Exogenous carbon monoxide and jasmonic acid mitigate salt stress in *Sorghum bicolor*

Gershwin Sias (3539430)

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Supervisor: Dr. Takalani Mulaudzi-Masuku

Co-supervisor: Prof. Emmanuel Iwuoha

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GENERAL PLAGIARISM & DECLARATION

University of the Western Cape

Private Bag X17, Bellville 7535, South Africa

Telephone: ++27-21- 959 2255/959 2762

Fax: ++27-21- 959 1268/2266

Email: jvanbeverdonker@uwc.ac.za

FACULTY OF NATURAL SCIENCES

Name: GERSHWIN SIAS

Student number: 3539430

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LIST OF ABBREVIATIONS

$O_2^{\bullet-}$	Superoxide	HRSEM-EDX	High resolution scanning electron microscopy-energy dispersive X-ray
µg	Microgram	Ht	Hematin
µM	Micromolar	JA	Jasmonic acid
$^{\bullet}HO$	Hydroxyl radical	JAs	Jasmonates
$^1O_2$	Singlet oxygen	JAZ	JASMONATE ZIM-DOMAIN
ABA	Abscisic acid	LOX	Lipoxygenase
ANOVA	Analysis of variances	MDA	Malondialdehyde
AOC	Allene oxide cyclase	MDHAR	Monodehydroascorbate reductase
AOS	Allene oxide synthase	MeJA	Methyl jasmonate
APX	Ascorbate peroxidase	mg	milligram
AsA	Ascorbate	MGT	Mean germination time
BV	Biliverdin	ml	Millilitre
CAT	Catalase	mM	Millimolar
cDNA	Complementary deoxyribonucleic acid	mm	Millimetre
CYP	Cytochrome P450	MYB	Myeloblastosis binding site
DAB	3',3' diaminobenzidine	MYC	BHLH transcription factor
ddH_2O	Double distilled water	NAC	NAC gene family transcription factor
DHAR	Dehydroascorbate reductase	SD	Standard deviation
DW	Dry weight	SL	Shoot length
ET	Ethylene	OPDA	Oxo-phytodienoic acid
FTIR	Fourier-transform infrared	PEG	Polyethylene glycol
FW	Fresh weight	PEPC	Phosphoenolpyruvate carboxylase
g	Gram	POX	Peroxidase
NADPH	Nicotinamide adenine dinucleotide phosphate	ROS	Reactive oxygen species
GR	Glutathione reductase	RT-qPCR	Real-time quantitative polymerase chain reaction
GSH	Glutathione	SA	Salicylic acid
GSSG	Oxidized glutathione	SOD	Superoxide dismutase
HbA	Haemoglobin	TBA	Thiobarbituric acid
TCA	Trichloroacetic acid	UBQ	Ubiquitin

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HO	Heme oxygenase	TSS	Total soluble sugar
TG	Total germination	VSP2	Vegetative storage protein

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ABSTRACT

The agricultural sector plays an important role in the world economy. Expanding research is piloting its improved contribution to the economic sector. Despite the economic gain, the increasing population has already put a strain on agriculture, but the effects of abiotic stresses are severely affecting crop production globally. Abiotic stress is not only affecting the immediate plants, but further inhibits soil fertility, which will affect future crop productivity. The damaging effects of salt stress is only expected to intensify, which further obscures the future prospect of food security. Salt stress management should be prioritised to alleviate food insecurity. Understanding the mechanism involved in conferring salt stress tolerance to plants is fundamental in the establishment of crops with improved tolerance. *Sorghum bicolor* is one of the most important crops globally that is moderately tolerant to drought and salt stress thus in addition to the availability of its genome, sorghum can be the perfect model crop to further elucidate the mechanism involved in salt stress tolerance. Phytohormones and other signalling molecules are elicitors at the forefront of plant defence in response to stress stimuli. This study was therefore, aimed at investigating the roles of methyl jasmonate (MeJA) and carbon monoxide [using hematin (Ht) as CO donor] in conferring salt stress tolerance to *S. bicolor* at physiological, biochemical and molecular level. Sorghum seeds that were pre-treated with MeJA (0, 10, 15 and 20 µM) and exogenously supplied with hematin (0, 1 and 1.5 µM) exposed to non-stress (0 mM NaCl) and salt stress (200 mM NaCl) conditions and left to germinate for 7 days at 25℃ in complete darkness. This was followed by conducting germination assays, including germination percentage (GP), mean germination time (MGT), germination index (GI), total germination (TG), root and shoot length. Salt stress inhibited germination by decreasing GP, MGT, GI, TG and growth (root and shoot length). MeJA pre-treated sorghum in combination with exogenous hematin ameliorated the salt stress induced seed germination

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inhibition by improving all germination parameters in addition to increasing root and shoot lengths. Findings indicated that MeJA and CO individually or in combination successfully alleviate seed germination inhibition caused by salt stress as indicated by improved germination and seedling growth under salt stress. Only MeJA pre-treated sorghum was selected for further investigation by growing plants in potting soil under summer conditions followed by exposure to varied salt concentrations (0, 100 and 200 mM NaCl). This was then followed by conducting further physiological, biochemical and gene expression assays. Pre-treatment with MeJA was more effective in reversing the damaging effects of salinity in sorghum during the vegetative stage of growth. Methyl jasmonate induced salt stress tolerance of sorghum by improving growth as indicated by increased shoot height and plant biomass. It also reversed most of the inhibitory effects of salinity by improving photosynthesis, while reducing oxidative damage and osmolyte accumulation in salt-stressed *S. bicolor*. Furthermore, pre-treated sorghum illustrated reduced shrivelling of epidermal surface with enhanced deposits of silica phytoliths. Sorghum pre-treated with MeJA had induced levels of secondary metabolites under salt stress. Additionally, pre-treatment down regulated the salt stress-induced gene expression levels of *SbLOX, SbAOS, SbAOC* and *SbOPDR*. Pre-treatment with 10 µM MeJA proved to be the most effective at enhancing salt stress tolerance to sorghum. Findings suggest MeJA signalling plays a crucial role in inducing salt stress tolerance in sorghum. Data from this study will aid in the elucidation of the mechanisms involved in conferring salt stress tolerance, which is fundamental in pioneering the establishment of transgenic crops with enhanced stress tolerance. Thus, improving crop productivity and the eventual alleviation of food insecurity.

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CHAPTER 1:

LITERATURE REVIEW

1.1 INTRODUCTION

Plants play a considerable role in the agricultural sector, which is continuously expanding and benefitting the livelihoods of many individuals worldwide. Agriculture, forestry and fisheries were the main sectors, which contributed a 1.3% growth to the country's economy in 2017 exceeding the expected 1% (Gibson, 2012). Regardless of the economic gain, the foremost problem persists due to the growing population and changing climate, which are continuously putting severe strain on food security. Food supply is required to increase by an estimated 70-100% in order to feed the expected ~9.8 billion population globally by the year 2050 (Rayfuse & Weisfelt, 2012).

Ubiquitously found cereal crops act as staple food for many individuals globally due to their high nutritional values and many usages (Wang et al., 2018). Underprivileged countries have been found to derive more than 80% of their calories from cereal crops (Awika, 2011). Cereal crops are not only used for animal feed and human consumption, but their high starch content is receiving considerable attention as sources for biofuel production (Gawande & Patil, 2014). As a C4 carbon cycle plant, sorghum, is the 5th largely produced cereal crop in the world rankings after maize, wheat, rice and barley and the 2nd most important food crop in Africa. More than 80% of sorghum plantation area consisting of about 42.12 million hectares of land can be found in developing countries of Asia and Africa. These countries still, however, produce only ~56% of the global sorghum (Hariprasanna & Rakshit, 2016). Sorghum has good adaptability and, therefore, grows widely in arid and semi-arid regions. Studies have shown sorghum to contain several quantitative trait loci associated with drought tolerance

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(Badigannavar et al., 2018), and is gaining increased interest due to its relatively higher tolerance to salt stress (Huang, 2018).

Harsh environmental conditions are the leading cause of low crop productivity and resulted to more than 50% reduction in crop yield globally (Hinojosa et al., 2018). Plants are very vulnerable to abiotic stresses including exposure to excess heavy metals, severe temperatures, ultraviolet radiation, water deficiency and the ever increasing saline conditions, among others (He et al., 2018). Abiotic stress is often as a result of conjunctive manifestations where constant exposure to increasing temperatures leads to water deficiency. When inadequate amounts of water are present within soils due to evaporation, the condition elevates the soil salinity levels increasing osmotic and ionic stress and therefore reducing crop growth and development drastically (Abhinandan et al., 2018). Due to constant exposure, plants developed specific mechanisms assisting in their adaptations and survival under these stressful conditions (Rejeb et al., 2014). Plants respond to stress by stimulating stress sensor signals, which allow for the activation of constitutively defensive mechanisms including signalling cascade of molecules, which varies upon exposure to different stress factors (Tuteja & Sopory, 2008; Rejeb et al., 2014; Chi et al., 2019). Plants will regulate specific kinases and ion channels, accumulate reactive oxygen species (ROS), induce the synthesis of phytohormones and modify the expression of certain genes in attempts to abate injury and enhance stress tolerance (Rejeb et al., 2014).

Signalling molecules are important for the growth and development of all plants and allow for the activation of defence mechanisms such as the antioxidative system within plants (Sewelam et al., 2016). Carbon monoxide (CO) and jasmonates (JAs) are some of the important signalling molecules gaining retrospection (Wang & Liao, 2016; Ruan et al., 2019). Previous studies have shown the effect of CO on seed germination, plant growth and development, and its impact on the growth of plants under abiotic conditions (Liu et al., 2007; Guo et al., 2009; Kong et al.,

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2010; Zhang et al., 2012). Jasmonates are usually activated within seconds in plants upon exposure to biotic stress, however, they have also been found to be activated upon exposure to abiotic stresses and, therefore, play a critical role in plant stress tolerance (Kepczyńska & Król, 2012; Singh & Shah, 2014; Howe et al, 2018).

A study by Cheng et al. (2018), suggested that there exists an underlying crosslink between CO and JAs in plant growth and development processes however, their relationship is yet to be strongly established. The purpose of this review was thus to attain understanding of plant responses to abiotic stress, mainly salt stress, and to further establish the link between signalling molecules; CO and JAs with the goal to fully elucidate the mechanism by which these signalling molecules work in conjunction to enhance plant stress tolerance.

1.2 ENVIRONMENTAL STRESS FACTORS

The growth and development of plants are highly dependent on environmental conditions such as carbon, sunlight, water, minerals and nutrients for optimal growth (Zhang et al., 2014; Guo et al., 2016; Schumann et al, 2017; Robbins & Dinneny, 2018). Environmental stress factors (abiotic stress) can, therefore, be defined as the negative effect that non-living conditions have on plants where growth and development are constrained below their optimal level (Cramer et al., 2011). In order to be classified as stress, the non-living variable should severely impact the environment's variation beyond its normal range and have a significantly harmful effect on the plant's physiology or performance of the plant population (Rolf et al., 2004). Inanimate stress factor is an inevitable occurrence, which affects animals and has a predominantly constraining effect on plants given its high dependency on environmental conditions. Abiotic stresses have

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a severe impact on crop production and have been found to be most detrimental when coinciding with other stressors (Mittler, 2006).

1.2.1 Excess heavy metals

Trace amounts are essential nutrients required for plant life. However, the absorption of these elements in excess can have detrimental effects on the nutritional value and yield of crops (Rizvi & Khan, 2018; Tchounwou et al., 2013). The growth and leaf area of *Helianthus annuus* were severely hindered with an increase in concentrations of Cd and Zn, leading to increased absorption in certain parts of the plant (Chaves et al., 2011). A similar trend was observed where increasing Pb concentrations limited the growth of *Ligustrum lucidum* seedlings while simultaneously increasing the level of Pb found in its roots, stems and leaves (Zhou et al., 2018). Excessive heavy metals severely reduced chlorophyll and carotenoid content in *Vigna mungo* and *L. lucidum* seedlings, which significantly affected these species' net photosynthesis, transpiration rate and stomatal conductance (Gurpreet et al., 2016; Zhou et al., 2018). Cadmium and nickel toxicity induced the accumulation of hydrogen peroxide, lipid peroxidation and proline content, and increased the activities of the antioxidant enzymes, glutathione reductase, catalase, superoxide dismutase, and ascorbate peroxidase of *Pisum sativum* (El-Amier, 2019). While similar responses to Zn, Cu, Ni and Cd were observed in *Zea mays*, AbdElgawad et al. (2020) also found that treatment with these heavy metals significantly increased the endogenous production of metals within the plant's organs.

1.2.2 Extreme temperature

Plants are very susceptible to thermal stress at their vegetative stage of development. Cold stress (below 12℃) inhibited germination, while high temperatures of 32℃ and above deteriorated the leaf expansion of *Brassica oleracea* L. (Rodríguez et al., 2015). Low temperatures significantly reduced the plant biomass and survival rate of *S. bicolor* seedlings

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as compared to *Zea mays* seedlings when grown under 12.5℃/9.5℃ (day/night) temperatures and below (Antony et al., 2019). Increasing temperatures deteriorated the morphology of *Oryza sativa* L. cultivars (Huanghuazhan and IR-64), in addition to reducing the leaf area, photosynthetic and water use efficiency (Fahad et al., 2016). High temperatures have been found to significantly increase the production of proline, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), while decreasing chlorophyll content and the production of oxygen (O_2) in *Vigna radiata L. cv. Binamoog-1* (Nahar et al., 2015). Increased temperatures induced the production of apoplastic H_2O_2 and decreased stomatal aperture in *Solanum lycopersicum* (Zhang et al., 2019). Heat stress can have varying effects on the expression and activity of lipoxygenase (LOX). While LOX activity decreased in response to heat stress in *V. radiata*, members of the LOX gene family responded differently in *S. lycopersicum* (Nahar et al., 2015; Upadhyay et al., 2019). Extreme temperatures are also detrimental to the plant's reproductive system. Heat stress increases plant sterility and have proven to be quite detrimental to pollen viability in *S. bicolor* and *O. sativa* (Djanaguiraman et al., 2018; Rezaul et al., 2019). Chiluwal et al. (2020) found heat stress to induce significant injury to the plant ovarian tissue causing cytoplasmic content build-up and nucleus disintegration in *S. bicolor*. Plants are prone to multiple stress factors that may occur in conjunction with one another, which can be seen as a common occurrence between temperature stress and water deprivation (Źróbek-Sokolnik, 2012).

1.2.3 Water deficiency

Water scarcity is a frequent climatological occurrence whereby inadequate water supply is made available due to the lack of precipitation. Changing environmental conditions induces water loss through evaporation or transpiration, which reduces the water availability within soil and plants. Damage caused to plants due to this phenomenon is known as drought stress and

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has proven to be very detrimental to crops worldwide (Jaleel et al., 2009). Drought inhibits cell expansion and therefore, rapidly decrease leaf-number and -size of *Zea mays* and *Saccharum officinarum* (Nelissen et al., 2018; Hoang et al., 2019). Limited water restricted plant growth by reducing root elongation and caused erected leaves in *S. bicolor* (Akbudak et al., 2018). Drought incapacitates the proficiency of many important enzymes such as Adenosine Diphosphate Glucose Pyrophosphorylase, Sucrose- and Starch Synthases and Starch Branching Enzymes, thus hindering the yield of many food crops (Fahad et al., 2017). Water scarcity most commonly induces ROS and MDA production in most plant species including sorghum plantlets (Nxele et al., 2017). While most *Achillea* species adhere to the common trend, Gharibi et al. (2016) illustrated a reduction in both ROS and MDA when exposed to severe drought conditions in *Achillea nobilis* and *A. millefolium.* Additionally, researchers found a positive correlation between drought stress severity and the levels of proline, flavonoid and antioxidant activity in all of their investigated *Achillea* species (*A. filipendulina*, *A. nobilis* and *A. millefolium*) (Gharibi et al., 2016). The plant's antioxidant system plays an important role in plant defence. A study have demonstrated the synergistic relationship between ascorbate peroxidase 4 (APX4) and glutathione peroxidase 2 (GPX2) due to their opposing expression levels under drought conditions in the roots and leaves of *S. bicolor* (Akbudak et al., 2018). The lack of water is detrimental to the plant's photosystem and have, therefore, proven to reduce the oxidoreductive activity and photochemical efficiency in *S. bicolor* (Guo et al., 2018). Drought stress is harmful to plant maturity and reproductive system as illustrated by the retardation in the development of the flag leaf and the grain head, in addition to a significant reduction in grain yield in *S. bicolor* (Dimkpa et al., 2019). Improper filtration of soils due to lack of precipitation raises soil salt concentrations, which induce further dehydration of cells and osmotic stress.

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1.2.4 Salinity stress

Subsidiary levels of Na^+ and Cl^- ions supply may act as essential nutrients to especially some C4 crops. However, the development of C4 species such as maize and sorghum, do not benefit from the addition of Na^+ (Kronzucker et al., 2013). Sodium facilitates the absorption of pyruvate into chloroplasts, and pyruvate acts as a precursor for important biochemical reactions such as isoprenoid metabolism and the synthesis of fatty acids in all plant species (Kronzucker et al., 2013). Chloride is an important micronutrient that plays a pivotal role in regulating cytoplasmic enzyme activity, acting as a co-factor for the process of photosynthesis, stabilizing membrane potential and regulating pH levels and turgor pressure (Teakle & Tyerman, 2010). However, excess amounts of NaCl can cause severe injury to plants. The negative impact salinity stress has on plant growth and development is mainly due to nutrient imbalance, cytotoxicity caused by the influx of Na^+ and Cl^- ions and due to the aforementioned water stress (Isayenkov & Maathuis, 2019).

Salinity stress has proven to be one of the most severe and tenacious abiotic stress due to the increased levels of salt found in arable land globally. Many plant species succumb when exposed to NaCl concentrations exceeding 200 mM. The nutritional deficiency, ionic toxicity, oxidative and osmotic stress as an effect of high salinity severely restrict the plant's life cycle from seedling establishment to its propagation (He et al., 2018). This abiotic stress is often interlinked with and results in common effects on the plant as the other severe global issues such as drought and extreme temperature. Therefore, enhancing a plant's tolerance to salt stress would most likely have increased tolerance to drought, heavy metals and extreme temperature.

1.2.4.1 Osmotic stress

Osmotic stress can be detrimental to the plant's metabolism and occurs when plants experience abrupt alterations in solute concentration from its surrounding that limits the available water

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potential within its cells (Hohmann, 2002). Salt may have an immediate or prolonged effect on plants. Osmotic stress occurs within minutes to days, which will inhibit cell expansion and result in stomatal closure (Isayenkov & Maathuis, 2019). It therefore restricts plant growth and development since it significantly reduces the root and shoot length, plant biomass, photosynthetic pigments while inducing MDA and H_2O_2 content as seen in many plant species such as *Solanum lycopersicum* L. (Ahmad et al., 2018), *Lemna aequinoctialis* (de Morais et al., 2019), *Zingiber officinale* (Yin et al., 2020), *Gossypium hirsutum* L. (Chen et al., 2020) among others. Salt stress-induced oxidative stress by resulting in the accumulation of MDA accompanied with increased phenolic compounds and antioxidant flavonoids in *Nerium oleander* L. High salt concentrations were also found to increase antioxidant activities of ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT) in attempts to scavenge reactive oxygen species and reduce further oxidative stress as seen in *N oleander* (Kumar et al., 2017), *Solanum lycopersicum* L. (Ahmad et al., 2018; Tanveer et al., 2020).

1.2.4.2 Ionic stress

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Ionic stress occurs over days to weeks accumulating cytotoxins that perturb the plant's metabolism leading to premature leaf senescence and eventual cell death (Isayenkov & Maathuis, 2019). Ionic toxicity occurs due to the competitive nature of Na^+ and Cl^- ions that disrupts the homeostasis of many important nutrient elements such as the ratios of Ca^{2+} to Mg^{2+}, Na^+ to K^+ and Na^+ to Ca^{2+} causing an imbalance of the plant's ionic composition. Increased Na^+ uptake due to salinization, particularly disrupts the Na^+/K^+ ratio by triggering the efflux of K^+ ions from cells resulting in K^+ leakage and ultimately cytosolic K^+ deficiency (Chokshi et al., 2017). Salt stress caused a significant decrease in K^+ content in *Brassica juncea* L., which thus caused up to 99% decrease in the Na^+/K^+ ratio in response to increasing NaCl concentrations (0 mM - 200 mM) (Pandey & Penna, 2017). The decrease in K^+ content with a

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concomitant increase in Na^+ and Cl^- content in response to increased salt concentrations was also later observed in *Solanum melongena*, *Cucumis sativus*, *Solanum lycopersicum* and *Punica granatum* (Hannachi & Van Labeke, 2018; Zhang et al., 2019; Abdelaziz et al., 2019; Liu et al., 2020). Increased saline condition further induces intracellular Na^+ content when Na^+ uptake replaces the Ca^{2+} ions from the cell membrane (Rahman et al., 2016). This occurrence can, therefore, give rise to many modifications in the plant's physiological traits (Evelin et al., 2019). Plants under stressful saline conditions often have alter fatty acid ratios and membrane fluidity to reduce the influx of harmful Na^+ and Cl^- ions into cells. Salt stress, therefore, increased the linolenic acid observed in *Brassica rapa* (Yepes et al., 2018). This was supported by the same effect being observed in *Brassica oleracea var. italica* under salinity stress (López-Pérez et al., 2009).

1.3 PLANT STRESS RESPONSIVE MECHANISMS

The sessile nature of plants makes it very important to survive under harsh environmental conditions. Plant survival is therefore highly dependent on the activation of essential mechanisms, which include changes in its metabolic or physiological profiles, triggering of important molecular pathways and transcription factors, and its adaptation to the changing environment. This can negatively impact agriculture as these responsive mechanisms that protect the plant, usually occurs at the expenditure of the plant development and yield (Bechtold & Field, 2018). Stress-responsive transcription factors triggered by signal transduction induces downstream response that aids in the activation of the suitable defence to abiotic stress, which allows for the regulation of plant growth and acclimatization (Bechtold & Field, 2018). Over-accumulation of ROS is detrimental to plants and its detection by plant cell

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walls activates a cascade of signalling events that are often interceded with plant hormones and antioxidant enzymes (Novaković et al., 2018).

1.3.1 Reactive Oxygen Species

Reactive oxygen species are primarily produced in peroxisomes, mitochondria and chloroplast, and may also be produced at secondary sites such as the apoplasts, cell wall, cell membrane and endoplasmic reticulum. ROS includes mainly ionic states such as superoxide ($O_2^{\bullet -}$) and hydroxyl radical ($^{\bullet}HO$), and molecular states such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Das & Roychoudhury, 2014). ROS plays a vital role as secondary messenger when cellular homeostasis is maintained, however when this homeostasis is disturbed due to an abiotic stresses, over-accumulation of these molecules occurs that inflicts great damage to cells by inducing oxidative stress and degrading pivotal lipids, carbohydrates, DNA and proteins, ultimately resulting in cell death (Das & Roychoudhury, 2014). The redox molecule, H_2O_2, is one of the most important ROS due to its remarkable stability and capability to rapidly inflict reversible oxidation of its target protein (Huang et al., 2019). It plays a key role in the regulation of cell signalling and have been found to partake the formation of cell walls, programmed cell death, senescence and cell differentiation (Huang et al., 2019). Furthermore, H_2O_2's regulatory role in plant growth and development, and plant response to stress could, however, be due to its interaction with other hormones (Huang et al., 2019).

1.3.2 Antioxidants

Plants can trigger a whole range of mechanisms in response to stress. The antioxidant system is one of the most important stress-alleviator activated mechanism in plant metabolism. It plays a crucial role in mediating oxidative damage that occurs due to over-accumulation of ROS in response to stress (Imam et al., 2017). These enzymes are highly effective in protecting the plant against damage by reducing lipid peroxidation and thus oxidative stress, which highlights

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their importance in plant metabolism. Many antioxidants have been characterised to have either enzymatic or non-enzymatic properties (Ahmad et al., 2010).

1.3.2.1 Enzymatic antioxidants

The major antioxidant enzymes that aid in plant stress defence include superoxide dismutase (SOD), guaiacol peroxidase (GPOX), catalase (CAT) and enzymes of the ascorbate (AsA)-glutathione (GSH) cycle - ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Gill & Tuteja, 2010; Foyer & Noctor, 2011; Ahmad et al., 2018; Hasanuzzaman et al., 2020). The enzyme catalysing the biosynthesis of endogenous carbon monoxide (CO), known as heme oxygenase (HO), may have antioxidative properties due to its role in synthesizing antioxidant pigments (biliverdin and bilirubin). This forms part of the plant's defence system and have been shown to serve a protecting role in assimilation and nitrogen fixation under harsh saline conditions (He & He, 2014). Over-accumulation of ROS due to salt stress firstly triggers SOD that will convert superoxide into oxygen and peroxide (H_2O_2). The H_2O_2 will further dismutate into water and oxygen by CAT in the peroxisomes while APX will scavenge H_2O_2 molecules in the chloroplasts with the aid of non-enzymatic antioxidant, ascorbic acid, as a reducing agent (Das & Roychoudhury, 2014). Salinity stress have been found to mostly activate APX, CAT and SOD in plants (Kharusi et al., 2019). Moderate to severe increments in soil salinity gradually increased the activities of CAT and POX in the leaves of *S. bicolor* (Desoky et al., 2018). Similar results were observed in *Allium cepa* with the addition to increased activities of SOD, APX and GR (Rady et al., 2018). A significant increase was observed in the activities of APX, SOD, CAT and POX when *Cicer arietinum* was exposed to 150 mM NaCl conditions (El-Esawi et al., 2019). Rakgotho et al. (2022) further demonstrated the importance of SOD in scavenging reactive oxygen species by observing greater activity of SOD as compared to the induced activities of CAT and APX in response to salt stress in *S. bicolor*. Contrary to this,

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Hurtado et al. (2020) found CAT and APX activities to slightly decrease when *Helianthus annuus* and *S. bicolor* is exposed to 100 mM NaCl after 30 days.

Gene expression of *Oryza sativa SOD*, *OsCAT*, *OsAPX* and *OsGR* isoforms were rapidly induced in *Oryza sativa* under salt stress (Rossatto et al., 2017). Similar expression profiles of the above-mentioned genes was observed with the addition of increased *BjMDHAR* and *BjDHAR* levels upon exposure to salt stress in *Brassica juncea* L. seedlings (Kaur et al., 2018). Increasing levels in the expression of *RoAPX* and *RoSOD* were also observed in *Rosmarinus officinallis* L. under increasing saline conditions (El-Esawi et al., 2017). Exposure of *Cucumis sativus* L to salinity stress significantly upregulated the expression of *CsAPX*. However, did not affect the expression levels of *CsGR* under similar conditions (Zhang et al., 2019). Furthermore, Mulaudzi et al. (2020) emphasised the protective roles of ascorbate peroxidase and catalase in the antioxidant system by observing a more than 10-fold upregulation in the gene expression levels of *SbAPX2* and *SbCAT3* in *S. bicolor* upon exposure to harsh levels of salt stress

1.3.2.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants includes the low molecular weight phenols, free proline, ascorbic acid, flavonoids and glutathione (Kharusi et al., 2019). Their synthesis is induced under harsh conditions as a means to aid in the detoxification of ROS molecules in plants. Drought stress elevated the levels of flavonoids, polyphenols and ascorbic acid in *Amaranthus tricolor* (Sarker & Oba, 2018). Salt stress significantly induced the oxidized glutathione (GSSG), reduced glutathione (GSH), dehydroascorbate (DHA) and ascorbic acid (AsA) content in *Cucumis sativus* L., which reduced, and therefore, negatively impacted the GSH/GSSG and AsA/DHA ratios which play an integral part in plant stress stimuli response (Zhang et al., 2019). Salinity

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stress also induced the levels of both AsA and GSH in *S. bicolor*, *A. cepa* and *C. arietinum* (Desoky et al., 2018; Rady et al., 2018; El-Esawi et al., 2019).

1.3.3 Plant Metabolites

1.3.3.1 Cyanogenic glucosides (dhurrin)

Cyanogenic glucosides are naturally produced plant toxins found in several plant species. These include amygdalin, taxiphyllin, linamarin, prunasin and dhurrin (Vetter, 2000), which have been found to make up 30% of sorghum's shoot tip dry mass depending on ontogeny (Halkier & Møller, 1989; Busk & Møller, 2002). Dhurrin is the cyanogenic glycoside produced in all vegetative tissues of sorghum plants and generally serves as response to herbivory attack. Dhurrin is synthesised from tyrosine when P450 cytochrome enzyme CYP79A1 acts as a catalyst in the formation of Z-*p*-hydroxyphenylacetaldehyde oxime. Subsequently, Z-*p*-hydroxyphenylacetaldehyde oxime gets converted to *p*-hydroxymandelonitrile by enzyme CYP71E1 followed by the conversion of *p*-hydroxy mandelonitrile to dhurrin by the glycosyltransferase UGT85BI (Figure 1.1) (Busk & Møller, 2002).

Hydrolysis of dhurrin by dhurrinase, activated by maceration and digestion releases the respiratory toxin, hydrogen cyanide (HCN), which, through acute intoxication, may ultimately damage important tissues from the central nervous system (O'Donnell et al., 2013). Dhurrin content is most prevalent at the start of germination in sorghum, and have been found to be directly correlated to the activities of both CYP7941 and CYP71E1 biosynthetic enzymes (Busk & Møller, 2002). This bioactive metabolite is induced with the exogenous application of nitrogen and have been found to play a role in the transport and storing of reduced nitrogen, and also act as osmolytes (Busk & Møller, 2002; O'Donnell et al., 2013; Nielsen et al., 2016).

Oxidative stress has proven to elevate the production of the cyanogenic glucoside, dhurrin. Forage sorghum timeously subjected to 20% PEG had significant increments of dhurrin levels

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in its shoots than its roots (O'Donnell et al., 2013). Salinity stress have been found to upregulate cyanogenesis in *Trifolium repens*, indicated by increased levels of cyanide found upon exposure to 0.021 mM to 0.048 mM NaCl (Ballhorn & Elias, 2014). The level of cyanogenesis is age and have therefore been found in lower levels in young *Manihot esculenta* upon exposure to high salt levels, while increasing in response to moderate saline levels (Gleadow et al., 2016). This finding was later emulated when exposure of younger (pre-flowering) *S. bicolor* to drought conditions significantly decreased leaf dhurrin, while older (post-flowering) had significantly increased dhurrin levels (Emendack et al., 2018). Although exposure to high cyanogenic glucoside levels can be lethal to animals and humans, its presence have been found to play a significant role in mitigating oxidative stress in plants (O'Donnell et al., 2013).

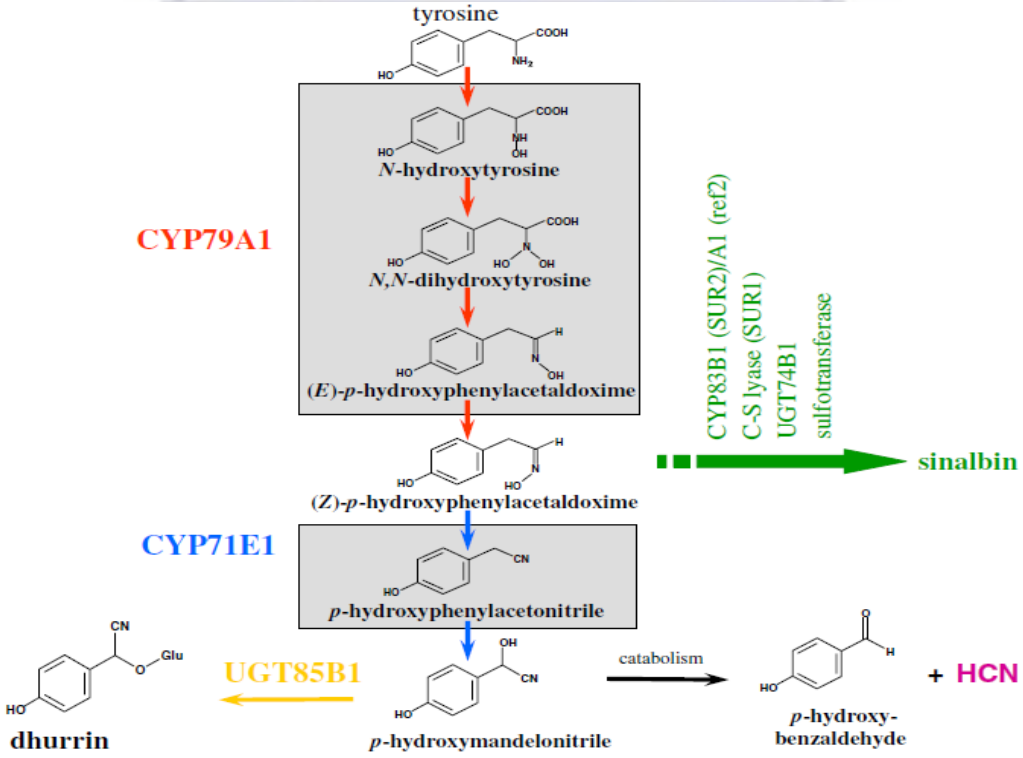

Figure 1.1: The biosynthetic pathway of dhurrin in sorghum. Tyrosine conversion to aglycone p-hydroxymandelonitrile is efficiently channelled by intermediate (gray) catalysed by membrane bound cytochromes P450 CYP79A1 and CYP71E1 encoded by single structural gene. The L-tyrosine is converted to z-p-hydroxyphenylacetaldoxime and subsequently to the labile cyanohydrin p-hydroxymandelonitrile followed by the conversion to dhurrin by CYP79A1, CYP71E1 and UGT85B1 respectively. The hydroxymandelonitrile commonly dissociates to form p-hydroxybenzaldehyde and hydrogen cyanide of which the overproduction is regulated by the glucosylation of cyanohydrin before dissociation. [Adapted from (Nielsen et al., 2008)].

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1.3.4 Osmoprotectants

Osmolytes are miniature neutrally charged organic compounds that serve an efficient role in the maintenance of osmotic pressure and in the stabilization of membranes and proteins under adverse conditions. Proline and glycine betaine are two of the extensively studied osmoprotectants worldwide. Proline may serve as a signalling molecule, antioxidant and metal chelator. This osmolyte may also aid in the modulation of mitochondrial functions and regulate cell proliferation or apoptosis, which are pivotal to plant stress recovery (Szabados & Savouré, 2010). It is induced within plants upon exposure to saline conditions in attempts to protect plants from oxidative injury caused by accumulating ROS (Iqbal & Nazar, 2015). Proline content levels increased when *S. bicolor* was exposed to 100 mM NaCl. However, a gradual decrease in the proline levels was observed in said plant upon longer exposure (after 2 days) to 100 mM NaCl (Reddy et al., 2015). Higher proline levels were associated with enhanced salinity stress tolerance as observed in transgenic *S. bicolor* cultivars, which had ameliorated growth and development under all subjected salt treatments (0 to 4 days of exposure to 100 mM NaCl) (Reddy et al., 2015). Previous studies supported this when Watanabe et al. (2000) also observed an induction in proline levels when *Populus euphratica* was exposed to 150 mM NaCl and higher, which led to the conclusion that proline is associated to osmotic and salinity stress tolerance.

Glycine betaine is the common form of betaines produced in plants in response to dehydration. It is a carboxylic acid containing quaternary ammonium compound and may function in conjunction with proline at maintaining the membrane structure (Iqbal & Nazar, 2015; Sharma et al., 2019). Glycine betaine scavenge ROS molecules and enhance stress tolerance by stabilizing the important rubisco proteins (Sharma et al., 2019).

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In addition to osmoprotectants, soluble sugars may act signalling molecules and substrates for various cell processes such as starch biosynthesis, secondary metabolite synthesis and cell respiration (Živanović et al., 2020). Soluble sugars play an important role in controlling several processes in diverse plant developmental phases with its primary function as ROS scavenging molecules (Afzal et al., 2021). These carbohydrates may form as a product of photosynthesis which will then act as energy sources for cell respiration (Alves et al., 2019). Soluble sugars are highly sensitive to environmental stress as observed with increased accumulation under drought stress in *Lycopersicon esculentum* (Živanović et al., 2020) and oxidative damage caused by salinity stress in *Spinacia oleracea* (Muchate et al., 2019) and *Triticum aestivum* (Nadeem et al., 2022). Osmoprotectants are vital molecules in plant metabolism and play an important role in plant stress tolerance mechanism. Harsh environmental conditions such as salt stress induces the biosynthesis of these osmoprotectants and can thus be regarded as key molecules in enhancing stress tolerance (Sharma et al., 2019).

1.3.5 Photosynthetic pigments

Photosynthetic pigments such as chlorophyll and carotenoids function as vital molecules in harvesting light energy from the sun to mediate photosynthesis. Fluctuation in their concentration has a significant effect on the metabolism of plants (Gong et al., 2018). Harsh environmental conditions such as salinity generally reduce the photosynthetic efficiency in plants by inhibiting photosynthesis. Salinity stress inhibits photosynthesis by decreasing rubisco activity found in the chloroplast's stroma and degrading photosynthetic pigments (Gong et al., 2018). Carotenoid not only contributes to photosynthesis; it has been described to play a crucial role in the dispersion of surplus light to protect plant metabolism from oxidative damage as well (Shah et al., 2017). Although salt stress amongst other abiotic stress typically

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decreases chlorophyll content, its increments due to this abiotic stress can however be assigned to plants possessing naturally higher tolerance to salinity stress (Shah et al., 2017).

1.4 Plant signalling molecules

Plants constantly have to respond and adapt to their surroundings. This is achieved by activating inducible defence mechanisms to aid in plant survival under adverse conditions. At the forefront of these responses are signalling molecules proven to be essential for plant growth and development. Such molecules include various chemicals, hormones, proteins, amino acids, nucleotides, polyamines and gases in particular nitric oxide (NO) and carbon monoxide (CO), among others (Tuteja & Sopory, 2008). Cell membrane receptors recognises stress stimuli, which transduce the information downstream triggering various genes, which codes for proteins that will ultimately promote stress tolerance and adaptation (Tuteja & Sopory, 2008). A plant's defence mechanism is derived from an intricate network and often requires the aid of many signalling molecules working in conjunction. Therefore, there persists an underlying crosstalk between phytohormones and signalling molecules and many other smaller gas-like signalling molecules (Wang & Liao, 2016).

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1.4.1 Phytohormones

Phytohormones plays an important role in regulating various processes within plant metabolism. They are produced in trace amounts and work as chemical messengers communicating critical biological processes to trigger signalling pathways and hence aid in the modulation of physiological and molecular reactions in response to abiotic stresses (Wani et al., 2016). A considerable volume of literature is highlighting the importance of phytohormone, abscisic acid (ABA) in the growth and development of plants under favourable and unfavourable conditions. However, jasmonates are other phytohormones attracting interest

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from researchers and have proven to enhance plant tolerance against both abiotic and biotic stress (Hassini et al., 2017; Singh et al., 2019).

1.4.1. 1 Jasmonates

Jasmonates are produced as a product of fatty acid metabolism, which primarily produce jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA) (Ahmad et al., 2016; Wani et al., 2016). The evolutionary origin of jasmonates is analogous to that of eicosanoids known to be vital in inflammatory and wound responses in mammals, which lead to the adoption of jasmonates as key signal transducers of tissue damage across different kingdoms (Koo, 2018). These compounds have been proven to play a crucial role in biological processes such as flowering, senescence, fruiting, secondary metabolism and defence responses (Wani et al., 2016). The best characterised derivative, JA, is highly induced in response to injury caused by pathogens and environmental stimuli such as low temperatures, water deficiency and salinity stress (Wani et al., 2016).

1.4.1.2 Biosynthesis of jasmonates

Chloroplasts are one of the most important organelles within plant cells and the main site for jasmonate biosynthesis (Figure 1.2). Its membranes contain galactolipids catalysed by phospholipases to produce an important amino acid such as α-linolenic acid (α-LeA) (Ahmad et al., 2016). The 13-lipoxygenase (LOX) stimulate the oxidation of α-LeA to form 13-hydroperoxy-9, 11, 15-octadecatrienoic acid (13-HPOT). Another oxygen molecule is then added by allene oxide synthase (AOS) to produce allene oxide a precursor for the synthesis of a more stable 12-oxo-phytodienoic acid (12-OPDA) as catalysed by Allene oxide cyclase (AOC). These reactions take place within the chloroplast whereby the enzymes, AOS and AOC will function in union within the plastids. The 12-OPDA will then be converted to JA in the peroxisomes with the aid of three cycles of β-oxidation (Ahmad et al., 2016). Jasmonic acid is therefore transferred to the cytoplasm, where it will be metabolised to form various chemical

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compounds including 12-hydroxyjasmonic acid (12-OH-JA), *cis*-jasmone (CJ), its isoleucine conjugate (JA-Ile), and most importantly, methyl jasmonate (MeJA), which is formed through methylation catalysed by JA methyltransferase (Ahmad et al., 2016; Ruan et al., 2019).

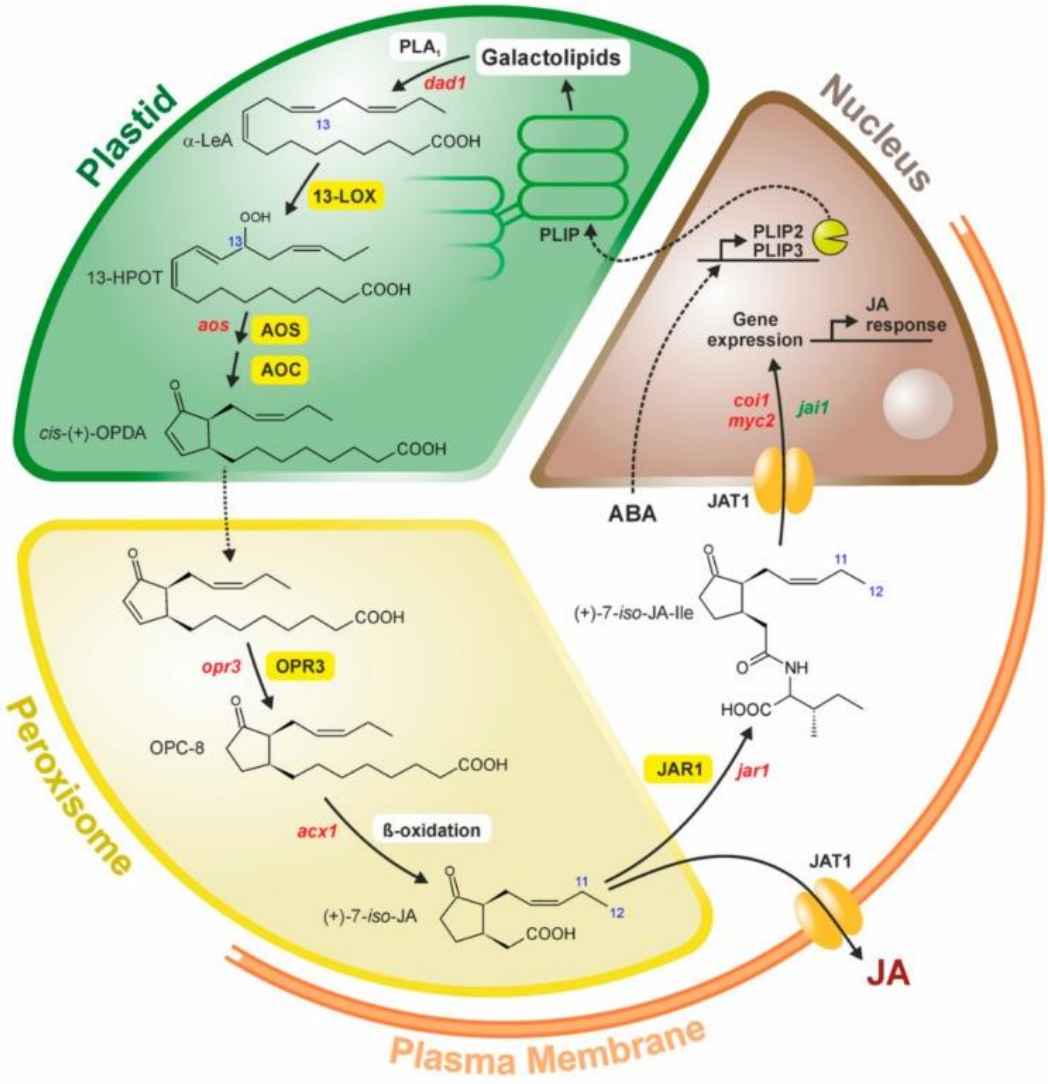

Figure 1.2: Endogenous biosynthesis of jasmonic acid: Biosynthesis involves various cell compartments. Linolenic acid (α-lea) released from lipases (PLA) is subsequently converted to OPDA in the plastid by enzymes LOX, AOS, AOC, respectively. OPDA is reduced by OPR3 to OPC in the peroxisomes where the initial configuration of JA ((+)-7-iso-JA) is formed. In the cytosol is where JA will be metabolised to form 12-OH-JA, CJ, JA-Ile, and most importantly, MeJA. JA-Ile can be transported back to the nucleus and induce gene expression [Adapted from: (Wasternack & Strnad, 2018)].

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1.4.1.3 Roles of jasmonic acid (JA) and Methyl jasmonate (MeJA) in plant growth and development

Jasmonates (JAs), including jasmonic acid and its derivative Methyl jasmonate, are important signalling hormones that have a role in the modulation of various plant physiological processes. They have been found to be involved in the accumulation of anthocyanin, trichome initiation, fertility, aging, pathogen and environmental stress response (Hu et al., 2017). Exogenously applied JAs have been proven to dose- and species dependently enhance plant stress tolerance (Ahmad et al., 2016). These hormones have been found to break seed dormancy, enhance storage proteins and upregulate important antioxidant enzymes. Transcriptional factors modulated by JA stimulated secondary metabolism in *Arabidopsis thaliana* (De Geyter et al., 2012). Additionally, exogenously applied MeJA enhanced the allelopathic potential, and subsequently increased phenolics and ROS scavenging enzyme activity in *Oryza sativa* L. (Bi et al., 2007).

1.4.1.4 Role of MeJA under stress stimuli

Overexpression of the jasmonic acid regulating cytochrome P450 monooxygenase, GmCYP82A3 from *Glycine max* L., enhanced pathogen defence and salinity stress tolerance in transgenic *Nicotiana benthamiana* (Yan et al., 2016). Exogenously applied MeJA have a role in improving plant stress tolerance. The exogenous application of MeJA on linolenic acid deficient mutant Arabidopsis plants lead to a substantial improvement in the survival and resistance to root pests such as *Bradysia impatiens* in Arabidopsis (McConn et al., 1997). In *Zea mays*, it enhanced activities of ascorbic acid and glyoxalases I and II, thus improving the redox status whilst counteracting increments in glutathione and proline levels in response to extreme alkaline salts (Ahmad et al., 2016; Mir et al., 2018). JAs may interact with various signalling molecules to improve plant stress response. It has thus been found that the exogenous application of JA and NO, individually or in combination ameliorated salt stress damage by

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inducing increments in metabolites, synthesis in osmolytes and antioxidant metabolism in *Solanum lycopersicum* L. (Ahmad et al., 2018).

1.4.1.5 Lipoxygenase (LOX) activity

Jasmonic acid and its derivative, MeJA are pivotal transducers of stress stimuli in plants and have particularly been associated with accumulated levels in response to mechanical damage of plants (Yu et al., 2019). Lipoxygenases are typically categorised as 9-LOX and 13-LOX based on the carbon atom binding site of oxygen and are non-heme containing iron dioxygenases (Koo, 2018). While wounding has been found to induce the expression levels of LOX2, chilling stress reduced LOX activity to regulate the fatty acid composition of the membranous lipids of *Eriobotrya japonica* cv L. Fuyang (Cao et al., 2009).

The lipoxygenase family oxidises linolenic, α-linolenic and linoleic acids. The resulting biologically active compound, oxylipins, produced are key regulators of plant development, programmed cell death and stress response (Vellosillo et al., 2007; Garcia-Marcos et al., 2013; Savchenko et al., 2014). The release of polyene fatty acid in the chloroplast is observed to be directly proportional to the activity of LOX (Babenko et al., 2017).

Elevated LOX activity enhanced pathogen resistance in *Oryza sativa* cv. Aichiasahi (Ohta et al., 1991). This activity is typically induced by the exogenous application of MeJA as observed in *Hordeum vulgare* cv. Salome (Feussner et al., 1995). Although LOX activity increased when *Oryza sativa* L. was stressed with cadmium, its activity decreased in response to treatment with MeJA, which is suggested to be as a result of reformed translation of the LOX gene in *Oryza sativa* L. (Singh & Shah, 2014). A significant increase in LOX activity in addition to increased MDA levels was observed when *Gossypium hirsutum* L. and *Zea mays* L. were subjected to salt stress (Zhang et al., 2013; Rohman et al., 2019). The increased lipid peroxidation in response to saline environments indicates a key role of LOX in the regulation of oxidative

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damage (Zhang et al., 2013). Mutant *Capsicum annuum* overexpressing *CaLOX1* elicited improved tolerance to high salinity stress. However, this was accompanied by lower levels of ROS and fatty acid peroxidation as compared to the wild-type plants (Lim et al., 2015).

1.4.2 Carbon monoxide

Carbon monoxide (CO) has been extensively known as a pollutant capable of causing acute or chronic health hazards to humans experienced when produced excessively (Wu & Wang, 2005). In animals CO molecules have a higher affinity to haemoglobin (HbA) than oxygen molecules, which will therefore, upon entry into blood, starve the HbA of its O_2, causing hypoxia leading to severe cardiovascular and neurological injury (Wu & Wang, 2005). Despite its detrimental effects on animals, previous studies have proven its importance to both animals and plants under physiological amounts (Xuan et al., 2007). In animals, CO is involved in platelet aggregation, vasodilation and neurotransmission (Wang & Liao, 2016). Although its role have not yet been fully elucidated in plants, it is emerging as a pivotal signalling molecule involved in various bio-processes such as delaying gibberellin induced programmed cell death by the upregulation in the transcript levels and activities of APX and CAT (Wu et al., 2011). The endogenous generation of CO within animals and plants are most prevalently produced enzymatically, but have been found to be produced non-enzymatically as well (Wu & Wang, 2005).

1.4.2.1 Enzymatic biosynthesis of CO

Carbon monoxide (CO) is endogenously produced as a by-product through the stereospecific heme cleavage and degradation by heme oxygenase. Heme plays an important role as prosthetic group of electron transport chain proteins, cytochrome *b*558, cytochrome *b*5, cytochrome *c,* cytochrome *c* oxidase, and cytochrome *c* reductase (Bose et al., 2013). The plant's transcriptome contains genes encoding four isoforms of the HO enzyme, namely, HO1, HO2, HO3 and HO4, which are divided into two sub-families. The HO1 sub-family, consisting of

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isoforms HO1, HO3 and HO4, which readily degrades heme to form BV, whereas HO2 isoform is the only member of the HO2 sub-family and do not possess the heme degradation activity (Shekhawat & Verma, 2010). Heme degradation is catalysed by the robust heme oxygenase (HO) in the presence of oxygen and co-factor NADPH, FNR or Fd to form BV-IXa resulting in the release of by-products, ferrous iron (Fe^{2+}) and CO (Shekhawat & Verma, 2010). Biliverdin-IXa and Fe^{2+}, are then further reduced to form antioxidant products, bilirubin and ferritin, respectively (Bose et al., 2013). Likewise, CO also possess the ability to act as an antioxidant and have been found to play a pivotal role in the regulation of ROS homeostasis. The ubiquitous enzyme, HO, is induced by various stress stimuli and play a key role in enhancing the efficiency of other antioxidants such as APX, CAT and SOD (Bose et al., 2013). Free heme molecules are readily found throughout cells and are most commonly released by the chloroplast or mitochondria, which accumulate in response to stress and are able to induce oxidative damage through the catalysis of the production of free radicals.

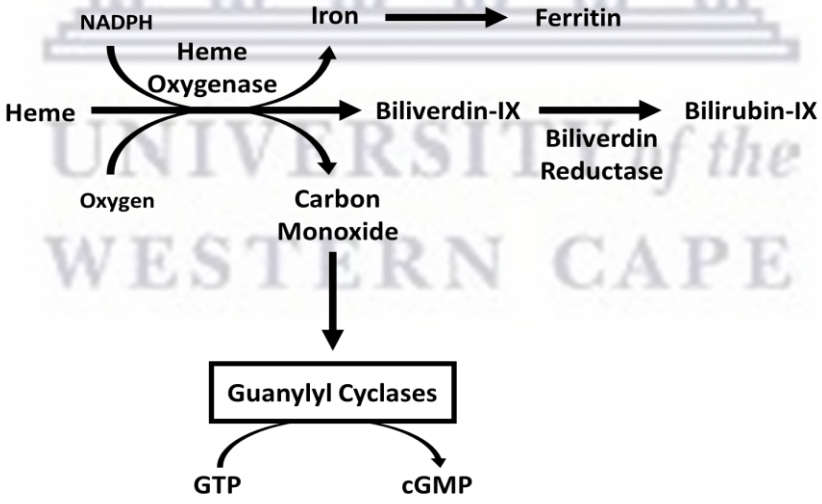

Figure 1.3: Overview of the endogenous enzymatic biosynthesis of carbon monoxide. Heme is metabolised to form the antioxidative BV with Fe and CO as by-products in a reaction driven by HO. The CO proposedly activates important cGMP molecules by binding to its iron atom of the heme moiety. The relevant cGMP content will then play an important role in the regulation of various physiological effects including stomatal conductance.

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1.4.2.2 Non-enzymatic biosynthesis of CO

Relatively low levels of CO are generated non-enzymatically via lipid peroxidation, ureide metabolism and the breakage of methylene bridges (Dulak & Józkowicz, 2003; Zilli et al., 2014). The former is supported by a study, which showed that CO produced through the enzymatic degradation of heme by HO is not the main source of CO production in *Glycine max*, instead primarily make use of lipid peroxidation and ureide metabolism to produce CO (Zilli et al., 2014). Little information is understood about the non-enzymatic synthesis of CO and therefore need further elucidation.

1.4.3 The role of CO in plant growth and development

A great deal of evidence persists in the involvement of CO in the modulation of important biological processes including the suppression of tumour growths on smooth muscle and inhibition of platelet accumulation, vasodilation and neurotransmission in animals (Wu & Wang, 2005; Liu et al., 2007). CO shows great similarities to nitric oxide (NO) in terms of functionality, and thus, possesses the ability to induce the production of pivotal cGMP by activating soluble guanylyl cyclase through binding to its iron atom (Figure 3) (K. Liu et al., 2007). Studies suggest that CO also play a vital role in plant growth and development. CO have been proven to be dose-dependently beneficial to the germination of *Setaria faberi*, *Oryza sativa* and *Brassica nigra* (Dekker & Hargrove, 2002; Liu et al., 2007; Amooaghaie et al., 2015). It has been found to regulate root development by enhancing elongation in *Triticum aestivum* L., promoting root hair density in *Solanum lycopersicum* and promoting adventitious rooting formation in *Cucumis Sativus* L. (Xuan et al., 2007; Guo et al., 2009; Chen et al., 2017). CO has also been found to positively regulate stomatal conductance in *Vicia faba* when exogenously applied (Cao et al., 2007).

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1.4.3.1 The role of CO under abiotic stress

The endogenous production of CO within plants is increased upon exposure to salinity stress and may, thus, play a pivotal role in enhancing plant tolerance to salinity stress (He & He, 2014). Studies suggests that CO plays a significant role in promoting seed germination due to its alleviating effect on salinity induced seed germination inhibition in *Triticum aestivum* L., *Oryza sativa*, and *Cassia obtusifolia* L. (Xu et al., 2006; Liu et al., 2007; Zhang et al., 2012). Exogenously applied CO attenuated the salinity-induced inhibition of seedlings growth of *T. aestivum*, *O. sativa* and *C. obtusifolia* by enhancing their photosynthetic efficiency through increasing chlorophyll content, increasing soluble sugars and by increasing the activities of antioxidants SOD and CAT, in addition to further counteracting oxidative damage by decreasing lipid peroxidation (Xu et al., 2006; Liu et al., 2007; Zhang et al., 2012). Exogenously applied CO was able to ameliorate Cd-induced oxidative injury in *Medicago sativa* and restore iron homeostasis in *Arabidopsis* and *Chlamydomonas reinhardtii* (Han et al., 2008; Kong et al., 2010).

1.4.3.2 CO's role in gene expression

The HO is induced upon the exposure to various stimuli including CO, ABA, ROS, iron deprivation, UV radiation and light (Shekhawat & Verma, 2010). These stimuli could therefore increase CO endogenously produced through the enzymatic degradation of heme within plants (Zhang et al., 2012). Osmotic stress significantly elevated the transcript levels in *S. bicolor*, suggesting the importance of HO in alleviating stress in plants (Mulaudzi-Masuku et al., 2019).

1.4.4 Crosstalk between MeJA and CO

Studies have shown that both MeJA and CO have positive effects on plant growth and development and may act as crucial signalling molecules. However, their relationship with one another is yet to be fully elucidated. Crosstalk between these signalling molecules ascended

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due to their induced concentrations, in addition to increased nicotine biosynthesis found in response to increased temperatures on *Nicotiana tabacum*. Furthermore, restriction of endogenous CO production in tobacco through the inhibition of HO activity decreased the levels of JA found in the plant (Cheng et al., 2018). This occurrence was supported in a previous study conducted where exogenously applied JA enhanced the activity and protein expression of HO, indicating that HO and JA might work in conjunction to ameliorate oxidative damages caused by Cd in *Glycine max* roots (Noriega et al., 2012).

1.4.4.1 Transcription level

Jasmonic acid exerts its effects due to the suppression of the JASMONATE ZIM-DOMAIN (JAZ) through the actions of the 26S proteasome. The JA molecule forms an E3-ubiquitin ligase adduct by binding to the F-box containing protein COI1, which inhibits JAZ from binding to the operator, thus allowing for the activation of plant defence through the emancipation of the MYC2 transcription factors (Cheng et al., 2018). The MYC2 transcription factor have been found to play an important role in the biosynthesis of nicotine within tobacco plants. The NtMYC2 of *Nicotiana tabacum* upregulates the enzyme, putrescine N-methyltransferase (NtPMT1), known to be a key catalyst in the biosynthesis of nicotine. The heme oxygenase 1 (HY1) in *Arabidopsis thaliana* have been deduced to play an important role in abiotic stimuli and hormonal signal transduction, and these gene is upregulated by salinity stress. Myeloblastosis (MYB) binding sites in the HY1 promoter have been concluded to be key to the upregulation of this enzyme when the *myb* mutants, lost its ability to induce the HY1 expression under saline conditions (Wang et al., 2016). The exogenously applied CO upregulated the expression levels of the JA-related transcription factors, and subsequently JA, in addition to inducing the biosynthesis of nicotine by acting as a signal and promoting the binding of NtMYC2 to the NtPMT1 promoter region (Cheng et al., 2018).

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1.5 The choice of the plant species: *Sorghum bicolor*

Sorghum is a sought-after cereal crop in Africa due to its relatively higher drought tolerative characteristics, digestibility potential and rich nutritional value (Zhao & Ambrose, 2017; Kaplan et al., 2020). The high levels of tannins and phenolic compounds found especially in the red and brown grain variety are highly beneficial to humans against diabetes, cardiovascular and hypertensive diseases (de Morais Cardoso et al., 2017). Sorghum acts as a staple food to many globally and has many uses such as a feedstock, human feed and in the production of biofuels (Shen et al., 2018). Therefore, its development will not only strengthen food security, it will also be highly beneficial for human health due to its high photochemical, dietary fibres and minerals (Kaplan et al., 2020). The sorghum genome comprises of a relatively miniscule 730 million base pairs, which makes it a vital model organism for the comparative genomic studies of other C4 crops (Mullet et al., 2002). Its perennial characteristic in conjunction with its stalk reserve retention and tiller-forming capabilities are sought after characteristics in cellulosic biomass crops. Additionally, sorghum's high inbreeding level and gene flow are highly attractive characteristics in genetic systems (Paterson et al., 2009)

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1.6 CONCLUSION

Countless attempts are being made at the establishment of food security worldwide. Increasing literature is aiding in achieving this objective. However, the inadequate food production rate due to the changing climate, declining soil fertility and reduced crop productivity, in addition to the increasing population are obscuring the future prospect of food security. Sorghum act as a staple food to many individuals especially in Asian and African countries. Therefore, it is of great importance upholding crop production levels under the harsh conditions of our country and the world. Streamlining attention to enhancing the growth and productivity of sorghum with limited resources could prove pivotal in ameliorating the strain on food security.

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Increasing salinization is diminishing the growth and productivity of food crops worldwide. Plants responds to stress by activating an array of defence mechanism including the regulation of ROS, antioxidants, metabolites, osmoprotectants, photosynthetic pigments and signalling molecules such as MeJA and CO. With the interconnected nature in response of various plants to abiotic stress, establishing techniques to enhance salinity stress tolerance in sorghum can possibly establish cross tolerance to multiple stressors of different plant species. Fundamental to establishing transgenic plants with enhanced stress tolerance is understanding the molecular mechanism involved. Signalling molecules are at the forefront of plant defence, said molecules include MeJA and CO, which are emerging as key molecules to exploit for the establishment of transgenic lines with improved stress tolerance. Although the phytohormone, MeJA is typically associated with plant tissue damage and biotic stress, it has been found to enhance tolerance to salinity stress in various plant species. Additionally, endogenously produced and exogenously applied CO proved to be vital in plant growth and development under physiological and harsh conditions including salinity stress. However, the mechanism at which both molecules induce stress tolerance needs further elucidation. Based on the literature cited, there is an underlying crosslink between these molecules possibly through the regulation of important MYB sites in response to stress stimuli. Little information is understood about plant response to exogenous MeJA and CO as combinatory treatment and further studies taking this into account needs to be undertaken. The novel information gained from this study will aid in elucidating the aforementioned defence mechanism and so doing aid in establishing food crops with enhanced stress tolerance. This will increase food productivity and allow researchers to meet the growing food demand of the world.

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1.7 Problem Statement

The unpredictable climate in addition to the exponential growth in the world population are putting severe strain on the entire agricultural sector and therefore, continues to pose great threat to its sustainability. Global warming is intensifying the impact of abiotic stressors, which aggravates the declining productivity, quantity and quality of food crops. Salt stress is one of the most detrimental abiotic stresses and its negative impact is being accentuated through agricultural malpractice, erosion of large areas of land, mineral weathering, the rising sea level and the intrusion of water from the sea to arable land (Chele et al., 2021). Hence, there remain a desperate need to improve crop growth and development under unfavourable conditions such as salinity. Although countless attempts are being made by research to achieve this prospect, the complex mechanism regulating salinity stress tolerance requires in depth knowledge and comprehensive understanding which are imperative in establishing improved crop productivity. Salinity has a major impact on plants from seed germination to plant development. Therefore, elucidating the stress mechanism involved in governing salt stress should be holistically approached throughout all plant life cycles. Phytohormones in addition to many other signalling molecules are crucial in plant metabolism throughout all stages of plant growth and are known to be induced upon stress stimuli. The accumulation of phytohormone, methyl jasmonate, and signalling molecule, carbon monoxide, in response to stress can be considered as a major mechanism, which requires extensive research. Elucidating their roles in conferring salt stress tolerance to *S. bicolor* and how these mechanisms persist in cohesion with one another would be crucial in the establishment of other cereal crops with improved tolerance to salt stress.

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1.8 Aims & Objectives

This study was aimed at understanding the effect of salt stress on the growth and development of *S. bicolor* during its germination and vegetative stage of growth, and investigating the role of MeJA and hematin (CO-donor) in conferring salt stress tolerance to sorghum. These aims were achieved through the following objectives:

- To germinate and grow *S. bicolor* in the presence and absence of MeJA and hematin (CO-donor), singly and in combination, under control and salinity stress.
- To conduct germination and physiological assays including growth attributes including root/shoot length and biomass measurements.
- To conduct biochemical assays including osmolyte accumulation, lipid peroxidation, ROS accumulation and photosynthetic pigments measurements.
- To investigate alteration in anatomical attributes using Scanning Electron Microscopy
- To investigate alteration in the metabolic profile using Fourier Transform Infrared spectroscopy
- To conduct Real time quantitative polymerase chain reaction gene quantification of jasmonate related genes (*SbLOX1, SbAOS, SbAOC, SbOPDR*).

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1.9 Significance of the study

The substantial data from this study would be important in further elucidating the mechanisms involved in salt stress tolerance in sorghum plants which would prove fundamental in establishing crops with improved tolerance to salt stress. Given the plants related responses to stress, conferring salt stress tolerance will most likely improve tolerance to all other environmental stresses. The role of MeJA and CO in conferring salinity stress tolerance

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remains elusive. Hence, this study availed newly refined and innovative information that could contribute to the expanding research which would ultimately allow for the generation of transgenic food crops with improved tolerance and better sustainability. The improved crop productivity would not only alleviate world-hunger but rather transforms South-Africa's agricultural sector by increasing its contribution and further boosting the economy.

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CHAPTER 2

MATERIALS AND METHODS

2.1 Plant material

Sorghum (*Sorghum bicolor* L. Moench) seeds were attentively selected for this study. These seeds were purchased from Agricol Brackenfell, Cape Town, South Africa.

2.2 Seed preparation

The *S. bicolor* seeds were surface decontaminated as previously described by Mulaudzi-Masuku et al. (2015). Seeds were washed with 70% ethanol while shaking for 1 minute before the ethanol solution was discarded. Seeds were thoroughly rinsed three times with autoclaved double distilled water (ddH_2O). Partially cleaned seeds were then soaked in 5% sodium hypochlorite solution (NaOCl) for 1 hour while shaking at 600 rpm. The NaOCl solution was removed, and the seeds were thoroughly rinsed three times with autoclaved ddH_2O. After surface decontamination seeds were left to imbibe by soaking in autoclaved ddH_2O overnight at 25℃ while shaking in the dark.

2.3 Germination and seedling growth

After imbibition, ddH_2O was removed and the decontaminated seeds were placed on sterile paper to dry at room temperature under the laminar flow until their original moisture content was obtained. Germination was induced by incubating the dried seeds in sterile water-imbibed

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paper towel for 7 days at 25℃ while being completely concealed from light. Germination of *S. bicolor* were analysed and seedlings cultivated as described in Section 2.3.1 and 2.3.2.

2.3.1 Germination parameters

Germination assays was performed by placing five pre-soaked [Methyl jasmonate at different concentrations of 0 µM (control), 10 µM, 15 µM and 20 µM MeJA (experiment)] seeds in petri dishes containing paper towel imbibed with 4 ml of treating solutions [Non-saline and saline solution supplemented with 0 µM (control), 1 µM and 1.5 µM hematin (experiment)]. Seeds were grown in the sterile petri dishes for 7-days at 25℃ concealed from light and seeds with a protruded radicle of >2 mm was considered to have germinated. Seedlings were carefully removed from the petri dishes followed by diligent measurement of root and shoot lengths on day 7. The germination was analysed by conducting several germination assays as described:

• Germination percentage (%) = n/N ×100. *n* represents the number of seeds that have germinated on the day and the *N* represents the total number of seeds sowed (Kader, 2005).

• Total germination (TG) (%) = ($total\ number\ of\ seeds\ that\ germinated/total\ seeds$)×100. This parameter was determined using the number of seeds that germinated on the last day of incubation (day 7) (Kader, 2005).

• Germination index (GI) = Σ($TiNi$). *Ni* represents the number of seeds germinated on the day and *Ti* represent the number of days after sowing. The maximum amount (day 7) is given to the seeds germinated on the 1st day and the minimum amount (day 1) is given to the seeds germinated on the 7th day (Kader, 2005)

• Mean germination time (MGT) = Σ($nidi$)/N. *ni* represents the number of seeds germinated. The *di* represents the day the germinated seeds were counted, and N = total number of seed germinated at the termination of the experiment (Kulkarni et al., 2007).

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2.3.2 Growth conditions

After 7 days of incubation, seedlings were transferred to seedling trays containing 35 g potting soil and vermiculite (2:1). The initial roots were firmly placed in the soil mix while the coleoptiles were directed upwards from the soil. Plantlets were irrigated with 25 ml Dr Fisher's Multifeed Classic 19:8:16 (43) (Gouws and Scheepers Pty, Ltd) every alternate day (3 times per week) until the plantlets reached their 3-leaf stage of development. The plantlets were grown under summer conditions where temperatures ranged between 26℃/22℃ (Day/night). Plantlets were harvested when the 5-leaf stage of development was reached. During harvesting the shoots were carefully separated and stored at -80℃ until required for assays. Immediately after separation, plant material was weighed to determine its fresh weight followed by drying in an oven at 80℃ for 24 hours to determine its dry weight.

2.4 Treatment application

After reaching the 3-leaf stage of growth in the soil, plantlets were stressed with varied concentrations of NaCl (0 mM, 100 mM, 200 mM). The plantlets were stressed until reaching the 5-leaf stage of development after 7 days. During imbibition (as described in section 2.2), seeds were pre-treated by priming them with different concentrations of MeJA in 50 ml solutions containing 0 µM (control), 10 µM, 15 µM and 20 µM MeJA concentrations overnight at 25℃ while shaking in total darkness. For the germination experiments, sorghum seeds were grown in various solutions prepared in double distilled H_2O_2. These included sodium chloride concentrations: 0 mM and 200 mM; MeJA concentrations of 0 µM, 10 µM, 15 µM and 20 µM; hematin (CO donor) concentrations of 1 µM and 1.5 µM. In addition to a combinatory treatment of both MeJA and CO under non-saline (0 mM NaCl) and saline (200 mM NaCl) conditions.

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2.5 Oxidative stress parameters

2.5.1 Histochemical detection of H_2O_2

Hydrogen peroxide localisation was determined in the leaves as previously described by Rahman et al. (2016). Briefly, leaves were submerged in 1 $mg.ml^{-1}$ 3',3' diaminobenzidine (DAB) at a pH 3.8 for 12 hours at 25℃ concealed from light. Following incubation, chlorophyll was extracted by boiling in 90% ethanol for 15 minutes for clear identification of formazan caused by the oxidation of DAB by H_2O_2.

2.5.2 Lipid peroxidation

Malondialdehyde (MDA) accumulation was selected as a biomarker of lipid peroxidation in this study. This was spectrophotometrically estimated as previously determined with slight modifications (Heath & Packer, 1968). Macerated plant material (0.1 g) was homogenized with 0.1% TCA (1 ml) and centrifuged for 10 minutes at 13 000 rpm (4℃). About 1 ml of 0.5% TBA prepared in 20% TCA was added to 0.4 ml of supernatant after centrifugation. To avoid pressure build-up, caps of the Eppendorf tubes were perforated before being incubated in a hot water bath set at 80℃ for 30 minutes. The reaction was terminated by immediately placing the samples on ice after incubation, followed by further centrifugation for 5 minutes at 13 500 rpm (4℃). Absorbance readings were measured at 532 nm and 600 nm with the Helios® Epsilon visible 8 nm bandwidth spectrophotometer (Thermo Fisher Scientific, USA). MDA content was further calculated using the following formula (Formula 5):

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

Where: $\Delta A_{corrected}$= A532 – A600 corrected with $\Delta A_{corrected}$ of the blank, b = light path length (0.56 cm for 200 µl), ε = millimolar extinction coefficient (155 mM^{-1}), 3.5 (dilution

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factor from 400 µl extract + 1 ml TBA/TCA solution), *x* (ml) = 0.1% TCA used from extraction (1 ml), y (g) = fresh weight (FW) used for extraction and 1000 as conversion factor.

2.6 Photosynthetic pigment

2.6.1 Chlorophyll content

Chlorophyll content were determined based on previously described methods (Mackinney, 1941; Lichtenthaler & Wellburn, 1983). Briefly, 0.1 g of ground plant material was homogenised in 10 ml of 80% acetone. The homogenates were thoroughly vortexed and centrifuged at 10 000 rpm for 10 minutes. About 1 ml was aliquoted into a glass cuvette and the pigment absorption was spectrophotometrically measured at 645 nm and 663 nm (Mackinney, 1941) using the Helios® Epsilon visible 8 nm bandwidth spectrophotometer (Thermo Fisher Scientific, USA). Chlorophyll content was calculated according to Arnon (1949) as follow:

- Chlorophyll *a* = 12.7 (A_{663}) – 2.69 (A_{645})
- Chlorophyll *b* = 22.9 (A_{645}) – 4.68 (A_{663})
- Total Chlorophyll = 20.2 (A_{645}) + 8.02 (A_{663})

2.7 Osmolyte accumulation

2.7.1 Proline content

Proline levels were measured as previously described with slight modifications (Carillo & Gibon, 2011). Macerated plant material (0.1 g) was homogenised with 3% sulfosalicylic acid (0.5 ml), vigorously mixed and centrifuged for 20 minutes at 13 000 rpm. The supernatant (0.3

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ml) was transferred to a reaction mixture containing 99% glacial acetic acid and acidic ninhydrin solution (2.5% ninhydrin, 60% acetic acid and 20% ethanol). The reaction was induced by heating the samples in a hot water bath set at 95℃ for 20 minutes and arrested by placing it on ice, followed by further centrifugation at 13 000 rpm. The chromophore (200 µl) was transferred to a 96-well microplate and its absorbance was measured at 520 nm using the FLUOstar® Omega microtiter plate reader (BMG LABTECH, Germany). Proline concentration was determined according to a calibration curve containing known proline concentrations expressed as µmol g^{-1} FW

2.7.2 Total soluble sugars

Total soluble sugar content was determined as previously described in the anthrone method with some modifications (Watanabe et al., 2000). Ground plant material (0.1 g) was homogenised with 80% acetone and centrifuged for 10 minutes at 10 000 rpm. About 1 ml of the supernatant was transferred to a 0.2% anthrone solution (0.2 g anthrone dissolved in 96% sulfuric acid) and placed in a hot water bath for 15 minutes at 80℃. The reaction was terminated by placing the samples on ice. Thereafter, 1 ml of sample was aliquoted to a glass cuvette and its absorbance was measured at 625 nm. Levels of total soluble sugars were quantified using a calibration curve containing known glucose concentrations and expressed as mg·g^{-1} FW.

2.10 Anatomic analysis using scanning electron microscopy

Macerated plant material was oven-dried at 80℃ overnight. The dried plant matter was subjected to high resolution scanning electron microscopy-energy dispersive X-ray spectroscopy (HRSEM-EDX) system. The plant matter was coated with carbon using the EMITECH-K950x carbon coater. Microphotographs were taken to obtain the morphological

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characteristics using the Tescan MIRA field emission gun scanning electron microscope using an in-lens secondary electron detector and set to an operating acceleration voltage of 5kV.

2.11 Fourier-transform infrared (FTIR) spectroscopic analysis of biomolecules

About 2 g of dried macerated plant material were subjected to FTIR analysis. Samples were analysed using the Perkin Elmer Spectrum 100-FTIR Spectrometer [PerkinElmer (Pty) Ltd., Midrand, South Africa] where a wider window frequency range of 450 to 4000 cm^{-1} was considered.

2.12 Gene expression analysis

2.10.1 RNA extraction and cDNA preparation

Total RNA was extracted from 0.1 g of macerated plant material (treated and untreated) using the FavorPrep™ Plant Total RNA Purification Mini Kit (FAPRK001-1, Favorgen Biotech Corp, Ping-Ting, Taiwan) following the manufacturer's protocol. The genomic DNA was removed by treating the resulting RNA with RNase-free DNase solution (0.5 U/µl) (New England Biolabs, Massachusetts). Complementary DNA was synthesised from ~1 µg of extracted RNA using the SuperScript™ III First-Strand Synthesis kit (Invitrogen, Carlsbad, California, USA) following the manufacturer's protocol. High quality and integrity of both RNA and cDNA were spectrophotometrically assured by quantification using NanoDrop™ 2000/2000c spectrophotometer (Thermo Scientific, USA).

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2.10.2 Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine expression profiles using the LightCycler® 480 SYBR Green I Master kit (Roche Diagnostics, SA) following the manufacturer's protocol. One reaction contained 1 µl DNA template, 5 µl 2X SYBR Green I Master Mix (Roche Applied Science, Germany) and optimized primer concentrations made up to a final volume of 10 µl with RNase free dH_2O. Primers were designed using the Primer3 online tool. Primer information and PCR cycling conditions are listed below. Transcript expression levels were normalised using beta actin, ubiquitin (UBQ) and phosphoenolpyruvate carboxylase (PEPC) as reference genes and analysed using the LightCycler® 480 Software version 1.5.1.62. Expression levels were quantified relative to a calibration curve of serially diluted cDNA. The results obtained serves as a representation of 3 biological and 3 independent repeats including non-template control.

Table 2.1: Primer information.

Primer	Forward	Reverse	Accession number
SbLOX1	GTACCGCTACGACGTCTACA	GTCAACTCTCGTGCAGCAAA	GQ369443.1
SbAOS	ACCATCACCTCGCTCAAGAA	TCACACAGTATCACGGCACT	XM_002463784.2
SbAOC	GTACGAGGCCATCTACAGCT	AGGGGAAGACGATCTGGTTG	XM_002465042.2
SbOPDR	GGGTATGATCGGGAGGAAGG	CAACGGGATCTTGCGTGTAG	XM_002438007.2
PEPC	GAAGAATATCGGCATCAA	CTATGTAATACTTGGTAACTTTC	XM_002438476
Beta actin	CCTTACCGACTACCTCAT	ATAGATCCTTCCTAATATCCA	AF369906.1
UBQ	GCCAAGATTCAGGATAAG′	TTGTAATCAGCCAATGTG′	XM_002452660

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Table 2.2: Real-time quantitative polymerase chain reaction conditions.

Programme		Target ℃	Hold	Cycles
Pre-incubation		95 ℃	10 min	1X
Amplification		95 ℃	10 s	47X
	SbLOX1	58 ℃		
	SbAOS	58 ℃		
	SbAOC	58 ℃		
	SbOPDR	58 ℃		
	PEPC	60 ℃		40X
	Beta actin	60 ℃		
	UBQ	60 ℃		46X
		72 ℃	20s	
Melting			Default	
Cooling				

2.13 Statistical analysis

All assays conducted in this study were sampled from equally pooled plant samples and conducted from at least three technical replicates. The data was statistically analysed by test, one-way or two-way ANOVA where appropriate using Graphpad Prism 9 (2021). Statistical significances were determined according to Bonferroni multiple comparison and represented as * = $P \leq 0.05$,** = $P \leq 0.01$ and *** = $P \leq 0.001$.

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CHAPTER 3

Analysis of the effect of methyl-jasmonate and carbon monoxide on the germination of *Sorghum bicolor* under salt stress

ABSTRACT

Research illustrated the beneficial role of phytohormones and signalling molecules to reduce the effects of abiotic stress in many plant species, yet their role on the germination of salt-stressed sorghum needs further elucidation. Hence, this study was aimed at investigating the effect of salt stress on the germination of *Sorghum bicolor* and the role of methyl jasmonate (MeJA) and carbon monoxide (CO) in alleviating seed germination inhibition caused by salinity stress. Sorghum seeds were primed with increasing concentration of MeJA (0, 10, 15, 20 µM) and germinated in petri dishes on a paper towel imbibed with solutions containing various NaCl (0; 200 mM) or CO [using hematin (Ht), a CO donor] (0, 1, 1.5 µM) concentration, and left to germinate at 25℃ in complete darkness. Salt stress delayed germination by decreasing germination percentage (GP), mean germination time (MGT), germination index (GI) and total germination (TG). Additionally, salinity stress hindered seedling growth by decreasing root and shoot length. Pre-treatment with MeJA and exogenous hematin individually or in combination proved successful in reversing seed germination inhibition caused by salt stress as indicated by increasing GP, MGT, GI and TG in addition to the increase in root length under salinity stress. Findings illustrated that the combination of MeJA and hematin effectively ameliorated the negative effects of stress on germination and seedling growth. Results further suggested that MeJA is more efficient in alleviating the effects of salinity stress on germination and seedling growth of *S. bicolor*.

Keywords: Carbon monoxide, hematin, germination, salinity, seedling growth, MeJA

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3.1 INTRODUCTION

The sessile nature of plants has enforced adaptation for survival under adverse conditions. Natural strategies are becoming inadequate due to the increasing intensity of abiotic stresses such as salt stress, which is only expected to exacerbate particularly in arid and semi-arid regions (Hadia et al., 2022). Salinization is on the increase in all parts of the world as approximately 20% of all agricultural areas are subjected to increased level of salt (Jovović et al., 2018; Hadia et al., 2022). It, therefore, requires prioritised intervention to antagonise its deleterious effects on crop productivity and ultimately food security.

The constraint on crop productivity is often initiated at the germination phase of growth where salt stress evokes poor emergence and inadequate seed establishment by disturbing metabolic processes due to inhibition in nutrient or water uptake (Zafar et al., 2022). Germination is defined as the developmental process initiated by the absorption of water by the quiescent dry seed terminating with the protrusion of the radicle from the seed coat to commence embryonic axis elongation (Gianinetti, 2020). Most species demonstrate their highest resistance to adverse condition during germination and is therefore the most critical stage in the plant's life cycle, crucial for species distribution (Anaya et al., 2018). Germination is a qualitative response of seeds relative to time that may exhibit complex patterns. Therefore, it relies on time course evaluation to elucidate timing, uniformity and the extent of germination in seed population (Talská et al., 2020). Research have adopted many parameters to quantify germination. These include mean germination time (MGT), which is a measure of the quickness of germination and its spread through time. Secondly, germination index (GI), which measures the combination of percentage and rate of germination, whereas total germination (TG) focuses on the cumulative percentage germination of the population at the end of the germination trail (Javaid et al., 2018; Gianinetti, 2020).

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Plants have developed natural strategies comprised of a complex network to survive seed germination inhibition caused by adverse conditions with various phytohormones and signalling molecules at the forefront in these biological strategies (Li et al., 2016). Phytohormones regulate many physiological processes such as growth and development, respiration and transpiration, and has often been induced in response to adverse conditions. Methyl jasmonate (MeJA) improved drought tolerance of *Glycine max* (Mohamed & Latif, 2017), mitigated arsenic stress in *Brassica napus* (Farooq et al., 2018) and ameliorated growth and oxidative damage under salt stress in *Citrus sinensis* (Mahmoud et al., 2021). Similarly, carbon monoxide improved cadmium tolerance in *Vigna radiata* (Mahawar et al., 2021), and mitigated salinity stress in *Medicago sativa* (Amooaghaie & Tabatabaie, 2017). Given the systemic nature of phytohormones, they often work in concert with many other signalling molecules to alleviate stress induced damage (Iqbal et al., 2017). Therefore, research have risen to an underlying crosstalk between phytohormone, MeJA, and signalling molecule CO. However, their individual or combinatorial role on the seed germination remains elusive. Therefore, the aim of this chapter was to investigate the role of both MeJA and CO (using hematin, a CO donor) on the germination and seedling growth of *S. bicolor* under control and salt stress conditions.

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3.2 RESULTS

3.2.1 Salt stress delays germination of S. bicolor

Percentage germination of sorghum seedlings exposed to non-saline (control) and saline (200 mM NaCl) conditions was observed for seven days. Under control conditions sorghum seedlings displayed full germination at day three. Salt stress negatively affected germination by significantly decreasing germination percentage from 96.667% to 26.667% at day two (*** = P≤0.001) of the germination period, followed by decreasing germination percentage from 100% to 50% at day three (** = P≤0.01) of the germination period (Figure 3.1). This equalled to a percentage decrease of 72.41% (day 2) and 50% (day 3) as compared to their respective controls. Due to considerable changes in germination percentage being mostly evident during the earlier days of germination, emphasis was placed on the difference in germination percentage obtained during day 1, day 2 or 3 and day 7 of germination.

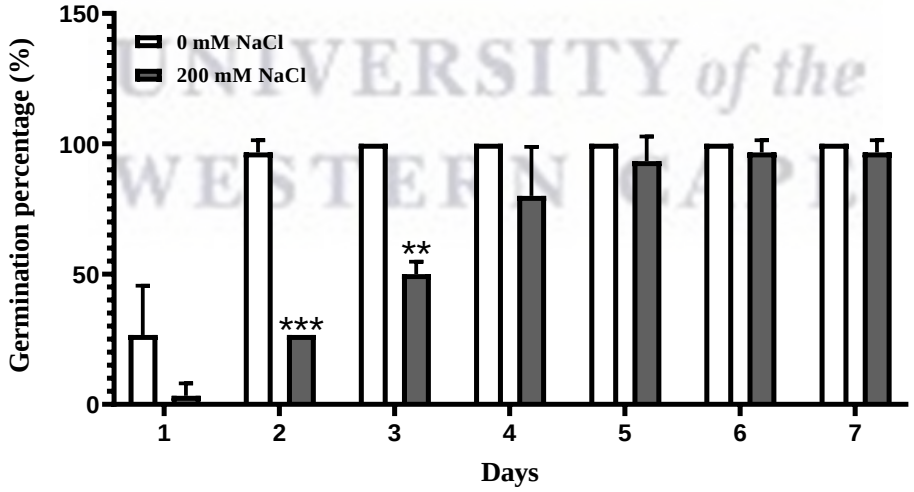

Figure 3.1: Germination percentage of *S. bicolor* in response to non-stress (0 mM NaCl) and salt stress (200 mM NaCl) condition. Seedlings were germinated in petri dishes for 7 days exposed to 0 mM and 200 mM NaCl stress. Error bars represents SD from 6 biological replicates. Statistical significance was achieved according to t-test (GraphPad Prism 9) where ** = P≤0.01 and *** = P≤0.001.

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3.2.2 The effect of MeJA and hematin on germination percentage of S. bicolor under non-saline stress

MeJA had no significant effect on the germination percentage of sorghum seedlings under non-stress condition (Figure 3.2 A). In addition to control seedlings, MeJA pre-treated seedlings reached peaked germination at day three as indicated by 100% germination percentage for all treatments. Exogenously applied hematin affected the germination percentage of sorghum under non-stress conditions (Figure 3.2 B). Lower concentration of hematin (1 µM) had no significant effect on germination percentage of sorghum under non-stress conditions. However, increased hematin concentration (1.5 µM) affected germination percentage of sorghum at physiological level as shown by a decreased germination percentage (93.333%) as compared to 100% obtained from control seedlings at day three of germination. This resulted in a 6.67% significant (*** = $P \leq 0.001$) decrease in germination percentage. The combinatory exposure to pre-treatment with MeJA (10, 15 and 20 µM) and incubation with 1 µM hematin solution had no significant effect on the germination percentage of sorghum under non-saline condition (Figure 3.2 C). Although not significant, sorghum subjected to the combinatory pre-treatment with MeJA (10, 15, and 20 µM) and hematin (1.5 µM) had increased variation in germination percentage at day two, which resulted in slightly lower average germination obtained as compared to control seedlings (Figure 3.2 D).

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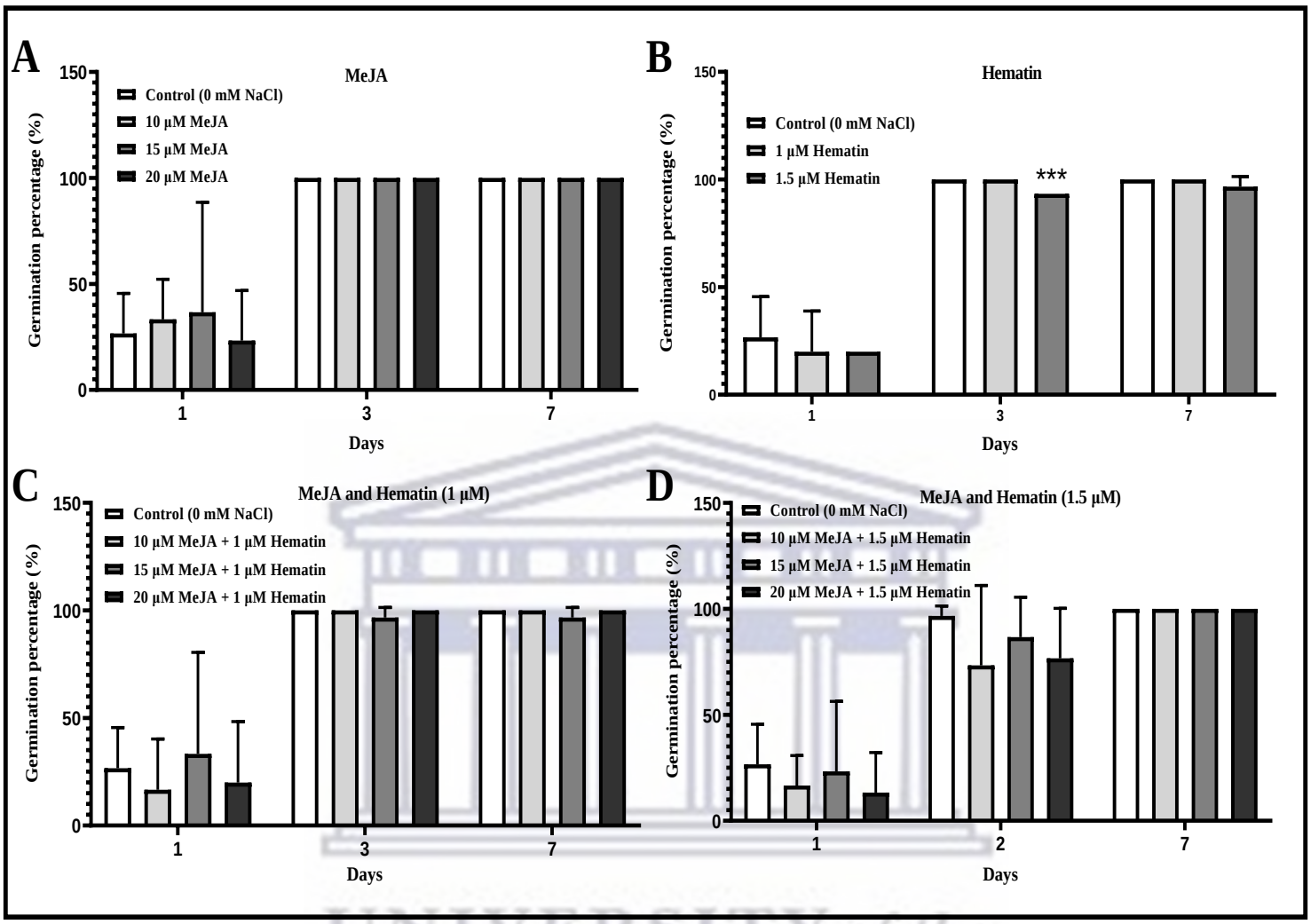

Figure 3.2: Germination percentage of *S. bicolor* in response to MeJA pre-treatment and exogenous hematin under non-stress (0 mM NaCl) condition. Seedlings were (**A**) pre-treated with 0, 10, 15 and 20 µM MeJA, (**B**) exposed to solution containing 0, 1 and 1.5 µM hematin, (**C**) pre-treated with MeJA (0, 10, 15 and 20 µM) and germinated in the presence of 1 µM hematin solution, (**D**) pre-treated with MeJA (0, 10, 15 and 20 µM) and germinated in the presence of 1.5 µM hematin solution. Error bars represents SD from 6 biological replicates. Statistical significance was achieved using two-way ANOVA (GraphPad Prism 9) where *** = P≤0.001 according to Bonferroni's multiple comparison.

3.2.3 Combinatory MeJA and hematin improves germination percentage of S. bicolor under salt stress

Pre-treatment with MeJA positively affected *S. bicolor*'s germination as shown by increasing the average germination percentage to 96.667% (10 µM MeJA), 86.667% (15 µM MeJA) and 76.667% (20 µM MeJA) as compared to 50% obtained from control seedlings under 200 mM

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NaCl stress at day three of germination (Figure 3.3 A). This resulted in a 93.33% significant increase (* = P≤0.05) in germination percentage for sorghum pre-treated with 10 µM MeJA, in addition to increases of 73.33% (15 µM MeJA) and 53.33% (20 µM MeJA) in average germination percentages observed at day three under salt stress. The exogenous application of hematin affected percentage germination by further inhibiting germination of sorghum under 200 mM NaCl stress at day two of germination (Figure 3.3 B). This was shown by decreases to 0% for both 1 µM and 1.5 µM hematin as compared to 26.67% in germination obtained from control seedlings. Clearly, equalling to 100% significant (*** = P≤0.001) decreases for both hematin concentration (1 µM and 1.5 µM hematin) in germination percentage at day two under salt stress. The combinatory pre-treatment with MeJA (10, 15 and 20 µM MeJA) in the presence of hematin solution (1 µM hematin) affected germination percentage of sorghum by increasing the average germination percentage to 60% (10 µM MeJA + 1 µM hematin), 90% (15 µM MeJA + 1 µM hematin) and 63.33% (20 µM MeJA + 1 µM) as compared to 3.33% obtained with control seedlings at day three of germination under 200 mM NaCl salt stress (Figure 3.3 C). Evidently, this resulted in average germination difference of 56.67% (10 µM MeJA + 1 µM hematin) and 60% (20 µM MeJA + 1 µM hematin), only 86.67% significant (* = P≤0.05) increase observed in germination percentage of sorghum treated with the combination of 15 µM MeJA and 1 µM hematin under salt stress. Conjunctive exposure to MeJA (10, 15 and 20 µM) and hematin (1.5 µM) had no significant effect on the germination percentage of sorghum under 200 mM NaCl stress (Figure 3.3 D). However, a considerable 60% increase in percentage germination was observed with sorghum seedlings subjected to treatment with 15 µM MeJA and 1.5 µM hematin at day three of germination under salt stress.

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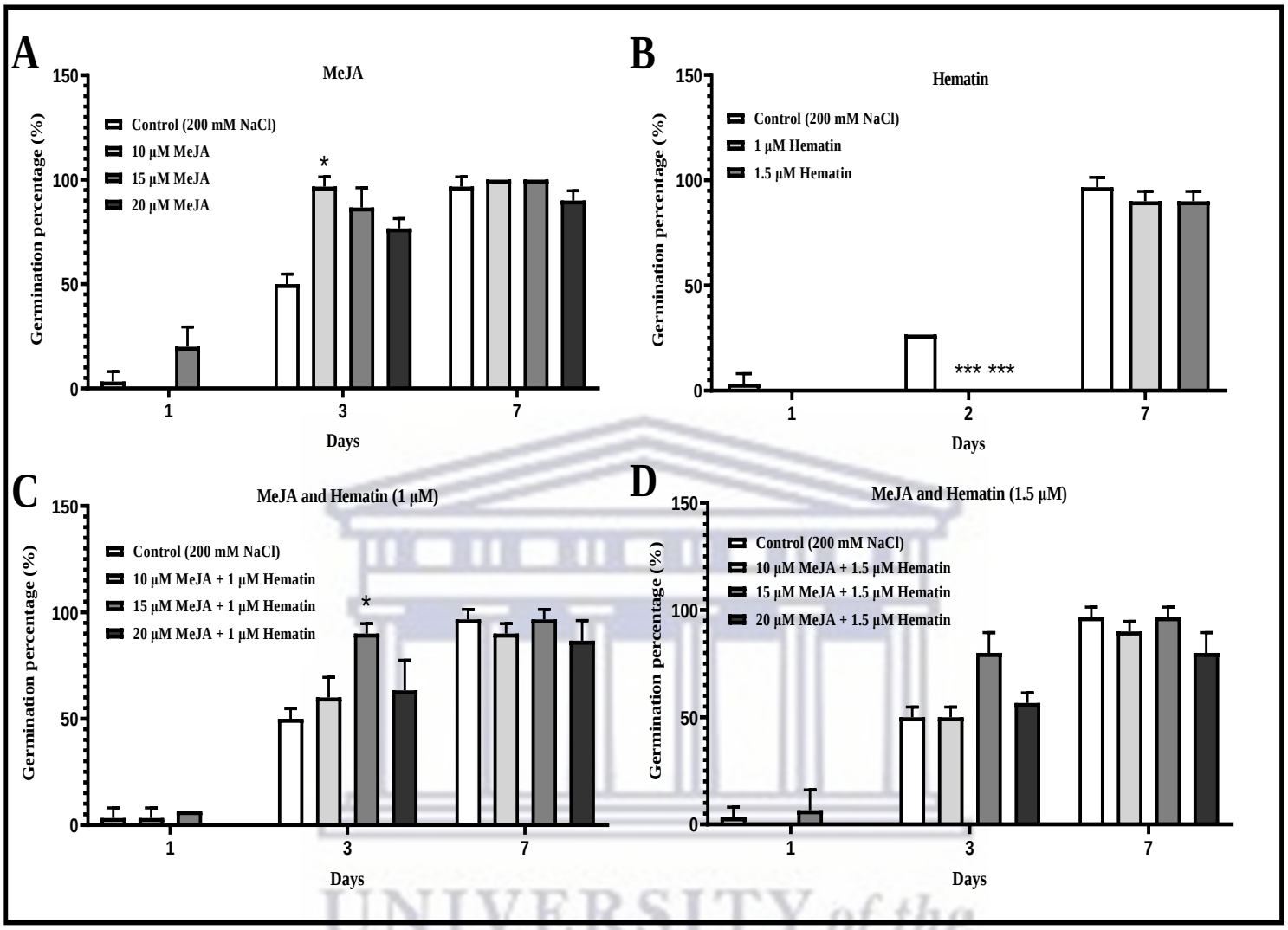

Figure 3.3: Germination percentage of *S. bicolor* in response to MeJA pre-treatment and exogenous hematin under salt stress (200 mM NaCl) condition. Seedlings were (**A**) pre-treated with 0, 10, 15 and 20 µM MeJA, (**B**) exposed to solution containing 0, 1 and 1.5 µM hematin, (**C**) pre-treated with MeJA (0, 10, 15 and 20 µM) and germinated in the presence of 1 µM hematin solution, (**D**) pre-treated with MeJA (0, 10, 15 and 20 µM) and germinated in the presence of 1.5 µM hematin solution. Error bars represents SD from 6 biological replicates. Statistical significance was achieved using two-way ANOVA (GraphPad Prism 9) where * = P≤0.05 and *** = P≤0.001 according to Bonferroni's multiple comparison.

3.2.4 Combinatory MeJA and hematin had no substantial effect on mean germination time (MGT), germination index (GI) and total germination (TG) of S. bicolor under salinity stress

Salt stress affected germination of *S. bicolor* by decreasing MGT from 27.2 days to 23.3 days as observed from control sorghum. Although non-significant, this resulted in average percentage decrease of 14.51%, that was evident as compared to control seedlings (Table 3.1).

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Pre-treated sorghum resulted in slightly decreased MGT under non-stress conditions. Additionally, similar observations were made in sorghum exposed to exogenous hematin and the combinatory application of MeJA and hematin. Contrary to this, pre-treatment with MeJA in addition to the combinatory treatment with MeJA and hematin resulted in slightly increased MGT under salt stress.

Salt affected the germination of sorghum by decreasing GI from 113.333 to 66.167 under 200 mM NaCl stress. This resulted in a 41.17% significant (* = $P \leq 0.05$) decrease as compared to control seedlings. Non-significant changes in GI were observed in sorghum seedlings pre-treated with MeJA in addition to the exogenous application of hematin, under non-stress condition. MeJA pre-treatment on sorghum had slightly improved GI under salt stress. Whereas decreased GI was observed in sorghum treated with hematin only under salt stress. Albeit non-significantly, the combinatory treatment of MeJA and hematin had slightly improved GI under 200 mM NaCl stress.

No significant effect on the TG of sorghum was observed in response to salt stress. Salt stress had a slight negative effect on TG of sorghum seedlings. Treatment with MeJA and hematin resulted in maximum germination under non-stress conditions. Furthermore, greater variation in TG was observed under salt stress (200 mM NaCl) condition where all treatments resulted in TG above 80%.

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Table 3.1: Germination parameters of *S. bicolor* exposed to pre-treatment with MeJA and exogenously applied hematin under non-stress and salinity stress conditions. Mean germination time (MGT), germination index (GI) and total germination (TG) were measured in *S. bicolor* pre-treated with 0, 10, 15 and 20 µM MeJA and in sorghum exposed to 1 and 1.5 µM hematin in response to control (0 mM NaCl) and salt stress (200 mM NaCl) conditions. Data represented as mean ± standard deviation.

NaCl (mM)	MeJA (µM)	Hematin (µM)	MGT (Days)	GI	TG (%)
0	**0**	**0**	27.2 ± 0.283	113.333 ± 8.014	100 ± 0
	10	**0**	26.933 ± 0.754	110.667 ± 15.085	100 ± 0
	15	**0**	27.233 ± 0.707	115.833 ± 20.977	100 ± 0
	20	**0**	26.967 ± 0.613	109.167 ± 13.906	100 ± 0
	0	**1**	27.067 ± 0.377	110 ± 9.428	100 ± 0
	0	**1.5**	26.902 ± 0.239	104.667 ± 2.357	96.667 ± 4.714
	10	**1**	26.967 ± 0.519	107.833 ± 12.492	100 ± 0
	15	**1**	27.157 ± 0.788	110.167 ± 15.792	96.667 ± 4.714
	20	**1**	26.667 ± 1.037	104 ± 21.213	100 ± 0
	10	**1.5**	26.633 ± 0.896	102.833 ± 16.263	100 ± 0
	15	**1.5**	26.967 ± 0.707	109.167 ± 17.206	100 ± 0
	20	**1.5**	26.667 ± 0.660	102.667 ± 13.671	100 ± 0
200	**0**	**0**	23.252 ± 0.963	66.167 ± 8.721*	96.667 ± 4.714
	10	**0**	25.200 ± 0.660	82 ± 4.243	100 ± 0
	15	**0**	25.567 ± 1.179	94.833 ± 11.078	100 ± 0
	20	**0**	24.951 ± 0.474	74.333 ± 7.071	90 ± 4.714
	0	**1**	22.986 ± 0.525	55.167 ± 5.421	90 ± 4.714
	0	**1.5**	22.618 ± 0.975	53 ± 0.943	90 ± 4.714
	10	**1**	23.060 ± 0.521	58.833 ± 7.307	90 ± 4.714
	15	**1**	25.543 ± 0.364	84.5 ± 7.307	96.667 ± 4.714
	20	**1**	24.321 ± 1.667	71 ± 4.243	86.667 ± 4.714
	10	**1.5**	23.352 ± 0.699	63 ± 1.414	90 ± 4.714
	15	**1.5**	24.050 ± 2.051	77.333 ± 8.957	96.667 ± 4.714
	20	**1.5**	23.385 ± 0.870	53.333 ± 3.771	80 ± 9.428

Statistical significance was achieved using t-test (salt stress only) and two-way ANOVA (salt stress vs treatment) (GraphPad Prism 9) where * = P≤0.05 and *** = P≤0.001 according to Bonferroni's multiple comparison.

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3.2.5 Salt stress perturbs root and shoot growth of S. bicolor

After seven days of germination the root and shoot lengths of sorghum was observed under control (0 mM NaCl) and salt stress (200 mM NaCl) conditions. The sorghum seedlings favoured growth in the germination conditions with growth averaging more than 60 mm for both root and shoot length under non-stress conditions. Salt stress affected the growth of sorghum during germination by causing a decrease in root length from 62.4 mm to 32 mm obtained from control and a decrease in shoot length to 3.611 mm from 79.6 mm obtained from control sorghum (Figure 3.4). This resulted in 48.72% and 95.46% significant (*** = P≤0.001) decreases in the root and shoot length of sorghum under 200 mM NaCl stress, respectively.

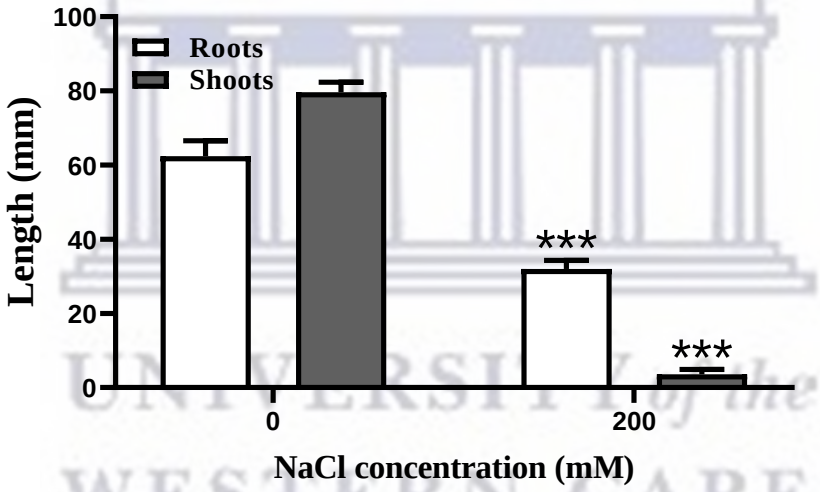

Figure 3.4: Root and shoot length of *S. bicolor* in response to non-stress (0 mM NaCl) and salt stress (200 mM NaCl) condition. Seedlings were germinated in petri dishes for 7 days exposed to 0 mM and 200 mM NaCl stress. Error bars represents SD from 3 biological replicates. Statistical significance was achieved according to t-test (GraphPad Prism 9) where ** = P≤0.01 and *** = P≤0.001.

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3.2.6 Combinatory MeJA and hematin improves the growth of S. bicolor under non saline conditions

MeJA pre-treatment affected growth of sorghum during germination by slightly increasing root length to 68.5 mm (10 µM MeJA) followed by decreasing root length to 52.95 mm (15 µM MeJA) and 48.267 mm (20 µM MeJA) from 62.4 mm obtained from control sorghum under non-stress conditions (Figure 3.5 A). This resulted in a slight 9.78% increase for 10 µM MeJA, and a 15.14% decrease for 15 µM MeJA, while a 22.65% significant (* = $P \leq 0.05$) decrease for 20 µM MeJA as compared to the control. Pre-treatment with MeJA had no significant effect on the shoot length of sorghum seedlings under control (0 mM NaCl) conditions. The exogenous application of hematin (Ht) had no significant effect on the root and shoot lengths of sorghum under non-stress conditions (Figure 3.5 B). Combinatory treatment of MeJA (10, 15 and 20 µM) and hematin (1 µM) affected root growth by increasing average length to 64.667 mm (10 µM MeJA+ 1 µM Ht), 81 mm (15 µM MeJA + 1 µM Ht) and 72.467 mm (20 µM MeJA + 1 µM Ht) from 62.4 mm obtained from control sorghum under non stress condition (Figure 3.5 C). This resulted in a 3.63% increase for the conjunctive application of 10 µM MeJA and 1 µM Ht, a 29.81% significant (** = $P \leq 0.01$) increase for 15 µM MeJA and 1 µM Ht and a 16.12% increase for 20 µM MeJA and 1 µM hematin as compared to control sorghum under non-saline condition. Furthermore, no significant effect was observed on the shoot lengths of sorghum at physiological level. Additionally, the combinatory application of MeJA (10, 15 and 20 µM) and hematin (1.5 µM) had no significant effect of the root and shoot lengths of sorghum under non-saline conditions (Figure 3.5 D).

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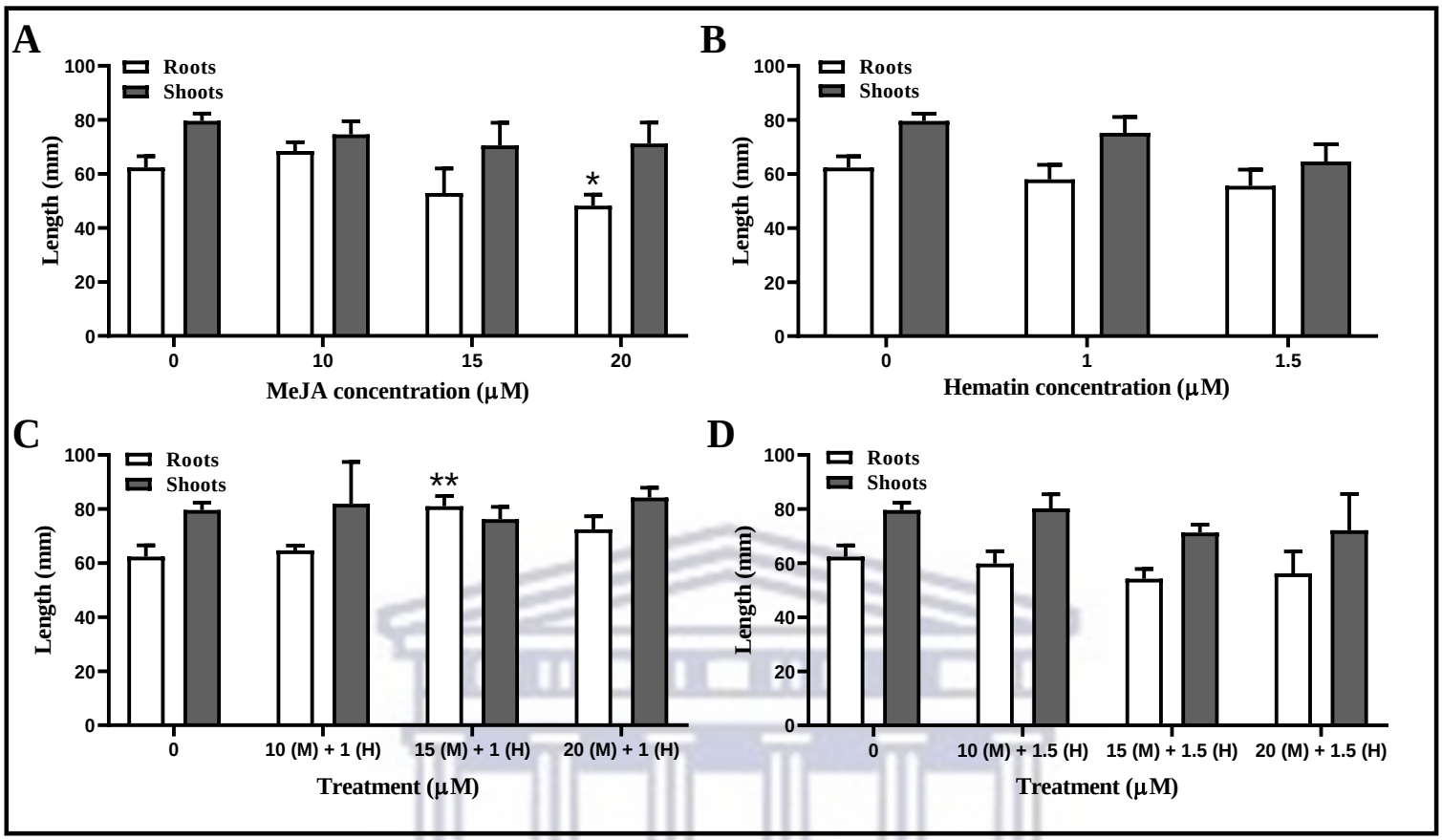

Figure 3.5: Root and shoot lengths of *S. bicolor* in response to MeJA pre-treatment and exogenous hematin under non-stress (0 mM NaCl) condition. Seedlings were (**A**) pre-treated with 0, 10, 15 and 20 µM MeJA, (**B**) exposed to solution containing 0, 1 and 1.5 µM hematin, (**C**) pre-treated with MeJA (0, 10, 15 and 20 µM) and germinated with hematin (1 µM) solution, (**D**) pre-treated with MeJA (0, 10, 15, 20 µM) and germinated with hematin (1.5 µM) solution. Error bars represents SD from 3 biological replicates. Statistical significance was achieved using two-way ANOVA (GraphPad Prism 9) where * = P≤0.05 and ** = P≤0.01 according to Bonferroni's multiple comparison.

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3.2.7 Combinatory MeJA and hematin improves the growth of S. bicolor under salt stress conditions

Pre-treatment with MeJA affected root growth of sorghum by increasing root length to 45.2 mm (10 µM MeJA), 47.4 mm (15 µM MeJA) and 44.567 mm (20 µM MeJA) from 32 mm obtained with control seedlings under salt stress (200 mM NaCl) conditions (Figure 3.6 A). This resulted in a significant increases of 41.25% (* = P≤0.05) for 10 µM MeJA, 48.13% (** = P≤0.01) for 15 µM MeJA and 39.27% (* = P≤0.05) for 20 µM MeJA as compared to the control. However, pre-treatment with MeJA had no significant effect on the shoot length of

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sorghum seedlings under salt stress conditions. Similarly, the exogenous application of hematin had no significant effect on the root and shoot lengths of sorghum exposed to salt stress (200 mM NaCl) (Figure 3.6 B). Combinatory application of both MeJA (10, 15 and 20 µM) and hematin (1 µM) affected root growth of sorghum by increasing average root length to 35.217 mm (10 µM MeJA + 1 µM Ht), 39.183 mm (15 µM MeJA + 1 µM) and 51.489 mm (20 µM MeJA + 1 µM Ht) from 32 mm obtained with control seedlings under salt stress condition (Figure 3.6 C). This equalled a slight 10.05% increase for 10 µM MeJA + 1 µM Ht and 22.45% increase for 15 µM MeJA +1 µM Ht, whereas a significant 60.9% increase (*** = $P \leq 0.001$) was observed in the root length for 20 µM MeJA + 1 µM Ht as compared to control. Although slightly increased averages were observed, this combinatory treatment had no significant effect on the shoot lengths of sorghum under 200 mM NaCl stress. Furthermore, sorghum seeds subjected to pre-treatment with MeJA (10, 15 and 20 µM) and exogenous hematin (1.5 µM) had no significant improvement on the root and shoot lengths under 200 mM NaCl stress (Figure 3.6 D).

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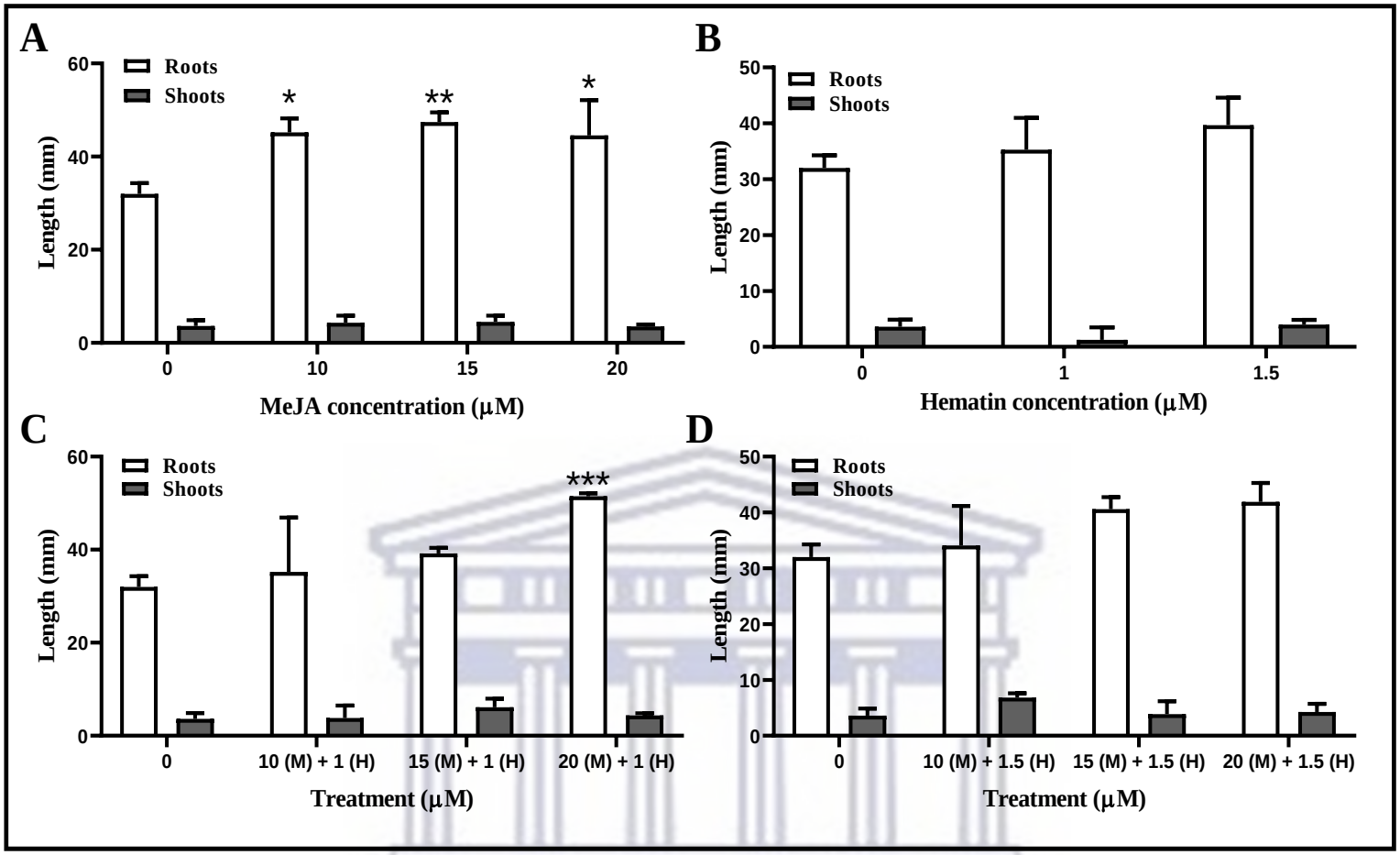

Figure 3.6: Root and shoot lengths of *S. bicolor* in response to MeJA pre-treatment and exogenous hematin under salt stress (200 mM NaCl) condition. Seedlings were (**A**) pre-treated with 0, 10, 15 and 20 µM MeJA, (**B**) exposed to solution containing 0, 1 and 1.5 µM hematin, (**C**) pre-treated with MeJA (0, 10, 15 and 20 µM) and germinated with hematin (1 µM) solution, (**D**) pre-treated with MeJA (0-20 µM) and germinated with hematin (1.5 µM) solution. Error bars represents SD from 3 biological replicates. Statistical significance was achieved using two-way ANOVA (GraphPad Prism 9) where * = P≤0.05 and *** = P≤0.001 according to Bonferroni's multiple comparison.

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3.3 DISCUSSION

Seed germination is the first and crucial step in the plant's life cycle. Understanding salt stress and its effects on plants during germination and early growth phase are fundamental to understanding the mechanisms involved in conferring stress tolerance for improved growth and development. Therefore, this chapter focused on the role of MeJA and carbon monoxide (CO) in alleviating salt stress tolerance in the moderately tolerant *Sorghum bicolor* at its germination phase of growth.

3.3.1 MeJA and hematin improves germination percentage of S. bicolor in response to salinity stress

Salt stress proved detrimental to sorghum's development by repressing germination during the early stage. This was indicated by the significant decrease in germination percentage evident from day two of germination under saline condition (Figure 3.1). Observations from this study coincides with results previously obtained from *S. bicolor* (Mulaudzi et al., 2020), *Sulla carnosa* (Bouzidi et al., 2021) and *Phaseolus vulgaris* L. (Tania et al., 2022) under similar salt stress conditions. The inhibiting effect of salt stress may be due to the role of salt in reducing water uptake during germination thus limiting imbibition and seed turgescence (Tarchoun et al., 2022). Delayed germination may also be due to salt stress negatively affecting seed viability during germination (Mbarki et al., 2020).

Seed priming with MeJA did not have a negative effect on seed germination at physiological level as indicated by similar germination percentages obtained from control sorghum under non saline conditions. However, high concentration of hematin (*e.g* 1.5 µM MeJA) had a slightly negative impact on the germination of *S. bicolor* at physiological level. This was indicated by a significant decrease in germination percentage of sorghum exposed to increased

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concentration of hematin under non-saline condition (Figure 3.2 A-B). This finding coincides with similar observation made with *Nicotiana tabacum* and *Medicago sativa* (Li & Gu, 2016; Amooaghaie & Tabatabaie, 2017). Indicating that excessive CO is toxic during the early stage of germination under non-stressed conditions. This deleterious effect was later restored as indicated by similar total germination percentages to control seedlings at the end of the germination period. Pre-treatment with MeJA in conjunction with exogenous hematin had no inhibitory effect on the germination of *S. bicolor* at physiological level as indicated by similar germination percentages to control sorghum seedlings under non-saline conditions (Figure 3.2 C-D).

Pre-treatment with MeJA proved efficacious in ameliorating salinity stress induced inhibition on the germination of sorghum seedlings. This was indicated by a significant increase in percentage germination of *S. bicolor* pre-treated with the lowest concentration of MeJA (10 µM) under salt stress condition (Figure 3.3 A). This result agreed with previous studies where MeJA enhanced the germination of *Brassica oleracea* and *Oryza sativa* under oxidative stress (Hassini et al., 2017; Sheteiwy et al., 2018). Findings from this study illustrated MeJA eliciting an important role of alleviating osmotic stress by altering physiological processes during germination (Ali et al., 2019). The exogenous application of hematin further impaired germination of *S. bicolor* under salt stress at the earlier stage of germination. This was indicated by sorghum seeds only germinating after day two of the experiment in response to hematin treatment (1 µM and 1.5 µM) under salt stress (Figure 3.3 B). A similar observation was made where higher concentration of hematin further reduced germination percentage of *M. sativa* (Amooaghaie & Tabatabaie, 2017). This inhibitory effect was later rescued at the termination of germination indicated by similar cumulative germination percentage as compared to the salt stressed control seedlings. Pre-treatment with MeJA in addition to exogenous hematin proved successful in enhancing germination of *S. bicolor* under salt stress. As observed by a

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significantly increased germination percentage of sorghum seedlings in response to 15 µM MeJA and 1 µM Ht under salt stress environment (Figure 3.3 C). However, treatment with MeJA and increased hematin concentration had no significant effect on the germination of sorghum under salt stress (Figure 3.3 D). Indicating the combinatory treatment of MeJA and hematin dose-dependently enhancing germination percentage of sorghum under salt stress.

3.3.2 MeJA and hematin improves mean germination time, germination index and total germination of S. bicolor under salinity stress

Salt stress insignificantly reduced the mean germination time (MGT) of *S. bicolor* (Table 3.1), which indicates the decreased relative emergence of sorghum seed population under salinity stress (Kader, 2005). This implied that salinity stress caused the population of seeds to reach maximum germination within the population faster as compared to the population of seeds under control condition. Similarly, salinity stress in addition to MeJA singly or in combination with hematin had insignificant effect on total germination (TG) of sorghum seedlings. Which illustrated the ability of sorghum to tolerate peculiar conditions that might be harmful by rescuing germination on a later stage. Furthermore, salt stress significantly reduced the germination index (GI) of *S. bicolor*. Germination index considers both the percentage and speed of germination (Kader, 2005). Therefore, this indicated that salt stress proved harmful to the germination of sorghum by reducing percentage and rate of germination. This harmful effect of salinity stress on germination index coincides with findings from *Solanum lycopersicum*, *Brassica chinensis* and *Salicornia bigelovii* (Tanveer et al., 2020; Ren et al., 2020; García-Galindo et al., 2021). The decreased germination index (GI) under NaCl stress further confirmed the deleterious effect of salt stress on the germination of *S. bicolor*, which is supported by the decreased germination percentages observed from Figure 3.1. Although non-significant, the combinatory treatment of MeJA and hematin resulted in slightly improved

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averages in MGT, GI and TG under salt stress, which demonstrate minor attenuation of the inhibitory effect caused by salinity stress.

3.3.3 The role of MeJA and hematin on the root and shoot length of S. bicolor under salinity stress

In addition to perturbed germination, salinity stress inhibited growth of *S. bicolor* during the germination phase of development. Evidently, sorghum had significantly decreased root and shoot length after seven days of exposure to salt stress (200 mM NaCl) (Figure 3.4). Findings coincide with previous observations made with *Trifolium repens* (Cheng et al., 2018), *Lens culinaris* (Foti et al., 2019) and *Festuca arundinacea* (Shiade & Boelt, 2020) as well as *S. bicolor* (Mulaudzi et al., 2020). The impaired growth in response to salt stress reflects toxic effects associated with disturbed water uptake and nutrient imbalance due to decreased osmotic potential causing ionic stress and therefore decreased water or nutrient transportation from the roots to the shoot (Foti et al., 2019; Mulaudzi et al., 2020).

Excessive MeJA concentration had a negative effect on the growth of sorghum during germination at physiological level. This was observed by the decreased root length of sorghum pre-treated with a higher concentration of MeJA (20 µM) and germinated under control conditions (0 mM NaCl) (Figure 3.5 A). A similar observation was made with *O. sativa* in response to higher concentration exogenous MeJA (2.5 mM) (Bhavanam & Stout, 2021). The decreased root length may be due to MeJA application in higher doses increasing the distribution of resources to resistant related pathways, processes and metabolites at the expense of growth (Bhavanam & Stout, 2021). Jasmonates often interfere with other hormonal pathways from which upregulated jasmonic acid causes downregulation of gibberellic acid, thus affecting seedling growth (Hou et al., 2013; Bhavanam & Stout, 2021). Exogenous hematin had no significant effect on the seedling growth of sorghum at physiological level as indicated by similar root and shoot lengths as compared to control seedlings upon exposure to

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hematin solution. However, combinatory MeJA and hematin proved advantageous to sorghum seedling growth at physiological level. This was indicated by significantly increased root length of sorghum upon exposure to 15 µM MeJA and 1 µM hematin under non-saline stress (Figure 3.5 C). These results demonstrated that a combination of MeJA and hematin has beneficial characteristics towards seedling growth of sorghum under normal (0 mM NaCl) condition in a concentration-dependent manner.

The inhibitory effect on the growth of sorghum caused by salinity stress was reversed by seed priming with MeJA. This was indicated by enhanced root and shoot lengths of sorghum seedlings pre-treated with all concentrations of MeJA under salt stress (Figure 3.6 A). Similar observations were made where MeJA enhanced root growth of salinity stressed *Brassica napus* and *Glycyrrhiza uralensis* (Ahmadi et al., 2018; Lang et al., 2020). It can therefore be deduced from this study that 200 mM NaCl significantly damaged growth whereas the addition of MeJA efficaciously promoted root growth of salt-stressed *S. bicolor*. Exogenous hematin had no significant effect on the root and shoot lengths of sorghum under salt stress, although slightly improved averages were evident upon exposure to hematin as compared to control seedlings (Figure 3.6 B). MeJA pre-treatment in addition to exogenous hematin ameliorated salt induced inhibition on the growth of sorghum. Seedlings pre-treated with 20 µM MeJA and supplemented with 1 µM hematin proved advantageous to the root length of sorghum under salt stress. Whereas seedlings subjected to conjunctive treatment of MeJA (10, 15 and 20 µM) and increased hematin concentration (1.5 µM) had no significant effect on the root and shoot lengths of sorghum exposed to salt stress (Figure 3.6 C-D). Indicating MeJA and hematin concentrations dose-dependently enhance the growth of sorghum during germination under harsh environments. Based on these results, MeJA in addition to hematin promoted growth under non-saline stress and mitigated salt stress by enhancing germination and seedling growth of *S. bicolor* under salinity stress.

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CHAPTER 4

Physiological and biochemical analysis of the effect of methyl jasmonate on *Sorghum bicolor* growth under salt stress

ABSTRACT

Salt stress is one of the most detrimental abiotic stressors at the forefront of deterring crop productivity globally. It is important to find different ways to improve crop tolerance to salinity stress. The exogenous application of organic compounds such as osmolytes, microelements and phytohormones have formerly proven efficacious to plants, however their effects on the growth and development of a moderately tolerant crop like sorghum remains elusive. This chapter, therefore, reports mainly on the physiological and biochemical effects of methyl jasmonate (MeJA) pre-treatment on *Sorghum bicolor* in conferring salinity stress tolerance. Sorghum seeds were pre-treated with various concentrations of MeJA (0, 10, 15, 20 µM) and grown in potting soil under summer conditions (±22℃/26℃) followed by exposure to increasing NaCl (0, 100 and 200 mM NaCl) concentrations. Salinity stress reduced growth as evident by reduced shoot lengths, fresh weights and photosynthetic pigments. Furthermore, high levels of oxidative stress biomarkers such as reactive oxygen species (ROS) accumulation and lipid peroxidation was observed. In particular, considerably increased formazan precipitation on histochemical stained leaves representation of H_2O_2 content formation in addition to a 45.76% increment in MDA content was observed in salt treated sorghum plants. Salt also increased osmolyte content by inducing the synthesis of proline and soluble sugars by more than 150%. Methyl jasmonate pre-treated *S. bicolor* showed improved growth and development under salinity stress, as indicated by induced biomass and shoot height. It further led to the reversal of many deleterious effects of salinity stress on plant metabolism such as reduced MDA content, H_2O_2 accumulation in leaves, proline content and soluble sugars,

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whereas chlorophyll content was increased. Findings suggest that pre-treatment with 10 µM MeJA proved highly effective in ameliorating the effect of salinity stress in sorghum as indicated by the aforementioned physiological and biochemical assays.

Keywords: Salinity stress, pre-treatment, stress tolerance, biomarkers, lipid peroxidation, ROS, osmolyte, photosynthetic pigments, biomass.

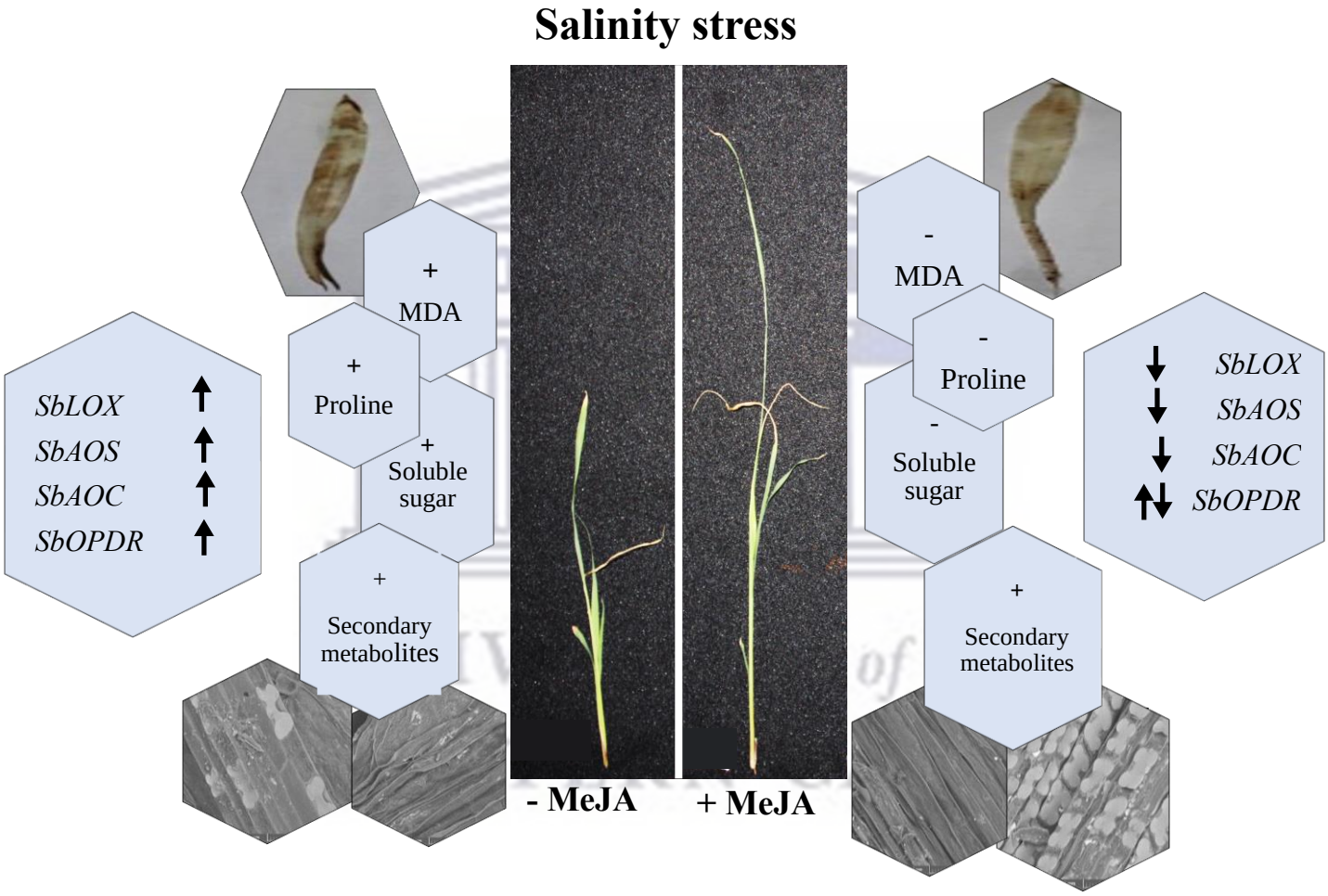

Plate: Graphical abstract of MeJA's role in salinity stress tolerance in sorghum. Salinity stress inhibited growth and increased oxidative damage, osmolyte accumulation and secondary metabolites. Additionally, increased epidermal shrivelling and induced transcripts of JA-biosynthesis pathway genes *SbLOX*, *SbAOS*, *SbAOC* and *SbOPDR*, while reducing silica phytoliths formation on epidermal tissue. MeJA pre-treated sorghum had improved growth and decreased oxidative damage, osmolytes and further increased secondary metabolites under salt stress. Additionally, smoother epidermis, increased silica phytoliths and predominantly down-regulated JA-biosynthesis pathway genes.

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4.1 INTRODUCTION

Salt stress is detrimental to plants and activates various coping mechanisms to deal with the damaging effects. Plants often have to make use of reserve energy to survive and therefore, induce peroxidation of its membrane lipids, which can be triggered by a range of factors including reactive oxygen species (ROS) formation and increased lipoxygenase activity as a result of stress (Morales & Munné-Bosch, 2019). Stressful environments induce the production of ROS molecules, which are harmful causing damages to lipids, proteins, carbohydrates, and nucleic acids (Du et al., 2014). Malondialdehyde (MDA) is one of the major products produced from lipid peroxidation and has therefore extensively been accepted as an oxidative stress marker (Kong et al., 2016). Plants counteract stressors by synthesizing signalling molecules such as osmolytes to stabilise the osmotic potential and inhibit the over-accumulation of free radicals. Organic solutes such as polyamines, proline and soluble sugars conserve cellular metabolism by protecting cell membranes and proteins (Sharma et al., 2019). The increase in cellular osmolality due to induced osmolytes has generally been accepted as cells maintaining adequate water to allow the necessary turgor for cell expansion (Hannachi & Van Labeke, 2018).

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Chloroplasts are imperative to plant metabolism and carry out essential functions such as fatty acid synthesis, stress signalling and are the main sites for the synthesis of osmolytes (Hameed et al., 2021). Photosynthesis is the main function of chloroplasts and is affected by elevated levels of salinity. Chlorophyll pigments are vital harvesters of light and largely determines plant photosynthetic capacity. High levels of salinity are known to adversely affect chlorophyll content and will ultimately induce photo-damage and capitulate reaction centres resulting in perturbed plant growth and yield (Hameed et al., 2021). Studies have suggested phytohormones as crucial in regulating chloroplast development and activity (Cortleven & Schmülling, 2015;

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Sharma et al., 2019). Phytohormones are essential regulators of plant response and may act synergistically and have antagonistic effects on growth and development such as the role that ethylene plays in inhibiting vegetative growth through its interaction with auxins (Liu & Hou, 2018). However, stressful conditions induce its activity, which will signal downstream developmental processes aimed at alleviating injury and is therefore pivotal in providing tolerance to plants under harsh conditions. Plant growth and yield are heavily reliant on auxins, cytokinins and gibberillins, whereas enhanced stress response largely involves the systemic interactions between abscisic acid, salicylates and jasmonates (Xu et al., 2018). Jasmonic acid (JA) play key roles in developmental processes including seed germination, plant maturity and senescence. Additionally, JA and its derivatives have been found to be highly responsive to salt stress and their concentrations increased in halophytes and glycophytes when exposed to salinity stress (Trifunović-Momčilov et al., 2021). Extensive research exists on the effect of jasmonates on plants under biotic and abiotic stresses. However, the role and mechanism by, which MeJA enhance salinity stress tolerance in *S. bicolor* remains elusive. Hence, in this chapter, the physiological and biochemical effects of pre-treating sorghum seeds with various concentrations of MeJA under non- saline and salinity stress conditions was assayed. The findings from this study are novel and will prove crucial in further elucidating the role of MeJA in salinity stress tolerance and ultimately establishing crops with improved abiotic stress tolerance.

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4.2 RESULTS

4.2.1 Effect of MeJA on the growth of S. bicolor under salt stress

In addition to the detrimental effect of the accumulating sodium and chlorine ions, salt stress hinders water and nutrient uptake. This perturbs cell expansion and significantly affect plant growth and biomass (Dastogeer et al., 2020). In this study salt stress affected *S. bicolor* growth as shown by a decrease in shoot length (SL) of 298 mm for 100 mM NaCl and 201 mm for 200 mM NaCl treated plants as compared to 408.3 mm obtained for control plants (Figure 4.1). This resulted in significant (*** = P≤0.001) decreases of 27.01% (100 mM NaCl) and 50.77% (200 mM NaCl) in SL. Salt stress also decreased sorghum fresh weight (FW) resulting in plant biomass of 0.662 g (100 mM NaCl) and 0.397 g (200 mM NaCl) as compared to 0.8 g for control plants (Table 4.1). A slight decrease in FW was observed when *S. bicolor* plants were stressed with 100 mM NaCl, however a significant (* = P≤0.05) decrease of 50.38% was observed in the FW of *S. bicolor* plants stressed with 200 mM NaCl as compared to the control. Additionally, salt stress decreased sorghum dry weight (DW) resulting in plant biomass of 0.089 g (100 mM NaCl) and 0.053 g (200 mM NaCl), as compared to control plants (0.053 g). Similarly, non-significant reduction was observed when plants were stressed with 100 mM NaCl, whereas a significant (* = P≤0.05) decrease of 41.76% in DW was observed in plants stressed with 200 mM NaCl.

To determine the role of MeJA in ameliorating the effects of salt stress in sorghum growth, morphological traits were also measured in MeJA pre-treated *S. bicolor* plants. Pre-treatment with MeJA showed no significance on the growth of *S. bicolor* under 100 mM NaCl stress (Figure 4.2 C). However, MeJA pre-treatment increased plant growth as evident by improved SL (Figure 4.2 D), FW and DW (Table 4.1) under 200 mM NaCl stress. Pre-treatment with 10 µM, 15 µM and 20 µM MeJA resulted in significant (*** = P≤0.001) increases in SL

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amounting to 60.7%, 53.53% and 74.13% under 200 mM NaCl stress, respectively. Additionally, MeJA pre-treatment (10, 15 and 20 µM) resulted in significant increases in FW of 83.88% (** = P≤0.01), 67.51% (* = P≤0.05) and 76.07% (** = P≤0.01) under 200 mM NaCl stress respectively. Significant (*** = P≤0.001) increases in DW were also observed, resulting in 107.55% (10 µM MeJA), 92.45% (15 µM MeJA) and 92.45% (20 µM MeJA) increases under 200 mM NaCl stress as compared to the control.

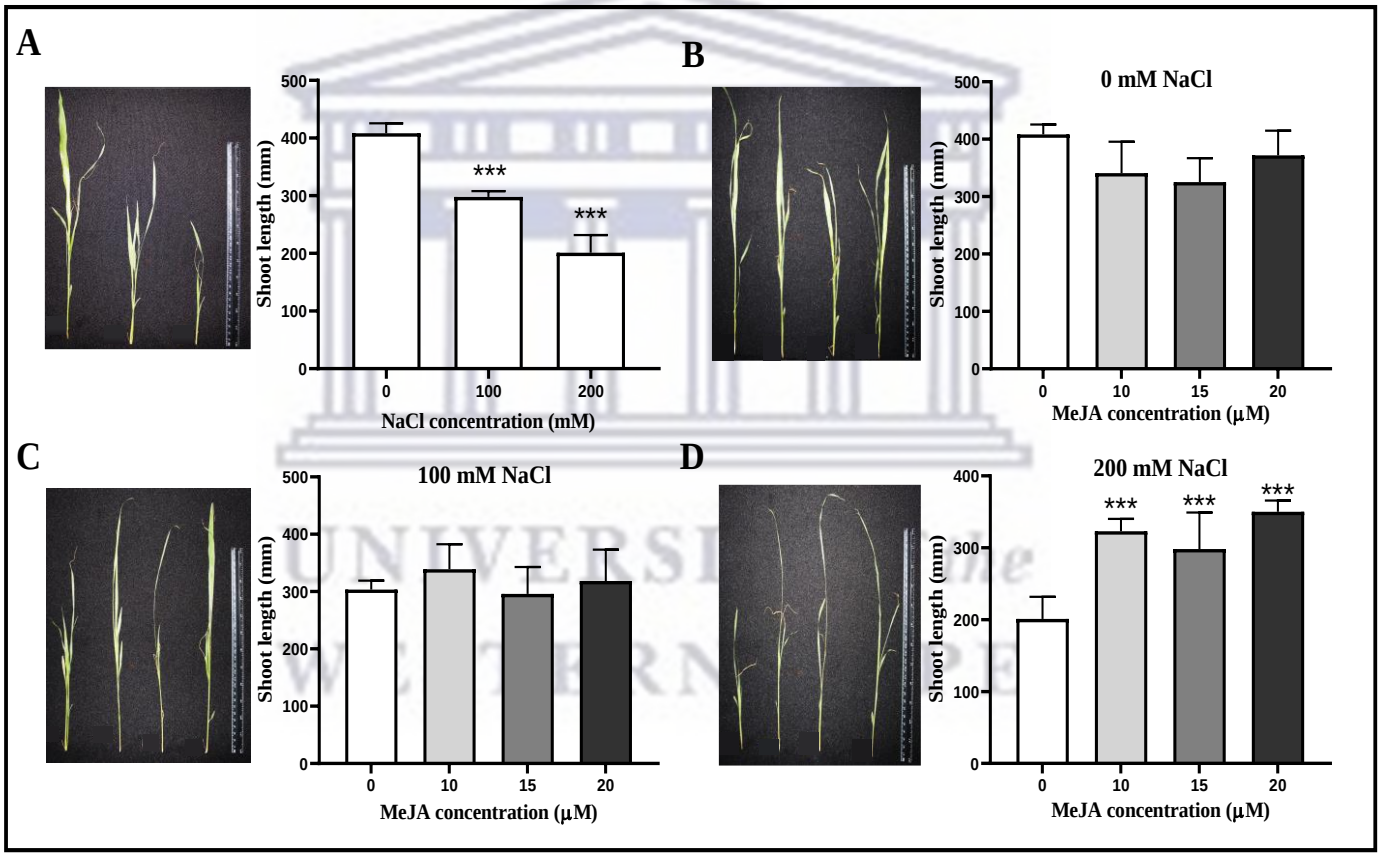

Figure 4.1: Shoot length of *S. bicolor* pre-treated with MeJA (10, 15 and 20 µM) in response to non-stress (0 mM NaCl) and salt stress (100 mM and 200 mM NaCl) conditions. A) Plantlets were exposed to increasing salt concentrations (100 mM and 200 mM NaCl). **B)** Plantlets were pre-treated in concentration of 10 µM, 15 µM and 20 µM MeJA under non-stress conditions. **C)** Plantlets were pre-treated with MeJA and grown in salt stress. Error bars represents SD from six biological replicates. Statistical significance was achieved using one-way ANOVA (Unstressed vs stressed) and two-way ANOVA (Salinity stress vs MeJA) (GraphPad Prism 9) where * = P≤0.05, ** = P≤0.01 and *** = P≤0.001 according to Bonferroni's multiple comparison.

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Table 4.1: Plant biomass of MeJA pre-treated *S. bicolor* in response to non-stress and salinity stress conditions. Fresh weight (FW) and dry weight (DW) were determined in *S. bicolor* pre-treated with 0 µM, 10 µM, 15 µM and 20 µM MeJA in response to control (0 mM NaCl) and salt stress conditions (100 mM and 200 mM NaCl). Data presented as mean ± standard deviation.

NaCl Concentration (mM)	MeJA concentration (µM)	Fresh weight (g)	Dry weight (g)
0	0	0,8±0,154	0,091±0,013
	10	0,773±0,005	0,084±0,005
	15	0,778±0,098	0,093±0,008
	20	0,914±0,130	0,108±0,015
100	0	0,662±0,102	0,089±0,014
	10	0,650±0,081	0,092±0,010
	15	0,680±0,110	0,095±0,011
	20	0,647±0,064	0,088±0,013
200	0	0,397±0,022*	0,053±0,005*
	10	0,730±0,025**	0,110±0,005***
	15	0,665±0,032*	0,102±0,004***
	20	0,699±0,036**	0,102±0,005***

Data represents the SD from three independent experiments from pooled plant material. Statistical significance was achieved using one-way ANOVA (Unstressed vs stressed) and two-way ANOVA (Salinity stress vs MeJA) (GraphPad Prism 9) where * = P≤0.05, ** = P≤0.01 and *** = P≤0.001 according to Bonferroni's multiple comparison.

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4.2.2 Effect of MeJA on the photosynthetic pigments in S. bicolor under salt stress conditions

Chlorophyll quantity is an important indicator of a plant's photosynthetic capacity (Li et al., 2018). Salinity stress had a slight impact on chlorophyll a where a non-significant decrease in chlorophyll a content was observed under salt stress (100 mM and 200 mM NaCl). However, increased chlorophyll a content was observed when *S. bicolor* was pre-treated with 15 µM (11.073 mg/ml) and 20 µM (10.985 mg/ml) MeJA in response to 100 mM NaCl resulting in significant (* = P≤0.05) increases of 194.42% and 192.08%, respectively, as compared to 3.761 mg/ml chlorophyll a content for control plants (100 mM NaCl only). Whereas increased chlorophyll a content for the 200 mM NaCl stressed plants was only observed when pre-treated

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with 10 µM MeJA (11.992 mg/ml) resulting in a 178.37% (* = P≤0.05) increase as compared to 4.308 mg/ml chlorophyll a content obtained in control plants (200 mM NaCl only). No significant effect was observed in chlorophyll b content for all treatments.

Table 4.2: Chlorophyll content of MeJA pre-treated *S. bicolor* in response to control and salinity stress conditions. Photosynthetic pigments (Chl a, Chl b and tot Chl) were determined in 21-day old *S. bicolor* pre-treated with 0 µM, 10 µM, 15 µM and 20 µM MeJA in response to control (0 mM NaCl) and salt stress conditions (100 mM and 200 mM NaCl). Data presented as mean ± standard deviation.

NaCl Concentration (mM)	MeJA concentration (µM)	Chlorophyll a (mg/ml)	Chlorophyll b (mg/ml)	Total Chlorophyll (mg/ml)
0	0	8.168±2.679	4.392±1.520	12.028±2.437
	10	7.510±0.753	4.402±0.584	11.686±1.310
	15	7.063±2.907	3.891±0.100	10.749±2.777
	20	6.167±0.289	3.656±0.875	9.636±0.561
100	0	3.761±0.499	3.202±1.007	6.236±1.447*
	10	10.6.86±1.841	5.182±0.036	15.580±1.785**
	15	11.073±4.523*	5.184±0.402	15.964±4.860**
	20	10.985±2.471*	4.934±0.022	15.635±2.463**
200	0	4.308±1.342	3.172±1.159	6.667±2.077*
	10	11.992±4.869*	5.011±0.729	16.705±5.518**
	15	9.104±0.928	4.585±0.726	13.438±0.214*
	20	10.453±2.280	4.515±0.135	14.703±2.123*

Data represents the SD from three independent experiments from pooled plant material. Statistical significance was achieved using one-way ANOVA (Unstressed vs stressed) and two-way ANOVA (Salinity stress vs MeJA) (GraphPad Prism 9) where * = P≤0.05 and ** = P≤0.01 according to Bonferroni's multiple comparison.

Control plants (0 mM NaCl) resulted in total chlorophyll content of 12.028 mg/ml, whereas salt stressed *S. bicolor* resulted in total chlorophyll content of 6.236 mg/ml (100 mM NaCl) and 6.667 mg/ml (200 mM NaCl). Evidently, salt-induced stress significantly decreased the total chlorophyll content of *S. bicolor* (Table 4.2) by 48.15% (* = P≤0.05) and 44.57% (* = P≤0.05) under 100 and 200 mM NaCl stress, respectively. A non-significant decrease in total chlorophyll content was observed in plants pre-treated with MeJA under control (0 mM NaCl)

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conditions. However, pre-treatment with MeJA significantly (** = P≤0.01) increased total chlorophyll content in plants stressed with 100 mM NaCl amounting to increases of 149.84% (10 µM MeJA), 155.99% (15 µM MeJA) and 150.72% (20 µM MeJA). Additionally, increased total chlorophyll content was also observed under 200 mM NaCl stress when pre-treated with 10, 15 and 20 µM MeJA resulting in total chlorophyll increases of 150.56% (** = P≤0.01), 101.56% and 120.53% (* = P≤0.05), respectively, as compared to 6.667 mg/ml total chlorophyll obtained for control plants (200 mM NaCl only).

4.2.3 Effect of MeJA on the oxidative damage in *S. bicolor* under salt stress

4.2.3.1 Effect of MeJA on ROS accumulation in S. bicolor under salt stress

ROS over-accumulation are known to be very detrimental to plants and may cause irreparable damage to biomolecules including DNA, protein, lipids and carbohydrates proving lethal when the plant's homeostasis gets disrupted. However, at physiological level these molecules are important signal transducers and regulators of plant growth and response (Choudhary et al., 2020). To determine the effect of salt stress in sorghum, this study examined the production of ROS using histochemical staining and lipid peroxidation by measuring the MDA content. For ROS, hydrogen peroxide (H_2O_2) levels were determined by histochemical staining based on its role in oxidising 3,3'-diaminobenzidene (DAB) in a reaction induced by horseradish peroxidase to form brown formazan at the location of enzyme activity (Daudi & O'brien, 2012). It is clear that salt stress induces the accumulation of H_2O_2 as indicated by increased levels of precipitation in response to 100 mM and 200 mM NaCl stress (Figure 4.2 A). *S. bicolor* pre-treated with 10 µM, 15 µM and 20 µM MeJA showed similar levels of precipitation under non-saline conditions (Figure 4.2 B). Pre-treatment with 10 µM, 15 µM and 20 µM MeJA resulted in substantial reductions in the formation of brown formazan (indicative of H_2O_2

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formation) on the leaves of *S. bicolor* exposed to 100 mM and 200 mM NaCl conditions (Figure 4.4 C-D).

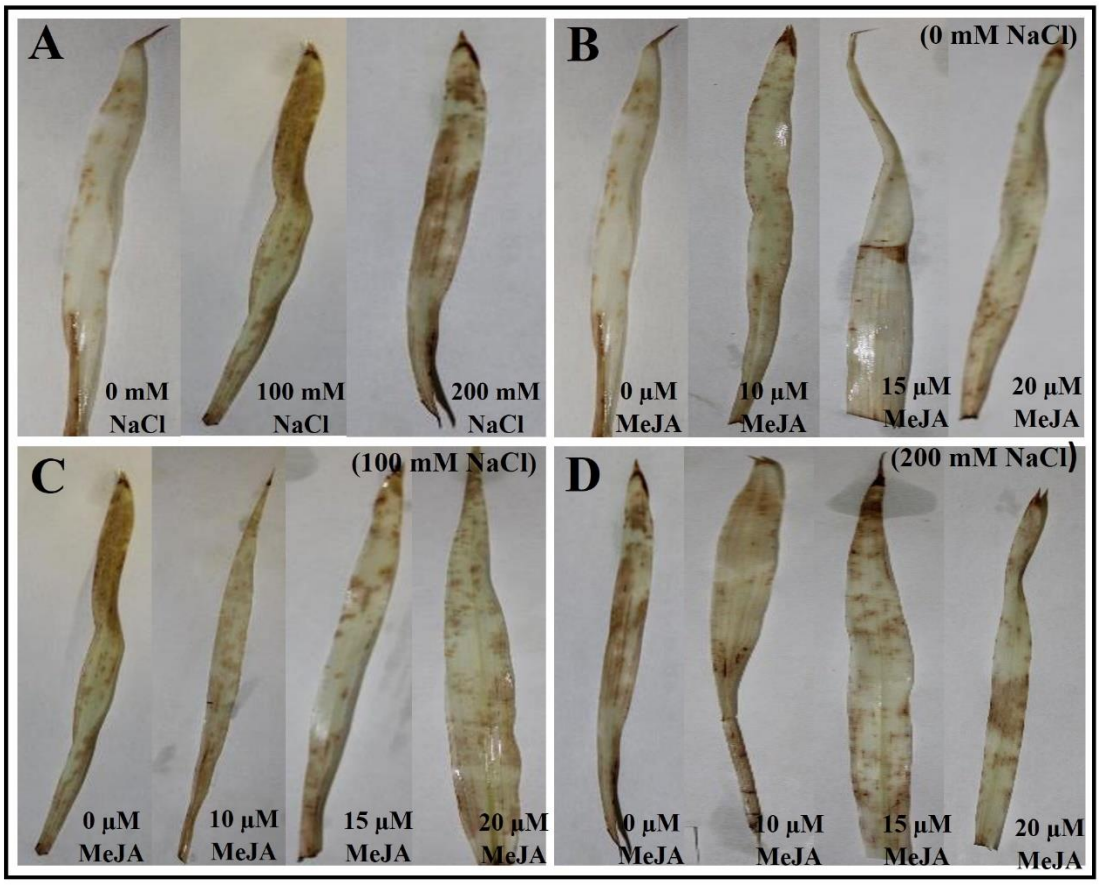

Figure 4.2: Histochemical detection of H_2O_2 in the leaves of *S. bicolor* in response to non-stress and salt stress when pre-treated with MeJA. (**A)** Plantlets were exposed to increasing salt concentrations (100 mM and 200 mM NaCl). Plantlets were pre-treated with 10 µM, 15 µM and 20 µM MeJA under (**B)** non-stress, (**C**) 100 mM NaCl and (**D**) 200 mM NaCl salt stress conditions.

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4.2.3.2 Effect of MeJA on lipid peroxidation in S. bicolor under salt stress

Lipid peroxidation is indicative of the oxidation of unsaturated fatty acids in the cell membrane whereby malondialdehyde is produced as a by-product (Vilcacundo et al., 2018). In this study, the traditional thiobarbituric acid (TBA) reaction method was utilised to determine the amount of MDA (nmol•g^{-1} FW) formed in sorghum plants. It is clear (Figure 4.3) that a gradual increase in salt concentration affected lipid peroxidation as indicated by an increase in MDA content of 24.66 nmol•g^{-1} FW for 100 mM NaCl and 26.15 nmol•g^{-1} FW for 200 mM NaCl stressed sorghum as compared to 17.94 nmol•g^{-1} FW MDA obtained for control plants (Figure 4.3 A). This amounted to significant increases of 37.46 % (100 mM) (*=P≤0.05) and 45.76% (200 mM NaCl) (** = P≤0.01) in MDA content in sorghum plants.

MDA levels in sorghum plants pre-treated with the various MeJA concentrations under non-stress conditions were slightly low (Figure 4.3 B). Sorghum subjected to MeJA pre-treatment resulted in low MDA levels of 19.641 nmol•g^{-1} FW (10 µM), 22.42 nmol•g^{-1} FW (15 µM) and 21.96 nmol•g^{-1} FW (20 µM) as compared to MDA content of 17.94 nmol•g^{-1} FW obtained in the control. Marginally increased levels in MDA content were observed in MeJA pre-treated *S. bicolor* under non-stress conditions. Evidently, pre-treatment with 10 µM MeJA had no significant effect on the MDA level, whereas pre-treatment with higher concentrations of MeJA showed significant (*=P≤0.05) increases of 24.97% (15 µM MeJA) and 22.4% (20 µM MeJA) in MDA levels of *S. bicolor* under non-stress conditions.

MeJA pre-treated *S. bicolor* plants resulted in decreased MDA content under saline conditions (Figure 4.3 C-D). Pre-treatment with MeJA in sorghum decreased MDA levels to 15.98 nmol•g^{-1} FW (10 µM MeJA), 23.24 nmol•g^{-1} FW (15 µM MeJA) and 24.49 nmol•g^{-1} FW (20 µM MeJA) from 24.67 nmol•g^{-1} FW MDA for plants treated with 100 mM NaCl only (Figure 4.3 C). Pre-treatment with MeJA resulted in MDA content of 20.34 nmol•g^{-1} FW (10 µM

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MeJA), 24.06 nmol•g^{-1} FW (15 µM MeJA) and 24.97 nmol•g^{-1} FW (20 µM MeJA) as compared to 26.15 nmol•g^{-1} FW obtained in plants treated with 200 mM NaCl stress (Figure 4.3 D). A significant decrease in MDA content was observed in *S. bicolor* pre-treated with 10 µM MeJA under both 100 mM (*** = P≤0.001) and 200 mM (** = P≤0.01) NaCl stress, amounting to decreases of 35.22% and 22.22%, respectively. Furthermore, non-significant reductions in MDA content were observed in plantlets pre-treated with 15 µM and 20 µM MeJA under both 100 mM and 200 mM NaCl stress.

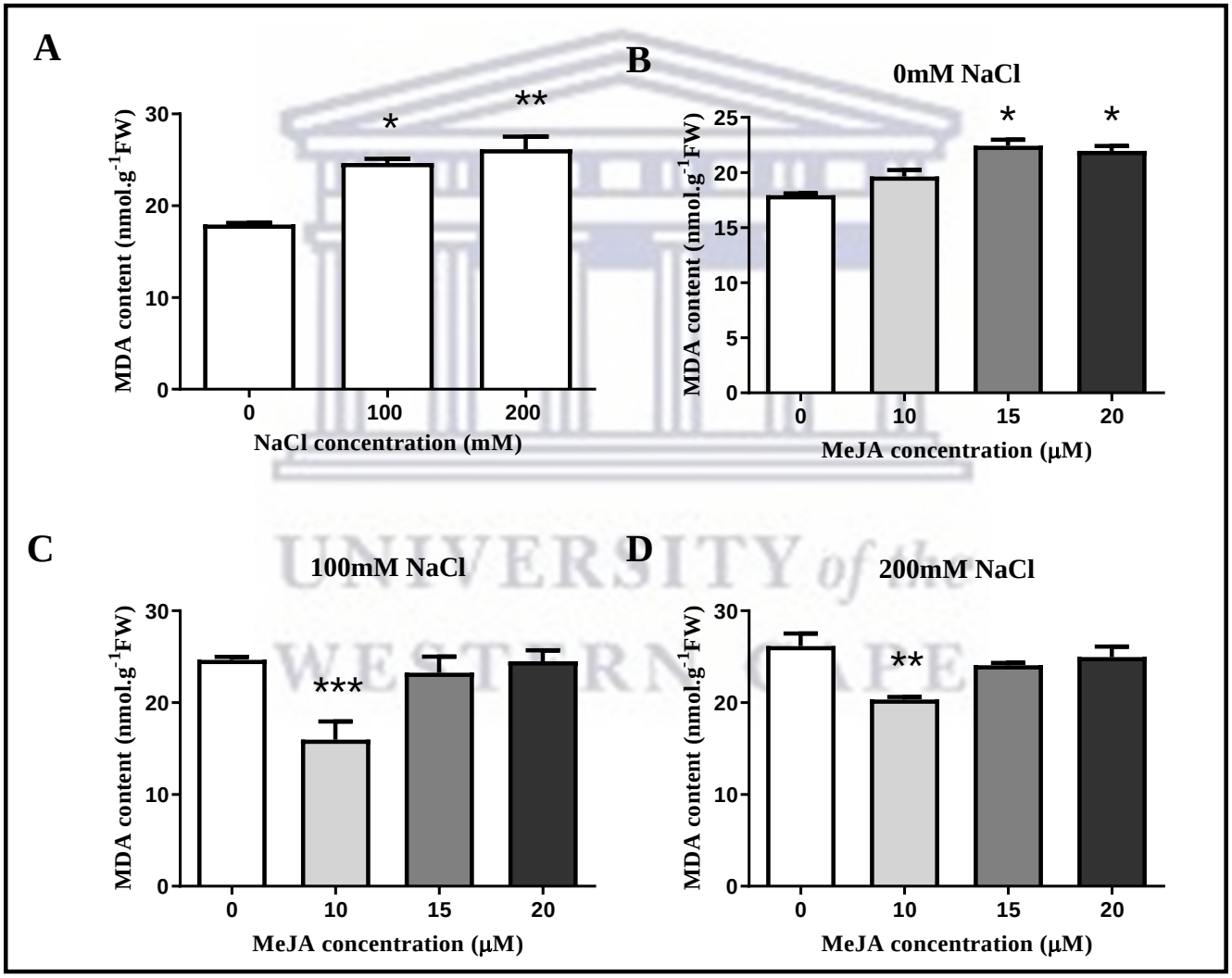

Figure 4.3: Lipid peroxidation of MeJA pre-treated (0, 10, 15 and 20 µM) *S. bicolor* in response to non-saline (0 mM NaCl) and salt stress (100 mM and 200 mM NaCl). (**A)** Plantlets were exposed to increasing salt concentrations (100 mM and 200 mM NaCl). Plantlets were pre-treated with 10 µM, 15 µM and 20 µM MeJA under (**B)** non-stress, (**C**) 100 mM NaCl and (**D**) 200 mM NaCl salt stress conditions. Error bars represents SD from three biological replicates from pooled plant samples. Statistical significance was achieved using one-way ANOVA (unstressed *S. bicolor* vs salt stressed *S. bicolor*) and two-way ANOVA (Salinity stress vs MeJA) (GraphPad Prism 9) where * = P≤0.05, ** = P≤0.01 and *** = ≤0.001 according to Bonferroni's multiple comparison.

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4.2.4 Effect of MeJA on osmolyte accumulation in *S. bicolor*

Osmolytes are rapidly triggered in response to abiotic stress aimed at protecting plants by regulating osmotic imbalance, sustaining cell turgor, and regulating redox potential without disrupting other important biochemical processes (Wang et al., 2019). Plants makes use of several osmolytes, but this study measured proline content and total soluble sugars. In addition to proline, soluble sugars function to prevent cellular dehydration by stabilising proteins and membranes. Furthermore, it plays a role in regulating metabolic processes including starch synthesis (de Morais et al., 2019; Živanović et al., 2020).

4.2.4.1 Effect of MeJA on proline levels in S. bicolor under salt stress

Proline is one of the important osmolytes in the plant's metabolism. It aids in plant survival, induces rapid recovery from stress and serves as an important indicator of the plant's physiological status (Hayat et al., 2012). Salt stress (100 mM NaCl and 200 mM NaCl) significantly increased proline content in sorghum plants as compared to the control plants (Figure 4.4 A). Proline content increased from 2.27 µmol/gFW (control) to 3.87 µmol/gFW under 100 mM (* = P≤0.05) NaCl treatment and 6.88 µmol/gFW under 200 mM (*** = P≤0.001) NaCl treatment, indicating increases of 70.48% (100 mM NaCl) and 203.08% (200 mM NaCl).

Control sorghum plants pre-treated with various MeJA concentrations were subjected to non-saline conditions to observe the effect of MeJA pre-treatment on plant development (Figure 4.4 B). Pre-treatment with MeJA resulted in slight increases of 2.47 µmol/gFW (10 µM MeJA), 3.04 µmol/gFW (15 µM MeJA) and 2.730 µmol/gFW (20 µM MeJA) µmol/g FW in proline content as compared to 2.27 µmol/g FW proline obtained in control plants. Non-significant increases in proline were observed in *S. bicolor* plants pre-treated with 10 and 20 µM MeJA.

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However, pre-treatment with 15 µM MeJA resulted in a 33.92% significant (* = P≤0.05) increase in proline content under non-stress conditions.

S. bicolor pre-treated with various MeJA concentration were subjected to stress condition in order to observe its response to salt stress (Figure 4.4 C-D). Increased proline content was observed in salt-stressed *S. bicolor* plants. However, *S. bicolor* plants pre-treated with MeJA had decreased proline content under salt stress. *S. bicolor* plants pre-treated with MeJA resulted in proline content of 2.43 µmol/gFW (10 µM MeJA), 3.63 µmol/gFW (15 µM MeJA) and 3.038 µmol/gFW (20 µM MeJA) in comparison to 3.87 µmol/gFW proline obtained from plants treated with 100 mM NaCl only (Figure 4.4 C). Non-significant reductions in proline content were clearly observed in *S. bicolor* plants under 100 mM NaCl stress. MeJA pre-treated *S. bicolor* plants resulted in proline content of 4.84 µmol/gFW (10 µM), 7.47 µmol/gFW (15 µM) and 4.73 µmol/gFW (20 µM) from 6.88 µmol/gFW obtained in control plants under 200 mM NaCl stress (Figure 4.5 D). Evidently, a significant reduction in proline content was observed in *S. bicolor* plants pre-treated with 10 µM (* = P≤0.05) and 20 µM (*** = P≤0.001) MeJA under 200 mM NaCl stress treatment, amounting to decreases of 26.65% (10 µM MeJA) and 31.25% (20 µM MeJA).

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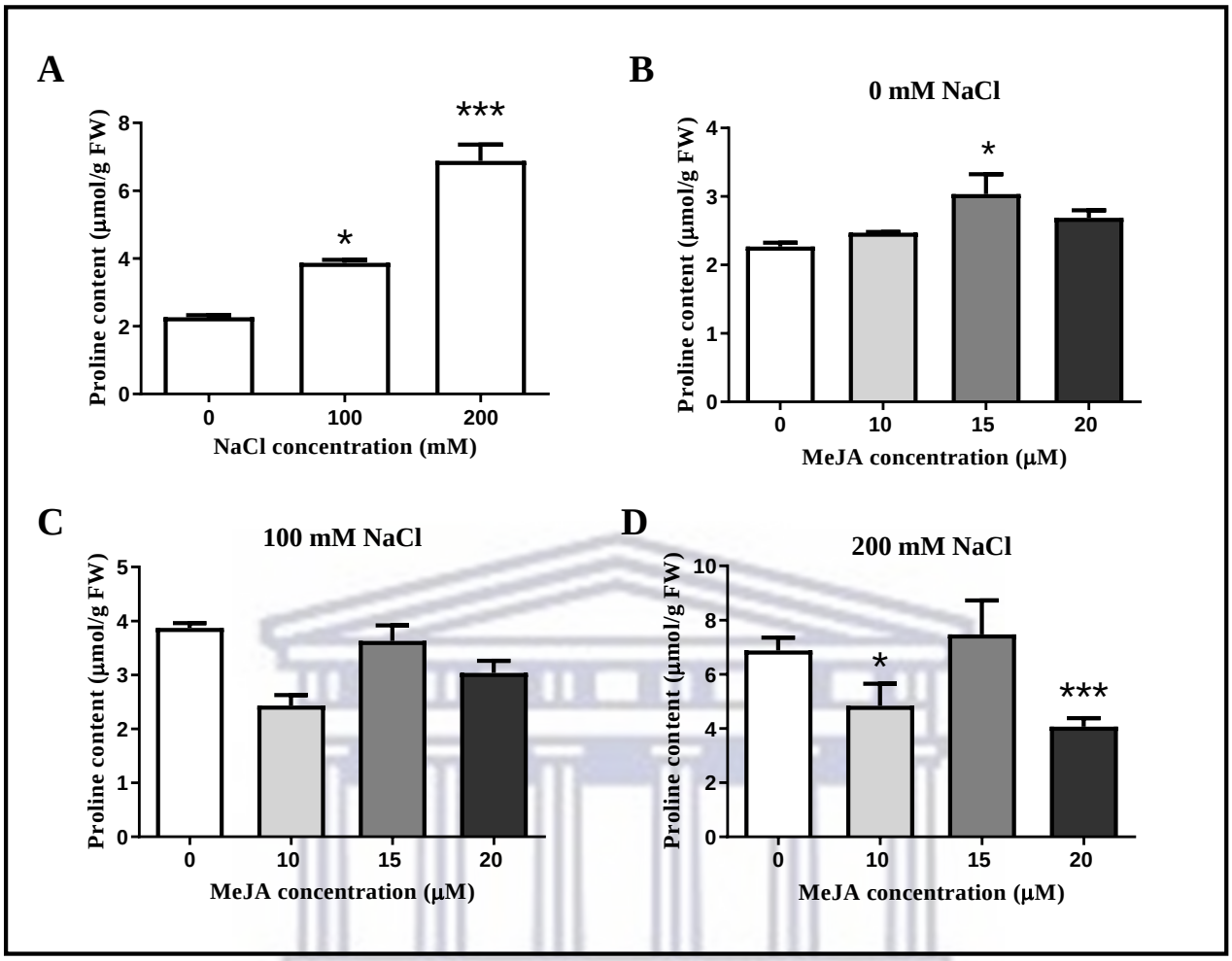

Figure 4.4: Proline content of MeJA pre-treated (0, 10, 15 and 20 µM) *S. bicolor* in response to non-saline (0 mM NaCl) and salt stress (100mM and 200 mM NaCl). (**A)** Plantlets were exposed to increasing salt concentrations (100 mM and 200 mM NaCl). Plantlets were pre-treated with 10 µM, 15 µM and 20 µM MeJA under (**B)** non-stress, (**C**) 100 mM NaCl and (**D**) 200 mM NaCl salt stress conditions. Error bars represents SD from three biological replicates from pooled plant samples. Statistical significance was achieved using one-way ANOVA (unstressed *S. bicolor* vs salt stressed *S. bicolor*) and two-way ANOVA (Salinity stress vs MeJA) (GraphPad Prism 9) where * = P≤0.05, 01 and *** P = ≤0.001 according to Bonferroni's multiple comparison.

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4.2.3.2 Effect of MeJA on soluble sugar content in S. bicolor under salt stress

Salt stress increased total soluble sugar (TSS) content in *S. bicolor* (Figure 4.5) resulting in increased TSS of 0.522 mg/ml (100 mM NaCl) and 0.767 mg/ml (200 mM NaCl) as compared to 0.296 mg.ml obtained from control plants (Figure 4.5 A). No significant increase in TSS was observed in *S. bicolor* under 100 mM NaCl stress. However, a 159.12% significant (* = P≤0.05) increase in TSS was observed in *S. bicolor* plants under 200 mM NaCl stress.

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To test the effect of MeJA pre-treatment on the TSS content of *S. bicolor* plants at physiological level, *S. bicolor* plants were subjected to pre-treatment with 0, 10, 15 and 20 µM MeJA and grown under non-saline conditions (Figure 4.5 B). Under non-stress conditions MeJA pre-treated *S. bicolor* plants resulted in TSS content of 0.303 mg/ml (10 µM MeJA), 0.305 mg/ml (15 µM MeJA) and 0.367 mg/ml (20 µM MeJA) as compared to 0.296 mg/ml TSS obtained in control plants. Evidently, MeJA pre-treatment resulted in no significant effect in TSS content in unstressed *S. bicolor* plants.

S. bicolor plants pre-treated with various MeJA concentration were subjected to stress condition in order to observe its effect on the levels of TSS in response to salt stress (Figure 4.6 C-D). Pre-treated *S. bicolor* plants exposed to 100 mM NaCl resulted in marginal decreases in TSS content of 0.472 mg/ml (10 µM MeJA), 0.346 mg/ml (15 µM MeJA) and 0.609 mg/ml (20 µM MeJA) as compared to 0.522 mg/ml TSS obtained from control plants (Figure 4.5 C). Evidently, no significant reductions in TSS content of pre-treated *S. bicolor* plants were observed under 100 mM NaCl stress. Furthermore, pre-treated *S. bicolor* plants exposed to 200 mM NaCl stress resulted in TSS content of 0.856 mg/ml (10 µM), 0.575 mg/ml (15 µM) and 0.412 mg/ml (20 µM) from 0.767 mg/ml TSS obtained in control plants (Figure 4.5 D). Although reduced level of TSS was observed in *S. bicolor* plants pre-treated with MeJA, only pre-treatment with 20 µM MeJA resulted in a 46.28% significant (* = $P \leq 0.05$) decrease in TSS under 200 mM NaCl stress.

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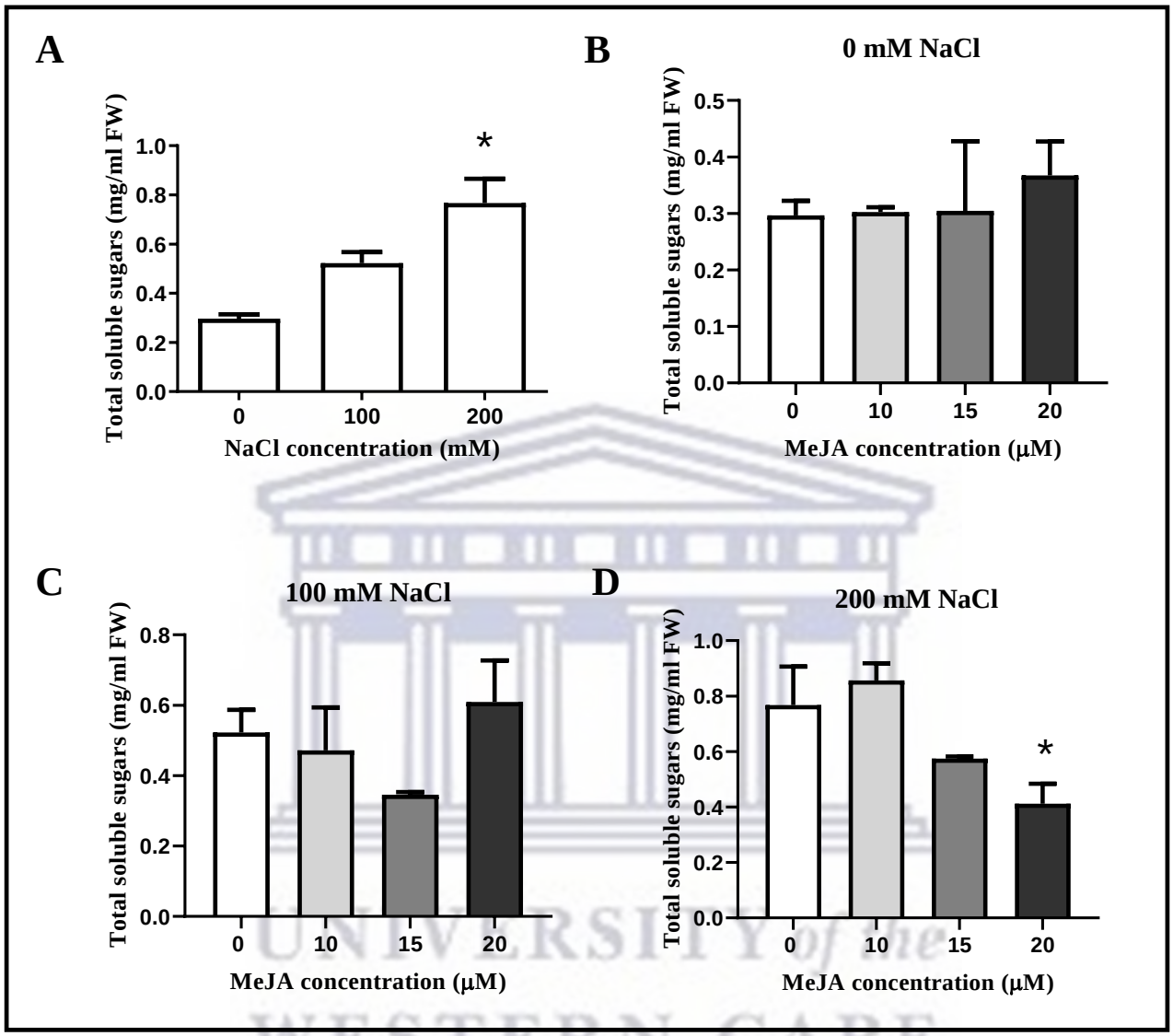

Figure 4.5: Total soluble sugar content of MeJA pre-treated (0, 10, 15 and 20 µM) *S. bicolor* in response to non-saline (0 mM NaCl) and salt stress (100mM and 200 mM NaCl). (**A)** Plantlets were exposed to increasing salt concentrations (100 mM and 200 mM NaCl). Plantlets were pre-treated with 10 µM, 15 µM and 20 µM MeJA under (**B)** non-stress, (**C**) 100 mM NaCl and (**D**) 200 mM NaCl salt stress conditions. Error bars represents SD from three biological replicates from pooled plant samples. Statistical significance was achieved using one-way ANOVA (unstressed *S. bicolor* vs salt stressed *S. bicolor*) and two-way ANOVA (Salinity stress vs MeJA) (GraphPad Prism 9) where * = P≤0.05, according to Bonferroni's multiple comparison.

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4.3 DISCUSSION

Salt stress is detrimental to plants and its management is of great importance in order to alleviate food insecurity. Understanding plant stress adaptation mechanisms is crucial to establishing crops with enhanced tolerance to different stresses. To respond to this, chapter four focused on the role MeJA in enhancing salt stress tolerance in *Sorghum bicolor* at physiological and biochemical level.

4.3.1 MeJA improves growth of Sorghum bicolor under salt stress

Salt stress caused significant reduction in the growth of *S. bicolor*. An increase in salt concentration resulted in the decrease of shoot length (Figure 4.1 A). Therefore, salt concentration proved indirectly proportional to the growth of sorghum. The inhibitory effect of salinity stress on the growth of plants are well documented and can be observed with the decreasing shoot lengths of *Solanum lycopersicum* L. (Ahmad et al., 2018), *Chenopodium quinoa* (Cai & Gao, 2020) and *Triticum aestivum* L. (Zhang et al., 2022) in response to salt stress. In addition to inhibiting cell division and elongation, the decrease in growth might also be due to decreased water uptake, energy diversion for survival and the decrease in carbon caused by salt stress (Ahmad et al., 2018).

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Pre-treatment with MeJA had no significant impact on shoot length of sorghum under physiological conditions and only slightly increased shoot length of *S. bicolor* under 100 mM salt stress. However, under harsh environment (200 mM NaCl) MeJA pre-treated (10, 15 and 20 µM MeJA) sorghum had significantly increased shoot length in response to salt stress. This finding aligned with results obtained in *Oryza sativa* L. (Sheteiwy et al., 2018) under polyethylene glycol (PEG) induced osmotic stress and in *Cicer arietinum* L. (Lalotra et al., 2019) and *Crithmum maritimum* L. (Labiad et al., 2021) under salinity stress. Furthermore, these results were emulated in the fresh and dry weights of sorghum under salt stress. This

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proved deleterious by greatly reducing biomass of sorghum plants as indicated by a decrease in both fresh and dry weight under harsh (200 mM) salt stress conditions (Table 4.1). Pre-treatment with MeJA proved beneficial to plant biomass under salt stress conditions. Sorghum plants pre-treated with MeJA (10, 15 and 20 µM MeJA) had significantly increased fresh and dry weights under harsh levels of salt stress. The exogenous application of MeJA also resulted in increased plant biomass in *Brassica napus* L. (Ahmadi et al., 2018), *Triticum aestivum* L. (Avalbaev et al., 2021) and *Phaseolus vulgaris* L. (Mohi-Ud-din et al., 2021) under abiotic stress. The enhancing growth under salt stress may be due to MeJA's role in inducing the plant's survival strategies and jasmonic acid's role in promoting cell expansion (Tayyab et al., 2020).

4.3.2 MeJA improves photosynthetic pigments of S. bicolor in response to salt stress

Chlorophyll is a vital pigment in photosynthesis and can indirectly be used as indicator of the plant's nutritional status (Liang et al., 2017). These pigments are susceptible to nutrient deficiency and environmental stress. To assess the effect that salinity stress has on the photosynthetic pigments (Chl_a, Chl_b and Chl_{tot}), *S. bicolor* was exposed to increasing salt (0 mM, 100 mM and 200 mM NaCl) concentration. Based on the result obtained (Table 4.1), salinity stress proved deleterious by significantly decreasing chlorophyll content in *S. bicolor,* and therefore inhibiting plant photosystem (Saddiq et al., 2021). This study is supported by previous studies where exposure to salinity stress reduced chlorophyll content in *Calendula officinalis* L., *Oryza sativa* and *Glycine max* (Baniasadi et al, 2018; Nounjan et al., 2020; Khan et al., 2021). The reduction of chlorophyll content could be indicative of restricted chlorophyll biosynthesis in conjunction with the induction of chlorophyllases (Taïbi et al., 2016).

The chlorophyll content was evaluated in *S. bicolor* pre-soaked in increasing MeJA concentration to determine its effect on photosynthetic pigments at physiological level. Results indicated that increasing MeJA concentration resulted in non-significant decreases in

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chlorophyll content under non saline conditions. The effect of MeJA on the chlorophyll content of *S. bicolor* was assessed in response to salt stress and indicated that MeJA was able to reverse the deleterious effect of salt stress on chlorophyll content by significantly inducing chlorophyll content under saline conditions. These results agreed with previous observations in *Brassica napus* (Ahmadi et al., 2018), *Anschusa italica* (Taheri et al., 2020), and *Citrus sinensis* (Mahmoud et al., 2021) when exposed to salinity stress. The increased chlorophyll content in MeJA pre-treated *S. bicolor* in response to salt stress might be due to MeJA playing a role in the formation of 5-aminolevulinic acid, which is a vital enzyme in the biosynthesis of chlorophyll (Taheri et al., 2020).

4.3.3 MeJA reduces oxidative stress in S. bicolor

Maintaining the steady state of ROS molecules, including H_2O_2, is important for regulating many molecular mechanisms in plant cells and over-accumulation is detrimental to plants causing severe oxidative damage (Nguyen et al., 2016). Salt stress strongly affected ROS homeostasis by inducing the abundance of H_2O_2 and therefore inducing oxidative damage in sorghum plants (Figure 4.2). This was illustrated by the over-accumulation of brown formazan, from a reaction catalysed by H_2O_2, on the leaves of *S. bicolor* in response to increasing salt stress conditions (100 mM and 200 mM NaCl). Pre-treatment with MeJA had no substantial effect on H_2O_2 content under non-stress conditions (Figure 4.2 B). This was indicated by similar formation of brown precipitate on pre-treated sorghum as compared to control under non-stress condition. Consistent with MeJA's role in reversing the deleterious effect of salt stress on the photosynthetic pigments of sorghum plants, pre-treated *S. bicolor* showed substantial decreases in ROS accumulation under saline conditions (100 mM and 200 mM NaCl) (Figures 4.2 C-D). Clear differences in H_2O_2 abundance were observed in pre-treated sorghum as indicated by substantial decreases in formazan formed on sorghum leaves under both salt stress conditions. These results illustrated MeJA's influence in scavenging H_2O_2 under

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harsh conditions. The ameliorating effect that MeJA has on the over-accumulation of H_2O_2 in response to abiotic stress was also observed in *Glycyrrhiza uralensis* (Lang et al., 2020), *Zea mays* (Tayyab et al., 2020) and *Vitis vinifera* L. (Karimi et al., 2022). The reduced ROS formation might be due to MeJA's role in maintaining the stability of the chloroplast by protecting the photosystem II (Fatma et al., 2021).

The cell membrane is a very important structure and houses intricate properties crucial for survival. Disruption in membrane properties can be deleterious due to the inhibition of ionic transport eventually leading to cell death (Vilcacundo et al., 2018). Increased levels of lipid peroxidation were observed when *S. bicolor* was subjected to salt stress (100 mM and 200 mM NaCl). This was indicated by the increasing level of malondialdehyde (MDA) in response to the increasing level of salt stress (Figure 4.3 A). The findings were previously supported by observations in *Oryza sativa* (Vighi et al., 2017), *Ocimum basilicum* (Farsaraei et al., 2020), *Zea mays* (Shah et al., 2021) and *Sorghum bicolor* (Rakgotho et al., 2022) when exposed to salinity stress. The elevated levels of lipid peroxidation could be as a result of limited stomatal conductance and increasing lipid membrane damage caused by ROS molecules due to osmotic stress (Wu et al., 2017).

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Pre-treatment with MeJA dose dependently increased lipid peroxidation of sorghum plants under normal conditions (Figure 4.3 B). Pre-treated sorghum had slight increments in MDA content, only significantly increasing MDA content at higher concentrations (15 µM and 20 µM MeJA). The increase in lipid peroxidation in resposnse to exogenous MeJA was also observed in *Solanum lycopersicum*, *Nitraria tangutorum* and *Triticum aestivum* (Wang et al., 2018; Gao et al., 2022; Javadipour et al., 2021). The increase in lipid peroxidation, which may be as a result of hormonal imbalance indicated excess MeJA as being a strong inducer of oxidative stress at physiological level (Zuñiga et al., 2020; Gao et al., 2022). Although MeJA

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induced lipid peroxidation under normal conditions, alleviated levels of lipid peroxidation were observed in sorghum plants pre-treated with 10 µM MeJA under salt stress (100 mM and 200 mM NaCl) (Figure 4.3 C-D). Significantly reduced level of MDA content was observed in MeJA pre-treated *S bicolor* plants under both concentrations of salt stress illustrating the dose-dependent ameliorating effect of MeJA on lipid peroxidation under harsh conditions. Decreasing MDA content under abiotic stress was also observed in *Brassica napus*, *Nitraria tangutorum* and *Pisum sativum* (Ahmadi et al., 2018; Gao et al., 2022; Manzoor et al., 2022). Pre-treatment with MeJA therefore played a beneficial role maintaining cell membrane complexity under stressful conditions. This ameliorating effect under stress conditions might be due to MeJA superior molecule's (jasmonic acid) role in scavenging free radicals (Kaya & Doganlar, 2016).

4.3.4 MeJA mediates osmoprotectants of S. bicolor under salt stress

Assaying the amount of free proline found in plants is another method of assessing plant tolerance to stress and over-accumulation is often associated with severely perturbed metabolism whereby these molecules would aid protection against disease and stress mitigation in plants (Hayat et al., 2012). In this study the proline content in *S. bicolor* was assessed in response to salt stress. Salt stress disturbed osmotic potential and thereby triggered the over-accumulation of proline in sorghum plants (Figure 4.4 A). Significant increments in proline content were observed with increasing salt concentration, illustrating a directly proportional relation between salt stress and proline content in *S. bicolor*. The increasing levels of proline in response to adverse conditions is well documented as observed in *Solanum lycopersicum* L., *Triticum turgidum* and *Hordeum vulgare* L. (De la Torre-González et al., 2018; Ami et al., 2020; Torun et al., 2022). This increasing proline content is as a result of osmotic adjustment in aid of survival under harsh environment (Torun et al., 2022).

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Pre-treatment with MeJA induced proline content under non-stress conditions (Figure 4.4 B). Sorghum plants pre-treated with 15 µM MeJA had significantly increased proline level under normal conditions. This increased proline level in response to exogenous MeJA at physiological level coincides with observations made in *Zea mays* L., *Anchusa italica* and *Cichorium pumilum* (Luo et al., 2020; Taheri et al., 2020; Sarabi & Arjmand-Ghajur, 2021). The elevated level of proline could be indicative of osmotic adjustment and the signalling of other metabolites including polyphenols and ascorbic acid (Luo et al., 2020; Tayyab et al., 2020). Similarly, even though increased proline content was observed under non stress conditions, sorghum plants pre-treated with 10 µM and 20 µM MeJA significantly decreased proline content under harsh salt stress conditions (200 mM NaCl) (Figure 4.4 D). In agreement with the results obtained, the decreasing levels of proline content under harsh environments was also observed in *Anschusa italica, Zea mays* and *Origanum vulgare* L (Taheri et al., 2020; He et al., 2021; Jafari et al., 2022). The increasing proline is generally accepted as decreasing osmotic potential. This result, therefore, illustrated the role of MeJA in alleviating disturbance in osmotic potential in aid of maintaining cell turgor in sorghum plants under salt stress (Taheri et al., 2020).

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In addition to proline, soluble sugars also play a crucial role in osmoregulation. Soluble sugars are crucial constituents of cellular respiration making it an important energy source for plants (Živanović et al., 2020). Salt stress induced soluble sugar content in sorghum plants (Figure 4.5 A). Based on the result obtained, salinity stress only significantly affected soluble sugar accumulation at higher concentration (200 mM NaCl). The increased soluble sugar in response to adverse conditions is a common occurrence that has been observed in *Triticum aestivum* L, *Pisum sativum* and *Vicia faba* L (Kanwal et al., 2018; Ahmad et al., 2020; Nasrallah et al., 2022). The increasing soluble sugar content is as a result of osmotic adjustment and scavenging

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of ROS molecules. Additionally, the increased soluble sugar content would aid in providing energy for survival under adverse conditions (Nasrallah et al., 2022).

Pre-treatment with MeJA had no effect on soluble sugar level under control condition (Figure 4.5 B). As observed with relatively stable levels of soluble sugar in MeJA pre-treated sorghum in response to non-saline conditions. Pre-treatment with MeJA had no significant effect on soluble sugar under 100 mM NaCl stress (Figure 4.5 C). However, it proved efficacious in reducing soluble sugar content under increased level of salt stress. From this study, MeJA and salt stress dose-dependently affected sugar metabolism in *S. bicolor* (Figure 4.5 D). Although decreasing soluble sugar content was observed with pre-treatment with increasing levels of MeJA, soluble sugar content only significantly reduced in 20 µM MeJA pre-treated sorghum under 200 mM NaCl stress. It is well documented that exogenous application of MeJA increase soluble sugar under abiotic stress, but as in this study the decreasing level of soluble sugar content due to MeJA under adverse conditions were consistent with those observed in *Glycyrrhiza uralensis* and *Anchusa italica* (Lang et al., 2020; Taheri et al., 2020). The increasing effect in response to salt stress and then diminishing effect in MeJA pre-treated sorghum plants could be as result of a shift to the usage of secondary metabolites or MeJA role in eliciting early response to adverse conditions during pre-treatment (Nguyen et al., 2020; Nasrallah et al., 2022).

Briefly, this chapter showed that MeJA mitigated salt stress in *S bicolor* by improving growth and photosynthesis as indicated by increased chlorophyll content under salt stress, while reducing oxidative damage as indicated by decreased ROS accumulation and lipid peroxidation. Additionally, MeJA played an important role in regulating osmotic stress by decreasing the levels of proline and soluble sugars under salt stress.

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CHAPTER 5

Microscopic and gene expression analysis of the effect of methyl jasmonate on *Sorghum bicolor* under salt stress

ABSTRACT

The ameliorating response of *S. bicolor* to pre-treatment with MeJA under salinity stress incited its investigation at ultrastructural and molecular levels. In this chapter, the anatomical structure, biomolecules and transcript levels of jasmonate related genes such as *Sorghum bicolor* lipoxygenase (*SbLOX*), allene oxide synthase (*SbAOS*), allene oxide cylase (*SbAOC*) and 12-oxophytodecanoic acid reductase (*SbOPDR)* were investigated in sorghum plants pre-treated with MeJA under salt stress. Based on the morphological and physiological results, emphasis was placed on the effect of 200 mM NaCl, whereas only 10 µM and 15 µM MeJA were considered effective in alleviating the effects of salt stress. Results were achieved using scanning electron microscopy (SEM), Fourier-transform infrared (FTIR) spectroscopy and real-time quantitative polymerase chain reaction (RT-qPCR). Salt stress caused severe damage to the structure of sorghum plants resulting in shrinkage and deformation of the vascular bundle tissue (xylem and phloem) and epidermis layers, in addition to decreased formation of silica phytoliths. However, these effects were reversed by treatment with MeJA. FTIR spectra confirmed reduced degradation of biomolecules correlating with alleviated oxidative injury in MeJA-pre-treated sorghum plants under salt stress. Salt stress induced *SbLOX, SbAOS, SbAOC* and *SbOPDR* transcripts. A further upregulation of *SbOPDR* was observed in salt-stressed *S. bicolor* plants pre-treated with 10 µM MeJA, whereas the transcripts level of the other genes decreased in MeJA-treated plants. Evidently, 10 µM MeJA proved effective in enhancing the tolerance of *S. bicolor* to salinity stress.

Keywords: Ultrastructural, molecular, SEM, FTIR, RT-qPCR, silica phytoliths

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5.1 INTRODUCTION

Research is expanding exponentially and requires the utilisation of high throughput technologies to contribute to data outputs due to its high processing speeds, accuracy, and reliability, which has proven pivotal in molecular biology. Ultrastructural responses of plants to stress stimuli are equally important in understanding stress tolerance mechanisms. Scanning electron microscopy (SEM) is highly recommendable in investigating the plant's ultrastructure due to its high resolution microphotographs from small samples. Microscopic analysis allows for in-depth investigation of the plant's initial response to stress including alterations in the vascular system and epidermis under unfavourable conditions. The plant's vascular system is important in the uptake and distribution of water and nutrients. Any obstructions thereof can limit the growth and development of distant tissues (Hwang et al., 2016; Pacheco-Silva & Donato, 2016; Li et al., 2021). The epidermis is the outermost photoderm-derived cell layers of the primary plant body protecting plants by playing crucial roles in water relation and defence (Glover, 2000). Leaf epidermis may control organ growth by restricting elongation due to interconnection between the rigid outer coat and inner cell layers (Zörb et al., 2015). Additionally, the epidermis of most grasses may also have silica bodies as adaptive strategy for survival formed by the uptake of non-essential nutrient, for example silicone (Si), to form microstructures known as phytoliths. These phytoliths may act as a mechanical barrier positively affecting the plant's fitness and productivity (Rudall et al., 2014; Nawaz et al., 2019).

Adaptive strategies are dependent on the concomitant interactions in the plant metabolism. Expanding technology has allowed for obtaining large amounts of information from a single sample. Fourier-transform infrared (FTIR) spectroscopy is one of the fast and precise technology to reflect the overall composition of samples by detecting alterations in biomolecules such as lipids, carbohydrates, lipopolysaccharides, nucleic acids and proteins

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through infrared spectra (Kazarian, 2007; Zimmermann et al., 2017; Vogt et al., 2019). Furthermore, Real-time quantitative polymerase chain reaction (RT-qPCR) is a highly efficient, accurate and relatively cost-effective method for quantifying large number of nucleic acids. Its extensive usage in gene expression studies proved vital in understanding adaptations of cells to developmental transitions or environmental changes (Exner, 2010; Furda et al., 2014).

Model crops are crucial in assessing plant responsive changes to stress. Sorghum is an important cereal crop for gaining better understanding and fully elucidating salt stress mechanisms at molecular levels, which will be crucial in generating crops with enhanced tolerance to stress. Extensive research is available on the genome of sorghum making it easy to measure the transcript levels of target genes. Several studies successfully demonstrated gene expression analysis of important pathways in sorghum including ion channel Salt Overlay Sensitive (Kumar et al., 2009; Mulaudzi et al., 2020), sodium-proton antiporter NHX (Kumari et al., 2018) antioxidant enzymes (Dudziak et al., 2019; Mulaudzi et al., 2020; Sujeeth et al., 2020), heme oxygenase genes (Mulaudzi-Masuku et al., 2019) and genes involved in the jasmonic acid biosynthesis pathway (Wang et al., 2016; Ye et al., 2017). Jasmonic acid is an important phytohormone for plant growth and development and can also serve as stress signalling molecule. Its biosynthesis is reliant on the concomitant action of enzymes including LOX, AOS, AOC and OPDR. Understanding the role of this pathway in salinity stress tolerance at transcript level will further elucidate its mechanism used in enhancing stress tolerance. The aim of this chapter was to investigate the effect of salt stress and pre-treatment with MeJA on the anatomical structure, biomolecules and the jasmonic acid biosynthesis pathway in *S. bicolor* at a genetic level.

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5.2 RESULTS

5.2.1 MeJA improved the anatomical structure of sorghum under salinity stress

5.2.1.1 Effect of MeJA pre-treatment on the vascular bundle of S. bicolor under salt stress

The response of the *Sorghum bicolor'*s plant tissue to pre-treatment with MeJA and exposure to salinity stress at microscopic level was examined using scanning electron microscopy (Figure 5.1). No great effect was observed in the xylem and phloem of *S. bicolor* under 200 mM NaCl stress treatment (Figure 5.1 D) as compared to control plants (Figure 5.1 A). Similarly, pre-treatment with 10 µM and 15 µM MeJA had no great effect on the xylem and phloem of *S. bicolor* under non-saline conditions (Figure 5.1 B & C) and 200 mM NaCl stress (Figure 5.1 E & F).

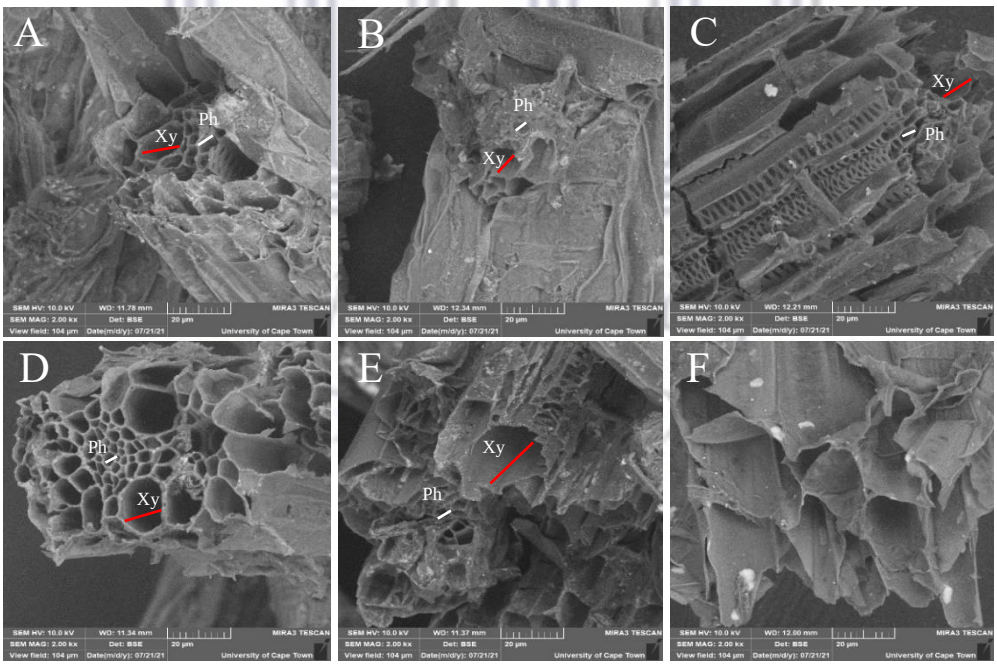

Figure 5.1: Scanning electron microscopy illustrating the vascular bundle tissue of MeJA pre-treated *S. bicolor* exposed to non-saline and salt stress. Cross section imaging *S. bicolor* xylem (Xy) indicated by the red bar and phloem (Ph) indicated by the white bar under (**A**) 0 mM NaCl, (**B**) 0 mM NaCl + 10 µM MeJA, (**C**) 0 mM NaCl + 15 µM MeJA, (**D**) 200 mM NaCl, (**E**) 200 mM NaCl + 10 µM MeJA and (**F**) 200 mM NaCl + 15 µM MeJA.

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5.2.1.2 Effect of MeJA on the epidermal structure in S. bicolor under salt stress

The epidermis forms part of the first line of plant defence, preventing rapid water loss and excessive uptake of harmful substances (Zörb et al., 2015; Pacheco-Silva & Donato, 2016; Brookbank et al., 2021). It is therefore crucial in understanding the plant's early responses and regulation of stress related alterations and adaptations (Hwang et al., 2016; Pacheco-Silva & Donato, 2016; Kollist et al., 2019). SEM microphotographs of *S. bicolor*'s epidermis indicated a slight rough and shrunken layers under 200 mM NaCl treatment (Figure 5.2 D) as compared control plants (Figure 5.2 A). *S. bicolor* plants pre-treated with 10 µM MeJA had no effect on the epidermal layers and remained relatively smooth under non-saline conditions (Figure 5.2 B), but the epidermis of the 15 µM MeJA-pre-treated plants (Figure 5.2 C), showed slight shrinkage as compared to the control (Figure 5.2 A). However, *S. bicolor* plants pre-treated with 15 µM MeJA had a considerably smooth epidermis under 200 mM NaCl treatment (Figure 5.2 F) as compared to those plants under salt stress only (Figure 5.2 D).

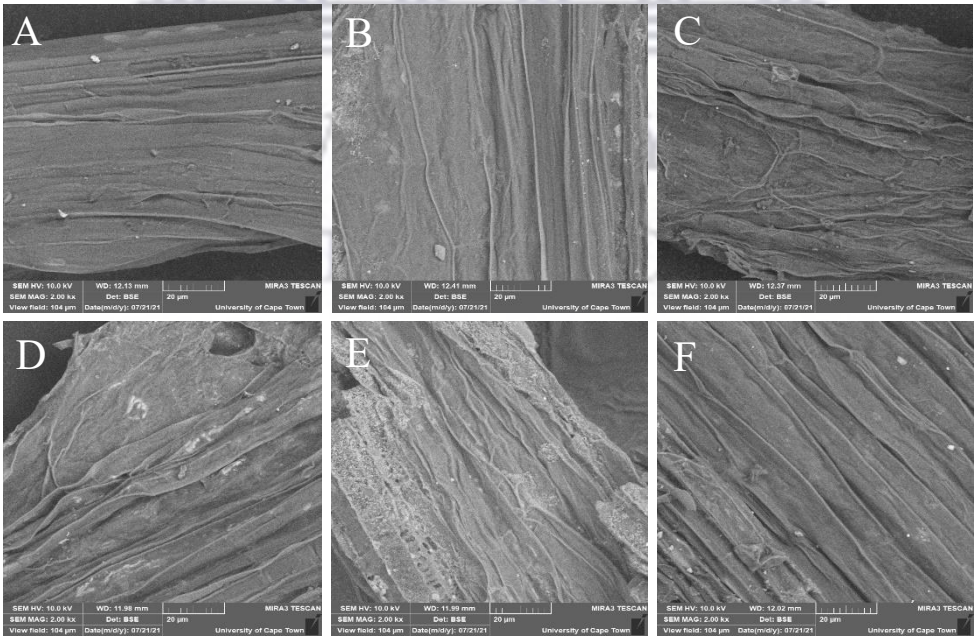

Figure 5.2: Scanning electron microscopy illustrating the epidermis of MeJA pre-treated *S. bicolor* exposed to non-saline and salt stress. Microphotographs of *S. bicolor* epidermis under (**A**) 0 mM NaCl, (**B**) 0 mM NaCl + 10 µM MeJA, (**C**) 0 mM NaCl + 15 µM MeJA, (**D**) 200 mM NaCl, (**E**) 200 mM NaCl + 10 µM MeJA and (**F**) 200 mM NaCl + 15 µM MeJA.

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5.2.1.3 Effect of MeJA on the epidermal stability in S. bicolor under salt stress

Silica phytoliths are microparticles that can accumulate on plant tissues due to the uptake of silicon from the soil (Zancajo et al., 2019). Phytoliths may contain organic molecules that lend support and structure to plants due to their stability and may be augmentative to stress tolerance in plants (Song et al., 2015; Zancajo et al., 2019). A considerable decrease in silica phytoliths was observed in *S. bicolor* exposed to 200 mM NaCl stress (Figure 5.3 D) as compared to the control (Figure 5.3 A). Evidently, pre-treatment with MeJA (10 µM and 15 µM) slightly decreased the formation of silica phytoliths in sorghum plants under non-stress conditions (Figure 5.3B-C). However, MeJA pre-treated (10 µM) *S. bicolor* had greatly improved amounts of silica phytoliths on its tissue under 200 mM NaCl stress (Figure 5.3 E) as compared to the plants under salt stress (Figure 5.3 D). Whereas the phytoliths were completely removed in sorghum plants pre-treated with 15 µM MeJA (Figure 5.3F).

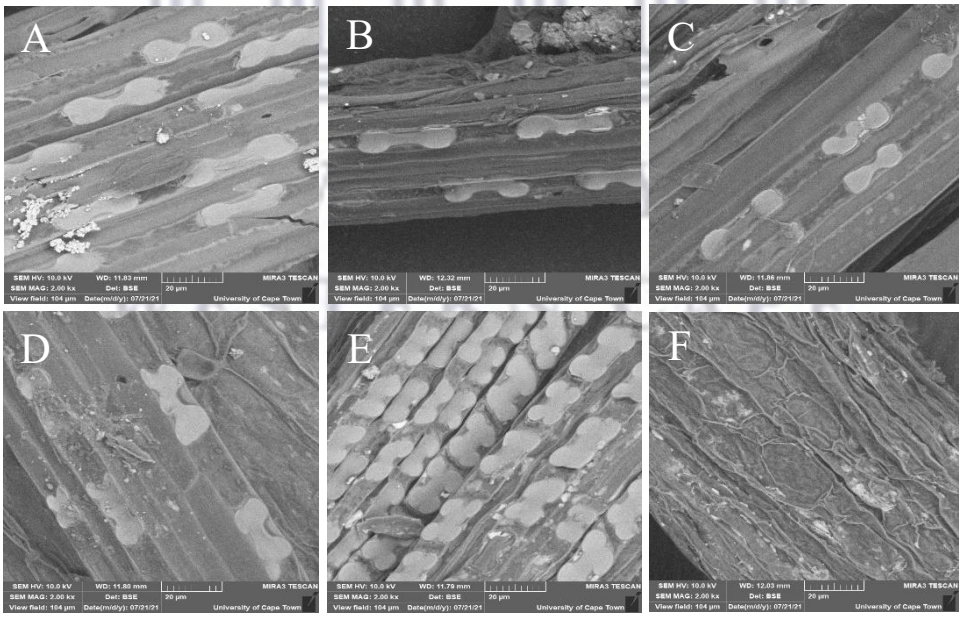

Figure 5.3: Scanning electron microscopy illustrating the silica phytolith formation of MeJA pre-treated *S. bicolor* exposed to non-saline and salt stress. The formation of silica phytoliths on the surface tissue of *S. bicolor* under (**A**) 0 mM NaCl, (**B**) 0 mM NaCl + 10 µM MeJA, (**C**) 0 mM NaCl + 15 µM MeJA, (**D**) 200 mM NaCl, (**E**) 200 mM NaCl + 10 µM MeJA and (**F**) 200 mM NaCl + 15 µM MeJA.

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5.2.2 Effect of MeJA on organic and inorganic active compounds in *S. bicolor*

5.2.2.1 Effect of salt stress on the biomolecules of S. bicolor

In this study, infrared radiation was used to identify changes caused in the active components of *S. bicolor* in response to pre-treatment with MeJA. Using FTIR technology, this study focused on a spectral region of 4000 – 500 cm^{-1}. FTIR analysis identified 10 different functional groups across all samples as indicated by frequency ranges of 3700 - 3200 cm^{-1}; 3100 - 2850 cm^{-1}; 2140 - 2100 cm^{-1}; 1740 - 1720 cm^{-1}; 1680 - 1620 cm^{-1}; 1400 - 1000 cm^{-1}; 1320 - 1000 cm^{-1}; 1250 - 1080; 910 - 665 cm^{-1}; 600 - 500 cm^{-1}. Spectral peaks 3604, 3527, 3326 and 3295 represented the O-H stretching vibrations (3700 - 3200 cm^{-1}), indicating the presence of different forms of phenolic groups (aromatic compounds). Spectral peaks 3068 and 2931 represented C-H stretching vibrations found within the frequency range 3100 - 2850 cm^{-1}, indicating the presence of alkanes. The peak at 1677 cm^{-1} under frequency range 1680 - 1620 cm^{-1} represents C=C stretching vibration indicating the presence of alkenes. Whereas the peak at 2135 cm^{-1} under the frequency range of 2140 - 2100 cm^{-1} represents the C≡C stretching vibrations indicating the presence of alkynes which, together with alkenes and alkanes make out aliphatic compounds such as carbohydrates. The peak at 1733 cm^{-1} represented C=O stretching vibration under the frequency range 1740 - 1720 cm^{-1}, indicating the presence of aldehyde containing aliphatic compounds. Spectral peaks at 1370 cm^{-1} represented C-F stretching vibration under frequency range 1400 - 1000 cm^{-1}, indicating the presence of alkyl halide containing flouro compounds. Spectral peak at 1041 cm^{-1} represented C-O stretching vibration, which may be assigned to alcohols, carboxylic acids, esters, or ethers. The peak at 1250 cm^{-1} represented C-N stretching vibrations under frequency range 1250 - 1080 cm^{-1}, indicating the presence of aliphatic amine containing proteins. Whereas peaks 824 cm^{-1} and 670 cm^{-1} represented N-H stretching vibrations under frequency range 910 - 665 cm^{-1}, indicating the presence of proteins with primary and secondary amines. Lastly, the peak at 551

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cm^{-1} represented C-F stretching vibration under frequency range 600 - 500 cm^{-1}, indicating the presence of halo compounds.

Salt stress disturbed the metabolism of *S. bicolor* by greatly altering the composition of organic molecules including carbohydrates, proteins, lipids, and secondary metabolites (Figure 5.4, red spectrum). Constant band patterns were observed throughout all samples analysed. However increased band absorption was observed in salinity stressed sorghum as compared to unstressed plants, which were clearly evident at peaks 3295, 2135, 1733, 1250, 824 and 551 cm^{-1}.

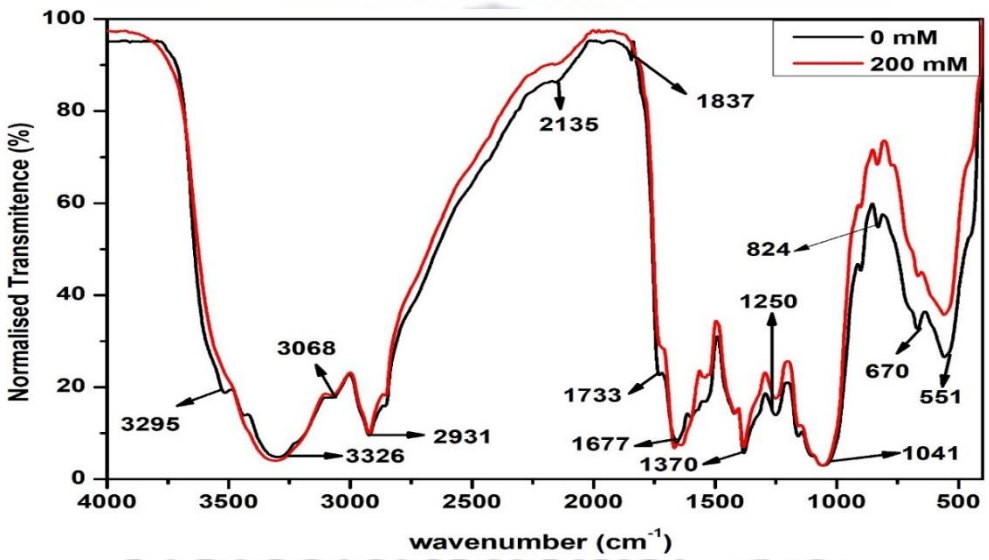

Figure 5.4: FTIR analysis of the effect of salt stress on the biomolecules in *S. bicolor*. Changes in functional groups were detected in sorghum plants exposed to 0 mM NaCl (black) and 200 mM NaCl (red) conditions.

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5.2.2.2 MeJA induced changes in organic molecules under non-saline conditions

To understand the effect of MeJA on the biomolecular composition of these organic molecules at physiological level, the absorption spectra of MeJA (10 and 15 µM) pre-treated *S. bicolor* grown in non-saline conditions was analysed (Figure 5.5). Major structural changes in band patterns were observed in MeJA pre-treated plants. Pre-treatment with 10 µM MeJA resulted in increased absorption at peak 3527 cm^{-1}(phenolic compounds), whereas a decrease in absorption strength was observed at peak 2135 cm^{-1}(carbohydrates). However, pre-treatment

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with 15 µM MeJA resulted in considerable increases in absorption strength at all peaks and increased bandwidth at the 3000 - 2750 cm^{-1} frequency range.

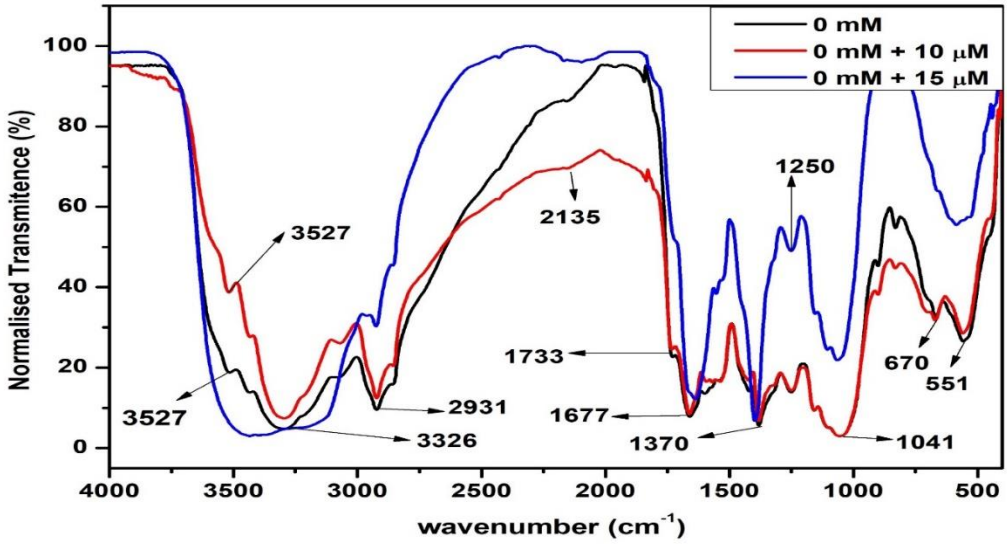

Figure 5.5: FTIR analysis of the effect of MeJA on the biomolecules in *S. bicolor* under control conditions. Changes in functional groups were detected in control sorghum plants (black) and pre-treated with 10 µM (red) and 15 µM (blue) under non saline conditions.

5.2.2.3 MeJA induced alterations in the organically active biomolecules under salt stress

To understand the effect of MeJA in the composition and alteration of these organic biomolecules under salt stress, the infrared absorption spectra of *S. bicolor* plants pre-treated with 10 µM and 15 µM MeJA when subjected to salt stress (200 mM NaCl) was analysed (Figure 5.6). A similar pattern in the peaks were observed in salt-stressed plants. Evidently, a major shift with increased absorption was observed in MeJA-pre-treated (10 µM MeJA) plants at the 3700 - 2750 cm^{-1} frequency range at maximum peak values 3604, 3295 (phenolic compounds) and 3068 cm^{-1} (carbohydrates). Whereas considerable decreases in absorption was observed at peaks 2135, 824, 670 and 551 cm^{-1} as compared to salt-stressed plants. *S. bicolor* plants pre-treated with 15 µM MeJA resulted in slight decreases in the absorption intensity at 2135 cm^{-1} (lipids) and 824 cm^{-1} (proteins) as compared to salt-stressed plants.

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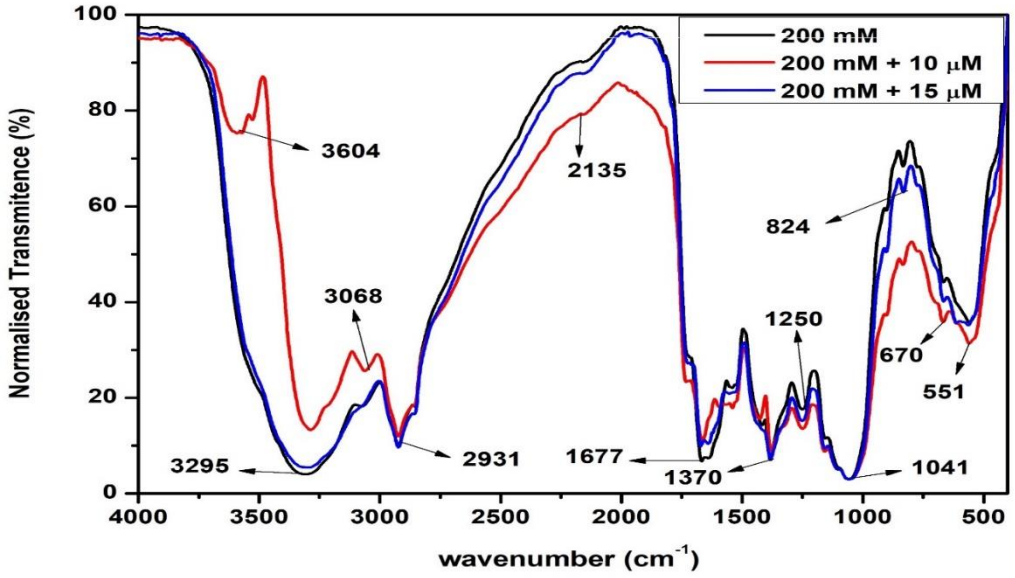

Figure 5.6: FTIR analysis of the effect of pre-treatment with MeJA on the biomolecules in *S. bicolor* under salt stress conditions. Changes in functional groups were detected in control sorghum plants (black) and pre-treated with 10 µM (red) and 15 µM (blue) under 200 mM NaCl stress conditions.

5.2.3 Effect of MeJA on jasmonic acid biosynthesis pathway in *S. bicolor*

5.2.3.1 Salinity stress induced the expression of jasmonate-related genes in S. bicolor

Response and adaptation to stress rely on intricate systemic regulatory mechanisms within plants and causes drastic alterations in plant physiology, morphology and gene expression (Sun & Zhou, 2018). Understanding changes at genetic level is thus crucial in fully elucidating stress tolerance mechanisms in plants. To understand the effect of salt stress on the synthesis of endogenous jasmonic acid, the expression level of genes involved in the jasmonic acid biosynthesis pathway were investigated in *S. bicolor* using quantitative polymerase chain reaction. These genes included *SbLOX*, *SbAOS*, *SbAOC* and *SbOPDR*, evidently (Figure 5.7), all abovementioned genes were constitutively expressed under physiological conditions. Expression levels of *SbLOX* and *SbAOC* were relatively high, followed by that of *SbAOS* and lastly *SbOPDR*. Significant upregulation in the gene expression levels of *SbLOX* (94.96%) (* = P≤0.05), *SbAOS* (65.3%) (* = P≤0.05), *SbAOC* (145.33%) (** = P≤0.01) and *SbOPDR*

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(31.12%) (** = P≤0.01) was observed in the cDNA of *S. bicolor* exposed to 200 mM NaCl stress.

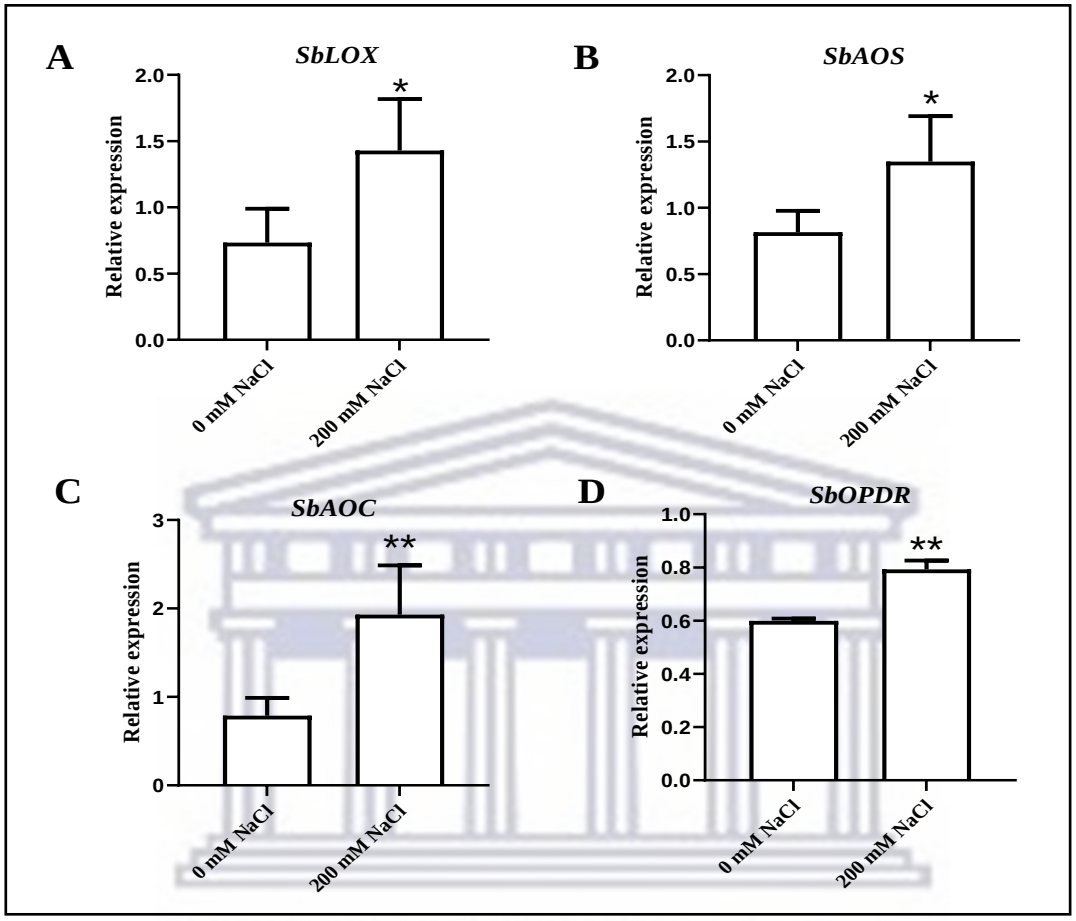

Figure 5.7: The relative expression levels of jasmonate-related genes in *S. bicolor* under non saline (0 mM NaCl) and salt stress (200 mM NaCl) conditions. Relative expression levels of *SbLOX* (**A**), *SbAOS* (**B**), *SbAOC* (**C**) and *SbOPDR* (**D**) was analysed in the cDNA of *S. bicolor*. Error bars represents SD from three biological replicates. Statistical significance was achieved using one-way ANOVA (GraphPad Prism 9) where * = P≤0.05 and ** = P≤0.01 according to Bonferroni's multiple comparison.

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5.2.3.2 Effect of MeJA on the expression of jasmonate related genes in S bicolor under salt stress

Under 200 mM NaCl, a significant downregulation of 42.4% (* = P≤0.05) and 58.01% (** = P≤0.01) in the transcript level of *SbLOX* was observed in *S. bicolor* pre-treated with 10 µM and 15 µM MeJA, respectively. Pre-treatment with 15 µM MeJA resulted in a 43.79% (* = P≤0.05), 63.9% (** = P≤0.01) and 26.69% (* = P≤0.05) decrease in the transcript levels of *SbAOS*, *SbAOC* and *SbOPDR*, respectively, as compared to the salt treatment only. However,

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S. bicolor plants pre-treated with 10 µM MeJA resulted in a further 67.24% (*** = P≤0.001) increase in the relative expression level of *SbOPDR* under 200 mM NaCl stress.

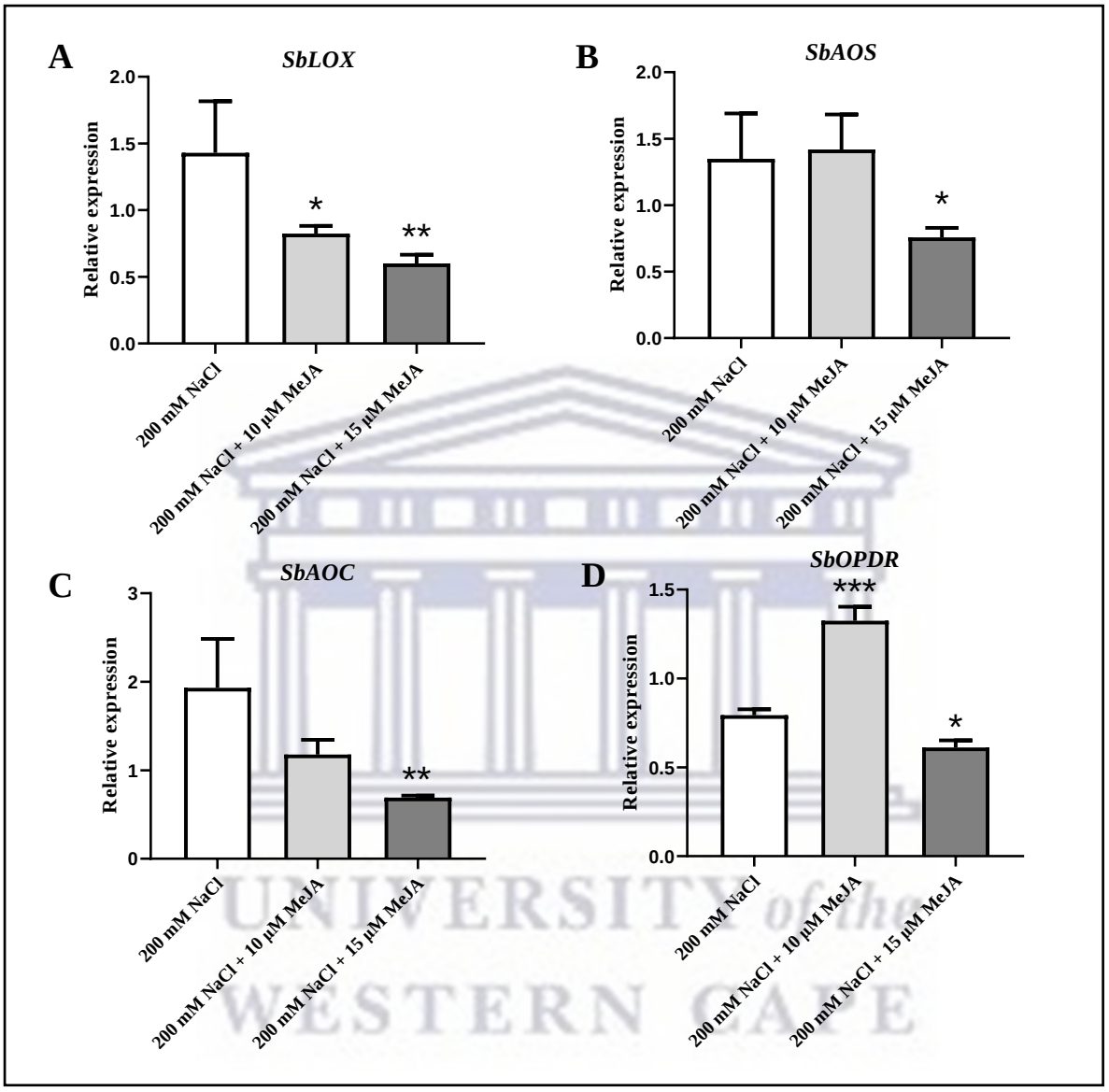

Figure 5.8: **The relative expression levels of jasmonate-related genes in *S bicolor* pre-treated with 10 µM and 15 µM MeJA and salt stress (200 mM NaCl) conditions**. Relative expression levels of *SbLOX* (**A**), *SbAOS* (**B**), *SbAOC* (**C**) and *SbOPDR* (**D**) was analysed in the cDNA of *S. bicolor* plants grown in potting soil under summer conditions. Error bars represents SD from three biological replicates. Statistical significance was achieved using one-way ANOVA (GraphPad Prism 9) where * = P≤0.05 and ** = P≤0.01 according to Bonferroni's multiple comparison.

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5.3 DISCUSSION

Plant stress mechanism exists from highly intricate intrinsic and extrinsic systemic events. Understanding these occurrences requires the usage of advanced technology available to study plant life. Therefore, this chapter focused on the ameliorating effect of exogenous MeJA on the anatomy, biomolecules and genetic profile of *S. bicolor* under saline conditions using high-throughput technology.

5.3.1 MeJA improves the anatomical attributes of S. bicolor

Plant survival is highly dependent on systemic acquired adaptation to environmental stressors of, which uptake and distribution of water and nutrients from plant vascular tissue are essential for productivity (Hwang et al., 2016; Perri et al., 2019). Adverse conditions are known to disturb vascular tissue by narrowing the xylem and phloem causing tension to increase water uptake (Aliche et al., 2020). Salinity caused slight thickening in the vascular tissue walls of sorghum (Figure 5.1). Although no great effect on the vascular tissue of *S. bicolor* plants under 200 mM NaCl stress, some lignification was present. In previous studies, substantial thickening and deformation occurred in the vascular tissue of *S. bicolor* under higher salt (300 mM NaCl) concentration in germinating seeds (Mulaudzi et al., 2020; Rakgotho et al., 2022), suggesting that extreme deformation of the vascular bundle in response to stress might be dose dependent. The lesser change observed in this study may be due to *S. bicolor*'s relatively higher stress tolerance trait (Yang et al., 2020; Mansour et. al., 2021). Similarly, no substantial differences were observed in the vascular bundle of MeJA pre-treated (10 µM and 15 µM) sorghum plants under normal conditions (Figure 5.1 B-C) and salinity stress conditions (Figure 5.1 D-E).

Alterations of plant epidermis forms a crucial part of the plant's systemic acclimation to environmental challenges (Liu et al., 2022). Abiotic stress is known to induce stiffening of plant epidermal cells, which may affects growth by limiting expanding cells (Zörb et al., 2015).

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Salinity stress caused deformation to the epidermal tissue of sorghum plants (Figure 5.2). This was indicated by increased shrivelling on the epidermis of *S. bicolor* plants under salt (200 mM NaCl) stress conditions. The stiffening and shrinking of the epidermis occur as a strategy to limit excessive water loss (Li et al., 2021). Pre-treatment with MeJA caused no deformation of the epidermis under physiological condition, which was indicated by the similar textures of the epidermis in MeJA pre-treated (10 µM and 15 µM MeJA) sorghum as compared to the control under non-stress condition. However, MeJA had an ameliorating effect on the epidermis of sorghum plants under salinity stress. Pre-treatment with MeJA proved successful in reversing the stress-induced restriction of the epidermal layers by having a considerably smooth epidermis under salt stress.

Grass-like plants such as sorghum are known for the deposition of phytoliths on the aerial parts of tissues, which occur through the absorption of silica from soil. The resilient nature of these structures may be equipped to resist destructive environmental forces (Nawaz et al., 2019; Badgal et al., 2022). Abiotic stress factors have been found to have a deleterious effect on the formation of silica phytoliths, which compromises the plant structure (Meunier et al., 2017). Salinity stress reduced the silica phytolith deposits on sorghum plants (Figure 5.3). This was indicated by a clear decrease in the amount of silica deposits observed in the epidermis in response to harsh salt (200 mM NaCl) stress. Previously, similar observation was made where PEG-induced osmotic stress resulted in decreased silica bodies on *Triticum turgidum* (Meunier et al., 2017). However, sorghum plants pre-treated with MeJA had slightly decreased silica phytoliths on the aerial tissue of sorghum plants under non-stress conditions, indicating that excessive MeJA might play a negative role in the sequestration of silica at physiological level. Contrary to this, sorghum plants pre-treated with 10 µM MeJA had significantly improved silica phytolith formation under stress conditions. As indicated by the greatly increased amount of silica bodies found on the aerial tissue of *S. bicolor* under 200 mM NaCl salt stress. Pre-

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treatment with MeJA proved beneficial to plant structure under adverse environment. The increased silica bodies thus provided better support by improving strength against epidermal shrinkage and deformation, which will allow for optimal photosynthetic activity (Nadeem et al., 2022). This was supported by the increased photosynthetic pigment in pre-treated sorghum under salt stress (Table 4.1).

5.3.2 MeJA induces the accumulation of secondary metabolites of S. bicolor

The biochemical changes in plant metabolism is highly dependent on the uptake and accumulation of inorganic and organic elements, which induces changes to lipids, proteins and carbohydrates (Westworth et al., 2019). Adverse conditions cause major alterations in the functional groups initiating strategies for plant survival (Figure 5.4). Salt stress induced the volume of various metabolites found in sorghum plants. These included phenolic compounds (3295 cm^{-1}), which may include flavonoids, phenolic acids or tannins (Valifard et al., 2014), alkyne containing aliphatic compounds (2135 cm^{-1}), which may include acetylenic fatty acids (Li et al., 2021), aldehyde containing aliphatic compounds (1733 cm^{-1}), which may have been formed during lipid peroxidation (Končitíková et al., 2015), alkyl halide containing fluoro-compounds (1250 cm^{-1}), primary or secondary amine containing proteins (824 cm^{-1}), which may be in conjunction with the induced proline levels (Kishor et al., 2015), and the presence of halo compounds (824 cm^{-1}) identified in samples.

The exogenous application of MeJA induced substantial structural shifts in the make-up of functional groups at physiological level (Figure 5.5). Pre-treatment with 10 µM MeJA resulted in increased volume of phenolic compounds whereas decreased volume of functional groups related to acetylenic fatty acids was detected in sorghum plants under non-saline conditions. Greater changes were observed with the higher concentration of MeJA, for example 15 µM MeJA resulted in substantial structural changes in the metabolism of sorghum plants under non-stress conditions. This indicated induced intensity in the peaks of all identified functional

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groups. Additionally, the increased bandwidth observed at the 3000 - 2750 cm^{-1} frequency range may be as a result of induced hybridisation in alkane containing aliphatic compounds (Almond et al., 2020).

Sorghum plants pre-treated with MeJA had substantially clear alteration in the metabolic make-up under salinity stress (Figure 5.6). In addition to different forms, a clear increased volume was identified in the phenolic compounds in *S. bicolor* plants pre-treated with 10 µM MeJA under salt stress (200 mM) condition. These may suggest that secondary metabolites such as flavonoids and phenolic acids are playing a major role in ameliorating the positive effect that 10 µM MeJA has on *S bicolor* under salt stress. Additionally, FTIR spectroscopy identified decreased volumes of acetylenic fatty acids, and primary or secondary amines as indicated by the decreased intensity in peaks found in frequency ranges 2140 - 2100 cm^{-1} and 910 - 665 cm^{-1}, respectively. Slight changes in the metabolic network were observed in sorghum plants pre-treated with 15 µM MeJA in response to salinity stress. This was indicated by decreased volume of fatty acids (2135 cm^{-1}) and primary or secondary amines (824 cm^{-1}) identified in pre-treated sorghum subjected to harsh salt stress condition.

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5.3.3 The role of NaCl and exogenous MeJA on transcripts of jasmonate-related genes in S. bicolor

Part of the multidisciplinary approaches used in this study to investigate the role of MeJA on the stress tolerance of *S. bicolor*, included comprehensive gene expression analysis to further elucidate stress mechanisms at genetic level. Research have demonstrated jasmonic acid (JA), playing a crucial role in mediating the effect of abiotic stress and plant survival under unfavourable conditions (Raza et al., 2021). Jasmonic acid ameliorated salt induced restriction on the growth and chlorophyll content in *Capsicum annuum* (Rezai et al., 2013) and *Prunus dulcis* (Tavallali & Karimi, 2019). Additionally, mitigating the salt induced damages in transgenic *Arabidopsis thaliana* (Gu et al., 2008) and *Arachis hypogaea* (Liu et al., 2015)

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overexpressing JA genes and therefore, having enhanced stress tolerance. Therefore, this study focussed on the role of MeJA in inducing the transcription profile of JA-biosynthesis genes *SbLOX*, *SbAOS*, *SbAOC* and *SbOPDR* in sorghum plants under salt (200 mM NaCl) stress.

Salinity stress induced the assumptive endogenous jasmonic acid (JA) in sorghum plants (Figure 5.7). All targeted genes were constitutively expressed at physiological level. However, significant upregulation in the transcript levels of *SbLOX*, *SbAOS*, *SbAOC* and *SbOPDR* were observed in response to salt stress. Findings coincides with literature as observed with the induced expression of JA-biosynthetic genes in response to salt stress in *Ipomoea batatas*, *Medicago truncatula* and *Solanum chilense* (De Domenico et al., 2019; Kashyap et al., 2020; Zhang et al., 2017).

Pre-treatment with MeJA reversed the salt induced upregulation in the gene expression of the JA-biosynthetic pathway in sorghum under adverse salt condition (Figure 5.8). This was indicated by a significant down regulation in the transcript levels of *SbLOX*, *SbAOS*, *SbAOC* and *SbOPDR* in MeJA pre-treated *S. bicolor* plants exposed to salt stress. This may be as a result of a negative feedback loop existing through the induction of JASMONATE ZIM DOMAIN (JAZ) proteins inhibiting the activity of MYC2, which is a key activator for JA response as observed in transgenic *A thaliana* (Chini et al., 2007; Thines et al., 2007). This was further supported by exogenous MeJA inhibiting JA biosynthesis through inhibiting the activity of OPDR in *Citrus sinensis* (Qiu et al., 2020). Contrary to this, this study showed a significant further upregulation in the transcript level of JA precursor gene *SbOPDR* in sorghum pre-treated with 10 µM MeJA under salt stress, which may indicate induced biosynthesis of JA. Furthermore, supporting the suggestion of JA independent pathways that could regulate the synthesis of OPDR precursor substrate, 12-oxo phytodienoic acid (OPDA) (Qiu et al., 2020).

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CHAPTER 6

Conclusion and Future Prospective

World population is increasing at an alarming rate and is thus expected to exceed 9 billion by 2050 (Tyczewska et al., 2018). Food production needs to rapidly be increased to reach the prospect of food security. Climate change is continuing to challenge the world's agricultural resources due to harsh environments and induced soil deterioration. Approximately 2.7 billion hectares of land are unsuitable for agricultural purposes due to harsh geoclimatic conditions (FAO, 2018). Therefore, innovative methods to improve crop productivity and tolerance to harsh environment needs to be prioritised to foresee in achieving food security. Expanding research is constantly illustrating the damaging effects of abiotic stress on plants. Yet, stressors such as drought and salt stress, in addition to population growth are prevailing to constrain the productivity of agricultural crops worldwide.

Although the damaging effect of salt stress on plants have been studied across various species including *Brassica oleracea* (Hassini et al., 2017), *Glycerrhiza uralensis* (Lang et al., 2020) and *Cucuribita maxima* (Tarchoun et al., 2022) in addition to *S. bicolor* (Mulaudzi et al., 2020; Mansour et al., 2021; Rakgotho et al., 2022), the roles of methyl jasmonate (MeJA) and carbon monoxide (CO) in conferring salinity stress tolerance still requires extensive research. Plants makes use of intricate natural strategies for survival under adverse conditions (Mansour et al., 2021), which therefore requires great depth of knowledge to fully elucidate the mechanisms involved in crop tolerance to stress. Plants respond to environmental stressors by utilizing elaborate signalling pathways. Organic compounds such as phytohormones and other signalling molecules including CO trigger specific cascades upon the perception of stress stimuli. Part of the early responses to stress include fluctuation in phytohormones such as abscisic acid (ABA), ethylene (ET), salicylic acid (SA) and jasmonic acid (JA), which will

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facilitate metabolic bioprocessing to induce physiological, biochemical and genetic changes that alter growth and developmental patterns suitable to withstand stressors (Verma et al., 2016). This study was therefore, aimed at investigating the roles of MeJA and CO in conferring salinity stress tolerance to *S. bicolor* at physiological, biochemical, microscopic, and genetic level through methods described in chapter 2.

In chapter 3, this study investigated the effect of MeJA and carbon monoxide [using hematin (Ht) as adonor] on the growth and development during the germination of sorghum under salinity stress. Findings further demonstrated the damaging effect of salinity stress on *S. bicolor* by delaying seed germination and inhibiting seedling growth. Although the combinatory treatment of both MeJA and carbon monoxide (CO) proved successful in alleviating the seed germination inhibition caused by salinity stress, pre-treatment with MeJA were more efficient in promoting germination and enhancing seedling establishment under salt stress. Hence, focus was placed on MeJA's role in alleviating salinity stress at the later stage of plant growth.

The deleterious effect of salt stress was later emulated in chapter 4, which investigated the role of MeJA in enhancing salinity stress tolerance to sorghum at the vegetative stage of growth. Salt stress inhibited growth by decreasing shoot height and disturbing photosynthesis by decreasing photosynthetic pigments. This was then followed by inducing oxidative damage indicated by increased levels of reactive oxygen species and lipid peroxidation. Part of the natural strategies of plants for survival was then demonstrated by the increased levels of osmoprotectants such as proline and total soluble sugars, which regulates the osmotic potential to aid in alleviating the toxicity caused by salt stress. Findings from this study showcased the resilience of *S. bicolor* to salt stress by mostly inflicting these metabolic changes upon exposure to harsher levels of saline conditions. Pre-treatment with MeJA effectively enhanced salinity stress tolerance by reversing many of the damaging effects of salt stress on sorghum. These might be as a result of the physiological changes induced at germination stage (Ali et al., 2019).

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The ameliorating effect of MeJA was indicated by improved growth and photosynthetic pigments, in addition to decreasing oxidative stress and osmolyte accumulation.

Chapter 5 was aimed at investigating the alleviating effect of MeJA on the anatomical attributes, biomolecular composition and transcript levels of the JA-biosynthesis pathway in salt stressed sorghum at microscopic and molecular level. Salt stress is known to negatively impact water potential and cause nutrient imbalance. In aid of preventing excessive water loss, *S. bicolor* conformed to shrivelling of epidermal layers and shrinking of vascular bundle tissue creating negative pressure during the uptake and transportation of water and nutrients for faster distribution throughout the plant (Zheng et al., 2021). Plants, particularly grass-like crops, often make use of environmental elements to strengthen their structure and aid in survival under abiotic stress. This study illustrated that *S. bicolor* have high amounts of silica phytoliths formed by the absorption of silica and its deposit on the plant surface tissue, which was negatively affected upon the exposure to salt stress. Moreover, salinity stress further altered sorghum's metabolism by inducing secondary metabolites and upregulating the gene expression levels of jasmonate-related genes *SbLOX*, *SbAOS*, *SbAOC* and *SbOPDR* indicating the importance of JA and its derivatives in enhancing stress tolerance in *S. bicolor*.

Pre-treatment with MeJA ameliorated salinity induced damage by resulting in smoother epidermal layers and increased amounts of silica phytoliths, which improved tolerance and anatomical structure under salt stress. Findings from this study suggests that MeJA conferred salt stress tolerance to sorghum by increasing the levels of secondary metabolites as indicated by FTIR spectra. Furthermore, pre-treatment with higher concentration of MeJA (15 µM) resulted in the down regulation of the expression levels of *SbLOX*, *SbAOS*, *SbAOC* and *SbOPDR* under salinity stress. This might be as a result of the activation of some JASMONATE ZIM DOMAIN (JAZ) proteins reducing JA responses by obstructing the activity of transcription factor, MYC2 (Chung et al., 2008; Liu & Timko, 2021). Additionally,

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gene expression level of *SbOPDR* was further induced in salinity stressed sorghum plants pre-treated with 10 µM MeJA, which may indicate elevated levels of endogenous JA initiating a signalling cascade by activating various downstream defence-related responsive genes such as the vegetative storage protein (VSP2) (Liu & Timko, 2021). However, further investigation is required to confirm its effects on regulatory transcription factors and on the endogenous production of JA. Due to its systemic nature, it will be crucial to determine the role of MeJA on the endogenous biosynthesis of other phytohormones such as ABA, SA, and ET in addition to signalling molecules such as endogenous CO, NO or H_2S. Additionally, investigating exogenous MeJA's role on important transcription factors downstream of JA-signalling, including MYC and NAC would prove vital in fully elucidating the mechanism involved in MeJA conferring salt stress tolerance plant. Furthermore, due the unique behaviour of the *SbOPDR* gene in response to 10 µM under salt stress, further analysis should include investigating exogenous MeJA's role on the ABC-transporter proteins facilitating the movement of OPDA to the peroxisomes during JA-biosynthesis.

Plant defence response is reliant on intricate cascade of events in plant metabolism, therefore, requiring multiple approaches to fully elucidate stress mechanisms. The increasing intensity of abiotic stresses in addition to the rapidly increasing population is continuing to worsen food security for the immediate and future generation, and further threatening the lives of people facing shortages. Data from this study will pioneer the establishment of transgenic plant cultivars with improved tolerance and therefore, increased crop productivity. From this study, MeJA enhanced salt stress tolerance of sorghum by improving germination and growth at the germination phase and vegetative stage of growth followed by alleviating salt induced toxicity as indicated by reduced oxidative stress and osmolyte content. Furthermore, MeJA reversed salt induced physiological changes by reducing constraint on the vascular bundle tissue, smoothening of the epidermis tissue, and inducing the level of silica phytoliths on the surface

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tissue of sorghum. Additionally, MeJA altered the biomolecules by further inducing secondary metabolites. Lastly, alleviating salinity stress by further inducing the gene expression level of JA precursor *SbOPDR*. This study, therefore, aids in fully elucidating the salinity stress mechanism of the moderately salt tolerant *S. bicolor* and further enlighten possibility of enhancing crop tolerance through improving MeJA signalling.

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