THE EFFECT OF CHITOSAN IN THE GERMINATION AND GROWTH OF *SORGHUM BICOLOR* UNDER SALT STRESS

By

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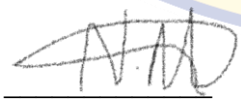
Signature:  **Date: 27 May 2022**

DEDICATION

This work is dedicated to my late grandmother (Nndwammbi Azwinndini Violet) may your soul continue to rest in eternal peace. To my family, I hope that this research will justify that hard work, discipline, and perseverance yield good results.

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MANUSCRIPT THESIS

This thesis was written in manuscript style in a manner that is suited for publication.

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LIST OF ABBREVIATION

$[Ca^{2+}]_{cyt}$	Calcium ion concentration in cytosol
ABA	Abscisic acid
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
ASC	Ascorbate
ATP	Adenosine triphosphate
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
$CaSO_4$	Calcium sulphate
CAT	Catalase
Chl a	Chlorophyll a
Chl b	Chlorophyll b
Cl^-	Chloride ion
CO_2	Carbon dioxide
CT	Chitin
DHAR	Dehydroascorbate reductase
ddH_2O	Double distilled water
EC_e	Electrical conductivity
EDX	Energy dispersive X-ray spectroscopy
Fe	Iron
G	Gram
GA	Gibberellin
GB	Glycine betaine
GI	Germination index

GLRs	Glutamate receptor-like channels
GP	Germination percentage
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
H_2SO_4	Phosphoric acid
K^+	Potassium ion
MDA	Malondialdehyde
MDHAR	Monodehydroascorbate dehydrogenase
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MGT	Mean germination time
mRNA	Messenger RNA
Na^+	Sodium ion
Na^+/H^+	Sodium/hydrogen ion
NAD	Nicotinamide adenine dinucleotide
NSCCs	Non-selective cations channels
O_2	Superoxide radical
$^1O_2^{\bullet-}$	Singlet oxygen
$OH^{\bullet}$	Hydroxyl radical
OPP	Oxidative pentose phosphate
POD	Peroxidase
PS I	Photosystem I
PS I	Photosystem II
ROS	Reactive oxygen species
RWC	Relative water content

SOS	Salt Overly Sensitive
SOS1	Salt Overly Sensitive 2
SOS3	Salt Overly Sensitive 3
TBA	Thiobarbutric acid
TCA	Trichloroacetic acid
TG	Total germination

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ABSTRACT

The agricultural sector has been facing enormous challenges including reduced crop yield and increased food production demand to cater for the growing population. Crop production is severely affected by abiotic and biotic factors, however, abiotic stresses especially salinity is a major factor that has contributed to more than 50% loss of important crops including rice, wheat, and maize. The use of fertilisers and pesticides played a vital role in improving crop yield and quality throughout the seasons. However, in the last decade, there has been a rising concern about their negative effects on human health and the environment due to their overuse. It is therefore necessary to develop novel technologies that will assist the agricultural sector to produce more crops in an environmental friendly manner and to overcome the negative effects of stress on crop growth. One of the promising targets is the application of small signalling molecules such as nitric oxide, carbon monoxide, and potential bio-stimulants including nutrients, (calcium, zinc, molybdenum, and iron), bio-polymers (cellulose, chitin, and chitosan amongst others). Chitosan is a natural compound obtained from the deacetylation of chitin. This compound is beginning to receive noticeable attention in agriculture due to its attractive features, which include biodegradability, non-toxicity and biocompatibility for application with different medium. A few studies showed the effects of chitosan to improve growth and tolerance to stress, but the mechanism of chitosan mediated stress tolerance remain elusive. This study reports the role of chitosan in conferring salinity stress tolerance in sorghum towards increasing crop production and unveiling the mechanism of chitosan-induced tolerance. This will be achieved by assessing the efficiency of chitosan on germination, growth and antioxidative defence capacity of sorghum under salt stress.

Decontaminated seeds were sown in mediums containing different NaCl (0, 100, 200, and 300 mM NaCl) and chitosan (0, 0.25, 0.5 mg/ml) concentrations. Samples were incubated in tissue culture for 7 days in the dark, while germination rate was measured daily. Germination

parameters included, germination percentage (GP), germination index (GI), mean germination time (MGT), and total germination (TG), while fresh and dry weights, and shoot length were measured on the 7th day. Seedlings germinated in ddH_2O only were further transferred into potting soil and after 14 days of growth were then subjected to different NaCl and chitosan treatments as applied every second day for 7 days. Seedlings were harvested, followed by assaying morphological (anatomical structure), physiological (growth assays) and biochemical (osmolytes and oxidative stress markers) responses.

Salt stress reduced germination and growth of sorghum as evident by low GP, GI, MGT, TG, fresh and dry weights. Salt-reduced growth was improved by the application of chitosan, whereas chitosan applied alone showed no significant effects on non-salt-stressed plants (control). Salt stress caused oxidative damage was evident in sorghum plants by the over production of H_2O_2 (~40%) under salt stress, which was sufficient to degrade polyunsaturated lipids as shown by an over 60% increase in malondialdehyde (MDA) content. Sorghum plants also accumulated high proline content (319.8%) and soluble sugars (40%) in response to salt stress. FTIR clearly showed the absorption peaks of functional groups corresponding to the presence of proteins, phenols and carbohydrates. These biomolecules were negatively affected in salt-stressed sorghum plants as was seen by shifts and disappearance of certain peaks and the development of new peaks representing the induction of defence related molecules. The negative effects of salt stress were reversed by the application of chitosan, which resulted in a significant decrease of both oxidative stress markers and osmolytes by more than 50% in all salt-treated plants. The antioxidant capacity was also induced as observed from high superoxide dismutase and ascorbate peroxidase activities, which increased by 66.7% and 180% in salt-stressed plants respectively as compared to the control. Interestingly, chitosan concentrations significantly reduced the antioxidant enzyme activities in salt-stressed plants by more than 50%.

This study showed that chitosan serves as an excellent bio-stimulant by improving sorghum germination, growth, and conferred a high degree of salt tolerance. Findings suggested that chitosan-induced stress tolerance in sorghum is related to the regulation of osmolytes and the antioxidant system, and in the long run, these results will lead to the use of chitosan on a larger scale to improve agricultural productivity and sustain food security globally.

Key words: Antioxidants, Chitosan, Germination, Osmolytes, Stress makers, Stress tolerance, *Sorghum bicolor*, and Salinity.

CHAPTER ONE

1. INTRODUCTION

Salinity is one of the environmental stresses that affects agriculture by decreasing growth and yield (Martinez *et al*., 2015). It does so by causing osmotic stress and ion poisoning, which lead to secondary stresses known as oxidative stress, due to damage of cell membrane, mitosis inhibition, loss of chloroplast activity and hence decreasing the efficiency of photosynthesis (Munna, 2002). It has been estimated that about 7% of the land around the world and close to 20% irrigated land areas are affected by salinity (Parihar *et al*., 2015; Negrão *et al*., 2017; Velmurugan *et al*., 2020; Ibrahim *et al*., 2021). This has a great impact on biomass production and yield of staple foods (Parihar *et al*., 2015). Therefore, to minimise the effects of salinity is very important to introduce sustainable agricultural practices and to meet present and future food demand globally (Arif *et al*., 2020).

Moreover, in arid and semi-arid areas manual or artificial irrigation is one of the ways to improve agricultural productivity (Formi *et al*., 2017). However, poor irrigation, lack of rainfall, salt ingression, water contamination and other environmental factors are the main drivers of salinity increase in our ecosystem (Mishra and Tanna, 2017). Salinity stress proved to negatively affect the growth and development of plants through alteration of physiological, and biochemical processes including chlorophyll synthesis, photosynthesis, respiration, and ion homeostasis. Furthermore, salinity stress is detrimental to metabolism, in particular nitrogen or carbon assimilation mechanisms. However, when plants sense any threat, they activate hormone biosynthetic pathway and hence accumulation of different hormones (Goche *et al*., 2020), and induction of the antioxidant system as well as synthesis of compatible solutes (Chen and Marata, 2002).

However, the effectiveness of the above-mentioned adaptive mechanisms differs from plant-to-plant and species, growth stage, period of exposure to stress, and level of the stress, among other factors (Guche *et al*., 2020). Different substances with elicitor properties, which mitigate salt stress linked to plants defence mechanisms have been identified as bio-stimulants for example chitosan (Jabeen and Ahmad, 2013).

Chitosan is the most naturally available polysaccharide and is used as a cheap, safe, and non-toxic product. Furthermore, chitosan is a well-known remediation agent in many fields such as environmental and industrial (Morais *et al*., 2008: Zhang *et al*., 2016). Chitosan can be produced from different sources including walls of exoskeletons of crabs, shrimp, lobster, insects, and fungi. However, chitosan produced through the deacetylation of chitin, is regarded as non-toxic, biodegradable, and biocompatible natural polymer (Mehmood *et al*., 2020). It has been utilised in several applications such as biomedicine, drug delivery systems, hydrogels, water treatments, and food packaging (Mehmood *et al*., 2020).

Due to the above properties, chitosan has gained potential application in other fields such as biotechnology, biomedicine, and cosmetics (Yuvaraja *et al*., 2020). It was first applied as a bio-stimulant in 1983 due to its proteinase inhibitor through the production of phytoalexin (Walker-Simmons *et al*., 1983). Since then, chitosan has been showing significant improvement of germination, growth, and flowering of different crop species such as cereals, fruits and medicinal crops (Turk, 2019). In addition to its antimicrobial activity, chitosan promotes germination parameters in *Begonia* and *Zea mays* (Chen *et al*., 2011; Mondal *et al*., 2013); encouraged early flowering in ornamental plants (Amiri *et al*., 2016) and improved biomass (fresh and dry weights) in *Solanum tuberosum* (Asghari-Zakaria *et al*., 2009). With all the interesting and significant characteristics of chitosan in agriculture, its mechanism of action remains elusive.

Sorghum [*Sorghum bicolor* (L.) Moench] is a naturally abiotic stress (drought and salinity) tolerant and high productive C_4 photosynthetic crop (Krishnamurt *et al*., 2007; Guche *et al*., 2021). In terms of area of cultivation and production yields, sorghum is ranked as the 5th most important crop in the world after maize, rice, wheat and barley and 2nd in Africa after maize (FAOSTAT, 2006). In addition, sorghum is more salt tolerant than maize (Ngara *et al*., 2020), and thus the availabilityof its genome sequence makes it a very attractive and relevant model crop to use when studying the mechanisms of stress tolerance in cereal crops.

1.2. PROBLEM STATEMENT

Abiotic stress, mainly extreme temperature, drought and salinity are the major contributing factors to over 50% of crop loss globally. Furthermore, salinity only has been estimated to be affecting about 20% cultivated land and 33% agricultural land worldwide (Shrivatava and Kumar, 2015). World's agriculture targets to feed about 10 billion people by the year 2050. To achieve that food production is expected to increase by at least 70% more than what is currently being produced. However, environmental factors including drought, and most importantly salinity, hinder crop growth and quality. Food security depends on the discovery of innovative strategies to help improve crop growth and productivity under saline stress. To achieve this, there is a need to understand the defence mechanisms inherent in plants such as induction of gene expression, hormone regulation, osmoprotectants, signalling and balanced ion homeostasis, for improved stress tolerance. It will make more scientific sense to obtain this information from a crop that is moderately tolerant to stresses such as sorghum.

1.3. AIM AND OBJECTIVES

The aim of this study was to elucidate the role of chitosan to confer tolerance in sorghum under salt stress. To achieve the aim of the study, the following objectives were pursued

I. To germinate sorghum seeds under salt stress in the absence (control) and presence of chitosan (treatment).

II. To evaluate the physiological response of sorghum seedlings to salt and chitosan by performing germination and growth assays.

III. To study the morphology, anatomy and element distribution of sorghum at a vegetative growth stage in response to salt and chitosan.

IV. To study the biochemical response of sorghum plants in response to salt and chitosan by evaluating the contents of osmolyte, oxidative stress markers, and activation of the antioxidant defence system.

1.4. SIGNIFICANCE OF THE STUDY

The agricultural sector is significantly affected by environmental factors such as drought and salinity. These stresses are the major cause of several physiological, morphological, biochemical as well as molecular changes in plants, which in turn results in quality and yield loss of most important agricultural crops (Shrivantava and Kumar, 2015). In the past, the use of pesticides and fertilisers played significant roles in ensuring good quality and yield of crops. However, overuse of these chemicals has resulted in negative effects on both human and animal health and the environment (Davydov *et al.*, 2018). *Sorghum bicolor* is one of the most important cereal crops in the world, with benefits such as a food source and biorefinery uses, it is therefore necessary to develop environmental friendly technologies (such as application of bio-stimulant) to improve productivity and quality of important agricultural crops using this model crop. Chitosan, being a potential bio-stimulant, which is naturally occurring, biodegradable, non-toxic and biocompatible, it is therefore necessary to take advantage of its properties to investigate its role in mitigating crop (*S. bicolor*) stress to improve quality and yield. This study is crucially relevant at this point of economic collapse in South Africa, since the findings will be applied in a larger scale (agricultural sector) to help improving crop production, hence increasing food in the market (beer, oats, and other food products) and animal feed for citizens and exportation to the neighbouring countries and boost the economy.

CHAPTER TWO (LITERATURE REVIEW)

UNDERSTANDING THE MECHANISM OF SALT TOLERANCE IN PLANT GROWTH: CHITOSAN AS THE BIO-STIMULANT

2.1 INTRODUCTION

Salinity is a major abiotic stress that severely affects plant growth development and crop yield. Detrimental effects of salinity are putting strain on the agricultural sector in arid and semi-arid regions with low rainfall (Jha *et al*., 2019). It is estimated that about 7% of total land and 20% of arable land are already affected by salinity (Zhu and Gong, 2014). By the year 2050 it has been predicted that about 50% of the arable land will be unproductive because of salinity. Moreover, the increase in human population requires an increase in food production to meet the demand and secure food for the future. There is an urgent need to utilise arable land to increase crop productivity even in unproductive lands by developing efficient and tolerant crops that are able to grow in saline environment (Safdar *et al*., 2019; Moukhtari *et al*., 2020).

Salinity affects growth and development by causing water deficit due to osmotic stress, ionic stress, nutritional imbalance, and oxidative stress leading to cellular damage and hence cell death (Shrivastava and Kumar, 2015). However, plants have evolved several mechanisms including the management of osmotic and ionic stress through osmoregulation and ion homeostasis and compartmentalisation (dos Santos *et al*., 2022). They also use several detoxification processes to scavenge toxic reactive oxygen species, which are not limited to the accumulation of non-enzymatic antioxidants (ascorbic acid, glutathione, catenoids, proline, flavonoids) and activation of enzymatic antioxidants and expression of key genes involved in stress response.

With all these important features, plant yield and growth are still affected in very high salinity conditions. It is therefore necessary to develop new scientific approaches to allow crops to

efficiently tolerate salt stress, while producing maximally. One of the recent developments to improve stress tolerance in crops is the utilisation of naturally available molecules. Among these compounds, chitosan and its derivatives deserve special attention (Quitadamo *et al*., 2021).

Chitosan (poly [1,4]-2-amino-2-deoxy-D-glucose) is a biopolymer, which is produced from the deacetylation of chitin, which can be extracted from fungi and the exoskeleton of crustaceans (Zhang *et al*., 2021). Chitosan has a unique structure, which is characterised by three functional groups including the amino group, primary and secondary hydroxyl group that are responsible for enhancing its affinity (Betchem *et al*., 2019). Due to its excellent properties including, non-toxicity, biodegradability, biocompatibility and affordability, chitosan has been applied in several fields including the agricultural sector. Since the discovery of chitosan, several studies have investigated its role in improving plant growth under environmental factors including salinity in *Lactuca sativa* L (Zhang *et al*., 2021), *Oryza sativa* L (Pongprayoon *et al*., 2013), *Zea mays* (Guan *et al*., 2009) and *Trifolium repens* (Li *et al*., 2017). Bittelli *et al*. (2001) indicated that chitosan's effectiveness in alleviating salinity stress is associated with increase in water use efficiency, mineral nutrients, improved photosynthesis and hence reduced oxidative stress. In addition, other studies suggested that mitigation of stress by chitosan is also achieved through the over accumulation of total sugars, soluble proteins, and improvement of antioxidant scavenging capacity in plants (Jabeen and Ahmad, 2013; Tourian *et al*., 2013). This review will describe the effects of salt stress on plants, some possible mechanisms of salt tolerance and the use of exogenous compunds to improve tolerance with special reference to chitosan. Finally, some recent data on chitosan as a nanomaterial will also be briefly discussed.

2.2 Effects of salinity on plant physiology

Through their lifetime, plants are confronted with different environmental stresses such as temperature, drought, flooding, pollutant, heavy metal stress, and salinity (Formi *et al*., 2017). All these stresses can reduce the growth and also alter the plant's ability to reach their full maturity (Mahajan and Tuteja, 2005). Salinity being a condition that is characterised by high salt concentration in the soil. Soils are regarded saline when the electrical conductivity (ECe) of a saturated paste soil extract is 4dS/m (40 mM NaCl) and generates an osmotic pressure of about 0.2 MPa (Munns and Tester, 2008). There are other dissolved salts, which contribute to soil salinisation, such as, Na_2SO_4, $MgSO_4$, $CaSO_4$, $MgCl_2$, KCl, and Na_2CO_3, whereas NaCl is the most prevalent solute salt (Munns and Tester, 2008; Formi *et al*., 2017).

According to Rahneshan *et al.* (2018) salts in the soil water may inhibit plant growth in two ways. Firstly, by affecting the plant's ability to absorb water leading to growth reduction, resulting in osmotic or water-deficit stress. Secondly salinity or excess salt causes ionic stress due to excess amount of salt entering the plant in the transpiration stream, this may lead to injury of cells in the transpiring leaves and this may cause further reductions in growth. This is called the salt specific or ion-excess effect of salinity (Parihar *et al*., 2015). This effect reduces water potential and causes ion imbalance or disturbances in ion homeostasis and toxicity; this altered water status leads to initial growth reduction and limitation of plant productivity. The detrimental effect is observed at the whole plant level as death of plants or decrease in productivity. Salt stress affects all the major processes, which promote germination, growth and development including hormone signalling, chlorophyll pigments and photosynthesis, water relation, nutrient balance, ion homeostasis, antioxidant capacity, and gene expression (Parida and Das, 2005).

2.2.1 Effects of salinity on seed germination, growth, and yield

Seed germination is one of the most important phases in the plant growth cycle that determines the yield. Fernández-Torquemada and Sánchez-Lizaso (2013) reviewed the effects of salinity in the germination of different plant species including *Posidonia, Oryza sativa, Triticum aestivum, Zea mays* and *Brassica* spp. Salinity affects the germination process, by altering the physiological activity of the seed. It hinders water potential, protein content, and reduction of food reserved in germinating seeds of several plants such as *Brassica oleracea var. botrytis* and *Brassica oleracea var. italica* (Wu *et al*., 2019). Furthermore, salinity stress results in significant damage to plant yield and its components. High saline soil caused a decline in the biomass, leaf area, yield, stem, and root length of plants (Zörb *et al.*, 2019).

2.2.2 Effects of salinity on the physiological processes, stomatal conductance, and transpiration

Over the years, it has been documented that high level of salinity affects photosynthesis due to stomata alteration, such as stomatal closure (Munns and Tester, 2008), non-stomatal restrictions comprising chlorophyll break down (Jiang *et al*., 2012), deprivation of enzymatic reaction and membranes of photosynthetic apparatus (Mittal *et al*., 2012), and chloroplast ultrastructure alteration (Gengmao *et al*., 2015). The leaf chlorophyll and total carotenoid contents of plants generally decrease when plants are under salt stress or affected by salinity, which is mostly due to protein instability. The oldest leaves start to develop chlorosis and fall with prolonged periods of salt stress. Contrary to that, chlorophyll content increases under salt conditions in *Amaranthus spp* as a result of reduction in leaf tissue water content (Wang and Nil, 2000). Protochlorophyll, chlorophyll and carotenoids are significantly reduced under NaCl stress, but the rate of decline of protochlorophyll and chlorophyll is greater than of Chl-a and carotenoids. High concentration of Na^+ in plants causes direct ionic effect, which stimulates

stomatal closure hence reducing the amount of CO_2 and results in photosynthesis reduction (Hidangmayum *et al.*, 2019).

2.2.3 Effects of salinity on nutrient's uptake

Nutrient disturbances due to salinity reduce plant growth by affecting the availability, transport, and partitioning of nutrients. Nutrient deficiencies or imbalances are due to the competition of Na^+ and Cl^- with essential nutrients such as K^+, Ca^{2+}, and NO_3^- (Niste *et al.*, 2014). Reduction of plant growth and yield on plants under salt stress is as a result of ion toxicities (Na^+ and Cl^-) and ionic imbalances, acting on biophysical and/or metabolic components of plant growth (Jouyban, 2012). Increased NaCl concentration induces an increase in Na^+ and Cl^-, while there is a decrease in transport of essential element N, P, Ca, K and Mg levels in fennel (Alh and Omer, 2011). Saline environment is generally deficient in nitrogen and in addition salinity interferes with NO_3^- uptake in many plant species hence limiting plants from nitrogen. The reduction in NO_3^- uptake could be due to high Cl^- content in saline soil (Yamshi *et al.*, 2020).

2.2.4 Effects of salinity effects on plant's water relation

Water potential is an important physiological parameter for determining the water status of plants (Parida and Das, 2005). According to Romero-Aranda *et al.* (2001), increased salt concentrations in the roots can lead to a decrease in leaf water potential, hence affecting many processes within the plant. At very low soil water potentials, this condition interferes with the plant's ability to extract water from the soil and maintain turgor. However, at low or moderate salt concentration (higher soil water potential), plants adjust osmotically by accumulating solutes and maintain a potential gradient for the influx of water. Khan *et al.* (2013) found that in *Cucumis sativa*, water potential decreased linearly with an increase in salinity level. Salinity reduces crop yield, costing billions of dollars annually (Parvaiz *et al.*, 2018). Globally, about

45 million hectors is affected by high Na^+ and Cl^- concentration as a results of salinity levels in the soil. Under salinity stress the osmotic pressure of the soil exceeds that of root cells, hence reducing the plant's ability to take up nutrients and water (Capula-Rodriguez *et al*., 2016). Salinity is also known to directly affect the metabolic activities in the cytosol and plasmalemma. According to Negrao *et al.* (2016) plants respond to salinity in two phases including shoot ion-independent response, which occurs within minutes to days and the ion-dependent response which occurs after days to weeks.

2.3 Mechanisms of salinity stress tolerance

Unlike animals with several strategies to move away from unfavourable conditions, plants are immobile, thus cannot relocate from one environment to another, thus they have developed several adaptive and defence mechanisms (Yadav *et al.,* 2020). These mechanisms include several physiological, biochemical, molecular, and cellular responses (Rejeb *et al*., 2014). However, ways in which plants respond to abiotic stress (salinity) depend on plant species, cultivar, development stage of plants as well as salt concentration, duration of exposure to stress and method of salt application (Oliveira *et al*., 2013). In glycophytes, the seedling stage is more sensitive to salinity compared to the germination stage. Munns (2002) found that at later stages of plant development, physiological processes such as photosynthesis, respiration, protein synthesis, water relation and enzyme reactions are affected by salinity. For example, in *S. bicolor*, response to salt stress has been investigated in different growth and development stages including germination (Olivera and Gomes-Filho, 2009; Mulaudzi *et al.*, 2020), seedling (Swami *et al*., 2011) and vegetative growth stage (Bavei *et al*., 2011; Rakgotho *et al.*, 2022), whereas Jafari *et al.* (2009) studied tolerance during final stages of physiological maturity. All these studies showed that *S. bicolor* is moderately tolerant to salt stress. Different mechanisms of salt tolerance in plants include hormone signalling, ion homeostasis and

compartmentalisation, biosynthesis of osmoprotectants and compatible solutes, activation of the antioxidant system and, polyamine synthesis, gas (NO, CO, H_2S) signalling and modulation (Gupta and Huang, 2014).

2.3.1 Hormone signalling

Throughout the plant's life cycle, phytohormones play an important role in controlling the interaction between plants and the environment, including plant responses to salinity stress (Tao *et al*., 2015). The major plant hormones are auxins, gibberellins (GA), cytokinins (CK), abscisic acid (ABA), ethylene (ET), salicylic acid (SA), jasmonates (JA), brassinosteroids (BR) and strigolactones (Peleg and Blumwald, 2011). However, among all these only ABA, SA, JA, and ET have been found to be play vital roles in mediating plant defence response to pathogens and abiotic stresses (Bari and Jones, 2009; Verma *et al*., 2016; Yu *et al*., 2020) and they will be briefly disscussed.

2.3.1.1 Abscisic acid

Abscisic acid is one of the vital stress response hormones, which plays a significant role in salt stress defence (Verma *et al*., 2016). It serves as a central integrator that connects and reprograms the complex developmental process and salt stress, especially osmotic stress and adaptive signalling cascades in plants (Goldack *et al*., 2014). In response to salinity and osmotic stress, endogenous level of ABA rapidly increases, and enhanced ABA signalling activates *sucrose nonfermenting 1-related protein kinase* (*SnRK2s*) (Umezawa *et al*., 2009). Furthermore, according to Thalmann *et al*. (2016), in response to salinity, ABA-activated SnRK2s regulates osmotic homeostasis through regulating the *β-amylase1* (*BAM1*) and *α-amylase* (*AMY3*)-dependent breakdown of starch into sugar and sugar-derived osmolytes. High level of ABA modulates the abiotic stress-regulation network, whereas biotic stress response is mediated by antagonism between other hormones such as SA and JA (Rejeb *et al.*, 2014). According to Sun *et al*. (2016), there is a great overlap in gene expression in response to salinity

and ABA treatments, which in turn indicated that ABA signalling pathway plays an important role in sorghum in response to salt stress. Furthermore, under salt stress, the ABA synthesis related gene (*Sb04g030640*) was up-regulated in the leaves of salt-tolerant sweet sorghum inbred lines and was down-regulated in salt sensitive inbred lines.

2.3.1.2 Salicylic acid

Salicylic acid (SA) plays an important role in plant salt tolerance. The exogenous application of SA was found to improve the antioxidant system and production of osmolytes as well as promoting the photosynthesis of salt stressed plants (Palma *et al*., 2013; Ahanger *et al*., 2019). Application of SA activates the expression of *P5CS*, which is responsible for proline accumulation and reduces the concentration of H_2O_2 under salt stress (Lee *et al*., 2010). It also has positive role in facilitating symbiosis and nitrogen fixation in saline environments, thus alleviate the negative effects from salt stress. Over expression of *MYB* on salt stressed plants supplemented with SA implies that ABA might have a role in the SA-mediated salt response (Zheng *et al*., 2018).

2.3.1.3 Jasmonate

Jasmonates are phytohormones that regulates plants response against biotic (herbivore and pathogen attack) as well as mitigate abiotic stress tolerance and regulates various plant development aspects such as root growth, flower formation, stamen development and leaf senescence (Wasternack and Hause, 2013; Chini *et al*., 2016). There are produced from α-linolenic acid (α-LeA/18:3) through the octadecanoid pathway, however, α-LeA is produced through the coordinated actions of fatty acid desaturase (FAD) and phospholipase A1 (PLA) within the plastids (Huang *et al*., 2017). Jasmonic acid (JA) and its methyl ester methyl jasmonate (MejA) have been reported to induce cell signalling and regulatory phenomenon responsible for seed germination, turerisation, senescence, root growth, reproductive and fruit ripening (Creelman and Mulpuri, 2002; Wasternack and Hause, 2002). MejA has been also

found to mitigate the detrimental effects of salinity and water stress in soybean (Anjum *et al*., 2011).

2.3.1.4 Ethylene

Ethylene (ET) is a simple gaseous hormone playing a significant role in regulating plant growth and development and also serves as a key modulator between plant responses to environmental stresses. Furthermore, in the past decades, research has also highlighted the role of ethylene in regulating plant responses to different biotic and abiotic stresses (Tao *et al*., 2015; Riyazuddin *et al*., 2020). Ethylene biosynthesis and signalling are implicated in salinity stress tolerance in plants, moreover, in *A. thaliana* ethylene was found to mediate salinity stress tolerance as well as in other crop plants including grapevines, maize, and tomato (Siddikee *et al*., 2012; Freitsa *et al*., 2018).

2.3.2 Ion homeostasis and compartmentalisation

Salinity creates one of the threats to plants by causing osmotic and water stress. Salt enters the plants using symplastic and apoplastic pathways based on the plant type (Arif *et al*., 2020). In monocot plants, apoplastic pathway is the most preferred pathway whereas, dicots depend mainly on the symplastic pathway for salt transport (Figure 2.1). For NaCl transport in symplastic pathways, this relies mainly on transporters and channels. Non-selective cation channels (NSCCs) and Na^+/H^+ antiporter (NHA) or Salt Overlay Sensitive 1 (SOS1) are required for the influx of Na^+. Two gene families (Glutamate receptor-like channels (GLRs) and Nucleotide-gated channels (CNGCs) are activated in NSCCs mediation and are inhibited by Ca^{2+}.

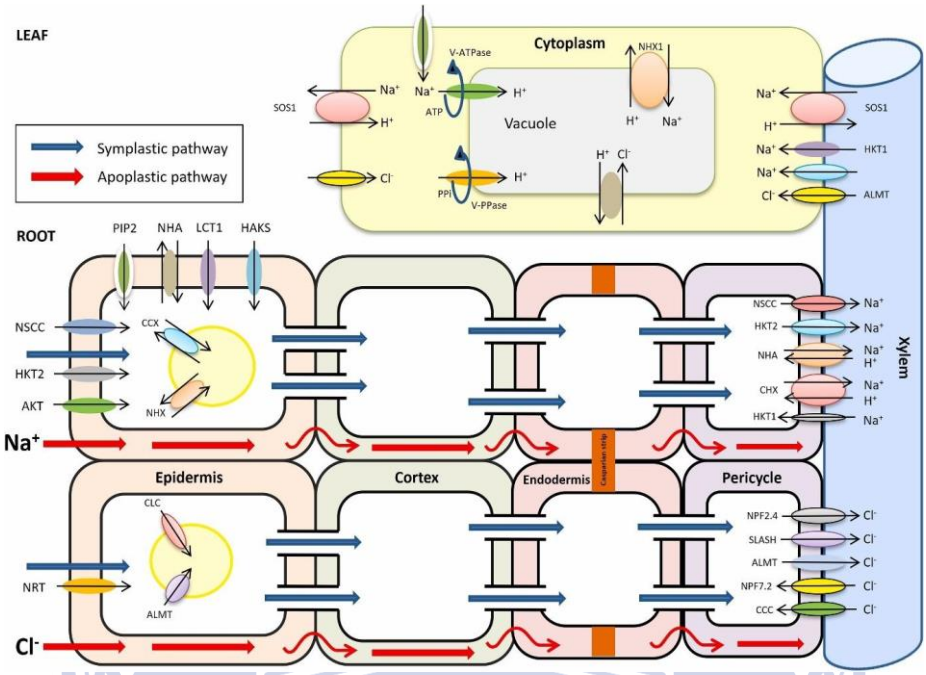

Figure 2.1: Symplastic and apoplastic pathway for salt transport in roots and leaf through transporters and channels. Transporters such as AKT, HKT2, NSCC, NHA, HAK, LCT, PIP2 are active in Na^+ influx and transport. For long-distance transport into the leaf NSCC, HKT, CHX, and NHA are activated. CCX and NHX are available on vacuole membranes and help in ion compartmentalization. For Cl^- other transporters are available for uptake and long-distance transport such as NRT, NPF, SLASH, ALMT and CCC (adapted from Arif *et al*., 2020).

The Salt overly sensitive (SOS) pathway is one of the well-studied pathways, which is involved in both ion homeostasis and salt tolerance. There are three important proteins involved in SOS signalling, namely SOS1, SOS2, and SOS3 (Gupta and Huang, 2014). SOS1 encodes a plasma membrane Na^+/H^+ antiporter and regulate Na^+ efflux at cellular level. Over-expression of SOS1 protein confers salt tolerance in plants. SOS2, encodes a serine/threonine kinase that is activated by saline stress elicited Ca^{2+} signals, whereas SOS3 gene, is a myristoylated Ca^{2+} binding protein and contains myristylation site at its N-terminus, which plays an important role in conferring salt tolerance. The C-terminal regulatory domain of SOS2 protein has a FISL motif (NAF domain), of 21 amino acid long sequence, and serves as a site of interaction for Ca^{2+} binding SOS3 protein. According to Keisham *et al.* (2018), when Na^+ enters the cell through the NSCCs and transporters (high affinity K^+ uptake transporter (HAK5) and high-

affinity K^+ transporters (HKTs), It is detected by an unknown sensory mechanism, and Ca^{2+} signalling is activated. Ca^{2+} modulates intracellular Na^+ homeostasis along with Salt Overlay Sensitive (SOS) proteins (Gupta and Huang, 2014). Salt stress induces $[Ca^{2+}]_{cyt}$, which is then detected by SOS3, which then interacts with and SOS2 to form an SOS2-SOS3 complex. The SOS2-SOS3 complex then phosphorylates SOS1 and activates its Na^+/H^+ antiporter activity facilitating Na^+ efflux from the cell. SOS1 protein is characterised by a long cytosolic C-terminal tail, about 700 amino acids long, comprising a putative nucleotide binding motif and an auto-inhibitory domain. This auto-inhibitory domain is the target site for SOS2 phosphorylation. The phosphorylated SOS1 results in the increased Na^+ efflux, through a novel efflux mechanism involving a putative ouabain (OU)-sensitive ATPase. Na^+ is then finally compartmentalised within vacuoles through the Na^+/H^+ exchange (NHX) signalling system as a response to salt stress (Gupta and Huang, 2014). Other than conferring salt tolerance, it also regulates pH homeostasis, membrane vesicle trafficking, and vacuole functions.

The plant maintains ion homeostasis and balance by ion influx and its compartmentalisation, which helps the plant for proper growth under saline environment. Plants cannot withstand increase in salt stress in thier cells, so it clears it from the cytosol as described through the SOS signalling pathway and demonstreated in Figure 2.2 (Gupta and Huang, 2014; Arif *et al*., 2020).

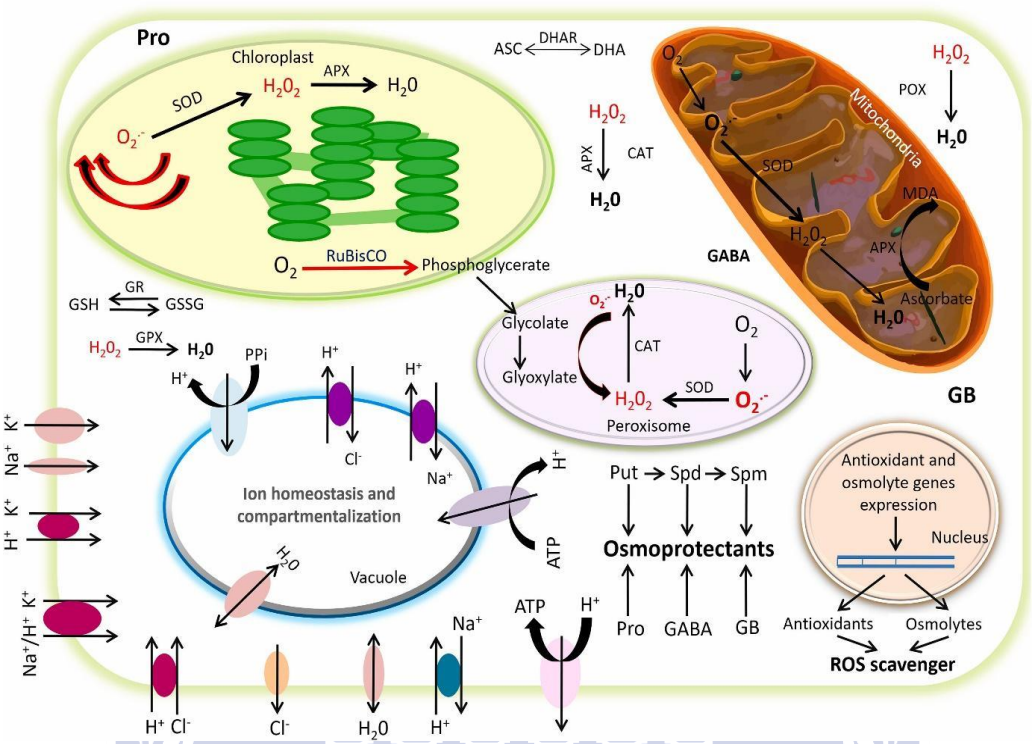

Figure 2.2: Cellular mechanism undergoing ion homeostasis and its compartmentalisation, induction of antioxidant and osmolytes in the cytosol and various organelles. Ion homeostasis takes place through different transporters that help influx of ion within the vacuole (adapted from Arif *et al.*, 2020).

2.3.3 Accumulation of osmoprotectants

Osmoprotectants, also known as compatible solutes or osmolytes, are a group of large organic compounds with a polar change, and dissolves well in water and do not inhibit cellular metabolism even at high concentration. Sugars or sugar alcohols and zwitter-ion compounds are two major classes of osmolytes. Examples of sugar alcohols include mannitol, sorbitol, pinitol, and D-inositol and oligosaccharides (trehalose and fractions), whereas others include amino acids such as proline and glycine betaine (Verslues *et al.*, 2006). Different plants produce different solutes, which are produced at different amounts based on particular plant species. In the cytoplasm, osmoprotectants raise osmotic pressure and stabilise proteins and membranes when plants are under harsh salt condition. Production of organic osmolytes in addition to proteins from the late embryogenesis abundant (LEA) superfamily, play an important role in maintaining the low intracellular osmotic potential of plants in hindering the

toxic effects of salt stress. Therefore, osmoprotectants have an important role in the adaptation of cells to different environmental conditions (Verslues *et al*., 2006).

2.3.4 Antioxidant defence

Abiotic stress such as salinity induces ionic and osmotic stress, hence causing a rapid overproduction of reactive oxygen species (ROS; Foyer and Noctor, 2005; AbdElgawad *et al*., 2016). ROS are chemical species that contain oxygen, and they commonly include superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), singlet oxygen (O_2) and hydroxyl radical ($OH^{\bullet}$). Plants produce ROS to avoid tissue damage, however, ROS are highly toxic to plants when produced at high levels (Xia *et al*., 2009; Choudhury *et al*., 2013). ROS are involved in signalling; hence this might also help in reducing the oxidative stress caused during abiotic stress (Rajeb *et al*., 2014).

Furthermore, ROS production can also play a vital role in cell-to-cell communication, which is possible when there is an amplified signal by the *Respiratory Burst Oxidase Homologue D* (*RBOHD)* (Miller *et al*., 2010). In addition, ROS can also be a secondary messenger that modifies protection structures and activate defence genes (Spoel *et al*., 2011). Interestingly, ROS respond to both biotic and abiotic stress, but responses differ from one stress to another and differs with species (Rajeb *et al*., 2014). These ROS also led to perturbation of basic metabolic pathways hence causing oxidative damage to membrane and organic molecules such as proteins, DNA, lipids, and pigments ultimately leading to cell death (Milleer *et al*., 2010; Zare and Pakniyat, 2012). In response to salinity, plants use enzymatic and non-enzymatic antioxidants to detoxify ROS and cope with oxidative stress.

Enzymatic antioxidants function by converting oxidised metabolic products in a multi-step process to hydrogen peroxide and then to water using cofactors such as iron, zinc, copper, and manganese (Moussa *et al*., 2019). Enzymatic antioxidants include superoxide dismutase

(SOD), catalase (CAT), and enzymes of ascorbate (ASC)-glutathione (GSH) cycle, which are ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate dehydrogenase (MDHAR) and dehydroascorbate reductase (DHAR) (Das and Roychoudhury, 2014). In peroxisomes, H_2O_2 is expressed when glycolate is oxidised to glyoxylic acid during photorespiration (Miller *et al*., 2010). According to Suzuki and Mittler (2006) the cytosol has about three different types of peroxidases that are responsible for H_2O_2 detoxification, these include APX, GPX and PrxR.

2.3.4.1 Superoxide Dismutase (SOD)

SOD (E.C.1.15.1.1) is a member of the metalloenzymes family that forms part of the first line of defence in detoxifying ROS-induced stress and are present in every organelle. SOD plays a significant role in scavenging superoxide radicals ($O_2^{\bullet -}$) by dismutating it into oxygen and H_2O_2 (Banerjee and Royehoudhury, 2018). This process is vital in inhibiting the Haber-Weiss reaction, which is the formation of $OH^{\bullet}$. SOD enzymes are grouped into several isozymes in plants based on the metal ion that they bind, and they include Mn-SOD that is localised in mitochondria, Fe-SOD localised in the chloroplast and Cu/Zn-SOD localised in the cytosol, peroxisomes, and chloroplasts (Mishra and Sharma, 2019; Rio *et al*., 2018). According to Zare and Pakniyat (2012), increased SOD activity confers oxidative stress tolerance furthermore, to endure salt-induced oxidative damage, constitutive or induced activity of SOD and other antioxidant enzymes (POX, APX, CAT, and GR) is crucial for stress tolerance.

2.3.4.2 *Catalase (CAT)*

CAT (E.C.1.11.1.6) is a "*tetrameric heme-containing enzyme responsible for catalysing the dismutation of H_2O_2 into H_2O and O_2*" (Das and Roychoudhury, 2014). Catalase has high affinity for H_2O_2, than for organic peroxide. According to Mittler (2002), peroxisomes are the main site for H_2O_2 production due to β-oxidation of fatty acids, photorespiration, purine

catabolism and oxidative stress. Contrary to that, other studies suggested that CAT is also available in the cytosol, chloroplast, and mitochondria (Mhamdi *et al*., 2010), however, ascorbate peroxidase has higher affinity for H_2O_2 in these organelles.

2.3.4.3 *Ascorbate peroxidase (APX)*

APX (E.C.1.1.11.1) is an integral component of the ascorbate-glutathione (ASC-GSH) cycle. Having catalase scavenging H_2O_2 in the peroxisome, on the other hand, APX carry out the same role in the cytosol and the chloroplast. It alleviates stress by reducing H_2O_2 to H_2O and DHA, in presence of ascorbic acid as the reducing agent (Figure 2.2). However, according to Sharma and Dubey, (2004), APX family is made up of isoforms based on several amino acids and locations such as mitochondria, peroxisome, and the chloroplast.

2.3.4.4 *Glutathione Reductase (GR)*

GR (E.C.1.6.4.2) is a flavo-protein oxidoreductase, which rely on NADPH as a reductant to reduce glutathione disulfide (GSSG) to glutathione (GSH) (Das and Roychoudhury, 2014). In its reduced form, GSH can scavenge ROS and reactive nitrogen species (RNS), as a result contribute to a balanced redox homeostasis (Couto *et al*., 2016). The main sites of generation of GR are chloroplast, mitochondria, and cytosol. However, GR enzymes are further grouped in to two groups (GR1 and GR2) depending on the N-terminal prolongation (Garcia-Caparros *et al*., 2021).

2.3.4.5 *Monodehydroascorbate Reductase (MDHAR)*

MDHAR (E.C.1.6.5.4) is an antioxidant enzyme responsible for regenerating ascorbic acid out of short-lived MDHA, using NADPH as a reducing agent. Due to its function, it is co-localised with APX in the peroxisome and mitochondria, in which APX catalyses H_2O_2 and oxidise ascorbic acid in the process. This makes MDHAR a very important enzyme also, it has several isozymes in the chloroplast, mitochondria, peroxisome, cytosol, and glyoxysomes.

2.3.4.6 *Dehydroascorbate Reductase (DHAR)*

DHAR (EC1.8.5.1) is involved in scavenging ascorbate, catalysing the glutathione (GSH)-dependent reduction of oxidised ascorbate called dehydroascorbate (DHA). ROS is detoxified by a pool of reduced ascorbate regenerated by DHAR (Yadav and Sharma, 2016). DHAR is important in regulating the ascorbic acid pool size in symplast and apoplast, as a result maintains redox state of the plant cell. Moreover, it is found in high concentrations in seeds, roots, and shoots (Das and Roychoudhury, 2014).

In summary, the peroxidation of membrane lipids, and the degree of peroxidative damage of cells happen because of abundant production of oxygen free radicals. The toxic superoxide radical is rapidly dissimulated by superoxide dismutase (SOD) to H_2O_2, which can be detoxified by catalase (CAT) and guaiacol peroxidases since H_2O_2 is stable (Grant and Loake, 2000). The balance between ROS production and activities of the antioxidat enzymes determines if oxidative signalling and damage will occur. It is reported that under different environmental stresses the activities of these enzymes are increased (Zare and Pakniyat, 2012). And this process works together with the non-enzymatic antioxidants, that are big part of the antioxidant mechanism, they comprise of asorbaic acid, flavonoids, proline, carotenoids, phenolics, amino acid, and α-tocopherol (Koyro *et al*., 2012; Das and Roychoudhury, 2014). They play a significant role in giving protection against cell damage and regulate plant growth and development by improving biological processes such as mitosis, cell elongation and cell death (Kumar *et al*., 2013).

2.3.4.7 *Ascorbic Acid (AA) (Vitamin C*)

This is the most abundant and extensively investigated antioxidant and is found in different plant cell type, organelles and apoplast. It is a powerful electron donor to both enzymatic and non-enzymatic reactions. The Smirnoff-Wheeler pathway result in the production of most AA in plants cells in the presence of L-galactano-y-lactase dehydrogenase in the plant

mitochondria, with the remaining being produced from D-galacturonic acid (Foyer and Noctor, 2005). About 30 to 40% of the total ascorbate is produced in the chloroplast. Furthermore, majority of AA pool is not only concentrated in the cytosol but also in the apoplast, thus acting as the first line of defence against ROS attack. Two steps have been found to oxidase AA, oxidation into MDHA, which if not reduced immediately to ascorbate, disproportionate to AA and DHA occurs. It protects the cell against oxidative membrane by reacting with H_2O_2, $OH^{\bullet}$ and $O_2^{\bullet-}$ and regenerate α-tocopherol from tocopheroxyl radical (Das and Roychoudhury, 2014).

2.3.4.8 *Flavonoids*

Flavonoids are compounds found in the plant kingdom and normally found in high level in the leaves and pollen grains. There are four classes of flavonoids based on structure and these include, flavones, isoflavones, flavonols and anthocyanins. It has been found that flavonoids promote salt stress tolerance by scavenging free radicals, thus reducing oxidative stress (Chandran *et al*., 2019). However, according to Das and Roychoudhury (2014), flavonoids have been found to scavenge secondary ROS in plants during photosynthetic apparatus damage.

2.3.4.9 *Carotenoids*

Carotenoids is a member of lipophilic antioxidants, which are found in the plastids of both non-photosynthetic and photosynthetic cells (Das and Roychoudhury, 2014). However, carotenoids are not only found in plants but also in microbial community (Maoka, 2020). They exhibit their antioxidative activity by protecting the photosynthetic machinery in several ways. They react with lipid peroxidase products to end the chain reaction, scavenging singlet oxygen and generating heat as a by-product, they also hinder the formation of $^1O_2$ by interacting with chlorophyll (Das and Roychoudhury, 2014).

2.3.5 Transcription factors

Transcription factors (TFs) are proteins that act together with other transcriptional regulators, including chromatin remodelling, to employ or obstruct RNA polymerases to the DNA template (Lata *et al*., 2011). According to Kavar *et al.* (2007), there are several genes, which are activated in response to abiotic stresses during transcriptional level, and their products promote stress tolerance through production of vital metabolic proteins. Among these genes, transcription factors such as *AP2/ERF*, *MYB*, *NAC*, *WRKY*, *bZIP*, and *bHLH* are reported to play a vital regulatory role in plants in response to abiotic stress (Tang *et al.*, 2017; Sun *et al*., 2018; Tang *et al*., 2019). In rice plant *bZIP*, subfamily OsABI5 were overexpressed during salinity stress (Zou *et al*., 2008), ERF family, *SODERF3* and *SNAC1* were reported to be expressed during drought and salinity in tobacco and rice plants respectively (Hu *et al*., 2006; Trujillo *et al*., 2008). *MYB* family genes, *MYB15* and *OsMYB3R*-2 were expressed in Arabidopsis (Dai *et al*., 2007; Ding *et al*., 2009).

Any environmental stress can trigger changes in gene expression, and the programming of the molecular mechanism is regulated by the action of transcription factors. The altered expression of genes is an important event in helping plants to have an effective defensive state, and there is strong evidence that many genes are multifunctional and able to induce tolerance in plants towards more than one stress (Rejeb *et al*., 2014). According to Mengiste *et al.* (2003) phytohormones such as ABA, SA, JA, and Ethylene mediate the activity of genes involved in defence mechanisms. For example, *BOTRYTIS SUSCEPTIBLE1* (*BOS1*) gene activity is mediated by both stress responsive hormones (ABA and JA) and induces resistance against osmotic stress and necrotrophic pathogens. Transcription factor, *MYB96* plays an important role in plant protection under pathogen infection by mediating the molecular link between ABA induced by drought stress and SA expressed following pathogen infection in Arabidopsis (Seo

et al., 2010). In tomato, *SLAIM1* responds to both abiotic stress and infection with *Botrytis cinerea* (Xiong and Yong, 2003).

2.4 Application of chitosan as a bio-stimulants to improve salt tolerance in crops

Chitosan is a natural biopolymer, which is derived from the deacetylation of chitin (Figure 2.3) (Krupa-Malkiewicz and Fornal, 2018). Chitosan has capacity in reticulation and cation exchange in acid solutions and has great affinity with metallic ions (Rinaudo, 2006). Chitosan is safe for the environment, and it is characterised by its biodegradability, bioactivity, and biocompatibility. Chitosan is applicable in different fields including agriculture, waste treatment, food, textile, and pharmaceutical sector, cosmetics development, and biomaterials such as gels, films, polymer membranes and nanofibers (Rufato *et al*., 2018). This section discusses the application of chitosan with special focus on its role in agriculture to improve plant germination, growth and tolerance to stress. The mechanism of its stress induction is also discussed.

2.4.1 CHITOSAN

Chitosan antioxidant activity depends on the molecular weight and degree of deacetylation. Several studies confirmed that chitosan promotes the increase in yield and production and induces a range of metabolic changes hence plants become more tolerant to abiotic stresses (Rinaudo, 2006). However, responses vary from plant to plant, and this is also determined by the type of chitosan, molecular weight and concentration of chitosan (Malerba and Cerana, 2016; Krupa-Malkiewicz and Fornal, 2018). According to Martinez *et al*. (2015), chitosan is used widely in agriculture for its antimicrobial activity and its ability to activate plant defence-related enzymes such as chitinases, glucanases and phenylalanine ammonia lyase (PAL). Upon

the initial oxidative burst with H_2O_2 accumulation in several species, chitosan also induces the production of secondary metabolites such as polyphenolics, lignin, flavonoids and phytoalexins.

Chitosan enhances the defence responses to both abiotic and biotic stress on several plant species including *Trifolium repens* (Ma *et al*., 2012; Zeng and Luo, 2012; Li *et al*., 2017), *Thymus daenensis* (Bistgani *et al*., 2027), *Ocium ciliatum* and *Ocium basilicum* (Bistgani *et al*., 2017) and *Solanum lycopersicum* (Attia *et al*., 2021).

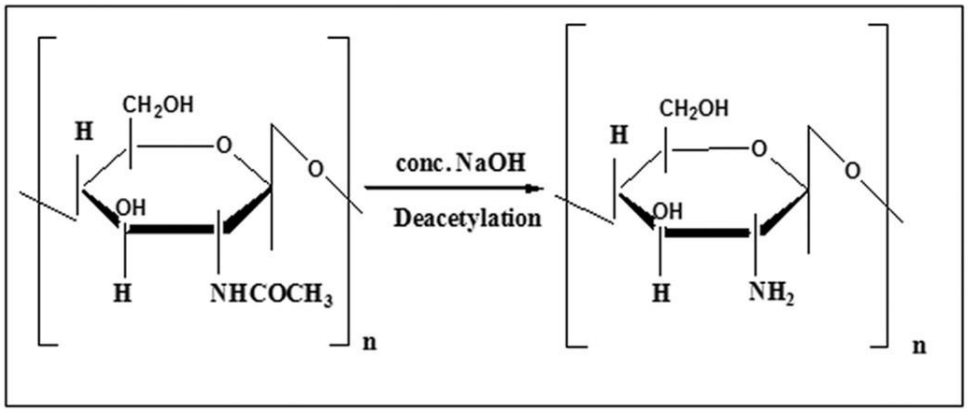

Figure 2.3: Chemical structure of Chitin and deacetylation of chitin to Chitosan (Adapted from Devil *et al*., 2017).

2.4.1.1 Chitosan in seed germination

In general, the use of oligo-chitosan improved wheat growth as observed by increased germination capacity, root length, seedling height as well as root activity (Ma *et al*., 2014). Batool and Asghar (2013) demonstrated an increase in germination percentage, germination rate, seedling vigour index, length, and dry weight on seeds of *Carum copticum* that were pre-treated with chitosan. Similar effects were also observed in other species including Zea *mays* (Shao *et al*., 2005), *Pennisetum glaucum* (Manjunatha *et al*., 2008), and *Brassica napus* (Sui *et al*., 2002). Chitosan also improved seed germination under salt stress in different crops. Pre-treatment of *Triticum aestivum* seeds with chitosan (0.0625%) also positively affected other

growth parameters including shoot length, root length, shoot dry weight and relative water content (RWC) for seedlings under saline environment (Ma *et al.*, 2012). Priming *Lens culinaris L.* seeds with chitosan (3 g/L) under salt stress (50, 100, 200, and 300 mM NaCl), had the highest germination percentage, hypocotyl length, radical length, hypocotyl dry weight and radical dry weight. (Al-Tawaha and Al-Ghzawi, 2013).

2.4.1.2 Chitosan on plant growth

The role of chitosan in promoting growth has been demonstrated in different crops including *Brassica oleracea var. capitata*, *Glycine max*, and *Ocimum basilicum* and *Vitis vinifera* L (Barka *et al.*, 2004). In a study conducted in 2018 by Al-Tawaha and co-workers, it was found that application of chitosan (120 mg/L) on *Oryza sativa* seedlings showed the highest growth on root dry weight. Chitosan also improved growth and yield of various agricultural important crops including *Cucumis sativus*, *Oryza sativa*, *Zea mays*, *Solanum lycopersicum*, *Helianthus*, *Abelmoschus esculentus*, *Freesia alba* and *Carum copticum.* Krupa-Malkiewicz and Fornal (2018) investigated the role of chitosan in minimising the effect of salinity on *Petunia atkinsiane* D. don and it was found that all treated plants showed improved growth irrespective of chitosan type and concentration. Chitosan with a molecular weight of 970 kDa was found to adjust salt toxicity on shoot and root length, fresh and dry mass, and water contents on *Petunia atkinsiane* D. don.

2.4.1.3 Induction of the antioxidant system through chitosan-mediated salt stress response

Under harsh conditions, reactive oxygen species (ROS) are related to light dependent processes taking place within the plants (Katiyar *et al.*, 2015). Oxidative stress harms photosynthetic cells easily, which result in injuries of biomolecules (Katiyar *et al.*, 2015). Chitosan activates the

accumulation of H_2O_2 in plants by causing an oxidative burst and the induction of the ROS scavenging enzymatic system. Ma *et al*. (2014) documented that oligo-chitosan induced SOD, peroxidase (POD), and CAT activities in *Triticum aestivum* leaves by 218%, 34% and 15% compared to the control, respectively. In addition, Geng *et al*. (2020) reported the significant increase in SOD, POD, and CAT on salt (100, 150 and 200 mmol/L NaCl) stressed *Agrostis stolonifera* pre-treated with chitosan (0.1, 0.2, 1, and 2 g/L). Whereas exogenous application chitosan showed no significant effects on POD activity under control and stressed conditions. In another study, priming *Carthamus litinctorius* and *Helianthus annuus* seeds with chitosan (0, 0.25, 0.5 and 0.75%) also improved antioxidant enzyme (CAT and POD) activities in salt-stressed plants (Jabeen and Ahmad, 2013), although, the increase was concentration dependent.

Plants treated with chitosan showed biochemical and molecular responses that are observed such as: callose apposition, increase in cytosolic Ca^{2+}, MAP kinases activation, plasma membrane H^+-ATPase inhibition, chromatin alterations, synthesis of alkaloids and phytoregulators especially JA and ABA (Malerba and Cerana, 2016).

2.4.1.4 Mechanism of chitosan induced stress tolerance

Elicitors may be used to improve the growth of different crops under harmful conditions. These molecules induce plant defence through biological signals perception and transduction (Krupa-Malkiewicz and Fornal, 2018). Chitin is the second most abundant polysaccharide in nature after cellulose (Younes and Rinaudo, 2015; Winkler *et al.*, 2017), which has been used on several agricultural crops, for its potential as an elicitor, antimicrobial, protective and water stress relief. These properties make chitosan the most important and safe biopolymer for application in agriculture. According to Hien (2004) chitosan triggers the defensive mechanisms in plants, improves root growth and activates certain enzymes (chitinases, pectinases and glucanases). Chitosan also adjusts cell osmotic pressure hence promoting plant

growth through increasing the availability and uptake of water and required nutrients (Guan *et al*., 2009).

Since chitosan is synthesised from chitin, most studies designed to understand the mechanism of chitosan were done using chitin or chitin-oligomers. Chitin oligomers are essential microbe-associated molecular patterns (MAMPs), which are recognised by plant cells and activate plant immune responses that will result in fungal resistance (Liang *et al*., 2014). Several studies (Miya *et al*., 2007; Wan *et al*., 2012; Cao *et al*., 2014) found that in Arabidopsis, chitin is recognised by a lysin motif (LysM) containing receptor complex, which include the chitin elicitor receptor kinase-1(*CERK1*), lysM-containing *receptor -like kinase 4* (*LYK4*) and *LYK5*. In a study conducted by Wan *et al*. (2012) it is documented that the *LYK1/ CERK1* and *CEBiP* (LysM-containg protein) are important for chitin recognition in Arabidopsis and rice respectively. It was found that *CERK1* expression was induced by NaCl treatment and the *cerk1* mutant was more susceptible to NaCl but not to osmotic stress. They also found that chitin and NaCl increased Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in the cytosol.

Effects of chitosan in inducing ABA activity was investigated in tobacco plants wherethe ABA inhibitor [nordihydroguaiaretic acid] pre-treated with chitosan was able to reduce callose production and induced resistance against tobacco necrosis virus (Iriti and Faoro, 2009). Furthermore, chitosan also induced stomatal closure through increasing ABA content on bean plant leaves. Even though the mechanism behind chitosan induced stomatal closure is not clear, Azinheiro *et al*. (2014) highlighted that, through transcription and phenotypic analysis on chitosan treated plants, the effect of chitosan is partially as a result of activation of ABA signalling pathway.

Abiotic stresses such as drought, high temperature, heavy metals as well as salinity affects whole plant, both physiologically and biochemically. These stressful conditions may also

disturb and hinder nutrients and water uptake as a result of osmotic stress and result in loss of crop productivity and yield (Waqas *et al*., 2017; Vaughan *et al*., 2018; Zafar *et al*., 2018). In plants growing under stressed environment, several studies found high accumulation of MDA as a result of lipid peroxidation of membranes caused by oxidative stress (Hidangmayum *et al*., 2019). However, in the study conducted by Jabeen and Ahmad (2013) on *Carthamus tinctorius* L. and *Helianthus annus* L, pre-treating seeds with low chitosan concentration (0.25%, 0.50% and 0.75%) managed to alleviate the oxidative stress caused by salinity stress through reduction of enzyme activities in both plants. Several studies found similar results on pre-treated seeds of *Zea mays* (Al-Tawaha *et al*., 2018), *Vigna radiata* (Ray *et al*., 2016), *Trachyspermum ammi* (Mahdavi and Rahimi, 2013) and *Plantago ovata* (Mahdavi, 2013). In hydroponic study, 0.0625% oligochitosan treated salt-stressed wheat seedlings showed positive effects by significantly increasing antioxidant enzymes (SOD, POD and CAT) and was able to alleviate the oxidative stress (Ma *et al*., 2012).

2.5 Application of Nanotechnology in agriculture

Nanotechnology is a new emerging broad field of advanced research, and its discoveries could open novel applications in biotechnology and agriculture (Chhipa, 2019). Nanoparticles are produced by size reduction using top-down methods such as milling, high-pressure homogenisation and sonication or by bottom-up processes such as reactive precipitation and solvent displacement (Diyva and Jisha, 2018). In recent years nanotechnology has been developed to improve stress resistance and nutrient uptake.

Currently, nanotechnology-derived devices are implemented to reduce crop pathogens, to minimise nutrient loss during fertilisation and to improve yield. Several nanoparticles including ZnONPs (Rakgotho *et al*., 2022), FeNPs (Rizwan *et al*., 2019), AgNPs (Sadak, 2019; Khan *et*

al., 2021), Hematite NPs (Boutchuen *et al*., 2019) have been shown to be effective to improve crop growth. Chitosan nanoparticles have also been synthesised and applied exogenously as fertilisers to supply plants with sufficient chemicals and nutrients (Malerba and Cerana, 2016). This is because chitosan can be easily absorbed to the epidermis of leaves and stems prolonging the contact time and facilitating the uptake of bioactive molecules. It is reported that, nanoparticles less than 100 pm with concentrations ranging from 10 to 40 mg/kg administered directly on the soil, foliarly, or pre-treatment of the seeds, have proved to have beneficial effects on growth whereas higher concentration have negative effects (Hernandez-Hernandez *et al*., 2018). However, these effects are dependent on the properties of nanoparticles, plant species, soil dynamics, and soil microbial communities.

2.5.1 Properties of chitosan nanoparticles

Chitosan nanoparticles are natural materials with very good physiochemical, antimicrobial, and biological properties, making them bioactive, environmentally friendly, and harmless to humans (Malmiri *et al*., 2012). Due to these properties, chitosan nanoparticles find vast array of applications including tissue engineering, cancer diagnosis, drug delivery, enzyme immobilisation support, antioxidant activity, encapsulation of biologically active compounds, water treatment, and in agriculture (Diyva and Jisha, 2018). Chitosan nanoparticles were first described in 1994 based on the chitosan nanoparticles prepared by emulsifying and crosslinking for intravenous delivery of 5-fluorouracil, anticancer drug. Ever since then there was a development of new methods to produce nano-chitosan such as ionotropic gelation, emulsification solvent diffusion, microemulsion, polyelectrolyte complex and reverse micellar method (Tiyaboonchai, 2003).

2.5.2 Application of chitosan nanoparticles in agriculture

In agriculture, over the years the use of fertilisers is very important for both plant growth and development. However, these fertilisers lead to several challenges such as leaching, degradation by photolysis, hydrolysis, and decomposition. Hence, nanotechnology has come into play to minimise nutrient losses in fertilisation and to improve crop yield through use of nanoparticles (Siddiqui *et al*., 2015). Pinedo-Guerrero *et al.* (2017) added that the use of nanotechnology will change the entire food production chain from production to post-harvest, even though the application of nanotechnology in plant science has not received much interest compared to nanomedicine and nano pharmacology.

Only little information regarding the utilisation of nanoparticles as bio-stimulants of bio-compounds in plant crops is known (Siddiqui *et al*., 2015; Pinedo-Guerrero *et al.,* 2017). However, the application of nanoparticles has been investigated and the effects of these differ with species and other factors such as dose and type of nanoparticles. Chitosan-based nanoparticles can easily penetrate through the chloroplast membrane due to the positive coating. Within the chloroplast these nanoparticles exhibit both confined diffusion and convection, then reach an irreversibly trapped state (Malerba and Cerana, 2016).

Effect of nano-chitosan (0.1%, 0.2%, 0.3%) was investigated on *Phaseolus vulgaris (*Zayed *et al*., 2017) under salinity stress and it was found that all concentrations significantly improved seed germination but 0.3% showed better results on germination. Growth characteristics such as plant height, leaf area, fresh and dry weights of the shoots also increased significantly. High increase was recorded in membrane stability index (MSI) and Chl a, even in CAT, proline, RWC, Chl b, carotenoids and at 0.1% antioxidant enzymes showed positive effects. In a study conducted by Oliveira *et al.* (2016) it was found that chitosan nanoparticles containing S-nitroson-mercaptosuccinic acid (S-nitroso-MSA), was able to alleviate effect of salinity on

maize plant. Chitosan is applicable in different methodologies and strategies. Other method of applying chitosan in plants is through application of chitosan hydrogels.

2.5.3 Hydrogels

Hydrogel (aqua gel) is a three-dimensional (3-D) hydrophilic macromolecular network of polymer chains. The 3-D networks are formed because of chemical or physical hydrophilic crosslinking polymer chains (Bahram *et al*., 2016). In chemical gel, polymer chains are linked by covalent bonds whereas in physical gel they are connected by noncovalent bonds. Natural and modified natural polymers such as chitosan, are used to produce hydrogels. Hydrogels swell in water and as a result, they absorb large amounts of water from about 10% to thousands of times of its own volume (Akakuru and Isiuku, 2017). According to Neethu *et al.* (2018), the effectiveness of the hydrogel is dependent on its chemical properties such as molecular weight and the hydrogel properties tend to have differing effects on different soil.

Hydrogels are super absorbent polymers and show the potential to retain substantial quality of water. There is a possibility of application of these substances in both industrial and environmental zones (Ahmed, 2015). In agriculture, water retention capacity of soil can be improved by the addition of hydrogels. Cellulose, pectin, chitin and carboxymethyl cellulose (CMC), are naturally occurring macro-molecules with high water absorption potential to form hydrogels. Hydrogels are used to sustain availability of nutrients necessary for crop productivity (Wu and Liu, 2008). In addition, availability of hydrogels in the soil increases water availability and reduces nutrients loss by percolation and leaching, hence enhancing soil aeration and drainage, which will lead to high growth rate of plants and shoots. Hydrogels improve plants performance under stress through enhancing permeability of soil, infiltration rate, reduction of irrigation frequency, decreasing soil erosion and lessen water loss (Khan *et al*., 2018).

According to Shariatinaia *et al.* (2018) hydrogels absorb high quality water without dissolving through its 3-D cross linked network. Recently, hydrogels have gained great attention due to their diverse features in response to abiotic stress. Chitosan-based hydrogels have found applications in different fields as biomedical materials (drug delivery devices, tissue engineering and biosensor membrane). Nowadays, preparation of physical chitosan hydrogels without residual crosslinkers have gained attention due to their low cost and high safety and is synthesised through evaporation of chitosan acetate salt in a hydro-alcoholic solution and gelation of chitosan solution under ammonia (NH_3) gaseous atmosphere (Shariatinaia and Jalali, 2018).

In 2018, Pinedo-Guerrero and co-workers investigated Cu nanoparticles in chitosan hydrogels on the post-harvest of *Capsicum annuum* and results suggested that application of Cu nanoparticles in chitosan-PVA hydrogels increased the capsaicin content, antioxidants (ABTS and DPPH), phenols, and flavonoids content by 51, 23.9, 1,54, and 17,2% respectively which led to improved post-harvest characteristics of *C. annuum*. Interestingly, the negative effects of abiotic stress on plant growth and development can be improved by hydrogels through reduced stress and formation of oxygen radicals hence promoting growth and yield even under harsh conditions (Neethu *et al*., 2018).

Based on the positive effects of chitosan that are discussed in this literature review so far, there is no study that tested these effects using *S. bicolor*. As an important cereal crop, it will be interesting and very beneficial to bring up knowledge on the mechanism of chitosan induced stress using sorghum. The following section will give brief description of sorghum highlighting its importance as a crop and potential as a model plant for cereal crops.

2.6 *Sorghum bicolor*

Sorghum is the 5th most important cereal crop in the world and the 2nd in Africa, serving as staple food for both humans and animals (Awika *et al*., 2003). *S. bicolor* has received a lot of attention for biomass production under salt stress due to its salt and drought tolerance mechanism (Calone *et al*., 2020). As a moderately stress tolerant crop, it serves as a good candidate for investigations under salinity and due to its C4 metabolism, sorghum can sustain photosynthetic activity, and dry matter production under stressful conditions (Calone *et al*., 2020; Reddy, 2019). Sorghum can tolerate up to 6.8 and 4.5 dS m^{-1} soil and water salinity electrical conductivity (EC) respectively and if grown under these conditions, there will be about 16% yield reduction per each soil salinity unit increase (Calone *et al*., 2020).

Although sorghum is native to central Africa, it is widely spread to Asia and India (Kimber, 2000). Sorghum is being produced in high numbers in countries like India, Nigeria, USA, Argentina, and Ethiopia (Schnitzenbaumer and Arendt, 2014). Sorghum does not contain gluten proteins, which are causative agent for coeliac diseases hence sorghum has a great potential to be used in the production of gluten-free foods such as bread, cakes, cookies, noodles, flatbreads, and tortilla chips (Schnitzenbaumer and Arendt, 2014). As a moderately salt tolerant crop, the presence of large genotypic variation for tolerance to salinity is reported in sorghum and these genetic variations should be monitored to search for the most saline tolerant genotypes (Rajabi *et al*., 2020).

2.6.1 History of sorghum

Sorghum originates from the southern part of the Sahara Desert in Africa and has different cultivated genotypes including grain, sweet, forage, and biomass sorghum. Sorghum is classified under the genus sorghum (Ananda *et al*., 2020). In 1978, De Wet recognised *S. bicolor* as a model of all annual cultivated, wild, and weedy sorghums along with other rhizomatous taxa: *S. halepense* and *S. propinquum*. *S. bicolor* is further broken down into three

subsp: *S. bicolor subsp. Bicolor*, *S. bicolor sub-species. Drummondii*, and *S. bicolor subsp. Verticilliflorum.* Cultivated sorghums are grouped as *S. bicolor subsp. bicolor* and in Europe are represented by agronomic types such as grain, sweet, sudangrass and broomcorn sorghum (Berennji *et al*., 2011).

2.6.2 Uses of sorghum

Sorghum is an important cereal crop with potential uses as food (grains), feed (grain and biomass), fuel (ethanol production), fibre (paper), fermentation (methane production) and fertiliser. In India, sorghum is used as feed for animals/poultry during the rainy period, whereas during the post rainy season is used for consumption. Sorghum is rich in energy, proteins, vitamins, and minerals (Roy *et al*., 2018). Protein content in sorghum is equal to that of wheat and maize since they are genotypically related. Starch content in seed ranges from 56 to 73% and is relatively rich in iron, phosphorus, and vitamin B-complex (Roy *et al*., 2018).

2.6.3 Biochemical and molecular response of sorghum to salt stress

Generally, many important metabolic processes are affected by detrimental effects of salinity at both physiological and biochemical levels. Moreover, photosynthesis and growth rate of several plant species have been found to be inhibited by salt stress (Ma *et al*., 2020). The regulation of these metabolic process in response to salinity stress would modulate the ability of salt stress tolerance in sorghum (Yang *et al*., 2020). In *S. bicolor*, salt tolerance differs among species and occur in different growth stages. Germination is the starting point of development and growth process for all crops; therefore, good germination ability is vital for all crops (Yang *et al*., 2020). Salt tolerant sweet sorghum (germplasms) maintained good germination under salt stress. However, Mulaudzi *et al.* (2020) found that salinity delayed germination, reduced growth, improve proline and hydrogen peroxide content, salt stress also induced over expression of antioxidant and *SOS1* genes. In addition, Yang *et al*. (2018) found expression of *SbHKT1* as well as expression of genes related to ROS scavenging and osmoregulation solutes

on sweet sorghum under salt stress. Expression of these genes resulted in the increase in proline, soluble proteins, catalase and peroxidase content in salt (NaCl) treatment. Very recent study investigated effects of salt stressed sorghum (Rakgotho *et al*., 2022), salt stress was found to induce stress markers (ROS, MDA), enzyme activities (SOD, CAT and APX) and osmolytes (proline and soluble sugars).

2.7 CONCLUSION

Chitosan induces several biological responses in plants such as stress tolerance and improved productivity, which rely on the composition of the chemical, timing and rate of application. Several studies conducted already have not been able to elucidate the mode of action of chitosan in plants under harsh conditions. There is still a need to conduct transcpritomic and proteomic studies of defence genes and proteins to fully understand the complex chitosan-mediated responses. However, application of chitosan is more effective in conferring abiotic stress tolerance than nanoparticles and hydrogels. Nanoparticles and hydrogels depend on concentration and nature of particles whereas chitosan improves plant's tolerance independent of concentration. There is a need to document the effects of chitosan on germination and growth of *S. bicolor* under saline environments since such knowledge is still elusive. In the long run, the results obtained will lead to the use of chitosan on a larger scale to improve agricultural productivity and hence sustain food security globally.

CHAPTER THREE

EVALUATING THE EFFECT OF CHITOSAN ON THE GERMINATION AND SEEDLING GROWTH OF *SORGHUM BICOLOR* UNDER SALT STRESS

ABSTRACT:

Salinity is one of the detrimental environmental factors affecting about 20% of the cultivated land area globally, leading to more than 50% crop loss. Thus, food security depends on the development of new agricultural technologies that will improve crop growth and productivity under salinity. Exogenous application of small molecules, serve as promising targets towards improving plant tolerance to different stresses. Chitosan is a natural biopolymer, which is widely used in agriculture mainly for its antimicrobial activity and has added characteristics as a bio-stimulant in plants under normal and stressful conditions. However, the mechanism of chitosan-induced stress tolerance remains elusive. This chapter aims to elaborate on the positive effect of chitosan on the germination and seedling growth of sorghum under salt stress. Sorghum seeds were decontaminated and soaked overnight in autoclaved distilled water, followed by air drying under the laminar flow. Decontaminated sorghum seeds were sown in petri dish containing different NaCl (0, 100, 200, and 300 mM NaCl) and Chitosan (0, 0.25, 0.5 mg/ml) concentrations. Samples were incubated in the tissue culture for 7 days in the dark, while germination rate was measured daily. Germination parameters including germination percentage (GP), germination index (GI), mean germination time (MGT), and total germination (TG), and other while other growth parameters (fresh and dry weights, shoot and root length) were measured on the 7th day. Salt stress reduced germination and growth of sorghum as evident by low GP, GI, MGT, TG, fresh and dry weights. However, salt-reduced germination and growth was reversed by the application of chitosan, but chitosan showed no significant effect on control plants.

Key words: Bio-stimulant, Chitosan, Germination parameters, Salt stress, Sorghum, Tolerance

3.1 INTRODUCTION

Seed germination among other developmental stages is a vital stage of the plant's life cycle. Successful seed germination begins when the seed absorbs enough water followed by degradation of macromolecular substances, repair of genetic material and expansion of the embryo and endosperm, which then lead to a seed coat rupture and production of a prominent radical (Li, 2008). During the germination phase, the dormant seed becomes highly active and eventually becomes a healthy seedling (Koornneef *et al*., 2002). Healthy seed germination greatly contributes to an early seedling's establishment and quality production. However, germination is greatly affected by environmental stresses such as salinity.

Germination percentage (GP) is described as a qualitative measure that indicates the viability of a population of seeds at a critical stage in the life cycle of a plant (Kader, 2005). On the other hand, mean germination time (MGT) is an accurate measure of the time it takes for a seed to germinate, however, there is no correlation with the time spread or uniformity of germination and it focusses more on the day when most germination events occurred (Kader, 2005). The germination index appears to be the most comprehensive measurement parameter combining germination percentage and speed.

Salinity affects so many areas around the world and is a major abiotic stress, which contributes to major losses in the agricultural sector (Toshio and Eduardo, 2005). Soil salinity adversely affects the seed germination, through the osmotic stress, which limit water absorption and causes ion toxicity as a result of high accumulation of toxic ions (Na^+ and Cl^-) inhibiting the absorption of essential nutrients. These events contribut to low seed germination rate and lower crop productivity. To ensure their own survival and that of their seeds, plants have developed a range of physiological, biochemical and molecular adaptive mechanisms to cope with adverse conditions. Plants are grouped as glycophytes and halophytes depending on the level of their tolerance to salt stress. To understand the mechanisms used by plants to adapt under stress,

several assays have been conducted and more attention comes from studying the halophytes. In this case, sorghum [*Sorghum bicolor* (L.) Moench] is a moderately stress (drought and salt) tolerant crop and it requires high temperature for its growth. Sorghum is one of the most important cereal crops, serving as a food source and major potential for bioenergy production. Though sorghum is regarded as a stress resistant crop, it is sensitive to salt at early growth stage and salt exposure can limit early seedling establishment and reduce growth and yields (Amelework *et al*., 2016).

Chitosan is a natural polysaccharide composed of two molecules of D-glucosamine and naturally available in the call walls of several organisms such as crabs, shrimps, fungi, and insect's external skeleton (Katiyar *et al*., 2015). In the agricultural sector, chitosan improves the morpho-physiological parameters and reverses the abiotic stress induced damage through the transduction pathway (Hidangmayum *et al*., 2019). Exogenous application of chitosan also improves stress tolerance (Kim *et al*., 2005; Al-Tawaha *et al*., 2006), growth, and germination of different plants (Al-Tawaha *et al*., 2006; Balal *et al*., 2017). Exogenous application of compounds and small molecules have been seen successful to improve germination of crops under salt stress (Šírová *et al*., 2011; Mulaudzi *et al*., 2020; Rakgotho *et al*., 2022). Chitosan has attractive properties as compared to most compounds, it is a natural biological polymer, which is environmentally friendly and actively used in several fields not limited to agriculture (Kim, 2018). Different chitosan concentration (10-80 mg/L) enhanced seed germination of *Dendrobium formosum* under normal growth condition (Kananont *et al*., 2010). Furthermore, under low temperature, chitosan showed improved seed germination of *Zea mays* (Guan *et al*., 2009). Therefore, this study considered the advantage of sorghum's tolerance to study mechanisms of stress response and the potential use of chitosan (0.25 and 0.5 mg/ml) to improve its germination under salt (0, 100, 200, 300 mM NaCl), stress.

3.2. Methods and Materials

3.2.1. Germination and growth media

The medium used for the germination in this study included different solutions prepared in autoclaved double distilled water (ddH_2O). The solutions included ddH_2O for the control (0 mM NaCl), different sodium chloride concentrations (100 mM, 200 mM, and 300 mM NaCl) and two chitosan concentrations (0.25 and 0.5 mg/ml).

3.2.2. Plant treatment and growth conditions

Sorghum (*Sorghum bicolor* (L.) *Moench)* seeds were purchased from Agricol, Brackenfell, Cape Town, South Africa. For decontamination, sorghum seeds were soaked in 70% ethanol and placed on a motor shaker at 600 rpm and allowed to shake for 60 seconds followed by rinsing with autoclaved ddH_2O. Seeds were then soaked in 5% Sodium hypochlorite (bleach) and placed on a motor shaker set at 600 rpm for 60 minutes while shaking, seeds were rinsed with distilled water and air dried under the lamina flow. After decontamination, seeds were soaked overnight in autoclaved ddH_2O at 25 ^oC with shaking at 600 rpm. Seeds were dried under 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 media as detailed in section 3.2.1. Petri dishes containing the seeds and medium were placed at 25 ^oC and seeds were germinated for 7 days in the dark. Seeds were inspected daily, and the number of germinated seeds were recorded. Both root and shoot length were measured on final day (day 7) and seedlings were harvested for fresh and dry weight measurements.

3.2.3. Germination assays

Germination assays conducted included, germination percentage (GP), mean germination time (MGT), germination index (GI) and total germination (TG) were calculated according to the equations shown below (Kader *et al.*, 2005):

3.2.3.1. Germination percentage

$$GP = \frac{n}{N} \times 100\ldots \quad (1)$$

Where n: is the total number of seeds germinated and N: is the total number of seeds sown

3.2.3.2. Germination index

$$GI = \sum \quad (n1 \times d7) + (n2 \times d6) + (n3 \times d5) \ldots\ldots (n7 \times d1) \quad (2)$$

Where, n1 is the number of seeds germinated on day 1, and d7 is number of seeds germinated on day 7

3.2.3.3. Total germination

$$TG = \frac{d7}{N} \times 100 \quad (3)$$

Where in, d7 the total number of seeds germinated on day 7 and N is is the number of seeds germinated

3.2.3.4. Growth attributes: Seedling lengths including roots and shoots were measured using a ruler (mm) on the final day (day 7).

Fresh weights were measured by weighing the whole seedling on day 7. Dry weights were measured after drying seedling in the oven (80 ^{o}C) overnight.

3.3. RESULTS

Seed germination is described as an important stage of plant growth, which results in seedling establishment and future plant growth. At this stage, seeds showing high tolerance towards salinity determines better plant establishment under a stressed environment (Abo-Kassem *et al*., 2007; Keshavarizi *et al.*, 2012). Generally, ever increasing salinity conditions significantly reduce germination percentage, germination rate, plant growth (roots and shoot length), and biomass (fresh and dry weights) of plants regardless of species type (Jamil *et al*., 2006).

3.3.1. Germination parameters

To demonstrate the efficiency of chitosan to improve plant adaptation to salt stress, the study investigated the GP, GI, MGT and TG.

3.3.1.2. Germination percentage

Germination percentage is described as a qualitative measure that indicates the viability of a population of seeds at a very critical stage in plant life cycle (Ranal and Santana, 2006). *S. bicolor* seeds were germinated in the absence (0 mM) and presence (100, 200 and 300 mM) of NaCl for 7 days as shown in figure 3.1A & B. Salt stress significantly decreased germination percentage by 50, 100 and 100% for seeds under 100, 200 and 300 mM NaCl respectively after day 1. After day 7 of sowing, only 200 and 300 mM NaCl had significant ($p \leq 0.001$ and $p \leq 0.01$) decrease in germination percentage. Interestingly, supplementing salt (200 mM NaCl) stressed sorghum seeds with chitosan (0.25 and 0.5 mg/ml) showed significant ($P \leq 0.01$) increase in germination percentage after day 1 and 7 of sowing. However, application of chitosan (0.25 and 0.5 mg/ml) had no significant effects on all seeds germinated under normal condition.

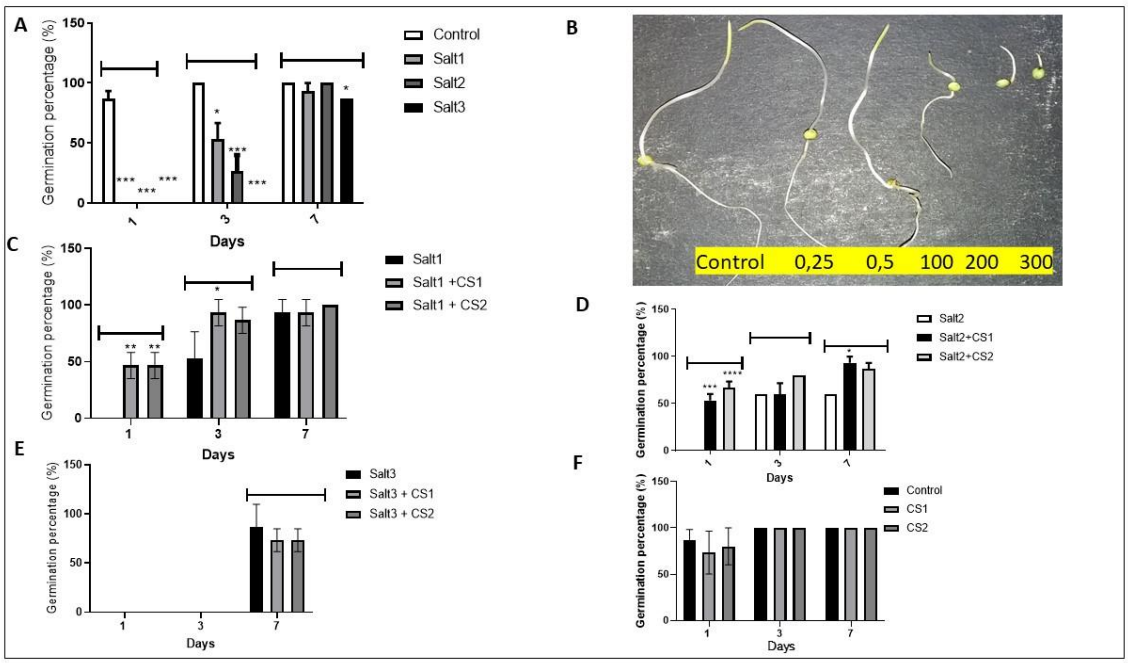

Figure 3.1: Effects of chitosan on the germination percentage of *Sorghum bicolor* seedlings under NaCl stress. Seeds germinated under different (A) salt [0 (Control), Salt1(100), Salt2 (200), and Salt3 (300 mM NaCl)] concentrations only, (B-F) salt and different Chitosan (CS1=0.25 and CS2=0.5 mg/ml) concentration, (F) chitosan only. Error bars represent the SD calculated from three biological replicates. Statistically significant between control and treated seedlings was determined using two-way ANOVA conducted on GraphPad Prism 9.00, (2021), shown as ****=p≤0.0001, ***=p≤0.0010, **=p≤0.01, and *=p≤0.05.

The germination index acts as an indicator of how fast the seed has germinated (Kader, 2005). Table 3.1 shows the effects of chitosan on the germination index (GI) and total germination of salt stressed sorghum seeds. Increases in salt stress resulted in a significant decrease in GI, however, 200 and 300 mM NaCl showed less GI (45 and 42 units) in comparison with the control (135.33 units). Interestingly, both chitosan concentrations (0.25 and 0.5 mg/ml) improved GI on salt (200 mM NaCl) stressed seeds from 45 units to 67 and 92 units, representing a 48, 89 and 104.44% increase. There was no significant difference in the total germination of all treatments, and seeds treated with 300 mM NaCl showed very low total germination (86%) as compared to other treatments.

Table 3.1: Effects of chitosan on the germination parameters of sorghum seeds germinated in different salt (0, 100, 200, and 300 mM NaCl) concentration. Data represented are mean ± SD.

Chitosan (mg/ml)	NaCl (mM)	Germination Index (GI)	Total germination
0	0	135.333 ± 4.041	100.00 ± 0.000
	100	97.667 ± 5.132**	93.333 ± 11.547
	200	45.000 ± 0.000***	100.00 ± 0.000
	300	42.666 ± 4.031***	86 ± 23.094
0.25	0	130.667 ± 8.083	100.00 ± 0.000
	100	106.667 ± 14.434	93.333 ± 11.547
	200	67.333 ± 2.887	80.00 ± 20.000
	300	22.000 ± 1.732**	66.667 ± 23.094
0.5	0	131.00 ± 10.149	100.00 ± 0.000
	100	90.00 ± 3.464	100.00 ± 0.000
	200	92.000 ± 3.464**	86.667 ± 11.547
	300	16.667 ± 4.041**	53.333 ± 0.094

Significant differences shown as ****=p≤0.0001, ***=p≤0.0010, and **=p≤0.01.

3.3.2. Growth analysis

Roots and shoots were measured on the last day of germination (day 7) and findings are summarised in Figure 3.2. As expected, both roots and shoots length (Fig 3.2A) significantly ($p \leq 0.05$ and $p \leq 0.001$) reduced gradually with an increase in NaCl concentration with 300 mM NaCl recording the lowest root (21.8 mm) and shoot (41.43 mm) length in comparison with control (roots:42 mm and shoots: 56.67 mm). Application of chitosan (0.25 and 0.5 mg/ml) on control seedlings had no significant differences on both roots and shoots length. However, chitosan (0.25 mg/ml) improved the shoot length of seedlings under 100 mM NaCl (100 mM) significantly ($p \leq 0.01$) while there was no significant difference on the length of the root and shoot length of other treatments (Figure 3.2C). Under 200 mM salt stress, chitosan (0.25 and 0.5 mg/ml) improved the shoots length by 239.51% (0.25 mg/ml chitosan) and 192.62 % (0.5 mg/ml chitosan), no significant difference was observed on the root length. Unexpectedly,

under 300 mM NaCl, chitosan (0.25 and 0.5 mg/ml) showed a decrease on the root length, chitosan decreased root length by 34.46% (0.25 mg/ml) and 60.30% (0.5 mg/ml).

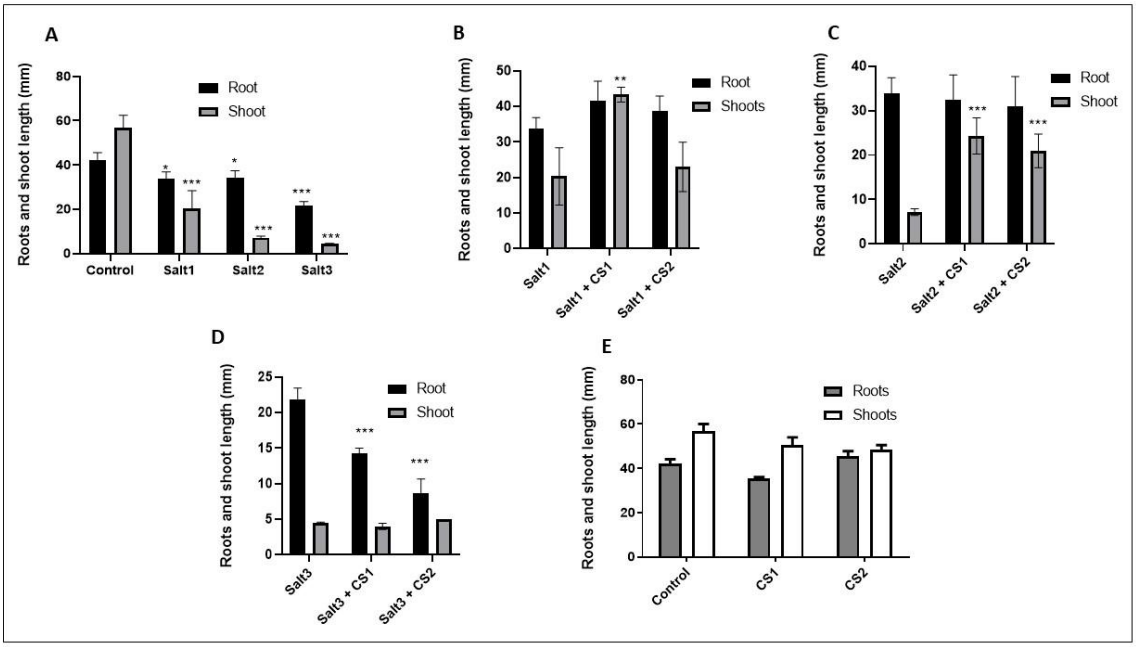

Figure 3.2: Effects of chitosan on the roots and shoot length of salt (NaCl) stressed sorghum seedlings measured on day 7 of germination. Seeds germinated in the presence of different (A) NaCl (0, 100, 200 and 300 mM) concentrations, (B-D) salt and different chitosan (0.25 and 0.5 mg/ml) concentration, (E) Chitosan only. Error bars represent the SD calculated from three biological replicates. Statistically significant between control and treated seedlings was determined using two-way ANOVA conducted on GraphPad Prism 9.00, (2021), shown as ***=p≤0.0010, **=p≤0.01, and *=p≤0.05.

3.3.3. Fresh and dry weight

Sorghum biomass was assayed by measuring fresh and dry weights (Figure 3.3) on the final day of germination. Salt stress significantly reduced the fresh weight as by 46.37% (100 mM NaCl), 54.59% (200 mM NaCl), and 60.84% (300 mM NaCl) compared to the control. Seedlings under normal conditions (control) showed high fresh weight of 0.33 g wheares 100, 200, and 300 mM NaCl showed decrease in fresh weight with salt stress increase with lowest weight (0.13 g) observed under 300 mM NaCl treatment. Under 100 mM NaCl, chitosan (0.25 and 0.5 mg/ml) significantly (P≤0.05) improved the seedling fresh weight by 54.84% (0.25 mg/ml) and 47.48% (0.5 mg/ml). Surprisingly, under 300 mM NaCl chitosan showed no

significant changes in comparison with salt-stressed (300 mM) seedlings. There was no significant difference in dry weights of all treatments.

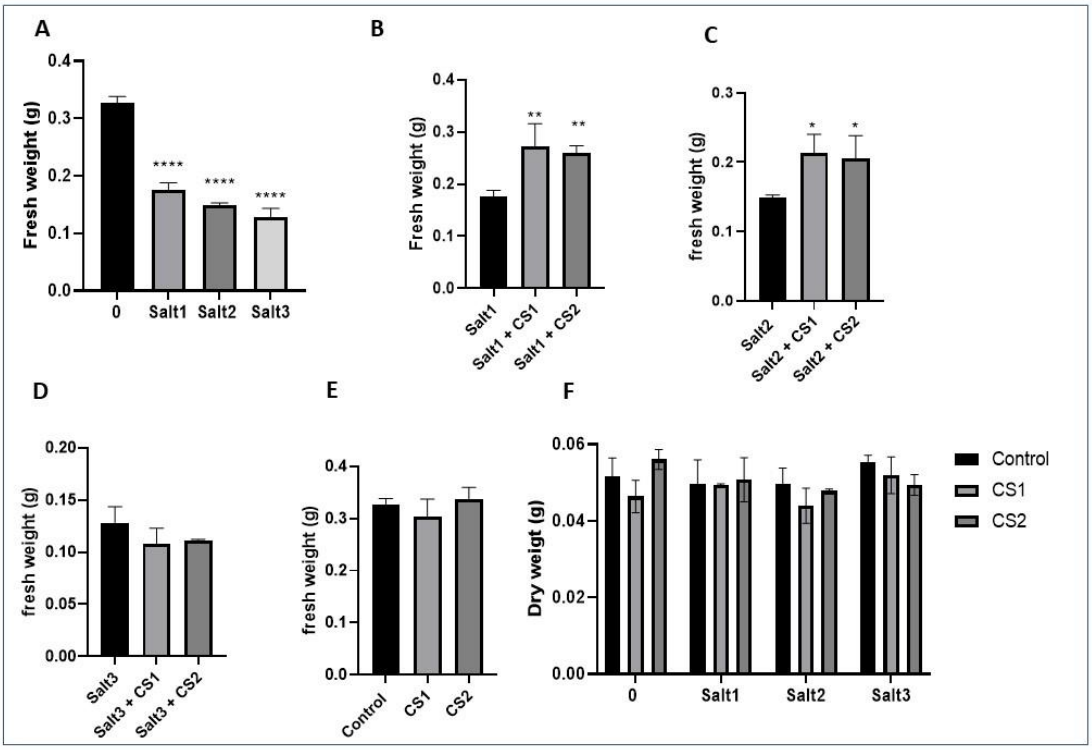

Figure 3.3: Effects of NaCl and Chitosan on fresh (A-E) and dry weight (F) of sorghum seed. Seeds germinated under different (A) salt (0, 100, 200, and 300 mM NaCl) concentrations only, (B-D) salt and under different Chitosan (0.25 and 0.5 mg/ml), (E) Chitosan only. Error bars represent the SD calculated from three biological replicates. Statistically significant between control and treated seedlings was determined using two-way ANOVA conducted on GraphPad Prism 9.00, (2021), shown as ****=p≤0.0001, ***=p≤0.0010, **=p≤0.01, and *=p≤0.05.

DISCUSSION

3.4.1. Effects of NaCl and chitosan on the germination of *S. bicolor*

Salt stress significantly reduced germination percentage and delayed germination of sorghum seeds with the highest salt concentrations (200 and 300 mM NaCl) showing significant decrease on day 7 after sowing (Figure 3.1). Germination is greatly affected by salt stress through the reduction of gemination rate, and seedling establishment as seen in *Zea mays* (Khayatnezhad and Gholamin, 2011), *Capsicum annuum* L. (Khan *et al*., 2009) as well as *S. bicolor* (Mulaudzi *et al*., 2020). In the study conducted by Kandil and others (2017), it was found that all germination parameters (GP, GR, GI, and energy of germination) were significantly reduced on NaCl (20-150 mM) treated sorghum seeds. Interestingly, chitosan improved germination percentage on all salt stressed sorghum seeds. Priming with 1.5% chitosan also improved germination of *Oryza sativia L* under salt stress (Songlin and Qingzhong, 2002). Chitosan had significant effects on improving seed germination of *Zea mays* seeds under low temperature stress (Guan *et al*., 2009).

The study further investigated the effects of salt on germination index and total germination time. According to Kader (2005), germination index combines the germination speed and percentage, and it shows how much time the seed took to germinate. In the present study it has been found that an increase in salt stress concentration results in the delay of germination process as evident by low GI (Table 3.1). These delays in germination might be as a result of osmotic stress, which causes impairment in the nutrient uptake and ionic stress due to the excess accumulation of ions leading to ion toxicity (Nawaz *et al*., 2010; Arif *et al*., 2020). Application of chitosan (0.25 and 0.5 mg/ml) significantly improved the germination time of sorghum seeds germinated under 200 mM NaCl.

3.4.2. Effects of NaCl and chitosan on the growth of *Sorghum bicolor*

Sodium chloride reduced roots and shoots length of sorghum seedlings with the shoots being more sensitive to salt as compared to the roots. This is due to excess salts in the roots, which cause ionic stress and decrease in root osmotic potential that hinders the root from absorbing enough water and its transport to the shoot (Aroca *et al*., 2012; Mulaudzi *et al*., 2020). Similar results were observed on a study of Pandey and Penna (2017) on *Brassica junce*, where in salt-stressed seedlings decreased growth and seedlings development was observed. Chitosan on its own had no significant changes on the root length of seedlings grown in the absence (0 mM NaCl) and presence (200 and 300 mM NaCl) of salt. However, chitosan improved shoot length of seedlings grown in the presence of 300 mM NaCl, with 0.25 mg/ml improving shoot of seedlings under 100 mM NaCl. Increasing salt concentration decreased fresh weight of sorghum seedlings indicating that cell division and elongation were hindered. Treatment with chitosan showed no significant improvement in the fresh and dry weight of seedlings germinated in absence (0 mM NaCl) and presence (300 mM NaCl). However, application of chitosan improved fresh weight of sorghum seedlings germinated in the presence of 100 and 200 mM NaCl.

This chapter illustrated the effects of salt stress on the germination of *S. bicolor* and the application of chitosan in amelioration of salinity effects. This study further confirmed that sorghum is moderately tolerant to salt stress, since seeds reached up to 86% germination percentage under high sanilty (300 mM NaCl). However, exogenous application of chitosan (0.25 and 0.5 mg/ml) improved germination resulting in much better yield under salinity.

CHAPTER FOUR

EVALUATING THE EFFECTS OF CHITOSAN ON THE GROWTH, MORPHO-PHYSIOLOGICAL AND BIOCHEMICAL RESPONSE OF *SORGHUM BICOLOR* UNDER SALT STRESS

ABSTRACT:

Salinity stress is one of the major environmental constraints responsible for the reductions in agricultural productivity. Salinity leads to growth reductions, by causing osmotic and ionic stress, which leads to the over accumulation of toxic molecules known as reactive oxygen species including superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl ion (OH^-) and singlet oxygen ($^1O_2$). These molecules induce oxidative damage to cellular components including membranes and biomolecules when accumulated at higher concentrations, thus affecting plant growth. But plants have developed several ROS scavenging machineries including no-enzymatic (cyclic nucleic acid, flavonoids, phenols, reduced glutathione, carotenoids, and proline) and enzymatic antioxidant systems. This chapter investigated the effects of exogenous chitosan (0.25 and 0.5 mg/ml) in protecting sorghum plants from the deleterious effects of salt stress by analysing the morphological, physiological, and biochemical traits of *S. bicolor* plants. This was achieved by treating seven days old sorghum plants with 300 mM NaCl (experiment) and 0 mM NaCl (control), whereas another group was treated with 0.25 and 0.5 mg/ml chitosan and the treatments continued for 7 days. Morph-physiological and biochemical traits included assaying anatomical structure, non-enzymatic (proline and soluble sugar) and enzymatic (SOD and APX) antioxidants. Application of 0.25 and 0.5 mg/ml chitosan improved shoot length by 33.87% and 24.32% respectively. Salt stress decreased fresh (66.92%) and dry (48.26%) weights, whereas application of chitosan reversed these effects by increasing fresh weight by 79.50% (0.25 mg/ml chitosan) and 30.95% (0.5 mg/ml chitosan) and dry weight by 53.02% (0.25 mg/ml chitosan) and 46.31% (0.5 mg/ml

chitosan). Anatomical structure analysis revealed that epidermis and vascular layers (xylem and phloem) under 300 mM NaCl were severely affected showing shrinkage and deformation and these structures were restored by exogenous chitosan. Salt stress also increased Na^+ (from 0 to 2.34 wt%) while there was a decrease in K^+ from 4.30 wt% to 1.823 wt% resulting in a Na^+/K^+ ratio of 1.28. Exogenous chitosan had no significant effects Na^+ and K^+distribution in sorghum plants under salt stress. Salt stress induced the formation of H_2O_2 (44%) and MDA (125%) contents, while these levels were reduced by more than 50% when sorghum plants were treated with chitosan. This was complemented by a higher osmolyte content including proline (318.67%) and soluble sugars (44.69%), and activities of SOD (36.04%) and APX (131.58%), indicating that sorghum has an efficient ROS scavenging capacity. Application of chitosan reduced the osmolyte content and the activities of the antioxidant enzymes by more than 50%. These results suggest that chitosan has a scavenging capacity and induces tolerance mainly by regulating the nutrient balance, osmolyte content and the antioxidant scavenging capacity.

Keyword: Anatomical structure, Antioxidants, Chitosan, Osmolytes, Oxidative stress, ROS, *Sorghum bicolor*

4.1 INTRODUCTION

Salinity is one of the most important issues affecting the agricultural sector. Each year, millions of tons of salt enter the arable land through irrigation, climate changes as well as human activities (Ramezani *et al*., 2011). Soil salinity is a detrimental abiotic stress that affects growth, productivity, and the physiological traits of plants (Ramezani *et al*., 2011; Katagiri and Kolluru, 2017). Salinity stress negatively affects growth through decreasing leaf water potential, morphological, physiological and biochemical changes (Zhang *et al*., 2013; Rahneshan *et al*., 2018).

According to Kotagiri and co-worker (2017), sodium chloride is regarded as one of the most well-known sources of salinity, which increases Na^+ and Cl^- ions, while reducing the microelements such as N^+, PO_4^{-3}, K^+, Mg^{2+} and Ca^{2+} ions in the upper part of the plant. High Na^+ and Cl^- ions content in plants resulting in ionic stress which affects functioning of enzyme function, imbalance in homeostasis due to K^+ transport leak (Mums *et al*., 2006). As a result reactive oxygen species (ROS) are excessively produced, causing disruptions in the antioxidant defence mechanism by the excessive production of reactive oxygen species (ROS) including singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($O_2^{\bullet-}$) which causes oxidative stress damage oxidative stress damage of different cellular macromolecules such as lipids, proteins, and nucleic acids, which will lead to deactivation of several cellular and metabolic processes (Rahman *et al*., 2016; El-Taher *et al*., 2022).

Plants have developed various mechanisms to survive under harsh environmental conditions which includes homeostasis and activation of the antioxidative scavenging capacity. For ion and osmotic homeostasis, several plants produce proline or any other compatible solutes to sustain water relationship, protein, and enzyme complexes (Gupta and Huang, 2014).

According to Kumar *et al*. (2021), plants activate the production of osmolytes (proline and soluble sugars) to protect cells against the detrimental effects of salt stress. These help in osmotic adjustment, and increase salinity tolerance (Rahneshan *et al*., 2018). Osmolytes in particular proline also act as ROS scavenger in the form of non-enzymatic antioxidant to counterbalance osmotic stress (Ashrafad and Floolad, 2007). Plants also accumulate other antioxidants including ascorbic acid (AsA), glutathione (GSH), phenolic compounds, alkaloids, and others. Enzymatic antioxidants including superoxide dismutase (SOD: EC 1.15.1.1), peroxidase (POD: EC 1.11.1.7), catalase (CAT: EC 1.11.1.6) and ascorbate peroxidase (APX: EC 1.11.1.1) among others (Rahman *et al*., 2016; Kumar *et al*., 2021). However, under very high salt concentrations, these inherent defence mechanisms are not sufficient especially for glycophytes. Thus, researchers have explored different strategies to improve the plants response and tolerance to abiotic stresses (*e.g* salinity stress) such as the induction of these inherent mechanisms. Some of the effects include the exogenous application of molecules that act as bio-stimulants such chitosan as proposed in this study. Although the effect of chitosan was demonstrated in sorghum growth under the influence of *Pseudomonas aeruginasa*-P17 in *S. bicolor*, (Kumar *et al*., 2019), to date there are no reports on the role of chitosan to improve sorghum's tolerance to salinity stress.

Salinity stress proved to be detrimental to the growth and development of existing crops (Ashraf and Hegazy, 2013; Hashim *et al*., 2020) including *Vicia faba* (El Nahhas *et al*., 2021), *Phaseolus vulgaris* (Shabana *et al*., 2020; Bargaz *et al*., 2021), *Tricum aestivum* (Alnusairi *et al*., 2021), *Ocimum basilicum* (Nassar *et al*., 2019), *Lupinus termis* (Rady *et al*., 2016), as well as *Zea mays* (Konuskan *et al*., 2017; Gebreegziabher and Qufa, 2017; Hafez *et al*., 2021).

Chitosan is a polysaccharide obtained from crab shells and other crustaceans with an alkaline or enzymatic deacetylation (Asghari-Zakaria *et al*., 2009; Mehmood *et al*., 2020). It is documented to influence the production of substances related to stress response. Chitosan

application have plant growth promoting effects, resulting in yield improvement and plant health (Asghari-Zakaria *et al*., 2009). Chitosan can also be applied commercially in controlling rot diseases in tomato, in addition, it was found that foliar spray application increased yield by 20% in mildew disease control (Walker *et al*., 2004).

This chapter aims to elucidate the effects of chitosan as a bio-stimulant in improving the response of *S. bicolor* to salt stress. The current chapter aims to elucidate the effects of chitosan as a bio-stimulant in improving the response of *S. bicolor* salt stress. Sorghum is an economically important crop through its several uses such as grain (food), stalks (building), broom-making and biofuel productions (Dahlberg *et al*., 2011) and thus its cultivation is important and will play as a future crop to ensure food security. To bring an understanding and a link between the morpho-physiological and the biochemical responses of sorghum to NaCl and chitosan treatment, the results and discussion in this chapter (Chapter 4) will be divided into two major sections: section A (evaluating the effect of chitosan on the morphological and physiological response of *S. bicolor* seedlings to NaCl) and section B (evaluating the effect of chitosan on the biochemical response of *S. bicolor* seedlings to NaCl).

4.2. Material and methods

4.2.1 Plant treatment and growth conditions

Sorghum [*Sorghum bicolor* (L.) Moench] seeds were prepared as described in chapter 3, section 3.2.2. On day 7 sorghum seedlings grown under normal conditions were transferred into soil consisting of 2:1 potting soil and vermiculite and growth was allowed to continue in the green house (26 ^{o}C) and continue with treatment. Seedlings were treated following method explained by Ahmad *et al*. (2018) with some modifications, briefly, the pots containing seedlings were watered with about 100 ml of multifeed nutrient solution 19:8:16 every second day for a week followed by watering with only water for a week. Then salt (300 mM NaCl) and chitosan (0.25 mg/ml and 0.5 mg/ml) treatments were initiated on day 14 after planting and was done every second day for 1 week. Plants were harvested on day 35 after sowing.

4.2.2 Growth attributes

Sorghum plants were harvested on day 20 after transferring to potting soils and growth attributes including shoot length, fresh and dry weights and moisture content were measured.

Fresh weights were measured by weighing the whole plant and dry weights were measured after drying seedling in the oven (80 ^{o}C) overnight or until constant weight.

Chlorophyll and carotenoids were determined using a spectrophotometric method of Goncalves *et al*. (2001). Briefly, leaf samples (0.1 g) were ground in liquid nitrogen and then extracted with aqueous acetone solution (80% *v/v*). After homogenising the samples were centrifuged at 5000 rpm for 5 minutes at 4 ^{o}C. The supernatant was used to determine absorbance at 645, and 663 nm to obtain carotenoid and chlorophyll concentration using Helios® Epsilon visible 8 nm bandwidth spectrophotometer (Thermo Fisher Scientific, USA).

4.2.3 Anatomic structure and element analysis

Anatomical analyses were done using Scanning Electron Microscopy-Energy dispersive X-ray spectroscopy (SEM-EDX). Analysis of *S. bicolor* was undertaken for both untreated and treated samples. Seedlings shoots were placed on aluminium stubs coated with conductive carbon tape. The plant samples were then coated with a thin layer of carbon using a carbon coater. All EDX spectra were collected with an Oxford X-Max silicon solid-state drift detector at an accelerating voltage of 20 kV for 60 seconds to ensure proper x-ray detection. All spectra were analysed using the build in Oxford Aztec software suite. Samples were then imaged, and images were collected using a Zeiss Auriga field emission gun scanning electron microscopes, operated at an accelerating voltage of 5 kV using an in-lens secondary electron detector (Mulaudzi *et al*., 2020; Rakgotho *et al*., 2022).

4.2.4 Hydrogen peroxide content

Hydrogen peroxide (H_2O_2) was analysed following an optimised method by Junglee *et al*. (2014). About 0.15 g ground plant material was homogenised with 0.25 ml trichloroacetic acid (TCA), 0.5 ml potassium iodide (1 M), and 0.25 ml potassium phosphate buffer (10 mM, pH 6.0). Tubes were then vortexed and centrifuged for 15 minutes at 10 000 rpm (at 4 ^{o}C). Samples were transferred to 96 microwell plates and allowed to incubate at room temperature for 20 minutes. Absorbances were read at 390 nm using FLUOstar® Omega (BMG LABTECH, Ortenberg, Germany) microtiter plate reader and H_2O_2 was measured by generating a standard curve.

4.2.5 Malondialdehyde (MDA)

Lipid peroxidation: lipid peroxidation was determined by measuring malondialdehyde (MDA) formation following the thiobarbituric acid method as explained by Maia *et al*. (2010). Fresh

shoot samples (50 mg) were homogenised with 2 ml of 1% trichloroacetic acid (TCA). The homogenate was then centrifuged for 10 minutes at 10 000 rpm (4 ^{o}C). Aliquots of 1 ml of the MDA extract was added to 2 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA). The mixture was boiled for 30 minutes at 95 ^{o}C and then allowed to cool in an ice bath. Mixture was then centrifuged at 10 000 rpm for 15 minutes and then absorbance of the supernatant was measured at 532 nm using the FLUOstar® Omega (BMG LABTECH, Ortenberg, Germany, microtiter reader. Measurement was corrected for nonspecific turbidity by subtracting the absorbance at 660 nm. The MDA concentration was then calculated using formula 5.

$$nmolMDA/gFW = \frac{\Delta Acorrected \times 3.5 \times 100}{\varepsilon \times b \times y}$$ Formula 5

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

4.2.6 Fourier-transform infrared spectroscopy (FTIR) analysis of biomolecules

The phytochemical composition and the newly formed chemical compositions were determined using Fourier-transform infrared (FTIR) spectroscopy as described by Rakgotho *et al*. (2022). FTIR spectrum of sorghum shoot was analysed using a PerkinElmer Spectrum 100-FTIR Spectrometer [PerkinElmer (Pty) Ltd., Midrand, South Africa]. About 2 g of dry sorghum shoot tissues where analysed, where a wider window between 450 to 4000 cm^{-1} was considered.

4.2.7 Determination of osmolytes content

In this study, two molecules were chosen including proline and soluble sugars in order to understand osmotic balance in sorghum. Both were chosen based on their ability to play an osmoprotection role and detoxification of ROS.

4.2.7.1 Proline content

Proline content was examined following methods of Carillo and Gibon (2011), with slight modification. About 100 mg of sorghum shoots were ground in liquid nitrogen and resuspended in 500 µl of 3% aqueous sulfosalicylic acid [3 g sulphuric acid with molecular weight of 218.185 g/mol, dissolved in 100 ml ddH_2O], followed by centrifugation at 13 000 rpm for 20 minutes. About 300 µl of the supernatant was mixed with 600 µl of reaction mixture [1.25 g of ninhydrin in 30 ml of 99% acetic acid and 20 ml 6 M H_3PO_4] and boiled for 10 minutes in a water bath set at 95 ^oC. The sample was placed in ice and allowed to cool, and then equal volumes of toluene was added, and the optical density was measured at 520 nm using using FLUOstar® Omega (BMG LABTECH, Ortenberg, Germany) microtiter plate reader The proline content was determined from a standard curve using pure proline as a standard.

4.2.7.2 Total soluble sugars

The total soluble sugars were determined as described (Watanabe *et al*., 2000) before with some modifications. About 100 mg grounded plants material was homogenised in ice cold 10 ml 80% acetone. The mixture was then centrifuged at 10 000 rpm for 10 minutes at 4 ^oC and 1 ml of the supernatant was added in a tube containing 3 ml of anthrone reagent [0.15 g anthrone, dissolved in 100 ml of 96% H_2SO_4]. Samples were then placed in a boiling water bath set at 95 ^oC for 15 minutes, followed by a cooling 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 and the content was expressed as mg $µl^{-1}$ FW.

4.2.8 Determination of activities of antioxidant enzyme activity

Samples for the determination of enzyme activities were prepared as previously discussed (Gunes *et al*., 2019). Plant material (0.5 g) was homogenised in mortar with 3 ml of 50 mM phosphate buffer (pH 7). The homogenate was filtered, followed by centrifugation at 18 000 rpm for 15 minutes using a refrigerated centrifuge set at 4 ºC. The supernatant was stored at -20 ºC until further assays.

4.2.8.1 Superoxide dismutase (SOD; EC 1.15.1.1)

SOD activity was measured by observing the reduction of photochemical of nitroblue tetra zolium (NBT) through a reaction mixture prepared by adding 0.2 ml potassium phosphate buffer (50 mM), 0.3 ml (12 mM methionine), 0.3 ml (75 µM NBT), 0.03 ml (1 µM riboflavin), pH 7 followed by adding 3 ml of the enzyme extract and then sodium carbonate (Na_2CO_3) was used as control. Absorbances were read FLUOstar® Omega (BMG LABTECH, Ortenberg, Germany) microtiter reader after exposing the plate to light for 20 min until colour change was observed.

$$Enzyme\ activity = \frac{A-A1}{A1} \times 100 \quad (6)$$

Where: A is Blank absorbance and A1 is sample absorbance (Zhang *et al*., 2016).

4.2.8.2 Ascorbate peroxidase (APX; EC 1.11.1.1)

APX was assayed by estimating the decrease in optical density due to ascorbate at 290 nm. Reaction was prepared by mixing 50 mM potassium phosphate (pH 7), 0.1 ml of 0.1 mM H_2O_2, EDTA (0.1 mM), ascorbate (0.5 mM), and 0.1 ml enzyme extract and water. The decrease of

absorbance was assayed and measured using FLUOstar® Omega (BMG LABTECH, Ortenberg, Germany) microtiter reader.

$$Enzyme\ activity\ (\frac{unit}{L}) = \frac{\Delta Abs \times Total\ assay\ volume}{\Delta t \times \varepsilon \times l \times Enzyme\ sample\ volume} \quad (7)$$

Where: ΔAbs is a change in absorbance, total assay volume (200µl), Δt is a change in time, ε= extinction coefficient of substrate (2.8 mM), l is acurvert diameter (1 cm) and enzyme sample volume (20 µl).

SECTION A

EVALUATING THE EFFECT OF CHITOSAN ON THE MORPHOLOGICAL AND PHYSIOLOGICAL RESPONSE OF *SORGHUM BICOLOR* TO SALT

4.3 RESULTS

4.3.1. Growth analysis

In the current study, to understand the morphological changes on salt-stressed sorghum plants, different growth parameters such as the overall plant growth (Figure 4.1), shoot length, plant biomass (fresh and dry weights) (Table 4.1) were measured. Sodium chloride (300 mM) significantly ($P \leq 0.001$) decreased shoot length by 52% resulting in 23 mm length as compared to the control (48 mm). Application of chitosan improved shoot length by 33.9% (0.25 mg/ml chitosan) and 24.3% (0.5 mg/ml chitosan) resulting in 51 mm and 48 mm shoot lengths respectively. Salt stress also decreased fresh weights (66.9%) and dry weights (48.3%) as compared to the control (0 mM NaCl), whereas these were restored by the exogenous application of chitosan. Application of 0.25 mg/ml chitosan increased fresh weight by 79.5%, whereas 0.5 mg/ml chitosan increased fresh weight by 30.9% in salt treated plants. Furthermore, salt stress decreased dry weights by 48.3%, whereas application of chitosan increased dry weights by 53.02% (0.25 mg/ml chitosan) and 46.31% (0.5 mg/ml chitosan). Application of chitosan on control sorghum plants had no significant changes in all growth parameters.

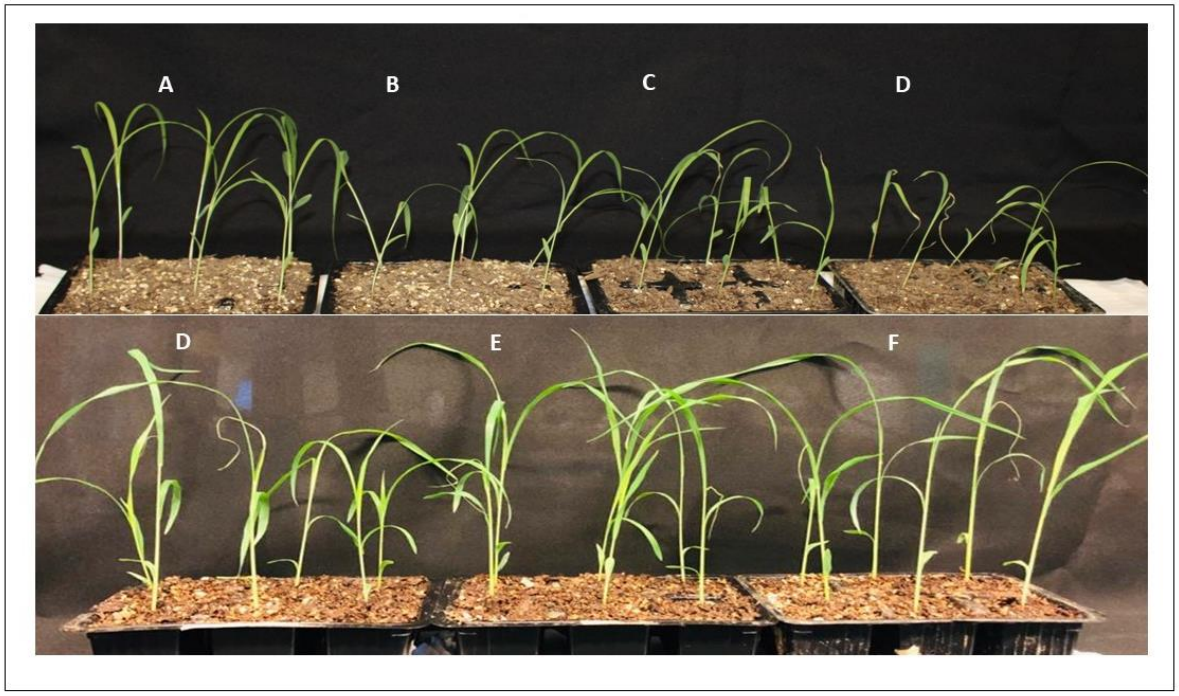

Figure 4.1: Effects of chitosan and salt stress on the morphology of *Sorghum bicolor* seedlings. (A) control [0 mM NaCl], (B) 0.25 mg/ml chitosan, (C) 0.5 mg/ml chitosan, (D) salt [300 mM NaCl], (E) 300 mM NaCl + 0.25 mg/ml chitosan and (F) 300 mM NaCl + 0.5 mg/ml chitosan.

Table 4.1: Effects of chitosan on growth parameters of salt stressed *Sorghum bicolor*. Data represented are mean ± SD.

Chitosan (mg/ml)	NaCl (mM)	Shoot length (mm)	Fresh weight (g)	Dry weight (g)
0	0	48.333 ± 6.506	2.920 ± 0.482	0.288 ± 0.045
0.25	0	51.00 ± 4.00	2.983 ± 0.207	0.263 ± 0.020
0.5	0	48.00 ± 1.00	2.292 ± 0.140	0.244 ± 0.039
0	300	24.667 ± 1.155***	0.966 ± 0.040****	0.1490± 0.003**
0.25	300	33.00 ± 1.00	1.734 ± 0.142	0.228 ± 0.038
0.5	300	30.667 ± 1.15	1.265 ± 0.232	0.218 ± 0.083

Significant differences shown as ****=p≤0.0001, ***=p≤0.0010, and **=p≤0.01.

4.3.2. Anatomical analysis

The anatomical structure (epidermis and vascular bundle) of sorghum seedlings was examined to determine the effects of chitosan on the growth of sorghum under salt stress using scanning electron microscopy (SEM) as shown in Figure 4.2 and 4.3. The Negative impact of salt stress were also analysed on the epidermal layers (Figure 4.2A) ofcontrol sorghum plants (0 mM NaCl), which showed smooth layers. Similar pattern was observed on the epidermis of sorghum shoots treated with chitosan only (Figure 4.2E-F). The epidermis of salt stressed (Figure 4.2B) plants showed rough layers as compared to the control (Figure 4.2A). However, chitosan application on salt stressed plants showed to have impact on the epidermis of sorghum, this has been seen by smooth layers as compared to salt stressed only.

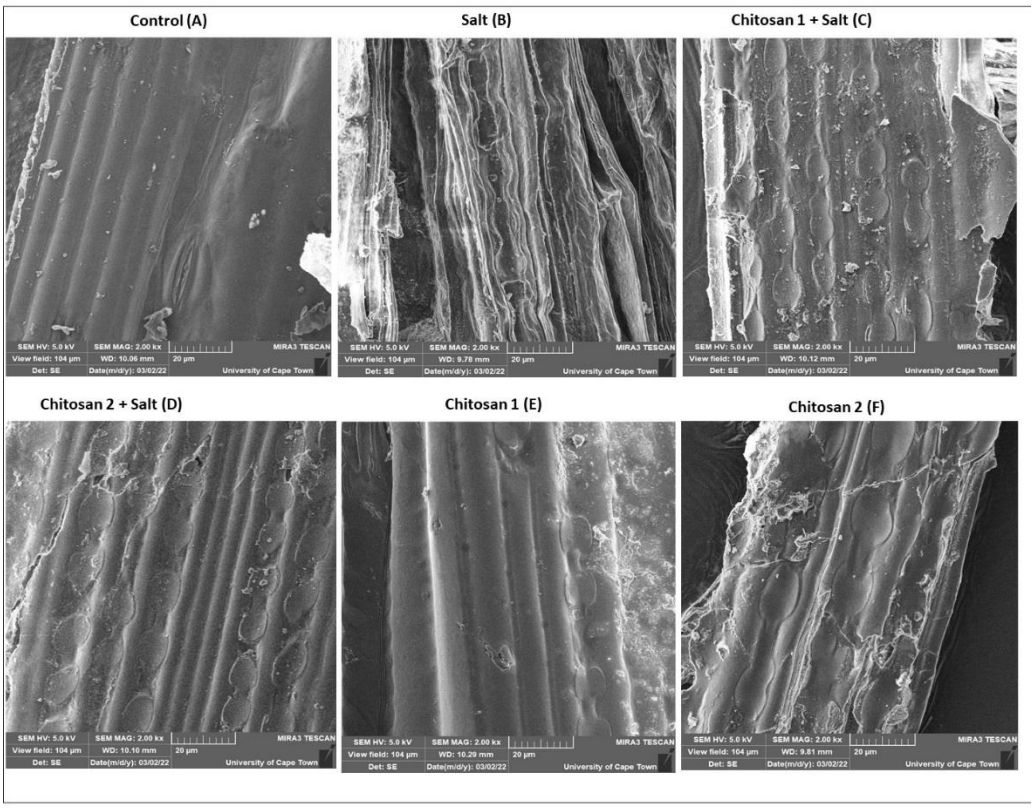

Figure 4.2: Anatomical image showing the epidermis of *Sorghum bicolor* analysed by SEM. (A) Control [0 mM NaCl], (B) Salt [300 mM NaCl], (C) Chitosan 1 + Salt [0.25 mg/ml + 300 mM NaCl], (D), Chitosan 2 + Salt [0.5 mg/ml + 300 mM NaCl], (E) Chitosan 1 [0.25 mg/ml], (F) Chitosan 2 [0.25 mg/ml].

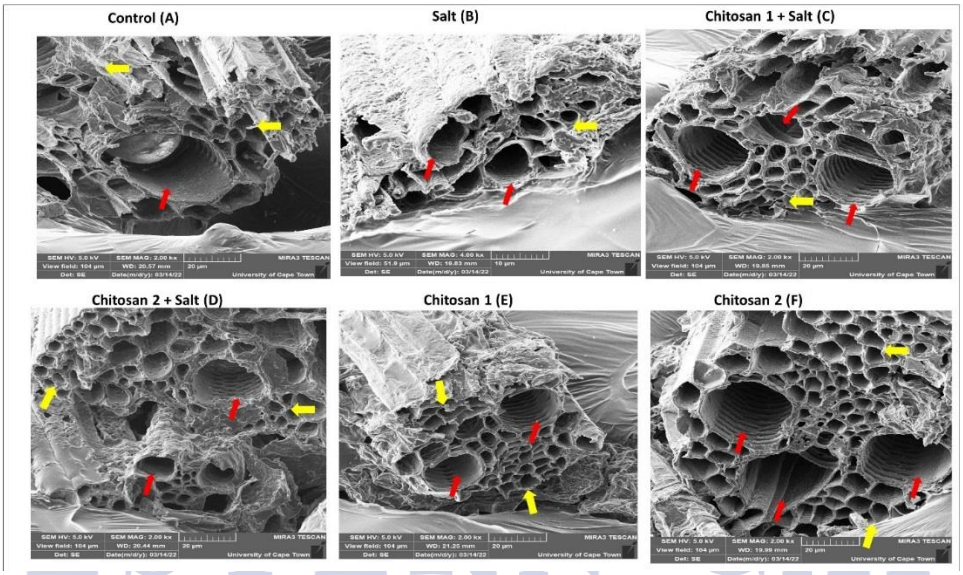

Figure 4.3: Anatomical image showing the xylem (red arrows) and phloem (yellow arrow) layer of *Sorghum bicolor* shoot analysed by SEM. (A) Control [0 mM NaCl], (B) Salt [300 mM NaCl], (C) Chitosan 1 + Salt [0.25 mg/ml + 300 mM NaCl], (D), Chitosan 2 + Salt [0.5 mg/ml + 300 mM NaCl], (E) Chitosan 1 [0.25 mg/ml], (F) Chitosan 2 [0.25 mg/ml].

4.3.3. Ion content

Ion content, especially Na^+, K^+, Cl^- and Ca^{2+} were analysed using scanning electron microscopy-energy dispersive X-ray Spectroscopy (SEM-EDX) for all the treatments. Under normal conditions (control: 0 mM NaCl), plants accumulated more Cl^- (2.283 wt %) and K^+ (4.307 wt %) ion than any other ions. In plants salt stress also causes the ionic imbalance through over accumulation of excess toxic ions including Na^+ and Cl^- and a decrease in essential elements K^+ and Ca^{2+} (Morgan *et al*., 2014). However, under salt stress, there was an increase in Na^+ (2.34 wt %) and Cl^- (4.73 wt%), whereas K^+ and Ca^{2+} decreased by 57.7% and 40% respectively in comparison to the control (Table 4.2). SEM images for the EDX investigated area showed significant morphological changes (Figure 4.4). SEM image for untreated (0 mM NaCl) plants revealed a smooth surface area (Figure 4.4A) as compared to salt stressed plants (300 mM NaCl), which showed severe damage (Figure 4.4B). SEM images from salt stressed plants treated with chitosan (0.25 mg/ml (Figure 4.4C) and 0.5 mg/ml chitosan (Figure 4.4D) revealed restoration by showing smooth epidermal layers.

Table 4.2. Overall ion content measured through the SEM-Energy dispersive X-ray (EDX) spectroscopy in *Sorghum bicolor*. Data represented are mean ± SD. Control (0 mM NaCl), CS1 (0.25 mg/ml chitosan), CS2 (0.5 mg/ml chitosan), Salt (300 mM NaCl), Salt + CS1 (300 mM NaCl + 0.25 mg/ml chitosan) and Salt + CS2 (300 mM NaCl + 0.5 mg/ml chitosan).

Element	Control	Salt	Salt + CS1	Salt + CS2	CS1	CS2
	Wt%	Wt%	Wt%	Wt%	Wt%	Wt%
Na	0.00	2.343 ± 0.327	2.30 ± 0.407	3.267 ± 0.532	0.00	0.00
Cl	2.283 ± 0.870	4.737 ± 1.076	4.553 ± 0.541	6.720 ± 1.650	1.823 ± 0.127	1.880 ± 1.410
K	4.307 ± 1.568	1.823 ± 0.509	1.513 ± 0.059	2.040 ± 0.382	4.20 ± 1.538	4.140 ± 2.735
Ca	0.1 ± 0.087	0.053 ± 0.092	0.080 ± 0.070	0.063 ± 0.055	0.00	0.060 ± 0.104
Total	100	100	100	100	100	100

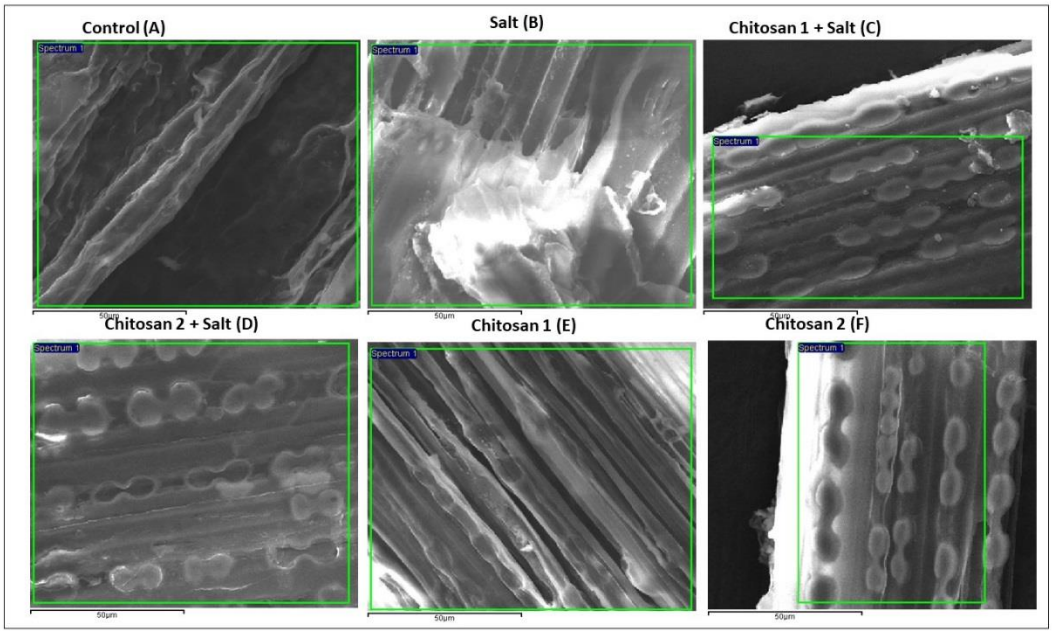

Figure 4.4: Anatomical image showing the SEM micrographs for the EDX-investigated area in *Sorghum bicolor* shoot. Non-stressed (A) Control [0 mM NaCl], (B) Salt [300 mM NaCl], (C) Chitosan 1 + Salt [0.25 mg/ml + 300 mM NaCl], (D), Chitosan 2 + Salt [0.5 mg/ml + 300 mM NaCl], (E) Chitosan 1 [0.25 mg/ml], (F) Chitosan 2 [0.25 mg/ml].

4.3.4. Photosynthetic pigments

Under normal conditions (0 mM NaCl), sorghum plants showed low chlorophyll (Chl) a content as compared to Chl b content, which resulted in high total Chl content. Salt stress (300 mM NaCl) resulted in non-significant increase in total Chl (18.7%), Chl b (51.8%) and decreased Chl a (9.6%) content as compared to the control (Figure 4.5A). However, supplementing salt stressed sorghum plants with chitosan (0.25 mg/ml) decreased total Chl (15.8%), and Chl b (32.4%), while there was an increase in Chl a (5.1%) as compared to salt-stressed plants. Application of 0.5 mg/ml chitosan showed a decrease in total Chl (11.1%) and Chl b (29.1%), while there was an increase in Chl a (13.3%) in salt stressed plant (Figure 4.5B).

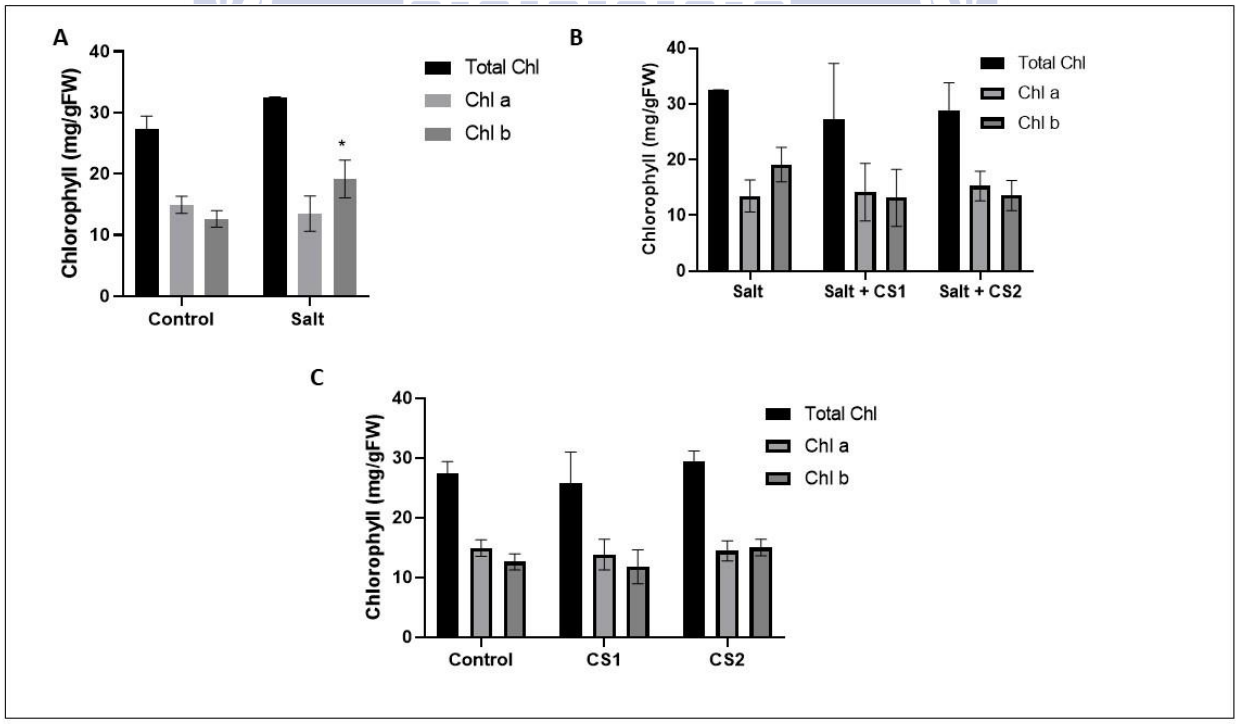

Figure 4.5: Effects of salt stress and chitosan on chlorophyll content in *Sorghum bicolor* seedlings. Seedling treated with (A) Salt [300 mM NaCl], (B) with NaCl + Chitosan (C) chitosan only [CS1:0.25 mg/ml and CS2:0.5 mg/ml]. Error bars represent the SD calculated from three biological replicates. Statistically significant between control and treated seedlings was determined using two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as ***=p≤0.001, **=p≤0.01, and *=p≤0.05.

4.4 DISCUSSION

4.4.1 *Growth analysis*

Looking at Figure 4.1 and Table 4.1, salt stress negativly affected sorghum growth, and this was evident by reduced shoot length, fresh and dry weight. These findings are in line with the findings of Memon *et al*. (2010), where it was found that an increase in salt concentration (0, 50, 100, 150, 200 and 250 mM NaCl) reduced biomass of *Brassica campestris* L. Similar effects were observed in a study conducted by Zhou *et al*. (2018), that reported decreases in shoot length and dry weight of *Schezonepeta tenuifolia* Briq under increasing NaCl concentration (0, 25, 50, 75 and 100 mM NaCl). The inhibitory effects of salt stress were as a result of salt on the photosynthesis process, as intercellular CO_2 concentration and photosynthetic enzymes were reduced (Zhou *et al*., 2018). Furthermore, other studies reported similar results on other important plants such as *Mentha pulegium* (Oueslati *et al*., 2010) and *Cuminum cyminum* L. (Rebey *et al*., 2017). In spite of the fact that several studies have highlited the negative effects of NaCl on biomass (fresh and dry weight) there are contrary findings highliting positive effects of NaCl on biomass such in *Lactuca sative* L (Andriolo *et al*., 2005), *Beta vulgaris* (L) (Niaz *et al*., 2005) and *Beta maritime* (L) (Qados, 2011) where in an increase in NaCl concentration increased fresh weights of *Vicia faba* L. Based on these studies it is possible that NaCl sress reduced plant growth by affecting water and nutrient absorption, which led to direct injury to the plant cells through accumulation of toxic ions causing a decline in plant growth (Verslues *et al*., 2006). Furthuremore, Akram *et al*. (2010) argued that the decline in biomass and yield might be due to the inhibition of cell expansion due to low turgor pressure in salinity stress resulting in a reduction in the shoot growth. However, application of chitosan reversed the effects of salt stress on *S. bicolor* plants at early vegegative growth stage resulting in increased shoot length and biomass (Table 4.1). These might be as a result of exogenous chitosan facilitating plant growth by regulating the nutritional

balance and reducing ion toxicity. Similar findings were recorded on salt stressed *Zea mays* (Al-Tawaha *et al*., 2018), wereby foliar application of chitosan ameliorated the detrimental effects of salinity on the shoot and root growth. In a study conducted by Younas *et al*. (2021), it was found that combining silicon and chitosan improved the growth of salt stressed maize. In onother study, exogenous application of chitosan (100 mg/l) mitigated the growth inhibition by increasing total leaf area, fresh and dry weight of salt stress lettuce seedlings (Zhang *et al*., 2021).

4.4.2 Anatomic and ion content analysis

Salt stress caused membrane damage as a result of oxidative stress by over production of Na^+ from phospholipid membrane binding sites (Garg and Manchanda, 2009). This study highlighted that vascular layer under 300 mM NaCl showed shrinkage of the tubes as compared to control (figure 4.3), even though at different plant growth stage, during germination (Mulaudzi *et al.*, 2020) and vegetative (Rakgotho *et al*., 2022) stages, salt stress had similar effects on sorghum. Salt stress is also responsible for the ionic imbalance due to the accumulation of excess Na^+ and decrease in K^+ and Ca^{2+} (Cambridge *et al*., 2017). In the present study, high Na^+ led to low K^+ under 300 mM NaCl. Similar results were documented by Mulaudzi *et al.* (2020) in sorghum seedlings germinated under salt stress (300 mM NaCl). These results will be analysed further using another approach to further understand the role of chitosan in ion homeostasis under salt stress.

4.4.3 Photosynthetic pigment

Generally, Na^+ and Cl^- are known to cause injuries to the plant that are exposed to salt stress. In the present study, application of 300 mM NaCl resulted in increase in total Chl , Chl b and decreased Chl a in comparison with the control (Figure 4.5A). These findings are in contradiction with those of Gomes *et al*. (2017), where it was found that an increase in salt stress (NaCl) resulted in decrease in total chlorophyll content of *Salvinia auriculata* Aubl

(Gomes *et al.*, 2017). It was further found in this study that, salt stress increased chlorophyll b in comparison to the control. Recent study found that an increase in salinity stress (50, 100, and 200 mM NaCl) with increase in exposure period negatively affected total chlorophyll contents of *Tetragonia decumbens* Mill (Sogoni *et al*., 2021). However, Houimli *et al*. (2010) reported the decrease in both chlorophyll a and b on *Capsicum annuum* CV. In the current study, application of chitosan on salt-stressed sorghum plants had no significant changes on photosynthetic pigments. However, in other studies, chitosan improved salt tolerance of *Oryza sativa* (Phothi and Theerakarunwong, 2017) and *Solanum lycopersicum* L (Attia *et al*., 2021) through improving photosynthetic rates.

SECTION B

EVALUATING THE EFFECT OF CHITOSAN ON THE BIOCHEMICAL RESPONSE OF *SORGHUM BICOLOR* TO SALT

4.5 RESULTS

In Section A, results showed that sorghum shoots accumulated high Na^+ content in salt treated plants. It is commonly known that excess Na^+ hinders the absorption and transport of essential elements, which eventually lead to the over production of ROS, causing oxidative stress. To obtain a link or a better understanding of the effect of high Na^+ content and oxidative stress, Section B investigated the extent of oxidative damage by measuring ROS content, lipid peroxidation and the extent of ROS scavenging capacity.

4.5.1 The effects NaCl and chitosan on oxidative stress markers

To understand the level of oxidative stress induced by salt on sorghum and the ability of chitosan in ameliorating the effects of salt stress, the levels of H_2O_2 and MDA were measured (Figure 4.6 and 4.7). Reactive oxygen species (ROS) are produced from excess dissolved molecular oxygen (O_2), which then gets energised to become superoxide before forming H_2O_2, this is followed by the formation of hydroperoxide, which eventually causes lipid peroxidation (Trchounian *et al.*, 2016*;* Figure 4.6A). Salt stress significantly ($p \leq 0.01$) increased H_2O_2 content (144 nmol/g FW) by 44% in sorghum shoots of salt-stressed plants compared to control (100 nmol/g FW) plants (Figure 4.6B). Application of chitosan (0.25 and 0.5 mg/ml) to salt-stressed plants significantly ($p \leq 0.01$) decreased H_2O_2 content by 52% in comparison to those treated with salt only (Figure 4.6C). There was no significant ($P > 0.05$) difference on the sorghum plants treated with chitosan only (Figure 4.6D).

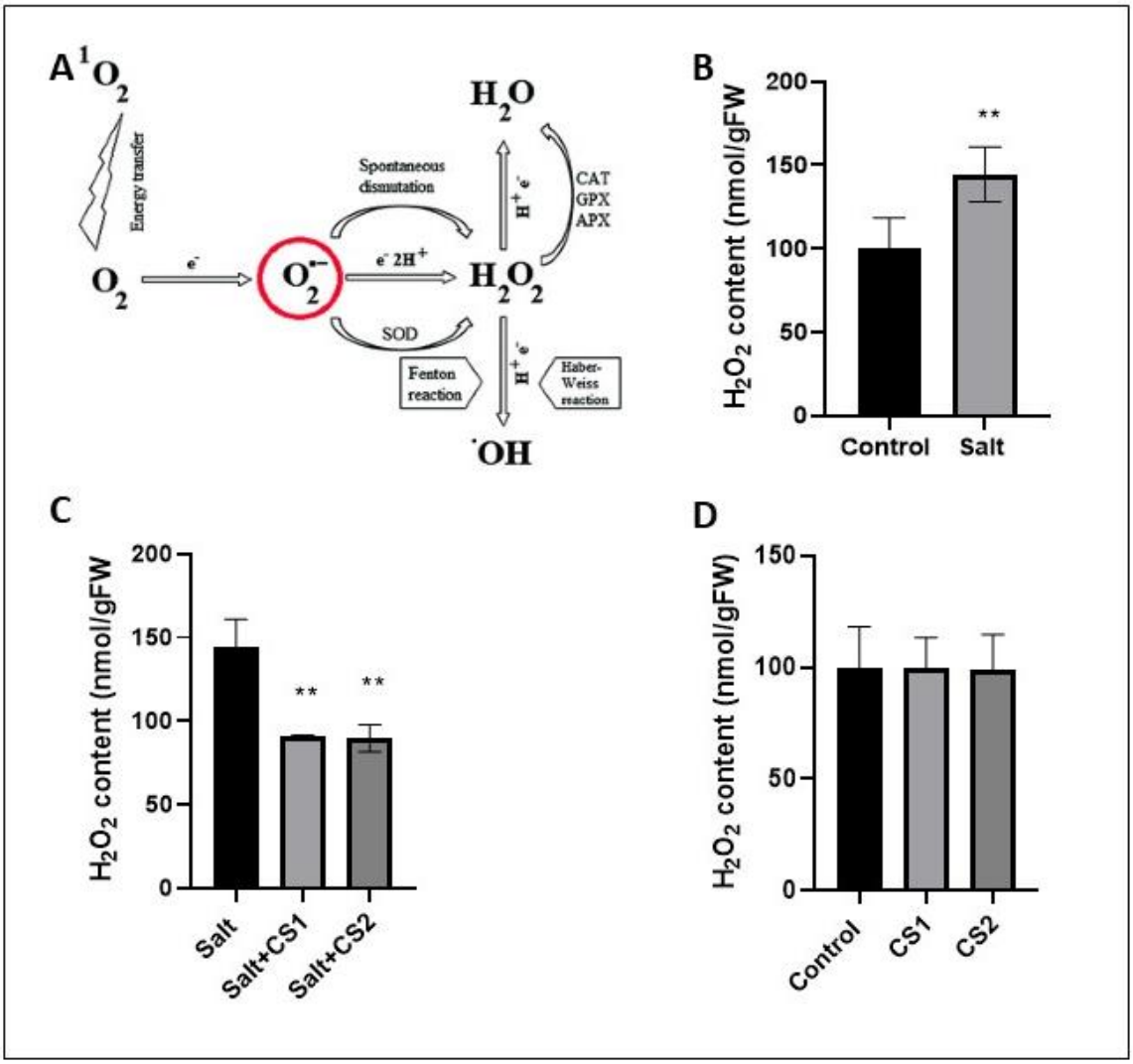

Figure 4.6: Effects of NaCl and Chitosan on the H_2O_2 on the sorghum seedlings at vegetative growth stage. (A) H_2O_2 regulation in plants under abiotic stress (adapted from Trchounian *et al.*, 2016). Sorghum plants treated with, (B) different salt concentration [0 and 300 mM NaCl], (C) salt + different chitosan concentration [CS1 (0.25 mg/ml) and CS2 (0.5 mg/ml) and, (D) chitosan only]. Error bars represent the SD calculated from three biological replicates. Statistically significant between control and treated seedlings was determined using two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as ***=p≤0.001, **=p≤0.01, and *=p≤0.05.

Lipid peroxidation of sorghum shoots was determined by quantifying malondialdehyde (MDA) content (Nakano *et al.*, 1981; Figure 4.7A). Sorghum plants under salt stress (300 mM NaCl), showed significant increase in MDA content (18 nmol MDA/g FW) by 50% in comparison to the control (8 nmol MDA/g FW) as observed in Figure 4.7B. Interestingly, supplementing salt-stressed sorghum plants with chitosan, significantly (p≤0.01) reduced MDA content by 50% as

compared to plants treated with salt only. There was no significant difference on MDA content of sorghum plants treated with 0.25 mg/ml chitosan only, however, 0.5 mg/ml chitosan significantly ($p \leq 0.05$) increased MDA content by 52% as compared to control plants.

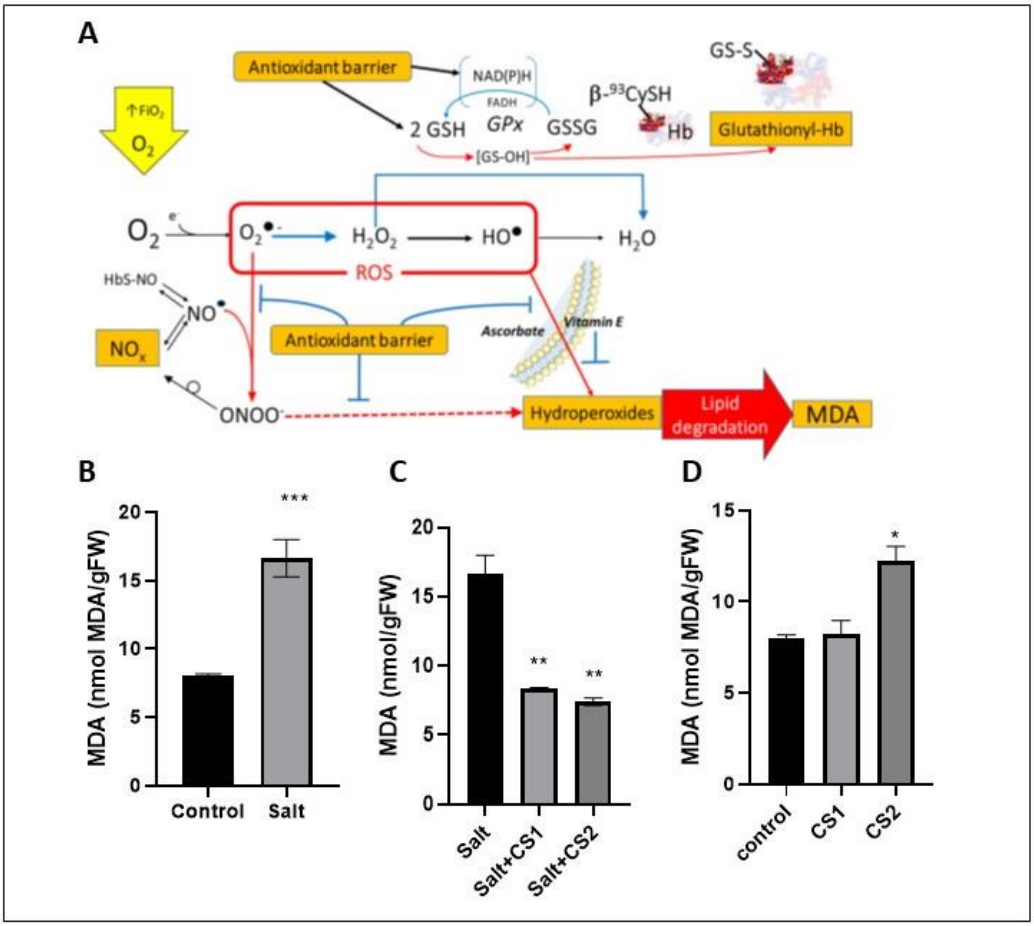

Figure 4.7 Effect of NaCl stress and Chitosan on the MDA content in sorghum seedlings at vegetative stage. (A) MDA chemical reaction in plants under oxidative stress (adapted from Ottolenghi *et al*., 2019). Sorghum plants treated with, (B) different salt concentration [0 and 300 mM NaCl], (C) salt + chitosan concentration (CS1:0.25 mg/ml and CS2:0.5 mg/ml) and (D) chitosan only. Error bars represent the SD calculated from three biological replicates. Statistically significant between control and treated seedlings was determined using two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as ***=$p \leq 0.001$, **=$p \leq 0.01$, and *=$p \leq 0.05$.

4.5.2 The effect of chitosan on the defense mechanism in sorghum under salt stress

To fully understand the positive effect of chitosan on salt-stressed sorghum plants, this study assayed the accumulation of biomolecules (Figure 4.8), osmolytes (Figure 4.9 & 4.10) and the activities of antioxidant enzyme activities (Figure 4.11 and Figure 4.12).

4.5.2.1 Fourier transform infrared spectroscopy analysis of biomolecules

The damage of biomolecules, such as carbohydrates, proteins, lipids and phenolics, were determined using Fourier transform infrared (FTIR) Spectroscopy and analysed a wide spectral region from 450 to 4000 cm^{-1}, which were consistent in all samples (Figure 4.8A-D). When comparing the control (0 mM NaCl) and the salt-stressed (300 mM NaCl) sorghum shoots, the spectra in Figure 4.8A (black line represents control and red line, salt stress), showed several peaks (3525, 2924, 2835, 1589, 1380, and 1058 cm^{-1}), were peak 1058 cm^{-1} is assigned to the C-N stretching, representing the presence of aliphatic amines, corresponding to proteins. Peaks, 1589 and 1380 cm^{-1} represent diketone and hydroxyl group compounds, thus corresponding to carbohydrates, however, under salt stress these peaks disappeared (Figure 4.8A; red line). Under salt stress (Figure 4.8A, red line), there was a formation of a new peak due to changes in compounds, peaks ranging from 910 to 665 cm^{-1} that are assigned to the N-H stretching vibration, representing the availability of primary and secondary amines (proteins), which are not present in the control sample (black line). Furthermore, application of 300 mM NaCl induced a shift resulting in the formation of new peaks (824, 663 and 558 cm^{-1}) that are assigned to the O-H bond represented by 824, 663 and 558 cm^{-1} indicating the presence of phenolic group as compared to that of the control. The positive effect of chitosan in salt-stressed sorghum plants showed that their peaks (Figure 4.8B; red and blue lines) are very close to those of the control (Figure 4.8B, black line), with a special reference to peaks at 910, 824, 663 558

and 665 cm^{-1}indicating the effectiveness of chitosan in protecting degradation of biomolecules. These results are further confirmed by comparing salt-stressed samples with those treated with chitosan (Figure 4.8C). It is clear that chitosan improved plants response to salt stress, since treatment with 0.25 mg/ml chitosan (red line, D) resulted in a shift forming O-H peak represented by 824, 663 and 558 cm^{-1} showing the presence of phenolic group.

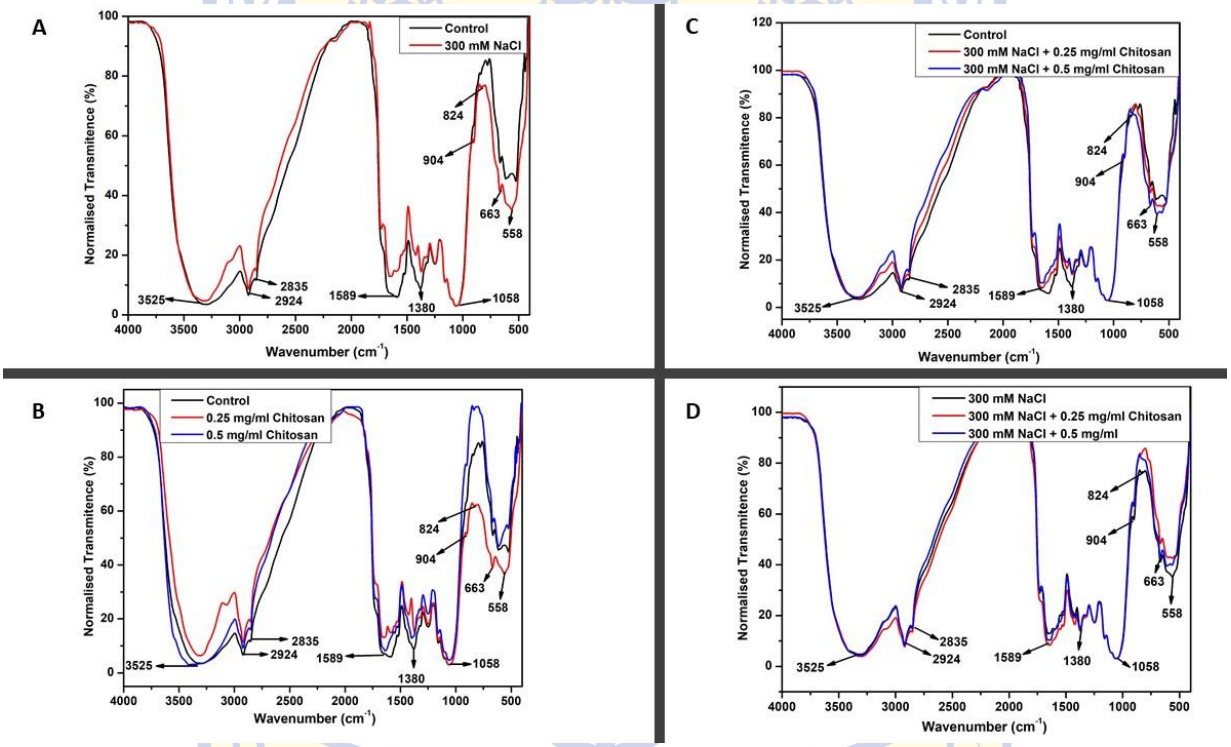

Figure 4.8: FTIR analysis of the effect of salt and chitosan on biomolecules in sorghum plants. Sorghum plant treated with, (A) 0 mM NaCl (Control, Red) and 300 mM NaCl (Salt, Black), (B) 300 mM NaCl + chitosan (0.25 mg/ml, Red and 0.5 mg/ml, Blue), (C) 300 mM NaCl [only; black], 300 mM NaCl + 0.25 mg/ml chitosan (Red), and 300 mM NaCl + 0.5 mg/ml chitosan (Blue).

4.5.2.2 ROS scavenging through the enzymatic antioxidant capacity

The effectiveness of chitosan in scavanging ROS activity was determined by analysing osmolytes (proline and total soluble sugars) accumulation, and level of osmotic balance induced in salt-stressed *S. bicolor* plants (Figure 4.9 and 4.10). Proline accumulation has been observed in different plant species in response to different kinds of environmental stresses, suggesting a positive correlation between proline accumulation and plant stress tolerance (Dar

et al., 2016). There was a significant (P<0.01) increase in proline by 319% in salt-stressed *S. bicolor* as compared to the control (0 mM NaCl) (figure 4.9). Chitosan effectively induced stress tolerance by mediating a decrease in proline content by more than 50% for both (0.25 and 0.5 mg/ml) chitosan concentrations. In salt-stressed plants, there was a significant drop in proline content when sorghum seedlings were supplemented with different chitosan concentrations (Figure 4.9B). There were no significant differences in the proline content of sorghum plants treated with chitosan (0.25 and 0.5 mg/ml) only.

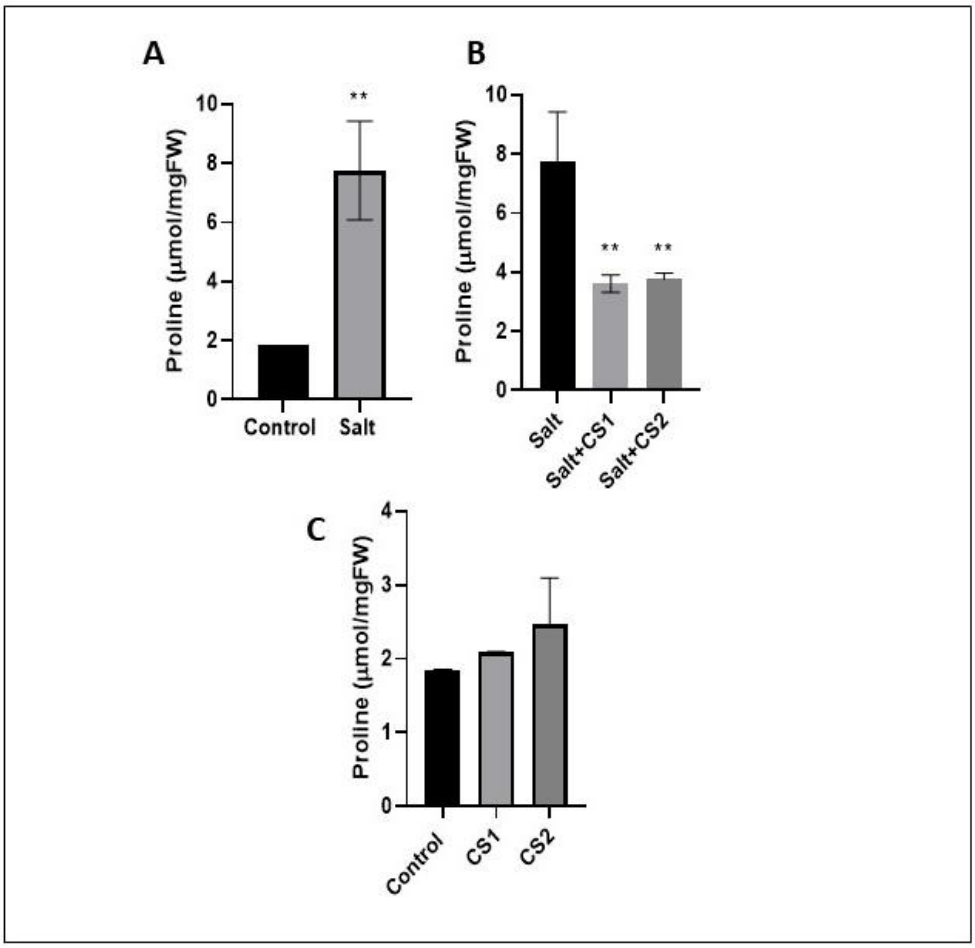

Figure 4.9 Effects of NaCl and Chitosan on the Proline content on sorghum seedlings at vegetative growth stage. Seedlings treated with, (A) different salt concentration [0 and 300 mM NaCl], (B) salt + different chitosan concentration (CS1:0.25 mg/ml and CS2:0.5 mg/ml), (C) treated with chitosan only. Error bars represent the SD calculated from three biological replicates. Statistically significant between control and treated seedlings was determined using two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as ***=p≤0.001, **=p≤0.01, and *=p≤0.05.

In the present study, the effects of salt stress and chitosan on osmolyte accumulation were also analysed by assaying soluble sugars content in sorghum were analysed (Figure 4.10). Salt stress significantly (P≤0.05) increased soluble sugars by 68.33% in sorghum plants in comparison to the control (0 mM NaCl). Interestingly, application of chitosan (0.25 and 0.5 mg/ml) on salt-stressed (300 mM NaCl) sorghum plants significantly (p≤0.01) reduced soluble sugars by 28.76% (0.25 mg/ml chitosan) and 33.28% (0.5 mg/ml chitosan). Application of chitosan to control plants also increased soluble sugars (0.25 and 0.5 mg/ml) by 19.98 (0.25 mg/ml chitosan) and 34.78% (0.5 mg/ml chitosan), but the increase was not significant (Figure 4.10C).

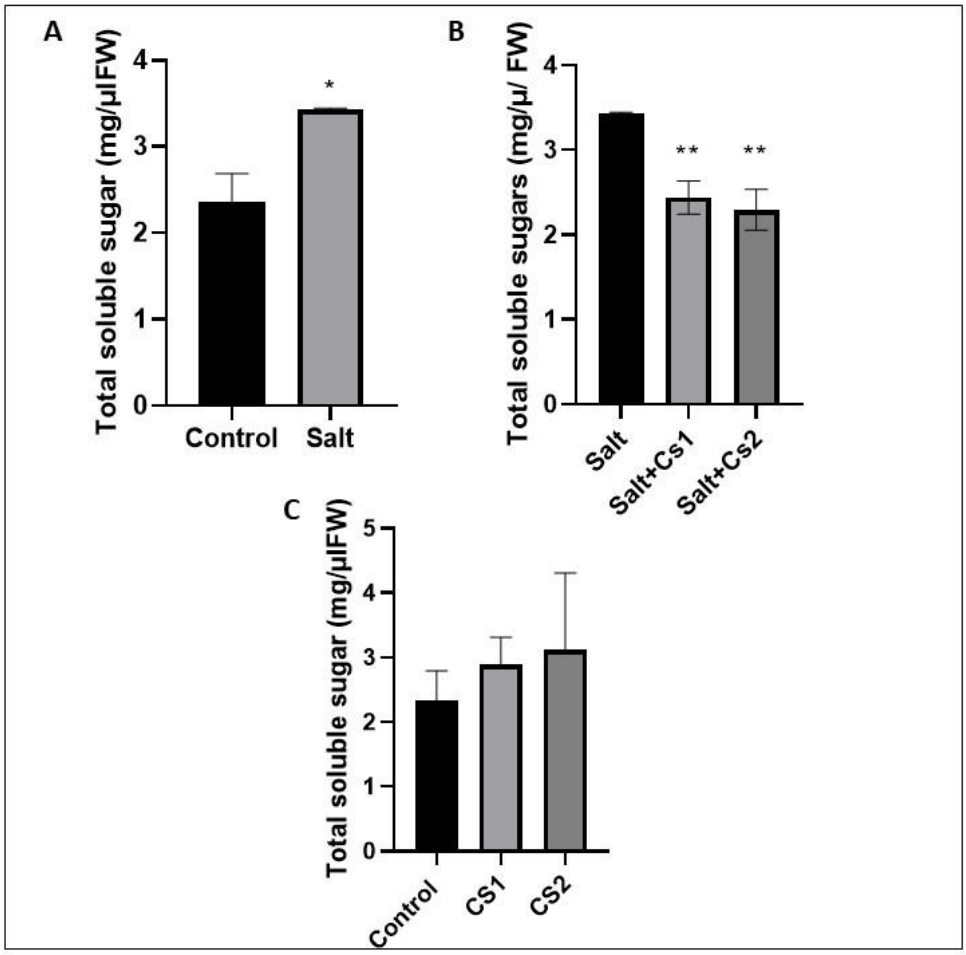

Figure 4.10 Effects of NaCl and Chitosan on the total soluble sugar content on sorghum seedlings at vegetative growth stage. Seedlings treated with, (A) different salt concentration [0 and 300 mM NaCl], (B) salt + different chitosan concentration [CS1 (0.25 mg/ml) and CS2 (0.5 mg/ml) and (C) chitosan only]. Error bars represent the SD calculated from three biological replicates. Statistically significant between control and treated seedlings was determined using two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as ***=p≤0.001, **=p≤0.01, and *=p≤0.05.

4.5.2.3 ROS scavenging through the enzymatic antioxidant capacity

Naturally plants contain both enzymatic and non-enzymatic antioxidant systems, which are major role players in ROS detoxification within the cells. SOD is known as the first line of defence by directly dismutating $O_2^{\bullet -}$ to H_2O_2 and H_2O, whereas CAT and APX are the main role players for H_2O_2 scavenging (Das and Roychoudhury, 2014).

To determine the nature of the antioxidant levels of sorghum in response to salt (300 mM NaCl) stress and chitosan (0.25 and 0.5 mg/ml), enzymatic activity of SOD and APX were analysed from the shoots and results are summarised in figure 4.11 and figure 4.12 respectively. Results showed that application of salt stress (300 mM NaCl) significantly ($P \leq 0.05$) increased SOD activity by 36% as compared to control (0 mM NaCl) plants (Figure 4.11B). However, supplementing salt-stressed plants with 0.25 mg/ml chitosan showed significant decrease in SOD activity for salt-stressed (41.88%) plants and control (57%) plants, whereas 0.5 mg/ml chitosan had not significant effects.

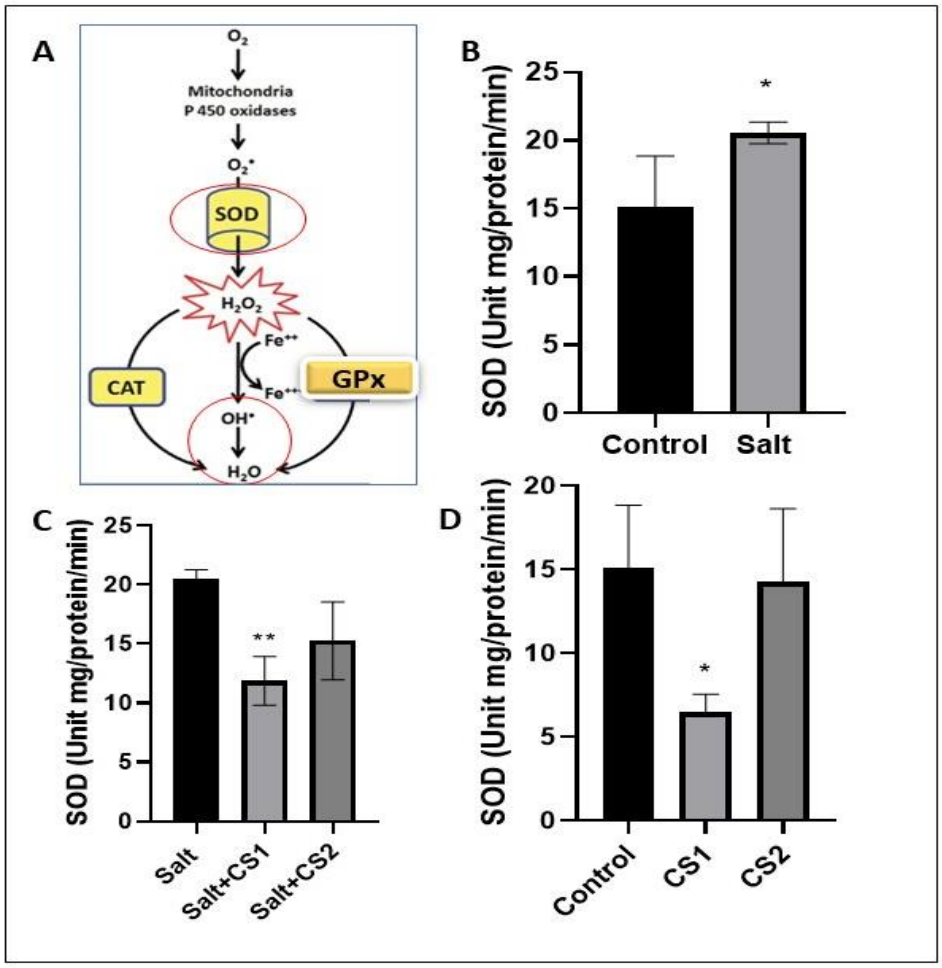

Figure 4.10 Effects of exogenous application of chitosan on antioxidant SOD activity of salt stressed sorghum seedlings. (A) SOD activity in scavenging radicals and H_2O_2 picture prepared on PowerPoint). (B) Seedlings treated with different salt concentration [0 and 300 mM NaCl]. (C), salt + different chitosan concentration [CS1 (0.25 mg/ml) and CS2 (0.5 mg/ml), (D) with chitosan only]. Error bars represent the SD calculated from three biological replicates. Statistically significant between control and treated seedlings was determined using two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as ***=p≤0.001, **=p≤0.01, and *=p≤0.05.

Ascorbate peroxidase is one of the enzymes involved in the detoxification of H_2O_2 (Anjum *et al*., 2016) as shown in Figure 4.12A. Salt stress significantly (P≤0.01) increased APX activity by 132% as compared to control plants (Figure 4.12B). Application of 0.5 mg/ml chitosan showed significant (P≤0.05) decreases in APX activity for salt-stressed (41.88%) plants, whereas 0.25 mg/ml showed no effects. Both chitosan concentrations had not significant effects on control plants (Figure 4.12D).

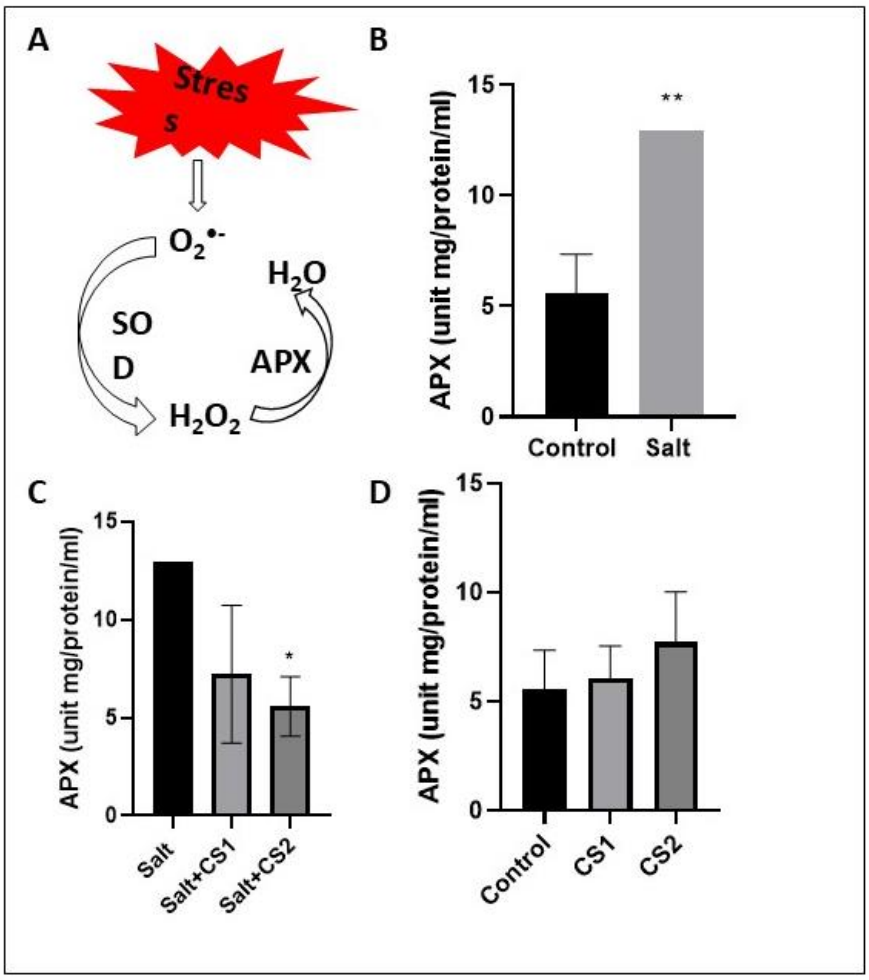

Figure 4.12 Effects of exogenous application of chitosan on antioxidants enzyme (APX) of salt stressed sorghum seedlings. (A) APX mechanism in detoxifying H_2O_2 (picture prepared on PowerPoint). Seedlings treated with different, (B) salt concentration [0 and 300 mM NaCl, (C) Salt + different chitosan concentration [CS1 (0.25 mg/ml) and CS2 (0.5 mg/ml) and, (D) chitosan only]. Error bars represent the SD calculated from three biological replicates. Statistically significant between control and treated seedlings was determined using two-way ANOVA conducted on GraphPad Prism 8.4.2, shown as ***=p≤0.001, **=p≤0.01, and *=p≤0.05.

4.6 DISCUSSION

4.6.1 Chitosan reduces the accumulation of oxidative stress markers

In the current study, the increase in salinity levels contributed to the formation of ROS (increased the H_2O_2) and hence caused lipid peroxidation (Mehmood *et al*., 2020). One way in which plants respond to abiotic stresses is through the overproduction of ROS. H_2O_2 plays a significant role as a signalling molecule in plants when available at physiological level (Slesak *et al*., 2007; Das and Roychoudhury, 2014). Whereas the accumulation of MDA is an indicator of membrane damage by the overaccumulation of ROS (Mehmood *et al*., 2020). A balance between production and detoxification of ROS is required to maintain cellular metabolic processes under stressed conditions, hence minimising oxidative damages (Farhangi-Abriz and Torabian, 2018). In this study, sorghum plants experienced oxidative stress caused by salt stress as evident by the high content of H_2O_2 and MDA in salt-stressed plants as compared to control plants (Figure 4.6 and 4.7). These results are in agreement with those of previous studies done in tomato (Attia *et al*., 2021), Soybean (Osman *et al*., 2021), Chickpea (Ahmad *et al*., 2016), and Rice (Mostofa *et al*., 2015), which showed significant increases of H_2O_2 and MDA content in plants treated with NaCl (50-150 mM NaCl). According to Hidangmayum *et al*. (2019), supplementing salt-stressed plants with chitosan induces different defensive responses. Furthermore, Attia *et al*. (2021) and Guan *et al*. (2009) explained that supplementing plants with chitosan lessened the production of H_2O_2 and MDA contents through increasing the level of antioxidant compounds that scavenges ROS and protect cellular membranes from oxidative damage. In corroboration with other studies, this study showed that application of chitosan on sorghum plants exposed to salt was able to reduce the oxidative stress by reducing H_2O_2 and MDA content (Figure 4.6 and Figure 4.7) regardless of the method of administration. For example, foliar application of chitosan on salt-stressed *Salanam lycopersicum* L. was also

found to reduce both H_2O_2 and MDA levels (Attia *et al*., 2021). These results suggest that chitosan can effectively ameliorate salt-induced oxidative stress.

4.6.2 Chitosan protects biomolecules from oxidative damage

Fourier Transform Infrared Spectroscopy is a very important technique in revealing different types of organic and inorganic compounds present in an organism (Chandra, 2019). In this study, the presence of different biomolecules was investigated using FTIR to inspect any shift in the spectral peaks of *S. bicolor* shoot samples (Figure 4.8). The FTIR spectra clearly showed the absorption peaks of functional groups that represent the presence of primary and secondary amines corresponding to proteins, alkane and ketone groups corresponding to carbohydrates. FTIR spectra revealed a major shift and disappearance of other peaks (1589 and 1380 cm^{-1}) was observed in salt-stressed plants as compared to control, which suggest the degradation of some biomolecules (Rakgotho *et al*., 2022). Additionally, samples from sorghum salt-stressed plants indicated the induction and formation of carbohydrates, proteins and phenols as represented by new peaks (824, 663 and 558 cm-1). These results are in agreement with the findings by Rakgotho *et al*., (2022), that showed shifts in FTIR spectral peak of salt-stressed (400 mM NaCl) sorghum plants. This result suggests the induction of a protective mechanism in the form of non-enzymatic (represented by sugars, amino acids and phenols), and enzymatic possibly heat shock proteins, however, will be further investigated. Interestingly, application of chitosan (0.25 and 0.5 mg/ml) on salt-stressed sorghum plants prevented degradation of biomolecules and reversed the formation of new peaks as evident by FTIR spectra showing similar trend as those of the control, suggesting that chitosan reduced salt-induced oxidative damage improving sorghum response to salt stress.

4.6.3 Chitosan improves osmoregulation of sorghum under salt stress

Proline is one of the well-researched osmolyte that plays a significant role in maintaining osmotic balance by improving osmotic potential during osmotic stress (Chen and Jiang, 2010; Hayat *et al*., 2012). Proline accumulation is a common observation in response to abiotic stress in plants (Liang *et al.*, 2013). Sorghum seedlings accumulated high proline content under salt stress (Figure 4.9). Proline accumulation was also observed in sorghum under salt stress in different plant growth stages, including germination (Mulaudzi *et al*., 2020) and vegetative stages (Heidari, 2009; El Omari *et al*., 2016; Nxele *et al*., 2017; Rakgotho *et al.*, 2022). Proline accumulation under stress is an important tolerance mechanism since proline act as an antioxidant that protects biomolecules from oxidative damage (Hayat *et al*., 2012). It also scavenges ROS, maintain membrane stability and controls the osmotic balance and homeostasis (Liang *et al*., 2013).

As part of the adaptive response to overcome the negative effects of ROS under stress, plants induce the synthesis of soluble sugars (Couée *et al.*, 2006). Total soluble sugars increased in salt-stressed sorghum plants (Figure 4.10). Similar responses were observed in sorghum plants stressed with 400 mM NaCl (Rakgotho *et al.*, 2022). These findings are also in line with those of Nedjimi (2011) in *Zygeum spartum* seedlings, where soluble sugars also increased under salt stress. However, application of chitosan (0.25 and 0.5 mg/ml) on salt stressed sorghum plants showed a positive effect by significantly decreasing the total soluble sugar level as compared to plants treated with salt only. Chitosan applied on control plants showed no significant difference, suggesting that the concentration used are not detrimental to the plants. These findings further indicated that sorghum plants exhibited higher adaptive osmotic potential under salinity stress as evident by the high accumulation of proline and soluble sugars (Farissi *et al*., 2011).

4.6.4 Chitosan reduced the antioxidant enzyme activity

Antioxidant enzymes play a significant role in salt stress through the prevention of oxidation damage (Mehmood *et al*., 2020). In line with other studies (Abd-Allah *et al*., 2015; Farhangi *et al*., 2018; Mehmood *et al.,* 2020; Rakgotho et al., 2022), salt stress (300 mM NaCl) significantly increased the activities of SOD and APX in sorghum as compared to the control (0 mM NaCl). Furthermore, salt stress significantly increased antioxidant enzyme activity in other cereals for example wheat (Erdal *et al*., 2011; Gercek and Erdal, 2015). The results of this study showed that SOD was induced at a greater extent than APX, which truly suggest that SOD contributes majorly to maintaining the antioxidative balance in sorghum as previously documented (Rakgotho *et al.,* 2022). In general, the increased activity of the antioxidant enzymes under salt stress indicates the level of ROS scavenging capacity mediated by sorghum. On the other hand, 0.25 mg/ml chitosan only significantly decreased SOD activity, while 0.5 mg/ml chitosan significantly decreased APX activity in salt-stressed plants. This reduction might be caused by the superoxide scavenging ability of chitosan as proposed by Raty *et al*. (2015). Previous studies reported significant increases in antioxidant enzyme activities in salt-stressed plants (Gorcek and Erdal, 2015; Erdal *et al*., 2011; Hulya, 2019) associated with tolerance. The findings of this study are contrary with previous studies, which reported a further increase in antioxidant enzyme activities in salt-stressed *Zea mays* (Hulya, 2019) and *Glycine max* (Mahmood *et al*., 2020) plants.

In summary, the results indicated that salt stress resulted in the over-accumulation of ROS, which caused oxidative damage of membranes as seen by damaged epidermis, xylem and lipid membrane layers and degradation of biomolecules. However, sorghum is known to be moderately tolerant to stress, and in this study, it was confirmed by high synthesis of osmolytes, proteins, phenolic compounds and the antioxidant enzyme activities. This study proved the positive effects of chitosan since its application in salt-stressed plants, decreased all the markers of oxidative stress and there was no need for the activation of the defence systems via the

osmolyte and the antioxidant regulation, when the salt-stressed plants were exogenously treated with chitosan. Furthermore, this study sugesst the use of 0.25 mg/ml chitosan since it showed the most positive effects with no significant damage on control plants.

FINAL REMARKS FUTURE WORK

In nature, plants are confronted with several environmental stresses, contributing to changes in their metabolism, resulting in a serious threat within the crop production sector. Among all these environmental stresses, salinity imposes major effects on the growth and development of plants and hence crop loss (Ibrahim *et al*., 2020; Alnusairi *et al*., 2021). Salinity also affects the agricultural lands mostly in arid and semi-arid regions. More than 6% of the world irrigated land are on the line to lose their sustainability for agricultural production as a result of salinity (Hasanuzzaman *et al*., 2014; Hossain, 2019). Several factors that contribute to soil salinity includes shortage of proper drainage, improper agricultural practices as well as excessive soluble salts accumulation (Yildiz *et al*., 2020). Therefore, it is important to develop environmental friendly technologies to combat the ongoing negative effects of salt stress on crops and develop crops that can produce maximally under severe stresses.

Several studies have already documented the effects of salinity on the growth and development of several plants including most important agricultural crops such as *Zea mays* (Ramadan and Flowers, 2004; Konuskan *et al*., 2017; Bose *et al*., 2018; Shahzad *et al*., 2019), *Triticum aestivum* L. (Harris *et al*., 2010; Datta *et al*., 2010; Hadia *et al*., 2022), *Oryza sativa* L. (Pattanagul and Thitisakul, 2008; Minh *et al*., 2016; Dolo, 2018) and *S. bicolor* (Dashti *et al*., 2009; Sharif *et al*., 2016; Mulaudzi *et al*., 2020; AL-Shoaibi *et al*., 2020; Rakgotho *et al*., 2022). Other studies documented the exogenous application of different compounds to improve stress tolerance including the application of chitosan in different crops such as *Zea mays* (Turk, 2019), *Carthamus tinetorius* L and *Helianthus annuus* L (Jabeen and Ahmad, 2013) and *S. bicolor* (Kumar *et al*., 2019).

Sorghum serves as a good model system for this study because it is naturally moderately tolerant to abiotic stresses with a wide genetic variation, and the availability of its genome sequence provides an important resource tool for molecular studies. To understand the effects

of salinity and chitosan on the germination and growth of sorghum, different assays were conducted as described in chapter 3 and chapter 4. This study (chapter 3) investigated the effect of different salt (0, 100, 200, 300 mM NaCl) and chitosan (0.25 and 0.5 mg/ml) concentrations on the germination and seedling growth of sorghum by employing several germination assays including germination percentage (GP), germination index (GI), mean germination time (MGT), and total germination (TG). Results clearly showed that increasing salt concentration negatively affected the germination as seen with decrease in GP, GI, decreased plant growth (roots and shoots length) and hence biomass (fresh and dry weights). However, exogenous application of chitosan reversed the severe effects of salinity by improving germination and growth of sorghum seedlings.

In chapter 4, Section A, the synergistic effects of salt and chitosan were investigated at the vegetative growth stage of *S. bicolor* plants grown in the greenhouse. Based on the germination results (Chapter 3), 300 mM NaCl was selected as the high salt stress and it was found that salt (300 mM NaCl) stress had noticeable effects on the morphological traits of sorghum (Figure 4.1), which resulted in reduced growth (shoot length) and biomass (fresh and dry weights). However, supplementing salt-stressed plants with chitosan showed improvement of the growth rate and shoot length, fresh and dry weights and this implies that indeed chitosan acted as a bio-stimulant in improving sorghum tolerance to salt stress. Furthermore, chitosan prevented damage to the epidermal layers and vascular budle tissue, which result in less water loss and improved nutrient and water transport. This restricted the transport of Na^+ to the shoots and K^+ content in sorghum shoots.

In chapter 4, Section B, this study further investigated the oxidative state of sorghum in response to salt and chitosan by assaying Hydrogen peroxide (H_2O_2) and lipid peroxidation (MDA) content. According to Bernstein *et al*. (2010), reactive oxygen species are signalling molecules that regulate ion channel activity and gene expression. Findings showed that

sorghum accumulated high H_2O_2 and MDA content in response to salt stress. However, application of chitosan showed a positive effect in mitigating the detrimental effects of salt stress on sorghum by reducing oxidative stress markers. Furthermore, sorghum also accumulated high level of osmolytes in response to high salt stress. However, since chitosan is considered a potential bio-stimulant, it can promote physiological responses to water stress tolerance (Katiyar *et al*., 2015). This was further confirmed in this study, since chitosan (0.25 and 0.5 mg/ml) managed to reverse the detrimental effects of salinity by reducing oxidative damage (low H_2O_2 and MDA contents) as well as osmotic stress and prevented damage to biomolecules and hence improved sorghum growth under salinity.

Plants undergo different physiological and biochemical responses to adapt to salinity depending on their characteristic either as halophytes or glycophytes. Different stress response mechanisms include ion homeostasis and compartmentalisation, ion transport, ion uptake, osmotic adjustments, modulation of hormones and induction of antioxidant enzyme mechanisms (Gupta and Huang, 2014; Zhang and Dai, 2019). Furthermore, plants under salt stress facilitate the over production and activation of antioxidant enzymes to scavenge ROS and this was further confirmed in this study by high SOD and APX activities on the sorghum plants under high salt (300 mM NaCl) stress (Chapter 4, Section B). However, application of chitosan reduced the level of oxidative stress by reducing the over production of ROS, hence there was reduction in the level of antioxidant enzyme (SOD and APX) activities.

In conclusion, findings of this study illustrated that exogenous application of chitosan could enhance plant growth and improve sorghum tolerance to salinity by increasing some germination parameters, biomass, reducing lipid peroxidation and production of ROS, osmolytes level as well as reducing antioxidant enzyme activity. These findings will shed light on the mode of action of chitosan in improving plant growth, ameliorating abiotic induced stress, and induction of tolerance and will serve as an important resource for the future

applicability of chitosan in agriculture worldwide. However, future work will try to deduce the mechanism of chitosan-induced salt tolerance by linking the physiological and biochemical response results with the molecular response analysis. And these will be achieved by profiling the transcripts of selected genes such as *S. bicolorSOD* (*SbSOD), SbAPX, SbNHX, SbCCAAT enhancer-binding protein 1 (SbCEBP1),* and *SbCCAAT enhancer-binding protein 2 (SbCEBP2)*. And in the long run, these findings will lead to the use of chitosan in a larger scale (field) to improve agricultural productivity and sustain food security globally.

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