Molecular, Biochemical and Physiological Responses of Wheat (*Triticum aestivum*) to Spot Blotch Disease and Salinity

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Abstract

Biotic and abiotic stresses are well known to significantly damage crop production and hence reduce yields. In wheat, which is the second major cereal crop and a major source of dietary carbohydrate and vegetable protein for humans, salt stress is recognised as a significant abiotic factor, while spot blotch disease, caused by the fungus *Bipolaris sorokiniana*, is an important biotic stressor. In order to protect crops from these threats, it is necessary to have a holistic understanding of the plant response towards these particular stress agents. In this study the responses of wheat to salt stress and spot blotch disease, both individually, and in combination with one another, were investigated. Using morphological, physiological, biochemical, and molecular parameters, the responses of four commercial spring wheat genotypes, two developed by KWS (Alderon, Cochsie), and two Saudi varieties (Najran, Sama) to stress, were investigated. The findings show that Najran, Alderon, and Cochise performed better to salt stress than Sama in terms of growth, proline production, and abundance of transcripts for several WRKY genes, when exposed to 80 and 160 mM NaCl for 21 days. *TaWRKY53-a*, *37*, *3*, and *71* were identified as key genes that respond differentially between Najran and Sama genotypes, when subjected to salinity (160 mM NaCl). In addition, the findings demonstrated that Najran, Alderon, and Cochsie exhibited less disease severity than Sama to spot blotch. Moreover, Alderon showed greater tolerance than Sama to the combination of salt and spot blotch disease. Biochemical changes (i.e., MDA, SA, JA content) in Alderon in response to *B. sorokiniana* infection and also to the dual stress were different to those exhibited by Sama. During fungal infection expression of *TaWRKY3* and *37* were upregulated in Alderon, while expression of the other WRKY genes investigated were downregulated. In contrast, expression of most WRKY genes were upregulated in Sama, although *TaWRKY19* and *45* were downregulated. The majority of glucan synthase (GLS) genes were shown to be upregulated after 48 hours of infection in Alderon, whereas most of these genes were downregulated in Sama. Alderon and Sama exhibited different expression patterns for *TaPAL*, *TaNPR1*, *TaAOS* and *TaLOX2*. Under the combined stress (salt and spot blotch disease) most WRKY genes, including *TaPAL*, *TaNPR1*, *TaAOS* and *TaLOX2*, were downregulated in Alderon, but upregulated in Sama. Furthermore, bio-informatic analysis suggests that W-box elements found in the promoter regions of *TaWKY53-a*, and *71* genes are regulated by WRKY genes. *TaWRKY71* was shown to contain motifs related to light response. In addition, *TaPAL* was shown to contain the highest frequency of motifs that were related to drought. Moreover, *TaNPR1* and *TaLOX2* were shown to have a high frequency for

cis- elements relating to wounding and biotic stress. Finally, studies showed that Alderon and Sama respond differently to spot botch disease and salinity, either when applied individually, or when combined. Knowledge gained will help inform the development or more resilient wheat varieties with enhanced tolerance to biotic and abiotic stress.

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List of Abbreviations

ABA	Abscisic Acid
ACS	1-Aminocyclopropane-1-Carboxylic Acid Synthase
ANOVA	Analysis of Variance
AOC	Allene Oxide Cyclase
AOS	Allene Oxide Synthase
APX	Ascorbate Peroxidase
BA	Benzoic Acid
BA2H	Benzoic-Acid-2-Hydroxylase
CAT	Catalase
CC	Chlorophyll Content
cDNA	Complementary DNA
CDPKs	Calcium-Dependent Protein Kinases
CRD	Complete Randomised Design
Ct	Cycle Threshold
CWA	Cell Wall Apposition
dpi	post-Inoculation
DW	Shoot Dry Weight
ET	Ethylene
ETI	Effector-Triggered Immunity.
FW	Shoot Fresh Weight
GA	Gibberellic Acid
GB	Glycine Betaine
GSAL	L-Glutamate-γ-Semialdehyde
GSL	Glucan Synthase-Like gene
H_2O_2	Hydrogen Peroxide
Hpi	Hours Post infection
HR	Hypersensitive Response
IC	Isochorismate

JA	Jasmonic Acid
LA	Leaf Area
LC-MS/MS	Liquid Chromatography Tandem-Mass Spectrometry.
LOX	Lipoxygenase
MAPK	Mitogen-Activated Protein Kinases
MDA	Malondialdehyde
MeJA	Methyl Jasmonate
MeSA	Methyl Salicylate
NL	Number of Leaves
NPR1	Non-expresser for Pathogenesis-Related genes1
NTC	No Template Control
$O_{2}^{\cdot -}$	Superoxide
OAT	Ornithine δ-Aminotransferase
OPDA	12-Oxo-Phytodienoic Acid
P5C	Δ1 -Pyrroline-5- Parboxylate
P5CS	Δ1 -Pyrroline-5- Parboxylate synthetase
PAL	Phenylalanine Ammonia-Lyase
PAMPs	Pathogen Associated Molecular Patterns
PD	Plasmodesmata
Phe	Phenylalanine
POD	Peroxidase
PR	Pathogenesis-Related Proteins
PRRs	Pattern Recognition Receptors
PTI	PAMP-Triggered Immunity
qPCR	Quantitative Polymerase Chain Reaction.
RCS	Reactive Carbonyl Species
RFW	RNase Free Water
ROS	Reactive Oxygen Species
RWC	Relative Water Content
SA	Salicylic Acid
SAD	Standard Area Diagram

SAR	Systemic Acquired Resistance
SEL	Size Exclusion Limit
SL	*S*hoot Length
SOD	Superoxide Dismutase
SOS	Salt Overly Sensitive
SOS_3	Calcium-Binding Protein
TAE	Tris-Acetate-EDTA
TaEF1α	Elongation Factor 1 Alpha in wheat
t-CA	Trans-Cinnamic Acid
TFs	Transcription factors
TW	Turgidity Weight
α-LeA	α-Linolenic Acid
ΔΔCt	Delta Delta

Chapter 1. General Introduction

1.1 Improvement of Crop Tolerance to Stress

Grain crops are one of the most important sources for human food and livestock feed; continued cereal production must increase to ensure global food security. There are however challenges that impact this food production. Firstly, global population is an important factor. According to a recent survey, the global population will reach approximately 9.7 billion by 2050, with the population rising 1.1% per year (Molotoks *et al.*, 2021). As a rising population will impact food production and security, it will be necessary to enhance crop yield by 70-100% in order to feed this predicted world population by 2050 (Iriti and Vitalini, 2020). Secondly, water shortages and the quality of the water will also negatively impact availability of food. Low amounts of water, seasonally or regionally, influence crop production. The quality of water can be affected by salinity, influencing crop development, and growth (Chakraborty and Newton, 2011). Thirdly, lands that are ideal for growing crops are important to sustainable production. Unfortunately, high quality agricultural land has diminished, resulting in an increased use of poor-quality soils for crop production resulting in lower yield. In fact, factors such as farming techniques, weather, infrastructure, and technology affect the quality of soil and therefore, cultivation of crops (e.g., climate change has been influential on variations in weather and soil). Moreover, factors such as hot weather and soil moisture have the capability to affect plant metabolism and reduce yield (Arora *et al.*, 2020).

As mentioned above, a significant threat to adequate food supplies is climate change. The consequences of climate change are twofold: Sea levels have risen due to melting ice. Additionally, oceans and air have warmed significantly. All these developments have already affected, and will continue to affect, humans and the environment. In that manner, the distribution of many plant species, animals, microbes, and soils are influenced by climate events and global warming (Iriti and Vitalini, 2020). Furthermore, plants have rapidly changed in respect to their growth and development due to temperature and rainfall. As an illustration, crops have experienced numerous side effects from climate change that end up modifying their distribution and their ability to be resistant towards associated pathogens. As a result, crop development and production has been declining. For example, an increased temperature could provide favourable conditions for pathogen communities and cause damaging effects on plant growth (Burdon and Zhan, 2020). Additional consequences arising from alterations in climate are increasing, affecting the duration and severity of abiotic and

biotic stress, which leads to constraints placed upon crop productivity. Abiotic stress is any environmental condition that is not favourable for plant growth such as salinity, drought, heat, nitrogen deficiency, high temperature, and heavy metals (Pereira, 2016). All these stresses can cause lower yield; for instance, 19.55% of irrigated land is classified as salt effected land globally (Yadav *et al.*, 2020).

Finding solutions is crucial in addressing those restrictions to increase crop tolerance towards abiotic and biotic stress and to increase adaptation to climate changes through traditional breeding, transgenesis/genetic engineering, plant transformation, and marker-assisted selection. For instance, the classical method (i.e. traditional breeding) is used for identifying a unique variety of species within a population by genetic variation that contains distinctive characteristics of their phenotypes and genotypes to have variety-tolerant stress (Shah *et al.*, 2018). Notably, a prominent factor that improves crop tolerance is an understanding of the molecular network response to different stresses, individually or simultaneously. It can be used to improve crop tolerance to cope with those stresses and their interactions (Mittler, 2006). A significant issue in addressing abiotic and biotic stress is understanding the resistance/tolerance processes of plants towards various types of stresses and gaining biological information of the target species (Babar *et al.*, 2014).

1.2 Wheat Production and Benefits

In terms of worldwide cultivation, wheat is the second major cereal crop after rice (Bousba *et al.*, 2012) . In temperate zones wheat is the most substantial crop and is increasing in demand in nations that are undergoing urbanization and industrialization (Shewry and Hey, 2015).Wheat bran contains abundant proteins (gluten), vitamins (e.g., thiamine, riboflavin, and vitamin E), carbohydrates, dietary fibres, and minerals (Zn , Fe ,P, Mn, and Mg) (Onipe *et al.*, 2015). It is also of some medicinal importance as its bran and germ protect humans from constipation, ischemic heart attack, diverticulitis, appendicitis, diabetes, and obesity (Kumar *et al.*, 2011). Remarkably, one-third of the global population consumes wheat as its main food source (Budhwar *et al.*, 2020). It is produced at an approximate rate of 737 million tons worldwide annually and 217 million hectors of land have been used for its growth (Ramadas *et al.*, 2019). As for production, almost 1.8 million hectares (40%) of the arable cropping land within the United Kingdom is allocated to production of wheat, contributing to 2% of the worldwide yield (Harkness *et al.*, 2020). As for the country of Saudi Arabia, wheat is the most important cereal which is planted on 215,700 hectares, or 26% of the overall farmland. Wheat has been receiving considerable government support since 1973, which has led to increased

production and productivity over the ensuing years (Al-Qunaibet and Ghanem, 2014). The wheat genome is more complex as compared to other plant species. For example, the genome size of rice is 430 Mb (Kurata *et al.*, 2002) with barley being 5.5Gb. However, the genome size for wheat has been known to be 17 Gb in hexaploidy species (Marcussen *et al.*, 2014; Sánchez-Martín *et al.*, 2016). In addition, diploid, tetraploid, and hexaploid are three structures of ploidy which exist within the genome of wheat (Hancock, 2012). The genome of bread wheat (*Triticum aestivum)* consists of three sub-genomes (AABBDD) that emerge from hybridization of three diploid species (hexaploidy). Moreover, every genome has three copies (one from A, B and D) (Zhou *et al.*, 2020). Several hundred thousand years ago, *Triticum turgidum* (2n = 28 AABB) which is tetraploid wheat was hybridized by two diploid species: The first one is *Triticum urartu* and the second from the *Sitopsis* segment of *Triticum*, which is considered linked to *Aegilops speltoides*. Thereafter, a hybridization occurred between *Triticum turgidum* (2n = 28 AABB) and *Aegilops tauschii* (DD) (belonging to diploid grass species), generating the ancestral allohexaploid *T. aestivum* (AABBDD) which has 21 pairs of chromosomes (2n = 42) (Marcussen *et al.*, 2014; Mirzaghaderi *et al.*, 2020).

1.3 Abiotic Stress

Stress, as stated, is an external factor influencing and decreasing plant growth potential and plant productivity. The response of plants can be shaped by stress and results in changes in cell metabolism, altering gene expression, growth rates, and crop yield. Stress is classified into two types: abiotic stress and biotic stress (Gull *et al.*, 2019). Abiotic stress refers to adverse environmental conditions around plants. Plants live in an environment where they experience continuous stresses due to their sessile life. Abiotic stresses cause major restraints to plant productivity and growth, leading to reduced yield (Acquaah, 2007). Drought (deficiency of water), extreme temperatures (cold and heat), salinity (salt excess), and minerals (metal toxicity) are some examples of abiotic stress that hinder growth, reduce quality of seed, and lessen yield of crops (Zhu, 2016). Factors such as heat stress and high temperature during the anthesis stage can cause sterility and substantial loss of production for wheat. What must be considered is that the critical temperature throughout reproduction is approximately 30°C. Additionally, there is risk from acute winter frosts (i.e., crop destruction such as leaf chlorosis) after experiencing temperatures lower than −20°C when there is no snow cover (Harkness *et al.*, 2020).

1.3.1 Plant response to abiotic stress

Overall impact from the majority of abiotic stresses are usually dependent on the species of plants, plants' stages, time of stress, stress intensity, and the genotype (Le Gall *et al.*, 2015). When plants receive stress signals from unfavourable environments, a process of adaptation to the adverse condition initiates. Understanding the manner which plants are able to resist stress is vital (Zhu, 2016). After being subjected to abiotic and/or biotic stress, the plant responds by activating specific kinase cascades and ion channels. Thereafter, hormone signalling mechanisms, transcription factors (TFs), and reactive oxygen species (ROS) are triggered. The following phytohormones (e.g., ethylene (ET), salicylic acid (SA), abscisic acid (ABA), and jasmonic acid (JA) are recognized as involved(antagonistically/synergistically), as a reaction against stress. For instance, ABA is highly responsive to osmotic stress, while SA, JA, and ET have significant roles in reaction to invasion by pathogens. These responses lead to plants being tolerant or resistant thus lowering the biololgical damage done by these stressors (Vlot *et al.*, 2009). A component of the plants' defence systems, after confronting many stresses, transcription factors, which are regulatory molecules. They are proteins which attach to a specific location of the gene and enhance or suppress gene expression. For example, for transcription factors that respond to abiotic and biotic stresses, A2P/EREBP, MYB, WRKY, NAC, and bZIP are well-documented families that play an imperative role (Chen *et al.*, 2012; Phukan *et al.*, 2016).

1.3.2 The effect of salt stress in plants

A well-known prominent environmental stress with destructive tendencies is soil salinity. This is due to the fact that it has negative impacts such as affecting cultivated land and decreasing yield and quality of crops. High levels of salinity have adversely affected approximately one-fifth of the overall cultivated land area in addition to one-third of the irrigated farming areas (Shrivastava and Kumar, 2015). The rate of increase in salinization of agricultural land, now estimated to be in the region of 10% annually, due to a range of different factors (e.g., sub-standard cultural practices, weathering of natural rocks, limited precipitation, and elevated surface evaporation), has attracted significant attention. This concern is growing as another approximation concludes that by 2050, there is the possibility that over half of the global arable land will be salinized (Jamil *et al.*, 2011). Regrettably, from the arable land currently available in the world today, salinization has impacted 800 million hectares (Acosta-Motos *et al.*, 2017).In addition, one million hectares of irrigated land in European and Mediterranean countries has been affected by salinization (Machado and

Serralheiro, 2017). In terms of Saudi Arabia, salinization has become a dominant stress generating factor in most of the agricultural lands, primarily as a consequence of excessive irrigation, which has also been the cause of waterlogging (Elhag, 2016).

The biochemical and morphological performance of plants is adversely affected by salinity as it hinders plant development, germination of seeds, and hampers productivity. More specifically, salinity also impedes the photosynthesis process of plants (e.g., negatively altering chloroplast ultrastructure and PSII system) (Arif *et al.*, 2020). Consequences from salt stress could be detected on the entire plant as it diminishes plant growth and may cause plant death. Salt stress has two effects on a plant: First, an osmotic effect t that decreases the water potential gradient between soil and root structure of the plant due to a high level of salt in the soil solution. As a result, the plant will continue transpiration whereas the rate of water absorption from the soil is reduced, causing a water deficit (water stress). Second, is the ionic toxicity that is mostly developed due to higher absorption of Na^+ and Cl^- inside the plant cell. Increasing the concentration of Na^+ competes with K^+ and decreases K^+ intake in the cell, causing disruption of cell metabolism, eventually resulting in a reduction of plant development and growth (Munns, 2002; Munns *et al.*, 2006). High amounts of salt and time period play a substantial role in causing salt injury by physiological, morphological, and biochemical variations (Farahmandfar *et al.*, 2013; Meriem *et al.*, 2014).

1.3.3 Signalling processes involved in salt stress and their markers

When plants are growing in a salty environment, there are several changes occurring in them, including morphological, physiological, and biochemical characters. It is important to understand how plant cells sense and respond to these adverse conditions. The response of plants starts from sensing the concentration salt in their root system, followed by many reactions inside the plant cells including perception and thereafter signal transduction to numerous sites, eventually resulting in metabolic changes. The timing of salt stress and attributes of plant reactions will play a role in salt tolerance or sensitivity. Components of osmotic effect and ionic type are major ways that enable the plant cell to perceive salt stress. When root cells are exposed to high concentrations of ions on the outside of the cell, the volume of the cell is decreased; this causes osmotic stress, leading to alternation in plasma membrane formation that facilitates influx of ions inside the cell via ionic channels known as osmo-sensors. The ions will get inside the cell and cause depolarization of the membrane of the cell; this results in the accumulation and increase of Ca^{2+} inside the cytoplasm (Gheyi *et al.*, 2016). The elevated Ca^{2+} will perform as a secondary messenger and cause perception of

stress and also initiate signal transduction pathways. The signal triggers expression of responsive genes to salt stress ultimately. This is termed as salt stress signal transduction. Salt stress signal transduction comprises ROS, ion channel activity, phospholipid dependent, phosphoprotein cascade, calcium/calmodulin dependent, mitogen-activated protein kinases (MAPK) cascade, and ABA dependent. Additionally, one of the signals used as the common pathway for ionic element is the salt overly sensitive (SOS) pathway (Figure 1) (Türkan and Demiral, 2009; Conde *et al.*, 2011; Gheyi *et al.*, 2016) When it comes to cell signalling, ABA is a key player. At times when there are significant changes in osmolarity, it has the potential to regulate and activate downstream pathways. The way ABA is perceived, communicated, and transported are all crucial. ABA receptors are a key for signal reception. One of these receptors are pyrabactin resistance 1/ Pyr-likes/regulatory component of ABA receptors (PYR1/PYLs/RCARs) that are proteins attached to ABA and precisely interact with two type 2C protein phosphatases (PP2Cs) which prevent phosphatase activity of the PP2Cs. As result , SNF1-related kinase 2 (SnRK2 kinases) are activated. Following that, the SnRK2s phosphorylate transcription factors(AREB/ABF) and the AREB/ABF interact directly with ABA-responsive elements that existed in promoter region of the ABA-responsive genes (Qin *et al.*, 2011; Gheyi *et al.*, 2016).The second initiation of the signal transduction pathway is Na^+, sensed by receptors of membrane cells. Afterwards, the Na^+ in the cell can be sensed by Na^+ sensitive enzymes and membranes proteins, resulting in activation of the SOS pathway (Zhu, 2003; Conde *et al.*, 2011). This pathway is initiated when Ca^{2+} are perceived by a calcium-binding protein (SOS3), that is defined as sodium hyper sensibility responsive which is one of the major elements of SOS regulation. After that, SOS3 interacts with SOS2(serine/threonine protein kinase) and the result of the interaction triggers their phosphorylation. The result of phosphorylation is SOS3 and SOS2 with PO4, a complex activating SOS1 (i.e., protein of membrane). SOS1 regulates Na^+ /H^+ antiport and leads ultimately to exclude excessive sodium ion from the cytosol. This pathway is important for ion homeostasis (Ji *et al.*, 2013; Gheyi *et al.*, 2016). In addition, SOS2 play important a role for interaction with vacuolar Na+/H+ antiporter (NHX) that influence on activity of Na+/H+ exchange. As a result, excessive Na+ ions are taken up and stored in the vacuolar compartment, which contributes to the maintenance of ion homeostasis. Furthermore, SOS2 without interaction with SOS3 regulate SOS1 and vacuolar H+/Ca^{2+} antiporter CAX1 which lead to maintain Ca^{2+} homeostasis(Qiu *et al.*, 2002). Furthermore, SOS2 without interaction with SOS3 regulate SOS1 and vacuolar H+/Ca^{2+} antiporter CAX1 which lead to maintain Ca^{2+} homeostasis. This shows a relationship between the homeostasis of Na+ and Ca^{2+} in

plants((Zhu, 2002). SOS2 has been found to involved in regulation of vacuolar V-ATPase (H+-ATPase) that facilitate protons influx into vacuolar (Batelli *et al.*, 2007). HKT(high-affinity K^+ transporters) are proteins participate in Na^+ and K^+ uptake and homeostasis and they are found all plant parts (shoot, leaves , roots and flowers)(Riedelsberger *et al.*, 2021). More importantly, it has been mentioned that SOS3-SOS2 complex regulate the expression of HKT1 negatively as result averting Na+ influx((Mahajan and Tuteja, 2005). SOS4(pyridoxal (PL) kinase) that is engaged in pyridoxal-5-phosphate (PLP) biosynthesis and it is an active form of vitamin B6(Mahajan *et al.*, 2008).It was stated that pyridoxal-5-phosphate has a role as a cofactor for SOS1 and SOS4 is involved in SOS1 regulation (Singh *et al.*, 2017b). SOS5 is located in plasm membrane protein that help to sustain cell wall and cell expansion under salt stress((Shi *et al.*, 2003; Mahajan *et al.*, 2008)

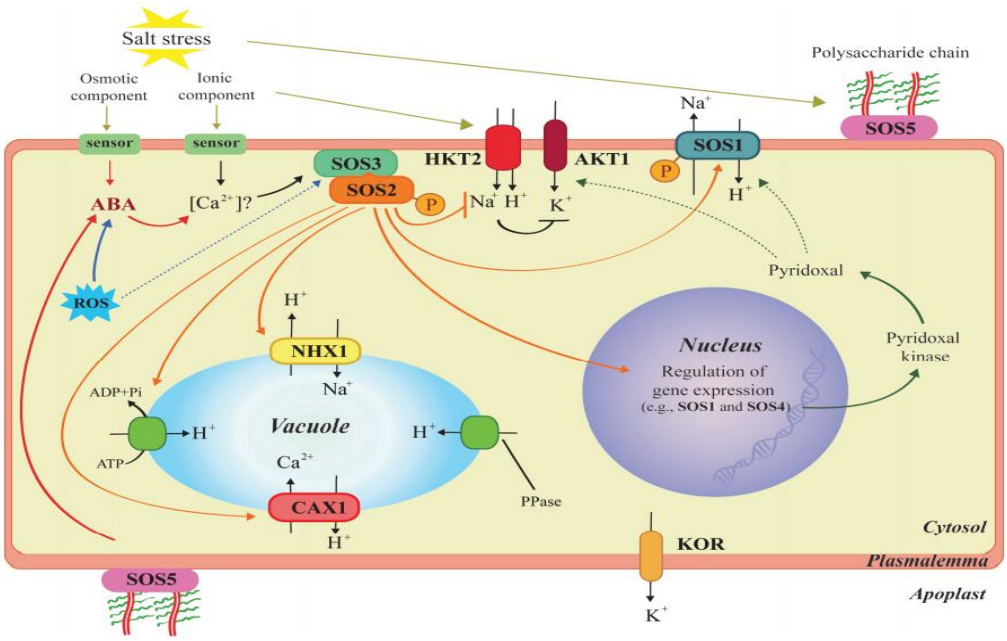

Figure 1 Illustration of the regulation of the SOS pathway. Ca^{2+} signalling is triggered by salt stress, which in turn activates the SOS3/SOS2 protein kinase complex. This subsequently phosphorylates a plasma membrane Na+/H+ antiporter called SOS1, which in turn influences the expression of several genes as well. Additionally, SOS2 activates the tonoplast $Na^{+/}H^+$ antiporter, which sequesters sodium within the vacuole (NHX1). CAX1, also known as the H^+/Ca^+ antiporter, is an additional target for the action of SOS2, which helps restore cytosolic Ca^{2+} homeostasis. Negative regulation of AtHKT1 activity is mediated by the SOS3/SOS2 complex. (Türkan and Demiral, 2009; Gheyi *et al.*, 2016).

1.3.4 Biochemical changes under salt stress

Antioxidant enzymes, (ROS), and osmoprotectants have been considered as markers in plants to indicate the occurrence of salt stress (Soltabayeva *et al.*, 2021). ROS, including hydrogen peroxide (H_2O_2) and Superoxide ($O_2^{\cdot-}$), is produced inside cells and increases

under salt conditions (Gémes et al., 2016). Enhanced ROS leads to lipid oxidation in the membrane of the cell and can generate reactive carbonyl species (RCS) which includes Malondialdehyde (MDA) that has been used as an oxidative marker to indicate cell injury under high salt concentration levels (Mano *et al.*, 2014). On the other hand, antioxidant enzyme activity (e.g., peroxidase (POD), peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) will be produced for scavenging ROS production. Different levels of salt concentrations can play a role to enhance or suppress antioxidant enzyme activity. Most studies have shown the level of antioxidant enzymes are changeable at high levels of salt concentration (Akyol *et al.*, 2020).

Finally, proline is one of the osmoprotectant compounds and is also known as compatible solute or osmolyte. It is an amino acid that gathers in plant tissue during abiotic stress. It had been studied in the plant as an osmoregulator under salt and drought stress (Ashraf and Foolad, 2007).

1.3.5 Proline accumulation

As a response to many abiotic/biotic factors such as, pathogen infection, shortage of nutrients, water deficit stress, extreme temperatures, UV-radiation, heavy metal toxicity and salinity stress, an overproduction of free proline occurs (Song *et al.*, 2005). Proline has the roles of ROS scavenger, stabilizer of protein structure, balancing cell redox status, and buffering cytosolic pH, behaving as a compatible osmolyte (Verbruggen and Hermans, 2008). Proline increases in content when the concentration of Na^+ and Cl^- ions have risen inside the vacuole of plant cells for maintaining osmotic pressure (Munns and Tester, 2008). Within plants, the main proline biosynthesis derives from glutamate by two NADPH-dependent enzymatic stages as follows: Firstly, the enzyme Δ1 -pyrroline-5- carboxylate (P5C) synthetase (P5CS) reduces glutamate into L-glutamate-γ-semialdehyde (GSAL). Thereafter, GSAL suddenly changes into P5C. P5C reductase (P5CR) then diminishes P5C in order to generate proline. Secondly, ornithine δ-aminotransferase (OAT) forms GSAL/P5C; thus, offering a path (ornithine) for proline generation (Verbruggen and Hermans, 2008; Tavakoli *et al.*, 2016; Furlan *et al.*, 2020).

1.3.6 Malondialdehyde (MDA) content

Plants stimulate the over-production of ROS and damage cell membrane by lipid peroxidation when under conditions of stress. As a result, accumulation of MDA content occurs. As a biochemical marker, MDA indicates salt stress and drought stress (Luna *et al.*, 2000; Singh *et al.*, 2012). In terms of abiotic stress, salt stress is a condition which leads to the

increased production of MDA content that depends on the following 2 factors: duration of salt stress and salt stress intensity. For instance, the duration of salt stress in rice has been shown to be a factor affecting its MDA accumulation, with an increase in sensitive genotypes and decrease in resistant cultivars (Lutts *et al.*, 1996). In addition, differing quantities of MDA content have been detected in varieties of wheat when affected by drought stress. Moreover, drought-sensitive cultivars showed greater MDA content in leaves and roots compared with drought-tolerant cultivars (Singh *et al.*, 2012).

In terms of biotic stress, the impact of pathogen attack on plants can alter the MDA content such as when the pathogen *Botrytis cinerea* infects leaves of *Arabidopsis thaliana*, MDA levels are reduced in infected leaves as compared to healthy leaves (Muckenschnabel *et al.*, 2002). In contrast, much research has shown that MDA content actually can increase during a pathogen attack such as *Botrytis cinerea* infecting bean (*Phaseolus vulgaris)* (Muckenschnabel *et al.*, 2001). The response of wheat lines is different between the resistant wheat variety and susceptible wheat variety to *Bipolaris sorokinian*, with the MDA content being higher in susceptible as compared to the resistant lines (Yusuf *et al.*, 2016).

Initiation of MDA in plants is from polyunsaturated fatty acids (PUFAs) that are the main elements of the bio membrane. As a response to oxidative stress, PUFAs are peroxidized easily (Yamauchi *et al.*, 2008). Lipid hydroperoxides or peroxyl radicals are known to be the key products of peroxidation which can be cleaved via enzymatic/non-enzymatic processes to form short-chain oxidation products such as various aldehydes (e.g., 4-hydroxy-2-nonenal (HNE), alkanes, and MDA) (Ayala *et al.*, 2014; Sousa *et al.*, 2017).

1.4 Biotic Stress

Crops can be bred for enhanced tolerance to biotic stress; however this is often dependent upon detailed knowledge of the underlying molecular responses of the crop in question to a particular stress agent (Roberts and Mattoo, 2018).Fungi, bacteria, nematodes, insects, viruses, parasites, and weeds are recognized as living organisms that cause stress and affect plant growth. This is known as biotic stress. Biotic stress adversely impacts crop development and hence productivity. A factor that impacts biotic stress is climate change as all around the world, cultivated crops are affected by it in two ways: First is increased crop *susceptibility* when exposed to pathogens, and second, assisting the *prevalence* of plant diseases, pests, and parasites, causing yield reduction (Chakraborty and Newton, 2011).The response of plants when exposed to stress is to enhance ROS, transcription factors (TFs), and hormones signalling pathways that play essential roles in the crosstalk between the abiotic

and biotic response (Fujita *et al.*, 2006). Phytohormones (e.g., ABA, SA, JA, and ET are involved synergistically as well as antagonistically in plant cells. ABA is extensively engaged in the response to osmotic stress but SA, ET, and JA also play central roles in reaction to pathogen challenge (Vlot *et al.*, 2009).

1.4.1 Fungal stress

Fungal infestation in plants causes a reduction of plant growth by affecting the digestion and absorbance of nutrition by plants. The fungi are heterotrophic organisms which can penetrate the plant tissue and absorb nutrition or complete their life cycle within the tissue. These are called biotrophic fungi. There are also fungi which obtain nutrition by degradation of plant residues, called saprotrophic fungi, while other types of fungi infect and kill plant tissue, referred to as necrotrophic fungi. Fungal attack can be through various ways. The fungus can enter through stomata, and also, by penetration after directly attaching on the surface of plants. The mechanism of fungal penetration in plant tissue is thorough hypha that secretes enzymes to break down substrates into small compounds (Mendgen *et al.*, 1996; Mendgen and Hahn, 2002).

1.4.2 Bipolaris sorokiniana

B. sorokiniana is a hemi biotrophic pathogen that have two stages in their development: first, is the biotrophic stage followed by a second stage called the necrotrophic stage that causes spot blotch and black points on wheat leaves. It also causes root rot and black spots in grains. Favourable conditions for the growth of *B. sorokinia* are higher humidity, warmer temperatures (20-30 °C), and lower light. It can grow and colonize on the hot tissue of wheat rapidly. As a consequence, it reduces and inhibits wheat productivity via yield loss (Kumar *et al.*, 2002). *B. sorokinia* infects the foliar part of wheat, causing spot blotch and black points. The distribution of *B. sorokinia* is worldwide and leads to potential yield loss (e.g., 19% yield loss of wheat in Australia, Canada, and Southern Brazil, 15 % in Bangladesh, and 16% in Nepal). The fungal pathogen *B. sorokinia* is dominant around the world and diminishes wheat production (Duveiller and Garcia Altamirano, 2000)

1.4.3 Spot blotch disease

Indications of spot blotch are usually displayed on the sheath, node, leaf, and glumes in the form of little light brown lesions whose shapes are largely oval, to oblong, to slightly elliptical, having a length of 5–10 mm and width of 3–5 mm. The above-mentioned lesions have brown margins, are commonly dispersed all over the leaves, and their size slowly

increases, coming together to create bigger necrotic patches. The impacted leaves quickly become deficient in chlorophyll, ultimately dying. Amidst the most difficult conditions, the spikes are impacted as well with dark brown-to-black stains appearing around the germinating seeds, referred to as a black-points (Gupta *et al.*, 2018).

The adhesion of conidial spores on leaves and forming germ tubes is the initiation of the infection within 4 hours. Thereafter, the germ tubes form appressorium 8 hours after infection. This is followed by the next step where hypha starts to develop inside the cell infection via spores on leave surfaces after 24 hours. Finally, hyphae generate conidiophores that rise from the host, resulting to a progression of conidia in four days. Figure 2 provides a diagram illustrating the different disease stages of *B. sorokiniana* in wheat (Sahu *et al.*, 2016).

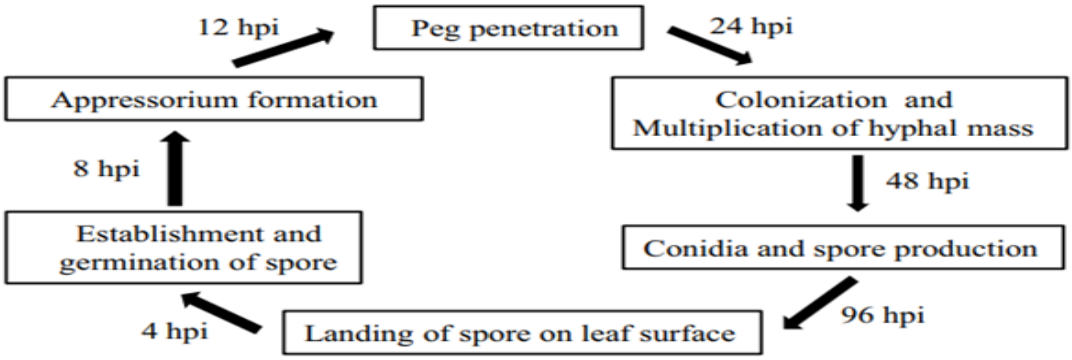

Figure 2 The diagram illustrating the different disease stages of *B.sorokiniana* in wheat (Sahu *et al.*, 2016).

1.4.4 Plant defence mechanisms

A short period (few mins) following attack on plants by pathogens, plants display a local response. However, a sophisticated response is triggered after a few hours in tissues of plants far away relative to the infection's original location (i.e., issue damage). The aforementioned stimulated systemic response depends on the kind of attacking organism which caused it. The response is different for pathogens when compared to herbivores. For pathogens, it is linked to a systemic acquired resistance (SAR), that causes synthesis of PR proteins (pathogenesis-related proteins). These PR proteins are engaged in the defence response of the plant during pathogens attack as illustrated in Figure 3 and include molecules for instance chitinase and β-1,3-glucanase are enzymes that break down fungal cell walls (Saboki Ebrahim and Singh, 2011; Lal *et al.*, 2018).

For the defence of plants from pathogenic diseases, numerous physical barriers exist like the cell wall and waxy cuticle of plant (Yeats and Rose, 2013). For most of the time, plant pathogens have to constantly attack host plants in order to absorb nutrients which facilitate pathogen development. Accordingly, plants have developed two defence mechanisms for detecting pathogens. Firstly, upon pathogen attack on the exterior surface of the host cell, pathogen associated molecular patterns (PAMPs), that are known to be conserved microbial elicitors, are released. Thereafter, they are detected by receptor proteins referred to as pattern recognition receptors (PRRs) in plant cell surface and this causes basal resistance mechanisms. Stimulation of PRRs cause PAMP-triggered immunity (PTI) (Jones and Dangl, 2006; Rasmussen *et al.*, 2012; Meng and Zhang, 2013; Lal *et al.*, 2018). This class of elicitors is critical for pathogens because it binds to receptors on the plasma membrane of plant and they do not present in the host such as bacterial flagellin, peptidoglycans , lipopolysaccharide, fungal xylanase and chitin. Chitin is a key component of fungal cell walls and is a well-known PAMP. This recognition is made possible by chitin elicitor-binding protein (CEBiP) and chitin elicitor receptor kinase 1(CERK1) which present on the plasma membrane of the plant and have an extracellular domain that includes LysM-motifs, that are capable of binding to chitin. Bacterial flagellin (flg22) is one of the elicitors that recognised via he flagellin-sensing (FLS2) that exist in plasma membrane of plant(Koeck *et al.*, 2011; Lal *et al.*, 2018).

In the range of mins to hours, PAMPs cause various responses; for example, activation of MAPKs, oxidative burst, quick ion movements across the plasma membrane, and calcium-dependent protein kinases (CDPKs), which stimulate defence-related genes that produce enzymes (e.g., defensin, chitinases, and glucanases) that play a role in affecting cell wall or cell membrane of pathogen. Additional responses might also be the generation of antimicrobial phytoalexins and plant cell wall formations (e.g, lignin and callose) (Jones and Dangl, 2006; Rasmussen *et al.*, 2012; Meng and Zhang, 2013; Lal *et al.*, 2018). After the plant recognised pathogen attack, MAPK activation is one of the first signalling events of PTI and ETI((Meng and Zhang, 2013). MAPK cascades are divided into three categories: Mitogen-activated protein kinase kinase kinase(MAPKKKs) as known MAPK/ERK kinase kinase(MEKKs), Mitogen-activated protein kinase kinase (MAPKKs/MKKs), and Mitogen-activated protein kinase(MAPKs/MPKs)(Ichimura *et al.*, 2002). The activation of MAPKs occurred via phosphorylation. MKKs are phosphorylated by MEKKs because of that phosphorylation and activation of MPKs. Following activation of MPKs, downstream

components including transcription factors and metabolic enzymes are phosphorylated to control their function (Bigeard *et al.*, 2015; Thulasi Devendrakumar *et al.*, 2018) For example, following pathogen attack (*Pseudomonas syrin*)and flagellin application in Arabidopsis lead to activate MPK4 which phosphorylate MKS1(MPK4 substrate). As result, MKS1 interact with WRKY33 and generate complex. MKS1-WRKY33 complex bind to PHYTOALEXIN DEFICIENT3 (PAD3) promoter region that is an enzyme involved in antimicrobial camalexin synthesis(Qiu *et al.*, 2008).

Secondly effectors, which are the intracellular receptors of pathogen virulence molecules, are involved in a process of recognition that stimulates effector-triggered immunity (ETI). The plants' immune response against particular pathogens is referred to as the ETI. It arises in response to certain molecules which are released from the pathogen (i.e., effectors). As the most effective way to control pathogens, ETI is capable of PTI (Jones and Dangl, 2006).

Previously referred to as vertical resistance or 'R gene-mediated' resistance, the mechanism by which ETIs work is by prompting the plant's defence signalling in consequence to interaction of the R gene products in the host plant and Avr products of the pathogen, being incompatible. Most of the R proteins, i.e., products of the R gene, which stimulate ETI and are mediating race-specific-resistance, contain diagnostic structural motifs. Varying R proteins play 2 fundamental functions. First, they detect effector molecules directly or indirectly. Second, the R proteins initiate downstream signalling, causing the stimulation of different defence responses. When there is an attack from the pathogen, the R genes' expression is increasing. Additionally, the role of R proteins is associated with detection of varied pathogens such as viruses, fungi, oomycetes, bacteria, nematodes, and insects. ETI, to a large part, depends on the endogenous NB-LRR protein products which the R genes (resistance genes) encode (Keane, 2012; Taiz *et al.*, 2015). ETI is triggered by plant intracellular resistance (R) proteins following a particular perception of pathogenic, and it is related with a kind of programmed cell death (PCD), a reaction that is also called as the hypersensitive response (HR)(Hou *et al.*, 2011). HR is similar to other types of cell death that have been studied in animals (such as pyroptosis, apoptosis, necroptosis), like mitochondrial swelling, chromatin condensation, and cytoplasmic shrinkage. However, HR also provides features that are unique to plants, such as chloroplast disruption and vacuolization in the last phases of the process(Salguero-Linares and Coll, 2019). The detection of pathogen effectors via nucleotide-binding leucine-rich repeat receptors (NLRs), which are constituted of a central nucleotide-binding site and C-terminal leucine-rich repeats (LRR) domains, is what

triggers the activation of HR during early stages of infection (ETI)(Chiang and Coaker, 2015). NLRs can either recognize a pathogen penetration directly, through the physical interaction between effectors and the relating NLRs, or indirectly, through the detection of modifications in target host proteins of effectors by the NLRs(Zhou and Zhang, 2020). NLRs quickly evolved to detect a wide variety of effectors by creating chimeric and point mutations in LRR domains(Tamborski and Krasileva, 2020). Additionally, NLRs are able to sense effectors indirectly throughout integrated domains (ID), that simulate the target of a pathogen, or from the interaction with guardees or decoys(Sun *et al.*, 2020). When an effector is recognized, an NLR will go through a structural change and begin to connect with other NLRs. The formation of dimers or oligomers of NLRs to signal to components of downstream, activates NLRs (helper) ultimately results in the ETI response(Nguyen *et al.*, 2021). Additionally, ETI is enhanced via a hormone signalling network and it involves transcriptional reprogramming that is triggered via the EDS1/PAD4 dimer entering the nucleus in TNL-mediated ETI(Thordal-Christensen, 2020). It is less apparent what causes the activation of transcriptional reprogramming in (coiled-coil domain CC-NLRs CNL)-mediated ETI, but one possibility is that it is driven by immune signals that disseminate from cells which have undergone HR. The interaction of N-hydroxypipecolic acid (NHP) , ethylene (Et), salicylic acid (SA), jasmonic acid (JA), and is essential to the functioning of the hormone network(Thordal-Christensen, 2020). Numerous enzymes and other proteins, altered during ETI, have been linked to these hormones and shown to play critical roles in immune function. A well-known enzyme in the shikimate-pathway, isochorismate synthase, SID2, is responsible for the synthesis of SA in chloroplasts of Arabidopsis plants, where it is involved in immunity. NHP, which is generated from lysine, works synergistically with SA to enhance immunity(Hillmer *et al.*, 2017; Hartmann and Zeier, 2018). Signalling through SA seems to sustain itself, which is an interesting phenomenon. Within the first hour after spraying, 1500 genes in Arabidopsis are up regulated in response to SA treatment. NPR1 and NPR3/4 control the majority of them. Many of the upregulated immunity-related genes encode pattern recognition receptors (PRRs) and nucleotide-binding domain and leucine-rich repeats (NLRs) 4, and in specific, over expression of NLRs can stimulate defence responses on its own, that illustrates how the loop enhances immunity (Thordal-Christensen, 2020).

Commonly, ETI and PTI trigger comparable responses, even though ETI is faster and stronger qualitatively, in addition to usually involving a type of localized cell death, indicated by a hypersensitive response (HR). PTI is normally effectual against non-adapted pathogens, generally referred to as non-host resistance, while ETI is effective against adapted pathogens

or host resistance (Dodds and Rathjen, 2010). When a signal is recognized via effectors or elicitors, it is transduced via the process of protein activation. Notably, a vital contribution is given by protein phosphorylation amidst an attack by pathogens. In that regard, the activation of two categories of protein kinases is conducted: CDPKs and MAPKs. In terms of location, MAPKs, which are signalling molecules, exist in all eukaryotes while CDPKs exist only in plants. In fact, the expression of the first group of genes that are responsible for protein signalling and regulation is activated by signalling cascades of CDPKs and MAPKs. Thereafter, the expression of the second group of genes which are responsible for regulating deterrence of attack by pathogens and defence proteins are activated by CDPKs and MAPKs as well (Meng and Zhang, 2013; Lal *et al.*, 2018).

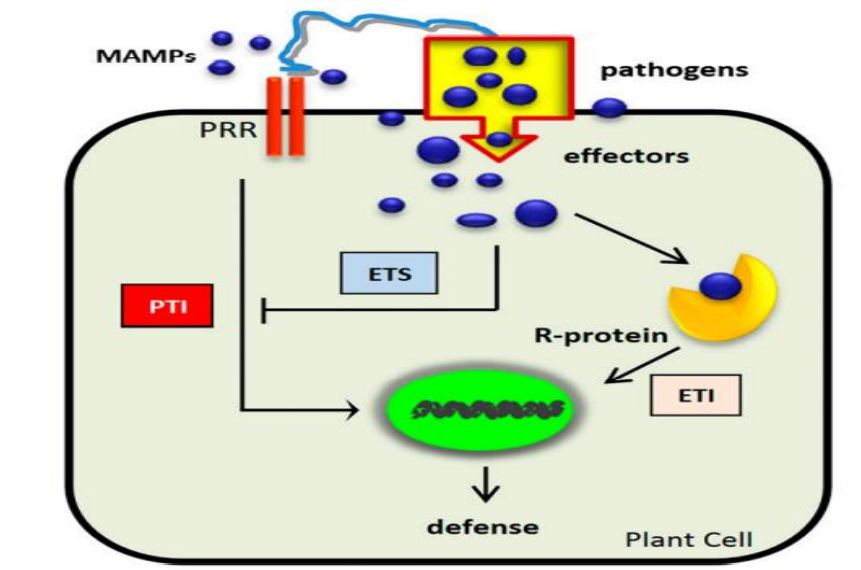

Figure 3 Plant defence mechanisms against pathogen attack (Kazan and Lyons, 2014).

1.4.5 Callose deposition and synthesis within the plant

The plant immune system initiates various defence mechanisms throughout fungal infection, including hypersensitive response (HR) for the swift collapse of attacked host. When the chemical and physical deterrents fail to avert fungal attacks in the plants' cells, the first line of defence of the plant is to form a structure at the infection spot, referred to as papillae or cell wall apposition (CWA) in order to delay fungal pathogen development in the cells. Formation of papillae in pathogen detection sites is believed to be performing the role of a physical obstacle so as to reduce pathogen entry to inside plant tissues. The difference in structure of the papillae and cell wall is that the papillae contain chemical barriers whilst the normal cell wall contains pectin and cellulose. However, the specific biochemical components of papillae seem to be somewhat different among plant species with categories of compounds

commonly found inside papillae being many (e.g., Proteins-peroxidases, enzyme inhibitors-callose, ROS (H_2O_2), silicon, phytoalexins, and phenolics (Hardham *et al.*, 2007; Voigt, 2014).

At the beginning, callose was recognized to be a crucial constituent within the composition of the plant cell wall during stages of plant development and growth. Additionally, callose has a significant role in the process of pollen formation. There is the possibility for Callose to be generated inside Plasmodesmata (PD) for controlling molecules' movements (cell-to-cell) via governing the size exclusion limit (SEL) of PD (Chen and Kim, 2009). Moreover, the local formation of callose is triggered via the following: physiological stress, wounding, and pathogen infection (Verma and Hong, 2001). Callose, which is β-1,3-glucan , is a plant polysaccharide found in plants that is made of glucose residues joined via β-1,3-glycosidic bonds, and exists inside cell walls of many plants (Chen and Kim, 2009; Piršelová and Matušíková, 2013). Callose synthase genes are involved in biotic stress and plant growth. Their production is because of the glucan synthase-like gene (GSL). Studies have been conducted on *AtGsl5* (glucan synthase-like 5), an Arabidopsis gene, showing that it encodes a protein that is associated with plasma membrane and is homologous to the catalytic component (β-1,3-glucan synthase) of fungus. It was found that β-1,3-glucan synthase activity along with *AtGsl5* mRNA levels are increased during pollen development and are present in floral tissues (Østergaard *et al.*, 2002).

In a study, wheat was selected for analysis since very little is known regarding its GSL gene regulation and callose synthase activity, in spite of having high agricultural significance. The expression of GSL was found in different tissues during plant development. Findings from this study demonstrate GSL genes being expressed differently in organs (leaves, spikes, and pollen) of wheat. Additionally, *TaGSL2*, *TaGSL3*, *TaGSL8*, *TaGSL10*, *TaGSL12*, *TaGSL19*, *TaGSL22*, and *TaGSL23* were investigated and found to be involved in plant development and growth (Voigt *et al.*, 2006). In another finding, *TaGSL8* and *TaGSL3* genes were shown to be engaged in resistance to the fungal pathogen named, *Fusarium graminearum* (Rana *et al.*, 2014).

1.4.6 Production of phytohormones during stress

This section will emphasis on the importance of phytohormones in salt tolerance within plants. When discussing biotic and/or abiotic stress, phytohormone responses are significant (Janda *et al.*, 2014). In terms of stress responses, ABA, a type of phytohormone, has been thought to have a substantial role (Sharma *et al.*, 2005). In addition, it has an

important role throughout numerous phases in the life cycle of plants. This includes dormancy, development of seeds, and mediating responses of plants towards different stresses of the environment (Eyidogan *et al.*, 2012). ABA is usually considered the stress hormone since it behaves as the main internal signal facilitating plants to survive in unfavourable environmental conditions (Keskin *et al.*, 2010).

During drought periods, ABA plays a vital function for managing the water status of plants via guard cells. In addition, it plays a critical function in induction of genes which regulate enzymes involved in cellular dehydration tolerance (Zhang *et al.*, 2006). Similarly, during conditions of salt stress in maize, research available has revealed that ABA accumulation assists in maintaining a high Na^+ homeostasis and cytosolic K^+ plus a more improved water status (Zongshuai *et al.*, 2017). Its mediated signalling also regulates the expression of drought and salt responsive genes when in conditions of salinity or drought (Narusaka *et al.*, 2003).

1.4.7 Salicylic acid (SA)

SA makes important contributions to defence of plants against pathogens (Janda *et al.*, 2014). Moreover, there exists substantial research nowadays suggesting that it is additionally engaged in responding to many abiotic stresses (e.g., salt stress, temperature, drought, and ozone) (Khan *et al.*, 2015). Other research observed that salt stressed maize, when treated with SA, resulted in stimulation of its salt tolerance by speeding up its carbohydrate metabolism and photosynthesis performance (Khodary, 2004). As mentioned, when with SA, salt tolerance is altered which is attributed to upregulation of antioxidative systems, and the build-up of compatible solutes-for example, glycine betaine and proline (Palma *et al.*, 2009; Nazar *et al.*, 2011).

SA, also recognised as 2-hydroxybenzoic acid, is among the many phenolic compounds (compounds having a benzene ring which hold a minimum of one hydroxyl group) which plants synthesize. SA and its derivatives, for example methyl salicylate (MeSA), are engaged in local and SAR (Vlot *et al.*, 2009). It gets generated inside plants through 2 varying routes: The first is the phenylalanine ammonia lyase pathway (PAL) with the second pathway being the isochorismate pathway (IC). The location for the pathway is inside the cytoplasm where chorismate-derived phenylalanine (Phe) is converted into *trans*-cinnamic acid (*t*-CA) via phenylalanine ammonia lyase (PAL). Thereafter, *t*-CA is oxidized to become benzoic acid (BA). SA is formed through the hydroxylation of the BA's aromatic ring which is catalysed by benzoic-acid-2-hydroxylase (BA2H) (Lee *et al.*, 1995). The second

route is within the chloroplast where SA is produced from chorismate by isochorismate in a 2-phased reaction: Firstly, chorismic acid is catalysed by isochorismate synthase (ICS), forming isochorismic acid. Secondly, isochorismic acid is catalysed by isochorismate pyruvate lyase (IPL), forming SA as shown in Figure 4. Most importantly, the second route has been found to be critical in defence of plants against pathogens (Wildermuth *et al.*, 2001; Vlot *et al.*, 2009).

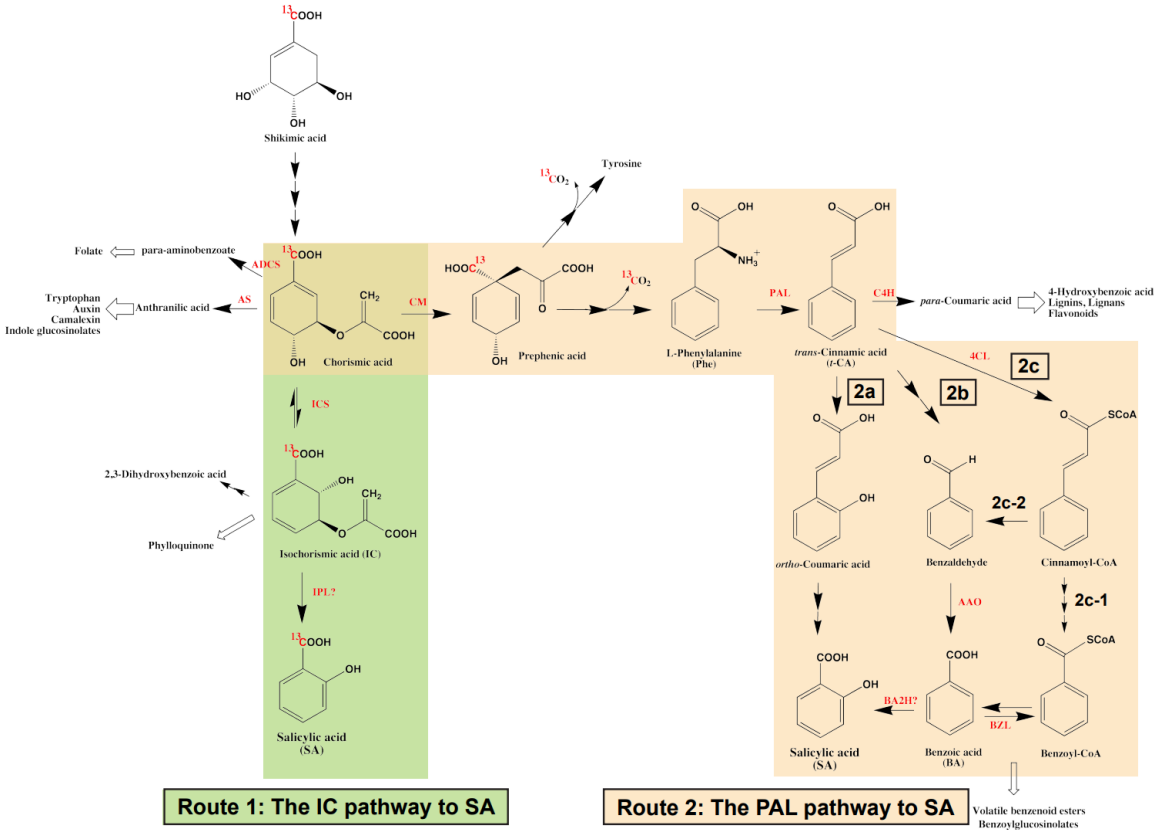

Figure 4 SA is synthesised via two different pathways (IC pathway and PAL pathway) (Dempsey *et al.*, 2011).

1.4.8 Jasmonic acid (JA)

JA are crucial cellular regulators which are engaged in varied processes relating to plant growth (e.g., flowering, gum formation, primary root growth, germination of seeds, callus growth, senescence, and bulb)(Fahad *et al.*, 2015). JA is understood to initiate the plants' defensive responses against pathogens (Mei *et al.*, 2006). Furthermore, JA has a role in abiotic stress (e.g., freezing, ozone, light, salinity, drought, and heavy metals) (Ali and Baek, 2020). For instance, within the roots and leaves of rice, when under salt stress, JA rises in response to higher salinity which leads to induction of JA biosynthetic genes (Tani *et al.*, 2008). However, it must be noted that the rise in JA induced by stress was merely investigated

for vegetative tissues. There needs to be further exploration on whether saline conditions boost JA within reproductive organs. Particularly, it is important to note that the JA concentration within a salt-tolerant rice cultivar was much more, compared to a cultivar which is sensitive to salt (Kang *et al.*, 2005). Another case was of a stressed sweet potato where transcript profile analysis showed that throughout salt stress, the JA levels rose substantially to adjust to the impact of salt stress (Zhang *et al.*, 2017).

JA is an organic compound existing in many plants such as jasmine. This hormone is a member of the jasmonate category of plant hormones. JA with its derivatives (e.g., methyl jasmonate (MeJA) and methyl esters) are involved in the regulation of plants. They participate in the following functions: Root elongation, response of plants towards wounds and abiotic stresses, defending against pathogens and insects, ripening of fruits, and viable pollen formation (Turner *et al.*, 2002). Biochemical stages preceding JA biosynthesis in plants are divided into peroxisomes and plastids, which are organelles (Figure 5). JA biosynthesis is initiated in the two mentioned organelles. The phospholipase PLA enzyme within the cell membrane releases galacto-lipids which in turn produces —α-linolenic acid. —α-linolenic acid is responsible for the secretion of JA in plastids. Firstly, 1,3-lipoxygenase (LOX) enzyme oxygenates —α-linolenic acid which will produce peroxide. Secondly, allene oxide cyclase (AOC) converts peroxide into allene oxide (2^{nd} conversion). Thirdly, allene oxide synthase (AOS) converts (3^{rd} conversion) allene oxide into oxo-phytodienoic acid (OPDA). By way of the ATP-binding cassette (ABC) transporter, oxo-phytodienoic acid (OPDA) is transported to the peroxisomes from the plastids. By means of β-oxidation stages and reduction, OPDA gets converted into (+)-7-iso-jasmonic acid (+)-7-iso-JA) in peroxisome. Isoleucine is coupled together with (+)-7-iso-JA to produce jasmonyl isoleucine (JA-Ile) (physiologically active form of JA) within cytosol (Wasternack, 2015).

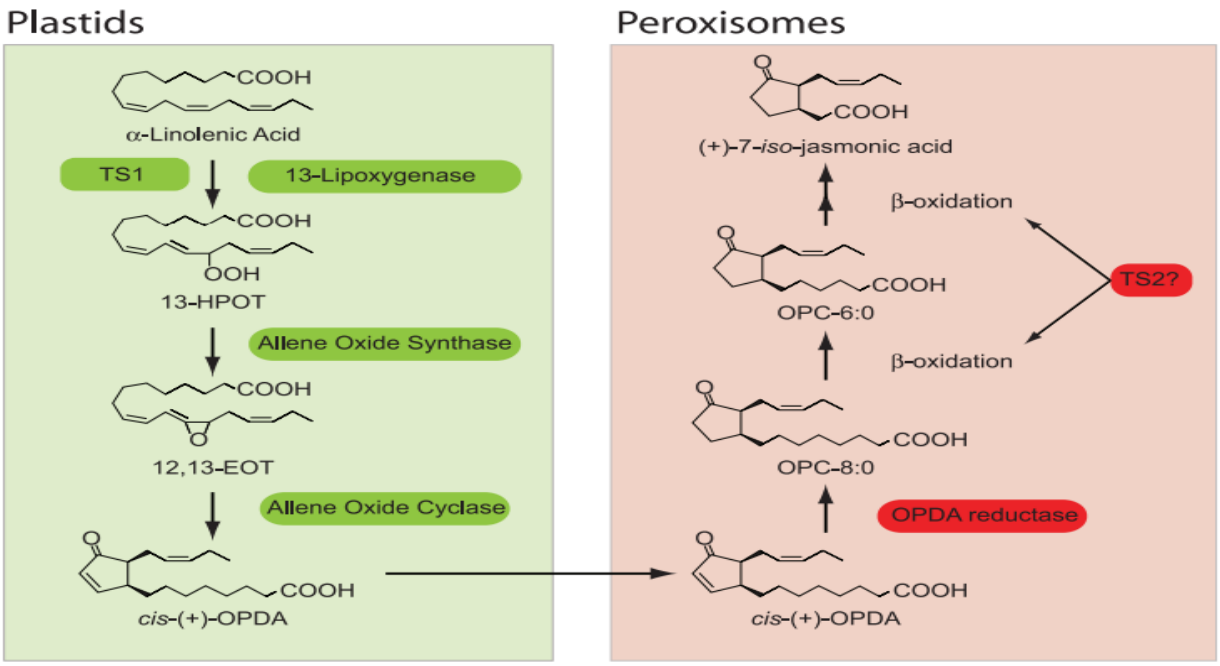

Figure 5 Initiation of the JA pathway (Acosta *et al.*, 2009)

1.4.9 Cross talk between plant hormones

JA, SA, ABA, and ET are phytohormones; low-molecular-weight molecules that are endogenous, mainly regulating plants' protective responses against abiotic/biotic stresses. This is done by antagonistic and synergistic activities denoted by signalling crosstalk (Fujita *et al.*, 2006). ABA (i.e., universal plant stress hormone) regulates abiotic stress responses through stimulating the expression of abiotic stress-related genes, acting as the main internal signal, supporting crops to survive under hostile environmental situations (e.g., drought, salt, and cold, salt, and stress) (Keskin *et al.*, 2010). In contrast, JA, SA, and ET have key roles in signalling pathways countering biotic stress. Actually, SA-mediated resistance is active against biotrophic pathogens as has been noted, while ET-mediated responses or JA are largely involved in countering herbivorous insects and necrotrophic pathogens (Spoel and Dong, 2008).

Furthermore, the aforementioned phytohormones are core actors to regulate signalling pathways that could crosstalk, either negatively or positively, resulting in antagonistic or synergistic responses, respectively (Koornneef and Pieterse, 2008). One instance of hormone crosstalk is when ABA suppresses expression of genes engaged in basal resistance against pathogens and SA accumulation (Yasuda *et al.*, 2008). In a different study, it was seen that NaCl treatment suppressed the initiation of SAR via activating ABA-mediated signal transduction, hampering signal transduction down/up-stream of SA (Hofmann, 2008). An investigation demonstrated that SA-mediated defences, which are stimulated when infected by

a biotrophic pathogen (*Pseudomonas syringae*), affected tissues to become more vulnerable to inoculation by the necrotrophic pathogen (*Alternaria brassicicola*) through inhibiting the JA-signalling pathway. Likewise, infection caused via *Hyaloperonospora parasitica* (biotrophic pathogen) firmly inhibited JA-mediated defences (Koornneef and Pieterse, 2008).

1.5 The Role of Transcription Factors of WRKY Genes under Stress

In order to survive under numerous adverse environmental stresses, a broad array of specific approaches is established by plants via molecular/cellular alterations and physical adaption (Ahuja *et al.*, 2010). DNA-binding TFs which control gene expression as a response to developmental change and environmental variation, which is a prominent regulatory plant process (Buscaill and Rivas, 2014).

WRKY transcription factors are considered to be significant constituents of a plant signalling that regulate numerous plant responses to abiotic/biotic stimuli. In addition, WRKY transcription factors do this in response to signals that direct growth processes. In wheat genome, Ye *et al.* (2021) found that 124 WRKY genes present in the genome and revealed that 294 copies of the WRKY were homologous gene. However, a further study has revealed that 116 WRKYs exist in bread wheat, describing 13 of the WRKY as stimulated by senescence (Zhang *et al.*, 2016). Moreover, Ning *et al.* (2017) conducted research which identified 171 WRKY genes existing in bread wheat. The structure of WRKY comprises 60 amino acids, which include WRKYGQK (the highly conserved amino acid sequence) at the N-terminus. Subsequently, there is a C_2H_2 ($C–X_{4–5}–C–X_{22–23}–H–X–H$) or C_2HC ($C–X_7–C–X_{23}–H–X–C$) zinc-finger structure at the location of the C-terminus. The WRKY family members govern gene expression by specifically attaching to TTGACC/T (W-box), that is recognised as a cis-element within promoter section belonging to the desired gene (Eulgem *et al.*, 2000; Bakshi and Oelmüller, 2014b; Meng *et al.*, 2016; Bai *et al.*, 2018b).

According to the type of zinc-finger-like motif and the number of WRKY domains, WRKY proteins are grouped into three categories. The proteins which have 2-WRKY domains are known as group I, whilst the majority of proteins that have 1-WRKY domain, are in group II or III. Specifically, members of group I and II contain the following finger motif: $C_2–H_2$ ($C–X_{4–5}–C–X_{22–23}–H–X–H$), while members of group II are additionally divided into 5 distinctive subgroups (IIa–e) according to 10 extra conserved motifs. In place of a $C_2–H_2$ pattern, group III have a $C_2–HC$ finger motif ($C–X_7–C–X_{23}–H–X_1–C$) (Eulgem *et al.*, 2000; Li *et al.*, 2010a).

WRKYs are parts of a complicated hormone signalling network. They have the ability to up/downstream of hormones, participate in antagonistic roles of SA, JA, and ET, and

regulate growth processes by brassinosteroids, cytokinins, and auxins. JA/ET signalling usually corresponds with response to necrotrophic pathogens whilst the SA signalling pathway is engaged with response to biotrophic and hemibiotrophic pathogens (Bakshi and Oelmüller, 2014b).

There are many examples for the involvement of WRKY TFs during biotic/abiotic stresses. An instance of WRKY involvement during abiotic stresses, rice can be mentioned, as the induction of the *OsWRKY 71* gene is stimulated by cold-related stress (Kim *et al.*, 2016). As a further example, participation of WRKY factors in the process of plant salt adaptation was demonstrated by WRKY33 and WRKY25, elevating ABA sensitivity and salt tolerance, once over-expressed within *Arabidopsis thaliana* (Jiang and Deyholos, 2009). Moreover, studying over-expression of *TaWRKY44* and *TaWRKY10* in tobacco has revealed that these genes enhanced drought and salt tolerance via controlling osmotic balance and ROS scavenging (Wang *et al.*, 2013a; Wang *et al.*, 2015). Similarly, over-expression of *TaWRKY19* and *TaWRKY2* within *Arabidopsis,* also improved tolerance to drought and salt stress (Niu *et al.*, 2012).

As mentioned, WRKYs also participate and have roles during biotic stresses. In fact, studies have shown that WRKYs have prominent roles in the defence responses of plants against invasions via numerous pathogens. For instance, in pepper (*Capsicum annum*) there is a WRKY protein named *CaWRKY27* which positively regulates the response against *Ralstonia solanacearum* infection by modulating SA, JA and ET signalling pathways in tobacco (*Nicotiana tabacum*) (Dang *et al.*, 2014). Another illustration is when 16 WRKY genes (57%) changed their expression levels in *Vitis vinifera* (grape), following infection by the fungal pathogen named *Coniothyrium diplodiella*, which lead to create white rot in grapes (Zhang, 2014).

Additionally, in terms of biotic stress in wheat, when affected by wheat leaf rust, the following genes *TaWRKY10*, *TaWRKY15*, *TaWRKY17*, and *TaWRKY56* differentially change their expression, suggesting that they may be good candidates for the purpose of molecular breeding programmes (Satapathy *et al.*, 2014). Moreover, in terms of wheat confronting a combination of biotic and abiotic stresses-for instance, high degree of heat plus a fungus (i.e., a wheat high-temperature seeding plant (HTSP) resistance and *Puccinia striiformis* f. sp. *tritici* (Pst) that cause wheat to have stripe rust), *TaWRKY70* was involved in enhancing tolerance, activating ET and SA signalling and also being involved in hypersensitive cell death. In fact, high temperatures induce defence mechanisms in wheat against *Puccinia striiformis* f. sp. *tritici* (Pst) (Wang *et al.*, 2017a).

It must be remembered that WRKY genes, which facilitate tolerance or resistance against many stresses, can be very beneficial for purposes such as breeding. Despite this, these genes are not always involved in enhancing stress tolerance. They can also have negative impacts as well. For instance, they could affect abiotic and biotic stress tolerance negatively because complicated signalling between signalling networks has the potential to impose antagonistic and synergistic influences on the control of plant responses against various stresses (Bai *et al.*, 2018b).

1.6 The Interaction between Abiotic and Biotic Stress

Plants subjected to abiotic stress can be under threat from not only one abiotic stress, but simultaneously to multiple stresses, leading to reduction in productivity and yield (Mittler, 2006). In fact, these crops are frequently facing various kinds of combined biotic and abiotic stresses which lead to changes in plant metabolism that affect both performance and growth (Rejeb *et al.*, 2014)

The response against stress which plants have is a unique signalling pathway dependent upon the kind of stress (Kissoudis *et al.*, 2014). A complex response occurs in plants exposed to concurrent biotic and abiotic stresses. It involves expression of a series of genes which trigger many signalling pathways which usually interact in antagonistic or synergistic ways (Asselbergh *et al.*, 2008). Growing proof exists for supporting the idea of crosstalk amongst signalling pathways and molecules (e.g., Many transcription factors, Ca^{2+} regulated proteins, calcium Ca^{2+}signalling, MAP kinases). These interactions create regulatory and signalling networks resulting in numerous responses, facilitating the ability of plants to adjust and adapt to hostile environmental circumstances (Fujita *et al.*, 2006).

The majority of research in this area has investigated the response of plants to individual stress factors, while far fewer studies have been carried out to investigate the plant response to multiple stress factors. Numerous factors exist showing the extent of plant vulnerability when they are threatened by many kinds of stress (e.g., development stages, duration of stress, plant species, and severity of stress) (Kissoudis *et al.*, 2014). Experiments which explored the outcomes of abiotic stress simultaneously with impacts of herbivore or pathogen damage show negative and positive interactions, according to intensity, nature, and duration of every stress. As an illustration, drought and high temperature can instigate a negative interaction in that the plant becomes more susceptible to biotic invaders (e.g., nematodes, pathogens, bacteria, fungi, and virus) (Atkinson and Urwin, 2012). However, a positive interaction between pathogen attack and abiotic stress has been described as well. In one case, salt-induced osmotic stress improves resistance of barley against powdery mildew

via enhancing of antioxidant activity (Wiese *et al.*, 2004). Furthermore, there are examples whereby a plant exposed to one form of stress becomes more tolerant to a series of other stresses; this is known as cross tolerance (Pastori and Foyer, 2002; Shah *et al.*, 2012)

1.6.1 The interaction of salinity and fungi on plant development

As stated above, not only has most research focused on the plant response to either abiotic or biotic stress, but usually only to a single stressor. Much less attention has been placed on the plant exposed to abiotic and biotic factors combined. In that regard, investigating the impact of fungal disease and salt stress on crops becomes critical. When these stress factors are combined, the impact could be very detrimental for the plant (DiLeo *et al.*, 2010; Kissoudis *et al.*, 2014). As mentioned, scholarships illuminating in plant interactions between biotic and abiotic stress responses are not many with most of them reaching the conclusion that negative impacts arise from abiotic stress (e.g., salinity stress and drought) on pathogen resistance (Suzuki *et al.*, 2014). Nevertheless, positive impacts have been observed particularly in relation to resistance against foliar pathogens (Kissoudis *et al.*, 2014).

A study conducted more than two decades ago found that the severity of maize susceptibility towards *Ustilago maydis* (smut disease) under various concentrations of salt stress diminished when salt stress rose to 9 dSm^{-1} (Souman and Kostandi, 1998). In another study related to plants under salt stress, it was found that fungal infection was repressed or heightened based on the degree of salt stress; at low concentrations (50 mM NaCl), the salt stress repressed the tomato's defence performance towards *Oidium neolycopersici* (causal agent of powdery mildew), but high concentrations of salt (150 mM NaCl) enhanced resistance towards the same pathogen (Kissoudis *et al.*, 2016). However, low/medium levels of NaCl could increase susceptibility of plants to fungal stress. For instance, the tomato crop showed heightened susceptibility to powdery mildew when under conditions of mild stress (50 or 100 mM NaCl) (Kissoudis *et al.*, 2016).

Currently there is very little scientific understanding of the changes in wheat response mechanisms, its growth and development, and biochemical changes during exposure to salinity stress and pathogenesis by *B. sorokiniana*, either when applied individually or simultaneously.

1.6 The Rationale of Research

The rational of the present study is to identify candidate WRKY genes that can be used as gene markers for salt stress in wheat. The study also seeks to provide more evidence

as to the use of proline and MDA as biochemical markers for salt tolerance. Selection of appropriate salt tolerance markers in wheat has the potential to aid wheat-breeding programmes so as to increase crop yield and productivity for this strategically important crop. In addition, Understanding the wheat-*B. sorokiniana* interaction is key to improving defence mechanisms deployed by wheat to disease.

1.7 Aim of the Current Study

The novelty and aim of the current study are to better understand the molecular and biochemical responses in wheat to the pathological effects of *B. sorokiniana* under salt stress. To achieve this over-arching aim, experiments were conducted to better understand the mechanisms of the wheat response to salinity stress and pathogenicity of *B. sorokiniana*, solely as well as simultaneously, on growth, physiological features, and biochemical attributes. The focus is to identify genes involved in the stress response to inform future wheat breeding programmes.

1.8 Objectives

Specific objectives of the current study are:

1. Assessing the physiological, biochemical, and molecular responses of spring wheat (cultivars: Alderon, Cochise, Najran and Sama) to salt stress (Chapter 3).
2. Understanding defence mechanisms at the biochemical and molecular levels in spring wheat (cultivars: Alderon, Cochise, Najran, and Sama) in response to infection by *B. sorokiniana* at seedling stage of plant growth (Chapter 4).
3- Understanding the level of complexity in the response observed in wheat against the combination of *B. sorokiniana* infection together with salt stress, with a focus on gene regulation, oxidative stress, and phytohormone changes (Chapter 5).
4. Conducting a bioinformatics analysis in wheat of WRKY genes and genes that are related to hormone biosynthesis and signalling, focusing on their potential roles in plant defence mechanisms (Chapter 6).

Chapter 2. Material and Methods

2.1 Plant Material

As to test response to salinity, two Saudi spring wheat *(Triticum aestivum*) varieties (Najran and Sama) were taken from Milling Company3–Kingdom of Saudi Arabia, Khamis Mushait Branch. The UK spring wheat (*Triticum aestivum)* varieties (Alderon and Cochise) obtained from the KWS UK were assessed. The justification for selecting wheat depends upon drought/salt stress (abiotic stress) and pathogenic resistance (biotic stress). It was proposed that Alderon and Cochise be used as varieties which is resistant against wheat diseases like *Septoria tritici*, Mildew, Brown rust, and Yellow Rust, in addition to which is a via (AHDB, 2020-21) . Additionally, the response of Saudi spring wheat cultivars (Almutairi, 2022)to environmental stresses were experimented. Najran was found to show tolerance to salt stress (Ahamed *et al.*, 2017) whereas Sama showed to its sensitivity to salt stress (Al-Otayk, 2020), sensitive to heat(Al-Otayk, 2010) and sensitive to drought (El-Nakhlawy *et al.*, 2015)

2.1.1 Sterilisation of seeds and Germination

This process has many stages as follows: Firstly, using ethanol (70%), 50 dry seeds are sterilised. Secondly, they are rinsed 3 times using water. Thirdly, the seeds are soaked inside distilled water for the duration of 15 minutes in the shaking condition. Fourthly, the distilled water is substituted with seeds imbibed via fresh distilled water inside a 50 ml cap Falcon tube for a period of 8 hours in room temperature. Fifthly, 30 similar sized seeds from the previous 50 dry seed batch are put onto 2 moist filter papers (Whatman) within petri dish plates and adding limited volume of distilled water on the seeds, sealed using Parafilm. Sixthly, the petri dishes are placed for the duration of 48-96 hours in a dark area at 25 °C.

2.1.2 Preparation of wheat plants for salt stress

2.1.3 Irrigation Medium for salt stress

The experiment was conducted to understand the wheat plant response to salt stress. Germinated seeds of wheats from four wheat genotypes noted above (Alderon, Cochise, Najran, Sama) of similar sizes were selected and transplanted into pots (9 cm size diameter =9 cm/height = 8.5 cm with silver sand). Before planting, sand culture had been sprayed with distilled water for moisturizing the surface of the sand. Similar sizes of three seedlings of wheat were chosen and planted into the pots at closely identical distances. After the first leaf of the 3 seedlings had expanded, two of the seedlings were taken out and one that is fully expanded was left in sand. The reason for utilising sand was removing plants and harvesting

the clean undamaged root material. For the purpose of preventing algae to grow on the surface of the sand, aluminium foil was used to cover every pot, whilst leaving a tiny hole in the middle for allowing emergence of shoot. A number of 6 pots had been placed in every tray with little plastic plates put underneath each of them for growth analysis and also 6 pots were used for biochemical and molecular studies. Each pot contains one individual wheat seedling for each wheat cultivar and the pot represented one biological replication. Using Hoagland solution (pH 6) of half strength, irrigation of seedlings is carried out (Hoagland and Arnon, 1950) every other day for the period of 2 weeks(Table 1). Thereafter, this was caried out with a solution which is full strength. Importantly, this investigation had been performed in a controlled growth room having the following requirements: 16:8 h day/ night length (photoperiod), 250-300 μmol m^{-2} s^{-1} illumination, and 25/20 °C day/night temperatures.

2.1.4 Irrigation and treatments for salt stress

In line with Hoagland and Arnon (1950), the modified Hoagland nutrition solution was made with Table 1 showing its composition. For the purpose of irrigating plants, in this experiment, 2 different Hoagland nutrition solutions were prepared. The first one contained numerous NaCl concentration levels (40, 80, 120,160 mM), whereas the second which was intended as a control solution, had 0 mM of NaCl (half strength and full-strength Hoagland solution) displayed in Table 1. Procedurally, the wheat seedling was first watered via the control solution (half strength Hoagland solution (pH 6) for the duration of 2-weeks every other day. Thereafter, the irrigation was altered to full strength until the emergence of the 3rd leaf of seedling wheat (Figure 6). After the 3rd leaf appeared, the plant underwent 2-treatments: irrigation by control treatment holding 0 mM NaCl in full Hoagland solution for the control plant, and irrigation by NaCl Hoagland solution for experimental plant. These plants were incrementally watered using saline Hoagland solution every two days (NaCl / irrigation (40, 80, 120, 160 mM NaCl) for reaching the ultimate concentration of salinity level (80 and 160 mM NaCl). The experimental and control wheat plants were watered every other day till the experiment was fully finalized. The overall duration of salt treatment (80 and 160 mM) for wheat plants was 21 days and, there was equal amount of water irrigation for the experimental and control plants (Figure 6). Importantly, for the purpose of growth analysis, seedlings were utilised as shown in Figure 6. Moreover, plants were collected and dried at 65°C in oven for the period of 48-72 hours. Then, the dried samples were put in storage (room temperature) intended for ion analysis, whereas the rest of the plants (fresh plants) were stored at -80 °C and later employed for molecular studies and biochemical analysis.

Table 1 Various chemicals employed for preparing Hoagland's solution

					Full strength Hoagland's solution		Half strength Hoagland's solution	
Compounds		**g/mol**	**g/400 ml stock**	**Stock concentration (M)**	**Volume of stock (ml) for 2.5 L Hoagland solution**	**Final concentration (M)**	**Volume of stock (ml) for 2.5 L Hoagland solution**	**Final concentration (M)**
Macronutrients	$Ca(NO_3)_{2.4}H_2O$	236.15	94.4	1M	12.5	5 mM	6.25	2.5 mM
	KNO_3	101.1032	40.4	1M	12.5	5 mM	6.25	2.5 mM
	KH_2PO_4	136.086	54.4	1M	5	2 mM	2.5	1.25 mM
	$MgSO_{4.7}H_2O$	246.48	98.6	1M	5	2 mM	2.5	1.25 mM
			g/200 ml stock					
Micronutrients	H_3BO_3	61.83	0.572	46.5 mM	2.5	46.5 uM	1.25	23.25 uM
	$MnCl_{2.4}H_2O$	197.92	0.362	9.15 mM	2.5	9.15 uM	1.25	4.57 uM
	$ZnSO_{4.7}H_2O$	287.6	0.044	0.765 mM	2.5	0.765 uM	1.25	0.382 uM
	$CuSO_{4.5}H_2O$	249.68	0.0102	0.204 mM	2.5	0.204 uM	1.25	0.102 uM
	$NaMoO_{4.2}H_2O$	241.95	0.024	0.5 mM	2.5	0.5 uM	1.25	0.25 uM
	FeEDTA				2.5		1.25	
	KOH	56.1056	11.22	1M				
	$EDTA._2H_2O$	292.2438	2.08	0.035 M				
	$FeSO_{4.7}H_2O$	278.01	1.56	0.028 M				

Notice: FeEDTA contains KOH dissolved in water with its water pH measured at 6. Later, $EDTA._2H_2O$ and $FeSO_{4.7}H_2O$ are added to the water.

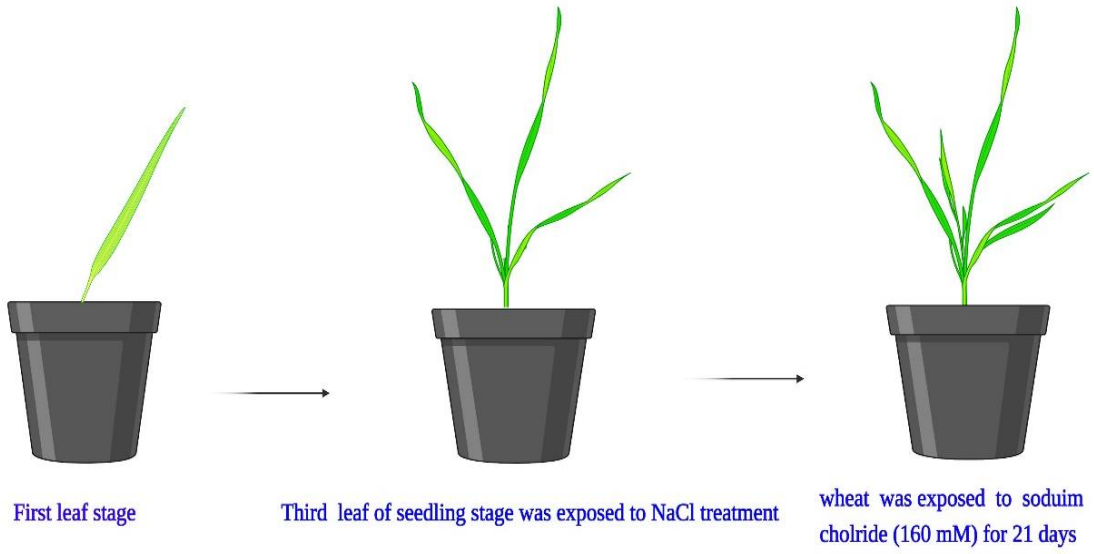

Figure 6 Irrigation of wheat plant with different levels of salt stress (done via BioRender)

2.1.5 Preparation of Bipolaris sorokiniana conidia

Bipolaris sorokiniana pure culture was obtained (from a colleague, a former Ph.D student Saddam), prior to planting the wheats. *Bipolaris sorokiniana* was then transferred into 2 dissimilar Fungal media within numerous petri dish plates. One medium is Corn Meal Agar (CMA) and the other is Potato dextrose agar (PDA). Both media have been dissolved in distilled water and later sterilized via autoclave. Prior to pouring both media inside petri dish plates, they were cooled down. Thereafter, 25ug/ml of antibiotic (chloramphenicol) was added in order to avert bacterial contamination. The *Bipolaris sorokiniana* fungal cultures have been incubated inside a dark place for the duration of a fourteen night at the temperature of 28 ℃. Afterwards, for preparing the inoculum, 5-10 ml of distilled water has been added to each petri dish plate. Subsequent to that, for scrapping the mycilia from the media, sterile forceps were utilised. This was conducted with the aim of releasing spores from the fungus. Later, 2-layers of cheese cloth (3.5 cm × 20 m) were used to filter these spores as to eradicate mycelial residues. A haemocytometer was employed for determining concentration of spore suspension. This was conducted through pipetting 200 µLof spore suspension into the channels of the haemocytometer and covering it using a cover slip. Next, the haemocytometer was placed into the microscope while having the following setting: 10× objective magnification for counting (Iftikhar *et al.*, 2008; Baba, 2019).

2.1.6 Preparation of wheat plants for Bipolaris sorokiniana pathogen and salt stress with spot blotch disease

Owing to complications associated with experiments overlaps, simultaneously measuring plant growth, biochemical changes, disease severity and gene expression for treatments was not feasible. Consequently, experiments were performed independently.

2.1.7 Preparation of wheat plants against Bipolaris sorokiniana pathogen

Two different experiments were conducted separately. The 1st experiment prepared the four wheat genotypes (Alderon, Cochise, Najran and Sama) for *B. sorokiniana* pathogen to measure disease severity and plant growth (shoot length and number of leaves).After the seeds were germinated and the seedlings were transported to the pots, these pots were watered via half-strength Hoagland solution with the frequency of 3 times/week up to the point where the 4th seedling leaf emerged in plant growth room: 16:8 h day, night length (photoperiod), 250-300 μmol m^{-2} s^{-1} illumination, and 25/20 °C day/night temperatures. *B. sorokiniana* was used to infect the leaves of seedlings in this phase using brush method as displayed at below (Figure 7). The features of the experiment were as follows: it was performed in a controlled growth room with the photoperiod regime being 16 hours light, 8 hours dark, the temperature was set 25/20 °C day/night temperatures. Sixteen plants of each wheat genotype (Alderon, Cochise, Najran and Sama) were used for disease severity measurements (third leaf) and 6 of them (6 for each genotype) were used for plant growth measurements such as leaf number and shoot length. In, addition, after determination the wheat variety resistance and sensitivity (Alderon and Sama, respectively) to spot blotch disease, time course experiments (0, 24, 48, 72 and 96-hours post inoculation) were conducted. Five pots (one plant/pot) for each wheat variety were used for MDA content (3rd leaf). Moreover, first leaf was used for phytohormones determination (SA and JA contents), and the second leaf was used for callose content and gene expression determinations. Samples were placed and stored at -80 °C.

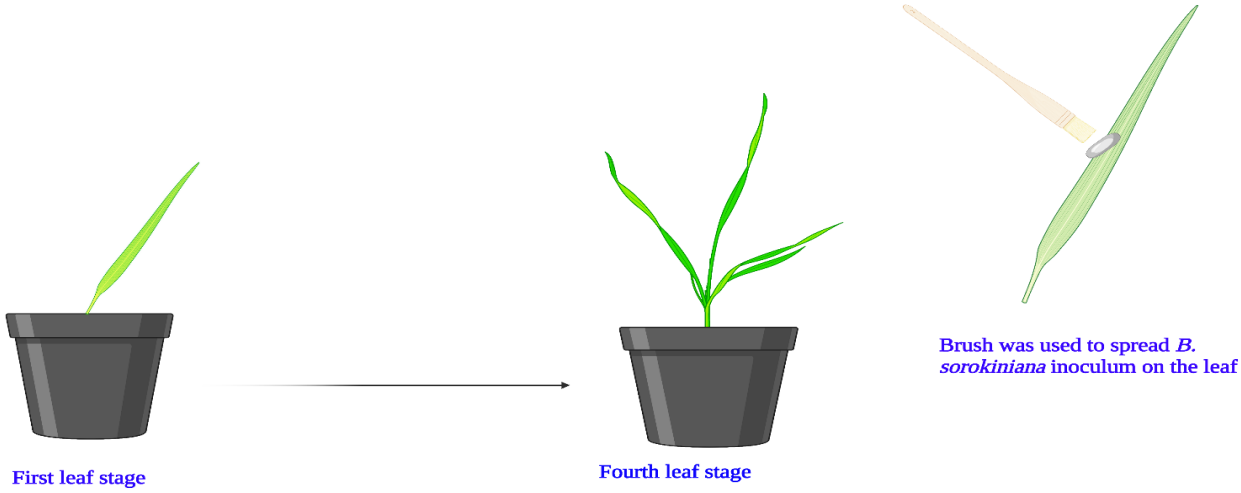

Figure 7 Wheat leaf infected by *Bipolaris Sorokiniana* by brush method (done via BioRender)

2.1.8 Preparation of wheat plants to salt stress with spot blotch disease in combination

The experiment was preparing the plant to salt and *B. sorokiniana* pathogen in combination. For these experiments, different wheat seed cultivars (Alderon resistant & susceptible Sama) which were identified for their resistance and vulnerability against spot blotch disease. In addition, Alderon exhibited a better growth than Sama under salt stress. These seed cultivars were germinated for the period of 48 hours in petri dishes. Subsequent to the germination, the wheat seedlings were moved to the pots. All these plants have been irrigated via half-strength Hoagland solution 3-times in each week up to the point that the 4^{th} seedling leaf emerged. Afterwards, wheat plants were exposed to salt stress via irrigation by Hoagland solution having 160 mM NaCl for the duration of eight days in glass house (12 h/12 day/night, minimum temperature was 15 ºC and a maximum one was 22-25 ºC, with an average of 18-20 ºC. light minimum value was around 60 µmol m^{-2} s^{-1} and the highest was 210 µmol m^{-2} s^{-1}) (Kissoudis *et al.*, 2016).During the next phase, plants were infected by *B. sorokiniana* using brush method (as described below) in control growth room 16 hours light, 8 hours dark, the temperature was set 25/20 °C day/night temperatures (Figure 8). During and subsequent to 4 days of infection, the plant growth, biochemical change, and molecular study were conducted. Ten plants (3^{red} leaf) for each genotype were used for disease severity and 6 plants of them for each wheat genotype were used for growth parameters shoot length and number of leaves). For ion content (Na^+ and K^+) 4 plants (3^{red} leaf) after 96 hpi were employed. Moreover, for time

course experiment, infected leaves of 5 plant of each wheat genotype were used SA, JA contents and 4 plants of them were used for MDA content Samples were placed and kept at -80°C

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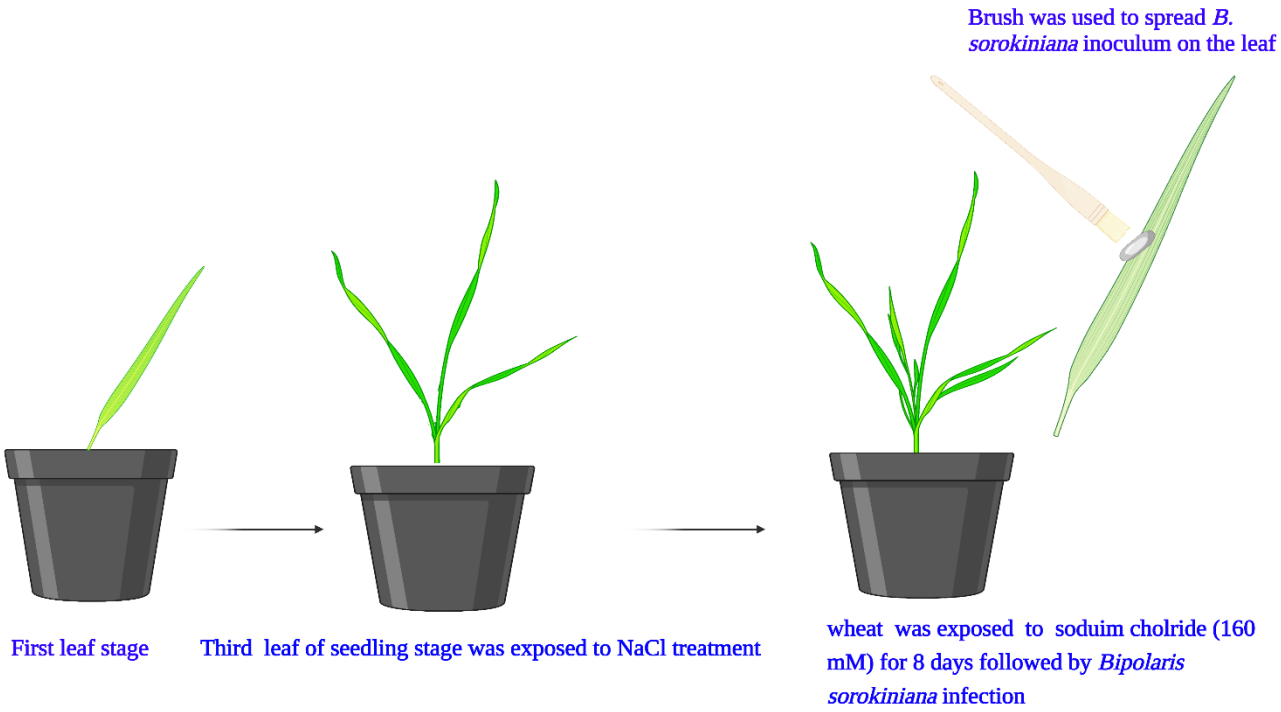

Figure 8 Wheat plants exposed to a combination of 160 mM NaCl and Bipolaris sorokiniana (done via BioRender)

2.1.9 Inoculation of wheat leaves with Bipolaris sorokiniana spores using brush method

As stated before, wheat genotypes have been planted and watered until the 4th leaf emerged for wheat plants against *B. sorokiniana* pathogen as shown in Figure 7 while wheat exposed to salt and pathogen in combination until 8 days of salt treatments as seen in Figure 8. Subsequently, the spore suspension was made with the spore concentration set to 3×10^4 spore ml^{-1} and soon after, 0.1% of Tween 20 was added as a surfactant. Leaves has been inoculated via brush soft bristle through spreading out the spores evenly onto the leaf to be made uniform and also, preventing run off. Every inoculated wheat plant has been put in a box and sprayed using distilled water. Afterwards, the boxes were covered by plastic bags for the period of 24 hours as to raise the moisture for effective *B. sorokiniana* infection. These plants have been grown for an extra period of 4-days-post-inoculation (dpi) as to observe the infection symptoms.(Renfro, 1955; Tu and Poysa, 1990; Baba, 2019)

2.2 Plant Analysis and Traits Measured

Firstly, wheat plants subjected to salt stress, the following were measured: leaf area, chlorophyll content, shoot length, and number of leaves, relative water content, dry shoot weight, and fresh shoot weight. In addition, proline and MDA contents were determined. Secondly, for evaluating the wheat response to pathogen (*B. sorokiniana*) the shoot length, leaf numbers, MDA content, callose content, SA and JA contents have been measured with the infected wheat plant and non-infected wheat plant. Thirdly, in order to evaluate the salt stress and pathogen in combination, the growth parameters were measured (e.g., shoot length and number of leaves) and MDA content, SA and JA content were quantified.

2.3 Salt Stress Experiments

2.3.1 Shoot length and number of leaves

In order to establish the shoot length (cm), the main shoot length beginning from the edge of the plastic pot until top of the main shoot has been measured. Because the sand might get compressed over time with the watering, measurement was not done from its top. Number of leaves were counted from treated and non-treated plants

2.3.2 Chlorophyll content

Using a SPAD meter (Opti-Sciences CCM-200), the chlorophyll content has been approximated at the 5^{th} and 4^{th} leaves in every plant. Subsequent to calibrating the empty clip, 3 random readings were taken at the middle, base, and tip of the leaf with the mean of the 3-readings considered as approximate chlorophyll content.

2.3.3 Leaf area (LA)

LA was measured on the same leaf as the chlorophyll content was quantified, for determining the leaf area. The leaf width (W) and length (L) have been measured. The calculation of this leaf area was done in line with Chanda and Singh (2002) seen in the equation below.

$$\mathrm{LA} = \mathrm{W} * \mathrm{L} * 0.75$$

2.3.4 Relative water content (RWC)

As to prepare relative water content (RWC), the 4^{th} leaf, which is the youngest and completely emerged, was chosen. For ascertaining that there is no change in humidity, every leaf was put in a plastic bag and then sealed. All of these samples were thereafter moved to the lab. In the laboratory, the fresh weight (FW) of samples was weighed and later, the whole leaf was cut to parts of 5 cm in length. Then immediately, all parts from one leaf were put into a clear glass vial

having distilled water. These leaf parts were floating in the distilled water for 3-4 hours at room temperature to achieve turgidity. This was followed by the overflowing water on the surface of leaf parts being absorbed by paper towel. The weight of all leaf parts was afterwards determined for finding turgidity weight (TW). All these leaf parts have been later put in an oven and dried at a temperature of 80 °C for duration of one night. Furthermore, all these parts have been weighed to get dry weight (DW). At the end, the RWC has been calculated via the equation below (Barrs and Weatherley, 1962; Smart and Bingham, 1974).

$$\mathrm{RWC}(\%) = \frac{\mathrm{FW} - \mathrm{DW}}{\mathrm{TW} - \mathrm{DW}} * 100$$

FW: sample fresh weight, DW= sample of dry weight, turgid weight

2.3.5 Ion content (Na^+ and K^+)

For measuring Na^+ and K^+ content, we used a microwave for digesting the plant materials. A mortar and pestle were used for the grinding of around 100- 500 mg dry wheat sample (shoots for salt stress, leaves for combined stress (spot blotch disease and 160 mM NaCl) until reaching fine powder condition. Thereafter, each sample was put inside a labelled vessel, with 10 ml of HNO_3 added later. This sample was mixed in the vessel and then, a cap, lid, and screw cap were used to close the vessel securely. After the samples were placed into all the vessels, they were put on a microwave carousal and later put inside a CEM Mars microwave. The CEM Mars microwave was adjusted as follows: heating done at 90 ºC for the duration of 10 min and subsequently, at 200 ºC temperature for a period of 15 min with a 25 minute cooling at the end (Huang *et al.*, 2004). Following this digestion procedure, every sample which has been digested is filtered via a Whatman filter paper into a 50 ml volumetric glass. Afterwards, deionized water has been added to the final volume. Subsequent to diluting all these samples, a flame photometer was utilised in order to measure the Na^+ and K^+ ions. The standard solution for Na^+ and K^+ comprises the following concentrations: 0, 5, 10, 20, 25 ug/ml. These were prepared from a stock solution of Na^+ and K^+. The stock solutions were prepared as follows: By dissolving 1.271g of dry NaCl (Sodium Chloride) inside distilled water until the whole becomes 500 ml, and, dissolving 1.74g of dry Potassium dihydrogen orthophosphate in distilled water until becoming 500 ml as well. The next step entails applying the working standards which recommend preparing volumes of 0.5, 0.10, 1.5, 2, 2.5 ml of Na^+ and K^+ stock solutions. Thereafter, for each volume of the stock solutions, 20 ml of HNO_3 is added along with distilled

water until reaching 100 ml of final volume (A colleague has been consulted for this procedure Fiona) (Munns *et al.*, 2010).

2.4 Pathogen Stress Alone and Salt plus Pathogen in Combination Experiments

2.4.1 Shoot length and number of leaves

As described in section 2.3.1

2.4.2 Disease severity (%)

The severity of the spot blotch disease has been measured according to a standard area diagram (SAD) (Domiciano *et al.*, 2014). In this diagram, severity of disease is graded according to disease symptoms which could be observed via the naked eye for the following periods: every 24, 48, 72, 96 hours after inoculation. At the 96-hour period, it was observed clearly that the chlorotic and necrotic lesions existed (Domiciano *et al.*, 2014). The SAD has been commonly used due to being its quick.

2.5 Biochemical Changes in Wheat under Stresses

2.5.1 Proline content

In order to measure the proline content, the following steps are taken: First, the samples are harvested, approximately 100 mg (3^{rd} leaf) of fresh leaves, placed in liquid nitrogen to be frozen, and stored in -80 ℃. Second, 100 mg of the frozen samples was grinded by mortar and pestle to reach fine powder with 3% of sulfosalicylic acid (5 μL/mg fresh weight) added afterwards. It is important to notice that the first and second stages mentioned are all conducted in ice condition. Additionally, , the tubes are now placed in centrifuges for 10 min at room temperature with a speed of 13,000 xg . 100 μLof upper phase was transported to a new centrifuge tube (2 ml) with 500 μL of extraction solution (acidic ninhydrin: 1.25 g ninhydrin (1,2,3-indantrionemonohydrate), 30 mL glacial acetic acid, and 20 mL of 6 orthophosphoric acid stored up to one week at 4 ℃) added later. Afterwards, every new tube was incubated for the duration of 60 minutes at the temperature of 96 ℃. Importantly, the lid of the microcentrifuge tube must be punctured via a needle for avoiding pressure which can open tubes in an ensuing reaction. Once the 60 min of incubation has passed, every sample is transported and put on ice for the termination of the reaction. Next, 1 mL of toluene has been added to the reaction mix, and the samples are vortexed for the duration of 20 secs. The tubes are then left on the bench for a period of 5 min at room temperature for facilitating the separation of the water and organic phases. Following 5 min, 1ml from the upper phase of the mixture is then transported to a quartz cuvette

for being put inside a spectrophotometer. Thereafter, the samples are measured for their absorbance at the wavelength of 520 nm via toluene as blank. The concentration of proline could be obtained by the help of a standard concentration curve and later attained on a fresh weight basis (typically stated in terms of micromole/ gram FW or microgram/gram FW). For the purpose of establishing standard curve, L- proline has been utilised with the following concentrations: 25, 50, 75, 100, 125, 175, 200 uM (Ábrahám *et al.*, 2010).

2.5.2 MDA content

Firstly, leaf tissue (0.5 to 0.1 g) is homogenised via mortar and pestle in liquid nitrogen until it becomes fine powder. Then, 0.5 ml of 0.1 % (w/v) TCA has been added and mixed by pestle. Thereafter, the homogenate is centrifuged for the duration of 10 minutes (15000xg, 4.0 ℃). That is when the supernatant is collected and then, 0.5 ml of it is mixed with 1.5ml 0,5% TBA diluted in 20% TCA. Next, the tubes are incubated in heat block for 25 min at 95℃. This reaction was then stopped through incubation in ice. After the incubation period, the samples' absorbance was measured in the following wavelengths: 532, 600 nm. At this point in time, the OD600 values are deducted from MDA-TBA complex values in the wavelength of 532 nm. Subsequently, the concentration of MDA was calculated via the Lambert-Beer law using the extinction coefficient ε M= 155 mM-1cm-1. The MDA content is displayed in terms of μmols MDA g-1 FW (Heath and Packer, 1968).

2.5.3 Callose content

Callose has been extracted from 50 mg of the leaf tissue. The leaf tissue has been soaked for a duration of 5 mins inside 1.2 ml of ethanol through shaking it in the 50 ℃ temperature. Subsequent to centrifuging the leaf tissue (5 min, 400×g, room temperature), the pellet was cleaned two times inside 600 μL of ethanol and centrifuged (5 min, 400×g) again. In order to solubilize callose, the cleaned pellet has been incubated for a 15 min period in 80 ℃ temperature inside 200 μl of 1 N NaOH within shaking conditions. A number of aliquots of 10 μL from the aforementioned solution were utilised for the purpose of determining callose content inside 96-well microtiter plates.

After extracting the callose, callose was determined via adding 100-200μLof aniline blue mix (This mixture has been made via mixing 59 volume of 1 M glycine/NaOH buffer-pH 9.5, 40 volume of 0.1% aniline blue, and 21 volume of 1 N HCl). 120 μL from the aforementioned mixture was put onto microplate with approximately 10-30 μL of extracted callose added later.

After that, the microplate was mixed and incubated in the temperature of 50 °C for duration of 30 min and later, in room temperature for a 30 min period in shaking conditions to facilitate decolorization of the aniline blue plus reaction with fluorochrome. The quantification of the callose content was accomplished by using fluorescence spectrophotometer (fluorescence plate reader) at the emission wavelength of 460 nm and excitation wavelength of 355nm. In order to generate standard curves, (1,3)-β-glucan from Euglena gracilis was used by the following procedure: Exact quantities of glucan were dissolved in 1 N NaOH through heating in 80 °C for 30 min. The obtained callose quantities have been stated as E. gracilis glucan-equivalents(Shedletzky *et al.*, 1997; Voigt *et al.*, 2006).

2.5.4 Phytohormone (SA and JA) contents

2.5.5 Sample preparation of wheat leaves

Two kinds of healthy plant leaves and those infected by *B. sorokiniana* were weighed approximately from 100 to 200 mg. Next, the leaf samples were grinded by mortar and pestle and mixed with liquid nitrogen. after the samples become fine powder, all of them are transferred to 2ml tubes and 400 µL of 10% methanol containing 1% acetic acid was added to them. All these tubes were then placed in tissuelyser for mixing. The settings of the TissueLyser were as follows: 2 min at 25 hz/s. all the tubes were then put in ice for a period of 30 min and centrifuged for the duration of 10 min at 13,000 g in the temperature of 4 °C. That is when the supernatant was transferred to new tubes where the pellets have been re-extracted using 400 µL of 10% methanol including 1% acetic acid. Subsequent to an extra 30 min of incubation in ice, this extract was also centrifuged with the supernatants collected and added to the same new tubes (Forcat *et al.*, 2008). After the extraction, 200 µL of the extraction was added to 2 ml clear glass vial and kept on cold condition to send to liquid chromatography tandem-mass spectrometry (LC-MS/MS).

2.5.6 Standard solution preparation for LC-MS/MS

High purity of abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA) were used to make calibration standards (> 98.5%). Every single calibration standard (10 mg) was dissolved in deionized water to prepare 400 µg/mL of stock solution. Dilution of specific volume of stock solutions with water in order to prepare mixture of working solutions of SA, JA, and ABA (from 0.031 to 10 µg/ mL). Standard solutions were made in following concentrations: 0, 0.01, 0.05, 0.1, 0.5, 1, 2.5, 5, 10, 25 and 50 uM. 200 µL was used as injected volume. All solutions were

placed and kept at at 4 ºC (A colleague has been consulted for this procedure Alex) (Alshegaihi, 2019).

2.5.7 LC-MS/MS conditions

LC-MS/MS technique for a highly sensitive detection of phytohormones was performed via Acquity UPLC coupled to Xevo TQ-S Triple Quadrupole Mass Spectrometer. The column was used an Acquity BEH-C_{18} (150 x 2.1 mm; Particle Size = 1.7 µm) at 40 ºC. The injected volume of each sample was 5 µL. The solvents that used were A (Water (0.1 % Formic Acid) and B (Methanol (0.1 % Formic Acid).The solvent gradient was utilised as follow : 5% B at time = 0, then hold for 0.5 min, this was increased to 20 % B at time = 1 min, 50 % B at 8 min, then 95 % B at 10 min. This was held for 5 min, before decreasing to 5 % B at 15.5 and holding for 6.5 min. The overall runtime was 22 min in total. the flow rate was 0.3 mL/min. Ionisation Source was electrospray ionisation (ESI). Compounds were detected using the following multiple reaction monitoring (MRM) fragmentations using both positive and negative ion mode: salicylic acid (negative ion mode): 137 > 93, jasmonic acid (negative ion mode): 209 > 59, abscisic acid (negative ion mode): 263 > 153, gibberellic acid (negative ion mode): 345 > 143 and 3-indole acetic acid (Positive Ion Mode): 176 > 130. Compounds were then quantified using the TargetLynx quantitation feature in MassLynx v4.1 software (A colleague has been consulted for this procedure Alex) (Alshegaihi, 2019).

2.6 Statistical Analysis

Data was showed as means ± SE. Through utilising analysis of variance (ANOVA) with subsequent Tukey test via SPSS software, means have been compared. Significant differences ($P<0.05$) amongst single means were found. All experiments were set up as Complete Randomised Design (CRD)

2.7 Genes Expression

2.7.1 RNA isolation and DNase treatment

The wheat leaf samples which have been stored in –80 ℃ and the leaves which have been freeze dried for 48 hours, were used for RNA extraction. For salt stress experiment, 100 mg sample of freeze-dried leaves has been transferred in a 2 ml microcentrifuge tube that has a screw cap. Then, the sample was grinded using a 0.2 mm diameter stainless steel at the frequency of 30 HZ/ s for 2 min utilising a TissueLyser II (QIAGEN). On the other hand, the frozen leaf sample (*B. sorokiniana* infection and salt with spot blotch disease experiments**)** of roughly 50 to

200 mg has been homogenised within a mortar and pestle in liquid nitrogen, until a fine powder is reached. This is later transported to a 2 ml microcentrifuge tube. A solution employed for lysing the leaf tissue (TRIzol Reagent (purelink) has been added (1ml for 100 mg of plant tissue) to the tube and left at room temperature for around 5-7 min so it is fully lysed prior to adding chloroform. 200 μL /1ml TRIzol of chloroform was then added to every extract sample and energetically shaken for a period of 15-25 secs. It was later left in room temperature for a 3 min period. This was performed to enable the process of phase separation and permit protein concentration in the layer solution's bottom. The solution mix was then spun by centrifuging for a duration of 15 min in a 4 ℃ temperature at 12000xg speed. Following this centrifugation, 3 layers of the solution were observed in the tube with the aqueous layer being transported to a 2 ml microcentrifuge tube. Next, the same volume taken of the aqueous layer was also taken from 70 % ethanol and mixed by using vortex during a 10 sec period. Afterwards, 700 μL of the mixture was transported to a spin-cartridge tube, spun via centrifuge for a 15 sec period in 25 ℃ and 12,000xg speed. Subsequently, the flow-through in the collection tube was disposed of and spin-cartridge reinserted within the same tube. At this time, 350 μL of wash buffer I has been added to the spin-cartridge and spun via centrifugation for a duration of 15 sec in 25 ℃ and 12,000xg speed. The obtained flow–through after the centrifugation process was also disposed of with the spin-cartridge reinserted in a fresh collection tube. Afterwards, 80 μL of DNase mixture (Purelink) is added onto the centre surface of the spin-cartridge and incubated for 15 min at 25 ℃ later. Subsequently, 350 μLof wash buffer I was added onto the spin cartridge with a 15 sec centrifugation and 12,000xg speed in 25 ℃following. After that, the flow-through has been removed with the spin cartridge reinserted in a fresh collection tube. 500 μL of wash buffer II was then added on the spin cartridge and spun using centrifugation for a 15 sec duration at 12,000xg speed in 25 ℃.

This time also, the flow–through was removed with the spin cartridge being placed inside the same collection tube as before. The aforementioned step has been repeated once again. Thereafter, the tube was spun via centrifugation for 2 min at 25 ℃ and 12,000xg speed in order for the spin cartridge to dry, all liquid to precipitate on the tube's bottom, and obtained RNA stuck onto the spin cartridge's surface. This was when the tube was disposed of with the spin cartridge placed inside a recovery tube. Finally, 50 μL RNase-FreeWater was placed onto the spin cartridge's central surface with the recovery tube incubation in 25 ℃ for 1 min following.

Then, the spin cartridge was centrifuged for a period of 2 min at 12,000xg speed in 25 ℃in order for RNA to be eluted from the spin cartridge's surface into the recovery tube. This process was carried out in accordance with the manufacturer's instructions (PureLink RNA Mini Kit, Life Technologies).

2.7.2 Quality assessment and quantification of RNA

Subsequent to RNA being fully extracted from the leaf sample, its quality/quantity has been measured by a NanoDrop spectrophotometer (ND-1000). Following the cleansing of NanoDrop spectrophotometer's pedestal using water and calibration using RNase-Free, extracted RNA (1 μL) was utilised for determining purity and concentration via the NanoDrop with results displayed simultaneously. The results show RNA concentration (ng/ μL) and additionally the purity revealed 2-various absorbances. The first ratio of the wavelength was 260/280, representing the absorbance of contamination such as phenol. The second ratio of the wavelength is 260/230 ratio which demonstrated that RNA is present within the sample and it indicate some contaminations such as polysaccharides, protein, salts (e.g, guanidine thiocyanate. RNA purity has to be in the close to 2.0 for the 260/280 ratio and 1.8 to 2.2 for 260/230. The sample has been divided to aliquots and kept in – 80 ℃ temperature (Thermo Fisher Scientific Inc, 2009; Koetsier and Cantor, 2019).

2.7.3 Synthesis of complementary DNA

Following the determination of RNA purity, the complementary DNA (cDNA) is synthesized according to SensiFASTTM cDNA Synthesis. On ice conditions, cDNA master mix was made with a maximum of 1 ug of RNA, 4 μL of 5x TransAmp Buffer, 1 μLof Reverse Transcriptase, and ultimately, RNase free water was added, so the final volume reaches 20 μLoverall. After making the mixture solution, it was mixed gently via pipetting up and down and spinning for few seconds. The PCR tube was placed, thereafter, in a thermal cycler with settings adjusted as follows: initially, 25 ℃ for 10 min; next, 42 ℃ for 15 min for reverse transcription; followed by 85 ℃ for 5min; lastly, 4 ℃ for hold. Finally, the cDNA was synthesised and measured by NanoDrop. The concentrated cDNA was diluted via RNase free water to obtain 100 ng/ μL which is recommend for usage in quantitative polymerase chain reaction (qPCR). This sample was kept at -20 ℃ (SensiFASTTM cDNA Synthesis Kit- from Scientific Laboratory Supplies Ltd (SLS).

2.7.4 Primer design

The selection of genes have been explained in chapter 3 and 4 while elongation factor 1 alpha in wheat (*TaEF1α*) have been used as reference gene (Metz *et al.*, 1992; Prasad *et al.*, 2020). The validation of *TaEF1α* has been tested under all stresses for gene expression (see Appendices). The primer sequence of *TaWRKY3*, *8*, *9*, *3*, *45*, *46* and *TaEF1* α genes have been obtained from previous colleagues (Alshegaihi, 2019; Baba, 2019) while *TaWRKY 53- a*, *TaWRKY 71*, *TaGSL2*, *3*, *8*, *10*, *12*,*19*, *22*, *23*, *TaPAL*, *TaNPR1*, *TaAOS* and *TaLOX2* were designed via primer3plus (Table 2). After searching for the mRNA nucleotide sequences of target genes from NCBI (National Center for Biotechnology Information), all primers were designed via Primer3Plus (http://primer3plus.com/) software. The sequence of the target gene was copied from NCBI and pasted in Primer3Plus software. Before selecting primers from the software, the general settings section on the software should be changed to the following: First, the product size should be ranging from 80-150 base pairs. Secondly, the primer length should be optimum from 18 to 23 base pairs. Thirdly, the primer melting temperature has to be from 55 ºC to 60 º C temperature. Finally, primers should have 45% to 55% of GC content. After adjusting the settings, primers were designed, selected, and then checked with NCBI for matching these primers to target genes. All primers must be checked using PCR Primer Stats (bioinformatics.org) (Chloe communication) for testing general properties of primer sequence and length, GC content and percentage, molecular weight, GC Clamp, melting temperature (Tm), Hairpin formation, and self- annealing (Chuang *et al.*, 2013; Hung and Weng, 2016). After verifying the primers, they were ordered from Sigma Life Science, either in dried powder or in solution (water or TE buffer).

Table 2 List of primers used in this study

Gene Names	Forward Primer (5'-3')	Reverse Primer (5'-3')	Gene size (bp)	Accession number
TaWRKY3	GTGCTGGACGACGGATACAA	TAGCTCCTGGGATGAAGGCT	79	EU665432
TaWRKY8	CCTACTTCCGGTGCTCCTTC	CGCCACGAGTATGGTCTTGT	83	DQ323885
TaWRKY19	TTGGCAACTTCAGTGCTGAC	ACCAGTGTGATGGCAAAG	90	EU665430
TaWRKY37	GCCAGAAGGCAGTTAAGGGT	CTTAACTGGACAGCTCGCCT	77	EU665452
TaWRKY44	GCAGTTCAGGGTGGAGAGTAT	CATATGCTGAGTGCTCCACTT	91	KR827395.1
TaWRKY45	CATGAGGAGCTTGGAGGACG	AGGCCTTTGAGTGCTTGGAG	80	EF397613
TaWRKY46	CGAGCACAACCAACCAACAG	GTGGACAGACACATCACCGT	72	EF368365
TaWRKY53-a	ATCGCCATGTCCTCCTTCAC	GCCAGACCCTGATAGAAGCTC	103	EF368357
TaWRKY71	CCGTAATCTCCAAGCGTTAC	GTTGTCCTTGGTCACCTTCTG	107	EF368356
TaGSL2	GCTCAGGTGATCAGGCCATT	GGACAGCAATGCAACTGGTG	120	DQ086483

TaGSL3	GCCCACATGACAGTCCTTGA	CATCTCGACGACAGGCTTGA	105	DQ086484
TaGSL8	GTGGCTCTGAACACCCAGTT	CCGTCAGGACACCTTCTTCC	94	DQ086485
TaGSL 10	CCAGCTCTTCGTGTTGCCTA	TGGGCCAGGCAGCTTTATAC	138	DQ086486
TaGSL 12	AGCGTGTGGTGAAGACTCTG	CGATGGGCGCAAAGATAACG	96	DQ086487
TaGSL 19	TGATCTCGCTGTGTCAGACG	AAACAGCGGTCCACACACTT	97	DQ086488
TaGSL 22	TGGGCTGGAGGAGTACATCA	AACAGGCCCAGCTGTATGAC	99	DQ086489
TaGSL23	CATTTCTTGCCCACCGGATG	CTCGGACAGATCCCCATAGC	91	DQ086490
TaLOX2	GAACCTGCCCTTCATCACCA	CGAGGGTGATGGTCTTGAGG	140	GQ166691
TaAOS	CGGAGAGAAGTTCCACCAGA	CTTGGTCAGCTCCATCTTCTC	106	KJ001800
TaPAL	ATCATGTCTTCCACGTTCCTG	TTCATGCTCAGGGTCTTCTTG	125	MT150275
TaNPR1	GAGCTTACTCAAGTGCGTGA	TCATCAGCGATTTCGGGAAG	101	KU736862
TaEF1 α	ACCTGAAGAAGGTCGGCTACAA	ATCTGGTCAAGCGCCTCAAG	139	M90077.2

2.7.5 Dilution of oligo primer

Once oligo primers were received from sigma, there was a procedure of primer preparation and dilution before using them in PCR and qPCR. Primers received were in dried powder form or in solution (water, TE buffer). Under sterilized conditions, the following transpired: Firstly, primers were centrifuged for 15 sec at 4 ºC at 12000xg to allow the dried powder to be at the bottom of the tube. Secondly, to make the stock solution concentration at 100 uM, RNase-free water was added and the quantity of RNase – free water varied with the number of nanomoles that were usually found on the instruction manual included with the order or the tube itself. Thirdly, the stock solution was vortexed and then, the primer stocks were kept at -20 ℃. Fourthly, all primer stock with 100 uM concentration had their concentration reduced to 10 uM. This was done by using working primer solution through adding 10 µL of primer stock solution to sterilised tube and then, 90 µLof RNase free water was added. Next, the solution was mixed together by pipetting. The last part of reducing the concentration was, the diluted primers were aliquoted into different tubes and kept at -20 ºC to avoid freeze thawing of the solution (Addgene, 2021).

2.7.6 Quantitative PCR (qPCR)

The qPCR experiment was performed using a reference gene as endogenous control, called elongation factor 1 alpha in wheat (*TaEF1α*) and a target gene. Three biological replicas from each condition (stressed plants or unstressed plants) were used and divided each to three technical replications. Importantly, SensiFAST SYBR® No-ROX Kit was used for preparing the qPCR master mix which is described in the Table 3

Table 3 Preparation of Mastermix reaction intended for analysis by qPCR

Reagent	Volume (µL)
2x SensiFAST SYBR® No-ROX Mix	10
10 uM forward primer	1
10 uM reverse primer	1
100 ng/ µL cDNA	1
RNase-Free-Water	7
Final volume	20

2.7.7 Gene expression's data analysis

As stated by Livak and Schmittgen (2001), an easy route for calculating relative fold gene expression level is the Delta-Delta Ct, which can be given as $2^{-\Delta\Delta Ct}$. Subsequent to the qPCR reaction, for finding gene expression, Ct values (cycle of threshold) of samples have been presented by normalization of every genes' (target gene) Ct value against the Ct value of wheat elongation factor 1 alpha-subunit (*TaEF1 α* (reference gene/house-keeping gene). The average of 3-technical replications (Ct-values) is deducted from the average of 3-technical replications of *TaEF1α* (Ct-values) from each biological replication condition (demonstrated in the next formula):

ΔCt=average Ct (target gene) - average Ct (*TaEF1 α*)

Thereafter, delta delta Ct (ΔΔCt) has been computed according to the untreated and treated groups as seen next:

ΔΔCt= ΔCt (treated samples) ΔCt (untreated samples)

Lastly, For graphic display of the relative transcription levels, log 2 scale is used (Caldana *et al.*, 2007). All figures for qPCR represent as $2^{-\Delta\Delta CT}$ however, the result description was explained as fold change

2.8 R Software

For data presentation of wheat genotypes and WRKY gene expression, R software was used to generate heatmap that represent visualization of data and dendrogram in. In addition, the data displayed as matrix (Holtz, 2018).

2.9 Phylogenetic Tree Analysis

In order to illustrate images of the phylogenetic tree for every WRKY protein, MEGA software has been employed. In fact, MEGA 7.0 was utilised as to draw the phylogenetic tree via

the neighbour-joining technique. The measurements within the tree have been computed with the assistance of the p-distance method (choosing 1000 bootstrap replications for testing support rate of every branch plus attaining the Bootstrap Value) (Saitou and Nei, 1987; Nei and Kumar, 2000; Kumar *et al.*, 2016).

2.10 Promoter Analysis

Plant care software was employed for recognition of regulation motifs in the promoter of the candidate genes (Lescot *et al.*, 2002). The quick progress in the field of bioinformatics has indeed enabled the search to identify amino acid and nucleotide sequences. Moreover, the NCBI data base was helpful for finding the sequence alignment, resulting in amino acid sequences to be found. Within the NCBI search engine, multiple keywords were inputted for finding the accession number of the target genes. Next, promoter regions of selected genes were determined via the DNA sequences (in FASTA format) with the help of Ensembl Plants data base (http://ensembl.gramene.org/Triticum_aestivum/Tools/Blast?db=core), which allowed a search to be conducted for identifying the selected genes against the *Triticum asetivum* wheat. The data obtained has been exported afterwards as 2000 bp upstream. Subsequently, the sequences were examined for locating putative regulatory elements.

The attained data and information were then stored in a text file. Deploying the Plant CARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), protein promoter regions have been found by submitting DNA sequences (including upstream/downstream sequences) (Martin communication).(Li *et al.*, 2020a).

Chapter 3. Morpho-Physiological, Biochemical and Molecular Response of Wheat Seedling to Salt Stress

3.1 Introduction

Soil salinity has been recognised as an important abiotic stress factor that adversely damages the agricultural environment. The affected regions by this stress constitute 20 % of the total cultivated land around the world. More specifically, the consequence of salinity comes to a 20 % reduction in yield. However, crops are also exposed to other abiotic stresses such as heavy metal, heat, and drought in addition to biotic stresses such as fungi and insects (Acosta-Motos *et al.*, 2017; Alkharabsheh *et al.*, 2021; Bakala *et al.*, 2021). The factors contributing to elevated soil salinity are complex and result in an ion imbalance in plants thus disturbing metabolic processes and inducing oxidative stress. The two main factors that disrupt numerous biochemical and physiological traits are considered to be osmotic and ionic stresses. These stresses affect plant physiology (water, chlorophyll, Na^+ and K^+ content) and reduce growth rate (leaf area, shoot length, number of leaves, biomass) (Acosta-Motos *et al.*, 2017). Therefore, the outcome is an overall reduction in crop productivity (Munns and Tester, 2008). For plants to adapt to salt stress, they respond via many molecular and biochemical processes. Notably, the end of the biochemical pathways providing products that enhance salt tolerance (Parida and Das, 2005). A known bio-chemical alteration inside plants which are under salt stress, has been producing compatible solutes (e.g., glycine betaine (GB), proline, polyols, and sugars). The function of the compounds is accommodating ionic equilibrium within cytoplasm and vacuoles of plant cell (Parida and Das, 2005). Another biochemical change which occurs in plants undergoing salt stress is the accumulation of malondialdehyde (MDA) through membrane lipid peroxidation. This is a useful marker to evaluate membrane damage and oxidative stress (Ashraf *et al.*, 2010).

Transcriptomics technologies such as integrating transcriptomics and genomics can be used to elucidate the molecular response to elevated salinity in plants. The variation of gene expression in response to salinity includes a broad range of ways which plants employ for upregulating/downregulating gene transcription. Gene expression regulations can be enhanced or suppressed by transcription factors (TFs) engaged in the production of mRNA and protein. This is used as a new method in plants to understand tolerance to salt stress (Arif *et al.*, 2020). Under all stress, TFs hold a significant function as to modulate and regulate various gene expression. These transcription factors regulate expression of genes via recognising and binding to *cis*-elements which are located within the promoter section of the gene. One group of TFs is the WRKY family which has a prominent function throughout many processes such

as plant development and plant stress tolerance. Importantly, it performs a necessary function for tolerance within plants against salt stress (Amin *et al.*, 2021). For instance, *TaWRKY10* was found a play role to raise the tolerance in transgenic tobacco lines (*Nicotiana tobaccum*) against drought and salt stresses. This, in turn, led to the preservation of soluble sugar and proline, in addition to lowering reactive oxygen species (ROS) and MDA contents in transgenic tobacco lines, when comparing to wild type (WT).(Wang *et al.*, 2013a).

The effect of the salinity on plant performance such as reduction in shoot length, leaf numbers, leaf area, chlorophyll content, shoot and dry weight are investigated due to the necessity of testing the tolerance and sensitivity of four wheat varieties under different salinity levels. Understanding the biochemical alterations under salt stress are important and could be potentially used as a biochemical marker to identify the tolerance and sensitivity under salt stress, such as MDA and Proline content. As mentioned before, recognising the candidate WRKY gene could pave the way for it to be used as a gene marker for selecting the tolerant genotype in crops against salt stress, potentially aiding to increase crop yield and productivity for this strategically important crop.

3.1.1 Aim of the study

The current research aims to understand the underlying process in which the wheat plant responds to salt stress

3.1.2 Objective of the study

- Understanding the effects and features of salt stress on 4 wheat varieties
- Contributing to the identification of the putative WRKY gene involved in plant's response to salinity stress
- Evaluation of plant growth after salt treatment and measuring the shoot length, leaf numbers, relative water content, biomass, leaf area, and chlorophyll content
- Evaluation of the biochemical trait after salt treatment

-Quantifying the proline accumulation and MDA

-Measuring the quantity of Na^+ content and K^+ content

- Analysing changes in gene expression and identifying the most significant WRKY TFs for response to salt stress.

3.1.3 Hypothesis

In this study, it is hypothesised that Saudi wheat varieties and UK spring wheat varieties have different responses to salt stress, resulting in variation in their plant growth at seedling stage, differences in the biochemical changes that are related to their response towards salinity, and disparity in their WRKY gene expression

3.2 Results

3.2.1 *Shoot length (SL)*

This experiment intended to measure the shoot length for Alderon, Sama, Cochise, and Najran under three different treatments of NaCl concentration (0, 80,160 mM) for 21 days exposure in order to explore the eventual effect of salinity on shoot length amongst the 4 aforementioned wheat varieties. First, the shoot length for of each wheat variety (Alderon, Cochise, Najran and Sama) under all salt level (0, 80, 160 Mm NaCl) was recorded. Alderon, Cochise and Sama were approximately similar (43.94 cm ± 0.96, 44.47 cm ± 0.80, and 41.38 cm ±1.28, respectively) while Najran was recorded only 28.68 cm ± 1.12 as seen in Figure 9 (A). In addition, the effect of each salt level (0, 80, 160 mM NaCl) on shoot length for all wheat varieties is shown in Figure 9 (B). The average of shoot length in all wheat varieties was recorded, with 37.01 cm ± 1.81 and 39.37 cm ± 1.35 at 80 and 160 mM, respectively, as compared to control at 0 mM (42.41 cm ± 1.41). Statistical analysis was conducted using two-way ANOVA considering the effect of salinity, varieties and their interaction. The effect of wheat varieties was highly significant ($F = 67.29$, $P < 0.0005$) in shoot length and the effect of salinity levels was deeply significant ($F = 11.66$, $P < 0.0005$) while there was no significant interaction between salinity and wheat varieties ($F= 1.14$, $P < 0.34$). The statistical analysis confirmed that Najran was significant different ($P < 0.0005$) with other varieties while there was no significant difference among Alderon, Cochise and Sama in shoot length. In addition, the shoot length for varieties at 0 mM was significant difference with 80 Mm($P < 0.021$) and 160 mM ($P < 0.0005$) but there was no significant difference between 80 Mm and 160 Mm.We can observe that the lower shoot length was recorded for Najran while other wheat varieties were similar in their shoot length as revealed in Figure 9 (A). In addition, we can find that shoot length for all wheat varieties were affected by salinity at 80 and 160 mM as shown in Figure 9 (B).

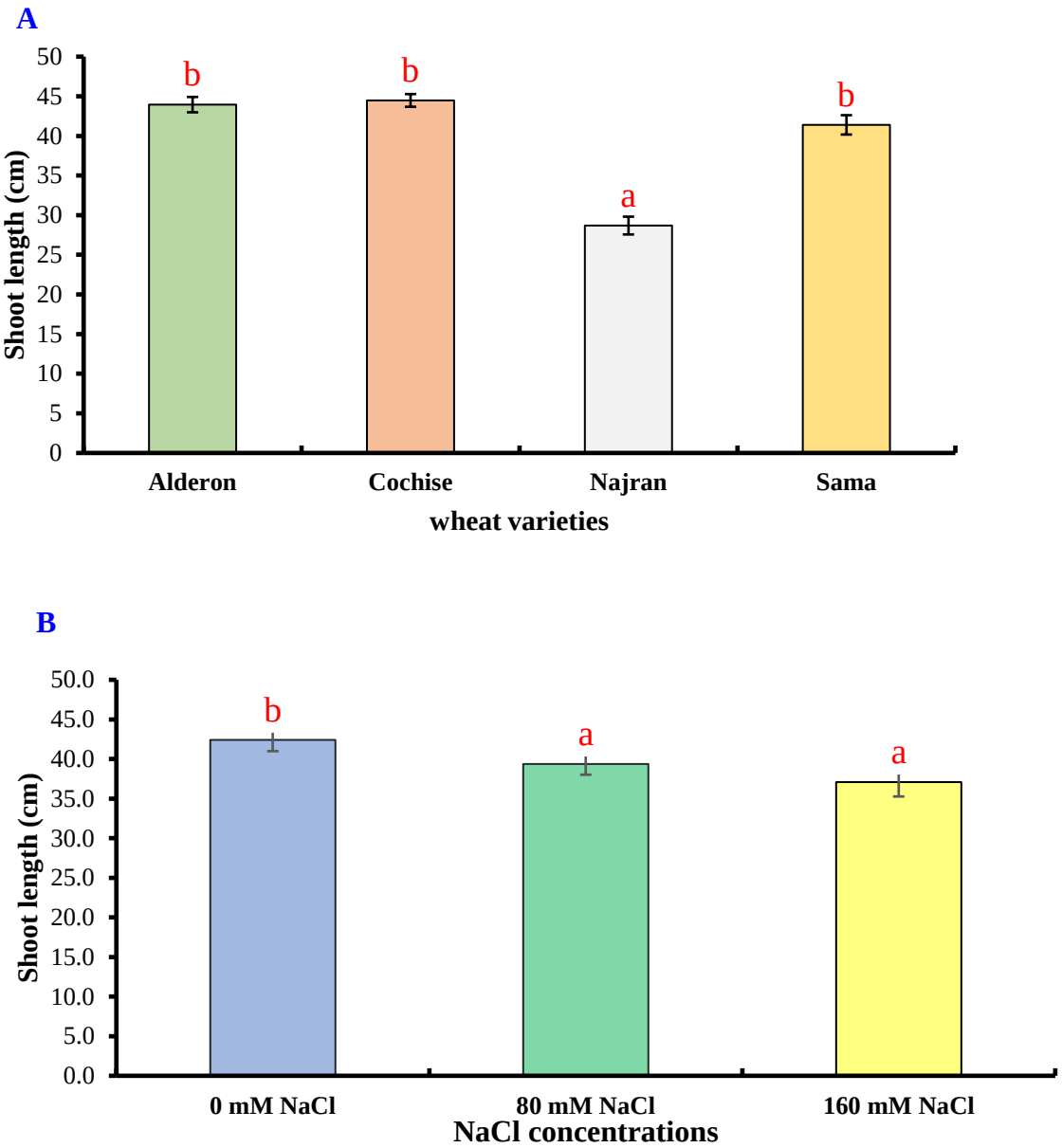

Figure 9: Shoot length variation according to wheat varieties (**A**) and NaCl concentration treatment (B). The variation in shoot length within 4 wheat varieties recorded after exposure to 0, 80, 160 mM NaCl for 21 days of growth in sand culture is displayed. Varietal effect (A), shoot length of each wheat variety under 3 NaCl concentrations represents mean ± standard error (6 biological replications/ treatment). NaCl concentration effect (B), shoot length of 4 wheat varieties through each salt level represents mean ± standard error (6 biological replications/ wheat variety). Using two-way ANOVA, values with different letters are significantly different at $P < 0.05$, based on Tukey multiple comparison test.

3.2.2 Number of leaves (NL)

The number of leaves was quantified under the same experimental conditions mentioned in the experiment. It was revealed that the number of leaves varied according to the wheat variety under all levels of salinity. Indeed, Najran produced the greatest number of leaves, whereas Alderon, Cochise, and Sama produced numbers of leaves that were similar as shown in Figure 10 (A). As the salt concentration increased, the number of leaves decreased as demonstrated in Figure 10 (B). Indeed, it was found that increasing levels of salt concentration had a negative effect on number of leaves for all wheat varieties. The maximum number of leaves was recorded at 0 Mm (8 ± 0.32), followed by 6 ± 0.16 and 4 ± 0.20 that were observed at 80 and 160 mM NaCl, respectively. Two-way ANOVA was used to examine the effect of variety, salinity and their interaction. Firstly, there was a significant effect of wheat varieties on number of leaves ($F = 7.75\ 29$, $P < 0.0005$). It was shown that number of leaves of Najran was significantly ($P < 0.05$) different from Alderon and Cochise, while there was no significant difference between Najran and Sama as shown in Figure 10 (A). In addition, there was no significant difference on number of leaves for both Alderon, Cochise and Sama varieties. Secondly, there was statistically significant differences in number of leaves for 0, 80 and 160 Mm NaCl ($F = 74$, $P < 0.000$). It was observed that number of leaves for all wheat varieties were significantly different ($P < 0.0005$) at 0 mM as compared to 80 and 160 mM NaCl, and it was also found significant difference ($P < 0.0005$) between 80 and 160 mM NaCl as shown in Figure 10 (B). Thirdly, no significant interaction could be found between salinity and wheat varieties ($F= 1.84$, $P < 0.106$). We found that number of leaves was influenced by all levels of salt concentration, and we observed that Najran maintains high number of leaves under all salt treatments, and it could be more tolerant than other varieties.

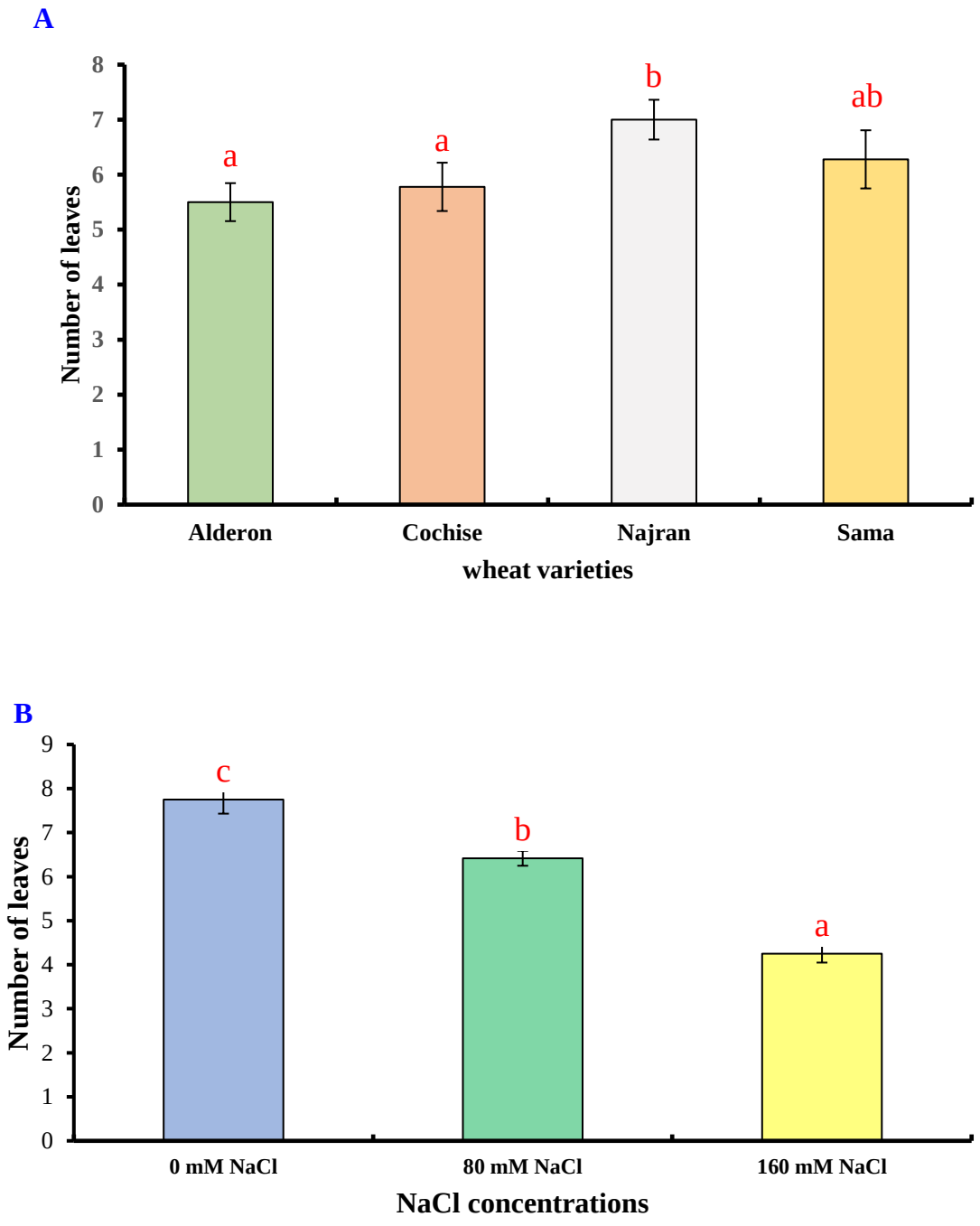

Figure 10 Leaves number of 4 wheat varieties (A) and NaCl concentration effect (B). The number of leaves of 4 wheat varieties measured after growth at 0, 80, and 160 mM NaCl for 21days in sand culture is displayed. Varietal effect (A), number of leaves of each wheat variety under 3 NaCl concentrations represents mean ± standard error (6 biological replications/ treatment). NaCl concentration effect (B), number of leaves of 4 wheat varieties through each salt level represents mean ± standard error (6 biological replications/ wheat variety). Using two-way ANOVA, values with different letters are significantly different at $P < 0.05$, based on Tukey multiple comparison test.

3.2.3 Leaf area (LA)

Changes in leaf area for the 4 wheat varieties in the experiment under the conditions stated before, it was measured via the methodology used by (Chanda and Singh, 2002). As seen in Figure 11 (A). The leaf area was found different in 4 wheat varieties. It was shown that the leaf area in Alderon under all three level of salt concentration had the highest value (18 cm ± 1.17) followed by Cochise and Sama (15 cm ± 1.11 and 15 cm ± 1.13, respectively) while the lowest value was recorded at Najran (7 cm ± 0.50). In addition, it was noted that the leaf area for all wheat varieties varied for each level of salt concentration and it was found that the leaf area was reduced when the level of salt was increased. The leaf area for all wheat varieties was recorded as the highest values at 0 mM (18 ± 1.25) followed by 80 mM (13 ± 0.91) and then the lowest value was found at 160 mM (11 ± 0.90) as displayed in Figure 11 (B). The two-way ANOVA was used to assess the effects of variety, salinity, and their interaction. The statistical analysis confirmed that there was significant difference on the leaf area in Alderon, Cochise, Sama and Alderon ($F = 60.85$, $P < 0.0005$). It was found that Alderon was significantly ($P < 0.05$) different from the other wheat varieties, while Cochise and Sama were not significantly different, but they were significantly different ($P < 0.0005$) from Alderon and Najran. Moreover, Najran appeared significantly different ($P < 0.0005$) from the remaining wheat varieties as displayed in Figure 11 (A). Furthermore, the statistical analysis displayed that there was significant difference in the leaf area for all wheat varieties among all levels of salt concentration (0, 80 and 160 mM NaCl). It was shown that the leaf area of all wheat varieties was significantly ($P<0.0005$) different at 0 mM from 80 and 160 Mm NaCl. In. In addition, the leaf area was significantly different ($P<0.027$) from 80 and 160 mM. In addition, there was no significant interaction between variety and salinity ($F = 1.54$, $P < 0.181$). We saw that Najran had the lowest value and it reflects the behaviour of the genotype (short leaf) and it may be semi dwarf wheat .

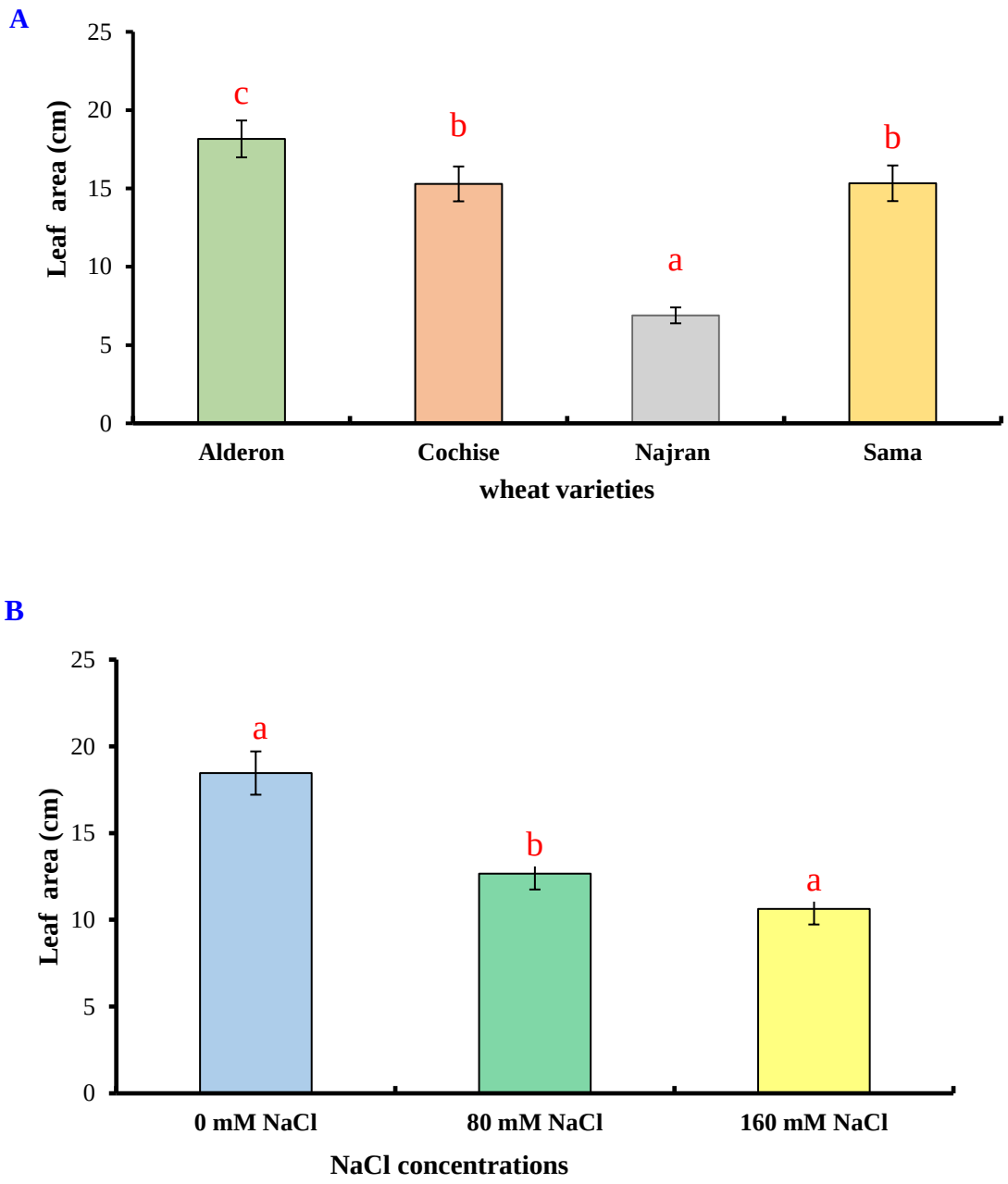

Figure 11 Leaf area variation according to wheat varieties (**A**) and NaCl concentration treatment (B) on leaf area. The leaf area of 4 wheat varieties measured after growth at 0, 80, and 160 mM NaCl for 21days in sand culture is displayed. Varietal effect (A), leaf area of each wheat variety under 3 NaCl concentrations represents mean ± standard error (6 biological replications/wheat variety). NaCl concentration effect (B), leaf area of 4 wheat varieties through each salt level represents mean ± standard error (6 biological replications/treatment). Using two-way ANOVA, values with different letters are significantly different at $P < 0.05$, based on Tukey multiple comparison test.

3.2.4 Chlorophyll content (CC)

The Chlorophyll content was measured as an important physiological parameter, under the same conditions discussed, by SPAD meter at leaves number 4 and 5 and the values recorded based on SPAD units. The chlorophyll content was influenced by wheat varieties as presented in Figure 12 (A). The chlorophyll content was recorded a maximum value with Alderon for all salinity levels (8 ± 1.10), while the chlorophyll content was similar in Cochise, Sama and Najran (7 ± 1.31, 6 ± 0.96 and 5 ± 0.61, respectively). Moreover, the chlorophyll content was higher at 0 mM NaCl for all wheat varieties (11 ± 0.79) it was reduced when salt concentration increased. The chlorophyll content in all wheat genotypes varieties recorded 6 ± 0.69 at 80 mM NaCl, while it was recorded 3 ± 0.27 at 160 mM NaCl as shown in Figure 12 (B). A two-way ANOVA analysis was carried out in order to investigate the effects of both variety and salinity, as well as the interaction between the two factors on chlorophyll content. Firstly, it was revealed that chlorophyll content was influenced significantly by variety factor ($F = 3.76$, $P < 0.020$). There was a significant difference between Alderon and Najran ($P < 0.020$), while there was no significant difference within Alderon, Cochise and Sama. In addition, there was no significant difference among Cochise, Sama and Najran. Secondly, in term of salinity effect, it was found that the chlorophyll content was impacted significantly by salinity ($F= 44.60$, $P < 0.0005$), the statistical analysis has shown that there was significant difference in chlorophyll content for 4 wheat genotypes at 0 mM with 80 and 160 mM NaCl ($P<0.0005$). in addition, there was significant difference between in Chlorophyll content for 4 wheat genotypes between 80 and 160 mM($P<0.0005$). In term of interaction, there was no significant interaction between variety and salinity ($F= 1.53$, $P < 0.182$). Alderon performed better than the other varieties.

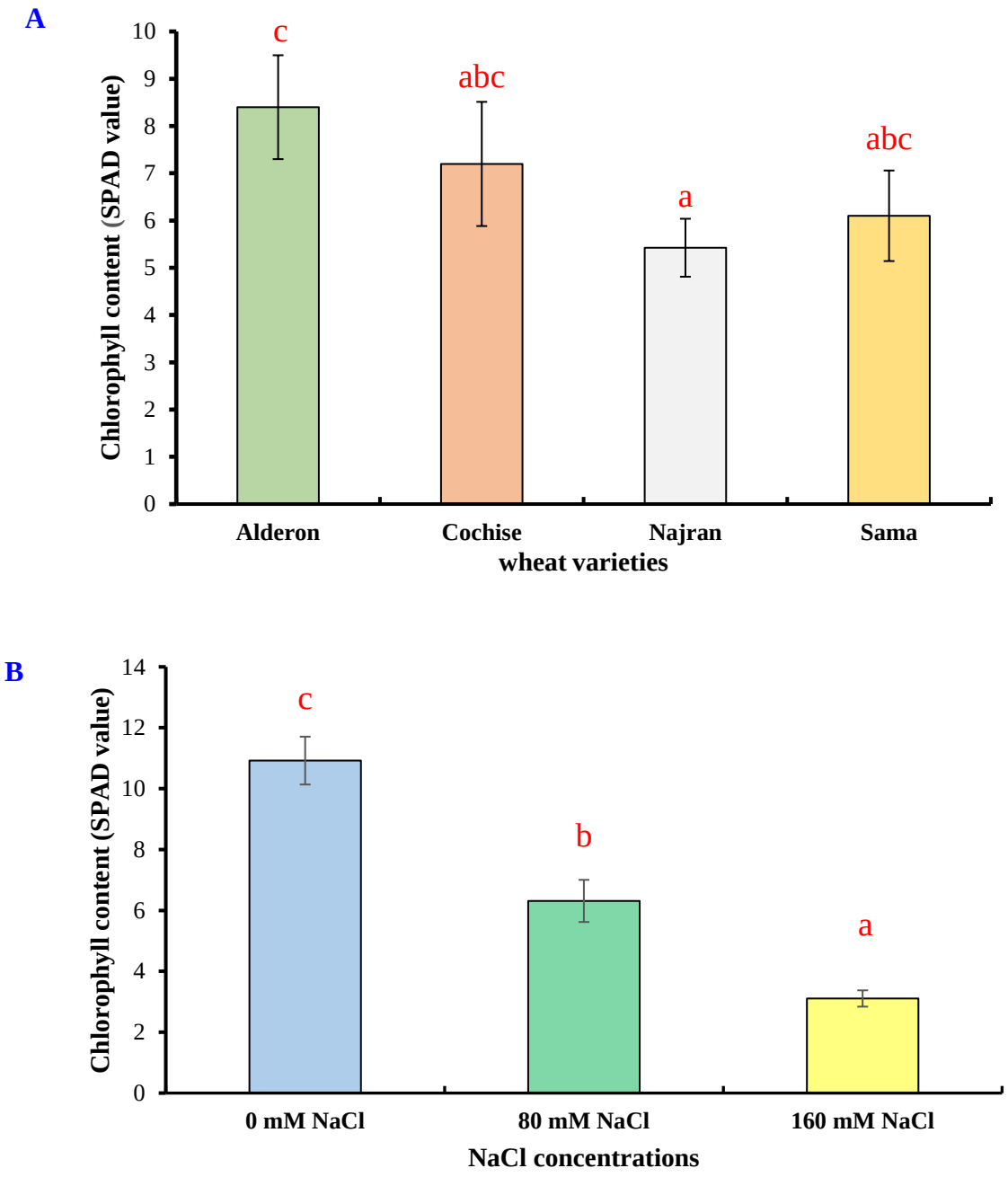

Figure 12 Chlorophyll content variation according to wheat varieties (**A**) and NaCl concentration treatment (B). The chlorophyll content of 4 wheat varieties measured after growth at 0, 80, and 160 mM NaCl for 21days in sand culture is displayed. Varietal effect (A), chlorophyll content of each wheat variety under 3 NaCl concentrations represents mean ± standard error (6 biological replications/treatment). NaCl concentration effect (B), chlorophyll content of 4 wheat varieties through each salt level represents mean ± standard error (6 biological replications/ wheat variety). Using two-way ANOVA, values with different letters are significantly different at $P < 0.05$, based on Tukey multiple comparison test.

3.2.5 Relative water content (RWC)

The RWC is an important parameter to consider when investigating the impact of salt stress on the wheat varieties (under same conditions in the same experiment). For that purpose, leaf number 4 was chosen across all varieties to measure the RWC. In term of wheat varieties, RWC content was shown similar for each wheat variety under all three salt levels. Alderon, Cochise, Najran and Sama were recorded 74.9, 70.6, 75.33 and 73.22%, respectively as shown in Figure 13 (A). However, there was difference in RWC under three different of salt levels, RWC for all wheat varieties across each level of salt concentration as presented Figure 13(B). At 0 mM NaCl, RWC content was found 82%, while at 80 and 160, it was recorded 74 and 65%. The statistical analysis (two-way ANOVA) confirmed that there was significant effect of salinity on RWC ($F = 53.18$, $P < 0.0005$). The statistical analysis has shown for RWC for all wheat varieties at 0 mM NaCl a significant difference ($P<0.0005$) when compared to 80 and 160 mM NaCl, and there was significant difference ($P < 0.0005$) between 80 and 160 mM. However, there was no significant difference ($F = 53.18$, $P < 2.57$) between wheat varieties in their RWC, and there was no significant interaction between variety and salinity ($F (6, 60) = 53.18$, $P < 0.073$).

Leaf RWC is decreased by osmotic stress (80 and 160 mM NaCl) for all wheat varieties. Indeed, the four varieties of wheat are equally affected by salt treatment. Particularly, 160 mM NaCl induces a reduction in Leaf RWC of about 20% for all wheat varieties, Consequently, the salt stress is perceived as an osmotic stress by the plants whose leaves tissues became clearly dehydrated. Leaf RWC reduction is commonly observed in plants submitted to salt stress, particularly susceptible ones.

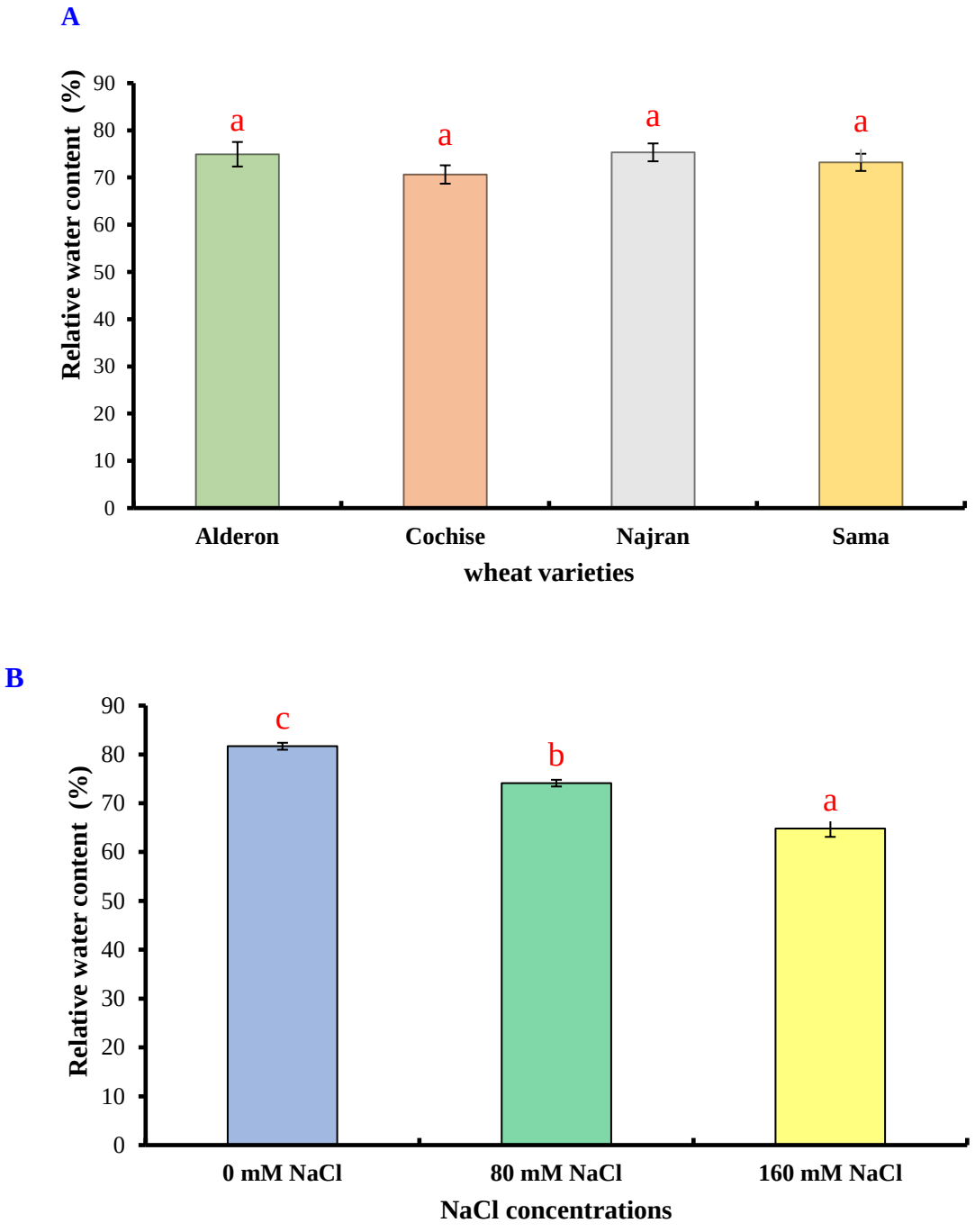

Figure 13 Relative water content (RWC) variation according to wheat varieties (**A**) and NaCl concentration treatment (B). The RWC of 4 wheat varieties measured after growth at 0, 80, and 160 mM NaCl for 21days in sand culture is displayed. Varietal effect (A), RWC of each wheat variety under 3 NaCl concentrations represents mean ± standard error (6 biological replications/ treatment). NaCl concentration effect (B), RWC of 4 wheat varieties through each salt level represents mean ± standard error (6 biological replications/ wheat variety). Using two-way ANOVA, values with different letters are significantly different at $P < 0.05$, based on Tukey multiple comparison test.

3.2.6 Shoot fresh weight (FW)

The shoot fresh weight was determined to see the impact of various quantities of salt upon wheat varieties in the previously stated experimental conditions. It was shown that the FW decreased across all varieties with an increase in salt concentration. For instance, in 80 mM NaCl treatment, Alderon, Cochise, Najran, and Sama declined to 46.5, 52.2, 23.9, and 69.2 %, respectively, relative to control. In addition, 160 mM NaCl, Alderon, Cochise, Najran, and Sama dropped noticeably to 69.4, 71, 62.4 and 84.9 %, respectively (Figure 14).

The statistical analysis (two-way ANOVA) was used to examine varieties effect, salt treatment effect and the interaction between varieties and salt treatments. Firstly, the statistical test provides that there was significant difference between shoot fresh weight among varieties ($F = 3.60\ 18$, $P < 0.018$). It was found that there was no significant difference between Alderon and each of Cochise, Najran and Sama on shoot fresh weight for all salinity levels. In addition, there was no significant difference between Cochise and each of Alderon and Najran on shoot fresh weight, but there was significant difference ($P < 0.018$) between Cochise and Sama. Additionally, Najran was no significantly different from each of Alderon, Cochise, Sama. Moreover, Sama has shown there was no significant difference for each of Alderon and Najran on shoot fresh weight but there was significant difference ($P < 0.018$) between Sama and Cochise. Secondly, the results of the statistical test indicated that there was a significant difference between salt concentration in terms of shoot fresh weight ($F = 143.89$, $P < 0.000$). There was a significant difference ($P < 0.0005$) in shoot fresh weight for all wheat varieties at 0 mM in comparison to each of 80 and 160 mM NaCl and there was a significant difference ($P < 0.0005$) between 80 and 160 mM NaCl. Thirdly, there was statistically a significant difference between interaction of wheat varieties and salt level ($F = 3.74$, $P < 0.003$).

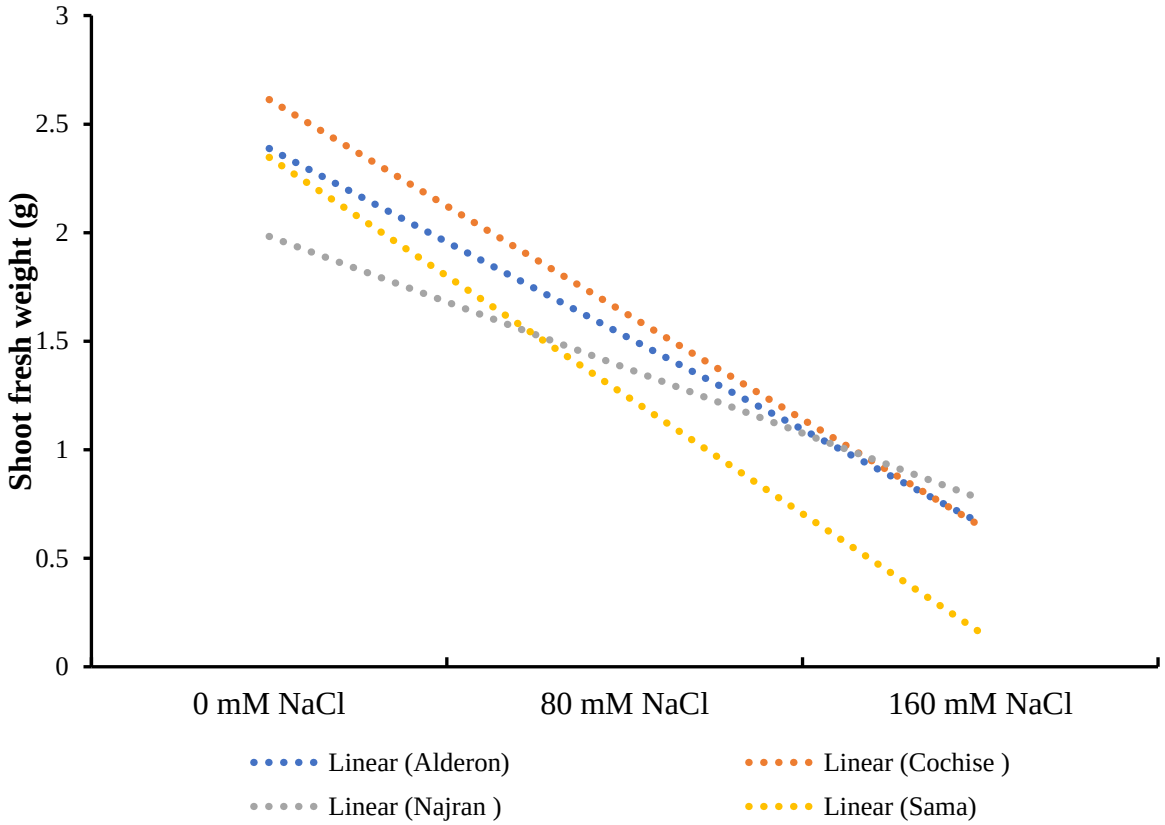

Figure 14 Impact of salt concentration on shoot fresh weight of 4 selected wheat varieties.The Figure presents the interactive effects of wheat varities and salt concentration on shoot fresh weight. Shoot fresh weight of wheat varieties have been measured 21 days after growth in various salt concentrations (0, 80, 160 mM NaCl). Statistical analysis was used by two-way ANOVA with Tukey multiple comparison tests (values are mean of n = 6)

3.2.7 Shoot dry weight (DW)

The parameter of shoot dry weight was explored to understand the impact of salinity on the four wheat varieties (in same experimental conditions). The shoot dry weight was found to be decreasing as the salinity levels increased for all varieties. For instance, at 80 mM NaCl, Alderon, Cochise, Najran and Sama had their shoot dry weight decreased drastically by 43.7, 57.5, 30.9 and 60, respectively. Secondly, it must be noted that the decline in shoot dry weight of Alderon, Cochise, Najran, and Sama dropped by 55.5, 67.3, 39.7 and 76.1%, respectively. Thirdly, it was observed that there was significant interaction between wheat varieties and salt levels (Figure 15).

The statistical analysis (two-way ANOVA) was used to assess the effect of varieties, the effect of salt treatments, and the interaction between the two factors on shoot dry weight. Firstly, statistical analysis revealed a considerable variation in shoot fresh weight among cultivars ($F = 6.13$, $P < 0.001$). It was found that Alderon was significantly different ($P < 0.001$) from Najran on shoot dry weight under all salt levels, and it was also significantly different ($P < 0.008$) with Sama in shoot dry weight, but there was no significant difference

between Alderon and Cochise. The shoot dry weight in Cochise was not significantly different from all other wheat varieties. In addition, Najran was significantly different ($P < 0.001$) from Alderon in shoot dry weight but it was no significant difference with Cochise and Sama. Moreover, Sama was only different with Alderon, while other varieties were not statistically different. Secondly, Statistical analysis revealed a significant difference between salt concentration on shoot dry weight ($F= 143.96$, $P < 0.0005$). There was significant difference ($P < 0.0005$) in shoot dry weight for all wheat varieties at 0 mM in comparison to each of 80 and 160 mM NaCl and there was a significant difference ($P < 0.008$) between 80 and 160 mM NaCl. Thirdly, there was statistically a significant difference between interaction of wheat varieties and salt level ($F = 5.30$, $P < 0.0005$). Our results provided information based on data and statistical analysis that Najran had the lowest reduction on shoot dry weight that can be tolerant to salt treatment while Sama has the highest reduction on shoot dry weight which indicates to be sensitive to salt treatment. Alderon and Cochise are close to each other in term of shoot dry weight.

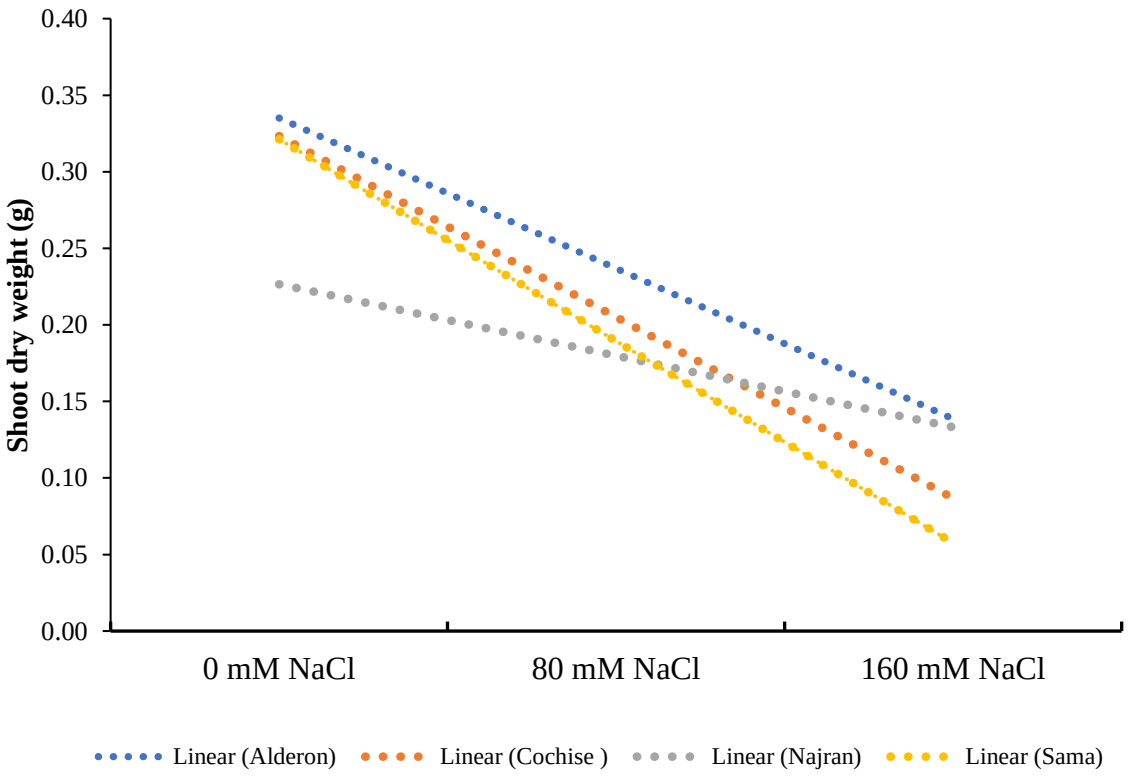

Figure 15 Impact of salt concentration on shoot dry weight of four selected wheat varieties. The Figure presents the interactive effects of wheat varieties and salt concentration on shoot dry weight. Shoot dry weight of four wheat varieties were recorded 21 days after being

exposed to 0, 80, 160 mM NaCl concentrations in sand. Statistical analysis was used by two-way ANOVA with Tukey multiple comparison tests (values are means of n = 6).

3.2.8 Na^+ content in shoot

The ion content was analysed by using flame photometer for the dry shoot to measure the Na^+ concentration for each variety after exposure to 21 days of different salt concentrations. As seen in Figure 16, the Na^+ concentration has increased as the salt concentration increased. It has been observed that there is a variation between Na^+ concentrations of wheat genotypes. Firstly, at 80 mM NaCl, Alderon, Cochise, Najran, and Sama contained 337, 272, 143 and 197 µmol/g DW Na^+ (Figure 16). Secondly, for 160 mM NaCl treatment, the Na^+ content in dry shoot of Alderon, Cochise, Najran, and Sama was seen to be 429, 483, 334, and 430 µmol/g DW, respectively. Thirdly, at 0 mM NaCl treatment, Na^+ accumulation in the control condition was 10, 69, 37, and 54 µmol/g DW, respectively.

Two-way analysis of variance (ANOVA) was used to examine the effect of varieties, salt treatment and the interaction between them on Na^+ content. Regarding varieties effect, there was significant difference amongst wheat genotypes in their Na^+ content ($F = 9.08$, $P < 0.0005$). Statistical analysis showed that Alderon under all salt level was significant difference ($P < 0.001$) with Najran in Na^+ content while Alderon did not show statistically significant difference in Na^+ content with each of Cochise and Sama. Moreover, the Na+ content of Cochise showed a statistically significant difference ($P < 0.0005$) with Najran, but the Na^+ content of Cochise did not exhibit a statistically significant difference with either Alderon or Sama. In addition, there was a statistically significant difference in Na^+ content between Najran and Alderon ($P < 0.001$) and between Najran and Cochise ($P < 0.0005$), however there was no statistically significant difference between Najran and Sama. In addition, there was no significant difference between Sama and each of Alderon, Cochise and Najran in Na^+ content. Concerning salt treatments, there was statistically significant difference in Na^+ content for salt treatments ($F = 208.17$, $P < 0.0005$). The statistical test confirmed that there was significant difference ($P < 0.0005$) in Na^+ content between 0 mM for all wheat varieties and each of 80 NaCl treatments and between 80 and 160 mM NaCl.There was a statistically significant interaction between wheat varieties and salt levels on Na^+ content $F= 4.00$, $P< 0.002$. We can see that Najran had the lowest value of Na^+ content which indicates its tolerance to salt stress.

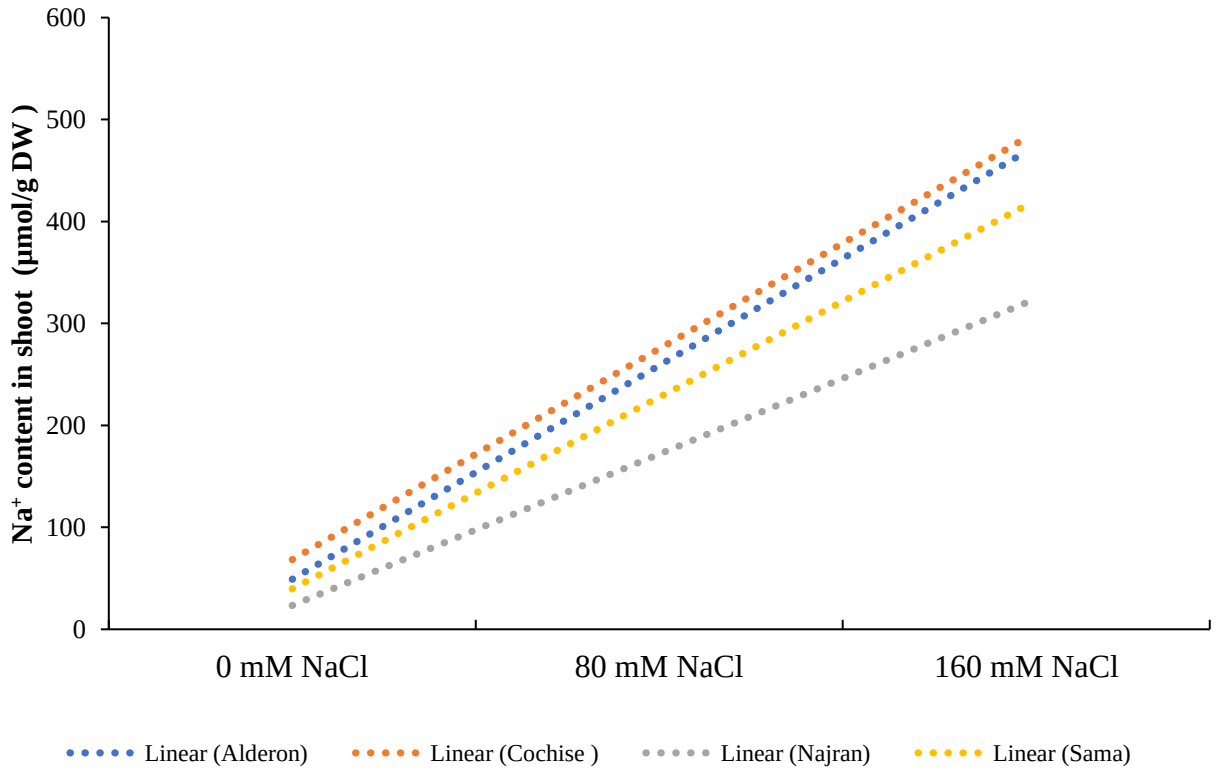

Figure 16 Effect of salt concentration on Na^+ content in shoots of four wheat varieties. The Figure presents the interactive effects of wheat varieties and salt concentration in Na^+ content. Effect of 21 days salinity stress (0, 80, 160 mM NaCl) in Na^+ accumulation in shoot of four wheat varieties. Statistical analysis was used by two-way ANOVA with Tukey multiple comparison tests (values are means of n = 6).

3.2.9 K^+ content in shoot

The potassium concentration was quantified via a flame photometer for dry shoot in order to measure the K^+ levels in every wheat genotype after experiencing 21 days of various salinity levels. As presented in Figure 17 (A), there was variation on K^+ content among wheat varieties. It was revealed that Najran, when tested with all three different levels of salt content, had the greatest concentration of K^+ (178 µmol/g DW ± 10.80) followed by Sama (154 µmol/g DW ± 16.41). In addition, the K^+ content in Cochise had 116 µmol/g DW ± 10.80 and it was determined that Alderon had the lowest concentration of K^+ content (76 µmol/g DW ± 10.78). In addition, it was noted that the K^+ content was variable for each level of salinity. The K^+ decreased when salt level is increased, for instance, it was noted that the K^+ content for all wheat varieties at 0 mM NaCl was 193 µmol/g DW ± 11.35 and the K^+ content at 80 and 160 mM NaCl was determined 116 µmol/g DW ± 8.89 and 84 µmol/g DW ± 10.84 as shown in Figure 17 (B). It was determined via two-way ANOVA, there was statistically significant main effect of wheat variety on K^+ content ($F = 37.76$, $P < 0.0005$). As displayed on Figure 17 (A), Najran was statistically different ($P < 0.0005$) when compared

with Alderon and Cochise but there was no significant difference between Najran and Sama ($P < 0.107$). Sama was significant difference ($P < 0.0005$) with Alderon and It was also significant difference with Cochise ($P < 0.002$). In addition, there was significant difference between ($P < 0.001$) Alderon and Cochsie. Furthermore, there was significant difference in salt treatments on K^+ content. As seen in Figure 17 (B), there was a significant difference ($P < 0.0005$) between 0 mM and each of 80 and 160 mM NaCl. There was also a significant difference ($P < 0.0005$) 80 and 160 mM ($P < 0.002$). However, there was no significant interaction between wheat varieties and salinity ($F = 1.98$, $P < 0.083$). It was shown that Najran contained the highest concentration of K^+ content while Alderon was shown to have the lowest K^+ content.

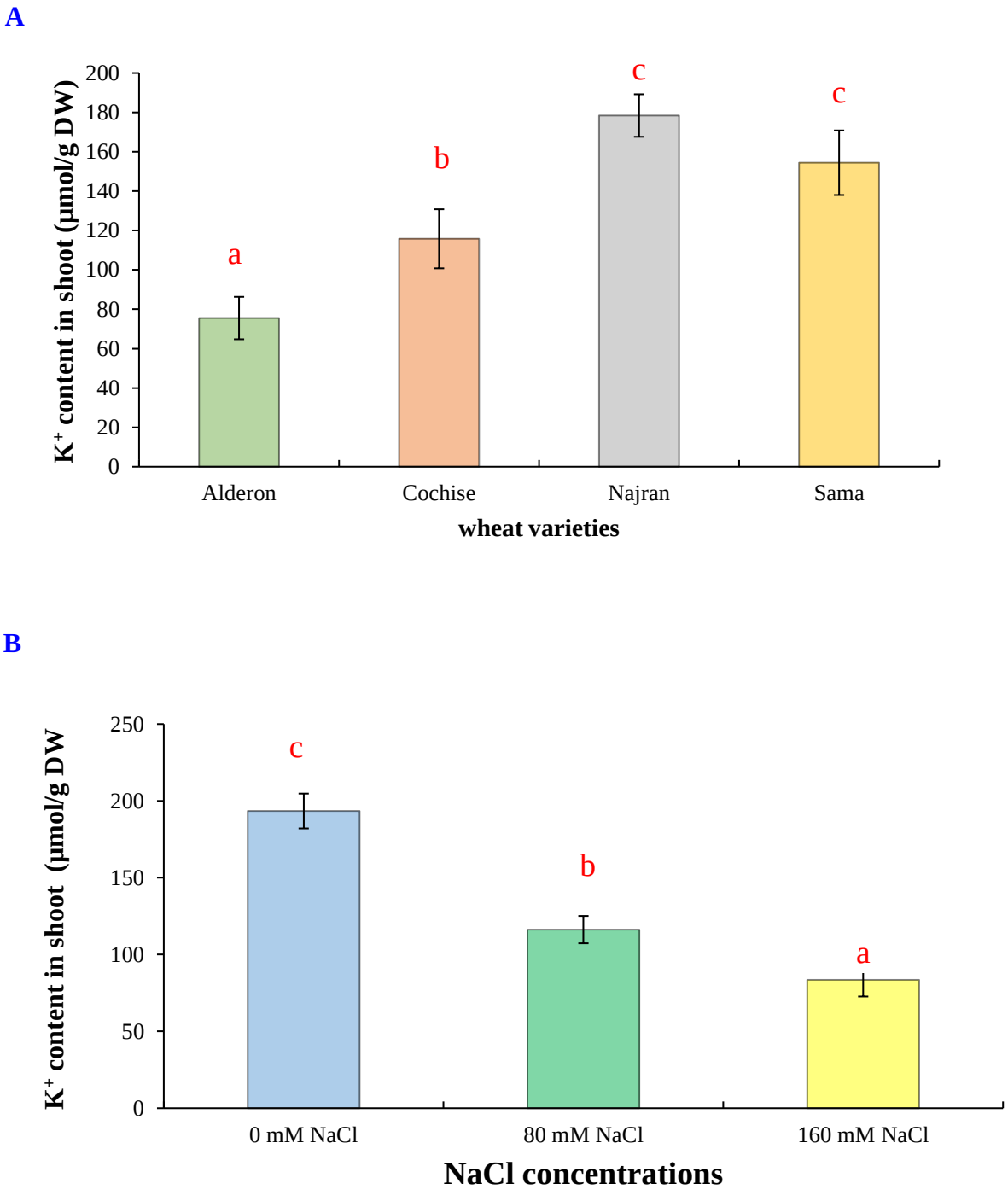

Figure 17 Potassium content variation according to wheat varieties (**A**) and NaCl concentration treatment (B). The K^+ content of 4 wheat varieties measured after growth at 0, 80, and 160 mM NaCl for 21days in sand culture is displayed. Varietal effect (A), K^+ content of each wheat variety under 3 NaCl concentrations represents mean ± standard error (6 biological replications/ treatment). NaCl concentration effect (B), K^+ content of 4 wheat varieties through each salt level represents mean ± standard error (6 biological replications/ wheat variety). Using two-way ANOVA, values with different letters are significantly different at $P < 0.05$, based on Tukey multiple comparison test.

3.2.10 Na^+/ K^+ ratio in shoot

The ratio (Na^+/K^+) of ions was calculated as to identify the effect of the ions under different salt conditions across wheat varieties. Figure 18 displays the relative content of Na^+ and K^+ ions in shoot. As seen, for the NaCl treatment, the lowest Na^+/K^+ ratio was revealed to be in Najran (0.85 for 80 mM and 2.44 for 160 mM NaCl) and the higher most in Alderon (5.42.for 80 mM and 16.87 for 160 mM). Sama and Cochise presented 1.72 and 2.73 for 80 mM NaCl, respectively, and 4.18 and 11.21 being detected for 160 mM NaCl, respectively (Figure 18).

Two-way analysis of variance (ANOVA) was used to explore the effects of varieties, salt treatment, and the interaction of varieties and salt treatments on ratio (Na^+/K^+). Regarding wheat varieties, there was statistically a significant difference in (Na^+/ K^+) for varieties ($F= 25.70$, $P < 0.0005$). The analysis has shown that Alderon was statistically significantly different ($P < 0.007$) from Cochise and each of Najran and Sama ($P < 0.0005$) in Na^+/K^+ ratio. In addition, Cochise was significantly different ($P < 0.007$) from Alderon in Na^+/K^+ ratio and with Najran ($P < 0.0005$) and Sama ($P < 0.006$). Moreover, Najran was significantly different ($P < 0.0005$) with each of Alderon and Cochise but not with Sama. Regarding Sama variety, it was significantly different with each of Alderon ($P < 0.0005$) and Cochise ($P < 0.006$) but not with Najran.

In term of salt effect, there was statistically significant difference in Na^+ content for salt treatments ($F = 80.17$, $P < 0.0005$). The statistical test confirmed that there was significant difference ($P < 0.0005$) in Na^+/ K^+ ratio between 0 mM NaCl treatment and each of 80 NaCl treatment ($P < 0.002$) and 160 mM NaCl ($P < 0.0005$) in Na^+/ K^+ ratio and there was a significant difference ($P < 0.0005$) between 80 and 160 mM NaCl. Moreover, there was a significant interaction between wheat varieties and salt level in Na^+/ K^+ ratio ($F = 12.51$, $P < 0.0005$). Overall, Najran, followed by Sama, can be considered to be more tolerant compared to Alderon and Cochise. Najran appears to be the tolerant variety in terms of Na^+/K^+ ratio in shoot, that has the lowest Na^+/K^+.

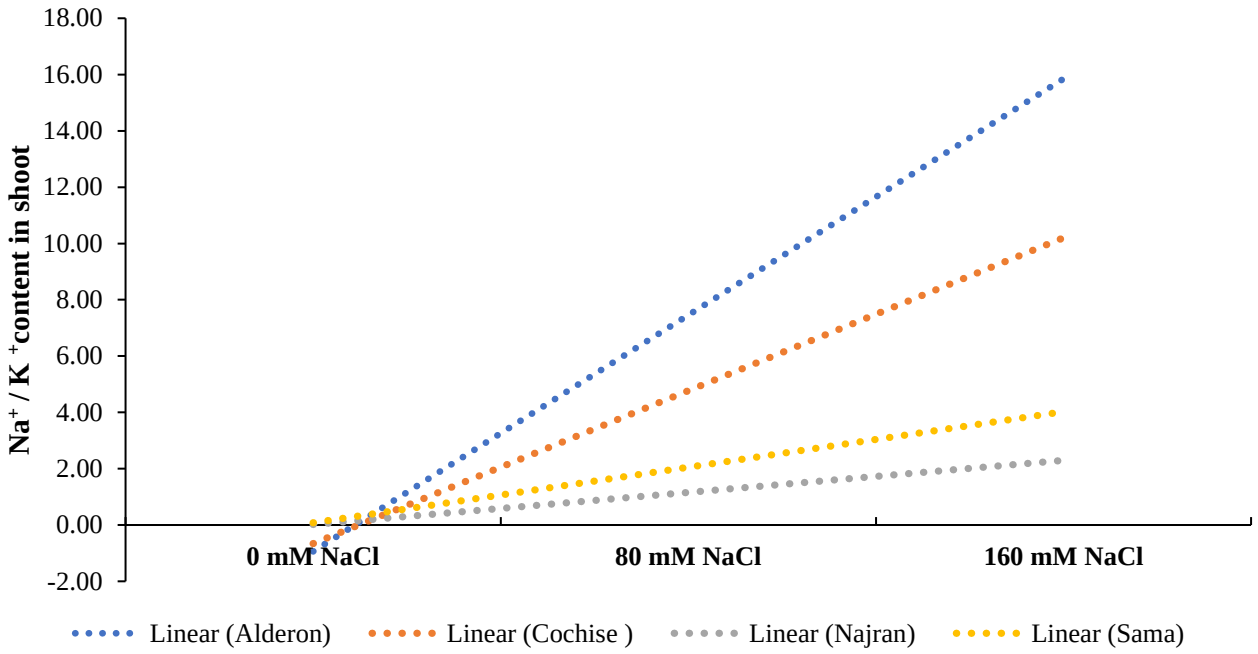

Figure 18 Effect of salt concentration on in Na^+/K^+ ratio content in shoots of four wheat varieties. The Figure presents the interactive effects of wheat varieties and salt concentration in Na^+/K^+ ratio. Effect of 21 days salinity stress (0, 80, 160 mM NaCl) in Na^+/K^+ ratio of shoot of four wheat varieties. Statistical analysis was used by two-way ANOVA with Tukey multiple comparison tests (values are means of n = 6).

3.2.11 Proline content

The proline content was measured in the experiment as a key parameter to find the accumulation of the proline when all wheat varieties were growing for 21days under salt condition. Within the 3rd leaf of all wheat varieties, the various proline amounts were found to be 80 mM NaCl and 160 mM NaCl treatment. At 80 mM NaCl treatment, Najran and Cochise were similar in production of proline as they increased to 232 µmol/g FW and 243 µmol/g FW, respectively, while Alderon and Sama increased to 168 µmol/gFW and 122 µmol/g FW. At 160 mM NaCl treatment, Najran showed the highest increased proline within wheat varieties as it grew to 567 µmol/g FW, whilst Sama was recorded to rise by 210 µmol/g FW. This was the lowest value within varieties. The proline content enhanced in the leaves of Cochise and Alderon by 344 µmol/g FW and 441µmol/g FW, respectively (Figure 19).

Two-way analysis of variance (ANOVA) was used to explore the effects of varieties, salt treatment, and the interaction between them on proline content. Firstly, the proline content had been impacted by wheat varieties ($F = 42.18$, $P < 0.0005$). The statistical analysis provides that proline content for all salt treatments in Alderon was significantly different ($P < 0.0005$) with each of Najran and Sama, but there was no significant difference between Alderon and Cochise. Moreover, Cochise was found to be significantly different with each of

Najran ($P < 0.003$) and Sama ($P < 0.0005$) in proline content, whereas Alderon and Cochise were not significant difference in proline content. Interestingly, Najran was shown to be significantly different with all wheat varieties (Najran with Alderon and Sama, $P < 0.0005$) and (Najran with Cochise, $P < 0.003$). Additionally, Sama was significantly different ($P < 0.0005$) with each of Alderon, Cochise and Najran. For salt treatments effect, the proline content was significantly different between 0, 80, 160 mM NaCl. The proline content for all varieties at 0 mM NaCl was significantly different ($P < 0.0005$) with each of those at 80 and 160 mM NaCl and there was a significant difference ($P < 0.0005$) between 80 and 160 mM NaCl. There was statistically significant an interaction between salt treatment and wheat varieties on proline content ($F = 13.61$, $P < 0.0005$). In Summary, we can find that Najran has the highest amount of proline content followed by Alderon and then Cochise while the Sama was shown to have the lowest amount of proline content particularly at 160 mM NaCl. Najran could be more tolerant in salt treatment while Sama may be sensitive to salinity.

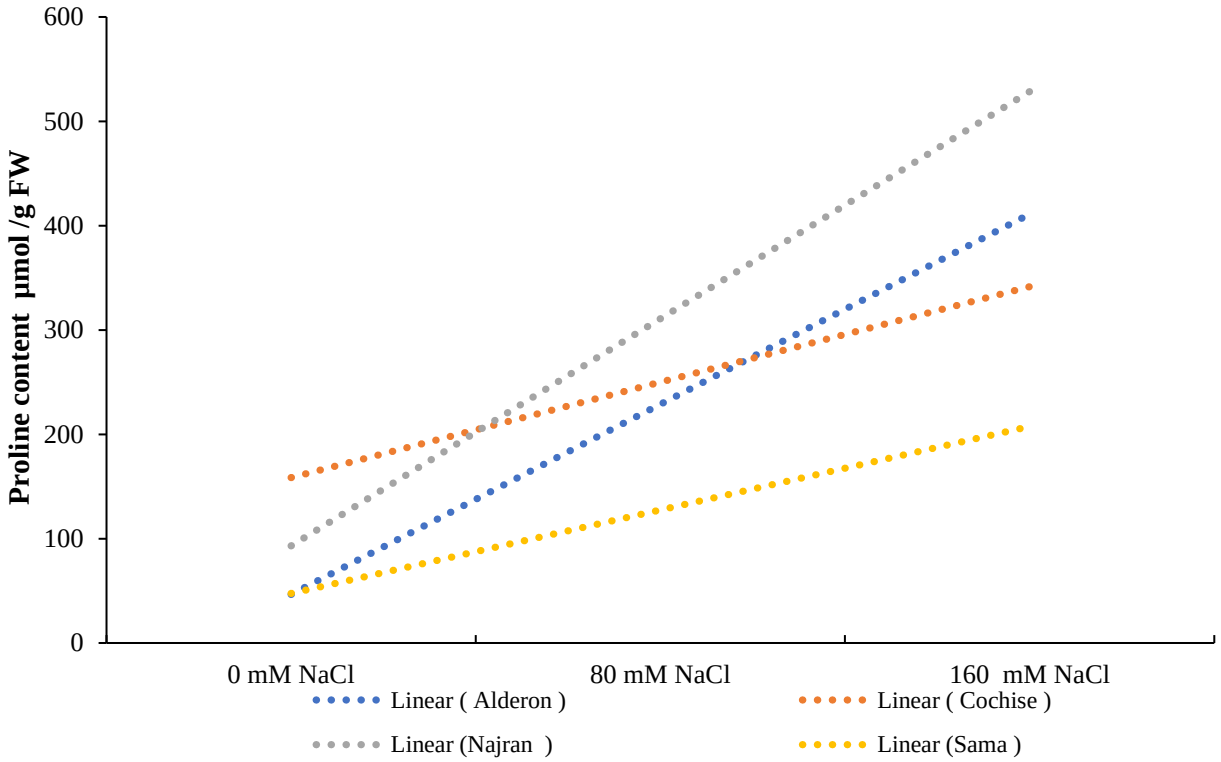

Figure 19 Impact of soil salinity on proline accumulation on four selected wheat varieties. The Figure presents the interactive effects of wheat varieties and salt concentration on proline content. Wheat genotypes were subjected to 0, 80, 160 mM NaCl for duration of 21 days in sand. Statistical analysis was used by two-way ANOVA with Tukey multiple comparison tests (values are means of n = 6).

3.2.12 MDA content

The MDA content, as a key parameter of lipid peroxidation indicator, was measured as to obtain the oxidative stress when wheat varieties were subjected to salt stress. The quantity of MDA was accumulated when all 4 wheat varieties experienced different levels of salt stress for 21days. As revealed in Figure 20 (A), the MDA content was found to be close for each wheat variety under all levels of salinity. Alderon, Cochise, Najran and Sama were recorded 12.09, 13.72, 12.27 and 13.56 nmol/g FW, respectively. Conversely, as seen in Figure 20 (B), there was a significant difference for MDA content under 0, 80, 160 mM NaCl. The MDA contents for all wheat varieties under each of 0, 80, 160 mM NaCl was 9.72, 12.55 and 16.47 nmol/g FW. The two-way ANOVA was used to test wheat varieties, salt treatment and their interaction as well. It was confirmed that there was statistically significant main effect of salt treatment on MDA content ($F = 31.08$, $P < 0.0005$). As shown in Figure 20 (B), there was significant difference ($P < 0.005$) on MDA content between 0 and 80 mM NaCl, and there was significant difference between 0 and 160 mM NaCl ($P < 0.005$). However, there was no significant difference ($F = 1.46$, $P < 0.233$) between wheat variety on their MDA content. Furthermore, there was no significant interaction between variety and salinity on MDA content ($F= 0.739$, $P < 0.620$). It seems all wheat varieties effect via salt level but there was no variation on MDA content amongst genotypes.

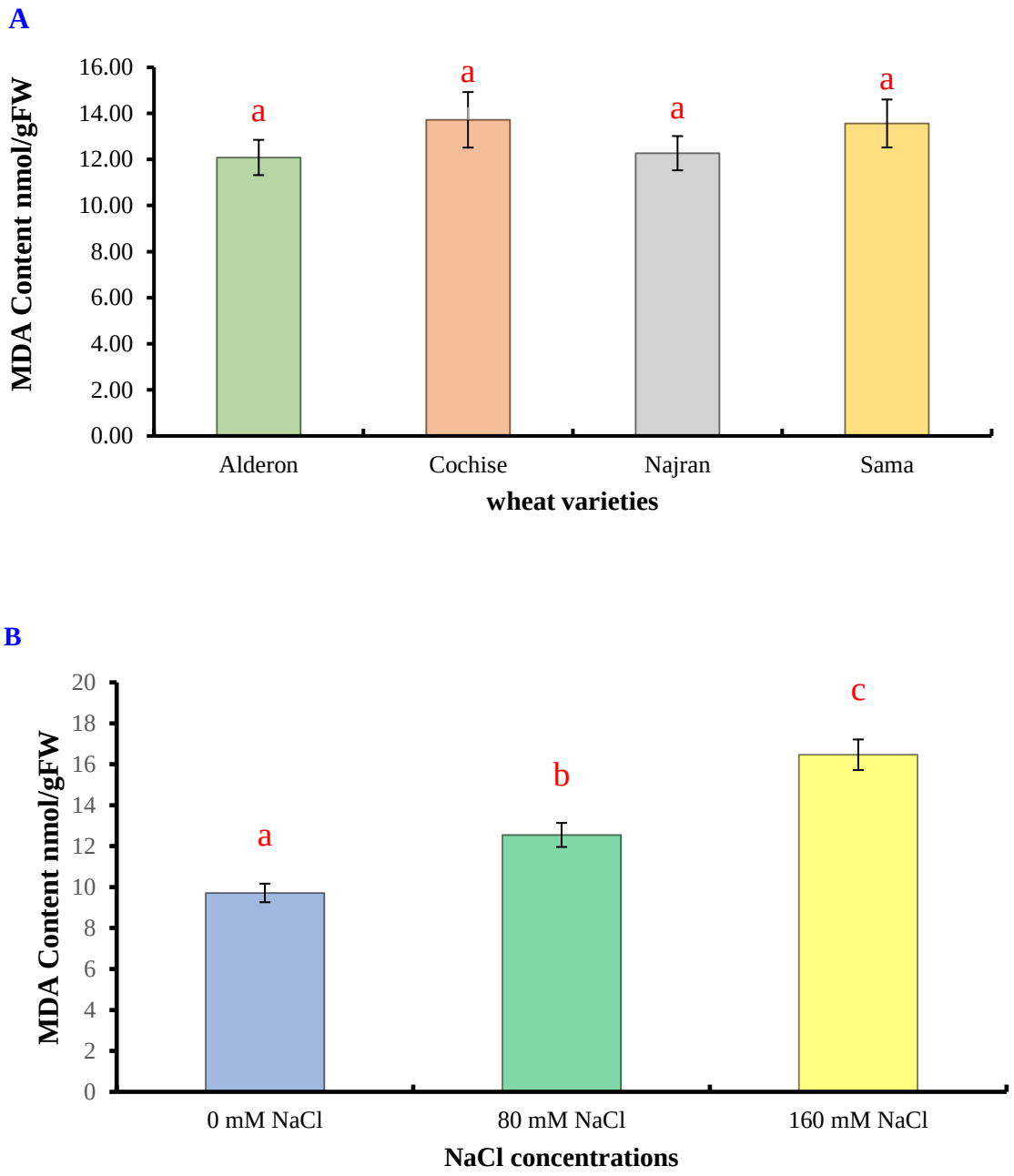

Figure 20 MDA content variation according to wheat varieties (**A**) and NaCl concentration treatment (B). The leaf area of 4 wheat varieties measured after growth at 0, 80, and 160 mM NaCl for 21days in sand culture is displayed. Varietal effect (A), leaf area of each wheat variety under 3 NaCl concentrations represents mean ± standard error (6 biological replications/ treatment). NaCl concentration effect (B), leaf area of 4 wheat varieties through each salt level represents mean ± standard error (6 biological replications/ wheat variety). Using two-way ANOVA, values with different letters are significantly different at $P < 0.05$, based on Tukey multiple comparison test.

Table 4 Correlations among different growth and physiological characters of the four selected wheat genotypes grown under increasing NaCl stress levels. Correlation is significant at the 0.01(**) and 0.05 level(*).Green colour displays positive correlation and blue colour represent negative correlation. The analysis performed by SPSS is a Correlation Coefficient

	SL	LN	LA	CC	RWC	FW	DW	Na^+	K^+	Na^+/K^+	Proline	MDA
SL	1											
LN	-0.048	1										
LA	0.769**	0.218	1									
CC	0.381**	0.500**	0.640**	1								
RWC	0.145	0.674**	0.388**	0.583**	1							
FW	0.276*	0.576**	0.536**	0.706**	0.656**	1						
DW	0.385**	0.481**	0.655**	0.707**	0.599**	0.800**	1					
Na^+	-0.061	-0.773**	-0.347**	-0.618**	-0.725**	-0.743**	-0.700**	1				
K^+	-0.187	0.685**	0.031	0.330**	0.499**	0.561**	0.451**	-0.697**	1			
Na^+/K^+	0.138	-0.626**	-0.093	-0.409**	-0.526**	-0.472**	-0.403**	0.756**	-0.738**	1		
Proline	-0.429**	-0.504**	-0.527**	-0.467**	-0.571**	-0.563**	-0.511**	0.606**	-0.435**	0.503**	1	
MDA	-0.118	-0.549**	-0.293*	-0.542**	-0.607**	-0.598**	-0.543**	0.604**	-0.407**	0.343**	0.447**	1

The subsequent section describes the types of correlation which different parameters had with each other under salt stress:

As observed in Table 4, the statistical analysis illustrated that the shoot length was strongly significant ($P<0.01$) and positively correlated with each of the following: leaf area, chlorophyll content, and dry weight. Contrarily, the shoot length was strongly significant ($P < 0.01$) and negatively correlated with proline content. Moreover, the leaf number was shown (Table 4) to have strong positive and negative correlations ($P<0.01$) with various different parameters individually, as are explained. There was significant positive correlation between leaf number and each of chlorophyll content, relative water content, fresh weight, dry weight, K^+ content. On the other hand, there was a strong significant ($P < 0.01$) negative correlation between leaf number and each of Na^+ content, Na^+/K^+ ratio, proline content, and MDA content.

Furthermore, the experiment's statistical analysis disclosed that the correlation between leaf area with each of the following parameters had been positive and significant ($P < 0.01$): shoot length, chlorophyll content, relative water content, fresh weight, and dry weight. On the contrary, there was a strong negative ($P < 0.01$) correlation between leaf area and each of the Na^+ content and proline content. Notably, concerning chlorophyll content, a very significant ($P < 0.01$) positive correlation had been seen between it and shoot length, leaf number, leaf area, relative water content, fresh weight, and dry weight, individually. In contrast, correlation between chlorophyll content and each of the following was highly significant ($P < 0.01$) and negative: Na^+ content, Na^+/K^+ ratio, proline content, and MDA content.

Based on the statistical analysis done within the current research, the correlation coefficient calculated between RWC and each of the studied parameters was highly significant ($P<0.01$) and positive such as leaf number, leaf area, chlorophyll content, fresh weight, dry weight, and K^+ content. On the contrary, it was significantly negative($P<0.01$) for every trait remaining such as Na^+ content, Na^+/K^+ ratio, proline content, and MDA content (Table 4). As can be seen in Table 4 there is a very significant ($P < 0.01$) and independent correlation among various traits under salt stress with fresh weight. Very positive correlations were observed between fresh weight and each of the following parameters: Leaf area, K^+ content, chlorophyll content, leaf number, and relative water content. In contrast, negative correlation between fresh weight and each of the following parameters had been seen: Na^+ content, Na^+/K^+ ratio, proline content, and MDA content. Noticeably, dry weight's correlations were very close to fresh weight when under salt stress

(Table 4). In fact, there are highly significant ($P < 0.01$) correlations observed between various traits under salt stress and dry weight. Positive correlations were observed between dry weight and each of the subsequent parameters: Relative water content, shoot length, chlorophyll content, leaf number, leaf area, and K+ content. On the contrary, there is negative correlation between dry weight and Na^+ content, Na^+/K^+ ratio, proline content, and MDA content. Particularly, Na^+ content was also under scrutiny in our experiments.

As can be seen in Table 4, it appears that there is a very significant ($P < 0.01$) correlation between Na^+ content and many traits under salt stress. There are existed independent positive correlations were between Na^+ content and each of the following parameters: Na^+/K^+ ratio, proline content, and MDA content. The rest of parameters such as leaf area, leaf number, chlorophyl content, relative water content, fresh weight, dry weight, and K^+ content were negatively correlated with sodium concentration (separately). As demonstrated by Table 4, a strong significant ($P < 0.01$) positive correlation exists between K^+ content and each of the leaf number, chlorophyll content, relative water content, and fresh weight, and dry weight. Conversely, it was noticed that negative independent correlations existed between K^+ content and each of the following parameters: Na^+ content, Na^+/K^+ content, proline content, and MDA content.

After analysing Na^+ and K^+, the Na^+/K^+ ratio was under examination. Table 4 showed that there is a high significant ($P < 0.01$) positive correlation between $Na^{+/}K^+$ ratio and each of Na^+, proline, and MDA content. The remaining traits namely leaf number, chlorophyll content, relative water content, fresh weight, dry weight, and K^+ content were negatively correlated with the Na^+/K^+ ratio (independently). During the time of intensified concentration of salinity, proline accumulation showed strong significant ($P < 0.01$) and positive correlation with Na^+ content, Na^+/K^+ ratio, and MDA content, independently. A negative correlation, on the other hand, was observed between proline content and each of the following parameters: relative water content, leaf number, chlorophyl content, dry weight, fresh weight, and K^+ content. Regarding MDA, a high significant ($P < 0.01$) positive correlation had been noticed observed between MDA and each of Na^+ content, Na^+/K^+ ratio, and proline content. The remaining studied parameters, namely leaf number, chlorophyll content, relative water content, fresh weight, dry weight, and K^+ content were negatively correlated with MDA content under increased salinity (independently)

3.2.13 The impact of salt stress on WRKY gene expression in wheat leaves revealed via quantitative PCR (qPCR)

3.2.14 Phylogenetic tree analysis

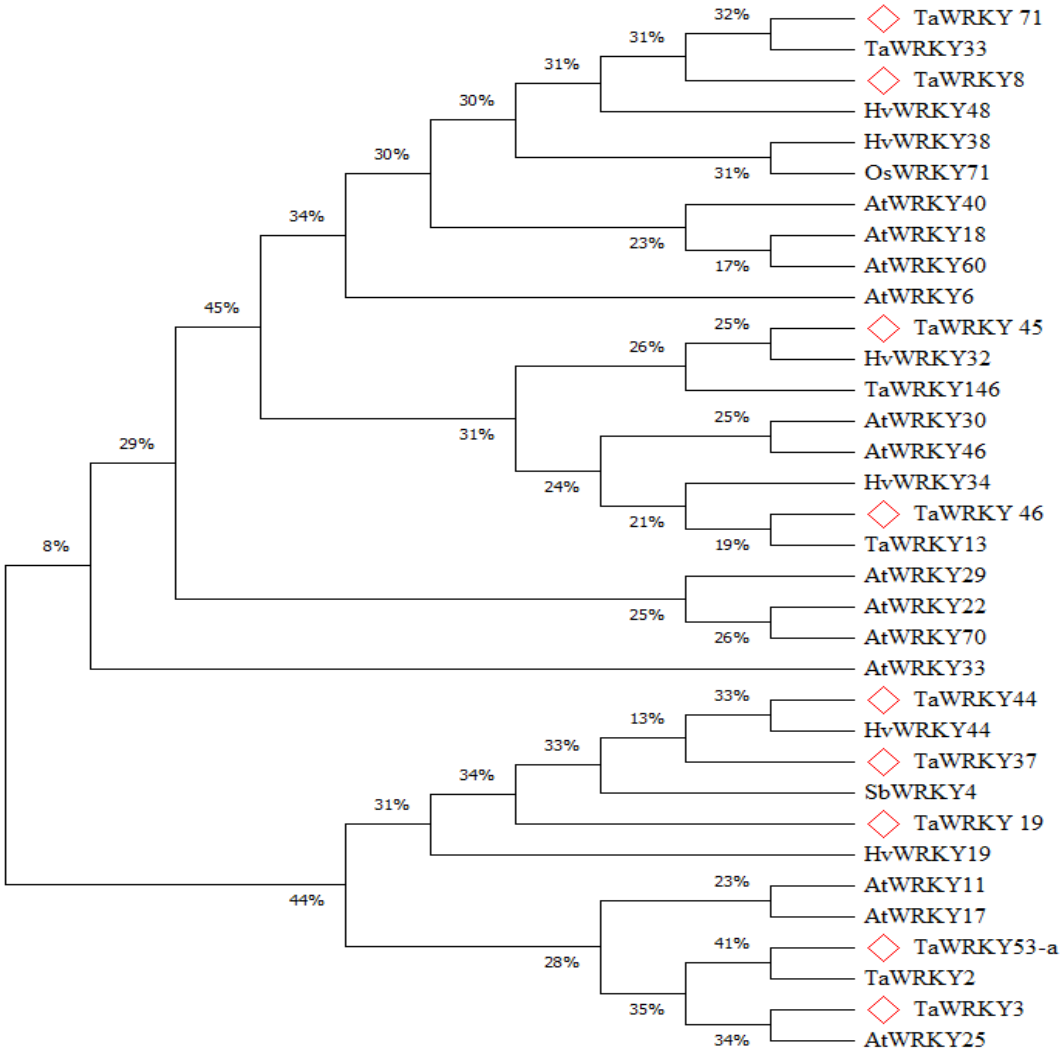

Figure 21 Phylogenetic tree of the WRKY domains different plant species illustrates WRKY's relationships within the wheat crop with other plant species (*Arabidopsis thalian, Hordeum vulgar , Sorghum biocolor, Oryza sativa*) that have WRKY proteins. In order to create the phylogenetic tree WRKY amino acid have been submitted to MEGA4.0 software. ◇ represented WRKY genes in wheat using for qPCR in this study.

In Figure 21, these have been indicated via a red diamond shape and were the ones chosen for qPCR. The obtained gene sequence was selected from National Centre for Biotechnology Information (NCBI) database. The following WRKY TFs in wheat were selected to examine salt tolerance: *TaWRKY3, TaWRKY8, TaWRKY19, TaWRKY37, TaWRKY44, TaWRKY45, TaWRKY46, TaWRKY53-a, and TaWRKY71* candidate genes were chosen based on how close these genes are with other genes in diverse plant species (e.g., *Arabidopsis thalian, Hordeum vulgar, Sorghum biocolor, Oryza sativa)* (Asai *et al.*, 2002; Mare *et al.*, 2004; Journot-Catalino *et al.*, 2006; Xie *et al.*, 2006; Knoth *et al.*, 2007; Mangelsen *et al.*, 2008; Wu *et al.*, 2008; Jiang and Deyholos, 2009; Chen *et al.*, 2010; Fu and Yu, 2010; Kasajima *et al.*, 2010; Zhou *et al.*, 2011; Besseau *et al.*, 2012; Niu *et al.*, 2012; Van Eck *et al.*, 2014; Ding *et al.*, 2015; Wang *et al.*, 2015; He *et al.*, 2016; Kloth *et al.*, 2016; Ma *et al.*, 2017; Ali *et al.*, 2018; Ahmadi *et al.*, 2019; El-Esawi *et al.*, 2019; Zhou *et al.*, 2019; Li *et al.*, 2020c; Gowayed and Abd El-Moneim, 2021; Zheng *et al.*, 2021). For example, *AtWRKY25* was found to be associated with salt stress (Jiang and Deyholos, 2009) , having close orthologs with *TaWRKY3*. TaWRKY2 that involved with salt response (Niu *et al.*, 2012) was homologous with *TaWRKY53-a.* The literature has revealed that *AtWRKY11* and *17* were engaged with ABA (Abscisic acid), salt stress, and osmotic stress (Ali *et al.*, 2018). In addition, it was observed that *HvWRKY38* has a role against drought and cold stresses (Mare *et al.*, 2004).(all close with *TaWRKY3* and *TaWRKY53-a*). *OsWRKY71* was seen to be involved in hormonal signalling responses such as ABA and GA (Xie *et al.*, 2006). Additionally, *HvWRKY48* had role in plant immunity (Meng and Wise, 2012). *AtWRKY18, 40,* and *60* were also engaged with ABA and other abiotic stresses (e.g., osmotic and salt stress) (Chen *et al.*, 2010) . Moreover, *AtWRKY6* contributed to the response against mineral deficiency (boron) (Kasajima *et al.*, 2010) (all close to *TaWRKY8* and *71*). *HvWRKY32* participated in the response to salt stress (Zheng *et al.*, 2021). This gene was orthologous with *TaWRKY45*. It was also found that *TaWRKY146* had a role in reactions towards drought stress (Ma *et al.*, 2017). In addition, studies showed that *TaWRKY13* had involvement in response to salt stress (Zhou *et al.*, 2019) (all close to *TaWRKY45*).

Furthermore, *HvWRKY34* was mentioned to be engaged in the plant reaction against sugar stress (Kumar *et al.*, 2020), *AtWRKY33* was response to salt, NaCl, osmotic stress and ABA (Fu and Yu, 2010). *TaWRKY13* was play role in salt stress (Zhou *et al.*, 2019) . all these genes are close to *TaWRKY46*. *HvWRKY44* was shown to have engagement in early developmental phase

for grain in barely (Zheng *et al.*, 2021) was close to *TaWRKY44*. In addition, *HvWRKY 19* was engaged in plant immunity (Meng and Wise, 2012) and *AtWRKY17*, *11* in abiotic stress (salt, ABA, osmotic stress) (Ali *et al.*, 2018) *SbWRKY4* has been identified in *Sorghum bicolor* which has ability to grow in dry and hot lands (Ahmadi *et al.*, 2019; Baillo *et al.*, 2020). All these genes close to *TaWRKY19* and *37,44*

3.2.15 The differential expression of WRKY genes following high salt concentration

3.2.16 WRKY gene expression of Alderon

To determine the expression of WRKY genes, as shown in Figure 21, in Alderon wheat variety, the real time quantitative PCR (RT-qPCR) was utilised to compare the expression patterns of these WRKY genes in Alderon, which can be observed in Figure 22. The results showed that there were changes in expression profiles of all genes after the variety was exposed to of 160 mM NaCl for 21 days compare to plants not exposed to elevated NaCl concentrations. Firstly, In Alderon, expression levels for 7 of the 9 WRKYs genes increased. For example, *TaWRKY53-a* showed the highest expression pattern with upregulation relative to *TaWRKY53-a* in unstressed wheat. The fold induction of *TaWRKY53-a* was greater than 4-fold upregulation. Additionally, the differential level of *TaWRKY3* and *TaWRKY8* expression was similar to each other, and their induction was upregulated in 3 and 3.6-fold changes, respectively, relative to the *TaWRKY3* and *TaWRKY8*, in unstressed wheat. Moreover, the expression of *TaWRKY46* and *TaWRKY45* levels had similar patterns in their expression. The relative expression of these genes was recorded 2.85and 2.4more fold change respectively, relative to the same genes in control plant (unstressed wheat). Firstly, the fold change detected for *TaWRKY19*, *37* and *44* were below 2.0 and therefore, due to the sensitivity of the qPCR assay, are considered not demonstrate expression levels that differ from those seen in the control plants. Secondly, only two WRKY genes had downregulation in their expression (*TaWRKY44*, *TaWRKY71*). The fold change in *TaWRKY71* was seen to be greater than 2-fold down (2.22-fold). Statistical analysis (t-test) confirmed that *TaWRKY 45*, *46,53-a* and 71 expressions were significant ($P<0.05$) difference with these genes in unstressed plants (using delta Ct values)

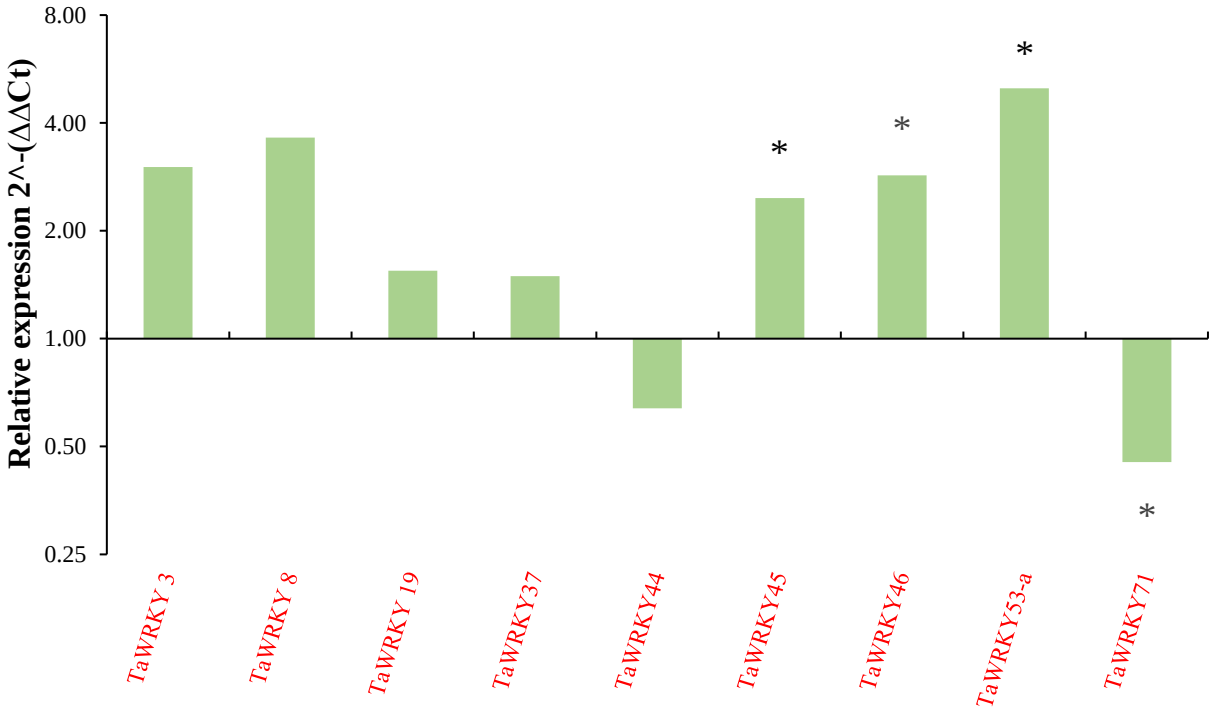

Figure 22 *TaWRKY* expression profiles in Alderon variety following exposure to 160 mM NaCl for 21 days.For the Alderon variety, different expression profiles of the following genes during 21days of being subjected to 160 mM NaCl have been normalised to *TaEF1α*: *TaWRKY3*, *TaWRKY8*, *TaWRKY19*, *TaWRKY37*, *TaWRKY44*, *TaWRKY45*, *TaWRKY46*, *TaWRKY53-a*, and *TaWRKY71*. Thereafter, the aforementioned genes were later normalised to the control treatment containing 0 mM NaC. It must be noted that averages/means of 3- biological replicates are signified by the values and the t-test was used to determine significant in gene expression between uninfected leaves and infected leaves using delta Ct value *: P<0.05, **:P<0.01,*** P<0.001).

3.2.17 The expression of the WRKY gene in Cochise wheat variety

In Figure 23, the expression of WRKY genes in Cochise wheat variety, after exposure to 160 mM NaCl for 21 days, showed differential gene expression under salt stress. The alteration of the gene expression showed upregulation and downregulation. The most interesting two genes with upregulation were *TaWRKY37* and *TaWRKY46*, which were the highest expressed among other WRKY genes. More than 6-fold increase in expression was

recorded, relative to their control. In addition, some other genes were similar in their expression profile pattern. *TaWRKY8*, *TaWRKY19*, *TaWRKY44*, and *TaWRKY45* showed an approximate fold change of 2-2.6, relative to the same genes in unstressed plant. In contrast, *TaWRKY3* and *TaWRKY53-a* showed down regulation in their expression pattern, 1.88 and 1.31-fold respectively, in comparison to unstressed tissue. These values are outside the sensitivity of the assay and might not indicate a change in gene expression. In addition, *TaWRKY71* was not expressed in stressed tissue, as compared unstressed tissue. The fold change detected for *TaWRKY3*, *53a*, *71*, and *44* were below 2.0 and therefore, due to the sensitivity of the qPCR assay, are considered not demonstrate expression levels that differ from those seen in the control plants. Statistical analysis (t-test) confirmed that *TaWRKY 37* and *46* expressions were significant (P<0.05) difference with *TaWRKY 37* and *46* expression unstressed plants (using delta Ct values)

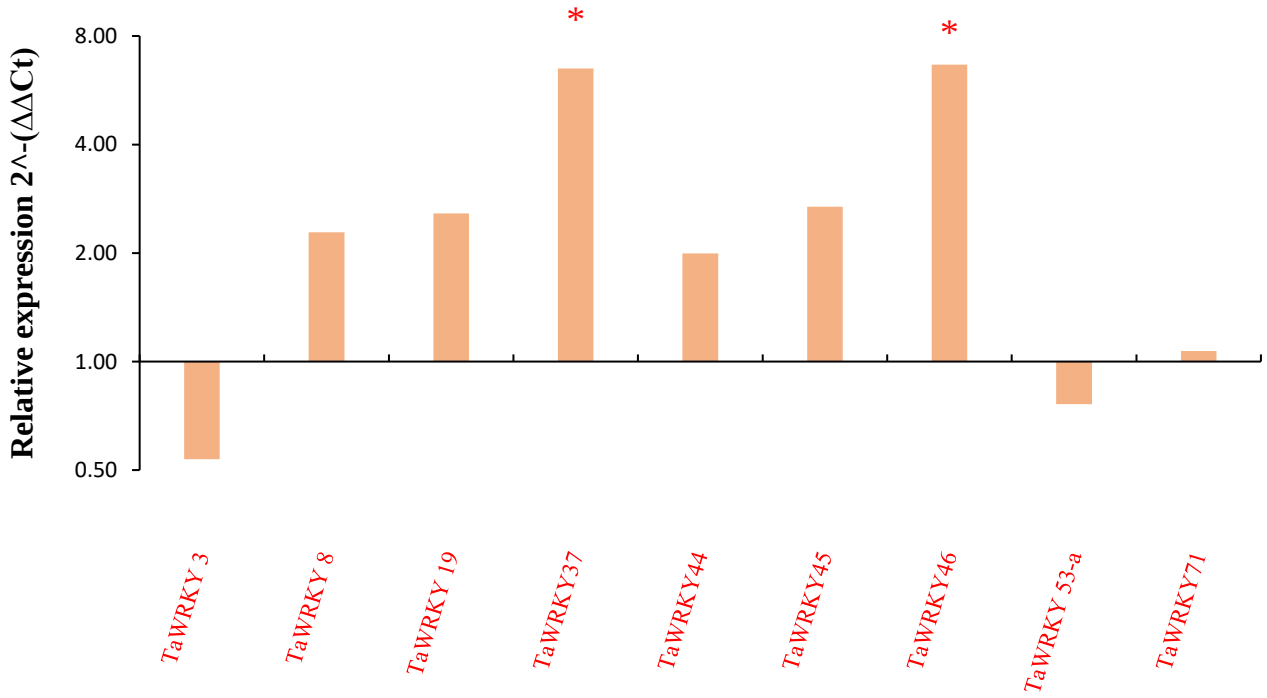

Figure 23 WRKY expressional profiles in Cochise variety following exposure to 160 mM NaCl for 21 days.Nine WRKY genes' expression levels under salt stress (160mM NaCl) for 21days within Cochise leaves, were normalised to the reference gene (*TaEF1α*), and also normalise to the control treatment It must be noted that averages/means of 3- biological replicates are signified by the values and the t-test was used to determine significant in gene expression between uninfected leaves and infected leaves using delta Ct value *: P<0.05, **:P<0.01,*** P<0.001) .

3.2.18 WRKY gene expression in Sama variety

In Figure 24 Sama wheat varieties, the findings revealed that *TaWRKY53-a* and *TaWRKY37* were negatively expressed with down regulation (relative to the control). To elaborate, there was a 25-fold change in the downregulation pattern for *TaWRKY53-a*, while, *TaWRKY37* showed 4-fold change. For some genes, the level of their expression was not very high in upregulation, such as *TaWRKY3* and *TaWRKY8*. In contrast, *TaWRKY19, TaWRKY71*, and *TaWRKY45* displayed the highest genes expressed with an upregulation. The level of their expression was recorded to 5.7 -, 4.8- and 3.23-fold change respectively, in stressed tissue, respectively (compared to their control in unstressed tissue). As for *TaWRKY 3, TaWRKY8*, and *TaWRKY46*, the increase was seen to be from 1 to 1.5-fold changes, relative to their control. The fold change detected for *TaWRKY3, 8,* and *46* were below 2.0 and therefore, due to the sensitivity of the qPCR assay, are considered not demonstrate expression levels that differ from those seen in the control plants. Based on t-test analysis we found that *TaWRKY 71* expression was significant (P<0.05) difference with *TaWRKY 71* in unstressed plants (using delta Ct values).

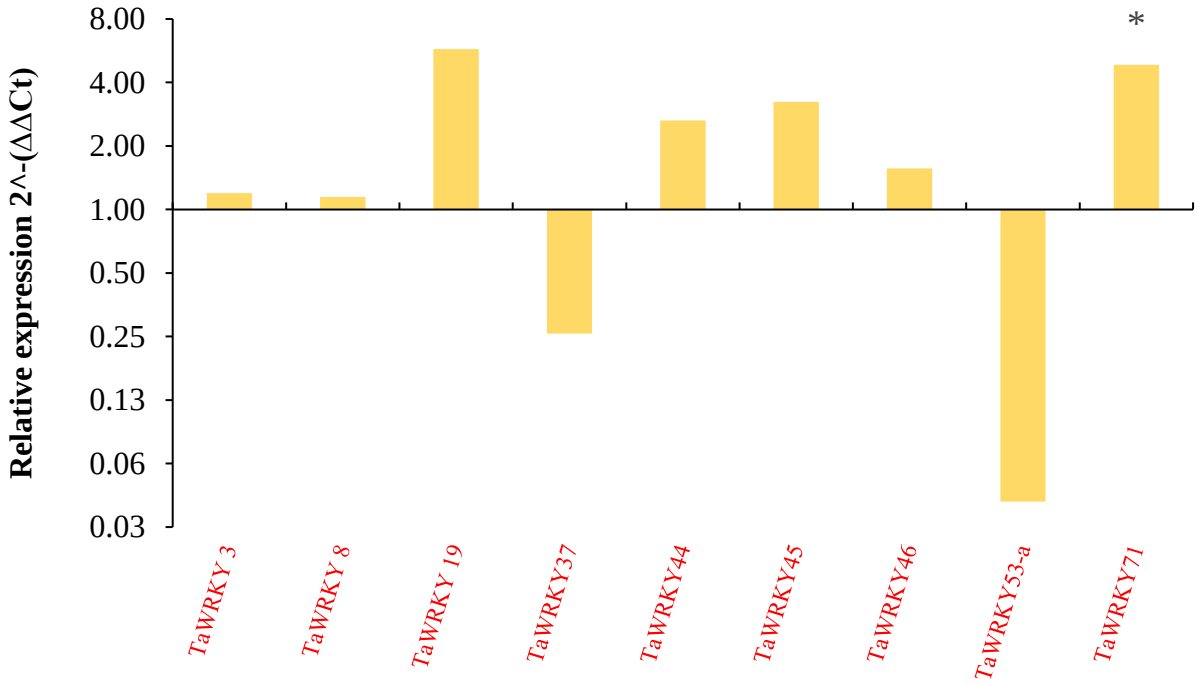

Figure 24 WRKY expressional profiles in Sama variety. Various expression profiles from the 9 WRKY genes of Sama were subjected to a period of 21days under salt condition (160 mM NaCl). The 9 genes were normalised to the reference gene (*TaEF1α*). Following that, all the WRKY

genes were normalised with the WRKY gene in the control treatment. It must be indicated that averages/means of 3- biological replicates are signified by the values and the t-test was used to determine significant in gene expression between uninfected leaves and infected leaves using delta Ct value *: P<0.05, **:P<0.01,*** P<0.001).

3.2.19 WRKY gene expression in Najran variety

The level of gene expression in the Najran genotype following 160 mM NaCl application throughout 21 days led to mostly upregulation, as a response to salt stress, except for the *TaWRKY71* gene that revealed to have down regulation (relative to control) as seen in Figure 25. The first interesting gene, *TaWRKY37*, showed the maximum expression pattern amongst WRKY genes. It revealed more than 20-fold change with upregulation (compared to control). The second interesting gene in its expression was *TaWRKY 46*, with more than a 12-fold change with upregulation (compared to control). The third interesting gene was *TaWRKY53-a*, having more than 8-fold change with upregulation (compared to control). The expression levels of *TaWRKY3*, *TaWRKY19*, and *TaWRKY45* had 3.44, 3.73, and 2.96-fold upregulation, respectively, as compared to their control. *TaWRKY44* showed the lowest (1.30-fold) upregulation compared to other WRKY gene expressions. The fold change detected for *TaWRKY44*, and 71 were below 2.0 and therefore, due to the sensitivity of the qPCR assay, are considered not demonstrate expression levels that differ from those seen in the control plants. The only WRKY gene that experienced downregulation was *TaWRKY71* at 0.63-fold. Based on t-test analysis we can see *TaWRKY 37* and *54-a* expression were significant (P<0.05) difference with the expression of these genes in unstressed plants (using delta Ct values).

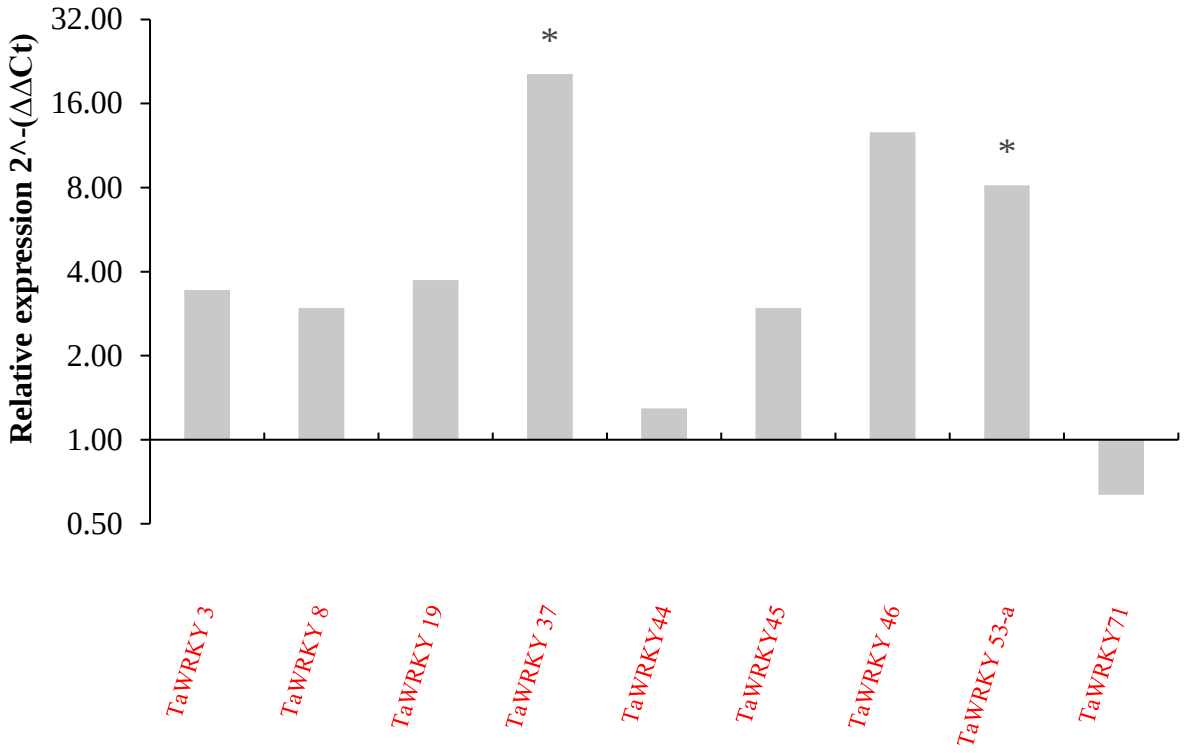

Figure 25 WRKY expressional profiles in Najran variety. In regard to the Najran wheat variety that has been exposed to salinity (160 mM NaCl, 21 days), the expression levels of 9 genes were analysed and normalised to *TaEF1α* (internal reference gene). Then, they were compared with the 0mM NaCl (control treatment) It must be indicated that averages/means of 3- biological replicatesare signified by the values. The t-test was used to determine significant in gene expression between uninfected leaves and infected leaves using delta Ct value *: P<0.05, **:P<0.01,*** P<0.001).

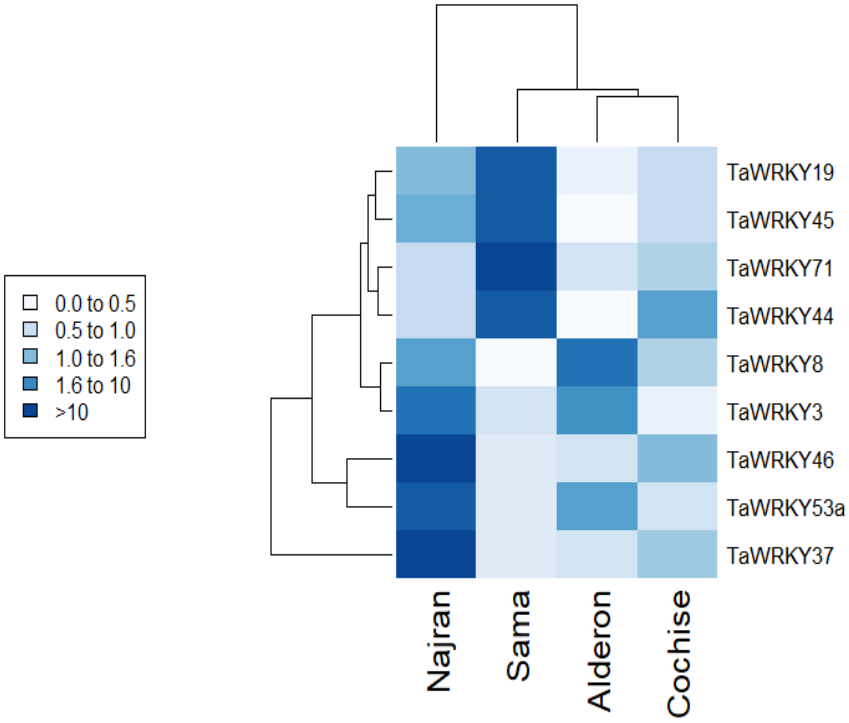

Figure 26 Heatmap represent 9 WRKY gene expression in four wheat varieties under salt condition. Heatmap display change in nine WRKY gene expression amongst varieties under 160 mM NaCl treatments. Blue Dark colour represent higher expression and blue bright colour, lower expression. The colour key signifies the values of fold change in expression of WRKY genes under salt stress.

The complex expression profiles displayed in Figure 22, Figure 23, Figure 24 and Figure can be summarised in a heatmap to quickly visualise the response of the four varieties to elevated salinity. The blue dark colour represents high expression, while light blue denotes low expression. Inside the heatmap, the rows stand for different WRKY genes, and the columns signify wheat varieties. The dendrograms indicate similarity of all gene expression. It was shown that the two UK varieties, Alderon and Cochise, were close in their gene expression while Sama was more similar to Alderon and Cochise than to the other Saudi wheat variety. Notably, gene expression in Najran was dissimilar to the other wheat varieties with *TaWRKY37*, *53a*, *46* and 3 showing consistently high expression (Figure 26).

3.3 Discussion

A prominent abiotic stress, known globally in the agricultural field, is salt stress as it hinders crop development, growth, and yield (Liang *et al.*, 2018). That is why for enhancing productivity in crops of strategic value, such as wheat, knowledge of molecular/physiological alterations in the response of wheat to this stress can contribute towards comprehending the processes engaged within the phenomenon, known as salt tolerance. This is particularly beneficial as to identify a candidate marker relevant to salt tolerance selection and breeding (Singh *et al.*, 2020). Moreover, variation in plant growth will lead to highlighting genetic diversity and how different wheat varieties can respond to salt stress (based on genetic variation) (Oyiga *et al.*, 2016).

The current research aims to describe the characteristics of plant behaviour through evaluating the effect of salt stress on many phenotypes such as water relations, growth, ion homeostasis, photosynthesis, and osmoregulation. In this study, numerous morphological, physiological, and biochemical parameters were used to assess the response and behaviour of Alderon, Cochise, Najran, and Sama wheat varieties under salinity conditions (i.e., shoot length, number of leaves, leaf area, chlorophyll content, RWC, shoot fresh weight and shoot dry weight, Na^+ content, K^+ content, Na^+/K^+ ratio, proline content, and MDA content).

3.3.3 The effect of salt concentration on shoot length

Adverse effect on shoot growth is a normal response to salt stress and water shortage. However, how much seedlings could withstand the stress is dependent on the natural genetic features of cultivars (Kaur *et al.*, 2018)**.** It was observed in the present research that salinity (i.e., salt stress) resulted in a significant ($P < 0.05$) decline in the shoot length of all four wheat varieties (compared to control). In that regard, the impact of salt stress was clear with both salinity applications (80, 160 mM NaCl) on shoot length for the 4 wheat varieties. Yet, the increase in shoot length reduction was not changed for 160 mM NaCl in all wheat varieties, when compared to 80 mM (Figure 9 B). This result, which showed salt concentration reducing the shoot length, is in accordance with findings obtained by Bacu *et al.* (2020), who described the impact of salt concentration in inhibiting shoot length (using 0, 50, 100, 200 mM NaCl in modified Hoagland solution). Moreover, the study by Bacu *et al.* (2020) also found a wheat variety, named Viktoria, which was constant in shoot length when the NaCl concentration increased from 50 to 200 mM NaCl (signifying medium tolerance) .In our findings, we found that

all wheat varieties were affected by salt stress in shoot length reduction. However, once salt concentration was raised from 80 mM to 160 mM, we could not find any variation between wheat varieties. In the regard of wheat varieties, we can observe Najran was recorded at the lowest value and this is related to the behaviour of the genotype (short steam) while other varieties were similar in their shoot length as seen in Figure 9 (A).

It is important to state the mechanisms behind reduction in shoot length, when plants are exposed to salt stress: First of all, the osmotic impact from the surrounding salt to the roots decreases the capability of root cells in absorbing nutrients and water. Furthermore, the toxic impact, due to salt build-up through time, in transpiring leaves, leads to damage in them (Munns, 2005; Munns and Tester, 2008).

3.3.4 The impact of salt concentration on number of leaves

The total amount of leaves is considered as one of the growth characteristics, which is influenced by salinity. The rate of leaf number, which rises in salinity, can lead the plant to potentially become more productive (Munns, 2002). Based on our observations, it has been shown that leaf numbers are reduced as salt rises in both of the treatments, for all wheat varieties (80, 160 Mm NaCl) as shown in Figure 10 (B). For salt treatments, the number of leaves, for every wheat variety, diminished. Nevertheless, Sama and Najran had more leaf numbers, as compared to Cochise and Alderon. Najran was found to have the maximum number of leaves, when compared to the rest of the varieties as shown in Figure 10 (A). These findings agree with the experiment done by Ouhaddach *et al.* (2018), which found that salinity decreased leaf numbers when the Salama variety was exposed to treatments (0, 50, 75, 100 mM NaCl) for the duration of 4-6 weeks. The hindrance for leaf growth which is salt sensitive seems to be due to another factor as well; the salt impeding symplastic xylem loading of Ca^{2+} in the root of plants (Carillo *et al.*, 2011). Another study that showed that the barley plant was exposed to 3-weeks of salt stress under 0, 100, and 200 mM NaCl. However, it was found that under 100 mM concentration (moderate salinity), the Giza cultivar had limited or approximately no decrease in overall leaf quantity, when compared to the 200 mM concentration, which had the highest reduction of leaf numbers (compared to control). The barley plant was exposed to 3-weeks of salt stress under 0, 100, and 200 mM NaCl. However, it was found that under 100 mM concentration (moderate salinity), the Giza cultivar had limited or approximately no decrease in overall leaf

quantity, when compared to the 200 mM concentration, which had the highest reduction of leaf numbers (compared to control) (Khalil *et al.*, 2021).

The Najran variety had shown the capability to preserve a number of leaves. It also enabled ion toxicity to be accumulated inside the lower leaves. Moreover, this variety might possess a high efficiency in its metabolism. The underlying processes has been explained by Munns (2002) who stated that the higher the species are in terms of salt tolerance, the less salt they absorb. Importantly, reduction in number of leaves might be a result of NaCl accumulating inside cytoplasm (of older leaves) and cell walls (Qados, 2011). The current study has confirmed this matter, expressing the correlation between Na^+ concentration and quantity of leaves (Table 4). This table displays a strong negative correlation ($P<0.001$) between number of leaves and Na^+ content ($R= -0.77$).

3.3.5 The effect of salt concentration on leaf area

The main impact coming from salt stress has been recorded to be placed upon total leaf area, for cereals (Munns and Tester, 2008). This diminution of leaf area, because of salt stress, can be interpreted as, photosynthesis per plant is always being lessened. If a water supply is secured, expansion of leaf area would become more productive (e.g., irrigated food production systems) (Munns and Tester, 2008). On that account, it can be stated that plant leaf area is a reliable marker for salinity and water stress, as expansion of leaf area usually needs great turgor pressure for the process of cell enlargement (Munns and Tester, 2008). In the present results, it was seen that all varieties experienced a decrease in leaf area as salt concentration increased (Figure 11 B). The most reduction in leaf area for wheat varieties was observed when they were exposed to 160 Mm NaCl. For instance, Najran had a maximum reduction (7 cm)while Alderon had a minimum reduction (18 cm)under all level of salt concentration (Figure 11 A).. This finding is in accord with Elhakem (2020) which saw a reduction in flag leaf area as salinity increased. This was evident in Elhakem (2020) found in his experiment with the increase from 80 to 160 mM NaCl for all wheat cultivars.

What must not be forgotten is that reduction in area of leaves may point to early senescence, declined growth rate, delayed emergence, or death (Netondo *et al.*, 2004)due to salts accumulating within the cytoplasm and preventing activity of enzymes. They could also accumulate within cell walls, dehydrating the cells. The rate of leaf death is critical for plant survival, if older leaves die quicker than new leaves emerge (i.e., the plant is in danger) (Munns,

2005). Decline in leaf area can adversely impact plant photosynthesis as well, which in turn, could cause plant development to be reduced (Qados, 2011). This point by Qados (2011) corresponds with the correlation analysis in my thesis (Table 4). It was shown that leaf area had a very significant ($P < 0.01$) positive correlation with chlorophyll content ($R=0.640$).

3.3.6 The effect of salt concentration on chlorophyll content

There is possibility for chlorophyll content to be utilised in order to examine salt tolerance of genotypes, due to corresponding with leaf damage and photosynthesis (Farhat *et al.*, 2020). In the results obtained within the current study (Figure 12 B), a reduction of chlorophyll content was observed as salinity escalated (80, 160 mM). However, difference in the decrease of chlorophyll content was seen among wheat varieties. One instance of tolerance was Alderon, which was affected less than the rest of the varieties, with lower reduction in chlorophyll content (8 SPAD unit), followed by Cochise, Sama and Najran (close to each other) (Figure 12 A). . This could be explained by the two mentioned varieties' attempt to preserve photosynthetic activity (Muranaka *et al.*, 2002). Najran followed by Sama and then Cochise were the most affected by all level of salt concentration (5, 6 and 7 SPAD unit respectively) in terms of chlorophyll content. This finding is in accord with the experiment done by Saddiq *et al.* (2021) which saw chlorophyl content index decreasing in all wheat genotypes with escalation of salt stress (100 mol/L and 200 mol/L).

As for an explanation to the reduction in chlorophyll content, the following applies: 1) Salts might accumulate within the apoplast, dehydrating the cells, 2) Salts can amass within the cytoplasm, hindering the enzyme engaged with carbohydrate metabolism, 3) There is a possibility for them to build up within the chloroplast, imposing a toxic impact upon photosynthesis (Munns and Tester, 2008). Concerning the variance of chlorophyll content amongst genotypes, certain genotypes having the capability for limiting salt ions from penetrating the plant system, could be a reason (genotypes that have low chlorophyll content are not successful in that regard) (Dong *et al.*, 2019).

As a matter of fact, there are species which are tolerant to salt, showing heightened/unaffected chlorophyll content among salt stress, whilst in species which are sensitive to salt, there is decrease in chlorophyll content. This implies that this factor can be seen as a sign, within plants, of tolerance towards salt (Stepien and Johnson, 2009; Ashraf and Harris, 2013).

Confirming the above explanation about salt accumulation and its impact on photosynthesis, our correlation analysis found that chlorophyll content was significantly and negatively correlated with Na+ ($P<0.001$; $R=-618$) as seen in Table 4. From the obtained results, it could be that Alderon appears more tolerant among the wheat varieties in terms of chlorophyll content. In addition, due to leaf area in Najran was shorter, it may be reflected to chlorophyll content.

3.3.7 The effect of salt concentration on RWC content

As a parameter which can be employed for inspecting salt tolerance in plants, RWC is an indicator of salt stress. In conditions wherein RWC is kept inside tissues/cells, a continuance of metabolism is enabled via osmotic modifications and further physiological characteristics related to salt stress (Mohamed *et al.*, 2020). Obtained results from this study (Figure 13 B) showed that every plant was seen to have a decrease of RWC when subjected to 80 mM treatment. Moreover, in the 160 mM treatment, not only was reduction of RWC witnessed, but also the reduction was much more than the 80 mM treatment. It must be noted that there was no disparity amongst varieties in terms of RWC as seen in Figure 13 (A). However, this observation is quite frequent, when considering decline of RWC within salt stress.

Our experimental results are in accord with those of Elhakem (2020), in terms of decrease in leaf relative water content of plants subjected to NaCl treatments and also, having higher reduction when salt concentration increases (compared to their control). In order to explain RWC decline, organic solutes can be considered. Owing to the rise in solute content of cells within plants that have been treated with salt, more water is absorbed compared to control leaves, which causes low RWC (Munns *et al.*, 2006). It can be understood, from our experiments, that RWC is a reliable indicator for salt concentration. However, the experiment does not show any obvious variation of RWC amongst genotypes. Providing more explanation regarding the relationship between solute content and RWC, our analysis found that the correlation existing between RWC and proline was negative and significant ($R=-0.571$), as shown in Table 4.

3.3.8 The effect of salt concentration on shoot fresh weight and dry weight (FW and DW)

This dissertation's experiments showed that fresh weight and dry weight reduced with salt concentration increasing in all wheat varieties (Figure 14 and Figure 15). These two parameters were reduced at 80 mM and also, the reduction was obvious for 160 mM NaCl. Sama was shown to have the most adverse effect in FW reduction while others had the same effect from salt stress.

In terms of dry weight, levels of salt concentration (80, 160 Mm NaCl) had effect on all varieties, but the reduction was obvious at 160 Mm NaCl. Regarding changes in dry weight, Najran and Alderon were shown to have better performance, as compared with others, while the remaining wheat varieties, Cochise and Sama, had more effects from salt stress.

Bhutto *et al.* (2019) in his research is also in alignment with this thesis as they observed that

shoot fresh weight was affected by the different concentrations of NaCl, with the results showing that it decreased significantly with increasing salinity levels in all wheat varieties (using distilled water as control; 0, 9, 12 and 16 dSm^{-1} treatment).

Another scholarship (Elhakem, 2020) evaluated the reduction in shoot FW and DW following NaCl treatment (160 mM NaCl) and discovered that there was significant decline. Specifically, the effect on FW was varied for all wheat plants. Notably, when compared to untreated, the smallest decrease was observed in Sakha69, followed by Giza168, and the greatest decrease was observed in Sakha8. Additionally, the highest decline in shoot DW was observed in Sakha8, with Giza168 coming after, and the lowest decline seen in Sakha69. These results were found to resonate with the current research's findings as Najran and Alderon displayed more tolerance for salt stress, while Sama was shown to be the most damaged variety (sensitive to salt).

Our findings indicated that for Najran and Alderon, which had better performance in response to salinity (potentially, could be more salt tolerant), salt stress is linked with dry biomass, water status preservation, and chlorophyll content. Additionally, observing Najran showed that physiological processes of salt tolerance are established upon capability for limiting Na^+ absorption and exclusion, retaining high degree of K^+ content to perform osmoregulation, while balancing the Na^+/K^+ ratio near 1. Notably, what was also seen in Najran was high amount of proline, while Sama was shown to be the cultivar which salt stress had the highest impact on.

3.3.8 The effect of salt concentration on Na^+ content

It is commonly recognised that Na^+ is not a necessary ion in crops. Due to hydrated ionic radii of K^+ and Na^+ being close, Na^+ toxicity happens in crops amidst salinity. The build-up of high amounts of Na^+ inside the cytosol could lead to a shortage of K^+, and consequently, cause disruption in numerous enzymatic processes. In addition, it can also apply an energetic burden upon the plant cells, due to the need of organic solute synthesis for compensating Na^+ export for the purpose of osmotic adjustment (Wu, 2018).

In our experiment(Figure 16), sodium contents increased due to salinity in all wheat genotypes; notably, Alderon and Cochise genotypes maintained the highest Na^+ in their shoot, followed by Sama, whereas Najran had the lowest Na^+ content in its shoot (indicating salt-tolerance). The study by Khan *et al.* (2009) also found that the variety, Lu-26s, which had the lowest Na^+ accumulation, was indeed the most tolerant, compared to varieties with high Na^+ in their leaves . A relevant phenomenon, referred to as Salt injury, is caused by Na^+ or Cl^- (or both) gathering in transpiring leaves to extreme concentrations, surpassing the capability of plant cells to compartmentalize the aforementioned ions in the vacuole. Thereafter, ions quickly accumulate within the cytoplasm, impeding activities of enzymes (or ions accumulate within plant cell walls) (Munns, 2002). Observing our experiment within the current study, it was understood that there is a possibility for sodium exclusion being a mechanism for salt tolerance used by the Najran variety, in order to protect its tissues from toxicity. Such strategy was also found in the varieties studied by Khan *et al.* (2009), where they believe that restricting the absorption of Na^+, as toxic ions, was done by plants to increase tolerance. In line with that, James *et al.* (2011) found that Nax1 (*TmHKT1;4-A2*) gene can remove Na^+ from the xylem within lower sections of leaves, roots, and leaf sheaths.

Our experiment conveyed that salinity gave rise to shoot Na^+ content (Figure 16), but decline in K^+ (Figure 17) (which varied considerably amongst wheat genotypes). Further, salt-sensitive genotypes (all except Najran) demonstrated more shoot Na^+ content along with less K^+ content (variant among genotypes), in response to salt stress. The reason why Najran had lower Na^+ content can be attributed to plant cells having sodium accumulation, due to the equilibrium between ion channel inflow and outflow via a putative Na^+/H^+ antiporter (Tester and Davenport, 2003). An explanation as to Najran having the highest K^+ levels amongst varieties can be suggested by Cuin *et al.* (2008)who also viewed a comparable escalation in leaf K^+ content among wheat varieties subjected to salt stress. They demonstrated that alterations of K^+ content in tissues are a marker of K^+ behaviour inside the vacuole.

3.3.9 The impact of salt concentration on K^+ content

In the process of maintaining cell turgor and osmotic adjustment, potassium ions have a vital contribution. The cytosol and vacuole are considered as the main pools for these ions inside cells of crops. When facing deficient situations due to salinity, ROS might be created and cells damaged (Wang *et al.*, 2013b). The present research saw an inverse relationship between salt

stress and K^+ content, as increase in salinity reduced potassium content. Figure 17 (B) describes that for 80 and 160 mM NaCl, K^+ content declined, with the 160 mM treatment being the cause for the maximum decline of potassium in wheat shoots. Also, K^+ build up was seen to be variant in this dissertation's experiment, with the highest K^+ content belonging to Najran, Sama following right after, and the lowest being of Alderon as shown in Figure 20 (A).

As seen in this thesis, Najran performed a better osmoregulation, when compared to the rest of the varieties, by absorbing potassium and amassing it (i.e., Najran is potentially tolerant to salt stress). Our observation is in line with the explanations given by Colmer *et al.* (2005), in terms of K^+ absorption/maintenance being fundamental to activities of enzymes and also, it being a core characteristic of salt tolerance. Such fluctuations in potassium level were also observed by Saddiq *et al.* (2021), with their results being in alignment with the current result . Their experiment proved that K^+ content in leaves had lowered with the heightening of salinity levels in every wheat genotype.

Potassium is involved in certain physiological processes. It can act as a pH stabilizer. In fact, it is a very abundant cation in the cytoplasm. It balances immobile anions in cytoplasm, mobile anions in vacuoles as well as mobile anions in xylem and phloem (Jeschke *et al.*, 1985). In addition, it intervenes in the accumulation of organic acids. Potassium also plays an essential osmo-regulating role in maintaining the status of water in cells. It intervenes in the reduction of perspiration. The opening of the stomata is conditioned by a high concentration of K^+ in the guard cells. (Dietrich *et al.*, 2001). In addition, Increased photosynthetic assimilation, improved nutrient absorption, and proper leaf inclination maintained by turgor regulation are all benefits of an adequate supply of potassium (Carroll *et al.*, 1994).When plant exposed to salt and K^+ deficiency , K^+ deficit considerably enhanced the detrimental impacts that were generated by salt in the photosynthesis of barley, and this was linked to an increase in salt sensitivity(Degl'Innocenti *et al.*, 2009) it was revealed that a lack of K+ greatly hindered nitrogen and photosynthetic carbon absorption in maize under salt stress, in addition to damaging the light response pathways of PS I and PS II(Qu *et al.*, 2011; Qu *et al.*, 2012).

3.3.10 The effect of salt concentration on Na^+/K^+ ratio

It is reliable that lower Na^+ absorb and higher K^+ absorb are the crucial indicators of salinity tolerance in higher plants. Furthermore, preserving minimal shoot Na^+/K^+ ratio is an significant stress tolerance characteristic in some halophytes and tolerant glycophytes (Assaha *et*

al., 2017). In the current dissertation's results, higher Na^+/K^+ ratio (Figure 18) was noted in shoots of Alderon, followed by Cochise, while lower Na^+/K^+ ratio was recorded in shoots of Sama, and the lowest recorded in Najran (that indicates more tolerance). These results agree those obtained by Iqra *et al.* (2020) , in terms of increasing salinity causing heightening of Na^+/K^+ ratio. They showed that the highest Na^+/K^+ ratio was for the Galaxy-13 and Anaj-17 wheat varieties, and lowest ratio was shown to be in Inqalab-91 and Shafaq-06 (Salt tolerance), under 15 dS/m NaCl treatment. For the 10dS/m NaCl treatment, highest Na^+/K^+ ratio was noted in shoots of Faisalabad-08, with Shafaq-06 coming after, while the lowest Na^+/K^+ ratio was seen in shoots of Galaxy-13 and Anaj-17.

Yassin *et al.* (2019) also found similar results to my research, in terms of the variation of Na^+ and K^+ within wheat varieties. Within their experiments, they found that the Na^+ /K^+ ratio in parts of genotype named Misr 2 such as stems, leaves , and roots were significantly lower than the genotype 'Sakha 95', when exposed to 150 mM for 12 days (Yassin *et al.*, 2019). Table 4 shows that there is a great significant ($P < 0.01$) and positive correlation between Na^+/K^+ ratio and Na+ (R=0.75), whereas K^+ content is negatively correlated with Na^+/K^+ ratio (R= -0.73). In fact, Sodium competes with potassium for access to the main binding sites, during metabolism (low and high affinity HKT transporter); thus, interfering in metabolic reactions of plants. Furthermore, high levels of Na^+/K^+ ratio can interrupt numerous enzymatic functions in cytoplasm. Besides, protein synthesis requires high amounts of K^+ in ribosomes to function; so, if Na^+ is elevated, it can disrupt protein synthesis(Tester and Davenport, 2003; Hussain *et al.*, 2021). Potassium absorption regulation, inhibition of sodium entry, and efflux of sodium from cells, are the plans commonly used by plants to preserve a necessary $Na^{+/}K^+$ ratio within cytosol (Khan *et al.*, 2009). Among wheat genotypes experimented within our research, it was found that Najran and Sama have the minimum Na^+/K^+ ratio, as Figure 18 illustrates (Najran is slightly lower). In contrast, Alderon has the maximum Na^+/K^+ ratio. This means that Najran is the tolerant genotype and Alderon the sensitive.

3.3.11 The effect of salt concentration on proline accumulation

There is a larger amassing of proline (more common for higher plants), as compared with other amino acids, in plants which are under salt stress (Ashraf, 2004).The findings of the current study show that at salt treatment, maximum proline content was for Najran while Alderon and Cochise were similar , and Sama having minimum content (Figure 19). This indicates that Najran

resist salinity much better than Sama, which is more sensitive to salt stress. On that account, it is evident that Cochise and Alderon has moderate tolerance towards salinity. The results from the current research are in accord with Momeni *et al.* (2021), who observed proline content being heightened by salinity in the Behrang salt-tolerant genotype amidst 200 mM NaCl for durum wheat

In addition, my results were also similar to the study by Borzouei *et al.* (2012), which saw plants' proline content increasing with salinity, but the more tolerant plants having higher accumulation of proline content, compared with the more sensitive ones (1.3, 6, 8, 10 and 12 dSm^{-1}).

The mechanism underpinning all this is osmolytes maintaining water status within cell/sub-cellular structures, plus offering protection against the denaturing impacts of osmotic stress. It must be emphasized that proline is a significant osmolyte with more contributions than just osmotic adjustments. Proline actually supports stability of sub-cellular structures (Rana *et al.*, 2017). In that regard, proline build up can be helpful to stress signalling and protecting cellular components(Arteaga *et al.*, 2020). For more explanation, our correlation analysis in Table 1, illustrates that there was significant correlation between proline and RWC negatively (R= -0.57). Since proline (Pro) is a common osmolyte, showing substantial increase in response to salinity, it can be used as an indicator of salt stress, which has also been observed in other plants such as beans(Arteaga *et al.*, 2020).

3.3.12 The effect of salt concentration on MDA content

Lipid peroxidation is considered as a commonly employed stress marker for plant membra (Taulavuori *et al.*, 2001). The product of lipid peroxidation, Leaf MDA content, is a leading marker for membrane impairment within plants exposed to salt stress (Katsuhara *et al.*, 2005). As for this research, salt stress had an impact on all wheat seedlings, causing an escalation of MDA content. This increase was gradual as seedlings experienced 80 and 160 mM NaCl treatment as seen in Figure 20(B). In this experiment, maximum MDA content was observed within the variety which was affected more by level salinity (Cochise and Sama). On the other hand, the minimum MDA content was found in Alderon and Najran (Figure 20 A) but all of varieties did not show their significancy in their MDA content under all salt concentrations. Agreeing with this, in the study by Ma *et al.* (2021),salt treatment led to an increase in MDA content due to 150

mM NaCl, throughout the duration of 48hrs in salt-tolerant mutant lines of wheat Another study by Yassin *et al.* (2019) gave similar results, discovering that MDA content in wheat genotypes, called Sakha 95 and Misr 2, had surged in the leaves after NaCl treatment of 150 mM, in the period of 12 days (MDA content was lower in the tolerant variety, Misr 2).Our experiment established that MDA levels (markers for harm to cell membrane) can be utilised as an indicator for cell membrane damage; meaning, plants with lower MDA content, frequently have higher resistance to abiotic stress; for instance, salt stress (Mandhania *et al.*, 2006; Tang *et al.*, 2017). For example, Najran and Alderon displayed a slightly lower MDA content, comparing to Cochise and Sama. This means that there is no higher significant tolerance towards salt stress in all wheat varieties

The root system is very important in salt tolerance but we could not study root system, because we lost some of root parts and the root system study need more and specific method to make sure we have the whole root parts such as hydroponic system(water solution). The decrease in leaf expansion is subject to a double control, including water balances and hormonal signals from the roots. Water losses induced by atmospheric demands are regulated by the stomata; but it is at the level of the roots that the weak link in the water flow chain is located (Jackson *et al.*, 2000) The regulation of water content is also linked to root mechanisms. In general, the presence of salt in the soil solution increases the osmotic potential of the soil and creates a water deficit for the plant whose roots are unable to absorb the water necessary for the growth of this solution. As a result, leaf water potential also decreases(Prisco *et al.*; Zhu *et al.*, 2015) In a similar manner, sodium is the primary harmful ion found in salty soils; hence, glycophytic plants should ideally not over accumulate sodium in their leaves when subjected to salinity stress. As a result, a crucial mechanism for the salt tolerance of glycophytes may be the restriction of sodium transport from the roots to the shoots(Ismail and Horie, 2017).In addition, previous investigations revealed that Electrophysiological studies utilising the microelectrode ion flux estimation (MIFE) technique on the net Na^+ flux in the roots part of bread and durum wheat cultivars revealed that the active Na+ efflux system energized by the P-type H+-ATPase is available in the epidermis of roots of wheat and that such activity was maximum in a salt-tolerant cultivar under salt stress(Cuin *et al.*, 2011).

3.3.13 WRKY gene expression level under salt conditions

Amongst plant transcriptional regulators, WRKY TFs are considered a huge group. They play many developmental/physiological tasks as a response against environmental stresses within crops like soybean, rice, cotton, soybean, wheat, and maize (Li *et al.*, 2020b). Even though it is well established that WRKY transcription factors are closely linked with numerous defence mechanisms of plants and respond to harmful environmental conditions, functional studies are just conducted for a limited number of WRKYs in wheat, with proof of associations between WRKY proteins and abiotic stresses continuing to be few (when compared to biotic stresses). In fact, WRKY TFs have fundamental functions in controlling plant response against salt stress. So far, 47 WRKY genes are seen to be expressed in salinity inside the wheat genome (Li *et al.*, 2020b) .

Within the current thesis, for highlighting the putative implications of *WRKY TFs* in wheat salt tolerance, nine candidate genes have been obtained based on former studies (Figure 21) and the synteny relationship. We have investigated, through qPCR, their pattern of expression under salt treatment (160 mM NaCl) after exposure to 21 days for all wheat varieties.

Firstly, upregulation of gene expression was observed, being always positively expressed in all wheat leaf varieties. The following genes were found to be upregulated for all varieties in their patterns of expression (Figure 22, Figure 23,Figure 24 and Figure25). *TaWRKY8*, *TaWRKY19*, *TaWRKY45*, and *TaWRKY46*. However, the level of expression for these 4 genes had different levels based on wheat variety response. Specifically, *TaWRK8* was shown to have the highest pattern of expression in the Alderon variety, and the lowest in Sama, while the patterns of expression for Najran and Cochise were nearby. As for *TaWRKY19*, it was found that the highest gene expression profile was in Sama, followed by Najran, with Cochise coming afterwards. On the other hand, the lowest gene expression profile was found in Alderon. Furthermore, in *TaWRKY45*, a close gene expression pattern was found in all wheat varieties. The gene expression pattern of *TaWRK*Y46 was recorded to be the highest in Najran, followed by Cochise, and Alderon coming thereafter. However, the lowest gene expression pattern for *TaWRKY*46 was recorded in Sama. It can be suggested from our observations that Sama had the lowest gene expression patterns in terms of upregulation among the genes, with the exception of *TaWRKY19* (highest in this gene).

Secondly, as for the downregulation of gene expression (Figure 22,Figure 23,Figure 24, and Figure25), it was observed that two genes (out of 9) had down regulation within the Sama variety (*TaWRKY*37 and *TaWRKY* 53-a). This was the lowest level of gene expression in terms of down regulation. In addition, two out of 9 genes downregulated in the Alderon variety (*TaWRKT44* and *TaWRKY*71). Two genes were also downregulated in the Cochise variety (*TaWRKY*3 and *TaWRKY*53-a). However, in Najran, only one gene out of 9 was down regulated (*TaWRKY*71). We suggest that the Najran variety, potentially had the most upregulated genes, compared to the rest of the varieties (except for *TaWRKY* 71), as seen in Figure 26. Curiously, it was found that *TaWRKY*53-a gene had the lowest level of expression amongst all varieties. Regarding differential gene expressions amongst varieties, the patterns of *TaWRKY*37, *TaWRKY* 53-a, and *TaWRKY*3 had differences in their expression, as seen in Figure 22,Figure 23,Figure 24, and Figure 25. From the varieties, *TaWRKY*37 was expressed in the highest level for Najran, with Cochise following after. However, *TaWRKY*37 in Sama was found to be down regulated. *TaWRKY*53-a had the highest level of expression in Najran and Alderon, while it was downregulated in Sama and Cochise. Additionally, *TaWRKY 3* was upregulated in its expression within the Najran and Alderon varieties; however, this gene in Cochise was down regulated and not expressed in Sama. We can suggest *TaWRKY*37, *TaWRKY 53*-a, and *TaWRKY*3 are putative candidate genes that have different profile patterns in their expression amongst the 4 varieties. Further, *TaWRKY* 71 was not expressed highly within the varieties, except in Sama (Figure 22,Figure 23,Figure 24 ,Figure 25, and Figure 26).

Our study found that *TaWRKY46* had the highest gene expression pattern, in terms of upregulation, in Najran. Moreover, our results regarding *TaWRKY8* showed that there was upregulation in all wheat varieties at 160 mM NaCl for 21 days treatment, which is somewhat in agreement with Gowayed and Abd El-Moneim (2021) who found that *TaWRKY 8* was upregulated in all wheat genotypes under different concentrations of salt (50, 150, and 250 mM NaCl). They saw that the *TaWRKY8* had higher upregulation at 50 mM and 150 mM most of wheat genotypes while lower at 250 mM, after one week of treatment. Considering our results and comparing them to the other aforementioned study, it can be suggested that *TaWRKY 8* is a putative candidate gene induced in upregulation after long duration (21 days) of high level of salt stress (160 mM NaCl).

Concerning the gene, *TaWRKY19*, it was found to be upregulated in all wheat varieties after being subjected to salt stress. This is in line with Niu *et al.* (2012) who observed that *TaWRKY19* induced the maximum gene expression at the 3rd and 6th hour, from the total 24hrs being exposed to salinity (200 mM) in wheat . They also found that *TaWRKY 19* can be attached to *DREB2A* gene promoters. It is important to note that the mentioned transcription factor, *DREB2A*, is engaged in the reaction of the plant towards cold and drought stress (Sakuma *et al.*, 2006). In the present result, *TaWRKY19* was expressed in leaves after 21 days of 160 mM NaCl. It is understood that there is a possibility that this gene had a role in the response to sever salinity, for long time of stress.

Moving in a relevant direction, in case two genes have similar ortholog or homology, there is a possibility for them to have similar functions (Huang *et al.*, 2015; Restrepo-Montoya *et al.*, 2021).In terms of *TaWRKY*45's gene expression within the current research, we constructed the phylogenetic tree analysis which revealed that the ortholog of *TaWRKY45* was closer to *HvWRKY32* (Figure 21). Consequently, this may indicate that *TaWRKY45* has similar function to *HvWRKY32*. The study by Zheng *et al.* (2021) saw *HvWRKY32* was significantly reduced in 200 mM NaCl during 24 hrs of exposure in barely. This is contrary to our findings which showed *TaWRKY45* had upregulation in all wheat varieties. In addition, phylogenetic tree analysis showed that *TaWRKY45* and *HvWRKY32* clustered together with *TaWRKY146* (Figure 21), which is expressed in leaves and roots of wheat for responding to osmotic stress. *TaWRKY146* had upregulation within leaves subject to polyethylene glycol (PEG-6000) treatments (15 % and 20 %-24h) (Ma *et al.*, 2017). Within the current research, I believe there is a probability that *TaWRKY45* was expressed with up regulation. It may have even been involved in response to high level of salt stress for a long period of time.

My analysis also revealed that *TaWRKY13* and *TaWRKY46* clustered together with *HvWRKY34*, according to the phylogenetic tree. On that note, the research done by Zhou *et al.* (2019) found that *TaWRKY13* had a higher upregulation (approximately, 22-fold) at the 1st hour of treatment. The treatment that they used had 150 mM NaCl for 24 hours. Another study by Kumar *et al.* (2020), mentioned that *HvWRKY34* was engaged in sugar stress. There was an upregulation of *TaWRKY46* at the 3rd hour by increasing 3.57-fold after PEG treatment (20 %), as well as the *TaWRKY46* gene being slightly upregulated by NaCl (200 mM NaCl for 24) at a maximum level of approximately 1.48-fold at the 3rd hour in wheat(Li *et al.*, 2020c). In our

project, it was understood that *TaWRKY46* was expressed under high level of salt for long period time.

Focusing on upregulation, our experiments observed that the first interesting gene that was found to be expressed at the highest level in the Najran variety, followed by Cochise, and thereafter Alderon was *TaWRKY 37* (Figure 22,Figure 23,Figure 24 ,Figure 25, and Figure 26).

Contrarily, it was observed that *TaWRKY 37* had downregulation within the Sama variety, which signifies differential pattern expression for this gene among different wheat varieties (in reaction against salinity). So as to observe comparable functions to the rest of the genes within wheat or various cereal crops, we utilised the phylogenetic tree analysis. It was revealed that *HvWRKY44* and *TaWRKY44* clustered together with *TaWRKY37* (Figure 21).

In support of our experiment, research conducted by Okay *et al.* (2014), who used RNA-seq database, found *TaWRKY37* within wheat tissues under drought stress. For that matter, wheat plants which were 10-days old had been exposed to dehydration stress for 24 hrs within plastic pots that held Murashige and Skoog medium and (20 % PEG 6000). Moreover, work done by Wang *et al.* (2015) showed that *TaWRKY44* gene was induced by salt stress 200Mm NaCl for 24h in wheat leaves. As for *HvWRKY 44* gene, Zheng *et al.* (2021) saw that its expression was induced in an early developmental phase for grain in barely. In our research, *TaWRKY37* was expressed in the tolerant variety (Najran) with the highest upregulation, followed by Cochise, and Alderon coming afterwards. This was while the same gene was down regulated in its expression within the sensitive variety (Sama). It is suggested that *TaWRKY37* may be associated in response to high salt stress for 160 mM NaCl at long periods of time. On this matter, El-Moneim (2020) stated that variations among wheat genotypes could be owing to using wheat genotypes that have dissimilar genetic backgrounds.

The expression level of *TaWRKY44* in all wheat varieties did not exceed more than 2.65-fold change, except Alderon which was down regulated. 1.30-, 2.65-, 1.99-, and (1.52down)-fold change was seen in Najran, Sama, Cochise, and Alderon, respectively as displayed in Figure 22, Figure 23,Figure 24 and Figure 25. This is in accordance with Wang *et al.* (2015) who showed that *TaWRKY44* gene was induced by salt stress (200 mM NaCl for 24 h). In our results, *TaWRKY44* was induced via salt stress, but the expression level was not high for most of the varieties. It could be changed based on intensity of salt and duration of salt stress.

Within the present research, the expression level of *TaWRKY71* was found to be down regulated in Alderon and Najran; however, it was not expressed in Cochise. On the other hand, there was upregulation of *TaWRKY71* in Sama, as shown in Figure 22,Figure 23, Figure 24, and Figure 25. The heat map in Figure 26 shows the differential pattern expression of *TaWRKY*71 amongst varieties. My observation suggests that *TaWRKY71* was not induced via salt stress (except in Sama variety). In order to investigate the function of *TaWRKY71*, the phylogenetic tree was generated and revealed that *TaWRKY71* was similar with *TaWRKY33*, in terms of homology Figure 21. Also, there is study indicating that the expression of *TaWRKY33* being involved in response to high temperature, low temperature, and jasmonic acid methylester (MeJA) in wheat (He *et al.*, 2016) . In another scholarship, Wu *et al.* (2008) stated *TaWRKY71*'s expression patterns in wheat have been different for a variety of stresses and that *TaWRKY71* was involved in response to low and high temperature. Overall, considering my experiment and the studies mentioned, it seems the *TaWRKY71* was involved with plant hormones and the response to temperature stress. It could also be understood that *TaWRKY71*'s upregulation in the Sama variety contributed to act as repressor to some salt-related genes that may involve in salt response. As Xie *et al.* (2006) demonstrated that *OsWRKY71* and *OsWRKY51* repress synergistically the alpha amylase in aleurone cell through ABA and GA signalling. The alpha amylase is important enzyme in sugar mobilization through starch hydrolysis during seed germination (Damaris *et al.*, 2019). In order to understand the role of *TaWRKY71*, further research is needed.

On another note, it was displayed in Figure 22, Figure 23,Figure 24, Figure 25, and Figure 26 that the level of expression for *TaWRKY44* gene was different among varieties. Importantly, the expression levels between Cochise and Sama are close, while Najran and Alderon had higher expression levels when compared with the rest of the varieties. The present research concluded that *TaWRKY44* was a close ortholog to *HvWRKY44* and close homolog to *TaWRKY37* in Figure 21. This finding was in line with the study by Wang *et al.* (2015) where *TaWRKY44* was evidently upregulated, subsequent to treatment with PEG and NaCl (200 mM NaCl solution, 20 % PEG6000 solution in wheat for 24 hrs) in wheat. Furthermore, regarding transgenic tobacco, proline and soluble sugar content increased in transgenic plant with *TaWRKY44* gene; however, MDA was decreasing under drought and salt stress (400 mM NaCl for 3 weeks in transgenic lines). After carefully observing my experiment and studying Wang *et al.* (2015), we can

conclude that *TaWRKY44* possibly played a role in response to long duration of salt stress and concentrated salt intensity.

The second interesting gene that had shown differential expression in Najran and Sama (Figure 22, Figure 23, Figure 24, Figure 25, and Figure 26) was *TaWRKY53-a*. Based on phylogenetic tree analysis (Figure 21), *TaWRKY53-a* was found to have a close homology with *TaWRKY2*. Relating to homology, Niu *et al.* (2012) found that *TaWRKY2*'s expression was induced under salt stress (200 mM NaCl for 24 h) in wheat. In contrast with our findings, Wu *et al.* (2008) described *TaWRKY53-a* not being induced by any one of the following abiotic stresses: low temperature, high temperature, high salinity, or osmotic stress. In our results, the transcript level of *TaWRKY*53-a was more abundant in tolerant varieties than the sensitive variety (Sama). It may be the candidate gene for long period of 160 mM NaCl. It must be remembered that the variation in expression levels could be because of genetic diversity in wheat.

The current research experiment found the *TaWRKY3* gene showing differential expression amongst wheat varieties, in upregulation or in downregulation. *TaWRKY3* had different profile expressions, as expressed in Figure 22, Figure 23, Figure 24, Figure 25, and Figure 26. The expression level was the highest in Najran, followed by Alderon (i.e., upregulation), while, it was not expressed highly in Sama. However, the expression pattern of *TaWRKY3* in Cochise was downregulated. It is possible that *TaWRKY3* is involved in salt stress for long periods of salinity. Also, the change of this gene's expression in the wheat varieties could be associated to genetic diversity.

3.4 Conclusions

Based on our research, Najran exhibited improved performance in reaction to salinity (potentially, could be more salt tolerant) linked the less reduction in shoot fresh weight and shoot dry weight. It contained the lowest Na+ concentration in dry shoot compared with other varieties. Najran showed the less reduction in its leave numbers. It showed the highest concentration in proline and K^+. In contrast, Sama wheat was shown the most variety has been impacted vis salinity (it could be more sensitive to salinity). Sama was shown the most variety that has highest reduction in shoot fresh weight and shoot dry weight. It contained the lowest proline concentration. Moreover, our study highlighted the importance of the nine genes in wheat response to 160 mM NaCl for 21 days. From the nine, *TaWRKY37*, *TaWRKY53-a*, and *TaWRKY3* had huge variation in their expression regarding their upregulation or down regulation, based on

variety (particularly, Najran and Sama). It is possible to use them as maker genes linked to salt stress for high levels of salt and long periods of stress to determine resistance of wheat to salt stress. In addition, *TaWRKY71* was not induced via salt in all three varieties, except for Sama. There is a possibility for *TaWRKY71* to be a negative regulator in salt -linked genes in salt stress. For improved understanding the role of *TaWRKY37*, *TaWRKY53*-a, and *TaWRKY3* in wheat, more research should be done.

Chapter 4. Phenotypic, Biochemical and Molecular Response of Seedling Wheats to *Bipolaris Sorokiniana* Infection

4.1 Introduction

Future demand for wheat requires a production rise of 70 % for satisfying global food security (Sharma *et al.*, 2018). Without careful control of diseases in the field, during harvest and in post-harvest storage we will struggle to keep up with this demand (Sharma *et al.*, 2018). There are more than 200 known pathogens of wheat, of those, 50 are globally distributed and are attributed to significant financial losses. It must be noted that every year, disease results in almost 1/5th of wheat production being lost (Al-Sadi, 2021). Spot blotch, rusts, smut, common root rot, tan spot, powdery mildew, Septoria blotch, blast, and fusarium head blight are the most economically important diseases affecting wheat (Al-Sadi, 2016; 2021).

Bipolaris sorokiniana, a hemibiotophic fungal pathogen, is the causative agent of spot blotch as described in chapter 1. Spot blotch disease is a well-known foliar disease which restricts the development and growth of wheat within warmer regions of the world (Kumar *et al.*, 2002). The current research project aimed to quantify the wheat response to spot blotch disease, to better understand the induced defence mechanisms, focusing on phenotypic parameters, biochemical changes of diagnostic metabolites, and alterations in gene expression. In our study, four wheat varieties were screened for their interaction with *B. sorokiniana* infection with shoot length, leave numbers, and disease severity being evaluated. The initial screen was used to identify a resistant variety (Alderon) and a susceptible variety (Sama) with contrasting responses towards the pathogen. malondialdehyde (MDA) content, callose content, salicylic acid (SA) and jasmonic acid (JA) content of the leaves were also evaluated over the time of infection. The same two varieties were examined for their response to pathogen at molecular level. Several genes have been selected to observe their expression during pathogen infection. Glucan synthase-like genes (2, 3, 8, 10, 12, 19, 22, and 23) responsible for the defence-related deposition of callose in the vascular tissue were explored as to understand their expression in the mentioned two wheat varieties after pathogen attack in 24 and 48 hours post infection (hpi).

Moreover, for identifying candidate genes as gene markers, WRKY genes (3, 8, 19, 37,44, 45, 46, 53-a and 71) were investigated in 24 and 48 hpi. We also carried out experiments to decipher expression of defence-signalling pathways, including SA biosynthesis such as *TaPAL* and *TaNPR1* (SA signalling), and JA, such as *TaAOS* and *TaLOX2.*

4.1.1 Aim of study

To demonstrate how wheat response against *B. sorokiniana* at three different levels: Phenotypic trait, biochemical reaction, and molecular response to the pathogen.

4.1.2 Objectives

1-Determining how the growth and development of wheat is affected by *B. sorokiniana* infection and assessing the disease severity for four wheat varieties.

2-Quantify the temporal effects of pathogen induced on Glucan Synthase-Like genes (GSL), SA (*TaPAL* and *TaNPR1*), JA (*TaAOS* and *TaLOX2*), and WRKY gene expression in two contrasting wheat varieties.

3-Investigate the temporal effects of *B. sorokiniana* infection on key wheat metabolites; MDA, callose, SA, and JA.

4.1.3 Hypotheses

1-The MDA content, callose concentration, JA content, and SA concentration are increased at early or late stage of infection in 2 different wheat varieties (resistant and susceptible)

2-The variation of WRKY gene expression (*TaWRKY3*, *8*, *19*, *37*, *44*, *45*, *46*, *53-a* and *71)*, Glucan synthase-like genes expression (*TaGSL2*, *3*, *8*, *10*, *12*, *19*, *22* and *23*) and SA (*TaPAL* and *TaNPR1*), JA (*TaAOS* and *TaLOX2*), expression between 24 and 48 hpi within the Alderon and Sama varieties is influenced by infection development

4.2 Results

Knowledge the mechanisms of resistance of wheat to spot blotch disease is a complex process. It will be described in our findings how the spot blotch disease affects the growth and development of wheat, the severity of the disease, the MDA content, the callose concentration, the SA, and the JA content. In addition, the influence of spot blotch disease on the expression of WRKY, GSL, and SA-related genes, as well as the expression of JA-related genes, will be characterized in this section.

4.2.1 Impact of spot blotch disease on growth trait determination.

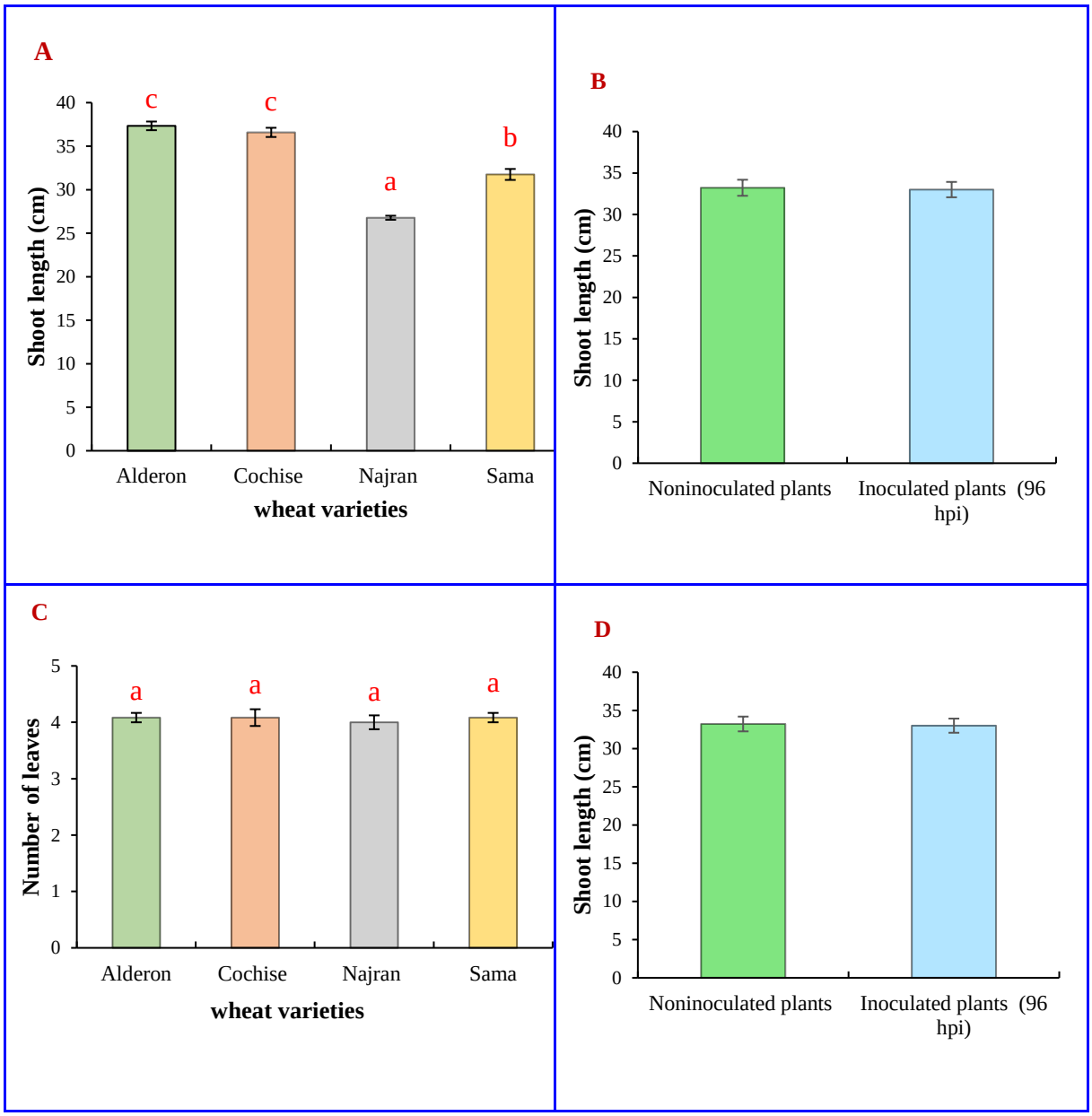

Figure 27 Impact of *B. sorokiniana* on shoot length and leaf number for 4 wheat varieties on Shoot length. Shoot length variation according to wheat varieties (**A**) and treatments (noninoculated and inoculated plants) (**B**). Leaves number of 4 wheat varieties (**C**) and treatments (noninoculated and inoculated plants) effect (**D**).Shoot length and Number of leaves were determined at 96 hpi for four wheat varieties at seedling stage via brush method. Varietal effect (**A** and **C**), shoot length and numbers of leaves of each wheat variety across noninoculated and inoculated treatments represents mean ± standard error (6 biological replications/treatment).Noninoculated and inoculated plants (treatments) effect (**B** and **D**), shoot length and number of leaves for 4 wheat varieties through each treatment (noninoculated and inoculated plants) represents mean ± standard error (6 biological replications/ wheat variety). Using two-way ANOVA, values with different letters are significantly different at $P < 0.05$, based on Tukey multiple comparison test.

Four wheat varieties were grown in sand and all plants were irrigated with Hoagland solution until 4th seedling leaf emerged. Third leaf of each plant was inoculated via one drop of 3×10^4 ml of spore suspension using brush method. Growth of plants was recorded at 96 hpi and disease severity were recorded at 48, 72, and 96 of hpi. The effect of *B. sorokiniana* on shoot length and number of leaves was observed at 96 hpi.

Firstly, the shoot length of each wheat variety (Alderon, Cochise, Najran and Sama) across all treatments (noninoculated and inoculated plants) was recorded. Alderon and Cochise were approximately similar (37.33cm ± 0.49, 36.58 cm ± 0.52 and, respectively) followed by Sama (31.75 cm ± 0.62), while Najran was recorded only 26.77 cm ± 0.62 as can be seen in Figure 27 (**A**). Secondly, the effect of each treatment (noninoculated and inoculated plants) on shoot length for all wheat varieties was shown in Figure 27 (**B**). The average of shoot length in all wheat varieties was recorded, with 33.22 cm ± 0.96 and 33.0 cm ± 0.92 at noninoculated and inoculated plants, respectively. Statistical analysis was conducted using two-way ANOVA considering the effect of treatments, varieties and their interaction on shoot length. The effect of wheat varieties was highly significant ($F = 96.29$, $P < 0.0005$) in shoot length. It was shown that shoot length of Alderon was significantly different ($P < 0.0005$) from Najran and Sama, while there was no significant difference between Alderon and Cochise. In addition, there was significant difference ($P < 0.0005$) on shoot length between Cochise and each of variety (Najran Sama) whereas there was no significant difference between Cochise and Alderon. Interestingly, significant differences ($P < 0.0005$) were found between Sama and all other varieties on shoot length. Najran was also showed its difference ($P < 0.0005$) significantly with all other varieties as shown in Figure 27 (**A**). The effect of treatments (noninoculated and inoculated plants) was not significant ($F = 0.196$, $P < 0.660$) and there was no significant interaction between treatments and wheat varieties ($F = 1.015$, P

< 0.396). Najran was shown the have lowest shoot length, Alderon and Cochsie were the highest in their shoot length.

Secondly, it was revealed that the number of leaves were similar according to the wheat variety under all treatment (noninoculated and inoculated plants). as shown in Figure 27(**C**). In addition, it was observed that all number of leaves for all 4 wheat varieties at each treatment (noninoculated / inoculated plants) were similar as seen in Figure 27(**D**) Two-way ANOVA was confirmed that there was no significant the effect on treatments, varieties and their interaction on number of leave.

4.2.2 Assessment of disease severity %

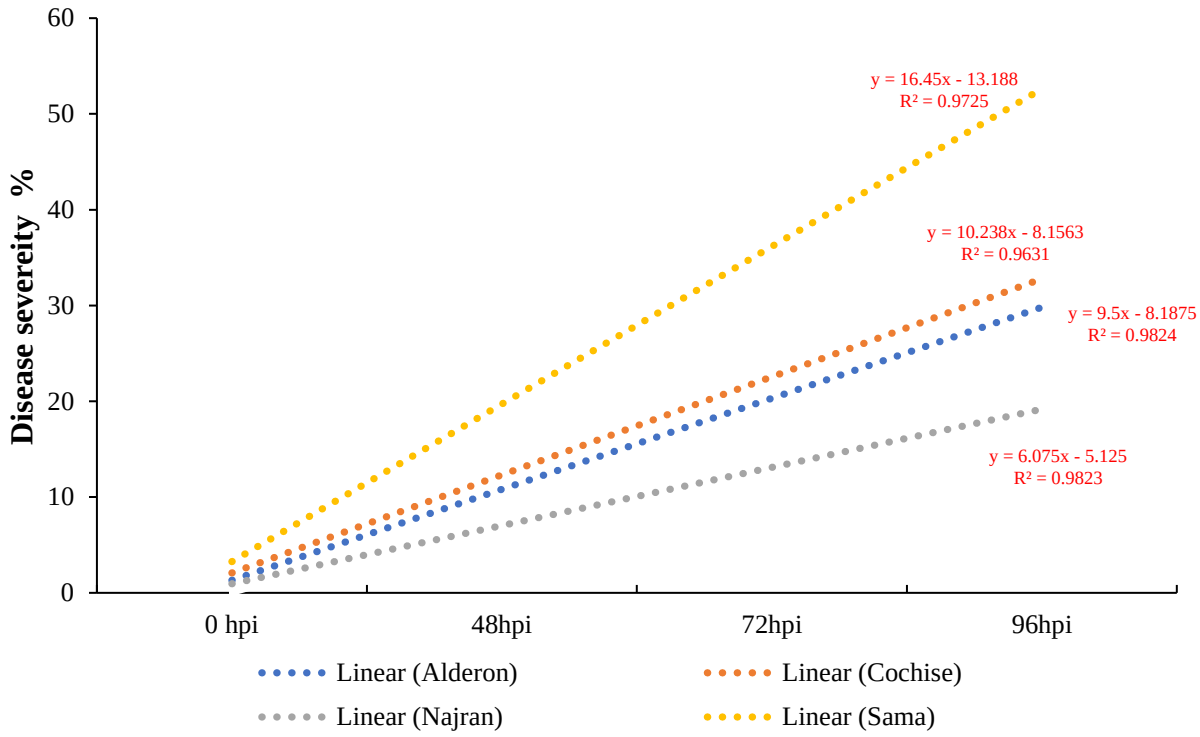

Figure 28 Spot blotch disease severity (%) for selected wheat varieties at different time points. Spot blotch disease severity (%) in top leaves for Alderon, Cochise, Najran, and Sama were assessed at 48, 72 and 96 hpi as compared to control healthy leave which is 0 value. The evaluation of disease severity was recorded. Data are means for 16-biological replications. The data showed fitted lines. ANCOVA was conducted to determine the difference in disease severity among four selected wheat varieties while controlling via hours post infection (covariate variable).

Using the same experimental conditions described previously, the four wheat varieties were evaluated for their disease severity (%) on third leaf of each plant (Figure 28), after being inoculated with *B. sorokiniana.* At 48, 72 and 96 hpi, necrotic and chlorotic lesions, as

seen in inoculated wheat leaves by using standard area diagram (SAD), were counted to evaluate the endogenous varietal resistance or susceptibility to the pathogen. Disease severity (%) demonstrated variability among the different wheat varieties. Najran was shown to have the lowest disease severity (%) after 48, 72 and 96 hpi. The percentage of disease severity increased to 8, 14, and 18% at 48, 72 and 96 hpi respectively, relative to control plants (non-inoculated leaf or healthy leaf which is recorded 0 value no symptoms appeared). The Alderon wheat variety followed Najran, in terms of having the lowest disease severity. It was recorded that disease severity increased to 13, 20 and 30 % after 48, 72 and 96 hpi, respectively. Moreover, the disease severity (%) of the Cochise wheat variety was logged after 48, 72 and 96 hpi, as it increased to 16, 21 and 32%, respectively. Finally, the Sama wheat variety was shown to hold the highest percentage of disease severity among all varieties. After 48, 72 and 96 hpi of *B. sorokiniana*; it was noted that disease severity increased to 23, 39, and 50%, respectively.

The statistical analysis (ANCOVA) was used to examine the difference in disease severity among four wheat varieties after controlling via hours post infection. The results showed significant difference in disease severity between the four selected varieties (variety main effect) with $F = 16.43$ and $P<0.0005$. However, there was significant increase in disease severity with hours post infection (time main effect) with $F= 167.33$ and $P<0.0005$. In addition, there was significant interaction between varieties and time after infection ($F = 7.67$, $P<0.0005$). Moreover, the statistical analysis (pairwise comparison) showed significant difference between Alderon and Sama ($P<0.0005$), but there was no significant difference between Alderon and each of Cochise and Najran in terms of disease severity. Furthermore, Cochise was significantly different with Najran ($P<0.03$) and Sama ($P<0.0005$). However, there was no significant difference between Alderon and Cochise. Furthermore, Najran was significantly different with each of Cochsie ($P<0.03$) and Sama($P<0.0005$) while there was no significant difference between Najran and Alderon on disease severity. Sama was shown significant difference with each of Alderon, Cochise and Najran on disease severity. As demonstrated above (Figure 28), Najran showed the lowest disease severity among all wheat varieties, followed by Alderon and Cochise, however, Sama was shown to have the highest disease severity. We selected Alderon (resistant to pathogens-UK variety) and Sama (susceptible to pathogens-Saudi variety) for subsequent experiments (Figure 29)

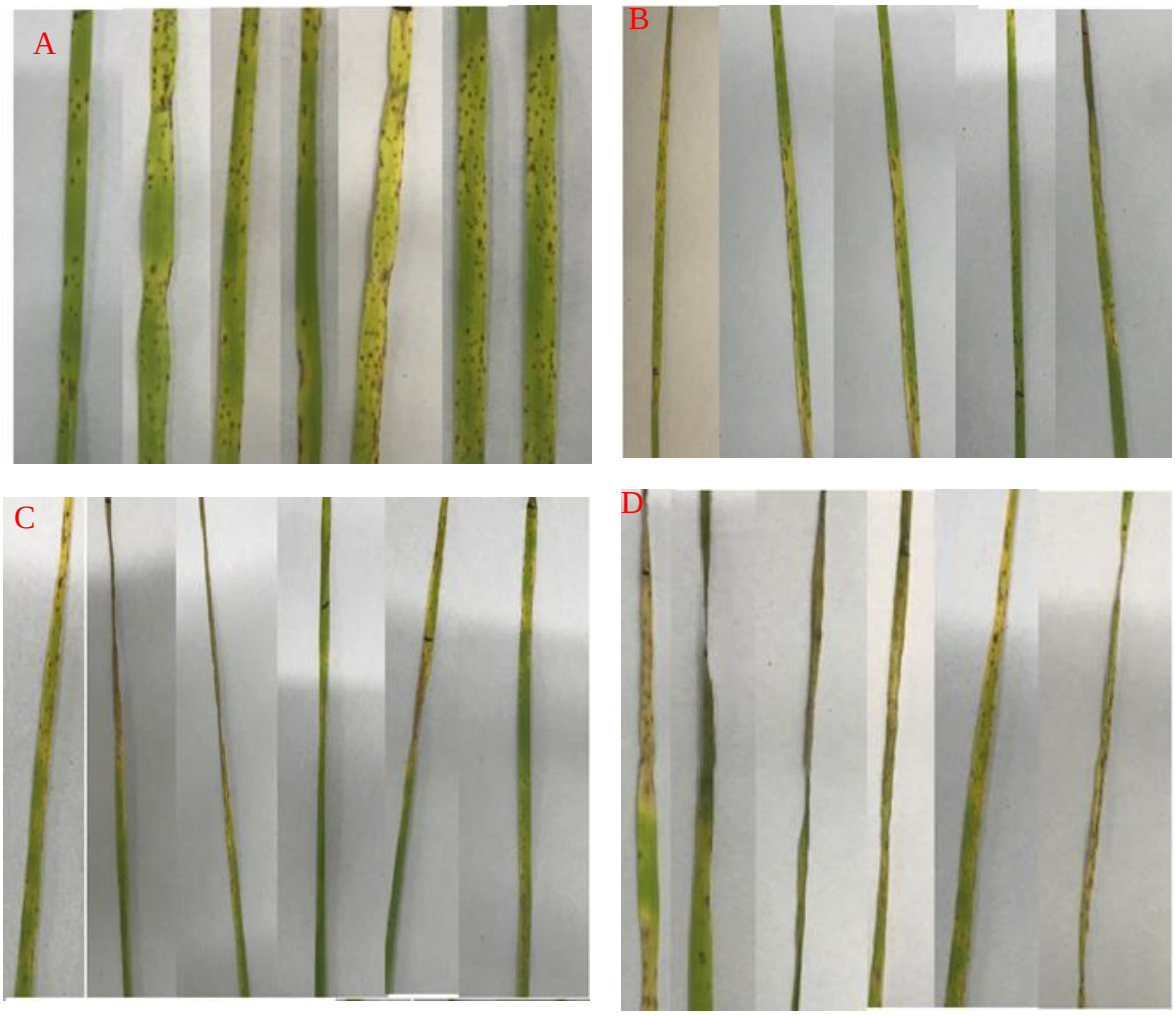

Figure 29 Spot blotch disease symptoms (necrosis and chlorosis) On the third leaf of four wheat varieties using brush method, Symptoms were more recorded at 96 hpi on (A) Alderon, (B) Najran, (C) Cochise and (D) Sama

4.2.3 Biochemical change in resistance and susceptible wheat genotypes after inoculated via B. sorokiniana

4.2.4 Estimation of Lipid peroxidation in form of malondialdehyde (MDA)

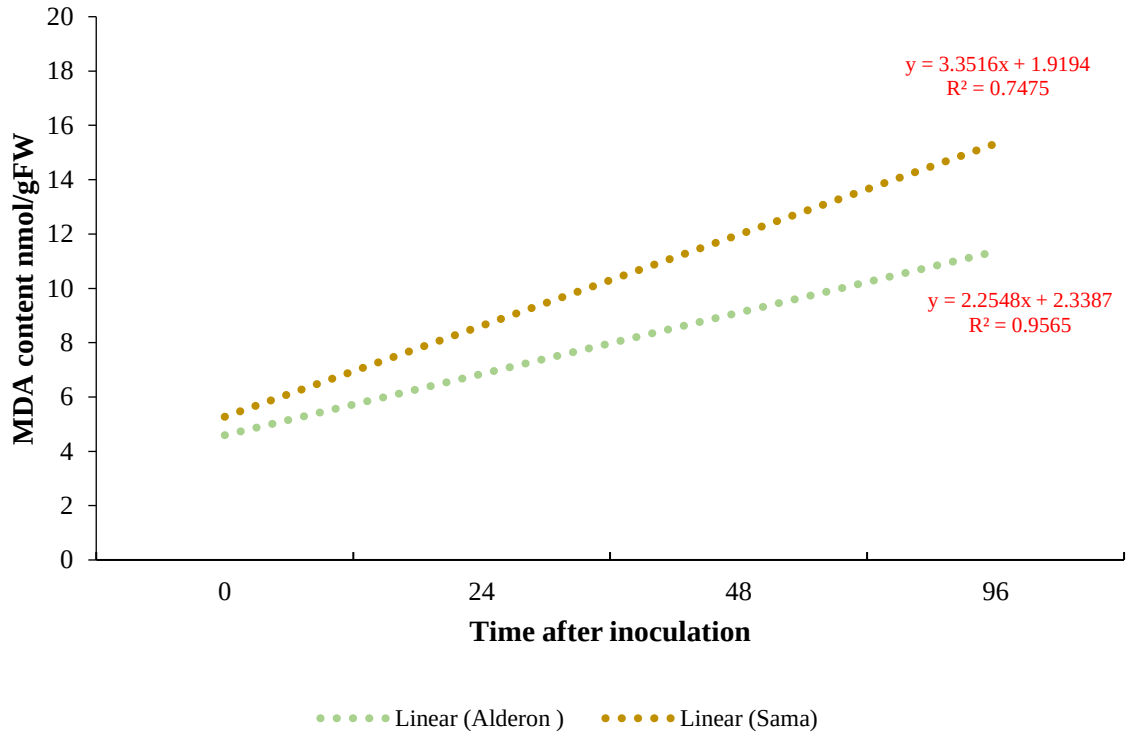

Figure 30 MDA content change in two selected wheat varieties at various time points after inoculation with the fungus *B. sorokiniana*. The MDA content of Sama and Alderon, in wheat leaves at different sampling times (0, 24, 48, 96 hpi). Mean MDA values represent 4-biological replicates. The data revealed fitted lines. ANCOVA was conducted to identfiy the difference between two selected wheat varieties in MDA concentration adjusted for time post inoculation (covariate variable).

The infected leaves were selected to determine MDA content. It was quantified across examined time at 24, 48 and 96 hpi in compression with control (at 0 hpi namely before *B. sorokiniana* infection, healthy leaf as control) for two selected wheat variety (Alderon and Sama). The MDA content of Alderon, a tolerant UK variety, and Sama, a susceptible Saudi variety, showed temporal variation compared to the controls. There was an increase in MDA content after inoculation for both wheat varieties. It was shown in Figure 30 that for Alderon variety, MDA content was recorded 6.48, 8.42 and 11.94 nmol/g FW at 24, 48 and 96 hpi, respectively as compared to control (5.06 nmol/gFW). For Sama variety, MDA content was found increased to 8.87, 8.48 and 17.61 nmol/gFW at 24, 48 and 96 hpi respectively as relative to control (6.29 nmol/gFW). Interestingly, Alderon was shown to have lower MDA content than Sama (11.94 ± 0.86 nmole/gFW for Alderon and 17.61 ± 1.05 nmol/gFW for

Sama. In line with the analysis, ANCOVA was used to determine the effect of two wheat varieties on MDA content after controlling via hours post infection (0, 24, 48 and 96 h). After adjustment via hours post infection, there was statistically significant difference on MDA content between Alderon and Sama, ($F = 7.59$, $P < 0.010$) and there was significant difference in MDA content with hours after infection, ($F = 62.15$, $P<0.0005$). In addition, there was no significant interaction between varieties and hours after infection, ($F = 3.40$, $P<0.076$). Statistical test (pairwise comparison) confirmed that Alderon was significantly ($P<0.010$) different with Sama in MDA content.

4.2.5 Callose concentration

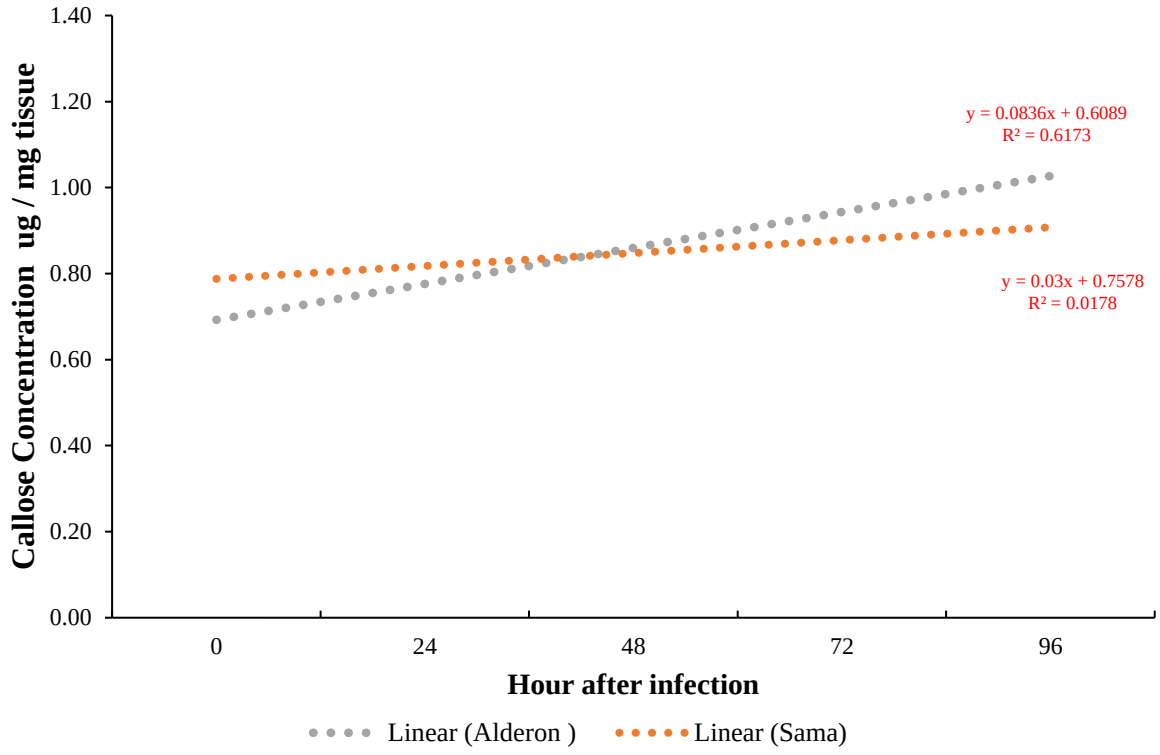

Figure 31 Temporal accumulation of callose in wheat leaves in response to *B. sorokiniana* infection. The callose concentration in wheat leaves (Alderon and Sama) at different sampling times (0, 24, 48,72 and 96 hpi. Mean callose concentration represent four biological replicates. The data show fitted lines. Analyses of covariance (ANCOVA) were used to assess whether or not there was a statistically significant difference in callose concentration between two different wheat varieties while adjusted for hour after infection (covariate variable).

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Callose concentration was measured in the two wheat varieties chosen previously, in order to demonstrate contrasting responses to infection. Changes in callose concentration were monitored over time course of the inoculation (0, 24, 48, 72 and 96 hpi). *B. sorokinana* infection caused an increase in callose content at all timepoints within the experiment. In Figure 31 the callose concentration at 24 hpi was shown to be 0.86 ± 0.04 ug/mg tissuefor Alderon and 1.28 ± 0.038 ug/mg tissue for Sama. The concentration of callose in both varieties after 24 hpi was higher than the concentration in the non-inoculated control plants. The callose concentration in Alderon did not differ throughout the remainder of the infection, 0.99 ± 0.13, 0.92 ± 0.06 and 0.96 ± 0.13 µg/mg tissue for 48, 72 and 96 hpi respectively. Callose concentration in Sama was recorded the highest level at 24 hpi and declined to 1.09 ± 0.12 µg/mg after 48 hpi and then plateaued at 0.74 ± 0.12 to 0.77 ± 0.05 µg/mg tissue at 72 and 96 hpi. An analysis of covariance (ANCOVA) was carried out to assess whether there was a statistically significant difference between two different wheat genotypes with respect to callose content while adjusting for the hours after infection (covariate). There was no statistically significant difference on callose content between Alderon and Sama, (F=.012, P<0.912) and there was no significant difference in callose content with hours after infection (F = 2.012, P<0.164). In addition, there was no significant interaction between varieties and hours after infection (F =3.40, P < 0.506).

4.2.6 SA content

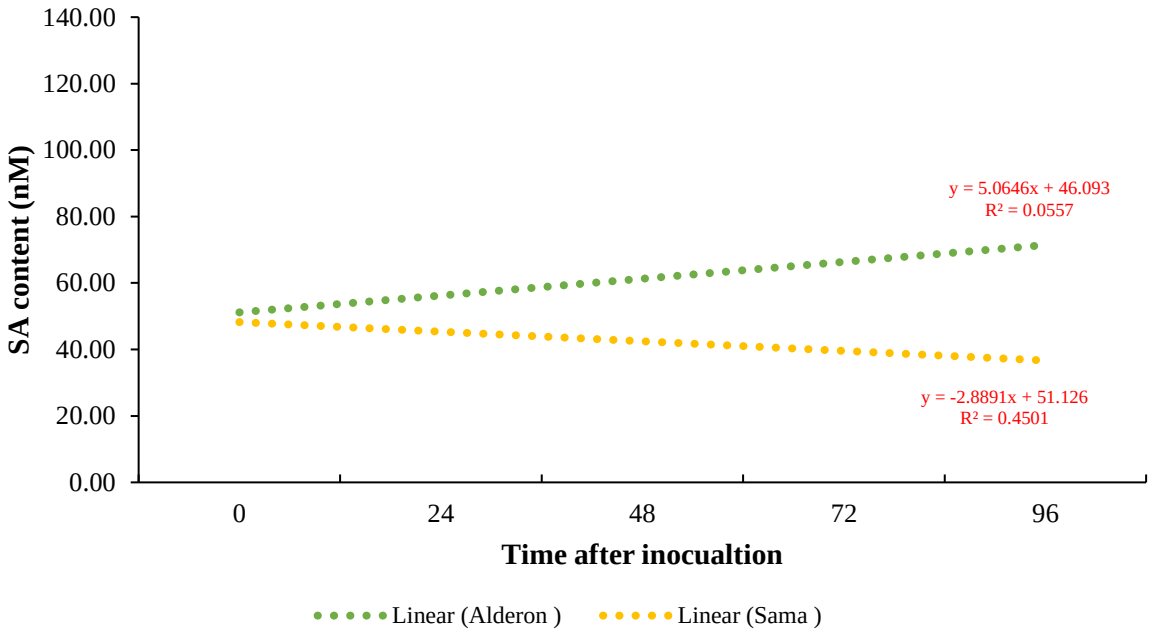

Figure 32 SA content in two selected wheat varieties before infection and at different hours after fungus *B. sorokiniana* infection The SA content was quantified in Alderon and Sama wheat varieties before infection and after infection by *B. sorokiniana* by LC-MS/MS. Mean SA content represent 5 biological replicates. The data show fitted lines. Analyses of covariance (ANCOVA) were used to assess SA content between Alderon and Sama and the SA content. The time after infection was used as (covariate variable).

To determine the effect of *Biopolaris* infection on the phytohormone biosynthesis, SA and JA hormones levels in Alderon and Sama wheat varieties grown under the conditions described previously were quantified using LC-MS/MS. SA content was quantified for Alderon and Sama wheat varieties throughout the pathogen infection at 24, 48, 72 and 96 hpi with 0 hpi as the non-infected leaf (control). Firstly, as shown in Figure 32, the concentration of SA at 24, 48, 72, 96 hpi was recorded 39.63, 120.17, 54.03 and 55.36 nM respectively as relative to the control (37.24 nM). The highest SA content was recorded at 48 hpi in Alderon. Secondly, SA content was determined 53.52, 42.29, 37.38 and 36.36 nM at 24, 48, 72, and 96 hpi respectively, compared to the control (42.74 nM)(Figure 32).

The analysis of covariance (ANCOVA) was used to determine a statistically significant difference between two different wheat genotypes in SA content with adjusting for the hours after infection (covariate). There was no statistically significant difference in SA content between Alderon and Sama, ($F= 3.74$, $P< 0.059$) and there was no significant difference in SA content with hours after infection, ($F=0.1$, $P<0.753$). In addition, there was significant interaction between varieties and hours after infection ($F =1.34$, $P<0.252$).

4.2.7 JA content

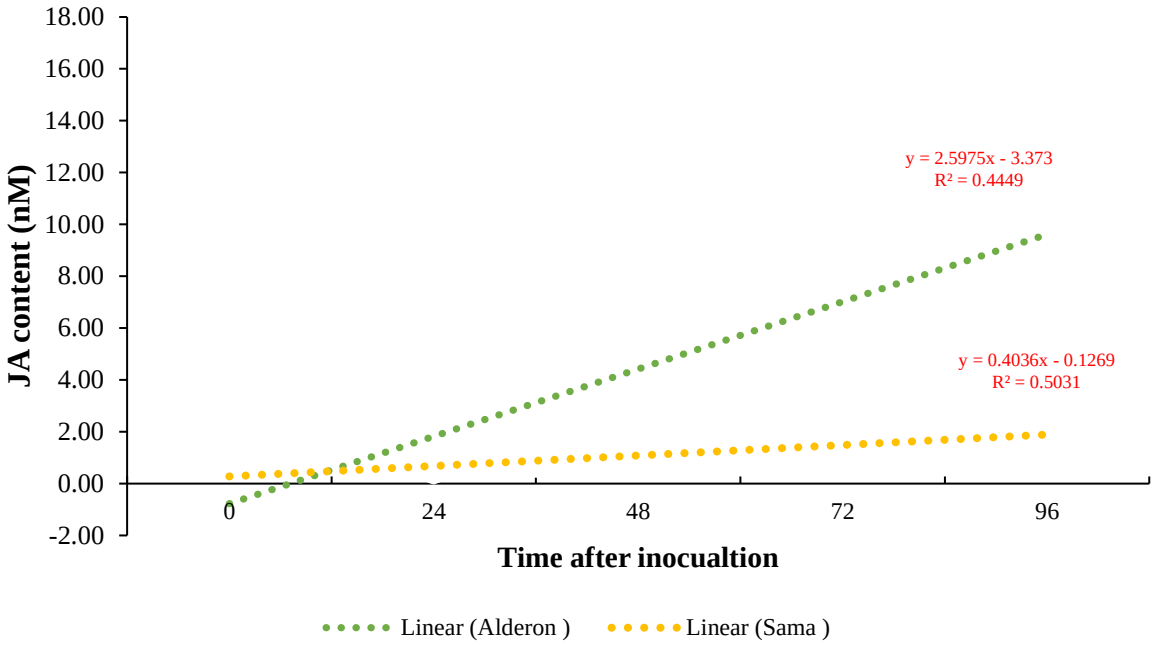

Figure 33 JA content in two selected wheat varieties before infection and at different hours after fungus *B. sorokiniana* infection The JA content was quantified in Alderon and Sama wheat varieties before infection and after infection by *B. sorokiniana* by LC-MS/MS. Mean SA content represent 5 biological replicates.The data show fitted lines. Analyses of covariance (ANCOVA) were used to assess JA content between Alderon and Sama and the JA content. The time after infection was (covariate variable).

JA content was measured during time after infection (24 ,48, 72 and 96 hpi) and 0 hpi as control for Alderon and Sama by method that was previously described, the most interesting time point was at 96 hpi as shown in Figure 33, Alderon was found to have higher JA content (15.21 nM) than Sama (2.53 nM). Statistcial analysis (ANCOVA) was used to determine the significant difference in JA content between Alderon and Sama during time after inoculation. After adjustment for time after inoculation, there was significant difference in JA conttent between Alderon and Sama($F=5.35$, $P< 0.025$).Moreover, there was significant differecne in JA content with hours after inoculation($F=8.67$, $P< 0.005$).In addtion, there was statistcally signficant between varieties and time after inoculation ($F=4.32$, $P< 0.030$). Statistical test (pairwise comparison) confirmed that Alderon was significantly ($P<0.025$) different with Sama in JA content.

4.2.8 The impact of gene expression during B. sorokiniana infection in two selected wheat varieties

4.2.9 Phylogenetic tree analysis

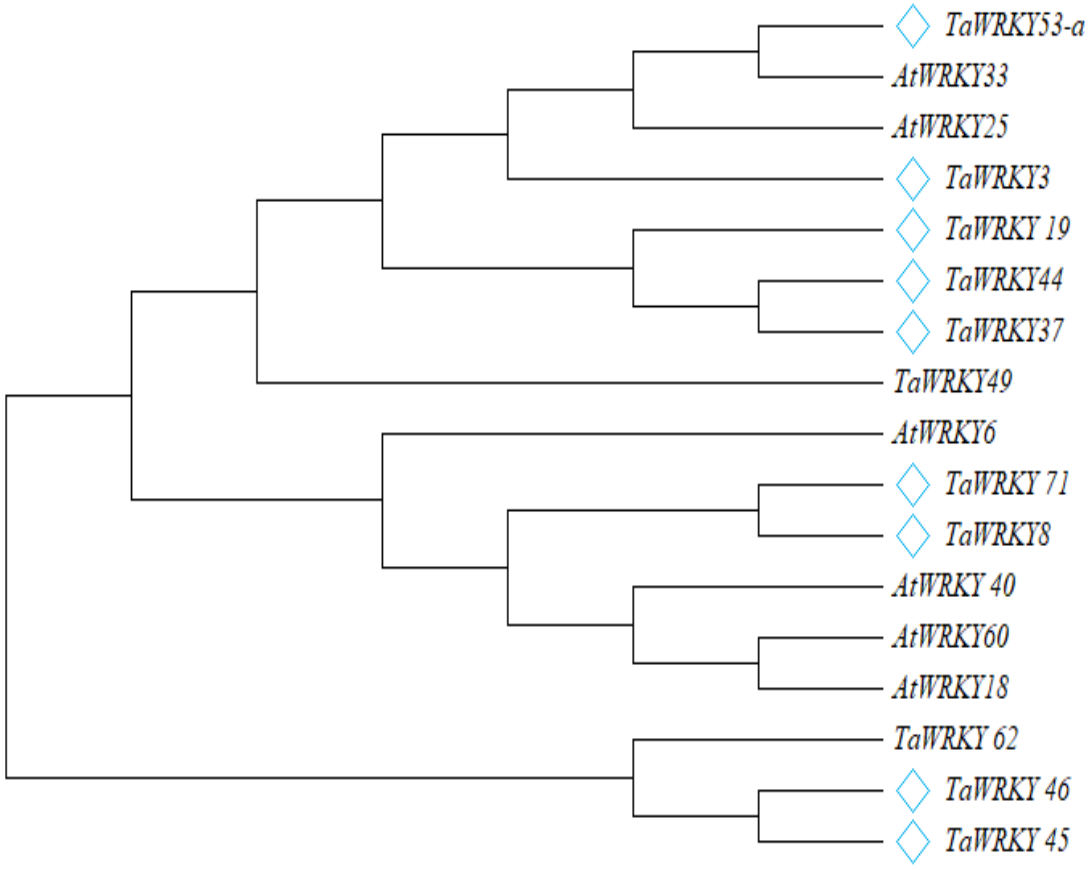

Figure 34 Phylogenetic tree of the WRKY domains different plant species. Phylogenetic tree representing the relationships among WRKY within wheat with other plant *Arabidopsis thalian.* phylogenetic tree WRKY amino acid was constructed using MEGA software. TaWRKY represent WRKY proteins from *Triticum aestivum* and AtWRKY are WRKY Proteins from *Arabidopsis thalian.* ◇ signify selected WRKY genes for this study

TaWRKY3, TaWRKY8, TaWRKY19, TaWRKY37, TaWRKY44, TaWRKY45, TaWRKY46, TaWRKY53-a, and TaWRKY71 were chosen for this study. The rationale of selection these genes was based on previous studies from WRKY genes in *Arabidopsis thalian*. In addition, the how is their relevant to WRKY genes in *Triticum aestivum* in regard of homology and ortholog. Firstly, *AtWRKY18* and *40 and 60* response to in bacterial and Fungal disease(Xu *et al.*, 2006; Pandey *et al.*, 2010) and *AtWRKY6* was response to resistant against pathogen and senescence (Robatzek and Somssich, 2002) were near to *TaWRKY8* and

71. Secondly, *TaWRKY62* response to with disease (stripe rust in wheat) and high temperature (Wang *et al.*, 2017b) was homologous with *TaWRKY45* and *46*. Thirdly, *AtWRKY25* engaged in plant reaction with bacterial disease (Zheng *et al.*, 2007). *AtWRKY33* response to fungal disease (Zheng *et al.*, 2006) both gene was orthologous with *TaWRKY53-a* and *AtWRKY25* and 33 were also close *TaWRKY3*, *19*, *37* and *44*. MEGA software has been utilised for constructing Phylogenetic tree via WRKY amino acid sequences (*Triticum aestivum* and *Arabidopsis thaliana)* which were obtained from NCBI database (Figure 34).

4.2.10 The effect of B. sorokiniana on WRKY gene expression at 24 and 48 hpi

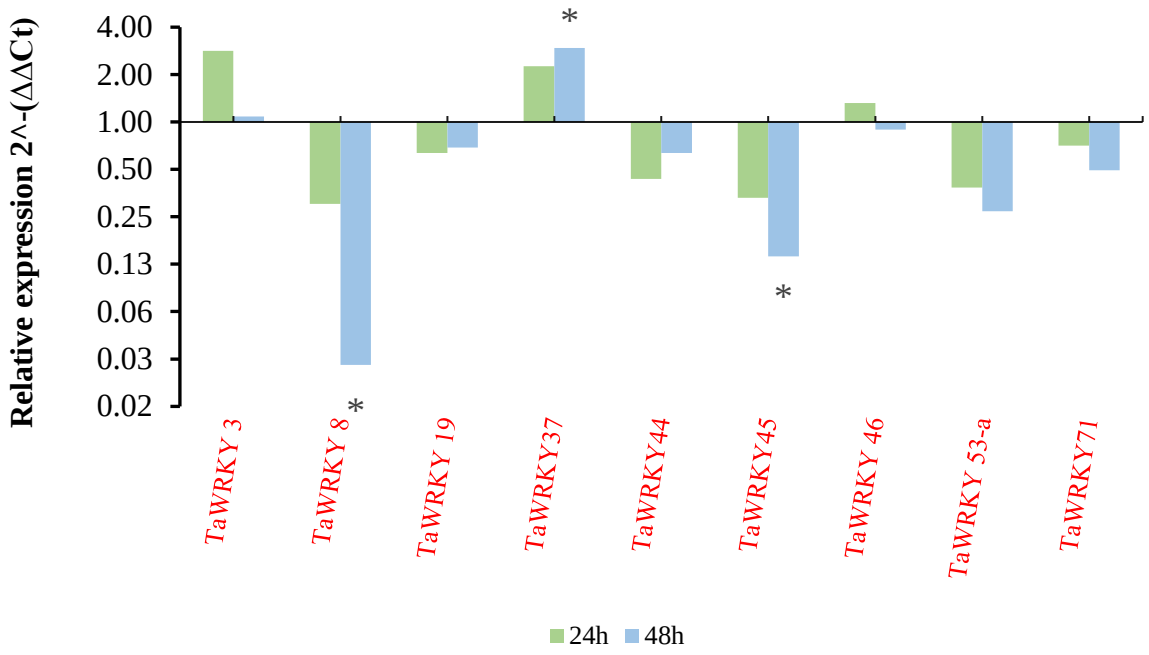

Figure 35 TaWRKY expression profiles in Alderon variety after two times of *B. sorokiniana.* For the Alderon variety, differential expression profiles of the following genes have been analysed in 24 and 48 hpi: *TaWRKY3, TaWRKY8, TaWRKY19, TaWRKY37, TaWRKY44, TaWRKY45, TaWRKY46, TaWRKY53-a,* and *TaWRKY71*. Relative levels of gene expression have been normalised to *TaEF1α*. Thereafter, the aforementioned genes were later normalised to the control (healthy uninfected leaves 0 hpi). It must be noticed that the value repersent using 3 replicates and the t-test was used to determine significant in gene expression between uninfected leaves and infected leaves using delta Ct value *: P<0.05, **:P<0.01,*** P<0.001.

To verify the expression of WRKY genes in the Alderon wheat variety, the real time quantitative PCR (RT-qPCR) has been employed for comparing expression patterns of the mentioned 9-WRKY genes after inoculation by the pathogen (*B. sorokiniana*). In Figure 35, the results showed that there were changes in expression profiles after the different varieties

of wheat were attacked by *B. sorokiniana* during 24 and 48 hpi. Concerning upregulation, firstly, at 24 hpi, there was upregulation in the expression of WRKY genes and the level of fold changes was altered within these genes. For example, *TaWRKY3* showed the highest expression pattern with upregulation compared to control. Its expression level was shown to undergo 2.83-fold change, followed by *TaWRKY37,* which showed its induction to have about 2.26-fold change. Secondly, at 48 hpi, the only gene that was upregulated with highest values amongst other genes was *TaWRKY37* (2.95-fold change). In terms of downregulation, firstly, there were WRKY genes which had downregulation in their expression after 24 and 48 hpi, where 2.63, 3.12, and 3.33-fold change down were observed in *TaWRKY53-a*, *TaWRKY45* and *TaWRKY8*, respectively at 24 hpi. In addition, *TaWRKY44* was seen its expression 2.32-fold change down. Secondly, at 48 hpi, *TaWRKY8* expression was found to have the highest down regulation of around 35-fold change down, followed by *TaWRKY45* and *TaWRKY53-a* (7.6 and 3.7 respectively). Moreover, *TaWRKY71* was induced in its expression with 2.0-fold change down. The genes that did not change expression by more than 2-fold were not deemed have different expression level relative to control due to limitations of the qPCR analysis, for example at 24 hpi, *TaWRKY19,71, 46* were expressed to be 1.58 down, 1.42 down and 1.31 up respectively, at 48 hpi the expression of *TaWRKY44* and *19* were 1.53, 1.47-fold change down. Results from a t-test showed that, in comparison to plants that were not subjected to stress, *TaWRKY8, 37*and 45 at 48 hpi differed significantly (P <0.05) (using delta Ct values).

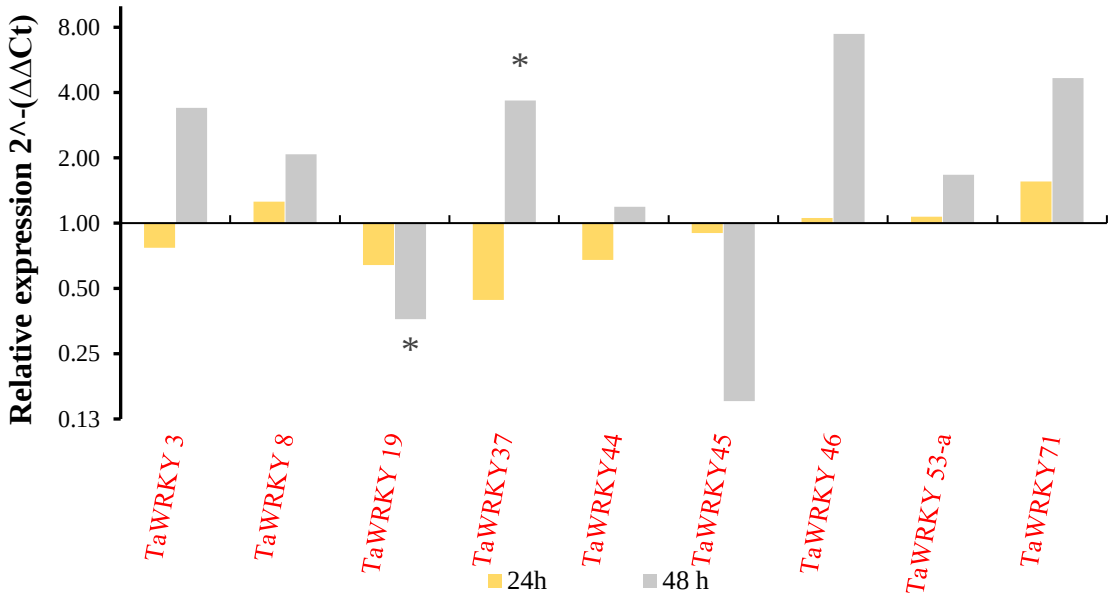

Figure 36 Expression of WRKY gene at two different time after pathogen infection in Sama variety. For the Sama variety, expression profiles of 9 putative WRKY genes (3, 8, 19, 37, 44, 45, 46, 53-a, and 71) have been investigated after 24 and 48 hours being inoculated by *B. sorokiniana*. The elongation factor *TaEF1α* has been used as a standard, and the aforementioned genes were further normalised to control (uninfected leaves 0 hpi). Values aremeans of 3 replicates and the t-test was used to determine significant in gene expression between uninfected leaves and infected leaves using delta Ct value *: P<0.05, **:P<0.01,*** P<0.001.

The level of expression for *TaWRKY3, TaWRKY8, TaWRKY19, TaWRKY37, TaWRKY44, TaWRKY45, TaWRKY46, TaWRKY53-a* and *TaWRKY71* genes have been analysed in response to *B. sorokiniana* at 24 and 48 hpi. In regard to upregulation, firstly, at 48 hpi, *TaWRKY46, TaWRKY71, TaWRKY37, TaWRKY3*, and *TaWRKY8* were expressed highly at 7.46, 4.66, 3.67, 3.39, and 2-fold change. Regarding downregulation, firstly, at 24 hpi, *TaWRKY37* gene was recorded to have fold changes down of 2.27. Secondly, at 48 hpi, *TaWRKY45* was downregulated in its pattern of expression (6.6 -fold change down) with *TaWRKY19* being expressed by 2.77-fold change down. Due to qPCR sensitivity, some genes were not clear in their expression relative to control for example, at 24 hpi, *TaWRKY8, 71,46,53-a,44, 19, 3,45*, were recorded less than 2-fold., At 48 hpi, *TaWRKY44* was recorded less than 2-fold (Figure 36). The statistically analysis (t-test) was showed that *TaWRKY19* and *37*at 48 hpi were significant (P<0.05) difference with unstressed plants (using delta Ct values).

4.2.11 Analysis of TaGSL genes in Inoculated wheat leaves via B. sorokiniana-expression at 24 and 48 hpi

A total of eight transcripts of genes expressed in infected leaves at the seedling stage for Alderon and Sama were analysed for their patterns, post 24 and 48 h, of being penetrated by *B. sorokiniana*.

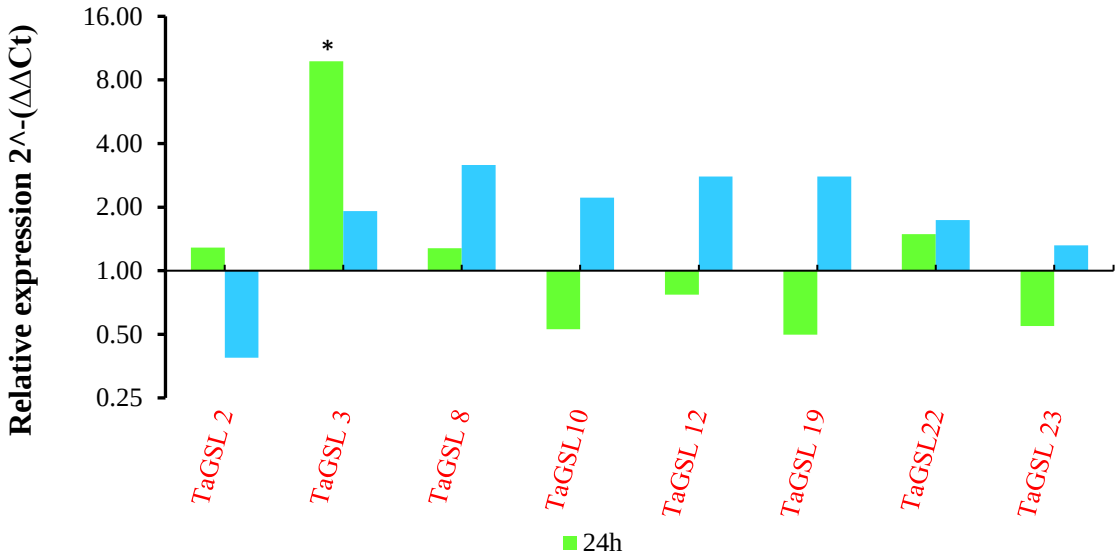

Figure 37 Expression level of *TaGSL* genes at 24 and 48 after pathogen penetration in Alderon. Eight putative *TaGSL* genes (*2,3,8,10,12,19,22,23*) has been conducted and measure their relative expression at the following times penetrated by *B. sorokiniana*: 24 and 48 hpi via qPCR. Further, the *TaGSL* genes have been normalised to the reference gene (*TaEF1α*). Subsequently, the stated genes had been normalised to control (uninfected leaves 0 hpi). It must be indicated that averages/means of 3- biological replicates and the t-test was used to determine significant in gene expression between uninfected leaves and infected leaves using delta Ct value *: P<0.05, **:P<0.01,*** P<0.001)..

Based on real time quantitative PCR (RT-qPCR), the level of expression for *TaGSL* genes in the Alderon wheat variety has been analysed. As displayed in Figure 37. Findings revealed changes in expression profiles after the variety was subjected to 24 and 48 hours, post inoculation via *B. sorokinian*. Focusing on upregulation, firstly, after 24 hpi, within the infected leaves, *TaGSL22*, *8*, and *2* were not showed expression clearly relative control due to sensitivity of qPCR however, there was one gene that expressed the highest amongst all genes; *TaGSL3*, which is recorded to have 9.8-fold change. Secondly, at 48hpi, most of the *TaGSL* genes were upregulated in their expression, except *TaGSL2*, which had 2.63 down-fold change. Additionally, *TaGSL8* was shown to have the highest expression level amongst others (3.16-fold change). *TaGSL12* and *19* had similar pattern in their expression (2.79 and

2.78-fold change respectively). Moreover, *TaGSL10* were close in their upregulation expression (2.21-fold change). *TaGSL3,22* and *23* were found to be similar in their profile expression (1.91.73 and 1.3-fold change, respectively) which were not clear in their expression. Concentrating on downregulation, firstly, at 24hpi, *TaGSL19* had 2.0-fold change down. *TaGSL23*, *10*, and *12* were 1.85, 1.92 and 1.29-fold change down, respectively which they were not considered as expression due qPCR sensitivity. Secondly, at 48hpi, the only gene that was downregulated was *TaGSL2* (2.63-fold change down). The statistically analysis (t-test) was displayed that *TaGSL3* at 24 hpi were significant (P<0.05) difference with unstressed plants (using delta Ct values).

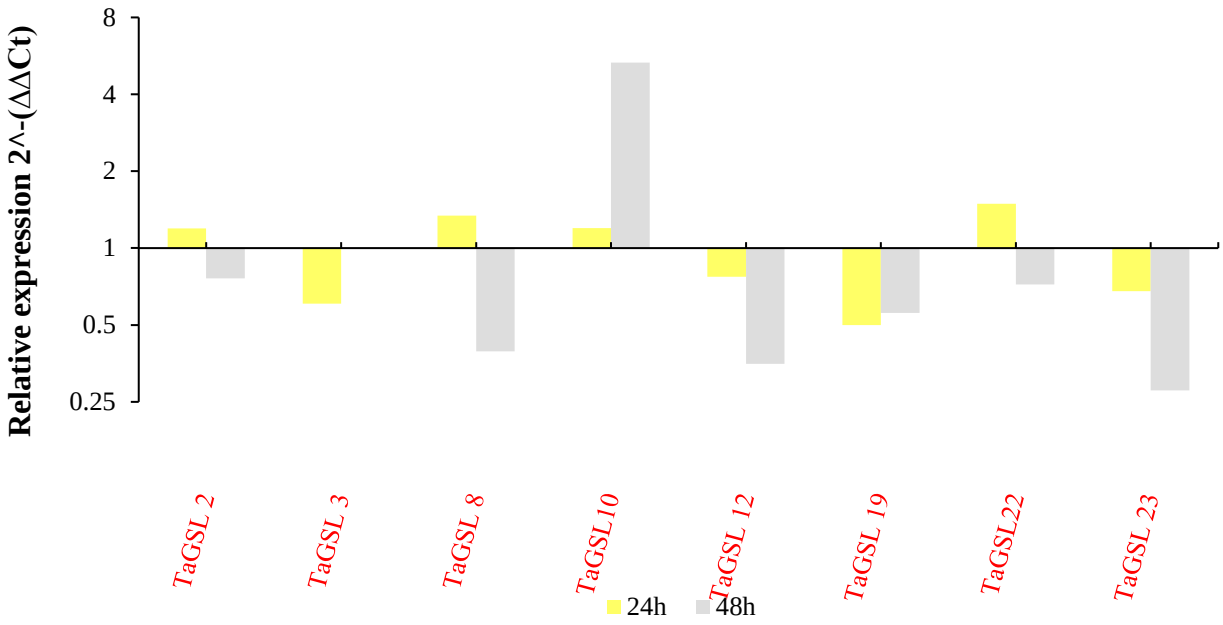

Figure 38 *TaGSL* genes Expression at 24 and 48 after *B. sorokiniana* infiltration. For the Sama variety, expression profiles of 8 candidate *TaGSL* genes (2, 3, 8, 10, 12, 19, 22, and 23) have been investigated after 24 and 48 hpi of *B. sorokiniana*. The elongation factor *TaEF1α* has been utilized as a reference gene. Moreover, the relative expression of the aforementioned genes was further calculated to control (uninfected leaves 0 hpi). Values are means of 3 replicates and the t-test was used to determine significant in gene expression between uninfected leaves and infected leaves using delta Ct value *: P<0.05, **:P<0.01,*** P<0.001)

In Figure 38, the expression level of GSL in Sama had various patterns in response to *B. sorokiniana* invasion. At early stage of infection for 24 and 48 hpi, all genes were analysed via qPCR. At 24 hpi, *TaGSL2*, *8, 10*, and *22* were not highly upregulated and their expression were not clear due to qPCR sensitivity (below at 2-fold change). However, *TaGSL3*, *12* and *23* were down regulated and were close in their expression patterns (1.66, 1.29 and 1.49-fold

change down, respectively) their expressions were not explained the change relative to control. However, *TaGSL19* was change 2-fold change down at 48 hpi, the only gene that were upregulated in its expression, was *TaGSL10* (5.32-fold change), while *TaGSL23*, *12*, and *8* were downregulated with expression profiles which were near to each other (3.70, 2.85, 2.56 -fold change down, respectively). *TaGSL22*, *19*, and *2* were downregulated to 1.38, 1.81, and 1.31-fold change down relative to control, respectively (0 hpi or healthy leaf). In addition, *TaGSL3* was not expressed. Their expressions were not explained their change clearly.

4.2.12 The expression level of SA -related genes (TaPAL, TaNPR1) and JA- related genes (TaAOS and TaLOX2) in wheat inoculated via spot blotch disease at 24 and 48 hpi

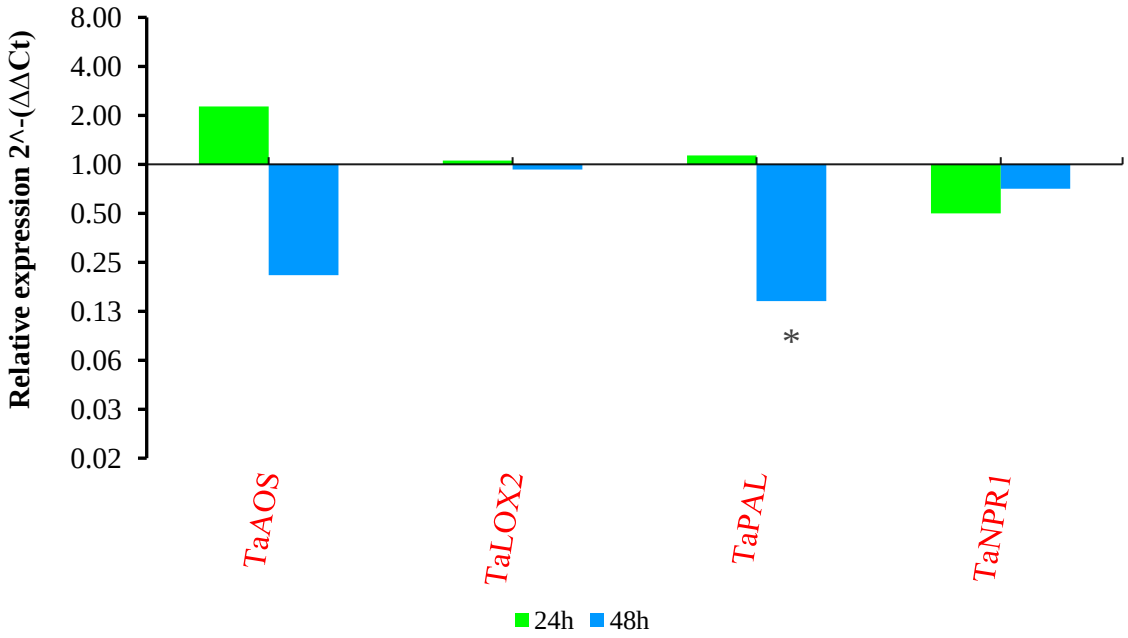

Figure 39 For Alderon genes expression study, selected genes namely *TaAOS*, *TaLOX2*, *TaPAL*, and *TaNPR1* were normalized with *TaEF1* α reference gene. Therefore, the relative gene expression levels were obtained for each candidate in leaveafter 24 and 48 hpi relative to 0 hpi (healthy leaf as control has been set to 1) Mean values are expressed from 3 biological replicates and t-test was used to determine significant in gene expression between uninfected leaves and infected leaves using delta Ct value *: P<0.05, **:P<0.01,*** P<0.001).

The transcript level expressions of *TaAOS*, *TaLOX2*, *TaPAL*, and *TaNPR1* were analysed via qPCR in the Alderon wheat variety after the pathogen infection (24 and 48hpi). As seen in Figure 39, in terms of upregulation (at 24 hpi), *TaAOS* was expressed with 2.27-fold change, relative to control. The rest of the genes were not expressed in upregulation at 24

hpi. However, there was no expression found in all genes at 48hpi, in terms of upregulation. As for down regulation (at 24 hpi), *TaNPR1*was regulated to 2-fold change down, while others were not induced. At 48hpi however, *TaAOS*, *TaPAL*, and were found to have 4.76, 7.14, respectively. *TaNPR1 was* 1.40-fold change down that its expression was not determined the expression change relative to control (regarding down regulation). Compared to non-stressed plants, the t-test revealed that *TaPAL* at 48 differed significantly (P<0.05) (using delta Ct values)

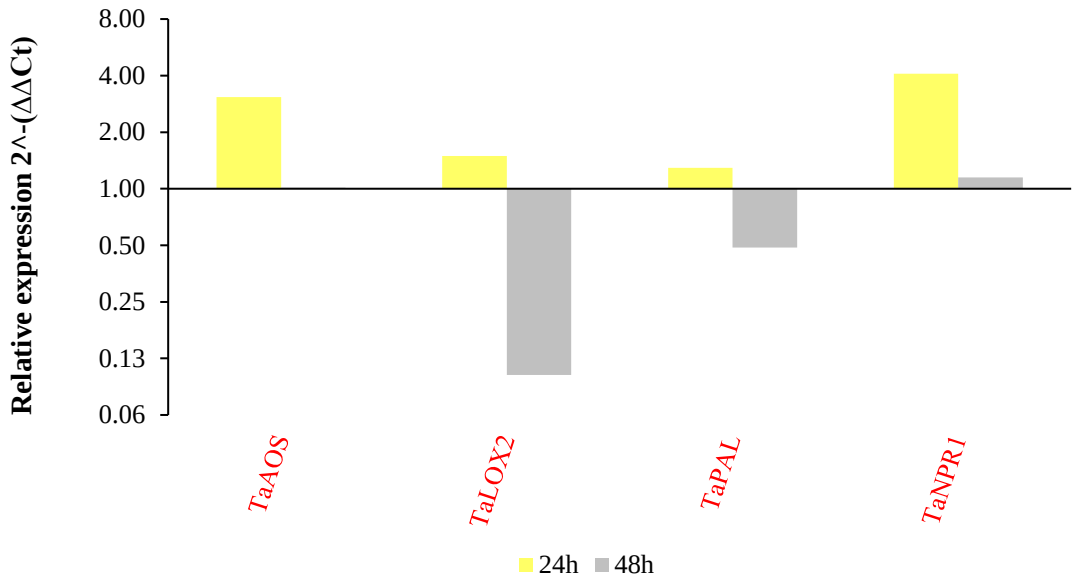

Figure 40 Expression levels of *TaAOS*, *TaLOX2*, *TaPAL*, and *TaNPR1* after pathogen infection (24, 48 hpi) in Sama. *TaAOS*, *TaLOX2*, *TaPAL*, and *TaNPR1* expression level Sama wheat leaves after 24 and 48hpi measured via qPCR. The reference gene *TaEF1α* was used for normalization of these genes, afterwards, the relative gene expression was calculated to on 0 hpi (healthy leaf, as control, has been set to 1). Mean values are expressed from 3 biological replicates and t-test was used to determine significant in gene expression between uninfected leaves and infected leaves using delta Ct value *: P<0.05, **:P<0.01,*** P<0.001)

In the Sama wheat variety, the level of expression for *TaAOS*, *TaLOX2*, *TaPAL*, and *TaNPR1* was analysed via qPCR after the pathogen infection (24 and 48 hpi). Regarding upregulation (at 24 hpi), *TaAOS* and *TaNPR1*were expressed at 3.07 and 4.0-fold change relative to control, respectively. *TaLOX2 and TaPAL were* 1.49, 1.29-fold, they were not considering their expressions.

At 48 hpi, *TaNPR1* was expressed to 1.15-fold change (upregulation); it was not expressed clearly relative to control. As for down regulation, at 48hpi, *TaLOX2* and *TaPAL*

were regulated to 9.8 and 2.0-fold change down, respectively. The gene *TaAOS* was not showed its expression at this time of infection (Figure 40).

Table 5 Expression level of selected genes in two wheat varieties over time of infection (24 ,48 hpi)

Gene	Across timepoint in Alderon (compared to 0 hpi)				Across timepoint in Sama (compared to 0 hpi)				Between varieties (Alderon Vs Sama) at24 and 48 hpi							
									Alderon		Sama		Alderon		Sama	
	24 hpi (Fold induction)		48hpi (Fold induction)		24 hpi (Fold induction)		48hpi (Fold induction)		24 hpi (Fold induction)		24 hp (Fold induction)		48 hpi(Foldinduction)		48 hpi (Fold induction)	
TaWRKY 3	2.83	↑	1.08	↑	1.29	↓	3.4	↑	2.83	↑	1.29	↓	1.08	↑	3.4	↑
TaWRKY 8	3.33	↓	35.71	↓	1.25	↑	2.08	↑	3.33	↓	1.26	↑	35.71	↓	2.08	↑
TaWRKY 19	1.58	↓	1.47	↓	1.56	↓	2.27	↓	1.58	↓	1.56	↓	1.47	↓	2.77	↓
TaWRKY37	2.26	↑	2.95	↑	2.27	↓	3.68	↑	2.26	↑	2.27	↓	2.95	↑	3.68	↑
TaWRKY44	2.32	↓	1.58	↓	1.49	↓	1.19	↑	2.32	↑	1.49	↓	1.58	↓	1.19	↑
TaWRKY45	3.12	↓	7.69	↓	1.11	↓	6.66	↓	3.12	↓	1.11	↓	7.69	↓	6.66	↓
TaWRKY 46	1.13	↑	1.12	↓	1.06	↑	7.46	↑	1.13	↑	1.06	↑	1.12	↓	7.46	↑
TaWRKY 53-a	2.63	↓	3.70	↓	1.07	↑	1.67	↑	2.63	↓	1.07	↑	3.70	↓	1.67	↑
TaWRKY71	1.42	↓	2 .0	↓	1.56	↑	4.67	↑	1.42	↓	1.56	↑	2 .0	↓	4.67	↑
TaGSL 2	1.29	↑	2.63	↓	1.19	↑	1.31	↓	1.29	↑	1.19	↑	2.63	↓	1.31	↓
TaGSL 3	9.81	↑	1.91	↑	1.66	↓	1	↑	9.81	↑	1.66	↓	1.19	↑	1	↑
TaGSL 8	1.28	↑	3.16	↑	1.34	↑	2.56	↓	1.28	↑	1.34	↑	3.16	↑	2.56	↓
TaGSL10	1.92	↓	2.22	↑	1.2	↑	5.32	↑	1.92	↓	1.2	↑	2.22	↑	5.32	↑
TaGSL 12	1.29	↓	2.79	↑	1.29	↓	2.85	↓	1.29	↓	1.29	↓	2.79	↑	2.85	↓
TaGSL 19	2.04	↑	2.79	↑	2 .0	↓	1.81	↓	2 .04	↓	2.0	↓	2.79	↑	1.81	↓
TaGSL22	1.49	↑	1.74	↑	1.49	↑	1.38	↓	1.49	↑	1.49	↑	1.74	↑	1.38	↓
TaGSL 23	1.85	↓	1.32	↑	1.49	↓	3.70	↓	1.85	↓	1.49	↓	1.32	↑	3.70	↓
TaAOS	2.27	↑	4.76	↓	3.07	↑	1.01	↑	2.27	↑	3.07	↑	4.76	↓	1.01	↑
TaLOX2	1.06	↑	1 .0	↑	1.49	↑	10	↓	1.06	↑	1.49	↑	1	↑	10	↓
TaPAL	1.13	↑	7.14	↓	1.29	↑	2	↓	1.13	↑	1.29	↑	7.14	↓	2	↓
TaNPR1	2	↓	1.40	↑	4.09	↑	1.15	↑	2	↓	4.09	↑	1.40	↓	1.15	↑

expression level of selected genes in this study. All genes expression values are shown in each variety across 24 and 48 hpi and between wheat varieties over time of infection (24 ,48 hpi). First group that seen as yellow colour are WRKY genes and the second group GSL genes represent green colour and third group genes SA/ JA- related genes are shown blue colour. ↓ mean down regulation ↑ mean upregulation

4.3 Discussion

The current research offers molecular, biochemical, and physiological data related to resistance against the spot blotch disease, affecting wheat genotypes. In terms of the physiological data.

4.3.3 The effect on phenotype trait from spot blotch disease

Effect of spot blotch disease on the growth trait was not obvious in this study. In (Figure 27 A, B,), shoot length was significantly different between varieties, but between inoculated and non-inoculated plants, there was no statistically significant variation in shoot length observed for all varieties at 96 hpi (Figure 27 Aand B). Moreover, there was no significant difference in number of leaves among all varieties and treatment (inoculated and non-non-inoculated itself) during seedling stage (4^{th} leaf fully expanded and following 4 days of spot blotch infection) using brush leaf method as presented in Figure 27(C and D). Due to the short time of the study, the effect of spot blotch disease on growth of four wheats was not clear. It may be that the growth of wheat plant is affected more, and that effect is clearer at 96 hpi. The wheat stage, inoculation method, fungal spore concentrations, and severity duration, are factors that impact the growth of wheat.

Evaluation of this disease is essential to numerous works of research which are connected to the *B. sorokiniana* pathogen. As a dependable estimate for spot blotch disease, SAD is used. It is also considered as a reference for comparison to other wheat samples (Domiciano *et al.*, 2014). In the present study, as displayed in Figure 28, all four wheat varieties that were inoculated against *B. sorokiniana,* showed different variations in disease severity (%) using SAD. Since the lowest disease severity occurred in the top leaves of Najran and thereafter, Alderon, they were considered as less disease severity. However, between them, they were close to each other, in terms of disease severity.

The next genotype that less disease severity the spot blotch disease after Najran and Alderon, was Cochise. The disease severity percentage in Alderon and Cochise was not significantly different. Surprisingly, the highest disease severity was recorded in the Sama variety. This indicates that the Sama wheat variety had more disease symptoms displayed, as compared to the rest of the wheat varieties. In contrast, Najran and Alderon had less severe symptoms than others (Sama and Cochise). These findings are in agreement with Baba (2019) who found that Rashid, Iraq, and Cordiale wheat varieties show significant high disease severity

compared to Latifia and Tamoz3. Subsequent to the spot blotch infection, the Rashid wheat variety showed higher sever disease symptoms than the Latifia. A further study by Singh *et al.* (2017a) saw that from 176 genotypes, the following examples were resistant wheat varieties against the spot blotch disease (*B. sorokiniana*): VL 892, MP 1277and HD 3043. They also found that the following are instances of wheat varieties which are susceptible to the same pathogen: AKDW 2997, DDK 1029 and HUW 234.

A possible explanation for Najran and Alderon being resistant against the *B.sorokiniana* pathogen, is improved photosynthesis plus antioxidant processes as shown in Figure 28.This explanation is in line with Rios *et al.* (2017) who believe that having less spot blotch severity in cv. BR-18 (barley variety) plants' leaves is due to maintaining the photosynthesis, in addition to the antioxidant system having efficient scavenging of ROS within infected leaves for preventing damage to cells (as a result of the pathogen). Another explanation which is probable for Najran and Alderon, they potentially resist to the pathogen, is that these two varieties were seen to exhibit less disease severity, (necrotic and chlorotic damage) as compared with other varieties. This lower level of damage has been attributed by Chand *et al.* (2008) ,who experimented on barley, to the *B. sorokiniana* pathotoxin being neutralised through the deployment of induced defence mechanisms.

One explanation for the Sama variety showing more severe symptoms is because of the plant tissue being exposed to more damage as a result of the necrosis and chlorosis, which has been pointed to in the research by Chand *et al.* (2008) . They clarify that a potential reason for the higher necrosis is the heightened production of endogenous ethylene, which has been detected in barely, and can be associated to the response against the *B. sorokiniana* toxin.

4.3.4 MDA content

Biotic stress causing overproduction in ROS (Reactive Oxygen Species) has the potential to negatively interrupt plant cell metabolism. Consequently, oxidative stress can be resulted that could damage DNA, proteins, and/or cellular lipids. This ultimately causes death of the cells (Spanic *et al.*, 2017). Malondialdehyde (MDA) content is considered in terms of a lipid peroxidation marker for a long time within research associated with redox signalling and oxidative stress, especially as part of studies which focus on plant exposure to biotic/abiotic stress (Morales and Munné-Bosch, 2019). In the present research in Figure 30 we explore the MDA content inside Alderon (resistant to the pathogen) and Sama (susceptible to the pathogen) during

time-course experiment (0, 24, 48, and 96 hpi). In that regard, there was increased in MDA content with time significantly in Sama variety, for example in comparison to control (6.29 nmol/gFW), MDA content was shown to rise to 8.87, 8.48, and 17.61 nmol/gFW after 24, 48, and 96 hpi, respectively.

In contrast, in Alderon, there was gradual increase over time of infection and it was increased with time significantly,for example, at 24, 48, and 96 hpi, the MDA concentration of the Alderon variety was 6.48, 8.42, and 11.94 nmol/g FW, respectively, compared to the control (5.06 nmol/gFW). . However, it was found that MDA content in both varieties was high at 96 hpi, relative to their control (0 hpi). The MDA content in Alderon was seen to be 11.94 nmole/gFW, which was lower than Sama (17.61) nmole/gFW. Results of our experiment at 96 (infected leaves) was similar to a study which found that MDA content increased at 72 hpi, following infection via *B. sorokiniana* in wheat (Sarkar *et al.*, 2018). In separate research, the Malondialdehyde (MDA) in cv. BR-18 was lower than cv. Guamirim. This research found that there was contrasting amount of resistance within wheat leaves (flag) of the two aforementioned wheat cultivars against spot blotch disease. Severity of Spot blotch disease was significantly lower within cv. BR-18 than cv. Guamirim (Rios *et al.*, 2017) . One of the possible explanations for Alderon having lower levels of MDA content, as compared with Sama, is that Alderon can have less oxidative stress and less disease symptoms while Sama could have more oxidative stress and higher disease severity. An additional explanation might be fungal biomass in Sama being higher than Alderon. Supporting what was just stated, mentions that there was a significant and positive correlation of MDA content with number of branches and number of appressoria in the spot blotch pathogen.

4.3.5 Callose content

Throughout fungal infections, the most powerful defence of the plant is considered to be rapid synthesis of callose. In these circumstances, cell wall appositions (papillae) receive a deposit of callose which is formed under locations of infections and support the papillae as a physical barrier against fungal invasion (Nishimura *et al.*, 2003). In our existing research, we measure the callose content between two varieties (Alderon and Sama) of the pathogen infection over time. As seen in Figure 31, in Sama, the highest level of callose concentration was at 24 hpi and then the concentration was reduced at 48 hpi and more reduction was found at 72 and 96 hpi. In Alderon, callose concentration was gradually increased until 48 hpi and that was reduced at 72

and 96 hpi Based on ANCOVA analysis, there was no statistically difference between two selected varieties and time after infection

Our result is comparable to the research done by Shetty *et al.* (2009) who observed callose deposition, as a reaction against inoculation via *Septoria tritici* in two wheat cultivars (Stakado-resistant and Sevin-susceptible), but with various quantities and rates. Throughout the current dissertation, results showed that in 24 and 48 hpi, the callose quantity reached the maximum level (not statistically different). One probable elucidation regarding this occurrence is that it can be related to *B. sorokiniana* lifecycle throughout infection. *B. sorokiniana* initiates the infection on the leaf, initially, through the biotrophic phase where the callose could be gathered. This has been mentioned in the research done by Basavaraju *et al.* (2009). Basavaraju *et al.* (2009) further clarifies that appositions like callose deposits and papillae structures can cause deficiency in nutrients throughout the biotrophic stage of hemi biotrophy. This is due to primary hyphae and infection vesicles taking nutrients from host plasma membrane.

One additional observation within the current thesis is that the level of callose was higher in Alderon, during 72 and 96 hpi, compared to Sama (not statistically different). It is possible that Alderon is more resistant than Sama against *B. sorokiniana.* This is similar with the study by Muhae-Ud-Din *et al.* (2020), in terms of the higher concentration of the callose deposition in the resistant cultivar than the susceptible one in wheat, when infected by *Tilletia controversa*.

4.3.6 Phytohormones SA, JA content

Phytohormones facilitate all features of responses in plants against abiotic/biotic stresses. One type of phytohormone, SA is a recognised signalling molecule that has a critical role for systemic and local acquired resistance in opposition to pathogens. In addition, it is involved with acclimation to particular abiotic stimuli (Janda *et al.*, 2014). A majority of plants keep a moderately low SA level throughout normal development and growth. However, infections from pathogens generally contribute to a speedy escalation of SA levels (Peng *et al.*, 2021). In Figure 32, the endogenous level of SA was quantified in Alderon and Sama wheat varieties before and after *B. sorokiniana* attack. Time course experiment was conducted to monitor SA content during development of *B. sorokiniana* infection. SA content was higher in Alderon than in Sama at 48, 72, and 96 hpi relative to noninfected leaves (not statistically different). Remarkably, the SA content in Alderon at 48 hpi was the highest (222 % compared to control). In addition, at 24, 72,

and 96 hpi, SA content was recorded to be 6 %, 45 %, and 48 % respectively, which is higher than control (not statistically different). In Sama, SA content was found to be higher at 24 hpi than Alderon, but the difference was not statistically significant. However, after 48, 72, and 96 hpi, it was lower than its control. Our finding is in accordance with Sahu *et al.* (2016), who observed that the Yangmai #6 wheat cultivar is resistant against spot blotch disease and demonstrated greater SA content compared with the Sonalika wheat cultivar, which is susceptible towards it, at 0, 8, 12, and 24 hpi.

In my research, it was noticed that Alderon is possibly more resistant against spot blotch disease and Sama is probably susceptible to it, based on the SA quantification level. This observation was also made in the study conducted by Sharma *et al.* (2018), who noticed that in wheat genotypes, subsequent to infection by spot blotch disease at 12 and 24 hpi, resistant lines exhibited a rise in SA levels, whilst every susceptible genotype (i.e., Sonalika and CIANO T79) did not display a significant rise in SA accumulation following infection via spot blotch disease. I believe that the high level of SA in Sama (24 hpi) and Alderon (48 hpi) could be linked to the lifecycle of *B. sorokiniana*, which comprises two phases during the infection process: the first phase is the biotrophic one which starts at the early stage of infection, while the second phase is the necrotrophic, initiating at late stages of infection. Generally, SA is involved with the biotrophic and hemobiotrophic pathogens (Attard *et al.*, 2010; Sahu *et al.*, 2016).

Jasmonic acid and SA-mediated signalling pathways duty is to prompt responses of plants against external harm such as ones coming from insects, herbivores, and mechanical damages, not to forget pathogen infection. This is because JA and SA-mediated signalling pathways are primarily associated with plant resistance (Ruan *et al.*, 2019). JA has a prominent position as to trigger plants against hemi biotrophic and necrotrophic pathogens, plus feeding from herbivorous insects (Yang *et al.*, 2019a). In the current study, as seen in Figure 33, JA content was measured in two wheat varieties, Alderon and Sama, during the pathogen infection. The measurement of the JA content was based on the following course times: 0, 24, 48, 72, and 96 hpi. It was noticed that the level of JA content did not increase during 24, 48, and 78 hpi for both varieties. However, remarkably, at 96 hpi, the level of JA content was higher in Alderon compared to Sama. Alderon was recorded reaching the highest level of JA content at 15.21 nM, relative to control (2.69 nM), while Sama was 2.53 nM relative to its control (0.95 nM). Our study is similar with the one done by Sun *et al.* (2016). They observed JA content within Wangshuibai, which

was a highly resistant wheat against FHB (*Fusarium Head Blight*), increasing at the 6th hour and returning to normality, afterwards. However, JA content had no growth for NAUH117, which was a susceptible mutant at *Fhb1* locus. Furthermore, JA content was seen to be reduced in the 24th hour for NAUH117.

During my study, JA quantity was obvious at 96 hpi for both wheat varieties, which could be an indicator of the lifecycle of *B. sorokiniana (*the pathogen that switches from biotrophic to necrotrophic phase at 96 hpi), as I view it. This understanding has also been pointed to in the lifecycle of *B. sorokiniana*, as described by Kumar *et al.* (2002). My study also had indications of the JA level being increased when *B. sorokiniana* switched to the necrotrophic phase, which is was mentioned by Yang *et al.* (2019a) , expressing JA being involved with the hemibiotrophic and necrotrophic pathogens.

4.3.7 Expression of WRKY genes when attacked via B. sorokiniana pathogen in early stage of infection at 24 and 48 hpi

Owing to the fact that they are in the position of being critical regulators of immune reactions of crops towards different biotic stresses, thorough research was performed on *WRKY TFs*. (Wani *et al.*, 2021). Understanding the *WRKY* gene expression at the early stage of infection makes it possible to explore wheat resistance to the pathogen. Generally, there was varied expression in *WRKY* genes between two contrasting wheat varieties after 24 and 48 hpi.

Firstly, *TaWRKY3* was expressed in Alderon (resistant to spot blotch disease) and Sama (susceptible to spot blotch disease) varieties during the interaction with the pathogen. This *TaWRKY3* is possibly important to the response against spot blotch disease. In our phylogenetic tree analysis, it was illustrated that *TaWRKY3* was found to be similar in orthology with *AtWRKY25* and *AtWRKY33*. Moreover, it was similar in homology with *TaWRKY53-a* as exhibited in Figure 34. In that regard, *AtWRKY25* has been explained in terms of negatively regulating SA-mediated defence responses countering *Pseudomonas syringae*, which is a bacterial pathogen (Zheng *et al.*, 2007) . Furthermore, mutations of the Arabidopsis *WRKY33* have been seen exhibiting intensified susceptibility towards *Alternaria brassicicola* and *Botrytis cinerea* (necrotrophic fungal pathogens), which were determined via increased disease symptoms plus heightened pathogen development within infected plants (Zheng *et al.*, 2006).

However, within my research, the pattern of expression was changed after 24 and 48 hpi in both wheat varieties. For example, at 24 hpi, *TaWRKY3* expression was upregulated (2.8-fold)

in Alderon, whereas it was downregulated in Sama (1.29-fold not considered it expression as mentioned above due to qPCR sensitivity) (Figure 35, Figure 36, and Table 5). This finding was similar with Baba (2019) who realised that *TaWRKY 3* within Latifia (wheat resistant cultivar to spot blotch disease) had higher expression as compared to the susceptible wheat cultivar (Rashid) subsequent to being infected via the *B. Sorokiniana*. Additionally, at 48 hpi, our result showed that *TaWRKY3* expression level in Sama (susceptible) was 3.39-fold higher than in Alderon (resistant) (Alderon was not expressed at 48 hpi). This change can be associated with other unknown signalling pathways and the *WRKY* genes can be induced or inhibited at specific time after inoculation/pathogen attack.

Secondly, in Table 5, *TaWRKY8, TaWRKY71*, and *TaWRK53-a* were expressed differentially between Alderon (resistant) and Sama (susceptible). Interestingly, in Alderon, the expression levels of these three genes were downregulated, whereas they were upregulated in Sama. Moreover, the level of expression, either upregulated or downregulated, was higher in 48hpi than 24 hpi. It was shown in Figure 35 ,Figure 36, and Table 5, based on the phylogenetic tree, that *TaWRKY8* and *TaWRKY71*were homologues to each other and could be similar in their function and response against spot blotch disease. *TaWRKY8* and *TaWRKY71* were nearly orthologous to *AtWRKY18, AtWRKY40*, and *AtWRKY60*. The reason of the choice for *AtWRKY18*, *AtWRKY40*, and *AtWRKY60* was that they were found in the study by Xu *et al.* (2006) as these genes have been shown to be induced by *Botrytis cinerea* (necrotrophic fungal pathogen) and *Pseudomonas syringae* (hemi biotrophic bacterial pathogen) and. In addition, my study saw *TaWRKY53*-a was similar in homology to *AtWRKY33*. The reason for choosing *AtWRKY33* was that Zheng *et al.* (2006) found that *AtWRKY33* was linked to disease resistance towards two fungal necrotrophic pathogens (*Alternaria brassicicola* and *Botrytis cinerea*).

In addition, the expression of *TaWRKY8, TaWRKY71*, and *TaWRKY53-a* was related to disease development. As we can see, at 48 hpi, the expression was higher than at 24 hpi in both varieties in terms of upregulation/down regulation. One possible explanation for the increased expression of these genes at 48 hpi, is that they may be associated with the progression of *B. sorokiniana* infection in wheat leaf tissue. Another probable explanation is that the downregulation of these three genes in Alderon (resistant) may be associated with the biotrophic phase of the *B. sorokiniana.* However, the upregulation of three genes in the Sama (susceptible) variety may be that they are engaged in the initial period of the necrotrophic phase of *B.*

sorokiniana. It is feasible that Alderon had a delay in exhibiting disease symptoms development when compared to Sama. Chowdhury *et al.* (2017) have explained that the period for the shift from biotrophic phase to the necrotrophic phase of the pathogen causing charcoal rot disease (*Macrophomina phaseolina*) had a substantial delay within Nirmala (resistant variety for sesame) in comparison to VRI-1 (susceptible variety).

Thirdly, *TaWRKY19, TaWRKY45*, and *TaWRKY44* expressions were downregulated; however, expression level was variant between variety and time point. *TaWRKY19, TaWRKY45*, and *TaWRKY44* downregulated subsequent to infection via the pathogen within both varieties of wheat (Alderon and Sama-24 hpi and 48 hpi). The only exception was in Sama for *TaWRKY44* at 48 hpi when it was somewhat upregulated by 1.18-fold which is considered was not clear in its expression relative to control. The level of expression was higher, mostly at 48 hpi, in terms of downregulation. It may be that *TaWRKY19, 45*, and *44* could change their expression after 48 hpi and the change be correlated with disease progression. Related to my study, Millyard (2019) scholarship saw that *TaWRKY2, TaWRKY9, TaWRKY19,* and *TaWRKY29* were upregulated inside wheat, after being subjected to *Septoria leaf blotch*. The upregulation of these genes occurred approximately near time of the shift of *Septoria* to necrotrophic development. This is signified by the beginning of noticeable symptoms in 12day-post-infection. Another comparable research has been done by Baba (2019) where *TaWRKY19* has been found to be greater in 72 h compared to 24 h and 48 h after infection via *B. sorokiniana*.

Fourthly, the expression of *TaWRKY37* was shown to be upregulated in both wheat varieties (except in Sama at 24 hpi, having a 2.27-fold down regulation). In Alderon (resistant), the expression of *TaWRKY37* was obvious in two different time points of infection; however, the expression of the gene was higher at 48 hpi (2.95-fold) than 24 hpi (2.26-fold). In Sama (susceptible), at 24 hpi, the *TaWRKY37* was downregulated (as mentioned), but at 48 hpi, the expression was upregulated to reach 3.67-fold. *TaWRKY37* could be involved in spot blotch disease. Additionally, its expression level can be changed any time, but can be expressed at early stage and late stage of infection. Moreover, it may be correlated with fungal development. On that issue, the explication by Yang *et al.* (2013a) describes a correlation between rise in fungal biomass with *Septoria tritici* transition to destructive necrotrophy from symptomless biotrophy. Fifthly, *TaWRKY46* was expressed slightly in Alderon (resistant) at 24 hpi. Similarly, in Sama, it was somewhat expressed. However, at 48 hpi in Alderon, *TaWRKY46* had been downregulated,

while in Sama it had highest expression (7.6-fold). The variation of this gene between time points and between varieties, could be a response against the *B. Sorokiniana* pathogen with unknown behaviour. As stated, the expression for the sensitive cultivar (Sama) was highest at 48 hpi, which may be linked to the progression of the disease. The expression of *TaWRKY46* could have suppressed/activated some of the linked genes.

Overall, there was differential expression in *WRKY* genes between Alderon and Sama at 24 hpi and 48 hpi. It was clear that the expression of *TaWRKY8, 71,* and *53-a* indicated that each wheat genotypes has distinctly different response to the spot blotch disease, based on genetic diversity. Due to upregulation of *TaWRKY8, 71,* and *53-a* being observed in Sama and the opposite seen in Alderon (downregulation), these genes can be putative candidates for further study. In addition, we can largely see the expression of *WRKY* genes being higher in 48 hpi than 24 hpi.

4.3.8 Relative expression levels of Glucan Synthase-Like (TaGSL) genes in wheat at 24 and 48 hpi by invading B. sorokiniana

Callose is extensively spread in a majority of plant tissues, having a critical function for defence of plants, particularly, in response to environmental signals and/or developmental cues (e.g., invasion by herbivores, infection via pathogens, and wounding) (Qian *et al.*, 2021). The pathogen stimulated callose build-up works as a physical/chemical defence process to strengthen walls of plant cells, playing a crucial function within defensive responses against pathogen challenges (Wang *et al.*, 2021b). An additional feature for differentiating amongst various GSL genes is expression level. Voigt *et al.* (2006) found that *TaGSL10* and *TaGSL3* have change in their expression in organ of wheat and that *TaGSL3* was described *that* might be contributing to creation of callose within pollen/pollen tubes.

In the current study, our selection of *TaGSL2, TaGSL 3, TaGSL 8, TaGSL 10, TaGSL 12, TaGSL 19, TaGSL 22, and TaGSL 23* has been based on various studies, already mentioned in the introduction. As seen in Figure 37 and Figure 38, and Table 5, the relative expression of GSL have been analysed in Alderon (resistant to spot blotch disease) and Sama (susceptible to spot blotch disease) after *B. sorokiniana* challenge (24 hpi and 48 hpi). Firstly, at 24 hpi, it was seen in Alderon in Figure 37 and Table 5 (resistant to spot blotch disease) that some of the GSL genes expressed, change in their expression. For example, we observed that *TaGSL2, TaGSL8,* and *TaGSL22* were closer in their expression level and their expression was not high due to qPCR

sensitivity (from 1.27 to 1.49-fold up regulation). However, expression level of *TaGSL3* was found to be the highest amongst all genes (9.8-fold up regulation). On the other hand, there was downregulation for *TaGSL10, 12, 19,* and *23*. Secondly, for Alderon at 48 hpi, the expression level of all *TaGSL 3, 8, 10, 12, 19, 22,* and *23* upregulated higher than 24 hpi (except for *TaGSL2* that had been induced during the period it was being downregulated). We can explain that the upregulation of most of *TaGSL* genes was clear after 48 hpi which may have induced the callose to be accumulated at this time. These genes also could be engaged in wheat resistance against the pathogen. A relevant research saw that *TaGSL3* and *TaGSL8* gene transcripts play a function in resisting *Fusarium graminiarum*, which is a fungal pathogen (Rana *et al.*, 2014).

Within the Sama variety, as displayed in Figure 38 and Table 5, the most the level of GSL expression was below 2 in upregulation or down regulation and their expression because qPCR sensitivity, however *TaGSL19* was downregulated 2-fold change. At 48 hpi, the expression level of GSL in Sama showed downregulation (*TaGSL8, 12* and *23* change to 2.5 ,2.85 and 3.70-fold, respectively) (except for *TaGSL10* which expressed upregulation 5.3-fold). The upregulation of *TaGSL10* could be due to the general callose synthesis within the leaf, performing an unknow function. We had already predicted the possibility of downregulation in most GSL genes within Sama at 48 hpi. Those genes may be suppressed due to the pathogen changing its strategies to invade the host, which switches to an early necrotrophic phase. The production of a toxin by *B. sorokiniana* to prevent callose synthesis throughout the necrotrophic stage has been explicated by scholars (Kumar *et al.*, 2001; Kumar *et al.*, 2002).

We can summarize that most of the *TaGSL* genes' expression, except for *TaGSL10* at 48 hpi which was expressed highly, were not expressed high or down regulated, which induced the increased susceptibility to spot blotch disease. However, in Alderon, we can observe that *TaGSL*s, except for *TaGSL2* which was induced in downregulation, were higher in their expression at 48 hpi than 24 hpi which induced them to be resistant to the pathogen. In term of *TaGSL* expression, a study conducted by Voigt *et al.* (2006) who demonstrated that GSL genes expressed in different organs of wheat such as *TaGSL3* was expressed in spike but it was not expressed in leaf and stem. In addition, *TaGSL10* was highly expressed in stem and *TaGSL22* was highly expressed in leaf

4.3.9 Expression of JA-related (TaAOS, TaLOX2) and SA-related (TaPAL, TaNPR1) genes affected by Spot Blotch disease at early stage of infection (24 and 48hpi)

For the purpose of controlling and managing resistance against various pathogens, SA is known to be a critical signaling molecule, as it stimulates SAR (Systemic Acquired Resistance), in addition to inducing expression for genes which encode PR (pathogenesis-related) proteins along with H_2O_2 buildup (Qi *et al.*, 2019). The SA biosynthetic pathway can have two different routes in plants: phenylalanine ammonia-lyase (PAL) and isochorismate (IC). In the phenylpropanoid pathway, phenylalanine ammonia-lyase (PAL) enzyme transforms phenylalanine (Phe) to NH_3 via a non-oxidative deamination reaction and trans-cinnamic acid (t-CA) (Dempsey *et al.*, 2011). A considerable amount of research has pointed to the expression of the *PAL* gene being a response to various environmental stimuli (e.g., wounds, pathogen invasions, nutrient impoverishment, temperatures which are extreme, UV irradiation, and other stress factors) (Huang *et al.*, 2010). Non-expresser for pathogenesis-related genes1 (*NPR1)* is necessary to transduce SA signals for the activation of genes which are pathogen related (PR) plus inducing systemic acquired resistance (SAR) (Ahangar *et al.*, 2017).

Acting in the role of a core regulator for SA-dependent pathway, NPR1 operates downstream of SA(Kumar, 2014). Within the current study, we attempted to determine the impact of *B. sorokiniana* infection on the expression of SA-related genes (*TaPAL* and *TaNPR1*) and JA-related genes (*TaAOS* and *TaLOX2*) at two different time points (24 and 48 hpi) for the 4 genes. This was done using two different wheat varieties, Alderon and Sama, as displayed in Figure 39 and Figure 40, and Table 5. Firstly, in Alderon, the expression level of *TaPAL* at 24 hpi was slightly increased, relative to the control. It is not clear its expression due qPCR sensitivity

However, the expression level of *TaPAL* at 48 hpi was reduced to 7.14 -fold down, relative the control. It is critical to highlight that this change of expression at different time points, potentially indicates that there was a response to *B. sorokiniana*. The *TaPAL* gene could be expressed higher before these two time points.

Secondly, in Sama, the level of expression for *TaPAL* was increased and it was not considered its expression (below 2) due to qPCR sensitivity at 24 hpi. However, at 48 hpi, the gene expression was reduced to 2.0-fold, relative to control. As we can see, the response of the *TaPAL* gene in both wheat varieties at 24 hpi was expressed. However, it was suppressed at 48

hpi. Overall and as partly mentioned, this pattern of change could indicate that there was response for this gene against the fungus at specific times, but its role in this research is unknown. The transcript level may be higher at an early time of infection as to avert the fungus penetrating the plant leaf. An instance where the over-expression of PAL significantly decreased susceptibility against *Cercospora nicotianae* within tobacco, was observed in the research performed by Shadle *et al.* (2003).

Regarding *TaNPR1*, firstly, the expression level of *TaNPR1* was observed to be reduced at 24 and 48hpi in Alderon, although at different levels 2 and (1.42-fold Due qPCR sensitivity), respectively. Secondly, in the Sama variety, the transcript level of *TaNPR1* reached 4.0-fold and then, was reduced to 1.15-fold. This value is not clear in its relative to control. at 24 and 48 hpi, respectively. The pattern of alteration in this gene, at two different times, revealed that *TaNPR1* is influenced by the pathogen. This variation in expression revealed how the pathogen can impact gene expression in an unknown manner, which may indicate the gene's involvement and association with other PR1 and SA pathways (Cao *et al.*, 1997; Gao *et al.*, 2018). Interestingly, we noticed certain results in the Sama variety as NPR1 expression was upregulated at 48 hpi (1.15-fold due to qPCR sensitivity), while PAL was down regulated at 48 hpi (2-fold). *TaNPR1* may act as negative feedback regulator for *TaPAL*. This was demonstrated within the research conducted by Wildermuth *et al.* (2001) where they saw NPR1 as a negative feedback regulator for *ICS1* expression.

As mentioned in introduction chapter 1 on JA, allene oxide synthase (AOS) and lipoxygenase (LOX) participate in its synthesis and its derivatives (Wasternack and Hause, 2013; Chini *et al.*, 2018). In our study, as far as JA-related genes were observed, various changes in the expression of *TaAOS* and *TaLOX2* were found in both wheat varieties. In Alderon, *TaAOS* expression level was upregulated to 2.27-fold at 24 hpi and then it was downregulated (4.76-fold) at 48 hpi. However, *TaLOX2* was not expressed at 24 and 48 hpi in the Alderon variety. For Sama however, the expression level of *TaAOS* increases 3-fold at 24 hpi, but was not expressed at 48 hpi. Expression of *TaLOX2* elevated by 1.49-fold which was not considered its expression due qPCR sensitivity at 24 hpi, but it was reduced to 10-fold down at 48 hpi. The changes and non-expression of these genes is possibly not related to JA-synthesis during the pathogen attack. The gene response may be expressed at a late stage of the infection. The alteration of expression

along with non-expression at 24 and 48 hpi, is possibly the priming response for plants (Galis *et al.*, 2009; Hilker *et al.*, 2016).

4.4 Conclusions

Findings from the analyses of this study reveal that the response from wheat towards spot blotch disease is quite a complicated process, starting in 1st stage of infection to the last stage of plant resistance. Combining physiological, biochemical, and molecular levels is required for improving the knowledge we hold regarding the resistance process and for understanding the whole reaction of wheat crops towards spot blotch disease. Determining MDA content, in addition to comparing the susceptible variety (Sama) with the resistant variety (Alderon) at various time points, has shown that Alderon was less impacted by oxidative stress than Sama.

Since JA and SA phytohormones participate within plants' resistance and immunity mechanisms in opposition to numerous diseases(Shigenaga *et al.*, 2017) , their content was quantified in the early phase and late phase of infection for Sama and Alderon. It seemed that the level of SA was higher for Alderon compared with Sama in the early infection phase, and, even higher JA level was shown for Alderon, in the late infection phase. For describing the temporal and spatial stages of pathogen infection, callose concentration was focused on in varying time points. It was noticed that callose content increased after fungal inoculation in both resistant and susceptible varieties. Moreover, *WRKY* genes were impacted by the pathogen for both varieties in upregulation and downregulation. In 48 hpi specifically, there was an upregulation of GSL, especially for Alderon. Additionally, SA and JA related genes were found to be induced in both varieties. Thus, the current study shows the complexity and differential scheme of biochemical and molecular responses of each variety to *B. sorokinia* infection, and the fine regulation of the disease resistance mechanism at the spatial-temporal level, starting from the inoculation to the end of the resistance mechanism.

Chapter 5. Combination of Salt Stress and Spot Blotch Disease in Wheat

5.1 Introduction

Due to potential consequences of climate change and global warming, abiotic/biotic stress factors appear in combination within crops are likely to occur more frequently, these combined stresses could result in a synergistic increase in their impact upon growth and yield (Pandey *et al.*, 2017). The responses and reactions of crops have against many simultaneous stresses are often dissimilar to when they are subjected to single stress factors, owing to the added complication and involvement of numerous signalling pathways (Atkinson and Urwin, 2012). Under a combination of biotic and abiotic factors, which could have positive or negative impacts upon the plant, crops could react differently, depending on susceptibility to each. Many factors contribute to the combination of stresses such as type of stress, level of the stress, virulency of a pathogen, stress duration, and vulnerability of crop (Rejeb *et al.*, 2014).

The co-occurrence of salinity and plant diseases is of great concern, this combination of stresses has a considerable detrimental effect upon health, safety, and quality of foods (Chojak-Koźniewska *et al.*, 2018). More specifically on salt stress, it should be mentioned that soil salinity causes ion imbalance within crops which disturbs metabolic activities and leads to oxidative damage, reducing plant productivity (Munns and Tester, 2008). A known hemibiotophic pathogen, *Bipolaris sorokiniana*, the causative agent of the foliar disease spot blotch, severely limits wheat growth and development in warmer areas of the planet (Kumar *et al.*, 2002). It is worth mentioning that the issue of salt stress has been researched by numerous scholars (Poustini and Siosemardeh, 2004; Yassin *et al.*, 2019) with spot blotch disease having been given the same considerable attention (Tembo *et al.*, 2017). However, the impact upon wheat from their combination has been under-researched and not received significant attention (to the best of the author's knowledge). Therefore, response to a combined *B. sorokiniana* together with salt stress is not known in detail. Within the current study, the aim of this chapter is focusing on the biochemical and molecular response and the physiological performance of two contrasting wheat varieties (i.e., Alderon and Sama) under combined high-level salinity stress and spot blotch infection.

Chapter 5. combination of salt and Spot blotch disease in wheat.

5.1.1 Chapter objectives

1. To evaluate growth and estimate disease severity in two contrasting wheat varieties (Alderon and Sama) under combined stresses of salt and spot blotch disease

2. To study the ion contents (Na^+ and K^+ concentration) in Alderon and Sama, upon exposure to combined salt stress and spot blotch disease

3. To study the quantity of MDA and hormonal modulation (SA, JA) under combined salt and spot blotch disease in both genotypes

4. Analysing the level of change in expression of putative WRKY genes exposed to a combination of stresses within 2 wheat cultivars

5- To observe the change in candidate gene expression related to SA biosynthesis and signalling and JA biosynthesis, in Alderon and Sama, under combined salt and spot blotch disease

5.1.2 Chapter hypothesis

1- The effect of Na^+ and K^+ content reduce spot blotch disease severity in wheat

2-The effect of a combination of salt and spot blotch disease, results in elevated levels of MDA in wheat.

3-The impact of combined salt with spot blotch disease, changes the abundance of SA and JA in wheat.

4-Expression of WRKY genes is influenced by both genotype and temporal regulation.

5-Genes of phytohormone biosynthetic pathways, *TaPAL* (SA biosynthesis), *TaNPR*1 (SA-downregulation), *TaAOS,* and *TaLOX2* (JA-biosynthesis), show genotype and temporal differences in expression profiles.

5.2 Results

To examine the effects of high levels of salt stress (160 mM NaCl) on spot blotch disease progression, we evaluated the response of two selected wheat varieties: Sama (susceptible to spot blotch disease and sensitive to salt stress) and Alderon (resistant to spot blotch disease and more tolerant than Sama to salt stress). Plants were precondition with 160 mM NaCl for 8 days and then inoculated with *B. sorokiniana* spores. All parameters were assessed over time of infection (0, 24, 48, 72, and 96 hrs). It must be noted that during time of infection, salt stress was maintained by plants with 160 mM NaCl solution. The growth of plants, ion content (Na^+ and K^+), MDA content, and plant hormonal concentrations (SA and JA) were assessed. Following that, the effect of the combined stresses of 160 mM NaCl and *B. sorokiniana* pathogen on WRKY gene expression and SA/JA-related gene biosynthetic pathways, during 24- and 48-hours post infection (hpi) were evaluated. The control condition in this experiment refers to the non-stressed plant at 0 hpi.

5.2.1 Assessment of disease severity and growth parameters under combined stresses

5.2.2 Disease severity

Under the experimental conditions mentioned above, spot blotch disease severity was observed and recorded under combined stresses of 160 mM NaCl and spot blotch infection (48, 72, and 96 hpi) for Alderon and Sama (relative to non-stressed plant at time=0, which is the control, with value of disease severity=0) as seen in Figure 41. The disease severity was recorded based on SAD method for both varieties and using the brush method. Differences were observed in disease severity in the 3rd leaf for each variety. Data for Sama shows that the disease severity rose more rapidly and reached a higher final level than Alderon. At 48, 72 and 96 hpi of combined stresses, the disease severity was recorded at 3, 9, 25 % when compared to control respectively in the Alderon genotype. In the Sama genotype, at 48, 72, and 96 hpi, the disease severity has been recorded at 5, 18, 43 43 %, when compared to control (Figure 42). Statistical analysis (ANCOVA) was used to see the difference between Alderon and Sama on disease severity and the time after infection was used as covariate variable. There was significant difference in disease severity between Alderon and Sama ($F= 6.77$, $P<0.002$) and there was significant increasing on disease severity with time main effect ($F = 91.38$, $P<0.0005$). In addition, there was significant interaction between varieties and time ($F =73.27$, $P<0.0005$). The statistical analysis (pairwise comparison) provides that there was significant difference ($P <0.015$) between Alderon and Sama on disease severity. All results confirmed that Alderon is more resistance than Sama to the pathogen.

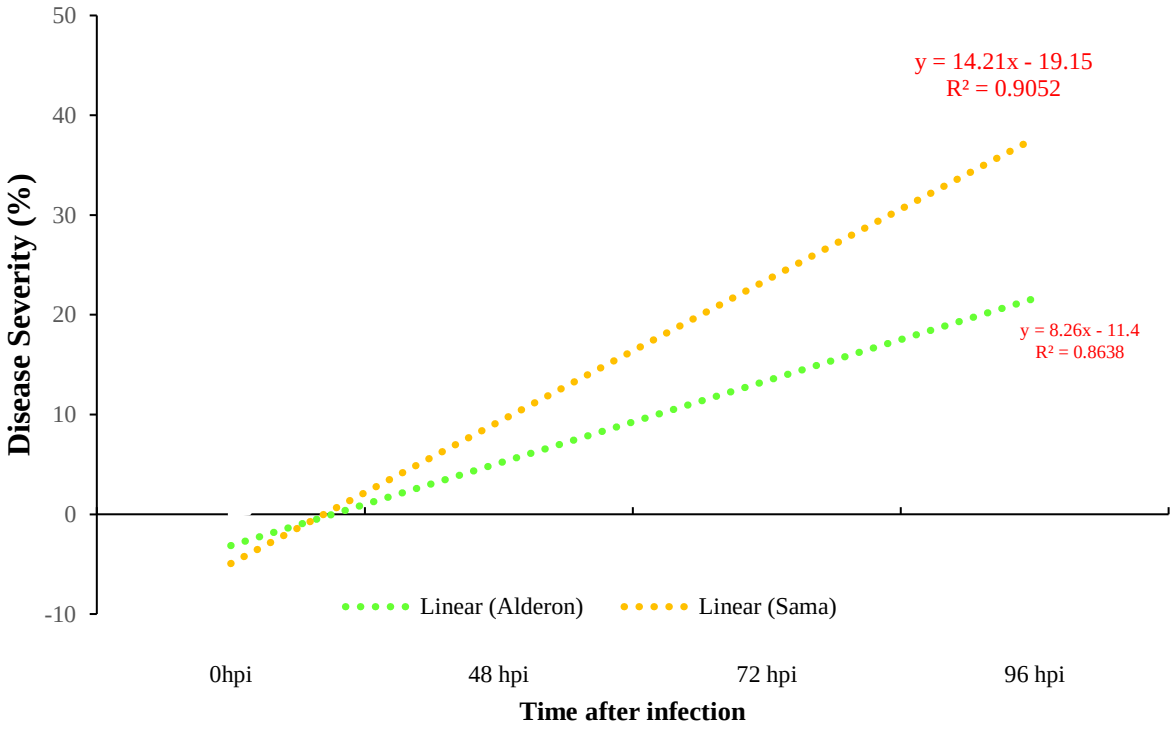

Figure 41 Disease severity (%) of two selected wheat varieties under salt and spot blotch disease combination. Alderon and Sama were grown in sand culture treated with Hoagland solution, containing 160 mM NaCl for 8 days following spot blotch disease infection. Disease severity (%) on top leaves of Alderon and Sama varieties were recorded at 48, 72, and 96 hrs after inoculation via *B. sorokiniana* and 160 mM NaCl exposure (relative to time=0, which is non-stressed plant). Data represents the mean values of 10 biological replications (n=10). The data was shown as fitted lines. ANCOVA was conducted to determine a statistically significant difference in disease severity between two selected wheat varieties during time post infection (covariate variable).

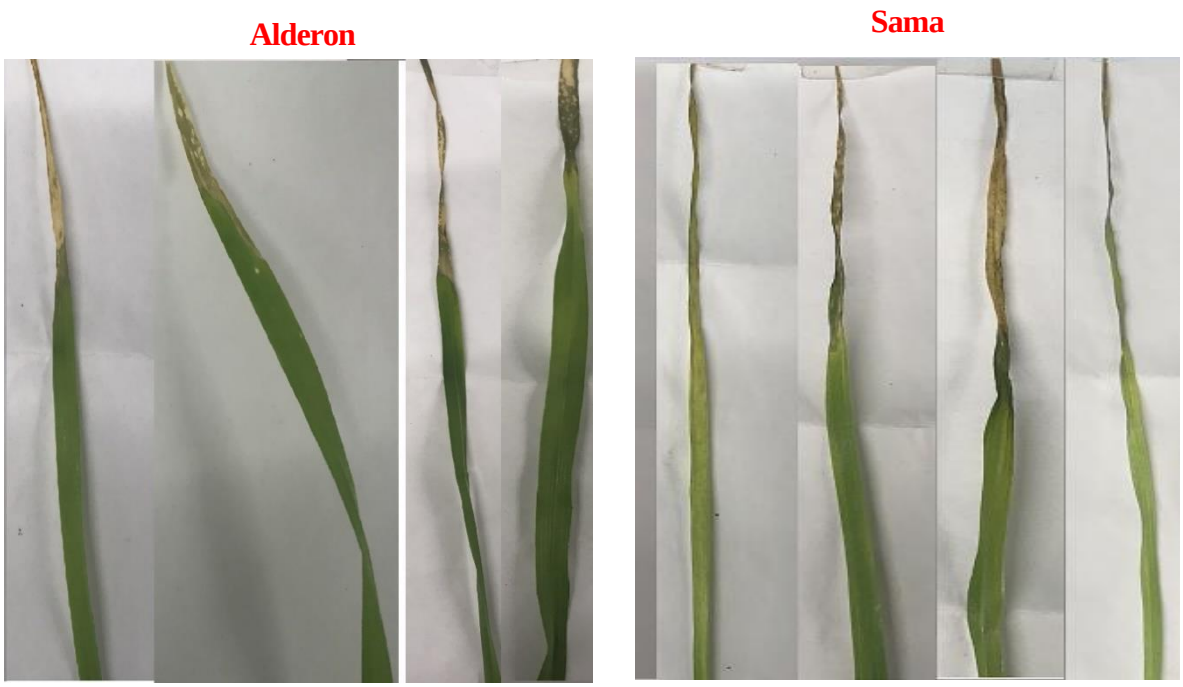

Figure 42 Spot blotch disease symptoms (necrosis and chlorosis) On the third leaf of four wheat varieties using brush method, Symptoms were more recorded at 96 hpi of combined stress Alderon, Sama

5.2.3 Shoot length and leaf numbers

Under the same experimental conditions as above, the growth parameters were measured from stressd plants grown under combined stress (160 mM NaCl and 96 hrs of infection) and unstressed plants. Shoot length was measured for Alderon and Sama. The shoot length was affected due to combined stresses in Sama. The shoot length of each wheat variety (Alderon and Sama) for all treatments (non-stressed plants and stressed plants) was recorded. Alderon was measured 46.33 cm ± 0.54 while Sama was recored only 35.58 cm ± 0.87 as displayed in Figure 43(**A**). Moreover, the impact of each treatment (non-stressed plants and stressed plants) on shoot length for all wheat varieties is shown in Figure 43 (B). The average of shoot length in all wheat varieties was recorded at non stressted plant (42.33 cm ± 1.09) while the shoot length was found 39.58 cm ± 1.33 in stressed plants. Statistical analysis was conducted using two-way ANOVA considering the effect of treatments, varieties and their interaction on shoot length. The effect of wheat varieties was highly significant ($F = 159.09$, $P < 0.0005$) in shoot length and the effect of treatment (non- stressed and stressed plants) was signifcant ($F = 10.41$, $P < 0.004$), However, there was no signficant interaction between genotypes and treatment ($F(1, 20) = 1.61$, $P < 0.218$).

It was revealed that the number of leaves were close in Alderon and Sama according to the wheat variety under all treatment (non- stressed and stressed plants) as shown in Figure 43 (**C**). However, it was observed that number of leaves for 2 wheat varieties at non- stressed treatment was higher than the number of leaves at stressed plants as can be seen in Figure 43 (D). The two-way ANOVA analysis was shown that there was significant effect on treatments ($F = 19.6$, $P < 0.0005$), but there was no significant effect on number of leaves on varieties and their interaction (varieties and treatments)

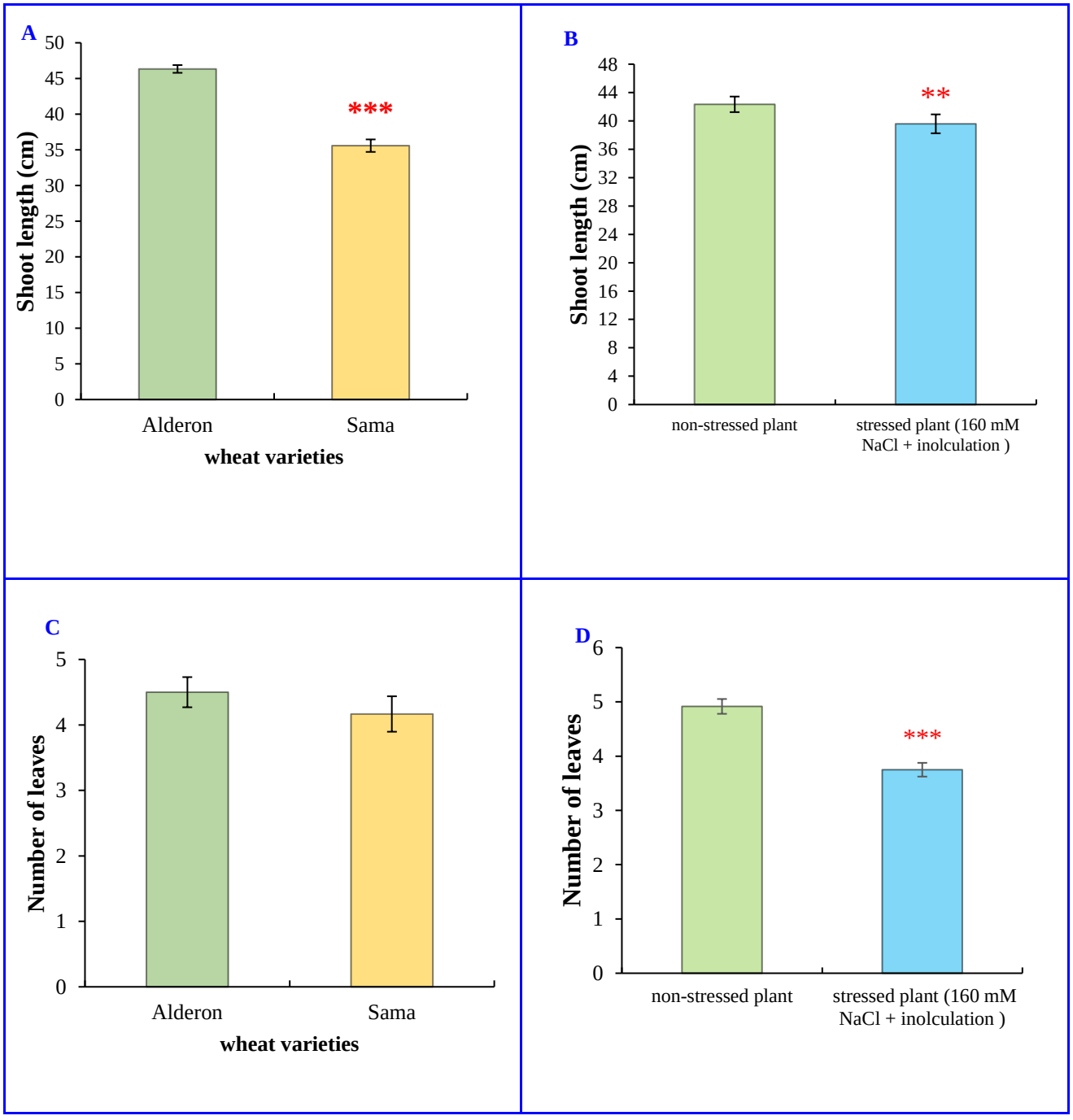

Figure 43 The effect of salt and B. sorokiniana infection on shoot length and number of leaves for two wheat varieties. Shoot length variation according to wheat varieties (**A**) and treatments (non-stressed plants and stressed plants) (**B**). Leaves number of 2 wheat varieties (**C**) and treatments (non-stressed plants and stressed plants effect(**D**). Alderon and Sama were planted in sand and watered with Hoagland solution until the 4th leaf was displayed, and the plant was exposed to 160 mM NaCl for 8 days. Afterwards, the plant was infected by B. sorokiniana for 96 hrs with continuous watering of 160 mM NaCl (Control, in this experiment, was the non-stressed plants, having 0 mM NaCl without inoculation). Varietal effect (A, C), shoot length and numbers of leaves of each wheat variety across non-stressed plants and stressed plants (treatments) represents mean ± standard error (6 biological replications/treatment). non-stressed plants and stressed plants (treatments) effect (B, D), shoot length and number of leaves for 2 wheat varieties through each treatment (non-stressed plants and stressed plants) represents mean ± standard error (6 biological replications/ wheat variety). Using two-way ANOVA. $P < 0.05$. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

5.2.4 Biochemical changes under the combination of salt stress and spot blotch disease

5.2.5 The effect of combined stresses leaf sodium and potassium ion content

Among same experimental conditions described above, the ion content of the 3rd leaf was measured by flame photometry. Na^+ and K^+ content was measured at 96 hpi. Firstly, Na^+ content was higher in Alderon than in Sama. The Na^+ content in Alderon under all treatment (non-stressed plants and stressed plants) was quantified at 314.03 mmol/l while in Sama was recorded at 234.08 mmol/l as shown in Figure 44(A). Secondly, the study showed that Na^+ content for Alderon and Sama in stressed treatment was higher than non- stressed treatment (526.08 and 22.03 mmol/l, respectively) as presented in Figure 44(B). The statistical analysis (two way-ANOVA) was used to determine the effect of variety, treatments and the interaction of variety and treatments. Obtained results from statistical analysis have shown that there was significant on Na^+ content between treatments ($F = 82.28$, $P < 0.0005$) while there was no significant effect on Na^+ content between varieties ($F = 2.06$, $P < 0.169$) and the interaction ($F = 3.13$, $P < 0.96$) between varieties and treatments. The K^+ content in Alderon under all treatment was recorded 101.61 mmol/l while it was found 81.63 mmol/l in Sama as can be seen in Figure 44 (C). In addition, the K^+ content in stressed plant (all varieties at stressed treatment) was 99.85 mmol/l while it was observed 83.39 mmol/l as presented in Figure 44 (D). Two- way ANOVA was shown there was no significant effect on variety, treatments and their interaction in K^+ content.

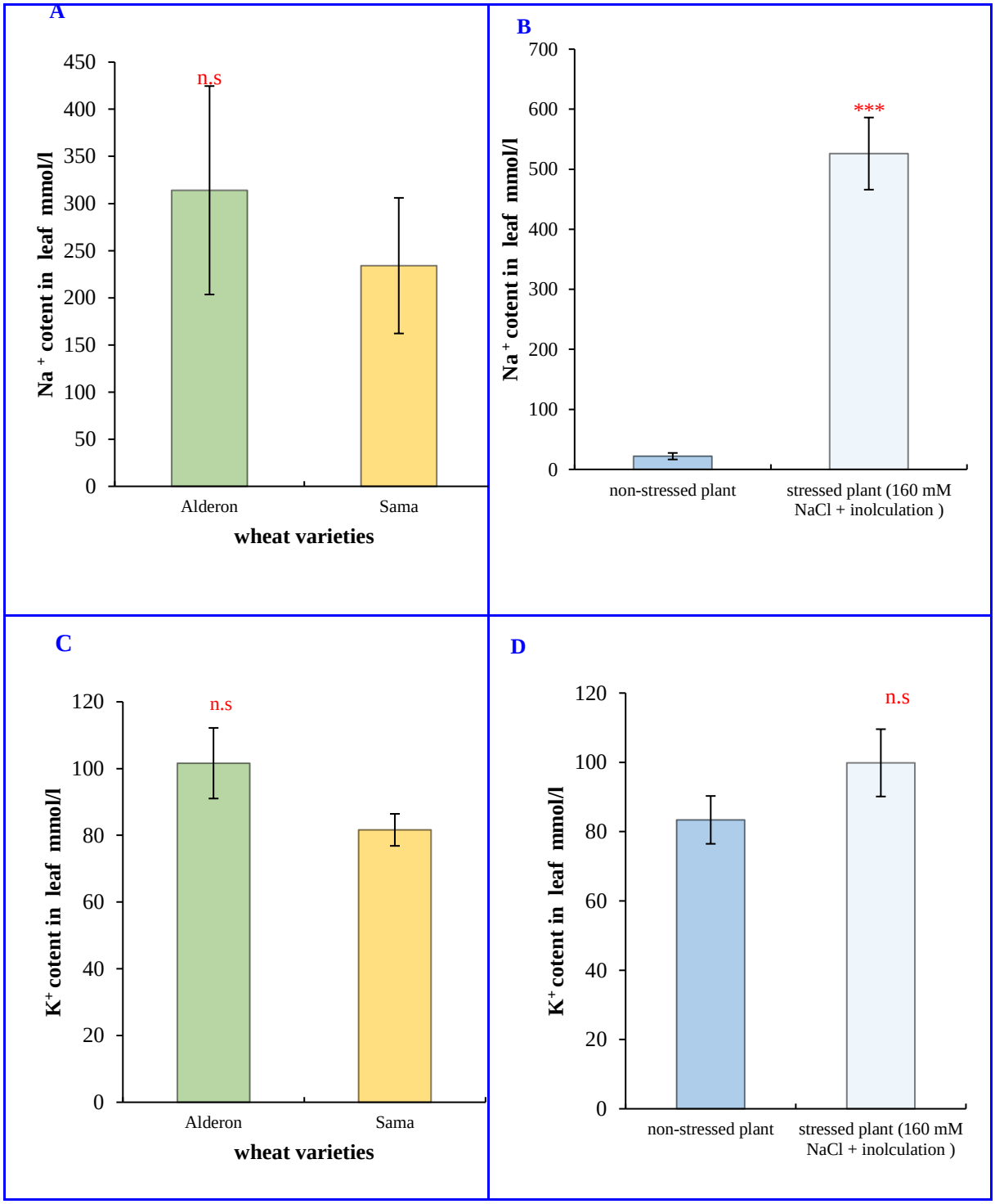

Figure 44 Ion content in leaves for two selected wheat varieties under combination of salt and spot blotch disease. Na^+ content according to wheat varieties (**A**) and treatments (non-stressed plants and stressed plants (B). K^+ content of 2 wheat varieties (**C**) and treatments (non-stressed plants and stressed plants) effect (D). Alderon and Sama were planted in sand and watered with Hoagland solution until the 4th leaf was displayed, and the plant was exposed to 160 mM NaCl for 8 days. Afterwards, the plant was infected by *B. sorokiniana* for 96 hrs with continuous watering of 160 mM NaCl (control, in this experiment, was the non-stressed plants, having 0 mM NaCl without inoculation). Varietal effect (**A**, **C**), Na^+ and K^+ contents of each wheat variety across non-stressed plants and stressed plants (treatments) represents

mean ± standard error (5 biological replications/treatment). Non-stressed plants and stressed plants (treatments) effect (**B**, **D**), Na^+ and K^+ contents for 2 wheat varieties through each treatment (non-stressed plants and stressed plants) represents mean ± standard error (5 biological replications/ wheat variety). Using two-way ANOVA: $P < 0.05$. *: $P<0.05$, **: $P<0.01$, *** $P<0.001$. n.s: not significant effect).

5.2.6 MDA content in two selected wheat varieties under salt and spot blotch infection

In plants exposed to the same conditions as described before, the difference in oxidative stress between Alderon and Sama, under combined stresses, was measured by changes in leaf MDA content at 24, 48, and 96 hpi. First, for Sama, MDA content at 96 hpi was recorded as 10.25 nmol/gFW, compared to control (0 hpi) (1.39 nmol/gFW). In contrast, Alderon was noted 6.63 nmol/gFW relative to control 1.11 nmol/gFW. For both genotypes the highest levels of MDA were obtained at 96 hrs of combined stresses. However, the level of MDA accumulation was higher in Sama than in Alderon for this time point. Secondly, in Alderon genotype, at 24 (2.41 nmol/gFW) and 48 hrs (2.77 nmol/gFW) of combined stresses, when compared to control (1.11 nmol/gFW). In addition, at 24 and 48 hpi, MDA content in Sama was recorded 2.89 and 2.05 nmol/gFW, respectively relative to control (1.39 nmol/gFW) (Figure 45).

Statistical analysis (ANCOVA) was used to find the difference in MDA content between Alderon and Sama varieties while controlling the time points (time after infection combined 160 mM NaCl). There was no significant difference on MDA content between Alderon and Sama ($F= 0.811$, $P < 0.375$) and there was significant increasing in MDA content with time main effect ($F = 27,59$, $P<0.0005$). In addition, there was no significant interaction between varieties and time ($F =1.78$, $P < 0.193$).

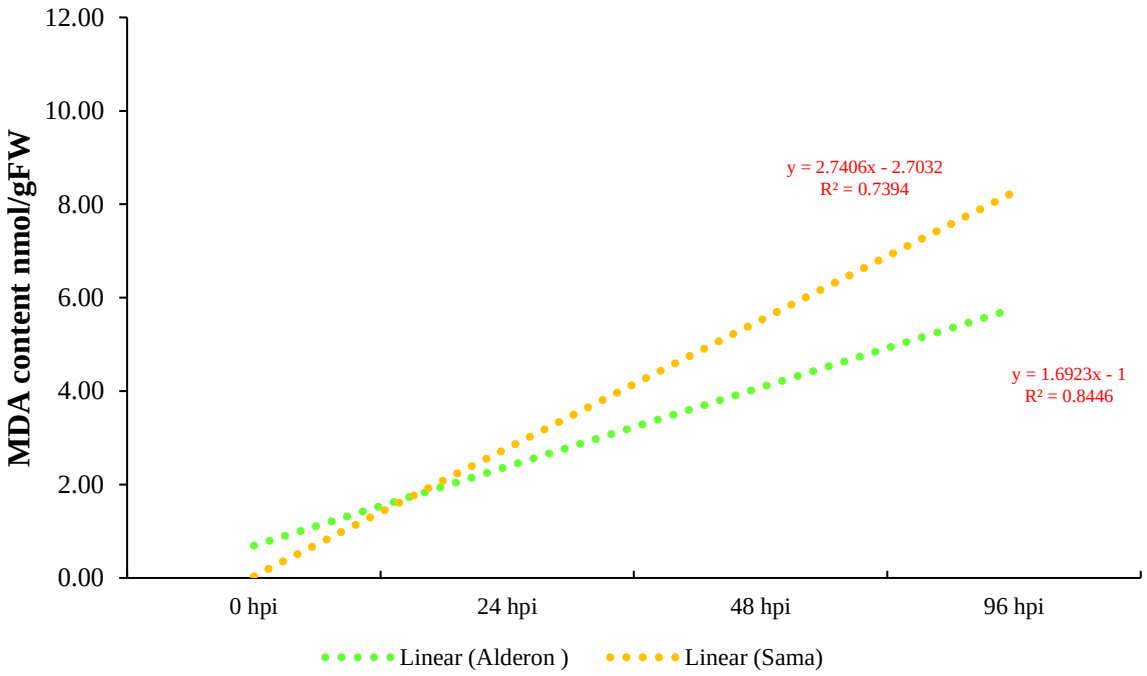

Figure 45 MDA accumulation in non-stressed and stressed leaves of Alderon and Sama, after exposure to combined 160 mM NaCl and *B. sorokiniana* infection. MDA accumulation was measured in leaves of Alderon and Sama under combined stress treatment (160 mM NaCl and B. sorokiniana infection during 24, 48, and 96 hrs) and non-stressed treatment. Data are means of 4 biological replications. The data was shown as fitted lines. ANCOVA was conducted to determine a statistically significant difference between two selected wheat varieties on MDA concentration controlling for time (covariate variable).

5.2.7 SA content in two selected wheat varieties under salt and spot blotch infection

For crops subjected to the same experimental conditions as mentioned above, SA content was quantified via the LC-MS/MS method, when both varieties were exposed to combined stresses at 0, 24, 48, 72, and 96 hpi. The level of SA content was recorded as being highest at the 72hpi (135 nM) timepoint in Alderon, compared to the rest of the time points. While in Sama, the highest SA level was recorded at the 96hpi (104 nM) timepoint (Figure 46).Using analysis of covariance (ANCOVA), we compared the impact of Alderon and Sama on SA levels while adjusting for time (time after infection with 160 mM NaCl). The analysis showed that there was no significant difference in SA among tested varieties ($F=1.09$, $P<0.30$). There was no significant difference for SA with time ($F=0.864$, $P<0.357$) and there was no significant interaction between time and varieties for SA ($F=1.06$, $P<0.308$).

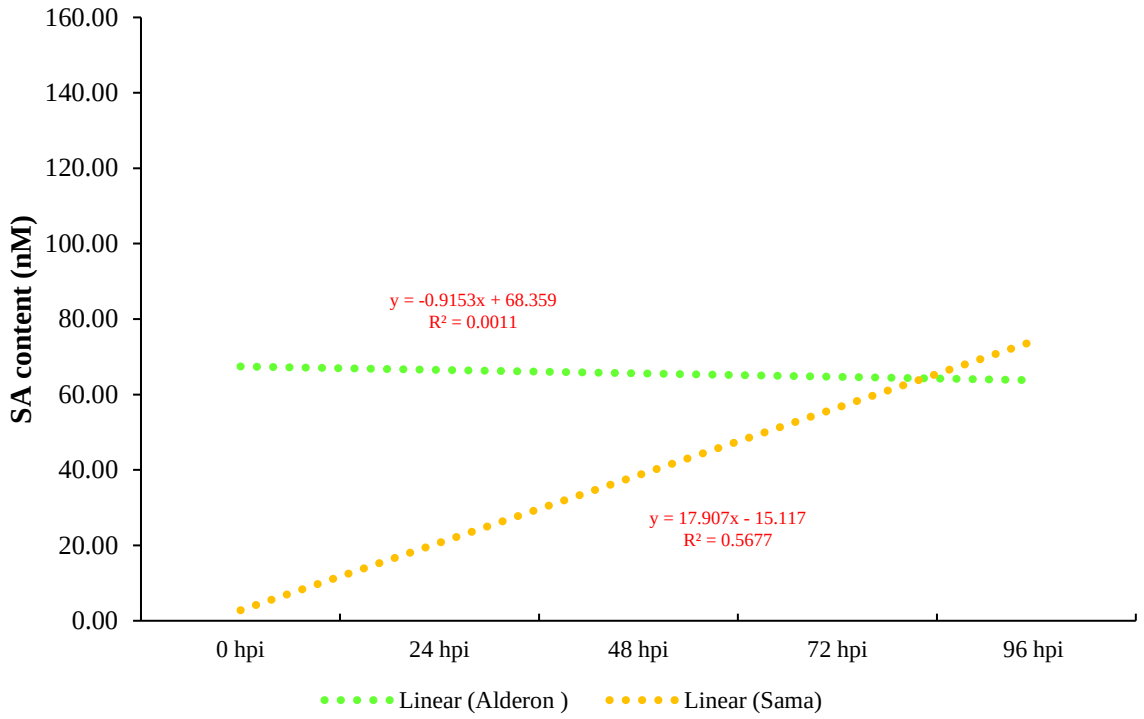

Figure 46 SA content amidst salt and spot blotch disease in combination. SA content in two chosen wheats following the combined stresses of salt and spot blotch disease were measured. Change in SA content in leaves of Alderon and Sama plants exposed to the combined treatment of 160 mM NaCl and infection by *B. sorokiniana* during 24, 48, 72, and 96 hpi was quantified (in addition to quantifying the control, which is represented at 0 hr), using LC-MS/MS. Values represent mean of 5 biological replications. The data was shown as fitted lines. Analyses of covariance (ANCOVA) were used to assess SA content between Alderon and Sama and the SA content was adjusted via time after infection (covariate variable).

5.2.8 JA content in two selected wheat varieties under salt and spot blotch infection

For crops subjected to the same experimental conditions as mentioned above, JA contents were quantified utilising the LC-MS/MS method, when both varieties were exposed to combined stresses at 0, 24, 48, 72, and 96 hpi. For Alderon, we can observe the level of JA was higher at 24 and 72 hpi (4.80 and 9.87nM respectively, relative to control (1.06 nM). However, in Sama, it was recorded that the highest level was at 96 hpi, followed by 72 hpi and then, 24 hpi and 48 hpi (4.85, 3.11, 2.94, and 0.96 nM, respectively, relative to control (0.42 nM) (Figure 47).

With time (time after infection with 160 mM NaCl) as a covariate, we utilized analysis of covariance (ANCOVA) to determine the difference between Alderon and Sama in JA content. The analysis demonstrated that there was no significant difference in JA content with variety effect ($F=0.435$, $P<0.513$), there was no significant on JA with time ($F=2.03$,

P<0.161) and there was no significant interaction between time and varieties on SA (F=0.207, P<0.652)

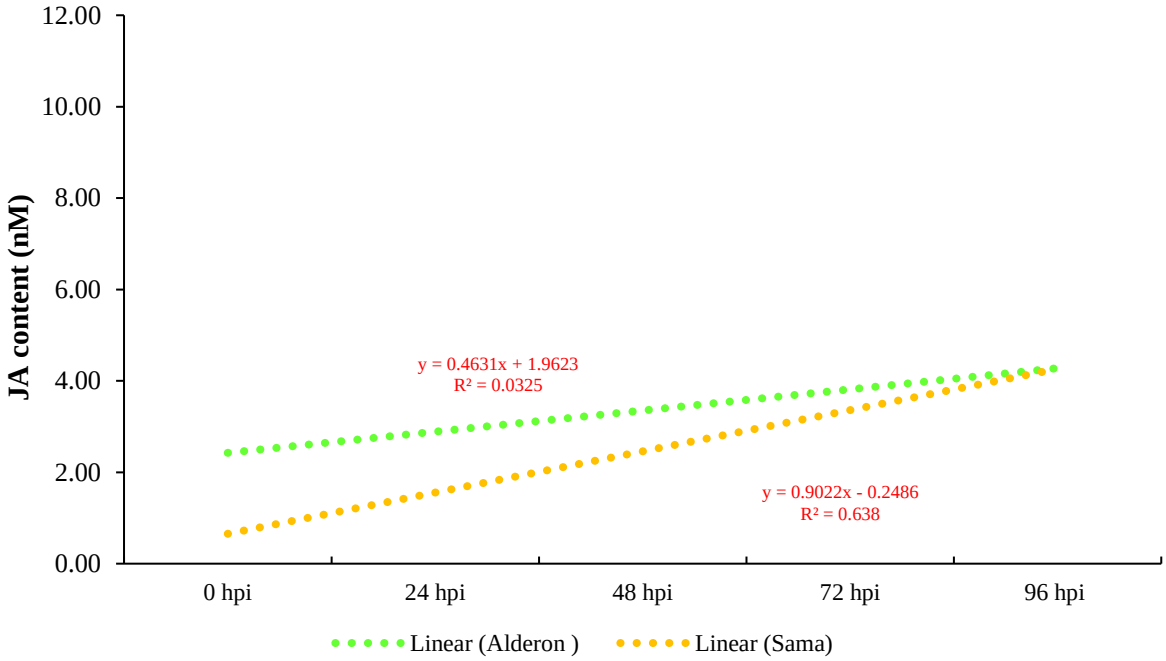

Figure 47 JA content in two chosen wheats following the combined stresses of salt and spot blotch disease were measured. Change in JA content in leaves of Alderon and Sama plants exposed to the combined treatment of 160 mM NaCl and infection by *B. sorokiniana* during 24, 48, 72, and 96 hpi was quantified (in addition to quantifying the control, which is represented at 0hr), using LC-MS. Values represent mean of 5 biological replications. The data was shown as fitted lines. Analyses of covariance (ANCOVA) were used to assess JA content between Alderon and Sama and the JA content was adjusted via time after infection (covariate variable).

5.2.9 Gene expression analysis

5.2.10 The combined stresses of salt and spot blotch disease on WRKY gene expression in Alderon at 24 and 48hpi

All WRKY genes have been chosen based on the phylogenetic tree, available in Chapters 2 and 3, respectively. The expression of WRKY genes in Alderon wheat leaves was evaluated after being subjected to combined treatment of 160mM NaCl and infection by *B. sorokiniana* during 24 and 48 hpi, using qPCR First, for expressions which can be detected by the qPCR device, at 24hpi, in terms of upregulation, *TaWRKY46* and *TaWRKY71* were expressed to reach 4.21 and 3.51-fold up change, respectively, compared to control (time=0, which is non-stressed plant. However, *TaWRKY53-a* was not expressed. Moreover, at 48hpi, there was no upregulation for all genes; for example, *TaWRKY3*, *TaWRKY8*, and *TaWRKY19*

were close in their expression (2.77, 2.63, and 3.12-fold down, respectively). Some of the genes did not change in expression compared to control their expression, such as *TaWRKY37*, *44*, and *46*(Figure 48) and Table 6.

Second, for fold changes, which cannot be considered expression due to being below 2 for qPCR sensitivity, at 24hpi, *TaWRKY8* and *TaWRKY45* were similar in their induction (1.86 and 1.66-fold up, respectively). The lowest expression level was in *TaWRKY3* (1.20-fold up). Regarding downregulation of gene expression, *TaWRKY19*, *TaWRKY44*, and *TaWRKY37* were downregulated (1.36, 1.35, and 1.0-fold down, respectively). For 48hpi, the expressions of *TaWRKY45*, *TaWRKY71*, and *TaWRKY53-a* were near each other (1.78, 1.69, and 1.31-fold down, respectively) (Figure 48) and Table 6..). The statistical analysis (t-test) showed that there was significant difference in genes expression *TaWERKY71*at 24 hpi in comparison to non-stressed plants

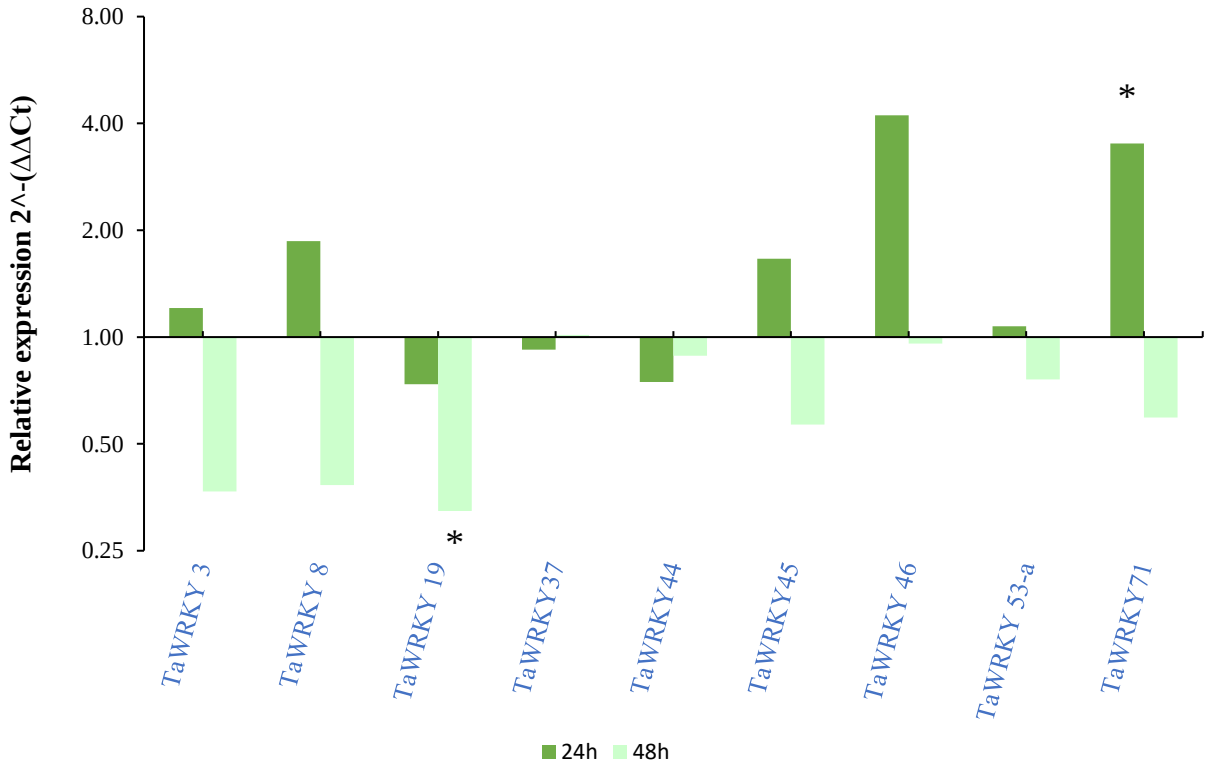

Figure 48 Relative expression of WRKY genes in the Alderon wheat variety after exposure to the combined stresses of salt and spot blotch disease. The gene expression of *TaWRKY3*, *8*, *19*, *34*, *44*, *45*, *46*, *53-a*, and *71* in Alderon leaves after being subjected to the combined stresses of 160mM NaCl (8days) and *B. sorokiniana* infection (24 and 48hrs) was assessed. The WRKY expressions are relative to *TaEF1α*, which was used as a reference gene. Thereafter, the expression of these genes was normalized to time=0 (control), which was set

to 1, and the relative expression was calculated. Mean values are expressed from 3 biological replicates and t-test was used to determine significant in gene expression between uninfected leaves and infected leaves using delta Ct value *: P<0.05, **:P<0.01, *** P<0.001).

5.2.11 The combined stresses of salt and spot blotch disease on WRKY gene expression in Sama variety at 24 and 48hpi

The expression level of WRKY genes in Sama wheat leaves, after experiencing combined treatment of 160 mM NaCl and infection by *B. sorokiniana*, during 24 and 48hpi, using qPCRwas assessed. In Figure 49and Table 6, most of the WRKY genes were expressed in upregulation. First, at 24hpi, *TaWRKY46* was upregulated at the highest level (6.11-fold). *TaWRKY3*, *TaWRKY19*, *TaWRKY53-a*, and *TaWRKY71* had similar trends in their expression (2.81, 2.23, 2.45, and 2.45-fold, respectively). For 48hpi, *TaWRKY37* was recorded as the highest gene, having 23.4-fold change followed by *TaWRKY19* (14.23-fold change). After that, *TaWRKY3*, *53-a, 7*1, and *44* genes were found to have expressions of 8.96, 6, 3, and 2.32-fold change, respectively.

Second, for fold changes that cannot be considered expression due to being below 2 for qPCR device sensitivity, at 24 hpi, the lowest expression level was recorded in *TaWRKY37* and *TaWRKY8* (1.48 and 1.35-fold, respectively). For 48 hpi however, the lowest level of expression was recorded in *TaWRKY8* and *TaWRKY46* (1.21 and 1.81-fold change, respectively). Nonetheless, *TaWRKY45* was shown to be downregulated (1.78-fold change). The statistical analysis (t-test) showed that there was significant difference in genes expression *TaWERKY45* (at 24 hpi), 71(at 24 hpi) and *46* (at 24and 48 hpi) in comparison to non-stressed plants

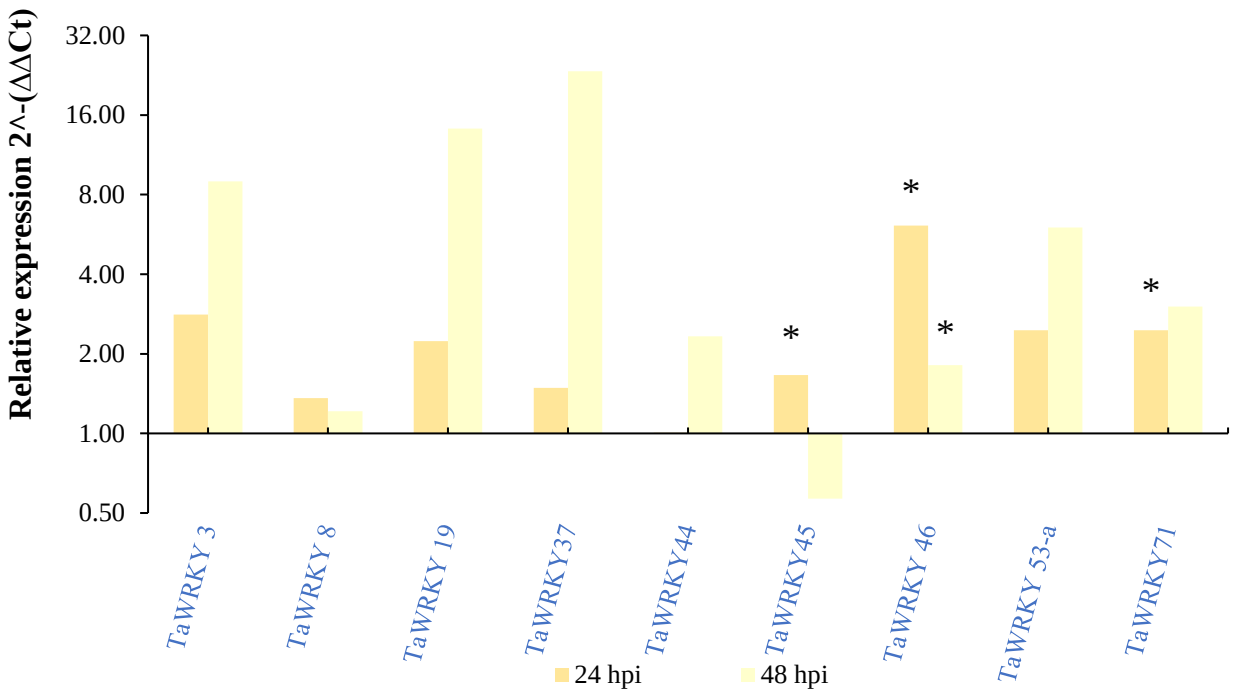

Figure 49 Relative expression of WRKY genes in the Sama wheat variety after being exposed to combined stresses of salt and spot blotch disease. The gene expression of TaWRKY3, 8, 19, 34, 44, 45, 46, 53-a, and 71 in Sama leaves, after being subjected to the combined stresses of 160mM NaCl (8 days) and B. sorokiniana infection (24 and 48hrs), was assessed. The WRKY expressions are relative to TaEF1α, which was used as a reference gene. Thereafter, the expression of these genes was normalized to time=0 (control), which was set to 1, and the relative expression was calculated. Mean values are expressed from 3 biological replicates and t-test was used to determine significant in gene expression between uninfected leaves and infected leaves using delta Ct value *: P<0.05, **:P<0.01, *** P<0.001).

5.2.12 Expression of genes regulating phytohormone synthesis and accumulation under combined stresses in Alderon and Sama at 24 and 48hpi

As for the Alderon genotype, the effect of relative gene expressions of *TaPAL (SA biosynthesis)*, *TaNPR1* (SA downregulation pathway), *TaAOS*, and *TALOX2* (markers for JA biosynthesis), under combined treatment, were assessed. All gene expressions were downregulated, but there was variation between timepoints. At 24hpi, the expression from *TaAOS* and *TaLOX2* were recorded at 3.44-fold down (0.29 2^-ddCt) and 6.66-fold down (0.15 2^-ddCt), respectively. However, expressions of both genes at 48hpi were similar, as *TaAOS* was 5.55-fold down (0.18 2^-ddCt) and *TALOX2*, 5-fold down (0.20 2^-ddCt). *TaPAL* underwent a change in its expression between two time points: at 24hpi, it was 3.33-fold down (0.30 2^-ddCt) and at 48hpi, it was reduced to 5.55-fold down (0.182^-ddCt). *TaNPR1* was not considered as expression due to being lower than 2 for the qPCR device sensitivity,

as it was stable in its expression between 24 and 48hpi (1.72 fold down (0.582^-ddCt) and 1.63 (0.61 2^-ddCt) fold down, respectively) Figure 50 (A) and Table 6

In the Sama variety, Figure 50 (B) and Table 6 display that the expression patterns of *TaPAL (SA biosynthesis)*, *TaNPR1* (SA downregulation pathway), *TaAOS*, and *TALOX2* (markers for JA biosynthesis) were changed and upregulated, except for *TaPAL* at 24hpi. In 24hpi, *TaLOX2*, *TaNPR1*, and *TaAOS* (not considered as a biologically significant change in expression due to being lower than 2 for the qPCR device sensitivity) were expressed to reach (4.49, 3, and 1.90-fold, respectively). However, *TaPAL* was downregulated to 1.66-fold (not considered as expression due to being lower than 2 for the qPCR device sensitivity). As for 48hpi, all genes were induced in upregulation. For example, the expressions of *TaPAL* and *TaNPR1* were recorded as being higher than *TaAOS* and *TALOX2*. To elaborate, *TaPAL* and *TaNPR1* had upregulation of 11.54 and 9.18-fold, respectively, while *TaAOS* and *TaLOX2*, were upregulated to 3.77 and 3.50-fold (respectively). The statistical analysis(t-test) provides that there was significant difference ($P<0.05$) in genes expression (*TaLOX* at 24 hpi and *TaPAL* at 48 hpi) in stressed plant in comparison with non-stressed plants in Alderon. In addition, there was significant difference($P<0.05$) in expression of *TaAOS* (at 24 and 48 hpi), *TaPAL* (48 hpi) and *TaNPR1*(24 hpi) in stressed plant relative to non-stressed plant in Sama (using delta Ct values)

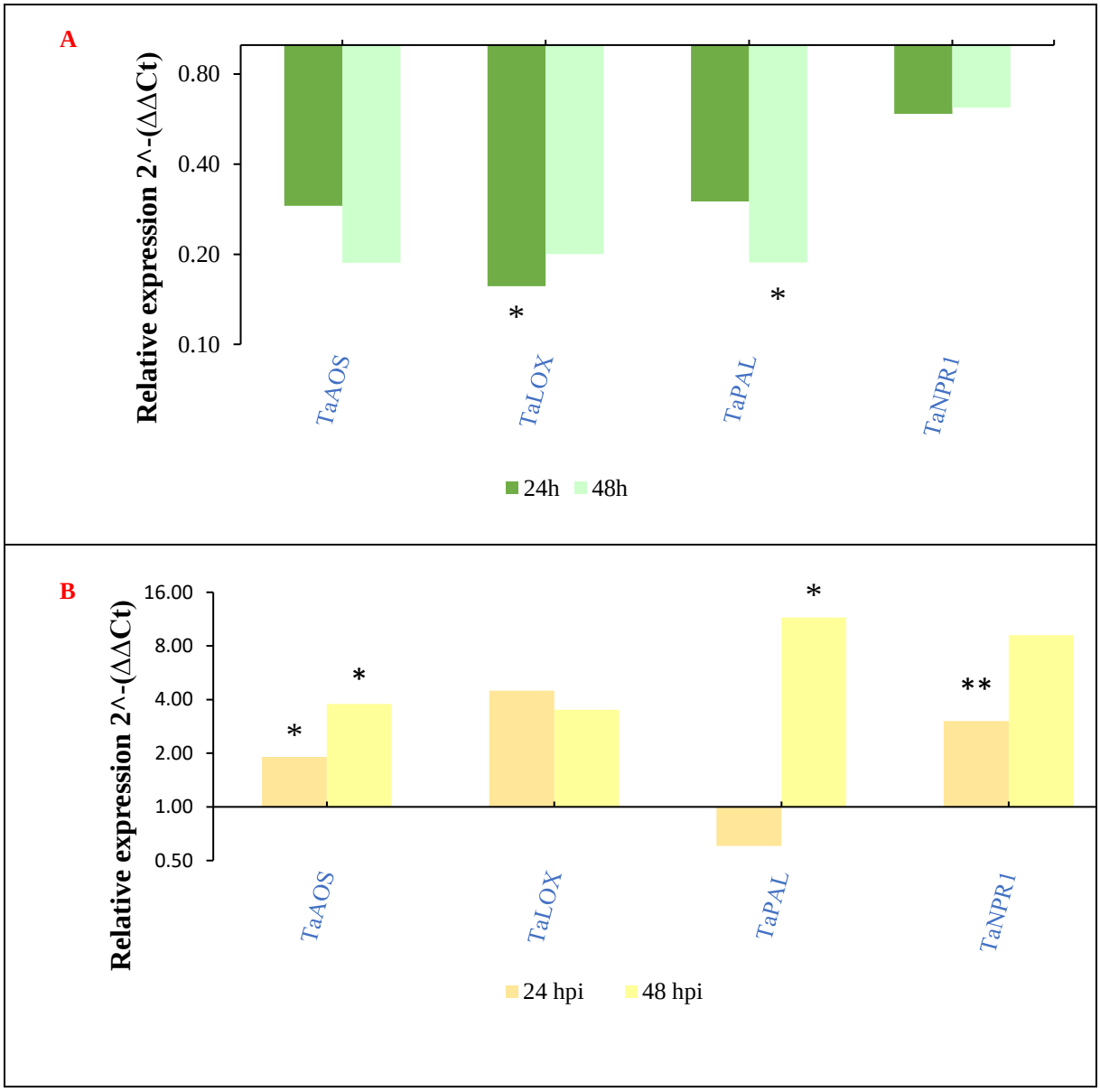

Figure 50 Expression of genes related to hormonal signalling pathways in two wheat varieties growing in combined stresses of salt and spot blotch disease. (A) represents the expression of genes in Alderon, and (B) represents the expression of genes in Sama. The relative expression of *TaPAL* (SA biosynthesis), *TaNPR1* (SA downregulation pathway), *TaAOS*, and *TalOX2* (markers for JA biosynthesis) in Alderon and Sama, after experiencing combined treatment of 160mM NaCl and infection by *B. sorokiniana*, during 24 and 48hpi, using qPCR was assessed. All expressions are relative to *Ta EF1α*, that was used as a reference gene. The expression of these genes was normalized to time=0, set to 1, and relative expression was calculated. Mean values are expressed from 3 biological replicates and t-test was used to determine significant in gene expression between uninfected leaves and infected leaves using delta Ct value *: P<0.05, **:P<0.01, *** P<0.001)

Table 6 Expression level of selected genes in two wheat selected varieties in combination of salt and spot blotch disease

Gene	Across timepoint in Alderon (compared to 0 hpi)				Across timepoint in Sama (compared to 0 hpi)				Between varieties (Alderon Vs Sama)at24 and 48 hpi							
									Alderon		Sama		Alderon		Sama	
	24 hpi		48 hpi		24 hpi		48 hpi		24 hpi		24 hpi		48 hpi		48 hpi	
TaWRKY 3	1.20	↑	2.77	↓	2.81	↑	8.90	↑	1.20	↑	2.81	↑	2.77	↓	8.90	↑
TaWRKY 8	1.86	↑	2.63	↓	1.35	↑	1.21	↑	1.86	↑	1.35	↑	2.63	↓	1.21	↑
TaWRKY 19	1.36	↓	3.12	↓	2.23	↑	14.23	↑	0.73	↓	2.23	↑	3.12	↓	14.23	↑
TaWRKY37	No change in gene expression		No change in gene expression		1.48	↑	23.44	↑	No change in gene expression		1.48	↑	No change in gene expression		23.44	↑
TaWRKY44	1.35	↓	No change in gene expression		No change in gene expression		2.32	↑	1.35	↓	No change in gene expression		No change in gene expression		2.32	↑
TaWRKY45	1.66	↑	1.78	↓	1.66	↑	1.78	↓	1.66	↑	1.66	↑	1.78	↓	1.78	↓
TaWRKY 46	4.21	↑	No change in gene expression		6.11	↑	1.81	↑	4.21	↑	6.11	↑	No change in gene expression		1.81	↑
TaWRKY 53-a	No change in gene expression		1.31	↓	2.45	↑	6.00	↑	no	↑	2.45	↑	1.31	↓	6.00	↑
TaWRKY71	3.50	↑	1.69	↓	2.45	↑	3.00	↑	3.50	↑	2.45	↑	1.69	↓	3.00	↑
TaAOS	3.44	↓	5.55	↓	1.90	↑	3.77	↑	3.44	↓	1.90	↑	5.55	↓	3.77	↑
TaLOX2	6.66	↓	5	↓	4.49	↑	3.50	↑	6.66	↓	4.49	↑	5	↓	3.50	↑
TaPAL	3.33	↓	5.55	↓	1.66	↓	11.54	↑	3.33	↓	1.66	↓	5.55	↓	11.54	↑
TaNPR1	1.72	↓	1.63	↓	3.00	↑	9.00	↑	1.72	↓	3.00	↑	1.63	↓	9.00	↑

All genes expression values are shown in each variety across 24 and 48 hpi of combined stress and between wheat varieties over time of infection (24 ,48 hpi) combined stress. First group that seen as yellow colour are WRKY genes and the second genes SA/ JA- related genes are shown blue colour. ↓ represent down regulation ↑ signify up regulation

5.3 Discussion

Impact of salinity stress upon pathogen infection within plants relies on various factors, such as pathogen type and severity of the imposed salt stress (Triky-Dotan *et al.*, 2005). In the present study, as referred to before, wheat plants were acclimatised in 160mM NaCl before exposure to *B. sorokiniana* spores. This level of NaCl stress was selected to allow unrestricted growth of the pathogen as high concentrations of salt stress can decrease the effect of fungal disease (Kissoudis *et al.*, 2016). Overall, the following parameters were evaluated under combined salt stress and spot blotch disease: gene expression, biochemical change, and wheat growth.

5.3.1 The disease severity and wheat performance under combined stresses

The disease severity was estimated using SAD (Standard Area Diagram), as mentioned (Chapter 4). In our results, the estimation of disease severity was assessed after 48, 72, and 96hpi, under combined treatment. As we can see in Figure 41 and Figure 42, the disease severity increased after 48hpi, and the progression of disease was recorded after 72 and 96hpi. In Sama, the disease severity percentage was recorded at 18 % and 43 %, respectively, for 72 and 96 hpi, while the disease severity in Alderon was recorded to be less than Sama during 72 and 96 hpi (9 % and 26 %, respectively). The variation of disease severity between Alderon and Sama reflects the behaviour of plant in responding to the combined treatment. Important to note that Alderon performed better than Sama under the individual stress from spot blotch disease (see Chapter 4). We can observe that in both genotypes, the disease severity was less in the combined treatment, when compared to the individual stress from spot blotch, as shown in chapter 4 (particularly at 96 hpi). It could be that the salt stress affects the plant by a specific way before the pathogen infects the wheat. There is a possibility that this could enhance the resistance of the plant to spot blotch disease.

Various research have disclosed that abiotic stresses could impose a beneficial and/or harmful impact upon plants exposed to pathogen to reduce effect of biotic stress or increase the impact of biotic stress, based on factors such as stress period, intensity, and kind of stress(Atkinson and Urwin, 2012). For a majority of instances, abiotic stress, in this case salt, has been recognised to influence the plant's ability to resist pathogen infection, leading to an unfavourable impact during the pathogen attack on the plant. It should be reminded that salt stress (100 mM NaCl) has been shown to have a beneficial effect on the triggering of pathogen resistance. In one study, salt stress (100 mM NaCl) was shown to significantly reduce biotrophic fungus susceptibility (i.e., *Oidium neolycopersici*) in tomato (*Lycopersicon*

esculentum) (Achuo *et al.*, 2006).The reduction of disease severity may be correlated with the accumulation of Na^+ content in infected leaves, as the level of Na^+ content in Alderon is higher than Sama, which may have led to less disease severity in Alderon, as shown in Figure 41. In that regard, it has been mentioned that NaCl might perhaps have a detrimental effect on the development of fungus, and that this has played a function as an antifungal agent via the accumulation of leaf material (Blomberg and Adler, 1993; Kissoudis *et al.*, 2016). More specifically, both an osmotic and an ionic component are present in the condition known as salinity stress. The first perception of osmotic stress leads to an inhibition of growth owing to a loss in turgor, as well as a decrease in photosynthesis as a consequence of stomatal closure(Munns and Tester, 2008). Because of the way pathogens are perceived, the presence of physical barriers like the cuticle, stomata, and cell walls is essential for prompt pathogen detection and interception(Kissoudis *et al.*, 2014). When a plant is exposed to unfavourable environmental circumstances that cause an osmotic imbalance and tissue desiccation, the hormone ABA plays a pivotal role in controlling the plant response. When plants are under stress from a lack of water, ABA acts as the major chemical signal that causes stomatal closure. In the presence of biotic stress, ABA has a beneficial effect on pre-invasive stomatal immunity by inducing stomatal closure, which limits the amount of bacterial and fungal pathogens that may enter the plant (Lee and Luan, 2012).

Salinity constraint may have a dual impact on plant disease susceptibility, whether increasing or decreasing, depending on pathogen and plant species(Bai *et al.*, 2018a; Velásquez *et al.*, 2018). Indeed, naturally combined abiotic and biotic stresses would implies a complex regulatory mechanism in plant response(Suzuki *et al.*, 2014). As per example, tomato powdery mildew resistance is weakened under salt stress(Bai *et al.*, 2018a), while it is enhanced for some Arabidopsis mutants through lowered cell death mechanism(Yang *et al.*, 2019b). Generally, salt stress would cause a stomata closure and a reduction in stomatal conductance, and an increase in cell membrane(Hniličková *et al.*, 2017; Ran *et al.*, 2021) permeability, which increase the potentiality of pathogens penetration, whether through stomata or cell membrane. While, for some cases, salinity could also increase the content of some protective contents against membrane peroxidation, like free proline and glycine betaine, improving therefore the plant membrane integrity against pathogens penetration(Hmidi *et al.*, 2018; Annunziata *et al.*, 2019).

5.3.2 Differences in growth parameters and ion content in Alderon and Sama under combined stresses

Salt stress is one of the reasons for ion toxicity (owing to ion build-up) and osmotic stress, as a result of reduction in plant growth, mentioned in Chapter 3. In our results, first, we can observe the reduction in shoot length in Sama as seen in Figure 43 (A), and there was effect in Aldron and Sama at treatment (160 mM NaCl and 96 hrs of infection) as presented in Figure 43 (B) Second, the reduction of leaf numbers was shown in both genotypes at treatment (160 mM NaCl and 96 hrs of infection) as seen in Figure 43 (D) ; however, each of Sama and Alderon under all treatments(unstressed plants and160 mM NaCl and 96 hrs of infection plants) was not shown the difference in leaf numbers as we can see in Figure 43 (C). This could be due to Sama being more affected by the combined treatment, as compared to Alderon. This research found, in Chapters 3 and 4, that the Sama variety was more sensitive to salt stress and more susceptible to spot blotch disease, than Alderon. One probable reason is that extreme salt stress resulted in a reduction in growth (Munns and Tester, 2008), while another possibility may be that the impaired growth is related to senescence stress. A relevant study found that senescence stress was displayed when tomato was exposed to dual stresses/combination of stresses (i.e., salt stress and powdery mildew disease) (Gregersen *et al.*, 2013; Kissoudis *et al.*, 2016).

Regarding the Na^+ ion, significant differences were observed in the effect of combined spot blotch disease and salt stress on the Na^+ content of leaves between non-stressed (without salt stress, without inoculation) and stressed (combined stresses→96 hours of infection and 160 mM NaCl presence) for all genotypes plants Figure 44(B). Na^+ content was higher in stressed and non-stressed leaves of Alderon than Sama (not significant difference) as seen in Figure 44 (A). The variation of Na^+ content between Alderon and Sama indicates that there was a relationship between Na^+ and disease severity, as we observed that the higher Na+ content was correlated with less disease severity in Alderson (96 hpi). However, for Sama, the lower level of Na+ was correlated with higher disease severity (96 hpi). Our study is similar with the one done by Kissoudis *et al.* (2016) who found that Na^+ levels escalated amidst salt stress and powdery mildew in combination, upon various tomato lines. Moreover, what had been observed was that the highest Na^+ level was detected for the following combination: 150 mM NaCl and Powdery mildew (Kissoudis *et al.*, 2016).

As for the K^+ ion, there was not a significant difference observed for each of Sama and Alderon wheat varieties, under stressed and non-stressed leaves Figure 44(C). However, minor increase of K^+ was seen in the stressed leaves for all in Alderon and Sama as seen in

Figure 44 (D) but there was no significant effect between treatment (stressed and non-stressed plant across all genotypes). Sama and Alderon, although it was considered insignificant. It must be noted that K^+ content could be decreased in case there is a lengthy period of the combined stresses.

5.3.3 The effect of dual stress on MDA content

As noted in previous chapters on determining the malondialdehyde content (MDA is an important biochemical parameter for assessing oxidative stress induced membrane damage by abiotic/biotic stress), in the current research project, the level of MDA was evaluated and seen to increase after the combined stress treatment as shown in Figure 45. To elaborate, MDA content increased gradually at 24hpi and 48hpi, with the combined treatment. However, in Sama and Alderon genotypes, it has been shown that the concentration of MDA reached maximum values at 96hpi, under the same combined treatment. What was observed regarding the two varieties was that MDA content was higher in Sama than Alderon (not statistically different). This suggests that the plant had less oxidative stress during 24 and 48hpi, in both genotypes, but for 96hpi, more oxidative stress occurred. However, Sama was more impaired by the stress than Alderon. This is linked to the accumulation of the combined stress treatment and its continuous effects on plants, which resulted in increased MDA. Our study is similar to Tani *et al.* (2018), who saw that the response of eggplants against the combined treatment of *Verticillium dahlia* (fungal plant pathogen) and drought, influenced MDA levels to grow, subsequent to exposure of 3-weeks in both cultivars (EMI and Skoutari), which have intermediate resistance (Tani *et al.*, 2018).

5.3.4 SA, JA contents

Measuring the concentration of phytohormone (Phytohormone profiling) in JA and SA, was done under combined stresses at different time points of infection using LC-MS/ MS. For SA content, we can see that its level was highest in 72 hpi of combined stress for Alderon, while in Sama, it was highest at 96 hpi (not statistically different). (Figure 46). We can also observe that the level of SA during 24 and 48 hpi was not highest. It can be suggested that SA content gets induced and increases with continual development of the pathogen and accumulation of the salt stress. Our results were comparable with Gupta *et al.* (2017) who found that SA content in *Arabidopsis thaliana* was not changed at 8 hpi with the combined stress treatment (drought stress following tomato DC3000 (bacterial inoculation). However, at 24 hpi, with combined stress, the SA level rose (Gupta *et al.*, 2017).

As for JA content, we can see that its level was induced at 24, 48, 72, and 96 hpi, with combined stresses in the Sama variety (as compared to control), while in Alderon, it was induced at 24 and 72 hpi, with combined stressed (not statistically different) Figure 47. Our result is similar to Gupta *et al.* (2017) , who found that JA content and JA-Ile content in *Arabidopsis thaliana* was induced at 8 and 24 hours post combined stress treatment (drought stress following *Pseudomonas syringae* pv. tomato DC3000 (bacterial inoculation). We can observe that the quantity for JA and SA was induced higher as the developing of disease progressed. It can be understood that the JA and SA content were possibly affected by salt stress, which preceded the infection from *B. Sorokiniana*. Moreover, the salt stress could have modulated the JA and SA biosynthesis and signalling pathways.

5.3.5 The effect of combined salt and spot blotch disease on WRKY gene expression and SA, JA-related gene expression

There is a possibility for WRKY genes to impose the opposite impact upon biotic/abiotic stress tolerance, due to complicated interactions amongst signalling networks, having the capacity to result in antagonistic/synergistic influences upon plant response regulation against various stresses (Bai *et al.*, 2018b).In order to understand the molecular basis of simultaneous disease and abiotic stress tolerance, and to identify putative genes involved in combined stress (fungal infection and 160 mM NaCl treatment), we carried out an expression study of WRKYs, *TaAOS*, *TaLOX2*, *TaPAL*, and *TaNPR1* genes under both *B. sorokiniana* infection and salt treatment (160 mM NaCl), at two time points (24 and 48h). The obtained results showed a complex pattern of gene expression.

At 24 hpi of combined stresses, in terms of upregulation, we observed that the highest increase in expression was for *WRKY46* and *WRKY71* (*WRKY37* and *53-a* were not expressed at this time). Notably, at 48 hpi of combined stresses, some of the selected *WRKY* genes (*3*,*8*, and *19*) were downregulated at 48 hpi, for the Alderon variety. Whereas for Sama variety, they were rather upregulated after both 24 and 48 hpi (except for *WRKY45*, at 48 hpi, which was downregulated). Moreover, for the Sama WRKY members mentioned, it looks like the time point 48 hpi is more important than 24 hpi, in terms of change and reactivity amplitude. For example, the expression of *WRKY37*, *19*, *3*, *53-a*, *71*, and *44* was increased at 24 hpi of combined stresses, whereas all these genes were higher and upregulated at 48 hpi (Figure 48 , Figure 49and Table 6). One possible explanation for these variations in WRKY gene expression may be that the WRKY genes are engaged in regulating some of the specific genes that are involved in the combined stress by activating or suppressing these target (specific)

genes at different time points. Two factors that could be affecting the alternation of this expression are progression of disease development and accumulation of salt. Another possible explanation could be that the WRKY expression change is related to the senescence response. It is possible that senescence response can occur in this combined stress. It was found in a study that the stress combination of salt and powdery mildew had led to a senescence effect within the tomato crop (Kissoudis *et al.*, 2016).

Furthermore, it was detected that in tomato, *SlWRKY8* provides positive regulation within the immune system, countering bacterial pathogen (increasing plant resistance) (*Pseudomonas syringae* pv. tomato DC3000 infection), in addition to plant reactions against salinity and drought. Scholars have witnessed *SlWRKY8* playing a role in salt and drought tolerance (Gao *et al.*, 2020).In fact ,Bai *et al.* (2018a) have mentioned that abiotic factors may incline plants towards senescence signalling and regulation that could, in turn, differentially impact resistance reaction based upon the pathogen lifecycle .

Returning to gene expressions, the gene expression of *TaAOS* and *TaLOX2* (related to JA) in additional to *TaPAL* and *TaNPR1* (related to SA), are downregulated upon the combined stresses at 24 and 48 hpi for Alderon, with differential expression compared to Sama, which has upregulation in those genes (except *TaPAL* at 24hpi) (Figure 50 A and B) and Table 6 . Therefore, for these genes, the trend is downregulation instead of upregulation, meaning it is possible for the existence of a mechanism of negative regulation and feedback control (in Aderon). A research saw that for both cultivars (Skoutari and EMI) of eggplant, there was low relative expression for the LOX gene, under combined drought and fungal plant pathogen (*Verticillium dahlia*) (Tani *et al.*, 2018). Moreover, in terms of *TaPAL* gene expression in Alderon, there was a study that stated genes (e.g., *PAL1*) which have been participating in previous stages of the phenylpropanoid pathway for monolignol biosynthesis were seen to experience downregulation inside the chickpea plants, when chickpea was subjected to a combination of rapid drought stress together with 2-days of *Ralstonia solanacearum* infection and, slow drought stress along with 4-days of *Ralstonia solanacearum* infection. The researchers suggested, as an explanation for this expression, that the genes engaged in lignin synthesis at the first stage, have undergone upregulation at an early point in time throughout the aforementioned stress combination, as to start the lignin synthesis pathway and might undergo downregulation in a later point in time for sustaining the metabolic activity. One reason as to why this occurs is the feedback regulation, which is facilitated via high monolignoltiter, at 2dpi in addition to 4 dpi (Sinha *et al.*, 2017). Another study on related genes to JA biosynthesis (*TaAOS* and *TaLOX1*) found both genes were

downregulated in two different wheat cultivars (Santiago and Lili) at examined time points 0, 24 and 48 hours, when exposed to JA- treatment. These researchers proposed that the reason for downregulation of these genes could be the negative feedback mechanism(Campanaro *et al.*, 2021).

Concerning the *TaNPR1* gene, which is related to SA signalling, we found that it experienced downregulation between the two examined points (24 and 48 hpi). The level of *TaPAL* expression was downregulated while the expression of *TaNPR1* was upregulated at 24hpi. It may be that *TaNPR1* gene controls the SA biosynthesis. It was suggested that *TaNPR1* gene plays the function of main downstream regulation for SA signalling, that in turn, imposes a negative feedback upon SA synthesis for averting the consequences of uncontrolled accumulation (Kunkel and Brooks, 2002; Savadi *et al.*, 2018). Moreover, in the current research, within the Sama variety, the expression profiles of *TaPAL* and *TaNPR1* were induced near to each other (11.54 and 9.18-fold change, respectively) at 48 hpi of combined stress. It is possible that the combined stress triggers and activates the signalling defence system for SA. Additional investigation was conducted by Savadi *et al.* (2018),where it was seen that high levels of induction for *TaNPR1* gene inside the resistant NIL cultivar (wheat) during exposure to leaf rust pathogen could lead to a more rapid and effectual PR protein mediated resistance. Moreover, high amounts of *TaPAL* expression induction, caused by interactions between *Lr24* (which is a gene that resists leaf rust pathogen in NIL wheat cultivar) and leaf rust pathogen, might cause greater SA build-up.

Pertaining to *TaAOS* and *TaLOX2*, their expressions increased in their upregulation at 24 and 48hpi. We can notice that both gene expressions had similar trends at 48hpi. This induction could be a response to senescence and JA biosynthesis, which makes Sama more susceptible to spot botch disease. It also may be linked to photosynthesis, as mentioned in the study by Bilgin *et al.* (2010), who found down regulation of photosynthesis in response to activation of ethylene and JA signalling. Our finding is in accordance with Kissoudis *et al.* (2016), who saw that the AOS gene in tomatoes was expressed when exposed to a combination of salt and powdery mildew stresses (Kissoudis *et al.*, 2016). As for *TaLOX2* expression in my experiment, a relevant study showed that *TaLOX6* is influenced by senescence process and MeJA-treatment. The authors of the study proposed that *TaLOX6* could be possibly linked to the JA-caused leaf senescence(Qiao *et al.*, 2021).

Overall, it was understood that the gene expressions between WRKY genes and hormone signalling pathways are very complex, in response to the combined stresses of salt and spot blotch disease. More specifically, we can see the two contrasting wheat genotypes (Alderon and Sama) behaving differently, which was reflected in their gene expression patterns.

5.4 Conclusions

We conclude that the effect of high salt concentration and spot blotch disease, in the two contrasting wheat genotypes appeared in the form of morphological, biochemical, and molecular responses. In this context, stress intensity might have heavily impacted interactions of pathogens with salinity, that would affect the level of salt build up within the wheat. Moreover, it was understood that various defence mechanisms of the host against ion toxicity could have the ability to change the consequential outcome of the interaction between plant and pathogen.

Remarkably, the interaction between salt and spot blotch disease could have a positive or negative effect on wheat plant. As stated before, our results provide two contrasting wheat genotypes responding differently at morphological, biochemical, and molecular levels. This effect could influence wheat development, specifically the Sama variety, which was identified as the sensitive variety to salt stress. Furthermore, during the combination of salt stress and pathogen infection, we exposed the wheat variety to salt stress for 8-days and thereafter, to pathogen infection. The effect of the salt stress that preceded the infection, could alter the metabolic pathway, gene expression, and ion imbalance, which reflects the resistance to pathogen interaction within the plant, causing the additive effect (reducing Fungal penetration).

In terms of positive interaction, it was observed that at 160 mM NaCl salt stress, the Na^+ content was increasing while disease severity (which would have normally increased at a high rate) was only slightly rising at an insignificant rate (particularly in the Alderon variety), at 96hpi. In terms of negative impact on plants, the long duration of 160mM NaCl could have a detrimental effect on the wheat plant. Moreover, biochemical change was observed in the combination of the salt stress together with the spot blotch disease, where MDA (oxidative marker), was seen to be rising as the duration (of the combined stresses) increased. The alteration of the JA and SA was observed amongst various times of infection with the combination of salt stress and pathogen present. The gene expression was affected by this

combined stress, whereas candidate WRKY expression and SA/JA–related gene biosynthesis changed at two different time points at early stages of infection.

The study provides insights on hormonal variation and MDA content (SA/JA) in various points in time throughout combined stresses. Additionally, the WRKY genes and SA/JA-related gene biosynthesis, in the present study, could be used as gene markers for employment in crop tolerance against combined stress. Future research should be considered for understanding the function of these genes. The cross-talk of signalling pathways participating in wheat responses to salinity and disease stresses discussed in the current research, indicate that a considerable challenge would be encountered for developing breeding techniques, as to achieve resilience in wheat subjected to stress combinations. Many factors should be considered in future research, such as different salt concentrations (various severities of stress), age of the plant, time of stress (short or long duration), and kinds of genotypes, which when combined with the spot blotch disease, can provide more information on how plants respond to stresses. Particularly, the induction of WRKY genes was demonstrated under both abiotic and biotic stresses, in this study, and should be further explored to elucidate their downstream regulatory functions which could lead to an association with increased crop tolerance.

Chapter 6. In Silico analysis of *TaPAL, TaNPR1, TaAOS, TaLOX2, TaWRKY3, 37, 53-a* and *71* Wheat Promoters.

6.1 Introduction

Globally, the wheat crop is subjected to a variety of biotic and abiotic challenges, which result in a drop in both yield and quality. Salt stress is one of the abiotic stresses which affects the growth and productivity of wheat (Ejaz *et al.*, 2020). Furthermore, disease, such as spot blotch disease is a severe concern that has a adverse effect on both production and growth of wheat crops (Jamil *et al.*, 2020). In order to better understand and shed the light on the molecular mechanisms of abiotic and biotic stresses tolerance in wheat, we have selected eight candidate genes (*TaPAL*, *TaNPR1*, *TaAOS*, *TaLOX2*, *TaWRKY3*, *37*, *53-a* and *71*), based on literature and from current works, that are recognised to have altered expression in response to salt, spot blotch disease and combination of salt and spot blotch disease. We analysed the promoter sequences of each gene to detect cis-regulatory elements to understating the global behaviour of each gene and to determine the role of transcription factors in regulating gene expression. Genes were selected based on results described in chapters 3, 4, and 5 and also their role in plant defence. Briefly, *PAL* genes are related to phenylalanine ammonia-lyase which is an important enzyme in phenylpropanoid biosynthesis and metabolism. This enzyme converts L-phenylalanine to cinnamic acid and it participates in lignin biosynthesis (Rui-Fang *et al.*, 2016). In addition to that, phenylalanine ammonia-lyase (PAL) play a central role in salicylic acid (SA) biosynthesis which phenylalanine ammonia-lyase enzyme transforms phenylalanine (Phe) to trans-cinnamic acid (t-CA) (Figure 51) (Dempsey *et al.*, 2011). In regard to environmental stresses, a study conducted by Savadi *et al.* (2018) who displayed that *TaPAL* expression in wheat was induced following interaction with leaf rust. Nonexpresser of pathogenesis-related protein 1(NPR1), which is a protein that is engaged in salicylic signalling and leads to the downstream regulation of the salicylic pathway (Figure 51) (Kumar, 2014). In terms of importance of NPR1 to stresses, researchers found that *TaNPR1* in wheat was expressed in response to leaf rust disease (Savadi *et al.*, 2018).

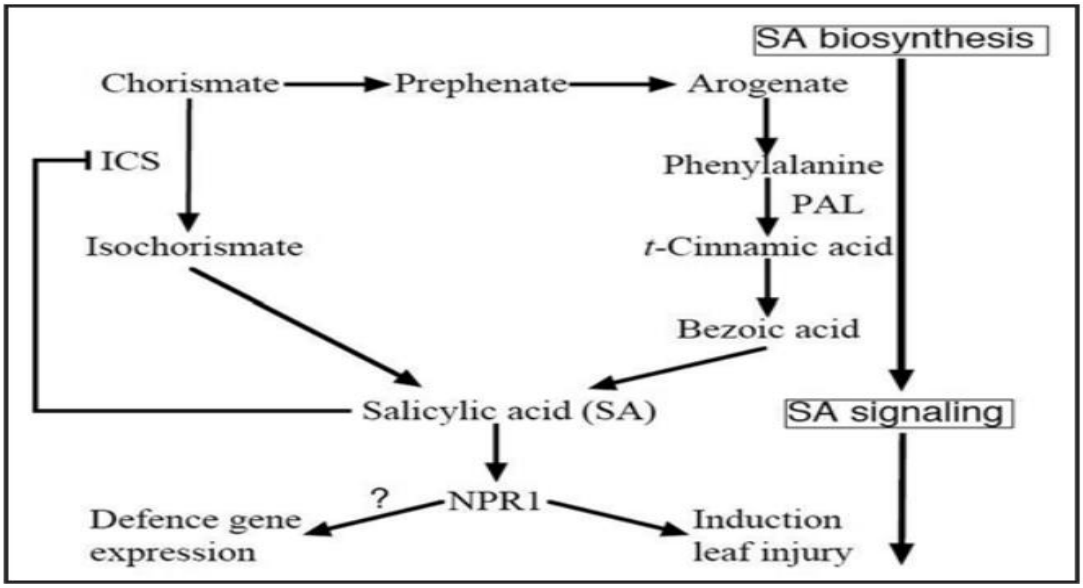

Figure 51 Salicylic acid biosynthesis and signalling in plants that have been exposed to ozone. Biosynthesis of salicylic acid (SA) in plants may occur through one of two different routes(isochorismate pathway or phenylalanine (PAL) pathway. The final product (SA) of this pathway has the ability to suppress the expression of ICS as well as the activity of ICS. NPR, also known as a nonexpresser of PR1 (Tamaoki, 2008).

Thirdly, as mentioned by Wang *et al.* (2021c), lipoxygenase (LOX) and allene oxide synthase (AOS) are two enzymes that contribute to the beginning of JA biosynthesis, and transform α-linolenic acid (α-LeA) to 12-oxo-phytodienoic acid (OPDA) (Figure52). In the face of abiotic and biotic stress, these two enzymes have demonstrated their relevance. According to Wang *et al.* (2018) who showed that once wheat is infected with fungal pathogens of the *Fusarium graminearum*, *TaLOX2* transcripts were enriched in wheat. Furthermore, research conducted by Liu *et al.* (2014) found that *TaAOS* is expressed in response to elevated NaCl and $ZnCl_2$

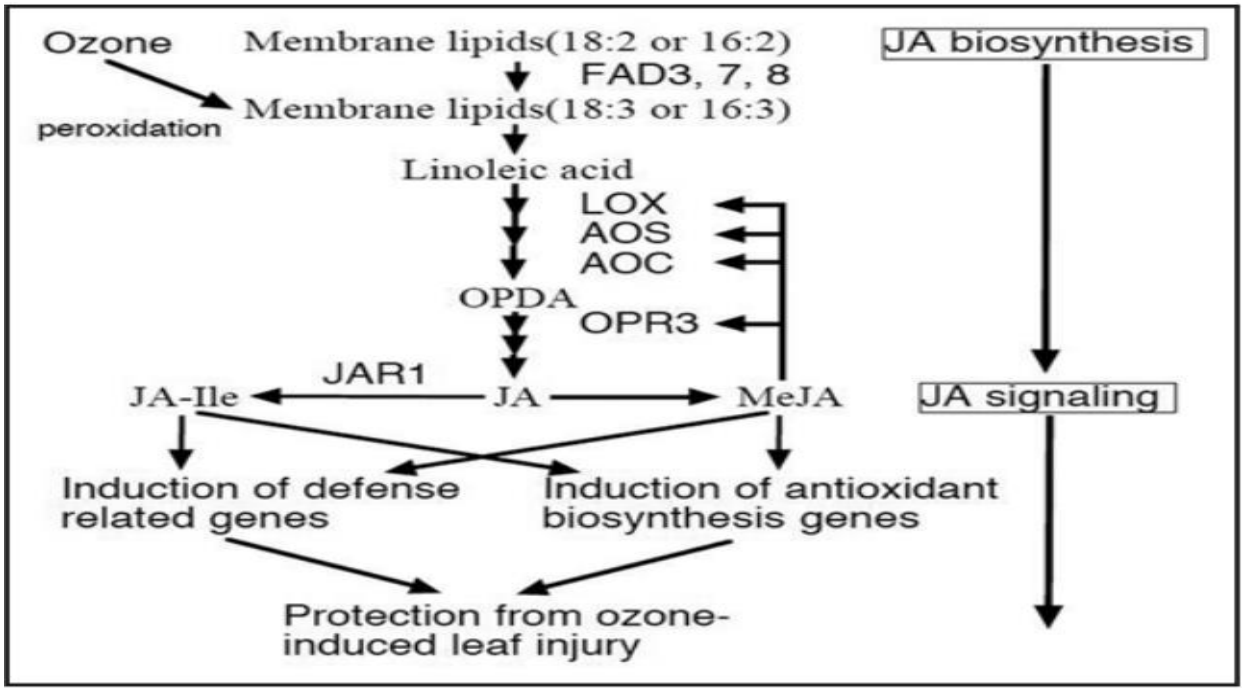

Figure52 Jasmoanic acid biosynthesis and signalling. Peroxodation of membrane lipids is the first step in the formation of jasmonic acid (JA) in plants that have been exposed to ozone. After being synthesized, JA undergoes further metabolism, which results in the formation of methyl JA (MeJA) or JA-isoleucine (JA-Ile). (Tamaoki, 2008).

Fourthly, WRKY, as a transcription factor (TF), plays a critical role in the resistance to biotic and abiotic stressors (Erpen *et al.*, 2018). In fact, WRKY have the ability for precisely binding to the W-box motifs ((T)TGAC(C/T)), which are found inside the promoter region of the target gene. Transcription factor binding controls transcription activities of the downstream gene (causing up/down regulation) (Jiang *et al.*, 2017). Intriguingly, these WRKY TFs play and important role in positive/negative regulation in response to stresses (Chen *et al.*, 2012). An instance of this is *OsWRKY24* in rice, which was seen to have a critical function for negatively regulating abscisic acid (ABA) signalling and gibberellic acid (GA) (Zhang *et al.*, 2009). Another illustration is *TaWRKY51*, which has a central role in root system via enhancing lateral root formation, within wheat (*Triticum aestivum L.*). It does this via negatively regulating ethylene biosynthesis through attaching to the W-box motifs inside the promoter region belonging to ethylene synthesis genes (1-aminocyclopropane-1-carboxylic acid synthase (ACS) (Hu *et al.*, 2018). Moreover, another example for positive regulation of the correlation between JA- and SA-activated signalling pathways, which enables responses towards heat stress, can be seen in *WRKY39* inside *Arabidopsis thaliana* (Li *et al.*, 2010b)

In eukaryotes, transcription factors govern the expression of target genes by combining with particular DNA sequences. Transcriptional modulation of gene expression is

regulated by the attachment of transcription factors to *cis*- regulatory motifs (Priest *et al.*, 2009). A specific emphasis has been placed on the *cis*-regulatory elements that present in promoters of genes because they give important insights into gene regulation and they engaged in plant response to abiotic and biotic stresses (Hernandez-Garcia and Finer, 2014).For instance , as mentioned by Hernandez-Garcia and Finer (2014) and Yamaguchi-Shinozaki and Shinozaki (2006) that (ACGTGG/T) named as ABRE which is ABA responsive motifs and it responses to salt stress and dehydration in rice and Arabidopsis. (A/GCCGAC) referred to DRE (dehydration response elements) in Arabidopsis, which was associated in the regulating of dehydration and low temperature. We considered it important to perform a global analysis of the transcriptional regulation of these candidate genes; and we have particularly focused on the study of their promoter regulatory regions. Therefore, *cis*-regulatory elements were found and analysed through the PLANTCARE databases.

6.2 Results

In our study, we analysed promoter region of *TaNPR1*, *TaPAL*, *TaAOS*, *TaLOX2* and also we analysed sequences of prompters for *TaWRKY3*, *TaWRKY53-a*, *TaWRKY37* and *TaWRKY71*. Promoter region is a sequence that are located upstream of each these genes and containing *cis*-acting regulatory elements which are important in gene expression regulation and these elements are engaged in response to abiotic / biotic stress. In current study, 2Kb of upstream 5' flanking sequence of each *TaNPR1*, *TaPAL*, *TaAOS*, *TaLOX2*, *TaWRKY3*, *TaWRKY53-a*, *TaWRKY37* and *TaWRKY71* was exported to PlantCARE database to locate and find *cis*-acting regulatory elements from positive strand of promoter. We only present abiotic or biotic stress related to cis- acting elements. As the CAAT-box and TATA-box elements are common amongst all eukaryotes and were identified in all chosen sequences, they are not presented in the following Tables (7-15). In term of scoring matrix, substitution matrix that employed for scoring similarity, within the sequence alignment and weight matrix or position-specific scoring matrix were utilise as statistical motif approaches. As to show the difference detected inside the aligned positions (Zheng, 2005)

6.2.1 Analysis of cis-acting elements in the promoter regions of TaPAL and TaNPR1 genes

As seen in Tables 7 and 8, *cis*-acting elements within the promoters of *TaPAL* (SA biosynthesis) and *TaNPR1* (SA signalling) are shown. Firstly, on motifs related to light response, G-Box, Sp1, and TCT-motif, which are illustrated in the aforementioned tables, are cis-elements. G-Box was detected four times in the promoter region for each of the *TaPAL* and *TaNPR1* genes. Moreover, Sp1 was recognised two times in the promoter region of *TaPAL* and also two times in *TaNPR1* promoter. It must be noted that TCT-motifs were found two times in *TaNPR1*, but did not exist in *TaPAL*.

Secondly, concerning motifs related to low temperature, LTR was found only one time in the *TaNPR1* (Tables 7 and 8). Thirdly, as for biotic and wound stress, both genes (*TaPAL*, *TaNPR1*) had responsive elements for wound (WRE3) that exist one time for each promoter of *TaPAL* and *TaNPR1*. Additionally, the stress-responsive element (STRE) existed four times in the promoter region of *TaNPR1*; however, this motif was not found in the promoter region of *TaPAL*. Fourthly, in terms of motifs related to drought stress, the presence of MBS, which plays a role in drought stress, was recognised two times in the promoter region of *TaPAL*, but was detected only one time in the *TaNPR1* promoter. Furthermore, MYB, MYC, and MYB-like sequence were shown to play a vital role in drought stress. More specifically,

MYB was found 5 times in the promoter of *TaPAL*, but occurred one time in the promoter region of *TaNPR1*. MYC was recognised one time in the promoter region of *TaPAL*, but was not found in *TaNPR1*. MYB was found four times in *TaPAL*, but did not exist in *TaNPR1*. MYB-like sequence was recorded two times in *TaPAL*, but was not found in *TaNPR1* as seen in Tables 7 and 8. We can see the most abundant motifs related to drought stress were found in the *TaPAL* promoter

Fifthly, regarding the motifs which are related to hormone response, we found various different hormonal responsive elements such as ABRE, which is involved in ABA response. This motif occurred two times in the promoter of *TaPAL*. However, in the promoter sequence of *TaNPR1*, the ABRE motif occurred five times. This motif is more abundant in *TaNPR1*, as shown in Tables 7 and 8. Other hormone response elements such as P-box and GARE-motif (gibberellin-responsive elements) were present one time each in the *TaPAL* promoter, but did not exist in *TaNPR1*. Moreover, some regulatory cis-elements were found in the promoter of *TaPAL*, but were not present in *TaNPR1*, such as AuxRR-core (one time occurred) and TGA-element which responds to auxin (one time occurred). It must be added that while other cis-elements were found in *TaNPR1*, they were not seen in *TaPAl* such as the TGACG-motif (existed two times) and the CGTCA-motif (recognised two times). These motifs (TGACG-motif, CGTCA-motif) are related to the MeJA-responsive elements. In addition, as-1 was responsible for SA and this element was presented three times in *TaNPR1*, was not present in *TaPAL* (Tables 7 and 8).

Table 7 *cis* – acting elements in promoter region of *TaPAL*

TaPAL						
Site name	**Organism**	**Position**	**Strand**	**Matrix Score**	**Sequence**	**Function**
ABRE	*Arabidopsis thaliana*	892	+	6	CACGTG	Abscisic acid responsiveness
	Arabidopsis thaliana	893	+	5	ACGTG	Abscisic acid responsiveness
AuxRR-core	*Nicotiana tabacum*	1701	+	7	GGTCCAT	Auxin responsiveness
G-Box	*Pisum sativum*	560	+	6	CACGTT	Light responsiveness
	Pisum sativum	892	+	6	CACGTT	
G-box	*Arabidopsis thaliana*	892	+	6	CACGTG	Light responsiveness
	Zea mays	1438	+	6	CACGTG	
GARE-motif	*Brassica oleracea*	1982	+	7	TCTGTTG	Gibberellin-responsive element
MBS	*Arabidopsis thaliana*	1396	+	6	CAACTG	MYB binding site involved in drought-inducibility
	Arabidopsis thaliana	786	+	6	CAACTG	MYB binding site involved in drought-inducibility
MYB	*Arabidopsis thaliana*	539	+	6	TAACCA	Drought response (Abe *et al.*, 2003)
	Arabidopsis thaliana	1222	+	6	TAACCA	
	Arabidopsis thaliana	1745	+	6	CAACCA	
	Arabidopsis thaliana	622	+	6	CAACCA	
	Arabidopsis thaliana	1079	+	6	CAACCA	
MYB-like sequence	*Arabidopsis thaliana*	539	+	6	TAACCA	Recognition site for MYB and involved in drought and salt (Yu *et al.*, 2012)
	Arabidopsis thaliana	539	+	6	TAACCA	
MYC	*Arabidopsis thaliana*	532	+	6	CAATTG	Drought response (Abe *et al.*, 2003)
Myb	*Arabidopsis thaliana*	22	+	6	TAACTG	MYB binding involved in salt (Cui *et al.*, 2013) dehydration ((Urao *et al.*, 1993)
	Arabidopsis thaliana	1105	+	6	TAACTG	
	Arabidopsis thaliana	786	+	6	CAACTG	
	Arabidopsis thaliana	1396	+	6	CAACTG	
P-box	*Oryza sativa*	1069	+	7	CCTTTTG	Gibberellin-responsive element (Heidari *et al.*, 2015)
Sp1	*Oryza sativa*	1547	+	6	GGGCGG	Light responsive element (Hima Kumari *et al.*, 2018; Shariatipour and Heidari, 2020)
	Oryza sativa	1789	+	6	GGGCGG	
TGA-element	*Brassica oleracea*	1230	+	6	AACGAC	Auxin-responsive element
WRE3	*Pisum sativum*	45	+	6	CCACCT	Wound-responsive element (Wang *et al.*, 2021a)

Table 8 *cis* – acting elements in promoter region of *TaNPR1*

TaNPR1						
Site name	**Organism**	**Position**	**Strand**	**Matrix Score**	**Sequence**	**Function**
ABRE	*Arabidopsis thaliana*	600	+	6	CACGTG	Abscisic acid responsiveness
	Arabidopsis thaliana	322	+	5	ACGTG	
	Arabidopsis thaliana	1834	+	5	ACGTG	
	Arabidopsis thaliana	1269	+	5	ACGTG	
	Arabidopsis thaliana	601	+	5	ACGTG	
CGTCA-motif	*Hordeum vulgare*	411	+	5	CGTCA	MeJA-responsiveness
	Hordeum vulgare	1024	+	5	CGTCA	MeJA-responsiveness
G-Box	*Pisum sativum*	770	+	6	CACGTT	Light responsiveness
	Pisum sativum	600	+	6	CACGTG	Light responsiveness
G-Box	*Zea mays*	207	+	6	CACGAC	Light responsiveness
	Arabidopsis thaliana	600	+	6	CACGTG	Light responsiveness
LTR	*Hordeum vulgare*	1734	+	6	CCGAAA	Low-temperature responsiveness
MBS	*Arabidopsis thaliana*	1596	+	6	CAACTG	MYB binding site involved in drought-inducibility
MYB	*Arabidopsis thaliana*	462	+	6	CAACCA	Drought response ((Abe *et al.*, 2003)
STRE	*Arabidopsis thaliana*	166	+	5	AGGGG	Stress-responsive element(Chauhan *et al.*, 2011)
	Arabidopsis thaliana	166	+	5	AGGGG	
	Arabidopsis thaliana	344	+	5	AGGGG	
	Arabidopsis thaliana	171	+	5	AGGGG	
Sp1	*Oryza sativa*	1762	+	6	GGGCGG	Light responsive element (Hima Kumari *et al.*, 2018; Shariatipour and Heidari, 2020)
	Oryza sativa	1766	+	6	GGGCGG	
TCT-motif	*Arabidopsis thaliana*	966	+	6	TCTTAC	Light responsive element
	Arabidopsis thaliana	1082	+	6	TCTTAC	Light responsive element
TGACG-motif	*Hordeum vulgare*	1267	+	5	TGACG	MeJA-responsiveness
	Hordeum vulgare	1750	+	5	TGACG	MeJA-responsiveness
WRE3	*Pisum sativum*	82	+	6	CCACCT	Wound-responsive element (Wang *et al.*, 2021a)
as-1	*Arabidopsis thaliana*	1832	+	5	TGACG	SA responsive element (Garretón *et al.*, 2002)
	Arabidopsis thaliana	1267	+	5	TGACG	
	Arabidopsis thaliana	1750	+	5	TGACG	

6.2.2 Analysis of cis-acting elements in the promoter regions of TaAOS and TaLOX2 genes

Cis-acting elements within the promoter regions of *TaAOS* and *TaLOX2* (JA biosynthesis) are shown (Tables 9 and 10). Firstly, regarding hormone response, the motifs (that are cis-regulatory elements) which were detected for the promoter regions of *TaAOS and TaLOX2* were ABRE (responsible for ABA). The occurrence of the ABRE motifs was recorded to be two times inside the promoter of *TaAOS* and one time in *TaLOX2.* Furthermore, CGTCA-motif occurred one time in the promoter region of *TaLOX2.* However, it was not present in *TaAOS*. TGACG-motif happened two times in *TaAOS* and was present one time in the promoter region of *TaLOX2.* Both motifs (CGTCA-motif, TGACG-motif) are engaged in MeJA. In addition, regarding hormone specific response to auxin, we found that AuxRR-core (auxin hormonal response) existed one time in *TaAOS,* but was not present in *TaLOX2.* Regarding hormone specific response to SA, we observed as-1 (SA response) occurring two times in *TaAOS,* but one time in *TaLOX2.*

Secondly, on issues of drought, dehydration, and salt response (Tables 9 and 10), DRE core is a motif which was present one time for each promoter in *TaAOS* and *TaLOX2.* Further, the presence of MBS that plays a role in drought stress was found only one time in the promoter of *TaLOX2.* However, it was not found in *TaAOS.* Additionally, MYB occurred two times in *TaAOS,* but did not exist in *TaLOX2.* Moreover, MYC was detected one time in *TaAOS* and four times in *TaLOX2*. Myb was found one time in *TaLOX2,* but was not present in *TaAOS.* Myb-binding site, MYB-recognition site, and MYB-like sequence were present, one time each, in *TaAOS,* but did not exist in *TaLOX2.* Thirdly, as for wounding and biotic stress, we found the motif, STRE (stress-responsive element), in *TaLOX2* three times and two times in *TaAOS.* Also, the WUN-motif was found to be present one time in *TaLOX2,* but did not exist in *TaAOS.*

Fourthly, on matters related to light response (Tables 9 and 10), it was found that the G-Box motif existed two times in *TaAOS,* but was not found in *TaLOX2*. Further, Box 4 existed one time in *TaAOS,* but was not seen in *TaLOX2.* Finally, I-box happened one time in *TaLOX2,* but was not present in *TaAOS*. Fifthly, concerning low temperature response, it was detected that LTR motifs were present one time in *TaAOS,* but did not exist in *TaLOX2*.

Table 9 *cis* – acting elements in promoter region of *TaAOS*

TaAOS						
Site name	**Organism**	**Position**	**Strand**	**Matrix Score**	**Sequence**	**Function**
ABRE	*Arabidopsis thaliana*	676	+	5	ACGTG	Abscisic acid responsiveness
	Arabidopsis thaliana	1160	+	5	ACGTG	
AuxRR-core	*Nicotiana tabacum*	1105	+	7	GGTCCAT	Auxin responsiven
Box 4	*Petroselinum crispum*	169	+	6	ATTAAT	Light responsiveness
DRE core	*Arabidopsis thaliana*	505	+	6	GCCGAC	Dehydration responsive elements / Salt response (Narusaka *et al.*, 2003)
G-Box	*Pisum sativum*	1147	+	6	CACGTT	Light responsiveness
	Pisum sativum	1687	+	6	CACGTT	
LTR	*Hordeum vulgare*	341	+	6	CCGAAA	Low-temperature responsiveness
MYB	*Arabidopsis thaliana*	981	+	6	TAACCA	Drought response ((Abe *et al.*, 2003)
	Arabidopsis thaliana	693	+	6	CAACAG	
MYB recognition site	*Arabidopsis thaliana*	1773	+	6	CCGTTG	Drought response (Abe *et al.*, 1997)
MYB-like sequence	*Arabidopsis thaliana*	981	+	6	TAACCA	Recognition site for MYB and involved in drought and salt (Yu *et al.*, 2012)
MYC	*Arabidopsis thaliana*	11	+	6	CAATTG	Drought response (Abe *et al.*, 2003)
Myb-binding site	*Nicotiana tabacum*	693	+	6	CAACAG	Drought response (Dutta *et al.*, 2021)
STRE	*Arabidopsis thaliana*	352	+	5	AGGGG	Stress-responsive element(Chauhan *et al.*, 2011)
	Arabidopsis thaliana	1881	+	5	AGGGG	
TGA-element	*Brassica oleracea*	672	+	6	AACGAC	Auxin-responsive element
TGACG-motif	*Hordeum vulgare*	433	+	5	TGACG	MeJA-responsiveness
	Hordeum vulgare	1421	+	5	TGACG	MeJA-responsiveness
as-1	*Arabidopsis thaliana*	433	+	5	TGACG	SA responsive element (Garretón *et al.*, 2002)
	Arabidopsis thaliana	1421	+	5	TGACG	

Table 10 *cis* – acting elements in promoter region of *TaLOX2*

TaLOX2						
Site name	**Organism**	**Position**	**Strand**	**Matrix Score**	**Sequence**	**Function**
ABRE	*Arabidopsis thaliana*	683	+	5	ACGTG	Abscisic acid responsiveness
CGTCA-motif	*Hordeum vulgare*	168	+	5	CGTCA	MeJA-responsiveness
DRE core	*Arabidopsis thaliana*	1601	+	6	GCCGAC	Dehydration responsive elements / salt (Narusaka *et al.*, 2003)
I-box	*Solanum tuberosum*	1291	+	9	TGATAATGT	Light responsive element
MBS	*Arabidopsis thaliana*	565	+	6	CAACTG	MYB binding site involved in drought-inducibility
MYC	*Arabidopsis thaliana*	275	+	6	CATGTG	Drought response (Abe *et al.*, 2003)
	Arabidopsis thaliana	321	+	6	CATTTG	
	Arabidopsis thaliana	1277	+	6	CATTTG	
	Arabidopsis thaliana	559	+	6	CATTTG	
Myb	*Arabidopsis thaliana*	565	+	6	CAACTG	Drought response (Dutta *et al.*, 2021)
STRE	*Arabidopsis thaliana*	1140	+	5	AGGGG	Stress-responsive element(Chauhan *et al.*, 2011)
	Arabidopsis thaliana	1859	+	5	AGGGG	
	Arabidopsis thaliana	1824	+	5	AGGGG	
TGACG-motif	*Hordeum vulgare*	1520	+	5	TGACG	MeJA-responsiveness
WUN-motif	*Nicotiana glutinosa*	1671	+	8	AAATTACT	Wound response (Luo *et al.*, 2020)
as-1	*Arabidopsis thaliana*	1520	+	5	TGACG	SA responsive element(Garretón *et al.*, 2002)

6.2.3 Analysis of cis-acting elements in promoter regions of TaWRKY3, TaWRKY37, TaWRKY53-a, and TaWRKY71

WRKY3, 37, 3, 53-a, and *71* were chosen based on their differential expression amongst Alderon, Najran, Cochise, and Sama after being affected by 160 mM NaCl stress (see Chapter 3) We investigated and analysed the promoter regions for each of these genes to identify regulatory elements(Tables 11, 12, 13 and 14). Firstly, on the matter of hormone responses, it was observed that ABRE4 was recognised once in *TaWRKY71* with ABRE present one time in *TaWRKY3* and three times in *TaWRKY53-a*. It must be noted that both of the aforementioned motifs are linked to ABA. Furthermore, we have found two motifs related to MeJA, CGTCA-motif and TGACG-motif. As for CGTCA, it was seen to be present four times in *TaWRKY3* and one time in of the following: *TaWRKY37 and 71.* As for TGACG, it was recognised one time for each of the following promoter regions: *TaWRKY37, 53-a,* and *71.* Moreover, as-1, TCA, and TCA-element were related to SA-response. Specifically, as-1 was present one time for each promoter of *TaWRKY37, 53-a, and 71,* whereas, TCA was seen only once in *TaWRKY3,* and TCA-element was seen one time only in *TaWRKY37*. Finally, ERE, a motif linked to ethylene hormone response, was detected once in *TaWRKY3*.

Secondly, in terms of motifs related to light response (Tables 11, 12, 13 and 14), the GT1-motif was found one time in *TaWRKY3* and *53-a*. Box 4 occurred two times in *TaWRKY37* and *71*, but it was found one time in *WRKY3*. The AE-box motif was only detected in *TaWRKY71* for one time. In addition, TCCC-motif was detected one time in *TaWRKY71* and *TaWRKY3*, each. It was also detected two times in *TaWRRKY53-a.* Moreover, the I-box motif was only seen two times in *TaWRKY71.* Sp1 was found one time in *TaWRKY53a*. It was additionally recognised that TCT-motif appeared one time in the promoter region of *TaWRKY3* and *53-a*. G-Box was found one time in *TaWRKY53-a* and two times in *TaWRKY71*. Finally, CAG-motif was present one time in *TaWRKY71*.

Thirdly, concerning motifs linked with drought, dehydration and salt stresses (Tables 11, 12, 13 and 14), MYC was present one time in the following promoter regions: *TaWRKY3, 37, 53-a, and 71*. Additionally, MYB appeared one time for each of the following: *TaWRKY37, 53-a, and 71*. MYB-like sequence was detected only for one time in *TaWRKY37*. (Abe *et al.*, 2003; Churin *et al.*, 2003; Jang *et al.*, 2004; Singh *et al.*, 2021). Related to drought, Myb-binding and MYB-recognitions site happened one time each, in *TaWRKY71.* DRE1 was seen to happen two times in *TaWRKY37* and one time in *TaWRKY53-a.* Further,

DRE core, which is connected to dehydration and salt stress, occurred one time in *TaWRKY37*, three times in *TaWRKY53-a*, and one time in *TaWRKY71*.

Fourthly, regarding low temperature response (Tables 11, 12, 13 and 14), we found that LTR motifs existed two times in *TaWRKY53-a* and one time in *TaWRKY71*. Fifthly, concerning wound and biotic response, we found STRE existed one time in *TaWRKY37* and one time in *71*. Moreover, TC-rich repeats existed one time only in *TaWRKY37*.Sixthly, related to transcription factors, specifically the *WRKY* family, W-box was present one time for each of *TaWRKY-53a and 71*.

Table 11 *cis* – acting elements in promoter region of *TaWRKY3*

TaWRKY3						
Site name	**Organism**	**Position**	**Strand**	**Matrix Score**	**Sequence**	**Function**
ABRE	*Arabidopsis thaliana*	1268	+	5	ACGTG	Abscisic acid responsiveness
Box 4	*Petroselinum crispum*	537	+	6	ATTAAT	Light responsiveness
CGTCA-motif	*Hordeum vulgare*	1093	+	5	CGTCA	MeJA-responsiveness
CGTCA-motif	*Hordeum vulgare*	1398	+	5	CGTCA	MeJA-responsiveness
CGTCA-motif	*Hordeum vulgare*	1314	+	5	CGTCA	
CGTCA-motif	*Hordeum vulgare*	1448	+	5	CGTCA	MeJA-responsiveness
ERE	*Nicotiana glutinos*	738	+	8	ATTTCATA	Ethylene response (Riaz *et al.*, 2021)
GT1-motif	*Arabidopsis thaliana*	560	+	6	GGTTAA	light responsive element
MYC	*Arabidopsis thaliana*	1605	+	6	CATGTG	Drought response (Abe *et al.*, 2003)
Myc	*Arabidopsis thaliana*	979	+	7	TCTCTTA	Drought response (Akram *et al.*, 2020)
TCA	*Pisum sativum*	788	+	9	TCATCTTCAT	SA response (Islam *et al.*, 2021)
TCCC-motif	*Spinacia oleracea*	329	+	7	TCTCCCT	Light responsive element
TCT-motif	*Arabidopsis thaliana*	461	+	6	TCTTAC	Light responsive element

Table 12 *cis* – acting elements in promoter region of *TaWRKY37*

TaWRKY37						
Site Name	**Organism**	**Position**	**Strand**	**Matrix score.**	**sequence**	**function**
Box 4	*Petroselinum crispum*	528	+	6	ATTAAT	Light responsiveness
Box 4	*Petroselinum crispum*	652	+	6	ATTAAT	Light responsiveness
CGTCA-motif	*Hordeum vulgare*	247	+	5	CGTCA	MeJA-responsiveness
DRE core	*Arabidopsis thaliana*	870	+	6	GCCGAC	Dehydration responsive elements / salt response (Narusaka *et al.*, 2003)
DRE1	*Zea mays*	221	+	7	ACCGAGA	Drought stress (Lata and Prasad, 2011)
DRE1	*Zea mays*	221	+	7	ACCGAGA	
MYB	*Arabidopsis thaliana*	562	+	6	TAACCA	Drought response (Abe *et al.*, 2003)
MYB-like sequence	*Arabidopsis thaliana*	562	+	6	TAACCA	Recognition site for MYB and involved in drought and salt (Yu *et al.*, 2012)
MYC	*Arabidopsis thaliana*	1452	+	6	CATTTG	Drought response (Abe *et al.*, 2003)
STRE	*Arabidopsis thaliana*	1529	+	5	AGGGG	Stress-responsive element (Chauhan *et al.*, 2011)
TC-rich repeats	*Nicotiana tabacum*	43	+	9	ATTCTCTAAC	Defence and stress responsiveness
TCA-element	*Nicotiana tabacum*	1495	+	9	CCATCTTTTT	Salicylic acid responsiveness
TGACG-motif	*Hordeum vulgare*	1398	+	5	TGACG	MeJA-responsiveness
as-1	*Arabidopsis thaliana*	1398	+	5	TGACG	SA responsive element (Garretón *et al.*, 2002)

Table 13 *cis* – acting elements in promoter region of *TaWRKY53-a*

TaWRKY53-a						
Site name	**Organism**	**Position**	**Strand**	**Matrix Score**	**Sequence**	**Function**
ABRE	*Hordeum vulgare*	1063	+	9	GCAACGTGTC	Abscisic acid responsiveness
ABRE	*Arabidopsis thaliana*	1066	+	5	ACGTG	
ABRE	*Arabidopsis thaliana*	1759	+	5	ACGTG	
DRE core	*Arabidopsis thaliana*	1039	+	6	GCCGAC	Dehydration responsive elements / salt response (Narusaka *et al.*, 2003)
DRE core	*Arabidopsis thaliana*	1272	+	6	GCCGAC	
DRE core	*Arabidopsis thaliana*	1180	+	6	GCCGAC	
DRE1	*Zea mays*	1226	+	7	ACCGAGA	Drought stress (Lata and Prasad, 2011)
G-Box	*Pisum sativum*	1717	+	6	CACGTT	Light responsiveness
GT1-motif	*Arabidopsis thaliana*	94	+	6	GGTTAA	Light responsive element
LTR	*Hordeum vulgare*	771	+	6	CCGAAA	Low-temperature responsiveness
LTR	*Hordeum vulgare*	824	+	6	CCGAAA	Low-temperature responsiveness
MYB	*Arabidopsis thaliana*	1870	+	6	CAACCA	Drought response (Abe *et al.*, 2003)
MYC	*Arabidopsis thaliana*	546	+	6	CAATTG	Drought response (Abe *et al.*, 2003)
Sp1	*Oryza sativa*	1018	+	6	GGGCGG	Light responsive element (Hima Kumari *et al.*, 2018; Shariatipour and Heidari, 2020)
TCCC-motif	*Spinacia oleracea*	1153	+	7	TCTCCCT	Light responsive element
TCCC-motif	*Spinacia oleracea*	1942	+	7	TCTCCCT	
TCT-motif	*Arabidopsis thaliana*	1750	+	6	TCTTAC	Light responsive element
TGACG-motif	*Hordeum vulgare*	1734	+	5	TGACG	MeJA-responsiveness
W box	*Arabidopsis thaliana*	1679	+	6	TTGACC	*cis*- element involved in WRKYTF (Yamamoto *et al.*, 2004)
as-1	*Arabidopsis thaliana*	1734	+	5	TGACG	SA responsive element (Garretón *et al.*, 2002)

Table 14 *cis* – acting elements in promoter region of *TaWRKY71*

TaWRKY71						
Site Name	**Organism**	**Position**	**Strand**	**Matrix score.**	**sequence**	**function**
ABRE4	*Zea mays*	78	+	6	CACGTA	ABA response (Liu *et al.*, 2021)
AE-box	*Arabidopsis thaliana*	1089	+	8	AGAAACAA	Light response
Box 4	*Petroselinum crispum*	400	+	6	ATTAAT	Light responsiveness
Box 4	*Petroselinum crispum*	806	+	6	ATTAAT	
CAG-motif	*Arabidopsis thaliana*	291	+	10	GAAAGGCAGAC	Light response element
CGTCA-motif	*Hordeum vulgare*	1529	+	5	CGTCA	MeJA-responsiveness
DRE core	*Arabidopsis thaliana*	1411	+	6	GCCGAC	Dehydration responsive elements / salt (Narusaka *et al.*, 2003)
G-box	*Zea mays*	1819	+	6	CACGAC	Light responsiveness
G-box	*Zea mays*	1622	+	6	CACGAC	
I-box	*Gossypium hirsutum*	600	+	10	AAGATAAGGCT	Light responsive element
I-box	*Larix laricina*	947	+	9	GTATAAGGCC	Light responsive element
LTR	*Hordeum vulgare*	289	+	6	CCGAAA	Low-temperature responsiveness
MRE	*Petroselinum crispum*	1106	+	7	AACCTAA	MYB binding site involved in light responsiveness
MYB	*Arabidopsis thaliana*	1501	+	6	CAACAG	Drought response (Abe *et al.*, 2003)
MYB recognition site	*Arabidopsis thaliana*	1444	+	6	CCGTTG	Drought response (Abe *et al.*, 1997)
MYC	*Arabidopsis thaliana*	668	+	6	CATTTG	Drought response (Abe *et al.*, 2003)
Myb-binding site	*Nicotiana tabacum*	1501	+	6	CAACAG	Drought response (Dutta *et al.*, 2021)
STRE	*Arabidopsis thaliana*	191	+	5	AGGGG	Stress-responsive element (Chauhan *et al.*, 2011)
TCCC-motif	*Spinacia oleracea*	1887	+	7	TCTCCCT	light responsive element
TGACG-motif	*Hordeum vulgare*	488	+	5	TGACG	MeJA-responsiveness
W box	*Arabidopsis thaliana*	1364	+	6	TTGACC	*cis*- element involved in WRKYTF (Yamamoto *et al.*, 2004)
as-1	*Arabidopsis thaliana*	488	+	5	TGACG	SA responsive element (Garretón *et al.*, 2002)

6.2.4 The occurrence of acting elements in promoter regions of TaNPR, TaPAL, TaAOS, TaLOX2, TaWRKY3, TaWRKY37 TaWRKY53-a, and TaWRKY7

Firstly, as for hormonal response as shown in Table 15, we found that *TaNPR1* is the most abundant (5 times) in terms of number of occurrence of motifs responding to ABA, followed by *TaWRKY53-a* (3 times), with *TaPAL* and *TaAOS* (two times) coming after. The next one is *TaWRKY 3* and *71* which were recognised as having one occurrence each, while *TaWRKY37* did not show any motifs related to ABA. In addition, in terms of MeJA motifs, we detected the highest occurrence being for *TaNPR1* and *TaWRKY3* (4 times each), followed by *TaAOS, TaWRKY37,* and *71,* which had an occurrence of two for each, and *TaLOX2* and *TaWRKY53-a* coming afterwards (1 occurrence each). However, *TaPAL* did not contain any motifs related to MeJA. Furthermore, as for gibberellin-responsive element and auxin responsive element, we found two motifs related to each present in *TaPAL*, followed by *TaAOS* that saw motifs related to them appear one time each. However, within the remaining genes, motifs related to gibberellin-responsive element and auxin responsive element did not exist. Moreover, for SA response, we found that *TaNPR1* has the most occurrence of motifs, compared to other genes (three times), followed by *TaAOS* and *TaWRKY37* (two times for each). Motifs within *TaLOX2, TaWRKY3,53-a,* and *71* were present one time only in each, but *TaPAL* did not contain any motifs related to SA response. Finally, we just recognised one occurrence of a motif for ethylene response in *TaWRKY3.*

Secondly, in terms of light response (Table 15) we found *TaWRKY71* has the highest occurrence of motifs (nine times), followed by *TaNPR1* (eight occurrences of motifs), with *TaPAL* and *TaWRKY 53-a* coming subsequently *(*six times of occurrences), while *TaWRKY3* came afterwards (4 times), followed by *TaAOS* (three times). *TaWRKY37* and *TaLOX2* came last containing two and one motif occurrences respectively. Thirdly, in terms of low temperature response, *TaWRKY53-a* (2 times) had the highest number of occurrences, with the following containing only one occurrence for motifs: *TaNPR1, TaAOS,* and *TaWRKY71*. However, *TaPAL, TaLOX2, TaWRKY3,* and *37* did not contain any motifs related to low temperature response. Fourthly, for drought response, *TaPAL* is the gene with the highest motif occurrence (fourteen times). After that, *TaLOX2* followed by *TaAOS* came, containing 8 and 7 times, respectively. *TaWRKY53-a* was found to have an occurrence of 6 times for motifs and then, *TaWRKY37* and *71,* having 5 times. *TaNPR1* and *TaWRKY3* were recognised to have two motif occurrences related to drought.

Fifthly, in terms of wound and biotic stress (Table 15), *TaNPR1* is the gene with the most motif occurrences (5 times), followed by *TaLOX2* (4 times). *TaAOS* and *TaWRKY37* were recognised to have two occurrences of motifs with T*aWRKY71* coming last (one occurrence). Sixthly, regarding transcription factors and WRKY, we detected one occurrence for the W-box motif in each of the following genes: *TaWRKY53-a* and *71*.

Table 15 Summary of *cis* – acting elements in promoter regions of *TaNPR, TaPAL, TaAOS, TaLOX2, TaWRKY3, TaWRKY37 TaWRKY53-a,* and *TaWRKY71*

Type of responses	*Cis*-Regulatory Element	Genes								Function
		SA biosynthesis and signalling		JA biosynthesis		Transcription factor (WRKY)				
		TaNPR1	*TaPAL*	*TaAOS*	*TaLOX2*	*TaWRKY 3*	*TaWRKY37*	*TaWRKY53-a*	*TaWRKY71*	
Hormonal responsive	ABRE and ABRE4	5	2	2	1	1	0	3	1	ABA response
	CGTCA-motif, TGACG-motif	4	0	2	1	4	2	1	2	MeJA-responsiveness
	P-box and GARE-motif	0	2	1	0	0	0	0	0	gibberellin-responsive element
	as-1, TCA, TCA-element	3	0	2	1	1	2	1	1	SA responsive element
	AuxRR-coreand TGA-element	0	2	1		0	0	0	0	auxin responsive element
	ERE	0	0	0	0	1	0	0	0	Ethylene response
Light responsive	G-Box, TCT-motif, AE-box, GT1-motif, TCCC-motif, I-box, Sp1, Box 4and CAG-motif	8	6	3	1	4	2	6	9	Light responsive element
Low responsive	LTR	1	0	1	0	0	0	2	1	low-temperature responsiveness
Drought, dehydration and salt	MBS, MYB, MYC, DRE1, Myc, Myb,Myb-binding site, MYB recognition site and MYB-like sequence	2	14	7	8	2	5	6	5	Drought, dehydration and salt responsive
Wound /biotic responsive	TC-rich repeats, WRE3, WUN-motif and STRE	5	1	2	4	0	2	0	1	Defence, wound and stress responsiveness
Regulation of other transcription factors	W BOX	0	0	0	0	0	0	1	1	cis- element involved in WRKY TF

6.3 Discussion

Transcription factors (TFs) are one of the regulatory proteins that are engaged in signalling transduction regulation and they play a critical role in controlling expression of genes under abiotic and biotic stresses (Agarwal *et al.*, 2006). One of the TFs that is engaged in plants when experiencing abiotic and biotic stresses is the WRKY family (Ng *et al.*, 2018). *Cis* – elements which are found in the promoter region of a target gene are recognised by TFs. These elements are important for gene regulation in response to stresses (Eulgem, 2005). For example, W-box (TGACC (A/T), one of cis-elements located in the promoter region of a specific gene, is recognised by the WRKY protein TFs in order to suppress or activate the specific gene expression related to stress response (Jiang *et al.*, 2017). The *cis*-regulatory elements found in promoters are given significant attention because they shed light on regulation of genes and are engaged in how plants conduct signalling to environmental stresses (Hernandez-Garcia and Finer, 2014).

In the present research, we investigated the *cis*-acting regulatory elements which were detected in WRKY genes and genes related to biosynthesis and signalling. The *cis*-acting regulatory elements were detected in the promoter regions of the following genes: *TaPAL*, *TaNPR1*, *TaAOS*, *TaLOX2*, *TaWRKY3*, *37*, *53-a*, and *71*. The aforementioned genes were explored to obtain knowledge about regulation of gene expression under different stresses. We found different motifs that are related to abiotic and biotic stresses and hormonal responses.

In regard to abiotic and biotic stresses, we recognised common motifs that were present in all gene promoters. As for biotic stresses, firstly, STRE, which is a stress-responsive element related to biotic stress in general, was detected in *TaNPR1*, *TaAOS*, *TaLOX2*, *TaWRKY37*, and *71*, as seen in Tables 8, 9, 10, 12 and 14. The highest frequency for STRE was found in *TaNPR1*. Previous research demonstrated that *TPP* (Trehalose-6-phosphate phosphatase) genes contain STRE cis-elements within their promoters via silico analysis. Moreover, the research reports that *TPP* genes partake in trehalose metabolism with these genes that play an significant a role in response to salt stress, drought stress, and senescence (Islam *et al.*, 2021). An additional paper exploring STRE stated that it was found within the promoter region of *AtWRKY75* gene via silico analysis (Abdullah-Zawawi *et al.*, 2021). The aforementioned gene has been shown to improve

resistance against *Sclerotinia sclerotiorum* (fungal disease) and oxalic acid stress in the Arabidopsis (Guo and Stotz, 2007; Chen et al., 2013).

Secondly, the WRE3 motif was identified in *TaPAL* and *TaNPR1*, as displayed in Tables 7 and 8 with the WUN-motif being present in *TaLOX2* (Table 10). Both of these *cis*-elements have a role in the regulation of wound response. One study stated that *TaDHN2* (a dehydrins gene in wheat) is triggered by PEG, cold, ABA, and salt stress. The study also stated that *TaDHN2* gene contained WUN-motif within its promoter location (Qin and Qin, 2016). An additional study on rice, exploring genes (Trehalose-6-phosphate phosphatase (*TPP*)) associated with Trehalose metabolism and biosynthesis, realised that the WRE3 cis-acting element was present within the promoters of 10 *OsTPP genes*, excluding *OsTPP1* and *OsTPP2*. Furthermore, the WUN-motif was detected inside the promoters of the following genes: *OsTPP9*, *OsTPP11*, *OsTPP3*, *OsTPP7*, and *OsTPP4*. It was indicated that Trehalose biosynthesis is linked to biotic/abiotic stresses like salt, pathogen, and drought (Rahman *et al.*, 2021).

Thirdly, TC-rich repeats were found only in *TaWRKY37* (Table 12). A study showed that as-1 cis-elements were also the most abundant regulatory signal in *TaTPS* genes that have role in the defence process for aphid (Zhao *et al.*, 2021). In the current research findings, it was interesting to find *TaNPR1* and *TaLOX2* containing the most occurrence of motifs related to biotic stress (Table 15).

On the other hand, our results found various *cis*-acting elements related to abiotic stress. Firstly, we found the distribution of various *cis*-acting elements that can be associated to light response in the promoter regions of the following genes (Tables 7, 8, 9, 10, 11, 12, 13 and 14): *TaPAL*, *TaNPR1*, *TaAOS, TaLOX2, TaWRKY3*, *37*, *53*-a, and *71*. For instance, G-Box existed in *TaNPR1*, *TaPAL*, *TaAOS, TaWRKY53-a*, and *TaWRKY71* with the highest occurrence of G-Box recognised in *TaNPR1* and *TaPAL*, as shown in (Tables 7and 8). In addition, TCT-motif occurred twice in *TaNPR1* and one time in each of *TaWRKY3* and *53-a*(Table 8, 11 and 13)(. AE-box was present in *TaWRKY71* (Table 14) with GT1-motif found in *TaWRKY3* and *TaWRKY53-a* (Table 10 and 13). Moreover, Box 4 occurred twice in each of *TaWRKY37* and *71* and appeared one time in each of *TaWRKY3* and *TaAOS* (Tables 12,14, 11 and 9). Furthermore, TCCC-motif was detected in *TaWRKY3*, *53-a*, and *71* (Tables 11, 13 and 14) with I-box found in *TaWRKY71* and *TaLOX2* (Tables 14 and 10). However, Sp1 was located in *TaNPR1*, *TaPAL*, and *TaWRKY53-a*

(Tables 7, 8 and 13). It must be noted that 'CAG-motif' was only present in the promoter of *TaWRKY71* as seen in Table 14.

Previous research has shown the location of Sp1, G-box, BOX I, Box 4, and GT1 elements in the promoter regions of *SbNHX* gene (related with sodium-proton antiporter (NHX)) and *SbNHE gene* (associated with sodium-proton exchanger (NHE)-type transporters) within the cereal crop *Sorghum bicolor*. This research project pointed to the two aforementioned genes participating in heat, cold, and salt stress (Hima Kumari *et al.*, 2018). In my research, I found that genes had various number of occurrences for motifs, in the following order: *TaWRKY71* was the highest, followed by *TaNPR1*, with *TaPAL* and *TaWRKY53-a* coming equally in third place (Table15). The aforementioned four genes could potentially be related to light stress.

Secondly, LTR motifs were found to be linked to low temperature response with the distribution of LTR being seen within the promoter region of the following genes: *TaNPR1*, *TaAOS*, *TaWRKY53-a*, and *TaWRKY71* as shown in (Tables 8, 9, 13, and 14). Previous research performed showed LTR existing inside the promoter regions of *GhWRKY6-like gene* (TF in cotton), which raises resistance countering salt stress in Arabidopsis and cotton (Ullah *et al.*, 2018).

Thirdly, some *cis*-regulatory elements are linked to drought, dehydration, and salt stresses. MYC binding site was recognized in the promoter regions of the following genes: *TaPAL*, *TaAOS*, *TaLOX2*, *TaWRKY3*, *37*, *53-a*, and *71* (Tables 7, 8, 9, 10, 11, 12, 13 and 14)Jang *et al.* (2004) explored MYB and MYC recognition sites, demonstrating that these sites were inside the promoter location of *TaLTP1* (Lipid transfer protein) within wheat. They found *TaLTP1* gene is responsible for tolerance to drought and salinity stress (Jang *et al.*, 2004). An additional group of scientists explored the promoter regions of 53 *TaOFP* genes (transcription factors responsible for plant growth and development-OVATE family proteins (OFPs), finding that they contain the MBS motif, which they propose is linked with response to drought stress (Wang *et al.*, 2020).

Furthermore, the DRE core motif has been found in *TaAOS*, *TaLOX2*, *TaWRKY37*, *TaWRKY 53-a*, and *71* (Tables 9, 10, 12,13 and 14) with DRE1, being in *TaWRKY37*, and *53-a*(Tables 12 and 13) Researchers stated that DRE, which stands for dehydration responsive elements are vital to regulation of genes in drought, low temperatures, and extreme salinity (Chen

et al., 2007). The same study realised that the *GmDREB2* gene, which is known to be a dehydration responsive element binding protein, inside soybean, has changed its expression under high salinity, low temperatures, and drought. After analysing G*mDREB2* gene, researchers recognised DRE motif existing within its promoters. They also saw the DRE motif connected to GmDREB2 in vitro. During my research, I found the highest occurrence for motifs related to drought and salt stresses for *TaPAL*, followed by *TaLOX2*, with *TaAOS* coming next. These genes could possibly play an unknown role in drought and salt stress (Table 15).

Fourthly, some *cis*-regulatory elements associated with hormonal signalling and response, such as ABRE were found in *TaPAL*, *TaNPR1* ,*TaAOS*, *TaLOX2*, *TaWRKY3*, and *53-a*, with ABRE4 existing within *TaWRKY71* (Tables 7, 8, 9, 10 ,11and 13). These elements (ABRE and ABRE4) are involved with ABA. Interestingly, ABRE was seen to be more abundant in *TaNPR1*, followed by *TaWRK53-a* (Table 15). The two motifs mentioned were not revealed in *TaWRKY37*. Chen *et al*. (2007), saw that DRE and ABRE recognition sequences were present within the promoter region of the G*mDREB2* gene (Chen *et al.*, 2007). *TaNPR1* was the gene with the most abundant ABRE, which is related to ABA. Moreover, specific motifs are related to hormone defence (SA) such as as-1 within *TaNPR1* (highest occurrence for motifs), *TaAOS*, *TaLOX2*, *TaWRKY 37*, *53-a*, and *71* (Tables 8, 9, 10, 12, 13,.14 and 15)

Additionally, a few *cis*-acting elements have been seen to be involved in the MeJA response, such as the CGTCA-motif, which exists in the following genes: *TaNPR1*, *TaLOX2*, *TaWRKY 3*, *37*, and *71*) (Tables 8, 10, 11,12and 14). Another cis-acting element, the TGACG-motif, was seen to be existing inside *TaNPR1*, *TaAOS*, *TaWRKY37*, *53-a*, and *71* as shown in Tables 8,.9, 12, 13and 14. However, *TaPAL* had not shown any of the aforementioned motifs associated with MeJA (Table 15). In fact, during my research in this chapter, the highest number of occurrences for the aforementioned two motifs (CGTCA-motif and TGACG-motif) was identified within *TaWRKY3* and *TaNPR1* (these two genes might be involved in MeJA) (Table 15). A study demonstrated that 139 transcripts of *TaTPS* genes (Terpene synthase) contained CGTCA and TGACG-motifs. It must be noted that *TaTPS* genes have a crucial contribution to plant defence mechanisms and are engaged in synthesizing and producing Terpenoids (Zhao *et al.*, 2021).

Continuing hormonal response, certain *cis*-regulatory elements are related to SA biosynthesis, such as the TCA-element, which is present in *TaWRKY37* (Tables 12). Another motif associated with SA-response is the TCA, which is present in *TaWRKY3* (Tables 11). We also recognised that some motifs were related to the gibberellin response, such as P-box found in *TaPAL* and GARE-motif in *TaPAL*. Moreover, ERE, which was involved in ethylene, was identified in *TaWRKY3* (Tables 11). In addition, some motifs related to auxin response, such as the TGA-elements were found within *TaPAL*.along with AuxRR-core, which was seen to be inside *TaPAL* and *TaAOS*. (Tables 7 and 9). Research has revealed that TPP (Trehalose-6-phosphate phosphatase) genes, particularly Group IV, include AuxRR-core and TGA-element, within their promoter regions related to auxin response (Islam *et al.*, 2021). The same research paper disclosed that SARE, TCA, and TCA-element, are linked to the SA response, while TATC-box, P-box, and GARE-motif have connection to gibberellin-responsive elements inside the promoter region of TPP. Moreover, it was uncovered that ERE (ethylene-responsive element) was recognised in TPP as well. The authors hypothesise that the TPP genes partake in the trehalose metabolism and some stress responses (e.g., Salinity, senescence, and drought).

Fifthly, in regards to motifs related to transcription factors and WRKY genes, one copy of W-box sequence was found in the promoter region of *TaWRKY53*, and one copy in *TaWRKY 71* as shown in Tables 13, 14, and 15. The *TaWRKY53-a* and *TaWRKY71* genes may be regulated by unknown WRKY genes or through self-regulation (Chi *et al.*, 2013; Yang *et al.*, 2013b). A research study exploring the tomato plant, found that W-box motifs exist in promoters of *SlProDH* (proline dehydrogenase) and *SlP5CS1* genes (encoding delta 1-pyrroline-5-carboxylate synthetase (P5CS); both of which contribute to synthesizing proline. The authors of the paper suggested that *SlWRKY81* gene can bond with *SlProDH* and *SlP5CS1* (Ahammed *et al.*, 2020)

6.4 Conclusions

Detection of *cis*-regulatory elements in the promoter regions of genes related to SA biosynthesis (*TaPAL*), genes associated with SA signalling (*TaNPR1*), genes that are responsible for JA biosynthesis (*TaAOS* and *TaLOX2*), and *WRKY* genes (*3*, *37*, *53-a*, and *71*) can provide more information on the regulation and controlling of these genes, and their response to abiotic and biotic stresses. The change in expression of these genes has been reviewed under salt and spot blotch disease in Chapters 3 and 4. In addition, we can observe that the distribution of *cis*-

regulatory elements is in the promoter regions of *TaPAL*, *TaNPR1*, *TaAOS*, *TaLOX2*, *TaWRKY3*, *37*, *71*, and *53-a*. Moreover, it was stated that *cis*-regulatory elements were correlated to abiotic/biotic stress and hormonal response. For drought / and salt response, *TaPAL* followed by *TaLOX2 then TaAOS* are highest occurrence for motifs related to drought / and salt. Moreover, for light response, *TaWRKY71* followed by *TaNPR1*, then *TaPAL* and *TaWRKY53-a* showed high occurrence for motifs linked to light. In addition, *TaNPR1* and *TaLOX2* have high frequency for *cis*- elements that connected to wound and biotic stress. Surprisingly, the regulation of the expression for the genes, *TaWRKY53-a* and *71*, could be done by w-box, which could bind with another WRKY gene. In addition, the identification of regulatory motifs would help in better understanding the potential biological roles and functionality of the genes of interest. After analysing the *cis*-regulatory elements detected within various promoter regions of genes, it was concluded that this assessment can facilitate the recognition and identification of potential elicitors for the purpose of gene transcription. It can be predicted that, since numerous hormonal compounds (e.g., ABA, MeJA, an SA) have been recognised to be acting in the capacity of cis-acting regulatory elements, they can be employed as triggers of gene expression regulation. In order to further examine the validation of *cis*-regulatory components, further research is required in future efforts.

Chapter 7. General Discussion

The response of crops to a given stress so as to maintain their growth, development, and productivity is highly complex. However, when subjected to a combination of abiotic and biotic stresses, the responses are even more complex. One of the major environmental factors is salt stress (i.e., abiotic stress) that reduces growth and productivity of crops. Additionally, spot blotch disease (i.e., biotic) is a factor that infects wheat and barley, causing damage to plant growth and consequently to yield (Kumar *et al.*, 2002). In the current research, we studied the response of wheat to salt stress and spot blotch disease, both individually and in combination, to better understand the mechanisms involved to these environmental factors at the physiological, biochemical, and molecular levels. It was observed that several WRKY transcription factor genes (*WRKY 3, 8, 19, 37, 44, 45, 46, 53-a* and *71*) were induced in wheat under salt stress and spot blotch disease, both singly and when combined. In addition, the SA-related genes (SA biosynthesis (*TaPAL*), SA signalling (*TaNPR1*) were induced differently in Sama and Alderon, JA-related genes (*TaAOX* and *TaLOX*2), and callose synthesis-related genes (*GSL 2, 3 ,8, 10, 12, 19, 22* and *23*) were upregulated or downregulated in their expression under spot blotch disease; JA/SA-related genes were also differentially expressed when plants were exposed to both spot blotch disease and a high level of salt stress compared to non-stressed plants.

Investigation of promoter regions in some WRKY genes (*TaWRKY3, 37, 53-a* and *71*), SA related genes (*TaPAL* and *TaNPR1*) and JA–related genes (*TaAOX* and *TaLOX*2) demonstrated the distribution of cis-regulatory elements that facilitate the regulation of these genes and are stimulated by abiotic/biotic stress, hormonal signalling, and some transcription factors. Our findings suggest that the wheat response to salt and spot blotch disease, applied individually, are different to when exposed to the dual stress.

7.1 Effects of Salt Stress on Wheat

7.1.1 Impact of salt stress on four wheat genotypes (morphological and biochemical)

Alderon, Cochise, Najran, and Sama genotypes were assessed for physiological parameters (shoot length, leave numbers, leaf area chlorophyll content, relative water content, shoot fresh weight, and shoot dry weight) and biochemical changes (Na^+, K^+, MDA, and proline contents) after plants were subjected to 0, 80, and 160 mM NaCl for 21 days. In addition, analysis of the changes in expression of *WRKY 3, 8,* 1*9, 37, 44, 45, 46, 53-a,* and *71* in these four wheat genotypes was investigated after long-term exposure to 160 mM NaCl (21 days). Our findings

verified that 80 and 160 mM NaCl cause reduction of shoot length, leaf numbers, leaf area, chlorophyll content, relative water content, shoot fresh weight, and shoot dry weight (see Chapter 3). Moreover, this high salt level also caused an increase in proline, MDA, and Na^+ contents, while leading to a reduction in K^+ content.

Our findings, however, indicated that there was variation among the four wheat genotypes in their response to the different levels of salt stress. The Najran wheat genotype showed optimal performance, when compared to the other three varieties, in terms of having lower reduction in number of leaves, and Na^+, but the highest accumulation of proline content (see Chapter 3). The next best performing wheat genotypes was Alderon and then Cochise, in terms of growth. Finally, Sama was shown to have the most impaired performance among the varieties tested, in terms of having the greatest reduction of shoot fresh weight and shoot dry weight, plus the least proline accumulation. As expected, the effects of salt on number of leaves, leaf area, chlorophyll content, relative water content, shoot fresh weight, shoot dry weight, Na^+ content, K^+ content, proline content and MDA content were more pronounced when plants were grown under 160 mM NaCl compared to 80 mM NaCl, irrespective of genotype.

The explanation for reduction in plant growth and perturbation in their metabolisms is that during plant growth in salt stress, osmotic stress and ion toxicity are major factors. (Chapter 1 and Chapter 3). Moreover, the increase in proline content in response to salt stress could be correlated with RWC and Na^+, as differences in proline levels was observed between salt-tolerant wheat and salt-sensitive wheat (see Chapter 3). It must be noted that the findings from my experiment were also comparable to the study by Borzouei *et al.* (2012), which observed that proline concentration of plants increased with salinity, but the more tolerant plants had higher build-up of proline content, relative to sensitive ones (1.3, 6, 8, 10 and 12 dSm^{-1}). The rise in proline content is known to be one of the responses of wheat to salt stress and is a well-described soluble osmoprotectant, which lessens the impact of osmotic stress due to salt stress (Nadeem *et al.*, 2020). In fact, the role of proline inside plant cells is to strengthen the cell capability for ionic alterations (Shahid *et al.*, 2020).

The rise in MDA due to increased salinity can potentially indicate that there is more oxidative stress in the plants, with tolerant plants having less oxidative stress than sensitive plants, which exhibit higher levels of oxidative stress. MDA content in plant cells, which is a result of lipid peroxidation, is a prominent indicator for membrane damage when they are

exposed to salinity (Katsuhara *et al.*, 2005). The reason why Najran had lower Na^+ content is thought to be due to its ability to exclude sodium and is therefore, proposed as a mechanism for salt tolerance used by the Najran variety. This could be related to a decline in the build-up of sodium, and hence, a rise in salt stress tolerance, which might be a result of influx reduction or efflux increase. There is a possibility that the Na^+/H^+ antiporter plays a substantial role in catalysing the mechanism of sodium efflux (Tester and Davenport, 2003).

7.1.2 WRKY gene expression under salt stress

Expression of *WRKY* genes can be influenced by salt stress, enabling signal transduction in addition to controlling expression of target genes (Li *et al.*, 2020b). In that regard, additional research is necessary both to exploit this characteristic for producing plants with enhanced resistance against salt stress, and to further identify transcription factors (TFs) involved in the response. These TFs are also recognised by way of transacting factors and have the role of binding particular DNA sequence motifs inside *cis*-acting elements, leading to the control of gene expression (Eulgem *et al.*, 2000; Malik *et al.*, 2020).

The impact of salinity in leaves of four wheat varieties after being exposed to 160mM NaCl revealed that there were changes in expression of *WRKY3*, *8*, 1*9*, *37*, *44*, *45*, *46*, *53-a*, and *71*. In addition, there were differences in the expression profiles of these 9 WRKY genes between Alderon, Cochise, Najran, and Sama, as shown in Chapter 3. The majority of WRKY genes in Najran were expressed at high levels in response to salt. For example, *TaWRKY37* was the most highly expressed gene of the 9 above genes, followed by *TaWRKY46* and *53-a*; in fact, *TaWRKY46* was the most highly expressed in all four genotypes studied. In Alderon, *WRKY53-a*, *8*, *46*, *3*, and *45* were upregulated whereas *WRKY71* was downregulated. Regarding Cochise, *WRKY37*, *46*, *45*, *19*, and *8* were shown to undergo upregulation while *WRKY3* was downregulated. In Sama, 4 of the WRKY genes were upregulated (i.e., *WRKY19*, *71*, *45*, and *44*) and *2* genes were downregulated (*WRKY53-a* and *37*) Concerning that, the investigation conducted by Zhou *et al.* (2019) saw that *TaWRKY*13 had a higher upregulation (approximately, 22-fold) in the first hour of treatment. The treatment that they used in their study had 150mM NaCl for a duration of 24hours. Furthermore, *TaWRKY3, 37, 53-a*, and *71* were shown to be differentially expressed between Sama and Najran (i.e., *WRKY 3*, *37*, and *53-a* were upregulated in Najran while *WRKY71* was downregulated. *TaWRKY53* and *37* were downregulated in Sama, *3* was not expressed, whereas *71* was upregulated). One possible explanation for the mentioned

changes in the expressions of these 9 WRKY genes is that these genes could be responsive to high levels of NaCl following long periods of salt exposure. This is relevant to the study done by Gowayed and Abd El-Moneim (2021) who determined that *TaWRKY8* was upregulated in all wheat genotypes amidst various salt concentrations (50, 150, and 250 mM NaCl). They observed that *TaWRKY8* had higher upregulation at 50 mM and 150 mM in a majority of wheat genotypes (gene expression for *TaWRKY*8 was lower at 250 mM) after one week of treatment (see Chapter 3).

Although there are many studies that have explained the effect of salt stress on wheat (Al-Ashkar *et al.*, 2020; Saddiq *et al.*, 2021), this study is more comprehensive in that it guides the reader on the effects of salt from the morphological level through to the molecular level. It was important to show how differences in salt concentration affect the seedling stages of wheat for long periods of stress (21 days) and to investigate the variations between wheat genotypes of differing levels of tolerance regarding their response in terms of physiological parameters and biochemical parameters (e.g. proline, MDA, Na^+, and K^+ content). In addition, WRKY genes that are highly expressed under high levels of salt concentration (160 mM NaCl) and their differential expression patterns amongst four wheat genotypes was also demonstrated.

7.2 Response of Wheat to *B. Sorokiniana* Infection

Bipolaris sorokiniana, the primary cause of spot blotch disease, infects many various tissue of the wheat crop, and is a hemibiotrophic fungal pathogen (Kumar *et al.*, 2002). In the present study, an investigation of the effects of spot blotch disease on wheat was conducted at the phenotypic, biochemical, and gene expression levels. The wheat genotypes Alderon and Sama, were chosen for comparison after resistance to spot blotch disease was assessed in the four genotypes (Alderon, Cochsie, Najran, and Sama). MDA content, callose content, SA, and JA content were measured across different times of infection. Furthermore, the results show the impact of pathogen infection over time (24 and 48 hpi) on the expression of TF (*WRKY3, 8, 37, 44, 45, 46, 53-a,* and *71*), glucan synthase-like (GSL) genes (*2,3,8,10,12,19,22, and 23*), in addition to genes involved in SA biosynthesis (*TaPAL*), SA signalling (*TaNPR1*), and JA biosynthesis (*TaAOX* and *TaLOX*2).

The results of this investigation show the variation in resistance amongst the four mentioned genotypes. In terms of the severity of the disease, it appears that Najran followed by Alderon, and Cochise exhibit the most resistance to the pathogen, while Sama was the most

susceptible. The variation of resistance amongst genotypes to spot blotch disease is related to their genetic background. Relevant research conducted by Baba (2019) indicated that the wheat varieties Rashid, Iraq and Cordiale exhibited substantial levels of disease severity, in comparison to Latifia and Tamoz3, which were more tolerant.

Alderon (resistant to *B. sorokiniana*) and Sama (susceptible to *B. sorokiniana*) were chosen to examine biochemical changes during the time course of infection (MDA, callose, SA and JA contents). MDA content measurement has long been recognised as a marker of lipid peroxidation in studies related to redox signalling and oxidative stress, particularly in investigations that concentrate on plant exposure to biotic/abiotic stress (Morales and Munné-Bosch, 2019). MDA was identified in high concentrations, especially at 96 hpi, in both Alderon and Sama; however, in Alderon, it was found in lower concentrations than Sama. This suggested that possibly two correlated and simultaneous phenomena were occurring: the development of disease in both wheat genotypes, and an increase in oxidative damage. Alderon exhibited less oxidative damage than Sama, at this specific period.

In the event of fungal infestation, fast callose production is considered the most potent defence mechanism of the plant. In some cases, papillae (cell wall appositions) get a deposit of callose, which is generated beneath infected areas and serves as a physical barrier to fungal invasion (Nishimura *et al.*, 2003). In the present study measuring callose concentration showed that it increased after time of infection in Alderon and Sama, and the amount of callose was higher at early stages of infection (24 and 48 hpi) than 72 and 96 hpi. Moreover, there was a variation in the amount of callose between the two genotypes. Within the late stages of infection, the amount of callose in Alderon was higher than in Sama. The results from my experiment are comparable to the study conducted by Shetty *et al.* (2009) who witnessed callose deposition, as a reaction against inoculation via *Septoria tritici* in two wheat cultivars (Stakado-resistant and Sevin-susceptible), but with various quantities and rates. Our result could demonstrate that the two wheat genotypes produced more callose to stop penetration at early stages of infection. Sama may not be able to produce callose after 48hpi, while callose concentration in Alderon was reduced at 72 and 96hpi. That would potentially mean that Alderon can resist the pathogen more than Sama.

SA and JA play a critical role in biotic stress after pathogen challenge (Fujita *et al.*, 2006). In our results, SA content was shown to be higher in Alderon compared with Sama; however, the

amount of SA content at the early stages of infection (48hpi) is higher than at later stages of infection (72 and 96hpi). In fact, Sahu *et al.* (2016) experiment is close to ours, as they observed the Yangmai #6 wheat cultivar being resistant against spot blotch disease, showing greater SA content relative to the Sonalika wheat cultivar, which is susceptible towards the pathogen, at 0, 8, 12, and 24 hpi.

Our finding suggests that Alderon produces SA during initial stages of infection as a signal to stimulate and activate biosynthesis pathways related to defence. Moreover, the amount of SA content being higher at 48hpi could be related to the lifecycle of *B. sorokiniana*, which is at its biotrophic stage at this time point (see Chapter 4). Additionally, JA was found to be higher in Alderon than Sama, at 96 hpi, which might be linked to the resistance of Alderon. There is also a likelihood that JA was active in Alderon at 96 hpi, because *B. sorokiniana* switched from the biotrophic phase to the necrotrophic phase (see Chapter 4).

At a molecular level, we observed changes in WRKY gene expression for Alderon and Sama at two examined time points (24 and 48hpi). In Alderon, expression of most of the WRKY genes was downregulated, except for *TaWRKY37* (upregulated) at 24 and 48hpi, and *TaWRKY3* (upregulated) at 24hpi. Furthermore, downregulation of *TaWRKY8*, *45*, *53-a*, and *71*, was greater at 48hpi than 24hpi. For the variety Sama, our findings showed changes in WRKY gene expression with most of the WRKY genes being upregulated, except for T*aWRKY19* and *45* at 24 and 48 hpi (downregulated), and *TaWRKY37* at 24hpi (downregulated). Surprisingly, at 48 hpi, we found that in both wheat genotypes, the expression of WRKY genes were more abundant than the expression of these genes at 24hpi.It is beneficial to consider the research by Millyard (2019) who saw *TaWRKY2, TaWRKY9, TaWRKY19,* and *TaWRKY29* being upregulated within wheat, subsequent to being exposed to *Septoria leaf blotch*. The upregulation of these genes happened almost near time of the shift of *Septoria* to necrotrophic development. It is noticeable by the start of visible symptoms in 12day-post-infection. Our results indicated that each wheat genotype responded differently to the spot blotch disease and importantly, both wheat genotypes experienced high expression at 48 hpi, in comparison to expression of WRKY genes at 24 hpi. We could explain the change of WRKY gene expression between genotypes and between the two examined points (24 and 48hpi), by the hypothesis that each genotype has a different genetic background, and the time of infection progression is important for both, due to the process of pathogen penetration in plant tissue.

Our study also investigated expression of genes involved in GSL biosynthesis in Alderon and Sama across time of infection at 24 and 48hpi. We found that most of GSL genes were induced in upregulation in Alderon while in Sama, most of the GSL genes were downregulated. Notably, the expression of most GSL genes in Alderon were higher at 48 hpi than in 24 hpi. A different, yet related study, observed *TaGSL3* and *TaGSL8* gene transcripts playing a role in resisting *Fusarium graminiarum*, that is a fungal pathogen (Rana *et al.*, 2014). Our results revealed that Alderon is probably able to synthesise callose at 48 hpi, and that Alderon was potentially more resistant to the pathogen at this time point than Sama.

The expression of genes related to SA biosynthesis (*TaPAL*) and signalling (*TaNPR1*) were not shown clearly in Alderon, but there were more changes in *TaPAL*, at 48hpi, in terms of downregulation, than 24 hpi. It could be that Alderon is responding to spot blotch disease and that it may have had higher expression in any time during infection. In Sama, we can see that *TaPAL* was downregulated and *TaNPR*1, upregulated after infection. Both of these genes could indicate a response to the pathogen with *TaNPR1* being conceivably involved with *TaPAL*. In Alderon, *TaAOS* was upregulated at 24hpi and downregulated at 48 hpi, while *TaLOX2* was not expressed at either time points. In Sama, *TaAOS* was expressed at 24 hpi but not at 48 hpi, while *TaLOX2* was downregulated at 48hpi, but showing no clear expression at 24 hpi. The expression patterns for JA could be related to the priming response and it might have been that these genes were expressed higher at late stages of infection.

7.3 Response to Salt and Spot Blotch Disease

As mentioned before, plants are severely limited in their ability to grow and develop due to salt stress, a primary abiotic stress. In the field, salt stress is a common problem for plants during their lifespan. In addition, pathogen infection is more likely to occur after salinity stress, although other stresses, such as drought and heat, may occur at any moment, either before or after pathogen infection. The consequence of these interactions will become more problematic as a result of the additional complexities (Bai *et al.*, 2018a). Since most research has concentrated just on single salt stress or spot blotch disease, a combination of salt stress and spot blotch disease is an uncommon study, and the current research tries to understand the wheat response to salt and spot blotch disease at phenotypic, biochemical, and molecular levels.

We subjected Alderon and Sama to a high salt concentration (160 mM NaCl) for eight days, followed by *B. sorokiniana* infection for four days, to study disease severity, shoot length,

leaf numbers, MDA, SA, and JA contents. The results show MDA was increased in both genotypes, but mostly at late stages of infection at 96 hpi (time effect is significant in MDA while the genotypes were not significant)
However, SA was higher at 72 hpi in Alderon than Sama, but was higher in Sama than Alderon at 96 hpi. JA-content was seen to be higher in Alderon than Sama at 72 hpi, but significantly higher in Sama than Alderon at 96 hpi, Sama was higher than Alderon in JA-content. It is important to note that Sama was higher at 96 hpi, compared with the control, and also higher than in Alderon (there was no significant effect for each of time, genotypes and their interaction statistically in SA and JA contents.

As a further step, we analysed gene expression of *TaWRKY3, 8, 19, 37, 44, 45, 46, 53-a,* and *71,* as well as *TaPAL, TaNPR1, TaAOX,* and *TaLOX2.* Based on disease severity, shoot length, and number of leaves, Sama was found to be more susceptible to salt and spot blotch disease than Alderon. Furthermore, the disease severity in both wheat varieties is lower than if they were infected by the pathogen alone (as explained in Chapter 4 and 5). Moreover, analysis of biochemical changes such ion content (Na^+ and K^+), was conducted where it was found that Alderon and Sama accumulated Na^+ in their stressed leaves with Alderon having higher concentrations than Sama.

Our results indicated that both wheat genotypes were affected by the combination of the stresses, in terms of their growth. Moreover, it was witnessed that within both genotypes, the disease severity was less in the wheat that was subjected to the combination of stresses than the one exposed to the pathogen alone (Sama had higher disease severity than Alderon). A possible explanation for this is the sodium accumulation which affected fungal penetration, as mentioned in Chapter 4,

One interesting observation within the experiment was MDA content being maximal at 96 hpi of combined stresses for both Alderon and Sama. However, Alderon contained less MDA than Sama. Our research is comparable to the one performed by Tani *et al.* (2018) , who witnessed that the response of eggplants against the combined treatment of *Verticillium dahlia* (fungal plant pathogen) and drought, affected MDA levels to rise, following exposure to 3-weeks in both cultivars (EMI and Skoutari), which have intermediate resistance the pathogen. Additionally, SA and JA were increased mostly at later stages of the infection i.e. at 72 and 96 hpi of combined stress. The results thus show that MDA, SA, and JA shared an increase in their

production at late stages of infection under combined stresses, which seems to be a very important time for fungus to be able to impact the plant. It is possible that salt stress may change the defence pathways in the crop (both genotypes) and metabolize them before infection, as described in Chapter 4.

The current study also investigated the expression of WRKY genes in Alderon and Sama. In Alderon, we found *TaWRKY46* and *71* were upregulated in their transcript levels, but *TaWRKY3*, *8*, and *19* were downregulated. In contrast, most of the WRKY genes were upregulated in Sama; for example, *TaWRKY37*, followed by *19*, and then *3*, were the most abundant in their transcript levels. *TaPAL*, *TaNPR1*, *TaAOS*, and *TaLOX2* were analysed for their expression in Alderon and Sama, where we found all genes in Alderon were downregulated, whereas in Sama, all were upregulated. The fact that expression of all genes in both Alderon and Sama were altered may be due to a stress response of senescence. A study determined that the stress combination of salt and powdery mildew caused a senescence effect inside the tomato crop (Kissoudis *et al.*, 2016).(See Chapter 5).

An additional occurrence that we could observe in the dual stress treatment was cross-talk, particularly Na^+ accumulation correlated with disease severity. Also, there was a negative impact of combined salt and spot disease on plant growth.

7.4 Analysis of Promoter Regions of WRKY Genes and Hormonal-Related Genes in Silico

We analysed the promoter regions of specific WRKY genes that respond to salt stress, such as *TaWRKY3, 37, 53-a,* and *71*. Furthermore, *TaPAL*, *TaNPR1*, *TaAOS,* and *TaLOX2*, which were impacted by spot blotch disease and a combination of salt and spot blotch disease were analysed to recognise *cis*-regulatory elements which could regulate gene expression. We detected that these elements are linked to hormonal signalling, abiotic stress (drought, salt, and dehydration), and biotic stress (wounds and pathogen). Importantly, we found that the W-box in *TaWRKY53-a* and *71*, may be controlled by WRKY genes or self-regulation. Moreover, *TaPAL, TaLOX2 and TaAOS* respond to drought and/or salt stresses. In addition, *TaWRKY71, TaNPR1, and TaPAL TaWRKY53-a* are involved in the light response within plants. Furthermore, *TaNPR1* and *TaLOX2* participate in wound and biotic stress.

In this context, our work focused on the characterization of some *TaWRKY* members under salt stress, which led to the identification of *TaWRKY37*, a potential candidate for salinity tolerance. Focusing on upregulation status, our experiments showed that the most interesting gene found to

be expressed at the highest level in Najran variety, followed by Cochise and thereafter Alderon, was *TaWRKY 37*. While, the latter was downregulated within Sama variety. So as to observe comparable functions to the rest of the genes within wheat or various cereal crops, we carried out phylogenetic tree analysis. It was revealed that *HvWRKY44* and *TaWRKY44* clustered together with *TaWRKY37*. In support of our experiment, research conducted by Okay *et al.* (2014) ,who used RNA-seq database, found *TaWRKY37* within wheat tissues under drought stress. Moreover, work done by Wang *et al.* (2015) showed that *TaWRKY44* was induced by salt stress (200Mm NaCl for 24h) in wheat leaves. As for *HvWRKY44* gene,Zheng *et al.* (2021) saw that its expression was induced in an early developmental phase for grain in barley. In addition, promoter sequence of *TaWRKY37* was abundantly reach in cis regulatory element related to drought and salinity such as DRE core and MYB-like motives. *TaWRKY37* could play a role in signalling and activation of downstream genes related to salt stress. It may activate downstream salt responsiveness genes via activation and initiation of ABA signalling pathway (Jiang *et al.*, 2017). It has been demonstrated for several WRKYs their implication in ABA signalling pathway and their responsiveness to this phytohormone (Bakshi and Oelmüller, 2014a) . They can play both positive or negative regulators in ABA mediated salinity response, constituting a complex network of WRKY transcriptions factors, like for AtWRKY18, 40, 80(Rushton *et al.*, 2012; Reddy *et al.*, 2013; Banerjee and Roychoudhury, 2015)Gene silencing and Knock-Out technologies have been already used in abiotic stress tolerance strategy within several plants and for different kind of genes(Meriç *et al.*, 2020; El-Sappah *et al.*, 2021). Indeed, WRKY gene knock-down approach would help determining the function and role of the silenced gene (El-Sappah *et al.*, 2021). It is possible to knock-down *TaWRKY37* and generate transgenic plants. In a study conducted on transgenic tomato plants carrying RNAi constructs, it was demonstrated for the WRKY12 and WRKY13 silenced lines, an upregulation of SOS1 transporter and APX enzyme respectively, leading to salt tolerance, and suggesting a negative regulation for these WRKYs (Birhanu and van der Linden, 2020) In the same way, genes functional characterization could be performed through transgenic approach in order to obtain a gain of function via gene overexpression (Adamski *et al.*, 2020). In this context, transgenic lines of rice overexpressing *TaWRKY13* displayed improved phenotype and better salt tolerance(Adamski *et al.*, 2020).

7.5 The Implications of the Study

Our results may contribute to providing knowledge for wheat breeding and wheat production, particularly in terms of how wheat can be sustained for long periods of exposure to salt stress, and what the biochemical markers are which show wheat tolerance to salt stress. Furthermore, the WRKY genes identified in current research may be employed as gene markers to develop wheat tolerance towards severe salt stress; specifically, *TaWRKY37, 53-a*, *3*, and *71* have shown their differential expression between tolerance and sensitivity to salt stress.

Moreover, understanding the biochemical changes and expression of selected genes in Alderon and Sama over the time of infection could offer us information on the behaviour of two contrasting wheat genotypes in response to Spot blotch disease. Notably, SA and JA are important hormonal responses for fungal penetration and wheat resistance to plant disease. Callose concentration can also be used as a biochemical marker for wheat resistance to plant disease. In terms of wheat transformation and breeding referred to earlier, most of the WRKY and GSL genes could be used as gene markers.

Overall, the interactions between the combination of salt and spot blotch disease with the wheat plant has many complexities, and many factors should be considered for future research on wheat, such as the timing of the stress, severity of the stress, and the wheat genotypes in question. We suggest investigating the potential for inducing a combination of spot blotch disease and salt stress for the purpose of finding new wheat performance enhancement methodologies via using abiotic stress. In fact, salt stress could be used for reducing disease within plant breeding (specific/limited periods), as long as special consideration is given to its concentration. However, high levels of salt tolerance should be considered beforehand to reduce the impact of salt on growth.

It is important to note that under a combination of stresses, wheat responds differently than when subjected to either salt or spot blotch individually; specifically, in terms of plant growth, biochemical changes, and gene expression (i.e., WRKY genes). This information is important to contribute towards wheat resistance against abiotic and biotic stresses, along with finding how cross-tolerance can be exploited to mitigate consequences of abiotic and biotic stresses on wheat.

Selected WRKY genes and hormonal related genes (SA and JA) could also be utilised as gene markers in wheat breeding and transgenic wheat. Future research should study the role of WRKY

and hormonal related genes (SA and JA) under individual stresses and their combinations (salt and spot blotch disease). These findings can potentially mean that these three genes are important for crop breeding programs, but further research should consider their function in response to abiotic and biotic stresses.

Ultimately, understanding the physiological, biochemical, and molecular mechanisms in wheat, under different salt concentrations, is important to improve new breeding approaches for crop tolerance. This information is critical to building wheat tolerance to plant diseases and identifying crops that are more tolerant to pathogens.

7.6 Conclusions

Wheat yield and growth are negatively affected by salt stress and spot blotch disease, with knowledge of its response to them helpful to preserve this important crop. In regard to salt stress, an examination of how four wheat genotypes (Alderon, Cochsie, Najran, and Sama) respond to this stress was provided in this study. We observed that Najran, followed by Alderon and Cochise performed better than Sama in terms of growth, proline production, and abundance of transcripts for several WRKY genes. The results demonstrated differential expression for *TaWRKY53-a*, *37*, *3*, and *7*1 between Najran and Sama. In addition, the findings suggested that Najran, followed by Alderon and Cochise, performed better than Sama in terms of disease severity. Based on MDA, SA, and JA levels, we found that the Alderon wheat genotype is more resistant than Sama to the spot blotch disease; callose content increased after infection in both genotypes. At the molecular level, we observed that *TaGSL* genes were expressed more at 48hpi in Alderon than in Sama (several WRKY genes were expressed differently in the two wheat genotypes). In addition, it was observed that the transcript profile of WRKY genes was more abundant at 48hpi. There were differences in gene expression related to SA-biosynthesis (*TaPAL*), SA-signalling (*TaNPR1*), and JA-biosynthesis (*TaAOS* and *TaLOX2*) between Alderon and Sama.

Our results indicated that Alderon and Sama respond differently to the combination of salt and spot blotch disease. Moreover, disease severity was shown to be less in Alderon than in Sama, when subjected to the combination of the two stresses. The MDA, JA, and SA levels changed in response to the combination of salt and spot blotch disease. During the gene expression analyses, we found that most WRKY genes together with *TaPAL*, *TaNPR1*, *TaAOS*, and *TaLOX2* were downregulated in Alderon, whereas in Sama, WRKY genes along with *TaPAL*, *TaNPR1*, *TaAOS*, and *TaLOX2* were upregulated. In regards to the aforementioned

genes, at 48hpi of combined stresses, more abundance is seen in transcript levels. Our findings demonstrated that the W-box elements found in the promoter regions, *TaWKY53-a,* and *71* are regulated by WRKY genes and also *TaWRKY71* was found to involved in light response. *TaPAL* one of the important genes due to it was found to contain high frequency of *cis*-regulatory elements. These elements are mostly related to drought stress. Additionally, *TaNPR1* and *TaLOX2* contribute biotic stress. Taken together, this information suggests that wheat responds differently to salt stress alone, spot blotch disease alone, and to the interaction of salt and spot blotch disease. This is supported by the findings that gene expression was found to be different in wheat plants when subjected to either of the stresses alone or to the dual stress, particularly for WRKY genes. This knowledge could help to develop wheat tolerance against abiotic stress and biotic stress and the interaction between these stresses. More research, however, needs to be performed to have a deeper understanding of how different wheat genotypes respond to salt stress and spot blotch disease. Finally, candidate WRKY genes (SA/JA-related genes and GSL genes) should be investigated in greater detail to better understand their precise function and how they can be regulated.

7.7 Future perspectives

- Exploitation of more wheat genotypes under different levels of salt stress at different stages of wheat development
- WRKY gene expression in wheat genotypes could be studied in relation to different levels of salt concentration across contrasting wheat varieties
- Exploring gene expression related to wheat salt tolerance, including ABA biosynthesis signalling, proline biosynthesis, MDA biosynthesis, and ion transporter genes
- Understanding the life cycle of *B. sorokiniana* from biotrophic phase to necrotrophic one, in addition to how wheat responds to each phase via microscopic and molecular studies
- Investigating different levels of salt concentration (i.e., low, medium, and high) along with differing severities of spot blotch disease on the wheat crop
- Identifying genes that are involved with the hormone ethylene and its biosynthesis under salt stress and spot blotch disease, individually, and the dual stress.
- Studying the function of *TaWRKY53-a, 71,* and *37* genes along with *TaLOX2* and *TaGSL* genes via gene knockout or gene overexpression.

- Validation of *cis*-regulatory elements and w-box found in the promoter regions of *TaWRKY53-a* and *71* genes.

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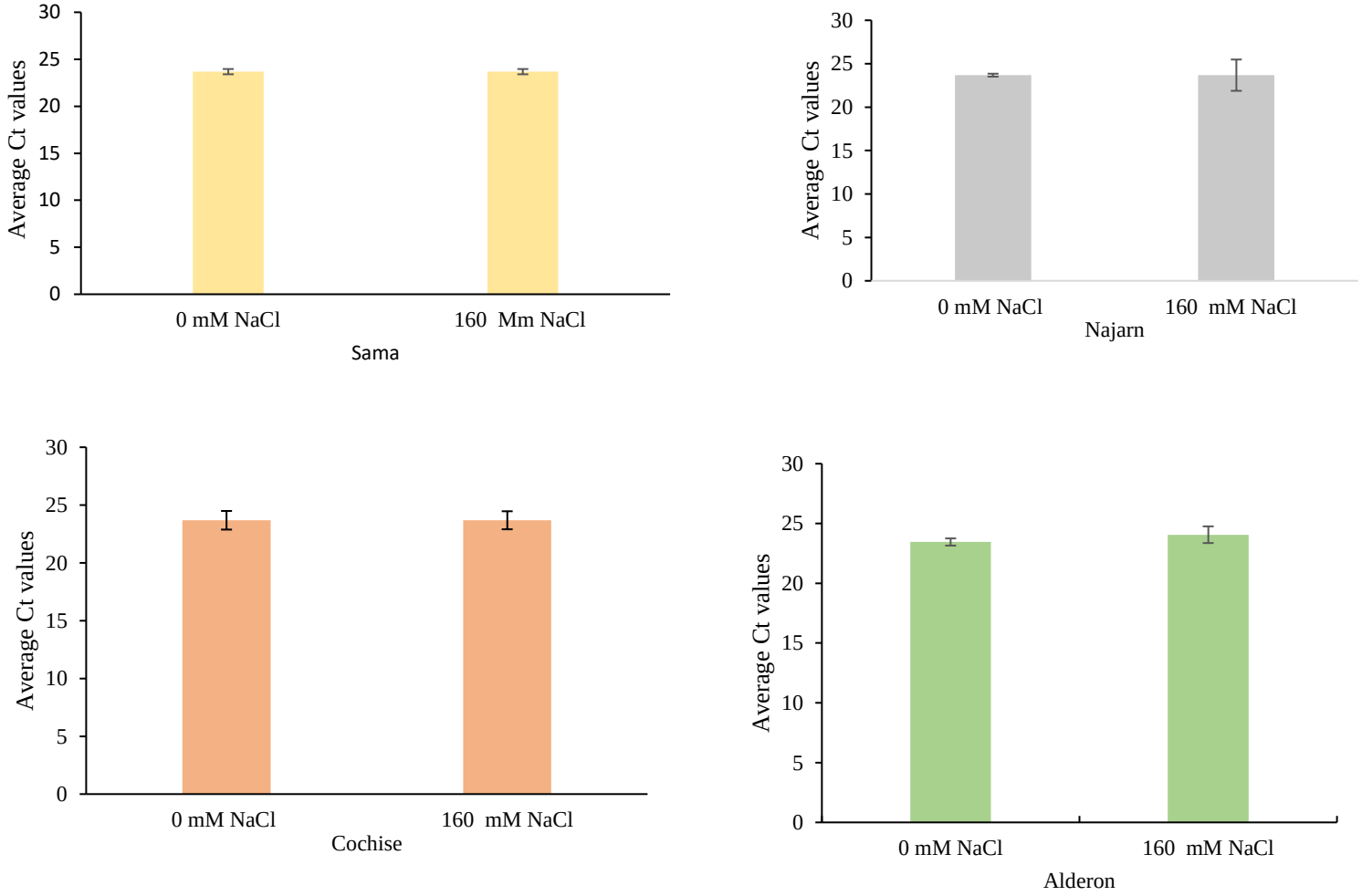
Appendices

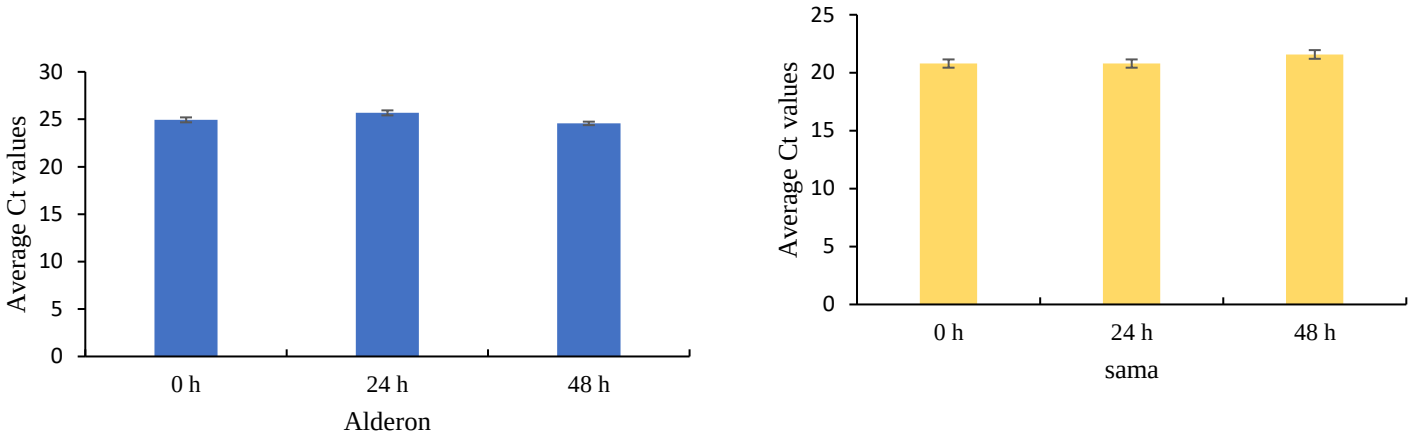
Appendix A1

Validation of reference gene under salt stress for selected wheat varieties

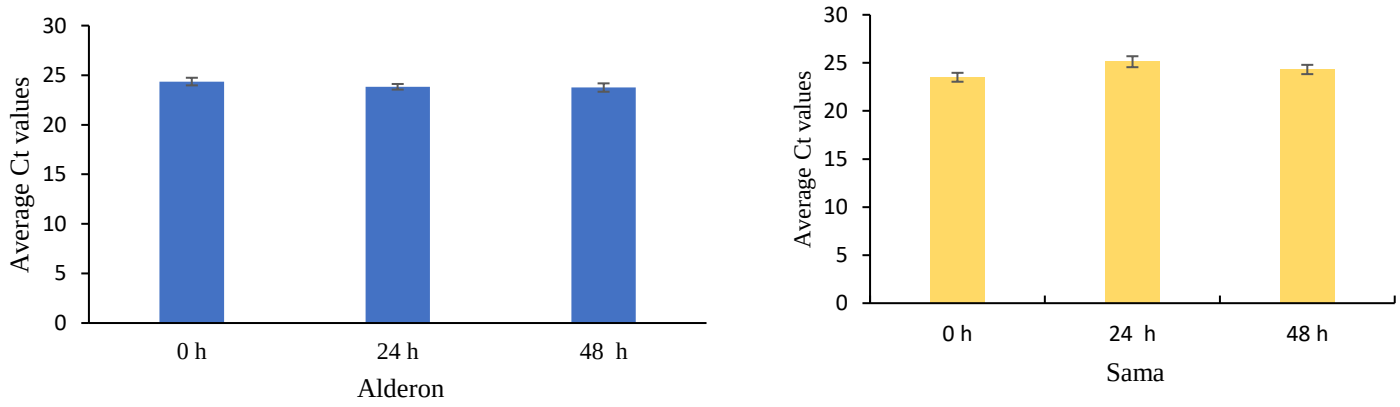


Expression of *TaEF1 α* at 0 Mm NaCl and 160 Mm NaCl concentrations in Sama , Najran , Cochise and Alderon wheat cultivars. Standard error was indicated by the error bar-3 replicates) using Ct values

Appendix A2

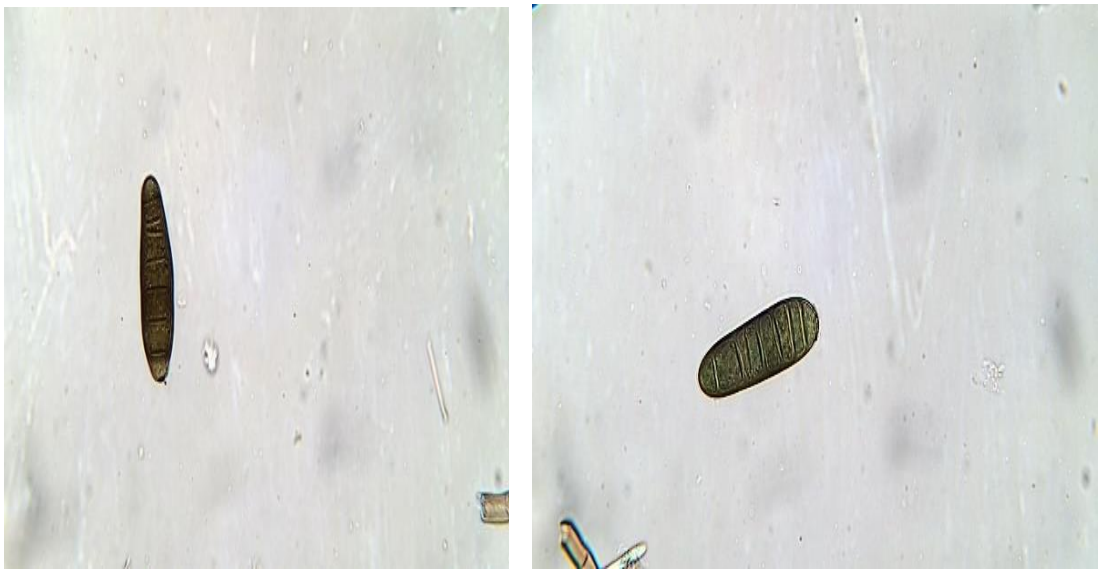


Expression of *TaEF1 α* at at different time after pathogen infection (24 and 48) and before infection (0 h)and in Alderon and Sama Sama cultivars. Values are means of 3 replicates and standard error is shown by error bars using delta Ct values



Expression of *TaEF1 α* at at different time after salt and pathogen infection (24 and 48) and before infection (0 h) and in Alderon and Sama Sama cultivars. Values are means of 3 replicates and standard error is shown by error bars using delta C

Appendix B



Spores of *Bipolaris sorokiniana* under light microscope