Mitigation of Salinity Stress Using Exogenously Applied Molybdenum in *Sorghum bicolor*

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A thesis submitted in fulfilment of the requirement for the degree, Doctor of Philosophy (PhD) in Biotechnology, Department of Biotechnology, University of the Western Cape.

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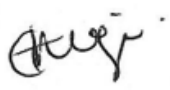
Signature:  Date: 23 May 2023

GENERAL ABSTRACT

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The agricultural sector is the main producer of food throughout the world. However, the constant changes in environmental conditions, such as extreme weather, droughts, and salinity have impacted this sector negatively over the years. These stresses cause nutritional imbalance, delayed seed germination and decreased growth resulting in reductions in crop yield and hence affect food prices. The food and agricultural organization (FAO), reported that the average increase rate in crop production is below the amount required to cater for the growing population. Thus, to meet the food demands, discovery of several strategies to improve crop growth and yield under severe environmental conditions are imperative. The overall aim of this study was to investigate the effects of exogenously applied molybdenum (Mo) to improve *Sorghum bicolor* growth under different levels of salinity. This was achieved by assaying certain traits in sorghum cultivated under salt and Mo treatment. Morpho-physiological attributes included assaying fresh and dry weights (FW and DW), water content (WC%), anatomical structure and photosynthetic pigments. Biochemical attributes including proline, soluble sugars, and carotenoids content and antioxidant enzymes [Superoxide dismutase (SOD) and Ascorbate Peroxidase (APX)] were assayed to further understand the morpho-physiological traits. Finally, the molecular response of sorghum to salt stress and Mo treatment was determined based on the expression of the heat shock protein-70 (HSP-70). Results (chapter 3), showed that exposure of sorghum plants to salt significantly affected their growth as observed by reductions in FW, DW, chlorophyll contents and changes in the structure of epidermal and vascular bundle layers. These changes were attributed to the

oxidative damage through increased ROS and lipid peroxidation. However, sorghum plants showed an efficient ROS-scavenging capacity by inducing high antioxidant targets, in particular, proline, soluble sugars, carotenoids, SOD and APX activities as well as the HSP-70 under salt treatment. Since Mo is used as part of the complex fertilizers in agriculture, and both Mo toxicity and deficiencies have been reported in literature. It was therefore crucial to determine the effects of Mo on sorghum plants under non-stress conditions. Results (chapter 4) showed that various Mo concentrations (0.5 µM, 1 µM and 2 µM) had differential effects on the growth of sorghum plants. The lowest (0.5 µM Mo) concentration did not induce any significant negative effects such as reduction in FW and overproduction ROS stains on sorghum leaves. In addition, SOD and APX activities were increased by all Mo treatments, but 0.5 µM was considered non-toxic for sorghum plants growing under non-stress conditions. The alleviatory effect of Mo on sorghum plants under different salt stress levels was demonstrated in chapter 5. Results showed that 0.5 µM Mo successfully reversed all the negative effects of salt stress on sorghum plants by reducing oxidative damage as seen by well arranged epidermal and vascular bundle layers, low ROS stains on sorghum leaves, low H_2O_2 and MDA contents. This was attributed to the increased SOD and APX activities as well as high HSP-70 expression under salt and Mo treatment. Results in (chapter 6) showed that Mo-induced salt stress tolerance in sorghum, was inhibited by tungsten, a known chemical antagonistic to Mo activity. This was observed by reduced growth attributes, increased ROS and lipid membrane damage and reduced soluble sugars and carotenoids due to a combination of tungsten and Mo, especially at high concentrations (1 µM and 2 µM). The study concluded that low Mo concentrations were effective in reducing salt-induced oxidative damage to sorghum plants by regulating ROS detoxification and induced HSP-70 expression.

Keywords: Antioxidants, HSP-70, Molybdenum, NaCl, Oxidative stress, ROS, *Sorghum bicolor.*

DEDICATION

This is a dedication to my daughters **Sisipho** and **Uyakona**, you came into this world and automatically became part of this journey with me. May you grow up to know that you can achieve anything in life with God by your side.

Philippians 4:13

Mommy loves you.

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CHAPTER ONE
RESEARCH RATIONALE AND LITERATURE REVIEW

1. Research rationale

1.1. Background

Sorghum [*Sorghum bicolor* (L.) Moench] is one of the most important cereal crops, and is ranked as the 5th in the world and the 2nd in Africa after *Zea mays.* It is mainly produced in tropical and sub-tropical regions (Andiku et al., 2021; Davis et al., 2019). The leading sorghum producers in the world are China, India, United States of America and Mexico (Andiku et al., 2021). In Sub-Saharan Africa (SSA), the fourth leading countries of sorghum production are Nigeria, Ethiopia, Sudan and Niger, with South Africa at the 19th position (FAOSTAT, 2022). Sorghum is grown for food, feed and bioenergy production (Kimber et al., 2013). Sorghum is mostly cooked and eaten, but it can also be processed and used as ingredients for other dishes. It is rich in calories, protein, fat, carbohydrates, fiber in addition to micronutrients such as iron, potassium, vitamin B6, phosphorus, manganese and magnesium (McGinnis et al., 2020). Despite the adaptability of this crop to drought and its importance as a diet ingredient, its production has been facing some challenges exerted by biotic and abiotic factors including diseases, insects, low soil fertility, heavy-metal, drought and salinity (Omoro, 2013; Derese et al., 2018).

Salinity is one of the major constraints of sorghum production in Sub-Saharan Africa since 5% of its land is already salinised (Tadele, 2017). High salinity levels negatively affect plant growth and development, especially in arid and semi-arid regions of the world (Hussain et al., 2019). It impacts plant growth and development by imposing osmotic and ionic stress, due to excessive uptake of toxic ions such as sodium and chloride (Isayenkov & Maathuis, 2019). Plants have developed various adaptive mechanisms to cope and prevent injury caused by

stressful environmental conditions, which is achieved by triggering a series of physiological and biochemical responses (Seleiman et al., 2021). These strategy includes the accumulation of low molecular mass scavengers or molecules (proline, soluble sugars, glycine betaines) together with the enzymatic and non-enzymatic (ascorbate, flavonoids, carotenoids and glutathione) antioxidant defence system, which play important roles in protecting cells from oxidative damage (Taïbi et al., 2016). Antioxidants especially, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidases (POD), are among the main antioxidant defence systems in plants (Hussain et al., 2019) and their levels are generally found to be increased in plants under stressful conditions (Hasanuzzaman et al., 2020).

In order to enhance productivity of plants exposed to saline environments, it is important to understand the mechanism of salinity tolerance in order to develop plants with a better response to this abiotic stress. The exposure of plants to stressful conditions such as salt stress can affect the physiological activities of plants, which can have deleterious effects on plant growth and development, hence lead to crop loss (Ma et al., 2019; Meringer et al., 2016). Unlike most cereals, sorghum has been found to be moderately tolerant to drought and salinity. This makes sorghum a great potential for it to serve as a food source in both dry and saline regions of the world. Several studies have been reported on the mitigation of salinity on sorghum by leaching through irrigation to reduce salt stress (Calone et al., 2020). Some of them include the use of biochar soil amendment methods (Videgain-Marco et al., 2020), exogenous application of other chemicals such as chitosan (Mulaudzi et al., 2022), zinc oxide nanoparticles (Rakgotho et al., 2022) calcium chloride (Mulaudzi et al., 2020), sulfuric acid, iron sulfate and aluminum sulfate (Provin & Pitt, 2001). Certain amounts of nitrogen fertilizers (75 kg/fed) improved the agronomic traits of sorghum under alkaline conditions as well (Adam & Taleim, 2018).

As a stress resistance element in higher plants, ammonium molybdenum ($[(NH_4)_6Mo_7O_{24}.4H_2O]$; Mo) has also been extensively reported to contribute to the improvement of plant tolerance to biotic and abiotic stresses (Al-Issawi et al., 2016; Rana et al., 2020). Molybdenum (Mo) is a micronutrient, usually required by plants in very small quantities for normal physiological and metabolic activities. It is generally used as a component of complex fertilizers in areas where the natural Mo content is not sufficient and plant development is affected; however, high concentrations can be detrimental to the plant. Molybdenum deficiency in soils is a widespread agricultural problem that results in low crop yields and crop loss (Bagale, 2021). Several studies have reported on the effective use of Mo to alleviate the effects of both biotic and abiotic stresses to plants (Babenko et al., 2015; Zhang et al., 2014). It is important to note that the role of exogenous Mo has never been demonstrated in sorghum.

1.1.2. Problem statement and significance of the study

Salinity is one of the major abiotic stresses that affect crop production across the world. The effects of salinity have been recorded to cause deleterious effects on agricultural productivity from seed germination to crop production. It is predicted that over 50% of the arable land will be salinized by the year 2050 (Butcher et al., 2016; Asad et al., 2021; Moukhtari et al., 2020). Crop production has been greatly affected by salinity with an average reduction yield loss of 50-80%, with great loss for most farmers over the years (Zorb et al., 2019). This calls for an urgent need to ensure food security by employing alternative approaches to increase crop productivity and fertility in saline environments. The use of exogenous compounds, which are eco-friendly, is one of the suitable approaches to overcome the negative effects of salt stress on crop productivity.

Molybdenum (Mo) is a micronutrient that has been widely used as a fertilizer to induce stress resistance when applied exogenously to the soil (Zhang et al., 2012). Molybdenum deficiency

in the soil is a widespread agricultural problem that results in low crop yields and crop loss (Rana et al., 2020; Bagale, 2021). As a moderately salt tolerant cereal crop, the exogenous application of Mo to salt-stressed sorghum plants will provide significant data towards the improvement of crop yield and quality. The results obtained from this study can be used as a building block for future studies in plant growth in order to improve productivity under stressful environments. It is important to note that this is the first study to investigate the potential of exogenously applied Mo on sorghum plants as a stress alleviator.

1.1.3. Hypothesis

- Salinity can have deleterious effects on *Sorghum bicolor* growth.
- Exogenous application of molybdenum can reverse the negative effects of salinity on sorghum plants.

1.1.4. Aims and objectives of the study

The aim of the study was to investigate the role of exogenously applied molybdenum (Mo) on the morphological, physiological, biochemical and molecular responses of *Sorghum bicolor* plants under salt stress conditions. The following objectives were conducted:

1. To assay *Sorghum bicolor* plants grown in the absence (untreated) and presence of different sodium chloride concentrations.

2. To assay *Sorghum bicolor* plants grown in the absence (untreated) and presence of different Mo concentrations.

3. To study the effect of Mo in *Sorghum bicolor* plants grown in the presence of different sodium chloride concentrations.

4. To study the effect of tungsten on the activity of Mo in *Sorghum bicolor* plants under salt stress.

1.2. LITERATURE REVIEW
EFFECT OF SALINITY ON PLANTS, TOLERANCE STRATEGIES AND THE ALLEVIATORY ROLE EXOGENOUSLY APPLIED MOLYBDENUM

1.2.1. Introduction

Plants are constantly exposed to a wide range of stresses mainly biotic factors such as pathogen, fungi and bacteria and abiotic factors including drought, salinity, heat, cold and heavy metals. Among the abiotic factors, salinity is one of the most detrimental environmental stresses, which cause a major reduction in crop production worldwide (El-Nahrawy, 2022). It has been estimated that 20% of total cultivated and 33% of irrigated agricultural lands are affected by salinity worldwide (Kumar & Sharma, 2020). Furthermore, it has been estimated that more than 50% of the arable land would be salinized by the year 2050 (Molotoks et al., 2021). South Africa has about 13-18% irrigated lands that are affected by salinity and sodicity (Mohanavelu et al., 2021), this will put a major strain on agriculture to come up with strategies of producing food under these environmental changes.

Salinity affects plant growth by inducing osmotic and ionic stress, this results in the overproduction of toxic reactive oxygen species (ROS). The overproduction of ROS can cause oxidative damage to cell organelles and membrane components and in severe cases can result in plant cell death (Hasanuzzaman et al., 2021). In order to minimize the toxic effects of ROS, plants have developed different coping strategies according to the level of stress in order to maintain cellular redox homeostasis (Huang et al., 2019). These include the accumulation of low molecular weight compounds known as osmo-protectants, which include proline, glycine betaine and soluble sugars among others. The activation of enzymatic and non-enzymatic antioxidant compounds, ion homeostasis, expression of stress responsive genes and

transcription factors (Huang et al., 2019; Ma et al., 2019; Mulaudzi et al., 2020; Mulaudzi et al., 2022) amongst other coping stratregies.

The application of various exogenous substances has been widely used to improve plant tolerance to salt stress conditions (Rakgotho et al., 2022; Rashid et al., 2021) and this is one of the strategies that require further attention. Molybdenum is a micronutrient that is required by plants in very small quantities for normal growth as well as enhancing the availability of nutrients to plants (Rana et al., 2020). Molybdenum deficiency can be lethal to plant growth, by decreasing photosynthesis resulting in pale green or yellowish green leaves resembling nitrogen deficiency.

The cultivation of crops with enhanced adaptations to a wide range of biotic and abiotic stresses is a great challenge for plant scientists around the world. This is crucial for the maintenance of food production in order to secure food supply for the world. Food insecurity is defined as the state when people do not have adequate physical, social or economic access to food (FAO, 2010). With the world population being estimated to increase by 2 billion in the next 28 years there is therefore, greater need to increase food production required to sustain the growing population (FAO, 2019; Chaves & Davies, 2010). Major climate changes affect agriculture and food production, leading to an increase in global hunger and starvation (Figure 1.1). The constant changing climates negatively affect food security as it has a direct impact on major crop production (FAO, 2018).

Cereal crops constitute a major role as staple food in the diet of people, especially in developing countries (Joshi et al., 2016). Food security depends on the increased production of important cereal crops such as *S. bicolor* that are well able to grow in unfavourable environments to fulfil the food supply for the projected population.

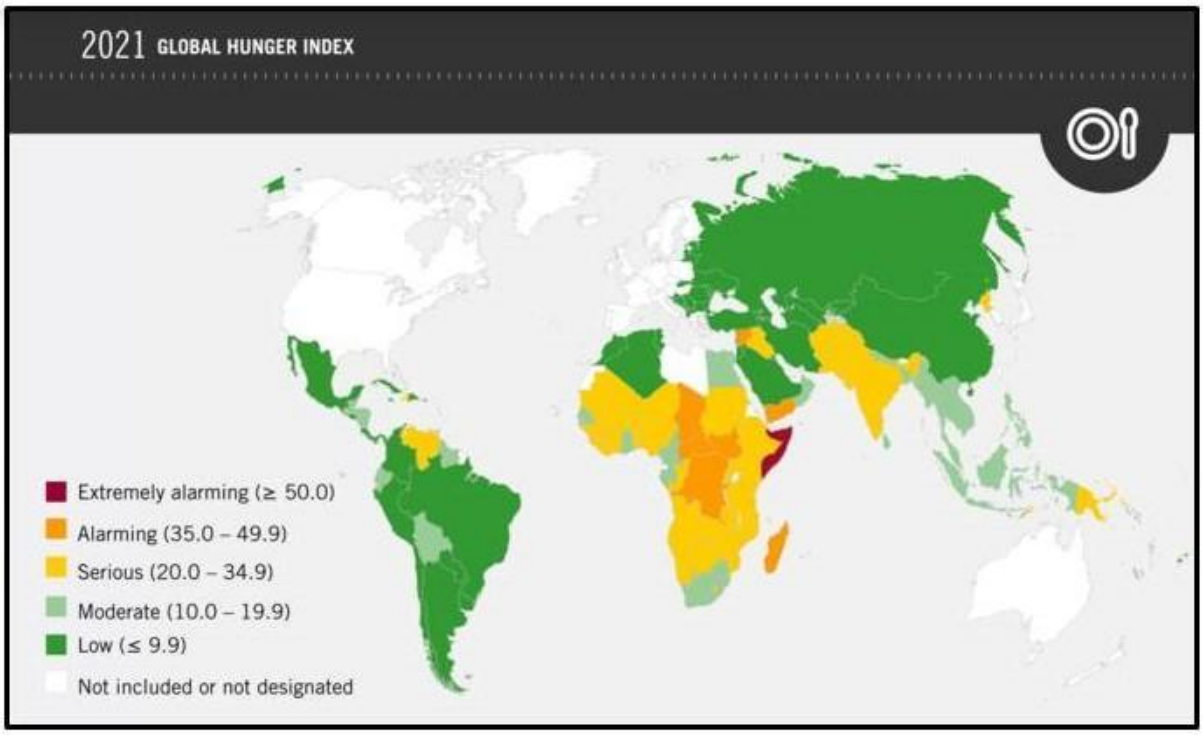

Figure 1.1. Global hunger index map. Showing the African continent to be most affected with Somalia suffering from extreme levels of hunger with moderate levels in South Africa (Adapted from Weltungerhilfe, 2021).

For the successful development of crops that are well adapted to a wide range of environmental conditions, it is important to understand the effects of stress on crops and their mechanism of stress tolerance towards improving food security. In the current review we focused on recent advances in understanding the mechanisms of plants response to environmental stresses (*e.g* salinity) at the morpho-physiological (plant height, biomass and relative water content, photosynthesis), biochemical (activation of osmolytes and antioxidants biosynthesis) as well as molecular (gene and protein expression) levels. In addition, this review will also highlight how the application of exogenous molybdenum enhances tolerance of crops to salt stress conditions.

1.2.2. *Sorghum bicolor*

Sorghum bicolor Moench (L) is a member of the Poaceae family commonly referred to as sorghum. Although sorghum is currently being cultivated throughout the world, it is believed to have originated in North East Africa (Prażak, 2016). Sorghum is ranked the 5th most important cereal crop in the world after *Oryza sativa, Zea mays*, *Hordeum vulgare* and *Triticum aestivum* and ranked 2nd in Africa after maize (Adebo, 2020). Sorghum is a naturally drought tolerant crop with moderate tolerant traits to salt conditions (Amombo et al., 2022; Ropelewska & Nazari, 2021). The crop is mostly cultivated in arid and semi-arid regions throughout the world (Ogbaga et al., 2014). Because of its small genome size of ~730 megabase (Mbp), that is also sequenced, these characteristics makes sorghum an attractive model in monocots for studies in understanding the molecular responses of cereal crops to different stresses (Paterson et al., 2008; Basso, 2021; Li et al., 2021).

1.2.2.1. Utilization of sorghum

Sorghum has also been reported as one of the main staple food crops for the world's poorest and food insecure population (Pereira & Hawkes, 2022). The crop has been used by humans for a variety of applications such as forage for livestock, starch, biofuel, paper and wax production. The grain of sorghum is processed to produce food products including porridge, flour, syrup, sugar and vegetable oil as shown in Figure 1.2 (Prażak, 2016). In addition, sorghum has also been used in other industries such as the health industry. Sorghum being a gluten free plant, it has been highly recommended for gluten intolerant patients. Being a low-calorie food makes it beneficial for individuals struggling with obesity, diabetes and cardiovascular diseases and having a rich source of antioxidants, which helps in reducing oxidative stress (Rezaee et al., 2021).

1.2.2.3. Tolerant traits of sorghum

Sorghum also possesses a variety of anatomical and physiological features that enable it to grow in water limited environments where other crops are not able to grow. The anatomical features include the development of deep roots system and the alterations in leaves that can roll up during moisture stress conditions, which help with the efficient water use of the crop (Mwamahonje et al., 2021). Furthermore, it also has internodes that are covered by thick waxy layers that reduce transpiration and increase drought tolerance. The stems of sorghum plants may reach up to 4 m height, with small grain of 3 - 4 mm in diameter (Figure 1.2b) (Dourado et al., 2022). The physiological adaptations are mediated by changes in stomatal density to maximize water uptake, and the ability to increase net carbon assimilation in stressful conditions via the C_4 photosynthesis (Dourado et al., 2022).

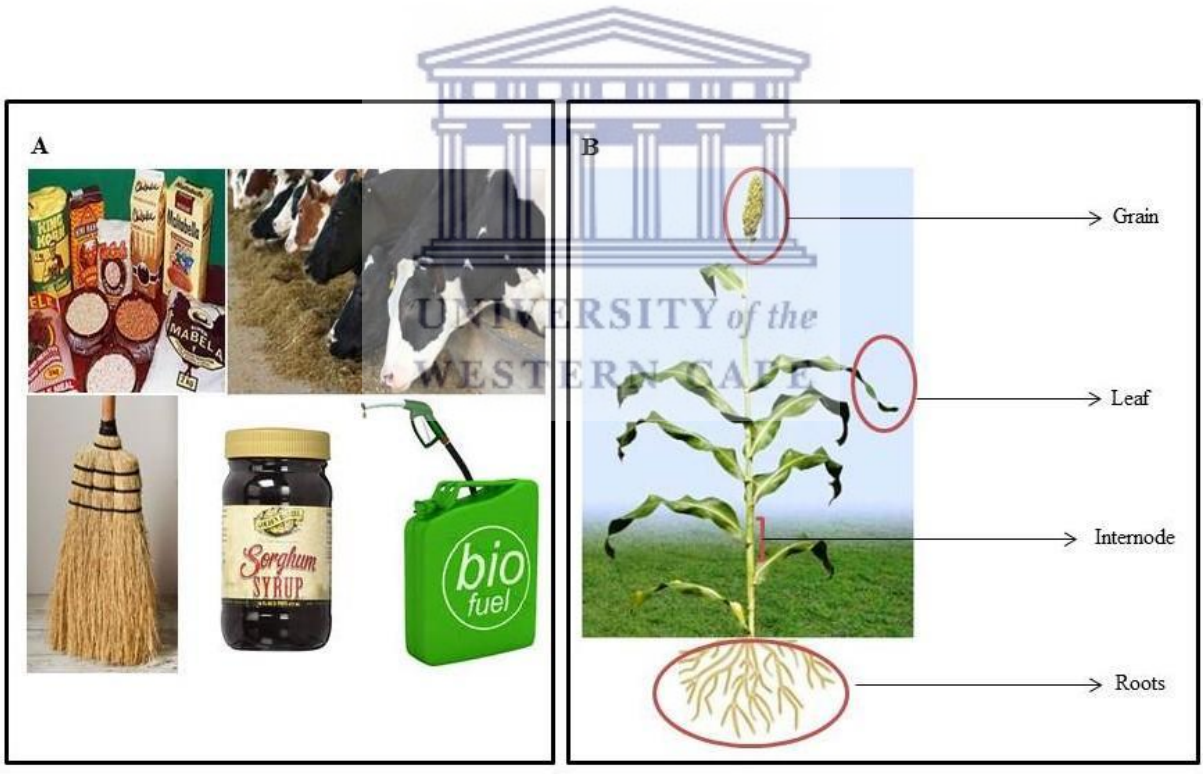

Figure 1.2. Products that can be obtained from sorghum and the growth of sorghum plants in the field. Different products obtained from *Sorghum bicolor* plants and (**A**) anatomical features of sorghum plants (**B**) (Modified image adapted from Upadhyaya et al., 2008).

1.3. Effect of stress in plants

Stress can be grouped into two main categories namely biotic and abiotic stress. Biotic stress is a stress that usually occurs when other living organisms such as pathogens (fungi and bacteria), insects, and plant parasites induce damage to plants resulting in the impairment of the normal metabolism of the plant. These can lead to reduced plant vigor and in extreme cases, death of the host plant (Anzano et al., 2021). Abiotic stress is the negative effect caused by non-living factors on living organisms, and pose a threat to agriculture as it negatively affects plant survival and productivity accounting for crop loss (Teshome et al., 2020). The abiotic stress factors include temperature, drought, water logging, salinity, heavy metals and other extreme environmental factors (He et al., 2018). Among these stresses, salinity is the most stubborn stress commonly seen as salinization of arable land. Based on the scope of this study, the effect of salinity will be further elaborated, to include its nature and the different tolerant mechanisms used by plants.

1.4. Salinity

Salinity is one of the severe environmental stresses affecting crop production in many parts of the world (Shrivastava & Kumar, 2015). High salt concentrations in the soil generate low water potential, reducing the ability of a plant to utilize water as well as nutrients. This causes a reduction in plant growth rate, decreases in the marketable yield, due to decreased productivity as well as changes in plant metabolic processes. The amount of the world's agricultural land affected by salt accumulation each year is estimated to be ~10 million ha (Machado & Serralheiro, 2017). The high rate of salinity can be accelerated by climate change, excessive use of ground water, increased use of low-quality water and introduction of irrigation from farming and poor drainage systems (Machado & Serralheiro, 2017). Salinity in the soil is usually composed of a range of dissolved salts, which include sodium chloride (NaCl), sodium sulfate (Na_2SO_4), magnesium sulfate ($MgSO_4$), calcium sulfate ($CaSO_4$),

magnesium chloride ($MgCl_2$), potassium chloride (KCI) and sodium carbonate (Na_2CO_3) each contributing to salinity stress (Provin & Pitt, 2001). Excess sodium (Na) in the soil will compete with other cations such as calcium ions (Ca) and potassium ions (K) to reduce their availability to crops and eventually reduce yield. Increased NaCl concentrations have been reported to induce increases in Na and Cl ions in a number of plants (Tavakkoli et al., 2011; Debouba et al., 2006; Viégas et al., 2001). The effect of salinity on crop production is more severe in arid and semi-arid regions where there is limited rainfall.

Salinity has been affecting the agricultural industry as it has major constraints on crop growth, development and production (Otayk, 2020). The impact of salinity on crops differs amongst species, growth and developmental stages. Several authors reported on the harmful effects of salt stress on plants, such as reductions in photosynthesis as observed in *Salix alba* leaves (Ran et al., 2021), delayed germination and reduced growth in *S. bicolor* seedlings (Mulaudzi et al., 2020; Rakgotho et al., 2022). Similarly, Rahnessha et al., (2018) also reported a reduction in soluble saccharides in the roots and shoots of two *Pistacia vera* cultivars at high salt concentrations. According to Ludwiczak et al., (2021), salt causes osmotic and ionic stress on plants and most of the response of plants to salinity is linked to these effects (Figure 1.3).

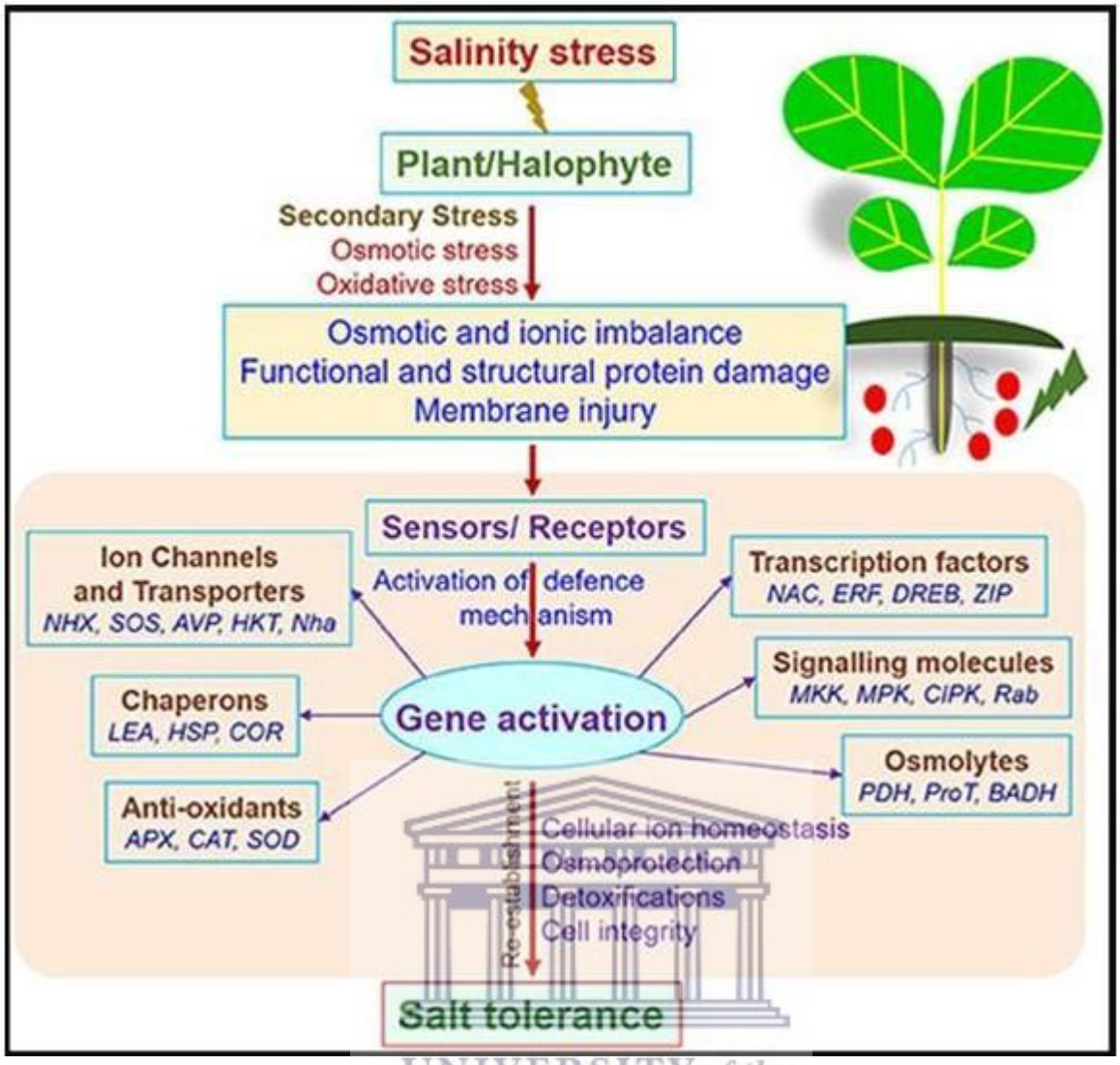

Figure 1.3. Salt stress tolerance in plants. A representation of salinity stress tolerance in plants (Adapted from Mishra & Tanna, 2017).

1.4.1. Osmotic and ionic stress

Salt causes two types of stress in plants, namely osmotic and ionic stress. Osmotic stress is the result of changes in the water potential in a plant's environment, which affects the normal activities of a plant and in extreme cases, can result in plant death (Darko et al., 2019). The roots are normally the first part of a plant to encounter salt stress and result in the inhibition of water uptake, cell expansion and lateral bud development, causing changes in water movement across the membrane (Munns & Tester, 2008). After these conditions, ionic stress

occurs in the plant, when toxic ions such as Na and Cl accumulate in cells causing an increase in leaf mortality, chlorosis, necrosis and decrease in the activity of cellular metabolism including photosynthesis. High concentrations of NaCl in the soil disturb the ionic balance of essential nutrients such as K, Mg, and Ca leading to nutrient deficiency, which can also result in reduced energy production in plants (Shrivastava & Kumar, 2015).

When plants are exposed to stressful conditions such as salinity, they result in increased levels of ROS. Reactive oxygen species include the free radicals like hydroxyl radical ($OH^{\bullet}$), superoxide ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$) as well as the non-radical, hydrogen peroxide (H_2O_2). Under favourable conditions ROS are generated at basal levels; however, their levels increase when plants are exposed to different types of biotic and abiotic stresses (Huang et al., 2019; Sachdev et al., 2021). When the levels of ROS exceed the range of scavenging, plant cells will experience oxidative stress (Huang et al., 2019; Sharma et al., 2012) and depending on the severity it can be very harmful. Oxidative stress results in the damage of macromolecules within cells such as proteins, lipids and nucleic acids resulting in the loss of physiological capacity leading to cell death (Chaki et al., 2020). For most part, ROS have been considered as the dangerous molecules in plants, when overproduced, however research advance has shown ROS to play important signalling roles when available at physiological levels (Checa & Aran, 2020; Turkan, 2018).

In order to maintain cellular homeostasis, the levels of ROS have to be tightly regulated through different mechanisms that allow adaptation and increase plant survival (Schützendübel & Polle, 2001).

1.5. Mechanism of salt tolerance

Upon exposure of plants to stressful conditions, plants exhibit a complex of responses, which include physiological, biochemical, genomic and molecular changes. Some of these responses of plants can be as a result of osmotic stress signals, while others may be due to secondary stress caused by primary signals such as phytohormones including, abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) (Xion & Zhu, 2002) amongst others.

The physiological appearance of plants exposed to salt stress share many similar features to drought stress, both drought and salt stress inhibits the absorption of water due to osmotic effects (Ma et al., 2020). Increased exposure of plants to salt stress conditions, reduces stomatal conductance, disrupt photosynthesis and results in decreased turgor pressure, causing cell walls to shrink and eventually reducing the size, fresh weight and water content of the plant (Hannachi et al., 2022; Munns & Tester, 2008). It can be stated that the general physiological response of plants to salt stress is the reduction in growth due to osmotic stress (Munns, 2002). Plants can adjust their water potential through osmotic adjustment by synthesizing compatible organic solutes known as osmo-protectants or osmolytes as a defence mechanism to cope with stress (Zulfiqar et al., 2019). The accumulation of antioxidants also plays critical roles in maintaining and reducing oxidative damage to plants (Mansoor et al., 2022).

1.5.1. Compatible solutes

Compatible solutes include amino acids, sugars, glycine betaines and polyols amongst others. They are low molecular weight and highly soluble compounds that are not toxic to plants even at high concentrations (He et al., 2018). Compatible solutes are one of the most common stress tolerance strategies in plants. They contribute towards osmotic adjustment, detoxification of ROS and stabilization of membranes (Hou et al., 2021). The accumulation of osmo-

protectants is usually favoured under water deficit or salt stress environments in plants and these compounds function to increase the ability of a cell to retain water without affecting the normal metabolism of the plant (Zulfiqar et al., 2019).

1.5.1.1. Proline

Proline is one of the osmolytes that has received much attention and it regulates osmotic adjustment and maintains cellular turgor under stressful conditions. Proline also plays an important role as a ROS scavenger, metal chelator, signalling molecule, protein stabilizer and inhibitor of programmed cell death (Hosseinifard et al., 2022). Proline biosynthesis in plants usually occurs in the chloroplast and cytoplasm, whereas degradation occurs in the mitochondria (Szepesi & Szőllősi, 2018). Biosynthesis of proline in plants takes place via two pathways namely the glutamic acid and ornithine pathway. The glutamic pathway is the main pathway, whereas the ornithine pathway is considered an alternative pathway. Biosynthesis of proline via the glutamate pathway occurs during osmotic stress in plants where two enzymes carry out biosynthesis of proline from glutamate. In this pathway, glutamate (Glu) is reduced to glutamate-1-semialdehyde (GSA) by delta-1-pyrroline-5-carboxylate synthetase (P5CS) and then converted to delta-1-pyrroline-5-carboxylic acid (P5C). This is then followed by the second enzyme delta-1-pyrroline-5-carboxylate reductase (P5CR) that reduces P5C to proline. Degradation of proline in the mitochondria occurs when proline dehydrogenase (PDH) and P5C dehydrogenase (P5CDH) convert proline to glutamate (Guan et al., 2020; Hosseinifard et al., 2022).

The positive influence of the exogenous application of proline has been widely reported in plants in response to various abiotic stresses, which include drought (Ghafoor et al., 2019), salinity (Tabssum et al., 2019) and heavy metals stress (Wang et al., 2022) amongst others. Such applications may trigger stress prevention mechanisms contributing to stress tolerance

of plants. For example, exogenous proline caused a significant increase in the physiological attributes, yield and anatomical traits of *Lupinus polyphyllus* varieties in comparison to their controls (Rady et al., 2016). Similarly, rapid accumulation of free proline in plants such as *Oryza australiensis* (Nguyen et al., 2021), *Sorghum bicolor* (Mulaudzi et al., 2020; Mulaudzi et al., 2022; Rakgotho et al., 2022), *Zea mays* (Perveen & Nazir, 2018) was observed in salt-stressed plants, which is mainly the result of better nutrient balance, water uptake and nitrogen fixation in the plants.

1.5.1.2. Soluble sugars

The accumulation of soluble sugars such as sucrose, glucose and fructose has also been widely studied in plants in response to environmental stresses (Kumar et al., 2017). Soluble sugars function as structural constituents of cells and like hormones they act as primary messengers and regulate signals that control expression of different genes that are involved in plant growth and metabolism (Rosa et al., 2009). Several studies have shown the accumulation of soluble sugars and other osmolytes to provide increased tolerance of plants to abiotic stresses (Saibi et al., 2020; Bouazzi et al., 2019). Their increased levels protect plants, minimize membrane damage and rescue plants from stress induced damage (Jogawat, 2019). Exogenous application of glucose and sucrose alleviated the inhibitory effects of salt stress on the growth of *Triticum* x *Secale* seedlings (Wang et al., 2019), also trehalose application enhanced salinity stress tolerance in *Triticum aestivum* plants by improving their growth and physiological attributes (Sadak, 2019), thus showing the protective role of soluble sugars in plants under stress.

1.5.2. Antioxidants

Plants have developed different coping strategies in order to overcome the harmful effects of ROS, through the activation of the antioxidant defence system, which maintains cellular redox status that is critical for various biological activities. The antioxidant defence system can be divided into two main systems; namely, enzymatic and non-enzymatic antioxidants.

1.5.2.1. Enzymatic antioxidants

The overproduction of ROS due to stress requires efficient detoxification to minimize the harmful effects for normal growth and increase plant survival (Schützendübel & Polle, 2002). The enzymatic antioxidant enzymes play important roles in scavenging free radicals and help maintain low levels of ROS. The major antioxidant defence enzymes include superoxide dismutase (SOD), catalase (CAT), ascorbic peroxidase (APX), peroxidase (POX), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Maury et al., 2020; Rajput et al., 2021).

1.5.2.1.1. Superoxide dismutase

Superoxide dismutase (SOD EC 1.15.1.1) is an antioxidant enzyme produced in both eukaryotic and prokaryotic cells. They are commonly found in the chloroplast, mitochondria, peroxisomes and cytoplasm of higher plants in three isoforms based on the metal ion they bind, Mn-SOD, Fe-SOD and Cu/Zn-SOD (Leonowicz et al., 2018). Superoxide dismutase provides the first line of defence against toxic ROS generation (Gill & Tuteja 2010), by scavenging superoxide radicals to ($O_2^{\bullet-}$) and H_2O_2, thus providing defence against ROS. Generally, antioxidant enzymes like SOD are normally found to increase in plants under

unfavourable environmental conditions such as salinity. Increased SOD activity was observed in a number of plants under salt stress conditions such as *Zea mays* (AbdElgawad et al., 2016), *Sorghum bicolor* (Rakgotho et al., 2022), *Hordeum vulgare* (Quertani et al., 2021), *Oryza sativa* (Rossatto et al., 2017) and *Triticum aestivum* (Tounsi et al., 2017) indicating a better adaptive response to salt stress.

1.5.2.1.2. Catalases

Catalases (CAT; EC 1.11.1.6) are haem containing enzymes present in almost all aerobic organisms. They are predominantly found in the peroxisomes, but also exist in the mitochondria and cytoplasm of cells. The haem group of CAT is the major component for enzyme activity (Sharma et al., 2012). Catalases function by degrading two molecules of H_2O_2 into water and oxygen (Karakus, 2019). The significance of CAT in antioxidant defense was observed in *Hordeum vulgare* salt-stressed plants (Zahra et al., 2020). Similarly, salt tolerant *Sorghum bicolor* cultivars showed enhanced antioxidant activities, including CAT, POX and SOD under salt stress conditions (Zhang et al., 2020). Catalases have the fastest turnover rate of all the enzymes (Smejkal & Kakumanu, 2019); however; they have a lower affinity to H_2O_2 than ascorbate peroxidase.

1.5.2.1.3. Ascorbate peroxidase

Ascorbate peroxidases (APX, EC 1.11.1.11) are other haem containing enzymes present in different cellular compartments including chloroplast, mitochondria, peroxisomes and cytosol with different isoforms classified according to their subcellular localization (Ozyigit et al., 2016; Caverzan et al., 2012). The overexpression of APX in each of these subcellular compartments confers tolerance against multiple stresses. It has been demonstrated that the overexpression of APX in the roots of *Zea mays* seedlings with increasing NaCl

concentrations, increased the plants tolerance to stress (AbdElgawad et al., 2016). Similarly, Kim et al., (2005) also showed the overexpression of APX in the roots and shoots of *Hordeum vulgare* plants to exhibit enhanced tolerance to salt stress after 24 hours of treatments. Ascorbate peroxidases are one of the most important enzymes for the detoxification of H_2O_2, which prevent toxic levels of ROS by reducing H_2O_2 into water using ascorbate as an electron donor (Kaur et al., 2021). The detoxification of H_2O_2 by APX involves a set of reactions that are catalysed by monodehydroascorbate reductase (MDHAR; EC. 1.6.5.4).

1.5.2.1.3.1. Ascorbate-Glutathione cycle (AsA-GSH)

The AsA-GSH pathway or Asada-Halliwell pathway is a major pathway in the antioxidant defense system for the detoxification of H_2O_2 (Hasanuzzaman et al., 2020). The pathway takes place in the cytosol, nucleus, chloroplast and mitochondria where both AsA and GSH are found. In this pathway APX converts H_2O_2 into H_2O with the help of AsA as an electron donor, which is also converted into monodehydroascorbate (MDHA). Monodehydroascorbate will regenerate AsA by the activity of monodehydroascorbate reductase (MDHAR), which will be converted into dehydroascorbate (DHA). Dehydroascorbate is reduced to AsA again by glutathione synthase (GSH), which will result in the oxidation to produce glutathione disulphide (GSSG). The GSSG produced will regenerate GSH by the activity of glutathione reductase (GR) using nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate + hydrogen [NADP (H)] as electron donor (Rajput et al., 2021).

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1.5.2.1.4. Peroxidase

Peroxidases (POX; EC. 1.11.1.7) are haem containing enzymes that are ubiquitous in nature. Plant peroxidases are mainly found in the apoplast and vacuoles (Takabe et al., 2001). Apart from their role in scavenging H_2O_2, plant peroxidases are involved in numerous cellular

processes such as plant growth and development, cell wall cross-linking, wound healing, lignification, suberization, ROS metabolism, defense against pathogen infection and auxin catabolism as well as fruit growth and ripening (Pandey et al., 2017).

1.5.2.1.5. Glutathione-S-transferase

Glutathione-S-transferase (GST; EC. 2.5.1.18) are antioxidant enzymes that are widely distributed in prokaryotes and eukaryotes. They can be divided into three distinct classes based on their cellular localization namely cytosolic, mitochondrial and microsomal. The cytosolic-GST class occurs in the cytoplasm. Mitochondrial-GST is found in the mitochondria and peroxisomes and the last class known as the microsomal-GST is localized in the mitochondria and endoplasmic reticulum. The function of the various GSTs has been found to protect plants from various abiotic stresses (Ding et al., 2017). They also play important roles in detoxification, cell signalling, regulating redox homeostasis in cells to protect them against ultra violet (UV) radiation and oxidative stress caused by lipid peroxidation (Zhuge et al., 2020).

1.5.2.2. Non-enzymatic antioxidants

The non-enzymatic antioxidants system includes carotenoids, phenolics, tocopherols, ascorbic acid and others. These antioxidants exhibit their antioxidant activities by interrupting with free radical chain reactions (Sachdev et al., 2021).

1.5.2.2.1. Carotenoids

Carotenoids are light harvesting pigments that can be found in plants, animals, algae as well as some non-photosynthetic fungi and bacteria (Maoka, 2020). In higher plants carotenoids

serve as accessory pigments that play important roles in photosynthetic organisms. They serve as accessory pigments in the photosystems, increasing light absorption in the blue spectral domain of 420-500 nm, and they protect the photosynthetic apparatus against toxic ROS (Ramel et al., 2012). Carotenoids act as antioxidants by protecting plants from damage caused by various environmental stresses (Kim et al., 2012). They function as photo-protectants by reducing ROS before oxidative damage occurs or disperse excess energy in the form of heat suppressing lipid peroxidation (McElroy & Kopsell, 2009). Carotenoids also play important roles in the regulation of growth and development of plants (Sun et al., 2022). Recent studies found the overexpression of carotenoids to improve plant tolerance to salt stress. For example, Li et al., (2020) showed the overexpression of *Lycium chinenses* phytoene desaturase (LcPDS), zeta carotene desaturase (ZDS) and carotene isomerase CRTISO genes to enhance carotenoid accumulation in salt-stressed transgenic *Nicotiana tabacum* plants.

1.5.2.2.2. Phenolic compounds

Phenolic compounds, which include flavonoids, phenolic acid, tannins, hydroxycinnamate esters and lignin are metabolites that are abundantly found in plant tissues (Albuquerque et al., 2020). The key role of phenolic compounds is to remove free radical reactions caused by ROS (Zargoosh et al., 2019). The content of phenolic compounds are enhanced in plants under stress conditions and act as a defense to provide plant disease resistance. They function as antioxidants by altering the peroxidation kinetics by modifying lipid packing order and decreasing fluidity of membranes. Their activity has been demonstrated by Brown et al., (2001) to regulate auxin transport in plants and hence play an important role in plant development.

1.5.2.2.3. Ascorbic acid

Ascorbic acid (AsA) also known as vitamin C, is one of the most studied antioxidants in plants (Arrigoni & de Tullio, 2000). Ascorbic acid does not only function to scavenge ROS, but it is also involved in a number of fundamental functions in plants. It plays important roles in cell division and cell expansion, acting as a cofactor for enzyme reactions, photosynthetic processes and membrane stability (Akram et al., 2017). Ascorbic acid has been found in different plant organelles including chloroplast and apoplast and it participates in numerous metabolic processes in plants under stressful conditions. Several studies have reported on the influence of AsA in plants under various stress conditions (Akram et al., 2019; Dolatabadian et al., 2010). Previously, the foliar application of AsA positively impacted the growth of *Carthamus tinctorious* cultivars under water stress conditions (Farooq et al., 2020). Similarly, Khan et al., (2006) also observed that the exogenous application of AsA improved the chlorophyll (chl) 'a' content in *Triticum aestivum* seedlings subjected to salt stress. As salt stress also causes ionic imbalance in plants, proper regulation of ion flux in cells is necessary to keep the concentration of toxic ions low and accumulation of essential ions through ion homeostasis.

1.5.3. Ion homeostasis

The effects of sodium chloride stress on plants impose water deficit and ion imbalance, and both must be alleviated for proper growth and survival of the plant. Homeostasis is therefore the ability of an organism to survive and maintain a steady internal state in response to environmental stress. The maintenance of ion homeostasis is crucial for improving tolerance under salt stress conditions (Ji et al., 2013). Under salinity, Na ion gains entry into the cell through cation channels and competes with K uptake and blocks K specific transporters, resulting in toxic levels of Na and insufficient K and Si for enzymatic reactions (Assaha et

al., 2017; Mulaudzi et al., 2020; Mulaudzi et al., 2022; Rakgotho et al., 2022). A suitable K/Na ratio can be attained by reducing cytoplasmic Na and increasing K to prevent cellular damage and nutrient deficiency of plants. The salt overly sensitive (SOS) signalling pathway plays an important role by controlling and regulating Na effluxes from the cells, therefore enhancing ion homeostasis and salt tolerance in plants (Ji et al., 2013; Ma et al., 2019). This pathway involves three important enzymes including SOS1, SOS2 and SOS3 and their activation partly results in plant tolerance to salt stress (Yang et al., 2009). Under salt stress Ca signal is induced and perceived by SOS3. Salt overly sensitive 3 interacts and activates SOS2, the serine/threonine protein kinase in the plasma membrane. The activated SOS2 will phosphorylate and activate plasma membrane Na/H antiporter also known as SOS1. The SOS1 is a key determinant of Na transported from the cytoplasm to the apoplast (Yang & Guo, 2018).

Plants have also developed complex regulatory mechanisms, including metabolic adjustment and gene expression for physiological and morphological adaptation to stressful conditions (Joshi et al., 2016). Activation of transcription factors, which may either be up or down regulated in plants in response to stress are amongst the molecular strategies employed by plants to withstand salt stress environments.

1.5.4. Transcription factors

The ability of plants to tolerate stressful conditions is regulated by several genes, including transcription factors (TFs) (Hu et al., 2015). Transcription factors function by regulating gene expression through binding to the *cis*-regulatory elements in the promoter region of different stress related genes by regulating their transcription and thus, enhance plant tolerance to harsh environmental conditions (Franco-Zorrila et al., 2014). The genetic modification of the expression of the regulatory genes will influence plants tolerance to stress (Wang et al., 2016).

Several TFs have been identified to be involved in plant adaptation to abiotic stresses such as '*worky*' WRKY, heat shock protein (HSP), apetala2/ethylene-responsive element binding protein (AP2/EREBP), myeloblastosis (MYB), N-acetyl cysteine (NAC) and basic region-leucine zipper (bZIP) (Li et al., 2018). The WRKY transcription factor is one of the largest families of TFs in plants and has been shown to participate in diverse processes, including plant growth, seed development, leaf senescence, and enhance tolerance to biotic and abiotic stresses (Rushton et al., 2010; Chen et al., 2018). In transgenic *Chrysanthemum indicum* seedlings overexpression of *Dg*WRKY*4* resulted in increased tolerance to salt conditions compared to the wild type (Wang et al., 2017) and the over-expression of wheat TF "*Ta*WRKY*146"* in transgenic *Arabidopsis thaliana* was shown to enhance drought tolerance (Ma et al., 2017). Similarly, *Gm*WRKY45 TFs from soybean improved salt tolerance in transgenic *Arabidopsis thaliana* plants (Li et al., 2019).

The Heat shock transcription factor has also been widely shown to enhance plant tolerance to diverse environmental conditions and not only under heat stress conditions as the name suggests.

1.5.4.1. Heat shock transcription factors

The heat shock transcription factors (HSF) are known to play important roles in plant response to several abiotic stresses by regulating the expression of stress responsive genes such as heat shock proteins (HSPs) (Hu et al., 2015). Most plant HSPs are up or down regulated by heat stress. The HSPs are well known molecular chaperons that facilitate the restoration of normal function of proteins by assisting in the refolding of denatured proteins, protein transport across cellular membranes and prevention of protein aggregation (Mulaudzi-Masuku et al., 2015). The function of HSPs are not limited to their definition, they are employed by all living organisms to counteract detrimental conditions. There are five different classes of HSPs

according to their molecular weight namely, HSP-100, HSP-90, HSP-70, HSP-60 and the small heat shock proteins (smHSPs). The HSP-70 is the most conserved HSP across different species, which consist of an N-terminal ATPase domain and a *c*-terminal substrate binding domain (He et al., 2018). Heat shock protein-70 respond to various stresses in plants including heat, cold, drought and other oxidative stresses (Ul Haq et al., 2019). A previous study showed that a *Sorghum bicolor* heat shock protein-70 (*Sb*HSP-70) was induced at both low and high temperatures (4, 37 and 45°C) (Mulaudzi-Masuku et al., 2015). Similarly, Ngara et al., (2012) showed enhanced levels of HSP-70 in salt-stressed sorghum leaf samples, these indicating the ability of HSP-70 to confer tolerance in plants.

1.6. Exogenous applications of substances

Recent advances in science have also shown the application of various exogenous substances to improve plant growth, yield and enhance tolerance under adverse environmental conditions. The agricultural industry has been under pressure to produce crops that are well able to grow in severe environmental conditions. Different approaches have been tested to increase plant tolerance to a wide range of abiotic stresses such as drought, salinity and extreme temperatures. The exogenous application of different substances such as, silicon (Mustafa et al., 2021; Somapala et al., 2016), proline (Tabssum et al., 2019), nitric oxide (Egbichi et al., 2014; Zhao et al., 2020), calcium (Hosseini et al., 2019; Mulaudzi et al., 2020), chitosan (Mulaudzi et al., 2022) and nanoparticles (Rakgotho et al., 2022; Singh et al., 2022) has proven effective to enhance tolerance in plants to adverse stresses. These exogenously applied substances help plants to detoxify harmful ROS, regulate osmotic adjustment and maintain membrane structure during stressful conditions (Seleiman et al., 2021). However, the extensive use of some of these substances can be hazardous to plants having a great environmental impact (Malerba et al., 2016). This has stimulated the search for the use of eco-friendlier micro and macro-nutrients that are effective at low concentrations.

1.6.1. Molybdenum

Molybdenum (Mo), a trace element found in the soil and is required for the growth of most biological organisms including plants and animals (Sigel & Siegel, 2002). Molybdenum has been widely used as a fertilizer in plants to boost the fertility of soil. The most commonly used Mo fertilizers include ammonium molybdate $(NH_4)_2MoO_4$, and sodium molybdate $(Na_2)MoO_4$. Molybdenum trioxide (MoO_3) is less soluble than $(NH_4)_2MoO_4$ and $(Na_2)MoO_4$, which makes it less available to the plant. Molybdenum is one of the essential trace elements in plants like boron (B), zinc (Z), chlorine (Cl), copper (Cu), iron (Fe), manganese (Mn), cobalt (Co), vanadium (V), sodium (Na) and silicon (Si) that are required in very small amounts. Their adequate concentrations in plants are below 100 parts per million (ppm) for physiological function. Molybdenum is essential for good plant growth and is also involved in many plant physiological processes (Mendel & Hansch, 2002; Sun et al., 2009); however, in order for it to be useful to plants it needs to be transformed into Mo-cofactor.

1.6.2. Molybdenum activity

Once Mo is inside the plant, it is not active, in order for it to be beneficial to the plant it is important for it to be transformed into Mo-cofactor for plant utilization. Molybdenum plays an important role in plants mainly via the activity of molybdo-enzymes (Seeda et al., 2020). The Mo-enzymes that exist in plants include nitrogen assimilation enzymes, nitrate reductase, xanthine oxidoreductase and aldehyde oxidase. Nitrate reductase (NR), the primary nitrogen assimilation enzyme that uses nitrite as a substrate to produce oxygen reactive species such as nitric oxide, an important signalling molecule in plants. Xanthine oxidoreductase (XO), catalyses the oxidation of hypoxanthine to xanthine and subsequently to uric acid the final steps of purine catabolism. Aldehyde oxidase (AO) functions by catalyzing the oxidation of

aldehyde into carboxylic acid. Sulfite oxidase (SO) plays an important role in converting sulfite to sulfate, an important step in the catabolism of sulphur-containing amino acids and the newly discovered amidoxime reducing component (ARC) function in the regulation of nitric oxide synthesis (Mendel & Hansch, 2002; Tejada-Jimenez et al., 2013). When plants have insufficient Mo, nitrates accumulate in the leaves and plants cannot use them to make proteins, which is the result of reduced molybdo-enzymes activity. The unavailability of Mo can be lethal to plants and deficiency can appear on the entire plant.

1.6.3. Nutrient deficiency

The availability of essential nutrients has become a challenge for proper plant growth and development due to the changing climate conditions. Nutrient deficiency occurs when the essential nutrient is not available to meet the requirement for proper plant growth and metabolism. The scarcity of nutrients to plants either from macronutrients, nitrogen (N), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg), sulphur (S), oxygen (O), carbon (C) and hydrogen (H) or micronutrients, boron (B), zinc (Z), chlorine (Cl), copper (Cu), iron (Fe), manganese (Mn), cobalt (Co), vanadium (V), sodium (Na), silicon (Si) and molybdenum (Mo) in the soil can adversely affect different plant components from physiological to molecular activities (Sawyer, 2004). In addition to being nutrients for crops, these mineral elements also participate in various processes in plants either directly or indirectly from physiological to molecular activities towards salt tolerance of crops (Gou et al., 2020). For example, some mineral nutrients such as Fe, Zn, Cu and Mn, are essential part of SOD antioxidant activity in plants and the deficiency of these nutrients can impair ROS scavenging capacity resulting in ROS over-accumulation (Sachdev et al., 2021). As more agricultural lands are becoming salty due to poor quality water used for irrigation, poor draining as well as the excessive use or scarcity of fertilizers, several plant improvement or management methods are necessary to also avoid toxicity.

1.6.4. Impact of Molybdenum on plant growth

When plants have insufficient Mo, nitrates accumulate in the leaves and plants cannot use them to make proteins. Symptoms of Mo deficiency vary in plants, but most often result in stunted growth, chlorosis or yellowing of the leaves (Mendel & Hansch, 2002). Molybdenum deficiency resembles those of nitrogen deficiency and produce symptoms similar to those of nitrogen starvation (Figure 1.4a). Nitrogen is the main element of many important organic compounds and is a key component of the chlorophyll molecule which is responsible for photosynthesis (Abriz et al., 2017; Haung et al., 2020). The leaves of affected plants usually show pale green or yellowish green colour, in severe cases young leaves wilt and necrotic tissue appears along their margins, resulting in leaf death with the overall decrease in plant growth (Weir, 2004). Most of these phenotypes are associated with molybdo-enzymes activity. One of the factors that determine Mo availability to plants is the pH of the soil, where the plants are growing. As an anion, Mo becomes more available when the pH increases and its deficiency is widely observed in acidic soils (pH <6.5). Molybdenum availability decreases as anion adsorption to soil oxides increases (Hamed et al., 2014; Seeda et al., 2020) (Figure 1.4b). Molybdenum fertilization through foliar sprays can supplement internal molybdenum deficiencies and rescue the activity of molybdo-enzymes. However, the excessive use of Mo fertilizers can also cause toxicity to plants. Earlier studies by Nautiyal & Chatterjee, (2004) showed that excess (>0.2 mgL^{-1}) Mo decreased biomass, seed yield, leaves size and decreased the quality of product as well as chlorosis on leaflets, which intensified to leaves drying. Molybdenum deficient (<0.02 mgL^{-1}) decreased biomass, produced less number of flowers, smaller plants and lower germination yield in *Cicer arietinum*. Progressive work has been done over the years on the impact of exogenous application of Mo in alleviating stress tolerance in plants.

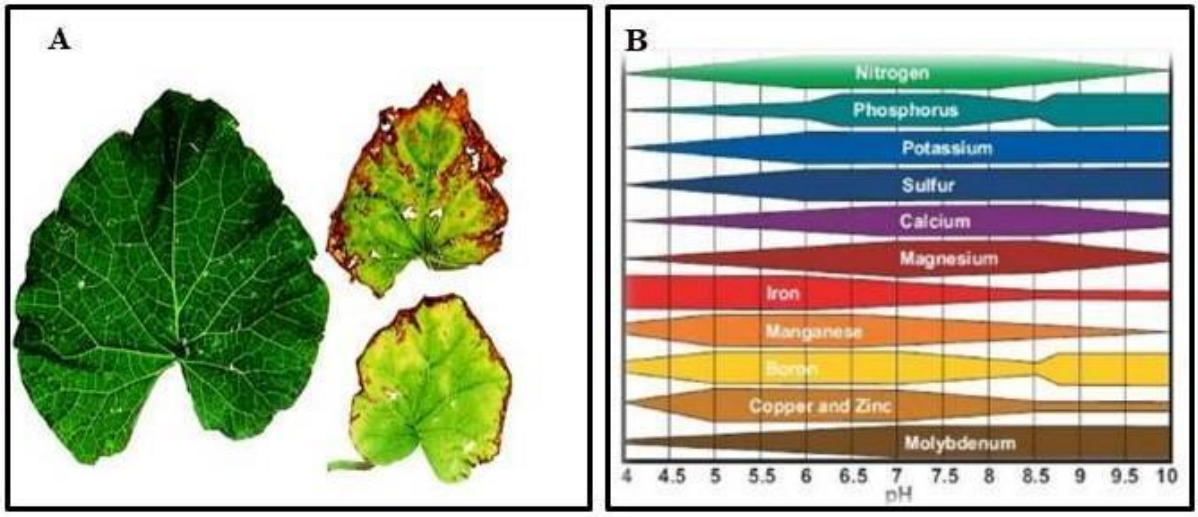

Figure 1.4. Molybdenum deficiency in plants and pH dependant mobility of Mo. Illustration of the leaves of plants treated with Mo and deficient of Molybdenum on the leaves showing yellowing and death on leave edges (**A**) pH mobility of Mo (**B**) (Adapted from Agfact, 2004; Seeda et al., 2020).

1.6.5. Molybdenum promotes stress tolerance

Molybdenum has been reported to be involved in multiple metabolic and cellular processes in plants (Rana et al., 2020). It has been extensively reported to play significant roles in mediating the resistance of plants to numerous stresses, such as salinity (Babenko et al., 2015), chemical (Han et al., 2020), drought (Hayyawi et al., 2020), cold (Al-issawi et al., 2015) amidst others. Although plants have developed adverse coping mechanisms to adapt to environmental stresses. The acquisition of micronutrients may become inefficient with the rapid increase in salinity. Salinity decreases the uptake of micronutrients by decreasing the solubility and mobility of micronutrients by the plant (Evelin et al., 2019).

The application of exogenous Mo can enhance a plant's tolerance to stress as well as increasing the availability of nutrients thereby promoting plant growth. For examples Zhang et al., (2012) reported the exogenous application of Mo to enhance salt stress tolerance in *Brassica campestris L. ssp Pekinensis* by increasing the fresh weight, activities of antioxidant enzymes (SOD, POD, CAT) and the non-enzymatic antioxidant contents of glutathione,

carotenoid and ascorbic acid. Similarly, *Agropyron cristatum* was not negatively affected by NaCl application, which resulted in visible improvement of plant state even after NaCl application in contrast to plants without Mo treatment (Babenko et al., 2015). It was also reported that Mo increased oxidative stress tolerance in *Triticum aestivum* under different nitrogen sources (Imran et al., 2019). These show the ability of Mo to mitigate the negative effects of stress on plants. Apart from this, Mo has also been found to play important roles in chlorophyll biosynthesis and ion homeostasis in plants (Zhang et al., 2014).

1.6.6. Molybdenum improves photosynthesis and ion homeostasis under salt stress

Molybdenum has been reported to play important roles in photosynthesis because of its involvement in chlorophyll biosynthesis (Yu et al., 2006). The formation of yellow colour in the leaves of Mo deficient plants is linked to lack of nitrogen as a result of decreased chlorophyll content (Peng et al., 2021). Chlorophyll is important in the adsorption and transmission of light energy in photosynthesis. When plants are deficient in nitrogen, chlorophyll content decreases resulting in decreased photosynthesis, plant growth and decline in crop yield (Gu et al., 2016). Plants that are deficient in Mo inhibit the biosynthesis of chlorophyll or enhance its decomposition (Yu et al., 2006). High salt concentrations in the soil also cause ion toxicity resulting in nutrient imbalance and thus disrupting ion homeostasis. Maintaining intracellular ionic homeostasis is important for plants in order to adapt to salt stress conditions (Gou et al., 2020). Earlier studies showed that the application of Mo in *Brassica pekinensis* under salt stress significantly increased the contents of chl ‘a’, ‘b’, carotene and total chl contents (Zhang et al., 2014). Molybdenum also significantly increased the ratios of K/Na; Ca/Na and Mg/Na ions under salt stress. Similarly Mo enhanced

photosynthesis of *Hordeum vulgare* plants under different salt concentration (5, 10 and 15 ds m^{-1}) (Bagheri & Jafari, 2012). This highlights the effects of exogenous application of Mo to enhance salt stress tolerance in plants by regulating photosynthesis.

1.7. Techniques used to study stress responses

There are different techniques that have been used by researchers to test the effectiveness of exogenous applications of substances/molecules in elevating stress tolerance in plants. Through the use of molecular techniques, including gene expression analysis using semi-quantitative methods such as western blot or dot blot analysis. To visualize and determine the up or down regulation of protein expression in cells or structural characterization of microstructure using microscopic tools such as the scanning electron microscope.

1.7.1. Western blot analysis

Immunoblotting, commonly referred to as western blot, is a highly sensitive method used for the detection of proteins as well as antigens, and viruses. Western blot analysis can detect proteins as small as 1 ng in a complex mixture of other proteins (Treindel et al., 2016). Immunoblotting is carried out in 4 stages as illustrated in Figure 1.5. The first stage is the separation of proteins based on size on a polyacrylamide gel this is then followed by stage 2 where proteins separated are transferred onto a nitrocellulose membrane. After transferring proteins to the membrane, the blot will be blocked by applying a primary antibody specific to the protein of interest and a secondary antibody that will recognise the primary antibody. The final stage is detection and visualization of the target protein. A substrate will react with the enzyme that is bound to the secondary antibody to generate a coloured substance. The bands corresponding to the protein of interest will appear as dark regions on the membrane showing the intensity of the protein of interest. Detection of proteins can be visualized using

colorimetric, radioactive, fluorescent or electro-chemiluminescent (ECL) detection methods (Mahmood & Yang, 2012).

The dot blot immunoblotting assay has also been commonly used for protein detection; this technique differs from western blot in that it allows for the direct transfer of protein samples onto the polyvinylidene fluoride (PVDF) membrane. Although the dot blot method is not as informative as the western blot, which allows for the separation of proteins by size (Mishra et al., 2017), the dot blot is a quick and easy method for the detection of simple proteins. It does not require electrophoretic separation of samples; proteins can directly be immobilized on to the membrane, which also avoids the transfer of gel to membrane (Faramarz, 2018). Both western and dot blot analysis has been used by several authors in plants. For example, western blot techniques were used to show how Mo influenced the upregulation of cold regulated-15 (COR15a) and C-repeat binding factor-14 (Cbf14) protein expression in *Triticum aestivum* cultivars subjected to cold stress (Al-Issawi et al., 2016). Similarly, Silva et al., (2011) showed a significant increase in the levels of formate dehydrogenase (FdhAB) protein in format and hydrogen-grown cells in the presence of tungsten in *Desulfovibrio vulgaris*. Both western and dot blot analysis were used for the detection of recombinant C-C chemokine ligand 21 (CCL21) expression in transformed leaves of *Solanum lycopersicum* (Beihaghi et al., 2017). No literature has been found yet on western blot or dot blot analysis of HSP-70 on Mo treated plants under salt stress conditions.

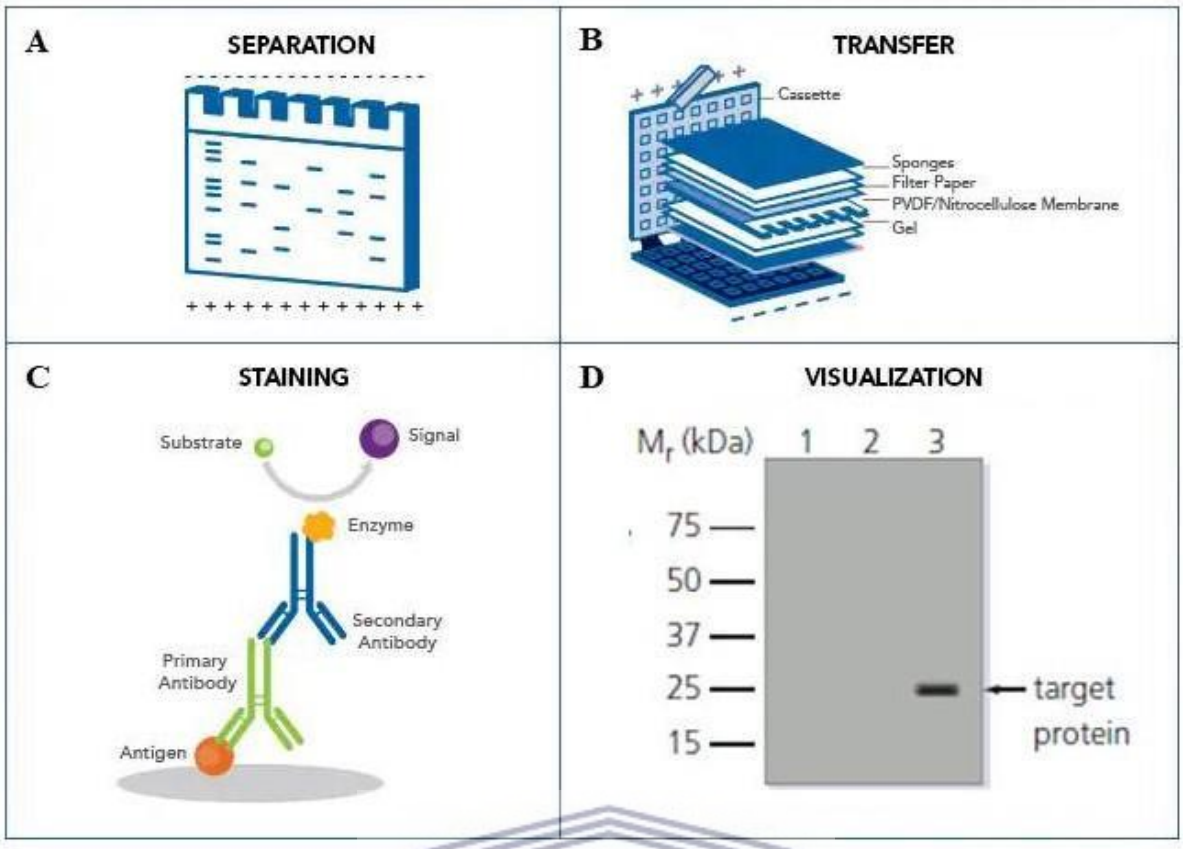

Figure 1.5. Steps involved in western blot techniques. Illustration of steps involved in western blot. Image credit: https://www.novusbio.com/application/western-blotting.

1.7.2. Scanning Electron Microscope

The scanning electron microscope (SEM) is a valuable technique that can be used to produce images of a sample by scanning the surface with a high energy beam of electrons (Omidi et al., 2017). When the electrons strike the specimen, signals will produce an image of the surface of the specimen (Yan et al., 2010). Scanning electron microscope analysis can be used to reveal information about the specimen (Webb et al., 2003) including, morphological characteristics such as size, shape, chemical composition, crystalline structure and materials making up the specimen (Luo et al., 2016; Goldstein, 2003). The scanning electron microscope can also be used to perform analysis of selected point locations of a specimen known as energy dispersive X-ray spectroscopy (EDS). The ability of SEM to produce images of such high magnification, while retaining high resolution, has resulted in its use in different

fields from commercial to research sectors (Lindenau et al., 2015; Primo et al., 2020; Kamikoriyama et al., 2019). For example, SEM analysis was used to determine the effects of salt stress and influence of calcium on the epidermis and xylem layers as well as SEM-EDX analysis to determine the ion content of Na/K on sorghum plants (Mulaudzi et al., 2020). Similarly, Rakgotho et al., (2022) used SEM-EDX to determine the effect of salinity stress and impact of zinc oxide nanoparticles on salt-stressed *Sorghum bicolor* plants. Furthermore, Mulaudzi et al., (2002) showed that using SEM-EDX Na ions accumulations hindered the distribution of essential elements (K, Si), but this was reversed by the exogenous chitosan.

1.8. Conclusion

Salinity has shown to disturb agricultural practices all over the world, and it is predicted to become an even bigger problem in the years to come. This will have a great impact on the production of crops. As observed with the current state of hunger, that is already at serious levels across the African continent. There is therefore a greater need to understand the physiological, biochemical and molecular responses of stress tolerance of crops and to introduce potential alleviating techniques that would enhance agricultural production.

The over accumulation of ROS in response to stress is found to be the prime factor for the destruction of cellular function. Reactive oxygen species have a dual function in plant cells: it acts as a signalling molecule that modulates the expression and activates multiple defensive gene responses when present at low concentration, whereas it's over accumulation can result in oxidative damage and even plant death (Hasanuzzaman et al., 2021). It is therefore important for plants to maintain appropriate levels of ROS, and this is possible through different coping mechanisms inherited in plants necessary to overcome environmental stresses (Seleiman et al., 2021). Recent research suggests that the exogenous application of substances is a promising field in crop stress management to increase their nutrition and

tolerance to various environmental stresses. As traditional breeding methods are not sufficient to overcome salinity imposed challenges, research scientist should work closely with breeders and present the implementation of exogenous substances that will not only improve crop productivity under unfavourable conditions, but also improve nutrition and health of plants with the emphasis of developing salt tolerant cultivars to cope with salinity stress and will thus ensure food security.

Molybdenum, an essential element required for healthy plant growth and development, is generally used as a component of complex fertilizers in plant areas where the natural Mo content is not sufficient and plant development is affected. Molybdenum is also known to facilitate the improvement of abiotic stress tolerance in plants.

CHAPTER TWO
MATERIALS AND METHODS

2.1. Plant growth and treatments

Red sorghum [*Sorghum bicolor* (L.) Moench] seeds were purchased from Agricol, Brackenfell, Western Cape, South Africa. Seeds were surface decontaminated as previously described (Mulaudzi et al., 2022). Briefly seeds were soaked for 1 minute in 70% ethanol while shaking at 600 rpm. This was then followed by three washes using autoclaved doubled distilled water (ddH_2O). After washing, seeds were soaked in 5% sodium hypochlorite solution and incubated for 1 hour while shaking at 600 rpm, followed by three washes with autoclaved ddH_2O_2. Decontaminated, seeds were imbibed overnight in ddH_2O at room temperature while shaking. The following day, seeds were dried under the laminar flow and five seeds were sown on BioPa MN 218 B blotting paper placed on sterilised petri dishes containing 4 ml ddH_2O. The petri dishes were placed in the dark and allowed to germinate for 7 days in the growth chamber (Bluepard MGC-350HP-2, Myanmar) set at 25˚C. After 7 days seedlings were individually transplanted in pots containing a mixture of double grow, all-purpose organic potting soil and vermiculite (2:1) [purchased from Stodels garden centre, Bellville, Western Cape, South Africa], and allowed to grow in the green house under controlled conditions [25˚C/22˚C day/night and 16 hours light/8 hours dark regime] for 14 days. Watering was done after every 2nd day with dH_2O. After 14 days of growth, plant seedlings were watered with different solutions containing NaCl (0 mM, 100 mM, 200 mM and 300 mM) for salinity stress with either Molybdenum (Mo) applied as $(NH_4)_6Mo_7O_{24}.4H_2O$ or Tungsten (W) applied as $Na_2WO_4.H_2O$ alone, and a combination of Mo and W (0.5 µM, 1 µM and 2 µM) for 7 days. Plants not subjected to any of these treatments

served as the control. After stress treatments plant roots and shoots were separated, flash frozen in liquid nitrogen and stored at -80˚C until further use.

2.2. Morphological analysis

Morphological parameters were measured from fresh seedlings from each treatment to include fresh weight (FW), dry weight (DW) and shoot length. For FW and DW, plants were weighed individually. Fresh weight represented the weight of fresh seedlings, while DW was determined on plants that were prepared by drying in oven at -80˚C for 24 hours or until constant weight was attained. Plants were measured on a Mettle Toledo AE50 analytical balance (Marshall Scientific, Hampton US) with an accuracy level of 0.0001 g. The water content was measured as percentage of fresh weight and dry weight (Sen & Alikamanoglu, 2013) as shown in equation 1.

Water content (%) = (FW - DW)/ (FW) x 100 Equation [1]

2.3. Photosynthetic pigments

To analyse the photosynthetic pigments, chlorophyll (chl) 'a' chl 'b' and total chl content were measured as previously described (Rajalaksmi & Banu, 2013). One hundred mg of shoots was homogenized in 10 ml of room temperature acetone 80% (v/v), followed by centrifugation at 10.000 rpm for 10 minutes at 4˚C. The absorbance of the supernatant was measured using Helious Epsilon spectrophotometer (Thermo scientific Waltham, Massachusettes US) at 663 nm and 645 nm for chl 'a', and chl 'b' content respectively. Carotenoid content was measured at 470 nm wavelength (Lichtenthaler & Welburn, 1983)

Total Chl, Chl-*a*, Chl-*b*, and carotenoids were calculated according to the equations given below.

Total chl = $20.2(A_{645}) + 8.02(A_{663})$ Equation [2]

[Chl-a] = $12.7\ (A_{663}) - 2.69\ (A_{645})$ Equation [3]

[Chl-b] = $22.9(A_{646}) - 4.68(A_{663})$ Equation [4]

Carotenoids = $(1000_{470} - 3.27[chl_a] - 104\ [Chl_b])/227$ Equation [5]

2.4. Anatomic structure

The damage of the membrane structure was determined based on the anatomical analysis of the epidermis and vascular bundles (xylem and phloem) layers using a High Resolution scanning electron microscope (HRSEM), located at the Electron Microscope Unit at the University of Cape Town, South Africa. All Spectra were analysed using the build in Oxford Inca software suite. Analysis were undertaken from *Sorghum bicolor* shoots which were prepared by oven drying grinded samples, which were placed on aluminium stubs coated with conductive carbon tape. Samples were imaged and collected using a Tescan MIRA field emission gun scanning electron microscope, operated at an accelerating voltage of 5 kV using an in-lense secondary electron detector.

2.5. Osmolytes determination

2.5.1. Proline content

Free Proline content was determined as described previously (Khare et al., 2012) with slight modifications. Three hundred mg of roots/shoots tissue was homogenized in 10 ml 3% sulfosalicylic acid, followed by centrifugation at 2.000 rpm for 20 minutes. The supernatant (2 ml) was treated with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid (1:1) and incubated for 30 minutes at 100˚C. The tubes were cooled on ice and 4 ml of toluene was added. The absorbance of the chromophore was measured at 520 nm using a Helios® Epsilon

visible 8 nm bandwidth spectrophotometer (Thermo scientific Waltham, Massachusettes US). The proline concentration was determined using standard curve using pure proline.

2.5.2. Soluble sugars

Soluble sugars were measured following the method of Pandey & Penna, (2016) with slight modifications. Briefly, 0.1 g of roots/shoots tissue was homogenized with 10 ml of 80% acetone. The samples were centrifuged at 10.000 rpm for 10 minutes and 1 ml of the supernatant was mixed with 3 ml anthrone reagent. The samples were placed in a boiling water bath for 15 minutes, followed by cooling on ice to stop the reaction; the absorption was measured at 625 nm using Helious Epsilon spectrophotometer (Thermo scientific Waltham, Massachusettes US). Soluble sugar contents were determined using pure glucose standard and expressed as mg g^{-1} FW.

2.6. Markers of Oxidative damage

2.6.1. Histochemical staining

Histochemical detection of ROS markers was done as described previously (Kumar et al., 2013) with slight modification. To detect $O_2^{\bullet-}$, leaves were excised and immersed in the reaction mixture [50 mM phosphate buffer (pH 7.5); 0.2% nitroblue tetrazolium (NBT)] and incubated for 4 hours at room temperature. For localization of H_2O_2, excised leaves were immersed in 1mg/ml of 3', 3'-diaminobezidine (DAB; pH 3.8) solution and incubated overnight at room temperature. From all samples, chlorophyll was removed by boiling leaves for 15 minutes in 80% (v/v) ethanol. The detection of $O_2^{\bullet-}$ was represented by dark blue spots resulting from the reaction of NBT and $O_2^{\bullet-}$ whereas H_2O_2 as detected based on the appearance of brown spots resulting from the reaction of DAB with H_2O_2.

2.6.2. Hydrogen peroxide content

Hydrogen peroxide content was determined as described previously (Junglee et al., 2014). One hundred and fifty mg roots/shoots tissues were homogenized in 1 ml reaction mixture [0.25 ml of 0.1% trichloroacetic (w/v) acid (TCA), 0.5 ml of 1 M potassium iodide and 0.25 ml of 10 mM potassium phosphate buffer (pH 7.5)]. Tubes were vortexed and centrifuged at 10.000 rpm for 15 minutes at 4˚C. Samples were then transferred to a 96 well microtiter plate and incubated at room temperature for 20 minutes. Absorbance was read at 390 nm using a FLUOstar® Omega (BMG LABTECH, Ortenberg, Germany) microtiter plate reader. Hydrogen peroxide content was determined from a standard curve using H_2O_2 solution as a standard.

2.6.3. Lipid Peroxidation

Oxidative damage of lipids as a result of salt stress was measured by estimating the total 2-thiobarbituric acid reactive substances (TBARS) and expressed as malondialdehyde (MDA) content (Egbichi et al., 2013). Plant material roots/shoots (0.1 g) were homogenized in 1 ml of 0.1% TCA (w/v) and centrifuged at 12.000 rpm for 10 minutes at 4˚C. An aliquot of 0.5 ml of the supernatant was mixed with 0.5 ml of TBA followed by heating at 90˚C for 30 minutes. The reaction was cooled on ice followed by centrifugation. The concentration of MDA was determined by observing the difference in absorbance at wavelengths of 532 nm and 600 nm measured on a FLUOstar® Omega microtiter plate reader (BMG LABTECH, Ortenberg, Germany) using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol of MDA g^{-1} FW.

2.7 Antioxidant enzyme activity assay

Samples for determination of enzyme activities were prepared as previously described (Gunes et al., 2019). Plant material roots/shoots (0.5 g) was ground in liquid nitrogen and

homogenized in 50 mM sodium phosphate buffer (pH 7.5) containing 2 mM EDTA and 5 mM β-mercaptoethanol and 4% (w/v) polyvinylpyrrolidine-40 (PVP-40). The homogenate was centrifuged at 30.000 rpm for 30 minutes at 4°C. The supernatant was used for superoxide dismutase and ascorbate peroxidase analysis analysed on a FLUOstar® Omega microtiter plate reader (BMG LABTECH, Ortenberg, Germany).

2.7.1. Superoxide dismutase

Activities of SOD were measured as previously described (Li et al., 2015). The reaction was initiated by adding 300 µl enzyme extract into the reaction solution [50 mM potassium phosphate buffer (pH 7.8), 12 mM methionine, 75 µM NBT and 1 µM riboflavin] and tubes were shaken followed by exposing them to fluorescent lamps for 20 minutes. The reduction of NBT was measured by monitoring the change in absorbance at 560 nm. The specificity of the protein was estimated as unit per mg^{-1} protein.

2.7.2. Ascorbate peroxidase

Ascorbate peroxidase activity was measured according to the method described (Scarpeci et al., 2008). Twenty microliters of enzyme extract were added to a solution containing 100 mM potassium phosphate buffer (pH 8.0), 10 mM ascorbic acid and 10 mM EDTA. The reaction was started by the addition of 100 mM H_2O_2. The decrease in absorbance was measured at 290 nm. The activity of APX was estimated using the extinction coefficient of 2.8 mM^{-1} cm^{-1} and expressed as mmol ascorbate oxidized per mg^{-1} of protein.

2.8. Dot blot analysis

Dot blot was done according to the method of (Beihaghi et al., 2017), with slight modifications. Briefly, 5 µl of extracted proteins were directly blotted on the nitrocellulose membrane that was previously soaked in methanol. The membrane was incubated for 1 hour

in cold casein with gentle shaking. After shaking, the membrane was washed briefly with 1x PBS-T. The mouse monoclonal anti-Hsp70 primary antibody (1:500) (Product# ab2787, Abcam, Cambridge, England) was added to the membrane and allowed to shake overnight on rotary shaker. The next day the membrane was washed with 1x PBS-T while shaking for 10 minutes; this was then followed by two times 5 minutes washed. Following the washes, the membrane was incubated with the secondary antibody StarBright Blue 520 goat anti-mouse IgG (1:1000) (Cat# 12005867; Bio-Rad Laboratories, Inc, Hercules, CA) for 1 hour, while shaking. This was then followed by washing for 10 minutes with 1x PBS-T followed by two washes at 5 minutes intervals. The blotted proteins were viewed using ChemiDoC ™ imaging system (Bio-rad, Hercules, U.S).

2.9. Statistical analysis

All experiments were done in triplicates for each tissue sample and data was statistically analysed by the one-way analysis of variance (ANOVA) for the controls and NaCl only and two-way ANOVA for the [controls and treated plants (NaCl + Mo only and NaCl + Mo + W combinations)] method using GraphPad Prism 9.2.0 (https://www.graphpad.com). Data in figures and tables represent the mean ± standard deviation. Statistical significance between control and treated plants were determined by the Bonferroni's multiple comparison test and represented as *** = $p \leq 0.001$, ** = $p \leq 0.01$, and * = $p \leq 0.05$.

CHAPTER THREE

EFFECTS OF SALT STRESS ON THE MORPHO-PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR ATTRIBUTES OF *SORGHUM BICOLOR*

3.1. ABSTRACT

Salinity is one of the major abiotic factors affecting plant growth, development and productivity especially in arid and semi-arid areas. The exposure of plants to salt stress disrupts the metabolic functioning of plants resulting in the excess production of reactive oxygen species (ROS), causing oxidative damage and hence cell death. This chapter aims to evaluate the responses of sorghum to different salt concentrations. This was achieved by subjecting fourteen-day old sorghum plants to different salt concentrations (0 mM, 100 mM, 200 mM and 300 mM NaCl) for seven days. Salt inhibited the growth of sorghum plants, as evident by reduced shoot length, fresh and dry weights by more than 2-fold. Salt caused severe damage of the epidermal and vascular bundle layers of plants especially under 300 mM NaCl treatments. Salt stress significantly increased the total chl and chl 'a' content under 100 mM NaCl treatment by over 1-fold, whereas the highest salt concentration (300 mM NaCl) reduced total chl and chl 'a' content by over 1-fold. The Na^+ toxicity was confirmed by detecting the overproduction of ROS stains observed as dark blue ($O_2^{\bullet -}$) and brown (H_2O_2) spots on sorghum leaves. Furthermore, H_2O_2 (7.3-fold), MDA (1.5-fold), proline (13-fold) and soluble sugars (5-fold) contents were significantly increased in the shoots under 300 mM NaCl treatment. The highest SOD activity (3-fold) was observed in the shoots, under 300 mM NaCl, whereas APX (3.9-fold) activity was highly increased under 200 mM NaCl treatments in addition to the induced HSP70 expression. These results suggest that sorghum has inherent salt stress adaptive mechanism by generating high responsive targets.

KEYWORDS: APX, Antioxidants, HSP-70, ROS, Salt stress, SOD, *Sorghum bicolor*

3.2. INTRODUCTION

It has been recorded by the Food and Agricultural Organization (FAO) that over 400 million hectares of the world's land is affected by salinity (FAO, 2009). Salinity is one of the main factors that result in the reduction in plant growth, development and productivity in irrigated lands (Babenko et al., 2015; Calone et al., 2020; Munns, 2002). High salinity is usually due to high concentrations of soluble salts in irrigation water and high rate of evaporation caused by high temperatures or soil type. Salinity affects plant growth by limiting the absorption of water from the soil due to osmotic stress and prevents the uptake of essential nutrients, due to ionic stress; as a result, from high concentrations of toxic Na ion within plant cells (Cheng et al., 2009; Isayenkov & Maathuis, 2019; Munns & Tester, 2008). Ion toxicity and osmotic stress lead to ROS generation, which causes oxidative damage of biomolecules and if not controlled; it can affect other developmental processes and may lead to cell death (Doyle et al., 2010; Hasanuzzaman et al., 2020; Tripathy & Oelmuller, 2012). Plants have developed numerous adaptive mechanisms to maintain cellular osmotic pressure and defence against toxic ROS (Hossain & Dietz, 2016; Huang et al., 2019). These include the accumulation of osmolytes such as proline and soluble sugars (Ahanger et al., 2017), photosynthetic pigments (Yang et al., 2020), expression of stress responsive proteins (Taïbi et al., 2016; Ul Haq et al., 2019) and enhanced antioxidant activities (Hasanuzzaman et al., 2020).

The photosynthetic pigments such as chlorophyll improve oxidative stress in plants by activating several photoprotection mechanisms when absorbed light energy surpasses its use in photosynthesis (Labudda et al., 2017). Proline is the most well studied osmolyte that plays important roles as a metal chelator, scavenging ROS and as a signalling molecule in plants under various stress conditions (Hayat et al., 2012). Soluble sugars also play major roles in

osmoregulation, osmotic adjustment and maintaining growth and structure of plant tissues (Yasseen et al., 2018). Stress responsive genes play important roles to enhance antioxidant activity and other tolerant inducing targets (Wang et al., 2020; Sevengor et al., 2011). The antioxidative defence system in plants is comprised of non-enzymatic antioxidants such as flavonoids, ß-carotenes, carotenoids, ascorbic acid and glutathione; whereas the main enzymatic antioxidants include SOD, APX, CAT and POD amongst others (Zhu et al., 2004; Yasar et al., 2007).

It has been reported that plants with high levels of antioxidants, have greater resistance to oxidative damage (Yasar et al., 2008; Siringam et al., 2011). Several publications reported on the positive effects of antioxidant enzyme activities on salinity tolerance in plants. Increases in SOD, CAT and APX activities were observed in *Phoenix dactylifera* under 240 mM NaCl (Al Kharusi et al., 2019), whereas in *Sorghum bicolor*, high SOD, CAT and APX activities were observed under 400 mM NaCl treatments (Rakgotho et al., 2022), SOD and APX were induced under 300 mM NaCl (Mulaudzi et al., 2022). Similarly POD activities significantly increased with increasing incubation time (6 to 24 hours) under different NaCl concentrations (150 mM, 300 mM and 450 mM) in *Kandelia candel* species (Wang et al., 2014). This study therefore took advantage of sorghum's tolerant trait to study the effect of different salt concentrations on its metabolic responses.

Sorghum bicolor is a well-known cereal crop that has been used as a staple food for people living in semi-arid and arid regions (Proietti et al., 2015). As a C_4 metabolism plant, sorghum is able to limit the rate of photorespiration and sustain photosynthetic activity in stressful conditions (Calone et al., 2020). Despite *Sorghum bicolor* being moderately tolerant to salt and drought environments, the growth of the plant have been greatly affected by high salinity (Hedayati-Firoozabadi et al., 2020). Thus, it is important to investigate the different levels of salinity tolerance in order to obtain broader response attributes of the crop.

3.3. RESULTS

3.3.1. Morpho-physiological response of sorghum to salt stress

3.3.1.1. Growth attributes

To understand the effects of increasing salt concentrations on *Sorghum bicolor* plants, the morphological and physiological attributes including phenotype (Figure 3.1), plant biomass (Table 3.1), anatomical structure (Figure 3.2) as well as photosynthetic pigments (Figure 3.3) were assayed. Salt stress gradually reduced shoot growth, with increasing NaCl concentrations (Figure 3.1).

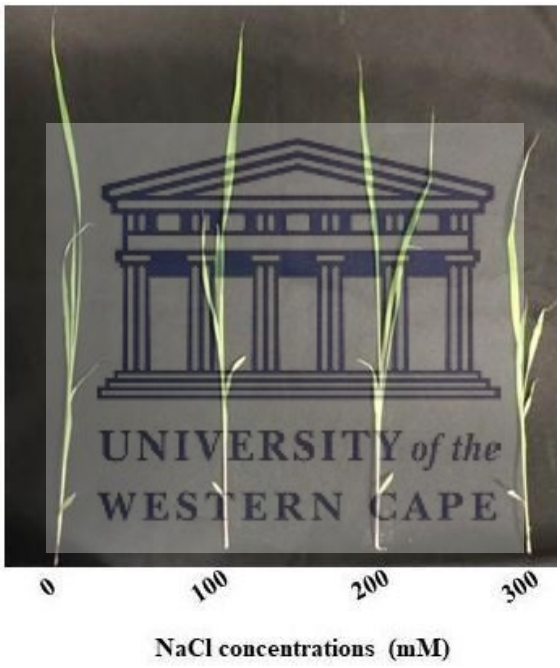

Figure 3.1. Shoot growth under salt stress conditions. Phenotypic representation of *Sorghum bicolor* shoots growth under the different NaCl treatments 0 mM, 100 mM, 200 mM and 300 mM NaCl respectively.

The results in Table 1 showed that salt stress affected the biomass of sorghum plants with increasing salt levels 0 mM (control), 100 mM, 200 mM and 300 mM NaCl. Sodium chloride treatment reduced the FW of plants from 0.52 mg to 0.45 mg (100 mM NaCl), 0.39 mg (200 mM NaCl) and 0.26 mg under (300 mM NaCl) representing a 1.2-fold, 1.3-fold and 2-fold

decrease respectively. Salt stress significantly reduced the DW of plants with increasing salt concentrations, from 0.05 mg to 0.03 mg (* = p≤0.05) for 100 mM NaCl, 0.03 mg (** = p≤0.01) for 200 mM NaCl to 0.03 mg (** = p≤0.01) and for 300 mM NaCl-treated plants to 0.02 mg (** = p≤0.01), representing a 1.4-fold, 1.8-fold and 2.6-fold reduction respectively. The WC% was increased under all salt concentrations by 1-fold for 100 mM, 200 mM and 300 mM NaCl.

Table 3.1. The effect of salt stress on the fresh weight, dry weight and water content of sorghum plants.

NaCl (mM)	FW (mg)	DW (mg)	WC%
0	0.521 ± 0.030	0.046 ± 0.004	91.180±0.312
100	0.445 ± 0.049	0.033 ± 0.003*	92.574±0.190
200	0.390 ± 0.014	0.025 ± 0.004**	93.730±0.679
300	0.260 ± 0.028**	0.018 ± 0.002**	93.184±1.557

** and * indicate significant differences at p≤0.01 and p≤0.05 respectively. FW = fresh weight, DW = dry weight and WC% = water content.

3.3.1.2. Anatomic structure

The anatomical structure analysis (Figure 3.2) showed the control plants to have well-arranged and smooth epidermis and vascular bundle (xylem and phloem) layers. Upon treatment with 100 mM, 200 mM and 300 mM NaCl, the scanning electron microscope (SEM) micrographs showed shrinkage of epidermal layers (black arrows) and thinning of xylem (red arrows) and phloem (blue arrows) walls.

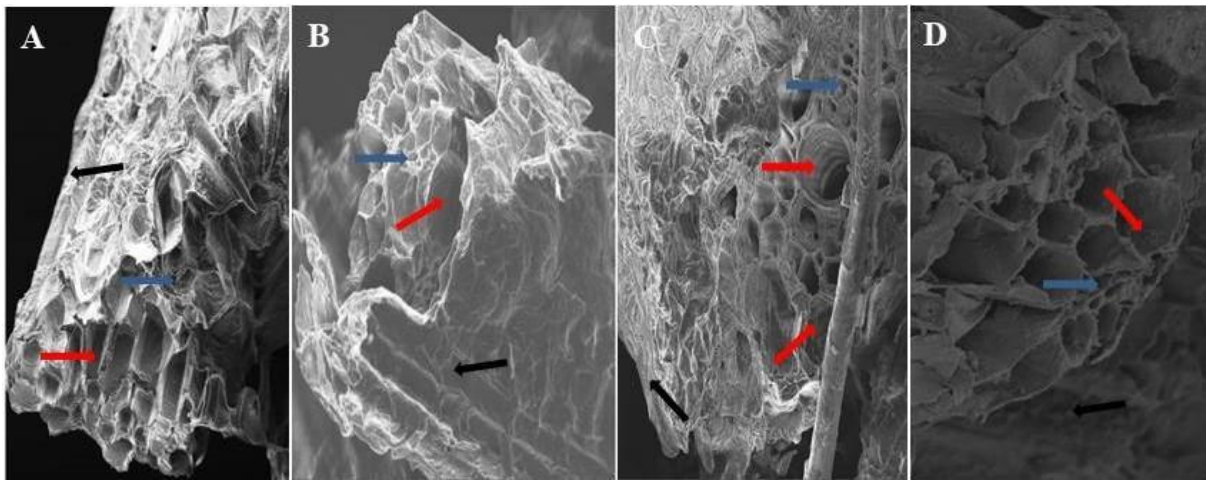

Figure 3.2. Scanning Electron Microscopy images showing the effects of salt stress on the epidermis and vascular bundle (xylem and phloem) layers of *Sorghum bicolor* plants. Control plants under 0 mM NaCl (**A**), 100 mM NaCl (**B**)**,** 200 mM and (**C**) 300 mM NaCl (**D**) concentrations respectively. Arrows show the different anatomical layers, epidermis (black), xylem (red) and phloem (blue).

3.3.1.3. Chlorophyll content

Chlorophyll is an important pigment in plants that determine the photosynthetic capacity and is highly correlated with plant growth (Li et al., 2018). Figure 3.3 showed a significant (* = $p \leq 0.05$) increase in total chl (1.2-fold) and chl 'a' (1.3-fold) contents under 100 mM NaCl treatment with no significant changes observed in chl 'b' content in comparison to that of the control (0 mM NaCl). No significant changes were observed in chl content under 200 mM NaCl treatments. Plants treated with 300 mM NaCl resulted in a significant decrease in total chl (1.4-fold) and chl 'a' (1.4-fold) content, with no significance changes observed in chl 'b' content as compared to that of the control.

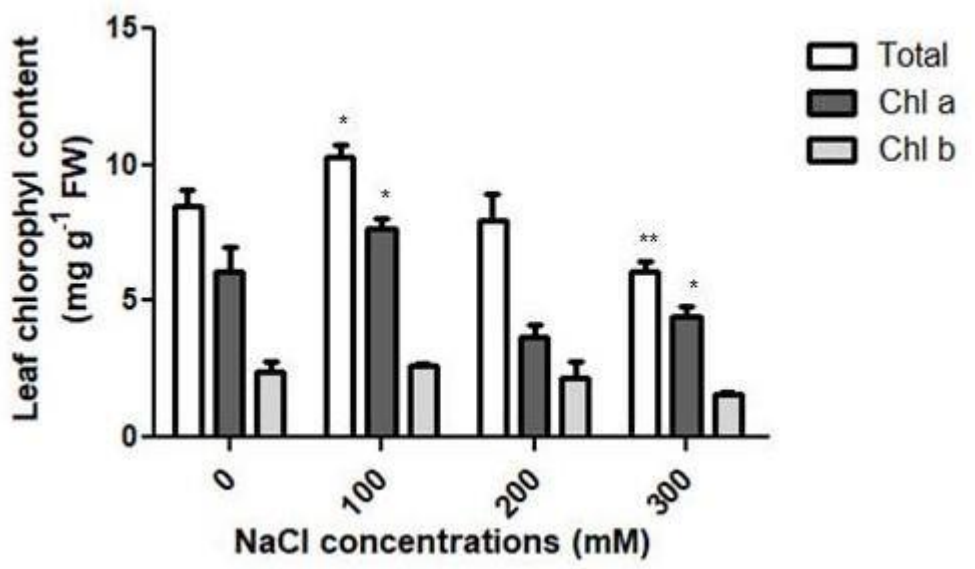

Figure 3.3. Effects of salt stress on chlorophyll content of sorghum plants. Chlorophyll content of sorghum plants with the application of different NaCl concentrations of 0 mM (control), 100 mM, 200 mM and 300 mM NaCl. Error bars represent the SD calculated from three biological replicates. Statistical significance between the different treated plants was determined using one-way ANOVA as conducted on GraphPad Prism and indicated as ** = $p \leq 0.01$, * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

3.3.2. Biochemical response of sorghum to salt stress

3.3.2.1. Oxidative stress markers

Oxidative damage due to salt stress was determined based on ROS ($O_2^{\bullet-}$ and H_2O_2) and malondialdehyde (MDA) content (Figure 3.4). Overproduction of ROS was determined through histochemical staining of sorghum leaves to reveal the accumulation of $O_2^{\bullet-}$ and H_2O_2 (Figure 3.4a and b). This was observed either as dark blue spots for $O_2^{\bullet-}$ and brown spots for H_2O_2 in the leaves of salt-stressed sorghum plants. For $O_2^{\bullet-}$ staining under 100 mM NaCl, salt-stressed sorghum plants barely showed any dark blue spots on the leaves. However, the increased NaCl concentrations of 200 mM NaCl slightly increased $O_2^{\bullet-}$ production, whereas 300 mM NaCl treatment resulted in the complete covering of sorghum leaves with $O_2^{\bullet-}$ spots. A similar trend was observed for H_2O_2 staining, which showed a minor increase in brown spots under 100 mM NaCl treatment in comparison to control leaves. Increased production of H_2O_2 spots was observed under 200 mM NaCl treatments, whereas under 300 mM NaCl

treatments a high intensity of brown spots was observed where the leaves were almost completely covered with brown spots indicating high level of H_2O_2 production.

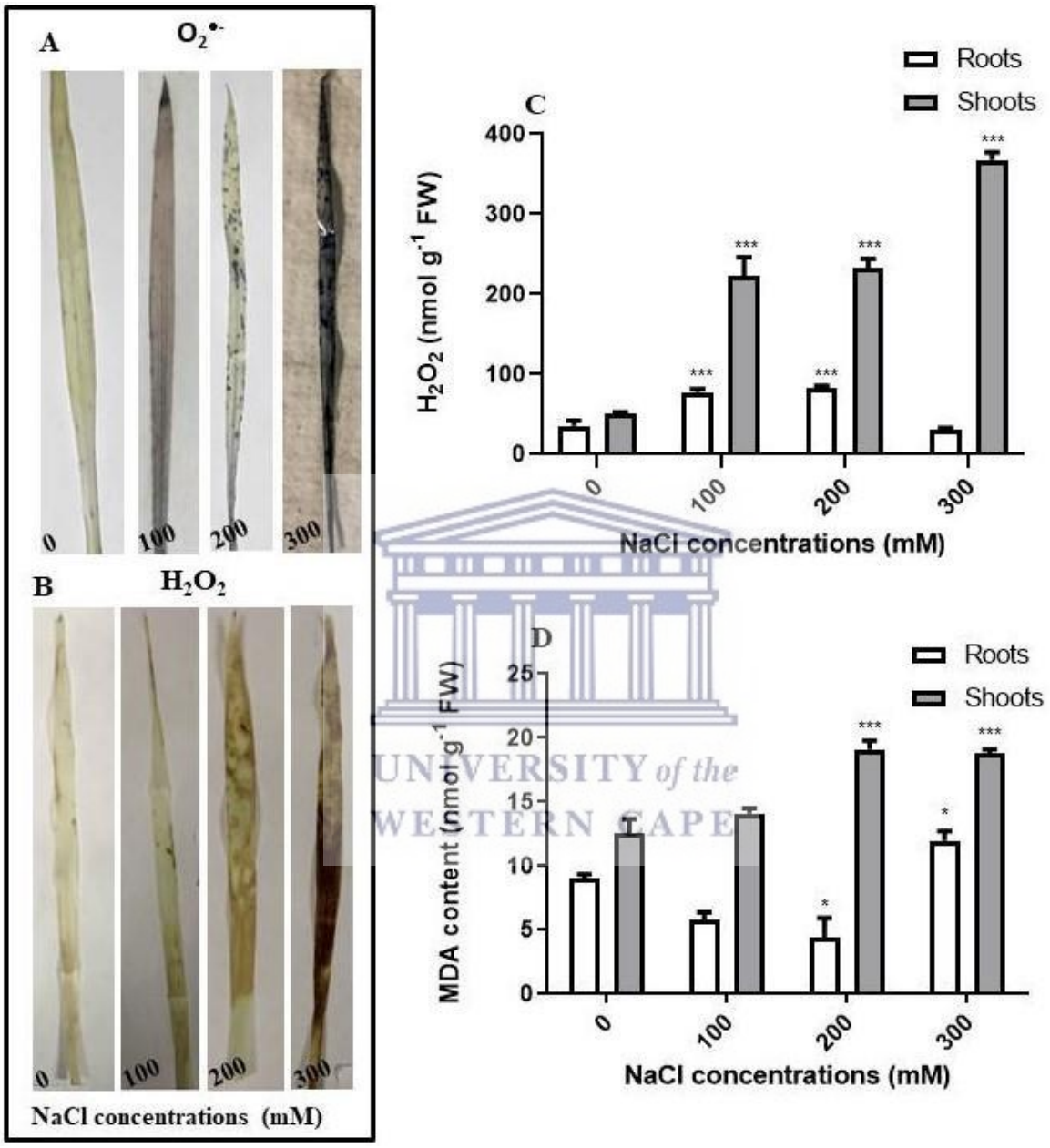

Figure 3.4. Effects of salt stress on oxidative stress markers ROS ($O_2^{\bullet-}$ and H_2O_2) and MDA content. Histochemical staining of $O_2^{\bullet-}$ (**A**), H_2O_2 (**B**), quantification of H_2O_2 and (**C**) MDA (**D**) content of sorghum plants under different NaCl concentrations (0 mM, 100 mM, 200 mM and 300 mM). Error bars represent the SD calculated from three biological replicates. Statistical significance between the control and treated plants was determined using one-way ANOVA as conducted on GraphPad Prism and indicated as *** = $p \leq 0.001$ and * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

Hydrogen peroxide and MDA contents were further quantified, where treatments of sorghum plants with different NaCl concentrations resulted in a significant (*** = p≤0.001) increase in H_2O_2 content in the roots and shoots except for treatments with 300 mM NaCl in the roots were no significant changes were observed as compared to that of the control (Figure 3.4c). Under100 mM NaCl treatment H_2O_2 content increased by 2.2-fold in the roots and 4.4-fold in the shoots. The application of 200 mM NaCl showed a significant increase (*** = p≤0.001) of 2.3-fold and 4.6-fold in the roots and shoots of sorghum plants respectively, with the highest increase (*** = p≤0.001) in H_2O_2 content observed under 300 mM NaCl (7.3-fold) application in the shoots. The levels of MDA a marker for lipid membrane peroxidase did not result in any significant changes under 100 mM NaCl treatments in both tissues (Figure 3.4d). A significant (* = p≤0.05) decrease in MDA content was observed in the roots (2-fold) and a significant (*** = p≤0.001) increase in the shoots (1.5-fold) under 200 mM NaCl treatments. Stressing sorghum plants with 300 mM NaCl significantly increased the MDA content in the roots by 1.3-fold (* = p≤0.05) and shoots by 1.5-fold (*** = p≤0.001) of plants.

3.3.3. Effect of salt stress on the defence mechanism

3.3.3.1. Non-enzymatic antioxidants

The non-enzymatic antioxidants play important roles in disrupting free radical chain reactions (Nimse & Pal, 2015). To determine the response of sorghum plants to salt stress, the non-enzymatic antioxidant contents of proline, soluble sugars and carotenoids were determined (Figure 3.5). A linear increase in proline content with increasing NaCl concentrations in the roots and shoots of sorghum plants, (except for roots under 300 mM NaCl treatment) was observed (Figure 3.5a). A significant (*** = p≤0.001) increase in proline content was observed in the roots (6.8-fold) and shoots (9.4-fold) under 100 mM NaCl treatment.

Furthermore under 200 mM NaCl treatment a significant (*** = p≤0.001) increase in proline content was observed in the roots and shoots by more than 11-fold. The highest salt (300 mM NaCl) concentration did not result in any significant changes in proline content in the roots as compared to that of the control, but a significant (*** = p≤0.001) increase was observed in the shoots (13.1-fold) under 300 mM NaCl treatment (Figure 3.5a).

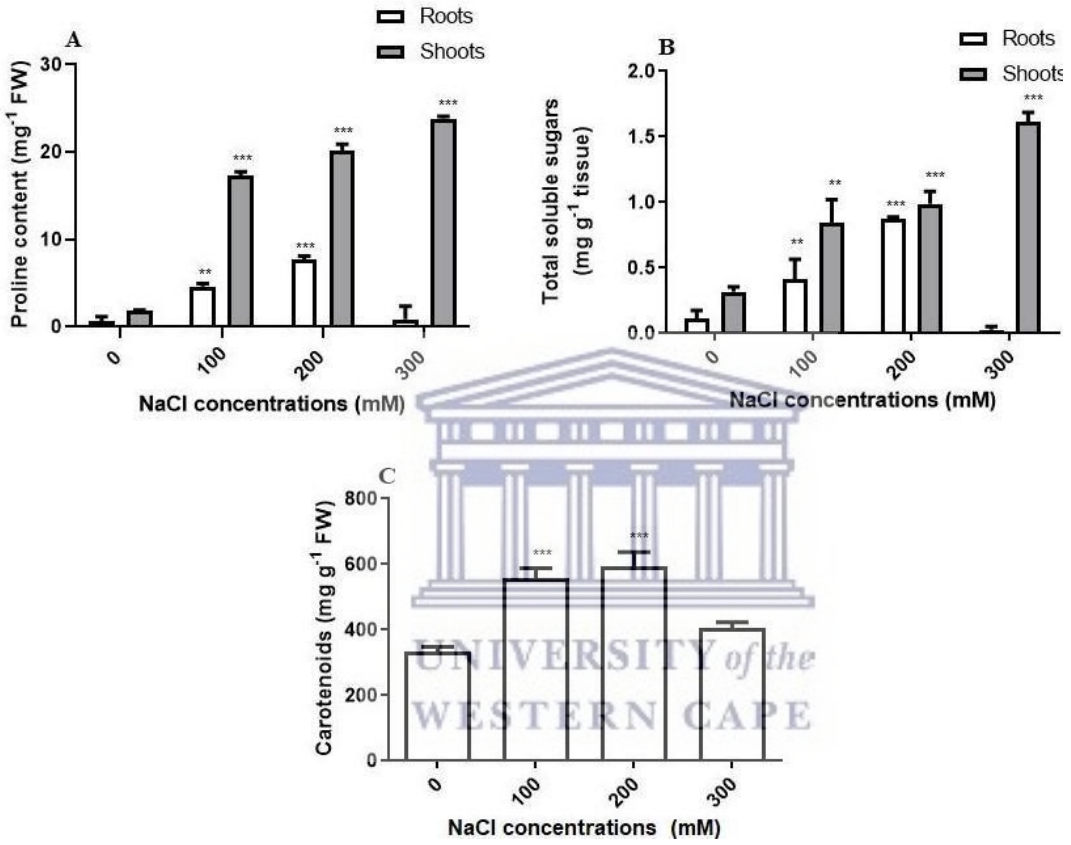

Figure 3.5. The effect of salt stress on the non-enzymatic antioxidants. Proline content (**A**), total soluble sugar content and (**B**) carotenoid content (**C**) measured under different NaCl treatments of (0 mM, 100 mM, 200 mM and 300 mM) respectively. Error bars represent the SD calculated from three biological replicates. Statistical significance between the control and treated plants was determined using one-way ANOVA as conducted on GraphPad Prism and indicated as *** = p≤0.001 and ** = p≤0.01 according to the Bonferroni's multiple comparison test.

A similar trend was observed with the soluble sugar content (Figure 3.5b). The soluble sugars increased by 2.6-fold in both the roots and shoots under 100 mM NaCl treatment, whereas under 200 mM NaCl a significant (*** = p≤0.001) increase in soluble sugars was observed in

the roots (5.4-fold) and shoots (3-fold) of sorghum plants. The application of 300 mM NaCl increased the soluble sugar content by 5-fold in the shoots.

Carotenoid content (Figure 3.5c) significantly (*** = p≤0.001) increased under 100 mM NaCl (1.7-fold) and 200 mM (1.8-fold) NaCl treatments, with no significant changes observed under 300 mM NaCl treatment.

3.3.3.2. Enzymatic antioxidant activity

To determine the antioxidant scavenging capacity of *Sorghum bicolor* plants in response to salt, the activities of ROS scavenging enzymes (SOD and APX) were assayed (Figure 3.6). The effects of salt stress on the antioxidant enzyme activities were observed to be more prominent in the roots than shoots of sorghum plants. Superoxide dismutase activity increased linearly with an increase in salt concentrations. A significant increase in the roots 2-fold (* = p≤0.05) and shoots 3.2-fold (*** = p≤0.001) was observed under 100 mM NaCl application. Treatment of sorghum plants with 200 mM NaCl significantly (*** = p≤0.001) increased SOD activity by 3-fold in both the roots and shoots. A significant (*** = p≤0.001) increase of 2.9-fold was observed in the roots under 300 mM NaCl treatments with no significant changes observed in the shoots as compared to the control.

The APX activity significantly (* = p≤0.05) increased in the roots by 1.3-fold with the application of 100 mM NaCl (Figure 3.6b). A significant (*** = p≤0.001) increase was observed in the roots (3.9-fold) and shoots (3.7-fold) of sorghum plants stressed with 200 mM NaCl, with no significant changes observed in APX activity under 300 mM NaCl treatments as compared to the control.

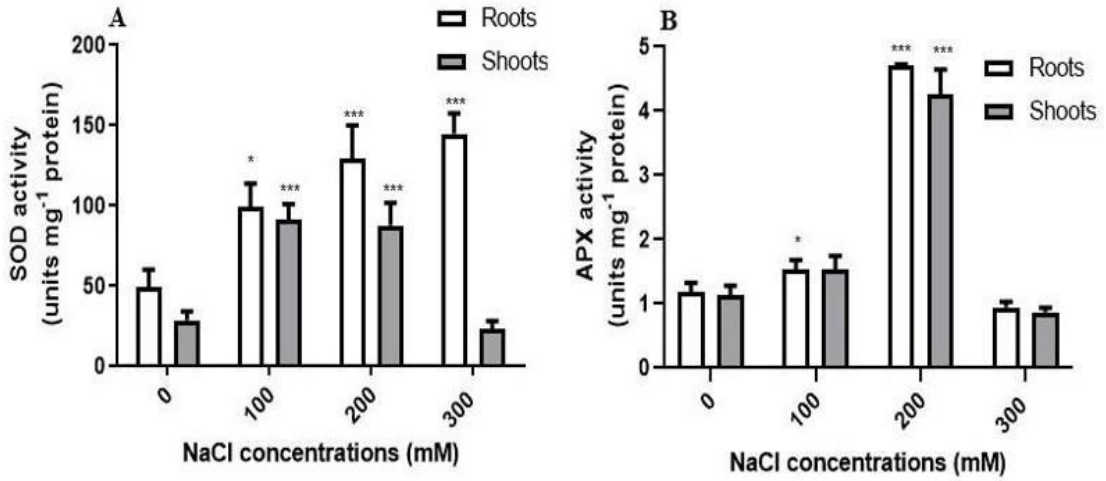

Figure 3.6. The effects of salt stress on enzymatic antioxidant activities. SOD (**A**) and APX (**B**) antioxidant activities of different NaCl concentrations (0 mM, 100 mM, 200 mM and 300 mM) applied to sorghum plants. Error bars represent the SD calculated from three biological replicates. Statistical significance between the control and treated plants was determined using one-way ANOVA as conducted on GraphPad Prism and indicated as *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

3.3.4. Heat Shock Protein-70 expression

The molecular response of sorghum plants exposed to salt treatment was investigated by determining the expression level of the heat shock protein-70 (HSP-70) in the shoots of sorghum plants using a dot blot assay (Figure 3.7). An increase in the expression levels of HSP-70 was observed with increasing salt treatments from 100 mM to 200 mM NaCl in comparison to the control (Figure 3.7a).

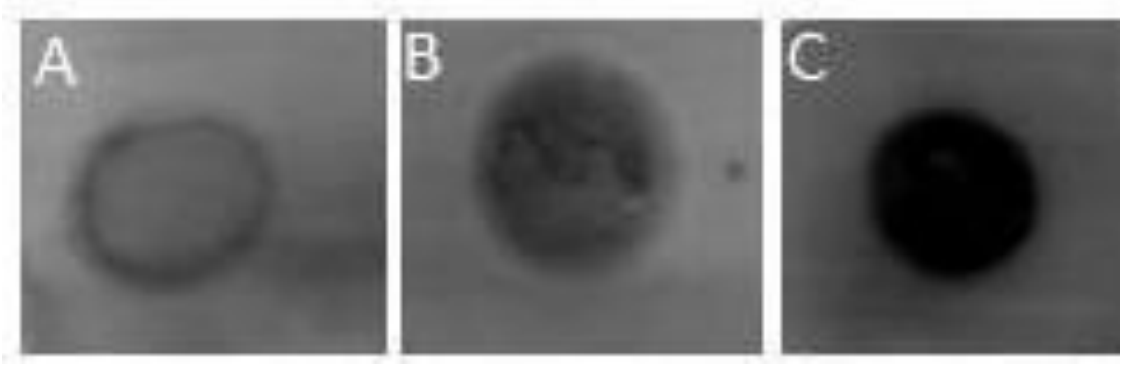

Figure 3.7. Dot blot analysis on the effect of salt stress on the expression of HSP-70 in sorghum shoots. Control (0 mM NaCl) (**A**), 100 mM NaCl and (**B**) 200 mM NaCl treatments (**C**).

3.4. DISCUSSION

Salinity is one of the most brutal environmental stresses that affect a plant's ability to absorb water by lowering the water potential in the soil (Otlewska et al., 2020). Although sorghum is considered a moderately salt tolerant crop, excess exposure to salt in the soil can have adverse effects on its growth and hence hinder crop yield (Calone et al., 2020).

3.4.1. Salt stress affects sorghum growth

Growth inhibition is one of the first and most general responses of plants to stress. In this study, at low salt concentrations (100 mM NaCl), the growth of sorghum plants was not affected (Figure 3.1). Under high salt concentrations from 200 mM to 300 mM NaCl, a significant reduction in sorghum growth was observed (Figure 3.1) as seen by reduced fresh and dry weights (Table 3.1). These results are consistent with the general trend of reduced plant growth under salt stress conditions (Zhao et al., 2021). Similar results were reported under salinity stress in *Sorghum bicolor* under 300 mM and 400 mM NaCl (Mulaudzi et al., 2022; Rakgotho et al., 2022), *Daucus carota* L under 50 mM NaCl (Inal et al., 2009) and *Solamum melongena* L. under 150 mM NaCl (Shaheen et al., 2012) treatments. The reduction in biomass under salt stress is indicative of growth limitations (Zhou et al., 2015) and could be due to the adverse effects of salinity on cell division and elongation. The results showed that the highest salt treatment of 300 mM NaCl caused a considerable reduction in sorghum plants growth and this agrees with similar results reported on *Vigna mungo* L (Kapoor & Srivasta, 2010), where increasing concentrations of NaCl resulted in reduced length of plants. This inhibitory effect was probably attributed to the influence of osmotic stress, which interferes with metabolic processes, reducing turgo and energy required for maintaining plant growth (de Oliveira et al., 2013). The high-water content might be due to enhance stomatal conductance in stressed sorghum plants. This also support the fact that sorghum is moderately

tolerant to salinity, hence the high-water content accumulation, as they are able to prevent water loss and hence led to adjusted osmotic pressure (Mansour et al., 2021). When plants are exposed to high salt environments, they undergo osmotic stress and ion toxicity, which in turn lead to changes in membrane permeability affecting the absorption of water by the plants. This can also destroy the structure of the membrane as observed with SEM micrographs in this study that showed deformation of epidermis and shrinkage of xylem and phloem layers (Figure 3.2).

3.4.2. Salt stress affects photosynthesis in sorghum

Photosynthesis is the key process that provides energy for plants. High salt concentrations affect photosynthesis through osmotic stress and ion toxicity (Qi et al., 2020). Plants that are exposed to high salt conditions result in ion imbalance in cells, thus affecting absorption of light energy. A decrease in chl content in salt treated sorghum plants was observed (Figure 3.3) under 200 mM and 300 mM NaCl treatments. The decrease in chl content agrees with other studies that reported a decrease in chl content under different salt stress conditions (Mostafa, 2011; Siddiqui et al., 2018; Zhang et al., 2014). The decrease in chl content due to salt stress can be attributed to the increased activity of chlorophyllase (Hameed et al., 2009) and ROS generation (Foyer et al. 1994; Mittler, 2002). Treatment of sorghum plants with 300 mM NaCl resulted in a significant reduction in total chl and chl content with no significant changes observed in chl 'b'. When plants are exposed to salt-stressed conditions, they experience water stress, which in turn reduces leaf expansion, thus photosynthesis is reduced. The reduced photosynthesis with increasing salinity is attributed to either stomatal closure, leading to reduction in intracellular CO_2 partial pressure or non-stomatal factors (Amirjani, 2011).

3.4.3. Salt stress causes oxidative damage in sorghum

The current study revealed that salt stress, stimulated oxidative stress in sorghum plants as evident from the high production of ROS ($O_2^{\bullet-}$ and H_2O_2) and lipid peroxidation (MDA) content (Figure 3.4). Under stress conditions, H_2O_2 is produced and accumulates in cell organelles, leading to oxidative stress in plants. The overproduction of ROS mainly $O_2^{\bullet-}$ and H_2O_2 on the leaves of salt-stressed sorghum plants were observed as dark blue spots for $O_2^{\bullet-}$ and brown spots for H_2O_2 (Figure 3.4a and b). The high production of ROS on the leaves of salt-stressed sorghum plants can be as a signal for adaptive response (Foyer et al., 2007) to salt stress. Our results showed that the production of ROS caused membrane damage, as observed in other plant species (Hasanuzzaman et al., 2021). This further correlates with the anatomical analysis showing rough epidermal, xylem and phloem layers of salt-stressed plants. Lipid peroxidation is another sign of oxidative stress, in this study it was estimated by assaying MDA content, a marker for oxidative damage (Morales & Munne-Bosch, 2019). Salt stress significantly increased MDA levels under 200 mM and 300 mM NaCl treatments. The high accumulation of MDA content observed represents the degree of cell membrane damage (Kumar et al., 2021; Taïbi et al., 2016).

3.4.4. Response of sorghum plants to salt stress via osmoregulation

Plants have evolved different responsive mechanisms in order to cope with salt stress conditions. Amongst these responses is the accumulation of low molecular compounds known as osmolytes such as proline and soluble sugars. The results from this study showed a gradual increase in proline and soluble sugar contents in the roots and shoot of sorghum plants with increasing NaCl concentrations. The uptake of high amounts of salt by plants leads to increased osmotic pressure in the cytosol and under these conditions cell homeostasis is maintained by osmotic adjustments, which is mediated by organic osmolytes (Munns, 2002).

It has been shown in many plant species that in addition to their roles as osmoregulators, elevated levels of osmolytes are produced to protect cells against the adverse effects of stress, in order to enhance stress tolerance by scavenging free radicals and protecting enzymes (Slama et al., 2015; Benhassaini et al., 2011). The high accumulation of osmolytes as observed in our study can be associated with tolerance of sorghum plants to salt stress conditions (Mulaudzi et al., 2022; Rakgotho et al., 2022; Mulaudzi et al., 2020). The increase in proline content can also be associated with high activities of pyrroline 5-caboxylate synthtase (P5CS) a key enzyme in proline biosynthesis (Chun et al., 2018). Similarly, many studies have shown that salt tress triggers the induction of genes involved in proline biosynthesis, which leads to proline accumulation (Heidari, 2014; Armenguad et al., 2004). The decrease in proline content observed in the roots (Figure 3.5a) under 300 mM NaCl treatments may suggest low activity of enzymes P5CS and glutamine dehydrogenase, which play important roles in the pathway of proline biosynthesis (Kumar et al., 2021).

Soluble sugars also play important roles in metabolic resources and the structural constitutes of cells. They act as signals regulating various processes associated with plant growth and development (Rosa et al., 2009). A linear increase in soluble sugar content was observed with increasing salt concentrations in both the roots and shoots (exception was observed under 300 mM NaCl treatment in the roots) of sorghum plants, in comparison to the control. Increased accumulation of soluble sugars has also been reported in different plant species exposed to salt stress including, *Oryza sativa* (Dubey & Singh, 1999), *Ficus carica* (Mascellani et al., 2021) *Pistica vera* L. (Benhassaini et al., 2011; Abbadpour et al., 2012) and *Oenanthe javanica* (Kumar et al., 2021). The high accumulations of osmolytes as a consequence of salt stress suggest a high osmotic adjustment of sorghum plants to salt stress conditions.

Carotenoids are light harvesting pigments that play important roles in photosynthetic tissue of plants by protecting plants from oxidative damage (Hashimoto et al., 2016). The increased

in carotenoid content observed were to alleviate oxidative damage induced by salt stress to sorghum plants. Numerous studies have shown that increase in carotenoid content can be to enhance plant tolerance to salt stress conditions (Kim et al., 2012; Li et al., 2017).

3.4.5. Enzymatic antioxidant response of sorghum to salt stress

The current study showed that the roots where more sensitive to oxidative stress as seen by higher antioxidant activities (Figure 3.6). The roots of plants play important roles in plant growth and development and are the first part of a plant to encounter salt stress and the most directly affected (Wang et al., 2014). When plants are exposed to salt stress conditions, the roots have to cope with osmotic stress and ion toxicity. These can cause reduction in water uptake, inhibition of root growth and induction of secondary stress (Munns & Tester, 2008). Superoxide dismutase is the most effective enzymatic antioxidant that is ubiquitous in all aerobic organisms and act as the first line of defence against oxidative stress in plants (Hassan et al., 2017). Superoxide dismutases function by catalysing the dismutation of $O_2^{\bullet-}$ into less toxic H_2O_2 and O_2 (Gill & Tuteja, 2010). Increased activities of SOD were observed with increasing NaCl concentrations. These results agree with previous reports, reporting an increase in SOD activities under 300 mM and 400 mM NaCl salt stress conditions (Rakgotho et al., 2022; Mulaudzi et al., 2022).

Ascorbate peroxidases are haem containing enzymes that also play important roles in scavenging free radical reactions (Kumar et al., 2021). The highest increase of APX activity was observed under 200 mM NaCl treatments, with no significant changes observed under 300 mM NaCl in comparison to control plants. This indicates that high salt concentrations (200 mM) in this case, stimulated APX activity to enhance basal antioxidant capacity to overcome oxidative stress. A decrease in APX activity under 300 mM NaCl treatments,

indicate that the exposure of plants to high salt concentrations might have destroyed cell membranes leading to lower antioxidant activities (Hnilickova et al., 2021).

Based on the antioxidant activities of APX that exhibited the highest expression at 200 mM NaCl treatments in both the roots and shoots of sorghum plants, we considered 200 mM NaCl to be the highest concentration to induce sorghum response to salt stress, therefore we investigated the expression of HSP-70 protein, a known stress responsive protein. Heat shock protein-70, was highly induced in the shoots of salt-stressed sorghum plants as compared to the control (Figure 3.7). These results are consistent with previous findings that showed increased expression of HSP-70 under salt stress in sorghum (Ngara et al., 2012). The induction of the HSP-70 under stress correlates with its role as a molecular chaperon by preventing protein mis-folding and help with proper folding of mis-folded proteins as well as preventing protein denaturation (Al Khateeb et al., 2020).

3.5. CONCLUSION

Salinity stress caused deleterious effects on the growth parameters of *Sorghum bicolor* through high accumulation of oxidative markers; $O_2^{\bullet-}$ and H_2O_2 and hence increased membrane damaged of salt-stressed plants. This study confirmed that sorghum is moderately tolerant to salt conditions, as it was shown under 100 mM NaCl, the lowest salt concentration tested since most parameters were not significantly affected. In addition, under high salt concentrations (200 mM and 300 mM NaCl), high accumulation of non-enzymatic (proline, soluble sugars and carotenoids) and enzymatic (SOD and APX) antioxidants was observed, as an indication of tolerance to stress. Similarly, high concentrations of salinity increased the expression of stressed responsive protein 'HSP-70'. This showed that ROS scavenging system is an essential part in protecting sorghum plants from salt stress and for maintaining cellular redox for plant growth and development.

CHAPTER FOUR
INFLUENCE OF MOLYBDENUM ON THE MOPHO-PHYSIOLOGICAL AND BIOCHEMICAL ATTRIBUTES OF *SORGHUM BICOLOR*

4.1. ABSTRACT

Molybdenum (Mo) is an essential micronutrient required for normal plant growth and development. Although required in trace amounts by plants, its overuse can affect crop production. This chapter investigated the morpho-physiological and biochemical response of unstressed sorghum plants to Mo treatment. Exogenous Mo negatively affected sorghum growth by reducing plant biomass especially at high concentrations. Exogenous Mo also significantly reduced the total chl content under treatment with 0.5 µM and 1 µM Mo, except for 2 µM Mo. The overproduction of ROS; $O_2^{\bullet-}$ and H_2O_2 stains were observed on the leaves of sorghum plants treated with high Mo (1 µM and 2 µM) concentrations. Furthermore, H_2O_2 content increased by 6.9-fold under 2 µM Mo treatment in the shoots. The highest MDA content was observed in sorghum plants treated with 1 µM Mo, which increased by 2-fold indicative of lipid peroxidation. Proline content significantly increased in both roots and shoots by more than 1-fold under the lowest Mo (0.5 µM) concentration, with the only significant increase observed in the roots (1.8-fold) under 1 µM Mo treatment and a reduction under 2 µM Mo treatment in the shoots (2.7-fold). Soluble sugars and carotenoid contents significantly increased under all Mo concentrations. The SOD activity increased in the roots under 0.5 µM Mo (5.9-fold) and 1 µM Mo (4.9-fold) treatments. The APX activity significantly increased in the roots under all applied Mo concentrations, where the highest increase was 2-fold observed under 0.5 µM Mo treatment. These findings indicate that low Mo (0.5 µM) concentration is effective in improving sorghum growth under salt stress.

KEYWORDS: Growth parameters, Micronutrient, Molybdenum, Sorghum.

4.2. INTRODUCTION

Molybdenum is one of the essential micronutrients in addition to boron (B), copper (Cu), Iron (Fe), chlorine (Cl), nickel (Ni), manganese (Mn) and zinc (Zn) required by plants for normal growth and development (Hansch & Mendel, 2009; White & Brown, 2010). Even though required in small quantities by plants, micronutrients cannot be neglected as they play various roles in plant metabolism and deficiency in any micronutrients can limit plant growth and development (Kumar et al., 2021). Except for its importance as a micronutrient in plants, Mo also play an important role as a catalytic metal for various enzymes in plants, which include nitrate reductase, aldehyde oxidase, xanthine dehydrogenase and aldehyde oxidase (Liu et al., 2019). It is one of the principal routes through, which inorganic nitrogen is incorporated into organic compounds in species such as algae and fungi (Salha et al., 2016). Molybdenum remains inactive in plants until it becomes complexed to molybdenum cofactor (Moco), to gain biological activity (Hayyawi et al., 2020; Wu et al., 2014).

The availability of micronutrients to plants is mostly dependent on the pH of the soil. Molybdenum becomes more available when the pH increases or is above neutrality (Valenciano et al., 2011). Molybdenum deficiency in plants induces reductions in Moco biosynthesis and Mo enzyme activities, which is displayed as chlorosis, necrosis, stunting and lack of vigor with dryness in leaves (Imran et al., 2019; Sun et al., 2009; Mendel & Hansch, 2002). Molybdenum deficiencies can be alleviated through the exogenous application of Mo to enhance crop growth and hence yield (Liu, 2001; Xue-Cheng et al., 2006) as observed in *Vitis vinifera* (Longbottom et al., 2010).

Molybdenum is usually used as a component of complex fertilizers for plants in areas where the natural Mo content is low. And this is important to manage nutrient balance in plants through application of fertilizers, to influence nutrient availability, induce plant tolerance to

stresses and increase crop yield or quality (FAO, 2009; Cheng et al., 2021). In this study *Sorghum bicolor* is used as a model plant to investigate the role of Mo to improve growth. Although sorghum is a well able to grow in different climates (Craufurd et al., 1999), its growth and development is still greatly affected by deficiencies of mineral nutrients in soils resulting in yield loss (Christin et al., 2009; Sarshad et al., 2021). Molybdenum plays an important role in different redox reactions in plants, achieved at very low concentrations (Tejada-Jimenez et al., 2013). But earlier studies have shown that high concentrations of Mo can have negative effects on plant growth and development (Rana et al., 2020). Thus, this chapter aims to determine the lowest Mo concentration necessary to improve plant growth under non-stress conditions without causing any toxicity.

4.3. RESULTS

4.3.1 Morpho-physiological response of sorghum to molybdenum

4.3.1.1. Growth attributes

In order to determine the effects of exogenously applied Mo on unstressed sorghum plants morphological and physiological parameters were analysed. Exogenous application of Mo (0.5 µM), did not result in any observable changes on sorghum plants, but high concentrations of 1 µM and 2 µM Mo reduced the shoot length (Figure 4.1).

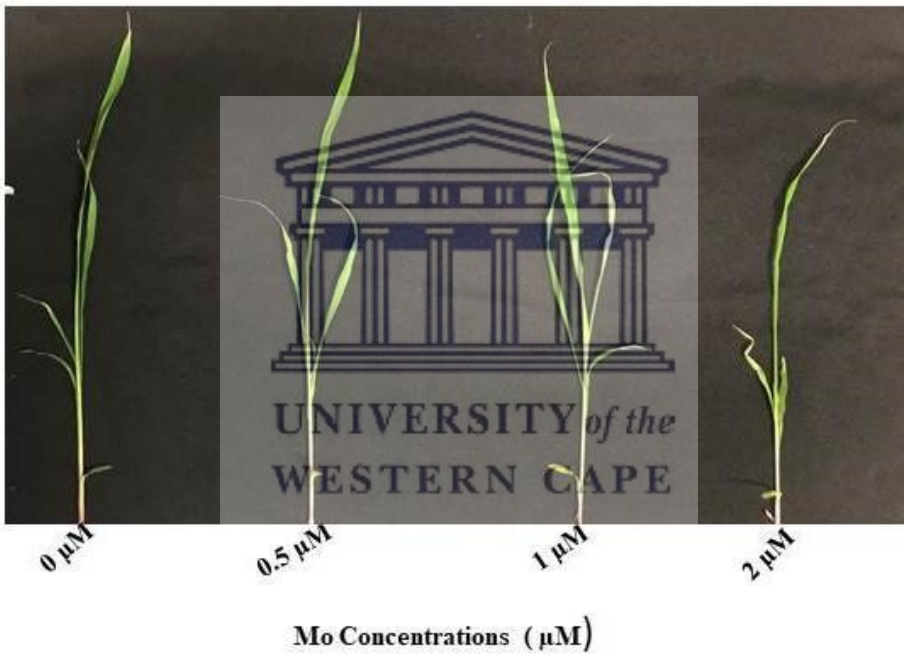

Figure 4. 1. Effects of Mo application on shoot growth of sorghum plants. Phenotypic representation of shoot growth of sorghum plants grown under normal growth conditions with different concentrations of exogenously applied Mo (0 µM, 0.5 µM, 1 µM. and 2 µM), respectively.

Table 1 showed a slight increase in the fresh weight (FW) of plants treated with 0.5 µM Mo applications from 0.52 mg to 0.57 mg. The application of 1 µM and 2 µM Mo significantly (** = $p \leq 0.01$) reduced the FW by 1.6-fold and 2.2-fold respectively. Exogenous application

of Mo significantly reduced the dry weight (DW) with increasing Mo concentrations from 0.05 mg to 0.03 mg (1.4-fold) for 0.5 µM Mo application, and 0.02 mg (2.3-fold) and 0.01 mg (3.8-fold) observed for 1 µM and 2 µM Mo applications respectively. All Mo concentrations increased the WC% of sorghum plants as compared to that of the control, with the only significance (* = $p \leq 0.05$) observed with the application of 2 µM Mo by 1-fold.

Table 4.1. Influence of Mo on the FW, DW and WC% of unstressed sorghum plants.

Mo (µM)	FW	DW	WC%
0	0.521±0.030	0.046±0.004	91.180±0.312
0.5	0.570±0.028	0.032 ± 0.005*	94.488±0.595
1	0.315±0.049**	0.020±0.001**	93.714±1.212
2	0.240±0.014**	0.012±0.002**	95.174±1.169*

** and * indicate significant differences at $p \leq 0.01$ and $p \leq 0.05$ respectively. FW = fresh weight, DW = dry weigtht and WC = water content.

4.3.1.2. Influence of Mo on chlorophyll content

The effect of Mo on chlorophyll content of sorghum plants was analysed as observed in Figure 4.2. Exogenous application of Mo to unstressed sorghum plants significantly (* = $p \leq 0.05$) reduced the total chl contents under 0.5 µM Mo treatment by 1.2-fold whereas no significant changes were observed in chl 'a' and chl 'b' content in comparison to that of the control. The application of Mo significantly (** = $p \leq 0.01$) reduced the total chl content by 1.3-fold and chl 'b' content by 1.6-fold under 1 µM Mo with no significant changes observed with 2 µM Mo application.

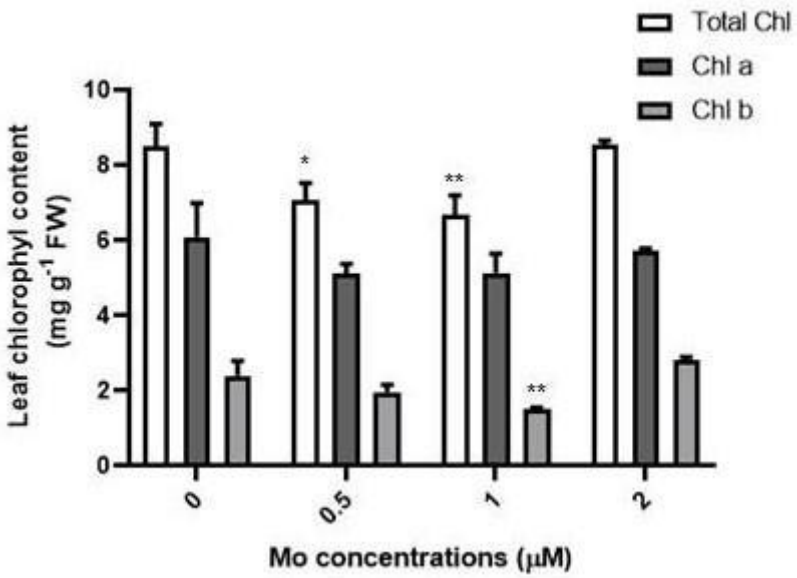

Figure 4.2. **The influence of Mo on the chlorophyll content of sorghum plants.** Representation of chlorophyll content in *Sorghum bicolor* plants under different Mo concentrations of 0 µM (control), 0.5 µM, 1 µM and 2 µM Mo respectively. Error bars represent the SD calculated from three biological replicates. Statistical significance between the different treated plants was determined using one-way ANOVA as conducted on GraphPad Prism and indicated as ** = $p \leq 0.01$, * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

4.3.2 Biochemical response of sorghum to molybdenum

4.3.2.1. Oxidative stress markers

To determine the effects of exogenously applied Mo on the biochemical traits of unstressed sorghum plants, the response of oxidative markers was determined based on ROS ($O_2^{\bullet-}$ and H_2O_2) and MDA contents. The production of ROS on the leaves of sorghum plants was determined through histochemical staining of $O_2^{\bullet-}$ and H_2O_2 as observed in Figure 4.3a and b. Figure 4.3a showed increased accumulation of $O_2^{\bullet-}$ seen as dark blue spots on the leaves of unstressed sorghum plants treated with Mo. The highest production of $O_2^{\bullet-}$ was observed with the highest application of 2 µM Mo. Application of 0.5 µM Mo did not result in any H_2O_2 toxicity to plants (Figure 4.3b). The exogenous application of 1 µM and 2 µM Mo resulted in the overproduction of H_2O_2 on the leaves of plants seen as brown spots.

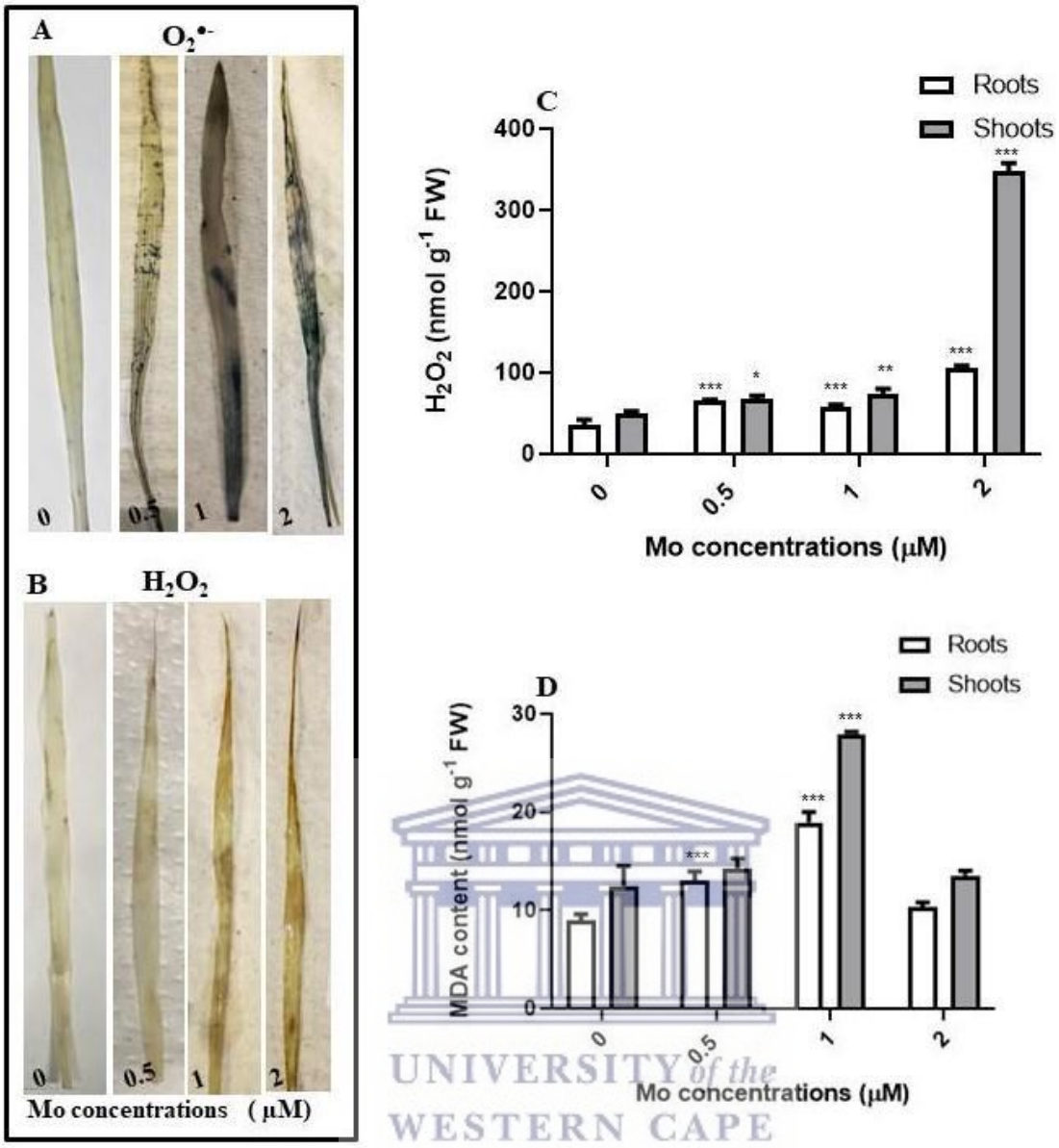

Figure 4.3. Effect of Mo application on oxidative stress markers. Histochemical staining of $O_2^{\bullet-}$ (**A**), H_2O_2 (**B**), quantification of oxidative stress markers H_2O_2 and (**C**) MDA content (**D**). Error bars represent the SD calculated from three biological replicates. Statistical significance between the control and treated plants was determined using one-way ANOVA as conducted on GraphPad Prism and indicated as *** = $p \leq 0.001$ and ** = $p \leq 0.01$ and * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

Analysis of H_2O_2 content showed significant increase in H_2O_2 content in the roots (1.8-fold) and shoots (1.3-fold) with the application of 0.5 µM Mo. Treatment of plants with 1 µM Mo significantly increased H_2O_2 content by 1.6-fold and 1.4-fold in the roots and shoots respectively. The highest Mo (2 µM Mo) concentration significantly (*** = $p \leq 0.001$) resulted in the highest H_2O_2 content in the roots (2.9-fold) and shoots (6.9-fold). The level of

membrane damage as determined by MDA content, which was significantly (*** = p≤0.001) increased in sorghum plants treated with 0.5 µM Mo by 1.4-fold in the roots, with no significant changes observed in the shoots of sorghum plants in comparison to the control. Treatment of sorghum plants with 1 µM Mo significantly (*** = p≤0.001) increased MDA content by 2.1-fold and 2.2-fold in the roots and shoots respectively. No significant changes were observed with Mo treatments of 2 µM in both the roots and shoots of sorghum plants.

4.3.3. Effect of molybdenum on the defence mechanism

4.3.3.1. Non- enzymatic antioxidants

To determine the effect of Mo to unstressed sorghum plants, the non-enzymatic antioxidant contents of proline, soluble sugars and carotenoids were determined. Figure 4.4a showed that the exogenous application of 0.5 µM Mo significantly (** = p≤0.01) increased proline content in the roots (2-fold) and shoots (1.5-fold) of sorghum plants. Treatment of plants with 1 µM Mo resulted in high proline content in the roots (1.8-fold) only, with no significant changes observed in the shoots as compared to that of the control. The highest application of 2 µM Mo significantly (*** = p≤0.001) reduced proline content (2.7-fold) in the shoots with no significant changes observed in the roots as compared to that of the control. Exogenous application of Mo significantly increased soluble sugar content at all concentration of Mo (0.5 µM, 1 µM and 2 µM) in the roots and shoots of sorghum plants. A significant (*** = p≤0.001) increase of 7.7-fold in the roots and 4.3-fold in the shoots was observed under 0.5 µM Mo application. Treatment of sorghum plants with 1 µM Mo significantly increased the soluble sugars contents in the roots by 5.1-fold and shoots by 4.2-fold. The application of 2 µM Mo significantly increased soluble sugars content in the roots and shoots by over 5-fold.

Figure 4.4c showed carotenoid content to significantly (*** = p≤0.001) increased by 1.5-fold (0.5 µM) 1.7-fold (1 µM) and 2.6-fold (2 µM) under Mo treatments.

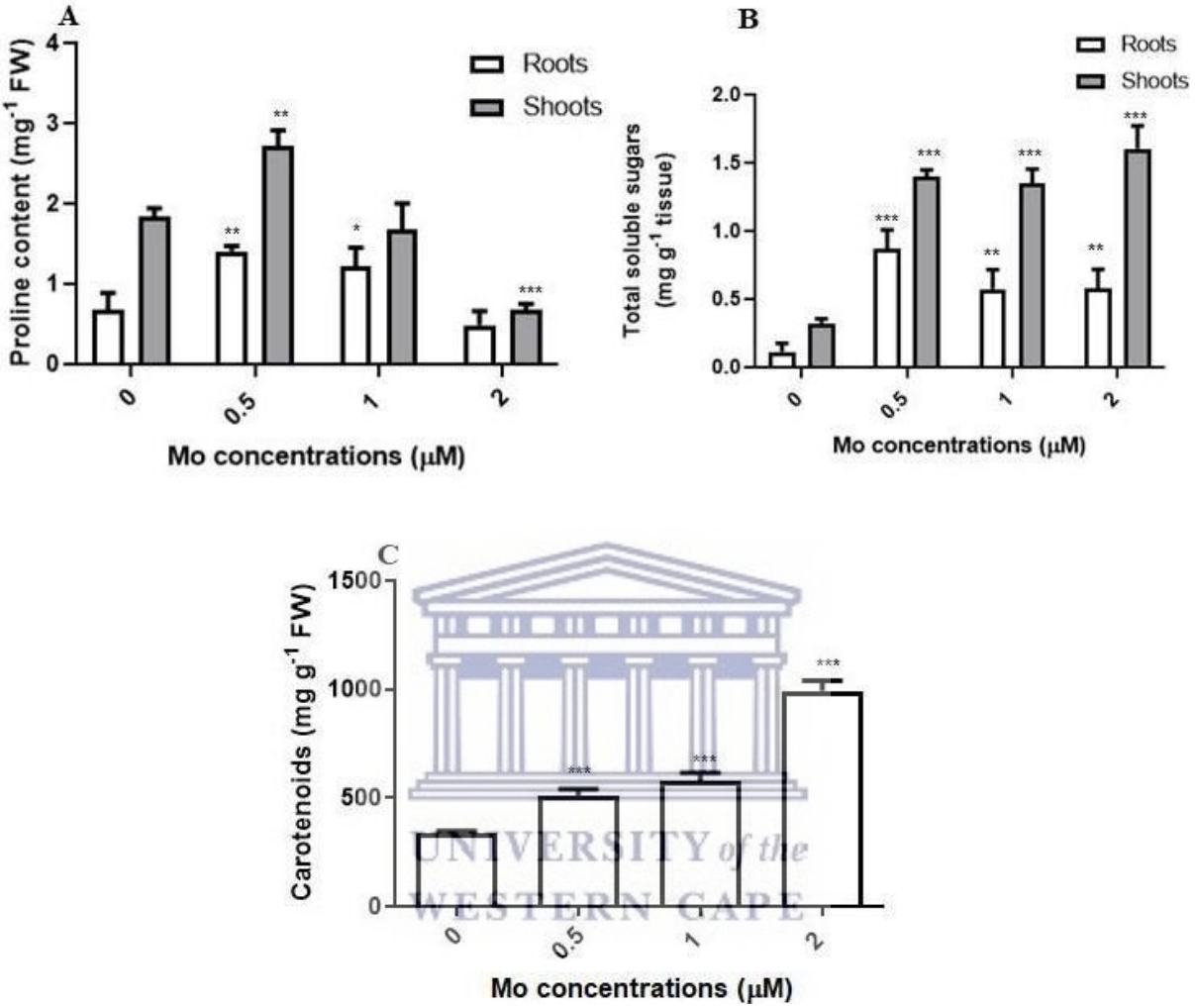

Figure 4.4. Non-enzymatic antioxidant content of sorghum plants in response to Mo application. Proline (**A**), soluble sugar and (**B**) carotenoid content (**C**) of sorghum plants under different Mo concentrations of (0 µM, 0.5 µM, 1 µM and 2 µM). Error bars represent the SD calculated from three biological replicates. Statistical significance between the control and treated plants was determined using one-way ANOVA as conducted on GraphPad Prism and indicated as *** = p≤0.001, ** = p≤0.01, and * = p≤0.05 according to the Bonferroni's multiple comparison test.

4.3.3.2. Enzymatic antioxidant activities

The antioxidant activities of SOD and APX were determined as shown in Figure 4.5. It can be observed in Figure 4.5a that the exogenous application of 0.5 µM Mo significantly (*** = p≤0.001) increased SOD activities in the roots by 5.9-fold with no significance changes

observed in the shoots of plants. Application of 1 µM significantly increased SOD activities in the roots by 4.9-fold and shoots by 1.6-fold, with no significant changes observed under the highest application of 2 µM Mo in the roots and shoots of sorghum plants. Ascorbate peroxidase significantly increased by 2-fold in the roots and shoots under 0.5 µM Mo treatment (Figure 4.5b). Application of 1 µM Mo to sorghum plants significantly (** = $p \leq 0.01$) increased APX activity in the roots only by 1.6-fold with no significant changes observed in the shoots as compare to that of the control plants. Similarly, 2 µM Mo significantly (** = $p \leq 0.01$) increased APX activities in the roots only by 1.7-fold.

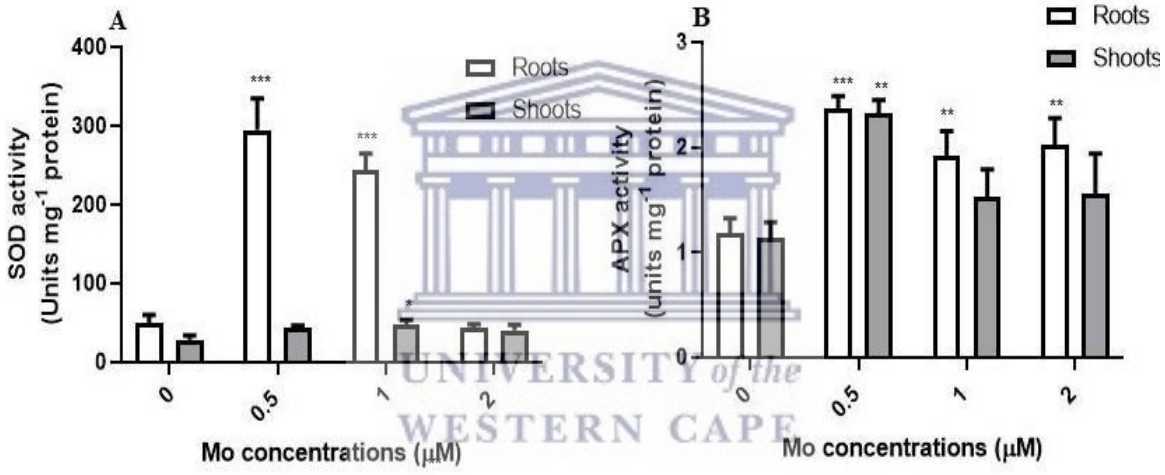

Figure 4.5. Effect of Mo on antioxidant activities of SOD and APX. SOD (**A**) and) APX (**B**) activity under different Mo concentrations of (0 µM, 0.5 µM, 1 µM and 2 µM). Error bars represent the SD calculated from three biological replicates. Statistical significance between the control and treated plants was determined using one-way ANOVA as conducted on GraphPad Prism and indicated as *** = $p \leq 0.001$, ** = $p \leq 0.01$, and * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

4.4. DISCUSSION

4.4.1. Molybdenum affects growth

Micronutrients such as Mo are important for normal plant growth and development and deficiencies can significantly reduce plant growth (Kumar et al., 2021). The overly absorption or use of essential nutrients can have toxic effects on plants (Khan et al., 2020).

In the current study the exogenous application of Mo to unstressed sorghum plants, reduced the shoot length, fresh and dry weight of sorghum plants with increasing Mo concentrations (Figure 4.1 and Table 4.1). The reduction in biomass with Mo treatments is attributed to the fact that plants require only small quantities of micronutrients and higher concentrations in the soil may act as stress and inhibit plant growth leading to yield loss (Alam et al., 2015), as evident in the current study. These results are supported by similar studies where excess Mo concentration of 10 mM affected *Brassica oleracea* seedling growth (Kumchai et al., 2013).

4.4.2. Molybdenum effects on chlorophyll content

The impact of Mo on the synthesis of photosynthetic pigments showed that the exogenous application of Mo to unstressed sorghum plants significantly reduced the total chl content under 0.5 µM Mo treatments and total chl and chl 'b' content of sorghum plants under 1 µM Mo applications (Figure 4.2). These results are contrary to results in which Mo induced photosynthetic pigments and improved the growth and biomass of unstressed *Canavalia* spp (Okla et al., 2021). Environmental stresses are generally expected to reduce the chl content in plants (Sherin et al., 2022). The reduction in chl content might suggest that the application of Mo to unstressed sorghum plants resulted in the inhibition of chlorophyll synthesis together with the degradation of chlorophyllase, an essential enzyme for chl metabolism (Santos,

2004). Previous studies showed that 1 µM Mo enhanced chlorophyll content and photosynthetic rate of unstressed *Triticum aestivum* (Imran et al., 2019).

4.4.3. Molybdenum induce oxidative damage in sorghum

It can be observed from the current study that the exogenous application of Mo to unstressed sorghum plants, stimulated oxidative response in sorghum plants as seen from the high accumulation of oxidative stress markers ($O_2^{\bullet-}$ and H_2O_2) and MDA content (Figure 4.3). Under normal conditions (0 µM Mo), the leaves of sorghum plants were observed to be clear, not showing any sign of dark blue spots (indication of $O_2^{\bullet-}$) and brown spots (indication of H_2O_2). However, the exogenous application of Mo resulted in the increased accumulation ROS ($O_2^{\bullet-}$ and H_2O_2) in the leaves of sorghum plants. The increased production of ROS can be as a signal for adaptive response to Mo toxicity of sorghum plants for maintenance of physiological functions (Huang et al., 2019; Schieber & Chandel, 2014). Lipid peroxidation an indicator of lipids deterioration as a result of ROS was estimated by assaying the levels of MDA, a product of lipid peroxidation used as a biomarker of oxidative stress (Cui et al., 2018; Morales & Munne-Bosch, 2019). The increased MDA content observed can be as a result of lipid deterioration as a result of Mo stress to unstressed sorghum plants.

4.4.4. Influence of exogenous Mo on osmoregulation

The elevated levels of omolytes such as proline and soluble sugars accumulation in plants has been correlated with enhanced tolerance of plants to unfavourable conditions through scavenging free radicals (Jogawat, 2019). The results from this study showed a significant increase in the accumulation of proline and soluble sugars contents with the exogenous application of Mo to unstressed sorghum plants. The high accumulation of osmolytes can be

attributed to the toxic effects of Mo. Although Mo plays an important role in different redox reactions in plants, it is required in very low concentrations (Kaiser et al., 2005). Molybdenum is a fertilizer applied in the form of salts and excess concentrations can cause toxicity to the plant (Shi et al., 2018), which was evident in the current study. These results are consistent with those of Soni et al. (2017) that showed increased proline levels under high Mo concentrations. Similarly, Qin et al. (2016) also found that the application of Mo to soil also increased soluble sugar content in *Brassica napus*. The decrease in proline content with the highest application of 2 µM Mo in the shoots may be as a result of deactivation of metabolic activities at high Mo concentrations (Kumar et al., 2021).

Carotenoids are synthesized in all organisms that are capable of photosynthesis including, prokaryotes. They function as light energy for photosynthesis and they act as chloroplast membrane stabilizers that partition between light harvesting complex and lipid phase of thylakoid membranes, reducing membrane fluidity and susceptibility to lipid peroxidation (Demmig-Adams et al., 1996; McElroy & Kopsell, 2009). Carotenoids have the potential to detoxify plants from the harmful effects of ROS. The result in Figure 4.4c, showed carotenoid content to significantly increase with increasing Mo applications to unstressed sorghum plants. It has been previously reported that the use of agrochemicals such as fertilizers, either in excess or deficit, can also be a cause of abiotic stress to plants by causing nutrient imbalance in plants (Uarrota et al., 2018). The increased carotenoid content observed can be related to its antioxidant role against excess Mo supplied to unstressed sorghum plants.

4.4.5. Influence of Mo on the antioxidant enzymatic response

Superoxide dismutase (SOD) and APX are one of the main antioxidant enzymes in plants that indicate the level of oxidative response by plants under stress factors (Han et al., 2020). Increased antioxidant activities have been associated with increased tolerance of plants to

stressful conditions, which help with plant tolerance. The results indicate that the exogenous application of Mo caused a significant increase in antioxidant activities of sorghum plants (Figure 4.5). Molybdenum significantly increased SOD activity in the roots under 0.5 µM and 1 µM Mo treatments. These results agree with similar findings that showed SOD activity to increase with the application of 0.15 mg Mo in *Triticum aestivum* under normal conditions (Liu et al., 2019).

Ascorbate peroxidase is one of the H_2O_2 scavenging enzymes that play an essential role in the protection of cells against environmental stresses, by catalysing the conversion of H_2O_2 into water using ascorbate as a specific electron donor (Caverzan et al., 2012). The exogenous application of Mo increased APX activities in the roots and shoots of sorghum plants, although no significant change was observed in the shoots with 1 µM and 2 µM Mo applications. The enhance antioxidant activities of sorghum plants can be as an attempt to protect plants from Mo-induced toxicity. These findings are consistent with other findings in *Cicer arietinum* that resulted in increased antioxidant activities under excess (<0.2 µM) Mo treatments under normal conditions (Gopal et al., 2017).

4.5. CONCLUSION

Plants require essential micronutrients like Mo for optimal growth and development, although Mo has been reported to have a positive impact on plants in relation to its application as a fertilizer. It was observed from this study that the exogenous application of high concentration of Mo to unstressed sorghum plants affected the growth and activated oxidative response in sorghum plants. We can thus conclude that the 0.5 µM Mo was the lowest Mo concentrations used in the current study with minimal negative effects on sorghum under non-stress conditions whereas 1 µM and 2 µM Mo, might have been too high for unstressed sorghum plants or the application of Mo is not necessary under normal conditions especially for this particular cultivar.

CHAPTER FIVE
INFLUENCE OF MOLYBDENUM ON THE MORPHO-PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR ATTRIBUTES OF *SORGHUM BICOLOR* UNDER SALT STRESS

5.1. ABSTRACT

Crop production in arid and semi-arid regions is heavily threatened by salinization. Salinity imposes deleterious effects at different developmental stages on crops, thus the discovery of salt management strategies to improve crop growth is imperative. This chapter investigates the role of Molybdenum (Mo) inmitigating salt stress adversities on *Sorghum bicolor* plants. This was achieved by analysing the morpho-physiological, biochemical and molecular traits of sorghum plants that were treated with different NaCl (100 mM, 200 mM and 300 mM) and Mo (0.5 µM, 1 µM and 2 µM) concentrations. The negative effects caused by salt stress on sorghum plants were reversed by Mo treatment. Thus, the highest fresh weight was observed with the application of 0.5 µM Mo under 100 mM NaCl (1.2-fold), 200 mM NaCl (1-fold) and 300 mM NaCl (1.4-fold) treatments. Whereas the highest dry weight was observed under 100 mM NaCl (1.1-fold) and 300 mM NaCl (1.2-fold) for plants treated with 0.5 µM Mo and 2 µM Mo respectively. There were no significant changes on the water content of sorghum for all Mo treatments. Molybdenum reversed the oxidative stress, resulting in well-arranged epidermal and vascular bundle layers and improved chlorophyll and osmolyte content. The SOD activity increased (≥2-fold) upon Mo application under all salt treatments, while the highest APX activity (6-fold) was observed under 300 mM NaCl in the roots, HSP-70 expression was reduced by Mo. Mo effectively alleviated the salt stress effects in sorghum plants, through improved photosynthesis, osmotic adjustment and the antioxidant defence system.

KEYWORDS: Salinity, Molybdenum, oxidative stress, Photosynthesis, SOD, APX.

5.2. INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] is amongst the most widely cultivated cereal crop worldwide, ranked the 2nd most important crop in Africa after maize (Mundia et al., 2019). Sorghum serves as a staple food for millions of people living in semi-arid regions of the African and Asian countries (Proietti et al., 2015). Although sorghum is moderately drought and salt tolerant (Amombo et al., 2022) than maize, the most widely produced grain, its growth is still affected by high salt stress (Krishnamurthy et al., 2007).

With food production that is expected to increase by 70% more than what is currently produced required to feed the world population by the year 2050 (FAO, 2009). Salinity poses a major threat to the agricultural production (Nandal et al., 2013). Irrigated lands provide the majority of the global food production (Munns & Tester, 2008) and more than 20% of cultivated land is affected by salinity. Salinity hinders plant growth by reducing water uptake, which result in nutrient deficiency due to increased accumulation of toxic Na and Cl ions (Kotagiri & Chaitanya Kolluru, 2017). Ion toxicity interferes with metabolic processes and reduce crop yield (Sogoni et al., 2021).

Plants have developed various adaptive response mechanisms to cope with stress, prevent injury and complete their life cycle (Cramer et al., 2011; Hussain et al., 2019). Although, plant breeding is crucial for agricultural production (Wu et al., 2009), is important to consider the application of exogenous substances that can induce stress tolerance in plants. Molybdenum is an essential micronutrient and stress resistant element that has been extensively reported to mediate stress tolerance in plants (Wu et al., 2014; Zhang et al., 2014). Reports showed that Mo enhanced chilling resistance of *Paspalum vaginatum* (Yu et al., 2005), alleviated cadmium stress effects in *Brassica napus* (Han et al., 2020) and salinity stress in many crops (Sun et al., 2009; Bagheri et al., 2012; Zhang et al., 2012). This chapter reports on the alleviatory effects of Mo in mitigating salt stress in *Sorghum bicolor*.

5.3. RESULTS

5.3.1. Morpho-physiological responses of sorghum to Mo under salt stress

5.3.1.1. Growth attributes

To study the effects of Mo on sorghum growth under salt stress, biomass (fresh and dry weight) and water content (WC%) were assayed as shown in Table 5.1. Increasing salt concentrations reduced the fresh weight (FW) and dry weight (DW) of sorghum plants as described in chapter 3. But the application of Mo to 100 mM NaCl-stressed sorghum plants improved the FW from 0.45 mg to 0.56 mg for 0.5 µM Mo, 0.55 mg for 1 µM Mo and 0.48 mg for 2 µM Mo treatments, indicating more than 1-fold increase for all Mo concentrations. The application of 0.5 µM Mo positively affected DW of 100 mM NaCl stressed plants, whereas other Mo concentrations (1 µM and 2 µM) led to a decrease in DW by more than 1-fold. A 1-fold increase was observed in WC% with all Mo concentrations under 100 mM NaCl stress treatments.

The application of Mo to 200 mM NaCl -stressed plants only increased the FW under 0.5 µM Mo from 0.39 mg to 0.42 mg by 1-fold. Higher Mo concentrations reduced FW to 0.28 mg (1 µM) by 1.4-fold and with the only significant (** = p≤0.01) reduction observed with 2 µM Mo treatments from 0.39 mg to 0.16 mg by 2.4-fold. Molybdenum reduced the DW for all treatment concentrations under 200 mM NaCl from 0.03 mg to 0.02 mg under 0.5 µM Mo treatment (1.3-fold), 0.01 mg for 1 µM Mo and 2 µM Mo treatments representing over 2-fold decrease. The WC% of sorghum plants stressed with 200 mM NaCl increased by 1-fold under 0.5 µM Mo and 1 µM Mo treatments. The application of Mo to 300 mM NaCl-stressed plants increased the FW at all Mo concentrations from 0.26 mg to 0.39 mg under 0.5 µM Mo (1.5-fold), 0.39 mg under 1 µM Mo (1.5-fold) and 0.36 mg under 2 µM Mo (1.2-fold). Similarly, the DW under 300 mM NaCl stress increased from 0.01 mg to 0.02 mg under 0.5 µM Mo, 0.02 mg under 1 µM Mo and 0.03 mg under 2 µM Mo, indicating more than 1-fold increase

for all Mo treatments. An increase in WC% by 1-fold was observed with the application of 0.5 µM Mo. The application of Mo reduced the WC% of sorghum plants by 1-fold when treated with 1 µM and 2 µM Mo in comparison to 300 mM NaCl treatment only.

Table 5.1. The influence of Mo on salt stress sorghum plants fresh weight, dry weight and water content (%).

NaCl (mM)	Mo(µM)	FW (mg)	DW (mg)	WC (%)
100	0	0.445±0.049	0.033±0.003	92.500±0.707
	0.5	0.556±0.004	0.038±0.004	95.500±2.121
	1	0.545±0.023	0.023±0.004	95.500±0.707
	2	0.479±0.013	0.020±0.003	95.500±0.707
200	0	0.390±0.014	0.025±0.004	93.500±0.707
	0.5	0.423±0.021	0.019±0.001	95.000±0.000
	1	0.284±0.037	0.009±0.005	97.000±1.414
	2	0.162±0.083**	0.011±0.000	92.500±4.950
300	0	0.260±0.028	0.018±0.002	93.000±1.414
	0.5	0.386±0.051	0.022±0.008	95.000±4.243
	1	0.387±0.009	0.024±0.002	91.500±0.707
	2	0.361±0.008	0.028±0.010	92.000±2.828

** indicate significant differences at p≤0.01. FW = fresh weight, DW = dry weigtht and WC = water content.

5.3.1.2. Anatomic structure

To further understand the role of Mo in alleviating salt stress effects on sorghum growth, the study analysed anatomical structure including the epidermis and vascular bundle (xylem and phloem) layers as shown in Figure 5.1. Treatment of sorghum plants with different NaCl concentrations (100 mM, 200 mM and 300 mM) caused anatomical alterations with severe damage of the epidermal layers (black arrows), whereas there was thinning and shrinking of phloem (blue) and xylem (red) layers. The application of 0.5 µM Mo under 100 mM NaCl-stressed plants slightly improved the epidermal layers (Figure 5.1b). Whereas the application of 1 µM Mo improved the epidermal layers as well as broadened the wells of xylem and phloem layers (Figure 5.1c). Sorghum plants stressed with 200 mM NaCl showed that the application of 0.5 µM Mo improved both the epidermal and vascular bundle layers (Figure 5.1e), whereas 1 µM Mo only improved the epidermal layers (Figure 5.1f). Furthermore, severe damage of the epidermal and vascular bundle layers of plants stressed with 300 mM NaCl was restored (Figure 5.1h-i) by the application of Mo (0.5 µM and 1 µM) showing less deformation of epidermal and broadening of xylem and phloem layers.

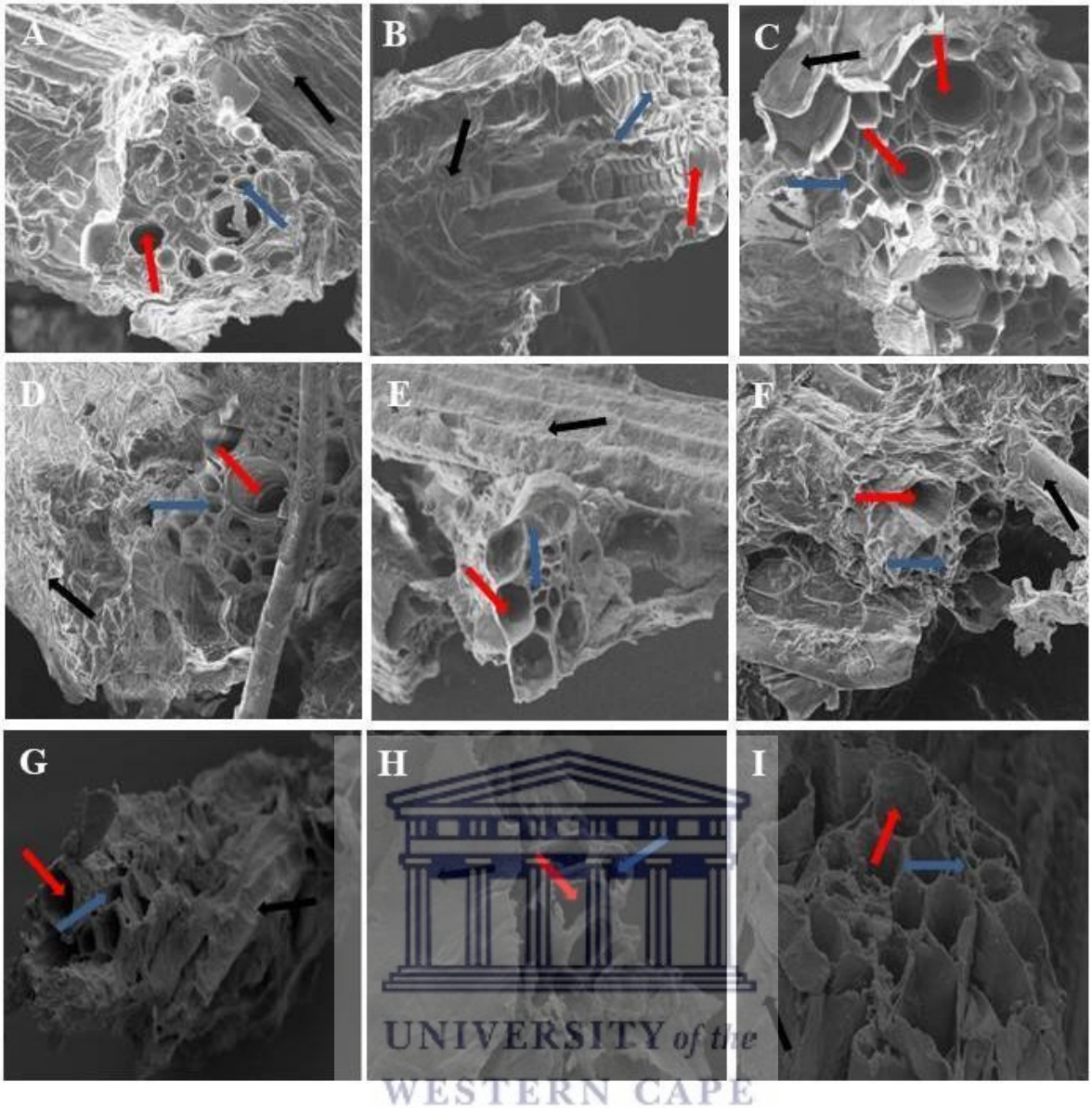

Figure 5.1. **Scanning Electron Microscope images showing the effect of Mo on the epidermis and vascular bundle layers (xylem and phloem) of salt-stressed sorghum plants.** Sorghum plants stressed with 100 mM NaCl, treated with different Mo (0.5 µM and 1 µM) concentrations (**A-C**), 200 mM NaCl-stressed plants treated with different Mo (0.5 µM and 1 µM) concentrations (**D-F**) 300 mM NaCl-stressed plants treated with different Mo (0.5 µM and 1 µM) concentrations (**G-I**). Xylem (red), phloem (blue) and epidermal (black) arrows show the different layers.

5.3.1.3. Effect of Mo on chlorophyll content

Photosynthetic pigments including total chlorophyll (chl), chl a and b were assayed to corroborate the results under growth attributes (Figure 5.2). Chlorophyll is an important photosynthetic pigment that helps in determining the capacity and growth of plants (Ying et al., 2018). Figure 5.2a showed that the exogenous application of 0.5 µM Mo to 100 mM NaCl-stressed plants significantly (*** = $p \leq 0.001$) increased the total chl (2.7-fold), chl 'a' (2.3-

fold) and chl ‘b’ (2.8-fold) content of sorghum plants. The exogenous application of 1 µM Mo only increased the total chl content by 1.4-fold with no other significant changes observed in chl ‘a’ and chl ‘b’ contents. The highest concentration of 2 µM Mo significantly (*** = p≤0.001) reduced chl ‘a’ (1.5-fold) content, whereas it significantly (*** = p≤0.001) increased chl ‘b’ (1.8-fold) content (Figure 5.2a).

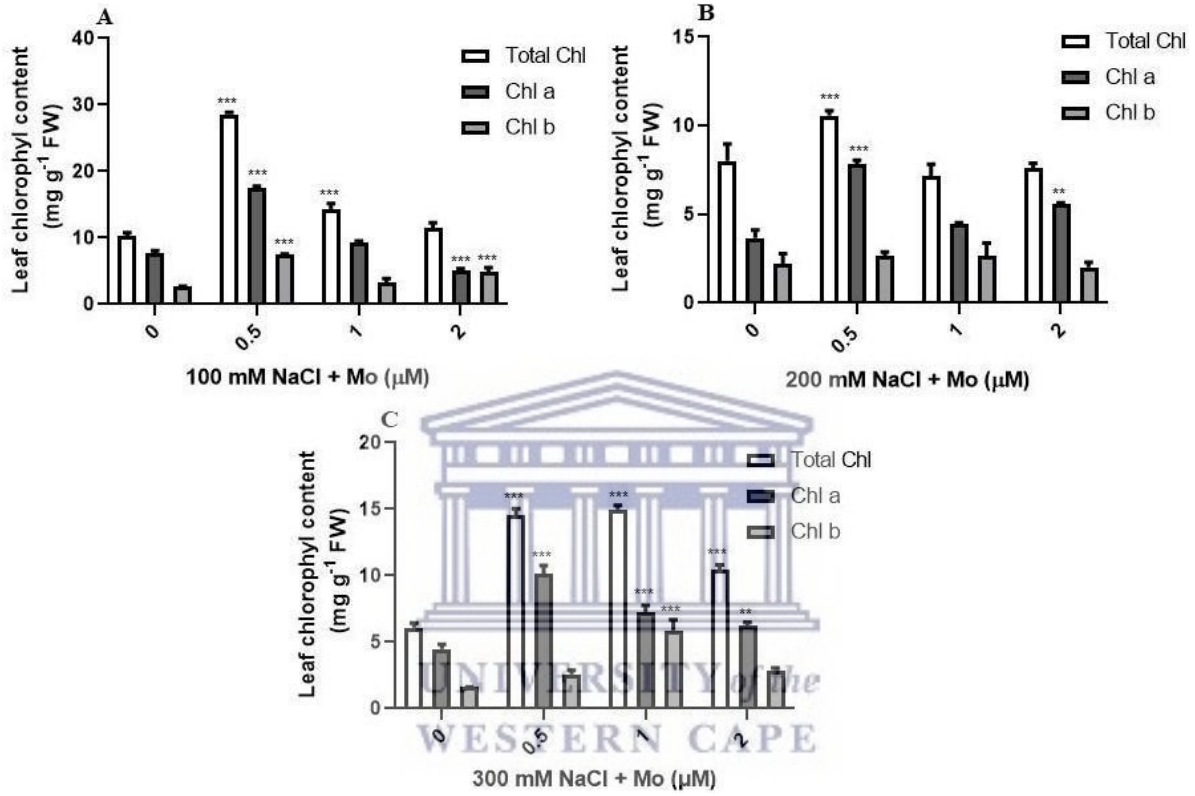

Figure 5.2. The effect of Mo on chlorophyll content of salt-stressed sorghum plants. Sorghum plants stressed with 100 mM NaCl **(A),** 200 mM NaCl and (**B**) 300 mM NaCl (**C**) with different Mo concentrations (0.5 µM, 1 µM and 2 µM). Error bars represent the SD calculated from three biological replicates. Statistical significance between the different treated plants was determined using two-way ANOVA as conducted on GraphPad Prism and indicated as *** = p≤0.001 and ** = p≤0.01, according to the Bonferroni’s multiple comparison test.

Sorghum plants stressed with 200 mM NaCl supplemented with 0.5 µM Mo, resulted in a significant (*** = p≤0.001) increase in total chl (1.3-fold) and chl ‘a’ (2-fold) contents, whereas the application of 1 µM Mo did not result in any significant changes in chlorophyll content (Figure 5.2b). Sorghum plants treated with 2 µM Mo significantly (** = p≤0.001) increased chl ‘a’ by 1.5-fold. It can be observed in Figure 5.2c that sorghum plants stressed

with 300 mM NaCl significantly (*** = p≤0.001) increased total chl (2.4-fold) and chl 'a' (2.3-fold) contents when treated with 0.5 µM Mo. Exogenously application of 1 µM Mo significantly (*** = p≤0.001) increased the total chl (2.5-fold), chl 'a' (1.6-fold) and chl 'b' (3.7-fold) contents of sorghum plants stressed with 300 mM NaCl. The application of 2 µM Mo also significantly increased the total chl (1.7-fold) and chl 'a' (1.4-fold) content only, with no significant changes observed in chl 'b' content.

5.3.2. Biochemical responses of sorghum to Mo under salt stress

5.3.2.1. Histochemical staining of ROS ($O_2^{\bullet-}$ and H_2O_2)

Superoxide radical ($O_2^{\bullet-}$) is one of the ROS produced in plants under stressful conditions (Sachdev et al., 2021). Histochemical staining showed that plants stressed with 100 mM NaCl caused slight toxicity to sorghum plants as observed by dark blue spots, which represents the formation of $O_2^{\bullet-}$ on the leaves of sorghum plants. The application of 0.5 µM Mo reversed the harmful effects of $O_2^{\bullet-}$ toxicity on the leaves of sorghum plants as seen by reduced dark blue spots, with no changes observed with 1 µM and 2 µM Mo applications (Figure 5.3a). Treatment of sorghum plants with 200 mM NaCl showed the effective use of 0.5 µM Mo in reducing $O_2^{\bullet-}$ toxicity on sorghum plants as observed by clear leaves whereas the application of 1 µM Mo slightly increased $O_2^{\bullet-}$ spots on the leaves with less damage observed for 2 µM Mo treatments (Figure 5.3b). All Mo concentrations applied to 300 mM NaCl-stressed plants successfully reversed the harmful effects of salt stress on sorghum plant leaves as less dark blue spots were observed on the leaves (Figure 5.3c).

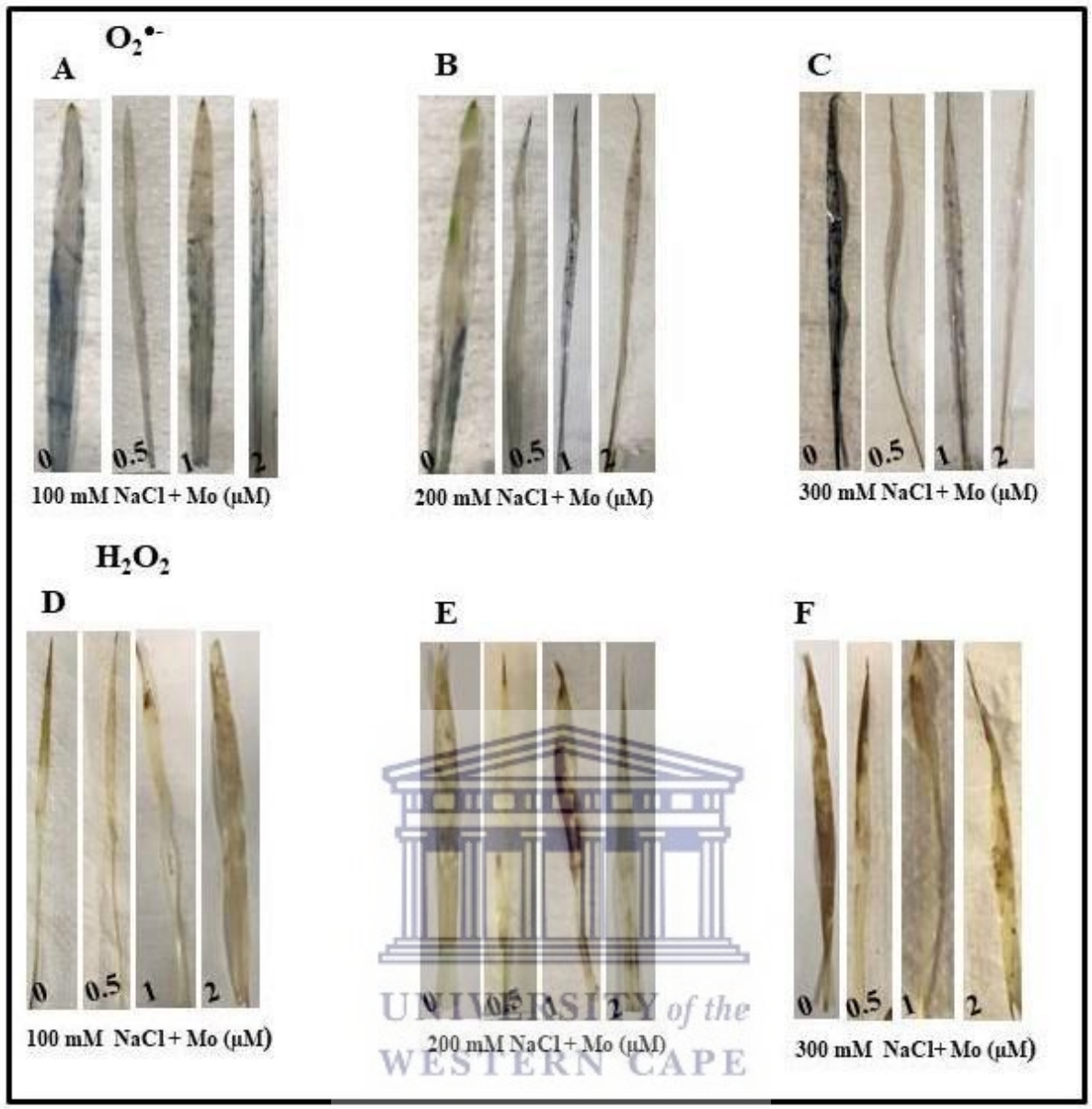

Figure 5.3. Histochemical staining of ROS ($O_2^{\bullet-}$ and H_2O_2) on the effects of Mo on salt-stressed plants. Histochemical staining of $O_2^{\bullet-}$ and the effects of different Mo concentrations (0.5 µM, 1 µM and 2 µM) on sorghum plants stressed with (100 mM, 200 mM and 300 mM) NaCl **(A-C**), histochemical staining of H_2O_2 on the effects of different Mo concentrations (0.5 µM, 1 µM and 2 µM) on sorghum plants stressed with (100 mM, 200 mM and 300 mM) NaCl (**D-F**).

Figure 5.3d-e showed the histochemical staining of the influence of Mo on the content of H_2O_2 in salt-stressed sorghum plants. Figure 5.3d showed that sorghum plants stressed with 100 mM NaCl resulted in a minimal production of brown spots as an indication of H_2O_2 on the leaves of sorghum plants. The application of 0.5 µM and 1 µM Mo reduced H_2O_2 production on the leaves of sorghum plants, whereas application of 2 µM Mo slightly

increased H_2O_2 production on the leaves (Figure 5.3d). Sorghum plants stressed with 200 mM NaCl resulted in the overproduction of H_2O_2 spots on the leaves. The exogenous application of 0.5 µM Mo to 200 mM salt-stressed plants effectively reversed the harmful effects of H_2O_2. The application of 0.5 µM and 1 µM Mo slightly reduced H_2O_2 accumulation of sorghum plants stressed with 300 mM NaCl; whereas 2 µM Mo applications did not reverse the harmful effects of H_2O_2 production on the leaves of stressed plants (Figure 5.3f).

5.3.2.2. Quantification of H_2O_2 and MDA content

Hydrogen peroxide plays an important role at physiological levels in plants as well as resistance to stress conditions (Quan et al., 2008). Figure 5.4a-c showed the influence of Mo on the production of H_2O_2 on salt-stressed sorghum plants. Figure 5.4a showed that the application of Mo to 100 mM NaCl-stressed sorghum plants significantly (*** = $p \leq 0.001$) reduced the levels of H_2O_2 in the roots and shoots under all Mo concentrations. Application of 0.5 µM Mo reduced H_2O_2 by 4.9-fold in the roots and 3.1-fold in the shoots (Figure 5.4a). The application of 1 µM Mo reduced H_2O_2 in the roots by 4.7-fold and in the shoots by 3.2-fold, whereas 2 µM Mo reduced H_2O_2 content in the roots by 14.2-fold and shoots by 10-fold. The application of Mo to 200 mM NaCl-stressed sorghum plants showed significant reduction in H_2O_2 content with 0.5 µM Mo in the roots leading to a 2.8-fold decrease and 1.7-fold decrease in the shoots and with 1 µM Mo in the roots resulting in 5.2-fold decrease (Figure 5.4b). For sorghum plants under 300 mM NaCl stress, Mo significantly (*** = $p \leq 0.001$) reduced H_2O_2 content in the shoots of sorghum plants treated with all Mo concentration resulting in 4.8-fold (0.5 µM Mo), 5.9-fold (1 µM Mo) and 6.5-fold (2 µM Mo) decrease (Figure 5.4c).

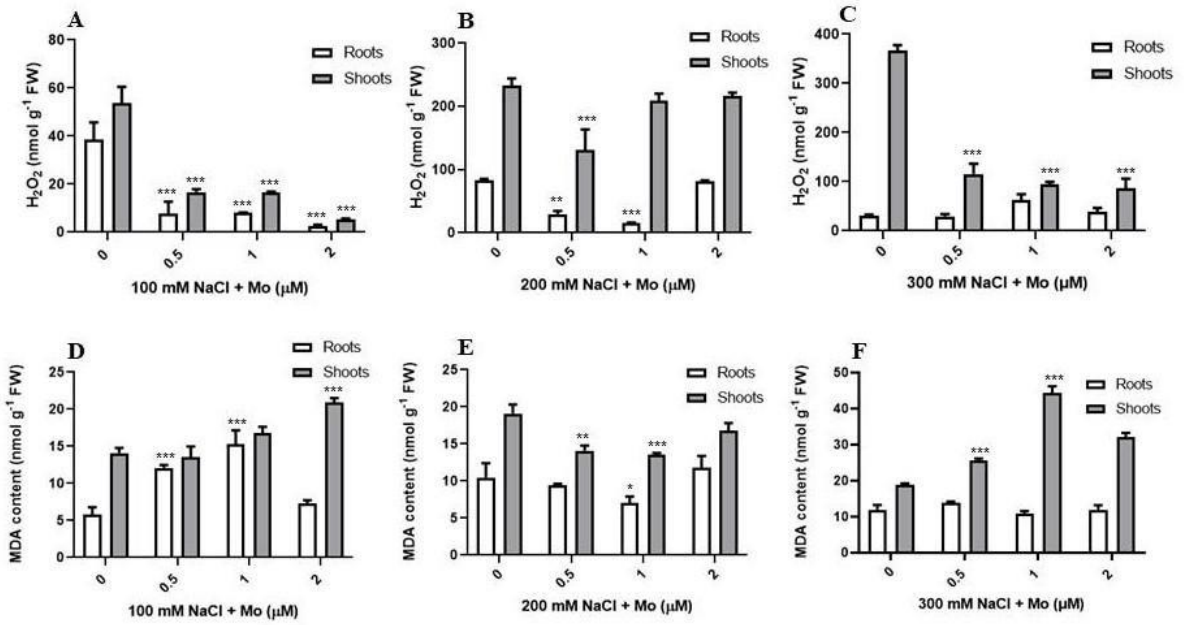

Figure 5.4. The effect of Mo on the influence of H_2O_2 and MDA content. Influence of Mo on H_2O_2 content of salt-stressed sorghum plants and **(A-C**) MDA content on the influence of Mo on salt-stressed sorghum plants (**D-F**). Error bars represent the SD calculated from three biological replicates. Statistical significance between the different treated plants was determined using two-way ANOVA as conducted on GraphPad Prism and indicated as *** = p≤0.001, ** = p≤0.01 and *= p≤0.05 according to the Bonferroni's multiple comparison test.

The exogenous application of Mo to 100 mM NaCl-stressed sorghum plants significantly (*** = p≤0.001) increased the MDA content in the roots by 2.1-fold (0.5 µM Mo) and 2.6-fold (1 µM Mo) treatments, with no significant changes observed in the shoots. The application of 2 µM Mo on 100 mM NaCl-stressed plants resulted in a significant (*** = p≤0.001) increase in MDA content in the shoots (1.4-fold). Exogenous application of Mo to 200 mM NaCl-stressed plants significantly (*** = p≤0.001) reduced the levels of MDA in the shoots only when treated with 0.5 µM Mo by 1.3-fold. The levels of MDA in the roots and shoots decreased by 1.4-fold when treated with 1 µM Mo, with no significant changes observed with 2 µM Mo treatment. Molybdenum significantly (*** = p≤0.001) increased the MDA content in the shoots of plants stressed with 300 mM NaCl by 1.3-fold (0.5 µM Mo) and 2.6-fold (1 µM Mo).

5.3.2.3 Non- enzymatic antioxidants

The antioxidant defence on the effects of exogenously applied Mo on salt-stressed sorghum plants were determined by analysing the non-enzymatic antioxidants; proline, soluble sugars and carotenoid content. It can be observed in Figure 5.5a that the exogenous application of Mo to sorghum plants stressed with 100 mM NaCl significantly (*** = $p \leq 0.001$) reduced proline content by 1.6-fold (0.5 µM Mo), 2.7-fold (1 µM Mo) and 1.7-fold (2 µM Mo). The only significant (** = $p \leq 0.01$) reduction in proline observed in the roots resulted in a 1.4-fold decrease when treated with 0.5 µM Mo.

Sorghum plants stressed with 200 mM NaCl significantly reduced proline content in the roots by 2.9-fold and by 4.3-fold in the shoots of sorghum plants treated with 0.5 µM Mo application. Proline content decreased by 2.6-fold in the roots and 1.1-fold in the shoots in sorghum plants treated with 1 µM Mo, whereas 2 µM Mo reduced proline content in the roots and shoots of sorghum plants under 200 mM NaCl by 4.4-fold and 2.2-fold respectively.

Sorghum plants stressed with 300 mM NaCl significantly increased proline content by 5.5-fold in the roots, while in the shoots, proline was reduced by 4.4-fold when the plants were treated with 0.5 µM Mo. Proline content increased significantly (*** = $p \leq 0.001$) in the roots by 17-fold under the application of 1 µM Mo. The application of 2 µM Mo significantly (*** = $p \leq 0.001$) increased proline content in the roots by 32.1-fold and shoots by 5.7-fold.

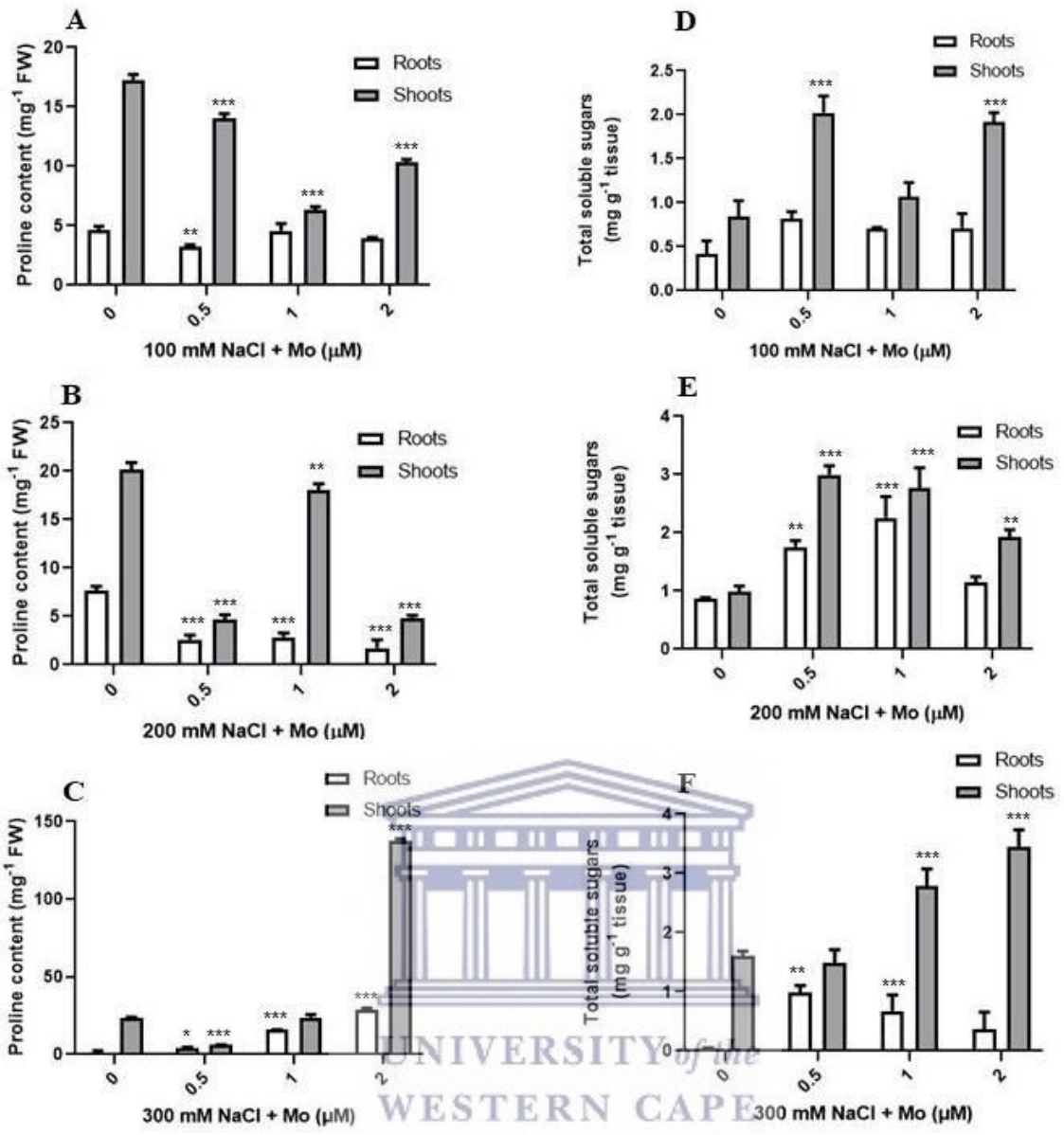

Figure 5.5. The effect of Mo on osmolyte accumulation of salt-stressed sorghum plants. Proline contents of the different NaCl treatments (100 mM, 200 mM and 300 mM) with different Mo concentration (0.5 µM, 1 µM and 2 µM) (**A**-**C**) soluble sugars contents under different NaCl treatments (100 mM, 200 mM and 300 mM) NaCl, with different Mo concentration (0.5 µM, 1 µM and 2 µM) respectively (**D**-**F**). Error bars represent the SD calculated from three biological replicates. Statistical significance between the different treated plants was determined using two-way ANOVA as conducted on GraphPad Prism and indicated as *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

The effect of Mo on the soluble sugars on 100 mM NaCl-stressed sorghum plants significantly (** = $p \leq 0.01$) increased soluble sugars in the shoots by 2.4-fold under 0.5 µM Mo treatment, with the only other significant increase (2.2-fold) observed in the shoots under 2 µM Mo treatment (Figure 5.5d).

Treatment of 200 mM NaCl-stressed sorghum plants with 0.5 µM Mo significantly increased soluble sugar content in the roots by 1.6-fold and in the shoots by 3-fold. Similarly, the application of 1 µM Mo significantly (*** = $p \leq 0.001$) increased soluble sugars content in the roots by 2.9-fold and shoots by 2.7-fold. The only significant (** = $p \leq 0.01$) increase under 2 µM Mo treatment was observed in the shoots, which resulted in a 2-fold increase.

Exogenous Mo application in sorghum plants stressed with 300 mM NaCl significantly (** = $p \leq 0.01$) increased soluble sugars content in the roots by 36-fold (0.5 µM Mo) and significantly (*** = $p \leq 0.001$) in the roots by 25-fold and shoots by 1.7-fold (1 µM), whereas the only significant increase (*** = $p \leq 0.001$) observed with 2 µM Mo was in the shoots by 2-fold.

Changes in carotenoid content after treating sorghum plants with the different concentrations of NaCl and Mo was analysed only in the shoots (Figure 5.6). The exogenous application of Mo to sorghum plants treated with 100 mM NaCl significantly (*** = $p \leq 0.001$) reduced the carotenoid content by 4.5-fold and 1.3-fold for treatments with 0.5 µM and 1 µM Mo respectively. No significant changes were observed with 2 µM Mo application. Sorghum plants stressed with 200 mM NaCl resulted in a significant (*** = $p \leq 0.001$) increase in carotenoid content by 1.5-fold when treated with 0.5 µM and 1 µM Mo, with a significant (* = $p \leq 0.05$) decrease of 1.2-fold observed with 2 µM Mo treatments. Application of Mo to 300 mM NaCl-stressed sorghum plants significantly (*** = $p \leq 0.001$) increased carotenoid content by 1.8-fold, 2-fold and 1.6-fold for 0.5 µM, 1 µM and 2 µM Mo treatments respectively.

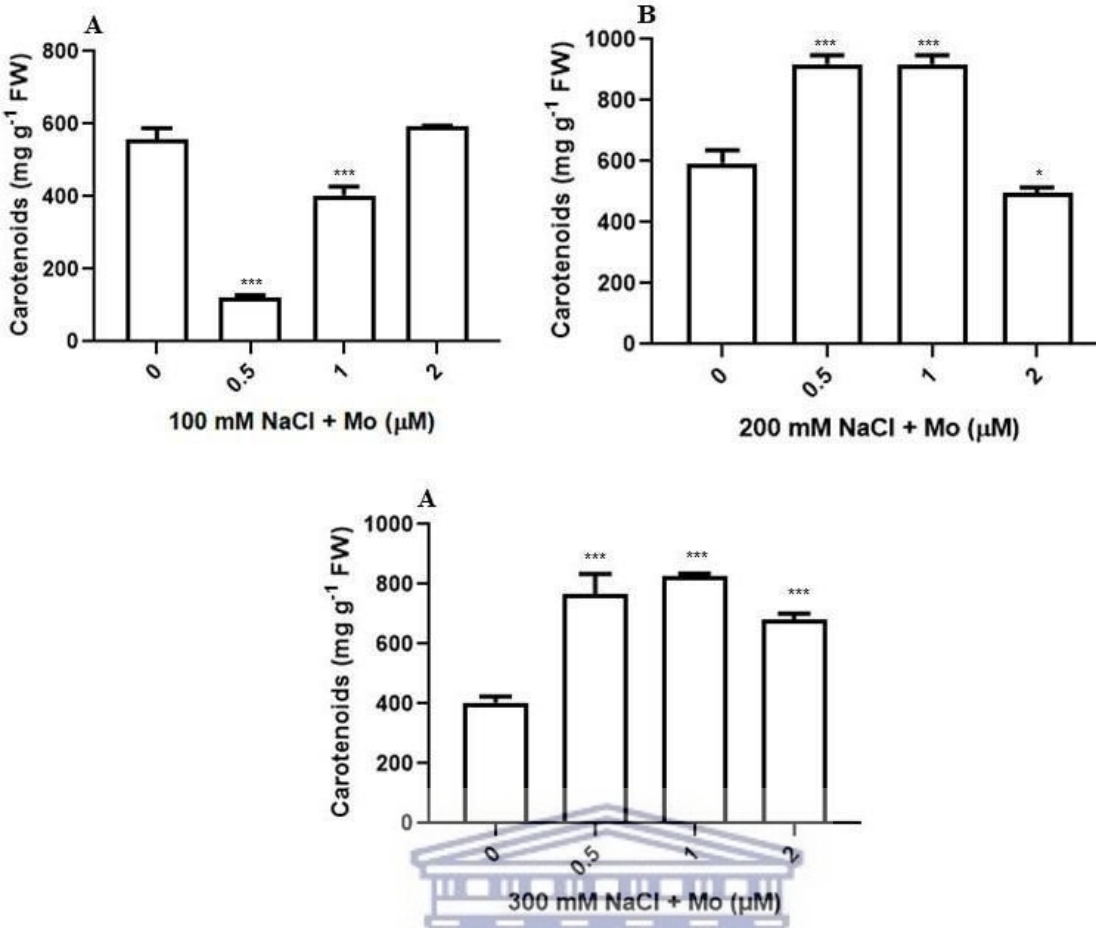

Figure 5.6. The influence of Mo on carotenoid content of salt-stressed plants. Carotenoid content of sorghum plants stressed with 100 mM NaCl and treated with different Mo concentrations of (0.5 µM, 1 µM, and 2 µM) (**A**), sorghum plants stressed with 200 mM NaCl and treated with different Mo concentrations and (**B**) sorghum plants stressed with 300 mM NaCl and treated with different Mo concentrations (**C**). Error bars represent the SD calculated from three biological replicates. Statistical significance between the different treated plants was determined using two-way ANOVA as conducted on GraphPad Prism and indicated as *** = $p \leq 0.001$, * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

5.3.2.3 Enzymatic antioxidant activities

Enzymatic antioxidant activities were determined by analysing SOD and APX activities of salt-stressed sorghum plants under the influence of different Mo concentration (Figure 5.7). It can be observed in Figure 5.7a that the exogenous application of 0.5 µM Mo to 100 mM NaCl-stressed sorghum plants significantly (*** = $p \leq 0.001$) increased SOD activity in the roots by 2.7-fold with no other significant changes observed with 1 µM Mo and 2 µM Mo treatments as compared to those treated with 100 mM NaCl only (Figure 5.7a). Exogenous application of Mo to 200 mM NaCl-stressed sorghum plants significantly increased SOD

activities in the roots by 2.6-fold, 1.6-fold and 1.8-fold for treatments with 0.5 µM, 1 µM and 2 µM Mo respectively (Figure 5.7b). The application of Mo significantly reduced SOD activity under 300 mM NaCl treatments in the roots by 2.2-fold when treated with 0.5 µM Mo and 1 µM Mo, whereas in the shoots SOD increased by 2.5-fold under 1 µM Mo treatments. But application of 2 µM Mo in the roots resulted in 2.9-fold decrease.

Antioxidant activities of APX in sorghum plants treated with 100 mM NaCl increased significantly (** = $p \leq 0.01$) in both the roots by 2.1-fold and shoots by 1.9-fold under 0.5 µM Mo treatments. The application of 1 µM Mo and 2 µM Mo significantly (*** = $p \leq 0.001$) increased APX activity in the roots by 2.2-fold and 2.9-fold respectively (Figure 5.7d).

The application of Mo to 200 mM NaCl sorghum stressed plants did not result in any significant changes with treatment of 0.5 µM Mo in the roots and shoots of plants (Figure 5.7e). The application of Mo in 200 mM NaCl-stressed plants significantly (*** = $p \leq 0.001$) reduced APX activities in the shoots by 2.3-fold and 2.4-fold when treated with 1 µM and 2 µM Mo respectively, with no significance change observed in the roots. The only significant (*** = $p \leq 0.001$) changes observed under 300 mM NaCl-stressed plants was in the roots when 0.5 µM Mo was applied resulting in 6-fold and a 5-fold increase when 1 µM Mo was applied (Figure 5.7f).

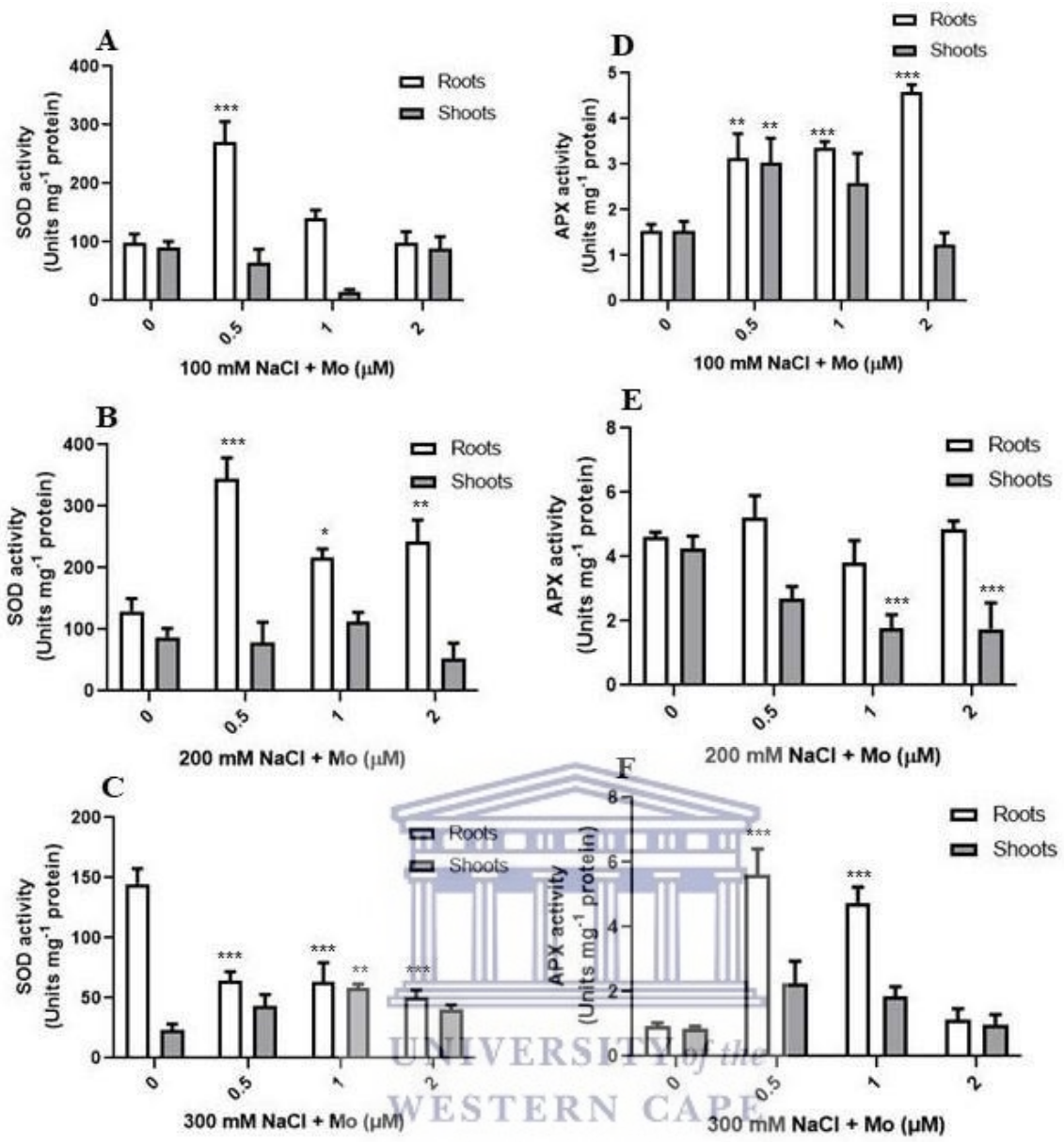

Figure 5.7. Antioxidant response of exogenous application of Mo to salt-stressed sorghum plants. SOD activity under different NaCl concentrations (100 mM, 200 mM and 300 mM) and different Mo treatments of (0.5 µM, 1 µM and 2 µM) and (**A-C**) APX activity under different NaCl concentrations (100 mM, 200 mM and 300 mM) and different Mo treatments of (0.5 µM, 1 µM and 2 µM) respectively. Error bars represent the SD calculated from three biological replicates. Statistical significance between the different treated plants was determined using two-way ANOVA as conducted on GraphPad Prism and indicated as *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$ according to the Bonferroni's multiple comparison test.

5.3.3 Molecular response of sorghum plants to Mo under salt stress

In order to determine the molecular response of sorghum to salt stress under the influence of Mo, this study quantified the expression of the heat shock protein 70 (HSP-70), a stress responsive protein. This was conducted on sorghum plants stressed with 100 mM and 200 mM NaCl concentrations by applying 0.5 µM and 1 µM Mo and analysed on a dot blot using the anti-heat shock protein-70 primary antibody (Figure 5.8). Sorghum plants stressed with 100 mM NaCl slightly induced HSP-70 expression (Figure 5.8a); but this induction was reduced by the exogenous application of 0.5 µM Mo (Figure 5.8b). Application of 1 µM did not show any difference in the expression of HSP-70 in comparison to 100 mM NaCl only (Figure 5.8c). It can be observed in Figure 5.8d that sorghum plants stressed with 200 mM NaCl showed high level of HSP-70 expression, which was considerably reduced by 1 µM Mo (Figure 5.8f).

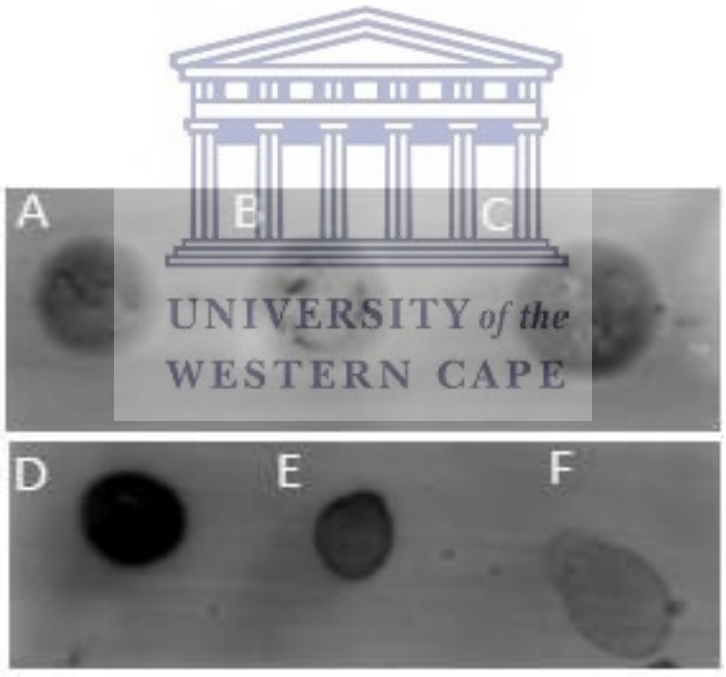

Figure 5.8. Dot blot analysis of the effects of Mo on the expression of HSP-70 to salt stressed plants. Sorghum plants stressed with 100 mM NaCl (**A**) 100 mM-stressed plants with different Mo concentrations (0.5 µM and 1 µM) (**B-C**). Sorghum plants stressed with 200 mM NaCl and (**D**) treated with different Mo treatments (0.5 µM and 1 µM) (**E-F**).

5.4 DISCUSSION

The production of crops in many arid and semi-arid regions of the world is threatened by salinity. Salinity is one of the most severe abiotic stresses that limit plant growth and has caused over 50% in crop loss. As a salt management strategy, this study investigated the effect of molybdenum to improve sorghum growth under NaCl-induced salt stress, by assaying sorghum's morphological, physiological, biochemical and molecular traits.

5.4.1. Molybdenum improves growth

It can be observed in Table 5.1 that salt stress caused a reduction in the biomass of sorghum plants with increasing salt concentrations as measured by fresh weight (FW) and dry weight (DW). The reduction observed is attributed to the influence of osmotic stress, which interferes with metabolic processes, reducing turgo and energy required for maintaining plant growth (de Oliveira et al., 2013). The application of various concentrations of exogenous Mo to salt-stressed sorghum plants increased the FW and DW. It has been reported by Cheong & Yun, (2007) that enhancing salinity resistance will improve plant biomass, which was evident in this study, where Mo application enhanced salinity tolerance in sorghum plants as compared to plants treated with NaCl only. Various studies reported that the application of Mo promotes the growth of plants under different abiotic stresses in *Brassica pekinensis* (Zhang et al., 2014), *Triticum aestivum* (Sun et al., 2006) and *Brassica napus* (Han et al., 2020).

5.4.2. Molybdenum improves photosynthesis and restores anatomy of stressed plants

Photosynthetic pigments play important roles in light harvesting and energy transduction in plants (Khan et al., 2018). Plants that are exposed to salt stress environments stimulates the activity of chlorophyllase, which promote degradation of chlorophyll (Zhang et al., 2014) and thus, results in reduced chl content. Our results showed that salt stress affected the chl content of sorghum plants; however, the exogenous application of Mo significantly improved chl content of sorghum plants. This is also supported by previous studies by Imran et al., (2019) where Mo increased the chl content in *Triticum aestivum.* Increasing photosynthetic capacity of plants can enhance productivity. Nautiyal and Chatterjee. (2004) also reported that high concentrations of Mo caused toxicity in plants; this explains the decrease in chl 'a' and 'b' content that was observed under 100 mM NaCl treatments in the presence of 2 µM Mo. Exposure of plants to salt stress conditions also affect stomatal conductivity by closing the stomata, which limits photosynthetic activities. This study showed that salinity caused severe damage to sorghum plants by damaging the epidermis and thinning of vascular bundle layers (Figure 5.1). Molybdenum is known to be responsible for various redox reactions in plants, such as those related to water relations and transpiration through stomatal control (Kaiser et al., 2005). The exogenous application of Mo reversed the harmful effects of salt stress to sorghum plants by improving structure of the epidermal and vascular bundle layers, showing less deformation and shrinkage. This might also explain the increased water content due to Mo application to salt-stressed plants, since the epidermal and vascular bundles play important roles in preventing moisture loss as previously reported for, *Suaeda maritima* (Hajibagheri et al., 1984), *Phaseolus vulgaris* (Bray & Reid, 2002*)* and *Imperata cylindrica* (Hameed et al., 2009) under salt-stressed conditions.

5.4.3. Molybdenum reduces oxidative damage in sorghum plants

To determine the effects of Mo on salt-stressed sorghum plants, histochemical staining of the two of the most important ROS molecules; $O_2^{\bullet-}$ and H_2O_2 was conducted (Figure 5.3). Salt stress caused the overproduction of free radicals on sorghum plants, causing ultra-structure and functional alterations in cell nuclei, DNA, lipids and proteins on leaves of stressed plants, seen as dark blue spots for $O_2^{\bullet-}$ and brown spots for H_2O_2 (Juan et al., 2021). The exogenous application of different Mo concentrations reversed the harmful effects of salt by diminishing the spots on leaves of salt-stressed sorghum plants. This is correlated with previous studies on *Triticum aestivum* (Wu et al., 2017). Furthermore, the study revealed that salt stress stimulated oxidative stress as evident from higher production of H_2O_2 and MDA contents in salt-stressed sorghum plants (Figure 5.4). The application of Mo reduced the H_2O_2 in the roots and shoots of salt treated sorghum plants. The application of Mo to protect plants from oxidative stress have also been reported in *Fragaria* x *ananassa* (Liu et al., 2017). The level of membrane damaged was measured based on MDA content, a secondary breakdown product of lipid peroxidation produced in plants under stress (Sun et al., 2006). Low levels of lipid peroxidation have been associated with increased tolerance of plants to stress (DaCosta & Huang, 2007). It can be observed from the current study that sorghum plants stressed with 200 mM NaCl were the only treatments that resulted in reduced levels of membrane damage upon the application of 0.5 µM Mo in the shoots and 1 µM Mo in both roots and shoots. The increased MDA content observed under 100 mM and 300 mM NaCl treatments, might be an indication of oxidative stress caused by high Mo concentration applied in sorghum plants due to excess ROS, thereby limiting cellular damage (El-Baky et al., 2003). Under physiological conditions ROS is effectively scavenged by the antioxidant system, however its overproduction surpasses the antioxidant system, leading to oxidative stress. Malondialdehyde is used as a biomarker for ROS mediated cellular damage after oxidative

stress (Cakmak, 2000). An increase in MDA estimates the intensity of the stress and the extent of its damaging effects on lipid membranes. It was found that under stress conditions, in the presence of Mo, increased antioxidant activities were induced to scavenge ROS in *Brassica oleracea var. capitate* (Kumchai et al., 2013).

5.4.4. Influence of exogenous Mo on non-enzymatic antioxidants

The biosynthesis of osmolytes plays an important role in the protection and stabilization of cellular membranes in plants and decrease water potential (Hasanuzzaman et al., 2021). Proline increased in sorghum plants under salt stress conditions, and this is consistent with previous studies done in sorghum (Mulaudzi et al., 2022; Rakgotho et al., 2022; Sarker & Oba, 2020). In the current study, the exogenous application of Mo to salt-stressed sorghum plants significantly reduced the levels of proline under 100 mM NaCl (Figure 5.5a) and 200 mM NaCl treatments (Figure 5.5b). However, the application of Mo to sorghum plants stressed with 300 mM NaCl significantly increased the levels of proline (Figure 5.5c). Proline has been reported to act as an environmental stress indicator (Kumar et al., 2015). It has been reported to increase in plants in response to different abiotic stresses (Sun et al., 2007; Yaish, 2015). The increased proline content in sorghum plants treated with 300 mM NaCl; provide evidence that Mo plays an important part inducing stress tolerance in plants.

The application of Mo significantly increased the levels of soluble sugars content in salt-stressed sorghum plants. This agrees with similar results by Zhange et al., (2012) who reported increased levels of soluble sugars with Mo application to salt-stressed *Brassica campestris L. ssp. Pekinensis*. The high accumulation of soluble sugars with the application of Mo in sorghum plants in response to salt stress is an adaptive response for alleviating the effects of salt stress (Hasegawa et al., 2000).

Carotenoids play important roles in plants to absorb light energy for photosynthesis and as antioxidants by deactivating free radicals (Maoka, 2020). The application of 0.5 µM Mo and 1 µM Mo significantly reduced the carotenoid content of sorghum plants stressed with 100 mM NaCl. Exogenous application of Mo to sorghum plants stressed with 200 mM and 300 mM NaCl significantly increased the activities of carotenoids, these results showed the effectiveness of Mo in reducing the toxic effects of salt stress to sorghum plants. This agrees with previous studies on *Brassica campestri*s (Zhang et al., 2014).

5.4.5. Molybdenun enhanced antioxidant enzyme response

Plants have developed different adaptive responsive mechanism to detoxify and eliminate toxic ROS as a result of stress (Jackson & Colmer, 2005; Zhang et al., 2012). Superoxide dismutase and APX are some of the major antioxidant enzymes that mediate defence system in many plants and their activities indicate the severity of stress on plants (Han et al., 2020). High activities of antioxidants are usually related to adaptive response of plants to unfavourable conditions (Fujita & Hasanuzzaman, 2022; Hashim et al., 2020). In this study, the exogenous application of various Mo concentrations to salt-stressed sorghum plants was effective in reducing the effects of oxidative stress in plants; this was observed by increased activities of SOD and APX (Figure 5.7). These results are consistent with previous studies by Zhang et al., (2011) who reported an increase in antioxidant activities under salt stress condition in *Brassica campestris.* However, the results from this study are contrary to the above statement for APX activity under 200 mM NaCl (1 µM and 2 µM Mo) in shoots and for SOD activity under 300 mM NaCl (0.5 µM, 1 µM and 2 µM Mo) treatments in the roots, where Mo treatments significantly reduced antioxidant activities. The reduction observed can

be due to damage in the antioxidant system caused by the high salt concentrations (Al Kharusi et al., 2019).

We further investigated the influence of Mo in regulating stress responsive protein "heat shock proteins-70" in sorghum plants stressed with 100 mM NaCl and 200 mM NaCl. Heat shock proteins, are proteins that are produced in cells in response to a variety of stresses (Al Khateeb et al., 2020). The HSP-70 was highly induced in the shoots of sorghum plants treated with 200 mM NaCl (Figure 5.8d). These results are consistent with previous findings (Ngara et al., 2012) under salt stress. Expression of stress responsive proteins under stress conditions plays important roles in protecting cells and developing tolerance against stress (Al-Whaibi, 2011). The induction of HSP-70 under salt stress correlates with its role as a molecular chaperon to limit the consequence of damage and facilitate cellular recovery under stress conditions (Yusof et al., 2022; Mayer & Bukau, 2005). Our results showed that Mo reduced HSP-70 expression in NaCl treated plants, suggesting that Mo contribute to the balance and stability of plants under stress.

5.5. CONCLUSION

Salt stress reduced the biomass of sorghum plants as well as stimulated the production of low photosynthetic pigments, high oxidative markers, and antioxidant activities as well as the induced expression of HSP-70, a stress responsive protein. Although some variations were observed with the different Mo concentrations applied to sorghum plants, there was consistency observed with the application of 0.5 µM Mo in alleviating the detrimental effects of salt stress under all NaCl treatments. These findings present for the first time that Mo application at low concentrations play an efficient role in reinforcing defense under salt-stressed conditions of sorghum plants and hence induce tolerance. To our knowledge, the present work offers the first demonstration of Mo in reducing the expression of HSP-70 in salt-stressed *Sorghum bicolor* plants.

CHAPTER SIX

INHIBITION OF MOLYBDENUM FUNCTION BY TUNGSTEN ON *SORGHUM BICOLOR* UNDER SALT STRESS

6.1. ABSTRACT

Tungsten (W) is a metal that has been widely used in industries, military and for household appliances. Tungsten shares similar chemical properties with Molybdenum (Mo) the plant micronutrient and it is proposed as an antagonistic against Mo enzyme activities. Chapter 5 results showed that 0.5 µM Mo effectively induced salt stress tolerance in sorghum, mediated through the increased antioxidant enzyme activities and the HSP-70 expression. Since, the mechanism of Mo-induced salt tolerance still remains elusive, this chapter provides preliminary data towards understanding this mechanism. Sorghum plants stressed with 200 mM NaCl were treated with a combination of W and Mo (0.5 µM, 1 µM and 2 µM) under the same growth conditions as described in chapter 3-5. Both metals improved shoot length, but increased $O_2^{\bullet-}$ and H_2O_2 accumulation on sorghum leaves. The highest H_2O_2 content (3.7-fold) was observed in the shoots under 1 µM W and Mo treatment, which correlated with a high MDA content of 4.5-fold observed under 2 µM W and Mo treatments. Soluble sugars significantly increased by 1-fold in the roots under 1 µM W and Mo treatments, whereas carotenoid content decreased significantly by 1.4-fold under 2 µM W and Mo treatment. Results confirmed the inhibitory effects of W on Mo activity and further affirmed that the use of low Mo concentrations is effective for stress adaptation in plants. This data will further be elaborated by assaying antioxidant enzymes activities and gene expression of analysis of stress responsive genes in addition to Mo related enzymes to complete the puzzle into unveiling the mechanism of Mo-induced salt tolerance.

KEYWORDS: Antagonist, Carotenoids, Molybdenum, Plant biomass, Toxicity, Tungsten.

6.2. INTRODUCTION

Tungsten (W) is one of the heavy metals that belong in group five on the periodic table of elements along with Molybdenum (Mo). Tungsten share similar chemical properties with Mo including, structure, electronegativity, atomic and ionic radii as well as range of oxidation states (Preiner et al., 2019). Despite their similarities, Mo is considered an essential micronutrient that is required by plants and animals, while W is a metal that has been widely used in different industries (Adamakis et al., 2012). The beneficial effect of low doses of W has been shown to promote root/shoot length and increased chl content in *Vigna unguiculata* (Kumar & Avery, 2012). At high concentrations, W has been reported to decrease root elongation in *Pisum sativum* and *Gossypium hirsutum (*Adamaskis et al., 2008). Due to their chemical identity, W is able to replace Mo in its various enzymes, including nitrate reductase (NR), xanthine dehydrogenase (XD), aldehyde oxidase (AO) and sulphite oxidase (SO) activity (Batyrshina et al., 2018; Mendel, 2007) and hence inhibits Mo-enzyme activity. The antagonist effect of W on Mo-enzyme activity has been reported by various studies including the inhibition of aldehyde oxidase activity in *Hordeum vulgare (*Batyrshina et al., 2018). The negative effects of W on Mo-enzyme activity in *Agropyron cristatum* subjected to salt stress has also been reported by (Babenko et al., 2015).

The antagonistic effect between W and Mo can be used to an advantage to understand the mechanism in, which Mo induces stress tolerance in plants. Results from this study showed that Mo-induced salt tolerance in sorghum is attributed to high antioxidant enzyme activities and HSP-70 expression when using low Mo (0.5 µM) concentration. Thus, it is expected that any inhibition to these responses by W will form part of understanding the mechanism of stress tolerance in plants. This chapter provides new data in order to better understand and elucidate the mechanism of Mo-induced salt tolerance by investigating the inhibitory effects of W on Mo activity in *Sorghum bicolor* plants under salt stress.

6.3. RESULTS

6.3.1. Combined effects of tungsten and molybdenum on sorghum growth

In order to determine the influence of W on Mo activity of *Sorghum bicolor*, the plants stressed with 200 mM NaCl were considered for this pilot study based on the consistency in the response of sorghum to this salt concentration as described in chapter 3 and 5. It was shown in chapter 5 that application of Mo to salt-stressed sorghum plants improved the shoot length under all Mo concentrations. Interesting, it can be observed that the combined treatments of W and Mo on salt-stressed sorghum plants increased shoot length (Figure 6.1). The highest shoot length was observed under 0.5 µM, followed by 1 µM and 2 µM (W and Mo) treatments.

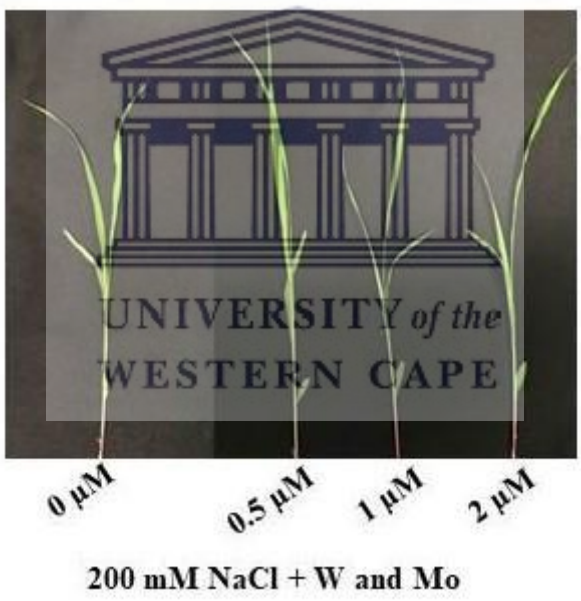

Figure 6.1. The influence of Tungsten (W) and Molybdenum (Mo) on sorghum growth. The phenotype of sorghum shoots under 200 mM NaCl salt stress in the presence of different W and Mo concentration (0.5 µM, 1 µM, and 2 µM).

6.3.2. Biochemical response of sorghum to tungsten and molybdenum

6.3.2.1. Histochemical staining of ROS ($O_2^{\bullet-}$ and H_2O_2)

Histochemical staining on the combined effects of W and Mo on the leaves of salt-stressed sorghum plants (Figure 6.2). Combined application of W and Mo induced toxicity on salt-stressed sorghum plants as observed by the over production of $O_2^{\bullet-}$ (dark blue spots) on the leaves of stressed sorghum plants (Figure 6.2a) and the complete covering of sorghum leaves with brown spots as an indication of H_2O_2 toxicity (Figure 6.2b).

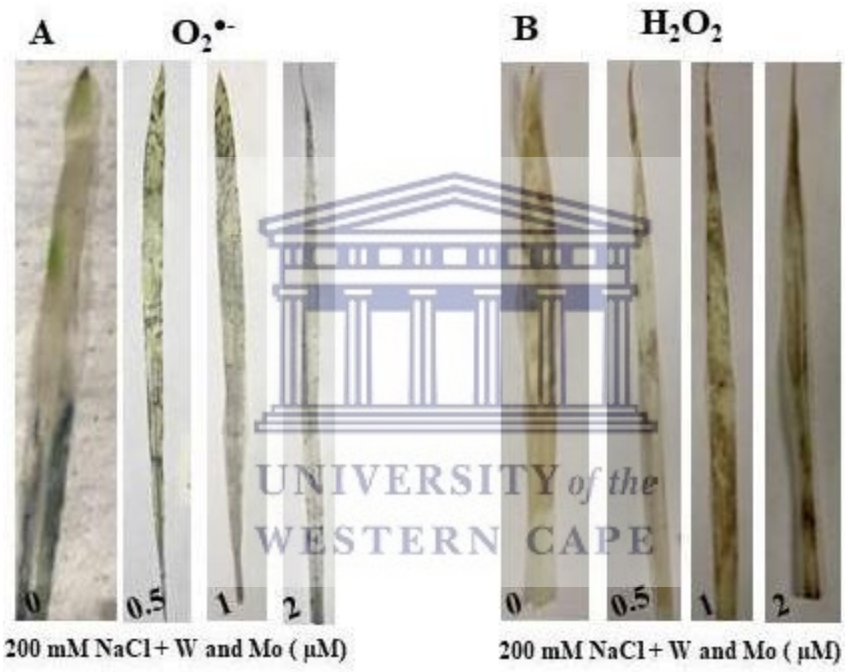

Figure 6.2. Histochemical staining of ROS ($O_2^{\bullet-}$ and H_2O_2) on the effects of W on Mo activity. Histochemical staining of $O_2^{\bullet-}$ and (**A**) H_2O_2 (**B**) in salt-stressed sorghum leaves treated with different concentrations of W and Mo (0.5 µM, 1 µM, and 2 µM).

6.3.2.2. Quantification of H_2O_2 and MDA content

The combined application of W and Mo significantly (*** = p≤0.001) increased H_2O_2 content in the shoots by 1.7-fold, 3.7-fold and 2.3-fold in the presence of 0.5 µM, 1 µM and 2 µM (W and Mo) treatments respectively, with no significant changes observed in the roots (Figure 6.3a). Similarly, W and Mo significantly (*** = p≤0.001) increased membrane damaged of

salt-stressed sorghum plants in the roots and shoots under all concentrations by 1.4-fold in the roots and 3.2-fold in the shoots under 0.5 µM W and Mo treatments. A further increase in MDA content was observed by 1.4-fold in the roots and 2.2-fold in the shoots under 1 µM W and Mo, 1.3-fold in the roots and 4.5-fold in the shoot under 2 µM W and Mo treatments.

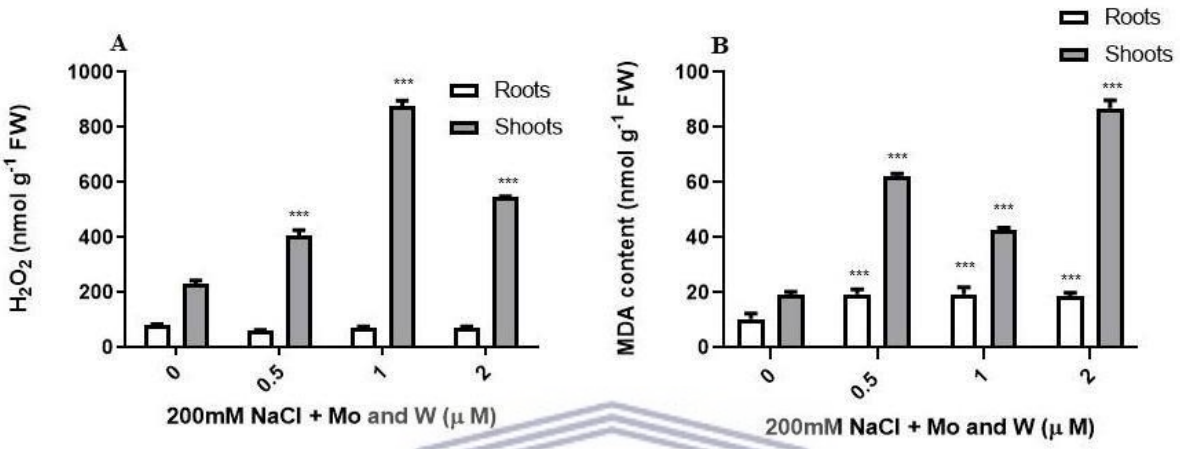

Figure 6.3. Effects of W and Mo application on the response of oxidative stress markers H_2O_2 and MDA. Tungsten and Mo on H_2O_2 content of salt-stressed sorghum plants (**A**), tungsten and Mo on MDA content of salt-stressed sorghum plants (**B**). Error bars represent the SD calculated from three biological replicates. Statistical significance between the different treated plants was determined using two-way ANOVA as conducted on GraphPad Prism and indicated as *** = $p \leq 0.001$according to the Bonferroni's multiple comparison test.

6.3.3. Influence of tungsten on molybdenum activity of non-enzymatic antioxidants

In order to determine the effects of W and Mo on the non-enzymatic antioxidants content of salt-stressed sorghum plants, the levels of soluble sugars and carotenoids were analysed. It can be observed in Figure 6.4a that the combined applications of W and Mo increased the soluble sugars content in the roots by 1-fold for 1 µM W and Mo treatments. But the combined W and Mo reduced carotenoid content in sorghum plants under salt stress conditions (Figure 6.4b). The decrease was only significant (** = $p \leq 0.01$) under 2 µM W and Mo concentration and this led to a 1.4-fold decrease.

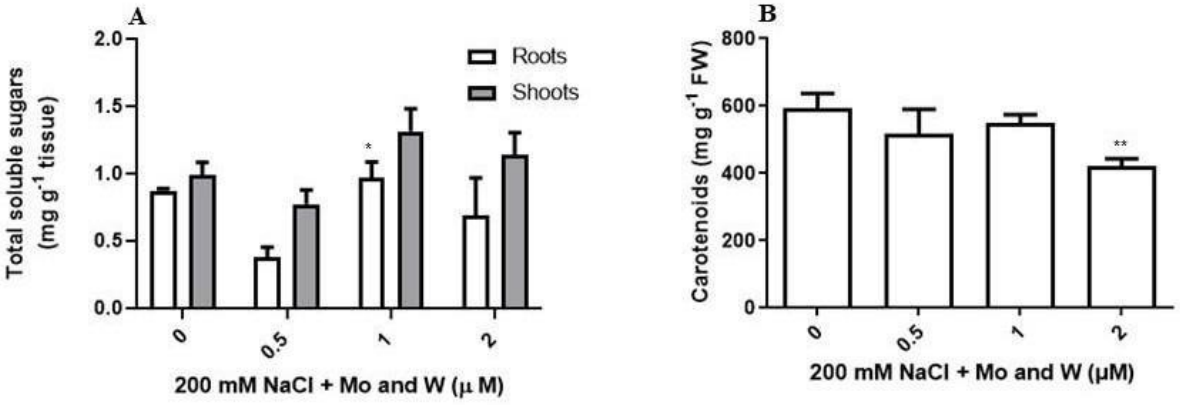

Figure 6.4. The combined effect of W and M on non-enzymatic antioxidants. Soluble sugar (**A**) carotenoid (**B)** contents on combined effects of different concentrations (0.5 µM, 1 µM, and 2 µM) of W and Mo on salt-stressed sorghum plants. Error bars represent the SD calculated from three biological replicates. Statistical significance between the different treated plants was determined using two-way ANOVA as conducted on GraphPad Prism and indicated as ** = p≤0.01, *= p≤0.05 according to the Bonferroni's multiple comparison test.

6.4. DISCUSSION

6.4.1. Effect of tungsten and molybdenum on growth

In this chapter, the influence of various concentrations of tungsten and molybdenum on the growth parameters and antioxidant content of *Sorghum bicolor* plants were studied. For the purpose of this study we considered 200 mM NaCl as the stress level of choice to demonstrate the effect of exogenous application of W on Mo activity. Although in chapter 5, results showed that the application of Mo only improved sorghum shoot growth with increasing Mo concentrations. Contrary to this, only 0.5 µM W + Mo, increased shoot length under 200 mM NaCl, but a combination of 1 µM and 2 µM W + Mo reduced shoot length gradually (Figure 6.1). Although, the incorporated W leads to defective Mo activity (Kumar et al., 1980), the results of this study might indicate that the increase in shoot growth under the treatment with both metals might be due to the fact that during the growth of sorghum plants, the concentration of incorporated W was low to cause any negative effect. Similarly, the

application of 0.1 mM W and Mo treatments did not show inhibitory effects on the shoot growth of *Hordeum vulgare* plants (Batyrshina et al., 2018), which corroborates the current results. But application of high W and Mo concentrations (1 µM and 2 µM) had negative growth effects, which might be due to W replacing Mo in Mo-related enzymes.

6.4.2. Effect of tungsten on molybdenum activity on ROS and MDA content

High accumulation of ROS is known to damage cellular membranes of plant tissues (Sachdev et al., 2021). Although Mo alone was able to reduce the toxicity of salt on the shoots of sorghum plants. The combination of W and Mo increased $O_2^{\bullet-}$ and H_2O_2 toxicity on the leaves seen as dark blue and brown spots on salt-stressed sorghum leaves (Figure 6.2), thus indicating the inhibitory effects of W on Mo-enzyme activity. The combined applications of W and Mo resulted in high H_2O_2 and MDA contents in sorghum plants. The increase in H_2O_2 may indicate that the ROS scavenging power in sorghum was somehow disrupted due to the unavailability of Mo on molybdo-enzymes, thus H_2O_2 served as a signalling molecule that under stress conditions (Gechev & Hille, 2005). Malondialdehyde content represent lipid peroxidation and oxidative injury mediated by ROS (Imran et al., 2020). The increased production of MDA observed in this chapter (Figure 6.3) indicates the level of membrane damage as a result of W toxicity as well as inhibitory effects of W on Mo activity, since the single application of Mo reduced MDA content in salt-stressed plants. The toxicity of W in plants has also been reported with the combined treatments of W and Mo in *Pisum sativum* (Adamakis et al., 2010).

6.4.3. Influence of tungsten and molybdenum on non-enzymatic antioxidants

It has been reported in many studies that osmolytes are produced in plants to protect plant cells from adverse effects of oxidative stress by inhibiting the production of harmful ROS (Ghosh et al., 2021; Sharma et al., 2019). Although the single application of Mo increased total soluble sugars and carotenoid content of salt-stressed sorghum plants as a result of adaptive response as observed in chapter 5. The combined application of metals (W and Mo) increased soluble sugar content in the roots under 1 µM treatments (Figure 6.4). An increase in soluble sugars may provide protection by chelating the metals in the cytoplasm and maintain water balance, which was disrupted by the metals (Xu et al., 2009). Similar results were reported by Kumar & Aery, (2012) in *Triticum aestivum* treated with both metals under normal conditions. Higher concentrations of both metals resulted in significant reductions in carotenoid content, which further confirms the inhibitory effects of W on Mo activity since Mo alone increased the carotenoid content of salt-stressed sorghum plants.

6.5. CONCLUSION

Taken together the results clearly indicated that tungsten (W) hindered Mo activity since Mo was unable to prevent oxidative damage as seen by high ROS accumulation, lipid membrane damage and reduced carotenoid content in salt-stressed sorghum plants. Since Mo is primarily used in plants in the production of molybo-enzymes that regulate various plant functions, thus disruptions of molybdo-enzymes cause conformation change and inhibit Mo activity. Therefore, this data provided groundwork for future studies. It would be useful for future studies to also look at the effects of tungsten only, in addition analyse gene expression profiles of molybdo-enzymes, towards fully understanding the mechanism of Mo-induced salt tolerance in plants.

GENERAL CONCLUSION AND FINAL REMARKS

The agricultural sector is under strain with the growing population as food insecurity has become a global crisis. The effects of abiotic stresses to agricultural crops are the primary cause of crop losses, reducing the potential yield in food production by more than 50% (Ahmad et al., 2014). The agricultural sector is the largest contributor to the economies and livelihoods of many African countries, and food security is dependent on the development of crops that are able to grow under stressful environments. With the constant change in climate the production of important commodities has declined severely. Salinity is one of the main abiotic stresses that causes a huge reduction in crop productivity and quality worldwide (Zhu, 2001). It is therefore a matter of urgency to develop crops that are well able to produce under stressful environmental conditions such as salinity in order to meet the food demands of the growing population.

In this study the effect of salt stress on *Sorghum bicolor* plants was investigated, by assaying morphological, physiological, biochemical and molecular parameters. It was demonstrated in chapter 3, that salt stress negatively affected the growth of sorghum plants with increasing salinity concentrations. This was observed by the reduction in shoot length with increasing salt concentrations, reduction in fresh and dry weight of plants, deformation of the epidermis and shrinkage of the vascular bundle layers. This correlated with increased ROS and antioxidant activities as well as increased expression of stress responsive protein "HSP-70'. The findings from this study are consistent with previous studies that salinity causes considerable damage to crops by elaborating toxic substances, which have deleterious effects on plants (Isayenkov & Maathuis, 2019).

Molybdenum (Mo) is an essential nutrient required for healthy plant growth and known to participate in various redox reactions in plants (Kaiser et al., 2005). This study investigated the effects of different concentrations of exogenous application of Mo to sorghum plants grown under normal conditions. Chapter 4 showed that exogenous application of Mo induced oxidative response in plants as a result of Mo-induced toxicity to unstressed sorghum plants. Although, exogenous application of Mo has been proven to be an important nutrient for plant growth and development (Sun et al., 2009), it is required by plants in very small quantities, and its overuse or high concentrations can become toxic to plants, which was evident in this study.

Molybdenum has also been reported to be a stress resistant element in plants to various abiotic stresses (Zhang et al., 2014). In chapter 5, the study demonstrated the ability of Mo to alleviate the effects of salinity stress on sorghum plants. This was evident by the increased FW and DW of plants, restoration of epidermal and vascular bundle layers and reduced ROS ($O_2^{\bullet-}$ and H_2O_2) production on the leaves of stressed plants. This also led to increased activities of antioxidants and reduced HSP-70 expression under high Mo concentrations. Although different concentrations of Mo were used in order to alleviate stress tolerance in salt-stressed *Sorghum bicolor* plants, the application of 0.5 µM Mo showed more prominent and consistent results in response to salt tolerance. With these interesting results, the mechanism of action of Mo-induced stress tolerance has not yet been elucidated. This can bring more understanding on how to manipulate the use of low concentrations of essential elements in agriculture without affecting the environment.

To try and bring this understanding, in chapter 6, this study investigated the relationship between Mo and tungsten (W). Since W is considered to be an inhibitor for Mo function, as it can replace molybdenum in the cofactor structure thus inactivating molydo-enzymes (Preiner et al., 2019). The study proved that W inhibited Mo activity, by negatively affecting

Mo-induced stress tolerance on sorghum salt-stressed plants. The results from this study opened a gap for future studies that would determine the individual effects of W on sorghum plants. Previous studies (Kumar & Aery, 2012) have shown that low concentrations of W to improve biomass and biochemical parameters of unstressed plants. It would also be of importance to perform gene expression analysis on the activity of both W and Mo on stressed and unstressed plants by analysing the expression of molybdo-enzymes including nitrate reductase (NR), xanthine dehydrogenase (XD), aldehyde oxidase (AO) and sulfite oxidase (SO). In addition, analysis of the expression of heat shock protein (HSP), sodium/hydrogen antiporter (NHX), dehydration responsive element binding protein (DREB) and ascorbate peroxidase (APX), which are amongst the genes that are instrumental for the adaptation and tolerance of plants to abiotic stresses will be valuable. To study the effects of of Mo and W under salt stress conditions using proteomics and transcriptomics. Future studies could also include investigating the positive effects of Mo under field conditions, which could help in understanding the possible mechanism of Mo in elucidating its protective role in plants under salt stress conditions.

APPENDICES

List of abbreviations

ABA	Abscisic acid
AP2/EREBP	Petala2/ethylene-responsive element binding protein
APX	Ascobic peroxidase
ARC	Amidoxime reducing component
AsA	Ascorbic acid
AOR	Aldehyde oxidase reductase
B	Boron
BSA	Bovine serum albumin
Bzip	Basic region-leucine zipper
C	Carbon
CaSO4	Calcium sulfate
CAT	Catalase
Cat#	Catalog number
Ca	Calcium ion
Chl	Chlorophyll
Cl	Chloride
Co	Cobalt
Cu/Zn-SOD	Copper Zinc superoxide dismutase
Cu	Copper
DAB	3'3'diaminobezidine (DAB)
dH_2O	Distilled water

ddH_2O	Doubled distilled water
Dehydrogenase reductase	DHAR
DTT	Dithiothreitol
DW	Dry weight
ECL	Electro-chemiluminescent
EDX	Energy dispersive X-ray spectroscopy
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and agricultural organization
Fe	Iron
Fe-SOD	Iron duperoxide dismutase
FW	Fresh weight
G	Grams
Glu	Glutamate
GR	Glutathione reductase
GPX	Glutathione peroxidase
GSH	Reductase glutathione
H	Hydrogen
HCl	Hydrogen chloride
HSP	Heat shock proteins
HSF	Heat shock transcription factors
H_2O_2	Hydrogen peroxide
JA	Jasmonic acid
KCI	Potassium chloride
KH_2PO	Potassium phosphate monobasic
K_2HPO_4	Potassium phosphate dibasic

MDA	Malondialdehyde
MDHAR	Monodehydrogenase reductase
Min	Minute
Mbp	Megabase
Mn-SOD	Manganese superoxide
Mn	Manganese
mM	Milli molar
Mg	Magnesium ion
Mg	Magnesium
MYB	Myeloblastosis
N	Nitrogen
NAC	N-acetyl cysteine
Na_2CO_3	Sodium carbonate
Na	Sodium
NADH	Nicotinamide adenine dinucleotide
NADH(H)	nicotinamide adenine dinucleotide + hydrogen
Na_2HPO_4	Na_2HPO_4
Na_2MoO4	Sodium molybdate
NaOH	Sodium hydroxide
$(NH_4)2MoO_4$	Ammonium molybdate
NBT	Nitrotetrazolium blue chloride
Ni	Nickel
Nm	Nano meters
$OH^{\bullet}$	Hydroxyl radical

$O_2^{\bullet-}$	Superoxide anion
$^1O_2$	Singled oxygen
O	Oxygen
P	Phosphorus
PBS-T	Phosphate buffer saline + Tween
pH	Potential of hydrogen
PMSF	Phenylmethylsulfonyl fluoride
Product#	Product number
POX	Guaiacol peroxidase
PDH	Proline dehydrogenase
PVDF	Polyvinylidene Fluoride
PVP	Polyvinylpyrrolidone
PC5	Delta-1-pyrroline-5-carboxylate
P5CDH	P5C dehydrogenase
P5CR	Pyrroline-5-carboxylate reductase
P5CS	Delta-1-pyrroline-5-carboxyli acid synthetase
ROS	Reactive oxygen species
Rpm	Revolutions per minute
S	Sulphur
SA	Salicyclic acid
*Sb*HSP-70	*Sorghum bicolor* heat shock protein-70
SDS	Sodium dodecyl sulfate
SEM	Scanning electron miscroscope
Si	Silicon

smHSP	Small heat shock proteins
SOD	Superoxide dismutase
SO	Sulfite oxidase
SOS	Salt overlay sensitive
SSA	sub-Saharan Africa
TBA	Thiobabituric acid
TCA	Trichloroacetic acid
TFs	Transcription factors
V	Vanadium
W	Tungsten
WC	Water content
XO	Xanthine oxidoreductase
Z	Zinc
µM	Micrometer
Ml	Millie litre
v/v	Volume to volume
w/v	Weight per volume
%	Percentage
ß	Beta

List of chemicals and suppliers

Chemical/Reagent	Supplier
2-Thiobabituric acid (TBA)	Inqaba
3’3’diaminobezidine (DAB)	Inqaba
ß-mercaptoethanol	Sigma-Aldrich
Acetone	Kimix
Ammonium Acetate	Sigma-Adrich
Ammonium Persulfate (APS)	Inqaba
Anti-his HSP70	BIOCOM Africa
Anthrone	CT labs
Bromophenol Blue	Inqaba
Bovine serum albumin (BSA)	Sigma-Aldrich
Casein bovine milk	Sigma-Aldrich
Coomassie brilliant blue R-250	Merck
Dithiothreitol (DTT)	Inqaba
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Ethanol	Kimix
Glacial acetic acid	Kimix
Glycine	Labchem
Goat anti-mouse	Lasec
Guaiacol	Sigma-Aldrich
Hydrogen peroxide	BIO-RAD
L- Ascorbic acid	Inqaba

Methanol	Kimix
Methionine	Inqaba
Ammonium heptamolybdate	Merch
Ninhydrin	Inqaba
Nitrotetrazolium blue chloride (NBT)	Inqaba
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich
Polyvinylpyrrolidone (PVP)-Mw- 40 000	Sigma-Aldrich
Potassium hydroxide pellets	Kimix
Potassium iodide (KI)	Kimix
Potassium cyanide (KCI)	Labchem
Potassium phosphate monobasic (KH_2PO_4)	Inqaba
Potassium phosphate dibasic (K_2HPO_4)	Inqaba
Potting soil	Stodels
Proline	Labchem
Propan-2-ol (isopropanol)	Kimix
PVDF membrane	Lasec
Riboflavin	Labchem
Sodium chloride (NaCl)	Kimix
Sodium dodecyl sulfate (SDS)	Merck
Sodium hydroxide (NaOH)	Kimix
Toluene	Inqaba
Trichloroacetic acid (TCA)	Inqaba
Tungsten	Ingaba
Tween® 20	Inqaba
Vermiculite	Stodels

List of stock solutions prepared

Buffer/Stock Solution	Composition
Acetone (80%)	80% (v/v) acetone in distilled water dH_2O_2
Anthrone	150 mg anthrone, 100 ml of 72 % v/v sulfuric acid).
BSA (10 mg/ml)	10 mg/ml BSA in extraction solution
DAB staining solution	1 mg/ml DAB in dH_2O_2 pH adjusted to 3.8
Ethanol 80% (v/v)	80% (v/v) ethanol in dH_2O_2
Extraction buffer	50 mM K_2HPO_4 at pH 7.5, 2 mM EDTA, , 4% PVP-40,
HCl (1M) for pH	1 M HCl in dH_2O_2
KH_2PO_4 (1M)	1M KH_2PO_4 in dH_2O_2
K_2HPO_4 (1M)	1 M K_2HPO_4 in dH_2O_2
KI (1M)	1 M KI in dH_2O_2
Molybdenum (0.1M)	0.61 g in dH_2O
NaCl (5M)	1.46 g NaCl in dH2O
NBT staining solution	0.2% (w/v) NBT in 50 mM KPO_4 pH adjusted to 7.5
Ninhydrin solution	1.2 g Ninhydrin in 30 ml glacial acetic acid and 20 ml phosphoric acid
Phosphate buffered saline-T (PBS-T) (10X)	137 mM NaCl; 2.7 mM KCI, 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4, 1% Tween-20 in dH_2O_2

Blocking buffer	PBS + 0.05% Tween + 0.05 % casein
Sulfosalicylic acid (3%)	3 g sulfosalicylic acid in dH_2O_2
TCA (20%)	0.1% (w/v) TCA in dH_2O_2
TCA (20%)/TBA (0.5%) solution	0.5% (w/v) TBA in 20% (v/v) TCA
Transfer Buffer	14.4 glycine, 3.03 g Tris-base, 200 ml methanol
Tungsten (0.1M)	0.33 g in dH_2O

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