 200 and 300 mM NaCl. Error bars represent the SD calculated from three biological replicates. Statistical significance between control and treated plants was determined using two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as *** = $p \leq 0.001$ and * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

3.1.2.3 The effect of NaCl and Ca^{2+} on the membrane structure of Sorghum bicolor seedlings

The anatomical structure (epidermis and xylem layers) of sorghum seedlings was examined to determine the effect of NaCl and Ca^{2+} on oxidative damage using Scanning Electron Microscopy (SEM). The epidermis of seedlings treated with 5 mM Ca^{2+} revealed a smooth epidermal layer (Figure 3.8B) as compared to the control (Figure 3.8A). Under 300 mM NaCl, the epidermis showed changes associated with shrinkage and the formation of several additional features (Figure 3.8C). The application of 5 mM Ca^{2+} improved the epidermis structure (Figure 3.8D). Xylem walls of control seedlings in the presence of Ca^{2+} only, showed slight changes (thin layers) (Figure 3.8F) as compared to the control (Figure 3.8E). In the presence of NaCl, damage to the xylem is clearly evident as shown by shrinkage and thinning of the walls (Figure 3.8G). Seedlings under 300 mM NaCl that were treated with 5 mM Ca^{2+} showed improvements *e.g.*, thickened xylem walls (Figure 3.8H).

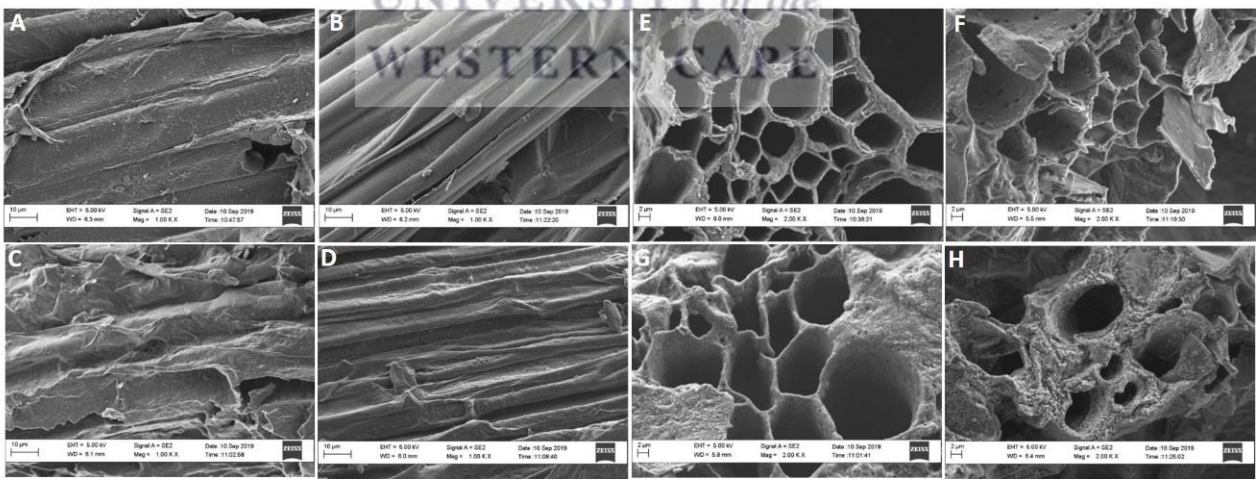

Figure 3.8. Anatomical image showing the epidermis and xylem layers of *Sorghum bicolor* analysed using Scanning Electron Microscopy. Cross section of epidermis layer of seedlings under (**A**) 0 mM NaCl, (**B**) 0 mM NaCl + 5 mM Ca^{2+}, (**C**) 300 mM NaCl, (**D**) 300 mM + 5 mM Ca^{2+}. Cross section of the xylem layers of seedlings under (**E**) 0 mM NaCl, (**F**) 0 mM NaCl + 5 mM Ca^{2+}, (**G**) 300 mM NaCl, (**H**) 300 mM + 5 mM Ca^{2+}.

3.1.2.4 The effect of NaCl and Ca^{2+} on ion homeostasis in Sorghum bicolor seedlings

Ion content, particularly Na^+ and K^+ (Figure 3.9; Table 3.3), was analysed using Scanning Electron Microscopy-Energy dispersive X-ray spectroscopy *(SEM-EDX)* for seedlings treated with 0 and 300 mM NaCl in the absence and presence of 5 mM Ca^{2+}. Na^+/K^+ ratio was calculated based on the Weight %, however, the Weight Sigma and Atomic % values are also provided in Table 3.3. Na^+ concentration in seedlings treated with 300 mM NaCl increased by 13.5 fold, whereas K^+ concentration decreased by 1.3 fold resulting in a high Na^+/K^+ ratio of 3.19 as compared to the control (Na^+/K^+ = 0.17). Treatment with 5 mM Ca^{2+} decreased Na^+ concentration by 0.8 fold, whereas K^+ increased by 2.4 fold resulting in a low Na^+/K^+ ratio of 1.5. SEM images for the investigated surface area (ion analysis) revealed significant changes in the sorghum morphology (Figure 3.9E–H). Seedlings treated with Ca^{2+} only (Figure 3.9F) showed a smooth surface area as compared to the control (Figure 3.9E). In the presence of NaCl, the surface area showed shrinkage (Figure 3.9G), corresponding to the increased Na^+ concentration (2.43) whereas seedlings treated with both 300 mM NaCl and 5 mM Ca^{2+} showed an improved surface area, with less deformation (Figure 3.9H)

Table 3.3 Overall ion content measured by the energy dispersive X-ray (EDX) spectroscopy in sorghum seedlings.

Element	0 mM NaCl Wt %	0 mM NaCl Wt sigma	0 mM NaCl At %	5 mM Ca^{2+} Wt%	5 mM Ca^{2+} Wt Sigma	5 mM Ca^{2+} At%	300 mM NaCl Wt %	300 mM NaCl Wt Sigma	300 mM NaCl At %	300 mM NaCl +5 mM Ca^{2+} Wt %	300 mM NaCl +5 mM Ca^{2+} Wt Sigma	300 mM NaCl +5 mM Ca^{2+} At%
C	65,66	0.58	72.58	58,43	0.57	65.54	70,24	0.5	78.52	51.69	1.13	61.59
O	31.78	0.57	26.37	40.32	0.57	33.96	21.03	0.45	17.65	32.6	0.8	29.16
Na	0.18	0.06	0.11	-	-	-	2.43	0.08	1.42	2.89	0.11	1.8
Mg	0.21	0.05	0.12	0.11	0.04	0.06	0.11	0.03	0.06	0.15	0.04	0.09
P	0.76	0.06	0.33	0.37	0.05	0.16	0.32	0.03	0.05	0.22	0.05	0.1
S	0.24	0.04	0.1	0.17	0.04	0.07	Si (0.1)	Si (0.03)	Si (0.05)	Si (0.75)	Si (0.05)	Si (0.35)
Cl	0.14	0.04	0.05	0.12	0.04	0.05	5	0.1	1.89	5.84	0.15	2.36
K	1.02	0.07	0.35	0.37	0.05	0.13	0.76	0.05	0.26	1.86	0.07	0.68
Ca	-	-	-	0.1	0.04	0.03	-	-	-	0.3	0.04	0.11
Total	**100**		**100**	**100**		**100**	**100**		**100**	**100**		**100**

Weight % = Wt %, Weight Sigma= Wt Sigma and Atomic % = At%.

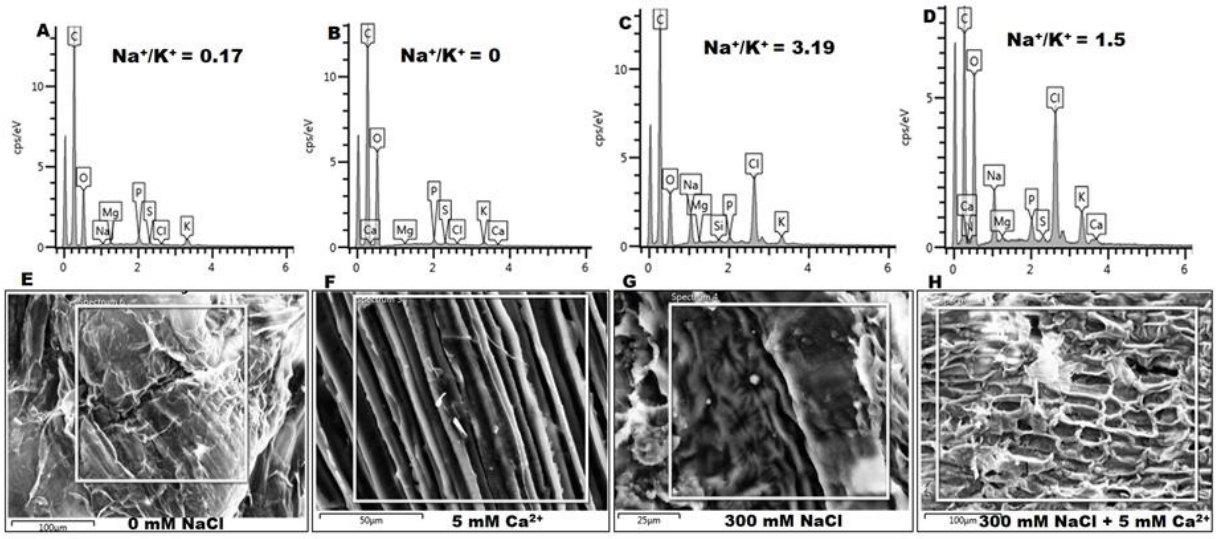

Figure 3.9. Energy dispersive X-ray (EDX) spectroscopy and SEM images of the effect of NaCl and Ca^{2+} on the concentration of Na^+ and K^+ of sorghum seedlings. (**A**–**D**) Spectra showing concentration of different elements, (**E**–**H**) Sorghum surface for mapped elements. (**A** & **E**) Na^+ and K^+ content in the absence of NaCl (0 mM), (**B** & **F**) presence of 5 mM Ca^{2+} only, (**C** & **G**) 300 mM NaCl only, (**D** & **H**) and 300 mM + 5 mM Ca^{2+}.

3.2 DISCUSSION

Salinity greatly affects seed germination and consequently induces a reduction in germination rate and a delay in the initiation of germination and seedling establishment (Misra and Dwivedi 2004; Almansouri *et al*., 2001). In this study the effects of salt stress and Ca^{2+} in the germination of *Sorghum bicolor* were demonstrated.

3.2.1 Effect of NaCl and Ca^{2+} on the germination of Sorghum bicolor

Salt stress significantly reduced germination percentage and delayed germination of sorghum seeds with significant effects observed at high NaCl concentrations (200 and 300 mM) within the first 3 days (Figure 3.1A). Similarly, salinity affected germination of *Suaeda salsa* (Song *et al*., 2008), a halophyte, *Halocnemum strobilaceum* (Qu *et al*., 2008), *Festuca ovina* L.

(Dianati-Tilaki *et al*., 2011; Salahshoor and Kazemi, 2016) and *Cucumis Sativus* L. (Fan *et al*., 2013). The delayed germination was evident by a decrease in germination index, which combines the percentage and speed of germination (Table 3.1) (Kader, 2005). The germination index was in agreement with the mean germination time, which explains the time taken for seeds to germinate (Table 3.1) (Kader, 2005), since these parameters decreased with increasing salt concentration. Results showed that the higher the NaCl concentration the longer it took for the seeds to germinate. Previous reports have also demonstrated that increasing salt concentration affects germination index of crops as observed in *Zea mays* (Khayatnez and Gholamin, 2011) and *Capsicum annuum* L. (Khan *et al*., 2009). This delay might be due to osmotic stress, which causes impairment in nutrient uptake and ionic stress due to the accumulation of ions causing ion toxicity (Rengel, 1992; Anvari *et al*., 2009). Application of Ca^{2+} reversed the effects of salinity on sorghum seed germination especially at low concentrations (5 mM Ca^{2+}) as compared to the control. Similarly, Ca^{2+} also improved seed germination of *Festuca ovina L.* (Salahshoor and Kazemi, 2016), *Phragmites karka* (Zehra *et al*., 2012), *Triticum aestivum* (LiYun and MingYou, 2010), *Urochondra setulosa* (Shaikh *et al*., 2007) and *Pisum sativum* (Bonilla *et al*., 2004).

3.2.2 Effect of NaCl and Ca^{2+} on the growth of Sorghum bicolor

Sodium chloride also reduced root and shoot length of sorghum seedlings in a concentration-dependent manner and shoots showed more sensitivity to NaCl than roots (Figure 3.3A). This is possibly due to excess salts in the roots causing ionic stress and a decrease in root osmotic potential that prevents the roots from absorbing water and water transport to the shoots. This would in turn affect embryo expansion and seedling emergence (Aroca *et al*., 2012). Similar effects were observed in *Brassica juncea*, which showed decreased growth and seedling emergence due to salt stress (Pandey and Penna, 2017). Ca^{2+} on its own decreased root length

of seedlings grown in the absence (0 mM NaCl) and presence (200 mM NaCl) of salt with significant effects observed for higher Ca^{2+} (35 mM) concentrations. However, Ca^{2+} improved both root and shoot length of seedlings grown in the presence of 300 mM NaCl (Figure 3.3D), and these responses are similar to those seen in *Festuca ovina* L. (Salahshoor and Kazemi, 2016). The highest root and shoot lengths were observed in seedlings that were treated with 5 or 15 mM Ca^{2+}, followed by a decrease at high Ca^{2+} concentration (35 mM) (Figure 3.3D). Given these observations, it was important to determine the Ca^{2+} concentrations best suitable to improve growth without causing toxicity and inhibitory growth effects (Cramer, 2002), thus 5 and 15 mM Ca^{2+} represent the best analysed in this study. Increasing NaCl concentration decreased fresh and dry weights of sorghum seedlings indicating that cell division and elongation were inhibited (Table 3.2) (Takemura *et al*., 2000; Li *et al*., 2010). Treatment with Ca^{2+} showed no significant improvement in the fresh and dry weight of seedlings germinated in the presence of NaCl (Table 3.2). Although the effect of Ca^{2+} on fresh and dry weights of NaCl-stressed sorghum seedlings was not significant, other studies reported positive effects (Zaman and Saleh, 2005; Salahsoor and Kazemi, 2016).

3.2.3 Effect of NaCl and Ca^{2+} on osmolyte accumulation

Proline is an important osmolyte that helps to maintain the osmotic balance by increasing the osmotic potential during osmotic stress (Mahajan *et al*., 2008). Sorghum seedlings accumulated high proline content under NaCl stress (Figure 3.4A). Although at different stages, these results are supported by other studies, which reported similar effects in sorghum (El Omari *et al*., 2016; El-Haddad and O'Leary, 1994) and other crops (Watanabe *et al*., 2000). A high concentration of Ca^{2+} (15 and 35 mM) significantly increased proline content in control seedlings (Figure 3.4B), whereas in NaCl treated seedlings, there were no significant changes (Figure 3.4C). This suggests that the protective effect of Ca^{2+} on sorghum might be independent

of proline regulation, since proline also acts as a molecular chaperone, protects photosynthesis, antioxidant enzymes, and prevents membrane damage (Szabados and Savouré, 2010; Surender *et al*., 2015). This might be the reason for its stable accumulation under NaCl stress and in combination with Ca^{2+} (Figure 3.4C), which can be linked to the induction of *pyrroline-5-carboxlyate synthetase* 1 (*P5CS1*), a key enzyme for proline biosynthesis that is dependent on Ca^{2+} signaling (Delauney and Verma, 1993; Verslues and Sharma, 2010). The results further suggest that sorghum seedlings adapt their osmotic potential under NaCl stress, consistent with the high accumulation of osmolytes. Salt stress affects carbohydrate partitioning, leading to the accumulation of various solutes, which might be of importance for the adjustment of the cellular water potential and they can act as scavengers of ROS (Sharma *et al*., 1990; Panuccio *et al*, 2014).

Total soluble sugars declined in seedlings under high NaCl (300 mM) concentrations compared to the control (without added NaCl) (Figure 3.5A). Since seedling growth and establishment relies on new compounds being synthesised and the consumption of ATP, the decline in sugars could be attributed to respiration, which requires ATP that gets channelled from sugars (Pandey and Penna, 2017). There was an increase in the total soluble sugars in seedlings treated with Ca^{2+} only (Figure 3.5B). Ca^{2+} further reduced the total soluble sugars of seedlings under 200 mM NaCl to the same levels as the control (without added NaCl) seedlings (Figure 3.5C). These results suggest the effectiveness of Ca^{2+} to reduce the effects of oxidative stress by maintaining the osmotic balance (Xu *et al*, 2017). The results further indicated that sorghum seedlings exhibited higher adaptive osmotic potential under salinity stress as seen by the high accumulation of proline.

3.2.4 The effect of NaCl and Ca^{2+} on H_2O_2 and MDA content

Sodium chloride stress causes oxidative stress through the accumulation of ROS, which can affect membrane structure and function as well as enzyme activities and with it, metabolic processes (Rahman *et al*., 2016). A significant accumulation of H_2O_2 in seedlings treated with 300 mM NaCl was observed and these levels were reduced by supplementation with Ca^{2+} (Figure 3.6A, C). Salt causes membrane damage due to oxidative stress and does so by displacing Ca^{2+} from the phospholipid membrane binding sites (Yermiyahu *et al*., 1997). However, this effect can be reversed, at least in parts, by the addition of Ca^{2+}, which affects the uptake and transport of ions and helps to maintain membrane integrity (Munns and Tester, 2008; Salahshoor and Kazemi,2016; Rahman *et al*., 2016).

The presence of free radicals induce lipid peroxidation of membranes, which is a sign for stress-induced oxidative damage in plants at the cellular level. Lipid peroxidation causes an increase in MDA content, an indicator of oxidative stress, which can somehow activate the antioxidant system (Jain *et al*., 2001; Hasanuzzaman *et al*. 2014; Pandey and Penna, 2017). It was suggested that at early stages of stress, plants perceive stress-induced damage but then immediately induce defence systems such as the anti-oxidative systems and osmolytes, which can reduce MDA content (Pandey and Penna, 2017). Similarly in this study, MDA content increased at 100 mM NaCl and declined at high NaCl (200 and 300 mM) concentrations (Figure 3.7A). The decline could be as a result of the plant modulating osmolytes and the antioxidant systems. An increased level of proline under 200 mM and 300 mM NaCl (Figure 3.4A) could have resulted in the low MDA content since proline is known to protect membrane integrity (Heuer, 2010). Salinity causes membrane damage by displacing Ca^{2+} from the phospholipid membrane binding sites (Leopold and Willing, 1984; Zhao and Mingliang, 1988) and this effect can be reversed by addition of excess Ca^{2+}, which controls the uptake and transport of ions and maintains membrane integrity (Greenway and Munns, 1980; Mokhtari *et al*., 2008). A slight

decrease in MDA content was observed for seedlings treated with 200 mM NaCl in the presence of Ca^{2+} suggesting that Ca^{2+} was able to stabilise the lipid bilayer of cellular membranes and provide structural integrity under the particular stress (Hepler, 2005) (Figure 3.7C). Ca^{2+} did not affect the MDA content of seedlings treated with 300 mM NaCl; this might be due to the fact that MDA levels were already low, similar to that of the control (without added NaCl) seedlings (Figure 3.7C).

3.2.5 The effect of NaCl and Ca^{2+} on membrane structure of Sorghum bicolor seedlings

In this study, NaCl triggered damage to the epidermis and xylem layers of sorghum seedlings as shown by the formation of structures and shrinkage on these layers (Figure 3.8C, G). Ca^{2+} partly stabilised the lipid bilayer of cellular membranes and provided structural integrity under these conditions (Figure 3.8H). This is supported by the smoothened epidermis and the thickened xylem layers of seedlings treated with both NaCl and Ca^{2+} (Figure 3.8D, H). These results indicated that Ca^{2+} can effectively alleviate NaCl-induced oxidative stress as previously reported (Cha-um *et al*., 2012; Tahjib-Ul-Arif *et al*., 2018).

3.2.6 The effect of NaCl and Ca^{2+} on ion homeostasis

Salt stress is also responsible for ionic imbalance due to the accumulation of excess Na^+ and a decrease of K^+ and Ca^{2+} (Munns and Tester, 2008; Morgan *et al*., 2014). In this study, this was evident by the high Na^+/K^+ ratio of 3.19 under 300 mM NaCl, and this effect was reversed by the exogenous application of 5 mM Ca^{2+} resulting in a low Na^+/K^+ ratio of 1.5 (Figure 3.9C, D). This is consistent with the effective maintenance of ion homeostasis by Ca^{2+}. Salt stress causes membrane damage due to oxidative stress by displacing Ca^{2+} from the phospholipid

membrane binding sites (Yermiyahu *et al*., 1997). This is true since seedlings treated with NaCl revealed shrinkage and some deformation as compared to the control (Figure 3.9G). However, this effect was reversed, at least in parts, by the addition of Ca^{2+} (Figure 3.9H), which affects the uptake and transport of ions and helps to maintain membrane integrity (Munns and Tester, 2008; Salahshoor and Kazemi, 2016; Rahman *et al*., 2016).

CHAPTER 4

TRANSCRIPTIONAL ANALYSIS OF THE EFFECTS OF NACL AND CA^{2+} ON THE GROWTH OF *SORGHUM BICOLOR*

ABSTRACT: The harmful effects of salt (NaCl) stress and the alleviatory effect of Ca^{2+} were determined on the biochemical, physiological and anatomical aspects of *Sorghum bicolor* as discussed in chapter 3. Thus, it was of equal importance to investigate the transcriptional aspect in order to determine the expression profiles of the antioxidant enzymes [superoxide dismutase (*SbSOD4A*), ascorbate peroxidase (*SbAPX*2) and catalase (*SbCAT3*)], the *Salt Overlay Sensitive* (*SOS1, 2* and *3*) pathway and the *vacuolar* Na^+/H^+ *exchanger antiporter2* (*NHX2*) genes in response to NaCl stress and Ca^{2+}. Physiochemical parameters such as the coding sequence (CDS), protein length (aa), molecular weight (M*w*) and isoelectric point (pI) of the target genes were determined using the ExPASy Proteomic server. *SbCAT3* had the longest CDS (1481 bp) and protein length (493 aa) and the highest molecular weight (56.55 kDa) amongst the other antioxidant enzymes, however all three antioxidant genes are acidic in nature. Similarly, among the *SOS* genes, *SbSOS1* had the longest CDS (3413 bp), protein length (1137 aa) and the highest molecular weight (126.39 kDa). All three SOS genes were acidic, whereas *SbNHX2* was basic. Quantitative real-time polymerase chain reaction revealed that salt induced the expression of *SbAPX2, SbCAT3* and *SbSOS1* genes, whereas *SbSOD4A*, *SbSOS2, SbSOS3* and *SbNHX2* genes were not affected by salt. In the presence of Ca^{2+} the expression of *SbAPX2*, *SbCAT3* and *SbSOS1* was reduced except for *SbNHX2* gene, which increased by 65-fold compared to the control.

Keywords: Antioxidant genes, physiochemical parameters, quantitative real-time polymerase chain reaction, *SOS* pathway genes, *vacuolar* Na^+/H^+ *exchanger antiporter2*.

4.1 RESULTS

4.1.1 Physiochemical parameters of Sorghum bicolor antioxidant, SOS pathway and the vacuolar Na^+/H^+ exchanger antiporter2 genes.

In order to gain a better understanding on the genes of interest, physiochemical properties were retrieved from the NCBI database and ExPASy Proteomic server. Among the antioxidant enzymes, *SbSOD4A* had the shortest CDS length of 458 bp, whereas *SbCAT3* (1481 bp) had the longest followed by *SbAPX2* (752 bp). The encoded protein lengths varied from 152 amino acid residues for *SbSOD4A* and 250 and 493 amino acid residues for *SbAPX2* and *SbCAT3* respectively. The molecular weights (M*w*) of the proteins in ascending order were 15.09 kDa, 27.16 kDa and 56.55 kDa for *SbSOD4A, SbAPX2* and *SbCAT3* respectively. *SbSOD4A* and *SbAPX2* had low pI's of 5.65 and 5.18 whereas *SbCAT3* had a slightly high pI of 6.50. The CDS lengths for the SOS pathway genes varied significantly. *SbSOS3* had the shortest CDS length of 641 bp, which encoded a protein of 213 amino acids and had the lowest M*w* of 24.39 kDa with a pI of 4.71. The CDS lengths for *SbSOS2* (1364 bp) was longer than that of *SbSOS3* but shorter than that of *SbSOS1* (3413 bp) and *SbNHX2* (1610 bp). The encoded protein lengths for *SbSOS2, SbNHX2* and *SbSOS1* were 454, 536 and 1137 amino acids residues with corresponding M*w* of 51.13 kDa, 58.87 kDa and 126.39 kDa and pI's of 6.70, 7.73 and 6.40 respectively.

Table 4.1. Physiochemical properties of various antioxidant and SOS pathway genes found in *Sorghum bicolor*.

Genes	CDS (bp)	Protein length (aa)	M*w* (kDa)	*pI*
SbSOD4A	458	152	15.09	5.65
SbAPX2	752	250	27,16	5.18
SbCAT3	1481	493	56.55	6.50
SbSOS1	3413	1137	126.39	6.40
SbSOS2	1364	454	51,13	6.70
SbSOS3	641	213	24.39	4.71
SbNHX2	1610	536	58.87	7.73

4.1.2 Transcriptional analysis of the effect NaCl and Ca^{2+} on antioxidant genes.

This study considered three antioxidant enzymes, namely; SOD, APX and CAT. They were selected based on their ability to detoxify Reactive Oxygen Species (ROS) and, therefore, allow for the analysis of their expression levels in response to the effects of NaCl and Ca^{2+}. All genes were constitutively expressed in sorghum seedlings at different levels, with *SbSOD4A* representing the highest transcript numbers followed by *SbAPX2* and *SbCAT3* (Figure 4.1A-C). The transcript levels of *SbAPX2* and *SbCAT3* increased significantly ($p \leq 0.01$) in seedlings under 300 mM NaCl (>10-fold relative to their controls) (Figure 4.1B, C), whereas a decrease was observed in the transcript levels of *SbSOD4A* (Figure 4.1A). Treatment of NaCl-stressed seedlings with different Ca^{2+} concentrations reduced their transcript levels in a concentration-dependent manner.

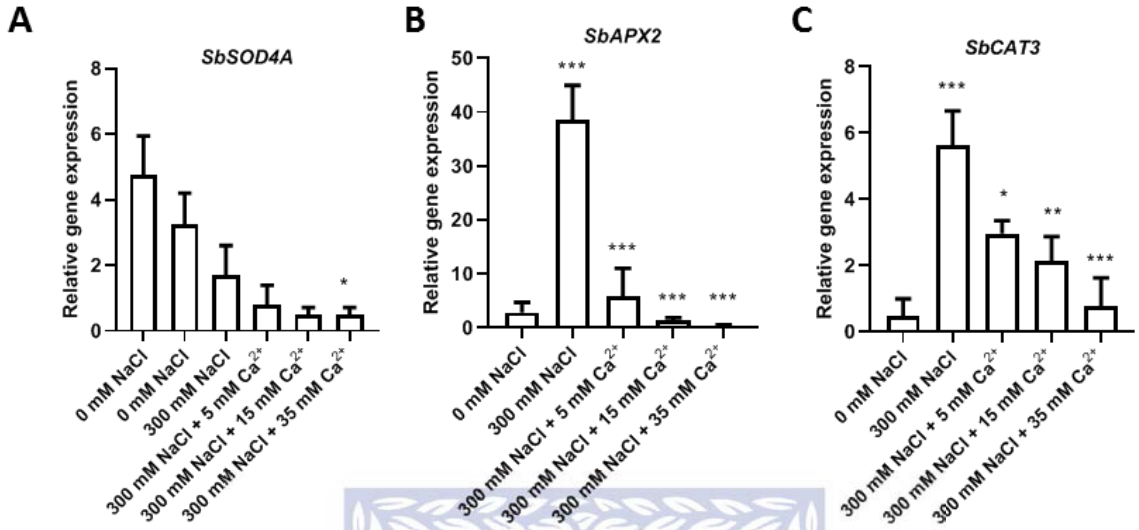

Figure 4.1.Transcript analysis of *Sorghum bicolor* antioxidant genes in the absence (0 mM) and presence (300 mM) of NaCl and treated with different Ca^{2+} (5, 15, and 35 mM) concentrations. Relative gene expression of (**A**) *SbSOD4A*, (**B**) *SbAPX2* and (**C**) *SbCAT3*. Error bars represent the SD calculated from three biological replicates. Statistical significance between control and treated plants were determined by two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as *** = $p \leq 0.01$, ** = $p \leq 0.01$, and * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

4.1.3 Transcriptional analysis of the effect NaCl and Ca^{2+} on SOS pathway genes

To understand the effect of NaCl and Ca^{2+} on the ion homeostasis of sorghum, the expression levels of the vacuolar Na^+/H^+ antiporter and SOS pathway genes were determined. The genes included the *SbSOS1* and *SbNHX2*, which are the plasma membrane and vacuolar Na^+/H^+ antiporters respectively, the *SbSOS2* a serine/threonine protein kinase and *SbSOS3,* a calcium binding protein were also included. All four genes were constitutively expressed, with *SbSOS2 and SbSOS3* representing the highest transcript level (Figure 4.2B, C). *SbSOS1* transcript was significantly ($p \leq 0.01$) induced in seedlings treated with 300 mM NaCl (~4-fold) as compared to *SbSOS2* and *SbSOS3*, which were significantly ($p \leq 0.001$) down-regulated. The presence of different Ca^{2+} concentrations decreased the expression of *SbSOS1* transcript in a concentration-

dependent manner (Figure 4.2A), whereas there were no changes in the expression of *SbSOS2* and *SbSOS3* transcripts (Figure 4.2B, C). The expression of the *vacuolar SbNHX2* was not significantly affected by 300 mM NaCl only, whereas treatment with different Ca^{2+} concentrations in the presence of NaCl increased its expression significantly ($p \leq 0.01$), with a 65-fold increase under 35 mM Ca^{2+} relative to the control (Figure 4.3).

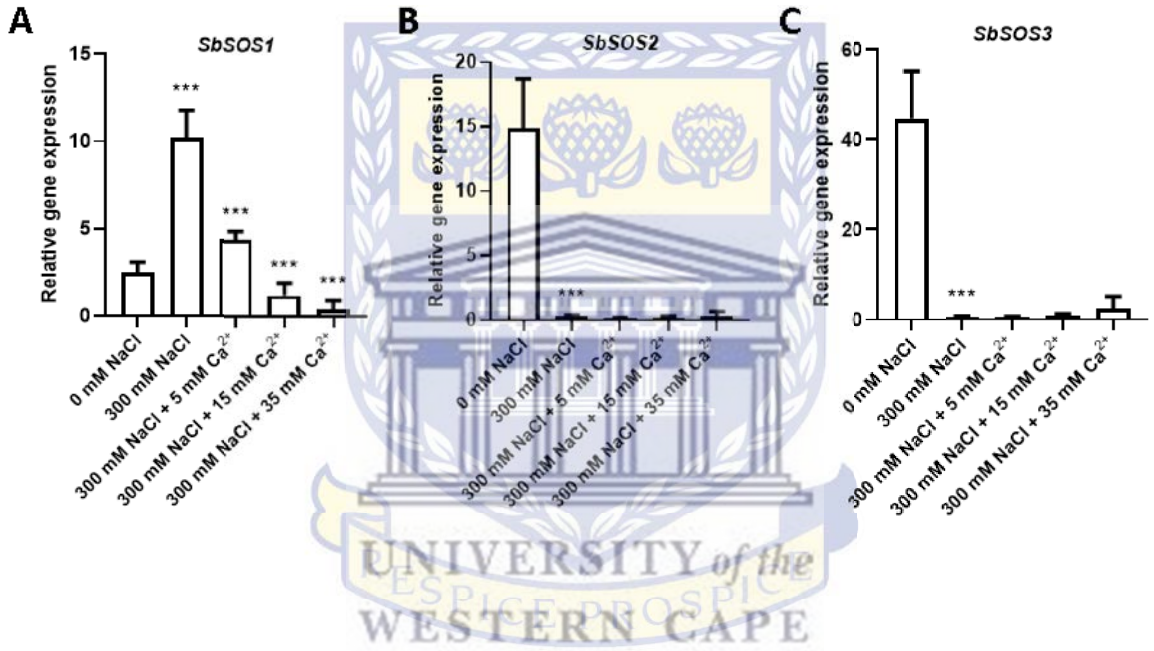

Figure 4.2.Transcript analysis of *Sorghum bicolor* SOS genes in the absence (0 mM) and presence (300 mM) of NaCl treated with different Ca^{2+} (5, 15, and 35 mM) concentrations. Relative gene expression of (**A**) *SbSOS1,* (**B**) *SbSOS2* and (**C**), *SbSOS3*. Error bars represent the SD calculated from three biological replicates. Statistical significance between control and treated plants were determined by two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as *** = $p \leq 0.001$, ** = $p \leq 0.01$, and * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

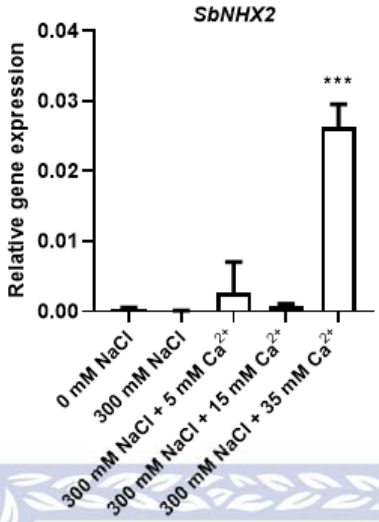

Figure 4.3.Transcript analysis of *Sorghum bicolor* vacuolar Na^+/H^+ exchanger antiporter gene in the absence (0 mM) and presence (300 mM) of NaCl treated with different Ca^{2+} (5, 15, and 35 mM) concentrations. Relative gene expression of the vacuolar *SbNHX*2. Error bars represent the SD calculated from three biological replicates. Statistical significance between control and treated plants were determined by two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as *** = $p \leq 0.001$ according to the Bonferroni's multiple comparison test.

4.2 DISCUSSION

4.2.1 Physiochemical analysis of antioxidant system genes

The antioxidant enzyme system is crucial to the plant because it plays a role in maintaining cellular homeostasis by detoxifying ROS, which accumulate when plants experience stress (Das and Roychoudhury, 2014). The CDS that is defined as the gene and mRNA portion that codes for protein varied among the three antioxidant enzymes. *SbCAT3* (1481 bp) had the longest CDS length followed by *SbAPX2* (752 bp) and *SbSOD4A* (458 bp). The protein length of *SbCAT3* (493 aa) closely correlated to its homolog in *Xerophyta viscosa* [*XvCAT3* (492 aa)], similarly the protein length of *SbAPX2* (250 aa) closely correlated to its homolog in *Selaginella moelendorffi* [*SmAPX2* (228 aa)] and lastly the homolog of *SbSOD4A* (152 aa) found in *Boea*

hygrometric [*BhSOD4A* (152 aa)], *Oropetium thomaeum* [*OtSOD4A* (153 aa)] and *Selaginella lepidophylla* [*SlSOD4A* (156 aa)] had corresponding protein lengths (Gupta *et al*., 2019). The same was observed for their molecular weights, *SbCAT3* (56.55 aa) homolog, *XvCAT3* had a M*w* of 57.02 kDa, while *SbAPX2* (27.16 kDa) homolog, *SmAPX2* had a M*w* of 24.82 kDa and *SbSOD4A* (15.09 kDa) homolog *BhSPD4A, OtSOD4A* and *SlSOD4A* had M*w* of 15.28 kDa, 15.17 kDa and 15.87 kDa respectively (Gupta *et al*., 2019). Finally, a similar trend was observed for the pI's of the homologs; all homologs had acidic pI's [*SmAPX2* (7.03), *XvCAT3* (6.6), *BhSOD4A* (5.94), *OtSOD4A* (5.65) and *SlSOD4A* (5.76)] (Gupta *et al*., 2019).

4.2.2 Physiochemical analysis of SOS pathway

The SOS pathway is known to play an important role in maintaining ion homeostasis and regulating plant tolerance in response to salinity stress (Cheng *et al*., 2019). The plasma membrane antiporter *SbSOS1* had the longest CDS of 3413 bp similar to that found in, *Aeluropus littoralis* (3421 bp), *Gossypium hirsutumvoucher* (3421 bp), *Mesembrayanthemum* (3421 bp) *Suaeda japonica* (3481 bp) and *Halogeton glomeratus* (3481 bp) (Fahmideh and Fooladvand, 2018). On the other hand, the vacuolar membrane antiporter, *SbNHX2* had a CDS length of 1610 bp, which was not far from the CDS lengths of NHX2 homologs in *Halostachys caspica*, *Suaeda pruinosa*, *Salsola suaeda*, *Suaeda corniculata* and *Atriplex halimus*, which all have a CDS length of 1621 bp (Fahmideh and Fooladvand, 2018). *SbSOS2* had a CDS length of 1364 bp closely related to its homolog found in *Brassica juncea* var. *Tumida* (1221 bp) while *SbSOS3* had the lowest CDS length of 641 bp similar to its homologs in *Brassica juncea* var. *Tumida SOS3-1* (660 bp), *SOS3-2* (657 bp) and *SOS3-3* (543 bp) (Cheng *et al*., 2019). *SbSOS1* had the longest protein length (1137 aa) followed by *SbNHX2* (536 aa), *SbSOS2* (454 aa) and *SbSOS3* (213 aa). *SbSOS1* had the highest molecular weight (126.39 kDa), whereas, *SbSOS3* had the lowest (24.39 kDa). All three SOS genes were considered acidic with pI's of 6.40, 6.07

and 4.71 for *SbSOS1, SbSOS2, SbSOS3* respectively, whereas the vacuolar Na^+/H^+ antiporter 2 was more basic with a pI of 7.73.

4.2.2 Transcriptional analysis of antioxidant genes

In this study the expression of antioxidant genes was assessed in response to salt stress and the effect of exogenously applied Ca^{2+}. The antioxidant system responsible for scavenging ROS was activated (Figure 4.1A–C) as seen by the induced expression of *Sorghum bicolor* antioxidant genes, *superoxide dismutase (SbSOD4A)*, *ascorbate peroxidase (SbAPX2) and catalase (SbCAT3)* under NaCl stress (Figure 4.1A–C). SOD is known as the first line of defense against oxidative stress, by converting the superoxide anion ($O_2^{\cdot-}$) into oxygen (O_2) and water with the formation of H_2O_2 (Mittler, 2002; Gill *et al*., 2015). APX and CAT are considered the most important enzymes in the detoxification of H_2O_2 (Gill *et al*., 2015; Parida and Das, 2005). *SbAPX2* and *SbCAT3* genes were constitutively expressed but their expression was highly induced under 300 mM NaCl treatment (Figure 4.1B, C). Thus, the increased expression of *SbAPX2* and *SbCAT3* indicates a protective mechanism since these genes are transcribed and translated into enzymes and hence, enable detoxification of excess H_2O_2. Additionally, overexpression of the *APX* gene in plants is associated with improved protection against oxidative stress (Wang *et al*., 1999). Similarly, microarray data indicated an increase in the expression of *CAT* and *APX* in *Arabidopsis thaliana* under abiotic stresses including salinity (100 mmol/L NaCl), cold (10 °C), heat (38 °C) and light (800 µmol photons $m^{-2}s^{-1}$) (Filiz *et al*., 2018). The expression of *APX* was also increased in *Oryza sativa* (Rossatto *et al*., 2017), whereas that of *CAT* was also increased in *Lotus japonicus* (Rubio *et al*., 2007) and *Cuminum cyminum* L. (Soleimani *et al*., 2017) in response to 50, 100, and 150 mM NaCl. These observations show a link between an increased antioxidant gene expression and low MDA content in sorghum seedlings under 300 mM NaCl stress in this study. Contrary to these results,

Mittal *et al*. reported a high MDA content and a decreased antioxidant enzyme activity in *Brassica juncea* seedlings treated with 200 mM NaCl (Mittal *et al*., 2012), suggesting that there is a direct relationship between lipid peroxidation and the antioxidant system. This suggests that a decrease in MDA content (Figure 3.7) of sorghum seedlings under high salt stress (200 and 300 mM NaCl) can be attributed to an increase in the expression of antioxidant genes (Figure 4.1). Although transcriptional levels do not necessarily always correlate with protein level or enzyme activity, other studies have reported on the increased activity of antioxidant enzymes under NaCl stress in germinating seeds (Xu *et al*., 2006; Jabeen and Ahmad, 2013). This expression (figure 4.1) occurred concomitantly with effective ROS scavenging and reduction of oxidative stress in seedlings under stress (Figure 3.7). Ca^{2+} significantly decreased the transcript levels of *SbSOD4, SbAPX2* and *SbCAT3* in salt treated seedlings in a concentration-dependent manner (Figure 4.1). The expression of *SbAPX2* was decreased to the same magnitude as that of the control, indicative of significant stimulation of detoxification by Ca^{2+}. The down-regulation of *SbSOD4A* in sorghum seedlings during germination in response to salt stress suggests that the oxidative stress might have been due to high H_2O_2 accumulation rather than other radicals, but further experiments are required to substantiate this statement. APX has a higher affinity (µM range) for H_2O_2 than CAT (mM range) (Mittler, 2002), which may explain why *SbAPX2* transcript levels were about ~7-fold higher than those of *SbCAT3* in seedlings treated with 300 mM NaCl, despite the fact that both enzymes are crucial for H_2O_2 detoxification.

4.2.2 Transcriptional analysis of SOS pathway genes

The expression of the *SOS1, SOS2, SOS3,* and the *vacuolar NHX2* antiporter genes that are responsible for maintaining ion homeostasis (Zhu, 2003; Moshaei *et al*., 2014) were measured in this study. Maintenance of ion homeostasis under salinity stress was elucidated by the

discovery of the SOS mutants in *Arabidopsis thaliana* (Zhu, 2003). Liu *et al*., demonstrated that *sos1* mutants were more sensitive to Na^+ and Li^+ stresses as compared to the *sos2* and *sos3* mutants (Liu *et al*., 1997) indicating the importance of *SOS1* in ion homeostasis. In this study, *SOS2* and *SOS3* genes were constitutively expressed under normal conditions, whereas under salt-stressed conditions (300 mM NaCl) their expression levels were very low. *SbSOS1* was upregulated under 300 mM NaCl stress in sorghum seedlings (Figure 4.2A). Similarly, in *Arabidopsis thaliana* under salt stress *SOS1* transcripts were higher than that of *SOS2* and *SOS3* (Rolly *et al*., 2020). This may be explained by its role in the exclusion of toxic Na^+ into the root apoplast region away from the cells delaying uptake into shoots and leaves (Apse *et al*., 1999; Tester and Davenport, 2003). Overexpression of *SOS1* was associated with increased salt tolerance in different species (Shi *et al*., 2000), suggesting that expression of *SbSOS1* in this study might have been sufficient to prevent ion toxicity and confer increased NaCl tolerance. Both, the constitutive and stress inductive expression of sorghum *SOS* pathway genes in this study correlate with the expression of *SOS* genes in wheat and Brassica genotypes (Kumar *et al*., 2009; Chakraborty *et al*., 2012; Sathee *et al*., 2015). In the presence of Ca^{2+}, the expression of *SbSOS1* decreased in a concentration-dependent way, whereas the expression of *SbSOS2* and *SbSOS3* transcripts was very low (Figure 4.2B, C). Thus, we propose that the application of exogenous Ca^{2+} induced tolerance may be through the binding of Ca^{2+} to the phospholipid bilayer of membranes to prevent the uptake and transport of Na^+ into cells (Liu and Zhu, 1997; Rubio *et al*., 2003). In turn, this may partly eliminate the need for strong *SOS1* expression.

The *vacuolar SbNHX2* was constitutively expressed in germinating seedlings, but the transcript level was reduced by salt stress (Figure 4.3). Incidentally, similar results were observed previously (Ohnishi *et al*., 2005; Hanana *et al*., 2007; Kumari *et al*., 2018). However, most studies reported an upregulation of other *NHX* genes under salt stress (Moshaei *et al*., 2014;

Yokoi *et al*., 2002; Fukuda *et al*., 2004; Zhang *et al*., 2008; Jha *et al*., 2011; Wang *et al*., 2011; Ababnejad *et al*., 2015). To our surprise, *vacuolar SbNHX2* was significantly induced by a combination of 300 mM NaCl and 35 mM Ca^{2+}. These results suggest that the sorghum vacuolar *NHX2* has a role in stress responses that might be mediated by Ca^{2+}. Overall gene expression of both the antioxidant and the *SOS* genes in sorghum showed a strong correlation with the alleviation of oxidative stress caused by ROS accumulation and this may be linked to Ca^{2+} signaling. The structural, physiological, and biochemical role of Ca^{2+} in *Sorghum bicolor* await further elucidation.

CHAPTER 5

CONCLUSION AND FUTURE PROSPECTS

Salinity stress is a severe abiotic stress that negatively affects plant growth and crop yield worldwide (Rao *et al*., 2019). The effect of salinity stress on food security is evident through the increasing world population (~ 820 million people) that is food insecure (FAO *et al*., 2020). These numbers are expected to increase with the rapidly growing population, which has been predicted to reach ~10 billion by 2050 (FAO, 2018). In addition to salt stress negatively affecting food security, the sudden global pandemic has further threatened food security, through decreased imports and exports and hence loss of jobs. Therefore, it is important to prepare and develop crops that are stress tolerant to combat ongoing and future issues.

The effect of salinity on the growth and development of important agricultural crops have been widely studied in *Hordeum vulgare* (Mozafar and Oertli, 1992); *Triticum aestivum* L. (Datta *et al*., 2010); *Zea mays* L. (Niu *et al*., 2012) and *Oryza sativa* L (Sajid *et al*., 2017). Although salinity studies were previously conducted in *Sorghum bicolor* (Francois *et al*., 1984; Igartua *et al*., 1995; Gill *et al*., 2003; Roy *et al*., 2018; Sun *et al*., 2019; Dehnavi *et al*., 2020), the effect of exogenously applied calcium (Ca^{2+}) in improving sorghum tolerance to stress has never been investigated. In addition to its tolerant nature, sorghum's genome has been sequenced, which makes it an ideal model plant for studying and understanding the molecular mechanisms of stress tolerance in cereal crops (Paterson *et al*.,2008; Krishnamurthy *et al*., 2007; Paterson *et al*., 2009).

In order to determine the effect of salt and Ca^{2+} on the germination and growth of sorghum, various assays were conducted as described in Chapter 2. Chapter 3 of this study demonstrated that high salt concentrations (300 mM NaCl) delayed germination and hindered the growth of sorghum, whereas low Ca^{2+} concentration (5 and 15 mM Ca^{2+}) was able to reverse these effects. This was demonstrated through several parameters including germination assays; growth assays; measurement of oxidative markers and analysis of the membrane structure and ion homeostasis. Concomitant to these results, previous studies have shown the negative effects of salt stress on the germination of *Suaeda salsa* (Song *et al*., 2008), a halophyte, *Halocnemum strobilaceum* (Qu *et al*., 2008), *Festuca ovina* L. (Dianati-Tilaki *et al*. 2011; Salahshoor and Kazemi, 2016) and *Cucumis sativus* L. (Fan *et al*., 2013). There was also an evident reduction in the root and shoot length as well as a decrease in the fresh and dry weight in sorghum seedlings in response to salt stress.

The study also investigated modulation of osmoprotectants in response to salt stress and Ca^{2+}, which included proline and total soluble sugars. The accumulation of the osmoprotectants is a good indicator of stress and the degree of stress tolerance in plants, since they accumulate under stressful conditions to maintain osmotic balance, stabilize enzymes and proteins and enable water absorption (Surender *et al*., 2015). In sorghum, proline content increased with an increase in NaCl concentration, whereas sugars declined at high NaCl concentrations. Seeds that were treated with high Ca^{2+} concentrations only showed an increase in both proline and soluble sugar content. However, seedlings under salt stress with added Ca^{2+} showed no changes in proline content but reduced soluble sugars to the same level as that of the control. This suggested that the protective effect of Ca^{2+} in sorghum might be independent of proline regulation. Furthermore, the results also indicated sorghum's ability to adapt to salt stress is mediated by the high accumulation of proline.

The study also investigated the oxidative state of sorghum in response to salt stress and Ca^{2+} by determining H_2O_2 and lipid peroxidation (MDA) contents. ROS are signalling molecules that regulate ion channel activity and gene expression (Mittler *et al*., 2004; Bernstein *et al*., 2010). However, abiotic stress generally causes an increase in ROS levels in plants, hence causing oxidative damage of important macromolecules including lipids, proteins and nucleic acids (Rodríguez and Taleisnik, 2012). Thus, it is important to measure ROS levels in response to salinity (Munns and Tester, 2008). H_2O_2 levels significantly increased under high NaCl (200 and 300 mM NaCl) concentrations whereas MDA decreased under high NaCl concentrations. The decline in MDA levels could have been attributed to the high levels of proline since proline is known to protect membrane integrity as well as the increase in the antioxidant transcript levels of ascorbate peroxidase (*SbAPX2)* and catalase (*SbCAT3)* under high NaCl concentrations. The addition of Ca^{2+} proved to have an alleviatory effect by the slight decrease in MDA observed in seedlings under 200 mM NaCl treatment.

Following the level of lipid peroxidation on sorghum, the effects of salt stress and Ca^{2+} were also assessed on the membrane structure using Scanning Electron Microscope (SEM). The membrane is crucial to the plant serving as a biological barrier as well as protecting cells and organelles from abiotic stress including salt stress (Guo *et al*., 2019). It is well known that under salt stress the membrane undergoes oxidative damage (Yermiyahu *et al*., 1997). Salt stress alters membrane lipids, which then has a direct effect on the properties of membrane proteins and activity of signalling molecules resulting in the adjustment of membrane permeability and fluidity (Mansour, 2013). Additionally, salt stress causes ionic imbalance due to the increase in Na^+, where excess Na^+ displaces Ca^{2+} from the phospholipid membrane binding site, which imposes membrane damage (Yermiyahu *et al*., 1997; Rahman *et al*., 2016). Salt stress damaged the sorghum structure and this was evident through the shrinkage of the epidermal and xylem layers of the seedlings. Ca^{2+} provided structural integrity for seedlings

that were stressed as displayed by the smoothened epidermis and the thickened xylem layers. Furthermore a high Na^+/K^+ (3.19) ratio was observed under 300 mM NaCl concentrations while exogenously applied Ca^{2+} reversed this effect resulting in a low Na^+/K^+ (1.5) ratio. This is consistent with the effective maintenance of ion homeostasis by Ca^{2+} as reported previously (Yermiyahu *et al*., 1997).

In chapter 4, the study brings a link between biochemical effects and the transcriptional levels in sorghum seedlings that were stressed with NaCl and observed the effects of Ca^{2+} in improving salt stress tolerance. To understand this relationship the transcripts of the antioxidant enzymes [*superoxide dismutase* (*SOD*), *ascorbate peroxidase* (*APX*) and *catalase* (*CAT*)], the *Salt Overly Sensitive* pathway (*SOS 1, 2* and *3*) and the *vacuolar* Na^+/H^+ *exchanger antiporter 2* (*NHX2*) genes from sorghum were measured using quantitative real-time polymerase chain reaction (qRT-PCR) in response to salt stress and Ca^{2+}. The physiochemical parameters of the aforementioned genes were investigated with the use of ExPASy Proteomic server. *SbCAT3* had the longest protein length among other antioxidant enzymes with the highest molecular weight, however, all three antioxidant genes were acidic. Similarly, among the *SOS* genes, *SbSOS1* had the longest protein length and the highest molecular weight. All three *SOS* genes were acidic, whereas *SbNHX2* was basic. All antioxidant genes including *SbSOD4A*, *SbAPX2* and *SbCAT3* were constitutively expressed in *Sorghum bicolor* and their existence in sorghum was confirmed experimentally by sequencing. In the presence of 300 mM NaCl, to our surprise *SbSOD4A* transcript decreased, whereas there was a significant increase in *SbAPX2* and *SbCAT3* transcript levels. The addition of Ca^{2+} decreased the transcript levels of all three genes under salt stress. When observing the expression patterns of the SOS pathway genes the study showed that *SOS2* and *SOS3* were constitutively expressed under normal conditions whereas there was no significant change in their expression levels under salt stress. Contrary to the expression patterns of *SOS2* and *SOS3*, *SOS1* was upregulated under salt stress. This may be

explained by the role of *SOS1* in the exclusion of toxic Na^+ into the root apoplast region away from the cells delaying uptake into shoots and leaves (Apse *et al*., 1999; Tester and Davenport, 2003). Ca^{2+} decreased the expression of *SbSOS1* in a concentration dependent manner whereas the expression of *SbSOS2* and *SbSOS3* transcripts was low with a slight increase under 35 mM Ca^{2+} for *SbSOS3*. Based on these results it was suggested that Ca^{2+} might have induced tolerance through binding of Ca^{2+} to the phospholipid bilayer of membranes to prevent the uptake and transport of Na^+ into cells as previously demonstrated (Rubio *et al*., 2003; Kumari *et al*., 2018), hence a high expression of *SbSOS1* was not necessary. Lastly, the study reported that the *vacuolar SbNHX2* was constitutively expressed under normal conditions but its expression was reduced by salt stress. On the other hand the highest concentration of Ca^{2+} used in this study in combination with NaCl significantly induced the transcript level of the *vacuolar SbNHX2*.

In conclusion, the study suggested that sorghum on its own has the ability to respond to high salt stress through modulating osmoprotectants and by regulating stress responsive genes. Finally, 5 mM and 15 mM Ca^{2+} were the most effective Ca^{2+} concentrations to enhance salt tolerance by improving the germination and growth of sorghum, while the decreased Na^+/K^+ ratio and ROS content, led to improved membrane structure. Future work could involve investigating the effect of salt stress and Ca^{2+} on abscisic acid, a key hormone involved in seed germination. It would also be of great value to investigate the effect of exogenously applied Ca^{2+} in combination with other signalling molecules such as nitric oxide, carbon monoxide, and hydrogen sulphide amongst others.

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