Physiological, biochemical and molecular characterization of *Zea mays* proteins in response to salt stress

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DECLARATION

I, Lerato Thamaga declare that this work entitled **"Physiological, biochemical and molecular characterization of *Zea mays* proteins in response to salt stress"** is my work and has not been submitted to any institution of higher learning other than at North-West University (Mafikeng campus) for examination or other purposes.

SIGNATURES

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Dr BT Dikobe **DATE**

(SUPERVISOR)

DEDICATION

This work is dedicated to myself and my family, Mrs Gadifele Rebecca Thamaga and Mr Thapelo Sylvester Thamaga for all the support and encouragement they have provided throughout my studies. To my supervisor Dr Dikobe for her guidance, support and patience throughout my academic years.

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DEFINITION OF TERMS

Abiotic Stress : The negative effects of non-living factors on living organisms in a specific environment.

Abscisic Acid : A lipid hormone that inhibits cell growth in plants, which also induces fruit drop, death and seed dormancy.

Antioxidants : Compounds that inhibit oxidation and chemical reactions, that can produce free radicals and chain reactions that result in organisms cell damage.

Catalase : An enzyme found in nearly all living organisms exposed to oxygen which catalyzes the decomposition of hydrogen peroxide to water and oxygen.

Glutathione Reductase : A part of antioxidant defense systems of plants and microbes, and it also takes part in both enzymatic and non-enzymatic oxidation reduction processes of the cell.

Maize : A large species of the American grass belonging to the genus Zea that is widely cultivated as a forage and food crop.

Osmotic Stress : Physiological dysfunction caused by a sudden change in the solute concentration around a cell, which result in a rapid change in the

movement of water across its cell membrane.

Oxidative Stress : The imbalance between the production and accumulation of by-products of oxygen metabolism called reactive oxygen species (ROS).

Reactive Oxygen Species : Chemically reactive molecules and ions of oxygen with unpaired electron.

Relative Water Content : The measure of a plant water status in terms of the physiological consequence of cellular water deficit.

Salt Stress : The accumulation of excessive salt in the soil that ultimately leads to plant growth inhibition and death.

SDS-PAGE : A technique used in molecular biology to separate different protein molecules according to their sizes and migration levels in polyacrylamide gel system subjected to a strong electrical field.

Superoxide Dismutase : An enzyme that alternately catalyzes the dismutation of the superoxide radical into ordinary molecular oxygen and hydrogen peroxide.

LIST OF ABBREVIATIONS

1D	**:**	One-dimensional
2D	**:**	Two-dimensional
2DE	**:**	Two-dimensional polyacrylamide gel electrophoresis
ABA	**:**	Abscisic acid
ACN	**:**	Acetonitrile
ANOVA	**:**	One-way analysis of variance
APS	**:**	Ammonium per sulfate
APX	**:**	Ascorbate peroxidase
CAT	**:**	Catalase
DDA	**:**	Data-dependent acquisition
DEPs	**:**	Differentially expressed proteins
DIA	**:**	Data-independent acquisition
DTT	**:**	Dithiothreitol
EDTA	**:**	Ethylenediamine-tetra acetic acid
FA	**:**	Formic acid
GO	**:**	Gene Ontology
GR	**:**	Glutathione reductase
IAA	**:**	Iodoacetamide
LC-MS-MS	**:**	Liquid-chromatography tandem mass-spectrometry
MDA	**:**	Malondialdehyde
NBT	**:**	*p*-Nitroblue tetrazolium chloride
NH_4HCO_3	**:**	Ammonium bicarbonate

PP	**:**	Protein precipitator
PVP	**:**	Polyvinyl-pyrrolidone
ROS	**:**	Reactive oxygen species
RWC	**:**	Relative water content
SDS	**:**	Sodium dodecyl sulphate
SEM	**:**	Standard errors of the means
SOD	**:**	Superoxide dismutase
TEMED	**:**	Tetramethylethylenediamine
TF	**:**	Transcription factor

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ABSTRACT

Maize (*Zea mays*) is an important cereal crop positioned third world-wide after wheat and rice due to its multiple uses such as in human diets. However, it is highly sensitive to salt stress and widely cultivated under a range of soil types and climatic conditions. Salt stress is a major abiotic stress that severely affects the morphological, biochemical, physiological, and molecular processes of plants. Thus, dissecting the molecular pathways utilized by plants when exposed to salt stress is significant for ensuring food security in the world's narrowing agricultural land and increasing population.

To evaluate the effects of salt stress in plants, this study examined the morphological, physiological, biochemical and molecular responses of maize exposed to varying concentrations of NaCl (0 - 400 mM) for 14 days. Salt stress inhibited the general morphology of maize plants, which resulted in reduced plant height, wilted leaves, and reduction in leaf number. Various physiological parameters such as shoot fresh and dry weights, root and shoot lengths, relative water content and chlorophyll content were assessed. The results of the study indicated that salt stress negatively impacted the growth and development of maize. A reduction in the shoot fresh and dry weights, root and shoot lengths, relative water content, and chlorophyll content was observed in plants for all stressed treatments. A slight increase in the relative water content was noted at 300 mM salt stress treatment. Moreover, biochemical parameters such as the antioxidant enzyme activities (catalase, glutathione reductase and superoxide dismutase) were also evaluated, and the results revealed that salt stress inhibited the catalase and superoxide dismutase activities of maize compared to the control. Notably, glutathione reductase activity significantly increased under salt stress. Protein separation demonstrated similar banding patterns for both the control and salt-treated maize with an overexpression at 300 and 400 mM NaCl treatment suggesting upregulation/downregulation of various proteins. Liquid chromatography tandem mass-spectrometry proteomic analysis was used to identify the changes in salt-stressed responsive leaf proteins in maize, where high salt concentration treatments, decreased the number of differentially expressed proteins (DEPs) compared to the control. Gene ontology was used to determine the biological process, molecular function and cellular component of the differentially expressed

proteins. Majority of the differentially expressed leaf proteins were involved in metabolic processes, followed by those with stimulus response. Various molecular functions of the salt responsive proteins were involved in catalytic, transport and binding activities. For the cellular component category, proteins were only associated with cellular anatomy. The common proteins identified for all treatments, were mainly involved in 38.7% metabolic process; followed by 38.7% involved in cellular process; 16.1% localization and 6.5% response to stimulus. Molecular functions of the commonly identified proteins were classified into three categories: whereby 57.9% of the proteins were involved in catalytic activity, while 31.6% were involved in transporter activity and 10.5% in binding activity. In combination, the physiological, biochemical and proteome profiling findings have proposed for possible response pathways underlying salt stress tolerance in maize. Additionally, this study has demonstrated the negative effects of salt stress on maize while on the other hand it has also shown some tolerance/adaptation abilities at the physiological, biochemical and proteomic levels. The data obtained here can provide insight in understanding the molecular basis of salt tolerance differences in maize as an essential agricultural crop and other related cereals.

Keywords: Antioxidants, biochemical pathways, protein identification, salt stress, *Zea mays*.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Plants are generally exposed to both biotic and abiotic stresses. Salinity appears to be one of the most significant abiotic stresses that cause significant losses in agricultural production with a huge impact on plant productivity that alters processes such as respiration, photosynthesis, nucleic acid synthesis and protein metabolism (Parihar *et al*., 2015). Salinity occurs in both irrigated and non-irrigated areas however; it affects plant production in many parts of the world particularly in irrigated land. Although the general perception is that salinization only occurs in arid and semi-arid regions, however, no climatic zone is free from this problem (Rengasamy, 2006). Thus, more than 800 million hectares of land worldwide is affected by either salinity (397 million hectares) or sodicity (434 million hectares) (FAO, 2005; Munns, 2005). Salinity and drought stress share numerous characteristics; the most notable being reduction in soil water potential around the roots in both cases. The expansion of salinity zones around the world occurs as a result of climate change, the overuse of chemical fertilizers and salt leaching in irrigated lands due to anthropogenic activities, which exacerbates the negative impact on plant productivity (Alsaeedi *.,* 2019*).*

Photosynthesis, respiration, starch metabolism and nitrogen fixation are different physiological processes that are affected by salinity, which then leads to crop productivity losses (Iqbal *et al.,* 2020). In addition, salinity affects growth and development of maize; however, the response of plants varies with the degree of stress and crop growth stage (Farooq *et al.,* 2015). Short-term exposure of maize to salt stress influences plant growth owing to osmotic stress in the first phase of salt stress without reaching toxic sodium concentrations (Fortmeier and Schubert, 1995).

Maize is a cross-pollinated, polymorphic plant in nature that is commonly salt-sensitive. In addition, it is the third most important cereal crop after wheat and rice that grows under a wide spectrum of soil and climatic conditions (Iqbal *et al.,* 2020). Although maize is a significant C_4 plant from the *Poaceae* family that is relatively vulnerable to salt stress, it has a lot of intraspecific genetic diversity for salt resistance (Hussain *et al.,* 2019). According to the biphasic model of salinity-induced growth reduction, lower growth in cereals notably wheat, is caused by osmotic stress in the first phase and ion toxicity in the second phase (Munns and Sharp, 1993). Fortmeier and Schubert (1995) validated a similar observation for salinity induced growth loss in maize, but ion toxicity and growth reduction can occur at a minor level during the first phase. Maize plants are harmed by saline levels of more than 0.25 M NaCl, which can restrict growth and result in severe wilting (Maqbool *et al.,* 2020). An increase in the accumulation of salts and ions in the upper layers of soil around the roots, causes osmotic stress and ion toxicity (Safdar *et al.,* 2019).

In a saline environment, root tips are the first organs to sense impaired water availability due to the osmotic effect sending a signal to shoots to adjust whole plant metabolism (Schubert, 2009). High abscisic acid levels in salt-tolerant maize help to minimize water loss and may even regulate growth promotion. Leaf growth sensitivity decreases as abscisic acid levels increase under such conditions. Maize plants facing salt stress undergo a variety of adaptive mechanisms at the molecular level to counteract the damaging effects of salinity stress. Accumulation or inhibition of several proteins and the upregulation and downregulation of many gene transcripts are important (Zorb *et al.,* 2004). Expression of antioxidant defence genes is triggered in maize to protect cells from salinity –induced oxidative damage. In photosynthesizing shoots of maize, catalase activity increased due to the induction of mRNA accumulation in response to higher reactive oxygen species levels under salt stress. In thepresent study, different NaCl concentrations were used to investigate the physiological, biochemical and molecular mechanisms underlying maize salinity tolerance.

1.1.1 Problem Statement

A major challenge faced by the world towards agriculture involves the need for production of about 70% more food that needs to be produced by 2050 to cater for an estimated additional 2.3 billion people (Misselhorn *et al.,* 2012). However, salinity has demonstrated to be a major constraint that causes an increase in food crop demand due to inhibition of various plant development processes. Soil salinity is prevalent in the arid and semi-arid regions worldwide (Sharma and Singh, 2015). The phenomenon reduces growth and development of various crops in the agricultural sector worldwide. This occurs due to the increasing use of poor-quality water for irrigation. Plant responses to salt stress involve various morphological, physiological, biochemical pathways and molecular or gene networks (Gupta and Huang, 2014). Maize being the world's number one staple food that is highly sensitive to salt stress, its effects are demonstrated by the limitation of growth and production rate which result in food insecurity to the increasing world population. Hence, investigations of the physiological, biochemical and molecular mechanisms underlying maize salinity tolerance are necessary to provide information on the development of salt-tolerant crop varieties in salt-affected areas. Information obtained from such studies including the present study could potentially contribute to the development of salt tolerant maize varieties and ultimately, provide applicable management strategies that would be beneficial to saline prone arid and semi-arid areas.

1.1.2 Research Aim

This study aimed to characterize salt stress responsive proteins physiologically, biochemically and molecularly from *Zea mays* var. WE 6206B.

1.1.3 Research Objectives

1. To generate and expose maize plant lines to different salt stress conditions.
2. To evaluate the growth and physiological responses of the maize plants to salt stress by determining the shoot fresh weight, shoot and root lengths, relative water content (RWC) and chlorophyll content.

3. To assess the biochemical effect of salt stress on *Zea mays* exposed to varying NaCl treatment levels.
4. To assess the expressional profile of the maize plants in response to induced salt stress.
5. To identify osmotic stress responsive proteins in maize leaves using LC-MS-MS.
6. To functionally categorize the identified maize proteins and annotate them to function bioinformatically.

1.1.4 Significance of the Research Study

After being successfully undertaken, the study was envisaged to provide the following benefits:

1. Contributing knowledge on the physiological and biochemical responses of maize- and other related crops to salinity.
2. A successful profiling of the proteins expressed under salt stress would contribute further knowledge on how to improve plant or crop tolerance and resistance to osmotic stress.
3. Assisting in providing the groundwork for further identification of osmotic stressed proteins in maize that would lead to a better understanding of their roles in stress tolerance mechanisms.

1.2 Literature Review

1.2.1 Overview of Salinity Stress in Plants

Salt stress is an accumulation of excessive salt in soil that leads to plant growth inhibition and death. It is a major stress that limits the increase in food crop demand, with more than 20% of the cultivated land worldwide (~45 hectares), affected by salt stress (Etesami and Noori, 2019). Plants can be divided into two groups based on their ability to withstand salinity namely, halophytes (which can tolerate salt stress) and glycophytes (which cannot tolerate salt stress and eventually die). The vast majority of significant crops belong to the second group; thus, salinity is regarded as one of the harshiest environmental stresses that hamper crop productivity worldwide (FAO, 2009; Flowers, 2004). Depending on the severity and periodic exposure of the stress, salinity stress induces alterations in different physiological and metabolic processes, which ultimately inhibit crop production (Sharma *et al.,* 2019).

Soil salinity inhibits plant growth in form of osmotic stress, which is then followed by ion toxicity. During the initial phase of salinity stress, water absorption capacity in the root system decreases and water loss from leaves is accelerated due to the high salt accumulation in soil and plants, and therefore, salinity is considered as a hyperosmotic stress (Rahnama *et al*., 2010; James *et al*., 2011). Osmotic stress, at the initial stage of salinity stress exposure causes various physiological changes such as interruption of membranes, nutrient imbalance, impairment of the ability to detoxify reactive oxygen species (ROS), differences in the antioxidant enzymes, decreased photosynthetic activity and stomatal aperture (Rahnama *et al*., 2010). The detrimental effects of salinity stress result in the accumulation of Na^+ and Cl^- ions in plant tissues exposed to high NaCl concentrations in soil. Entry of both Na^+ and Cl^- into the cells causes severe ion imbalance and an excess uptake might cause physiological disorders. High Na^+ concentrations inhibit the uptake of K^+ ions, which is an essential element for growth and development, inducing low productivity and consequently crop death (Carillo., 2018). Furthermore, salinity degrades soil fertility, which is a detrimental component for cultivation of most agricultural crops such as maize.

1.2.2 Utilization of Maize and Salinity Stress

Maize is one of the most important crops in the world today. This crop is both food and feed and provides essential raw materials for pharmaceuticals and other industrial products. Environmental stresses such as heat, cold, drought and high salt concentrations affect the growth and yield of maize. Furthermore, it is widely grown in a range of agro-ecological environments and its growth is greatly reduced by salinity. The rapid increase in the world's population requires an expansion of cropping areas to improve food production. While there are numerous cereal crops used for food only such as wheat, maize and rice are amongst the most important human food sources, accounting for 94% of cereal consumption (Ranum *et al.,* 2014). Consumption of these cereals varies widely by region, wheat being the most preferred cereal in Central Asia, Middle East, South and North America and Europe. Rice is a major cereal in Asia, while maize is preferred in Southern and Eastern Africa, Central America and Mexico (Ranum *et al.,* 2014). The way in which maize is processed and consumed varies greatly from country to country, while maize flour and maize meal are two of the most popular products. Maize provides essential raw materials for food, forage, pharmaceuticals and other industrial products. Furthermore, it is widely grown in a range of agro-ecological environments and its growth is greatly reduced by salinity.

The production of maize differs and fluctuates greatly depending on the region of cultivation and the season. Maize worldwide is estimated to be 197 million ha, producing 1.14 million tons of maize per year due to environmental stresses such as heat, cold, drought and high salt concentrations that often affect the growth and yield of maize worldwide (Suzuki *et al.,* 2014). Salinization is spreading rapidly in irrigated lands due to the inappropriate management of irrigation and drainage systems. The ability of seeds to germinate under high salt concentrations in soil is of crucial importance for the survival of many plant species. Although salinity delays the onset of germination and reduces germination percentage, its effects are modified by interactions with other environmental factors such as temperature and light (Bojovic *et al*., 2010). The high absorption of Na^+ and Cl^- ions during germination can result in cell toxicity, which then inhibits or slows down the rate of germination and thus decreasing the germination percentage (Sozharajan and Natarajan, 2014). In saline habitats, satisfactory seed germination takes place after high

precipitation (Gul *et al*., 2013) when the soil saline concentration has been reduced (Khan and Rizvi, 1994). Therefore, the seedlings from the primed seeds grow more vigorously and perform better in adverse conditions (Cramer *et al.,* 2002).

1.2.3 The Effects of Salinity on the Morphological Traits of Various Crops

Salt stress is one of the most detrimental abiotic stress factors that affect the growth, productivity, and physiology of plants. In addition, salinity imposes negative effects on plant growth by decreasing leaf water potential, inducing morphological and physiological changes, causing the production of reactive oxygen species (ROS), increasing osmotic stress and ion toxicity, and altering biochemical processes (Khan *et al*., 2014). Salt stress increases the concentration of Na^{+}, PO_4^{-3}, K^{+}, Mg^{+2} and Ca^{+2} ions in the leaves (Khalid and Cai, 2011). Sodium is responsible for the dispersion of secondary clay minerals in the soil (Paul and Lade, 2014). Chloride is utilized by plants as a micronutrient and mineral nutrient by many halophytes, but excessive accumulation of these ions results in low biodiversity and reduced growth, and the emergence of salt-sensitive plants (Parida and Das, 2005).

The two main phases of salt stress-induced responses of plants are shoot ion independence and shoot ion dependence responses (Mann *et al.,* 2019). The first phase occurs within minutes or few days, caused by sodium ions, which leads to the closure of stomata and inhibition of leaf expansion (Safdar *et al.,* 2019). During this phase, plant water relations are affected by salinity (Choi *et al*., 2014; Roy *et al*., 2014). In the second phase, the accumulation of toxic ions takes place particularly in older leaves, causing premature senescence, reduced yield, and plant death (Munns and Tester, 2008). The uptake of water, cell turgor and other physiological processes such as cell expansion, photosynthesis and stomatal opening are well controlled by osmotic adjustment in both leaves and roots of plants in response to salt (Serraj *et al*., 2002). In general, plants exhibit various resistance pathways when exposed to salinity by inducing the production

of secondary metabolites and avoiding the toxic effects caused by ions (Chen *et al*., 2009).

The effects of salt stress on plant morphology are manifested by dry or fresh total biomass, plant height and through other morphological markers. The germination rate of seeds is also affected by salinity because germination is also an informative marker of salt stress (Soltabayeva *et al.,* 2021). Since germination is among the foremost morphological processes, it is a useful indicator of stress as compared to biomass as stress can be noted as early as 2–14 days depending on a plant species (Soltabayeva *et al*., 2021). Figure 1.1 indicates the germination percentage of different crops under salt stress. Mostly salt stress among various crops is indicated by an inhibition of total germination at 400 mM NaCl, although below this concentration, some crops such as wheat can have a 3% germination rate with retarded growth (Hasanuzzaman *et al.,* 2013). Various plant growth markers are sensitive to salt stress at 100 – 400 mM NaCl concentrations (Deivanai *et al*., 2011).

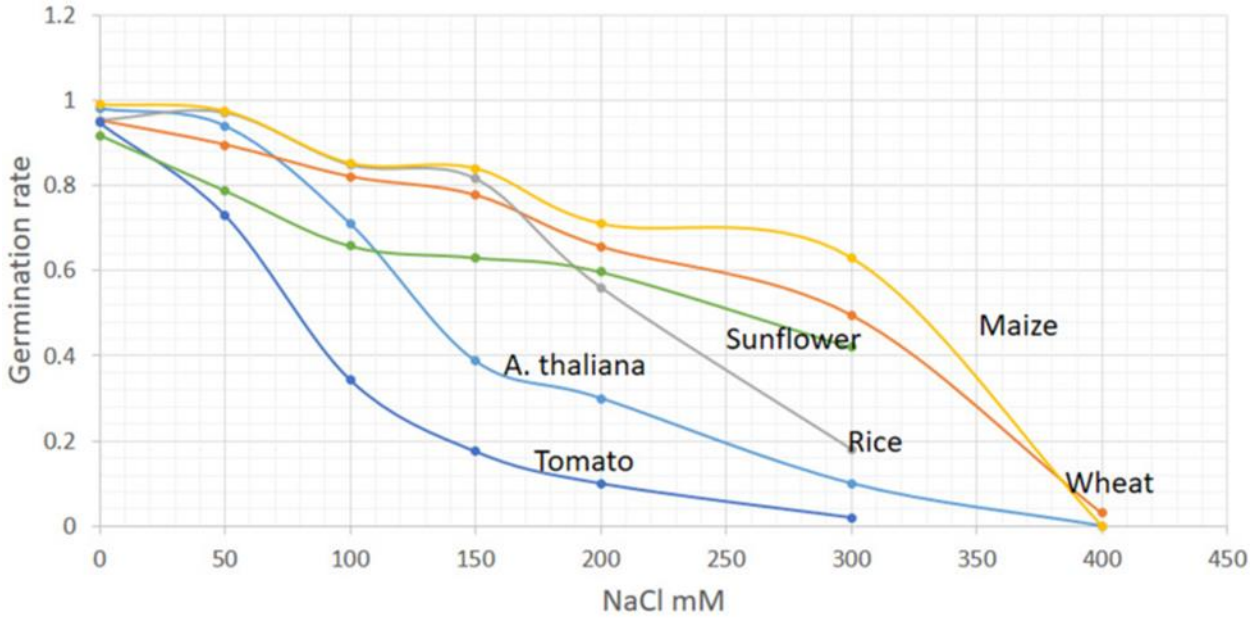

Figure 1.1: Changes in the germination rate of crop plants at different NaCl levels. Germination rate of plants grown under different NaCl concentrations after 7 days of treatment. Figure adapted from Soltabayeva *et al*. (2021).

1.2.4 Effects of Salinity on the Physiological Traits of *Zea mays*

Salt stress hinders the physiological characteristics and plant growth of maize. In addition, it causes the osmotic effect, associated with an increase in phytotoxic ions, oxidative stress by increased ROS production and ionic effect in the cytosol (Iqbal *et al*., 2020). Thus, the osmotic parameters of plants change rapidly in response to salt stress. This is usually expressed by evaluating the changes in turgor pressure, osmotic pressure, relatve water content (RWC) and water potential (Boyer *et al*., 2008). Toxic levels of sodium in various plant organs damage biological membranes and subcellular organelles, consequently reducing growth and causing abnormal development before plant mortality (Iqbal *et al*., 2020). Several physiological processes such as photosynthesis, respiration, starch metabolism and nitrogen fixation are also affected under saline conditions, leading to losses in productivity (Salah *et al.,* 2009). Seed germination is the most critical stage in a seedling establishment which determines the success of crop production. Generally, salt stress during germination delays the start, reduces the rate and enhances the dispersion of germination events (Farooq *et al*., 2015). Germination and early seedling growth are more sensitive to salinity than later developmental stages, hence seed germination is an important event during plant development. Seed germination is genetically programmed via the regulation, production and destruction of gibberellic acid (GA) and abscisic acid (ABA) (Bailly *et al*., 2008), and is strongly affected by environmental factors. Salt stress influences seed germination primarily by sufficiently lowering down the osmotic potential of the soil solution to retard water absorption by the seeds, causing sodium and/or chloride toxicity to the embryo or by altering protein synthesis (Roy *et al*., 2018).

Although the root is the first organ exposed to salt stress, shoots are more sensitive to salt stress than roots. Salinity promotes the suberization of the hypodermis and endodermis, and again, it initiates the casparian strip development closer to the root tip unlike in non-saline roots (Degenhardt and Gimmler, 2000). Furthermore, it reduces shoot growth by suppressing leaf initiation and expansion, as well as internode growth and accelerating leaf abscission (Sabagh *et al*., 2021). The leaf growth rate is rapidly reduced by salt stress due to a reduction in the number of elongating cells and/or the rate

of cell elongation (Farooq *et al*., 2015). As a salt-sensitive crop, shoot growth in maize is strongly inhibited in the first phase of salt stress (Uddin *et al.,* 2013).

The build-up of Na^+ and Cl^- ions in tissues of plants exposed to soils with high NaCl concentrations is one of the negative impacts of salinity stress. Entry of both Na^+ and Cl^- into the cells causes severe ion imbalance and excess uptake, which may cause physiological disorders such as wilting, dull leaves and grey leaf tips even when there is adequate soil moisture (Nadeem *et al.,* 2019). A high Na^+ concentration inhibits the uptake of K^+ ions, which is an essential element for growth and development, while its insufficiency results in lower productivity and may even lead to crop death (Nawaz *et al.,* 2010).

1.2.5 Effects of Salinity on the Biochemical Traits of *Zea mays*

Salt stress inhibits plant growth in multiple ways that result in osmotic stress, such as ion toxicity, nutritional and hormonal imbalance, and oxidative stress in maize (Basile *et al*., 2011). Higher concentrations of salt decrease the water potential, thereby restricting water and nutrient uptake by roots. Furthermore, it causes nutrient imbalance due to the competition of Na^+ and Cl^- with essential nutrients such as K^+, Ca^{2+} and NO^{3-} which leads to extreme ratios of Na^+/Ca^+ and Na^+/K^+ (Mousa *et.al*., 2014). Elevated levels of these ratios in plants under saline conditions, suppress plant growth in various ways, for example, adequate amount of K^+ is required for the activity of different cellular enzymes (Nounjan *et al*., 2012). Toxic levels of Na^+ in the cytosol induce many changes in the biochemical reactions (Tuteja *et al*., 2012). Higher endogenous levels of Na^+ induce oxidative stress in terms of production of the ROS such as superoxide radicals, singlet oxygen, hydrogen peroxides and hydroxyl radicals (Sharma *et al*., 2012). Reactive oxygen species are strong oxidizing agents that cause cell death by lipid peroxidation, oxidation of proteins and the induction of substantial damage to DNA (Gill and Tuteja, 2010).

In order to contain the inhibitory effects of ROS, plants activate their antioxidant system that is involved in the detoxification of ROS (Sytar *et al.,* 2013). This system comprises of the enzymatic and non-enzymatic antioxidants. Various enzymatic antioxidants such

as superoxide dismutase (SOD) converts superoxide to H_2O_2 in different sub-cellular compartments (Evelin *et al.,* 2019). Hydrogen peroxide is further scavenged by catalase (CAT), ascorbate peroxidase (APX) and other non-enzymatic antioxidants including tocopherols, carotenoids, ascorbate among others (Weisany *et al*., 2012). Malondialdehyde (MDA), a by-product of lipid peroxidation, is a good measure of cell membrane integrity. Moreover, MDA levels are higher in salt-sensitive plants than those of salt-tolerant plants (Arora *et al*., 2008).

1.2.6 Effects of Salinity Stress on Maize at a Proteomic Level

Analysis of the plant proteome is an important adjustment to the genome analysis approach because gene expression is altered under salinity stress (Ahmad *et al.,* 2016). In contrast to genome analysis, the proteome is not static but rather dependent on several responses influenced by both the internal and external factors (Zörb *et al*., 2004). Proteins that are identified in multiple studies are those involved in protein synthesis, processing, turnover, and degradation, as well as cytoskeleton stability (Basisty *et al*., 2018). For instance, in photosynthetic processes, a general decrease in the levels of chlorophyll biosynthesis related to proteins was noted, while an increase in proteins involved in the light-dependent reactions were observed (Reinbothe and Reinbothe, 1996). Plant lamina or root membrane proteomics, including those of plasma membrane, mitochondrial and thylakoid membrane, have revealed the up/down-regulation of a plethora of proteins. These include receptor proteins that perceive stress, membrane bound signaling and regulatory proteins that function in relaying the stress, vesicle trafficking, and transport proteins that are involved in maintaining ion and water homeostasis and drive the sequestration and/or removal of toxic compounds from the cell, membrane bound kinases and intrinsic proteins (Barkla *et al*., 2013).

The molecular, physiological, and morphological changes in plants follow an order, where the first changes by stress will be visualized via molecular, followed by biochemical and physiological and lastly, by morphological markers (Figure 1.2) by Soltabayeva *et al*., 2021. ROS molecules play an important role in signaling for stress and thus changes in these oxidative stress markers are detectable simultaneously with molecular changes

after an exposure to salt stress (Noctor *et al.,* 2015). Each stress marker has an order in terms of time observation after stress, where the oxidative and molecular stress markers are early sensors for stress compared to other markers (Soltabayeva *et al*., 2021). Figure 1.2 below demonstrates how some morphological, physiological, and molecular changes of plants under salt stress are highly detectable in older (mature) leaves as compared to other organs or whole plant (Soltabayeva *et al*., 2021). The measurement of these stress markers on a specific area of plants would increase efficiency of deep learning approaches on phenotyping a salt stressed plant. The environmental impact in plants is assessed through machine learning approaches, which mostly focus on the identification of visual symptoms caused by abiotic damage. These visual symptoms are described as morphological stress markers and appear in plants later than physiological, oxidative, and molecular stress markers (Soltabayeva *et al*., 2021).

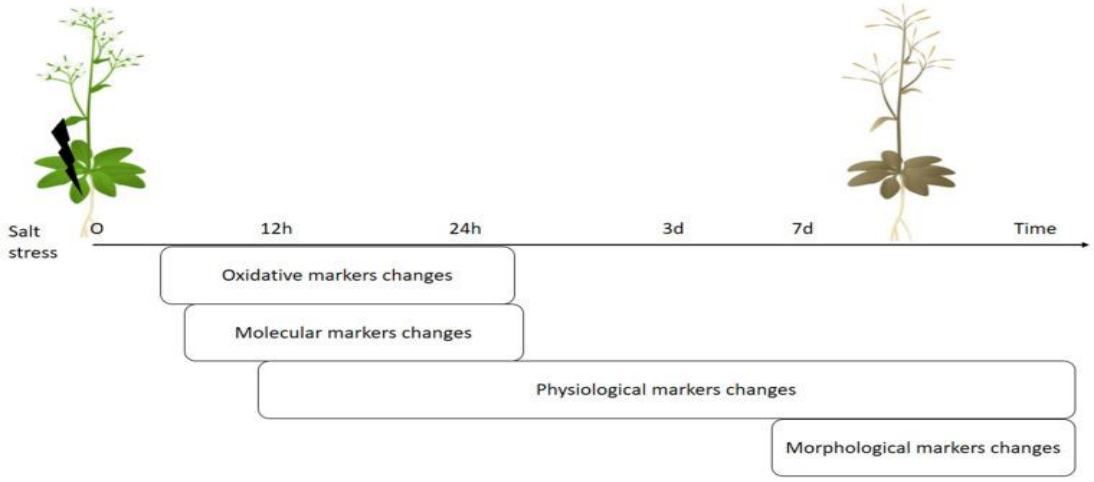

Figure 1.2: A sequential changes of plant responses to different levels of salt stress (Soltabayeva *et al*., 2021).

1.2.7 Plant Resistance Mechanisms to Salt Stress

Higher plants, including maize, have developed sophisticated genetic and molecular regulatory networks to respond and adapt to varying environmental stresses. Numerous studies have been conducted to better understand the stress tolerance mechanisms at physiological and biochemical levels. Multiple genes involved in regulating abiotic stress

responses have been identified in a variety of plants, including those that encode protein kinases, protein phosphatases and transcription factor (TF) families, as well as functional downstream genes that encode ionic and osmotic balance maintenance (Abe *et al*., 2003, Yamaguchi- Shinozaki and Shinozaki 2006, Huang *et al.,* 2012, Zhu 2016, Samad *et al.,* 2017, Viana *et al*., 2018). Some of the identified genes have been used as genetic or molecular markers to genetically engineer crops with improved stress tolerance without sacrificing the yield and quality. Among the various components of signaling pathways, TFs play pivotal roles in stress tolerance regulation because a single TF can coordinate the expression of many genes to improve stress tolerance (AbdElgawad *et al.,* 2016).

Anthocyanin synthesis is one of the subsequent production and localization of anthocyanin in root, stem and especially leaf tissues that may allow the plant to develop resistance to a number of environmental stresses (Chalker-Scott, 1999; Winkle-Shirley, 2002; Steyn *et al*., 2002.) Anthocyanins are purple flavonoid pigments that are synthesized in many vegetative plant organs including the leaves, stems, anthers, glumes of the cob tassel, coleoptiles and the aleurone layer of maize (Singh *et al*., 2019). Some other mechanisms that enable plants to survive under high salinity have been identified and these include compartmentalization, osmolytes accumulation, osmotic adaptation, selective transport and uptake of ions, ion homeostasis and leaf salt excretion (Flowers and Colmer, 2008). For instance, many plants under salt stress accumulate soluble osmolytes such as proline (Szabados and Savoure, 2010) and soluble sugars (Feng *et al*., 2002). Proline plays an important role as an osmolyte and contributes to the scavenging of ROS, stabilizing membrane and proteins, buffering cellular redox potential and inducing the expression of salt-stressed responsive genes (Carillo, 2018).

Plant resistance to salt stress is a complex phenomenon that involves biochemical and physiological processes as well as morphological development at different growth stages. Since gene expression is altered under salt stress, a combination of biochemical and proteomic analysis of plants is a crucial addition to genome analysis (Jiang *et al*., 2020). In contrast to genome analysis, proteome analysis approach is dynamic, relying on a variety of reactions impacted by both the internal and external influences. The plant's

response to environmental stress, such as soil salinity, is likely to exert a significant impact on proteins and their ultimate functions.

1.2.8 Recent Advances in Plant Proteomics Analysis

Proteomics refers to the study or complete analysis of the entire complements of proteins available in a cell, including the identification, quantification, interactions, and functions of proteins in a living cell (Ngara and Ndimba, 2014). Proteomics is widely used to unravel the biological processes and obtain detailed information about the mechanisms by which plants respond to various stress conditions. Two-dimensional polyacrylamide gel electrophoresis (2-DE) established by O'Farrel, coupled with mass spectrometry (MS) is a cost-effective and widely used proteomic technique (Robinson *et al.,* 2011). However, relatively expensive, alternative gel-free proteomic approaches such as isotopic labeling (iTRAQ and TMT) and data independent acquisition (DIA/SWATH) are rapidly emerging (Hu *et al*., 2015). Recent advances in proteomics have made it possible to perform large-scale studies to elucidate salt-tolerance mechanisms in roots. Plant adaptation to environmental stresses such as salinity exert a strong influence on proteins. Literature suggests that different common stress responsive proteins are expressed in response to various abiotic stresses in different plant species (Zandalinas *et al*., 2018). About 2171 proteins from 34 plant species have been identified and characterized as salt-responsive proteins, which are either up-regulated or down-regulated by salt stress (Gupta *et al*., 2014). Zorb et al. (2010) studied proteomic changes in maize roots after a short-term adjustment to saline growth conditions and detected a set of phosphoproteins such as fructokinase, UDP-glucosyl transferase BX9, 2-cys-peroxyredoxi ne and 40-S-ribosomal protein in maize. The expression of these proteins was different after exposure to saline conditions.

CHAPTER TWO

RESEARCH METHODOLOGIES

2.1 Generation and Maintenance of *Zea mays*

2.1.1 Seed Sterilization and Viability Testing

Zea mays var. WE 6206B used in this study was procured from Quality Seed (Dalton, KwaZulu-Natal, RSA). This variety is a white GMO noted to be drought tolerant (Quality Seed). The seeds were carefully inspected and selected based on size homogeneity and pest free damage. A total of 75 healthy seeds were selected, and about 5 per plant pot were collected in a 50 ml sterile falcon tube, where 2 ml of 70% (v/v) ethanol was added and vortexed for 1 minute to sterilize seed surfaces. The seeds were left to settle through gravity and the ethanol was discarded. The seeds were then submerged in 2 ml of 1.25% sodium hypochlorite solution (commercial bleach) and vortexed for 10 minutes. Immediately after surface decontamination, bleach was removed followed by repeated rinses of the seeds (3 times) with 3 ml of sterile distilled water to remove ethanol and bleach traces. After rinsing, sterilized seeds (5 per plant pot) were set for germination following a standard germination method with modifications by sowing them in petri dishes with sterile wet vermiculite and incubated in a growth chamber set at 25°C, 8/16 hours night/day at 10 000 light lux for 5 days (Mangena and Mokwala, 2019).

2.1.2 Plant re-generation, Growth and Treatment Conditions

On the 6th day after seed incubation in the growth chamber, sprouted seeds were manually transplanted (5 seeds per pot) into each of the prepared 15 plastic pots (16 cm diameter) with sterile potting soil composed of 3 parts peat-based soil (Culterra potting mix, Builders, South Africa) and 2 parts vermiculite (serial# SMC-9001, Rajasthan, India). The sown seeds were watered with 200 ml of sterilized tap water every second day and left to fully germinate, and seedlings allowed to grow for two weeks under greenhouse conditions at 25°C, 8/16 hours night/day at 10 000 light lux. After 14 days of maintaining

maize seedlings, the seedlings were divided into five independent batches (3 plant pots per treatment). Plant treatment was then carried out for 14 days, where the control seedlings were watered every second day with 200 ml of sterilized water and the experiment plantlets were similarly irrigated with 200 ml of either 100 mM, 200 mM, 300 mM, or 400 mM of NaCl solutions under the same growth conditions. Both the control and experimental treatments were performed in four replicates. Immediately after the treatment period (14 days), plant tissues (leaf, root, and shoot) were harvested to measure the physiological, biochemical, and molecular parameters. The harvested leaf tissues were ground in liquid nitrogen and stored at -80°C until further use, except for those that were used for physiological measurements.

2.3 Assessment of Maize Physiological Parameters

2.3.1 Measurement of Plant Growth Traits

The harvested material from each treatment was weighed for shoot fresh and dry weights using a weighing balance (Radwag, Model# PS 750/C/2, Radom, Poland). The root and shoot lengths of the seedlings from each experimental unit were measured using a ruler in centimeters (cm). All plant growth parameters were measured in three independent biological replicates.

2.3.2 Measurement of Chlorophyll Content

Chlorophyll content was measured in four replicates for each plant leaf from all the experimental settings. One gram of a freshly cut leaf was macerated in 90% acetone using a mortar and pestle. Absorbances were measured with a Helios spectrophotometer Epsilon (Thermo Electron Corporation, USA), and chlorophyll concentrations were measured at 663 and 645 nm wavelengths using the method described by Faramarzi and Faramarzi (2018), and determined following these calculations:

Chlorophyll a (mg/ml) = $12.7\ A_{663} - 2.69\ A_{645}$

Chlorophyll b (mg/ml) = 22.9 A_{645} – 4.68 A_{663}

Total Chlorophyll (mg/ml) = Chlorophyll a + Chlorophyll b

Where:

A_{645} = absorbance at a wavelength of 645 nm

A_{663} = absorbance at a wavelength of 663 nm.

2.3.3 Measurement of the Relative Water Content

Relative water content (RWC) was measured in four replicates using fully expanded leaves from the different maize plant groups. It was estimated as described by Qui *et al.* (2016) using the equation:

RWC = [(fresh weight – dry weight)] / [(turgid weight – dry weight)] × 100

Turgid weight was determined by weighing the leaf segments after 24 hours of immersion in distilled water in a sealed flask at room temperature. Furthermore, dry weight was determined by weighing the leaf segments after 48 hours of incubation at 70°C in an oven (Optolabor (Pty) Ltd, serial # SE 8482, Bromhof, South Africa).

2.4 Assessment of the Biochemical Parameters

2.4.1 Preparation of the Crude Enzyme Extract

About 0.5 g portion of the powdered harvested leaf tissue in four biological replicates was homogenized in 1.5 ml of an ice-cold extraction buffer containing 1% of polyvinyl-pyrrolidone (PVP) and 0.1% of ethylenediamine-tetra acetic acid (EDTA) and 100 µl of 0.1 M potassium phosphate (pH 7.4) for 5 minutes. The homogenized sample was transferred into an Eppendorf tube. The homogenized sample was then centrifuged at a maximum speed of 14 800 × *g* for 30 minutes and the supernatant collected into fresh tubes. Protein concentration for each sample was measured using a 2000 Nanodrop

spectrophotometer (Themo Scientific Inc., California, USA) and the supernatant was stored at -20°C for further biochemical tests.

2.4.2 Determination of the Antioxidant Activities

Catalase Activity

Catalase (CAT) activity was determined by measuring the absorption of hydrogen peroxide. The activity was determined by adding 50 µl of the enzyme extract to a solution that contained 2 ml of 20 mM sodium phosphate buffer pH 7.5 and 1 ml of 20 mM hydrogen peroxide (H_2O_2). This was followed by measuring the decrease in H_2O_2 (ε = 39.4 mM^{-1} cm^{-1}) absorbance at 240 nm at an interval of 30 seconds for 3 minutes per sample, using a spectrophotometer (Thermo Fisher Scientific., catalog # 9A1Y297118, Carlifornia, USA) at 25°C. The catalase activity was expressed in units, where one unit of the enzyme converts 1 µmole of H_2O_2 per minute (Azimian and Roshandel, 2016).

Superoxide Dismutase Activity

Superoxide diamutase activity was determined by adding 50 µl of the enzyme extract to a solution that contained 3 ml of [0.1 mM EDTA, 10 mM L-methionine, 0.1 mM *p*-nitroblue tetrazolium chloride (NBT), 0.005 mM riboflavin and 20 mM sodium phosphate buffer pH 7.5] solution. The reaction was initiated by placing the mixture under fluorescent light conditions for 5 minutes, followed by termination through switching off the light. The blue formazane (blue colour formed) produced was measured in an Accu-Reader (model # 96501555, Taiwan, China) as an increase in absorbance at 560 nm at an interval of 30 seconds for 3 minutes. In contrast, the control reaction mixture with no enzyme extract (the blank solution) but the same reaction mixture, was kept in the dark. An SOD unit activity was defined as the amount of enzyme that inhibits the nitroblue tetrazolium photoreduction by 50%, as compared to the reaction without the enzyme extract, and the SOD activity values were expressed in units per mg of protein (Kaur *et al.,* 2015).

Glutathione Reductase Activity

Glutathione reductase was determined by preparing a reaction mixture of 100 µl of 100 mM potassium phosphate pH 7.8 containing 2 ml of 2 mM EDTA, 2 ml of 0.5 mM GSSG and 50 µl of enzyme extract, followed by subsequent addition of 2 ml of 0.2 mM NADPH to initiate the reaction. A decrease in absorbance was measured at 340 nm, 30 seconds interval for 3 minutes. The GR activity was expressed in units, where a unit of GR activity was expressed as units (µmol of NADPH oxidized per minute) per mg of protein (Dontha, 2016).

2.5 Total Protein Extraction from Leaf Tissues

The powdered maize leaf tissues from section 2.1.2 were used for total protein extraction following the NucleoSpin® TriPrep kit (Catalog# 740966, Macherey-Nagel, Düren, Germany) protocol. Protein extraction was conducted in four biological replicates of the control (non- treated) and experiments (100 – 400 mM NaCl treated). In brief, about 1 g of the powdered maize leaf tissues was homogenized in 350 µl lysis buffer (RP1) and 3.5 µl β-mercaptoethanol then vortexed vigorously for 20 seconds. The homogenate mixture was the briefly centrifuged for 1 minute at 11,000 *x g* to separate the leaf debris from supernatant through a nucleospin filter. After centrifugation, the collected supernatant was transferred to a sterile 2 ml centrifuge tube and 350 µl of 70% ethanol was added and vortexed for 10 seconds. The obtained lysate was loaded into a nucleospin column placed in a collection tube and centrifuged at 11,000 *x g* for 30 seconds. About 700 µl of the flow-through was collected into a fresh tube then one volume of protein precipitator (PP) was added to it and mixed vigorously. The precipitated mixture was then centrifuged for 5 minutes at 11,000 *x g.* The resultant supernatant was removed by pipetting, then 500 µl of 50% ethanol was added and centrifuged at 11,000 *x g* for 1 minute. Subsequently, the supernatant was discarded while the pellet was air-dried for 15 minutes at room temperature. Two sets of the dried pellets were stored in separate 2 ml centrifuge tubes (one set for 1SDS-PAGE and the other set for liquid chromatography mass spectrometry analysis) at -20°C.

2.5.1 Determination of Protein Concentrations

Total soluble protein concentrations were subsequently determined from all sets of pellets (section 2.5) for each treatment condition using a 2000 Nanodrop spectrophotometer (Thermo Scientific Inc., California, USA). One set of known protein concentration per sample (about 40 µl) was kept aside for SDS-PAGE analysis, to evaluate and determine its purity and quality.

2.6 SDS-PAGE Analysis

Total extracted protein samples were analyzed on a discontinuous buffer system made up of a 12% running (resolving) and 4% stacking gel. The compositions of resolving and stacking gel are described below in Table 2.1.

Table 2.1: Resolving and stacking gel components in preparation for the sodium dodecyl sulphate polyacrylamide gel electrophoresis of proteins from the salt stressed maize plants.

Resolving gel components	Stacking gel components
4.0 ml 30% acrylamide (Merck)	0.8 ml 30% acrylamide (Merck)
1.25 ml 0.8% sodium dodecyl sulphate (SDS) (Merck)	0.625 ml 0.8% sodium dodecyl sulphate (SDS) (Merck)
1.25 ml 3 M Tris-HCI buffer (pH 8.8) (Merck)	0.625 ml 1 M Tris-HCI buffer (pH 6.8) (Merck)
3.43 ml sterile distilled water	2.905 ml sterile distilled water
50 µl 10% ammonium per-sulphate (APS) (Sigma)	25 µl 10% ammonium per-sulphate (APS) (Sigma)
20 µl 100% N, N, N', N'-tetramethylethylenediamine (TEMED) (Sigma)	20 µl 100% N, N, N', N'-tetramethylethylenediamine (TEMED) (Sigma)

2.6.1 Sample Preparation and SDS-PAGE Analysis

The previously stored pellets, containing total soluble leaf proteins (section 2.5.1), were thawed on ice to then evaluate both the quality, and loading quantities of proteins in their extracts. About 10 µg of the leaf protein extracts were re-suspended in 20 µl of protein solving buffer, containing TCEP (PSB-TCEP) (Macherey-Nagel, Düren, Germany) and boiled at 95°C on a digital dry bath (model #1660562, Bio-Rad Laboratories., California, USA) for 5 minutes then pulsed for 10 seconds on a microcentrifuge. Five microliters of the unstained protein marker (Catalog# P7704S New England Biolabs Inc., Massachusetts, USA) and 10 µl of each sample were then loaded into a gel and electrophoresed at 200 volts for 50 minutes or until the dye front had reached the bottom of the gel. After electrophoresis was completed, the gel was stained with the pierce silver stain kit (catalog# 24612; Thermo Fisher scientific, Rockford IL, USA), following the manufacturer's instructions, whereby a gel was first stained in 0.5 ml enhancer with 25 ml stain solution for 30 minutes. The gel was rinsed twice with ultrapure water for 20 seconds per wash. Subsequently, the gel was developed with the silver stain developer solution (0.5 ml enhancer with 25 ml developer) for 2 – 3 minutes until the protein bands were visible. The developed protein bands were stopped by adding 5% acetic acid to the gel, shaking on an ultra-rocker (Bio-Rad Laboratories., USA) for 10 minutes. Afterward, the produced gel was inspected for visible osmotic stress induced proteins. Images were captured with a Chemi DOC^{TM} Imaging system (Bio-Rad Laboratories Inc., California, USA) using the Bio-Image Lab^{TM} Software.

2.7 Identification of the Salinity Stressed Proteins using Liquid Chromatography Tandem Mass-spectrometry (LC-MS-MS)

A set of the previously stored (section 2.5.1) proteins for all the tested experimental conditions were used to evaluate differential protein expression in maize under salt stress via liquid chromatography tandem mass-spectrometry (LC-MS-MS). A total of 20 maize samples (4 biological replicates) were used for the proteomic analyses.

2.7.1 In-gel digestion of the Salinity Stressed Proteins

The targeted maize leaf extract samples were incubated with the Bolt™ LDS sample buffer and the sample reducing agent before being electrophoresed (stacked) on a Bolt™ 4 to 12%, Bis-Tris, 1.0 mm, mini protein gel. The polyacrylamide gels were stained with the GelCode™ blue stain reagent and de-stained with Milli-Q water. Resolved Proteins were digested from the stacked gel bands according to Shevchenko *et al.* (2007). Briefly, the proteins were reduced in gel with 10 mM dithiothreitol (DTT) in 25 mM ammonium bicarbonate (NH_4HCO_3) for one hour at 60°C. Samples were cooled at room temperature, then 100% acetonitrile (ACN) was added and incubated for 10 minutes. The supernatant was discarded while 55 mM iodoacetamide (IAA) in 25 mM was added to the gel pieces. The reaction was carried out in the dark for 20 minutes at room temperature. After incubation, the supernatant was discarded and gels dehydrated with 25 mM ammonium bicarbonate (NH_4HCO_3) in 50% acetonitrile, followed by vortexing and removal of the supernatant. The gel pieces were dried, and a freshly prepared trypsin was added. Protein digestion was then allowed to proceed overnight at 37°C. The digestion was quenched by adding 0.1% formic acid (FA) and the samples were dried under vacuum. The dried samples were re-suspended in 2% acetonitrile (ACN) and 0.2% formic acid (FA) for mass spectrometry analysis.

2.7.2 Proteomic Analysis of the Proteins using LC-MS-MS

The generated tryptic peptides from each gel fraction were analyzed using a Dionex Ultimate 3000 RSLC system coupled to an AB Sciex 6600 TripleTOF mass spectrometer. The injected peptides were inline de-salted using an Acclaim PepMap C18 trap column (75 µm × 2 cm; 2 minutes at 5 µl·min^{-1} using 2% acetonitrile (ACN)/0.2% formic acid (FA)). Trapped peptides were gradient eluted and separated on a Waters nanoEase CSH C18 column (75 µm × 25 cm, 1.7 µm particle size) at a flow rate of 0.3 µl.min^{-1} with a gradient of 10-55% B versus A over 30 min (solvent A: 0.1% formic acid (FA); solvent B: 80% acetonitrile (ACN)/0.1% formic acid (FA)). The 6600 TripleTOF mass spectrometer was operated in positive ion mode. Data-dependent acquisition (DDA) was employed; precursor (MS) scans were acquired from the *m/z* 400-1500 (2^+-5^+ charge states), using

an accumulation time of 100 ms followed by 40 fragment ion (MS/MS) scans, acquired from the *m/z* 100-1800 with a 20 ms accumulation time each.

2.7.3 Data Collection and Bioinformatics Analysis

A total of 20 LC-MS/MS experiments (4 biological replicates of each treatment conditions (non-salt stressed and salt stressed) in maize were acquired from the Dionex Ultimate 3000 RSLC system coupled to an AB Sciex 6600 TripleTOF mass spectrometer at the Council for Scientific and Industrial Research (CSIR, Pretoria, South Africa). Raw data files (.wiff) were searched with the Protein Pilot V5.0 software (SCIEX), using a database containing sequences from *Zea mays*, downloaded from UniProt (Reference proteome downloaded on 17 February 2022) and common contaminants. Trypsin was set as the digestion enzyme, cysteine alkylation (iodoacetamide) was allowed as a fixed modification and biological modifications allowed in the search parameters. A 1% false discovery rate filter was applied at the protein level for refinement of identifications. In addition, to gain functional insights on the identified proteins, proteins were annotated according to functions using Gene Ontology (GO) (Gene Ontology Consortium, 2004). Differentially retrieved proteins were further classified according to their molecular function, biological function and then into subcellular localization using UniProtKB, OMA orthology, and WoLF PSORT. Then Venny 2.1 software was used to demonstrate the relationships between identified proteins for various treatment conditions (the non-salt and salt treated).

2.8 Statistical Analysis

All experiments were performed in four independent biological replicates. A one-way analysis of variance (ANOVA) was used to compare physiological, biochemical, and proteomic data obtained between the samples. The means were compared using Turkey-Kramer test at 5% level of significance.

CHAPTER THREE

RESULTS

3.1 Germination and Treatment of Maize

Zea mays seeds were germinated and allowed to grow for 14 days under greenhouse conditions in sterile potting soil before being subjected to various experimental treatments. Control plants were irrigated with sterile distilled water while experimental plants were watered with saline solutions of varying NaCl concentrations (100, 200, 300 and 400 mM) for 14 days. Various growth parameters including shoot length, root length, shoot fresh weight and shoot dry weight were measured and are shown in Table 3.1. Figure 3.1 shows the induced morphological appearance between the non-treated and treated maize plants after the treatment period. The general growth rate of maize plants was highly affected by salinity resulting in diminished plant height and wilting of leaves with increasing salt treatment.

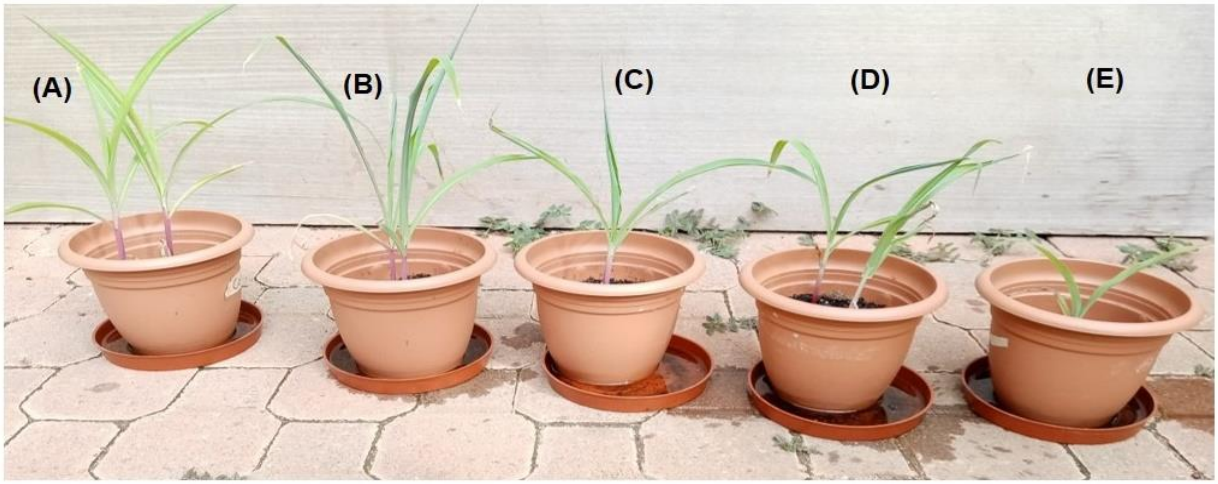

Figure 3.1: Phenotypic changes in maize seedlings under salt stress after 14 days. **(A)** represents control maize seedlings treated with sterile distilled water only, **(B)** represents experiment 1 maize seedlings treated with 100 mM NaCl solution, **(C)** represents experiment 2 maize seedlings treated with 200 mM NaCl solution, **(D)** represents experiment 3 maize seedlings treated with 300 mM NaCl solution, and **(E)** represents experiment 4 maize seedlings treated with 400 mM NaCl solution.

3.2 The Effects of Salt Stress on Growth and Physiological Parameters of Maize

Maize leaf material was harvested 14 days after treatment and shoot fresh weight, shoot dry weight, root length, shoot length, relative water content and chlorophyll content were measured and recorded.

3.2.1 Shoot Fresh and Dry Weights

The shoot fresh and dry weights (g) of maize seedlings were measured and recorded after 14 days of treatment as indicated in Table 3.1 and Figures 3.2A – B. A variation in the shoot fresh weight was observed as the control was higher compared to the treatment variables (Figure 3.2A). However, salt stress somewhat reduced the shoot fresh weight of maize for all treatment concentrations (100 – 400 mM). Figure 3.2B demonstrates a decline in shoot dry weight for all treatments as compared to the control. Generally, salt stress demonstrated a negative influence on both the shoot fresh and dry weights of maize as NaCl concentration increases (Figure 3.2A - B).

Table 3.1: Shoot fresh and dry weights of maize plants exposed to varying salt treatments for 14 days.

Treatment conditions	**Shoot fresh weight (g)**				**Shoot dry weight (g)**			
NaCl concentrations	**Plant 1**	**Plant 2**	**Plant 3**	**Mean ± SD**	**Plant 1**	**Plant 2**	**Plant 3**	**Mean ± SD**
0 mM (control)	1,90	1,80	0,70	1,47 ± 0,67	1,30	1,10	0,10	0,83 ± 0,64
100 mM	1,00	0,90	0,70	0,87 ± 0,15	0,50	0,40	0,30	0,40 ± 0,10
200 mM	1,30	1,30	1,10	1,23 ± 0,15	0,60	0,40	0,50	0,50 ± 0,10
300 mM	2,20	0,75	1,70	1,55 ± 0,74	0,10	0,30	0,60	0,33 ± 0,25
400 mM	0,70	0,80	0,70	0,73 ± 0,06	0,60	0,40	0,30	0,43 ± 0,15

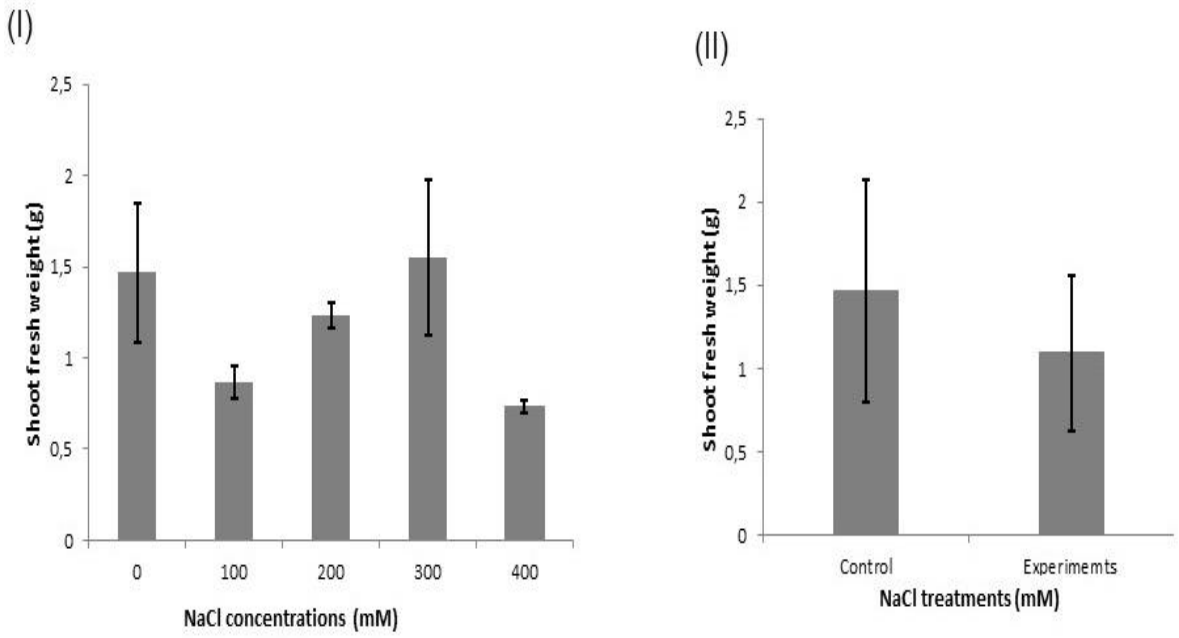

Figure 3.2A: The effects of salt stress on maize shoot fresh weight treated with water only (0 mM, control) and experiments at different NaCl concentrations (100 – 400 mM), where (I) is for individual responses and (II) for average responses. Error bars indicate the standard errors of the means (SEM) of four (n = 4) independent seedling treatments.

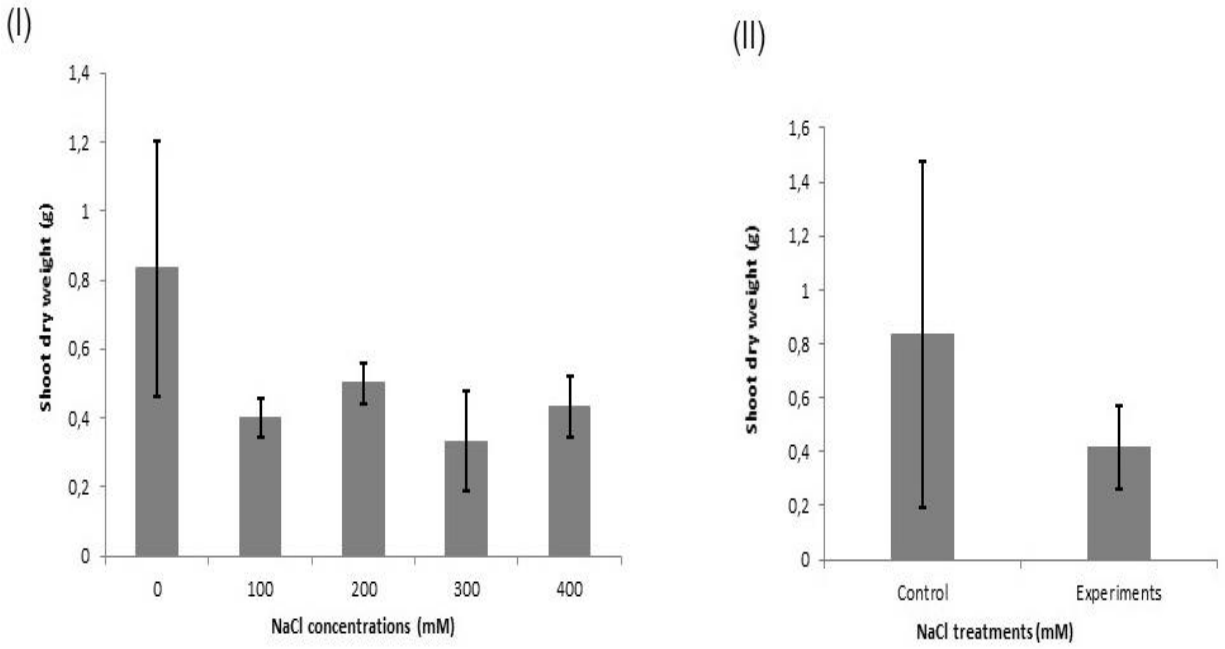

Figure 3.2B: The effects of salt stress on maize shoot dry weights treated with water only (0 mM, control) and experiments at different NaCl concentrations (100 - 400 mM), where (I) is for individual responses and (II) for average responses. Error bars indicate the standard errors of the means (SEM) of four (n = 4) independent seedling treatments.

3.2.2 Shoot and Root Lengths

The shoot and root lengths (cm) of maize seedlings were measured and recorded after 14 days of treatment as indicated in Table 3.2 and Figures 3.3A – B. The shoot length of maize declined for the experiments (100, 200, 300 and 400 mM) as compared to the control (Figure 3.3A). Furthermore, maize root length significantly decreased with an increase in NaCl concentration compared to the control for all treatment conditions (Figure 3.3B).

Table 3.2: Shoot and root lengths of maize plants subjected to salt stress for 14 days.

Treatment conditions	**Shoot length (cm)**				**Root length (cm)**			
NaCl concentrations	**Plant 1**	**Plant 2**	**Plant 3**	**Mean ± SD**	**Plant 1**	**Plant 2**	**Plant 3**	**Mean ± SD**
0 mM (control)	40	44	56	46,67 ± 8,33	38	44	68	50,00 ± 15,87
100 mM	46	50	32	42,67 ± 9,45	33	18	24	25,00 ± 7,55
200 mM	35	58	68	53,67 ± 16,92	27	21	30	26,00 ± 4,58
300 mM	29	39	33	33,67 ± 5,03	17	15	10	14,00 ± 3,61
400 mM	21	27	23	23,67 ± 3,06	9	5	11	8,33 ± 3,06

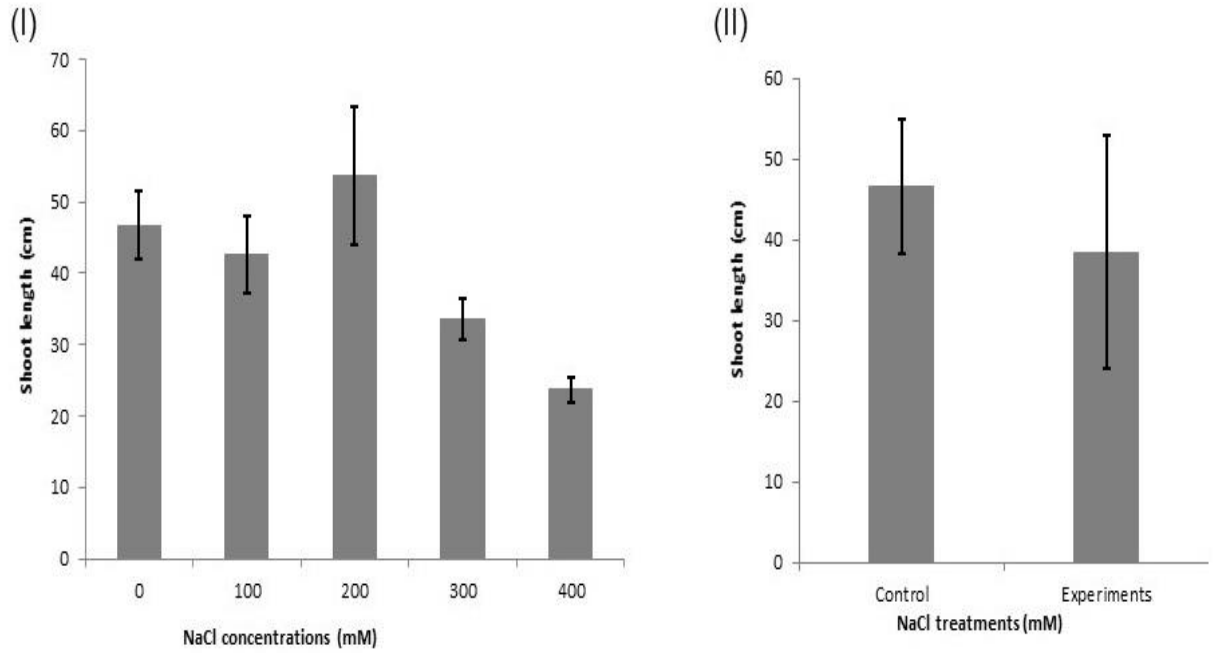

Figure 3.3A: The effects of salt stress on maize shoot length. Treatments were treated with water only (0 mM, control) and salt solution (100 - 400 mM, experiments), where (I) is for individual responses and (II) for average responses. Error bars indicate the standard errors of the means (SEM) of four (n = 4) independent seedling treatments.

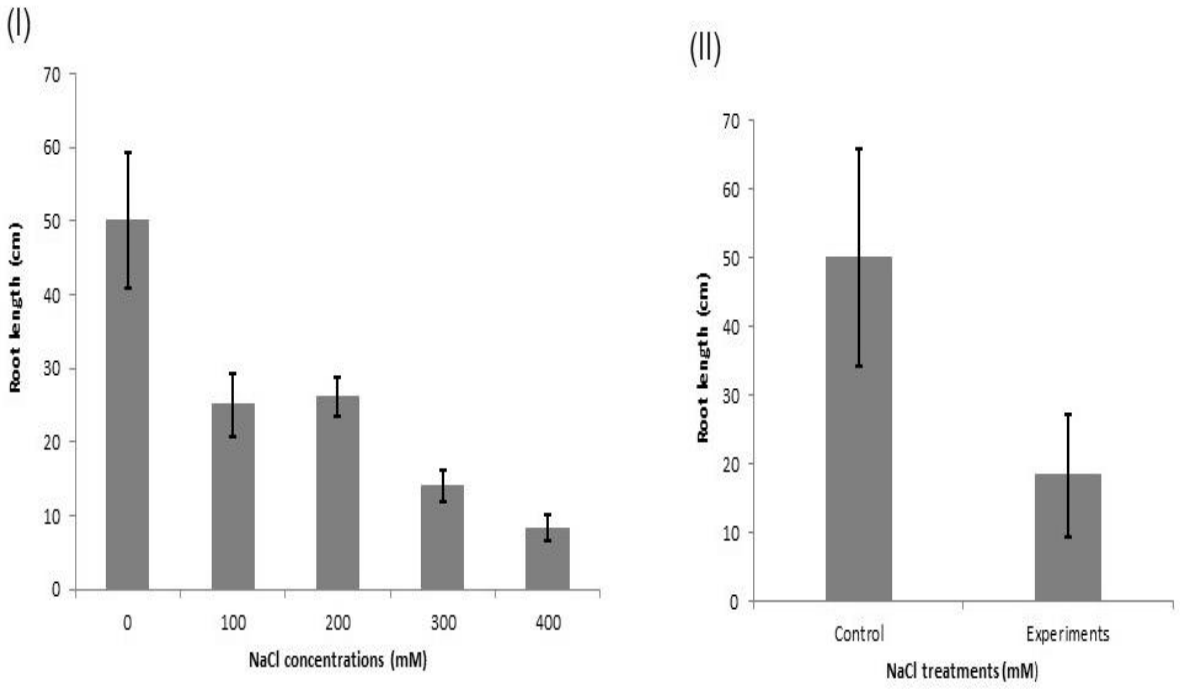

Figure 3.3B: The effects of salt stress on maize root length treated with water only (0 mM, control) and experiments at different NaCl concentrations (100 - 400 mM), where (I) is for individual responses and (II) for average responses. Error bars indicate the standard errors of the means (SEM) of four (n = 4) independent seedling treatments.

3.2.3 Chlorophyll Content

Salt stress on maize plants subjected to 100 – 400 mM NaCl differentially influenced the total chlorophyll content. Maize chlorophyll content gradually decreased with an increase in salt treatment compared to the control variable. Figure 3.4 demonstrates that as NaCl treatment increases, a decrease in the chlorophyll content is observed. However, a 400 mM NaCl concentration showed a highly significant decline in chlorophyll content when compared to the control (Figure 3.4).

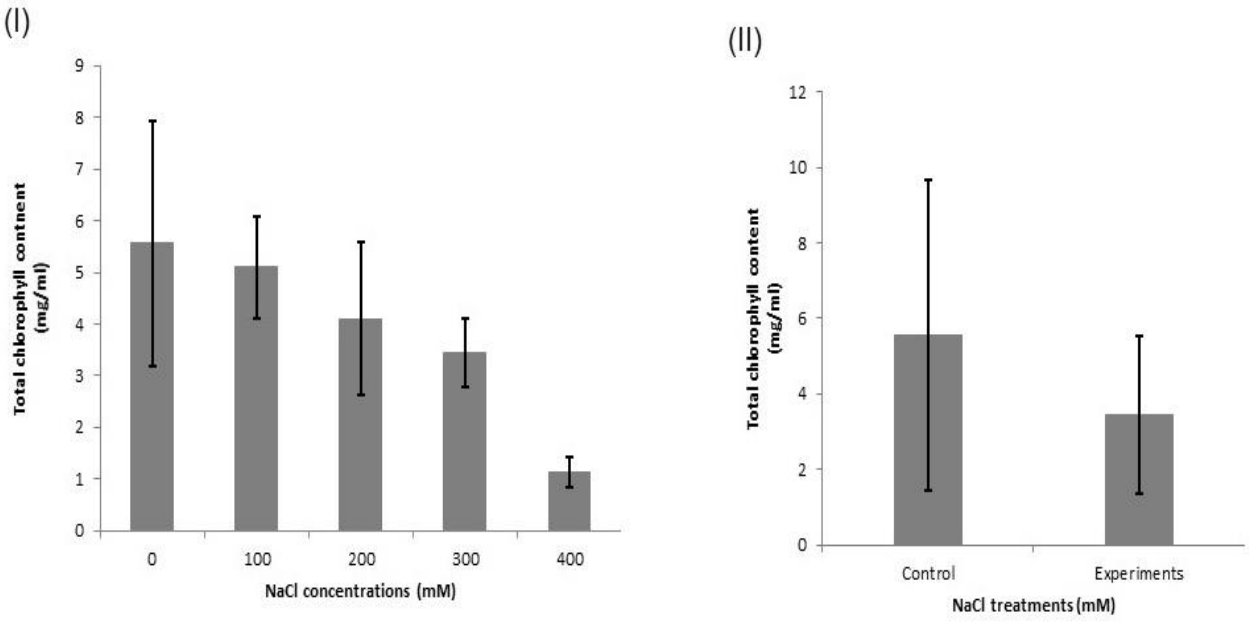

Figure 3.4: The effects of salt stress on total chlorophyll content after 14 days of treatment with water only (0 mM, control) and NaCl (100 - 400 mM, experiments), where (I) is for individual responses and (II) for average responses. Error bars indicate the standard errors of the means (SEM) of four (n = 4) independent seedling treatments.

3.2.4 Relative Water Content (RWC)

Relative water content (RWC) response of maize seedlings under salt stress (100 – 400 mM NaCl) is depicted in Figure 3.5 below. At 100, 200, 300 and 400 mM NaCl concentrations, a gradual decrease in the RWC was observed compared to the control. However, an increase in NaCl concentrations decreased the RWC of maize.

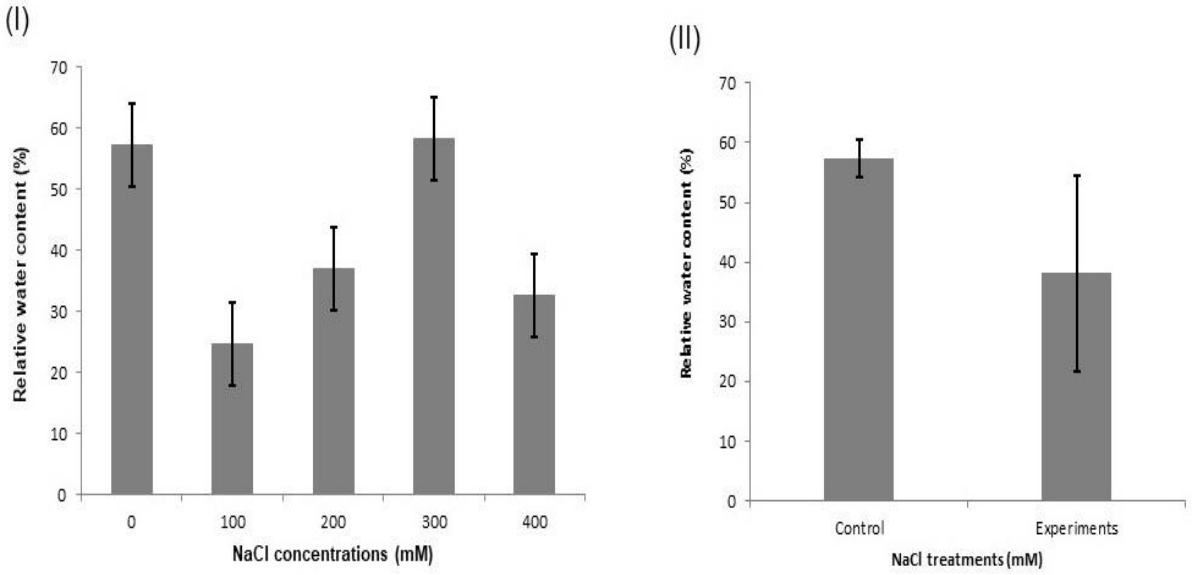

Figure 3.5: The effects of salt stress on relative water content of maize seedlings treated with water only (0 mM, control) and NaCl (100 - 400 mM NaCl treatments), where (I) is for individual responses and (II) for average responses. Error bars indicate the standard errors of the means (SEM) of four (n = 4) independent seedling treatments.

3.3 Effects of Salt Stress on Maize Biochemical Parameters

Different biochemical processes in terms of enzyme activities were assessed for 14-day-old maize seedlings exposed to salt stress. The processes assessed included catalase, glutathione reductase and superoxide dismutase activities. The results obtained from such different assessments are presented on the three graphs below (Figure 3.6 – 3.8).

3.3.1 Catalase Activity

A spectrophotometer-based assay was used to measure the catalase enzymatic activity from maize leaves (Figure 3.6). Figure 3.6 demonstrates the effect of various NaCl concentrations on the activity. The activity significantly decreased in response to varying salt stress effects when compared to the control. However, a strong catalase activity inhibition was observed at higher salt concentration (400 mM NaCl) unlike the other treatment concentrations. Overall, salinity stress strongly inhibited the catalase activity in maize leaves.

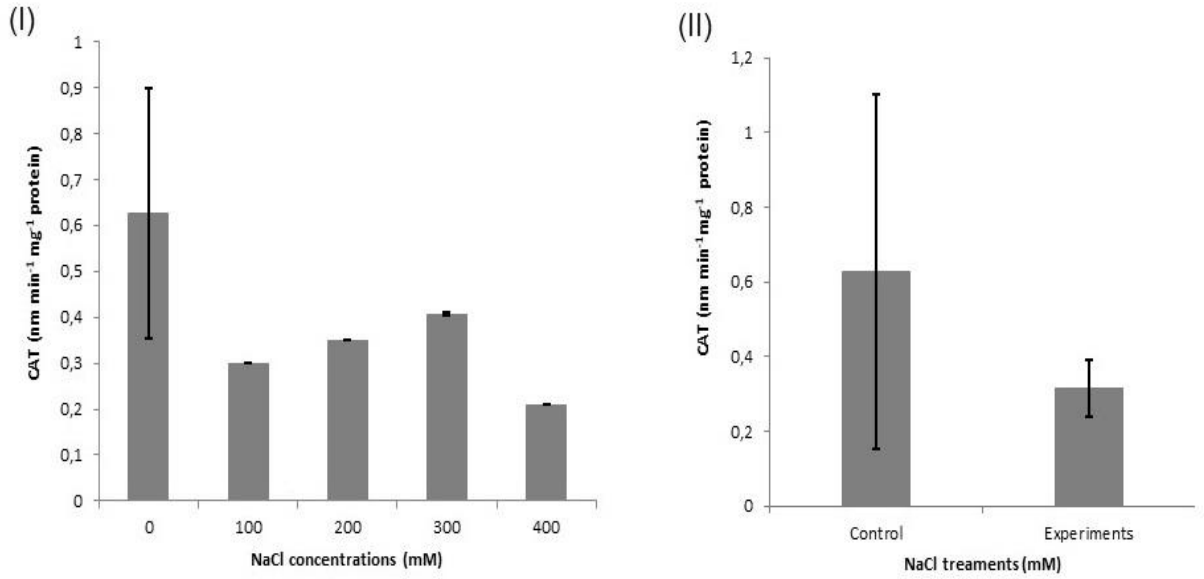

Figure 3.6: The effects of salinity on catalase activity of maize plants after 14 days of treatment with 100 – 400 mM NaCl (experiments) and water only (0 mM, control), where (I) is for individual responses and (II)

for average responses. Measurements were taken at an absorbance of 240 nm to determine the activity. Error bars indicate the standard errors of the means (SEM) of four (n = 4) independent seedling treatments.

3.3.2 Glutathione Reductase Activity

Spectrophotometer measurements for the glutathione reductase (GR) activity was conducted on maize leaves (Figure 3.7). Of particular interest, increased GR activities were observed at all salt treatment conditions compared to the control. Furthermore, at treatment concentrations of 200 and 300 mM, the GR activities were highly induced (Figure 3.7).

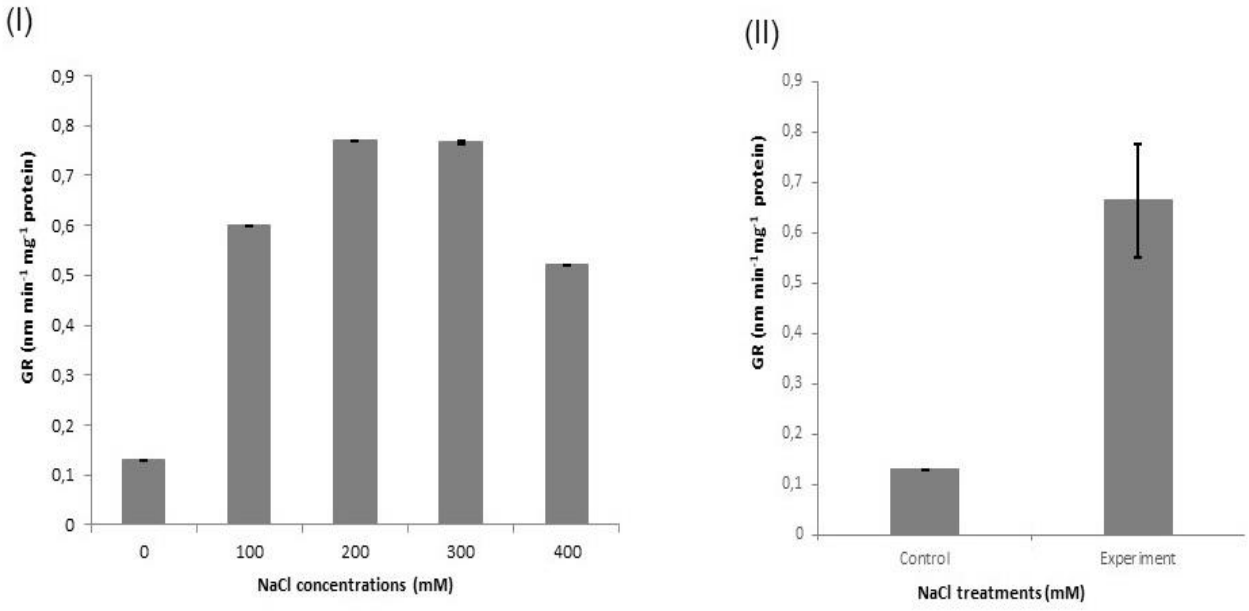

Figure 3.7: The effects of NaCl concentrations on glutathione reductase (GR) activity in maize plants after 14 days of treatment with salt (100 – 400 mM NaCl) and control (0 mM, water only) measured at an absorbance of 240 nm, where (I) is for individual responses and (II) for average responses. Error bars indicate the standard errors of the means (SEM) of four (n = 4) independent seedling treatments.

3.3.3 Superoxide Dismutase Activity

A spectrophotometric assay was used to determine the superoxide dismutase (SOD) activity in maize plants exposed to various levels of salt stress (Figure 3.8). As displayed in Figure 3.8, the SOD activity gradually decreased under salt stress when compared to control.

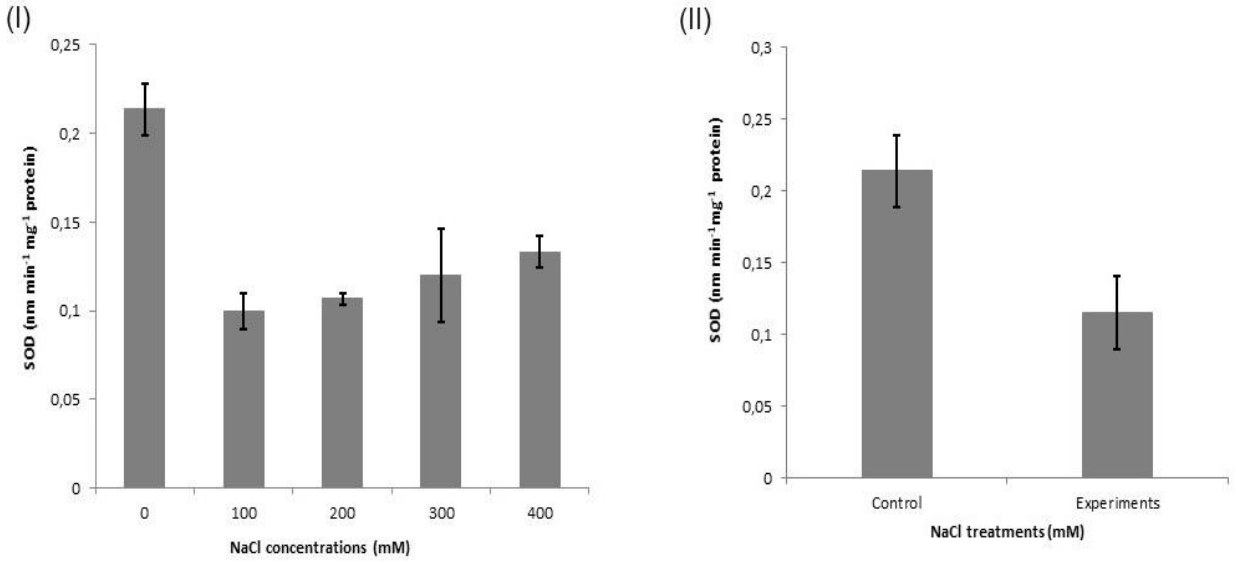

Figure 3.8: The effects of salt stress on superoxide dismutase (SOD) activity in maize plants after 14 days of treatment with 100 – 400 mM NaCl and water only (0 mM, control) measured at an absorbance of 240 nm, where (I) is for individual responses and (II) for average responses. Error bars indicate the standard errors of the means (SEM) of four (n = 4) independent seedling treatments.

3.4 Assessment of Maize Expressional Protein Profiles in Response to Salt Stress

One dimensional SDS-PAGE was used to assess the quality of total extracted proteins from maize leaves under varying NaCl concentrations (100 – 400) as compared to the control (water only). Overall, all the biological replicates resembled similar banding patterns for both the control and experiments, with an overexpression at 300 and 400 mM NaCl concentrations (E3 and E4) (Figure 3.9).

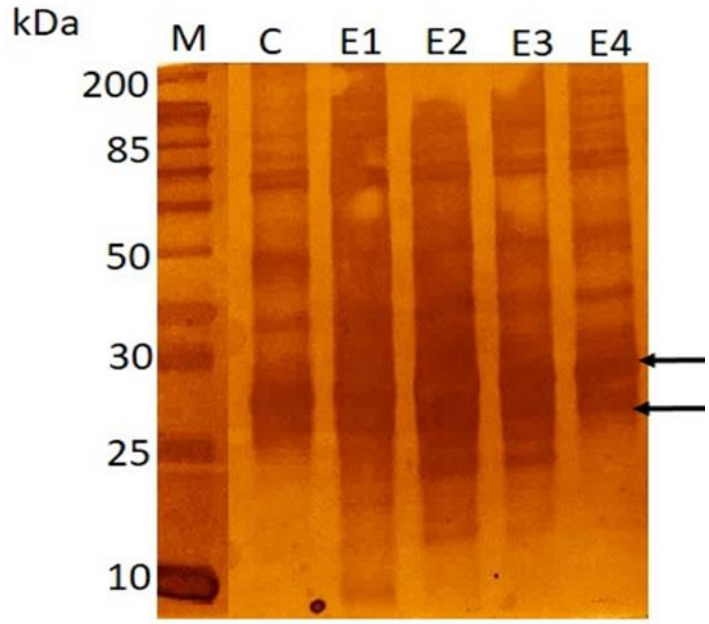

Figure 3.9: Expression profiles of the salt stressed proteins. A 12% SDS-PAGE resolution of proteins from the salt stressed maize seedlings, where lane 1 (M) represents the unstained protein marker (Catalog # P7704S New England Biolabs Inc., Massachusetts., USA); lane 2 (C) represents the control (water treatment only); lane 3 (E1) represents experiment 1 (100 mM NaCl treatment); lane 4 (E2) represents experiment 2 (200 mM NaCl treatment); lane 5 (E3) represents experiment 3 (300 mM NaCl treatment) and lane 6 (E4) represents experiment 4 (400 mM NaCl treatment) treated with NaCl concentrations. The gel was stained with silver nitrate. Error bars indicate the standard errors of the means (SEM) of four (n = 4) independent seedling treatments.

3.5 Identification and Classification of Salt Responsive Proteins in *Zea mays*

3.5.1 Identification of Salinity Stress Responsive Proteins in Maize

To identify salt stress responsive proteins in maize leaves subjected to varying concentrations of NaCl (0, 200, 300 and 400 mM), protein level identifications from those conditions were successfully analyzed by LC-MS-MS and listed in Appendices A-D (Tables 3.3- 3.6), indicating the varying accession numbers, protein names, species names and subcellular locations. Furthermore, information from all the positively identified maize proteins indicated in Tables 3.3-3.5 (Appendices A-C) were used to plot a Venn diagram. Venny 2.1 software was used to do a comparative assessment of the identified differentially expressed proteins between the control and treatment conditions (200, 300 and 400 mM NaCl), as well as the common proteins present amongst all the conditions. Three treatment conditions (200, 300 and 400 mM NaCl) were selected to identify the abundant/varying salt stress signaling pathways in maize. Figure 3.10 shows the protein expression relationship between varying treatment conditions. A general trend was observed, whereby as salt concentration increased, the number of differentially expressed proteins (DEPs) decreased compared to the control. In addition, protein overlap was also noted amongst the various treatments. Under 200 mM NaCl treatment, 14.9% (40) differentially expressed proteins were observed compared to 23% (62) in the control, while 3.3% (9) of proteins were commonly abundant in both the control and 200 mM treatment. At 300 mM NaCl treatment, (33) 12.3% proteins were differentially expressed compared to (62) 23% in the control, while (8) 3% of proteins were commonly present in both the control and 300 mM NaCl treatment. In addition, (32) 11.9% differentially expressed proteins were found in the 400 mM NaCl treatment compared to (62) 23% in the control while (7) 2.6% of proteins were abundant in both the control and 400 mM treatment. Only (3) 1.1% of the proteins were common amongst all the conditions (0, 200, 300 and 400 mM treatment).

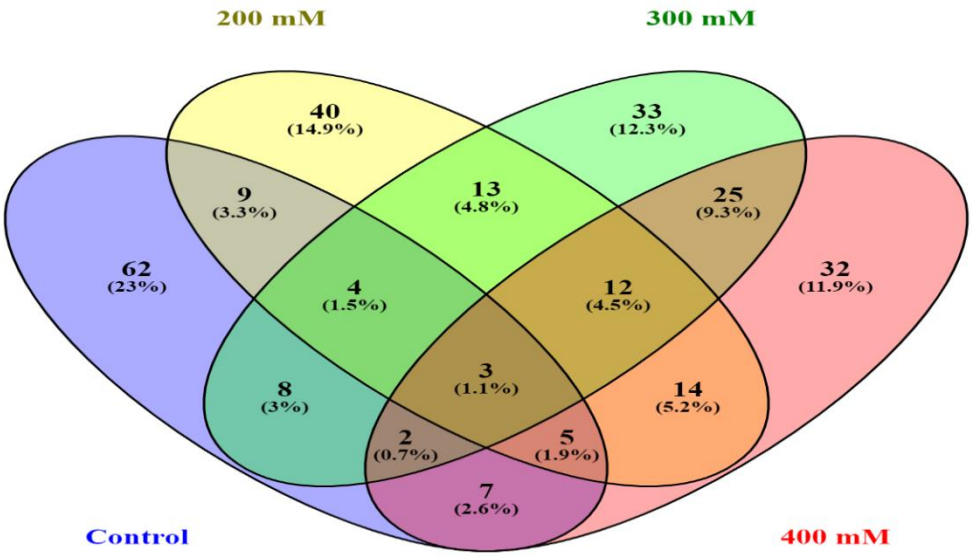

Figure 3.10: A Venn diagram representation of the different maize overlapping proteins under salt stress. Differentially expressed proteins from varying conditions: control (0 mM NaCl) and treatments (200, 300 and 400 mM NaCl) were used to assess and depict the relationship between common and different proteins present in the control and experimental treatments.

3.5.2 Functional Classification of Maize Proteins under Salt Stress

Panther Gene ontology (GO) was used to determine the biological, molecular functions and cellular components of the differentially expressed maize salt stress responsive leaf proteins. The GO annotation results are presented in pie-charts, which represent the different protein percentages mapped under various categories as shown in Figures 3.11 – 3.15 respectively. The common proteins identified for all treatments, GO, biological processes (Table 3.6; Figure 3.11) were classified into four categories according to their functions: where 38.7% proteins were mainly involved in metabolic process; followed by 38.7% involved in cellular process; 16.1% localization and 6.5% response to stimulus (Figure 3.11A). Moreover, molecular functions of the commonly identified proteins were classified into three categories: whereby the first class had 57.9% of the proteins involved in catalytic activity; while 31.6% were involved in transporter activity and 10.5% in binding activity (Figure 3.11B). Cellular component had only one category, which contained 100% of cellular anatomical entity (Figure 3.11C). Furthermore, the identified proteins for

individual treatments were classified into functional groups as indicated in Appendices E-H (Figures 3.12 – 3.15).

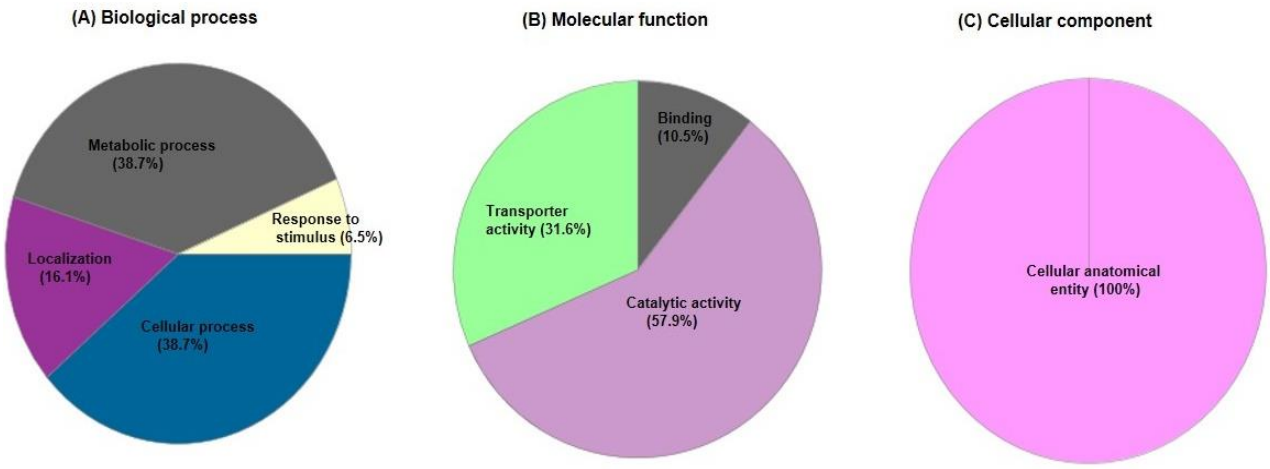

Figure 3.11: Functional classification of Gene ontology (GO) annotation for common proteins expressed between the control and treatments (200, 300 and 400 mM NaCl) classified to various: **(A)** biological processes, **(B)** molecular functions and **(C)** cellular components in maize.

CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1 Discussion

Salinity or salt stress is among the major abiotic stresses that limit plant growth and productivity (Sabir *et al*., 2011). This was evident in a number of crops, where salt stress induced a reduction of various growth attributes including *Proso millet* (Sabir *et al.,* 2011) and sunflower (Akram and Ashraf, 2011). The decline in growth and productivity of maize (*Zea mays* L.) under salt stress can be explained in various ways such as the disturbed osmotic relations, nutritional and hormonal imbalance, and lastly, oxidative stress. Plants grown under elevated salt concentration are not able to take up water and minerals like K^+ and Ca^{2+} that ultimately leads to a reduction in growth (Parida and Das, 2005). Although salinity has a negative impact on maize development and yield attributes during its life cycle, the final impact on plant productivity is determined by the length, severity, and growth phase during which the stress occurs (Hatfield and Prueger, 2015).

Maize is an important cereal crop, which serves as a basic source of food and oil for human intake. Moreover, it is a significant staple diet that feeds about 1.2 billion people worldwide, providing for more than 20% of total calories in 21 countries. It is also used as feed for livestock (Nawaz *et al*., 2010). However, cultivation of this crop is highly challenged since it is mostly exposed to various abiotic stresses such as salinity and high temperature worldwide. It is estimated that about 20% of the irrigated land is affected by salt stress that is exclusively classified as arid and desert lands, comprising 25% of the total land on earth (Rasool *et al.,* 2013). Several plant scientists have led studies seeking answers on the mechanisms that are exhibited by plants when exposed to various abiotic stresses, including salinity and hoping to address the major crop productivity constraints that threatens food security. Thus, this study investigated the effects of salt stress on the physiological, biochemical and molecular responses of the maize crop.

To pursue this, maize plant lines were grown under varying salt stress conditions (100, 200, 300 and 400 mM) using sodium chloride (NaCl) as an inducer for 14 days under greenhouse conditions. Evident changes in growth and physiological traits were observed after 14 days of salt stress exposure. A negative impact of salt stress was notably observed on the general morphology of the maize plants, which then resulted in reduced plant height, wilted leaves, and reduction in leaf number (Figure 3.1). In addition, salt stress also led to a significant reduction in growth traits including shoot fresh weight as well as the shoot and root lengths (Table 3.1 - 3.2; Figures 3.2A; Figure 3.3A and B). An observed decrease in root and shoot lengths of the maize plants may be due to the high accumulation of Na^+ ions in the plant tissues (Shao *et al*., 2021). Furthermore, the observed reduction in maize shoot growth may be attributed to a number of factors such as diminished leaf initiation, poor cell expansion, lower intermodal distance and leaf abscission, which are all directly influenced by high Na^+ ionic content (Shahzad *et al*., 2012). The observed high reduction in leaf number per plant is mainly attributed to a decrease in water potential and stomatal conductance, increased root senescence, reduced shoot and root growth and decreased gaseous exchange (Barret-Lennard, 2003). An increase in the shoot fresh weight at 300 mM shows that maize was tolerant to salinity under 300 mM concentration. Salt tolerant plants generally exclude Na^+ from their shoots to prevent Na^+ toxicity (Munns, 2005). The results obtained in our study have revealed that shoot and root lengths as well as the shoot fresh weights of maize were significantly affected by salinity (Figures 3.2 and 3.3).

Typically, increased salinity inhibits root and shoot elongation, and this occurs due to the decreased water uptake and lowered essential mineral nutrition absorption from the soil by the plant (Neumann, 1997). Our data for shoot and root lengths has shown a length reduction in maize plants treated with the salt as compared to the control (Figures 3.3A and 3.3B). Apparently, shoot length reduction was moderate across nearly all the used concentrations, however, a contrary increase in length was noted at 200 mM NaCl concentration (Figure 3.3A). This anomalous increase may be an indication of tolerance at different salt stress concentrations, which perhaps could then promote growth and yield in plants (de Azevedo Neto *et al.,* 2006; Farre and Faci, 2006; Kanwal *et al.,* 2019). The observed inhibited root length may be due to salt stress damage since the root is an initial

organ to be exposed to stress, resulting in water transportation difficulty from them to shoots and the other various parts of the plants (Munns and Sharp, 1993). In addition to the assessed growth attributes, shoot fresh weight displayed a fluctuation between the control (water only) and salt treatments (100, 200, 300 and 400 mM) with a great decrease observed at 100 and 400 mM NaCl, while a transient increase was noted at 300 mM NaCl (Figure 3.2A). Shoot dry weight indicated a decline on salt treated maize plants compared to the non-treated group (Figure 3.2B). This is an indication that the accumulation of Na^+ ions has a negative effect on the growth and productivity of maize and our obtained results are consistent with other previously published findings (Zheng *et al.,* 2009; Falakboland, 2016). A similar trend was also reported in the salt-tolerant maize (Zahra *et al*., 2020), and *Brassica campestris* L. (Bohra and Vyas, 2006) varieties.

It is generally known that in salt-tolerant species, chlorophyll content increases while it decreases in salt-sensitive species under salt stress (Khan *et al*., 2009; Akram *et al*., 2011). Maize, as a salt-sensitive crop, has demonstrated a gradual decline in the total chlorophyll content as the concentration of NaCl increases compared to the control (Figure 3.4) and in this regard, observations from this study do concur with those of other previous studies (Khan *et al*., 2009; Akram *et al*., 2011). Salt stress is known to cause a significant degradation of chlorophyll pigments (Jamil *et al*., 2012) and thus affect chlorophyll content, which is fundamental in understanding plant's response to varying environmental conditions especially salinity.

The relative water content (RWC) of a leaf is a measurement of its hydration status (actual water content) relative to its maximal water holding capacity at full turgidity. According to Lugojan and Ciulca (2011), RWC is an important indicator of the water status in plants, which reflects the balance between water supply to the leaf tissue and transpiration rate. In our study, the analysis of various salinity levels on RWC showed that, increase in salinity concentration caused a significant reduction in the RWC (Figure 3.5) for the 100, 200, and 400 mM NaCl treatments but an incline for the 300 mM NaCl treatment. A decrease in the RWC of maize involves important and key physiological and morphological traits such as leaf enlargement, stomatal opening, and the associated leaf photosynthesis, which directly affect the leaf turgor potential and result in water loss

(Jones and Turner, 1978). An increase of the RWC might be as a result of low uptake of Na^+ and less reduction in K^+ in this cultivar, thus being able to keep a high salt concentration and absorb more water and consequently to adjust osmotic pressure (Kumar *et al.,* 2021).

Salt stress always triggers the excessive generation of reactive oxygen species (ROS) as a response mechanism in plants, however, to counteract this effect; plants use multiple biochemical strategies to avoid damage (Mittler, 2002; Scandalios, 2002). One mechanism that may be involved in the resistance of many types of stress is the activity of the antioxidant pathways. Enzymes involved in these pathways include superoxide dismutase (SOD, peroxidase (POD), catalase (CAT) as well as those of the ascorbate-glutathione cycle. High activity levels of these enzymes have been studied in response to heat, chilling, freezing, salt, drought, wounding as well as in response to oxidative stress (Foyer *et al.,* 1994).

In the present study, biochemical activities of the CAT, glutathione reductase (GR) and SOD antioxidants were assessed. CAT is an antioxidant noted to increase with an increase in NaCl concentrations in maize plants (Arora *et al.,* 2008). An increase in CAT activity acts as an adaptive mechanism which plays a significant role in overcoming the destruction of tissue metabolism by reducing the venomous levels of H_2O_2 (Sekmen *et al.,* 2017). CAT activity in our study has shown a decrease in the salt stressed plants as compared to the control (Figure 3.6). The CAT activity decrease may be associated with a degradation caused by the induced peroxisomal proteases or due to the photo-inactivation of the enzyme (Abedi and Pakniyat, 2010). However, another study has demonstrated that severe salt stress caused an inhibition of the antioxidative enzymes CAT and POD in *Pancratium maritimum* L. (Khedr *et al*., 2003). The results of the present study concurred with those of Khedr and colleagues (2003), which could be due to the destruction of plant tissues by salt stress.

Glutathione (GSH) enhances plant tolerance to various abiotic stresses including salinity, drought, high temperature and toxic stress (Hasanuzzaman and Fujita, 2011). An increase in GR activity is caused by the accumulation of GSH and results in plant

tolerance. It was reported in several studies that abiotic stresses increased GR activity in pea (Hernandez *et al.,* 2001), cowpea (Contour-Ansel *et al.,* 2006) and *Reamuria soongorica* (Bai *et al.,* 2009). Incidentally, our study demonstrated an increase in GR activity in maize seedlings under salt stress (Figure 3.7), which clearly corresponds with observations of the other previously conducted studies. Our results thus pointed out to the aspect that this studied maize cultivar had some level of tolerance to salt stress.

Superoxide dismutase (SOD) has been reported as one of the major antioxidative enzymes present in all aerobic organisms and largely in subcellular elements that generate activated oxygen, where it is involved in the dismutation of superoxide radicals to hydrogen peroxide and oxygen (Sajjad and Pakniyat, 2009). According to Ahmad and Umar (2011), an increased SOD activity enables plants to protect themselves against the oxidative damage caused by exposure to salt stress. Results obtained from the present study have shown a decrease in SOD activity at different NaCl concentrations when compared to the control (Figure 3.8). These observations have clearly demonstrated an intense oxidative damage caused by the salt stress exposure to maize and in that instance, the results were not consistent with those of Ahmad and Umar (2011). Another reason for SOD activity decrease in maize leaves could be due to an increase in the production of superoxide.

Proteomic assessment was carried out to further understand the effect of salt stress on *Zea mays.* The extracted total leaf proteins expressed during the induced salt stress (100 – 400 mM) compared with the control (water only), were analyzed using a one-dimensional sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) (1DE). Separation of proteins by 1DE is essential for resolving total proteome according to the molecular weights of the resolved proteins (Gallagher, 2012). Figure 3.9 shows that protein molecules ranging from 25 to 85 kDa in both the control and experiments were more pronounced and less visible from the 10 to 20 kDa range. Proteomic analysis provides new insights into plants' responses to salt stress at the protein level (Zhang *et al*., 2012). Several proteomic studies have revealed which proteins are responsible for cell differentiation in *Arabidopsis thaliana* under salt stress and osmotic stress (Ndimba *et al*., 2005), and drought stress in the maritime pine (Costa *et al*., 1998); maize (Riccardi *et al*., 1998) and the wild watermelon (Kawasaki *et al*., 2000).

In the present study, differential proteins were successfully identified using liquid chromatography tandem mass-spectrometry (LC-MS-MS). This technique was used to detect changes in the levels of protein expression, estimate protein abundance and reveal the major regulating networks based on computational bioinformatics (Neilson *et al.*, 2011). All the positively identified maize proteins from varying treatment conditions (200, 300 and 400 mM NaCl) showed changes in their abundance under salt stress compared to the control (Figure 3.10). Furthermore, an increase in salt concentration strongly inhibited the expression of differential proteins. A similar trend in the abundance and differential expression of proteins was also observed in a bean exposed to salt stress (de Abreu *et al*., 2014).

The detected proteins are summarized in Appendices A-D (Table 3.3 - 3.6) with their subcellular locations. Table 3.3 shows an extracted comparison between the 200 mM NaCl treatment and the control, where heat shock (HSP) and chaperonin proteins were both detected in the cytoplasm. For the 200 mM NaCl treatment 5 HSPS were detected, whereby two (2) were found in the chloroplast and one (1) in the cytoplasm while non-subcellular locations were predicted for other two (2). The HSPS detected showed that the maize plant was able to cope with the salt stress, whereas the chaperonin proteins were expressed in response to elevated stress (Timperio *et al*., 2008). Under the 300 mM concentration group (Table 3.4); 2 chaperonin proteins were detected in the cytoplasm; this may be a protective mechanism, which prevents the damage caused by salt stress; 2 proteasome proteins in the nucleus and the other with no predicted subcellular location; and 1 HSP without a predicted subcellular location.

The proteasome protein in the nucleus means that the plant's response to salt stress was controlled by all the metabolic activities of the cell (Yu *et al*., 2020). When comparing the control with the 400 mM NaCl treatment (Table 3.5), three (3) HSPS were detected on the treatment variable in the chloroplast, cytoplasm and cytosol while in the other subcellular locations they were not predicted. Four (4) proteasome proteins were also detected, whereby two (2) were found in the cytoplasm and cytosol, one (1) in the nucleus while the other subcellular locations were not predicted. Only one (1) chaperonin protein was detected in the cytoplasm and cytosol. The detection of these proteins revealed that

the studied maize plant was able to stabilize other cellular proteins and adjust cellular metabolism to cope with salt stress (Parida and Das, 2005).

For the common proteins found amongst all the treatment conditions (0, 200, 300 and 400 mM NaCl) seven (7) HSPS were detected in the chloroplast, cytosol, and cytoplasm, while a chaperonin protein was detected in the cytosol (Appendix D, Table 3.6). In the cytosol of a plant cell, a high ratio of K^+ /Na^+ is an important factor that maintains ion homeostasis under salt stress. It has been reported that under high salinity, Na^+ may inhibit the potassium transporters by competing with the K^+ for influx into plants (Assaha *et al.,* 2017). The findings of the present study are consistent with the results of previous studies in maize (Friso *et al*., 2010), whereby most of the identified proteins were localized in the chloroplast, cytoplasm, cytosol, and few in other organelles such as the nucleus. Chloroplasts are very important organelles because they facilitate metabolic functions that control plant growth and development (Zybailov *et al*., 2008).

The functional classification of salt-responsive proteins can provide clue on the physiological and metabolic pathways that maize use in response to salt stress. According to Gene Ontology (GO), the first 50 selected salt-responsive proteins were classified into functional categories as shown in Figures 3.11 - 3.15. These proteins were grouped into functional categories and the most dominant differentially expressed leaf proteins were associated with metabolism and cellular processes in maize (Figure 3.11A). In various plant species, it has been observed that the binding activity is closely associated with the regulation of salt-stress-modulated gene expression (Lin *et al*., 2009). It has also been stated that proteins involved in various chemical activities like kinases, pyrophosphates and hydrogenases play significant roles in detecting and relaying salt-stress signals for the regulation of specific genes and thus mediate cellular responses to salt stress (Hasegawa *et al.,* 2000). The biological process functional classification in this study has shown that many of the proteins were involved in cellular processes, metabolic processes, and response to stimulus amongst all the treatment conditions and the common proteins were detected (Figure 3.11 - 3.15).

Molecular functional activities that are responsible for salt stress mechanism were detected and presented in Appendices E - H (Figure 3.12 - 3.15) and the key categories

were: binding activity, catalytic activity, and transporter activity. These categories were abundant in all treatment conditions (0, 200, 300 and 400 mM NaCl). This indicates that salinity stress did induce antioxidation and transcription factor activities, which in turn, generated the synthesis of solutes such as proline, trehalose, mannitol, and lysine that are mainly used as adaptive mechanisms underlying salinity tolerance in maize roots (Luo *et al.,* 2013; Mittler, 2002).

Out of the GO pertaining to cellular component category, about 98% of proteins occupied the cellular anatomical entity. Moreover, the protein containing complex category was between 2% to 7% for all the treatment conditions as shown in Figures 3.11 - 3.15. The proteins detected in the maize nucleus were plant proteasome, and chaperonin, and all play important roles in increasing maize tolerance to salt stress and osmotic stress.

4.2 Conclusion

The present study evaluated the morphological, physiological, biochemical, and proteomic profiles of the maize plants in response to various salt stress exposures. Salt stress negatively affected the treated maize plants at the morphological, physiological, biochemical, and molecular levels when compared to the control plants, thus the response pathways of maize under varying salinity stress conditions was determined through these processes. Our study, has successfully linked maize salinity response to key morphological and physiological traits such as plant height, shoot length, root length, shoot fresh weight and shoot dry weight, and important physiological parameters including chlorophyll content and relative water content. Furthermore, the study has demonstrated a negative response to biochemical traits such as catalase, superoxide dismutase and glutathione reductase of maize under salinity. The results have fairly demonstrated that the maize plant does undergo varying changes at both the physiological and biochemical levels when subjected to salt stress. In addition, maize leaf proteome analysis was carried out to monitor salt stress response and a variety of proteins were positively identified. The most differentially expressed leaf proteins were associated with metabolic and cellular processes, followed by an increased catalytic activity, with the majority occupying the cellular anatomical entity.

Collectively, our combined physiological, biochemical, and proteomic analyses of maize in this study have demonstrated that the plant was highly affected by salt stress but on the other hand has also shown some tolerance/adaptation abilities to this stress at the biochemical and proteomic levels. From this information, our findings can therefore, serve as a source of background / baseline information for future studies on salt tolerance in cereal grain crops.

4.3 Recommendations

The undertaken study has highlighted several topics into which further research would be beneficial. Future studies on maize salt stress responses are hereby recommended to understand how this crop adapts to this stress type. Confirmatory studies using gene expression analysis of few target gene identities from the current study could be carried out. Possible future investigations may also be on profiling maize metabolites under varying NaCl treatment concentrations.

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APPENDICES

Appendix A

Table 3.3: Differentially expressed proteins of the control and 200 mM NaCl treatment in *Z. mays* identified by LC-MS-MS.

Accession	Protein Name	Subcellular location	Species Name	Accession	Protein Name	Subcellular location
Control				**200 mM NaCl**		
tr|C4J410	Heat shock 70 kDa protein	Cytoplasm	*Z. mays*	tr|A0A1D6LAW0	Elongation factor 2	Intracellular membrane-bounded organelle
tr|B4FAL9	Fructose-bisphosphate aldolase	Cytosol	*Z. mays*	sp|P19023	ATP synthase subunit beta, mitochondrial	Mitochondrion
tr|A0A1D6LJS9	Chaperonin 60 subunit beta 2 chloroplastic	Cytoplasm	*Z. mays*	tr|A0A1D6PJL0	Aconitate hydratase	Cytosol
sp|P08735	Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	Cytoplasm and cytosol	*Z. mays*	tr|A0A1D6LSP5	Elongation factor gamma1	None predicted
sp|P04709	ADP, ATP carrier protein 1, mitochondrial	Mitochondrion	*Z. mays*	tr|B4G031	L-ascorbate peroxidase	Chloroplast
tr|A0A1D6F3N9	Elongation factor Ts, mitochondrial	Mitochondrion	*Z. mays*	tr|B6TS21	Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial	Mitochondrion
sp|P25709	NAD(P)H-quinone oxidoreductase subunit H, chloroplastic	Chloroplast	*Z. mays*	tr|B4FSA7	ADP/ATP translocase	Membrane
tr|B6SSU6	Fructose-bisphosphate aldolase	None predicted	*Z. mays*	tr|B6UG10	40S ribosomal protein S3a	Cytoplasm and cytosol
tr|B4FBA4	9,10-9,10 carotenoid cleavage dioxygenase 1	Chloroplast	*Z. mays*	tr|O22453	40S ribosomal protein S4	Ribosome

tr|O24561	Chlorophyll a-b binding protein, chloroplastic	Chloroplast	*Z. mays*	tr|B4FZU8	Malate dehydrogenase	Mitochondrion
sp|Q9ZT00	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	Chloroplast	*Z. mays*	tr|A0A1D6JXJ7	D-fructose-1,6-bisphosphate 1-phosphohydrolase	Cytosol
sp|Q8VXG7	Phenylalanine/tyrosine ammonia-lyase	Cytoplasm and cytosol	*Z. mays*	tr|A0A1D6GQ42	Cell division cycle protein 48	Cytosol
tr|B6U5I1	Peptidylprolyl isomerase	Thylakoid	*Z. mays*	tr|Q9SLP5	Ferredoxin--NADP reductase, chloroplastic	Chloroplast
tr|B4FRR1	Inorganic diphosphatase	Chloroplast	*Z. mays*	tr|C0P8J4	22.0 kDa heat shock protein	None predicted
tr|B4F8M8	Exoglucanase1	Secreted	*Z. mays*	tr|K7UUB7	Elongation factor 1-alpha	Intracellular membrane-bounded organelle
tr|B8A0V4	Beta-galactosidase	Secreted	*Z. mays*	tr|B4FU39	PKS_ER domain-containing protein	None predicted
tr|B6TCK3	NADH-cytochrome b5 reductase	None predicted	*Z. mays*	tr|B4FQK0	Monodehydroascorbate reductase 5 mitochondrial	Mitochondrion
tr|C0HEG7	Putative plastid-lipid-associated protein 13 chloroplastic	None predicted	*Z. mays*	tr|C0P5X6	Chloroplast ribulose bisphosphate carboxylase/oxygenase activase	Chloroplast
sp|B4G072	DIMBOA UDP-glucosyltransferase BX9	None predicted	*Z. mays*	tr|B6TI78	Peptidylprolyl isomerase	Thylakoid
tr|Q5IBC6	DANA2	Chloroplast	*Z. mays*	tr|B6T398	Putative plastid-lipid-associated protein 13 chloroplastic	None predicted
tr|K7VH58	Peroxidase	Secreted	*Z. mays*	tr|A0A1D6F9C2	Oxygen-evolving enhancer protein 2-1 chloroplastic	Chloroplast
tr|B4FMX4	Glutamine synthetase	None predicted	*Z. mays*	tr|B6SIS5	Ribosomal protein	Cytosol

tr|A0A1D6F5U0	Alpha-mannosidase	None predicted	*Z. mays*	tr|K7UXK5	Putative alcohol dehydrogenase superfamily protein	None predicted
tr|C4J4B7	Rhodanese/Cell cycle control phosphatase superfamily protein	None predicted	*Z. mays*	tr|A0A1D6I6H5	Heat shock protein 90-5 chloroplastic	Chloroplast
tr|A0A1D6H703	Aminopeptidase	Endoplasmic reticulum	*Z. mays*	tr|B4G1C2	GH18 domain-containing protein	None predicted
tr|B6T7G7	Elongation factor 1-gamma 3	None predicted	*Z. mays*	tr|B4F836	Lactoylglutathione lyase	None predicted
tr|K7VQ65	Putative translation elongation factor family protein	None predicted	*Z. mays*	tr|K7TJV6	Oxoglutarate dehydrogenase (succinyl-transferring)	Mitochondrion
tr|B4FL09	Adenylate kinase	Chloroplast	*Z. mays*	tr|B4FT31	Dehydroascorbate reductase	None predicted
tr|B4FVH1	Malate dehydrogenase	None predicted	*Z. mays*	tr|B4F8L7	Glyceraldehyde-3-phosphate dehydrogenase	Chloroplast
tr|B4FUM2	Ferredoxin--NADP reductase, chloroplastic	Chloroplast	*Z. mays*	tr|A0A1D6K2G2	Sucrose synthase	None predicted
tr|B4FWG2	Photosynthetic NDH subunit of subcomplex B 2 chloroplastic	Cytoplasm	*Z. mays*	tr|B4FH62	NAD(P)-binding Rossmann-fold superfamily protein	None predicted
tr|A0A096PQR7	Cytochrome P450 CYP74A19	Mitochondrion	*Z. mays*	tr|B7ZXR4	Heat shock 70 kDa protein 8	Cytoplasm
tr|B6T171	Alanine--glyoxylate transaminase	Peroxisome	*Z. mays*	tr|B4FT59	17.4 kDa class I heat shock protein	None predicted
tr|C4J4E4	Monodehydroascorbate reductase homolog1	Peroxisome	*Z. mays*	tr|B6SZT9	Chlorophyll a-b binding protein, chloroplastic	Chloroplast
tr|B4F7T9	Peroxidase	Secreted	*Z. mays*	tr|B4FA27	Alpha-galactosidase	Cell wall

tr|Q9FQC5	Glutathione transferase	None predicted	*Z. mays*	tr|B4FM07	Thioredoxin-dependent peroxiredoxin	None predicted
tr|B6T9P0	UDP-glucose 6-dehydrogenase	Cytosol	*Z. mays*	tr|Q41815	Heat shock protein 26	Chloroplast
tr|B6T4J1	50S ribosomal protein L6	Ribosome	*Z. mays*	tr|B4FB72	rRNA N-glycosidase	Chloroplast
tr|Q9SLP6	Ferredoxin--NADP reductase, chloroplastic	Chloroplast	*Z. mays*	tr|B6SKI1	Photosystem I reaction center subunit II	Chloroplast
tr|B4FS17	Thylakoid lumenal 29 kDa protein chloroplastic	None predicted	*Z. mays*	tr|B6T769	60S ribosomal protein L7a	Cytosol
tr|A0A1D6M323	Ribosomal protein	Mitochondrion	*Z. mays*	tr|B6U7Y1	Activator of 90 kDa heat shock protein ATPase	Nucleus
tr|B4FUA	Chlorophyll a-b binding protein, chloroplastic	Chlorophyll	*Z. mays*	tr|C4J3Y5	Glutamate decarboxylase	None predicted
tr|C0PC61	Transaldolase	Cytoplasm and cytosol	*Z. mays*	tr|B4G1D2	CBS domain protein	None predicted
tr|B4FZ35	CHL-Zea mays Chloroplastic lipocalin	Thylakoid lumen	*Z. mays*	tr|C4J4W3	Hsp70-Hsp90 organizing protein 3	Thylakoid
tr|A0A1D6I1V3	Phosphoenolpyruvate carboxylase	None predicted	*Z. mays*	tr|B4FZB8	Signal recognition particle 54 kDa protein chloroplastic	Endoplasmic reticulum
tr|Q5QJA2	Harpin binding protein 1	None predicted	*Z. mays*	tr|B6T2T0	DNA binding activity2	None predicted
tr|B4F9M9	Isocitrate dehydrogenase [NADP]	None predicted	*Z. mays*	tr|B4G019	Hydroxyproline-rich glycoprotein family protein	None predicted
tr|B6T7B2	30S ribosomal protein S4, chloroplastic	Cytosol	*Z. mays*	tr|A0A1D6HT76	Protein containing PDZ domain a K-box domain and a TPR region	Thylakoid
tr|A0A1D6GDM6	DPP6 N-terminal domain-like protein	None predicted	*Z. mays*	tr|A0A1D6GWZ8	Sucrose synthase	None predicted

tr|B6TDR5	Geranylgeranyl reductase	None predicted	*Z. mays*	sp|P19023	ATP synthase subunit beta, mitochondrial	Mitochondrion

Appendix B

Table 3.4: Differentially expressed proteins of the control and 300 mM NaCl treatment in *Z. mays* identified by LC-MS-MS.

Accession	Protein Name	Subcellular location	Species Name	Accession	Protein Name	Subcellular location
Control				**300 mM NaCl**		
tr|C4J410	Heat shock 70 kDa protein	Cytoplasm	*Z. mays*	tr|A0A1D6K5Y7	Chaperone protein ClpB3 chloroplastic	Cytoplasm
tr|B4FAL9	Fructose-bisphosphate aldolase	Cytosol	*Z. mays*	tr|A0A1D6M7C2	Phosphoglycerate kinase	Cytosol
tr|A0A1D6LJS9	Chaperonin 60 subunit beta 2 chloroplastic	Cytoplasm	*Z. mays*	tr|A0A0B4J303	Eukaryotic initiation factor 4a	None predicted
sp|P08735	Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	Cytoplasm and cytosol	*Z. mays*	tr|B6T7G7	Elongation factor 1-gamma 3	None predicted
sp|P04709	ADP, ATP carrier protein 1, mitochondrial	Mitochondrion	*Z. mays*	tr|C4J4W3	Hsp70-Hsp90 organizing protein 3	Thylakoid
tr|A0A1D6F3N9	Elongation factor Ts, mitochondrial	Mitochondrion	*Z. mays*	tr|A0A1D6FKV6	Ketol-acid reductoisomerase	None predicted
sp|P25709	NAD(P)H-quinone oxidoreductase subunit H, chloroplastic	Chloroplast	*Z. mays*	tr|A0A1D6K2E8	Sucrose synthase	None predicted
tr|B6SSU6	Fructose-bisphosphate aldolase	None predicted	*Z. mays*	tr|A0A1D6N0R5	Protein RETICULATA-RELATED 4 chloroplastic	Chloroplast
tr|B4FBA4	9,10-9,10 carotenoid cleavage dioxygenase 1	Chloroplast	*Z. mays*	tr|A5GZ73	Glucose-1-phosphate adenylyltransferase	Chloroplast
tr|O24561	Chlorophyll a-b binding protein, chloroplastic	Chloroplast	*Z. mays*	tr|C0PG07	Dehydroascorbate reductase	Chloroplast

sp|Q9ZT00	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	Chloroplast	*Z. mays*	tr|K7UKK5	Elongation factor G, chloroplastic	Chloroplast
sp|Q8VXG7	Phenylalanine/tyrosine ammonia-lyase	Cytoplasm and cytosol	*Z. mays*	tr|B4FQ03	60S ribosomal protein L7-2	Cytosol
tr|B6U5I1	Peptidylprolyl isomerase	Thylakoid	*Z. mays*	tr|A1XCI1	Lipoxygenase	Chloroplast
tr|B4FRR1	Inorganic diphosphatase	Chloroplast	*Z. mays*	tr|A0A1D6EXK9	Oxygen-evolving enhancer protein 3-1	Chloroplast
tr|B4F8M8	Exoglucanase1	Secreted	*Z. mays*	tr|B6TH64	30S ribosomal protein S4, chloroplastic	Plastid
tr|B8A0V4	Beta-galactosidase	Secreted	*Z. mays*	tr|A0A096RG04	SOUL heme-binding family protein	Cellular anatomical entity
tr|B6TCK3	NADH-cytochrome b5 reductase	None predicted	*Z. mays*	tr|B4G143	Chlorophyll a-b binding protein, chloroplastic	Chloroplast
tr|C0HEG7	Putative plastid-lipid-associated protein 13 chloroplastic	None predicted	*Z. mays*	tr|B4FT54	HSP40/DnaJ peptide-binding protein	Cytosol
sp|B4G072	DIMBOA UDP-glucosyltransferase BX9	None predicted	*Z. mays*	tr|K7U1C6	NADH:ubiquinone reductase (non-electrogenic)	Mitochondrion
tr|Q5IBC6	DANA2	Chloroplast	*Z. mays*	tr|A0A1D6L9Y9	NAD-dependent epimerase/dehydratase	None predicted
tr|K7VH58	Peroxidase	Secreted	*Z. mays*	tr|C0PFK3	Proteasome subunit alpha type	None predicted
tr|B4FMX4	Glutamine synthetase	None predicted	*Z. mays*	tr|B6TVG1	Malic enzyme	Chloroplast
tr|A0A1D6F5U0	Alpha-mannosidase	None predicted	*Z. mays*	tr|B6TGL3	Proteasome subunit beta	Nucleus
tr|C4J4B7	Rhodanese/Cell cycle control phosphatase superfamily protein	None predicted	*Z. mays*	tr|C0P5C0	Importing subunit alpha	Nucleus

tr|A0A1D6H703	Aminopeptidase	Endoplasmic reticulum	*Z. mays*	tr|B4FQ64	Isovaleryl-CoA dehydrogenase	None predicted
tr|B6T7G7	Elongation factor 1-gamma 3	None predicted	*Z. mays*	tr|K7VGZ6	40S ribosomal protein SA	None predicted
tr|K7VQ65	Putative translation elongation factor family protein	None predicted	*Z. mays*	tr|B4F989	Actin-7 OS=Zea mays	None predicted
tr|B4FL09	Adenylate kinase	Chloroplast	*Z. mays*	tr|C4J9M7	Thioredoxin-dependent peroxiredoxin	None predicted
tr|B4FVH1	Malate dehydrogenase	None predicted	*Z. mays*	tr|Q5EUE1	Protein disulfide-isomerase	Endoplasmic reticulum
tr|B4FUM2	Ferredoxin--NADP reductase, chloroplastic	Chloroplast	*Z. mays*	tr|Q84TL7	Legumin-like protein	None predicted
tr|B4FWG2	Photosynthetic NDH subunit of subcomplex B 2 chloroplastic	Cytoplasm	*Z. mays*	tr|B6T1D8	60S ribosomal protein L18a	Cytosol
tr|A0A096PQR7	Cytochrome P450 CYP74A19	Mitochondrion	*Z. mays*	sp|Q9FQ11	Sucrose-phosphatase 1	None predicted
tr|B6T171	Alanine--glyoxylate transaminase	Peroxisome	*Z. mays*	tr|C0P558	UBP1-associated protein 2C	Nucleus
tr|C4J4E4	Monodehydroascorbate reductase homolog1	Peroxisome	*Z. mays*	tr|B4G1Q6	SERPIN domain-containing protein	Secreted
tr|B4F7T9	Peroxidase	Secreted	*Z. mays*	tr|B4FTT2	Regulator of chromosome condensation2	Cytosol
tr|Q9FQC5	Glutathione transferase	None predicted	*Z. mays*	tr|A0A1D6JX93	Peroxisomal nicotinamide adenine dinucleotide carrier	Cytoplasm
tr|B6T9P0	UDP-glucose 6-dehydrogenase	Cytosol	*Z. mays*	tr|B4FT85	Isochorismate synthase 1	Membrane
tr|B6T4J1	50S ribosomal protein L6	Ribosome	*Z. mays*	tr|G2XK63	CCT-beta	None predicted
tr|Q9SLP6	Ferredoxin--NADP reductase, chloroplastic	Chloroplast	*Z. mays*	tr|A0A1D6KR27	Prolyl carboxypeptidase like protein	None predicted

tr|B4FS17	Thylakoid lumenal 29 kDa protein chloroplastic	None predicted	*Z. mays*	tr|B6TB97	40S ribosomal protein S3	Chloroplast
tr|A0A1D6M323	Ribosomal protein	Mitochondrion	*Z. mays*	tr|B4F976	17.4 kDa class I heat shock protein	None predicted
tr|B4FUA	Chlorophyll a-b binding protein, chloroplastic	Chlorophyll	*Z. mays*	tr|A0A1D6K5D2	Nucleoredoxin1	None predicted
tr|C0PC61	Transaldolase	Cytoplasm and cytosol	*Z. mays*	tr|B4FBC2	GDP-mannose 35-epimerase	None predicted
tr|B4FZ35	CHL-Zea mays Chloroplastic lipocalin	Thylakoid lumen	*Z. mays*	tr|Q9SLP5	Ferredoxin--NADP reductase, chloroplastic	Chloroplast
tr|A0A1D6I1V3	Phosphoenolpyruvate carboxylase	None predicted	*Z. mays*	sp|P80607	Probable UDP-arabinopyranose mutase 1	Golgi apparatus
tr|Q5QJA2	Harpin binding protein 1	None predicted	*Z. mays*	tr|B4FV03	Glutathione transferase11	Cytoplasm
tr|B4F9M9	Isocitrate dehydrogenase [NADP]	None predicted	*Z. mays*	tr|B6SLX1	Chaperonin	None predicted
tr|B6T7B2	30S ribosomal protein S4, chloroplastic	Cytosol	*Z. mays*	tr|A0A1D6IJP9	Alanine aminotransferase9	None predicted
tr|A0A1D6GDM6	DPP6 N-terminal domain-like protein	None predicted	*Z. mays*	tr|C0PGG5	Pyruvate kinase	Cytoplasm
tr|B6TDR5	Geranylgeranyl reductase	None predicted	*Z. mays*	tr|Q43264	Alcohol dehydrogenase 1	Cytosol

Appendix C

Table 3.5: Differentially expressed proteins of the control and 400 mM NaCl treatment in *Z. mays* identified by LC-MS-MS.

Accession	Protein Name	Subcellular location	Species Name	Accession	Protein Name	Subcellular location
Control				**400 mM NaCl**		
tr|C4J410	Heat shock 70 kDa protein	Cytoplasm	*Z. mays*	tr|A0A1D6GK64	Heat shock 70 kDa protein 6 chloroplastic	Chloroplast
tr|B4FAL9	Fructose-bisphosphate aldolase	Cytosol	*Z. mays*	sp|Q08277	Heat shock protein 82	cytoplasm and cytosol
tr|A0A1D6LJS9	Chaperonin 60 subunit beta 2 chloroplastic	Cytoplasm	*Z. mays*	tr|A0A1D6K2G2	Sucrose synthase	None predicted
sp|P08735	Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	Cytoplasm and cytosol	*Z. mays*	tr|A0A1D6JMZ9	Aconitate hydratase	None predicted
sp|P04709	ADP, ATP carrier protein 1, mitochondrial	Mitochondrion	*Z. mays*	tr|A0A1D6HFC3	Tripeptidyl-peptidase II	None predicted
tr|A0A1D6F3N9	Elongation factor Ts, mitochondrial	Mitochondrion	*Z. mays*	tr|B6U284	14-3-3-like protein	Cytoplasm
sp|P25709	NAD(P)H-quinone oxidoreductase subunit H, chloroplastic	Chloroplast	*Z. mays*	tr|C0PHD8	Aldehyde dehydrogenase (NAD+)	None predicted
tr|B6SSU6	Fructose-bisphosphate aldolase	None predicted	*Z. mays*	tr|B4FWU6	Glutathione reductase	None predicted
tr|B4FBA4	9,10-9,10 carotenoid cleavage dioxygenase 1	Chloroplast	*Z. mays*	tr|A0A1D6H6F1	Citrate synthase	Mitochondrion
tr|O24561	Chlorophyll a-b binding protein, chloroplastic	Chloroplast	*Z. mays*	tr|C0P732	Hsp70-Hsp90 organizing protein 3	Thylakoid

sp|Q9ZT00	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	Chloroplast	*Z. mays*	tr|Q8W0Q2	Putative aldehyde dehydrogenase MIS1	None predicted
sp|Q8VXG7	Phenylalanine/tyrosine ammonia-lyase	Cytoplasm and cytosol	*Z. mays*	tr|B4F8L7	Glyceraldehyde-3-phosphate dehydrogenase	Chloroplast
tr|B6U5I1	Peptidylprolyl isomerase	Thylakoid	*Z. mays*	tr|Q41815	Heat shock protein 26	Chloroplast
tr|B4FRR1	Inorganic diphosphatase	Chloroplast	*Z. mays*	tr|A0A1D6LSP5	Elongation factor gamma1	None predicted
tr|B4F8M8	Exoglucanase1	Secreted	*Z. mays*	tr|B4FRZ2	Pyridoxal 5'-phosphate synthase-like subunit PDX1.2	None predicted
tr|B8A0V4	Beta-galactosidase	Secreted	*Z. mays*	tr|B6UAK0	Probable 6-phosphogluconolactonase	Cytoplasm
tr|B6TCK3	NADH-cytochrome b5 reductase	None predicted	*Z. mays*	tr|C4J4W3	Hsp70-Hsp90 organizing protein 3	Thylakoid
tr|C0HEG7	Putative plastid-lipid-associated protein 13 chloroplastic	None predicted	*Z. mays*	tr|K7VEB9	Importin subunit alpha	Cytoplasm
sp|B4G072	DIMBOA UDP-glucosyltransferase BX9	None predicted	*Z. mays*	tr|B4FBC2	GDP-mannose 35-epimerase	None predicted
tr|Q5IBC6	DANA2	Chloroplast	*Z. mays*	tr|B6SZT9	Chlorophyll a-b binding protein, chloroplastic	Chloroplast
tr|K7VH58	Peroxidase	Secreted	*Z. mays*	tr|B4FT59	17.4 kDa class I heat shock protein	None predicted
tr|B4FMX4	Glutamine synthetase	None predicted	*Z. mays*	tr|B4FPB7	60S ribosomal protein L7a	Cytosol
tr|A0A1D6F5U0	Alpha-mannosidase	None predicted	*Z. mays*	tr|B6TNR8	40S ribosomal protein S2	Cytosol
tr|C4J4B7	Rhodanese/Cell cycle control phosphatase superfamily protein	None predicted	*Z. mays*	tr|O50018	Elongation factor 1-alpha	Intracellular-membrane bounded organelle

tr|A0A1D6H703	Aminopeptidase	Endoplasmic reticulum	*Z. mays*	tr|B4FI86	Proteasome subunit beta	cytoplasm and cytosol
tr|B6T7G7	Elongation factor 1-gamma 3	None predicted	*Z. mays*	tr|K7TL05	General regulatory factor2	Cytoplasm
tr|K7VQ65	Putative translation elongation factor family protein	None predicted	*Z. mays*	tr|B4FTW5	60S ribosomal protein L7-1	Cytosol
tr|B4FL09	Adenylate kinase	Chloroplast	*Z. mays*	tr|B4FL55	Chlorophyll a-b binding protein, chloroplastic	Chloroplast
tr|B4FVH1	Malate dehydrogenase		*Z. mays*	tr|A0A1D6MSC0	Peroxidase	Secreted
tr|B4FUM2	Ferredoxin--NADP reductase, chloroplastic	Chloroplast	*Z. mays*	tr|B8A0E5	Omega-amidase chloroplastic	None predicted
tr|B4FWG2	Photosynthetic NDH subunit of subcomplex B 2 chloroplastic	Cytoplasm	*Z. mays*	tr|B4FEH8	Epimerase domain-containing protein	None predicted
tr|A0A096PQR7	Cytochrome P450 CYP74A19	Mitochondrion	*Z. mays*	tr|K7TM64	Pyruvate kinase	Cytoplasm
tr|B6T171	Alanine--glyoxylate transaminase	Peroxisome	*Z. mays*	tr|B4F7U0	Proteasome subunit alpha type	Nucleus
tr|C4J4E4	Monodehydroascorbate reductase homolog1	Peroxisome	*Z. mays*	tr|A0A1D6KJ07	N-acyl-L-amino-acid amidohydrolase	cytoplasm and cytosol
tr|B4F7T9	Peroxidase	Secreted	*Z. mays*	tr|B6TBW4	ERBB-3 BINDING PROTEIN 1	None predicted
tr|Q9FQC5	Glutathione transferase	None predicted	*Z. mays*	tr|B6SSU6	Fructose-bisphosphate aldolase	None predicted
tr|B6T9P0	UDP-glucose 6-dehydrogenase	Cytosol	*Z. mays*	tr|B4FE30	10 kDa chaperonin	None predicted
tr|B6T4J1	50S ribosomal protein L6	Ribosome	*Z. mays*	tr|K7V1I3	E1 ubiquitin-activating enzyme	None predicted

tr|Q9SLP6	Ferredoxin--NADP reductase, chloroplastic	Chloroplast	*Z. mays*	tr|K7UKK5	Elongation factor G, chloroplastic	Chloroplast
tr|B4FS17	Thylakoid lumenal 29 kDa protein chloroplastic	None predicted	*Z. mays*	tr|B6TCK3	NADH-cytochrome b5 reductase	None predicted
tr|A0A1D6M323	Ribosomal protein	Mitochondrion	*Z. mays*	tr|B4G0G4	Protein BOBBER 1	None predicted
tr|B4FUA	Chlorophyll a-b binding protein, chloroplastic	Chlorophyll	*Z. mays*	tr|B4FRQ7	Proteasome subunit beta	cytoplasm and cytosol
tr|C0PC61	Transaldolase	Cytoplasm and cytosol	*Z. mays*	tr|Q9SLP6	Ferredoxin--NADP reductase, chloroplastic	Chloroplast
tr|B4FZ35	CHL-Zea mays Chloroplastic lipocalin	Thylakoid lumen	*Z. mays*	tr|B4F8M8	Exoglucanase1	Secreted
tr|A0A1D6I1V3	Phosphoenolpyruvate carboxylase	None predicted	*Z. mays*	tr|A0A1D6F8K6	RNA helicase	None predicted
tr|Q5QJA2	Harpin binding protein 1	None predicted	*Z. mays*	tr|B6TH55	Photosystem I reaction center subunit IV A	Chloroplast
tr|B4F9M9	Isocitrate dehydrogenase [NADP]	None predicted	*Z. mays*	tr|C4JAX7	UDP-sulfoquinovose synthase chloroplastic	Chloroplast
tr|B6T7B2	30S ribosomal protein S4, chloroplastic	Cytosol	*Z. mays*	tr|Q5EUD7	Protein disulfide-isomerase	Endoplasmic reticulum
tr|A0A1D6GDM6	DPP6 N-terminal domain-like protein	None predicted	*Z. mays*	sp|P02355	30S ribosomal protein S4, chloroplastic	Chloroplast
tr|B6TDR5	Geranylgeranyl reductase	None predicted	*Z. mays*	tr|B4FN21	Proteasome subunit beta	Nucleus

Appendix D

Table 3.6: Maize salt stressed common proteins expressed in all treatment conditions identified by LC-MS-MS.

Accession	Protein Name	Species Name	Subcellular location
tr|A0A1D6E4M0	Elongation factor G, chloroplastic	*Z. mays*	Chloroplast
tr|C0P702	(S)-2-hydroxy-acid oxidase	*Z. mays*	None predicted
tr|B4F9E2	Carbonic anhydrase	*Z. mays*	None predicted
tr|A0A1D6MY43	Beta-glucosidase 17	*Z. mays*	None predicted
tr|K7U6M0	NAD(P)-binding Rossmann-fold superfamily protein	*Z. mays*	None predicted
tr|Q9SLP6	Ferredoxin--NADP reductase, chloroplastic	*Z. mays*	Chloroplast
sp|Q6XZ79	Fructokinase-1	*Z. mays*	Cytosol
tr|Q9SAZ6	Phosphoenolpyruvate carboxylase	*Z. mays*	Cytoplasm and cytosol
tr|A0A096R6Z8	Heat shock 70 kDa protein 6 chloroplastic	*Z. mays*	Chloroplast
tr|C0P4Q3	Heat shock protein 90 kDa	*Z. mays*	Cytosol
tr|K7TL05	General regulatory factor2	*Z. mays*	Cytoplasm
tr|Q947B9	Glucose-1-phosphate adenylyltransferase	*Z. mays*	None predicted
tr|B6U0S1	Elongation factor 2	*Z. mays*	None predicted
tr|A0A096RZN2	Carbonic anhydrase	*Z. mays*	None predicted
tr|A0A1D6GQ42	Cell division cycle protein 48	*Z. mays*	None predicted
tr|B6TS21	Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial	*Z. mays*	Mitochondrion
tr|K7U9C9	RNA helicase	*Z. mays*	None predicted
tr|A0A1D6I6H5	Heat shock protein 90-5 chloroplastic	*Z. mays*	Chloroplast
tr|B4FZU8	Malate dehydrogenase	*Z. mays*	Mitochondrion
tr|B6TI78	Peptidylprolyl isomerase	*Z. mays*	Thylakoid
tr|B7ZXR4	Heat shock 70 kDa protein 8	*Z. mays*	Cytoplasm
tr|A0A1D6N1Z8	6-phosphogluconate dehydrogenase, decarboxylating	*Z. mays*	None predicted
tr|Q41815	Heat shock protein 26	*Z. mays*	None predicted
tr|Q9LKL4	Lipoxygenase	*Z. mays*	None predicted

tr|C0PDC7	Chaperone protein ClpB1	*Z. mays*	Cytosol
tr|C0P530	Chaperonin 60 subunit beta 2 chloroplastic	*Z. mays*	Cytosol
tr|B6U237	Heat shock 70 kDa protein 14	*Z. mays*	None predicted
sp|P04712	Sucrose synthase 1	*Z. mays*	None predicted
tr|A0A1D6ES19	Heat shock protein 90-2	*Z. mays*	Cytosol
tr|A0A1D6GDM6	DPP6 N-terminal domain-like protein	*Z. mays*	None predicted
tr|A0A1D6F5U0	Alpha-mannosidase	*Z. mays*	None predicted
tr|A0A1D6H703	Aminopeptidase	*Z. mays*	Endoplasmic reticulum
tr|A0A1D6N7I4	Putative mediator of RNA polymerase II transcription subunit 37c	*Z. mays*	Cytoplasm
tr|A0A096PQR7	Cytochrome P450 CYP74A19	*Z. mays*	None predicted
tr|B4FWP0	Fructose-bisphosphate aldolase	*Z. mays*	Cytosol
tr|C0PHR4	Adenosylhomocysteinase	*Z. mays*	Cytosol
sp|Q09054	Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic	*Z. mays*	Cytoplasm and cytosol
tr|A0A1D6EGC1	Aconitate hydratase	*Z. mays*	None predicted
tr|A0A1D6KCZ2	Alanine aminotransferase 2 mitochondrial	*Z. mays*	None predicted
tr|C4J4E4	Monodehydroascorbate reductase homolog1	*Z. mays*	Peroxisome
tr|B4FUH2	Aspartate aminotransferase	*Z. mays*	Mitochondrion
tr|B4F988	ATP-dependent zinc metalloprotease FTSH 6 chloroplastic	*Z. mays*	Chloroplast
tr|Q9SWR9	Acetyltransferase component of pyruvate dehydrogenase complex	*Z. mays*	Mitochondrion
tr|A0A1D6NFC1	ATP synthase subunit beta	*Z. mays*	Mitochondrion
tr|A0A1D6JXJ7	D-fructose-1,6-bisphosphate 1-phosphohydrolase	*Z. mays*	Cytosol
tr|B4F8L7	Glyceraldehyde-3-phosphate dehydrogenase	*Z. mays*	Chloroplast
tr|A1XCI1	Lipoxygenase	*Z. mays*	Chloroplast
tr|A0A1D6NVZ7	Transketolase	*Z. mays*	Cytosol
tr|A5GZ73	Glucose-1-phosphate adenylyltransferase	*Z. mays*	Chloroplast
tr|A0A1D6N0R5	Protein RETICULATA-RELATED 4 chloroplastic	*Z. mays*	Chloroplast

Appendix E

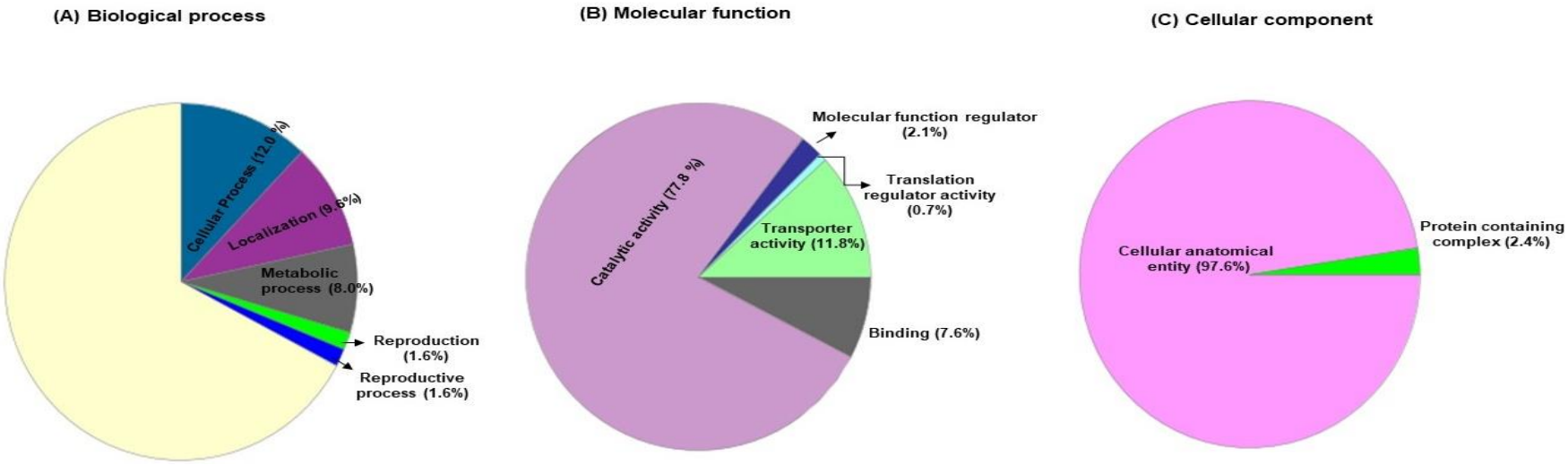

Figure 3.12: The functional classification of the control (water only) variable in maize proteins. **(A)** Biological process, **(B)** Molecular function and **(C)** Cellular component.

Appendix F

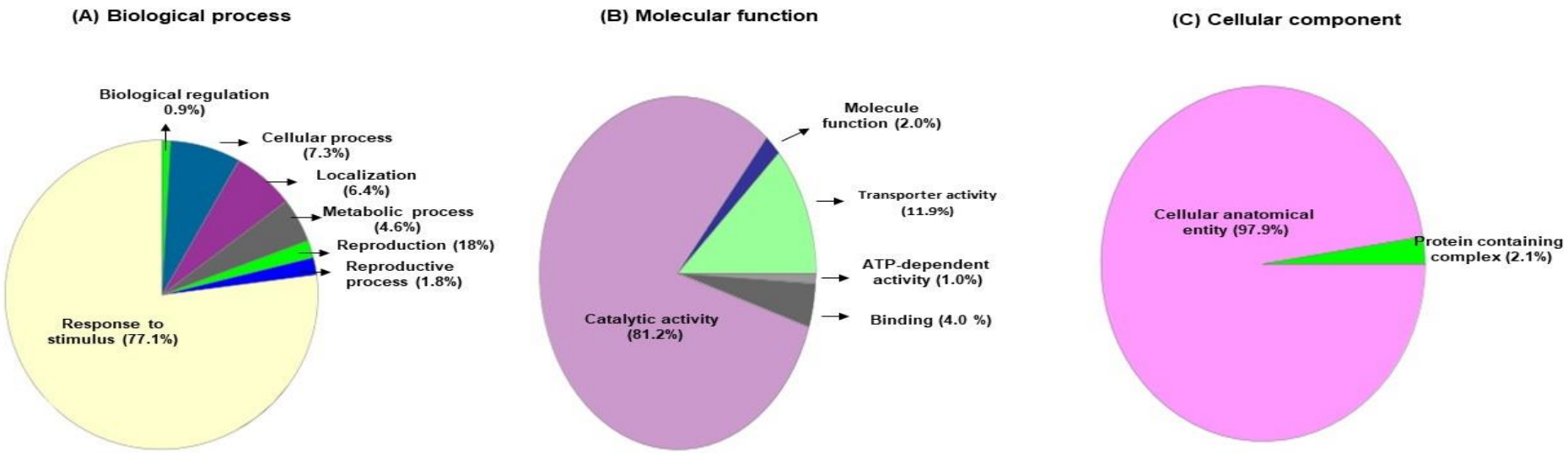

Figure 3.13: **The functional classification of maize proteins under 200 mM NaCl Concentration,** whereby: **(A)** Biological process, **(B)** Molecular function and **(C)** Cellular component.

Appendix G

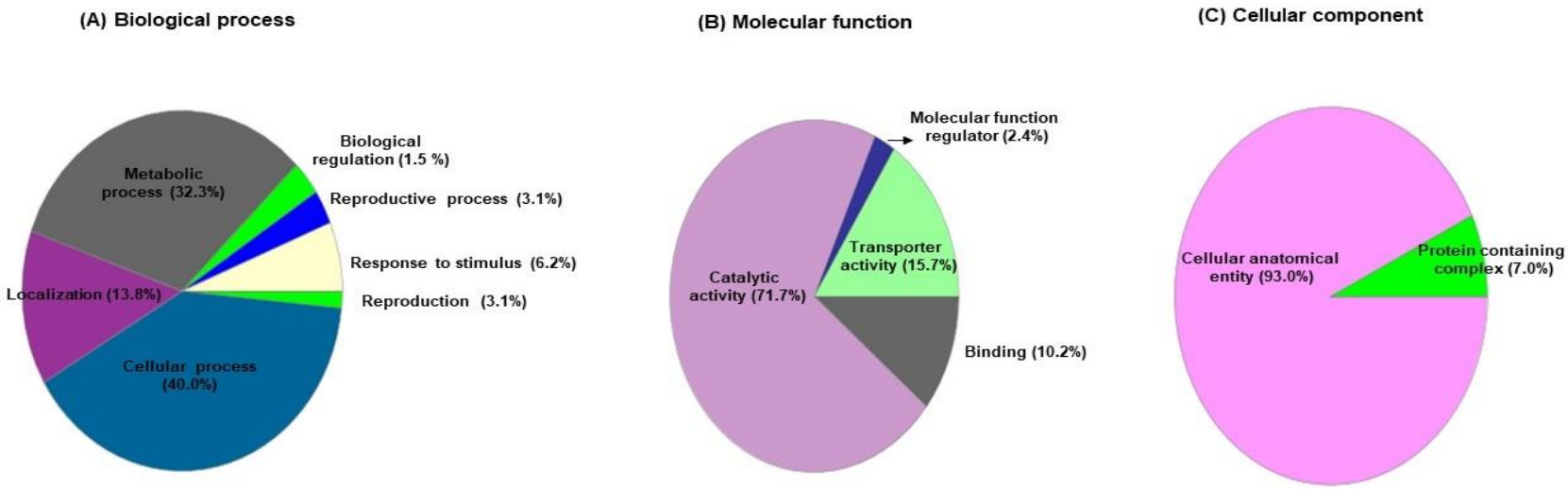

Figure 3.14: The functional classification of maize proteins under 300 mM NaCl Concentration, whereby: **(A)** Biological process, **(B)** Molecular function and **(C)** Cellular component.

Appendix H

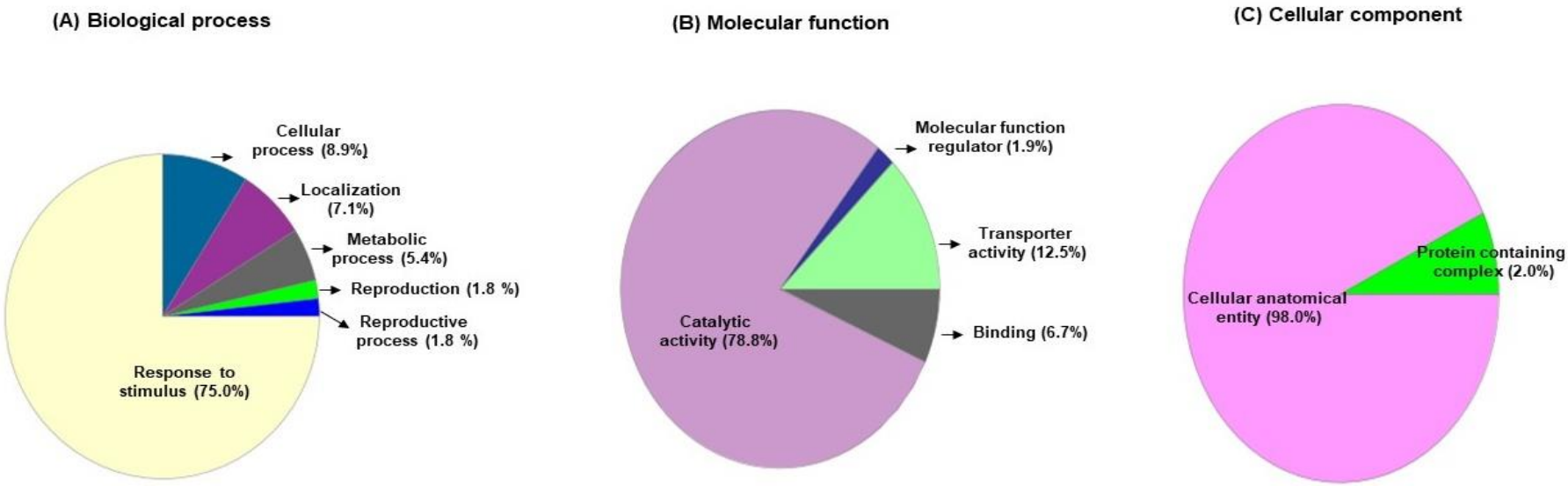

Figure 3.15: The functional classification of maize proteins under 400 mM NaCl Concentration, whereby: **(A)** Biological process, **(B)** Molecular function and **(C)** Cellular component.