The effects of gallic acid on the membrane proteome and antioxidant system of wheat plants under salt stress

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A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae in the Department of Biotechnology, University of the Western Cape

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KEYWORDS

Antioxidant enzymes

Gallic acid (GA)

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Photosynthesis

Protein identification

Protein synthesis

Proton transport

Reactive oxygen species

Salt – induced

Salt stress tolerance

Wheat (*Triticum aestivum* L.)

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ABSTRACT

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MSc Thesis, Department of Biotechnology, University of the Western Cape

Salt stress is a major abiotic stress that accounts for huge agricultural losses worldwide, which in turn threaten food security and sustainable agriculture. Salt triggers the excessive production of reactive oxygen species (ROS) which accumulate to levels that become toxic to plants, resulting in cell death and reduced plant growth. Part of the plant's mechanisms to counteract ROS-induced cell death involves the scavenging ability of the antioxidant defense system to maintain redox homeostasis. Gallic acid (GA) is an antioxidant that has been shown to reduce salt-induced ROS in legume plants. However, its effects on wheat plants have not been elucidated. This study thus investigated the role of exogenous GA (250 µM) on the physiological responses and antioxidant system of wheat plants under salt stress (150 mM). In addition, this study also investigated how GA and salt stress influenced changes in the membrane proteome of wheat plants using LC-MS proteomic analysis.

Salinity stress significantly reduced plant biomass, growth, relative water content (RWC) and chlorophyll content and simultaneously increased ROS content (hydrogen peroxide and superoxide) and augmenting the extent of lipid peroxidation. However, the opposite response was observed in plants treated with GA, whereby improved plant biomass, growth, RWC and chlorophyll content were observed. In addition, ROS accumulation and the extent of lipid peroxidation was reduced in the presence of GA. It was noted that when plants under salt stress were supplemented with GA, the adverse effects caused by salinity stress were reversed although not to the level of the untreated control.

Furthermore, salt stress and GA differentially altered antioxidant enzyme activity. Although salt stress induced the activity of antioxidant enzymes, the increase was not sufficient to counteract the

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adverse effects caused by salinity. However, the exogenous application of GA to salt stressed plants notably reversed the negative effects to the level of the untreated control.

A total of 28 proteins were identified using LC-MS proteomic analysis. Of the proteins identified, 50 % were membrane bound and classified as peripheral membrane proteins (2) and integral membrane proteins (12). The proteins identified showed varying degrees of abundance across the different treatments. The proteins were functionally classified into eight categories, which included photosynthesis and energy metabolism (39 %), transport (18 %), DNA binding (14 %), defense (3 %), signal transduction (4%), translation (11 %), pollen development (4 %) and unknown function (7 %). Interestingly, 13 proteins (46 %) were unique to the GA-treated plants whereas only one unique protein was identified in response to salinity. A total of five proteins were also identified in the combined treatment when compared to the salt stressed plants. Majority of the proteins identified were localised to the chloroplast thylakoid membrane. Based on the results of this study, we suggest that GA may play a key role in the protection of plants against salt stress and may enhance salinity tolerance in wheat. Moreover, GA-induced proteins identified in this study could potentially serve as biomarkers to enhance salt tolerance in wheat.

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DECLARATION

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- First and foremost, I would like to thank my creator **Allah** for granting me the strength, patience, knowledge and ability to complete this study through the most difficult times.
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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AmBic	Ammonium bicarbonate
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
AsA	Ascorbic acid
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAT	Catalase
CBB	Coomassie brilliant blue
Chl a	Chlorophyll a
Chl b	Chlorophyll b
Chl a + b	Total chlorophyll
Cu/Zn-SOD	Copper zinc superoxide dismutase
DAB-POD	Diaminobenzadine peroxidase
DTT	Dithiothreitol
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FA	Formic acid
FDR	False discovery rate
Fe-SOD	Iron superoxide dismutase
FW	Fresh weight
GA	Gallic acid
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
HBA	Hydroxybenzoic acid
HCA	Hydroxycinnamic acid

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HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
IMPs	Integral membrane proteins
KCN	Potassium cyanide
KI	Potassium Iodide
LC-MS/MS	Liquid chromatography- tandem mass spectrometry
MDA	Malondialdehyde
MMTS	Methyl methanethiosulphonate
Mn-SOD	Manganese superoxide dismutase
NaOH	Sodium hydroxide
NBT	Nitrotetrazolium blue chloride
PAGE	Polyacrylamide gel electrophoresis
PMPs	Peripheral membrane proteins
POD	Peroxidase
PS I	Photosystem I
PS II	Photosystem II
PSM	Peptide-spectrum matches
PVP	Polyvinylpyrrolidone
Q-TOF	Quadrupole-time-of-flight
ROS	Reactive oxygen species
RPM	Revolutions per minute
RWC	Relative water content
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TEMED	N, N, N’, N’-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TW	Turgid weight
2-DE	2-dimensional gel electrophoresis

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CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Cereals form a significant part of the human diet in both developed and developing countries (Laskowski *et al.*, 2019). The importance of cereals is based on the fact that global food security largely depends on its production to feed billions of people. According to Eurostat (2016), cereals represent one of the world's major food sources, contributing around 300 million tons annually. In developing countries, 60 % of calories are derived from cereals, with calorie intake exceeding 80 % in poorer African countries. Although agriculture is the dominant sector in Africa, providing employment for more than half the continent's working population and contributing 30 % – 60 % of gross domestic product, the produce from this sector is unable to provide food security to its citizens (Tadele, 2017). Hence, food insecurity is extremely worrisome within the African continent (Figure 1.1), with eleven African countries experiencing acute food insecurity.

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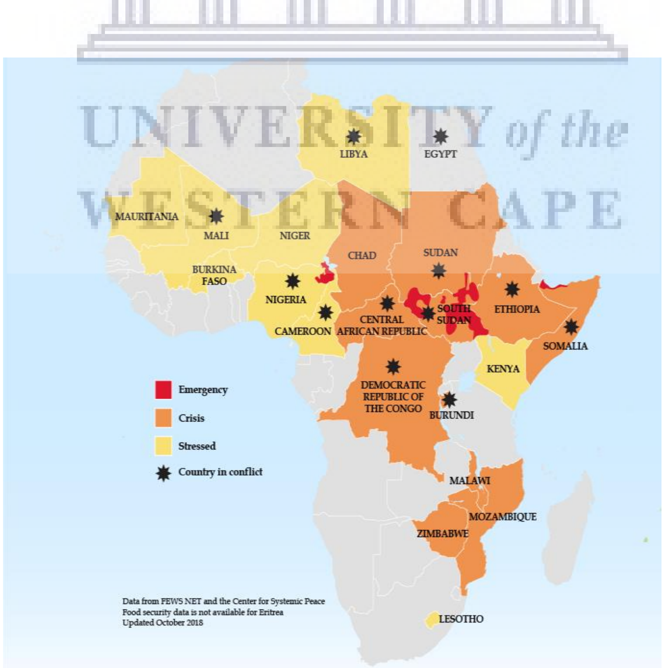

Figure 1.1. The distribution of food insecurity in Africa 2018. The map was adapted from Africa center of strategic studies (https://africacenter.org/spotlight/africas-unresolved-conflicts-a-key-driver-of-food-insecurity/).

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Although South Africa is known to be food secure at national level, the country remains overwhelmed with poverty and unemployment. This affects many households, deeming the country to be food insecure at household level as many do not have access to adequate food (Naicker *et al*., 2015), leaving 6,8 million South Africans hungry in 2017 (STATS SA, 2019).

In addition to the economic impacts faced within South Africa, the country is affected by climate change (WWF, 2013). While one third of South Africa receives adequate rain for crop production, only 12 % of the country has fertile land. Thus, the majority of crop farmers are reliant on fertilizers to increase soil fertility in order to achieve good crop yields. The increase in inputs to compensate for reduced soil fertility therefore increases the countries food prices (National Agricultural Marketing Council of South Africa, 2005). As a consequence, the nation continuously suffers from hunger and malnutrition (Naicker *et al*., 2015).

South Africa and many African countries are therefore dependent on the import of cereals every year. Cereals such as rice, maize and wheat are important crops that contribute over 50 % of the calories consumed by the population (Figure 1.2) (Awika, 2011).

Therefore, in order to improve food security in South Africa, this research focused on addressing the effect of salt stress on the major commercial crop, wheat (*Triticum aestivum* L.) in order to improve food security.

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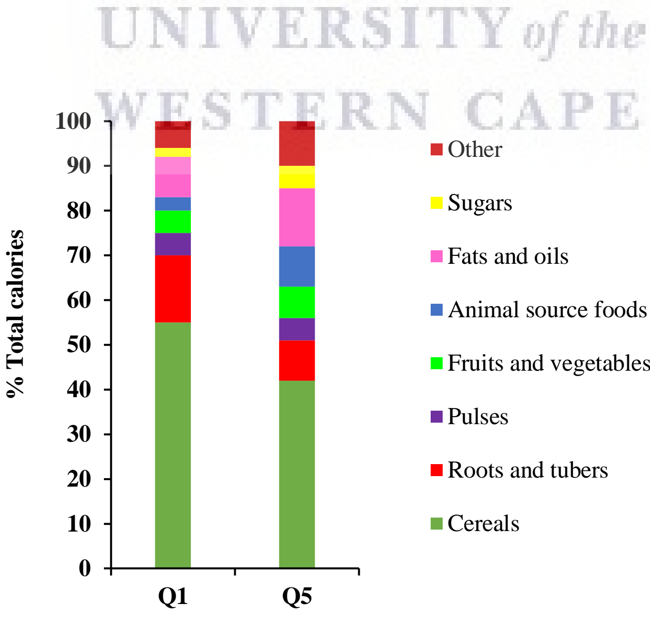

Figure 1.2. Total calorie intake in the African continent between low-income groups (Q1) and higher-income groups (Q5) FAO (2015).

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1.2 Economic importance and nutritional value of wheat

Triticum aestivum L., also known as the common bread wheat, is the second major agricultural crop produced globally (Rusmania, 2015). It is an important staple food for two billion people, accounting to 36 % of the world's population. Wheat is a widely cultivated crop, able to grow in dry (spring wheat) and cold (winter wheat) climates owing to the vast number of species and their adaptability. Over 700 million tons of wheat is produced annually (FAO 2015) (Oyiga *et al.*, 2016). The production of wheat on 217 million hectares (Mha) of land is estimated to increase to 838.5 million tons (Mt) by the year 2029 (Table 1.1).

Table 1.1. Major cereal grain production statistics (2020). The table was adapted from OECD/FAO (2020).

Commodity	Production area (Mha)		Production (Mt)	
	2020	2029	2020	2029
Rice	166.2	165.6	532.5	581.8
Maize	188.9	197.6	1122.1	1315.2
Wheat	217.0	217.6	764.9	838.5

At a global level, bread wheat accounts for 95 % of wheat production. The production of this wheat type is also dominant in Sub-Saharan Africa, accounting for 1.4 % (7.5 Mt) of the wheat produced at global levels (Tadesse *et al*., 2019). Two of the most important wheat producing countries in Sub-Saharan Africa are Ethiopia and South Africa.

Although South Africa is a relatively small wheat producing country, the wheat industry contributes around 4 billion rand to the gross domestic product (GDP) of the country and provides job opportunities for 28 000 people (Dikgwatlhe *et al*., 2008). Preceded by maize, wheat is the second most important cereal crop produced in South Africa. Despite South Africa having 4000 commercial wheat growers, the country still has to import wheat every year in order to meet its domestic consumption requirements. Findings in a study by Rusmania (2015) demonstrated that import levels also has a significant impact on the price of wheat worldwide.

Due to the rapid population growth along with the challenges wheat production encounters such as increased costs of inputs, climate change and increased intensity of biotic (pests and diseases) and abiotic (salinity, drought and heat) stresses, the global demand for wheat is expected to increase in developing countries by 60 % in the year 2050 (Lucas, 2012; Rosegrant and Agcaoili,

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2010). Total wheat (44.7 Mt) imports for Africa amounted to 11.2 billion USD in 2018 (FAOSTAT, 2020) (Figure 1.3). This indicates that African governments expend huge amounts of their budget to meet the demand for food.

The fact that wheat is such a major crop for a large proportion of the population is owing to its nutritional value (Călinoiu and Vodnar, 2018). It provides 21 % of protein and 19 % of the calorie needs of the population. Wheat is a high source of dietary fibre and minerals including manganese, folate and magnesium.

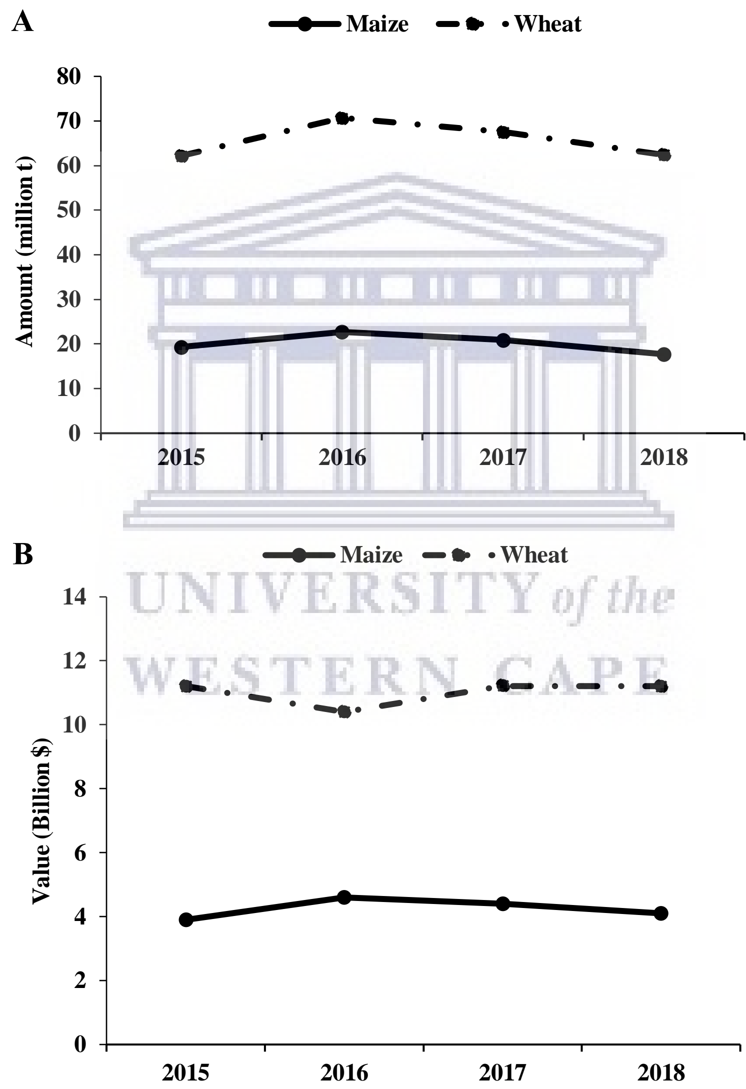

Figure 1.3. Crops imported by Africa from 2015 to 2018. (A) amount of maize and wheat imported and (B) total value of maize and wheat imported (FAOSTAT, 2020).

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Wheat is also high in B vitamins (B1-B3, B6 and B9), phytochemicals and contain essential amino acids like tryptophan.

Major antioxidant and anti-inflammatory compounds present in wheat include salicylic acid, sinapic acid, ellagic acid, vanillic acid , caffeic acid, ferulic acid, *p*-coumaric acid, quercetin and catechins (Călinoiu and Vodnar, 2018). These nutrients, together with fibre and manganese are noted for their health advantages and play a role in safeguarding against heart diseases, type-2-diabetes and colorectal cancer (Călinoiu and Vodnar, 2018). Numerous studies have demonstrated that wheat is good for cardiovascular and coronary heart diseases (Zhang *et al*., 2018; Aune *et al*., 2016; Benisi-Kohansal *et al*., 2016, Wu *et al*., 2015).

1.3 How plants respond to salinity stress

Plants, being sessile organisms are continuously exposed to biotic and abiotic stresses that may become unfavorable for their development and growth. Abiotic stresses, such as salinity affects plant productivity particularly in the arid and semi-arid regions (Husain *et al*., 2009). Soils are considered saline when the soil solution reaches an electrical conductivity of 4 dS m^{-1} (equivalent to 40 mM NaCl), generating an osmotic pressure around 0.2 MPa (Acosta-Motos *et al*., 2017). Salinity is a global problem affecting more than 800 million hectares (6 %) of arable land (FAO 2010) which contributes to major agricultural losses and poses a threat to sustainable agriculture and global food security (Oyiga *et al*., 2016).

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The harmful effects of salt stress vary depending on the plant species, climatic conditions, soil conditions and light intensity. Plants that withstand saline environments and grow under these harsh conditions, are classified as glycophytes (salt-sensitive) or halophytes (salt-tolerant) and their responses to salinity stress differs in terms of osmotic regulation, photosynthetic electron transport, toxic ion uptake, CO_2 assimilation, chlorophyll content, ion compartmentalization and/or exclusion, reactive oxygen species (ROS) production and antioxidant defences. Glycophytes, which includes most of the agricultural crops, are unable to withstand high salt levels. Their growth is inhibited by NaCl concentrations ranging from 100 to 200 mM. In contrast, halophytes can grow and survive under high salt concentrations (300–500 mM NaCl) due to their salt resistance mechanisms.

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Salinity affect crops in two ways; either by the osmotic effect or specific ion effect. Short-term salt stress induces osmotic stress, which restricts water availability. Exposure of plants to long-term salt stress results in ion toxicity due to nutrient imbalances in the cytosol. It has been shown that saline conditions negatively affect all stages of plant development including germination, leaf growth and total yield and biomass (Muchate and Nikalje, 2016). Symptomatically, the plant displays scorched leaf tips, premature discoloration, reduced vegetation and underdeveloped root systems under these conditions (Figure 1.4). These stress responses are mainly due to the osmotic effect where high salt levels reduces the plants ability to take up water. Thus, salt in the soil or water impairs the development and growth of plants via water stress, nutritional imbalance and cytotoxicity due to sodium (Na^+) and chloride (Cl^-) ions that accumulate in plant tissues (Figure 1.4) (Isayenkov and Maathuis, 2019). Additionally, salinity reduces the K^+ uptake by plants and increases the shoot Na^+/K^+ ratio, which results in the disruption of metabolic processes such as respiration, photosynthesis and N_2-fixation (Kumar *et al.*, 2017; Klein, 2012). Plants have the ability to exclude salts from entering their cells or may compartmentalize them into the vacuole. The compartmentalization of toxic ions is one of the mechanisms involved in plant salt-tolerance. Studies on salt tolerant plants have shown that they tend to keep their cytosolic K^+ concentrations high and Na^+ concentrations low through the regulation of K^+ and Na^+ transporters. This selective uptake of K^+ ions over Na^+ ions is a well-recognized mechanism for salt tolerance of the plants (Abbas *et al.*, 2013).

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Exposure of plants to prolonged periods of salt stress also causes changes in leaf anatomy. In general, salt-tolerant species may respond by increasing leaf thickness. Anatomical changes in leaves include a decrease in spongy parenchyma and an increase in palisade parenchyma, serving to facilitate CO_2 diffusion when stomatal aperture has been reduced (Acosta-Motos *et al.*, 2017). Long-term salt exposure also affects photosynthetic processes, which leads to a reduction of chlorophylls and the inhibition of Calvin cycle enzymes. As a mechanism to protect the photosynthetic processes, salt-tolerant species respond by increasing or maintaining their chlorophyll content (Sairam *et al.*, 2005).

Furthermore, the drastic changes in water and ion homeostasis due to salt stress causes oxidative stress within the plant systems thus resulting in excessive generation/accumulation of reactive oxygen species (ROS). This interference in homeostasis occurs at both cellular and whole plant level. ROS molecules such as hydroxyl radicals ($OH^{\cdot}$), superoxide anion (O_2^-) and hydrogen

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peroxide (H_2O_2) are produced at basal levels required for metabolic processes and function as signaling molecules under normal conditions (Anjum *et al*., 2011). Exposure of plants to salt stress increase ROS to levels that may become toxic to plants cells. Numerous studies have shown that excess ROS leads to the disruption of DNA strands, inactivation of various essential enzymes, protein degradation and lipid peroxidation (Jones, 2017; Ozfidan-Konakci *et al*., 2014; Klein, 2012).

According to Muchate and Nikalje (2016), salinity induce the production of H_2O_2 and other toxic oxygen species in cellular compartments. This disrupts normal metabolic processes and leads the oxidation of bio-macromolecules (proteins, enzymes, DNA and lipids). The overproduction of H_2O_2 and O_2^- has been observed in plants exposed to various stresses (Nxele *et al*., 2017; Chakraborty and Pradhan, 2012). The electron transport chains in the mitochondria and chloroplasts intracellularly produce these ROS molecules. Other sites of H_2O_2 production includes the peroxisomes. If not quenched or kept under control, these ROS molecules can be converted to highly toxic hydroxyl radicals that damage important cellular components that are required for maintenance of normal growth. Therefore, to compensate for their lack of mobility and regulate excess ROS levels produced under stressed conditions, plants evolved scavenging mechanisms that keep ROS levels at controllable and safe concentrations (Jones, 2017).

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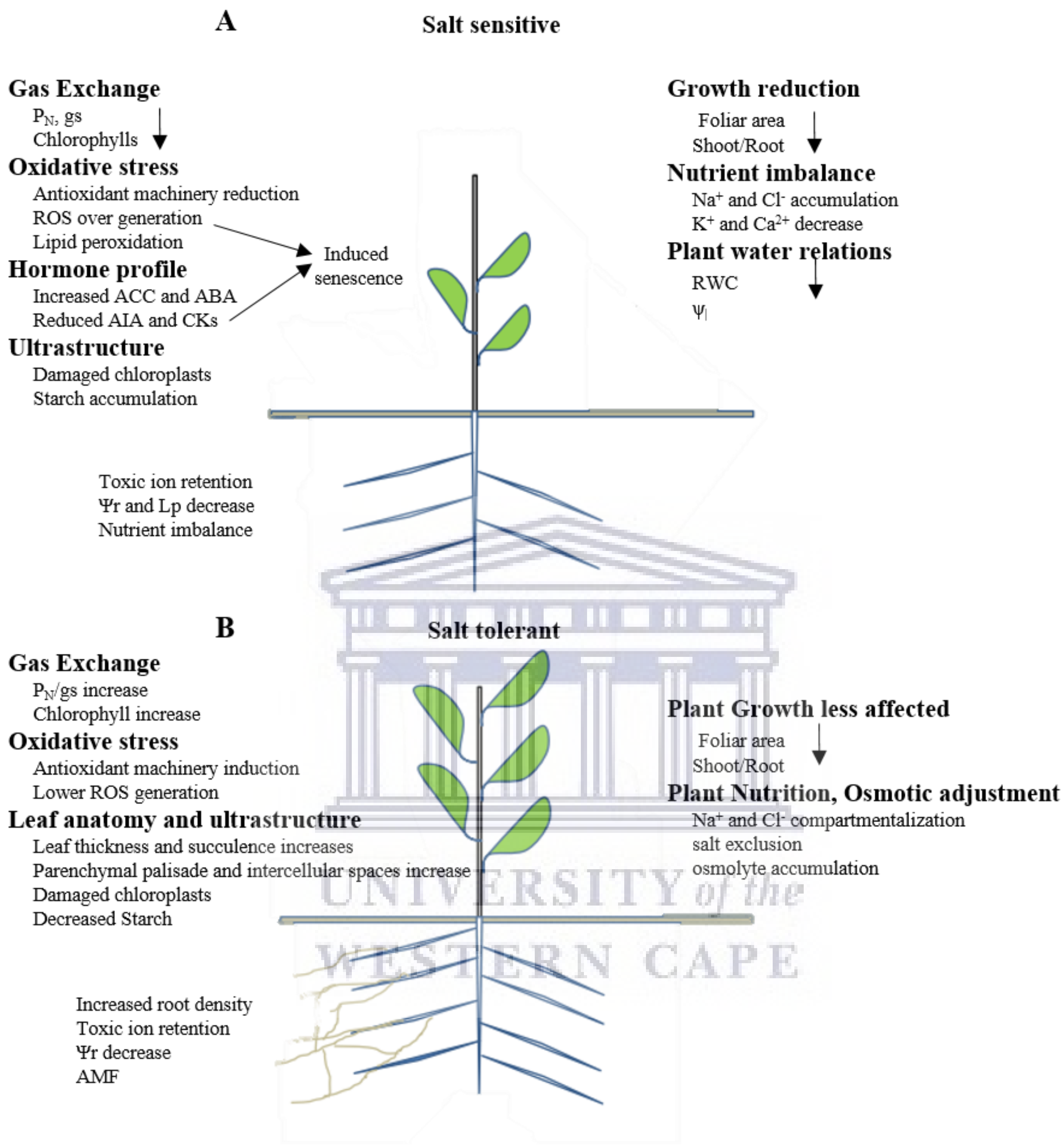

Figure 1.4. Schematic diagram showing the effect of salt stress on (A) salt-sensitive and (B) salt-tolerant plants. Salt stress decreases plant growth in salt-sensitive plants. The accumulation of Na^+ and Cl^- ions results in nutrient imbalances reflected in reduced K^+ and Ca^{2+}. Salt stress affects the photosynthesis rate (P_N). In parallel, chlorophyll content decreases. In addition, salinity induces oxidative stress leading to increased synthesis of abscisic acid (ABA) and ethylene and decreasing cytokinin (CK) and indol-3-acetic acid (IAA), favouring the progression of senescence in salinized leaves. Damage to chloroplasts is accompanied by starch accumulation. Salt accumulation in the root zone causes a decrease in root hydraulic conductivity (Lp) and root water potential (ψr), thus provoking a nutrient imbalance in roots. This figure was adapted from Acosta-Motos et al. (2017).

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1.4 Defense against ROS by the ROS scavenging mechanisms

ROS homeostasis is reached when a balance exists between ROS production and ROS scavenging, regulated by the plants antioxidant defense system, which include enzymatic and non-enzymatic antioxidants. Plants grown under normal conditions experiences low ROS concentrations in cells (240 µM s^{-1} O_2^- and 0.5 µM H_2O_2 in chloroplast) (Racchi, 2013). Non-enzymatic antioxidants include compounds such as glutathione, ascorbate (vitamin C), flavonoids, phenolic compounds, a-tocopherols (vitamin E) and carotenoids. Antioxidants act as electron donors and reduce toxic ROS to less harmful molecules. Enzymatic antioxidants include a variety of scavengers such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), peroxidases (PODs) and glutathione peroxidase (GPX) which protect cells by scavenging O_2^- and H_2O_2 (Table 1.2).

Table 1.2. Some natural antioxidants. The table was adapted and modified from Racchi (2013).

Non-enzymatic antioxidants		**Primary ROS**	**Location**
Ascorbate (vitamin C)		$O_2^{\bullet-}$, H_2O_2	Cyt, Chl, Mit, Apo, Per
α-tocopherol (vitamin E)		ROOH, $^1O_2$	Cell and plastid membrane
Glutathione reduced (GSH)		H_2O_2	Mit, Chl, Cyt, Nuc, Per, Apo, Vac
Enzymatic antioxidants	EC number		
Superoxide dismutase (SOD)	1.15.1.1	$O_2^{\bullet-}$	Apo, Cyt, (Cu/ZnSOD)
			Chl, (Cu/ZnSOD; FeSOD)
			Mit, (MnSOD)
			Per, (Cu/ZnSOD)
Ascorbate peroxidase (APX)	1.11.1.11	H_2O_2	Chl, Per, Apo, Cyt, Mit,
Glutathione peroxidase (GPX)	1.11.1.9	H_2O_2, ROOH	Cyt, Mit,
Peroxidase (non-specific) (POD)	1.11.1.7	H_2O_2	Cyt; CW
Catalase (CAT)	1.11.1.6	H_2O_2	Per

Abbreviations: Chl, chloroplast; Cyt, cytosol; Mit, mitochondria; Per, peroxisome; Apo, apoplast; Nuc, nucleus; Vac, vacuole.; CW, cell wall (Racchi, 2013).

When homeostatic defenses become overwhelmed under stress conditions, ROS production increases (240-720 µM s^{-1} O_2^- and 5-15 µM H_2O_2). Thus, in order to reduce the toxic and damaging levels of ROS, plants activate the enzymatic and non-enzymatic antioxidant defense system to minimize oxidative stress.

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1.4.1 Enzymatic components of ROS Scavenging

Enzymatic antioxidants such as SOD, APX, GPX and POD detoxify ROS molecules that are produced during stressed conditions (Hlatshwayo, 2018). Additionally, other enzymes that function in the Halliwell – Asada pathway (commonly referred to as the ascorbate–glutathione pathway) reduce toxic oxygen derivatives. These enzymes include glutathione reductase (GR), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR). Numerous studies have indicated the importance of the enzymatic antioxidants to reduce toxic ROS produced under salinity stress.

SOD (EC 1.15.1.1) belongs to a group of metalloproteins and functions to reduce toxic O_2^- into H_2O_2 and molecular oxygen (O_2) [$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$] (Chakraborty and Pradhan, 2012). SOD therefore serves as the first line of enzymatic antioxidant defense against ROS. The peroxide product (H_2O_2) is then reduced by APX (EC 1.11.1.7), using ascorbate as an electron donor (Lee *et al*., 2001) to water and monodehydroascorbate (MDHA). H_2O_2 is also scavenged by CAT (EC 1.11.1.6) and POD (EC 1.11.1.7) to form water and oxygen (Racchi, 2013). CAT, a tetrameric heme-containing enzyme is mainly located in the peroxisomes. Although CAT has high turnover rates, they have a lower affinity for H_2O_2 than APX (Hlatshwayo, 2018). This enzymes degrades H_2O_2 into water and oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$).

GPX (EC 1.11.1.9) is a thiol-dependant enzyme that reduce H_2O_2 and cytotoxic peroxides (phospholipid hydroperoxides and peroxynitrite) into alcohols at the expense of thioredoxins (Castella *et al.*, 2017). The enzyme is considered the main line of defense against oxidative membrane damage and is capable of membrane lipid peroxidation repair (Yoshimura *et al.*, 2004). However, the precise physiological role of plant GPXs is still poorly understood.

1.4.2 Non-enzymatic components of antioxidative defense systems

Among the non-enzymatic antioxidants, glutathione (GSH), ascorbic acid (AsA) and phenolic acids are particularly important (Sgherri *et al*., 2003). GSH acts as a ROS-scavenging antioxidant protecting plants during oxidative stress (Ali, 2019). In the Halliwell – Asada pathway, GSH is utilized in order to reduce dehydroascorbate (DHA). Simultaneously, during the reaction, GSH gets oxidized to glutathione disulphide (GSSG). Glutathione reductase (GR) may then convert GSSG back to GSH using NADPH as the electron donor (Yannarelli *et al*. 2007). Although GSH

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mostly serves as a substrate in the ascorbate-glutathione cycle, it has antioxidant properties which allows it to scavenge ROS, resulting in the reduction of ROS molecules (Jones, 2017).

AsA acts as a reducing agent in the ascorbate–glutathione cycle and assists in plant protection against H_2O_2 (Ali, 2019). APX utilizes two AsA molecules to reduce H_2O_2 to water. Simultaneously, monodehydroascorbate (MDHA) is produced during this reaction in which it can dissociate into dehydroascorbate (DHA) and AsA.

1.5 Phenolic compounds

One of the most studied phytochemicals are phenolic compounds (Engert, 2011). They are an important group of secondary metabolites that is largely distributed throughout plant tissues (Michalak, 2006). More than 8000 phenolic compounds exist in various plant species. Phenolic compounds are derived through the shikimate, pentose phosphate and phenylpropanoid pathways of plants (Kumar *et al.*, 2014). These compounds are essential to the plants physiology because of their involvement in several important functions such as structural support, defense against biotic and abiotic stress, plant growth and development, pigmentation and improved plant metabolism (Sharma *et al.*, 2019). Structurally, the compounds are composed of an aromatic ring with one or more hydroxyl groups attached and are classified based on the amount of aromatic rings and structural elements that bind to them, rendering them ideal for radical scavenging activities (Table 1.3). Phenolic compounds are divided into four classes: phenolic acids, flavonoids, lignans and stilbenes. An important function of phenolic compounds is their mode of action in plant defense mechanisms (Metsämuuronen and Sirén, 2019). Stress conditions such as salinity induces the synthesis of flavonoids and phenolic acids, which is associated with the plant's ability to cope under environmental constraints. Thus, external stimuli may significantly enhance the levels of various phenolic compounds in plants. These compounds have antioxidative properties which scavenges free radicals to protect plants against oxidative damage.

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Table 1.3. Chemical structure of some phenolic compounds. The table was adapted and modified from Kumar *et al.* (2014).

Class	Structure	Compound
Phenolic acid	Hydroxycinnamic acid (HCA)	caffeic acid 3,4–OH ferulic acid 4–OH; 3–OCH3
	Hydroxybenzoix acid (HBA)	gallic acid 3, 4, 5–OH p-HBA 4–OH
Flavonol		Quercetin 3',4',5,7-OH kaempferol 4',5,7-OH Naringenin 4',5,7-OH hesperetin 3',5,7-OH 4'-OCH3

1.5.1 Phenolic acids

Phenolic acids are found in all plant tissues and account for one third of the phenolic compounds in our diet. These compounds are associated with important metabolic events such as photosynthesis, nutrient uptake, enzymatic activity, protein synthesis and allelopathy effects. In response to stress, phenolic acids act as signaling molecules or play a direct role in defense against stress, preventing the generation of harmful ROS products. Phenolic acids are divided into hydroxybenzoic acids (HBA - seven carbon atoms) and hydroxycinnamic acids (HCA - nine carbon atoms). HCA and HBA may serve as allelopathic compounds (Klein, 2012). Cinnamic acid derivatives include caffeic acid, ferulic acid and sinapic acid whereas benzoic acid derivatives include vanillic acid, syringic acid and gallic acid.

1.5.2 Gallic acid (GA) as a phenolic compound

Gallic acid (GA), also known as 3,4,5-trihydroxy-benzoic acid, is a naturally occurring phenolic acid found in grapes, green tea, cherries, pomegranate and several other plants (Choubey *et al.*, 2015). It has been reported that GA has several beneficial health effects including antifungal,

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antimicrobial, antiviral, anti-inflammatory, antimutagenic and anticarcinogenic properties. Additionally, it has been demonstrated that GA acts as a potent antioxidant, protecting cells against oxidative stress. Oxidative stress is triggered by an excess of ROS molecules and involves chemical reactions between free radicals and biologically important macromolecules such as proteins, lipids and nucleic acids. The antioxidative activity of GA is due to its redox properties, which play a role in adsorbing and eliminating diverse free radicals such as singlet oxygen, hydroxyl radical, peroxyl, alkyl peroxyl and long-lived mutagenic radicals. Some reports performed in animal trials showed that GA reduced oxidative damage, enhanced GSH levels, induced GPX and GR and inhibited polyunsaturated fatty acids. GA may directly scavenge ROS through coupled reactions with enzymatic antioxidants (Ozfidan-Konakci *et al*. 2014). It has been reported that GA improves plant growth in various plant species (Menzi *et al*. 2018; Singh *et al*. 2017). Contrary to this, GA was reported to decrease germination rate, growth of radicle and the fresh weight and dry weight of cucumber seedlings (Muzaffar *et al*., 2012).

1.5.3 Biosynthesis of gallic acid (GA)

Three alternative pathways have been proposed for the synthesis of GA. Biosynthesis of hydroxybenzoic acids such as GA may start from the shikimate pathway (Figure 1.5) (Widhalm and Dudareva, 2015). The first step in the pathway is the synthesis of 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) through the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) by the enzyme DAHP synthase. Secondly, the intermediate for gallic acid, 3-dehydroshikimate (3-DHS) is formed via the removal of phosphate and water from 3-dehydroquinate. This reaction is catalyzed by 3-dehydroquinate dehydratase (DHD). Studies suggest that GA is synthesized from 3-DHS by shikimate dehydrogenase to produce 3,5-didehydroshikimate (de la Rosa *et al.*, 2019). This compound then tautomerizes to its redox equivalent, GA, which can further produce gallotannins and ellagitannis. GA may also be synthesized by the hydroxylation of protocatechuic acid (3,4-dihydroxybenzoic acid) or β-oxidation of 3,4,5-trihydroxycinnamic acid (Taylor and Saijo, 2014).

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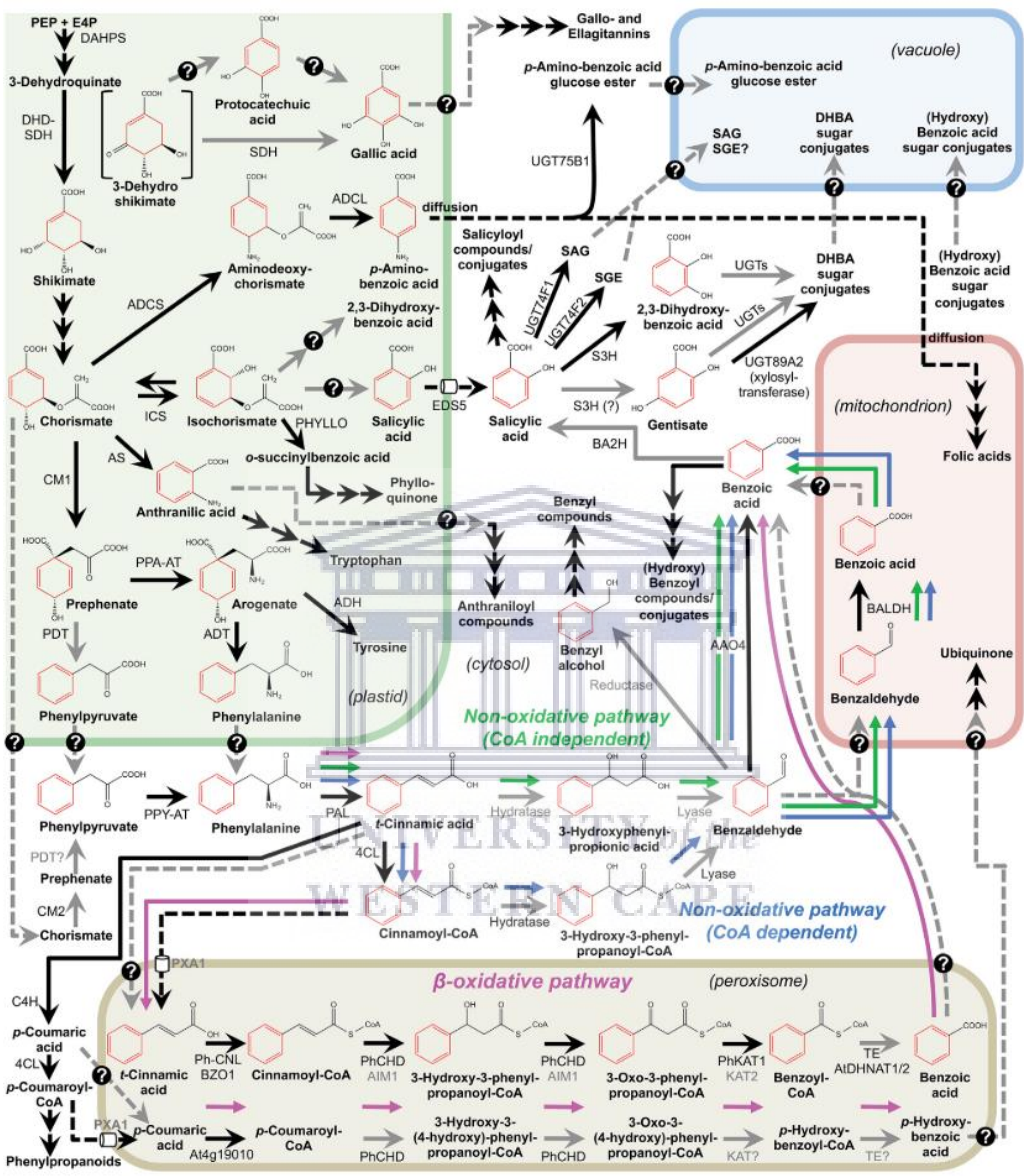

Figure 1.5. The Biosynthesis of plant benzoic acids (Widhalm and Dudareva, 2015)

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1.6 Proteomics advances in crop research

The term "proteomics" refers to the study of proteins expressed in a cell, tissue or subcellular compartment at a given time under specifically defined conditions. Proteomics studies become essential to understanding the cellular responses that occur during unfavorable conditions. High-throughput proteomics techniques using mass spectrometry (MS) as a platform have been utilized in plant research over the past decade and have enabled researchers to study the proteome responses of several plant species to various biotic and abiotic stress stresses (Li *et al.*, 2015; Bernfur *et al.*, 2013; Kosová *et al.*, 2011).

Improvements in proteomic technologies such as the separation of proteins by 2-dimensional gel electrophoresis (2-DE), downstream trypsinisation and peptide analysis using MS have allowed the detection, quantification and identification of a large number of proteins (Fernández-Acero *et al.*, 2006). These traditional methods have been used to analyse changes in protein expression subsequent to changes in environmental conditions (Ali, 2019). However, 2-DE poses limitations for identification of specific protein classes (Nouri and Komatsu, 2010). These mainly include the low-abundant and poorly soluble membrane proteins, making them difficult to analyse using conventional techniques (Blomqvist *et al*., 2006). In recent years, alternative proteomics techniques referred to as label free liquid chromatography-mass spectrometry (LC-MS) have been developed for comparative proteome analysis (Katz *et al*., 2010). These improvements in proteomic technologies have made the field an active area of research for biomarker discovery and validation (Trinh *et al.*, 2013).

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1.6.1 Membrane proteins

Membrane proteins carry out several essential functions that are vital for the organism's survival. These include molecular ion transport, cell-cell communication, signal transduction and cell motility (Tai *et al.*, 2011). Studies on membrane proteins are particularly difficult due to their poor solubility in aqueous solutions and low abundance (Seddon *et al*., 2004). This class of proteins are usually under-represented in many large scale proteomic studies (Zhang, 2015). Furthermore, about 25 % of all annotated proteins are predicted as membrane proteins, but less than 2 % is represented in the protein data bank (PDB) database.

Membrane proteins are classified into two groups, integral membrane proteins (IMPs) and peripheral membrane proteins (PMPs). PMPs are lightly bound to the membrane through non-

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covalent bonds with extended parts of the IMP. PMPs mostly represent the proteins that associate with multicomponent complexes such as photosynthetic proteins and H^+-ATPase (Shahzad *et al.*, 2016). IMPs are attached to the cell membrane through transmembrane domains that span the region. These proteins functions as transporters, receptors for signaling cascades or ion channels.

Various strategies have been developed to improve the solubility of membrane proteins. Addition of detergents or organic solvents have shown to increase the number of protein identifications from membrane fractions. Amongst the commonly used detergents, sodium-dodecyl-sulfate (SDS) is one of the powerful solubilizing reagents (Speers and Wu, 2007). Although SDS concentrations used in shotgun proteomics varies (0.1% - 1% SDS), the molecules are known to interfere with LC separation of peptides and has poor MS compatibility (Min *et al.*, 2016). Thus, prior to digestion (with trypsin) and MS/MS acquisition, the SDS concentration is diluted to 0.1 % or completely removed using C_{18} clean-up stage tips (Millipore Ziptips). Despite these inherent challenges when working with membrane proteins, they serve as an important area for study because of their role in fundamental biochemical processes (Seddon *et al.*, 2004).

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Project aim and objectives

The aim of this study was to investigate the effect of exogenously applied gallic acid on the physio-biochemical responses and membrane protein abundance in wheat (*Triticum aestivum* L.) plants under salt stress.

The specific objectives for this study were as follows:

- To measure changes in plant growth parameters including shoot and root length, relative water content and biomass and chlorophyll pigments.
- To monitor changes in ROS accumulation and antioxidant enzyme activities
- To identify and functionally characterize shoot membrane proteins using LC-MS/MS

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CHAPTER TWO

MATERIALS AND METHODS

All absorbance measurements were recorded using a UV-visible FLUstar Omega spectrophotometer (BMG LabTech GmbH, Orthenberg, Germany).

2.1 Plant material and growth conditions

Wheat (*Triticum aestivum* L., cv. SST015) seeds, obtained from the Research and Technology Development Services (Department of Agriculture, South Africa) were surface sterilized in 0.35 % sodium hypochlorite for 20 minutes and washed five times with sterile distilled water. The seeds were imbibed in sterile distilled water for 45 minutes prior to germination on wet filter paper in a dark environment for two days. The germinated seedlings were transplanted (6 seedlings per pot) into 3-litre plastic pots containing a potting soil and silica sand mixture (1:3). Seedlings were irrigated every third day with 200 mL distilled water and grown under controlled temperature conditions (26 $^{\circ}$C) with ambient light until the V2 stage of development before the start of treatments.

2.2 Plant treatments

Nitrosol®, a commercially available organic plant fertilizer was used in this study. For treatments, plants at the V2 stage of development were treated with half strength Nitrosol® supplemented with either 250 µM gallic acid (GA-treated plants), 150 mM salt (NaCl-treated plants) or a combination of 150 mM salt and 250 µM gallic acid (NaCl + GA-treated plants). The control plants (untreated) were supplemented with half-strength Nitrosol® only (no GA or NaCl). Treatments (150 mL per pot) were applied at three-day intervals over a period of 28 days before the plants were harvested. The experiment was carried out in a completely randomized design (rotated weekly) throughout the growth period with eight replicates per treatment.

2.3 Analysis of growth parameters

At the end of the treatment period (after 28 days), plants were carefully removed from the soil. Roots and shoots from six plants of each treatment were separated and scored for their root length, shoot height, root and shoot fresh weight (FW) and root and shoot dry weight (DW). For measuring

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DW, samples were dried in an oven at 55 °C for 48 hours and weighed as described by Tarchoune *et al.* (2012).

2.4 Relative water content

Relative water content (RWC) was measured as described by Yildirim *et al*. (2009) with slight modifications. The third eldest leaf blade from each treatment were weighed to determine FW. For turgid weight (TW), the leaf blades were rehydrated for 2 hours in distilled water, blotted dry to remove surface water and the weights recorded. DW was recorded after incubation of the leaf blades at 55 °C for 48 hours. Values of FW, DW and TW were used to calculate RWC according to the following equation:

$$\text{RWC} = \frac{\text{Fresh weight (FW)} - \text{Dry weight (DW)}}{\text{Turgid weight (TW)} - \text{Dry weight (DW)}} x\ 100$$

2.5 Measurement of photosynthetic pigments

Chlorophyll and carotenoid content was determined by submerging leaf tissue (0.1 g) in 1 mL of 80 % acetone. The samples were vortexed for 1 minute and centrifuged at 13 000 rpm for 10 minutes. This process was repeated until a clear pellet was observed. The absorbance was measured at wavelengths of 470 nm, 663 nm, and 646 nm.

Chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid content were measured and calculated as described by Lichtenthaler and Buschmann (2005) and total chlorophyll (Chl a + b) according to Arnon (1949) with modifications proposed by Palta (1990) using the following equations:

$$\text{Chl a (mg/g FW)} = [12.25\ (A_{663}) - 2.79\ (A_{646})] \text{ x } [V/W/1000]$$

$$\text{Chl b (mg/g FW)} = 21.50\ (A_{646}) - 5.10\ (A_{663}) \text{ x } [V/W/1000]$$

$$\text{Chl a + b (mg/g FW)} = 20.2\ (A646) + 8\ (A663) \text{ x } [V/W/1000]$$

$$\text{Carotenoids (mg/g FW)} = [(1000\ (A_{470}) - 1.82\ [\text{Chl a}] - 85.02\ [\text{Chl b}]) / 198] \text{ x } [V/W/1000]$$

where:

V= Total volume of filtrate (mL)

W= Weight of plant material (g)

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2.6 Evaluation of cell viability using Evans Blue

Cell viability was measured in wheat plants using a modified method described by Vijayaraghavareddy *et al*. (2017). Fresh plant material (leaf and root tissue, 2 cm in length from the tip) was immersed in 0.25 % Evans blue solution for 1 hour at room temperature. The leaves and roots were thoroughly washed with distilled water until all un-bound dye was removed. Evans blue dye (taken up by dead cells) was then extracted from leaf and root tissue by incubating the plant material in 1 mL of 1 % sodium dodecyl sulfate (SDS) for 1 hour at 55 °C. The solution was cooled at room temperature and absorbance of the extract measured at 600 nm to determine the level of Evans blue uptake by the cells.

2.7 Biochemical analysis for determination of oxidative stress

Plant material (shoots and roots) from all treatments were ground into a fine powder using liquid nitrogen and stored at - 80 °C until further analysis.

2.7.1 Trichloroacetic acid (TCA) extraction

Plant material (0.2 g) was homogenized in 1 mL of cold 6 % TCA. The homogenate was centrifuged at 13 000 rpm for 20 minutes at 4 °C and the supernatant (TCA extract) was used for estimation of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content as previously described by Klein (2012).

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2.7.2 Lipid peroxidation

The oxidative stress biomarker and end product of lipid peroxidation (reflective as MDA content) was measured following the thiobarbituric acid (TBA) method of Chakraborty and Pradhan (2012) with slight modifications. For these measurements, 200 µL of TCA extract was added to 400 µL of 20 % TCA containing 0.5 % TBA. The assay mixture was heated in a 95 °C water bath for 10 minutes, cooled on ice and centrifuged at 13 000 rpm for 5 minutes. The absorbance was measured at 532 nm and 600 nm to correct for nonspecific turbidity. The MDA content was calculated using an extinction coefficient $\varepsilon = 155\ mM^{-1}\ cm^{-1}$ and results expressed as nmol g^{-1} FW.

2.7.3 Hydrogen peroxide (H_2O_2) measurement

The H_2O_2 content was measured according to Egbichi *et al*. (2014). The reaction mixture consist of 50 µL TCA extract, 5 mM dipotassium phosphate (K_2HPO_4, pH 5.0) and 0.5 M potassium iodide (KI) in a final volume of 200 µL. The reaction was incubated in the dark for 20 minutes at 25 °C and the absorbance measured at 390 nm. The H_2O_2 content was calculated and expressed as nmol. g^{-1} FW by using a standard curve prepared with H_2O_2 standards of known concentrations.

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2.7.4 Superoxide (O_2^-) measurement

The O_2^- content was estimated by its ability to reduce nitroblue tetrazolium chloride (NBT). A modified method of Bates *et* al. (1973) was used. Fresh plant material (leaf and root tissue, 2 cm in length from the tip) were immersed in a reaction buffer containing 78 mM potassium phosphate (KPO_4, pH 7.0), 10 mM potassium cyanide (KCN), 10 mM H_2O_2 and 80 µM NBT in a final volume of 800 µL. After incubation of the reaction mixture at room temperature for 20 minutes, plant material was crushed and the homogenate centrifuged at 13 000 rpm for 20 minutes at 4 $^{\circ}$C. The absorbance of the supernatant was measured at 600 nm and the O_2^- content was calculated according to its extinction coefficient $\varepsilon = 12.8\ mM^{-1}\ cm^{-1}$.

2.8 Preparation of antioxidant enzyme extracts

Enzyme extracts were prepared from plant material (shoots and roots) of the various treatments according to Jones (2017) with slight modifications. Using a pre-cooled mortar and pestle, 0.2 g of plant material was homogenized in 600 µL of cold extraction buffer [40 mM K_2HPO_4 (pH 7.4); 5 % polyvinylpyrrolidone (PVP); 5 % glycerol; 1 mM ethylenediaminetetraacetic acid (EDTA)]. The homogenates were centrifuged at 13 000 rpm for 20 minutes at 4 $^{\circ}$C and the supernatants (enzyme extracts) were used for antioxidant analysis. The protein concentration of the enzyme extract was determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

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2.9 Measurement of antioxidant enzyme activity

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Ascorbate peroxidase (APX) activity was assayed following a modified method of Nakano and Asada (1981). The reaction buffer contained 50 mM K_2HPO_4 (pH 7.0), 0.2 mM EDTA, 0.25 mM ascorbic acid (AsA), 4.5 µM H_2O_2 and 17.5 µg enzyme extract in a final volume of 200 µL. The reaction was initiated with the addition of H_2O_2 and the enzymatic activity was measured by observing the decrease in absorbance at 290 nm for 1 minute. Total APX activity was determined using the extinction coefficient $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$.

Superoxide dismutase (SOD) activity was measured using a modified method of Giannopolitis and Ries (1977). The reaction mixture contained 50 mM KPO_4 (pH 7.4), 13 mM L-methionine, 75 µM NBT, 0.1 mM EDTA and 10 µL of enzyme extract in a final volume of 200 µL. The reaction was initiated by the addition of 2 µM riboflavin and exposure the mixture to light until a colour change was observed. The decrease in absorbance of the superoxide-nitro blue tetrazolium complex by

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the enzyme was measured at 560 nm. One unit of SOD activity represented the amount of enzyme causing 50 % inhibition of NBT under assay conditions.

2.10 Separation and detection of antioxidant isoforms by activity staining

For each antioxidant enzyme a fraction of each sample (per treatment) were separated on discontinuous non-denaturing polyacrylamide gels (5 % stacking gel and 10 % resolving gel) and electrophoresed at 10 mA in the stacking gel followed by 15 mA in the resolving gel at 4 °C using running buffer [25 mM Tris; 192 mM glycine] unless otherwise stated. Individual isoforms for each antioxidant enzyme were detected using different staining methods as described below.

2.10.1 SOD activity staining

SOD isoforms were detected following a modified method by Beauchamp and Fridovich (1971) using 40 µg protein. The SOD isoforms were differentiated by incubating gels for 20 minutes in staining solution I [50 mM KPO_4 (pH 7.8) containing 5 mM KCN (to inhibit Cu/Zn-SOD) or 6 mM H_2O_2 (to inhibit Cu/Zn-SOD and Fe-SOD)] as described by Vitória *et al*. (2001). The gels were further incubated in staining solution II [50 mM KPO_4 (pH 7.8); 0.25 mM NBT] for 20 minutes in the dark with slight agitation followed by a final incubation in staining solution III [50 mM KPO_4 (pH 7.8); 0.5 mM riboflavin; 28 mM N, N, N, N-tetramethylethylenediamine (TEMED)] on a light box until SOD isoforms were detected.

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2.10.2 APX activity staining

APX isoforms were detected using a modified method previously described by Seckin *et al.* (2010). Prior to loading samples (40 µg protein), gels were equilibrated in running buffer containing 2 mM AsA for 30 minutes at 4 °C. Subsequently, the electrophoresed gels were incubated in staining solution I [50 mM KPO_4 (pH 7.0); 2 mM AsA] for 20 minutes followed by staining solution II [50 mM KPO_4 (pH 7.8); 4 mM AsA; 2 mM H_2O_2] for 20 minutes. The gels were then washed with 50 mM KPO_4 (pH 7.8) for 1 minute prior to a final incubation in staining solution III [50 mM KPO_4 (pH 7.8); 0.5 mM NBT; 28 mM TEMED) for 1 minute. All the incubation steps were done in the dark with slight agitation. The gels were exposed to light until the APX activity was observed as clear zones on a blue background.

2.10.3 Peroxidase (POD) activity staining

For the detection of individual POD isoforms each sample (40 µg protein) was separated on a discontinuous non-denaturing polyacrylamide gel (4 % stacking gel and 8 % resolving gel) as

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described by Tewari *et al*. (2013) with slight modifications. Following electrophoresis, individual POD isoforms were detected by incubating each gel in 50 mM KPO_4 (pH 7.0) containing 8.8 mM H_2O_2 for 10 minutes. After the initial incubation each gel was incubated in 50 mM KPO_4 (pH 7.0) containing 0.02 % 3,3'- Diaminobenzidine (DAB) until DAB-POD activity was visible.

2.10.4 Glutathione-like peroxidase (GPX) activity staining

GPX isoforms were detected on a 10 % polyacrylamide gel using a modified method previously described by Seckin *et al.* (2010). Prior to loading samples (40 µg protein),the gels were equilibrated in running buffer containing 4 mM GSH for 30 minutes at 4 °C. Following electrophoresis, the gels were incubated in KPO_4 buffer (pH 7.0) containing 2 mM GSH for 20 minutes. The gels were then transferred to 50 mM KPO_4 (pH 7.8) containing 4 mM GSH and 0.01 % cumene hydroperoxide for a further 20 minutes. Finally, the gel was washed with distilled water and incubated in 50 mM KPO_4 (pH 7.8) containing 0.37 mM NBT and 28 mM TEMED for 15 minutes with gentle agitation under light conditions.

2.11 Densitometry analysis of ROS scavenging enzymes

The Native PAGE gels for SOD, APX, POD and GPX antioxidant enzymes were analyzed by densitometry analysis using the Alpha Ease FC imaging software (Alpha Innotech Corporation). The enzymatic activity of each isoform from three independent gels were measured according to Klein (2012). The average of the pixel intensities was expressed as relative arbitrary units. This was achieved by assigning the isoforms in the untreated plants to a value of 1 and expressing the pixel intensities of the rest of the isoforms in the various treatments relative to this isoform.

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2.12 Profiling the membrane proteome of wheat

2.12.1 Isolation of membrane fraction

The total membrane fraction (organelle membrane and plasma membrane) was isolated using the minute™ plasma membrane protein isolation kit for plants (Invent Biotechnologies, Inc., Plymouth, MN 55441) with slight modifications. Shoot material (0.2 g) from each treatment were transferred to pre-chilled filter tubes containing 0.02 g dissociation beads and homogenized (using a plastic rod) for 1 minute in 100 µL buffer A. An additional 100 µL buffer A was added and the samples were incubated on ice for 5 minutes, centrifuged at 13 200 rpm for 10 minutes and the filter discarded. Pellets were re-suspended in solution by vigorously vortexing the samples followed by centrifugation at 3 000 rpm for 3 minutes to pellet nuclei and larger debri. The

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supernatants were transferred to 1.5 mL eppendorf tubes and centrifuged at 13 200 rpm for 45 minutes. The supernatants were discarded and the pellets (total membrane fraction) were stored at - 80 °C until further analysis.

2.12.2 Membrane-associated protein extraction and precipitation

Membrane proteins were extracted from the total membrane fraction by re-suspending the pellets from each treatment in 50 µL of extraction buffer [100 mM Tris (pH 8.0); 1 % TritonX-100; 4 M guanidine hydrochloride]. The samples were sonicated for 3 minutes and incubated on ice for 1 minute. After four cycles, the supernatants (protein extracts) were transferred to 2 mL eppendorf tubes and the proteins precipitated using the water/chloroform/methanol precipitation method as described by Fic *et al.* (2010) with slight modifications.

To precipitate the proteins, 200 µL of methanol was added to 50 µL protein extract. The mixture was vortexed and 50 µL chloroform was added. Phase separation was induced by adding 150 µL of water before mixing and the samples were centrifuged at 13 200 rpm for 5 minutes. The upper aqueous methanol phase was removed and discarded before 200 µL of methanol was added to the mixture. The samples were vortexed and centrifuged at 13 200 rpm for 5 minutes. The supernatant was discarded and the protein pellets were air dried.

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2.13 Protein solubilisation

Proteinswere solubalised and purified using the method by Arefian *et al*. (2019)with modifications. Protein pellets were resuspended in 20 µL of 50 mM Tris (pH 8.0) containing 3 % SDS. The samples were incubated at 70 °C for 10 minutes and centrifuged at 13 000 rpm for 5 minutes. The supernatants (total soluble membrane proteins) were recovered and transferred to 2 mL eppendorf tubes and stored at - 80 °C until further analysis.

2.13.1 Acetone precipitation, alkaline hydrolysis and protein quantification

For purification, the protein samples (1 µL) were diluted 10 times in ultrapure water and 5 volumes of cold acetone was added. Proteins were precipitated overnight at - 20 °C and centrifuged for 30 minutes at 11 800 rpm. The pellets were re-suspended in 20 µL of 1 N NaOH prior to incubation at 100 °C. The total soluble membrane protein concentration was quantified using the Pierce microplate BCA protein assay kit (Thermo Scientific) according to the manufacturer's instructions using ovalbumin as a positive control.

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2.13.2 On-bead HILIC digest

All reagents used for this part of the study were analytical grade or equivalent.

Protein samples were digested using the MagReSyn HILIC workflow (Resyn Biosciences) with slight modifications. Protein samples (1.28 µg) were made up to a final volume of 25 µL in 50 mM ammonium bicarbonate (AmBic) before reduction with 10 mM dithiothreitol (DTT) (prepared in 50 mM AmBic) for 30 minutes at room temperature. The cystein residues were carbamidomethylated with 30 mM iodoacetamide (IAA) for 30 minutes in the dark and an equal volume of binding buffer [200 mM ammonium acetate (pH 4.5); 30 % acetonitrile (ACN)] added. The protein solutions were then added to 20 µL of pre-equilibrated MagReSyn® HILIC magnetic microparticles (Resyn Biosciences) that were prepared according to manufacturer's instructions. The proteins were allowed to bind to the microparticles by incubation at 4 °C for 4 hours. After binding, the supernatants were removed and the microparticles washed twice with 200 µL of 95 % ACN for 1 minute.

For digestion, the magnetic particles were suspended in 20 µL of 50 mM AmBic containing trypsin (New England Biosystems) to a final ratio of 1:50 total protein and was incubated overnight at 27 °C with constant shaking. Following digestion, the supernatant containing the peptides was transferred to glass vials. Further peptide recovery was achieved by adding 10 µL of 1 % trifluoroacetic aicd (TFA) to the microparticles prior to incubation at room temperature for 3 minutes. The process was repeated once more before combining the supernatants to the glass vial.

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2.13.3 Solid - phase extraction

Following trypsin digestion, residual digest reagents were removed and peptides purified via solid - phase extraction (SPE) using an in-house manufactured C_{18} stage tip (Empore™ Octadecyl C_{18} 47 mm extraction discs; Supelco) according to Rappsilber *et al*. (2007) with modifications. The C_{18} membrane was activated with 30 µL methanol and equilibrated with 30 µL of 2 % ACN containing 0.1 % formic acid (FA), followed by sample loading. The bound sample was then washed with 30 µL of 2 % ACN containing 0.1 % FA. Peptides were eluted with 30 µL of 50 % ACN containing 0.05 % FA and the eluate evaporated to dryness. The dried peptides were then dissolved in 2 % ACN containing 0.1 % FA for LC-MS analysis.

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2.13.4 Liquid chromatography - mass spectrometry (LC-MS) analysis

LC-MS analysis was performed according to Nouri and Komatsu (2010) with modifications. Peptides were injected into a Dionex Ultimate 3000 RSLC nano-HPLC system coupled to a Fusion mass spectrometer equipped with a nanospray Flex ionization source (Thermo Fisher Scientific, USA). The peptides were dissolved in 0.1 % FA containing 2 % ACN and loaded onto a C_{18} trap column (5 mm x 300 µm) for 5 minutes at a flow rate of 2 µL/min from a temperature controlled autosampler set at 7 °C. Chromatographic peptide separation was performed at 40 °C on a 25 cm x 75 µm (1.7 µm particle size) CSH C_{18} analytical column (Waters) at a flow rate of 250 nL/min described below.

The solvent system employed was solvent A: 2 % ACN; 0.1 % FA and solvent B: 100 % ACN; 0.1 % FA. The gradient was generated as follows: 5 % - 35 % solvent B for 60 minutes and 35 % - 50 % solvent B from 60 – 75 minutes.

The outflow was then introduced to the mass spectrometer through a stainless steel nano-bore emitter. Data acquisition was collected in positive ion mode with an applied electrospray voltage set to 1.8 kV and ion transfer capillary set to 280 °C. Spectra were internally calibrated using polysiloxane ions at mass – charge ratios of m/z = 45.12003 and 371.10024. MS1 acquisitions were performed using an orbitrap detector set at 120 000 resolutions over the m/z scan range of 350 – 1650 with AGC target at 3e5 and maximum injection time of 40 milli seconds.

MS2 acquisitions were performed using monoisotopic precursor ion selection with +2 - +7 charges and an error tolerance set to +/- 10 ppm. Precursor ions were excluded from fragmentation for a period of 60 seconds and then fragmented using the quadrupole mass analyser in HCD mode with an HCD energy set to 30 %. Fragmented ions were detected in the orbitrap mass analyzer set to 30 000 resolutions. The AGC target was set to 5e4 with maximum injection time of 80 milli seconds. Data was acquired in centroid mode.

2.13.5 Data analysis

The raw MS files generated were imported into Proteome Discoverer v1.4 (Thermo Scientific) and further processed using the Amanda and Sequest algorithms. Database interrogation was performed using the uniprot *Triticum* database concatenated with the common repository of adventitious proteins (cRAP) contaminant database (https://www.thegpm.org/crap/). The semi-tryptic digest of 2 missed cleavages was allowed. The precursor mass tolerance was set to 10 ppm

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while the fragment mass tolerance was set to 0.02 Da. Variable modifications such as the deamidation (NQ), acetylation and oxidation (M) of the N-terminus as well as static modifications such as carbimidomethyl of C were also included as parameters. Peptide validation was performed using the Target-Decoy PSM validator node and search results imported into the Scaffold Q + for further validation (www.proteomesoftware.com).

2.14 Functional classification, transmembrane domain identification and subcellular localization

Proteins were functionally characterized using the data available in the UniProt database (www.uniprot.org) as well as literature sources. The sequences for the proteins identified by LC-MS analysis were entered in HMMTOP (http://www.enzim.hu/hmmtop/), which returned output consisting of the number of transmembrane domains (TMDs) for each protein. The subcellular localization was predicted based on Uniprot (www.uniprot.org) and Aramemnon database searches (http://aramemnon.botanik.uni-koeln.de/).

2.15 Statistical analysis

All experiments were performed as three independent studies. For growth measurements, eight plants per treatment was analysed. For all other experiments, three plants per treatment were used For statistical purposes, the one-way analysis of variance (ANOVA) test was used for all data, and means (for three independent experiments) were compared according to the Tukey–Kramer test at 5 % level of significance.

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CHAPTER THREE

GALLIC ACID REGULATES THE PHYSIOLOGICAL RESPONSES OF WHEAT UNDER SALT STRESS

3.1 Introduction

Soil salinity is a major environmental stress condition limiting the growth and productivity of agricultural crops worldwide (Volschenk, 2005). The presence of excess ions (Mg_2^+, Ca_2^+, Na^+, Cl^- and SO_4^{2-}) in the soil interferes with several physiological processes including plant nutrition, stomatal behavior and water relations, consequently disturbing normal metabolic functions required for plant growth, development and survival (Butcher *et al.*, 2016; Lopez *et al.*, 2002). The negative effects of soil salinity may also reduce CO_2 intake which results in the reduction of chlorophyll content and overall photosynthesis (Bose *et al.*, 2014). The effects of short and long term salt stress on different wheat cultivars (tolerant and susceptible) revealed a significant reduction in photosynthetic rate (Zheng *et al.*, 2008), stomatal conductance, plant height, root length (Qiu *et al.*, 2014), water uptake (Sairam *et al.*, 2005) and shoot and root dry weight (Abdella *et al.*, 2017). The same phenomenon was observed in other important agricultural crops such as soybean (Egbichi *et al.*, 2014) and maize (Valentovič *et al.*, 2006).

Salt-induced stress triggers the over production of reactive oxygen species (ROS) including superoxide ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2). Accumulation of these ROS molecules, to toxic levels, cause a series of responses including DNA mutation, protein denaturation and lipid peroxidation, which results in severe damage to plant structures (Akram and Ashraf, 2009). ROS scavenging is sustained by plant antioxidant defense systems, which comprises the activation of key antioxidant enzymes or the synthesis of various antioxidant compounds. There is a considerable amount of evidence revealing that phenolic compounds are able to scavenge toxic ROS levels due to their antioxidative capacity (Ozfidan-Konakci *et al.*, 2014; Yen *et al.*, 2002). Phenolic compounds can act as free radical scavengers or assist in ROS-scavenging by increasing the activity of key antioxidant enzymes (Nkomo *et al.*, 2019; Varela *et al.*, 2016).

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Gallic acid (GA), a naturally occurring phenolic compound present in most plants, is known to have strong antioxidant properties (Marino *et al*., 2014) and exhibit several other important biological (Silva *et al.*, 2010) and pharmacological (Gao *et al.*, 2019) activities. Evidence of its protective role against stress induced oxidative damage has been well studied, but is restricted to *in vitro* experiments with human and animal cells (Padma *et al*., 2011). The impact of exogenous GA application in a few plant species have been investigated, however the information available in the public domain remains limited and contradictory. For instance, Muzaffar *et al*. (2012) showed that the presence of GA in growth media had inhibitory effects on seed germination and seedling growth of cucumber plants. The opposite was observed in the study conducted by Reigosa *et al*. (1999), where GA showed stimulatory effects on the germination of different weed species. To date, no reports on the impacts of GA in wheat plants under salt stress have been elucidated. Therefore, this study aims to evaluate the role of GA on wheat plants under salt stress by monitoring growth, biomass and photosynthetic pigments in order to better understand the impact of GA on salt stress responses of plants.

3.2 Results

3.2.1 Effects of exogenous GA on growth and biomass of wheat plants under salt stress

The effect of exogenous GA on the growth and development of wheat plants under salt stress was evaluated. The results show that GA and NaCl differentially influences wheat growth (shoot and root length) and biomass (shoot and root fresh and dry weight) (Table 3.1).

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GA treatment increased shoot length by 4 %, whereas NaCl treatment reduced the shoot length by 13 % when compared to the untreated control. However, GA application to salt stressed plants (NaCl + GA) showed an increase of 12 % in shoot length (albeit not to the level of the untreated control) when compared to NaCl-treated plants. On the other hand, root length increased by 9 % in response to NaCl treatment when compared to the untreated control, whereas no significant differences were observed in the other treatments. However, NaCl-treated plants supplemented with GA displayed an 8 % reduction in root length when compared to the NaCl treated plants. When compared to the untreated control no significant changes in root length was observed.

Furthermore, exogenous application of GA significantly increased shoot fresh and dry weights by 14 % and 27 % respectively, whereas NaCl-treatment significantly decreased shoot fresh and dry weights by 20 % and 13 % in comparison to the untreated controls (Table 3.1). However, the

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reduction in shoot fresh and dry weight observed in the NaCl treatment was reversed (to the level of the untreated plants) when NaCl-treated plants were supplemented with GA. Combined treatment (NaCl + GA) showed a significant increase in shoot fresh and dry weight of 30 % and 18 % respectively in comparison to the NaCl treated plants.

Similar results were observed for root fresh and dry weights in response to the various treatments. As compared to the untreated controls, after NaCl treatment, root fresh and dry weights significantly decreased by 48 % and 37 % respectively. On the other hand, application of GA to NaCl stress plants improved root fresh and dry weights by 73 % and 41 % respectively (albeit not to the level of the untreated control) when compared to NaCl-treated plants. Similarly, GA treatment alone improved the root fresh and dry weights by 16 % and 20 % respectively when compared to the untreated control (Table 3.1).

Table 3.1. The effect of GA on wheat growth parameters under salt stress.

Treatment	SL (cm)	RL (cm)	SFW ($g.plant^{-1}$)	RFW ($g.plant^{-1}$)	SDW ($g.plant^{-1}$)	RDW ($g.plant^{-1}$)
Untreated	34.5 ± 0.333^b	10.30 ± 0.667^b	0.60 ± 0.027^b	0.08 ± 0.003^b	0.072 ± 0.001^b	0.0066 ± 0.0002^b
NaCl	30.0 ± 1.000^d	11.25 ± 0.250^a	0.48 ± 0.029^c	0.04 ± 0.007^d	0.063 ± 0.003^c	0.0041 ± 0.0002^d
GA	36.0 ± 0.333^a	10.40 ± 0.245^b	0.69 ± 0.004^a	0.09 ± 0.004^a	0.092 ± 0.002^a	0.0079 ± 0.0003^a
NaCl + GA	33.5 ± 0.500^c	10.40 ± 0.258^b	0.61 ± 0.006^b	0.07 ± 0.004^c	0.074 ± 0.001^b	0.0059 ± 0.0004^c

Values shown are representative of the mean (± SE) of three independent experiments (n=3). Within columns, means followed by different letters are significantly different from each other ($p < 0.05$). SL (shoot length); RL (root length); SFW (shoot fresh weight); RFW (root fresh weight); SDW (shoot dry weight); RDW (root dry weight).

3.2.1 The effects of exogenous GA on water retention in wheat plants under salt stress

Relative water content (RWC) significantly declined in the leaves of wheat plants treated with NaCl (Figure 3.1). This decrement was 24 % compared to the untreated plants. A slight but significant decrease of 4 % was observed in the GA treated plants when compared to the untreated plants. However, the combined treatment (NaCl + GA) significantly improved RWC (albeit not to the level of the untreated plants) by 8 % when compared to the NaCl-treated plants.

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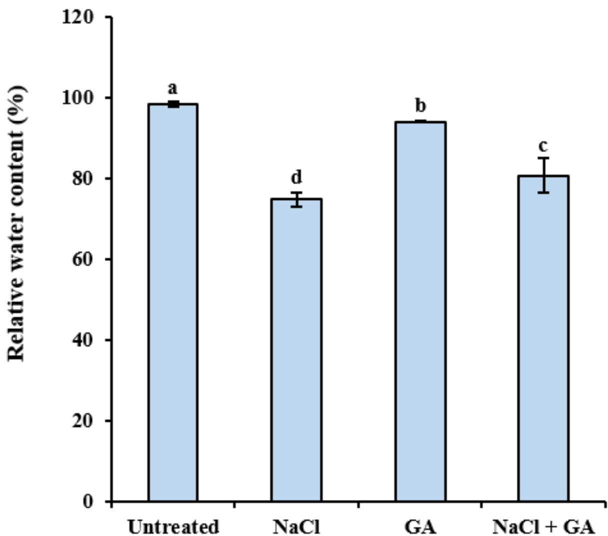

Figure 3.1. The effect of exogenous gallic acid and salinity on water retention in wheat leaves. Data presented are means (± SE) of three independent experiments (n=3). Different letters above the error bars indicate statistically differences in the means at $P < 0.05$

3.2.3 The effects of exogenous GA on photosynthetic pigments

Various photosynthetic pigments were investigated in wheat plants treated for 28 days. Significant changes were observed in chlorophyll (chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total chlorophyll (Chl *a* + *b*)) and carotenoid contents in response to the different treatments (Figure 3.2). Exogenous application of GA and NaCl differentially alters the photosynthetic metabolism and carotenoid biosynthesis in wheat leaves.

Exogenous GA enhanced total chlorophyll content by 58 % relative to the control (Figure 3.2 C). This was evident with the marked increases observed for both Chl *a* (61 %) *and* Chl *b* (41 %) (Figure 3.2 A-B). In response to NaCl-induced salt stress, total chlorophyll content was reduced by 11 % when compared to the control. This reduction in total chlorophyll was attribute to the reduction in Chl *a*, Chl *b* respectively. Interestingly, supplementation of GA to NaCl-induced salt stressed plants enhanced total chlorophyll contents in wheat leaves by 26 % relative to the control.

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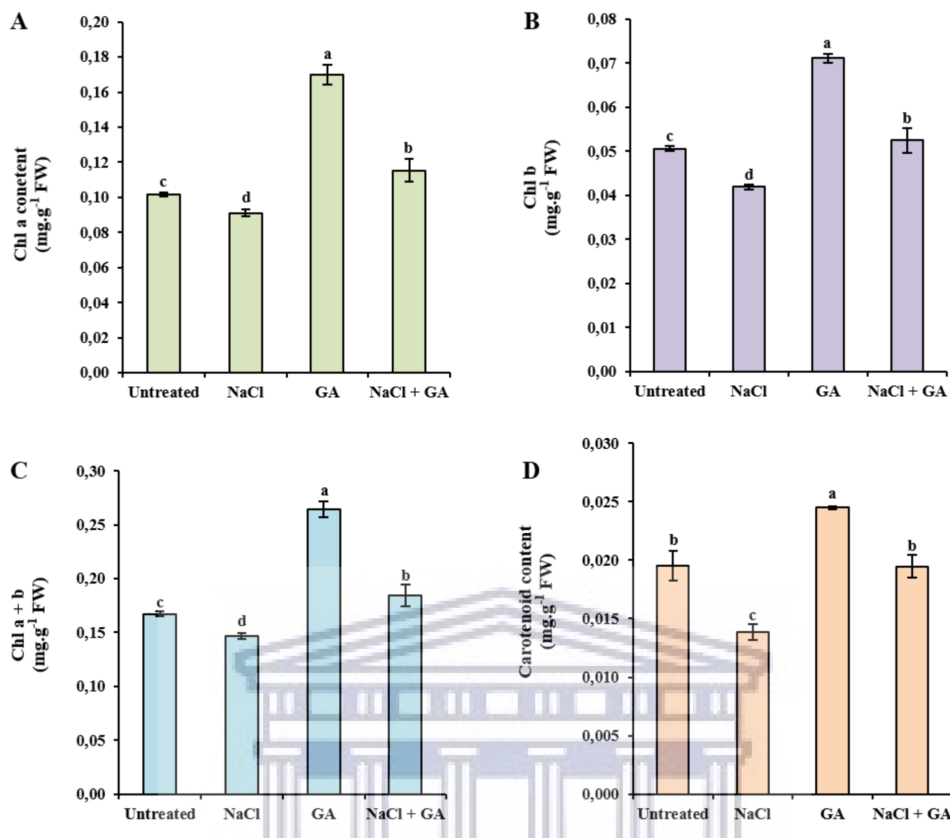

Figure 3.2. Changes in photosynthetic pigments of wheat leaves in response to various treatments. (A) Chlorophyll a; (B) Chlorophyll b; (C) Total Chlorophyll and (D) Carotenoid content. The values are representative of the mean (± SE) of three independent experiments (n=3). The different letters above the error bars indicate statistically significant differences in the means at $P < 0.05$

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The carotenoid content in the leaves of wheat plants was increased by 26 % in response to exogenous GA when compared to the control. The opposite was observed when wheat plants was treated with NaCl. A significant reduction (29 %) in carotenoid content was observed in the leaves of wheat plants treated with NaCl relative to the control. However, when GA was supplemented to NaCl treated plants, the increase (40 %) in carotenoid content was significantly higher than was observed for the NaCl treatment and to the same level as that of the control (Figure 3.2 D).

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3.3 Discussion

3.3.1 GA improves the growth and biomass of wheat plants under salt stress

The general pattern of a plants response to salinity stress is reduced growth and yield (Mariani and Ferrante, 2017; Hand *et al.*, 2017). Similarly, from the results in this study (Table 3.1), salinity stress drastically inhibited wheat growth and biomass as manifested by the reduction in shoot length as well as shoot and root fresh and dry weights. However, exogenous application of GA improved wheat growth and biomass under saline and non-saline conditions. This was evident as wheat plants under salt stress supplemented with GA or treated with GA alone displayed an increase in shoot length and shoot and root fresh and dry weights. These results are in agreement with recent studies by Singh *et al*. (2017) and Menzi *et al.* (2018) who reported that exposure of plants to exogenously applied GA alleviated salinity-induced growth inhibition of rice and soybean respectively. Similarly, the study conducted by Ozfidan-Konakci and Kabakci (2020) showed that exogenous GA application affects the plants physiology by increasing the growth rate and biomass of wheat plants under cadmium toxicity.

GA-induced enhancement in growth of plants under salt stress may be due to several changes that occur in the physiological and biochemical processes upon GA exposure (Akram and Ashraf, 2009). For example, Ozfidan-Konakci *et al*. (2014) reported that salt-stressed plants had an efficient antioxidant metabolism after supplementation of GA. This was supported by a decrease in H_2O_2 content, lipid peroxidation and increased antioxidant activities, thus increasing relative growth rate. Several other studies have also shown that negative effects associated with oxidative damage can be alleviated by the exogenous application of other phenolic acids although to a lesser extent (Habibi, 2012; Noreen and Ashraf, 2008).

Furthermore, it is known that plants increase their specific root length in order to facilitate the access of available water under nutrient limitation (Schippers and Olff, 2000) or water deprivation (Suralta *et al.*, 2010). According to Kano *et al.* (2011), promotion of root development and elongation is considered as a kind of phenotypic plasticity, defined by the plants ability to alter its phenotype and adapt under stressed environments. An increase in root length under stress conditions is likely the result of cell division and expansion in the root apex or reduced osmotic potential (West and Inze, 2004). Similar to the study by Kano *et al.* (2011), our findings showed that salinity stress increased the root length of wheat plants. The fact that supplementation of GA

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was able to reduce the root length of wheat plants under salt stress or maintain its root length upon GA application alone suggests that GA may play a role in the root elongation metabolic pathway allowing adequate water or nutrient availability around plant roots. This is correlated with the higher RWC (Figure 3.1) and greater biomass (Table 3.1) observed in the GA treatments. Hence, GA protects the root system under salt stress.

Therefore, from the results of this study, together with previous reports (Ozfidan Konakci, 2019; Ozfidan-Konakci *et al*., 2014), it is clear that exogenously applied GA has positive effects on growth and biomass of plants under salt stress. The fact that exogenous application of GA improved salt-induced inhibition of wheat growth and biomass implies that GA protects the plant tissues from oxidative damage, thus it may confer salt stress tolerance in wheat, promoting crop yield. Since limited studies have been conducted on the role of GA in plants under abiotic conditions, this is the first study elucidating the positive effects of GA on growth and yield of wheat plants under salt stress.

3.3.2 Exogenous GA improves water retention of wheat plants under salt stress

The ability of a plant to control hydration of their cells has important implications in physiological and metabolic processes (Acosta-Motos *et al*., 2017). Plant growth and development is mainly affected by the water status of the plant (Qin *et al.*, 2010). Salt stress causes a kind of physiological drought (Das *et al*., 1990), which leads to osmotic stress and cellular dehydration. According to Munns and Tester (2008), plant growth parameters is significantly reduced when the salt concentration around the roots increases to its threshold value of 40 mM NaCl. This is the case for most plants and is largely (but not entirely) caused by the osmotic effect of the salt in the soil, limiting the plant's ability to take up soil water (Sheldon *et al*., 2004). Similarly, in this study, by analyzing the water relations in the leaves of wheat plants, we observed a common response to salinity as those reported for other species (Razzaghi *et al*., 2011; Stępień and Kłobus, 2006). A significant reduction in RWC was observed as a result of the high salinity levels used in this study (150 mM NaCl) (Figure 3.1). However, this hindrance was counteracted by the exogenous application of GA (at a final concentration of 250 µM). Wheat plants treated with GA under saline or non-saline conditions maintained a higher water content than those under salt stress (Figure 3.1). These results are supported by a study conducted by Singh *et al*. (2015) that showed that the

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treatment of a similar phenolic acid to that of gallic acid, salicylic acid, was able to counteract the toxic effects generated by salt stress which resulted in a higher RWC of two week old maize plants.

Furthermore, in a study comparing plant yield and leaf water relations, Gupta *et al*. (2001) showed that leaf water relations as well as transpiration rate had a positive correlation with wheat yield under drought conditions. The drought stressed plants showed significantly lower turgor potential than control plants as well as reduced yields. It was also evident in this study that a direct relationship exists between RWC (Figure 3.1) and plant biomass and yield (Table 3.1), suggesting enhanced salt tolerance could partially be attributed to higher RWC. This suggests that GA reverse the effects of salt stress by improving water retention and promoting plant survival.

3.3.3 Photosynthetic metabolism is differentially altered by exogenous GA and salt stress

Plant growth is a measure of net photosynthesis. Any environmental stresses that affects plant biomass production also affects photosynthesis (Parida and Das, 2005). Chlorophylls and carotenoids are essential pigments involved in photosynthetic processes. These photosynthetic pigments are important for leaf function and the first target of defense when plants are exposed to stressful environments (Qiu *et al.*, 2014). Variations in chlorophyll and carotenoid content provides information on the physiological state of leaves (Sims and Gamon, 2002). It is well documented that chlorophyll and total carotenoid contents of leaves generally decrease under salt stress, leading to chlorosis of older leaves (which eventually fall off) and overall retarded growth (Abbas *et al.*, 2018; Tarchoune *et al.*, 2012; Parida and Das, 2005). This was evident from the results in this study which showed that the chlorophyll contents (Chl *a* and Chl *b*) (Figure 3.2 A-B) was significantly reduced under salt stress, leading to an overall decrease in the total chlorophyll (Chl *a* + *b*) (Figure 3.2 C) and carotenoid content (Figure 3.2 D). However, these negative effects observed under salt stress were mitigated upon GA treatment. In the presence of GA, the chlorophyll contents were significantly increased beyond that of the control under saline conditions, whereas carotenoid content increased to the same level as observed in the control plants. Furthermore, both chlorophyll and carotenoid contents were enhanced due to GA treatment under non-saline conditions beyond that of the control plants. This suggests that plants treated with GA were more tolerant to salt stress. The results observed in this study was similar to that of Ozfidan-Konakci *et al*. (2014) who reported that the exogenous application of higher GA concentrations (0.75 mM and 1.5 mM) than that used in this study (0.25 mM) alleviated

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chlorophyll and carotenoid reductions of two rice cultivars under salt (120 mM NaCl) and osmotic (20 % PEG6000) stress.

An increase in photosynthesis under normal or stressed conditions in response to hydroxybenzoic acid polyphenols have been linked to increased rubisco activity (Singh *et al*., 2015, Lee *et al*., 2014), stabilization of photosystem II (PSII) and electron transport (Bukhat *et al*., 2020). Xuan (2018) suggested that an increase in chlorophyll content may be due to the inhibitory effects of the phenolic acids (protocatechuic and vanillic acid) on the chlorophyllase enzyme or the increase of endogenous levels of flavonoids and other phenolic acids. Therefore, GA may improve the photosynthetic metabolism by increasing rubisco activity, decreasing chlorophyllase activity or stabilizing the PSII system, thus increasing the tolerance of wheat plants to salt stress.

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CHAPTER FOUR

EXOGENOUSLY APPLIED GALLIC ACID ENHANCES SALT STRESS TOLERANCE IN WHEAT BY MEDIATING ROS SCAVENGING

4.1 Introduction

Soil salinity is one of the major abiotic stresses that cause severe crop losses every year. Worldwide, about 30 % of irrigated land have already been damaged by salt, which accounts for the loss of 20 % - 69 % agricultural yield of a wide variety of crops (Qadir *et al.*, 2019). According to Jamil *et al.* (2011), salt-affected areas are increasing annually by 10 % and more than 50 % of global arable land will be salinized by 2050, rendering useless for crop production.

Elevated levels of salt in the soil restricts the process of water absorption by osmosis causing osmotic stress (cell dehydration). Additionally, the intracellular concentration of ions are disrupted, causing metabolic imbalances resulting in ion toxicity and excessive generation of reactive oxygen species (ROS) (Mandhania *et al.*, 2006). Excess ROS which consists of hydroxyl radicals ($OH^{\cdot}$), singlet oxygen ($^{1}O_2$), superoxide (O_2^{-}) and hydrogen peroxide (H_2O_2) are extremely harmful to plants and interfere with essential biological processes (Chernane *et al.*, 2015). If not carefully regulated, these reactive species cause oxidative stress and damage to cell structure and function (Hefny and Abdel-Kader, 2009; Gómez *et al.*, 1999).

To mitigate ROS-induced damage, plants possess enzymatic antioxidative defense systems including superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), glutathione peroxidase (GPX) and enzymes of the ascorbate-glutathione cycle such as ascorbate peroxidase (APX) (Hefny and Abdel-Kader, 2009). Besides these enzymes, plants also produce several antioxidant compounds such as ascorbic acid (AA), α-tocopherol, proline and various phenolic compounds that assist in maintaining optimal ROS levels required for signaling (Bose *et al.*, 2014).

In recent years, research has focused on phenolic compounds due to their antioxidant capacity (Metsämuuronen and Sirén, 2019; Krishnaiah *et al.*, 2010). These compounds, synthesized as the

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first line of defense against abiotic and biotic stress conditions play an important role in plant growth (Chandrasekara, 2019). A few lines of research have shown how different phenolic compounds ameliorate the adverse effects associated with oxidative damage in plants (Varela *et al*., 2016; Noreen and Ashraf, 2008).

One of the most widely distributed phenolic compounds present in plants are phenolic acids. Phenolic acids form part of an important group of allelochemicals whose contents are strongly associated with the scavenging of free radicals in plants (Bhardwaj *et al*., 2017). The efficacy of phenolic acids to function as free radical scavengers is influenced by the amount of hydroxyl groups present on the compound allowing them to modulate enzyme activity, alter signal transduction pathways and act as reducing agents, hydrogen atom-donors or metal ion chelators (Cheng *et al*., 2007; Amarowicz *et al*., 2004). Phenolic acids which contain a greater number of hydroxyl groups, as in the case of gallic acid (GA), exhibits higher antioxidant capacity (Adefegha *et al*., 2015; Roh, 2003).

GA and its derivatives, have gained much interest due to their strong antioxidant (Serrano *et al*., 2010), anti-carcinogenic (Gao *et al*., 2019), antimicrobial (Silva *et al*., 2010), anti-inflammatory, anti-mutagenic and anti-tumour activities (Subramanian *et al*., 2015). Recent studies have investigated the antioxidative effects of GA in maize (Yetişsin and Kurt, 2019), rice (Ozfidan-Konakci *et al*., 2014) and soybean (Menzi *et al*., 2018) under abiotic stressed conditions. These reports showed that GA relieved oxidative damage under copper, osmotic and salt stress respectively. The inhibitory effects of GA on lipid peroxidation has also been shown (Cho *et al*., 2003). However, the impact of GA on wheat plants under salt stress has not been . Therefore, the present study aims to evaluate the role of exogenously applied GA on the extent of lipid peroxidation, ROS content, cell death and antioxidant enzyme activity to determine whether GA can contribute to the protection of wheat plants and enhance its tolerance under salt stressed conditions.

4.2 Results

4.2.1 Effects of GA on the O_2^- content in wheat plants under salt stress

As salt stress is known to cause excessive ROS accumulation, the effect of the exogenous GA on O_2^- content in the leaf and root tissue of wheat plants under salt stress was investigated. Relative to the untreated controls, exogenous application of GA significantly reduced O_2^- content in leaves

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by 47 % (Figure 4.1 A). Plants treated with 150 mM NaCl (to impose salinity stress) significantly enhanced the leaf O_2- content by 189 %. However, when exogenous GA was supplemented to NaCl treated plants, the O_2^- content in the leaves was significantly reduced when compared to the NaCl-treated plants. This reduction was 21 % lower compared to the plants treated with 150 mM NaCl albeit still higher (128 %) than observed for the untreated control plants (Figure 4.1 A).

Similar results were observed for the root O_2^- content in response to salt stress. Relative to the untreated controls, NaCl treatment significantly increased the root O_2^- content by 200 % (Figure 4.1 B). However, GA had no effect on root O_2^- content when compared to the untreated control as no change was observed. On the other hand, application of GA to NaCl stress plants significantly reduced root O_2^- content when compared to the NaCl-treated plants. This reduction was 42 % lower in comparison to the salt treatment albeit still higher (76 %) than that observed for the untreated controls.

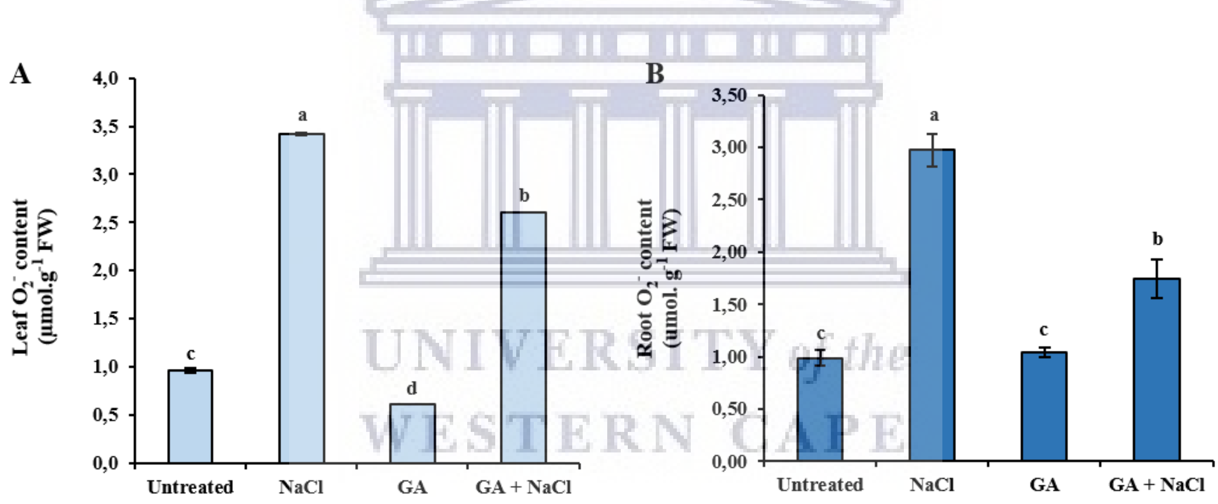

Figure 4.1. The effect of exogenous gallic acid on O_2^- content in (A) leaves and (B) roots of wheat plants under salt stress. Data presented are means (± SE) of three independent experiments (n=3). Statistically significant differences ($P < 0.05$) are indicated by different letters above error bars.

4.2.2 Effects of GA on the extent of lipid peroxidation in wheat plants under salt stress

For H_2O_2 content, a similar profile to O_2^- content was observed in the shoots and roots (Figure 4.2 A-B). Relative to the untreated plants, GA treatment reduced H_2O_2 content of shoots by 24 %, whereas NaCl stress significantly increased the H_2O_2 content in the shoots by 24 % (Figure 4.2 A). For NaCl-treated plants supplemented with GA, the H_2O_2 content of shoots was reduced by 5 % in comparison to the salt treatment although not to the levels observed for the untreated plants.

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A similar trend was observed for the H_2O_2 content in roots. GA treatment reduced root H_2O_2 content by 3 %, whereas NaCl stress significantly increased the H_2O_2 content in the roots by 27 % (Figure 4.2 B). The H_2O_2 content for NaCl-treated plants supplemented with GA was reduced by 13 % in comparison to the salt treatment although not to the levels observed for the untreated plants.

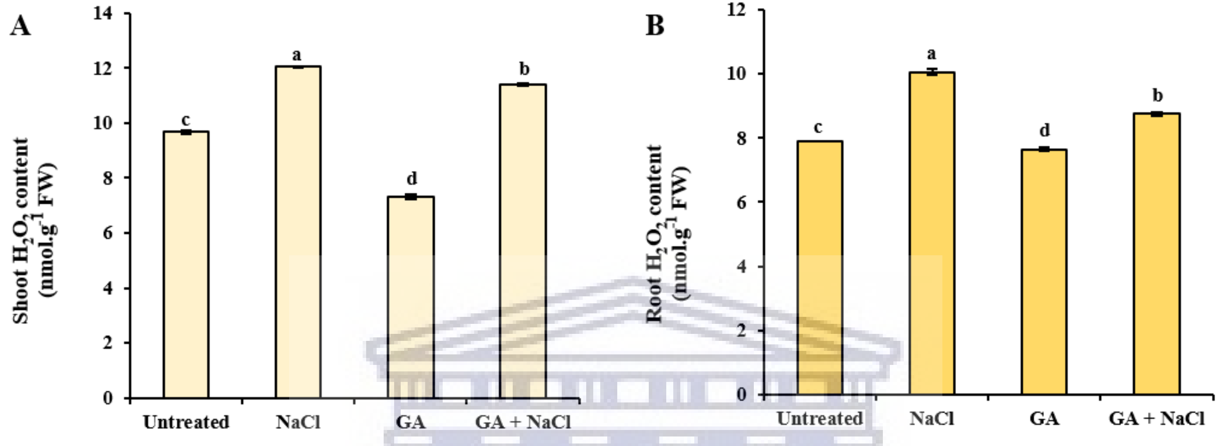

Figure 4.2. The effect of exogenous gallic acid on H_2O_2 content in (A) shoots and (B) roots of wheat plants under salt stress. Data presented are means (± SE) of three independent experiments (n=3). Statistically significant differences ($P < 0.05$) are indicated by different letters above error bars.

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4.2.3 Effects of GA on the extent of lipid peroxidation in wheat plants under salt stress

The level of lipid peroxidation (assessed as MDA content) was measured in the shoots and roots as an indication of membrane damage (Figure 4.3 A-B). The shoot MDA content of GA-treated plants was reduced by 13 % whereas NaCl treatment caused an induction in the MDA content of 23 % in comparison to the untreated plants (Figure 4.3 A). However, the combined treatment (NaCl + GA) reduced the shoot MDA content by 11 % in comparison to the plants receiving NaCl treatment alone, although not to the level observed for the untreated plants.

A similar trend was observed for the roots. GA reduced root MDA content by 13 % whereas NaCl treatment enhanced MDA content by 43 % in comparison to the untreated plants (Figure 4.3 B). However, the combined treatment (NaCl + GA) reduced the root MDA content by 22 % in comparison to the plants receiving NaCl treatment alone, although not to the level observed for the untreated plants (Figure 4.3 B).

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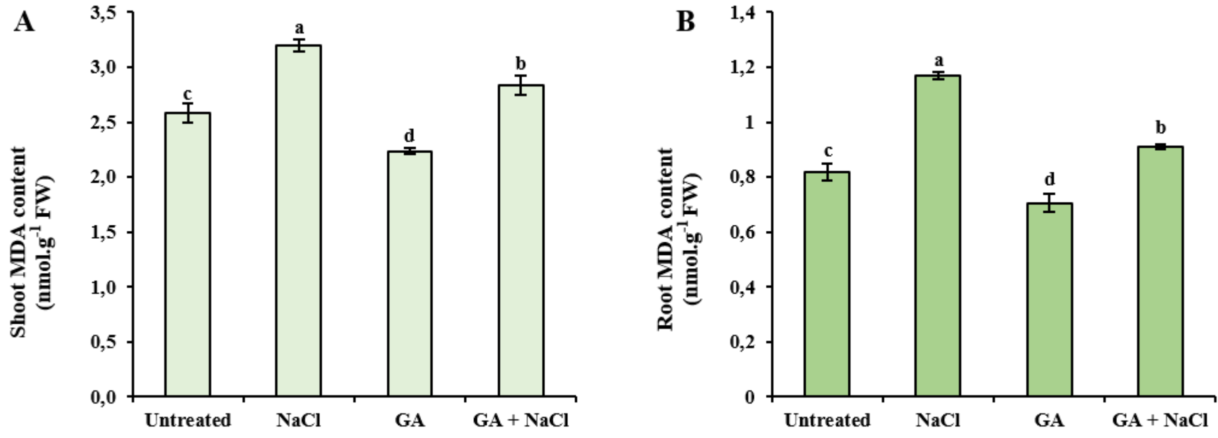

Figure 4.3.The effect of exogenous gallic acid on the extent of lipid peroxidation in (A) shoots and (B) roots of wheat plants under salt stress. Data presented are means (± SE) of three independent experiments (n=3). Statistically significant differences (P < 0.05) are indicated by different letters above error bars.

4.2.4 Exogenous GA restricts salt-induced cell death

The level of cell death (estimated by Evans Blue uptake) was measured in the leaves and roots of wheat plants under various treatments (Figure 4.4 A-B). Relative to the untreated plants, cell death in the leaves was significantly reduced by 15 % in response to GA treatment whereas a significant increase of 196 % was observed in response to NaCl treatment (Figure 4.4 A). However, application of GA to NaCl-treated plants significantly reduced leaf cell death by 49 % in comparison to NaCl-treated plants, although not to the level of the untreated plants.

Cell death in the roots of wheat plants exhibited the same response to the various treatments (Figure 4.4 B). A significant increase of 73 % in root cell death was observed in the plants exposed to salt stress, whereas GA application significantly reduced root cell death by 44 % in comparison to the untreated plants. However, combined treatment reduced the root cell death by 39 % compared to the NaCl-treated plants, although not the level observed for the untreated plants.

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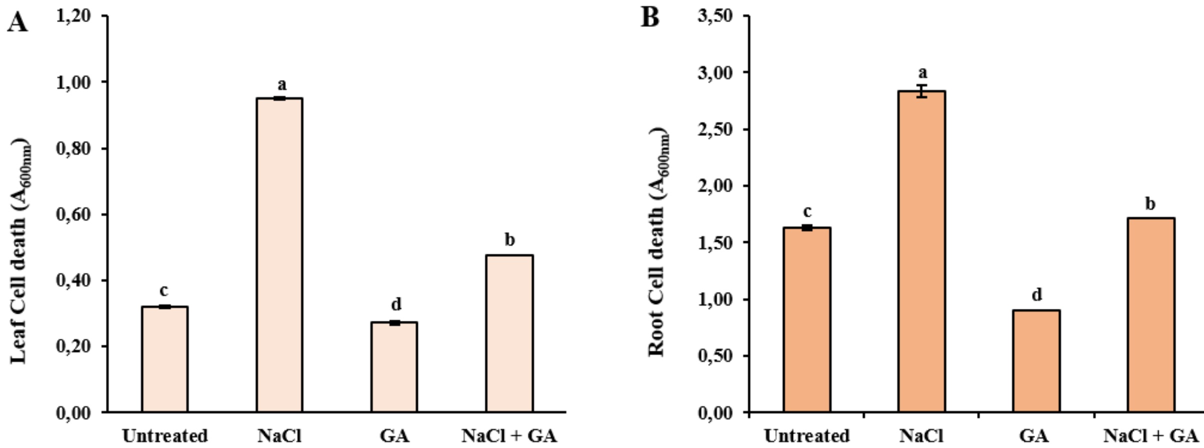

Figure 4.4. The effect of gallic acid and salt stress on the extent of cell death in (A) leaves and (B) roots of wheat plants. Data presented are means (± SE) of three independent experiments (n=3). Statistically significant differences ($P < 0.05$) are indicated by different letters above error bars.

4.2.5 Effects of exogenous GA on antioxidant enzyme activity of wheat plants under salt stress

The effects of exogenous application of GA on the antioxidant enzyme activities of SOD and APX in the shoots of wheat plants are shown in Figure 4.5. GA and NaCl differentially regulated total SOD activity. Relative to the untreated plants, GA treatment significantly reduced total SOD activity by 25 %, whereas NaCl treatment caused a significant increase of 41 % (Figure 4.5 A). However, NaCl-treated plants supplemented with GA displayed a significant reduction in SOD activity (19 %) when compared to the NaCl-treated plants, although not to the level of the untreated plants (Figure 4.5 A).

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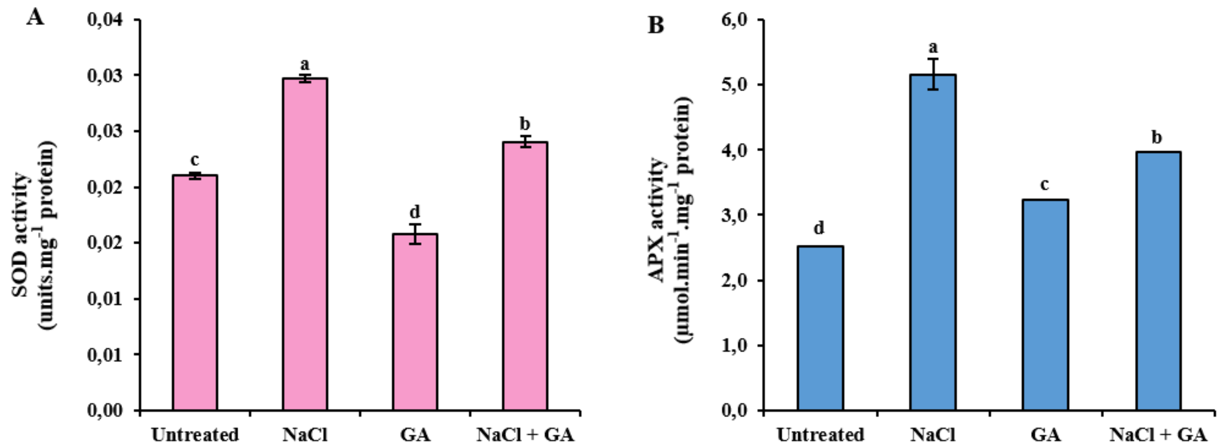

Figure 4.5. Antioxidant activity in the shoots of wheat plants. (A) SOD activity and (B) APX activity. Data presented are means (± SE) of three independent experiments (n=3). Statistically significant differences ($P < 0.05$) are indicated by different letters above error bars.

Interestingly, total APX activity generally increased in response to the various treatments in comparison to the untreated plants (Figure 4.5 B). Relative to the untreated plants, NaCl treatment and GA treatment augmented APX activity by 105 % and 58 % respectively. However, in salt stressed plants supplemented with GA, APX activity was reduced by 23 % in comparison to the plants treated with NaCl alone, although still higher to what was observed in response to GA treatment (Figure 4.5 B).

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4.2.6 Effects of exogenous GA and salt stress on antioxidant isoforms of wheat plants

4.2.6.1 Effects of salt stress and GA on SOD isoforms

To determine whether the changes in the SOD activity as observed in section 4.2.5 (Figure 4.5 A) was due to changes in the constitutive SOD isoform activity or to an induction of new isoforms, enzyme extracts were subjected to native PAGE. Five SOD isoforms were observed in the shoots of wheat plants in response to all treatments (Figure 4.6 A). The SOD isoforms were differentiated from one another by using 5 mM KCN and 6 mM H_2O_2 as inhibitors. The isoforms were identified as two Mn-SOD's (uninhibited by KCN and H_2O_2), two Cu/Zn-SOD's (inhibited by KCN and H_2O_2) and one Fe-SOD (inhibited by H_2O_2 only).

Based on densitometry analysis (Table 4.1.), the activity of Mn-SOD1 was inhibited by 17 % in response to GA treatment. It was observed that even though NaCl treatment had no effect on Mn-SOD1 activity, the combined treatment inhibited the activity by 13 % when compared to untreated

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or NaCl-treated plants. Relative to the untreated plants, the activity of Mn-SOD2 was significantly reduced in response to all treatments. This reduction in response to NaCl, GA and NaCl + GA treatment was 31 %, 17 % and 27 % respectively. Interestingly, a significant enhancement was observed in the intensities of Cu/Zn-SOD1, Cu/Zn-SOD2 and Fe-SOD isoforms under the various treatments when compared to the untreated plants. Cu/Zn-SOD1 isoform activity increased by 33 %, 34 % and 20 % respectively in response to NaCl, GA and NaCl + GA treatment in comparison to the untreated plants. However, a reduction of ± 10 % in Cu/Zn-SOD1 was observed in NaCl-treated plants supplemented with GA when compared to NaCl-treated plants. The same trend as Cu/Zn-SOD1 was observed for Cu/Zn-SOD2. Cu/Zn-SOD2 increased by 9 %, 22 % and 6 % respectively in response to NaCl, GA and NaCl + GA treatment in comparison to the untreated plants. However, a reduction of 3 % in Cu/Zn-SOD2 was observed in the combined treatment when compared to NaCl-treated plants. Furthermore, for Fe-SOD isoforms, a slight but significant increase (± 1 %) was observed in GA and NaCl + GA treatment groups, whereas under NaCl treatment the Fe-SOD activity was augmented by 4 % relative to the untreated plants. However, a 3 % reduction in Fe-SOD was observed in the combined treatment when compared to NaCl-treated plants. From these results it may be concluded that GA treatment significantly reduced the activity of Mn-SOD1, Cu/Zn-SOD1 and Fe-SOD of wheat plants under salt stress.

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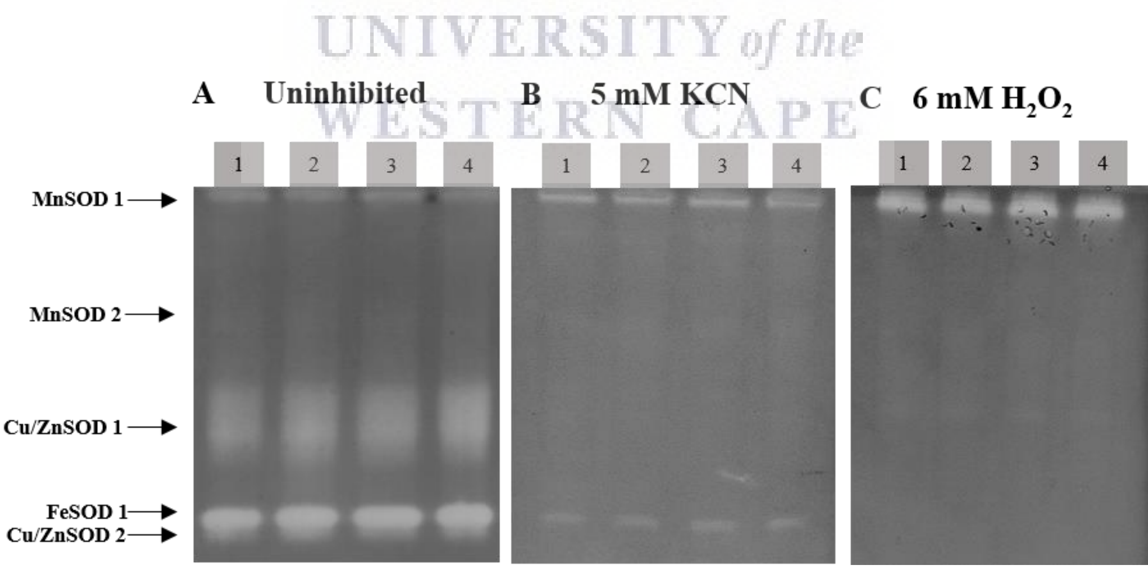

Figure 4.6. Changes in SOD isoform activity in the shoots of wheat plants. Detection of SOD isoforms in response to the various treatments with (A) no inhibitors, (B) 5 mM KCN and (C) 6 mM H_2O_2.

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Table 4.1. Tabular representation of relative SOD isoforms in wheat shoots.

SOD isoforms	Treatments			
	Untreated	NaCl	GA	NaCl + GA
Mn-SOD1	1.000 ± 0.000[a]	1.008 ± 0.000[a]	0.831 ± 0.004[c]	0.880 ± 0.001[b]
Mn-SOD2	1.000 ± 0.000[a]	0.693 ± 0.000[c]	0.831 ± 0.000[b]	0.727 ± 0.000[c]
Cu/Zn-SOD1	1.000 ± 0.000[c]	1.334 ± 0.013[a]	1.345 ± 0.002[a]	1.196 ± 0.002[b]
Cu/Zn-SOD2	1.000 ± 0.000[c]	1.041 ± 0.000[b]	1.011 ± 0.002[a]	1.014 ± 0.001[b]
Fe-SOD	1.000 ± 0.000[d]	1.090 ± 0.001[a]	1.222 ± 0.000[c]	1.059 ± 0.002[b]

The relative pixel intensity values were measured using the Alpha Ease FC software and the SOD activities are expressed as relative arbitrary units. All SOD isoforms were normalized to the untreated plants. Values shown are representative of the mean (± SE) of three independent experiments (n=3). Within columns, means followed by different letters are significantly different from each other ($p < 0.05$)

4.2.6.2 Effects of salt stress and GA on APX isoforms

To analyze the changes in APX isoforms in the shoots of wheat plants under various treatments, enzyme extracts were subjected to native PAGE. Three APX isoforms were observed across all treatments (Figure 4.7). A general increase in the enzymatic activity of APX1 was observed in response to the various treatments. The exogenous application of GA and NaCl significantly increased APX1 activity by 8 % and 19 % respectively when compared to the untreated plants (Table 4.2.). Interestingly, APX1 enzymatic activity was enhanced even further by 14 % in the combined treatment in comparison to the NaCl-treated plants, to the levels beyond that of the untreated plants.

Relative to the untreated plants, a reduction of 8 % in the APX2 enzymatic activity was observed in response to NaCl treatment. On the contrary, exogenous application of GA increased the enzymatic activity by 9 % compared to the untreated plants. The enzymatic activity was enhanced even further in the combined treatment with a 26 % increase in comparison to the NaCl-treated plants, to levels beyond that of the untreated plants. However, no significant changes were observed in the enzymatic activity of APX3 for the various treatments.

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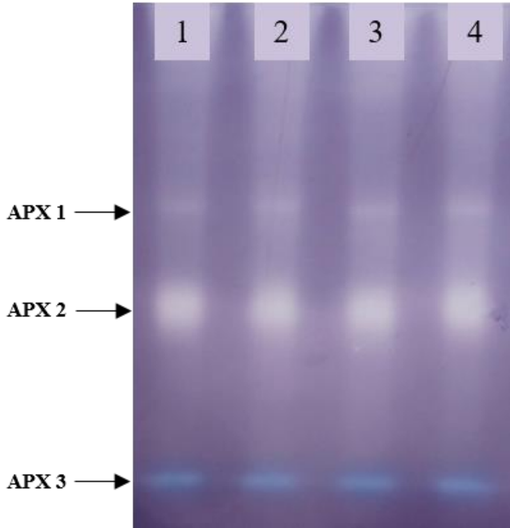

Figure 4.7. The effects of NaCl and GA on ascorbate peroxidase (APX) isoform activity in the shoots of wheat plants. Changes in APX activity was detected in response to (1) untreated, (2) 150 mM NaCl, (3) 250 µM GA and (4) 150 mM NaCl + 250 µM GA treatment groups.

Table 4.2. Tabular representation of relative APX isoforms in wheat shoots.

APX isoforms	Treatments			
	Untreated	NaCl	GA	NaCl + GA
APX1	1.000 ± 0.000^{c}	1.078 ± 0.022^{b}	1.193 ± 0.018^{a}	1.226 ± 0.044^{a}
APX2	1.000 ± 0.000^{c}	0.922 ± 0.025^{d}	1.094 ± 0.001^{b}	1.160 ± 0.033^{a}
APX3	1.000 ± 0.000^{a}	1.016 ± 0.020^{a}	0.985 ± 0.020^{a}	1.030 ± 0.103^{a}

The relative pixel intensity values were measured using the Alpha Ease FC software and the APX activities are expressed as relative arbitrary units. All APX isoforms were normalized to the untreated plants. Values shown are representative of the mean (± SE) of three independent experiments (n=3). Within columns, means followed by different letters are significantly different from each other ($p < 0.05$)

4.2.6.3 Effects of salt stress and GA on POD isoforms

Five POD isoforms were identified in all treatments (Figure 4.8). Although GA treatment did not alter the enzymatic activity of POD1, an increase in activities of POD4 (6 %) and POD5 (25 %) isoforms were observed when compared to the untreated control (Table 4.3.). Furthermore, GA treatment decreased the enzymatic activities of POD2 and POD3 by 15 % and 4 % respectively relative to the untreated control.

As with GA treatment, NaCl treatment did not have any effect on the enzymatic activity of POD1. However, relative to the untreated plants, an increase in the enzyme activity of POD3 (14 %), POD

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4 (3 %) and POD5 (13 %) as well as a decrease in POD2 (5 %) was observed in response to NaCl treatment (Table 4.3.). Interestingly, when plants under salt stress were supplemented with GA, the enzymatic activities of POD2, POD3 and POD4 isoforms were differentially regulated compared to the NaCl-treated plants. The enzymatic activity of POD2 increased by 7 %, whereas a decrease in POD3 (16 %) and POD4 (5 %) isoforms was detected.

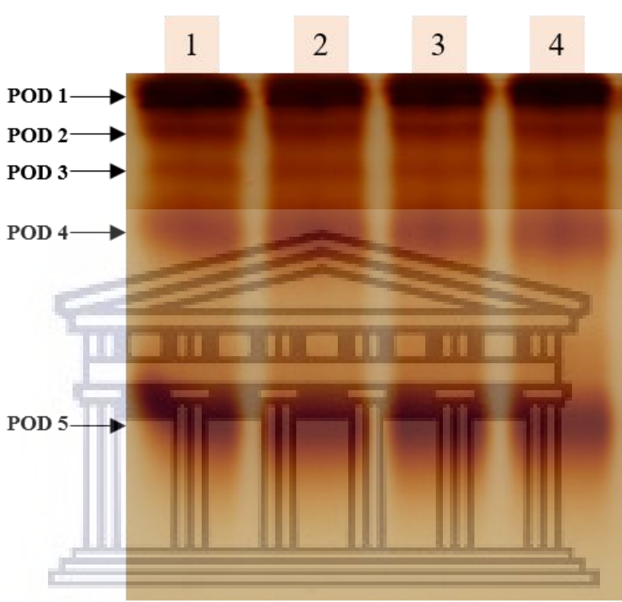

Figure 4.8. Exogenous GA and salt stress differentially alters peroxidase (POD) isoform activity in the shoots of wheat plants. DAB-POD activities in the shoots of wheat plants was detected in response to (1) untreated, (2) 150 mM NaCl, (3) 250 µM GA and (4) the combination treatment (150 mM NaCl + 250 µM GA)

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Table 4.3. Tabular representation of relative POD isoforms in wheat shoots.

POD isoforms	Treatments			
	Untreated	NaCl	GA	NaCl + GA
POD1	1.000 ± 0.000[a]	1.007 ± 0.067[a]	0.956 ± 0.063[a]	0.913 ± 0.009[a]
POD2	1.000 ± 0.000[a]	0.946 ± 0.005[b]	0.854 ± 0.101[b]	1.014 ± 0.057[a]
POD3	1.000 ± 0.000[b]	1.137 ± 0.018[a]	0.957 ± 0.015[c]	0.960 ± 0.022[c]
POD4	1.000 ± 0.000[c]	1.032 ± 0.012[b]	1.065 ± 0.014[a]	0.981 ± 0.002[d]
POD5	1.000 ± 0.000[c]	1.133 ± 0.010[b]	1.253 ± 0.032[a]	1.231 ± 0.030[a]

The relative pixel intensity values were measured using the Alpha Ease FC software and the POD activities are expressed as relative arbitrary units. All POD isoforms were normalized to the untreated plants. Values shown are representative of the mean (± SE) of three independent experiments (n=3). Within columns, means followed by different letters are significantly different from each other ($p < 0.05$)

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4.2.6.4 Effects of salt stress and GA on GPX-like isoforms

The activity changes of six GPX-like isoforms in the shoots of wheat plants under different treatments were observed by activity staining of native PAGE (Figure 4.9). Relative to the untreated plants, an increase in the enzyme activities of GPX-like 1 (19 %), GPX-like 2 (23 %), GPX-like 3 (12 %), GPX-like 4 (38 %), GPX-like 5 (24 %) and GPX-like 6 (25 %) was observed in response to GA treatment (Table 4.4.). A similar pattern was observed for NaCl-treated plants, whereby there was an increase in GPX-like 1 (11 %), GPX-like 2 (26 %), GPX-like 3 (22 %), GPX-like 4 (49 %) and GPX-like 5 (19 %). However, the GPX-like 6 isoform was reduced by 6 % under NaCl treatment when compared to the untreated plants. Furthermore, it was interesting to note that when NaCl-treated plants were supplemented with GA, the GPX-like enzyme activities for all isoforms were enhanced to levels beyond the NaCl-treated plants.

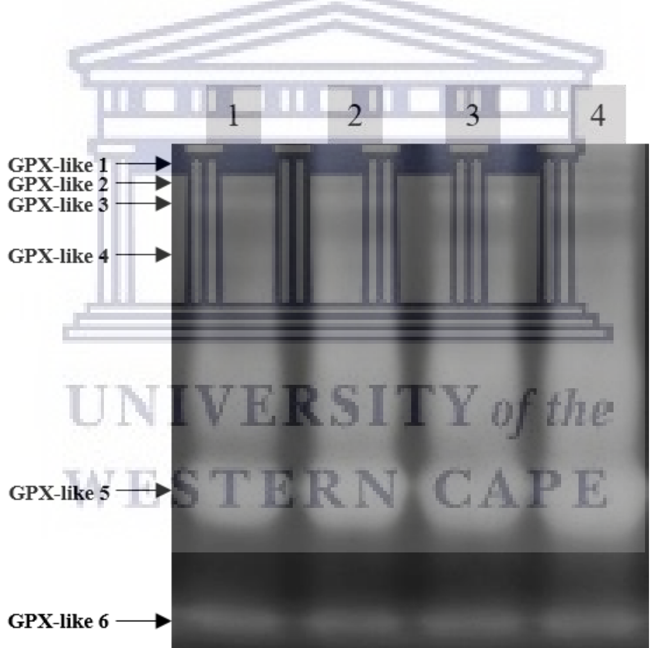

Figure 4.9. Changes in glutathione-like peroxidase (GPX) isoform activity in the shoots of wheat plants. GPX-like activities in the shoots of wheat plants was detected in response to (1) untreated, (2) 150 mM NaCl, (3) 250 µM GA and (4) the combination treatment (150 mM NaCl + 250 µM GA).

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Table 4.4. Tabular representation of relative GPX-like isoforms in wheat shoots.

GPX isoforms	Treatments			
	Untreated	NaCl	GA	NaCl + GA
GPX-like 1	1.000 ± 0.000[d]	1.108 ± 0.009[c]	1.191 ± 0.000[b]	1.298 ± 0.011[a]
GPX-like 2	1.000 ± 0.000[b]	1.258 ± 0.105[a]	1.228 ± 0.010[a]	1.400 ± 0.072[a]
GPX-like 3	1.000 ± 0.000[d]	1.221 ± 0.039[b]	1.122 ± 0.000[c]	1.308 ± 0.017[a]
GPX-like 4	1.000 ± 0.000[c]	1.487 ± 0.010[b]	1.381 ± 0.132[b]	1.876 ± 0.081[a]
GPX-like 5	1.000 ± 0.000[d]	1.186 ± 0.000[c]	1.238 ± 0.034[b]	1.393 ± 0.044[a]
GPX-like 6	1.000 ± 0.000[b]	0.935 ± 0.005[c]	1.253 ± 0.040[a]	1.163 ± 0.099[a]

The relative pixel intensity values were measured using the Alpha Ease FC software and the GPX-like activities are expressed as relative arbitrary units. All GPX-like isoforms were normalized to the untreated plants. Values shown are representative of the mean (± SE) of three independent experiments (n=3). Within columns, means followed by different letters are significantly different from each other ($p < 0.05$)

4.3 Discussion

4.3.1 GA alleviates oxidative damage induced by salt stress and restricts cell death

Abiotic stress conditions such as salt stress is known to be associated with increased oxidative stress (Zhan *et al.*, 2019; (Chakraborty and Pradhan, 2012). Salinity disrupts cellular homeostasis by inducing the production and accumulation of ROS molecules (particularly O_2^- and H_2O_2) (Prisco *et al.*, 2016). Excess ROS, being highly reactive, becomes toxic to the cells and causes damage to vital macromolecules (carbohydrates, proteins, nucleic acids and lipids) which ultimately results in cell death (Sahu *et al.*, 2012; Seckin *et al.*, 2009).

Our findings showed that salt stress caused an increase in the ROS content of O_2^- and H_2O_2 as well as elevated levels of MDA in shoots and roots of wheat plants suggesting the disturbance of normal metabolic pathways. Furthermore, an increase in cell death (Figure 4.4 A-B) was also evident in the study, which may be attributed to oxidative damage to the cell membranes observed as the enhanced lipid peroxidation caused by an accumulation of free radicals and inadequate activities of antioxidant enzymes (Seckin *et al.*, 2009). These findings are in agreement with several studies that have reported increased oxidative damage in response to excessive ROS accumulation in a variety of crops including soybean (Klein, 2012), maize (Singh *et al.*, 2015; De Azevedo Neto *et al.*, 2006), sugar beet (Bor *et al.*, 2003) and wheat (Ashraf *et al.*, 2012). Sairam *et al.* (2002) reported a significant increase in the amount of H_2O_2 which further lead to increased lipid peroxidation in two wheat genotypes under salinity stress. The occurrence of lipid peroxidation in response to treatment with NaCl (100 – 150 mM) has also been reported in the leaves and roots of wheat plants (Qiu *et al.*, 2014; Ma *et al.*, 2012; Seckin *et al.*, 2009). The MDA content was also

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found to be elevated under cold stress in bread wheat and durum wheat (Nejadsadeghi *et al.*, 2014). Several other literature reported that excessive ROS production induces oxidative damage in wheat (Bali and Sidhu, 2019).

Nevertheless, results in the present study showed a decrease in ROS levels (Figure 4.1 A-B and Figure 4.2 A-B) and lipid peroxidation (Figure 4.3 A-B) in the shoots and roots of wheat plants supplemented with GA alone or in the combined treatment (NaCl + GA – treated plants). This suggests that GA acts as a ROS-scavenger and alleviates oxidative damage, thus has a protective role in wheat plants under salt stress as observed by reduced cell death (Figure 4.4 A-B). Consistent with this idea, Yetişsin and Kurt (2019) demonstrated that exogenously applied GA protects the cells from membrane damage by preventing ROS formation. Exogenous application of GA was also reported to reduce the effects of ROS in soybean under salt stress and improve the plant biomass (Menzi *et al.*, 2018). Recently, Ozfidan Konakci (2019) showed that besides directly scavenging O_2^- and H_2O_2 , GA also increases the proline content, which has been associated with the direct scavenging of ROS molecules or indirect scavenging of ROS by enhancing the plants antioxidant defense systems (Ben Rejeb *et al*., 2014).

From these results and the supported reports on GA, we can conclude that GA protects wheat plants under salt stressed conditions by scavenging ROS formation, reducing membrane damage and restricting cellular death.

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4.3.2 Salinity tolerance in wheat plants is mediated by the antioxidant capacity of GA

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Restricting excessive ROS accumulation is critical for plant survival. Under normal and oxidative stress conditions, plants possess non-enzymatic and enzymatic antioxidant systems. Activation of the enzymatic antioxidant system is important for maintaining cellular redox homeostasis (Nikalje *et al.*, 2018). Enzymatic antioxidants include SOD, APX, CAT and others that eliminate excess ROS (Wang *et al.*, 2016). Alteration of these antioxidant enzyme activities in response to various abiotic stress conditions is well – documented and reported to be important for protection (Saed-Moucheshi *et al.*, 2017; Miller *et al.*, 2010; Gill and Tuteja, 2010; De Azevedo Neto *et al.*, 2006).

Results of the present study revealed that wheat plants had higher total SOD activity in response to salt stress as compared to the other treatments (Figure 4.5 A). This result is in agreement with the study done by Sairam *et al*. (2002), which found that SOD activity increased under salt stress in tolerant and susceptible wheat genotypes. In addition, the authors observed that the SOD activity

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was higher in the tolerant genotype than the susceptible one, suggesting that a higher SOD activity plays a role in imparting tolerance to plants under salt stress. A higher SOD activity is indicative of higher O_2^- scavenging ability under stressed conditions. However, given that O_2^- (Figure 4.1 A) levels remained significantly higher in NaCl-treated plants and the combined treatment as compared to the untreated or GA-treated plants, it is hypothesized that the increase observed in SOD activity was inefficient to counteract the excessively high levels of O_2^- that accumulated in response to salt stress.

On the other hand, when salt stressed plants were supplemented with GA, the total SOD activity was reduced under saline and non- saline conditions. This result could suggest that GA was able to act as a scavenger of O_2^- and reduce the demand for enhanced enzymatic activity since the plant experiences reduced signs of stress in the presence of GA (Figures 4.1-Figure 4.4). A study by Yang *et al.* (2014) showed that the GA derivatives (methyl gallate, ethyl gallate and pentagalloylglucose) extracted from the young leaves of roemer (*Toona sinensis*) exhibited antioxidant properties that were able to scavenge O_2^- and other free radicals. Microscopic analysis also confirmed a reduction of O_2^- and H_2O_2 by exogenous application of GA (Singh *et al.*, 2017).

Furthermore, results from this study also showed that the total APX activity increased across all treatments, with the highest APX activity observed under salt stress (Figure 4.5 B). However, this salinity - induced increase in APX activity was significantly reduced in the presence of GA. Again, this result could suggest that GA assists in the scavenging of H_2O_2, thus reducing the demand for enhanced APX activity. This is supported by the fact that GA has been described as a powerful antioxidant capable of eliminating O_2^-, H_2O_2 and hydroxyl radicals (Samuel *et al.*, 2017; Yen *et al.*, 2002).

Moreover, results of this study also showed that in addition to directly scavenging H_2O_2, GA assisted in the indirect scavenging of H_2O_2 by enhancing the APX activity under non-saline conditions beyond that of the control. The upregulation of APX under high salt concentrations has been shown to be one of the response mechanisms of salt adaptation in halophytes (Bose *et al.*, 2014). Therefore, GA may play a role in protecting wheat plants under salt stress or improving wheat plants grown under normal conditions.

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4.3.3 Exogenous GA application regulates antioxidant system

Many stress situations cause an increase in the total antioxidant activity. Here, we explore how long term exposure of GA (250 µM GA) and salt stress (150 mM NaCl) influence the enzymatic activity of isoforms of four antioxidant enzymes (SOD, APX, POD and GPX) in the shoots of wheat plants that were treated for a period of 28 days.

4.3.3.1 SOD isoforms are differentially regulated under GA and salt stress

The dismutation of O_2^- into H_2O_2 and O_2 by a group of SOD antioxidant enzymes is an important step in cellular defense against ROS toxicity to protect cells from oxidative damage (Ozgur *et al.*, 2013). SOD enzymes are categorized into three main groups depending on their metal cofactor binding at the active site, which may contain manganese (Mn-SODs), copper/zinc (Cu/Zn-SODs) or iron (Fe-SODs). Generally, Mn-SOD localizes in the mitochondria and peroxisomes, while Cu/Zn-SODs and Fe-SODs are located in cytosol, mitochondria, chloroplast and peroxisomes (Saibi and Brini, 2018). The regulation of different SOD isoforms have been observed in many plant species and considerable variation exists in response to various abiotic stresses (Ozfidan Konakci, 2019; Mohamed *et al.*, 2015; Klein, 2012; Sairam *et al.*, 2005). The level of response of SOD activity is affected by several conditions, depending on the plant organ used for measurements, the plant species (susceptible or tolerant) and furthermore, the duration and concentration of the stress applied.

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In this study, five SOD isoforms were detected and differentially regulated in response to GA and NaCl treatment. The SOD isoforms observed in Figure 4.6 consisted of two Mn (Mn-SOD1-2), one Fe (Fe-SOD) and two Cu/Zn (Cu/Zn-SOD1-2) isoforms. Apart from MnSOD1 which remain unchanged in response to salt stress, expression of all the other SOD isoforms were either up-regulated (Cu/Zn-SOD1, Cu/Zn-SOD2 and Fe-SOD) or down-regulated (MnSOD2). These findings were in line with the results of Sairam *et al.* (2005). While investigating the long-term exposure of salt stress in salt-sensitive lines (HD2687 and HD2009) and salt-tolerant lines (KRL19, Kharchia65) of wheat, the authors observed enhanced activities in chloroplastic Cu/Zn-SOD and Fe-SOD isoforms, while chloroplastic Mn-SOD activity was deemed to be rudimentary. Salinity-induced increase in Cu/Zn-SOD and Fe-SOD isoforms has also been reported in salt sensitive and salt tolerant *Oryza sativa* (Mishra *et al.*, 2013) and *Pisum sativum* (Gómez *et al.*, 1999).

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The results of this study further showed that induction in SOD activity was reversed by GA application. The SOD isoform analysis of NaCl-treated plants supplemented with GA revealed that Mn-SOD1, Cu/Zn-SOD1, Cu/Zn-SOD2 and Fe-SOD were down-regulated, while Mn-SOD2 was up-regulated in comparison to the NaCl-treated plants. The down-regulation of the four SOD isoforms may be as a result of the scavenging ability of GA that reduces the need for enhanced activity of SOD enzymes. Our data therefore suggests that Cu/Zn-SOD and Fe-SOD isoforms constituted the major part of total SOD activity (Figure 4.5 A) and that the distinct isoforms play a role in scavenging the salinity induced O_2^- radical.

4.3.3.2 GA-induced increase in ascorbate peroxidase activity contributes to H_2O_2 scavenging and plant survival under salinity stress

Accumulation of excess ROS, specifically H_2O_2, is highly toxic for cells as it readily passes through biological membranes. In the presence of divalent cations, H_2O_2 may also rapidly degrade to highly oxidizing hydroxyl radicals through oxidation or reduction reactions and cause damage to plant cells (Queval *et al.*, 2008). Detoxification of H_2O_2 into H_2O and O_2 via ascorbate peroxidase (APX) is tremendously important to maintain redox homeostasis in plants exposed to environmental stresses (Pandey *et al.*, 2017). The role of APX in regulating the ROS levels is supported by specific isoforms of the enzyme being functional within the cytosol and various sub-cellular locations (Ishikawa and Shigeoka, 2008). To date, five APX isoforms namely, chloroplastic, mitochondrial, peroxisomal/glyoxysomal and cytosolic have been identified in plants based on amino acid composition (Pandey *et al.*, 2015). In the case of this study, three APX isoforms were identified across all treatments in the shoots of wheat plants (Figure 4.7). The exogenous application of GA significantly enhanced the expression levels of two APX isoforms (APX1 and APX2) under saline and non-saline conditions, while no significant change was observed in the enzymatic activity of APX3. Furthermore, the highest APX activities were detected in the combined treatment compared to plants treated with 150 mM NaCl only. Moreover, the combined treatment caused an even greater increase in the APX activities when compared to the untreated plants. These results indicate that the exogenous application of GA induce APX activities to scavenge H_2O_2 in wheat plants under salt stress and contributes to salt tolerance and plant survival as observed by reduced cell death (Figure 4.4 A).

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4.3.3.3 GA regulate the expression of POD isoforms in wheat plants under salt stress

Peroxidases (PODs) are multifunctional enzymes that perform key roles in detoxification of H_2O_2. Increased POD activity is a common response to environmental stresses (Sreenivasulu *et al.*, 1999; Fieldes and Gerhardt, 1998). Similarly, in this study, the increased accumulation of H_2O_2 in wheat plants under salt stress induced POD activities as observed by the intensified POD isoforms (POD3- POD5) (Figure 4.8). However, these enzymatic isoforms failed to overcome the stress-induced oxidative load as indicated by the enhanced level of lipid peroxidation (Figure 4.3 A). Recently, it has been suggested that POD may act as an efficient H_2O_2 scavenging system in the presence of phenolic acids (Ozfidan-Konakci *et al*., 2014). Contrary to this, our study showed that in the presence of GA, the enzymatic activities of POD isoforms (POD1 and POD3 - POD4) decreased under saline and non - saline conditions, with the exception of POD 5 which was induced, while no significant change was observed in POD 2. Down-regulation of the POD isoforms may be due to the scavenging ability of GA (Marino *et al*., 2014). Furthermore, our data suggest that different isoforms may play a role in salt tolerance of wheat plants.

4.3.3.4 GA-induced increase in glutathione peroxidase (GPX) activity contributes to H_2O_2 scavenging and plant survival under salinity stress

According to Yoshimura *et al.* (2004), GPX is an important H_2O_2 scavenging enzyme protecting cell membranes from oxidative damage. Several reports demonstrated that the expression of GPX genes and/or their enzymatic activities were often induced by various environmental stresses, contributing to stress tolerance (Wang *et al*., 2017; Yoshimura *et al.*, 2004). Apart from GPX6, which was downregulated in response to NaCl, the enzymatic activities for all other GPX isoforms (GPX1 - GPX5) were enhanced in comparison to the untreated plants (Figure 4.9). However, the GPX activity was not sufficient for scavenging of salt-induced H_2O_2 content (Figure 4.2 A). Furthermore, the enzymatic activities of all GPX isoforms were substantially induced in response to exogenous application of GA, with even higher activities observed in the salt stressed plants supplemented with GA. From these results, we suggest that GA contributes to H_2O_2 scavenging by inducing the activity of GPX-like enzyme isoforms of wheat plants under salt stress, which may be associated with salt stress tolerance.

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CHAPTER FIVE

GALLIC ACID AND SALT STRESS ALTERS THE SHOOT MEMBRANE PROTEOME OF WHEAT PLANTS

5.1 Introduction

Phenolic acids are a group of secondary metabolites widely distributed throughout the plant kingdom (Chandrasekara, 2019). They have antioxidant functions in response to environmental stresses (Michalak, 2006) and complement the roles of the enzymatic antioxidant defense system, scavenging free radicals and reducing excessive ROS levels in plant tissues (Varela *et al.*, 2016).

Among various phenolic acids, gallic acid (GA), a triphenolic compound, has been suggested to be the most versatile (Badhani *et al*., 2015) and biologically active compound of plant origin (Karamać *et al*., 2006). Several studies have shown the potential of GA as a strong antioxidant against ROS (Badhani *et al*., 2015; Marino *et al*., 2014), protecting cells against oxidative damage. Recent studies by Menzi *et al.* (2018) and Ozfidan-Konakci *et al*. (2014) have shown that the exogenous application of GA enhanced salt tolerance of soybean and rice respectively. These authors focused on the mode of action of GA at physiological and biochemical levels, such as growth rate, photosynthesis, ROS scavenging and antioxidant enzyme production.

Similarly, we have shown that exogenous GA improved wheat tolerance to salt stress by reducing excess ROS and modulating antioxidant enzyme activities (Chapter 3 and 4 of this study). However, to date, no information is available about the molecular mechanisms underlying GA-induced salt tolerance in plants at the proteomic level, making this the first study of its kind.

Proteomics studies have become increasingly important in plant research (Vanderschuren *et al.*, 2013). Plant membrane proteomics provides valuable information on plant-specific biological processes as proteins are directly linked to cellular functions. Therefore, this part of the study focused on investigating the changes in the shoot membrane proteome of GA-treated wheat under salt stress conditions to better understand plant tolerance mechanisms.

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5.2 Results

5.2.1 Membrane protein identification and subcellular localization

This part of the study focused on isolating membrane proteins from the shoots of wheat plants using a commercialized membrane protein kit (Section 2.12.1) and identifying the membrane proteins under various treatments using label-free mass spectrometry analysis. A total of 28 proteins were successfully identified using a probability score greater than 98 % with two or more peptides being unique to a particular protein (Table 5.1). Distinct differences were observed in protein identifications between the various treatments. For example, photosystem II proteins (A0A3B6NX57 and M7YKG6) were only identified in the GA-treated plants, while a chlorophyll a-b binding protein (W5GFA4) was only identified in the NaCl-treated plants.

The identified proteins were classified into sub-groups (i.e. integral, peripheral or non-membrane proteins) using a the Universal Protein Sequence database (uniprot) (http://www.uniprot.org/) alongside the Aramemnon database (which combines results of a number of prediction programs), whereby 50 % (14 proteins) of the proteins identified were found to be membrane proteins. In addition, 43 % (12 proteins) were predicted to have transmembrane domains using HMMTOP algorithms and were further classified as integral membrane proteins (Figure 5.1 A).

Furthermore, membrane proteins were isolated across all treatments with the highest number of membrane proteins (12 proteins) identified in the GA treatment (Figure 5.1 B). The total number of proteins identified in the shoots of wheat plants under salt stress were significantly reduced (5 proteins). However, in the presence of GA, wheat plants under salt stress expressed more proteins as observed by the increase in number of proteins identified in the combined treatment (7 proteins).

The subcellular localizations of the positively identified proteins were predicted using Uniprot and Arememnon databases as well as WoLF PSORT software (https://wolfpsort.hgc.jp) and various literature sources. Most of the proteins were localized to the chloroplast thylakoid membrane (32 %) (Figure 5.1 C). Proteins identified in the study were also found to be localized in the cytoplasm (7 %) and other subcellular organelles such as ribosome (7 %), mitochondria (7 %), nucleus (14 %), plasma membrane (11 %), mitochondrial inner membrane (4 %), chloroplast (7 %), chloroplast thylakoid lumen (4 %), chloroplast envelope membrane (3 %) and chloroplast envelope (4 %).

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Table 5.1. Proteins identified using label-free LC-MS/MS.

Identified proteins	[a]Accession number	Cellular component	Protein type	Molecular weight (kDa)	Probability of protein present			
					Untreated	NaCl	GA	GA + NaCl
Photosynthesis and energy metabolism								
Photosystem I reaction center subunit II	R7W2W9	Chloroplast thylakoid membrane	PMP	11	100 %	100 %	100 %	100 %
Uncharacterized protein	W5AQE7	Chloroplast thylakoid membrane	IMP	35	100 %	-	100 %	100 %
PsbP domain-containing protein	A0A3B6AYY2	Chloroplast thylakoid membrane	IMP	27	100 %	-	100 %	100 %
Uncharacterized protein	A0A3B6JKJ5	Chloroplast thylakoid lumen	NMP	19	100 %	-	100 %	100 %
Uncharacterized protein	W4ZSM1	Chloroplast thylakoid membrane	IMP	15	100 %	-	100 %	-
Glyceraldehyde-3-phosphate dehydrogenase	A5YVV3	Cytosol	NMP	37	*33 %	*83 %	100 %	-
Putative photosystem I subunit n	A0A0M5J8Z4	Chloroplast	NMP	8	-	-	100 %	-
Photosystem II 10 kDa polypeptide	A0A3B6NX57	Chloroplast thylakoid membrane	IMP	14	-	-	100 %	-
Chlorophyll a-b binding protein	W5GFA4	Chloroplast thylakoid membrane	IMP	28	-	100 %	*82 %	-
Photosystem II reaction center protein H	M7YKG6	Chloroplast thylakoid membrane	IMP	8	-	-	100 %	-
Acid phosphatase 1	M8AJA6	Cytosol	NMP	44	-	-	100 %	-
Transport proteins								
Photosystem I iron-sulfur center	A0A218LG23	Chloroplast thylakoid membrane	PMP	9	100 %	100 %	100 %	100 %
Cytochrome b-c1 complex subunit 7	A0A3B6KP84	Inner mitochondrial membrane	IMP	15	100 %	-	100 %	100 %
Cytochrome f	S4Z3G1	Chloroplast thylakoid membrane	IMP	35	*20 %	-	100 %	100 %
Proton gradient regulation 5	H9C8A5	Chloroplast	NMP	13	100 %	-	*12 %	-

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uncharacterized protein	M7Z1Y4	Plasma membrane	IMP	65	-	-	100 %	-
DNA binding proteins								
Histone H2A.2.1	M8BSG9	Nucleus	NMP	16	-	100 %	100 %	-
Histone H2B.3	A0A3B6MKX9	Nucleus	NMP	15	-	100 %	100 %	-
Histone H2A	A0A3B6H4D4	Nucleus	NMP	18	-	-	100 %	-
H15 domain-containing protein	A0A3B6KRY4	Nucleus	NMP	25	-	-	100 %	-
Translation-related proteins								
Putative 50S ribosomal protein L15	N1R1R5	Chloroplast envelope	NMP	17	*19 %	-	100 %	-
60S ribosomal protein L36	A0A096UNN1	Ribosome	NMP	13	-	-	99 %	-
Uncharacterized protein	A0A080YUM7	Ribosome	NMP	149	100 %	-	-	-
Signal transduction								
Adenylate kinase	M8CA41	Mitochondrion	NMP	26	-	-	100 %	-
Defense proteins								
Disease resistance protein RPP13	M8A6K1	Plasma membrane	IMP	105	100 %	-	-	-
Pollen development								
Mitochondrial ATP synthase	Q6IY71	Mitochondrion	NMP	27	-	-	100 %	-
Unknown								
Uncharacterized protein	M8AUW8	Chloroplast envelope membrane	IMP	33	-	-	100 %	-
Uncharacterized protein	M7ZYZ9	Membrane	IMP	27	100 %	-	100 %	-

*Proteins with a probability score less than 98 % were not regarded as positive identifications.
- No proteins detected
a) Accession number in the Uniprot database

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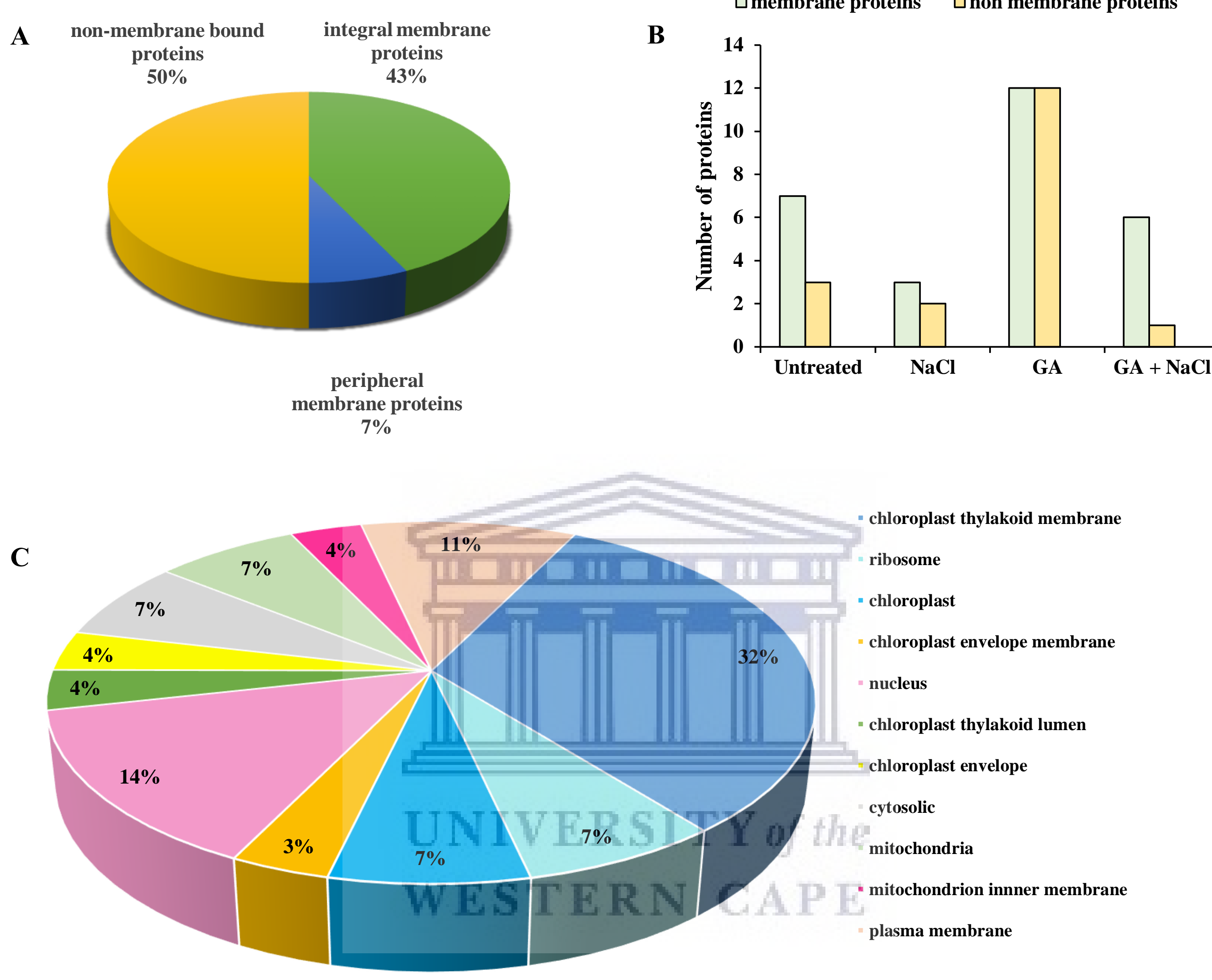

Figure 5.1. Proteins identified in the shoots of wheat plants. (A) Classification of proteins according to hydrophobicity, (B) Total number of membrane and non-membrane proteins identified in the various treatments and (C) Subcellular localizations of proteins identified in wheat shoots as predicted by Uniprot and Arememnon databases as well as WoLF PSORT software and literature sources.

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5.2.2 Functional characterization of proteins identified in wheat shoots

The identified proteins were functionally characterized based on their molecular and biological functions obtained using a combination of the uniprot database and literature sources. The 28 proteins identified were organized into eight broad categories including photosynthesis and energy metabolism, transport, defense, DNA binding, translation, pollen development, signal transduction and unknown function (Figure 5.2). Majority of the proteins that were identified in this study were involved in photosynthesis and energy metabolism (39 %) followed by transport (18 %) and DNA binding (14 %). In addition, some proteins were also shown to be involved in defense (3 %), translation (11 %), pollen development (4 %), signal transduction (4 %) and unknown function (7 %) (Figure 5.2).

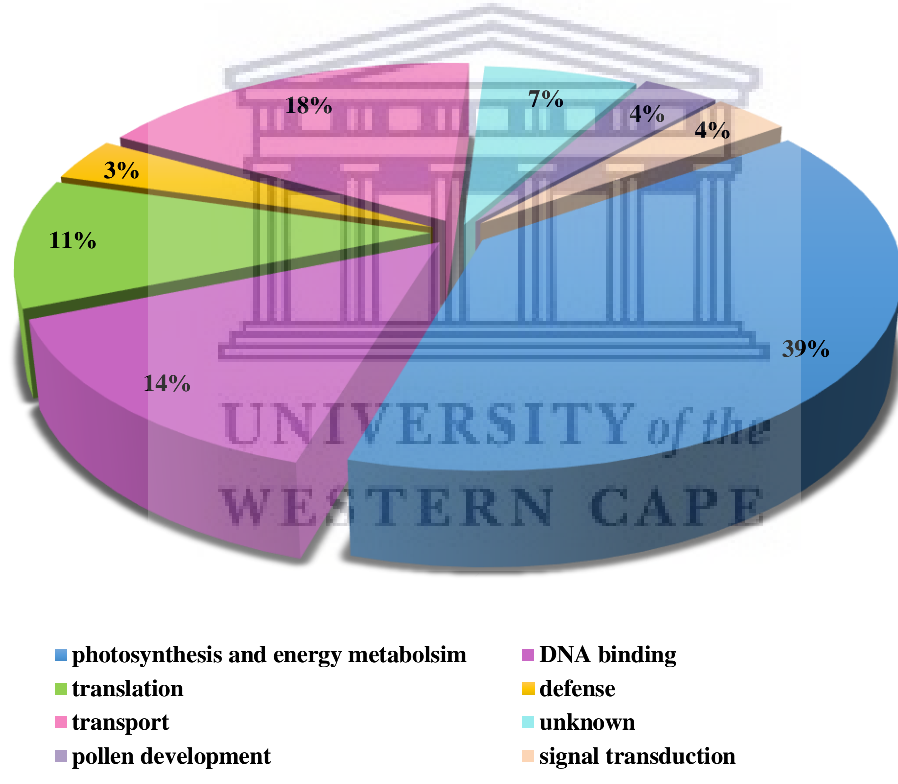

Figure 5.2. Functional classification of identified proteins. Eight protein groups (including the "unknown" group) were categorized based on the biological functions of the proteins. Different colour codes represent different functional groups. The percentages of each protein group is indicated.

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5.2.3 Common and unique proteins identified in wheat plants under GA and salt stress

To elucidate the proteomic response of wheat plants to GA treatment during salt stress, the shoot membrane proteomes of the untreated, 150 mM NaCl treatment, 250 µM GA treatment and 150 mM NaCl + 250 µM GA-treatment groups were compared. Unique proteins were only identified in the untreated (3 proteins), NaCl-treated (1 protein) and GA-treated (13 proteins) plants (Figure 5.3).

Common proteins were also shared between the various treatments. Of the 11 proteins that were identified in the untreated plants, 8 proteins were shared in the GA-treated plants, 2 proteins in the NaCl-treated plants and 6 proteins were shared in the combined treatment.

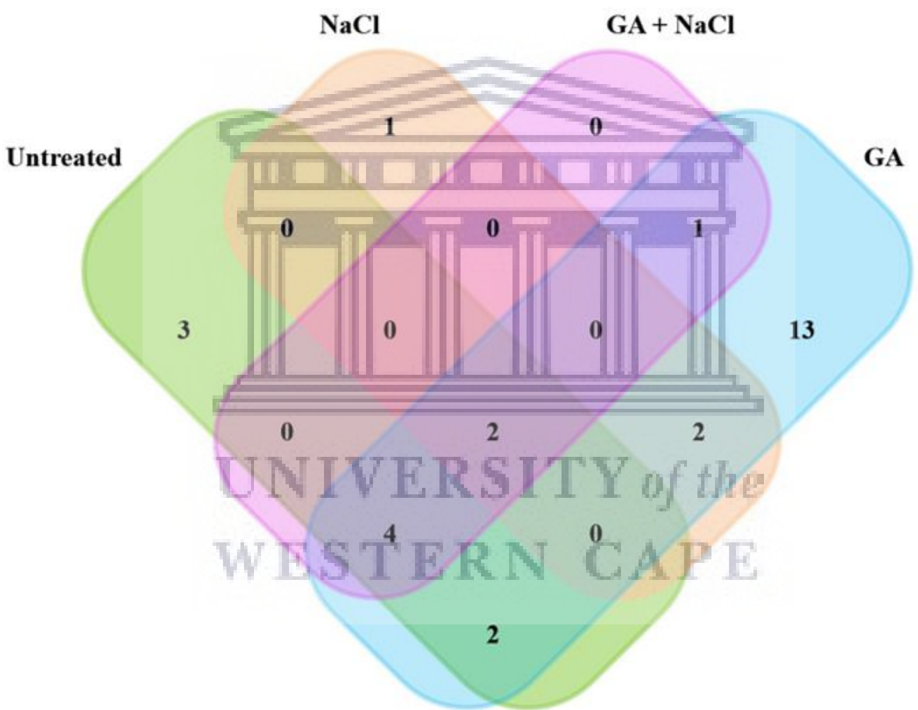

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Figure 5.3. Venn diagram depicting the common and unique proteins identified in the various treatment groups as identified by mass spectrometry (http://www.funrich.org).

Of the 24 proteins identified in the GA-treated plants, 4 proteins were shared in the NaCl-treated plants and 7 proteins were shared in the combined treatment. No unique proteins were identified in the combined treatment (NaCl + GA).

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5.2.4 Qualitative analysis of proteins expressed in wheat plants under GA and salt stress

The heatmap shows the expression levels of the proteins identified in the various treatments (Figure 5.6).

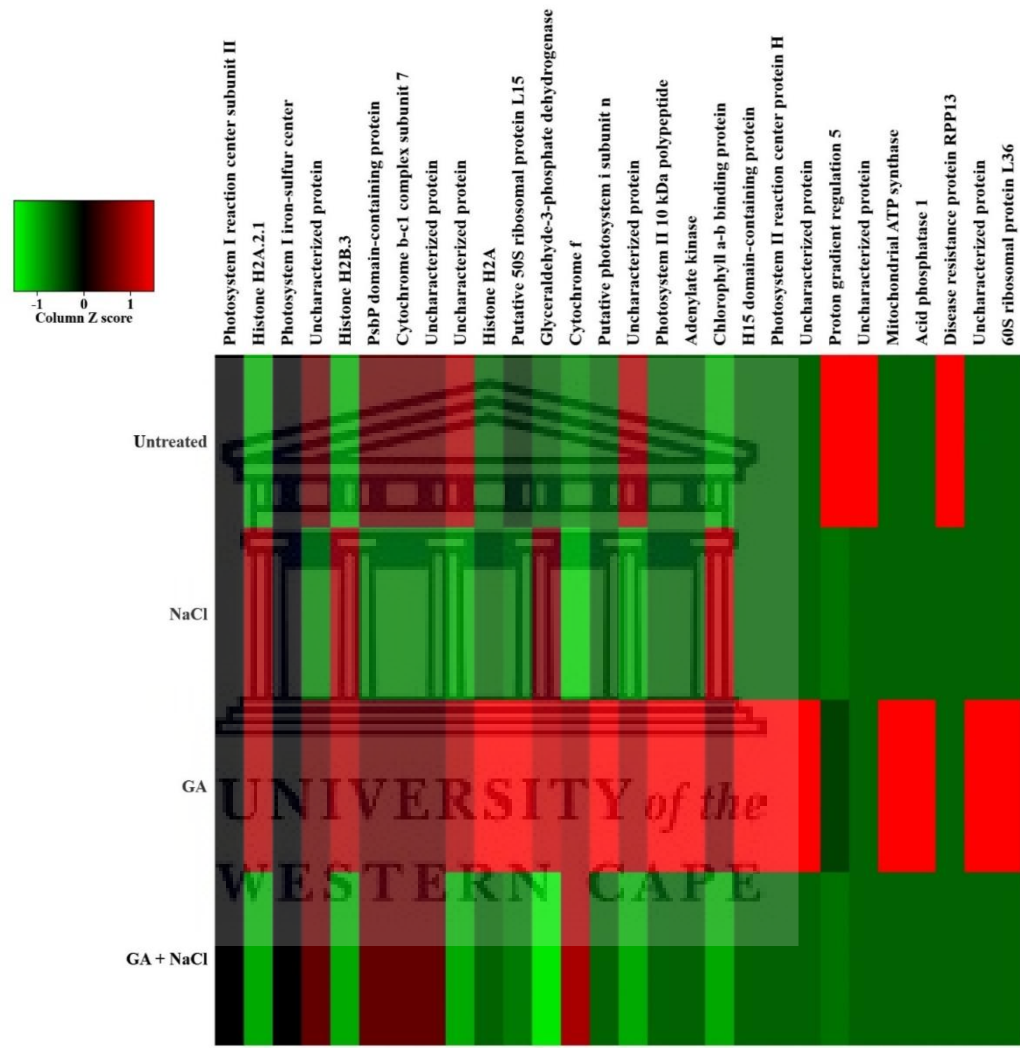

Figure 5.4. Protein expression patterns of wheat shoots exposed to various treatments. Wheat shoots were treated with 150 mM NaCl with or without 250 µM GA and the proteins were qualitatively identified by LC-MS analysis. The colors correspond to the Z score values.

From the results, a few proteins showed differential expression across treatments. These proteins included the chlorophyll a-b binding protein, glyceraldehyde-3-phosphate dehydrogenase, proton gradient regulation 5 and putative 50S ribosomal protein L15 (Table 5.1, Figure 5.4). Chlorophyll a-b binding protein was highly expressed in wheat plants under salt stress and partially expressed

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under GA treatment. The same protein was found to be absent under controlled conditions as well as in the combined treatment. On the other hand, glyceraldehyde-3-phosphate dehydrogenase and putative 50S ribosomal protein L15 were highly expressed in wheat plants treated with exogenous GA. However, these proteins were only partially expressed under controlled conditions. Although glyceraldehyde-3-phosphate dehydrogenase was also partially expressed under salt stress, the protein was expressed at a higher level than under controlled conditions. Proton gradient regulation 5 was the only protein found to be highly expressed in the untreated plants with partial expression under salt stress. Proteins that were only found to be highly expressed in the GA-treated plants included Histone H2A, putative photosystem I subunit n, photosystem II 10 kDa polypeptide, adenylate kinase, H15 domain containing protein, photosystem II reaction center protein H, mitochondrial ATP synthase, acid phosphatase 1, 60S ribosomal protein L36 and two uncharacterised proteins. Two proteins that were also only highly expressed in the untreated plants included an uncharacterised protein and the disease resistance protein RPP13.

5.3 Discussion

5.3.1 Identification of membrane proteins in wheat plants under salt stress and GA

Despite the high sensitivity of modern mass spectrometry, identification of extremely low abundant proteins, such as membrane proteins, are more challenging due to their hydrophobicity (Tai *et al.*, 2011), with only a few membrane proteomes being analyzed successfully (Friso *et al.*, 2004). Currently, there is no powerful method that is able to isolate, extract, solubilize and identify membrane proteins from low amounts of starting material. In this study, we isolated membrane proteins from 200 mg of plant material from each treatment and reduced contamination of soluble proteins using a commercial plasma membrane isolation kit with further downstream processing and identification using LC-MS analysis.

The degree to which proteins associate with cellular membranes varies, being classified as integral or peripheral membrane proteins. Integral membrane proteins (IMPs) comprise one or multiple membrane spanning regions, thereby serving as highly selective connections between intracellular compartments and providing several important functions such as cell-cell recognition, signal transduction, generation of electrical or chemical energy and catalysis of active transport (Redeby, 2006). Peripheral membrane proteins (PMPs) are associated with other membrane proteins or by reversible interactions with lipids. From the results of this study, a total of 28 proteins were

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identified, of which 12 were IMPs and 2 PMPs. This accounted for 50 % of the total proteins identified.

However, 50 % of the proteins identified were soluble proteins, which suggests contamination of other plant cellular organelles such as the nucleus and ribosomes. Hence, the extraction method used in the study along with the plasma membrane protein isolation kit wasn't as efficient in separating the nucleus, cytosol and membrane fractions from one another and these might be difficult to eliminate during preparation.

Furthermore, the isolation of membrane proteins usually requires large amounts of fresh starting material (10 g – 50 g) (Song *et al.*, 2015; Bernfur *et al.*, 2013). In this study a maximum amount of 200 mg plant material (previously ground in liquid nitrogen) was used. This may account for the low number of proteins identified in the study. Nonetheless, distinct differences were observed in the number of proteins identified in the various treatments (Figure 5.1 B). The majority of the proteins identified were present in the GA-treated plants, whereas the NaCl-treated plants had the fewest number of proteins present. This suggests that exogenous GA influence protein abundance/changes in wheat shoots exposed to salt stress.

5.3.2 Subcellular localization of proteins identified in the shoots of wheat plants

Protein subcellular localization is of substantial importance to biological research as it infers the potential functions and interactions for a protein (Ali, 2019). All identified proteins were analyzed and its subcellular localizations predicted using Arememnon (http://aramemnon.uni-koeln.de/) and UniProt (https://www.uniprot.org/) databases along with WoLF PSORT (https://wolfpsort.hgc.jp) and various literature sources (Ali, 2019; Jones, 2017).

It has been suggested that proteins identified from leaf microsomal fractions are usually dominant in chloroplast membranes (Bernfur *et al.*, 2013). Similarly, in this study majority of the proteins were localized in the chloroplast thylakoid membrane (Figure 5.1 C). The thylakoid membranes of chloroplast functions in light reactions of photosynthesis and are enriched in photosystem I and photosystem II complexes (Simpson and Von Wettstein, 1989). This was also evident in this study whereby the protein components that make up the photosynthetic electric transport chain were localized to the chloroplast thylakoid membrane. These proteins included Photosystem I reaction center subunit II, Photosystem II 10 kDa polypeptide, Photosystem II reaction center protein,

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Photosystem I iron-sulfur center and Cytochrome f (Table 5.1). These findings were consistent with an observation made in maize and sorghum (Ali, 2019).

Besides being responsible for photosynthesis which is vital for plant development, growth and crop yield, chloroplast displays several other essential functions such as stress signaling in plants, amino acid synthesis and fatty acid synthesis (Chan *et al.*, 2016). From this study, it was noted that proteins involved in transport of electrons were also localized in the chloroplast (Proton gradient regulation 5) or chloroplast thylakoid membrane (Photosystem I iron-sulfur center and Cytochrome f). Therefore, from the results of this study, the dominance of the chloroplast correlates with the fact that it is an essential organelle in photosynthesizing plants.

5.3.3 Functional characterization and expression of proteins identified in wheat shoots

Photosynthesis and energy metabolism

Of the 28 proteins identified, 11 were involved in photosynthesis and energy metabolism. The proteins associated with this category also represented most of the proteins identified in this study (39 %; Figure 5.2). Of the 11 proteins, 8 were present and highly expressed in the GA-treated plants and absent under salt stress conditions. These proteins included three uncharacterized proteins, PsbP domain-containing protein, putative photosystem I subunit n, photosystem II 10 kDa polypeptide, photosystem II reaction center protein H and acid phosphatase 1 (Table 5.1, Figure 5.3 and Figure 5.4). It is known that under salt stressed conditions, plants may accumulate Na^+ ions in chloroplasts which is often associated with a decrease in photosynthesis due to inhibition of photosynthetic electron transport activities (Sudhir and Murthy, 2004). Similarly, in this study salt stress inhibited most of the proteins involved in photosynthesis. In higher plants, salinity stress causes disorganization of PSII complex (Ahmad *et al.*, 2013) and inhibits PSII activity (Parida *et al.*, 2003), as observed in our study. According to Sudhir and Murthy (2004), this may be due to the dissociation of a 23 kDa polypeptide bound extrinsically to PSII.

Although glyceraldehyde-3-phosphate dehydrogenase and chlorophyll a-b binding protein were both expressed under GA and NaCl, the expression levels varied. Glyceraldehyde-3-phosphate dehydrogenase was highly expressed in GA-treated plants and partially expressed in the NaCl-treated plants, whereas the opposite was observed for chlorophyll a-b binding protein. Chlorophyll a-b binding protein was highly expressed in the NaCl-treated plants and partially expressed in the GA-treated plants. The expression and upregulation of chlorophyll a-b binding proteins in response

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to salinity or shade stress has also been observed in chickpea (Arefian *et al.*, 2019) and maize (Gao *et al.*, 2020).

Apart from this study, the influence of GA on membrane protein expression has not been described. Salt stress in known to alter the abundance of proteins involved in photosynthesis and energy metabolism (Li *et al.*, 2015). The reduction in protein abundance in response to salt stress is consistent with cotton (Li *et al.*, 2015), wheat (Guo *et al.*, 2012) and rice (Yan *et al.*, 2005).

Transport proteins

In order to survive under environmental stresses, plants are required to re-establish homeostasis in the stressed environment. Hence, the regulation of electron or ion transport through changes in expression levels of transporting proteins are important for achieving this. Transport proteins (18 %; Figure 5.2) represented the second major category of proteins identified. Of these proteins, photosystem I iron-sulfur center, cytochrome f and proton gradient regulation 5 are involved in regulating the electron flow around PSI, whereas cytochrome b-c1 complex subunit 7 forms part of the mitochondrial electron transport chain that drives oxidative phosphorylation (Dowhan and Bogdanov, 2002). An uncharacterized plasma membrane associated protein, based on uniprot searches, was also functionally characterized as a metal ion transporter.

Membrane organelles function in the fluidity of various biological substances such as lipids, ions, and membrane proteins (Sade *et al.*, 2014). However, environmental stresses alter the composition and fluidity of the membrane, evident in the changes in the expression levels observed for these particular proteins in response GA and salt stress. Cytochrome b-c1 complex subunit 7, cytochrome f and the uncharacterized protein were highly expressed in the GA-treated plants, while salinity stress negatively affected these proteins due to their absence in wheat plants under salt stress. The regulation of cytochrome proteins is important for energy generation (Furtado *et al.*, 2019). An increase in the expression of these proteins under GA treatment indicate increased energy production within the plant systems, which in turn may drive a variety of energy consuming cellular processes during the developmental stages of the plant.

Signal transduction-related proteins

In the signal transduction category, one protein, namely adenylate kinase was identified. Adenylate kinase is involved in ATP biosynthesis and plays an important role in adenine nucleotide metabolism and cellular energy homeostasis (Gong *et al.*, 2010; Kosová *et al*., 2013). This protein

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was only found to be expressed in the GA-treated plants (Figure 5.4). Thus, increased expression of adenylate kinases provides the cells with more ATP for maintaining cellular activities (Gong *et al.*, 2010).

Protein synthesis

The function of protein synthesis is to provide the cell with proteins and enzymes needed for the cellular processes (Jones, 2017). This category constituted 11 % of the proteins identified (Figure 5.2) and included two ribosomal proteins (Table 5.1). These proteins, which constitute the ribosome, functions in translation and were uniquely identified and highly expressed in the plants supplemented with GA, while salinity stress decreased the protein synthesis of these proteins as evident by the proteins being absent under salt stress (Shokri-Gharelo and Noparvar, 2018). Thus, GA increased protein synthesis. It is a common observation for protein synthesis to decrease under salinity stress (Shokri-Gharelo and Noparvar, 2018). However, other studies have shown that salt-tolerant cultivars induce protein synthesis under salt stress (Banaei-Asl *et al*., 2016; Bandehagh *et al*., 2011). The proper functioning of protein synthesis machinery is essential for plants under any environmental constraint. It was interesting to note that the exogenous application of GA to wheat plants under salt stress did not improve protein synthesis, suggesting that salt stress highly affects protein synthesis.

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CHAPTER SIX

CONCLUDING REMARKS AND FUTURE OUTLOOK

Salinity stress causes major agricultural losses in areas where rainfall is low such as in arid and semi-arid regions like South Africa. Although South Africa is classified as an upper-middle income country, food security remains a constant concern as evident in 2015 when 22 % of households were left food insecure (Nalley *et al*., 2018). The wheat industry plays an essential role in establishing food security within the country as majority of South Africans consume wheat as their staple diet (De Wet and Liebenberg, 2018). However, wheat production has steadily declined over the past three decades despite the increase in local consumption with 1.5 million tons of wheat having to be imported in 2019 to meet the local demand. Thus, it is vital to improve wheat production under saline conditions in order to increase yield and mitigate future food insecurity.

This study explored the antioxidant effects of GA in response to long term salinity stress. The results obtained in this study is divided into three research chapters.

Chapter three explored the effects of exogenous GA on the growth, biomass, relative water content and photosynthetic pigments of wheat plants under salt stress. Here, we have illustrated that GA and salt stress (induced by NaCl) differentially alters total chlorophyll and carotenoid contents as well as influences plant growth. Salinity stress showed inhibitory effects on the growth parameters whereas GA improved photosynthetic pigments, plant growth and biomass. Interestingly in the combined treatment (GA + salt stress), GA reversed the harmful effects caused by salt stress.

Chapter four explored the regulatory role of GA in enhancing salt stress tolerance in wheat through the differential modulation of ROS metabolism and antioxidant capacity. Here, we have illustrated that salt stress significantly enhanced ROS molecules (H_2O_2 and O_2), induced lipid peroxidation and ultimately lead to high cell death in wheat plants. However, GA was able to control the increase of these ROS molecules by inducing the antioxidant system to scavenge ROS to levels that are no longer toxic to plants. This in turn inhibited the extent of lipid peroxidation and ultimate cell death. It was also noted that GA reduced the activity of some enzymatic antioxidants suggesting that GA may directly act as a scavenging molecule, thus reduces the demand for enhanced antioxidant enzyme activity.

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Chapter five explored the effects of GA on the membrane proteome of wheat plants under salt stress. Here, we illustrated that GA regulates changes in protein expression. Using label-free LC-MS/MS analysis, we have identified 28 proteins, some of which could potentially serve as biomarkers to improve salt stress tolerance in wheat and other cereal crops. These proteins include the GA induced proteins identified in Table 5.1.

In conclusion, the identification of photosynthesis and energy metabolism related proteins together with improved plant growth and biomass observed in the study suggest that GA could potentially serve as a signaling molecule or a growth promoting agent under salt stress conditions. However, more in depth studies is required to support this hypothesis. The role of GA should not only be limited to modifications of the antioxidant defense system and changes in protein abundance, but extended to transcriptome and metabolome changes as well. This would provide significant insight and pave the way for engineering salt tolerance in sensitive food and feed crops.

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